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Preparation of 3-Aminonocardicinic Acid and Its Acyl Derivatives

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3-Aminonocardicinic acid (3-ANA, **2**) was prepared by removal of the N-acyl group of nocardicin A (**1**). Reacylation of **2** with some typical side-chain acids gave the corresponding semisynthetic nocardicins **6**.

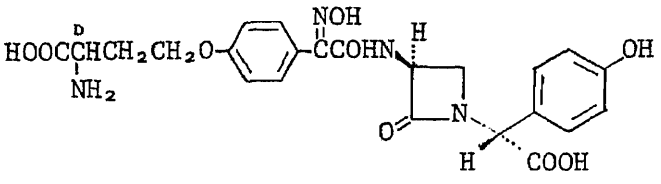
Keywords——3-aminonocardicinic acid; nocardicin; antibacterial activity; structure–activity relationship; the Edman degradation

Nocardicin A (**1**)[1] and its congeners[2] were the first monocyclic β-lactam antibiotics with quite different structures[3] from the classical β-lactam antibiotics, penicillins and cephalosporins. Although nocardicins have a broad antibacterial spectrum, the level of activity is only moderate, especially against gram-positive bacteria.[4] It seemed useful, therefore, to study the structure–activity relationships as a preliminary step toward designing more potent analogues of nocardicins. For the preparation of scuh semisynthetic nocardicins, the removal of the N-acyl group of nocardicin A was required to give 3-aminonocardicinic acid (3-ANA, **2**), the basic nucleus of this family of antibiotics. We previously reported the side-chain cleavage of nocardicin A in a review of the chemistry of nocardicins.[5] This paper is devoted to a full account of the work, also describing the preparation of some new acyl derivatives of 3-ANA.

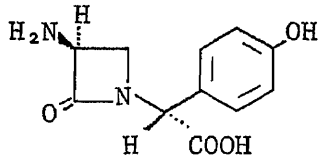
The Edman method is the most common procedure for peptide bond cleavage.[6] We applied this procedure to the preparation of 3-ANA (**2**) from nocardicin A (**1**). For this purpose, the oxime group of **1** was reduced to the corresponding amino group. Catalytic reduction of **1** on 10% Pd–C gave a 1 : 1 mixture of the diastereomeric amino derivatives **3**, one of which was shown to be identical with nocardicin C.[2] This mixture was then treated with phenylisothiocyanate to yield the dithiourea **4** in a ratio reflecting that of the starting amino derivatives **3**. After treatment of **4** with concentrated HCl in AcOH at room temperature followed by dilution with H_2O and adjustment to pH 4 with Amberlite IR-45 under ice-bath cooling, the reaction mixture was concentrated *in vacuo*. The resulting precipitate was collected by filtration and washed with H_2O to afford **2**. Concentration of the mother liquor followed by cooling gave the piperazone **5** as a by-product, which was probably formed from **2** during the reaction and isolation processes, seemingly *via* cyclization of the 3-

amino group with the carboxy group in the 1-substituent, followed by cleavage of the β-lactam ring.[7] Compound **5** was found to be quantitatively obtained by treatment of **2** with concentrated HCl in AcOH. 3-ANA (**2**) was thus highly labile to acids and, therefore, the yield varied widely with slight alterations of the reaction conditions, being at best 40%. Alkaline conditions (*e.g.* K_2CO_3 in aqueous MeOH) were also examined for the Edman degradation of **4**, but gave in poorer yields (*ca.* 25%) of **2**.

For the preparation of semisynthetic nocardicins, **2** was reacylated with typical side-chain acids. Compounds **6a** and **6b** were prepared by acylation with phenoxyacetyl chloride and phenylacetyl chloride, respectively, in aqueous acetone in the presence of $NaHCO_3$. Compounds **6c** and **6d** were obtained by the acid chloride method. The acids used for **6c** and **6d**, in which the oxime function was protected by the dichloroacetyl group, were converted on treatment with PCl_5 into the corresponding acid chlorides which were immediately reacted with a solution of the trimethylsilyl ester of **2** in CH_2Cl_2. The oxime protecting group was then removed by treatment with aqueous $NaHCO_3$. The same procedure was used for the preparation of **6e** and **6f** starting from 2-(2-trifluoroacetamido-1,3-thiazol-4-yl)-2-methoxyiminoacetic acid and 2-(4-hydroxyphenyl) glyoxylic acid, respectively. The N-protecting group of the former was removed by treatment with aqueous AcONa. Compounds **6g** and **6h** were prepared by the active ester procedure using *N*-hydroxysuccinimide. Thus, the *N*-*tert*-butoxycarbonyl derivatives of *p*-hydroxyphenylglycine and phenylglycine were converted to the active esters, which were reacted with **2** in aqueous acetone in the presence of $NaHCO_3$. Deprotection of the products by treatment with trifluoroacetic acid gave **6g** and **6h**.



1



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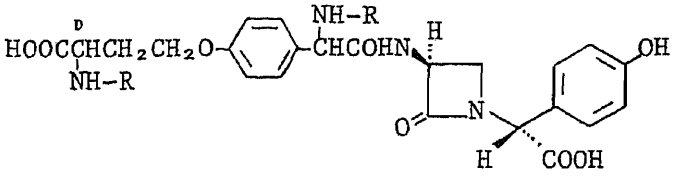

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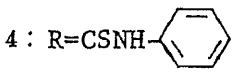

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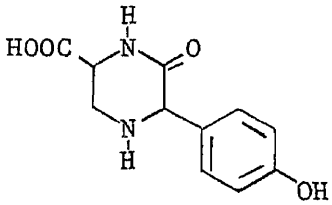


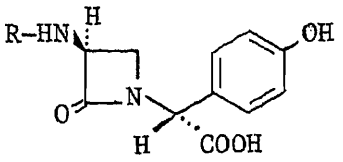
5

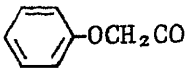
The antibacterial activity of the derivatives described above was determined by the agar dilution method. The data are summarized in Table I. All the derivatives were found to be less active than the parent nocardicin A. In particular, the compounds lacking oxime and keto groups (**6a**, **b**, **g** and **h**) were considerably less active or devoid of activity. The oxime and keto derivatives (**6c**, **d**, **e** and **f**) were active against *Pseudomonas aeruginosa*, *Escherichia coli*, and *ES*-114, indicating that these functions are desirable for biological activity. However, even these compounds showed reduced activity as compared to nocardicin A. This indicates that the D-homoserine side-chain in nocardicin A is also important for antibacterial activity.

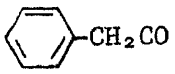
TABLE I. MIC's of Nocardicins[a]

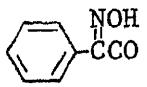
Compound	Organism					
	Pseudomonas aeruginosa 10490	*Escherichia coli* NIHJ, JC-2	*Escherichia coli* 114[b]	*Proteus vulgaris*	*Staphylococcus aureus*	*Bacillus subtilis*
6a	0.375	>3	3	>3	3	0.75
6b	>10	>10	>10	>10	>10	2.5
6c	0.6	10	0.6	2.5	0.6	2.5
6d	0.1	3.2	0.4	10	2.5	—
6e	2.5	1.25	0.6	10	—	—
6f	2.5	5	1.25	2.5	>10	>10
6g	0.1	>3.2	>3.2	>10	>10	1.25
6h	1.25	>5	>5	>5	>5	>5
Nocardicin A	0.025	0.8	0.0063	0.25	—	0.1

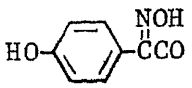
a) Agar dilution method (mg/ml). *b*) A mutant strain of *E. coli* NIHJ: sensitive to β-lactam antibiotics.

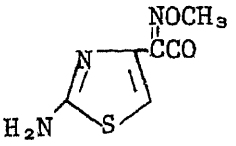


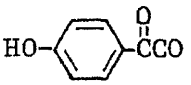
6 a: R = 

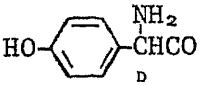
6 b: R = 

6 c: R = 

6 d: R = 

6 e: R = 

6 f: R = 

6 g: R = 

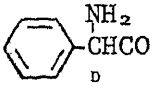
6 h: R = 

Fig. 1

Experimental

Melting points were measured on a Thomas–Hoover apparatus and are uncorrected. Infrared (IR) and proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded using a Hitachi 260-10 spectrophotometer and JEOL PS-100 spectrometer, respectively. Optical rotations were measured on a JASCO automatic polarimeter.

Minimum inhibitory concentrations (MIC's) of the nocardicin analogues were determined by the agar dilution method. One loopful of an overnight culture of each test organism in Tripticase broth (about 10^8 viable cells/ml) was streaked on heart infusion agar containing graded concentrations of the drugs and was incubated at 37 °C for 18 h.

Diastereomeric Nocardicin C (3)——A mixture of nocardicin A (**1**) sodium salt (100.0 g) and 10% Pd–C (30.0 g) was shaken with hydrogen under atmospheric pressure until the absorption of hydrogen ceased. The catalyst was filtered off, and the filtrate was adjusted to pH 3 with 10% HCl under ice-cooling, decolorized with activated charcoal, and diluted with acetone (1.9 l). A crystalline solid separated out, and was collected by filtration and washed with acetone to give **3** (78.2 g, 84.8%): mp 196—200 °C (dec.). IR (Nujol): 3400, 3300, 1735 (β-lactam C=O), 1610 cm^{-1}. ^{1}H-NMR (D_2O + $NaHCO_3$) δ: 2.52 (2H, m, CCH_2C), 3.05 and 3.07 (1H, two dd, J=3, 5 Hz, β-lactam 4β-H), 3.66—3.93 (2H, m, β-lactam ring 4α-H and NCHCOO), 4.12 and 4.16 (2H, two t, J=6 Hz, CCH_2O), 5.13 (1H, m, β-lactam 3α-H), 6.86—7.40 (8H, m, ArH). *Anal.* Calcd for $C_{23}H_{26}N_4O_8 \cdot 1/2H_2O$: C, 55.75; H, 5.49; N, 11.31. Found: C, 55.53; H, 5.39; N, 11.28.

Diastereomeric Bis(phenylthioureido)nocardicin C (4)——Phenylisothiocyanate (47.8 g) was added to a solution of 50% aqueous pyridine (1.1 l) and diastereomeric nocardicin C (**3**, 57.8 g) over a period of 5 min at room temperature. The reaction mixture was stirred for 1 h, during which time the pH was maintained at 9 by adding $NaHCO_3$. The reaction mixture was cooled to room temperature and extracted with Et_2O. The aqueous layer was cooled to approximately 10 °C and adjusted to pH 2 with 20% HCl to precipitate a solid, which was collected by filtration and washed with H_2O to give **4** (69.7 g, 78.1%): mp 144—145 °C (dec.). IR (Nujol): 3300—3200, 1735 (β-lactam C=O) cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 2.26 (2H, m, CCH_2C), 2.98 and 3.10 (1H, two dd, J=3, 5 Hz, 4β-H), 3.78 and 3.80 (1H, two t, J=5 Hz, 4α-H), 4.05 (2H, t, J=6 Hz, CCH_2C), 4.33 (1H, m, NCHCOO), 5.32 and 5.34 (1H, two s, ArCHCO), 5.45 (1H, s, ArCHCOO), 6.62—7.45 (18H, m, ArH). *Anal.* Calcd for $C_{37}H_{36}N_6O_8S_2 \cdot H_2O$: C, 57.35; H, 4.94; N, 10.85. Found: C, 57.33; H, 5.11; N, 10.63.

3-Aminonocardicinic Acid (3-ANA, 2)——Method 1: Diastereomeric bis(phenylthioureido)nocardicin C (**4**, 30.0 g) was dissolved in AcOH (80 ml). The solution was then cooled to 10 °C and concentrated HCl (6.4 ml) was added in one portion with vigorous stirring. Stirring was continued for 30 min at the same temperature, then the reaction mixture was poured into ice water (300 ml) and extracted with AcOEt. The aqueous layer was stirred under ice-cooling and Amberlite IR-45 (about 200 ml) was added to adjust the pH to 2.4—4.4. After the resin had been removed by filtration, the filtrate was evaporated under high vacuum at 35—40 °C. The crystalline solid was washed with acetone to give **2** (3.77 g, 40.0%): mp 198—200 °C (dec.). $[\alpha]_D^{25}$ −252° (c=1.1, 0.1 N $NaHCO_3$). IR (Nujol): 1763 (β-lactam C=O), 1742 cm^{-1}. ^{1}H-NMR (D_2O + NaOD) δ: 2.89 (1H, dd, J=3, 5 Hz, 4β-H), 3.79 (1H, t, J=5 Hz, 4α-H), 4.22 (1H, dd, J=3, 5 Hz, 3α-H), 5.26 (1H, s, ArCHCOO), 6.91 (2H, d, J=8 Hz, ArH), 7.23 (2H, d, J=8 Hz, ArH). *Anal.* Calcd for $C_{11}H_{12}N_2O_4$: C, 55.93; H, 5.12; N, 11.86. Found: C, 56.05; H, 5.01; N, 11.58.

Method 2: Diastereomeric bis(phenylthioureido)nocardicin C (**4**, 1.44 g) was suspended in H_2O (10 ml) and K_2CO_3 (0.56 g) was added. The reaction mixture was stirred at 30 °C for 24 h. After the precipitate had been removed by filtration, the filtrate was adjusted to pH 2 with 5% HCl and extracted with AcOEt. The aqueous layer was adjusted to pH 4 with $NaHCO_3$. The mixture was evaporated and the residue was subjected to column chromatography on activated charcoal with H_2O. The eluate was evaporated and the resulting residue was washed with acetone to give **2** (0.133 g, 25.7%).

The Piperazone 5——A solution of 3-ANA (**2**, 0.1 g) in AcOH (3 ml) was cooled to 10 °C, and concentrated HCl (0.2 ml) was added. The reaction mixture was allowed to stand overnight. The solvent was evaporated off and the residue was dissolved in hot H_2O (20 ml). The aqueous solution was cooled at 5 °C for 2 h, during which time crystals separated out; they were collected and washed with cold H_2O and acetone to give **5** as colorless needles (0.087 g, 87.0%): mp 206—209 °C. IR (Nujol): 3475, 3295, 1670 (amide C=O), 1630 cm^{-1}. ^{1}H-NMR (D_2O) δ: 3.57 (1H, dd, J=4, 12 Hz, 6-H), 3.71 (1H, dd, J=4, 12 Hz, 6-H), 4.34 (1H, br t, J=4 Hz, 5-H), 5.19 (1H, s, 2-H), 6.96 (2H, d, J=8 Hz, ArH), 7.36 (2H, d, J=8 Hz, ArH). *Anal.* Calcd for $C_{11}H_{12}N_2O_4$: C, 55.93; H, 5.12; N, 11.86. Found: C, 55.79; H, 5.31; N, 11.74.

3-Phenoxyacetamidonocardicinic Acid (6a)——A solution of phenoxyacetyl chloride (0.89 g) in acetone (10 ml) was added dropwise at −5 °C to a solution of 3-ANA (**2**, 0.94 g) and $NaHCO_3$ (0.74 g) in 50% aqueous acetone (20 ml). The reaction mixture was stirred at the same temperature for 2 h and then the acetone was evaporated off. The remaining aqueous layer was washed with Et_2O, adjusted to pH 2 with 10% aqueous HCl and extracted with AcOEt. The organic layer was washed with H_2O, dried over $MgSO_4$ and evaporated to give a residue, which was crystallized from a mixture of AcOEt and Et_2O to give **6a** as colorless needles (0.78 g, 40.0%): mp 138—140 °C. IR (Nujol): 1745 (β-lactam C=O), 1690, 1660 cm^{-1}. ^{1}H-NMR (D_2O + $NaHCO_3$) δ: 2.99 (1H, dd, J=3, 5 Hz, 4β-H), 3.79 (1H, t, J=5 Hz, 4α-H), 4.56 (2H, s, $PhOCH_2$), 5.02 (1H, dd, J=3, 5 Hz, 3α-H), 5.35 (1H, s, ArCHCOO), 6.86—7.39 (9H, m, ArH). *Anal.* Calcd for $C_{19}H_{18}N_2O_6$: C, 61.61; H, 4.90; N, 7.56. Found: C, 61.69; H, 4.77; N, 7.40.

3-Phenylacetamidonocardicinic Acid (6b)——This compound was prepared from 3-ANA (0.94 g) and phenylacetyl chloride (0.80 g) in a manner similar to that used for **6a**, (0.97 g, 69.0%): mp 165—167 °C. $[\alpha]_D^{25}$ −206° (c=1.0, MeOH). IR (Nujol): 1740 (β-lactam C=O), 1695, 1640 cm^{-1}. ^{1}H-NMR (D_2O + $NaHCO_3$) δ: 2.85 (1H, dd, J=3, 5 Hz, 4β-H), 3.45 (2H, s, $ArCH_2CO$), 3.64 (1H, t, J=5 Hz, 4α-H), 5.26 (1H, s, ArCHCOO), 6.82 (2H, d, J=8 Hz, ArH), 7.07 (2H, d, J=8 Hz, ArH), 7.20 (5H, m, ArH). *Anal.* Calcd for $C_{19}H_{18}N_2O_5 \cdot 1/4H_2O$: C, 63.59; H, 5.19; N,

7.80. Found: C, 63.70; H, 5.07; N, 7.82.

3-(2-Phenyl-2-hydroxyiminoacetamido)nocardicinic Acid (6c)——PCl_5 (0.25 g) was added to a suspension of 2-phenyl-2-(2,2-dichloroacetoxyimino) acetic acid (0.355 g) in benzene (7 ml) at 0—5 °C, and the mixture was stirred for 1 h. The solvent was evaporated off and benzene (7 ml) was added to the residue and evaporated. After this operation had been repeated three times, the residue was dissolved in CH_2Cl_2 (10 ml). 3-ANA (0.236 g) was suspended in CH_2Cl_2 (20 ml), then *N,N*-bis(trimethylsilyl)acetamide (0.87 g) was added and the mixture was stirred at ambient temperature. The resulting solution was added to the solution obtained above under cooling at 0—5 °C, and the mixture was stirred for 1 h. The reaction mixture was washed with H_2O and evaporated to give an oily residue, to which AcOEt and 5% aqueous $NaHCO_3$ were added. The aqueous layer was separated, adjusted to pH 1—2 with 10% HCl and then extracted with AcOEt. The extract was washed with H_2O, dried over $MgSO_4$ and evaporated to give an oily residue. The oil was triturated with a small amount of $CHCl_3$ to give **6c** as crystals (0.095 g, 27.0%): mp 197—199 °C (dec.). IR (Nujol): 1730 (β-lactam C=O), 1660 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.09 (1H, dd, J=3, 5 Hz, 4β-H), 3.78 (1H, t, J=5 Hz, 4α-H), 5.01 (1H, m, 3α-H), 5.12 (1H, s, ArCHCOO), 7.28—7.52 (10H, m, ArH). *Anal.* Calcd for $C_{19}H_{17}N_3O_6 \cdot 1/2H_2O$: C, 58.16; H, 4.62; N, 10.71. Found: C, 58.38; H, 4.57; N, 10.69.

3-[2-(4-Hydroxyphenyl)-2-(hydroxyimino)acetamido]nocardicinic Acid (6d)——This compound was prepared from 3-ANA (0.47 g) and 2-(4-hydroxyphenyl)-2-(2,2-dichloroacetoxyimino)acetic acid (0.58 g) in a manner similar to that used for **6c** (0.24 g, 31.0%): mp 228—231 °C (dec.). $[\alpha]_D^{24}$ −192° (c=1.0, H_2O). IR (Nujol): 1745 (β-lactam C=O), 1690 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 3.25 (1H, dd, J=3, 5 Hz, 4β-H), 3.90 (1H, t, J=5 Hz, 4α-H), 5.50 (1H, s, ArCHCOO), 6.80 (2H, d, J=8 Hz, ArH), 6.85 (2H, d, J=8 Hz, ArH), 7.28 (2H, d, J=8 Hz, ArH), 7.50 (2H, d, J=8 Hz, ArH). *Anal.* Calcd for $C_{19}H_{17}N_3O_7$: C, 57.14; H, 4.29; N, 10.52. Found: C, 56.95; H, 4.20; N, 10.48.

3-[2-(2-Amino-1,3-thiazol-4-yl)-2-(methoxyimino)acetamido]nocardicinic Acid (6e)——3-ANA (0.24 g) was acylated with 2-(2-trifluoroacetamido-1,3-thiazol-4-yl)-2-methoxyiminoacetic acid (0.27 g) in a manner similar to that used for **6c** to give an oil (0.28 g), which was used in the following reaction without further purification. This oil (0.24 g) and AcONa (0.34 g) were dissolved in H_2O (4 ml) and the solution was stirred for 8 h at room temperature. The reaction mixture was adjusted to pH 3.2 with 5% HCl and after the precipitate had been removed by filtration, the filtrate was evaporated and the residue was dissolved in H_2O (3 ml). The solution was subjected to column chromatography on XAD2 eluted with H_2O. The fractions containing the target compound were collected and evaporated. The residue was triturated with EtOH to give **6e** as a pale yellow powder (0.17 g, 40.5%): mp 144—148 °C. IR (Nujol): 1740 (β-lactam C=O), 1660 cm^{-1}. ^{1}H-NMR ($D_2O + NaHCO_3$) δ: 3.16 (1H, dd, J=3, 5 Hz, 4β-H), 3.88 (1H, t, J=5 Hz, 4α-H), 5.06 (1H, dd, J=3, 5 Hz, 3α-H), 5.32 (1H, s, ArCHCOO), 6.82 (1H, s, thiazole ring H), 6.92 (2H, d, J=8 Hz, ArH), 7.24 (2H, d, J=8 Hz, ArH). *Anal.* Calcd for $C_{17}H_{17}N_5O_6S \cdot 2H_2O$: C, 44.83; H, 4.64; N, 15.38. Found: C, 45.27; H, 4.21; N, 15.41.

3-[(4-Hydroxyphenyl)glyoxyloylamino]nocardicinic Acid (6f) Sodium Salt——3-ANA (0.47 g) was acylated with (4-hydroxyphenyl)glyoxylic acid (0.37 g) in a manner similar to that described for **6c** to give a crude powder (0.46 g). This powder was dissolved in acetone and then treated with a solution of sodium 2-ethylhexanonate in acetone. The mixture was evaporated to give an oil, which was triturated with Et_2O to give **6f** as a pale yellow powder (0.17 g, 20.8%): mp 220—225 °C. IR (Nujol): 1740 (β-lactam C=O), 1660, 1600 cm^{-1}. ^{1}H-NMR (D_2O) δ: 3.08 (1H, dd, J=3, 5 Hz, 4β-H), 3.87 (1H, t, J=5 Hz, 4α-H), 5.08 (1H, dd, J=3, 5 Hz, 3α-H), 5.43 (1H, s, ArCHCOO), 6.77 (2H, d, J=8 Hz, ArH), 6.95 (2H, d, J=8 Hz, ArH), 7.32 (2H, d, J=8 Hz, ArH), 7.38 (2H, d, J=8 Hz, ArH). *Anal.* Calcd for $C_{19}H_{15}N_2NaO_7 \cdot H_2O$: C, 53.77; H, 4.04; N, 6.60. Found: C, 53.75; H, 3.81; N, 6.55.

3-[D-(4-Hydroxyphenyl)glycylamino]nocardicinic Acid (6g)——*N*-(*tert*-Butoxycarbonyl)-D-(4-hydroxyphenyl)-glycine succinimidyl ester (0.95 g) was added to a solution of 3-ANA (0.472 g) and $NaHCO_3$ (0.17 g) in 50% aqueous acetone (20 ml). The mixture was stirred for 8 h at room temperature and then the acetone was evaporated off. The remaining aqueous layer was washed with AcOEt, adjusted to pH 2 with 10% HCl and extracted with AcOEt. The extract was washed with H_2O, dried over $MgSO_4$ and evaporated to give a powder (0.62 g, 65.0%). This powder (0.50 g) was dissolved in trifluoroacetic acid (3 ml) under ice-bath cooling and the mixture was stirred for 1 h at the same temperature. After evaporation of the trifluoroacetic acid, the residue was dissolved in H_2O. The solution was subjected to column chromatography on XAD2 eluted with H_2O. The fractions containing the target compound were collected and evaporated. The residue was triturated with Et_2O and AcOEt to give **6f** as a powder (0.31 g, 70.0%): mp 205—209 °C. $[\alpha]_D^{24}$ −205° (c=1.0, 1% $NaHCO_3$). IR (Nujol): 1745 (β-lactam C=O), 1670, 1600 cm^{-1}. ^{1}H-NMR (D_2O) δ: 3.07 (1H, dd, J=3, 5 Hz, 4β-H), 3.82 (1H, t, J=5 Hz, 4α-H), 4.97 (1H, dd, J=3, 5 Hz, 3α-H), 5.28 (1H, s, ArCHCOO), 6.80 (2H, d, J=8 Hz, ArH), 6.90 (2H, d, J=8 Hz, ArH), 7.10 (2H, d, J=8 Hz, ArH), 7.28 (2H, d, J=8 Hz, ArH). *Anal.* Calcd for $C_{19}H_{19}N_3O_6 \cdot 1/2H_2O$; C, 57.87; H, 5.11; N, 10.65. Found: C, 57.62; H, 5.14; N, 10.39.

3-(D-Phenylglycylamino)nocardicinic Acid (6h)——This compound was prepared from 3-ANA (0.47 g) and *N*-(*tert*-butoxycarbonyl)-D-phenylglycine succinimidyl ester (0.91 g) in a manner similar to that used for **6g**, (0.25 g, 35.0%): mp 193—196 °C. IR (Nujol): 1740 (β-lactam C=O), 1695, 1610 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 2.89 (1H, dd, J=3, 5 Hz, 4β-H), 3.81 (1H, t, J=5 Hz, 4α-H), 5.27 (1H, s, ArCHCOO), 6.74 (2H, d, J=8 Hz, ArH), 7.16 (2H, d, J=8 Hz, ArH), 7.40 (5H, s, ArH). *Anal.* Calcd for $C_{19}H_{19}N_3O_5$: C, 61.78; H, 5.19; N, 11.38. Found: C, 61.99; H, 5.19; N, 11.51.

References and Notes

1) H. Aoki, H. Sakai, M. Kohsaka, T. Konomi, J. Hosoda, Y. Kobuchi, E. Iguchi, and H. Imanaka, *J. Antibiot.*, **29**, 492 (1976).
2) J. Hosoda, T. Konomi, N. Tani, H. Aoki, and H. Imanaka, *Agric. Biol. Chem.*, **41**, 2013 (1977); M. Kurita, K. Jomon, T. Komori, N. Miyairi, H. Aoki, S. Kuge, T. Kamiya, and H. Imanaka, *J. Antibiot.*, **29**, 1243 (1976).
3) M. Hashimoto, T. Komori, and T. Kamiya, *J. Am. Chem. Soc.*, **98**, 3023 (1976); *idem*, *J. Antibiot.*, **29**, 890 (1976).
4) M. Nishida, Y. Mine, S. Nonoyama, H. Kojo, S. Goto, and S. Kuwahara, *J. Antibiot.*, **30**, 917 (1977).
5) T. Kamiya, "Recent Advances in the Chemistry of β-Lactam Antibiotics," Special Publication No. 28, ed. by J. E. Elks (The Proceedings of an International Symposium arranged by the Fine Chemicals and Medicinal Group of the Industrial Division of the Chemical Society, Cambridge, June 1976), The Chemical Society, London, 1977, p. 281.
6) P. Edman, *Acta Chem. Scand.*, **4**, 277, 283 (1950).
7) After we reported this experimental result,[5] Wasserman and Robinson proposed a somewhat different mechanism.[8]
8) H. H. Wasserman and R. P. Robinson, *Heterocycles*, **21**, 279 (1984).

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Enantioselective Synthesis of Monocyclic β-Lactams Related to Nocardicins *via* a [2+2] Cycloaddition Reaction

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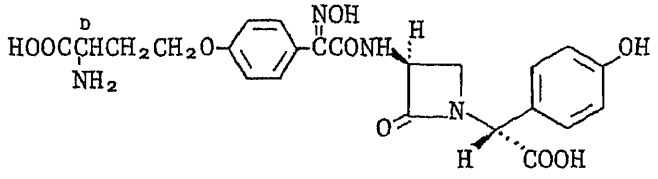
Monocyclic β-lactams related to nocardicins were enantioselectively synthesized from phthalimidoacetyl chloride and hexahydro-1,3,5-triazines (4) *via* a [2+2] cycloaddition reaction. The preparation and biological activity of some typical acyl derivatives are also described.

Keywords——stereochemistry; ketene–imine cycloaddition; 3-aminonocardicinic acid; nocardicin; antibacterial activity; structure–activity relationship

Nocardicins, represented by nocardicin A (**1**), are a group of naturally occurring monocyclic β-lactam antibiotics. They show chemical and biological parallels to penicillins and cephalosporins[1,2] in respect of having (*R*)-carboxyl and (*S*)-acylamino groups and being inhibitors of bacteria cell-wall biosynthesis. In the preceding paper, we reported the preparation of 3-aminonocardicinic acid (3-ANA, **2**), the basic framework of this group of antibiotics, and the synthesis of some semisynthetic nocardicins by acylation of 3-ANA.[3] As a continuation of this series of investigations, we subsequently focused on modifications of the *p*-hydroxyphenyl group in the 3-ANA structure. In a previous communication,[4] we reported a versatile synthetic method for 3-ANA. Herein we present a full account of the work and an application to the synthesis of other analogous compounds.



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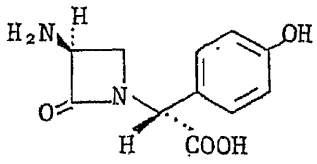

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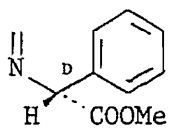

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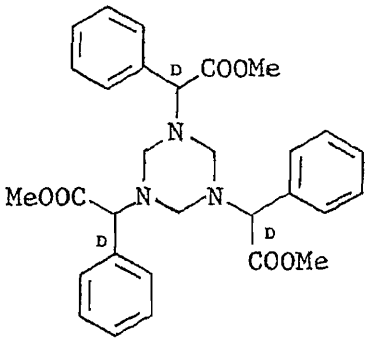
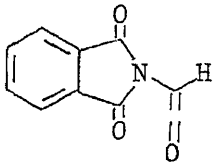


4a



5

Our synthesis of β-lactams of the nocardicin type can be conceptually classified as a [2+2] cycloaddition reaction between ketenes and imines,[5] which is the best known procedure in β-lactam synthesis. The synthesis of nocardicins by this cycloaddition approach requires preparation of the appropriate formaldimine precursors (*e.g.*, **3**), which usually exist as trimers, hexahydro-*s*-triazines (*e.g.*, **4a**).[6] We conjectured that the monomeric precursors might be regenerated *in situ* by treatment of the trimers with a Lewis acid and, on reaction with ketenes, could afford the corresponding β-lactams. Thus, hexahydro-*s*-triazine **4a**, prepared from methyl phenylglycinate by the known procedure,[7] was treated with $BF_3 \cdot OEt_2$ in CH_2Cl_2 at room temperature and the mixture was added to a cooled (−78 °C) solution of phthalimidoacetyl chloride and pyridine in CH_2Cl_2. The temperature was then raised to 0 °C. The reaction seemed to proceed at temperatures near 0 °C. The usual work-up gave, after a short silica gel column chromatography, a 3 : 1 mixture of β-lactams **6** and **7** in good yield. A

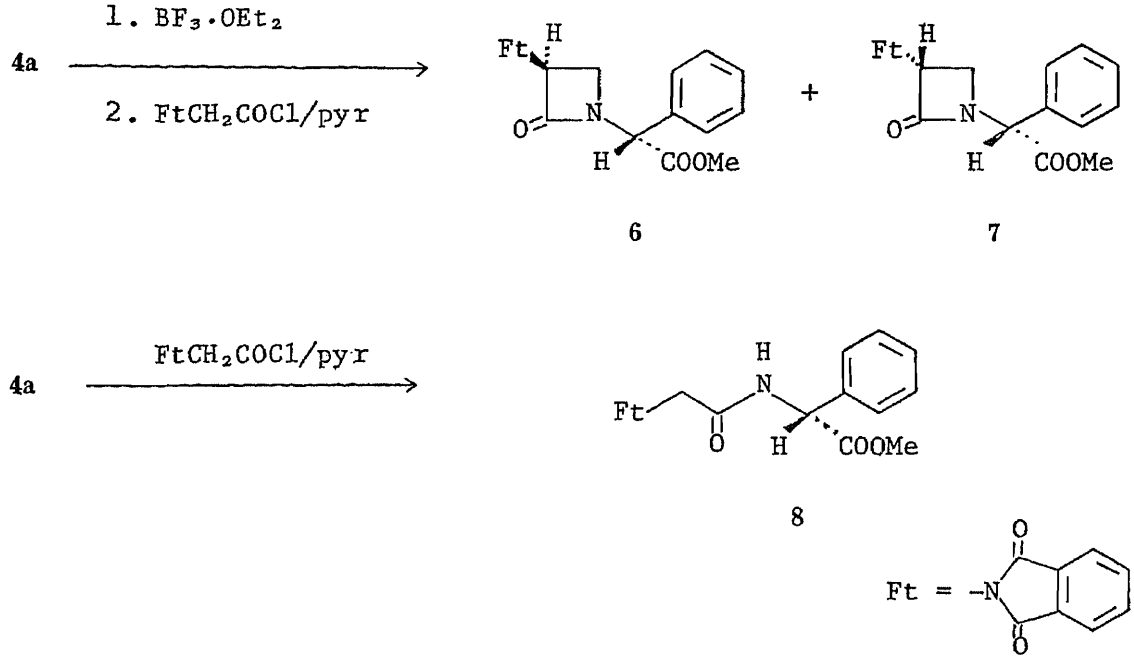


Chart 1

more careful chromatography yielded the major product **6** in 35% yield, while the minor product **7** was isolated from the mother liquor by high performance liquid chromatography (HPLC) in low yield.

The configurations of the phthalimido (Ft) groups in these products were assigned on the basis of the proton nuclear magnetic resonance (^{1}H-NMR) spectral data in comparison with those of nocardicins. In the major product **6**, the 4β proton, which appeared at a higher field (δ 3.46) than the 4α proton (δ 3.94) in agreement with the data on nocardicins,[8] was *trans*-coupled (J=3 Hz) to the 3α proton (δ 5.48). In the minor product **7**, on the other hand, the corresponding 4β proton, which also appeared at a higher field (δ 3.60) than the 4α proton (δ 4.10), was *cis*-coupled (J=5 Hz) to the 3β proton (δ 5.34). The structures of these major and minor products were thus assigned as **6** and **7**, respectively.

The mechanism of the β-lactam formation in the above reaction can be explained as follows. In the presence of BF_3, the trimer **4a** might be transformed to some extent to the monomer **3**, as expected, while phthalimidoacetyl chloride might be converted to the ketene **5** in the presence of pyridine. The ketene **5** would undergo cycloaddition to the imine **3** to give the β-lactams **6** and **7**. The requirement for BF_3 in this reaction was shown by the following

observation. When the trimer **4a** was directly reacted with phthalimidoacetyl chloride–pyridine in the absence of $BF_3 \cdot OEt_2$, only the acyl derivative **8** of methyl phenylglycinate was obtained. The enantioselectivity in the above reaction would be governed by the substituents of both reactants. It is thought that the cycloaddition reaction of ketenes and imines proceeds *via* a HOMO (ketenophile)/LUMO (ketene) interaction. In our case, overlap between the π-orbitals of the formaldimine **3** and those of the phthaliminoketene **5** might be as shown in Chart 2. The phenylglycyl moiety of **3** would be situated so as to be away from the oxygen of **5**

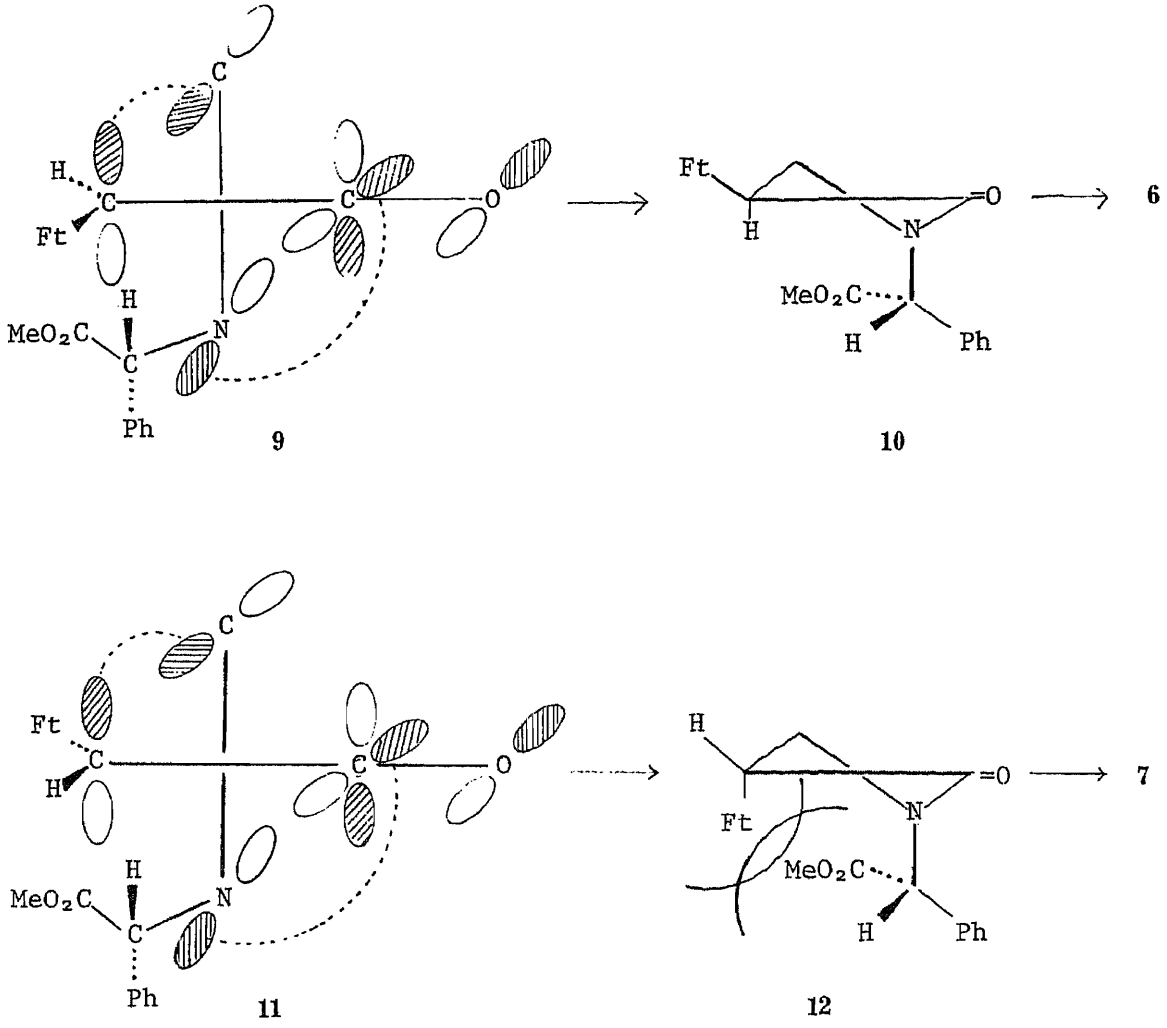


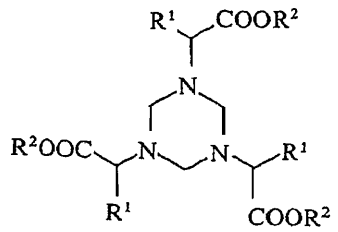
Chart 2

due to their steric (or electronic) repulsion and, further, the phenyl (Ph) group of the phenylglycyl moiety would be oriented apart from the Ft group of **5**. When **5** develops an overlap to **3** as shown in **9**, the Ft group is allowed to take an *exo* configuration in the intermediate **10**. On the other hand, overlap of **5** to **3** as shown in **11** compels the Ft group to adopt an *endo* configuration in the intermediate **12**. It is therefore clear that, on comparison of **10** and **12**, the steric interaction of the Ft group and the phenylglycyl moiety is less in **10** than **12**. The major product **6** would thus be favorably formed *via* the intermediate **10**.

In order to ascertain the stereochemical outcome in the above reaction, we examined the reaction using other ketenes and ketenophiles under the same conditions as those used for the above reaction. The results are summarized in Tables I and II. When azidoacetyl chloride was

used in place of phthalimidoacetyl chloride and allowed to react with **4a** (entry **2**), the β-lactams **13a** and **14a** were obtained in a ratio of 3:2. The poorer stereoselectivity in this reaction is ascribed to the smaller bulk of the azido group as compared with the Ft group. On the other hand, the trimer **4b** derived from α-naphthylglycine (having the naphthyl group, with a larger steric interaction than the Ph group), when reacted with phthalimidoacetyl chloride (entry **3**), gave a product with greater stereoselectivity (10:1 of **13b** and **14b**). These results support the above mechanistic considerations. The reactions of phthalimidoacetyl chloride

Table I. Structures of **4**, **13** and **14**



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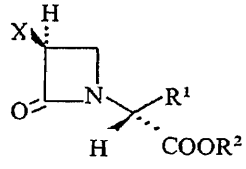
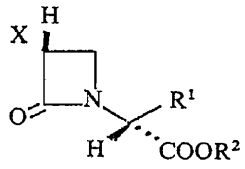


13



14

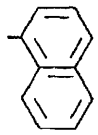
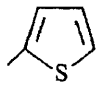
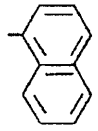
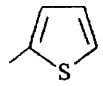
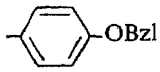
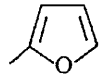
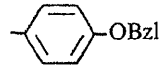
4	R^1	R^2	13, 14	R^1	R^2	X
4b		Me	**13a, 14a**		Me	N_3
4c		Me	**13b, 14b**		Me	Ft
4d		Me	**13c, 14c**		Me	Ft
4e		Me	**13d, 14d**		Me	Ft
4f	H	Bzl	**13e, 14e**		Me	Ft
			13f, 14f	H	Bzl	Ft

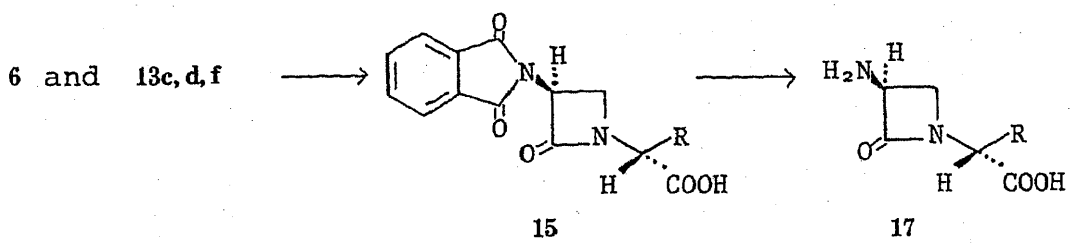
Table II. Product Yields and Ratios (See Text)

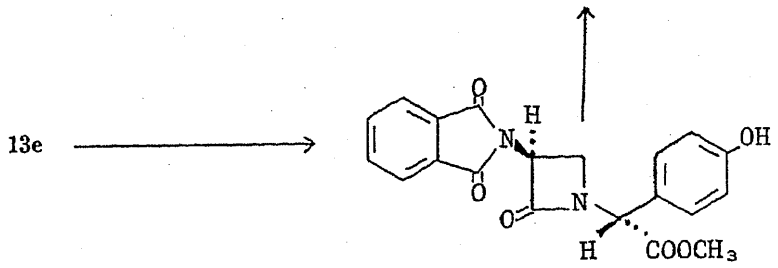
Entry	Compounds	Total yield (%) of **6** (**13**) and **7** (**14**)	Ratio of **6** (**13**) and **7** (**14**)[a]
1	**6** and **7**	80	3:1
2	**13a** and **14a**	42	3:2
3	**13b** and **14b**	51	10:1
4	**13c** and **14c**	65	7:2
5	**13d** and **14d**	39	4:1
6	**13e** and **14e**	87	3:1
7	**13f** and **14f**	35	—

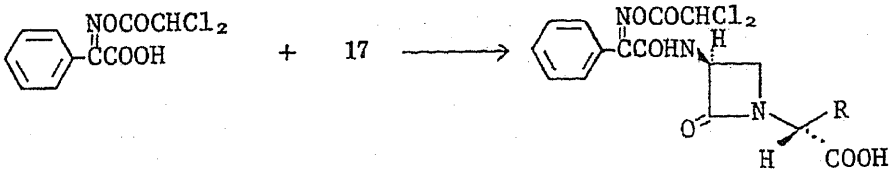
a) The ratios of **6** (**13**) and **7** (**14**) were calculated by measuring the integration values of their methyl signals in the ^{1}H-NMR spectrum.

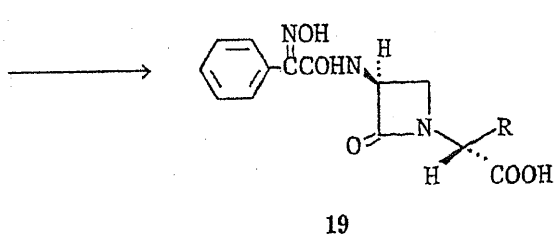
with the trimers **4c—e**, derived from 2-thienylglycine, 2-furylglycine, and (*p*-hydroxyphenyl)glycine having a similar steric bulk to phenylglycine, gave products with practically the same stereoselectivity as in the case of **4a** (entries **4**, **5**, and **6**).

With these results in hand, we turned to removal of the protecting groups in the *β*-







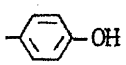


A : R = 

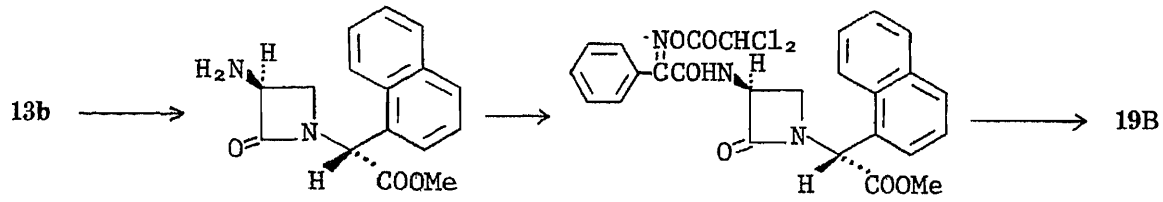
B : R =  (*dl*)

C : R = 

D : R =  (*dl*)

E : R = 

lactams **6** and **13** and acylation of the resulting amino acids **17**. According to the procedures reported in our previous report,[8] **6** was first subjected to demethylation using LiI in pyridine[9] to give the corresponding acid **15A**, which was then treated with dimethylaminopropylamine in MeOH to yield the amino acid **17A**. Similarly, **13c** and **13d** were converted to **17C** and **17D**, respectively. Conversion of **13e** *via* **15E** to 3-ANA (**17E**) was reported previously. Acylation of the resulting amino acids **17A**, C, D and E with 2-phenyl-2-(2,2-dichloroacetoxyimino) acetic acid by the acid chloride procedure using PCl_5, followed by removal of the dichloroacetyl protecting group in the products **18A**, C, D and E, provided the compounds **19A**, C, D and E. The derivative **19B** was prepared *via* an alternative route. Thus, the Ft group of **13b** (*dl*-mixture) was first removed in a similar manner and the resulting amine **20** was acylated to give **21**. Deprotection of **21** by hydrolysis with NaOH gave the derivative **19B** as a *dl*-mixture.



20 **21**

Chart 3

Antibacterial activity of **19A**, B, C, D, and E was examined by the agar dilution method and the results are summarized in Table III. All the derivatives except the naphthyl compound **19B** were active against both gram-negative and -positive bacteria. It is noteworthy that **19A** and **19C** were more active than the parent *p*-hydroxyphenyl compound **19E** against *Escherichia coli* and *Staphylococcus aureus*. The thienyl compound **19C** was the most active against all the bacteria except *Proteus vulgaris*.

TABLE III. Minimum Inhibitory Concentrations of Nocardicins[a]

Compounds	Organism					
	Pseudomonas aeruginosa 10490	*Escherichia coli* NIHJ, JC-2	*Escherichia coli* 114[b]	*Proteus vulgaris*	*Staphylococcus aureus*	*Bacillus subtilis*
19A	1.25	2.5	0.3	5	0.075	0.6
19B	>10	>10	>10	>10	0.6	>10
19C	0.15	2.5	0.15	>10	0.0375	0.15
19D	2.5	10	1.25	>10	0.6	1.25
19E	0.6	10	0.6	2.5	0.6	2.5

a) Agar dilution method (mg/ml). *b*) A mutant strain of *E. coli* NIHJ: sensitive to β-lactam antibiotics.

Experimental

Melting points were measured on a Thomas–Hoover apparatus and are uncorrected. Infrared (IR) and ^{1}H-NMR spectra were recorded using a Hitachi 260-10 spectrophotometer and a JEOL-Ps-100 spectrometer, respectively. Optical rotations were measured on a JASCO automatic polarimeter.

Minimum inhibitory concentrations (MIC's) of the nocardicin analogues were determined by the agar dilution method. One loopful of an overnight culture of each test organism in Trypticase broth (about 10^8 viable cells/ml) was

streaked on heart infusion agar containing graded concentrations of drugs and incubated at 37 °C for 8 h.

Trimethyl [$\alpha R,\alpha' R,\alpha'' R$]-$\alpha,\alpha',\alpha''$-Triphenylhexahydro-1,3,5-triazine-1,3,5-triacetate (4a)——Methyl D-phenylglycinate hydrochloride (24.2 g) was dissolved in H_2O (100 ml) and benzene (250 ml) was added. To this mixture, 1 N NaOH (120 ml) was added dropwise under ice-cooling and then a 37% aqueous solution of formaldehyde (9.9 ml) was added. The mixture was stirred for 2 h at the same temperature and the organic layer was separated, washed with H_2O, and dried over $MgSO_4$. The solvent was evaporated off to give a residue, which was crystallized from diisopropyl ether to give **4a** as white needles (18.5 g, 86.5%): mp 148—155 °C. IR (Nujol): 1730 cm^{-1} (ester C=O). ^{1}H-NMR ($CDCl_3$) δ: 3.49 (9H, s, $COOCH_3$), 3.51 (6H, s, NCH_2N), 4.50 (3H, s, ArCHCOO), 6.90—7.42 (15H, m, ArH). *Anal.* Calcd for $C_{30}H_{33}N_3O_6$: C, 67.78; H, 6.23; N, 7.91. Found: C, 67.99; H, 6.10; N, 7.83.

The following compounds were prepared by reacting the corresponding amine derivatives with formaldehyde in substantially the same manner as described above.

Trimethyl α,α',α''-Tri(1-naphthyl)hexahydro-1,3,5-triazine-1,3,5-triacetate (**4b**): Yield 96.5%, oil. MS *m/z* 681 (M^+). IR (film): 1735 (ester C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.28 (9H, s, $COOCH_3$), 3.82 (6H, s, NCH_2N), 5.16 (3H, s, ArCHCOO), 7.03—7.83 (21H, m, ArH).

Trimethyl [$\alpha R,\alpha' R,\alpha'' R$]-$\alpha,\alpha',\alpha''$-Tri(2-thienyl)hexahydro-1,3,5-triazine-1,3,5-triacetate (**4c**): Yield 92.9%, mp 131—134 °C. IR (Nujol): 1739 (ester C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.69 (9H, s, $COOCH_3$), 3.78 (6H, s, NCH_2N), 4.89 (3H, s, ArCHCOO), 6.80—7.43 (9H, m, ArH). *Anal.* Calcd for $C_{24}H_{27}N_3O_6S_3$: C, 52.46; H, 4.95; N, 7.65. Found: C, 52.11; H, 4.92; N, 7.57.

Trimethyl α,α',α''-Tri(2-furyl)hexahydro-1,3,5-triazine-1,3,5-triacetate (**4d**): Yield 83.5%, oil. MS *m/z* 501 (M^+). IR (film): 1740 (ester C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.50—3.80 (15H, m, $COOCH_3$ and NCH_2N overlapping), 7.32 (9H, m, ArH).

Trimethyl [$\alpha R,\alpha' R,\alpha'' R$]-$\alpha,\alpha',\alpha''$-Tri(4-benzyloxyphenyl)hexahydro-1,3,5-triazine-1,3,5-triacetate (**4e**): Yield 58.0%, mp 141—145 °C. IR (Nujol): 1725 (ester C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.58 (15H, s, $COOCH_3$ and NCH_2N overlapping), 4.50 (3H, s, ArCHCOO), 5.04 (6H, s, CH_2Ph), 6.80 (6H, d, J=9 Hz, ArH), 7.29 (6H, d, J=9 Hz, ArH), 7.40 (15H, s, ArH). *Anal.* Calcd for $C_{51}H_{51}N_3O_9$: C, 72.06; H, 6.05; N, 4.94. Found: C, 71.83; H, 5.96; N, 4.98.

Tribenzyl Hexahydro-1,3,5-triazine-1,3,5-triacetate (**4f**): Yield 39.5%, oil. MS *m/z* 531 (M^+). IR (film): 1740 (ester C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.44 (6H, s, NCH_2COO), 3.69 (6H, s, NCH_2N), 5.10 (6H, s, CH_2Ph), 7.40 (15H, s, ArH).

Methyl (3*S*,αR)-2-(2-Oxo-3-phthalimido-1-azetidinyl)-2-phenylacetate (6)——A solution of pyridine (0.96 g) in CH_2Cl_2 (4 ml) was added to a solution of phthalimidoacetyl chloride (2.68 g) in CH_2Cl_2 (40 ml) over 6 min at −30—−35 °C. After 15 min of stirring, the mixture was cooled to −78 °C and a mixture of triazine (**4a**, 1.06 g), $BF_3 \cdot OEt_2$ (0.86 g) and CH_2Cl_2 (20 ml) was added over 20 min. The whole was stirred for 2 h at the same temperature and for 1 h at 0 °C and then washed successively with H_2O, 5% HCl, 5% $NaHCO_3$, and H_2O. Drying over $MgSO_4$ and evaporation gave an oil (3:1 mixture of **6** and **7**, 3.50 g, 80.3%). These two isomers were separated by column chromatography on silica gel with $CHCl_3$. The fractions containing the major compound **6** were collected and evaporated to give an oily residue, which was crystallized from a mixture of EtOH and Et_2O to give colorless needles (**6**, 35.2%): mp 133—134 °C. $[\alpha]_D^{25}$ −253° (c=0.98, $CHCl_3$). IR (Nujol): 1775, 1755, 1735 (β-lactam C=O), 1720 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.46 (1H, dd, J=3, 5 Hz, 4β-H), 3.77 (3H, s, $COOCH_3$), 3.94 (1H, t, J=5 Hz, 4α-H), 5.48 (1H, dd, J=3, 5 Hz, 3α-H), 5.78 (1H, s, ArCHCOO), 7.38 (5H, s, ArH), 7.57—7.96 (4H, m, ArH). *Anal.* Calcd for $C_{20}H_{16}N_2O_5$: C, 65.93; H, 4.43; N, 7.69. Found: C, 66.11; H, 4.32; N, 7.72. The fractions containing the minor product were combined and evaporated to give a crude oil, which was further purified by HPLC using Reporasol to give **7** as crystals (47.0 mg): mp 96—98 °C. $[\alpha]_D^{25}$ 20° (c=0.08, MeOH). IR (Nujol): 1775—1760, 1735 (β-lactam C=O), 1725 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.60 (1H, t, J=5 Hz, 4β-H), 3.84 (3H, s, $COOCH_3$) 4.10 (1H, dd, J=3, 5 Hz, 4α-H), 5.34 (1H, dd, J=3, 5 Hz, 3β-H), 5.72 (1H, s, ArCHCOO), 7.36 (5H, s, ArH), 7.57—7.96 (4H, m, ArH). *Anal.* Calcd for $C_{20}H_{16}N_2O_5$: C, 65.93; H, 4.43; N, 7.69. Found: C, 66.01; H, 4.29; N, 7.69.

The following compounds were prepared by reacting the corresponding perhydro-1,3,5-triazines with acyl chlorides in substantially the same manner as described aobve.

Methyl (3*R*,αR)- and (3*S*,αR)-2-(3-Azido-2-oxo-1-azetidinyl)-2-phenylacetate (**13a**, **14a**): Yield 42.0%. Major product (**13a**, 13.4%): oil. MS *m/z* 232 (M^+ −28). IR (film): 2100 (N_3), 1765 (β-lactam C=O), 1730 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.93 (1H, dd, J=3, 5 Hz, 4β-H), 3.73 (3H, s, $COOCH_3$), 3.86 (1H, t, J=5 Hz, 4α-H), 4.63 (1H, dd, J=3, 5 Hz, 3α-H), 5.56 (1H, s, ArCHCOO), 7.28 (5H, s, ArH). Minor product (**14a**, trace): oil. MS *m/z* 232 (M^+ −28). IR (film): 2100 (N_3), 1760 (β-lactam C=O), 1735 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.26—3.76 (2H, m, 4α, 4β-H overlapping), 3.80 (3H, s, $COOCH_3$), 4.50 (1H, dd, J=3, 5 Hz, 3β-H), 5.60 (1H, s, ArCHCOO), 7.33 (5H, s, ArH).

Methyl 2-(1-Naphthyl)-2-(2-oxo-3-phthalimido-1-azetidinyl)acetate (**13b**, **14b**): Yield 51.3%. Major product (**13b**, 43.3%): mp 192 °C. IR (Nujol): 1780, 1750, 1740 (β-lactam C=O), 1742 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.16 (1H, dd, J=3, 5 Hz, 4β-H), 3.80 (3H, s, $COOCH_3$), 3.89 (1H, t, J=5 Hz, 4α-H), 5.50 (1H, dd, J=3, 5 Hz, 3α-H), 6.45 (1H, s, ArCHCOO), 7.30—8.30 (7H, m, ArH). *Anal.* Calcd for $C_{24}H_{18}N_2O_5$: C, 69.56; H, 4.38; N, 6.76. Found: C, 69.84; H, 4.35; N, 6.68. The minor isomer was not isolated.

Methyl (3*R*,αS)- and (3*S*,αS)-2-(2-Oxo-3-phthalimido-1-azetidinyl)-2-(2-thienyl)acetate (**13c**, **14c**): Yield 65.2%.

Major product (**13c**, 32.1%): mp 167—170 °C. $[\alpha]_D^{25}$ −116° (c=0.38, $CHCl_3$). IR (Nujol): 1765, 1735 (β-lactam C=O), 1710 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.62 (1H, dd, J=3, 5 Hz, 4β-H), 3.81 (3H, s, $COOCH_3$), 3.96 (1H, t, J=5 Hz, 4α-H), 5.52 (1H, dd, J=3, 5 Hz, 3α-H), 5.98 (1H, s, ArCHCOO), 6.90—7.42 (3H, m, ArH), 7.75 (4H, m, ArH). *Anal.* Calcd for $C_{18}H_{14}N_2O_5S$: C, 58.38; H, 3.81; N, 7.56. Found: C, 58.21; H, 3.99; N, 7.57. Minor product (**14c**, trace): mp 152—154.5 °C. $[\alpha]_D^{25}$ −10° (c=0.40, $CHCl_3$). IR (Nujol): 1770, 1735 (β-lactam C=O), 1715 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.73 (1H, t, J=5 Hz, 4β-H), 3.88 (3H, s, $COOCH_3$), 4.09 (1H, dd, J=3, 5 Hz, 4α-H), 5.38 (1H, dd, J=3, 5 Hz, 3β-H), 5.98 (1H, s, ArCHCOO), 6.92—7.42 (3H, m, ArH), 7.78 (4H, m, ArH). *Anal.* Calcd for $C_{18}H_{14}N_2O_5S$: C, 58.38; H, 3.81; N, 7.56. Found: C, 58.27; H, 3.92; N, 7.44.

Methyl 2-(2-Furyl)-2-(2-oxo-3-phthalimido-1-azetidinyl)acetate (**13d**, **14d**): Yield 39.2%. Major product (**13d**, 17.5%): mp 176—178 °C. IR (Nujol): 1760, 1730 (β-lactam C=O), 1710 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.64 (1H, dd, J=3, 5 Hz, 4β-H), 3.80 (3H, s, $COOCH_3$), 4.02 (1H, t, J=5 Hz, 4α-H), 5.52 (1H, dd, J=3, 5 Hz, 3α-H), 5.84 (1H, s, ArCHCOO), 6.64 (1H, m, ArH), 7.47 (1H, d, J=3 Hz, ArH), 7.77 (4H, m, ArH). *Anal.* Calcd for $C_{18}H_{14}N_2O_6$: C, 61.01; H, 3.98; N, 7.71. Found: C, 61.22; H, 3.91; N, 7.91. The minor isomer was not isolated.

Methyl (3*R*,α*R*)- and (3*S*,α*R*)-2-(2-Oxo-3-phthalimido-1-azetidinyl)-2-(4-benzyloxyphenyl)acetate (**13e**, **14e**): Yield 87.2%. Major product (**13e**, 40.4%): oil. MS m/z 470 (M^+). IR (film): 1780, 1760, 1740 (β-lactam C=O), 1720 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.42 (1H, dd, J=3, 5 Hz, 4β-H), 3.78 (3H, s, $COOCH_3$), 3.90 (1H, t, J=5 Hz, 4α-H), 5.04 (2H, s, CH_2Ph), 5.46 (1H, dd, J=3, 5 Hz, 3α-H), 5.72 (1H, s, ArCHCOO), 7.00 (2H, d, J=9 Hz, ArH), 7.33 (2H, d, J=9 Hz, ArH), 7.37 (5H, s, ArH), 7.70 (4H, m, ArH). Minor product (**14e**, trace): oil. MS m/z 470 (M^+). IR (film): 1775, 1760, 1735 (β-lactam C=O), 1720 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.66 (1H, t, J=5 Hz, 4β-H), 3.78 (3H, s, $COOCH_3$), 4.06 (1H, dd, J=3, 5 Hz, 4α-H), 4.97 (2H, s, CH_2Ph), 5.32 (1H, dd, J=3, 5 Hz, 3β-H), 5.65 (1H, s, ArCHCOO), 6.98 (2H, d, J=9 Hz, ArH), 7.35 (2H, d, J=9 Hz, ArH), 7.37 (5H, s, ArH), 7.70 (4H, m, ArH).

Benzyl 2-(2-Oxo-3-phthalimido-1-azetidinyl)acetate (13f)——A solution of pyridine (0.48 g) in CH_2Cl_2 (5 ml) was added to a solution of phthalimidoacetyl chloride (1.34 g) in CH_2Cl_2 (15 ml) over 10 min at −30°——35 °C.

A solution of tribenzyl 1,3,5-triazine-1,3,5(2*H*,4*H*,6*H*)-triacetate (**4f**, 0.531 g) and $BF_3 \cdot OEt_2$ (0.43 g) in CH_2Cl_2 (15 ml) was then added dropwise at −15 °C over 15 min, and the whole was stirred for 2 h at the same temperature and then for 1 h under ice-cooling. The reaction mixture was washed with 10% HCl, 5% $NaHCO_3$, and H_2O, dried over $MgSO_4$ and evaporated to give a residue (1.4 g), which was subjected to column chromatography on silica gel. Elution was carried out with $CHCl_3$ and the fractions containing the target compound were collected. The solvent was removed to give an oil, which was crystallized from Et_2O to give **13f** as colorless crystals (0.375 g, 35.4%): mp 129—135 °C (dec.). IR (Nujol): 1780, 1750, 1730 (β-lactam C=O), 1710 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.81—3.94 (2H, m, 4α-H, 4β-H overlapping), 5.08 and 5.36 (2H, ABq, J=18 Hz, NCH_2COO), 5.19 (2H, s,$COOCH_2Ph$), 5.52 (1H, dd, J=3, 5 Hz, 3α-H), 7.68—7.92 (9H, m, ArH). *Anal.* Calcd for $C_{20}H_{16}N_2O_5$: C, 65.93; H, 4.43; N, 7.69. Found: C, 65.94; H, 4.34; N, 7.66

Reaction of Perhydro-1,3,5-triazine with Phthalimidoacetyl Chloride in the Absence of BF_3——A solution of pyridine (0.48 g) in CH_2Cl_2 (2 ml) was added to a solution of phthalimidoacetyl chloride (1.34 g) in CH_2Cl_2 (20 ml) over 5 min at −30——35 °C. After 15 min, the mixture was cooled to −78 °C and a solution of the triazine (**4a**, 0.50 g) in CH_2Cl_2 (10 ml) was added over 20 min. The mixture was stirred for 2 h at the same temperature and for 1 h at 0 °C. After removal of the solvent, the residue was dissolved in AcOEt and this solution was washed with H_2O, 5% HCl, and 5% $NaHCO_3$. Drying over $MgSO_4$ and evaporation gave a residue, which was crystallized from EtOH to give **8** as colorless needles (0.64 g, 62.2%): mp 262—263 °C. IR (Nujol): 3400, 1775, 1740, 1725, 1670 (amide C=O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.62 (3H, s, $COOCH_3$), 4.35 (2H, s, NCH_2CON), 5.48 (1H, d, J=8 Hz, ArCHCOO), 7.40 (5H, s, ArH), 7.93 (4H, m, ArH), 9.23 (1H, d, J=8 Hz, CONH). *Anal.* Calcd for $C_{19}H_{16}N_2O_5$: C, 64.76; H, 4.58; N, 7.95. Found: C, 65.01; H, 4.61; N, 7.78.

Methyl (3*S*,α*R*)-2-(2-Oxo-3-phthalimido-1-azetidinyl)-2-(4-hydroxyphenyl)acetate (16)——A mixture of methyl (3*R*,α*R*)- and (3*S*,α*R*)-2-(2-oxo-3-phthalimido-1-azetidinyl)-2-(4-benzyloxyphenyl)acetate (**13e**, 0.27 g) in EtOH (20 ml) and 10% Pd–C (0.10 g) was shaken with H_2 under atmospheric pressure until the absorption ceased. After the catalyst had been filtered off, the filtrate was evaporated to give an oil, which was crystallized from ether to give **16** as colorless needles (0.11 g, 60.0%): mp 203—204 °C (dec.). $[\alpha]_D^{25}$ −238° (c=0.025, MeOH). IR (Nujol): 1780, 1740 (β-lactam C=O), 1720 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.47 (1H, dd, J=3, 5 Hz, 4β-H), 3.80 (3H, s, $COOCH_3$), 3.95 (1H, t, J=5 Hz, 4α-H), 4.89 (1H, dd, J=3, 5 Hz, 3α-H), 5.71 (1H, s, ArCHCOO), 6.80 (2H, d, J=8 Hz, ArH), 7.20 (2H, d, J=8 Hz, ArH), 7.75 (4H, m, ArH). *Anal.* Calcd for $C_{20}H_{16}N_2O_6$: C, 63.15; H, 4.24; N, 7.37. Found: C, 63.18; H, 4.11; N, 7.31.

(3*S*,α*R*)-2-(2-Oxo-3-phthalimido-1-azetidinyl)-2-phenylacetic Acid (15A)——Anhydrous LiI (0.42 g) was added to a solution of methyl 2-(2-oxo-3-phthalimido-1-azetidinyl)-2-phenylacetate (**6**, 0.36 g) in dry pyridine (5 ml), and the mixture was refluxed for 2 h. After cooling, the reaction mixture was poured into a mixture of ice-water and AcOEt. The organic layer was separated, washed with 5% HCl and H_2O, dried over $MgSO_4$, and evaporated to give an oil, which was crystallized from AcOEt to give **15A** as colorless needles (0.26 g, 74.0%): mp 191.5 °C (dec.). $[\alpha]_D^{25}$ −244° (c=1.01, acetone). IR (Nujol): 1780, 1720 (β-lactam C=O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.54 (1H, dd, J=3, 5 Hz, 4β-H), 3.94 (1H, t, J=5 Hz, 4α-H), 5.48 (1H, dd, J=3, 5 Hz, 3α-H), 5.68 (1H, s, ArCHCOO), 7.45 (5H, s, ArH), 7.84 (4H, s, ArH). *Anal.* Calcd for $C_{19}H_{14}N_2O_5$: C, 65.13; H, 4.02; N, 8.00. Found: C, 65.04; H, 4.21; N, 7.98.

The following compounds were prepared by reacting the corresponding β-lactam esters with LiI in substantially the same manner as described above.

(3*S*,α*S*)-2-(2-Oxo-3-phthalimido-1-azetidinyl)-2-(2-thienyl)acetic Acid (**15C**): Yield 78.5%, mp 199—201 °C. $[\alpha]_D^{25}$ −192° (*c*=0.5, MeOH). IR (Nujol): 1770, 1740, 1720 (β-lactam C=O) cm^{-1}. ^{1}H-NMR ($D_2O+NaHCO_3$) δ: 3.36 (1H, dd, *J*=3, 5 Hz, 4β-H), 3.91 (1H, t, *J*=5 Hz, 4α-H), 5.38 (1H, dd, *J*=3, 5 Hz, 3α-H), 5.73 (1H, s, ArCHCOO), 7.00—7.80 (7H, m, ArH). *Anal.* Calcd for $C_{17}H_{12}N_2O_5S$: C, 57.31; H, 3.39; N, 7.86. Found: C, 57.25; H, 3.59; N, 7.74.

(3*RS*,α*SR*)-2-(2-Oxo-3-phthalimido-1-azetidinyl)-2-(2-furyl)acetic Acid (**15D**): Yield 70.8%, mp 187—190 °C (dec.). IR (Nujol): 1780, 1730 (β-lactam C=O), 1710 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.59 (1H, dd, *J*=3, 5 Hz, 4β-H), 3.97 (1H, t, *J*=5 Hz, 4α-H), 5.42 (1H, dd, *J*=3, 5 Hz, 3α-H), 5.76 (1H, s, ArCHCOO), 6.53 (1H, m, ArH), 6.62 (1H, d, *J*=3 Hz, ArH), 7.71 (1H, d, *J*=3 Hz, ArH), 7.86 (4H, s, ArH). *Anal.* Calcd for $C_{17}H_{12}N_2O_6$: C, 60.00; H, 3.55; N, 8.23. Found: C, 59.75; H, 3.49; N, 8.04.

(3*S*,α*R*)-2-(2-Oxo-3-phthalimido-1-azetidinyl)-2-(4-hydroxyphenyl)acetic Acid (**15E**): Yield 63.1%, mp 202—203 °C (dec.). $[\alpha]_D^{25}$ −301° (*c*=0.59, MeOH). IR (Nujol): 1780, 1740, 1720 (β-lactam C=O), 1700 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.47 (1H, dd, *J*=3, 5 Hz, 4β-H), 3.86 (1H, t, *J*=5 Hz, 4α-H), 5.39 (1H, dd, *J*=3, 5 Hz, 3α-H), 5.46 (1H, s, ArCHCOO), 6.88 (2H, d, *J*=8 Hz, ArH), 7.17 (4H, s, ArH), 7.26 (2H, d, *J*=8 Hz, ArH). *Anal.* Calcd for $C_{19}H_{14}N_2O_6$: C, 62.29; H, 3.85; N, 7.65. Found: C, 62.40; H, 3.88; N, 7.43.

(3*S*,α*S*)-2-(3-Amino-2-oxo-1-azetidinyl)-2-phenylacetic Acid (17A)——*N*,*N*-Dimethyl-1,3-propanediamine (0.31 g) was added to a solution of D-2-(2-oxo-3-phthalimido-1-azetidinyl)-2-phenylacetic acid (**15A**, 0.35 g) in MeOH (6 ml) and the mixture was stirred for 8 h at ambient temperature. The reaction mixture was poured into H_2O (6 ml), and Amberlite IRC-50 (about 20 ml) was added to adjust the pH to 5.8—6.0. After removal of the resin by filtration, the filtrate was evaporated to give a residue (0.19 g), which was crystallized from CH_3CN to give **17A** as needles (0.12 g, 54.0%): mp 143—147 °C (dec.). IR (Nujol): 1780 (β-lactam C=O), 1620 cm^{-1}. ^{1}H-NMR (D_2O) δ: 3.04 (1H, dd, *J*=3, 5 Hz, 4β-H), 3.83 (1H, t, *J*=5 Hz, 4α-H), 4.35 (1H, dd, *J*=3, 5 Hz, 3α-H), 5.30 (1H, s, ArCHCOO), 7.42 (5H, s, ArH). *Anal.* Calcd for $C_{11}H_{12}N_2O_3$: C, 59.99; H, 5.49; N, 12.72. Found: C, 59.71; H, 5.68; N, 12.61.

The following compounds were prepared by reacting the corresponding phthaloyl β-lactams with *N*,*N*-dimethyl-1,3-propanediamine in substantially the same manner as described above.

(3*S*,α*R*)-2-(3-Amino-2-oxo-1-azetidinyl)-2-(2-thienyl)acetic Acid (**17C**): Yield 57.8%, mp 144—149 °C (dec.). IR (Nujol): 1760 (β-lactam C=O), 1745, 1620 cm^{-1}. ^{1}H-NMR (D_2O) δ: 3.13 (1H, dd, *J*=3, 5 Hz, 4β-H), 3.92 (1H, t, *J*=5 Hz, 4α-H), 4.31 (1H, dd, *J*=3, 5 Hz, 3α-H), 5.53 (1H, s, ArCHCOO), 7.10—7.46 (3H, m, ArH). *Anal.* Calcd for $C_9H_{10}N_2O_3S \cdot 1/2H_2O$: C, 45.96; H, 4.71; N, 11.91. Found: C, 46.24; H, 4.67; N, 11.82.

(3*RS*,α*SR*)-2-(3-Amino-2-oxo-1-azetidinyl)-2-(2-furyl)acetic Acid (**17D**): Yield 20.0%, mp 185—189 °C (dec.). IR (Nujol): 1725 (β-lactam C=O), 1640 cm^{-1}. ^{1}H-NMR (D_2O) δ: 3.36 (1H, dd, *J*=3, 5 Hz, 4β-H), 3.90 (1H, t, *J*=5 Hz, 4α-H), 5.41 (1H, s, ArCHCOO), 6.49 (2H, m, ArH), 7.37 (1H, m, ArH). *Anal.* Calcd for $C_9H_{10}N_2O_4$: C, 51.42; H, 4.79; N, 13.33. Found: C, 51.64; H, 4.77; N, 13.15.

3-Aminonocardicinic Acid (3-ANA) (**17E**): Yield 60.4%, mp 194—199 °C (dec.). $[\alpha]_D^{25}$ −239° (*c*=1.0, 0.1 N $NaHCO_3$). IR (Nujol): 1763, (β-lactam C=O), 1742 cm^{-1}. ^{1}H-NMR ($D_2O+NaHCO_3$) δ: 2.89 (1H, dd, *J*=3, 5 Hz, 4β-H), 3.79 (1H, t, *J*=5 Hz, 4α-H), 4.22 (1H, dd, *J*=3, 5 Hz, 3α-H), 5.26 (1H, s, ArCHCOO), 6.88 (2H, d, *J*=8 Hz, ArH), 7.26 (2H, d, *J*=8 Hz, ArH). *Anal.* Calcd for $C_{11}H_{12}N_2O_4$. C, 55.93; H, 5.11; N, 11.86. Found: C, 56.11; H, 5.00; N, 11.68.

2-[3-(2-Hydroxyimino-2-phenylacetamido)-2-oxo-1-azetidinyl]-2-phenylacetic Acid (19A)——PCl_5 (0.25 g) was added to a suspension of 2-(2,2-dichloroacetoxyimino)-2-phenylacetic acid (0.35 g) in benzene (7 ml), and the mixture was stirred for 40 min at ambient temperature. The resulting solution was evaporated to dryness to give a residue, which was dissolved in CH_2Cl_2 (5 ml). This solution was cooled to 0 °C, and the silyl ester of 2-(3-amino-2-oxo-1-azetidinyl)phenylacetic acid [prepared from **17A** (0.22 g) and *N*,*N*-bis(trimethylsily)acetamide (0.87 g) in CH_2Cl_2 (20 ml) by stirring for 30 min at ambient temperature] was added. The reaction mixture was stirred for 1 h at 0 °C, then washed with 5% HCl, and the solvent was removed by evaporation. The residue was dissolved in AcOEt and extracted with 5% $NaHCO_3$. The aqueous layer was separated, adjusted to pH 1—2 with 10% HCl and extracted with AcOEt. The extract was washed with H_2O, dried over $MgSO_4$, and evaporated to give a residue, which was triturated with a small amount of $CHCl_3$ to give **19A** as crystals (0.11 g, 30.1%): mp 147—150 °C (dec.). IR (Nujol): 1745 (β-lactam C=O), 1700, 1665 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 3.28 (1H, dd, *J*=3, 5 Hz, 4β-H), 3.39 (1H, t, *J*=5 Hz, 4α-H), 5.05 (1H, dd, *J*=3, 5 Hz, 3α-H), 5.59 (1H, s, ArCHCOO), 7.12—7.72 (10H, m, ArH). *Anal.* Calcd for $C_{19}H_{17}N_3O_5$: C, 62.12; H, 4.66; N, 11.44. Found: C, 62.31; H, 4.55; N, 11.21.

The following compounds were obtained by the reaction of the corresponding 3-amino-β-lactam derivatives and 2-(2,2-dichloroacetoxyimino)-2-phenylacetyl chloride in substantially the same manner as described above.

2-[3-(2-Hydroxyimino-2-phenylacetamido)-2-oxo-1-azetidinyl]-2-(2-thienyl)acetic Acid (**19C**): Yield 64.7%, mp 144.5—148 °C. IR (Nujol): 1755 (β-lactam C=O), 1700, 1650 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 3.43 (1H, dd, *J*=3, 5 Hz, 4β-H), 3.95 (1H, t, *J*=5 Hz, 4α-H), 5.71 (1H, dd, *J*=3, 5 Hz, 3α-H), 5.84 (1H, s, ArCHCOO), 6.90—7.73 (8H, m, ArH). *Anal.* Calcd for $C_{17}H_{15}N_3O_5S$: C, 54.68; H, 4.05; N, 11.25. Found: C, 54.89; H, 3.39; N, 10.98.

2-[3-(2-Hydroxyimino-2-phenylacetamido)-2-oxo-1-azetidinyl]-2-(2-furyl)acetic Acid (**19D**): Yield 41.4%, mp

142—145 °C (dec.). IR (Nujol): 1755 (β-lactam C=O), 1735, 1710, 1660 cm^{-1}. ^{1}H-NMR (acetone-d_6) δ: 3.41 (1H, dd, J=3, 5 Hz, 4β-H), 3.99 (1H, t, J=5 Hz, 4α-H), 5.25 (1H, m, 3α-H), 5.71 (1H, s, ArCHCOO), 6.46 (1H, m, ArH), 6.58 (1H, d, J=3 Hz, ArH), 7.30—7.70 (6H, m, ArH), 8.26 (1H, d, J=8 Hz, CONH). *Anal.* Calcd for $C_{17}H_{15}N_3O_6$: C, 57.14; H, 4.23; N, 11.76. Found: C, 57.16; H, 4.19; N, 11.51.

2-[3-(2-Hydroxyimino-2-phenylacetamido)-2-oxo-1-azetidinyl]-2-(4-hydroxyphenyl)acetic Acid (**19E**): Yield 59.3%, mp 197—199 °C (dec.). IR (Nujol): 1730 (β-lactam C=O), 1660 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.09 (1H, dd, J=3, 5 Hz, 4β-H), 3.78 (1H, t, J=5 Hz, 4α-H), 5.01 (1H, m, 3α-H), 5.12 (1H, s, ArCHCOO), 7.28—7.52 (9H, m, ArH). *Anal.* Calcd for $C_{19}H_{17}N_3O_6 \cdot 1/2H_2O$: C, 58.15; H, 4.62; N, 10.71. Found: C, 58.11; H, 4.77; N, 10.48.

Methyl (3*RS*,α*SR*)-2-[3-(2,2-Dichloroacetoxyimino-2-phenylacetamido)-2-oxo-1-azetidinyl]-2-(1-naphthyl)acetate (21)——*N*,*N*-Dimethyl-1,3-propanediamine (1.08 g) was added to a solution of methyl (3*RS*,α*SR*)-2-(2-oxo-3-phthalimido-1-azetidinyl)-2-(1-naphthyl)acetate (**13b**, 2.07 g) in a mixture of MeOH (30 ml) and $CHCl_3$ (40 ml), and the mixture was stirred overnight at ambient temperature. After evaporation of the reaction mixture, the residue was dissolved in AcOEt and extracted with 10% HCl. The aqueous layer was adjusted to pH 8 with $NaHCO_3$ and extracted with AcOEt. The extract was washed with H_2O, dried over $MgSO_4$ and evaporated to give a residue (1.53 g), which was subjected to column chromatography on silica gel. Elution was carried out with $CHCl_3$ and the fractions containing the desired compound were collected and evaporated to give **20** as a crude oil. This product was reacted with 2-(2,2-dichloroacetoxyimino)-2-phenylacetyl chloride (1.91 g) in substantially the same manner as described above to give **21** (1.55 g, 74.3%): oil. MS m/z 431 (M^+). IR (film): 1740 (β-lactam C=O), 1660 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.89 (1H, dd, J=3, 5 Hz, 4β-H), 3.67 (3H, s, $COOCH_3$), 3.84 (1H, t, J=5 Hz, 4α-H), 5.07 (1H, m, 3α-H), 6.32 (1H, s, ArCHCOO), 7.12—7.96 (12H, m, ArH).

2-[3-(2-Hydroxyimino-2-phenylacetamido)-2-oxo-1-azetidinyl]-2-(1-naphthyl)acetic Acid (19B)——A solution of **21**, (0.20 g) in MeOH (10 ml) was treated with 1 N NaOH (9.3 ml) under ice-cooling, and the mixture was stirred for 3 h at the same temperature. After evaporation of the mixture, the residue was dissolved in H_2O, and this solution was adjusted to pH 2 with 10% HCl and extracted with AcOEt. The extract was washed with H_2O, dried over $MgSO_4$, and evaporated to give an oil, which was triturated with a small amount of AcOEt to give **19B** as crystals (0.14 g, 72.4%): mp 124—129 °C. IR (Nujol): 1730 (β-lactam C=O), 1675 cm^{-1}. ^{1}H-NMR (acetone-d_6) δ: 3.06 (1H, dd, J=3, 5 Hz, 4β-H), 4.07 (1H, t, J=5 Hz, 4α-H), 6.35 (1H, s, ArCHCOO), 7.08—8.02 (12H, m, ArH). *Anal.* Calcd for $C_{23}H_{19}N_3O_5$: C, 66.18; H, 4.59; N, 10.07. Found: C, 65.91; H, 4.55; N, 10.13.

References and Notes

1) H. Aoki, H. Sakai, M. Kohsaka, T. Konomi, J. Hosoda, Y. Kubochi, E. Iguchi, and H. Imanaka, *J. Antibiot.*, **29**, 492 (1976).
2) M. Hashimoto, T. Komori, and T. Kamiya, *J. Am. Chem. Soc.*, **98**, 3023 (1976); *idem*, *J. Antibiot.*, **29**, 890 (1976).
3) O. Nakaguchi, T. Oku, M. Hashimoto, and T. Kamiya, *Chem. Pharm. Bull.*, **35**, 3979 (1987).
4) T. Kamiya, T. Oku, O. Nakaguchi, H. Takeno, and M. Hashimoto, *Tetrahedron Lett.*, **1978**, 5119.
5) H. Stauginger, *Justus Liebigs Ann. Chem.*, **356**, 51 (1907); A. K. Bose, G. Spiegelman, and M. S. Manhas, *J. Am. Chem. Soc.*, **90**, 4506 (1968).
6) E. M. Smolin and L. Rapopot, "The Chemistry of Heterocyclic Compounds," Vol. 13, ed. by A. Weissberger, Interscience Publication Inc., New York, 1959.
7) D. D. Reynolds and B. C. Cossar, *J. Heterocycl. Chem.*, **8**, 597 (1971).
8) T. Kamiya, M. Hashimoto, O. Nakaguchi, and T. Oku, *Tetrahedron*, **35**, 323 (1979).
9) Hydrolysis of **6** with NaOH caused epimerization of the methoxycarbonyl group in **6**.

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Synthesis of Some Polyhydroxylated Pyrrolidine Derivatives

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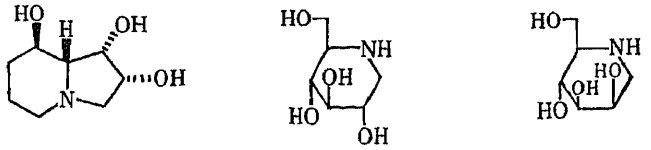
Polyhydroxylated pyrrolidine derivatives, **7**, **11**, **15**, and **18**, were synthesized from **4**, a key intermediate for our total synthesis of swainsonine (**1**). The immunostimulating activities of these new derivatives were found to be moderate and less than that of swainsonine.

Keywords——swainsonine; azamannofuranose; enantiospecific synthesis; D-mannose; immunostimulating activity

Polyhydroxylated pyrrolidine, piperidine, and indolizidine alkaloids are of great interest because of their specific glycosidase inhibitory activity.[1] Moreover, it has recently been found in our laboratories that swainsonine (**1**), a representative of the polyhydroxylated indolizidine alkaloids, has an immunostimulating activity, possibly as a result of its glycosidase inhibitory activity.[2] This has prompted us to explore related pyrrolidine and piperidine alkaloids. In the preceding paper,[3] we reported the syntheses of two piperidine alkaloids, deoxynojirimycin (**2**) and deoxymannojirimycin (**3**). Herein we report the syntheses and biological activity of some pyrrolidine derivatives **7**, **11**, **15**, and **18**.



1 2 3

Chart 1

During the course of our studies on the total synthesis of swainsonine,[4] we found that the intermediates **4** and **5** derived from D-mannose could be conveniently adopted for the synthesis of such pyrrolidine derivatives. For example, pyrrolidine ring formation starting from **4** and **5** would give compounds **6** and **12**, respectively, from which the pyrrolidine derivatives **7**, **11**, **15**, and **18** could be prepared.

For the cyclization of **4** to the pyrrolidine derivatives, **4** was treated with NaH in *N*,*N*-dimethylformamide (DMF) to give compound **6** in 54% yield. The protecting groups in **6** were removed by catalytic hydrogenation on 10% Pd–C in EtOH and subsequent acid treatment (6 N HCl) to give **7** in 80% yield. On the other hand, partial hydrolysis of the acetonide protecting groups in **6** with TsOH in aqueous MeOH gave a 68% yield of the diol **8**, which was then oxidized with $NaIO_4$ in aqueous tetrahydrofuran (THF) and subsequently reducted with $NaBH_4$ in MeOH to afford, *via* the aldehyde **9**, the alcohol **10** in 84% yield. Catalytic hydrogenation of **10** on 10% Pd–C in EtOH, followed by treatment with 6 N HCl gave the

trihydroxypyrrolidine **11** in 71% yield.

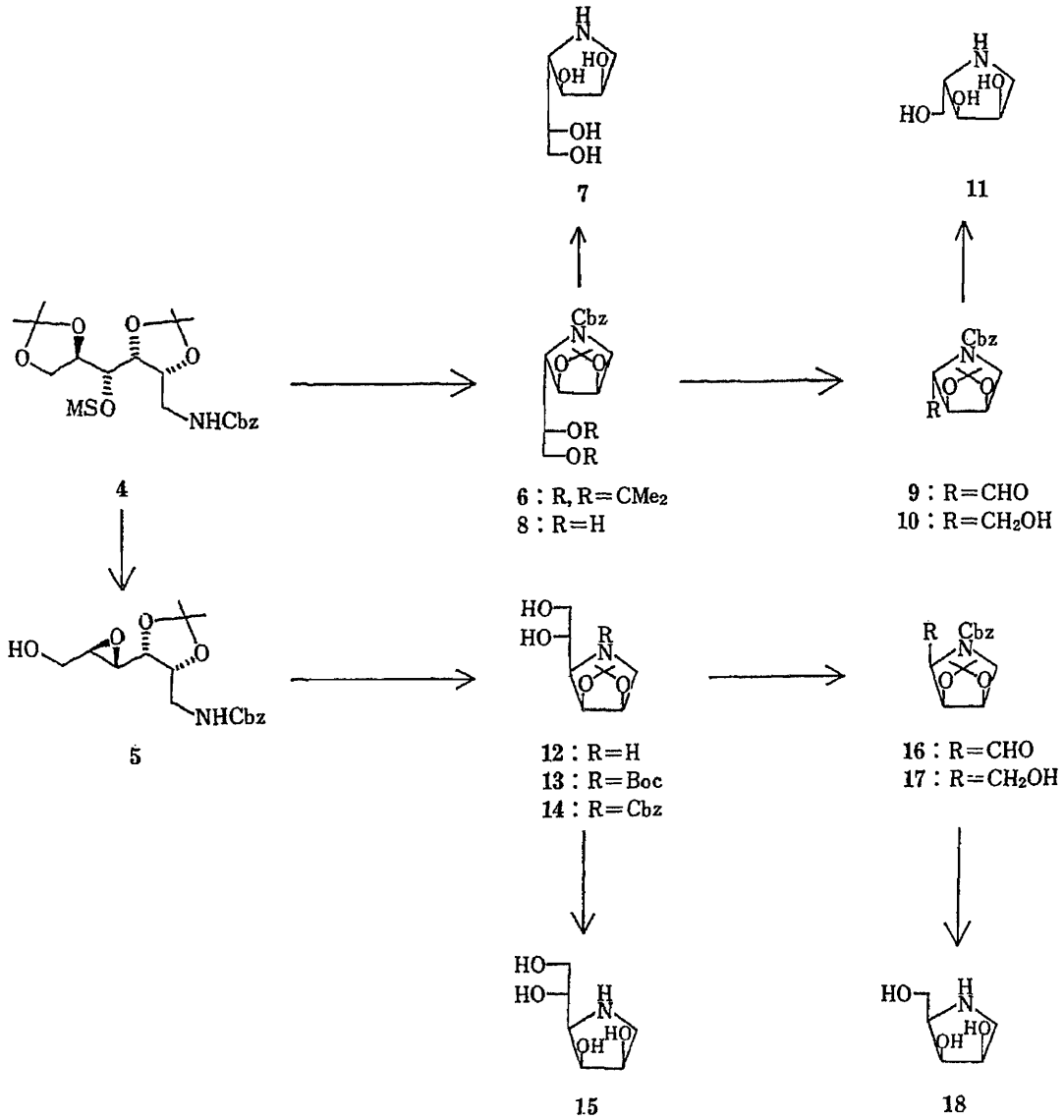


Chart 2

For the preparation of the stereoisomers **15** and **18**, the mesylate **4** was converted to the epoxy-alcohol **5** as described in the preceding paper.[4] Catalytic reduction of **5** as described above for the removal of the Cbz group directly gave the cyclized product **12**, which was successively converted to the Boc derivative **13** by treatment with $(Boc)_2O$ in THF in the presence of Et_3N and purified by silica gel chromatography (81% from **5**). The pure **13** was then deprotected by treatment with 6 N HCl in THF to afford **15** in 78% yield. The crude amine **12**, on the other hand, was acylated with CbzCl in aqueous THF to give a 68% yield of the Cbz derivative **14**, which in turn was oxidized with $NaIO_4$ in aqueous THF, and the intermediary aldehyde was subjected to reduction with $NaBH_4$ in MeOH to provide the alcohol **17** in 90% yield. The Cbz group in **17** was removed by catalytic hydrogenation as described above and then the acetonide group was removed by treatment with 6 N HCl to afford **18** in 95% yield.

The immunostimulating activity of **7**, **11**, **15**, and **18** was determined in terms of the

TABLE I. Competitive Effect (MEC, μg/ml) against Immunosuppressive Factors Obtained from Tumor-Bearing Mouse Serum in Con A-Induced Stimulation of [^{3}H]Thymidine Incorporation by Mouse Spleen Cells

Compound	MEC (μg/ml)
1	0.01
7	16
11	16
15	12.5
18	4

MEC, minimal effective concentration.

capacity to restore the depression of mitogenic responses of mouse spleen cells by immunosuppressive factors in tumor-bearing mouse serum.[2] The data are summarized in Table I. All the new derivatives showed moderate activity but were considerably less active than swainsonine (**1**). It was found, however, that all the new pyrrolidine derivatives were more active than the piperidine derivatives **2** and **3**.[3] These results suggested that the pyrrolidine derivatives corresponding to the five-membered part of swainsonine are more effective than the piperidine compounds. Moreover, the configuration at the 2 position in the pyrrolidine compounds seemed to be important for the immunostimulant activity. Thus, the 2*R* derivatives **15** and **18** are more active than the 2*S* counterparts **7** and **11** as shown in the table. This result suggests that the *R* configuration at C-8a of swainsonine is important for the activity.

Experimental

All melting points are uncorrected. The proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL FX-270 spectrometer using tetramethylsilane (TMS) or 3-(trimethylsilyl)propionic acid-d_4 sodium salt (TSP-d_4) as an internal reference. The infrared (IR) spectra were taken with a JASCO A-102 spectrometer. The optical rotations were measured with JASCO automatic polarimeter. The fast atom bombardment (FAB) high-resolution mass spectra (MS) were recorded on a VG ZAB spectrometer.

(1*R*,5*S*,6*S*,4′*S*)-7-Benzyloxycarbonyl-3,3-dimethyl-6-4′-2′,2′-dimethyl-1′,3′-dioxanyl-2,4-dioxa-7-azabicyclo-[3.3.0]octane (6)——NaH (60 mg, 60% in oil) was added to a solution of **4** (440 mg, 0.93 mmol) in DMF (15 ml), and the mixture was heated at 70 °C for 6 h. After quenching with H_2O, the reaction mixture was extracted with AcOEt and the extract was washed with H_2O and brine and dried over $MgSO_4$. The solvent was removed by evaporation to give an oil, which was chromatographed on silica gel (*n*-hexane–AcOEt 1 : 1) to give **6** (206 mg, 54%) as a pale yellow oil. IR (neat): 1695, 1365 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.32 (9H, s), 1.50 (3H, s), 3.5—4.4 (6H, m), 4.6—4.8 (2H, m), 5.18 (2H, s), 7.37 (5H, s). MS *m/z*: Calcd for $C_{20}H_{27}NO_6$ 378.1917 (M+H), obsd. 378.1951 (M+H).

(2*S*,3*S*,4*R*,1′*S*)-3,4-Dihydroxy-2-1′,2′-dihydroxyethylpyrrolidine (7)——A solution of **6** (189 mg, 0.50 mmol) in EtOH (12 ml) was shaken under H_2 in the presence of 10% Pd–C (30 mg) at room temperature for 4 h. After removal of the catalyst by filtration, the filtrate was added to 6 N HCl (10 ml) and the mixture was stirred at room temperature overnight. After evaporation of the mixture, the residue was dissolved in H_2O and passed through a column of Amberlite IRA-400 (OH^-) with H_2O. The eluate was evaporated to give **7** (65.5 mg, 80%) as a pale yellow syrup. IR (neat): 3300, 1345, 1110, 1065 cm^{-1}. ^{1}H-NMR (D_2O) δ: 2.80 (1H, dd, J=3.5, 12 Hz), 2.96 (1H, dd, J=4, 7.5 Hz), 3.21 (1H, dd, J=5, 12 Hz), 3.60 (1H, dd, J=8, 12 Hz), 3.71 (1H, dd, J=4, 12 Hz), 3.82 (1H, dt, J=4, 8 Hz), 3.97 (1H, dd, J=5, 8 Hz), 4.14 (1H, dt, J=3.5, 5 Hz). $[\alpha]_D^{20}$ −40° (c=0.2, H_2O). MS *m/z*: Calcd for $C_6H_{13}NO_4$ 164.0924 (M+H), obsd. 164.0918 (M+H).

(1*R*,5*S*,6*S*,1′*S*)-7-Benzyloxycarbonyl-6-1′,2′-dihydroxyethyl-3,3-dimethyl-2,4-dioxa-7-azabicyclo[3.3.0]octane (8)——A mixture of **6** (4.40 g, 11.6 mmol) and TsOH·H_2O (220 mg, 1.16 mmol) in a mixture of MeOH (44 ml) and H_2O (4.9 ml) was stirred at room temperature overnight. The mixture was treated with Amberlite IRA-400 (OH^-) and after removal of the resin by filtration, the filtrate was concentrated to give a crude oil, which was purified by chromatography on silica gel. Elution with *n*-hexane–AcOEt (1 : 1) gave **8** (2.57 g, 68%) as an oil. IR (neat): 3420, 1665, 1420, 1370, 1235 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.34 (3H, s), 1.41 (3H, s), 3.4—4.3 (8H, m), 4.80 (2H, m), 5.21

(2H, s), 7.40 (5H, s). MS m/z: Calcd for $C_{17}H_{23}NO_6$ 338.1604 (M+H), obsd. 338.1638 (M+H).

(1*R*,5*S*,6*S*)-7-Benzyloxycarbonyl-3,3-dimethyl-6-hydroxymethyl-2,4-dioxa-7-azabicyclo[3.3.0]octane (10)——A solution of $NaIO_4$ (3.20 g, 14.8 mmol) in H_2O (50 ml) was added dropwise to a solution of **7** (2.50 g, 7.41 mmol) in THF under ice-bath cooling. After being stirred at the same temperature for 1.5 h, the mixture was extracted with $CHCl_3$ and washed successively with H_2O, aqueous $NaHCO_3$ and brine. Drying over $MgSO_4$ and evaporation of the solvent gave **9** (2.26 g) as a crude oil. This oil was used for the next step without further purification.

A solution of **9** in MeOH (50 ml) was cooled in an ice bath and $NaBH_4$ (290 mg, 7.67 mmol) was added. The mixture was stirred at the same temperature for 30 min, then diluted with $CHCl_3$, washed with H_2O and brine, dried over $MgSO_4$, and evaporated to give an oil, which was purified by column chromatography on silica gel (*n*-hexane–AcOEt 1:1) to give **10** (2.06 g, 84% from **8**) as a colorless oil. IR (neat): 3450, 1685, 1425, 1125 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.30 (3H, s), 1.43 (3H, s), 3.57 (1H, dd, J=5, 12 Hz), 3.6—3.9 (4H, m), 4.17 (1H, m), 4.71 (2H, m), 5.14 (2H, s), 7.36 (5H, s). MS *m/z*: Calcd for $C_{16}H_{21}NO_5$ 308.1498 (M+H), obsd. 308.1489 (M+H).

(2*S*,3*S*,4*R*)-3,4-Dihydroxy-2-hydroxymethylpyrrolidine (11)——Compound **10** (900 mg, 2.93 mmol) was treated in a manner similar to that used for the preparation of **7** from **8** to give **11** (343 mg, 88%) as a pale yellow solid. mp 114—117 °C (EtOH–Et_2O). IR (Nujol): 3280, 1320, 1140, 1100, 1035 cm^{-1}. ^{1}H-NMR (D_2O) δ: 2.82 (1H, dd, J=4, 12 Hz), 3.06 (1H, ddd, J=4.5, 6, 7 Hz), 3.18 (1H, dd, J=5.5, 12 Hz), 3.63 (1H, dd, J=6, 11.5 Hz), 3.74 (1H, dd, J=4.5, 11.5 Hz), 3.87 (1H, dd, J=5.5, 7 Hz), 4.15 (1H, dt, J=4, 5.5 Hz). $[\alpha]_D^{22}$ −20.7° (c=0.5, H_2O). MS *m/z*: Calcd for $C_5H_{11}NO_3$ 134.0819 (M+H), obsd. 134.0811 (M+H).

(1*R*,5*S*,6*R*,1′*S*)-7-*tert*-Butoxycarbonyl-6-1′,2′-dihydroxyethyl-3,3-dimethyl-2,4-dioxa-7-azabicyclo[3.3.0]octane (13)——A solution of **5** (2.20 g, 6.52 mmol) in EtOH (60 ml) was shaken under H_2 (3 atm) in the presence of 10% Pd–C (300 mg) at room temperature for 5 h. After removal of the catalyst, the solvent was evaporated to give **12** as a crude oil. Di-*tert*-butyldicarbonate (Boc_2O) (1.04 ml, 4.53 mmol) and Et_3N (0.63 ml, 4.53 mmol) were added to a solution of **12** (760 mg) in a mixture of THF (20 ml) and H_2O (5 ml) under ice-bath cooling, and the mixture was stirred at room temperature for 3 h. THF was evaporated off and the resulting aqueous solution was extracted with $CHCl_3$. The organic layer was washed with H_2O, dried over $MgSO_4$, and evaporated to give an oil, which was purified by chromatography on silica gel (*n*-hexane–AcOEt 1:1) to give **13** (916 mg, 81% from **5**) as a pale yellow oil. IR (neat): 3420, 1670, 1400 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.34 (3H, s), 1.43 (9H, s), 1.53 (3H, s), 3.0—4.3 (8H, m), 4.6—5.0 (2H, m). MS *m/z*: Calcd for $C_{14}H_{25}NO_6$ 304.1760 (M+H), obsd. 304.1713 (M+H).

(1*R*,5*S*,6*R*,1′*S*)-7-Benzyloxycarbonyl-6-1′,2′-dihydroxyethyl-3,3-dimethyl-2,4-dioxa-7-azabicyclo[3.3.0]octane (14)——A crude sample of **12** prepared from 6.20 g (18.4 mmol) of **5** was acylated in a manner similar to that used for the preparation of **13** to give **14** (3.17 g, 51%) as an oil. IR (neat): 3430, 1680, 1405, 1245 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.32 (3H, s), 1.53 (3H, s), 2.9—4.3 (8H, m), 4.82 (2H, m), 5.11 (2H, s), 7.30 (5H, s). MS *m/z*: Calcd for $C_{17}H_{23}NO_6$ 338.1604 (M+H), obsd. 338.1621 (M+H).

(2*R*,3*S*,4*R*,1′*S*)-3,4-Dihydroxy-2-1′,2′-dihydroxyethylpyrrolidine (15)——A solution of **13** (800 mg, 2.64 mmol) in a mixture of THF (20 ml) and 6 N HCl (20 ml) was stirred at room temperature overnight and the solvent was evaporated off to give an oil. This oil was treated in a manner similar to that used for **7** to give **15** (335 mg, 78%) as a pale yellow powder. mp 125—128 °C (EtOH–Et_2O). IR (Nujol): 3450, 3260, 3210, 1335, 1085 cm^{-1}. ^{1}H-NMR (D_2O) δ: 2.75 (1H, dd, J=7, 12 Hz), 3.09 (1H, dd, J=4, 10 Hz), 3.15 (1H, dd, J=8, 11.5 Hz), 3.55 (1H, dd, J=7, 12 Hz), 3.75 (1H, dd, J=3.5, 12 Hz), 3.85 (1H, ddd, J=3.5, 7, 10 Hz), 4.20 (1H, t, J=4 Hz), 4.32 (1H, dt, J=4, 8 Hz). $[\alpha]_D^{25}$ −12.4° (c=0.7, H_2O). [Lit.[1c] mp 137 °C. $[\alpha]_D^{20}$ −10.4° (c=0.12, H_2O)].

(1*R*,5*S*,6*R*)-7-Benzyloxycarbonyl-3,3-dimethyl-6-hydroxymethyl-2,4-dioxa-7-azabicyclo[3.3.0]octane (17)——Compound **13** (3.00 g, 8.89 mmol) was treated with $NaIO_4$ (3.24 g, 15.0 mmol) and then with $NaBH_4$ (336 mg, 8.89 mmol) in the same way as described for **6** to provide **17** (2.45 g, 90%) as an oil. IR (neat): 3420, 1685, 1415, 1080 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.33 (3H, s), 1.47 (3H, s), 3.66 (2H, d, J=2 Hz), 3.8—4.0 (4H, m), 4.75 (2H, m), 5.13 (2H, s), 7.31 (5H, s) MS *m/z*: Calcd for $C_{16}H_{21}NO_5$ 308.1498 (M+H), obsd. 308.1502 (M+H).

(2*R*,3*S*,4*R*)-3,4-Dihydroxy-2-hydroxymethylpyrrolidine (18)——A solution of **17** (1.09 g, 3.55 mmol) in EtOH (50 ml) was treated in the same manner as described for **10** to give **18** (450 mg, 95%) as an oil. IR (neat): 3300, 1415, 1125, 1055 cm^{-1}. ^{1}H-NMR (D_2O) δ: 2.77 (1H, dd, J=7, 12 Hz), 3.11 (1H, dd, J=7, 12 Hz), 3.25 (1H, dt, J=4, 7 Hz), 3.64 (1H, dd, J=7, 11 Hz), 3.79 (1H, dd, J=7, 11 Hz), 4.18 (1H, t, J=4 Hz), 4.28 (1H, dt, J=4, 7 Hz). $[\alpha]_D^{25}$ −9.3° (c=1.3, H_2O). MS *m/z*: Calcd for $C_5H_{11}NO_3$ 134.0819 (M+H), obsd. 138.0793 (M+H).

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References

1) Piperidine alkaloids: *a*) S. V. Evans, L. E. Fellows, and E. A. Bell, *Phytochemistry*, **22**, 768 (1983); *b*) G. W. J. Fleet, A. N. Shaw, S. V. Evans, and L. E. Fellows, *J. Chem. Soc., Chem. Commun.*, **1985**, 841. Pyrrolidine alkaloids: *c*) D. W. C. Jones, R. J. Nash, E. A. Bell, and J. M. Williams, *Tetrahedron Lett.*, **26**, 3125 (1985); *d*) G. W. J. Fleet, S. J. Nicholas, D. W. Smith, S. V. Evans, L. E. Fellows, and R. J. Nash, *Tetrahedron Lett.*, **26**, 3127

(1985); *e*) G. W. J. Fleet, P. W. Smith, S. V. Evans, and L. E. Fellows, *J..Chem. Soc., Chem. Commun.*, **1984**, 1240.
2) T. Kino, N. Inamura, K. Nakahara, S. Kyoto, T. Goto, H. Terano, M. Khosaka, H. Aoki, and H. Imanaka, *J. Antibiot.*, **38**, 936 (1985).
3) H. Setoi, H. Takeno, and M. Hashimoto, *Chem. Pharm. Bull.*, **34**, 2642 (1986).
4) H. Setoi, H. Takeno, and M. Hashimoto, *J. Org. Chem.*, **50**, 3948 (1985).

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Synthesis and Anti-platelet Aggregating Activity of 3-Hetero Analogues of (+)-9(*O*)-Methano-$\Delta^{6(9\alpha)}$-prostaglandin I_1

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Optically active 3-hetero analogues of isocarbacyclin (**34a**, **38**, **39**, **40** and **42**) as well as ω-chain analogues have been synthesized from the bicyclic alcohol (**1**). Compound **34d** had more potent anti-platelet aggregating activity than prostacyclin in human platelet-rich plasma.

Keywords——isocarbacyclin; 3-oxaisocarbacyclin; 3-thiaisocarbacyclin; 3-azaisocarbacyclin; 3-heteroisocarbacyclin; platelet aggregation inhibitor; regioselective deprotonation

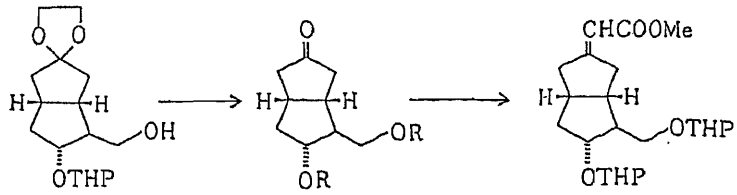
Prostaglandins are metabolized very quickly in the body. One of their main metabolic pathways is the β-oxidation reaction[1] of the α-carboxylic side chain, which results in the loss of biological activity. It is, therefore, of interest to block the β-oxidation reaction from the medicinal point of view. In order to block the β-oxidation reaction, the introductions of an oxygen atom at the C_3 position into both prostaglandin E_1[2] and carbacyclin derivative[3] have been reported.

As one of our synthetic programs on stable prostacyclin analogues, we have already reported the synthesis of 3-oxa-9(*O*)-methano-$\Delta^{6(9\alpha)}$-prostaglandin I_1 (3-oxaisocarbacyclin) in an optically inactive form.[4] It had a quite potent anti-platelet aggregating activity; its IC_{50} value was 23 ng/ml against adenosine-5′-diphosphate (ADP)-induced platelet aggregation in rabbit platelet-rich plasma (*in vitro*). This finding prompted us to prepare an optically active 3-oxaisocarbacyclin, its ω-chain analogues, and other 3-hetero analogues of isocarbacyclin such as 3-thia-, 3-sulfinyl-, 3-sulfonyl- and 3-azaisocarbacyclins. In this paper, we describe the synthesis and anti-platelet aggregating activity of optically active 3-heteroisocarbacyclins (**34a**, **38**, **39**, **40** and **42**) and some ω-chain analogues.

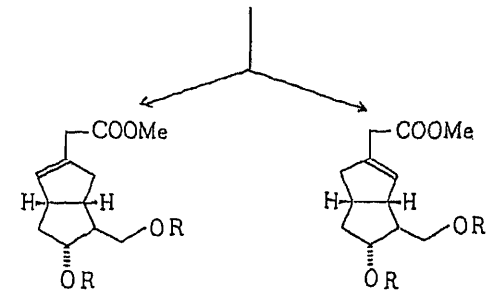
Synthesis of (+)-3-Oxaisocarbacyclin and Its ω-Chain Analogues

In order to synthesize (+)-3-oxaisocarbacyclin (**34a**), we selected the alcohol (**10a**) as a key intermediate. This compound was synthesized from the optically active alcohol (**1**) *via* three routes as shown in Charts 1, 4 and 5. The alcohol (**1**) is readily available from *cis*-bicyclo[3.3.0]octane-3,7-dione.[5]

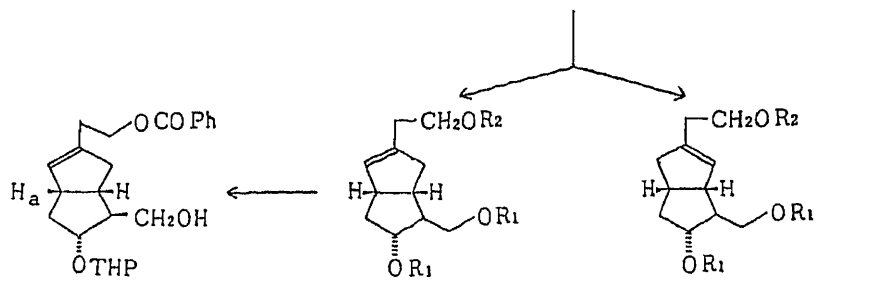
The first route utilized the crystalline diol (**9a**) as a key intermediate. Compound **9a** was prepared through the following sequence of reactions (Chart 1). Deprotection of **1** with *p*-toluenesulfonic acid (*p*-TsOH) in aqueous acetone, followed by protection of the resulting diol (**2**), mp 95—99 °C, with dihydropyran and *p*-TsOH afforded the known tetrahydropyranyl ether (**3**)[6] in 80% yield from **1**. The Wittig–Horner reaction of **3** with trimethyl phosphonoacetate gave the ester (**4**) as an inseparable mixture of *E*- and *Z*-isomers in 90% yield. The ratio of *E*- to *Z*-isomer was determined to be 51 to 49 by reverse-phase high-performance liquid chromatographic (HPLC) analysis of the diol (**11a**, **b**) derived from **4** by



1 2 : R=H 3 : R=THP 4



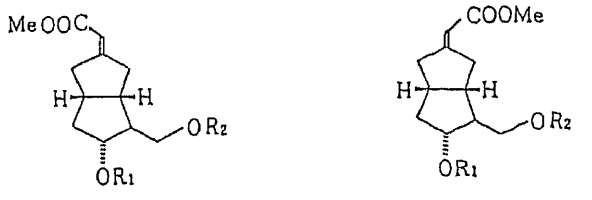
5a : R=THP 6a : R=H 5b : R=THP 6b : R=H



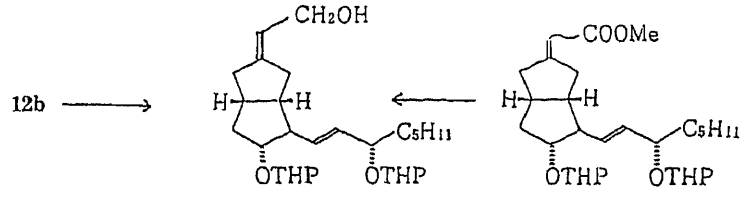
10a 7a : R_1=THP, R_2=H 8a : R_1=THP, R_2=COPh 9a : R_1=H, R_2=COPh 7b : R_1=THP, R_2=H 8b : R_1=THP, R_2=COPh 9b : R_1=H, R_2=COPh

THP: tetrahydropyran-2-yl

Chart 1



11a : R_1=R_2=H 12a : R_1=H, R_2=CPh_3 11b : R_1=R_2=H 12b : R_1=H, R_2=CPh_3



13 14

Chart 2

acid treatment (Chart 2). The *E*- and *Z*-configurations of **11a** and **11b** were assigned on the basis of the following data; treatment of a mixture of the diol (**11a, b**) with trityl chloride and triethylamine (Et_3N), followed by careful chromatographic separation afforded the less polar alcohol (**12b**) and the more polar alcohol (**12a**). The structures of **12a** and **12b** were assigned on the basis of the fact that the alcohol (**12b**) was led, by a conventional method, to **13**. Authentic **13** having *Z*-configuration[3] was alternatively prepared by diisobutylaluminum hydride (DIBAL) reduction of **14**.[7] The product (**13**) was identical with the authentic sample. Deprotection of **12a** yielded the diol (**11a**), which corresponded to the peak having shorter retention time on reverse-phase HPLC. From these data, the *E*- and *Z*-configurations of **11a** and **11b** were determined.

Next, we investigated the deconjugation reaction of the α,β-unsaturated ester moiety in 4. Thus, treatment of **4** with lithium dicyclohexylamide in tetrahydrofuran (THF) in the presence of hexamethylphosphoric triamide (HMPA)[8a] quantitatively afforded the β,γ-unsaturated methyl ester (**5a, b**) as an inseparable mixture of the double bond isomers. The isomeric ratio (**5a** : **5b** = 66 : 34) was determined by HPLC analysis of the diol (**6a, b**) derived from **5a, b** by treatment with aqueous methanolic *p*-TsOH. The major isomer having shorter retention time was assigned as the $\Delta^{6(9\alpha)}$-isomer (**6a**) (prostaglandin numbering)[9] on the basis of the relative HPLC retention time, as in the case of the benzoates (**9a, b**); the $\Delta^{6(9\alpha)}$-regioisomer (**9a**) had shorter retention time than the Δ^{6}-regioisomer (**9b**). Preferential formation of **5a** may be due to regioselective deprotonation of the allylic proton on the bicyclo[3.3.0]octane ring through electronic and remote steric control.[8b-d] Deprotonation of the allylic proton in a simple acyclic α,β-unsaturated ester occurs exclusively at the position

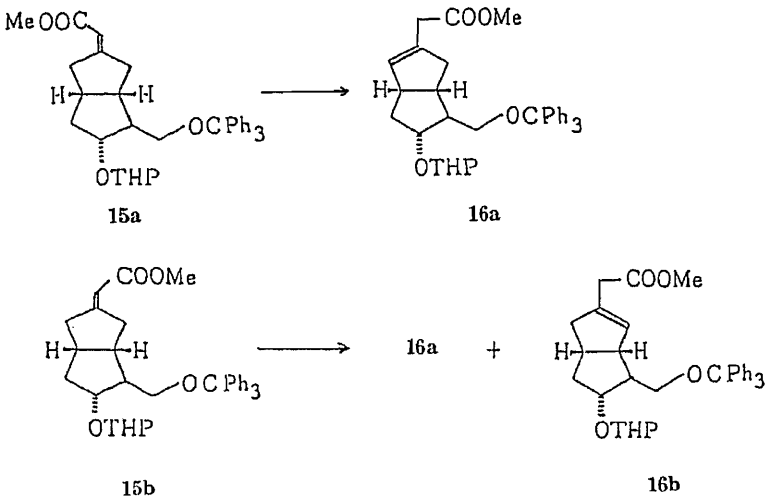


Chart 3

syn to the ester group through electronic control.[8b] The electronic and remote steric control in the case of *cis*-bicyclo[3.3.0]octane derivatives was more clearly demonstrated by the following results (Chart 3): treatment of the *E*-ester (**15a**) having a bulky trityl protecting group as described for **4** gave exclusively the $\Delta^{6(9\alpha)}$-isomer (**16a**). On the other hand, the corresponding *Z*-iomer (**15b**) afforded a mixture of **16a, b** in a ratio of 53 to 47 (**16a** to **16b**). It seemed to be better, from the viewpoint of stereoselectivity, to use **16a** for further elaboration of the synthesis. In practice, however, we used the ester mixture (**5a, b**) because these double bond isomers could be easily separated, in a later step, by recrystallization. Accordingly, a mixture of **5a, b** was led to **10a** through the following sequence of reactions (Chart 1).

Firstly, a mixture of **5a, b** was treated with lithium aluminum hydride ($LiAlH_4$) to yield

the alcohol (**7a**, **b**). Benzoylation of **7a**, **b** with benzoyl chloride in pyridine afforded the benzoate (**8a**, **b**) in 84% yield from **5a**, **b**. Deprotection of **8a**, **b** with aqueous methanolic *p*-TsOH yielded a mixture of **9a**, **b** in a ratio of 66 to 34 (**9a** to **9b**) as judged by HPLC analysis. The major isomer (**9a**) had a shorter retention time than **9b** on HPLC. Several recrystallizations of the above mixture gave the crystalline diol (**9a**), mp 87—89 °C, as a single isomer in 32% yield from **8a**, **b**. Compound **9a** was confirmed to be the $\Delta^{6(9\alpha)}$-isomer by leading it to **10a** and **34a**. The desired alcohol (**10a**) was finally prepared from **9a** in three steps through a conventional method[10] in 66% yield: 1) treatment with one molar equivalent of trichloroacetyl chloride and Et_3N, 2) protection with dihydropyran and *p*-TsOH, 3) hydrolysis with aqueous sodium bicarbonate. Compound **10a** showed a signal at δ3.0 (multiplet) due to the H_a proton, a characteristic of the $\Delta^{6(9\alpha)}$-double bond isomer, in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum.[4]

The second route used the sulfide (**21**) as a key intermediate, whose synthesis was achieved in several steps from **1** (Chart 4).

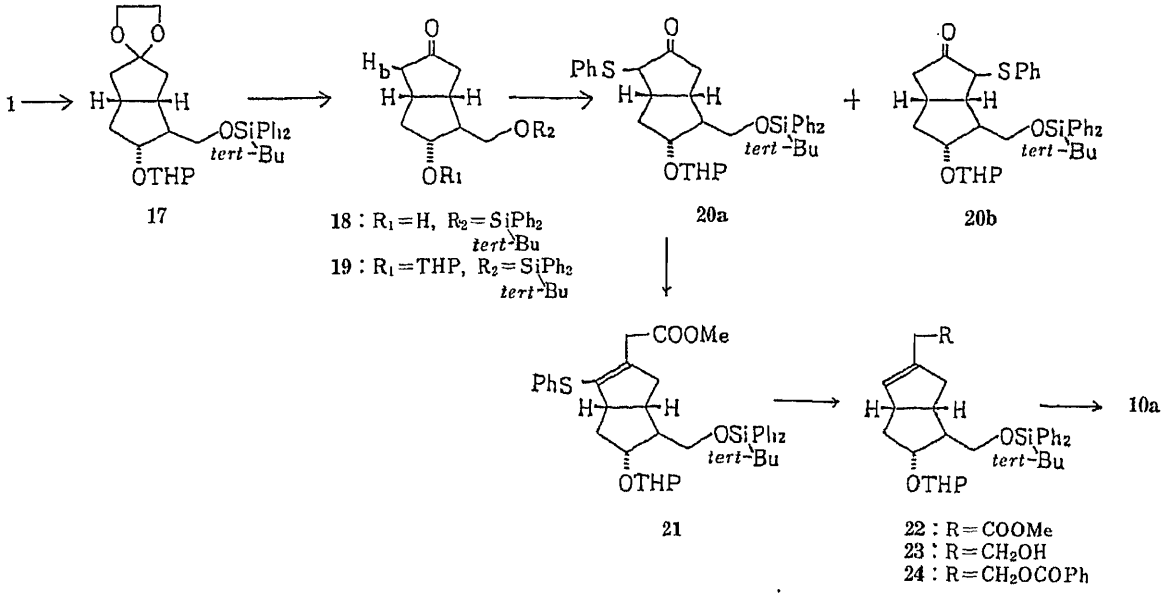


Chart 4

Treatment of **1** with *tert*-butyldiphenylchlorosilane and imidazole, followed by deprotection with aqueous acetic acid afforded the ketone (**18**), mp 103—104 °C. Protection of the hydroxy group in **18** with dihydropyran and *p*-TsOH gave **19** in 83% yield from **1**. Reaction of **19** with lithium dicyclohexylamide and HMPA in THF, followed by addition of diphenyl disulfide afforded the sulfide (**20a**) and its regio-isomer (**20b**) in 61 and 29% yields, respectively, after purification by silica gel column chromatography. Selective formation of **20a** may be based on the selective deprotonation of the H_b proton in **19** through the remote steric effect of the *tert*-butyldiphenylsilyloxymethyl moiety. The structure of **20a** was confirmed by leading **20a** to **10a** through the following sequence of reactions. This completed the second route to **10a**. The Wittig–Horner reaction of **20a** with trimethyl phosphonoacetate yielded the sulfide (**21**) in 57% yield together with recovery of **20a** (13%). Deconjugation reaction of an *exo*-double bond to an *endo*-double bond occurred during this reaction. This was easily determined from the fact that the compound (**21**) showed no olefinic proton signal in the ^{1}H-NMR spectrum. Desulfurization of **21** with Raney nickel, followed by reduction of the product (**22**) with $LiAlH_4$ afforded the alcohol (**23**) in 90% yield from **21**. Benzoylation of **23** with benzoyl chloride in pyridine gave **24**, which was deprotected with tetrabutylam-

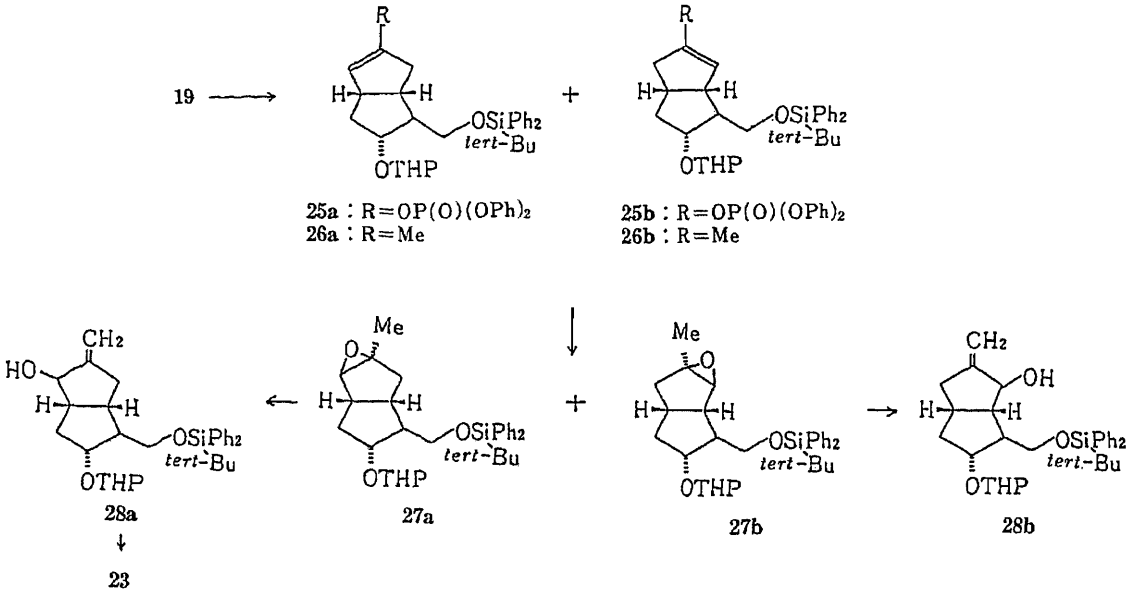


Chart 5

monium fluoride to afford the alcohol (**10a**) in 80% yield from **23**.

The third route started with the ketone (**19**) (Chart 5). Deprotonation of **19** with lithium dicyclohexylamide in THF, followed by the addition of diphenyl phosphorochloridate afforded the enol phosphate (**25a, b**) as an inseparable mixture of double bond isomers (*vide infra*). The reaction of **25a, b** with trimethylaluminum in the presence of tetrakis(triphenylphosphine)palladium[11] gave the olefin (**26a, b**) in 47% yield. This product showed two close peaks on HPLC, due probably to double bond isomers. Epoxidation of **26a, b** with *m*-chloroperbenzoic acid (MCPBA) in methylene chloride, followed by careful chromatographic purification yielded the epoxide (**27a** and **27b**, in 42 and 32% yields, respectively). The β-configurations of the epoxide ring in **27a** and **27b** were assigned on the basis of the steric effects due to the *cis*-bicyclo[3.3.0]octane structure including the α-tetrahydropyranyloxy group. The structure of **27a** was further confirmed by leading **27a** to the alcohol (**23**) as described below.

Isomerization of **27a** with diethylaluminum 2,2,6,6-tetramethylpiperidinylamide[12] afforded the allyl alcohol (**28a**) as a single isomer in 72% yield. Similarly, treatment of **27b** afforded exclusively the isomeric allyl alcohol (**28b**). Sequential treatment of **28a** with potassium hydride, tributyltiniodomethane and 15% *n*-butyllithium in THF in the presence of 18-crown-6[13] afforded the alcohol (**23**) in 24% yield along with recovery of **28a** (18%). The alcohol (**23**) was led to **10a** as described in route 2 (Chart 4).

Among the three routes described above, the first route was considered to be the most practical.

With the required intermediate in hand, we then converted the alcohol (**10a**) to 3-oxaisocarbacyclin (**34a**) through the following sequence of reactions. Oxidation of **10a** with pyridine sulfur trioxide (SO_3) complex and Et_3N in dimethyl sulfoxide (DMSO),[14] followed by reaction with an ylide, tributyl 2-oxoheptylidenephosphorane, gave the enone (**29**) in 97% yield from **10a**. Sodium borohydride reduction of **29** yielded the alcohol (**30a**) and its 15(*R*)-epimer (**30b**) in a ratio of 2 to 1. The 15(*S*)-configuration of **30a** was assigned on the basis of the circular dichroism (CD) spectrum[15] of the benzoate (**31**) derived from **30a**; the benzoate (**31**) exhibited a positive Cotton effect showing a positive chirality. This result showed the configuration at C_{15} in **30a** to be *S*. The hydroxy group in **30a** was protected with

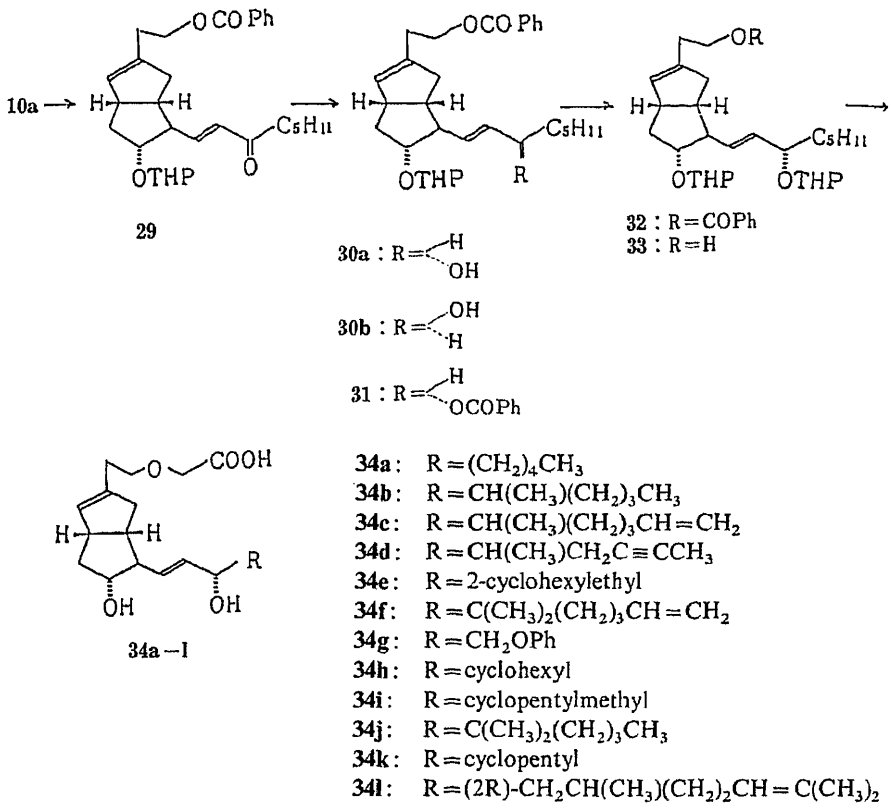


Chart 6

dihydropyran and *p*-TsOH to give **32**. Methanolysis of **32** with potassium carbonate in methanol afforded the alcohol (**33**) in 96% yield from **30a**. The alcohol (**33**) was led to **34a**, mp 62—64 °C, through the sequence of reactions described previously[4]: 1) *n*-butyllithium and lithium chloroacetate, 2) camphorsulfonic acid in aqueous acetone. Compound **34a** was identical (infrared (IR), ^{1}H-NMR and mass (MS) spectra) with *dl*-**34a** synthesized previously.[4]

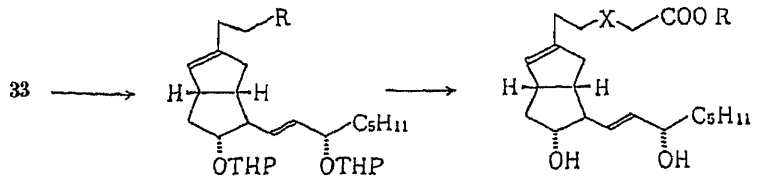
By using the same sequence of reactions as described for the synthesis of **34a**, the alcohol (**10a**) was led to various 3-oxaisocarbacyclins (**34b—l**) with a modified ω-side chain.

Synthesis of 3-Thia-, 3-Sulfinyl-, 3-Sulfonyl- and 3-Azaisocarbacyclins

The alcohol (**33**) was also converted to 3-heteroisocarbacyclins (**38**, **39**, **40** and **42**) through the following sequence of reactions (Chart 7).

Mesylation of **33** with mesyl chloride and Et_3N gave the mesylate (**35**) in 97% yield. Treatment of **35** with thioglycolic acid in DMSO in the presence of NaH, followed by esterification with diazomethane, afforded the ester (**36**). Deprotection of **36** with aqueous acetic acid yielded the diol (**37**) in 43% yield from **35**. The ester group in **37** was hydrolyzed with sodium hydroxide (NaOH) in aqueous methanol to give 3-thiaisocarbacyclin (**38**) in 95% yield. Oxidation of **37** with one molar equivalent of MCPBA, followed by hydrolysis of the ester group gave 3-sulfinylisocarbacyclin (**39**) in 93% yield from **37**. On the other hand, oxidation of **37** with two molar equivalents of MCPBA, followed by hydrolysis of the ester group afforded 3-sulfonylisocarbacyclin (**40**) in 84% yield from **37**.

Oxidation of **33** with trifluoroacetic anhydride and MDSO in dichloromethane,[16] followed by sequential treatment with methyl glycinate and sodium cyanoborohydride[17] afforded the amine (**41**). Hydroxy protecting groups in **41** were removed by treatment with aqueous acetic acid to give the methyl ester of 3-azaisocarbacyclin (**42**) in 29% yield from **33**.



35: R=OMs
36: R=SCH_2COOMe
41: R=$NHCH_2COOMe$
37: R=Me, X=S
38: R=H, X=S
39: R=H, X=SO
40: R=H, X=SO_2
42: R=Me, X=NH

Chart 7

TABLE I. Anti-platelet Aggregating Activity of 3-Hetero Analogues of Isocarbacyclin (IC_{50}: ng/ml)

Compd.	Rabbit	Human
34a	11	4.6
34b	2.4	6.1
34c	1.9	2.9
34d	2.4	0.42
34e	347	770
34f	56	128
34g	103	>1000
34h	3.7	1.4
34i	9.5	5.5
34j	87	697
34k	3.5	1.4
34l	6.3	6.2
38	132	52
39	>1000	>1000
40	>1000	>1000
42	>1000	>1000
Prostacyclin	5.2	0.9

Anti-platelet Aggregating Activity

Anti-platelet aggregating activities of the 3-heteroisocarbacyclins are shown in Table I. The introduction of an oxygen atom in place of the 3-methylene group slightly decreased the activity. Accordingly, 3-oxaisocarbacyclin (**34a**) was still quite a potent inhibitor of platelet aggregation. This decrease was fully compensated by a modification of the ω-side chain, and **34d** was found to be more potent than prostacyclin in human platelet-rich plasma (*in vitro*). On the other hand, 3-thiaisocarbacyclin (**38**) was a weak inhibitor and 3-sulfinyl-, 3-sulfonyl- and 3-azaisocarbacylins (**39**, **40**, **42**) were inactive.

Some of these compounds were tested for oral activity. An *ex vivo* experiment showed that **34b—d** were orally active in the rabbit. Furthermore the anti-platelet aggregating activity of **34d** lasted for more than five hours after oral administration of 0.3 mg/kg in the rabbit.[18] Further pharmacological investigation of **34d** is in progress. Details will be published elsewhere.

Experimental

Melting points are uncorrected. IR spectra were recorded with a JASCO A-102 spectrophotometer. ^{1}H-NMR spectra were recorded with a Varian T-60A (60 MHz) or EM-390 (90 MHz) spectrometer in deuteriochloroform, with tetramethylsilane as an internal reference. MS spectra were obtained with a JEOL JMS-01SG or JMS-G300 mass

spectrometer. Optical rotation was measured with a Perkin Elmer model 141 polarimeter. Ultraviolet (UV) spectra were taken with a Cary 118C spectrophotometer and CD spectra with a JASCO J-500C spectrophotometer. Removal of solvents *in vacuo* was accomplished with a rotating flash evaporator at 20—30 mmHg and usually at 35—50 °C. Plates for thin layer chromatography (TLC) were Silica gel 60 F-254 (E. Merck AG) and spots were visualized by spraying a solution of 0.5% vanillin in 20% ethanol in sulfuric acid (v/v), followed by heating. Columns for ordinary chromatography were prepared with Silica gel 60 (70—230 mesh or 230—400 mesh, E. Merck AG). In general, reactions were carried out under a nitrogen stream.

(1*R*,5*S*,6*S*,7*R*)-3-Oxo-6-hydroxymethyl-7-hydroxybicyclo[3.3.0]octane (2)——*p*-TsOH (1.0 g) was added to a solution of **1** (8.00 g), $[\alpha]_D^{24}$ −18.6° (*c*=1.0, $CHCl_3$), in a mixture of acetone (80 ml) and water (30 ml). The whole was heated at 40 °C for 2 h, then diluted with saturated $(NH_4)_2SO_4$, and extracted with AcOEt. The extract was dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a residue, which was purified by silica gel column chromatography. Elution with AcOEt to 2% MeOH in AcOEt (v/v) afforded **2** (4.03 g) as crystals. Recrystallization from AcOEt gave an analytical sample, mp 95—97 °C. *Anal.* Calcd for $C_9H_{14}O_3$: C, 65.31; H, 8.29. Found: C, 65.25; H, 8.21. IR (Nujol): 3200, 1733 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.13 (1H, q, *J*=4.5 Hz, –CHOH). MS *m/z*: 170 (M^+), 152, 134. $[\alpha]_D^{26}$ −11.9° (*c*=1.0, $CHCl_3$).

(1*R*,5*S*,6*S*,7*R*)-3-Oxo-6-(tetrahydropyran-2-yl)oxymethyl-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]octane (3)——A catalytic amount of *p*-TsOH was added to a mixture of **2** (4.03 g) in CH_2Cl_2 (15 ml) and dihydropyran (DHP) (5.4 ml) at room temperature. The whole was stirred for 30 min, quenched with dilute $NaHCO_3$, and extracted with AcOEt. The extract was washed with brine, and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a residue, which was purified by silica gel column chromatography. Elution with 10—15% AcOEt in hexane (v/v) afforded **3** (7.25 g) as a colorless oil. IR (neat): 1743, 1030 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.65 (2H, br s, OCHO×2). MS *m/z*: 338 (M^+), 254. $[\alpha]_D^{26}$ −19.5° (*c*=1.0, MeOH).

(1*S*,5*S*,6*S*,7*R*)-3-Methoxycarbonylmethylene-6-(tetrahydropyran-2-yl)oxymethyl-7-(tetrahydropyran-2-yl)-oxybicyclo[3.3.0]octane (4)——A solution of **3** (82.1 g) in THF (145 ml) was added at 20—30 °C to a solution of the sodium salt of trimethyl phosphonoacetate [prepared from 55% NaH in oil (14.3 g) and trimethylphosphonoacetate (66.3 g) in THF (675 ml) and dimethylformamide (DMF) (490 ml)]. After being stirred at 20—30 °C for 4 h, the reaction mixture was diluted with ice-water and extracted with AcOEt. The extract was washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a residue, which was purified by silica gel column chromatography. Elution with 10—15% AcOEt in hexane (v/v) afforded **4** (86.2 g) as a colorless oil. IR (neat): 1715, 1656, 1430, 1130, 1032 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.79 (3H, s, COOMe), 4.65 (2H, br s, OCHO×2), 5.80 (1H, br s, olefinic-H). MS *m/z*: 263 (M^+ −31), 310, 226.

A Mixture of (1*S*,5*S*,6*S*,7*R*)-3-Methoxycarbonylmethyl-6-(tetrahydropyran-2-yl)oxymethyl-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]oct-2-ene (5a) and (1*S*,5*R*,6*S*,7*R*)-3-Methoxycarbonylmethyl-6-(tetrahydropyran-2-yl)-oxymethyl-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]oct-3-ene (5b)——HMPA (38.9 ml) was added to a solution of lithium dicyclohexylamide [prepared from 15% *n*-butyllithium in hexane (118 ml) and dicyclohexylamine (40.2 ml) in THF (650 ml)]. A solution of **4** (50.0 g) in THF (150 ml) was added to the above solution at −73——65 °C. After being stirred at the same temperature for 20 min, the reaction mixture was quenched with saturated NH_4Cl, diluted with water and extracted with AcOEt. The extract was washed with brine, 3% HCl (filtration of the precipitate), and water, and dried over Na_2SO_4. Removal of the solvent afforded an oily residue, which was purified by silica gel chromatography. Elution with 15—25% AcOEt in hexane (v/v) gave **5a, b** (50.0 g) as a colorless oil. IR (neat): 1742, 1038 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.68 (3H, s, COOMe), 4.67 (2H, m, OCHO×2), 5.53 (1H, br s, olefinic-H). MS *m/z*: 263 (M^+ −31), 310, 226. The ratio of **5a** to **5b** (66 to 34) was determined by HPLC analysis of **6a, b** derived from **5a, b**; *p*-TsOH (100 mg) was added to a solution of **5a, b** (520 mg) in a mixture of MeOH (10 ml) and water (4 ml). The whole was stirred at 35 °C for 2 h, then diluted with saturated $(NH_4)_2SO_4$, and extracted with AcOEt. The extract was washed with saturated $(NH_4)_2SO_4$, and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a residue, which was purified by silica gel column chromatography. Elution with 30—90% AcOEt in hexane gave **6a, b** (371 mg). HPLC analysis showed that the ratio of **6a** to **6b** (**5a** to **5b**) was 66 to 34. HPLC conditions: column, ERC-silica-1161 (ERMA); solvent, 1% MeOH in a mixture of AcOEt : hexane=4 : 6 (v/v); flow rate, 2.5 ml/min; t_R 3.89 min (**6a**), 4.27 min (**6b**).

A Mixture of (1*S*,5*S*,6*S*,7*R*)-3-(2-Hydroxyethyl)-6-(tetrahydropyran-2-yl)oxymethyl-7-(tetrahydropyran-2-yl)-oxybicyclo[3.3.0]oct-2-ene (7a) and (1*S*,5*R*,6*S*,7*R*)-3-(2-Hydroxyethyl)-6-(tetrahydropyran-2-yl)oxymethyl-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]oct-3-ene (7b)——A solution of **5a, b** (50.9 g) in Et_2O (150 ml) was added to a suspension of $LiAlH_4$ (9.8 g) in Et_2O (870 ml) at 5—10 °C. The mixture was stirred at 5—10 °C for 0.5 h, then 4% NaOH solution (39.2 ml) was added dropwise under stirring. Stirring was continued for 2 h, then the precipitate was filtered off and the filtrate was evaporated to dryness. The residue obtained was purified by silica gel column chromatography. Elution with 20—35% AcOEt in hexane (v/v) afforded **7a, b** (41.1 g) as a colorless oil. IR (neat): 3450, 1030 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.65 (2H, br s, OCHO×2), 5.47 (1H, br s, olefinic-H). MS *m/z*: 366 (M^+), 348, 282.

A Mixture of (1*S*,5*S*,6*S*,7*R*)-3-(2-Benzoyloxyethyl)-6-(tetrahydropyran-2-yl)oxymethyl-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]oct-2-ene (8a) and (1*S*,5*R*,6*S*,7*R*)-3-(2-Benzoyloxyethyl)-6-(tetrahydropyran-2-yl)oxymethyl-

7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]oct-3-ene (8b)——Benzoyl chloride (19.1 ml) was added to a solution of **7a, b** (46.3 g) in pyridine (140 ml) at 20—30 °C, and the reaction mixture was allowed to stand for 15 min. The reaction mixture was quenched with ice-water, diluted with brine and extracted with AcOEt. The extract was washed with brine, 3% HCl, brine, dilute $NaHCO_3$ and then brine, and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a residue, which was purified by silica gel column chromatography. Elution with 5—15% AcOEt in hexane (v/v) gave **8a, b** (57.0 g) as a colorless oil. IR (neat): 1720, 1590, 1280, 1030, 1005 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.44 (2H, t, J= 6 Hz, $CH_2\underline{CH}_2O$), 4.64 (2H, br s, OCHO × 2), 5.47 (1H, br s, olefinic-H). MS *m/z*: 470 (M^+), 386, 302, 180.

(1*S*,5*S*,6*S*,7*R*)-3-(2-Benzoyloxyethyl)-6-hydroxymethyl-7-hydroxybicyclo[3.3.0]oct-2-ene (9a)——Water (100 ml) and *p*-TsOH (15.8 g) were added to a solution of **8a, b** (59.0 g) in MeOH (630 ml). The whole was stirred for 1.5 h, then diluted with water, and extracted with AcOEt. The extract was washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a crystalline residue (36.0 g). HPLC analysis showed that the ratio of **9a** to **9b** was 66 to 34. HPLC conditions: column, ERC-silica-1161 (ERMA); solvent, 1% MeOH in a mixture of AcOEt : hexane = 4 : 6 (v/v); flow rate, 2.4 ml/min; t_R 4.60 min (**9a**), 4.93 min (**9b**). Four recrystallizations from a mixture of CH_2Cl_2 and cyclohexane (1 : 4—5) gave pure **9a** (12.6 g), mp 87—88 °C. *Anal.* Calcd for $C_{18}H_{22}O_4$: C, 71.45; H, 7.28. Found: C, 71.51; H, 7.32. IR (KBr): 3220, 1715, 1280, 1120, 1080 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.43 (2H, t, J = 6 Hz, $CH_2\underline{CH}_2O$), 5.46 (1H, br s, olefinic-H), 7.48 (3H, m, arom.-H), 8.05 (2H, m, arom.-H). $[\alpha]_D^{24}$ −2.8° (c = 1.0, $CHCl_3$).

(1*S*,5*S*,6*S*,7*R*)-3-(2-Benzoyloxyethyl)-6-(hydroxymethyl)-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]octane (10a)——a) Synthesis from **9a**: Et_3N (1.75 ml) and then CCl_3COCl (0.78 ml) in benzene (20 ml) were added to a solution of **9a** (2.00 g) in benzene (40 ml) at 10—20 °C. After being stirred for 10 min, the reaction mixture was diluted with ice-water, and extracted with Et_2O. The extract was washed with water and dried over Na_2SO_4. Removal of the solvent gave a residue, which was purified by silica gel column chromatography. Elution with 20—35% AcOEt in hexane (v/v) gave the trichloroacetate (2.36 g) as a colorless oil. Dihydropyran (0.72 ml) and a catalytic amount of *p*-TsOH were added to a solution of the trichloroacetate (2.36 g) in CH_2Cl_2 (7.4 ml). Treatment as described for the synthesis of **19** afforded 2.72 g of product as an oil. A mixture of the crude product (2.72 g) in MeOH (55 ml) and saturated $NaHCO_3$ solution (3.8 ml) was stirred at 40—45 °C for 2 h. The reaction mixture was diluted with water, and extracted with AcOEt. The extract was washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a residue, which was purified by silica gel column chromatography. Elution with 20—40% AcOEt in hexane (v/v) gave **10a** (1.69 g) as a colorless oil. IR (neat): 3420, 2940, 1715, 1270 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.02 (1H, m, C_1-H), 4.45 (2H, t, J = 6 Hz, $CH_2\underline{CH}_2O$), 4.68 (1H, br s, OCHO), 5.46 (1H, br s, olefinic-H), 7.2—7.6 (3H, m, arom.-H), 7.8—8.1 (2H, m, arom.-H). MS *m/z*: 303 (M^+ − 57), 180, 162. $[\alpha]_D^{26}$ −28.5° (c = 1.0, MeOH).

b) Synthesis from **24**: A 1 M solution of Bu_4NF in THF (0.5 ml) was added to a solution of **24** (30 mg) in THF (1.0 ml), and the whole was stirred at room temperature for 7 h. The reaction mixture was poured into water and extracted with AcOEt. The extract was washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 18—25% AcOEt in hexane (v/v) afforded **10a** (18 mg) as a colorless oil.

A Mixture of (1*S*,5*S*,6*S*,7*R*)-3-[(*E*)-Methoxycarbonylmethylene]-6-hydroxymethyl-7-hydroxybicyclo[3.3.0]-octane (11a) and (1*S*,5*S*,6*S*,7*R*)-3-[(*Z*)-Methoxycarbonylmethylene]-6-hydroxymethyl-7-hydroxybicyclo[3.3.0]-octane (11b)——*p*-TsOH (2.0 g) was added to a solution of **4** (3.00 g) in a mixture of MeOH (50 ml) and water (8 ml). After being stirred at room temperature for 1 h, the reaction mixture was diluted with saturated $(NH_4)_2SO_4$, and extracted with AcOEt. The extract was washed with saturated $(NH_4)_2SO_4$ and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 50—90% AcOEt in hexane (v/v) afforded **11a, b** as a colorless oil (1.52 g). IR (neat): 3380, 1708, 1658, 1130 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.70 (3H, s, COOMe), 5.84 (1H, br s, olefinic-H). MS *m/z*: 226 (M^+), 208. HPLC analysis showed that the ratio of **11a** to **11b** was 51 to 49. HPLC conditions: column, ERC-ODS-1161 (ERMA); solvent, MeOH : H_2O = 1 : 1 (v/v); flow rate, 1.2 ml/min; t_R 3.05 min (**11a**), 3.50 min (**11b**).

A Mixture of (1*S*,5*S*,6*S*,7*R*)-3-[(*E*)-Methoxycarbonylmethylene]-6-trityloxymethyl-7-hydroxybicyclo[3.3.0] octane (12a) and (1*S*,5*S*,6*S*,7*R*)-3-[(*Z*)-Methoxycarbonylmethylene]-6-trityloxymethyl-7-hydroxybicyclo[3.3.0]-octane (12b)——A mixture of **11a, b** (1.81 g) in toluene (50 ml), trityl chloride (2.45 g) and Et_3N (1.29 ml) was heated under reflux for 0.5 h. The reaction mixture was cooled, stirred with dilute $NaHCO_3$ (10 ml), diluted with brine, and extracted with AcOEt. The extract was washed with dilute HCl (cooled), and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a residue, which was purified on a Lobar column [Merck, silica gel, size A, hexane : AcOEt = 2 : 1 (v/v)] to afford less polar **12b** (1.01 g) and more polar **12a** (0.82 g), both as oils. **12a**: IR (neat): 3550, 1710, 1660, 1500 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.68 (3H, s, COOMe), 5.80 (1H, br s, olefinic-H), 7.0—7.7 (15H, m, arom.-H). MS *m/z*: 468 (M^+), 450, 391. $[\alpha]_D^{24}$ +90.3° (c = 1.0, $CHCl_3$). **12b**: IR (neat): 3550, 1710, 1660, 1500 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.69 (3H, s, COOMe), 5.80 (1H, br s, olefinic-H), 7.0—7.7 (15H, m, arom.-H). MS *m/z*: 468 (M^+), 450, 391. $[\alpha]_D^{24}$ +36.3° (c = 1.0, $CHCl_3$).

(1*S*,5*S*,6*S*,7*R*)-3[(*Z*)-2-Hydroxyethylene]-6-[3(*S*)-(tetrahydropyran-2-yl)oxy-1(*E*)-octenyl]-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]octane (13)——a) Synthesis from **14**[5]: A solution of DIBAL in toluene (25.9 ml) was added at 0—5 °C to a solution of **14** (2.47 g) in toluene (62 ml) at 0—5 °C. After being stirred for 1.5 h, the reaction mixture was

quenched by addition of aqueous THF and stirred for 30 min. The precipitate was filtered off and the filtrate was evaporated to dryness. The residue was purified on a Lobar column [Merck, silica gel, size C, 35% AcOEt in hexane (v/v)] to give less polar **13** (940 mg) and the more polar *E*-isomer (930 mg), both as oils. TLC *Rf* 0.40 (**13**), 0.36 (*E*-isomer of **13**) [AcOEt : hexane = 1 : 2 (v/v)]. **13**: IR (neat): 3440, 1024 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, brt, Me), 4.71 (2H, brs, OCHO × 2), 5.2—5.8 (3H, m, olefinic-H). MS *m/z*: 360 ($M^+ - 102$), 342. $[\alpha]_D^{26}$ −13.4° (*c* = 1.2, MeOH). *E*-Isomer of **13**: IR (neat): 3440, 1024 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, brt, Me), 4.71 (2H, brs, OCHO × 2), 5.2—5.8 (3H, m, olefinic-H). MS *m/z*: 360 ($M^+ - 102$), 342. $[\alpha]_D^{26}$ −10.6° (*c* = 1.1, MeOH).

b) Synthesis from **12b**: Treatment of **12b** (490 mg) as described in the following procedures 1, 2 and 3 gave **13** (78 mg) as an oil; 1) deprotection with *p*-TsOH in aqueous methanol as described for the synthesis of **11a, b**, 2) conversion reaction as described for the synthesis of **32** from **9a**, and 3) DIBAL reduction as described for the synthesis of **13** from **14**.

(1*S*,5*S*,6*S*,7*R*)-3-[(*E*)-Methoxycarbonylmethylene]-6-trityloxymethyl-7-(tetrahydropyran-2-yl)oxybicyclo-[3.3.0]octane (15a)——DHP (0.22 ml) and a catalytic amount of *p*-TsOH were added to a solution of **12a** (733 mg) in CH_2Cl_2 (1.5 ml). Treatment as described for the synthesis of **3**, except for the use of 10—15% AcOEt in hexane (v/v) as the chromatographic eluent, afforded **15a** (895 mg) as a colorless oil. IR (neat): 1719, 1660, 1500 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.68 (3H, s, COOMe), 5.80 (1H, brs, olefinic-H), 7.0—7.7 (15H, m, arom.-H). MS *m/z*: 552 (M^+), 475, 309. $[\alpha]_D^{24}$ +25.3° (*c* = 1.0, $CHCl_3$).

(1*S*,5*S*,6*S*,7*R*)-3-[(*Z*)-Methoxycarbonylmethylene]-6-trityloxymethyl-7-(tetrahydropyran-2-yl)-oxybicyclo-[3.3.0]octane (15b)——Reaction and treatment of **12b** (723 mg) as described for the synthesis of **15a** yielded **15b** (770 mg) as a colorless oil. IR (neat): 1720, 1660, 1500 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.68 (3H, s, COOMe), 5.77 (1H, brs, olefinic-H), 7.0—7.7 (15H, m, arom.-H). MS *m/z*: 552 (M^+), 475, 309. $[\alpha]_D^{24}$ −20.4° (*c* = 1.0, $CHCl_3$).

(1*S*,5*S*,6*S*,7*R*)-3-Methoxycarbonylmethyl-6-trityloxymethyl-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]oct-2-ene (16a) and (1*S*,5*S*,6*S*,7*R*)-3-Methoxycarbonylmethyl-6-hydroxymethyl-7-hydroxybicyclo[3.3.0]oct-2-ene (6a)——Reaction and treatment of **15a** (200 mg) as described for the synthesis of **5a, b** afforded pure **16a** (190 mg) as a colorless oil. IR (neat): 1741, 1600, 1020 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.65 (3H, s, COOMe), 5.40 (1H, brs, olefinic-H), 7.0—7.6 (15H, m, arom.-H). MS *m/z*: 552 (M^+), 475, 309. *p*-TsOH (100 mg) was added to a solution of **16a** (190 mg) in MeOH (5 ml). The whole was stirred for 1 h, then diluted with saturated $(NH_4)_2SO_4$, and extracted with AcOEt. The extract was washed with saturated $(NH_4)_2SO_4$, and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a residue, which was purified by silica gel column chromatography. Elution with 30—90% AcOEt in hexane (v/v) afforded 57 mg of a crystalline solid. HPLC analysis showed that it was 99% homogeneous (**6a** : **6b** = 99 : 1). HPLC conditions: column, ERC-silica-1161 (ERMA); solvent, 1% MeOH in a mixture of AcOEt : hexane = 4 : 6 (v/v); flow rate, 2.5 ml/min; t_R 3.87 min (**6a**). Recrystallization from AcOEt–hexane mixture gave **6a** (32 mg), mp 96—99°C. *Anal.* Calcd for $C_{12}H_{18}O_4$: C, 63.66; H, 7.95. Found: C, 63.53; H, 8.04. IR (KBr): 3300, 1740, 1215, 1080 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.69 (3H, s, COOMe), 5.54 (1H, brs, olefinic-H). MS *m/z*: 226 (M^+), 208, 190. $[\alpha]_D^{24}$ −22.1° (*c* = 1.0, MeOH).

Deconjugation and Deprotection Reaction of 15b——Reaction and treatment of **15b** (237 mg) as described for the synthesis of **5a, b** afforded a mixture of **16a, b** (225 mg) as a colorless oil. IR (neat): 1741, 1600, 1020 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.65 (3H, s, COOMe), 5.60 (1H, brs, olefinic-H), 7.0—7.6 (15H, m, arom.-H). MS *m/z*: 552 (M^+), 475, 309. Acid treatment of **16a, b** as described in the synthesis of **6a** gave the diol products (**6a, b**). HPLC analysis showed that the ratio of **6a** to **6b** (**16a** to **16b**) was 53 : 47. The same HPLC conditions as described for the analysis of **6a** were used.

(1*R*,5*S*,6*S*,7*R*)-3,3-Ethylenedioxy-6-(tert-butyldiphenylsilyloxymethyl)-7-tetrahydropyran-2-yl)oxybicyclo[3.3.0]-octane (17)——A mixture of **1** (10.0 g), *tert*-butyldiphenylchlorosilane (13.8 g) and imidazole (3.42 g) in DMF (100 ml) was stirred at room temperature for 1 h. The reaction mixture was poured into water and extracted with AcOEt. The extract was washed with water and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 6—10% AcOEt in hexane (v/v) afforded **17** (17.8 g) as a colorless oil. IR (neat): 2940, 2860, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (9H, s, *tert*-Bu), 3.88 (4H, s, OCH_2CH_2O), 7.3—7.5 (6H, m, arom.-H), 7.6—7.8 (4H, m, arom.-H). MS *m/z*: 479 ($M^+ - 57$), 395, 283, 199. $[\alpha]_D^{26}$ −7.5° (*c* = 1.1, MeOH).

(1*R*,5*S*,6*S*,7*R*)-3-Oxo-6-(*tert*-butyldiphenylsilyloxymethyl)-7-hydroxybicyclo[3.3.0]octane (18)——A mixture of **17** (1.53 g), AcOH (6 ml), THF (4 ml) and water (6 ml) was stirred at 45—50°C for 7 h. The reaction mixture was poured into water and extracted with AcOEt. The extract was washed with 5% $NaHCO_3$ and brine, and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave **18** (0.982 g) as crystals. Recrystallization from AcOEt–hexane gave an analytical sample, mp 103—104°C. *Anal.* Calcd for $C_{25}H_{32}O_3Si$: C, 73.44; H, 7.83. Found: C, 73.33; H, 7.93. IR (KBr): 3520, 1730, 1105 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.09 (9H, s, *tert*-Bu), 4.0—4.4 (1H, m, –CHOH), 7.3—7.5 (6H, m, arom.-H), 7.6—7.9 (4H, m, arom.-H). MS *m/z*: 351 ($M^+ - 57$), 333. $[\alpha]_D^{26}$ −14.6° (*c* = 1.0, MeOH).

(1*R*,5*S*,6*S*,7*R*)-3-Oxo-6-(*tert*-butyldiphenylsilyloxymethyl)-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]octane (19)——A mixture of **18** (800 mg), DHP (0.27 ml) and a catalytic amount of *p*-TsOH in CH_2Cl_2 (16 ml) was stirred under ice-cooling for 30 min. The reaction mixture was diluted with AcOEt, washed with 5% $NaHCO_3$ and brine, and then dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 6—10% AcOEt in hexane (v/v) afforded **19** as a colorless oil (960 mg). IR (neat): 2940,

2860, 1740, 1150 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (9H, s, *tert*-Bu), 4.50 (1H, m, OCHO), 7.3—7.5 (6H, m, arom.-H), 7.6—7.8 (4H, m, arom.-H). MS *m/z*: 435 (M^+-57), 351, 333. $[\alpha]_D^{26}$ $-10.9°$ ($c=1.1$, MeOH).

(1*S*,5*R*,6*S*,7*R*)-2-Phenylthio-3-oxo-6-(*tert*-butyldiphenylsilyloxymethyl)-7-(tetrahydropyran-2-yl)oxybicyclo-[3.3.0]octane (20a) and (1*R*,5*S*,6*S*,7*R*)-3-Oxo-4-phenylthio-6-(*tert*-butyldiphenylsilyloxymethyl)-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]octane (20b)——A solution of **19** (340 mg) in THF (4 ml) was added to lithium dicyclohexylamide solution [prepared from dicyclohexylamine (0.34 ml) and 15% *n*-BuLi in hexane (1.0 ml) in THF (3 ml) and HMPA (0.5 ml)] at -78 °C. The mixture was stirred under the same conditions for 1 h, then a solution of $(PhS)_2$ (370 mg) in HMPA (4 ml) was added under ice-cooling, and the whole was stirred for 45 min. The reaction mixture was poured into water, and extracted with Et_2O. The extract was washed with water and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was chromatographed on a Lobar column [Merck, silica gel, size B, hexane : AcOEt = 7 : 2 (v/v)] to give the less polar sulfide (**20a**) (253 mg), and the more polar sulfide (**20b**) (120 mg), both as colorless oils. **20a**: IR (neat): 3080, 2940, 1740, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.07 (9H, s, *tert*-Bu), 4.4—4.7 (1H, m, OCHO), 7.1—7.8 (15H, m, arom.-H). MS *m/z*: 600 (M^+), 516, 459, 381. $[\alpha]_D^{26}$ $+10.9°$ ($c=1.0$ MeOH). **20b**: IR (neat): 3060, 2930, 1735, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.07 (9H, s, *tert*-Bu), 4.4—4.7 (1H, m OCHO), 7.1—7.8 (15H, m, arom.-H). MS *m/z*: 600 (M^+), 516, 459, 381. $[\alpha]_D^{26}$ $-2.1°$ ($c=1.0$, MeOH).

(1*S*,5*R*,6*S*,7*R*)-2-Phenylthio-3-(methoxycarbonylmethyl)-6-(*tert*-butyldiphenylsilyloxymethyl)-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]oct-2-ene (21)——Trimethyl phosphonoacetate (0.15 ml) was added to NaH [prepared from 55% NaH in oil (42 mg) by hexane washing] in a mixed solvent of THF (3 ml), DMF (3 ml) and HMPA (0.6 ml). The mixture was stirred for 30 min, then a solution of **20a** (120 mg) in THF (1 ml) was added, and the whole was stirred at room temperature for 3 h. The reaction mixture was poured into water and extracted with AcOEt. The extract was washed with water and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 4—5% AcOEt in hexane (v/v) afforded **21** (75 mg) as a colorless oil. Further elution with 6—8% AcOEt in hexane (v/v) gave recovered **20a** (15 mg). **21**: IR (neat): 2940, 1740, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.05 (9H, s, *tert*-Bu), 3.35 (2H, s, CH_2COOMe), 3.67 (3H, s, COOMe), 4.62 (1H, brs, OCHO), 7.2—7.8 (15H, m, arom.-H). MS *m/z*: 656 (M^+), 571, 515. $[\alpha]_D^{26}$ $-46.6°$ ($c=1.0$, MeOH).

(1*S*,5*S*,6*S*,7*R*)-3-(Methoxycarbonylmethyl)-6-(*tert*-butyldiphenylsilyloxymethyl)-7-(tetrahydropyran-2-yl)-oxybicyclo[3.3.0]oct-2-ene (22)——A mixture of **21** (64 mg) and Raney Ni [Kawaken Fine Chemical, suspended in EtOH] (0.2 ml) in EtOH (2 ml) was refluxed for 1 h, and then the Raney Ni was filtered off. Removal of the solvent of the filtrate *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 3—5% AcOEt in hexane (v/v) afforded **22** (52 mg) as a colorless oil. IR (neat): 2950, 1740, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.07 (9H, s, *tert*-Bu), 3.05 (2H, s, CH_2COOMe), 3.67 (3H, s, COOMe), 5.50 (1H, brs, olefinic-H), 7.2—7.9 (10H, m, arom.-H). MS *m/z*: 491 (M^+), 407, 283. $[\alpha]_D^{26}$ $-13.6°$ ($c=0.8$, MeOH).

(1*S*,5*S*,6*S*,7*R*)-3-(2-Hydroxyethyl)-6-(*tert*-butyldiphenylsilyloxymethyl)-7-(tetrahydropyran-2-yl)oxybicyclo-[3.3.0]oct-2-ene (23)——a) Synthesis from **22**: A solution of **22** (51 mg) in THF (2 ml) was added to a stirred suspension of $LiAlH_4$ (5 mg) in THF (2 ml) under ice-cooling, and the whole was stirred for 30 min. The mixture was poured into water and extracted with Et_2O. The extracts were washed with water and dried over Na_2SO_4. Removal of the solvent gave an oily residue, which was purified by silica gel column chromatography. Elution with 10—15% AcOEt in hexane (v/v) afforded **23** (45 mg) as a colorless oil. IR (neat): 3420, 2920, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (9H, s, *tert*-Bu), 4.62 (1H, brs, OCHO), 5.40 (1H, brs, olefinic-H), 7.3—7.5 (6H, m, arom.-H), 7.6—7.8 (4H, m, arom.-H). MS *m/z*: 463 (M^+-57), 379, 361. $[\alpha]_D^{26}$ $-15.9°$ ($c=1.1$, MeOH).

b) Synthesis from **28a**: A solution of **28a** (202 mg) in THF (4 ml) was added to KH [prepared from 35% KH dispersion in oil (82 mg) by hexane washing] in THF (6 ml) at room temperature. The mixture was stirred for 15 min, then 18-crown-6 (190 mg) followed by ICH_2SnBu_3 (0.2 ml) were added, and the whole was stirred for a further 3 h. The reaction mixture was cooled to -78 °C, and 15% *n*-BuLi in hexane (0.6 ml) was added. The whole was stirred at the same temperature for 20 min, and then allowed to stand at ambient temperature. The reaction mixture was poured into water and extracted with AcOEt. The extract was washed with water and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 7—8% AcOEt in hexane (v/v) afforded **23** (50 mg). Further elution with 12—15% AcOEt in hexane (v/v) gave recovered **28a** (36 mg).

(1*S*,5*S*,6*S*,7*R*)-3-(2-Benzoyloxyethyl)-6-(*tert*-butyldiphenylsilyloxymethyl)-7-(tetrahydropyran-2-yl)oxybicyclo-[3.3.0]oct-2-ene (24)——A mixture of **23** (32 mg) in pyridine (0.5 ml) and benzoyl chloride (0.05 ml) was stirred at room temperature for 1 h. The reaction mixture was poured into water and extracted with AcOEt. The extract was washed with 3% HCl and brine, and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 4—6% AcOEt in hexane (v/v) afforded **24** (32 mg) as a colorless oil. IR (neat): 2920, 1720, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.05 (9H, s, *tert*-Bu), 4.38 (2H, t, $J=7$ Hz, CH_2CH_2O), 4.65 (1H, brs, OCHO), 5.42 (1H, brs, olefinic-H), 7.2—8.2 (15H, m, arom.-H). MS *m/z*: 567 (M^+-57), 483, 405. $[\alpha]_D^{26}$ $+22.4°$ ($c=0.7$, MeOH).

A Mixture of (1*S*,5*S*,6*S*,7*R*)-3-Methyl-6-(*tert*-butyldiphenylsilyloxymethyl)-7-(tetrahydropyran-2-yl)oxybicyclo-[3.3.0]oct-2-ene (26a) and (1*S*,5*R*,6*S*,7*R*)-3-Methyl-6-(*tert*-butyldiphenylsilyloxymethyl)-7-(tetrahydropyran-2-yl)-oxybicyclo[3.3.0]oct-3-ene (26b)——A solution of **19** (10.0 g) in THF (100 ml) was added to lithium dicyclohexyl-

amide solution [prepared from dicyclohexylamine (9.5 ml) in THF (300 ml) and 15% *n*-BuLi in hexane (25 ml)] under ice-cooling. The mixture was stirred at the same temperature for 10 min, the $ClP(O)(OPh)_2$ (9.0 ml) was added, and the whole was stirred at room temperature for 20 min. The reaction mixture was poured into water and extracted with Et_2O. The extract was washed with brine and dried over Na_2SO_4. Removal of the solvent gave an oily residue, which was purified by silica gel chromatography. Elution with 50% Et_2O in hexane (v/v) afforded a mixture of **25a, b** (14.5 g) as an oil. IR (neat): 2940, 1490, 1190, 1160, 965 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (9H, s, *tert*-Bu), 5.42 (1H, br s, olefinic-H), 7.1—8.1 (20H, m, arom.-H). Next, 15% Me_3Al in hexane (60 ml) was added to a solution of the phosphate (14.5 g) and $Pd(PPh_3)_4$ (2.00 g) in $ClCH_2CH_2Cl$ (200 ml) at room temperature, and the whole was stirred for 3 h. The reaction was quenched by addition of water–saturated ether, and the precipitate was filtered off. Removal of the solvent of the filtrate *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 3—4% AcOEt in hexane (v/v) afforded a mixture of **26a, b** (4.68 g) as a colorless oil. HPLC analysis showed that this was a 63:37 mixture of **26a, b**. HPLC conditions: column, ERC-silica-1161 (ERMA); solvent, 1% AcOEt in hexane (v/v); flow rate, 1.4 ml/min; t_R 5.6 min (**26a**), 6.0 min (**26b**). **26a, b**: IR (neat): 2920, 1425, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (9H, s, *tert*-Bu), 1.67 (3H, s, Me), 4.70 (1H, br s, OCHO), 5.28 (1H, br s, olefinic-H), 7.3—7.5 (6H, m, arom.-H), 7.6—7.8 (4H, m, arom.-H). MS *m/z*: 433 ($M^+ - 57$), 404, 348.

(1*S*,2*R*,3*S*,5*R*,6*S*,7*R*)-2,3-Epoxy-3-methyl-6-(*tert*-butyldiphenylsilyloxymethyl)-7-(tetrahydropyran-2-yl)-oxybicyclo[3.3.0]octane (27a) and (1*R*,3*R*,4*S*,5*S*,6*S*,7*R*)-3,4-Epoxy-3-methyl-6-(*tert*-butyldiphenylsilyloxymethyl)-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]octane (27b)——MCPBA (85% purity, 150 mg) was added to a solution of a 3:2 mixture of **26a, b** (300 mg) in CH_2Cl_2 (6 ml) under ice-cooling, and the whole was stirred for 1 h. The reaction mixture was diluted with AcOEt, washed with 5% $NaHCO_3$ and brine, and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was chromatographed on a Lobar column [Merck, silica gel, size B, hexane : AcOEt = 4 : 1 (v/v)] to give the less polar epoxide (**27b**) (100 mg), and the more polar epoxide (**27a**) (130 mg), both as colorless oils. **27a**: IR (neat): 2940, 1430, 1115 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (9H, s, *tert*-Bu), 1.41 (3H, s, Me), 7.3—7.5 (6H, m, arom.-H), 7.6—7.8 (4H, m, arom. H). MS *m/z*: 449 ($M^+ - 57$), 421, 365. $[\alpha]_D^{26}$ −7.1° (*c* = 1.1, MeOH). **27b**: IR (neat): 2940, 1430, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (9H, s, *tert*-Bu), 1.42 (3H, s, Me), 7.3—7.5 (6H, m, arom.-H), 7.6—7.8 (4H, m, arom.-H). MS *m/z*: 449 ($M^+ - 57$), 421, 365. $[\alpha]_D^{26}$ −4.8° (*c* = 1.1, MeOH).

(1*S*,2*R*,5*R*,6*S*,7*R*)-2-Hydroxy-3-methylene-6-(*tert*-butyldiphenylsilyloxymethyl)-7-(tetrahydropyran-2-yl)-oxybicyclo[3.3.0]octane (28a)——A solution of **27a** (50 mg) in benzene (0.5 ml) was added to diethylaluminum 2,2,6,6-tetramethylpiperidinylamide [prepared[12] from 2,2,6,6-tetramethylpiperidine (0.11 ml) in benzene (3 ml), 15% *n*-BuLi in hexane (0.37 ml) and 15% diethylaluminum chloride in hexane (0.73 ml)] under ice-cooling, and the whole was stirred for 30 min. The reaction mixture was poured into water and extracted with Et_2O. The extract was washed with water and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 12—14% AcOEt in hexane (v/v) afforded **28a** (36 mg) as a colorless oil. IR (neat): 3400, 2930, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (9H, s, *tert*-Bu), 4.94 and 5.06 (each 1H, br s, olefinic-H), 7.3—7.5 (6H, m, arom.-H), 7.6—7.8 (4H, m, arom.-H). MS *m/z*: 449 ($M^+ - 57$), 365, 347. $[\alpha]_D^{26}$ −22.6° (*c* = 1.1, MeOH). For the large scale experiment, a 3:2 mixture of **26a, b** (3.60 g) was epoxidized with MCPBA to afford a mixture of **27a, b** (3.72 g), which was treated with diethylaluminum 2,2,6,6-tetramethylpiperidinylamide. The obtained product was purified by silica gel column chromatography. Elution with 10—14% AcOEt in hexane (v/v) afforded **28b** (1.25 g), and further elution with 16—20% AcOEt in hexane (v/v) afforded **28a** (1.56 g).

(1*S*,4*S*,5*S*,6*S*,7*R*)-4-Hydroxy-3-methylene-6-(*tert*-butyldiphenylsilyloxymethyl)-7-(tetrahydropyran-2-yl)-oxybicyclo[3.3.0]octane (28b)——Compound **27b** (48 mg) was converted into oily **28b** (32 mg) by the same procedure as used for the synthesis of **28a**. IR (neat): 3400, 2930, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (9H, s, *tert*-Bu), 4.90 and 5.02 (each 1H, br s, olefinic-H), 7.3—7.5 (6H, m, arom.-H), 7.6—7.8 (4H, m, arom.-H). MS *m/z*: 449 ($M^+ - 57$), 365, 347. $[\alpha]_D^{26}$ +10.6° (*c* = 1.1, MeOH).

(1*S*,5*S*,6*S*,7*R*)-3-(2-Benzoyloxyethyl)-6-[3-oxo-1(*E*)-octenyl]-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]oct-2-ene (29)——A solution of pyridine–SO_3 complex (6.00 g) in DMSO (20 ml) was added to a stirred mixture of **10a** (3.00 g) and Et_3N (16.2 ml) in DMSO (30 ml) at room temperature. After being stirred for 1 h, the reaction mixture was poured into ice-water and extracted with AcOEt. The extract was washed with water and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a practically pure aldehyde (3.10 g) as a pale yellow oil. The crude material was used for the subsequent step without purification. IR (neat): 2720, 1720, 1280 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.44 (2H, t, *J* = 7 Hz, $CH_2\underline{CH_2}O$), 4.64 (1H, br s, OCHO), 5.46 (1H, br s, olefinic-H), 7.3—7.7 (3H, m, arom.-H), 8.0—8.2 (2H, m, arom.-H), 9.80 (1H, d, *J* = 3 Hz, CHO). Tributyl 2-oxoheptylidenephosphorane (3.00 g) in ether (30 ml) was added to a solution of the aldehyde (3.00 g) obtained above in ether (60 ml), and the whole was stirred at room temperature for 3 h, then evaporated to dryness. The residue was purified by silica gel column chromatography. Elution with 8—9% AcOEt in hexane (v/v) afforded **29** (3.61 g) as a colorless oil. IR (neat): 1725, 1675, 1630, 1275 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.43 (2H, t, *J* = 7 Hz, $CH_2\underline{CH_2}O$), 4.63 (1H, br s, OCHO), 5.47 (1H, br s olefinic-H), 6.18 (1H, dd, *J* = 15, 3 Hz, olefinic-H), 6.7—7.1 (1H, m, olefinic-H), 7.3—7.7 (3H, m, arom.-H), 8.0—8.2 (2H, m, arom.-H). MS *m/z*: 398 ($M^+ - 84$), 378, 352, 232. $[\alpha]_D^{26}$ +16.8° (*c* = 1.1, MeOH).

(1*S*,5*S*,6*S*,7*R*)-3-(2-Benzoyloxyethyl)-6-[3(*S*)-hydroxy-1(*E*)-octenyl]-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]-oct-2-ene (30a) and (1*S*,5*S*,6*S*,7*R*)-3-(2-Benzoyloxyethyl)-6-[3(*R*)-hydroxy-1(*E*)-octenyl]-7-(tetrahydropyran-2-yl)-

oxybicyclo[3.3.0]oct-2-ene (30b)——$NaBH_4$ (425 mg) was added to a stirred solution of **29** (3.61 g) and $CeCl_3 \cdot 7H_2O$ (3.40 g) in methanol (60 ml) under ice-cooling. The mixture was stirred for 30 min, then excess reagent was decomposed by addition of AcOH, and the reaction mixture was diluted with brine and extracted with AcOEt. The extract was washed with water and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was chromatographed on a Lobar column [Merck, silica gel, size C, toluene : AcOEt = 3 : 2 (v/v)] to give the less polar 15(*R*)-alcohol (**30b**) (1.10 g) and the more polar 15(*S*)-alcohol (**30a**) (2.12 g), both as colorless oils. **30a**: IR (neat): 3430, 1725, 1275 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.43 (2H, t, J = 7 Hz, $CH_2\underline{CH}_2O$), 4.65 (1H, br s, OCHO), 5.45 (1H, br s, olefinic-H), 5.5—5.8 (2H, m, olefinic-H), 7.3—7.7 (3H, m, arom.-H), 8.0—8.2 (2H, m, arom.-H). MS *m/z*: 464 ($M^+ - 18$), 380, 336. $[\alpha]_D^{25}$ +4.3° (*c* = 1.0, MeOH). **30b**: IR (neat): 3440, 1725, 1275, 1115 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.43 (2H, t, J = 7 Hz, $CH_2\underline{CH}_2O$), 4.67 (1H, br s, OCHO), 5.45 (1H, br s, olefinic-H), 5.5—5.7 (2H, m, olefinic-H), 7.3—7.7 (3H, m, arom.-H), 8.0—8.2 (2H, m, arom.-H). MS *m/z*: 464 ($M^+ - 18$), 380, 336. $[\alpha]_D^{25}$ +2.0° (*c* = 1.0, MeOH).

(1*S*,5*S*,6*S*,7*R*)-3-(2-Benzoyloxyethyl)-6-[3(*S*)-benzoyloxy-1(*E*)-octenyl]-7-(tetrahydropyran-2-yl)oxybicyclo-[3.3.0]oct-2-ene (31)——Reaction and treatment of of **30a** (150 mg) as described for the synthesis of **8a, b**, except for the use of 4—10% AcOEt in hexane (v/v) as the chromatographic eluent, afforded **31** (181 mg) as a colorless oil. IR (neat): 2920, 1720, 1270 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.45 (2H, t, J = 7 Hz, $CH_2\underline{CH}_2O$), 5.3—5.9 (3H, br s, olefinic-H), 7.3—7.7 (6H, m, arom.-H), 7.9—8.1 (4H, m, arom.-H). MS *m/z*: 484 ($M^+ - 102$), 464, 380, 362. $[\alpha]_D^{26}$ −10.0° (*c* = 1.0, MeOH). UV λ_{max}^{MeOH} nm (ε): 228.3 (26600), 271.5 (1800). CD (*c* = 0.0039, MeOH) $[\theta]^{25}$ (nm): +14900 (226.5) (positive maximum).

(1*S*,5*S*,6*S*,7*R*)-3-(2-Benzoyloxyethyl)-6-[3(*S*)-(tetrahydropyran-2-yl)oxy-1(*E*)-octenyl]-7-(tetrahydropyran-2-yl)-oxybicyclo[3.3.0]oct-2-ene (32)——A mixture of **30a** (1.97 g), DHP (0.57 ml) and a catalytic amount of *p*-TsOH in CH_2Cl_2 (20 ml) was stirred under ice-cooling for 30 min. The reaction mixture was diluted with AcOEt, washed with 5% $NaHCO_3$ and brine, and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 6—9% AcOEt in hexane (v/v) afforded **32** (2.30 g) as a colorless oil. IR (neat): 2960, 1725, 1175 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.47 (2H, t, J = 7 Hz, $CH_2\underline{CH}_2O$), 4.72 (2H, br s, OCHO × 2), 5.45 (1H, br s, olefinic-H), 5.5—5.7 (2H, m, olefinic-H), 7.3—7.7 (3H, m, arom.-H), 8.0—8.2 (2H, m, arom.-H). MS *m/z*: 464 ($M^+ - 102$), 380, 338. $[\alpha]_D^{25}$ −15.1° (*c* = 1.1, MeOH).

(1*S*,5*S*,6*S*,7*R*)-3-(2-Hydroxyethyl)-6-[3(*S*)-(tetrahydropyran-2-yl)oxy-1(*E*)-octenyl]-7-(tetrahydropyran-2-yl)-oxybicyclo[3.3.0]oct-2-ene (33)——A mixture of **32** (2.29 g) and anhydrous K_2CO_3 (1.18 g) in methanol (50 ml) was stirred at 40—45 °C for 1 h. The reaction mixture was poured into water, and extracted with AcOEt. The extract was washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 14—18% AcOEt in hexane (v/v) afforded **33** (1.81 g) as a colorless oil. IR (neat): 3470, 1030 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.70 (2H, br s, OCHO × 2), 5.42 (1H, br s, olefinic-H), 5.5—5.7 (2H, m, olefinic-H). MS *m/z*: 360 ($M^+ - 102$), 316, 276. $[\alpha]_D^{25}$ −31.0° (*c* = 1.1, MeOH).

(+)-3-Oxaisocarbacyclin (34a)——The alcohol (**33**) (603 mg) was led to **34a** (260 mg), mp 62—64 °C, through the sequence of reactions described in the previous report.[4] *Anal.* Calcd for $C_{20}H_{35}O_5$: C, 68.15; H, 9.15. Found: C, 68.20; H, 9.30. IR (KBr): 3400, 1730, 972 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, br t, Me), 3.00 (1H, m, C_9-H), 3.67 (2H, t, J = 6 Hz, $CH_2\underline{CH}_2O$), 4.07 (2H, s, OCH_2COOH), 5.40—5.55 (3H, m, olefinic-H). $[\alpha]_D^{25}$ +6.2° (*c* = 1.0, MeOH).

Synthesis of 34b—l——Through a sequence of reactions similar to that described for the synthesis of **34a**, **10a** was led to **34b—l** using the corresponding phosphonates. In the case of **34g**, the Wittig–Horner reaction (step 2) was done by heating the THF solution at reflux for 1.5 h. Physical data are summarized in Table II.

(1*S*,5*S*,6*S*,7*R*)-3-(2-Mesyloxyethyl)-6-[3(*S*)-(tetrahydropyran-2-yl)oxy-1(*E*)-octenyl]-7-(tetrahydropyran-2-yl)-oxybicyclo[3.3.0]oct-2-ene (35)——Mesyl chloride (0.36 ml) was added to a solution of **33** (1.79 g) and Et_3N (0.81 ml) in CH_2Cl_2 (35 ml) under ice-cooling, and the whole was stirred for 30 min. The reaction mixture was poured into water and extracted with AcOEt. The extract was washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave **35** (2.03 g) as a colorless oil. IR (neat): 2950, 1360, 1180 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.98 (3H, s, $MeSO_3$), 4.70 (2H, br s, OCHO × 2), 5.40 (1H, br s, olefinic-H), 5.5—5.7 (2H, m, olefinic-H). MS *m/z*: 354 ($M^+ - 102 - 84$), 336, 310, 283, 258. $[\alpha]_D^{25}$ −27.5° (*c* = 1.1, MeOH).

(1*S*,5*S*,6*S*,7*R*)-3-(2-Methoxycarbonylmethylthioethyl)-6-[3-(*S*)-(tetrahydropyran-2-yl)oxy-1(*E*)-octenyl]-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]oct-2-ene (36)——Thioglycolic acid (0.6 ml) was added dropwise to a mixture of NaH [55% NaH in oil (600 mg) was washed with hexane] in DMSO (20 ml) at 18—20 °C, and the whole was stirred for 30 min. A solution of **35** (1.92 g) in DMSO (20 ml) was added, and the whole was stirred at room temperature for 2 h. The reaction mixture was poured into ice-water, acidified with 10% HCl and then extracted with AcOEt. The extract was washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was treated with excess CH_2N_2 in ether, and purified by silica gel column chromatography. Elution with 6—10% AcOEt in hexane (v/v) afforded **36** (1.03 g) as a colorless oil. IR (neat): 2950, 1740, 1020 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.73 (3H, s, COOMe), 4.68 (2H, br s, OCHO × 2), 5.32 (1H, br s, olefinic-H), 5.4—5.8 (2H, m, olefinic-H). MS *m/z*: 448 ($M^+ - 102$), 364, 320, 258. $[\alpha]_D^{25}$ −15.1° (*c* = 1.1, MeOH).

3-Thiaisocarbacyclin Methyl Ester (37)——A mixture of **36** (503 mg) in AcOH (5 ml) and water (2.5 ml) was stirred at room temperature overnight. The reaction mixture was poured into water and extracted with AcOEt. The

TABLE II. Physical and Spectral Data for **34b—1**

Compd.	IR (neat)	^{1}H-NMR ($CDCl_3$)	MS	$[\alpha]_D^{24}$ ($c=1.0$)
34b	3350, 1738	0.7—1.1 (6H, m), 3.68 (2H, t, $J=6$ Hz), 4.07 (2H, s), 5.42 (1H, brs), 5.55 (2H, m)	348 (M^+-18)	+5.2 (EtOH)
34c	3380, 1740, 1645	0.89 (3H, m), 3.66 (2H, t), 4.07 (2H, s), 4.7—5.2 (2H, m), 5.3—6.1 (4H, m)	360 (M^+-18)	+7.6 ($CHCl_3$)
34d	3380, 1734	1.78 (3H, t, $J=1$ Hz) 3.68 (2H, t), 4.09 (2H, s), 5.42 (1H, s), 5.56 (2H, m)	344 (M^+-18)	+20.7 (EtOH)
34e	3350, 1738	3.68 (2H, t), 4.09 (2H, s), 5.43 (1H, brs), 5.54 (2H, m)	374 (M^+-18)	−0.8 ($CHCl_3$)
34f	3400, 1735	0.7—1.1 (6H, m), 4.09 (2H, s), 4.8—5.2 (2H, m), 5.2—6.1 (4H, m)	374 (M^+-18)	+16.4 ($c=0.25$, EtOH)
34g	3350, 1740, 1600, 1590	3.65 (2H, t), 4.07 (2H, s), 5.52 (1H, brs), 5.72 (2H, m), 6.8—7.5 (5H, m)	384 (M^+-18)	−1.7 (EtOH)
34h[a]	(Nujol) 3550, 3450, 1741	3.68 (2H, t), 4.08 (2H, s), 5.40 (1H, brs), 5.52 (2H, m)	346 (M^+-18)	+6.5 ($CHCl_3$)
34i	3350, 1740	3.67 (2H, t), 4.08 (2H, s), 5.42 (1H, brs), 5.54 (2H, m)	346 (M^+-18)	+11.4 (EtOH)
34j	3400, 1738	0.7—1.1 (9H, m), 3.77 (2H, t), 4.08 (2H, s), 5.42 (1H, brs), 5.56 (2H, m)	362 (M^+-18)	+17.2 ($c=0.25$, EtOH)
34k[b]	(Nujol) 3410, 3350, 1705, 1660	3.67 (2H, t), 4.07 (2H, s), 5.40 (1H, brs), 5.52 (2H, m)	332 (M^+-18)	+4.2 ($CHCl_3$)
34l	3350, 1730	0.94 (2H, t), 4.07 (2H, s), 1.06 (3H, s), 1.69 (3H, s), 3.68 (2H, t), 4.08 (2H, s), 5.11 (1H, brt), 5.41 (1H, brs), 5.54 (2H, m)	388 (M^+-18)	−1.9 ($CHCl_3$)

a) mp 92—94 °C. *Anal.* Calcd for $C_{21}H_{32}O_5$: C, 69.20; H, 8.85. Found: C, 69.38; H, 8.78. *b*) mp 98—101 °C. *Anal.* Calcd for $C_{20}H_{30}O_5$: C, 68.54; H, 8.63. Found: C, 68.43; H, 8.59.

extract was washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 40—70% AcOEt in hexane (v/v) afforded **37** (287 mg) as a colorless oil. IR (neat): 3360, 2940, 1735, 1280 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.20 (2H, s, SCH_2COOMe), 3.73 (3H, s, COOMe), 5.2—5.6 (3H, m, olefinic-H). MS *m/z*: 364 (M^+-18), 346, 320, 258, 214. $[\alpha]_D^{25}$ +17.2° ($c=1.1$, MeOH).

3-Thiaisocarbacyclin (38)——A mixture of **37** (130 mg) and 5% NaOH (1.0 ml) in methanol (2 ml) was stirred under ice-cooling for 1 h. The reaction mixture was poured into water, acidified with 10% HCl, and then extracted with AcOEt. The extract was washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by acid-washed silica gel column chromatography. Elution with 70% AcOEt in hexane (v/v) to AcOEt afforded **38** (119 mg) as a colorless oil. IR (neat): 3350, 2950, 1710 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.20 (2H, s, SCH_2COOH), 5.07 (3H, s, OH×2 and COOH), 5.2—5.7 (3H, m, olefinic-H). MS *m/z*: 350 (M^+-18), 332, 306, 291, 258. HR-MS *m/z*: Calcd for $C_{20}H_{30}O_3S$ (M^+-H_2O): 350.1916. Found: 350.1924. $[\alpha]_D^{25}$ +17.0° ($c=1.1$, MeOH).

Methyl Ester of 3-Sulfinylisocarbacyclin (39)——MCPBA (85% purity, 85 mg) was added to a solution of **37** (151 mg) in methanol (10 ml) at −50 °C, and the whole was stirred under the same conditions for 1.5 h. The reaction

mixture was diluted with AcOEt, washed with 5% $NaHCO_3$ and brine, and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by acid-washed silica gel column chromatography. Elution with AcOEt to 10% methanol in AcOEt (v/v) afforded the methyl ester of **39** (149 mg) as a colorless oil. IR (neat): 3400, 2940, 1740, 1145 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.72 (2H, s, CH_2COOMe), 3.79 (3H, s, COOMe), 5.2—5.6 (3H, m, olefinic-H). MS *m/z*: 380 (M^+-18), 362, 345, 289, 214. $[\alpha]_D^{27}$ +21.1° (*c*=1.0, MeOH).

3-Sulfinylisocarbacyclin (39)——The methyl ester of **39** (127 mg) was hydrolyzed to **39** (120 mg) by the same procedure as used for the synthesis of **38**. IR (neat): 3350, 1720 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.74 (2H, s, CH_2COOH), 4.75 (3H, s, OH×2 and COOH), 5.2—5.6 (3H, m, olefinic-H). MS *m/z*: 304 (M^+-80), 292, 274, 248, 214. HR-MS *m/z*: Calcd for $C_{19}H_{28}OS$ ($M^+-CH_4O_4$): 304.1860. Found: 304.1840. $[\alpha]_D^{27}$ +20.4° (*c*=1.0, MeOH).

Methyl Ester of 3-Sulfonylisocarbacyclin (40)——MCPBA (85% purity, 115 mg) was added to a solution of **37** (102 mg) in methanol (10 ml) at −50 °C, and the whole was stirred under ice-cooling for 6 h. The reaction mixture was diluted with AcOEt, washed with 5% $NaHCO_3$ and brine, and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by acid-washed silica gel column chromatography. Elution with 50—70% AcOEt in hexane (v/v) afforded the methyl ester of **40** (59 mg) as a colorless oil. IR (neat): 3400, 2940, 1740, 1320 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.82 (3H, s, COOMe), 3.98 (2H, s, CH_2COOMe), 5.3—5.6 (3H, m, olefinic-H). MS *m/z*: 396 (M^+-18), 352, 325, 258. $[\alpha]_D^{27}$ +12.7° (*c*=1.0, MeOH). Further elution with AcOEt to 10% MeOH in AcOEt (v/v) gave **39** (40 mg).

3-Sulfonylisocarbacyclin (40)——The methyl ester of **40** (84 mg) was hydrolyzed to **40** (80 mg) by the same procedure as used for the synthesis of **38**. IR (neat): 3400, 1725, 1320 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.97 (2H, s, CH_2COOH), 4.47 (3H, s, OH×2 and COOH), 5.3—5.6 (3H, m, olefinic-H). MS *m/z*: : 338 (M^+-62), 320, 267. HR-MS *m/z*: Calcd for $C_{19}H_{30}O_3S$ ($M^+-CH_2O_3$): 338.1916. Found: 338.1904. $[\alpha]_D^{27}$ +13.2° (*c*=1.0, MeOH).

(1*S*,5*S*,6*S*,7*R*)-3-(2-Methoxycarbonylmethylazaethyl)-6-[3(*S*)-(tetrahydropyran-2-yl)oxy-1(*E*)-octenyl]-7-(tetrahydropyran-2-yl)oxybicyclo[3.3.0]oct-2-ene (41)——A solution of trifluoroacetic anhydride (0.35 ml) in CH_2Cl_2 (1 ml) was added to a solution of DMSO (0.27 ml) in CH_2Cl_2 (2 ml) at −78 °C. The mixture was stirred for 5 min, then a solution of **33** (580 mg) in CH_2Cl_2 (5 ml) was added at −78 °C, and the whole was stirred for 10 min. Et_3N (0.75 ml) was added and the reaction mixture was stirred at −70——30 °C for 30 min, then diluted with ice-water, and extracted with AcOEt. The extract was washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a residue, which was purified by silica gel column chromatography. Elution with 10—15% AcOEt in hexane (v/v) gave the aldehyde (336 mg) as a colorless oil. IR (neat): 2720, 1730 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, br t, Me), 4.67 (2H, br s, OCHO×2), 5.2—5.6 (3H, br s, olefinic-H), 9.66 (1H, t, *J*=2 Hz, CHO). MS *m/z*: 358 (M^+-102). A solution of the aldehyde (330 mg) obtained above in MeOH (1 ml) was added to a solution of methyl glycinate hydrogen chloride salt (540 mg) in MeOH (5 ml) at room temperature. The mixture was stirred for 10 min, then $NaBH_3(CN)$ (58 mg) was added. The whole was further stirred at room temperature for 30 min and stored at −20 °C for 48 h, then diluted with dilute $NaHCO_3$ and brine, and extracted with AcOEt. The extract was washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a residue, which was purified by silica gel column chromatography. Elution with 25—45% AcOEt in hexane (v/v) gave **41** (262 mg) as a colorless oil. IR (neat): 1748, 1025 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, br t, Me), 3.37 (2H, s, NCH_2COOMe), 3.69 (3H, s, COOMe), 4.66 (2H, br s, OCHO×2), 5.2—5.7 (3H, m, olefinic-H). MS *m/z*: 533 (M^+).

3-Azaisocarbacyclin Methyl Ester (42)——Camphorsulfonic acid (326 mg) was added to a solution of **41** (232 mg) in a mixture of acetone (23 ml) and water (10 ml). The whole was stirred at room temperature for 10 h, then heated at 35 °C for 1 h, diluted with dilute $NaHCO_3$, and extracted with AcOEt. The extract was washed with brine and dried over Na_2SO_4. Removal of the solvent gave a residue, which was purified by silica gel column chromatography. Elution with AcOEt gave **42** (117 mg) as a colorless oil. IR (neat): 3350, 1745, 973 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, br t, Me), 3.41 (2H, s, NCH_2COOMe), 3.73 (3H, s, COOMe), 5.42 (1H, br s, olefinic-H), 5.56 (2H, m, olefinic-H). MS *m/z*: 365 (M^+). HR-MS *m/z*: Calcd for $C_{21}H_{35}NO_4$ (M^+): 365.5919. Found: 365.5924.

Biological Test Method——All manipulations were carried out at room temperature. For the isolation of platelet-rich plasma (PRP) from human blood or rabbit blood, the following procedure was used. Blood was obtained from human volunteers and Japanese white rabbits, and was anticoagulated with 1/10 volume of 3.8% trisodium citrate. PRP was prepared by centrifugation of whole blood at 95×*g* for 15 min. Platelet-poor plasma (PPP) was prepared by centrifugation at 1000×*g* for 15 min. The number of platelets in the PRP was adjusted to $3.0\times10^5/\mu l$ (human) or $6.0\times10^5/\mu l$ (rabbit) by addition of an appropriate volume of PPP. Platelet aggregation was measured with human and rabbit platelets by using the method of Born.[19] Twenty-five microliters of a test compound was added to 250 μl of stirred PRP in the aggregometer. After 2 min, 25 μl of ADP, at a final concentration of 2—5 μM, was added and the platelet aggregation response was recorded. To evaluate control platelet aggregation, 25 μl of saline without any test compound was added to the PRP, and an identical procedure was performed. The IC_{50} value of each test compound was calculated as the concentration required to reduce the aggregation by 50% of the control value.

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References and Notes

1) *a*) B. Samuelsson, E. Granstrom, K. Green and M. Hamberg, *Ann. N. Y. Acad. Sci.*, **180**, 138 (1977); *b*) F. F. Sun and B. M. Taylor, *Prostaglandins*, **21**, 307 (1983).
2) G. Bundy, F. Lincoln, N. Nelson, J. Pike and W. Schneider, *Ann. N. Y. Acad. Sci.*, **180**, 76 (1977).
3) W. Skuballa, E. Schillinger, C-St. Sturzebecher and H. Vorbrüggen, *J. Med. Chem.*, **29**, 313 (1986).
4) K. Kojima, K. Koyama, S. Amemiya and S. Saito, *Chem. Pharm. Bull.*, **35**, 948 (1987).
5) K. Kojima, S. Amemiya, K. Koyama and K. Sakai, *Chem. Pharm. Bull.*, **33**, 2688 (1985).
6) K. Ueno, H. Suemune and K. Sakai, *Chem. Pharm. Bull.*, **32**, 3768 (1984).
7) S. Amemiya, K. Koyama, S. Saito and K. Kojima, *Chem. Pharm. Bull.*, **34**, 4403 (1986).
8) *a*) N. Petragnani and M. Yonashiro, *Synthesis*, **1982**, 521; *b*) F. L. Harris and L. Weiler, *Tetrahedron Lett.*, **25**, 1333 (1984); *c*) J. A. Katzenellenbogen and A. L. Crumrin, *J. Am. Chem. Soc.*, **98**, 4925 (1976); *d*) J. P. Gesson, J. C. Jacquesy and M. Mondon, *Tetrahedron Lett.*, **21**, 2509 (1980).
9) Final structural proof of **6a** was done by leading **6a** to **34a**.
10) E. J. Corey, H. Shirahama, H. Yamamoto, S. Terashima, A. Venkateswarlu and T. K. Schaaf, *J. Am. Chem. Soc.*, **93**, 1490 (1971).
11) *a*) K. Takai, K. Oshima and H. Nozaki, *Tetrahedron Lett.*, **21**, 2531 (1980); *b*) M. Sato, K. Takai, K. Oshima and H. Nozaki, *ibid.*, **22**, 1609 (1981).
12) A. Yasuda, S. Tanaka, K. Oshima, H. Yamamoto and H. Nozaki, *J. Am. Chem. Soc.*, **96**, 6513 (1974).
13) W. C. Still and A. Mitra, *J. Am. Chem. Soc.*, **100**, 1927 (1978).
14) J. R. Parikh and W. von E. Doering, *J. Am. Chem. Soc.*, **89**, 5505 (1967).
15) N. C. Gonnella and K. Nakanishi, *J. Am. Chem. Soc.*, **104**, 3775 (1982).
16) S. L. Huang, K. Omura and D. Swern, *J. Org. Chem.*, **41**, 3329 (1976).
17) R. F. Borsh, M. D. Bernstein and H. D. Durst, *J. Am. Chem. Soc.*, **93**, 2897 (1971).
18) K. Kojima, S. Amemiya, K. Koyama, S. Kobayashi and T. Oshima, Abstracts of Papers, 6th Int. Conf. on Prostaglandins and Related Compounds Florence, 1986, p. 272.
19) G. V. R. Born, *Nature* (London), **194**, 927 (1962).

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Chemical and Chemotaxonomical Studies of Filices. LXXI.[1] Chemical Studies on the Constituents of *Cheiropleuria bicuspis* (BL.) PR.

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Three new hopane-type triterpenes, **1**, **2** and **3**, were isolated from the fronds of *Cheiropleuria bicuspis* (BL.) PR. The structures were determined as 22,25-dihydroxyhopan-1-one, 1α,11α,22-trihydroxyhopane and 1α,11α,22,25-tetrahydroxyhopane, respectively, on the basis of spectral data, X-ray analysis and chemical correlations.

Keywords——*Cheiropleuria bicuspis*; Cheiropleuriaceae; fern; triterpene; 22,25-dihydroxyhopan-1-one; 1α,11α,22-trihydroxyhopane; 1α,11α,22,25-tetrahydroxyhopane; X-ray analysis

Hopane-type triterpenes without an oxygen atom at C-3 have been isolated from large numbers of ferns.[2] In a continuation of our studies, three new members of this group, **1**, **2** and **3**, were isolated from the fronds of *Cheiropleuria bicuspis* (BL.) PR. (Japanese name: suji-hitotsuba, Cheiropleuriaceae). In this paper, we report the structure elucidation of these compounds.

Compound **1**, $C_{30}H_{50}O_3$, colorless needles, mp 255—256 °C, $[\alpha]_D^{20}$ +67° (c=0.6, pyridine), showed infrared (IR) absorption bands due to a carbonyl group (ν_{max}^{KBr}: 1695 cm^{-1}) and hydroxyl groups (3400, 1165, 1045 cm^{-1}). In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** in C_5D_5N, proton signals due to a hydroxymethyl group were observed at δ 4.36 (1H, d, J=12 Hz) and 4.56 (1H, d, J=12 Hz), together with seven methyl signals at δ 0.91 (6H, s), 1.06, 1.13, 1.17, 1.37 and 1.41 (each 3H, s). Among these methyl signals, the last two are assignable to those of a hydroxyisopropyl group. Based on these spectral data, **1** was expected to be a hydroxyhopane-type (**4**) triterpene bearing a ketonic group and a primary hydroxyl group.

In the mass spectrum (MS) of **1**, characteristic fragment peaks of hydroxyhopane-type triterpenes were observed at m/z 207, 189 and 149 (Fig. 1).[3,4] The occurrence of these peaks not only supported the anticipated structure, but also indicated that the other oxygen functions are located on the A- and/or B-rings. Further, the appearance of the $[M-CH_2O]^+$ peak at m/z 428 indicated that the hydroxymethyl and ketonic groups are located in sufficiently close proximity to form a six-membered ring at the transition state of the cleavage (Fig. 1). Therefore, the position of the ketonic group is limited to C-1 or C-3 or C-7.

Compound **1** gave a monoacetate **5**, mp 204—205 °C, $[\alpha]_D^{20}$ +64° (c=1.0, $CHCl_3$), on treatment with acetic anhydride in pyridine. To confirm the structure, the carbon-13 nuclear magnetic resonance (^{13}C-NMR) data of **5** were compared with those of 22-hydroxyhopan-1-one (**6**).[4] As shown in Table I, almost all the chemical shifts of **5** are in good agreement with

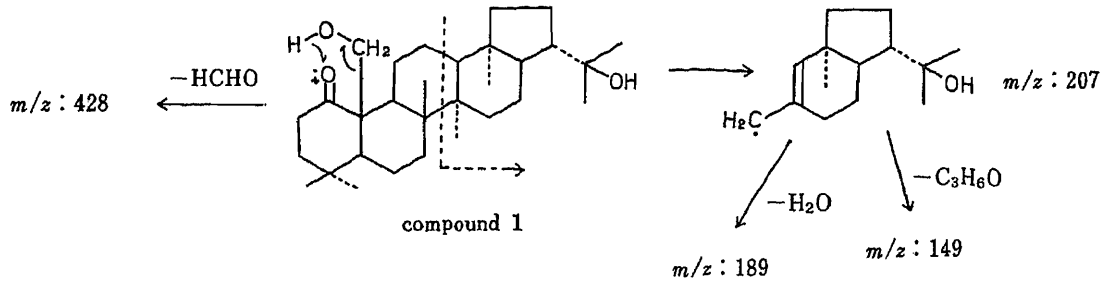
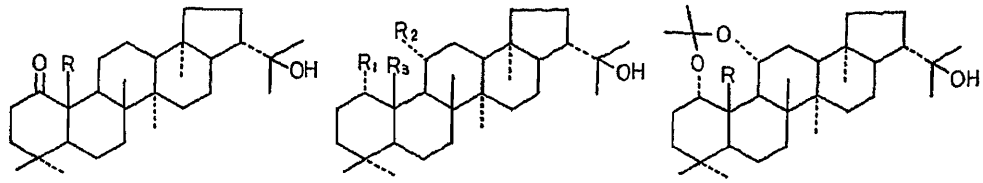


Fig. 1



1: R=CH_2OH
5: R=CH_2OAc
6: R=CH_3

2: $R_1=R_2=OH$, $R_3=CH_3$
3: $R_1=R_2=OH$, $R_3=CH_2OH$
4: $R_1=R_2=H$, $R_3=CH_3$

7: R=CH_3
8: R=CH_2OH
9: R=CHO

Fig. 2

TABLE I. ^{13}C Chemical Shifts

	2 C_5D_5N	3 C_5D_5N	4 C_5D_5N	5 $CDCl_3$	6[4] $CDCl_3$		2 C_5D_5N	3 C_5D_5N	4 C_5D_5N	5 $CDCl_3$	6[4] $CDCl_3$
C-1	73.0	71.5	40.7	213.4	217.4	C-17	54.7	54.7	54.8	54.0	54.1
C-2	25.8	26.3	19.1	36.9	35.5	C-18	44.2	44.2	44.5	44.1	44.2
C-3	35.5[a]	36.2[a]	42.4	41.9	43.0	C-19	41.5	41.6	41.9	41.2	41.3
C-4	33.8	33.4	33.6	33.2	33.3	C-20	27.0	27.0	27.0	26.6	26.6
C-5	48.3[b]	48.9[b]	56.5	56.9	57.4	C-21	51.4	51.5	51.7	51.1	51.2
C-6	19.0	19.0	19.1	20.7	19.3	C-22	72.4	72.4	72.6	73.9	73.9
C-7	35.5[a]	35.0[a]	33.6	32.5	32.7	C-23	33.9	34.1	33.8	32.4	32.0
C-8	42.3	42.9	42.4	42.5	42.3	C-24	21.7	22.8	21.8	23.2	22.6
C-9	47.4[b]	48.4[b]	50.9[a]	41.2	41.5	C-25	18.1	63.3	16.6	63.5	29.7
C-10	36.6	48.2	37.7	55.4	52.4	C-26	17.5[c]	18.5	17.1[b]	17.3	15.1
C-11	68.5	68.8	21.4	23.8	23.8	C-27	17.4[c]	17.7	17.4[b]	17.0	17.1
C-12	34.6[a]	35.0[a]	24.6	24.2	24.1	C-28	16.2	16.4	16.1	16.1	16.2
C-13	49.1[b]	49.0[b]	50.4[a]	50.1	50.5	C-29	29.9	29.8	29.9	28.7	28.7
C-14	43.3	43.5	40.7	41.9	41.9	C-30	31.4	31.4	31.4	30.9	30.8
C-15	34.6	34.6	34.9	34.4	34.5	$CH_3\underline{C}O$–				170.8	
C-16	22.3	22.3	22.5	21.9	22.0	$\underline{C}H_3CO$–				21.1	

a—*c*) Assignments with the same superscript for each compound may be interchanged.

those of **6** except for C-1, C-2, C-3, C-5, C-6, C-10, C-24, C-25 and C-26. These data indicated that **5** is 25-acetoxy-22-hydroxyhopan-1-one.

To confirm the structure deduced from the spectral data, X-ray analysis of **5** was carried out. The crystal data and the course of the X-ray structure determination are summarized in Table II.[5] The relative molecular structure of **5** is illustrated in Fig. 3, which is consistent with the above-mentioned structure. The possibility of the antipodal structure was excluded by the circular dichroism (CD) spectrum. Compound **5** showed the same negative Cotton effect ($[\theta]^{20}_{292}$ −2140°) as compound **6** ($[\theta]^{20}_{295}$ −707°)[6] indicating that both compounds have the

TABLE II. X-Ray Structure Determination

Compound name	Compound 5	Compound 2
Formula	$C_{32}H_{52}O_4$	$C_{30}H_{52}O_3$
Molecular weight	500.8	460.7
Crystallizing solutions	Ethanol	Pyridine
Habit	Prisms elongated along *c*	Plates flattened on 010
Size of X-ray specimen (mm)	$0.30\times0.35\times0.40$	$0.12\times0.28\times0.52$
Radiation	Graphite-monochromated CuK_α	
2θ range (°)	6° through 156°	6° through 156°
No. of reflections		
Possible	3391	3307
Observed as above 2σ (I)	3221	2656
Sym. equivalent (*R* fac.)	148 (0.032)	—
Crystal system	Monoclinic	Orthorhombic
Space group	$P2_1$	$P2_12_12_1$
Cell dimensions		
a (Å)	17.851 (10)	12.903 (7)
b (Å)	7.583 (5)	31.098 (18)
c (Å)	10.704 (6)	6.645 (6)
β (°)	104.34 (5)	
U (Å^3)	1404	2666
Z	2	4
D_{cal} (g cm^{-3})	1.185	1.148
μ for CuK_α (cm^{-1})	5.6	5.2
Structure determination	MULTAN	MULTAN
No. of heavier atoms (anisotropic)	36	33
No. of hydrogen atoms (isotropic)	52	52
Final *R* factor	0.065	0.098
for No. of reflections	3073	2656

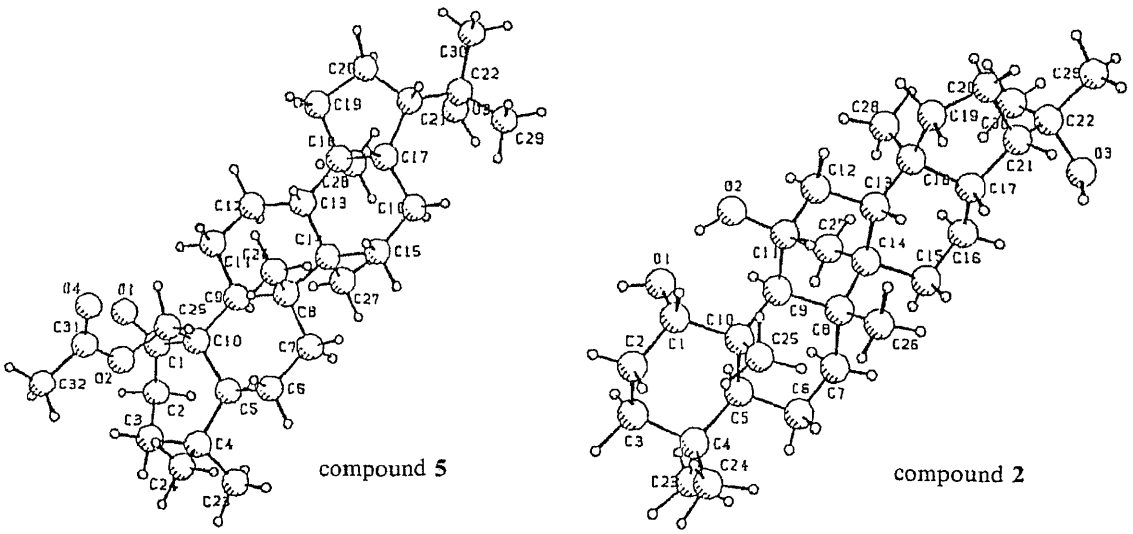
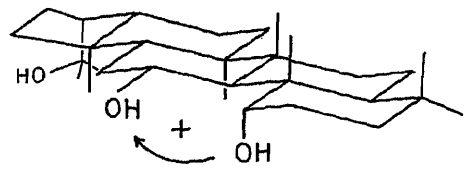


Fig. 3[7]

same skeleton. Thus, the structure of **1** was established as 22,25-dihydroxyhopan-1-one.

Compound **2**, $C_{30}H_{52}O_3$, colorless plates, mp 280—281 °C, $[\alpha]_D^{20}$ +52° (c=0.6, pyridine), showed IR absorption bands due to hydroxyl groups at 3400, 1070 and 1005 cm^{-1}. In the MS



compound 2

Fig. 4

of **2**, three dehydration peaks at *m/z* 442, 424 and 406 and the same peaks as in the case of **1** at *m/z* 207, 189 and 149 were observed, indicating the structure of **2** to be a hydroxyhopane-type compound bearing two more hydroxyl groups among the A-, B- and C-rings. In the ^{1}H-NMR spectrum of **2** (in C_5D_5N), two hydroxy-bearing methine proton signals were observed at δ4.28 (1H, dt, J=6 and 10 Hz) and 4.53 (1H, br s). The coupling pattern of the former signal indicates that the proton is an axial one and couples to two axial protons and one equatorial proton on the neighboring carbons. Therefore, one of the secondary hydroxyl groups was considered to be at C-6α or C-11α or C-12β. As compound **2** formed an acetonide **7** on treatment with acetone and sulfuric acid, the only possible positions for the two hydroxyl groups were deduced to be at C-11α and C-1α.[8] A comparison of the ^{13}C-NMR data of **2** with those of hydroxyhopane (**4**) also suggested the structure of **2** to be 1α,11α,22-trihydroxyhopane (Table I).

The final confirmation of the structure of **2** was achieved by X-ray analysis (see Table II). The relative molecular structure of **2** is illustrated in Fig. 3, which is consistent with the suggested structure. Compound **2** showed a positive maximum at 293 nm and a negative maximum at 305 nm in the CD spectrum using nickel(II) acetylacetonate.[9] Though the method has not been established well for cyclic glycol systems, a right-handed screw rotation of the glycol system of **2** may be deduced from the result (Fig. 4). Considering that compound **1** co-exists in this fern, the result seems to reflect the true configuration of **2**. Thus, the structure of **2** was established as 1α,11α,22-trihydroxyhopane.

Compound **3**, $C_{30}H_{52}O_4$, colorless needles, mp 227—228 °C, $[\alpha]_D^{20}$ +48° (c=1.0, pyridine), showed a ^{1}H-NMR spectrum similar to that of **2** except that one of the methyl signals of **2** was replaced by the hydroxymethyl signals at δ4.32 and 4.50 (each 1H, d, J= 11 Hz) indicating that **3** is a tetraol having a similar structure to **2**. As the characteristic peaks of a hydroxyhopane-type triterpene at *m/z* 207, 189 and 149 were observed in the MS of **3**, the hydroxymethyl group was considered to be on the A- or B-ring. In the ^{13}C-NMR spectrum of **3** (Table I), almost all the chemical shifts are in good agreement with those of **2** except around C-25. The differences of the data indicate that **3** is the 25-hydroxyl derivative of **2**.

Confirmation of the structure of **3** was achieved by chemical correlation as follows. Compound **3** was converted into an acetonide **8** by treatment with acetone and sulfuric acid. Oxidation of **8** with CrO_3 in pyridine yielded an aldehyde **9**. Huang–Minlon reduction of **9** gave **7**. Compound **7** was shown to be identical with the acetonide of **2** by direct comparison. Thus, **3** was concluded to be 1α,11α,22,25-tetrahydroxyhopane.

Experimental

The instruments, materials and experimental conditions were the same as described in Part LXI[10] of this series.

Isolation Procedure——The air-dried fronds (2 kg) of *Cheiropleuria bicuspis* (BL.) PR., collected in December at Tashulinsan, Ping Tung county, Taiwan, China, were extracted twice with 10 l of MeOH under reflux for 6 h. The combined extracts (20 l) and then 10 l of MeOH were passed over activated charcoal (200 g) packed in a column of 7 cm diameter. The resulting solution was concentrated to a syrup under reduced pressure. The syrup was chromatographed on silica gel (120 g) using $CHCl_3$ and ether as eluents to yield **3** (120 mg) and a mixture of **1** and **2**. The mixture was rechromatographed on silica gel to yield **1** (1.3 g) and **2** (230 mg).

Compound 1 [22,25-Dihydroxyhopan-1-one]——Colorless needles from a mixture of EtOH and $CHCl_3$, mp 255—256 °C, $[\alpha]_D^{20}$ +67° (c=0.6, C_5H_5N). IR ν_{max}^{KBr} cm^{-1}: 3400, 2940, 2870, 1695, 1165, 1045. ^{1}H-NMR (100 MHz, in C_5D_5N) δ: 0.91 (6H, s), 1.06 (3H, s), 1.13 (3H, s), 1.17 (3H, s), 1.37 (3H, s), 1.41 (3H, s), 4.36 (1H, d, J=12 Hz), 4.56 (1H, d, J=12 Hz). MS m/z: 458 (M^+), 440 (M^+-H_2O), 428 (M^+-CH_2O), 410 (428−H_2O), 369 (428−C_3H_7O), 207, 189, 149. Calcd for $C_{30}H_{50}O_3$: 458.3760 (M). Found: 458.3755 (M^+).

Compound 2 [1α,11α,22-Trihydroxyhopane]——Colorless plates from pyridine, mp 280—281 °C, $[\alpha]_D^{20}$ +52° (c=0.6, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3400, 2940, 2870, 1070, 1005. ^{1}H-NMR (100 MHz, in C_5D_5N) δ: 0.92 (3H, s), 0.97 (3H, s), 0.99 (3H, s), 1.11 (3H, s), 1.14 (3H, s), 1.27 (3H, s), 1.37 (3H, s), 1.42 (3H, s), 4.28 (1H, dt, J=6, 10 Hz), 4.53 (1H, brs). MS m/z: 460 (M^+), 442 (M^+-H_2O), 424 (442−H_2O), 406 (424−H_2O), 383 (442−C_3H_7O), 207, 189, 149. Calcd for $C_{30}H_{52}O_3$: 460.3917 (M). Found: 460.3920 (M^+).

Compound 3 [1α,11α,22,25-Tetrahydroxyhopane]——Colorless needles from a mixture of EtOH and $CHCl_3$, mp 227—228 °C, $[\alpha]_D^{20}$ +48° (c=1.0, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3370, 2940, 2870, 1065, 1045, 1030. ^{1}H-NMR (100 MHz, in C_5D_5N) δ: 0.91 (3H, s), 0.96 (3H, s), 1.00 (3H, s), 1.15 (3H, s), 1.36 (3H, s), 1.38 (3H, s), 1.41 (3H, s), 4.32 (1H, d, J=11 Hz), 4.50 (1H, d, J=11 Hz), 5.06 (1H, m), 5.24 (1H, m). MS m/z: 476 (M^+), 458 (M^+-H_2O), 440 (458−H_2O), 422 (440−H_2O), 381 (440−C_3H_7O), 207, 189, 149. Calcd for $C_{30}H_{52}O_4$: 476.3866 (M). Found: 476.3867 (M^+).

Monoacetate of 1 (5)——A mixture of **1** (50 mg), pyridine (10 ml) and Ac_2O (5 ml) was stirred at room temperature for 15 h. The reaction mixture was poured into ice-water and extracted with EtOAc. The extract was washed with 5% HCl solution, 5% Na_2CO_3 solution and water, then dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was crystallized from EtOH to yield 45 mg of **5**, colorless needles, mp 204—205 °C, $[\alpha]_D^{20}$ +64° (c=1.0, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3550, 2940, 2870, 1735, 1695, 1240, 1035. ^{1}H-NMR (100 MHz, in C_5D_5N) δ: 0.87 (3H, s), 0.91 (3H, s), 1.01 (3H, s), 1.05 (3H, s), 1.10 (3H, s), 1.35 (3H, s), 1.41 (3H, s), 1.98 (3H, s), 4.85 (2H, brs). MS m/z: 500 (M^+), 482 (M^+-H_2O), 440 (482−CH_3CO).

Acetonide of 2 (7)——**2** (20 mg) was suspended in anhydrous acetone (30 ml) containing one drop of concentrated H_2SO_4. The mixture was stirred for 1.5 h at room temperature. The resulting solution was poured into ice-water, and the products were extracted with ether. The extract was washed with water, dried over anhydrous Na_2SO_4 and concentrated. The residue was chromatographed on silica gel using $CHCl_3$ as an eluent to yield 8 mg of **7**, colorless syrup, $[\alpha]_D^{20}$ +57° (c=0.4, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3500, 2935, 2860, 1455, 1380, 1365, 1065. ^{1}H-NMR (60 MHz, in $CDCl_3$) δ: 0.81 (3H, s), 0.84 (3H, s), 0.91 (3H, s), 0.93 (3H, s), 1.06 (3H, s), 1.07 (3H, s), 1.19 (3H, s), 1.21 (3H, s), 1.26 (3H, s), 3.6—4.1 (2H, m). MS m/z: 500 (M^+), 485 (M^+-CH_3), 467 (485−H_2O).

Acetonide of 3 (8)——**3** was converted to its acetonide **8** in the same way as **2**. Colorless syrup, $[\alpha]_D^{20}$ +60° (c=0.4, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3380, 2940, 2870, 1465, 1380, 1370, 1080. ^{1}H-NMR (60 MHz, in $CDCl_3$) δ: 0.78 (3H, s), 0.82 (3H, s), 0.91 (3H, s), 1.08 (3H, s), 1.16 (3H, s), 1.19 (3H, s), 1.21 (3H, s), 1.39 (3H, s), 3.72 (1H, d, J=12 Hz), 3.9—4.5 (3H). MS m/z: 501 (M^+-CH_3), 498 (M^+-H_2O), 458 ($M^+-C_3H_6O$), 440 (458−H_2O).

Oxidation of 8——**8** (20 mg) was added to a solution of CrO_3 (100 mg) in pyridine (1 ml). The mixture was allowed to stand at room temperature for 1 h and poured into ice-water. The products were extracted with ether. The extract was washed with 5% HCl solution and water, dried over anhdrous Na_2SO_4 and concentrated. The residue was crystallized from acetone to yield 13 mg of **9**, colorless needles, mp 261—262 °C, $[\alpha]_D^{20}$ +76° (c=0.3, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3350, 2950, 2870, 1700, 1385, 1375, 1080, 1045. ^{1}H-NMR (60 MHz, in $CDCl_3$) δ: 0.77 (3H, s), 0.80 (3H, s), 0.84 (3H, s), 0.97 (3H, s), 1.09 (3H, s), 1.18 (3H, s), 1.20 (3H, s), 1.26 (3H, s), 1.29 (3H, s), 4.02 (1H, m), 4.68 (1H, t, J=4 Hz), 10.27 (1H, s). MS m/z: 499 (M^+-CH_3), 456 ($M^+-C_3H_6O$), 438 (456−H_2O).

Huang-Minlon Reduction of 9——A mixture of **9** (10 mg), 100% hydrazine hydrate (0.2 ml), KOH (120 mg), *n*-BuOH (2 ml) and diethylene glycol (2 ml) was refluxed for 2 h. The water, *n*-BuOH and excess hydrazine were removed by distillation, and the temperature of the solution was allowed to rise to 200 °C, when refluxing was continued for 4 h. The cooled solution was diluted with water and extracted with ether. The extract was washed with water, dried over anhydrous Na_2SO_4 and concentrated. The residue was chromatographed on silica gel using $CHCl_3$ as an eluent to yield 3 mg of **7**. This product was found to be identical with an authentic sample on direct comparison.

References and Notes

1) Part LXX: N. Tanaka, T. Noguchi, K. Kawashima, K. Kurihara, T. Matsudo, T. Murakami, Y. Saiki, and C.-M. Chen, *Yakugaku Zasshi*, **107**, 586 (1987).
2) Recent reports; H. Ageta and Y. Arai, *Phytochemistry*, **22**, 1801 (1983); H. Ageta, K. Shiojima, and K. Masuda, *Chem. Pharm. Bull.*, **30**, 2272 (1982).
3) J. A. Elix, A. A. Whitton, and A. J. Jones, *Aust. J. Chem.*, **35**, 641 (1982).
4) S. Huneck, A. Preiss, J. Schmidt, and A. M. Mendez, *Phytochemistry*, **22**, 2027 (1983).
5) Lists of F_O and F_C, anisotropic temperature factors, bond lengths and bond angles may be obtained from one of the authors (Y. Iitaka) upon request. Atomic coordinates will be deposited with the Cambridge Crystallographic Database.
6) The authors are grateful to Dr. S. Huneck, Research Center for Molecularbiology and Medicine of the Academy of Sciences of the GDR, for the generous gift of a sample of 22-hydroxyhopan-1-one.

7) The hydrogen bonds in the crystal structure were found to be as follows;
Compound **5**: From O3 to O4 of the molecule at x, $1+y$, $1+z$ 3.032(8) Å with HO3···O4 2.07(7) Å.
Compound **2**: From O2 to O1 of the same molecule 2.859(8) Å with HO2···O1 1.90(10) Å [intramolecular hydrogen bond]. From O1 to O3 of the molecule at $-1/2+x$, $1/2-y$, $1-z$ 2.839(8) Å with HO1···O3 2.10(10) Å.
It is interesting that the hydroxyisopropyl groups take different conformations depending on the intermolecular hydrogen bonds. The authors wish to thank Prof. H. Ageta, Showa College of Pharmaceutical Sciences, for a valuable discussion of these conformational changes.
8) As the proton at C-1 showed a broad singlet signal, $W_{1/2}=6$ Hz, in the ^{1}H-NMR spectrum, the hydroxy group was considered to be axial.
9) J. Dillon and K. Nakanishi, *J. Am. Chem. Soc.*, **96**, 4059 (1974).
10) N. Tanaka, T. Murakami, H. Wada, A. B. Gutierez, Y. Saiki, and C.-M. Chen, *Chem. Pharm. Bull.*, **33**, 5231 (1985).

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Characterization of Directly Acting Mutagens Produced from *N*-Nitroso-*N*-(formylmethyl)alkylamines: Their Possible Involvement in the Carcinogenesis of *N*-Nitrosamines Having a 2-Hydroxyethyl Group

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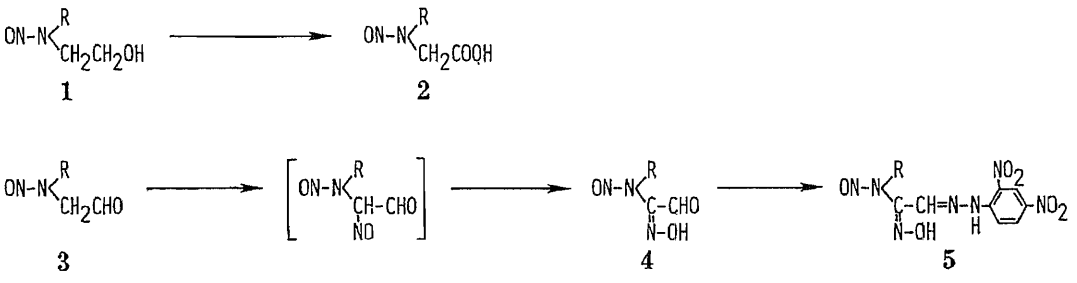
Directly acting mutagens formed from *N*-nitroso-*N*-(formylmethyl)alkylamines (**3**) were isolated and identified as *N*-nitroso-*N*-alkyl-1-hydroxyimino-2-oxoethylamines (**4**). Their structures were elucidated on the basis of nuclear magnetic resonance spectra and confirmed by derivatization to the crystalline 2,4-dinitrophenylhydrazones (**5**). Compounds **4** (alkyl=ethyl and butyl) were strongly mutagenic to *Salmonella typhimurium* TA1535 and *Escherichia coli* WP2 *hcr*⁻ without metabolic activation, while **4** with a *tert*-butyl group was not mutagenic. The formation of **4** is considered to proceed by the nitrosation of **3**, indicating a possible involvement of the formylmethyl metabolite in carcinogenesis by *N*-nitrosamines with a 2-hydroxyethyl group.

Keywords——*N*-nitroso-*N*-(2-hydroxyethyl)alkylamine; *N*-nitroso-*N*-(formylmethyl)alkylamine; *N*-nitroso-*N*-alkyl-1-hydroxyimino-2-oxoethylamine; *N*-nitroso-*N*-(oxoalkyl)alkylamine; directly acting mutagen; β-hydroxylation activation; nitrosamine activation; mutagenicity

N-Nitroso-*N*-(2-hydroxyethyl)alkylamines (**1**) (alkyl=ethyl and butyl) are potent carcinogens which induce tumors of the liver and esophagus in rats.[2,3] Since their principal urinary metabolites, *N*-nitroso-*N*-(carboxymethyl)alkylamines (**2**) (alkyl=ethyl and butyl), are not carcinogenic,[4] *N*-nitroso-*N*-(formylmethyl)alkylamines (**3**), the obligatory metabolic intermediates between **1** and **2**, are suspected to be involved in carcinogenesis by **1**. Previously[5] we prepared *N*-nitroso-*N*-(formylmethyl)butylamine (**3b**), *N*-nitroso-*N*-(formylethyl)butylamine, and *N*-nitroso-*N*-(formylpropyl)butylamine in connection with our studies on the correlation of chemical structure and *in vivo* metabolism with urinary bladder carcinogenicity of *N*-nitrosodialkylamines. They were all mutagenic in the presence of rat-liver 9000×*g* supernatant toward *Salmonella typhimurium* TA1535, *Escherichia coli* WP2



1 2 3 4 5

3a: R=C_2H_5 **4a**: R=C_2H_5 **5a**: R=C_2H_5
3b: R=C_4H_9 **4b**: R=C_4H_9 **5b**: R=C_4H_9
3c: R=*tert*-C_4H_9 **4c**: R=*tert*-C_4H_9 **5c**: R=*tert*-C_4H_9

Chart 1

hcr⁻ and *E. coli* WP2.[6] Among these three formylalkyl compounds only the formlymethyl compound (**3b**) was chemically unstable, and produced directly acting mutagen(s) upon standing at room temperature.[5] This paper deals with the isolation and characterization of the directly acting mutagens formed from **3b** and its homolog, *N*-nitroso-*N*-(formylmethyl)ethylamine (**3a**) and a nonmutagen formed from *N*-nitroso-*N*-(formylmethyl)-*tert*-butylamine (**3c**). Their possible role in carcinogenesis by **1** is discussed.

Experimental

Instrumental Analyses——Ultraviolet (UV) spectra were measured in CH_3CN solution with a Hitachi 200-20 spectrophotometer. Infrared (IR) spectra were determined on a Hitachi EPI-G3 spectrometer. Nuclear magnetic resonance (NMR) spectra were taken at 60 MHz on a Hitachi R-24B spectrometer. Chemical shifts are expressed in δ with tetramethylsilane as an internal standard; s = singlet, d = doublet, t = triplet, q = quartet, and br s = broad singlet. Melting points were determined on a micro hot-stage apparatus and are uncorrected.

Chromatography——Thin-layer chromatography (TLC) was performed on pre-coated TLC plates with a 0.25 mm layer of Silica gel 60 F_{254} (E. Merck AG, Darmstadt, G.F.R.). Spots were visualized with UV light (254 nm) and the reagent reported earlier[7] or with 2,4-dinitrophenylhydrazine solution [dissolve 2,4-dinitrophenylhydrazine (400 mg) in a mixture of 95% H_2SO_4 (2 ml), H_2O (3 ml) and ethanol (10 ml)]. For column chromatography, silica gel (230—400 mesh, E. Merck AG.) and Sephadex LH-20 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) were used. The following solvent systems were used: S_1, hexane–ether–methylene chloride (8 : 3 : 2); S_2, hexane–ether–chloroform (5 : 3 : 2); S_3, hexane–ether–chloroform (4 : 3 : 2); S_4, hexane–ether–chloroform (3 : 3 : 2); S_5, chloroform–acetonitrile (9 : 1).

***N*-Nitroso-*N*-(formylmethyl)butylamine (3b)**——**3b** diethyl acetal was synthesized according to the method reported earlier,[5] and **3b** was prepared from the diethyl acetal with some modifications of the previous procedure.[5] Thus, **3b** diethyl acetal (1.5 g) was added to 1 N HCl (300 ml) and the mixture was stirred at room temperature for 1 h and then extracted with ethyl acetate (300 ml × 3). The organic layer was washed with water and dried over anhydrous Na_2SO_4. The solvent was evaporated off under reduced pressure below 25 °C and the residue (953 mg, 96% yield) was used without purification for further experiment. A part of the residue was treated with 2,4-dinitrophenylhydrazine to give the hydrazone as described previously.[5]

***N*-Nitroso-*N*-(formylmethyl)ethylamine (3a)**——Bromoacetaldehyde diethyl acetal (94% purity, 25 g) was added to 70% ethylamine aqueous solution (50 ml), and the mixture was left at room temperature with occasional stirring for 2 d. Then it was heated at 65 °C in a sealed glass tube for 6 h. After cooling of the reaction mixture, excess of ethylamine was evaporated off. After usual work-up,[5] *N*-(formylmethyl)ethylamine diethyl acetal was obtained as a colorless oil (13 g, 60% yield), bp 76.5—78 °C (19.5 mmHg). Nitrosation of this diethyl acetal with sodium nitrite and acetic acid was carried out in the usual way and the reaction mixture was extracted with hexane. The residue obtained after evaporation of hexane was subjected to silica gel column chromatography and elution with S_1 to give **3a** diethyl acetal as a pale yellow oil (97% yield). *Anal.* Calcd for $C_8H_{18}N_2O_3$: C, 50.50; H, 9.54; N, 14.73. Found: C, 50.60; H, 9.55; N, 14.71.

3a diethyl acetal (2.0 g) was added to 1 N HCl (60 ml) and the mixture was stirred at room temperature for 1 h, then processed as described above for **3b**. **3a** (1.05 g, 86% yield) obtained was used without further purification: ^{1}H-NMR ($CDCl_3$) δ: 1.08, 1.41[8] (3H, t, J=7 Hz, CH_3), 3.64, 4.28 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 4.21, 4.97 (2H, s, $C\underline{H}_2CHO$), 9.40, 9.78 (1H, s, CHO). Treatment of **3a** (70 mg) with 2,4-dinitrophenylhydrazine reagent gave **3a** 2,4-dinitrophenylhydrazone as yellow needles (128 mg, 82% yield), mp 151—152 °C (from hexane–methylene chloride). *Anal.* Calcd for $C_{10}H_{12}N_6O_5$: C, 40.54; H, 4.08; N, 28.37. Found: C, 40.43; H, 4.05; N, 28.13. ^{1}H-NMR ($CDCl_3$) δ: 1.13, 1.48 (3H, t, J=7 Hz, CH_3), 3.67, 4.30 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 4.42, 5.03 (2H, d, J=5 Hz, $C\underline{H}_2CH$), 7.40, 7.64 (1H, t, J=5 Hz, CH), 7.8—9.2 (3H, aromatic H).

***N*-Nitroso-*N*-(formylmethyl)*tert*-butylamine (3c)**——*tert*-Butylamine (58.4 g) was added in portions to the bromoacetaldehyde diethyl acetal (94% purity, 64 g). The mixture was left at room temperature for 3 d with occasional stirring and then refluxed for 4 h in an oil bath (95 °C). After usual work-up, the residue was distilled to give a colorless oil, bp 67—72 °C (12 mmHg), which was nitrosated with sodium nitrite and acetic acid in the usual manner to give the product as a pale yellow oil. It was purified on a column of silica gel with S_1 to give **3c** diethyl acetal (overall yield was 24% starting from bromoacetaldehyde diethyl acetal). *Anal.* Calcd for $C_{10}H_{22}N_2O_3$: C, 55.02; H, 10.10; N, 12.83. Found: C, 54.91; H, 10.24; N, 12.97.

3c diethyl acetal (1.0 g) was added to 0.1 N HCl (200 ml) and the mixture was stirred at room temperature for 25 min. After processing as described above, **3c** was obtained as a pale yellow oil (522 mg, 79% yield), which crystallized in a freezer (−20 °C). ^{1}H-NMR ($CDCl_3$) δ: 1.57 (9H, s, $C(CH_3)_3$), 4.09 (2H, s, CH_2), 9.31 (1H, s, CH). Treatment of **3c** (300 mg) with 2,4-dinitrophenylhydrazine reagent gave the 2,4-dinitrophenylhydrazone as yellow needles (206 mg, 46% yield), mp 162 °C (from methanol–chloroform). *Anal.* Calcd for $C_{12}H_{16}N_6O_5$: C, 44.44; H, 4.97;

N, 25.92. Found: C, 44.35; H, 5.00; N, 26.06. ^{1}H-NMR ($CDCl_3$) δ: 1.63 (9H, s, $C(CH_3)_3$, 4.38 (2H, d, J=5 Hz, CH_2), 7.46 (1H, t, J=5 Hz, CH).

2,4-Dinitrophenylhydrazine Reagent——2,4-Dinitrophenylhydrazine (1 g) was dissolved in acetonitrile (450 ml) and 0.1 N HCl (1 ml) was added to the solution (9 ml) before use.

Isolation and Identification of Directly Acting Mutagens (4)——A solution (100 mg/ml) of *N*-nitroso-*N*-(formylmethyl)alkylamine (**3**) in methyl acetate was left at room temperature for several days with occasional monitoring by TLC. Several spots besides that of **3** were observed on thin-layer chromatograms, using S_3 as the solvent. When a product having Rf=0.4, 0.5 or 0.5, which is less polar than the starting material (**3**), reached its maximal amount in the methyl acetate solution of **3a**, **3b**, or **3c**, respectively, the residue obtained after evaporation of the solvent was chromatographed on a silica gel column using S_4 for **3a** and S_3 for **3b** and **3c** as eluting solvents. The fraction containing the product having Rf=0.4, 0.5 or 0.5, respectively, was collected in a flask containing acetonitrile. Each fraction was concentrated under reduced pressure below 20 °C and the concentrate (acetonitrile solution) was chromatographed on a Sephadex LH-20 (20 g) column with S_5. The concentrated fraction containing the product (**4**) was used for mutation assay. **4a** (8% yield). ^{1}H-NMR (CD_3CN) δ: 0.98, 1.28 (3H, t, J=7 Hz, CH_3), 3.71, 4.33 (2H, q, J=7 Hz, CH_2), 9.26, 9.54 (1H, s, CHO). **4b** (11% yield). ^{1}H-NMR (CD_3CN) δ: 0.90 (3H, t, CH_3), 1.1—1.7 (4H, m, $C\underline{H}_2C\underline{H}_2CH_3$), 3.70, 4.26 (2H, t, J=7 Hz, NCH_2), 9.31, 9.60 (1H, s, CHO). Of these three compounds, **4c** was much more stable than the others and could be concentrated to dryness without decomposition to give colorless crystals (18% yield), mp 104 °C (dec.). *Anal.* Calcd for $C_6H_{11}N_3O_3$: C, 41.61; H, 6.40; N, 24.27. Found: C, 41.77; H, 6.36; N, 24.04. UV $\lambda_{max}^{CH_3CN}$ nm (ε): 223 (12000), 363 (77), 375 (84), 385 (65).[9] IR ν_{max}^{KBr} cm^{-1}: 3100 (OH), 2800 (CHO), 1695 (C=O), 1465 (N=O), 1035 (C–O). ^{1}H-NMR (CD_3CN) δ: 1.52 (9H, s, $C(CH_3)_3$), 9.40 (1H, s, CHO).

4a, **4b** and **4c** in acetonitrile were treated with 2,4-dinitrophenylhydrazine reagent and kept at room temperature for 1 h. After evaporation of the solvent, the yellow crystalline products were dissolved in ethyl acetate. Each ethyl acetate solution was washed with water, and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was purified on a column of silica gel with S_3 or S_4 as the solvent in the usual way to give the respective hydrazones, **5a**, **5b** and **5c**, as yellow needles. **5a**, mp 132—133 °C (dec.) (from chloroform–methanol). *Anal.* Calcd for $C_{10}H_{11}N_7O_6$: C, 36.93; H, 3.41; N, 30.15. Found: C, 37.07; H, 3.43; N, 30.31. UV $\lambda_{max}^{CH_3CN}$ nm (ε): 365 (33000).[9] IR ν_{max}^{KBr} cm^{-1}: 3350 (OH), 3250 (NH), 1610 (C=N). ^{1}H-NMR (CD_3COCD_3) δ: 1.14, 1.46 (3H, t, J=7 Hz, CH_3), 3.88, 4.51 (2H, q, J=7 Hz, CH_2), 8.34, 8.59 (1H, s, CH), 7.7—9.0 (3H, aromatic H). **5b**, mp 119 °C (dec.) (from acetonitrile). *Anal.* Calcd for $C_{12}H_{15}N_7O_6$: C, 40.80; H, 4.28; N, 27.75. Found: C, 40.84; H, 4.23; N, 27.71. UV $\lambda_{max}^{CH_3CN}$ nm (ε): 365 (31000).[9] IR ν_{max}^{KBr} cm^{-1}: 3270 (NH, OH), 1610 (C=N). ^{1}H-NMR (CD_3COCD_3) δ: 0.96 (3H, t, J=6 Hz, CH_3), 1.1—1.9 (4H, m, $C\underline{H}_2C\underline{H}_2CH_3$), 2.87 (1H, br s, NH or OH), 3.90, 4.47 (2H, t, J=7 Hz, NCH_2), 8.33, 8.58 (1H, s, CH=N), 7.7—9.0 (3H, aromatic H). **5c**, mp 137.5 °C (dec.) (from methanol). *Anal.* Calcd for $C_{12}H_{15}N_7O_6 \cdot CH_3OH$: C, 40.51; H, 4.97; N, 25.45. Found: C, 40.46; H, 4.94; N, 25.51. UV $\lambda_{max}^{CH_3CN}$ nm (ε): 365 (34000).[9] IR ν_{max}^{KBr} cm^{-1}: 3270 (NH), 3150 (OH), 1610 (C=N). ^{1}H-NMR (CD_3OD) δ: 1.68 (9H, s, $C(CH_3)_3$), 4.79 (2H, s, NH, OH), 8.07 (1H, s, CH=N), 7.6—9.0 (3H, aromatic H).

Formation of 4c from 3c by Nitrosation——Nitrosation by Sodium Nitrite and HCl: **3c** (118 mg, 0.82 mmol) was dissolved in 0.1 N HCl (8.2 ml). After the solution had been made acidic (pH 2—3) with 12 N HCl, sodium nitrite (56.6 mg, 0.82 mmol) was added and the reaction mixture was stirred for 3 h at room temperature, then extracted with ethyl acetate. The organic layer was processed in the usual way to give **4c** (9 mg, 6% yield) after chromatographic purification of the product on a silica gel column (42% of **3c** was recovered).

Nitrosation by Nitrosyl Chloride: Nitrosyl chloride (1.1 mmol) in chloroform (0.4 ml) was added to **3c** (156 mg, 1.1 mmol) in methyl acetate (1.5 ml) and the whole was left for 3 h at room temperature. After evaporation of the solvents, the residue was chromatographed on a column of silica gel to give **4c** (17 mg, 9% yield) (42% of **3c** was recovered).

Nitrosation by Amyl Nitrite: Amyl nitrite (0.1 ml, 0.1 mmol) was added to **3c** (109 mg, 0.8 mmol) in methyl acetate (1.1 ml) and the mixture was stirred for 3 h at room temperature. After usual work-up, **4c** (0.3 mg, 0.2% yield) and **3c** (96 mg, 88% recovery) were obtained.

Nitrosation of *N*-Nitroso-*N*-(2-oxopropyl)butylamine (6), *N*-Nitroso-*N*-(2-oxobutyl)butylamine (7) and *N*-Nitroso-*N*-(3-oxobutyl)butylamine (8)——**6**, **7** and **8** were synthesized according to the procedure described previously.[7] A methyl acetate solution of these compounds (100 mg/ml) was treated at room temperature with nitrosyl chloride (1—1.5 mol eq) in chloroform. After the brown color of nitrosyl chloride had disappeared, the organic solvents were evaporated off and the residue was chromatographed on a column of silica gel to isolate the products.

Products Formed from **6** by Nitrosation: Thin-layer chromatographic examination with S_3 of the products obtained from **6** (2.26 g) by nitrosation showed the presence of three products (**9**, Rf 0.6; compound X, Rf 0.41; **10**, Rf 0.38) besides **6** (Rf 0.27). **9** (401 mg, 20% yield) was obtained from the first fraction after chromatographic separation on a column of silica gel (120 g) with S_2. It was identified as *N*-butyl-1,2-dioxopropylamine (**9**) on the basis of IR and ^{1}H-NMR spectral data. IR ν_{max}^{film} cm^{-1}: 3320 (NH), 1725 (C=O), 1675, 1530 (CONH), 1170 (C–O). ^{1}H-NMR (CD_3CN) δ: 0.88 (3H, t, J=6 Hz, $CH_2C\underline{H}_3$), 1.2—1.8 (4H, m, $C\underline{H}_2C\underline{H}_2CH_3$), 2.33 (3H, s, $COCH_3$), 3.17 (2H, q, J=6 Hz, NCH_2), 7.4 (1H, br s, NH). **9** was derivatized to the 2,4-dinitrophenylhydrazone in the usual way to give yellow

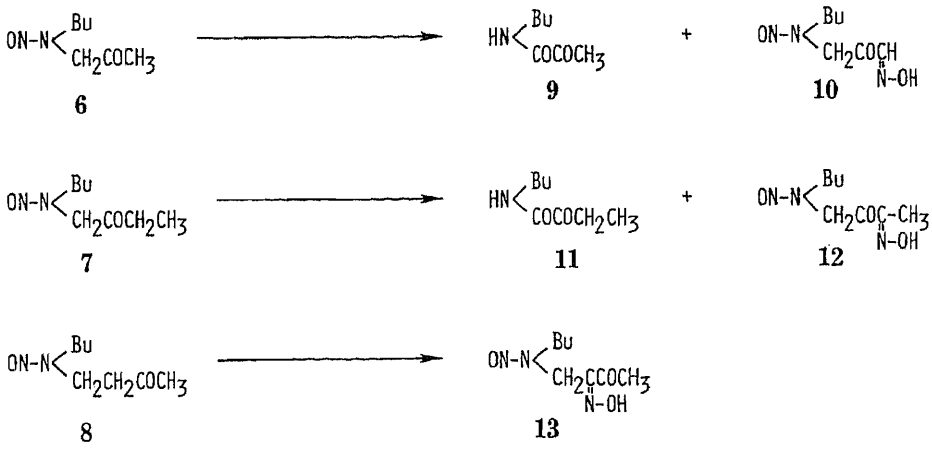
plates (61% yield); mp 150—151 °C (from ethyl acetate). *Anal.* Calcd for $C_{13}H_{17}N_5O_5$: C, 48.29; H, 5.30; N, 21.66. Found: C, 48.36; H, 5.16; N, 21.65. UV $\lambda_{max}^{CH_3CN}$ nm (ε): 356 (23000).[9] IR ν_{max}^{KBr} cm^{-1}: 3325, 3290 (NH), 1650, 1540 (CONH), 1610 (C=N). ^{1}H-NMR (CD_3COCD_3) δ: 0.92 (3H, t, J=6 Hz, CH_2CH_3), 1.1—1.9 (4H, m, $CH_2CH_2CH_3$), 2.27 (3H, s, C-CH_3), 3.30 (2H, q, J=6 Hz, CH_2NH), 8.0 (1H, br s, NH), 8.2—9.1 (3H, aromatic H).

The fraction containing compound X and **10** was further subjected to LH-20 column chromatography with S_5 to give compound X (68 mg) and **10** (64 mg, 2% yield). The latter compound was identified as *N*-nitroso-*N*-butyl-2-oxo-3-hydroxyiminopropylamine (**10**) based on the following data. *Anal.* Calcd for $C_7H_{13}N_3O_3$: C, 44.91; H, 7.00; N, 22.45. Found: C, 44.86; H, 7.02; N, 22.61. UV $\lambda_{max}^{CH_3CN}$ nm (ε): 225 (18000), 353 (145). IR ν_{max}^{KBr} cm^{-1}: 3150 (OH), 1710 (C=O), 1620 (C=N), 1415 (N=O), 1020 (C-O). ^{1}H-NMR (CD_3CN) δ: 0.94 (3H, t, J=6 Hz, CH_3), 1.1—1.8 (4H, m, $CH_2CH_2CH_3$), 3.52, 4.18 (2H, t, J=7 Hz, N-CH_2CH_2), 4.60, 5.34 (2H, s, CH_2CO), 7.50, 7.60 (1H, s, CH), 10.0 (1H, br s, N-OH). The structure of compound X has not yet been elucidated. IR ν_{max}^{film} cm^{-1}: 2950, 1745, 1690, 1450, 1425. ^{1}H-NMR (CD_3CN) δ: 0.94 (3H, t, J=6 Hz, CH_3), 1.1—1.8 (4H, m, $CH_2CH_2CH_3$), 4.17 (2H, t, J=7 Hz, N-CH_2), 4.33, 4.34 (3H, s). Approximately 10% of **6** was recovered unchanged after the chromatographic separation on a silica gel column.

Products Formed from **7** by Nitrosation: Examination by TLC with solvent S_3 of the reaction products obtained from **7** (1.35 g) by nitrosation revealed the presence of three main products (**11**, *Rf* 0.69; compound Y, *Rf* 0.53; **12**, *Rf* 0.37). Chromatography on a column of silica gel (120 g) with S_2 gave three fractions containing **11**, compound Y, and **12**. Each fraction was further purified on a column of LH-20 with S_5 to give **11**, compound Y, and **12**. **11** (158 mg, 13% yield) was identified as *N*-butyl-1,2-dioxobutylamine. IR ν_{max}^{film} cm^{-1}: 3310 (NH), 1720 (C=O), 1675, 1525 (CONH), 1130 (C-O). ^{1}H-NMR (CD_3CN) δ: 1.0 (6H, t, J=7 Hz, CH_3 × 2), 1.2—1.8 (4H, m, $CH_2CH_2CH_3$), 2.86 (2H, q, J=7 Hz, $COCH_2CH_3$), 3.19 (2H, q, J=7 Hz, N-CH_2), 7.32 (1H, br s, NH). For confirmation of the structure of **11**, it was derivatized quantitatively to its 2,4-dinitrophenylhydrazone; yellow needles, mp 173 °C (from ethyl acetate). *Anal.* Calcd for $C_{14}H_{19}N_5O_5$: C, 49.84; H, 5.68; N, 20.76. Found: C, 49.62; H, 5.55; N, 20.75. UV $\lambda_{max}^{CH_3CN}$ nm (ε): 371 (30000).[9] IR ν_{max}^{KBr} cm^{-1}: 3400 (NH), 1640, 1580 (CONH), 1615 (C=N). ^{1}H-NMR ($CDCl_3$) δ: 0.95 (3H, t, J=6 Hz, $CH_2CH_2CH_3$), 1.1—1.8 (4H, m, $CH_2CH_2CH_3$), 1.29 (3H, t, J=7 Hz, C-CH_2CH_3), 2.58 (2H, q, J=7 Hz, CCH_2CH_3), 3.43 (2H, q, J=7 Hz, N-CH_2), 6.0 (1H, br s, NH), 7.9—9.1 (3H, aromatic H).

12 (360 mg, 23% yield) was obtained as colorless needles, mp 104—105.5 °C (from acetonitrile). It was identified as *N*-nitroso-*N*-butyl-2-oxo-3-hydroxyiminobutylamine (**12**) on the basis of ^{1}H-NMR spectral and other data. *Anal.* Calcd for $C_8H_{15}N_3O_3$: C, 47.75; H, 7.51; N, 20.88. Found: C, 47.73; H, 7.53; N, 20.86. UV $\lambda_{max}^{CH_3CN}$ nm (ε): 228 (17000), 355 (120). IR ν_{max}^{KBr} cm^{-1}: 3150 (OH), 1700 (C=O), 1630 (C=N), 1415 (N=O), 1030 (C-O). ^{1}H-NMR (CD_3CN) δ: 0.94 (3H, t, J=7 Hz, CH_2CH_3), 1.1—1.8 (4H, m, $CH_2CH_2CH_3$), 1.84 (3H, s, C-CH_3), 4.12 (2H, t, J=7 Hz, N-CH_2CH_2), 4.63, 5.30 (2H, s, N-CH_2CO), 10.00 (1H, s, N-OH). Compound Y (14 mg, 1.5% yield) was tentatively identified as 2-oxo-3-hydroxyiminobutanol based on its ^{1}H-NMR spectrum. ^{1}H-NMR (CD_3CN) δ: 1.91 (3H, s, CH_3), 2.30 (2H, s, CH_2OH), 3.80 (2H, s, CH_2), 10.57 (1H, N-OH). No further study has been done on this compound.

Products Formed from **8** by Nitrosation: Thin-layer chromatographic examination with S_3 of the products formed from **8** (150 mg) by nitrosation showed the presence of a single product (**13**), which was purified by chromatography on a column of silica gel (30 g) with S_3 as a solvent. **13** (101 mg, 66% yield) was obtained as colorless crystals, mp 62—63 °C. This compound was identified as *N*-nitroso-*N*-butyl-2-hydroxyimino-3-oxobutylamine (**13**)



Bu = $CH_2CH_2CH_2CH_3$

Chart 2. Products Formed from *N*-Nitroso-*N*-(oxoalkyl)butylamines by Treatment with Nitrosyl Chloride

on the basis of the following data. *Anal.* Calcd for $C_8H_{15}N_3O_3$: C, 47.75; H, 7.51; N, 20.88. Found: C, 47.40; H, 7.50; N, 21.03. UV $\lambda_{max}^{CH_3CN}$ nm (ε): 223 (14500), 356 (100). IR ν_{max}^{KBr} cm^{-1}: 3120 (OH), 1690 (C=O), 1625 (C=N), 1450 (N=O), 1000 (C–O). ^{1}H-NMR (CD_3CN) δ: 0.98 (3H, t, $CH_2C\underline{H}_3$), 1.1—1.9 (4H, m, $C\underline{H}_2C\underline{H}_2CH_3$), 2.27, 2.33 (3H, s, $COCH_3$), 3.46, 4.12 (2H, t, J=7 Hz, N–$C\underline{H}_2CH_2$), 4.34, 4.97 (2H, s, N–$C\underline{H}_2C$), 10.06, 10.4 (1H, s, N–OH).

Nitrosation of 8 with 3c——Treatment of **8** with **3c** Alone: **8** (86 mg, 0.5 mmol) and **3c** (144 mg, 1 mmol) were dissolved in methyl acetate (1.6 ml) and the solution was left at room temperature for 5 d. After evaporation of the solvent, the residue was chromatographed on a column of silica gel (60 g) with S_3 as a solvent to give **4c** (18 mg, 20% yield) after usual work-up. No **13** was produced, and **8** was mostly recovered unchanged.

Treatment of **8** with **3c** and Nitrosyl Chloride: **8** (172 mg, 1 mmol) and **3c** (144 mg, 1 mmol) were dissolved in methyl acetate (1.5 ml). Nitrosyl chloride (1 mmol) in chloroform (350 μl) was added to this solution and the mixture was left at room temperature for 85 min. After evaporation of the solvents, the residue was subjected to column chromatography on silica gel (70 g) to give **4c** (3 mg, 2% yield) and **13** (100 mg, 50% yield), recovering **8** (53 mg, 31%) and **3c** (51 mg, 35%).

Mutation Assay——The mutation assay using *S. typhimurium* TA1535 and TA1538, and *E. coli* WP2 *hcr*$^-$ was carried out as described previously in the absence[10] and in the presence of rat-liver 9000 × *g* supernatant.[11]

Results

The IR spectrum of **4c** showed a strong absorption band at 3100 cm^{-1} due to OH, bands at 2800, 1695 and 1035 cm^{-1} ascribable to the aldehyde group and a band at 1465 cm^{-1} due to the N=O group. The UV spectrum of **4c** with absorption at 223 nm (ε=12000) also indicated the presence of the *N*-nitroso group. The NMR spectrum of **4a** taken in CD_3CN showed two sets of signals indicating the existence of a mixture of *E*- and *Z*-isomers because of the partial double bond character of the N–N bond[8a]: signals at 0.98 and 3.71 ppm for the *E*-form and 1.28 and 4.33 ppm for the *Z*-form were assignable to the ethylnitrosamino protons. A similar spectral characteristic was noticed with **3a** (in $CDCl_3$): 1.08 and 3.64 ppm for *E*-form and 1.41 and 4.28 ppm for *Z*-form. Singlet peaks assignable to the aldehyde proton were also observed at 9.26 (*Z*-form) and 9.54 (*E*-form) ppm for **4a** and at 9.40 (*Z*-form) and 9.78 (*E*-form) ppm for **3a**. However, singlet peaks observed with **3a** at 4.21 (*Z*-form) and 4.97 (*E*-form) ppm due to two protons adjacent to the aldehyde group disappeared in **4a**, indicating substitution at this position.

Quite similar NMR spectral results were observed with **4b** and **3b**. On the other hand, **4c** and **3c** exist as *Z*- and *E*-form exclusively, respectively, because of the bulkiness of the *tert*-butyl group.[8b] Besides these spectral data, the result of elemental analysis of **4c** supported its identification as *N*-nitroso-*N*-*tert*-butyl-1-hydroxyimino-2-oxoethylamine. Analogously the structure of **4a** and **4b** could be assigned as indicated in Chart 1, although they were not stable enough for elemental analysis. For further confirmation of their structures, **4a**, **4b** and **4c** were derivatized into their crystalline 2,4-dinitrophenylhydrazones (**5**). The NMR spectrum of **5a** taken in CD_3COCD_3 also showed two sets of signals assignable to the ethylnitrosamino protons, indicating the presence of *E*- and *Z*-isomers due to the *N*-nitroso group; 1.14 and 3.88 ppm for *E*-form and 1.46 and 4.51 ppm for *Z*-form. The aldehyde proton signals observed in **4a** disappeared in **5a** while new singlet peaks appeared at 8.34 (*Z*-form) and 8.59 (*E*-form) in addition to peaks due to phenyl protons, indicating that **4a** is derivatized to the 2,4-dinitrophenylhydrazone (**5a**) with retention of its structure. A similar conclusion was reached when the NMR spectra of **4b** and **5b** were compared. The NMR spectrum of **5c** indicated that it exists as the *Z*-form exclusively. Based on these spectral and elemental analysis data for **5a**, **5b** and **5c**, their structures were elucidated to be as shown in Chart 1.

The IR spectrum of **9** showed absorption bands at 3320, 1675 and 1530 cm^{-1} attributable to a secondary amido group and at 1725 and 1170 cm^{-1} due to a carbonyl group. The NMR spectrum of **9** taken in CD_3CN showed peaks at 0.88, 1.2—1.8 and 3.17 ppm assignable to a butylamino group. No indication of the presence of *E*- and *Z*-isomers due to the *N*-nitroso group was observed. On derivatization of **9** to its 2,4-dinitrophenylhydrazone, the absorption

bands due to a carbonyl group observed in the IR spectrum disappeared, while a new absorption band due to C=N appeared at 1610 cm^{-1}. The NMR spectrum indicated that the butylamino group remained intact (0.92, 1.1—1.9 and 3.30 ppm) and a singlet peak assignable to C–CH_3 appeared at 2.27 ppm instead of that at 2.33 ppm assignable to $COCH_3$. Based on these spectral findings, the structure of **9** was assigned as *N*-butyl-1,2-dioxopropylamine. The elemental analysis data for its 2,4-dinitrophenylhydrazone also supported this structure.

The IR spectrum of **10** exhibited absorptions at 3150 and 1620 cm^{-1}, 1710 and 1020 cm^{-1}, and 1415 cm^{-1} due to hydroxyimino, carbonyl, and nitroso groups, respectively. The presence of the *N*-nitroso group was also apparent from the UV spectrum. The NMR spectrum of **10** taken in CD_3CN showed the existence of *E*- and *Z*-isomers arising from the *N*-nitroso group. The presence of a butylnitrosamino grouping was presumed on the basis of the observed peaks at 0.94, 1.1—1.8, and 3.52 (*E*-form) & 4.18 (*Z*-form) ppm. When the NMR spectra of **10** and **6** taken in CD_3CN were compared, the singlet peaks at 2.07 (*Z*-form) and 2.18 (*E*-form) ppm observed in **6** due to $COCH_3$ had disappeared in the spectrum of **10**, while new singlet peaks appeared at 7.50 (*Z*-form) and 7.60 (*E*-form) ppm. The singlet peaks at 4.27 (*Z*-form) and 4.98 (*E*-form) ppm of **6**, assignable to methylene protons adjacent to the oxo group, were shifted to 4.60 (*Z*-form) and 5.34 (*E*-form) ppm in **10**, indicating substitution of two protons of $COCH_3$ with an electron-withdrawing group. Based on these spectral and elemental analysis data, the structure of **10** was determined to be *N*-nitroso-*N*-butyl-2-oxo-3-hydroxyiminopropylamine.

Compound **11** was considered to be a homolog of **9** from a comparison of their IR, UV and NMR spectral data. Since **11** showed triplet and quartet peaks at 1.0 and 2.86 ppm, respectively, an ethyl group adjacent to the carbonyl group remained intact, thus identifying **11** as *N*-butyl-1,2-dioxobutylamine. Compound **11** was derivatized to its 2,4-dinitrophenylhydrazone, whose IR, UV, NMR and elemental analysis data also supported the assigned structure.

Quite similar IR and UV spectra were obtained with **10** and **12**. However, instead of a singlet peak observed in the ^{1}H-NMR spectrum of **10** due to CH, a singlet peak assignable to C–CH_3 appeared in that of **12** at 1.84 ppm. Thus **12** was identified as *N*-nitroso-*N*-butyl-2-oxo-3-hydroxyiminobutylamine.

Comparison of the IR, UV and NMR spectral data of **13** with those of **8** indicated that methylene protons of **8** adjacent to a carbonyl group were replaced with a hydroxyimino group. The structure of **13** was thus assigned as *N*-nitroso-*N*-butyl-2-hydroxyimino-3-oxobutylamine, which was supported by elemental analysis data.

Compounds **4a**, **4b** and **4c** were tested for mutagenicity using acetonitrile as a solvent.

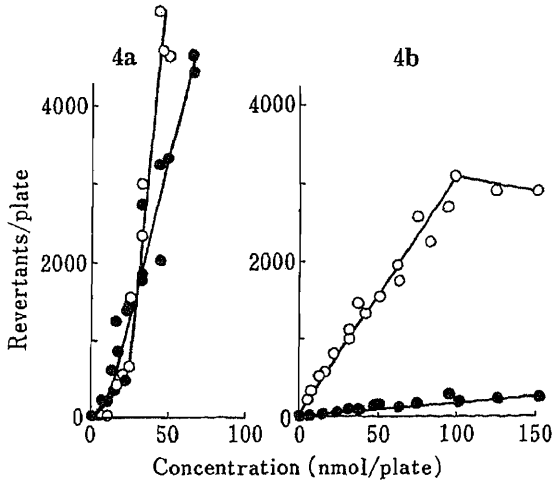


Fig. 1. Mutagenicity of **4a** and **4b** without Metabolic Activation

S. typhimurium TA1535 (○); *E. coli* WP2 *hcr*$^-$ (●). Data from three different experiments are shown.

Since **4a** and **4b** were unstable in the absence of acetonitrile, their content in the solution was determined by HPLC after conversion to the 2,4-dinitrophenylhydrazones (**5a** and **5b**). The formation of **5c** from **4c** was quantitative. The retention times (min) of **5a**, **5b** and **5c** were 6.5 & 6.8,[12] 12.0 & 13.0 and 10.0, respectively.

Compounds **4a** and **4b** were strongly mutagenic to *S. typhimurium* TA1535 and *E. coli* WP2 *hcr*$^-$ without metabolic activation (Fig. 1), while **4c** was not mutagenic to either of the bacterial strains with or without metabolic activation. These three compounds were not mutagenic to *S. typhimurium* TA1538.

The mutagenic activities of **9**—**13** and compound X were tested without metabolic activation. None of them was mutagenic to *E. coli* WP2 *hcr*$^-$ and compound X alone was mutagenic to *S. typhimurium* TA1535, its potency being about one-third of that of **4b**.

Discussion

N-Nitrosoformylmethyl compounds (**3**) are chemically unstable and, upon standing at room temperature, they (**3a** and **3b**) produced directly acting mutagens in the Ames assay.[5] We tried to isolate and characterize those mutagens since they are presumably relevant to carcinogenesis by *N*-nitrosodialkylamines (**1**) containing a 2-hydroxyethyl group, which would form **3**.

By following the mutagenic activity of a methyl acetate solution of **3** upon standing, we were able to isolate a new type of *N*-nitroso compounds and identify them as **4** (Chart 1). Compounds **4a** and **4b** showed potent direct mutagenicity toward *S. typhimurium* TA1535 and *E. coli* WP2 *hcr*$^-$ (Fig. 1), while **4c** did not show any mutagenic activity with or without metabolic activation. Most of the mutagenic activity (99%) toward *S. typhimurium* TA1535 of the methyl acetate solution containing **3b** could be accounted for by **4b**, while about half of the mutagenic activity of the solution against *E. coli* WP2 *hcr*$^-$ was explainable in terms of **4b**, indicating the formation of unidentified mutagenic products.

The potent direct mutagenic activities of **4a** and **4b** are evident from the finding that their mutagenic activities are ten times more potent than those of the corresponding α-hydroxy *N*-nitroso compounds, that is *N*-nitroso-*N*-(hydroxymethyl)alkylamines (alkyl=ethyl and butyl).[13] Compounds **4a** and **4b** were not mutagenic to *S. typhimurium* TA1538, indicating that they act as alkylating agents.

Loeppky *et al.*[14] reported that *N*-nitrosoformylmethylamines readily transfer their nitroso group to an acceptor such as dimethylamine to give *N*-nitrosodimethylamine. There are reports that nitrosation of ketones lead to the formation of oximes *via* addition of NO^+ to an enol tautomer.[15] Thus, it is conceivable that upon standing of **3** at room temperature, a nitrosating agent(s) is produced slowly, which attacks **3**, followed by isomerization to oximes (**4**). In order to demonstrate the formation of NO^+ during storage of **3**, the effect of three nitrosating agents on the formation of **4c** was studied, since **4c** is stable enough for isolation. The formation of **4c** was greatly accelerated by addition of nitrosyl chloride or nitrite under acidic conditions to a solution of **3c**, while little effect was observed with amyl nitrite under neutral conditions in accordance with the order of easiness of NO^+ formation. Compound **4c** was not formed within 3 h in the absence of a nitrosating agent.

Compounds **6**, **7** and **8** are also potent carcinogens that induce tumors of the liver and esophagus in rats.[3,4] Although these compounds are not considered to produce nitrosating agents by themselves upon standing at room temperature, they might give *N*-nitroso-*N*-alkyl-1-hydroxyiminoalkylamine-type compounds by addition of nitrosating agents. Addition of nitrosyl chloride to methyl acetate solutions of **6**, **7** and **8** gave **9** and **10**, **11** and **12**, and **13**, respectively (Chart 2). The formation of **9** and **10** is explainable as follows. After acid-catalyzed enolization of **6**, addition of NO^+ to enol tautomers occurs,[15b] followed by

isomerization to form oximes, namely *N*-nitroso-*N*-butyl-1-hydroxyimino-2-oxopropylamine and **10**. The former oxime would be further changed to *N*-nitroso-*N*-butyl-1,2-dioxopropylamine by the action of excess nitrosyl chloride to afford *N*-nitroso-*N*-butyl-2-oxopropamide,[16] which would not be stable in an acidic solution due to the electron-withdrawing effect of the 1,2-dioxo group, resulting in cleavage of the *N*-nitroso bond to give **9**.[17] The production of **11** and **12** from **7** would occur through a similar mechanism. Compound **8** gave only **13** in a good yield.

These compounds (**9**, **10**, **11**, **12** and **13**) are not 1-hydroxyimino-type compounds, and showed no mutagenic activity toward *S. typhimurium* TA1535 or *E. coli* WP2 *hcr*$^-$. Compound X (obtained from **6**) was mutagenic to *S. typhimurium* TA1535. Although its structure has not been elucidated yet, it has no hydroxyimino group based on its IR spectrum. The mutagenic activation of **6**, **7** and **8** might be different from that of **1** or **3**.

In order to study the ability of **3c** to produce nitrosating agents during storage in methyl acetate, nitrosation of **3c** was investigated in the presence of **8**. Compound **4c** was obtained in 20 or 18% yield with or without **8**, while **13** was not produced and **8** was recovered unchanged. On the other hand, **13** was produced in 50% yield when nitrosyl chloride was added as a nitrosating agent to a methyl acetate solution of **3c** and **8**. These results showed that **3** is much more easily nitrosated than **8** by nitrosating agents formed from itself.

In addition to the generally accepted α-hydroxylation pathway for the metabolic activation of *N*-nitrosamines, an activation through β-hydroxylation has been proposed especially for *N*-nitrosamines with a 2-hydroxyethyl group.[14,18] The formation of a directly acting mutagen with a unique structure from the formylmethyl compound may be another possible pathway for activation through β-hydroxylation. To determine which of these pathways is mostly involved in the initiation of carcinogenesis, further work will be necessary.

Since **4c** is formed even in aqueous media with nitrite and hydrochloric acid, it may be expected that **4** is formed *in vivo* in the stomach from **3** and nitrite.

References and Notes

1) Present address: *Kyoritsu College of Pharmacy, Shibakōen, Minato-ku, Tokyo 105, Japan.*
2) H. Druckrey, R. Preussmann, S. Ivankovic and D. Schmähl, *Z. Krebsforsch.*, **69**, 103 (1967).
3) M. Okada and Y. Hashimoto, *Gann*, **65**, 13 (1974).
4) M. Okada, E. Suzuki and Y. Hashimoto, *Gann*, **67**, 825 (1976).
5) E. Suzuki and M. Okada, *Chem. Pharm. Bull.*, **27**, 541 (1979).
6) E. Suzuki, Y. Akaike and M. Okada, *Mutat. Res.*, **54**, 254 (1978).
7) M. Okada, E. Suzuki and M. Iiyoshi, *Chem. Pharm. Bull.*, **26**, 3891 (1978).
8) *a*) G. J. Karabatsos and R. A. Taller, *J. Am. Chem. Soc.*, **86**, 4373 (1964); *b*) M. Okada, E. Suzuki and M. Iiyoshi, *Chem. Pharm. Bull.*, **28**, 979 (1980); *idem, ibid.*, **28**, 1612 (1980).
9) The most intense absorption is shown.
10) M. Mochizuki, E. Suzuki, T. Anjo, Y. Wakabayashi and M. Okada, *Gann*, **70**, 663 (1979).
11) T. Yahagi, M. Nagao, Y. Seino, T. Matsushima, T. Sugimura and M. Okada, *Mutat. Res.*, **48**, 121 (1977).
12) Because of the existence of *E*- and *Z*-isomers, **5a** and **5b** gave two peaks on HPLC.[8b]
13) M. Mochizuki, T. Anjo, K. Takeda, E. Suzuki, N. Sekiguchi, G.-F. Huang and M. Okada, "*N*-Nitroso-Compounds: Occurrence and Biological Effects: IARC Scientific Publications," No. 41, ed. by H. Bartsch, I. K. O'Neill, M. Castegnaro and M. Okada, International Agency for Research on Cancer, Lyon, 1982, pp. 553—559.
14) R. N. Loeppky, W. Tomasik, D. A. Kovacs, J. R. Outram and R. H. Byington, "*N*-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer: IARC Scientific Publications," No. 57, ed. by I. K. O'Neill, R. C. Von Borstel, C. T. Miller, J. Long and H. Bartsch, International Agency for Research on Cancer, Lyon, 1984, pp. 429—436; R. N. Loeppky, W. Tomasik, G. Eisenbrand and E. Denkel, The 9th International Meeting on *N*-Nitroso Compounds: Relevance to Human Cancer, Vienna, Austria, September 1986.
15) *a*) M. C. Archer, S. D. Clark, J. E. Thilly and S. R. Tannenbaum, *Science*, **174**, 1341 (1971); *b*) J. R. Leis, M. E. Pẽna and D. L. H. Williams, *J. Chem. Soc., Chem. Commun.*, **1987**, 45.

16) S. B. Shim, K. Kim and Y. H. Kim, *Tetrahedron Lett.*, **28**, 645 (1987).
17) C. N. Berry and B. C. Challis, *J. Chem. Soc., Chem. Commun.*, **1972**, 627.
18) C. J. Michejda, M. B. Kroeger-Koepke, S. R. Koepke and D. H. Sieh, "*N*-Nitroso Compounds," ed. by R. A. Scanlan and S. R. Tannenbaum, American Chemical Society, Washington, D.C., 1981, pp. 3—20; L. Airoldi, M. Bonfanti, R. Fanelli, B. Bove, E. Benfenati and P. Gariboldi, *Chem.-Biol. Interact.*, **51**, 103 (1984); W. Sterzel and G. Eisenbrand, *J. Cancer Res. Clin. Oncol.*, **111**, 20 (1986); E. Denkel, W. Sterzel and G. Eisenbrand, The 9th International Meeting on *N*-Nitroso Compounds: Relevance to Human Cancer, Vienna, Austria, September 1986; C. J. Michejda, S. R. Koepke, M. B. Kroeger-Koepke and W. Bosan, The 9th International Meeting on *N*-Nitroso Compounds: Relevance to Human Cancer, Vienna, Austria, September 1986.

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Syntheses, Antitumor Activity and Vascular Relaxing Effect of Purino[7,8-*g*]-6-azapteridines and [1,2,4]Triazino-[3,2-*f*]purines

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Novel heterocycles, purino[7,8-*g*]-6-azapteridines and [1,2,4]triazino[3,2-*f*]purines, were synthesized and their biological activities were examined. Heating of 7,8-diamino-1,3-dimethylxanthine (**2**), which was obtained by the reaction of 8-aminotheophylline with hydroxylamine-*O*-sulfonic acid, with hydrochloric acid gave 2,4,7,9-tetramethylpurino[7,8-*g*]-6-azapteridine-1,3,8,10(2*H*,4*H*,7*H*,9*H*)-tetrone (**4**) in 96% yield; this product was identical with the compound obtained by the reaction of **2** with alloxan followed by methylation. Treatment of **4** with alkylamines gave 3-alkylamino-2-alkylcarbamoyl-6,8-dimethyl[1,2,4]triazino[3,2-*f*]purine-7,8(6*H*,8*H*)-diones (**10a—g**) in 63—95% yields. Compound **4** was active against P 388 leukemia. Vascular relaxing effects of some of the triazino[3,2-*f*]purines (**10a, b, e, f**) were examined, but none showed potent activity.

Keywords——xanthine; hydroxylamine-*O*-sulfonic acid; 7,8-diamino-1,3-dimethylxanthine; purino[7,8-*g*]-6-azapteridine; [1,2,4]triazino[3,2-*f*]purine; alloxan; alkylamine; hydrochloric acid; vascular relaxing effect; antitumor activity

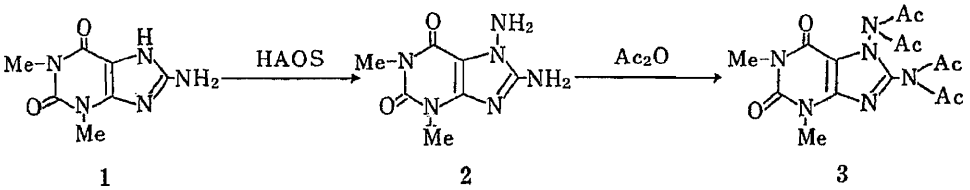
Xanthine derivatives such as caffeine, theophylline and theobromine are widely distributed in nature and show some important biological activities. Previously we synthesized 1-aminoxanthines from caffeine and its analogues and examined their biological activities.[1] In connection with that work it seemed interesting to synthesize 7,8-diamino-1,3-dimethylxanthine (**2**) and its derivatives. During the investigation of their syntheses and reactivities we have found the formation of two novel classes of heterocycles, purino[7,8-*g*]-6-azapteridines and [1,2,4]triazino[3,2-*f*]purines, which we report in this paper. Some of the new compounds were examined for vascular relaxing effect and antitumor activity.

Chemistry

As show in Chart 1, the synthesis of **2** was carried out by the amination of 8-aminotheophylline[2] (**1**) with hydroxylamino-*O*-sulfonic acid in 56% yield. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **2** showed a new signal of N–NH_2 at δ 6.56 ppm.[3] The signal of C–NH_2 was observed at δ 5.55 ppm. Reflux of **2** with acetic anhydride gave 7,8-bis(*N*,*N*-diacetylamino)-1,3-dimethylxanthine (**3**). In order to examine the stability of **2** under acidic conditions, **2** was heated in 5% hydrochloric acid for 3 h, giving yellow crystals of 2,4,7,9-tetramethylpurino[7,8-*g*]-6-azapteridine-1,3,8,10 (2*H*,4*H*,7*H*,9*H*)-tetrone (**4**) in 74% yield (Chart 2). When **2** was heated in a solution of glycol-concentrated hydrochloric acid (10 : 1) at 130 °C, the yield of **4** was increased to 96%. The structural assignment was carried out as follows. The ^{1}H-NMR spectrum of **4** disclosed only four N-methyl groups and no other signals were observed. Mass spectrum (MS) [*m/z*: 344 (M^+)] and

elemental analysis gave the formula $C_{13}H_{12}N_8O_4$. Thus, it was assumed that the formation of **4** might involve the reaction of **2** with dimethyl alloxan which was probably generated in part by the hydrolysis and oxidation of **2**. However, another possible structure 1,3,7,9-tetramethylpurino[8,7-*g*]-7-azapteridine-2,4,8,10 (1*H*,3*H*,7*H*,9*H*)-tetrone (**4'**) had to be considered; that is, if the amino group at the 7-position of **2** reacted faster than that at the 8-position with the C_5-carbonyl group of dimethylalloxan, **4** would be formed, while in the opposite case **4'** would be obtained. Therefore we examined which amino group of **2** would be more reactive. As depicted in Chart 3, the reaction of **2** with *N*,*N*-dimethylformamide (DMF) dimethylacetal, *n*-propylaldehyde or pyruvic aldehyde dimethylacetal gave 8-amino-7-[(*N*,*N*-dimethylamino)formylimino]-1,3-dimethylxanthine (**5**), 8-amino-7-[(*n*-butylidene)amino]-1,3-dimethylxanthine (**6**) or 8-amino-7-(2,2-dimethoxy-1-methylideneamino)-1,3-dimethylxanthine (**7**), respectively. The ^{1}H-NMR spectra of these compounds showed signals of C_8–NH_2 at δ 5.17, 5.88 or 4.96 ppm (signals of N–NH_2 would have been observed at around δ 6.5 ppm). The infrared (IR), MS and elemental analyses were consistent with the assigned structures. These results suggest that the NH_2-group at the 7-position is more reactive than that at the 8-position and the structure **4** is likely to be correct. In order to confirm the structure of **4** and the reaction pathway, the reaction of **2** with alloxan[4] was carried out in water at 70 °C for 30 min to give 8-amino-1,3-dimethyl-7-(2,4,6-trioxopyrimidine-5-imino)xanthine (**8**), which was cyclized to 7,9-dimethylpurino[7,8-*g*]-6-azapteridine-1,3,8,10(2*H*,4*H*,7*H*,9*H*)-tetrone (**9**) by the use of boric acid[5] in acetic acid in a good yield. Methylation of **9** with dimethyl sulfate gave **4** in 72% yield. This compound was identical with that obtained by the heating of **2** in hydrochloric acid (Chart 4).

Pyrimidinones or fused pyrimidinones are generally susceptible to nucleophilic reagents.[6] Thus it seemed interesting to examine the reaction of **4** or **9** with alkylamines. As shown in Chart 5, the reaction of **4** with a large excess of benzylamine gave 3-benzylamino-2-benzylcarbamoyl-6,8-dimethyl[1,2,4]triazino[3,2-*f*]purine-7,9(6*H*,8*H*)-



HAOS = hydroxylamine-*O*-sulfonic acid

Chart 1

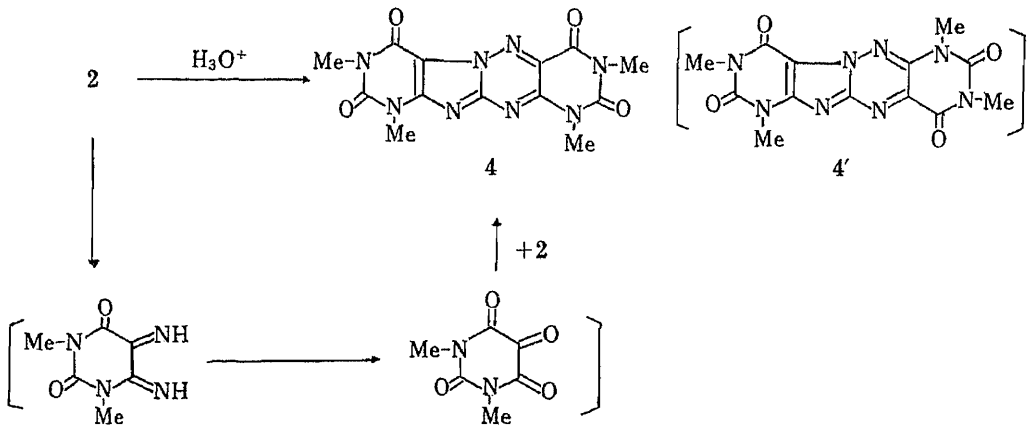


Chart 2

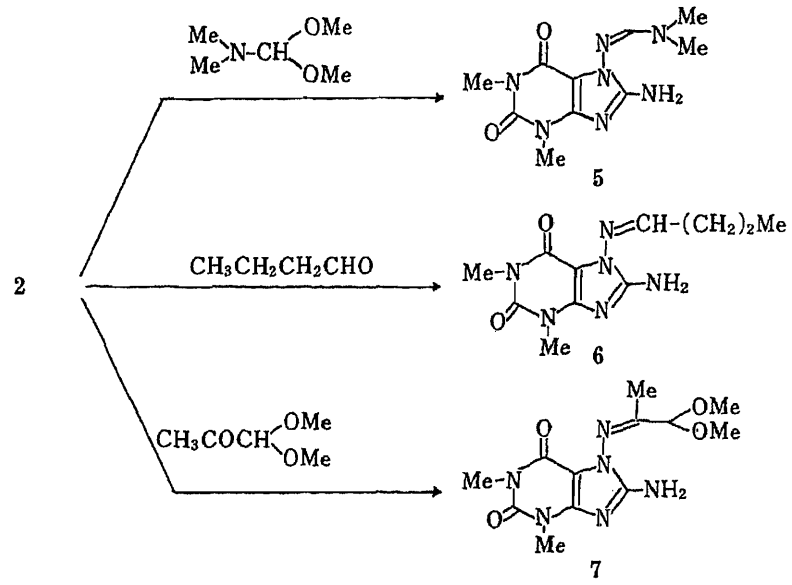


Chart 3

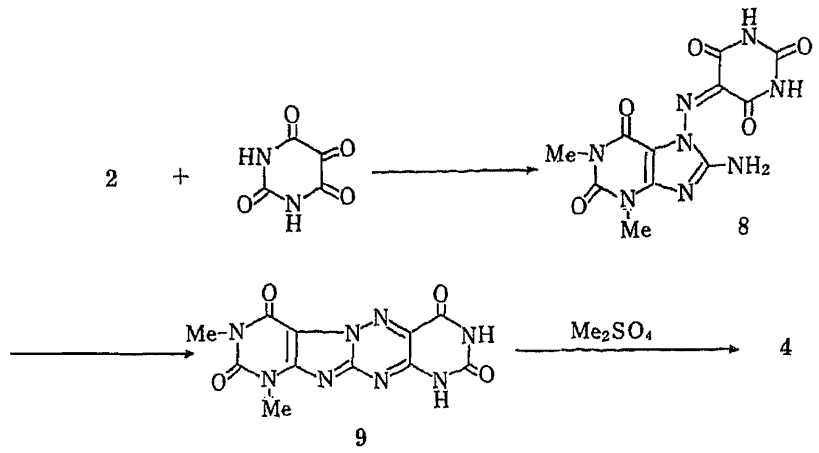


Chart 4

dione (**10a**) in 94% yield; this compound was also obtained by the reaction of **9** with benzylamine. The IR spectrum of **10a** showed signals of secondary amines at 3430 and 3250 cm^{-1}, and the ^{1}H-NMR spectrum also showed signals of two secondary amines at δ 9.40 and 10.03 ppm. Absorptions of two benzyl groups were observed at δ 4.56 (2H, CH_2), 4.80 (2H, CH_2), and 7.15—7.40 (10H, 2×Ph) ppm. The MS and elemental analyses were consistent with the assigned structure. Compounds **10b**—**g** were similarly obtained by the reaction of **4** with *n*-butylamine, *n*-hexylamine, *n*-octylamine, *n*-decylamine, 2-hydroxyethylamine, or 3-hydroxypropylamine, respectively. Acetylation of **10f**, **g** with acetic anhydride gave 3-(2-acetoxyethylamino)-2-(2-acetoxyethylcarbamoyl)-6,8-dimethyl[1,2,4]triazino[3,2-*f*]purine-7,9(6*H*,8*H*)-dione (**11a**) and 3-(3-acetoxypropylamino)-2-(3-acetoxypropylcarbamoyl)-6,8-dimethyl[1,2,4]triazino[3,2-*f*]purine-7,9(6*H*,8*H*)-dione (**11b**).

A plausible mechanism accounting for the formation of **10a**—**g** by the reaction of **4** with alkylamines may be formulated as shown in Chart 6. Nucleophilic addition of alkylamines seems to take place initially at C_{4a} in **4**. However, this unstable intermediate appears to undergo rupture of the pyrimidine ring and successive nucleophilic substitution of an alkylamine at the amide carbamoyl may give **10a**—**g**.

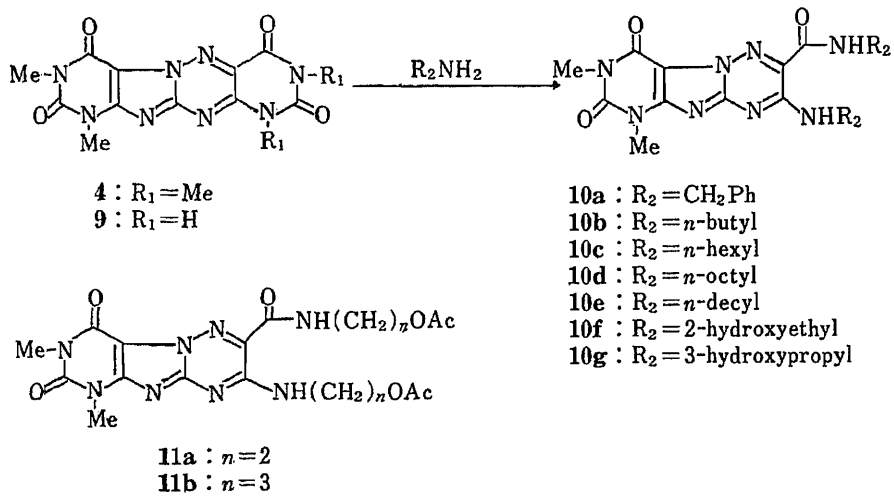


Chart 5

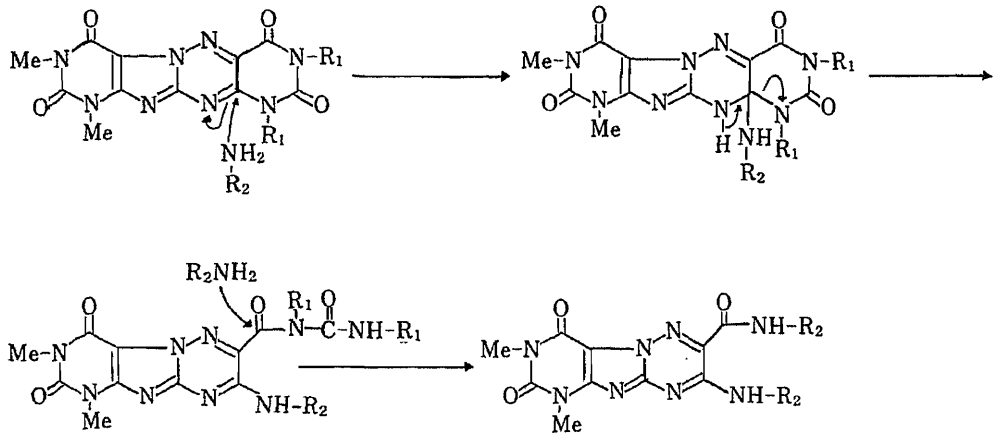


Chart 6

A number of purine derivatives show antitumor activity.[7] Since the newly synthesized purino[7,8-*g*]-6-azapteridine (**4**) and [1,2,4]triazino[3,2-*f*]purines (**10**) are purine derivatives, we examined their antitumor activity. Furthermore, compounds **10**, which contain structural features similar to those of theophylline, were examined for vascular relaxing activity, one of the known physiological activities of theophylline.[8]

Antitumor Activity Test

The *in vivo* activity of 2,4,7,9-tetramethylpurino[7,8-*g*]-6-azapteridine-1,3,8,10-(2*H*,4*H*,7*H*,9*H*)-tetrone (**4**) and 3-alkylamino-2-alkylcarbamoyl-6,8-dimethyl[1,2,4]-triazino[3,2-*f*]purine-7,8(6*H*,8*H*)-diones (**10a**) against P 388 leukemia was examined.

Animals——Female BDFI strain mice, aged 6 weeks, were used. The mice were maintained on a laminar air-flow shelf and were fed an ordinary pellet diet (Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*.

Tumors——P 388 leukemia tumor was initially supplied by the National Cancer Center Research Institute of Japan and was maintained in female DBA/2 strain mice in the Research Laboratories of Taisho Pharmaceutical Co., Ltd.

Evaluation of Antitumor Activity——Seven-day-old P 338 tumor cells (1×10^6 cells/0.2 ml) were inoculated i.p. into the mice on day 0. The required amount of the drug was

TABLE I. Antitumor Activity Test of 2,4,7,9-Tetramethylpurino[7,8-*g*]-6-azapteridine-1,3,8,10(2*H*,4*H*,7*H*,9*H*)-tetrone (**4**) and 3-Alkylamino-2-alkylcarbamoyl-6,8-dimethyl[1,2,4]triazino[3,2-*f*]purine-7,9(6*H*,8*H*)-diones (**10a, b, c, e, f**) on P388 Lymphocytic Leukemia

Compd. No.	Dose (mg/kg, i.p.)	Schedule	Number of mice	*T/C* (%)
Control	0	Days 1—5	16	100
4	12.5	Days 1—5	8	116 (−)
4	25	Days 1—5	8	145 (+)
4	50	Days 1—5	8	167 (+)
10a	25	Days 1—5	8	91 (−)
10b	50	Days 1—5	8	98 (−)
10c	50	Days 1—5	8	98 (−)
10e	50	Days 1—5	8	98 (−)
10f	80	Days 1—5	8	100 (−)
Mitomycin	0.8	Days 1—5	8	209 (++)
Mitomycin	1.6	Days 1—5	8	233 (++)

(+), ≧125; (++), ≧175.

TABLE II. Vascular Relaxing Effects of 3-Alkylamino-2-alkylcarbamoyl-6,8-dimethyl-[1,2,4]triazino[3,2-*f*]purine-7,9(6*H*,8*H*)-diones (**10a, b, c, f**) on Strips of Mesenteric Artery Isolated from 13-Week-Old Spontaneously Hypertensive Rats

Compd.	*N*[a]	Percent relaxation in strips induced by the compound at a concentration of	
		3 μM	10 μM
10a	4	3.6±2.3	7.6±3.9
10b	4	4.1±2.0	12.8±3.8
10c	4	11.4±0.8	26.8±1.7
10f	3	0.4±0.2	0.9±0.5

a) *N* indicates the number of arterial strips used. Data are expressed as mean±S.E.

suspended in 0.5% gum arabic. Mitomycin was dissolved in saline. Antitumor activity of compounds was expressed as follows:

$$\frac{\text{median survival time of treated group}}{\text{median survival time of control}} \times 100\ (T/C)$$

Results——As can be seen from Table I, compound **4** (25 mg, 50 mg/kg) exhibited antitumor activity against P 388 leukemia. However, its activity was much weaker than that of mitomycin and a decrease of body weight was observed.

Vascular Relaxing Activity

Vascular relaxing effects of 3-alkylamino-2-alkylcarbamoyl-6,8-dimethyl[1,2,4]-triazino[3,2-*f*]purine-7,9(6*H*,8*H*)-diones (**10a, b, c, f**) were determined, and the results are summarized in Table II.

To demonstrate the relaxing effects of these compounds, helical strips of mesenteric arteries isolated from 13-week-old spontaneously hypertensive rats (SHR) were used. The rats were stunned and exsanguinated. Distal portions of the superior mesenteric arteries (0.6—

0.8 mm outside diameter) were quickly disected out, and cut helically into strips of 0.8 mm in width and 7 mm in length, according to the method described by Asano *et al.*[9] The strips were mounted for isometric recording of tension in a water-jacketed (37 ± 0.5 °C) 20 ml tissue bath containing oxygenated Krebs' bicarbonate solution. Relaxing responses of the strips to the compounds were examined by means of the procedures described by Asano and Hidaka.[10] Briefly, the strips were contracted with 30 mM KCl. After the contraction had reached a plateau, one of the test compounds at a concentration of 3 or 10 μM was cumulatively added. The relaxation induced by the compound is expressed as a percentage of the contraction developed by 30 mM KCl.

Among the test compounds, **10b**, and **10c** showed arterial relaxing activities, whereas **10a** and **10f** were less effective.

Experimental

The melting points were determined with a Yanagimoto micro melting point apparatus and are uncorrected. The IR spectra were measured with an IR-810 machine from Nihon Bunko Spectroscopic Co., Ltd. Mass spectra were measured with a Japan Electron Optics Laboratory Co. JMS-DX 300 mass spectrometer. The ^{1}H-NMR spectra were recorded with Japan Electron Optics Laboratory Co. JNM-MH-100 and JNM-FX-100 instruments. Abbreviations are as follows: s, singlet; d, doublet; q, quartet; br, broad; m, multiplet.

7,8-Diamino-1,3-dimethylxanthine (2)——Hydroxylamino-*O*-sulfonic acid (30 g) was added dropwise to a solution of 8-aminotheopylline (20 g) in 400 ml of 1.2 N KOH, and the mixture was allowed to stand overnight at room temperature. The resulting crystals were collected by filtration and recrystallized from water. Yield 12.2 g (56%), mp 279—283 °C. ^{1}H-NMR ($CDCl_3$–DMSO-d_6 (1 : 1)) δ: 3.24 (3H, s, N–Me), 3.36 (3H, s, N–Me), 5.55 (2H, s, C–NH_2), 6.56 (2H, s, N–NH_2). IR ν_{max}^{KBr} cm^{-1}: 3330, 3440 (NH_2), 1690, 1650 (C=O). MS *m/z*: 210 (M^+). *Anal.* Calcd for $C_7H_{10}N_6O_2$: C, 40.00; H, 4.80; N, 39.98. Found: C, 40.30; H, 5.01; N, 39.76.

7,8-Bis(*N*,*N*-diacetylamino)-1,3-dimethylxanthine (3)——A mixture of **2** (0.21 g) and acetic anhydride (10 ml) was refluxed for 6 h. Excess acetic anhydride was distilled off. The residue was column-chromatographed on silica gel using $CHCl_3$–MeOH (100 : 1) as an eluent. The eluate was collected and the solvent was distilled off. The residue was recrystallized from ethanol to give colorless prisms, mp 168—170 °C. Yield 0.26 g (69%). ^{1}H-NMR ($CDCl_3$) δ: 2.38 (6H, s, 2 × $COCH_3$), 2.43 (6H, s, 2 × $COCH_3$), 3.38 (3H, s, N–CH_3), 3.59 (3H, s, N–CH_3). IR ν_{max}^{KBr} cm^{-1}: 1750, 1740, 1700, 1670 (C=O). MS *m/z*: 378 (M^+). *Anal.* Calcd for $C_{15}H_{18}N_6O_6$: C, 47.62; H, 4.80; N, 22.22. Found: C, 47.78; H, 4.52; N, 22.15.

2,4,7,9-Tetramethylpurino[7,8-*g*]-6-azapteridine-1,3,8,10(2*H*,4*H*,7*H*,9*H*)-tetrone (4)——Method A: A mixture of **2** (0.5 g), concentrated HCl (1 ml) and ethylene glycol (10 ml) was heated at 130 °C for 2.5 h, then allowed to cool. Water was added and the mixture was extracted with chloroform. The extract was dried over anhydrous magnesium sulfate. Chloroform was distilled off and the residue was recrystallized from acetonitrile to give a yellow crystalline powder, mp > 290 °C. Yield 0.4 g (48%). ^{1}H-NMR (pyridine-d_5) δ: 3.48 (3H, s, N–CH_3), 3.52 (3H, s, N–CH_3), 3.72 (3H, s, N–CH_3), 3.82 (3H, s, N–CH_3). IR ν_{max}^{KBr} cm^{-1}: 1730, 1710, 1670 (C=O). MS *m/z*: 344 (M^+). *Anal.* Calcd for $C_{13}H_{12}N_8O_4$: C, 45.35; H, 3.51; N, 32.55. Found: C, 45.30; H, 3.44; N, 32.82.

Method B: A solution of dimethyl sulfate (1 g) in acetone (5 ml) was added dropwise to a suspension of **9** (0.5 g) and anhydrous potassium carbonate (0.45 g) in acetonitrile. The mixture was stirred for 2 h at 0—5 °C and then for 5 h at room temperature. The solvent was distilled off and water was added to the residue, which was extracted with chloroform. The extract was dried over anhydrous magnesium sulfate. Chloroform was distilled off and the residue was recrystallized from acetonitrile, mp > 290 °C. Yield 0.37 g (72%). The IR, MS and ^{1}H-NMR spectra of this compound were identical with those of the compound prepared by method A.

8-Amino-7-[(*N*,*N*-dimethyl)aminomethyleneamino]-1,3-dimethylxanthine (5)——A mixture of **2** (0.21 g, 1 mmol) and *N*,*N*-dimethylformamide dimethylacetal (0.18 g, 3 mmol) in DMF (5 ml) was refluxed for 1 h. The solvent was distilled off and the residue was recrystallized from ethanol to give colorless fine needles, mp 221—222 °C, yield 0.17 g (64%). ^{1}H-NMR ($CDCl_3$) δ: 3.12 (3H,s, N–CH_3), 3.15 (3H, s, N–CH_3), 3.36 (3H, s, N–CH_3), 3.48 (3H, s, N–CH_3), 5.17 (2H, s, NH_2), 8.48 (1H, s, N=CH–). IR ν_{max}^{KBr} cm^{-1}: 3350—3200 (NH_2), 1690, 1660 (C=O). MS *m/z*: 265 (M^+). *Anal.* Calcd for $C_{10}H_{15}N_7O_2$: C, 45.28; H, 5.70; N, 36.96. Found: C, 45.45; H, 5.87; N, 36.68.

8-Amino-7-[(*n*-butylidene)amino]-1,3-dimethylxanthine (6)——A mixture of **2** (0.1 g, 0.48 mmol) and *n*-butyraldehyde (0.5 g, 7 mmol) in acetic acid (5 ml) was heated for a few minutes at 80—90 °C. Acetic acid was distilled off. The residue was washed with *n*-hexane and recrystallized from ethanol to give colorless prisms, mp 180 °C (dec.), yield 0.1 g (80%). ^{1}H-NMR ($CDCl_3$) δ: 1.00 (3H, t, CH_3), 1.46 (2H, m, CH_2), 2.37 (2H, m, CH_2), 3.36 (3H, s, N–CH_3), 3.45 (3H, s, N–CH_3), 5.88 (2H, s, NH_2), 8.97 (1H, t, –N=CH–). IR ν_{max}^{KBr} cm^{-1}: 3410, 3250 (NH_2), 1690, 1640 (C=O). MS *m/z*: 264 (M^+). *Anal.* Calcd for $C_{11}H_{16}N_6O_2$: C, 49.99; H, 6.10; N, 31.80. Found: C, 50.28; H, 5.81;

N, 31.48.

8-Amino-7-(2,2-dimethoxy-1-methylethyleneamino)-1,3-dimethylxanthine (7)——A mixture of **2** (0.15 g, 0.7 mmol and pyruvic aldehyde dimethylacetal (1.5 g, 12.7 mmol) in acetic acid (5 ml) was heated for 10 min at 100 °C. Acetic acid was distilled off and the residue was recrystallized from benzene to give pale yellow needles, mp 184—186 °C, yield 0.18 (81%). ^{1}H-NMR ($CDCl_3$) δ: 2.16 (3H, s, C–CH_3), 3.58 (3H, s, N–CH_3), 3.64 (6H, s, 2 × O–CH_3), 4.84 (1H, s, –$CH(OCH_3)_2$), 4.96 (2H, s, C_8–NH_2). IR ν_{max}^{KBr} cm^{-1}: 3340, 3150 (NH_2), 1700, 1650 (C=O). MS *m/z*: Calcd for $C_{12}H_{18}N_6O_4$: 310.13914. Found: 310.13880.

Table III. Analytical Data for 3-Alkylamino-2-alkylcarbamoyl-6,8-dimethyl[1,2,4]-triazino[3,2-*f*]purine-7,9(6*H*,8*H*)-diones (**10a—g**)

Compd. No.	mp (°C) (recryst. solvent)	Yield (%)	Formula	Analysis (%) Calcd (Found)		
				C	H	N
10a	241—242 (benzene–*n*-hexane (1 : 1))	94	$C_{24}H_{22}N_8O_3 \cdot H_2O$	59.01 (59.18	4.95 4.88	22.94 22.90)
10b	132—134 (benzene–*n*-hexane (1 : 2))	81	$C_{18}H_{26}N_8O_3$	53.72 (53.37	6.51 6.38	27.84 27.83)
10c	96—98 (pet. ether–EtOH (10 : 1))	63	$C_{22}H_{34}N_8O_3$	57.62 (57.47	7.47 7.69	24.44 24.40)
10d	71—73 (pet. ether)	75	$C_{26}H_{42}N_8O_3$	514.33836 (514.33408)		
10e	107—109 (*n*-hexane–ether (10 : 1))	65	$C_{30}H_{50}N_8O_3$	63.13 (63.44	8.83 8.93	19.63 19.30)
10f	280 (dec.) (H_2O–EtOH (1 : 1))	84	$C_{14}H_{18}N_8O_5$	44.45 (44.75	4.80 4.61	29.62 29.31)
10g	290 (H_2O–EtOH (1 : 1))	95	$C_{16}H_{22}N_8O_5$	47.29 (46.96	5.46 5.22	27.57 27.36)

Table IV. IR, Mass and ^{1}H-NMR Spectral Data for 3-Alkylamino-2-alkylcarbamoyl-6,8-dimethyl[1,2,4]triazino[3,2-*f*]purine-7,9(6*H*,8*H*)-diones (**10a—g**)

Compd.	ν_{max}^{KBr} cm^{-1}	MS *m/z* (M^+)	^{1}H-NMR $\delta_{ppm}^{CDCl_3}$
10a	3430, 3250 (NH) 1700, 1660 (C=O)	470	3.42 (3H, s, N–Me), 3.66 (3H, s, N–Me), 4.56 (2H, d, J=6 Hz, CH_2), 4.80 (2H, d, J=6 Hz, CH_2), 7.30 (10H, m, 2 × Ph), 9.40 (1H, t, J=6 Hz, NH), 10.03 (1H, t, J=6 Hz, NH)
10b	3450, 3250 (NH) 1700, 1660, 1640 (C=O)	402	3.44 (3H, s, N–Me), 3.66 (3H, s, N–Me), 0.96 (6H, m, 2 × Me), 1.20—1.80 (8H, m, 4 × CH_2), 3.36 (2H, m, N–CH_2), 3.54 (2H, m, N–CH_2), 8.24 (1H, br, NH), 9.68 (1H, br, NH)
10c	3450, 3230 (NH) 1700, 1660, 1640 (C=O)	458	3.38 (3H, s, N–Me), 3.66 (3H, s, N–Me), 0.96 (6H, m, 2 × Me), 1.20—1.80 (16H, m, 8 × CH_2), 3.50 (4H, m, 2 × N–CH_2), 8.44 (1H, br, NH), 9.68 (1H, br, NH)
10d	3520, 3440 (NH) 1700, 1660 (C=O)	514	3.42 (3H, s, N–Me), 3.64 (3H, s, N–Me), 0.83 (6H, m, 2 × Me), 1.20—1.80 (24H, m, 12 × CH_2), 3.34 (4H, m, 2 × N–CH_2), 8.32 (1H, br, NH), 9.68 (1H, br, NH)
10e	3400, 3440 (NH) 1700, 1660 (C=O)	570	3.44 (3H, s, Me), 3.66 (3H, s, Me), 0.86 (6H, m, 2 × Me), 1.20—1.70 (32H, m, 16 × CH_2), 3.58 (4H, m, 2 × N–CH_2), 8.32 (1H, br, NH), 9.68 (1H, br, NH)
10f	3120—3610 (OH) 3240, 3370 (NH) 1700, 1660 (C=O)	378	
10g	3600—3050 (OH) 3350, 3220 (NH) 1700, 1660 (C=O)	406	

8-Amino-1,3-dimethyl-7-[(2,4,6-trioxopyrimidine-5-ylidene)amino]xanthine (8)——A mixture of **2** (0.84 g, 4 mmol) and alloxan (1.28 g, 8 mmol) was dissolvd in hot water (70 °C, 50 ml) and the solution was stirred for 30 min. The resulting crystals were collected by filtration and washed with ethanol to give colorless crystals, mp 265—267 °C. Yield 0.84 g (63%). IR ν_{max}^{KBr} cm^{-1}: 3400, 3350 (NH_2), 1730, 1680, 1640 (C=O). This compound was used for the next reaction without further purification.

7,9-Dimethylpurino[7,8-*g*]-6-azapteridine-1,3,8,10(2*H*,4*H*,7*H*,9*H*)-tetrone (9)——Compound **8** (0.5 g) and boric acid (0.95 g) were dissolved in hot acetic acid (100 °C, 60 ml) and the mixture was stirred for 1 h. The solvent was distilled off and the residue was recrystallized from acetonitrile to give yellow fine crystals, mp >290 °C. Yield 0.38 (80%). ^{1}H-NMR ($CDCl_3$–DMSO-d_6) δ: 3.21 (3H, s, N–CH_3), 3.44 (3H, s, N–CH_3), 6.72 (1H, s, NH), 10.78 (1H, br, NH). IR ν_{max}^{KBr} cm^{-1}: 3090, 3190 (NH), 1710, 1660 (C=O). *Anal.* Calcd for $C_{11}H_8N_8O_4 \cdot H_2O$: C, 39.53; H, 3.02; N, 33.52. Found: C, 39.94; N, 33.22.

General Procedure for the Synthesis of 3-Alkylamino-2-alkylcarbamoyl-6,8-dimethyl[1,2,4]triazino[3,2-*f*]purine-7,9(6*H*,8*H*)-diones (10a—g)——A mixture of **4** (0.5 g) and an alkylamine (30 ml) was refluxed for 8 h, then allowed to cool. Water was added and the mixture was neutralized with hydrochloric acid to give crystals, which were collected by filtration. When crystals were not obtained, the mixture was extracted with chloroform. The extract was washed with water and dried over anhydrous magnesium sulfate. The solvent was distilled off and the residue was column-chromatographed on silica gel and eluted with chloroform. The solvent was distilled off and the residue was recrystallized from a suitable solvent. Physical data are listed in Tables III and IV.

3-(2-Acetoxyethylamino)-2-(2-acetoxyethylcarbamoyl)-6,8-dimethyl[1,2,4]triazino[3,2-*f*]purine-7,9(6*H*,8*H*)-dione (11a)——A mixture of **10f** (100 mg), acetic anhydride (4 ml) and acetic acid (20 ml) was refluxed for 1.5 h. The solvent was distilled off and the residue was subjected to silica gel column chromatography with chloroform as the eluent. The eluate was collected and the solvent was distilled off. The residue was recrystallized from ethanol to give prisms, mp 84—87 °C, yield 120 mg (98%). IR ν_{max}^{KBr} cm^{-1}: 3380, 3220 (NH), 1740, 1700, 1640 (C=O). ^{1}H-NMR ($CDCl_3$) δ: 2.08 (6H, s, 2 × $COCH_3$), 3.44 (3H, s, N–CH_3), 3.64 (3H, s, N–CH_3), 3.76 (4H, m, 2 × CH_2), 4.30 (4H, m, 2 × CH_2). MS *m/z*: 462 (M^+). *Anal.* Calcd for $C_{18}H_{22}N_8O_7$: C, 46.76; H, 4.80; N, 24.23. Found: C, 46.74; H, 4.97; N, 24.20.

3-(3-Acetoxypropylamino)-2-(3-acetoxypropylcarbamoyl)-6,8-dimethyl[1,2,4]triazino[3,2-*f*]purine-7,9(6*H*,8*H*)-dione (11b)——A mixture of **10g** (100 mg), acetic anhydride (4 ml) and acetic acid (20 ml) was refluxed for 1.5 h and treated as described for **11a**; mp 161—162 °C. Yield 112 mg (93%). IR ν_{max}^{KBr} cm^{-1}: 3250, 3420 (NH), 1740, 1700, 1660 (C=O). ^{1}H-NMR ($CDCl_3$) δ: 2.12 (3H, s, $COCH_3$), 2.08 (3H, s, $COCH_3$), 3.44 (3H, s, N–CH_3), 3.66 (3H, s, N–CH_3), 3.62 (4H, m, 2 × N–CH_2), 2.02 (4H, m, 2 × CH_2–), 4.20 (4H, m, 2 × –OCH_2–), 8.76 (1H, br, NH), 9.80 (1H, br, NH). MS *m/z*: 490 (M^+). *Anal.* Calcd for $C_{20}H_{26}N_8O_7$: C, 48.98; H, 5.34; N, 22.85. Found: C, 48.69; H, 5.35; N, 22.82.

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References and Notes

1) J. Sakakibara, T. Ueda, T. Ohsaki, K. Takeya, and S. Ando, *Yakugaku Zasshi*, **105**, 730 (1985).
2) J. W. Jones and R. K. Robins, *J. Am. Chem. Soc.*, **82**, 3773 (1960).
3) 7-Aminotheophylline has been synthesized; its NH_2 signal in the ^{1}H-NMR (DMSO-d_6) spectrum is at δ 6.33 ppm [E. M. Karpitschka, G. Smole, and W. Kloetze, *Sci. Pharm.*, **49**, 453 (1981)].
4) This compound was purchased from Tokyo Kasei Kogyo Co., Ltd.
5) R. Kuhn and F. Weygand, *Chem. Ber.*, **68**, 1282 (1935).
6) D. J. Brown, "Fused Pyrimidines, Part I," Interscience Publisher, New York, 1967, p. 112; R. M. Hoskinson, *Aust. J. Chem.*, **21**, 1913 (1968); T. Ueda, N. Oda, and I. Ito, *Chem. Pharm. Bull.*, **28**, 2144 (1980).
7) A. Burger, "Medicinal Chemistry," 3rd ed., Part I, Wiley-Interscience, New York, 1970, pp. 680—783.
8) C. O. Wilson, O. Gisveld, and R. F. Doerge, "Textbook of Organic Medicinal and Pharmaceutical Chemistry," 7th ed., T. B. Lippin cott Co., Philadelphia, 1977, p. 578.
9) M. Asano, K. Aoi, and T. Matsuda, *J. Pharmacol. Exp. Ther.*, **223**, 207 (1982).
10) M. Asano and H. Hidaka, *J. Pharmacol. Exp. Ther.*, **234**, 476 (1985).

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Synthesis of Macrocyclic Terpenoids by Intramolecular Cyclization. XI.[1] Total Synthesis of Zerumbone

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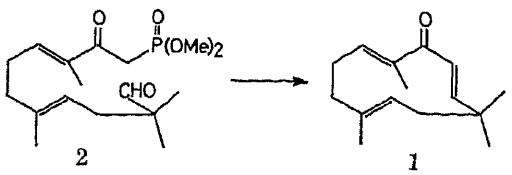
Zerumbone (1), an eleven-membered ring sesquiterpene isolated from *Zingiber zerumbet*, was synthesized by means of an intramolecular Wittig-type reaction of the keto-phosphonate **2**.

Keywords——zerumbone; synthesis; humulane sesquiterpene; intramolecular Wittig reaction; eleven-membered ring formation; 2,6,9,9,13,17,20,20-octamethyl-2,6,10,13,17,21-cyclodocosahexaene-1,12-dione

Zerumbone (**1**) is a humulane-type sesquiterpene which is the major component of the essential oil of *Zingiber zerumbet*[2] and is known to exhibit plant growth-regulatory and cytotoxic activities.[3,4] This structurally and biologically interesting compound has been synthesized only by the oxygenation of humulene.[5]

In a previous paper[1] we disclosed that an intramolecular Wittig-type reaction is an effective method of synthesizing oxygenated macrocyclic terpenoids. Considering the α,β-unsaturated ketone functionality in **1**, this compound should be a suitable target for synthesis by this methodology. Furthermore, it is of interest to know whether the intramolecular olefination takes place as expected in the case of medium-sized ring formation, since a severe ring strain would exist in the transition state.

Although the yield was not satisfactory, we achieved the synthesis of **1** by means of the following intramolecular olefination. The present synthesis reveals that the method is applicable to the formation of an eleven-membered ring.



2 1

Chart 1

Reaction of the lithio derivative of methyl isobutyrate with geranyl bromide (**3**) afforded the ester **4** in 60% yield. The Δ^9-double bond in **4** was cleaved by the following sequence in 39% overall yield: (i) reaction of **4** with *N*-bromosuccinimide in aqueous tetrahydrofuran (THF) followed by treatment with potassium carbonate to yield the epoxide **5**; (ii) acid-catalyzed epoxide ring opening to the diol **6**; (iii) reduction with lithium aluminum hydride to the triol **7**, and subsequent cleavage of diol moiety to furnish the aldehyde **8**. An α,β-unsaturated ester group was then introduced by means of a Wittig reaction using (ethoxycarbonylethylidene)triphenylphosphorane to give the *E*-enoate **9** selectively in 83% yield. After oxidation with pyridinium chlorochromate (PCC) to **10**, the resulting aldehyde

group was protected as an acetal and the product **11** was treated with the lithium anion of dimethyl methylphosphonate to afford the keto-phosphonate **12** in 59% overall yield. The key intermediate **2** was obtained by hydrolysis of the acetal group in **12**. The intermediate **2** thus obtained was treated with sodium hydride at high dilution in dimethoxyethane (DME) at 60 °C.[6] The product was a mixture of several compounds from which zerumbone (**1**) was isolated in 3% yield. The identity of the synthetic compound with the natural product was confirmed by infrared (IR), proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectral comparisons and a mixed melting point test. The major product formed in 55% yield was found to be a 22-membered macrocyclic dimer **13** on the basis of the mass spectrum (MS) (M^+, *m/z* 436) and ^{1}H- and ^{13}C-NMR spectra, which closely resembled those of **1**. The formation of the dimer **13** could not be suppressed even under conditions of higher dilution. Other conditions[7] examined so far afforded similar or smaller amounts of **1**. Interestingly, 400 MHz ^{1}H-NMR spectroscopy of the reaction products failed to detect any of the geometrical isomer of **1**.

The present results provide a new entry to the synthesis of humulane-type sesquiterpenes,[8] although they suggest a limitation of the intramolecular Wittig reaction approach with respect to ring size.

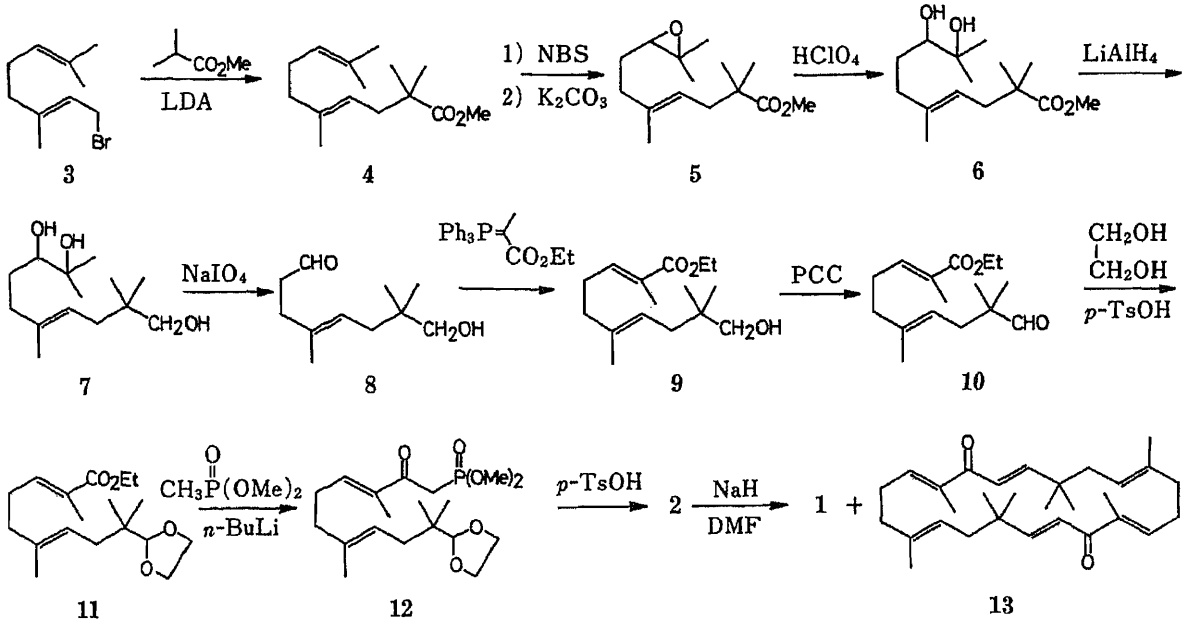


Chart 2

Experimental

^{1}H- and ^{13}C-NMR were recorded on a JEOL GX-400 or JEOL FX90Q spectrometer in $CDCl_3$ solution with $(CH_3)_4Si$ as an internal standard. IR were taken on a Shimadzu IR-27G spectrometer. MS were measured on a Shimadzu LKB-9000 spectrometer. High-resolution mass spectra (HRMS) were obtained on a JEOL HX-100 spectrometer.

Methyl (*E*)-2,2,5,9-Tetramethyl-4,8-decadienoate (4)——A hexane solution of *n*-butyllithium (0.123 mol) was added dropwise to a stirred solution of diisopropylamine (10.5 g, 0.104 mol) in dry THF (300 ml) under argon at −30 °C. After being stirred at 0 °C for 30 min, the mixture was cooled to −70 °C with a dry ice-acetone bath. Methyl isobutyrate (11.9 ml, 0.104 mol) was then added dropwise to the mixture over 1 h, followed by addition of geranyl bromide (**3**) (20.5 g, 0.095 mol) in dry THF (50 ml) over 1 h. The mixture was stirred for 30 min at −70 °C, then the cooling bath was removed and stirring was continued overnight at room temperature. The reaction mixture was concentrated to one-third of its original volume, diluted with water and extracted with ether. The combined ether layers were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo*. The residue was chromatographed on 200 g

of silica gel (benzene–hexane, 1 : 1) to give 13.5 g (60%) of **4** as a colorless oil. IR (film): 1738 cm^{-1}. ^{1}H-NMR δ: 1.14 (6H, s), 1.59 (6H, br s), 1.68 (3H, br s), 3.65 (3H, s), 5.08 (2H, m). MS *m/z*: 238 (M^+), 109 (base peak). HRMS *m/z*: 238.1918 (Calcd for $C_{15}H_{26}O_2$, 238.1933).

Methyl (*E*)-8,9-Epoxy-2,2,5,9-tetramethyl-4-decenoate (5)——*N*-Bromosuccinimide (8.97 g, 0.050 mol) was added in small portions over 30 min to an ice-cooled solution of **4** (8.0 g, 0.034 mol) in THF (90 ml) and water (30 ml) with stirring. The reaction mixture was concentrated *in vacuo* to one-third of its original volume, diluted with water and extracted with ether. The combined extracts were washed with saturated $NaHCO_3$ solution and brine, then dried ($MgSO_4$) and concentrated *in vacuo*. The residue was chromatographed on 50 g of silica gel (benzene) to give 6.65 g (59%) of the bromohydrin as a colorless oil, which was immediately used for the next reaction.

A mixture of the bromohydrin (6.65 g, 0.02 mol) and anhydrous potassium carbonate (11.0 g, 0.08 mol) in methanol (100 ml) was vigorously stirred at room temperature for 30 min. The precipitate was filtered off and washed with methanol. The combined filtrates were concentrated *in vacuo* and the residue was taken up in 150 ml of ether. The ether solution was washed with brine, dried ($MgSO_4$) and concentrated *in vacuo* to afford 4.65 g (92%) of **5** as a colorless oil. IR (film): 1746 cm^{-1}. ^{1}H-NMR δ: 1.14 (6H, s), 1.25 (3H, s), 1.30 (3H, s), 1.62 (3H, br s), 2.70 (1H, t, J = 6.4 Hz), 3.63 (3H, s), 5.14 (1H, br t, J = 7.4 Hz). MS *m/z*: 254 (M^+), 109 (base peak). HRMS *m/z*: 254.1872 (Calcd for $C_{15}H_{26}O_3$, 254.1882).

Methyl (*E*)-8,9-Dihydroxy-2,2,5,9-tetramethyl-4-decenoate (6)——A stirred solution of **5** (4.6 g, 0.018 mol) in THF–water (3 : 1, 200 ml) was treated with 0.1 ml of 70% perchloric acid. The mixture was stirred at room temperature for 4 h and then concentrated *in vacuo* to one-third of its original volume. The residue was taken up in 200 ml of ether and the ether solution was washed with brine, dried ($MgSO_4$) and then concentrated *in vacuo*. The residue was chromatographed on 40 g of silica gel ($CHCl_3$) to afford 4.64 g (95%) of the diol **6** as a colorless oil. IR (film): 3450, 1732 cm^{-1}. ^{1}H-NMR δ: 1.16 (12H, s), 1.60 (3H, br s), 3.32 (1H, ddd, J = 9.5, 5.1, 2.5 Hz, changed to dd (J = 9.5, 2.5 Hz) on treatment with D_2O), 3.66 (3H, s), 5.15 (1H, br t, J = 7.4 Hz). MS *m/z*: 254 ($M^+ - H_2O$), 102 (base peak).

(*E*)-8,9-Dihydroxy-2,2,5,9-tetramethyl-4-decenol (7)——A solution of **6** (4.6 g, 0.02 mol) in 20 ml of dry THF was added over 30 min to a suspension of $LiAlH_4$ (0.8 g) in 80 ml of dry THF with stirring at 0 °C. The mixture was stirred overnight at room temperature, and then excess reagent was decomposed by careful addition of water. The mixture was acidified with 2 N HCl and extracted with ether. The combined ether layers were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo*. The residue was chromatographed on 40 g of silica gel ($CHCl_3$–methanol, 96 : 4) to give 3.8 g (92%) of **7** as a colorless oil. IR (film): 3400, 2500 cm^{-1}. ^{1}H-NMR δ: 0.86 (3H, s), 1.15 (3H, s), 1.19 (3H, s), 1.62 (3H, br s), 5.29 (1H, br t, J = 7.7H). MS *m/z*: 226 ($M^+ - H_2O$), 71 (base peak).

(*E*)-8-Hydroxy-4,7,7-trimethyl-4-octenal (8)——Sodium periodate (3.66 g, 0.017 mol) was added in small portions over 1 h to a stirred solution of **7** (3.80 g, 0.016 mol) in THF–water (3 : 1, 80 ml) at room temperature. After being stirred for 3.5 h, the reaction mixture was poured into water and extracted with ether. The combined extracts were dried ($MgSO_4$) and evaporated *in vacuo*. The residue was chromatographed on 50 g of silica gel (CH_2Cl_2) to afford 2.35 g (82%) of the aldehyde **8** as a colorless oil. IR (film): 3400, 1720 cm^{-1}. ^{1}H-NMR δ: 0.84 (6H, s), 1.62 (3H, br s), 3.30 (2H, br s), 5.25 (1H, br t, J = 7.4 Hz), 9.78 (1H, t, J = 2.6 Hz); MS *m/z*: 166 (M^+), 109 (base peak).

Ethyl (*E,E*)-10-Hydroxy-2,6,9,9-tetramethyl-2,6-decadienoate (9)——A mixture of **8** (2.30 g, 12.5 mmol) and (ethoxycarbonylethylidene)triphenylphosphorane (4.52 g, 12.5 mmol) in 50 ml of dry benzene was stirred at room temperature for 3 h. The reaction mixture was evaporated *in vacuo* and the residue was chromatographed on 60 g of silica gel (benzene–CH_2Cl_2, 1 : 1) to give 2.80 g (83%) of the ester **9** as a colorless oil. IR (film): 3450, 1710, 1650 cm^{-1}. ^{1}H-NMR δ: 0.85 (6H, s), 1.28 (3H, t, J = 7.2 Hz), 1.62 (3H, br s), 1.82 (3H, br s), 3.32 (2H, br d, J = 5.6 Hz, changed to br s on treatment with D_2O), 4.18 (2H, q, J = 7.2 Hz), 5.23 (1H, br t, J = 7.7 Hz), 6.72 (1H, br t, J = 6.5 Hz). MS *m/z*: 268 (M^+), 128 (base peak). HRMS *m/z*: 268.2033 (Calcd for $C_{16}H_{28}O_3$, 268.2038).

Ethyl (*E,E*)-9-Formyl-2,6,9-trimethyl-2,6-decadienoate (10)——Pyridinium chlorochromate (13.0 g, 60.2 mmol) was added to a solution of **9** (2.70 g, 10.1 mmol) in CH_2Cl_2 (150 ml) and the mixture was stirred for 1 h at room temperature. The reaction mixture was filtered through a bed of celite and the residue was washed with CH_2Cl_2. The combined filtrates were washed successively with 2 N HCl, saturated $NaHCO_3$ and brine, and then dried ($MgSO_4$). Evaporation of the solvent and chromatography of the residue on 50 g of silica gel (benzene) yielded 1.90 g (70%) of **10** as a colorless oil. IR (film): 1725, 1710, 1650 cm^{-1}. ^{1}H-NMR δ: 0.99 (6H, s), 1.28 (3H, t, J = 7.2 Hz), 1.60 (3H, br s), 1.82 (3H, br s), 4.18 (2H, q, J = 7.2 Hz), 5.10 (1H, br t, J = 7.7 Hz), 6.70 (1H, br t, J = 6.4 Hz), 9.48 (1H, s). MS *m/z*: 266 (M^+), 128 (base peak). HRMS *m/z*: 266.1864 (Calcd for $C_{16}H_{26}O_3$, 266.1882).

Ethyl (*E,E*)-9-(1,3-Dioxolan-2-yl)-2,6,9-trimethyl-2,6-decadienoate (11)——A mixture of **10** (1.80 g, 6.8 mmol), ethylene glycol (3.0 g) and *p*-toluenesulfonic acid (20 mg) in 100 ml of benzene was heated under reflux for 1.5 h with stirring, during the time water was removed by a Dean–Stark water separator. After cooling, the reaction mixture was washed with saturated $NaHCO_3$, dried ($MgSO_4$). Evaporation of the solvent yielded the acetal **11** (2.00 g, 95%) as a colorless oil. IR (film): 1710, 1650 cm^{-1}. ^{1}H-NMR δ: 0.86 (6H, s), 1.29 (3H, t, J = 7.2 Hz), 1.62 (3H, br s), 1.84 (3H, br s), 3.90 (4H, m), 4.18 (2H, q, J = 7.2 Hz), 4.54 (1H, s), 5.23 (1H, br t, J = 7.7 Hz), 6.75 (1H, br t, J = 6.5 Hz). MS *m/z*: 310 (M^+), 183 (base peak). HRMS *m/z*: 310.2149 (Calcd for $C_{18}H_{30}O_4$, 310.2144).

Dimethyl (*E,E*)-10-(1,3-Dioxolan-2-yl)-3,7,10-trimethyl-2-oxo-3,7-undecadienylphosphonate (12)——A hexane

solution of *n*-butyllithium (6.40 mmol) was added dropwise to a stirred solution of dimethyl methylphosphonate (0.7 ml, 6.45 mmol) in dry THF (20 ml) under argon at −78 °C, and the mixture was stirred for 15 min at −78 °C. A solution of **11** (1.0 g, 3.23 mmol) in dry THF (4 ml) was added over 15 min. After being stirred at −78 °C for 30 min, the mixture was poured into water and extracted with ether. The combined ether layers were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo*. The residue was chromatographed on 20 g of silica gel. Elution with benzene afforded recovered **11**. Further elution with CH_2Cl_2 gave 0.68 g (89%) of **12** as a colorless oil. IR (film): 1670, 1645 cm^{-1}. ^{1}H-NMR δ: 0.85 (6H, s), 1.62 (3H, br s), 1.80 (3H, br s), 3.35 (2H, d, J=22.8 Hz), 3.76 (6H, d, J=11.0 Hz), 3.89 (4H, m), 4.54 (1H, s), 5.28 (1H, br t, J=7.7 Hz), 6.72 (1H, br t, J=6.5 Hz). MS *m/z*: 388 (M^+), 206 (base peak). HRMS *m/z*: 388.2007 (Calcd for $C_{19}H_{33}O_6P$, 388.2015).

Dimethyl (*E*,*E*)-10-Formyl-3,7,10-trimethyl-2-oxo-3,7-undecadienyl phosphonate (2)——A mixture of **12** (650 mg, 1.68 mmol) and *p*-toluenesulfonic acid (20 mg) in acetone–water (2 : 1, 20 ml) was heated under gentle reflux for 2 h. After cooling, the mixture was diluted with water and extracted with ether. The combined extracts were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo* to give 550 mg (95%) of the aldehyde **2** as a colorless oil. IR (film): 1725, 1665, 1640 cm^{-1}. ^{1}H-NMR δ: 1.04 (6H, s), 1.63 (3H, br s), 1.80 (3H, br s), 3.38 (2H, d, J=22.8 Hz), 3.78 (6H, d, J=11.0 Hz), 5.12 (1H, br t, J=7.7 Hz), 6.67 (1H, br t, J=6.5 Hz), 9.46 (1H, s). MS *m/z*: 344 (M^+), 206 (base peak). HRMS *m/z*: 344.1751 (Calcd for $C_{17}H_{29}O_5P$, 344.1752).

Intramolecular Wadsworth–Emmons Olefination of 2——A solution of **2** (300 mg, 0.87 mmol) in 500 ml of dry DME was heated at 60 °C under argon and 52 mg of sodium hydride (60% mineral oil dispersion, 1.30 mmol) was added with stirring. The mixture was stirred at 60 °C for 16 h, then allowed to cool. The solvent was evaporated off *in vacuo* and the residue was taken up in 200 ml of ether. The ether solution was washed with brine, dried ($MgSO_4$) and concentrated *in vacuo*. The residue was chromatographed on 30 g of silica gel. Elution with benzene gave a crystalline solid (6 mg, 3%) which was recrystallized from ethanol to yield **1** as colorless needles, mp 64—65 °C (lit.[2] 67—69 °C). The ^{1}H-NMR and ^{13}C-NMR spectra were identical with those of natural zerumbone (**1**). Further elution with benzene–CH_2Cl_2 (1 : 1) afforded a crystalline solid (104 mg, 55%) which was recrystallized from ethanol to give the dimer **13** as colorless needles, mp 123—125 °C. IR (Nujol): 1662, 1616 cm^{-1}. ^{1}H-NMR δ: 1.07 (6H, s), 1.62 (3H, br s), 1.79 (3H, br s), 5.09 (1H, br t, J=7.8 Hz), 6.39 (1H, d, J=15.6 Hz), 6.42 (1H, br t, J=7.2 Hz), 6.66 (1H, d, J=15.6 Hz). ^{13}C-NMR δ: 11.7 (q), 15.8 (q), 26.5 (t), 26.7 (q × 2), 37.9 (s), 38.7 (t), 40.8 (t), 121.9 (d), 122.6 (d), 135.6 (s), 138.1 (s), 142.3 (d), 154.6 (d), 192.8 (s). MS *m/z*: 436 (M^+), 151 (base peak). HRMS *m/z*: 436.3333 (Calcd for $C_{30}H_{44}O_2$, 436.3342).

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References

1) Part X: M. Kodama, Y. Shiobara, H. Sumitomo, K. Fukuzumi, Y. Miyamoto, and H. Minami, *Tetrahedron Lett.*, **27**, 2157 (1986).
2) S. Dev, *Tetrahedron*, **8**, 171 (1960); N. P. Damodaran and S. Dev, *Tetrahedron Lett.*, **1965**, 1977.
3) P. S. Kalsi, O. S. Singh, and B. R. Chhabra, *Phytochemistry*, **17**, 576 (1978); *idem*, *Experientia*, **35**, 481 (1979).
4) H. W. P. Mathes, B. Luu, and G. Ourisson, *Phytochemistry*, **19**, 2643 (1980).
5) H. Shirahama, B. R. Chhabra, and T. Matsumoto, *Chem. Lett.*, **1981**, 717.
6) K. C. Nicolau, S. P. Seitz, M. R. Pavia, and N. A. Petasis, *J. Org. Chem.*, **44**, 4011 (1979).
7) K. C. Nicolau, S. P. Seitz, and M. R. Pavia, *J. Am. Chem. Soc.*, **104**, 2030 (1982); M. A. Blanchette, W. Choy, J. T. Davis, A. P. Essenfeld, S. Masamune, W. R. Roush, and T. Sakai, *Tetrahedron Lett.*, **25**, 2183 (1984).
8) For the synthesis of humulane-type compounds, see E. J. Corey and E. Hamanaka, *J. Am. Chem. Soc.*, **89**, 2758 (1964); O. P. Vig, B. Ran, K. S. Atwal, and S. S. Bari, *Ind. J. Chem.*, **14B**, 855 (1976); Y. Kitagawa, A. Itoh, S. Hashimoto, H. Yamamoto, and H. Nozaki, *J. Am. Chem. Soc.*, **99**, 3864 (1977); J. E. McMurry and J. R. Matz, *Tetrahedron Lett.*, **23**, 2723 (1982); T. Takahashi, K. Kitamura, and J. Tsuji, *ibid.*, **24**, 4695 (1983).

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Synthesis of 2-(5-Cholesten-3β-yloxy) Glycosides of *N*-Acetyl-D-neuraminic Acid Derivatives[1)]

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2-(5-Cholesten-3β-yloxy) α- and β-glycosides of *N*-acetyl-D-neuraminic acid were prepared under various conditions through a Koenigs–Knorr-like reaction. The stereochemistry of the products was confirmed by analysis of the nuclear magnetic resonance and circular dichroism spectra.

Keywords——*N*-acetylneuraminic acid; *O*-glycoside; cholesterol; NMR; CD; stereochemistry

N-Acetyl-D-neuraminic acid is widely distributed in membrane glycoprotein and glycolipids. Recently, we reported the stereochemistry,[2,3)] syntheses,[4)] and some biological activities[5,6)] of 2-*O*-glycosyl-[7)] and 2-*N*-substituted derivatives of *N*-acetyl-D-neuraminic acid.[8)] The stereochemistry of these compounds was studied by comparing the chemical shifts of 3-H (eq) in the nuclear magnetic resonance (NMR) spectra, and measuring the circular dichroism (CD) spectra, and the rate of hydrolysis of the glycosidic bonds with water.[7,8)]

In this paper, we wish to report the synthesis of cholesterol glycosides of *N*-acetyl-D-neuraminic acid derivatives for examination of neuritogenesis.[9)] Some cholesteryl glycosides have previously been prepared for studies on the effects of external carbohydrate determinants on liposome distribution[10)]: for example, 5-acetamido-2-*S*-[6-(5-cholesten-3β-yloxy)-hexyl]-3,5-dideoxy-2-thio-D-*glycero*-β-D-*galacto*-2-nonulopyranosonic acid was synthesized from methyl 5-acetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonate and 6-(5-cholesten-3β-yloxy)-1-thiohexane in the presence of boron trifluoride etherate.

Koenigs–Knorr-like reaction of methyl *N*-acetyl-4,7,8,9-tetra-*O*-acetyl-2-chloro-β-D-neuraminate[11)] (**1**) and cholesterol (**2**) under various conditions (Table I) gave α- and β-

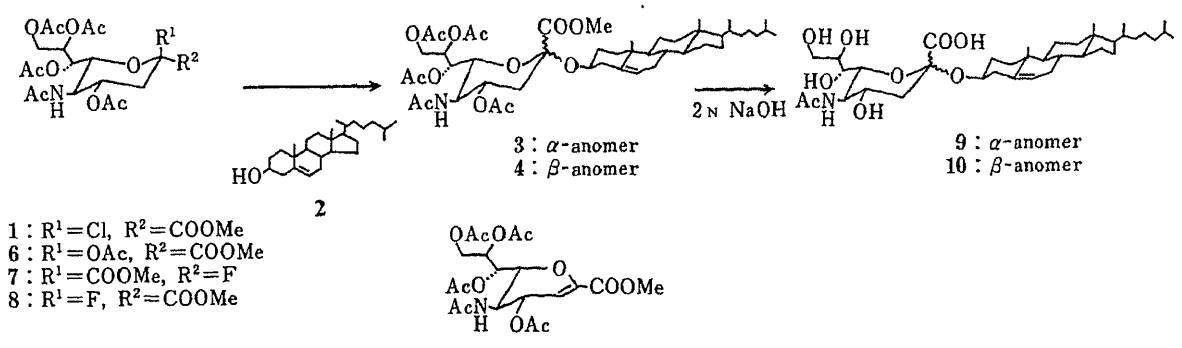

Chart 1

TABLE I. Isolated Yields of Cholest-5-en-3-yloxy Glycoside of *N*-Acetyl-D-neuraminic Acid Tetra-*O*-acetate after Koenigs–Knorr Reaction at 20—25 °C

Run	Compound No.	R^1	R^2	Catalyst	Solvent	Reaction time (d)	Total yield (%)[a]	Ratio of products 3	4	By-product 5 (%)[b]
1	1	Cl	COOMe	Ag_2CO_3	Benzene	3	20	4	1	19
2	1	Cl	COOMe	Ag_2CO_3/I_2	Benzene	3	22	11.5	1	21
3	1	Cl	COOMe	Ag salicylate	Benzene	1	27	7.2	1	35
							(34	12	1	42)[c]
4	1	Cl	COOMe	$AgClO_4$	CH_2Cl_2	1	58	1	1.5	10
5	1	Cl	COOMe	$AgOSO_2CF_3$	CH_2Cl_2	1	60	1	1	10
6	1	Cl	COOMe	$AgOCOCF_3$	CH_2Cl_2	1	21	2	1	10
7	1	Cl	COOMe	$Hg(CN)_2/HgBr_2$	CH_3NO_2	1	12	1	1	50
8	8	F	COOMe	$AgClO_4/SnCl_2$	Ether	1	8	1	1.3	—
9	8	F	COOMe	$BF_3 \cdot Et_2O$	CH_2Cl_2	1 (h)	56	0	1	42
10	7	COOMe	F	$BF_3 \cdot Et_2O$	CH_2Cl_2	1 (h)	16	1	6	10
11	7	COOMe	F	$AgOSO_2CF_3/SnCl_2$	Benzene	1	42	1	1.3	33
12	6	OAc	COOMe	$TMSOSO_2CF_3$	CH_2Cl_2	1	5	0	1	—

a) Isolated yield calculated from cholesterol. *b*) Isolated yield calculated from *N*-acetylneuraminic acid derivatives. *c*) Data of the best run.

anomers of methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-(5-cholesten-3β-yloxy)-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranosonate (**3** and **4**).

As can be seen from Table I, when silver perchlorate or silver trifluoromethanesulfonate was used as a catalyst, the yield was about 60% after chromatographic separation. When silver trifluoroacetate was used, the yield was low (21%), but the α-anomer (**3**) was obtained in 2 : 1 ratio. When a sparingly soluble catalyst, silver carbonate, was used, the α-anomer (**3**) was formed in about 16% yield with 4% of the β-anomer (**4**) after 3 d. When iodine was added to this reaction mixture, the α-anomer (**3**) was obtained in 11.5 : 1 ratio. In a case of silver salicylate catalyst, the α-anomer (**3**) was obtained in 7.2 : 1 ratio. Mercury salts were not good catalysts, because the anomeric mixture (1 : 1) was formed in 12% yield together with a large amount of the 2,3-dehydro derivative[11] (**5**; 50%).

Koenigs–Knorr-like reaction of methyl *N*-acetyl-4,7,8,9-tetra-*O*-acetyl-2-fluoro-α- (and -β-)-D-neuraminate[12,13] (**7**, **8**) and cholesterol (**2**) gave α- and β-anomeric mixture (**3**, **4**) as shown in Table I. This is very different from the known sugar chemistry.[14] On the other hand, the reaction of methyl *N*-acetyl-2,4,7,8,9-penta-*O*-acetyl-β-D-neuraminate[8] (**6**) with trimethylsilyl trifluoromethanesulfonate or that of methyl *N*-acetyl-4,7,8,9-tetra-*O*-acetyl-2-fluoro-β-D-neuraminate (**8**) with boron trifluoride etherate or tin(II) chloride–silver perchlorate gave only the β-anomer. In the latter reactions, methyl *N*-acetyl-4,7,8,9-tetra-*O*-acetyl-2,3-dehydro-2-deoxyneuraminate (**5**) was formed in 10—40% yield.

In conclusion, in view of the 1C conformational structure of *N*-acetylneuraminic acid, the β-anomer is more stable than the α-anomer. For this reason, formation of a β-glycosidic linkage is easier (for example, only the β-glycoside was formed from **8** by using boron trifluoride etherate *via* formation of an intimate ion pair). However, α-glycosidic linkage could be formed with high selectivity by using an insoluble catalyst, such as silver carbonate or silver salicylate, *via* a S_N2-like reaction mechanism.

Saponification of these acetates (**3**, **4**) with 2 N sodium hydroxide afforded the α- and β-anomers of *N*-acetyl-2-(5-cholesten-3β-yloxy)-D-neuraminic acid (**9**, **10**) in fair yields, and their sodium salts (**11**, **12**) were prepared with an equimolar amount of sodium hydroxide.

The stereochemistry of these compounds was evaluated from the proton nuclear

TABLE II. Selected ^{1}H-NMR Chemical Shifts of Sialic Acid Derivatives ($CDCl_3$; δ ppm)

Anomeric configuration	Methyl *N*-acetyl-2,4,7,8,9-penta-*O*-acetyl-D-neuraminate			**3**	**4**	*Δ*	**9**	**10**	*Δ*	**11**	**12**	*Δ*
	α	*β*	*Δ* (*α*−*β*)	*α*	*β*	*α*−*β*	*α*	*β*	*α*−*β*	*α*	*β*	*α*−*β*
3-H_{ax}	1.930	—	—	—	—	—	—	—	—	—	—	—
3-H_{eq}	2.718	2.550	+0.168	2.596	2.525	+0.071	2.43	2.39	+0.04	2.839	2.482	+0.357
4-H	4.924	5.258	−0.334	4.854	—	—	—	—	—	—	—	—

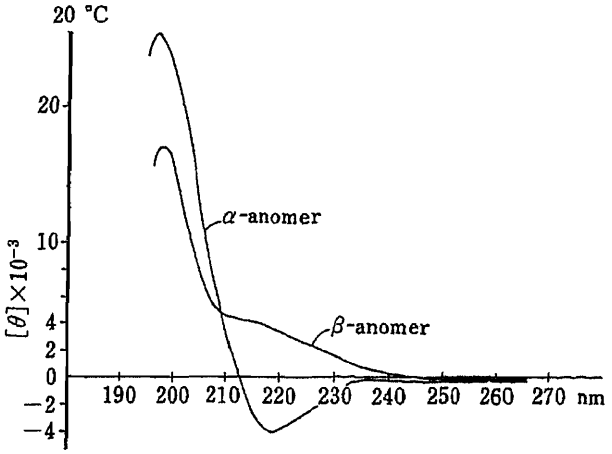

Fig. 1. CD Curves of 5-Acetamido-2-(5-cholesten-3*β*-yl)-3,5-dideoxy-D-*glycero*-*α*- and -*β*-D-*galacto*-nonulopyranosonic Acid (**9** and **10**) in Methanol

magnetic resonance (^{1}H-NMR) spectra and the CD spectra. The differences of the chemical shifts of the 3-H (eq) double-doublet of *α*-anomers (**3**, **9**, **11**) and *β*-anomers (**4**, **10**, **12**) are +0.07, +0.04 and +0.36 ppm, respectively, as shown in Table II. The anomeric configuration of *N*-acetylneuraminic acid derivatives can be inferred from the lower chemical shifts of 3-H (eq) in the range from δ 2.5 to 2.8. In conclusion, the stereochemistry at the anomeric position could be assessed from the NMR data.

We have already reported the CD spectra of a number of *N*-acetylneuraminic acid derivatives, and the peak at *ca.* 220 nm was assigned to the n–π^* Cotton effect of the carboxyl group. The negative sign of the Cotton effect is attributed to *α*-glycoside and the positive sign is attributed to *β*-glycoside.[2] Figure 1 shows the CD spectra of the *α*- and *β*-anomer of *N*-acetyl-2-(5-cholesten-3*β*-yloxy)-D-neuraminic acid (**9**, **10**). The *α*-anomer (**9**) shows a negative n–π^* Cotton effect around 220 nm, whereas the *β*-anomer (**10**) shows a positive n–π^* Cotton effect.

In conclusion, the stereochemistry at the C-2 position of the *N*-acetylneuraminic acid moiety was confirmed on the basis of the NMR and CD spectra.

Experimental

All temperatures are uncorrected. Infrared (IR) spectra were recorded with a JASCO A-2 spectrometer and NMR spectra on a Varian XL-400 spectrometer. Tetramethylsilane (TMS) in $CDCl_3$ or sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS) in D_2O was used as an internal reference. Field desorption (FD) mass spectra (MS) were obtained on a JEOL JMS-DX 300 spectrometer. Optical rotations were measured in a 50 mm cell with a JASCO DIP-181 automatic polarimeter, and CD data were obtained with a JASCO J-20 recording polarimeter.

Methyl 5-Acetamide-4,7,8,9-tetra-*O*-acetyl-2-(5-cholesten-3*β*-yl)-3,5-dideoxy-D-*glycero*-*α*- and -*β*-D-*galacto*-nonulopyranosonate (3, 4) from Methyl *N*-Acetyl-4,7,8,9-tetra-*O*-acetyl-2-chloro-*β*-D-neuraminate (1)—— Dichloromethane or benzene (10 ml), **1** (2 mmol) and silver salts (2.4 mmol) were added to a solution of cholesterol (**2**;

TABLE III. ^{1}H-NMR Data for 3 and 4 in $CDCl_3$ (400 MHz, δ ppm)

Compound	3		4	
Sialic acid moiety				
3-H_{ax}	—		—	
3-H_{eq}	2.596	1H, dd, J=5.2, 12.8 Hz	2.525	1H, dd, J=4.9, 13.1 Hz
4-H	4.853	1H, ddd, J=5.2, 9.8, 12.0 Hz	5.22—5.27	1H, m
5-H	4.02—4.09	2H, m	4.04—4.13	2H, m
6-H				
7-H	5.33—5.37	2H, m	5.34—5.38[a]	2H, m
8-H			5.07	1H, tt, J=2.0, 8.2 Hz
9-H[a]	4.166	1H, dd, J=5.8, 12.5 Hz	4.146	1H, dd, J=7.6, 12.5 Hz
9-H[b]	4.347	1H, dd, J=2.5, 12.8 Hz	4.880	1H, dd, J=1.8, 12.5 Hz
$NHCOCH_3$	1.883	3H, s	1.871	3H, s
$NHCOCH_3$	5.205	1H, d, J=10.1 Hz	5.34—5.38[a]	2H, m
$(COCH_3)_4$	2.026		2.021×2	
	2.031	3H×4, s×4	2.077	3H×4, s×3
	2.126		2.130	
	2.145			
$COOCH_3$	3.790	3H, s	3.798	3H, s
Cholesterol moiety				
18-CH_3	0.669	3H, s	0.670	3H, s
19-CH_3	0.985	3H, s	0.999	3H, s
3-H	3.650	1H, m	3.572	1H, m

a) Values may be interchanged.

2.4 mmol) in dried tetrahydrofuran (THF) under an argon atmosphere. The reaction mixture was stirred for 24 h at room temperature (20—25 °C), then filtered through Celite. The filtrate was evaporated to dryness under reduced pressure, and the residue was extracted with ethyl acetate. The extract was purified by silica gel column chromatography with chloroform–methanol to yield the α-anomer (**3**) and β-anomer (**4**), and methyl *N*-acetyl-4,7,8,9-tetra-*O*-acetyl-2,3-dehydro-2-deoxyneuraminate (**5**).[11] **5** was assigned from the ^{1}H-NMR spectrum (90 MHz, $CDCl_3$) δ_H (ppm): 1.87 (3H, s, NAc), 2.04, 2.08, 2.10, 2.15 (12H, s, OAc), 3.74 (1H, s, COOMe), 5.98 (1H, d, J=1.9 Hz, H-3). The data are summarized in Table I.

α-Anomer (**3**): Colorless needles, mp 113—115 °C (ether–pet. ether). IR ν_{max}^{film} cm^{-1}: 3250 (NH), 2940, 1745 (OAc), 1660 (NHCO), 1540. $[\alpha]_D^{25}$ −23.8° (c=1, $CHCl_3$). The NMR ($CDCl_3$) data are summarized in Table III. MS (EI; in-beam method) *m/z*: 860 (M^++1), 800 (M^+−59). *Anal.* Calcd for $C_{47}H_{73}NO_{13}$: C, 65.63; H, 8.55; N, 1.63. Found: C, 65.41; H, 8.61; N, 1.60.

β-Anomer (**4**): Colorless needles, mp 138—140 °C (ether–pet. ether). IR ν_{max}^{film} cm^{-1}: 3420, 3250 (NH), 2930, 1740, (OAc), 1660 (NHCO), 1540. $[\alpha]_D^{25}$ −40.2° (c=1, $CHCl_3$) data are summarized in Table III. MS (EI; in-beam method) *m/z*: 860 (M^++1), 800 (M^+−59). *Anal.* Calcd for $C_{47}H_{73}NO_{13}$: C, 65.63; H, 8.55; N, 1.63. Found: C, 65.89; H, 8.58; N, 1.66.

Methyl 5-Acetamido-4,7,8,9-tetra-*O*-acetyl-2-(5-cholesten-3β-yl)-3,5-dideoxy-D-*glycero*-β-D-*galacto*-nonulopyranosonate (4)——Tetramethylsilyl trifluoromethanesulfonate (16 μl, 0.83 μmol) was added to a stirred mixture of methyl *N*-acetyl-penta-*O*-acetyl-D-neuraminate (**6**; 1.2 mmol),[8] cholesterol (**2**; 1.2 mmol), and molecular sieves (2 g) in dried dichloromethane (40 ml). After being stirred for 24 h at room temperature, the whole mixture was filtered through Celite. The filtrate was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel, giving the β-anomer (**4**), 0.043 g (yield; 5%).

Methyl 5-Acetamido-4,7,8,9-tetra-*O*-acetyl-2-(5-cholesten-3β-yl)-3,5-dideoxy-D-*glycero*-α- and -β-D-*galacto*-nonulopyranosonate (3, 4) from Methyl *N*-Acetyl-4,7,8,9-tetra-*O*-acetyl-2-fluoro-α-and -β-D-neuraminate (7, 8)——a) A solution of cholesterol (**2**; 0.1 mmol) in dried dichloromethane (5 ml) was stirred with molecular sieves 4A (0.05 g) under an argon atmosphere. After 0.5 h, methyl *N*-acetyl-4,7,8,9-tetra-*O*-acetyl-2-fluoro-β-D-neuraminate[12] (**8**; 0.15 mmol) and boron trifluoride etherate (0.6 mmol) were added to the mixture. After 1 h, the whole was filtered through Celite, the filtrate was evaporated to dryness under reduced pressure, and the residue was purified by silica gel column chromatography to yield the α-anomer (**3**), β-anomer (**4**), and **5**. These data are summarized in Table I. In the case of run No. 9, methyl *N*-acetyl-4,7,8,9-tetra-*O*-acetyl-β-D-neuraminate[15] (10.3% from **8**) was separated.

b) A solution of cholesterol (**2**; 0.1 mmol) in dried benzene (5 ml) was stirred with molecular sieves 4A (0.05 g) under an argon atmosphere. After 1 h, methyl *N*-acetyl-4,7,8,9-tetra-*O*-acetyl-2-fluoro-α-D-neuraminate[12] (**7**;

TABLE IV. ^{1}H-NMR Data (400 MHz) and ^{13}C-NMR Data (100 MHz) for **11** and **12** in CD_3OD (δ ppm, TMS)

Compound	**11**		**12**	
Cholesterol moiety				
6-H	5.332	1H, d, J=5.5 Hz	5.282	1H, d, J=5.3 Hz
18-CH_3	0.704	3H, s	0.700	3H, s
19-CH_3	0.992	3H, s	0.991	3H, s
21-CH_3	0.936	3H, d, J=6.5 Hz	0.928	3H, d, J=6.5 Hz
26-CH_3	0.870	3H×2, d, J=1.7 Hz	0.861	3H×2, d, J=1.5 Hz
27-CH_3	0.885		0.880	
Sialic acid moiety				
2-H_{eq}	2.839	1H, dd, J=4.2, 12.0 Hz	2.482	1H, dd, J=4.5, 13.0 Hz
NAc	2.010	3H, s	1.972	3H, s
^{13}C-NMR (100 MHz, CD_3OD)				
C-3	70.50		72.37	
C-5	142.87		143.08	
C-6	122.59		122.46	
C-1′	102.57		101.37	
C-2′	41.00		43.82	
1′-COONa	175.26		174.51	
NAc	175.91		176.95	

0.12 mmol), tin(II) chloride (0.12 mmol), and silver triflate (0.12 mmol) were added to the mixture. After continued stirring for 1 d in the dark, the whole was filtered through Celite, the filtrate was evaporated to dryness under reduced pressure, and the residue was purified by silica gel column chromatography to yield the α-anomer (**3**), β-anomer (**4**) and **5** (Table I). In the case of run No. 11, methyl *N*-acetyl-4,7,8,9-tetra-*O*-acetyl-β-D-neuraminate[15] (13.6% from **7**) was separated.

5-Acetamido-2-(5-cholesten-3β-yl)-3,5-dideoxy-D-*glycero*-α- and -β-D-*galacto*-nonulopyranosonic Acid (9, 10)——A stirred solution of **3** or **4** (0.05 g) in methanol (2 ml) was treated with 1 N NaOH (3 ml). After continued stirring overnight at room temperature, water (2 ml) was added and the solution was neutralized with Dowex 50 (H^+), and filtered. The filtrate was evaporated to dryness under reduced pressure to give **9** (yield; 79.7%) or **10** (yield; 76.1%) as colorless needles from ethanol.

α-Anomer (**9**): mp 207—208.5 °C. IR ν_{max}^{KBr} cm^{-1}: 2750, 1575. $[\alpha]_D^{22}$ −12.58° (c=0.41, MeOH). ^{1}H-NMR (CD_3OD) δ (90 MHz): 0.71 (3H, s, 18-CH_3), 0.84, 0.91 (6H, 26- and 27-CH_3), 0.95 (3H, d, J=4.5 Hz, 21-CH_3), 1.00 (3H, s, 19-CH_3), 2.01 (3H, s, NAc), 2.43 (1H, dd, J=4.5, 12.6 Hz, 3-H_{eq}). *Anal.* Calcd for $C_{38}H_{63}NO_9$: C, 67.36; H, 9.31; N, 2.07. Found: C, 67.41; H, 9.53; N, 2.09.

β-Anomer (**10**): mp 269—271 °C. IR ν_{max}^{KBr} cm^{-1}: 2870, 1620, 1550. $[\alpha]_D^{20}$ −31.77° (c=0.78, MeOH). ^{1}H-NMR (CD_3OD) δ (90 MHz): 0.71 (3H, s, 18-CH_3), 0.86, 0.92 (6H, 26- and 27-CH_3), 0.95 (3H, d, J=4.5 Hz, 21-CH_3), 1.00 (3H, s, 19-CH_3), 2.00 (3H, s, NAc), 2.39 (1H, dd, J=4.5, 12.6 Hz, 3-H_{eq}). *Anal.* Calcd for $C_{38}H_{63}NO_9$: C, 67.36; H, 9.31; N, 2.07. Found: C, 67.62; H, 9.55; N, 1.87.

Sodium 5-Acetamido-2-(5-cholesten-3β-yl)-3,5-dideoxy-D-*glycero*-α- and -β-D-*galacto*-nonulopyranosonate (11, 12)——A stirred solution of **3** or **4** (0.05 g) in methanol (100 ml) was treated with 2 N NaOH (20 ml). After continued stirring for 24 h at room temperature, the solution was neutralized with Dowex 50 (H^+) and the filtrate was evaporated to dryness under reduced pressure. The resulting white powder was dissolved in 0.02 N NaOH, and chromatographed on Diaion HP 20 with 75% methanol. The eluate was evaporated under reduced pressure. Freeze-drying of the residue gave **11** (90%) and **12** (88%) as a white powder.

α-Anomer (**11**): IR ν_{max}^{KBr} cm^{-1}: 3250, 2940, 1605. $[\alpha]_D^{24}$ +2.2° (c=1, MeOH). The NMR (CD_3OD) data are summarized in Table IV. MS (FD) *m/z*: 722 (M^++Na), 700 (M^++1), 386, 336, 314. *Anal.* Calcd for $C_{38}H_{62}NNaO_9 \cdot 2H_2O$: C, 61.96, H, 8.42; N, 1.90. Found: C, 61.92; H, 8.71; N, 2.04.

β-Anomer (**12**): IR ν_{max}^{KBr} cm^{-1}: 3270, 2950, 1608. $[\alpha]_D^{24}$ −10.6° (c=1.0, MeOH). The NMR (CD_3OD) data are summarized in Table IV. MS (FD) *m/z*: 722 (M^++Na), 700 (M^++1), 386. *Anal.* Calcd for $C_{38}H_{62}NNaO_9 \cdot H_2O$: C, 63.52; H, 8.91; N, 1.95. Found: C, 63.81; H, 9.25; N, 2.13.

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References and Notes

1) This constitutes Part X in the series entitled "Studies on Sialic Acids."
2) H. Ogura and K. Furuhata, *Tetrahedron Lett.*, **22**, 4265 (1981).
3) H. Ogura, K. Furuhata, H. Saito, G. Izumi, M. Itoh, and Y. Shitori, *Chem. Lett.*, **1984**, 1003.
4) H. Ogura and K. Furuhata, *Yuki Gosei Kagaku Kyokaishi*, **42**, 536 (1984).
5) I. Kijima, K. Ezawa, S. Toyoshima, K. Furuhata, H. Ogura, and T. Osawa, *Chem. Pharm. Bull.*, **30**, 3278 (1982).
6) I. Kijima-Suda, S. Toyoshima, M. Itoh, K. Furuhata, H. Ogura, and T. Osawa, *Chem. Pharm. Bull.*, **33**, 730 (1985).
7) K. Furuhata, K. Anazawa, M. Itoh, Y. Shitori, and H. Ogura, *Chem. Pharm. Bull.*, **34**, 2725 (1986).
8) H. Ogura, H. Fujita, K. Furuhata, M. Itoh, and Y. Shitori, *Chem. Pharm. Bull.*, **34**, 1479 (1986).
9) M. Tanaka, S. Tuji, T. Yamashita, and K. Nagai, *Bulletin of the Japanese Neurochemical Society*, **25**, 97 (1986).
10) M. M. Ponpipom, R. L. Bugianesi, and T. Y. Shen, *Can. J. Chem.*, **58**, 214 (1980).
11) H. Ogura, K. Furuhata, M. Itoh, and Y. Shitori, *Carbohydr. Res.*, **158**, 37 (1986).
12) M. N. Sharma and R. Ely, *Carbohydr. Res.*, **127**, 201 (1984).
13) H. Kunz and H. Waldmann, *J. Chem. Soc., Chem. Commun.*, **1985**, 638.
14) T. Mukaiyama, Y. Hashimoto, and S. Shoda, *Chem. Lett.*, **1983**, 935.
15) R. Kuhn, P. Lutz, and D. L. MacDonald, *Chem. Ber.*, **99**, 611 (1966).

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Absolute Configurations of Chaetochromin A and Related Bis(naphtho-γ-pyrone) Mold Metabolites

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The absolute configuration of chaetochromin A (**1**) was established by X-ray analysis of the 6-*O*-*p*-bromobenzoate (**15**) of the 5,5′,6′,8,8′-pentamethyl ether (**14**). The stereochemistry of the 9–9′ bond was proved to be *S*, in agreement with the result obtained by the application of the exciton chirality method. From the circular dichroism spectra, the absolute configurations of chaetochromins B, C and D (**2**—**4**) and cephalochromin (**8**) were proved to be *S*, while those of ustilaginoidins A, B, and C (**5**—**7**) were *R*. The stereochemistry of other bis(naphtho-γ-pyrones) is discussed.

Keywords——Chaetochromin; cephalochromin; ustilaginoidin; bis(naphtho-γ-pyrone); mycotoxin; CD; X-ray analysis; absolute configuration; exciton chirality

Chaetochromin A (**1**) is a mycotoxin, first isolated from *Chaetomium virescens* UDAGAWA (*C. thielavioideum* CHEN),[1] and exhibits toxicity to experimental animals[2] and antitumor activities.[3] The structure and the relative stereochemistry were established by the previous studies[1,4] and three related compounds (**2**—**4**) were also isolated from *C. gracile* UDAGAWA,[4] but the absolute configurations (including the atropisomerism) have not been established.

As related bis(naphtho-γ-pyrone) mold metabolites, ustilaginoidins A, B, and C (**5**—**7**) from *Claviceps virens* (anamorph state: *Ustilaginoidea virens* (COOKE) TAKAHASHI),[5] cephalochromin (**8**) from *Cephalosporium* sp.,[6] *Verticillium* sp.,[7] and *Nectoria* spp.,[8] and isoustilaginoidin A (**9**) (the antipode of **5**) and dihydroisoustilaginoidin A (**10**) from *Verticillium* sp.[7] are known. Among these compounds, the absolute configurations of ustilaginoidins (**5**—**7**) were proposed to be *R* based on the positive Cotton effect at around 400 nm in the optical rotatory dispersion (ORD) compared to those of biaryls,[9] but the application of the results to the absolute configuration of nigerone (**11**) from *Aspergillus niger*[10] was contradictory, and an unambiguous assignment of the stereochemistry of these compounds was required.[10] The circular dichroism (CD) data for **8**—**10**[7] and the ORD data for aurasperone A (**12**) from *A. niger*[11] were given but the absolute configurations were not discussed in the above papers.

Recently we have recognized the antitumor activities of chaetochromins and related compounds,[12] and the establishment of the absolute configurations of these compounds was required to confirm the structure–activity relationship of bis(naphtho-γ-pyrone) derivatives.

In order to determine the absolute configuration of chaetochromin A, the *O*-*p*-bromobenzoate (**15**) of chaetochromin A pentamethyl ether (**14**) was prepared from its tetramethyl ether[4] (**13**). Dark-yellow thick plate crystals of (**15**) were grown in hexane–ethyl acetate solution and a small crystal with approximate dimensions of $0.15 \times 0.3 \times 0.45$ mm was used for the X-ray diffraction study. The crystal was mounted on a Philips PW1100 diffractometer and the lattice parameters and intensity data were measured with the use of

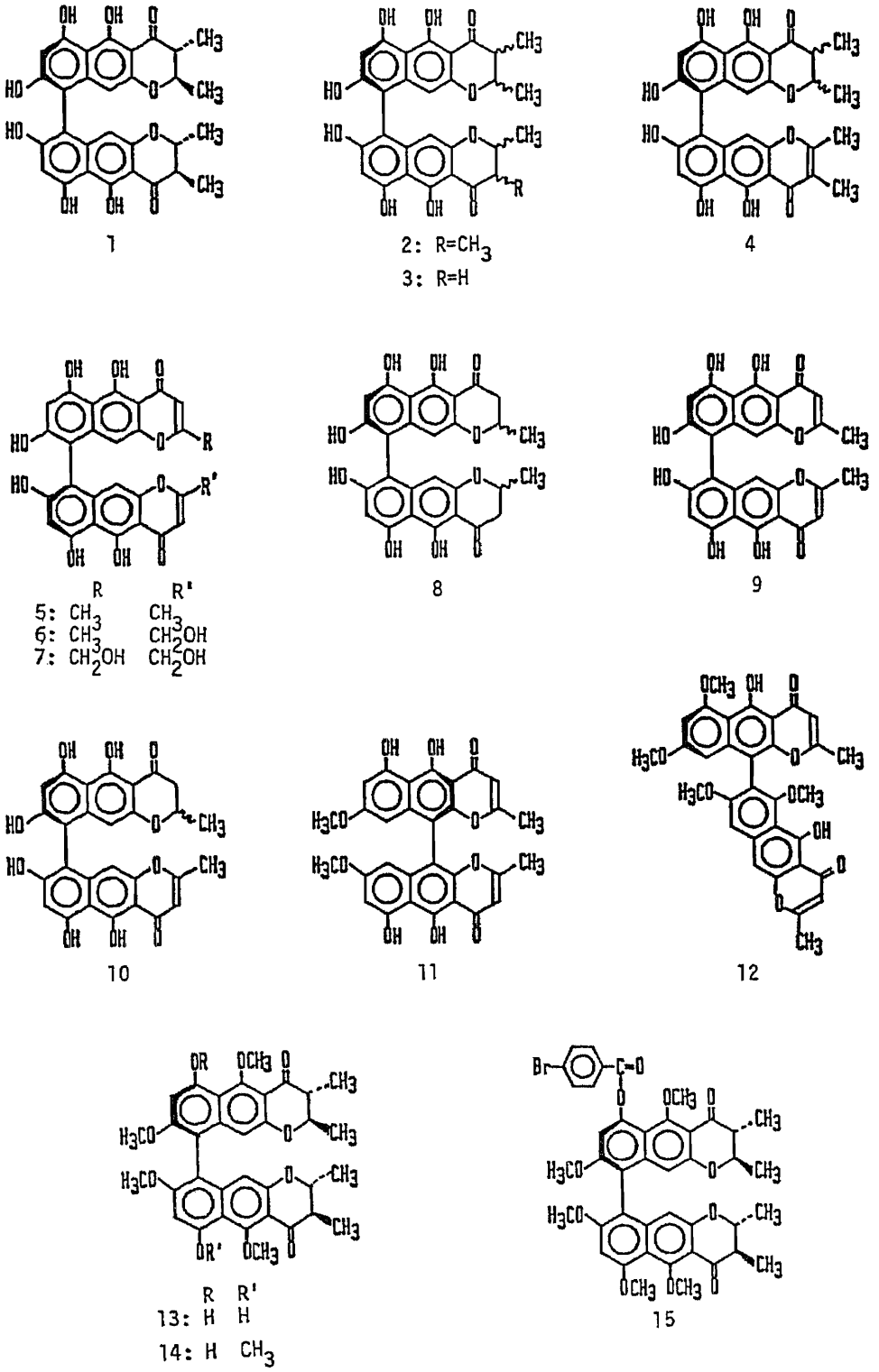

Chart 1

graphite-monochromated CuK_α radiation. The crystal data are: 6-*O*-*p*-bromobenzoyl-chaetochromin A 5,5′,6′,8,8′-pentamethyl ether, $C_{42}H_{39}O_{11}Br$, M_r 799.7; orthorhombic, space group $P2_12_12_1$, $Z=4$, $D_{cal}=1.377\,g\,cm^{-3}$; lattice constans, $a=14.642(8)$, $b=24.147(12)$, $c=10.908(6)$ Å, $U=3857$ Å^3; μ for Cu$K_\alpha=19.5\,cm^{-1}$.

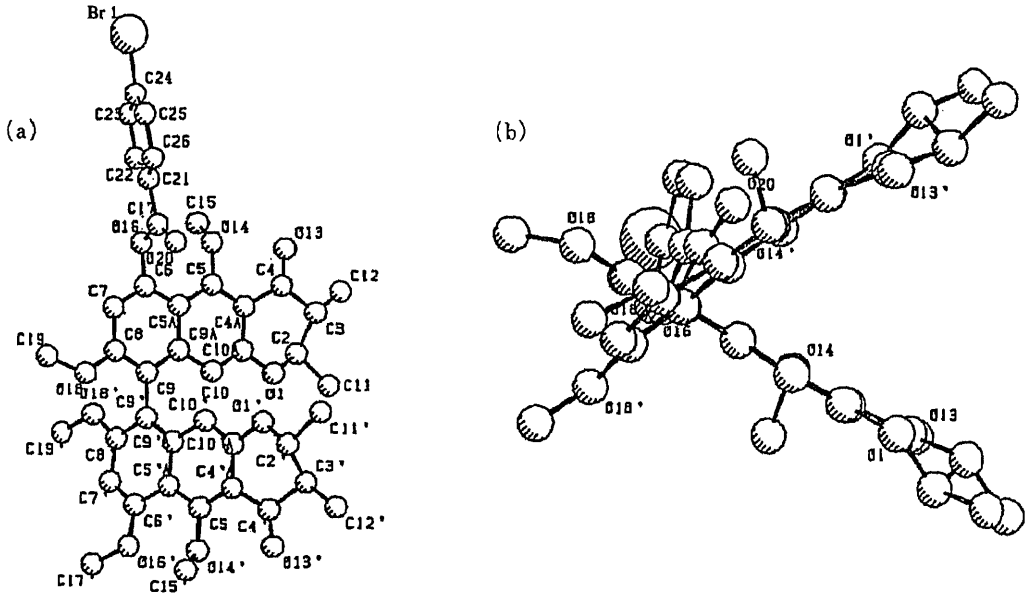

Fig. 1. The Molecular Structure of 6-*O*-*p*-Bromobenzoylchaetochromin A 5,5′,6′,8,8′-Pentamethyl Ether (**15**) Showing the Absolute Configuration

The figures were drawn by PLUTO. (a) Viewed down to the mean molecular plane. (b) Viewed along the C9–C9′ bond.

Intensities of 5229 reflections in the 2θ range of 6° through 156° were measured as above the $2\sigma(I)$ level, including 835 Friedel pairs and 93 symmetry equivalent reflections. The number of possible reflections in the same angular range was 4580. The difference of the intensities of Friedel pairs was estimated as $R(F)=\sum||F(hkl)|-|F(\bar{h}\bar{k}\bar{l})||/\sum|F(hkl)|$, where the sum was taken for 835 pairs, giving an $R(F)$ value of 0.061.

The crystal structure was determined by the heavy atom method and the atomic parameters were refined by the block-diagonal-matrix least-squares calculation. Hydrogen atoms were not included since most of the hydrogen atoms are methyl hydrogens and it was not possible to locate many of them on the difference electron-density map due probably to the rotational oscillation of methyl groups. Anisotropic refinement gave an R value of 0.074 for 4301 reflections.[13]

Absolute structure was determined by the anomalous dispersion method. Dispersion correction terms for C, O and Br scattering factors for CuK_α radiation were taken from International Tables for X-Ray Crystallography (1972). The observed and calculated structure factor ratios r_{obs} and r_{cal} were compared for the Friedel pairs, where $r_{obs}=|F_{obs}(hkl)|/|F_{obs}(\bar{h}\bar{k}\bar{l})|$, a^*, b^* and c^* axes were taken in the right-handed system and $r_{cal}=|F_{cal}(hkl)|/|F_{cal}(\bar{h}\bar{k}\bar{l})|$; F_{cal}'s were calculated with the refined atomic parameters and anomalous dispersion corrections for C, O and Br. Of the total of 279 Friedel pairs for which both r_{obs} and r_{cal} differ by more than 3% from unity and the difference in observed structure factors, $||F_{obs}(hkl)|-|F_{obs}(hkl)||$ is estimated to be greater than $2\sigma[F(hkl)]$, 244 pairs showed consistently the absolute configuration given in Fig. 1.

Subsequent least-squares refinement allowing the dispersion corrections gave R values of 0.070 and 0.076, the latter being for the inverted structure.

The corresponding bond lengths and angles for the two naphtho-γ-pyrone moieties are in good overall agreement with each other and are close to those expected for the chemical structure. The differences between two skeletons are almost within the limits of experimental error. The largest differences are found in C3–C12 = 0.067 Å and C7–C6–C5A = 3.4°. The conformations of the two skeletons are also not very different. The puckering of the γ-pyrone

TABLE I. The Least-Squares Plane through the Naphthol Skeleton Atoms and the Deviations of Atoms from the Plane

Least-squares plane (Å) formed by				Least-squares plane (Å) formed by			
C4A	−0.004 (6)	C9A	−0.005 (6)	C4'A	0.099 (6)	C9'A	−0.038 (6)
C5	−0.002 (6)	C10	0.019 (6)	C5'	0.021 (6)	C10'	−0.075 (6)
C5A	−0.016 (6)	C10A	0.011 (6)	C5'A	−0.037 (6)	C10'A	−0.008 (6)
C6	−0.010 (6)	*A*	0.6633	C6'	−0.086 (6)	*A*	0.9476
C7	0.034 (6)	*B*	−0.5668	C7'	0.015 (7)	*B*	0.3071
C8	−0.001 (6)	*C*	0.4886	C8'	0.084 (7)	*C*	−0.0880
C9	−0.026 (6)	*D*	10.953	C9'	0.025 (6)	*D*	4.642
Distance (Å) from the above plane				Distance (Å) from the above plane			
O1	−0.009 (5)	C17	−1.201 (7)	O1'	0.009 (6)	C17'	−0.393 (8)
C4	−0.127 (7)	O18	−0.049 (6)	C4'	0.488 (7)	O18'	0.232 (6)
O14	−0.040 (5)	C19	0.423 (8)	O14'	0.112 (6)	C19'	0.251 (9)
C15	1.283 (7)	C9'	−0.030 (6)	C15'	−1.082 (8)	C9	−0.081 (7)

A, *B*, *C* and *D* are the coefficients of the equation of the least-squares plane $AX+BY+C=D$, where *X*, *Y* and *Z* are the Cartesian coordinates taken as $X \| a$, $Y \| b$ and $Z \| c$ measured in Å unit.

TABLE II. Torsional Angles (°) of the γ-Pyrone Part of 6-*O*-*p*-Bromobenzoyl-chaetochromin 5,5',6',8,8'-Pentamethyl Ether (**15**)

C3–C2–O1–C10A	58.3 (7)	C3'–C2'–O1'–C10'A	56.6 (7)
C11–C2–O1–C10A	178.7 (5)	C11'–C2'–O1'–C10'A	177.1 (5)
C4–C3–C2–O1	−59.8 (7)	C4'–C3'–C2'–O1'	−64.0 (7)
C12–C3–C2–O1	179.3 (5)	C12'–C3'–C2'–O1'	175.3 (6)
C4–C3–C2–C11	−174.2 (5)	C4'–C3'–C2'–C11'	−178.9 (5)
C12–C3–C2–C11	64.8 (8)	C12'–C3'–C2'–C11'	60.5 (9)
C4A–C4–C3–C2	32.3 (9)	C4'A–C4'–C3'–C2'	37.9 (9)
C4A–C4–C3–C12	153.0 (6)	C4'A–C4'–C3'–C12'	159.7 (6)

ring may be characterized by the torsional angles, C4A–C10A–O1–C2 and C4A–C4–C3–C2. They are 32.3(9)° and −26.6(8)° respectively for the unprimed ring and 37.9(9)° and −17.8(8)° respectively for the primed ring. Although the bond C4A–C10A has a partial double bond character, C4A–C4 and C10A–O1 are twisted due to the puckering. The torsion angles, C4–C4A–C10A–O1 and C4'–C4'A–C10'A–O1' are −3.8(9)° and −11.5(9)°, respectively, for the two γ-pyrone rings. To illustrate the distortion of the naphtho-γ-pyrone moiety, the least-squares plane through the naphthyl skeleton atoms has been calculated and the deviations of atoms from the plane are listed in Table I.

The configurations at the 2-, 2'-, 3- and 3'-positions were proved to be *R*, *R*, *R*, and *R*, respectively. The relative configuration of the 2,3-dimethyl group was shown to be *trans* by proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) analyses. The torsion angles shown in Table II gave support to the NMR assignments.[1,4]

The two planes (formed by the unprimed and primed atoms, respectively) make an angle of 65.7° which can be compared to 45° in the gas-phase biphenyl molecule,[14] 76.6° in (+)-2,2'-dihydroxy-1,1'-binaphthalene-3,3'-dicarboxylic acid dimethyl ester,[15] 58° in 1,4,5,8-tetraphenylnaphthalene,[16] 77.6° in binaphthylbis(18-crown-6)[17] and 78.7° in 3,3'-(1,1'-bi-2-naphthol)-21-crown-5.[18] The aryl–aryl bond length, C9–C9' of 1.487(10) Å is also in good agreement with those in the above-mentioned compounds: 1.48 Å,[14] 1.49(3) Å,[15] 1.504(4) Å,[16] 1.465(7) Å[17] and 1.491(4), Å,[18] respectively. The torsion angles about the bond connecting the two naphthyl groups, C9A–C9–C9'–C9'A and C8–C9–C9'–C8' are 72.2(8)° and 65.1(8)°, respectively. The sign of the angle indicates the absolute configuration of the

atropisomer and it is proved to be *S*.

It is well known that the CD Cotton effects due to chiral exciton coupling between two or more chromophores suggest the absolute stereochemistry in a nonempirical manner.[19] The application of the method to binaphthyl chromophores has been published.[20,21] The observed dihedral angle (65.7°) of the two aromatic moieties guarantees the applicability of the method.[21]

The CD curves of chaetochromin A (**1**)[4] exhibit strong positive first ([θ] 50×10^4 (294 nm)) and second negative ([θ] -43×10^4 (266 nm)) Cotton effects due to the couplings between the ^1B_b transitions of the two naphthalene chromophores and this shows that the two long axes are twisted in a clockwise manner. This conclusion is in accord with the result established by X-ray analysis. Since the CD spectra of chaetochromins A—D (**1**—**4**) are superimposable, all these naphtho-γ-pyrones from *Chaetomium* spp. have *S*-configurations of the 9–9′ bond.

Cephalochromin (**8**) has been isolated from several fungal sources.[6-8] As described in Experimental, the compound was obtained from *Acremonium butyri* (syn. *Nectoria viridescens*[8]) in this work. As shown in Fig. 2, the compound shows strong positive first ([θ] 51×10^4 (294 nm)) and negative second ([θ] -49×10^4 (266 nm)) Cotton effects, indicating the same *S*-configuration as chaetochromins. It was reported that the treatment of cephalochromin (**8**) with iodine in acetic acid gave ustilaginoidin A (**5**),[6] but the product must be isoustilaginoidin A (**9**) (*vide infra*) or the racemate of **5**.

The structures (**5**—**7**) of ustilaginoidins A, B, and C, red coloring matters obtained from the smutted balls formed by the infection of *Claviceps virens* (anamorph state: *Ustilaginoidea virens*) on the spikes of rice plant, were firmly established by chemical, spectroscopic, and synthetic methods.[5] Empirical application of the ORD behavior of biaryls to the absolute configurations was done and the observed positive Cotton effect at the higher wavelength region (around 400 nm) suggested the *R*-configuration. We tried to isolate the pigments.[22] The CD spectrum measured for a sample of ustilaginoidin A showed negative first ([θ] -46×10^4 (290 nm)) and positive second ([θ] 31×10^4 (261 nm)) Cotton effects (Fig. 2). This and the reported data[7] showed that the two long axes of the naphthalene chromophores in ustilaginoidins A, B, and C (**5**—**7**) are twisted in a counter-clockwise manner, contrary to that in chaetochromins (**1**—**4**) and cephalochromin (**8**), and indicate *R*-configuration of the compounds.

The antipode of ustilaginoidin A named isoustilaginoidin (**9**), the dihydro compound (dihydroisoustilaginoidin A) (**10**), and the tetrahydro compound (identical with cephalo-

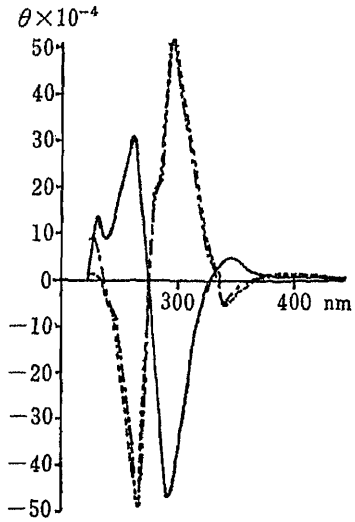

Fig. 2. CD Spectra of Chaetochromin A, Cephalochromin, and Ustilaginoidin A (in Dioxane)

–·–·–, chaetochromin A (**1**); ------, cephalochromin (**8**); ——, ustilaginoidin A (**5**).

chromin) (**8**) were isolated from *Verticillium* sp.[7] The reported CD data of these compounds[7] clearly indicated strong split bands around 280 nm with first positive and second negative signs, which suggest *S*-configuration.

From these facts it is clear that the stereochemistry of the binaphthyl moiety of the 9,9′-binaphtho-γ-pyrones is the same in the compounds from *Chaetomium* sp. (**1**—**4**), *Verticillium* sp. (**8**—**10**), and *Cephalosporium* sp. (**8**) but is antipodal in those from *Ustilaginoidea* (**5**—**7**).

Nigerone (**11**) is a 10,10′-bisnaphtho-γ-pyrone from *Aspergillus niger*.[10] The compound shows negative ORD and CD Cotton effects in the long wavelength region (about 430 nm), opposite to ustilaginoidins, and strong negative first and positive second effects in the short wavelength region (around 280 nm), similar to ustilaginoidins.[10] The latter observations clearly indicated the negative chirality of the chromophores, suggesting *R*-configuration.

Aurasperone A (**12**) is a 10,7′-bisnaphtho-γ-pyrone from *A. niger*.[11] The ORD curve, showing a clear Davydov splitting, was recorded for the compound though the stereochemistry was not discussed.[11] The curve clearly indicates positive first and negative second effects, indicating positive chirality of the chromophores and suggesting *S*-configuration of the compound.

Racemization involving binaphthyl bonds subject to restricted rotation has been reported for the binaphthopyrones.[5,10,11] In the case of cephalochromin (**8**) and chaetochromin A (**1**) with chiral carbons in the γ-pyran ring, epimerization of the methyl groups occurs simultaneously. Examination of the racemization of the binaphthyl bond and characterization of the products are in progress.

Experimental

All melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. The ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL GX-400 (^{1}H 400 MHz and ^{13}C 100 MHz) spectrometer in $CDCl_3$ with tetramethylsilane as an internal standard. Chemical shifts are recorded in ppm (δ). Mass spectra (MS) were taken on a JEOL JMS-D300. Ultraviolet (UV) and infrared (IR) spectra were measured with a Shimadzu UV-240 spectrophotometer and a JASCO A-102 infrared spectrophotometer. The $[\alpha]_D$ values were measured with a JASCO DIP-140 digital polarimeter.

Kiesel gel $60F_{254}$ (Merck) precoated plates were used for thin-layer chromatography (TLC) and the spots were detected by UV illumination. Column chromatography was carried out on 70—230 mesh silica gel (Merck). High performance liquid chromatography (HPLC) was carried out by using a Waters M45J pump with an Oyo-Bunko Uvilog-5 IIIA UV detector.

Chaetochromin A 5,5′,6,8,8′-Pentamethyl Ether (14)——A CH_2Cl_2 solution of chaetochromin A 5,5′,8,8′-tetramethylether[4] (**13**) (28.4 mg) was methylated with ethereal CH_2N_2 at room temperature for 2 h and the reaction mixture was purified by HPLC (Nucleosil 50-5) with hexane–EtOAc (1 : 1) to give **14** (8.3 mg), yellow leaflets (from MeOH–H_2O), mp 154—160 °C, $[\alpha]_D^{20}$ +386° (c = 0.09, dioxane). MS m/z: 616.2310 (M^+, Calcd for $C_{35}H_{36}O_{10}$: 616.2308). UV $\lambda_{max}^{dioxane}$ nm (ε): 231 (47700), 259 (47700), 290 (77500), 330 (11000), 341 (9300), 391 (8600). IR ν_{max}^{KBr} cm^{-1}: 3350, 1690, 1625, 1613, 1560, 1370, 1355, 1330, 1280, 1130, 1065. CD (dioxane) $[\theta]^{20}$ (nm): −476500 (253), −40800 (272), 0 (276), +925700 (290), 0 (321), −68100 (342), 0 (360), +8200 (400). ^{1}H-NMR ($CDCl_3$) δ: 1.17, 1.19 (3H × 2, each d, J = 6.7, 7.0, 3,3′-C$\underline{H}_3$), 1.38, 1.39 (3H × 2, each d, J = 6.1, 5.8, 2,2′-C$\underline{H}_3$), 2.51, 2.51 (1H × 2, each dq, J = 11.0, 6.7; 11.0, 7.0, 3,3′-$\underline{H}$), 3.76, 3.79 (3H × 2, each s, 8,8′-OC$\underline{H}_3$), 4.00 (3H, s, 6′-OC$\underline{H}_3$), 4.07, 4.14 (3H × 2, each s, 5,5′-OC$\underline{H}_3$), 4.11, 4.13 (1H × 2, each dq, J = 11.0, 6.1; 11.0, 5.8, 2,2′-$\underline{H}$), 6.63, 6.69 (1H × 2, each s, 7,7′-$\underline{H}$), 6.32, 6.32 (1H × 2, each s, 10,10′-$\underline{H}$), 10.18 (1H, s, 6-O$\underline{H}$). ^{13}C-NMR ($CDCl_3$) δ: 10.47, 10.59 (3,3′-$\underline{C}H_3$), 19.63, 19.75 (2,2′-$\underline{C}H_3$), 48.32, 48.48 (3,3′-$\underline{C}$), 56.12, 56.27, 56.37 (6′,8,8′-O$\underline{C}H_3$), 62.91, 64.32 (5,5′-O$\underline{C}H_3$), 77.54, 77.59 (2,2′-$\underline{C}$), 93.75, 97.05 (7,7′-$\underline{C}$), 105.15, 105.69 (10,10′-$\underline{C}$), 108.51, 109.03, 109.30, 109.58, 112.23, 112.57 (4a,4′a,5a,5′a,9,9′-$\underline{C}$), 139.71, 140.70 (9a,9′a-$\underline{C}$), 156.84, 157.34 (10a,10′a-$\underline{C}$), 158.35, 158.35 (8,8′-$\underline{C}$), 159.68, 160.22, 160.30, 161.29 (5,5′,6,6′-$\underline{C}$), 192.92, 193.10 (4,4′-$\underline{C}$).

6-*O*-*p*-Bromobenzoylchaetochromin A 5,5′,6′,8,8′-Pentamethyl Ether (15)——A solution of **14** (24.7 mg) and *p*-bromobenzoyl chloride (25 mg) in pyridine (3.5 ml) was stirred gently under N_2 in the dark for 2 h. After filtration, the reaction mixture was evaporated to dryness. The residue was chromatographed over silica gel using benzene–EtOAc (10 : 1) as the developing solvent. Purification by HPLC (Develosil 60-3) with benzene–EtOAc (8 : 1) gave **15** (10.5 mg), dark yellow prisms (from hexane–EtOAc), mp 285—290 °C, $[\alpha]_D^{20}$ +262° (c = 0.077, dioxane). MS m/z: 798 (M^+), 616. UV $\lambda_{max}^{dioxane}$ nm (ε): 235 (61600), 250 (71700), 273 (69900), 284 (89300), 325 (14800), 339 (12900), 381 (9800). IR ν_{max}^{KBr} cm^{-1}: 1740, 1692, 1615, 1590, 1567, 1345, 1270, 1260, 1125, 1097, 1010, 750. CD (dioxane) $[\theta]^{20}$ (nm):

−459600 (255), −271600 (266), 0 (274), +1211600 (285), 0 (315), −91900 (337), 0 (363), +14600 (394). ^{1}H-NMR ($CDCl_3$) δ: 1.14, 1.18 (3H×2, each d, J=6.7, 7.0, 3-, 3′-C$\underline{H}_3$), 1.38, 1.39 (3H×2, each d, J=5.8, 5.8, 2,2′-C$\underline{H}_3$), 2.50, 2.52 (1H×2, each dq, J=11.0, 6.7; 10.7, 7.0, 3-, 3′-$\underline{H}$), 3.79, 3.79, 3.80, 4.09 (3H×4, each s, 5,5′,8,8′-OC$\underline{H}_3$), 4.01 (3H, s, 6-OC$\underline{H}_3$), 4.11, 4.12 (1H×2, each dq, J=11.0, 5.8; 10.7, 5.8, 2,2′-$\underline{H}$), 6.32, 6.44 (1H×2, each s, 10,10′-$\underline{H}$), 6.64, 7.03 (1H×2, each s, 7,7′-$\underline{H}$), 7.71 (2H, d, J=8.5, 23, 25-$\underline{H}$), 8.19 (2H, d, J=8.5, 22, 26-$\underline{H}$). ^{13}C-NMR ($CDCl_3$) δ: 10.54, 10.56 (3,3′-$\underline{C}H_3$), 19.73, 19.81 (2,2′-$\underline{C}H_3$), 48.55, 48.55 (3,3′-$\underline{C}$), 56.25, 56.35, 56.46 (6′,8,8′-O$\underline{C}H_3$), 63.02, 63.71 (5,5′-O$\underline{C}H_3$), 77.68, 77.71 (2,2′-$\underline{C}$), 93.50, 107.07 (7,7′-$\underline{C}$), 105.09, 105.87 (10,10′-$\underline{C}$), 109.02, 112.37, 112.47, 112.54, 113.60, 115.94 (4a,4′a,5a,5′a,9,9′-$\underline{C}$), 128.54, 128.75 (21,24-$\underline{C}$), 131.83, 131.95 (22,23,25,26-$\underline{C}$), 140.30, 140.32 (9a,9′a-$\underline{C}$), 157.43, 157.46, 157.56, 158.15 (8,8′,10a,10′a-$\underline{C}$), 149.40 (6-C), 159.61, 160.60, 161.39 (5,5′,6,6′-$\underline{C}$), 164.74 (17-$\underline{C}$), 193.19, 193.29 (4,4′-$\underline{C}$).

Isolation of Cephalochromin (8) from *Acremonium butyri*——The strain (CBS 479.69) was incubated in stationary culture on sterilized wheat (110 g) at 26 °C for 30 d. The moldy wheat was extracted three times with EtOAc (200 ml) for 24 h at room temperature. The extract was chromatographed over silica gel (treated with 3% oxalic acid) using CH_2Cl_2 as the developing solvent, and purification by HPLC (Nucleosil 50-5, treated with 3% oxalic acid) using hexane–EtOAc (4:1) as the developing solvent gave **8** (120 mg), yellow powder (from CH_2Cl_2–hexane), mp 214—215 °C. MS m/z: 518.1224 (M^+, Calcd for $C_{28}H_{22}O_{10}$: 518.1213). UV $\lambda_{max}^{dioxane}$ nm (ε): 210 (52100), 232 (60200), 269 (53100), 292 (61200), 325 (15800), 338 (10200), 413 (10200). IR ν_{max}^{KBr} cm^{-1}: 3400, 1640, 1630, 1588, 1445, 1383, 1365, 1340, 1148, 1125, 1084, 873, 840. CD (dioxane) $[\theta]^{20}$ (nm): +11230 (226), 0 (236), −51000 (240), −489900 (266), 0 (274), +209200 (282), +510300 (294), +49000 (325), 0 (333), −56100 (339), 0 (354), +11200 (415). Its identity with **8** was confirmed by MS, ^{1}H- and ^{13}C-NMR, and CD.[6-8]

Ustilaginoidin A (5)——Isolation and identification will be reported in a forthcoming paper.[22] CD (dioxane) $[\theta]^{20}$ (nm): 0 (222), +133600 (231), +308400 (261), 0 (275), −469800 (290), 0 (328), +48300 (345), +3100 (425). UV $\lambda_{max}^{dioxane}$ nm (ε): 210 (71600), 220 (71400), 250 (40400), 270 (46800), 289 (55300), 329 (4300), 346 (2800), 410 (6400).

CD——CD spectra were measured in 0.01—0.1 mg/ml dioxane solution using a 100 mm cell on a JASCO J-20 spectropolarimeter.

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References and Notes

1) S. Sekita, K. Yoshihira, and S. Natori, *Chem. Pharm. Bull.*, **28**, 2428 (1980).
2) K. Ohtsubo, *Proc. Assoc. Mycotoxicol.*, **12**, 28 (1980); *idem*, *ibid.*, **15**, 25 (1982); Y. Ito and K. Ohtsubo, *ibid.*, **16**, 22 (1982).
3) The screening was performed under the auspices of the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland, U.S.A.
4) K. Koyama and S. Natori, *Chem. Pharm. Bull.*, **35**, 578 (1987).
5) S. Shibata, A. Ohta, and Y. Ogihara, *Chem. Pharm. Bull.*, **11**, 1174 (1963); S. Shibata, Y. Ogihara, and A. Ohta, *ibid.*, **11**, 1179 (1963); S. Shibata and Y. Ogihara, *ibid.*, **11**, 1576 (1963); E. Morishita and S. Shibata, *ibid.*, **15**, 1765, 1772 (1967).
6) G. Tertzakian, R. H. Haskins, G. P. Slater, and L. R. Nesbitt, *Proc. Chem. Soc. London*, **1964**, 195.
7) M. Matsumoto, H. Minato, E. Kondo, T. Mitsugi, and K. Katagiri, *J. Antibiot., Ser. A*, **28**, 602 (1975).
8) S. T. Carey and M. S. R. Nair, *Lloydia*, **38**, 448 (1975).
9) S. Shibata and Y. Ogihara, *Tetrahedron Lett.*, **1963**, 1777.
10) C. P. Gorst-Allman, P. S. Steyn, and C. J. Rabie, *J. Chem. Soc., Perkin Trans. 1*, **1980**, 2474.
11) H. Tanaka, P.-L. Wang, and M. Namiki, *Agric. Biol. Chem.*, **36**, 2511 (1972).
12) Unpublished data from our laboratory.
13) A list of final atomic parameters will be sent to the Cambridge Crystallographic Centre.
14) O. Bastiansen, *Acta Chem. Scand.*, **3**, 408 (1949).
15) H. Akimoto and Y. Iitaka, *Acta Crystallogr.*, **B25**, 1491 (1969).
16) G. Evrard, P. Piret, and M. Van Meerssche, *Acta Crystallogr.*, **B28**, 497 (1972).
17) I. Goldberg, *Acta Crystallogr.*, **B33**, 472 (1977).
18) I. Goldberg, *Acta Crystallogr.*, **B34**, 3387 (1978).
19) N. Harada and K. Nakanishi, *Acc. Chem. Res.*, **5**, 257 (1972); N. Harada, S. L. Chen, and K. Nakanishi, *J. Am. Chem. Soc.*, **97**, 5345 (1975); N. Harada, Y. Takuma, and H. Uda, *ibid.*, **98**, 5408 (1976); *idem*, *ibid.*, **100**, 4029 (1978).
20) M. Endo and H. Naoki, *Tetrahedron*, **36**, 2449 (1980).
21) S. F. Mason, R. H. Seal, and D. R. Roberts, *Tetrahedron*, **30**, 1671 (1974).
22) The details of the experiments including structure elucidation of the new congeners will be reported in a forthcoming paper.

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Synthesis of 5-Carbon-Substituted 1-β-D-Ribofuranosylimidazole-4-carboxamides *via* Lithiation of a Primary Carboxamide

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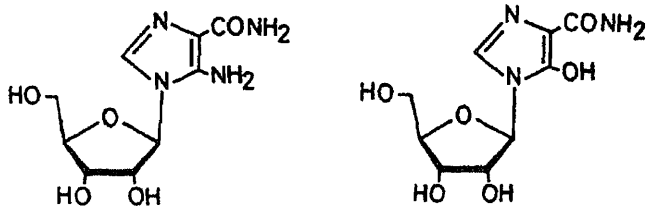
Several 5-carbon-substituted 1-β-D-ribofuranosylimidazole-4-carboxamides were synthesized *via* the direct C-5 lithiation of a protected 4-carboxamide derivative as the key reaction step. Wittig reaction of a 5-formyl derivative was also examined.

Keywords——lithiation; imidazole nucleoside; 5-substituted 1-β-D-ribofuranosylimidazole-4-carboxamide; lithium diisopropylamide; lithium 2,2,6,6-tetramethylpiperidide; Wittig reaction

Due to the considerable potential chemotherapeutic importance of 5-substituted 1-β-D-ribofuranosylimidazole-4-carboxamides as analogues of 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamide (AICAR, **1**), several compounds including bredinin (**2**)[1] have



1 **2**

Fig. 1

been prepared so far. Among the compounds in this class, the 5-chloro, 5-bromo, and 5-iodo derivatives were synthesized by Sandmeyer reaction of 5-amino-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)imidazole-4-carbonitrile followed by hydrolysis.[2] Another route to the 5-substituted derivatives is the classical condensation method, though formation of a mixture of regio-isomers is always anticipated in this case.[3]

Recently, we reported a lithiation approach to C-5 substitution of methyl 2-chloro-1-(2,3-*O*-methoxymethylidene-5-*O*-*tert*-butyldimethylsilyl-β-D-ribofuranosyl)imidazole-4-carboxylate (**3**)[4] wherein the chlorine atom served as a protecting group during the metallation. Hydrogenolysis of the chlorine atom followed by concurrent deprotection of the methoxymethylidene and *tert*-butyldimethylsilyl (TBDMS) groups furnished the corresponding 5-substituted products, which seemed to be easily convertible to the 4-carboxamide derivatives. However, drastic conditions are required to effect ammonolysis of the ester function. For example, the ammonolysis of ethyl 5-methyl-1-β-D-ribofuranosylimidazole-4-carboxylate has been carried out in a sealed tube with liquid NH_3 for 3 d.[3d]

Consideration of the above-mentioned background led us to examine C-5 lithiation of a preformed imidazole-4-carboxamide, in the hope that it would provide a general method for the preparation of 5-substituted 1-β-D-ribofuranosylmidazole-4-carboxamides.

The method used for the ester–amide conversion, which is relatively mild, and therefore

enabled us to employ the protected nucleoside **3** as a starting material, is as follows. When **3** was reacted with diisobutylaluminium hydride (DIBAL) in tetrahydrofuran (THF)–toluene at below −70 °C for 2 h, the formyl derivative (**4**) was obtained in 95% yield. By adopting

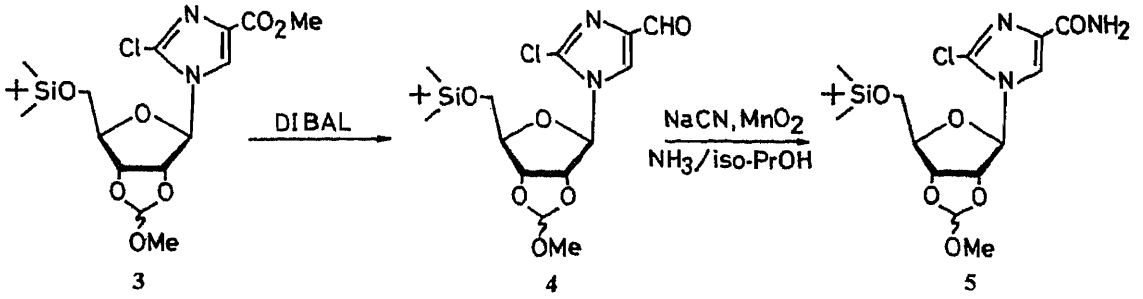


Chart 1

Gilman's method,[5] **4** was converted to the requisite carboxamide **5**. Thus, treatment of **4** with a suspension of NaCN in isopropanol saturated with NH_3 and then with activated MnO_2 at 0 °C for 2 h provided a 95% yield of **5** in a one-pot process (Chart 1).

Lithiation at the C-5 position of **5** was carried out by using lithium diisopropylamide (LDA), which has been successfully used for the metallation of nucleosides having a halogen substituent.[4,6–8] Compound **5** was treated with 3.8 eq of LDA and then reacted with MeI at below −80 °C for 6 h, after which 2-chloro-5-methyl-1-(2,3-*O*-methoxymethylidene-5-*O*-TBDMS-β-D-ribofuranosyl)imidazole-4-carboxamide (**6**) was obtained in 32% yield by

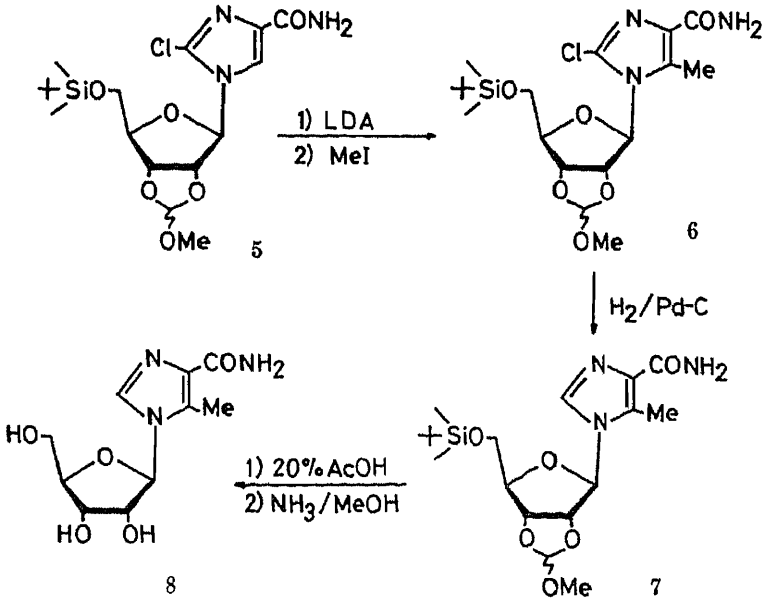


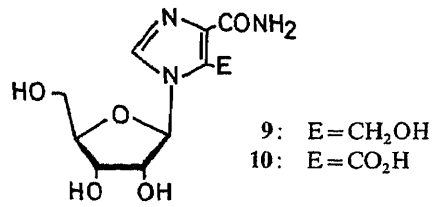
Chart 2

column chromatography on silica gel. Addition of hexamethylphosphoric triamide (HMPA) in the above reaction or the use of lithium 2,2,6,6-tetramethylpiperidide (LTMP) instead of LDA appeared to give no significant increase in the yield of **6**, and more than 40% of the starting material (**5**) was recovered in all three cases examined.

The chlorine atom thus used to protect the C-2 position from metallation was removed by hydrogenolysis in MeOH in the presence of 10% Pd–C and Et_3N (3 atm of H_2, room temperature, 24 h) to give **7** in 76% yield. Deprotection of the sugar moiety was performed as

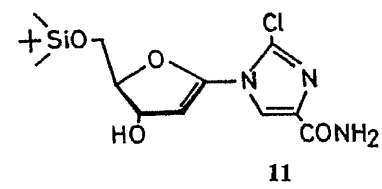
reported earlier[4] to furnish 5-methyl-1-β-D-ribofuranosylimidazole-4-carboxamide (**8**) in 74% yield as crystals (mp 178—179 °C). Physical constants of **8** were identical with those reported.[3d]

When HCO_2Me was employed as an electrophile in the reaction of the C-5 lithiated species of **5**, the 5-formylated product resulted, and this was reduced by $NaBH_4$ in a one-pot process. By following the reaction sequence illustrated in Chart 2, 5-hydroxymethyl-1-β-D-ribofuranosylimidazole-4-carboxamide (**9**: mp 155—156 °C) was isolated in 19% overall yield from **5**. Similarly, by using carbon dioxide as an electrophile, the 5-carboxylic acid **10** (mp 179—180 °C) was prepared in 17% overall yield.



9: E=CH_2OH
10: E=CO_2H

Fig. 2

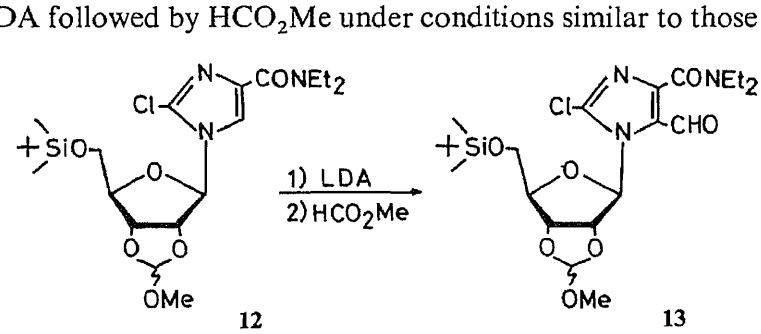


11

Fig. 3

During the LDA lithiation of **5**, we observed in the reaction mixture the presence of a polar by-product, in addition to the remaining **5** and the desired product. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of the by-product in dimethyl sulfoxide (DMSO)-d_6 showed no signals corresponding to the methoxymethylidene group and showed one D_2O-exchangeable doublet (δ 5.51 ppm) which coupled with a multiplet at δ 4.83 ppm. By comparison of the ^{1}H-NMR data with those reported for 6-amino-9-(2-deoxy-D-*erythro*-pent-1-enofuranosyl)purine[9] and from HOMO-SD experiments, the structure of this by-product was determined as 2-chloro-1-(2-deoxy-5-*O*-TBDMS-D-*erythro*-pent-1-enofuranosyl)imidazole-4-carboxamide (**11**).[10] In the mass spectrum (MS) of **11**, a fragment ion peak corresponding to [M^+ − Bu-*tert* − H_2O] (*m/z*: 298 and 300) was observed with an intensity of *ca.* 15 times higher than that of [M^+ − Bu-*tert*] (*m/z*: 316 and 318), which is also indicative of its structure. It should be noted that **11** was also formed during the LDA lithiation of 2-chloro-1-(2,3-*O*-isopropylidene-5-*O*-TBDMS-β-D-ribofuranosyl)imidazole-4-carboxamide.

On the other hand, when 2-chloro-1-(2,3-*O*-methoxymethylidene-5-*O*-TBDMS-β-D-ribofuranosyl)imidazole-4-*N*,*N*-diethylcarboxamide (**12**), a tertiary carboxamide, was reacted with 3.8 eq of LDA followed by HCO_2Me under conditions similar to those used for **5**, the 5-



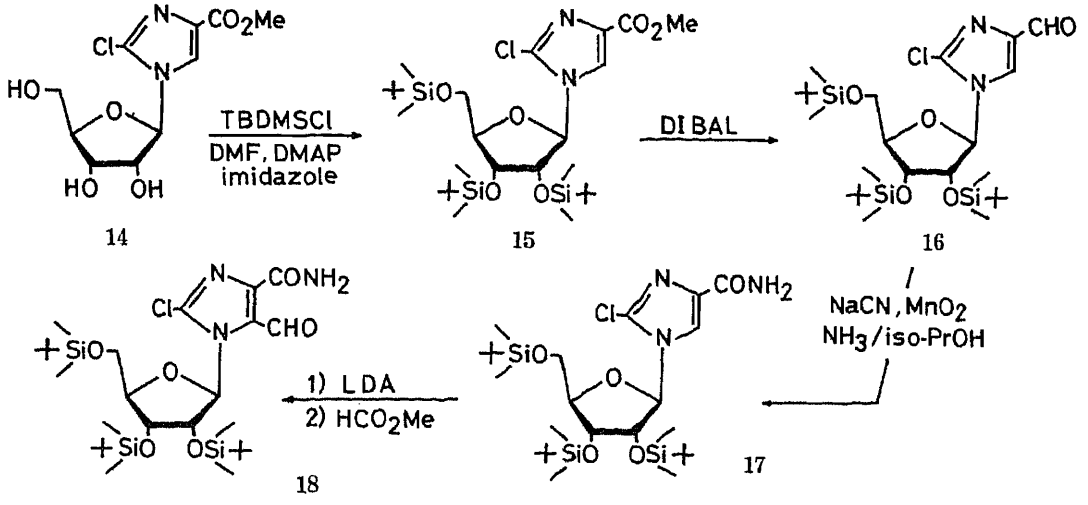
12 **13**

Chart 3

formyl derivative (**13**) was obtained in 63% yield along with a 34% recovery of **12** and the formation of the corresponding 1′,2′-unsaturated nucleoside was not observed. From these results, it became apparent that the formation of **11** and the low yield of the product in the reaction of **5** were associated with deprotonation of the 4-carboxamide group in **5**, though the

actual mechanism leading to **11** is still unknown.

We next investigated Wittig reaction of the 5-formyl 1-β-D-ribofuranosylimidazole-4-carboxamide derivative to synthesize other 5-carbon-substituted derivatives. For the preparation of the starting material for this reaction, we turned to the use of 2′,3′,5′-tris-*O*-TBDMS protection to preclude the above-mentioned elimination pathway observed in the lithiation of 2′,3′-*O*-alkylidene derivatives.



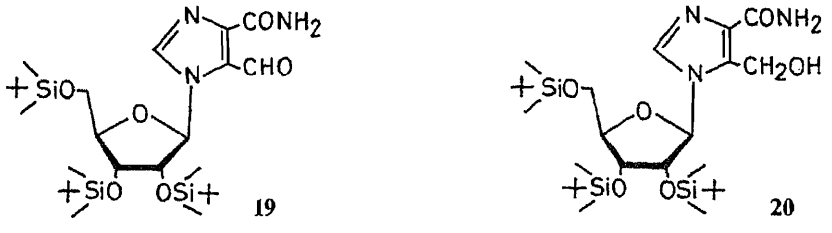
14 15 16 17 18

Chart 4

Methyl 2-chloro-1-β-D-ribofuranosylimidazole-4-carboxylate (**14**)[4] was treated with 6 eq of TBDMSCl in dimethylformamide (DMF) in the presence of imidazole (6 eq) and 4-*N*,*N*-dimethylaminopyridine (DMAP: 6 eq) to afford the 2′,3′,5′-tris-*O*-TBDMS derivative (**15**) in almost quantitative yield. Compound **15** was converted, *via* its 4-formyl derivative (**16**), to the corresponding 4-carboxamide (**17**) as shown in Chart 4.

When **17** was lithiated with 3.8 eq of LDA and then reacted with HCO_2Me, formation of the by-product, a 1′,2′-unsaturated nucleoside, was not detected on thin layer chromatography (TLC) (benzene : EtOAc = 3 : 1). However, the 5-formylated product (**18**) was obtained in only 13% yield and most of the starting material (**17**: 83%) was recovered. The use of LTMP (5 eq), a more basic lithiating agent than LDA,[11] gave a slightly increased yield (38%) which was comparable to the LDA lithiation level of **5**.

When the hydrogenolytic removal of the C-2 chlorine atom in **18** was carried out in MeOH in the presence of 10% Pd–C and Et_3N (3 atm of H_2, 3 h), both the 5-formyl (**19**) and the 5-hydroxymethyl (**20**) derivatives were formed with a preponderance of the latter (**19**: 36%

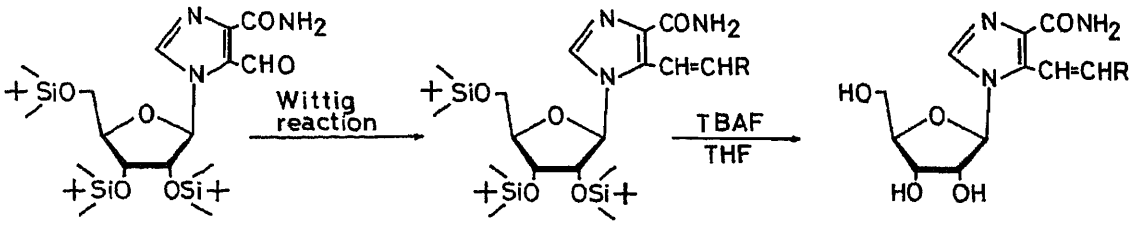


19 20

Fig. 4

vs. **20**: 48%). This unfavorable situation was easily overcome by changing the solvent to benzene and using 5% Pd–C as a catalyst. Under these conditions, the yield of **19** rose to 70%, while the formation of **20** was suppressed to 10%.

The Wittig reaction of **19** was performed in THF by using methylenetriphenylphosphorane and (carbethoxymethylene)triphenylphosphorane, respectively, to furnish **21** (77%) and **22** (59%). In the ^{1}H-NMR spectrum of **21** in $CDCl_3$, a characteristic ABX pattern (J_{AX} = 18.1 Hz, J_{BX} = 11.7 Hz, J_{AB} = 1.0 Hz) was observed, showing the presence of a vinyl group. (*E*)-Stereochemistry of **22** was deduced from its ^{1}H-NMR spectrum, the coupling constant



19

21: R=H
22: R=CO_2Et

23: R=H
24: R=CO_2Et

Chart 5

between vinylic protons being 16.6 Hz.

Finally, deprotection of **21** and **22** was carried out with tetrabutylammonium fluoride (TBAF) in THF to give the corresponding free nucleosides (**23** and **24**) in high yields.

In conclusion, the present work provides a general method for the preparation of various types of 5-carbon-substituted 1-β-D-ribofuranosylimidazole-4-carboxamides. Although the C-5 lithiation level is not high, presumably due to dissociation of the primary carboxamide group at the C-4 position, this method has certain advantages over the classical condensation method in that it causes no regio- and stereochemical problems. It should also be emphasized that no successful beta (or *ortho*) lithiation of a primary carboxamide has previously been reported to the best of our knowledge.[12,13]

Experimental

Melting points were determined with a Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H-NMR spectra were measured with tetramethylsilane as an internal standard, with either a JEOL JNM-FX 100 or a JEOL JNM-GX 400 NMR spectrometer. The abbreviations used are as follows: s, singlet; d, doublet; dd, double doublet; t, triplet; q, quartet; m, multiplet; br, broad. In the cases of 2′,3′-*O*-methoxymethylidene derivatives, ^{1}H-NMR signals of the major diastereomer are shown. MS were taken on a JEOL JMS-D 300 spectrometer. Ultraviolet (UV) spectra were recorded on a Shimadzu UV-240 spectrophotometer. Reactions at low temperature were performed using a CryoCool CC-100 apparatus (NESLAB Instrument, Inc.). Butyllithium in hexane was titrated before use by using diphenylacetic acid in THF. THF was distilled from benzophenone ketyl. Column chromatography was carried out either on silica gel (Wakogel® C-200) or on magnesium silicate (Florisil®). TLC was performed on precoated Silica gel plates 60 F_{254}, Merck.

2-Chloro-4-formyl-1-(2,3-*O*-methoxymethylidene-5-*O*-TBDMS-β-D-ribofuranosyl)imidazole (4)——A 1 M solution of DIBAL in toluene (5.6 ml) was added to a solution of **3** (687 mg, 1.5 mmol) in THF (5 ml) at below −70 °C under an Ar atmosphere. The mixture was stirred for 2 h at below −70 °C and then quenched with AcOH. The whole mixture was chromatographed on a silica gel column (benzene : EtOAc = 10 : 1) to give **4** (611 mg, 95%) as a syrup. MS *m/z*: 387, 389 (M−OMe), 361, 363 (M−Bu-*tert*), 289 (M−B). UV λ_{max}^{MeOH} nm: 256. ^{1}H-NMR ($CDCl_3$) δ: 0.11 (6H, s, SiMe), 0.89 (9H, s, SiBu-*tert*), 3.45 (3H, s, OMe), 3.91 (2H, m, CH_2-5′), 4.54 (1H, m, H-4′), 4.72 (1H, dd, H-3′), 4.91 (1H, dd, H-2′), 5.95 (1H, s, CHOMe), 6.12 (1H, d, *J* = 3.4 Hz, H-1′), 7.93 (1H, s, H-5), 9.77 (1H, s, CHO).

2-Chloro-1-(2,3-*O*-methoxymethylidene-5-*O*-TBDMS-β-D-ribofuranosyl)imidazole-4-carboxamide (5)——A suspension of NaCN (74 mg, 1.5 mmol) in isopropanol (15 ml) saturated with NH_3 at 0 °C was stirred for 10 min at 0 °C, after which a solution of **4** (132 mg, 0.3 mmol) in isopropanol (5 ml) was added dropwise. Activated MnO_2[14] (522 mg) was added to the above mixture and the whole was stirred for 2 h at 0 °C. Filtration through Celite followed by chromatographic purification of the filtrate on a silica gel column (2% MeOH in $CHCl_3$) gave **5** (130 mg, 95%). MS *m/z*: 402, 404 (M−OMe), 376, 378 (M−Bu-*tert*), 289 (M−B), 145, 147 (B+1). UV λ_{max}^{MeOH} nm: 220. ^{1}H-NMR ($CDCl_3$) δ: 0.11 (6H, s, SiMe), 0.90 (9H, s, SiBu-*tert*), 3.44 (3H, s, OMe), 3.87 (2H, m, CH_2-5′), 4.38—4.52 (1H, m, H-4′), 4.70—5.00 (2H, m, H-2′, H-3′), 5.60, 6.85 (2H, each br, NH_2), 5.95 (1H, s, CHOMe), 6.08 (1H, d, *J* = 3.4 Hz, H-1′), 7.82 (1H, s, H-5).

2-Chloro-5-methyl-1-(2,3-*O*-methoxymethylidene-5-*O*-TBDMS-β-D-ribofuranosyl)imidazole-4-carboxamide (6)——LDA (3.8 mmol) in THF (12 ml) was placed in a three-necked flask equipped with a gas-inlet adaptor, thermometer, and rubber septum. To this, a solution of **5** (437 mg, 1.0 mmol) in THF (5 ml) was added, under positive pressure of dry Ar, at such a rate that the temperature did not exceed −80 °C. The mixtures was stirred for 1.5 h at below −80 °C, after which MeI (0.24 ml, 3.8 mmol) was added and the whole was stirred for 4 h. Another 0.14 ml (0.4 mmol) of MeI was added to the above mixture and stirring was continued for a further 2 h below −80 °C. After being quenched with AcOH, the reaction mixture was evaporated and the residue was chromatographed on a silica gel column (benzene : EtOAc = 5 : 1) to give **6** (144 mg, 32%). MS *m/z*: 416, 418 (M−OMe), 390, 392 (M−Bu-*tert*), 289 (M−B). ^{1}H-NMR ($CDCl_3$) δ: 0.09 (6H, s, SiMe), 0.91 (9H, s, SiBu-*tert*), 2.67 (3H, s, 5-Me), 3.43 (3H, s, OMe), 3.93 (2H, m, CH_2-5′), 4.24 (1H, m, H-4′), 4.90—5.10 (2H, m, H-2′, H-3′), 5.28, 6.86 (2H, each br, NH_2), 5.98 (1H, d, J = 3.9 Hz, H-1′).

5-Methyl-1-(2,3-*O*-methoxymethylidene-5-*O*-TBDMS-β-D-ribofuranosyl)imidazole-4-carboxamide (7)——A mixture of **6** (140 mg, 0.3 mmol), Et_3N (0.1 ml, 0.8 mmol), and 10% Pd–C (50 mg) in MeOH (10 ml) was subjected to hydrogenolysis (3 atm of H_2) for 24 h at room temperature. After removal of the catalyst, the reaction mixture was purified through a silica gel column (4% MeOH in $CHCl_3$) to give **7** (98 mg, 76%). MS *m/z*: 382 (M−OMe), 356 (M−Bu-*tert*), 289 (M−B). ^{1}H-NMR ($CDCl_3$) δ: 0.09 (6H, s, SiMe), 0.90 (9H, s, SiBu-*tert*), 2.66 (3H, s, 5-Me), 3.44 (3H, s, OMe), 3.90 (2H, m, CH_2-5′), 4.41 (1H, m, H-4′), 4.70 (1H, dd, H-3′), 4.94 (1H, dd, H-2′), 5.91 (1H, d, J = 3.9 Hz, H-1′), 5.96 (1H, s, CHOMe), 5.27, 7.03 (2H, each br, NH_2), 7.62 (1H, s, H-2).

5-Methyl-1-β-D-ribofuranosylimidazole-4-carboxamide (8)——Compound **7** (75 mg, 0.2 mmol) in 20% aqueous AcOH was stirred for 24 h at room temperature. After evaporation of the solvent, the residue was treated with NH_3/MeOH (5 ml) for 5 min. Column chromatographic purification (8% MeOH in $CHCl_3$) of the mixture gave **8**, which was crystallized from EtOH to afford an analytical sample (35 mg, 74%, mp 178—179 °C). *Anal.* Calcd for $C_{10}H_{15}N_3O_5$: C, 46.69; H, 5.88; N, 16.33. Found: C, 46.96; H, 5.97; N, 16.05. MS *m/z*: 257 (M^+), 125 (B+1). UV $\lambda_{max}^{H_2O}$ nm (ε): 239 (12000), $\lambda_{min}^{H_2O}$: 205 (7600). ^{1}H-NMR (DMSO-d_6, after addition of D_2O) δ: 2.51 (3H, s, 5-Me), 3.58 (2H, m, CH_2-5′), 3.89—4.25 (3H, m, H-2′, H-3′, H-4′), 5.50 (1H, d, J = 5.9 Hz, H-1′), 7.90 (1H, s, H-5).

5-Hydroxymethyl-1-β-D-ribofuranosylimidazole-4-carboxamide (9)——The C-5 formylation of **5** (522 mg, 1.2 mmol) with HCO_2Me (0.3 ml, 4.9 mmol) was carried out for 2 h by the same procedure as described for the preparation of **6**. After being quenched with AcOH, the reaction mixture was diluted with MeOH (10 ml) and treated with $NaBH_4$ until TLC indicated complete reduction. Short-column chromatography gave the crude mixture, which was subjected to hydrogenolysis followed by deprotection as described for the preparation of **7** and **8**, respectively. This afforded a syrup, which was crystallized from MeOH to give analytically pure **9** (63 mg, 19%, mp 155—156 °C). *Anal.* Calcd for: $C_{10}H_{15}N_3O_6$: C, 43.96; H, 5.53; N, 15.38. Found: C, 44.22; H, 5.56; N, 15.56. MS *m/z*: 273 (M^+), 141 (B+1). UV $\lambda_{max}^{H_2O}$ nm (ε): 237 (17000), $\lambda_{shoulder}^{H_2O}$: 221 (10700), $\lambda_{min}^{H_2O}$: 207 (9600). ^{1}H-NMR (DMSO-d_6, after addition of D_2O) δ: 3.62 (2H, m, CH_2-5′), 3.93—4.24 (3H, m, H-2′, H-3′, H-4′), 4.53, 4.95 (2H, each d, J = 13.2 Hz, 5-CH_2OH), 5.76 (1H, d, J = 4.9 Hz, H-1′), 8.02 (1H, s, H-2).

5-Carbamoyl-3-β-D-ribofuranosylimidazole-4-carboxylic Acid (10)——The C-5 carboxylation of **5** (261 mg, 0.6 mmol) with CO_2 gas (large excess) was carried out for 30 min by the same procedure as described for the preparation of **6**. After short-column chromatography, the crude mixture was subjected to hydrogenolysis followed by deprotection as described for the preparation of **7** and **8**, respectively. Purification through DEAE cellulofine AL® (bicarbonate form, a linear gradient of 0—0.03 M $Et_3NH \cdot HCO_3$) and successive acidification through Dowex 50® (H^+ form) gave **10** (29 mg, 17%) as crystals (mp 179—180 °C). *Anal.* Calcd for $C_{10}H_{13}N_3O_7$: C, 41.82; H, 4.56; N, 14.63. Found: C, 41.65; H, 4.63; N, 14.45. MS *m/z*: 155 (B+1). UV $\lambda_{max}^{H_2O}$ nm(ε): 251 (6800), $\lambda_{min}^{H_2O}$: 235 (5900). ^{1}H-NMR (DMSO-d_6, after addition of D_2O) δ: 3.63, 3.81 (2H, each dd, CH_2-5′), 3.92—3.94 (1H, m, H-4′), 4.04—4.08 (2H, m, H-2′, H-3′), 6.49 (1H, s, H-1′), 8.69 (1H, s, H-2).

2-Chloro-1-(2-deoxy-5-*O*-TBDMS-D-*erythro*-pent-1-enofuranosyl)imidazole-4-carboxamide (11)——Physical data of this compound are as follows. MS *m/z*: 316, 318 (M−Bu-*tert*), 298, 300 (M−Bu-*tert*-H_2O). UV $\lambda_{shoulder}^{MeOH}$ nm: 250. ^{1}H-NMR (DMSO-d_6) δ: 0.02, 0.05 (6H, each s, SiMe), 0.84 (9H, s, SiBu-*tert*), 3.49 (2H, m, CH_2-5′), 4.41 (1H, m, H-4′), 4.83 (1H, m, H-3′), 5.44 (1H, d, H-2′), 5.51 (1H, d, 3′-OH), 7.60, 7.80 (2H, each br, NH_2), 7.80, (1H, s, H-5).

2-Chloro-1-(2,3-*O*-methoxymethylidene-5-*O*-TBDMS-β-D-ribofuranosyl)imidazole-4-*N*,*N*-diethylcarboxamide (12)——A solution of **4** (165 mg, 0.4 mmol) in isopropanol (5 ml) and activated MnO_2 (696 mg) were added to a suspension of NaCN (98 mg, 2.0 mmol) in isopropanol (10 ml) and Et_2NH (0.8 ml, 8.0 mmol). The mixture was stirred for 2 h at room temperature and then two 0.8 ml portions of Et_2NH were added at 2 h intervals. Filtration through Celite followed by chromatographic purification of the filtrate on a silica gel column (2% MeOH in $CHCl_3$) gave **12** (105 mg, 55%). MS *m/z*: 458, 460 (M−OMe), 432, 434 (M−Bu-*tert*), 289 (M−B). UV λ_{max}^{MeOH} nm: 224. ^{1}H-NMR ($CDCl_3$) δ: 0.10 (6H, s, SiMe), 0.89 (9H, s, SiBu-*tert*), 1.21 (6H, t, NCH_2CH_3), 3.35—3.91 (9H, m, OMe, NCH_2CH_3, CH_2-5′), 4.27—4.46 (1H, m, H-4′), 4.72—5.01 (2H, m, H-2′, H-3′), 6.06 (1H, d, J = 3.4 Hz, H-1′), 5.95 (1H, s, CHOMe), 7.68 (1H, s, H-5).

2-Chloro-5-formyl-1-(2,3-*O*-methoxymethylidene-5-*O*-TBDMS-β-D-ribofuranosyl)imidazole-4-*N*,*N*-diethylcarboxamide (13)——The C-5 formylation of **12** (95 mg, 0.2 mmol) with HCO_2Me (0.05 ml, 0.8 mmol) was carried out

for 1 h by the same procedure as described for the preparation of **6**. After being quenched with AcOH, the reaction mixture was purified by column chromatography on silica gel (benzene : EtOAc = 10—5 : 1). This afforded **13** (64 mg, 63%) and **12** (32 mg, 34%). Physical data of **13** are as follows. *Anal.* Calcd for $C_{22}H_{36}ClN_3O_7Si$: C, 51.00; H, 7.00; N, 8.11. Found: C, 51.22; H, 7.24; N, 7.93. MS *m/z*: 486, 488 (M − OMe), 460, 462 (M − Bu-*tert*). UV λ_{max}^{MeOH} nm (ε): 276 (8500), $\lambda_{shoulder}^{MeOH}$: 236 (8900), λ_{min}^{MeOH}: 256 (6900). ^{1}H-NMR ($CDCl_3$) δ: 0.08 (6H, s, SiMe), 0.90 (9H, s, SiBu-*tert*), 1.25 (6H, t, $NCH_2\underline{CH_3}$), 3.31—3.91 (10H, m, OMe, $NC\underline{H_2}CH_3$, H-4′, CH_2-5′), 5.00—5.13 (2H, m, H-2′, H-3′), 5.96 (1H, s, C<u>H</u>OMe), 6.37 (1H, d, *J* = 3.4 Hz, H-1′), 9.93 (1H, s, 5-CHO).

Methyl 2-Chloro-1-(2,3,5-tris-*O*-TBDMS-β-D-ribofuranosyl)imidazole-4-carboxylate (15)——Compound **14** (2.1 g, 7.0 mmol) was added to a mixture of TBDMSCl (6.4 g, 42 mmol), imidazole (2.9 g, 42 mmol), and DMAP (5.2 g, 42 mmol) in DMF (10 ml). The mixture was stirred for 40 h at room temperature and then poured into EtOAc–H_2O. The organic layer was separated, dried (Na_2SO_4), and evaporated to dryness. Silica gel column chromatographic purification (benzene) of the residue gave **15** (4.5 g, 99%). MS *m/z*: 577, 579 (M − Bu-*tert*). UV λ_{max}^{MeOH} nm: 232. ^{1}H-NMR ($CDCl_3$) δ: 0.10, 0.11, 0.17, 0.20 (18H, each s, SiMe), 0.84, 0.93, 0.97 (27H, each s, SiBu-*tert*), 3.67—3.92 (2H, m, CH_2-5′), 3.87 (3H, s, CO_2Me), 4.09—4.29 (3H, m, H-2′, H-3′, H-4′), 5.77 (1H, d, *J* = 5.9 Hz, H-1′), 8.02 (1H, s, H-5).

2-Chloro-4-formyl-1-(2,3,5-tris-*O*-TBDMS-β-D-ribofuranosyl)imidazole (16)——This compound was prepared from **15** (4.6 g) by the same procedure as used for the preparation of **4**. Silica gel short-column chromatography (benzene : EtOAc = 10 : 1) gave **16** (2.4 g, 55%). MS *m/z*: 547, 549 (M − Bu-*tert*). UV λ_{max}^{MeOH} nm: 254. ^{1}H-NMR ($CDCl_3$) δ: 0.03, 0.11, 0.17, 0.20 (18H, each s, SiMe), 0.85, 0.94, 0.98 (27H, each s, SiBu-*tert*), 3.68—4.00 (2H, m, CH_2-5′), 4.12—4.28 (3H, m, H-2′, H-3′, H-4′), 5.79 (1H, d, *J* = 5.4 Hz, H-1′), 8.07 (1H, s, H-5), 9.78 (1H, s, 4-CHO).

2-Chloro-1-(2,3,5-tris-*O*-TBDMS-1-β-D-ribofuranosyl)imidazole-4-carboxamide (17)——This compound was prepared from **16** (2.4 g) by the same procedure as used for the preparation of **5**. Silica gel column chromatography (benzene : EtOAc = 5 : 1) gave **17** (2.0 g, 81%), which was crystallized from MeOH–H_2O (mp 133—134 °C). *Anal.* Calcd for $C_{27}H_{54}ClN_3O_5Si_3$: C, 52.27; H, 8.77; N, 6.77. Found: C, 52.55; H, 8.86; N, 7.01. MS *m/z*: 562, 564 (M − Bu-*tert*). UV λ_{max}^{MeOH} nm (ε): 223 (14300). ^{1}H-NMR ($CDCl_3$) δ: −0.27, −0.05, 0.11, 0.16, 0.19 (18H, each s, SiMe), 0.84, 0.94, 0.96 (27H, each s, SiBu-*tert*), 3.79 (2H, m, CH_2-5′), 4.07—4.27 (3H, m, H-2′, H-3′, H-4′), 5.34, 6.80 (2H, each br, NH_2), 5.76 (1H, d, *J* = 5.9 Hz, H-1′), 7.91 (1H, s, H-5).

2-Chloro-5-formyl-1-(2,3,5-tris-*O*-TBDMS-β-D-ribofuranosyl)imidazole-4-carboxamide (18)——This compound was prepared from **17** (134 mg, 0.2 mmol), LTMP (1.0 mmol), and HCO_2Me (0.17 ml, 2.8 mmol) by the same procedure as used for the preparation of **6**. Silica gel column chromatography (benzene : EtOAc = 10 : 1) gave **18** (54 mg, 38%). MS *m/z*: 590, 592 (M − Bu-*tert*). UV λ_{max}^{MeOH} nm: 254. ^{1}H-NMR ($CDCl_3$) δ: −0.07, 0.09, 0.12 (18H, each s, SiMe), 0.78, 0.92, 0.95 (27H, each s, SiBu-*tert*), 3.62—4.20 (4H, m, H-3′, H-4′, CH_2-5′), 4.82 (1H, dd, H-2′), 5.59, 7.24 (2H, each br, NH_2), 6.45 (1H, d, *J* = 7.8 Hz, H-1′), 10.60 (1H, s, 5-CHO).

5-Formyl-1-(2,3,5-tris-*O*-TBDMS-β-D-ribofuranosyl)imidazole-4-carboxamide (19) and 5-Hydroxymethyl-1-(2,3,5-tris-*O*-TBDMS-1-β-D-ribofuranosyl)imidazole-4-carboxamide (20)——A mixture of **18** (105 mg, 0.16 mmol), 5% Pd–C (50 mg), and Et_3N (0.2 ml) in benzene (5 ml) was hydrogenated (3 atm of H_2) at room temperature for 3 h. After removal of the catalyst, the mixture was evaporated to dryness. Silica gel column chromatography gave **19** (elution with benzene : EtOAc = 10 : 1, 69 mg, 70%) and **20** (elution with 2% MeOH in $CHCl_3$, 10 mg, 10%).

Physical data of **19** are as follows. MS *m/z*: 556 (M − Bu-*tert*). UV λ_{max}^{MeOH} nm: 275. ^{1}H-NMR ($CDCl_3$) δ: 0.06, 0.08, 0.11, 0.16, 0.18 (18H, each s, SiMe), 0.90, 0.98 (27H, each s, SiBu-*tert*), 3.76—4.12 (5H, m, H-2′, H-3′, H-4′, CH_2-5′), 5.56, 8.55 (2H, each br, NH_2), 6.31 (1H, d, *J* = 2.5 Hz, H-1′), 7.26 (overlapped with $CHCl_3$, H-2), 10.63 (1H, s, 5-CHO).

Physical data of **20** are as follows. MS *m/z*: 558 (M − Bu-*tert*). ^{1}H-NMR ($CDCl_3$) δ: −0.05, 0.10, 0.14 (18H, each s, SiMe), 0.83, 0.93, 0.95 (27H, each s, SiBu-*tert*), 3.79—3.84 (2H, m, CH_2-5′), 4.07—4.27 (3H, m, H-4′, 5-C<u>H</u>$_2$OH), 4.77—4.86 (2H, m, H-2′, H-3′), 5.53—5.75 (3H, m, H-1′, CONH, 5-CH_2O<u>H</u>), 7.16 (1H, br, CONH), 7.74 (1H, s, H-2).

5-Vinyl-1-(2,3,5-tris-*O*-TBDMS-β-D-ribofuranosyl)imidazole-4-carboxamide (21)——A suspension of methyltriphenylphosphonium bromide (429 mg, 1.2 mmol) in THF (3 ml) was treated with NaH (48 mg, 1.2 mmol) at 0 °C under an Ar atmosphere, and the mixture was stirred for 0.5 h at 0 °C. After being stirred for 1 h at room temperature, the mixture was again cooled to 0 °C and treated with a solution of **19** (184 mg, 0.3 mmol) in THF (5 ml). The whole reaction mixture was stirred for 13 h at room temperature and then quenched with MeOH. The mixture was partitioned between ether and H_2O. The organic layer was separated, dried (Na_2SO_4), and chromatographed on a silica gel column (benzene: EtOAc = 8 : 1) to give **21** (142 mg, 77%). MS *m/z*: 569 (M − Me), 554 (M − Bu-*tert*). UV λ_{max}^{MeOH} nm: 264. ^{1}H-NMR ($CDCl_3$) δ: 0.10, 0.11, 0.14 (18H, each s, SiMe), 0.80, 0.93, 0.96 (27H, each s, SiBu-*tert*), 3.80 (2H, m, CH_2-5′), 4.16 (1H, m, H-4′), 4.19 (1H, dd, H-3′), 4.33 (1H, dd, H-2′), 5.39, 7.05 (2H, each br, NH_2), 5.61, 5.95, 7.22 (3H, each dd, vinyl protons), 5.92 (1H, d, *J* = 6.8 Hz, H-1′), 7.83 (1H, s, H-2).

5-(2-Ethoxycarbonyl)vinyl-1-(2,3,5-tris-*O*-TBDMS-β-D-ribofuranosyl)imidazole-4-carboxamide (22)——A THF (2 ml) solution of **19** (111 mg, 0.2 mmol) was added to a suspension of (carbethoxymethylene)triphenylphosphorane (251 mg, 0.7 mmol) in THF (1.5 ml) at 0 °C under an Ar atmosphere. The mixture was stirred for 1 h at 0 °C and then for 48 h at room temperature. The reaction mixture was partitioned between ether and H_2O. The organic layer was

separated, dried (Na_2SO_4), and chromatographed on a silica gel column (benzene : EtOAc = 10 : 1) to give **22** (73 mg, 59%). MS *m/z*: 626 (M − Bu-*tert*). UV λ_{max}^{MeOH} nm: 265, 300. ^{1}H-NMR ($CDCl_3$) δ: 0.10, 0.13, 0.14 (18H, each s, SiMe), 0.80, 0.94, 0.96 (27H, each s, SiBu-*tert*), 1.29 (3H, t, $CH_2C\underline{H}_3$), 3.83 (2H, m, CH_2-5′), 4.23 (2H, q, $C\underline{H}_2CH_3$), 5.37, 7.13 (2H, each br, NH_2), 5.90 (1H, d, J = 7.3 Hz, H-1′), 6.93, 8.12 (2H, each d, vinylic protons), 7.96 (1H, s, H-2).

5-Vinyl-1-β-D-ribofuranosylimidazole-4-carboxamide (23)——A THF (3 ml) solution of **21** (109 mg, 0.2 mmol) was treated with TBAF·$3H_2O$ (199 mg, 0.6 mmol) for 0.5 h at room temperature. The whole mixture was evaporated to dryness and the residue was chromatographed on a Florisil column (20% MeOH in $CHCl_3$) to give **23** (42 mg, 86%). MS *m/z*: 269 (M^+), 137 (B + 1), 136 (B^+). UV $\lambda_{max}^{H_2O}$ nm: 260, $\lambda_{min}^{H_2O}$: 238. ^{1}H-NMR (DMSO-d_6, after addition of D_2O) δ: 3.51—3.66 (2H, m, CH_2-5′), 3.92—3.94 (1H, t, H-4′), 4.09 (1H, t, H-3′), 4.30 (1H, t, H-2′), 5.52, 5.87, 7.23 (3H, each dd, vinyl protons), 5.64 (1H, d, J = 5.1 Hz, H-1′), 8.14 (1H, s, H-2).

Compound **23** was converted to its triacetate, and the high-resolution MS was measured. High-resolution MS *m/z*: 395.1342 (M^+) Calcd for $C_{17}H_{21}N_3O_8$: 395.1329. ^{1}H-NMR ($CDCl_3$) δ: 2.09, 2.14, 2.17 (9H, each s, Ac), 4.35 (2H, m, CH_2-5′), 4.40 (1H, m, H-4′), 5.38, 7.08 (2H, each br, NH_2), 5.43 (1H, t, H-3′), 5.59 (1H, t, H-2′), 5.66, 5.84 (2H, each d, vinyl protons), 5.96 (1H, d, J = 6.2 Hz, H-1′), 7.26 (1H, dd, vinyl proton), 7.76 (1H, s, H-2).

5-(2-Ethoxycarbonyl)vinyl-1-β-D-ribofuranosylimidazole-4-carboxamide (24)——This compound was prepared from **22** (32 mg, 0.05 mmol) by the same procedure as used for the preparation of **23**. Preparative TLC ($CHCl_3$: MeOH = 5 : 1) of the reaction mixture gave **24** (15 mg, 92%), which was crystallized from EtOH (mp 104—106 °C). *Anal.* Calcd for $C_{14}H_{19}N_3O_7 \cdot 3/4H_2O$: C, 47.39; H, 5.82; N, 11.84. Found: C, 47.18; H, 5.59; N, 11.74. UV $\lambda_{max}^{H_2O}$ nm (ε): 217 (18600), 300 (15000), $\lambda_{min}^{H_2O}$: 256 (4900). ^{1}H-NMR (DMSO-d_6, after addition of D_2O) δ: 1.27 (3H, t, $CH_2C\underline{H}_3$), 3.52—3.67 (2H, m, CH_2-5′), 3.98 (1H, m, H-4′), 4.09 (1H, t, H-3′), 4.21 (2H, q, $C\underline{H}_2CH_3$), 4.36 (1H, t, H-2′), 5.67 (1H, d, J = 5.5 Hz, H-1′), 6.71, 8.21 (2H, each d, vinylic protons), 7.34, 7.61 (2H, each br, $N\underline{H}_2$), 8.29 (1H, s, H-2).

References and Notes

1) K. Mizuno, M. Tsujino, M. Takada, M. Hayashi, K. Atsumi, K. Asano, and T. Matsuda, *J. Antibiot.*, **27**, 775 (1974); K. Fukukawa, S. Shuto, T. Hirano, and T. Ueda, *Chem. Pharm. Bull.*, **32**, 1644 (1984); *idem*, *ibid.*, **34**, 3653 (1986).
2) P. C. Srivastava, D. G. Streeter, T. R. Matthews, L. B. Allen, R. W. Sidwell, and R. K. Robins, *J. Med. Chem.*, **19**, 1020 (1976).
3) *a*) J. C. Reepmeyer, K. L. Kirk, and L. A. Cohen, *Tetrahedron Lett.*, **47**, 4107 (1975); *b*) P. C. Wyss and U. Fischer, *Helv. Chim. Acta*, **61**, 3149 (1978); *c*) P. D. Cook, R. J. Rousseau, A. M. Mian, P. Dea, R. B. Meyer, Jr., and R. K. Robins, *J. Am. Chem. Soc.*, **98**, 492 (1976); *d*) R. Alonso, J. I. Andrés, M. T. García-López, F. G. de las Heras, R. Herranz, B. Alarcón, and L. Carrasco, *J. Med. Chem.*, **28**, 834 (1985); S. G. Wood, K. G. Upadhya, N. K. Dalley, P. A. McKernan, P. G. Canonico, R. K. Robins, and G. R. Revankar, *ibid.*, **28**, 1198 (1985).
4) H. Tanaka, M. Hirayama, A. Matsuda, T. Miyasaka, and T. Ueda, *Chem. Lett.*, **1985**, 589; H. Tanaka, M. Hirayama, M. Suzuki, T. Miyasaka, A. Matsuda, and T. Ueda, *Tetrahedron*, **42**, 1971 (1986).
5) N. W. Gilman, *J. Chem. Soc., Chem. Commun.*, **1971**, 733.
6) H. Tanaka, Y. Uchida, M. Shinozaki, H. Hayakawa, A. Matsuda, and T. Miyasaka, *Chem. Pharm. Bull.*, **31**, 787 (1983).
7) H. Tanaka, A. Matsuda, S. Iijima, H. Hayakawa, and T. Miyasaka, *Chem. Pharm. Bull.*, **31**, 2164 (1983).
8) H. Tanaka, H. Hayakawa, S. Iijima, K. Haraguchi, and T. Miyasaka, *Tetrahedron*, **41**, 861 (1985).
9) M. J. Robins and R. A. Jones, *J. Org. Chem.*, **39**, 113 (1974).
10) For other reports on the formation of 1′,2′-unsaturated nucleosides: M. J. Robins and E. M. Trip, *Tetrahedron Lett.*, **38**, 3369 (1974); M. Kawana, K. Takeuchi, T. Ohba, and H. Kuzuhara, *Nucleic Acids Symposium Series*, **17**, 37 (1986).
11) LTMP is reported to be 1.6 pK units more basic than LDA: R. R. Fraser, A. Baignée, M. Bress, and K. Hata, *Tetrahedron Lett.*, **23**, 4195 (1982).
12) W. H. Puterbaugh and C. R. Hauser, *J. Org. Chem.*, **29**, 853 (1964).
13) The present method should be regarded as an alpha lithiation of a nitrogen-activated system.
14) J. Attenburrow, A. F. B. Cameron, J. H. Chapman, R. M. Evans, B. A. Hems, A. B. A. Jansen, and T. Walker, *J. Chem. Soc.*, **1952**, 1094.

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Identification de Nouvelles Structures Benzoyl-1 Isoquinoleiniques Obtenues par Oxydation de la Papavérine

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Papaverine **1** was oxidized by vanadium pentoxide in 2.5 M aqueous sulfuric acid media to give four new compounds: 4-hydroxy-6-demethylpapaveraldine (**3**), 4-hydroxy-4'-demethylpapaveraldine (**4**), 3-hydroxy-6-demethylpapaveraldine (**5**) and 3-hydroxy-4'-demethylpapaveraldine (**6**). The structures were established on the basis of mass spectrometry, ultraviolet–infrared spectrometry, nuclear magnetic resonance measurements and also chemical correlation with papaveraldine **2**.

Keywords——papaverine; 1-benzoylisoquinoline; papaveraldine 4-hydroxy-6-demethylpapaveraldine; 4-hydroxy-4'-demethylpapaveraldine; 3-hydroxy-6-demethylpapaveraldine; 3-hydroxy-4'-demethylpapaveraldine

La papavérine **1** constitue le chef de file des molécules à activité antispasmodique musculotrope. Son oxydation par divers réactifs conduit à de nombreux dérivés dont le principal est le dérivé benzoylé correspondant: la papavéraldine **2**.[1)]

L'oxydation de solutions de papavérine ou de son chlorhydrate par un courant d'oxygène ou par l'oxygène atmosphérique a été étudiée dès 1959 par Machovicova et Parrak qui reportent l'isolement d'un précipité brun foncé de composition inconnue[2)]; puis Pawelczyk et Hermann s'intéressent plus précisément à une substance X_3 dont ils ne parviennent pas à établir la structure.[3)] Enfin, plus récemment Gundermann et Pohloudek-Fabiani signalent l'existence de 3 composés inconnus dans les produits d'oxydation de la papavérine.[4)]

Lors d'études analytiques sur les possibilités de dosage de la papavérine par des solutions sulfovanadiques, nous avons remarqué la formation de composés colorés. Afin d'élucider leur structure nous avons réalisé à l'échelle préparative l'oxydation de la paravérine par des solutions sulfovanadiques selon un protocole précédemment décrit.[5)]

Comme nous l'avons constaté,[6)] la première étape de l'oxydation vanadique de la papavérine conduit à la papavéraldine **2** que l'on peut isoler, mais dont l'oxydation continue si l'on poursuit la réaction. Les composés colorés obtenus résultent par conséquent de l'oxydation de la papavéraldine **2**. Après précipitation *in situ* des produits d'oxydation, leur séparation et leur purification ont été réalisées par chromatographie liquide sur colonne de silice et cristallisation dans l'éther. Quatre nouveaux composés colorés ont ainsi été mis en évidence et leur structure déterminée grâce à la concordance des résultats analytiques. Ce sont les hydroxy-4 déméthyl-6 papavéraldine (**3**), hydroxy-4 déméthyl-4' papavéraldine (**4**), hydroxy-3 déméthyl-6 papavéraldine (**5**) et hydroxy-3 déméthyl-4' papavéraldine (**6**).

Ces composés ont en effet en commun une même formule brute: $C_{19}H_{17}NO_6$ déterminée à partir des résultats microanalytiques et confirmée par spectrométrie de masse. Pour **3**, de couleur verte, le spectre ultra-violet (UV)-visible en solution dans l'éthanol présente 6 maximums d'absorption à 280, 336, 348, 438, 464 et 596 nm. L'enregistrement des spectres

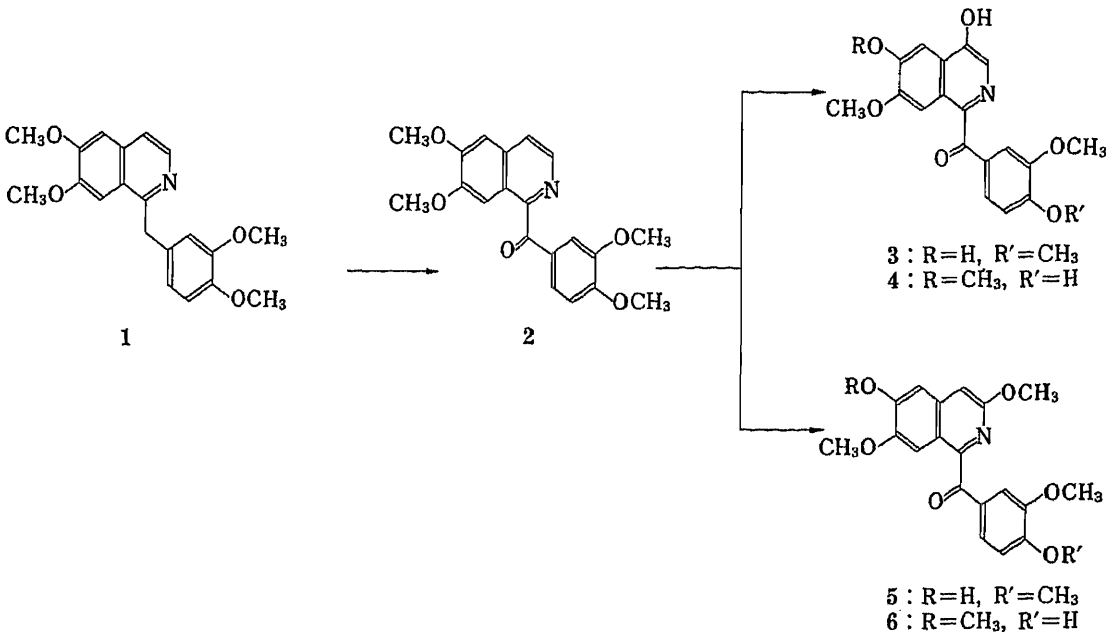

Fig. 1

dans des solutions éthanoliques acides et alcalines a permis de mettre en évidence les caractéristiques suivantes:

pH < 4 solution jaune—maximum d'absorption à 400 nm

pH > 10 solution incolore—maximum d'absorption à 370 nm

Ce composé a donc 2 valeurs de constante d'ionisation (pK_a) déterminées spectrophotométriquement[7]: 4,07 correspondant à la basicité de l'azote hétérocyclique et 9,54 correspondant à l'acidité de la fonction phénol.

Le spectre infra-rouge (IR) permet de mettre en évidence des groupements phénol (3400, 1210 et 1340 cm^{-1}) et de s'assurer de l'existence d'un groupement carbonyle (1680 cm^{-1}).

L'étude de l'influence de la constante diélectrique sur le pK_{a2} a permis de déterminer le nombre de fonctions phénol de la molécule. Le spectre de résonance magnétique nucléaire du proton (^{1}H-RMN) à 250 MHz présente 3 singulets à 3,84, 3,85 et 3,96 ppm dus aux 3 méthoxyles, 2 doublets dus aux protons C_5' et C_6' à 8,3 et 7,0 ppm, enfin 4 singulets à 6,4, 7,2, 7,6 et 7,9 ppm correspondant aux protons portés par C_2', C_3, C_5 et C_8. L'absence de doublets correspondant au couplage des protons portés par C_3 et C_4 distingue largement ce spectre de celui de la papavéraldine. En effet pour cette dernière, les protons en C_3 et C_4 sortent respectivement à 7,57 et 8,38 ppm sous forme de doublets (J=5,54 Hz). Le spectre de masse (SM) est en bon accord avec cette hypothèse; outre le pic moléculaire à m/z 355, les fragments m/z 337 (100), 322, 309, 293 et 292 sont caractéristiques des benzoyl-isoquinoléines[8]; le spectre présente également des pics à m/z 354 et 353 qui résultent d'une fragmentation spécifique des phénols.[9]

Nous avons pu mettre en évidence le caractère de base aminée organique du composé en identifiant par extraction chloroformique de la solution aqueuse acide la paire d'ions formée entre la molécule colorée (jaune) et le lauryl sulfate de sodium. La position du phénol en 6 est objectivée par une délocalisation électronique très importante et par l'existence de nombreuses formes tautomères (3 neutres et 2 amphioniques), ce qui ne serait pas le cas pour un phénol en position en 4'. La protonation (milieu acide) bloque la tautomérie, le composé présente donc une délocalisation électronique moindre, d'où une coloration jaune. La formation d'un ion

phénate en milieu alcalin empêche toute délocalisation électronique, ce qui explique l'absence de coloration du composé.

Les trois autres composés colorés isolés possèdent une structure très proche de celle de **3**.

Analogies Structurales

Les analyses élémentaires donnent des résultats très voisins de ceux trouvés pour le composé vert, ce qui nous conduit à émettre l'hypothèse d'isomères de position dont la structure de base reste celle de la papavéraldine **2**.

Les SM, réalisés par impact électronique à 20 eV et par ionisation chimique témoignent également d'une analogie structurale très importante: identification des fragments de même masse que pour **3**.

Les spectres IR nous permettent d'observer des structures identiques ou voisines, tout particulièrement entre les deux composés de coloration jaune (**5** et **6**).

Différences Structurales

Les spectres d'absorption UV-visible sont identiques pour **5** et **6** et présentent une forte analogie avec celui du composé correspondant à la forme protonée de **3** (maximums d'absorption à 258, 314 et 404 nm). Cette observation témoigne d'une faible délocalisation électronique de ces composés. Le spectre d'absorption de **4**, de coloration violette, est beaucoup plus proche de celui enregistré pour **3**. Les longueurs d'onde des maximums d'absorption sont de 298, 336, 412, 504 et 654 nm et le spectre visible présente un effet hypsochrome. Ces deux observations permettent de confirmer la position en 4′ du phénol (délocalisation électronique inférieure à **4**).

La Détermination des p*K* a Donné les Résultats Suivants——**4** (Violet): pH < 4—jaune, pH > 10—incolore. $pK_{a1} = 3{,}44$; $pK_{a2} = 10{,}88$.

5 et **6**: pH < 10—jaune, pH > 10—incolore. $pK_a = 10{,}0$.

La formation de paires d'ions avec le laurylsulfate n'a pu être réalisée qu'avec le composé violet **4**.

Les composés jaunes **5** et **6** présentent un caractère d'amide, ce qui implique que l'oxydation se soit effectuée sur le carbone 3 de l'hétérocycle.

Discussion

Oxydations en 3 et 4

En 1977 Waechter *et al.*[10] ont décrit l'oxydation privilégiée des diméthoxy-6,7 benzoyl-1

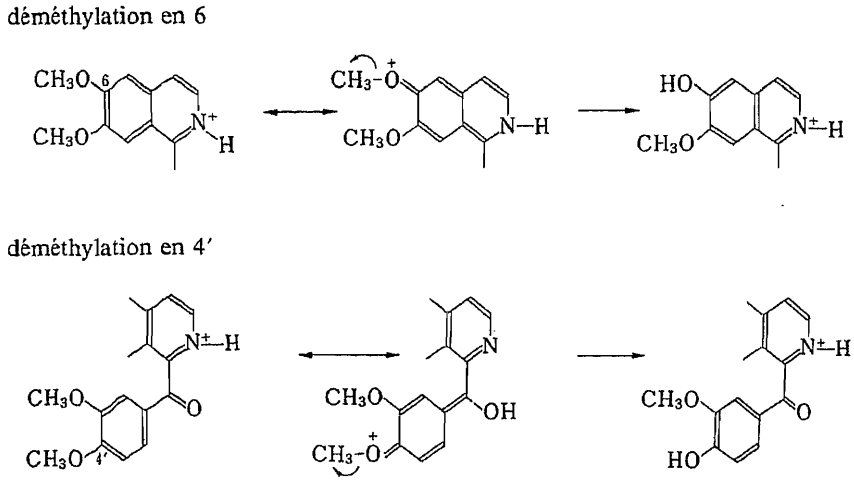

Fig. 2

isoquinoléines en position 3 et 4, mécanisme que nous avons par ailleurs pu affirmer lors de l'oxydation de benzyl-4 isoquinoléines.[11]

Déméthylation en 6 et 4'

L'oxydation périodique de diméthoxy-6,7 isoquinoléines substituées en 1 conduit à une réaction mettant en jeu, par l'intermédiaire d'un carbocation, la déméthylation du groupement éther-oxyde de phénol situé en 6.[12]

Un mécanisme analogue a déjà été décrit dans l'oxydation vanadique de diméthoxy-6,7 isoquinoléines.[13] C'est par un mécanisme identique que la déméthylation en 4' peut être envisagée (Fig. 2).

Partie Expérimentale

Les spectres UV sont enregistrés sur spectrophotomètre Hewlett Packard HP 8451 A et les spectres IR enregistrés en pastille de KBr sur appareil Perkin-Elmer 1420.

Les spectres de RMN ont été réalisés dans le diméthyl sulfoxide (DMSO) deutéré sur appareil Brucker. Les SM ont été enregistrés sur appareil NERMAG R 10-10, par impact électronique à 20 eV.

Oxydation de la Papavérine——2,5 g de chlorhydrate de papavérine (Fluka) sont mis à oxyder au bain marie bouillant dans 500 ml de réactif vanadique (19 g V_2O_5 Merck, dissous à chaud dans 300 ml de NaOH 1 N + 175 ml H_2SO_4 5 M + 29,1 ml H_2SO_4 36 N + H_2O déminéralisée QSP 500 ml). Le précipité (2,3 g) est récupéré par filtration sur verre fritté n°4. Le filtrat est lavé à l'eau et séché à l'étuve à 100 °C.

Séparation et Identification des Isoquinoléines——La séparation des composés est réalisée à l'aide d'une colonne de silice (40—60 μm), avec un mélange chloroforme–méthanol (80:20, v/v) comme éluant et un débit de 5,5 ml·min^{-1}; la purification totale étant obtenue par cristallisation dans l'éther.

Nous avons obtenu:

L'Hydroxy-4 déméthyl-6 Papavéraldine (**3**) (121 mg): $C_{19}H_{17}NO_6$. SM *m/z* (%): 355 (M^+, 5,2) 354 (1,7) 353 (11,3) 337 (100) 322 (61,0) 321 (2,7) 309 (6,7) 308 (18,7) 293 (12,3) 292 (5,3). UV λ_{max}^{EtOH} nm: 280, 336, 348, 438, 464, 596. ^{1}H-RMN δ ppm: 8,3; 7,9, 7,6, 7,2, 7,0, 6,4; 3,96; 3,85; 3,84. pK_{a1} = 4,07. pK_{a2} = 9,54. IR cm^{-1}: 3400, 2920, 2840, 1680, 1600, 1500, 1340, 1210, 1060, 890, 870. Analyse élémentaire %: C (64,12); H (4,68); N (3,98); O (27,22).

L'Hydroxy-3 déméthyl-6 Papavéraldine (**5**) (62 mg): $C_{19}H_{17}NO_6$. SM *m/z* (%): 355 (M^+, 5,1), 354 (1,9), 353 (8,2), 338 (21,2), 337 (5,6), 322 (13,2), 308 (4,1). UV λ_{max}^{EtOH} nm: 298, 336, 412, 504, 654. pK_{a1} = 3,44. pK_{a2} = 10,0. IR (cm^{-1}) 2920, 1700, 1590, 1490, 1420, 1310, 1210, 1140, 1050, 890, 850, 750. Analyse élémentaire %: C (64,13); H (4,72); N (4,02); O (27,13).

L'Hydroxy-4 déméthyl-4' Papavéraldine (**4**) (25 mg): $C_{19}H_{17}NO_6$. SM *m/z* (%): 355 (M^+, 16,6), 354 (23,3), 353 (67,7), 338 (52,0), 337 (16,1), 322 (10,2), 321 (3,2), 309 (4,3), 308 (9,4), 293 (5,9), 292 (7,0). UV λ_{max}^{EtOH} nm: 314,404. pK_a = 10,0. IR (cm^{-1}): 3400, 2920, 2840, 1700, 1590, 1480, 1390, 1210, 1060, 890, 860. Analyse élémentaire %: C (64,32); H (4,80); N (4,04); O (26,84).

L'Hydroxy-3 déméthyl-4' Papavéraldine (**6**) (23 mg): $C_{19}H_{17}NO_6$. SM *m/z* (%): 355 (M^+, 12,2), 354 (11,1), 353 (33,8), 338 (41,8), 377 (20,8), 308 (11,2). UV λ_{max}^{EtOH} nm: 314, 404. pK_a = 10,0. IR (cm^{-1}): 3420, 3010, 2920, 1700, 1590, 1500, 1420, 1310, 1210, 1140, 1050, 890, 850, 750. Analyse élémentaire %: C (64,21); H (4,70); N (3,91); O (27,18).

Bibliographie

1) A. Burger, "The Alkaloids Chemistry and Physiology," Vol. IX, ed. by R. H. F. Manske, Academic Press, New York, 1967, pp. 32—41.
2) F. Machovicova et V. Parrak, *Pharmazie*, **14**, 10 (1959).
3) E. Pawelczyk et T. Hermann, *Chemist-Analyst*, **13**, 617 (1968).
4) P. Gundermann et R. Pohloudek-Fabiani, *Pharmazie*, **33**, 205 (1978).
5) M. J. Waechter, J. Likforman et M. Hamon, *Analusis*, **5**, 34 (1977).
6) E. Postaire, D. Martinez, C. Viel, M. Chastagnier et M. Hamon, A paraître.
7) A. J. Waring, *Anal. Chim. Acta*, **153**, 213 (1983).
8) S. Pfeifer, G. Behnsen, L. Kuhn et R. Kraft, *Pharmazie*, **27**, 734 (1972).
9) H. Budzikiewicz, C. Djerassi et D. Williams, "Interpretation of Mass Spectra of Organic Compounds," Holden Day, San Francisco, 1964.
10) M. J. Waechter, J. Likforman et M. Hamon, *Analusis*, **5**, 34 (1977).
11) E. Postaire, M. Tsitini-Tsamis, M. Hamon et C. Viel, *Talanta*, **30**, 193 (1983).
12) G. Mahuzier et M. Hamon, *Bull. Soc. Chim. Fr.*, 684 (1969).
13) M. Tsitini-Tsami, M. J. Waechter, J. Likforman, J. P. Delcroix et M. Hamon, *Analusis*, **9**, 283 (1981).

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The Mechanism of the Reaction of Nicotinic Acid 1-Oxide with Acetic Anhydride

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In order to elucidate the mechanism of the 2-acetylation in the reaction of nicotinic acid 1-oxide (**2a**) with boiling acetic anhydride, thermal reactions and reactions with hot acetic anhydride have been explored with 3-X-pyridine 1-oxides (**2**). The former reactions of **2d** (X=CONHAc), **2f** (X=CONMeAc), **2h** (X=CH_2OAc) and **2j** [X=$CH(OAc)_2$] result in recovery or decomposition. The latter reactions of **2c** (X=$CONH_2$), **2d**, **2e** (X=CONHMe), **2h** and **2j** bring about mainly deoxygenative α-acetoxylation, no 2-acetylation being noticed. However, the reaction of **2f** with acetic anhydride affords 6,7-dihydro-6-methyl-7-methylene-5*H*-pyrrolo[3,4-*b*]pyridin-5-one 1-oxide (**7**) as an initial product, which further undergoes deoxygenative β-acetoxylation to give 7-acetoxy-7-acetoxymethyl-6,7-dihydro-6-methyl-5*H*-pyrrolo[3,4-*b*]pyridin-5-one (**8**) and 7-acetoxymethylene-6,7-dihydro-6-methyl-5*H*-pyrrolo[3,4-*b*]pyridin-5-one (**9**). On the basis of these results we propose a new electrophilic pathway for the 2-acetylation of **2a** and **2f**.

Keywords——pyridine 1-oxide 3-substituted; nicotinic acid 1-oxide; nicotinamide 1-oxide *N*-acetyl-*N*-methyl; pyrrolo[3,4-*b*]pyridine; 2-acetylation; pyridone formation

In 1960, Bain and Saxton found that nicotinic acid 1-oxide (**2a**) reacted with boiling acetic anhydride (Ac_2O) to give 2-acetylnicotinic acid 1-oxide (**5**) as a major product, and they explained the reaction in terms of an intramolecular electrophilic reaction of the mixed anhydride of **2a** and acetic acid, promoted by the electron-donating effect of the *N*-oxide function, as shown in Chart 1.[1] Although such an effect is well known with the free aromatic *N*-oxide group,[2] the operation of this effect seems improbable in this case taking into account the reaction medium consisting of a large excess of Ac_2O. We have recently reinvestigated this reaction in some detail and shown that the primary product is not **5** but instead the acetate of the ring tautomer of **5** (**6**); **6** is easily hydrolyzed to **5**, and **5** is readily transformed to **6** by heating with Ac_2O.[3]

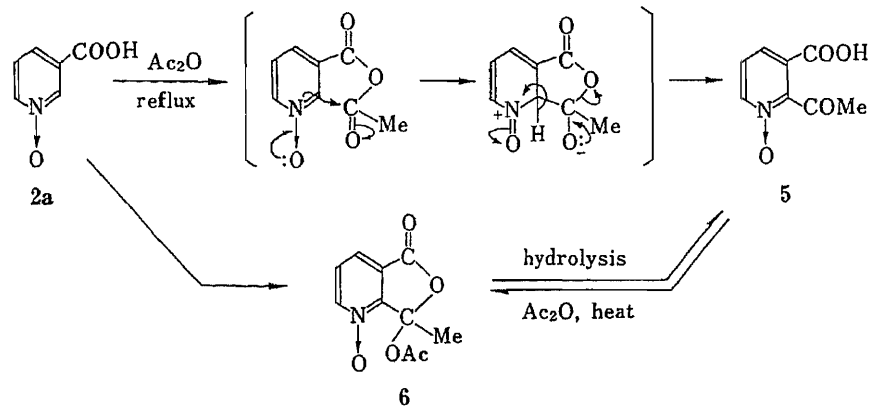


Chart 1

However, this finding gives no insight into the possible mechanism. In order to elucidate the mechanism of this unique electrophilic reaction, we carried out the following experiments.

At first we tried to prepare the mixed anhydride of **2a** and acetic acid under various conditions, but all attempts failed. On the other hand, 3-carboxy-*N*-acetoxypyridinium perchlorate[4] (**2a'**) was isolated in high yield when **2a** was treated with Ac_2O and perchloric acid in acetic acid according to the procedure of Muth and Darlark[5] (Chart 2). Attempted preparation of the mixed anhydride with benzoic acid was also unsuccessful. Treatment of nicotinic anhydride[6] with *m*-chloroperbenzoic acid (*m*-CPBA) in chloroform resulted in the formation of nicotinic acid (**1a**) and its *N*-oxide (**2a**), the expected *N*,*N'*-dioxide or *N*-monooxide of the anhydride being not obtained (Chart 2).

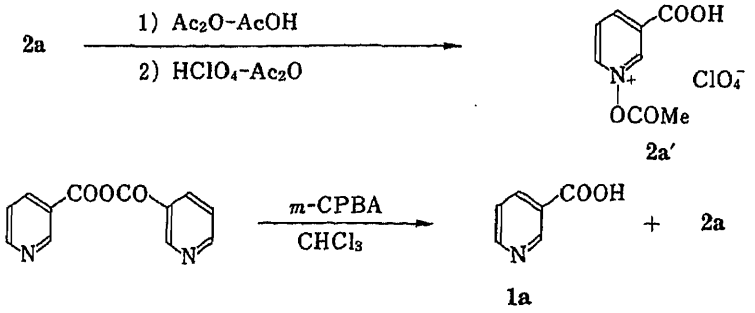
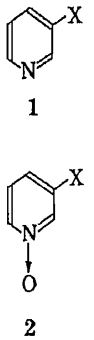


Chart 2

To examine the reactions of related 3-substituted pyridine 1-oxides, a series of 3-substituted pyridine 1-oxides (**2a—k**) was prepared mainly by *m*-CPBA oxidation of the corresponding 3-substituted pyridines (**1**) (Chart 3). Acetylation of *N*-methylnicotinamide (**1e**) was rather difficult and *N*-acetyl-*N*-methylnicotinamide (**1f**) was obtained in a low yield of 17.9% only upon treatment with 60% sodium hydride in tetrahydrofuran followed by warming with acetyl chloride at 60 °C for 4 h. While **1f** was fairly unstable and, for instance, was transformed readily to methyl nicotinate (**1b**) with boiling methanol, *N*-oxidation with *m*-CPBA proceeded smoothly to give *N*-acetyl-*N*-methylnicotinamide 1-oxide (**2f**) in 66.6% yield. The *N*-oxide **2f** was similarly unstable, and readily gave **2a** or **2b** when heated in water or methanol, respectively. 3-Pyridinecarbaldehyde 1-oxide (**2i**) was prepared by hydrolysis of 3-pyridinecarbaldehyde diacetate 1-oxide (**2j**) obtained by oxidation of 3-pyridinecarbaldehyde diacetate (**1j**) (Chart 3).



	X		X
a	COOH	**g**	CH_2OH
b	COOMe	**h**	CH_2OAc
c	$CONH_2$	**i**	CHO
d	CONHAc	**j**	$CH(OAc)_2$
e	CONHMe	**k**	CN
f	CONMeAc		

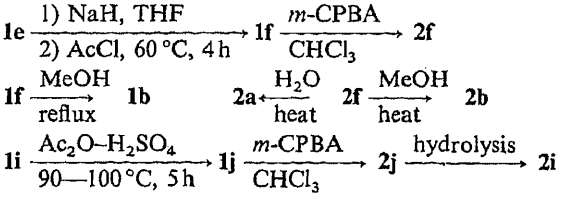
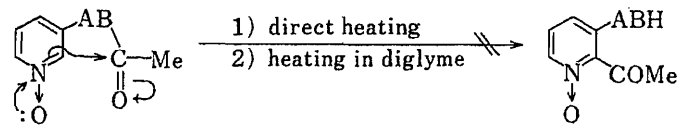


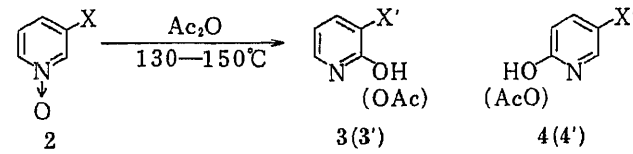
Chart 3

TABLE I. Thermal Reactions of **2d**, **2f**, **2h**, and **2j**



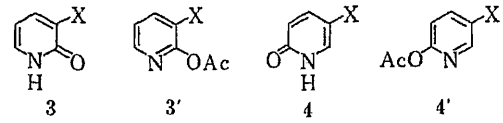
2d: AB=CONH	1) 160 °C	Rec. (recovery)
(mp 235—236 °C)	2) 150—160 °C, 3 h	Rec. (65%)
2f: AB=CONMe	1) 140—150 °C, 3 h	Dec.
(mp 117—119 °C)	2) 145—155 °C, 3 h	Rec. (41.2%)
2h: AB=CH_2O	1) 140—150 °C, 3 h	Rec. (90%)
(mp 71.9 °C)	2) 150—160 °C, 3 h	Rec. (80%)
2j: AB=CH(OAc)O	1) 140—150 °C, 3 h	Rec. (55.17%)+**2i** (20.3%)
(Oil)	2) 140—150 °C, 3 h	Rec. (64.0%)+**2i** (14.6%)

TABLE II. Reactions of **2c**, **2d**, **2e**, **2h**, **2j**, and **2k** with Ac_2O



2 3(3') 4(4')

N-Oxide 2	Reaction		Product (%)		
	Temp. (°C)	Time (h)	2-OH-deriv.	6-OH-deriv.	Other
2c[8]	130—140	4	**3d**[a]: 30		**2k**[b]: 25
2d	140—150	3	**3d**: 60		
2e[9]	140	2	**3e**: 14.6		
			3f[a]: 56.3		
2h	140—160	3	**3h**: 60.8	**4h**: 13.7	
2j	140—150	7	**3j**: 18.8	**4'j**: 14.0	
			3'j: 27.4		
2k	140—150	8	**3k**: 33.3		



3 3' 4 4'

d: X=CONHAc, **e**: X=CONHMe
f: X=CONMeAc, **h**: X=CH_2OAc
j: X=$CH(OAc)_2$, **k**: X=CN

a) 3-Substituent was also acetylated. *b*) 3-Pyridinecarbonitrile 1-oxide.

In exploring the feasibility of the reaction pattern suggested by Bain and Saxton[1] (Chart 1), pyridine 1-oxides having 3-β-acetyl-substituents, **2d**, **2f**, **2h** and **2j** were heated alone or at 140—160 °C in diglyme. In spite of detailed examination, the rearrangement of the acetyl group from the 3-substituent to the 2-position was not noticed at all, only recovery or decomposition being observed (Table I).

Heating with Ac_2O was next examined. Reactions of **2c**, **2d**, **2e**, **2h**, **2j** and **2k** did not produce 2-acetylation products, analogous to **5** or **6**, but gave deoxygenative 2- and 6-acetoxylation products, with a preponderance of 2-substitution[7] (Table II). In general, these reactions were largely dependent upon the reaction conditions; thus, Prachayasittikul and Bauer[8] isolated 2-acetoxy-*N*,*N*-diacetylnicotinamide (0.8%), 2-hydroxy-*N*-acetylnicotinamide (**3d**, 61%) and 2-hydroxy-3-pyridinecarbonitrile (**3k**, 0.54%) upon refluxing **2c** with Ac_2O for 3 h, whereas **3d** (30%) and **2k** (25%) were formed in the reaction at 130—140 °C for 4 h. The reaction of **2e** gave also the *N*-acetylated 2-pyridone (**3f**, 56.3%) together with **3e**

(14.6%). In reactions of **2h** and **2j**, the 6-acetoxylation also occurred in addition to the 2-acetoxylation. The structures of these products were established by elemental analyses, spectral examinations and some chemical evidence (see Experimental).

In contrast with the reactions given in Table II, the reaction of *N*-acetyl-*N*-methylnicotinamide 1-oxide (**2f**) with Ac_2O followed an alternate pathway which led to products of quite different types. When **2f** was heated with Ac_2O at 140—150 °C for 2 h, there were obtained 6,7-dihydro-6-methyl-7-methylene-5*H*-pyrrolo[3,4-*b*]pyridin-5-one 1-oxide (**7**), 7-acetoxy-7-acetoxymethyl-6,7-dihydro-6-methyl-5*H*-pyrrolo[3,4-*b*]pyridin-5-one (**8**) and the deacetic acid product of **8** (**9**) in 9.5, 12.0 and 30.0% yields, respectively (Chart 4). The structures of **7**, **8** and **9** were assigned on the basis of elemental analyses, and proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra. Further, the structures of **7** and **9** were confirmed by X-ray diffraction studies.

Product **7**, an analogue of **6**, apparently originates from 2-acetylation, and **8** and **9** are most likely formed by further action of Ac_2O on **7**. In order to confirm these aspects, the following examinations were carried out. Treatment of the methyl ester of **5** (**10**) or the ring chloride of **5**[3] (**11**) with 40% methylamine in methanol gave rise to 6,7-dihydro-7-hydroxy-6,7-dimethyl-5*H*-pyrrolo[3,4-*b*]pyridin-5-one 1-oxide (**12**) in fairly good yield. Warming of **12** with Ac_2O at 60—80 °C for 1 h afforded **7** in 81.3% yield, and **7** reverted to **12** upon heating with 1 N hydrochloric acid for 30 min on a steam bath. When **7** was heated with Ac_2O at 140—150 °C for 3 h, **8** and **9** were obtained in 5 and 57.3% yields, respectively; heating **8** with Ac_2O readily gave **9** (Chart 4).

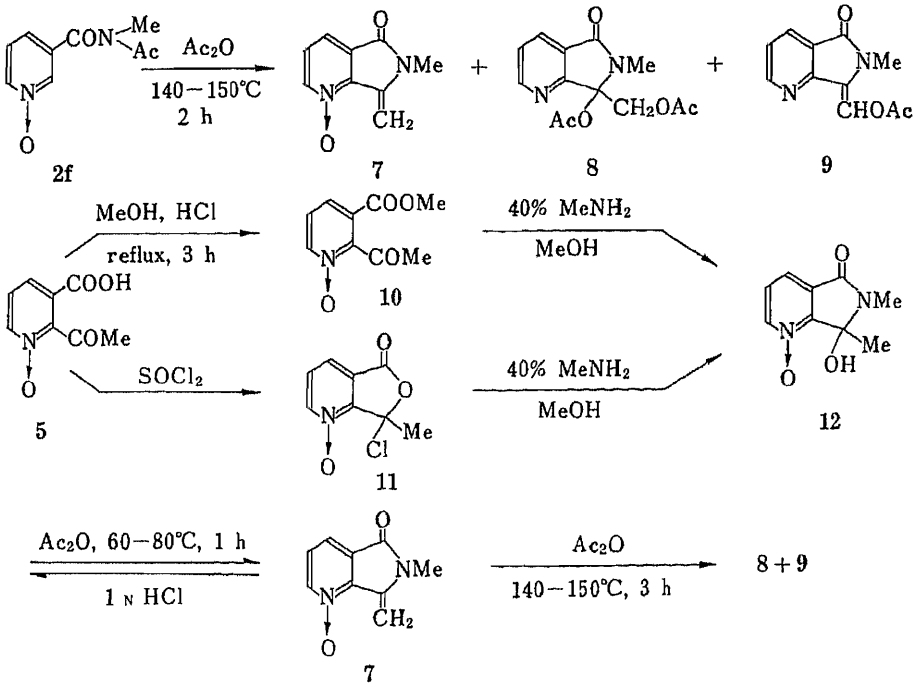


Chart 4

These results demonstrate that the reaction of **2f** with hot Ac_2O initially affords product **7**, which reacts further with Ac_2O to give **8** and **9**. The formation of **8** and **9** from **7** can be rationalized in terms of the reaction sequence illustrated in Chart 5. While the introduction of an acetoxy group into the β-position of the pyridine ring was not observed, probably due to the electron-attracting effect of the 5-oxo group, this is the same pattern of reaction as seen with 2-styrylpyridine[10] and 4-styrylquinoline[11] 1-oxides in their reactions with Ac_2O

(deoxygenative β-acetoxylation).[12]

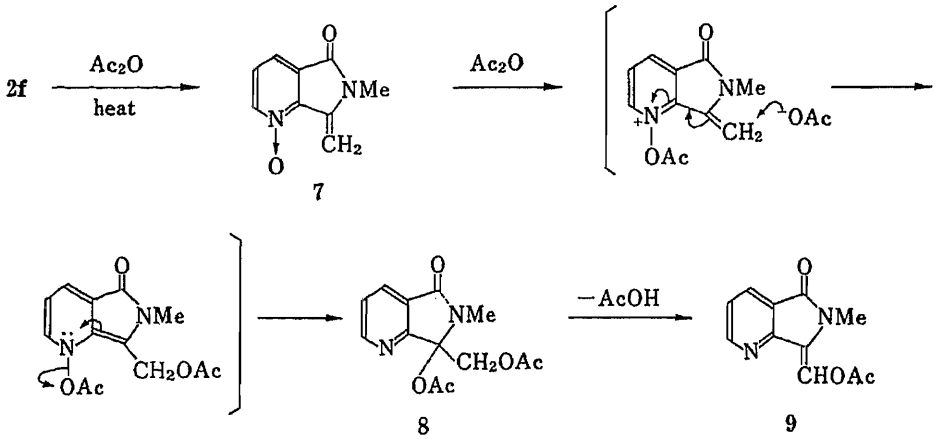


Chart 5

From the above-mentioned observations, it was revealed that, while **2c**, **2d**, **2e**, **2h** and **2j** gave mainly deoxygenated α-acetoxylation products (**3**, **3'** and **4**, **4'**) upon heating with Ac_2O, as in the reaction of pyridine 1-oxide itself, the reactions of **2a** and **2f** followed a different course to afford 2-acetylation products, **6** and **7**. The pathway in which the free *N*-oxide function participates as an electron-donating group (Chart 1) can be ruled out because there was no formation of 2-acetylation products in the thermal reactions of pyridine 1-oxides having 3-β-acetyl-substituents, **2d**, **2f**, **2h** and **2j**. We now propose a new electrophilic course for the specific 2-acetylation of **2a** and **2f** (Chart 6).

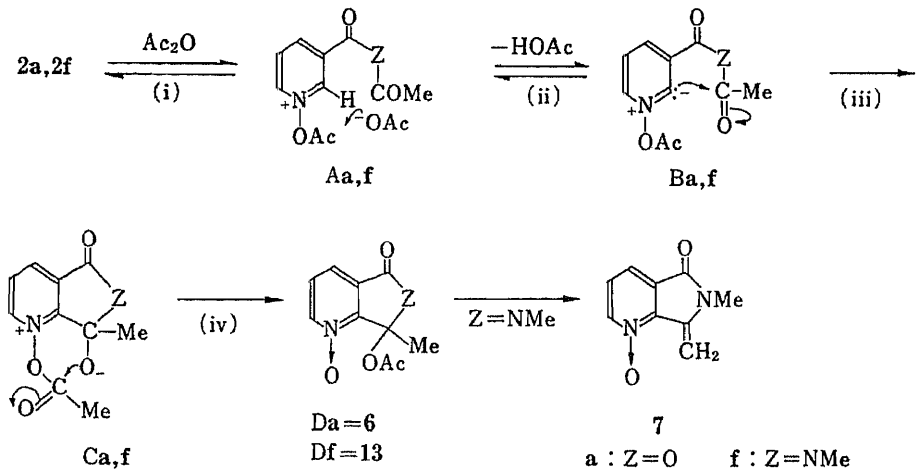


Chart 6

It may be reasonably considered that the *N*-acetoxypyridinium species, A, is initially formed (step i). Although the mixed anhydride of **2a** with acetic acid could not be prepared, the mixed anhydride (A**a**) should be also assumed as the initial active species in the reaction of **2a** in the same way for **2f**. Subsequently, the 2-proton of A is abstracted by an acetate anion to give B (step ii), followed by the intramolecular attack of the anionic center of B at the acetyl group of the side chain to form the cyclic intermediate C (step iii). The formation of D**a** (**6**) and D**f** (**13**) occurs readily by the intramolecular rearrangement of the acetyl group, and **13** undergoes immediate conversion to **7** by loss of acetic acid. The crucial step is the

transformation of A to B (step ii), for which strong electron-withdrawing character of the adjacent 3-substituent seems to be required in addition to the adjacent electron-withdrawing N^+-OAc group; thus, step ii may not proceed in the reactions of **2h** and **2j**. While species B can also be regarded as a carbene, it is inherently a nucleophilic one. In the case of **2d**, the NH group may prevent step iii owing to the electron-releasing character of the N–H bond. As described before, the reaction of **2e** with Ac_2O gave the *N*-acetyl-*N*-methylnicotinamide derivative **3f**; this result may be explained by assuming that 2-acetoxylation precedes acetylation of the NHMe group of **2f**.

Abramovitch and co-workers have reported on base-catalyzed α-deprotonation of pyridine 1-oxides[13] and of *N*-aryloxypyridinium salts,[14] and electrophilic reactions of the resultant 2-carbanions. Although the details are not necessarily clear, the above reaction can be regarded as being of a similar pattern. Further development of this type of reaction is now under investigation.

Experimental

Melting points were measured with a Yanagimoto micro melting apparatus and a Mettler FP61 apparatus, and are uncorrected. Spectral data were recorded on the following instruments: infrared spectrum (IR), Hitachi infrared spectrophotometer 260-30; mass spectrum (MS), Shimadzu LKB 9000; ^{1}H-NMR and ^{13}C-NMR, JEOL FX-200. Column chromatography was carried out on Wakogel C-200 (100—200 mesh) with appropriate solvent systems, the compositions of which were determined by thin layer chromatography (TLC) [Kieselgel 60F_{254} (0.25 mm, Merck)] checking. Compounds **1b**, **1c**, **1g** and **1k** were obtained from Tokyo Kasei Kogyo Co., Ltd., and **2a**, **2c** and **3a** from Aldrich Chemical Co.

3-Carboxy-*N*-acetoxypyridinium Perchlorate (2a')——To an ice-cooled suspension of nicotinic acid 1-oxide (**2a**, 1.39 g) in Ac_2O (20 ml)–AcOH (10 ml), a solution of 70% $HClO_4$ (1.87 g) in Ac_2O (5 ml) was added dropwise with stirring. The resulting solution was stirred for 0.5 h to deposit crystals, which were filtered and washed with ether to give 2.64 g (93.8%) of **2a'**, colorless prisms, mp 135—140 °C (MeCN–ether). *Anal.* Calcd for $C_8H_8ClNO_8$: C, 34.12; H, 2.86; N, 4.97. Found: C, 33.98; H, 2.86; N, 4.86. IR (KBr): 1840, 1825, 1730 (C=O), 1097 (N–O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.49 (3H, s, CH_3), 8.0—10.0 (4H, br, Py-H), 14.12 (1H, OH).[4]

Oxidation of Nicotinic Anhydride——A mixture of nicotinic anhydride[6] (2.28 g), *m*-CPBA (2.1 g) and $CHCl_3$ (100 ml) was stirred overnight at room temperature. Insoluble materials were filtered, the filtrate was concentrated *in vacuo*, and the residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 0.738 g (60%) of nicotinic acid (**1a**), mp 236—239 °C, and 0.417 g (30%) of **2a**, mp 260—262 °C (dec.).

***N*-Acetylnicotinamide 1-Oxide (2d)**——A solution of *N*-acetylnicotinamide (**1d**, 1.64 g) and *m*-CPBA (2 g) in $CHCl_3$ (60 ml) was stirred at room temperature for 12 h. Precipitates were filtered, the filtrate was concentrated *in vacuo*, and the residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 1.44 g (80%) of **2d**, colorless needles, mp 235—236 °C (MeOH). *Anal.* Calcd for $C_8H_8N_2O_3$: C, 53.33; H, 4.48; N, 15.55. Found: C, 53.30; H, 4.50; N, 15.54. MS *m/z*: 180 (M^+). ^{1}H-NMR (DMSO-d_6) δ: 2.33 (3H, s, CH_3), 7.38—7.93 (2H, m, Py-H), 8.28—8.55 (1H, m, Py-H), 8.75 (1H, brs, H-2), 11.0—11.5 (1H, brs, NH). ^{13}C-NMR (DMSO-d_6) δ: 25.4 (q, CH_3), 124.6 (d), 126.3 (d), 132.9 (s), 138.3 (d), 141.5 (d), 163.1 (s, CO), 171.5 (s, CO).

***N*-Acetyl-*N*-methylnicotinamide (1f)**——1) To a solution of *N*-methylnicotinamide (**1e**, 10 g) in tetrahydrofuran (THF) (100 ml) was added 60% NaH (3.24 g) under a nitrogen atmosphere, and the mixture was stirred at room temperature for 1 h, then at 40 °C for 1 h. AcCl (5.75 ml) was added under water-cooling and the mixture was warmed at 60 °C for 4 h. The reaction mixture was poured onto ice and extracted with THF. The THF was evaporated *in vacuo*, and the residue was chromatographed on silica gel with $CHCl_3$–ether to give 2.34 g (17.9%) of **1f** as an oil. ^{1}H-NMR ($CDCl_3$) δ: 2.35 (3H, s, CH_3), 3.22 (3H, s, CH_3), 7.41 (1H, dd, $J_{4,5}$=8.0 Hz, $J_{5,6}$=6.0 Hz, H-5), 7.94 (1H, dd, $J_{4,5}$=8.0 Hz, $J_{4,6}$=2.0 Hz, H-4), 8.64—8.94 (2H, m, H-2, H-6). ^{13}C-NMR ($CDCl_3$) δ: 25.7 (q, CH_3), 34.0 (q, CH_3), 123.5 (d), 131.6 (s, C_3), 135.9 (d), 148.7 (d), 152.2 (d), 171.7 (s, CO), 173.1 (s, CO).

2) A solution of **1f** (356 mg) in MeOH (10 ml) was refluxed for 1 h to give 219 mg (80%) of **1b**.

***N*-Acetyl-*N*-methylnicotinamide 1-Oxide (2f)**——1) A solution of **1f** (2.34 g) and *m*-CPBA (3 g) in $CHCl_3$ (30 ml) was stirred at room temperature for 2 h. The reaction mixture was worked up in the usual way and the product was purified by chromatography on silica gel with $CHCl_3$–ether–MeOH to give 1.7 g (66.6%) of **2f**, colorless prisms, mp 117—119 °C (MeOH–ether). *Anal.* Calcd for $C_9H_{10}N_2O_3$: C, 55.67; H, 5.19; N, 14.43. Found: C, 55.46; H, 5.24; N, 14.42. IR (KBr): 1700, 1685 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.38 (3H, s, CH_3), 3.22 (3H, s, CH_3), 7.24—7.51 (2H, m, Py-H), 8.11—8.41 (2H, m, Py-H). ^{13}C-NMR ($CDCl_3$) δ: 25.5 (q, CH_3), 33.8 (q, CH_3), 124.1 (d), 126.0 (d), 135.5 (s, C_3), 138.4 (d), 141.1 (d), 168.9 (s, CO), 172.9 (s, CO).

2) A suspension of **2f** (388 mg) in H_2O (5 ml) was heated on a steam bath for 1 h to given 195 mg (70%) of **2a**.

3) A solution of **2f** (582 mg) in MeOH (10 ml) was refluxed for 1 h to give 344 mg (75%) of **2b**, mp 101–102 °C.

3-Hydroxymethylpyridine 1-Oxide (2g)——A solution of 3-hydroxymethylpyridine (**1g**, 4.36 g) and *m*-CPBA (7 g) in $CHCl_3$ (50 ml) was stirred at room temperature for 12 h. The solvent was evaporated *in vacuo*, AcOEt (50 ml)–H_2O (50 ml) was added, and the aqueous layer was separated and concentrated *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 3.8 g (76%) of **2g**,[15] hygroscopic crystals. ^{1}H-NMR ($CDCl_3$–DMSO-d_6) δ: 4.61 (2H, d, J=6.0 Hz, CH_2), 5.69 (1H, t, J=6.0 Hz, OH), 7.17—7.57 (2H, m, Py-H), 7.87—8.37 (2H, m, Py-H).

3-Acetoxymethylpyridine 1-Oxide (2h)——1) A solution of **2g** (2.5 g) and Ac_2O (3.1 g) in $CHCl_3$ (5 ml) was stirred overnight at room temperature. The reaction mixture was poured onto ice, neutralized with $NaHCO_3$ and extracted with $CHCl_3$. The extract was washed with a saturated NaCl solution, dried over Na_2SO_4, and concentrated *in vacuo*. Hexane was added to the residue to give 3.34 g (100%) of **2h**, colorless hygroscopic crystals, mp 71.9 °C (MeOH–ether). *Anal.* Calcd for $C_8H_9NO_3$: C, 57.48; H, 5.43; N, 8.38. Found: C, 57.47; H, 5.48; N, 8.32. IR (KBr): 1730 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.21 (3H, s, CH_3), 5.07 (2H, s, CH_2), 7.20—7.42 (2H, m, Py-H), 8.02—8.37 (2H, m, Py-H). ^{13}C-NMR ($CDCl_3$) δ: 20.7 (q, CH_3), 62.2 (t, CH_2), 125.2 (d), 126.6 (d), 135.8 (s, C_3), 138.4 (d), 138.6 (d), 170.2 (s, CO).

2) Addition of Ac_2O (10 ml) to **1g** (4.36 g) caused a violent exothermic reaction. The mixture was stirred for 30 min, then warmed at 60 °C for 1 h and concentrated *in vacuo*. The residue was dried under vacuum to give 6.1 g of crude 3-acetoxymethylpyridine (**1h**) as an oil. A solution of **1h** (6.1 g) and *m*-CPBA (7.59 g) in $CHCl_3$ (60 ml) was stirred at room temperature for 2 h. The solvent was evaporated *in vacuo*, and the residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 5.34 g (80%) of **2h**.

3-Pyridinecarbaldehyde Diacetate 1-Oxide (2j)——1) A mixture of 3-pyridinecarbaldehyde (**1i**, 10 g), Ac_2O (30 ml) and conc. H_2SO_4 (2 ml) was heated at 90—100 °C under stirring for 5 h. The reaction mixture was concentrated *in vacuo*, and ice-water was added to the residue, which was basified with $NaHCO_3$ and extracted with $CHCl_3$. The extract was washed twice with aqueous NaCl and concentrated *in vacuo*, and the residue was chromatographed on silica gel with $CHCl_3$–ether to give 13.01 g (66.1%) of 3-pyridinecarbaldehyde diacetate as an oil. ^{1}H-NMR ($CDCl_3$) δ: 2.13 (6H, s, 2 × CH_3), 7.40 (1H, dd, $J_{4,5}$=8.0 Hz, $J_{5,6}$=5.2 Hz, H-5), 7.72 (1H, s, –CH<), 7.79—8.06 (1H, m, H-4), 8.68 (1H, dd, $J_{5,6}$=5.2 Hz, $J_{4,6}$=2.0 Hz, H-6), 8.82 (1H, d, J=2.0 Hz, H-2).

2) A solution of **1j** (6 g) and *m*-CPBA (5.94 g) in $CHCl_3$ (50 ml) was stirred at room temperature for 12 h. The product was purified by silica gel chromatography with $CHCl_3$–MeOH to give 6.0 g (93%) of **2j** as a colorless oil. MS *m/z*: 225 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.12 (6H, s, 2 × CH_3), 7.20—7.47 (2H, m, Py-H), 7.59 (1H, s, –CH<), 8.05—8.27 (1H, m, Py-H), 8.31 (1H, brs, Py-H). ^{13}C-NMR ($CDCl_3$) δ: 20.6 (q, CH_3), 86.6 (d, –CH<), 124.2 (d), 126.1 (d), 135.5 (s, C_3), 138.0 (d), 139.9 (d), 168.3 (s, CO).

3-Pyridinecarbaldehyde 1-Oxide (2j)——A mixture of **2j** (2.25 g) and AcOH (12.5 ml)–H_2O (12.5 ml) was refluxed for 8 h, then the reaction mixture was concentrated *in vacuo*. The deposited crystals were filtered and washed with ether to give 0.86 g (70%) of **2i**,[16] colorless needles, mp 139—141 °C ($CHCl_3$–ether). *Anal.* Calcd for $C_6H_5NO_2$: C, 58.54; H, 4.09; N, 11.38. Found: C, 58.38; H, 4.15; N, 11.26. ^{1}H-NMR (DMSO-d_6) δ: 7.4—7.9 (2H, m, Py-H), 8.31—8.57 (1H, m, Py-H), 8.63 (1H, s, Py-H), 9.93 (1H, s, CHO). ^{13}C-NMR (DMSO-d_6) δ: 124.2 (d), 127.1 (d), 134.8 (s, C_3), 139.9 (d), 143.1 (d), 190 (d, CO).

Reaction of Nicotinamide 1-Oxide (2c) with Ac_2O——A mixture of **2c** (1.38 g) and Ac_2O (30 ml) was heated at 130—140 °C for 4 h. The reaction mixture was concentrated *in vacuo* and the residue was heated with H_2O on a steam bath. THF was added and saturated with NaCl, then the THF layer was separated, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 0.54 g (30%) of 2-hydroxy-*N*-acetylnicotinamide[8] (**3d**) and 0.3 g (25%) of 3-pyridinecarbonitrile 1-oxide[17] (**2k**).

3d: Pale yellow needles, mp 222—224 °C (dec.) (MeOH–ether). *Anal.* Calcd for $C_8H_8N_2O_3$: C, 53.33; H, 4.48; N, 15.55. Found: C, 53.37; H, 4.44; N, 15.42. IR (KBr): 1710, 1685, 1650 cm^{-1}.

2k: Colorless needles, mp 177.6 °C (iso-PrOH). This product was identical with an authentic sample prepared by *m*-CPBA oxidation of 3-pyridinecarbonitrile (**1k**).

Reaction of *N*-Acetylnicotinamide 1-Oxide (2d) with Ac_3O——A mixture of **2d** (1 g) and Ac_2O (15 ml) was heated at 140—150 °C for 3 h. The reaction mixture was worked up as described above to give 0.6 g (60%) of 2-hydroxy-*N*-acetylnicotinamide (**3d**).

Reaction of *N*-Methylnicotinamide 1-Oxide (2e) with Ac_2O——A mixture of **2e**[9] (1.52 g) and Ac_2O (20 ml) was heated at 140 °C for 2 h. The reaction mixture was concentrated *in vacuo*, and the residue was warmed with H_2O (20 ml) at 40—50 °C for 20 min, then concentrated again *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 1.092 g (56.3%) of 2-hydroxy-*N*-acetyl-*N*-methylnicotinamide (**3f**) and 0.222 g (14.6%) of 2-hydroxy-*N*-methylnicotinamide (**3e**).

3e: Colorless needles, mp 217—218 °C (iso-PrOH–ether). *Anal.* Calcd for $C_7H_8N_2O_2$: C, 55.26; H, 5.30; N, 18.41. Found: C, 55.30; H, 5.23; N, 18.38.

3f: Pale yellow needles, mp 152—154 °C (iso-PrOH). *Anal.* Calcd for $C_9H_{10}N_2O_3$: C, 55.67; H, 5.19; N, 14.43. Found: C, 55.65; H, 5.13; N, 14.42. MS *m/z*: 194 (M^+). IR (KBr); 1695 (sh), 1680, 1650 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.35 (3H, s, CH_3), 3.16 (3H, s, CH_3), 6.21 (1H, dd, $J_{4,5}$=7.2 Hz, $J_{5,6}$=6.0 Hz, H-5), 7.35 (1H, dd, $J_{4,5}$=7.2 Hz, $J_{4,6}$=

2.0 Hz, H-4), 7.60 (1H, dd, $J_{4,6}$=2.0 Hz, $J_{5,6}$=6.0 Hz, H-6), 11.7—12.2 (1H, br, OH).

Heating of **3f** (194 mg) with a saturated $NaHCO_3$ solution (5 ml) on a steam bath for 30 min gave 106 mg (70%) of **3e**.

Reaction of 3-Acetoxymethylpyridine 1-Oxide (2h) with Ac_2O——A mixture of **2h** (1.67 g) and Ac_2O (10 ml) was heated at 140—160 °C for 3 h. The reaction mixture was concentrated *in vacuo*, and the residue was heated with water on a steam bath for 30 min, and THF and NaCl were added. The residue from the THF layer was chromatographed on silica gel with $CHCl_3$–MeOH to give 1.015 g (60.8%) of 3-acetoxymethyl-2-pyridone (**3h**) and 0.23 g (13.7%) of the 6-pyridone (**4h**).

3h: Colorless needles, mp 147—148 °C (iso-PrOH–hexane). *Anal.* Calcd for $C_8H_9NO_3$: C, 57.48; H, 5.43; N, 8.38. Found: C, 57.31; H, 5.36; N, 8.46. IR (KBr): 1720 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.10 (3H, s, CH_3), 5.02 (2H, s, CH_2), 6.24 (1H, t, J=6.0 Hz, H-5), 7.21—7.65 (2H, m, H-4, H-6), 13.05—13.75 (1H, br, OH). ^{13}C-NMR ($CDCl_3$) δ: 21.0 (q, CH_3), 61.5 (t, CH_2), 106.6 (d), 126.7 (s, C_3), 134.6 (d), 140.2 (d), 164.2 (s), 170.8 (s, CO).

4h: Colorless needles, mp 150 °C ($CHCl_3$–hexane). *Anal.* Calcd for $C_8H_9NO_3$: C, 57.48; H, 5.43; N, 8.38. Found: C, 57.53; H, 5.35; N, 8.40. IR (KBr): 1730 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.05 (3H, s, CH_3), 4.86 (2H, s, CH_2), 6.57 (1H, d, J=8.0 Hz, H-5), 7.24—7.67 (2H, m, H-4, H-2), 12.3—13.3 (1H, br, OH). ^{13}C-NMR ($CDCl_3$) δ: 20.9 (q, CH_3), 62.9 (t, CH_2), 115.3 (s, C_3), 120.4 (d), 135.1 (d), 142.8 (d), 165.1 (s), 170.8 (s).

Reaction of 3-Pyridinecarbaldehyde Diacetate 1-Oxide (2j) with Ac_2O——1) A mixture of **2j** (1.125 g) and Ac_2O (25 ml) was heated at 140—150 °C for 7 h. The reaction mixture was concentrated *in vacuo* and the residue was chromatographed on silica gel with $CHCl_3$–MeOH to give successively 187 mg (14%) of 6-acetoxy-3-pyridinecarbaldehyde diacetate (**4'j**), 366 mg (27.4%) of 2-acetoxy-3-pyridinecarbaldehyde diacetate (**3'j**) and 251 mg (18.8%) of 2-hydroxy-3-pyridinecarbaldehyde diacetate (**3j**).

4'j: A colorless oil. MS *m/z*: 267 (M^+). IR (neat): 1750 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.11 (6H, s, 2×CH_3), 2.32 (3H, s, CH_3), 7.08 (1H, d, $J_{4,5}$=8.2 Hz, H-5), 7.67 (1H, s, –CH<), 7.91 (1H, dd, $J_{4,5}$=8.2 Hz, $J_{2,4}$=2.4 Hz, H-4), 8.51 (1H, d, $J_{2,4}$=2.4 Hz, H-2). ^{13}C-NMR ($CDCl_3$) δ: 2.07 (q, CH_3), 21.2 (q, CH_3), 87.6 (d, –CH<), 116.3 (d), 130.0 (s), 138.3 (d), 147.4 (d), 158.6 (s), 168.5 (s, CO), 168.7 (s, CO).

3'j: A colorless oil. IR (neat): 1760 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.01 (6H, s, 2×CH_3), 2.31 (3H, s, CH_3), 7.27 (1H, dd, $J_{4,5}$=8.0 Hz, $J_{5,6}$=5.2 Hz, H-5), 7.80 (1H, s, –CH<), 8.0 (1H, dd, $J_{4,5}$=8.0 Hz, $J_{4,6}$=2.0 Hz, H-4), 8.40 (1H, dd, $J_{5,6}$=5.2 Hz, $J_{4,6}$=2.0 Hz, H-6). ^{13}C-NMR ($CDCl_3$) δ: 20.6 (q, CH_3), 21.0 (q, CH_3), 84.9 (d, CH), 122.5 (d), 123.6 (s), 138.0 (d), 149.8 (d), 155.5 (s), 168.2 (s, CO), 168.5 (s, CO).

3j: Colorless needles, mp 148—150 °C (MeOH–ether). *Anal.* Calcd for $C_{10}H_{11}NO_5$: C, 53.33; H, 4.92; N, 6.22. Found: C, 53.35; H, 4.86; N, 6.21. IR (KBr): 1755 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.11 (6H, s, 2×CH_3), 6.31 (1H, dd, $J_{4,5}$=8.0 Hz, $J_{5,6}$=5.2 Hz, H-5), 7.41—7.78 (2H, m, H-4, H-6), 8.01 (1H, s, –CH<), 12.7—13.5 (1H, br, OH).

2) A suspension of **3j** (500 mg) in H_2O (5 ml) was heated with stirring on a steam bath for 30 min. The product was purified by chromatography on silica gel with $CHCl_3$–MeOH to give 185 mg (75.2%) of 2-hydroxy-3-pyridinecarbaldehyde (**3i**), colorless needles, mp 225—226 °C (MeOH–ether). *Anal.* Calcd for $C_6H_5NO_2$: C, 58.54; H, 4.09; N, 11.38. Found: C, 58.54; H, 4.05; N, 11.32. MS *m/z*: 123 (M^+). IR (KBr): 1700 (sh), 1665 (C=O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 6.34 (1H, t, J=7.0 Hz, H-5), 7.66—8.16 (2H, m, H-4, H-6), 10.06 (1H, s, CHO), 12.0—12.70 (1H, br, NH or OH). ^{13}C-NMR (DMSO-d_6) δ: 105.2 (d), 124.4 (s, C_3), 142.7 (d), 143.4 (d), 162.1 (s, C_2), 189.1 (d, CHO).

3) A suspension of **4'j** (267 mg) in H_2O (5 ml) was heated on a steam bath for 30 min to give 74 mg (60.2%) of 2-hydroxy-3-pyridinecarbaldehyde (**4i**), colorless needles, mp 207—211 °C (dec.) (iso-PrOH–hexane). *Anal.* Calcd for $C_6H_5NO_2$: C, 58.54; H, 4.09; N, 11.38. Found: C, 58.60; H, 4.05; N, 11.31. MS *m/z*: 123 (M^+). IR (KBr): 1700 (sh), 1670 (C=O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 6.37 (1H, d, $J_{4,5}$=10 Hz, H-5), 7.20 (1H, dd, $J_{2,4}$=3.0 Hz, $J_{4,5}$=10.0 Hz, H-4), 8.19 (1H, d, $J_{2,4}$=3.0 Hz, H-2), 9.56 (1H, s, CHO), 11.7—12.7 (1H, br, NH or OH). ^{13}C-NMR (DMSO-d_6) δ: 117.3 (s, C_3), 120.4 (d), 135.9 (d), 146.3 (d), 162.6 (s, C_6), 187.4 (d, CHO).

Reaction of 3-Pyridinecarbonitrile 1-Oxide (2k) with Ac_2O——A mixture of **2k** (600 mg) and Ac_2O (5 ml) was heated at 140—150 °C for 8 h. The reaction mixture was worked up as described above to give 200 mg (33.3%) of 2-hydroxy-3-pyridinecarbonitrile (**3k**), pale yellow needles, mp 233 °C (iso-PrOH–ether). *Anal.* Calcd for $C_6H_4N_2O$: C, 60.00; H, 3.36; N, 23.32. Found: C, 60.16; H, 3.41; N, 23.13. ^{1}H-NMR (DMSO-d_6–$CDCl_3$) δ: 6.30 (1H, t, J=7.0 Hz, H-5), 7.70 (1H, dd, $J_{4,6}$=2.0 Hz, J=6.2 Hz, Py-H), 8.03 (1H, dd, $J_{4,6}$=2.0 Hz, J=7.0 Hz, Py-H), 12.55 (1H, br s, OH).

Preparation of 3e and 3f——1) A solution of **3a** (500 mg) in MeOH (10 ml) was treated with $SOCl_2$ (0.32 ml), and the mixture was stirred overnight at room temperature, then concentrated *in vacuo*. The residue was treated with aqueous $NaHCO_3$ and extracted with AcOEt. Addition of ether to the residue from the AcOEt extract gave 500 mg (90%) of methyl 2-hydroxynicotinate (**3b**), mp 153.5 °C (MeOH–hexane).

2) A solution of **3b** (500 mg) and 40% $MeNH_2$ (4 ml) in MeOH (30 ml) was stirred overnight at room temperature. The solvent was evaporated *in vacuo*, and then THF and a saturated NaCl solution were added to the residue. The separated THF layer was dried over Na_2SO_4 and concentrated *in vacuo*. Addition of ether to the residue gave 424 mg (85.4%) of **3e**, colorless needles, mp 217—218 °C (iso-PrOH–ether).

3) A mixture of **3e** (304 mg) and Ac_2O (5 ml) was heated at 140 °C for 2 h. The reaction mixture was concentrated

in vacuo, and the residue was heated with water on a steam bath for 30 min and concentrated *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 310 mg (80%) of **3f**, pale yellow needles, mp 152—154 °C (iso-PrOH).

Reaction of *N*-Acetyl-*N*-methylnicotinamide 1-Oxide (2f) with Ac_2O——A mixture of **2f** (582 mg) and Ac_2O (10 ml) was heated at 140—150 °C for 2 h. The reaction mixture was concentrated *in vacuo* and the residue was chromatographed on silica gel with $CHCl_3$–ether–MeOH to give successively 196 mg (30%) of 7-acetoxymethylene-6,7-dihydro-5*H*-pyrrolo[3,4-*b*]pyridin-5-one (**9**), 100 mg (12%) of 7-acetoxy-7-acetoxymethyl-6,7-dihydro-6-methyl-5*H*-pyrrolo[3,4-*b*]pyridin-5-one (**8**) and 50 mg (9.5%) of 6,7-dihydro-6-methyl-7-methylene-5*H*-pyrrolo[3,4-*b*]pyridin-5-one 1-oxide (**7**).

7: Yellow needles, mp 215 °C (MeOH–ether). *Anal.* Calcd for $C_9H_8N_2O_2$: C, 61.36; H, 4.58; N, 15.90. Found: C, 60.96; H, 4.51; N, 15.76. MS *m/z*: 176 (M^+), 160 ($M^+ - 16$). IR (KBr); 1705 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.19 (3H, s, CH_3), 5.43 (1H, s, $=C\langle^{\underline{Ha}}_{Hb}$ or $=C\langle^{Ha}_{\underline{Hb}}$), 6.50 (1H, s, $=C\langle^{\underline{Ha}}_{Hb}$ or $=C\langle^{Ha}_{\underline{Hb}}$), 7.34—7.92 (2H, m, H-3, H-4), 8.53 (1H, dd, $J_{2,3}$=6.0 Hz $J_{2,4}$=1.8 Hz, H-2). ^{13}C-NMR (DMSO-d_6) δ: 25.4 (q, CH_3), 98.5 (dd, CH_2), 119.9 (d), 126.3 (d), 128.2 (s), 136.5 (s), 143.2 (d), 162.1 (s, CO).

8: A pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 1.84 (3H, s, CH_3), 2.05 (3H, s, CH_3), 3.02 (3H, s, CH_3), 4.67 (2H, ABq, J=10.8 Hz, CH_2), 7.43 (1H, $J_{2,3}$=5.8 Hz, $J_{3,4}$=8.0 Hz, H-3), 8.10 (1H, $J_{2,4}$=1.8 Hz, $J_{3,4}$=8.0 Hz, H-4), 8.72 (1H, $J_{2,3}$=5.8 Hz, $J_{2,4}$=1.8 Hz, H-2). ^{13}C-NMR ($CDCl_3$) δ: 20.3 (q, CH_3), 21.1 (q, CH_3), 24.1 (q, CH_3), 61.1 (t, CH_2), 90.4 (s), 124.6 (d), 126.7 (s), 131.4 (d), 152.5 (d), 161.6 (s), 166.4 (s), 167.7 (s), 169.6 (s, CO).

9: Yellow prisms, mp 188—190 °C (dec.) (MeOH). *Anal.* Calcd for $C_{11}H_{10}N_2O_3$: C, 60.55; H, 4.62; N, 12.84. Found: C, 60.46; H, 4.55; N, 12.81. MS *m/z*: 218 (M^+). IR (KBr): 1755, 1710, 1695 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.30 (3H, s, CH_3), 3.58 (3H, s, CH_3), 7.33 (1H, dd, $J_{2,3}$=5.2 Hz, $J_{3,4}$=8.0 Hz, H-3), 7.93 (2H, m, H-4, =CH–), 8.68 (1H, dd, $J_{2,3}$=5.2 Hz, $J_{2,4}$=1.8 Hz, H-2). ^{13}C-NMR ($CDCl_3$) δ: 20.7 (q, CH_3), 28.7 (q, CH_3), 119.1 (d), 122.0 (s), 122.2 (s), 123.4 (d), 131.1 (d), 153.0 (d), 155.7 (s), 164.7 (s), 166.2 (s).

6,7-Dihydro-6,7-dimethyl-7-hydroxy-5*H*-pyrrolo[3,4-*b*]pyridin-5-one 1-Oxide (12)——1) A mixture of 2-acetylnicotinic acid 1-oxide (**5**, 1 g), HCl-saturated MeOH (50 ml) and toluene (50 ml) was refluxed for 3 h. The reaction mixture was slowly concentrated and the residue was dissolved in THF. The THF solution was neutralized with $NaHCO_3$ and saturated with NaCl. The THF layer was separated, and the residue from the THF solution was chromatographed on silica gel with $CHCl_3$–MeOH to give 500 mg (46.5%) of methyl 2-acetylnicotinate 1-oxide (**10**), pale yellow needles, mp 116—117 °C (MeOH–ether–hexane). *Anal.* Calcd for $C_9H_9NO_4$: C, 55.39; H, 4.65; N, 7.18. Found: C, 55.24; H, 4.57; N, 7.40. MS *m/z*: 195 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.64 (3H, s, CH_3), 3.88 (3H, s, CH_3), 7.37 (1H, dd, $J_{4,5}$=8.0 Hz, $J_{5,6}$=6.0 Hz, H-5), 7.80 (1H, dd, $J_{4,5}$=8.0 Hz, $J_{4,6}$=1.8 Hz, H-4), 8.27 (1H, dd, $J_{4,6}$=1.8 Hz, $J_{5,6}$=6.0 Hz, H-6).

2) A solution of **10** (390 mg) and 40% $MeNH_2$ (4 ml) in MeOH (10 ml) was stirred at room temperature for 10 min, then heated on a steam bath for 10 min. The solvent was evaporated *in vacuo*, and the residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 310 mg (80%) of **12**, pale yellow prisms, mp 222—224 °C (MeOH–ether). *Anal.* Calcd for $C_9H_{10}N_2O_3$: C, 55.67; H, 5.19; N, 14.43. Found: C, 55.55; H, 5.17; N, 14.35. IR (KBr): 1690 (CO), 3430 (OH) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.77 (3H, s, CH_3), 2.94 (3H, s, CH_3), 6.54 (1H, s, OH), 7.42—7.63 (2H, m, Py-H), 8.31 (1H, t, J=4.0 Hz, Py-H). ^{13}C-NMR (DMSO-d_6) δ: 19.2 (q, CH_3), 22.6 (q, CH_3), 86.2 (s, C_7), 119.0 (d), 127.4 (d), 129.9 (s, C_{4a}), 142.7 (d), 151.3 (s, C_{7a}), 161.8 (s, CO).

3) A mixture of 3-acetoxy-4-aza-3-methyl-1(3*H*)-isobenzofuranone 4-oxide[3] (**6**, 2.8 g) and a saturated $NaHCO_3$ solution was heated on a steam bath for 0.5 h, then acidified with 2 N HCl to pH 1. Insoluble materials were filtered. The filtrate was concentrated *in vacuo* to give crude **5**, which was treated with $SOCl_2$ (2 ml) and 3 drops of dimethylformamide (DMF) in $CHCl_3$ (100 ml) at room temperature for 12 h, then refluxed for 2 h. The solvent was evaporate to give crude 4-aza-3-chloro-3-methyl-1(3*H*)-isobenzofuranone 4-oxide[3] (**11**). A solution of **11** in $CHCl_3$ (50 ml) was added dropwise with stirring to an ice-cooled solution of 40% $MeNH_2$ (5 ml) in MeOH (10 ml), and the mixture was stirred at room temperature for 30 min, then concentrated *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 1.71 g (70%) of **12**.

Reaction of 12 with Ac_2O——A mixture of **12** (194 mg) and Ac_2O (2 ml) was warmed at 60—80 °C for 1 h, then concentrated *in vacuo*. Addition of ether to the residue gave 143 mg (81.3%) of **7**.

Reaction of 7——1) THF (2 ml) was added to a solution of **7** (88 mg) in 1 N HCl adjusted to pH 1, and the mixture was heated on a steam bath for 30 min. The solvent was evaporated *in vacuo*, and the residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 48 mg (49.5%) of **12**.

2) A mixture of **7** (176 mg) and Ac_2O (2 ml) was heated at 140—145 °C for 3 h. Upon cooling, deposited crystals were filtered and washed with ether to give 85 mg (40%) of **9**. The filtrate was concentrated *in vacuo*, and the residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 40 mg (17.3%) of **9** and 14 mg (5%) of **8**.

3) A mixture of **8** (0.075 g) and Ac_2O (1 ml) was heated at 140 °C for 2 h to give 0.035 g (59.3%) of **9**.

References and Notes

1) *a*) B. M. Bain and J. E. Saxton, *Chem. Ind.* (London), **1960**, 402; *b*) *Idem*, *J. Chem. Soc.*, **1961**, 5216.

2) E. Ochiai, "Aromatic Amine Oxides," Elsevier Publishing Co., Amsterdam, 1967, p. 14 and p. 212; A. R. Katritzky and J. M. Lagowski, "Chemistry of Heterocyclic *N*-Oxides," Academic Press, London and New York, 1971, p. 231; R. A. Abramovitch and E. M. Smith, "Pyridine and Its Derivatives," Supplement Part II, Wiley and Sons Ltd., 1973, p. 95.
3) H. Nagano, M. Hamana, Y. Nawata, S. Prachayasittikul, A. N. Abdel-Sayed, and L. Bauer, *Heterocycles*, **26**, 1263 (1987).
4) The structure assignment of **2a'** was based on the IR absorptions at 1840 and 1825 cm^{-1} (characteristic of the carbonyl group of the N^+-OAc moiety)[5] and also the anomalously broad pattern of aromatic signals in the ^1H-NMR spectrum. *N*-Acetoxypyridinium and 3-methoxycarbonyl-*N*-acetoxypyridinium perchlorates give similar spectra which are not readily analyzable, whereas the spectra of the perchlorates of 1-oxides of pyridine, nicotinic acid and methyl nicotinate are normal and easily assignable.
5) C. W. Muth and D. S. Darlark, *J. Org. Chem.*, **30**, 1909 (1965).
6) A. W. Schrecker and P. B. Maury, *J. Am. Chem. Soc.*, **76**, 5803 (1954).
7) E. Ochiai, "Aromatic Amine Oxides," Elsevier Publishing Co., Amsterdam, 1967, p. 290; A. R. Katritzky and J. M. Lagowski, "Chemistry of Heterocyclic *N*-Oxides," Academic Press, London and New York, 1971, p. 281; R. A. Abramovitch and E. M. Smith, "Pyridine and Its Derivatives," Supplement Part II, Wiley and Sons Ltd., 1973, p. 130.
8) S. Prachayasittikul and L. Bauer, *J. Heterocycl. Chem.*, **22**, 771 (1985).
9) H. Nagano and M. Hamana, *Yakugaku Zasshi*, **106**, 872 (1986).
10) S. Furukawa, *Yakugaku Zasshi*, **79**, 487 (1959).
11) M. Hamana and H. Noda, *Yakugaku Zasshi*, **83**, 342 (1963).
12) E. Ochiai, "Aromatic Amine Oxides," Elsevier Publishing Co., Amsterdam, 1967, p. 311; A. R. Katritzky and J. M. Lagowski, "Chemistry of Heterocyclic *N*-Oxides," Academic Press, London and New York, 1971, p. 287; R. A. Abramovitch and E. M. Smith, "Pyridine and Its Derivatives," Supplement Part II, Wiley and Sons Ltd., 1973, p. 131.
13) *a*) R. A. Abramovitch, G. M. Singer, and A. R. Vinutha, *Chem. Commun.*, **1967**, 55; *b*) R. A. Abramovitch, M. Saha, and R. T. Coutts, *J. Am. Chem. Soc.*, **89**, 1537 (1967); *c*) R. A. Abramovitch and E. E. Knaus, *J. Heterocycl. Chem.*, **6**, 987 (1969); *d*) R. A. Abramovitch, E. M. Smith, E. E. Knaus, and M. Saha, *J. Org. Chem.*, **37**, 1690 (1972); *e*) R. A. Abramovitch, R. T. Coutts, and E. M. Smith, *ibid.*, **37**, 3584 (1972); *f*) R. A. Abramovitch, J. Campbell, E. E. Knaus, and A. Silhankova, *J. Heterocycl. Chem.*, **9**, 1367 (1972).
14) *a*) R. A. Abramovitch, S. Kato, and G. M. Singer, *J. Am. Chem. Soc.*, **93**, 3074 (1971); *b*) R. A. Abramovitch, A. L. Miller, T. A. Radzikowska, and P. Tomesik, *J. Org. Chem.*, **44**, 464 (1979).
15) H. Hata, *Bull. Chem. Soc. Jpn.*, **31**, 224 (1958).
16) W. Mathes and W. Sauermilch, *Justus Liebigs Ann. Chem.*, **618**, 1512 (1958).
17) R. Jujo, *Yakugaku Zasshi*, **66**, 49 (1946).

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Studies on Pyrazolo[3,4-*d*]pyrimidine Derivatives. XV.[1] Reactions Involving the Formation of the Anion of the Reissert Compound Derived from 1*H*-Pyrazolo[3,4-*d*]pyrimidine

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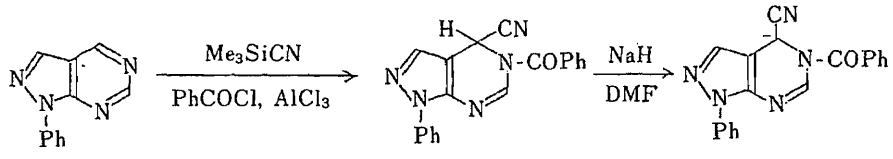
The anion (A) of the Reissert compound (**1**, 5-benzoyl-4,5-dihydro-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine-4-carbonitrile) was found to react with electrophiles in two ways. One is nucleophilic attack of the anion A. The other is self-decomposition of the anion A.

Thus, A underwent nucleophilic attack with aromatic and aliphatic aldehydes (**4a—j**), resulting in the formation of the corresponding α-aryl (or alkyl)-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylmethyl benzoates (**6a—j**) together with 1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine (**2**), the 4,4'-dimer (**7**) of **2**, *O*-benzoylaroins (**8a—d**), and *O*-benzoylcyanohydrins (**9e—j**). Nucleophilic substitution took place in the reaction of A with 2,4-dinitrochlorobenzene (**14a**) and methyl iodide (**14d**), giving the 4-(2,4-dinitrophenyl) derivative (**17a**) of **2** and 5,7-dibenzoyl-4-methyl-1-phenyl-4-(1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl)-4,5,6,7-tetrahydro-1*H*-pyrazolo[3,4-*d*]pyrimidine-6-carbonitrile (**19**), respectively.

However, the anion A did not react with other aryl (or alkyl) halides (**14b, c, e**), ketones (**11a, b**), 2-alkenonitriles (**21a, b**), and dimethyl acetylenedicarboxylate (**22**), and underwent the known self-decomposition, resulting in the formation of products such as **2**, **7**, the cyano derivative (**18**) of **2**, and the ester **6a**.

Keywords——pyrazolo[3,4-*d*]pyrimidine; Reissert compound; aldehyde; aryl halide; nucleophilic attack; self-decomposition; pyrazolopyrimidinemethanol

In connection with our interest in generating the anion (A) by the removal of the acidic hydrogen at the 4-position of 5-benzoyl-5,6-dihydro-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine-4-carbonitrile[1] (the pyrazolopyrimidine Reissert compound, **1**), which was derived from 1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine[2] (the pyrazolopyrimidine, **2**), with sodium hydride in dimethylformamide (DMF), we report here the reactions of the anion A with various electrophiles in DMF.



2 1 A

Chart 1

The Reaction with Aldehydes

It was reported[3] that reaction of the anion of 3-benzoyl-3,4-dihydro-4-quinazolinecarbonitrile (the quinazoline Reissert compound, **3**) with aldehydes (**4**) resulted in the

formation of α-aryl (or alkyl)-4-quinazolinylmethyl benzoates (**5**) in excellent yields.

In the case of the pyrazolopyrimidine Reissert compound **1**, the reaction yielded a complex mixture of products. Thus, when **1** reacted with aromatic aldehydes (**4a—g**) in the presence of sodium hydride in DMF, α-aryl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl benzoates (the esters, **6a—g**), **2**, and 4,4′-bis[1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine] (the dimer, **7**),[4] were obtained together with *O*-benzoylaroins (**8a—d**)[5,6] and *O*-benzoylcyanohydrins (**9a**, **e—g**).[7,8] It is noteworthy that the use of *p*-substituted benzaldehydes (**4b—d**) in the reaction gave the corresponding *O*-benzoylaroins (**8b—d**), and that of *o*-substituted benzaldehydes (**4e—g**) mainly afforded the corresponding *O*-benzoylcyanohydrins (**9e—g**). The results are summarized in Chart 2 and Table I.

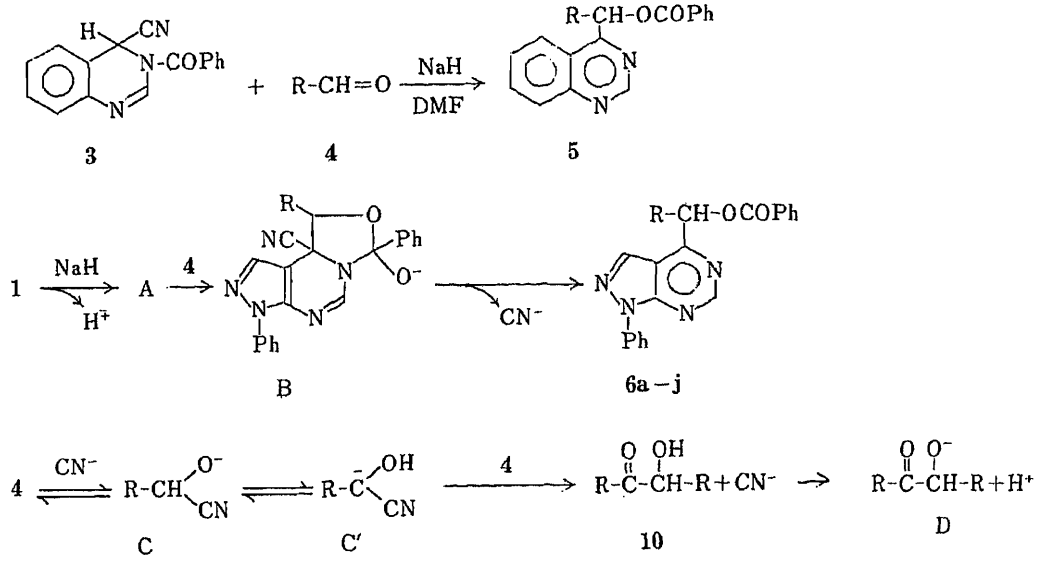


Chart 2

The formation of the pyrazolopyrimidine **2**, the esters **6**, the dimer **7**, *O*-benzoylaroins **8**, and *O*-benzoylcyanohydrins **9** may be explained by the following processes, as shown in Chart 2.

The first step is undoubtedly nucleophilic addition of the anion A to the carbonyl carbon of the aldehydes **4** to form a cyclic intermediate (B), similar to the cyclic intermediate[9] observed in the reaction of the anion of 1-benzoyl-1,2-dihydro-2-quinolinecarbonitrile (the

TABLE I. Reaction of 1 with 4 in the Presence of NaH in DMF

		Product									
1	4	2	Yield (%)	6	Yield (%)	7	Yield (%)	8	Yield (%)	9	Yield (%)
1	4a	2	15	6a	27	7	Trace	8a	15	9a	Trace
1	4b	2	13	6b	21	7	5	8b	5		
1	4c	2	9	6c	33	7	6	8c	15		
1	4d	2	33	6d	12	7	2	8d	8		
1	4e	2	6	6e	51	7	2			9e	33
1	4f	2	4	6f	43	7	4			9f	26
1	4g	2	6	6g	43	7	5			9g	38
1	4h	2	28	6h	3	7	12			9h	37
1	4h[a]	2	46	6h	5					9h	64
1	4i	2	33			7	16			9i	60
1	4j[a]	2	67	6j	10					9j	82

a) Reaction in refluxing THF–TMU mixture.

quinoline Reissert compound) with benzaldehyde (**4a**). Then B gives the esters **6** with loss of a cyanide ion. The resulting cyanide ion easily reacts with **4**, leading to the *O*-anion (C) of the cyanohydrins, which then undergoes the benzoin condensation with a second molecule of **4**, resulting in the generation of the *O*-anion (D) of the aroins (**10**). In the case of the reaction with *p*-substituted benzaldehydes (**4b—d**), the *O*-anion D, once formed, attacks the carbonyl carbon of the pyrazolopyrimidine Reissert compound **1** to give **2** and *O*-benzoylaroins **8** with loss of a cyanide ion. In the case of the reaction with *o*-substituted benzaldehydes (**4e—g**), nucleophilic attack of the *O*-anion C on the carbonyl carbon of **1** results in the formation of **2** and *O*-benzoylcyanohydrins **9e—g** with loss of a cyanide ion. The pyrazolopyrimidine **2** gives the dimer **7** by catalytic action of a cyanide ion. The dimerization mechanism was previously proposed by Higashino *et al.*[4,10]

Under the same conditions as used for the reaction with aromatic aldehydes **4a—g**, reaction of **1** with aliphatic aldehydes (**4h—j**) in DMF or in a mixture of tetrahydrofuran (THF)–tetramethylurea (TMU)[11] gave mainly **2**, **7**, and the *O*-benzoylcyanohydrins (**9h**[12]—**j**) together with the esters (**6h—j**) as by-products, as shown in Table I. These results show that the pyrazolopyrimidine Reissert compound **1** may favor the reaction with the *O*-anion C, once formed, leading to the *O*-benzoylcyanohydrins **9h—j** rather than the formation of the esters **6h—j**.

It was reported that the quinazoline Reissert compound **3** reacts with various ketones (**11**) in the presence of sodium hydride to give α,α-dialkyl-4-quinazolinylmethyl benzoates (**12**) in less than 50% yield.[3]

Attempts were made to form the desired α,α-dialkyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4-ylmethyl benzoates (**13**) by the reaction of **1** with acetone (**11a**) or acetophenone (**11b**), in the presence of sodium hydride under the same conditions as used for the reaction with the aldehydes **4**, affording the pyrazolopyrimidine **2** and the dimer **7**. The products **2** and **7** undoubtedly originate from the known self-decomposition of the anion A.[1]

The Reaction with Aryl or Alkyl Halides

It was reported that the reaction of the quinazoline Reissert compound **3** with 2,4-dinitrochlorobenzene (**14a**), 4-chloroquinazoline (**14c**), and methyl iodide (**14d**) in the presence of sodium hydride in DMF gives the corresponding 4-aryl (or alkyl)-3-benzoyl-3,4-dihydro-4-quinazolinecarbonitriles (**15a**, **c**, and **d**), which can then be easily hydrolyzed to the

TABLE II. Melting Points, Elemental Analyses, and Mass Spectral Data for **6**, **8**, and **9**

Compd.	mp (°C)	Formula	Analysis (%) Calcd (Found)		
			C	H	N
6b[d]	151—153[b]	$C_{26}H_{20}N_4O_2$	74.27	4.79	13.33
			(74.39	4.86	13.28)
6c[e]	93—95[a]	$C_{26}H_{20}N_4O_3$	71.55	4.62	12.84
			(71.50	4.65	12.67)
6d[c]	136—138[a]	$C_{25}H_{17}ClN_4O_2$	68.11	3.89	12.71
			(68.07	3.88	12.76)
6e[d]	117—118[b]	$C_{26}H_{20}N_4O_2$	74.27	4.79	13.33
			(74.25	4.80	13.33)
6f[e]	131—132[a]	$C_{26}H_{20}N_4O_3$	71.55	4.62	12.84
			(71.49	4.64	12.84)
6g[f]	116—118[b]	$C_{25}H_{17}ClN_4O_2$	68.11	3.89	12.71
			(67.71	3.84	12.69)
6h	—[h]	$C_{22}H_{20}N_4O_2$	MS *m/z* Calcd: 372.1586 (M^+)		
			Found: 372.1602		
6j	—[h]	$C_{26}H_{28}N_4O_2$	MS *m/z* Calcd: 428.2234 (M^+)		
			Found: 428.2212		
8c[g]	105—106[b]	$C_{23}H_{20}O_5$	73.39	5.36	
			(73.28	5.33)	
8d[e]	96—97[b]	$C_{21}H_{14}Cl_2O_3$	65.47	3.66	
			(65.41	3.66)	
9e	—[h]	$C_{16}H_{13}NO_2$	MS *m/z*: 251 (M^+)		
9i[d]	48—49[a]	$C_{13}H_{15}NO_2$	71.86	6.96	6.45
			(71.74	6.98	6.54)
9j	—[h]	$C_{16}H_{21}NO_2$	74.10	8.16	5.40
			(74.13	8.22	5.56)

a) Colorless prisms. *b*) Colorless needles. *c*) Recrystn. from MeOH. *d*) Recrystn. from petr. benzin. *e*) Recrystn. from MeOH–ether. *f*) Recrystn. from MeOH–petr. benzin. *g*) Recrystn. from petr. benzin–benzene. *h*) Colorless oil.

corresponding 4-aryl (or alkyl)quinazolines (**16a**, **c**, and **d**).[3]

Under the same conditions, the reaction of **1** with **14a** proceeded in the same way as that of **3**, yielding 4-(2,4-dinitrophenyl)-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine (**17a**) as a main product together with 1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine-4-carbonitrile (the cyanopyrazolopyrimidine, **18**) as a by-product (one of the self-decomposition products[1] of the anion A). While the reaction with **14d** took a different route from that of **3**, giving 5,7-dibenzoyl-4-methyl-1-phenyl-4-(1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl)-4,5,6,7-tetrahydro-1*H*-pyrazolo[3,4-*d*]pyrimidine-6-carbonitrile (**19**) as a main product together with the 4-cyanopyrazolopyrimidine **18** as a by-product. The structure of **19** was suggested by the following spectroscopic data and experimental result. The infrared absorption (IR) spectrum of **19** showed a carbonyl peak (1680 cm^{-1}), and did not show any peak due to the cyano group. The latter result is compatible with the reported absence of the absorption peak of a cyano group located at an electron-deficient carbon, such as in Reissert compounds **3**[13] and **1**.[1] The proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed two characteristic singlets due to a methyl group (2.44 ppm) and $C^{6'}$-H of the pyrazolopyrimidine ring (9.10 ppm). Moreover, the ^{13}C-NMR spectrum showed a quartet due to a methyl group (31.3 ppm), a doublet due to C^6 (70.8 ppm), and four singlets due to C^4 (50.5 ppm), cyano (111.8 ppm), and two carbonyl (168.1 and 171.2 ppm) carbons. The elemental analyses and the molecular ion

TABLE III. IR and ^{1}H-NMR Spectral Data for **6**, **8**, and **9**

Compd.	IR ν_{max}^{KBr} cm^{-1} C=O	^{1}H-NMR ($CDCl_3$) ppm
6b	1720	8.90 (1H, s, C^6–H), 8.35 (1H, s, C^3–H), 8.30—7.10 (15H, m, aromatic H and C^α–H), 2.30 (3H, s, CH_3)
6c	1720	9.00 (1H, s, C^6–H), 8.35 (1H, s, C^3–H), 8.30—6.90 (15H, m, aromatic H and C^α–H), 3.70 (3H, s, OCH_3)
6d	1720	9.00 (1H, s, C^6–H), 8.35 (1H, s, C^3–H), 8.30—7.10 (15H, m, aromatic H and C^α–H)
6e	1720	8.92 (1H, s, C^6–H), 8.20 (1H, s, C^3–H), 8.40—7.10 (15H, m, aromatic H and C^α–H), 2.59 (3H, s, CH_3)
6f	1725	8.95 (1H, s, C^6–H), 8.25 (1H, s, C^3–H), 8.30—6.70 (15H, m, aromatic H and C^α–H), 3.78 (3H, s, OCH_3)
6g	1730	8.95 (1H, s, C^6–H), 8.30 (1H, s, C^3–H), 8.30—7.00 (15H, m, aromatic H and C^α–H)
6h	1720[a]	9.05 (1H, s, C^6–H), 8.32 (1H, s, C^3–H), 8.40—7.20 (10H, m, aromatic H), 6.10 (1H, d, J=5.8 Hz, C^α–H), 2.66 (1H, m, C<u>H</u>Me_2), 1.10 (6H, d, J=6.1 Hz, CH(C<u>H</u>$_3$)$_2$)
6j	1720[a]	9.00 (1H, s, C^6–H), 8.30 (1H, s, C^3–H), 8.40—7.20 (10H, m, aromatic H), 6.20 (1H, t, J=6.0 Hz, C^α–H), 2.20—1.10 (12H, m, $(CH_2)_6$), 0.90 (3H, t, J=6.0 Hz, $(CH_2)_6$C<u>H</u>$_3$)
8c	1705, 1685	8.20—7.75 (4H, m, aromatic H), 7.70—7.20 (5H, m, aromatic H and Ar–C<u>H</u>(O)–CO), 7.10—6.70 (5H, m, aromatic H), 3.79 (3H, s, OCH_3), 3.75 (3H, s, OCH_3)
8d	1715, 1695	8.30—7.70 (4H, m, aromatic H), 7.70—7.10 (9H, m, aromatic H), 6.95 (1H, s, Ar–C<u>H</u>(O)–CO)
9i	1720	8.20—7.90 (2H, m, aromatic H), 7.70—7.10 (3H, m, aromatic H), 5.21 (1H, s, C–C<u>H</u>(CN)–O), 1.15 (9H, s, $C(CH_3)_3$)
9j	1729[a]	8.20—7.90 (2H, m, aromatic H), 7.70—7.10 (3H, m, aromatic H), 5.49 (1H, t, J=6.1 Hz, CH_2–C<u>H</u>(CN)–O), 2.30—1.10 (12H, m, (C<u>H</u>$_2$)$_6$Me), 0.88 (3H, t, J=6.0 Hz, $(CH_2)_6$C<u>H</u>$_3$)

a) IR ν_{max}^{neat} cm^{-1}.

(M^+) in the mass spectrum (MS) were consistent with the structural formula $C_{38}H_{27}N_9O_2$ of **19**. Compound **19** was easily converted to the dimer **7** by alkaline hydrolysis.

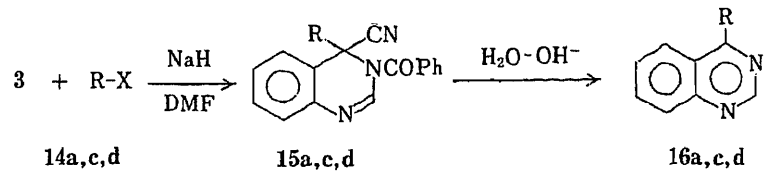
The formation of **19** is considered to occur through compound **20d**, as shown in Chart 3. The initial step is undoubtedly nucleophilic substitution of **14d** by the anion A, leading to a substitution product **20d** corresponding to **15**, but the mechanism of the formation of **19** from the resulting **20d** is not yet clear. Further studies on the mechanism are required.

However, in the case of 4-nitrochlorobenzene (**14b**), the reaction of **1** did not give any reaction product, and a tarry material was obtained. The reaction of **1** with other aryl (or alkyl) halides such as **14c** and ethyl iodide (**14e**) favored self-decomposition of the anion A rather than nucleophilic substitution, resulting in the formation of the pyrazolopyrimidine **2**, the ester **6a**, the dimer **7**, and the cyanopyrazolopyrimidine **18**, although the yields were poor. Therefore, arylation (alkylation) takes place only in the case of activated aryl (or alkyl) halides such as **14a** and **14d**.

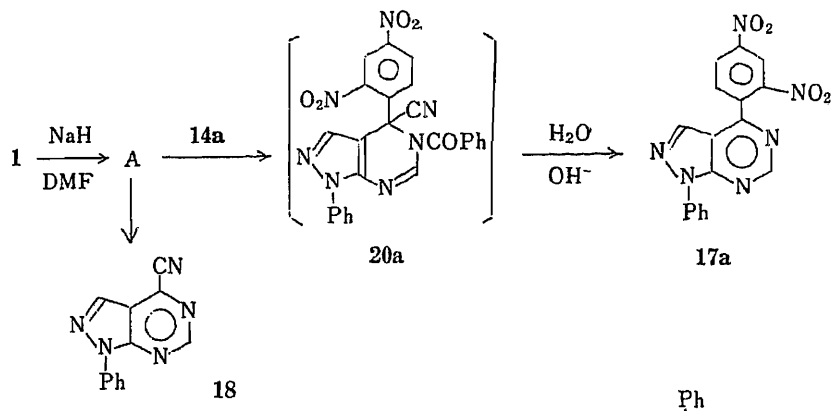
Other Reactions

In the previous paper, we reported that the quinazoline Reissert compound **3** reacted with 2-alkenonitriles (**21**) and dimethyl acetylenedicarboxylate (**22**) in the presence of sodium hydride, giving rise to Michael-type adducts (**23**), dimethyl 3-phenylpyrrolo[1,2-*c*]quinazoline-1,2-dicarboxylate (**24**), and dimethyl 3-benzoyl-4-cyano-1,2,3,4-tetrahydro-2,4-etheno-quinazoline-9,10-dicarboxylate (**25**), respectively.[3]

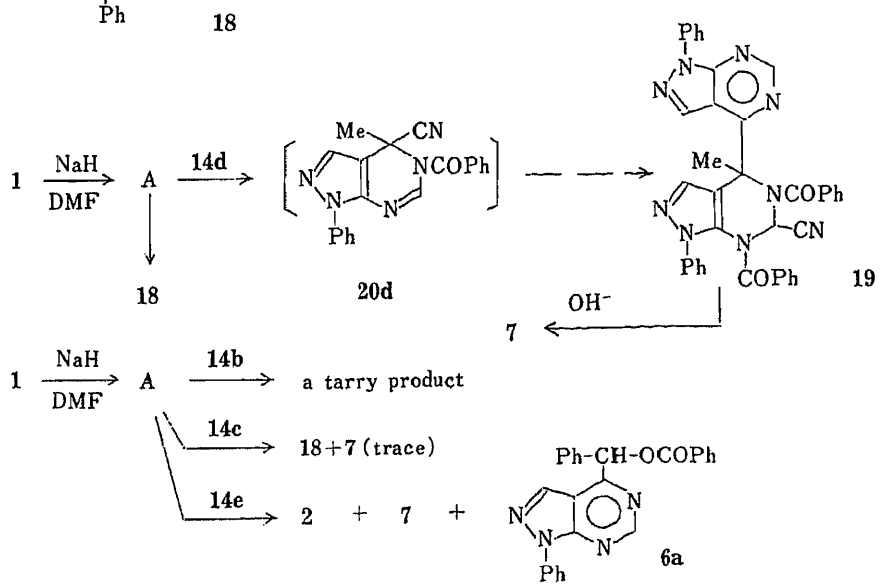
In the case of the pyrazolopyrimidine Reissert compound **1**, it was found that the reaction with **21** and **22** did not lead to the adducts corresponding to **23** and **24** observed in the reaction of **3**, but self-decomposition of the anion A proceeded, giving the pyrazolopyrimidine



14a,c,d 15a,c,d 16a,c,d



20a 17a 18



18 20d 19 7

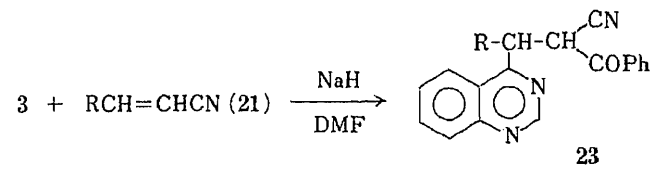
a tarry product

18+7 (trace)

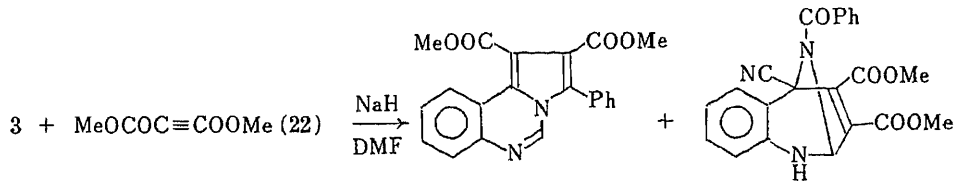
2 + 7 + 6a

a: R=2,4-$C_6H_3(NO_2)_2$, **b**: R=*p*-C_6H_4-NO_2, **c**: R=4-quinazolinyl, **d**: R=Me, **e**: R=Et

Chart 3



23



24 25

Chart 4

2, the dimer **7**, and the cyanopyrazolopyrimidine **18**.

The experimental results may be summarized as follows. i) On reaction with the aldehydes **4**, the anion A undergoes nucleophilic addition to the aldehyde group in **4** rather than self-decomposition of A, resulting mainly in the formation of the esters **6**. ii) In the reaction with the aryl (or alkyl) halides **14**, 2-alkenonitriles **21**, and dimethyl acetylenedicarboxylate **22**, the anion A prefers self-decomposition to nucleophilic attack, leading to self-decomposition products such as the pyrazolopyrimidine **2**, the ester **6a**, the dimer **7**, and the cyanopyrazolopyrimidine **18**. Therefore, arylation (or alkylation) takes place only in the case of activated aryl or alkyl halides such as 2,4-dinitrochlorobenzene **15a** and methyl iodide **15d**.

Experimental

All melting points are uncorrected. IR spectra were recorded on a Jasco A-102 diffraction grating IR spectrometer. ^{1}H-NMR spectra were measured at 60 MHz on a Hitachi R-24B high-resolution NMR spectrometer, and ^{13}C-NMR spectra were taken at 90 MHz on a JEOL JNM-FX90Q FTNMR spectrometer. Chemical shifts are quoted in parts per million (ppm) with tetramethylsilane as an internal standard, and coupling constants (*J*) are given in hertz (Hz). The following abbreviations are used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, and br s=broad singlet. MS were recorded on a JEOL JMS D-100 mass spectrometer. Exact mass measurement was carried out on a JEOL JMS-01SG-2 mass spectrometer combined with a JEC-6 spectrum computer. Samples were vaporized in a direct inlet system. Column chromatography was carried out on SiO_2, Wakogel C-200 (200 mesh). High-performance liquid chromatography (HPLC) was carried out on a Hitachi 655A-11 liquid chromatograph equipped with a Hitachi 655A variable-wavelength monitor. A column (20 mm × 250 mm) of CHEMCOSORB 7-ODS-H was used for reversed-phase chromatography.

Reaction of 1 with Aromatic Aldehydes (4a—g)——NaH (36 mg, 1.5 mmol) was slowly added to a solution of **1** (490 mg, 1.5 mmol) and an aldehyde (**4a—g**, 1.5 mmol) in DMF (3.0 g) under ice cooling, and the mixture was then stirred for 30 min under the same conditions. The reaction mixture was poured onto a large amount of ice, neutralized with AcOH, and extracted with AcOEt. The extract was washed with H_2O, dried over Na_2SO_4, and concentrated to dryness. The residue was chromatographed on a column of SiO_2. The first, second, third, and fourth fractions eluted with benzene gave the corresponding *O*-benzoylcyanohydrin (**9a, e—g**), *O*-benzoylaroin (**8a—d**), the dimer (**7**), and the ester (**6a—g**), respectively. The fraction subsequently eluted with benzene–$CHCl_3$ (1 : 1) gave the pyrazolopyrimidine (**2**).

Yields, elemental analysis, IR, MS, and ^{1}H-NMR spectral data for **6**, **8**, and **9** are listed in Tables I, II, and III.

Reaction of 1 with Aliphatic Aldehyde (4h—j)——i) In DMF: The procedure for the reaction of **1** (490 mg, 1.5 mmol) with isobutyraldehyde (**4h**, 108 mg, 1.5 mmol) in the presence of NaH (36 mg, 1.5 mmol) in DMF (3 g) was essentially the same as that described for the reaction with aromatic aldehydes **4**, and gave **2**, the ester **6h**, **7**, and the *O*-benzoylcyanohydrin **9h**.

On similar work-up of the reaction mixture, reaction of **1** (490 mg, 1.5 mmol) with pivaldehyde (**4i**, 129 mg, 1.5 mmol) did not give the ester **6i**, but yielded **2**, **7**, and *O*-benzoylcyanohydrin **9i**.

ii) In THF–TMU: A mixture of **1** (327 mg, 1 mmol), **4h** (90 mg, 1.25 mmol), and NaH (24 mg, 1 mmol) in a mixture of THF (10 ml) and TMU (0.3 ml) was refluxed for 1 h. The same work-up of the reaction mixture as described for the reaction of **1** with aromatic aldehydes **4** afforded **2**, **6h**, and **9h**.

By the same procedure, the reaction of **1** (327 mg, 1 mmol) with octanal (**4j**, 160 mg, 1.25 mmol) gave **2**, **6j**, and **9j**.

The yields of **2**, **6**, **7**, and **9** are shown in Table I, and elemental analysis, MS, IR, and ^{1}H-NMR spectral data for **6** and **9** are summarized in Tables II and III.

Reaction of 1 with Ketones (11)——The procedure for the reaction of **1** (490 mg, 1.5 mmol) with ketones (**11**, 1.5 mmol) in the presence of NaH (36 mg, 1.5 mmol) in DMF (3 g) was essentially the same as that described for the reaction of **1** with aromatic aldehydes **4**.

From the reaction with acetone (**11a**), traces of **2** and **7** in 16% yeild (47 mg) were obtained. From the reaction with acetophenone (**11b**), **2** and **7** were obtained in 3% (8 mg) and 9% yields (26 mg), respectively.

Reaction of 1 with 2,4-Dinitrochlorobenzene (14a)——NaH (36 mg, 1.5 mmol) was slowly added to a stirred solution of **1** (490 mg, 1.5 mmol) and **14a** (304 mg, 1.5 mmol) in DMF (3 ml) under ice cooling, and the mixture was then stirred for 30 min under the same conditions. The reaction mixture was poured onto a large amount of ice, neutralized with AcOH, and extracted with AcOEt. The extract was washed with H_2O, dried over Na_2SO_4, and concentrated to dryness. The residue was chromatographed on a column of SiO_2 with benzene. The first and second fractions gave the cyanopyrazolopyrimidine **18** and 4-(2,4-dinitrophenyl)-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine (**17a**) in 4% (93 mg) and 17% yields (93 mg), respectively.

Compound **17a** was recrystallized from acetone to give yellow prisms, mp 206—207 °C. *Anal.* Calcd for

$C_{17}H_{10}N_6O_4$: C, 56.36; H, 2.78; N, 23.20. Found: C, 56.42; H, 2.79; N, 23.39. MS *m/z*: 362 (M^+). IR ν_{max}^{KBr} cm^{-1}: 1540, 1350 (NO_2). ^{1}H-NMR (in $(CD_3)_2SO$): 9.20 (1H, s, C^6-H), 8.92 (1H, d, J=3.0 Hz, $C^{3'}$-H), 8.70 (1H, s, C^3-H), 8.80—8.00 (4H, m, aromatic H), 7.85—7.30 (3H, m, aromatic H).

Reaction of 1 with MeI (14d)——NaH (36 mg, 1.5 mmol) was slowly added to a stirred solution of 1 (490 mg, 1.5 mmol) and **14d** (230 mg, 1.5 mmol) in DMF (3 ml) under ice cooling, and the mixture was then stirred for 1 h under the same conditions. The reaction mixture was poured onto a large amount of ice and neutralized with AcOH. The separated crystals were dissolved in benzene and chromatographed on a column of SiO_2 with benzene. The first, second, and third fractions gave **18**, **7**, and 5,7-dibenzoyl-4-methyl-1-phenyl-4-(1-phenyl-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4-yl)-4,5,6,7-tetrahydro-1*H*-pyrazolo[3,4-*d*]pyrimidine-6-carbonitrile (**19**) in 4% (12 mg), 4% (11 mg), and 45% yields (215 mg), respectively.

Compound **19** was purified by HPLC with 90% MeOH to give colorless needles, mp 238—240 °C, from acetone. *Anal.* Calcd for $C_{38}H_{27}N_9O_2$: C, 71.13; H, 4.24; N, 19.65. Found: C, 70.74; H, 4.30; N, 19.41. MS *m/z*: 641 (M^+). IR ν_{max}^{KBr} cm^{-1}: 1680 (C=O). ^{1}H-NMR ($CDCl_3$): 9.10 (1H, s, $C^{6'}$-H), 8.30—8.05 (4H, m, aromatic H), 7.82 (1H, s, $C^{3'}$-H), 7.70—6.75 (18H, m, aromatic H and C^6-H), 2.44 (3H, s, CH_3). ^{13}C-NMR ($CDCl_3$): 31.3 (q, CH_3), 50.5 (s, C^4), 70.8 (d, C^6), 111.8 (s, CN), 112.0 (s), 118.5 (s), 121.6 (d), 123.4 (d), 126.9 (d), 127.3 (d), 128.1 (d), 128.7 (d), 129.0 (d), 129.1 (d), 129.2 (d), 131.2 (d), 132.2 (s), 132.4 (d), 132.8 (d), 133.0 (s), 134.2 (s), 136.2 (d), 137.9 (s), 138.3 (s), 153.2 (s), 155.0 (d), 168.1 (s, C=O), 171.2 (s, C=O).

Reaction of 1 with *p*-Nitrochlorobenzene (14b) and 4-Chloroquinazoline (14c)——By the same procedure as described for the reaction with **14a**, the reaction using **1** (490 mg, 1.5 mmol), **14c** (247 mg, 1.5 mmol), and NaH (36 mg, 1.5 mmol) in DMF (6 ml) gave traces of **7** and **18** in 13% yield (44 mg).

From the reaction with **14b**, a tarry material was obtained.

Reaction of 1 with EtI (14e)——By the same procedure as described for the reaction with **14d**, the reaction using **1** (490 mg, 1.5 mmol), **14e** (234 mg, 1.5 mmol), and NaH (36 mg, 1.5 mmol) in DMF (3 ml) gave **2**, **7**, and **6a** in 9% (25 mg), 12% (34 mg), and 3% yields (19 mg), respectively.

Alkaline Hydrolysis of 19——An aqueous solution of 10% NaOH was slowly added to a stirred suspension of **19** (64 mg) in a mixture of MeOH (6 ml) and $CHCl_3$ (3 ml) and the mixture was then stirred for 1 h. After removal of the solvent under reduced pressure, H_2O was added to the residue. The insoluble crystals were collected by suction, washed with MeOH, dried, and recrystallized from MeOH to give **7**, mp above 300 °C, in 41% yield (16 mg).

The filtrate was neutralized with 5% HCl and extracted with $CHCl_3$. The extract was dried over Na_2SO_4 and concentrated to dryness. The residue was crystallized from petroleum benzin to give benzoic acid, mp 121—122 °C, in 25% yield (3 mg).

Reaction of 1 with 2-Alkenonitrile (21)——i) With Acrylonitrile (**21a**): NaH (36 mg, 1.5 mmol) was slowly added to a stirred solution of **1** (490 mg, 1.5 mmol) and **21a** (80 mg, 1.5 mmol) in DMF (3 g) under ice cooling, and the mixture was then stirred for 30 min under the same conditions. The reaction mixture was poured onto a large amount of ice and extracted with AcOEt. The extract was washed with H_2O, dried over Na_2SO_4, and concentrated to dryness. The residue was chromatographed on a column of SiO_2. The first and second fractions eluted with benzene gave traces of **18** and **7** in 4% yield (12 mg), respectively. The fraction subsequently eluted with $CHCl_3$ gave **2** in 31% yield (90 mg).

ii) With Crotononitrile (**21b**): NaH (24 mg, 1 mmol) was added to a stirred solution of **1** (327 mg, 1 mmol) and **21b** (73 mg, 1 mmol) in THF (5 ml) and the mixture was then refluxed for 50 min. After cooling, the reaction mixture was poured onto a large amount of ice, neutralized with AcOH, and extracted with $CHCl_3$. The extract was washed with H_2O, dried over Na_2SO_4, and concentrated to dryness under reduced pressure. The residue was chromatographed on a column of SiO_2. The fraction eluted with benzene gave **18** in 25% yield (55 mg). The fraction subsequently eluted with $CHCl_3$ afforded **2** in 60% yield (117 mg).

Reaction of 1 with Dimethyl Acetylenedicarboxylate (22)——NaH (36 mg, 1.5 mmol) was slowly added to a stirred solution of **1** (490 mg, 1.5 mmol) and **22** (213 mg, 1.5 mmol) in DMF (3 g) under ice cooling and the mixture was then stirred for 30 min under the same conditions. The reaction mixture was poured onto a large amount of ice and neutralized with AcOH. The separated crystals were collected by suction, washed with H_2O, and dried. The crystals were dissolved in benzene and chromatographed on a column of SiO_2. The first fraction eluted with benzene gave **18** in 6% yield (19 mg) and the second fraction gave recovered **1** in 15% yield (73 mg). The fraction subsequently eluted with benzene–$CHCl_3$ (1 : 1) afforded **2** in 24% yield (70 mg).

Preparation of 1-Cyanooctyl Benzoate (9j)——A mixture of octanal (640 mg, 5 mmol), benzoyl chloride (700 mg, 5 mmol), and KCN (325 mg, 5 mmol) in H_2O (2 ml) was vigorously shaken for 10 min, then heated for 30 min on a steam bath. The reaction mixture was extracted with benzene. The extract was washed with H_2O and dried over Na_2SO_4. After removal of the benzene, the residue was chromatographed on a column of SiO_2 with benzene. The first fraction gave **9j**, colorless oil, in 68% yield (886 mg). The elemental and spectroscopic data for **9j** are listed in Tables II and III.

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References

1) Part XIV: T. Higashino, S. Sato, A. Miyashita, and T. Katori, *Chem. Pharm. Bull.*, **34**, 4569 (1986).
2) T. Higashino, Y. Iwai, and E. Hayashi, *Yakugaku Zasshi*, **94**, 666 (1974).
3) T. Higashino, H. Kokubo, and E. Hayashi, *Chem. Pharm. Bull.*, **33**, 950 (1985).
4) T. Higashino, M. Goi, and E. Hayashi, *Chem. Pharm. Bull.*, **24**, 238 (1976).
5) E. P. Kohler and J. L. E. Erickson, *J. Am. Chem. Soc.*, **53**, 2301 (1931).
6) E. Stierlin, *Ber.*, **22**, 381 (1889).
7) F. Francis and O. C. M. Davis, *J. Chem. Soc.*, **95**, 1404 (1909).
8) T. Higashino, Y. Matsushita, M. Takemoto, and E. Hayashi, *Chem. Pharm. Bull.*, **31**, 3951 (1983).
9) L. R. Walters, N. T. Iyer, and W. E. McEwen, *J. Am. Chem. Soc.*, **80**, 1177 (1958).
10) A. Goto and T. Higashino, *Bull. Chem. Soc. Jpn.*, **59**, 395 (1986).
11) A. Lüttringhaus and H. W. Dirksen, *Angew. Chem. Int. Ed. Engl.*, **3**, 260 (1964).
12) J. Aloy and C. Rabaut, *Bull. Soc. Chim. Fr.*, [4] **13**, 459 (1913).
13) T. Higashino, H. Kokubo, and E. Hayashi, *Chem. Pharm. Bull.*, **32**, 3900 (1984).

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Pregnanes and Pregnane Glycosides from the Roots of *Apocynum venetum* var. *basikurumon* (Apocynum. I)

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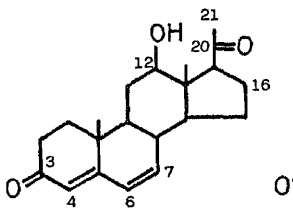
Three piscicidal pregnanes including two known defense substances of water beetles and four pregnane glycosides having the same aglycone as that of teikaside A were isolated from the roots of *Apocynum venetum* L. var. *basikurumon* HARA and their structures were determined.

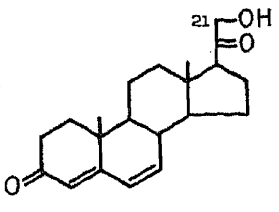
Keywords——Apocynaceae; *Apocynum venetum* var. *basikurumon*; pregnane; piscicidal pregnane; neridienone A; 6,7-didehydrocortexone; teikagenin bis-desmosidic glycoside; basikoside

Genus *Apocynum* is classified into a group containing cardenolides in Apocynaceae. In Japan, only one species, *Apocynum venetum* L. var. *basikurumon* HARA grows in Hokkaido and in the northern coastal area of the Japan Sea. The presence of five cardenolides, along with *p*-hydroxyacetophenone from the roots, was reported by Imai and Ikeda,[1] although the cardenolides were not identified. In the course of our studies on the constituents of Apocynaceae plants, we examined the steroidal constituents of this plant, and reported the presence of k-strophanthin-β and cymarin in the stem and leaves.[2] This paper deals with the isolation and structure determinations of pregnanes and pregnane glycosides from the roots.

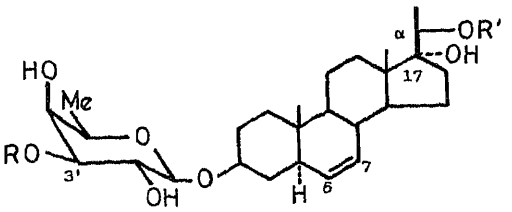
The air-dried roots were percolated with MeOH, and the MeOH percolate was partitioned with benzene and then with $CHCl_3$. Three compounds from the benzene layer and four compounds from the $CHCl_3$ layer were obtained and designated as I, II, III, and IV, V, VI, VII, respectively.



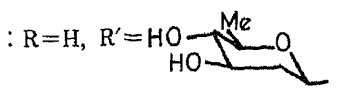
I (Δ^{16}) and II



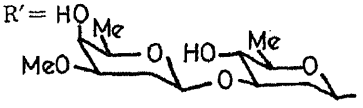
III



IV : R=H, R′=H
V : R=Ac, R′=H



VI : R=H, R′=



VII : R=H, R′=

Chart 1

TABLE I. ^{1}H Chemical Shifts of Pregnanes, δ (ppm) from TMS in Pyridine-d_5[a]

	I	II	III	IV[b]	V[b]	VI	VII
H-3α				3.99 (m)	3.99 (m)	4.00 (m)	3.99 (m)
H-4	5.88 (s)	5.89 (s)	5.88 (s)				
H-6	6.16	6.15	6.13	5.58	5.58	5.59	5.57
	(dd, 9, 2)	(dd, 10, 3)	(dd, 10, 3)	(d, 10)	(d, 10)	(d, 10)	(d, 10)
H-7	6.04	6.03	6.00	5.35	5.35	5.37	5.36
	(dd, 9, 1)	(dd, 10, 2)	(dd, 10, 2)	(d, 10)	(d, 10)	(d, 10)	(d, 10)
H-12α	3.82	3.75					
	(dd, 11, 5)	(dd, 11, 5)					
H-18,19	0.98	0.97	0.70	0.77	0.75	0.77	0.75
	1.02	1.01	0.98	0.80	0.81	0.78	0.77
H-20				4.13	4.13	4.02	4.01
				(q, 6)	(q, 6)	(q, 6)	(q, 6)
H-21	2.32	2.44	4.45 (d, 19)	1.50	1.50	1.65	1.65
			4.52 (d, 19)	(d, 6)	(d, 6)	(d, 6)	(d, 6)
H-1′				4.84	4.91	4.84	4.80
				(d, 8)	(d, 8)	(d, 8)	(d, 7)
H-2′				4.34	4.57	4.34	4.32
				(dd, 8, 9)	(dd, 8, 9)	(dd, 8, 9)	(dd, 7, 9)
H-3′				4.14	5.46	4.14	
				(dd, 9, 3)	(dd, 9, 3)	(dd, 9, 3)	
H-4′				4.08	4.29	4.08	
				(d, 3)	(d, 3)	(d, 3)	
H-5′				3.88	3.92	3.88	3.87
				(q, 6)	(q, 6)	(q, 6)	(q, 6)
H-6′				1.59	1.54	1.59	1.60
				(d, 6)	(d, 6)	(d, 6)	(d, 6)
Others	6.93	2.76			1.96	4.97	4.97
	(t, 2, H-16)	(t, 9, H-17α)			(–OAc)	(dd, 9, 1, H-1″)	(brd, 10, H-1″)
						3.56	4.82
						(t, 9, H-4″)	(brd, 10, H-1‴)
						1.65	3.27 (–OMe)
						(d, 6, H-6″)	1.65
							(d, 6, H-6″)
							1.54
							(d, 6, H-6‴)

a) Signal pattern and J value (Hz) are given in parentheses. b) Assignments of the proton signals due to the fucosyl moiety were carried out by ^{1}H–^{1}H COSY.

Compound I was obtained as crystals and was identified as neridienone A, based on the characteristic signals of olefinic protons due to the 4,6-dien-3-one and 16-en-20-one moieties, methyl protons due to 17-methyl ketone, and a 12α-carbinyl proton in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum and the corresponding signals in the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum, as well as direct comparison with an authentic sample.[3]

Compound II showed similar ^{1}H-NMR signals to those of I, except for the peaks due to the 16-olefinic proton. In the olefinic region of the ^{13}C-NMR spectrum, the signals due to C-4, C-5, C-6, and C-7 were observed. Since the molecular ion peak of II was observed at m/z 328 in the electron impact mass spectrum (EI-MS), II was assignable as 12β-hydroxypregna-4,6-diene-3,20-dione, which has been isolated along with I from *Nerium odorum*.[3a]

Compound III also retained a 4,6-dien-3-one system but had no 21-methyl group. Instead, the presence of a primary carbinol group was observed in the ^{1}H-NMR spectrum (1H each at δ 4.45 and 4.52, each as a doublet, J = 19 Hz) and in the ^{13}C-NMR spectrum (δ 70.1).

TABLE II. ^{13}C Chemical Shifts of the Pregnanes, δ (ppm) from TMS in Pyridine-d_5

C	I	II	III	IV[c]	V	VI	VII	C	IV[c]	V	VI	VII
1	34.2[a]	34.2[a]	34.3[a]	34.9	34.8	34.9	34.8	1′	102.7	102.7	102.7	102.7
2	33.8[a]	34.0[a]	34.1[a]	30.0	29.9	30.0	29.9	2′	72.3	69.1	72.3	72.4
3	198.1	198.1	198.1	77.6	77.8[a]	77.6	77.6	3′	75.4	78.0[a]	75.4	75.4
4	139.3	140.2	140.6	33.0	32.8	33.0	32.9	4′	72.7	70.2	72.7	72.9
5	162.6	162.8	163.0	45.1	45.1	45.1	45.1	5′	71.2	71.0	71.2	71.2
6	124.3	124.1	124.0	129.5	129.5	129.5	129.5	6′	17.4	17.1	17.3	17.4
7	128.3	128.3	128.1	131.0	131.0	131.1	131.1	1″ (1‴)		–OAc	102.4	102.4 101.8
8	35.0	36.8	37.6	38.2	38.2	38.2	38.2	2″ (2‴)		170.7	41.1	41.1 32.4
9	51.5	52.1	53.7	52.7	52.7	52.7	52.6	3″ (3‴)		21.0	72.1[a]	78.5[b] 79.1
10	36.1	36.1	36.1	34.5	34.5	34.5	34.5	4″ (4‴)			78.5	78.3[b] 67.1
11	31.7	30.6	20.7	20.9	20.9	21.0	20.9	5″ (5‴)			72.9[a]	72.1 70.7
12	73.9	77.9	38.4	38.5	38.5	37.9	37.9	6″ (6‴)			18.7	18.7[a] 17.8
13	53.1	51.2	45.2	47.0	47.0	47.0	46.9	–OMe				55.1
14	49.1	49.1	50.7	49.5	49.5	49.1	49.1					
15	29.1	23.8	23.3	23.5	23.5	23.5	23.4					
16	150.2	25.3	24.1	32.0	32.0	31.9	31.9					
17	155.1	62.4	58.5	85.3	85.3	85.0	84.9					
18	11.7	8.1	13.3	11.5	11.4	11.5	11.4					
19	16.0	16.1	16.1	14.4	14.4	14.6	14.6					
20	199.5	211.8	210.7	71.8	71.8	82.7	82.7					
21	26.6	31.8	70.1	19.4	19.4	18.0	18.0[a]					

a, b) Signal assignments marked *a*) or *b*) in each column may be reversed. *c*) Assignments of the carbon signals due to the fucosyl moiety were carried out by ^{13}C–^{1}H COSY.

Since the molecular formula was $C_{21}H_{28}O_3$, based on the EI-MS, III was considered to be 21-hydroxypregna-4,6-diene-3,20-dione (6,7-didehydrocortexone). The structure was finally confirmed by the direct comparison of III-acetate with an authentic sample prepared from 21-*O*-acetylcortexone by oxidation with chloranil.

Compound IV, mp 260—265 °C, gave the molecular formula $C_{27}H_{44}O_7$, based on the $M^+ + Na$ peak at *m/z* 503.299 in the fast atom bombardment mass spectrum (FAB-MS), suggesting IV to be a pregnane monohexoside. In the ^{1}H-NMR spectrum, a doublet peak observed at δ 1.50 ($J = 6$ Hz) was assignable to 21-methyl protons, suggesting the presence of a 20-hydroxyl group. Characteristic olefinic proton signals due to a Δ^6 linkage were observed at δ 5.58 and 5.35, each as a doublet. Since three carbinyl carbon signals which are assignable to C-3, C-17, and C-20, were observed at δ 77.6, 85.3, and δ 71.8 in the ^{13}C-NMR spectrum, the aglycone was considered to be an aglycone of teikaside A (5α-pregn-6-en-3β,17α,20α-triol; designated as teikagenin), formerly isolated from *Trachelospermum asiaticum* NAKAI.[4] The component sugar was considered to be fucose, based on the coupling constants between protons of the sugar moiety, as well as the ^{13}C-NMR signals. Based on a comparison of the chemical shifts of IV with those of the known teikaside A and other teikagenin glycosides, the fucose was allocated to the 3-hydroxyl of the aglycone.

In order to confirm the structure, IV was subjected to hydrolysis with acetone–HCl, and the aglycone moiety was identified as teikagenin-17,20-acetonide. The sugar moiety was subjected to gas liquid chromatography (GLC) after preparation of the methyl 2-(polyhydroxyalkyl)-thiazolidine-(4*R*)-carboxylate,[5] and the component sugar was determined to be D-fucose, from a comparison with the derivatives from D-fucose and L-fucose. Compound IV was named basikoside A.

Compound V, mp 240—246 °C, gave the molecular formula $C_{29}H_{46}O_8$, based on the FAB-MS. Compound V was considered to be a monoacetate of a pregnane glycoside, and the

acetyl group was allocated to 3′-OH of IV, since downfield shifts of C-3 and H-3 of the fucose (+2.6 and +1.32 ppm, respectively) were observed in the ^{13}C- and ^{1}H-NMR spectra. On deacetylation with KOH in MeOH, V was converted into IV. Compound V was named basikoside B.

Compound VI was considered to be a bioside having the same aglycone as IV, and one mole each of 6-deoxyhexose and 2,6-dideoxyhexose, based on the M^++Na peak at *m/z* 633.358 in the FAB-MS, and the ^{1}H- and ^{13}C-NMR spectra. On acid hydrolysis with 0.05 N H_2SO_4–50% dioxane, VI afforded IV and D-canarose, which is linked to the 20-hydroxyl group on the basis of the downfield shift of C-20 (+10.9 ppm) in comparison with that of IV. Compound VI was named basikoside C.

Compound VII seemed to be a trioside, based on the M^++Na peak at *m/z* 777, three anomeric proton peaks (δ 4.80, J=7 Hz; δ 4.82, J=10 Hz; δ 4.97, J=10 Hz) and three anomeric carbon peaks (δ 101.8, 102.4, 102.7) observed in the ^{1}H- and ^{13}C-NMR spectra. Since hydrolysis of VII with 0.05 N H_2SO_4–50% dioxane afforded IV, D-canarose, and D-diginose, the aglycone was assignable as teikagenin and the sugar moiety was considered to be composed of D-fucose, D-canarose, and D-diginose. The presence of the fragment peaks at *m/z* 609 (M−145 (diginosyl)) and *m/z* 479 (M−145−130 (canarosyl)) but no M−130 peak in the negative FAB-MS suggested that the terminal sugar is D-diginose. In the EI-MS of permethyl-VII (VII-1), a fragment ion peak due to a permethyl fucosyl moiety was observed at *m/z* 189, indicating that D-fucose is one of the terminal sugars, attached to the 3-hydroxyl as in the cases of IV, V, and VI, and consequently, diginosyl-canarose is linked to the 20-hydroxyl group. On the basis of ^{13}C-NMR considerations, the D-diginose was allocated to the 3-hydroxyl of the D-canarose. Thus, VII was determined to be teikagenin-3-*O*-β-D-fucosyl-20-*O*-β-D-diginosyl-(1→3)-β-D-canaroside, and was named basikoside D.

Three families containing cardenolides, Apocynaceae, Asclepiadaceae, and Scrophulariaceae (*Digitalis*) are each known to contain characteristic pregnanes. In Apocynaceae, most of the pregnanes are present as steroidal amines, and a pregnane having a 4,6-dien-3-one system has been isolated from *Paravallaris*.[6] Previously, we have isolated I, II, and neridienone B from the root bark of *Nerium odorum*,[3a] along with pregnenolone glucosides.[7] Compound I and its derivatives were also isolated from *Anodendron affine*.[3b] On the other hand, teikaside A was isolated from *Trachelospermum asiaticum* as the only example of a bis-desmosidic glycoside of pregnane in this family.[4] In this study, both free pregnanes having a 4,6-diene-3-one system and bis-desmosidic glycosides of teikagenin were isolated from the roots.

Previously, we described the piscicidal activity of I.[3a] Compounds II and III are known as defense substances of water beetles, *Cybister*[8] and *Acilius*,[9] respectively, and this is the first isolation of III from a plant source. The structures of the cardenolide glycosides from this plant will be reported shortly.

Experimental

Melting points were measured on a Kofler block and are uncorrected. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL GX-400 spectrometer in pyridine-d_5. Chemical shifts are given in δ values referred to internal tetramethylsilane, and the following abbreviations are used; s=singlet, d=doublet, t=triplet, m=multiplet, dd=doublet of doublets, and brd=broad doublet. EI-MS and FAB-MS were recorded on a JEOL D-300-FD spectrometer. Optical rotations were measured on a JASCO DIP 360 in MeOH solution. High performance liquid chromatography (HPLC) was run with a Waters model ALC 200, equipped with a radial pack C_{18}, and eluted with CH_3CN in H_2O (16%—45%). For reversed-phase column chromatography, an ODS column (RQ-1 and RQ-2, Fuji gel) was employed with CH_3CN in H_2O as a solvent. The following solvent systems were applied for silica gel column chromatography and thin layer chromatography (TLC); solv. 1, $CHCl_3$–MeOH–H_2O (bottom layer); solv. 2, EtOAc–MeOH–H_2O (top layer); solv. 3, hexane–AcOEt; solv. 4, benzene–acetone. TLC plates were heated until the spots appeared after being sprayed with 10% H_2SO_4. GLC was run on a Shimadzu GC-8A apparatus equipped with a

capillary column using N_2 as a carrier gas.

Extraction and Isolation——*Apocynum venetum* L. var. *basikurumon* HARA was cultivated in the medicinal plant gardens of Fukuoka University and Kobe Gakuin University and was harvested in 1985 and 1986. The air-dried and powdered roots (2.9 kg) were percolated with MeOH, and the methanolic solution was concentrated to 2 l *in vacuo*. To this solution, 2 l of H_2O was added and the mixture was filtered. The filtrate was extracted with benzene, and then with $CHCl_3$, successively. The benzene layer (3 g) was chromatographed repeatedly on a silica gel column with solv. 4 (5 : 1—3 : 1), and then with solv. 3 (1 : 1). Compound I (130 mg) was crystallized from acetone–ether–EtOAc–hexane to give prisms (22 mg). Compound II was separated from I on a silica gel column with solv. 3 (1 : 1) and finally purified by HPLC to give a solid (16 mg). Compound III was further purified by HPLC (solv. 45% CH_3CN) and was obtained as a solid.

The $CHCl_3$ layer (12.7 g) was chromatographed first on an MCI-gel column (Mitsubishi Chem. Ind. Co., CHP-20P) eluted with H_2O containing increasing amounts of MeOH. The effluents with 80% MeOH and MeOH were combined and chromatographed on silica gel columns with solv. 1 (7 : 2 : 1—7 : 3 : 1) and solv. 2 (4 : 1 : 5—4 : 1 : 3), alternately. Compounds IV—VII were then purified by HPLC. Yields of IV, 30 mg; V, 8 mg; VI, 20 mg; VII, 15 mg.

Neridienone A (I)——mp 202—204 °C, $[\alpha]_D^{23}$ +88.6° (c=0.44). EI-MS m/z: 326. On admixture with an authentic sample, no melting point depression was observed, and the ^1H- and ^{13}C-NMR spectra were in good agreement with those of an authentic sample.

12β-Hydroxypregna-4,6-diene-3,20-dione (II)——A solid, $[\alpha]_D^{19}$ +54.8° (c=0.56). EI-MS m/z: 328. The ^1H- and ^{13}C-NMR spectra were in good agreement with those of an authentic sample.

21-Hydroxypregna-4,6-diene-3,20-dione (6,7-Didehydrocortexone) (III)——mp 185—186 °C, $[\alpha]_D^{26}$ +122.7° (c= 1.80). EI-MS m/z: 328.203 (Calcd for $C_{21}H_{28}O_3$: 328.204). Acetylation of III was carried out with pyridine and Ac_2O at room temperature, and III-acetate (mp 105—108 °C) showed no melting point depression on mixed melting point measurement with an authentic sample (mp 105—106 °C) prepared from 21-*O*-acetylcortexone (100 mg) by reflux in *tert*-BuOH (1 ml) with chloranil (200 mg) for 8 h.

Teikagenin-3-*O*-β-D-fucopyranoside (IV)——A crystalline powder from MeOH mp 260—265 °C, $[\alpha]_D^{26}$ −101.8° (c=0.39). FAB-MS m/z: 503.299 (Calcd for $C_{27}H_{44}O_7$+Na: 503.299). Compound IV (25 mg) was allowed to stand in 1% HCl in acetone (2 ml) for 9 d at room temperature, then the mixture was deacidified with IRA-410 and partitioned with $CHCl_3$–H_2O. The $CHCl_3$-soluble fraction was purified on a silica gel column with solv. 4 (5 : 1), followed by crystallization from MeOH to give needles, mp 171—172 °C. On admixture with teikagenin-17,20-acetonide (mp 170—175 °C), no melting point depression was observed. The H_2O layer was concentrated *in vacuo* and the residue showed the same Rf value as D-fucose on TLC (solv. 1, 7 : 3 : 1). The component sugar was then converted into a methyl 2-(polyhydroxyalkyl)-thiazolidine-(4*R*)-carboxylate according to the procedure of Hara *et al.*[5] and examined on GLC (column G-SCOT, temperature 210 °C, carrier gas, N_2 at 1.4 kg/cm^2). The sample was confirmed to be D-fucose: t_R 12.8 min (the derivative of D-fucose 12.8 min; the derivative of L-fucose 13.1 min).

3′-*O*-Acetylteikagenin-3-*O*-β-D-fucopyranoside (V)——Prisms from MeOH, mp 240—246 °C, $[\alpha]_D^{26}$ −47.7° (c= 0.35). FAB-MS m/z: 545.308 (Calcd for $C_{29}H_{46}O_8$+Na: 545.309). After reflux of V (3 mg) with 5% KOH in dilute EtOH (1 ml) for 30 min, the product was identical with IV on TLC (solv. 1, 7 : 3 : 1; solv. 2, 4 : 1 : 0.5).

Teikagenin-3-*O*-β-D-fucopyranosyl-20-*O*-β-D-canaroside (VI)——Crystalline powder, mp 215—220 °C, $[\alpha]_D^{26}$ −92.2° (c=0.74). FAB-MS m/z: 633.362 (Calcd for $C_{33}H_{54}O_{10}$+Na: 633.361), negative FAB-MS m/z: 609 $(M-1)^-$, 479 $(M-C_6H_{10}O_3)^-$. After reflux of VI (5 mg) with 0.05 N H_2SO_4 in 50% dioxane (1 ml) for 1 h, IV and canarose were detected on TLC (solv. 1, 7 : 3 : 1, VI-sugar: Rf 0.41, canarose: Rf 0.41; solv. 2, 4 : 1 : 0.5, VI-sugar: Rf 0.45, canarose: Rf 0.45).[10]

Teikagenin-3-*O*-β-D-fucopyranosyl-20-*O*-β-D-diginopyranosyl-(1→3)-β-D-canaropyranoside (VII)——A solid, $[\alpha]_D^{26}$ −97.0° (c=0.40). FAB-MS m/z: 777 ($C_{40}H_{66}O_{13}$+Na), negative FAB-MS m/z: 753 $(M-1)^-$, 609 $(M-C_7H_{12}O_3)^-$, 479 $(609-C_6H_{10}O_3)^-$. After reflux of VII (5 mg) with 0.05 N H_2SO_4 in 50% dioxane (1 ml) for 1 h, IV, diginose, and canarose were detected on TLC (solv. 1, 7 : 3 : 1, VII-sugar: Rf 0.61 and 0.41, diginose: Rf 0.61, canarose: Rf 0.41; solv. 2, 4 : 1 : 0.5, VII-sugar: Rf 0.51 and 0.45, diginose: Rf 0.51, canarose: Rf 0.45). Compound VII (9 mg) was dissolved in dimethylsulfoxide (0.5 ml) and stirred with NaH (50 mg) and MeI (0.5 ml) for 2.5 h at room temperature. The mixture was diluted with $CHCl_3$ and washed with H_2O. The $CHCl_3$ layer was then purified on a silica gel column with solv. 1 (7 : 1 : 4) to give VII-1 as a homogeneous solid. FAB-MS m/z: 847 ($C_{45}H_{76}O_{13}$+Na), EI-MS m/z: 189, 159, 127.

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References and Notes

1) K. Imai and N. Ikeda, *Takamine Kenkyu Nenpo*, **9**, 31 (1957).

2) T. Yamauchi, Meeting of the Kyushu Branch, Pharmaceutical Society of Japan, Nagasaki, July 1982; T. Yamauchi, *Yakugaku Zasshi*, **105**, 695 (1985).
3) *a*) T. Yamauchi, F. Abe, Y. Ogata, and M. Takahashi, *Chem. Pharm. Bull.*, **22**, 1680 (1974); F. Abe and T. Yamauchi, *Phytochemistry*, **15**, 1745 (1976); *b*) T. Yamauchi, F. Abe, Y. Nishishita, H. Okabe, K. Shima, and S. Nishibe, *ibid.*, **18**, 1240 (1979).
4) F. Abe and T. Yamauchi, *Chem. Pharm. Bull.*, **29**, 416 (1981).
5) S. Hara, H. Okabe, and K. Mihashi, *Chem. Pharm. Bull.*, **34**, 1843 (1986); *idem*, *ibid.*, **35**, 501 (1987).
6) L. Zechmeister, "Progress in the Chemistry of Organic Natural Products," Vol. XXIV, Springer-Verlag, Vienna/New York, 1966, p. 112.
7) T. Yamauchi, M. Hara, and K. Mihashi, *Phytochemistry*, **11**, 3345 (1972).
8) M. C. Charda, N. K. Joshi, V. R. Mamdapur, and A. T. Sipahimalani, *Tetrahedron*, **26**, 2061 (1970).
9) H. Schildknecht, D. Hotz, and U. Maschwitz, *Z. Naturforsch.*, **22b**, 938 (1967).
10) D-Canarose of teikagenin glycosides isolated from *Trachelospermum asiaticum*[11] was identified by comparison with an authentic sample by Prof. J. Shoji of Showa University.
11) F. Abe and T. Yamauchi, The 106th Annual Meeting of the Pharmaceutical Society of Japan, Chiba, April 1986.

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Reactions of Nicotinic Acid 1-Oxide with Propionic, Phenylacetic and Benzoic Anhydrides

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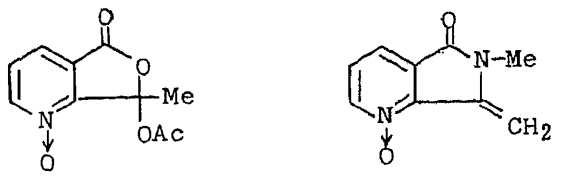
Nicotinic acid 1-oxide (**1**) reacts with hot propionic anhydride to give 4-aza-3-ethyl-3-propionyloxy-1(3*H*)-isobenzofuranone 4-oxide (**2**), 5-aza-3-methyl-4-propionyloxyisocoumarin (**3**), 4-aza-3-ethyl-3-propionyloxy-1(3*H*)-isobenzofuranone (**4**), 4-aza-3-ethyl-3,5-dipropionyloxy-1(3*H*)-isobenzofuranone (**5**) and a mixture of 2- and 6-hydroxynicotinic acids (**6** and **7**). Product **2** is a 2-propionylation product of **1**, and **3**, **4** and **5** arise from further action of the anhydride on **2**. Phenylacetic anhydride undergoes oxidative decarboxylation with **1** to give benzaldehyde and nicotinic acid. The reaction of **1** with benzoic anhydride affords a 2-benzoylation product, 4-aza-3-benzoyloxy-3-phenyl-1(3*H*)-isobenzofuranone 4-oxide (**12**).

Keywords——nicotinic acid 1-oxide; 4-aza-1(3*H*)-isobenzofuranone 4-oxide; 5-azaisocoumarin; propionic anhydride; phenylacetic anhydride; benzoic anhydride; 2-propionylation; 2-benzoylation; deoxygenative β-propionyloxylation; oxidative decarboxylation

In the preceding paper, we described the reactions of nicotinic acid 1-oxide and related compounds with acetic anhydride and showed that the reactions of nicotinic acid and *N*-acetyl-*N*-methylnicotinamide 1-oxides afford 2-acetylation products (I and II) by a novel electrophilic process[1] (Chart 1). As an extension of this work, we further investigated the reactions of nicotinic acid 1-oxide with propionic, phenylacetic and benzoic anhydrides.

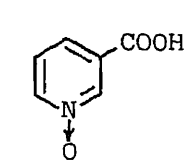


I II

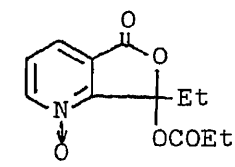
Chart 1

When a mixture of nicotinic acid 1-oxide (**1**) and propionic anhydride [$(EtCO)_2O$] was heated at 160 °C for 4 h and the mixture of products was chromatographed on silica gel with chloroform–methanol, we obtained 4-aza-3-ethyl-3-propionyloxy-1(3*H*)-isobenzofuranone 4-oxide (**2**), 5-aza-3-methyl-4-propionyloxyisocoumarin (**3**), 4-aza-3-ethyl-3-propionyloxy-1(3*H*)-isobenzofuranone (**4**), 4-aza-3-ethyl-3,5-dipropionyloxy-1(3*H*)-isobenzofuranone (**5**) and a mixture of 2- and 6-hydroxynicotinic acids (**6** and **7**) in 8.3, 39.8, 16.4, 2.1 and 9.4% yields, respectively. It is evident that **2** is a 2-propionylation product analogous with I and II, and products **3**, **4** and **5** seem to arise from further action of $(EtCO)_2O$ on **2**. In exploring this aspect, we followed the reaction at a range of temperatures (100—160 °C) by thin layer chromatographic monitoring, and found that the formation of **2** was initiated at around 115 °C and markedly accelerated at 130 °C, whereas the formation of **3**, **4** and **5** required higher temperatures. It was further confirmed that heating **2** with $(EtCO)_2O$ at 160—170 °C

TABLE I. Reactions of **1** and **2** with $(EtCO)_2O$



1



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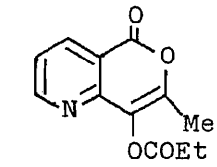

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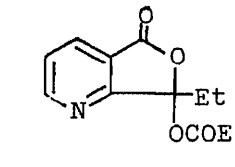

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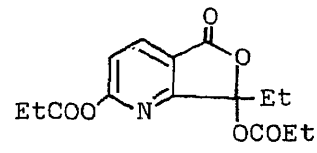

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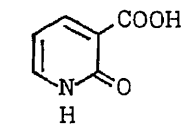

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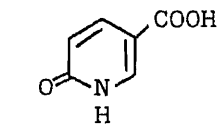
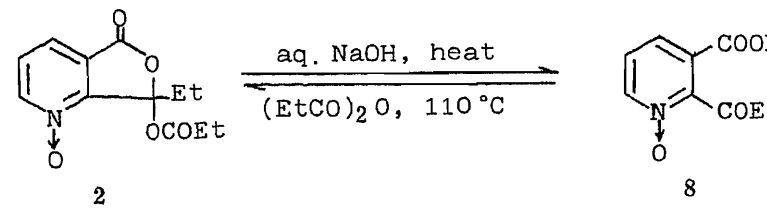
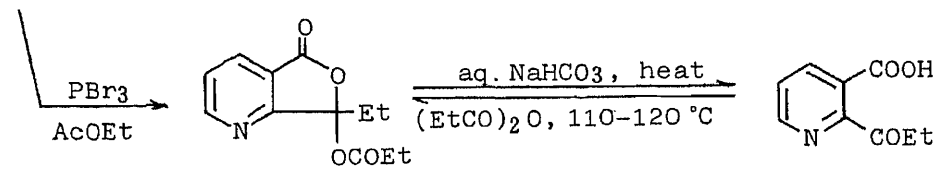


7

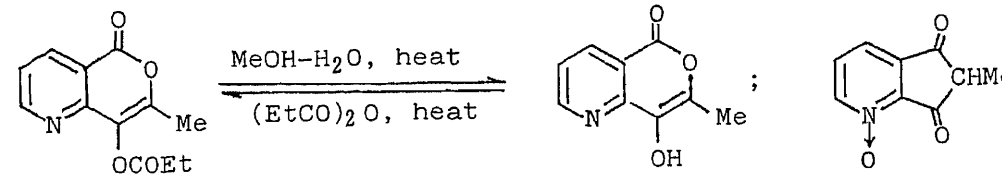
N-Oxide	Reaction		Products (%)				
	Temp. (°C)	Time (h)	**2**	**3**	**4**	**5**	**6** and **7**
1	125 130	6 8	54.2	12.9	Trace	—	4.3
1	160	4	8.3	39.8	16.4	2.1	9.4
2	160—170	6	—	24.9	20.0	11.4	—



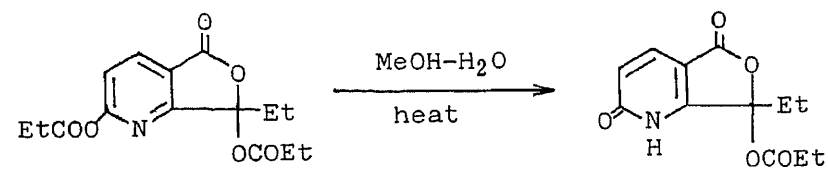
2 **8**



4 **10**



3 **9** **III**



5 **11**

Chart 2

for 6 h gave **3**, **4** and **5** in 24.9, 20.0 and 11.4% yields, respectively (Table I).

The structures of these products were assigned on the basis of elemental analyses, spectral examinations and some chemical reactions illustrated in Chart 2. Further, the structure of **3** was unambigously established by an X-ray diffraction study.

In the same way as I,[2] **2** was hydrolyzed in warm sodium hydroxide solution to 2-propionylnicotinic acid 1-oxide (**8**, 85%), which was convertible back to **2** upon heating with $(EtCO)_2O$. Compound **2** was smoothly deoxygenated with phosphorus tribromide in ethyl acetate to furnish product **4** (80.9%). The interconversion between **4** and 2-propionylnicotinic acid (**10**) was effected quite similarly by treatment with saturated sodium hydrogen carbonate solution or by heating with $(EtCO)_2O$, respectively. Refluxing a solution of **3** in methanol–water led to 5-aza-4-hydroxy-3-methylisocoumarin (**9**, 70%), which readily reverted to **3** upon heating at 100 °C with $(EtCO)_2O$. When **5** was heated in methanol–water (1 : 1) on a steam bath for 1 h, only hydrolysis of the 5-propionyloxy group occurred to afford the corresponding 5-hydroxy derivative (**11**, 60%), the 3-propionyloxy group being inert.

Bain and Saxton[3] reported that a minute amount of **8** (5%) could be isolated together with **6** (5%) upon refluxing **1** with $(EtCO)_2O$ for 1 h followed by hydrolysis of the reaction mixture, but another product was formed in a low yield (7.8%) instead of **8** when refluxing was continued for 3 h, and they tentatively assigned a diketone structure (III) to this product. However, its composition, $C_9H_7NO_3$, and melting point, 160—161 °C, were the same as those of **9** derived from **3** of unequivocally established structure. On the basis of these facts and also mechanistic considerations, particularly on the formation of **3** as discussed below, we concluded that the structure of III should be revised to the 5-aza-isocoumarin structure **9**.

The formation of **2** should be considered to follow the same course as the formation of I from **1** and acetic anhydride[1] (Chart 3).

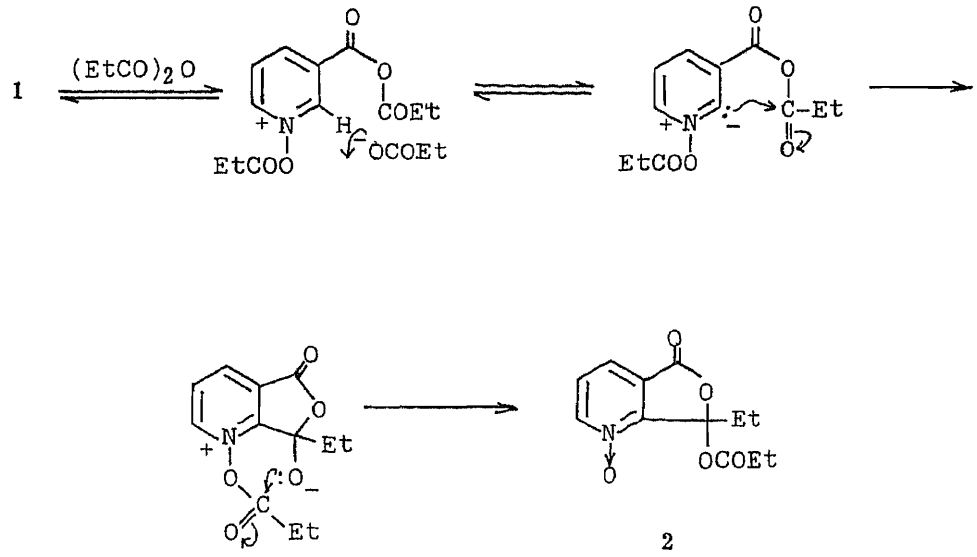


Chart 3

As for the transformation of **2** to **3**, two courses may be considered as formulated in Chart 4 [a) and b)]. Both courses involve two crucial steps, that is, A→B or A′→B′ and B→C or B′→C′. The first step is the conjugate addition of a propionyloxy anion or 3-carboxy anion to the β-position of the α-acyloxypropenyl side chain of A or A′ to give the anhydro base intermediate B or B′, respectively. The second step is the rearrangement of the propionyloxy group from the ring nitrogen of B or B′ to the α-position of the side chain, giving C or C′. While the participation of A in pathway a) seems to be reasonable in view of the isolation of II

and its reaction with acetic anhydride,[1] the mode of ring expansion of the lactone ring in C is not necessarily clear; the Wagner–Meerwein type α,β-shift of hydride (C→D) shown in Chart 4 is one plausible explanation. On the other hand, the ring expansion can be more plainly visualized in pathway b). However, the details have not yet been established. In any event, the reaction evidently falls into the category of deoxygenative β-acyloxylation[4] and is essentially

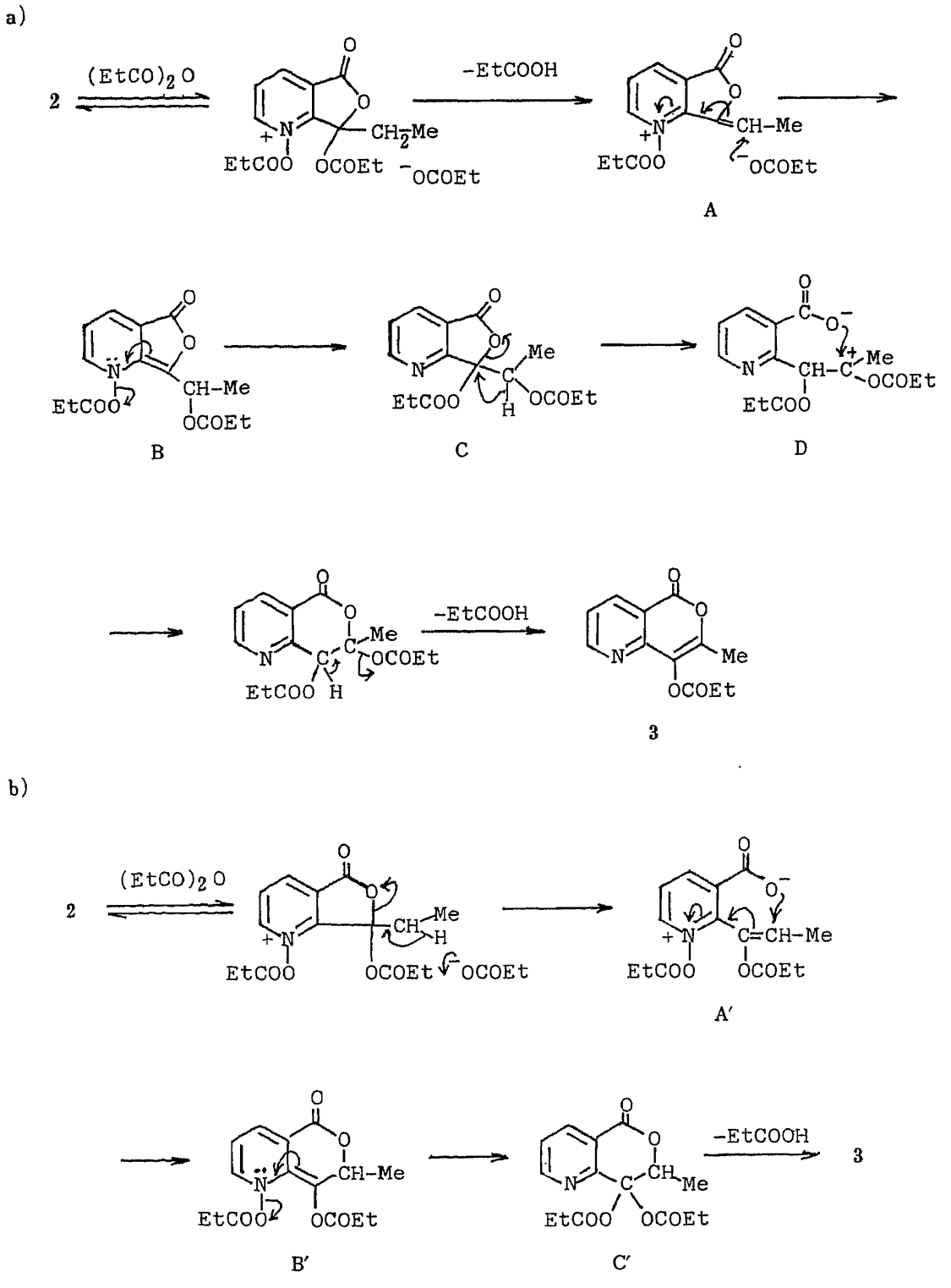


Chart 4

the same as the reactions of 2-styrylpyridine 1-oxide[5] and 4-styrylquinoline 1-oxide[6] with acetic anhydride; no formation of the 7-propionyloxy derivative was noticed, probably due to the electron-withdrawing effect of the 1-oxo group.

Products **4** and **5** are apparently formed by different types of reaction of **2** with $(EtCO)_2O$. The formation of **4** likely originated from oxidative decarboxylation[7-9] of $(EtCO)_2O$ with **2**. In exploring the ability of $(EtCO)_2O$ to undergo this type of reaction under the above conditions, we carried out the reaction of pyridine 1-oxide with excess $(EtCO)_2O$ at 150—160 °C for 2.5 h and obtained pyridine (26%) as the hydrochloride in addition to 2-propionyloxypyridine (47.9%). Obviously, **5** is the product of deoxygenative α-acyloxylation of **2** with $(EtCO)_2O$.[10]

The reaction of **1** with phenylacetic anhydride [$(PhCH_2CO)_2O$] was next examined. When **1** was heated with excess $(PhCH_2CO)_2O$ at 140—150 °C for 1 h, only oxidative decarboxylation of $(PhCH_2CO)_2O$ by **1** took place to give nicotinic acid in 42.9% yield as the sole product (Chart 5). From the reaction performed at 100—120 °C for 1 h, benzaldehyde was isolated as the *p*-nitrophenylhydrazone though in a poor yield of 5.2%. The established course of this type of reaction[7a,8b-d,9b] is also formulated in Chart 5.

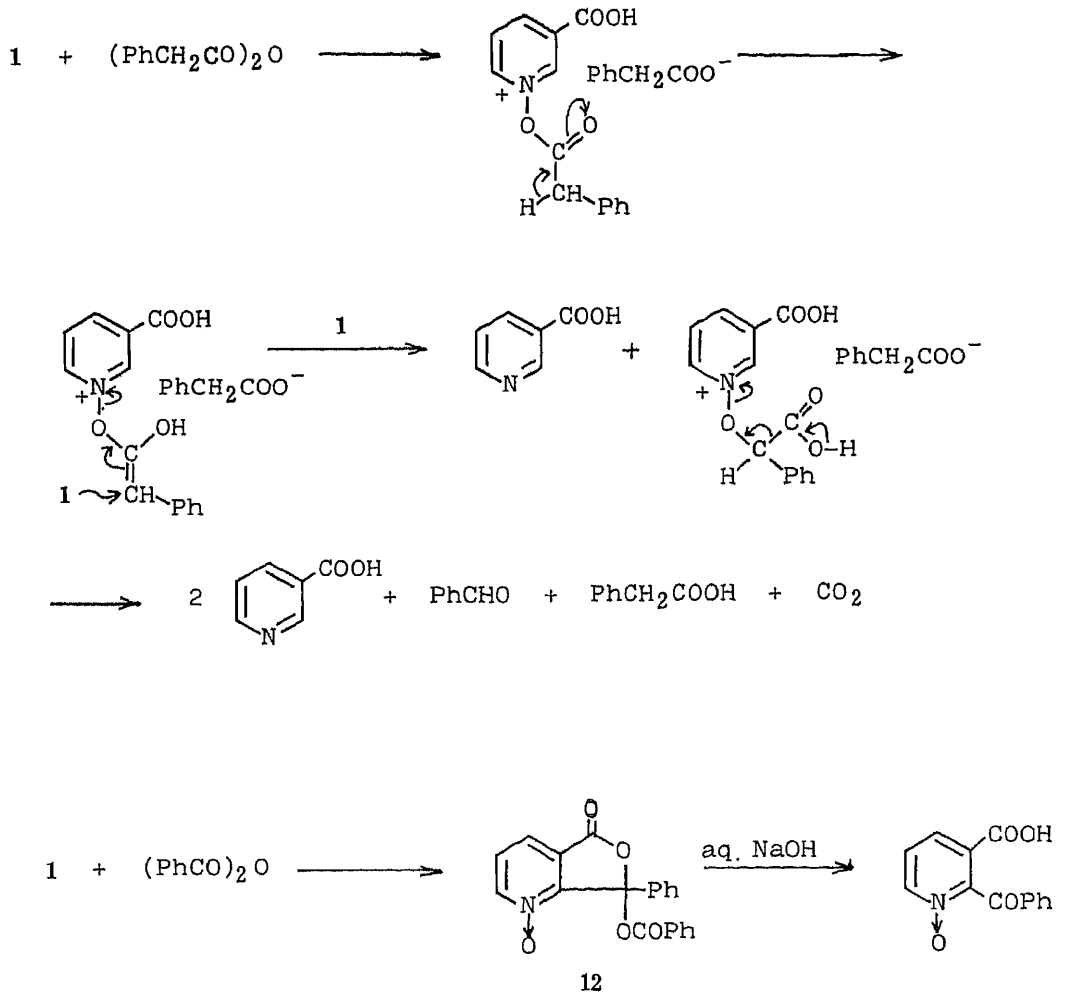


Chart 5

Finally the reaction of **1** with benzoic anhydride was found to follow the same path as that with acetic anhydride.[1] Thus, treatment of **1** with an excess of the anhydride at 150—160 °C for 3 h produced 4-aza-3-benzoyloxy-3-phenyl-1(3*H*)-isobenzofuranone 4-oxide (**12**)

in 13.5% yield. While the yield of **12** was not satisfactory, further examinations of the reaction conditions may improve the reaction yield. Hydrolysis of **12** to 2-benzoylnicotinic acid 1-oxide was readily effected upon warming with dil. sodium hydroxide solution (Chart 5).

Experimental

Melting points were measured with a Yanagimoto micro melting point apparatus and a Mettler FP61 apparatus, and are uncorrected. Spectral data were recorded on the following instruments. Infrared (IR), Hitachi 260-30 infrared spectrophotometer; mass spectrum (MS), Shimadzu LKB 9000; proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR), JEOL FX-200. Column chromatography was carried out on Wakogel C-200 (100—200 mesh).

Reaction of Nicotinic Acid 1-Oxide (1) with $(EtCO)_2O$——1) A mixture of **1** (1.39 g) and $(EtCO)_2O$ (13.9 ml) was refluxed at 160—170 °C with stirring for 4 h. The reaction mixture was concentrated *in vacuo*, and the residue was chromatographed on silica gel with $CHCl_3$–MeOH to give successively 4-aza-3-ethyl-3,5-dipropionyloxy-1(3*H*)-isobenzofuranone (**5**, 0.065 g, 2.1%), 4-aza-3-ethyl-3-propionyloxy-1(3*H*)-isobenzofuranone (**4**, 0.385 g, 16.4%), 5-aza-3-methyl-4-propionyloxyisocoumarin (**3**, 0.927 g, 39.8%), 4-aza-3-ethyl-3-propionyloxy-1(3*H*)-isobenzofuranone 4-oxide (**2**, 0.209 g, 8.3%) and a mixture of 2- and 6-hydroxynicotinic acids (**6** and **7**, 0.131 g, 9.4%). The last fraction was confirmed to consist of nearly equal amounts of **6** and **7** by thin layer chromatography in comparison with authentic samples.

2: Pale yellow needles, mp 67—68 °C (ether–hexane). *Anal.* Calcd for $C_{12}H_{13}NO_5$: C, 57.37; H, 5.22; N, 5.58. Found: C, 57.36; H, 5.12; N, 5.59. MS *m/z*: 251 (M^+), 223, 207. IR (neat): 1795, 1760 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.78—1.36 (6H, m, 2 × CH_3), 2.26—2.78 (4H, m, 2 × CH_2), 7.28—7.83 (2H, m, H-6, H-7), 8.33 (1H, d, J=6.4 Hz, H-5). ^{13}C-NMR ($CDCl_3$) δ: 6.6 (q, CH_3), 8.4 (q, CH_3), 27.2 (t, CH_2), 103.7 (s, C_3), 121.8 (d), 127.4 (d), 127.8 (s), 144.2 (d), 151.6 (s), 163.4 (s), 172.3 (s).

3: Pale yellow needles, mp 90 °C (ether–hexane). *Anal.* Calcd for $C_{12}H_{11}NO_4$: C, 61.80; H, 4.75; N, 6.01. Found: C, 61.67; H, 4.73; N, 5.93. IR (KBr): 1770, 1755, 1735 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.31 (3H, t, J=8.0 Hz, CH_3), 2.22 (3H, s, CH_3), 2.72 (2H, q, J=8.0 Hz, CH_2), 7.33 (1H, dd, J=8.0, 5.2 Hz, Py-H), 8.41 (1H, dd, J=2.0, 8.0 Hz, Py-H), 8.80 (1H, dd, J=2.0, 5.2 Hz, Py-H). ^{13}C-NMR ($CDCl_3$) δ: 9.1 (q, CH_3), 14.9 (q, CH_3), 27.1 (t, CH_2), 116.6 (s), 122.9 (d), 129.6 (s), 137.9 (d), 149.9 (s), 151.2 (s), 155.9 (d), 160.9 (s), 172.3 (s).

4: A pale yellow oil. *Anal.* Calcd for $C_{12}H_{13}NO_4$: C, 61.27; H, 5.57; N, 5.95. Found: C, 60.85; H, 5.52; N, 5.92. MS *m/z*: 235 (M^+), 207, 206, 190, 178, 162. IR (neat): 1785, 1755 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.60—1.40 (6H, m, 2 × CH_3), 1.85—2.65 (4H, m, 2 × CH_2), 7.49 (1H, dd, J=5.0, 7.8 Hz, Py-H), 8.15 (1H, dd, J=1.8, 7.8 Hz, Py-H), 8.82 (1H, dd, J=1.8, 5.0 Hz, Py-H). ^{13}C-NMR ($CDCl_3$) δ: 6.6 (q, CH_3), 8.4 (q, CH_3), 27.7 (t, CH_2), 30.2 (t, CH_2), 105.6 (s, C_3), 121.8 (s), 124.8 (d), 133.6 (d), 155.0 (d), 165.9 (s), 166.0 (s), 172.0 (s).

5: A pale yellow oil. MS *m/z*: 263, 236, 235, 234, 233. IR (neat): 1780, 1755, 1700 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.70—1.60 (9H, m, 3 × CH_3), 1.85—3.20 (6H, m, 3 × CH_2), 7.24 (1H, d, J=7.8 Hz, Py-H), 7.95 (1H, d, J=7.8 Hz, Py-H).

2) A mixture of **1** (1.39 g) and $(EtCO)_2O$ (26 ml) was heated at 125 °C for 6 h and then at 130 °C for 8 h to give 1.36 g (54.2%) of **2**, 0.3 g (12.9%) of **3**, a trace of **4** and 0.9 g (4.3%) of a mixture of **6** and **7**.

Reactions of 2——1) A solution of **2** (502 mg) in $(EtCO)_2O$ (6 ml) was heated at 160—170 °C for 6 h. The reaction mixture was worked up as described above to give 70 mg (11.4%) of **5**, 94 mg (20.0%) of **4** and 116 mg (24.9%) of **3**.

2) A mixture of **2** (2.51 g) and 4% NaOH (20 ml) was warmed on a steam bath for 0.5 h. The cooled reaction mixture was acidified to pH 2 with conc. HCl and concentrated *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 1.66 g (85%) of 2-propionylnicotinic acid 1-oxide (**8**), pale yellow prisms, mp 191 °C (H_2O). *Anal.* Calcd for $C_9H_9NO_4$: C, 55.39; H, 4.65; N, 7.18. Found: C, 55.09; H, 4.62; N, 7.05. IR (neat): 1720 (C=O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.06 (3H, t, J=7.0 Hz, CH_3), 2.76 (2H, q, J=7.0 Hz, CH_2), 7.44—8.0 (2H, m, H-4, H-5), 8.49 (1H, J=6.0 Hz, H-6). ^{13}C-NMR (DMSO-d_6) δ: 7.1 (q, CH_3), 31.0—36.3 (br, CH_2), 126.6 (d), 127.6 (s), 142.6 (d), 148.0 (br s), 164.1 (s), 190.8—199.2 (br s).

3) A mixture of **8** (1.95 g) and $(EtCO)_2O$ (10 ml) was heated at 110 °C for 0.5 h to give 2.26 g (90%) of **2**.

4) To an ice-cooled solution of **2** (251 mg) in AcOEt (5 ml) was added PBr_3 (0.2 ml), and the reactants were stirred at 5—10 °C for 1 h. The reaction mixture was poured onto ice and NaCl, then extracted with tetrahydrofuran (THF). The extract was washed with a saturated NaCl solution and concentrated *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$–ether to give 190 mg (80.9%) of **4**.

Reactions of 3——1) A solution of **3** (466 mg) in H_2O (10 ml)–MeOH (10 ml) was heated on a steam bath for 1 h. The solvent was evaporated, and the residue was chromatographed on silica gel with $CHCl_3$–MeOH. The eluate was concentrated *in vacuo* and triturated with ether to give 248 mg (70%) of crystalline 5-aza-4-hydroxy-3-methylisocoumarin (**9**), pale yellow needles, mp 161—162 °C (iso-PrOH). *Anal.* Calcd for $C_9H_7NO_3$: C, 61.02; H, 3.98; N, 7.91. Found: C, 61.17; H, 3.93; N, 7.90. MS *m/z*: 177 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.38 (3H, s, CH_3), 6.49 (1H, br s, OH), 7.41 (1H, dd, $J_{6,7}$=5.0 Hz, $J_{7,8}$=7.8 Hz, H-7), 8.49 (1H, dd, $J_{6,8}$=1.8 Hz, $J_{7,8}$=7.8 Hz, H-8), 8.87 (1H, dd, $J_{6,7}$=5.0 Hz, $J_{6,8}$=1.8 Hz, H-6). ^{13}C-NMR ($CDCl_3$) δ: 14.2 (q, CH_3), 116.3 (s), 122.8 (d), 132.6 (s), 138.5

(d), 141.1 (s), 149.1 (s), 154.7 (d), 160.9 (s).

2) A mixture of **9** (177 mg) and $(EtCO)_2O$ (2 ml) was heated at 100 °C for 15 min. The reaction mixture was concentrated *in vacuo* and the residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 163 mg (70%) of **3**.

Reactions of 4——1) A mixture of **4** (235 mg) and a saturated $NaHCO_3$ solution (5 ml) was warmed on a steam bath for 0.5 h. The reaction mixture was acidified to pH 2 with conc. HCl and extracted with THF. The extract was washed with a saturated NaCl solution and the residue from the extract was chromatographed on silica gel with $CHCl_3$–MeOH to give 148 mg (83%) of 2-propionylnicotinic acid (**10**), pale yellow prisms, mp 125—126 °C (MeOH–ether). *Anal.* Calcd for $C_9H_9NO_3$: C, 60.33; H, 5.06; N, 7.82. Found: C, 60.21; H, 5.04; N, 7.78. IR (KBr): 1765 (C=O) cm^{-1}. ^{1}H-NMR [DMSO-d_6–$CDCl_3$(1 : 1)] δ: 1.03 (3H, t, J=7.0 Hz, CH_3), 2.64 (2H, q, J=7.0 Hz, CH_2), 7.48 (1H, dd, $J_{4,5}$=7.8 Hz, $J_{5,6}$=5.0 Hz, H-5), 8.10 (1H, dd, $J_{4,5}$=7.8 Hz, $J_{4,6}$=1.8 Hz, H-4), 8.71 (1H, dd, $J_{4,6}$=1.8 Hz, $J_{5,6}$=5.0 Hz, H-6), 9.5—11.0 (1H, br, COOH).

2) A mixture of **10** (179 mg) and $(EtCO)_2O$ (5 ml) was heated at 110—120 °C for 1 h. The reaction mixture was concentrated *in vacuo* and the residue was chromatographed on silica gel with $CHCl_3$–ether to give 189 mg (80.4%) of **4** as an oil.

Hydrolysis of 5——A solution of **5** (307 mg) in H_2O (5 ml)–MeOH (5 ml) was heated on a steam bath for 1 h. The solvent was evaporated *in vacuo* and the residue was extracted with THF. The residue from the extract was chromatographed on silica gel with $CHCl_3$–MeOH to give 151 mg (60%) of 4-aza-3-ethyl-5-hydroxy-3-propionyloxy-1(3*H*)-isobenzofuranone (**11**), colorless needles, mp 182—183 °C (ether–hexane). *Anal.* Calcd for $C_{12}H_{13}NO_5$: C, 57.37; H, 5.22; N, 5.58. Found: C, 57.00; H, 5.07; N, 5.64. MS *m/z*: 251 (M^+). IR (KBr): 1800, 1765 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.78—1.40 (6H, m, 2×CH_3), 2.0—2.70 (4H, m, 2×CH_2), 6.62 (1H, d, J=9.8 Hz, Py-H), 7.78 (1H, d, J=9.8 Hz, Py-H). ^{13}C-NMR ($CDCl_3$) δ: 6.6 (q, CH_3), 8.5 (q, CH_3), 27.7 (t, CH_2), 30.0 (t, CH_2), 102.1 (s, C_3), 107.5 (s), 121.0 (d), 135.9 (d), 159.4 (s), 164.3 (s), 166.1 (s), 172.3 (s).

Reaction of Pyridine 1-Oxide with $(EtCO)_2O$——A solution of pyridine 1-oxide (0.95 g) in $(EtCO)_2O$ (20 ml) was heated at 150—160 °C with stirring for 2.5 h. The reaction mixture was concentrated *in vacuo*, and the residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 0.27 g (47.9%) of 2-propionyloxypyridine as an oil. This was heated with H_2O (10 ml) on a steam bath for 1 h to give 0.37 g (81.2%) of 2-pyridone, mp 107 °C (iso-PrOH–hexane).

The distillate from the reaction mixture was mixed with MeOH (10 ml) saturated with HCl gas, the mixture was again concentrated *in vacuo*, and the residue was triturated with ether to give 0.3 g (26%) of pyridine hydrochloride, which was identified by comparison of its IR spectrum with that of an authentic sample.

Reactions of 1 with Phenylacetic Anhydride——1) A mixture of **1** (1 g) and $(PhCH_2CO)_2O$ (22.5 g) was heated at 140—150 °C with stirring for 1 h. The reaction mixture was concentrated *in vacuo* and the residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 0.38 g (42.9%) of nicotinic acid, mp 235 °C, which was identified by comparison with an authentic sample.

2) A mixture of **1** (278 mg) and $(PhCH_2CO)_2O$ (5.08 g) was heated at 100—120 °C for 1 h. Excess ether and saturated $NaHCO_3$ solution were added with stirring to the cooled reaction mixture. The ether layer was separated and dried over Na_2SO_4, then the ether was evaporated under reduced pressure. The residue was warmed with a solution of *p*-nitrophenylhydrazine (400 mg) in MeOH (5 ml) on a steam bath for 20 min. The mixture was concentrated and the residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 25 mg (5.2%) of benzaldehyde *p*-nitrophenylhydrazone, yellow crystals, mp 195.6 °C (MeOH). It was identified by comparison with an authentic sample.

Reaction of 1 with Benzoic Anhydride——A mixture of **1** (1.39 g) and benzoic anhydride (15 g) was heated at 150—160 °C with stirring for 3 h. The reaction mixture was concentrated *in vacuo* and the residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 0.47 g (13.5%) of 4-aza-3-benzoyloxy-3-phenyl-1(3*H*)-isobenzofuranone 4-oxide (**12**), pale yellow prisms, mp 178—179 °C ($CHCl_3$–ether). *Anal.* Calcd for $C_{20}H_{13}NO_5$: C, 69.16; H, 3.77; N, 4.03. Found: C, 69.09; H, 3.83; N, 4.02. IR (KBr): 1795, 1740 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 7.1—8.35 (13H, m, Ar-H).

Hydrolysis of 12——A mixture of **12** (347 mg) and 8% NaOH (10 ml) was warmed on a steam bath for 0.5 h. The cooled reaction mixture was acidified to pH 2 with conc. HCl and concentrated *in vacuo*, and the residue was chromatographed on silica gel with $CHCl_3$–MeOH to give 194 mg (80%) of 2-benzoylnicotinic acid 1-oxide, pale yellow prisms, mp 282 °C (dec.) (MeOH–$CHCl_3$). *Anal.* Calcd for $C_{13}H_9NO_4$: C, 64.20; H, 3.73; N, 5.67. Found: C, 64.11; H, 3.74; N, 5.91. IR (KBr): 1695 (strong), 1700—2000 (br) cm^{-1}.

References

1) H. Nagano, Y. Nawata, and M. Hamana, *Chem. Pharm. Bull.*, **35**, 4068 (1987).
2) H. Nagano, M. Hamana, Y. Nawata, S. Prachayasittikul, A. N. Abde-Sayed, and L. Bauer, *Heterocycles*, **26**, 1263 (1987).
3) *a*) B. M. Bain and J. E. Saxton, *Chem. Ind.*, **1960**, 402; *b*) *Idem*, *J. Chem. Soc.*, **1961**, 5216.

4) E. Ochiai, "Aromatic Amine Oxides," Elsevier Publishing Co., Amsterdam, 1967, p. 259; A. R. Katritzky and J. M. Lagowski, "Chemistry of Heterocyclic N-Oxides," Academic Press, London, New York, 1971, p. 258; R. A. Abramovitch and E. M. Smith, "Pyridine and Its Derivatives," Supplement Part II, Wiley & Sons Ltd., New York, 1973, p. 105.
5) S. Furukawa, *Yakugaku Zasshi*, **79**, 487 (1959).
6) M. Hamana and H. Noda, *Yakugaku Zasshi*, **83**, 342 (1963).
7) *a*) C. Rüchardt, S. Eichler, and O. Krätz, *Tetrahedron Lett.*, **1965**, 233; *b*) C. Rüchardt, O. Krätz, and S. Eichler, *Chem. Ber.*, **102**, 3922 (1969).
8) *a*) T. Cohen, I. H. Song, and J. H. Fager, *Tetrahedron Lett.*, **1965**, 237; *b*) T. Cohen and I. H. Song, *J. Org. Chem.*, **31**, 3058 (1966); *c*) T. Cohen, I. H. Song, J. H. Fager, and G. L. Deets, *J. Am. Chem. Soc.*, **89**, 4968 (1867); *d*) T. Cohen, G. L. Deets, and J. A. Jenkins, *J. Org. Chem.*, **34**, 2550 (1969).
9) *a*) T. Koenig, *Tetrahedron Lett.*, **1965**, 3127; *b*) *Idem*, *ibid.*, **1967**, 2751.
10) E. Ochiai, "Aromatic Amine Oxides," Elsevier Publishing Co., Amsterdam, 1967, p. 290; A. R. Katritzky and J. M. Lagowski, "Chemistry of Heterocyclic N-Oxides," Academic Press, London, New York, 1971, p. 281; R. A. Abramovitch and E. M. Smith, "Pyridine and Its Derivatives," Supplement Part II, Wiley & Sons Ltd., New York, 1973, p. 130.

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Studies on Diazepines. XXVIII.[1] Syntheses of 5*H*-1,3-Diazepines and 2*H*-1,4-Diazepines from 3-Azidopyridines

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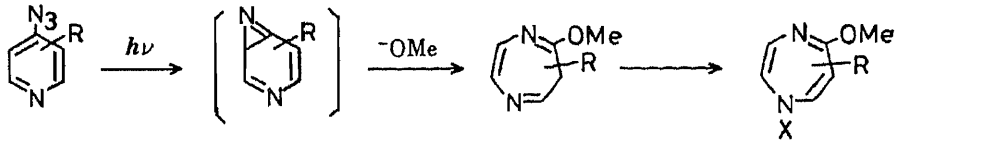
Photolysis of 2-unsubstituted (**5a—f**) and 2,4-disubstituted (**5k,l**) 3-azidopyridines in the presence of sodium methoxide resulted in ring expansion to give the 4-methoxy-5*H*-1,3-diazepines (**8** and **18**), presumably *via* the azirine intermediates **6** and **17** derived from the initially formed singlet 3-pyridylnitrenes by cyclization at the 2-position of the pyridine ring. On the other hand, in the photolysis of 2-substituted 3-azidopyridines (**5g—j**), the cyclization of the nitrenes occurred predominantly at the vacant 4-position giving rise to the 3-methoxy-2*H*-1,4-diazepines (**13**).

Keywords——3-azidopyridine; photolysis; pyridylnitrene; ring expansion; 5*H*-1,3-diazepine; 2*H*-1,4-diazepine; azirine intermediate

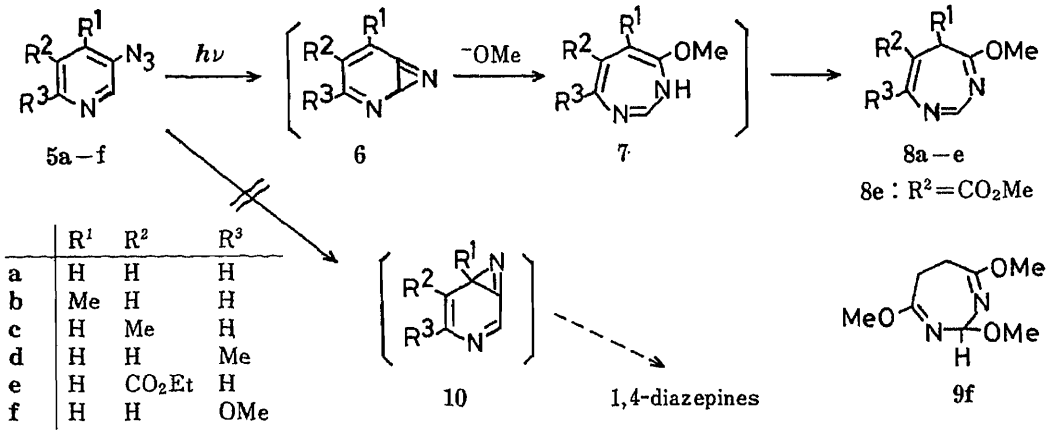
Thermal and photochemical ring expansion of aryl azides such as phenyl,[2] pyridyl,[3,4] and benzopyridyl azides[1,5,6] to seven-membered *N*-heterocyclic rings *via* the singlet nitrenes under basic conditions have recently been widely studied. In previous papers,[3] we have reported that the photolysis of 4-azidopyridines (**1**) in the presence of sodium methoxide resulted in the formation of the 6*H*-1,4-diazepines (**3**) *via* the azirine intermediates **2** and the acylation of **3** gave the 1-acyl-1*H*-1,4-diazepines (**4**). These results prompted us to examine the photochemical behavior of 3-azidopyridines. We report here that 2-unsubstituted and 2,4-disubstituted 3-azidopyridines, upon irradiation under basic conditions, afforded the corresponding novel 5*H*-1,3-diazepines, whereas the photolysis of 2-substituted 3-azidopyridines having no substituent at the 4-position gave the novel 2*H*-1,4-diazepines.[7]



1 (R=H, Me) 2 3 4 : X=COPh, COMe, CO_2Et

Chart 1

The starting 3-azidopyridines (**5a—c**, **5e—h**, and **5l**) were synthesized from the corresponding known 3-aminopyridines, prepared according to the reported methods (see Experimental), by diazotization followed by treatment with sodium azide. 3-Azido-6-methylpyridine (**5d**) was obtained from 3-amino-6-methylpyridine, prepared by the Hofmann rearrangement reaction of 6-methylnicotinamide.[8] Treatment of 3-azidopyridine 1-oxide[9] with phosphorus oxychloride gave 3-azido-2-chloropyridine (**5j**), which was treated with sodium methoxide to afford 3-azido-2-methoxypyridine (**5i**). Nitration of 6-amino-2,4-lutidine[10] gave 6-hydroxy-3-nitro-2,4-lutidine, which was treated with phosphorus oxychloride to give 6-chloro-3-nitro-2,4-lutidine. The chloro compound was hydrogenated over 5% palladium on carbon to afford 3-amino-2,4-lutidine, from which 3-azido-2,4-lutidine (**5k**)

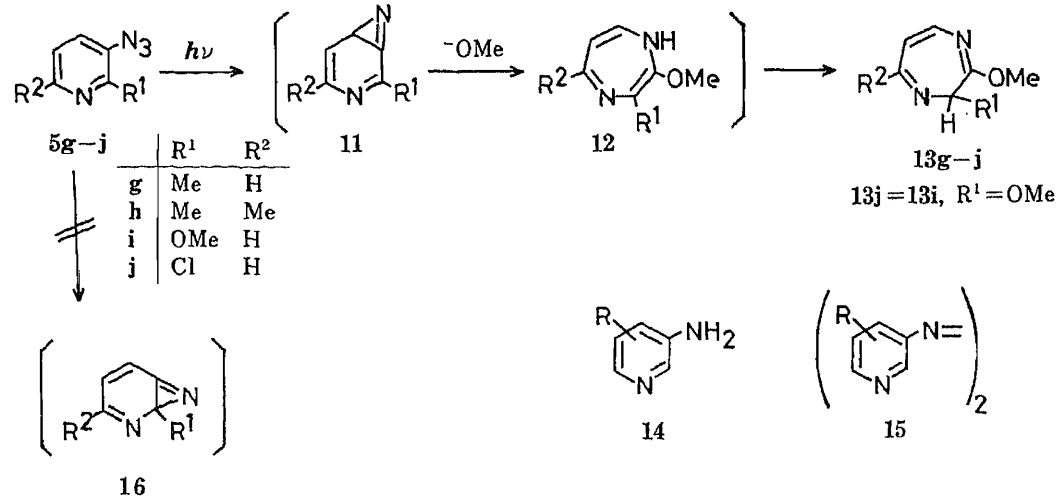


	R¹	R²	R³
a	H	H	H
b	Me	H	H
c	H	Me	H
d	H	H	Me
e	H	CO_2Et	H
f	H	H	OMe

Chart 2

was prepared.

Irradiation (400 W, high-pressure Hg lamp) of the 2-unsubstituted 3-azidopyridines (**5a**—**e**: 0.5—1.0 g) in methanol–dioxane (1 : 1)[1,3] containing a large excess of sodium methoxide for 1—2 h under ice cooling gave the corresponding 4-methoxy-5*H*-1,3-diazepines (**8a**—**e**) in 40—60% yields, as the sole characterizable products. The formation of the 1,3-diazepines (**8**) from **5a**—**e** may involve the azirine intermediates **6** formed from the initially generated 3-pyridylnitrenes by cyclization at the 2-position, and the products **8** are then formed *via* the unstable NH-diazepines (**7**). In the case of **5e** ($R^2=CO_2Et$), the ester group was exchanged to give **8e** ($R^2=CO_2Me$). However, 3-azido-2-methoxypyridine (**5f**), upon irradiation under similar conditions, afforded 2,4,7-trimethoxy-5,6-dihydro-2*H*-1,3-diazepine (**9f**) in *ca.* 60% yield, presumably *via* the initially formed 5*H*-1,3-diazepine (**8f**), which may undergo addition with methanol to the N=C bond followed by a 1,3-hydrogen shift. In all cases of **5a**—**f**, no products derived from the other possible azirine intermediates **10** such as 1,4-diazepines could be isolated.



	R¹	R²
g	Me	H
h	Me	Me
i	OMe	H
j	Cl	H

Chart 3

In contrast, it should be noted that irradiation of the 2-substituted 3-azidopyridines (**5g**—**j**), having no substituent at the 4-position, afforded the 3-methoxy-2*H*-1,4-diazepines (**13g**—**j**) in 15—40% yields, as well as small amounts of the reduction products **14** and/or the dimers **15**. The formation of the 1,4-diazepines from **5g**—**j** may involve different azirine

intermediates (**11**) from those for **5a**—**f**. These azirines **11** were derived by the cyclization of the 3-pyridylnitrenes at the 4-position. The formation of 1,3-diazepines from the azirines **16** was not observed, in contrast to the cases of **5a**—**f**.

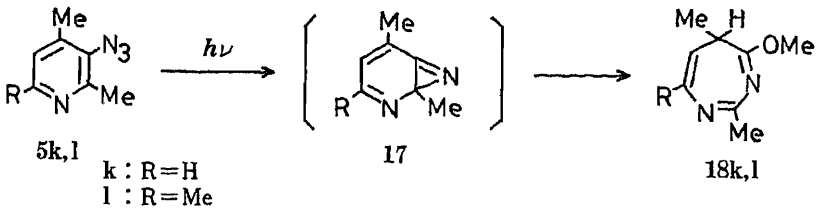


Chart 4

However, the 2,4-disubstituted 3-azidopyridines (**5k**, **l**) afforded only the 6*H*-1,3-diazepines (**18k**, **l**), presumably *via* the azirine intermediates **17**, by analogy with the case of **5a**—**f**. All the diazepines (**8**, **13**, and **18**) thus obtained are relatively unstable and gradually decomposed on standing, like the 6*H*-1,4-diazepines (**3**); this may account for the decrease in the isolated yields of the diazepines.

The above results indicate that in 2-unsubstituted (**5a**—**f**) and 2,4-disubstituted (**5k**, **l**) 3-azidopyridines, the initial intramolecular cyclization of the 3-pyridylnitrenes generated from the starting azides takes place predominantly at the 2-position of the pyridine ring rather than the 4-position to form the azirines **6** and **17**, respectively. This direction of azirine formation is analogous to that of 3-substituted phenylnitrenes (**19**), in which electron-withdrawing groups favor cyclization at the 2-position.[2,11] In the present case, the electron-withdrawing effect of the ring nitrogen would favor cyclization at the 2-position of the pyridine ring, as shown in the strucutre **20**, even in the case of **21** having an electron-withdrawing group at the 5-position; the nitrene **21** gave the 1,3-diazepine (**8e**). In contrast, in 2-substituted 3-azidopyridines (**5g**—**j**), the cyclization occurred predominantly at the 4-position to give the 1,4-diazepines (**13**) *via* the azirines **11**. This behavior is analogous to that of 2-substituted phenylnitrenes, which are known to cyclize preferentially at the vacant α-position.[11]

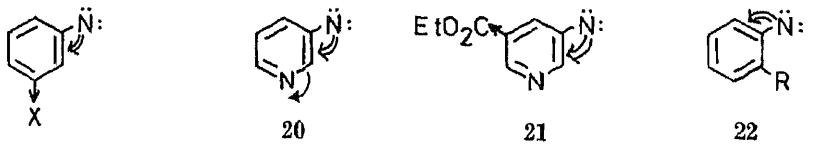


Chart 5

The aza-cycloheptatrienes such as azepines and diazepines can in theory display annular tautomerism between one or more unstable anti-aromatic NH forms and relatively stable CH forms.[12] Among monocyclic 1,3-[13] and 1,4-diazepines,[14] the NH forms are also unstable and have been isolated only as their N-substituted derivatives in which the substituent is an electron-withdrawing group such as an acyl group. The 5*H*-1,3-diazepines (**8** and **18**) are the first examples of the possible CH-forms of 1,3-diazepines. The products **13** are also the first examples of 2*H*-1,4-diazepines, although the 6*H*-tautomers have been reported.[3,15]

The structures of the new diazepines obtained were elucidated mainly from their proton nuclear magnetic resonance (^{1}H-NMR) (Table I) and ^{13}C-NMR spectral data (see Experimental). For example, the ^{1}H-NMR spectrum of **8a** showed an AB pair of doublets (J=7 Hz) at δ 5.04 and 6.74 assignable to 6-H and 7-H, respectively, and the former coupled further with the methylene protons (δ 2.70, d, J=7 Hz, 5-H_2), in addition to the signals at

TABLE I. ^{1}H-NMR Spectral Data for the Diazepines (**8**, **13**, and **18**)

8a	2.70 (2H, d, J=7, 5-H_2), 3.72 (3H, s, 4-OMe), 5.04 (1H, dt, J=7 and 7, 6-H), 6.74 (1H, d, J=7, 7-H), 7.92 (1H, s, 2-H)
8b	1.34 (3H, d, J=6, 5-Me), 2.1—2.4 (1H, m, 5-H), 3.72 (3H, s, 4-OMe), 4.76 (1H, dd, J=6 and 6, 6-H), 6.70 (1H, dd, J=6, 7-H), 7.91 (1H, s, 2-H)
8c	1.90 (3H, s, 6-Me), 2.60 (2H, s, 5-H_2), 3.72 (3H, s, 4-OMe), 6.60 (1H, s, 7-H), 7.84 (1H, s, 2-H)
8d	1.96 (3H, s, 7-Me), 2.56 (2H, d, J=7, 5-H_2), 3.74 (3H, s, 4-OMe), 4.88 (1H, t, J=7, 6-H), 7.88 (1H, s, 2-H)
8e	3.04 (2H, s, 5-H_2), 3.76 (6H, s, 4-OMe and CO_2Me), 7.76 (1H, s, 7-H), 8.02 (1H, s, 2-H)
13g	1.72 (3H, d, J=7, 2-Me), 3.04 (1H, qd, J=7 and 2, 2-H), 3.70 (3H, s, 3-OMe), 6.08 (1H, dd, J=8 and 4, 6-H), 7.12 (1H, dd, J=8 and 2, 5-H), 7.7—7.9 (1H, m, 7-H)
13h	1.64 (3H, d, J=7, 2-Me), 2.10 (3H, s, 7-Me), 3.06 (1H, q, J=7, 2-H), 3.66 (3H, s, 3-OMe), 6.02 (1H, d, J=8, 6-H), 6.90 (1H, d, J=8, 5-H)
13i	3.66 and 3.86 (each 3H, s, 2- and 3-OMe), 4.02 (1H, d, J=2, 2-H), 6.22 (1H, dd, J=7 and 4, 6-H), 7.26 (1H, dd, J=7 and 2, 5-H), 7.8—8.0 (1H, m, 7-H)
18k	1.30 (3H, d, J=7, 5-Me), 2.1—2.3 (1H, m, 5-H), 2.24 (3H, s, 2-Me), 3.70 (3H, s, 4-OMe), 4.70 (1H, dd, J=7 and 6, 6-H), 6.64 (1H, d, J=7, 7-H)
18l	1.24 (3H, d, J=7, 5-Me), 1.88 (3H, s, 7-Me), 2.1—2.3 (1H, m, 5-H), 2.18 (3H, s, 2-Me), 3.62 (3H, s, 4-OMe), 4.44 (1H, d, J=6, 6-H)

δ ($CDCl_3$), J=Hz.

δ7.92 (s, 2-H) and 3.72 (s, OMe); indicating the presence of the =N–CH=CH–CH_2– function. The methyl signals in the 5-methyldiazepines (**8b** and **18k**, **l**) were observed as doublets (J=6 or 7 Hz), while that in either **8c** (R^2=Me) or **8d** (R^3=Me) appeared as a singlet. The ^{13}C-NMR spectrum of **8a** showed signals due to two sp^3 carbons at 33.29 (t, 5-C) and 55.65 (q, OMe-C), and four sp^2 carbons at δ 109.36 (d, 6-C), 138.95 (d, 7-C), 151.89 (d, 2-C), and 159.01 (s, 4-C). On the other hand, the ^{1}H-NMR spectrum of **13g** showed signals assignable to 2-Me (δ 1.72, d, J=7 Hz), 2-H (δ 3.04, qd), 5-H (δ 7.12, dd), 6-H (δ 6.08, dd), 7-H (δ 7.7—7.9, m), $J_{2,7}$=2, $J_{5,6}$=8, $J_{5,7}$=2, and $J_{6,7}$=4 Hz, respectively. In the ^{13}C-NMR spectrum of **13g**, the signal due to 2-C appeared at δ 59.39 (d); indicating that the sp^3 carbon is adjacent to a nitrogen atom. These spectral data and the results of the following chemical studies are consistent with the proposed 5*H*-1,3-diazepine and 2*H*-1,4-diazepine structures, respectively, and rule out other possible CH forms.

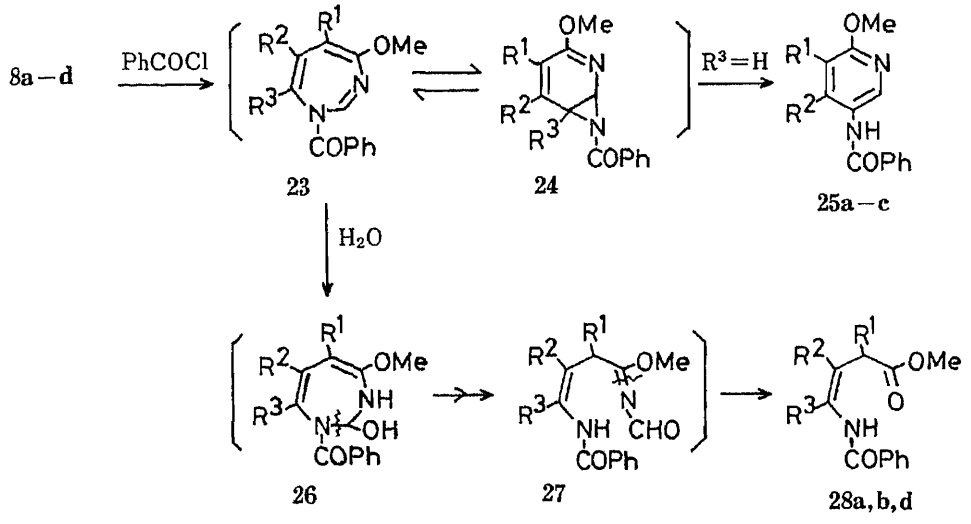


Chart 6

Treatment of the 1,3-diazepines (**8a**—**c**) with benzoyl chloride afforded 5-benzoylamino-2-methoxypyridines (**25**) in 25—40% yields. In the cases of **8a** and **8b**, the ring-opened products **28** were also obtained in *ca.* 10% yields. However, the 7-methyldiazepine (**8d**) gave only **28d** in 38% yield under similar reaction conditions and no pyridine derivative. A possible mechanism for the reaction is shown in Chart 6. This reaction may proceed by initial formation of the expected 1-benzoylamino-1*H*-1,3-diazepines (**23**), analogous to the acylation of 4*H*-1,2-diazepines,[16] 6*H*-1,4-diazepines,[3] and 5*H*-2,3-benzodiazepines,[17] although attempts to isolate the key diazepines (**23**) failed. The 1*H*-diazepines (**23**) might rearrange to the pyridines (**25**) *via* the aziridine intermediates **24**. Such rearrangement was widely observed in the thermolysis of various diazepines and oxazepines.[12,18] In the case of **8d**, the aziridine **24d** (R^3 = Me) could not rearrange to **25** and thus afforded only **28d**. The ring-opened products **28** may be formed from **23** *via* the intermediates **26** and **27** successively during aqueous work-up.

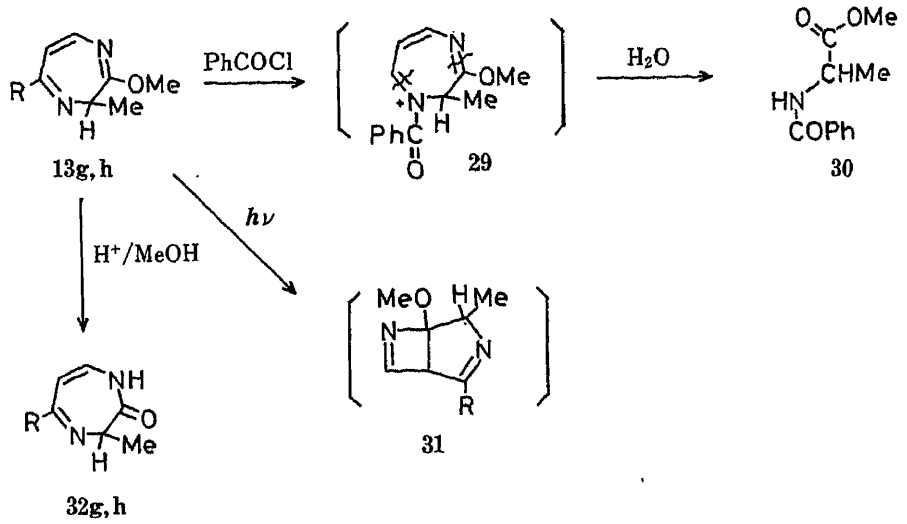


Chart 7

On the other hand, treatment of the 1,4-diazepine (**13g**) with benzoyl chloride resulted in decomposition to give methyl 2-benzoylaminopropanoate (**30**) in *ca.* 20% yield as a fragment product, presumably *via* the intermediate **29**. Irradiation of **13g** in benzene for 2 h afforded the bicyclic compound **31**, however, which was readily decomposed even at room temperature, so its structure was characterized only by ^{1}H-NMR spectral analysis. The formation of a similar bicyclic compound was also observed in the photolysis of the 3-azidopyridine (**5a**) and thus may cause the decrease in yields of the 1,4-diazepines (**18**). Similar intramolecular cyclization is observed in the photolysis of aza-cycloheptatrienes.[12,18] Finally, hydrolysis of **13g, h** with hydrochloric acid in methanol gave the 1,4-diazepinones (**32g, h**) in *ca.* 40% yields.

Experimental

Melting points were measured on a Yanagimoto micro melting point hot stage apparatus and are uncorrected. Infrared (IR) spectra were determined with a Hitachi 270-30 spectrometer and mass spectra (MS) were measured with a JEOL DX-300 instrument. ^{1}H-NMR spectra were recorded on a JEOL JNM-MH100 spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard unless otherwise stated; spectral assignments were confirmed by spin-decoupling experiments and, in the case of NH protons, by exchange with D_2O. ^{13}C-NMR spectra were recorded on a JEOL FX-100 spectrometer in $CDCl_3$. Microanalyses were performed in the Microanalytical Laboratory of this school by Mrs. R. Igarashi. Photolyses were carried out under a nitrogen atmosphere in an immersion apparatus equipped with a 400 W high-pressure Hg lamp, which was cooled internally with running ice-cold water.

Preparation of 3-Azidopyridines (5)——The starting azidopyridines used were synthesized from the correspond-

ing 3-aminopyridines, prepared by the reported methods, by the following general procedure unless otherwise stated.

A solution of sodium nitrite (*ca.* 1.2 mol eq) in water (10—15 ml) was added dropwise to a solution of an aminopyridine (4—8 g) in 10% HCl (30—50 ml) with stirring at 0—5 °C in an ice bath. After stirring for a further 10—20 min, a solution of sodium azide (1.05 mol eq) in water (10 ml) was added dropwise over a 30—40 min period to the reaction mixture at *ca.* 0 °C. The reaction mixture was stirred for an additional 30—60 min at room temperature, then made alkaline with Na_2CO_3 and extracted with CH_2Cl_2. The extract was washed with water, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was chromatographed on alumina (eluent: benzene) or silica gel (eluent: benzene–CH_2Cl_2, 1 : 1) to give a 3-azidopyridine (**5**).

3-Azidopyridine (5a)[19]——Oil, 78% yield from 3-aminopyridine. MS *m/z*: 120 (M^+). IR (neat): 2100 and 2150 (N_3) cm^{-1}. ^{1}H-NMR δ: 7.16—7.40 (2H, m, 4- and 5-H), 8.24—8.40 (2H, m, 2- and 6-H). *Anal.* Calcd for $C_5H_4N_4$: C, 49.99; H, 3.36; N, 46.65. Found: C, 49.91; H, 3.17; N, 46.37.

3-Azido-4-methylpyridine (5b)——Oil, 70% yield from 3-amino-4-methylpyridine.[20] MS *m/z*: 134 (M^+). IR (neat): 2150 (N_3) cm^{-1}. ^{1}H-NMR δ: 2.22 (3H, s, 4-Me), 7.10 (1H, d, J=5 Hz, 5-H), 8.28 (1H, d, J=5 Hz, 6-H), 8.45 (1H, s, 2-H). *Anal.* Calcd for $C_6H_6N_4$: C, 53.72; H, 4.51; N, 41.77. Found: C, 53.84; H, 4.45; N, 41.59.

3-Azido-5-methylpyridine (5c)——Oil, 65% yield from 3-amino-5-methylpyridine.[21] MS *m/z*: 134 (M^+). IR (neat): 2110 (N_3) cm^{-1}. ^{1}H-NMR δ: 2.34 (3H, s, 5-Me), 7.16 (1H, br s, 4-H), 8.16—8.28 (2H, m, 2- and 6-H). *Anal.* Calcd for $C_6H_6N_4$: C, 53.72; H, 4.51; N, 41.77. Found: C, 53.66; H, 4.52; N, 41.56.

5-Azido-2-methylpyridine (5d)——Bromine (9 g) was added dropwise to 5% NaOH (150 ml) with stirring in an ice bath and 6-methylnicotinamide[8] (7 g) was added in small portions over a 20 min period to the alkaline solution with stirring at 0—5 °C. The reaction mixture was heated at 70—80 °C with stirring and then extracted with CH_2Cl_2. The extract was washed with water, dried, and evaporated to dryness. The resulting solid residue was recrystallized from *n*-hexane–CH_2Cl_2 to give 5-amino-2-methylpyridine: 4.15 g, 75% yield, mp 93—94 °C. ^{1}H-NMR δ: 2.40 (3H, s, 2-Me), 3.64 (2H, br, NH_2), 6.87—6.90 (2H, m, 3- and 4-H), 7.96 (1H, s, 6-H). *Anal.* Calcd for $C_6H_8N_2$: C, 66.64; H, 7.46; N, 25.91. Found: C, 66.48; H, 7.10; N, 26.67.

The azide **5d** was prepared from 5-amino-2-methylpyridine thus obtained in 74% yield as an oil. MS *m/z*: 134 (M^+). IR (neat): 2120 (N_3) cm^{-1}. ^{1}H-NMR δ: 2.64 (3H, s, 2-Me), 7.44—7.64 (2H, m, 3- and 4-H), 8.64 (1H, d, J= 3 Hz, 6-H). *Anal.* Calcd for $C_6H_6N_4$: C, 53.72; H, 4.51; N, 41.77. Found: C, 53.68; H, 4.28; N, 41.51.

3-Azido-5-ethoxycarbonylpyridine (5e)——Oil, 57% yield based on ethyl 5-aminonicotinate prepared from 5-aminonicotinic acid.[22] MS *m/z*: 192 (M^+). IR (neat): 2120 (N_3), 1725 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.44 and 4.44 (3H, t, and 2H, q, CO_2Et), 7.94 (1H, dd, J=3 and 2 Hz, 4-H), 8.52 (1H, d, J=3 Hz, 2-H), 9.00 (1H, d, J=2 Hz, 6-H). *Anal.* Calcd for $C_8H_8N_4O_2$: C, 49.99; H, 4.20; N, 29.16. Found: C, 49.82; H, 4.27; N, 29.02.

5-Azido-2-methoxypyridine (5f)——Oil, 88% yield from 5-amino-2-methoxypyridine.[23] MS *m/z*: 150 (M^+). IR (neat): 2130 (N_3) cm^{-1}. ^{1}H-NMR δ: 3.90 (3H, s, 2-OMe), 6.72 (1H, d, J=8 Hz, 3-H), 7.24 (1H, dd, J=8 and 3 Hz, 4-H), 7.88 (1H, d, J=3 Hz, 6-H). *Anal.* Calcd for $C_6H_6N_4O$: C, 48.00; H, 4.03; N, 37.32. Found: C, 47.98; H, 4.31; N, 37.15.

3-Azido-2-methylpyridine (5g)——Oil, 51% yield from 3-amino-2-methylpyridine.[24] MS *m/z*: 134 (M^+). IR (neat): 2120 (N_3) cm^{-1}. ^{1}H-NMR δ: 2.45 (3H, s, 2-Me), 7.16 (1H, dd, J=8 and 4 Hz, 5-H), 7.40 (1H, d, J=8 Hz, 4-H), 8.28 (1H, d, J=4 Hz, 6-H). *Anal.* Calcd for $C_6H_6N_4$: C, 53.72; H, 4.51; N, 41.77. Found: C, 53.86; H, 4.23; N, 41.53.

3-Azido-2,6-dimethylpyridine (5h)——Oil, 81% yield from 3-amino-2,6-lutidine.[25] MS *m/z*: 148 (M^+). IR (neat): 2130 (N_3) cm^{-1}. ^{1}H-NMR δ: 2.44 and 2.57 (each 3H, s, 2- and 6-Me), 7.00 (1H, d, J=8 Hz, 5-H), 7.22 (1H, d, J=8 Hz, 4-H). *Anal.* Calcd for $C_7H_8N_4$: C, 56.74; H, 5.44; N, 37.82. Found: C, 56.78; H, 5.18; N, 37.72.

3-Azido-2-methoxypyridine (5i) and 3-Azido-2-chloropyridine (5j)——A mixture of 3-azidopyridine 1-oxide[9] (9 g) and $POCl_3$ (30 ml) was heated at 80—90 °C for 10 h. After removal of excess reagent *in vacuo*, the residue was poured into ice-water and the aqueous mixture was made alkaline with Na_2CO_3, then extracted with CH_2Cl_2. The extract was washed with water, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using *n*-hexane as an eluent to give **5j**: 4.5 g, 44% yield, mp 32—33 °C, colorless needles (from CH_2Cl_2–*n*-hexane). IR (KBr): 2120 (N_3) cm^{-1}. ^{1}H-NMR δ: 7.42 (1H, dd, J=8 and 5 Hz, 5-H), 7.68 (1H, dd, J=8 and 2 Hz, 4-H), 8.32 (1H, dd, J=5 and 2 Hz, 6-H). *Anal.* Calcd for $C_5H_3ClN_4$: C, 38.86; H, 1.96; N, 36.25. Found: C, 38.61; H, 1.83; N, 36.00.

A solution of **5j** (3 g) in MeOH (20 ml) containing sodium methoxide (2 g) was refluxed for 2 h and then evaporated *in vacuo*. Ice-water was added to the residue and the aqueous mixture was extracted with CH_2Cl_2. The extract was washed with water, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using benzene as an eluent to give **5i**: 2.6 g, 85% yield, oil. MS *m/z*: 150 (M^+). IR (neat): 2110 (N_3) cm^{-1}. ^{1}H-NMR δ: 4.00 (3H, s, 2-OMe), 6.88 (1H, dd, J=7 and 5 Hz, 5-H), 7.24 (1H, dd, J=7 and 2 Hz, 4-H), 7.96 (1H, dd, J=5 and 2 Hz, 6-H). *Anal.* Calcd for $C_6H_6N_4O$: C, 48.00; H, 4.03; N, 37.32. Found: C, 47.68; H, 4.14; N, 37.15.

3-Azido-2,4-dimethylpyridine (5k)——6-Amino-2,4-lutidine (37.5 g) was treated with concentrated HNO_3 in concentrated. H_2SO_4 and worked up according to the procedure for the nitration of 2-amino-3-methylpyridine, which gives 2-hydroxy-3-methyl-5-nitropyridine,[21] to give a mixture of 3-nitro- and 5-nitro-6-hydroxy-2,4-dimethylpyridine. A solution of the mixture (22.4 g) in $POCl_3$ (100 ml) was refluxed for 7 h. After removal of excess reagent *in vacuo*, the residue was poured into ice-water and the aqueous mixture was made alkaline with Na_2CO_3, then extracted with CH_2Cl_2. The extract was washed with water, dried, and evaporated *in vacuo*. The residue was

chromatographed on silica gel. Elution with *n*-hexane gave 6-chloro-2,4-dimethyl-3-nitropyridine: 8.12 g, 33% yield, mp 54—55 °C, colorless needles (from *n*-hexane). ^{1}H-NMR δ: 2.32 (3H, s, 4-Me), 2.52 (3H, s, 2-Me), 7.00 (1H, s, 5-H). *Anal.* Calcd for $C_7H_7ClN_2O_2$: C, 45.06; H, 3.78; N, 15.01. Found: C, 44.89; H, 3.79; N, 14.88. In addition, further elution with benzene gave the 5-nitro isomer: 4.4 g, 18% yield, mp 47—48 °C.

A solution of the 3-nitropyridine (4.37 g) thus obtained in MeOH (40 ml) containing concentrated ammonia (10 ml) was hydrogenated over 5% Pd–C (400 mg) at 3 atm of hydrogen. After removal of the catalyst by filtration, the filtrate was concentrated *in vacuo* and the residue was diluted with 5% Na_2CO_3 (10 ml). The alkaline mixture was extracted with CH_2Cl_2 and the extract was evaporated *in vacuo*. The residue was chromatographed on alumina using benzene–CH_2Cl_2 as an eluent to give 3-amino-2,4-dimethylpyridine: 2.64 g, 92% yield, oil. MS *m/z*: 122 (M^+). IR (neat): 3350 (NH) cm^{-1}. ^{1}H-NMR δ: 2.10 (3H, s, 4-Me), 2.40 (3H, s, 2-Me), 3.80 (2H, br, NH_2), 6.78 (1H, d, J=6 Hz, 5-H), 7.80 (1H, d, J=6 Hz, 6-H). **5k** was obtained from this compound by the general procedure: 68% yield, oil. MS *m/z*: 148 (M^+). IR (neat): 2130 (N_3) cm^{-1}: ^{1}H-NMR δ: 2.40 (3H, s, 4-Me), 2.62 (3H, s, 2-Me), 7.06 (1H, d, J=5 Hz, 5-H), 8.32 (1H, d, J=5 Hz, 6-H). *Anal.* Calcd for $C_7H_8N_4$: C, 56.74; H, 5.44; N, 37.82. Found: C, 56.54; H, 5.43; N, 37.68.

3-Azido-2,4,6-trimethylpyridine (5l)——Oil, 92% yield from 3-amino-2,4,6-trimethylpyridine.[25] MS *m/z*: 162 (M^+). IR (neat): 2120 (N_3) cm^{-1}. ^{1}H-NMR δ: 2.44 (3H, s, 4-Me), 2.60 and 2.70 (each 3H, s, 2- and 6-Me), 7.20 (1H, s, 5-H). *Anal.* Calcd for $C_8H_{10}N_4$: C, 59.24; H, 6.21; N, 34.55. Found: C, 59.43; H, 6.28; N, 34.27.

Photolysis of the Azidopyridines (5a—l)——General Procedure: A solution of **5** (0.5—1.0 g) in MeOH–dioxane (1 : 1, 150 ml) containing sodium methoxide (2.5—3 g) was irradiated (400 W, high-pressure Hg lamp, Pyrex filter, under N_2). This photolysis was followed in terms of the disappearance of the spot of the starting azide on thin-layer chromatography, and was complete in 1—2 h. After removal of the solvent *in vacuo*, ice-water (10—20 ml) was added to the residue and the aqueous mixture was extracted with *n*-hexane. The extract was washed with water, dried, and evaporated *in vacuo* to give a diazepine (**8**, **13**, or **18**) as an oil in a nearly pure state. The product was further purified by chromatography on Sephadex using benzene as an eluent to give an analytical sample.

4-Methoxy-5*H*-1,3-diazepines (8a—e)——^{1}H-NMR spectral data for the diazepines (**8**) are collected in Table I.

8a: *ca.* 40% yield. IR (neat): 1620 (C=N) cm^{-1}. High-resolution MS *m/z*: M^+ Calcd for $C_6H_8N_2O$: 124.0637. Found: 124.0635. ^{13}C-NMR δ: 33.29 (t, 5-C), 55.65 (q, OMe), 109.36 (d, 6-C), 138.95 (d, 7-C), 151.89 (d, 2-C), 159.01 (s, 4-C).

8b: 56% yield. IR (neat): 1610 (C=N) cm^{-1}. High-resolution MS *m/z*: M^+ Calcd for $C_7H_{10}N_2O$: 138.0793. Found: 138.0789. ^{13}C-NMR δ: 16.65 (q, 5-Me), 55.18 (q, OMe), 59.42 (d, 5-C), 114.36 (d, 6-C), 144.54 (d, 7-C), 159.97 (d, 2-C).

8c: 42% yield. IR (neat): 1620 (C=N) cm^{-1}: High-resolution MS *m/z*: M^+ Calcd for $C_7H_{10}N_2O$: 138.0793. Found: 138.0786. ^{13}C-NMR δ: 21.06 (q, 6-Me), 38.47 (t, 5-C), 55.53 (q, OMe), 119.59 (s, 6-C), 134.01 (d, 7-C), 150.18 (d, 2-C), 158.06 (s, 4-C).

8d: 43% yield. IR (neat): 1615 (C=N) cm^{-1}. High-resolution MS *m/z*: M^+ Calcd for $C_7H_{10}N_2O$: 138.0793. Found: 138.0792. ^{13}C-NMR δ: 21.62 (q, 7-Me), 32.33 (t, 5-C), 54.92 (q, OMe), 105.15 (d, 6-C), 150.39 (d, 2-C), 160.57 (s, 4-C), 159.05 (s, 4-C).

8e: *ca.* 10% yield. IR (neat): 1710 (C=O) cm^{-1}. High-resolution MS *m/z*: M^+ Calcd for $C_8H_{10}N_2O_3$: 182.0692. Found: 182.0695. ^{13}C-NMR δ: 32.65 (t, 5-C), 52.30 and 56.18 (each q, OMe), 112.89 (s, 6-C), 145.48 (d, 7-C), 154.84 (d, 2-C), 161.01 and 165.89 (each s, 4-C and C=O).

2,4,7-Trimethoxy-5,6-dihydro-2*H*-1,3-diazepine (9f)——57% Yield, mp 84—85 °C (from *n*-hexane). MS *m/z*: 186 (M^+). ^{1}H-NMR δ: 2.24—2.90 (4H, m, 5- and 6-H_2), 3.45 (3H, s, 2-OMe), 3.66 (6H, s, 4- and 7-OMe), 5.85 (1H, s, 2-H). ^{13}C-NMR δ: 26.12 (t, 5- and 6-C), 52.42 (q, 2-OMe), 52.89 (q, 4- and 7-OMe), 94.18 (d, 2-C), 163.13 (s, 4- and 7-C). *Anal.* Calcd for $C_8H_{14}N_2O_3$: C, 51.60; H, 7.58; N, 15.04. Found: C, 51.73; H, 7.77; N, 15.32.

3-Methoxy-2*H*-1,4-diazepines (13g—j)——^{1}H-NMR spectral data for the diazepines are collected in Table I.

13g: 45% yield. IR (neat): 1615 (C=N) cm^{-1}. High-resolution MS *m/z*: M^+ Calcd for $C_7H_{10}N_2O$: 138.0793. Found: 138.0791. ^{13}C-NMR δ: 16.62 (q, 2-Me), 55.15 (q, OMe), 59.39 (d, 2-C), 114.33 (d, 6-C), 144.51 (d, 5-C), 153.98 (s, 3-C), 159.51 (d, 7-C).

13h: 15% yield. IR (neat): 1615 (C=N) cm^{-1}. High-resolution MS *m/z*: M^+ Calcd for $C_8H_{12}N_2O$: 152.0950. Found: 152.0958. ^{13}C-NMR δ: 16.71 (q, 2-Me), 24.29 (q, 7-Me), 54.94 (q, OMe), 58.06 (d, 2-C), 116.30 (d, 6-C), 143.66 (d, 5-C), 149.78 (s, 3-C), 157.36 (s, 7-C).

13i (=**13j**): 8—10% yield from **8i**, *ca.* 15% yield from **8j**. IR (neat): 1615 (C=N) cm^{-1}. High-resolution MS *m/z*: M^+ Calcd for $C_7H_{10}N_2O_2$: 154.0742. Found: 154.0745. ^{13}C-NMR δ: 55.77 (q, 2- and 3-OMe), 91.42 (d, 2-C), 114.42 (d, 6-C), 144.77 (d, 5-C), 151.19 (s, 3-C), 156.25 (d, 7-C).

4-Methoxy-2-methyl-5*H*-1,3-diazepines (18k, l)——^{1}H-NMR spectral data for the diazepines are collected in Chart 1.

18k: 25% yield. IR (neat): 1620 (C=N) cm^{-1}. High-resolution MS *m/z*: M^+ Calcd for $C_8H_{12}N_2O$: 152.0950. Found: 152.0946.

18l: 18% yield. IR (neat): 1615 (C=N) cm^{-1}. High-resolution MS *m/z*: M^+ Calcd for $C_9H_{14}N_2O$: 166.1106. Found: 166.1117.

Treatment of 8a—d with Benzoyl Chloride——General Procedure: Benzoyl chloride (1.05 mol eq) was added to a solution of **8** (100—200 mg) in pyridine (1—2 ml) with stirring in an ice bath. The reaction mixture was allowed to stand in a refrigerator for 20—30 h, and then diluted with ice-water (*ca.* 10 ml) and the aqueous mixture was extracted with CH_2Cl_2. The extract was washed with water, dried, and evaporated *in vacuo*. The residue was chromatographed on alumina. Elution with benzene gave methyl 4-benzoylamino-3-butenoates (**28**) and further elution with benzene–CH_2Cl_2 (1 : 1) afforded 5-benzoylamino-2-methoxypyridines (**25**). The diazepines **8a** and **8b** gave both products, but, **8c** gave only **25c** and **8d** afforded only **28d**.

25a: 35% yield, mp 139—140 °C, colorless needles (from isopropyl ether). This compound was identical with an authentic sample.[3)]

25b: 25% yield, mp 137—138 °C, colorless needles (from isopropyl ether). MS *m/z*: 242 (M^+). IR (KBr): 3220 (NH), 1640 (C=O) cm^{-1}. ^{1}H-NMR δ: 2.12 (3H, s, 3-Me), 3.94 (3H, s, 2-OMe), 7.3—7.6 (3H, m, Ph-H), 7.75—7.95 (3H, m, 4-H and Ph-H), 8.14 (1H, d, J=2 Hz, 6-H), 8.56 (1H, br, NH). *Anal.* Calcd for $C_{14}H_{14}N_2O_2$: C, 69.41; H, 5.82; N, 11.56. Found: C, 69.17; H, 5.71; N, 11.52.

25c: 39% yield, mp 183—184 °C, colorless needles (from isopropyl ether). MS *m/z*: 242 (M^+). IR (KBr): 3260 (NH), 1640 (C=O) cm^{-1}. ^{1}H-NMR δ: 2.20 (3H, s, 4-Me), 3.84 (3H, s, 2-OMe), 6.62 (1H, s, 3-H), 7.3—7.6 and 7.85—8.0 (3H, m, and 2H, m, Ph-H), 8.40 (1H, s, 6-H). *Anal.* Calcd for $C_{14}H_{14}N_2O_2$: C, 69.41; H, 5.82; N, 11.56. Found: C, 69.54; H, 5.73; N, 11.58.

28a: 6% yield, oil. IR (neat): 3310 (NH), 1735 (C=O), 1650 (C=O) cm^{-1}. ^{1}H-NMR δ: 3.14 (2H, d, J=8 Hz, 2-H_2), 3.76 (3H, s, OMe), 4.96 (1H, dt, J=8 and 8 Hz, 3-H), 7.16 (1H, dd, J=8 and 8 Hz, 4-H), 7.4—7.6 and 7.9—8.1 (3H, m, and 2H, m, Ph-H), 9.30 (1H, br d, J=8 Hz, NH). ^{13}C-NMR δ: 32.06 (t, 2-C), 52.53 (q, OMe), 105.59 (d, 3-C), 132.25 (d, 4-C), Ph-C [126.65 (d), 127.54 (d), 128.89 (d), 133.65 (s)], 164.83 and 173.12 (each s, C=O). High-resolution MS *m/z*: M^+ Calcd for $C_{12}H_{13}NO_3$: 219.0896. Found: 219.0892.

28b: 12% yield, oil. IR (neat): 3320 (NH), 1730 (C=O), 1650 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.36 (3H, d, J=7 Hz, 2-Me), 3.42 (1H, qd, J=7 and 8 Hz, 2-H), 3.80 (3H, s, OMe), 4.80 (1H, dd, J=8 and 8 Hz, 3-H), 7.10 (1H, dd, J=8 and 8 Hz, 4-H), 7.4—7.7 and 7.9—8.1 (3H, m, and 2H, m, Ph-H), 9.23 (1H, br d, J=8 Hz, NH). High-resolution MS *m/z*: M^+ Calcd for $C_{13}H_{15}NO_3$: 233.1052. Found: 233.1051.

28d: 30% yield, oil. IR (neat): 3300 (NH), 1730 (C=O), 1640 (C=O) cm^{-1}. ^{1}H-NMR δ: 2.20 (3H, s, 4-Me), 3.04 (2H, d, J=8 Hz, 2-H_2), 3.00 (3H, s, OMe), 3.10 (1H, t, J=8 Hz, 3-H), 7.4—7.6 and 7.8—8.0 (3H, m, and 2H, m, Ph-H), 8.60 (1H, br, NH). ^{13}C-NMR δ: 21.12 (q, 4-Me), 32.65 (t, 2-C), 52.30 (q, OMe), 109.07 (d, 3-C), 127.48 (d), 128.83 (d), 131.95 (d), 134.60 (s), 137.36 (s), 165.72 (s), 173.43 (s). High-resolution MS *m/z*: M^+ Calcd for $C_{13}H_{15}NO_3$: 233.1052. Found: 233.1047.

Treatment of 13g with Benzoyl Chloride——The 1,4-diazepine (**13g**: 200 mg) was treated with benzoyl chloride (210 mg) and worked up as described for **8a—d** to give methyl 2-benzoylaminopropanoate (**30**): 45 mg, 15% yield, oil. IR (neat): 3300 (NH), 1730 (C=O), 1630 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.40 (3H, d, J=6 Hz, 2-Me), 3.52 (3H, s, OMe), 4.46 (1H, qd, J=6 and 6 Hz, 2-H), 6.36 (1H, br, NH), 6.8—7.1 and 7.2—7.4 (3H, m, and 2H, m, Ph-H). ^{13}C-NMR δ: 18.59 (q, 2-Me), 48.59 (d, 2-C), 52.59 (q, OMe), Ph-C [127.24 (d), 128.77 (d), 131.89 (d), 134.13 (s)], 167.07 (C=O), 173.95 (C=O). High-resoluton MS *m/z*: M^+ Calcd for $C_{11}H_{13}NO_3$: 207.0896. Found: 207.0903.

Photolysis of 13g——A solution of **13g** (250 mg) in benzene (150 ml) was irradiated (400 W, high-pressure Hg lamp; Pyrex filter) for 2 h and then evaporated *in vacuo*. The residue was extracted with *n*-hexane and the extract was evaporated *in vacuo* to give 5-methoxy-4-methyl-3,6-diazabicyclo[3.2.0]hepta-2,6-diene (**31**) in *ca.* 200 mg, 80% yield. However, the product was too unstable to be isolated, so its structure was confirmed only by ^{1}H-NMR spectral analysis: δ 1.25 (3H, d, J=8 Hz, 4-Me), 3.40 (3H, s, 5-OMe), 3.96 (1H, q, J=8 Hz, 4-H), 4.25 (1H, d, J=2 Hz, 1-H), 7.50 (1H, d, J=2 Hz, 7-H), 8.35 (1H, s, 2-H).

Hydrolysis of 13g, h——A solution of **13** (150 mg) in 10% HCl (3 ml) was stirred for 10 h at room temperature, then made alkaline with K_2CO_3 and the alkaline mixture was evaporated *in vacuo*. The residue was extracted with benzene and the extract was evaporated to dryness *in vacuo*. The resulting solid residue was recrystallized from CH_2Cl_2–isopropyl ether to give 2-methyl-3,4-dihydro-2*H*-1,4-diazepin-3-ones (**32**).

32g: 66 mg, 45% yield, mp 128—129 °C, colorless needles. IR (KBr): 3100 (NH), 1670 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.64 (3H, d, J=7 Hz, 2-Me), 3.44 (1H, qd, J=7 and 2 Hz, 2-H), 5.72 (1H, dd, J=8 and 2 Hz, 6-H), 5.26 (1H, d, J=8 Hz, 5-H), 7.70 (1H, m, 7-H), 9.50 (1H, br, NH). ^{13}C-NMR δ: 16.88 (q, 2-Me), 61.24 (d, 2-C), 111.42 (d, 6-C), 132.65 (d, 5-C), 160.30 (d, 7-C), 168.19 (s, 3-C). *Anal.* Calcd for $C_6H_8N_2O$: C, 58.05; H, 6.50; N, 22.57. Found: C, 57.83; H, 6.44; N, 22.76.

32h: 45 mg, 33% yield, mp 157—158 °C, colorless needles. IR (KBr): 3050 (NH), 1680 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.62 (3H, d, J=6 Hz, 2-Me), 2.16 (3H, s, 7-Me), 3.56 (1H, q, J=6 Hz, 2-H), 5.92 (1H, d, J=8 Hz, 6-H), 6.40 (1H, d, J=8 Hz, 5-H), 8.9 (1H, br, NH). *Anal.* Calcd for $C_7H_{10}N_2O$: C, 60.85; H, 7.29; N, 20.27. Found: C, 60.67; H, 7.34; N, 19.98.

References and Notes

1) Part XXVII: H. Sashida, A. Fujii, and T. Tsuchiya, *Chem. Pharm. Bull.*, **35**, 3182 (1987).

2) R. Purvis, R. K. Smalley, H. Suschitzky, and M. A. Alkhader, *J. Chem. Soc., Perkin Trans. 1*, **1984**, 248; Y. Ohba, S. Kudo, T. Nishiwaki, and N. Aratani, *Heterocycles*, **22**, 457 (1984) and references cited therein.
3) H. Sawanishi, K. Tajima, M. Osada, and T. Tsuchiya, *Chem. Pharm. Bull.*, **32**, 4694 (1984); H. Sawanishi, K. Tajima, and T. Tsuchiya, *ibid.*, **35**, 3175 (1987).
4) Y. Ohba, I. Matsukura, Y. Fukazawa, and T. Nishiwaki, *Heterocycles*, **23**, 287 (1985).
5) F. Hollywood, Z. U. Khan, E. F. V. Scriven, R. K. Smalley, H. Suschitzky, D. R. Thomas, and R. Hull, *J. Chem. Soc., Perkin Trans. 1*, **1982**, 431.
6) H. Sawanishi, H. Sashida, and T. Tsuchiya, *Chem. Pharm. Bull.*, **33**, 4564 (1985).
7) A part of this work has been published in a preliminary communication: H. Sawanishi and T. Tsuchiya, *Chem. Pharm. Bull.*, **33**, 5603 (1985).
8) P. A. Plattner, W. Keller, and A. Boller, *Helv. Chem. Acta*, **162**, 1379 (1954).
9) T. Itai and S. Kamiya, *Chem. Pharm. Bull.*, **11**, 1059 (1963).
10) This compound was obtained from Aldrich Chemical Co.
11) F. R. Atharton and R. W. Lambert, *J. Chem. Soc., Perkin Trans. 1*, **1973**, 1079; T. Boer, J. I. G. Cadogan, H. M. McWilliam, and A. G. Rowley, *J. Chem. Soc., Perkin Trans. 2*, **1975**, 554.
12) For a review, see T. Mukai, T. Kumagai, and Y. Yamashita, *Heterocycles*, **15**, 1569 (1981).
13) T. Tsuchiya, J. Kurita, and H. Kojima, *J. Chem. Soc., Chem. Commun.*, **1980**, 444; J. Kurita, H. Kojima, and T. Tsuchiya, *Chem. Pharm. Bull.*, **29**, 3688 (1981); J. Kurita, H. Kojima, M. Enkaku, and T. Tsuchiya, *ibid.*, **29**, 3696 (1981).
14) J. Kurita, K. Iwata, and T. Tsuchiya, *Chem. Pharm. Bull.*, **35**, 3166 (1987).
15) R. W. Begland, D. R. Hartter, F. N. Jones, D. J. Sam, W. A. Sheppard, O. W. Webster, and F. J. Weigert, *J. Org. Chem.*, **29**, 2341 (1974); Y. Ohtsuka, *ibid.*, **41**, 629 (1976); G. Reisenweber and J. Sauer, *Tetrahedron Lett.*, **1977**, 4389.
16) D. J. Harris, G. Kan, V. Snieckus, and O. Buchardt, *Synthesis*, **1975**, 603.
17) J. Kurita, M. Enkaku, and T. Tsuchiya, *Chem. Pharm. Bull.*, **30**, 3764 (1982).
18) For reviews, see T. Tsuchiya, *Yuki Gosei Kagaku Kyokai Shi*, **39**, 99 (1981); *idem*, *ibid.*, **41**, 641 (1983).
19) T. Okamoto and S. Hayashi, *Yakugaku Zasshi*, **86**, 766 (1966).
20) E. Köenig and A. Fulde, *Chem. Ber.*, **60**, 2109 (1927).
21) G. F. Hawkins and A. Roe, *J. Org. Chem.*, **14**, 328 (1949).
22) G. B. Bachman and D. D. Micucci, *J. Am. Chem. Soc.*, **70**, 2381 (1948).
23) O. Magidson and G. Mensohikoff, *Chem. Ber.*, **58**, 117 (1925).
24) E. D. Parker and W. Shive, *J. Am. Chem. Soc.*, **69**, 63 (1947).
25) E. Plazek, *Chem. Ber.*, **72**, 577 (1939).

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Studies on Diazepines. XXIX.[1] Syntheses of 3*H*- and 5*H*-1,4-Benzodiazepines from 3-Azidoquinolines

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Irradiation of the 3-azidoquinolines (**6a—c**) in the presence of sodium methoxide resulted in ring expansion to give the fully unsaturated 3-methoxy-3*H*-1,4-benzodiazepines (**8**). Further treatment of the 2-unsubstituted 3*H*-1,4-benzodiazepine (**8a**) with sodium methoxide in methanol gave the solvent adduct **7**, which reverted to **8a** on being refluxed in benzene, whereas the 2-substituted 3*H*-1,4-benzodiazepines (**8b, c**), upon treatment with sodium methoxide, underwent tautomerization to afford the 5*H*-1,4-benzodiazepines (**15**). Some reactions of the new 1,4-benzodiazepines (**8** and **15**) thus obtained were also examined.

Keywords——3-azidoquinoline; 3*H*-1,4-benzodiazepine; 5*H*-1,4-benzodiazepine; photolysis; ring expansion; tautomerization; base treatment; quinolylnitrene

The syntheses of 1,4-benzodiazepine derivatives have been most widely investigated[2] among the six benzodiazepine isomers, owing to their biological activities. However, only a few examples of fully unsaturated compounds have been reported.[3] Therefore, we were interested in finding new synthetic routes to 1,4-benzodiazepines as part of our continuing studies on the syntheses of novel diazepine ring systems.[4] We have very recently reported[5] that the photolysis of 4-azidoquinolines (**1**) in the presence of sodium methoxide resulted in the formation of the unstable 1*H*-1,4-benzodiazepines (**2**), which were tautomerized to the stable 3*H*-isomers (**3**) by further treatment with the base. The conversion of **3** into the first isolated examples of 1*H*-1,4-benzodiazepines (**4**) was also reported. These results and those of similar ring expansion reactions of 3-[1] and 4-azidopyridines[6,7] prompted us to examine such reactions of 3-azidoquinolines, and we report here the formation of 3*H*- and 5*H*-1,4-benzodiazepines.

1 2 3 4

Chart 1

The starting 3-azidoquinolines (**6a—c**) were prepared from the corresponding 3-aminoquinolines (**5a—c**) by diazotization followed by treatment with sodium azide in *ca.* 90% yields. Irradiation (400 W, high-pressure Hg lamp; Pyrex filter) of 3-azidoquinoline (**6a**: 0.5 g) in dioxane–methanol (1 : 1) containing a large excess of sodium methoxide for 30 min under ice-cooling gave 2,3-dimethoxy-2,3-dihydro-1*H*-1,4-benzodiazepine (**7**) in 40—50% yield as the sole characterizable product. Heating the dihydro compound **7** in refluxing benzene in the presence of molecular sieve (type 3A) resulted in the elimination of methanol to afford the

desired fully unsaturated 3-methoxy-3*H*-1,4-benzodiazepine (**8a**) in 75% yield. On the other hand, the 2-unsubstituted 3-azidoquinolines (**6b, c**), upon irradiation under similar conditions, gave directly the 3-methoxy-3*H*-1,4-benzodiazepines (**8b, c**) in *ca.* 40% yields with no methanol adducts.

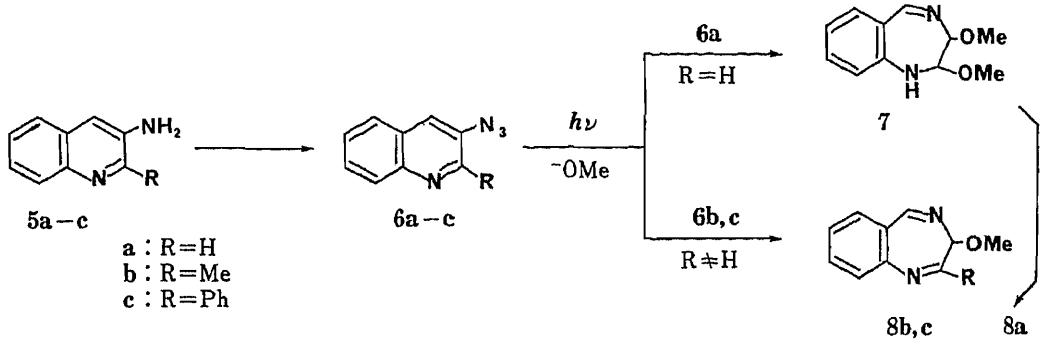

Chart 2

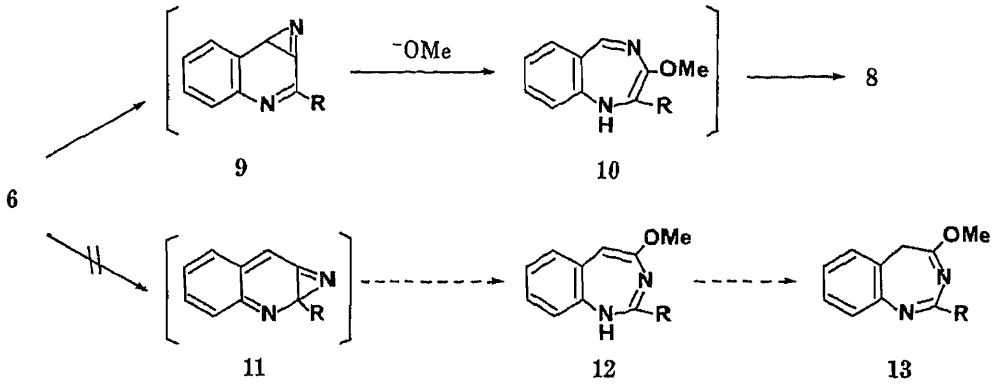

Chart 3

The formation of the 1,4-benzodiazepines from the azides (**6**) may involve the azirine intermediates **9**. The azirines (**9**) may undergo ring expansion to give the unstable anti-aromatic NH-benzodiazepines (**10**), which then tautomerize to the relatively stable CH-benzodiazepines (**8**). In all cases, the formation of the 1,3-benzodiazepines (**12** or **13**) derived from the other possible azirine intermediate **11** was not observed. This result indicates that the singlet 3-quinolylnitrenes initially formed from the azides (**6**) cyclize predominantly at the 4-position of the quinoline ring over the 2-position, even in the case of the 2-unsubstituted 3-azidoquinoline (**6a**), probably because the azirines (**9**) are more stable than **11**. This behavior is somewhat different from that of 3-pyridylnitrenes,[1] in which the intramolecular cyclization of 2-unsubstituted 3-pyridylnitrenes takes place predominantly at the 2-position of the pyridine ring to give 1,3-diazepines, whereas 2-substituted 3-pyridylnitrenes cyclize at the vacant 4-position to give 1,4-diazepines.

The structures of **8a—c** were elucidated mainly from their spectral data. In the proton nulcear magnetic resonance (^{1}H-NMR) spectra of **8a—c**, the signals due to 3-H appeared at around δ 4.0 and coupled with the signals of 5-H (δ 8.2—8.4, d, J=2 Hz); in the case of the 2-unsubstituted compound **8a**, the signal of 3-H coupled further with that of 2-H (J=2 Hz). The ^{13}C-NMR spectrum of **8a** showed a signal due to 3-C at δ 90.12, in addition to a methoxy carbon and eight sp^2 carbon signals. These spectral data and the results of the following reactions are consistent with the proposed 3*H*-1,4-benzodiazepine structure and rule out other possible structures such as 1*H*- and 5*H*-1,4-benzodiazepines and 1,3-benzodiazepines (**12** and **13**).

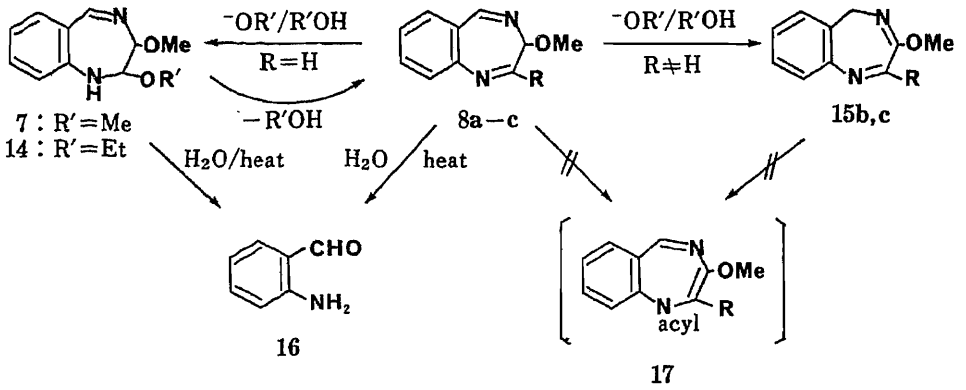

Chart 4

Treatment of the 2-unsubstituted 3*H*-1,4-benzodiazepine (**8a**) with sodium methoxide or ethoxide at room temperature gave the adduct **7** or **14** in *ca.* 55% yield as the sole product. Either the adduct **7** or **14** readily reverted to the 3*H*-diazepine (**8**) on being heated in refluxing benzene in the presence of molecular sieve. These results clearly indicate that the photolysis of the azide (**6a**) proceeds by initial formation of **8a**, which is then converted to the adduct **7** under the basic conditions. On the other hand, it should be noted that treatment of the 2-substituted 3*H*-benzodiazepines (**8b, c**) with sodium alkoxides resulted in tautomerization to afford the 5*H*-1,4-benzodiazepines (**15b, c**) in high yields with no adducts. The structures of **15** were confirmed by their spectral data. For example, in the ^{1}H-NMR spectrum of **15b**, a singlet signal assignable to 5-H_2 appeared at δ 3.96, and the ^{13}C-NMR spectrum of **15b** showed a signal due to 5-C at δ 53.30 (t).

Hydrolysis of either **7** or **8a** in refluxing water–dioxane (1 : 1) resulted in decomposition to give 2-aminobenzaldehyde (**16**) in *ca.* 50% yield, as a fragment product. Attempts to obtain the 1-acyl-1*H*-1,4-benzodiazepines (**17**) from the 3*H*- (**8**) and 5*H*-benzodiazepines (**15**) by treatment with acylating reagents such as acetyl chloride, acetic anhydride, and benzoyl chloride were unsuccessful, in contrast to the cases of 4*H*-1,2-diazepines,[8] 6*H*-1,4-

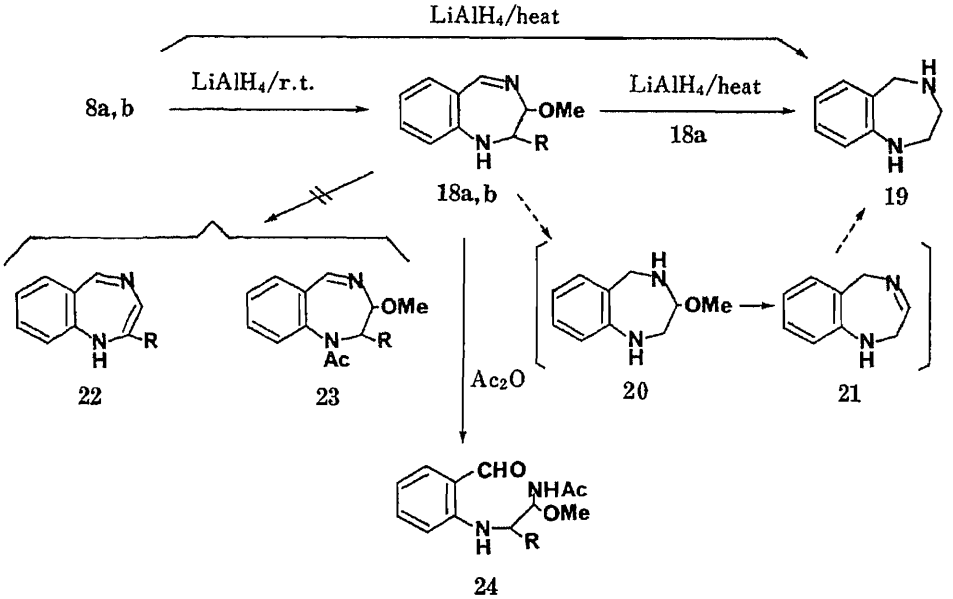

Chart 5

diazepines,[6] 5-methoxy-3*H*-1,4-benzodiazepines,[5] and 5*H*-2,3-benzodiazepines,[9] all of which are known to undergo acylation with tautomerization to give the corresponding *N*-acyldiazepines. On treatment with acylating reagents, the 3*H*-benzodiazepines (**8a—c**) were decomposed, whereas the 5*H*-isomers (**15b, c**) did not react.

Treatment of the 3*H*-benzodiazepines (**8a, b**) with $LiAlH_4$ at room temperature afforded the 1,2-dihydro compounds **18a, b** in high yields, whereas when **8a** was treated with $LiAlH_4$ in refluxing tetrahydrofuran (THF), the known fully unsubstituted tetrahydro-1,4-benzodiazepine (**19**)[10] was unexpectedly formed in 85% yield. This compound (**19**) was also obtained from **18a** by further treatment with $LiAlH_4$ in refluxing THF in 90% yield. The formation of **19** from **18a** may involve the 3-methoxy-tetrahydrodiazepine intermediate **20**, which undergoes elimination of methanol to form the 2,5-dihydro compound **21**.

Compound **21** may be reduced to give the product **19**, although the key intermediates **20** and **21** could not be isolated. However, attempts to prepare fully unsaturated 1,4-benzodiazepines (**22**) having no methoxy group from **18** by base-induced elimination of methanol were unsuccessful, resulting in decomposition. The synthesis of 1-acetyl-1*H*-1,4-benzodiazepines *via* **23** was also unsuccessful, because the acetylation of **18a, b** gave only the ring-opening products **24a, b** and none of the desired 1-acetyl compound **23**.

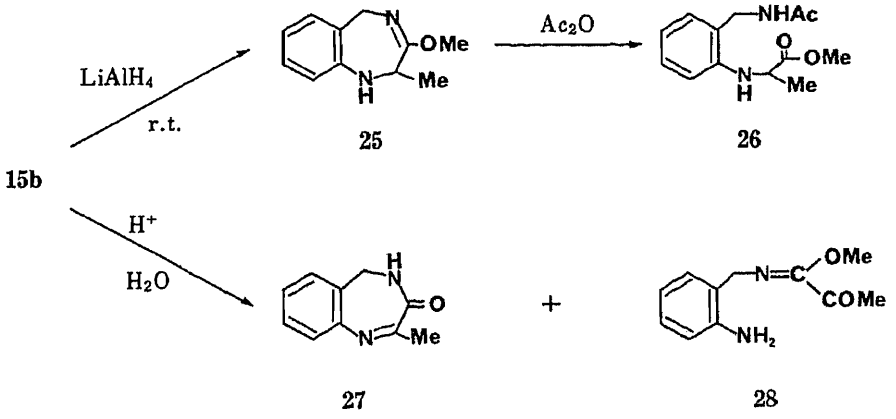

Chart 6

Finally, several reactions of the 5*H*-benzodiazepine (**15b**) were examined. $LiAlH_4$ reduction of **15b** gave the unstable 1,2-dihydro compound **25**, which was treated with acetic anhydride to afford the ring-opening product **26** in 32% yield, but no 1-acetyl derivative of **25**. Hydrolysis of **15b** in the presence of *p*-toluenesulfonic acid gave the 1,4-benzodiazepin-3-one (**27**) and the ring-opening product **28**, each in *ca.* 20% yield.

Experimental

The general experimental procedures were the same as in Part XXVIII.[1]

Starting Materials——3-Aminoquinoline (**5a**) was obtained from Tokyo Kasei Kogyo Co., Ltd. 3-Amino-2-methylquinoline (**5b**)[11] and 3-amino-2-phenylquinoline (**5c**)[12] were prepared by the reported methods.

3-Azidoquinolines (6a—c)——General Procedure: A solution of sodium nitrite (3.8 g, 1.1 mol eq) in water (30 ml) was added dropwise to a solution of **5** (50 mmol) in 10% HCl (50 ml) with stirring at 0—5 °C. Stirring was continued for an additional 1 h in an ice bath, then a solution of sodium azide (7 g, *ca.* 2 mol eq) in water (30 ml) was added dropwise to the reaction mixture with stirring and the reaction mixture was stirred for a further 1 h at room temperature. The reaction mixture was made alkaline with $NaHCO_3$ and extracted with CH_2Cl_2. The extract was washed with satd. NaCl, dried over $MgSO_4$, and evaporated *in vacuo*. The resulting solid residue was recrystallized from acetone–*n*-hexane to give **6**.

6a: 7.74 g, 91% yield, mp 79—80 °C (lit.[13] mp 81—82 °C), yellow needles.

6b: 8.65 g, 94% yield, mp 115—117 °C, yellow prisms. MS *m/z*: 184 (M^+). IR (KBr): 2100 (N_3) cm^{-1}. ^{1}H-NMR δ: 2.68 (3H, s, 2-Me), 7.84 (1H, s, 4-H), 7.4—7.9 and 8.23 (3H, m, and 1H, d, J=8 Hz, Ph-H). *Anal.* Calcd for $C_{10}H_8N_4$: C, 65.20; H, 4.38; N, 30.42. Found: C, 65.22; H, 4.53; N, 30.55.

6c: 10.45 g, 85% yield, mp 88—89 °C, pale yellow prisms. MS *m/z*: 246 (M^+). IR (KBr): 2130 (N_3) cm^{-1}. ^{1}H-NMR δ: 7.64 (1H, s, 4-H), 7.3—8.2 (9H, m, Ph-H). *Anal.* Calcd for $C_{15}H_{10}N_4$: C, 73.17; H, 4.07; N, 22.76. Found: C, 73.43; H, 3.98; N, 22.49.

2,3-Dimethoxy-2,3-dihydro-1*H*-1,4-benzodiazepine (7)——A solution of **6a** (500 mg) and sodium methoxide (4.0 g) in methanol–dioxane (1 : 1, 140 ml) was irradiated (400 W, high-pressure Hg lamp; Pyrex filter) for 30 min, then evaporated *in vacuo*. The residue was diluted with ice-water (*ca.* 20 ml) and the aqueous mixture was extracted with CH_2Cl_2. The extract was washed with water, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–acetone (20 : 1) as an eluent to give **7**: 250 mg, 41% yield, mp 145—147 °C, pale yellow prisms (from CH_2Cl_2–*n*-hexane). MS *m/z*: 206 (M^+). IR (KBr): 3230 (NH), 1630 (C=N) cm^{-1}. ^{1}H-NMR δ: 3.35 (3H, s, 2-OMe), 3.61 (3H, s, 3-OMe), 4.50 (1H, d, J=2 Hz, 3-H), 4.95 (1H, d, J=6 Hz, 2-H), 5.95 (1H, br d, J=6 Hz, NH), 6.7—6.9 and 7.2—7.4 (each 2H, m, Ph-H), 8.25 (1H, d, J=2 Hz, 5-H). ^{13}C-NMR δ: 54.47 (q, OMe), 55.71 (q, OMe), 86.72 (d, 2-C), 94.24 (d, 3-C), 158.06 (d, 5-C), Ph-C [117.54 (d), 118.18 (d), 119.66 (s), 132.36 (d), 134.66 (d), 143.89 (s)]. *Anal.* Calcd for $C_{11}H_{14}N_2O_2$: C, 64.08; H, 6.80; N, 13.59. Found: C, 63.86; H, 6.71; N, 13.52.

3-Methoxy-3*H*-1,4-benzodiazepine (8a)——A mixture of **7** (200 mg), molecular sieve (3A type, 1/16 inch pellets: 1 g), and benzene (40 ml) was refluxed for 6 h. After removal of the molecular sieve by filtration, the filtrate was evaporated to dryness *in vacuo*. The solid residue was recrystallized from acetone–*n*-hexane to give **8a**: 130 mg, 75% yield, mp 46—49 °C, pale yellow prisms. MS *m/z*: 174 (M^+). IR (KBr): 1610 and 1640 (C=N) cm^{-1}. ^{1}H-NMR δ: 3.61 (3H, s, 3-OMe), 3.96 (1H, dd, J=2, 2 Hz, 3-H), 7.2—7.6 (5H, m, 2-H and Ph-H), 8.35 (1H, d, J=2 Hz, 5-H). ^{13}C-NMR δ: 55.36 (q, 3-OMe), 90.12 (d, 3-C), 157.54 and 157.77 (each d, 2- and 5-C), Ph-C [125.66 (d), 127.59 (s), 128.01 (d), 129.71 (d), 131.19 (d), 147.89 (s)]. *Anal.* Calcd for $C_{10}H_{10}N_2O$: C, 68.95; H, 5.79; N, 16.08. Found: C, 69.03; H, 5.78; N, 15.88.

3-Methoxy-3*H*-1,4-benzodiazepines (8b, c)——The azides (**6b**, **c**: 500 mg) were irradiated and worked up as described for the photolysis of **6a** to give **8b**, **c**.

8b: 194 mg, 38% yield, mp 78—79 °C, pale yellow prisms (from acetone–*n*-hexane). MS *m/z*: 188 (M^+). IR (KBr): 1610 and 1640 (C=N) cm^{-1}. ^{1}H-NMR δ: 2.30 (3H, s, 2-Me), 3.46 (3H, s, 3-OMe), 3.77 (1H, d, J=2 Hz, 3-H), 7.0—7.4 (4H, m, Ph-H), 8.20 (1H, d, J=2 Hz, 5-H). *Anal.* Calcd for $C_{11}H_{12}N_2O$: C, 70.18; H, 6.43; N, 14.88. Found: C, 69.97; H, 6.46; N, 15.10.

8c: 183 mg, 36% yield, mp 90—92 °C, pale yellow prisms (from acetone–*n*-hexane). MS *m/z*: 250 (M^+). IR (KBr): 1615 (C=N) cm^{-1}. ^{1}H-NMR δ: 3.45 (3H, s, 3-OMe), 4.13 (1H, d, J=2 Hz, 3-H), 7.1—7.6 and 8.0—8.2 (7H, m, and 2H, m, Ph-H), 8.42 (1H, d, J=2 Hz, 5-H). *Anal.* Calcd for $C_{16}H_{14}N_2O$: C, 76.78; H, 5.64; N, 11.19. Found: C, 76.57; H, 5.81; N, 10.98.

Treatment of 8a with Sodium Methoxide in Methanol——A solution of **8a** (100 mg) and NaOMe (1 g) in MeOH (10 ml) was stirred for 12 h at room temperature and then evaporated *in vacuo*. The residue was extracted with CH_2Cl_2 and the extract was washed with water, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–acetone (20 : 1) as an eluent to give **7**: 68 mg, 57% yield.

Treatment of 8a with Sodium Ethoxide in Ethanol——A solution of **8a** (100 mg) and NaOEt (500 mg) in EtOH (10 ml) was worked up as described for the treatment with NaOMe to give 2-ethoxy-3-methoxy-2,3-dihydro-1*H*-1,4-benzodiazepine (**14**): 57 mg, 55% yield, mp 125—128 °C, pale yellow prisms (from acetone–*n*-hexane). MS *m/z*: 220 (M^+). IR (KBr): 3400 (NH), 1635 (C=N) cm^{-1}. ^{1}H-NMR δ: 1.11 and 3.55 (3H, t, and 2H, q, J=7 Hz, 2-OEt), 3.64 (3H, s, 3-OMe), 4.57 (1H, d, J=2 Hz, 3-H), 5.10 (1H, d, J=6 Hz, 2-H), 5.85 (1H, d, J=6 Hz, NH), 6.8—7.0 and 7.3—7.5 (each 2H, m, Ph-H), 8.35 (1H, d, J=2 Hz, 5-H). *Anal.* Calcd for $C_{12}H_{16}N_2O_2$: C, 65.43; H, 7.32; N, 12.72. Found: C, 65.63; H, 7.45; N, 12.55.

Treatment of 8b, c with Sodium Methoxide in Methanol: Formation of 3-Methoxy-5*H*-1,4-benzodiazepines (15b, c)——A solution of **8** (0.5 mmol) and NaOMe (1 g) in MeOH (20 ml) was refluxed for 8 h and then evaporated *in vacuo*. The residue was extracted with CH_2Cl_2, and the extract was washed with water, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–acetone (100 : 1) as an eluent to give **15**.

15b: 87 mg, 93% yield, mp 42—43 °C, pale yellow prisms (from *n*-hexane). MS *m/z*: 188 (M^+). IR (KBr): 1660 (C=N) cm^{-1}. ^{1}H-NMR δ: 2.38 (3H, s, 2-Me), 3.53 (3H, s, 3-OMe), 3.96 (2H, s, 5-H_2), 6.8—7.2 (4H, m, Ph-H). ^{13}C-NMR δ: 25.24 (q, 2-Me), 49.06 (q, 3-OMe), 53.30 (t, 5-C), 157.01 (s, 2-C), 161.20 (s, 3-C), Ph-C [126.07 (d), 126.95 (d), 127.66 (d), 128.48 (d), 132.07 (s), 147.24 (s)]. *Anal.* Calcd for $C_{11}H_{12}N_2O$: C, 70.18; H, 6.43; N, 14.88. Found: C, 70.01; H, 6.37; N, 14.62.

15c: 104 mg, 8.3% yield, mp 128—129 °C, pale yellow prisms (from *n*-hexane). MS *m/z*: 250 (M^+). IR (KBr): 1650 (C=N) cm^{-1}. ^{1}H-NMR δ: 3.63 (3H, s, 3-OMe), 4.0—4.2 (2H, br, 5-H_2), 6.9—7.4 and 7.6—7.8 (7H, m, and 2H, m, Ph-H). *Anal.* Calcd for $C_{16}H_{14}N_2O$: C, 76.78; H, 5.64; N, 11.19. Found: C, 76.87; H, 5.81; N, 10.99.

Hydrolysis of 8a——A solution of **8a** (550 mg) in dioxane–water (1 : 1, 20 ml) was refluxed for 20 h. After cooling, the solution was extracted with CH_2Cl_2, and the extract was dried and evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2 as an eluent to give 2-aminobenzaldehyde (**16**): 207 mg, 54% yield.

Hydrolysis of 7——A solution of **7** (200 mg) in dioxane–water (1 : 1, 10 ml) was refluxed for 15 h and worked up as described for the hydrolysis of **8a** to give **16**: 61 mg, 52% yield.

3-Methoxy-2,3-dihydro-1*H*-1,4-benzodiazepines (18a, b)——General Procedure: $LiAlH_4$ (50 mg) was added in small portions to a solution of **8** (0.5 mmol) in THF (15 ml) with stirring in an ice bath. The reaction mixture was stirred for a further 1 h at room temperature. After addition of saturated Na_2SO_4 (0.5 ml) to decompose excess reagent, the reaction mixture was dried over $MgSO_4$ and filtered. The filtrate was evaporated *in vacuo* to give **18** as a viscous oil in a nearly pure state. However, the products **18** were unstable and gradually decomposed during isolation by chromatography, so the resulting residue was used in the following reaction without further purification.

18a: 80 mg, 90% yield. ^{1}H-NMR δ: 3.63 (3H, s, 3-OMe), 3.6—4.0 (2H, m, 2-H_2), 4.4—4.6 (1H, m, 3-H), 5.6 (1H, br d, J=7 Hz, NH), 6.6—6.8 and 7.0—7.3 (each 2H, m, Ph-H), 8.12 (1H, d, J=2 Hz, 5-H).

18b: 93 mg, 98% yield. ^{1}H-NMR δ: 0.96 (3H, d, J=7 Hz, 2-Me), 3.55 (3H, s, 3-OMe), 3.6—4.0 (1H, m, 2-H), 4.4—4.5 (1H, m, 3-H), 5.3 (1H, br d, J=7 Hz, NH), 6.5—6.8 and 6.9—7.3 (each 2H, m, Ph-H), 8.12 (1H, d, J=2 Hz, 5-H).

2,3,4,5-Tetrahydro-1*H*-1,4-benzodiazepine (19)——i) From **8a**: $LiAlH_4$ (100 mg) was added in small portions to a solution of **8a** (140 mg) in THF (20 ml) with stirring at room temperature and then the reaction mixture was refluxed for 1 h. After cooling of the mixture excess reagent was decomposed with saturated Na_2SO_4, and the mixture was dried over $MgSO_4$ and filtered. The filtrate was evaporated *in vacuo* and the residue was chromatographed on alumina using CH_2Cl_2–MeOH (100 : 1) as an eluent to give **19** (100 mg, 85% yield) as a colorless oil. This product **19** was identical with an authentic sample prepared by the reported method.[10]

ii) From **18a**: Compound **18a** (50 mg) was treated with $LiAlH_4$ (50 mg) and worked up as described for i) to give **19** (46 mg, 90% yield).

Treatment of 18a, b with Acetic Anhydride——A mixture of **18** (0.4 mmol), Ac_2O (2 ml), and pyridine (4 ml) was stirred for 12 h at room temperature and then poured into ice-water. The aqueous mixture was made alkaline with $NaHCO_3$ and extracted with CH_2Cl_2. The extract was chromatographed on silica gel using CH_2Cl_2–acetone (20 : 1) as an eluent to give *o*-(2-acetylamino-2-methoxyethylamino)-benzaldehydes (**24**).

24a: 39 mg, 41% yield, pale yellow oil. MS *m/z*: 236 (M^+). IR (neat): 3420 and 3300 (NH), 1680 and 1660 (C=O) cm^{-1}. ^{1}H-NMR δ: 2.10 (3H, s, NAc), 3.48 (3H, s, OMe), 3.4—3.6 (2H, m, 1-H_2), 5.46 (1H, dt, J=9, 6 Hz, 2-H), 7.23 (1H, br d, J=9 Hz, 2-NHAc), 6.7—7.0 and 7.4—7.6 (each 2H, m, Ph-H), 8.64 (1H, br t, J=6 Hz, Ph-NH–), 9.67 (1H, s, CHO). *Anal.* Calcd for $C_{12}H_{16}N_2O_3$: C, 61.00; H, 6.83; N, 11.86. Found: C, 59.83; H, 6.87; N, 11.66.

24b: 26 mg, 26% yield, mp 127—129 °C, pale yellow prisms (from ether–*n*-hexane). MS *m/z*: 250 (M^+). IR (KBr): 3430 and 3300 (NH), 1670 and 1650 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.24 (3H, d, J=6 Hz, 1-Me), 1.93 (3H, s, OMe), 3.4—3.9 (1H, m, 1-H), 5.02 (1H, dd, J=10, 2 Hz, 2-H), 5.9 (1H, br d, J=10 Hz, NHAc), 6.4—6.6 and 7.1—7.3 (each 2H, m, Ph-H), 8.1 (1H, br d, J=9 Hz, Ph-NH–), 9.48 (1H, s, CHO). *Anal.* Calcd for $C_{13}H_{18}N_2O_3$: C, 62.38; H, 7.25; N, 11.19. Found: C, 62.25; H, 6.97; N, 11.18.

3-Methoxy-2-methyl-2,5-dihydro-1*H*-1,4-benzodiazepine (25)——The 5*H*-diazepine (**15b**) (188 mg) was treated with $LiAlH_4$ (50 mg) and worked up as described for **18** to give **25** (*ca.* 180 mg, 95% yield) in a nearly pure state. The structure of **25** was characterized by the ^{1}H-NMR spectral data: δ 1.52 (3H, d, J=7 Hz, 2-Me), 3.70 (3H, s, OMe), 3.92 (2H, s, 5-H_2), 3.8—4.4 (2H, m, 2-H and NH), 6.4—6.7 and 7.0—7.2 (each 2H, m, Ph-H). However, **25** was unstable and readily decomposed during isolation by chromatography, so the residue was used in the following reaction without further purification.

Treatment of 25 with Acetic Anhydride——A mixture of **25** (180 mg), Ac_2O (2 ml), and pyridine (4 ml) was stirred for 10 h at room temperature and worked up as described for **18** to give methyl α-(*o*-acetylaminomethylanilino) propionate (**26**): 80 mg, 32% yield, mp 92—93 °C, colorless prisms (from acetone–*n*-hexane). MS *m/z*: 250 (M^+). IR (KBr): 3450 and 3350 (NH), 1740 and 1660 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.48 (3H, d, J=7 Hz, CH-Me), 1.96 (3H, s, NAc), 3.65 (3H, s, CO_2Me), 4.0—4.5 (3H, m, Ph-CH_2–N and CHMe), 5.15 (1H, br d, J=6 Hz, Ph-NH–), 6.0 (1H, br, NHAc), 6.3—6.7 and 6.9—7.2 (each 2H, m, Ph-H). *Anal.* Calcd for $C_{13}H_{18}N_2O$: C, 62.38; H, 7.25; N, 11.19. Found: C, 62.14; H, 7.27; N, 11.05.

Hydrolysis of 15b in the Presence of *p*-Toluenesulfonic Acid——A mixture of **15b** (100 mg), TsOH (200 mg), water (10 ml), and dioxane (10 ml) was stirred for 12 h at room temperature. The reaction mixture was made alkaline with saturated $NaHCO_3$ and extracted with CH_2Cl_2. The extract was washed with saturated NaCl, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–acetone (30 : 1) as an eluent to give **27** and **28** successively.

2-Methoxy-4,5-dihydro-3*H*-1,4-benzodiazepin-3-one (**27**): 18 mg, 20% yield, mp 85—87 °C, colorless prisms (from *n*-hexane). MS *m/z*: 174 (M^+). IR (KBr): 3350 (NH), 1700 (C=O), 1660 (C=N) cm^{-1}. ^{1}H-NMR δ: 2.56 (3H, s, 2-Me), 4.73 (2H, br d, J=10 Hz, 5-H_2), 6.5—7.1 (5H, m, NH and Ph-H). *Anal.* Calcd for $C_{10}H_{10}N_2O$: C, 68.95; H, 5.79; N, 16.08. Found: C, 68.73; H, 5.78; N, 15.90.

N-(*o*-Aminobenzyl)-acetylmethoxymethylenimine (**28**): 22 mg, 20% yield, pale yellow oil. MS *m/z*: 206 (M^+). IR ($CHCl_3$): 3450 and 3400 (NH), 1730 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.55 (3H, s, COMe), 3.74 (3H, s, OMe), 3.95 (2H, br s, Ph-CH_2–N), 4.5 (1H, br, NH), 6.5—7.1 (5H, m, Ph-H and NH). *Anal.* Calcd for $C_{11}H_{14}N_2O_2$: C, 64.06; H, 6.84; N, 13.58. Found: C, 65.31; H, 7.01; N, 13.52.

References and Notes

1) Part XXVIII: H. Sawanishi, K. Tajima, and T. Tsuchiya, *Chem. Pharm. Bull.*, **35**, 4101 (1987).
2) For reviews, see G. A. Archer and L. H. Sternbach, *Chem. Rev.*, **69**, 747 (1969); L. H. Sternbach, *Angew. Chem., Int. Ed. Engl.*, **10**, 34 (1971); J. T. Sharp, "Comprehensive Heterocyclic Chemistry," Vol. 7, ed. by R. A. Katritzky and C. W. Rees, Pergamon Press, Oxford, 1984, p. 608.
3) P. Nedenkov and M. Mandrup, *Acta Chem. Scand., Ser. B*, **31**, 701 (1977); K. R. Randle and R. C. Storr, *J. Chem. Soc., Chem. Commun.*, **1984**, 1485.
4) For reviews, see T. Tsuchiya, *Yuki Gosei Kagaku Kyokai Shi*, **39**, 99 (1981); *idem, ibid.*, **41**, 641 (1983).
5) H. Sashida, A. Fujii, and T. Tsuchiya, *Chem. Pharm. Bull.*, **35**, 3182 (1987).
6) H. Sawanishi, K. Tajima, M. Osada, and T. Tsuchiya, *Chem. Pharm. Bull.*, **32**, 4694 (1984); H. Sawanishi, K. Tajima, and T. Tsuchiya, *ibid.*, **35**, 3175 (1987).
7) Y. Ohba, I. Matsukura, Y. Fukazawa, and T. Nishiwaki, *Heterocycles*, **23**, 287 (1985).
8) D. J. Harris, G. Kan, V. Snieckus, and O. Buchardt, *Synthesis*, **1975**, 603.
9) J. Kurita, M. Enkaku, and T. Tsuchiya, *Chem. Pharm. Bull.*, **30**, 3764 (1982).
10) M. Uskokovic, J. Iacobelli, and W. Wenner, *J. Org. Chem.*, **27**, 3606 (1962).
11) W. Lawson, W. H. Perkin, and R. Robinson, *J. Chem. Soc.*, **125**, 626 (1924).
12) V. A. Petrow, M. V. Steck, and W. R. Wragg, *J. Chem. Soc.*, **1943**, 316.
13) S. Kamiya, *Yakugaku Zasshi*, **81**, 1743 (1961).

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Benzylpiperazine Derivatives. V.[1] Quantitative Structure–Activity Relationships of 1-Benzyl-4-diphenylmethylpiperazine Derivatives for Cerebral Vasodilating Activity

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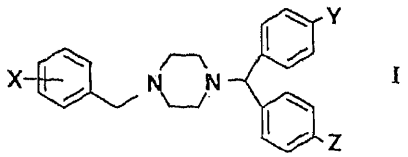
The quantitative structure–activity relationships of 1-benzyl-4-diphenylmethylpiperazines for cerebral vasodilating activity and duration of action were examined. The analyses indicate that increase of the electron density on the benzylic nitrogen atom and the introduction of sterically small substituents at the *para* position of the diphenylmethyl moiety bring about strong interactions of the molecule with the active site and result in high potency as well as prolonged action.

Keywords——quantitative structure–activity relationship; regression analysis; adaptive least-squares calculation; benzylpiperazine; cerebral vasodilator; action duration

In the course of our search for novel cerebral vasodilators, we have synthesized a series of 1-benzyl-4-diphenylmethylpiperazine derivatives (I) and tested them for vertebral blood flow-increasing activity in anesthetized dogs.[1] From the standpoint of stronger activity as well as longer-lasting effect than papaverine, 1-[bis(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxybenzyl)piperazine dihydrochloride (**16**: KB-2796) was selected for clinical evaluation. A consideration of structure–activity relationships suggested that bulky substituents on the diphenylmethyl moiety decrease the activity. Other factors are also presumed to influence the potency as well as the duration of action. These results prompted us to attempt a quantitative structure–activity relationship analysis with the aim of providing a basis for the design of better cerebral vasodilators.



I

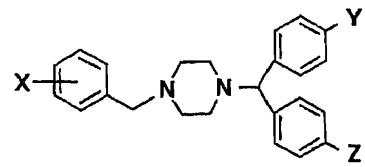
Chart 1

Method

Cerebral Vasodilating Activity——The cerebral vasodilating activities (CVA) of the compounds reported previously[1] were expressed in terms of the ratio of the maximum change of blood flow in vertebral arteries after intravenous administration of the test compound at 1 mg/kg to that in the case of papaverine at the same dose. For either the extrathermodynamic or the *de novo* method, the potency of compounds should be properly expressed on a molar basis. Therefore, the relative potencies (log RP) of the compounds were estimated as reported previously.[2] The dose–response curves of two potent derivatives (**10** and **16**) had been determined. The log(dose)–response plots of these analogs gave parallel lines.[3] Therefore the dose–response curves for a series of analogs were assumed to be parallel lines having a slope estimated as the mean of those for the two compounds ($=1.36$). This assumption may be expressed in equation form:

$$\log(\text{dose}) = a_x + b\ \text{response}_x \qquad (1)$$

TABLE I. Cerebral Vasodilating Activities and Parameters of 1-Benzyl-4-diphenylmethylpiperazines Used for Derivation of Eqs. 6—19



Compd. No.	X	Y	Z	$\Sigma\sigma^{a)}$	$MR^{b)}$	log RP Duration	
						Obs.	Obs.
1	4-OAc	H	H	(0.31)	1.03	6.54	1
2	4-Cl	H	H	(0.23)	1.03	6.43	0
3	4-F	H	H	(0.06)	1.03	6.49	0
4	H	H	H	0	1.03	6.48	0
5	4-NHAc	H	H	0	1.03	6.53	0
6	3,4,5-$(OMe)_3$	H	H	−0.03	1.03	6.57	0
7	3,4-$(OMe)_2$	H	H	−0.15	1.03	6.52	0
8	4-Me	H	H	−0.17	1.03	6.79	1
9	3,4-OCH_2O	H	H	−0.32	1.03	6.67	0
10	4-OH	H	H	−0.37	1.03	7.01	0
11	2,3,4-$(OMe)_3$	H	H	−0.42	1.03	7.32	0
12	2,4-$(OMe)_2$	H	H	−0.54	1.03	7.06	1
13	2,4,6-$(OMe)_3$	H	H	−0.81	1.03	7.67	0[c)]
14	4-NH_2	H	H	−0.66	1.03	6.79[d)]	0
15	4-NMe_2	H	H	−0.83	1.03	6.64[d)]	1
16	2,3,4-$(OMe)_3$	F	F	−0.42	0.92	7.41	1
17	2,3,4-$(OMe)_3$	Me	Me	−0.42	5.65	7.17	0
18	2,3,4-$(OMe)_3$	Cl	Cl	−0.42	6.03	6.66	0
19	2,3,4-$(OMe)_3$	OMe	OMe	−0.42	7.87	6.31	0
20	2,3,4-$(OMe)_3$	F	H	−0.42	1.03	7.17	1
21	2,3,4-$(OMe)_3$	Me	H	−0.42	5.65	6.41	0
22	2,3,4-$(OMe)_3$	Cl	H	−0.42	6.03	6.73	1
23	2,3,4-$(OMe)_3$	OMe	H	−0.42	7.87	6.13	0
24	3,4,5-$(OMe)_3$	F	F	−0.03	0.92	6.56	0
25	2,4,6-$(OMe)_3$	F	F	−0.81	0.92	7.18	0[c)]
26	2,4-$(OMe)_2$	F	F	−0.54	0.92	7.18	1
27	3,4-OCH_2O	F	F	−0.32	0.92	6.66	0
28	4-OH	F	F	−0.37	0.92	6.79	1
29	H	F	F	0	0.92	6.46	0
30	4-Me	F	F	−0.17	0.92	6.50	1
31	4-NMe_2	F	F	−0.83	0.92	7.31	1
32	4-OAc	F	F	(0.31)	0.92	6.60	0
33	2,4,6-$(OMe)_3$	F	H	−0.81	1.03	7.00	0[c)]
34	2,4-$(OMe)_2$	F	H	−0.54	1.03	7.04	1
35	3,4,5-$(OMe)_3$	F	H	−0.03	1.03	6.61	0
36	3,4-OCH_2O	F	H	−0.32	1.03	6.47	0
37	4-NMe_2	F	H	−0.83	1.03	7.13	1

a) In Eqs. 13—19 $\Sigma\sigma=0$ was used when $\Sigma\sigma>0$. *b*) MR stands for the larger value of MR_Y and MR_Z. *c*) Not included in the calculation. *d*) Not included in the correlation.

in which x refers to the analog x, and b is the slope of the log(dose)–response curve (=1.36). The value of interest for the structure–activity analysis is the negative logarithm of the dose required to produce a given response. From Eq. 1 it may be seen that at constant response and constant slope b, the logarithm of the dose of x is proportional to the value of $-a_x$; *i.e.*, $-a_x$ is the relative potency. The relative potencies of the compounds were calculated by using Eq. 2.

$$\log \mathrm{RP} = -\log(\mathrm{dose}) + 1.36\mathrm{CVA} \qquad (2)$$

Parameters——The classical physicochemical parameters of this type of analysis have been used, such as the π of Hansch for lipophilic effects, the MR for steric ones and the σ of Hammett for electronic effects. The values are taken from the literature.[4]

Regression Analysis——A regression analysis was carried out to investigate the relationships between the various parameters of the substituents (X, Y and Z) in 1-benzyl-4-diphenylmethylpiperazines and the cerebral vasodilating activities.

Adaptive Least-Squares (ALS) Calculation——The ALS method of Moriguchi *et al.*[5] (ALS 81) was applied to investigate the relationships between the various parameters of the substituents (X, Y and Z) in 1-benzyl-4-diphenylmethylpiperazines and the duration of action.

The ALS method, which is a nonparametric pattern classification method, has been devised to categorize multidimensional structural patterns into multiple ordered classes by means of a single equation. The equation (discriminant function) is formulated by a feedback procedure in a linear form, as in Eq. 3.

$$Y=w_0+w_1x_1+w_2x_2+\cdots+w_px_p \tag{3}$$

where Y is the discriminant score for the classification, x_k ($k=1, 2, \ldots, p$) is the k th descriptor for the structure, and w_k ($k=0, 1, 2, \ldots, p$) is the weight coefficient. The value of w_k is determined by the least-squares adaptation using the starting score, a_j ($j=1, 2, \ldots, m$ in the m-group case) and the correction term, $C_i(t)$, for misclassified substance i at the t-th iteration, which is given by Eq. 4

$$C_i(t)=0.1/(\delta_i(t)+0.45)^2+0.1 \tag{4}$$

where δ is the deviation defined by Eq. 5.

$$\delta_i(t)=|\,Y_i(t)-b_k\,| \tag{5}$$

In Eq. 5, b_k is the cutting point (nearer to $Y_i(t)$). With the data set of 34 compounds in this study, the starting score and the cutting point are as follows, $a_1=-0.765$, $a_2=1.235$, $b_1=0.235$. The adaptive iteration was performed 10 times, and the best discriminant function was selected. As the criteria of the best discrimination, the Spearman rank correlation coefficient with many ties, Rs, and the ε value[5] were used.

Results and Discussion

Initially, the relationships between structure and cerebral vasodilating activity (log RP) were examined by regression analysis.

With compounds that have no substituents on the diphenylmethyl moiety (**1**—**15**), $\sum\sigma$ seemed to correlate with the potency, when two analogs (**14** and **15**) were excluded (Fig. 1). This relation can be formulated as Eq. 6.

$$\log \mathrm{RP}=-1.083(\pm 0.370)\sum\sigma+6.591 \tag{6}$$

$$n=13,\quad r=0.889,\quad s=0.184,\quad F=41.42$$

In Eq. 6 the number in parentheses is the 95% confidence interval, n is the number of data points used in deriving the equation, r is the correlation coefficient, s is the standard deviation and F is the F-ratio between the variance of calculated and observed activities.

Figure 1 shows that the potency seems to be constant in the range of $\sum\sigma>0$, so these compounds were re-analyzed using $\sum\sigma=0$ when $\sum\sigma>0$ to give Eq. 7.

$$\log \mathrm{RP}=-1.388(\pm 0.340)\sum\sigma+6.475 \tag{7}$$

$$n=13,\quad r=0.938,\quad s=0.139,\quad F=80.73$$

Next, 1-[bis(fluorophenyl)methyl]piperazine derivatives (**16**, **24**—**32**) were considered, and Eq. 8 was obtained as the best equation.

$$\log \mathrm{RP}=-0.806(\pm 0.502)\sum\sigma+6.609 \tag{8}$$

$$n=10,\quad r=0.794,\quad s=0.235,\quad F=13.71$$

Re-analysis of these compounds using $\sum\sigma=0$ when $\sum\sigma>0$ gave Eq. 9.

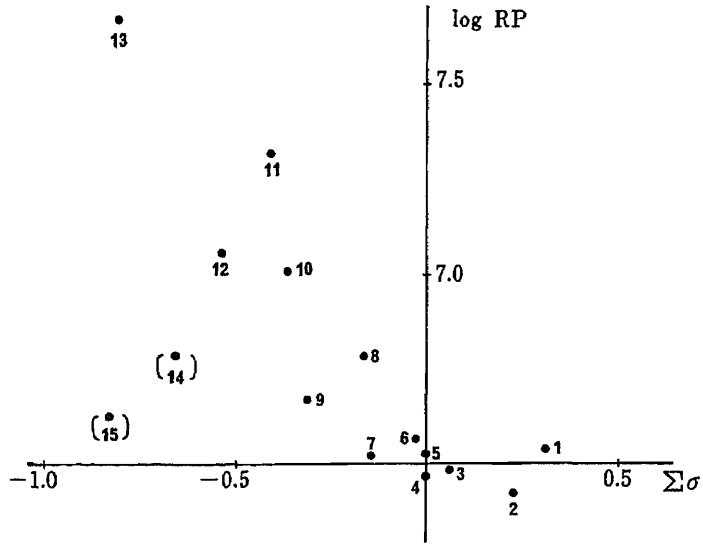


Fig. 1

$$\log RP = -0.994(\pm 0.517)\Sigma\sigma + 6.518 \quad (9)$$

$n=10,\ r=0.842,\ s=0.208,\ F=19.63$

Addition of a steric factor and/or lipophilic factor to Eqs. 6—9 did not give a significantly better results. These results suggest that the electron-donating effect of the substituent on the benzyl moiety is predominantly important for high activity.

In terms of the polar substituent constant of Taft (σ^*),[6] the electron withdrawing power is the following order: diphenylmethyl ($\sigma^*=0.405$) > benzyl ($\sigma^*=0.215$) > methyl ($\sigma^*=0$). The benzylic nitrogen atom is more basic in our compounds and the electron-donating group on the benzyl moiety increases the basicity. The second dissociation constants (pK_{a2}) of our molecules are expected to be slightly lower than that of 1-diphenylmethyl-4-methylpiperazine ($pK_{a1}=2.5$ and $pK_{a2}=7.9$)[7] owing to the electron-withdrawing groups on the benzyl moiety. In such a case, there would be no diprotonated species and little of the monoprotonated species in blood, which is controlled to nearly neutral pH. The reason why Eqs. 7 and 9 gave better results than Eqs. 6 and 8, respectively, may be as follows: the monoprotonated species, which increases in amount with increase of basicity of the benzylic nitrogen atom, is important for high activity, and the mechanism of action may be slightly different between the monoprotonated species and the free base.

A bulky substituent on the diphenylmethyl moiety seems to decrease the potencies of the compounds in which X=2,3,4-tri-OMe (**11**, **16**—**23**). This tendency can be formulated in Eq. 10.

$$\log RP = -0.143(\pm 0.070)MR + 7.480 \quad (10)$$

$n=9,\ r=0.876,\ s=0.243,\ F=22.99$

In Eq. 10, MR stands for the larger value between MR_Y and MR_Z. Analyses using Verloop's steric parameters (L, B_4)[8] gave similar results (Eqs. 11 and 12).

$$\log RP = -0.631(\pm 0.326)B_4 + 8.010 \quad (11)$$

$n=9,\ r=0.864,\ s=0.253,\ F=20.78$

$$\log RP = -0.618(\pm 0.338)L + 8.762 \quad (12)$$

$n=9,\ r=0.853,\ s=0.263,\ F=18.72$

Addition of an electronic factor and/or a lipophilic factor to Eqs. 10—12 did not give a significantly better result. Next, 35 compounds were subjected to the analysis (**14** and **15** were excluded again) and Eqs. 13—15 were obtained.

$$\log RP = -0.996(\pm 0.285)\sum\sigma - 0.073(\pm 0.036)MR + 6.593 \quad (13)$$

$$n=35,\quad r=0.809,\quad s=0.220,\quad F=30.27$$

$$\log RP = -1.064(\pm 0.272)\sum\sigma - 0.371(\pm 0.157)B_4 + 6.939 \quad (14)$$

$$n=35,\quad r=0.831,\quad s=0.208,\quad F=35.91$$

TABLE II. Results of the Regression Analysis and the ALS Recognition and Prediction

Compd. No.	Potency log RP		Duration Recognition				Prediction		
	Obs.	Eq. 13	Obs.	Eq. 17	Eq. 18	Eq. 19	Eq. 17	Eq. 18	Eq. 19
1	6.54	6.52	1	0	0	0	0	0	0
2	6.43	6.52	0	0	0	0	0	0	0
3	6.49	6.52	0	0	0	0	0	0	0
4	6.48	6.52	0	0	0	0	0	0	0
5	6.53	6.52	0	0	0	0	0	0	0
6	6.57	6.55	0	0	0	0	0	0	0
7	6.52	6.67	0	0	0	0	0	0	0
8	6.79	6.69	1	0	0	0	0	0	0
9	6.67	6.84	0	0	0	0	0	1	0
10	7.01	6.89	0	0	1	0	1	1	1
11	7.32	6.94	0	1	1	1	1	1	1
12	7.06	7.06	1	1	1	1	1	1	1
13	7.67	7.33	0[a]	—	—	—	—	—	—
14	6.79[b]	—	0	1	1	1	1	1	1
15	6.64[b]	—	1	1	1	1	1	1	1
16	7.41	6.95	1	1	1	1	1	1	1
17	7.17	6.60	0	0	0	0	0	0	0
18	6.66	6.57	0	0	0	0	0	0	0
19	6.31	6.44	0	0	0	0	0	0	0
20	7.17	6.94	1	1	1	1	1	1	1
21	6.41	6.60	0	0	0	0	0	0	0
22	6.73	6.57	1	0	0	0	0	0	0
23	6.13	6.44	0	0	0	0	0	0	0
24	6.56	6.56	0	0	0	0	0	0	0
25	7.18	7.33	0[a]	—	—	—	—	—	—
26	7.18	7.07	1	1	1	1	1	1	1
27	6.66	6.85	0	0	0	0	0	0	0
28	6.79	6.90	1	1	0	0	1	1	1
29	6.46	6.53	0	0	0	0	0	0	0
30	6.50	6.70	1	0	0	0	0	0	0
31	7.31	7.35	1	1	1	1	1	1	1
32	6.60	6.53	0	0	0	0	0	0	0
33	7.00	7.33	0[a]	—	—	—	—	—	—
34	7.04	7.06	1	1	1	1	1	1	1
35	6.61	6.55	0	0	0	0	0	0	0
36	6.47	6.84	0	0	1	0	0	0	0
37	7.13	7.35	1	1	1	1	1	1	1

a) Not included in the calculation. *b*) Not included in the correlation.

TABLE III. Correlation Matrix of Variables Used in Eqs. 13—15

	$\Sigma\sigma$	MR	B_4	L
$\Sigma\sigma$	1.000			
MR	−0.138	1.000		
B_4	−0.274	0.834	1.000	
L	−0.226	0.898	0.944	1.000

$$\log \mathrm{RP} = -1.087(\pm 0.286)\Sigma\sigma - 0.315(\pm 0.146)L + 7.235 \quad (15)$$

$n=35,\ r=0.818,\ s=0.215,\ F=32.42$

The correlation coefficient is not so high. There seem to be several reasons for this: the range of potency is very narrow, the slope of log(dose)–response curve (=1.36) is too steep, introducing error in measuring the potency, and the mechanisms of action of all compounds were assumed to be the same, even though, some exhibit prolonged action and others do not. The calculated potencies using Eq. 13 are summarized in Table II.

Next, the relationships between structure and duration of action were examined. For such discrete data, regression analysis is not appropriate. The ALS method was thought to be more appropriate to analyze our data. From the data, the compounds of stronger potency appeared to exhibit long-lasting effects. The ALS analysis between potency and duration of all 37 compounds gave unsatisfactory results ($n_{\mathrm{mis}}=11$, $Rs=0.317$, $t=1.977$, $p>0.05$). However, when the compounds which have a 2,4,6-tri-OMe group on the benzyl moiety (**13**, **25** and **33**) were excluded, a highly significant result was obtained (Eq. 16).

$$Y = 0.532 \log \mathrm{RP} - 3.340 \quad (16)$$

$n=34,\ Rs=0.571,\ \varepsilon=0.921,\ n_{\mathrm{mis}}=7,\ t=3.93,\ p<0.001$

Equation 16 was obtained after 9 iterative calculations. A possible justification for the exclusion is that the conformations of **13**, **25** and **33** may be different from the others because of the di-*ortho* substituents on the benzyl moiety.

Relative potency (log RP) was explained well with $\Sigma\sigma$ and a steric parameter (Eqs. 13—15), so analysis using these parameters instead of log RP was performed to give Eqs. 17—19.

$$Y = -1.774\Sigma\sigma - 0.128MR - 0.302 \quad (17)$$

$n=34,\ Rs=0.620,\ \varepsilon=0.773,\ n_{\mathrm{mis}}=6,\ t=4.47,\ p<0.001$

$$Y = -2.175\Sigma\sigma - 0.512B_4 - 0.044 \quad (18)$$

$n=34,\ Rs=0.491,\ \varepsilon=0.734,\ n_{\mathrm{mis}}=8,\ t=3.18,\ p<0.01$

$$Y = -1.031\Sigma\sigma - 0.055L - 0.051 \quad (19)$$

$n=34,\ Rs=0.555,\ \varepsilon=0.868,\ n_{\mathrm{mis}}=7,\ t=3.77,\ p<0.001$

In Eqs. 17—19, 82, 76 and 79% of the compounds were correctly classified, respectively. To confirm the validity of the ALS results, the leave-one-out technique was applied and the predictive results classified 76—79% of the compounds correctly. The results are summarized in Table II.

Duration of action of a drug depends on various factors such as elimination, distribution and in some cases the biological activity of the metabolites. However, Eqs. 13—16 and 17—19 suggest that increase of the electron density on the benzylic nitrogen atom and the introduction of a sterically small substituent at the *para* position on the diphenylmethyl moiety bring about

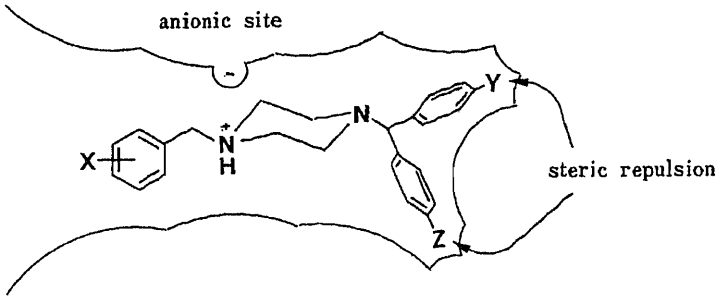


Fig. 2. A Model of the Active Site

strong interactions of the compound with the active site, resulting in strong potency as well as prolonged action. The mechanism of action of our compounds is likely to be a direct calcium-antagonistic action on vascular smooth muscle.[1] These results suggest that a compound with strong cerebral vasodilating activity has a strong affinity for the active site; such a compound may bind too tightly to be easily washed out by the blood flow, so that it would show a long-lasting action, while opposite considerations would apply to a compound with weak activity.

From the above results, a model of the active site is proposed as shown in Fig. 2. The putative active site may be like a hydrophobic pocket, and there is an anionic site which interacts electrostatically with the positively charged benzylic nitrogen atom. The effect of the diphenylmethyl moiety does not appear explicitly in the equations, but the introduction of a diphenylmethyl moiety resulted in a high activity compared to the activity of trimetazidine. Thus, there may be a strong hydrophobic interaction between the diphenylmethyl moiety and the wall of the pocket. The depth of the pocket from the anionic site is limited, leading to steric repulsion, which lowers the binding interaction, with the bulky substituents Y and Z. The lead development based on these assumptions will be reported separately.

References and Notes

1) *a*) This work was presented at the 8th Symposium on Medicinal Chemistry, Osaka, Nov. 1986; *b*) H. Ohtaka, T. Kanazawa, K. Ito and G. Tsukamoto, *Chem. Pharm. Bull.*, **35**, 3270 (1987).
2) H. Ohtaka and G. Tsukamoto, *Chem. Pharm. Bull.*, **35**, 2792 (1987).
3) H. Ohtaka, T. Kanazawa, K. Ito and G. Tsukamoto, Unpublished results.
4) *a*) C. Hansch, A. Leo, S. H. Unger, K. H. Kim, D. Nikaitani and E. J. Lien, *J. Med. Chem.*, **16**, 1207 (1973); *b*) A. Leo, C. Hansch and D. Elkins, *Chem. Rev.*, **71**, 525 (1971).
5) *a*) I. Moriguchi, K. Komatsu and Y. Matsusita, *J. Med. Chem.*, **23**, 20 (1980); *b*) I. Moriguchi and K. Komatsu, Abstracts of Papers, 8th Symposium on Structure-Activity Relationships, Tokyo Oct. 1981, p. 55.
6) R. W. Taft, Jr., "Steric Effects in Organic Chemistry," ed. by M. S. Newman, John Wiley, New York, 1956, pp. 556—675.
7) R. Baltzly, S. Dubreuil, W. S. Ide and E. Lorz, *J. Org. Chem.*, **14**, 775 (1949).
8) A. Verloop, W. Hoogenstraaten and J. Tipker, "Drug Design," Vol. 7, ed. by E. J. Ariens, Academic Press, New York, 1976, pp. 165—207.

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Benzylpiperazine Derivatives. VI.[1] Design and Syntheses of Vinylogs of 1-Benzyl-4-diphenylmethylpiperazine Derivatives and Their Cerebral Vasodilating Activities

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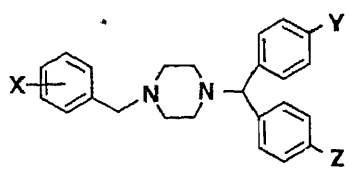
As a lead development based on the previous quantitative structure–activity relationship (QSAR) results on cerebral vasodilating activities of 1-benzyl-4-diphenylmethylpiperazines, 1-cinnamyl-4-diphenylmethylpiperazines having electron-donating groups on the cinnamyl moiety were designed. Two methods of synthesis were developed. As expected from the QSAR results, these compounds exhibited stronger potency and longer-lasting effect than cinnarizine and flunarizine.

Keywords——lead development; cinnamylpiperazine; diphenylmethylpiperazine; cinnarizine; flunarizine; cerebral vasodilating activity

In the previous paper, we described quantitative structure–activity relationships (QSAR) of 1-benzyl-4-diphenylmethylpiperazine derivatives (**1**) for cerebral vasodilating activity.[1] The results indicate that increase of the electron density on the benzylic nitrogen atom and the introduction of a sterically small substituent at the *para* position of the diphenylmethyl moiety bring about strong interaction of the molecule with the active site, resulting in high potency and prolonged action. Among the analogs, we selected 1-[bis(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxybenzyl)piperazine dihydrochloride (KB-2796) for clinical evaluation.

The above results prompted us to attempt a lead development. Although there may be several means to increase the electron density on the nitrogen atom, we selected the structure with a double bond between the phenyl residue and the methylene group to transmit the electronic effect of the substituents on the phenyl ring to the nitrogen atom. The substituent on the diphenylmethyl moiety was selected to be a hydrogen or a fluorine atom. Namely, 1-(substituted cinnamyl)-4-diphenylmethylpiperazines (**2**) were designed. These compounds correspond to substituted cinnarizine (**3**) or flunarizine (**4**), which were selected as references to modify the structure of trimetazidine at the beginning of this series of studies to search for a novel cerebral vasodilator.[2] In this paper, we describe the synthesis of these compounds as well as their cerebral vasodilating activities.



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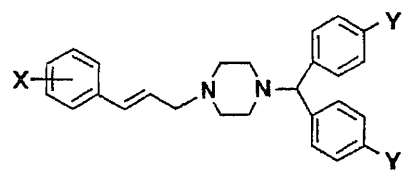

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3 (cinnarizine: X=Y=H)

4 (flunarizine: X=H, Y=F)

Chart 1

Chemistry

At first, the compounds (**2**) were synthesized by the route shown in Chart 2 (method A). Thus, a cinnamic acid derivative was converted to an acid chloride then condensed with a diphenylmethylpiperazine derivative. The product was converted to an acid addition salt and recrystallized in order to purify it (Table I). After reversion to the free base, the amide was reduced with lithium aluminum hydride in ether. The products are summarized in Table II.

As the above method gave very poor yields, other methods shown in Chart 3 (method B) were investigated. Thus, a cinnamic acid derivative was dissolved in *tert*-butanol–acetonitrile (5 : 1) in the presence of triethylamine then ethyl chloroformate was added to prepare a mixed anhydride. A diphenylmethylpiperazine derivative in the same solvent system was added to the mixed anhydride. After the reaction was completed, water was added to the mixture and

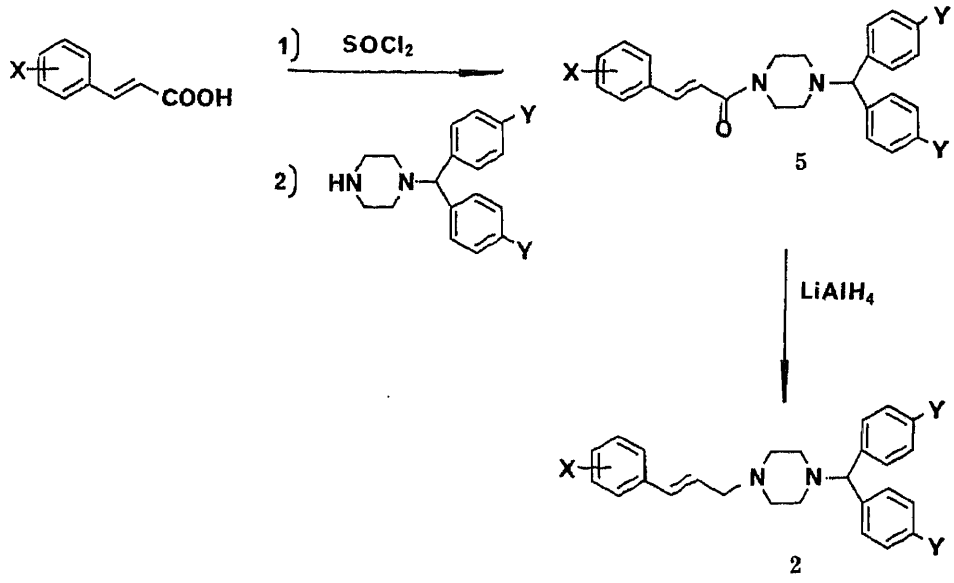
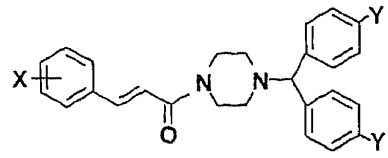


Chart 2

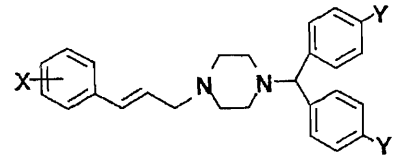
TABLE I. 1-Cinnamoyl-4-diphenylmethylpiperazines Obtained by Method A



Compd. No.	X	Y	Yield (%)	mp (°C)	Recrystn.[a] solvent	Formula[b]	Analysis (%) Calcd (Found)		
							C	H	N
5a	2,3,4-$(OMe)_3$	F	28	225—229 (dec.)	M	$C_{29}H_{30}F_2N_2O_4 \cdot HCl$	63.91 (63.69	5.73 5.89	5.14 5.08)
5b	2,3,4-$(OMe)_3$	H	44	207—209 (dec.)	E	$C_{29}H_{32}N_2O_4 \cdot HCl$	68.43 (68.32	6.53 6.60	5.50 5.56)
5c	2,4-$(OMe)_2$	F	29	195—197	C–M	$C_{28}H_{28}F_2N_2O_3 \cdot 0.5FA$	67.15 (67.11	5.64 5.68	5.22 5.27)
5d	2,4-$(OMe)_2$	H	40	239—241 (dec.)	M	$C_{28}H_{30}N_2O_3 \cdot HCl$	70.21 (70.07	6.52 6.53	5.85 5.87)

a) C=$CHCl_3$, E=EtOH, IP=iso-PrOH, M=MeOH, W=water. *b*) FA stands for fumaric acid.

TABLE II. 1-Cinnamyl-4-diphenylmethylpiperazines



Compd. No.	X	Y	Yield (%)[a] A	Yield (%)[a] B	mp (°C)	Recrystn.[b] solvent	Formula[c]	Analysis (%) Calcd (Found) C	H	N	Potency[d]
2a	2,3,4-$(OMe)_3$	F	24	56	205—212 (dec.)	E	$C_{29}H_{32}F_2N_2O_3 \cdot 2HCl$	61.38 (61.52)	6.04 (5.89)	4.94 (5.08)	1.25 (D)[e]
2b	2,3,4-$(OMe)_3$	H	20	58	230—234 (dec.)	E	$C_{29}H_{34}N_2O_3 \cdot 2HCl$	65.53 (65.35)	6.83 (6.79)	5.27 (5.42)	0.98 (D)
2c	2,4-$(OMe)_2$	F	33	51	194—195	E-W	$C_{28}H_{30}F_2N_2O_2 \cdot FA$	66.20 (66.12)	5.90 (5.91)	4.82 (4.73)	1.25 (D)
2d	2,4-$(OMe)_2$	H	26	59	192—195 (dec.)	E-W	$C_{28}H_{32}N_2O_2 \cdot FA$	70.57 (70.75)	6.66 (6.63)	5.14 (5.21)	0.99 (D)
2e	4-$N(Me)_2$	F	35[f]		186—189 (dec.)	IP	$C_{28}H_{31}F_2N_3 \cdot FA$	68.19 (68.40)	6.26 (6.29)	7.46 (7.48)	1.65 (D)
Cinnarizine											0.71
Flunarizine·2HCl											0.79 (D)

a) Yields are based on isolated amides. A and B mean method A and method B, respectively. *b*) and *c*) See footnotes *a*) and *b*) of Table I, respectively. *d*) The potency is expressed as the ratio of cerebral vasodilating activity to that of papaverine taken as 1. (D) stands for prolonged duration of action. *e*) Data obtained with a dose of 0.3 mg/kg, i.v. Other compounds were administered at a dose of 1 mg/kg, i.v. *f*) See the text.

the amide that precipitated was collected by filtration in high yield. In the case of **5b**, which is an oil, the product was extracted with ethyl acetate. The amides obtained by this procedure were pure enough to use in the next step without further purification. The amides were reduced in toluene with sodium bis(2-methoxyethoxy)aluminum hydride (Vitride) to give compounds **2a–d** in high yields (Table II).

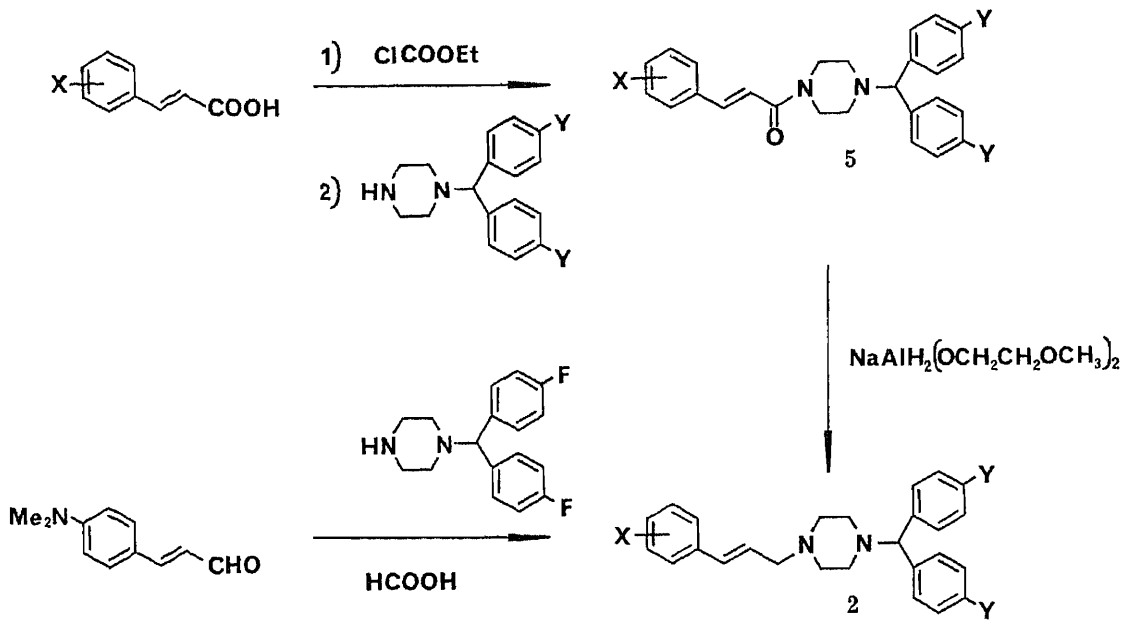


Chart 3

The *p*-dimethylamino derivative (**2e**) was obtained in a single step by using the Leuckart–Wallach reaction from the corresponding cinnamaldehyde, which was available commercially.

TABLE III. Free Base of 1-Cinnamoyl-4-diphenylmethylpiperazines Obtained by Method B

Compd. No.	X	Y	Yield (%)	mp (°C)	Recryst. solvent	Formula	Analysis (%) Calcd (Found)		
							C	H	N
Free **5a**	2,3,4-$(OMe)_3$	F	80	175—177	Benzene	$C_{29}H_{30}F_2N_2O_4$	68.49 (68.36	5.95 6.00	5.51 5.53)
Free **5b**	2,3,4-$(OMe)_3$	H	100[a]						
Free **5c**	2,4-$(OMe)_2$	F	82	171—173	Benzene	$C_{28}H_{28}F_2N_2O_3$	70.28 (70.30	5.90 5.77	5.85 5.85)
Free **5d**	2,4-$(OMe)_2$	H	85	175—177	Benzene	$C_{28}H_{30}N_2O_3$	75.99 (75.78	6.83 6.77	6.33 6.26)

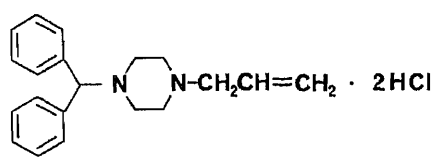
a) Free **5b** was an oil. Physical data of the HCl salt of this compound were the same as those of **5b** of Table I.

Results and Discussion

The compounds listed in Table II were tested for cerebral vasodilating activity by the method reported previously.[2] As expected from the previous QSAR results, these compounds, which have electron-donating substituents on the cinnamyl moiety, exhibited stronger activity and longer duration of action than **3** and **4**. The bis(4-fluorophenyl)methyl derivatives (**2a** and **2c**) were more potent than the corresponding diphenylmethyl analogs (**2b** and **2d**).

The *p*-dimethylamino derivative (**2e**) was one of the most active compounds, but its acute toxicity ($LD_{50}<45$ mg/kg) was very strong. Similar results were reported for the *p*-dimethylaminobenzyl derivative previously.[3] These results suggest that 1-benzyl-4-diphenylmethylpiperazines (**1**) and 1-cinnamyl-4-diphenylmethylpiperazines (**2**) exhibit not only cerebral vasodilating activity but also toxicity through similar mechanisms. Among these derivatives, the most active **2a** was selected for further study.

Many substituted cinnarizine derivatives have been reported,[4,5] but there seems to be no compound which is more potent than **3** and **4**, because some have an electron-withdrawing group on the cinnamyl moiety and some have a bulky substituent on the diphenylmethyl residue. Aligeron[6] (**6**) and Cinepazide[7] (**7**), cerebral vasodilators containing a piperazine moiety, can be regarded as analogs of **3**. The potencies of these compounds evaluated by the same method were 0.4 for **6** and 0.04 for **7**. Thus, **2a** is thought to be one of the most potent analogs of **3**.



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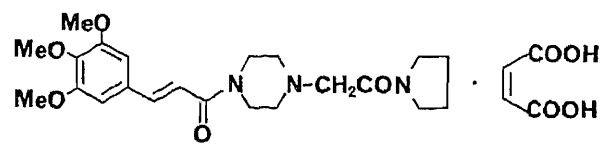

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Chart 4

Experimental

Melting points were determined on a Yamato capillary melting point apparatus, model MP-21, and are uncorrected. ^{1}H-Nuclear magnetic resonance (^{1}H-NMR) spectra were determined on a Hitachi R-24A NMR spectrometer with tetramethylsilane (TMS) as an internal standard. Silica gel 60 F_{254} (Merck) TLC plates were used for thin layer chromatography (TLC). For column chromatography, Silica gel 60 (Merck) was used.

Typical Procedures of Method A——1-Diphenylmethyl-4-(2,3,4-trimethoxycinnamoyl)piperazine Hydrochloride (**5b**): 2,3,4-Trimethoxycinnamic acid (5.0 g) was suspended in $CHCl_3$ (20 ml), and $SOCl_2$ (7.0 ml) was added dropwise under ice cooling. The mixture was stirred for 0.5 h then the excess $SOCl_2$ and $CHCl_3$ were distilled off under reduced pressure. The residue was diluted with CH_2Cl_2 (40 ml) and added dropwise under ice cooling to a solution of diphenylmethylpiperazine (4.0 g) in CH_2Cl_2 (60 ml). The mixture was stirred for 0.5 h under ice cooling, 10% $NaHCO_3$ was added to the mixture, and then the organic layer was separated, washed with water and dried over $MgSO_4$. After the solvent had been distilled off, the residue was dissolved in EtOH (40 ml). Concentrated HCl (2 ml) was added to the solution and then Et_2O was added. The precipitated solid was collected by filtration and recrystallized from EtOH to give **5b** (4.4 g).

Compounds **5a**, **5c** and **5d** were obtained in the same manner as described for **5b**, but **5c** was obtained as the hemifumarate. The yield, melting point and elementary analysis data are given in Table I.

1-Diphenylmethyl-4-(2,3,4-trimethoxycinnamyl)piperazine Dihydrochloride (**2b**): **5b** (3.7 g) was suspended in a mixture of AcOEt and water, and with stirring, 20% NaOH was added to adjust the pH of the aqueous layer to 9—10. The AcOEt layer was separated, washed with water, and dried over $MgSO_4$. The solvent was distilled off under reduced pressure and the resulting free base was dissolved in dry Et_2O (60 ml). Lithium aluminum hydride (0.3 g) was added portionwise to the mixture at room temperature, and the whole was stirred for 4 h at room temperature. Water was added portionwise, and then 3 N HCl was added to make the mixture nearly neutral. The organic layer was separated, washed with water, and dried over $MgSO_4$. After the solvent had been distilled off, the residue was diluted with EtOH (10 ml). Concentrated HCl (1 ml) was added to the solution and the precipitated solid was collected by filtration and recrystallized from EtOH to give **2b** (0.8 g).

Compounds **2a**,**2c** and **2d** were obtained in the same manner as described for **2b**, but **2c** and **2d** were obtained as the fumarates. The yield, melting point and elementary analysis data are given in Table II.

Typical Procedures of Method B——1-[Bis(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxycinnamoyl)piperazine (Free Base of **5a**): A solution of 2,3,4-trimethoxycinnamic acid (30.0 g) and triethylamine (25.5 g) in *tert*-BuOH–MeCN (5 : 1, v/v) (300 ml) was cooled in an ice-bath. A solution of ethyl chloroformate (13.7 g) in *tert*-BuOH–MeCN (5 : 1, v/v) (40 ml) was added dropwise, then the mixture was stirred for 0.5 h. A solution of 1-[bis(4-fluorophenyl)methyl]piperazine (36.3 g) in *tert*-BuOH–MeCN (5 : 1, v/v) (150 ml) was added dropwise, and the mixture was stirred for 1 h at room temperature. The reaction mixture was poured into ice-water (450 ml) and the deposited solid was collected by filtration, washed with water (400 ml) and then dried to give the free base of **5a** (51 g).

1-[Bis(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxycinnamyl)piperazine Dihydrochloride (**2a**): A toluene (10 ml) solution of sodium bis(2-methoxyethoxy)aluminum hydride (70% toluene solution, Vitride, Hexcel Corporation) (8.66 g) was added to a toluene (100 ml) solution of the free base of **5a** (10.16 g) and the mixture was stirred for 0.5 h. Water (30 ml) was added and the deposited solid was filtered off. The organic layer was separated and washed with water, then dried over $MgSO_4$. After evaporation of the solvent, the residue was diluted with $ClCH_2CH_2Cl$ (100 ml) and concentrated HCl (4 ml) was added. The precipitated solid was collected by filtration and recrystallized to give **2a** (6.36 g).

Compounds **2c** and **2d** were obtained as the fumarate in the same manner as described for **2a**. In the case of **2b**, the intermediate, the free base of **5b**, was not a solid. Therefore the free base of **5b** was obtained by extraction. The reduction process was similar to that for **2a**. The results are summarized in Table II.

1-(4-Dimethylaminocinnamyl)-4-[bis(4-fluorophenyl)methyl]piperazine Fumarate (2e)——4-Dimethylaminocinnamaldehyde (1.8 g) and 1-[bis(4-fluorophenyl)methyl[]piperazine (2.9 g) were melted in an oil bath at 120 °C and formic acid (0.5 ml) was added dropwise. The mixture was stirred for 1 h under heating, and then allowed to cool to room temperature. The mixture was diluted with EtOH (10 ml), a solution of fumaric acid (2.3 g) in EtOH (40 ml) was added, and the deposited solid was collected. Recrystallization from iso-PrOH gave **2e** (2.0 g). The yield, melting point and elementary analysis data are given in Table II.

Biological Testing Method[2]——The cerebral blood flow-increasing activity was measured by using the amount of vertebral blood flow as an index.[8] Mongrel dogs of either sex (body weight 11 to 18 kg) were anesthetized with sodium pentobarbital (30 mg/kg, by intravenous injection) and artificially ventilated. The right vertebral artery was isolated from the surrounding tissues and a flow probe was attached to it and led to an electromagnetic flow meter (MVF-2100, Nihon Koden Co., Ltd.). The blood flow was periodically measured.

Each of the test compounds was dissolved in a 2% tartaric acid solution containing 20% dimethylacetamide, and administered to the right femoral vein at a dose of 1 mg/kg. The potency was expressed in terms of the ratio of the maximum change of blood flow induced by the test compound to that induced by papaverine.

References and Notes

1) *a*) This study was presented at the 107th Meeting of the Pharmaceutical Society of Japan, Kyoto, April 1987; *b*) H. Ohtaka and G. Tsukamoto, *Chem. Pharm. Bull.*, **35**, 4117 (1987).
2) H. Ohtaka, M. Miyake, T. Kanazawa, K. Ito and G. Tsukamoto, *Chem. Pharm. Bull.*, **35**, 2774 (1987).
3) H. Ohtaka, T. Kanazawa, K. Ito and G. Tsukamoto, *Chem. Pharm. Bull.*, **35**, 3270 (1987).
4) Laboratoria Pharmaceutica Dr. C. Janssen, Belg. Patent 556791 [*Chem. Abstr.*, **54**, 590*c* (1960)].
5) P. A. J. Janssen, Ger. Offen., 1929330 [*Chem. Abstr.*, **73**, 14874*g* (1970)].
6) M. Nikolova, *Drugs of the Future*, **5**, 13 (1980).
7) C. Fauran and M. Turin, *Chim. Ther.*, **4**, 290 (1969).
8) K. Kubo, A. Karasawa, K. Yamada, M. Nito, K. Shuto and N. Nakamizo, *Nippon Yakurigaku Zasshi*, **79**, 383 (1982); J. Roca, M. Grau and J. Balasch, *Methods Find. Exptl. Clin. Pharmacol.*, **3**, 397 (1981).

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Studies on Hypolipidemic Agents. V.[1d] Synthesis and Esterase-Inhibitory Activity of 2-(1,4- and 4,4-Dialkylcyclohexyl)-2-oxoethyl Arenesulfonates

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2-(1,*t*- and *c*-4-Dialkylcyclohex-*r*-1-yl)-2-oxoethyl arenesulfonates, 2-(4,4-dialkylcyclohex-1-yl)-2-oxoethyl arenesulfonates and related compounds were synthesized and evaluated for esterase- and chymotrypsin-inhibitory activities *in vitro* and for hypolipidemic effect *in vivo*. The *trans*-isomers of 2-(1,4-dialkylcyclohex-1-yl)-2-oxoethyl arenesulfonates showed much more potent esterase-inhibitory action (about 13 to 6200 times) than the *cis*-isomers as well as more potent hypolipidemic action (about 1.5 to 10 times) but the chymotrypsin-inhibitory actions of the two isomers were similarly low. On the other hand, the 2-oxoethyl arenesulfonates having a 4,4-disubstituted cyclohexane ring mostly exhibited potent esterase-inhibitory action (order of IC_{50}; 10^{-8} to 10^{-9} M) and marked hypolipidemic effect (78% to 95% reductions of plasma triglyceride).

Keywords——4-alkylcyclohexanecarboxylic acid; 1,4-dialkylcyclohexanecarboxylic acid; 4,4-dialkylcyclohexanecarboxylic acid; diazoketone; arenesulfonate; esterase-inhibitory activity; chymotrypsin-inhibitory activity; hypolipidemic activity; structure–activity relationship

It is well known[2] that after meals, pancreatic lipase and esterase play important roles in the process of absorption of triglyceride and cholesterol ester from foods in the digestive organs. We previously reported[1a] the synthesis and novel mechanism of hypolipidemic action of various 2-oxoalkyl arenesulfonates,[1] which reduced the uptake of triglyceride and cholesterol ester by inhibiting the above enzymes in the small intestinal lumen. Among the reported 2-(4-alkylcyclohexyl)-2-oxoethyl arenesulfonates,[1d] remarkable enhancement of the esterase-inhibitory activity was observed when an alkyl substituent was introduced at the 4-position on the cyclohexane ring. A considerable difference of the potencies of esterase inhibition and the hypolipidemic effect was observed between the stereoisomers (*trans* and *cis*). These observations prompted us to synthesize 2-(1,4-dialkyl of 4,4-dialkylcyclohex-1-yl)-2-oxoethyl arenesulfonates and related compounds, which have another alkyl substituent at the 1- or 4-position on the cyclohexane ring of 2-(4-alkylcyclohexyl)-2-oxoethyl arenesulfonates,[1d] and to examine their esterase-inhibitory activity and hypolipidemic action. In this paper, we wish to report the preparation and biological activities of the stereoisomers of 2-(1,4-dialkylcyclohex-1-yl)-2-oxoethyl arenesulfonates (*t*-**4a**—**e** and *c*-**4a**—**e**) and 2-(4,4-dialkylcyclohex-1-yl)-2-oxoethyl arenesulfonates (**7a**—**e**).

Synthesis

The two stereoisomers of 2-(1,4-dialkylcyclohex-1-yl)-2-oxoethyl arenesulfonates (*t*-**4** and *c*-**4**) were prepared from the corresponding diazoketones (*t*-**3** and *c*-**3**) in the manner shown in Chart 1. Alkylation of 4-alkylcyclohexanecarboxylic acids (**1**)[1c] with alkyl iodide in the presence of lithium diisopropylamide[3] (LDA) afforded a stereoisomeric mixture of 1,4-

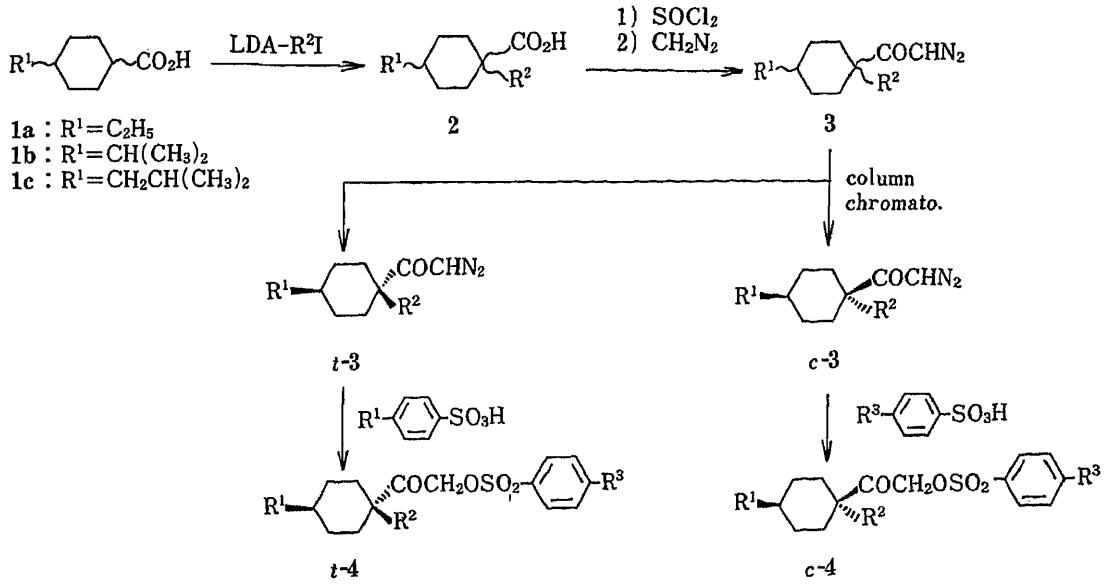

Chart 1

TABLE I. Physical Data for *t*-3 and *c*-3

Compd.[a] No.	R^1	R^2	MS (M^+)	^{1}H-NMR ($CDCl_3$) δ ppm
t-3a	C_2H_5	CH_3	194	0.60—1.90 (14H, m), 1.12 (3H, s), 5.41 (1H, s)
c-3a	C_2H_5	CH_3	194	0.70—1.75 (12H, m), 1.07 (3H, s), 2.06 (2H, br d, J=11.5 Hz), 5.45 (1H, s)
t-3b	C_2H_5	C_2H_5	208	0.70—1.90 (19H, m), 5.40 (1H, s)
c-3b	C_2H_5	C_2H_5	208	0.70—1.80 (17H, m), 2.10 (2H, br d, J=11.5 Hz), 5.44 (1H, s)
t-3c	$CH(CH_3)_2$	CH_3	208	0.75—1.80 (10H, m), 0.87 (6H, d, J=6.5 Hz), 1.12 (3H, s), 5.40 (1H, s)
c-3c	$CH(CH_3)_2$	CH_3	208	0.75—1.74 (8H, m), 0.82 (6H, d, J=6.3 Hz), 1.05 (3H, s), 2.11 (2H, br d, J=11.5 Hz), 5.45 (1H, s)
t-3d	$CH_2CH(CH_3)_2$	CH_3	222	0.80—1.90 (12H, m), .085 (6H, d, J=6.3 Hz), 1.12 (3H, s), 5.42 (1H, s)
c-3d	$CH_2CH(CH_3)_2$	CH_3	222	0.60—1.80 (10H, m), 0.83 (6H, d, J=6.3 Hz), 1.06 (3H, s), 2.06 (2H, br d, J=12 Hz), 5.45 (1H, s)

a) All compounds were yellowish oils. All infrared (IR) spectra in $CHCl_3$ of these compounds showed bands at 2100 (CHN_2) and 1620 (CO) cm^{-1}.

dialkylcyclohexanecarboxylic acids (**2**) in fair yield. The ratio of the *trans* and *cis* isomers was about 1 : 1 on the basis of the proton nuclear magnetic resonance (^{1}H-NMR) spectra. The subsequent treatment of the acid chlorides of **2** with diazomethane afforded the *cis*-diazoketones (*c*-**3**) and the *trans*-diazoketones (*t*-**3**) after column chromatographic separation. These structures were assigned as follows; the ^{1}H-NMR spectra of *c*-**3** showed two broad downfield peaks at δ 2.06—2.11 ppm (2H, br d, J=11.5—12 Hz), which did not appear in those of *t*-**3**. This feature apparently arises from deshielding of the axial protons at C-3 and C-5 by the C-1 axial carbonyl function on the cyclohexane ring and is consistent with the observation of Schindel and Pincock.[4] Furthermore, in carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra, there is no difference between the chemical shift of the methine carbon on the isopropyl group of *t*-**3c** (δ 32.5 ppm) and that of *c*-**3c** (δ 32.7 ppm). These findings demonstrate that the isopropyl group in both *t*-**3c** and *c*-**3c** may be equatorial. The spectral data of *t*-**3** and *c*-**3** are listed in Table I.

Treatment of *t*-**3** and *c*-**3** with the arenesulfonic acids afforded the corresponding *trans*-

TABLE II. Inhibitory Activities on Enzymes, and Hypolipidemic Effect of *t*-4 and *c*-4

Compd. No.	R^1	R^2	R^3	Yield[b] (%)	mp (°C)	Recryst.[d] solv.	Inhibitions: Esterase[e] IC_{50} (μM)	Inhibitions: Chymotry.[f] 1×10^{-4} M (%)	Reduction[g]: Trigly.[h]
t-4a	C_2H_5	CH_3	H	65	Oil[c]	—	0.58	29	—[i]
c-4a	C_2H_5	CH_3	H	66	Oil[c]	—	>100.0	8	—[i]
t-4b	C_2H_5	C_2H_5	H	74	48—49	PE–E	0.11	25	—[i]
c-4b	C_2H_5	C_2H_5	H	77	Oil[c]	—	35.0	15	—[i]
t-4c	C_2H_5	C_2H_5	CH_3	83	57—58	Et	7.4	44	—[i]
c-4c	C_2H_5	C_2H_5	CH_3	89	45—46	E	>100.0	29	—[i]
t-4d	$CH(CH_3)_2$	CH_3	H	66	49—50	Et–W	0.016	26	72
c-4d	$CH(CH_3)_2$	CH_3	H	74	Oil[c]	—	>100.0	14	49
t-4e	$CH_2CH(CH_3)_2$	CH_3	H	76	48—49	PE–E	2.2	15	71
c-4e	$CH_2CH(CH_3)_2$	CH_3	H	88	47—48	PE–E	>100.0	16	7
trans[a]	$CH(CH_3)_2$	H	H				0.065	62	90

a) Ref., 1*d*. *b*) Yield from the corresponding diazoketone (*t*-3 or *c*-3). *c*) Purified by column chromatography. *d*) Recrystallization solvents; Et=ethanol, E=diethyl ether, PE=petroleum ether, W=H_2O. *e*) Methyl butyrate was used as a substrate. *f*) ATEE was used as a substrate. The result was expressed as percentage inhibition of chymotrypsin-inhibitory activity at 1×10^{-4} M. *g*) The activity was expressed as percentage deviation from the control value. *h*) Plasma triglyceride. *i*) Not tested.

TABLE III. Physical Data for *t*-4 and *c*-4

Compd.[a] No.	Formula	Analysis (%) Calcd (Found) C	H	^{1}H-NMR ($CDCl_3$) δ ppm
t-4a	$C_{17}H_{24}O_4S$	62.93	7.46	0.75—1.80 (14H, m), 1.12 (3H, s), 4.92 (2H, s), 7.40—7.74 (3H, m),
		(62.97	7.64)	7.84—8.04 (2H, m)
c-4a	$C_{17}H_{24}O_4S$	62.93	7.46	0.50—1.76 (12H, m), 1.04 (3H, s), 2.08 (2H, br d, J=13 Hz), 4.91 (2H, s),
		(63.03	7.65)	7.40—7.75 (3H, m), 7.85—8.04 (2H, m)
t-4b	$C_{18}H_{26}O_4S$	63.88	7.74	0.56—1.90 (16H, m), 0.67 (3H, t, J=7.5 Hz), 4.88 (2H, s), 7.40—7.78
		(63.47	7.62)	(3H, m), 7.90—8.10 (2H, m)
c-4b	$C_{18}H_{26}O_4S$	63.88	7.74	0.50—1.80 (14H, m), 0.69 (3H, t, J=7.5 Hz), 2.12 (2H, br d, J=12 Hz),
		(64.03	7.93)	4.88 (2H, s), 7.40—7.76 (3H, m), 7.85—8.10 (2H, m)
t-4c	$C_{19}H_{28}O_4S$	64.74	8.01	0.58—1.86 (16H, m), 0.67 (3H, t, J=7.5 Hz), 2.45 (3H, s), 4.85 (2H, s),
		(64.94	8.05)	7.34 (2H, d, J=8.3 Hz), 7.84 (2H, d, J=8.3 Hz)
c-4c	$C_{19}H_{28}O_4S$	64.74	8.01	0.55—1.84 (14H, m), 0.68 (3H, t, J=7.5 Hz), 2.10 (2H, br d, J=12.5 Hz),
		(64.85	8.03)	2.44 (3H, s), 4.84 (2H, s), 7.34 (2H, d, J=8.3 Hz), 7.85 (2H, d, J=8.3 Hz)
t-4d	$C_{18}H_{26}O_4S$	63.88	7.74	0.75—1.80 (10H, m), 0.85 (6H, d, J=6.3 Hz), 1.12 (3H, s), 4.92 (2H, s),
		(63.95	7.77)	7.40—7.78 (3H, m), 7.88—8.10 (2H, m)
c-4d	$C_{18}H_{26}O_4S$	63.88	7.74	0.58—1.76 (8H, m), 0.79 (6H, d, J=6.3 Hz), 1.04 (3H, s), 2.12 (2H, br d,
		(64.01	7.85)	J=12 Hz), 4.91 (2H, s), 7.40—7.78 (3H, m), 7.90—8.10 (2H, m)
t-4e	$C_{19}H_{28}O_4S$	64.74	8.01	0.76—1.80 (12H, m), 0.84 (6H, d, J=6.3 Hz), 1.12 (3H, s), 4.92 (2H, s),
		(64.67	8.22)	7.40—7.78 (3H, m), 7.85—8.08 (2H, m)
c-4e	$C_{19}H_{28}O_4S$	64.74	8.01	0.58—1.78 (10H, m), 0.82 (6H, d, J=6.3 Hz), 1.04 (3H, s), 2.08 (2H, br d,
		(64.83	8.02)	J=13 Hz), 4.90 (2H, s), 7.40—7.78 (3H, m), 7.85—8.08 (2H, m)

a) All IR spectra in $CHCl_3$ of these compounds showed bands at 1720 (CO) and at 1345 and 1185 (SO_3) cm^{-1}.

and the *cis*-arenesulfonates (*t*-4 and *c*-4), respectively, in fair yields. The structual assignments of *t*-4 and *c*-4 were performed in the same way as those of *t*-3 and *c*-3.

2-(4,4-Dialkylcyclohex-1-yl)-2-oxoethyl arenesulfonates (7a—e) were prepared by the

treatment of the acid chlorides of the corresponding 4,4-dialkylcyclohexanecarboxylic acids (**5**) with diazomethane followed by reaction with arenesulfonic acids in the same manner as used for the preparation of **4** (Chart 2).

In the arenesulfonates (**7**), the conformation of the carbonyl function at the 1-position on the cyclohexane ring was assigned as equatorial on the basis of the following observations; the ^{1}H-NMR spectrum of **7a** showed a peak at 2.40 ppm (1H, br m) due to the axial proton at the 1-position on the cyclohexane ring, and this value is very similar to that of the axial proton of the previously reported 2-(*trans*-4-alkylcyclohexyl)-2-oxoethyl arenesulfonate[1d] (δ *ca.* 2.46 ppm). Moreover, the ^{13}C-NMR spectrum of **7a** showed a peak at 205.5 ppm(s) due to the equatorial carbonyl function at the 1-position on the cyclohexane ring, and this value was similar to that of *t*-**4d** (δ 205.7 ppm).

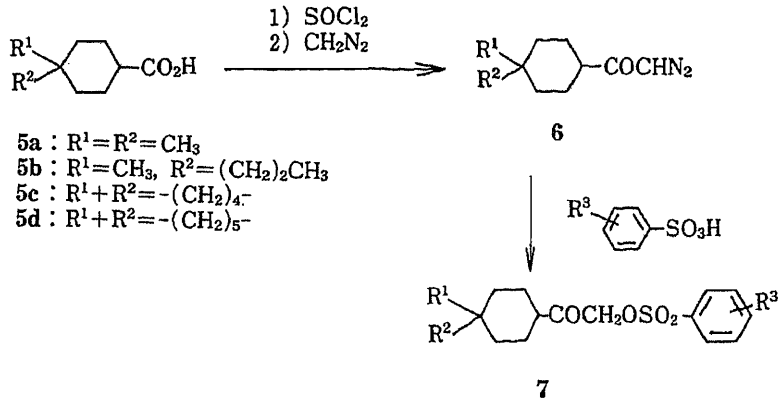

Chart 2

TABLE IV. Physical Data for **6**

Compd.[a] No.	R^1	R^2	MS (M^+)	^{1}H-NMR ($CDCl_3$) δ ppm
6a	CH_3	CH_3	180	0.91 (6H, s), 1.00—1.80 (8H, m), 2.20 (1H, br m), 5.26 (1H, s)
6b[b]	CH_3	$CH_2CH_2CH_3$	208	0.80—1.00 (6H, m), 1.00—1.80 (12H, m), 2.20 (1H, br m), 5.27 (1H, s)
6c	$-(CH_2)_4-$		206	1.00—2.00 (16H, m), 2.21 (1H, br m), 5.27 (1H, s)
6d	$-(CH_2)_5-$		220	0.80—1.96 (18H, m), 2.21 (1H, br m), 5.28 (1H, s)

a) All compounds were yellowish oils. All IR spectra in $CHCl_3$ of these compounds showed bands at 2100 (CHN_2) and 1620 (CO) cm^{-1}. *b*) This product is a diastereoisomeric mixture.

TABLE V. Inhibitory Activities on Enzymes, and Hypolipidemic Effect of **7**

Compd. No.	R^1	R^2	R^3	Yield[b] (%)	mp (°C)	Recryst.[d] solv.	Inhibition: Esterase[e] IC_{50} (μM)	Inhibition: Chymotry.[f] 1×10^{-4} M (%)	Reduction[g] Trigly.[h]
7a	CH_3	CH_3	H	78	34—35	PE–E	0.0058	71	78
7b	CH_3	CH_3	2,4,6-$(CH_3)_3$	54	70—71	Et	0.0020	18	—[i]
7c[a]	CH_3	$(CH_2)_2CH_3$	H	68	Oil[c]	—	0.0042	34	95
7d	$-(CH_2)_4-$		H	67	67—68	Et–W	0.022	41	84
7e	$-(CH_2)_5-$		H	70	60—61	Et–W	0.0054	69	94

a) This product is a diastereoisomeric mixture. *b*) Yield from the corresponding diazoketone (**6**). *c*—*i*) See the corresponding footnotes in Table II.

TABLE VI. Physical Data for 7

Compd.[a] No.	Formula	Analysis (%) Calcd (Found) C	H	^{1}H-NMR ($CDCl_3$) δ ppm
7a	$C_{16}H_{22}O_4S$	61.90 (61.62)	7.14 (7.11)	0.87 (3H, s), 0.91 (3H, s), 1.00—1.78 (8H, m), 2.40 (1H, br m), 4.63 (2H, s), 7.50—7.79 (3H, m), 7.90—8.00 (2H, m)
7b	$C_{19}H_{28}O_4S$	64.74 (64.69)	8.00 (7.67)	0.87 (3H, s), 0.91 (3H, s), 1.10—1.80 (8H, m), 2.32 (3H, s), 2.50 (1H, br m), 2.64 (6H, s), 4.54 (2H, s), 6.99 (2H, s)
7c	$C_{18}H_{26}O_4S$	63.87 (63.97)	7.74 (7.81)	0.80—1.00 (6H, m), 1.00—1.80 (12H, m), 2.41 (1H, br m), 4.52, 4.64 (2H, ss), 7.50—7.69 (3H, m), 7.90—8.00 (2H, m)
7d	$C_{18}H_{24}O_4S$	64.25 (64.68)	7.19 (7.08)	1.00—1.80 (16H, m), 2.42 (1H, br m), 4.63 (2H, s), 7.51—7.77 (3H, m), 7.90—8.00 (2H, m)
7e	$C_{19}H_{26}O_4S$	65.11 (64.70)	7.47 (7.70)	0.80—1.80 (18H, m), 2.40 (1H, br m), 4.63 (2H, s), 7.50—7.69 (3H, m), 7.90—8.00 (2H, m)

a) All IR spectra in $CHCl_3$ of these compounds showed bands at 1730 (CO) and at 1360 and 1180 (SO_3) cm^{-1}.

Enzyme Inhibitory Activity (*in Vitro* Experiments)

Methyl butyrate and *N*-acetyltyrosine ethyl ester (ATEE) were used as substrates for determination of the activities of esterase[5] and chymotrypsin,[5] respectively (Tables II and V).

Pharmacological Examination (*in Vivo* Experiments)

Male Wistar rats (7 weeks old) were used, with five animals in each experimental group. A test compound (0.3 mmol) was mixed with 5 ml of olive oil, and the mixture was orally administered to rats at the dose of 0.3 mmol per kg. Blood for determination of the plasma triglyceride was taken from the orbital vein of the rats at 2 h after the administration. Plasma triglyceride was analyzed by using a commercially available analysis kit (Determiner TG-S Kyowa[6]). Decreases of triglyceride were expressed as percentage values with respect to the control value obtained by using olive oil containing no test compound (Tables II and V).

Results and Discussion

The physical and biological data for *t*-**4**, *c*-**4** and **7** are listed in Tables II, III, V and VI.

i) In the series of the arenesulfonates (**4**) (Table II), potency of esterase inhibition by *t*-**4** was rather similar to that (IC_{50}; 6.5×10^{-8} to 4.6×10^{-6} M) reported for *trans*-arenesulfonates.[1d] However, there was a remarkable difference of esterase-inhibitory activity between *t*-**4** and *c*-**4**, as shown in Table II. In the case of **4d**, the *trans*-isomer exhibited esterase-inhibitory activity about 6200 times more potent than that of the *cis*-isomer. Moreover, as regards the plasma triglyceride-reducing effect *in vivo*, the *trans*-isomers (*t*-**4d** and *t*-**4e**) showed a hypolipidemic action about 1.5 and 10 times more potent, respectively, than those of the *cis*-isomers (*c*-**4d** and *c*-**4e**). Chymotrypsin-inhibitory activity was low in both cases (*t*-**4** and *c*-**4**).

ii) In the series of the arenesulfonates (**7**) (Table V), most of the compounds except for **7d** showed about 10 times more potent esterase-inhibitory activity as well as potent hypolipidemic action (**7c** and **7e** gave 95% and 94% reductions of plasma triglyceride) as compared with the reported *trans*-arenesulfonates.[1d]

Conclusion

A large difference in biological activities between *trans*- and *cis*-2-(1,4-dialkylcyclohex-1-

yl)-2-oxoethyl arenesulfonates (*t*-**4** and *c*-**4**) was observed. Further studies on the relationships between the stereo-structure and the esterase-inhibitory activity in these compounds are continuing, and we believe that investigations of the three-dimensional structure of the esterase active site would also be fruitful. Moreover, the series of 2-(4,4-dialkylcyclohex-1-yl)-2-oxoethyl arenesulfonates (**7**) showed more potent biological activities than the reported arenesulfonates.[1d] Among the compounds **7**, detailed studies on the hypolipidemic action of **7c** and **7e** in dogs are in progress.

Experimental

All melting points were recorded with a Yanagimoto micromelting point apparatus and are uncorrected. Spectral data were obtained as follows: IR spectra with a Hitachi 260-50 spectrophotometer; mass spectra (MS) with a JEOL LMS-01G-2 spectrometer; ^{1}H-NMR and ^{13}C-NMR spectra with a JEOL JMN-FX 100 spectrometer (using tetramethylsilane as an internal standard). Chemical shifts of ^{1}H-NMR signals are given in δ values (ppm).

Starting Materials: 4-Methyl-4-propylcyclohexanecarboxylic Acid (5b)——The bromination of 1,5-dihydroxy-3-methyl-3-propylpentane[7] (73.0 g) with phosphorus tribromide (90.0 g) according to Bartleson *et al.*[8] gave 1,5-dibromo-3-methyl-3-propylpentane. Yield, 58.0 g (44%). bp 130-132 °C/30 mmHg. MS *m/z*: 284 (M^+). ^{1}H-NMR ($CDCl_3$): 0.70—1.18 (6H, m), 1.18—2.06 (8H, m), 4.20—4.54 (2H, m), 4.56—4.80 (2H, m). The title compound (**5b**) was prepared by alkylation of diethyl malonate (16.0 g) with 1,5-dibromo-3-methyl-3-propylpentane (29.0 g) followed by hydrolysis and decarboxylation according to Rice *et al.*[9] Yield, 6.0 g (32%). bp 133—138 °C/2 mmHg. MS *m/z*: 184 (M^+). ^{1}H-NMR ($CDCl_3$): 0.84 (3H, s), 0.87 (3 H, t, J=7.5 Hz), 1.00—1.90 (12H, m), 2.26 (1H, br m), 9.82 (1H, br s). 4,4-Dimethylcyclohexanecarboxylic acid (**5a**),[10] spiro[4.5]decane-8-carboxylic acid (**5c**)[9] and spiro[5.5]undecane-3-carboxylic acid (**5d**)[9] were prepared from 1,5-dibromo-3,3-dimethylpentane,[11] 1,1-bis(2-bromoethyl)-cyclopentane[12] and 1,1-bis(2-bromoethyl)cyclohexane,[12] respectively.

Stereoisomeric Mixture of 4-Isopropyl-1-methylcyclohexanecarboxylic Acid (2c)——Typical Procedure for the Preparation of **2a—d**: A solution of butyllithium in hexane (160 ml of 1.42 M) was added dropwise to a solution of diisopropylamine (32.5 ml) and tetrahydrofuran (200 ml) at below −20 °C under a nitrogen atmosphere. The mixture was stirred for 0.5 h, then 4-isopropylcyclohexanecarboxylic acid (**1b**)[1c] (18.0 g) was added dropwise while maintaining the reaction temperature at below 0 °C. A milky white solution was formed and became homogeneous after the addition of hexamethylphosphorictriamide (HMPA) (24.0 g). After being stirred for 1 h at room temperature, the reaction mixture was cooled again to below −20 °C and then methyl iodide (18.2 g) was added dropwise. The mixture was stirred for 2 h at room temperature, then 10% HCl (300 ml) was added and the whole was extracted with chloroform (100 ml × 2). The chloroform layer was dried over sodium sulfate and evaporated under reduced pressure to give **2c**, which was purified by distillation. Yield, 15.0 g (77%). bp 122-123 °C/2 mmHg (lit.,[13] bp 79—80 °C/0.3 mmHg). MS *m/z*: 184 (M^+). Similar procedures were used for the preparation of 4-ethyl-1-methylcyclohexanecarboxylic acid (**2a**), 1,4-diethyl-cyclohexanecarboxylic acid (**2b**) and 4-isobutyl-1-methylcyclohexanecarboxylic acid (**2d**).

2a: Yield, 91%. bp 123-124 °C/4 mmHg (lit.,[13] bp 87—90 °C/1 mmHg). MS *m/z*: 170 (M^+). **2b**: Yield, 33%. bp 125—128 °C/4 mmHg. ^{1}H-NMR ($CDCl_3$): 0.86, 0.87 (6H, tt, J=6.3 Hz), 1.00—2.40 (13H, m), 10.17 (1H, br s). IR ($CHCl_3$): 1690 (CO) cm^{-1}. MS *m/z*: 184 (M^+). *Anal.* Calcd for $C_{11}H_{20}O_2$: C, 71.70; H, 10.94. Found: C, 71.81; H, 10.87. **2d**: Yield, 76%. bp 126—128 °C/2.5 mmHg (lit.,[13] bp 110—115 °C/0.5 mmHg). MS *m/z*: 198 (M^+).

***r*-1-Diazoacetyl-*t*-4-isopropyl-1-methylcyclohexane (*t*-3c) and *r*-1-Diazoacetyl-*c*-4-isopropyl-1-methylcyclohexane (*c*-3c)**——Typical Procedure for the Preparation of the Stereoisomeric mixture (**3a—d**) and Separation into the *trans*-(*t*-**3a—d**) and the *cis*-Isomer (*c*-**3a—d**): A mixture of thionyl chloride (50 ml) and **2c** (3.6 g) was stirred for 2 h under reflux, and then the reaction mixture was evaporated under reduced pressure. The residue (stereoisomeric mixture of 4-isopropyl-1-methyl-1-cyclohexanecarbonyl chloride) was added dropwise to an ethereal solution (150 ml) of diazomethane (obtained from 14 g of nitrosomethylurea) under stirring with ice-cooling. After being stirred for 1 h, the reaction mixture was evaporated under reduced pressure to give **3c** as a crude oil (stereoisomeric mixture). **3c** (4.0 g) was chromatographed on a long silica gel column with chloroform as an eluent. From the first eluate, the *cis*-isomer (*c*-**3c**) was obtained as a yellowish oily product. Yield, 1.8 g (45%). IR ($CHCl_3$): 2100 (CN_2), 1620 (CO) cm^{-1}. ^{13}C-NMR ($CDCl_3$): 19.9 (q), 27.0 (t), 28.9 (q), 32.7 (d), 36.1 (t), 43.7 (d), 47.2 (s), 52.2 (d), 200.4 (s). From the second eluate, the *trans*-isomer (*t*-**3c**) was obtained as a yellowish oily product. Yield, 1.6 g (40%). IR ($CHCl_3$): 2100 (CN_2), 1620 (CO) cm^{-1}. ^{13}C-NMR ($CDCl_3$): 19.9 (q), 20.3 (q), 24.8 (t), 32.5 (d), 33.8 (t), 43.6 (d), 45.7 (s), 51.8 (d), 202.0 (s). Similar procedures were used for the preparation of other stereoisomeric mixtures (**3a**, **3b** and **3d**) and their separation into *t*-**3a**, *t*-**3b** and *t*-**3d** and *c*-**3a**, *c*-**3b** and *c*-**3d**. Other data are listed in Table I.

2-(*t*-4-Isopropyl-1-methylcyclohex-*r*-1-yl)-2-oxoethyl Benzenesulfonate (*t*-4d) and 2-(*c*-4-Isopropyl-1-methylcyclohex-*r*-1-yl)-2-oxoethyl Benzenesulfonate (*c*-4d)——Typical Procedure for the Preparation of *t*-**4a—e** and *c*-**4a—e**: Benzenesulfonic acid monohydrate (1.6 g) was added to an ethereal solution (50 ml) of the *trans*-diazoketone

(*t*-**3c**) (1.5 g) under ice-cooling. After being stirred for 1 h at room temperature, the reaction mixture was washed with water and dried over sodium sulfate. The ethereal layer was evaporated under reduced pressure. The crude product was purified by chromatography on a silica gel column with chloroform as an eluent, followed by recrystallization from aqueous ethanol to give the *trans*-arenesulfonate (*t*-**4d**). Yield, 1.6 g (66%). Similar procedures were used for the preparation of *t*-**4a**—**c** and *t*-**4e** and *c*-**4a**—**e**. ^{13}C-NMR ($CDCl_3$): *t*-**4d**: 19.0 (q), 19.8 (q), 24.2 (t), 32.3 (d), 32.6 (t), 43.3 (d), 46.3 (s), 68.5 (t), 128.0 (d), 129.2 (d), 134.0 (d), 135.9 (s), 205.7 ppm (s). *c*-**4d**: 19.8 (q), 26.9 (t), 27.6 (q), 32.4 (d), 35.0 (t), 43.3 (d), 47.6 (s), 68.8 (t), 128.0 (d), 129.1 (d), 134.0 (d), 136.0 (s), 204.7 ppm (s). Other data are listed in Tables II and III.

1-Diazoacetyl-4,4-dimethylcyclohexane (6a)——Typical Procedure for the Preparation of **6a**—**d**: The title compound (**6a**) was prepared starting from **5a** (3.0 g) in the same manner as described for **3c**. Yield, 3.2 g (92%). MS *m/z*: 180 (M^+). IR ($CHCl_3$): 2100 (CN_2), 1620 (CO) cm^{-1}. Similar procedures were used for the preparation of **6b**—**d**. Other data are listed in Table IV.

2-(4,4-Dimethylcyclohex-1-yl)-2-oxoethyl Arenesulfonate (7a)——Typical Procedure for the Preparation of **7a**—**e**: The title compound (**7a**) was prepared from **6a** (2.0 g) and benzenesulfonic acid monohydrate (3.0 g) in the same manner as described for *t*-**4**. Yield, 2.7 g (78%). MS *m/z*: 310 (M^+). ^{13}C-NMR ($CDCl_3$): 23.9 (t), 24.3 (q), 29.7 (s), 32.3 (q), 38.1 (t), 46.8 (d), 69.9 (t), 128.0 (d), 129.3 (d), 134.2 (d), 135.7 (s), 205.5 (s). Similar procedures were used for the preparation of **7b**—**e**. Other data are listed in Tables V and VI.

Enzyme-Inhibitory Activities——The esterase- and chymotrypsin-inhibitory activities were determined by the methods described in previous paper.[1]

Pharmacology——The triglyceride level in plasma was measured by the method described in a preceding paper.[1c]

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References

1) *a*) K. Ogawa, T. Terada, Y. Muranaka, T. Hamakawa, S. Hashimoto, and S. Fujii, *Chem. Pharm. Bull.*, **34**, 1118 (1986); *b*) *Idem*, *ibid.*, **34**, 3252 (1986); *c*) K. Ogawa, T. Terada, Y. Muranaka, T. Hamakawa, and S. Fujii, *ibid.*, **35**, 2426 (1987); *d*) Part IV: K. Ogawa, T. Terada, Y. Muranaka, T. Hamakawa, S. Ohta, M. Okamoto, and S. Fujii, *ibid.*, **35**, 3276 (1987).
2) *a*) A. C. Frazer, *Physiol. Revs.*, **26**, 103 (1946); *b*) A. F. Hofmann and B. Borgström, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **21**, 43 (1962).
3) P. E. Pfeffer, L. S. Silbert, and J. M. Chirinko, *J. Org. Chem.*, **37**, 451 (1972).
4) W. G. Schindel and R. E. Pincock, *J. Org. Chem.*, **35**, 1789 (1970).
5) *a*) M. Muramatu and S. Fujii, *J. Biochem.* (Tokyo), **64**, 807 (1968); *b*) M. Muramatu, T. Onishi, S. Makino, Y. Hayakumo, and S. Fujii, *J. Biochem.* (Tokyo), **58**, 214 (1965); *c*) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949).
6) S. Okubo, F. Masige, S. Kamei, A. Okubo, and M. Yamanaka, *Rinshyo Kensa*, **27**, 329 (1983).
7) G. J. Handley, E. R. Nelson, and T. C. Somers, *Aust. J. Chem.*, **13**, 127 (1960).
8) J. D. Bartleson, R. E. Burk, and H. P. Lankelma, *J. Am. Chem. Soc.*, **68**, 2513 (1946).
9) L. M. Rice, B. S. Sheth, and T. B. Zalucky, *J. Med. Chem.*, **15**, 548 (1972).
10) Wellcome Foundation Ltd., Japan. Patent 205341 (1984) [*Chem. Abstr.*, **102**, 113082*w* (1985)].
11) L. Schmerling and J. P. West, *J. Am. Chem. Soc.*, **74**, 2885 (1952).
12) M. E. Freed and L. M. Rice, *J. Heterocycl. Chem.*, **2**, 214 (1965).
13) Hisamitsu Pharmaceutical Co., Ltd., Japan. Patent 98745 (1979) [*Chem. Abstr.*, **92**, 214972*s* (1980)].

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A Facile Method for Synthesis of *N*-Acyloxymethyl-5-fluorouracils, as a Class of Antitumor Agents[1)]

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Antitumor-active derivatives of 5-fluorouracil were prepared *via* a new method by introducing an acyloxymethyl group at the 1-, 3-, or 1,3-position(s). These derivatives were obtained by condensing 1,3-bis(hydroxymethyl)-5-fluorouracil with various short-/long-chain carboxylic acids or their derivatives, in the presence of dicyclohexylcarbodiimide and a catalytic amount of *N,N*-dimethylaminopyridine. Some of the derivatives showed strong antitumor activity against the leukemia L1210 system when administered orally.

Keywords——5-fluorouracil; 1,3-bis(hydroxymethyl)-5-fluorouracil; acyloxymethyl-5-fluorouracil; dicyclohexylcarbodiimide; antitumor agent; 1-undecenoyloxymethyl-5-fluorouracil

Various types of 5-fluorouracil derivatives have been used as antitumor agents in recent years. For example, 1,3-bis(hydroxyalkyl)-5-fluorouracils (Fig. 1) have been described as anticancer compounds[3)] and long-chain alkenoyl-5-fluorouracils as blood platelet aggregation and neoplasm inhibitors.[4)] Buur *et al.*[5)] have also studied various *N*-acyloxymethyl-5-fluorouracils to assess their potential as prodrugs with the aim of enhancing the delivery characteristics of the parent drug, 5-fluorouracil. In the course of our synthetic studies on 5-fluorouracil derivatives, aimed at obtaining more biologically active and less toxic compounds than 5-fluorouracil itself, we have synthesized and tested a series of compounds such as 1-carbamoyl-,[6)] 1-acyloxyalkyl-,[7)] 1-alkylthiocarbonyl-,[8)] and α-alkoxyalkyl-5-fluorouracils[9)] (Fig. 2). Among our synthesized compounds, 1-hexylcarbamoyl-5-fluorouracil (HCFU, Mifurol) is in clinical use in Japan. HCFU is remarkably effective on colorectal, lung, breast, and gastric cancers,[10-12)] but its side effects, such as hot sensation and pollakiuria syndrome, mean that it is still desirable to develop a better drug.

During our previous studies it was found that *N*-acyloxyalkyl-5-fluorouracil derivatives showed very strong antitumor activity. Among them the compounds having an acyloxymethyl group at the N-1 position were markedly active. These results prompted us to develop a new and convenient method for the preparation of 1-acyloxymethyl-5-fluorouracils.

These acyloxymethyl derivatives are known[5)] to be easily hydrolyzed under slightly basic conditions to afford 5-fluorouracil (**1**) in quantitative yield *via* an unstable *N*-hydroxymethyl-

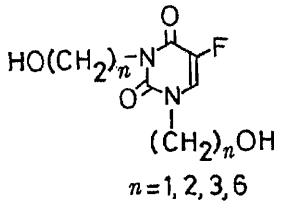
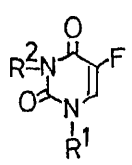


Fig. 1



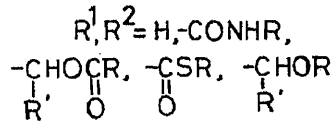


Fig. 2

5-fluorouracil intermediate. Consequently these compounds are thought to be suitable for use as antitumor drugs because the generation of **1** is necessary for antitumor activity after administration of the drug. It has also been reported[5] that the hydrolytic rate of acyloxymethyl-5-fluorouracil derivatives increases in the presence of human plasma or rat liver homogenate, which further suggests the utility of these derivatives as prodrugs. These derivatives have high lipophilicity, which is the predominant factor controlling the permeability of the drug through the biomembrane. It may also be possible to obtain prodrugs with various physicochemical properties such as lipophilicity just by varying the acyl part of these derivatives.

Keeping these points in mind, we have been studying the syntheses of *N*-acyloxymethyl-5-fluorouracil derivatives, especially those with an acyloxymethyl group at the N-1 position. Now we wish to describe a new synthesis of *N*-acyloxymethyl-5-fluorouracil derivatives and the antitumor activity of these compounds against the leukemia L1210 system.

Results and Discussion

This method of acyloxymethylation involves a two-step process (Chart 1). The first step was the preparation of 1,3-bis(hydroxymethyl)-5-fluorouracil (**2**). Compound **1** was heated with formalin for about 45 min below 70 °C to give **2** in quantitative yield. In the second step the various carboxylic acids (**3a—m**) were coupled with **2** in the presence of dicyclohexylcar-

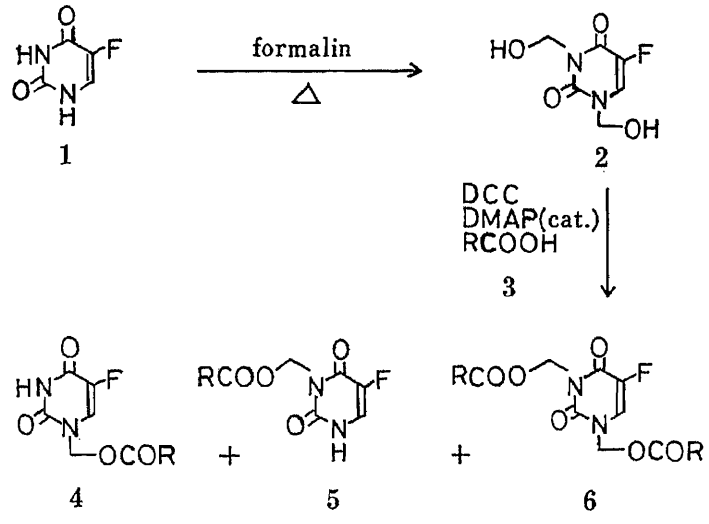
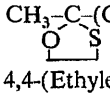
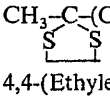


Chart 1

bodiimide (DCC) and a catalytic amount of *N*,*N*-dimethylaminopyridine (DMAP). The choice of solvent depended upon the solubility of the carboxylic acids (**3**). In most cases, CH_3CN was used. In a few cases, mixed solvents were used. The reaction was usually completed in 4—6 h with stirring at room temperature, but in some cases, the reaction was slow at room temperature. In these cases, raising the temperature (40 °C) and increasing the reaction time improved the yield.

The advantage of the present method over the previous one[7] is that long-chain carboxylic acids could be condensed easily with **2** even in the presence of many functional groups such as hydroxyl and keto groups, oxathiolane and dithiolane, and a carbon–carbon double bond in the carbon chain. Since the reaction conditions were very mild, these functionalities remained intact. The previous method for the preparation and the purification of α-chloroalkyl carboxylates of medium- and long-chain acids was troublesome, especially in

TABLE I. Synthesis of *N*-Acyloxymethyl-5-fluorouracil Derivatives

	Carboxylic acid	Conditions	Product	Yield (%)	mp (°C)
3a	$CH_2=CHCH_2COOH$ 3-Butenoic acid	CH_3CN 0—r.t., 4h	**4a**	59	87—88
3b	$CH_3CH_2CH=CHCH_2COOH$ 3-Hexenoic acid	CH_3CN r.t., 4h	**4b**	75	88
3c	$CH_3(CH_2)_4CH=CHCH_2COOH$ 3-Nonenoic acid	CH_3CN r.t., 4h	**4c**	65	92—93
3d	$CH_2=CH(CH_2)_8COOH$ 10-Undecenoic acid	CH_3CN r.t., 4h	**4d** **5d** **6d**	79 Trace 11	100 67—68 Liquid
3e	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$ Oleic acid	CH_3CN r.t., 4h	**4e** **6e**	61 11	91 Liquid
3f	$CH_3(CH_2)_3(CH_2CH=CH)_2(CH_2)_7COOH$ Linoleic acid	CH_3CN r.t., 4h	**4f**	65	Semisolid
3g	$CH_3(CH_2)_{16}COOH$ Stearic acid	$DMF+CH_2Cl_2$ r.t.—40°C, 24h	**4g**	54	114
3h	$C_6H_5CH_2OCONHCH_2COOH$ *N*-Carbobenzyloxyglycine	CH_3CN r.t., 4h	**4h**	65	128—129
3i	$HOOC(CH_2)_8COOH$ Sebacic acid	CH_3CN r.t., 6h	**4i**	51	177—178
3j	$CH_3(CH_2)_5CH(OH)(CH_2)_{10}COOH$ 12-Hydroxystearic acid	$DMF+CH_3CN$ r.t.—40°C, 24h	**4j**	49	98
3k	$CH_3CO(CH_2)_2COOH$ Levulinic acid	CH_3CN r.t., 4h	**4k** **6k**	66 10	119 Liquid
3l	 4,4-(Ethyleneoxothio)-pentanoic acid	CH_3CN r.t., 2h	**4l**	75	114
3m	 4,4-(Ethylenedithio)-pentanoic acid	CH_3CN r.t., 2h	**4m**	79	142

r.t., room temperature.

the case of carboxylic acids having different types of functional groups in the chain.

A further advantage of our method is that 1-substituted derivatives (**4**) were obtained as a major product and in some cases as the sole product. The yield of **4** varied in the range of 49—79% and was maximum when 1.1—1.2 eq of **3** was used. A very small amount of bis product was formed, because there is a large difference in reactivity between the two *N*-hydroxymethyl groups of **2**. Since the hydroxymethyl group was very unstable and hydrolyzed easily to leave the free N–H group, we obtained 1-acyloxymethyl-5-fluorouracil as a major product after work-up of the reaction mixture.

The *N*-acyloxymethylated products (**4**, **5**, and **6**) were obtained in a pure state and gave elemental analysis, infrared (IR), and nuclear magnetic resonance (NMR) data (detailed in Table II) in agreement with their proposed structures. The 3-substituted products were characterized by the NMR and IR data as described earlier.[9]

The antitumor activity of thirteen compounds against the leukemia L1210 system was examined, and the results are summarized in Table III. We found that 5-fluorouracil derivatives having a 10-undecenoyloxymethyl group at the N-1 and N-3 positions (**4d** and **5d**, respectively) were the best antitumor agents among the tested derivatives. These two

TABLE II. Elemental Analysis and Spectral Data of *N*-Acyloxymethyl-5-fluorouracils

Compd.	Molecular formula	Analysis (%) Calcd (Found) C	H	N	IR (Nujol) cm^{-1}	^{1}H-NMR *J* (Hz)	NMR solvent[a]
4a	$C_9H_9FN_2O_4$	47.38 (46.06)	3.98 4.16	12.28 11.98)	3200, 1735, 1700, 1645	3.16 (2H, d, J=6, $C\underline{H}_2CH=CH_2$), 5.2 (2H, m, $CH=C\underline{H}_2$), 5.62 (2H, s, N–CH_2), 5.8—6.0 (1H, m, $C\underline{H}=CH_2$), 7.62 (1H, d, J=5.6, C_6-H), 9.85 (1H, br, NH)	C
4b	$C_{11}H_{13}FN_2O_4$	51.56 (51.42	5.11 5.07	10.93 11.08)	3120, 1705, 1650, 1370	0.99 (3H, t like, CH_3), 2.19 (2H, m, $C\underline{H}_2-CH_3$), 3.12 (2H, d, J=6, $OCOCH_2$), 5.5 (2H, m, $C\underline{H}=C\underline{H}$), 5.6 (2H, s, N–$CH_2$), 7.73 (1H, d, J=5.5, C_6-H), 10.06 (1H, br, NH)	C
4c	$C_{14}H_{19}FN_2O_4$	56.37 (56.48	6.42 6.46	9.39 9.47)	3200, 1720, 1700, 1650	0.88 (3H, t like, CH_3), 1.28 (6H, brs, chain CH_2), 2.05 (2H, m, $C\underline{H}_2CH=CH$), 3.1 (2H, d, J=5.6, $OCOCH_2$), 5.5 (2H, m, $C\underline{H}=C\underline{H}$), 5.6 (2H, s, N–$CH_2$), 7.6 (1H, d, J=5.5, C_6-H), 9.8 (1H, br, NH)	C
4d	$C_{16}H_{23}FN_2O_4$	58.89 (58.77	7.10 7.17	8.58 8.51)	3150, 1750, 1730, 1700, 1650	1.28 (12H, s, chain CH_2), 2.05 (2H, m, $C\underline{H}_2CH=CH_2$), 2.4 (2H, t, J=7, $OCOCH_2$), 4.9—5.1 (2H, m, $CH=C\underline{H}_2$), 5.62 (2H, s, N–CH_2), 5.65—6.0 (1H, m, $C\underline{H}=CH_2$), 7.6 (1H, d, J=5.6, C_6-H), 9.4 (1H, br, NH)	C
5d	$C_{16}H_{23}FN_2O_4$	58.89 (58.70	7.10 6.95	8.58 8.35)	3200, 1760, 1740, 1720, 1680, 1640	1.25 (12H, s, chain CH_2), 2.02 (2H, m, $C\underline{H}_2CH=CH_2$), 2.3 (2H, t, J=7, $OCOCH_2$), 4.69—5.12 (2H, m, $CH=C\underline{H}_2$), 5.6 (2H, s, N–CH_2), 5.7—6.11 (1H, m, $C\underline{H}=CH_2$), 7.43 (1H, d, J=4.5, C_6-H), 9.98 (1H, brs, NH)	C
6d	$C_{28}H_{43}FN_2O_6$	64.35 (64.10	8.29 8.30	5.36 5.52)	1740, 1700, 1650	0.98—2.58 (32H, m, chain CH_2), 4.68—5.15 (4H, m, 2×($CH=C\underline{H}_2$)), 5.52—6.08 (6H, m, 2×(N–$C\underline{H}_2$, $C\underline{H}=CH_2$)), 7.63 (1H, d, J=5.5, C_6-H)	C
4e	$C_{23}H_{37}FN_2O_4$	65.07 (64.95	8.78 8.81	6.59 6.49)	3250, 1740, 1700, 1660	0.92 (3H, t like, CH_3), 1.3 (22H, brs, chain CH_2), 1.9—2.3 (6H, m, $OCOC\underline{H}_2$, $C\underline{H}_2CH=CHC\underline{H}_2$), 5.25 (2H, m, $C\underline{H}=C\underline{H}$), 5.6 (2H, s, N–$CH_2$), 7.6 (1H, d, J=6, C_6-H), 9.75 (1H, br, NH)	C
6e	$C_{42}H_{71}FN_2O_6$	70.16 (70.27	9.95 9.78	3.89 3.89)	1740, 1695, 1640	0.95 (6H, t like, CH_3), 1.3 (44H, brs, chain CH_2), 1.85—2.4 (12H, m, 2×($OCOC\underline{H}_2$, $C\underline{H}_2CH=CHC\underline{H}_2$)), 5.3 (4H, m, 2×($C\underline{H}=C\underline{H}$)), 5.7 (2H, s, N–$CH_2$), 5.9 (2H, s, N–$CH_2$), 7.7 (1H, d, J=7, C_6-H)	C
4f	$C_{23}H_{35}FN_2O_4$	65.38 (64.98	8.35 8.35	6.63 6.59)	2920, 1735, 1700, 1640, 1140	0.9 (3H, t like, CH_3), 1.24 (16H, brs, chain CH_2), 2.04 (4H, m, α to double bonds), 2.4 (2H, t like, $OCOCH_2$), 2.75 (2H, m, $=CH-C\underline{H}_2CH=$), 5.3 (4H, m, 2×$C\underline{H}=C\underline{H}$), 5.6 (2H, s, N–$CH_2$), 7.6 (1H, d, J=6, C_6-H), 9.6 (1H, br, NH)	C
4g	$C_{23}H_{39}FN_2O_4$	64.76 (65.01	9.22 9.30	6.57 6.43)	3150, 1730, 1690, 1650	0.9 (3H, t like, CH_3), 1.25 (30H, brs, chain CH_2), 2.35 (2H, m, $OCOCH_2$), 5.6 (2H, s, N–CH_2), 7.8 (1H, d, J=6, C_6-H), 9.7 (1H, br, NH)	C+D
4h	$C_{15}H_{14}FN_3O_6$	51.29 (51.15	4.02 4.08	11.96 12.01)	3400, 3150, 1745, 1705, 1680, 1650, 1490, 860	3.98 (2H, d, J=6, $COC\underline{H}_2NH$), 5.1 (2H, s, $C_6H_5C\underline{H}_2$), 5.62 (2H, s, $OCOCH_2$–N), 6.5 (1H, brs, $CH_2N\underline{H}CO$), 7.3 (5H, s, aromatic), 7.6 (1H, d, J=5.6, C_6-H), 11.6 (1H, br, NH)	C+D

TABLE II. (continued)

Compd.	Molecular formula	Analysis (%) Calcd (Found) C	H	N	IR (Nujol) cm^{-1}	^{1}H-NMR J (Hz)	NMR solvent[a]
4i	$C_{20}H_{24}F_2N_4O_8$	49.38 (49.64)	4.97 (5.07)	11.52 (11.24)	3300, 3200, 1740, 1720, 1700, 1670, 1640	1.3 (12H, s, chain CH_2), 2.28 (4H, t, J=7, 2×$OCOCH_2$), 5.6 (4H, s, 2×($N-CH_2$)), 7.85 (2H, d, J=6, 2×C_6-H), 11.92 (2H, br, 2× NH).	C+D
4j	$C_{23}H_{39}FN_2O_5$	62.42 (62.60	8.88 9.06	6.33 6.31)	3400, 3200, 1730, 1695, 1650	0.9 (3H, like, CH_3), 1.25 (28H, br s, chain CH_2), 2.4 (2H, t, J=7, $OCOCH_2$), 3.58 (1H, br s, CH–OH), 5.6 (2H, s, $N-CH_2$), 7.6 (1H, d, J=6, C_6-H), 9.7 (1H, br, NH)	C
4k	$C_{10}H_{11}FN_2O_5$	46.52 (46.62	4.29 4.31	10.85 10.53)	3150, 1740, 1720, 1700 1655	2.18 (3H, s, CH_3), 2.5—2.9 (4H, m, $(CH_2)_2$), 5.6 (2H, s, $N-CH_2$), 7.66 (1H, d, J=5.6, C_6-H), 11.66 (1H, br, NH)	C+D
6k	$C_{16}H_{19}FN_2O_8$	49.74 (49.78	4.96 4.85	7.25 7.30)	1740, 1705, 1690, 1665	2.2 (6H, s, 2×CH_3), 2.45—2.9 (8H, m, 2× $(CH_2)_2$), 5.68 (2H, s, $N-CH_2$), 5.96 (2H, s, $N-CH_2$), 7.68 (1H, d, J=6, C_6-H)	C
4l	$C_{12}H_{15}FN_2O_5S$	45.28 (45.47	4.75 4.84	8.80 8.73)	3200, 1710, 1680, 1650, 1435, 1250, 1060	1.58 (3H, s, CH_3), 2.18 (2H, t, J=7, CH_2 α to ring), 2.58 (2H, t, J=7, $OCOCH_2$), 3.05 (2H, t like, CH_2–S of ring), 4.1 (2H, m, CH_2–O of ring), 5.65 (2H, s, $N-CH_2$), 7.65 (1H, d, J=6, C_6-H), 9.9 (1H, br, NH)	C
4m	$C_{12}H_{15}FN_2O_4S_2$	43.12 (43.44	4.52 4.53	8.38 8.34)	3250, 1720, 1700, 1695, 1650, 1445, 1260	1.76 (3H, s, CH_3), 2.18 (2H, t, J=7, CH_2 α to ring), 2.64 (2H, t, J=7, $OCOCH_2$), 3.3 (4H, s, CH_2 of ring), 5.6 (2H, s, $N-CH_2$), 7.84 (1H, d, J=6, C_6-H), 12.24 (1H, br, NH)	C+D

a) C and D represent $CDCl_3$ and DMSO-d_6, respectively.

compounds showed the highest ILS (increase in life span) percentage, when administered orally. When we compared these derivatives with Tegafur, we found that in the case of Tegafur the ILS decreased from 31 to 13 when its dose was increased from 100 to 300 mg/kg/d. This indicated that it had some toxicity at higher dose. However, in the case of **5d** the ILS increased from 20 to 28 at the same doses.

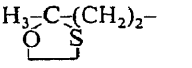
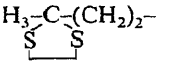
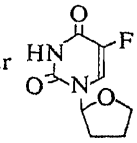
In conclusion, we can say that the *N*-acyloxymethylation of 5-fluorouracil by this new method is a potentially useful approach to obtain effective antitumor agents. The advantages of the present method are the very mild reaction conditions, the easy isolation, and the high yield of the 1-substituted product. 1-Undecenoyloxymethyl and 3-undecenoyloxymethyl-5-fluorouracils, which showed very good results, may serve as better antitumor agents than Tegafur and HCFU. Further testing of some of the *N*-acyloxymethyl-5-fluorouracils is in progress.

Experimental

All melting points were determined on a Buchi melting point apparatus and are uncorrected. ^{1}H-NMR spectra were recorded in $CDCl_3$ and dimethyl sulfoxide-d_6 (DMSO-d_6) (E. Merck) on Hitachi R-24 (60 MHz) and JEOL FX-100 (100 MHz) spectrometers with tetramethylsilane as an internal standard. IR spectra were obtained on a Hitachi EPI G-3 spectrometer.

General Procedure for the Preparation of *N*-Acyloxymethyl-5-fluorouracils: 1-(4-Oxopentanoyloxymethyl)-5-fluorouracil (4k) and 1,3-Bis(4-oxopentanoyloxymethyl)-5-fluorouracil (6k)——5-Fluorouracil (**1**) (1.3 g, 10 mmol) and formalin (1.78 g, 22 mmol) were stirred in a 100 ml round-bottomed flask on an oil bath (about 60 °C; above this temperature the 1,3-bis(hydroxymethyl)-5-fluorouracil decomposed to 5-fluorouracil or polymerized) for about

TABLE III. Antitumor Activity of *N*-Acyloxymethyl-5-fluorouracils against the Leukemia L1210 System

Compd.	R	Dose[a] mg/kg/d	ILS[b] %
4a	$CH_2=CH-CH_2-$	*p.o.* 100	9
4b	$CH_3CH_2CH=CH-CH_2-$	*p.o.* 30	7
4c	$CH_3(CH_2)_4CH=CH-CH_2-$	*p.o.* 100	18
4d	$CH_2=CH(CH_2)_8-$	*p.o.* 30	16
		p.o. 100	28
		p.o. 300	18
5d	$CH_2=CH(CH_2)_8-$	*p.o.* 30	3
		p.o. 100	20
		p.o. 300	28
4e	$CH_3(CH_2)_7CH=CH(CH_2)_7-$	*p.o.* 100	11
		i.p. 100	44
4g	$CH_3(CH_2)_{16}-$	*p.o.* 100	0
		p.o. 300	0
		i.p. 100	53
4h	$C_6H_5CH_2OCONHCH_2-$	*p.o.* 30	0
		p.o. 100	26
		p.o. 300	−2
4i	5-FU-$CH_2OCO(CH_2)_8-$	*p.o.* 100	0
		p.o. 300	6
		i.p. 100	63
4j	$CH_3(CH_2)_5CH(OH)(CH_2)_{10}-$	*p.o.* 100	4
		p.o. 300	29
		i.p. 100	42
4k	$CH_3CO(CH_2)_2-$	*p.o.* 100	23
		i.p. 100	19
4l		*p.o.* 100	4
		p.o. 300	41
		i.p. 100	31
4m		*p.o.* 100	0
		p.o. 300	48
		i.p. 100	25
	HCFU	*p.o.* 30	21
		p.o. 100	50
		p.o. 300	23
	Tegafur 	*p.o.* 30	0
		p.o. 100	31
		p.o. 300	13

a) Abbreviations: i.p. and *p.o.* mean intraperitoneal injection and *per os* (oral) administration, respectively. *b*) ILS means increase in life span; see Experimental.

45 min. The traces of water evolved were removed under reduced pressure. The resulting viscous **2**[13] was dissolved in 30 ml of dry acetonitrile (CH_3CN). To this solution, 4-oxopentanoic (levulinic) acid (1.4 g, 12 mmol) was added followed by DCC (2.47 g, 12 mmol) and a catalytic amount of DMAP. The reaction was monitored by thin layer chromatography (TLC). The reaction mixture was stirred at room temperature for 4 h, then filtered to remove dicyclohexylurea and washed with dichloromethane. The organic layer was wshed with 1 N HCl (3 times), 2:5% sodium bicarbonate and saturated NaCl solution, and dried over anhydrous sodium sulfate. Evaporation of the solvent *in vacuo* afforded a mixture of two major products (2.2 g). The compounds were purified by flash column chromatography on silica gel using methanol and dichloromethane (1:20) as the eluent. The first fraction gave **6k** (0.38 g, 10%) as a viscous oil. The second fraction gave **4k** (1.7 g, 66%) as white crystals. All the reactions were carried out similarly. The reaction time, temperature, solvent, and yield are summarized in Table I. Elemental analysis, IR and ^{1}H-NMR data are given in Table II.

Carboxylic Acids as Starting Materials——All the carboxylic acids used here were commercially available except for **31** and **3m**, which were synthesized from levulinic acid.

Preparation of 4,4-(Epoxyethanothio)pentanoic Acid (31)——A mixture of levulinic acid (2.9 g, 25 mmol) and 2-mercaptoethanol (1.95 g, 25 mmol) was allowed to react in the presence of boron trifluoride etherate (10 ml) at room temperature.[14] TLC monitoring of the reaction mixture showed that reaction completed within 1 h. After addition of a few drops of methanol to stop the reaction, the mixture was worked up with dichloromethane. The organic layer was washed with water and dried over anhydrous sodium sulfate. After evaporation of the solvent, the crude oil was crystallized in hexane–ether, affording **31** as white crystals (4.18 g, 95%). mp 63—64 °C. IR (Nujol): 1680, 1430, 1200, 1040 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.65 (3H, s, CH_3), 2.25 (2H, t, J=7 Hz, CH_2 α to ring), 2.55 (2H, t, J=7 Hz, CH_2COO), 3.0 (2H, t like, CH_2–S of ring), 4.15 (2H, m, CH_2–O of ring), 10.3 (1H, brs, COOH). *Anal.* Calcd for $C_7H_{12}O_3S$: C, 47.71; H, 6.86. Found: C, 47.94; H, 7.05.

Preparation of 4,4-(Ethylenedithio)pentanoic Acid (3m)——In the same manner as described above, levulinic acid (2.33 g, 20 mmol) and ethane 1,2-dithiol (1.88 g, 20 mmol) were allowed to react for 1 h. After final work-up and evaporation of the solvent, the crude oil was crystallized in ethanol, giving **3m** as white crystals (3.76 g, 98%). mp 52—53 °C. IR (Nujol): 1700, 1440, 1265 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.8 (3H, s, CH_3), 2.2 (2H, t, J=7 Hz, CH_2 α to ring), 2.6 (2H, t, J=7 Hz, CH_2COO), 3.3 (4H, s, CH_2 of ring), 11.55 (1H, s, COOH). *Anal.* Calcd for $C_7H_{12}O_2S_2$: C, 43.72; H, 6.29. Found: C, 43.67; H, 6.38.

Activity Test——The tumor system and animals used for the evaluation of the antitumor activity of these derivatives were the same as described in our previous publication[9]:

$$\text{ILS}\ (\%)=(T-C)/C\times 100$$

where T is the average number of days before death in the test group and C is the number of days before death in the control group.[15]

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References and Notes

1) 5-Fluorouracil Derivatives. XIII. Part XII: S. Ozaki, T. Nagase, H. Tamai, H. Mori, A. Hoshi, and M. Iigo, *Chem. Pharm. Bull.*, **35**, 3894 (1987).
2) Postdoctoral Research Fellow (1986—87) of the Ministry of Education, Science and Culture, Japan.
3) F. Changlie, M. Caisong, Y. Hui, X. Jianing, D. Qizhu, and Z. Renxi, *Wuhan Daxue Xuebao Ziran Kexueban*, **1984**, 115 [*Chem. Abstr.*, **102**, 6404*f* (1985)].
4) Terumo Corp, Japan. Patent Open 59-204175 (1984) [*Chem. Abstr.*, **102**, 166771*b* (1985)].
5) A. Buur, H. Bundgaard, and E. Falch, *Int. J. Pharm.*, **24**, 43 (1985).
6) S. Ozaki, Y. Ike, H. Mizuno, K. Ishikawa, and H. Mori, *Bull. Chem. Soc., Jpn.*, **50**, 2406 (1977).
7) S. Ozaki, Y. Watanabe, T. Hoshiko, H. Mizuno, K. Ishikawa, and H. Mori, *Chem. Pharm. Bull.*, **32**, 733 (1984).
8) T. Hoshiko, S. Ozaki, Y. Watanabe, T. Ogasawara, S. Yamauchi, K. Fujiwara, A. Hoshi, and M. Iigo, *Chem. Pharm. Bull.*, **33**, 2832 (1985).
9) S. Ozaki, Y. Watanabe, T. Hoshiko, T. Nagase, T. Ogasawara, H. Furukawa, A. Uemura, K. Ishikawa, H. Mori, A. Hoshi, M. Iigo, and R. Tokuzen, *Chem. Pharm. Bull.*, **34**, 150 (1986).
10) Y. Koyama, *Gan To Kagakuryoho*, **7**, 1181 (1980).
11) M. Nakanishi, Y. Tsutanishi, T. Nakamura, I. Hashimoto, Y. Sawata, M. Kawai, T. Okuno, S. Fujisawa, and M. Taneda, *Yakuri To Chiryo*, **8**, 1211 (1980).
12) M. Yoshida, H. Murai, and S. Miura, *Gan To Kagakuryoho*, **7**, 1203 (1980).
13) The ^{1}H-NMR spectrum of an acetonitrile solution of **2** showed no peak due to the NH proton in the uracil ring, which suggested that **2** contained not more than about 10% 1- or 3-hydroxymethyl-5-fluorouracil, that is, the purity of **2** was better than 90%. Direct acetylation of **2** with excess acetic anhydride in pyridine gave 1,3-bis(acetoxymethyl)-5-fluorouracil in 68% yield, T. Nagase, H. Tamai, and S. Ozaki, unpublished results.
14) L. F. Fieser, *J. Am. Chem. Soc.*, **76**, 1945 (1954).
15) A. Hoshi, M. Iigo, A. Nakamura, and K. Kuretani, *Chem. Pharm. Bull.*, **26**, 161 (1978).

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Synthesis and Antihypertensive Activities of 1,4-Dihydropyridine-5-phosphonate Derivatives. II[1)]

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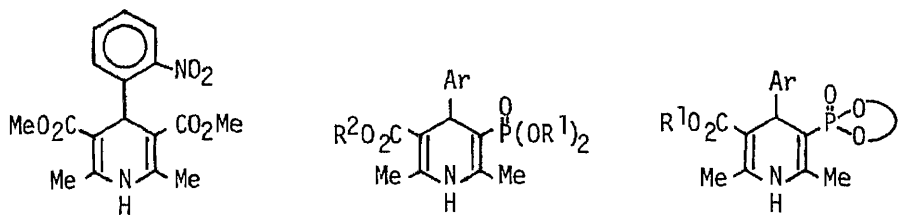
A series of 1,4-dihydropyridine-5-cyclic phosphonate derivatives, designed as analogues of 1,4-dihydropyridine-3,5-dicarboxylate calcium antagonists, was synthesized and examined for antihypertensive activity. Several compounds were proved to have activities superior or comparable to that of nifedipine in lowering blood pressure in normotensive and spontaneously hypertensive rats (SHR). Among these compounds, methyl 2,6-dimethyl-5-(2-oxo-1,3,2-dioxaphosphorinan-2-yl)-4-(2-nitrophenyl)-1,4-dihydropyridine-3-carboxylate (**31**, DHP-218) was approximately 7 times more active than nifedipine in SHR and was selected for further development and clinical evaluation. The structure–activity relationships are discussed.

Keywords——1,4-dihydropyridine derivative; cyclic phosphonate derivative; calcium antagonist; antihypertensive activity; nifedipine; structure–activity relationship

In a previous paper,[2)] the authors reported the synthesis and antihypertensive activities of 1,4-dihydropyridine-5-phosphonates (I), of these compounds which were expected to have long-lasting activity and good bioavailability. The results showed that the phosphonate function is a good replacement for the carboxylate group in the 1,4-dihydropyridine calcium antagonists, such as nifedipine.[3)] This led us to search for novel and more potent analogues, and we selected 4-aryl-5-(2-oxo-1,3,2-dioxaphosphorinan-2-yl)-1,4-dihydropyridine-3-carboxylates and their derivatives (II) as candidates. The cyclic phosphonate moiety instead of dialkyl phosphonate could be expected to increase the hydrophilicity of the molecule. Consequently, these compounds with the cyclic phosphonate group were expected to show good bioavailability and long-lasting antihypertensive activity. In this paper, we describe the synthesis and pharmacological evaluations of the title compounds and the structure–activity relationships of various 1,4-dihydropyridine-5-cyclic phosphonates.



nifedipine I II

Fig. 1

Chemistry

The 1,4-dihydropyridines (II) listed in Tables I to III were synthesized *via* the routes shown in Chart 1. In the same manner as described in the previous paper,[2)] the 1-

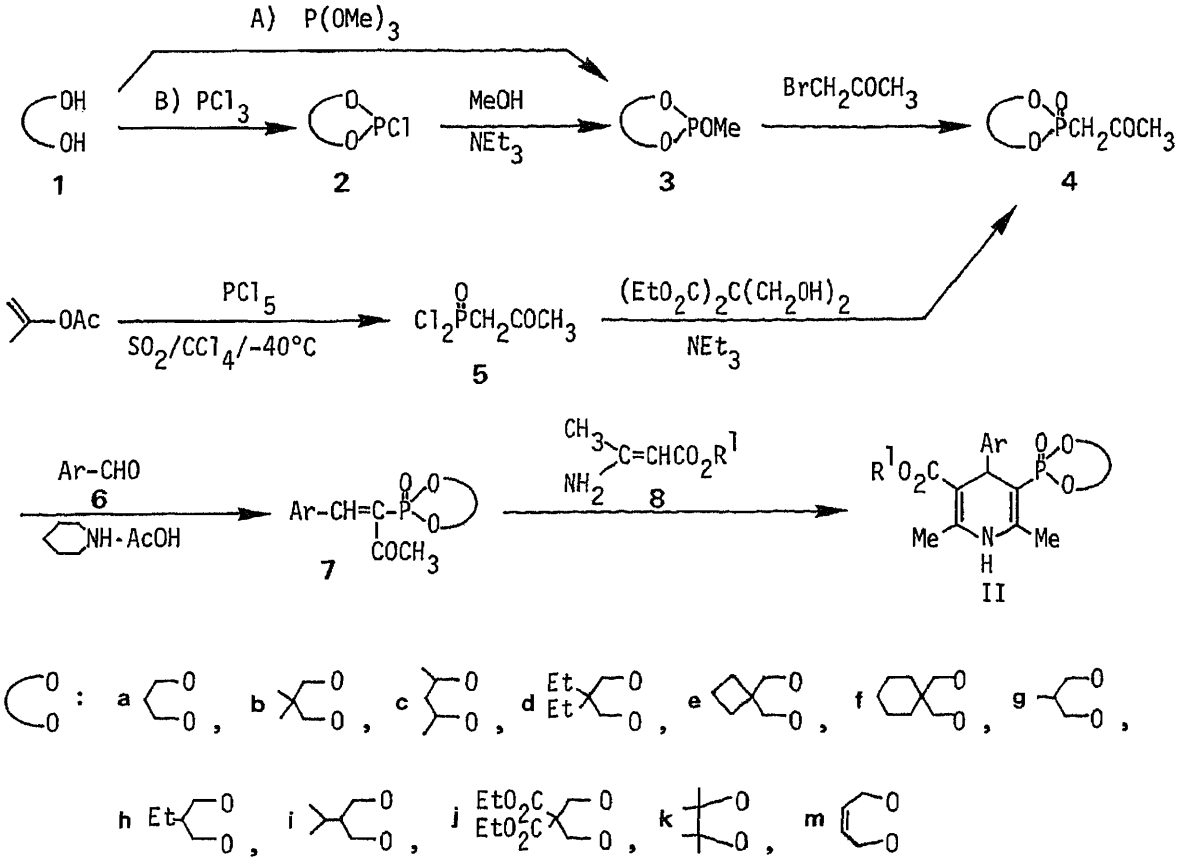


Chart 1

arylideneacetonylphosphonates (**7**) were allowed to react with the appropriate 3-aminocrotonates (**8**) in 2-propanol under reflux to afford the 1,4-dihydropyridines (II) in 10—86% yields. The 1-arylideneacetonylphosphonates (**7**) were prepared by the Knoevenagel reaction of the cyclic acetonylphosphonates **4** with the appropriate arylaldehydes (**6**) in the presence of piperidine–AcOH salt as a catalyst in benzene, with 14—46% yields (Table IV). In this reaction, a side reaction (Horner–Emmons reaction) took place resulting in the formation of benzalacetones.

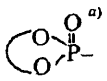
The intermediates **4**[4] were prepared as follows. The Arbuzov reaction of cyclic phosphites **3** with bromoacetone afforded cyclic phosphonates **4** in 10—55% yields (Table V). The only cyclic phosphonate **4j** which was not obtained under the Arbuzov reaction conditions was prepared by reaction of the acetonylphosphonodichloridate (**5**), obtained from propenyl acetate in two steps,[5] with diethyl 2,2-bis(hydroxymethyl)malonate. The cyclic phosphites **3** were synthesized by two known methods[6] (Chart 1). Method A was mainly used as a one-step reaction, but the phosphites which were obtained by method B were of better purity than those obtained by method A.

The 3-aminocrotonates (**8**) were prepared by the reaction of the appropriate acetoacetates with ammonia in methanol in good yields.[7]

Pharmacology

The compounds listed in Tables I to III were examined for antihypertensive activity. Blood pressure was measured in unanesthetized rats with normal blood pressure (normotensive rats) and spontaneously hypertensive rats (SHR). Male normotensive Wistar rats weighing 310—450 g (18 weeks or older) were placed in a supine position under ether

TABLE I. Physical and Biological Properties of 1,4-Dihydropyridines (II); (Ar = R^2-C_6H_4)

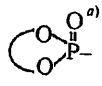
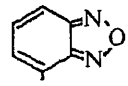
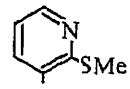
Compd. No.	 [a]	R^1	R^2	Yield[b] (%)	mp (°C)	Crystn. solvent[c]	Formula[d]	Antihypertensive potency[e]
9	a	Me	3-NO_2	26	221—223	a	$C_{18}H_{21}N_2O_7P$	3
10	a	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	60	139—140	b	$C_{27}H_{32}N_3O_7P$	4—5
11	b	Me	3-NO_2	64	208—209	a	$C_{20}H_{25}N_2O_7P$	3
12	b	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	28	125—126	c	$C_{29}H_{36}N_3O_7P^{f)}$	4
13	c	Me	3-NO_2	41	198—200	c	$C_{20}H_{25}N_2O_7P$	3—4
14	c	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	39	(Oil)		$C_{29}H_{36}N_3O_7P$	5
15	d	Me	3-NO_2	73	223—224	c	$C_{22}H_{29}N_2O_7P$	1
16	d	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	42	171—172	a	$C_{31}H_{40}N_3O_7P$	2
17	e	Me	3-NO_2	54	217—219	a	$C_{21}H_{25}N_2O_7P$	1
18	e	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	44	188—189	a	$C_{30}H_{36}N_3O_7P$	3
19	f	Me	3-NO_2	76	199—200	d	$C_{23}H_{29}N_2O_7P$	1
20	f	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	76	198—200	d	$C_{32}H_{40}N_3O_7P$	2—3
21	g	Me	3-NO_2	44	177—179	c	$C_{19}H_{23}N_2O_7P$	5
22	g	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	52	156—158	a	$C_{28}H_{34}N_3O_7P$	4
23	h	Me	3-NO_2	31	122—123	a	$C_{20}H_{25}N_2O_7P$	4
24	h	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	29	145—146	a	$C_{29}H_{36}N_3O_7P$	3
25	i	Me	3-NO_2	63	101—102	e	$C_{21}H_{27}N_2O_7P^{g)}$	3
26	i	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	60	161—162	a	$C_{30}H_{38}N_3O_7P$	2
27	j	Me	3-NO_2	72	216—217	f	$C_{24}H_{29}N_2O_7P$	1
28	j	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	57	142—143	c	$C_{30}H_{38}N_3O_7P$	1
29	k	Me	3-NO_2	31	236—237	a	$C_{21}H_{27}N_2O_7P$	1
30	k	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	17	186—188	c	$C_{30}H_{38}N_3O_7P$	3
31	a	Me	2-NO_2	54	245—247 (dec.)	g	$C_{18}H_{21}N_2O_7P$	6
32	b	Me	2-NO_2	45	249—251	h	$C_{20}H_{25}N_2O_7P$	4
33	c	Me	2-NO_2	39	125—135	e	$C_{20}H_{25}N_2O_7P$	4
34	d	Me	2-NO_2	36	235 (dec.)	i	$C_{22}H_{29}N_2O_7P$	1
35	e	Me	2-NO_2	10	174—177	a	$C_{21}H_{25}N_2O_7P^{g)}$	4
36	f	Me	2-NO_2	29	137 (dec.)	d	$C_{23}H_{29}N_2O_7P$	2
37	h	Me	2-NO_2	31	122—123	a	$C_{20}H_{25}N_2O_7P$	4
38	i	Me	2-NO_2	43	182—184	a	$C_{21}H_{27}N_2O_7P^{f)}$	4
39	j	Me	2-NO_2	39	198—199	j	$C_{24}H_{29}N_2O_7P$	1
40	m	Me	2-NO_2	46	221—222.5	g	$C_{19}H_{21}N_2O_7P$	4

a) See Chart 1. *b*) Yield is after recrystallization or isolated (an oil). *c*) Solvents for recrystallization: a, AcOEt; b, ether; c, AcOEt–ether; d, AcOEt–iso-Pr_2O; e, MeOH–ether; f, 2-propanol–$CHCl_3$; g, AcOEt–$CHCl_3$; h, MeOH; i, EtOH; j, 2-propanol–ether; k, AcOEt–MeOH. *d*) All compounds were analyzed for C, H and N; the analytical results were within ±0.4% of the calculated values. *e*) Potency was evaluated as follows: 1, little or no effect at 30 mg/kg; 2, effective at 30 mg/kg; 3, effective at 10 mg/kg; 4, effective at 3 mg/kg; 5, effective at 1 mg/kg; 6, effective at 0.3 mg/kg (nifedipine: 6). The effective dose causes 25% lowering of blood pressure. *f*) 1/2 H_2O. *g*) 1/4 H_2O.

anesthesia and a polyethylene catheter filled with heparinized saline was inserted into the femoral artery and connected with a pressure transducer (Nihon Kohden MPU-0.5). Blood pressure measurements were started under restraint after the rats had recovered from anesthesia, and blood pressure levels were recorded continuously on a potentiometric recorder (Toa EPR-10A or EPR) *via* a preamplifier (Nihon Kohden AP-621G). After the blood pressure had become stable, the test compounds prepared as suspensions in 0.5% methylcellulose (MC) solution, were administered orally at various doses (0.3, 1, 3, 10, or 30 mg/kg).

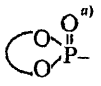
Some of the compounds with potent antihypertensive activity in normotensive rats were tested for antihypertensive activity in SHR. Male SHR weighing 280—300 g were purchased at the age of 15 weeks and housed in our laboratory. Systolic blood pressure was measured by

TABLE II. Physical and Biological Properties of 1,4-Dihydropyridines (II); (Ar = R^2–C_6H_4 or Heterocycles)

Compd. No.		R^1	R^2 (Ar)	Yield[b] (%)	mp (°C)	Crystn. solvent[c]	Formula[d]	Antihypertensive potency[e]
41	**a**	Me	2-CF_3	35	209—211	c	$C_{19}H_{21}F_3NO_5P$	6
42	**a**	$CH_2CH_2N(Me)CH_2C_6H_5$	2-CF_3	40	(Oil)		$C_{28}H_{32}F_3N_2O_5P$	4—5
43	**b**	Me	2-CF_3	26	210—212	a	$C_{21}H_{25}F_3NO_5P$	2
44	**b**	$CH_2CH_2N(Me)CH_2C_6H_5$	2-CF_3	31	(Oil)		$C_{30}H_{36}F_3N_2O_5P$	4—5
45	**a**	Me	2-$OCHF_2$	48	192—193	a	$C_{19}H_{22}F_2NO_6P$	5
46	**a**	$CH_2CH_2N(Me)CH_2C_6H_5$	2-$OCHF_2$	66	(Oil)		$C_{28}H_{33}F_2N_2O_6P$[f]	4
47	**b**	Me	2-$OCHF_2$	39	217—218	a	$C_{21}H_{26}F_2NO_6P$	1
48	**b**	$CH_2CH_2N(Me)CH_2C_6H_5$	2-$OCHF_2$	41	(Oil)		$C_{30}H_{37}F_2N_2O_6P$	3—4
49	**a**	Me	2,3-Cl_2	53	275—277	k	$C_{18}H_{20}Cl_2NO_5P$	3
50	**b**	Me	2,3-Cl_2	40	167—168	a	$C_{20}H_{24}Cl_2NO_5P$	3
51	**b**	$CH_2CH_2N(Me)CH_2C_6H_5$	2,3-Cl_2	39	197—198	a	$C_{29}H_{35}Cl_2N_2O_5P$	4
52	**a**	Me		57	238—250	i	$C_{18}H_{20}N_3O_6P$	4
53	**a**	Me		36	106 (dec.)	a	$C_{18}H_{28}N_2O_5SP$	4

a) to *f*): See footnotes *a*) to *f*) in Table I.

TABLE III. Physical and Biological Properties of 1,4-Dihydropyridines (II); (Ar = R^2–C_6H_4)

Compd. No.		R^1	R^2	Yield[b] (%)	mp (°C)	Crystn. solvent[c]	Formula[d]	Antihypertensive potency[c]
54	**a**	Et	2-NO_2	48	173—175	a	$C_{19}H_{23}N_2O_7P$	6
55	**a**	iso-Pr	2-NO_2	47	159—161	c	$C_{20}H_{25}N_2O_7P$	4
56	**a**	iso-Bu	2-NO_2	79	187 (dec.)	a	$C_{21}H_{27}N_2O_7P$	4
57	**a**	*tert*-Bu	2-NO_2	55	120—124	c	$C_{21}H_{27}N_2O_7P$	3
58	**a**	$CH_2CH=CH_2$	2-NO_2	86	(Oil)		$C_{20}H_{23}N_2O_7P$	4
59	**a**	CH_2–	2-NO_2	49	206—208	c	$C_{21}H_{25}N_2O_7P$	4
60	**a**	CH_2CH_2O-Pr	2-NO_2	76	(Oil)		$C_{22}H_{29}N_2O_8P$[f]	3
61	**a**	$CH_2CH_2OCH_2C_6H_5$	2-NO_2	72	(Oil)		$C_{26}H_{29}N_2O_8P$[f]	5
62	**a**	Et	2-CF_3	64	175—177	c	$C_{20}H_{23}F_3NO_5P$	5
63	**a**	$CH_2CH=CH_2$	2-CF_3	58	192—193	c	$C_{21}H_{23}F_3NO_5P$	4
64	**a**	CH_2–	2-CF_3	52	225—225.5	a	$C_{22}H_{25}F_3NO_5P$	2
65	**a**	–	2-CF_3	56	236—238	g	$C_{23}H_{27}F_3NO_5P$	2

a) to *e*): See footnotes *a*) to *e*) in Table I. *f*) H_2O.

the tail cuff plethysmographic method under mild restraint with a programmable sphygmomanometer (PS-802; Riken Kaihatsu). Before blood pressure measurements, rats were placed in a warm box at 35—37 °C for 10 min. The test compounds were administered orally as described above. The dose in mg/kg which produced a 30% drop in blood pressure was calculated from the regression line as the ED_{30} value (Table VI).

Results and Discussion

The antiyhypertensive activities of the new 1,4-dihydropyridine derivatives (II) having a

TABLE IV. Cyclic 1-Arylideneacetonylphosphonates (7); (Ar = R^2–C_6H_4 or Heterocycles)

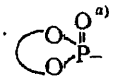
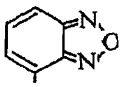
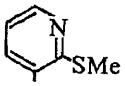
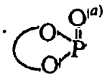
Compd. No.	 [a]	R^2 (Ar)	Yield[b] (%)	mp (°C)	IR[c] (cm^{-1}) $COCH_3$	NMR ($CDCl_3$) ($COCH_3$) (E/Z)	Analysis (%) Calcd (Found)		
							C	H	N
7an	**a**	3-NO_2	19	144—145	1700	2.31, —	50.17 (50.26	4.53 4.66	4.50 4.54)
7ap	**a**	2-NO_2	34	134—143	1700	2.32, 2.60 (10/1)	50.17 (50.21	4.53 4.58	4.50 4.50)
7aq	**a**	2-CF_3	44	(Oil)	1700	2.12, 2.58 (4/1)	50.31 (50.36	4.22 4.03)	
7ar	**a**	2-$OCHF_2$	44	85—87	1700	2.24, —	50.61 (50.60	4.55 4.63)	
7as	**a**	2,3-Cl_2	18	93—95	1705	2.19, —	46.59 (46.71	3.91 4.03)	
7at	**a**		8	(Oil)	1695			[d]	
7au	**a**		7	(Oil)	1700	2.22, 2.59 (3/1)		[d]	
7bn	**b**	3-NO_2	24	150—151	1705	2.33, —	53.10 (52.95	5.34 5.38	4.12 4.01)
7bp	**b**	2-NO_2	29	144—146	1695	2.34, 2.61 (5/4)	53.10 (53.06	5.34 5.33	4.12 4.19)
7bq	**b**	2-CF_3	35	80—87	1700	2.15, 2.58 (5/1)	53.05 (53.06	5.01 5.09)	
7br	**b**	2-$OCHF_2$	27	(Oil)	1700	2.25, 2.56 (8/1)		[d]	
7bs	**b**	2,3-Cl_2	25	117—119	1710	2.21, —	49.61 (49.63	4.72 4.76)	
7cn	**c**	3-NO_2	27	120—122	1705	2.27, —	53.10 (53.22	5.34 5.40	4.12 4.04)
7cp	**c**	2-NO_2	25	115—117	1705	2.17, —	53.10 (53.08	5.34 5.43	4.12 4.17)
7dn	**d**	3-NO_2	46	96—97	1710	2.31, —	55.59 (55.53	6.04 6.26	3.81 3.77)
7dp	**d**	2-NO_2	46	(Oil)	1700	2.32, 2.60 (7/3)		[d]	
7en	**e**	3-NO_2	24	152—154	1700	2.33, —	54.71 (54.62	5.17 5.22	3.99 4.02)
7ep	**e**	2-NO_2	26	121—123	1675	2.32, —	54.71 (54.77	5.17 5.25	3.99 4.05)
7fn	**f**	3-NO_2	26	125—129	1700	2.31, —	56.99 (56.89	6.00 5.92	3.69 3.68)
7fp	**f**	2-NO_2	46	(Oil)	1700	2.32, 2.60 (7/3)		[d]	
7gn	**g**	3-NO_2	18	123—125	1700	2.39, —	51.70 (51.62	4.96 5.06	4.31 4.38)
7gp	**g**	2-NO_2	19	83—85	1690	2.23, —	51.70 (51.72	4.96 4.93	4.31 4.28)
7hn	**h**	3-NO_2	21	99—101	1690	2.31, —	53.10 (53.10	5.35 5.47	4.13 4.18)
7hp	**h**	2-NO_2	40	(Oil)	1700	2.30, 2.60 (3/1)		[d]	
7in	**i**	3-NO_2	15	91—94	1695	2.29, 2.41 (3/1)	54.39 (54.46	5.71 5.79	3.96 4.01)

TABLE IV. (continued)

Compd. No.		R^2 (Ar)	Yield[b] (%)	mp (°C)	IR[c] (cm^{-1}) $COCH_3$	NMR ($CDCl_3$) ($COCH_3$) (*E*/*Z*)	Analysis (%) Calcd (Found)		
							C	H	N
7ip	**i**	2-NO_2	20	107—108	1700	2.25, —	54.39	5.71	3.96
							(54.35	5.85	3.94)
7jn	**j**	3-NO_2	37	100—101	1695	2.25, —	50.12	4.87	3.08
							(50.07	4.99	2.91)
7jp	**j**	2-NO_2	33	(Oil)	1700	2.19, —		[d]	
7kn	**k**	3-NO_2	14	180—183	1680	2.36, 2.61 (3/1)	54.39	5.71	3.96
							(54.63	5.75	4.07)
7mp	**m**	2-NO_2	23	(Oil)	1710	2.26, —		[d]	

a) See footnote *a*) in Table I. *b*) Isolated yield. Yield of an oil which was first isolated by chromatography. *c*) KBr (crystals) or film (an oil). *d*) A sample sufficiently pure for analysis was not obtained.

TABLE V. Cyclic Acetonylphosphonates 4

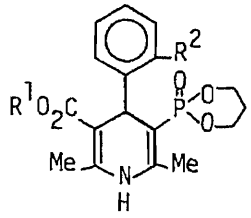
Compd. No.	bp (°C) (mmHg) or mp (°C)[a]	Yield[b] (%)	IR[c] max (cm^{-1})	NMR ($CDCl_3$) $-CH_2COCH_3$	Formula[d]	MS (*m*/*z*) M^+[e]	Analysis (%) Calcd (Found)	
							C	H
4a	163—165	35	1710	2.38 (3H, s)	$C_6H_{11}O_4P \cdot 1/2H_2O$	178	38.50	6.46
	(1)		1270	3.21 (2H, d)		(25)	(38.67	6.59)
4b	91—92	51	1705	2.39 (3H, s)	$C_8H_{15}O_4P$	206	46.60	7.33
	(Ether)		1270	3.20 (2H, d)		(16)	(46.48	7.01)
4c	146—148	55	1710	2.35 (3H, s)	$C_8H_{15}O_4P \cdot 1/2H_2O$	206	44.64	7.49
	(1)		1265	3.15 (2H, d)		(35)	(45.04	7.12)
4d	161—165	46	1710	2.38 (3H, s)	$C_{10}H_{19}O_4P \cdot 1/2H_2O$	234	49.38	8.29
	(0.5)		1275	3.19 (2H, d)		(6)	(49.27	8.26)
4e	105—106	46	1710	2.37 (3H, s)	$C_9H_{15}O_4P$	218	49.54	6.93
	(AcOEt–ether)		1270	3.17 (2H, d)		(5)	(49.41	6.87)
4f	178—183	53	1710	2.38 (3H, s)	$C_{11}H_{19}O_4P \cdot 1/4H_2O$	246	52.69	7.84
	(0.5)		1280	3.19 (2H, d)		(3)	(52.82	7.90)
4g	81—82	30	1710	2.39 (3H, s)	$C_7H_{13}O_4P$	192	43.76	6.82
	(Ether)		1260	3.21 (2H, d)		(35)	(43.67	6.92)
4h	152—156	40	1710	2.36 (3H, s)	$C_8H_{15}O_4P \cdot 3/4H_2O$	206	43.74	7.57
	(0.5)		1280	3.18 (2H, d)		(9)	(44.03	7.93)
4i	158—162	51	1710	2.36 (3H, s)	$C_9H_{17}O_4P \cdot 3/4H_2O$	220	46.25	7.98
	(0.5)		1280	3.17 (2H, d)		(10)	(46.60	7.69)
4j	(Oil)[f]	20	1760—1730	2.31 (3H, s)				
			1710	3.19 (2H, d)				
4k	151—153	38	1710	2.36 (3H, s)	$C_9H_{17}O_4P$	220	49.09	7.78
	(0.5)		1265	3.22 (2H, d)		(33)	(49.05	7.86)
4m	150—153	10	1715	2.40 (3H, s)	$C_7H_{11}O_4P \cdot 1/2H_2O$	190	42.20	6.07
	(0.5)		1270	3.23 (2H, d)		(16)	(42.16	5.43)

a) Solvents for recrystallization. *b*) Isolated yield after chromatography. *c*) KBr (crystals) or film (an oil). *d*) The oil show low hygroscopicity. *e*) M^+ (relative intensity, %). *f*) The oil, which was isolated by chromatography, could not be distilled and a sample sufficiently pure for analysis was not obtained.

cyclic phosphonate group at the 5-position in the 1,4-dihydropyridine (1,4-DHP) ring are shown in Tables I to III.

The 1,4-dihydropyridine-5-phosphonates (I), as mentioned in the previous paper,[2] had milder antihypertensive activity than the corresponding carboxylate compounds, but several

TABLE VI. Antihypertensive Activity of the Active Compounds, **31** (DHP-218), **41**, **45**, **54**, **61**, and Nifedipine in SHR



Compound	R^1	R^2	ED_{30}[a] (mg/kg)	Relative[b] potency
31 (DHP-218)	Me	2-NO_2	0.2	7.5
41	Me	2-CF_3	0.6	2.5
45	Me	2-$OCHF_2$	1.5	1.0
54	Et	2-NO_2	0.4	3.8
61	$CH_2CH_2OCH_2C_6H_5$	2-NO_2	0.7	2.1
Nifedipine			1.5	1.0

a) See pharmacology section. *b*) Potency relative to that of nifedipine.

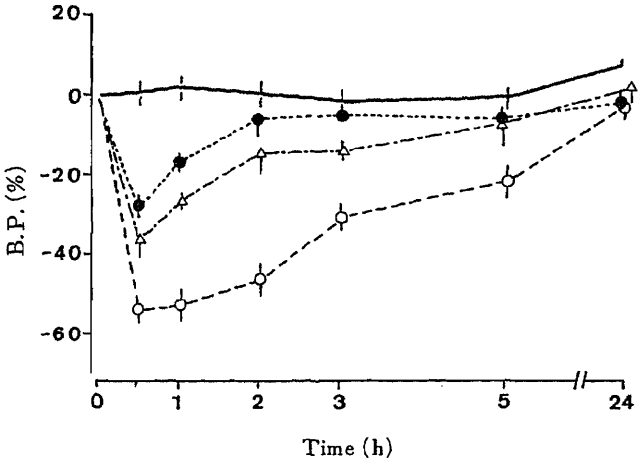


Fig. 2. Antihypertensive Activity of **31** (DHP-218), **41** and Nifedipine in SHR (1 mg/kg, *p.o.*)
——, control ---○, DHP-218; - - - -△, 41; ------●, nifedipine.

compounds in the present group of cyclic phosphonate derivatives (II) surprisingly showed excellent antihypertensive activity, superior to that of nifedipine.

Table I lists the 4-(2- or 3-nitrophenyl)-1,4-dihydropyridines which contain various cyclic phosphonates at the 5-position. Of the compounds with a 3-nitro substituent, 4,6-dimethyl (**c**), 5-methyl (**g**) and nonsubstituted (**a**) 2-oxo-1,3,2-dioxaphosphorinan-2-yl derivatives were strongly active, and of the compounds with a 2-nitro substituent, the phosphonate (**a**) (**31**) showed the most potent antihypertensive activity in this series. In both cases, the 5,5-dimethyl (**b**), 5-ethyl (**h**) and 5-isopropyl (**i**) derivatives were moderately active, and the 5,5-diethyl (**d**), 5,5-trimethylene (**e**), and 5,5-pentamethylene (**f**) derivatives were less active, while the 5,5-bis(ethoxycarbonyl) (**j**) derivative was essentialy inactive. The compounds having five-membered (**k**) or seven-membered (**m**) ring phosphonates showed mild activity. In general, the activity decreased with the size of the substituents of cyclic phosphonates, especially in the disubstituted six-membered cyclic phosphonates.

Of the substituents (Ar) at the 4-position in 1,4-DHP, seven substituents containing phenyl and heterocyclic rings were selected on the basis of the result with dialkyl phosphonates (I)[2] (Tables I and II). Among the substituents (R^2) on the phenyl ring, ortho

substituents were most active in the compounds having phosphonate (**a**) and a methyl ester at R^1, the order of potency being 2-NO_2, 2-CF_3, 2-$OCHF_2$, 2,3-Cl_2, and 3-NO_2. Heterocyclic substituents showed moderate activity. On the other hand, the order of potency of compounds possessing cyclic phosphonate (**b**) was 2-NO_2, 3-NO_2, 2,3-Cl_2, 2-CF_3, and 2-$OCHF_2$. However, with a 2-(*N*-benzyl-*N*-methylamino)ethyl ester moiety at R^1, variation of the substituent (R^2) did not appreciably change the activity of the compounds.

Varying the ester substituents (R^1) at the 3-position of 1,4-DHP, with a cyclic phosphonate (**a**) and 2-nitrophenyl substituent, weakened the activity with increasing substituent size, except for 2-benzyloxyethyl ester (Table III). The order of activity was methyl, ethyl, allyl, 2-benzyloxyethyl, iso-Pr, iso-Bu, cyclopropylmethyl, *tert*-Bu, and 2-propoxyethyl. With 2-trifluoromethylphenyl as a substituent, similar results were obtained. The 2-(*N*-benzyl-*N*-methylamino)ethyl ester, which showed potent activity in the dialkyl phosphonates (I), had weaker activity than the methyl and ethyl esters.

Some of the effective 1,4-dihydropyridines (II) were selected from those listed in Tables I to III, and their antihypertensive activities were examined in unanesthetized SHR. Among them, the five compounds listed in Table VI showed activity superior or comparable to that of nifedipine. The most active compound **31** (DHP-218) was approximately seven times more potent than nifedipine and had longer lasting action. DHP-218 also exhibited potent antihypertensive activities in other hypertensive rats, as well as cats, and dogs with normal blood pressure.[7] The time courses of antihypertensive effects of DHP-218, **41**, and nifedipine are shown in Fig. 2, after an oral dose of 1 mg/kg. DHP-218 was also shown to have a selective vasodilatory effect due to calcium antagonism with the pA_2 value of 9.11[8] (nifedipine: pA_2 9.58). Despite its weaker vasodilatory action than that of nifedipine *in vitro*, the oral administration of DHP-218 produced a much more potent and long-lasting antihypertensive effect. This might be due partly to its good bioavailability (dogs) and its lower serum protein binding (rats and dogs).[9] The structure of DHP-218 differs from that of nifedipine only in that the methoxycarbonyl moiety at the 5-position is replaced by the cyclic phosphonate (**a**). This presumably makes DHP-218 more hydrophilic than nifedipine. Actually, the water solubility of DHP-218 was approximately 10 times greater than that of nifedipine, and the log *P* values[10] of DHP-218 and nifedipine were 1.3 and 2.4, respectively. The *Rm* values,[11,12] which are parameters of lipophilicity, are −0.32 (DHP-218) and 0.00 (nifedipine); drugs with lower values are less lipophilic. These values substantiate the above discussion. Similar characteristics have been demonstrated in the β-blockers,[13] atenolol and nadolol.

The results of acute oral toxicity tests[9] of DHP-218 in mice and rats were very similar to those of nifedipine; consequently the safety margin is wide. DHP-218 was therefore selected for further development and clinical evaluation.

Experimental

Melting points were determined with a Büchi melting point apparatus and are uncorrected. Infrared (IR) spectra were measured on a Hitachi IR-215 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian YX-200 spectrometer. Chemical shifts are given in δ (ppm) with tetramethylsilane as an internal standard. The following abbreviations are used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad. Mass spectra (MS) were measured on a Hitachi RMU-6M instrument. Column chromatography was performed on Wakogel C-200.

General Procedure for Cyclic Phosphites (3)[6]——Method A: Equimolar quantities of trimethylphosphite and the appropriate diol (**1**) were heated at about 100 °C until reflux began. The methanol was removed by distillation at atmospheric pressure in an oil bath at temperatures up to 130 °C, and the desired product was obtained by reduced pressure distillation. [**3**, bp °C (mmHg), yield (%)]: **3a**, 63—68 (20), 26; **3b**, 65—70 (13), 49; **3c**, 85—88 (25), 34; **3d**, 135—136 (15), 32; **3e**, 105—115 (26), 34; **3f**, 107—118 (10), 31; **3g**, 75—85 (45), 38; **3h**, 83—88 (18), 42; **3i**, 85—97 (15), 46; **3k**, 58—60 (8), 31; **3m**, 60—65 (20), 14.

Method B: A diol (**1**) (1 mol) and triethylamine (202 g, 2 mol) were added dropwise to a well-stirred, ice-cooled

ether–CH_2Cl_2 solution (1000 ml–300 ml) of phosphorus trichloride (87 ml, 1 mol). The mixture was stirred for an additional 2 h at room temperature. The triethylamine hydrochloride was removed by filtration and washed with ether, and the filtrate and washing were combined and concentrated to dryness. The residue was distilled to give the corresponding **2** in *ca.* 40% yield. A solution of absolute methanol (14.1 g, 0.44 mol) and triethylamine (40.5 g, 0.4 mol) in 400 ml of dry ether was added dropwise to a well-stirred, ice-cooled dry ether solution of **2** (0.4 mol). The mixture was stirred for an additional 2 h at room temperature and then filtered. The residue was washed with ether, and the filtrate and washing were combined and were concentrated to dryness. The residue was distilled to give the corresponding cyclic phosphite (**3**) in high yield.

The cyclic phosphites **3** were prepared by one of the two methods described above.

General Procedure for Cyclic Acetonylphosphonates (4)——Bromoacetone (0.5 mol) and a cyclic phosphite **3** (0.5 mol) were mixed and stirred at room temperature. An exothermic reaction occurred, and the reaction temperature rose to 80—120 °C. After the evolution of methyl bromide gas had almost ceased, the mixture was heated to 80 °C for an additional 1 h, then evaporated *in vacuo*. The residual oil was diluted with AcOEt (500 ml) and extracted with cold 1 N NaOH (3 × 250 ml). The aqueous phase was neutralized with AcOH, extracted with $CHCl_3$ (3 × 250 ml) by salting-out, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was chromatographed on silica gel with hexane–AcOEt to afford the corresponding **4** in 10—55% yield as an oil or crystals. The oil obtained was distilled *in vacuo*, and the crystals were recrystallized from the solvents listed in Table V. All these cyclic acetonylphosphonates **4** were characterized by IR and NMR analyses.

A typical example is described below.

2-Acetonyl-2-oxo-1,3,2-dioxaphosphorinane (4a)——Bromoacetone (61.7 g, 0.45 mol) and phosphite **3a** (61.2 g, 0.45 mol) were mixed and stirred at 0 °C. An exothermic reaction occurred, and then reaction temperature rose to 80 °C. When the evolution of methyl bromide gas had almost ceased, the mixture was heated to 60 °C for 1 h, then evaporated *in vacuo*. The residual oil was diluted with AcOEt (400 ml) and extracted with water (3 × 250 ml). The aqueous phase was extracted with $CHCl_3$ (3 × 250 ml) by salting-out, dried ($MgSO_4$), and evaporated *in vacuo*. The residue obtained was chromatographed on silica gel with hexane–AcOEt (1 : 1—AcOEt only) to afford **4a** (35%) as an oil. The product (**4a**) obtained was purified by distillation, bp 163—165 °C (1 mmHg). MS *m/z* (%): 178 (M^+, 25.7), 136 (100). NMR ($CDCl_3$) δ: 1.96—2.22 (2H, m, CH_2), 2.38 (3H, s, $COCH_3$), 3.21 (2H, d, J=22 Hz, PCH_2), 4.26—4.65 (4H, m, 2 × OCH_2).

Preparation of 2-Acetonyl-5,5-bis(ethoxycarbonyl)-2-oxo-1,3,2-dioxaphosphorinane (4j)——The known intermediate **5** was prepared according to the procedure described by Lutsenko and Kirilov.[5] A solution of 2,2-bis(hydroxymethyl)malonate (30.5 g) and triethylamine (28 g, 2 eq) in dry ether (100 ml) was added dropwise to a stirred solution of **5** in dry ether (250 ml) at −5 °C over a period of 1 h. After the addition was complete, the mixture was removed from the ice-bath and stirred for 15 h at room temperature. The precipitate was collected by filtration and, washed with ether. The filtrate and washing were combined and concentrated to dryness. The residue obtained was chromatographed on silica gel with hexane–AcOEt to afford **4j** (8.9 g, 20%) as a pale yellow oil. IR (film): 1760—1730 (C=O, CO_2Et) cm^{-1}. NMR ($CDCl_3$) δ: 1.29 (3H, t, J=7.5 Hz, $CH_2\underline{CH_3}$), 1.31 (3H, t, J=7.5 Hz, $CH_2\underline{CH_3}$), 2.31 (3H, s, $COCH_3$), 3.19 (2H, d, J=23 Hz, PCH_2), 4.25 (2H, q, J=7.5 Hz, $\underline{CH_2}CH_3$), 4.28 (2H, q, J=7.5 Hz, $\underline{CH_2}CH_3$), 4.78 (2H, d, J=11 Hz, OCH_2), 4.79 (2H, d, J=12 Hz, OCH_2).

General Procedure for Cyclic 1-Arylideneacetonylphosphonates (7)——A solution of a cyclic acetonylphosphonate (**4**, 0.1 mol) and an arylaldehyde (**6**, 0.1 mol) in benzene (200 ml) containing a catalytic amount of piperidine·AcOH salt was refluxed for 12—24 h with continuous removal of water by means of a Dean–Stark apparatus. The benzene solution was washed with water, aqueous NaOH, aqueous $NaHSO_3$, and water, then dried ($MgSO_4$) and concentrated to dryness. The residue obtained was purified by chromatography on silica gel with hexane–AcOEt to give the corresponding **7** in 14—46% yield as a mixture of *E* (major) and *Z* isomers (listed in Table IV). The crystals obtained were recrystallized from the indicated solvents. All these compounds **7** were characterized by IR and NMR analyses.

A typical example is given below.

2-[1-(2-Nitrobenzylidene)acetonyl]-2-oxo-1,3,2-dioxaphosphorinane (7ap)——A solution of **4a** (8.5 g), 2-nitrobenzaldehyde (7.2 g) and piperidine·AcOH (1.72 g, 0.25 eq) in benzene (100 ml) was azeotropically refluxed for 16 h with stirring. The benzene solution was treated as mentioned above. The residue obtained was chromatographed on silica gel (150 g) with hexane–AcOEt (4 : 1—1 : 4, v/v) to give **7ap** (3.66 g, 34%) as crystals. An analytical sample was recrystallized from AcOEt, mp 134—143 °C (a mixture of *E* and *Z* isomers, 10/1). IR (KBr): 1700 (C=O), 1575, 1350, 1270, 1070 cm^{-1}. NMR ($CDCl_3$) δ: 1.90—2.04 (1H, m, >CH), 2.20—2.45 [1H, m, >CH, and total 3H, 2.32 (s, $COCH_3$, *E* isomer) and 2.61 (s, $COCH_3$, *Z* isomer), ratio of 10/1], 4.10—4.25 and 4.48—4.65 (total 4H, m, 2 × OCH_2), 7.23—7.28 (1H, m, ArH), 7.57—7.73 (2H, ArH), 7.90 [*ca.* 1H, d, J=24 Hz, PC=CH(*E*)], 8.22—8.27 (1H, m, ArH).

In the above chromatography, the first component was the *E*-isomer, mp 155—156 °C (from AcOEt), and the final component was the *Z*-isomer, mp 174—175 °C (from AcOEt). **7ap** (*E*): *Anal.* Found: C, 50.06; H, 4.59; N, 4.49. IR (KBr): 1695, 1600 cm^{-1}. NMR ($CDCl_3$) δ: 2.32 (3H, s, $COCH_3$), 7.90 [1H, d, J=23.5 Hz, =CH–(*E*)[14]]. **7ap** (*Z*): *Anal.* Found: C, 50.07; H, 4.44; N, 4.52. IR (KBr): 1680, 1595 cm^{-1}. NMR ($CDCl_3$) δ: 2.61 (3H, s, $COCH_3$), 8.32

[1H, d, J=48 Hz, =CH– (*Z*)].

General Procedure for 1,4-Dihydropyridines (II)——A solution of a cyclic 1-arylideneacetonylphosphonate (**7**, 0.01 mol) and a 3-aminocrotonate (**8**, 0.01 mol) in 2-propanol (20 ml) was refluxed for 3—30 h with stirring. The solvent was removed, and the residue was purified by crystallization from a suitable solvent (AcOEt or ether) or by chromatography on silica gel with hexane–AcOEt to give the corresponding 1,4-dihydropyridine (II) in 10—86% yields. When R^1 of the carboxylate was a basic ester [2-(*N*-benzyl-*N*-methylamino)ethyl or 2-(*N*,*N*-dimethylamino)-ethyl], the residue obtained was extracted with aqueous HCl, the acid extract was extracted with $CHCl_3$, and the $CHCl_3$ layer was washed with aqueous K_2CO_3, then water, dried ($MgSO_4$) and concentrated to give crude II. The residue obtained was purified as described above.

Typical examples are given below.

2-(*N*-Benzyl-*N*-methylamino)ethyl 2,6-Dimethyl-5-(5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinan-2-yl)-4-(3-nitrophenyl)-1,4-dihydropyridine-3-carboxylate (12)——A solution of **7bn** (1.19 g) and 2-(*N*-benzyl-*N*-methylamino)-ethyl 3-aminocrotonate (0.868 g) in 2-propanol (20 ml) was refluxed for 5 h with stirring. The solvent was removed *in vacuo*, then the residue was diluted with AcOEt (20 ml) and first extracted with 15 ml of 1 N HCl then twice with 10 ml each of water. The aqueous phase was extracted twice with $CHCl_3$, and the $CHCl_3$ layer was washed with aqueous K_2CO_3 and water, then dried ($MgSO_4$) and concentrated to dryness. The residue was chromatographed on silica gel (70 g) with hexane–AcOEt to give **12** (0.875 g, 44%) as an oil. The oil obtained was crystallized from ether, and recrystallization from AcOEt–ether gave yellow crystals (0.56 g, 28%), mp 125—126 °C. *Anal.* Calcd for $C_{29}H_{36}N_3O_7P \cdot 1/2H_2O$: C, 60.20; H, 6.45; N, 7.07. Found: C, 60.21; H, 6.64; N, 7.07. MS *m/z* (%): 569 (M^+, 5.6), 134 (100). IR (KBr): 3290, 3225, 3105, 1700, 1650, 1535, 1355, 1250, 1065 cm^{-1}. NMR ($CDCl_3$) δ: 0.87 (3H, s, CH_3), 1.04 (3H, s, CH_3), 2.22 (3H, s, C_2-CH_3), 2.30 (3H, s, $\underline{N}CH_3$), 2.39 (3H, d, $J_{P\text{-}H}$=2.5 Hz, C_6-CH_3), 2.68 (2H, t, J=6 Hz, $\underline{N}CH_2$), 3.41—3.74 [4H, (2H, m, OCH_2), 3.52 (2H, s, $\underline{N}CH_2$)], 4.14—4.28 (4H, m, 2×$POCH_2$), 4.94 (1H, d, $J_{P\text{-}H}$= 11 Hz, C_4-H), 6.28 (1H, br, $\underline{N}H$), 7.26—7.36 (6H, m, ArH), 7.64—7.69 (1H, m, ArH), 7.96—8.01 (1H, m, ArH), 8.11 (1H, t, J=2 Hz, ArH).

Methyl 2,6-Dimethyl-5-(5-methyl-2-oxo-1,3,2-dioxaphosphorinan-2-yl)-4-(3-nitrophenyl)-1,4-dihydropyridine-3-carboxylate (21)——A solution of **7gn** (1.15 g) and methyl 3-aminocrotonate (0.41 g) in 2-propanol (20 ml) was refluxed for 5 h with stirring. The solvent was removed *in vacuo*, and the residue was crystallized from ether to give **21**. Recrystallization from 2-propanol–ether gave yellow crystals (0.65 g, 44%), mp 177—179 °C. *Anal.* Calcd for $C_{19}H_{23}N_2O_7P$: C, 54.03; H, 5.49; N, 6.63. Found: C, 53.72; H, 5.63; N, 6.45. IR (KBr): 3300, 3250, 3210, 1685, 1645, 1530, 1350, 1250 cm^{-1}. NMR ($CDCl_3$) δ: 0.83 (3H, d, J=6 Hz), 2.15—2.40 [7H, m, 2.32 (3H, s), 2.34 (3H, d, J= 2.5 Hz)], 3.67 (3H, s), 3.95—4.27 (4H, m), 4.86 (1H, d, J=12 Hz), 6.72 (1H, br), 7.37 (1H, t, J=8 Hz), 7.62 (1H, d, J= 8 Hz), 7.95—8.04 (1H, m), 8.09 (1H, t, J=2 Hz).

Methyl 2,6-Dimethyl-5-(4,4,5,5-tetramethyl-2-oxo-1,3,2-dioxaphospholan-2-yl)-4-(3-nitrophenyl)-1,4-dihydropyridine-3-carboxylate (29)——A solution of **7kn** (0.75 g) and methyl 3-aminocrotonate (0.244 g) in 2-propanol (10 ml) was refluxed for 3 h with stirring. The solvent was removed *in vacuo*, and the residue was chromatographed on silica gel to afford **29** as crystals (0.36 g). Recrystallization from AcOEt gave yellow crystals (0.295 g, 31%), mp 236—237 °C. *Anal.* Calcd for $C_{21}H_{27}N_2O_7P$: C, 56.00; H, 6.04; N, 6.22. Found: C, 56.06; H, 6.18; N, 6.25. IR (KBr): 3290, 3220, 3110, 1700, 1655, 1530, 1355, 1235 cm^{-1}. NMR ($CDCl_3$) δ: 1.22 (3H, s), 1.26 (3H, s), 1.46 (3H, s), 1.48 (3H, s), 2.28 (3H, d, J=2.5 Hz), 2.32 (3H, s), 3.67 (3H, s), 4.94 (1H, d, J=11 Hz), 6.31 (1H, d, J=5 Hz), 7.38 (1H, t, J=8 Hz), 7.64 (1H, d, J=8 Hz), 7.98— 8.03 (1H, m), 8.08 (1H, t, J=2 Hz).

Methyl 2,6-Dimethyl-4-(2-nitrophenyl)-5-(2-oxo-1,3,2-dioxaphosphorinan-2-yl)-1,4-dihydropyridine-3-carboxylate (31, DHP-218)——A solution of **7ap** (4.04 g) and methyl 3-aminocrotonate (1.49 g) in 2-propanol (80 ml) was refluxed for 20 h with stirring. After removal of the solvent, the residue was chromatographed on silica gel with hexane–AcOEt to afford **31** (3.16 g) as crystals. Recrystallization from $CHCl_3$–AcOEt gave yellow prisms (2.86 g, 54%), mp 245—247 °C (dec.). *Anal.* Calcd for $C_{18}H_{21}N_2O_7P$: C, 52.95; H, 5.18; N, 6.86. Found: C, 52.86; H, 5.33; N, 6.73. MS *m/z* (%): 408 (M^+, 8.7), 391 (100). IR (KBr): 3280, 3200, 3080, 1690, 1645, 1520, 1350, 1250, 1100 cm^{-1}. NMR ($CDCl_3$) δ: 1.65—1.78 (1H, m), 2.27 (3H, s), 2.34—2.64 [4H, m, 2.47 (3H, d, J=2.4 Hz)], 3.57 (3H, s), 3.82— 4.68 (4H, m), 5.60 (1H, d, J=10 Hz), 6.01 (1H, br), 7.18—7.30 (1H, m), 7.45 (1H, t, J=8 Hz), 7.52 (1H, d, J=8 Hz), 7.70 (1H, d, J=8 Hz).

Methyl 2,6-Dimethyl-4-(2-nitrophenyl)-5-(2-oxo-4,7-dihydro-1,3,2-dioxaphosphepin-2-yl)-1,4-dihydropyridine-3-carboxylate (40)——A solution of **7mp** (1.30 g) and methyl 3-aminocrotonate (0.461 g) in 2-propanol (15 ml) was refluxed for 10 h with stirring. After removal of the solvent, the residue was chromatographed on silica gel to afford **40** (0.774 g, 46%) as yellow prisms (from $CHCl_3$–AcOEt), mp 221—222.5 °C. *Anal.* Calcd for $C_{19}H_{21}N_2O_7P$: C, 54.29; H, 5.04; N, 6.66. Found: C, 54.33; H, 5.04; N, 6.59. IR (KBr): 3280, 3220, 3090, 1695, 1650, 1535, 1365, 1260, 1050 cm^{-1}. NMR ($CDCl_3$) δ: 2.27 (3H, s), 2.31 (3H, d, J=2.6 Hz), 3.56 (3H, s), 4.04—4.30 (1H, m), 4.48—4.76 (3H, m), 5.55—5.78 (3H, m), 6.34 (1H, br), 7.18—7.32 (1H, m), 7.40—7.62 (2H, m), 7.69 (1H, dd, J=1.5, 8 Hz).

Methyl 2,6-Dimethyl-5-(2-oxo-1,3,2-dioxaphosphorinan-2-yl)-4-(2-trifluoromethylphenyl)-1,4-dihydropyridine-3-carboxylate (41)——A solution of **7aq** (1.70 g) and methyl 3-aminocrotonate (0.586 g) in 2-propanol (20 ml) was refluxed for 22 h with stirring. After removal of the solvent, the residue was chromatographed on silica gel to afford **41** (0.76 g, 35%) as colorless prisms (from AcOEt–ether), mp 209—211 °C. *Anal.* Calcd for $C_{19}H_{21}F_3NO_5P$: C, 52.91;

H, 4.91; N, 3.25. Found: C, 52.94; H, 5.15; N, 3.16. IR (KBr): 3280, 3220, 3100, 1695, 1645, 1240, 1105 cm^{-1}. NMR ($CDCl_3$) δ: 1.62—1.80 (1H, m), 2.10—2.30 [4H, m, 2.23 (3H, s)], 2.32 (3H, d, J=2.4 Hz), 3.60 (3H, s), 3.90—4.25 (2H, m), 4.32—4.62 (2H, m), 5.31 (1H, d, J=10 Hz), 6.98 (1H, d, J=5 Hz), 7.18—7.57 (4H, m).

Methyl 4-(2,1,3-Benzoxadiazol-4-yl)-2,6-dimethyl-5-(2-oxo-1,3,2-dioxaphosphorinan-2-yl)-1,4-dihydropyridine-3-carboxylate (52)——A solution of **7at** (0.85 g) and methyl-3-aminocrotonate (0.32 g) in 2-propanol (20 ml) was refluxed for 20 h with stirring. After removal of the solvent, the residue was chromatographed on silica gel with hexane–AcOEt to afford **52** (0.64 g, 57%) as crystals, mp 248—250 °C (from ethanol). *Anal.* Calcd for $C_{18}H_{20}N_3O_6P$: C, 53.34; H, 4.97; N, 10.37. Found: C, 53.47; H, 5.01; N, 10.22. IR (KBr): 3290, 3210, 3095, 1700, 1645, 1240, 1215, 1050 cm^{-1}. NMR ($CDCl_3$) δ: 1.62—1.78 (1H, m), 2.06—2.27 (1H, m), 2.31 (3H, s), 2.35 (3H, d, J=2.5 Hz), 3.59 (3H, s), 3.64—3.86 (1H, m), 4.12—4.63 (3H, m), 5.30 (1H, d, J=10 Hz), 6.16 (1H, br), 7.23—7.34 (2H, m), 7.63 (1H, dd, J=1.5, 8 Hz).

2-Benzyloxyethyl 2,6-Dimethyl-4-(2-nitrophenyl)-5-(2-oxo-1,3,2-dioxaphosphorinan-2-yl)-1,4-dihydropyridine-3-carboxylate (61)——A solution of **7ap** (1.56 g) and 2-benzyloxyethyl 3-aminocrotonate (1.41 g) in 2-propanol (20 ml) was refluxed for 20 h with stirring. After removal of the solvent, the residue was purified by chromatography on silica gel with hexane–AcOEt to afford **61** (1.91 g, 72%) as an oil. *Anal.* Calcd for $C_{26}H_{29}N_2O_8P \cdot H_2O$: C, 57.14; H, 5.72; N, 5.13. Found: C, 57.18; H, 5.36; N, 5.15. MS *m/z* (%): 528 (M^+, 3.1), 91 (100). IR (film): 3400, 3280, 3200, 3080, 1695, 1640, 1530, 1355, 1230, 1050 cm^{-1}. NMR ($CDCl_3$) δ: 1.62—1.78 (1H, m), 2.18—2.48 [7H, m, 2.24 (3H, s), 2.37 (3H, d, J=2.5 Hz)], 3.56—3.76 (2H, m), 3.88—4.62 [8H, m, 4.50 (2H, s)], 5.69 (1H, d, J=11 Hz), 6.91 (1H, d, J=5 Hz), 7.16—7.36 (6H, m), 7.43 (1H, t, J=8 Hz), 7.53 (1H, d, J=8 Hz), 7.70 (1H, d, J=8 Hz).

References and Notes

1) This work was presented at the 106th Annual Meeting of the Pharmaceutical Society of Japan, Chiba, April 1986.
2) Part I: I. Morita, S. Tada, K. Kunimoto, M. Tsuda, M. Kise, and K. Kimura, *Chem. Pharm. Bull.*, **35**, 3898 (1987).
3) W. Vater, G. Kroneberg, F. Foffmeister, H. Kaller, K. Meng, A. Oberdorf, W. Puls, K. Schlossmann, and K. Stoepel, *Arzneim.-Forsch.*, **22**, 1 (1972).
4) Reaction of Cyclic Phosphites with Haloacetones: I. Morita, M. Tsuda, M. Kise, and M. Sugiyama, *Chem. Pharm. Bull.*, printed.
5) I. F. Lutsenko and M. Kirilov, *Godishnik Sofiskiya Unive., Fiz.-Mat. Fak. Khim.*, **55**, 135 (1960/61) [*Chem. Abstr.*, **59**, 6433*f* (1963)].
6) *a*) B. A. Arbuzov, V. M. Zoroastrova, and M. K. Saikiva, *Izv. Akad. Nauk SSSR*, **1959**, 1579 [*Chem. Abstr.*, **54**, 8619*i* (1960)]; *b*) D. W. White, R. D. Bertrand, G. K. McEwen, and J. G. Verkade, *J. Am. Chem. Soc.*, **92**, 7125 (1970); *c*) G. M. Kosolapoff and L. Maier, "Organic Phosphorus Compounds," Vol. 5, John Wiley and Sons, Inc., New York, 1973, p. 155, 185.
7) Y. Kimura, H. Fukui, M. Tanaka, M. Okamoto, A. Morino, A. Miura, K. Kimura, and H. Enomoto, *Arzneim.-Forsch.*, **36**, 1329 (1986).
8) A. Miura, Y. Ukai, T. Matsuzaki, S. Hayashi, S. Kano, Y. Kimura, K. Kimura, and H. Enomoto, *Arzneim.-Forsch.*, **36**, 1323 (1986).
9) Data are not presented.
10) *a*) The log *P* value, which is the partition coefficient, was determined in a 1-octanol–water (phosphate buffer, pH 6.8) system; *b*) C. Hansch, "Drug Design," Vol. I, ed. by E. J. Ariens, Academic Press, New York, 1971, p. 271.
11) The *Rm* value was calculated by means of the formula $Rm = \log(1/Rf - 1)$. The *Rf* value was determined by reversed-phase thin-layer chromatography on silica gel (60 F_{254} silanized by Merck) in acetone–water (60 : 40), and represents the mean of 5 determinations.
12) C. B. C. Boyce and B. V. Milborrow, *Nature* (London), **208**, 537 (1965); R. Kaliszan, *J. Chromatogr.*, **220**, 71 (1981); N. Shirai, J. Sakakibara, T. Kaiya, S. Kobayashi, Y. Hotta, and K. Takaya, *J. Med. Chem.*, **26**, 851 (1983).
13) J. M. Cruickshank, *Am. Heart J.*, **100**, 160 (1980); A. J. McLean, D. Wilhelm and B. G. Heinzow, *Drugs*, **25** (Suppl. 2), 131 (1983).
14) L. M. Jackman and S. Sternhell, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry," 2nd ed., Pergamon Press, Inc., Braunschweig, 1969, p. 351.

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Comparative Studies on the Constituents of a Parasitic Plant and Its Host. III.[1] On the Constituents of *Boschniakia rossica* FEDTSCH. *et* FLEROV. (2)[2]

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Two iridoid glucosides, namely boschnaloside (**1**), and boschnaside (**2**), (+)-pinoresinol-β-D-glucopyranoside (**3**), a new oligosaccharide (=β-D-glucopyranosyl(1→4)-α-L-rhamnopyranosyl-(1→3)-D-(4-*O*-caffeoyl)-glucopyranose) (**4**), and a new phenylpropanoid glycoside named rossicaside A (**5**) have been isolated from *Boschniakia rossica* FEDTSCH. *et* FLEROV (Orobanchaceae) and their structures have been determined.

Keywords——*Boschniakia rossica*; Orobanchaceae; boschnaloside; boschnaside; (+)-pinoresinol-β-D-glucoside; acylated oligosaccharide; rossicaside A; phenylpropanoid glycoside

In the previous paper,[2] we reported the isolation and structure elucidation of three new phenylpropanoid glycosides, namely rossicasides B, C and D, as well as *p*-coumaric acid, methyl *p*-coumarate, β-sitosterol, oleanolic acid and 3-epioleanolic acid, from fresh plants of *Boschniakia rossica* (CHAM. *et* SCHLTDL.) FEDTSCH. *et* FLEROV (Orobanchaceae). *B. rossica* is a parasitic plant growing on the root of *Alnus maximowiczii* CALLIER (Betulaceae), and the dried herb or stem has been used as a tonic in Japan (Japanese name: oniku). The present paper deals mainly with the isolation and structure elucidation of two iridoid glucosides, namely boschnaloside (**1**)[3,4] and boschnaside (**2**),[5] (+)-pinoresinol-β-D-glucopyranoside (**3**),[6] an acylated oligosaccharide (**4**), and a phenylpropanoid glycoside named rossicaside A (**5**) from the butanol extract described in the previous paper.[2]

The butanol extract was treated by the method described in the experimental section. Compounds **1** and **2** have been isolated from the same plant by Sakan *et al.*[3] and Murai and Tagawa,[4,5] respectively, and the structures of both compounds have been established by stereochemical investigation. Compounds **1** and **2** were identified as boschnaloside (**1**)[3,4] and boschnaside (**2**)[5] by comparing the spectral data of the glucosides and their acetates.

Compound **3**, $C_{26}H_{32}O_{11}$, shows absorption bands due to hydroxyl groups and aromatic rings in the infrared (IR) spectrum. On acetylation with acetic anhydride and pyridine, **3** gave a pentaacetate (**3a**), $C_{36}H_{42}O_{16}$. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **3a** shows four aliphatic acetoxyl signals at δ 2.03 and 2.07 ppm, one aromatic acetoxyl signal at δ 2.30 ppm, two aromatic methoxyl signals at δ 3.83 and 3.84 ppm and the signals corresponding to six aromatic protons. The mass spectrum (MS) of **3a** exhibited the molecular ion peak at *m/z* 730 and a fragment ion peak at *m/z* 331 corresponding to an acetylated hexose. Accordingly, **3a** was suggested to contain a hexose, which was deduced to be β-D-glucose by analysis of the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **3** (Table I). Based on the ^{13}C-NMR spectral data, the aglycone part of **3** was deduced to be (+)-pinoresinol.[6c] Finally, **3** was identified as (+)-pinoresinol-β-D-glucopyranoside[6a] by compar-

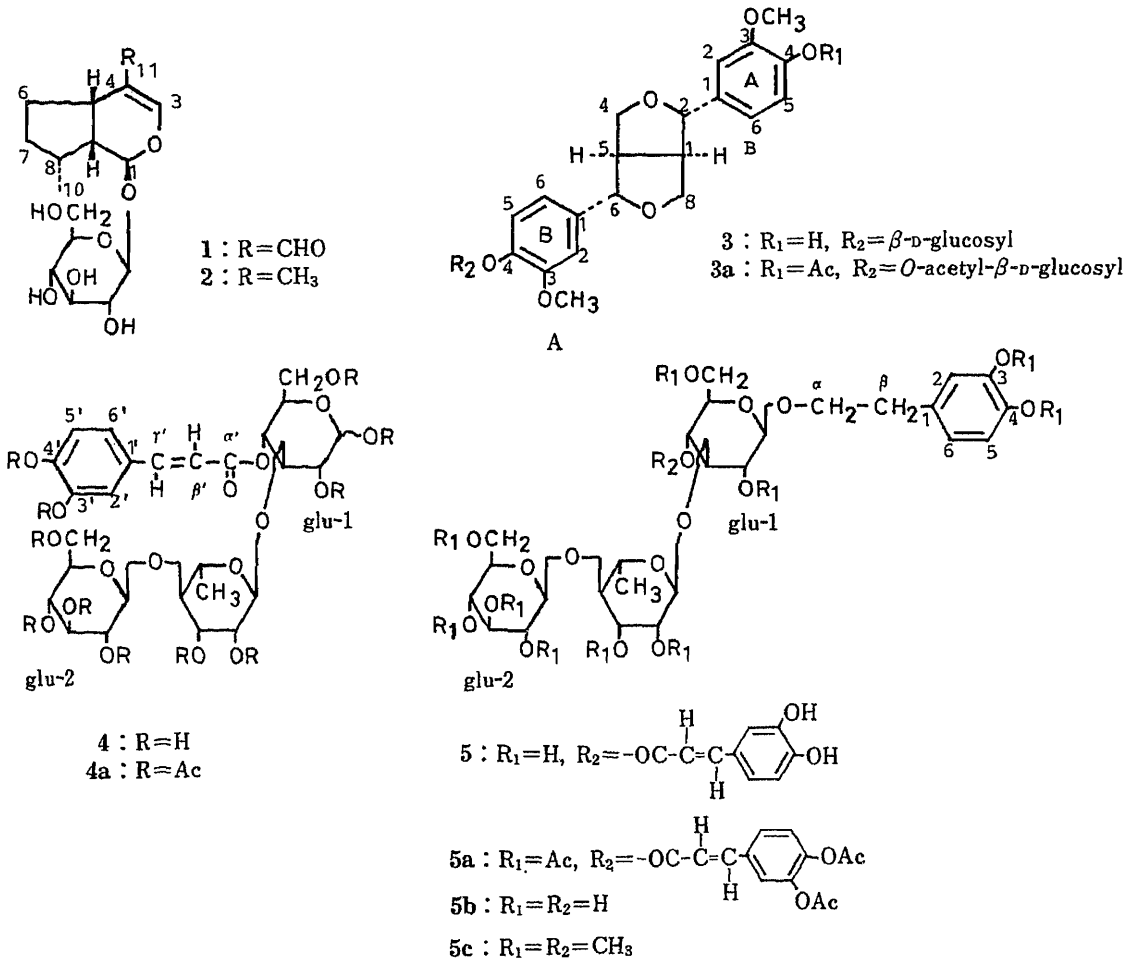

Chart 1

ing the physical data with reported values and by direct comparison with an authentic sample.

Compound **5**, $C_{35}H_{46}O_{20}$, was positive to the ferric chloride test, as in the cases of rossicasides B, C and D,[2] and showed absorption bands due to hydroxyl, ester, aromatic ring and olefinic double bond moieties in the IR spectrum. The ^{1}H-NMR spectrum also supported the presence of these functional groups in compound **5**. On acetylation in the usual way, compound **5** yielded a dodecaacetate (**5a**), $C_{59}H_{70}O_{32}$. On acidic hydrolysis, compound **5** gave glucose and rhamnose, while on enzymatic hydrolysis with crude hesperidinase, **5** gave glucose, rhamnose, caffeic acid and 3,4-dihydroxyphenethyl alcohol. When compound **5** was treated with 5% sodium methoxide in methanol, methyl caffeate and a deacyl product (**5b**), $C_{26}H_{40}O_{17}$, were formed. On methylation of compound **5b** by Hakomori's method,[7] **5b** gave a permethylate (**5c**), $C_{37}H_{62}O_{17}$. The ^{1}H-NMR spectrum of **5c** showed the signals of two aromatic methoxyl groups at δ 3.60 and 3.80 ppm. On methanolysis with methanolic 5% hydrogen chloride, **5c** afforded 3,4-dimethoxyphenethyl alcohol, methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside, methyl 2,4,6-tri-*O*-methyl-D-glucopyranoside and methyl 2,3-di-*O*-methyl-L-rhamnopyranoside. The field desorption mass (FDMS) and electron impact mass (EIMS) spectra of **5c** exhibited the $M^+ + H$ ion at *m/z* 779 and fragment ion peaks at *m/z* 219 and 393 corresponding to *O*-methylated terminal glucose and *O*-methylated glucosyl-rhamnose, respectively. Based on the foregoing results, the structure of **5b** was deduced to be 3,4-dihydroxyphenethyl alcohol 1-*O*-β-D-glucopyranosyl(1→4)-α-L-rhamnopyranosyl(1→3)-β-D-glucopyranoside. The location of the acyl group in **5** was determined as follows. In the

TABLE I. ^{13}C-NMR Chemical Shifts of **3**, **4**, **5** and **5b**[a]

Carbon	4	5	5b	Carbon	3
α		70.2	71.1	A	
β		36.1	36.1	1	133.0
1		130.6	130.3	2	111.1[b]
2		116.4[b]	116.4	3	148.7
3		146.9	146.8	4	147.5[c]
4		145.4	145.2	5	116.3
5		117.3	117.3	6	119.6[d]
6		120.5	120.3		
α′	166.9	167.0		B	
β′	115.5	115.9		1	136.1
γ′	146.8	146.8		2	110.9[b]
1′	126.6	127.0		3	150.0
2′	114.6	114.9		4	147.1[c]
3′	150.1	150.3		5	116.3
4′	147.2	147.4		6	119.0[d]
5′	116.4	116.7[b]			
6′	122.0	122.2		1	54.6
Glu-1	α β			2	85.9[e]
1	93.6 98.3	104.2	104.1 ($J_{C_1-H_1}$=156.4)[f]	4	71.9
2	74.6 75.8[b]	75.7[c]	76.4	5	54.6
3	84.5 84.5	84.8	85.3	6	86.2[e]
4	70.4 70.5	70.4	69.5	8	71.9
5	77.0 79.6	76.2	78.5[b]		
6	62.4 62.4	62.2[d]	62.4	Glu	
Glu-2				1	102.0
1	106.1	106.3	106.4 ($J_{C_1-H_1}$=162.5)[f]	2	74.6
2	75.6[b]	75.8[c]	75.6	3	78.0
3	77.9	77.9	78.2[b]	4	71.1
4	71.2[c]	71.8	71.2[c]	5	78.3
5	78.1	78.4	78.2[b]	6	62.2
6	62.0	62.9[d]	62.4		
Rham				OMe	56.0
1	102.9	102.8	101.9 ($J_{C_1-H_1}$=172.6)[f]		
2	71.8[c]	72.2	71.9[c]		
3	72.2	72.3	72.4		
4	82.0	81.5	82.7		
5	68.2	68.4	68.0		
6	18.7	18.8	18.6		

a) Chemical shifts were measured in pyridine-d_5. *b*—*e*) These assignments may be interchanged in each case. *f*) $J_{C_1-H_1}$ values were measured in the NOE mode.

previous report[2] the ^{1}H-NMR spectrum of rossicaside B revealed a triplet signal due to a methine proton bearing an ester group at δ5.02 ppm (J=8 Hz). Based on the ^{1}H-NMR spectrum of **5**, which shows a triplet signal (J=8 Hz) corresponding to one proton at δ5.00 ppm, it is suggested that caffeic acid of **5** is linked to the C-4 hydroxyl group of the glucose linked directly to the aglycone. Furthermore, ^{13}C-NMR spectral analysis of **5** and **5b** also suggested the location of caffeic acid. In the ^{13}C-NMR spectra of **5** and **5b**, the C-4 signal of the glucose linked directly to the aglycone is shifted 0.9 ppm downfield from that of **5b**, while the C-3 and C-5 signals are shielded by 0.5 and 2.3 ppm , respectively, and the other carbon signals of the two compounds are almost identical. The configurations of the monosaccharides of **5** were also assigned from the ^{13}C-NMR spectra, and the coupling constants of the anomeric carbon with the anomeric proton ($J_{H_1-C_1}$) of the two glucoses

(156.4, 162.5 Hz) and a rhamnose (172.6 Hz) revealed that the configurations of two glucoses are β-form and that of rhamnose is α-form.[8] In conclusion, the present results suggest that compound **5** is 3,4-dihydroxyphenethyl alcohol 1-*O*-β-D-glucopyranosyl(1→4)-α-L-rhamnopyranosyl(1→3) (4-*O*-caffeoyl)-β-D-glucopyranoside (**5**).

Compound **4**, $C_{27}H_{38}O_{18}$, gives a positive ferric chloride test, and the IR, ultraviolet (UV) and ^{1}H-NMR spectra indicated the presence of hydroxyl groups, an ester group, an aromatic ring and an olefinic double bond in **4**. On acidic hydrolysis compound **4** gave glucose and rhamnose. The presence of a reducing-end group in the molecule was suggested by the positive Benedict reaction.[9] When compound **4** was treated with 5% sodium methoxide in methanol, methyl caffeate was formed. By comparative studies on the ^{1}H-NMR spectrum of **4** with that of **5**, it was found that the number of aromatic protons were less than that of **5** and the methylene proton signal due to the phenethyl group was absent. A triplet proton signal at δ 4.99 ppm was observed in the spectrum of **4**, as in the case of **5**. Based on the ^{1}H-NMR spectrum of **4**, it is suggested that caffeic acid of **4** is linked to a secondary hydroxyl group of the sugar, and **4** was deduced to be an acylated oligosaccharide. The SIMS spectrum of **4** exhibited the $M^+ + H$ ion at *m/z* 651 and the EIMS spectrum of the acetate (**4a**) shows a fragment ion peak of *O*-acetylated terminal glucose at *m/z* 331 and that of *O*-acetylated glucosyl-rhamnose at *m/z* 561. Consequently, the acyl group of **4** was concluded to be linked to the reducing terminal sugar. By comparing the ^{13}C-NMR spectra of **4** and **5**, it could be seen that the former lacks the carbon signals corresponding to the "aglycone" of **5**, but the signals corresponding to the terminal glucose, rhamnose and caffeic acid coincide well with each other. The location of the terminal glucose was concluded to be the C-4 hydroxyl group of rhamnose, and the assignment of signals of reducing terminal glucose was carried out by comparing the chemical shifts with those of α,β-laminaribiose.[10] Furthermore, the location of caffeic acid in **4** was revealed by the ^{13}C-NMR spectrum to be the C-4 hydroxyl group of the terminal reducing glucose based on the acylation shift value.[11] In conclusion, the structure of **4** was elucidated to be β-D-glucopyranosyl(1→4)-α-L-rhamnopyranopyranosyl(1→3)-(4-*O*-caffeoyl)-D-glucopyranose.

Further studies on other constituents and on the pharmacological activities of *Boschniakia rossica* are in progress.

Experimental

All melting points were determined on a Yanagimoto micromelting point apparatus (hot stage type) and are uncorrected. The optical rotations were measured with a NEP-2 (Rex) automatic polarimeter. The IR spectra were recorded with a Shimadzu IR-27G spectrometer, UV spectra with a Shimadzu UV-200S spectrometer, ^{1}H-NMR spectra with Hitachi R-22 (90 MHz) and Varian CFT-20 (80 MHz) spectrometers, and ^{13}C-NMR spectra with a JEOL PFT-100 (22.15 MHz) spectrometer. Chemical shifts are given on a δ (ppm) scale with tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; dd, double doublet; m, multiplet; br, broad). Off-resonance spectra of each compound were also taken in order to aid assignment. Gas liquid chromatography (GLC) was run on a Shimadzu GC-6A unit with a flame ionization detector. MS were recorded on a Hitachi M-80 mass spectrometer. Thin layer chromatography (TLC) was performed on precoated Kieselgel F_{254} plates (Merck) and detection was achieved by spraying ethanolic $FeCl_3$ solution or 10% H_2SO_4 followed by heating.

Isolation of 1, 2, 3, 4 and 5——The BuOH extract (200 g)[2] was subjected to column chromatography on cellulose powder (Avicel) with $CHCl_3$–MeOH–H_2O (70 : 18 : 10, v/v; lower phase) and the eluate was separated into three fractions, fr. 1 (15 g), fr. 2 (100 g) and fr. 3 (75 g). Fr. 1 was rechromatographed on silica gel using $CHCl_3$–MeOH–H_2O (9 : 1 : 0.1, v/v) to afford boschnaloside **1** (2.4 g), boschnaside **2** (500 mg) and a lignan **3** (200 mg). Fr. 2 was repeatedly chromatographed on silica gel to afford rossicasides B, C and D. Fr. 3 was subjected to column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5, v/v) to yield fr. 4 (10 g) and fr. 5 (35 g). Fr. 4 was chromatographed on silica gel with $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5, v/v) then on Sephadex LH-20 with MeOH to afford rossicaside A, **5** (2.5 g). Fr. 5 was chromatographed on polystyrene gel (TSK-GEL LS-110) with MeOH–H_2O (5 : 95, v/v) to afford **4** (800 mg).

Properties of 1, 2, 3, 4 and 5——Compound **1**: Colorless needles from water, mp 96—97 °C (dec.), $[\alpha]_D^{23}$ −122.2°

($c=1.1$, MeOH) [lit.[3,4] mp 102—103 °C (dec.), $[\alpha]_D^{16}-134.5°$ ($c=1.0$, MeOH)]. UV λ_{max}^{EtOH} nm ($\log\varepsilon$): 248 (4.25). IR ν_{max}^{KBr} cm^{-1}: 3300—3500 (OH), 1660 (C=O), 1625 (olefin). ^{1}H-NMR (D_2O) δ: 1.01 (3H, d, $J=7$ Hz, CH_3), 4.87 (1H, d, $J=8$ Hz, anomeric H), 5.68 (1H, d, $J=2$ Hz, C_1-H), 7.48 (1H, s, C_3-H), 9.08 (1H, s, CHO). ^{13}C-NMR (pyridine-d_5) δ: 96.6 (C_1), 162.0 (C_3), 124.9 (C_4), 35.9 (C_5), 32.8 (C_6 or C_7), 30.5 (C_7 or C_6), 31.4 (C_8), 43.1 (C_9), 16.3 (C_{10}), 190.4 (C_{11}), glucose: 100.1 (C_1), 74.4 (C_2), 78.1 (C_3 or C_5), 71.3 (C_4), 78.5 (C_5 or C_3), 62.6 (C_6). *Anal.* Calcd for $C_{16}H_{24}O_8\cdot 1/3H_2O$: C, 54.85; H, 7.10. Found: C, 54.89; H, 7.23. Acetylation of **1** with Ac_2O–pyridine gave a tetraacetate of **1**, colorless needles from aq. MeOH, mp 137.5—138 °C, $[\alpha]_D^{23}-123.5°$ ($c=1.0$, $CHCl_3$) [lit.[3,4] mp 144—145 °C, $[\alpha]_D^{26.5}-131.0°$ ($c=1.0$, $CHCl_3$)]. UV λ_{max}^{EtOH} nm ($\log\varepsilon$): 244 (4.23). IR ν_{max}^{Nujol} cm^{-1}: 1760 (ester), 1670 (C=O), 1635 (olefin). ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, d, $J=7$ Hz, CH_3), 1.96, 2.02, 2.05, 2.10 (each s, OAc), 2.90 (2H, m), 3.80 (2H, m), 5.15 (1H, d, $J=8$ Hz, anomeric H), 5.44 (1H, d, $J=2$ Hz, C_1-H), 7.11 (1H, s, C_3-H). EIMS (*m/z*): 513 (M^++H), 331 (*O*-acetylated terminal glucose). *Anal.* Calcd for $C_{24}H_{32}O_{12}$: C, 56.24; H, 6.29. Found: C, 56.44; H, 6.33.

Compound **2**: Colorless needles from aq. MeOH, mp 102—103.5 °C, $[\alpha]_D^{20}-178.4°$ ($c=1.0$, MeOH). UV λ_{max}^{EtOH} nm ($\log\varepsilon$): 209 (3.52). IR ν_{max}^{KBr} cm^{-1}: 3300—3500 (OH), 1680 (olefin). ^{1}H-NMR (D_2O) δ: 0.99 (3H, d, $J=5$ Hz, CH_3), 1.52 (3H, s, CH_3), 4.70 (1H, d, $J=7$ Hz, anomeric H), 5.30 (1H, s, C_1-H), 6.01 (1H, s, C_3-H). *Anal.* Calcd for $C_{16}H_{26}O_7\cdot 1/4H_2O$: C, 57.38; H, 7.98. Found: C, 57.48; H, 8.04. Acetylation of **2** with Ac_2O–pyridine gave the tetraacetate of **2**, colorless needles from aq. MeOH. mp 132—134.5 °C (dec.), $[\alpha]_D^{24}-137.4°$ ($c=1.71$, $CHCl_3$) [lit.[5] 131—132 °C, $[\alpha]_D^{24}-140.8°$ ($c=1.25$, $CHCl_3$)]. UV λ_{max}^{EtOH} nm ($\log\varepsilon$): 209 (3.34). IR ν_{max}^{Nujol} cm^{-1}: 1760, 1745 (ester), 1675 (olefin). ^{1}H-NMR ($CDCl_3$) δ: 1.00 (3H, d, $J=7$ Hz, CH_3), 1.56 (3H, s, CH_3), 1.96, 1.99, 2.01, 2.07 (each 3H, s, OAc), 5.89 (1H, s, C_3-H). *Anal.* Calcd for $C_{24}H_{34}O_{11}\cdot 1/3H_2O$: C, 57.13; H, 6.93. Found: C, 57.17; H, 6.88.

Compound **3**: Colorless needles from water, mp 111—113 °C (dec.), $[\alpha]_D^{30}+12.4°$ ($c=1.0$, MeOH) [lit.[6a] mp 112—114 °C, $[\alpha]_D^{24}+10.8°$ ($c=1.0$, MeOH)]. IR ν_{max}^{KBr} cm^{-1}: 3200—3500 (OH), 1605, 1615 (aromatic ring). EIMS (*m/z*): 358 $(M+H-163)^+$, 163 (glucose), 179 $(M+H-163)^{++}$. ^{1}H-NMR (DMSO-d_6) δ: 3.05 (2H, m, C_1 and C_5-H), 3.76 (6H, s, OCH_3), 4.09 (2H, m, C_{4e} and C_{8e}-H), 4.61 (1H, d, $J=4.4$ Hz, C_2 or C_6-H), 4.66 (1H, d, $J=4.3$ Hz, C_6 or C_2-H), 4.85 (1H, d, $J=7$ Hz, anomeric H), 6.73—7.11 (6H, aromatic H), 8.86 (1H, s, aromatic OH). *Anal.* Calcd for $C_{26}H_{32}O_{11}\cdot 2H_2O$: C, 56.11; H, 6.52. Found: C, 56.00; H, 6.25. ^{13}C-NMR (pyridine-d_5): Table I. Acetylation of **3** with Ac_2O–pyridine gave an acetate of **3**, colorless needles from MeOH, mp 115.5—117 °C [lit.[6b] colorless crystalline powder from MeOH, mp 109—110 °C]. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1760 (ester), 1605, 1510 (aromatic). EIMS (*m/z*): 730 (M^+), 688 (M^+-COCH_2), 331, 169, 109. ^{1}H-NMR ($CDCl_3$) δ: 2.03, 2.07 (each 6H, s, OAc×2), 2.30 (3H, s, aromatic OAc), 3.07 (2H, br, C_1 and C_5-H), 3.83, 3.84 (each 3H, s, aromatic OCH_3), 6.85—7.04 (6H, aromatic H). *Anal.* Calcd for $C_{36}H_{42}O_{16}$: C, 59.17; H, 5.79. Found: C, 59.24; H, 5.91.

Compound **4**: Colorless needles from water, positive to the Benedict reagent,[9] mp 182—187 °C (dec.), $[\alpha]_D^{19.5}-56.3°$ ($c=1.0$, MeOH). UV λ_{max}^{EtOH} nm ($\log\varepsilon$): 220 (3.04), 248 (2.92), 300 (sh, 3.08), 330 (3.24). IR ν_{max}^{Nujol} cm^{-1}: 3200—3500 (OH), 1690 (C=O), 1630, 1600, 1520 (olefin, aromatic ring). ^{1}H-NMR (CD_3OD) δ: 1.19 (3H, d, $J=6$ Hz, CH_3 of rham), 4.49 (1H, d, $J=8$ Hz, anomeric H), 4.58 (1/2H, d, $J=8$ Hz, anomeric α-H), 4.99 (1H, t, $J=8$ Hz, C_4-H of glu-1), 5.15 (1/2H, d, $J=3$ Hz, anomeric β-H), 5.18 (1H, s, anomeric H), 6.25 (1H, d, $J=16$ Hz, Ar–CH=CH–CO–), 6.79 (1H, d, $J=8$ Hz, aromatic H), 6.99 (1H, d, $J=8$ Hz, aromatic H), 7.06 (1H, s, aromatic H), 7.95 (1H, d, $J=16$ Hz, Ar–CH=CH–CO–). SIMS (*m/z*): 743 (M^++glycerin+H), 673 (M^++Na), 651 (M^++H). EIMS (*m/z*): 650 (M^+), 163 ($C_6H_{11}O_5$), 136 (base peak, $C_7H_6O_3$). Elemental analysis was not done on account of the strongly hygroscopic nature of **4**.

Compound **5**: A white powder from AcOEt–MeOH, (mp 173—175 °C (dec.)), $[\alpha]_D^{11}-101.4°$ ($c=0.9$, MeOH). UV λ_{max}^{EtOH} nm ($\log\varepsilon$): 220 (4.46), 245 (sh, 4.18), 290 (sh, 4.20), 305 (4.23), 330 (4.28). IR ν_{max}^{KBr} cm^{-1}: 3200—3500 (OH), 1690 (C=O), 1630, 1605, 1515 (olefin, aromatic ring). ^{1}H-NMR (CD_3OD) δ: 1.22 (3H, d, $J=6$ Hz, CH_3 of rham), 2.81 (2H, t, $J=7$ Hz, CH_2), 4.39 (2H, d, $J=7$ Hz, anomeric H), 5.00 (1H, t, $J=8$ Hz, C_4-H of glu-1), 5.25 (1H, s, anomeric H), 6.25 (1H, d, $J=16$ Hz, Ar–CH=CH–CO–), 6.65—7.10 (6H, aromatic H), 7.58 (1H, d, $J=16$ Hz, Ar–CH=CH–CO–), *Anal.* Calcd for $C_{35}H_{46}O_{20}\cdot 1/2H_2O$: C, 52.83; H, 5.95. Found: C, 52.67; H, 5.72. ^{13}C-NMR data were shown in Table I.

Acetylation of 4 and 5——Compounds **4** (60 mg) and **5** (100 mg) were each dissolved in pyridine–acetic anhydride (1 : 1, v/v) and the solution was allowed to stand overnight at room temperature. The reaction mixture was poured into ice-cold water and the precipitate was filtered off. Each crude acetate was purified by column chromatography, on silica gel with benzene–acetone (3 : 1, v/v) for **5a** and on Sephadex LH-20 with MeOH for **4a**. **4a**: colorless needles from MeOH, mp 179—181 °C, $[\alpha]_D^{30}-26.0°$ ($c=1.0$, $CHCl_3$). UV λ_{max}^{EtOH} nm ($\log\varepsilon$): 210 (3.05), 285 (3.36). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1750 (ester), 1640, 1500 (aromatic ring). EIMS (*m/z*): 561 (*O*-acetylated glucosyl-rhamnose), 501 (561 – AcOH), 331 (*O*-acetylated terminal glucose). ^{1}H-NMR ($CDCl_3$) δ: 1.09 (3H, d, $J=5.5$ Hz, CH_3), 1.97—2.16 (27H, OAc×9), 2.29, 2.32 (each 3H, s, aromatic OAc), 6.36 (1H, d, $J=16$ Hz, Ar–CH=CH–CO–), 7.24—7.45 (3H, aromatic H), 7.65 (1H, d, $J=16$ Hz, Ar–CH=CH–CO–). *Anal.* Calcd for $C_{49}H_{60}O_{29}$: C, 52.87; H, 5.43. Found: C, 52.85; H, 5.44. **5a**: a white powder from MeOH, mp 103—106 °C (dec.), $[\alpha]_D^{30}-47.0°$ ($c=1.0$, $CHCl_3$). UV λ_{max}^{EtOH} nm ($\log\varepsilon$): 210 (4.17), 280 (4.20). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1750 (ester), 1640, 1605, 1503 (olefin, aromatic ring). EIMS (*m/z*): 561 (*O*-acetylated glucosyl-rhamnose), 501 (561 – AcOH), 331 (*O*-acetylated terminal glucose). ^{1}H-NMR ($CDCl_3$) δ: 1.08 (3H, d, $J=5$ Hz, CH_3), 1.98—2.11 (24H, OAc×8), 2.26, 2.27, 2.31, 2.39 (each 3H, s, aromatic OAc), 2.85 (1H, t, $J=$

7 Hz, CH_2), 6.34 (1H, d, J=16 Hz, Ar–CH=CH–CO–), 6.94—7.44 (6H, aromatic H), 7.62 (1H, d, J=16 Hz, Ar–CH=CH–CO–). *Anal.* Calcd for $C_{59}H_{70}O_{32}$: C, 54.88; H, 5.46. Found: C, 54.60; H, 5.51.

Acidic Hydrolysis of 5 and 4——Compound **5** (10 mg) was refluxed with 2 N HCl–50% dioxane (5 ml) for 2 h on a water bath. The reaction mixture was diluted with water and extracted with ether. The aqueous layer was neutralized with Amberlite IRA-410 and the neutral solution was evaporated to dryness under reduced pressure. The residue was examined by TLC and GLC. TLC (solvent: $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5, v/v). *Rf* 0.10 (glucose), 0.40 (rhamnose). GLC (column, 5% SE-52 on Chromosorb W 3 mm × 2 m; column temp., 165 °C; injection temp., 240 °C; carrier gas, N_2 1.0 kg/cm^2; sample, TMS derivative): t_R (min) 21.2, 32.7 (glucose), 6.6, 8.8 (rhamnose). Compound **4** (50 mg) was hydrolyzed by the procedure described above to afford glucose and rhamnose.

Enzymatic Hydrolysis of 5——Compound **5** (500 mg) was incubated with crude hesperidinase (200 mg) for 48 h at 32 °C. The precipitate was filtered off. The filtrate was extracted with BuOH and the aqueous layer was evaporated to dryness under reduced pressure. The residue was examined by TLC and GLC, and glucose and rhamnose were detected. The BuOH layer was evaporated to dryness and the residue (300 mg) was subjected to chromatography on Sephadex LH-20 with MeOH followed by silica gel with $CHCl_3$–MeOH–H_2O (7 : 3 : 0.3, v/v) as the solvent to afford caffeic acid (30 mg) and an aglycone (20 mg). The former was identified by comparison with an authentic sample, while the latter, an oily substance, was identified as 3,4-dihydroxyphenethyl alcohol from the ^{1}H-NMR spectrum. ^{1}H-NMR (CD_3OD) δ: 2.66 (2H, t, J=7 Hz, CH_2), 3.69 (2H, t, J=7 Hz, CH_2), 6.50 (1H, dd, J=2, 8 Hz, aromatic C_6-H), 6.62 (1H, d, J=2 Hz, aromatic C_2-H), 6.67 (1H, d, J=8 Hz, aromatic C_5-H).

Hydrolysis of 5 with 5% Sodium Methoxide——A solution of **5** (500 mg) in methanolic 5% NaOMe was allowed to stand at room temperature for 10 h. The reaction mixture was extracted with ether. The ether extract was dried over Na_2SO_4 and evaporated to dryness. The residue was chromatographed on Sephadex LH-20 with MeOH to afford colorless needles from MeOH. This compound was identified as methyl caffeate by direct comparison with an authentic sample.

The aqueous layer was neutralized with Amberlite IRA-410 and evaporated to dryness under reduced pressure. The residue was subjected to column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5, v/v) then on Sephadex LH-20 with MeOH to give a deacylate of **5** (**5b**, 200 mg). **5b**: a white powder from aq. MeOH, (mp 138—140 °C (dec.)), $[\alpha]_D^{30}$ −25.7° (c=1.2, MeOH). UV λ_{max}^{EtOH} nm (log ε): 220 (3.94), 280 (3.60). IR ν_{max}^{KBr} cm^{-1}: 3200—3600 (OH), 1610, 1520 (aromatic ring). ^{1}H-NMR (CD_3OD) δ: 1.35 (3H, d, J=6 Hz, CH_3), 2.78 (1H, t, J=7 Hz, CH_2), 4.29 (1H, d, J=7 Hz, anomeric H), 4.58 (1H, d, J=7 Hz, anomeric H), 5.14 (1H, s, anomeric H), 6.50 (1H, dd, J=2, 8 Hz, aromatic C_6-H), 6.67 (1H, d, J=8 Hz, aromatic C_5-H and 1H, d, J=2 Hz, aromatic C_2-H). *Anal.* Calcd for $C_{26}H_{40}O_{17}\cdot 2/3H_2O$: C, 49.05; H, 6.54. Found: C, 48.84; H, 6.53.

Methylation of 5b——Compound **5b** (110 mg) was methylated by Hakomori's method. The reaction mixture was diluted with water and extracted with ether. The ether layer was washed with water, dried over Na_2SO_4 and then evaporated to dryness. The residue was chromatographed on silica gel with benzene–acetone (10 : 1, v/v) to give a permethylate of **5b** (**5c**). **5c**: colorless oil, $[\alpha]_D^{30}$ −50° (c=2.4, $CHCl_3$). UV λ_{max}^{EtOH} nm (log ε): 220 (4.01), 270 (3.80). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1585, 1505 (aromatic ring). ^{1}H-NMR (CCl_4) δ: 1.19 (3H, d, J=5 Hz, CH_3), 3.36—3.55 (27H, aliphatic $OCH_3\times 9$), 3.60, 3.80 (each 3H, s, aromatic OCH_3), 4.15 (1H, d, J=7 Hz, anomeric H), 4.50 (1H, d, J=7 Hz, anomeric H), 5.15 (1H, s, anomeric H), 6.60—6.70 (3H, aromatic H). FDMS (m/z): 779 (M^++H). EIMS (m/z): 778 (M^+), 393 (*O*-methylated glucosyl-rhamnose), 219 (*O*-methylated terminal glucose). *Anal.* Calcd for $C_{37}H_{62}O_{17}$: C, 57.05; H, 8.02. Found: C, 57.28; H, 7.92.

Methanolysis of 5c——A solution of **5c** (10 mg) in methanolic 5% HCl (2 ml) was refluxed for 3 h. The reaction mixture was neutralized with Ag_2CO_3 and filtered. The filtrate was evaporated to dryness under reduced pressure. The residue was chromatographed on Sephadex LH-20 with MeOH to yield methylated monosaccharides and an aglycone. The *O*-methylated monosaccharides were examined by TLC (solvent: benzene–acetone 3 : 1, v/v) and GLC (5% NPGS on Chromosorb W, 3 mm × 2 m; column temp., 170 °C; injection temp., 200 °C; carrier gas, N_2 1.2 kg/cm^2). *Rf*: 0.42, 0.53; t_R (min) 2.8, 3.7 (methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside). *Rf*: 0.12, 0.23; t_R (min) 7.3, 10.7 (methyl 2,4,6-tri-*O*-methyl-D-glucopyranoside). *Rf*: 0.25, 0.35; t_R (min) 3.7, 5.0 (methyl 2,3-di-*O*-methyl-L-rhamnopyranoside). The aglycone was identified as 3,4-dimethoxyphenethyl alcohol by TLC (solvent: benzene–acetone (3 : 1, v/v); *Rf*: 0.38).

Alkali Hydrolysis of 4——Compound **4** (160 mg) was hydrolyzed and worked up as described for **5** to give methyl caffeate (20 mg), which was identified by direct comparison with an authentic sample.

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References and Notes

1) Part II: S. Sanada, K. Inaba (née Usui), Y. Ida and J. Shoji, *Shoyakugaku Zasshi*, **41**, 80 (1987).
2) T. Konishi and J. Shoji, *Chem. Pharm. Bull.*, **29**, 2807 (1981).

3) T. Sakan, F. Murai, S. Isoe, S. Hyeon, and Y. Hayashi, *Nippon Kagaku Zasshi*, **90**, 507 (1969).
4) F. Murai and M. Tagawa, *Chem. Pharm. Bull.*, **28**, 1730 (1980).
5) F. Murai and M. Tagawa, *Planta Medica*, **46**, 45 (1982).
6) *a*) K. Kudo, T. Nohara, T. Komori, T. Kawasaki and H. R. Schulten, *Planta Medica*, **40**, 250 (1980); *b*) M. Chiba, S. Hisada and S. Nishibe, *Shoyakugaku Zasshi*, **32**, 194 (1978); *c*) S. Nishibe, H. Tsukamoto and S. Hisada, *Chem. Pharm. Bull.*, **32**, 4653 (1984).
7) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 209 (1964).
8) O. Tanaka, *Yakugaku Zasshi*, **105**, 332 (1985).
9) "Identification of Organic Compounds," ed. by N. D. Cheronis and J. B. Entrikin, Interscience Publishers Inc., New York, 1963, p. 135.
10) *a*) T. Usui, N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama and S. Seto, *J. Chem. Soc., Perkin Trans. 1*, **1973**, 2425; *b*) P. Colson, H. J. Jennings and I. P. Smith, *J. Am. Chem. Soc.*, **96**, 8081 (1974).
11) *a*) V. M. Chan, M. Jordan, H. Wagner and P. W. Thies, *Phytochemistry*, **16**, 1110 (1977); *b*) H. Ishii, S. Seo, K. Tori, T. Tojyo and Y. Yoshimura, *Tetrahedron Lett.*, **1977**, 1227; *c*) K. Yamasaki, R. Kasai, Y. Masaki, M. Okihara, O. Tanaka, H. Oshio, S. Takagi, M. Yamaki, K. Masuda, G. Nonaka, M. Tsuboi and I. Nishioka, *ibid.*, **1977**, 1231.

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Studies on the Constituents of the Seeds of *Hernandia ovigera* L. VI.[1] Isolation and Structural Determination of Three Lignans

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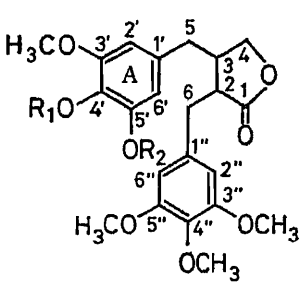
Three kinds of lignans, including a new lignan, named hernolactone, were isolated from the seeds of *Hernandia ovigera* L. besides six previously reported lignans, desoxypodophyllotoxin (DPT), desoxypicropodophyllin, bursehernin, podorhizol, hernandin and dehydro-DPT. The structure of hernolactone was determined as (2*R*,3*R*)-3-(4′-hydroxy-3′,5′-dimethoxybenzyl)-2-(3″,4″,5″-trimethoxybenzyl)-γ-butyrolactone (IV) and the other two lignans were identified as (−)-yatein ((−)-deoxypodorhizon) (V) and dehydropodophyllotoxin (IX).

Keywords——*Hernandia ovigera*; hernolactone; (−)-yatein; (−)-deoxypodorhizon; dehydropodophyllotoxin; dehydro-β-peltatin methyl ether; dehydrohernandin; ^{13}C-NMR spectrum; lignan; dibenzylbutyrolactone-type lignan

In the previous paper of this series,[2] the authors carried out the systematic extraction and isolation of the components of the seeds of *Hernandia ovigera* L.; six lignans, desoxypodophyllotoxin (DPT), desoxypicropodophyllin, bursehernin, podorhizol, hernandin and dehydro-DPT were isolated and their structures were confirmed. This time we reexamined the components of the original seeds and isolated a new lignan as well as two other known lignans. This paper describes the structural elucidation of these compounds. The three compounds A, B and C were isolated as described in Experimental.

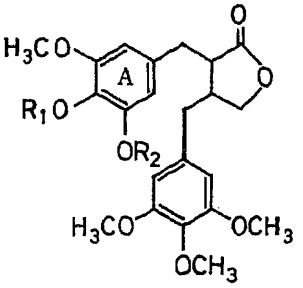
Compound A, named hernolactone, was obtained in a small quantity as a pale yellow solid, $[\alpha]_D -30°$ ($CHCl_3$). A molecular formula of $C_{23}H_{28}O_8$ and a molecular weight of 432.1765 were deduced by means of high-resolution mass spectrometry. In the ultraviolet (UV) spectrum, absorption maxima were seen at 225 and 272 nm. In the infrared (IR) spectrum, hydroxy group and carbonyl group absorptions were observed at 3585 and 1775 cm^{-1}, respectively. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum revealed the presence of four aromatic protons (δ 6.21 (singlet), 6.35 (singlet)), a proton deduced to be that of a hydroxy group (disappears on addition of D_2O) (δ 5.46), lactone methylene (δ 3.92, 4.20), five methoxy groups (δ 3.81, 3.82), two benzylmethylene groups (δ 2.94, 2.46—2.68) and two protons of lactone junctions C_2 and C_3 (δ 2.46—2.68). The facts that the signals of four protons including $C_{2,3}$-H and C_5-benzylmethylene appeared at δ 2.46—2.68 as a multiplet, C_6-benzylmethylene appeared at δ 2.94 as a multiplet and C_4-lactone methylene appeared at δ 3.92 and 4.20 as two double doublets show that this compound is a *trans*-2,3-dibenzylbutyrolactone-type compound, like hinokinin,[3] dimethylmatairesinol[4] and (−)-yatein. In the case of (−)-yatein, the *trans* structure was clearly confirmed by comparison with the *cis*-type compound.[5] From the above results, four possible structures, Ia, Ib, IIa and IIb, can be presumed for the structure of hernolactone.

In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of hernolactone, one methoxy group signal was shifted to lower magnetic field (60.9 ppm). The chemical shifts of

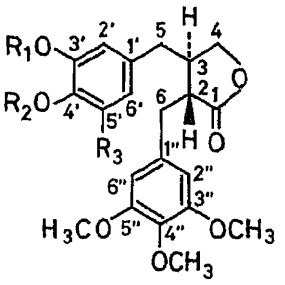


Ia: $R_1=H$, $R_2=CH_3$
Ib: $R_1=CH_3$, $R_2=H$

Fig. 1



IIa: $R_1=H$, $R_2=CH_3$
IIb: $R_1=CH_3$, $R_2=H$



III: $R_1=R_2=CH_3$, $R_3=OCH_3$
IV: $R_1=CH_3$, $R_2=H$, $R_3=OCH_3$
V: R_1–$R_2=CH_2$, $R_3=H$

Fig. 2

TABLE I. ^{13}C-NMR Data for Hernolactone (ppm)

Carbon	Chemical shifts	Carbon	Chemical shifts
1	178.7	4′	133.6
2	46.5	4″	137.0
3	41.3	5′	147.2
4	71.3	5″	153.3
5	38.8	6′	105.2
6	35.2	6″	106.3
1′	129.0	3″-OCH_3	56.1
1″	133.5	3′-OCH_3	56.3
2′	105.2	5″-OCH_3	56.1
2″	106.3	5′-OCH_3	56.3
3′	147.2	4″-OCH_3	60.9
3″	153.3		

the methoxy groups were examined in comparison with those of simple compounds such as 1,2,3-trimethoxybenzene, 2,3-dimethoxyphenol and 2,6-dimethoxyphenol.[6] In these compounds, the signal of a methoxy group situated between two methoxy groups or between methoxy and hydroxy groups is slightly shifted to lower magnetic field (approx. 60 ppm) as compared with ordinary methoxy groups (approx. 55 ppm). If hernolactone has the structure Ib or IIb in which a methoxy group of ring A is situated between methoxy and hydroxy groups, two such signals due to the methoxy groups at C-4′ and C-4″ should be seen. Accordingly the structures Ib and IIb were rejected. The assignment of every carbon was deduced by means of distortionless enhancement by polarization transfer (DEPT) and heteronuclear correlated 2D spectroscopy (HETCOR)[7] (Table I). In the mass spectrum (MS), fragment peaks were seen at 167, 181, 194, 238 and 251 and every fragment ion was assigned as shown in Chart 1.[3,4,8]

The peak at 238 shows that the position of the carbonyl group of the lactone ring is as in Ia, and hence the alternative structure IIa is rejected. As regards the absolute configuration of lactone junctions, negative Cotton effects appeared at 237 and 277 nm in the circular dichroism (CD) spectrum, as in the case of thujaplicatin methyl ether and analogous compounds,[9] suggesting that the configurations of C-2 and C-3 are 2*R* and 3*R*. In addition, the spectral data of the methyl ether of compound A coincided well with those of (−)-cubebininolide (III).[10] In conclusion, compound A, hernolactone, is confirmed to be (2*R*,3*R*)-3-(4′-hydroxy-3′,5′-dimethoxybenzyl)-2-(3″,4″,5″-trimethoxybenzyl)-γ-butyrolactone (IV).

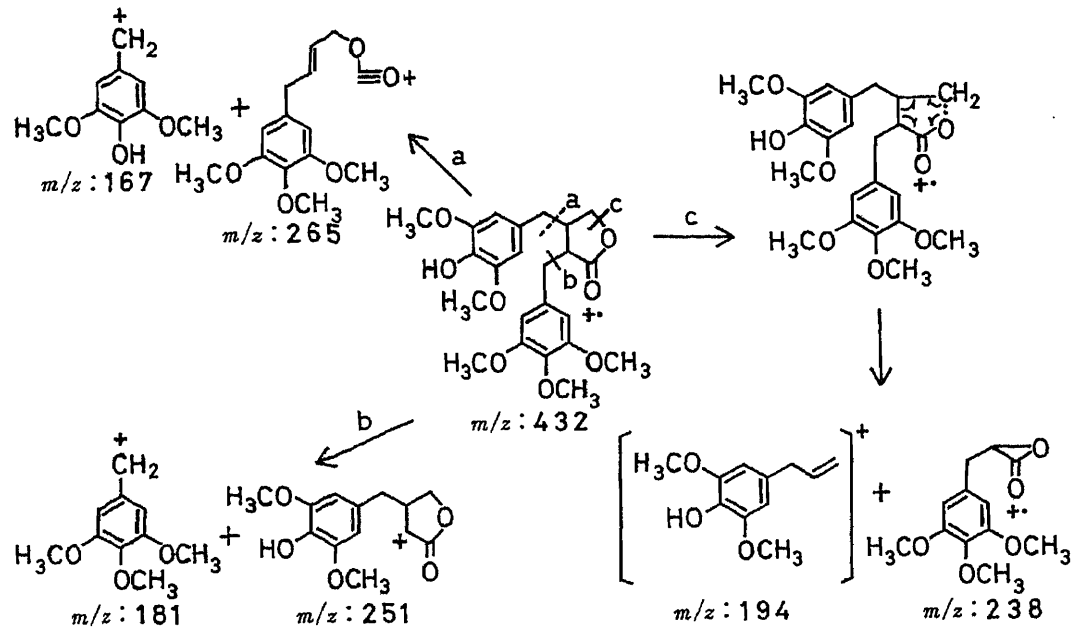
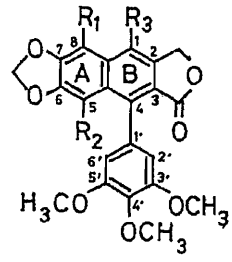


Chart 1

TABLE II. ^{1}H-NMR Chemical Shifts of VI and Related Compounds (VII and VIII)[a] (δ ppm, $CDCl_3$)



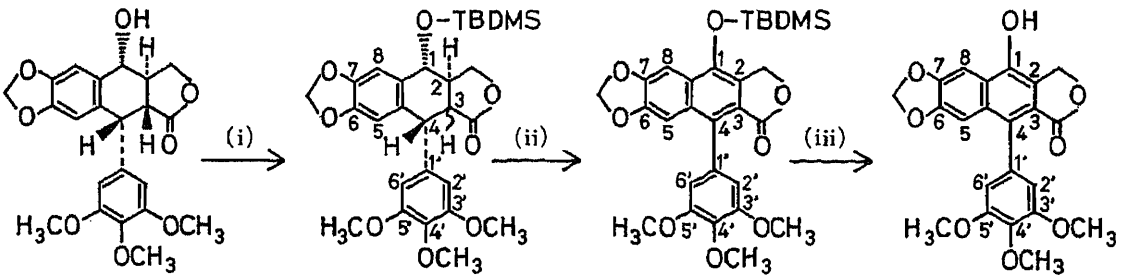
	VII ($R_1=OCH_3$, $R_2=R_3=H$)	VIII ($R_1=R_3=H$, $R_2=OCH_3$)	VI
C_1-H	8.15	7.64	7.57
C_8-H	—	6.98	7.07
C_5-H	6.85	—	
C_x-OCH_3[b]	4.20	3.47	4.09

a) VI was measured at 300 MHz, and VII and VIII were measured at 90 MHz. b) Methoxy group on ring A or ring B.

Compound B was obtained as a pale yellow glassy solid, $[\alpha]_D$ −29.5° ($CHCl_3$). A molecular formula of $C_{22}H_{24}O_7$ and a molecular weight of 400 were confirmed by means of elemental analysis and MS. The spectral data of IR, UV, ^{1}H-NMR, ^{13}C-NMR and CD agree well with those of (−)-yatein[11] ((−)-deoxypodorhizon) (V). Thus, the structure of compound B was confirmed as V. (−)-Yatein is widely distributed in many plants such as *Bursera schlechtendalli* (Burseraceae),[12] Umbelliferae,[13] Cupressaceae,[14] Piperaceae[8b,15] and Hernandiaceae.[16] It was synthesized by Koga *et al.*[17]

Compound C was obtained as needle crystals, mp 275—280 °C (dec.)[18]; it showed no optical rotation and was almost insoluble in ordinary solvents. It has a molecular formula of

$C_{22}H_{18}O_8$. In the UV spectrum, it shows absorption maxima at 222, 262, 322 and 355 nm. Hydroxy group and carbonyl group absorptions were observed at 3500 and 1770 cm^{-1}, respectively, in the IR spectrum. In the ^{1}H-NMR spectrum, four aromatic protons, two protons of a methylenedioxy group, two protons of a lactone methylene, nine protons of three methoxy groups and one proton of a hydroxy group (disappeared on addition of D_2O) were observed. These results suggest that compound C is a phenylnaphthalene-type lignan. Compound C was methylated with diazomethane and this methyl ether (VI) was compared with analogous known lignans. In the ^{1}H-NMR spectra of these lignans, VI differs from dehydro-β-peltatin methyl ether (VII)[19] and dehydrohernandin (VIII),[2b] as shown in Table II. From the above results, compound C was assumed to be dehydropodophyllotoxin (IX). Although IX is a well-known compound,[20] no precise spectral data have been given except in one report.[20b] We decided to synthesize IX in order to make a direct comparison with an authentic sample. Although Gensler *et al.*[20a] derived IX from podophyllotoxone in one step by means of selenium dioxide treatment, their method gave an unsatisfactory result in our reexamination and we prepared IX by another route as shown in Chart 2. By direct compari-



(i) TMDMSCl–imidazole/DMF, 75 °C[21]; (ii) DDQ/benzene, reflux;
(iii) Bu_4NF/THF, room temp. (TBDMS–: *tert*-butyldimethylsilyl group)

Chart 2

son of compound C with this authentic sample, the structure of compound C was confirmed as IX.

In conclusion, three kinds of lignans, including a new lignan named hernolactone (IV), were isolated from the seeds of *Hernandia ovigera* L. besides the previously reported six kinds of lignans, and their structures were clarified.

Experimental

All melting points were determined on a Yanaco micromelting point apparatus and are uncorrected. The instruments used in this study were as follows; optical rotations, Jasco DIP-181 digital polarimeter; UV spectra, Hitachi 200-10 spectrometer; IR spectra, Jasco IR A-1 spectrometer; CD spectra, Jasco J-500 A spectropolarimeter; MS, Hitachi M-80; ^{1}H- and ^{13}C-NMR spectra, Varian XL-300 instrument (with tetramethylsilane as an internal standard; chemical shifts are recorded in δ values). Column chromatography was carried out on Merck silica gel (Kieselgel 60; 70—230 mesh). Precoated silica gel plates used in preparative thin layer chromatography (PTLC) were Merck Kieselgel 60 F_{254}, 0.5 mm thickness. The original seeds of *Hernandia ovigera* L. (Hernandiaceae) were collected in Okinawa.

Extraction and Isolation——A benzene extract of the powdered seeds was prepared as described in ref. 2*a*). The extracts were crystallized from EtOH to yield DPT. The mother liquor was evaporated and the residue was defatted by treatment with *n*-hexane. The remaining part was submitted to column chromatography on silica gel ($CHCl_3$: AcOEt = 10 : 1), affording fraction (Fr.) 1-6. Fraction 2 was recrystallized from EtOH to give DPT and hernandin, and the mother liquor was concentrated *in vacuo*. The residue was further rechromatographed on silica gel with a chloroform–*n*-hexane (1 : 1) system to give compound B (=(−)-yatein). Fraction 6 was subjected to

rechromatography on silica gel with a mixture of *n*-hexane–AcOEt to afford Fr. 6-1—Fr. 6-5. Fraction 6-3 was crystallized from EtOH to give podorhizol and the mother liquor was evaporated. The residue was subjected to PTLC (*n*-hexane : AcOEt = 1 : 1; *Rf* = 0.21), affording compound A (=hernolactone). Fraction 6-5 was recrystallized repeatedly from MeOH and AcOEt to give compound C (=dehydropodophyllotoxin).

Compound A (Hernolactone) (IV)——Pale yellow solid. $[\alpha]_D$ −30° (*c* = 0.36, $CHCl_3$). High-resolution MS Calcd for $C_{23}H_{28}O_8$ (M^+): 432.1781. Found: 432.1765. UV $\lambda_{max}^{95\%\,EtOH}$ nm (log ε): 272 (3.28), 225 (4.19) sh. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3585 (OH), 1775 (C=O). CD (*c* = 5.213 × 10^{-5}, 95% EtOH) Δε: −0.58 (277), −0.12 (255), −2.91 (237) (negative maximum). MS *m/z* (rel. int.): 432 (87.6, M^+), 251 (2.5), 238 (1.2), 208 (0.4), 194 (2.6), 181 (100), 167 (54.1), 151 (15.3). ^{1}H-NMR ($CDCl_3$) δ: 2.46—2.68 (4H, m, $C_{2,3}$-H and C_5-H_2), 2.94 (2H, m, C_6-H_2), 3.81 (s), 3.82 (s) (15H, $C_{3',5'}$-OCH_3 and $C_{3'',4'',5''}$-OCH_3), 3.92 (1H, dd, *J* = 8.3, 7.5 Hz, $C_{4\beta}$-H), 4.20 (1H, dd, *J* = 8.7, 7.2 Hz, $C_{4\alpha}$-H), 5.46 (1H, s, $C_{4'}$-OH, disappeared on addition of D_2O), 6.21 (2H, s, $C_{2',6'}$-H), 6.35 (2H, s, $C_{2'',6''}$-H). The ^{13}C-NMR data are summarized in Table I.

Methylation of IV——A solution of IV (20 mg) in ether–MeOH was methylated with CH_2N_2–Et_2O for 16 h. After removal of the solvent, the residue was subjected to PTLC (*n*-hexane : AcOEt = 1 : 1) to give a pale yellow viscous mass (15 mg). $[\alpha]_D$ −35.9° (*c* = 0.41, $CHCl_3$) (ref.[10]: $[\alpha]_D^{25}$ −17.6° (*c* = 0.23, $CHCl_3$)). High-resolution MS Calcd for $C_{24}H_{30}O_8$ (M^+): 446.1938. Found: 446.1936. UV λ_{max}^{MeOH} nm (log ε): 270 (3.86), 226 (4.05) sh. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1770 (C=O). CD (*c* = 6.323 × 10^{-5}, 95% EtOH) Δε: −0.30 (276), −0.08 (260), −3.16 (237) (negative maximum). MS *m/z* (rel. int.): 446 (76.7, M^+), 265 (3.0), 238 (1.3), 223 (3.6), 219 (2.4), 182 (67.9), 181 (100). ^{1}H-NMR ($CDCl_3$) δ: 2.47—2.69 (4H, m, $C_{2,3}$-H and C_5-H_2), 2.97 (2H, d, *J* = 6.6 Hz, C_6-H_2), 3.80 (s), 3.81 (s), 3.82 (s) (18H, $C_{3',4',5'}$-OCH_3 and $C_{3'',4'',5''}$-OCH_3), 3.90 (1H, dd, *J* = 9.3, 7.2 Hz, $C_{4\beta}$-H), 4.19 (1H, dd, *J* = 9.0, 6.6 Hz, $C_{4\alpha}$-H), 6.21 (2H, s, $C_{2',6'}$-H), 6.39 (2H, s, $C_{2'',6''}$-H). ^{13}C-NMR ($CDCl_3$) δ: 35.1 (C-6), 39.0 (C-5), 41.1 (C-3), 46.6 (C-2), 56.1, 56.2 ($C_{3'',5''}$-O$\underline{C}H_3$ and $C_{3',5'}$-O$\underline{C}H_3$), 60.9 ($C_{4',4''}$-O$\underline{C}H_3$), 71.2 (C-4), 105.7 (C-2′ and C-6′), 106.4 (C-2″ and C-6″), 133.5, 133.7 (C-1′ and C-1″), 137.1 (C-4′ and C-4″), 153.4, 153.5 (C-3″, 5″ and C-3′, 5′), 178.5 (C-1). It was identified by comparison of the physical and spectral data with those of (−)-cubebininolide (III).[10]

Compound B ((−)-Yatein) (V)——Pale yellow glassy solid. $[\alpha]_D$ −29.5° (*c* = 0.48, $CHCl_3$). *Anal.* Calcd for $C_{22}H_{24}O_7$: C, 65.99; H, 6.04. Found: C, 65.74; H, 6.13. High-resolution MS Calcd for $C_{22}H_{24}O_7$ (M^+): 400.1520. Found: 400.1509. UV $\lambda_{max}^{95\%\,EtOH}$ nm (log ε): 230 (3.91), 287 (3.46). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1770 (C=O). CD (*c* = 7.315 × 10^{-5}, 95% EtOH) Δε: −0.50 (275), ±0 (255), −1.66 (239) (negative maximum). MS *m/z* (rel. int.): 400 (88.3, M^+), 182 (44.1), 181 (100), 167 (8.4), 151 (8.3), 148 (6.7), 135 (45.3), 131 (7.1). ^{1}H-NMR ($CDCl_3$) δ: 2.42—2.64 (4H, m, $C_{2,3}$-H and C_5-H_2), 2.91 (2H, m, C_6-H_2), 3.83 (9H, s, $C_{3'',4'',5''}$-OCH_3), 3.88 (1H, dd, *J* = 9.3, 7.5 Hz, $C_{4\beta}$-H), 4.18 (1H, dd, *J* = 9.3, 7.2 Hz, $C_{4\alpha}$-H), 5.93, 5.94 (each d, *J* = 1.3 Hz, $-OCH_2O-$), 6.36 (2H, s, $C_{2'',6''}$-H), 6.46 (1H, d, *J* = 1.5 Hz, $C_{2'}$-H), 6.48 (1H, dd, *J* = 6.9, 1.8 Hz, $C_{6'}$-H), 6.70 (1H, d, *J* = 8.7 Hz, $C_{5'}$-H). ^{13}C-NMR ($CDCl_3$) δ: 35.3 (C-6), 38.4 (C-5), 41.1 (C-3), 46.5 (C-2), 56.1 ($C_{3'',5''}$-O$\underline{C}H_3$), 60.9 ($C_{4''}$-O$\underline{C}H_3$), 71.2 (C-4), 101.1 ($-OCH_2O-$), 106.3 (C-2″ and C-6″), 108.3 (C-5′), 108.8 (C-2′), 121.6 (C-6′), 131.6 (C-1′), 133.4 (C-1″), 137.0 (C-4″), 146.5 (C-4′), 148.0 (C-3′), 153.3 (C-3″ and C-5″), 178.5 (C-1). It was identified by comparison of the physical and spectral data with those of (−)-yatein (V).[11]

Compound C (Dehydropodophyllotoxin) (IX)——Needle crystals. mp 275—280 °C (dec.). *Anal.* Calcd for $C_{22}H_{18}O_8 \cdot 1/2\ H_2O$: C, 63.00; H, 4.57. Found: C, 63.09; H, 4.76. High-resolution MS Calcd for $C_{22}H_{18}O_8$ (M^+): 410.1000. Found: 410.1013. UV λ_{max}^{MeOH} nm (log ε): 222 (4.48), 262 (4.60), 322 (3.99), 355 (3.70). IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1770 (C=O). CD (*c* = 2.5 × 10^{-5}, MeOH) Δε: ±0. MS *m/z* (rel. int.): 410 (100, M^+), 395 (30.2), 367 (8.1), 350 (3.6), 337 (3.2), 281 (3.9), 139 (4.7). ^{1}H-NMR ($CDCl_3$) δ: 3.83 (6H, s, $C_{3',5'}$-OCH_3), 3.95 (3H, s, $C_{4'}$-OCH_3), 5.37 (2H, s, lactone-CH_2), 6.10 (2H, s, $-OCH_2O-$), 6.52 (2H, s, $C_{2',6'}$-H), 7.10 (1H, s, C_5-H), 7.48 (1H, s, C_8-H), 12.00 (1H, s, C_1-OH, disappeared on addition of D_2O). ^{1}H-NMR (acetone-d_6) δ: 3.82 (6H, s, $C_{3',5'}$-OCH_3), 3.83 (3H, s, $C_{4'}$-OCH_3), 5.39 (2H, s, lactone-CH_2), 6.17 (2H, s, $-OCH_2O-$), 6.59 (2H, s, $C_{2',6'}$-H), 6.98 (1H, s, C_5-H), 7.66 (1H, s, C_8-H), 9.38 (1H, s, C_1-OH, disappeared on addition of D_2O).

Methylation of IX (Formation of VI)——A suspension of IX (29 mg) in ether–acetone (1 : 2) was methylated with CH_2N_2–Et_2O. After usual work-up, the residue was purified by column chromatography on silica gel with $CHCl_3$–acetone (9 : 1). The obtained product was recrystallized from EtOH to give VI as colorless needles. mp 268—270 °C. $[\alpha]_D$ ±0° (*c* = 0.39, $CHCl_3$). *Anal.* Calcd for $C_{23}H_{20}O_8 \cdot 1/4\ H_2O$: C, 64.41; H, 4.76. Found: C, 64.44; H, 4.70. High-resolution MS Calcd for $C_{23}H_{20}O_8$ (M^+): 424.1157. Found: 424.1161. UV λ_{max}^{MeOH} nm (log ε): 206 (4.57), 261 (4.60), 319 (3.99), 353 (3.69). IR ν_{max}^{KBr} cm^{-1}: 1770 (C=O), 930 ($-OCH_2O-$). CD (*c* = 2.46 × 10^{-5}, MeOH) Δε: ±0. MS *m/z* (rel. int.): 424 (100, M^+), 409 (27.9), 381 (3.8), 350 (8.7), 224 (8.5), 179 (8.3). ^{1}H-NMR ($CDCl_3$) δ: 3.84 (6H, s, $C_{3',5'}$-OCH_3), 3.96 (3H, s, $C_{4'}$-OCH_3), 4.09 (3H, s, C_1-OCH_3), 5.52 (2H, s, lactone-CH_2), 6.09 (2H, s, $-OCH_2O-$), 6.52 (2H, s, $C_{2',6'}$-H), 7.07 (1H, s, C_5-H), 7.57 (1H, s, C_8-H). ^{13}C-NMR ($CDCl_3$) δ: 56.0 (q, $C_{3',5'}$-O$\underline{C}H_3$), 59.9 (q, C_1-O$\underline{C}H_3$), 60.9 (q, $C_{4'}$-O$\underline{C}H_3$), 66.4 (t, lactone-CH_2), 98.4 (d, C-8), 101.8 (t, $-OCH_2O-$), 103.9 (d, C-5), 107.3 (d, C-2′, 6′), 119.5 (s), 125.8 (s), 127.8 (s), 130.4 (s), 132.2 (s), 135.4 (s), 137.7 (s), 148.5 (s), 148.9 (s), 150.0 (s), 152.9 (s), 169.3 (C=O).

Synthesis of Dehydropodophyllotoxin (IX)——i) *tert*-Butyldimethylsilylpodophyllotoxin: A mixture of *tert*-butyldimethylsilyl chloride (TBDMSCl) (147.3 mg, 0.98 mmol) and imidazole (128.1 mg, 1.88 mmol) in dimethylformamide (DMF) (2.5 ml) was added to a solution of podophyllotoxin (169.5 mg, 0.41 mmol) in absolute DMF (0.5 ml), and the resulting solution was stirred at 75 °C for 3 h. The reaction mixture was poured into water and

extracted with $CHCl_3$. The organic layer was washed with brine, and dried over anhydrous Na_2SO_4. Column chromatographic purification (silica gel; $CHCl_3$:AcOEt=10:1) gave *tert*-butyldimethylsilylpicropodophyllin (27 mg, 12.5%) and *tert*-butyldimethylsilylpodophyllotoxin (141 mg, 65.3%).

tert-Butyldimethylsilylpicropodophyllin: Needles (recrystallized from MeOH). mp 169—171 °C (ref.[21]: mp 169—170 °C). $[\alpha]_D$ + 56.6° (c=0.47, $CHCl_3$). *Anal.* Calcd for $C_{28}H_{36}O_8Si$: C, 63.61; H, 6.86. Found: C, 63.33; H, 6.84. High-resolution MS Calcd for $C_{28}H_{36}O_8Si$ (M^+): 528.2177. Found: 528.2179. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1780 (C=O), 940 (-OCH_2O-). MS *m/z* (rel. int.): 528 (47.4, M^+), 471 (34.4), 412 (34.5), 397 (29.7), 351 (19.9), 313 (60.7), 282 (36.7), 259 (23.7), 185 (63.3), 181 (26.2), 168 (20.6). ^{1}H-NMR ($CDCl_3$) δ: 0.15, 0.20 (each 3H, s, -Si-(CH_3)$_2$), 1.01 (9H, s, -C-(CH_3)$_3$), 2.60 (1H, m, C_2-H), 3.21 (1H, dd, J=8.0, 6.0 Hz, C_3-H), 3.84 (6H, s, $C_{3',5'}$-OCH_3), 3.87 (3H, s, $C_{4'}$-OCH_3), 3.93, 4.37 (each 1H, lactone-CH_2), 4.47 (1H, d, J=10.0 Hz, C_4-H), 4.51 (1H, d, J=10.0 Hz, C_1-H), 5.90, 5.92 (each 1H, d, J=1.5 Hz, -OCH_2O-), 6.23 (1H, s, C_5-H), 6.48 (2H, s, $C_{2',6'}$-H), 6.98 (1H, s, C_8-H).

tert-Butyldimethylsilylpodophyllotoxin: Colorless amorphous powder. $[\alpha]_D$ −86.2° (c=0.62, $CHCl_3$). High-resolution MS Calcd for $C_{28}H_{36}O_8Si$ (M^+): 528.2177. Found: 528.2174. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1780 (C=O), 935 (-OCH_2O-). MS *m/z* (rel. int.): 528 (100, M^+), 471 (7.8), 397 (17.3), 351 (23.6), 313 (48.1), 282 (19.4), 229 (17.4), 185 (55.8), 181 (11.3), 168 (20.6). ^{1}H-NMR ($CDCl_3$) δ: 0.11, 0.28 (each 3H, s, -Si-(CH_3)$_2$), 0.95 (9H, s, -C-(CH_3)$_3$), 2.85 (2H, m, $C_{2,3}$-H), 3.75 (6H, s, $C_{3',5'}$-OCH_3), 3.81 (3H, s, $C_{4'}$-OCH_3), 4.00, 4.51 (each 1H, lactone-CH_2), 4.57 (1H, d, J=3.0 Hz, C_4-H), 4.80 (1H, d, J=9.0 Hz, C_1-H), 5.95, 5.98 (each 1H, d, J=1.5 Hz, -OCH_2O-), 6.38 (2H, s, $C_{2',6'}$-H), 6.48 (1H, s, C_5-H), 6.94 (1H, s, C_8-H).

ii) *tert*-Butyldimethylsilyldehydropodophyllotoxin: 2,3-Dichloro-5,6-dicyanobenzoquinone (DDQ) (258.4 mg, 1.12 mmol) was added to a solution of *tert*-butyldimethylsilylpodophyllotoxin (257.3 mg, 0.49 mmol) in dry benzene (10 ml), and the mixture was refluxed for 29 h, then allowed to cool. The precipitate was filtered off. The residue was washed with benzene. The filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (*n*-hexane:AcOEt=7:3). The product was recrystallized from EtOH to give *tert*-butyldimethylsilyldehydropodophyllotoxin as colorless needles. mp 245—248 °C. $[\alpha]_D \pm 0°$ (c=0.43, MeOH). High-resolution MS Calcd for $C_{28}H_{32}O_8Si$ (M^+): 524.1865. Found: 524. 1878. UV λ_{max}^{MeOH} nm (log ε): 206 (4.89), 264 (4.74), 318 (4.12), 352 (3.78). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1770 (C=O), 945 (-OCH_2O-). MS *m/z* (rel. int.): 524 (100, M^+), 509 (11.1), 423 (14.7), 409 (4.1), 350 (4.1). ^{1}H-NMR ($CDCl_3$) δ: 0.28 (6H, s, -Si-(CH_3)$_2$), 1.14 (9H, s, -C-(CH_3)$_3$), 3.84 (6H, s, $C_{3',5'}$-OCH_3), 3.96 (3H, s, $C_{4'}$-OCH_3), 5.33 (2H, s, lactone-CH_2), 6.08 (2H, s, -OCH_2O-), 6.53 (2H, s, $C_{2',6'}$-H), 7.08 (1H, s, C_5-H), 7.46 (1H, s, C_8-H).

Desilylation of *tert*-Butyldimethylsilyldehydropodophyllotoxin (Formation of IX)——Tetrabutylammonium fluoride in tetrahydrofuran (THF) solution (1.0 M; 0.12 ml, 0.12 mmol) was added to a mixture of the silylated compound (30 mg, 0.057 mmol) and molecular sieve (4 Å) in THF (0.5 ml) at room temperature, and the whole was stirred overnight. The reaction mixture was poured into ice-water and extracted with Et_2O. The extract was washed and dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was subjected to high performance liquid chromatography (HPLC) (column, microporasil 3 i.d. × 250 mm; eluent, $CHCl_3$; flow rate, 0.5 ml/min). The product was identical with compound C on direct comparison of all spectral data.

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References and Notes

1) H. Yamaguchi, M. Arimoto, S. Nakajima, M. Tanoguchi and Y. Fukada, *Chem. Pharm. Bull.*, **34**, 2056 (1986).
2) *a*) H. Yamaguchi, M. Arimoto, K. Yamamoto and A. Numata, *Yakugaku Zasshi*, **99**, 674 (1979); *b*) H. Yamaguchi, M. Arimoto, M. Tanoguchi, T. Ishida and M. Inoue, *Chem. Pharm. Bull.*, **30**, 3212 (1982).
3) J. E. T. Corrie, G. H. Green, E. Ritchie and W. C. Taylor, *Aust. J. Chem.*, **23**, 133 (1970).
4) D. Takaoka, N. Takamatsu, Y. Saheki, K. Kohono, C. Nakaoka and M. Hiroi, *Nippon Kagaku Kaishi*, **1975**, 2192.
5) W. M. Kamil and P. M. Dewick, *Phytochemistry*, **25**, 2093 (1986).
6) E. Wenkert, H. E. Gottlieb, O. R. Gottlieb, M. O. S. Pereira and M. D. Formiga, *Phytochemistry*, **15**, 1547 (1976).
7) A. Bax, *J. Magn. Reson.*, **53**, 517 (1983).
8) *a*) D. Takaoka, M. Imooka and M. Hiroi, *Bull. Chem. Soc. Jpn.*, **50**, 2821 (1977); S. Nishibe, K. Okabe and S. Hisada, *Chem. Pharm. Bull.*, **29**, 2078 (1981); *b*) L. P. Badheka, B. R. Prabhu and N. B. Mulchandani, *Phytochemistry*, **25**, 487 (1986).
9) S. Nishibe, S. Hisada and I. Inagaki, *Yakugaku Zasshi*, **94**, 522 (1974).
10) S. K. Koul, S. C. Taneja, K. L. Dhar and C. K. Atal, *Phytochemistry*, **23**, 2099 (1984); B. R. Prabhu and N. B. Mulchandani, *ibid.*, **24**, 329 (1985).
11) J. Harmatha, M. Buděšinský and A. Trka, *Collect. Czech. Chem. Commun.*, **47**, 644 (1982).

12) P. B. McDoniel and J. R. Cole, *J. Pharm. Sci.*, **61**, 1992 (1972).
13) M. Kozawa, N. Morita and K. Hata, *Yakugaku Zasshi*, **98**, 1486 (1978).
14) H. Erdtman and J. Harmatha, *Phytochemistry*, **18**, 1495 (1979).
15) S. K. Koul, S. C. Taneja, K. L. Dhar and C. K. Atal, *Phytochemistry*, **22**, 999 (1983).
16) P. Richomme, J. Bruneton, P. Cabalion and M. M. Debray, *J. Nat. Prod.*, **47**, 879 (1984); M.-C. Chalandre, C. Pareyre and J. Bruneton, *Ann. Pharm. Fr.*, **42**, 317 (1984); P. Richomme, J. Bruneton and A. Cavé, *Heterocycles*, **23**, 309 (1985).
17) K. Tomioka, H. Mizuguchi and K. Koga, *Chem. Pharm. Bull.*, **30**, 4304 (1982).
18) The melting point was measured in an unsealed tube. The melting point depends on the rate of heating, see ref. 20*a*.
19) H. Yamaguchi, M. Arimoto, M. Tanoguchi and A. Numata, *Yakugaku Zasshi*, **101**, 485 (1981).
20) *a*) W. J. Gensler, F. Johnson and A. D. B. Sloan, *J. Am. Chem. Soc.*, **82**, 6074 (1960); *b*) B. J. Arnold, S. M. Mellows and P. G. Sammes, *J. Chem. Soc., Perkin Trans. 1*, **1973**, 1266; *c*) H. Kofod and C. Jørgensen, *Acta Chem. Scand.*, **8**, 1296 (1954); L. Fa, S. Tianmin and F. Fengyong, *Yao Hsueh Hsueh Pao*, **14**, 241 (1979); C. Xingrue, H. Zhibi and Z. Guangfang, *ibid.*, **15**, 158 (1980); H. P. Plaumann, J. G. Smith and R. Rodrigo, *J. Chem. Soc., Chem. Commun.*, **1980**, 354.
21) A. S. Kende, M. L. King and D. P. Curran, *J. Org. Chem.*, **46**, 2826 (1981).

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Isolation and Identification of New Oligomers in Aqueous Solution of Glutaraldehyde

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Commercially available aqueous solutions of glutaraldehyde (GA) sometimes contain GA polymers as impurities which can cause undesirable effects. We found new type oligomers present in a GA solution as impurities and isolated them by reverse-phase high performance liquid chromatography. These compounds were identified as GA trimer, pentamer and heptamer having a 1,3,5-trioxane skeleton. In particular, GA trimer, 2,4,6-tris(4-oxobutyl)-1,3,5-trioxane, was named "paraglutaraldehyde" (para-GA). These compounds showed absorption maxima at 280 nm (the same position as GA), and hence could not be distinguished from GA itself by the conventional ultraviolet absorption method. These impurities were not detected in highly purified GA solution after storage under the conditions of practical use. Accordingly, these oligomers seemed to be formed by the intermolecular cyclization of GA monomer during the processes of synthesis.

Keywords——glutaraldehyde solution; oligomer; impurity; paraglutaraldehyde; HPLC; disinfectant; chemosterilization

Introduction

In recent years, application of glutaraldehyde (GA) has been expanded to various fields such as histochemistry,[1] biochemistry,[2] polymer science,[3] and pharmaceutical science,[4] because of its bifunctional nature and high reactivity with amino and hydroxyl groups. However, the commercially available products sometimes contain GA polymers as impurities which can cause the following undesirable effects in practical use: white precipitates appear on dilution of concentrated GA solution with distilled water,[5] or with buffer solutions of physiological pH,[6] and enzyme activity in histochemical preparations for electron microscopy is reduced.[7] To deal with these problems, investigations have been carried out on the stability and impurities of aqueous GA solution.[8–10] It has been reported that certain impurities lower the pH of aqueous GA solution, absorb ultraviolet (UV) light at near 235 nm, and impart a yellow color to the solution.[11] Several reports postulated that the impurities of GA solution comprised acrolein, glutaric acid, glutaraldoxime, α,β-unsaturated aldehyde polymers and 3,4-dihydro-2-ethoxy-2*H*-pyran.[7,11–13]

On the other hand, the purity of GA solution has been evaluated by employing a purification index (P.I.) defined as A_{235}/A_{280}, where A_{280} is the UV absorbance at 280 nm assigned to n–π^* transition of the C=O bond in the aldehyde group of GA and A_{235} is that assigned to π–π^* transition of the C=C bond in α,β-unsaturated aldehyde polymers.[14] Naturally, a lower P.I. value corresponds to a higher purity. However, we found that a certain grade of commercially available GA solution contained appreciable amounts of impurities in spite of its low P.I. value. In order to resolve this inconsistency and to assess the quality of GA solution, we conducted high performance liquid chromatography (HPLC) analysis of the GA

solution and discovered new-type oligomers which exhibited λ_{max} only at 280 nm, where GA exhibits its λ_{max}.

This paper describes the isolation, spectral characterization and structural elucidation of these oligomers.

Experimental

Materials——Aqueous GA solutions of reagent grade were obtained from Wako Pure Chemicals, Nakarai Chemicals and Tokyo Kasei Kogyo. The GA solution from which GA oligomers were isolated was the electron microscopy grade of Ishidzu Pharmaceutical; this solution produced a large amount of precipitate under a weakly alkaline condition (pH about 8) in spite of its low P.I. value (about 0.2). Carbon tetrachloride used for infrared (IR) absorption spectroscopy and deuteriochloroform containing 1% tetramethylsilane used for nuclear magnetic resonance (NMR) spectroscopy were purchased from Wako Pure Chemicals and Merck, respectively. Acetonitrile of HPLC grade was obtained from Kanto Chemicals, and water used for HPLC was purified by distillation. Ammonia gas and pyridine used as reagent gases for chemical ionization mass (CI-MS) spectrometry were supplied by Seitetsu Chemical and Wako Pure Chemicals, respectively. Other chemicals of reagent grade were used without further purification.

Isolation of Impurities——A 50 ml portion of the 25% GA solution was shaken with 50 ml of ethyl acetate. The organic layer was washed with distilled water (3 × 50 ml) and evaporated *in vacuo* at room temperature. The residue was weighed and dissolved in a mixture of acetonitrile and distilled water (5 : 1). A 300 μl portion of the solution was subjected to HPLC. HPLC separation was carried out on a JASCO model TRIROTAR V liquid chromatograph by using a glass packed column (CPO-HS-221-20, 20μm ODS, 100 × 20 mm i.d.) supplied by Kusano Kikai Co. Elution was done at 55 °C with the mobile phase of 20% acetonitrile for the first 10 min followed by a linear gradient to 68% acetonitrile for the next 32 min, then isocratic elution was continued for 35 min. The flow rate was 3 ml/min. The eluate was monitored at 280 nm and the chromatograms were recorded on a PANTOS model U-329 chart recorder. The fractions containing impurities were collected.

High-Performance Liquid Chromatography——HPLC analyses of aqueous GA solutions and the fractions obtained from the above isolation procedure were carried out by using a Shimadzu model LC-6A chromatograph and a Photal model MCPD-3500 multichannel photodiode array UV detector. A 50μl portion of sample solution was injected. Separation was performed by using an Inertsil ODS column (5 μm, 250 × 4.6 mm i.d.) supplied by Gasukuro Kogyo. Elution was carried out at 55 °C in a gradient mode with solvent A (0.02 M phosphate buffer mixed with acetonitrile in a volume ratio of 3 : 7, pH 2.5) and solvent B (0.02 M phosphate buffer mixed with acetonitrile in a volume ratio of 9 : 1, pH 2.5). The ratio of A to B was 40 : 60 (v/v) for the first 5 min, followed by a linear gradient to 90 : 10 over the next 16 min, and the isocratic elution was continued thereafter. The flow rate was 1.5 ml/min. The absorption of the eluate was monitored in the range from 200 to 350 nm.

Gas Chromatography (GC)——Capillary GC analysis was performed by using a Shimadzu model GC-9A gas chromatograph equipped with a flame ionization detector (FID), connected with a model C-R2AX reporting integrator (Shimadzu). Samples were injected onto a 5% phenyl methyl silicone cross-linked capillary column (ULTRA #2, Hewlett-Packard, 25 m × 0.31 mm i.d., d_f 0.52 μm) in a split mode (splitting ratio *ca.* 1/50) by using a model SPL-G9 split/splitless injection system (Shimadzu). The injection port temperature was maintained at 260 °C and the oven temperature was programed from 80 to 250 °C at 5 °C/min. Nitrogen was used as the carrier gas at a flow rate of 23 cm/s and as the make-up gas at 60 ml/min.

Thermal and Elemental Analyses——Thermal gravimetry (TG) and differential scanning calorimetry (DSC) were carried out by using Rigaku TG-DSC thermal analysis units. Elemental analyses were performed on a Yanaco MT-3 CHN corder elemental analyses instrument.

Spectrometries——The fractions obtained by preparative HPLC separation were concentrated by evaporation of the solvent, and the residue was redissolved for the subsequent measurements of spectra. In order to avoid polymerization, those operations were carried out as quickly as possible, and the spectral data were obtained immediately after the sample preparation.

The IR absorption spectra in carbon tetrachloride solution were measured on a Hitachi model 260-30 spectrometer. The 60 MHz ^{1}H-NMR spectra in deuteriochloroform were recorded on a JEOL model PMX-60 SI spectrometer. The UV absorption spectra in dioxane solution were measured by using a Hitachi model 200-20 spectrophotometer.

The electron impact mass (EI-MS) and CI-MS spectra were measured on a JEOL model DX-303 mass spectrometer. In CI-MS of the GA heptamer (peak No. 4 in Fig. 3), the ion source temperature was controlled at 330 °C. Other conditions for EI-MS and CI-MS were as follows: (EI-MS) electron energy, 70 eV; emission current, 300 μA; ion source temperature, 250 °C: (CI-MS) electron ionization energy, 200 eV; emission current, 300 μA; ion source temperature, 200 °C. The accelerating voltage was 3 kV in both EI-MS and CI-MS measurements.

Results and Discussion

Figures 1(A) and (B) show the HPLC separation of GA solution, which gave a large amount of white precipitate under weakly alkaline conditions, with detection at 235 and 280 nm, respectively. Similarly, Fig. 2 shows the chromatograms of another GA solution which gave a smaller amount of precipitate than the GA solution of Fig. 1. In Figs. 1 and 2, GA monomer was eluted with a retention time of 2.2 min. The comparison between Fig. 1(A) and 2(A) indicates that the GA solution of Fig. 2 contains a large amount of impurities detectable by UV absorption measurement at 235 nm. On the other hand, the GA solution of Fig. 1 seems to have a large amount of impurities detectable at 280 nm, as shown in Figs. 1(B) and 2(B). Therefore, three compounds X, Y and Z in Fig. 1(B) may be responsible for the precipitation observed in weakly alkaline GA solution. In order to determine the chemical structures of these compounds, preparative HPLC was carried out by using the GA solution of Fig. 1. Figure 3 shows preparative HPLC separaion of the ethyl acetate extracts of aqueous GA solution with detection at 280 nm. Among the peaks separated, the peak with a retention time near 10 min was identical with that of GA, and the peaks 2 to 4 were therefore assignable to impurities. The peak intensity ratio of GA to the impurities found in Fig. 3 is much smaller than that expected from the chromatogram of the initial GA solution (Fig. 1(B)). This is

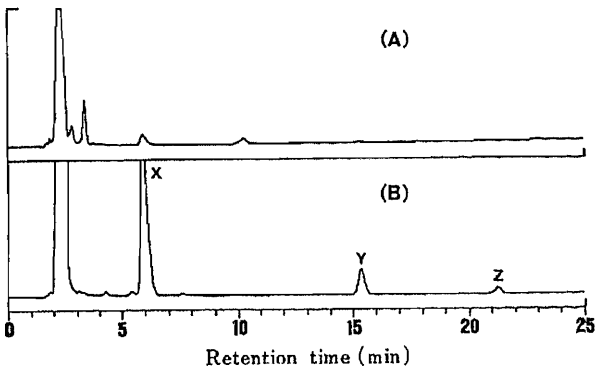


Fig. 1. HPLC Chromatograms of Aqueous Glutaraldehyde Showing Precipitate Formation under Alkaline Conditions (pH about 8) at Two Different Wavelengths

(A) 235 nm, (B) 280 nm.

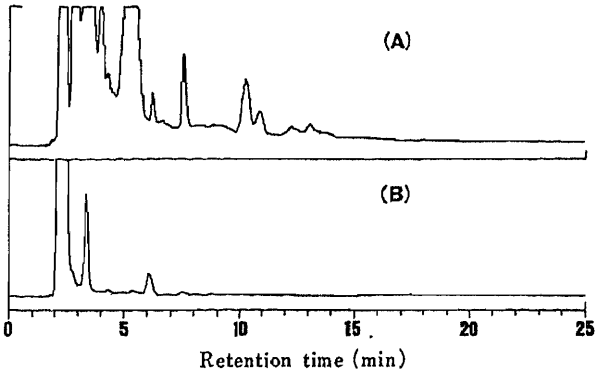


Fig. 2. HPLC Chromatograms of Aqueous Glutaraldehyde Giving a Smaller Amount of Precipitate than That in Fig. 1 at Two Different Wavelengths

(A) 235 nm, (B) 280 nm.

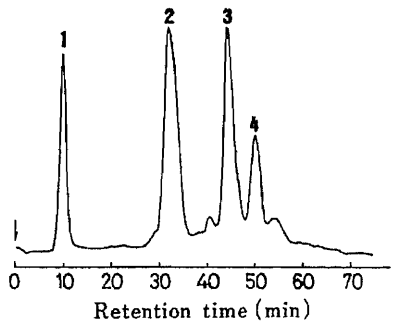


Fig. 3. Preparative HPLC Chromatogram of the Ethyl Acetate Extract of Aqueous Glutaraldehyde with Detection at 280 nm

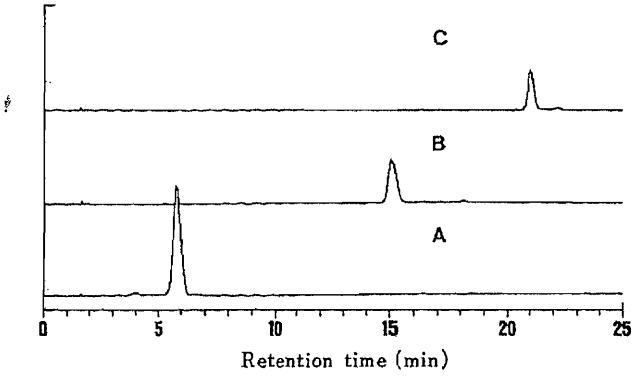


Fig. 4. HPLC Chromatograms of the Isolated Components 2, 3 and 4 in Fig. 3 with Detection at 280 nm

(A), (B) and (C) correspond to components 2, 3 and 4 in Fig. 3, respectively.

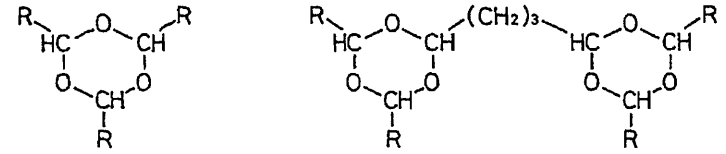
because GA monomer is readily hydrated[15] and remained mostly in the aqueous layer. Figure 4 shows the rechromatograms of the fractionated components of peaks 2 to 4 in Fig. 3, indicating that these components had been isolated in high purity and corresponded to X, Y and Z in Fig. 1(B). However, these components did not give peaks when rechromatographed with detection at 235 nm. The homogeneity of the isolated components of peaks 2 to 4 in Fig. 3 thus confirmed by HPLC analysis justified the use of these fractions in the subsequent structural investigations.

From the following results, the chemical structures of compounds X, Y and Z mentioned above were found to be as shown in Fig. 5(A), (B) and (C), respectively.

Thermal and Elemental Analyses

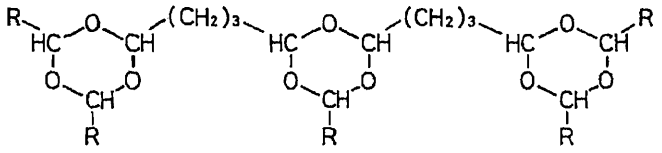
Figure 6 shows the results of TG and DSC analyses. The three compounds X to Z gave almost the same results; they lost approximately 85—90% of their weight in the temperature range between 150 and 300 °C, and showed an endothermal peak at around 250 °C. These results suggested that the three compounds may have similar bond character, that is, the ether linkage in their structures may be cleaved during heating.

The structural analogy was also suggested by the results of elemental analysis. Table I shows that GA monomer and the three compounds have almost the same carbon contents (57.2—59.4%) and hydrogen contents (7.9—8.2%). In addition, compounds X to Z were found not to contain nitrogen. These results suggested that the three compounds have the same elemental composition $(C_5H_8O_2)_n$ as GA monomer ($n=1$).



(A) compound X

(B) compound Y



(C) compound Z

R: $-(CH_2)_3CHO$

Fig. 5. Proposed Structures of Compounds X, Y and Z in Fig. 1(B)

TABLE I. Carbon and Hydrogen Contents of Glutaraldehyde, Compound X, Compound Y and Compound Z

Species	Carbon (%)	Hydrogen (%)
Glutaraldehyde	59.4	7.9
Compound X	57.8	8.1
Compound Y	57.2	8.0
Compound Z	58.1	8.2

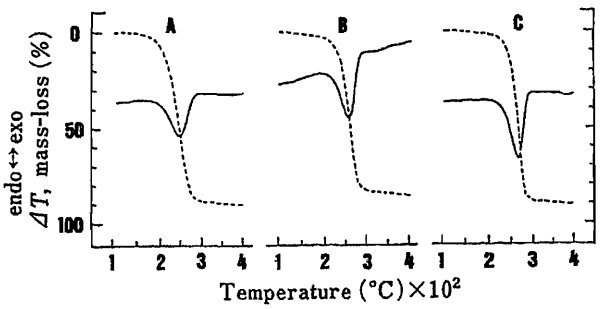


Fig. 6. TG and DSC Curves of Compounds X, Y and Z

Heating rate of 10 °C/min under an atmosphere of nitrogen (flow rate, 50 ml/min). TG curve; (------), DSC curve; (——). (A) Compound X, (B) compound Y, (C) compound Z.

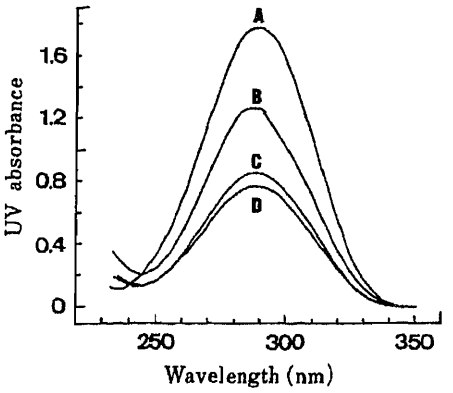


Fig. 7. UV Absorption Spectra of (A) 0.4% Glutaraldehyde, (B) 0.5% Compound X, (C) 0.5% Compound Y and (D) 0.5% Compound Z in Dioxane

TABLE II. Molar Absorbance Coefficients[a] and Numbers[b] of Aldehyde Group of Glutaraldehyde, Compound X, Compound Y and Compound Z

Species	Molar absorbance coefficient	Number of aldehyde groups
Glutaraldehyde	44	2.0
Compound X	73	3.3
Compound Y	86	3.9
Compound Z	110	5.0

a) These values were obtained by calculation from the absorbances of the four compounds (0.5% in dioxane) at 290 nm. *b*) The number of aldehyde unit in each molecule was estimated on the assumption that the absorption coefficient per one aldehyde group is 22.

UV Spectrometry

The UV absorption spectra of the three compounds X to Z in dioxane are shown in Fig. 7(B) to (D), respectively, together with that of GA monomer (Fig. 7(A)). Each compound exhibited an absorption maximum at near 290 nm. It is known that GA monomer in aqueous solution has an absorption maximum at 280 nm which is due to $n-\pi^*$ transition of the C=O bond,[16] and that this absorption band undergoes a red shift in less polar solvents.[17] Thus, the three isolated compounds seem to have an aldehyde group in their structures, but not an α,β-unsaturated aldehyde group, since an absorption maximum at around 235 nm assignable to $\pi-\pi^*$ band transition of C=C bond[16] is not apparent in Fig. 7.

Table II shows the values of the molar absorption coefficients of monomeric GA and compounds X to Z, where the latter three values were calculated on the assumption that the molecular weights of compound X (300), compound Y (500) and compound Z (700) are those corresponding to the structures (A), (B) and (C) in Fig. 5, respectively. If the molar absorption coefficient for monomeric GA (44) is allotted to two aldehyde groups, the absorption coefficient per one aldehyde group is 22. Then, by dividing the molecular absorption coefficients of compounds X (73), Y (86) and Z (110) by 22, we obtained the numbers of aldehyde groups in their structures as shown in Table II, *i.e.*, 3, 4 and 5, respectively. These values agree with those of the structures in Fig. 5.

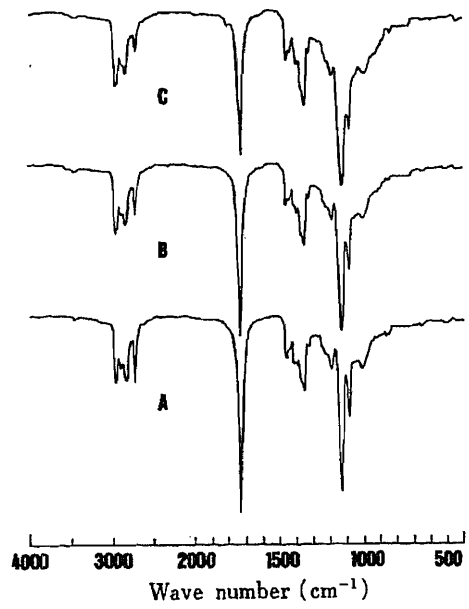


Fig. 8. IR Spectra of Compounds X, Y and Z in Carbon Tetrachloride

(A) Compound X, (B) compound Y, (C) compound Z.

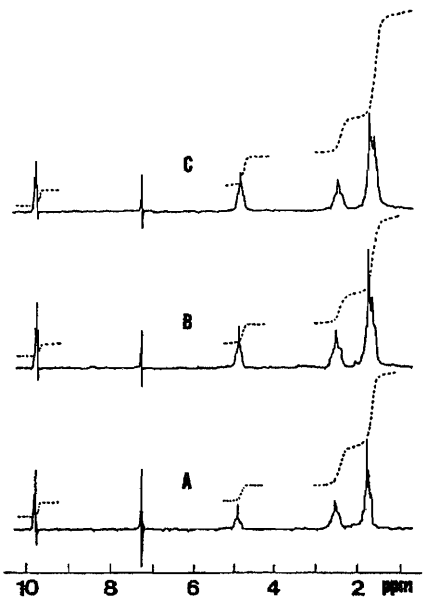


Fig. 9. ^{1}H-NMR Spectra of Compounds X, Y and Z in Deuteriochloroform

(A) Compound X, (B) compound Y, (C) compound Z.

IR and ^{1}H-NMR Spectrometries

Figure 8 shows the IR spectra of compounds X to Z in chloroform solution. The absence of an absorption band at around 3400 cm^{-1} assignable to ν_{O-H} indicates that these compounds do not contain a hydroxyl group. Characteristic absorption bands expected for the structures in Fig. 5 are found at 2950, 2720, 1730 cm^{-1} and around 1100 cm^{-1}. These absorption bands are assignable, respectively, to ν_{C-H} of methylene, ν_{C-H} of aldehyde, $\nu_{C=O}$ of aldehyde and ν_{C-O-C} of ether. Further, the ratio of absorption intensity of the aldehyde group to that of the methylene or ether group decreases in the order of compound X > compound Y > compound Z. This result also supports the structures in Fig. 5.

The ^{1}H-NMR spectra of the three compounds are shown in Fig. 9. Four signals are observed at near δ 9.8, 4.9, 2.5 and 1.8 ppm in each spectrum. Based on the previous NMR spectroscopic investigations of GA[18,19] and the above-mentioned results of IR spectrometry, these four signals were assignable to aldehyde (–CHO), methine (–O–CH–O–), α-methylene (–CH$_2$–CHO) and central methylene (–CH–CH$_2$–CH$_2$– and –CH$_2$–CH$_2$–CH$_2$–) protons, respectively. The signal near 7.3 ppm was due to chloroform. Integration (Fig. 9) indicated that the area ratios of these signals in the above order were approximately 1 : 1 : 2 : 4 in compound X, 1 : 1.5 : 2 : 5.5 in compound Y and 1 : 2 : 2 : 6.5 in compound Z. In accordance with the IR results, the proportion of aldehyde to central methylene decreased gradually in the order of compound X > compound Y > compound Z. Furthermore, the ratio of aldehyde to methine decreased in the same order. The ratio of aldehyde and α-methylene protons in the three compounds was always constant (1 : 2). These results further confirmed that compounds X to Z have the structures (A) to (C) shown in Fig. 5, respectively.

CI-MS Spectrometry

The CI-MS spectrometry was carried out by using ammonia as a reagent gas to confirm the molecular weights. The results are shown in Fig. 10. Compounds X to Z gave m/z 318 or 518 as a base peak. In addition, a peak was found at m/z 718 for compound Z. It is well known

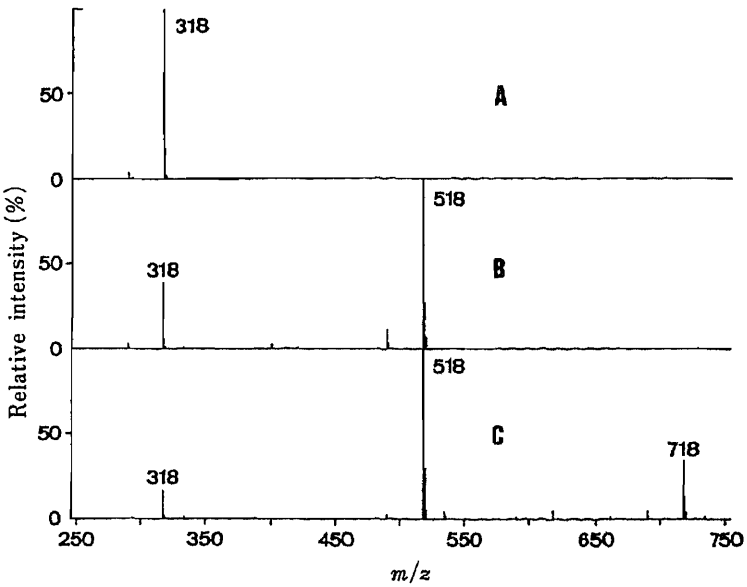


Fig. 10. Ammonia CI-MS Spectra of Compounds X, Y and Z
(A) Compound X, (B) compound Y, (C) compound Z.

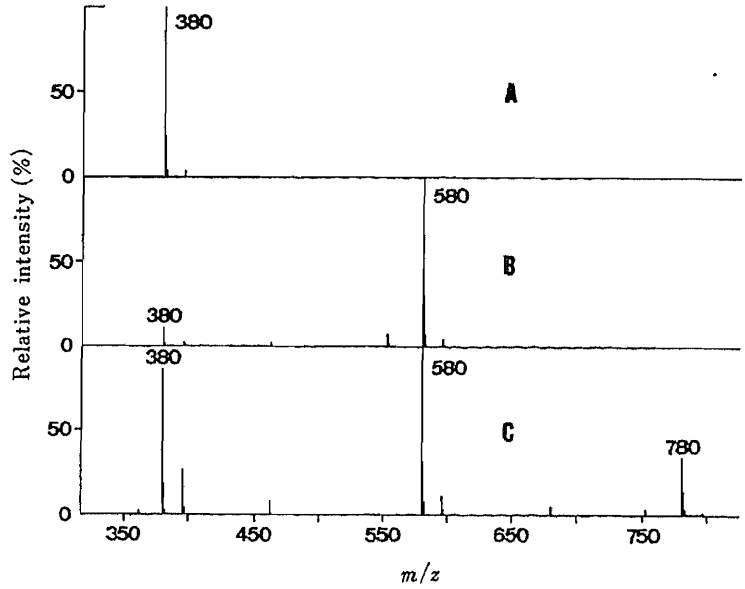


Fig. 11. Pyridine CI-MS Spectra of Compounds X, Y and Z
(A) Compound X, (B) compound Y, (C) compound Z.

that the ammonium adduct molecular ion, $(M+NH_4)^+$, is observed in ammonia-mediated CI-MS spectra of oxygen-containing compounds.[20] Therefore, the molecular weights of compounds X to Z can be presumed to be 300, 500 and 700, respectively. However, there still remained a possibility that the ion peaks at *m/z* 318, 518 and 718 may be due to the protonated molecular species, $(M+H)^+$. To answer this question, CI-MS spectra were measured by using pyridine as a reagent gas. The results obtained are shown in Fig. 11. It has been reported[21] that the use of pyridine as a reagent gas provides a signal at *m/z* $(M+80)^+$, formed by addition of a protonated pyridine. Therefore, the ion peaks at *m/z* 380, 580 and 780 in Fig. 11 confirm the molecular weights to be 300, 500 and 700, respectively. Furthermore, ion species corresponding to successive loss of 200 atomic mass units (amu) from molecular

adduct ions were found in each of the spectra. In contrast, it is expected that the previously proposed GA polymers[22-26] would show peaks due to the loss of 100 amu by elimination of $C_5H_8O_2$ or the loss of fragments of mass number different from 100 and 200 amu. Therefore, the observed loss of 200 amu, which corresponds to two molecules of GA monomer can be reasonably explained by assingning the structures (A) to (C) in Fig. 5 to compounds X to Z, respectively.

EI-MS Spectrometry

EI-MS spectrometry was also performed to confirm the chemical structures of these compounds. No ion peak indicating the molecular weight could be found in the spectra. However, as can be seen in Fig. 12, the similarity in the spectra suggests a structural resemblance. In the EI-MS spectra of 1,3,5-trioxane and paracetoaldehyde (having a trioxane skeleton), the ion species $CH_2=O^+-H$ and $CH_3-CH=O^+-H$ were also seen as a base peak at *m/z* 31 and 45, respectively.[27] McLafferty reported[28] that the ions at *m/z* 31 and 61 in the MS spectrum of 1,3,5-trioxane are produced by molecular rearrangement. Therefore, the ion peaks at *m/z* 101 (as a base peak) and *m/z* 201 in Fig. 12 suggest that compounds X to Z may have a trioxane skeleton. The fragment ions corresponding to $\cdot CH_2-CH=O^+-H$ and $^+CH_2-CH_2-CHO$ can be observed at *m/z* 44 and 57, respectively, analogously with the reported spectrum of GA.[27] The above odd-electron ion peak at *m/z* 44 may be formed through the McLafferty rearrangement reaction. Furthermore, fragment peaks can be found at *m/z* 229 and 129 in Fig. 12. The appearance of these ions (*m/z* 229 and 129) seems to be reasonable, if the three compounds have the structures shown in Fig. 5. In the cases of compounds Y and Z, no ion peak could be obtained at mass numbers higher than *m/z* 229. Therefore, compounds Y and Z may be decomposed to form compound X in the ion source under heating. This thermal degradation of compounds Y and Z is supported by the results of thermal analysis (Fig. 6). In addition, the ion peak at *m/z* 183 was presumed to be produced by the elimination of a water molecule from the ion species at *m/z* 201. The proposed fragmentation mechanisms of the three compounds in EI-MS spectrometry are summarized in Fig. 13.

Based on the results mentioned above, we propose that the compounds X, Y and Z

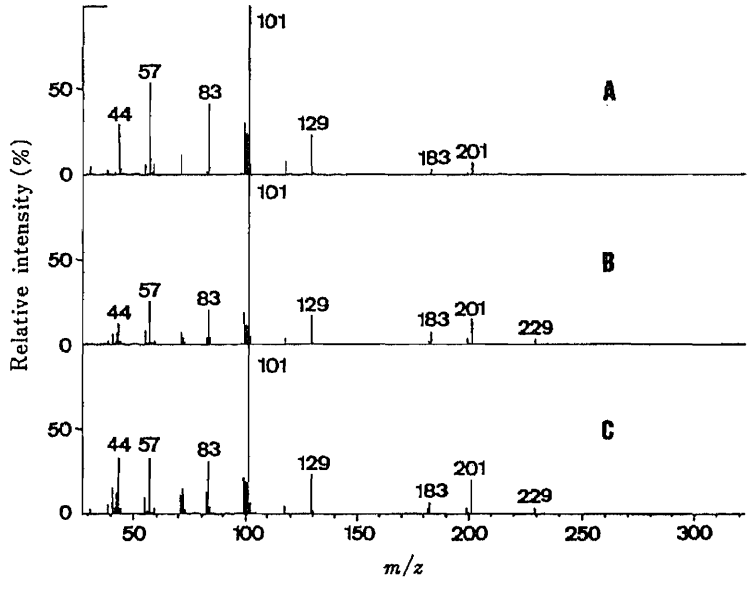


Fig. 12. EI-MS Spectra of Compounds X, Y and Z
(A) Compound X, (B) compound Y, (C) compound Z.

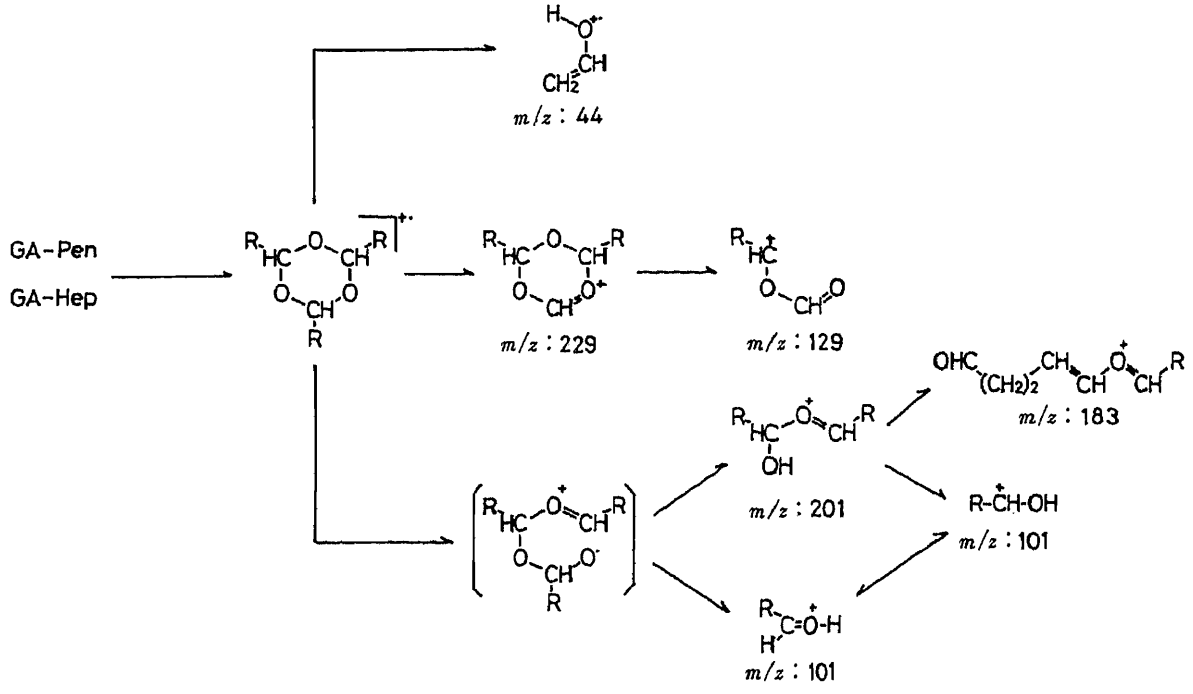


Fig. 13. Proposed Fragmentation Mechanism of Compounds X, Y and Z in the Electron Impact Ionization Mode

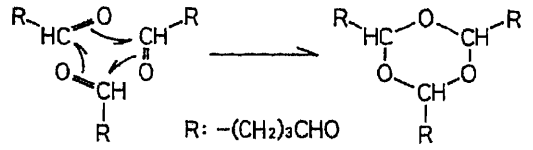


Fig. 14. Proposed Mechanism for Formation of Paraglutaraldehyde

isolated as impurities from the GA solution are trimer, pentamer and heptamer, respectively, of GA having the chemical structures shown in Fig. 5. In particular, compound X, determined as 2,4,6-tris(4-oxobutyl)-1,3,5-trioxane (named paraglutaraldehyde (para-GA)), may be formed by the intermolecular cyclization of three GA monomers as shown in Fig. 14. The other two oligomers of GA (pentamer and heptamer) may have bis- and tris-(trioxane) skeletons formed through reaction mechanisms similar to that of para-GA. Previous investigators have postulated the various structures of GA polymers shown in Fig. 15. It is known that GA changes into a glassy solid on standing at room temperature.[29] Overberger *et al.*[22] proposed that the solid product may be formed by the cyclic polymerization of GA (Fig. 15(A)). Yokota *et al.*[25] prepared linear polyesters of GA by means of the Tischenko condensation reaction (Fig. 15(B)). Aso and Aito[23] obtained a polymer formed through the intramolecular–intermolecular propagation polymerization of GA by using an organometallic catalyst such as triethyl aluminum (Fig. 15(C)). In the presence of a cationic catalyst such as BF_3Et_2O, GA polymerizes to give polymers having recurring $-(CH(RCHO)O)_n$-units (Fig. 15(D)).[30] This mode of polymerization is called pendant polymerization.[24] Furthermore, α,β-unsaturated aldehyde polymer is produced by aldol condensation under alkaline conditions (Fig. 15(E)).[26] However, no compound including a structure produced by linking of trioxane-like structure as shown in Fig. 5 has yet been reported. This finding of new oligomers of GA implies the existence of a new mechanism of polymerization of GA.

As mentioned before, we found that a certain class of commercially available aqueous GA solution was not always pure even though its P.I. value was small.[31] This apparent

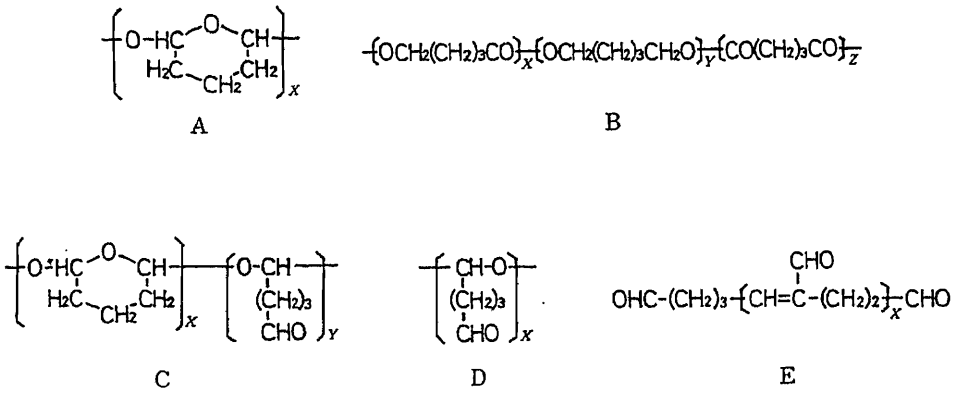


Fig. 15. The Suggested Chemical Structures of Glutaraldehyde Polymers Formed through Different Polymerization Reactions

(A) Cyclic polymerization, (B) Tischenko polymerization, (C) intramolecular–intermolecular propagation polymerization, (D) pendant polymerization, (E) aldol polymerization.

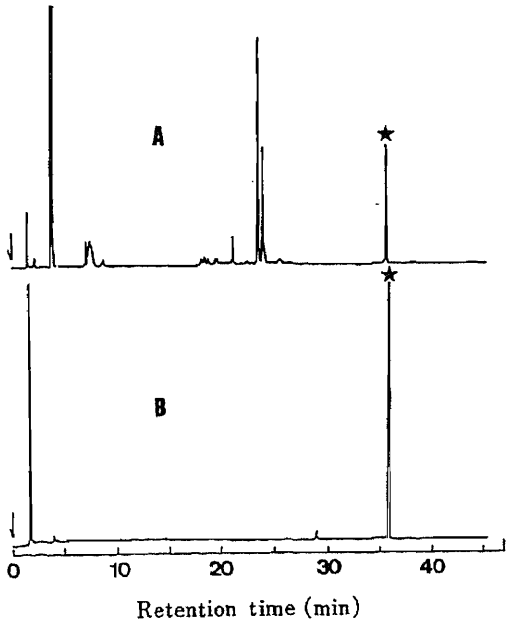


Fig. 16. GC Chromatograms of Aqueous Glutaraldehyde and Isolated Compound X

(A) Commercially available glutaraldehyde solution, (B) compound X.

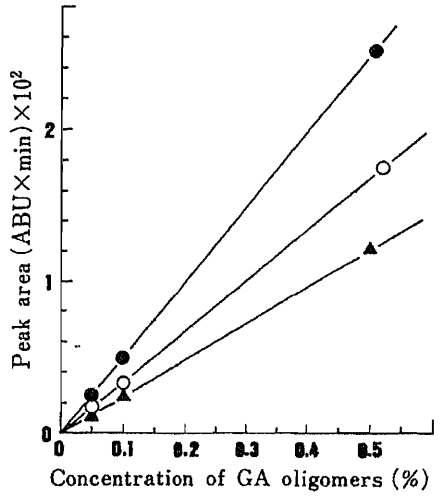


Fig. 17. Calibration Curves of the Three Oligomers of Glutaraldehyde in HPLC Determination with Detection at 280 nm

●, paraglutaraldehyde; ○, glutaraldehyde pentamer; ▲, glutaraldehyde heptamer. ABU: absorbance unit.

inconsistency is resolved by the finding of these three oligomers of GA, since they have no functional groups absorbing at around 235 nm (see Fig. 7). Commercially available aqueous solution of GA has pH about 3 to 4 generally, and the pH of the solution decreases during prolonged storage. This may accelerate the production of trioxane-type oligomers in GA solution, since formaldehyde and acetaldehyde are known to polymerize rapidly in the presence of acid catalysts to form trimers having a trioxane ring. However, the amount of the above oligomers in GA solution did not increase during storage for one year at room temperature. This suggests that trioxane-type oligomers of GA are not produced easily under acidic conditions. Furthermore, the contents of these oligomers gradually decreased under

TABLE III. Concentrations of Trioxane-Type GA Oligomers[a] and Turbidity[b] of GA Solution

Sample No.	Para-GA (%)	GA-Pen (%)	GA-Hep (%)	Turbidity (ppm)
1	0.09	N.D.	N.D.	9
2	0.14	N.D.	N.D.	75
3	0.15	N.D.	N.D.	60
4	0.24	N.D.	N.D.	184
5	0.14	0.06	0.03	120

a) The concentration of GA oligomers was measured in a 20% aqueous solution of GA. *b*) The turbidity of 2% alkaline GA solution (pH about 8) stored for 8 d at 40 °C was obtained by using a turbidimeter with integrated sphere.

weakly alkaline conditions, probably owing to a reaction such as aldol condensation. Therefore, it is likely that these oligomers are impurities produced during the synthesis of GA solution.

It has been reported that white precipitates are sometimes formed when a GA solution is buffered to physiological pH.[32] This presents a problem in the sterilization of hospital instruments and the fixation of tissues for electron microscopy in histochemistry. Previously, we detected the compound responsible for the precipitation by GC analysis of various commercially available GA solutions[33]; it is eluted as the asterisked GC peak shown in Fig. 16(A). In order to examine the correlation of this asterisked compound to the three GA oligomers, GC analyses were carried out. It was found that para-GA was identical with the asterisked compound in Fig. 16(B). This is also supported by the results of EI-MS and CI-MS spectrometries in GC-MS analysis. The other two GA oligomers (pentamer and heptamer) gave no GC peak. Therefore, para-GA seemed responsible for the precipitation in alkaline GA solution. However, there remains the possibility that GA pentamer and heptamer may also be involved in the precipitation, since they have the analogous structures to para-GA. To deal with this question, further investigation was carried out on the relation between the concentration of oligomers and the turbidity of GA solution. The results obtained are shown in Table III. The concentrations of GA oligomers could be obtained by HPLC analysis (calibration curves shown in Fig. 17). Para-GA was found to be responsible for the precipitation in the GA solution which did not contain GA pentamer and heptamer (samples No. 1 to 4). However, in sample No. 5, a larger value of turbidity than in samples No. 2 and 3 was obtained even at the same content of para-GA. Therefore, GA pentamer and heptamer can also cause precipitation in weakly alkaline aqueous GA solution. However, in practice it was rare to find trioxane-type oligomers other than para-GA in reagent-grade GA solution, as shown in Table III. Accordingly, the precipitation which is sometimes observed in GA solution during chemosterilization seems to be attributed mainly to para-GA.

References

1) D. D. Sabatini, K. Bensch and R. J. Barrnett, *J. Cell Biol.*, **17**, 19 (1963).
2) R. Kowal and R. G. Parsons, *Anal. Biochem.*, **102**, 72 (1980).
3) B. Gebben, H. W. A. van den Berg, D. Bargeman and C. A. Smolders, *Polymer*, **26**, 1737 (1985).
4) S. P. Gorman and E. M. Scott, *Int. J. Pharmaceut.*, **4**, 57 (1979).
5) H. D. Fahimi and P. Drochmans, *J. Histochem. Cytochem.*, **16**, 199 (1968).
6) W. W. Franke, S. Krien and R. M. Brown, *Histochemie*, **19**, 162 (1969).
7) P. J. Anderson, *J. Histochem. Cytochem.*, **15**, 652 (1967).
8) G. J. Jones, *J. Histochem. Cytochem.*, **22**, 911 (1974).

9) R. Gillett and K. Gull, *Histochemie*, **30**, 162 (1972).
10) K. E. Rasmussen and J. Albrechtsen, *Histochemistry*, **38**, 19 (1974).
11) E. A. Robertson and R. L. Schultz, *J. Ultrastruct. Res.*, **30**, 275 (1970).
12) H. D. Fahimi and P. Drochmans, *J. Histochem. Cytochem.*, **14**, 756 (1966).
13) B. J. Grenon and A. J. Tousimis, Abstracts of Papers, 33rd Annual Meeting of the Electron Microscopic Society of America, 1975, pp. 620—621.
14) F. Dijk, J. A. Oosterbaan and C. E. Hulstaert, *Histochemistry*, **83**, 573 (1985).
15) E. B. Whipple and M. Ruta, *J. Org. Chem.*, **39**, 1666 (1974).
16) S. Margel and A. Rembaum, *Macromolecules*, **13**, 19 (1980).
17) E. M. Kosower, *J. Am. Chem. Soc.*, **80**, 3261 (1958).
18) F. M. Richards and J. R. Knowles, *J. Mol. Biol.*, **37**, 231 (1968).
19) P. M. Hardy, A. C. Nicholls and H. N. Rydon, *J. Chem. Soc., Chem. Commun.*, **1969**, 565.
20) H. Nakata, Y. Hoshino, N. Takeda and A. Tatematsu, *Org. Mass Spectrom.*, **20**, 467 (1985).
21) L. P. Johnson, S. C. S. Rao and C. Fenselau, *Anal. Chem.*, **50**, 2022 (1978).
22) C. G. Overberger, S. Ishida and H. Ringsdorf, *J. Polym. Sci.*, **62**, S1 (1962).
23) C. Aso and Y. Aito, *Bull. Chem. Soc. Jpn.*, **35**, 1426 (1962).
24) K. Yokata, Y. Suzuki and Y. Ishii, *Kogyo Kagaku Zasshi*, **68**, 2459 (1965).
25) K. Yokota, Y. Ito and Y. Ishii, *Kogyo Kagaku Zasshi*, **66**, 1112 (1963).
26) P. Monsan, G. Puzo and H. Mazarguil, *Biochimie*, **57**, 1281 (1975).
27) E. Stenhagen, S. Abrahamsson and F. W. McLafferty, "Registry of Mass Spectral Data," Vol. 1, John Wiley & Sons, Inc., New York, 1974.
28) F. W. McLafferty, *Anal. Chem.*, **31**, 82 (1959).
29) C. Harries and L. Tank, *Ber. Deutsch. Chem. Gesell.*, **41**, 1701 (1908).
30) J. N. Koral and H. R. Lucas, U. S. Patent 3215675 (1965) [*Chem. Abstr.*, **64**, 3723 (1966)].
31) T. Tashima, U. Kawakami, N. Satoh, T. Nakagawa and H. Tanaka, *J. Electron Microsc.*, **36**, 136 (1987).
32) H. de F. Webster and G. H. Collins, *J. Neuropath. Exp. Neurol.*, **233**, 109 (1964).
33) T. Tashima, U. Kawakami, M. Harada, N. Satoh, T. Nakagawa and H. Tanaka, *Int. J. Pharmaceut.*, accepted for publication (1987).

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Phosphorylation of S-II, a Eukaryotic Transcription Factor, by Casein Kinase II

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Recently, casein kinase II was suggested to play a role in accurate transcription *in vitro* (R. Zandomeni, M. C. Zandomeni, D. Shungar, and R. Weinmann, *J. Biol. Chem.*, **261**, 3414 (1986)). In the present study, we examined whether transcription factor S-II is a target of casein kinase II, because the phosphorylated form of S-II, termed S-II′, is known to be present *in vivo*. We found that S-II was phosphorylated by casein kinase II purified from Ehrlich ascites tumor cells, and showed that this reaction was inhibited by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). DRB also inhibited accurate transcription of the adenovirus major late gene in a nuclear lysate of Ehrlich ascites tumor cells, as it has been found to do in a HeLa cell lysate. This inhibition of transcription was partly restored by addition of purified casein kinase II, but not S-II′, to the reaction mixture.

Keywords——transcription; S-II; stimulatory factor; RNA polymerase II; casein kinase II; phosphorylation; 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; Ehrlich ascites tumor cell

Some nuclear proteins are known to be phosphorylated and dephosphorylated with changes in the level of transcription, and these events are thought to be important in the regulation of selective gene expression.[1,2] Recently, several factors for ribonucleic acid (RNA) polymerase II-mediated transcription have been characterized.[3-16] One of these is S-II,[17-19] which is the only one that has been purified to homogeneity. The phosphorylated form of S-II, termed S-II′, has been shown to be present in cells at half the level of S-II.[19,20] Recently, we found that S-II is actively phosphorylated in cultured mouse L cells.[21] Thus, phosphorylation and dephosphorylation of S-II are thought to be involved in the processes of transcription. For determination of how the function of S-II is regulated through phosphorylation and dephosphorylation, the enzymes catalyzing its phosphorylation and dephosphorylation must be identified.

Casein kinase II is a protein kinase that is not dependent on cyclic nucleotide or calcium and that has been purified from a variety of cells and tissues.[22-32] It phosphorylates several acidic proteins, such as casein and phosvitin, preferentially, but also phosphorylates basic proteins such as histones to lesser extents.[30] However, its target proteins *in vivo* are unknown. Recently, Zandomeni *et al.* showed that calf thymus casein kinase II partially reversed the inhibition by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) of *in vitro* transcription in a HeLa cell lysate, suggesting the involvement of phosphorylation of certain proteins by casein kinase II in accurate transcription in the *in vitro* system.[33]

We examined whether casein kinase II phosphorylates S-II *in vitro*, using a preparation of enzyme purified to homogeneity from Ehrlich ascites tumor cells, and found that S-II is one of the target proteins of casein kinase II *in vitro*.

Materials and Methods

Assay of Protein Kinase——Protein kinase activity was determined with casein as the phosphate acceptor by the method of Dahmus.[34] The standard reaction mixture (0.08 ml) contained 25 mM Tris–HCl, pH 7.9, 5 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.0625 mM [γ-^{32}P] adenosine triphosphate (ATP), 100 mM NaCl, and 100 μg of casein. One unit of protein kinase activity was defined as the amount of enzyme catalyzing the transfer of 1 pmol of phosphate from ATP to casein per min.

Purifications of Casein Kinase II and S-II from Ehrlich Ascites Tumor Cells——Ehrlich ascites tumor cells were grown in male ddY mice as described before.[35] About 65 g of cells was harvested from the ascites fluids of 29 mice. Casein kinase II was purified essentially by the method of Dahmus and Natzle.[24] Transcription factor S-II was purified to homogeneity as described elsewhere.[19]

Protein Determination——During purification of casein kinase II, protein was determined by the method of Bradford[36] with bovine serum albumin as a standard. Proteins in other preparations were determined by a modification of the method of Lowry *et al.*[37] as described before.[19]

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis——SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli[38] in 12.5% acrylamide gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 by the method of Fairbanks *et al.*[39] Autoradiography was performed with Kodak X-Omat AR film using an intensifying screen at −80 °C.

Preparation of a Nuclear Lysate of Ehrlich Ascites Tumor Cells and *in Vitro* Transcription Assay——A nuclear lysate of Ehrlich ascites tumor cells was prepared by the method of Dignam *et al.*[4] except that proteins were solubilized from nuclei with 0.2 M $(NH_4)_2SO_4$ instead of 0.42 M NaCl. Starting from 10 g of fresh cells, we routinely obtained about 10 ml of lysate with a protein concentration of 6—8 mg/ml. The lysate was stored at −80 °C and was stable for at least 6 months. *In vitro* transcription assay was performed as described previously[40] with 1.0 μg of *Sma*I-digested pSmaF[41] as the deoxyribonucleic acid (DNA) template and 20 μl of nuclear lysate in 50 μl of reaction mixture. The lysate could transcribe the adenovirus 2 (Ad 2) major late gene, indicating that accurate transcription mediated by RNA polymerase II took place in the nuclear lysate of Ehrlich ascites tumor cells, as observed in a whole cell lysate of the same cells.[40]

Chemicals——[α-^{32}P] uridine triphosphate (UTP) (400 Ci/mmol) and [γ-^{32}P] guanosine triphosphate (GTP) (10 Ci/mmol) were purchased from Amersham Japan Corp. [γ-^{32}P]ATP (3000 Ci/mmol) was prepared from [^{32}P]phosphoric acid and *GammaPrep* (Promega Biotec) according to the supplier's instructions. Casein was purchased from Sigma, and DRB from Calbiochem.

Results

Purification of Casein Kinase II

To examine the effect of casein kinase II on the phosphorylation of S-II *in vitro*, we purified casein kinase II from Ehrlich ascites tumor cells essentially by the method of Dahmus and Natzle.[24] At the final purification step, the protein kinase activity phosphorylating casein was eluted from hydroxylapatite as a single major peak with 0.15 M KH_2PO_4, as shown in Fig. 1A. When each fraction of this peak was subjected to SDS-polyacrylamide gel electrophoresis, three proteins with molecular masses of 43, 40 and 26 kDa were detected, and the intensities of the bands of these three proteins coincided well with the enzyme activity as shown in Fig. 1B, indicating that these proteins are subunits of the intact enzyme. The small peak eluted with a lower phosphate concentration was probably a phosphorylated form of the same enzyme.[30] A densitometric scan of the stained gel revealed that the three subunits were present in a molar ratio of 1 : 1 : 2 in order of molecular mass and that the purity of the final preparation was more than 90%. The molecular mass of the purified enzyme was determined to be 140 kDa (7.5 S) by sucrose density gradient centrifugation, and this value was consistent with that calculated from the molecular masses of the three subunits.

No significant cyclic nucleotide- or calcium-dependent protein kinase activity was detected in the final preparation. The subunit structure of this enzyme coincided well with that of casein kinase II from rat ascites tumor cells[24] or calf thymus,[30] and several enzymatic characteristics of the purified enzyme revealed that it was casein kinase II (data not shown). We thus concluded that the final preparation of casein kinase II from Ehrlich ascites tumor cells was almost homogeneous. This is the first report of purification of casein kinase II from

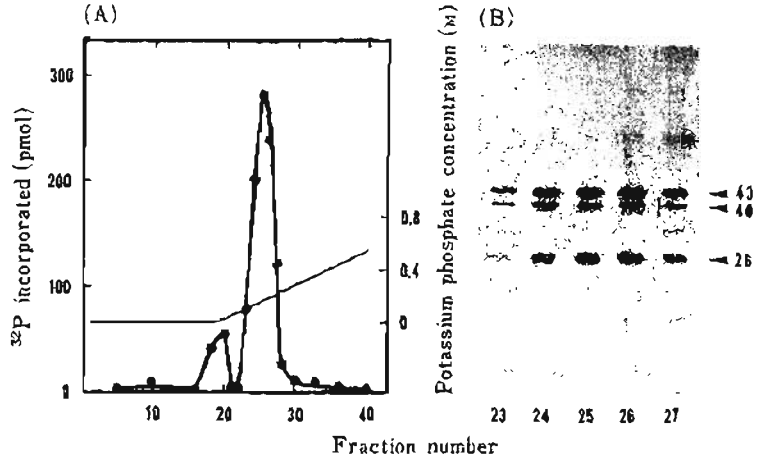

Fig. 1. Hydroxylapatite Chromatography of Casein Kinase II from Ehrlich Ascites Tumor Cells

(A) Active fractions from phosphocellulose were combined and loaded on a column of hydroxylapatite. Activity was eluted from the column with a linear gradient of 0.01—0.08 M potassium phosphate, pH 7.2. Activity for phosphorylating casein (thick line) and the concentration of potassium phosphate (thin line) in fractions were determined.

(B) Proteins in fractions with activity (fractions No. 23—27) were analyzed by SDS-polyacrylamide gel electrophoresis. The molecular weights of the three subunits of casein kinase II are shown in kilodaltons.

TABLE I. Purification of Casein Kinase II from Ehrlich Ascites Tumor Cells

Step	Protein (mg)	Activity (units)	Specific activity (units/mg protein)	Recovery (%)	Purification (fold)
30—50% Saturated $(NH_4)_2SO_4$ fraction	1500	1400000	920	100	1.0
DEAE-cellulose	260	390000	1500	28	1.6
DEAE-Sephadex	30	170000	5600	12	6.1
CM-cellulose	25	140000	5400	10	5.9
Phosphocellulose	0.70	40000	57000	2.9	62
Hydroxylapatite	0.14	25000	180000	1.8	200

Enzyme was purified from 65 g of freshly prepared cells.

mouse cells. The structural and functional properties of the enzyme were found to be similar to those of casein kinase II's from other sources. A typical purification of casein kinase II from Ehrlich ascites tumor cells is summarized in Table I. This purified casein kinase II was used in subsequent experiments.

Phosphorylation of S-II by Casein Kinase II *in Vitro*

We then examined whether purified casein kinase II phosphorylated S-II. S-II was incubated with casein kinase II in the presence of [γ-^{32}P]ATP as the phosphate donor, and phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. As shown in Fig. 2, radioactivity was detected at positions corresponding to 40 and 26 kDa (lane 1). The radioactivity at 40 kDa was found to be due to S-II phosphorylated by casein kinase II, because it was not found in the reaction mixture without S-II (lane 2) or without casein kinase II (lane 3). The radioactivity at 26 kDa in lanes 1 and 2 was the result of autophosphorylation of the 26 kDa subunit of casein kinase II as reported pveviously.[30] Consistent with the report of Zandomeni *et al.* that DRB inhibits casein kinase II,[33] increasing amounts of DRB progressively inhibited the phosphorylation of S-II by

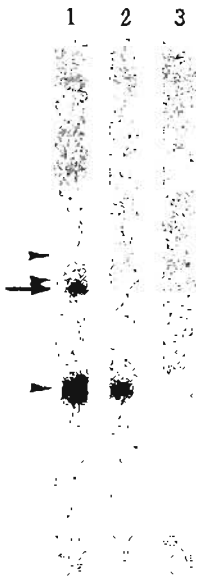

Fig. 2. *In Vitro* Phosphorylation of S-II by Casein Kinase II

A mixture of 1.3 μg of S-II and 0.11 μg of casein kinase II (lane 1), 0.11 μg of casein kinase II only (lane 2), and 1.3 μg of S-II only (lane 3) were incubated with [γ-^{32}P]ATP under the conditions described in Materials and Methods. Phosphorylated proteins were separated by SDS-polyacrylamide gel electrophoresis and subjected to autoradiography. The position of S-II is shown by an arrow, and arrowheads indicate those of the three subunits of casein kinase II.

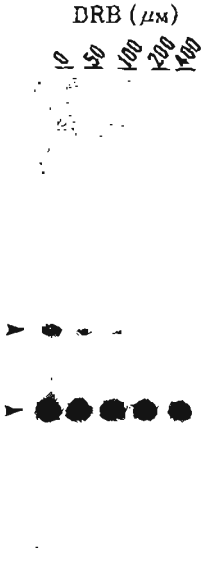

Fig. 3. Effect of DRB on *in Vitro* Phosphorylation of S-II by Casein Kinase II

Mixtures of 1.3 μg of S-II and 0.11 μg of casein kinase II were incubated with [γ-^{32}P]ATP as described in Materials and Methods in the presence or absence of DRB. Phosphorylated proteins were separated by SDS-polyacrylamide gel electrophoresis and located by autoradiography. Concentrations of DRB are indicated at the top of each lane. The positions of phosphorylated S-II and the 26 kDa subunit of casein kinase II are shown by arrowheads.

purified casein kinase II, as shown in Fig. 3. DRB also inhibited the autophosphorylation of the 26 kDa subunit of casein kinase II, although much less than that of S-II. Therefore, we conclude that S-II was phosphorylated by homologous casein kinase II *in vitro*.

Inhibition of Accurate Transcription by DRB and Partial Reversal of This Inhibition by Casein Kinase II *in Vitro*

DRB is also known to inhibit accurate transcription mediated by RNA polymerase II in a HeLa cell lysate.[42,43] Therefore, Zandomeni *et al.* suggested that casein kinase II plays a role in accurate transcription in a HeLa cell lysate, although its target protein in the lysate is unknown.[33] To test the possibility that phosphorylation of S-II by casein kinase II is essential for accurate transcription, we investigated the effects of purified casein kinase II and S-II′ on accurate transcription in a nuclear lysate of Ehrlich ascites tumor cells using *Sma*I-digested pSmaF as the template, to examine the production of run-off RNA of 536 bases.

We first examined the effect of DRB on accurate transcription *in vitro* in a nuclear lysate of Ehrlich ascites tumor cells. As shown in Fig. 4, DRB significantly inhibited *in vitro* transcription from the Ad 2 major late promoter. Usually 80 μM DRB was sufficient for maximum inhibition, but the maximal percentage inhibition varied in different experiments, and part of the transcription was always resistant to DRB. Then we examined whether purified casein kinase II could reverse the inhibition of transcription by DRB. As shown in Fig. 5a, exogenously added casein kinase II partly reversed this inhibition, confirming the results of Zandomeni *et al.* This reversal was clearer when the intensity of the band of accurate transcript was plotted against the dose of casein kinase II, arbitrarily defining the intensity without purified casein kinase II as 1, as shown in Fig. 5b. These results suggest that the phosphorylation of some transcription factor(s) by casein kinase II is involved in accurate

Fig. 4. Effect of DRB on Accurate Transcription in a Nuclear Lysate of Ehrlich Ascites Tumor Cells

Transcription was carried out in the presence or absence of DRB, and transcripts were analyzed by 5% polyacrylamide–7 M urea gel electrophoresis followed by autoradiography as described [lit. 40]. An autoradiogram of the dried gel is shown. The concentration of ethanol (2%) was the same in all reactions. The concentrations of DRB are indicated at the top of each lane. The position of the run-off product of 536-base RNA from the Ad 2 major late gene is shown by an arrowhead.

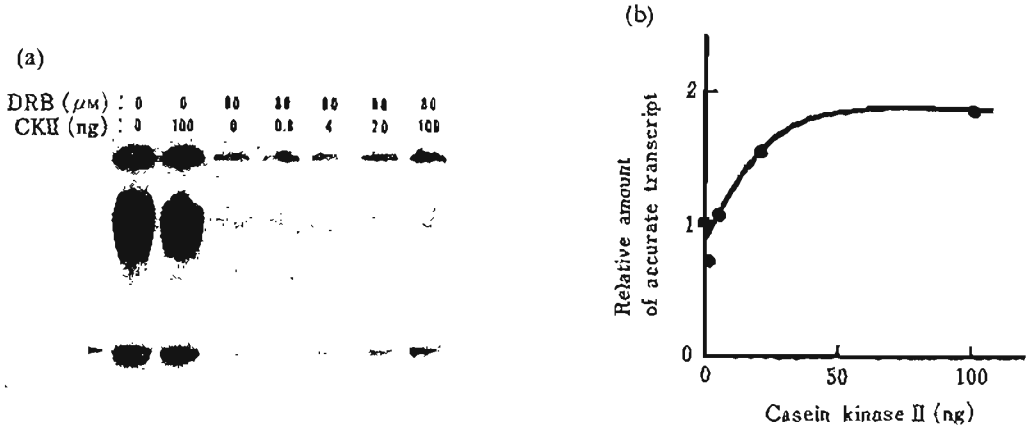

Fig. 5. Effect of Purified Casein Kinase II on Accurate *in Vitro* Transcription

Increasing amounts of casein kinase II purified from Ehrlich ascites tumor cells were added to the transcription reaction mixture in the presence or absence of DRB.

(a) Transcripts were analyzed as described in the legend to Fig. 4. The concentrations of DRB and amounts of casein kinase II added are indicated at the tops of the lanes. The run-off product of 536-base RNA is shown by an arrowhead.

(b) The intensity of the band of accurate transcript in (a) was measured by densitometric scanning, and the relative amount is plotted against the dose of casein kinase II, arbitrarily defining the amount of transcript without casein kinase II as 1.

transcription in a nuclear lysate of Ehrlich ascites tumor cells. To test whether this factor is S-II, we examined whether exogenously added S-II′ reversed the inhibition of transcription by DRB, but detected no significant reversal, even in the presence of excess S-II′ (data not shown).

Discussion

This paper describes the phosphorylation of S-II, a stimulatory protein of RNA polymerase II, by casein kinase II. An experiment using antibody against S-II demonstrated that S-II is a transcription factor of RNA polymerase II that is essential for accurate transcription.[17]

As we reported before, a phosphorylated form of S-II, termed S-II′ was found in Ehrlich

ascites tumor cells and purified to homogeneity.[19] Therefore, the function of S-II is apparently regulated by its phosphorylation and dephosphorylation. We examined the functions of S-II and S-II′ in terms of stimulation of RNA polymerase II, finding no appreciable difference between the two.[44]

Recently, S-II was shown to consist of two domains, one essential for stimulation of RNA polymerase II, and the other containing phosphorylation sites, and a 21 kDa chymotryptic fragment of S-II that did not contain the phosphorylation sites was found to be sufficient for stimulation of RNA synthesis *in vitro*.[45] Therefore, conceivably S-II has dual functions: stimulation of RNA polymerase II and an unknown function in accurate transcription. Probably, the latter function of S-II is regulated by phosphorylation and dephosphorylation. For insight into this function of S-II, the enzymes participating in the phosphorylation and dephosphorylation of this protein should be identified. In this paper we have demonstrated that purified homologous casein kinase II phosphorylated S-II *in vitro*, and that this phosphorylation was inhibited by DRB. Therefore, casein kinase II may be the enzyme responsible for the phosphorylation of S-II *in vivo*.

Independently, Zandomeni *et al.* showed that DRB, an inhibitor of casein kinase II, inhibited accurate transcription in a HeLa cell lysate, and claimed that the inhibition was partially reversed by casein kinase II purified from calf thymus.[33] We confirmed their results using a nuclear lysate of Ehrlich ascites tumor cells and homologous casein kinase II. However, we found that casein kinase II caused only partial reversal. If the effect of DRB were restricted to the inhibition of casein kinase II in the lysate, inhibition of accurate transcription by DRB should have been reversed much more by exogenously added casein kinase II. It was indicated that DRB inhibited transcription initiation in *Chironomus*[46] and in HeLa cells.[47] Tamm reported that DRB enhanced premature termination of SV40 gene transcription.[48] Therefore, DRB probably inhibits the activity of several factors participating in transcription, including casein kinase II, and this may be the reason why exogenously added casein kinase II could not completely reverse the inhibition of accurate transcription by DRB.

Although exogenously added casein kinase II partly restored the inhibition of DRB, S-II′ did not. It seems likely that phosphorylation of S-II *in situ* is essential for transcription. Phosphorylation of another factor(s) by casein kinase II may also be involved in this reaction.

It is technically difficult to demonstrate directly that casein kinase II phosphorylates S-II in a nuclear lysate of Ehrlich ascites tumor cells and that this step is essential for accurate transcription, because of the presence of other protein kinase activity. DRB inhibited the phosphorylation of S-II by casein kinase II *in vitro*, but we could not detect appreciable inhibition of phosphorylation of S-II in mouse L cells when DRB was added to their culture medium, although RNA synthesis was greatly suppressed.[21] These observations suggest that DRB inhibits various steps of transcription, and the phosphorylation of S-II by casein kinase II is one such step. Therefore, DRB is likely to inhibit other steps of transcription in preference to the phosphorylation of S-II in living cells.

References

1) I. R. Phillips, E. A. Shephard, J. L. Stein and G. S. Stein, *Cell Different.*, **10**, 23 (1981).
2) G. H. Murdock, M. G. Rosenfeld and R. M. Evans, *Science*, **218**, 1315 (1982).
3) T. Matsui, J. Segall, P. A. Weil and R. G. Roeder, *J. Biol. Chem.*, **255**, 11992 (1980).
4) J. D. Dignam, P. L. Martin, B. D. Sharsty and R. G. Roeder, *Methods Enzymol.*, **101**, 582 (1983).
5) S. Y. Tsai, M.-J. Tsai, L. E. Kops, P. P. Minghetti and B. W. O'Malley, *J. Biol. Chem.*, **256**, 13051 (1981).
6) A. Fire, M. Samuels and P. A. Sharp, *J. Biol. Chem.*, **259**, 2509 (1984).
7) M. Sawadogo and R. G. Roeder, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 4394 (1985).
8) M. Samuels and P. A. Sharp, *J. Biol. Chem.*, **261**, 2003 (1986).

9) W. S. Dynan and R. Tjian, *Cell*, **32**, 669 (1983).
10) W. S. Dynan and R. Tjian, *Cell*, **35**, 79 (1983).
11) J. T. Kadonaga, K. A. Jones and R. Tjian, *Trends Biochem. Sci.*, **11**, 20 (1986).
12) C. S. Parker and J. Topol, *Cell*, **36**, 357 (1984).
13) C. S. Parker and J. Topol, *Cell*, **37**, 273 (1984).
14) M. Sawadogo and R. G. Roeder, *Cell*, **43**, 165 (1985).
15) R. W. Carthew, L. A. Chodosh and P. A. Sharp, *Cell*, **43**, 439 (1985).
16) N. G. Miyamoto, V. Moncollin, J. M. Egly and P. Chambon, *EMBO J.*, **4**, 3563 (1985).
17) K. Sekimizu, H. Yokoi and S. Natori, *J. Biol. Chem.*, **257**, 2719 (1982).
18) S. Natori, *Mol. Cell. Biochem.*, **46**, 173 (1982).
19) K. Sekimizu, Y. Nakanishi, D. Mizuno and S. Natori, *Biochemistry*, **18**, 1582 (1979).
20) K. Sekimizu, Y. Kubo, K. Segawa and S. Natori, *Biochemistry*, **20**, 2286 (1981).
21) S. Hirashima, Y. Nakanishi, K. Sekimizu and S. Natori, *Biochem. Biophys. Res. Commun.*, **131**, 524 (1985).
22) O. Walinder, *Biochim. Biophys. Acta*, **293**, 140 (1973).
23) W. Thornbury and T. J. Lindell, *J. Biol. Chem.*, **252**, 6660 (1977).
24) M. E. Dahmus and J. R. Natzle, *Biochemistry*, **16**, 1901 (1977).
25) G. M. Hathaway and J. A. Traugh, *J. Biol. Chem.*, **254**, 762 (1979).
26) A. Kumon and M. Ozawa, *FEBS Lett.*, **108**, 200 (1979).
27) P. Boivin and C. Galand, *Biochem. Biophys. Res. Commun.*, **89**, 7 (1979).
28) K. M. Rose, L. E. Bell, D. A. Siefken and S. T. Jacob, *J. Biol. Chem.*, **256**, 7468 (1981).
29) C. Cochet, D. Job, Fj. Pirollet and E. M. Chambaz, *Biochim. Biophys. Acta*, **658**, 191 (1981).
30) M. E. Dahmus, *J. Biol. Chem.*, **256**, 3319 (1981).
31) C. Cochet, J. C. Feige and E. Chambaz, *Biochem. Biophys. Acta*, **743**, 1 (1983).
32) C. V. C. Glover, E. R. Shelton and D. L. Brutlag, *J. Biol. Chem.*, **258**, 3258 (1983).
33) R. Zandomeni, M. C. Zandomeni, D. Shugar and R. Weinmann, *J. Biol. Chem.*, **261**, 3414 (1986).
34) M. R. Dahmus, *Biochemistry*, **15**, 1821 (1976).
35) S. Natori, K. Takeuchi and D. Mizuno, *J. Biochem.* (Tokyo), **73**, 345 (1973).
36) M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
37) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
38) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
39) G. Fairbanks, T. L. Steck and D. F. H. Wallech, *Biochemistry*, **10**, 2606 (1971).
40) M. Ohtsuki, K. Sekimizu and S. Natori, *J. Biochem.* (Tokyo), **96**, 1267 (1984).
41) P. A. Weil, D. S. Luse, J. Segall and R. G. Roeder, *Cell*, **18**, 469 (1979).
42) R. Zandomeni, B. Mittlemann, D. Bunick, S. Ackerman and R. Weinmann, *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 3167 (1982).
43) B. Mittleman, R. Zandomeni and R. Weinmann, *J. Mol. Biol.*, **165**, 461 (1983).
44) Y. Nakanishi, Y. Mitsuhashi, K. Sekimizu, H. Yokoi, Y. Tanaka, M. Horikoshi and S. Natori, *FEBS Lett.*, **130**, 69 (1981).
45) M. Horikoshi, K. Sekimizu, S. Hirashima, Y. Mitsuhashi and S. Natori, *J. Biol. Chem.*, **260**, 5739 (1985).
46) E. Egyhazi, *Nature* (London), **262**, 319 (1976).
47) I. Tamm, R. Hand and L. A. Coligure, *J. Biol. Chem.*, **69**, 229 (1976).
48) I. Tamm, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 5011 (1977).

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Intraparticulate Localization of Peroxisomal Carnitine Acyltransferases in Chick Embryo Liver

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Hepatic peroxisomes from 20-d-old chick embryo were purified by sucrose density gradient centrifugation. The specific activities of catalase and D-amino acid oxidase (DAAO) in the peroxisomal fraction were 24- and 32-fold, respectively, higher than those in the homogenate. The isolated peroxisomes included activities of 85.5 and 416 nmol/min/mg protein of carnitine palmitoyltransferase (CPT) and carnitine acetyltransferase (CAT), respectively. CPT was easily solubilized by treatment with Tween 20 or sonication from the peroxisomes, being similar in this respect to catalase. On the other hand, CAT was more resistant to solubilization, behaving similarly to DAAO. These results indicate that CPT as well as catalase is located in the peroxisomal matrix in a soluble form, and that CAT as well as DAAO is weakly associated with some insoluble components.

Keywords——intraparticulate localization; chick embryo; peroxisome; carnitine acyltransferase; liver

Carnitine palmitoyltransferase (CPT) is the best known carnitine acyltransferase. CPT in rodent liver is located on both the inner and outer surfaces of the inner mitochondrial membrane[1] and plays an important role in the transport of long-chain acyl-CoA into the mitochondrial matrix.[2,3] Recently it was reported that CPT activity is also present in peroxisomes in the livers of human[4] and chick embryo.[5] In previous paper,[6] we showed that the peroxisomes in chick embryo liver contained CPT and carnitine acetyltransferase (CAT), but not carnitine octanoyltransferase (COT), and we suggested that peroxisomal CPT is different from the mitochondrial enzymes in several respects.

The purpose of the present study was to clarify the intraparticulate localization of peroxisomal carnitine acyltransferases in chick embryo liver. We purified the peroxisomes from 20-d-old chick embryo liver and examined the solubilization of these enzymes from the peroxisomes.

Materials and Methods

Palmitoyl-CoA, acetyl-CoA, reduced nicotinamide adenine dinucleotide phosphate (NADPH) and D-alanine were purchased from Sigma. L-(−)-Carnitine was kindly donated by Earth Pharmaceutical Co., Japan. All other reagents were of analytical grade. Fertilized eggs (White Leghorn line) were obtained from a poultry farm.

Subcellular Fractionation——The livers (6 g) removed from 20-d-old chick embryo were immediately homogenized in 60 ml of ice-cold homogenizing medium (0.25 M sucrose, 20 mM glycylglycine, pH 7.4) with a Potter-Elvehjem type Teflon-glass homogenizer (4 strokes at 1300 rpm). The homogenates were centrifuged at $5300 \times g$ (10 min) and the supernatant was further centrifuged at $12500 \times g$ (20 min). High-speed pellets (light mitochondrial fraction) were suspended in the homogenizing medium. Peroxisomes were isolated by discontinuous sucrose density gradient centrifugation of the light mitochondrial fraction. Three mililiters of the light mitochondrial fraction was layered over

a 49-ml discontinuous sucrose density gradient: 10 ml of 56.0% sucrose in 20 mM glycylglycine, pH 7.4, 5 ml of 48.5%, 6 ml of 46.0%, 6 ml of 44.5%, 6 ml of 36.5%, 8 ml of 30.0% and 8 ml of 23.5% from bottom to top, as shown in Fig. 1, and centrifuged at 24000 rpm for 2.5 h in an RPS-25-2 rotor (Hitachi, Japan) at 4 °C. The fractions 1 (8 ml), 2 (4 ml), 3 to 7 (6 ml each) and the last portion (10 ml) were collected from the bottom with a micropump.

Treatment with Tween 20——The isolated peroxisomes (0.2 mg of protein) were incubated with 20 mM glycylglycine buffer (pH 7.4) containing various concentrations of Tween 20 for 30 min at 4 °C and then centrifuged at 20000 × *g* for 30 min. The precipitates were separated from the supernatant and were suspended in homogenizing medium. The enzyme activities of the precipitate fraction and the supernatant were determined.

Sonication——An aliquot (6 ml) of the peroxisomal fraction was mixed with the same volume of 20 mM glycylglycine buffer (pH 7.4) and sonicated in Ohtake Works sonicator at 50 W (100 V, 500 mA) at 4 °C. After 0, 1, 2, 3, and 4 min of the treatment, 2-ml portions of peroxisomal protein solution were transferred to centrifuge tubes and then centrifuged at 20000 × *g* for 30 min. The precipitates were suspended in the homogenizing medium. The enzyme activities in the precipitate and the supernatant were determined.

Sucrose Density Gradient Centrifugation of Peroxisomes after the Treatment with Tween 20——Peroxisomal fraction (3 ml) was gently added to the same volume of 20 mM glycylglycine buffer (pH 7.4) with or without Tween 20 at a final concentration of 15 mg/mg protein and incubated at 4 °C. After 30 min, the suspensions were layered over 46-ml linear sucrose density gradient (25.0—55.0% sucrose in 20 mM glycylglycine buffer, pH 7.4) and centrifuged at 24000 rpm for 2.5 h in an RPS-25-2 rotor (Hitachi, Japan) at 4 °C. The fractions 1 to 11 (4 ml each) and the last portion (8 ml) were collected from the bottom with a micropump.

Enzyme and Protein Assay——The activities of carnitine acyltransferases were determined by the DTNB method of Bieber *er al.*[7] D-Amino acid oxidase (DAAO) activity was determined by measuring D-alanine-dependent H_2O_2 formation by the method of Allain *et al.*[8] with some modifications. One unit of the activity was defined as the amount of enzyme that produced 1 nmol of reaction product/min. Acid phosphatase activity was determined by the method of Appelmans *et al.*[9] One unit of the activity was defined as the formation of 1 mol of reaction product/min. NADPH–cytochrome c reductase activity was determined by the method of Beaufay *et al.*[10] One unit of the activity was defined as the amount of enzyme that reduced 1 nmol of cytochrome c/min. Catalase,[11] urate oxidase[12] and cytochrome c oxidase[13] were assayed according to the cited procedures. Units of enzyme activities are expressed as previously described.[14] Protein content was determined by the method of Lowry *et al.*[15] and by using a Bio-Rad protein assay kit (for protein assay in a sucrose density gradient) with bovine serum alubumine as a standard.

Results

Distribution of Peroxisomes after Sucrose Density Gradient Centrifugation

The light mitochondrial fraction from the livers of 20-d-old chick embryos was centrifuged in a discontinuous sucrose density gradient (Fig. 1). The distributions of peroxisomal and other enzymes are shown in Fig. 2. Catalase and DAAO as markers of peroxisomes were mainly distributed in fraction 2. Cytochrome c oxidase and acid phosphatase as markers of mitochondria and lysosomes, respectively, were mainly distributed in fraction 5. NADPH–cytochrome c reductase as a marker of microsomes was broadly distributed from fraction 2 to 5 (density 1.20—1.22), and a significant activity was also found in fraction 2. However, CPT and CAT were mainly distributed in fraction 2, corresponding to peroxisomes, though parts of these activities were also seen in fraction 5 (mitochondria).

Table I shows the properties of peroxisomal fraction (fraction 2 in Fig. 2). Peroxisomes were purified 24- and 32-fold on the bases of the activities of catalase and DAAO, respectively. Urate oxidase was not detectable in chick embryo liver. The relative specific activities of cytochrome c oxidase, NADPH–cytochrome c reductase and acid phosphatase were much less than those of peroxisomal enzymes. Therefore, this fraction was considered to be usable as isolated peroxisomes for the following experiments. The isolated peroxisomes had CPT activity (85.5 nmol/min/mg protein) and CAT activity (416 nmol/min/mg protein).

Solubilization of Peroxisomal Enzymes

Figure 3 shows the results of solubilization studies of peroxisomal enzymes by treatment with Tween 20 or sonication. CPT was easily solubilized and the patterns were consistent with those of catalase. More than 50% of CPT and catalase activities were released when the

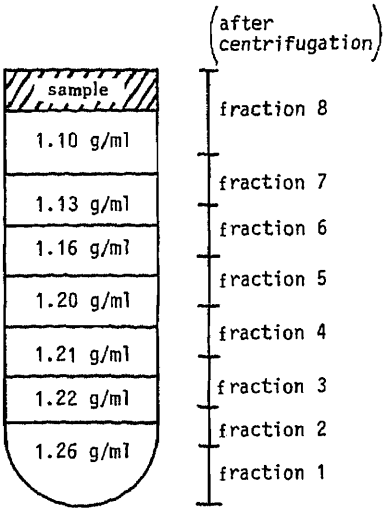

Fig. 1. Fractionation after Discontinuous Sucrose Density Gradient Centrifugation of the Light Mitochondrial Fraction

The fractions 1 (8 ml), 2 (4 ml), 3 to 7 (6 ml each), and the last portion (10 ml) were collected from the bottom with a micropump.

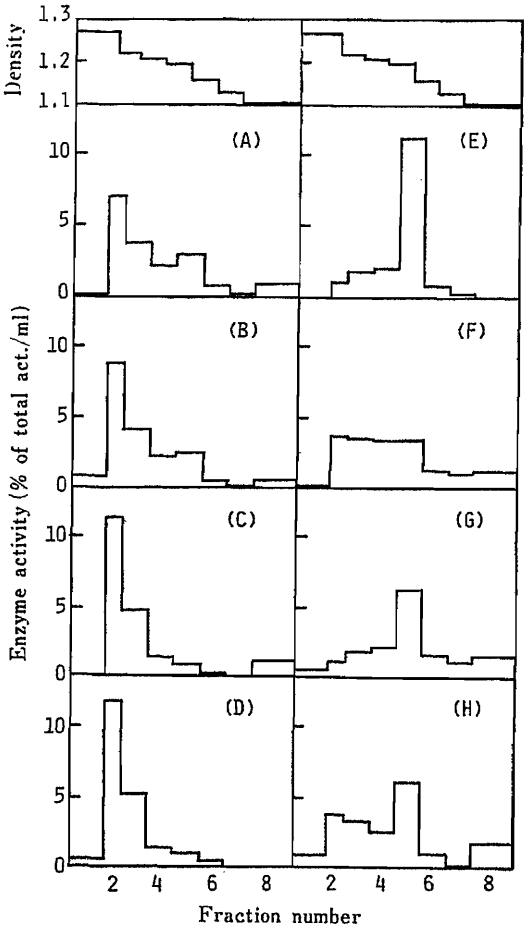

Fig. 2. Distribution of Various Marker Enzyme Activities after Sucrose Density Gradient Centrifugation of the Light Mitochondrial Fraction

A, CPT; B, CAT; C, catalase; D, DAAO; E, cytochrome c oxidase; F, NADPH-cytochrome c reductase; G, acid phosphatase; H, protein.

peroxisomal fraction was simpl diluted solely with 20 mM glycylglycine buffer (pH 7.4). On the other hand, CAT was more resistant to the solubilizations than CPT. Though the extent of the solubilization of CAT was different from that of DAAO, the patterns of CAT solubilization by both treatments were basically similar to those of DAAO.

Sucrose Density Gradient Centrifugation of Peroxisomes after Treatment with Tween 20

Figure 4 shows the sucrose density gradient centrifugation patterns of peroxisomal enzymes with or without Tween 20 treatment. In the control, about half of the CPT activity was released from peroxisomes to the lower density fractions, and the other still remained in fractions 2 and 3. This enzyme, however, was completely released to the lower density fractions by the treatment with Tween 20. These features resemble those of catalase. On the other hand, the centrifugel patterns of CAT activity under both conditions were similar to those of DAAO activity: almost all of the activities of CAT and DAAO in the control were distributed to higher density fractions, and most of those shifted to the lower density fractions after Tween 20 treatment, but showing broad distributions.

TABLE I. Properties of Isolated Peroxisomes

	Homogenate SA[a)]	Peroxisome SA	Peroxisome SA / Homogenate SA
CPT	10.1±1.5	85.5±4.2	8.5
CAT	20.5±1.8	416±75	20.3
Catalase	0.084±0.004	2.02±0.18	24.0
DAAO	2.26±0.06	71.2±10.4	31.5
Urate oxidase	Not detectable	Not detectable	—
Cytochrome c oxidase	4.32±1.08	1.98±0.16	0.46
NADPH–cytochrome c reductase	3.35±0.23	7.12±1.48	2.13
Acid phosphatase	31.6±2.2	9.96±1.0	0.32

a) SA, specific activity in unit/mg protein; units were defined as described under Materials and Methods. Fraction 2 in Fig. 2 was used as the enzyme source. The activities of carnitine acyltransferases and marker enzymes were assayed as described under Materials and Methods. Results are expressed as the means±S.D. of 3 preparations.

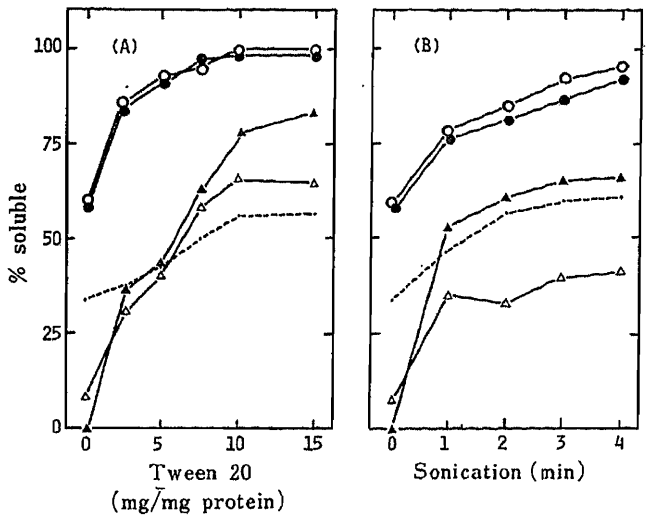

Fig. 3. Solubilization of Peroxisomal CPT and CAT by the Treatment with Tween 20 and by Sonication

In A, isolated peroxisomal fraction (0.2 mg of protein) was incubated with 20 mM glycylglycine buffer (pH 7.4) containing the indicated concentrations of Tween 20 for 30 min at 4 °C and then centrifuged at 20000×*g* for 30 min. In B, 6 ml of isolated peroxisomal fraction was mixed with the same volume of 20 mM glycylglycine buffer (pH 7.4) and sonicated in an Ohtake Works sonicator at 50 W (100 V, 500 mA) at 4 °C. After the indicated times, 2-ml aliquots were transferred to centrifuge tubes, and then centrifuged as in A. CPT (○); CAT (△); catalase (●); DAAO (▲); protein (----).

The values of % soluble: $\left[\frac{act_{(sup)}}{act_{(pellet)}+act_{(sup)}}\right]\times 100$

Discussion

The peroxisomal fraction obtained by sucrose density gradient centrifugation contained 24- and 32-fold higher specific activities of catalase and DAAO, respectively, than the homogenate of chick embryo liver, and included significant microsomal contamination of microsomes (see Table I). It was impossible to separate peroxisomes from microsomes more completely, because the density of the microsomes in chick embryo liver (1.20—1.22 g/ml) was higher than that in rat liver (medium density 1.17 g/ml).[16] However, we have demonstrated

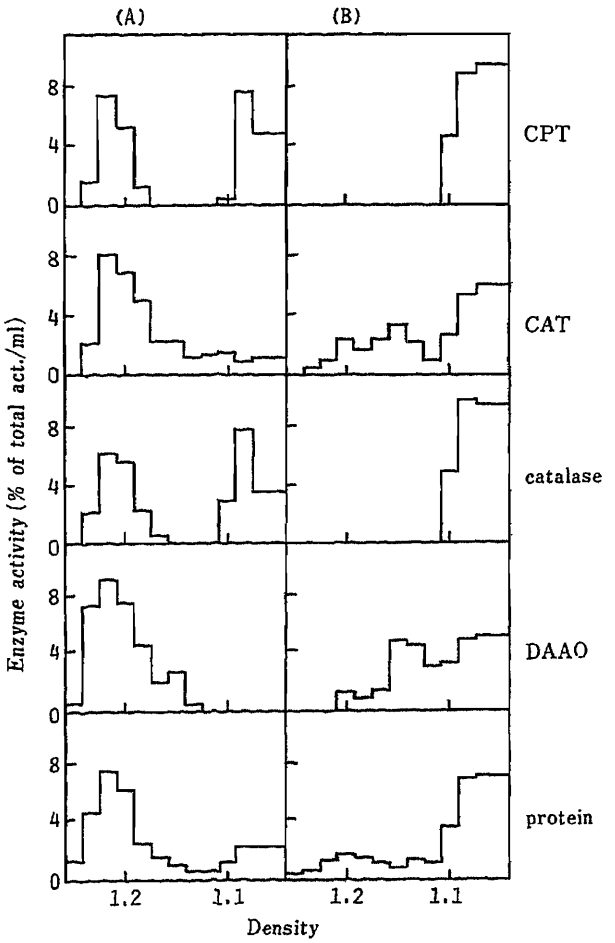

Fig. 4. Sucrose Density Gradient Centrifugation of Peroxisomes after the Treatment with Tween 20

Isolated peroxisomal fraction (3 ml) was added to the same volume of 20 mM glycylglycine buffer (pH 7.4) with or without Tween 20 at a final concentration of 15 mg/mg protein and incubated for 30 min at 4 °C. The suspensions were centrifuged in a linear sucrose density gradient at 24000 rpm for 2.5 h. (A) Control. (B) Tween 20.

previously[6] that almost all of the carnitine acyltransferases in chick embryo liver were present in both peroxisomes and mitochondria. Therefore, the activities of carnitine acyltransferases in this fraction were considered to be due to those of peroxisomal enzymes.

Markwell *et al.*[17] have reported from solubilization experiments that the peroxisomal CAT in rat liver had a similar distribution to catalase. In chick embryo liver, however, the solubilization of peroxisomal CAT with Tween 20 was similar to that of DAAO, being clearly different from that of catalase (see Fig. 4). Hayashi *et al.*[12,18-20] suggested that DAAO was weakly associated with the core of peroxisomes in rat liver and that urate oxidase was tightly bound to the core. In this experiment, no urate oxidase activity was detectable in chick embryo liver, though it has been reported that the core protein exists in peroxisomes of the early embryo liver.[21] Therefore, CAT may be associated with some other insoluble component such as the membrane, though its binding seems to be weak.

The solubilization study indicated that peroxisomal CPT was easily solubilized and had a similar distribution to catalase (see Figs. 3 and 4). Thus, peroxisomal CPT seems to be located in the matrix in a soluble form. In mitochondria, it is known that CPT is located on the inner membrane[1] and plays a role on the uptake of long-chain acyl-CoAs into the matrix,[2,3] because long-chain acyl-CoAs do not penetrate the mitochondrial inner membrane. In contrast, since carnitine is not required for the uptake of long-chain acyl-CoAs into the peroxisomal matrix, it is suggested that the peroxisomal membrane contains a direct carrier or a permease for long-chain acyl-CoAs.[22,23] Peroxisomal CPT must play some role other than the uptake of long-chain acyl-CoAs into the matrix. We have reported previously[5] that the activity of peroxisomal β-oxidation in chick embryo liver increased markedly before hatching,

and that the changes in the activity were consistent with those in the contents of hepatic lipids. Therefore, a large amount of long-chain acyl-CoAs may be supplied to hepatic peroxisomes at the hatching stage. Though higher concentrations of long-chain acyl-CoAs caused substrate inhibition of fatty acyl-CoA oxidase which is the first step enzyme of peroxisomal β-oxidation,[24,25] CPT present in the matrix of peroxisomes in chick embryo liver might play some important role, because peroxisomal CPT has high affinity for long-chain acyl-CoAs and its substrate specificity is similar to that of fatty acyl-CoA oxidase.[6] From these findings, it is suggested that the physiological role of peroxisomal CPT is to maintain the active oxidation of fatty acid in peroxisomes by the transforming excess long-chain acyl-CoAs to the corresponding acylcarnitines.

References

1) C. L. Hoppel and R. J. Tomec, *J. Biol. Chem.*, **247**, 832 (1972).
2) J. Bremer, *J. Biol. Chem.*, **237**, 2228 (1962).
3) I. B. Fritz and K. T. N. Yue, *J. Lipid Res.*, **4**, 279 (1963).
4) M. Bronfman and F. Leighton, *Biochem. J.*, **224**, 721 (1984).
5) H. Ishii, S. Ishii, T. Suga and M. Kazama, *Arch. Biochem. Biophys.*, **237**, 151 (1985).
6) S. Ishii, H. Ishii and T. Suga, *J. Biochem.* (Tokyo), **98**, 747 (1985).
7) L. L. Bieber, T. Abraham and T. Helmrath, *Anal. Biochem.*, **50**, 509 (1972).
8) C. C. Allain, L. S. Poon, C. S. G. Chan, W. Richmond and C. Fu, *Clin. Chem.*, **20**, 470 (1974).
9) F. Appelmans, R. Wattiaux and C. De Duve, *Biochem. J.*, **59**, 438 (1955).
10) H. Beaufay, A. Amar-Costesec, E. Feytmans, D. Thines-Sempoux, M. Wibo, M. Robbi and J. Berthet, *J. Cell Biol.*, **61**, 188 (1974).
11) H. Luck, "Methods of Enzymatic Analysis," ed. by H. U. Bergmeyer, Academic Press, New York, 1963, p. 885.
12) H. Hayashi, T. Suga and S. Niinobe, *Biochim. Biophys. Acta*, **252**, 58 (1971).
13) C. C. Wharton and A. Tzagoloff, "Methods in Enzymology," Vol. 10, ed. by R. W. Estabrook, Academic Press, New York, 1967, p. 245.
14) H. Ishii, S. Ishii, M. Kazama and T. Suga, *Arch. Biochem. Biophys.*, **226**, 484 (1983).
15) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
16) F. Leighton, B. Poole, H. Beaufay, P. Baudhuin, J. W. Coffey, S. Fowler and C. de Duve, *J. Cell Biol.*, **37**, 482 (1968).
17) M. A. K. Markwell, E. J. McGroaty, L. L. Bieber and N. E. Tolbert, *J. Biol. Chem.*, **248**, 3426 (1973).
18) H. Hayashi, T. Suga and S. Niinobe, *Biochim. Biophys. Acta*, **297**, 110 (1973).
19) H. Hayashi, K. Taya, T. Suga and S. Niinobe, *J. Biochem.* (Tokyo), **79**, 1029 (1976).
20) H. Hayashi, T. Suga and S. Niinobe, *J. Biochem.* (Tokyo), **82**, 607 (1977).
21) Z. Hruban and M. Rechigl, Jr., "Microbodies and Related Particles; Morphology, Biochemistry and Physiology," ed. by G. H. Bourne and J. F. Danielli, Academic Press, New York, 1969, p. 20.
22) E. L. Appelkvist and G. Dallner, *Biochim. Biophys. Acta*, **617**, 156 (1980).
23) H. Osmundsen and C. E. Neat, *FEBS Lett.*, **107**, 81 (1979).
24) T. Osumi, T. Hashimoto and N. Ui, *J. Biochem.* (Tokyo), **87**, 1735 (1980).
25) M. Walusimbi-Kisitu and E. H. Harrison, *J. Lipid Res.*, **24**, 1077 (1983).

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Purification and Characterization of Sarcosine Oxidase of *Streptomyces* Origin

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Sarcosine oxidase (EC 1.5.3.1) produced by *Streptomyces* sp. KB210-8SY was purified by ion exchange chromatography on DEAE-sepharose CL-6B, affinity chromatography on sarcosyl-AH-sepharose 4B and gel filtration on sephadex G-150 to electrophoretic homogeneity.

The molecular weight of the enzyme was estimated to be 44000 by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and gel filtration on Sephadex G-150. The enzyme showed the maximum activity at pH 8 and was stable at pH 7—9. In addition to sarcosine, the enzyme oxidized *N*-methyl-L-leucine, *N*-methyl-DL-alanine and *N*-methyl-DL-valine to lesser extents. The apparent K_m values for sarcosine, *N*-methyl-L-leucine, *N*-methyl-DL-alanine and *N*-methyl-DL-valine were 0.91, 0.58, 1.6 and 6.7 mM, respectively. The enzyme was inactivated by *N*-bromosuccinimide, hydroxylamine hydrochloride, SDS, Zn^{2+}, Ni^{2+} and Hg^{2+} but not by ethylenediaminetetraacetate, *p*-chloromercuribenzoate or *p*-toluenesulfonyl chloride.

The taxonomic characteristics of strain KB210-8SY are closely related to those of *Streptomyces flavovirens* and *S. misakiensis*.

Keywords——*Streptomyces*; sarcosine oxidase (EC 1.5.3.1); sarcosine; creatinine; creatine; clinical diagnosis

The Folin method[1] based on the chemical reaction reported by Jaffe[2] is most commonly used in the clinical diagnostic analysis of creatinine in serum and urine. However, the Folin method has the disadvantages of poor specificity and requirement for deproteinization. Recently, enzymatic measurement systems using specific enzymes, especially of microbial origin, have been gradually replacing the chemical method,[3-6] although they are still rather expensive. For example, the three creatinine catabolic enzymes, creatininase, creatinase and sarcosine oxidase, can be used in appropriate combinations for the determination of creatinine and /or creatine. The first one catalyzes interconversion between creatinine and creatine, the second hydrolyzes creatine to sarcosine and urea and the last further hydrolyzes sarcosine to glycine and formaldehyde with the simultaneous generation of hydrogen peroxide. The amounts of creatinine and /or creatine can be estimated by the determination of either formaldehyde or hydrogen peroxide.

$$\text{creatinine} + H_2O \xrightarrow{(1)} \text{creatine}$$

$$\text{creatine} + H_2O \xrightarrow{(2)} \text{sarcosine} + \text{urea}$$

$$\text{sarcosine} + O_2 + H_2O \xrightarrow{(3)} \text{glycine} + HCHO + H_2O_2$$

(1) creatinine amidohydrolase (creatininase, EC 3.5.2.10)

(2) creatine amidinohydrolase (creatinase, EC 3.5.3.3)
(3) sarcosine oxidase (EC 1.5.3.1)

During a search for novel creatinine catabolic enzymes, the production of sarcosine oxdase by a strain of genus *Streptomyces*, designated as KB210-8SY, was observed. To date, the sarcosine oxidases from genera *Corynebacterium*,[7] *Cylindrocarpon*[8] and *Bacillus*[9] have been extensively studied. Since there has been no report about the production of sarcosine oxidase by *Streptomyces*, the nature of the enzyme was of interest. The details of the purification and characterization of sarcosine oxidase produced by strain KB210-8SY are presented in this paper along with the results of taxonomic studies of the producing organism.

Materials and Methods

Materials——Sarcosine oxidase of *Bacillus* origin was purified as reported previously.[7] Horseradish peroxidase (EC 1.11.1.7) was obtained from Wako Pure Chemical Ind., Ltd. Formaldehyde dehydrogenase (EC 1.2.1.1) was a product of Oriental Yeast Co., Ltd. DEAE-Sepharose CL-6B, AH-Sepharose 4B and Sephadex G-150 Superfine were purchased from Pharmacia Fine Chemicals. *N*-Methyl-L-leucine, *N*-methyl-DL-valine and *N*-methyl-DL-alanine were products of Sigma Chemical Co., Ltd. All other materials were commercial products of analytical grade.

Cultivation——Seed medium was composed of 2.0% corn steep liquor, 1.5% soluble starch, 1.5% glucose, 0.3% yeast extract, 0.2% NaCl, 0.32% $CaCO_3$ and one drop of PRONAL ST-1 (antifoaming agent, Toho Chemical Ind., Co.) per 110 ml. Seed medium supplemented with 0.5% choline chloride was used as the production medium. The cultivation was conducted in 500 ml Sakaguchi flasks containing 110 ml of the production medium on a rotary shaker (amplitude 75 mm, 120rpm) at 27 °C for 5 d.

Assay Methods for Sarcosine Oxidase——Assay Method I: Formaldehyde formed by enzymatic reaction was measured according to the method of Nash.[10] A mixture of an enzyme solution (0.1 ml) and 0.1 M Tris-HCl buffer (0.9 ml, pH 8.0) containing 0.27 mmol of sarcosine was incubated at 37 °C for 10 min. The reaction was terminated by adding 1.0 N acetic acid (0.25 ml). The whole was further incubated with 20% ammonium acetate (1.5 ml) containing 0.04% acetylacetone at 37 °C for 40 min and the absorbance at 410 nm was read against the no-enzyme blank.

Assay Method II: Hydrogen peroxide was used for the enzymatic condensation of phenol and 4-aminoantipyrine by horseradish peroxidase, forming a quinone imine dye with an absorption maximum at 480 nm. An enzyme solution (0.05 ml) was incubated with a mixture of 0.1 M Tris-HCl buffer (0.6 ml, pH 8.0) containing 0.27 mmol of sarcosine, 0.03 M 4-aminoantipyrine (0.25 ml), 1.5%phenol (0.05 ml) and 150 units/ml horseradish peroxidase (0.05 ml) at 37 °C for 10 min. The reaction was terminated by adding ethanol (1 ml) and the absorbance at 480 nm was read against the blank.

In both methods, one unit was defined as the amount of the enzyme which catalyzes the oxidation of 1 μmol of substrate per min under the conditions described above. Assay method II is less time consuming than assay method I. The former could not be employed when the direct effect on sarcosine oxidase was to be examined, however, because the system consisted of two enzymes.

Protein Determination——Protein concentration was measured according to the method of Lowry *et al.*[11] using bovine serum albumin as a standard.

Electrophoresis——Electrophoresis was conducted in 10% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS) according to the method of Weber and Osborn.[12] Bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), α-chymotrypsinogen (M_r 25700) and lysozyme (M_r 14300) were used as standards to determine the molecular weight of the enzyme. Polyacrylamide gel electrophoresis was performed according to the method of Davis.[13] Specific staining of sarcosine oxidase in the gel was carried out by the formation of diformazan as follows. After electrophoresis, the gel was soaked in 0.1 M Tris-HCl buffer, pH 8.0 (7.5 ml), containing 5 mg of nicotinamide adenine dinucleotide, 2.8 mg of nitrotetrazolium blue, 0.15 mg of phenazine methosulfate, 15 units of formaldehyde dehydrogenase and 1.5 mmol of substrate at 37 °C for 1 h in the dark.

Preparation of Sarcosyl-AH-Sepharose——A mixture of H_2O-swollen AH-Sepharose 4B (16 ml packed volume), which had been successively washed with 0.5 M NaCl (800 ml) and distilled H_2O (150 ml, pH adjusted to 4.5 with diluted HCl), 100 mM aqueous sarcosine solution (32 ml, pH 4.5) and 1.6 M aqueous 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride solution (2.0 ml) was kept at room temperature for 19 h with gentle shaking. The resulting gel was washed with 0.5 M NaCl, 0.1 M $NaHCO_3$ containing 0.5 M NaCl, distilled H_2O, 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl and distilled H_2O in that order and stored at 4 °C until use.

Taxonomic Studies——Taxonomic studies were conducted in accordance with the methods described by Shirling and Gottlieb,[14] and Waksman.[15] Color names were determined according to the Color Harmony Manual published by Container Corporation of America.[16] The utilization of carbon sources was tested by growth at 27 °C on Pridham and Gottlieb's medium containing 1% carbon source.[14] The analysis of 2,4-diaminopimelic acid in the whole cell hydrolysate was carried out by the method of Becker *et al.*[17]

Results

Taxonomic Studies

Strain KB210-8SY was isolated from a soil sample collected in Wakayama Prefecture in 1978 and was maintained on agar slants containing 1% starch and 0.2% yeast extract by transfer every 6 months.

The cultural and physiological characteristics of strain KB210-8SY are shown in Tables I and II, respectively. Strain KB210-8SY developed pale yellowish brown or yellowish white vegetative growth and aerial mycelium colored white to light brownish gray (gray color-series of Tresner & Backus[18]). Melanoid or other pigments were not detected on any media tested. D-Glucose, D-xylose, D-raffinose and D-mannitol were used for growth, but L-arabinose, *i*-inositol or L-rhamnose could not support the growth of this strain and the utilization of sucrose was doubtful (Table II).

Mature spore chains are moderately long with 10 to 50 spores per chain. Spore chain morphology was mainly flexuous (Fig.1) and was classified in the section Rectus-Flexibilis (RF).[14] The spores were ellipsoidal or oval in shape with a smooth surface and measured 0.56—0.72 × 0.58—0.80 μm in size (Fig.2).

LL-Diaminopimelic acid was detected in the whole cell hydrolysate. Fragmentation in vegetative mycelium, sporangia or flagellated spores were not observed on any of the media tested. Based on these taxonomic observations, strain KB210-8SY was considered to belong to genus *Streptomyces*.

By consulting Bergey's Manual of Determinative Bacteriology[19] and ISP reports by Shirling and Gottlieb,[20] *Streptomyces flavovirens* and *S. misakiensis* appeared to be the most similar species to strain KB210-8SY. However, *S. flavovirens* bears long spore chains (often with more than 50 spores per chain) on yeast–malt extract agar, oatmeal agar, inorganic

TABLE I. Cultural Characteristics of Strain KB210-8SY

Agar medium	Amount of growth Degree of sporulation	Spore mass color	Color of reverse substrate mycelium	Soluble pigment
Yeast extract–malt extract (ISP-2)	Good growth; abundant sporulation	Mostly white (a); with patches of light brownish gray (3fe)	Pale yellowish brown	None
Oatmeal (ISP-3)	Moderate growth; poor sporulation	Light brownish gray (3dc); with edges of light brownish gray (3fe)	Pale yellowish brown	None
Inorganic salts–starch (ISP-4)	Good growth; good sporulation	Light brownish gray (3dc); with edges and tufts of light brownish gray (3fe)	Pale yellowish brown to yellowish brown	None
Glycerol–asparagine (ISP-5)	Good growth; good sporulation	Mostly light brownish gray (3fe); white (a) to pale-orange yellow (3ba) at center	Grayish yellow to pale yellowish brown	None
Peptone–yeast extract–iron (ISP-6)	Poor growth; moderate sporulation	Pale orange-yellow (3ba)	Colorless	None
Tyrosine (ISP-7)	Good growth; good sporulation	Light brownish gray (3fe); with edges of pale orange-yellow (3ba)	Yellowish brown	None
Sucrose–nitrate (Waksman-1)	Poor growth; poor sporulation	Hard to describe	Colorless	None
Glucose–asparagine (Waksman-2)	Moderate growth; moderate sporulation	Light brownish gray (3fe); with tufts of white (a)	Yellowish white	None
Nutrient (Waksman-8)	Good growth; poor sporulation	Hard to describe	Yellowish white	None

TABLE II. Physiological Properties of Strain KB210-8SY

Melanin formation	
Tyrosine agar (ISP-6)	Negative
Peptone–yeast extract agar (ISP-7)	Negative
Tryptone–yeast extract broth (ISP-1)	Negative
Gelatin liquefaction	Negative
Starch hydrolysis	Positive
Action on skimmed milk	
Coagulation	Negative
Peptonization	Positive
Temperature range for growth	20—43 °C
Carbon utilization	
L-Arabinose	Negative
D-Xylose	Positive
D-Glucose	Positive
Sucrose	Doubtful
D-Fructose	Negative
l-Inositol	Negative
L-Rhamnose	Negative
Raffinose	Positive
D-Mannitol	Positive

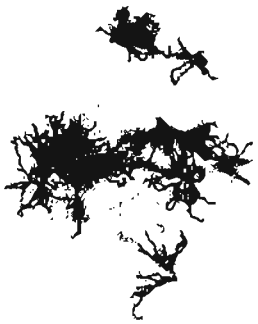

Fig. 1. Optical Micrograph of 10-Day Old Culture of Strain KB210-8SY Grown on Inorganic Salts–Starch Agar (ISP-4)

×100.

Fig. 2. Scanning Electron Micrograph of 10-Day Old Culture of Strain KB210-8SY Grown on Inorganic Salts–Starch Agar (ISP-4)

The bar represents 1 μm.

salts–starch agar and glycerol–asparagine agar and the type culture strain of this species has been reported to grow on L-arabinose and L-rhamnose, which could not support the growth of the strain KB210-8SY. As for *S. misakiensis*, red pigment is observed in the medium in yeast–malt agar, oatmeal agar and salts–starch agar. D-Xylose and D-mannitol can not support the growth of the type culture strain of this species, whereas these carbohydrates were used for growth of strain KB210-8SY. Unlike strain KB210-8SY, *S. misakiensis* can grow on sucrose.

Purification of Sarcosine Oxidase

All procedures were carried out at 4 °C unless otherwise specified. The enzyme activity was measured by assay method I. The cultured broth was centrifuged at 2500 rpm for 20 min to remove the mycelial cake. The supernatant was 70% saturated with ammonium sulfate and allowed to stand overnight. The precipitate obtained by centrifugation at 6500 prm for 15 min was dissolved in and dialyzed against 0.01 M Tris–HCl buffer (pH 8.0). The dialysate (55 ml, 60 units or 171 mg as protein) was charged on a column of DEAE–Sepharose CL-6B (2.5 × 12 cm) equilibrated with 0.01 M Tris–HCl buffer (pH 8.0). After the column had been washed with a sufficient amount of 0.01 M Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl, the enzyme was eluted with a linear gradient of NaCl (0.1—0.5 M) in the same buffer. The active fractions were combined, dialyzed against 0.01 M Tris–HCl buffer (pH 8.0) and charged on a column (1.6 × 5.4 cm) of DEAE–Sepharose CL-6B, from which the enzyme was eluted with a linear gradient of NaCl (0.05—0.4M). The active fractions from the second DEAE–Sepharose CL-6B column chromatography were combined and dialyzed against 0.01 M Tris–HCl buffer (pH 8.0). The crude enzyme was further subjected to affinity chromatography on sarcosyl-AH-Sepharose 4B (Fig. 1). The active fractions were combined, dialyzed against 1 mM Tris–HCl buffer (pH 8.0) overnight, lyophilized and redissolved in 4.0 ml of 0.01 M phosphate buffer (pH 8.0). The partially purifed sample was charged on a column of Sephadex G-150 Superfine (Fig. 2). The active fractions were combined and stored in a frozen state until use.

A summary of purification is given in Table III.

Estimation of Molecular Weight

The most highly purified sample gave a single band on SDS–polyacrylamide gel electrophoresis (Fig. 5). The molecular weight of the enzyme was estimated to be 44000 by both SDS–polyacrylamide gel electrophoresis and Sephadex G-150 gel filtration.

Effect of pH on Enzyme Activity and Stability

The effect of pH on the enzyme activity and stability was studied by assay method I. The optimum activity was observed at pH 8.0 (Fig. 6), and the enzyme proved to be stable in the pH range of 7—9 (Fig. 7).

Effect of Various Chemicals and Metal Salts

The effects of chemicals and metal salts on the enzyme activity were tested by assay

TABLE III. Purification of Sarcosine Oxidase

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Ammonium sulfate ppt.	171	60	0.35	100
DEAE–Sepharose, 1st	18	48	2.7	80
DEAE–Sepharose, 2nd	8.1	44	5.4	73
Sarcosyl-AH-Sepharose	1.1	24	22	40
Sephadex G-150	0.76	19	25	32

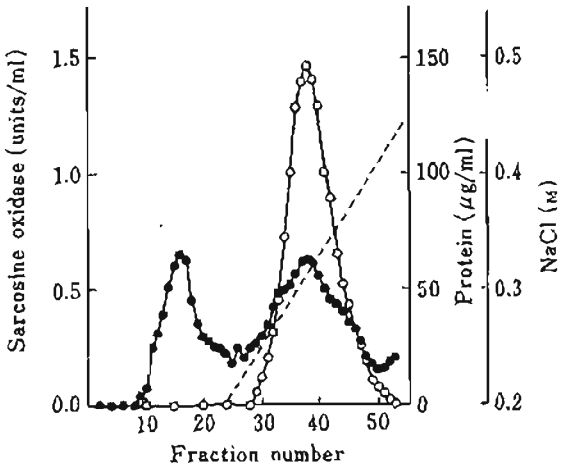

Fig. 3. Chromatography of Sarcosine Oxidase on Sarcosyl-AH-Sepharose

The crude enzyme obtained by DEAE-Sepharose chromatography (44 units, 8.1 mg as protein) was charged on a sarcosyl-AH-Sepharose column (1.2×2.4 cm) equilibrated with 0.01 M Tris-HCl buffer (pH 8.0). The column was washed with a sufficient amount of the same buffer, then the enzyme was eluted with a linear gradient of NaCl (0.2–0.5 M) in the starting buffer. The eluate was collected in 4.0 ml fractions and the enzyme activity was measured by assay method 1.

○, sarcosine oxidase activity; ●, protein; ------, NaCl.

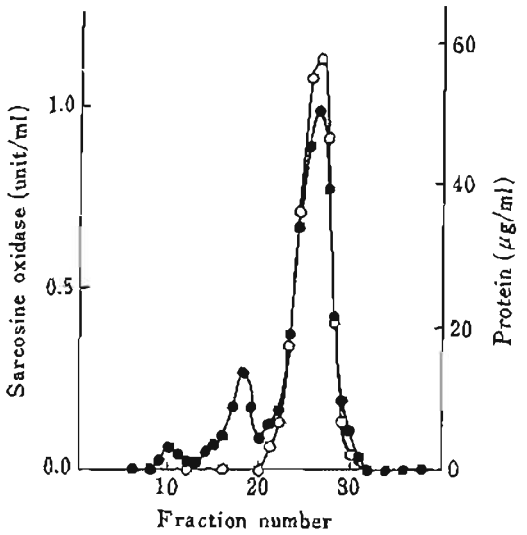

Fig. 4. Chromatography of Sarcosine Oxidase on Sephadex G-150

The partially purified enzyme from sarcosyl-AH-Sepharose chromatography (24 units, 1.1 mg as protein) was charged on a Sephadex G-150 Superfine column (1.9×84 cm). Ten mM phosphate buffer (pH 8.0) was used for elution of the enzyme and the eluate was collected in 4.0 ml fractions.

○, sarcosine oxidase activity; ●, protein.

Fig. 5. SDS-Polyacrylamide Gel Electrophorogram of Purified Sarcosine Oxidase

The purified enzyme (15 μg as protein) was subjected to electrophoresis at a constant current of 10 mA per gel for 4 h, and then the gel was stained with 0.25% Coomassie Brilliant Blue R-250.

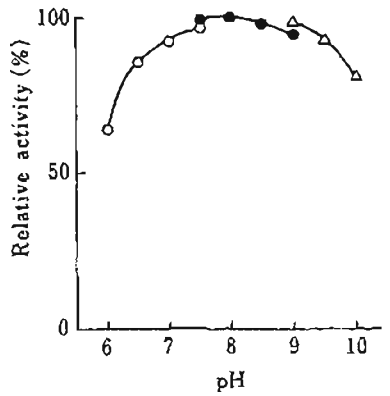

Fig. 6. Effect of pH on Sarcosine Oxidase Activity

The sarcosine oxidase activity was measured by assay method I, in which aqueous enzyme solution (0.03 units/0.1 ml) was incubated with either 0.1 M phosphate buffer (○, pH 6.0—7.5), 0.1 M Tris-HCl buffer (●, pH 7.5—9.0) or 0.1 M carbonate buffer (△, pH 9.0—10.0). The enzyme activity under the standard conditions was defined as 100%.

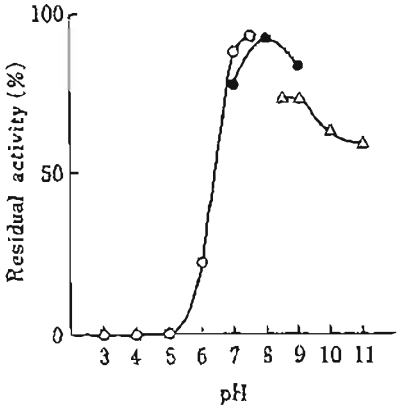

Fig. 7. Effect of pH on the Stability of Sarcosine Oxidase

A mixture of enzyme solution (0.1 unit/0.1 ml) and 0.1 M buffer solution (0.1 ml) was allowed to stand at room temperature for 1 h. The pH was adjusted to 8.0 with 1.0 M Tris or 1.0 N HCl and the volume to 1.0 ml with distilled H_2O, then the residual activity was measured by assay method I.

○, citrate buffer (pH 3.0—7.5); ●, Tris-HCl buffer (pH 7.0—9.0); △, carbonate buffer (pH 8.5—11.0).

TABLE IV. Effect of Various Chemicals on Sarcosine Oxidase Activity

Chemical	Residual activity (%)	
	1 mM	10 mM
None	100	100
Ethylenediaminetetraacetate	100	98
8-Hydroxyquinoline	90	67
o-Phenanthroline hydrochloride	98	83
α,α′-Dipyridyl	98	102
p-Chloromercuribenzoate	95	NT
Monoiodoacetate	99	99
Glutathione (oxidized)	102	96
5,5′-Dithio-bis(2-nitrobenzoate)	87	85
N-Ethylmaleimide	98	83
p-Toluenesulfonyl chloride	99	102
Diisopropyl fluorophosphate	118	122
Phenylmethanesulfonyl fluoride	100	80
N-Bromosuccinimide	0	NT
Glutathione (reduced)	98	76
Dithiothreitol	94	62
2-Mercaptoethanol	95	71
Hydroxylamine hydrochloride	50	9
Sodium dodecyl sulfate	26	0

A mixture of 0.2 ml of enzyme solution (0.3 unit/ml) in 0.1 M Tris–HCl buffer (pH 8.0) and 0.2 ml of chemical solution (2 or 20 mM) in the same buffer was incubated at 37 °C for 30 min and the residual activity was measured by assay method I. NT: not tested.

TABLE V. Effect of Various Metal Ions on Sarcosine Oxidase Activity

Metal salt (1 mM)	Residual activity (%)	Metal salt (1 mM)	Residual activity (%)
None	100	$FeCl_3$	62
$ZnSO_4$	11	$FeSO_4$	95
$MnCl_2$	89	$CuSO_4$	88
$CoCl_2$	67	$CaCl_2$	100
$NiCl_2$	46	$HgCl_2$	1

A mixture of 0.2 ml of enzyme solution (0.3 unit/ml) in 0.1 M Tris–HCl buffer (pH 8.0) and 0.2 ml of metal salt solution (2 mM) in the same buffer was incubated at 37 °C for 30 min and the residual activity was measured by assay method I.

method I. As shown in Table IV, the enzyme was markedly inactivated by *N*-bromosuccinimide, SDS and hydroxylamine hydrochloride, but not by ethylenediaminetetraacetate, monoiodoacetate, *p*-chloromercuribenzoate (PCMB) or *p*-toluenesulfonyl chloride. The enzyme was adversely affected by Zn^{2+} and Hg^{2+}, but the other metal ions had relatively little or no effect (Table V).

Substrate Specificity and Kinetics

The relative rate of oxidation of various compounds was measured by assay method II. Besides sarcosine, *N*-methyl-L-leucine, *N*-methyl-DL-alanine and *N*-methyl-DL-valine were preferred as substrates of the enzyme, though α-amino acids, β-alanine, *N*-methylethanolamine, *N*,*N*-dimethylalanine, betaine, *N*-methylhydantoin, 2-methylalanine, 1,3-dimethylurea, 1-methylguanidine, methoxyacetate, creatine and creatinine were not hydro-

TABLE VI. Kinetic Parameters of Sarcosine Oxidases

Substrate	*Streptomyces*[a]		*Bacillus*[b]	
	K_m (mM)	Relative V_{max} (%)	K_m (mM)	Relative V_{max} (%)
Sarcosine	0.91	100	11	100
N-Methyl-L-leucine	0.58	54	60	97
N-Methyl-DL-alanine	1.6	57	6.2	37
N-Methyl-DL-valine	6.7	41	170	172

a) The K_m and V_{max} values were calculated by assay method I, using a reaction mixture composed of 0.9 ml of substrate solution (0.3—8.0 mM) in 0.1 M Tris–HCl buffer (pH 8.0) and 0.1 ml of aqueous enzyme solution (0.21, 0.21, 0.29 and 0.29 unit/ml, respectively, on going from sarcosine to *N*-methyl-DL-valine). *b*) A reaction mixture consisted of 0.9 ml of substrate solution (10—300 mM) in 0.1 M Tris–HCl buffer (pH 8.0) and 0.1 ml of aqueous enzyme solution (0.5, 1.5, 0.2 and 1.5 units/ml, respectively, on going from sarcosine to *N*-methyl-DL-valine).

lyzed by the enzyme. The kinetic parameters for *N*-methylamino acids were obtained from Lineweaver–Burk plots,[21] and the results are shown in Table VI along with those obtained for sarcosine oxidase of *Bacillus* origin.

Specific Staining of Sarcosine Oxidase on Polyacrylamide Gel After Electrophoresis

Electrophoresis was conducted in 7% polyacrylamide gel according to the method of Davis.[13] The purified enzymes (50 μg as protein) of *Streptomyces* and *Bacillus* origins were charged on the gel columns in combination and individually. After electrophoresis, specific staining of sarcosine oxidase was carried out according to the method described in the Materials and Methods section. Two diformazan-stained bands were observed in the gel loaded with the two enzymes. Further, the mobilities of the diformazan bands were identical to those of the bands stained with coomassie brilliant blue R-250.

Discussion

Sarcosine oxidase has been reported in genera *Corynebacterium*,[7] *Cylindrocarpon*[8] and *Bacillus*.[9] Although the former two enzymes were inactivated by PCMB and monoiodoacetate, the last one was not inactivated by these chemicals, as was also the case for the *Streptomyces* enzyme. Further, the molecular weights of sarcosine oxidases from *Bacillus* and *Streptomyces* were very similar; 42000 and 44000, respectively. However, these enzymes differed in electrophoretic behavior, substrate specificities and kinetic parameters.

The K_m values of the *Bacillus* and *Streptomyces* enzymes for sarcosine were 11 and 0.9 mM, respectively. Both enzymes were charged on a column of sarcosyl-AH-Sepharose. Contrary to expectation (affinity chromatography), this adsorbent works as a strong anion exchanger due to the basicity of the terminal methylamino group, which also seems to be essential for the specific interaction with the enzyme. Sarcosyl-AH-Sepharose did partially purify the *Streptomyces* enzyme. As for the *Bacillus* enzyme, however, its affinity for the adsorbent is much lower than that of the *Streptomyces* enzyme, resulting in elution at lower NaCl concentrations (0.15—0.2 M), within the elution range of impurities (0—0.25 M). In contrast, the relative mobility of the *Streptomyces* enzyme with respect to the *Bacillus* enzyme on polyacrylamide gel electrophoresis was 0.8, showing the more anionic nature of the latter. Therefore, the results in the affinity chromatography are partly attributable to the difference in the K_m values of the enzymes.

The use of sarcosine oxidase of *Streptomyces* origin as a diagnostic reagent for the determination of creatinine and/or creatine in clinical samples in combination with other creatinine catabolic enzymes is now under investigation in our laboratory.

References

1) O. Folin, *Z. Physiol. Chem.*, **41**, 223 (1904).
2) M. Jaffe, *Z. Physiol. Chem.*, **10**, 399 (1886).
3) I. Oka, S. Watanabe, H. Ishida and S. Asano, *Rinsho Kagaku*, **8**, 81 (1979).
4) T. Kinoshita and T. Hiraga, *Chem. Pharm. Bull.*, **28**, 3501 (1980).
5) M. Yasuhara, S. Fujita, K. Arisue, K. Kohda and C. Hayashi, *Clin. Chim. Acta*, **122**, 181 (1982).
6) M. Suzuki and M. Yoshida, *Clin. Chim. Acta*, **140**, 289 (1984).
7) M. Suzuki, *J. Biochem.* (Tokyo), **89**, 599 (1981).
8) N. Mori, M. Sano, Y. Tani and H. Yamada, *Agric. Biol. Chem.*, **44**, 1391 (1980).
9) Y. Matsuda, H. Hoshika, Y. Inouye, S. Ikuta, K. Matsuura and S. Nakamura, *Chem. Pharm. Bull.*, **35**, 711 (1987).
10) T. Nash, *Biochem. J.*, **55**, 416 (1953).
11) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
12) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
13) B. J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, art., 2, 404 (1964).
14) E. B. Shirling and D. Gottlieb, *Intern. J. Syst. Bacteriol.*, **16**, 313 (1966).
15) S. A. Waksman, "The Actinomycetes," Vol. 2, The Williams and Wilkins Co., Baltimore, 1961.
16) "Color Harmony Manual," 4th ed., Color Standards Department, Container Corporation of America, Chicago, 1958.
17) B. Becker, M. P. Lechevalier, R. E. Gordon and H. A. Lechevalier, *Appl. Microbiol.*, **12**, 421 (1964).
18) H. D. Tresner and E. J. Backus, *Appl. Microbiol.*, **11**, 335 (1963).
19) T. G. Pridham and H. D. Tresner, "Bergey's Manual of Determinative Bacteriology," 8th ed., The Williams and Wilkins Co., Baltimore, 1974, pp. 748—829.
20) E. B. Shirling and D. Gottlieb, *Intern. J. Syst. Bacteriol.*, **18**, 69 (1968); *idem*, *ibid.*, **18**, 279 (1968); *idem*, *ibid.*, **19**, 391 (1969); *idem*, *ibid.*, **22**, 265 (1972).
21) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

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Reactivities of Various Amines in the Modifications of Acetic Acid and Aspartic Acid-101 of Lysozyme in the Carbodiimide Reaction

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Reactivities of various amines in the modifications of acetic acid and Asp-101 of hen egg-white lysozyme in the carbodiimide (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC)) reaction were investigated at pH 5.0 and room temperature. The reactivity of an amine towards EDC-activated Asp-101 was at least 50 times higher than that towards EDC-activated acetic acid. Such a high efficiency of modification of Asp-101 could not be explained only by the EDC-binding mechanism in which the EDC molecule binds to the active site cleft of lysozyme close to Asp-101 to activate Asp-101 selectively [R. Kuroki, H. Yamada, and T. Imoto, *J. Biochem.* (Tokyo), **99**, 1493 (1986)]. Therefore, in addition to the above mechanism, an amine-binding mechanism in which an amine molecule binds to lysozyme close to the EDC-activated Asp-101 residue so as to increase the effective concentration of the amine by more than 50 times is proposed.

Keywords——lysozyme; 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; carboxylic acid amine modification; amine reactivity

Introduction

1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), a water-soluble carbodiimide, has been widely used for the modification of carboxyl groups of proteins with amine nucleophiles.[1,2] Previously, we have reported the selective modification of Asp-101 in hen egg-white lysozyme with various amine nucleophiles by EDC reaction.[3,4] Asp-101 is located at the edge of the active site cleft of lysozyme and is involved in substrate binding.[5] We have shown that the EDC molecule binds to the active site cleft of lysozyme and activates Asp-101 specifically to lead the selective modification of this residue with an amine nucleophile.[6] We are interested in the alteration of lysozyme function by introduction of a new catalytic function into Asp-101. In order to establish whether any kind of amines can be used for this purpose or not, we examined the reactivities of various amines towards EDC-activated Asp-101 of lysozyme, and compared them with those against EDC-activated acetic acid.

Experimental

Materials——Five-times-recrystallized hen egg-white lysozyme was donated by QP Company (Japan). EDC was purchased from the Protein Research Foundation (Osaka, Japan). *N*-Ethylacetamide, *N*-(2-hydroxyethyl)acetamide, *N,N*-dimethylacetamide, *N,N*-diethylacetamide, ethylamine, 2-hydroxyethylamine, dimethylamine, diethylamine, and bis(2-hydroxyethyl)amine were obtained from Nakarai Chemicals Ltd. (Kyoto, Japan). *N,N*-Bis(2-hydroxyethyl)acetamide was prepared as follows. *N,N*-Bis(2-acetoxyethyl)acetamide (3 g, 13 mmol), synthesized by the method of Mann,[7] was dissolved in 26 ml of 1 N NaOH and stirred overnight at room temperature. The reaction mixture was applied to the column of Dowex 50W-X2 (H^+ form, 2 × 40 cm) and the column was eluted with water. The iodine-positive fraction was concentrated, and dried under vacuum, to give 1.06 g (52.5%) of *N,N*-bis(2-hydroxyethyl)acetamide as a colorless liquid. ^{1}H-NMR (D_2O): 2.27 (3H,s), 3.45—4.03 (8H,m). *Anal.* Calcd for $C_6H_{13}NO_3$: C, 48.97; H, 8.90; N, 9.52. Found: C, 48.22; H, 8.90; N, 9.25.

Simultaneous Reaction of Various Amines with EDC-Activated Acetic Acid under Competitive Conditions——Five kinds of amines (ethylamine, 2-hydroxyethylamine, dimethylamine, diethylamine, and bis(2-hydroxyethyl)amine; the final concentration of each amine is listed in Table I) and acetic acid (final concentration 1 M) were dissolved in water and the pH of the solution was adjusted to 5.0 with HCl. The volume of the solution was adjusted to 10 ml with water, then 30 mg of EDC (0.156 mmol or 15.6 mM) was added. The mixture was stirred at room temperature (22 °C) for 1 d and then the products were analyzed by high-performance liquid chromatography (HPLC) on a reversed-phase column.

Coupling of Lysozyme with Amine by EDC-Reaction——Lysozyme (20 mg; final concentration, 0.14 mM) and an amine (0.1 M) were dissolved in water and the pH of the solution was adjusted to 5.0 with HCl. The volume of the solution was adjusted to 10 ml, then 1.0 mg of EDC (5.2 μmol or 0.52 mM) was added with stirring at room temperature (22 °C). The pH of the solution was maintained at 5.0 for 2 h by addition of dilute HCl. Thereafter, the pH remained constant. After 1 d of stirring, the mixture was dialyzed against distilled water and then analyzed by cation-exchange HPLC.

Analytical Methods——HPLC was accomplished with a Hitachi 655 liquid chromatograph. Quantitative analyses of acetamide derivatives produced in the simultaneous reaction of various amines against EDC-activated acetic acid under competitive conditions were performed on a 0.4 × 30 cm-column of TSK gel ODS-120A (5 μm, Toyo Soda, Japan). An aliquot (20 μl) of the reaction mixture was injected and the column was eluted with a gradient formed from 40 ml of 0.1% concentrated HCl and 40 ml of 20% acetonitrile containing 0.1 % concentrated HCl at a flow rate of 0.8 ml/min. The elution of each acetamide was monitored by measuring the absorbance of the effluent at 210 nm. Yields of acetamide derivatives were determined from the peak areas, which were calibrated with authentic acetamides.

The yields of Asp-101 modified lysozyme with each amine was determined by ion-exchange HPLC on a 0.4 × 30 cm-column of TSK gel CM-2SW (5 μm, Toyo Soda), carboxylic cation-exchanger. Dialysate of the reaction mixture was injected, and the column was eluted with a gradient of 24 ml of 0.05 M phosphate buffer (pH 7) containing 0.1 M NaCl and 24 ml of the same buffer containing 0.76 M NaCl at a flow rate of 0.8 ml/min. The elution of protein was monitored by measuring the absorbance at 280 nm. Asp-101 modified lysozyme was eluted just after the native lysozyme peak in every case, as observed previously.[3] The yields of unreacted and Asp-101 modified lysozymes were calculated from the peak areas assuming that the molar extinction coefficient of lysozyme at 280 nm was not changed by the modification of Asp-101.

Results and Discussion

The reactivities of various amines [ethylamine, 2-hydroxyethylamine, dimethylamine, diethylamine and bis(2-hydroxyethyl)amine] towards two kinds of EDC-activated carboxylic acids, acetic acid and Asp-101 in lysozyme, were investigated at pH 5.0 and room temperature. The acetamide derivatives produced were all separated simultaneously by reversed-phase HPLC as shown in Fig. 1. Therefore, in the case of acetic acid, the reaction was carried out under competitive conditions. That is, acetic acid (1 M) was activated by 15.6 mM EDC and reacted with a mixture of various amines (total concentration of amines, 1 M) for 1 d. The concentration of each amine used is indicated in Table I. The reaction mixture was analyzed by reversed-phase HPLC and the yields of the respective derivatives were determined from the calibration lines of authentic samples. Two independent experiments gave almost the same results. The results are shown in Table I, where the pK_a value of each amine and its calculated relative reactivity are also shown. The yield of each acetamide derivative was extremely low. Under the conditions employed ([acetic acid] = 1 M, [EDC] = 15.6 mM, and pH 5), the direct decomposition of EDC with water could be neglected.[8] Therefore, all of the EDC (15.6 mM) was considered to have been converted to EDC-activated acetic acid. Nevertheless, the total yield of acetamide derivatives was only 23.5 μM, which corresponds to a yield of 0.15% on the basis of the amount of EDC used. These results indicated that the reactivities of amines towards EDC-activated acetic acid were extremely low compared with that of water, and consequently most of the EDC-activated acetic acid (99.85%) was decomposed by water.

As shown in Table I, the relative reactivities of the amines towards EDC-activated acetic acid were widely spread [from diethylamine (1) to dimethylamine (188)]. Difference in structure and /or pK_a of the amines may be responsible for the range of reactivities observed.

TABLE I. Relative Reactivities of Various Amines towards EDC-Activated Acetic Acid under Competitive Conditions at pH 5.0 and Room Temperature[a]

Amine (pK_a)	Concentration (M)	Yield of corresponding acetamide		Relative reactivity
		(μM)	(%)[b]	
Ethylamine (10.63[c])	0.10	3.2	0.020	20
2-Hydroxyethylamine (9.50[c])	0.10	10.8	0.069	69
Dimethylamine (10.64[c])	0.02	5.9	0.038	188
Diethylamine (10.98[c])	0.70	1.1	0.007	1
Bis(2-hydroxyethyl)amine (8.88[d])	0.08	2.5	0.016	20

a) 1 M acetic acid and 15.6 mM EDC. Details are given in the text. *b*) Based on EDC. *c*) H. K. Hall, Jr., *J. Am. Chem. Soc.*, 79, 5441 (1957). *d*) N. F. Hall and M. R. Sprinkle, *J. Am. Chem. Soc.*, 54, 3469 (1932).

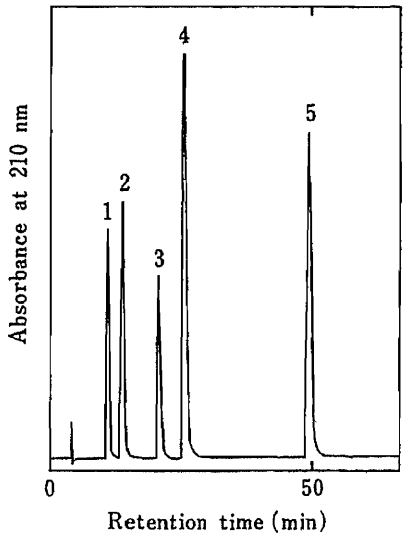

Fig. 1. Reversed-Phase HPLC of Acetamide Derivatives

A mixture of authentic acetamides was injected into a 4 × 300 mm column of TSK gel ODS-120A (5 μm; Toyo Soda, Japan). The column was eluted with a gradient formed from 40 ml of distilled water and 40 ml of 20% acetonitrile containing 0.1% concentrated HCl at a flow rate of 0.8 ml/min. 1, *N*-(2-Hydroxyethyl)acetamide; 2, *N*,*N*-bis(2-hydroxyethyl)-acetamide; 3, *N*-ethylacetamide; 4, *N*,*N*-dimethylacetamide; 5, *N*,*N*-diethylacetamide.

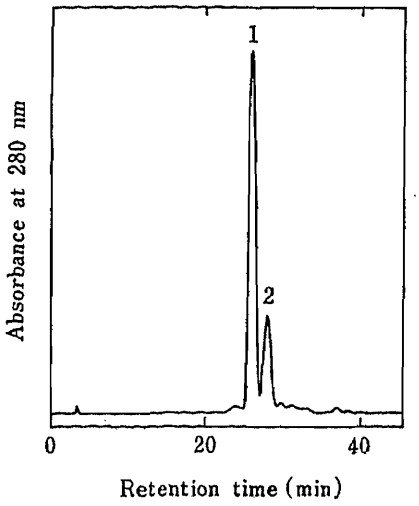

Fig. 2. Ion-Exchange HPLC of the Reaction Mixture of Lysozyme with Ethylamine Catalyzed by EDC on TSK Gel CM-2SW

The column (0.4 × 30 cm) was eluted with a gradient of 24 ml of 0.05 M phosphate buffer containing 0.1 M NaCl and 24 ml of the same buffer containing 0.76 M NaCl at a flow rate of 0.8 ml/min. 1, unreacted lysozyme; 2, Asp-101 modified lysozyme with ethylamine.

In the case of lysozyme, the reaction under competitive conditions could not be utilized because there was no effective method to separate the Asp-101 modified lysozymes from each other simultaneously. However, each Asp-101 modified lysozyme was well separated from unreacted native lysozyme by ion-exchange HPLC. Therefore, the coupling reaction of lysozyme (0.14 mM) with each amine (0.1 M) by EDC (0.52 mM) was carried out separately. The chromatographic pattern obtained from ethylamine is shown in Fig. 2 as a representative example. Asp-101 modified lysozyme, a main product, appeared clearly as a single peak and other by-products were not obvious. For all other amines, the situations were almost the same except for the yields of the respective Asp-101 modified lysozymes. These observations

TABLE II. Yield of Asp-101 Modified Lysozyme and Recovery of Unreacted Lysozyme in the Coupling Reaction of Lysozyme (0.14 mM) with Various Amines (0.1 M) by EDC (0.52 mM) at pH 5.0 and Room Temperature for 1 d

Amine	Recovery of unreacted lysozyme (%)[a]	Yield of Asp-101 modified lysozyme		
		(%)[a]	(%)[b]	Relative yield
Ethylamine	78	20	5.4	7
2-Hydroxyethylamine	62	38	10.2	13
Dimethylamine	61	36	9.7	12
Diethylamine	96	3	0.8	1
Bis(2-hydroxyethyl)amine	95	4	1.1	1.3

a) Based on lysozyme. *b*) Based on EDC.

indicated that among 10 carboxyls[9] in lysozyme, only Asp-101 was selectively modified with an amine in the carbodiimide reaction, as observed previously.[3] The yield of Asp-101 modified lysozyme based on lysozyme used was determined from the relative peak area of Asp-101 modified lysozyme to the total peak area. The results are summarized in Table II. In Table II, the relative yields of Asp-101 modified lysozymes for each amine are also shown. If the relative yields of the Asp-101 modified lysozymes were assumed to be the same as the relative reactivities of the respective amines towards EDC-activated Asp-101, the results indicated that the relative reactivities of amines against EDC-activated Asp-101 were not so widely spread [from diethylamine (1) to 2-hydroxyethylamine (13)] as those against EDC-activated acetic acid.

In order to compare the reactivity of an amine towards EDC-activated Asp-101 with that towards EDC-activated acetic acid, the yields of respective Asp-101 modified lysozymes based on EDC may be more convenient than those based on lysozyme. Therefore, they are also shown in Table II. As shown above, in the case of acetic acid, the total yield of acetamide derivatives was only 0.15% based on EDC at 1 M concentration of total amine. On the other hand, in the case of lysozyme, the yield of Asp-101 modified lysozyme was in the range of 0.8-10.2% based on EDC at 0.1 M concentration of an amine. Since water should compete with an amine in the reaction with EDC-activated carboxylic acid, these results indicated that the amines used in this study reacted with EDC-activated Asp-101 at least 50 times more effectively than with EDC-activated acetic acid. Since the concentration of acetic acid used (1 M) was more than 7000 times higher than that of lysozyme (0.14 mM), this value is considered to be an underestimate.

In order to explain such high reactivity of an amine towards EDC-activated Asp-101, we must consider some mechanism to enhance the effective concentration of an amine in the case of lysozyme. Thus, we propose that an amine molecule could bind to the active site cleft of EDC-activated lysozyme close to the activated Asp-101 residue. This mechanism would increase the effective concentration of amine and also explains the observations that the relative reactivities of amines against EDC-activated Asp-101 (Table II) were considerably different from those against EDC-activated acetic acid (Table 1). Namely, the yields of Asp-101 modified lysozymes would be affected by differences in the binding ability of amines to EDC-activated lysozyme and by differences in the orientation of the amines in the complex.

Previously,[6] we have shown that the EDC molecule binds to the saccharide binding site of the lysozyme molecule close to Asp-101 and selectively activates Asp-101 among 10 carboxyls[9] in lysozyme. Thus, the present results and the results reported previously[3,6] would indicate that the extremely high reactivity of Asp-101 of lysozyme in the modification using EDC and an amine nucleophile is not only due to the specific activation of Asp-101 with EDC

by the EDC-binding mechanism but also due to the effective attack of an amine molecule on EDC-activated Asp-101 through the amine-binding mechanism.

The modification of Asp-101 of lysozyme with amines in the EDC reaction was so effective that we need not use a large excess of EDC over lysozyme in this reaction. This may be a great advantage in the modification of Asp-101 of lysozyme, because many side reactions have been reported on using an excess of EDC.[2] Therefore, this reaction would be very useful when the conversion of lysozyme to some new functional enzyme is attempted by the introduction of an amine with substituent(s) showing a new catalytic ability into Asp-101. As there seems to be a tendency that primary amines are more reactive than secondary amines (Table II), primary amines possessing functional substituents would be more suitable for this purpose.

References

1) D. G. Hoare and D. E. Koshland, Jr., *J. Am. Chem. Soc.*, **88**, 2057 (1966).
2) K. L. Carraway and D. E. Koshland, Jr., "Methods in Enzymology," Vol. 25B, ed. by C. H. W. Hirs and S. N. Timasheff, Academic Press, New York, 1972, pp. 616—623.
3) H. Yamada, T. Imoto, K. Fujita, K. Okazaki, and M. Motomura, *Biochemistry*, **20**, 4836 (1981).
4) K. Okazaki, T. Imoto, H. Yamada, R. Kuroki, and K. Fujita, *J. Biol. Chem.*, **257**, 12559 (1982).
5) C. C. F. Blake, L. N. Johnson, G. A. Mair, A. T. C. North, D. C. Phillips, and V. R. Sarma, *Proc. R. Soc. London, Ser. B*, **167**, 378 (1967).
6) R. Kuroki, H. Yamada, and T. Imoto, *J. Biochem.* (Tokyo), **99**, 1493 (1986).
7) F. G. Mann, *J. Chem. Soc.*, 461 (1934).
8) I. T. Ibrahim and A. Williams, *J. Am. Chem. Soc.*, **100**, 7420 (1978).
9) T. Imoto, K. Okazaki, H. Yamada, K. Fujita, T. Yamato, and D. Koga, *J. Biochem.* (Tokyo), **90**, 991 (1981).

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Effect of Ginsenoside-Rb_2 on Nitrogen Compounds in Streptozotocin-Diabetic Rats

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The effects of ginsenoside-Rb_2 on nitrogen compounds in streptozotocin-diabetic rats were investigated. A marked decrease in blood urea nitrogen level was observed in the ginsenoside-Rb_2 administered group. Administration of ginsenoside-Rb_2 induced increases of total protein and Lys, Gly, Glu, Arg, *etc.* in serum, while no significant change of serum albumin was observed. In the liver, the urea content was decreased with a concomitant increase of ribonucleic acid content. A quantitative study on the distribution of free and membrane-bound ribosomes indicated that the increase in ribosomes caused by the administration of ginsenoside-Rb_2 is mainly due to the increase in membrane-bound ribosomes. Ginsenoside-Rb_2 exhibited a normalizing action on the hepatic concentrations of Glu, Thy, Phe, and Tyr. Furthermore, body weight significantly increased in diabetic rats receiving ginsenoside-Rb_2, even though they took less food than the control group. Ginsenoside-Rb_2 remarkably improved diabetic symptoms such as over-eating, polyuria, *etc.*

Keywords——ginsenoside-Rb_2; streptozotocin-induced diabetic rat; urea; protein; RNA; membrane-bound ribosome; amino acid

In our previous paper, we showed that among the various kinds of ginsenosides isolated from ginseng, ginsenoside-Rb_2 exhibited a wide variety of biochemical activities in normal rats, accelerated glycogenolysis, glycolysis, and lipogenesis in the liver, and increased the content of triglyceride in the adipose tissue.[1,2] Moreover, we reported that ginsenoside-Rb_2 treatment of streptozotocin-induced diabetic rats produced a significant decrease in blood glucose level and a marked improvement of hyperlipemia.[3-5] In addition, the mechanism of decrease in blood glucose level was examined by successive intraperitoneal administrations of ginseng to rats.[4] These experiments prompted us to examine whether or not ginsenoside-Rb_2 is a useful agent for the treatment of diabetes. In the present paper, further studies were carried out to investigate its effect on nitrogen compounds and to clarify further the action mechanism of ginsenoside-Rb_2 in streptozotocin-induced diabetic rats.

Materials and Methods

Animals——Male rats of the JCL: Wistar strain (Hokuriku Labour, Ltd., Toyama, Japan), initially weighing 110—120 g, were maintained in an air-conditioned room with a 12-h dark-light cycle. The room temperature (22 ± 2 °C) and humidity (about 60%) were controlled automatically. Throughout the experimental period, a laboratory pellet chow (purchased from CLEA Japan Inc., Tokyo; CE-2) and water were given freely.

Streptozotocin-Induced Diabetic Rats[6]——Diabetes was induced by intraperitoneal injection of streptozotocin (65 mg/kg body weight) dissolved in 10 mM citrate buffer (pH 4.5). Several days after the injection, the blood glucose level was determined and rats with a glucose level between 670 to 770 mg/dl were used.

Saponin——Ginsenoside-Rb_2 was isolated and purified from the extract of roots of *Panax ginseng* C.A. MEYER according to the procedure of Shibata and co-workers.[7] This preparation was found to be pure by means of various

chemical and physicochemical analyses.

Treatment with Ginsenoside-Rb_2——Ginsenoside-Rb_2 (10 mg/rat/d) dissolved in saline was administered intraperitoneally to rats at 9—10 a.m. for 6 d, while control rats were treated with an equal volume of saline. At 8 h after the last treatment, rats were sacrificed by means of a sharp blow on the head and exsanguinated. The blood was collected in a conical centrifuge tube for the determination of glucose, urea nitrogen, total protein, albumin, and free amino acids. The liver was removed quickly, cooled on ice, weighed rapidly and used for the determination of urea, protein, ribonucleic acid (RNA), deoxyribonucleic acid (DNA), ribosomes, and free amino acids.

Analytical Methods

Serum Constituents——Glucose was determined by using a commercial reagent (Glucose B-Test Wako obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan) based on the glucose-oxidase method.[8] Urea nitrogen was determined by using a commercial reagent (BUN KAINOS obtained from Kainos Laboratories, Inc., Tokyo, Japan) based on the urease–indophenol method.[9] Total protein and albumin were determined using a commercial reagent (A/G B-Test Wako) based on the biuret method[10] and BCG method,[11] respectively. Free amino acids were determined with a Hitachi 835 high-speed amino acid analyzer. Before this determination, the serum was deproteinized by adding 3 volumes of 2% sulfosalicylic acid.

Protein and Urea in the Liver——A portion of the liver was homogenized with 9 volumes of ice-cold distilled water in a Potter-Elvehjem-type glass homogenizer. The homogenate obtained was used for the estimation of protein by the biuret method.[10] A part of the homogenate was diluted with ice-cold distilled water and deproteinized by the addition of 0.15 M $Ba(OH)_2$ and 5% $ZnSO_4$. After centrifugation at 3000 rpm for 15 min, the supernatant obtained was determined by the procedure of Archibald[12] for assay of urea.

RNA and DNA in the Liver——A portion of the liver was homogenized with 0.5 volumes of 2.1 N perchloric acid (PCA) solution. After centrifugation at 29000 rpm for 60 min, the precipitate obtained was washed twice with 0.7 N PCA solution. The residue was mixed with 0.3 N KOH and incubated at 37 °C for 60 min. The sample was neutralized with 10 N PCA solution, and then acidified by the addition of an equal volume of 1 N PCA solution. After centrifugation at 3000 rpm for 20 min, the precipitate was washed twice with 0.5 N PCA solution. These combined supernatants were used for RNA determination by the method of Fleck and Munro.[13] DNA in the precipitate was determined by the method of Ceriotti.[14,15]

Free and Membrane-Bound Ribosomes in the Liver——The ribosomal fractions were isolated by a modification of the method of Redman,[16] as described previously.[17] Ribosomal RNA prepared was determined by the method of Fleck and Munro.[13]

Free Amino Acids in the Liver——The 10% homogenate of liver was deproteinized by the addition of 3% sulfosalicylic acid. The supernatant obtained by centrifugation at 12000 rpm for 20 min was analyzed with a Hitachi 835 high-speed amino acid analyzer.

Statistics——The significance of differences between the non-diabetic and diabetic rats (control or ginsenoside-Rb_2-treated group) was tested by means of Student's *t*-test.

Results

Diabetic Symptoms

Compared to non-diabetic rats, streptozotocin-induced diabetic rats exhibited a marked

TABLE I. Effect of Ginsenoside-Rb_2 on Urea Nitrogen, Total Protein, and Albumin Levels in the Serum

	Urea nitrogen (mg/dl)	T. protein (g/dl)	Albumin (g/dl)
Non-diabetic rat	13.5±0.6 (100)	4.85±0.04 (100)	3.65±0.01 (100)
Diabetic rat			
Control	37.2±1.7 (276)[c] [100]	4.45±0.09 (92)[b] [100]	3.35±0.04 (92)[c] [100]
Rb_2	21.3±1.0 (158)[c] [57]**	4.68±0.06 (96)[a] [105]*	3.43±0.05 (94)[b] [102]

Values are means±S.E. of 7 rats. Figures in parentheses are percentages of the non-diabetic or diabetic control value. *a*), *; Significantly different from the non-diabetic or diabetic control value, $p<0.05$, *b*) $p<0.01$, *c*), **; $p<0.001$.

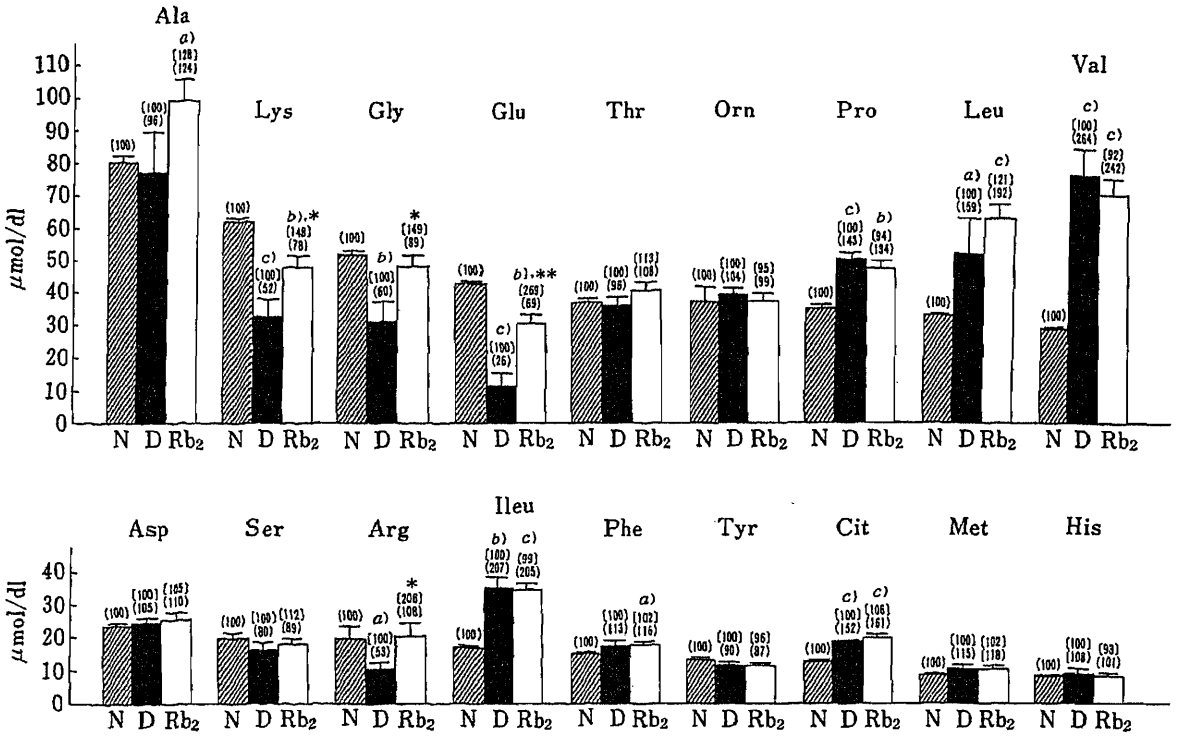

Fig. 1. Effect of Ginsenoside-Rb_2 on Free Amino Acid Concentrations in the Serum

N, non-diabetic rat; D, diabetic rat (control group); Rb_2, diabetic rat (ginsenoside-Rb_2-treated group). Values are means ± S.E. of 7 rats. Figures in parentheses are percentages of the non-diabetic or diabetic control value. *a*), *; Significantly different from the non-diabetic or diabetic control value, $p<0.05$, *b*) $p<0.01$, *c*), **; $p<0.001$.

elevation of blood glucose level (116.9±4.6 mg/dl *vs.* 718.9±36.6 mg/dl) and a tendency toward a loss of body weight and liver weight. When ginsenoside-Rb_2 was successively given at a dose of 10 mg once a day, the blood glucose level fell to 539.8 mg/dl after 6 d of administration (significantly decreased by 25% of the control value), as reported previously.[3,4] Body weight and liver weight increased significantly more in the group receiving ginsenoside-Rb_2. However, daily intake of food was smaller, in spite of the larger weight gain. Daily intake of drinking water was remarkably low in the ginsenoside-Rb_2 administered group. Similarly, urinary output was remarkably low, at a near-normal level, in the ginsenoside-Rb_2 administered group.

Urea Nitrogen, Total Protein, and Albumin in the Serum

Table I shows the serum constituent levels of the ginsenoside-Rb_2-treated and control groups in streptozotocin-induced diabetic rats. The urea nitrogen was increased about 2.8-fold in streptozotocin-induced diabetic rats as compared with the non-diabetic rats. The rats of the ginsenoside-Rb_2-treated group showed a significant decrease of urea nitrogen; as shown in Table I, the blood urea nitrogen level was 43% less at the 6th d in the ginsenoside-Rb_2-treated group as compared with the control group. The total protein level in the serum was increased by 5% at a dose of 10 mg/rat/d for 6 d, as compared with the control value. On the other hand, the albumin level showed no appreciable change after the administration of ginsenoside-Rb_2.

Free Amino Acids in the Serum

As shown in Fig. 1, the concentrations of free amino acids in serum were significantly decreased in streptozotocin-induced diabetic rats as compared with the non-diabetic rats, as

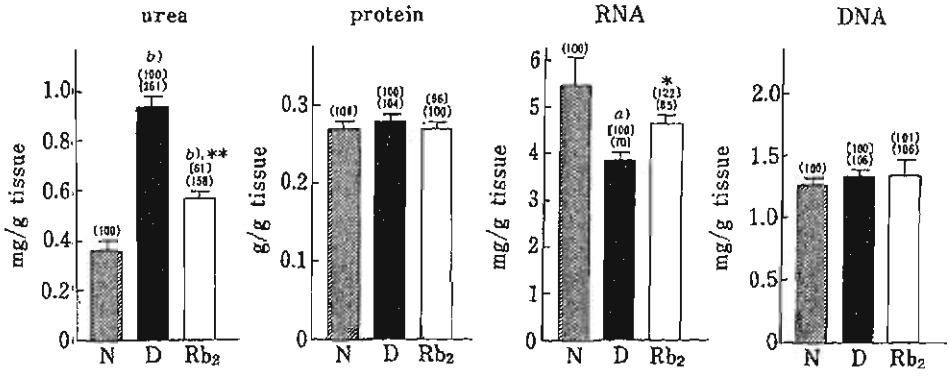

Fig. 2. Effect of Ginsenoside-Rb_2 on Urea, Protein, RNA, and DNA Contents in the Liver

N, non-diabetic rat; D, diabetic rat (control group); Rb_2, diabetic rat (ginsenoside-Rb_2-treated group). Values are means ± S.E. of 7 rats. Figures in parentheses are percentages of the non-diabetic or diabetic control value. *a*), *; Significantly different from the non-diabetic or diabetic control value, $p<0.01$, *b*), **; $p<0.001$.

TABLE II. Effect of Ginsenoside-Rb_2 on Ribosome Contents in the Liver

		Total ribosomes	Membrane-Bound ribosomes	Free ribosomes
Non-diabetic rat	mg RNA/g tissue	5.52±0.47	3.90±0.55	1.63±0.13
		(100)	(100)	(100)
	Ratio	100	71	29
Diabetic rat				
Control	mg RNA/g tissue	3.87±0.22	3.12±0.21	0.74±0.07
		(70)[a)]	(80)	(45)[b)]
		[100]	[100]	[100]
	Ratio	100	81	19
Rb_2	mg RNA/g tissue	4.71±0.17	3.99±0.23	0.72±0.17
		(85)	(102)	(44)[b)]
		[122]**	[128]*	[97]
	Ratio	100	85	15

Values are means±S.E. of 7 rats. Figures in parentheses are percentages of the non-diabetic or diabetic control value. *; Significantly different from the non-diabetic or diabetic control value, $p<0.05$, *a*), **; $p<0.01$, *b*) $p<0.001$.

follows: Lys (48%), Gly (40%), Glu (74%), and Arg (47%). The concentrations of Pro, Leu, Val, Ileu, and Cit were increased by 43, 59, 164, 107, and 52%, respectively. However, the Lys, Gly, Glu, and Arg levels of the ginsenoside-Rb_2-treated group in diabetic rats were significantly higher than those of the control group. No change was seen in the levels of Pro, Leu, Val, Ileu, and Cit.

Urea, Protein, RNA, and DNA in the Liver

As shown in Fig. 2, a remarkable increase in hepatic urea concentration was observed in diabetic rats as compared with the non-diabetic rats. The rats treated with ginsenoside-Rb_2 showed a significant decrease from 0.94 to 0.57 mg/g tissue. The data in Fig. 2 further indicate that the ginsenoside-Rb_2-treated group of streptozotocin-induced diabetic rats showed a significant increase in RNA content. Protein and DNA in the liver showed no appreciable changes.

Membrane-Bound and Free Ribosomes in the Liver

As shown in Table II, the ratio of the distribution of membrane-bound and free

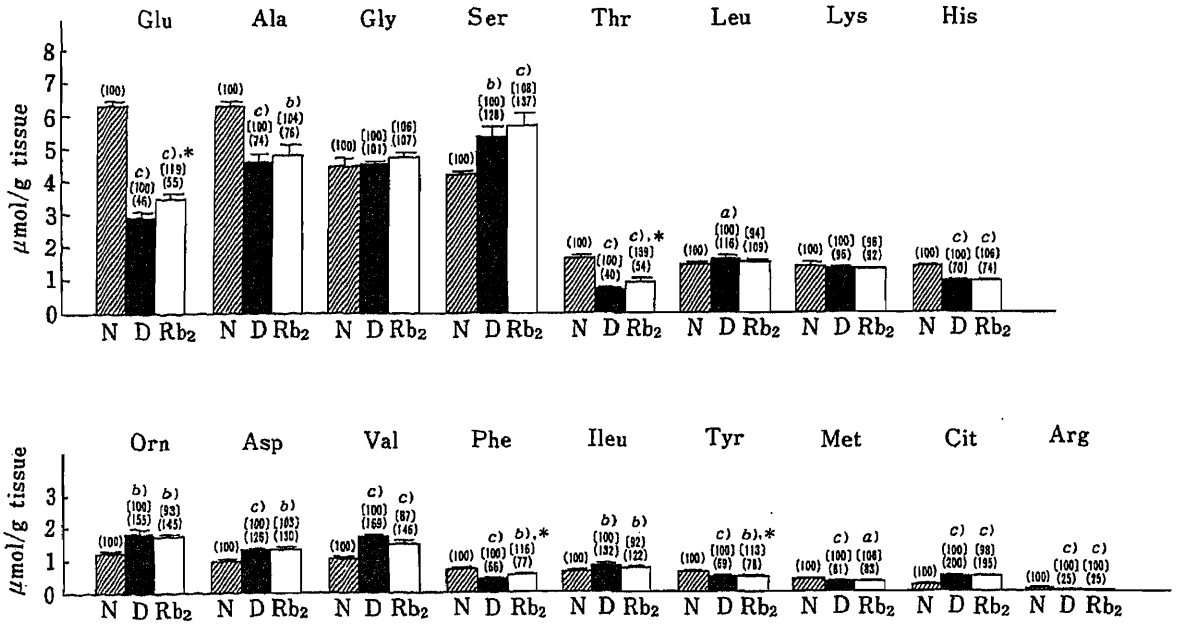

Fig. 3. Effect of Ginsenoside-Rb_2 on Free Amino Acid Concentrations in the Liver

N, non-diabetic rat; D, diabetic rat (control group); Rb_2, diabetic rat (ginsenoside-Rb_2-treated group). Values are means ± S.E. of 7 rats. Figures in parentheses are percentages of the non-diabetic or diabetic control value. *a*), *; Significantly different from the non-diabetic or diabetic control value, $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

ribosomes was found to be 71 : 29 in the normal rats. In diabetic rats, total and free ribosomes were decreased significantly as compared with non-diabetic rats. Membrane-bound ribosomes also showed a moderate decrease (but it was statistically insignificant). The amount of total ribosomes showed a 22% increase in the ginsenoside-Rb_2-treated group over the control animals. Similarly, administration of ginsenoside-Rb_2 to rats resulted in a significant increase in the membrane-bound ribosomes from 3.12 to 3.99 mg/g wet weight. The amount of membrane-bound ribosomes was increased 28% by ginsenoside-Rb_2 administration. However, a significant difference in free ribosome content was not observed between the control and ginsenoside-Rb_2-treated group (3% decrease).

Free Amino Acids in the Liver

Figure 3 shows the effect of ginsenoside-Rb_2 on free amino acid concentrations in the liver. The concentrations of Glu (54%), Ala (26%), Thr (60%), His (30%), Phe (34%), Tyr (31%), Met (19%), and Arg (75%) were significantly decreased in diabetic rats as compared with the non-diabetic rats. The concentrations of Ser, Leu, Orn, Asp, Val, Ileu, and Cit were increased by 28, 16, 55, 26, 69, 32, and 100%, respectively. Ginsenoside-Rb_2 had a normalizing effect on the hepatic concentrations of Glu, Thr, Phe, and Tyr.

Discussion

In diabetes, a high level of blood glucose produces an increase of plasma osmotic pressure and results in a deficiency of water in the body due to osmotic diuresis. Finally, diabetic symptoms such as over-eating, over-drinking, polyuria, and dehydration may develop.[18] In our experiment, rats with streptozotocin-induced diabetes showed abnormally high urinary output, delayed weight gain, over-eating, and over-drinking, compared with normal rats. However, diabetic rats receiving ginsenoside-Rb_2 showed a singnificant increase in body weight, although they took less food than diabetic rats with no treatment. Moreover,

their intake of drinking water and urinary output were lower than those of the control group. It is apparent that ginsenoside-Rb_2 can improve diabetic symptoms.

On the other hand, diabetic rats receiving ginsenoside-Rb_2 showed a significant decrease in blood urea nitrogen. Serum protein was increased significantly in the ginsenoside-Rb_2-treated group. The protein content in the blood might be expected to reflect diabetes-induced alterations of liver protein synthesis since most of the blood proteins are synthesized in and exported from the liver.[19] These observations suggest that rats with diabetes have a reversible alteration in liver protein synthesis that is modulated, in part, by ginsenoside-Rb_2 treatment.

Furthermore, although there were no appreciable changes in protein and DNA contents in the liver, the RNA content of the ginsenoside-Rb_2-treated group was significantly increased. The experimental result on RNA content led us to examine directly the changes in the ribosomes of hepatic cells that occur in ginsenoside-Rb_2-treated rats. Ribosomes in rat liver cells are present either free or attached to endoplasmic reticulum membrane, and in both states they exist largely as polysomal aggregates. A quantitative study of the distribution of ribosomes between these two states was carried out with and without deoxycholic acid (DOC)-treatment of each fraction of liver by a modification of the method of Redman.[16] The ratio of free and membrane-bound ribosomes was changed by the ginsenoside-Rb_2 treatment, resulting in an increase in the amount of membrane-bound ribosomes as determined by quantitative studies of the distribution of ribosomes between these two states. However, the free ribosome content was not significantly different between the control and ginsenoside-Rb_2-treated rats. Clearly, the results support the validity of the conclusion that the effect of ginsenoside-Rb_2 is connected with the stimulation of RNA synthesis, ribosome formation, and heavy polysome accumulation. These actions are very similar to those of fraction 3 prepared from ginseng extract, as reported previously.[17]

In addition, in an examination of the effect of ginsenoside-Rb_2 on the free amino acid concentrations, it was observed that the concentrations of Lys, Gly, Glu, and Arg in serum and Glu, Thr, Phe, Tyr, *etc.* in the liver were normalized after the administration of ginsenoside-Rb_2. Umpleby *et al.*[20] demonstrated that the levels of Gly and branched-chain amino acids in the serum of diabetic patients were normalized after an overnight insulin infusion. The effect of ginsenoside-Rb_2 on branched-chain amino acids was not observed, but the normalization of Gly level in the serum by the ginsenoside-Rb_2 treatment is in accord with the effect of insulin. However, diabetic rats receiving ginsenoside-Rb_2 showed no significant change in serum insulin level (data not shown). Based on this result, we are planning a further study on whether ginsenoside-Rb_2 acts as a kind of modifier which enhances the action of insulin at the insulin receptor or whether ginsenoside-Rb_2 reduces the secretion of adrenaline, glucagon, or adrenocorticotropic hormone which acts as an antagonist against insulin.

References

1) T. Yokozawa, T. Kobayashi, H. Oura, and Y. Kawashima, *Chem. Pharm. Bull.*, **32**, 2766 (1984).
2) T. Yokozawa, T. Kobayashi, A. Kawai, H. Oura, and Y. Kawashima, *Chem. Pharm. Bull.*, **32**, 4490 (1984).
3) T. Yokozawa, T. Kobayashi, H. Oura, and Y. Kawashima, *J. Med. Pharm. Soc. WAKAN-YAKU*, **1**, 22 (1984).
4) T. Yokozawa, T. Kobayashi, H. Oura, and Y. Kawashima, *Chem. Pharm. Bull.*, **33**, 869 (1985).
5) T. Yokozawa, T. Kobayashi, H. Oura, and Y. Kawashima, *Chem. Pharm. Bull.*, **33**, 3893 (1985).
6) A. Junod, A. E. Lambert, L. Orci, R. Pictet, A. E. Gonet, and A. E. Renold, *Proc. Soc. Exp. Biol. Med.*, **126**, 201 (1967).
7) S. Sanada, N. Kondo, J. Shoji, O. Tanaka, and S. Shibata, *Chem. Pharm. Bull.*, **22**, 421 (1974).
8) Y. Kameno, "Rinsyo Kagaku Bunseki," Vol. III, ed. by M. Niwa, M. Kitamura, and M. Saito, Tokyo Kagaku Dojin, Tokyo, 1981, p. 1.
9) T. Sasaki, "Rinsyo Kagaku Bunseki," Vol. II, ed. by M. Saito, M. Kitamura, and M. Niwa, Tokyo Kagaku Dojin, Tokyo, 1979, p. 1.
10) K. Sano, "Rinsyo Kagaku Bunseki," Vol. II, ed. by M. Saito, M. Kitamura, and M. Niwa, Tokyo Kagaku

Dojin, Tokyo, 1979, p. 140.
11) F. L. Rodkey, *Clin. Chem.*, **11**, 478 (1965).
12) R. M. Archibald, *J. Biol. Chem.*, **157**, 507 (1945).
13) A. Fleck and H. N. Munro, *Biochim. Biophys. Acta*, **55**, 571 (1962).
14) G. Ceriotti, *J. Biol. Chem.*, **198**, 297 (1952).
15) G. Ceriotti, *J. Biol. Chem.*, **214**, 59 (1955).
16) C. M. Redman, *J. Biol. Chem.*, **244**, 4308 (1969).
17) H. Oura, S. Hiai, S. Nabetani, H. Nakagawa, Y. Kurata, and N. Sasaki, *Planta Medica*, **28**, 76 (1975).
18) T. Tanaka, "Gendai Seibutsu Kagaku," Vol. 16, ed. by Y. Yamamura, Iwanami Shoten, Tokyo, 1975, p. 43.
19) F. R. Blattner, A. E. Blechl, K. Denniston-Thompson, H. R. Faber, J. L. Richards, P. W. Slightom, O. Tucker, and O. Smithies, *Science*, **202**, 1279 (1978).
20) A. M. Umpleby, M. A. Boroujerdi, P. M. Brown, E. R. Carson, and P. H. Sonksen, *Diabetologia*, **29**, 131 (1986).

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Calcitonin Stimulates Adenosine-5′-triphosphate Synthesis Calcium-Dependently in the Hepatic Mitochondria of Rats

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The effect of calcitonin (CT) on adenosine-5′-triphosphate (ATP) synthesis in the hepatic mitochondria of rats was investigated. Administration of CT (80 MRC mU/100 g body weight) produced a marked elevation of ATP content in the hepatic cytosol. This increase was completely inhibited by administration of 2,4-dinitrophenol (0.1 mg/100 g), an inhibitor of oxidative phosphorylation of mitochondria. Verapamil (1.0 mg/100 g) also inhibited the hormonal effect on hepatic ATP content. Moreover, administration of calcium chloride (2.0 and 4.0 mg Ca^{2+}/100 g) elevated the cytosolic ATP content by about 1.8- and 2.3-fold, respectively. Meanwhile, administration of CT produced a remarkable increase of ATPase activity in the hepatic mitochondria. Calcium contents in both the liver and the mitochondria were raised by CT administration. The removal of the mitochondrial calcium by washing with 10 mM ethyleneglycol-bis(2-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, pH 7.4) caused complete loss of the increase in mitochondrial adenosine triphosphatase (ATPase) activity induced by CT administration. The CT-increased ATPase activity was appreciably increased by addition of 10 and 50 μM Ca^{2+}, but this increase was not altered by addition of 10 μM trifluoperazine or calmodulin (2.5 μg/ml). These results suggest that CT stimulates ATP synthesis in the hepatic mitochondria, and that the hormonal effect may be mediated through calcium, but not calmodulin.

Keywords——Calcitonin; ATP synthesis; ATPase; calcium; rat liver mitochondria

Introduction

It is well known that calcitonin (CT), a calcium-regulating hormone, inhibits bone resorption.[1] In recent years, it has been demonstrated that CT acts on liver metabolism; the hormone stimulates glycogenolysis,[2] gluconeogenesis,[3] and fatty acid synthesis[4] in the liver of rats. This hormonal action is mediated through calcium entry into liver cells by the specific binding of CT to the plasma membranes.[5]

From previous investigations,[2-4] it is assumed that CT stimulates energy metabolism in liver cells. Oxidative phosphorylation is the synthesis of adenosine-5′-triphosphate (ATP) by energy liberated during substrate oxidation.[6] This function is localized in the inner membrane of mitochondria. The energy transfer system in mitochondria includes ATPase, which synthesizes ATP.[6] The present investigation was therefore undertaken to clarify the effect of CT on the ATP content in the cytosol and the adenosine triphosphatase (ATPase) activity in the liver mitochondria of rats. It was found that the mechanism by which CT stimulates ATP synthesis involves an increase of calcium in the hepatic mitochondria.

Materials and Methods

Animals——Male Wistar rats, weighing 100—130 g, were used. They were obtained from the Nippon Bio Supply Center, Tokyo, Japan. The animals were fed commercial laboratory chow containing 1.1% Ca, 1.1% P and 57.4% carbohydrate (Oriental Test Diet, Tokyo, Japan) and distilled water freely until use.

Hormone, Drugs and Treatment——Calcitonin (synthetic [$Asu^{1,7}$]eel calcitonin, 4000 MRC U/mg) was supplied

through the courtesy of Toyo Jozo Research Laboratories, Shizuoka, Japan. The hormone was dissolved in ice-cold distilled water to a concentration of 80 MRC mU/0.5 ml. This solution (0.5 ml/100 g body weight) was subcutaneously administered to rats. The animals were sacrificed at various times after the hormone administration. 2,4-Dinitrophenol (DNP) or verapamil hydrochloride was dissolved in distilled water to a concentration of 0.2 mg/ml or 2.0 mg/ml, respectively. DNP (0.5 ml/100 g) was intraperitoneally injected immediately in rats administered CT. Likewise, verapamil (0.5 ml/100 g) was injected immediately. The rats were bled 30 min after drug injection. Calcium chloride was dissolved in distilled water to concentrations of 4.0 and 8.0 mg/ml. These solutions were intraperitoneally injected into rats, which were bled 10 min later.

Preparation of Mitochondria and Cytosol——The liver was perfused with ice-cold 0.25 M sucrose solution, frozen immediately, cut into small pieces, suspended 1 : 9 in 0.25 M sucrose solution, and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was immediately spun at $105000 \times g$ for 60 min and its supernatant fraction (cytosol) was collected. The volume was measured. The cytosol was immediately used to determine ATP concentration. In another experiment, the homogenate was spun at $600 \times g$ in a refrigerated centrifuge for 10 min and the supernatant fraction was spun at $5500 \times g$ for 20 min to obtain the mitochondrial fraction.[7] This mitochondrial fraction was suspended in ice-cold distilled water for analyses of ATPase and calcium. In separate experiments, the pellet of mitochondria was stirred in an ice-cold 0.25 M sucrose solution containing 10 mM ethyleneglycol-bis(2-aminoethylether)-*N*,*N*, *N*′,*N*′-tetraacetic acid (EGTA, pH 7.4) for 10 min at 4 °C in order to remove calcium. This suspension was washed twice by centrifugation with an ice-cold 0.25 M sucrose solution.

Analytical Methods——The enzymatic determination of ATP in the cytosol with 3-phosphoglycerate kinase (ATP: 3-phospho-D-glycerate-1-phosphotransferase, EC 2.7.2.3.) was done by the method of Jawork *et al.*[8] ATP content in the cytosol was expressed as μmol of ATP per g wet liver tissue.

Enzyme assay was carried out under optimal conditions. ATPase activity in the mitochondria was measured by incubation for 10 min at 37 °C in a final volume of 1.0 ml containing 5 mM ATP, 5 mM $MgSO_4$, 20 mM potassium phosphoenolpyruvate, 32 μg pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40), 20 mM Tris–H_2SO_4 (pH 7.4) and the protein (30—70 μg)[6] with or without the appropriate additions of 0.1—100 μM calcium ion and/or 10—25 μM trifluoperazine (pH 7.4) and 2.5 μg of calmodulin (bovine brain, from Sigma Chemical Co., St. Louis. Mo. U.S.A.). Trichloroacetic acid (50%, 0.1 ml) was added, and the supernatant fraction (0.5 ml) after centrifugation was mixed with 3.25 ml of water, 1.0 ml of 2.5% ammonium molybdate in 5 N H_2SO_4, and 0.25 ml of aminonaphthol sulfonate solution. The mixture was incubated at 30 °C for 10 min, and the sbsorbance was expressed as μmol of phosphate released per min per mg protein. Protein concentration was determined by the method of Lowry *et al.*[9]

The calcium content in the liver tissue and the fraction of mitochondria was determined by atomic absorption spectrophotometry after digestion with nitric acid. The amount of calcium in the liver and the mitochondria were expressed as μg of calcium per g wet of liver and nmol of calcium per mg protein of the mitochondria.

Statistical Methods——The significance of the difference between values was estimated by means of Student's *t*-test; *p* values less than 0.05 were considered to indicate statistically significant differences.

Results

Effect of CT on ATP Content in the Hepatic Cytosol

The time course of alteration of ATP content in the hepatic cytosol after a single subcutaneous administration of CT (80 MRC mU/100 g) in rats is shown in Fig. 1A. ATP content in the cytosol was significantly increased 10 min after CT administration. This increase reached a maximum at 30 min after the hormone administration. This level was maintained until 60 min, and then began to decrease. The effect of increasing doses of CT (20, 40 and 80 MRC mU/100 g) on ATP content in the cytosol was examined 30 min after the hormone administration, and the result is shown in Fig. 1B. The dose of 40 MRC mU/100 g caused about 2-fold increase over the control value. The hormonal effect was saturated at the dose of 80 MRC mU/100 g.

DNP, an inhibitor of oxidative phosphorylation of mitochondria, was injected immediately after administration of CT (80 MRC mU/100 g), and 30 min later the rats were bled. Administration of DNP (0.1 mg/100 g) caused a complete inhibition of the hormonal effect on the cytosolic ATP content of control rats.

The effect of verapamil, a blocker of calcium entry into liver cells, on the increase of ATP content in the hepatic cytosol of rats administered CT is shown in Fig. 3. Verapamil (1.0 mg/100 g) was injected immediately after CT (80 MRC mU/100 g) administration, and 30 min later the rats were bled. The increasing effect of CT on the cytosolic ATP content was

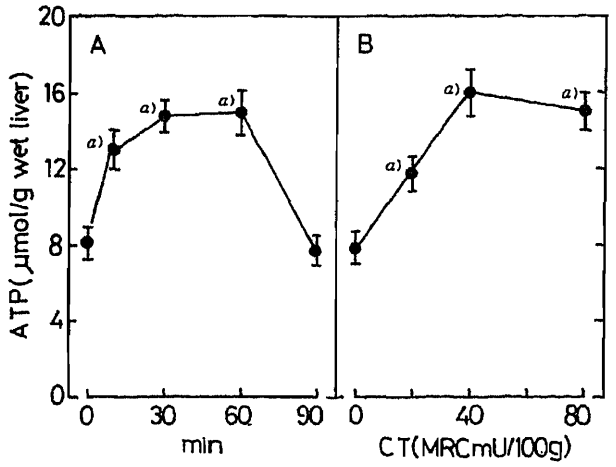

Fig. 1. Effect of Calcitonin (CT) on ATP Content in the Hepatic Cytosol of Rats

A; Time course of alteration of the cytosolic ATP content after a single subcutaneous administration of CT (80 MRC mU/100 g) in rats. At 30 min after control (vehicle) administration, the ATP content was not significantly altered in comparison with the zero time value. B; Effect of increasing doses of CT (20, 40 and 80 MRC mU/100 g) on the cytosolic ATP content. The rats were bled 30 min after CT administration. Each bar is the mean of five rats. Vertical lines represent the S.E.M. *a*) $p<0.01$, compared with the control value.

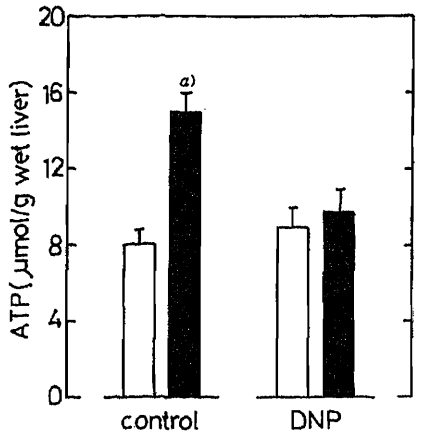

Fig. 2. Effect of 2,4-Dinitrophenol (DNP) on ATP Content in the Hepatic Cytosol of Rats Given a Single Subcutaneous Administration of CT

DNP (0.1 mg/100 g) was intraperitoneally injected immediately after administration of CT (80 MRC mU/100 g), and 30 min later the rats were bled. Each bar is the mean of five rats. Vertical lines represent the S.E.M. *a*) $p<0.01$, compared with the control value. □, control; ■, CT.

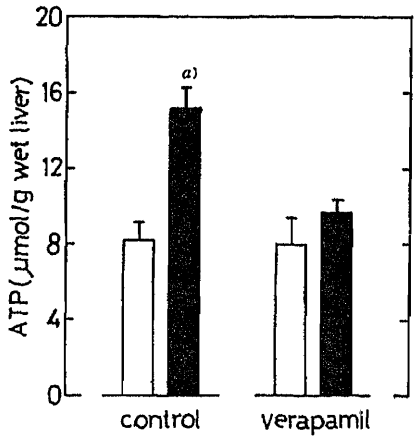

Fig. 3. Effect of Verapamil on ATP Content in the Hepatic Cytosol of Rats Given a Single Subcutaneous Administration of CT

Verapamil (1.0 mg/100 g) was subcutaneously injected immediately after administration of CT (80 MRC mU/100 g), and 30 min later the rats were bled. Each bar is the mean of five rats. Vertical lines represent the S.E.M. *a*) $p<0.01$, compared with the control value. □, control; ■, CT.

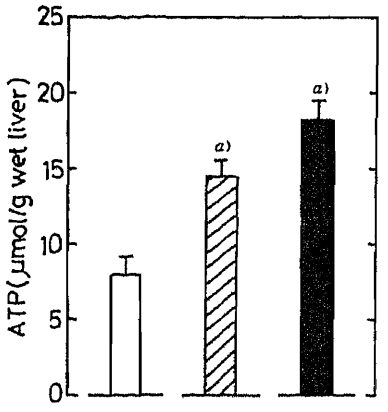

Fig. 4. Effect of Calcium Administration on ATP Content in the Hepatic Cytosol of Rats

The animals received a single intraperitoneal administration of calcium chloride (2.0 and 4.0 mg Ca/100 g), and 10 min later they were bled. Each bar is the mean of five rats. Vertical lines represent the S.E.M. *a*) $p<0.01$, compared with control value. □, control; ▨, Ca (2.0 mg/100 g); ■, Ca (4.0 mg/100 g).

completely blocked by verapamil treatment. Verapamil alone did not cause a significant alteration of the cytosolic ATPcontent of control rats.

The effect of calcium chloride administration on the cytosolic ATP content was also examined. Calcium chloride (2.0 and 4.0 mg Ca/100 g) was injected into rats, and 10 min later the animals were bled. Administration of calcium caused a significant increase in ATP content in the hepatic cytosol of rats (Fig. 4).

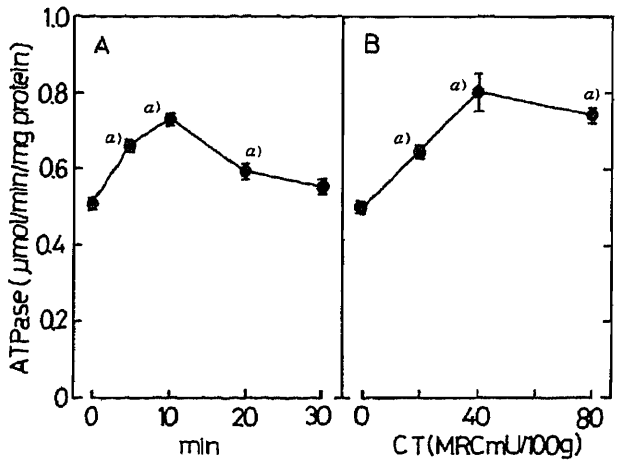

Fig. 5. Effect of CT on ATPase Activity in the Hepatic Mitochondria of Rats

A; The animals were bled at various times after a single subcutaneous administration of CT (80 MRC-mU/100 g). B; The rats were given CT (20, 40 or 80 MRC mU/100 g), and 10 min later they were bled. Each point represents the mean of five rats. Vertical lines represent the S.E.M. *a)* $p<0.01$, compared with the control value.

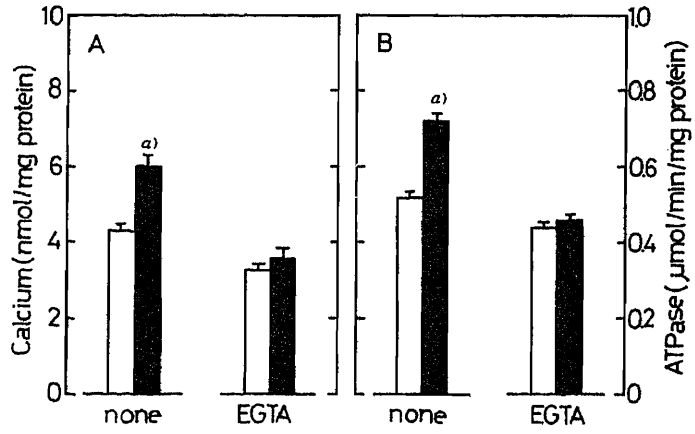

Fig. 6. Changes in Calcium Content and ATPase Activity Caused by EGTA Washing of the Hepatic Mitochondria Obtained from CT-Treated Rats

The animals received a single subcutaneous administration of CT (80 MRC mU/100 g), and 10 min later they were bled. The hepatic mitochondria were washed with 10 mM EGTA solution. Each bar represents the mean of five rats. Vertical lines represent the S.E.M. *a)* $p<0.01$, compared with the control value. □, control; ■, CT.

Effect of CT on ATPase Activity in the Hepatic Mitochondria

The effect of CT on ATPase activity in the hepatic mitochondria of rats is shown in Fig. 5. Administration of CT (80 MRC mU/100 g) produced a significant increase of ATPase activity in the hepatic mitochondria. The activity reached a maximum at 10 min after the hormone administration, and then began to decrease (Fig. 5A). ATPase activity was not significantly altered by the vehicle administration in comparison with the zero time control (data not shown). The effect of increasing doses of CT (20, 40 and 80 MRC mU/100 g) on ATPase activity was examined 10 min after the hormone administration (Fig. 5B). A significant increase in the enzyme activity was obsered at 20 MRC mU/100 g. The dose of 40 MRC mU/100 g gave the maximal effect.

Administration of CT (80 MRC mU/100 g) caused a significant increase of calcium content (free and bound) in the liver of rats from 14.1 ± 1.0 to 19.9 ± 1.4 (μg/g wet tissue) at 10 min after the hormone administration. Calcium content in the hepatic mitochondria was significantly increased by CT administration (Fig. 6A). This increase was reduced by washing of the mitochondria with 10 mM EGTA. Likewise, CT-increased ATPase activity in the mitochondria was reduced to control level by washing of the mitochondria with 10 mM EGTA (Fig. 6B). Thus, the increase in the mitochondrial ATPase activity by CT administration might be related to the elevation of the mitochondrial calcium.

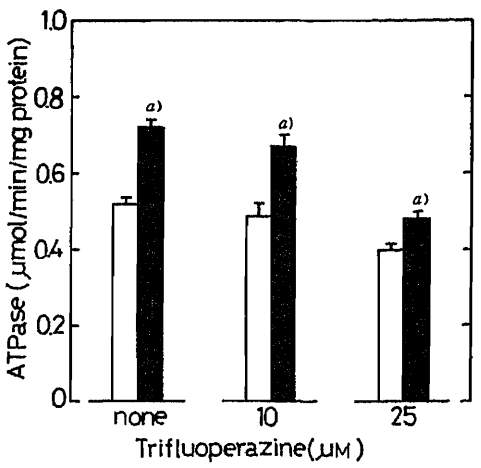

Fig. 7. Effect of Trifluoperazine, a Calmodulin Inhibitor, on ATPase Activity of the Hepatic Mitochondria Obtained from CT-Treated Rats

The animals were bled 10 min after a single subcutaneous administration of CT (80 MRC mU/100 g). The hepatic mitochondria were incubated with 10 and 25 μM trifluoperazine. Each bar represents the mean of five rats. Vertical lines represent the S.E.M. *a*) $p<0.01$, compared with the control value. □, control; ■, CT.

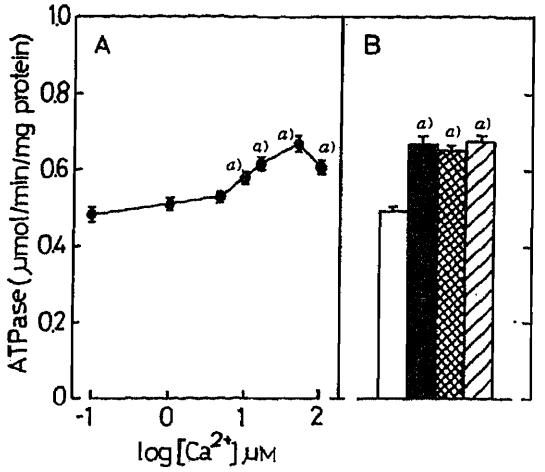

Fig. 8. Effect of Calcium Addition on ATPase Activity in the Hepatic Mitochondria Obtained from Control Rats

The hepatic mitochondria were incubated with calcium ion (Ca^{2+}) in the range of 0.1—100 μM (A), and with 50 μM Ca^{2+} plus 10 μM trifluoperazine or calmodulin (2.5 μg/ml) (B). Each point and bar represents the mean of five rats. Vertical lines represent the S.E.M. *a*) $p<0.01$, compared with the control value. □, control; ■, Ca^{2+}; ▩, Ca^{2+} + trifluoperazine; ▨, Ca^{2+} + calmodulin.

The effect of calcium ion (Ca^{2+}) addition on ATPase activity in the hepatic mitochondria is shown in Fig. 8. Ca^{2+} (0.1—100 μM final concentration) was added to the enzyme reaction system containing the mitochondria prepared from control rat liver. Addition of 10 μM Ca^{2+} caused a significant increase in ATPase activity (Fig. 8A). With 50 μM Ca^{2+}, the effect was maximum. The activation of ATPase activity by addition of 50 μM Ca^{2+} was not inhibited by 10 μM trifluoperazine (Fig. 8B). The Ca^{2+}-increased ATPase activity was not enhanced by addition of calmodulin (2.5 μg/ml).

Discussion

In the present study, it has been demonstrated that administration of CT produced a remarkable increase of ATP content in the hepatic cytosol of rats. It was reported that CT stimulates glycogenolysis in the liver cells of rats.[2] This action of CT may induce the increase of ATP content in liver cells. Meanwhile, CT stimulates fatty acid synthesis in the liver cells of rats.[4] ATP is utilized by stimulation of fatty acid synthesis. It is unlikely, therefore, that the increase of ATP content in the liver cells by CT administration is a result of a decrease of utilization and decomposition of cellular ATP. It is reasonable to conclude that CT stimulates ATP synthesis in the liver of rats. Thus, CT may play a role in the stimulation of energy metabolism in liver cells.

Administration of 2,4-dinitrophenol, an inhibitor of mitochondrial oxidative phosphorylation, caused a complete inhibition of the increase in the cytosolic ATP content caused by CT administration. This may indicate that the hormonal ability to elevate the cytosolic ATP content is based on the stimulation of mitochondrial oxidative phosphorylation. Futhermore, administration of verapamil, a blocker of calcium entry into liver cells,[10,11] completely prevented the augmentation of the cytosolic ATP content following CT administration. This result suggests that the action of CT to increase the cytosolic ATP content involves an

increase of calcium in liver cells by the hormone. In fact, CT elevates calcium content in the liver cells of rats and this hormonal action is completely blocked by verapamil.[11]

Oxidative phosphorylation is the synthesis of ATP by energy liberated during substrate oxidation.[12] This function is localized in the inner membrane of mitochondria, which is mainly composed of many kinds of proteins and several phospholipids. Administration of CT produced a significant increase in the activity of ATPase, which synthesizes ATP,[6] in the hepatic mitochondria of rats. This increase was observed during 20 min after administration of CT. Thus, the time course of CT action on the mitochondrial ATPase activity preceded the elevation of the cytosolic ATP content. The increase of ATP induced by CT was maintained during about 30 min (Fig. 1). This may indicate that the increased ATP is not consumed promptly.

Administration of CT caused a significant increase in calcium content in the hepatic mitochondria of rats. Washing of the hepatic mitochondria with EGTA caused a complete loss of the increase in calcium content and a corresponding fall in ATPase activity in the hepatic mitochondria of rats treated with CT. This result suggests that the increase in ATPase activity caused by CT administration may be based on the increase of calcium in the hepatic mitochondria by the hormone. CT action on mitochondrial ATPase may be mediated through calcium. It has been reported that calmodulin is located in hepatic mitochondria,[13] and trifluoperazine is a calmodulin inhibitor. However, the CT-increased ATPase activity was not inhibited by addition of trifluoperazine (10 or 25 μM) to the enzyme reaction system. Furthermore, ATPase activity in the hepatic mitochondria of control rats was increased by addition of Ca^{2+} in the range of 10—100 μM. This activation was not inhibited by trifluoperazine, and it was not enhanced by calmodulin addition. Thus, the mitochondrial ATPase may be a Ca^{2+}-sensitive enzyme, independently of calmodulin. It is concluded that the CT action on the mitochondrial ATPase depends on calcium.

In conclusion, the present study demonstrates that CT induces ATP synthesis calcium-dependently in the hepatic mitochondria of rats. This CT effect may have a cell physiological significance in liver metabolism.

References

1) M. A. Aliapoulis, P. A. Goldhaber, and P. L. Munson, *Science*, **151**, 330 (1966).
2) M. Yamaguchi and K. Momose, *Endocrinol. Jpn.*, **29**, 553 (1982).
3) M. Yamaguchi and K. Momose, *Horm. Metabol. Res.*, **16**, 650 (1984).
4) M. Yamaguchi, K. Momose, and K. Takahashi, *Horm. Metabol. Res.*, **17**, 346 (1985).
5) M. Yamaguchi and M. Ito, *Acta Endocrinol.*, **110**, 124 (1985).
6) Y. Kagawa, "Methods in Membrane Biology," Vol. 1, ed. by E. D. Korn, Plenum Press, New York-London, 1974, p. 201.
7) G. H. Hogeboom, "Methods in Enzymology," Vol. 1, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York, 1974, p. 16.
8) D. Jawork, W. Gruber, and H. U. Bergmeyer, "Methods of Enzymatic Analysis," Vol. 4, ed. by H. U. Bergmeyer, Academic Press, New York, 1974, p. 2097.
9) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
10) B. P. Hughes, S. E. Milton, G. J. Barritt, and A. M. Auld, *Biochem. Pharmacol.*, **35**, 3045 (1986).
11) M. Yamaguchi and H. Yoshida, *Acta Endocrinol.*, **110**, 239 (1985).
12) H. A. Lardy and S. M. Ferguson, "Annual Reviews of Biochemistry," Vol. 38, Annual Reviews, Inc., Palo Alto, 1969, p. 991.
13) O. Hatase, A. Doi, T. Itano, H. Matsui, and Y. Ohmura, *Biochem. Biophys. Res. Commun.*, **132**, 63 (1985).

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Purification and Characterization of the Product of Chemically Synthesized Human Growth Hormone Gene Expression in *Escherichia coli*

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The efficient purification and characterization of the product of chemically synthesized human growth hormone (hGH) gene expressed in *Escherichia coli* are described. The product was purified from the cell lysates of the *E. coli* by means of ammonium sulfate precipitation, DE-52 chromatography, chromatofocusing chromatography and Ultrogel AcA 54 chromatography. The purified hGH gene product was homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, non-denaturing polyacrylamide gel electrophoresis, gel electrofocusing and high-performance liquid chromatography (HPLC). The purified product and an authentic methionyl hGH (m-hGH) showed identical behavior in these systems. The structural features of the purified product were examined by means of amino acid composition analysis, NH_2-terminal sequence analysis and tryptic peptide mapping. The experimental values of the amino acid composition of the purified product were in agreement with the theoretical values for m-hGH. Its NH_2-terminal sequence (39 amino acid residues) was identical with that of the published sequence of hGH, except for an additional amino-terminal methionine residue immediately preceding phenylalanine at residue 1. The elution profile of the tryptic peptides of the purified product on HPLC was identical with that of authentic m-hGH. These elution profiles were nearly identical with that of a pituitary-derived hGH, with the exception of one peak due to NH_2-terminal peptide. On the basis of these results, the purified product was identified as m-hGH.

Keywords——chemically synthesized gene; human growth hormone gene; human growth hormone; purified human growth hormone gene product

Recently a gene coding for human growth hormone (hGH) has been totally synthesized by chemical means.[1] A recombinant plasmid carrying the hGH gene produced the polypeptide in *Escherichia coli*. It was identical with hGH in size and immunological properties.[1] We partially purified the hGH gene product from the *E. coli* extract and showed that it was as active as hGH in the tibia test in hypophysectomized rats.[2] For further development of the product as a pharmaceutical agent, it is essential to establish an efficient purification procedure, through which adequate quantities of the product for laboratory use or clinical studies can be obtained. Definitive identification and extensive characterization of the purified product are also of the utmost importance, because it is the product of fundamentally artificial manipulation. In the present work, the hGH gene product was purified efficiently to homogeneity and its physicochemical properties were thoroughly analyzed in comparison with those of methionyl hGH (m-hGH).

Materials and Methods

Materials——Purified pituitary-derived hGH and m-hGH produced by recombinant deoxyribonucleic acid

(DNA) technology[3,4] were gifts from Sumitomo Chemicals Co., Ltd. Polybuffer exchanger, Polybuffer and disposable PD-10 columns containing Sephadex G-25 were purchased from Pharmacia Fine Chemicals. Constant-boiling hydrochloric acid, trifluoroacetic acid (sequential grade) and HPLC-grade acetonitrile were obtained from Wako Pure Chemicals. Molecular weight markers for sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis were from Bio-Rad Laboratories. PI markers for gel electrofocusing were purchased from Oriental Yeast Co., Ltd.

Methods——Protein was measured by the method of Bradford[5] using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories). The amount of immunoreactive hGH (IM hGH) was measured by radioimmunoassay using an EIKEN-hGH-1 kit (double-antibody method) from Eiken Immunochemicals. One milligram of the standard hGH (m-hGH) was tentatively defined as 1 IM hGH unit. SDS–polyacrylamide gel electrophoresis was performed by the method of Laemmli.[6] Non-denaturing polyacrylamide gel electrophoresis was performed by the method of Davis[7] with the pH 8.3 buffer system. Gel electrofocusing was performed as described previously.[8] High-performance liquid chromatography (HPLC) of the purified product was performed as follows. The purified hGH gene product was injected onto a Synchropak RP-P column (25.0 cm × 4.1 mm, Synchrom Inc.) on a Gilson high-performance liquid chromatograph and the effluent was monitored at 280 nm. Tryptic digests of hGH samples were prepared as follows. The purified product was concentrated to a final concentration of 1.0 mg/ml in a Centricon-10 (Amicon). The pituitary-derived hGH and the m-hGH were each dissolved at a final concentration of 1.0 mg/ml in saline, pH 8.0. These solutions were desalted on a PD-10 column equilibrated in 1% ammonium bicarbonate, pH 9. Trypsin, dissolved in 1% ammonium bicarbonate, pH 9, at a concentration of 2.0 mg/ml was added to each sample in a ratio of 3 to 100 by weight and incubated at room temperature. After 8 h, a second aliquot of trypsin was added to the reaction mixture in a ratio of 2 to 100 by weight. After a further incubation overnight, aliquots of 100 μg were injected into the HPLC column as described above. Amino acid analysis of the purified product was performed as follows. Pooled and dried samples from the HPLC were taken up in 1 ml of constant-boiling HCl with one crystal of phenol, and transferred to acid-washed hydrolysis tubes, which were sealed under reduced pressure. Hydrolysis was carried out at 110 °C.[9] The amino acid composition of the sample was analyzed on a Hitachi 835 amino acid analyzer equipped with an SIC 7000A integrator. Amino acid sequence analysis of the purified product was performed as follows. Pooled and dried samples (4.5 nmol) from the HPLC were suspended in 30 μl of distilled water containing 0.1% SDS and transferred into the cup of an Applied Biosystem 470A sequencer. Phenylthiohydantoin (PTH) amino acids were identified on an ABI PTH (22 cm × 2.1 mm) column at a flow rate of 200 μl/min with the regular program.

Results and Discussion

The hGH gene carried by plasmid phGH-1 was expressed in *E. coli* (HB 101) as described previously.[1] The hGH gene product was purified from the cell lysates of the *E. coli* by means of ammonium sulfate precipitation, DE-52 chromatography, chromatofocusing chromatography and Ultrogel AcA 54 chromatography.

Crude lysates were prepared by lysis in 50 mM Tris–HCl buffer, pH 8.0, containing 50 mM ethylenediaminetetraacetic acid (EDTA) and egg white lysozyme (1 mg/ml) followed by treatment with deoxyribonuclease (DNase) I as described previously.[2] The crude lysates were sonicated for 30 s 3times using a W-225 sonicator (Heat System Co., Ltd.). The combination of the lysis by lysozyme and the sonication resulted in efficient extraction of the induced polypeptide from the cells. When the crude lysates were treated with 4.5 M NaCl in place of the sonication or sonicated in phosphate buffer without lysozyme and DNase I according to Goeddel *et al.*,[3] the yield of the hGH gene product was very poor (data not shown). The sonicates were mixed with polyethyleneimine (0.2%) and centrifuged at 100000 × *g* for 30 min. The supernatant was fractionated by ammonium sulfate precipitation (50%). The pellet was then dissolved in 20 ml of 20 mM Tris–HCl, pH 7.5, and dialyzed against the same buffer at 4 °C. The dialyzate was charged on a DE-52 column (1.6 × 33 cm) equilibrated with the same buffer and the column was eluted with a linear gradient of NaCl concentration from 0 to 200 mM using the same buffer. Immunoreactive hGH in the eluate was measured by radioimmunoassay and found in fractions containing about 100 mM NaCl. The fractions containing immunoreactive hGH were concentrated by ammonium sulfate precipitation (50%), dissolved in 5 ml of 20 mM Tris–HCl, pH 7.5, and then dialyzed against 25mM histidine–HCl, pH 6.25, at 4 °C. The dialyzate was charged on a Polybuffer exchanger (PBE 94) column (1 × 55 cm) equilibrated with 25 mM histidine–HCl, pH 6.25, and the column was

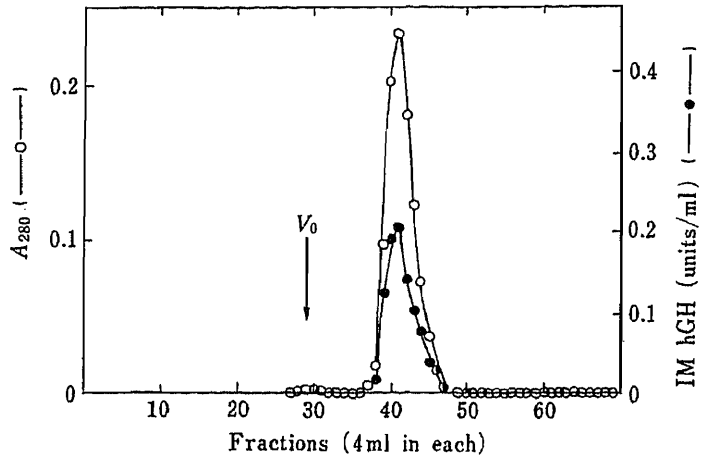

Fig. 1. The Elution Profile of the Gene Product on Ultrogel AcA 54 Gel Filtration Chromatography

Immunoreactive (IM) hGH was measured by radioimmunoassay as described in Materials and Methods.

TABLE I. Summary of the Purification of hGH Gene Product

Step	Total protein (mg)	Specific activity (IM hGH units/mg)	Total immuno-reactivity (IM hGH units)	Yield (%)
Extract	315.5	0.123	38.7	100
Ammonium sulfate fraction	87.0	0.379	33.0	85.3
DE-52	14.0	0.541	7.58	19.6
Chromatofocusing	7.93	0.932	7.39	19.1
Ultrogel AcA 54	7.13	1.030	7.34	19.0

IM hGH was defined and measured as described in Materials and Methods.

eluted with 390 ml of 12.5% Polybuffer 74–HCl, pH 4. The immunoreactive hGH was found in fractions at about pH 5.1. The fractions were concentrated by ammonium sulfate precipitation (50%), dissolved in 3 ml of 5 mM sodium phosphate, pH 7.3 containing 500 mM glycine and then further fractionated on an Ultrogel AcA 54 (LKB) column (2.2 × 78 cm) equilibrated with the same buffer. The elution profiles are shown in Fig. 1. A major peak of protein was eluted after a minor peak of contaminant at the void volume. When each fraction was analyzed by radioimmunoassay, the elution profiles of the immunoreactivity were closely correlated with the profiles of the major protein. Table I summarizes the results of the purification of the hGH gene product.

Among these steps, chromatofocusing chromatography was found to be effective to purify the product. The specific activity of the product was increased by 1.7-fold by this process and the recovery of the protein was 97%. Starting from about 10 g (wet weight) of *E. coli* cells, 7.13 mg of the purified product was obtained. The specific activity of the purified product was 1.03 IM hGH unit/mg, as determined by radioimmunoassay, being almost the same as that of an authentic m-hGH (1 IM hGH unit/mg).

Besides our results described here, the purification of m-hGH or hGH from *E. coli* has been studied by others.[4,10–12] Olson *et al.*[4,10] purified m-hGH by means of ammonium sulfate precipitation, and DE-52, CM-52 and Sephacryl S-200 chromatographies. And Jónsdóttir *et al.*[11] purified m-hGH by employing an immunoadsorbant using monoclonal antibody to hGH and ion-exchange chromatography. In these reports, the yield and the specific activity of m-hGH at each purification step were not described, so it is difficult to compare our

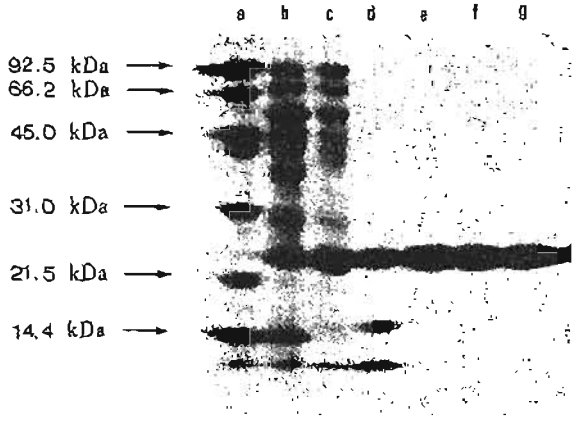

Fig. 2. The Protein Pattern of the Sample from Purification Steps of the hGH Gene Product in SDS–Polyacrylamide Gel Electrophoresis

The gel was stained with Coomassie brilliant blue in 10% acetic acid and 25% methanol.

a, molecular weight standards containing lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin and phosphorylase B; b, crude extract of *E. coli*; c, ammonium sulfate precipitation (50%) fraction; d, DE-52 fraction; e, chromatofocusing fraction; f, Ultrogel AcA 54 fraction; g, authentic m-hGH.

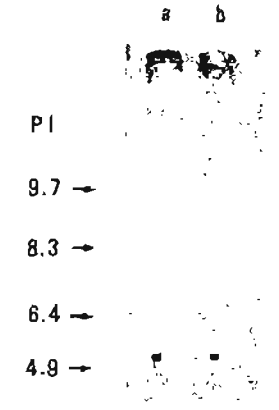

Fig. 3. Gel Electrofocusing of the Purified Product and Authentic m-hGH

a, the purified product; b, m-hGH.

Fig. 4. Non-denaturing Polyacrylamide Gel Electrophoresis of the Purified Product and Authentic m-hGH

a, the purified product; b, m-hGH.

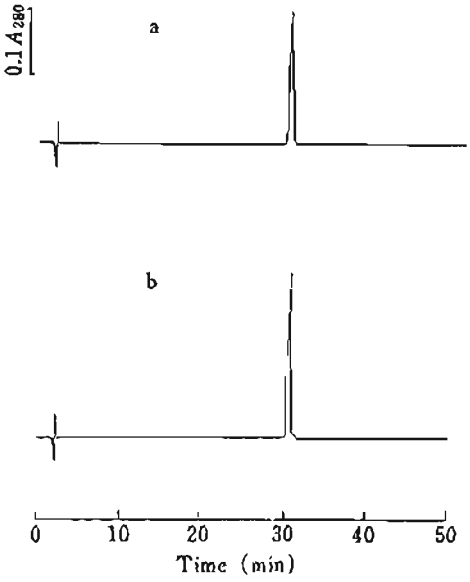

Fig. 5. HPLC of the Purified Product and Authentic m-hGH

The elution solvents were 0.1% trifluoroacetic acid (TFA) in water (solvent 1) and 0.1% TFA in acetonitrile (solvent 2). The elution conditions consisted of a linear gradient from solvent 1 to solvent 2 at 25°C and a flow rate of 1 ml/min. The effluent was monitored at 280 nm.

a, the purified product; b, m-hGH.

results with theirs. On the other hand, Gerald and Hansen[12] purified hGH by means of anion exchange and size exclusion chromatographies. The overall purification and the recovery at the final step were 15-fold and 71%, respectively. However, the segment coding for an *E. coli* signal peptide fused to hGH was designed so as to secrete hGH into the periplasmic space. In our case, the desired protein was produced and accumulated in the cytoplasm. Therefore, it may not be meaningful to compare the efficiencies of purification because of the difference in the accumulation sites of the desired product.

Figure 2 shows the protein pattern of the sample from each purification step in SDS-polyacrylamide gel electrophoresis. *E. Coli* proteins were removed step by step. Finally, a

TABLE II. Amino Acid Composition of the Purified Product

	Experimental values	Theoretical values
Aspartic acid	20	20
Threonine	9.9	10
Serine	18.4	18
Glutamic acid	26.8	27
Proline	7.8	8
Glycine	8.5	8
Alanine	6.7	7
Cysteine	N.D.	4
Valine	7.5	7
Methionine	3.6	4
Isoleucine	8.1	8
Leucine	25.4	26
Tyrosine	7.4	8
Phenylalanine	13.1	13
Tryptophan	N.D.	1
Lysine	8.8	9
Histidine	3.3	3
Arginine	10.9	11

The experimental value of aspartic acid was defined as 20. Other experimental values are expressed relatively to the value of aspartic acid. The experimental values presented are the average values of triplicate analyses performed after hydrolysis for 24, 48 and 72 h as described in Materials and Methods. The values for serine and threonine were obtained by extrapolation to zero time. The 72 h values for isoleucine and leucine were used. Cysteine and tryptophan contents were not determined. The theoretical values for the amino acid composition of hGH are based upon the data of Li.[16] N.D.: not determined.

TABLE III. NH_2-Terminal Sequence of the Purified Product

Cycle No.	PTH-amino acid (pmol)	Theoretical	Cycle No.	PTH-amino acid (pmol)	Theoretical
1	Met (2510)	Met	21	Leu (360)	Leu
2	Phe (1760)	Phe	22	His (70)	His
3	Pro (1520)	Pro	23	Gln (410)	Gln
4	Thr (610)	Thr	24	Leu (370)	Leu
5	Ile (1640)	Ile	25	Ala (300)	Ala
6	Pro (810)	Pro	26	Phe (270)	Phe
7	Leu (1260)	Leu	27	Asp (40)	Asp
8	Ser (590)	Ser	28	Thr (110)	Thr
9	Arg (280)	Arg	29	Tyr (30)	Tyr
10	Leu (1260)	Leu	30	Gln (250)	Gln
11	Phe (780)	Phe	31	Glu (30)	Glu
12	Asp (440)	Asp	32	Phe (70)	Phe
13	Asn (540)	Asn	33	Glu (160)	Glu
14	Ala (770)	Ala	34	Glu (160)	Glu
15	Met (850)	Met	35	Ala (180)	Ala
16	Leu (850)	Leu	36	Tyr (210)	Tyr
17	Arg (180)	Arg	37	Ile (100)	Ile
18	Ala (650)	Ala	38	Pro (150)	Pro
19	His (130)	His	39	Lys (80)	Lys
20	Arg (260)	Arg			

NH_2-Terminal sequence analysis of the purified product was performed as described in Materials and Methods. The numbers in parentheses indicate the recovery of each PTH-amino acid at each Edman cycle. The theoretical values for the NH_2-terminal sequence of hGH are based upon the data of Li.[16]

single protein band was found on the chromatogram. The molecular weight of the product was estimated as about 22 kDa, which corresponds to that of m-hGH.

The purity of the final preparation was further assessed by gel electrofocusing, non-denaturing polyacrylamide gel electrophoresis and HPLC, and the chromatographic behavior was compared with that of an authentic m-hGH. Figure 3 shows the chromatogram of the purified product on gel electrofocusing. The purified product migrated as a single band and its PI was found to be about 5.1, being the same as that of authentic m-hGH. Figures 4 and 5 show the behavior of the purified product on non-denaturing gel electrophoresis and HPLC, respectively. The purified product was found to be homogeneous and to have identical mobilities with the authentic m-hGH in both systems. These results indicate that the hGH gene product has been highly purified to homogeneity and is identical with m-hGH.

The integrity of the purified product was further confirmed by amino acid composition analysis, NH_2-terminal sequence analysis and tryptic peptide mapping. The ratios between the amino acids were consistent with the theoretical ones for m-hGH (Table II). The NH_2-terminal amino acid sequence of the purified product was determined by Edman degradation followed by HPLC analysis of released PTH amino acids. PTH-methionine and PTH-phenylalanine were released at the first cycle and second cycle, respectively (Table III). Recovery of PTH-methionine on the first cycle of degradation was approximately 98%. There was no significant amount of PTH-phenylalanine on the first cycle. Further sequence analysis of the PTH-amino acid derivatives for 39 stages has shown that the sequence is exactly the same as expected for hGH. In other words, the NH_2-terminal sequence of the purified product was identical with that of hGH with the exception of the additional amino-terminal

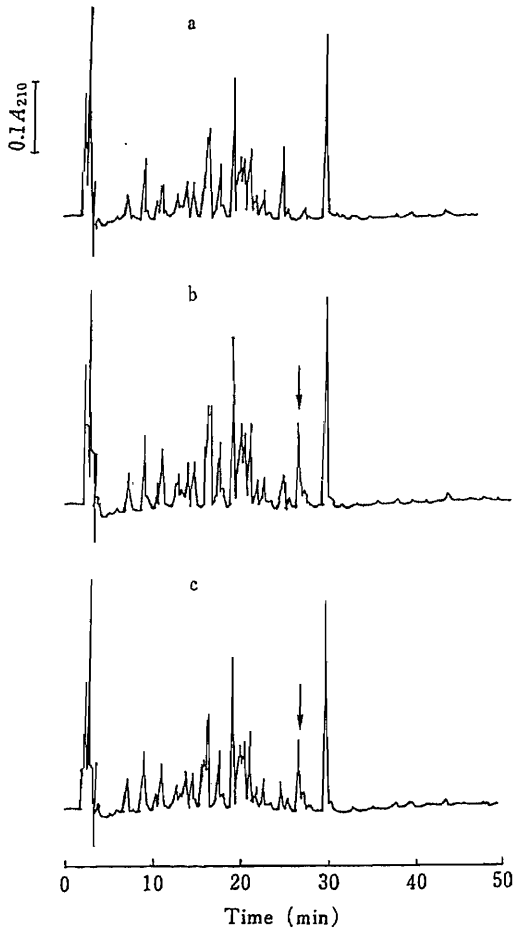

Fig. 6. Tryptic Peptide Mapping of the Purified Product, Pituitary-Derived hGH and Authentic m-hGH

Tryptic digestion was performed as described in Materials and Methods. The elution solvents were the same ones as described in Fig. 5. The elution conditions consisted of a linear gradient from 100% solvent 1 to 50% solvent 1 plus 50% solvent 2 at 25 °C and at a flow rate of 1 ml/min. The effluent was monitored at 210 nm..

a, hGH; b, m-hGH; c, the purified product.

methionine residue immediately preceding phenylalanine at residue 1. The presence of methionine at the NH_2-terminus is consistent with the evidence that formylmethionine initiates protein synthesis in *E. coli*[13,14] and that this bacterium contains an enzyme which removes the formyl group.[15] The identity of the purified product with m-hGH was further confirmed by tryptic peptide mapping. Tryptic peptides were prepared from the purified product, authentic m-hGH and pituitary-derived hGH and subjected to HPLC. Figure 6 shows the elution profiles of the tryptic peptides of the hGH, the m-hGH and the purified product. The elution profile of the tryptic peptides from the m-hGH was essentially identical to that from the hGH with the exception of one peak labeled with an arrow. The primary structural difference of m-hGH from hGH is the presence of an additional NH_2-terminal methionine. Therefore it is evident that the non-coincident peaks correspond to the NH_2-terminal tryptic peptides of the m-hGH and the hGH. The difference in elution time is due to the presence of NH_2-terminal methionine. When the elution profile from the purified product was compared with that from the m-hGH, every peak including the peak labeled with the arrow was coincident in the two profiles. On reduction of the tryptic digests of the purified product and the m-hGH with dithiothreitol, the elution profile of the reduced product formed from the purified product was also identical to that from the authentic m-hGH (data not shown). On the basis of these results, the purified product was concluded to be identical with m-hGH in terms of physicochemical features. Further investigations on the biological activity of the purified product are in progress and will be reported elsewhere.

The development of recombinant DNA technology should enable a wide variety of new medically useful peptides and proteins to be produced in adequate quantities for laboratory use or clinical studies. Ikehara *et al.* synthesized a gene coding for hGH by chemical means.[1] The gene consists of 584 base pairs, the longest gene so far synthesized chemically. The chemically synthesized gene was inserted into an *E. coli* plasmid downstream from the *E. coli trp* promoter, with a modified ribosome-binding region carried on pBR322. A recombinant plasmid carrying the promoter and the hGH gene produced the hormone in *E. coli* at a high level. To make the bast use of the product, efficient purification and extensive characterization of the product are essential. In this work, we developed a procedure for the purification of the hGH gene product from the cytoplasm of the *E. coli*. We also established the fidelity of protein translation in the bacterial protein synthesis system and the identity of the purified product.

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References

1) M. Ikehara, E. Ohtsuka, T. Tokunaga, Y. Taniyama, S. Iwai, K. Kitano, S. Miyamoto, T. Ohgi, Y. Sakuragawa, K. Fujiyama, T. Ikari, M. Kobayashi, T. Miyake, S. Shibahara, A. Ono, T. Ueda, T. Tanaka, H. Baba, T. Miki, A. Sakurai, T. Oishi, O. Chisaka, and K. Matsubara, *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 5956 (1984).
2) T. Oshizawa, T. Terao, H. Sato, T. Kimura, H. Fukuda, T. Tanimoto, S. Niimi, J. Kawamura, T. Hayakawa, E. Ohtsuka, and M. Ikehara, *Chem. Pharm. Bull.*, **33**, 3561 (1985).
3) D. V. Goeddel, H. L. Heyneker, T. Hozumi, R. Arentzen, K. Itakura, D. G. Yansura, M. J. Ross, G. Miozzari, R. Crea, and P. H. Seeburg, *Nature* (London), **281**, 544 (1979).
4) K. C. Olson, J. Fenno, N. Lin, R. N. Harkinsa, C. Sinder, W. H. Kohr, M. J. Ross, D. Fodge, G. Prender, and N. Stebbing, *Nature* (London), **293**, 408 (1981).
5) M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
6) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
7) B. J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).
8) R. F. Ritche, J. G. Harter, and T. B. Bayles, *J. Lab. Clin. Med.*, **68**, 842 (1966).

9) W. J. Kohr, R. Keck, and R. N. Harkins, *Anal. Biochem.*, **122**, 348 (1982).
10) M. J. Ross, "Insulins, Growth Hormone, and Recombinant DNA Technology," ed. by J. Gueriguian, Raven Press, New York, 1981, pp. 33—48.
11) I. Jónsdóttir, B. Skoog, H.-P. T. Ekre, B. Pavlu, and P. Perlmann, *Mol. Cell. Endocrinol.*, **46**, 131 (1986).
12) W. B. Gerald and M. H. Hansen, *FEBS Lett.*, **204**, 145 (1986).
13) B. F. C. Clark and K. A. Marcher, *J. Mol. Biol.*, **17**, 394 (1966).
14) M. R. Cappech, *Proc. Natl. Acad. Sci. U.S.A.*, **55**, 1517 (1966).
15) J. M. Adams, *J. Mol. Biol.*, **33**, 571 (1968).
16) C. H. Li, "Hormonal Proteins and Peptides," Vol. 3, ed. by C. H. Li, Academic Press, London, 1977, pp. 1—40.

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Studies on Superoxide Dismutase. I. Purification and Properties of Superoxide Dismutase from *Azotobacter vinelandii*-230

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In a cell-free extract of *Azotobacter vinelandii*-230, at least four electromorphologically different components possessing superoxide dismutase activity were detected. One component was purified to homogeneity. The purified enzyme (molecular weight 43000) consists of two non-covalently interacting subunits of equal size. Metal analysis indicated the presence of iron, 2 mol/mol-dimer. The amino acid composition (*ca.* 153 residues) was determined.

Keywords——iron-superoxide dismutase; *Azotobacter vinelandii*-230; purification; amino acid composition; superoxide dismutase isoenzyme; metalloenzyme; pyrogallol autoxidation inhibition assay

Superoxide dismutases (SOD) are a family of metalloenzymes essential for the survival of aerobic cells; they scavenge the superoxide radical, which appears to be a toxic species of oxygen. SOD catalyzes the dismutation of the superoxide radicals to hydrogen peroxide and oxygen.[1] The SOD first isolated from bovine erythrocytes is a Cu/Zn-SOD.[2] Subsequently two more classes of SOD (Fe-SOD and Mn-SOD) have been identified from diverse biological sources.[1,3] Of these, Cu/Zn-SOD has been studied extensively, and the amino acid sequences of SODs from several organisms have been determined (human erythrocyte,[4] bovine erythrocyte,[5] horse liver,[6] swordfish liver,[7] yeast,[8] the fungus *Neurospora crassa*,[9] and fruit fly[10]). However, little information on Mn-SOD, especially Fe-SOD, is available at the present time.[11,12] Moore and his coworkers[12] reported that crude cell-free extracts of *Azotobacter vinelandii* (5 strains) contained a single component of Fe-SOD, but so far no further purification has been reported.

We found that *Azotobacter vinelandii*-230 (A.v.-230) has at least three SOD active components. One of them has been purified to homogeneity and some of its properties have been examined.

Materials and Methods

A.v.-230 was grown in a modified Burk's N_2-free medium.[13] Sephadex G-75 (lot. No. 830314) and a mixture of standard proteins (bovine serum albumin, ovalbumin, α-lactalbumin, carbonic anhydrase, and soybean trypsin inhibitor) in a vial (lot. No. 4099) were purchased from Pharmacia Fine Chemicals Company. Hydroxyapatite (lot. No. 840424) was purchased from Bio-Rad Company. Diethylaminoethyl (DEAE)-52 cellulose (lot. No. 820216), nitro blue tetrazolium (NBT), acrylamide, Coomassie Brilliant Blue R-250, and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Company. All other chemicals were of analytical grade. Dialysis tubings were purchased from Nakarai Chemical Co. (Code No. 411-07). An ultrasonic generator (model CFS-250X, China) and a centrifuge (J2-21M, Beckman Co.) were used to obtain lysates. An ultraviolet (UV) spectrometer (Shimadzu, model UV-250) and a grating spectrograph (model WPI, China) were used for isolation and metal analysis of SOD respectively.

Enzyme and Protein Assays——SOD activity was determined in each purification step by using a modification of the reported method.[14] One unit of SOD activity was defined as the amount of enzyme required for the inhibition of autoxidation of pyrogallol by 50%, under the assay conditions. In the steps of column chromatographic purification, fractions were monitored by measuring the UV absorption at 280 and 260 nm[15] and the amount of purified enzyme was determined by the method of Lowry *et al.*[16] using bovine serum albumin as a standard.

Metal Analysis——The metal content of purified SOD was determined quantitatively by Massey's method.[17] A grating spectrometer was used for qualitative analysis.

Polyacrylamide Gel Electrophoresis——The purity of isolated SOD was analyzed by polyacrylamide gel electrophoresis (the gel was stained with Coomassie Brilliant blue R-250 for proteins). SOD active principles were stained photochemically as a negative image by the method of Beauchamp and Fridovich[18] using nitro blue tetrazolium. The molecular weights of the enzyme and its subunits were determined by gradient and SDS polyacrylamide gel electrophoresis according to the methods of Margolis and Wrigley[19] and Weber and Osborn,[20] respectively.

Results and Discussion

Purification of the Enzyme

Preliminary Treatment——Wet cells of A.v.-230 (120 g) were suspended in 1.4 volumes of 0.025 M Tris–HCl buffer (pH 7.2) and ruptured with an ultrasonic generator. The crude extract was obtained by spinning the lysate at 8000 rpm (7000 × *g*) for 30 min and then heating to 60 °C for 6—8 min under stirring. After this treatment, all operations were carried out at 4 °C. The precipitate was removed by centrifugation at 20000 rpm (40000 × *g*) for 20 min. The supernatant (*ca.* 200 ml) was applied to a DEAE-cellulose column (5 × 30 cm) pre-equilibrated with pH 7.2, 0.025 M Tris–HCl buffer. The column was eluted with the same buffer (1000 ml), then with 0.15 M NaCl in the same buffer (500 ml) at a flow rate of 0.6 ml/min. The colored band was collected, placed in dialysis tubing and dialyzed thoroughly against pH 8.2, 0.02 M Tris–HCl buffer (500 ml × 7). The dialysate was clarified by centrifugation.

Column Chromatographic Purification on DEAE-52 Cellulose——The above dialysate (*ca.* 165 ml) was applied to a column of DEAE-52 (2 × 30 cm) pre-equilibrated with the above dialysis buffer. Gradient elution was carried out with 0.2 M NaCl (300 ml) through a mixing flask containing the above dialysis buffer (300 ml) at a flow rate of 0.4 ml/min. The fractions containing the SOD activity (3 ml each, tube Nos. 90—103, Fig. 1) were combined and the combined eluate was concentrated to *ca.* 3 ml by lyophilization.

Purification by Gel-Filtration on Sephadex G-75——The sample solution obtained above (*ca.* 3 ml) was applied to a column of Sephadex G-75 (2.5 × 90 cm) pre-equilibrated with pH 8.2, 0.02 M Tris–HCl buffer. The column was then eluted with the same buffer at a flow rate of 0.2 ml/min. The fractions containing the SOD activity (3 ml each, tube Nos. 41—48, Fig. 2) were pooled and the combined solution was dialyzed against pH 7.2, 0.005 M phosphate buffer (1000 ml).

Column Chromatographic Purification of the Sephadex G-75-Purified Sample of A.v.-230 SOD on Hydroxyapatite——The above dialysate (21 ml) was applied to a column of hydroxyapatite (1.5 × 10 cm) pre-equilibrated with pH 7.2, 0.005 M phosphate buffer. The column was then eluted with pH 7.2, 0.2 M phosphate buffer (200 ml) through a mixing flask containing pH 7.2, 0.005 M phosphate buffer (200 ml) at a flow rate of 15 ml/h. The fractions (2.5 ml each, tube Nos. 30—58, Fig. 3) containing the SOD activity coincided with a peak of the protein concentration measured at UV 280 nm. The combined fractions were dialyzed against pH 7.2, 0.005M phosphate buffer (1000 ml) overnight and the dialysate was lyophilized. The purified sample was stored at −30 °C. The results of purification are summarized in Table I.

Purity of the Isolated Enzyme

The purity of A.v.-230 SOD isolated above was examined by gel electrophoresis. After

TABLE I. Summary of A.v.-230 SOD Purification

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude extract	250		14825		
DEAE-cellulose (batch elution)	165	30690	548	56	100
DEAE-52 cellulose	38	26524	104	254	86
Sephadex G-75	21	18900	33	566	62
Hydroxyapatite	70	10920	12	918	36

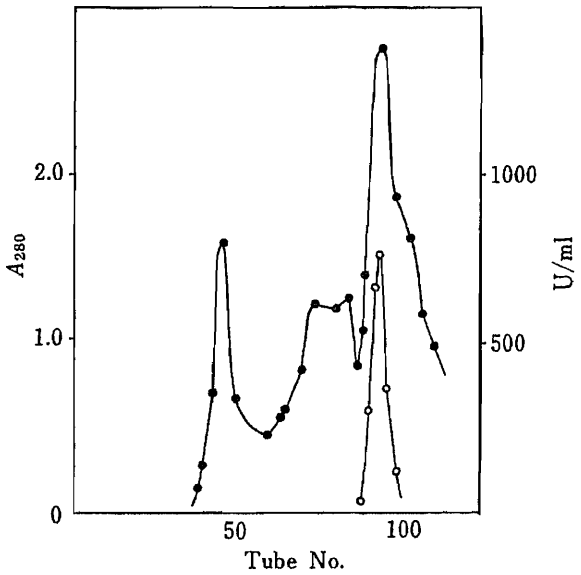

Fig. 1. DEAE-52 Cellulose Column Chromatographic Purification of A.v.-230 SOD

○, SOD activity (U/ml); ●, protein concentration.

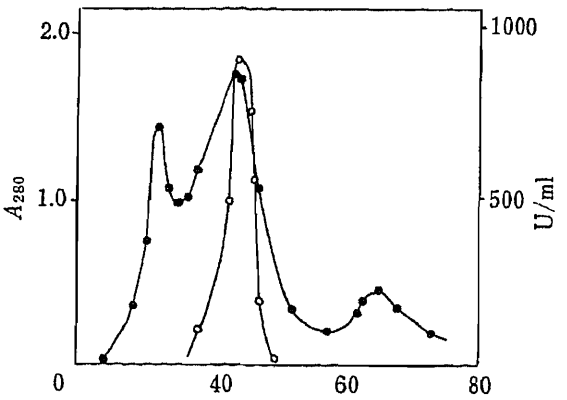

Fig. 2. Gel-Filtration of the DEAE-Purified Sample of A.v.-230 SOD on Sephadex G-75

○, SOD activity (U/ml); ●, protein concentration.

electrophoresis at 150 V (10 mA per tube) for 10 h in pH 8.8, 0.38 M Tris–HCl buffer according to Beauchamp and Fridovich,[18] the gel was stained with Coomassie brilliant blue R-250. The crude extract exhibited many protein bands, but the purified sample gave a single band (Fig. 4, A-b), which coincided with the zone of enzymatic activity determined by the photochemical procedure[18] (Fig. 4, B-b). For further confirmation of its homogeneity, a sample reduced with 0.01 M β-mercaptoethanol (at 37 °C for 2 h) was electrophoretically examined on SDS–polyacrylamide gel. When run under the same conditions as above, the reduced sample yielded a single band. Thus, we concluded that the enzyme we have isolated possesses a high degree of homogeneity.

When gels were stained for SOD activity in the above experiment, we observed, besides the band corresponding to the component that we isolated, three other bands in the crude preparation (Fig. 4, B-a). The results imply the presence of SOD isozymes in A.v.-230. However, the possibility can not be excluded that these components might be derived from modifications in the isolation steps. Isolation and characterization of these components are in progress.

Molecular Weight and Subunit Composition

The molecular weight (M.W.) of the enzyme was estimated by polyacrylamide gradient gel electrophoresis, using the following standard proteins (M.W.); bovine serum albumin

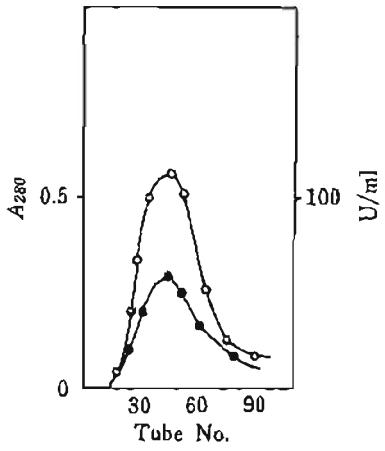

Fig. 3. Column Chromatographic Purification of the Sephadex G-75-Purified Sample of A.v.-230 SOD on Hydroxyapatite

○, SOD activity (U/ml); ●, protein concentration.

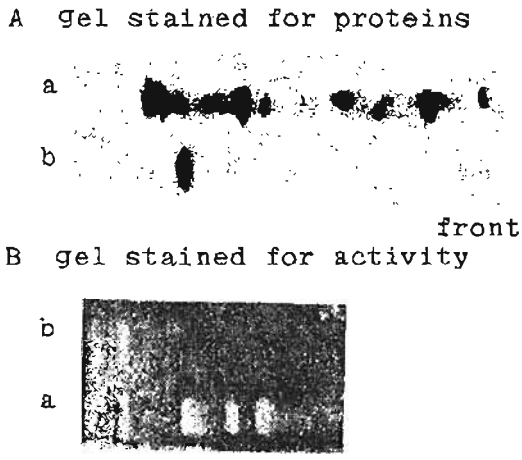

Fig. 4. Gel Electrophoresis of A.v.-230 SOD

a, crude dialyzed sample; b, purified sample.

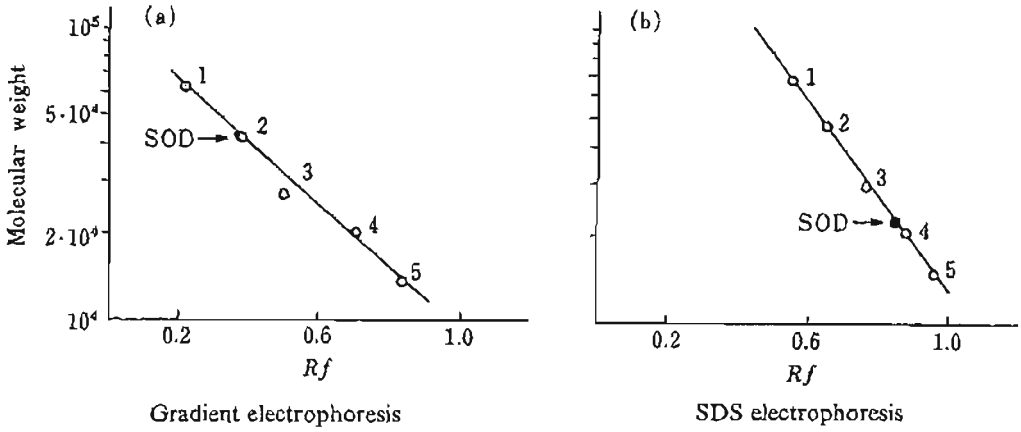

Fig. 5. Molecular Weight Determination of A.v.-230 SOD by Polyacrylamide Gel Electrophoresis

Standards: 1, bovine serum albumin (67000); 2, ovalbumin (43000), 3, carbonic anhydrase (30000); 4, soybean trypsin inhibitor (20100); 5, α-lactalbumin (14400).

(67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100) and α-lactalbumin (14400). By comparison of the mobility of the sample with those of the standards, the calculated M.W. of this sample was 43000 (Fig. 5-a).

The subunit structure was examined by SDS–polyacrylamide gel electrophoresis using the same standards mentioned above. As shown in Fig. 5-b, the purified enzyme ran as a single component with a molecular wight of 20500 in the presence or absence of β-mercaptoethanol. From these experimental data, it can be concluded that the SOD preparation from A.v.-230 is composed of two subunits of equal size held together by noncovalent interaction.

Metal Content

When examined by using a grating spectrometer, the enzyme was found to contain only Fe. The content of Fe was determined by Massey's method.[17] The average value for Fe, obtained after seven repeated experiments, was 1.57 mol per mol of enzyme. Thus, it can be estimated that SOD isolated from A.v.-230 contains 2 mol of Fe per mol of enzyme (M.W. 43000).

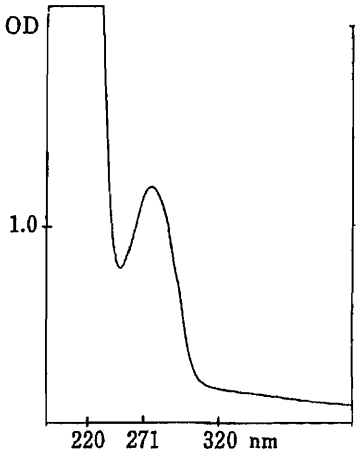

Fig. 6. UV Absorption Spectrum of the Purified Enzyme

Sample (0.2 mg/ml) in 0.005 M phosphate buffer, pH 7.2.

Other Physical Constants

As shown in Fig. 6, the maximum UV absorption of the purified enzyme is at 271.1 nm. When examined by isoelectrofocusing (Pharmalyte, pH 3—10), its isoelectric point was estimated to be 4.4.

Amino Acid Composition

The purified sample was subjected to amino acid analysis. Amino acid ratios in a 6 N HCl hydrolysate [110 °C for 24 h; Phe (8 residues) was taken as the basis of calculation, and values were not corrected for destruction]: Asp 16.76, Thr 9.06, Ser 6.68, Glu 12.80, Pro 8.36, Gly 14.52, Ala 16.92, Cystine 0.38, Val 7.44, Met 0.26, Ile 6.28, Leu 13.42, Tyr 7.66, Phe 8.00, Lys 8.40, His 8.70, Arg 2.42 (sum of the nearest integers, *ca.* 153 ± 2). As the N-terminal amino acid, Tyr was detected by Edman's method.[21]

In the present studies, we have isolated a major component of SOD from A.v.-230 and characterized it as a dimer consisting of two subunits of equal molecular size. It seems interesting to note that this SOD possesses Fe, instead of Cu/Zn or Mn. A.v.-230 is a nitrogen-fixing and obligately aerobic bacterium that possesses the highest cellular respiratory rate of any organism so far known. For this purpose, Fe may play an important role.

A.v.-230 contains a relatively large amount of SOD. This is convenient for further studies, such as structural elucidation, characterization of isozymes and elucidation of the functions of Fe-containing SOD. The results of further studies on A.v.-230 SOD will be published in due course.

References and Notes

1) I. Fridovich, *Ann. Rev. Biochem.*, **44**, 147 (1975); Y. M. Lee, D. J. Friedman, and F. J. Ayala, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 824 (1985).
2) J. M. McCord and I. Fridovich, *J. Biol. Chem.*, **244**, 6049 (1969).
3) I. Fridovich, *Science*, **201**, 875 (1978).
4) J. R. Jabusch, D. L. Fab, D. A. Kerschensteiner, and H. F. Deutsch, *Biochemistry*, **19**, 2310 (1980).
5) H. M. Steinman, V. R. Naik, J. L. Avernethy, and R. L. Hill, *J. Biol. Chem.*, **249**, 7326 (1974).
6) K. Lerch and D. Ammer, *J. Biol. Chem.*, **256**, 11545 (1981).
7) H. A. Rocha, W. H. Bannister and J. V. Bannister, *Eur. J. Biochem.*, **145**, 477 (1984).
8) J. T. Johansen, C. Overballe-Peterson, B. Martin, V. Hasemann, and I. Svendsen, *Carlsberg Res. Commun.*, **44**, 201 (1979).
9) K. Lerch and E. Schank, *J. Biol. Chem.*, **260**, 9559 (1985).
10) Y. M. Lee, D. J. Friedman, and F. J. Ayala, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 824 (1985).
11) P. Jurtshuk, Jr., *Appl. Environ. Microbiol.*, **47**, 1185 (1984).
12) E. R. B. Moore, E. P. Norrod, and P. Jurtshuk, Jr., *TFEMS Macrobiol. Lett.*, **24**, 261 (1984).

13) J. W. Newton, P. W. Wilson, and R. H. Burris, *J. Biol. Chem.*, **204**, 445 (1953).
14) S. Marklund and G. Marklund, *Eur. J. Biochem.*, **47**, 469 (1974).
15) E. Layne, "Methods in Enzymology," Vol. 3, Academic Press, New York, 1957, p. 447.
16) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
17) V. Massey, *J. Biol. Chem.*, **229**, 763 (1957).
18) C. Beauchamp and I. Fridovich, *Anal. Biochem.*, **44**, 276 (1971).
19) J. Margolis and C. W. Wrigley, *J. Chromatogr.*, **106**, 204 (1975).
20) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
21) P. Edman, *Acta Chem. Scand.*, **4**, 277, 283 (1950); *idem*, *ibid.*, **7**, 700 (1953).

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Calcitonin Increases Succinate Dehydrogenase Activity Dependently on Calcium in the Hepatic Mitochondria of Rats

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The regulatory effect of calcitonin (CT) on succinate dehydrogenase in the hepatic mitochondria of rats was investigated. Administration of CT (80 MRC mU/100 g body weight) produced a significant increase in calcium content and a corresponding elevation of succinate dehydrogenase activity in the hepatic mitochondria. These increases were dose-dependent. CT also increased succinate dehydrogenase activity and calcium content in the hepatic mitochondria of thyroparathyroidectomized rats. The CT-induced increase in enzyme activity was completely reversed by treatment with 1.0 mM ethylene glycol bis-(2-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA). The increased activity was restored by addition of Ca^{2+} (2.5—25 μM). Trifluoperazine (25 and 50 μM), a calmodulin inhibitor, completely prevented the increase in succinate dehydrogenase activity caused by CT. Insulin (1.0 U/100 g) also increased succinate dehydrogenase activity in the hepatic mitochondria by about 30% ($p < 0.01$), while the enzyme activity was not elevated by thyroxine (10 μg/100 g). The effect of insulin on the enzyme activation was attributed to the elevation of mitochondrial calcium. The present result indicates that succinate dehydrogenase activity is regulated by CT, and that the effect may be mediated through an increase of calcium in the mitochondria.

Keywords——calcitonin; succinate dehydrogenase; calcium; calmodulin; liver mitochondria

Introduction

It is well known that calcitonin (CT), a calcium-regulating hormone, inhibits bone resorption.[1] In recent years, it has been reported that CT also acts on liver metabolism.[2-4] CT stimulates glycogenolysis,[2] gluconeogenesis[3] and fatty acid synthesis[4] in the liver of rats. This hormonal action is mediated through calcium entry into liver cells by the specific binding of CT to the plasma membranes.[5-8] The action of CT on liver metabolism, however, remains to be elucidated.

More recently, much interest has been focussed on the hormonal regulation of the function of mitochondria in liver cells.[9-11] Since CT increases calcium content in liver cells,[5] the hormone may affect mitochondrial function. Succinate dehydrogenase, located on the inner membranes of hepatic mitochondria, is a physiologically significant enzyme related to electron transport. Whether this enzyme activity is hormonally regulated, however, has not been clarified so far. We found that CT increases succinate dehydrogenase activity in the hepatic mitochondria of rats, and that this hormonal action is mediated through the increase of calcium in the mitochondria.

Materials and Methods

Animals——Male Wistar rats weighing 100—130 g were used in the experiments. The animals were fed commercial laboratory chow containing 1.1% Ca, 1.1% P and 57.4% carbohydrate and distilled water freely.

Hormone, Drugs and Treatment——Calcitonin (synthetic [$Asu^{1,7}$] eel calcitonin, 4000 MRC U/mg) was supplied

through the courtesy of Toyo Jozo Research Laboratories, Shizuoka, Japan. Insulin (bovine pancreas, 26.2 I.U./mg) and L-thyroxine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trifluoperazine dimaleate was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of reagent grade. All water used was glass-distilled. The hormone solutions (0.5 ml/100 g body weight) were subcutaneously administered to rats. The animals were bled by cardiac puncture at various times after the hormone administration. Control rats received the vehicle alone.

Preparation of Mitochondria——The liver was perfused with an ice-cold 0.25 M sucrose solution and immediately cut into small pieces, suspended 1 : 4 in 0.25 M sucrose solution and homogenized in a Potter-Elvejem homogenizer with a Teflon pestle. The homogenate was spun at $600 \times g$ in a refrigerated centrifuge for 10 min and the supernatant was spun at $5500 \times g$ for 20 min to obtain the mitochondria.[12] The $5500 \times g$ pellet was washed twice with ice-cold 0.25 M sucrose solution by centrifugation for 10 min at $5500 \times g$. Mitochondrial preparations for enzyme analysis were resuspended in ice-cold distilled water. In separate experiments, the mitochondrial pellet was gently stirred in an ice-cold 0.25 M sucrose solution containing 1.0 mM ethylene glycol bis-(2-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA) for 10 min at 4 °C in order to remove calcium. This suspension was washed by centrifugation with an ice-cold 0.25 M sucrose solution. This mitochondrial fraction was suspended in ice-cold distilled water for enzyme analyses.

Analytical Methods——Enzyme assays were carried out under optimal conditions. Succinate dehydrogenase activity was measured by incubation of the reaction mixture for 15 min at 37 °C in a final volume of 1.0 ml containing 50 mM potassium phosphate (pH 7.4), 0.1% 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, 50 mM sodium succinate, 25 mM sucrose and the mitochondrial protein (5—20 μg).[13] In other experiments, the reaction mixture contained 2.5—250 μM Ca^{2+} and/or 10—50 μM trifluoperazine (pH 7.4) as final concentrations. The reaction was stopped by addition of trichloroacetic acid (10%, 1.0 ml), then the formazan was extracted with 4.0 ml of ethyl acetate and its absorbance was measured at 490 nm. Enzyme activity was expressed as absorbance at 490 nm per min per mg protein. The protein concentration was determined by the method of Lowry *et al.*[14]

Calcium contents in the liver tissue and in the mitochondria were determined by atomic absorption spectrophotometry after digestion with nitric acid, and expressed as the amount of calcium per g wet liver or mg protein of the mitochondria.

Statistical Methods——The significance of the difference between values was estimated by means of Student's *t*-test; a p value of less than 0.05 was considered to indicate a statistically significant difference.

Results

Effect of CT on Calcium Content and Succinate Dehydrogenase Activity in the Hepatic Mitochondria

Administration of CT (80 MRC mU/100 g body weight) causes a significant fall in serum calcium concentration and a corresponding elevation of liver calcium content in intact and thyroparathyroidectomized rats.[5] This was also observed in the present experiment; serum calcium level was lowered ($p<0.01$) from 10.1 ± 0.15 to 8.1 ± 0.21 (mg/100 ml) and liver calcium content was increased ($p<0.01$) from 15.0 ± 1.0 to 20.9 ± 1.4 (μg/g wet tissue) at 30 min after a single subcutaneous administration of CT (80 MRC mU/100 g) to intact rats (5 animals). Calcium content in the hepatic mitochondria was significantly increased by administration of CT (80 MRC mU/100 g) (Fig. 1). This elevation was observed at 10 min after the hormone administration and reached a maximum at 30 min. With the lowest dose of CT (20 MRC mU/100 g), the mitochondrial calcium content was significantly increased. Meanwhile, succinate dehydrogenase activity in the hepatic mitochondria was significantly increased 10 min after administration of CT (80 MRC mU/100 g) (Fig. 2). This increase was maximum at 30 min after the hormone administration, and then began to decrease. This hormonal effect was dose-dependent (20, 40 and 80 MRC mU/100 g).

The effect of CT on calcium content and succinate dehydrogenase activity was also examined in thyroparathyroidectomized rats. The thyroparathyroid glands complex was removed with fine forceps under light ether anesthesia. At 24 h after thyroparathyroidectomy, the serum calcium concentration was significantly reduced from 10.1 ± 0.12 to 7.8 ± 0.23 (mg/100 ml) for six rats. The animals received a single subcutaneous administration of CT (80 MRC mU/100 g) 24 h after thyroparathyroidectomy, and were sacrificed 30 min after the hormone administration. The serum calcium concentration was significantly decreased from 7.8 ± 0.23 to 6.7 ± 0.11 (mg/100 ml). The calcium content and succinate dehydrogenase

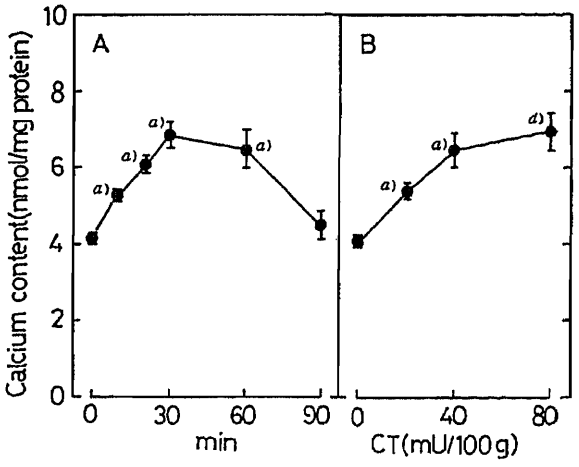

Fig. 1. Effect of CT on Calcium Content in the Hepatic Mitochondria of Rats

A: Rats received a single subcutaneous administration of CT (80 MRC mU/100 g), and they were sacrificed at various times. B: Rats were sacrificed 30 min after a single subcutaneous administration of CT (20, 40, or 80 MRC mU/100 g). Each value represents the mean ± S.E.M. of 5 animals. *a)* $p<0.01$, compared with the control value.

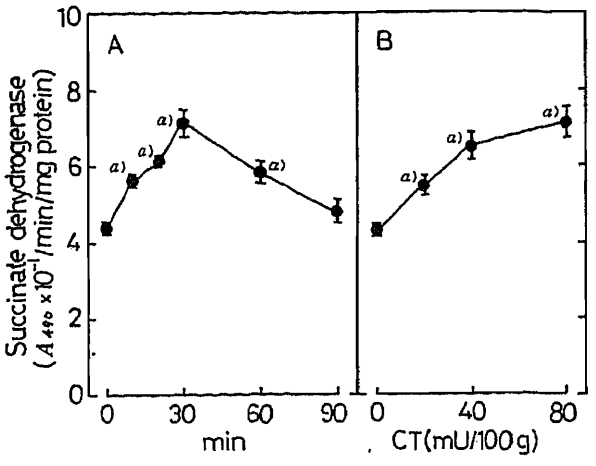

Fig. 2. Effect of CT on Succinate Dehydrogenase Activity in the Hepatic Mitochondria of Rats

The experimental procedures are described in the legend to Fig. 1. Each value represents the mean ± S.E.M. of 5 animals. *a)* $p<0.01$, compared with the control value.

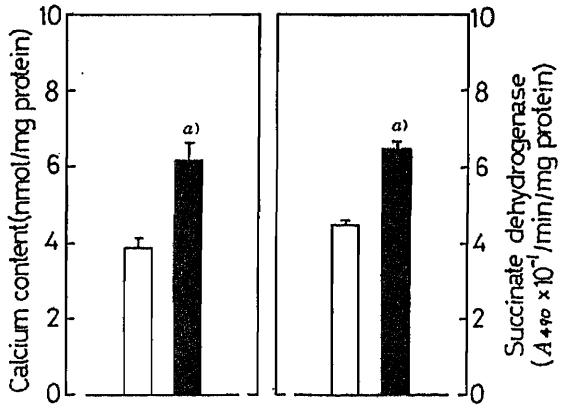

Fig. 3. Effect of CT on Succinate Dehydrogenase Activity in the Hepatic Mitochondria of Thyroparathyroidectomized Rats

The animals were thyroparathyroidectomized surgically, and 24 h later they received a single subcutaneous administration of CT (80 MRC mU/100 g). The rats were sacrificed 30 min after the hormone treatment. Each value represents the mean ± S.E.M. of 6 animals. *a)* $p<0.01$, compared with the control value. □, control; ■, CT.

activity in the hepatic mitochondria were significantly increased by CT administration (Fig. 3).

Effect of EGTA on CT-Increased Succinate Dehydrogenase Activity in the Hepatic Mitochondria

When the hepatic mitochondria of control rats or CT-treated rats were washed with 1.0 mM EGTA solution, the mitochondrial calcium content was significantly reduced from 4.11 ± 0.13 to 3.59 ± 0.11, or from 6.90 ± 0.44 to 4.52 ± 0.27 (nmol/mg protein) for six animals, respectively. Succinate dehydrogenase activity in the hepatic mitochondria of control rats was not significantly altered by washing with 1.0 mM EGTA (Fig. 4A). The CT-induced increase of succinate dehydrogenase activity was completely reversed to the control level by washing of the mitochondria with 1.0 mM EGTA. The increased enzyme activity was clearly restored by addition of Ca^{2+} in the range of 2.5—250 μM (Fig. 4B). Maximal elevation was observed at 25 μM $Ca.^{2+}$ Thus, the CT-induced increase of succinate dehydrogenase activity in the hepatic mitochondria was related to the increase of calcium in the mitochondria.

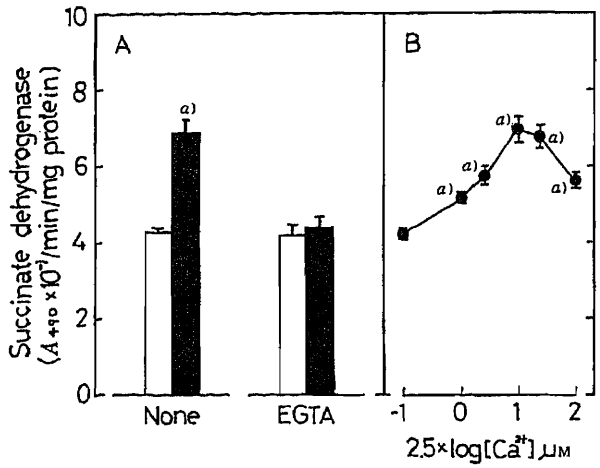

Fig. 4. Change of Succinate Dehydrogenase Activity by EGTA Washing of the Hepatic Mitochondria Obtained from Rats Administered with CT

A: Rats received a single subcutaneous administration of CT (80 MRC mU/100 g), and 30 min later they were sacrificed. The hepatic mitochondria were washed with 1.0 mM EGTA. B: Calcium ion (0.25—250 μM as final concentration) was added to the enzyme reaction mixture containing the mitochondria washed with 1.0 mM EGTA. Each value represents the mean ± S.E.M. of 6 experiments. *a)* $p<0.01$, compared with the control value. □, control; ■, CT.

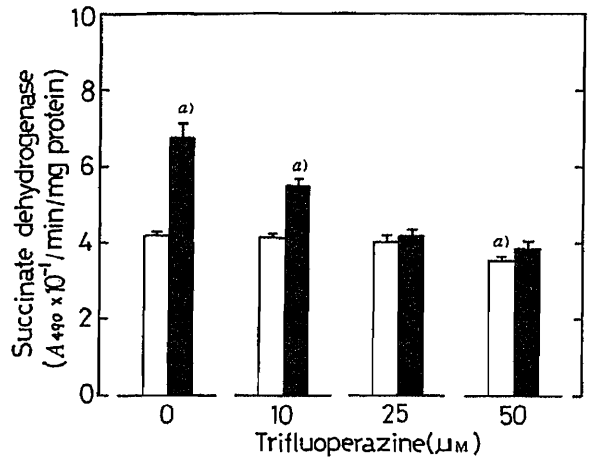

Fig. 5. Effect of Trifluoperazine on Succinate Dehydrogenase Activity Increased by Administration of CT

Rats received a single subcutaneous administration of CT (80 MRC mU/100 g), and 30 min later they were sacrificed and the hepatic mitochondria were obtained. Trifluoperazine (10—50 μM as final concentration) was added to the enzyme reaction mixture containing the mitochondria. Each value represents the mean ± S.E.M. of 6 animals. *a)* $p<0.01$, compared with the value in the absence of trifluoperazine. □, control; ■, CT.

TABLE I. Effects of Various Hormones on Succinate Dehydrogenase Activity in the Hepatic Mitochondria of Rats

Treatment	Dose per 100 g body weight	Succinate dehydrogenase ($A_{490}\times10^{-1}$/min/mg protein)	
		10 min	30 min
Control		4.46±0.09	4.40±0.11
CT	80 MRC mU	5.58±0.05[a)]	7.07±0.34[a)]
Insulin	0.1 U	5.47±0.18[a)]	5.95±0.07[a)]
	1.0 U	5.66±0.05[a)]	6.06±0.05[a)]
Thyroxine	1.0 μg	4.66±0.05	5.31±0.30
	10.0 μg	4.78±0.09	4.53±0.15
CT+insulin	1.0 U	5.69±0.13[a)]	7.21±0.22[a)]

Each value represents the mean ± S.E.M. of 5 animals. Rats received a single subcutaneous administration of various hormones, and 30 min later they were sacrificed. *a)* $p<0.01$, compared with the control value.

Effect of a Calmodulin Inhibitor on Succinate Dehydrogenase Activity in the Hepatic Mitochondria

The effect of trifluoperazine, a calmodulin inhibitor, on succinate dehydrogenase activity in the hepatic mitochondria was examined (Fig. 5). The mitochondrial succinate dehydrogenase activity of control rats was not significantly decreased by the presence of 10 or 25 μM trifluoperazine in the enzyme reaction mixture, although the enzyme activity was significantly inhibited by 50 μM trifluoperazine. In the hepatic mitochondria obtained from CT (80 MRC mU/100 g)-administered rats, addition of 25 and 50 μM trifluoperazine caused a complete suppression of the enzyme activity.

TABLE II. Effect of EGTA Washing on Succinate Dehydrogenase Activity in the Hepatic Mitochondria Obtained from Rats Administered with Insulin

Treatment		Calcium (nmol/mg protein)	Succinate dehydrogenase ($A_{490} \times 10^{-1}$/min/mg protein)
Control		4.35±0.37	4.51±0.09
Insulin	None	6.12±0.47[a]	6.01±0.12[a]
	EGTA washing	4.70±0.39	4.43±0.17

Each value represents the mean±S.E.M. of 5 animals. Rats received a single subcutaneous administration of insulin (1.0 U/100 g), and 30 min later they were sacrificed. The hepatic mitochondria was washed with or without 1.0 mM EGTA. *a*) $p < 0.01$, compared with the control value.

Effect of Other Hormones on Succinate Dehydrogenase Activity in the Hepatic Mitochondria

The change of succinate dehydrogenase activity was examined at 10 and 30 min after administration of insulin or thyroxine (Table I). Administration of insulin (0.1 and 1.0 U/100 g) produced a significant increase in the enzyme activity at 10 and 30 min after the treatment. Administration of thyroxine (1.0 and 10 μg/100 g) did not cause a significant increase in succinate dehydrogenase activity. Simultaneous administration of CT (80 MRC mU/100 g) and insulin (1.0 U/100 g) could not enhance succinate dehydrogenase activity either additively or synergistically in the hepatic mitochondria.

Administration of insulin (1.0 U/100 g) caused a significant increase of calcium content in the hepatic mitochondria (Table II). The increase in mitochondrial succinate dehydrogenase activity by insulin (1.0 U/100 g) was completely prevented by 1.0 mM EGTA washing of the mitochondria. Thus, the effect of insulin on the enzyme activity were related to the increase of calcium in the mitochondria by the hormone.

Discussion

It has been suggested that vasopressin or α-agonist induces an increase in the tricarboxylic acid cycle flux in hepatic mitochondria.[15,16] Currently, much interest is focussed on the possible stimulation of isocitrate dehydrogenase or 2-oxoglutarate dehydrogenase by an increased matrix free Ca^{2+} concentration in hepatic mitochondria.[16,17] Thus, mitochondrial enzyme may be regulated by hormone(s). CT can increase the clacium content in liver cells by stimulating calcium influx and inhibiting calcium efflux.[6] The effect of CT on hepatic mitochondrial function, however, has not been investigated fully. In the present study, it has been found that administration of CT produced an increase in calcium content and a corresponding elevation of succinate dehydrogenase activity in the hepatic mitochondria of intact and thyroparathyroidectomized rats. Since this enzyme is important in regulation of the electron transport system in the mitochondria of rat liver, it was suggested that CT may stimulate the electron transport system in the hepatic mitochondria of rats.

The increase in the hepatic mitochondrial succinate dehydrogenase activity induced by CT administration might be involved in the augmentation of calcium content in the mitochondria, because there was a corresponding elevation in the enzyme activity and the calcium content. The removal by EGTA of calcium in the hepatic mitochondria obtained from CT-administered rats completely suppressed the increase in succinate dehydrogenase activity. The increase of enzyme activity was clearly restored by addition of Ca^{2+} (2.5–25 μM). These results indicate that the increase in the hepatic mitochondrial succinate dehydrogenase activity following CT administration may be a result of the elevation of calcium content in the mitochondria. At present, we do not know the mechanism of CT action to increase mitochondrial calcium content. However, this increase may be attributed to the augmentation

of mitochondrial calcium uptake through stimulation of calcium entry into liver cells by CT.[6]

Trifluoperazine, a calmodulin inhibitor, completely inhibited the increase of succinate dehydrogenase activity in the hepatic mitochondria following CT administration to rats, although this drug is not a specific inhibitor for calmodulin. This result suggests that the CT effect on succinate dehydrogenase may depend on calmodulin, since it has been reported that rat hepatic mitochondria contain calmodulin.[18] Thus far, it is not known whether the hepatic mitochondrial enzyme is regulated by Ca^{2+}-calmodulin. It is possible that succinate dehydrogenase located on the inner membranes of hepatic mitochondria may be activated by Ca^{2+}-calmodulin. However, this remains to be elucidated.

Administration of insulin caused an increase in succinate dehydrogenase activity, while thyroxine had no effect. The effect of insulin might be mediated through calcium, since the hormone increased calcium in the mitochondria and EGTA treatment caused a complete suppression of the increase in succinate dehydrogenase activity. However, we do not know the mechanisms of insulin action to increase calcium content in the hepatic mitochondria of rats. Insulin may stimulate calcium entry into liver cells. Like CT, insulin could also regulate enzyme activity related to calcium. However, the combination of CT and insulin did not further enhance the hormonal effects. These results suggest that succinate dehydrogenase activity in the hepatic mitochondria is regulated by peptide hormone, and that a possible regulatory mechanism may be mediated through calcium.

In the previous study, it was found that CT can stimulate adenosine triphosphate synthesis dependently on calcium in the hepatic mitochondria of rats.[19] CT stimulated the mitochondrial oxidative phosphrylation.[19] The present finding, that CT increases succinate dehydrogenase activity dependently on calcium in the hepatic mitochondria, demonstrates that the hormone can also stimulate the mitochondrial electron transport system. These investigations support the view that CT plays a physiologically important role in the stimulation of hepatic mitochondrial function.

References

1) M. A. Aliapoulis, P. A. Goldhaber, and P. L. Munson, *Science*, **151**, 330 (1966).
2) M. Yamaguchi and K. Momose, *Endocrinol. Jpn.*, **29**, 553 (1982).
3) M. Yamaguchi and K. Momose, *Horm. Metabol. Res.*, **16**, 650 (1984).
4) M. Yamaguchi, K. Momose, and K. Takahashi, *Horm. Metabol. Res.*, **17**, 346 (1985).
5) M. Yamaguchi, Y. Takei, and T. Yamamoto, *Endocrinology*, **96**, 1004 (1975).
6) M. Yamaguchi and J. R. Williamson, *Horm. Metabol. Res.*, **15**, 176 (1983).
7) M. Yamaguchi and M. Ito, *Endocrinol. Jpn.*, **31**, 327 (1984).
8) M. Yamaguchi and M. Ito, *Acta Endocrinol.*, **110**, 124 (1985).
9) A. P. Halestrap, P. T. Quinlan, A. E. Armaton, and D. E. Whipps, *Biochem. Soc. Trans.*, **13**, 659 (1985).
10) J. G. McCormack and R. M. Denton, *Biochem. Soc. Trans.*, **13**, 664 (1985).
11) W. N. Taylor, E. Van De Pol, and F. L. Bygrave, *Biochem. J.*, **233**, 321 (1986).
12) G. H. Hogeboom, "Methods in Enzymology," Vol. 1, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York, 1955, p. 16.
13) R. J. Pennington, *Biochem. J.*, **80**, 649 (1961).
14) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
15) R. M. Denton, J. G. McCormack, and N. J. Edgell, *Biochem. J.*, **190**, 107 (1980).
16) R. S. Ochs, *J. Biol. Chem.*, **259**, 13004 (1981).
17) J. G. McCormack, *FEBS Lett.*, **180**, 259 (1985).
18) O. Hatase, A. Doi, T. Itano, H. Matsui, and Y. Ohmura, *Biochem. Biophys. Res. Commun.*, **132**, 63 (1985).
19) M. Yamaguchi and N. Ogawa, *Chem. Pharm. Bull.*, **35**, 4215 (1987).

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Conjugative Metabolism of 4-Methylumbelliferone in the Rat Liver: Verification of the Sequestration Process in Multiple Indicator Dilution Experiments

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By means of the multiple indicator dilution (MID) method, the sequestration process of 4-methylumbelliferone (4-MU), which is well known to be conjugated to glucuronide (4-MUG) and sulfate (4-MUS) in the rat liver, was found to be saturated as the dose was increased (*J. Pharmacokin. Biopharm.*, **15**, 25 (1987)). This observation might be attributed to the saturation of the conjugative metabolism. In the present study, using an *in vivo* tissue sampling single injection technique, we determined whether the sequestration process obtained by means of the MID method reflected the conjugative metabolism process. When the dose of 4-MU was increased from a low dose (110 μg/rat) to a high dose (3200 μg/rat), $FD_{app,4\text{-MUG}}$ and $FD_{app,4\text{-MUS}}$, which represent the fractions of the amounts of the formed 4-MUG and 4-MUS remaining in the liver, respectively, remarkably decreased. Since 4-MUG and 4-MUS formed from 4-MU have low diffusional clearances between the blood and hepatocytes, they are effectively trapped in the hepatocytes and therefore $FD_{app,4\text{-MUG}}$ and $FD_{app,4\text{-MUS}}$ represent the extents of the conjugative metabolism of 4-MU. Therefore, the dose-dependent change in the sequestration process was found to be attributable to the saturation of the conjugative metabolism. This observation confirms our previous hypothesis that the saturation of the sequestration process observed by using the MID method reflected that of the conjugative metabolism. Furthermore, according to the "sinusoidal" model, we simulated the time profiles of the amounts of drugs remaining in the liver, using parameters obtained from the MID data. The model, which has 20 sinusoidal compartments and can be regarded as identical to a "parallel tube" model, could reliably predict the time profiles of the amounts of drugs remaining in the liver.

Keywords——multiple indicator dilution method; sequestration; conjugative metabolism; sinusoidal model

Introduction

The rapid, single injection multiple indicator dilution (MID) method developed by Goresky *et al.*[1-3] is a very useful and elegant technique for the investigation of the uptake of materials from blood into the liver. It has provided an insight into the resistance to the influx and efflux processes at the cell membranes, in both the absence and presence of sequestration processes (intracellular metabolism, biliary excretion and/or intracellular transport). However, the parameters of the sequestration process obtained from the MID data in the case of low-clearance drugs, such as warfarin[4] and tolbutamide,[5] were usually much larger than those obtained from *in vivo* experiments,[6,7] since this method is performed within such a short period (30 s) that neither metabolism nor biliary excretion can be entirely determined for such low-clearance drugs. Unfortunately, it is still not clear what sequestration process is being measured, but in the case of a high-clearance drug, the sequestration process observed by means of the MID method might be considered to reflect metabolism and/or excretion processes. The purpose of the present study was to determine whether the sequestration

process obtained by using the MID method does reflect these processes in the case of a high-clearance drug.

4-Methylumbelliferone (4-MU), which is used therapeutically as a cholagogue, is well known to be substantially cleared by conjugative metabolism in the liver. It has often been used as a model compound in conjugation studies.[8,9] In our previous study, we found a remarkable dose-dependency in the hepatic elimination of 4-MU.[10] Moreover, utilizing the MID method, we found that the limiting step of the apparent hepatic intrinsic clearance of 4-MU changed with increasing dose owing to the saturation of the sequestration process.[11] Although we considered that the saturation of the sequestration process might be attributed to that of the conjugative metabolism, we have not demonstrated yet whether the metabolism of 4-MU actually occurs in such a short period (30 s). In the present study, using an *in vivo* tissue sampling single injection technique developed by Pardridge *et al.*,[12-15] we examined whether conjugative metabolism occurred within such a short period. Furthermore, using the parameters obtained from the MID data, we simulated the time profiles of the amounts of drugs remaining in the liver, according to the "sinusoidal" model,[16] which breaks up the sinusoid into a large number of sequentially perfused compartments with accompanying cellular compartments.

Experimental

Materials——4-MU, its glucuronide (4-MUG) and sulfate (4-MUS) and bovine serum albumin (BSA) (fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). Beta-Glucuronidase/arylsulfatase was purchased from Boehringer Mannheim GmbH (Mannheim, West Germany). All other reagents were commercial products of analytical grade.

***In Vivo* Tissue-Sampling Single Injection Technique**——The technique used in the present study was basically according to Pardridge *et al.*[12-15] Male Wistar rats (weighing 250—300 g) maintained on a normal laboratory diet, were used throughout. Under light ether anesthesia, a midline abdominal incision was made and the hepatic artery was ligated. Immediately after ligation of the hepatic artery, the portal vein was canulated with a 25-gauge needle and 300 μl of the injection solution (low dose, 110 μ/rat; high dose, 3200 μg/rat) was rapidly injected. The injection solution was made by mixing equal volumes of rat plasma and Krebs-Ringer buffer containing the test substance. At particular times (20, 40 and 60 s) after portal injection, the portal vein was rapidly cut off and the whole liver tissue was excised. Immediately after excision, the whole tissue was frozen in a bath of acetone cooled to -70 °C with dry ice and kept at -40 °C until assayed. The amounts of 4-MU taken up into the liver and its metabolites generated intracellularly were determined individually by using thin layer chromatography as described in detail previously.[17]

The fractions of the dose for 4-MU, 4-MUG and 4-MUS remaining in the liver after injection of 4-MU are designated as $FD_{app,4\text{-MU}}$, $FD_{app,4\text{-MUG}}$ and $FD_{app,4\text{-MUS}}$, respectively. It is assumed that no biliary excretion of the parent drug (4-MU) or its metabolites (4-MUG and 4-MUS) occurs during such a short period. No metabolism other than sulfation and glucuronidation occurs in the body.[10] Since 4-MUG and 4-MUS formed from 4-MU may be effectively trapped in the liver due to their low diffusional clearances between the blood and hepatocytes,[17] $FD_{app,4\text{-MUG}}$ and $FD_{app,4\text{-MUS}}$ may reflect the extents of the conjugative metabolism of 4-MU. Therefore, the sum of $FD_{app,4\text{-MUG}}$ and $FD_{app,4\text{-MUS}}$ was designated as the extent of the conjugative metabolism of 4-MU (E_{seq}). In this method, the recirculation of 4-MU might slight even at 60 s, since more than 90% of the injected 4-MU was still taken up in the liver at 40 s and the steady-state distribution volume of 4-MU (290 ml/kg) was very large.[10]

Furthermore, according to the "sinusoidal" model,[16] we simulated the time profiles of the amounts of drugs remaining in the liver, using parameters obtained from the MID data. The "sinusoidal" model breaks up the sinusoid into a large number of sequentially perfused compartments with accompanying cellular compartments. The system used for simulation was a portal vein bolus injection. For the sake of simplicity, several assumptions were made, as follows; no biliary excretion is assumed for the parent drug (4-MU) and its metabolites (4-MUG and 4-MUS) during such a short period. The volumes of blood and the liver, and the intrinsic clearance for the influx, efflux and sequestration processes are identical with regard to all compartments.

In the case of the n-compartment sinusoidal model, in which the sinusoid is broken up into n sequentially perfused compartments with accompanying cellular compartments, the following mass-balance equations hold in the i-th compartment: for the extracellular space

$$(V_B/n)\frac{dC_{B,i}}{dt} = -(Q + f_B \cdot CL_{int,n}^{inf}) \cdot C_{B,i} + CL_{int,n}^{eff} \cdot C_{L,i} + Q \cdot C_{B,i-1} \quad (1)$$

and for the intracellular space

$$(V_L/n)\frac{dC_{L,i}}{dt}=f_B\cdot CL_{int,n}^{inf}\cdot C_{B,i}-(CL_{int,n}^{eff}+CL_{int,n}^{seq})\cdot C_{L,i} \quad (2)$$

where Q is the hepatic blood flow; t is the time; $C_{B,i}$ and $C_{L,i}$ are the concentrations in the extracellular and intracellular spaces of the i-th compartment ($i=1\rightarrow n$), respectively; f_B represents the unbound fraction in the blood ($f_B=0.91$; *in vivo* experiments[17]) or the perfusate ($f_B=0.88$; perfusion experiments[11]); V_E and V_L are the volumes of the extracellular and intracellular spaces, respectively; $CL_{int,n}^{inf}$, $CL_{int,n}^{eff}$ and $CL_{int,n}^{seq}$ are the intrinsic clearances for the influx, efflux and sequestration processes in the n-compartment sinusoidal model, respectively. In the case of a portal vein injection, the above equations are integrated from $t=0$ to ∞. Subsequently, rearranging all equations, we obtained the dose-normalized AUC_n (area under the outflow fraction of dose (per ml) *v.s.* time) of the n-th compartment as follows:

$$AUC_n=\frac{Q^n}{(Q+f_B\cdot CL_{int,n}^{app})^n} \quad (3)$$

$$CL_{int,n}^{app}=\frac{CL_{int,n}^{inf}\cdot CL_{int,n}^{seq}}{CL_{int,n}^{eff}+CL_{int,n}^{seq}} \quad (4)$$

where $CL_{int,n}^{app}$ represents the apparent intrinsic clearance in the n-compartment sinusoidal model. Since AUC_n is identical to the instantaneous hepatic availability (F), the instantaneous hepatic extraction ratio (E) is expressed as follows:

$$E=1-\frac{Q^n}{(Q+f_B\cdot CL_{int,n}^{app})^n} \quad (5)$$

Rearranging Eq.5 to solve for $CL_{int,n}^{app}$ yields:

$$f_B\cdot CL_{int,n}^{app}=\frac{1-\sqrt[n]{1-E}}{Q\sqrt[n]{1-E}} \quad (6)$$

Using the previously reported E values ($E=0.89$ and 0.28 at low and high doses, respectively),[11] the values of $f_B\cdot CL_{int,n}^{app}$ were calculated at the low and high doses according to Eq.6. Although in our previous experiment,[11] the low dose was 50 μg/rat, we chose 110 μg/rat as the low dose in the present study, taking account of the sensitivity of the determination method of drugs in the liver. According to our preliminary experiments (unpublished observations), the extraction ratio of 4-MU was almost constant when the dose was increased to 300 μg/rat. Therefore, in the present case (110 μg/rat), kinetic parameters obtained from the MID analysis at the dose of 50 μg could be used for the simulation. Subsequently, assuming that the ratio among $f_B\cdot CL_{int,n}^{inf}$: $CL_{int,n}^{eff}$: $CL_{int,n}^{seq}$ is identical to that among the influx : efflux : sequestration intrinsic clearances ($CL_{int,inf}$, $CL_{int,eff}$ and $CL_{int,seq}$, respectively) obtained by means of the MID method, we obtained the parameters ($f_B\cdot CL_{int,n}^{inf}$, $CL_{int,n}^{eff}$ and $CL_{int,n}^{seq}$), using the following ratios[11]:

in the case of the low dose: $f_B\cdot CL_{int,n}^{inf}$: $CL_{int,n}^{eff}$: $CL_{int,n}^{seq}=4.7:1:1$

in the case of the high dose: $f_B CL_{int,n}^{inf}$: $CL_{int,n}^{eff}$: $CL_{int,n}^{seq}=15:5.0:1$

The fraction of the dose for 4-MU ($FD_{app,4\text{-}MU}$) and the extent of the conjugative metabolism (E_{seq}) were obtained by numerically solving all differential equations as described above from $t=0$ to 60 s, by the Runge–Kutta–Gill method at a suitable interval (1/1200 of 60 s) using a microcomputer. Parameters used in this simulation are as follows:

(1) $f_B\cdot CL_{int,n}^{inf}$, $CL_{int,n}^{eff}$ and $CL_{int,n}^{seq}$ were calculated as described above.
(2) Q is 0.025 ml/s g liver.[18,19]
(3) V_E and V_L are 0.227 ml/g liver and 0.593 ml/g liver, respectively.[11]

Results

In Vivo Tissue Sampling Single Injection Technique

The time courses of the fractions of the amount of 4-MU ($FD_{app,4\text{-}MU}$) and the sum of those of its formed metabolite (4-MUG and 4-MUS) remaining in the liver (E_{seq}) at 20, 40 and 60 s after the portal vein injection are summarized in Table I. The value of E_{seq} decreased remarkably as the dose was increased. Previously, we could not detect the conjugative

TABLE I. Results of the Uptake Study by an *in Vivo* Tissue-Sampling Single Injection Technique

Dose	Time	$FD_{app,4\text{-}MU}$ [a]	$FD_{app,4\text{-}MUG}$ [a]	$FD_{app,4\text{-}MUS}$ [a]	E_{seq} [b]
Low (110)	20	0.250±0.021	0.507±0.135	0.183±0.001	0.690±0.100
	40	0.195±0.019	0.419±0.088	0.224±0.014	0.643±0.066
	60	0.078±0.021	0.484±0.086	0.187±0.051	0.671±0.112
High (3200)	20	0.841±0.088[c]	0.062±0.005[c]	0.018±0.002[c]	0.080±0.005[c]
	40	0.705±0.056[c]	0.095±0.004[c]	0.017±0.002[c]	0.109±0.002[c]
	60	0.448±0.135[c]	0.124±0.009[c]	0.018±0.002[c]	0.142±0.006[c]

a) $FD_{app,4\text{-}MU}$, $FD_{app,4\text{-}MUG}$ and $FD_{app,4\text{-}MUS}$ represent the fractions of 4-MU, formed 4-MUG, and formed 4-MUS remaining in the liver, respectively. The parameters were expressed as mean±S.E. ($n=3$). *b*) E_{seq} was designated as the sum of $FD_{app,4\text{-}MUG}$ and $FD_{app,4\text{-}MUS}$. This value was assumed to be the extent of the conjugative metabolism of 4-MU. The parameters were expressed as mean±S.E. ($n=3$). *c*) Statistically significant ($p<0.01$) difference from the data at low dose.

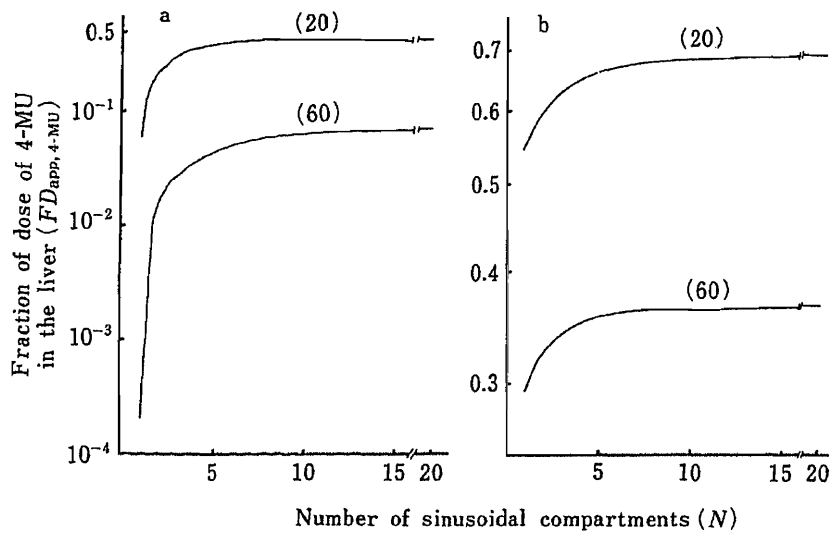

Fig. 1. Relationship between the Fraction of the Amounts of 4-MU in the Liver ($FD_{app,4\text{-}MU}$) and the Number of the Sinusoidal Compartments (N)

Panel a, low dose (110 μg); panel b, high dose (3200 μg). The numbers on figures show the time (s) after the portal vein bolus injection.

metabolites in the effluent perfusate within a short period (<30 s) by means of the MID method.[11] 4-MUG and 4-MUS were found to show low diffusional clearance between the blood and hepatocytes by using isolated hepatocytes. Consequently, the fractions of the amounts of 4-MUG and 4-MUS remaining in the liver ($FD_{app,4\text{-}MUG}$, $FD_{app,4\text{-}MUS}$, respectively) were considered to reflect the extents of the conjugative metabolism, on the ground that 4-MUG and 4-MUS formed from 4-MU were effectively trapped in the hepatocytes by the diffusional barrier between the blood and hepatocytes.[17] Thus, it was suggested that saturation of the conjugative metabolism actually occurred for a short period when the dose was increased, and the saturation of the sequestration process observed by means of the MID method might reflect that of the conjugative metabolism. The values of $FD_{app,4\text{-}MUG}$ at the low dose and $FD_{app,4\text{-}MUS}$ at the low and high doses were almost constant irrespective of time, whereas the value of $FD_{app,4\text{-}MU}$ decreased with time at both doses. On the other hand, the values of $FD_{app,4\text{-}MUG}$ at the high dose gradually increased with time, whereas the value of $FD_{app,4\text{-}MU}$ decreased.

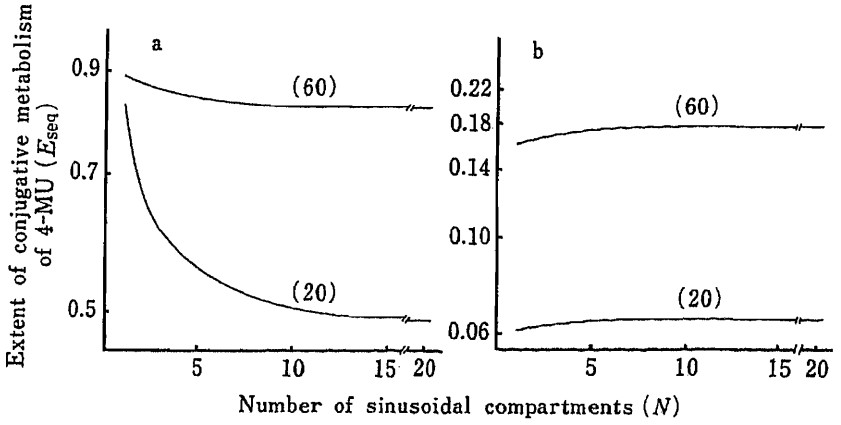

Fig. 2. Relationship between the Extent of Conjugative Metabolism (E_{seq}) and the Number of the Sinusoidal Compartments (N)

Panel a, low dose (110 μg); panel b, high dose (3200 μg). The numbers on figures show the time (s) after the portal vein bolus injection.

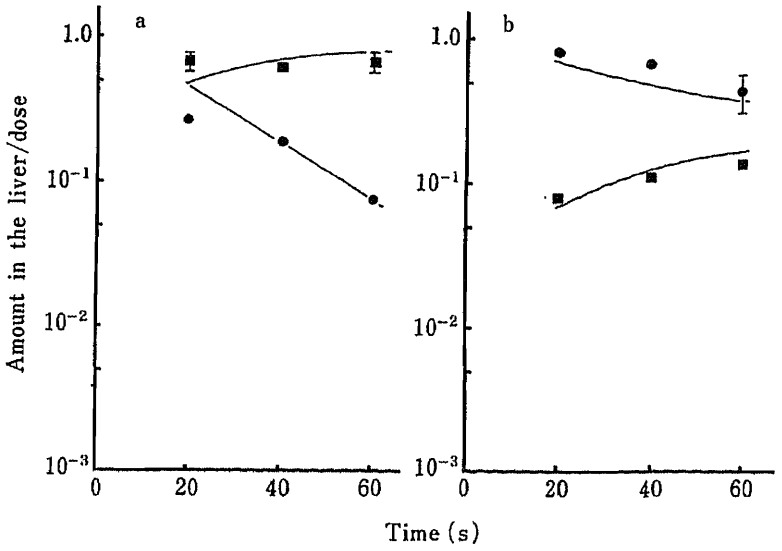

Fig. 3. Prediction of the 4-MU, Formed 4-MUG and Formed 4-MUS Remaining in the Liver Using the Parameters Obtained from the MID Data

Panel a, low dose injection (110 μg); panel b, high dose injection (3200 μg). The lines represent the simulation curves of the model, in which the sinusoid has 20 sinusoidal compartments. Each point represents the mean $\pm$S.E. of three experiments. (●), 4-MU; (■), 4-MUG+4-MUS.

Simulation Study

According to the "sinusoidal" model, we simulated $FD_{app,4\text{-}MU}$ and E_{seq}, using the parameters obtained from the MID data.[11] The relationships between $FD_{app,4\text{-}MU}$ and E_{seq} (20 and 60 s after bolus portal vein injection) and the sinusoidal compartment number (N) are depicted in Figs. 1 and 2. When the N value became more than 7, the values of $FD_{app,4\text{-}MU}$ and E_{seq} were almost constant irrespective of the N value. Since the concentration gradient along the hepatic blood flow path in the "sinusoidal" model becomes sharper as the N value is increased, this model might be considered to be identical with the "parallel" tube model, in the range of larger N value (more than 7). The results of simulation with the model, in which the sinusoid has 20 sinusoidal compartments, at low and high doses are depicted in Fig. 3. This model could predict the values of $FD_{app,4\text{-}MU}$ and E_{seq} well at both low and high doses.

Discussion

The MID method has been extensively used to clarify the physiology of various organs such as the heart,[20] kidney,[21] and liver.[1-3] This method has the advantage that three parameters with regard to the influx, efflux and sequestration (metabolism, biliary excretion and/or intracellular transport) processes are determined simultaneously and individually. However, the experimental period is so short that we cannot easily determine what the sequestration process is.

We will consider the relationship between the intrinsic clearance for the sequestration process observed by means of the MID method and that *in vivo*. The hepatic clearance (CL_H) is a function of the hepatic blood flow (Q_H), the blood unbound fraction (f_B) and the apparent intrinsic clearance ($CL^{app}_{int,H}$), and can be expressed according to the following two models[22]; namely, the "well-stirred" model, which describes the liver as a well-stirred compartment, and the "parallel tube" model, which describes the liver as a series of identical and parallel tubes with enzymes distributed evenly within hepatocytes lining the tubes. In these two models, CL_H is expressed by the following equations.[23] For the well-stirred model:

$$CL_H = \frac{Q_H \cdot f_B \cdot CL^{app}_{int,H}}{Q_H + f_B \cdot CL^{app}_{int,H}} \tag{7}$$

For the parallel tube model:

$$CL_H = Q_H \left(1 - \exp\left(-\frac{f_B \cdot CL^{app}_{int,H}}{Q_H}\right)\right) \tag{8}$$

For either model, $CL^{app}_{int,H}$ is expressed by

$$CL^{app}_{int,H} = \frac{CL^{inf}_{int} \cdot CL^{seq}_{int}}{CL^{eff}_{int} + CL^{seq}_{int}} \tag{9}$$

where CL^{inf}_{int}, CL^{eff}_{int} and CL^{seq}_{int} represent the intrinsic clearances for the influx, efflux and sequestration processes, respectively.

Using the reported values obtained from an *in vivo* experiment,[6] we estimated the value of $f_B \cdot CL^{app}_{int,H}$ for a low-clearance drug, warfarin, which is metabolized only by the liver and is used therapeutically as an anticoagulant drug. The total body clearance (CL_{tot}) calculated from AUC_{iv} (area under plasma concentration *vs.* time curve after intravenous injection) was very small (0.0025—0.010 ml/min) relative to Q_H (12—16 ml/min). The values of $f_B \cdot CL^{app}_{int,H}$ for such a low-clearance drug (warfarin) in both models are identical to that of CL_{tot}. On the other hand, the value of $f_B \cdot CL^{app}_{int,H}$ for warfarin calculated using parameters obtained from the MID data[7] according to Eq. 9, was much larger (10.1—25.4 ml/min) than that obtained from *in vivo* experiment. This overestimation might be related to the uncertainty in the kinetic parameter for the sequestration process during such a short experiment period (<30 s), since warfarin is a slowly metabolized drug. We found a similar overestimation in $f_B \cdot CL^{app}_{int,H}$ obtained from the MID method in the case of tolbutamide.[5,7]

Recently, we used the MID method to investigate the dose-dependency of the hepatic elimination for 4-MU, which is well known to be conjugated to glucuronide (4-MUG) and sulfate (4-MUS). In this experiment, we found that the sequestration process was saturated as the dose was increased, suggesting that the saturation of the sequestration process might be due to that of the conjugative metabolism. However, we could not measure the conjugative metabolites (4-MUG and 4-MUS) in the effluent perfusate and in the liver within such a short period (<30 s) and therefore, strictly speaking, we could not determine what the sequestration process reflected. In the present study, we examined whether conjugative metabolism occurred in the liver within such a short period by employing an *in vivo* tissue-sampling single injection

technique. As shown in Table I, the value of E_{seq} at the low dose (0.69) was much higher than that at the high dose (0.08) at 20 s. Thus, it was suggested that conjugative metabolism actually occurred during this short period and was saturated at the high dose. At the low dose, the value of E_{seq} was constant independent of time, whereas the value of $FD_{app,4\text{-}MU}$ decreased with time (Table I). In contrast, at the high dose, E_{seq} gradually increased, whereas the value of $FD_{app,4\text{-}MU}$ decreased as in the case of the low dose, although the absolute values of $FD_{app,4\text{-}MU}$ were different between the low and high doses (Table I). The difference in E_{seq} between the low and high doses might be explained as follows. At the low dose, 4-MU was removed to a large extent by the conjugative metabolism. On the other hand, at the high dose, a large amount of 4-MU remained in the liver due to the saturation of the conjugative metabolism. At the high dose, the conjugative metabolism might continue for 60 s and therefore, the value of E_{seq} gradually increased with time. In contrast, the value of $FD_{app,4\text{-}MU}$ decreased with time due to both wash-out with the hepatic blood flow and conjugative metabolism.

Furthermore, according to the "sinusoidal" model,[16] we simulated the fraction of 4-MU remaining in the liver and the extent of its conjugative metabolism in the liver, using the parameters obtained from the MID data. The "sinusoidal" model breaks up the sinusoid into a large number of sequentially perfused compartments with accompanying cellular compartments. The number of perfused compartments (N) within the sinusoid is an index of the degree of axial mixing in the model. Thus, as the N value increases to infinity, this model may approach the "parallel tube" model. When the N value was more than 7, the value of $FD_{app,4\text{-}MU}$ was constant irrespective of the N value (Fig. 1). The value of E_{seq} was also constant irrespective of the N value, when the N value was more than 7 (Fig. 2). Therefore, the sinusoidal model with more than 7 perfused compartments mimicks the "parallel tube" model. As shown in Fig. 3, the simulation curves based on the "sinusoidal" model with 20 perfused compartments predicted well the time-courses of the values of $FD_{app,4\text{-}MU}$ and E_{seq} at both low and high doses. In the present study, the kinetic parameters obtained from the MID data were used to simulate the *in vivo* data according to a first order kinetic model. The best model in this simulation is a "flow-limited distributed" model, incorporating nonlinearity of the efflux and sequestration processes. However, such a calculation for the intracellular concentrations of drugs has not been successful so far, due to the mathematical difficulties. Therefore, a simpler "sinusoidal" model with first-order kinetics was used in the present study. A similar approach, in which the dose-dependent hepatic uptake of sulfobromophthalein,[24] indocyanine green[25] or bile acid[26] was analyzed according to a first-order kinetic model, has often been used. Although such an approximation is convenient, we should be cautious in giving a quantitative interpretation to the kinetic parameters.

In conclusion, it is suggested that the sequestration rate constant obtained by means of the MID method for a highly cleared compound, 4-methylumbelliferone (4-MU), for a short period (<30 s) reflects the conjugative metabolic rate constant.

References and Notes

1) C. A. Goresky, W. H. Ziegler, and G. G. Bach, *Circ. Res.*, **17**, 739 (1970).
2) C. A. Goresky and B. E. Nadeau, *J. Clin. Invest.*, **53**, 634 (1973).
3) A. W. Wolkoff, C. A. Goresky, J. Sellin, Z. Gatmaitan, and I. M. Arias, *Am. J. Physiol.*, **236**, E638 (1979).
4) S. C. Tsao, Y. Sugiyama, Y. Sawada, S. Nagase, T. Iga, and M. Hanano, *J. Pharmacokinet. Biopharm.*, **14**, 51 (1986).
5) S. C. Tsao, Y. Sugiyama, Y. Sawada, T. Iga, and M. Hanano, Proceedings of the 104th Annual Meeting of Japanese Pharmaceutical Association, Sendai, 1984, p. 690.
6) A. Yacobi and G. Levy, *J. Pharm. Sci.*, **66**, 567 (1977).
7) O. Sugita, Y. Sawada, Y. Sugiyama, T. Iga, and M. Hanano, *Drug Metab. Dispos.*, **12**, 131 (1984).

8) G. J. Mulder, S. Brouwer, J. G. Weitering, E. Scholtens, and K. S. Pang, *Biochem. Pharmacol.*, **34**, 1325 (1985).
9) B. Andersson, M. Berggren, and P. Moldeus, *Drug Metab. Disposit.*, **6**, 611 (1978).
10) K. Morita, Y. Sugiyama, and M. Hanano, *J. Pharmacobio-Dyn.*, **9**, 117 (1986).
11) S. Miyauchi, Y. Sugiyama, Y. Sawada, K. Morita, T. Iga, and M. Hanano, *J. Pharmacokinet. Biopharm.*, **15**, 25 (1987).
12) W. M. Pardridge and L. S. Jefferson, *Am. J. Physiol.*, **228**, 1155 (1975).
13) W. M. Pardridge, *Am. J. Physiol.*, **232**, E492 (1977).
14) W. M. Pardridge and L. J. Mietus, *Am. J. Physiol.*, **237**, E367 (1979).
15) W. M. Pardridge, A. J. Van Herle, R. T. Naruse, G. Flierer, and A. Costin, *J. Biol. Chem.*, **258**, 990 (1983).
16) R. A. Weisiger, C. M. Mendel, and R. R. Cavalieri, *J. Pharm. Sci.*, **75**, 233 (1986).
17) S. Miyauchi, Y. Sugiyama, Y. Sawada, T. Iga, and M. Hanano, *J. Pharm. Sci.*, submitted.
18) C. V. Greenway and R. D. Stark, *Physiol. Rev.*, **51**, 23 (1971).
19) F. W. Ossenberg, P. Devis, and J. P. Benhamou, *J. Appl. Physiol.*, **37**, 806 (1974).
20) D. Cousineau, C. P. Rose, and C. A. Goresky, *Circ. Res.*, **49**, 501 (1981).
21) N. Ito, Y. Sawada, Y. Sugiyama, T. Iga, and M. Hanano, *Am. J. Physiol.*, **251**, F103 (1986).
22) K. S. Pang and M. Rowland, *J. Pharmacokinet. Biopharm.*, **5**, 625 (1977).
23) H. Sato, Y. Sugiyama, S. Miyauchi, Y. Sawada, T. Iga, and M. Hanano, *J. Pharm. Sci.*, **75**, 3 (1986).
24) C. A. Goresky, *Am. J. Physiol.*, **207**, 13 (1964).
25) G. Paumgartner and J. Reichen, *Experientia*, **31**, 306 (1975).
26) J. Reichen and G. Paumgartner, *Am. J. Physiol.*, **231**, 734 (1976).

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Preparation of Sustained-Release Suppositories Containing Microencapsulated Indomethacin and Bioavailability of Indomethacin in Rabbits[1)]

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The absorption of indomethacin (IM) from suppositories containing surface-modified IM microcapsules (MC) after rectal administration in rabbits was investigated with the aim of producing sustained-release suppositories. The IM-MC were prepared by phase separation of ethylcellulose (EC) from cyclohexane, and the IM surface was modified with a carboxy-vinyl polymer (Hiviswako® 104, HW) by a dry blend technique before encapsulation.

As a sustained plasma level of IM was not obtained when IM-MC-containing suppositories which showed a zero-order release profile were administered, the factors affecting the IM absorption, that is, the EC and HW contents in the MC, and the suppository base and type, were investigated. The EC and HW contents in the MC affected the IM release rate but had little effect on the IM absorption. When the IM-MC were directly administered, the IM plasma level was significantly lower than that after administration of the macrogol base suppository containing the IM-MC. The area under the concentration–time curve (AUC) in the case of the Witepsol® base was smaller than that in the case of the macrogol one. The plasma concentration–time curve of the hollow-type suppository showed a lag time, and the T_{max} was delayed by 1 h compared with that of the conventional suppository. Thus, it was found that a suitable kind and amount of suppository base and an appropriate suppository type should be selected to prepare sustained-release suppositories containing IM-MC.

Keywords——indomethacin; microcapsule; suppository; bioavailability; rabbit; sustained release; suppository base; surface modification

In the previous paper,[2)] we reported the preparation of sustained-release suppositories containing surface-modified indomethacin (IM) microcapsules (MC) and the release profile of IM from the suppository was shown to be zero-order. The IM surface was modified with a carboxy-vinyl polymer (Hiviswako® 104, HW) by a dry blend technique before encapsulation with ethylcellulose (EC) as a wall material. In this study, we investigated the absorption of IM from suppositories containing IM-MC in rabbits. Since there are some reports[3-7)] on the effects of suppository bases on drug absorption in animals, we used not only the macrogol base but also the Witepsol® base. In addition, hollow-type[3)] as well as conventional suppositories were used to investigate the effect of suppository type on the IM absorption in rabbits.

Experimental

Materials——The sources of materials used in this work were as follows: IM from Sumitomo Chemical Co., Ltd., EC (standard type, 100 cP) from Hercules Co., Ltd., polyethylene (Sun Wax® 131P, M.W. 3500) from Sanyo Kasei Co., Ltd., and HW from Wako Pure Chemical Industries Ltd. All other chemicals were reagent-grade commercial products.

Surface Modification of IM——This procedure was carried out as reported previously.[2] Thirty grams of IM and either 15 or 30 g of HW were mixed using an automatic ceramic mortar (Yamato-Nitto, Labo-mill UT-21, Yamato Kagaku Co., Ltd.) for 1 h at room temperature.

Preparation of the IM-MC——The IM-MC were prepared as reported previously.[2] Thirty grams of HW-modified IM [HW/IM = 1/2 or 1/1 (w/w)], 6—8 g of EC and cyclohexane (300 ml) containing 1% (w/v) polyethylene were placed in a 500 ml flask. The flask was heated to 80—82 °C, then cooled to 40 °C with continuous stirring (324 rpm) for 60 min, and finally cooled quickly to 25 °C. The microcapsules that formed were recovered by decantation, washed with cyclohexane and dried under reduced pressure. The MC were passed through JIS standard sieves.[2] The MC with a particle size range of 177 to 250 μm were used in the subsequent experiments.

Determination of IM Content——The IM content in IM-MC was determined spectrophotometrically.[2] The EC content was obtained by applying the following equation: $EC(\%) = [(MC - IM - HW)/MC] \times 100$ where MC, IM and HW are the weights of the substances. When IM and HW in the IM-MC were extracted with 0.2 M phosphate buffer and then the wall of the IM-MC was dried, the weight of the wall was almost the same as that obtained from the above equation. Thus, the weight of HW was calculated from its ratio to IM. The values of EC content of the MC were 20.1 ± 0.9 (S.D.)%, and 13.9 ± 1.0 (S.D.)% for the HW-modified IM-MC [EC 20%, HW/IM = 1/1 (w/w)], [IM-MC (I)] and [EC 14%, HW/IM = 1/2 (w/w)], [IM-MC (II)], respectively.

Preparation of Conventional and Hollow-Type Suppositories——Table I shows the suppository formulae. The conventional suppositories were made by the fusion method.[2] The bases (about 45 g) were fused in a beaker on an oil bath at 70 °C and cooled to 50 °C. Then, intact IM or IM-MC (1.5 g IM) was added to the bases and dispersed by stirring for 30 min. The fused bases containing intact IM or IM-MC were poured into suppository molds (1.5 ml in volume), which were quickly placed in a refrigerator at 5 °C. The hollow-type suppositories (macrogol base) were made by the method of Matsumoto *et al.*[3] The bases without IM-MC were melted and poured into smaller suppository containers (1.0 ml in volume). The containers were cooled to form hollow suppositories, IM-MC (IM 25 mg) were put into the hollow part, and the same melted bases were added to complete the hollow-type suppositories.

Release of IM from Suppositories——Release of IM was measured by the method of Muranishi *et al.*[8] as described previously.[2] The test solution was 0.2 M phosphate buffer solution (pH 7.2). A suppository was placed in 3 ml of the test solution in a cylindrical cell equipped with a Millipore filter (pore size 3.0 μm) and stirred with a rod at 25 rpm. The cell was connected with a glass vessel containing 300 ml of the test solution which was stirred with a magnetic stirrer at 100 rpm. The IM concentration was assayed spectrophotometrically at 318 nm.

Animal Experiments——White male rabbits each weighing 2.8—3.6 kg were fasted for 48 h prior to the experiments but allowed free access to water. About a half of the conventional suppository or about three-quarters of the hollow-type suppository (750 mg, 25 mg content of IM) was inserted by hand. The IM-MC (IM 25 mg) was directly inserted with a 2 ml syringe whose needle had been cut off. Retention of the suppositories and IM-MC by the rabbits was ensured by fastening the anus with a clip after insertion. Blood (2 ml) was taken by cardiac puncture from rabbits at different time intervals. The plasma was obtained by centrifugation at 3000 rpm for 10 min.

Assay of IM in Plasma——The plasma (0.7 ml) was pipeted into a glass stoppered centrifuge tube containing 2 ml of 0.2 M citrate buffer (pH 3.6) and 10 ml of ethyl acetate. The test tube was mechanically shaken for 10 min and then centrifuged at 3000 rpm for 10 min. Eight milliliters of the ethyl acetate phase was pipeted into the other centrifuge tube and evaporated to dryness under reduced pressure. The residue was dissolved in 250 μl of a mobile phase. A 20 μl sample was injected into the high performance liquid chromatography apparatus (Hitachi 655-12 liquid chromatograph with a Hitachi 655A variable-wavelength UV monitor). The conditions for analysis were as

TABLE I. Formulae of Suppositories

Macrogol type		Witepsol® type	
Component	mg	Component	mg
Microencapsulated IM	50.0[a]	Microencapsulated IM	50.0[a]
Glycerine	120.0	Dibutylhydroxytoluene	1.2
Dibutylhydroxytoluene	0.8	Light anhydrous silicic acid	30.0
Distilled water	30.0	Witepsol® E85	210.0
Hydrogenated castor oil	6.0	Witepsol® S55 Total	1500.0
POE[b] (40) monostearate	30.0		
Macrogol 1540	13.0		
Macrogol 6000	64.0		
Macrogol 4000 Total	1500.0		

a) IM content. *b*) Polyoxyethylene.

follows: column, 15cm×4mm i.d.; packing, TSK-LS 410 (5 μm) ODS; mobile phase, methanol–water–acetic acid–triethanolamine (74.3:25:0.5:0.2); flow rate, 0.5ml/min; wavelength, UV at 260nm; column temperature, 50°C.

Results and Discussion

Release and Absorption Studies on Suppositories Containing IM-MC (I)

In the release test, the conventional macrogol base suppository containing IM-MC (I) gave a zero-order release profile, as shown in Fig. 1A. In the absorption test, however, the IM plasma level was not well sustained, as shown in Fig. 2A and Table II. The area under the concentration–time curve (AUC) was calculated by means of the trapezoidal method. The AUC_0^8 value after administration of the suppositories containing IM-MC (I) was 26.91 μg·h/ml and it was about 76% ($p<0.05$) of the value of the suppositories containing intact IM. Although the suppositories containing IM-MC (I) showed a zero-order release profile in the release test, the IM plasma concentration was not well sustained; T_{max} was delayed only 30min compared with the suppositories containing intact IM. These results

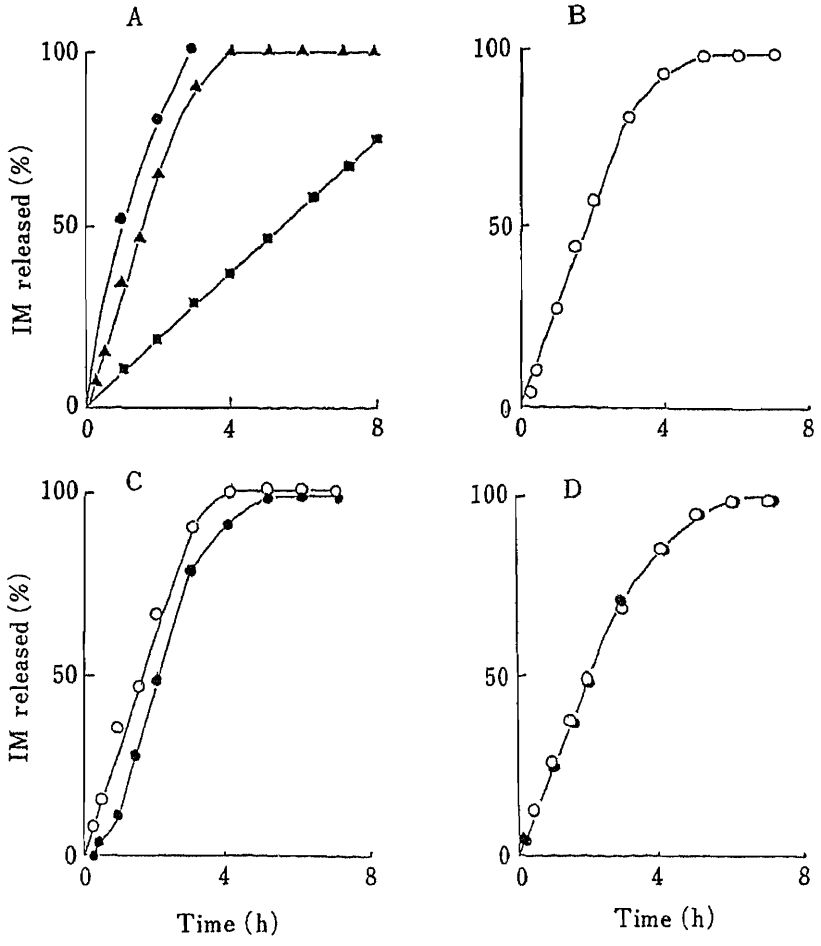

Fig. 1. Release Profiles of IM from Various Suppositories and IM-MC (II)

A: Conventional-type macrogol base suppositories. Content: ●, intact IM; ■, IM-MC (I) (EC 20%, HW/IM=1/1); ▲, IM-MC (II) (EC 14%, HW/IM=1/2).
B: IM-MC (II), ○.
C: Hollow- and conventional-type macrogol base suppositories containing IM-MC (II). Type: ●, hollow; ○, conventional.
D: Conventional-type Witepsol® base suppositories. Content: ●, intact IM; ○, IM-MC (II).
Each value represents the mean ($n=2$).

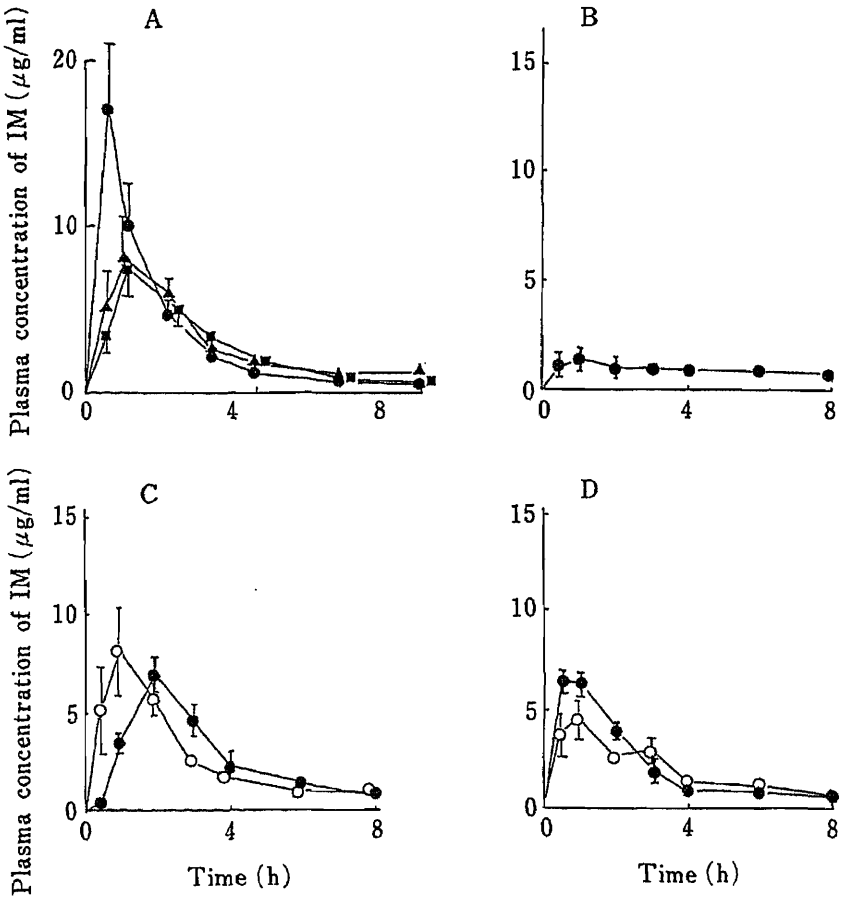

Fig. 2. Plasma Concentration of IM after Rectal Administration of Various Suppositories and IM-MC (II)

A: Conventional-type macrogol base suppositories. Content: ●, intact IM; ■, IM-MC (I) (EC 20%, HW/IM = 1/1); ▲, IM-MC (II) (EC 14%, HW/IM = 1/2).
B: IM-MC (II), ●.
C: Hollow- and conventional-type macrogol base suppositories containing IM-MC (II). Type: ●, hollow; ○, conventional.
D: Conventional-type Witepsol® base suppositories. Content: ●, intact IM; ○, IM-MC (II).
Each value represents the mean ± S.E. (n = 3—6).

suggest that the amount of the rabbit rectal fluid was less than that of the medium of the release test, and thus not all of the IM in IM-MC was released in the rabbit rectum.

Release and Absorption Studies on Conventional Macrogol Base Suppositories Containing IM-MC (II)

To obtain sufficient release of IM from the suppositories in the rabbit rectum, the EC and HW contents were reduced when the MC was prepared. Figures 1A and 2A and Table II also show the results of the release and absorption tests of the macrogol base suppositories containing IM-MC (II). The rate of the release of IM from the suppositories containing IM-MC (II) was faster than that from the suppositories containing IM-MC (I), and the time required to release 50% of IM from the suppository (t_{50}) was about 90 min. Umeda *et al.*[9] reported a sustained-release suppository containing microencapsulated IM with gelatin as a wall material. They found that the IM plasma level was sustained when a suppository with a t_{50} of about 90 min was administered to a rabbit, although the release test was not exactly the same as in our experiment. Thus, we used IM-MC (II) to obtain complete release of IM from the suppositories in the rabbit rectum. As shown in Fig. 2B, however, the IM plasma levels of

TABLE II. Pharmacokinetic Parameters of IM after Rectal Administration in Rabbits

Base	Type	Containing	C_{max} (μg/ml)	T_{max} (h)	AUC_0^8 (μg·h/ml)	BA[d] (%)	n
Macrogol	Conventional	Intact IM	17.11±3.91	0.5	26.91±5.42	100	6
		IM-MC (I)[a]	7.27±1.61	1	20.70±0.81	76.9	3
		IM-MC (II)[b]	9.07±1.63	1	23.45±2.52	87.1	3
	Hollow	IM-MC (II)	6.83±0.93	2	21.13±3.17	78.5	3
Witepsol®	Conventional	Intact IM	6.61±0.62	0.5	17.50±0.84	65.0	3
		IM-MC (II)	4.97±0.73	1	15.87±0.38	59.0	3
Microcapsule[c]		IM-MC (II)	1.26±0.64	1	5.66±2.20	21.0	3

a) HW-modified IM-MC (EC 20%, HW/IM = 1/1). *b*) HW-modified IM-MC (EC 14%, HW/IM = 1/2). *c*) IM-MC (II) were directly administered into the rectum. *d*) BA, the ratio to the AUC_0^8 of the conventional-type macrogol base suppository containing intact IM. Each value of C_{max} and AUC_0^8 represents the mean±S.E. (n = 3—6).

the suppositories containing IM-MC (II) were not well sustained, and the AUC_0^8 did not increase significantly compared with the case of IM-MC (I). The reduction in EC and HW contents in IM-MC (II) did not result in a sustained IM plasma level after rectal administration.

Release and Absorption Studies on IM-MC (II)

The IM release test and the IM absorption test were carried out with IM-MC (II) to avoid the effect of suppository bases. Figures 1B and 2B and Table II show the results. The release rates were almost the same as those of the suppositories containing IM-MC (II). On the other hand, the IM plasma levels showed a sustained-release pattern, although the IM plasma levels and the AUC_0^8 were lower and smaller than those of the suppositories containing IM-MC (II). These results indicate that the IM release was suppressed by the microencapsulation of IM, but that the suppository bases considerably affected the IM release and absorption in the rabbit rectum.

Release and Absorption Studies on Hollow-Type Suppositories Containing IM-MC (II)

The IM plasma levels of the suppositories containing IM-MC (II) were higher than those of IM-MC (II), as shown in Fig. 2A and 2B. One of the reasons was thought to be the effect of the suppository base on the IM absorption. Another reason might be that part of the IM in the IM-MC (II) was released in the suppository base while the suppositories were being prepared. Thus, the application of the hollow-type suppository was investigated to eliminate the putative release of IM into the suppository base during preparation. Figures 1C and 2C and Table II show the results of the release and absorption studies of hollow-type macrogol base suppositories containing IM-MC (II). The IM release profiles of the hollow-type suppositories showed a short lag time but were not very different from those of the conventional suppositories containing IM-MC (II); the t_{50} was delayed about 30 min compared with that of the conventional suppositories.

The IM plasma concentration–time curves of the hollow-type suppositories also showed a lag time corresponding to the release profiles. The T_{max} of the hollow-type and the conventional suppositories were 2 and 1 h, respectively. The amount of release of IM from IM-MC (II) while the suppositories were being prepared was not considered to be significant because the AUC_0^8 of the conventional suppositories containing IM-MC (II) was not significantly different from that of the hollow-type ones, and the patterns of the IM plasma concentration–time curves were almost the same except for the lag time. The hollow-type suppositories containing IM-MC (II) gave a delayed T_{max} in the absorption test.

Release and Absorption Studies on Conventional-Type Witepsol® Base Suppositories Containing IM-MC (II)

Watanabe *et al.*[3] reported that the IM serum level obtained with the water-soluble base suppository was higher than that with the Witepsol® base suppository, and Vidras *et al.*[6] reported that the IM serum level obtained with a Witepsol® base suppository was higher than that with a macrogol base suppository up to 30 min after rectal administration. Thus, Witepsol® base suppositories containing IM-MC (II) were also examined. Figures 1D and 2D and Table II show the results of the release and absorption tests on Witepsol® base suppositories containing intact IM and IM-MC (II). The IM release profile of the Witepsol® base suppository containing IM-MC (II) was almost the same as that of the suppository containing intact IM. The IM release rate from the Witepsol® base suppositories containing intact IM or IM-MC (II) was somewhat slower than that from the macrogol base suppositories, as shown in Fig. 1A. The IM plasma level of the Witepsol® base suppositories containing IM-MC (II) was lower than that of the suppositories containing intact IM and was not well sustained; T_{max} was delayed only 30 min. The AUC_0^8 of the Witepsol® base suppositories was lower ($p < 0.05$) than that of the macrogol base ones containing intact IM or IM-MC (II). These results correspond to those of Watanabe *et al.*,[3] but not to those of Vidras *et al.*[6]

Tsuchiya *et al.*[7] reported that the secretion of the rectal fluid varied with the kind and amount of the suppository base; the rectal fluid volume increased with increase in the amount of the macrogol base, and there was no detectable amount of fluid but some mucus in the rectum after administration of an oleaginous base suppository.

These findings help to explain our experimental results. As hardly any rectal fluid was secreted, the IM plasma level and the AUC_0^8 were low when IM-MC (II) and the Witepsol® base suppository containing IM-MC (II) were administered. On the other hand, when the macrogol base suppository containing IM-MC (II) was administered, enough rectal fluid was secreted. Thus the IM plasma level was high because IM was released well. It seems clear that when IM-MC are used to prepare sustained-release suppositories, a suitable kind and amount of suppository base and an appropriate suppository type should be selected. For instance, the combinations of conventionl- and hollow-type suppositories with Witepsol® and macrogol base could be useful for the production of sustained-release suppositories containing HW-modified IM-MC.

References and Notes

1) This work was presented at the 2nd Annual Meeting of the Academy of Pharmaceutical Science and Technology of Japan, Tokyo, September 1986.
2) T. Nakajima, Y. Takashima, K. Iida, H. Mitsuta and M. Koishi, *Chem. Pharm. Bull.*, **35**, 1201 (1987).
3) Y. Watanabe, K. Yokoyama, M. Yamaji, F. Tanaka and M. Matsumoto, *Yakugaku Zasshi*, **105**, 278 (1985); Y. Watanabe, Y. Tone, M. Suzuki and M. Matsumoto, *ibid.*, **105**, 284 (1985).
4) T. Ogiso, M. Iwaki and E. Tamaki, *J. Pharmacobio-Dyn.*, **7**, 392 (1984).
5) T. Fukuda, Y. Takeuchi, Y. Hanyu, M. Yamashita, I. Kaneko, K. Tahira and Y. Fukumori, *Yakuzaigaku*, **40**, 167 (1980).
6) N. J. Vidras, V. E. Reid, N. R. Bohidar and F. M. Plakogiannis, *J. Pharm. Sci.*, **71**, 945 (1982).
7) S. Tsuchiya, M. Hiura and H. Matsumaru, *Chem. Pharm. Bull.*, **25**, 667 (1977).
8) S. Muranishi, Y. Okubo and H. Sezaki, *Yakuzaigaku*, **39**, 1 (1979).
9) T. Umeda, A. Matsuzawa, T. Yokoyama, K. Kuroda and T. Kuroda, *Chem. Pharm. Bull.*, **31**, 2793 (1983); T. Kuroda, T. Yokoyama, T. Umeda, A. Matsuzawa, K. Kuroda and S. Asada, *ibid.*, **31**, 3319 (1983).

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Measurement of Acid Strength of Excipients by Photoacoustic Spectroscopy

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Acid–base properties of excipient surfaces have been studied by photoacoustic spectroscopy (PAS) in the visible absorption range using seven acid–base indicators. The acid strength (H_0) of each excipient was determined from the PA spectral patterns of the indicators. The H_0 values of silica–alumina, silica–magnesia and crystalline cellulose were determined to be between 3.3 and −3.0, between 4.8 and 2.0, and between 4.8 and 3.3, respectively. In the cases of some excipients, the acid strength measured by PAS was weaker than the value which was measured from the color changes of the indicators on the surface of the excipients in benzene solution. These differences are ascribed to water adsorption on the surface during PAS sample preparation. In the case of crystalline cellulose, the hydrogen bonding network was so tight in benzene solution that the indicators could not enter the network.

Keywords——photoacoustic spectroscopy; acid strength; excipient; silica–alumina; silica–magnesia; talc; crystalline cellulose; methylcellulose

Photoacoustic spectroscopy (PAS) is an effective technique to study solid samples, because the absorption spectra of opaque samples can be easily measured by PAS.[1,2] Recently, Jagannathan *et al.* studied the acid–base properties of catalysts by PAS and estimated the acidic sites of oxide catalysts quantitatively.[3]

The physicochemical properties of drug excipients have significant effects on the stability and the bioavailability of drugs in the dosage forms. The surface acid–base properties of excipients are known to affect strongly the drug stability. In this study, we investigated the application of PAS to the determination of the surface acid–base properties of drug excipients. The acid strength (H_0) was used for the quantitative evaluation of acid–base properties.[4]

Experimental

Materials——Methyl red (MR), methyl yellow (MY), 2-amino-5-azotoluene (AAT), benzeneazodiphenylamine (BAD), dicinnamalacetone (DIC), benzalacetophenone (BAP), anthraquinone (ATQ), methylcellulose (400 cP), talc, kaolin and silica–alumina were purchased from Wako Pure Chemical Industries Ltd. Magnesium oxide and montmorillonite were purchased from Nakarai Chemicals Co., Ltd. Crystalline cellulose (PH-101, Asahi Chemical Industries), carbon black (Koso Chemical Co., Ltd.) and silica–magnesia (Kyowa Chemical Industrial Co., Ltd.) were also used. Sodium montmorillonite and sodium kaolin were prepared as follows. Montmorillonite and kaolin were washed with 1 N sodium chloride solution five times, then with distilled water, and dried.

All excipients were dried in the following ways. Silica–alumina and silica–magnesia were dried at 550 °C for 3 h. Sodium montmorillonite and sodium kaolin were dried at 100 °C overnight. Magnesium oxide was dried at 900 °C for 3 h. Talc was dried at 105 °C for 3 h. Crystalline cellulose and methylcellulose were dried at 100 °C for 3 h *in vacuo*. Other reagents were used without further purification.

Absorption Spectra——A Hitachi type 557 spectrometer was used for the measurements of absorption spectra of each indicator in the acidic solvents and in the basic solvents. The compositions of the acidic solvents and the basic

TABLE I. Acidic Solvents and Basic Solvents for Each Indicator

Acid–base indicator	Acidic solvent	Basic solvent
MR	1 N HCl and C_2H_5OH (50% v/v)	0.1 N NaOH and C_2H_5OH (50% v/v)
MY	1 N HCl and C_2H_5OH (50% v/v)	0.1 N NaOH and C_2H_5OH (50% v/v)
AAT	20% H_2SO_4	0.1 N NaOH and C_2H_5OH (50% v/v)
BAD	1 N HCl and C_2H_5OH (50% v/v)	0.1 N NaOH and C_2H_5OH (50% v/v)
DIC	95% H_2SO_4	2% H_2SO_4
BAP	95% H_2SO_4	5% H_2SO_4
ATQ	95% H_2SO_4	5% H_2SO_4

solvents used are shown in Table I. Each indicator takes the acid form in the acidic solvents and the base form in the basic solvents. The concentration of indicators was usually 5 mg/l. The sample solutions were stored in the dark for 3 h before the measurements.

Photoacoustic Spectroscopy——The single-beam photoacoustic spectrometer was constructed in our laboratory out of the following parts.[5] The light source was a high pressure 500 W xenon arc lamp (UXL-500D, Ushio Inc.) equipped with a monochromator (G250, Nikon). The light was modulated by a light chopper (CH-353, NF Circuit Design Block Co., Ltd.) and the modulation frequency was 16 Hz. A microphone (EPM-100, Nippon Chemicon Co.) with a lock-in amplifier (LI-574A, NF Circuit Design Block Co., Ltd.) was used as the detector. Sample powder (10—40 mg) was loaded in the sample holder, which was put in the PAS cell (made of brass).[6] The scanning speed of the monochromator was 30 nm/min. From the amplitude of the photoacoustic (PA) signal, the relative PA signal was calculated every 5 nm by means of the following equation.[7]

relative PA signal = (PA signal of the indicator on the excipient surface
− PA signal of the excipient)/(PA signal of carbon black
− PA signal of calcium fluoride)

As reference materials, carbon black and calcium fluoride with particle diameters of less than 63 μm were used.

Measurement of PA Spectra of Excipients——One gram of a dried excipient and 5 ml of benzene solution of an indicator were put into the test tube, and incubated for 24 h at 30 °C. The excipient powder was collected by filtration and dried at 30 °C for 1 h *in vacuo*. Then the PA spectrum of the indicator on the excipient surface was measured in the visible region (350—600 nm).

Measurement of PA Spectra of Ground Mixtures of Indicator and Excipient——A mixture of dried excipient (2 g) and indicator (0.5 mg) was ground for 10 min in a vibrating mill (TI-200, Heiko Seisakusho, Ltd.), then the PA spectrum of the ground mixture was measured.

Measurement of Acid Strength of Excipients in Benzene Solution——The acid strength of excipients in benzene solution was measured according to Benesi's method.[8]

Results and Discussion

Absorption spectra of the seven indicators in the acidic and basic solvents were measured as shown in Fig. 1. The wavelengths of absorption maxima (λ_{max}) are presented in Table II. Although the azo indicators are known to be light-sensitive,[9,10] the effects of *trans–cis* isomerization were negligible in this experiment. In the cases of ATQ and BAP, the wavelengths of absorption peaks in the basic solvents were shorter than 350 nm, and they were not determined in this experiment.

Photoacoustic spectra of seven indicators adsorbed on the silica–alumina surface are shown in Fig. 2. For ATQ, BAP and DIC, no peaks were observed in this wavelength region. From the spectral coincidence between the PA and the absorption of the base form in solution, it was found that ATQ, BAP and DIC molecules (Fig. 2e—g) on the silica–alumina surface existed in the base form. The PA peaks of BAD and AAT were observed at 525 and 500 nm, respectively (Fig. 2c and d). These peak positions show that the indicator molecules of BAD and AAT mainly take the acid form on the silica–alumina surface, although the presence of the base form of BAD and AAT molecules on the silica–alumina surface was also

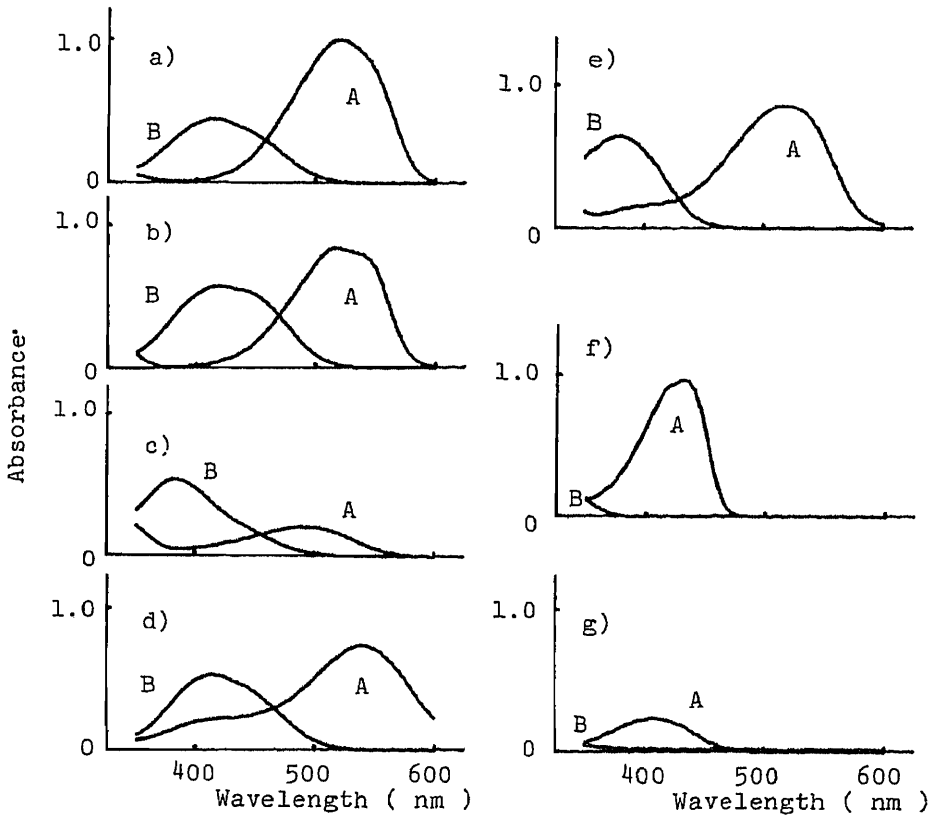

Fig. 1. Absorption Spectra of Indicators

a) MR; b) MY; c) AAT; d) BAD; e) DIC; f) BAP; g) ATQ. Curves A show the absorption spectra in acidic solvents, and curves B show the absorption spectra in basic solvents.

TABLE II. The pK_a Values and the Wavelength of Absorption Maxima (λ_{max}) of Each Indicator

Acid–base indicator	pK_a	λ_{max} in the acidic solvent (nm)	λ_{max} in the basic solvent (nm)
MR	4.8	519	415
MY	3.3	517	418
AAT	2.0	494	384
BAD	1.5	540	415
DIC	−3.0	519	380
BAP	−5.6	432	—
ATQ	−8.2	410	—

clear. The PA peaks of PA signal of MR and MY were observed at 515 and 510 nm, respectively (Fig. 2a and b), and therefore the MR and MY molecules on the silica–alumina surface were considered to be in the acid form. These results led to the conclusion that the Hammett acid strength, H_0, of the silica–alumina surface was between 3.3 and −3.0. This range is somewhat different from the value which was measured by Benesi using the color changes of indicators in benzene solution.[8] He reported that the H_0 of silica–alumina was stronger than −8.2. As this difference in H_0 values could be due to the different sources of silica–alumina samples, the H_0 value of silica–alumina was measured by the same method as reported by Benesi. The H_0 value of the silica–alumina used in this experiment was determined to be stronger than −8.2 by the benzene solution method, and it was found that

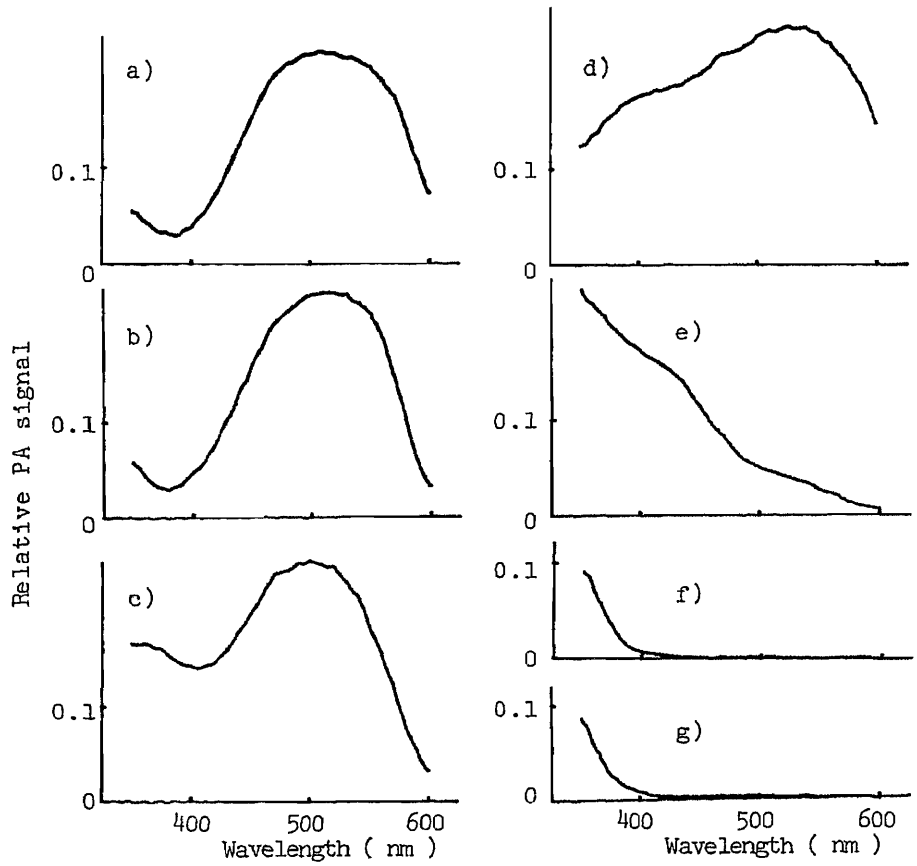

Fig. 2. Photoacoustic Spectra of Indicators on a Silica–Alumina Surface
a) MR; b) MY; c) AAT; d) BAD; e) DIC; f) BAP; g) ATQ.

the PAS method generally tended to give a weaker acid strength value than the conventional benzene solution method. Uma *et al.* reported that when silica–alumina wetted with benzene was exposed to the air, the acidic sites of silica–alumina were affected by moisture.[11] Therefore the difference of the acid strength determined by the PAS method and by the benzene solution method could be attributed to the adsorption of water at the acidic sites of silica–alumina during the sample preparation for PAS.

Previously, Vorob'ev *et al.* measured the visible spectra of some indicators on a silica–alumina surface using the reflectance method.[12] The reflectance spectra of MR and BAD on silica–alumina showed the same pattern as the PA spectra. They reported that MR took the acid form and BAD was present as a mixture of the acid and the base forms on a silica–alumina surface, which is in agreement with the PAS results.

Photoacoustic spectra of indicators on the silica–magnesia surface are shown in Fig. 3. The photoacoustic spectrum of MR on the silica–magnesia surface had a peak at 530 nm, and the absorption spectrum of MR in an acidic solvent had a peak at the same wavelength. This might be due to the formation of the acid form of MR molecules on the silica–magnesia. From a comparison of PA and absorption spectra, it can be said that AAT took the base form on the silica–magnesia surface. Consequently, the H_0 value was determined to be between 4.8 and 2.0 for the silica–magnesia.

Photoacoustic spectra of indicators on sodium montmorillonite are shown in Fig. 4. The PA spectra of MR, MY and AAT had peaks at 565, 500 and 535 nm, respectively. These three indicators on the sodium montmorillonite surface were found to be present in the acid form by comparison with the spectral patterns in acidic solvents for each indicator. While DIC,

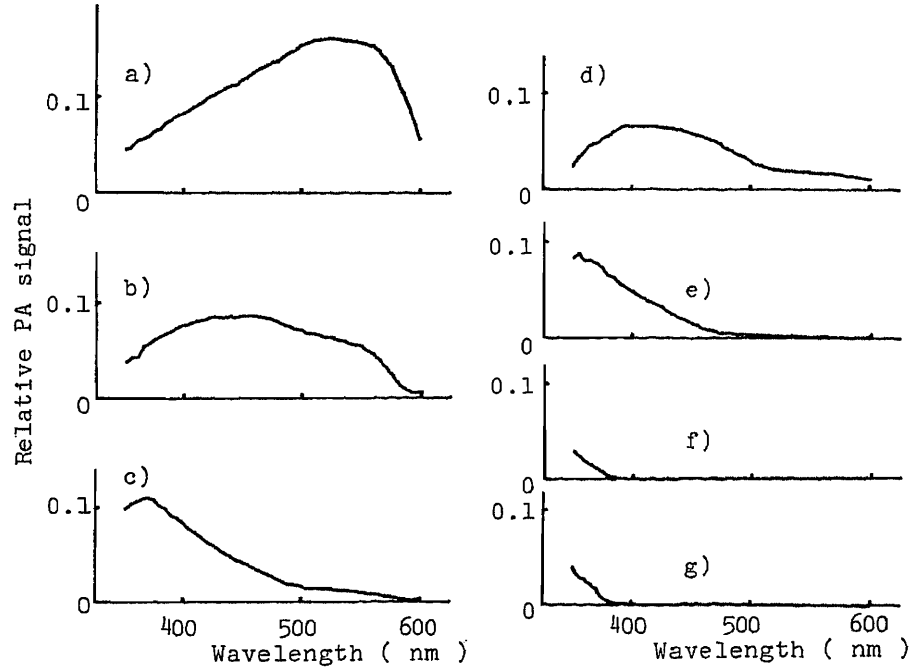

Fig. 3. Photoacoustic Spectra of Indicators on a Silica–Magnesia Surface
a) MR; b) MY; c) AAT; d) BAD; e) DIC; f) BAP; g) ATQ.

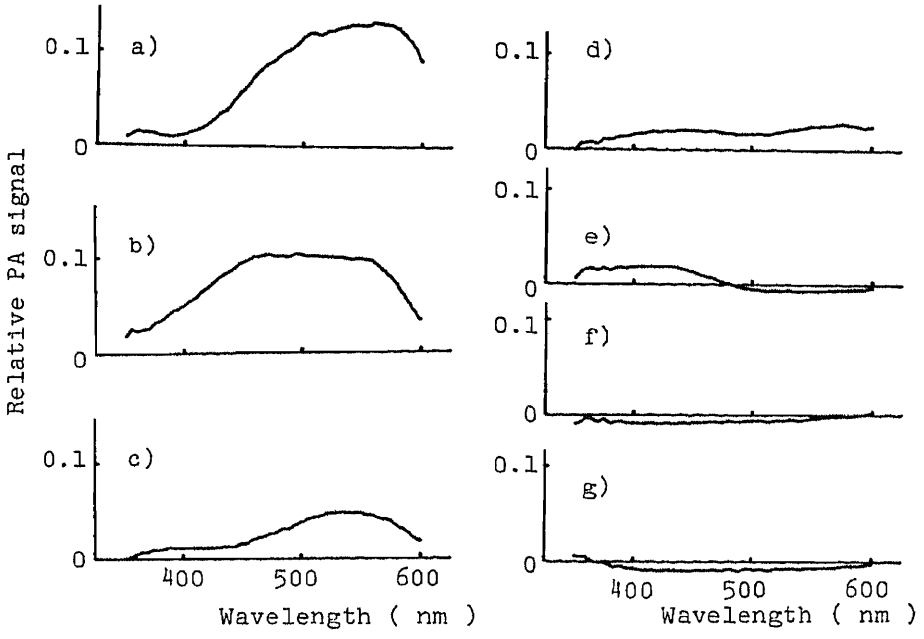

Fig. 4. Photoacoustic Spectra of Indicators on a Sodium Montmorillonite Surface
a) MR; b) MY; c) AAT; d) BAD; e) DIC; f) BAP; g) ATQ.

BAP and ATQ were in the base form on the sodium montmorillonite surface, BAD molecules were considered to be in both acid and base forms on the sodium montmorillonite surface. The acid strength of sodium montmorillonite, H_0, was estimated to be between 2.0 and -3.0.

Table III shows the peak positions in the PA spectra of excipients, including other excipients which were not mentioned above. The peak positions of each indicator showed significant variations between excipients. Table IV shows the molecular forms of indicators

TABLE III. The Wavelength of Maximum Relative Photoacoustic Signals for Each Indicator

Excipient	Wavelength (nm)						
	MR	MY	AAT	BAD	DIC	BAP	ATQ
Silica–alumina	510	515	500	525	—	—	—
Silica–magnesia	530	455	370	415	—	—	—
Na-montmorillonite	565	500	535	580	420	—	—
Na-kaolin	525	495	500	540	360 540	—	—
Talc	525	525	380	425 560	365	—	—
Magnesium oxide	410	405	410	430	375	—	—
Crystalline cellulose	500 525[a]	400	380	415	365	—	—
Methylcellulose	460	375 440	355 430	380 445	370	—	—

a) MR was adsorbed in ethanol.

TABLE IV. Molecular Forms of Indicators and H_0 Values of the Excipients Determined by Photoacoustic Spectroscopy

Excipient	Indicators							H_0
	MR	MY	AAT	BAD	DIC	BAP	ATQ	
Silica–alumina	A	A	AB	AB	B	B	B	3.3——3.0
Silica–magnesia	A	AB	B	B	B	B	B	4.8—2.0
Na-montmorillonite	A	A	A	AB	B	B	B	2.0——3.0
Na-kaolin	A	A	A	A	AB	B	B	1.5——5.6
Talc	A	AB	AB	AB	B	B	B	4.8——3.0
Magnesium oxide	B	B	B	B	B	B	B	>4.8
Crystalline cellulose	A	B	B	B	B	B	B	4.8—3.3
Methylcellulose	B	B	B	B	B	B	B	>4.8

A, acid form; B, base form; AB, mixture of acid and base forms.

TABLE V. Molecular Forms of Indicators and H_0 Values of the Excipients Determined by Benesi's Method

Excipient	Indicators							H_0
	MR	MY	AAT	BAD	DIC	BAP	ATQ	
Silica–alumina	A	A	A	A	A	A	A	<−8.2
Silica–magnesia	A	A	A	A	B	B	B	1.5——3.0
Na-montmorillonite	A	A	A	A	B	B	B	1.5——3.0
Na-kaolin	A	A	A	A	A	B	B	−3.0——5.6
Talc	A	A	A	A	B	B	B	1.5——3.0
Magnesium oxide	B	B	B	B	B	B	B	>4.8
Crystalline cellulose		B	B	B	B	B	B	>3.3
Methylcellulose		B	B	B	B	B	B	>3.3

A, acid form; B, base form.

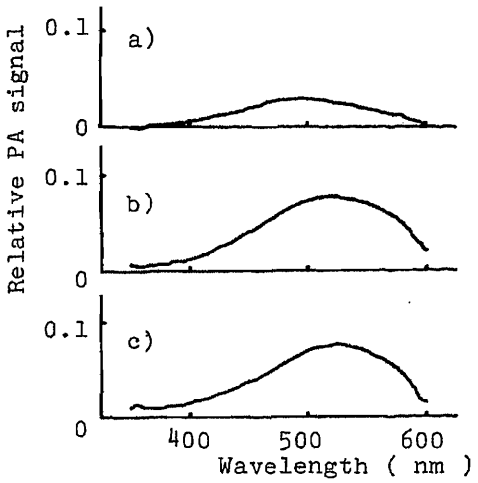

Fig. 5. Photoacoustic Spectra of the MR–Crystalline Cellulose System

a) MR adsorbed on crystalline cellulose in benzene solution; b) MR adsorbed on crystalline cellulose in ethyl alcohol; c) ground mixture of MR and crystalline cellulose.

on the excipient surfaces which were determined from a comparison of Tables II and III. The acid strengths of the excipients were also determined and are shown in the last column of Table IV. The acid strengths of excipients in benzene solution determined by Benesi's method[8] are indicated in Table V.

For silica–alumina and silica–magnesia, the acid strengths measured by PAS were considerably weaker than those measured in benzene solution. The differences are ascribed to water adsorption at the acidic sites of the excipients during preparation of the samples for PAS.

In the case of MR and crystalline cellulose, particular attention should be paid to the molecular form determination. Although MR molecules appear to have the acid form on the crystalline cellulose surface from a comparison of the PA spectra and absorption spectra, the peak position of MR on the crystalline cellulose surface in the PA spectra varied with the solvent used, that is, from 500 nm in benzene to 525 nm in ethanol (Fig. 5a and b).[5]

As the cellulose molecules form a hydrogen bonding network in crystalline cellulose, widely differing chemical properties of solvents could influence the nature of the hydrogen bonding network. It is known that in a polar solvent, such as ethanol, the hydrogen bonding network of crystalline cellulose is loosened, while in a non-polar solvent, the hydrogen bonding network remains tight.[13] Therefore, it was considered that the MR molecules could enter deeply into the hydrogen bonding network of crystalline cellulose in ethanol, while in benzene solution, the MR molecules could not enter the hydrogen bonding network. Thus, the solvent used may affect the PA peak of MR.

Nakai *et al.* reported that drug molecules were dispersed mono-molecularly in the hydrogen bonding network of crystalline cellulose in a ground mixture.[14] In this experiment, a ground mixture of crystalline cellulose with MR was prepared and the PA spectrum of the ground mixture was measured in order to investigate the effects of the dispersed state of MR on the PA spectra. The peak position of the PA signal of the ground mixture of MR with crystalline cellulose was observed at 525 nm (Fig. 5c) and this coincided with that of MR adsorbed on crystalline cellulose in ethanol. The molecular state of indicators was consequently explained in terms of the dissociation of the hydrogen bonding network by the solvent used. The acid strength of crystalline cellulose was determined to be between 3.3 and 4.8 by the PAS method.

In the case of methylcellulose, the PA spectra of MY, AAT and BAD on the methylcellulose surface had two peaks between 350 and 450 nm. The indicators which had two peaks in the PA spectra, however, seemed to take the base forms on methylcellulose, because the PA signal of the acid forms at about 500 nm was negligible. Therefore, the acid strength of

methylcellulose was determined to be weaker than 4.8 by the PAS method.

The molecular states of MR on the crystalline cellulose surface and on the methylcellulose surface were not determined by Benesi's method (Table V), as the color of MR in benzene solution with crystalline cellulose and methylcellulose is orange, which is different from the red color of the acid form and the yellow of the base form.

References and Notes

1) Y. Pao (ed.), "Optoacoustic Spectroscopy and Detection," Academic Press, Inc., New York, 1977.
2) A. Rosencwaig, "Photoacoustics and Photoacoustic Spectroscopy," John Wiley and Sons, Inc., New York, 1980.
3) K. Jagannathan, P. Ganguly, and C. N. R. Rao, *J. Catalysis*, **75**, 262 (1982).
4) L. P. Hammett and A. J. Deyrup, *J. Am. Chem. Soc.*, **54**, 2721 (1932).
5) Y. Nakai, K. Yamamoto, K. Terada, and M. Sakai, *Chem. Pharm. Bull.*, **33**, 3068 (1985).
6) T. Sawada and M. Yamamoto, *Bunseki*, **1979**, 172.
7) E. M. Monahan, Jr. and A. W. Nolle, *J. Appl. Phys.*, **48**, 3519 (1977).
8) H. A. Benesi, *J. Am. Chem. Soc.*, **78**, 5490 (1957).
9) J. Dijkstra, *J. South African Chem. Inst.*, **13**, 27 (1960).
10) W. R. Brode, J. H. Gould, and G. M. Wyman, *J. Am. Chem. Soc.*, **74**, 4641 (1952).
11) V. Uma, M. Subrahmanyam, and J. C. Kuriacose, *Indian J. Chem.*, **16A**, 830 (1978).
12) V. N. Vorob'ev, D. R. Kadyrova, G. Sh. Talipov, and A. S. Sultanov, *Kinet. Kataliz.*, **15**, 170 (1974).
13) K. Kawano and Y. Nakai, *Yakugaku Zasshi*, **103**, 1060 (1983).
14) Y. Nakai, E. Fukuoka, S. Nakajima, and K. Yamamoto, *Chem. Pharm. Bull.*, **25**, 3340 (1977).

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Percutaneous Absorption of Dexamethasone Acetate and Palmitate, and the Plasma Concentration

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To investigate quantitatively the percutaneous absorption of dexamethasone acetate (DA) and palmitate (DP), gel and petrolatum ointments were prepared and applied to rat skin. DA in the presence of absorption enhancers (Azone® and sorbitan monooleate) was rapidly absorbed, in the form of dexamethasone, through the skin, whereas the absorption after application of DA ointment without enhancers was relatively poor. DP was also efficiently absorbed in the presence and absence of enhancers, with bioavailability of 5.3 and 4.1%, respectively. The absorbed DP was slowly hydrolyzed in the systemic circulation. The percutaneous absorption of drugs from the petrolatum ointments with enhancers was slower than that from the gel ointments. The *in vitro* release rates of DA and DP from the gel ointments with enhancers were 7.3 and 6.1 mg/h$^{1/2}$, respectively and were much higher than those from the petrolatum ointments. The partition coefficient of DP was much larger than that of DA. Thus, it is suggested that DA and DP gel ointments with enhancers are effective in terms of high absorption into the circulation.

Keywords——dexamethasone acetate; dexamethasone palmitate; percutaneous absorption; rat; Azone®; absorption enhancer; plasma concentration; gel ointment; petrolatum ointment; *in vitro* release rate

Systemic administration of cortisone and its synthetic analogs gives excellent results in clearing inflammatory skin lesions. Unfortunately, corticosteroids carry the risk of side effects such as peptic ulceration, hypokalemic alkalosis and edema.[1] Corticosteroids have been frequently used in topical dosage forms for cutaneous diseases. It has been found that the esterification of the 17- and 21-hydroxy groups produces increased efficacy and improved penetration through skin, as shown in the case of betamethasone dipropionate and hydrocortisone butyrate propionate.[2,3] The changes of molecular structure and technical advances in drug delivery systems have thus allowed increased utilization of transdermal formulations in corticosteroid therapy. Consequently, a kinetic study of esterified steroids in the systemic circulation would provide important information for practical application. Despite many studies on dermatologic preparations of corticosteroids, surprising little work has been carried out to define quantitatively the percutaneous absorption based on measuring plasma concentration. In this work, the *in vivo* percutaneous absorption of dexamethasone acetate and palmitate was investigated in rats, using gel and petrolatum ointments with or without absorption enhancers (Azone® and sorbitan monooleate). In addition, various factors (the drug release rate, partition coefficient and hydrolysis in skin) which influence the penetration of drugs were investigated in relation to the percutaneous absorption.

Experimental

Materials——Dexamethasone acetate (DA, pharmaceutical grade) and dexamethasone palmitate (DP) and stearate were generous gifts of Nippon Merck-Banyu Co., Ltd. and the Green Cross Co., Ltd., respectively.

Prednisolone, an internal standard (I.S.) for high-performance liquid chromatography (HPLC) was purchased from Nakarai Chemicals Co., Ltd. Azone® and sorbitan monooleate were kindly supplied by Nelson Research and Development Co. and Dai-ichi Industries Co., Ltd., respectively. Hiviswako 104® was purchased from Wako Pure Chemical Industries Co., Ltd. Other chemicals were of reagent grade. The chromatographic solvents were of HPLC grade.

Animals——Male Wistar albino rats (180—250 g) were used throughout this experiment. Animals had free access to MF diet (Oriental Yeast Co., Ltd.) and water before experiments.

Preparation of Ointments——The gel ointments were prepared by dissolving the corticosteroid in a mixture of dimethylsulfoxide (DMSO) and propylene glycol and then mixing the solution with a gel base containing water, ethanol, diisopropyl adipate, diisopropanolamine and/or absorption enhancers. The white petrolatum ointments were prepared by a fusion method after dissolving corticosteroids in a mixture of DMSO, Azone® and sorbitan monooleate. Therefore, these preparations were emulsion-type or solution-type ointments. Sorbitan monooleate, an enhancer, was mainly used as an emulsifier for Azone®. Details of the ointment composition are listed in Table I.

Single Intravenous (i.v.) Administration to Rats——DA or DP (500 μg/kg, dexamethasone equivalent) dissolved in DMSO, after dilution with saline, was administered intravenously into the jugular vein through a silicone tubing cannula.[4] Blood samples (0.2 ml) were collected periodically from the jugular vein with a heparinized syringe.

***In Vivo* Percutaneous (p.c.) Absorption Studies**——On the day before the experiment, the rat jugular vein was cannulated with tubing,[4] and the hair of the abdominal area was carefully removed with electric clippers and an electric razor to prevent damage to the *stratum corneum* (*s.c.*). On the next day, 1 g of ointment was uniformly spread over the shaved abdominal skin (3 × 3 cm area, designated by attaching an adhesive tape with a cut-out area) under pentobarbital anesthesia (32 mg/kg), and immediately occluded with a sheet of aluminum foil and adhesive tape. The ointment remained in contact with the skin for 8 h, and then the unabsorbed ointment was wiped off with absorbent cotton soaked in warm water. Blood samples (0.2 ml) were collected periodically for 48 h after dosing through the silicone tubing. The plasma was separated immediately by centrifugation and stored frozen until assay. In some experiments, the drug remaining within the skin 8 h after application of ointment was estimated by HPLC after extraction with methanol followed by hydrolysis in 1 N NH_4OH–methanol (1 : 9, v/v) for 36 h at 60 °C.

Determination of DA and DP——DA and DP in plasma or sample solution were determined by means of HPLC, with prednisolone and dexamethasone stearate as internal standards (I.S.), respectively. Samples were extracted with 3 ml of methylene chloride and centrifuged. The methylene chloride layer was aspirated and evaporated *in vacuo*. The residue was dissolved in 30 μl of elution solvent (acetonitrile–isopropanol, 85 : 15, v/v) and then injected into a column (25 × 0.4 cm i.d.) packed with Lichrosorb RP-18, using a Shimadzu LC-3A liquid chromatograph equipped with an injector (model SIL-1A), a 254 nm ultraviolet (UV) monitor (model SPD-2A), and a Chromatopac C-R1B.

Determination of Dexamethasone——Dexamethasone in sample solution was extracted and determined by the method of Alvinerie and Toutain.[5] A chromatograph equipped with a 254 nm UV monitor was used with a 10 × 0.6 cm i.d. column containing ERC-silica.

Measurement of Partition Coefficient——The drug (5 mg) was dissolved in 10 ml of *n*-octanol or chloroform, which was saturated with water, and the solution was added to 10 ml of water (saturated with *n*-octanol or chloroform). After vigorous shaking for 1 h at about 25 °C, the phases were separated by centrifugation. The drug concentration in both phases was measured by means of a spectrophotometer at the excitation maximum (246 nm for drug in the aqueous phase and 253 nm in the organic phase). The partition coefficient (P) was calculated by using the following equation:

TABLE I. Composition of DA and DP Ointments

Rp.	Drug[a]	Solvent			Azone® (%)	Sorbitan monooleate (%)	Base
		PG	DMSO	EtOH			
1	DA	26	4	12	—	—	Hiviswako 104®[b] (1%)
2	DA	26	4	12	5	5	
3	DP	26	4	12	—	—	
4	DP	26	4	12	5	5	
5	DA	25	—	—	5	5	Petrolatum (65%)
6	DP	25	—	—	5	5	

a) 1.7% as dexamethasone. *b*) Containing diisopropanolamine (1.1%) and diisopropyl adipate (2%) in the base. DA, dexamethasone acetate; DP, dexamethasone palmitate; PG, propylene glycol; DMSO, dimethylsulfoxide; EtOH, ethanol.

$$P=\frac{\text{concentration of drug in organic phase}}{\text{concentration of drug in aqueous phase}}$$

Drug Release from Ointment——The drug release from ointment was estimated by using a Franz diffusion cell with a 3.5 cm i.d. O-ring flange.[6] A Toyo filter paper (No. 2) spread with 1 g of ointment was mounted on a diffusion assembly and the drug that penetrated into the receptor fluid (90% ethanol–10 mM sodium phosphate buffer, pH 7.4) at 30 °C was determined by the HPLC method described above. The quantity (mg) of each ester released from the ointment was plotted against the square root of time, and the release rate (mg/h$^{1/2}$) was calculated from the slope of the straight line obtained.

Pharmacokinetic and Statistical Analyses——The plasma dexamethasone concentration data for individual animals after a single i.v. adminstration of DA were fitted to the equation:

$$C=A\cdot e^{-\alpha t}+B\cdot e^{-\beta t}$$

where C is the drug concentration at time t, α and β are the rate constants during the initial rapid and terminal slower phases of the curve, respectively, with A and B being the respective zero-time intercepts. The area under the plasma drug concentration–time curve (AUC) was determined by applying the following equation:

$$AUC=\frac{A}{\alpha}+\frac{B}{\beta}$$

The logarithmic plasma concentration of dexamethasone after a single i.v. dosing of DP was plotted against time after administration. The elimination rate constant (k_e) was calculated from the linear part of the plots using the least-squares method. The apparent hydrolysis rate constant (k) was calculated by the method of residuals.

The AUC after i.v. dosing of DP and percutaneous administration of ointments was calculated by the linear trapezoidal method up to the last sampling point (C_n) and the area to infinite time was added by integration (C_n/k_e). The absolute bioavailability was calculated by using the following equation:

$$\text{bioavailability (\%)}=\frac{AUC_{\text{p.c.}}\times \text{dose}_{\text{i.v.}}}{AUC_{\text{i.v.}}\times \text{dose}_{\text{p.c.}}}\times 100$$

where $AUC_{\text{p.c.}}$ and $AUC_{\text{i.v.}}$ are AUC after p.c. and i.v. administrations, respectively.

The means of all data are presented with their standard deviation (S.D.). Statistical analysis was performed by using the non-paired Student's t-test, and a p-value of 0.05 or less was considered to be significant.

Results

Plasma Concentration of Dexamethasone after Single i.v. Administration of DA or DP

The plasma concentration–time curves for dexamethasone after a single i.v. dosing of DA and DP are shown in Fig. 1. The plasma decay curve after dosing of DA showed biexponential kinetics, whereas the curve after DP dosing gave a delayed peak plasma concentration of dexamethasone, at about 1.5—2 h, and a terminal first-order elimination, suggesting relatively slower hydrolysis of DP in the circulation. The biexponential decline of dexamethasone concentration after administration of DA agreed well with that after dexamethasone phosphate shown in our previous paper.[7] This also indicated that the conversion of DA to dexamethasone was extremely rapid *in vivo*. The pharmacokinetic parameters of dexamethasone produced in the systemic circulation are shown in Table II. The elimination rate constants (β and k_e) after dosing of DA and DP were 0.179 and 0.238 h^{-1}, respectively.

In Vivo p.c. Absorption Studies

Gel Ointments——The plasma concentration–time curves for dexamethasone after application of gel ointments with and without absorption enhancers are shown in Fig. 2. These results demonstrated that DA in the presence of Azone® and sorbitan monooleate was rapidly absorbed through the skin and a considerable part of the drug absorbed passed directly into the systemic circulation. On the other hand, DP was apparently slowly absorbed

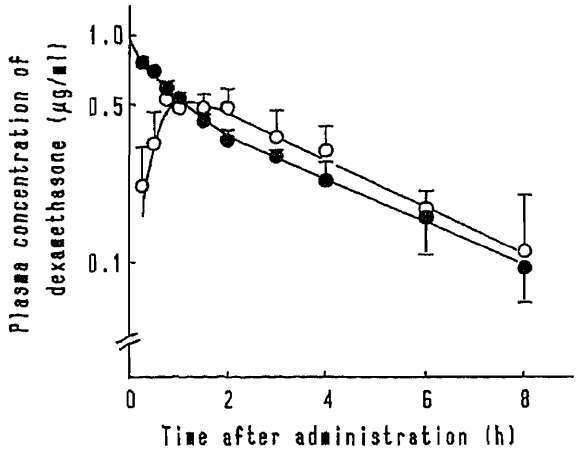

Fig. 1. Semilogarithmic Plots of Plasma Dexamethasone Concentration after a Single i.v. Administration of DA or DP

●, DA; ○, DP. Dose: 500 μg/kg as dexamethasone. Each point represents the mean ± S.D. of 4 rats.

TABLE II. Pharmacokinetic Parameters after a Single i.v. Administration of DA or DP

Parameter	DA	DP
A (μg/ml)	0.408 ± 0.033	
α (h^{-1})	1.138 ± 0.343	
B (μg/ml)	0.497 ± 0.037	
β (h^{-1})	0.179 ± 0.020	
k (h^{-1})		2.077 ± 0.553
k_e (h^{-1})		0.238 ± 0.024
AUC (μg·h/ml)	3.188 ± 0.410	2.552 ± 0.446

k, apparent hydrolysis rate constant. Each value represents the mean ± S.D. of 3 experiments.

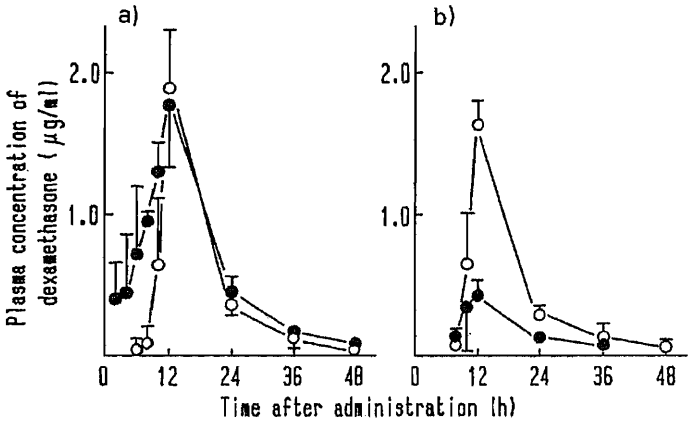

Fig. 2. Plasma Concentration of Dexamethasone after Single Application of DA or DP Gel Ointment

The applied amount of ointment was 1.0 g/9 cm². *a*) Ointment with enhancers, *b*) ointment without enhancers. ●, DA ointment; ○, DP ointment.

from the ointment and the maximum concentration (C_{max}, 1.9 μg/ml) of dexamethasone was reached about 12 h after application, as shown in Fig. 2a). The elimination pattern of dexamethasone in plasma was similar in both cases (DA and DP ointments with enhancers).

The plasma dexamethasone concentrations after application of the gel ointments without enhancers are depicted in Fig. 2b). The C_{max} of dexamethasone after application of DP ointment without enhancers was 1.6 μg/ml and the value was similar to that of DP ointment with enhancers. The plasma concentrations after application of DA ointment without enhancers were very much lower than those in the presence of enhancers, indicating poor absorption of DA in the absence of enhancers and a smaller effect of enhancers on the absorption of DP.

The AUC, bioavailability and the amount of durg remaining within the skin after application for 8 h are listed in Table III. The AUC (29.8 μg·h/ml) after application of DA ointment with enhancers was significantly larger than that (19.4 μg·h/ml) after DP ointment ($p < 0.025$), leading to bioavailability of 6.2 and 5.3%, respectively. In the absence of

TABLE III. Pharmacokinetic Parameters for Percutaneous Absorption of DA and DP from Gel or Petrolatum Ointments

Rp.	C_{max} (μg/ml)	Lag time (h)	AUC (μg·h/ml)	Bioavailability (%)	Drug within skin (μg/cm²)	
					Free	Total
1	0.44±0.08	6	5.5±0.9	0.96±0.16	9.01±1.94	11.49±3.82
2	1.73±0.21[a)]	<1	29.8±5.8[a)]	6.19±1.68[a)]	10.82±1.23	12.55±2.61
3	1.62±0.14	8	18.8±2.2	4.12±0.48	3.49±0.89	22.67±3.41
4	1.88±0.19[a)]	3	19.4±5.4	5.32±0.73	2.22±1.24	11.45±2.72
5	1.71±0.36	3	21.5±1.5	4.18±0.09	—[b)]	9.68±2.39
6	0.87±0.09[a)]	6	14.4±4.1	2.33±0.68	—[b)]	4.50±1.00

Each value represents the mean±S.D. of 3—4 experiments. Rp. 1, DA gel; Rp. 2, DA gel with enhancers; Rp. 3, DP gel; Rp. 4, DP gel with enhancers; Rp. 5, DA petrolatum with enhancers; Rp. 6, DP petrolatum with enhancers. *a*) $p<0.05$ compared with Rp. 1, Rp. 3 or Rp. 5. *b*) Not determined.

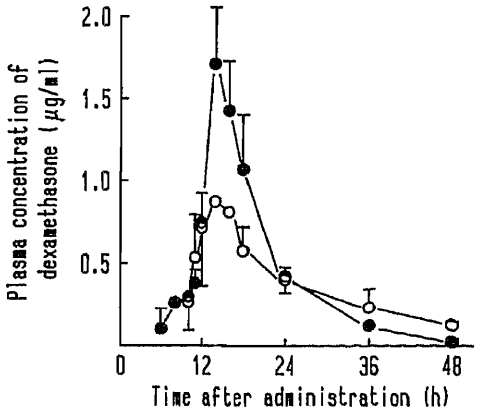

Fig. 3. Plasma Concentration of Dexamethasone after Single Application of DA or DP Petrolatum Ointment

The applied dose of each ointment was 1.0 g/9 cm². ●, DA ointment with enhancers; ○, DP ointment with enhancers.

enhancers, the total amount of drug remaining within the skin at 8 h after application was much greater for DP ointment than for DA ointment, and the greater part of DP within the skin existed in the ester form, while DA was found mainly as dexamethasone, the cleavage product of the ester linkage. Thus, it would be reasonable to assume that the *in vivo* skin penetration might occur chiefly in the form of dexamethasone in DA ointment and in the form of the ester in DP ointment.

Petrolatum Ointments——The plasma dexamethasone concentrations after application of the petrolatum ointments of DA and DP are shown in Fig. 3. The p.c. absorption was measured only for the ointments with absorption enhancers. There was a trend for slower absorption of DA and DP compared with that in gel ointment with enhancers. The lag time, determined graphically, was about 3 h for DA and 6 h for DP, as shown in Table III, which were longer than those after the corresponding gel ointments. The AUC and bioavailability of dexamethasone after application of DA ointment were larger than those after DP ointment ($p<0.05$).

In Vitro Release of DA and DP from Ointments

DA and DP release from the gel and petrolatum ointments with enhancers was examined. When the amounts of DA and DP released from the gel ointments were plotted against the square root of time, straight lines were obtained, as shown in Fig. 4, while in the case of the petrolatum ointment, a linear relationship was only observed at the initial period (within 1 h) and was followed by a decreased rate. The release rates (7.27 and 6.06 mg/h$^{1/2}$ for DA and DP, respectively) of drugs were much higher in the gel ointment than those (1.82 and

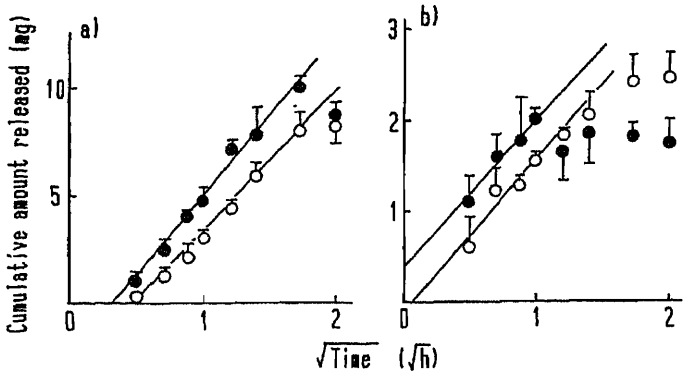

Fig. 4. Release of DA and DP from Gel and Petrolatum Ointments at 30 °C

The applied amount of ointments was 1.0 g/9.6 cm^2. *a*) Gel ointment, *b*) petrolatum ointment. ●, DA; ○, DP.

TABLE IV. Partition Coefficients of DA and DP

Solvent	DA	DP
$CHCl_3$–H_2O	599	>700
n-Octanol–H_2O	173	500

Each value represents the mean of 3 experiments.

1.74 mg/h$^{1/2}$ for DA and DP respectively) in the corresponding petrolatum ointment.

Partition Coefficients (*P*) of DA and DP

The calculated *P* values are shown in Table IV. The values of DP were much higher than those of DA in both solvent systems, suggesting high lipophilicity of DP compared with DA.

Discussion

Transdermal medication is intended to be applied to the skin but to elicit systemic effects. In order to clarify the systemic absorption of corticosteroids applied to skin and to define quantitatively the relationship between the formulation and drug absorption, gel and petrolatum ointments of DA and DP were prepared and applied to rat skin.

In the absence of absorption enhancers, good absorption of DP from the gel ointment was observed, with a relatively long lag time (about 8 h) (Fig. 2b). The good penetration of DP through skin may be partly due to its high lipophilicity, as suggested by the *P* values (Table IV). Guy *et al.*[8] have shown that for a range of 12 compounds the effective partition coefficient between the s.c. and the viable epidermis was a linear function of the octanol–water partition coefficient.

The bioavailability of glucocorticoids in various topical drug delivery systems has received attention from both the pharmaceutical and medical communities. A percutaneous penetration study of radioactively labeled hydrocortisone in 18 subjects at a dose of 4 μg/cm^2 showed that only 1% of the dose was actually absorbed from the forearm of a normal subject.[9] This problem of low bioavailability of glucocorticoids means that there is an urgent need to develop more efficient systems for delivering these therapeutically useful agents. It is reported that Azone® enhances the biological activity of various topically active agents.[10,11] Stoughton reported that percutaneous penetration of triamcinolone acetonide in the presence of 10% Azone® was 8-fold higher than that without the agent after 7h.[10] From this point of view, the effect of the enhancers on the percutaneous absorption of DA and DP was

estimated. The enhancers increased the percutaneous absorption of these corticosteroids, especially that of DA, from the gel ointment (Fig. 2). The smaller effect of enhancers on the absorption of DP may be ascribed to the high lipophilicity of the drug and its long fatty acid chain. There was an apparently delayed percutaneous absorption of DP from the gel ointment in the presence of enhancers, as shown by the longer lag time, followed by a sharp increase in plasma level 6—12 h after dosing. The apparently delayed absorption probably resulted from the slow hydrolysis of the ester linkage in the circulation, as suggested from the data after i.v. injection of the drug (Fig. 1). Thus, DP would penetrate through the skin as quickly as in the case of DA ointment in the presence of enhancers. The bioavailability presented was obtained by an 8 h application (Table III); if the ointments are applied to the skin for a prolonged time, the bioavailability may be considerably increased.

Although these ointments were removed 8 h after application, the peak plasma concentration time (T_{max}) was about 12 h. This suggests that a significant amount of drug was held in reserve within the skin during application (Table III), and was continuously released into the systemic circulation after the removal of the ointments. The so-called "reservoir effect" has been reported quantitatively or semiquantitatively for various topically applied glucocorticoids.[12] Our data agreed well with the results reported.

The lower C_{max} and longer lag time after application of DP petrolatum ointment compared with those after the gel ointment might be attributed to the slow release of DP, a highly lipophilic drug, from the lipophilic base, as demonstrated by the *in vitro* release rate. This would also contribute to the slow elimination of dexamethasone after DP petrolatum ointment (Fig. 3). The delayed absorption after dosing of DA petrolatum ointment may also be related to the slow release rate of DA from the ointment.

Comparison of the amounts of remaining, free and ester drugs in the skin indicated that the greater part of DA was absorbed as dexamethasone after hydrolysis in the skin, and that DP mainly penetrated through the skin in the form of the ester. These results suggest the presence of strong esterase activity in skin, but the enzyme would hardly hydrolyze the long fatty acid ester, DP, and the DP in the systemic circulation was only gradually hydrolyzed, probably in the liver.

It is known that the amount of drug released from suspension-type creams and ointments is approximately proportional to the square root of time.[13,14] When the amounts of DA and DP released from gel ointments were plotted against the square root of time, straight lines were obtained (Fig. 4). Thus, it was demonstrated that the release of these drugs from gel ointments fitted well to Higuchi's equation.[15]

In conclusion, DP was efficiently absorbed in the form of the ester from both gel and petrolatum ointments in the presence or absence of absorption enhancers, Azone® and sorbitan monooleate. DA, after hydrolysis in the skin, also effectively penetrated through rat skin in the presence of these enhancers, and a rapid elevation of the plasma concentration was seen, while the drug was poorly absorbed from ointment without the promoters. The difference of skin penetration between DA and DP might be mainly due to the differences of physicochemical properties, such as melting point, lipophilicity and solubility, of the drugs and ointment bases.

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References

1) R. C. Haynes, Jr. and F. Murad, "The Pharmacological Basis of Therapeutics," Vol. VII, ed. by A. G. Gilman, L. S. Goodman, T. W. Rall and F. Murad, Macmillan Publishing Co., New York, 1985, pp. 1459—1489.

2) A. W. McKenzie and R. M. Atkinson, *Arch. Dematol.*, **89**, 741 (1964).
3) A. Kukita and S. Harada, *Nishinippon Hifuka*, **44**, 657 (1982).
4) R. A. Upton, *J. Pharm. Sci.*, **64**, 112 (1975); S. K. Bakarand and S. Niazi, *ibid.*, **72**, 1027 (1983).
5) M. Alvinerie and P. L. Toutain, *J. Pharm. Sci.*, **71**, 816 (1982).
6) Y. W. Chien, P. R. Keshary, Y. C. Huang and P. P. Sarpotdar, *J. Pharm. Sci.*, **72**, 968 (1983).
7) T. Ogiso, M. Iwaki and A. Ohtori, *J. Pharmacobio-Dyn.*, **8**, 1032 (1985).
8) R. H. Guy, J. Hadgraft and H. I. Maibach, *Toxicol. Appl. Pharmacol.*, **78**, 123 (1985).
9) H. I. Maibach, *Dermatologica*, **152** (Suppl. 1), 11 (1976).
10) R. B. Stoughton, *Arch. Dermatol.*, **118**, 474 (1982).
11) R. B. Stoughton and W. O. McClure, *Drug Dev. Ind. Pharm.*, **9**, 725 (1983).
12) R. J. Scheuplein and I. H. Blank, *Physiol. Rev.*, **51**, 702 (1971).
13) T. Higuchi, *J. Soc. Cosmet. Chem.*, **11**, 85 (1960).
14) F. Bottari, G. Di Colo, E. Nannipieri, M. F. Saettone and M. F. Serafini, *J. Pharm. Sci.*, **63**, 1779 (1974).
15) W. I. Higuchi and T. Higuchi, *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 598 (1960).

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Use of Hydrogenated Soya Phospholipids as a Diluent: Preparation of Sustained-Release Tablets of Theophylline and Sodium Diclofenac

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Sustained-release tablets (SR-tablet), prepared with hydrogenated soya phospholipids, gave slow release of theophylline and sodium diclofenac into a solvent at pH 6.8, in an *in vitro* study. The release profile of theophylline from the SR-tablet remained unchanged at pH values between 2.0 and 6.8. However, the release of diclofenac from the SR-tablet into a solvent at pH 2.0 was very slight, due to the low solubility at pH 2.0. The release of both drugs seems to occur predominantly by a leaching mechanism, as proposed by Higuchi. The oral administration of SR-tablets to dogs avoided a transient peak of drug concentration in the plasma and maintained plasma drug concentrations at higher levels for a longer period, in comparison with the oral administration of theophylline in suspension form or a commercial tablet of sodium diclofenac.

Keywords——tablet; theophylline; sodium diclofenac; hydrogenated soya phospholipid; *in vitro* release study; acidic pH; neutral pH; dog; oral administration; plasma concentration

Although the intensity of pharmacological effect is related to the drug concentration at the site of action, which in turn is often also related to the drug plasma concentration, it is desirable to maintain the drug concentration in the body continuously between the minimal effective and the maximal safe levels. Drugs, such as theophylline, sodium diclofenac and indomethacin, may require the use of a sustained-release formulation to avoid a transient peak of drug concentration in plasma and to maintain an effective plasma drug concentration for a long period.

As sustained-release formulations of oral administration, osmotic pump systems of indomethacin, theophylline and antipyrine are available,[2-4] but are rather complicated to produce, so that a simple sustained-release tablet (SR-tablet) would be preferable from a commercial point of view.

Recently, we have reported[5] that addition of hydrogenated soya phospholipids (H-phospholipids) to a triglyceride base controlled the release of sodium diclofenac from a suppository. We have also reported that sustained-release granules of sodium diclofenac, which were prepared with phospholipids.[6] In the granules, the release rate of sodium diclofenac was regulated by the addition of cholesterol to the granules. Since phospholipids are hydrated, in spite of their poor solubility,[7] the mechanism might be dependent on the permeation rate of solvent through the suppository matrix containing phospholipids.

In the present study, we investigated the release of theophylline and sodium diclofenac from SR-tablets as a single-unit dosage form, prepared with phospholipids alone as a diluent. Further, we examined plasma drug concentrations after oral administration of the tablets to dogs.

Experimental

Materials——H-Phospholipids, supplied by Nikko Chemicals Co., Ltd. (Tokyo, Japan), contains more than

98% phospholipids (about 70% phosphatidylcholine), and had an iodine value of about 6%. Sodium diclofenac and theophylline were supplied by Ciba-Geigy Japan (Takarazuka, Japan) and Shiratori Pharmaceutical Industry (Chiba, Japan), respectively. The particle size of these substances was less than 75 μm. Commercial tablets of sodium diclofenac were obtained from a public hospital. Other reagents used were of analytical grade.

Preparation of SR-Tablet——Two grams of theophylline or sodium diclofenac was mixed well with 18 g of H-phospholipids. Ethanol was then added to the mixture to obtain a paste by thorough mixing after gradual addition of 5 ml portions of ethanol. After drying of the paste under reduced pressure, the solid mass was pulverized with a mortar and a pestle. Granules with a size of 150 to 350 μm were compressed at 500 kg/cm^2 to obtain SR-tablets. Tablets of sodium diclofenac, with 8 mm diameter and 3 mm thickness, weighed 252.7 ± 3.2 mg (n=60) and the content of sodium diclofenac was 26.2 ± 1.4 mg (n=20). Tablets of theophylline, with 8 mm diameter and 3 mm thickness, weighed 247.1 ± 4.6 mg (n=60) and the content of theophylline was 24.9 ± 1.1 mg (n=20).

An *in Vitro* Release Study——A tablet wrapped with gauze was immersed in a beaker containing 200 ml of solvent at 37 °C. The beaker was shaken at 40 cycle/min or at 100 cycle/min and 100 μl aliquots were collected at designated time intervals, passed through a Millipore filter (pore size; 0.45 μm), and assayed to determine the release of theophylline or sodium diclofenac. As a solvent, 0.01 N HCl saline solution (pH 2.0) and 0.1 M sodium phosphate buffer (pH 6.8) was used. For comparison, commercial tablets of sodium diclofenac and theophylline powder were examined. The powder was immersed directly in solvent. The solubility of each drug in the solvent at pH 2.0 and at pH 6.8 was determined after incubation of 1 g of drug in 5 ml of solvent at 37 °C for 48 h (Table I).

When 1 μM drug solution was incubated at 37 °C for 48 h, recovery of each drug was more than 98% [for theophylline, 99.2 ± 1.7% (n=4) at pH 2.0 and 99.7 ± 1.9 (n=4) at pH 6.8; for sodium diclofenac, 98.1 ± 1.1% (n=4) at pH 2.0 and 99.1 ± 1.2% at pH 6.8]; thus, degradation of each drug in the solvent during the experimental period was ignored.

Data Analyses of Drug Release in the *in Vitro* Study——Release of drugs from an SR-tablet can be described in terms of the fraction of drug released (F) by Eq. 1.

$$F=(\text{amount of drug released from an SR-tablet at time } t)\ /(\text{initial amount of drug in SR-tablet}) \quad (1)$$

When a linear relationship is observed between F and the square root of time (as shown in Figs. 1 and 2), the release of drugs from SR-tablet seems to occur by the leaching mechanism proposed by Higuchi,[8] according to Eq. 2.

$$Q=[D\varepsilon(2A-\varepsilon Cs)Cs\cdot t/\tau)^{1/2} \quad (2)$$

Where Q=the amount of drug released after time t per unit exposed area; D=the diffusivity of the drug in the permeating fluid; τ=the tortuosity factor of the capillary system; A=the total amount of drug present in the matrix per unit volume; Cs=the solubility of drug in the permeating fluid; and ε=the porosity of the matrix.

Thus, the amount of drug released from an SR-tablet after time t can be represented by Eq. 3.

$$Q'=Q\cdot Sq \quad (3)$$

Where Sq=the total exposed area of an SR-tablet. When the total volume of an SR-tablet is presented by V_0, F in Eq. 1 is given by Eq. 4.

$$F=Q'/(AV_0)=(Q\cdot Sq)/(AV_0) \quad (4)$$

Thus, Eq. 5 is obtained from Eqs. 2 and 4.

$$F=(Sq/AV_0)[D\varepsilon(2A-\varepsilon Cs)Cs\cdot t/\tau]^{1/2}=kt^{1/2} \quad (5)$$

In Eq. 5, k represents the slope of the straight line obtained when the F values are plotted against the square root of time, and it may represent an apparent overall control factor for the release of drug from an SR-tablet; *i.e.*, a small value of k implies slow release of drug from the tablet.

Table I. Solubility of Theophylline and Sodium Diclofenac in Solvent at pH 6.8 and at pH 2.0, at 37 °C

Drug	Solubility[a] (mM)	
	pH 6.8	pH 2.0
Theophylline	58.4 ± 2.2	62.6 ± 3.4
Sodium diclofenac	18.2 ± 1.2	0.011 ± 0.002

a) Each value represents the mean ± S.D. (n=4).

An *in Vivo* Absorption Study in Dogs——Three male beagle dogs, weighing 9.0 to 9.8 kg, were fasted (but water was given freely) for 16 h prior to experiments and used in a cross-over study. Dogs were walked for 30 min prior to the oral administration of drug and were used in a conscious state. After oral administration of tablet followed by administration of 20 ml of water *via* a gastric catheter, blood was collected from the femoral vein at designated time intervals for 30 h. Theophylline powder was administered in suspension form with 2 ml of distilled water through a gastric catherter. During the experimentation, water was given freely. After centrifugation of the blood, plasma was collected to assay drug concentrations. The area under the curve of drug concentrations in plasma for 30 h was determined by trapezoidal integration after the oral administration.

Assays——Assays of diclofenac[9] and theophylline[10] were performed by high-performance liquid chromatography as described previously. The detection limits were 0.04 μg/ml for diclofenac and 0.10 μg/ml for theophylline.

Statistical Analyses——Statistical analyses were performed by using Student's *t*-test.

Results and Discussion

An *in Vitro* Release Study

Complete dissolution of theophylline in the powder form was observed both at pH 2.0 and at pH 6.8 within 0.5 h (Fig. 1).

In terms of the release of theophylline from an SR-tablet, which occurred slowly, there were only slight differences between the patterns at pH 2.0 and 6.8, and those at 40 and 100 cycle/min, up to 8 h (Fig. 1). A linear relationship was also observed between the fraction of theophylline released (F in Eqs. 1 and 5) and the square root of time (immersion time) up to 8 h, in both solvent (Fig. 1); the k value (slope of the line) are given in Table II. These results may indicate that release of theophylline from an SR-tablet occurs by the leaching mechanism, proposed by Higuchi.[8] The fact that the k value for theophylline release is independent of the pH of the solvent (Table II) may be related to the fact that the solubility of theophylline is similar at pH 2.0 and 6.8 (Table I).

Further, no significant disintegration of SR-tablet was observed, but swelling occurred at pH 2.0 during the experimental period (naked eye observation). At pH 6.8, swelling was also observed during the experimental period at 40 cycle/min, but a gradual disintegration of the SR-tablet occurred at 100 cycle/min after 8 h. Thus, the disintegration of the SR-tablet may result in a surge of theophylline release after 8 h at pH 6.8 and at 100 cycle/min, in comparison

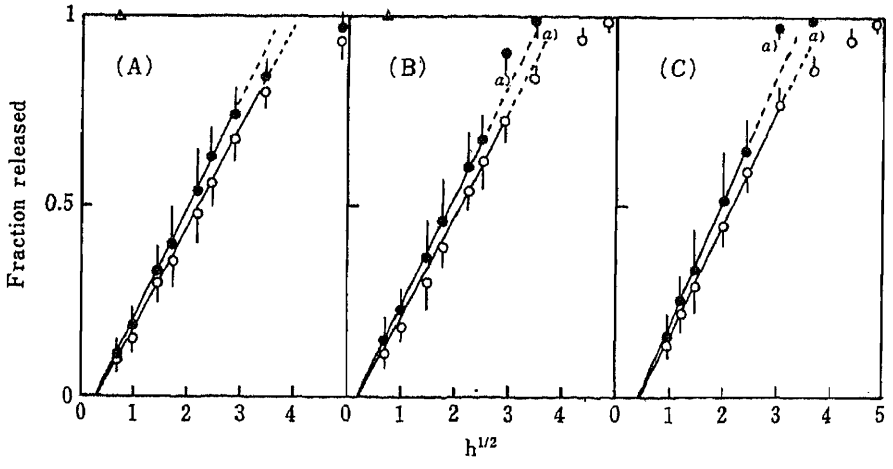

Fig. 1. Fraction of Theophylline Released from an SR-Tablet *versus* Square Root of Shaking Time

The release profile of theophylline from an SR-tablet at pH 2.0 (A), at pH 6.9 (B), and at pH 6.8 after immersion for 1 h at pH 2.0 (C). Open symbols and closed symbols represent the results when tablets were shaken at 40 cycle/min and at 100 cycle/min, respectively. Triangles represent the dissolution of theophylline after addition of its powder to the solvent. The amount of theophylline used was 25 mg. Each value represents the mean ± S.D. ($n=4$). *a*) $p<0.05$ *versus* at 40 cycle/min.

TABLE II. Values of k,[a] an Apparent Overall Control Factor for Drug Release from an SR-Tablet, in Solvent at pH 2.0 and 6.8

Drug	pH 2.0	pH 6.8	pH 6.8[b]
Theophylline			
at 40 cycle/min	0.312 ± 0.029 ($r=0.093 \pm 0.002$, $p<0.05$)	0.309 ± 0.031 ($r=0.990 \pm 0.005$, $p<0.05$)	0.304 ± 0.027 ($r=0.986 \pm 0.002$, $p<0.05$)
at 100 cycle/min	0.341 ± 0.074 ($r=0.982 \pm 0.011$, $p<0.1$)	0.336 ± 0.057 ($r=0.990 \pm 0.006$, $p<0.05$)	0.351 ± 0.042 ($r=0.971 \pm 0.012$, $p<0.1$)
Sodium diclofenac			
at 40 cycle/min		0.148 ± 0.012 ($r=0.994 \pm 0.004$, $p<0.05$)	0.162 ± 0.036 ($r=0.990 \pm 0.006$, $p<0.05$)
at 100 cycle/min		0.174 ± 0.042 ($r=0.982 \pm 0.009$, $p<0.1$)	0.182 ± 0.039 ($r=0.977 \pm 0.014$, $p<0.1$)

a) Value obtained from the slope of the solid straight line in Figs. 1 and 2. *b*) The k value was obtained after changing the solvent to pH 6.8, following immersion of the tablet in the solvent to pH 2.0 for 1 h. Each value represents the mean $\pm$ S.D. ($n=4$).

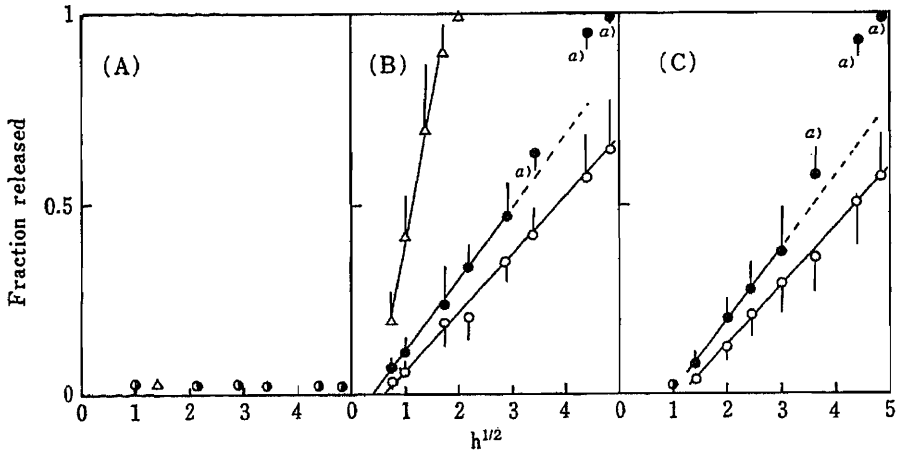

Fig. 2. Fraction of Diclofenac Released *versus* Square Root of Shaking Time

The release profile of diclofenac from a conventional tablet (triangles) and from an SR-tablet (circles) at pH 2.0 (A), at pH 6.8 (B), and at pH 6.8 after immersion for 1 h at pH 2.0. Open symbols and closed symbols represent the results when tablets were shaken at 40 cycle/min and at 100 cycle/min, respectively. Each value represents the mean $\pm$ S.D. ($n=4$). *a*) $p<0.05$ *versus* at 40 cycle/min.

with the release expected on the basis of a leaching mechanism (shown in a dashed line in Fig. 1).

When an SR-tablet of theophylline was immersed in a solvent at pH 6.8 after 1 h in a solvent at pH 2.0, the k value was not changed in comparison with that in pH 6.8 alone (Fig. 1 and Table II).

Release of about 75% of diclofenac from the commercial tablet along with disintegration was observed within 2 h in a solvent at pH 6.8, but only slight release of diclofenac was observed in a solvent at pH 2.0 (Fig. 2). The apparently poor release of diclofenac at pH 2.0 is due to its poor solubility at pH 2.0 (Table I).

Slight release of diclofenac an SR-tablet at pH 2.0 was also observed (Fig. 2A). For the release of diclofenac at pH 6.8, a linear relationship (the value of slope, k, are summarized in

Table II) was observed between the fraction of diclofenac released (F in Eqs. 1 and 5) and the square root of immersion time up to 24 h, when the tablet was shaken at 40 cycle/min (Fig. 2B). It is considered that the release of diclofenac from an SR-tablet may also occur by the leaching mechanism. The release of diclofenac under the above conditions was about 60% at 24 h, though complete release of theophylline was observed (Fig. 2). This slow release of diclofenac is due to the low solubility of diclofenac, since the release of the drug from the formulation in the leaching system according the Eq.5 is dependent on the drug solubility. When the tablet was shaken at pH 6.8 and at 100 cycle/min, a linear relationship was obtained between the fraction of diclofenac released and the square root of time up to 8 h. However, after 8 h, the release of diclofenac from the tablet under these conditions was greater than that expected from a leaching system, as observed with theophylline. This unexpectedly large release of diclofenac also seemed to be due to the disintegration of SR-tablets by vigorous shaking.

When an SR-tablet was immersed in the solvent at pH 6.8 after incubation for 1 h in the solvent at pH 2.0, the release profile of diclofenac similar to that in the solvent of pH 6.8 alone after a lag time (Fig. 2C). The observation of a lag time on changing the solvent from pH 2.0 to 6.8 may be due to a period of displacement of the initial solvent of pH 2.0 by the solvent of pH 6.8

An *in Vivo* Absorption Study in Dogs

After an oral administration of theophylline in suspension form at a dose of 25 mg to a dog, a transient high theophylline concentration in the plasma was observed with a maximum level of more than 30 μg/ml at 4 h (Fig. 3). However, administration of theophylline in an SR-tablet avoided the transient high theophylline concentration, and a maximum plasma theophylline concentration of about 10 μg/ml was observed at about 6 h. Further, the concentration of theophylline at 30 h was about 3 μg/ml in the case of the SR-tablet but less

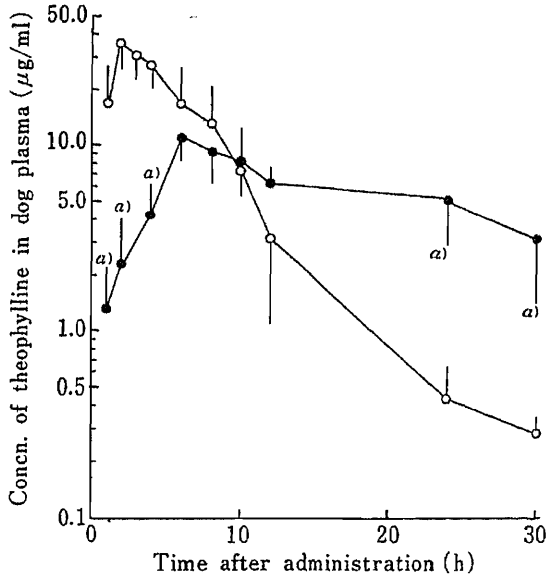

Fig. 3. Plasma Theophylline Concentration in Dogs as a Function of Time after Administration of Suspension (Open Symbols) or an SR-Tablet (Closed Symbols) at a Dose of 25 mg of Theophylline

Three dogs were used in a cross-over study. Each value represents the mean ± S.D. ($n=3$). *a*) $p<0.05$ *versus* a suspension form.

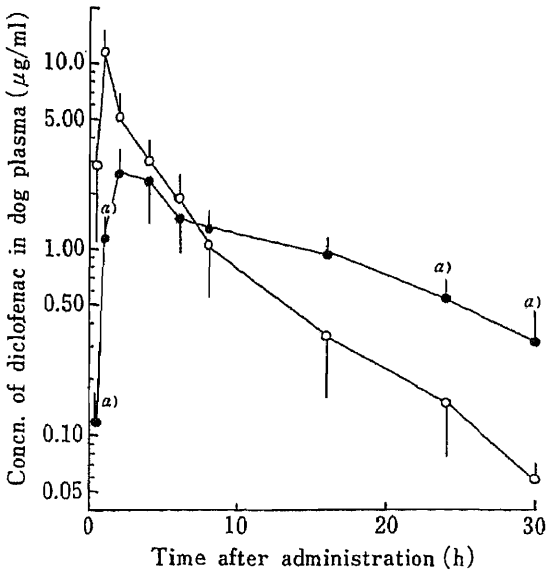

Fig. 4. Plasma Diclofenac Concentration in Dogs as a Function of Time after Administration of a Conventional Tablet (Open Symbols) and SR-Tablet (Closed Symbols) at a Dose of 25 mg of Sodium Diclofenac

Three dogs were used in a cross-over study. Each value represents the mean ± S.D. ($n=3$). *a*) $p<0.05$ *versus* the conventional tablet.

TABLE III. $AUC^{a)}$ of Plasma Drug Concentration up to 30 h, C_{max} and T_{max} after Oral Administration

Drug	Formulation	AUC (μg·h/ml)	C_{max} (μg/ml)	T_{max} (h)
Theophylline				
	Suspension	430±102	39.4±8.6	2.3±0.58
	SR-tablet	222±62[b)]	10.9±1.1[b)]	8.0±2.0[b)]
Sodium diclofenac				
	Commercial tablet	51.9±12.2	11.7±4.1	1
	SR-tablet	25.4±7.9[b)]	2.8±0.9[b)]	2.9±1.1[b)]

a) AUC was obtained from the plasma drug concentration in Figs. 3 and 4. Each value represents the mean±S.D. ($n=3$). *b*) $p<0.05$ *versus* suspension for theophylline and *versus* commercial tablet for sodium diclofenac.

than 0.5 μg/ml with the suspension. These observations also indicate that the administration of an SR-tablet maintained the plasma theophylline concentration for a long period. The relative bioavailability (area under the blood concentration curve (AUC) up to 30 h) was greater in the suspension than in the test tablet (Table III). The low bioavailability of theophylline after the administration of an SR-tablet may be due to incomplete release of theophylline *in vivo*, in spite of the observation that almost complete release of theophylline from an SR-tablet occurred whthin 24 h in the *in vitro* study (Fig. 1).

Similar results were obtained for diclofenac (Fig. 4). Oral administration of a commercial tablet resulted in a high transient diclofenac concentration in plasma, followed by rapid elimination. However, the administration of the SR-tablet did not give such a transient peak and the plasma diclofenac concentration was maintained for a long period. After 30 h, the plasma diclofenac concentration was about 0.35 μg/ml in the case of the SR-tablet, but was less than 0.1 μg/ml with the commercial tablet. The relative bioavailability of diclofenac (AUC up to 30 h) was greater from the commercial tablet than from the SR-tablet (Table III).

In spite of the rapid release of theophylline from an SR-tablet in comparison with the release of sodium diclofenac in the *in vitro* study, the T_{max} for the sodium diclofenac was faster than that for theophylline (Figs. 3 and 4, and Table III). Although we did not investigate the pharmacokinetic behavior of the drugs, this discrepancy may be due to differences of the absorption and elimination rates of the two drugs.

In the present study, it can be concluded that the SR-tablets of theophylline and sodium diclofenac, prepared with H-phospholipids, avoided a transient peak of drug concentration in plasma and maintained high plasma drug concentrations in dogs after oral administration, owing to the slow release of the drug from the SR-tablet.

References and Notes

1) Present address: *Pharmaceutical Chemistry Department, University of Kansas, Lawrence, Kansas 66045, U.S.A.*
2) F. Theeuwes, *J. Pharm. Sci.*, **64**, 1987 (1975).
3) L. G. J. de Leed, A. G. de Boer, and D. D. Breimer, *Biopharm. Drug Disposition*, **2**, 131 (1981).
4) L. G. J. de Leed, A. G. de Boer, A. van Velzer, and D. D. Breimer, *J. Pharmacokinet. Biopharm.*, **10**, 525 (1982).
5) T. Nishihata, M. Sudoh, A. Kamada, M. Keigami, T. Fujimoto, S. Kamide, and N. Tatsumi, *Int. J. Pharmaceut.*, **33**, 181 (1986).
6) Y. Hirotani, Y. Arakawa, Y. Maeda, A. Yamaji, A. Kamada, and T. Nishihata, *Chem. Pharm. Bull.*, **35**, 3049 (1987).
7) B. Lunberg, E. Sevens, and S. Ekman, *Chem. Phys. Lipids*, **22**, 285 (1978).
8) T. Higuchi, *J. Pharm. Sci.*, **52**, 1145 (1963).
9) H. Yaginuma, T. Nakata, H. Toya, T. Murakami, M. Yamazaki, and A. Kamada, *Chem. Pharm. Bull.*, **29**, 2974 (1982).
10) T. Nishihata, J. H. Rytting, and T. Higuchi, *J. Pharm. Sci.*, **70**, 71 (1981).

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Constituents of *Murraya exotica* L. Structure Elucidation of New Coumarins

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Eight new coumarins, peroxyauraptenol (**1b**), *cis*-dehydroosthol (**2**), murraol (**3**), murranganon (**4a**), isomurranganon senecioate (**5**), murrangatin acetate (**6b**), isomurralonginol acetate (**7b**), and chloticol (**8**), were isolated from leaves of *Murraya exotica* L. (Rutaceae), and their structures were elucidated. Seventeen known coumarins were also characterized. However, no alkaloid was detected.

Keywords——coumarin; Rutaceae; *Murraya exotica*; peroxyauraptenol; *cis*-dehydroosthol; murraol; murranganon; isomurranganon senecioate; isomurralonginol acetate; chloticol

Introduction

We have previously shown that the root bark of *Murraya euchrestifolia* HAYATA contains many kinds of carbazole alkaloids and coumarins.[1] This time, we studied the constituents of leaves of *M. exotica* L., and eight new coumarins were isolated together with seventeen known coumarins. However, no alkaloid was detected. The plant of *M. exotica* has been considered to be a synonym of *M. paniculata*. However, recent investigation led to the proposal that this species be re-instated as a distinct taxon.[2] In these circumstances, studies of the constituents of this plant are of interest from the viewpoint of chemical taxonomy.

Results and Discussion

The acetone extract of the leaves of *M. exotica* was treated as shown in Fig. 1, and eight new coumarins, named peroxyauraptenol (**1b**), *cis*-dehydroosthol (**2**), murraol (**3**), murranganon (**4a**), isomurranganon senecioate (**5**), murrangatin acetate (**6b**), isomurralonginol acetate (**7b**), and chloticol (**8**), were isolated. Together with these new coumarins, 7-methoxy-8-(1′-acetoxy-2′-oxo-3′-methylbutyl)coumarin (**4c**), murrangatin (**6a**), *trans*-dehydroosthol (**9**), phebalosin (**10**), minumicrolin (**11**), osthol (**12**), meranzin (**13**), meranzin hydrate (**14a**) murrayatin (**14b**), casegravol (**15**), isomeranzin (**16**), osthenon (**17**), murralongin (**18**), 7-methoxy-8-formylcoumarin (**19**), umbelliferone (**20**), scopoletin (**21**), and sibiricol (**22**) were also isolated and characterized.

Structure of Peroxyauraptenol (1b)

Peroxyauraptenol was obtained from fresh leaves in 0.002% yield as colorless prisms, mp 114—116 °C, $[\alpha]_D$ +3.53° ($CHCl_3$). The chemical ionization mass spectrum (CI-MS) using ammonia or isobutane as reactant gas established the molecular weight as 276. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum (see Experimental) showed six singlets including one carbonyl carbon, five doublets, two triplets, and two quartets. The ultraviolet (UV) [λ_{max} 248, 257, and 322 nm], infrared (IR) [ν_{max} 1730 and 1610 cm^{-1}], and proton nuclear magnetic resonance (^{1}H-NMR) spectra [δ 3.95 (3H, s, OCH_3), 7.64 (1H, d, J=9.4 Hz), 6.26

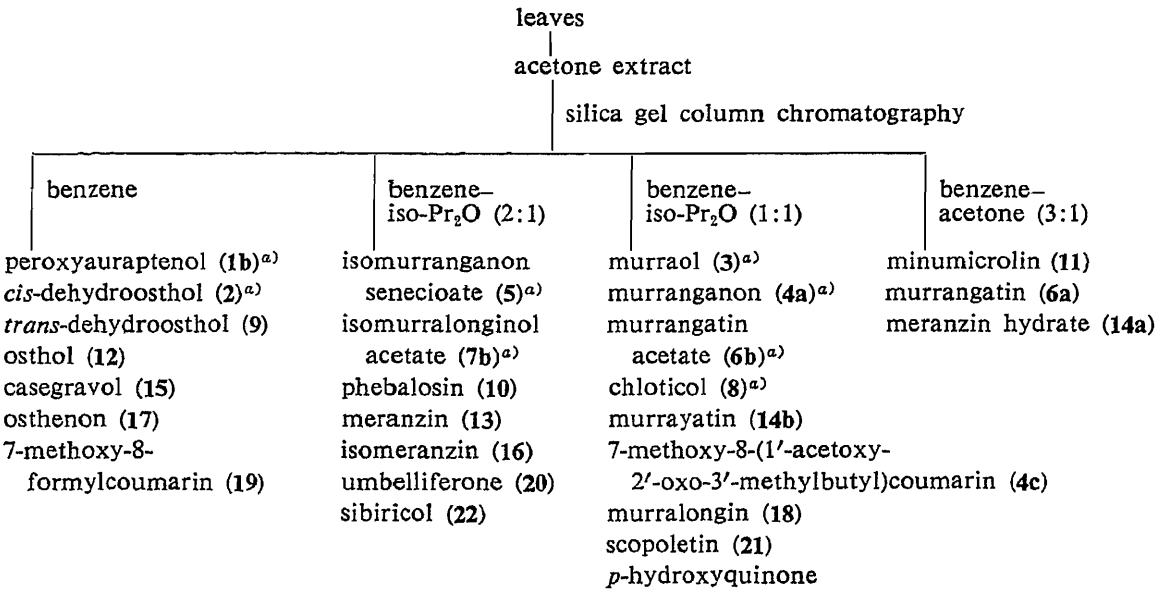

Fig. 1. Isolation of Coumarins from *Murraya exotica*

a) New compounds.

(1H, d, $J=9.4$ Hz), 7.36 (1H, d, $J=8.7$ Hz), and 6.87 (1H, d, $J=8.7$ Hz)] indicated the presence of a 7-methoxy-8-substituted coumarin nucleus in this molecule.[3] Further, in the ^{1}H-NMR spectrum, ABC-type signals at δ 3.16 (1H, dd, $J=5.4$, 13.8 Hz), 3.27 (1H, dd, $J=7.7$, 13.8 Hz), and 4.60 (1H, dd, $J=5.4$, 7.7 Hz), two 1H singlets at δ 4.88 and 4.94 assignable to *exo*-methylene protons, and a 3H singlet at δ 1.91 due to an allyl methyl group were observed. The appearance of the signals at δ_H 4.60 (1H, dd, $J=5.4$, 7.7 Hz), one of the ABC-type protons, and δ_C 87.3 (d) in ^{1}H- and ^{13}C-NMR spectra, respectively, together with the occurrence of fragments corresponding to $[M^+ - \cdot OH]$ and $[M^+ - \cdot OOH]$ in the electron impact mass spectrum (EI-MS), and the IR band at 3520 cm^{-1}, suggested the presence of a hydroperoxy moiety in this coumarin. These data coupled with the observation of a significant mass fragment peak at *m/z* 189 (100%) ascribed to an ion [a], showed the structure $[-CH_2-CH(OOH)-C(CH_3)=CH_2]$ for the side chain. Treatment of this coumarin with triphenylphosphine in methanol at room temperature gave a colorless syrup, $[\alpha]_D$ $-4.6°$ ($CHCl_3$), which was found to be identical with auraptenol (**1a**), which was isolated first from *Citrus aurantium*,[4] and subsequently from *M. exotica*[5] collected in India. The yield of peroxyauraptenol from the plant was so small that we could not obtain enough **1a** to get a satisfactory $[\alpha]_D$ value [lit.[5] $[\alpha]_D$ $-11.83°$ ($CHCl_3$)]. However, the negative $[\alpha]_D$ value in chloroform of **1a** derived from peroxyauraptenol suggested *S*-configuration for the asymmetric center according to the proposal by Shoolery *et al.*[5] These results led us to conclude the structure of peroxyauraptenol to be **1b**.

Since the first isolation of a coumarin hydroperoxide from plants of the genus *Mammea* by Crombie *et al.*,[6] this is only the second example of isolation of a hydroperoxygenated coumarin from a natural source.

Structure of *cis*-Dehydroosthol (2)

cis-Dehydroosthol was obtained (0.024% yield) as a colorless oil. The presence of a 7-oxygenated coumarin nucleus was presumed from the UV [λ_{max} 220 (sh), 277, and 322 nm] and IR [ν_{max} 1725 and 1600 cm^{-1}] spectra.[3] The ^{1}H-NMR spectrum contained signals assignable to a methoxy [δ 3.91 (3H, s)], an allyl methyl [δ 1.60 (3H, s)], and an *exo*-methylene group

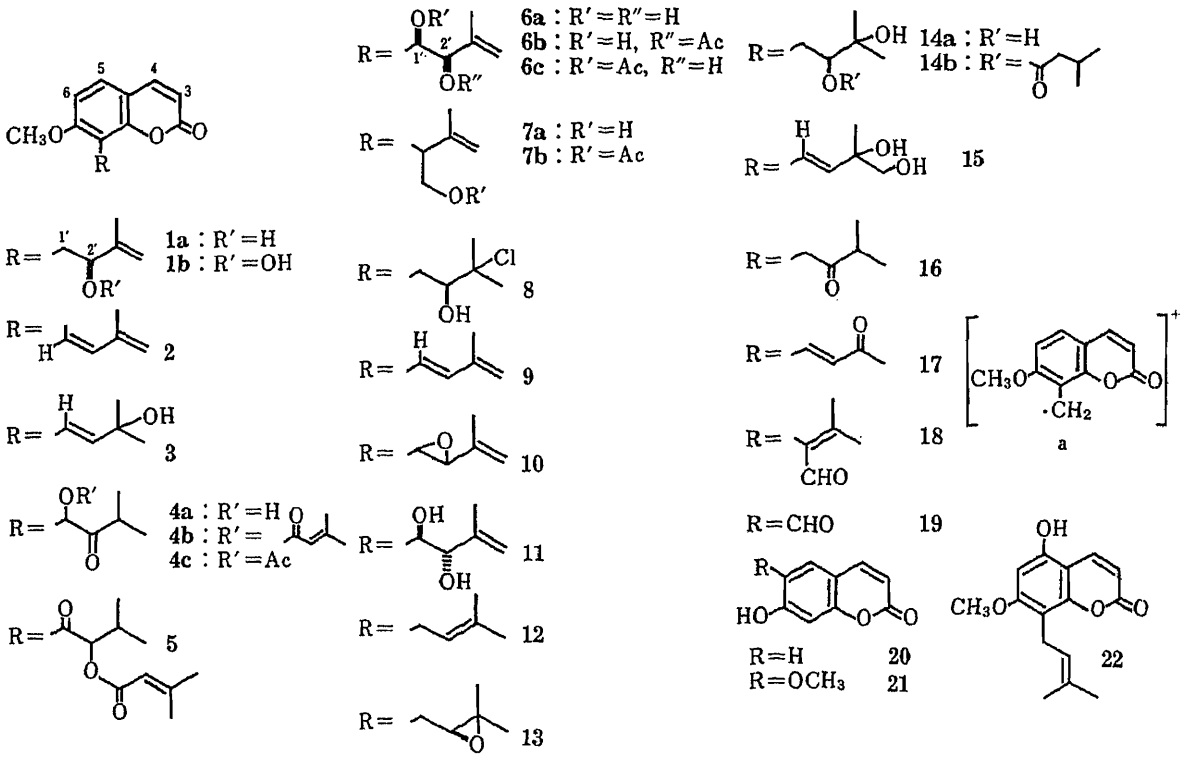

Fig. 2

[δ 4.84 (2H, s)] as well as three pairs of doublets [δ 7.62 (1H, d, J=9.4 Hz), 7.36 (1H, d, J=8.7 Hz), 6.84 (1H, d, J=8.7 Hz), 6.45 (1H, d, J=12 Hz), 6.24 (1H, d, J=9.4 Hz), 6.20 (1H, d, J=12 Hz)]. Among these pairs of doublets, two at δ 7.62 and 6.24, and δ 7.36 and 6.84 were easily assignable to H-4 and -3, and H-5 and -6, respectively, in the coumarin nucleus.[3] The observation of another pair of doublets having a larger coupling constant (J=12 Hz) at δ 6.45 and 6.20 revealed the presence of (*Z*)-disubstituted double bond in the molecule. On the basis of these spectral data coupled with a similar mass fragment pattern (see Experimental) to that of *trans*-dehydroosthol (**9**)[7] which was also isolated from the same plant source and characterized, the structure of *cis*-dehydroosthol was proposed to be as shown by formula **2**.

Structure of Murraol (3)

Murraol was isolated as colorless prisms (0.013% yield), mp 105—107 °C. The molecular formula $C_{15}H_{16}O_4$ was established by analysis of the high-resolution MS. Observation of two characteristic pairs of doublets [δ 7.62 and 6.26 (each 1H, d, J=9.4 Hz), and δ 7.30 and 6.86 (each 1H, d, J=8.7 Hz)] in the ^{1}H-NMR spectrum, coupled with the UV [λ_{max} 244 (sh), 254 (sh), 274 (sh), 282, and 318 nm] and IR [ν_{max} 1720 and 1600 cm^{-1}] features, suggested that murraol possesses the same 7-oxygenated-8-substituted coumarin nucleus[3] as **1b**. The ^{1}H-NMR spectrum indicated the presence of a methoxy group [δ 3.95 (3H, s)] and two methyls [δ 1.47 (6H, s)] attached to a carbon atom bearing a hydroxy group [δ 1.82 (1H, br s, exchangeable with D_2O); ν_{max} 3450 cm^{-1}]. Remaining AB-type signals at δ 7.02 and 6.93 (each 1H, d) having a large coupling constant (J=16.4 Hz) were attributable to an (*E*)-disubstituted double bond, as in **9**.[8] In the MS, murraol furnished ions at *m/z* 242 (100%, $M^+ - H_2O$), 227, 211, 183, 155, 131, 128, and 115, corresponding to the behavior of **9**, in addition to fragments at *m/z* 260 (M^+), 245 ($M^+ - \cdot CH_3$), and so on. Based on these results, we assigned the structure **3** to murraol.

Structure of Murranganon (4a)

Murranganon was obtained as a colorless oil, $[\alpha]_D$ +105.8° ($CHCl_3$), and the molecular formula $C_{15}H_{16}O_5$ was determined by high-resolution MS. The UV absorptions at λ_{max} 247, 257, and 320 nm, IR bands at ν_{max} 1730 and 1610 cm^{-1}, and ^{1}H-NMR signals at δ 3.87 (OCH_3), 7.65 and 6.29 (each 1H, d, J=9.4 Hz), and δ 7.46 and 6.86 (each 1H, d, J=8.7 Hz) were consistent with a 7-methoxy-8-substituted coumarin skeleton.[3] Strong IR bands at 1730 and 3500 cm^{-1} indicated the presence of an isolated carbonyl group in addition to a carbonyl in the coumarin nucleus, and a hydroxy group, respectively. The 1H quintet signal at δ 2.61 (J= 6.7 Hz) together with two 3H doublets at δ 1.13 and 0.98 (both J=6.7 Hz) were assignable to an isopropyl moiety attached to a carbonyl carbon. Among two doublets at δ 5.90 and 4.23 coupled with each other (J=5.7 Hz), the former signal collapsed to a singlet and later disappeared on addition of deuterium oxide. These spectral data together with the appearance of the base fragment peak at m/z 205 due to $[M^+ - \cdot CO\text{–}CH(CH_3)_2]$ led us propose the structure **4a** for murranganon, leaving the absolute stereochemistry undetermined.

Structure of Isomurranganon Senecioate (5)

Colorless oil, $[\alpha]_D$ +60.6° ($CHCl_3$). The molecular weight (358) of this compound was indicated by CI-MS using NH_3 or isobutane as the reactant gas. The presence of a 7-methoxy-8-substituted coumarin nucleus was also suggested by the UV, IR, and ^{1}H-NMR spectra (see Experimental). The ^{13}C-NMR spectrum revealed nineteen carbon signals including three lower singlet signals at δ 198.6, 165.5, and 159.5 assignable to carbonyl carbons of benzylic ketone, ester, and the coumarin nucleus, respectively. The ^{1}H-NMR spectrum showed a doublet at δ 5.79 (J=3.4 Hz) ascribed to a proton attached to a carbon bearing an ester oxygen and adjacent to a carbonyl moiety, coupled with a methine proton [δ 2.44 (multiplet)] which was further coupled with two sets of methyl protons at δ 1.04 and 1.02. This led to the following structure of the side chain: –CO–CH(O–COR)–$CH(CH_3)_2$. The presence of a senecioic acid ester moiety in the molecule was indicated by the following ^{1}H- and ^{13}C-NMR signals, and mass fragment: δ_H 5.67 (1H, s), 2.12 (3H, s), and 1.87 (3H, s); δ_C 165.5 (s), 27.4 (q), and 19.8 (q); m/z 274 $[M^+ - \cdot COCH=C(CH_3)_2 + \cdot H]$. On the basis of these spectral data together with ^{1}H-NMR spectral comparisons of this coumarin and **4b** derived from murranganon (**4a**) and senecioic acid chloride, the structure **5** was proposed for isomurranganon senecioate, leaving the stereochemistry undetermined.

Structure of Murrangatin Acetate (6b)

Murrangatin acetate was isolated as a colorless oil. The 7-methoxy-8-substituted coumarin skeleton in this molecule was indicated by the UV, IR, and ^{1}H-NMR spectra [δ 7.61 and 6.26 (each 1H, d, J=9.4 Hz, H-4, 3), 7.39 and 6.87 (each 1H, d, J=8.7 Hz, H-5, 6), and 4.00 (3H, s, OCH_3)].[3] Signals at δ 4.77 (1H, s), 4.74 (1H, s), 2.14 (3H, s), and 1.75 (3H, s) in the ^{1}H-NMR spectrum were easily assignable to two *exo*-methylene, acetyl methyl, and allyl methyl protons, respectively. Among the ABC-type signals at δ 5.73 (1H, d, J=7.7 Hz), 5.53 (1H, dd, J=11.1, 7.7 Hz), and 3.56 (1H, d, J=11.1 Hz), the double-doublet at 5.53 collapsed to a doublet (J=7.7 Hz), and the doublet at δ 3.56 disappeared on addition of deuterium oxide. An observation of long-range coupling between the *exo*-methylene protons and the lower signal at δ 5.73 (see Experimental) suggested the location of the *O*-acetyl moiety at C-2′ in the glycol side chain. On the basis of these data, this coumarin was considered to have the structure corresponding to murrangatin (**6a**)[7,9] monoacetate. This was confirmed by acetylation of murrangatin (**6a**) with acetyl chloride in pyridine to afford **6b** as a major product (56% yield), which was found to be identical with the natural product. Regioisomer **6c** was also formed as a minor product (10% yield), and in the ^{1}H-NMR spectrum, the proton (H-1′) attached to the carbon having the ester moiety was observed at δ 6.42 as a doublet (J= 7.7 Hz) which was at lower field as compared with the corresponding proton (H-2′) of **6b**.

These data led us to the structure **6b** for murrangatin acetate isolated from the natural source.

Structure of Isomurralonginol Acetate (7b)

Isomurralonginol acetate was obtained as a colorless oil, $[\alpha]_D$ +18.8° ($CHCl_3$). The molecular formula $C_{17}H_{18}O_5$ was established by high-resolution MS. The 7-methoxy-8-substituted coumarin skeleton of this compound was suggested by the following spectroscopic data[3]: λ_{max} 220 (sh), 248, and 320 nm; ν_{max} 1730 and 1600 cm^{-1}; δ_H 7.63 and 6.25 (each 1H, d, J=9.4 Hz) and δ_H 7.36 and 6.85 (each 1H, d, J=8.7 Hz); MS *m/z* 189. The presence of an *O*-acetyl moiety in the molecule was revealed by a signal at δ 1.96 (3H, s), and fragments at *m/z* 260 ($M^+ \cdot COCH_3$) and 242 ($M^+ - HOCOCH_3$) in ^{1}H-NMR spectrum and MS, respectively. The ^{1}H-NMR spectrum of isomurralonginol acetate showed two 1H singlets at δ 4.91 and 4.92, and a 3H singlet at δ 1.70, assignable to an *exo*-methylene and allyl methyl protons, respectively. Among ABC-type signals at δ 4.83 (1H, dd, J=10.8, 7.4 Hz), 4.59 (1H, dd, J=10.8, 7.4 Hz), and 4.48 (1H, t, J=7.4 Hz), two lower signals at δ 4.83 and 4.59 could be assigned to a methylene protons having an acetoxy group. The triplet at δ 4.48 was further shown to be long-range-coupled with the *exo*-methylene protons. On the basis of these spectral data and the co-occurrence of murralongin (**18**)[10] in the same plant, isomurralonginol acetate can be represented by formula **7b**.[11]

Structure of Chloticol (8)

Chloticol was isolated as a colorless oil, $[\alpha]_D$ −10.4° ($CHCl_3$). In the EI-MS, the appearance of the molecular ion peak at *m/z* 296 accompanied with an isotope ion at *m/z* 298 in the ratio 3 : 1 suggested the presence of a chlorine atom in the molecule, and the high-resolution MS showed the molecular formula to be $C_{15}H_{17}O_4Cl$. The UV and IR spectra (see Experimental), and ^{1}H-NMR signals at δ 7.64 (1H, d, J=9.4 Hz), 7.36 (1H, d, J=8.7 Hz), 6.88 (1H, d, J=8.7 Hz), 6.26 (1H, d, J=9.4 Hz), and 3.95 (3H, s), suggested the 7-methoxy-8-substituted coumarin skeleton.[3] The ABC-type signals at δ 3.22 (1H, dd, J=3.4, 13.5 Hz), 3.14 (1H, dd, J=10.1, 13.5 Hz), and 3.75 (1H, m) along with the observation of mass fragments at *m/z* 219 [$M^+ - \cdot C(CH_3)_2Cl$] and 189 [$M^+ - \cdot CH(OH)-C(CH_3)_2Cl$] suggested the structure of the side chain to be $-CH_2-CH(OH)-C(CH_3)_2Cl$. On the basis of these results, we proposed the structure **8** for chloticol, leaving the stereochemistry undetermined. The artifactual formation of chloticol can not be ruled out.

Other coumarins isolated from the same plant material were fully characterized as 7-methoxy-8-(1′-acetoxy-2′-oxo-3′-methylbutyl)coumarin (**4c**),[12] murrangatin (**6a**),[7,9] *trans*-dehydroosthol (**9**),[8] phebalosin (**10**),[13] minumicrolin (**11**),[9,14] osthol (**12**),[15] meranzin (**13**),[16] meranzin hydrate (**14a**),[16] murrayatin (**14b**),[17] casegravol (**15**),[18] isomeranzin (**16**),[19] osthenon (**17**),[20] murralongin (**18**),[11] 7-methoxy-8-formylcoumarin (**19**),[21] umbelliferone (**20**),[22] scopoletin (**21**),[23] and sibiricol (**22**)[24] by analyses of the UV, IR, MS and/or NMR spectra.

Experimental

All melting points were measured on a micromelting point hot-stage apparatus (Yanagimoto). ^{1}H- and ^{13}C-NMR spectra were recorded on GX-270 (JEOL) and GX-400 (JEOL) spectrometers, respectively, in $CDCl_3$, unless otherwise stated. Chemical shifts are shown in δ values (ppm) with tetramethylsilane (TMS) as an internal reference. EI-MS were taken with an M-52 (Hitachi) spectrometer having a direct inlet system, and CI-MS and high-resolution MS with an M-80 (Hitachi) spectrometer. UV spectra were determined in methanol and IR spectra were recorded in $CHCl_3$.

Extraction and Separation——The fresh leaves (340 g) of *Murraya exotica* L. cultivated at Higashiyama Zoo & Botanical Garden (Nagoya) were collected in November, 1986, and extracted with acetone at room temperature. The acetone extract (27 g) was subjected to silica gel column chromatography. Successive elution with benzene, benzene–isopropyl ether (2 : 1), benzene–isopropyl ether (1 : 1), and benzene–acetone (3 : 1) gave four fractions. Each fraction

was further subjected to silica gel preparative thin layer chromatography (prep. TLC) (solvent: appropriate combinations of acetone, benzene, hexane, isopropyl ether, and chloroform) to give the following components (values in parentheses are yields from fresh leaves): from the first fraction: peroxyauraptenol (**1b**) (0.002%), *cis*-dehydroosthol (**2**) (0.024%), *trans*-dehydroosthol (**9**) (0.004%), osthol (**12**) (0.03%), casegravol (**15**) (0.0004%), osthenon (**17**) (0.002%), 7-methoxy-8-formylcoumarin (**19**) (0.0004%). From the second fraction: isomurranganon senecioate (**5**) (0.003%), isomurralonginol acetate (**7b**) (0.006%), phebalosin (**10**) (0.21%), meranzin (**13**) (0.002%), isomeranzin (**16**) (0.004%), umbelliferone (**20**) (0.0009%), sibiricol (**22**) (0.0009%). From the third fraction: murraol (**3**) (0.013%), murranganon (**4a**) (0.002%), murrangatin acetate (**6b**) (0.0009%), chloticol (**8**) (0.0064%), murrayatin (**14b**) (0.0009%), 7-methoxy-8-(1′-acetoxy-2′-oxo-3′-methylbutyl)coumarin (**4c**) (0.005%), murralongin (**18**) (0.0009%), scopoletin (**21**) (0.0009%), and *p*-hydroquinone. From the last fraction: minumicrolin (**11**) (0.004%), murrangatin (**6a**) (0.012%), and meranzin hydrate (**14a**) (0.007%).

Peroxyauraptenol (1b)——Colorless prisms from ether, mp 114—116 °C, $[\alpha]_D$ +3.53° (c=0.085, $CHCl_3$). UV λ_{max} nm: 248, 257, 322. IR ν_{max} cm^{-1}: 3500, 1730, 1610. ^{1}H-NMR δ: 8.46 (1H, br s, OH), 7.64 (1H, d, J=9.4 Hz), 7.36 (1H, d, J=8.7 Hz), 6.87 (1H, d, J=8.7 Hz), 6.26 (1H, d, J=9.4 Hz), 4.94 (1H, s), 4.88 (1H, s), 4.60 (1H, dd, J=5.4, 7.7 Hz), 3.95 (3H, s), 3.27 (1H, dd, J=7.7, 13.8 Hz), 3.16 (1H, dd, J=5.4, 13.8 Hz), 1.91 (3H, s). ^{13}C-NMR δ: 160.9 (s), 160.7 (s), 153.6 (s), 143.8 (d), 127.2 (d), 114.3 (s), 113.4 (t), 113.2 (d), 113.2 (s), 113.0 (s), 107.4 (d), 87.3 (d), 24.3 (t), 56.3 (q), 18.6 (q). CI-MS (iso-C_4H_{10}) m/z: 277 $(M+\cdot H)^+$, 259, 243, 219, 189. CI-MS (NH_3) m/z: 294 $(M+NH_4)^+$, 276, 260, 224, 208, 191. EI-MS m/z (%): 259, 244, 243, 190 (67), 189 (100), 175 (16), 159, 146, 131 (84), 118, 103.

Treatment of 1b with Triphenylphosphine——A methanolic solution (1 ml) of peroxyauraptenol (**1b**) (2 mg) and triphenylphosphine (1 mg) was stirred overnight at room temperature. The mixture was evaporated to dryness *in vacuo*. The residue was submitted to silica gel prep. TLC to afford a colorless syrup (1.7 mg). $[\alpha]_D$ −4.6° (c=0.087, $CHCl_3$). UV λ_{max} nm: 248, 257, 322. IR ν_{max} cm^{-1}: 1720, 1610. ^{1}H-NMR δ: 7.62 (1H, d, J=9.8 Hz), 7.43 (1H, d, J=8.7 Hz), 6.86 (1H, d, J=8.7 Hz), 6.24 (1H, d, J=9.8 Hz), 4.90 (1H, br s), 4.80 (1H, br s), 4.35 (1H, m), 3.93 (3H, s), 3.20 (dd, J=5.1, 13.8 Hz), 3.09 (1H, dd, J=13.8, 8.4 Hz), 1.90 (3H, s). EI-MS m/z (%): 243 $(M^+ - \cdot OH)$, 190 (100), 189, 175, 161, 131. The above data are in agreement with those listed in the literature[4,5] for auraptenol (**1a**).

***cis*-Dehydroosthol (2)**——Colorless oil. High-resolution MS: Calcd for $C_{15}H_{14}O_3$: 242.0941. Found: 242.0919. UV λ_{max} nm: 220 (sh), 277, 322. IR ν_{max} cm^{-1}: 1725, 1600. ^{1}H-NMR δ: 7.62 (1H, d, J=9.4 Hz), 7.36 (1H, d, J=8.7 Hz), 6.84 (1H, d, J=8.7 Hz), 6.45 (1H, d, J=12 Hz), 6.24 (1H, d, J=9.4 Hz), 6.20 (1H, d, J=12 Hz), 4.84 (2H, s), 3.91 (3H, s), 1.60 (3H, s). EI-MS m/z (%): 242 (M^+, 100), 227 (26), 211 (76), 189 (27), 183 (41), 155 (29), 131 (10), 128 (20), 115 (20).

Murraol (3)——Colorless prisms from acetone, mp 105—107 °C. High-resolution MS: Calcd for $C_{15}H_{16}O_4$: 260.1047. Found: 260.1029. UV λ_{max} nm: 244 (sh), 254 (sh), 274 (sh), 282, 318. IR ν_{max} cm^{-1}: 1600, 1720, 3450. ^{1}H-NMR δ: 7.62 (1H, d, J=9.4 Hz), 7.30 (1H, d, J=8.7 Hz), 7.02 (1H, d, J=16.4 Hz), 6.93 (1H, d, J=16.4 Hz), 6.86 (1H, d, J=8.6 Hz), 6.26 (1H, d, J=9.4 Hz), 3.95 (3H, s), 1.82 (1H, br s, OH), 1.47 (6H, s). EI-MS m/z (%): 260 (M^+, 27), 245 (25), 242 (100), 218 (12), 227, 217 (45), 211 (42), 203 (13), 189 (19), 183 (25), 155 (17), 131, 128, 115.

Murranganon (4a)——Colorless oil. $[\alpha]_D$ +105.8° (c=0.06, $CHCl_3$). High-resolution MS: Calcd for $C_{15}H_{16}O_5$: 276.0996. Found: 276.1044. UV λ_{max} nm: 247, 257, 320. IR ν_{max} cm^{-1}: 3500, 1730, 1610. ^{1}H-NMR δ: 7.65 (1H, d, J=9.4 Hz), 7.46 (1H, d, J=8.7 Hz), 6.86 (1H, d, J=8.7 Hz), 6.29 (1H, d, J=9.4 Hz), 5.90 (1H, d, J=5.7 Hz), 4.23 (1H, d, J=5.7 Hz, OH), 3.87 (3H, s), 2.61 (1H, quintet, J=6.7 Hz), 1.13 (3H, d, J=6.7 Hz), 0.98 (3H, d, J=6.7 Hz). EI-MS m/z (%): 276 (M^+), 259, 206 (13), 205 (100), 203 (13), 190, 175 (26), 162.

Isomurranganon Senecioate (5)——Colorless oil, $[\alpha]_D$ +60.6° (c=0.078, $CHCl_3$). UV λ_{max} nm: 214, 297 (sh), 321, 335 (sh). IR ν_{max} cm^{-1}: 1738, 1600, 1300, 1250. ^{1}H-NMR δ: 7.60 (1H, d, J=9.4 Hz), 7.46 (1H, d, J=8.7 Hz), 6.84 (1H, d, J=8.7 Hz), 6.25 (1H, d, J=9.4 Hz), 5.79 (1H, d, J=3.4 Hz), 5.67 (1H, s), 3.88 (3H, s), 2.44 (1H, m), 2.12 (3H, s), 1.87 (3H, s), 1.04 (3H, d, J=2.7 Hz), 1.02 (3H, d, J=2.7 Hz). ^{13}C-NMR δ: 198.6 (s), 165.5 (s), 159.5 (2s), 157.6 (s), 152.0 (s), 142.7 (d), 130.2 (d), 115.5 (d), 114.1 (d), 113.0 (d), 107.5 (d), 81.7 (d), 56.3 (q), 29.8 (d), 27.4 (q), 19.8 (q), 16.8 (2q). CI-MS m/z: 359 $(M+\cdot H)^+$; 376 $(M+NH_4)^+$. EI-MS m/z (%): 288, 204 (12), 203 (100), 188, 175, 160.

Murranganon Senecioate (4b)——A pyridine (0.5 ml) solution of murranganon (**4a**) (7 mg) [derived from **4c** by hydrolysis with aqueous $NaHCO_3$ in MeOH] and 3,3-dimethylacryloyl chloride (20 mg) was left in room temperature overnight, and then evaporated to dryness. The residue was purified by prep. TLC (silica gel; iso-Pr_2O–Me_2CO=20:1) to afford **4b** as a colorless oil (7 mg). **4b**: High resolution MS: Calcd for $C_{20}H_{22}O_6$: 358.1414. Found: 358.1397. UV λ_{max} nm: 220, 247 (sh), 257, 296 (sh), 320. IR ν_{max} cm^{-1}: 1730, 1610, 1270, 1250. ^{1}H-NMR δ: 7.61 (1H, d, J=9.4 Hz), 7.46 (1H, d, J=8.7 Hz), 7.03 (1H, s), 6.87 (1H, d, J=8.7 Hz), 6.26 (1H, d, J=9.4 Hz), 5.79 (1H, br s), 3.91 (3H, s), 2.96 (1H, quintet, J=7.1 Hz), 2.19 (3H, s), 1.89 (3H, s), 1.20 (3H, d, J=7.1 Hz), 1.04 (3H, d, J=7.1 Hz). EI-MS m/z (%): 358 (M^+), 287 (88), 205 (100), 189 (14), 175 (10), 162 (16), 149 (10), 131 (12).

Murrangatin Acetate (6b)——Colorless oil, $[\alpha]_D$ −6.3° (c=0.08, $CHCl_3$). UV λ_{max} nm: 219 (sh), 247, 257, 298 (sh), 320, 336 (sh). IR ν_{max} cm^{-1}: 1730, 1610, 1250. ^{1}H-NMR δ: 7.61 (1H, d, J=9.4 Hz), 7.39 (1H, d, J=8.7 Hz), 6.87 (1H, d, J=8.7 Hz), 6.26 (1H, d, J=9.4 Hz), 5.73 (1H, d, J=7.7 Hz), 5.53 (1H, dd, J=11.1, 7.7 Hz), 4.77 (1H, s), 4.74 (1H, s), 4.00 (3H, s), 3.56 (1H, d, J=11.1 Hz, OH), 2.14 (3H, s), 1.75 (3H, s). On irradiation at the middle of the frequences of the *exo*-methylene protons at δ 4.77 and 4.74, the signal at δ 5.73 [H-2′, half-band width, $W_{1/2}$=3.3 Hz)] sharpened ($W_{1/2}$=2.2 Hz), as did the allyl methyl signal at δ 1.75, and no change of the signal at δ 5.35 (H-1′) was

observed. CI-MS *m/z*: 319 $(M+\cdot H)^+$; 336 $(M+NH_4)^+$. EI-MS *m/z* (%): 248, 242, 206 (16), 205 (100), 175 (20), 162, 148, 134.

Acetylation of Murrangatin (6a)——A solution of murrangatin (**6a**) (14 mg) and acetyl chloride (20 mg) in dry pyridine (1 ml) was stirred for 10 min at room temperature, then three drops of water were added, and the mixture was evaporated to dryness. The residue was subjected to silica gel prep. TLC (solvent: benzene : acetone = 4 : 1; $CHCl_3$: acetone = 20 : 1) to afford **6b** (5 mg) and **6c** (1 mg) together with the starting material (**6a**) (6 mg). The major product (**6b**) was found to be identical with murrangatin acetate isolated from the natural source by IR, ^{1}H-NMR, and MS comparisons. **6c**: ^{1}H-NMR δ: 7.60 (1H, d, J=9.4 Hz), 7.38 (1H, d, J=8.7 Hz), 6.84 (1H, d, J=8.7 Hz), 6.42 (1H, d, J=7.7 Hz), 6.25 (1H, d, J=9.4 Hz), 4.92 (1H, br d, J=7.7 Hz), 4.77 (1H, br s), 4.72 (1H, br s), 3.95 (3H, s), 2.61 (1H, br s, OH), 2.11 (3H, s), 1.75 (3H, s).

Isomurralonginol Acetate (7b)——Colorless oil. $[\alpha]_D$ +18.8° (c=0.2, $CHCl_3$). High-resolution MS: Calcd for $C_{17}H_{18}O_5$: 302.1153. Found: 302.1149. UV λ_{max} nm: 220 (sh), 248, 257, 320. IR ν_{max} cm^{-1}: 1730, 1600. ^{1}H-NMR δ: 7.63 (1H, d, J=9.4 Hz), 7.36 (1H, d, J=8.7 Hz), 6.85 (1H, d, J=8.7 Hz), 6.25 (1H, d, J=9.4 Hz), 4.92 (1H, s), 4.91 (1H, s), 4.83 (1H, dd, J=10.8, 7.4 Hz), 4.59 (1H, dd, J=10.8, 7.4 Hz), 4.48 (1H, t, J=7.4 Hz), 3.88 (3H, s), 1.96 (3H, s), 1.70 (3H, s). EI-MS *m/z* (%): 302 (M^+, 4), 260, 242 (100), 230 (22), 229 (21), 227 (30), 211 (26), 199 (40), 190 (42), 189 (45), 187 (41), 175 (16), 171 (15), 159 (16), 149 (18), 131 (36), 110 (25).

7-Methoxy-8-(1′-acetoxy-2′-oxo-3′-methylbutyl)coumarin (4c)[12]——Pale yellow oil. UV λ_{max} nm: 219 (sh), 247, 256, 296 (sh), 320. IR ν_{max} cm^{-1}: 1740, 1610. ^{1}H-NMR δ: 7.64 (1H, d, J=9.4 Hz), 7.49 (1H, d, J=8.7 Hz), 6.97 (1H, s), 6.89 (1H, d, J=8.7 Hz), 6.28 (1H, d, J=9.4 Hz), 3.91 (3H, s), 2.85 (1H, quintet, J=7.1 Hz), 2.16 (3H, s), 1.18 (3H, d, J=7.1 Hz), 1.03 (3H, d, J=7.1 Hz). EI-MS *m/z* (%): 318 (M^+), 258, 247, 231, 215, 206, 205 (100), 189, 175, 162.

Murrangatin (6a)[7]——Colorless needles from ether, mp 116 °C. $[\alpha]_D$ −7.4° (c=0.04, $CHCl_3$). UV λ_{max} nm: 248, 258, 321. IR ν_{max} cm^{-1}: 3500, 1730, 1605. ^{1}H-NMR δ: 7.62 (1H, d, J=9.4 Hz), 7.39 (1H, d, J=8.7 Hz), 6.87 (1H, d, J=8.7 Hz), 6.25 (1H, d, J=9.4 Hz), 5.30 (1H, t, J=8.7 Hz), 4.65 (1H, s), 4.58 (1H, s), 4.51 (1H, d, J=8.7 Hz), 3.97 (3H, s), 3.74 (1H, br s), 3.19 (1H, br s), 1.78 (3H, s). EI-MS *m/z* (%): 206, 205 (100), 191, 175.

Chloticol (8)——Colorless oil, $[\alpha]_D$ −10.4° (c=0.087, $CHCl_3$). High-resolution MS: Calcd for $C_{15}H_{17}O_4Cl$: 296.0813. Found: 296.0802. UV λ_{max} nm: 246, 257, 322. IR ν_{max} cm^{-1}: 1730, 1610. ^{1}H-NMR δ: 7.64 (1H, d, J=9.4 Hz), 7.36 (1H, d, J=8.7 Hz), 6.88 (1H, d, J=8.7 Hz), 6.26 (1H, d, J=9.4 Hz), 3.95 (3H, s), 3.75 (1H, m), 3.22 (1H, dd, J=3.4, 13.5 Hz), 3.14 (1H, dd, J=10.1, 13.5 Hz), 2.31 (1H, br, OH), 1.74 (3H, s), 1.71 (3H, s). MS *m/z* (%): 298 (M^++2), 296 (M^+) [(M+2) : M = 1 : 3], 219, 190 (100), 189, 175, 161, 131.

***trans*-Dehydroosthol (9)**[8]——Colorless oil. High-resolution MS: Calcd for $C_{15}H_{14}O_3$: 242.0941. Found: 242.0919. UV λ_{max} nm: 218 (sh), 262, 275, 290 (sh), 300, 316, 340 (sh). IR ν_{max} cm^{-1}: 1725, 1600. ^{1}H-NMR δ: 7.63 (1H, d, J=9.4 Hz), 7.49 (1H, d, J=16.8 Hz), 7.30 (1H, d, J=8.7 Hz), 6.90 (1H, d, J=16.8 Hz), 6.87 (1H, d, J=8.7 Hz), 6.27 (1H, d, J=9.4 Hz), 5.19 (1H, s), 5.14 (1H, s), 3.97 (3H, s), 2.03 (3H, s). EI-MS *m/z* (%): 242 (M^+, 100), 227 (14), 211 (60), 183 (24), 155 (18), 131 (13), 128 (15), 115 (13).

Phebalosin (10)[13]——Colorless needles from ether, mp 118—120 °C. UV λ_{max} nm: 246, 256, 320. IR ν_{max} cm^{-1}: 1732, 1608. ^{1}H-NMR δ: 7.61 (1H, d, J=9.4 Hz), 7.41 (1H, d, J=8.7 Hz), 6.87 (1H, d, J=8.7 Hz), 6.26 (1H, d, J=9.4 Hz), 5.30 (1H, s), 5.08 (1H, s), 3.99 (1H, d, J=2.0 Hz), 3.97 (3H, s), 3.92 (1H, d, J=2.0 Hz), 1.87 (3H, s). EI-MS *m/z* (%): 258 (M^+), 229, 213, 199, 189, 175.

Minumicrolin (11)[14]——Colorless needles from ether, mp 129—132 °C, $[\alpha]_D$ −3.0° (c=0.06, $CHCl_3$). UV λ_{max} nm: 248, 258, 320. IR ν_{max} cm^{-1}: 3500, 1730, 1608. ^{1}H-NMR δ: 7.63 (1H, d, J=9.4 Hz), 7.40 (1H, d, J=8.7 Hz), 6.89 (1H, d, J=8.7 Hz), 6.25 (1H, d, J=9.4 Hz), 5.41 (1H, dd, J=10, 7 Hz), 4.98 (1H, s), 4.97 (1H, s), 4.51 (1H, d, J=7 Hz), 3.97 (3H, s), 3.64 (1H, d, J=10 Hz), 2.23 (1H, br s), 1.88 (3H, s). EI-MS *m/z* (%): 206, 205 (100), 191, 175.

Osthol (12)[15]——Colorless prisms from ether, mp 79—81 °C. UV λ_{max} nm: 249, 258, 322. IR ν_{max} cm^{-1}: 1720, 1610. ^{1}H-NMR δ: 7.61 (1H, d, J=9.4 Hz), 7.29 (1H, d, J=8.7 Hz), 6.83 (1H, d, J=8.7 Hz), 6.23 (1H, d, J=9.4 Hz), 5.22 (1H, t, J=7.4 Hz), 3.92 (3H, s), 3.52 (2H, d, J=7.4 Hz), 1.84 (3H, s), 1.67 (3H, s). EI-MS *m/z* (%): 244 (M^+, 100), 229 (38), 213 (23), 201 (28), 189 (38), 131 (25).

Meranzin (13)[16]——Colorless prisms from ether, mp 89—92 °C. $[\alpha]_D$ −46.0° (c=0.063, $CHCl_3$). UV λ_{max} nm: 246, 256, 321. IR ν_{max} cm^{-1}: 1730, 1610. ^{1}H-NMR δ: 7.64 (1H, d, J=9.4 Hz), 7.36 (1H, d, J=8.7 Hz), 6.87 (1H, d, J=8.7 Hz), 6.25 (1H, d, J=9.4 Hz), 3.94 (3H, s), 3.26—2.98 (3H, overlapped m), 1.50 (3H, s), 1.29 (3H, s). EI-MS *m/z* (%): 260 (M^+), 242, 217, 202 (100), 189, 187, 174, 159, 131.

Meranzin Hydrate (14a)[16,19]——Colorless oil. $[\alpha]_D$ −29.1° (c=0.11, $CHCl_3$). UV λ_{max} nm: 248, 257, 322. IR ν_{max} cm^{-1}: 3500, 1720, 1610. ^{1}H-NMR δ: 7.64 (1H, d, J=9.4 Hz), 7.36 (1H, d, J=8.7 Hz), 6.88 (1H, d, J=8.7 Hz), 6.25 (1H, d, J=9.4 Hz), 3.94 (3H, s), 3.65 (1H, dd, J=2.7, 10 Hz), 3.11 (1H, dd, J=2.7, 13.8 Hz), 3.00 (1H, dd, J=13.8, 10 Hz), 2.14 (2H, br s, OH), 1.34 (6H, s). EI-MS *m/z* (%): 278 (M^+), 263, 261, 245, 220, 219, 205, 203, 190, 189, 178, 177 (100), 175, 160, 147, 131.

Murrayatin (14b)[17]——Colorless prisms, mp 101—103 °C. $[\alpha]_D$ +60.5° (c=0.18, $CHCl_3$). UV λ_{max} nm: 247, 257, 323. IR ν_{max} cm^{-1}: 1720, 1610. ^{1}H-NMR δ: 7.60 (1H, d, J=9.4 Hz), 7.32 (1H, d, J=8.7 Hz), 6.82 (1H, d, J=8.7 Hz), 6.23 (1H, d, J=9.4 Hz), 5.18 (1H, dd, J=10.4, 2.4 Hz), 3.94 (3H, s), 3.34 (1H, dd, J=10.4, 13.8 Hz), 3.04 (1H, dd, J=13.8, 2.4 Hz), 2.10—1.76 (3H, overlapped m), 1.37 (3H, s), 1.31 (3H, s), 0.72 (3H, d, J=6.7 Hz), 0.67 (3H, d, J=6.7 Hz). EI-MS *m/z* (%): 260, 245 (24), 220 (100), 189 (33), 131 (31).

Casegravol (15)[18]——Colorless prisms, mp 159—162 °C. $[\alpha]_D$ −10.1 ° (c=0.079, $CHCl_3$). UV λ_{max} nm: 246 (sh), 256 (sh), 271 (sh), 284, 320, 340 (sh). IR ν_{max} cm^{-1}: 1720, 1600. ^{1}H-NMR δ: 7.64 (1H, d, J=9.4 Hz), 7.33 (1H, d, J=8.7 Hz), 7.04 (1H, d, J=16.5 Hz), 6.87 (1H, d, J=8.7 Hz), 6.86 (1H, d, J=16.5 Hz), 6.27 (1H, d, J=9.4 Hz), 3.95 (3H, s), 3.69 (1H, d, J=11.1 Hz), 3.56 (1H, d, J=11.1 Hz), 2.50 (2H, br s, OH), 1.41 (3H, s). EI-MS m/z (%): 245 (63), 205 (78), 203 (52), 167 (63), 149 (100). CI-MS m/z 294 $(M+NH_4)^+$.

Isomeranzin (16)[19]——Colorless oil. UV λ_{max} nm: 217 (sh), 247, 256, 321. IR ν_{max} cm^{-1}: 1720, 1610. ^{1}H-NMR δ: 7.63 (1H, d, J=9.4 Hz), 7.37 (1H, d, J=8.7 Hz), 6.85 (1H, d, J=8.7 Hz), 6.22 (1H, d, J=9.4 Hz), 4.01 (2H, s), 3.87 (3H, s), 2.82 (1H, m), 1.21 (6H, d, J=7.06 Hz). EI-MS m/z (%): 260 (M^+), 242, 203, 190, 189 (100), 175, 161, 146, 131, 118.

Osthenon (17)[20]——Colorless prisms from ether, mp 134—136 °C. High-resolution MS: Calcd for $C_{14}H_{12}O_4$: 244.0735. Found: 244.0763. UV λ_{max} nm: 240, 304, 331 (sh). IR ν_{max} cm^{-1}: 1740, 1600. ^{1}H-NMR δ: 7.98 (1H, d, J=16.8 Hz), 7.65 (1H, d, J=9.4 Hz), 7.46 (1H, d, J=8.7 Hz), 7.34 (1H, d, J=16.8 Hz), 6.91 (1H, d, J=8.7 Hz), 6.30 (1H, d, J=9.4 Hz), 4.00 (3H, s), 2.43 (3H, s). EI-MS m/z (%): 244 (17), 229 (50), 213 (100), 201 (38), 186 (18), 173 (15), 158 (19), 115 (20).

Murralongin (18)[11]——Colorless prisms from ether, mp 132—134 °C. UV λ_{max} nm: 235, 322. IR ν_{max} cm^{-1}: 1720, 1670, 1600. ^{1}H-NMR δ: 10.22 (1H, s), 7.65 (1H, d, J=9.4 Hz), 7.45 (1H, d, J=8.7 Hz), 6.90 (1H, d, J=8.7 Hz), 6.23 (1H, d, J=9.4 Hz), 3.83 (3H, s), 2.43 (3H, s), 1.79 (3H, s). EI-MS m/z (%): 258 (M^+, 100), 215 (90), 201 (30), 199 (50), 187 (68), 171 (40), 159 (25).

7-Methoxy-8-formylcoumarin (19)[21]——Colorless oil. UV λ_{max} nm: 258, 279 (sh), 315, 341 (sh). IR ν_{max} cm^{-1}: 1738, 1690, 1600. ^{1}H-NMR δ: 10.68 (1H, s), 7.66 (1H, d, J=9.4 Hz), 7.64 (1H, d, J=8.7 Hz), 6.95 (1H, d, J=8.7 Hz), 6.34 (1H, d, J=9.4 Hz), 4.02 (3H, s). EI-MS m/z (%): 204 (M^+), 176, 161, 147, 118.

Umbelliferone (20)[22]——Colorless prisms from acetone, mp 223—225 °C. UV λ_{max} nm: 216, 245 (sh), 258 (sh), 279, 322. IR ν_{max} cm^{-1}: 3300, 1730, 1710, 1610. ^{1}H-NMR (acetone-d_6) δ: 7.87 (1H, d, J=9.4 Hz), 7.51 (1H, d, J=8.7 Hz), 6.84 (1H, dd, J=8.7, 2.4 Hz), 6.75 (1H, d, J=2.4 Hz), 6.16 (1H, d, J=9.4 Hz). EI-MS m/z (%): 162 (M^+), 134, 105.

Scopoletin (21)[23]——Colorless prisms from ether, mp 202—203 °C. UV λ_{max} nm: 227, 252, 257, 298, 341. IR ν_{max} cm^{-1}: 3530, 1720, 1610, 1580. ^{1}H-NMR δ: 7.60 (1H, d, J=9.4 Hz), 6.92 (1H, s), 6.85 (1H, s), 6.27 (1H, d, J=9.4 Hz), 6.15 (1H, s, OH), 3.95 (3H, s). EI-MS m/z (%): 192 (M^+, 90), 177 (73), 164 (45), 149 (100), 121 (60).

Sibiricol (22)[24]——Colorless prisms from acetone, mp 197—199 °C. UV λ_{max} nm: 262, 329. IR ν_{max} cm^{-1}: 3400, 1720, 1600. ^{1}H-NMR (acetone-d_6) δ: 7.99 (1H, d, J=9.4 Hz), 6.49 (1H, s), 6.06 (1H, d, J=9.4 Hz), 5.24 (1H, t, J=7.4 Hz), 3.88 (3H, s), 3.75 (1H, br s, OH), 3.42 (2H, d, J=7.4 Hz), 1.82 (3H, s), 1.65 (3H, s). EI-MS m/z (%): 260 (M^+), 245, 217, 205 (100), 189, 176.

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References and Notes

1) H. Furukawa, T.-S. Wu, T. Ohta, and C.-S. Kuoh, *Chem. Pharm. Bull.*, **33**, 4132 (1985); A. T. McPhail, T.-S. Wu, T. Ohta, and H. Furukawa, *Tetrahedron Lett.*, **24**, 5377 (1983); H. Furukawa, T.-S. Wu, and T. Ohta, *Chem. Pharm. Bull.*, **31**, 4202 (1983); H. Furukawa, M. Yogo, C. Ito, T.-S. Wu, and C.-S. Kuoh, *ibid.*, **33**, 1320 (1985); H. Furukawa, T.-S. Wu, and C.-S. Kuoh, *ibid.*, **33**, 2611 (1985).
2) Y.-C. Kong, G. K. H. Ng, C. K. H. Wat, and P. P.-H. But, *Int. J. Crude Drug Res.*, **24**, 167 (1986); Y.-C. Kong, K.-F. Cheng, K.-H. Ng, P. P.-H. But, Q. Li, S.-X. Yu, H.-T. Chang, P. C. Cambie, T. Kinoshita, W.-S. Kan, and P. G. Waterman, *Biochem. Syst. Biol.*, **14**, 491 (1986); P. P.-H. But, Y.-C. Kong, K.-H. Ng, H.-T. Chang, Q. Li, S.-X. Yu, and P. G. Waterman, *Acta Phytotaxon. Sin.*, **24**, 186 (1986).
3) R. D. H. Murray, J. Mendez, and S. A. Brown, "The Natural Coumarins," John Wiley & Sons Ltd., New York, 1982, p. 27.
4) W. L. Stanley, A. C. Waiss, Jr., R. E. Lundin, and S. H. Vannier, *Tetrahedron*, **21**, 89 (1965).
5) B. R. Barik, A. K. Dey, P. C. Das, A. Chatterjee, and J. N. Shoolery, *Phytochemistry*, **22**, 792 (1983).
6) L. Crombie, D. E. Games, N. J. Haskins, G. F. Reed, R. A. Finnegan, and K. E. Merkel, *Tetrahedron Lett.*, **1970**, 3975; L. Crombie, D. E. Games, N. J. Haskins, and G. F. Reed, *J. Chem. Soc., Perkin Trans. 1*, **1972**, 2241.
7) S. K. Talapatra, L. N. Dutta, and B. Talapatra, *Tetrahedron*, **29**, 2811 (1973).
8) F. Bohlmann, H. Franke, and C. Zdero, *An. Quim.*, **68**, 765 (1972).
9) Murrangatin and minumicrolin were isolated from *M. paniculata* by us and H. Ishii *et al.* (Chiba University), and we proposed that the relative stereochemistry[7,14] of the glycol moieties of these coumarins was such that murrangatin=*threo* (**6a**), and minumicrolin=*erythro* (**11**) based on differential nuclear Overhauser effect

experiments on the acetonides of these coumarins. Further, the absolute stereochemistry of murrangatin (**6a**) was also proposed as [*R*, *R*] based on the results of the exciton chirality method applied to the tetrahydrodibenzoate of murrangatin (**6a**). Full details were presented at the 107th Annual Meeting of the Pharmaceutical Society of Japan, Kyoto, April 1987 (The Abstract of the Meeting, May 1987, p. 344).

10) F. Imai, T. Kinoshita, A. Itai, and U. Sankawa, *Chem. Pharm. Bull.*, **34**, 3978 (1986).
11) Recently, we isolated a double-bond regioisomer of isomurralonginol (**7a**) named murralonginol as the isovalerate from *M. paniculata*, and characterized it (unpublished data).
12) J. Yang and M. Du, *Huaxue Xuebao*, **42**, 1308 (1984) [*Chem. Abstr.*, **102**, 128816*n* (1985)].
13) J. T. Pinhey and I. A. Southwell, *Aust. J. Chem.*, **26**, 409 (1973).
14) S. Das, R. H. Baruah, R. P. Sharma, J. N. Barua, P. Kulanthaivel, and W. Herz, *Phytochemistry*, **23**, 2317 (1984).
15) M. Murayama, E. Seto, T. Okubo, I. Morita, I. Dobashi, and M. Maehara, *Chem. Pharm. Bull.*, **20**, 741 (1972).
16) M. F. Grundon and I. S. McColl, *Phytochemistry*, **14**, 143 (1975).
17) B. R. Barik, A. K. Dey, and A. Chatterjee, *Phytochemistry*, **22**, 2273 (1983).
18) S. K. Talapatra, S. Goswami, N. C. Ganguly, and B. Talapatra, *Chem. Ind.* (London), **1980**, 154.
19) E. Atkinson, D. R. Boyd, and M. F. Grundon, *Phytochemistry*, **13**, 853 (1974).
20) Recently, this coumarin was also isolated from a *Citrus* plant by one of the authors (H. F.) and M. Ju-ichi *et al.* (Mukogawa Women's University). Full details of the structure elucidation of this coumarin will be reported elsewhere.
21) A. G. Gonzalez, J. T. Barroso, R. J. Cardona, J. M. Medina, and F. Rodriguez Luis, *An. Quim.*, **73**, 1188 (1977).
22) A. Hashimoto and T. Kawana, *Yakugaku Zasshi*, **55**, 183 (1935); W. Steck and M. Mazurek, *Lloydia*, **35**, 418 (1972).
23) C. W. Moore, *J. Chem. Soc.*, **97**, 2223 (1910).
24) R. Kumar, B. D. Gupta, S. K. Banerjee, and C. K. Atal, *Phytochemistry*, **17**, 2111 (1978); R. D. H. Murray, M. M. Ballantyne, T. C. Hogg, and P. H. McCabe, *Tetrahedron*, **31**, 2960 (1975).

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Polysaccharides in Fungi. XX.[1] Structure and Antitumor Activity of a Branched (1→3)-β-D-Glucan from Alkaline Extract of Yū ěr[2]

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A water-insoluble glucan (N-5P), $[\alpha]_D^{23}$ +2.3° (c=0.51, 0.5 M sodium hydroxide), was isolated from the alkaline extract of the fruit bodies of Yū ěr (Chinese name) (*Auricularia* species). N-5P was homogeneous as judged by gel filtration and electrophoresis. By gel filtration, the molecular weight of N-5P was estimated to be 5.6×10^5. The ^{13}C-nuclear magnetic resonance and infrared spectra, together with the result of chromium trioxide oxidation, indicated that glucosidic linkages in N-5P have the β-D configuration. From the results of methylation analysis, periodate oxidation, Smith degradation (complete, as well as partial), partial acetolysis, and enzymic hydrolysis, it was concluded that N-5P has a main chain composed of β-(1→3)-linked D-glucopyranosyl residues, with single, β-(1→6)-linked D-glucopyranosyl groups attached as side chains, which account for a quarter of the total sugar residues. In addition, the results of enzymic hydrolysis indicated that the β-(1→6)-linked D-glucosyl side chains are mainly localized in the neighborhood of the nonreducing end of the main chain. Furthermore, N-5P showed potent antitumor activity against the solid form of sarcoma 180 in mice, and exhibited significant carbon clearance-enhancing activity in mice.

Keywords——branched (1→3)-β-D-glucan; Yū ěr (*Auricularia* species); molecular weight; methylation analysis; ^{13}C-NMR; structure of polysaccharide; antitumor activity; phagocytic activity; carbon clearance; Smith degradation

The fruit bodies of Yū ěr (*Auricularia* species)[3] belonging to the Auriculariaceae have been used as a food and as a crude drug in China. Previously, we reported[4,5] the characterization, antitumor, and anti-inflammatory activities of acidic polysaccharide isolated from the hot water extract of the fungus. Now we have isolated a β-D-glucan (N-5P) from the alkaline extract of fruit bodies of this fungus. The present paper deals with the purification, characterization, structural analysis, and antitumor activity of N-5P.

The fruit bodies were successively extracted with hot methanol, hot 70% aqueous ethanol, and hot water as reported previously.[5] The residue was treated with 5% sodium carbonate,[6] and then finally extracted with 1 M sodium hydroxide at room temperature. The sodium hydroxide extract was made neutral, and dialyzed. The nondialyzable solution was mixed with 3 volumes of ethanol, and the precipitate obtained on centrifugation was washed with water, dispersed in water, and lyophilized to give N-5P in 1.2% yield; this product was homogeneous, as determined by gel filtration on Toyopearl HW-65 with sodium hydroxide solution (see Fig. 1). N-5P was also found to be pure by glass-fiber-paper electrophoresis. The polysaccharide (N-5P) was composed solely of D-glucosyl residues, as shown by paper chromatography (PPC) of the hydrolyzate, and also by gas-liquid chromatography (GLC) of the alditol acetate[7] prepared from the hydrolyzate.

The glucan was soluble in alkaline solutions, but insoluble in neutral or acidic solutions. It slowly dissolved in dimethyl sulfoxide (DMSO). N-5P had a low, positive specific rotation,

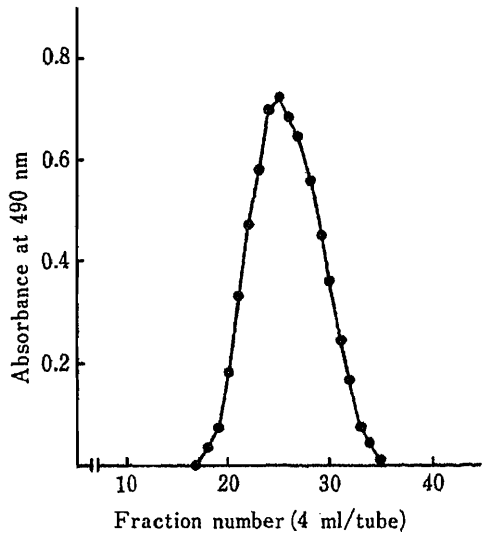

Fig. 1. Chromatogram of N-5P in 0.5N NaOH on Toyopearl HW-65

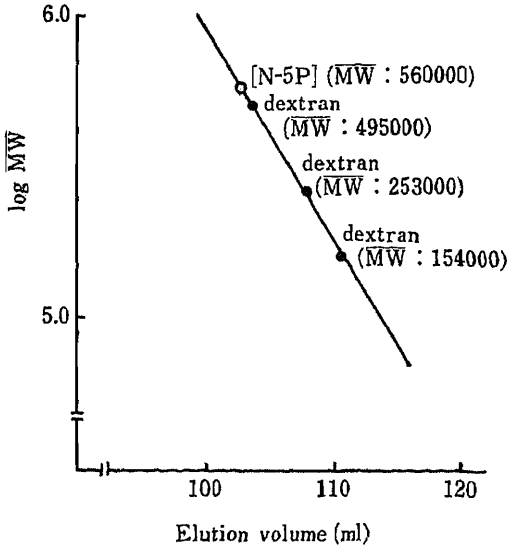

Fig. 2. Determination of Molecular Weight of N-5P by Gel Filtration on Toyopearl HW-65 with Standard Dextrans

TABLE I. GLC and GLC-MS of Partially Methylated Alditol Acetates

Peak	Methylated sugar (as alditol acetate)	Relative retention time[a]	Main mass-fragments (*m/z*)	Molar ratio	Mode of linkage
a	2,3,4,6-Me_4-Glc	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	1.0	[Glc*p*]1→
b	2,4,6-Me_3-Glc	1.60	43, 45, 87, 101, 117, 129, 161, 233	1.9	→3[Glc*p*]1→
c	2,4-Me_2-Glc	2.87	43, 87, 117, 129, 189	0.9	$\rightarrow^{3}_{6}$[Glc*p*]1→

a) Relative to authentic 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. 3% ECNSS-M column, 170°C.

$[\alpha]_D^{23}$ +2.3° (c=0.51, 0.5 M NaOH), and showed characteristic absorbance at 890 cm^{-1} in the infrared (IR) spectrum, indicating the presence of the β-D configuration.[8] N-5P contained no nitrogen (by elementary analysis), and the total sugar content was found to be 98.1% (as hexosyl residues) by the phenol–sulfuric acid method.[9] The calibration curve shown in Fig. 2 was made by gel filtration of standard dextrans on Toyopearl HW-65 with 0.5 M sodium hydroxide; the molecular weight of N-5P was estimated to be approximately 5.6×10^5.

The glucan was methylated by the method of Hakomori,[10] and the fully methylated glucan was hydrolyzed with acid. The partially methylated sugars were analyzed as the alditol acetate derivatives[7] by GLC and GLC–mass spectrometry (GLC-MS), and identified by comparing their retention times in GLC, and their fragment patterns in MS, with those of authentic samples, or with the values in the literature.[11] As shown in Table I, the methylation analysis indicated the presence of 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl-D-glucoses in the molar ratios of 1.0 : 1.9 : 0.9.

Periodate oxidation of N-5P was conducted, with monitoring by the Fleury–Lange method.[12] N-5P consumed 0.52 mol of periodate per hexosyl residue. The oxidized polysaccharide was treated with sodium borohydride, and the resulting polyalcohol (N-5P polyol) was hydrolyzed with acid in accordance with the description of Hamilton and Smith.[13] The hydrolyzate was analyzed by GLC as the alditol acetate derivatives,[7] and glycerol and glucose were detected in the molar ratio of 1.00 : 2.84. The glycerol must have arisen from terminal

residues, and the occurrence of glucose must be due to the presence of oxidation-resistant D-glucose, such as (1→3)-linked residues. These results are in good agreement with those of the methylation analysis.

The mild acid hydrolysis of the polyalcohol just described gave a water-insoluble product (the controlled Smith-degradation product: N-5PS). N-5PS was methylated by the methods of Haworth[14] and Hakomori.[10] The methylation analysis showed the presence of 2,4,6-tri-*O*-methyl-D-glucose (>99%), and the result indicated that N-5PS was essentially composed of linear, (1→3)-linked D-glucosyl residues. From these results, it is concluded that N-5P has a backbone of (1→3)-linked D-glucosyl residues; some of the residues are substituted at *O*-6, and (1→3)-linked residues are absent from the side chains.

On partial acetolysis,[15] followed by deacetylation, N-5P gave glucose, disaccharide, and a number of oligosaccharides in PPC. The disaccharide fraction isolated by gel filtration on Bio-gel P-2 was identified as laminarabiose by GLC of the trimethylsilyl derivative of the disaccharide-alditol.[16] Gentiobiose was not detected because (1→6)-linkages were preferentially cleaved during acetolysis. Moreover, a linear relationship existed between the presumed degree of polymerization of the oligosaccharides detected and their $\log[Rf/(1-Rf)]$ values, as proposed by French and Wild.[17] These results indicated that partial acetolysis of N-5P yielded a homologous series of β-(1→3) linked D-gluco-oligosaccharides.

The ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectrum of N-5P is shown in Fig. 3. This spectrum characteristically showed a triplet signal (86.56, 86.18, 85.87 ppm) due to substituted C-3 of a glucosyl unit and was similar to those of other branched (1→3)-β-D-glucans.[18-20] The resonance at 102.85 ppm corresponds to the anomeric carbon atoms of D-glucopyranosyl residues in N-5P, and the $^1J_{CH}$ value of 162.3 Hz indicated that the glucosyl residues were in β-D configurations (see ref. 21).

Chromium trioxide oxidation[22] of N-5P acetate resulted in the loss of 98.6% of the D-glucose residues, supporting the presence of the β-D configuration, because a fully acetylated aldopyranoside in which the aglycone is equatorially attached in the most stable chair form (generally the β anomer) is oxidized by chromium trioxide in acetic acid.

The glucan was digested with *exo*-(1→3)-β-D-glucanase (Lysing enzymes) from Basidiomycetes, and enzymic degradation products of N-5P were examined by gel filtration on Bio-gel P-2; only two peaks, corresponding to mono- and disaccharide, were revealed (Fig. 4). The products recovered from the eluates at 5 h were analyzed by PPC, and glucose and gentiobiose were detected. In the earlier stage (at 5 h), gentiobiose was mainly liberated, but the molar ratio of Gen/Glc gradually decreased to reach a constant value (0.38) at 48 and 72 h (Fig. 5).

The foregoing data indicate that the glucan N-5P has a main chain composed of (1→3)-β-D-glucopyranosyl residues, and has single, β-(1→6)-linked D-glucopyranosyl groups attached as side chains, which account for a quarter of the total sugar residues. Furthermore, from the results of enzymic hydrolysis, it was indicated that the β-(1→6)-linked D-glucosyl groups are mainly localized in the neighborhood of the nonreducing end of the main chain, as shown in Fig. 6.

The conformational behavior of linear and branched (1→3)-β-D-glucans, including our previously reported β-D-glucans (T-4-N, T-5-N, and T-3-G), has been discussed in regard to change in the visible absorption spectra of the complexes formed with Congo red at various concentrations of alkali.[23-26] At low alkali concentration (0.10 M), the absorption maximum (λ_{max}) of Congo red (488 nm) was markedly shifted to longer wavelength (510 nm) in the presence of N-5P, whereas at high alkali concentration (0.25 M), the λ_{max} was not shifted. A large shift to longer wavelength (510 nm) was also observed in a 0.1 M sodium hydroxide solution of N-5P polyol. Such a shift in the value of λ_{max} of Congo red is similarly observed in other linear and branched β-(1→3)-D-glucans.[23-26] This result suggested that N-5P has an

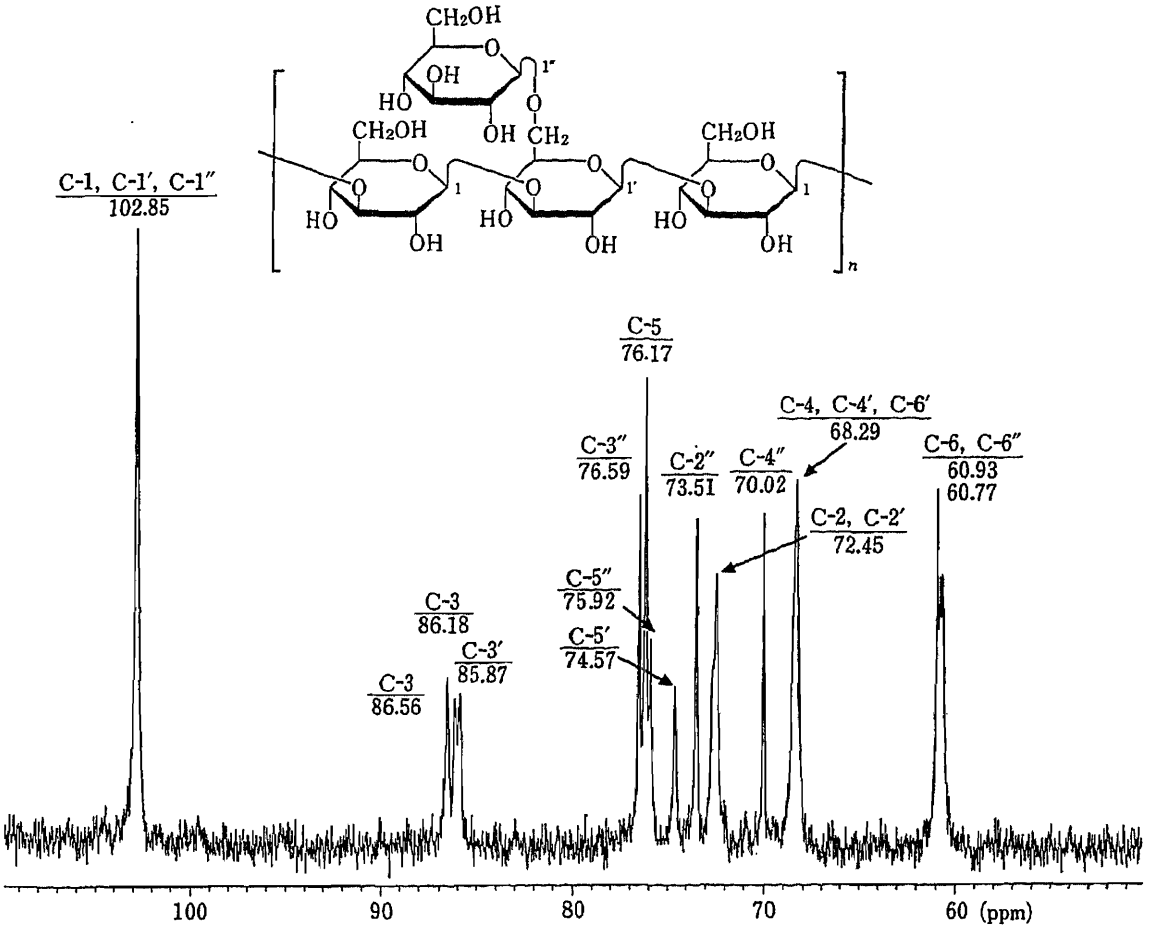

Fig. 3. ^{13}C-NMR Spectrum of N-5P in DMSO-d_6 at 60 °C

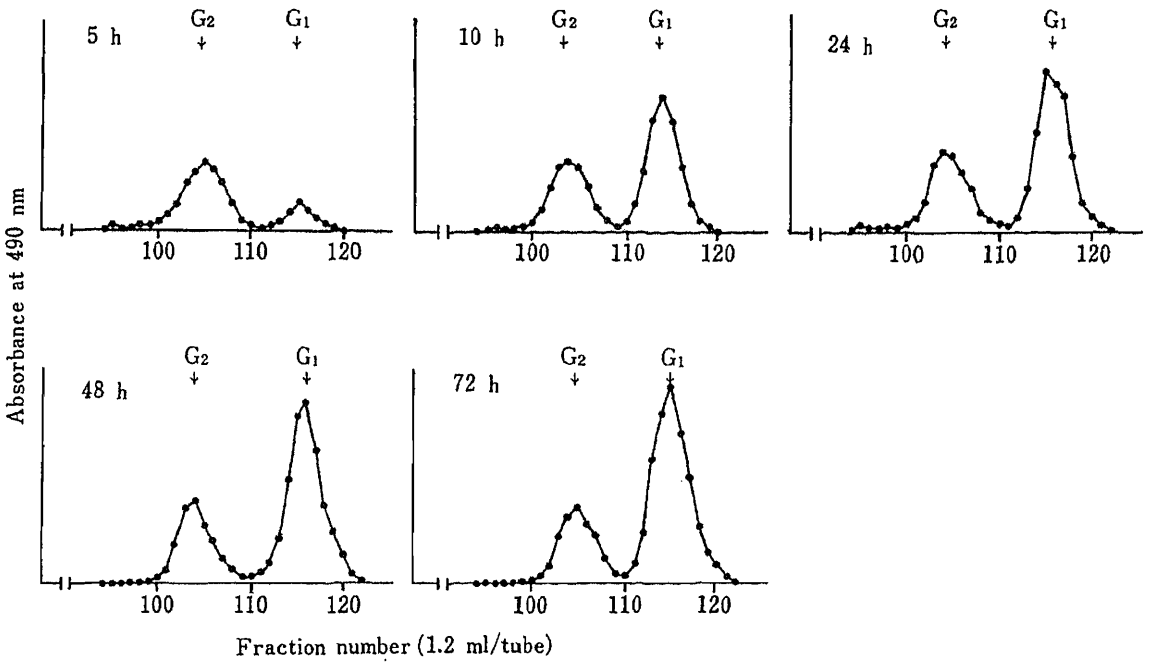

Fig. 4. Chromatograms of Enzyme Degradation Products of N-5P on Bio-Gel P-2

G_1, glucose; G_2, gentiobiose.

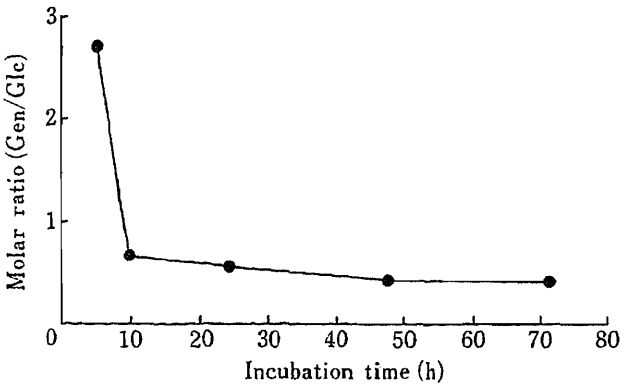

Fig. 5. The Ratio of Gentiobiose to Glucose in Enzyme Degradation Products of N-5P

The ratio was calculated on the basis of the peak areas in gel filtration on Bio-Gel P-2.

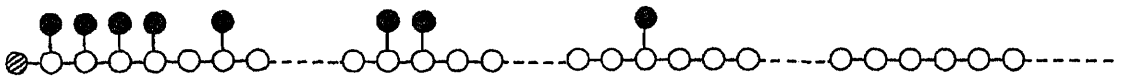

Fig. 6. Possible Structure of N-5P

◍, nonreducing end-group of the main chain; ●, (1→6)-linked, β-D-glucosyl group; ○, (1→3)-linked, β-D-glucosyl residue (main chain).

TABLE II. Antitumor Activity of the Polysaccharides against Sarcoma 180 Solid Tumor

Sample	Dose (mg/kg/d ×10)	Mean tumor wt. ±S.D.[a] (g)	Inhibition ratio (%)	Mortality at 4 weeks[b]	Complete regression
Exp-1					
N-5P	1	2.72±2.54[c]	72	0/6	1/6
	10	1.81±1.43[c]	81	0/7	1/7
PSK	200	0.94±1.15[c]	90	1/5	2/4
Control	—	9.64±3.48	—	0/7	0/7
Exp-2					
N-5P polyol	1	1.01±1.36[c]	87	0/10	2/10
	5	3.31±2.80[d]	56	0/10	0/10
	10	5.39±1.31[e]	28	0/10	0/10
Control	—	7.50±2.55	—	0/9	0/9

a) Significant difference from the control (*c*) $p<0.001$, *d*) $p<0.01$, *e*) $p<0.05$). *b*) All the deaths from tumors occurred later than the 3rd week after the tumor transplantation.

ordered, triple-helical structure in weakly alkaline solution, as previously observed with T-4-N, T-5-N, and T-3-G.[24-26]

Branched (1→3)-β-D-glucans having side chains of single D-glucosyl group at *O*-6, such as lentinan,[27] schizophyllan,[28] sclerotan,[29] and others[24-26,30] have been reported to exhibit antitumor activity against sarcoma 180 implanted in mice, and our present glucan N-5P from Yū ěr was structurally similar to these glucans. The antitumor activities of N-5P and N-5P polyol against sarcoma 180 in mice are listed in Table II. N-5P showed antitumor activity at doses of 10 and 1 mg/kg. Its activity at a dose of 10 mg/kg was similar to that of PSK at a dose of 200 mg/kg. N-5P polyol showed strong activity at a dose of 1 mg/kg, but its activity was weak at a dose of 10 mg/kg. The reason for the difference in the dose–response relationships of these glucans is not yet clear.

Next, the effect of N-5P on the phagocytic activity of the mouse reticuloendothelial system was examined by the carbon clearance method *in vivo*. As shown in Table III, the clearance rate of carbon was significantly faster at 48 h after the administration of N-5P (50 mg/kg, i.p.). It is known that schizophyllan shows carbon clearance-enhancing activity,

TABLE III. Effect of N-5P and Zymosan on the Clearance Rate of Carbon from the Circulating Blood of Mice

Treatment[a]	Dose (mg/kg)	No. of mice	Clearance rate of carbon[b] ($t_{1/2}$, min)
Control (Saline)		7	15.5±0.8
N-5P	50	7	12.4±0.4[c]
Zymosan	50	6	10.1±0.7[d]

a) Test substances were injected i.p. at 48 h before the i.v. injection of carbon solution. *b*) Each value represents the mean±S.E. Significant difference from the control (*c*) $p<0.01$, (*d*) $p<0.001$).

but lentinan does not.[31,32] The mechanism of the antitumor effect of N-5P has not yet been elucidated, but from the result in the carbon clearance test, it was suggested that the mechanism involved in the antitumor activity of N-5P may be similar to that of schizophyllan.

We previously demonstrated the existence of heterogeneity in the branching of the (1→6)-branched (1→3)-β-D-glucan (T-4-N, T-5-N, T-3-G).[24-26] Recently Misaki *et al.*[33] reported the significance of the presence of side-chains in (1→3)-β-D-glucan main chain in relation to the antitumor activity. It would be interesting to compare the antitumor and other biological activities of glucans having different distributions of side-chains.

Experimental

Materials——The dried fruit-bodies of Yŭ ĕr (*Auricularia* species) are commercially available in Hong Kong. A sample of the fruit bodies is stored as a specimen (No. 821002) at the Herbarium of Gifu Pharmaceutical University. Lysing enzymes were purchased from Sigma Chemical Company. Toyopearl HW-65 and standard dextrans were respectively purchased from Toyo Soda Industry Co., Ltd., and Pharmacia Fine Chemicals. Bio-gel P-2 was purchased from Bio-Rad Laboratories.

General——All evaporations were conducted under reduced pressure at bath temperatures not exceeding 40 °C. Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. IR spectra were recorded with a JASCO A-102 spectrometer. PPC was performed by the triple-ascending method, using Toyo Roshi No. 51A filter-paper and the following solvent systems (v/v): (A) 1-butanol–pyridine–water (6:4:3), (B) 1-propanol–ethyl acetate–water (6:1:3). Sugars were detected with alkaline silver nitrate reagent. GLC was performed in a Shimadzu GC-4CM apparatus equipped with a flame-ionization detector. Glass columns (0.3×200 cm) were used, with nitrogen as the carrier gas at a flow rate of 40 ml/min. The column packings used were (1) 3% ECNSS-M on Gaschrom Q (100—120 mesh) at 170 °C, (2) 2% EGSS-X on Chromosorb W (60—80 mesh) at 185 °C, and (3) 2% Silicon DC QF-1 on Chromosorb W (80—100 mesh) at 200 °C. Peak areas were measured with a Shimadzu E1A Chromatopac. GLC-MS was conducted with a JEOL JMS-D 300 apparatus equipped with a glass column (0.2×100 cm) packed with 3% ECNSS-M, at 190 °C, at a pressure of helium of 0.8 kg/cm^2. The MS were recorded at an ionizing potential of 70 eV, an ionizing current of 50 μA, and a temperature of the ion source of 250 °C.

Isolation of the Polysaccharide——After the successive extraction of the dried, pulverized fruit bodies (85 g) with hot methanol, hot 70% aqueous ethanol, hot water, and 5% sodium carbonate, the residue was extracted twice with 1 M sodium hydroxide (each 0.5 l) for 24 h at room temperature under a nitrogen atmosphere. The alkaline suspension was centrifuged for 30 min at 5000 rpm, and the extract was made neutral with 2 M hydrochloric acid, and then dialyzed against distilled water for 5 d. Ethanol (3 vol.) was added to the nondialyzable solution. The resulting precipitate was collected by centrifugation, washed with water, dispersed in water, and lyophilized to afford the purified polysaccharide (N-5P), in 1.2% yield.

Electrophoresis——Paper electrophoresis was conducted on Whatman GF-81 glass-fiber paper (9×41 cm) with 0.1 M sodium hydroxide containing 0.05 M sodium tetraborate for 3 h at 210 V. N-5P showed one spot at a distance of 3.5 cm from the origin.

Gel Filtration and Estimation of Molecular Weight——Gel filtration of N-5P and standard dextrans on a column of Toyopearl HW-65, with 0.5 M sodium hydroxide as the eluent, was performed as previously reported.[24] A calibration curve, constructed by the use of standard dextrans, is shown in Fig. 2, and the molecular weight was estimated therefrom.

Analysis of Component Sugars——The polysaccharide (N-5P) was hydrolyzed with 2 M trifluoroacetic acid for 8 h at 100 °C. The hydrolyzate was evaporated to remove the trifluoroacetic acid, and analyzed by PPC (solvent A).

An aqueous solution of the hydrolyzate was reduced with sodium borohydride, and, after acetylation,[7] the product was identified as glucitol acetate by GLC (condition 1).

Methylation Analysis——N-5P (3.0 mg) was methylated three times by Hakomori's method[10] as previously described.[25] The final methylation product showed no hydroxyl absorption band in the IR spectrum. The fully methylated glucan (2.0 mg) was heated successively with 90% formic acid (1 ml) for 5 h at 100 °C, and with 0.25 M sulfuric acid (1 ml) for 15 h at 100 °C. After neutralization of the acid with barium carbonate, the suspension was filtered. The filtrate was passed through a column of Amberlite CG-120 (H^+) resin, and the hydrolyzate was converted into the alditol acetates.[7] The resulting, partially methylated alditol acetates were analyzed by GLC (condition 1) and GLC-MS.

Periodate Oxidation and Smith Degradation——A solution of N-5P (11.3 mg) in 0.5 M sodium hydroxide (1 ml) was neutralized with 0.5 M hydrogen chloride (1 ml), and water (18 ml) and 20 mM sodium metaperiodate (20 ml) were added. The mixture was kept in the dark, with stirring, for 8 d at 4 °C. An aliquot (1 ml) was taken at various times, and the periodate consumption was determined by the Fleury–Lange method.[12] The oxidation was stopped by addition of ethylene glycol, the mixture was dialyzed, and the contents were reduced with sodium borohydride (20 mg) for 24 h at room temperature. The mixture was treated with acetic acid, dialyzed, and then lyophilized to give N-5P polyol; this (2.0 mg) was treated successively with 90% formic acid for 5 h at 100 °C, and with 0.25 M sulfuric acid for 15 h at 100 °C. The hydrolyzate was analyzed by GLC as the alditol acetates, as already described.

Controlled Smith Degradation——The polyalcohol (20.0 mg) obtained by a procedure similar to that just described was hydrolyzed with 50 mM sulfuric acid (30 ml), with stirring, for 26 h at 25 °C. The mixture was centrifuged for 30 min at 8000 rpm, then the precipitate was washed with water, and dried to give N-5PS (10.1 mg). A portion (5.0 mg) of N-5PS was methylated once by Haworth's method[14] and 3 times by Hakomori's method.[10] The fully methylated product was hydrolyzed, the sugars were reduced with sodium borohydride, the alditols acetylated, and the acetates analyzed by GLC (condition 1) as already described.

Partial Acetolysis——N-5P (19.6 mg) was suspended in a mixture of acetic anhydride (5 ml), acetic acid (3 ml), and sulfuric acid (0.5 ml), and the suspension was allowed to stand, with occasional shaking, for 3 d at room temperature. The reaction mixture was poured into ice-water (50 ml), made neutral with sodium hydrogen carbonate, and then extracted with chloroform. The acetates were deacetylated in methanol (10 ml) containing 0.05 M sodium methoxide as previously reported,[25] and a portion of the products was analyzed by PPC (solvent B). The other fraction of the products was applied to a column of Bio-gel P-2, and the disaccharide fraction recovered was treated with sodium borohydride, followed by trimethylsilylation[16] with anhydrous pyridine–hexamethyldisilazane–trimethylchlorosilane (2 : 2 : 1) for 2 min at 75 °C on an oil bath and for 15 min at room temperature. The resulting trimethylsilylate of the disaccharide-alditol was analyzed by GLC (condition 3). The retention time of the derivatives of the sample and of laminarabiose were both 50.4 min.

^{13}C-NMR Spectroscopy——^{13}C-NMR spectra was recorded with a JEOL GX-270 spectrometer operated at 67.70 MHz in the pulsed, Fourier-transform mode at 60 °C in DMSO-d_6. The spectrum is shown in Fig. 3.

Chromium Trioxide Oxidation——A solution of N-5P (20.1 mg) in formamide (4.0 ml) was treated with acetic anhydride (2.0 ml)–pyridine (2.0 ml) for 48 h at room temperature, and then for 3 h at 65—70 °C. A mixture of the resulting fully acetylated N-5P (5.6 mg) with *myo*-inositol hexaacetate (5.0 mg), as an internal standard, was dissolved in chloroform (3 ml). A part (1 ml) of the solution was kept as a control. The rest was dissolved in glacial acetic acid (0.3 ml), dry chromium trioxide (30 mg) was added to the solution, and the mixture was sonicated in an ultrasonic bath for 1 h at 50 °C. The resulting solution was poured into water, and the mixture was extracted 5 times with chloroform. The extracts were combined, and evaporated to dryness. The control sample was treated similarly, but without adding chromium trioxide. The hydrolyzates of the products thus obtained were converted into the alditol acetates,[7] and analyzed by GLC (condition 2).

Enzymic Hydrolysis——N-5P (8.0 mg) was treated with *exo*-(1→3)-β-D-glucanase (Lysing enzymes; 3.5 mg) in 17 mM McIlvaine buffer, pH 4.88 (30 ml), for 72 h at 38 °C. A portion (4 ml) of the reaction mixture was taken at various times (5, 10, 24, 48, and 72 h), and each was heated for 15 min at 100 °C. Then ethanol (4 vol.) was added to the solution, the supernatant liquor obtained by centrifugation was evaporated to dryness, the residue was dissolved in water (0.3 ml), and the solution was applied to a column (1.5 × 87.5 cm) of Bio-gel P-2. The column was eluted with water, and fractions (1.2 ml each) were collected, and analyzed by the phenol–sulfuric acid method.[9] The molar ratio (Gen/Glc) of the products was calculated from the peak areas. When the products (5 h) were analyzed by PPC (solvent A), two spots, corresponding to glucose and gentiobiose (R_{Glc} 0.57), were detected.

Interaction with Congo Red in Aqueous Sodium Hydroxide——N-5P (1 mg/ml) was dissolved in sodium hydroxide (0.10, 0.25 M) containing Congo red (50 μM), and λ_{max} was measured using a Hitachi 323 spectrophotometer.

Antitumor Activity——The animals used in the experiment were ddY male mice (4 weeks old). They were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Hamamatsu. Sarcoma 180 ascites cells, maintained by serial i.p. transplantation into ddY male mice, were used. Tumor cells (7 d old) were employed for the experiment. The cells were transplanted subcutaneously at a dose of 0.1 ml (2×10^6 cells) into the right groin of mice. Starting 1 d after the transplantation, 0.25 ml of physiological saline or saline solution of

each test material was given i.p. once a day for 10 d. The saline solution of each test material was prepared as followed. Each test material was dissolved in 0.5 M NaOH, then the solution was neutralized with 0.5 M HCl and diluted to the desired saline concentration. The growth of tumors was observed for 30 d, then the mice were killed, and tumors were extirpated and weighed. The inhibition ratios were calculated by use of the formula: inhibition ratio (%) $=[(A-B)/A]\times 100$, where A is the average tumor weight of the control group, and B is that of the tested group.

Phagocytic Activity——The clearance rate of carbon was measured by the method of Biozzi *et al.*[34] Mice (5-week-old ddY male mice) were injected with colloidal carbon at a dose of 0.1 ml/10 g *via* the tail vein (the mixture consisted of Pelikan drawing ink 17 (black) 3 ml, saline 4 ml, and 3% gelatin solution 4 ml) at 48 h after i.p. injection of N-5P, and 44.7 μl blood samples were taken from the orbital vein at 5, 10, and 15 min. The blood was discharged into 4 ml of 0.1% (w/v) Na_2CO_3 solution and the absorbance at 675 nm was determined. The clearance rate is expressed as the half-life period of carbon in the blood ($t_{1/2}$, min), calculated by means of the following equation:

$$K=\frac{\ln OD_1-\ln OD_2}{t_2-t_1} \qquad t_{1/2}=\frac{0.693}{K}$$

where OD_1 and OD_2 are the optical densities at times t_1 and t_2, respectively.

References and Notes

1) Part XIX: C. Hara, Y. Yokomori, T. Kiho, K. Nagai, and S. Ukai, *Carbohydr. Res.*, submitted.
2) This work was presented at the 106th Annual Meeting of the Pharmaceutical Society of Japan, Chiba, April 1986.
3) Chinese name: Yū ěr (榆耳); K. Akamatsu, "Wakanyaku," Ishiyaku, Tokyo, 1970, p. 695.
4) S. Ukai, T. Kiho, C. Hara, M. Morita, A. Goto, N. Imaizumi, and Y. Hasegawa, *Chem. Pharm. Bull.*, **31**, 741 (1982).
5) T. Kiho, M. Sakai, S. Ukai, C. Hara, and Y. Tanaka, *Carbohydr. Res.*, **142**, 344 (1985).
6) The carbonate extract contained very small amounts of other polysaccharides.
7) J. H. Sloneker, *Methods Carbohydr. Chem.*, **6**, 20 (1972).
8) S. A. Barker, E. J. Bourne, and D. H. Whiffen, *Methods Biochem. Anal.*, **3**, 213 (1956).
9) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
10) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
11) H. Björndal, C. G. Hellerqvist, B. Lindberg, and S. Svensson, *Angew. Chem., Int. Ed. Engl.*, **9**, 610 (1970).
12) P. F. Fleury and J. Lange, *J. Pharm. Chem.*, **17**, 107 (1933).
13) J. K. Hamilton and F. Smith, *J. Am. Chem. Soc.*, **78**, 5907 (1956).
14) W. H. Haworth, *J. Chem. Soc.*, **107**, 8 (1915).
15) K. Fujimoto, K. Matsuda, and K. Aso, *Nippon Nōgeikagaku Kaishi*, **36**, 346 (1962).
16) H. Nakamura and Z. Tamura, *Chem. Pharm. Bull.*, **18**, 2314 (1970).
17) D. French and G. M. Wild, *J. Am. Chem. Soc.*, **75**, 2612 (1953).
18) A. Misaki, K. Kawaguchi, H. Miyaji, H. Nagae, S. Hokkoku, M. Kakuta, and T. Sasaki, *Carbohydr. Res.*, **129**, 209 (1984).
19) N. Ohno, I. Suzuki, and T. Yadomae, *Chem. Pharm. Bull.*, **34**, 1362 (1986).
20) H. Saito, R. Tabeta, T. Sasaki, and Y. Yoshioka, *Bull. Chem. Soc. Jpn.*, **59**, 2093 (1986).
21) K. Bock, I. Lundt, and C. Pedersen, *Tetrahedron Lett.*, **1973**, 1037.
22) J. Hoffman, B. Lindberg, and S. Svensson, *Acta Chem. Scand.*, **26**, 661 (1972).
23) K. Ogawa, T. Watanabe, J. Tsuruji, and S. Ono, *Carbohydr. Res.*, **23**, 399 (1972).
24) C. Hara, T. Kiho, and S. Ukai, *Carbohydr. Res.*, **110**, 77 (1982).
25) C. Hara, T. Kiho, and S. Ukai, *Carbohydr. Res.*, **117**, 201 (1983).
26) C. Hara, T. Kiho, and S. Ukai, *Carbohydr. Res.*, **145**, 237 (1986).
27) T. Sasaki and N. Takasuka, *Carbohydr. Res.*, **47**, 99 (1976).
28) K. Tabata, W. Ito, and T. Kojima, *Carbohydr. Res.*, **89**, 121 (1981).
29) M. Kitahara and Y. Takeuchi, *Nippon Nōgeikagaku Kaishi*, **35**, 474 (1961).
30) J. Johnson, S. Kirkwood, A. Misaki, T. E. Nelson, J. V. Scaletti, and F. Smith, *Chem. Ind.* (London), **1963**, 820.
31) N. Komatsu, N. Nagumo, S. Okubo, and K. Kike, *Jpn. J. Antibiot.*, **26**, 277 (1973).
32) Y. Akiyama and J. Hamuro, *Tanpakushitu Kakusan Koso*, **26**, 208 (1981) (Y. Maeda and G. Chihara, *Int. J. Cancer*, **11**, 153 (1973)).
33) K. Matsuzaki, T. Sato, K. Enomoto, I. Yamamoto, R. Oshima, K. Hatanaka, T. Uryu, H. Kaku, Y. Sone, and A. Misaki, *Carbohydr. Res.*, **157**, 171 (1986).
34) G. Biozzi, B. Benacerraf, and B. N. Halperm, *Br. J. Exp. Pathol.*, **34**, 441 (1953).

Notes

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Facile Conversions of Carboxylic Acids into Amides, Esters, and Thioesters Using 1,1′-Oxalyldiimidazole and 1,1′-Oxalyldi(1,2,4-triazole)[1)]

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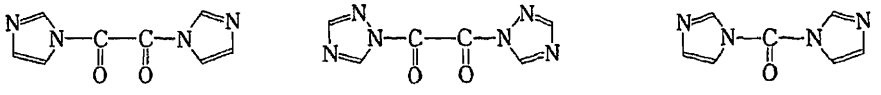
Aliphatic, aromatic, and heteroaromatic carboxylic acids react with 1,1′-oxalyldiimidazole (**1**) or 1,1′-oxalyldi(1,2,4-triazole) (**2**) in acetonitrile for 40 min at 40 °C to give the corresponding 1-acylazole intermediates (**11**), which promptly undergo aminolysis and alcoholysis to form amides (**13**) including dipeptides (**14**), esters (**16**), and thioesters (**19**). These findings show that both **1** and **2** can be utilized as condensing reagents for the synthesis of carboxylic acid derivatives.

Keywords——1,1′-oxalyldiimidazole; 1,1′-oxalyldi(1,2,4-triazole); 1,1′-carbonyldiimidazole; 1-acylazole; condensing reagent; amidation; esterification; dipeptide; aminolysis; alcoholysis

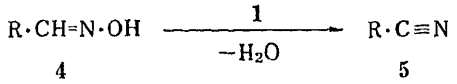
The development of efficient and convenient reagents for functional group conversions is still a major goal in synthetic organic chemistry, and it is also desirable that such conversions for the construction of target molecules should proceed smoothly under mild conditions. In the course of synthetic studies on dehydrating reagents, we have demonstrated that 1,1′-oxalyldiimidazole (**1**) can be used for the conversion of aldehyde oximes (**4**) into the corresponding nitriles (**5**) under neutral conditions,[2)] and dehydration utilizing **1** also serves as a convenient method for the synthesis of carboxylic acid anhydrides (**7**).[3)] Murata has separately shown that the reaction of aliphatic carboxylic acid derivatives such as lithium or sodium linolate with methanol using **1** takes place in chloroform to give the corresponding methyl ester (**9**) in 79% yield.[4)]

As indicated in the literature,[2,4)] the key compounds,**1** and **2** are easily prepared on a laboratory scale by the reaction of 1-(trimethylsilyl)imidazole[5)] and 1-(trimethylsilyl)-(1,2,4-triazole)[5)] with oxalyl chloride in benzene at room temperature.[6)] Incidentally, 1,1′-carbonyldiimidazole (Staab's reagent; **3**),[7)] which is structurally analogous to **1**, is frequently used as a condensing or dehydrating reagent for the preparations of amides (**13**),[8)] dipeptides (**14**),[9)] esters (**16**),[10)] nitriles (**5**),[11)] and acid anhydrides (**7**).[12)] Staab's reagent (**3**), though commercially available, is too expensive for application on a large scale. Furthermore, toxic phosgene, used as a starting material for the preparation of **3**, requires very careful handling. To overcome these shortcomings in the synthesis of amides (**13**) and esters (**16** or **19**), we examined the possibility that substitution of **1** or **2** for **3** as a condensing reagent may be effective for amidations or esterifications.

We now wish to report that compounds **1** and **2** are generally useful for the transformation of carboxylic acids (**6**) into amides (**13**) including peptides (**14**), esters (**16**), and thioesters (**19**) under mild conditions. The condensation reactions described herein were carried out basically as a two-step, one-pot procedure, as shown in Chart 2; step 1, formation



1 2 3



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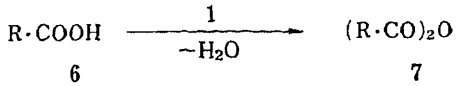
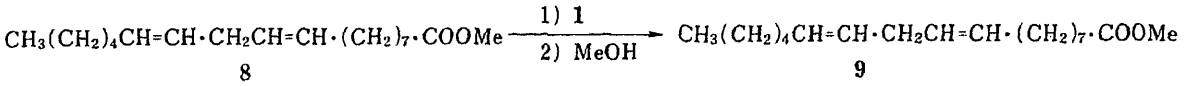


6 7



8 9

Met : Li or Na

Chart 1

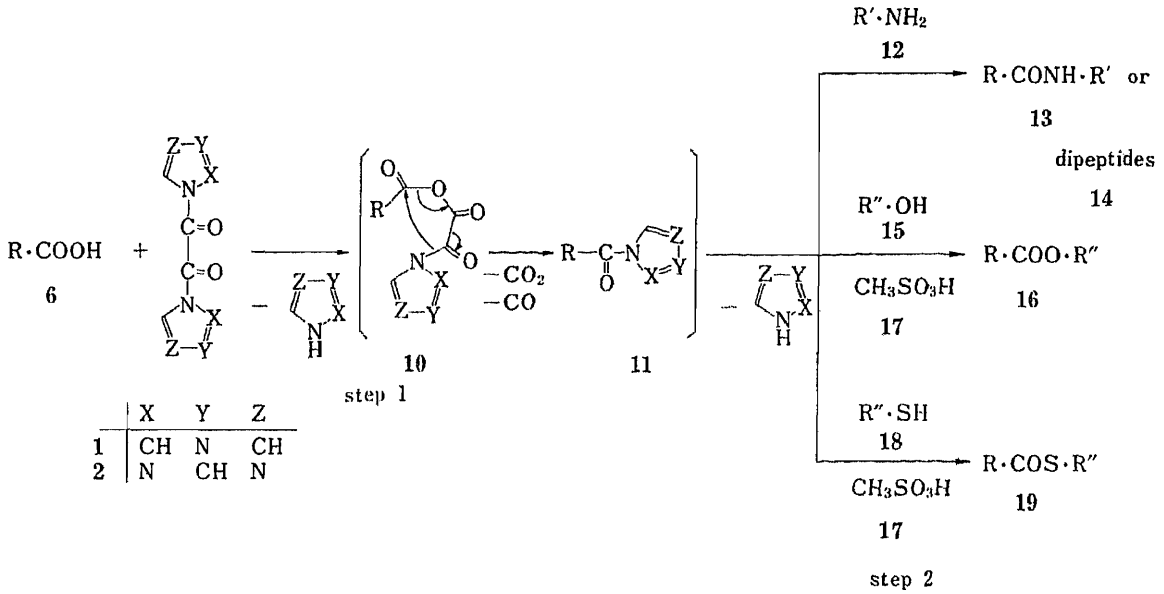


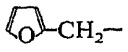
Chart 2

of active 1-acylazoles (**11**) from the reaction of carboxylic acids (**6**) with **1** or **2** for 40 min at 40 °C accompanied with liberation of carbon dioxide and carbon monoxide, and step 2, the 1-acylazoles (**11**) react with amines (**12**) or alcohols (**15** or **18**) as nucleophiles to afford amides (**13**), esters (**16**) or thioesters (**19**) as the final products.

Syntheses of Amides (13) and Dipeptides (14)

First of all, an attempt to prepare benzanilide (**13g**) from the condensation of benzoic acid and aniline using **1** was very successful (91% yield). Similarly, other aromatic and heteroaromatic carboxylic acids (**6**) were also derived as expected into the corresponding amides (**13**) in acceptable yields. Moreover, we found (Table I) that hindered carboxylic acids

TABLE I. Preparation of Amides (13) Using 1,1′-Oxalyldiazoles (1 and 2)
R·CO·NH·R′

No.	R	R	Reagent 1 or 2[a]	Yield (%)	mp [°C] (bp/mmHg) Found	Reported
13a	$CH_3(CH_2)_5$–	Ph	1	59	69—70	71[19]
			2	88		
13b	$CH_3-\underset{CH_3}{\overset{CH_3}{C}}-$	Ph	1	77	130—132	131—132[20]
			2	80		
13c	$CH_3-\underset{CH_3}{\overset{CH_3}{C}}-$	Cyclohexyl	1	83	123—124	125[21]
13d	$CH_3-\underset{CH_3}{\overset{CH_3}{C}}-$	*n*-Butyl	1	63	(95—98/3)	(58—59/0.2)[22]
13e	Cyclohexyl	Ph	1	73	128—131	131—132[23]
			2	67		
13f		Ph	1	91	205—206	197[24]
			2	87		
13g	Ph	Ph	1	91	161—162	162—163[25]
			2	83		
13h	CH_3– 	Ph	1	81	143—145	145—146[26]
13i	O_2N– 	Ph	1	96	211—213	214[27]
13j		Ph	1	64	121—122	124[28]
			2	43		
13k		Cyclohexyl	1	47	110—111	112[29]
13l		*n*-Butyl	1	43	40—41	40—41[30]
13m		Ph	1	72	76—78	79—80[31]
13n	O_2N– 	Ph	1	77	174—177	178—180[32]
13o		Ph	1	75	140—142	142—143[33]
				61		
13p		Cyclohexyl	1	84	153—155	155—156[33]
13q		*n*-Butyl	1	78	69—71	67—68[33]
13r	O_2N– 	Ph	1	79	181—183	179—180[34]
13s		Ph	1	90	122—123	123—124[33]

a) Compounds, **13a—b**, **13e—f**, and **13j**, which were prepared using 1,1′-oxalyldi(1,2,4-triazole) (**2**), were characterized by comparison of their mp (bp) and IR data with those of authentic samples.

such as 2,2-dimethylpropanoic acid readily react with aniline using **1** to give the corresponding anilide in good yields (**13b** in 77% yield in the case mentioned).

Judging from the yields of amides (**13**) in Table I, it is clear that reagent **1** as well as **2** is capable of achieving amide formation through the reaction of carboxylic acids (**6**) with amines (**12**).

Next, the successful application of **1** and **2** as condensing reagents led us to investigate their usefulness in peptide bond formation. A typical dipeptide synthesis was started by

TABLE II. Preparation of *N*-Protected Dipeptide Esters (**14**) Using 1,1′-Oxalyldiazoles (**1** and **2**)

No.	Protected amino acid	Amino acid ester	Product	Reagent (**1**) Yield (%)	mp (°C)	$[\alpha]_D$ (°C, *c*, solv.) [lit.]	Reagent (**2**) Yield (%)	mp (°C)	$[\alpha]_D$ (°C, *c*, solv.)
14a	Z-Ala	Gly-OEt	Z-Ala-Gly-OEt	84	97—98 [99—102	−20.9 (25, 2.19, EtOH) −22.1 (3.08, EtOH)][35a]	84	98—99	−21.5 (25, 1.94, EtOH)
14b	Z-Ala	Phe-OMe	Z-Ala-Phe-OMe	79	98—99 [99—100	−8.7 (25, 1.45, EtOH) −9.3 (22, 1.0, EtOH)][35b]	83	97—98	−9.0 (25, 1.67, EtOH)
14c	Z-Phe	Gly-OEt	Z-Phe-Gly-OEt	92	105—107 [110—112	−16.2 (26, 2.03, EtOH) −17.4 (2.01, EtOH)][35a]	97	106—107	−16.9 (26, 2.17, EtOH)
14d	Z-Phe	Leu-OMe	Z-Phe-Leu-OMe	71	105—108 [110—111	−25.2 (26, 1.99, MeOH) −24.7 (20, 3.1, MeOH)][37]	90	107—108	−24.0 (26, 2.1, MeOH)
14e	Z-Val	Val-OMe	Z-Val-Val-OMe	96	99—102 [116	−22.6 (25, 2.05, MeOH) −24.3 (25, 0.3, MeOH)][36]	99	103—105	−25.4 (25, 1.79, MeOH)
14f	Z-Val	Gly-OEt	Z-Val-Gly-OEt	66	165—166 [170—171	−33.6 (25, 2.14, dioxane) −32.4 (20, 1.85, dioxane)][35a]	66	163—164	−32.5 (25, 1.89, dioxane)
14g	Boc-Phe	Val-OMe	Boc-Phe-Val-OMe	85	115—117 [117—118	−11.0 (25, 2.90, DMF) −11.0 (1.89, DMF)][36]	95	115—117	−12.2 (25, 1.90, DMF)

TABLE III. Preparation of Esters (**14**) and Thioesters (**18**) Using 1,1′-Oxalyldiazoles (**1** and **2**)

$$R{\cdot}\overset{\overset{O}{\|}}{C}{\cdot}X{\cdot}R''$$

No.	R	X	R″	Reagent 1 or 2[a]	Yield (%)	mp [°C] (bp/mmHg) Found	Reported
16a	$CH_3(CH_2)_5$–	O	Me	1	61	(82—85/30)	(170—173)[38]
				2	72		
16b	Cyclohexyl	O	Me	1	65	(78—81/22)	(181—182)[39]
				2	60		
16c		O	Me	1	73	36—37	38—39[40]
				2	68		
16d	Ph	O	Me	1	67	(90—94/20)	(195—199)[41]
				2	61		
19a	Ph	S	Ph	1	78	59—60	56—57[42]
				2	75		
19b	Ph	S		1	63	49—50	50—51[43]
				2	70		
16e	O_2N- 	O	Me	1	83	90—92	96[44]
				2	76		
19c	O_2N- 	S	Ph	1	54	158—160	160—161[45]
				2	66		
16f		O	Me	1	74	(71—74/13)	(181.3)[46]
				2	53		
16g	O_2N- 	O	Me	1	69	78—79	81[47]
				2	73		
16h		O	Me	1	76	(90—93/11)	(40/0.5)[48]
				2	68		
16i	O_2N- 	O	Me	1	80	74—76	75[49]
				2	74		

a) Compounds **16a—i** and **19a—c**, which were prepared using 1,1′-oxalyldi(1,2,4-triazole) (**2**), were characterized by comparison of their mp (bp) and IR data with those of authentic samples.

treating an *N*-protected amino acid with **1** or **2** in acetonitrile for 40 min at room temperature. Effervescent was immediately observed. An appropriate amino acid ester was reacted for 2 h at, or slightly above, room temperature to give the corresponding dipeptide (**14**) in a yield comparable to those obtained by using known condensing reagents, *e.g.*, Staab's reagent (**3**).[13] Racemization was not detectable by nuclear magnetic resonance (NMR) analysis as described in the literature.[14]

Syntheses of Esters (16) and Thioesters (19)

The procedure demonstrated in our laboratory was extended to the synthesis of esters (**16**) and thioesters (**19**). According to the literature,[10a] esterification of carboxylic acids (**6**) using Staab's reagent (**3**) usually requires a base or active halide as a catalyst. For example, it has been reported that sodium or potassium *tert*-butoxide,[15] 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU),[10b] *N*-bromosuccinimide (NBS),[16] and active halides[17] such as allyl bromide, methyl iodide, or benzyl bromide can effect the alcoholysis of 1-acylimidazole (**11**) derived from the reaction of the carboxylic acid (**6**) with Staab's reagent (**3**). Some nitro compounds such as 5-nitro-2-furoic acid or 5-nitro-2-thenoic acid, however, showed great instability when allowed to stand in contact with a basic catalyst such as an alkali metal *tert*-butoxide or DBU. Thus, it seemed desirable to examine whether acidic catalysts, as well as basic or neutral ones,

also effect the alcoholysis of 1-acylazoles (**11**). Hence, we employed methanesulfonic acid (**17**) as an acidic catalyst.[18] To get a better picture of the course of the esterification, the reaction of benzoic acid with **1** in acetonitrile was carried out for 40 min at 40 °C to provide 1-benzoylimidazole, which was subsequently reacted with methanol for 2 h at 60 °C in the presence of **17** to afford methyl benzoate (**16d**) in 67% yield. Next, one of the heteroaromatic nitro compounds in question, 5-nitro-2-furoic acid, was directly converted to the desired methyl ester (**16g**) in 69% yield. On the other hand, 2-mercaptopyridine or thiophenol can be used in place of methanol as a nucleophile for the thioesterification. The results of representative cases are summarized in Table III. As judged from the yields (Table III) of reactions so far examined, **2** appears to give similar results to **1**.

A speculative explanation for the major role of **17** is as follows. The formation of 1-acylazole methanesulfonates from the reaction of 1-acylazoles (**11**) with **17** is essential to enhance the leaving ability of the azole ring from **11**, so that concerted nucleophilic attack of the alcohol on the carbonyl carbon atom of **11** can occur readily to form the corresponding esters (**16**), as shown in Chart 3.

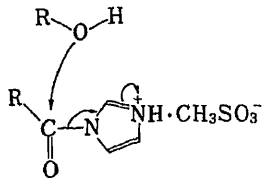


Chart 3

In conclusion, compounds **1** and **2** can be conveniently used as condensing reagents for the direct conversion of carboxylic acid (**6**) into amides (**13**) including dipeptides (**14**), esters (**16**), and thioesters (**19**) under mild reaction conditions.

Experimental

Melting points were taken on a Yanagimoto melting point apparatus. All melting and boiling points are uncorrected. NMR spectra were measured on a Bruker AM-400 spectrometer (400 MHz) using tetramethylsilane as an internal reference, and chemical shifts were recorded as δ-values. Optical rotations were measured on a JASCO DIP-180 polarimeter (Japan Spectroscopic Co., Ltd.). Reagents 1 and 2 were prepared by the known procedures.[6] *N*-Protected amino acids and amino acid ester hydrochlorides were commercial products, and were used without further purification.

Synthesis of Amides (13)——General Procedure Using **1** as a Condensing Reagent: **1** (2.09 g, 11 mmol) was added rapidly in a single portion to a stirred solution of the carboxylic acid (**6**) (10 mmol) in acetonitrile (30 ml). The mexture was stirred at room temperature for 15 min, heated for 40 min at 40°C, and then cooled to room temperature. The amine (**12**) (10 mmol) was added, and the whole was heated again for 2 h at 60 °C. The solvent was removed *in vacuo*, the resulting residue was poured into ice-water, and the mixture was extracted with ethyl acetate. The extract was washed with 5% $NaHCO_3$, 2% HCl, and water, then dried over anhydrous Na_2SO_4. The organic solvent was evaporated off *in vacuo* to give the crude amide (**13**), which was purified by distillation or recrystallization.

Synthesis of *N*-Protected Dipeptide Esters (14)——General Procedure Using **1** as a Condensing Reagent: A well-stirred solution of *N*-protected α-amino acid (10 mmol) in acetonitrile (30 ml) was cooled to 10 °C and treated with 1 (2.09 g, 11 mmol) while maintaining the reaction temperature at below 15 °C. Stirring was continued for 1 h at 15—20 °C, then a mixture of α-amino acid ester hydrochloride (10 mmol) and triethylamine (10 mmol) in acetonitrile (5 ml) was added. The resultant mixture was stirred for 2 h at 10—15 °C. After removal of the solvent *in vacuo*, the residue was dissolved in ethyl acetate and the organic solution was washed with 5% $NaHCO_3$ 2% HCl, and saturated brine, then dried over anhydrous Na_2SO_4. The organic solvent was evaporated off *in vacuo* to give the crude dipeptide, which was purified by recrystallization.

Synthesis of Esters (16 and 19)——General Procedure Using **1** as Condensing Reagent:**1** (2.09 g, 11 mmol) was added rapidly in a single portion to a stirred solution of carboxylic acid (10 mmol) in acetonitrile (20 ml). The mixture was stirred for 15 min at room temperature, heated for 40 min at 40 °C, then cooled to room temperature. A solution of methanol (10 mmol) or the thiol (**18**) (10 mmol) and **17** (2.1 g, 20 mmol) in acetonitrile (10 ml) was added dropwise. The resultant mixture was heated for 2 h at 60 °C. After removal of the solvent *in vacuo*, the resulting residue was poured into ice-water, and the mixture was extracted with ethyl acetate. The extract was washed with 5% $NaHCO_3$

and water, and then dried over anhydrous Na_2SO_4. The organic solvent was evaporated off *in vacuo* to give the crude ester (**16**) or thioester (**19**), which was purified by distillation or recrystallization.

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References and Notes

1) Parts of this work were presented at the 35th local meeting of the Pharmaceutical Society of Japan at Kyoto, November 2, 1985. Abstracts p. 36.
2) T. Kitagawa, H. Sasaki, and N. Ono, *Chem. Pharm. Bull.*, **33**, 4014 (1985).
3) T. Kitagawa, H. Kuroda, and H. Sasaki, *Chem. Pharm. Bull.*, **35**, 1262 (1987).
4) *a*) S. Murata, *Chem. Lett.*, **1983**, 1819; *b*) *Idem*, *Bull. Chem. Soc. Jpn.*, **57**, 3597 (1984).
5) L. Brikofer, P. Richter, and A. Ritter, *Chem. Ber.*, **93**, 2804 (1960).
6) W. Walter and M. Radke, *Justus Liebigs Ann. Chem.*, **1979**, 1756.
7) *a*) H. A. Staab, *Angew. Chem. Int. Ed. Engl.*, **1**, 351 (1962); *b*) S. Ohta and M. Okamoto, *Yuki Gosei Kagaku Kyokai Shi*, **41**, 38 (1983) [*Chem. Abstr.*, **98**, 124969*r* (1983)].
8) H. A. Staab and K. Wendel, *Chem. Ber.*, **93**, 2910 (1960).
9) *a*) R. Paul and G. W. Anderson, *J. Org. Chem.*, **27**, 2094 (1962); *b*) H. A. Staab, *Justus Liebigs Ann. Chem.*, **609**, 75 (1959).
10) *a*) von H-J. Gais, *Angew. Chem.*, **89**, 251 (1977); *b*) T. Kamijio, H. Harada, and K. Iizuka, *Chem. Pharm. Bull.*, **32**, 5044 (1984); *c*) S. Ohta, A. Shimabayashi, M. Aono, and M. Okamoto, *Synthesis*, **1983**, 833.
11) *a*) T. Kamijio, H. Harada, and K. Iizuka, *Chem. Pharm. Bull.*, **32**, 2560 (1984); *b*) H. G. Foley and D. R. Dalton, *J. Chem. Soc., Chem. Commun.*, **1973**, 628.
12) H. A. Staab, G. Walther, and W. Rohr, *Chem. Ber.*, **95**, 2073 (1962).
13) *a*) G. W. Anderson and R. Paul, *J. Am. Chem. Soc.*, **80**, 4423 (1958); *b*) R. Paul and G. W. Anderson, *J. Am. Chem. Soc.*, **82**, 4596 (1960).
14) For instance, in the NMR spectrum of Z–Ala–Phe–OMe (**14b**), the methyl signal of the alanyl group appeared as a doublet ($J=7$ Hz) centered at δ 1.32, which is similar to the δ value (1.30) of Z–L-Ala–L-Phe–OMe reported by Weinstein *et al.* Conversely, in the case of Z–D-Ala–L-Phe–OMe, the corresponding methyl signal appeared as a doublet at higher field (δ 1.27). *a*) B. Weinstein and A. E. Pritchard, *J. Chem. Soc., Perkin Trans. 1*, **1972**, 1015; *b*) B. Halpern, L. F. Chew, and B. Weinstein, *J. Am. Chem. Soc.*, **89**, 5051 (1967); *c*) K. Kawasaki, S. Okusada, and T. Tsuji, "Peptide Chemistry 1984," ed. by N. Izumiya, Protein Research Foundation, Osaka, 1985, p. 71.
15) H. A. Staab and A. Mannschreck, *Chem. Ber.*, **95**, 1284 (1962); see also ref. 4*a*.
16) T. Katsuki, *Bull. Chem. Soc. Jpn.*, **49**, 2019 (1976).
17) T. Kamijio, R. Yamamoto, H. Harada, and K. Iizuka, *Chem. Pharm. Bull.*, **30**, 4242 (1982); see also ref. 10*a*.
18) Methanesulfonic acid (**17**) was first used for activation of **3** in the dehydration of α-formylamino acid into the corresponding isocyanide by Ugi and his co-workers. G. Giesemann, E. von Hinrichs, and I. Ugi, *J. Chem. Res. (S)*, **1982**, 79.
19) J. S. Lumsden, *J. Chem. Soc.*, **87**, 90 (1905).
20) H. E. Ungnade, *J. Am. Chem. Soc.*, **67**, 5133 (1954).
21) G. T. Tisue, S. Linke, and W. Lwowski, *J. Am. Chem. Soc.*, **89**, 6303 (1967).
22) A. Pelter, T. E. Levitt, and P. Nelson, *Tetrahedron*, **26**, 1539 (1970).
23) E. K. Harvill, R. M. Herbst, E. C. Schreiner, and C. W. Roberts, *J. Org. Chem.*, **15**, 662 (1950).
24) H. Stetter and E. Rauscher, *Chem. Ber.*, **93**, 1161 (1960).
25) R. Mestres and C. Palomo, *Synthesis*, **1982**, 288.
26) P. Krishnamurti, *Chem. Zentralbl.*, **1929** I, 2156 [*Chem. Abstr.*, **23**, 2164 (1929)].
27) O. L. Brady and R. P. Mehta, *J. Chem. Soc.*, **125**, 2297 (1924).
28) L. Vargha and F. Gönczy, *J. Am. Chem. Soc.*, **72**, 2738 (1950).
29) C. V. Bowen and L. E. Smith, *J. Am. Chem. Soc.*, **62**, 3522 (1940).
30) W. M. Degnan and F. B. Pope, *J. Am. Chem. Soc.*, **62**, 1960 (1940).
31) M. M. Runde, E. W. Scott, and J. R. Johnson, *J. Am. Chem. Soc.*, **52**, 1284 (1930).
32) H. R. Snyder, Jr., *J. Med. Chem.*, **10**, 737 (1967).
33) T. Kametani, T. Takano, O. Umezawa, H. Agui, K. Kanno, Y. Konno, S. Satoh, H. Nemoto, and K. Yamaki, *Yakugaku Zasshi*, **86**, 823 (1966) [*Chem. Abstr.*, **65**, 20093 *g* (1966)].
34) M. Bellenghi, G. Carrara, F. Fava, E. Ginoulhiac, C. Martinuzzi, A. Vecchi, and G. Weitnauer, *Gazz. Chim. Ital.*, **82**, 773 (1952) [*Chem. Abstr.*, **48**, 2029*i* (1954)].

35) *a*) M. Furukawa, N. Hokama, and T. Okawara, *Synthesis*, **1983**, 42; *b*) E. Schröder, *Justus Liebigs Ann. Chem.*, **679**, 207 (1964).
36) M. Ueda, H. Oikawa, N. Kawaharasaki, and Y. Imai, *Bull. Chem. Soc. Jpn.*, **56**, 2485 (1983).
37) M. Ueda and H. Oikawa, *J. Org. Chem.*, **50**, 760 (1985).
38) A. I. Vogel, *J. Chem. Soc.*, **1948**, 624.
39) S. S. Nametkin and M. A. Volodira, *J. Gen. Chem. USSR*, **17**, 325 (1947) [*Chem. Abstr.*, **42**, 527*g* (1948)].
40) H. Koch and W. Haaf, "Organic Syntheses," Vol. 44, ed. by W. E. Parham, John Wiley and Sons, Inc., New York, 1964, p. 1.
41) G. Graebe, *Justus Liebigs Ann. Chem.*, **340**, 244 (1905).
42) Y. Kanaoka, K. Tanizawa, E. Sato, O. Yonemitsu, and Y. Ban, *Chem. Pharm. Bull.*, **15**, 593 (1967).
43) M. Araki, S. Sakata, H. Takei, and T. Mukaiyama, *Bull. Chem. Soc. Jpn.*, **47**, 1777 (1974).
44) K. Alder, He. Söll, and Ha. Söll, *Justus Liebigs Ann. Chem.*, **565**, 73 (1949).
45) A. Takamizawa, Y. Sato, and S. Tanaka, *Yakugaku Zasshi*, **85**, 298 (1965) [*Chem. Abstr.*, **63**, 9940*h* (1965)].
46) K. Auwers, *Chem. Ber.*, **44**, 3679 (1911).
47) S. Nishida, T. Sato, and Y. Sato, *Repts. Sci. Research Inst.* (Japan), **31**, 430 (1955) [*Chem. Abstr.*, **50**, 15504*a* (1956)].
48) B. Weinstein, *J. Am. Chem. Soc.*, **77**, 6709 (1955).
49) P. Fournari and J. P. Chane, *Bull. Soc. Chim. Fr.*, **1963**, 479 [*Chem. Abstr.*, **59**, 1569*e* (1963)].

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Bitter Principles of *Ailanthus altissima* SWINGLE.[1] Structure Determination of Shinjuglycosides E and F

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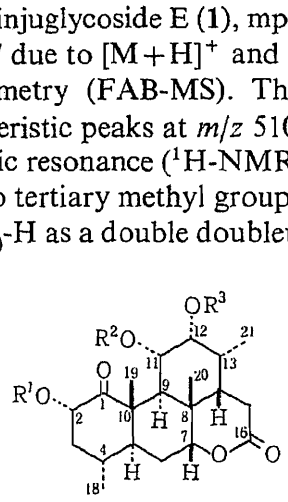
Two new quassinoid glycosides, shinjuglycosides E and F, were isolated from the root bark of *Ailanthus altissima* SWINGLE and their structures were established to be 2-*O*-(β-D-glucopyranosyl)-2α,11α,12α-trihydroxypicrasane-1,16-dione and 2-*O*-(β-D-glucopyranosyl)-2α,12β-dihydroxypicrasane-1,11,16-trione, respectively.

Keywords——quassinoid glycoside; bitter principle; Simaroubaceae; *Ailanthus altissima*; ^{1}H-NMR; ^{13}C-NMR

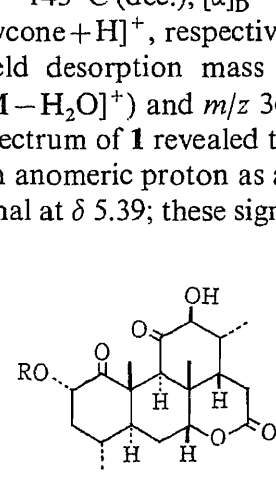
In previous papers we have reported the isolation and structure elucidation of fifteen quassinoids[2-8] and four quassinoid glycosides[9] from a plant of the Simaroubaceae family, *Ailanthus altissima* SWINGLE (Shinju or Niwaurushi in Japanese). This paper describes the structure determination of two new bitter quassinoid glycosides, shinjuglycosides E and F (**1** and **2**), isolated from the same plant.

The aqueous extract of the root bark of *A. altissima* was extracted with dichloromethane and the aqueous layer was subjected to chromatographic separation to afford shinjuglycosides E (**1**; 0.0002%) and F (**2**; 0.00005%) together with ailanthone,[3] $\Delta^{13(18)}$-dehydroglaucarubolone,[10] shinjulactones A,[3] D,[5] M and N,[8] and shinjuglycoside D.[9]

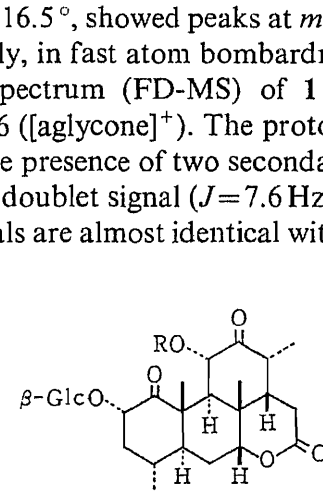
Shinjuglycoside E (**1**), mp 137—143 °C (dec.), $[\alpha]_D^{20}$ −16.5°, showed peaks at *m/z* 529 and *m/z* 367 due to $[M+H]^+$ and $[aglycone+H]^+$, respectively, in fast atom bombardment mass spectrometry (FAB-MS). The field desorption mass spectrum (FD-MS) of **1** exhibited characteristic peaks at *m/z* 510 ($[M-H_2O]^+$) and *m/z* 366 ($[aglycone]^+$). The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** revealed the presence of two secondary methyl and two tertiary methyl groups, an anomeric proton as a doublet signal ($J=7.6$ Hz) at δ 4.81 and $C_{(2)}$-H as a double doublet signal at δ 5.39; these signals are almost identical with those of



1: $R^1=\beta$-Glc, $R^2=R^3=H$
5: $R^1=R^2=R^3=H$
6: $R^1=R^3=H$, $R^2=Ac$
7: $R^1=R^2=H$, $R^3=Ac$



2: R=β-Glc
8: R=H



3: R=Ac
4: R=H

TABLE I. ^{1}H-NMR Spectra of Shinjuglycosides (SG) E (**1**), F (**2**), C (**3**), and D (**4**) in C_5D_5N (Coupling Constants in Parenthesis)

	SG E (**1**)[a]	SG F (**2**)[b]	SG C (**3**)[b]	SG D (**4**)[b]
2-H	5.39 dd (11.4, 7.5)	5.35 dd (12.2, 7.4)	5.39 dd (11.3, 7.6)	5.48 dd (11.3, 7.6)
3-H	2.26 ddd (12.2, 7.5, 4.5)			
6α-H		1.83 ddd (14.7, 3, 2)		
6β-H		1.71 ddd (12.2, 2, 14.7)		
7-H		4.29 dd (2, 2)	4.37 brs	4.30 t (3.7)
9-H	3.39 d (11.5)	3.22 s	3.40 d (13.4)	3.09 d (12.8)
11-H		—	5.62 d (13.4)	4.67 d (12.8)
12-H		4.51 d (11.3)	—	—
13-H			3.13 quint (6.4)	3.08 quint (6.4)
15α-H		3.23 dd (18.6, 13.1)		2.28 dd (18.6, 12.5)
15β-H	2.68 dd (19.1, 7.0)	2.91 dd (18.6, 6.7)	2.74 dd (18.6, 6.7)	2.67 dd (18.6, 6.7)
4-Me	0.67 d (6.8)	0.65 d (6.4)	0.97 d (6.7)	1.00 d (6.7)
8-Me	1.10 s	1.62 s	1.30 s	1.45 s
10-Me	1.43 s	1.04 s	1.49 s	1.50 s
13-Me	1.10 d (6.1)	1.26 d (6.7)	0.63 d (6.4)	0.63 d (6.4)
11-OAc	—	—	2.19 s	—
1′-H	4.81 d (7.6)	4.94 d (7.6)	4.85 d (7.6)	4.97 d (7.6)
2′-H		4.07 dd (7.6, 8.2)	4.10 dd (7.6, 8.6)	4.09 dd (7.6, 8.9)
3′-H			4.18 dd (8.6, 8.9)	4.15 dd (8.9, 8.5)
4′-H			4.23 dd (8.9, 8.9)	4.24 dd (8.5, 9.6)
5′-H		3.8 m	3.89 ddd (8.9, 5.5, 2.4)	3.76 ddd (9.6, 5.2, 2.4)
6′-H		4.37 dd (11.9, 5.2)	4.38 dd (11.9, 5.5)	4.35 dd (11.9, 5.2)
6′-H′		4.52 dd (11.9, 2.1)	4.38 dd (11.9, 2.4)	4.35 dd (11.9, 2.4)

a) Measured at 270 MHz. *b*) Measured at 400 MHz.

shinjuglycosides C and D (**3** and **4**) (Table I). These spectral data and the cabon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum (Table II) indicate that shinjuglycoside E is a hexoside with the molecular formula, $C_{26}H_{40}O_{11}$, and the aglycone, $C_{20}H_{30}O_6$, was inferred to be 2α,11,12-trihydroxypicrasane-1,16-dione, the configurations at C-11 and C-12 being undetermined.

Acid hydrolysis of **1** with 1.5 M sulfuric acid in methanol gave the aglycone (**5**) and the hexose. The sugar part was identified as D-glucose by gas chromatography (GC) after

TABLE II. ^{13}C-NMR Spectra of Shinjuglycosides (SG) E (1), F (2), D (4), and Shinjulactones (SL) I (6), K (7), and H (8)

No. of carbon	SG E (1)[a]	SG F (2)[b]	SG D (4)[a]	SL I (6)[c]	SL K (7)[c]	SL H (8)[d]
1	216.9	211.4	213.1	214.3	221.9	213.0
2	75.7	75.8	75.7	70.1	69.1	69.9
3	45.1	46.6	45.2	49.0	47.9	45.4
4	29.0	28.2	29.1	28.8	28.7	27.9
5	32.8	39.3	40.1	30.5	31.9	39.4
6	26.9	27.0	26.9	26.4	26.4	26.7
7	75.7	77.1	75.7	73.4	77.1	76.2
8	50.4	50.2	50.7	48.6	49.7	48.8
9	32.5	46.8	47.5	32.1	33.8	46.8
10	35.9	39.2	35.4	35.9	35.9	39.2
11	82.6	209.4	82.0	82.3	82.5	208.2
12	70.8	81.9	210.3	72.8	70.6	81.9
13	47.8	47.3	48.0	48.0	47.2	47.0
14	45.4	44.2	42.7	44.8	44.9	45.3
15	29.9	28.1	29.1	29.4	28.9	27.6
16	171.1	169.8	168.9	170.0	170.3	169.3
18	18.5	18.3	18.4	18.3	18.5	18.2
19	13.3	15.6	12.9	13.4	13.4	15.2
20	14.3	15.1	10.6	13.9	13.9	15.2
21	21.6	23.6	21.5	21.7	21.8	23.8
1′	103.3	103.8	103.1	—	—	—
2′	76.2	76.3	76.0	—	—	—
3′	78.6	78.6	78.6	—	—	—
4′	71.4	71.4	71.5	—	—	—
5′	78.2	78.3	78.2	—	—	—
6′	62.7	62.8	62.8	—	—	—
11-OAc	—	—	—	171.1	—	—
	—	—	—	21.1	—	—
12-OAc	—	—	—	—	170.7	—
	—	—	—	—	21.0	—

a) Measured at 22.5 MHz in C_5D_5N. *b*) Measured at 100 MHz in C_5D_5N. *c*) Measured at 22.5 MHz in $CDCl_3$. *d*) Measured at 100 MHz in $CDCl_3$.

trimethylsilylation. The configurations at C-11 and C-12 of the aglycone (**5**) were determined easily by comparison with the hydrolyzates of shinjulactones I and K (**6** and **7**).[6] Shinjulactones I and K possess 11α-acetoxy-2α,12α-dihydroxy- and 12α-acetoxy-2α,11α-dihydroxypicrasane structures, respectively. Acid hydrolysis of a mixture of **6** and **7** afforded the 2α, 11α, 12α-trihydroxy derivative, which was identical with the aglycone (**5**).

When the ^{13}C-NMR spectrum of shinjuglycoside E (**1**) was compared with those of shinjulactones I and K (**6** and **7**), a glycosylation shift (*ca.* 6 ppm) was observed at C-2 (Table II). Therefore the structure of shinjuglycoside E (**1**) was determined to be 2-*O*-(β-D-glucopyranosyl)-2α,11α,12α-trihydroxypicrasane-1,16-dione.

Shinjuglycoside F (**2**), mp 200—205 °C (dec.), $[\alpha]_D^{22}$ −9.6° showed the presence of two secondary methyl (δ 0.65 and 1.26) and two tertiary methyl groups, an anomeric proton at δ 4.94 as a doublet signal (J=7.6 Hz), a proton on a hydroxy-bearing carbon atom as a doublet at δ 4.51, and a proton due to C-2 as a double doublet at δ 5.35 in the ^{1}H-NMR spectrum. The ^{13}C-NMR spectrum (Table II) and a fragment peak at m/z 364 due to the aglycone in the electron impact mass spectrum (EI-MS) suggest a hexoside structure with the molecular formula, $C_{26}H_{38}O_{11}$, for shinjuglycoside F (**2**). In the ^{1}H-NMR spectrum, the doublet signal

ascribable to 13-Me appeared at low field (δ 1.26), which indicates the presence of a carbonyl group at C-11, as in the case of shinjulactone H (**8**)[4] or chaparrolide.[11] Since the coupling constant, $J_{12,13}$, of the doublet signal due to C-12 at δ 4.51 was 11.3 Hz, the structure of the C-ring was revealed and therefore the aglycone of **2** was suggested to be shinjulactone H (**8**).[4]

On acid hydrolysis, shinjuglycoside F (**2**) afforded shinjulactone H (**8**) and D-glucose; the former was identified by infrared (IR), MS and high-performance liquid chromatography (HPLC) and the latter was identified as its trimethylsilyl derivative by GC. The glycosylation shift, Δ5.9 ppm, was observed at C-2, indicating that D-glucose is attached to C-2 of the aglycone (Table II). Thus the structure of shinjuglycoside F (**2**) was concluded to be 2-*O*-(β-D-glucopyranosy)-2α,12β-dihydroxypicrasane-1,11,16-trione.

Experimental[12]

Isolation of Shinjuglycosides E (1) and F (2)——The air-dried root bark[13] (*ca.* 7 kg) was extracted with hot water (95 °C) twice (15 and 10 l) and the aqueous extract was evaporated *in vacuo* to afford a concentrated extract (*ca.* 5 l), which was continuously extracted with dichloromethane (1 l × 3, each for 12 h). The aqueous layer was separated from the organic layer and concentrated to give a dark brown solution (2 l). Insoluble materials which formed on addition of methanol (3 l) were removed by filtration, and the filtrate was evaporated to give a residue (*ca.* 420 g). A part (317 g) of the residue was absorbed on silica gel (316 g), which was placed on the top of a silica-gel column (2 kg). Elution was performed successively with the lower layer of chloroform–methanol–water in the following ratio; 50 : 12 : 3 (6.5 l), 65 : 35 : 10 (12 l), and 50 : 50 : 18 (5 l). Forty fractions (each 500 ml) were collected (column A). The remaining part (103 g) of the residue was subjected to separation under the same conditions as above to afford 38 fractions (each 200 ml) (column B).

Fractions 19 and 20 of column A and fractions 17 and 18 of column B were combined and evaporated to afford a residue (4 g), which was separated by column chromatography on silica gel (80 g). Elution was carried out with 18% methanol in ethyl acetate (200 ml), and then with 20, 23, 26, 29, and 32% methanol in ethyl acetate (each 100 ml) in turn. Eight fractions (each 100 ml) were collected (column C). Fraction 4 exhibited a strong bitter taste.

The residue (581 mg) of fraction 4 of column C was separated by partition chromatography on silicic acid (430 g) pretreated with water (287 g). Elution was performed with 0,1,3,6,...24% ethanol in chloroform and 33 fractions (each 250 ml) were collected (column D).

Fractions 22—28 of column D were combined and evaporated to afford a residue (117 mg), which was separated by reversed-phase chromatography using LiChroprep RP-8 with methanol–water (3 : 7). Forty fractions (each 10 g) were collected (column E). Fractions 13—17 yielded shinjulactone M (12.6 mg).

The residue (29 mg) obtained from fractions 18—33 of column E was separated by gel column chromatography using Toyopearl HW-40S (2.4 × 100) with methanol as an eluent. Seventy fractions (each 10 g) were collected (column F). From fractions 50—52, shinjulactone N (11 mg) was obtained.

Fractions 42—49 of column F were evaporated to give a residue (9 mg), which was separated by reversed-phase chromatography under the same conditions as column E. Seventy fractions (each 5 g) were collected. Fractions 57—68 afforded crude shinjuglycoside F (**2**; 5.8 mg), which crystallized from methanol–acetone to give pure **2** (3.4 mg).

Fractions 21—23 of column A and fractions 19—22 of column B gave a residue (6.48 g), which was subjected to column chromatography on silica gel (130 g). Elution was performed with 10—20% methanol in chloroform and forty fractions (each 100 ml) were collected (column G). Shinjuglycoside D was obtained from fractions 17—24.

The residue (902 mg) obtained from fractions 25—28 of column G was separated by gel column chromatography under the same conditions as described for column F. Methanol was used as an eluent and 70 fractions (each 10 g) were collected (column H). Fraction 63 afforded crystalline needles, mp 236—238 °C. This product was identified as adenosine.

Fractions 38 and 39 of column H were combined and evaporated to yield a residue (105 mg). The residue was absorbed on silica gel (300 mg) and placed on top of a silica-gel column (20 g). Twenty fractions (each 20 ml) were collected by elution with 10—20% methanol in ethyl acetate. Shinjuglycoside E (**1**; 11.8 mg) was obtained from fractions 13—18.

Shinjuglycoside E (1)——mp 137—143 °C (MeOH–AcOEt) (dec.). $[\alpha]_D^{20}$ −16.5° (c=0.2, EtOH). IR (neat): 3360, 1710, 1230, 1080, 1040 cm^{-1}. ^{1}H-NMR (Table I). ^{13}C-NMR (Table II). FAB-MS (MeOH + glycerol) m/z (%): 621 ([M + H + glycerol]$^+$; 3), 529 ([M + H]$^+$; 4), 367 ([aglycone + H]$^+$; 40). FD-MS m/z (%): 510 ([M − H_2O]$^+$; 100), 366 ([aglycone]$^+$; 22).

Shinjuglycoside F (2)——mp 200—205 °C (EtOH–AcOEt) (dec.). $[\alpha]_D^{22}$ −9.6° (c=0.27, MeOH). IR (neat): 3350, 1740, 1735, 1720, 1230, 1220, 1070, 1040 cm^{-1}. ^{1}H-NMR (Table I). ^{13}C-NMR (Table II). EI-MS m/z (%): 364 ([M − $C_6H_{10}O_5$]$^+$; 7), 362 (20), 346 (17), 334 (22), 55 (100).

Acid Hydrolysis of Shinjuglycoside E (1)——A solution of **1** (9.7 mg) in methanol (2 ml) and 1.5 M sulfuric acid

(1 ml) was refluxed for 7 h. The usual work-up and chromatographic separation on silica gel afforded shinjuglycoside E aglycone (**5**; 1.5 mg). IR (neat): 3400, 2920, 1705, 1225 cm^{-1}. ^{1}H-NMR (90 MHz, C_5D_5N) δ: 0.70 (3H, d, J= 5.5 Hz; 4-Me), 1.10 (3H, d, J=6.0 Hz; 13-Me), 1.14 (3H, s; 8-Me), 1.47 (3H, s; 10-Me), 2.71 (1H, dd, J=7.7, 18.0 Hz; 15β-H). EI-MS m/z (%): 366 (M^+; 13), 351 (9), 348 (4), 330 (3), 322 (12), 302 (5), 258 (17), 57 (100).

Acid Hydrolysis of a Mixture of Shinjulactones I (6) and K (7)——A mixture (8 mg) of shinjulactones I (**6**) and K (**7**) obtained from *A. altissima*[6] was dissolved in methanol (4 ml) and the solution was refluxed for 30 h with 1.5 M sulfuric acid (2.0 ml). The usual work-up and chromatography on silica gel yielded 2α,11α,12α-trihydroxypicrasane-1,16-dione (**5**; 5.3 mg). IR (KBr): 3550, 3400, 2960, 2940, 1725, 1705, 1250 cm^{-1}. ^{1}H-NMR (90 MHz, C_5D_5N) δ: 0.69 (3H, d, J=5.5 Hz; 4-Me), 1.10 (3H, d, J=6.2 Hz; 13-Me), 1.14 (3H, s; 8-Me), 1.47 (3H, s; 10-Me), 2.70 (1H, dd, J=7.3, 19.1 Hz; 15β-H), 3.44 (1H, d, J=11.2 Hz; 9-H), 5.11 (1H, dd, J=11.2, 7.9 Hz; 2-H). EI-MS m/z (%): 366 (M^+; 9), 348 (4), 57 (100).

Acid Hydrolysis of Shinjuglycoside F (2)——Shinjuglycoside F (**2**; 4 mg) in methanol (1 ml) was hydrolyzed with 1.5 M sulfuric acid (0.5 ml) at reflux temperature for 6 h and the usual work-up and purification gave shinjulactone H (**8**; 0.4 mg), IR (neat): 3350, 1740, 1730, 1700, 1215, 1080, 1050 cm^{-1}. EI-MS m/z (%): 364 (M^+; 62), 346 (94), 336 (78), 321 (77), 206 (61), 55 (100). HPLC conditions: column, Gasukro Kogyo Unisil Q C_{18}; solvent, MeOH–H_2O (1 : 1); flow rate, 2 ml/min. The t_R values of shinjuglycoside F aglycone and shinjulactone H (**8**) were 9.3 and 9.4 min, respectively.

References and Notes

1) Part XIX of this series. Part XVIII: M. Ishibashi, T. Furuno, H. Hietter, T. Tsuyuki, and T. Takahashi, *Chem. Pharm. Bull.*, **35**, 3011 (1987).
2) M. Ishibashi, T. Tsuyuki, T. Murae, H. Hirota, T. Takahashi, A. Itai, and Y. Iitaka, *Bull. Chem. Soc. Jpn.*, **56**, 3683 (1983).
3) H. Naora, M. Ishibashi, T. Furuno, T. Tsuyuki, T. Murae, H. Hirota, and T. Takahashi, *Bull. Chem. Soc. Jpn.*, **56**, 3694 (1983).
4) M. Ishibashi, S. Yoshimura, T. Tsuyuki, T. Takahashi, and K. Matsushita, *Bull. Chem. Soc. Jpn.*, **57**, 2013 (1984).
5) T. Furuno, M. Ishibashi, H. Naora, T. Murae, H. Hirota, T. Tsuyuki, T. Takahashi, A. Itai, and Y. Iitaka, *Bull. Chem. Soc. Jpn.*, **57**, 2484 (1984).
6) M. Ishibashi, S. Yoshimura, T. Tsuyuki, T. Takahashi, A. Itai, and Y. Iitaka, *Bull. Chem. Soc. Jpn.*, **57**, 2885 (1984).
7) M. Ishibashi, T. Tsuyuki, and T. Takahashi, *Bull. Chem. Soc. Jpn.*, **58**, 2723 (1985).
8) Y. Niimi, T. Tsuyuki, T. Takahashi, and K. Matsushita, *Bull. Chem. Soc. Jpn.*, **59**, 1638 (1986).
9) S. Yoshimura, M. Ishibashi, T. Tsuyuki, T. Takahashi, and K. Matsushita, *Bull. Chem. Soc. Jpn.*, **57**, 2496 (1984).
10) J. Moron, J. Rondest, and J. Polonsky, *Experientia*, **22**, 511 (1966); S. M. Kupchan and J. A. Lacadie, *J. Org. Chem.*, **40**, 654 (1975).
11) R. E. Mitchel, W. Stöcklin, M. Stefanović, and T. A. Geissman, *Phytochemistry*, **10**, 411 (1971).
12) General procedures are the same as those described in Ref. 3. FAB- and FD-MS were run on a JEOL JMS-DX300.
13) *A. altissima* was collected at the Botanical Gardens, Faculty of Science, the University of Tokyo, in October 1984. The authors wish to thank Mr. Eiichi Ishii of the Botanical Gardens for collection of the plant.

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Synthesis and Analgesic Activity of C-Terminal Fragment Peptides of Cholecystokinin[1)]

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Eleven C-terminal fragment peptides of cholecystokinin (CCK) which contain a O-sulfated tyrosine residue [CCK(27—32), CCK(27—31), CCK(27—31)amide, CCK(27—30), CCK(27—30)amide, CCK(27—29), CCK(27—29)amide, CCK(26—27), CCK(26—27)amide, CCK(25—27) and CCK(25—27)amide] were prepared by the solution method. The syntheses of these peptides were carried out by stepwise condensation in combination with fragment condensation. Analgesic effects of these fragment peptides were measured by means of the writhing test. The ED_{50} value of CCK(27—32) was 9.1 mg/kg and that of CCK(25—27) was 7.5 mg/kg, but the other synthetic peptides produced no statistically significant response at a dose of 8 or 10 mg/kg.

Keywords——cholecystokinin (CCK); C-terminal CCK fragment peptide; peptide synthesis; O-sulfated tyrosine residue; CCK(27—32); CCK(25—27); writhing test; analgesic effect

Zetler[2)] reported that cholecystokinin octapeptide (CCK-8) and caerulein produced potent analgesic effects after subcutaneous injection in mice. In our previous study[3)] on some C-terminal CCK-related peptides, it was found that CCK-7 [H–Tyr(SO_3H)–Met–Gly–Trp–Met–Asp–Phe–NH_2] produced analgesic effect, and the O-sulfated tyrosine residue was considered to be essential for this effect. However, excepting the finding that CCK(27—32)amide did not show analgesic effect at a dose of 8 mg/kg,[3)] there exists no information on analgesic effects of CCK-related peptides which are smaller than CCK-7 and contain a sulfate residue at the N-terminal or C-terminal. In order to obtain such information, we intended to synthesize eleven C-terminal fragment peptides of CCK [CCK(27—32), CCK(27—31), CCK(27—31)amide, CCK(27—30), CCK(27—30)amide, CCK(27-29), CCK(27—29)amide, CCK(26—27), CCK(26—27)amide, CCK(25—27) and CCK(25—27)amide] by the solution method, and to examine their analgesic effects using the writhing method according to the procedure of Zetler.[2)] The synthesis of these peptides except CCK(27—29) and CCK(26—27)amide has not been reported. CCK(27—29) has been synthesized by Kádár *et al.*[4)] in their study on the structure–activity relationships of anticonvulsive activity of CCK-8, but the synthetic method was not reported in detail. CCK(26—27)amide has been synthesized by Crawley *et al.*[5)] by the solid-phase method in their study on the behavioral activity of C- and N-terminal fragments of CCK-8. The present report deals with the synthesis of eleven C-terminal fragment peptides of CCK and the determination of their analgesic effects.

The synthetic scheme for CCK(27—32) [H–Tyr(SO_3^-)–Met–Gly–Trp–Met–Asp–OH] is shown in Fig. 1.

The α-amino functions of amino acid residues and peptides were protected with the Boc group. The two carboxyl groups of the aspartic acid residue at the C-terminal were protected as methyl esters. The C-terminal peptide fragment, Boc–Met–Asp(OMe)–OMe (I) was prepared by the coupling of H–Asp(OMe)–OMe[6)] with Boc–Met–OCp.[7)] Boc–Met–Gly–Trp–OMe (II) was synthesized by the coupling of Boc–Met–Gly–OH[3)] with H–Trp–OMe[8)] using

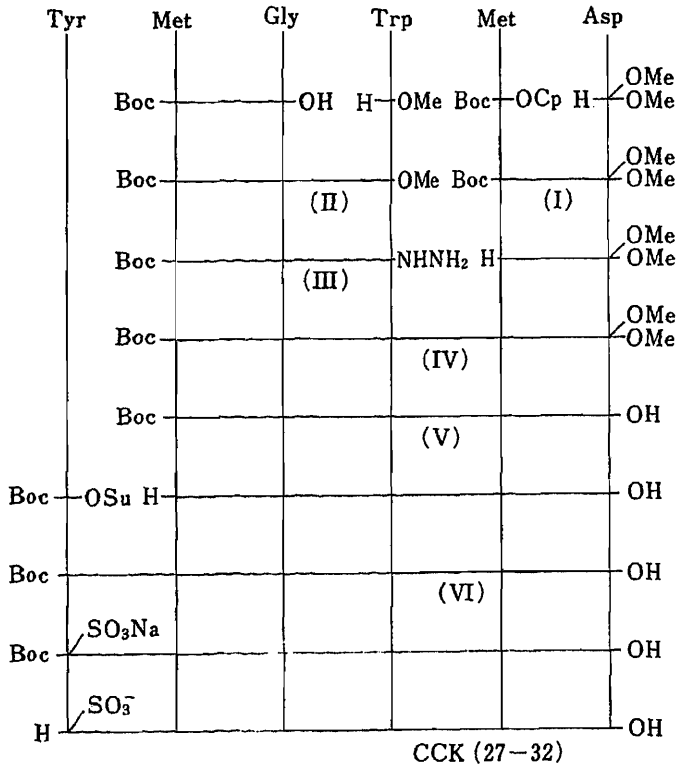

Fig. 1. Synthetic Scheme for CCK (27—32)

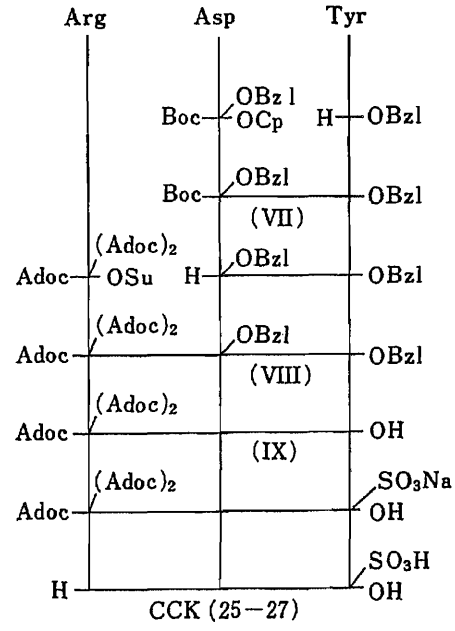

Fig. 2. Synthetic Scheme for CCK (25—27)

DCC in the presence of HOBt, then converted to the corresponding hydrazide (III) by treatment with hydrazine hydrate. After deprotection of the Boc group of I with TFA, the resulting H–Met–Asp(OMe)–OMe was condensed with hydrazide III by the azide procedure to afford the Boc-protected pentapeptide dimethyl ester (IV), which was saponified to produce Boc–Met–Gly–Trp–Met–Asp–OH (V). This compound V was deprotected with TFA, and a tyrosine residue was linked at the N-terminal using Boc–Tyr–OSu[9] to afford Boc–Tyr–Met–Gly–Trp–Met–Asp–OH (VI). Compound VI was sulfated with pyridine–sulfur trioxide complex[10] in anhydrous pyridine solution, and then treated with sodium carbonate. Deprotection of the Boc group with TFA provided crude CCK(27—32), which was purified by column chromatography on diethylaminoethyl (DEAE) Sephadex A-25 using a linear gradient of aqueous ammonium carbonate solution as the eluting solvent, and lyophilized.

The synthetic scheme for CCK(25—27) [H–Arg–Asp–Tyr(SO_3^-)–OH] is outlined in Fig. 2.

The C-terminal carboxyl group of the tyrosine residue and the β-carboxyl group of the aspartic acid residue were protected as benzyl esters. However, these benzyl groups were removed by catalytic hydrogenolysis before sulfation, because the removal of these protecting groups by catalytic hydrogenolysis after sulfation was considered to be hindered by the negative catalysis of the sulfur atom of the sulfate. The α-amino function and the guanidino function of the arginine residue were protected with the Adoc groups, in order to increase the solubility of protected CCK(25—27) and in order to have protecting groups that could be removed under mild acidic conditions. H–Tyr–OBzl[11] was coupled with Boc–Asp(OBzl)–OCp to obtain Boc–Asp(OBzl)–Tyr–OBzl (VII). After removal of the N-terminal Boc-group of VII, an arginine residue was linked using Adoc–Arg$(Adoc)_2$–OSu[12] to produce the tripeptide derivative (VIII). Catalytic hydrogenolysis of VIII produced the Adoc-tripeptide deriva-

TABLE I. Analgesic Effect of CCK-Related Peptides

Peptides	Dose mg/kg, s.c.	Number of mice	Writhing (%) mean ± S.E.
CCK(27—32)	2.5	6	90.9 ± 19.2
	5	6	72.7 ± 8.1
	10	6	45.5 ± 12.1[a]
CCK(27—31)	10	5	82.8 ± 13.8
CCK(27—31)amide	10	5	86.2 ± 17.2
CCK(27—30)	10	5	91.0 ± 14.2
CCK(27—30)amide	10	5	89.6 ± 15.7
CCK(27—29)	8	5	67.5 ± 6.0
CCK(27—29)amide	8	5	72.3 ± 16.9
CCK(26—27)	10	6	109.3 ± 21.3
CCK(26—27)amide	10	6	67.6 ± 11.1
CCK(25—27)	2.5	6	106.3 ± 17.7
	5	6	62.5 ± 19.8
	10	6	39.6 ± 10.4[b]
CCK(25—27)amide	10	6	72.3 ± 12.8
CCK(27—32)amide	10	6	72.9 ± 11.4

a) $p<0.05$, *b*) $p<0.01$ (*t*-test).

tive (IX). Compounds IX was sulfated, treated with sodium carbonate, and than Adoc groups were removed with TFA to afford crude CCK(25—27), which was purified by DEAE Sephadex A-25 column chromatography.

CCK(27—31), CCK(27—31)amide, CCK(27—30), CCK(27—30)amide, CCK(27—29), CCK(27—29)amide, CCK(26—27), CCK(26—27)amide and CCK(25—27)amide were synthesized analogously.

The eleven CCK-related peptides thus synthesized were shown to be homogeneous by thin-layer chromatography (TLC) on silica gel. Elemental analyses and amino acid analyses of acid hydrolysates of these peptides were in good agreement with the theoretically expected values. In the infrared (IR) spectra of these CCK-related peptides, the characteristic band ($1050\,cm^{-1}$)[10] due to a sulfate ester was observed.

The analgesic effects of these eleven CCK-related peptides and CCK(27—32)amide[3] were examined by means of the writhing test according to Zetler.[2] Writhing was elicited by intraperitoneal injection of 0.6% (v/v) acetic acid (10 ml/kg) into ddY male mice weighing 18—22 g. Solutions of the peptides in distilled water or 0.05 M sodium bicarbonate were administered subcutaneously 10 min before the acetic acid injection. The number of writhings occurring between 10 and 20 min after injection of acetic acid was counted. Data were expressed as the ratio (%) with respect to the control value (Table I). CCK(27—32) and CCK(25—27) reduced the number of writhings dose-dependently and the ED_{50} values were calculated from the regression analysis of the dose–response curves to be 9.1 and 7.5 mg/kg, respectively. The other synthetic CCK-related peptides gave no statistically significant response at a dose of 10 or 8 mg/kg [in the case of CCK(27—29) and CCK(27—29)amide].

In the present study, small peptides, CCK(27—32) and CCK(25—27) were found to retain the analgesic effect ($ED_{50}=9.1$, 7.5 mg/kg, respectively), notwithstanding the fact that the other synthetic CCK-related peptides produced no statistically significant response at a dose of 8 or 10 mg/kg. These phenomena are very interesting, since they seem to suggest that two active centers for analgesic effect exist in the C-terminal of CCK and that structural change at the C-terminal of these active centers is reflected in the analgesic activity.

Experimental

In order to prevent oxidation of the methionine residue, every reaction was performed under a nitrogen atmosphere and peroxide-free ether stored over ferrous sulfate was used.[13] The melting points are uncorrected. Optical rotations were measured with a DIP-181 polarimeter (Japan Spectroscopic Co.). Amino acid analyses of acid hydrolysates were performed with a JEOL JLC-6AH amino acid analyzer. IR spectra were measured with a Shimadzu IR-400 infrared spectrophotometer. Elementary analyses were carried out with a Yanagimoto MT-3 CHN Corder. Ascending TLC was performed on a silica gel TLC plate (Kieselgel 60 F_{254}, Merck) using the following solvent systems: Rf^1 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2); Rf^2 AcOEt–pyridine–AcOH–H_2O (60:20:6:11); Rf^3 *n*-BuOH–AcOH–pyridine–H_2O (30:20:6:24); Rf^4 *n*-BuOH–AcOH–pyridine–H_2O (60:20:6:24); Rf^5 AcOEt; Rf^6 $CHCl_3$–AcOEt (2:1); Rf^7 ether.

Boc-Met-Asp(OMe)-OMe (I)——Et_3N (0.7 ml) was added to a solution of H-Asp(OMe)-OMe·HCl[6] (988 mg) in DMF (10 ml) at 0 °C and the mixture was stirred for 30 min, then Boc-Met-OCp[7] (2.04 g) was added and stirring was continued for 3 d at 8 °C. The solvent was evaporated off *in vacuo*. The residue was dissolved in AcOEt (150 ml). This solution was washed successively with 5% sodium bicarbonate (2 × 50 ml), 5% citric acid (2 × 50 ml) and brine (2 × 50 ml), and then dried over Na_2SO_4. After evaporation of the solvent *in vacuo*, the residue (2.8 g) was purified by silica gel column chromatography (Kieselgel 60, Merck, 100 g) with AcOEt–$CHCl_3$ (2:1) as the eluent and the product was crystallized with AcOEt–petroleum ether. Yield 1.4 g (75%), mp 64.5—66 °C, $[\alpha]_D^{21}$ −20.0 (c=1.0, MeOH), Rf^1 0.78, Rf^5 0.62, Rf^6 0.50. *Anal.* Calcd for $C_{16}H_{28}N_2O_7S$: C, 48.97; H, 7.19; N, 7.14. Found: C, 49.02; H, 7.14; N, 7.25.

Boc-Met-Gly-Trp-OMe (II)——Et_3N (1.4 ml) was added to a solution of H-Trp-OMe·HCl[8] (2.55 g) at 0 °C. The mixture was stirred for 30 min, then Boc-Met-Gly-OH[3] (3.06 g), HOBt (1.35 g) and DCC (2.27 g) were added. The whole was stirred overnight at 8 °C, dicyclohexylurea was removed by filtration, and the solvent was evaporated off *in vacuo*. The residue was dissolved in AcOEt (200 ml). This solution was washed successively with 5% sodium bicarbonate (2 × 50 ml), 5% citric acid (2 × 50 ml) and brine (2 × 50 ml), and then dried over Na_2SO_4. The solvent was evaporated off *in vacuo* and the resulting residue was purified by silica gel column chromatography (Kieselgel, 60, Merck, 70 g). The column was developed with AcOEt, and the desired fractions were collected. The solvent was evaporated off *in vacuo* to give a white powder. Yield 4.5 g (88.8%), mp 45—47°C (soften), $[\alpha]_D^{22}$ +33.6° (c=1.0, AcOEt), Rf^2 0.93, Rf^5 0.37. Amino acid ratio in an acid hydrolysate: Gly, 1.02; Met, 0.98 (average recovery, 99%). *Anal.* Calcd for $C_{24}H_{34}N_4O_6S$: C, 56.90; H, 6.76; N, 11.06. Found: C, 56.96; H, 6.91; N, 10.88.

Boc-Met-Gly-Trp-$NHNH_2$ (III)——Hydrazine hydrate (1.5 g) was added to a solution of II (1.53 g) in *n*-PrOH (10 ml). The mixture was left to stand at room temperature for 4 d to yield a precipitate. This was collected by filtration and recrystallized from *n*-PrOH. Yield 1.26 g (80%), mp 76—78 °C, $[\alpha]_D^{21}$ −11.9° (c=1.0, MeOH), Rf^1 0.68, Rf^2 0.74. *Anal.* Calcd for $C_{23}H_{34}N_6O_5S \cdot H_2O$: C, 52.66; H, 6.72; N, 16.02. Found: C, 52.97; H, 6.52; N, 15.96.

Boc-Met-Gly-Trp-Met-Asp(OMe)-OMe (IV)——Compound I (1.89 g) was added to a solution of TFA (5 ml) containing thioanisole (0.5 ml) at 0 °C and the mixture was stirred for 1 h. Ether (30 ml) was added to the mixture to cause a precipitate, which was collected by filtration, washed with ether (50 ml) and dried over KOH *in vacuo* to give H-Met-Asp(OMe)-OMe·TFA (542 mg). On the other hand, 5.6 N HCl in dioxane (0.5 ml) and isoamyl nitrite (0.14 ml) were added to a solution of III (481 mg) in DMF (18 ml) at −50 °C, and the mixture was stirred at −30—−20 °C for 30 min to prepare a solution of Boc-Met-Gly-Trp-N_3. H-Met-Asp(OMe)-OMe·TFA (540 mg) was added to a solution of Et_3N (0.37 ml) in DMF (10 ml) at 0 °C and the mixture was added to the solution of Boc-Met-Gly-Trp-N_3 at −50 °C. The reaction mixture was stirred for 2 d at 8 °C and the solvent was evaporated off *in vacuo*. The residue was dissolved in AcOEt (200 ml). The solution was washed successively with 5% citric acid (2 × 50 ml), 5% sodium bicarbonate (2 × 50 ml), H_2O (2 × 50 ml) and brine (2 × 50 ml), then dried over Na_2SO_4. Evaporation of the solvent *in vacuo* gave an amorphous residue, which was recrystallized from EtOH–H_2O. Yield 454 mg (63.6%), mp 125—128 °C, $[\alpha]_D^{21}$ −27.5° (c=1, MeOH), Rf^1 0.79, Rf^2 0.84, Rf^3 0.76. Amino acid ratio in an acid hydrolysate: Asp, 1.03; Gly, 1.00; Met, 1.96 (average recovery 89%). *Anal.* Calcd for $C_{34}H_{50}N_6O_{10}S_2 \cdot 0.5\ H_2O$: C, 52.63; H, 6.63; N, 10.83. Found: C, 52.70; H, 6.50; N, 10.91.

Boc-Met-Gly-Trp-Met-Asp-OH (V)——A 1 N NaOH solution (1.54 ml) was added to a solution of IV (537 mg) in MeOH (10 ml) and the mixture was stirred at room temperature for 5 h. After evaporation of the solvent *in vacuo*, water (50 ml) was added to the residue. The aqueous layer was washed with AcOEt (2 × 50 ml), acidified with citric acid and extracted with AcOEt (3 × 50 ml). The extract was washed with H_2O (50 ml) and brine (2 × 50 ml), then dried over Na_2SO_4. The solvent was evaporated off *in vacuo* and the residue was recrystallized from EtOH–H_2O. Yield 327 mg (64%), mp 191—192 °C(dec.), Rf^1 0.59, Rf^2 0.37, Rf^3 0.59, $[\alpha]_D^{22}$ −23° (c=1, MeOH). Amino acid ratio in an acid hydrolysate: Asp, 1.03; Gly, 0.99; Met, 1.98 (average recovery, 92%). *Anal.* Calcd for $C_{32}H_{46}N_6O_{10}S_2$: C, 52.02; H, 6.28; N, 11.37. Found: C, 52.31; H, 6.19; N, 11.44.

Boc-Tyr-Met-Gly-Trp-Met-Asp-OH (VI)——Compound V (270 mg) was treated with TFA to give the TFA salt of the deprotected pentapeptide (280 mg, Rf^1 0.42, Rf^2 0.49) in the same manner as described for the deprotection of I in the synthesis of IV. This product (248 mg) was added to a solution of Et_3N (34 mg) in DMF (5 ml) and the mixture was stirred at 0 °C for 20 min, then Boc-Tyr-OSu[9] (125 mg) was added. The whole was stirred at

8 °C for 7 d and the solvent was evaporated off *in vacuo*. The residue was dissolved in AcOEt (150 ml). This solution was washed successively with 5% citric acid (3 × 50 ml), H_2O (2 × 50 ml) and brine (2 × 50 ml), then dried over Na_2SO_4. After evaporation of the solvent, the residue was applied to a Sephadex G-25 column (3 × 37 cm). The column was equilibrated and developed with 0.05 M ammonium carbonate. Fractions No. 10—16 (15 ml each) were collected and lyophilized to give a white powder. This material was added to a solution of 3% citric acid and extracted with AcOEt (100 ml). The extract was washed with H_2O (2 × 50 ml) and dried over Na_2SO_4. The solvent was evaporated off *in vacuo* and the residue was triturated with ether. Yield 205 mg (64%), mp 120—130 °C (dec.), $[\alpha]_D^{20}$ −19.0° (c=1.4, 1 N NH_4OH), Rf^1 0.74, Rf^2 0.36, Rf^3 0.60. Amino acid ratio in an acid hydrolysate: Asp, 1.00; Gly, 0.99; Met, 1.96; Tyr, 1.04 (average recovery, 90%). *Anal.* Calcd for $C_{41}H_{55}N_7O_{12}S_2 \cdot 0.5AcOEt \cdot H_2O$: C, 53.57; H, 6.38; N, 10.17. Found: C, 53.81; H, 6.49; N, 10.19.

CCK(27—32) [H–Tyr(SO_3^-)–Met–Gly–Trp–Met–Asp–OH]——Pyridine–sulfur trioxide complex (130 mg) was added to a solution of VI (150 mg) in anhydrous pyridine (5 ml) and the mixture was stirred at 8 °C for 2 d. After removal of the solvent *in vacuo*, a solution of 10% sodium bicarbonate solution (10 ml) was added to the residue at 0 °C and the mixture was stirred for 30 min. The aqueous layer was washed with *n*-BuOH (5 ml) and concentrated to about 5 ml *in vacuo*. This was applied to a Sephadex G-25 column (3 × 37 cm). The column was equilibrated and eluted with 0.05 M ammonium carbonate. Fractions No. 11—14 (14.6 ml each) were collected. The solvent was evaporated off *in vacuo* and MeOH (2 ml) was added to the residue. After removal of insoluble material by filtration, ether (10 ml) was added to the filtrate to form a precipitate, which was collected by filtration and washed with ether to give Boc–CCK(27—32). Yield 85 mg, mp 240 °C (dec.), $[\alpha]_D^{20}$ −11° (c=1.4, 1 N NH_4OH), Rf^1 0.56, Rf^2 0.13. Amino acid ratio in an acid hydrolysate: Asp, 1.02; Gly, 1.01; Met, 1.96; Tyr, 1.01, NH_3, 0.88 (average recovery, 86%). Boc–CCK(27—32) (60 mg) was added to a solution of TFA (2 ml) containing thioanisole (0.1 ml) at 0 °C, followed by stirring for 1.5 h. After the addition of ether (50 ml), the resulting precipitate was collected by filtration and washed with ether to give crude CCK(27—32). This was purified by chromatography on a DEAE Sephadex A-25 column. The column (1.2 × 15 cm) was eluted with a linear gradient of ammonium carbonate (from 0.1 to 0.7 M). Fractions (13 ml each) were checked for UV absorption at 254 nm. Fractions No. 35—41 were collected and lyophilized. The lyophilization was repeated to give CCK(27—32) as a hygroscopic powder. Yield 20 mg (37%), mp 170—175 °C (dec.), $[\alpha]_D^{20}$ −8.0° (c=1.1, H_2O), Rf^1 0.27, Rf^2 0.12. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). Amino acid ratio in an acid hydrolysate: Asp, 1.01; Gly, 0.99; Met, 1.98; Tyr, 1.02; NH_3, 0.89 (average recovery, 88%). *Anal.* Calcd for $C_{36}H_{46}N_7NaO_{13}S_3 \cdot NH_3 \cdot 2.5H_2O$: C, 44.76; H, 5.63; N, 11.60. Found: C, 45.01; H, 5.97; N, 11.38.

Boc–Asp(OBzl)–Ocp——2,4,5-Trichlorophenol (4.94 g) and DCC (5.42 g) were added to a solution of Boc–Asp(OBzl)–OH[14] (8.08 g) in dichloromethane (50 ml) under cooling with ice, and the mixture was stirred at 7—8 °C overnight. After removal of dicyclohexylurea by filtration, the solvent was evaporated off *in vacuo*. The residue was recrystallized from AcOEt–petroleum ether. Yield 8.4 g (66%), mp 82—83 °C, $[\alpha]_D^{21}$ −29.1° (c=1, DMF). *Anal.* Calcd for $C_{22}H_{22}Cl_3NO_6$: C, 52.56; H, 4.41; N, 2.79. Found: C, 52.75; H, 4.36; N, 2.68.

Boc–Asp(OBzl)–Tyr–OBzl (VII)——This compound was obtained from H–Tyr–OBzl·TsOH[11] and Boc–Asp–(OBzl)–OCp according to the procedure described for I. The crude product was purified by silica gel column chromatography with $CHCl_3$–AcOEt (3 : 1) as the eluent and crystallized from ether–petroleum ether to give a white powder. Yield 81%, mp 101—102 °C, $[\alpha]_D^{21}$ −10.8° (c=1, AcOEt), Rf^6 0.47, Rf^7 0.54. *Anal.* Calcd for $C_{32}H_{36}N_2O_8$: C, 66.65; H, 6.29; N, 4.86. Found: C, 66.39; H, 6.31; N, 4.88.

Adoc–Arg(Adoc)$_2$–Asp(OBzl)–Tyr–OBzl (VIII)——Compound VII (865 mg) was treated with TFA in the same manner as described for the deprotection of I in the synthesis of IV. After evaporation of the TFA *in vacuo*, the residue was washed with ether and dried over KOH *in vacuo* to give H–Asp(OBzl)–Tyr–OBzl·TFA (567 mg). From this compound and Adoc–Arg(Adoc)$_2$–OSu,[12] VIII was obtained according to the procedure described for I. Crude VIII was purified by silica gel column chromatography with AcOEt–$CHCl_3$ (1 : 1) as the eluent and crystallized from ether–petroleum ether. Yield 44%, mp 78—85 °C (dec.) $[\alpha]_D^{20}$ −13.6° (c=1, MeOH), Rf^6 0.61, Rf^7 0.39. *Anal.* Calcd for $C_{66}H_{82}N_6O_{13}$: C, 67.90; H, 7.08; N, 7.20. Found: C, 67.63; H, 7.37; N, 7.03.

Adoc–Arg(Adoc)$_2$–Asp–Tyr–OH (IX)——A solution of VIII (413 mg) in MeOH (50 ml) was hydrogenated under the pressure of 40 psi over a palladium catalyst (palladium black, 50 mg) at room temperature for 8 h using a Parr hydrogenation apparatus. After removal of the catalyst, the solvent was evaporated off *in vacuo* and the residue was crystallized from AcOEt–ether. Yield 250 mg (72%), mp 178—182 °C (dec.), $[\alpha]_D^{20}$ +8.9° (c=1, AcOEt), Rf^2 0.81, Rf^4 0.71. Amino acid ratio in an acid hydrolysate: Arg, 1.00; Asp, 0.98, Tyr, 1.02 (average recovery, 90%). *Anal.* Calcd for $C_{52}H_{69}N_6O_{12} \cdot 2H_2O$: C, 62.07; H, 7.31; N, 8.35. Found: C, 62.13; H, 7.36; N, 8:23.

CCK(25—27) [H–Arg–Asp–Tyr(SO_3^-)–OH]——Compound IX was sulfated then treated with aqueous sodium carbonate in the same manner as described for CCK(27—32). Water was evaporated off *in vacuo*, the residue was extracted with MeOH, and then the extract was concentrated *in vacuo* to give crude protected CCK(25—27). This material was deprotected and the resulting crude CCK(25—27) was purified according to the procedure described for CCK(27—32), except that the DEAE Sephadex A-25 column was eluted with a linear gradient of ammonium carbonate from 0.01 to 0.1 M. Yield 65%, mp 190—205 °C (dec.), $[\alpha]_{365}^{20}$ −5.5° (c=0.5, H_2O), Rf^1 0.08, Rf^4 0.17. IR ν_{max}^{KBr} cm^{-1} : 1045 (SO_3). Amino acid ratio in an acid hydrolysate: Arg, 1.01; Asp, 0.98; Tyr, 1.01 (average recovery, 84%).

Nine other C-terminal fragment peptides of CCK were prepared analogously. The physical properties are as follows.

CCK(27—31) [H-Tyr(SO_3^-)-Met-Gly-Trp-Met-OH]: mp 140—145 °C (dec.), $[\alpha]_{365}^{20}$ +3° (c=0.1, H_2O), Rf^1 0.43, Rf^2 0.09. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). Amino acid ratio in an acid hydrolysate: Gly, 1.01; Met, 1.99; Tyr, 1.00; NH_3, 0.92 (average recovery, 83%). *Anal.* Calcd for $C_{32}H_{42}N_6O_{10}S_2 \cdot NH_3 \cdot 3.5H_2O$: C, 45.38; H, 6.19; N, 11.58. Found: C, 45.15; H, 6.35; N, 11.92.

CCK(27—31)amide [H-Tyr(SO_3^-)-Met-Gly-Trp-Met-NH_2]: mp 160—166 °C (dec.), $[\alpha]_D^{20}$ +22° (c=1, MeOH), Rf^1 0.62, Rf^2 0.35. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). Amino acid ratio in an acid hydrolysate: Gly,1.05; Met, 1.95; Tyr, 1.00; NH_3, 1.24 (average recovery, 81%). *Anal.* Calcd for $C_{32}H_{43}N_7O_9S_3 \cdot 3H_2O$: C, 46.87; H, 6.02; N, 11.96. Found: C, 46.74; H, 5.89; N, 12.04.

CCK(27—30) [H-Tyr(SO_3^-)-Met-Gly-Trp-OH]: mp 67—75 °C (soften), 105—112 °C (dec.), $[\alpha]_D^{21}$ −10° (c=0.1, 1 N NH_4OH), Rf^1 0.36, Rf^2 0.06. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). Amino acid ratio in an acid hydrolysate: Gly, 0.99; Met, 0.97; Tyr, 1.03; NH_3, 0.95 (average recovery, 90%). *Anal.* Calcd for $C_{27}H_{33}N_5O_9S_2 \cdot NH_3 \cdot 2H_2O$: C, 47.08; H, 5.85; N, 12.20. Found: C, 46.92; H, 5.46; N, 11.88.

CCK(27—30)amide [H-Tyr(SO_3^-)-Met-Gly-Trp-NH_2]: mp 194—197 °C (dec.), $[\alpha]_D^{20}$ +2° (c=0.1, 1 N NH_4OH), $[\alpha]_{365}^{20}$ +12° (c=0.1, 1 N NH_4OH), Rf^1 0.44, Rf^2 0.15. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). Amino acid ratio in an acid hydrolysate: Gly, 0.95; Met, 1.01; Tyr, 1.02; NH_3, 1.02 (average recovery, 89%). *Anal.* Calcd for $C_{27}H_{34}N_6O_8S_2 \cdot 2.5H_2O$: C, 47.70; H, 5.78; N, 12.36. Found: C, 47.66; H, 5.58; N, 12.51.

CCK(27—29) [H-Tyr(SO_3^-)-Met-Gly-OH]: mp 178—182 °C (dec.), $[\alpha]_D^{22}$ +6° (c=1, H_2O), Rf^1 0.3, Rf^2 0.02. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). Amino acid ratio in an acid hydrolysate: Gly, 0.99; Met, 1.02; Tyr, 0.99; NH_3, 0.99 (average recovery, 89%). *Anal.* Calcd for $C_{16}H_{23}N_3O_8S_2 \cdot NH_3 \cdot H_2O$; C, 39.66; H, 5.82; N, 11.56. Found: C, 39.78; H, 5.94; N, 11.57.

CCK(27—29)amide [H-Tyr(SO_3^-)-Met-Gly-NH_2]: mp 124—127 °C (dec.), $[\alpha]_D^{22}$ +25°(c=1, H_2O), Rf^1 0.40, Rf^2 0.06. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). Amino acid ratio in an acid hydrolysate: Gly, 0.99; Met, 1.02; Tyr, 0.99; NH_3, 1.05 (average recovery, 98%). *Anal.* Calcd for $C_{16}H_{26}N_4O_8S_2 \cdot H_2O$: C, 41.19; H, 5.61; N, 12.00. Found: C, 41.29; H, 5.64; N, 12.13.

CCK(26—27) [H-Asp-Tyr(SO_3^-)-OH]: mp 122—126 °C (dec.), $[\alpha]_D^{21}$ +5.2° (c=1, H_2O), Rf^1 0.08, Rf^2 0.01, Rf^4 0.21. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). Amino acid ratio in an acid hydrolysate: Asp, 0.98; Tyr, 1.01; NH_3, 2.02 (average recovery, 90%). *Anal.* Calcd for $C_{13}H_{16}N_2O_9S \cdot 2NH_3 \cdot 0.5H_2O$: C, 37.23; H, 5.53; N, 13.36. Found: C, 37.45; H, 5.37; N, 13.02.

CCK(26—27)amide [H-Asp-Tyr(SO_3^-)-NH_2]: mp 72—77 °C (soften), 128—130 °C (dec.), $[\alpha]_D^{21}$ +15.6° (c= 0.5), Rf^1 0.14, Rf^2 0.01, Rf^4 0.29. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). Amino acid ratio in an acid hydrolysate: Asp, 0.99; Tyr, 0.99; NH_3, 2.03 (average recovery, 88%). *Anal.* Calcd for $C_{13}H_{17}N_3O_8S \cdot NH_3 \cdot 0.5H_2O$: C, 38.90; H, 5.27; N,13.96. Found: C, 38.99; H, 5.20; N, 13.78.

CCK(25—27)amide [H-Arg-Asp-Tyr(SO_3^-)-NH_2]: mp 205—215 °C (dec.), $[\alpha]_D^{20}$ +12.6° (c=0.46, H_2O), Rf^1 0.11, Rf^4 0.21. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_3). Amino acid ratio in an acid hydrolysate: Arg, 0.99; Asp, 0.99; Tyr, 1.03; NH_3, 1.10 (average recovery, 92%).

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References and Notes

1) The customary L indication for amino acid residues is omitted. Standard abbreviations for amino acids and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [*Biochemistry*, **5**, 2485 (1966); **6**, 362 (1967); **11**, 1726 (1972)]. Other abbreviations used are: Boc, *tert*-butyloxycarbonyl; Adoc, adamantyl-1-oxycarbonyl; OCp, 2,4,5-trichlorophenyl ester; OSu, *N*-hydroxysuccinimido ester; OMe, methyl ester; OBu^t, *tert*-butyl ester; OBzl, benzyl ester; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; TFA, trifluoroacetic acid; AcOEt, ethyl acetate; AcOH, acetic acid; MeOH, methanol; *n*-PrOH, 1-propanol; *n*-BuOH, 1-butanol; HOBt, 1-hydroxybenzotriazole.
2) G. Zetler, *Neuropharmacology*, **19**, 415 (1980).
3) K. Iuchi, M. Nitta, K. Ito, Y. Morimoto and G. Tsukamoto, *Chem. Pharm. Bull.*, **34**, 115 (1986).
4) T. Kádár, A. Pesti, B. Penke, G. Tóth, M. Zarándi and G. Telegdy, *Neuropharmacology*, **22**, 1223 (1983).
5) J. N. Crawley, S. St-Pierre and P. Gaudreau, *J. Pharmacol. Exp. Ther.*, **230**, 438 (1984).
6) W. Grassmann and E. Wünsh, *Chem. Ber.*, **91**, 449 (1958).
7) P. H. Bentley, H. Gregory, A. H. Laird and J. S. Morley, *J. Chem. Soc.*, **1964**, 6130.
8) H. Peter, M. Brugger, J. Schreiber and A. Eschenmoser, *Helv. Chim. Acta*, **46**, 577 (1963).
9) Y. Wolman, A. Schejter and M. Sokolovsky, *J. Am. Chem. Soc.*, **94**, 1720 (1972).

10) M. A. Ondetti, J. Pluščec, E. F. Sabo, J. T. Sheehan and N. Williams, *J. Am. Chem. Soc.*, **92**, 195 (1970).
11) L. Zervas, M. Winitz and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957).
12) G. Jäger and R. Geiger, *Chem. Ber.*, **103**, 1727 (1970).
13) Y. Mori and H. Yajima, *Chem. Pharm. Bull.*, **24**, 2781 (1976).
14) E. Bayer, G. Jung and H. Hagenmaier, *Tetrahedron*, **24**, 4853 (1968).

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Inhibition of Xanthine Oxidase by Anthraquinones[1)]

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Inhibitory activities of nine anthraquinone derivatives against xanthine oxidase (EC 1.2.3.2) were assayed *in vitro*. Anthragallol (1,2,3-trihydroxyanthraquinone, **8**) was the most active inhibitor among them. The concentration of **8** in the assay mixture required to give 50% inhibition (IC_{50}) was 1.2×10^{-5} M. The type of inhibition by **8** with respect to xanthine as a substrate was uncompetitive.

Keywords——xanthine oxidase; inhibitor; uncompetitive inhibition; xanthine; anthragallol; anthraquinone

Inhibitors of xanthine oxidase (XO, EC 1.2.3.2) may be candidate drugs for the treatment of gout.[2)] We have already reported some XO inhibitors, such as flavonoids[3)] and xanthones.[1)] Anthraquinones are a large group of naturally occurring quinones, which have many physiological activities and applications; for example, they show aperient, antibiotic,[4)] mutagenic,[5)] antimutagenic,[6)] genotoxic,[7)] carcinogenic,[8)] phytoalexin,[9)] antifeedant[10)] and enzyme-inhibitory activities. Enzymes that are inhibited by anthraquinones include adenosine 3′,5′-cyclic monophosphate phosphodiesterase,[11,12)] guanosine 3′,5′-cyclic monophosphate phosphodiesterase,[12)] glutathione *S*-transferase,[13)] prolyl 4-hydroxylase,[14)] 15-hydroxyprostagrandin dehydrogenase,[15)] prostaglandin synthetase,[16)] nicotinamide adenine dinucleotide linked dehydrogenases[17)] and Na-K-activated adenosine triphosphatase.[18)] This paper deals with the inhibitory activities of anthraquinones against XO.

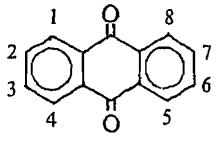
Results and Discussion

The activity of XO *in vitro* towards xanthine as a substrate was assayed spectrometrically at 290 nm by the method described in the previous paper.[3)] Anthragallol (**8**)[19)] and rufigallol (**9**)[20)] were synthesized as described in Experimental. Emodin (**7**) and other anthraquinones (**1**—**6**) used in this experiment were commercial materials. These nine anthraquinones were assayed for inhibitory activity against XO. The results (Table I) suggested that the presence of *ortho* hydroxy groups as in **2**, **6**, **8** and **9** is required for inhibitory activity in anthraquinones. The weaker activity of **9** may be a result of poor solubility in the assay mixture. The most active compound in this experiment was **8**. The concentration of **8** in the assay mixture required to give 50% inhibition (IC_{50}) was 1.2×10^{-5} M. Kinetic data on the effect of **8** on the oxidation of xanthine by XO under the assay conditions are shown as Lineweaver–Burk plots[21)] in Fig. 1. The type of inhibition by **8** was uncompetitive.

Experimental

The following instruments were used to obtain physical data. Melting points were determined on a Yanagimoto melting point apparatus and are uncorrected. The infrared (IR) spectra were recorded on a JASCO IRA-202 infrared

TABLE I. XO Inhibitory Activities of Anthraquinones



Compound	1	2	3	4	5	6	7	8	Inhibition (%)	
									10 μg/ml	1 μg/ml
Anthraquinone (1)	H	H	H	H	H	H	H	H	0.9	0.0
Alizarin (2)	OH	OH	H	H	H	H	H	H	29.2	1.7
Quinizarin (3)	OH	H	H	OH	H	H	H	H	0.6	0.0
Anthrarufin (4)	OH	H	H	H	OH	H	H	H	1.0	0.0
Chrysazin (5)	OH	H	H	H	H	H	H	OH	0.2	0.0
Purpurin (6)	OH	OH	H	OH	H	H	H	H	37.2	6.5
Emodin (7)	OH	H	OH	H	H	Me	H	OH	17.5	4.1
Anthragallol (8)	OH	OH	OH	H	H	H	H	H	93.2	7.0
Rufigallol (9)	OH	OH	OH	H	OH	OH	OH	H	22.0	0.5

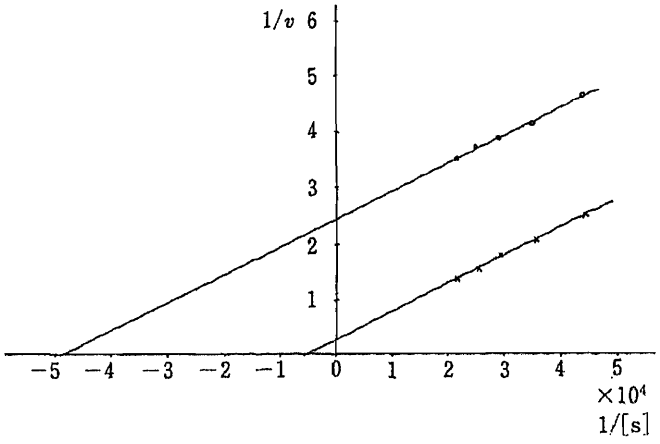


Fig. 1. Inhibitory Effects of Anthragallol (8) on XO

Lineweaver–Burk plots in the absence (0 M, ×—×) and in the presence of 8 (7.80×10^{-5} M, O—O) with xanthine as the substrate. v: μM substrate metabolized/mg enzyme/min. s: substrate.

spectrophotometer. The proton nuclear magnetic resonance (^{1}H-NMR) spectra and the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were recorded on a JEOL FX-90Q NMR spectrometer (89.55 and 22.50 MHz, respectively) with tetramethylsilane as an internal standard (δ value). Mass spectra (MS) were recorded on a JEOL JMS-01 SG-2 mass spectrometer. Silica gel 60 GF_{254} (Merck) was used for thin layer chromatography (TLC) and detection was achieved by illumination with an ultraviolet lamp and by spraying 20% aq. H_2SO_4 followed by heating, or by spraying 3% $FeCl_3$ in EtOH. The spectrophotometric measurements were carried out with a Hitachi model 101 spectrophotometer.

Enzyme and Chemicals——Xanthine oxidase (XO, EC 1.2.3.2) from cow's milk was obtained from Boehringer Mannheim Co., Ltd. Xanthine was obtained from ICN Pharmaceutical Inc. Emodin (7) and Tween 80 were obtained from Wako Pure Chemical Industries, Ltd. Sodium phosphate dibasic 12 hydrate and potassium phosphate monobasic were obtained from Kanto Chemical Co., Inc. Anthraquinone (1), alizarin (2), quinizarin (3), anthrarufin (4), chrysazin (5) and purpurin (6) were obtained from Tokyo Kasei Kogyo Co., Ltd. The buffer, the substrate solution and the enzyme solution were prepared as described previously.[3]

Test Solution——All test samples were purified by recrystallization, and the test solutions were prepared as described previously.[3]

Assay of XO Activity——For the assay of XO activity, the method described previously[3] was employed, but the preincubation and incubation temperatures were 35 °C in this experiment.

Estimation of XO-Inhibitory Activity——The same method[3] as described previously was used for the estimation of XO-inhibitory activity.

Lineweaver–Burk Plots——The Lineweaver–Burk plots[21] for XO with xanthine as the substrate under our assay conditions in the absence and in the presence of **8** are shown in Fig. 1.

Anthragallol (8)——**8** was synthesized by the method of Seuberlich.[19] Orange needles from EtOH. mp 315 °C (lit. mp 312—313 °C[19]). MS *m/z*: 256(M^+). *Anal.* Calcd for $C_{14}H_8O_5$: C, 65.63; H, 3.15. Found C, 65.39; H, 3.09. The structure was confirmed by the spectral data.

Rufigallol (9)——**9** was synthesized by the method of Robiquet.[20] Red needles from EtOH. mp > 350 °C (lit. mp > 300 °C[20]). MS *m/z*: 304(M^+). *Anal.* Calcd for $C_{14}H_8O_8$: C, 55.27; H, 2.65. Found C, 55.06; H, 2.57. The structure was confirmed by the spectral data.

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References and Notes

1) This forms Part IV of "Studies of Enzyme Inhibitors." Part III: T. Noro, A. Ueno, M. Mizutani, T. Hashimoto, T. Miyase, M. Kuroyanagi, and S. Fukushima, *Chem. Pharm. Bull.*, **32**, 4455 (1984).
2) R. W. Rundles, *Trans. Assoc. Am. Physicians*, **76**, 126 (1963); M. Isa, G. Hayashi, and Y. Kowa, *Nippon Yakurigaku Zasshi*, **64**, 108 (1968).
3) T. Noro, Y. Oda, T. Miyase, A. Ueno, and S. Fukushima, *Chem. Pharm. Bull.*, **31**, 3984 (1983); S. Nishibe, A. Sakushima, T. Noro, and S. Fukushima, *Shoyakugaku Zasshi*, **41**, 116 (1987).
4) S. Matsueda, K. Takagaki, M. Shimoyama, and A. Shiota, *Yakugaku Zasshi*, **100**, 900 (1980).
5) T. Masuda, K. Ishii, and Y. Ueno, Abstracts of Papers, The 103rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1983, p. 307; T. Matsushima, M. Muramatsu, O. Yagase, A. Araki, L. Tikkanen, and S. Natori, *Prog. Clin. Biol. Res.*, **209B**, 133 (1986).
6) A. Kushi, A. Koiwai, D. Yoshida, and F. Goto, *Agric. Biol. Chem.*, **44**, 2513 (1980).
7) H. Mori, K. Kawai, F. Ohbayashi, T. Kuniyasu, M. Yamazaki, T. Hamasaki, and G. M. Williams, *Cancer Research*, **44**, 2918 (1984).
8) H. Mori, S. Sugie, K. Niwa, N. Yoshimi, T. Tanaka, and I. Hirono, *Gann*, **77**, 871 (1986).
9) R. Wijnsman, I. N. van Weerden, R. Verpoorte, P. A. A. Harkes, C. B. Lugt, J. J. C. Scheffer, and A. B. Svendsen, *Planta Medica*, **1986**, 211.
10) D. K. Reed, M. Jacobson, J. D. Warthen, Jr., E. C. Uebel, N. J. Tromley, L. Jurd, and B. Freedman, *Tech. Bull. U. S. Dep. Agric.*, **1981**, 1641 [*Chem. Abstr.*, **96**, 16023c (1982)].
11) T. Nikaido, T. Ohmoto, U. Sankawa, S. Kitanaka, and M. Takido, *Chem. Pharm. Bull.*, **32**, 3075 (1984).
12) M. Ruckstuhl and Y. Landry, *Biochem. Pharmacology*, **30**, 697 (1981).
13) M. Das, D. R. Bickers, and H. Mukhtar, *Biochem. Biophys. Res. Commun.*, **120**, 427 (1984).
14) C. J. Cunliffe and T. J. Franklin, *Biochem. J.*, **239**, 311 (1986).
15) S. Yamanouchi, F. Kimura, M. Takido, and U. Sankawa, Abstracts of Papers, the 107th Annual Meeting of the Pharmaceutical Society of Japan, Kyoto, April 1987, p. 304.
16) Y. Gohda, Y. Kawasaki, S. Yamazaki, K. Yoshihira, Y. Hirose, M. Shibuya, and U. Sankawa, Abstracts of Papers, the 33rd Annual Meeting of the Japanese Society of Pharmacognosy, Saitama, October 1986, p. 4.
17) Y. Zhang and Q. H. Chen, *Shengwu Huaxue Yu Shengwu Wuli Xuebao*, **18**, 239 (1986).
18) I. S. Koh, *Taehan Saengri Hakhoe Chi*, **11**, 1 (1977).
19) Deutschen Chemischen Gesellschaft, "Beilsteins Handbuch der Organischen Chemie," Vol. 8, Verlag von Julius Springer, Berlin, 1925, p. 505.
20) Deutschen Chemischen Gesellschaft, "Beilsteins Handbuch der Organischen Chemie," Vol. 8, Verlag von Julius Springer, Berlin, 1925, p. 567.
21) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934); M. Dixon and E. C. Weff, "Enzymes," 3rd ed., Longman Group Ltd., London, 1979, p. 341.

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Isotachophoresis of Flavonoids

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A new system for the rapid and microscale analysis of flavonoids has been established using capillary isotachophoresis. Under the conditions employed, authentic flavonoids examined were clearly separated into individual zones, and the compounds in plant extracts could also be identified on the basis of enhancement of the appropriate zones in runs involving mixed charging of extracts with the authentic samples. This new technique seems to be useful for the detection of rutin (quercetin-3-*O*-rhamnoglucoside), which is a taxonomical marker in the genus *Calamagrostis* (Poaceae), in large numbers of plant specimens of this taxa on a micro scale.

Keywords——capillary isotachophoresis; flavonoid; rutin; *Calamagrostis*; taxonomical marker

Introduction

Isotachophoresis (ITP) has become a powerful tool for rapid and reproducible microanalysis of a variety of electrically charged substances.[1] However, this technique has not so far been exploited widely in the field of plant biochemistry. For the separation of flavonoids and other plant phenolic constituents, it has been generally accepted that paper electrophoresis offers no advantage over chromatographic techniques.[2] The authors recently succeeded in separating and identifying anthocyanins in plant extracts by ITP.[3] We describe here the application of this method to the analysis of flavonoids.

Results and Discussion

The isotachopherograms of 10 authentic flavonoids under neutral (Fig. 1) and alkaline (Fig. 2) conditions indicated that these compounds were successfully separated into individual zones on the basis of differences in molecular weight and negative charge. As summarized in Table I, flavonoids with lower molecular weight and larger numbers of free hydroxyls in the flavonoid skeleton generally had larger mobility (*e.g.* kaempferol > kaempferol-3-*O*-glucoside > kaempferol-3-*O*-diglucoside *etc.*), although luteolin-7-*O*-glucoside and kaempferol-3-*O*-glucoside, as well as the 3-*O*-glucoside and 3-*O*-galactoside of quercetin, having the same molecular weight and the same number of free phenolic hydroxyls, were not distinguished from each other on the isotachopherograms. Kaempferol and quercetin-3-*O*-glucoside were separated only under neutral conditions, while the zone of naringenin-7-*O*-rhamnoglucoside was detected only under alkaline conditions. On the other hand, the potential unit (PU) value of quercetin-3-*O*-glucuronide was smaller than that of quercetin-3-*O*-glucoside with smaller molecular weight and the same number of free phenolic

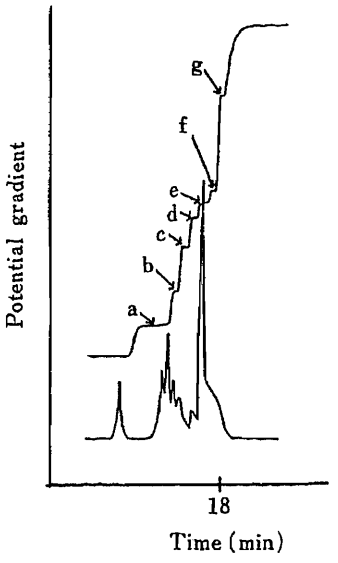

Fig. 1. Isotachophoretic Separation of Flavonoids under Neutral Conditions

Leading electrolyte, 0.01 M HCl–L-histidine (pH 5.8); terminating electrolyte, 0.01 M MES–Tris (pH 7.2). Sample: combined methanolic solution (2—5 μl of 0.1—0.5 mg/ml each) of quercetin (a; PU value 0.10), quercetin-3-*O*-glucuronide (b; 0.20), kaempferol (c; 0.35), quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside (d; 0.41), luteolin-7-*O*-glucoside and kaempferol-3-*O*-glucoside (e; 0.44), quercetin-3-*O*-rhamnoglucoside (f; 0.50) and kaempferol-3-*O*-diglucoside (g; 0.77). Applied current, 75 μA; chart speed, 20 mm/min.

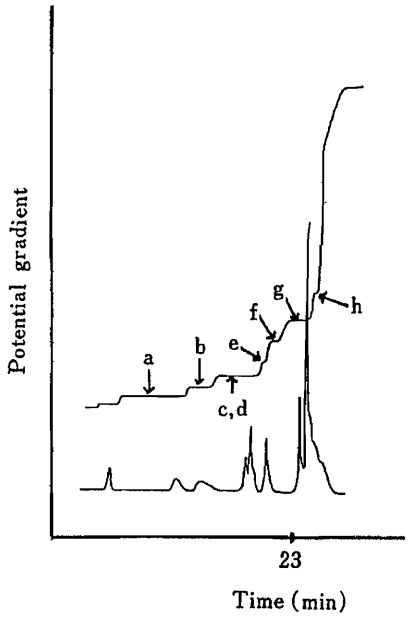

Fig. 2. Isotachophoretic Separation of Flavonoids under Alkaline Conditions

Leading electrolyte, 0.01 M HCl–amediol (pH 8.4); terminating electrolyte, 0.01 M 6-aminocapronic acid–$Ba(OH)_2$ (pH 10.8). Samples: combined methanolic solution (5—10 μl of 0.1—0.5 mg/ml each) of quercetin (a; PU value 0.03), quercetin-3-glucuronide (b; 0.06), kaempferol, quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside (c—d; 0.10), luteolin-7-*O*-glucoside and kaempferol-3-*O*-glucoside (e; 0.12), quercetin-3-*O*-rhamnoglucoside (f; 0.19), kaempferol-3-*O*-diglucoside (g; 0.27) and naringenin-7-*O*-rhamnoglucoside (h; 0.36). Applied current, 75 μA; chart speed, 40 mm/min.

TABLE I. PU Values (×100) of Flavonoids

Compounds	Positions of free flavonoid OH	MW	PU value	
			Natural conditions[a]	Alkaline conditions[b]
Luteolin-7-*O*-glucoside	3′, 4′, 5	448	44	12
Kaempferol	4′, 3, 5, 7	286	35	10
Kaempferol-3-*O*-glucoside	4′, 5, 7	448	44	12
Kaempferol-3-*O*-diglucoside	4′, 5, 7	610	77	29
Quercetin	3′, 4′, 3, 5, 7	302	10	03
Quercetin-3-*O*-glucoside	3′, 4′, 5, 7	464	41	10
Quercetin-3-*O*-galactoside	3′, 4′, 5, 7	464	41	10
Quercetin-3-*O*-glucuronic acid	3′, 4′, 5, 7	478	20	06
Quercetin-3-*O*-rhamnoglucoside	3′, 4′, 5, 7	610	50	19
Naringenin-7-*O*-rhamnoglucoside	4′, 5	579	—	36

a) L, 0.01 M HCl–L-histidine (pH 5.8); T, 0.01 M MES–Tris (pH 7.2). *b*) L, 0.01 M, HCl–amediol (pH 8.4); T, 0.01 M 6-aminocapronic acid–$Ba(OH)_2$ (pH 10.8).

hydroxyls, possibly due to the negative charge of glucuronic acid. However, much more work seems to be needed in order to establish the relation between chemical structure and isotachophoretic behavior of flavonoids.

The zones of flavonoids appeared clearly after the injection of 1 μl of 0.1 mg/ml methanolic solution, and one analysis required less than 25 min. These ITP analysis systems were applied to the separation and identification of flavonoids in plant extracts whose constituents had previously been studied by conventional chromatographic methods. The isotachopherograms of plant extract samples under alkaline conditions exhibited a rather continuous elevation of the potential gradient, resulting in incomplete isotachophoretic separation, presumably due to the coexistence of a variety of anionic substances. Therefore, all the examined extracts from leaf blades of 9 *Calamagrostis* (Poaceae) species as well as authentic flavonoids, were treated only under neutral conditions, and zones were monitored not only for potential gradient, but also for changes in the ultraviolet (UV) absorption at 254 nm. Several specific zones, including 2 or 3 major ones, all of which corresponded to UV absorption peaks, were observed on the isotachopherograms of 4 (*C. masamurae* MAXIM., *C. nana* TAKEDA, *C. hakonensis* FR. *et* SAV. and *C. langsdorffi* TRIN.) and 5 (*C. gigas* TAKEDA, *C. fauriei* HACK, *C. masamurai* HONDA, *C. tashiroi* OHWI and *C. sachalinensis* FR. SCH.) species, respectively. The compound responsible for the major zone with the PU value of 0.50 detected only on the isotachopherograms of the latter 5 species was identified as quercetin-3-*O*-rhamnoglucoside (rutin) on the basis of enhancement of the appropriate zone after mixed charging with an authentic sample (Fig. 3). The other 2 major zones of aromatic compounds (PU values: 0.39 and 0.72) commonly present in all 9 species examined were hardly distinguishable from those of authentic quercetin-3-*O*-glucoside (0.41) and kaempferol-3-*O*-diglucoside (0.77) respectively, in similar experiments. On the other hand, it was shown by our previous studies that isoorientin and its 7-*O*-glucoside occur universally in the species of this genus as the main flavonoid components of the leaf blades. Rutin was also contained as another major glycosidic constituent in some species or strains of this taxa.[4-6] Therefore, it has been considered that rutin may be an important marker in taxonomical studies of this genus, especially for solving problems relevant to natural hybridization. Thus, the presence *vs.* absence of rutin in various species and its strains has been examined by the use of a reversed-phase high-performance liquid chromatography (HPLC).[7,8] The results of the present study concerning the presence *vs.* absence of rutin are in agreement with the HPLC findings, and the other 2 major zones were assigned to isoorientin and its 7-*O*-glucoside based on the results of previous studies mentioned above and the general tendency of isotachophoretic behavior of flavonoids shown in Table I. The zone of rutin was clearly detected using 5 μl of 1 ml

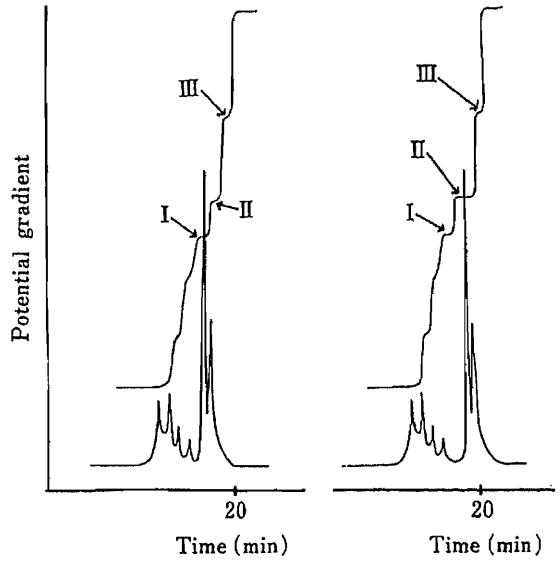

Fig. 3. Isotachopherograms of *Calamagrostis sachalinensis* Leaf Extract (Left) and a Mixture of It and Authentic Rutin (Right) under Neutral Conditions

Leading electrolyte, 0.01 M HCl–L-histidine (pH 5.8); terminating electrolyte, 0.01 M MES–Tris (pH 7.2); applied current, 75 μA, chart speed, 40 mm/min. Samples: 5 μl each of *C. sachalinensis* leaf extract (1 ml of MeOH solution of the extract from 0.3 g dry weight) (A) and the authentic sample of rutin (0.3 mg/ml) (B). The left and right isotachopherograms were obtained by the ITP analysis of A (left) and A+B (right), respectively. Among the 3 major zones (I—III), zone II (PU value 0.50) corresponds to rutin. Zones I (0.39) and III (0.72) were assigned to isoorientin and its 7-*O*-glucoside, respectively (see the text).

methanolic solution of the extract from 0.3 g of dried leaf blades, and one examination took about 20 min, so that the sample size and time requirement, as well as reproducibility, were almost the same as those of HPLC. Therefore, the present ITP system, as well as HPLC, should allow us to detect rutin in large numbers of plant extract samples with rapidity and simplicity on a microscale. A survey employing this system is in progress.

Experimental

Samples——The authentic samples of quercetin, rutin and naringenin-7-*O*-rhamnoglucoside were purchased from Wako Pure Chemicals. Quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronide were isolated from the petals of *Epimedium cremeum* NAKAI and *Polygonum perfoliatum* L., respectively. The other authentic flavonoids were obtained in previous studies in connection with the chemotaxonomy of ferns.[9-11] The plant extracts containing flavonoid glycosides were prepared from 0.3 g samples of dried leaf blades of *Calamagrostis masumurae* MAXIM., *C. nana* TAKEDA, *C. hakonensis* FR. *et* SAV., *C. langsdorffi* TRIN., *C. gigas* TAKEDA, *C. fauriei* HACK, *C. masumurai* HONDA, *C. tashiroi* OHWI and *C. sachalinensis* FR. SCH., in the same manner as in the case of the HPLC analysis.[7,8] The dried extracts thus obtained, as well as the authentic samples (0.1—0.5 mg each), were dissolved in 1 ml of MeOH.

ITP of Flavonoids——ITP was carried out with a Shimadzu IP-2A isotachophoretic analyzer equipped with a PGD-2 potential detector and UVD-10A UV absorption detector. An aliquot (1—10 μl) of methanolic solution of a sample to be analyzed was injected, and the separation was run in a PTFE capillary tube (1 mm × 4 cm + 0.5 mm × 15 cm) maintained at 20 °C. The current was first stabilized at 200 μA, then maintained at 75 μA. Chart speed was 20 or 40 mm/min. The combinations of leading (L) and terminating (T) electrolytes were 0.01 M HCl adjusted to pH 5.8 with L-histidine (L) and 0.01 M 2-(*N*-morpholino)ethanesulfonic acid (MES) adjusted to pH 7.2 with tris-hydroxymethylaminomethane (Tris) (neutral conditions) or 0.01 M HCl adjusted to pH 8.4 with Tris (L) and 0.01 M 6-aminocapronic acid adjusted to pH 10.8 with amediol (T) (alkaline conditions).

References

1) A. Adam and C. Schot, "Analytical Chemistry Symposia Series, Vol. 5, Biochemical and Biological Applications of Isotachophoresis," ed. by A. Adam and C. Schot, Elsevier, Amsterdam, Oxford and New York, 1980, p. VII.
2) J. B. Harborne, "Chromatography," ed. by E. Heftmann, Von Nostrand Reinhold Company, New York, 1975, p. 767.
3) A. Hiraoka and K. Yoshitama, *Chem. Pharm. Bull.*, **34**, 2257 (1986).
4) T. Tateoka, A. Hiraoka and T. N. Tateoka, *Bot. Mag.* (Tokyo), **90**, 193 (1977).
5) T. Tateoka, *Species Biology*, **4**, 68 (1980).
6) T. Tateoka and T. N. Tateoka, *Bot. Mag.* (Tokyo), **94**, 355 (1981).
7) M. Maeda, A. Hiraoka, T. N. Tateoka and T. Tateoka, *Bot. Mag.* (Tokyo), **97**, 271 (1984).
8) T. Tateoka, T. N. Tateoka, M. Maeda and A. Hiraoka, *J. Phytogeogr. Taxonomy*, **35**, 13 (1987).
9) A. Hiraoka and M. Hasegawa, *Bot. Mag.* (Tokyo), **88**, 127 (1975).
10) A. Hiraoka, *Biochem. Syst. Ecol.*, **6**, 171 (1978).
11) A. Hiraoka and M. Maeda, *Chem. Pharm. Bull.*, **27**, 3130 (1979).

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Solanum Alkaloids as Inhibitors of Enzymatic Conversion of Dihydrolanosterol into Cholesterol

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The effects of several solanum steroidal alkaloids on cholesterol biosynthesis from 24,25-dihydrolanosterol by 10000 × *g* supernatant fluid of rat liver were examined. Solacongestidine (I), solafloridine (II) and solasodine (III) (40 μM each) exhibited considerable inhibitory effects (59, 51, 37% inhibition, respectively) on the synthesis of cholesterol from [24,25-^{3}H]-24,25-dihydrolanosterol (18 μM). The biological importance of the inhibitory properties of the steroidal alkaloids is discussed.

Keywords——cholesterol biosynthesis; [24,25-^{3}H]-24,25-dihydrolanosterol; rat hepatic subcellular S_{10} fraction; solacongestidine; solafloridine; solasodine; solanum steroidal alkaloid

One of the present authors, Sato, and his coworkers have shown that some lanosterol and cholesterol analogs and oxygenated sterols are potent inhibitors of the enzymatic conversion of lanosterol and 24,25-dihydrolanosterol into cholesterol.[1-3] This finding prompted us to examine whether other steroidal alkaloids, some of which show potent antifungal activity against *Candida albicans*, *Trichophyton rubrum*, *etc.*,[4] are also inhibitors of cholesterol biosynthesis. We found that solacongestidine (I) is a potent inhibitor of cholesterol biosynthesis from dihydrolanosterol, compared with the other solanum alkaloids and oxygenated sterols already reported by Sato *et al.*

Experimental

Solacongestidine (I), solafloridine (II), solasodine (III), tomatidine (IV), tomatillidine (V) and solanocapsine (VI) were obtained as a result of the research on solanum alkaloids in the Steroids Section of the National Institute of Arthritis, Metabolic and Digestive Disease (NIAMDD) of the National Institutes of Health (NIH) in the U.S.A. The substrate, [24,25-^{3}H]dihydrolanosterol, was prepared as described previously.[5] Experiments to examine the effects of solanum alkaloids on cholesterol biosynthesis from 18 μM [24,25-^{3}H]dihydrolanosterol rat hepatic subcellular fraction (S_{10} fraction) were performed as described previously.[5]

Results and Discussion

In the previous studies,[1,2] the substrate and test compounds were used at the concentrations of 18 and 40 μM, respectively, and the structure–activity relationship was examined. The studies demonstrated that both the side chain and skeleton of the test compounds are important for the inhibitory activity on cholesterol synthesis. In the present study, the influence of solacongestidine (I) and solafloridine (II) along with solasodine (III), tomatidine (IV), tomatillidine (V) and solanocapsine (VI) on enzymatic conversion of dihydrolanosterol into cholesterol was examined. By the same method as described previously,[5] [24,25-^{3}H]dihydrolanosterol (18 μM) was incubated with the rat liver homogenate

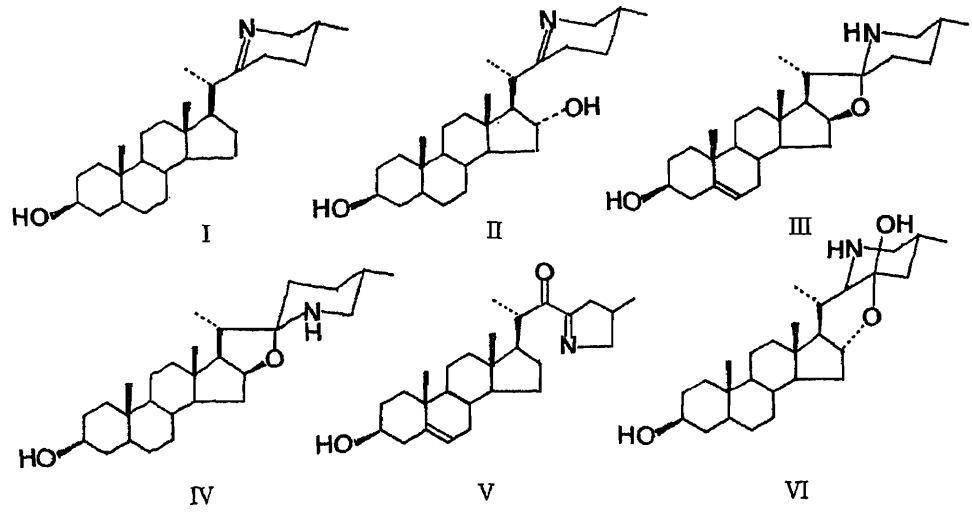

Fig. 1

TABLE 1

Compound		Dihydrolanosterol Fr. (%)	Cholesterol Fr. (%)	Inhibition (%)
None (control)		26.0	21.9	—
Solacongestidine	(I)	62.3	9.0	59
Solafloridine	(II)	51.8	10.7	51
Solasodine	(III)	42.9	13.7	37
Tomatidine	(IV)	37.8	19.2	12
Tomatillidine	(V)	33.1	17.6	20
Solanocapsine	(VI)	32.2	23.4	0

[24,25-^{3}H]-24,25-Dihydrolanosterol (3.84×10^7 dpm; 185.08 μCi/μmol, 18 μM) was incubated with rat liver S_{10} fraction at 37 °C for 3 h. The incubation mixture contained, in a total volume of 5 ml, 4 ml of S_{10} fraction (19.9—20.0 mg protein/ml) and cofactors. Analytic methods for incubation products and the calculation of percentage inhibition were described previously. Each incubation was carried out in triplicate and the standard deviation of each value listed was less than 5%. S_{10} fraction means supernatant fluid at $10000 \times g$.

S_{10} fraction in the presence of solanum alkaloids (I-VI, 40 μM each). The results are summarized as Table I. It is clear that some of the alkaloids (I, II, III) inhibited cholesterol synthesis from dihydrolanosterol. Solacongestidine (I) was found to be the most potent, followed by solafloridine (II). The potency of I (59% inhibition) was almost the same as that of 3β-hydroxy-5α-cholest-8(14)-en-15-one (64%), one of the most potent oxygenated cholesterol derivatives, examined by the same method.[3] It is of interest that solanum alkaloids such as solacongestidine (I) and solafloridine (II) are inhibitors of cholesterol biosynthesis from dihydrolanosterol. The recovery yield of the substrate, dihydrolanosterol, from the incubation medium with I and II was high (62.3 and 51.8%) compared with that from the control (26.0%). This suggests that one of the sites of inhibition by I and II is the step of 14α-demethylation of dihydrolanosterol.

Solacongestidine (I) and solafloridine (II) have been obtained as aglycones after hydrolysis of glycosides from *Solanum congestiflorum*.[6] Both alkaloids, especially I, showed potent antifungal activity against *Candida albicans, Trichophyton rubrum, Cryptococcus albidus, C. neoformans, Torulopsis candida* and *Trichosporon cutaneum*, while other related compounds showed much lower activity.[4] Neither biological nor pharmacological studies have been carried out on solacongestidine (I) except for our studies on the antifungal activity.

Therefore, it is noteworthy that I is a potent inhibitor of cholesterol biosynthesis from dihydrolanosterol. More systematic pharmacological and biological studies may provide greater insight into the biological activities of I and related compounds.

Ketoconazole, an N-substituted imidazole derivative, shows significant therapeutic effects upon some fungal diseases and it inhibits ergosterol synthesis in fungal cells with accumulation of 14α-methyl sterols, indicative of an interaction with the 14α-demethylase system.[7] This reaction is carbon monoxide-sensitive, indicating that a cytochrome P-450-containing enzyme system is required to initiate oxidation of the 14α-methyl group of lanosterol and dihydrolanosterol.[8] The accumulation of 14α-methyl sterols in fungal cells has been suggested to lead to functional changes in cell membranes and to cell death. The biosynthesis of cholesterol and ergosterol from lanosterol involves similar steps for the removal of the three methyl groups.[9] It has been established in the case of ketoconazole that the dose required to influence cholesterol synthesis in rat liver is at least six times that required to inhibit ergosterol synthesis in *Candida albicans*.[10] It has also been shown that cholesterol synthesis in a subcellular fraction of rat liver is about 20—27 times less sensitive to ketoconazole than ergosterol synthesis in a similar fraction obtained from *C. albicans*.[10]

Solacongestidine (40 μM) exhibited 59% inhibition of cholesterol synthesis from [24,25-^{3}H]dihydrolanosterol (18 μM). On the other hand, the minimum inhibitory concentration (MIC) of solacongestidine (I) against *C. albicans* is less than 1 μg/ml (2.5 nM/ml). The influence of solacongestidine (I) on ergosterol synthesis should be examined.

The structure of solacongestidine (I) is similar to that of 25-azasteroids, *e.g.*, 25-azacholesterol [24-dimethylamino-chol-5-en-3β-ol]. This azasteroid and 25-azacoprostane [24-dimethylamino-5β-cholan] are highly active inhibitors of insect molting and metamorphosis,[11] and they also show inhibitory effects on the growth and development of the free-living stages of nematodes such as *Nippostrongylus brasiliensis* and *Nematospiroides dubius*.[12] These azasteroids, though their mode of inhibitory action is different from that of the compounds tested in the present studies, have been established to inhibit the Δ^{24}-sterol reductase enzyme system, resulting in the disturbance of essential sterol synthesis and the inhibition of growth of insects and nematodes, which lack *de nove* sterol biosynthesis and require exogenous sterols, utilizing sterols of both plant and animal origin.[13,14] As judged from the above discussion, our finding that solanum alkaloids, especially solacongestidine (I), are potent inhibitors of cholesterol biosynthesis from dihydrolanosterol suggests that some solanum alkaloids and related compounds may be biological modulators of sterol biosynthesis (anabolic and catabolic).

References and Notes

1) Y. Sato and Y. Sonoda, *Chem. Pharm. Bull.*, **29**, 2604 (1981).
2) Y. Sato, Y. Sonoda, M. Morisaki and N. Ikekawa, *Chem. Pharm. Bull.*, **31**, 1765 (1983).
3) Y. Sato, Y. Sonoda, M. Morisaki and N. Ikekawa, *Chem. Pharm. Bull.*, **32**, 3305 (1984).
4) G. Kusano, A. Takahashi, K. Sugiyama and S. Nozoe, *Chem. Pharm. Bull.*, submitted.
5) Y. Sato and Y. Sonoda, *Chem. Pharm. Bull.*, **32**, 1912 (1984).
6) Y. Sato, Yo. Sato, H. Kaneko, E. Bianchi and H. Kataoka, *J. Org. Chem.*, **34**, 1577 (1969).
7) M. Borgers, H. Van den Bossche and M. De Brabander, *Am. J. Med.*, **74-1B**, 2 (1983).
8) H. Van den Bossche and G. Willemsens, *Arch. Int. Physiol. Biochim.*, **90-B**, 218 (1982).
9) K. Alexander, M. Akhtar, R. B. Boar, J. F. McGhie and D. H. R. Barton, *J. Chem. Soc., Chem. Commun.*, **1972**, 383.
10) G. Willemsens, W. Cools and H. Van den Bossche, "The Host Invader Interplay," ed. by H. Van den Bossche, Elsevier, Amsterdam, 1980, p. 691.
11) M. A. J. Al-Izzi and T. L. Hopkins, *J. Insect. Physiol.*, **28**, 267 (1982).
12) K. P. Bottjer, P. P. Weinstein and M. J. Thompson, *Comp. Biochem. Physiol.*, **78-B**, 805 (1984).
13) J. A. Svoboda, M. J. Thompson and W. E. Robbens, *Lipids*, **6**, 113 (1972).
14) M. Rothstein, *Comp. Biochem. Physiol.*, **27**, 309 (1968).

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Inhibition of Adenosine Triphosphatase Activity in Brain Microtubule Proteins by S100 Protein

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S100 protein exhibited an inhibitory effect on the adenosine triphosphatase activity of brain microtubule proteins in a concentration-dependent manner. In the presence of Ca^{2+}, maximal inhibition was about 90% (only about 10% of the original activity remained), while the maximum inhibition in the presence of Mg^{2+} was about 30%. Zn^{2+}, which can bind both microtubule proteins and S100 protein, had little effect on the inhibitory action of S100 protein in the presence or absence of Ca^{2+}. The inhibition by S100 protein was reduced by chlorpromazine.

Keywords——S100 protein; microtubule protein; ATPase; chlorpromazine; zinc; calcium

Microtubules have architectural and contractile roles in nearly all eucaryotic cells; for example, they are involved in axonal transport, chromosomal movement and hormonal secretion.[1] The motive force of cell motility depends on the hydrolysis of adenosine 5′-triphosphate (ATP). It has been reported that an ATPase activity is present in brain microtubule proteins.[2–4] The activity is insensitive to several inhibitors of membrane ATPase and remains bound to the microtubule proteins through successive cycles of polymerization and depolymerization.[3,4] These results suggest that ATPase in microtubule proteins is associated with the motility of cytoplasm.

S100 protein has been shown to regulate microtubule assembly and disassembly[5–7] and the phosphorylation of several proteins.[8,9] These regulations are Ca^{2+} dependent. S100 protein also binds to Zn^{2+} with high affinity and induces the inhibition of microtubule assembly.[6,10–12] However, the functions of S100 protein have not been established in detail.

We report here that S100 protein inhibits Ca^{2+}-ATPase activity in porcine brain microtubule proteins, and the inhibition by S100 protein is reduced by chlorpromazine.

Materials and Methods

Materials——Diethylaminoethyl(DEAE)-Sephadex A-50, and Sephadex G-75 were purchased from Pharmacia, 2-(*N*-morpholino)ethanesulfonic acid (MES) from Boehringer, Chlorpromazine from Sigma and [γ-^{32}P]ATP from ICN Radiochemicals. Other chemicals used were of the highest quality commercially available.

Preparation of Proteins——S100 protein and calmodulin were purified from porcine brain by ammonium sulfate fractionation followed by column chromatography on DEAE-Sephadex A-50 and Sephadex G-75.[11,13] Microtubule proteins were prepared from porcine brain by three cycles of temperature-dependent assembly and disassembly as described previously.[14]

Assay for ATPase——ATPase activity was assayed by measuring the amount of inorganic phosphate released. In the standard assay of ATPase activity, the reaction mixture contained 60 mM MES–KOH (pH 6.5), 2 mM 2-mercaptoethanol, 12.5% (v/v) glycerol, 0.5 mM [γ-^{32}P]ATP (4—6 μCi/μmol) and 20 μg of microtubule proteins in a final volume of 50 μl. Reactions were initiated by the addition of substrate and stopped by the addition of 20 mM silicotungstic acid in 0.02 N H_2SO_4 after incubation for 30 min at 37 °C. The liberation of inorganic phosphate was

determined with a liquid scintillation spectrometer (Aloka, LSC-651).[15]

Protein Concentration——Protein was determined by the method of Lowry *et al.*[16] with bovine serum albumin as a standard.

Results

The effect of S100 protein on the ATPase activity of porcine brain microtubule proteins in the presence of 5 mM Ca^{2+} or 2 mM Mg^{2+} is shown in Fig. 1. Ca^{2+}-ATPase activity was remarkably decreased with increasing concentration of S100 protein. The activity was only about 10% of the original level at over 0.8 mg/ml S100 protein. On the other hand, in the presence of Mg^{2+} the ATPase activity was decreased to a lesser extent by adding S100 protein. The concentrations of S100 protein required for half-maximal inhibition were 0.31 mg/ml in the presence of Ca^{2+} and 0.41 mg/ml in the presence of Mg^{2+}.

Calmodulin is a Ca^{2+}-binding protein which is evolutionarily related to S100 protein and is present in large amounts in the brain.[16] When porcine brain calmodulin (0—0.7 mg/ml) was added to the reaction mixture instead of S100 protein, calmodulin had no effect on the Ca^{2+}-ATPase activity of microtubule proteins (data not shown).

Figure 2 illustrates the dependence of the ATPase activity of microtubule proteins on Ca^{2+} concentration. ATPase activity without S100 protein was slightly stimulated by the addition of Ca^{2+}. When S100 protein (0.45 mg/ml) was present, the ATPase activity decreased with increasing Ca^{2+} concentration, showing a plateau level at over 2 mM Ca^{2+}. These results suggest that high concentrations of Ca^{2+} are required for effective ATPase inhibition by S100 protein.

Zn^{2+} has been shown to bind both S100 protein and microtubule proteins.[10-12,17] We therefore examined the effect of Zn^{2+} on the ATPase activity (Table I). In the absence of Ca^{2+} and S100 protein, the ATPase activity was found to be inhibited slightly by adding Zn^{2+} (S100 protein also did not effectively inhibit the ATPase activity in the absence of Ca^{2+}). In the presence of 5 mM Ca^{2+}, addition of Zn^{2+} did inhibit the ATPase activity, but the extent of

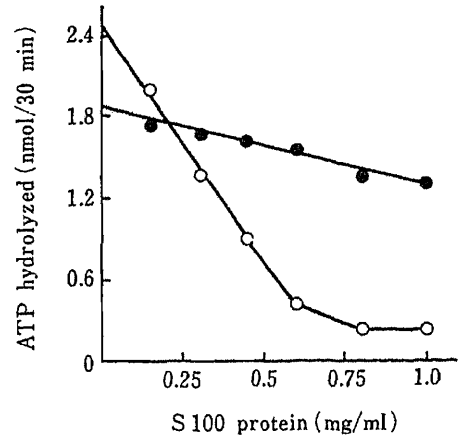

Fig. 1. Effect of S100 Protein on the ATPase Activity in Microtubule Proteins

ATPase activity was measured as described in Materials and Methods, in media containing 60 mM MES-KOH (pH 6.5), 2 mM 2-mercaptoethanol, 12.5% glycerol and 0.5 mM [γ-^{32}P]ATP, made up to contain the indicated concentration of S100 protein. Results each represent the average of three individual experiments. The concentration of microtubule proteins was 0.4 mg/ml. ○, 5 mM $CaCl_2$; ●, 2 mM $Mg(CH_3COO)_2$.

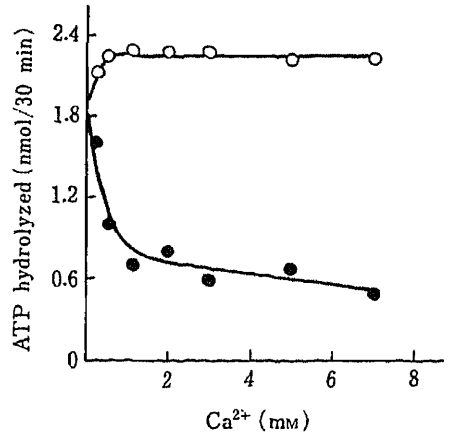

Fig. 2. Dependence on Ca^{2+} Concentration of ATPase Activity in the Presence or Absence of S100 Protein

Enzyme activity was measured under the standard conditions modified to the indicated concentrations of Ca^{2+}. The concentrations of microtubule proteins and S100 protein were 0.4 and 0.45 mg/ml (21 μM), respectively. ○, (−) S100 protein; ●, (+) S100 protein.

TABLE I. Effect of Zn^{2+} on ATPase Activity in the Presence or Absence of S100 Protein

Conditions	Zn^{2+} (mM)	(−)S100 nmol/30 min	(+)S100 nmol/30 min	Inhibition[a] (%)
(−)Ca^{2+}	0	1.74	1.24	29
	0.1	1.52	1.28	16
	0.2	1.48	1.26	15
	0.4	1.38	1.30	6
(+)Ca^{2+}	0	3.00	0.74	75
	0.1	1.50	0.56	63
	0.2	1.14	0.30	74
	0.4	1.02	0.22	78

Experimental conditions were the same as for Fig. 1 except that the concentration of S100 protein was 0.45 mg/ml. *a*) "Inhibition" is the percent inhibition caused by S100 protein with respect to the ATPase activity under the same conditions except for the absence of S100 protein.

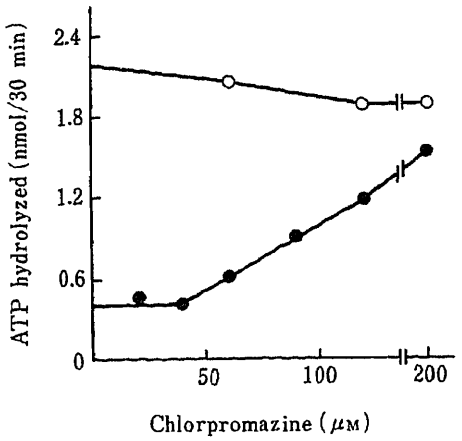

Fig. 3. Effect of Chlorpromazine on Ca^{2+}-ATPase Activity in the Presence or Absence of S100 Protein

Experimental conditions were the same as for Fig. 2 except that the concentration of Ca^{2+} was 5 mM and that of chlorpromazine was varied as indicated. ○, (−) S100 protein; ●, (+) S100 protein.

inhibition by S100 protein was high and almost constant, irrespective of Zn^{2+} concentration.

Chlorpromazine reduced the inhibitory effect of S100 protein in a dose-dependent manner under conditions where the ATPase activity was inhibited about 80% by S100 protein (Fig. 3). In the absence of S100 protein, the drug had little effect on the ATPase activity. The inhibition by S100 protein was markedly reduced by over 5 mol of chlorpromazine per mole of S100 protein. This value is about 5 times the molar ratio of chlorpromazine bound to S100 protein reported by Donato.[18,19]

Discussion

The present study is the first to find that S100 protein inhibits the ATPase activity contained in brain microtubule proteins. The ATPase activity has been shown to be activated by non-physiologically high concentrations of Ca^{2+}.[15,20] Potent inhibition by S100 protein was found at higher concentrations of Ca^{2+} (over 1 mM) (Fig. 2), while S100 protein had little effect on the activity at lower Ca^{2+} concentrations. The inhibition of microtubule assembly by S100 protein, on the other hand, was observed when the concentration of Ca^{2+} was 10^{-5}—10^{-4} M.[5-7] The binding affinity of S100 protein for Ca^{2+} has been calculated to be 0.1—2×10^{-4} M.[19] Tubulin, a main component of microtubule proteins, also contains one high-affinity Ca^{2+} site (3.3×10^{-6} M) and 16 low-affinity sites (2.8×10^{-4} M).[21] Thus, the inhibitory effect of S100 protein may due to Ca^{2+} binding to tubulin as well as to S100 protein.

As shown previously,[6,11,19] S100 protein in the presence of Zn^{2+} inhibits microtubule

assembly. However, Zn^{2+} has no effect on the inhibition of microtubule ATPase activity by S100 protein (Table I). The molar ratio of S100 protein to tubulin required for half-maximal inhibition is about 5, assuming that the content of tubulin in microtubule proteins is 75% (Fig. 1). On the other hand, we have reported that the molar ratio required to suppress the microtubule assembly is about 2 at 0.2 mM Ca^{2+}.[7] In addition, Ca^{2+} is one of the most potent inhibitors of microtubule assembly at high concentrations.[22] These results suggest that S100 protein has different effects on ATPase of microtubule proteins from those on the assembly.

It has been reported that chlorpromazine, an antipsychotic drug belonging to the phenothiazine group, binds to S100 protein.[18,19,23] S100 protein can interact with phenothiazine-conjugated Sepharose 4B in a Ca^{2+}-dependent fashion and the chlorpromazine binding results in a conformational change of S100 protein. Although the molar ratio of chlorpromazine bound to S100 protein has been reported to be about 1,[18,19,23] low concentrations of chlorpromazine showed little effect on ATPase activity in the absence or presence of S100 protein under the conditions employed in the present study (Fig. 3). High concentrations of chlorpromazine were required to reverse the S100 protein-dependent inhibition of ATPase activity, indicating that low-affinity binding sites for chlorpromazine may be present on S100 protein.

Like S100 protein, calmodulin has been demonstrated to modulate the assembly of microtubule proteins in a Ca^{2+}-dependent fashion,[24,25] but it has no effect on the ATPase activity. Thus, S100 protein may have a regulatory function on ATPase in microtubules.

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References

1) P. Dustin, "Microtubules," 2nd ed., Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, 1984.
2) V. I. Gelfand, F. K. Gyoeva, V. A. Rosenblat, and N. A. Shanina, *FEBS Lett.*, **88**, 197 (1978).
3) K. Prus and M. Wallin, *Histochemistry*, **78**, 181 (1983).
4) H. D. White, B. A. Coughlin, and D. L. Purich, *J. Biol. Chem.*, **255**, 486 (1980).
5) T. Endo and H. Hidaka, *FEBS Lett.*, **161**, 235 (1983).
6) R. Donato, T. Isobe, and T. Okuyama, *FEBS Lett.*, **186**, 65 (1985).
7) T. Fujii, N. Gocho, V. Akabane, Y. Kondo, T. Suzuki, and K. Ohki, *Chem. Pharm. Bull.*, **34**, 2261 (1986).
8) J. Patel and P. J. Marangos, *Biophys. Res. Commun.*, **109**, 1089 (1982).
9) J. Patel, P. J. Marangos, W. E. Heydorn, G. Chang, A. Verma, and D. Jacobowitz, *J. Neurochem.*, **41**, 1040 (1983).
10) J. Baudier, N. Glasser, K. Haglid, and D. Gerard, *Biochim. Biophys. Acta*, **790**, 164 (1984).
11) T. Fujii, N. Gocho, Y. Akabane, M. Fujii, Y. Kondo, T. Suzuki, and K. Ohki, *Chem. Pharm. Bull.*, **34**, 5040 (1986).
12) J. Bandier, N. Glasser, and D. Gerard, *J. Biol. Chem.*, **261**, 8192 (1986).
13) N. Ishioka, T. Isobe, T. Okuyama, Y. Numata, and H. Wada, *Biochim. Biophys. Acta*, **625**, 281 (1980).
14) T. Fujii, Y. Kondo, M. Kumasaka, and K. Ohki, *J. Neurochem.*, **39**, 1587 (1982).
15) Y. Ihara, T. Fujii, T. Arai, R. Tanaka, and Y. Kaziro, *J. Biochem.* (Tokyo), **86**, 587 (1979).
16) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
17) V. D. Lee, R. H. Himes, and L. Wilson, *J. Mol. Biol.*, **171**, 457 (1983).
18) R. Donato, *J. Neurochem.*, **42**, 1468 (1984).
19) R. Donato, *Cell Calcium*, **7**, 123 (1986).
20) B. C. Webb, *Arch. Biochem. Biophys.*, **198**, 296 (1979).
21) F. Solomon, *Biochemistry*, **16**, 358 (1977).
22) L. Karr, D. Kristofferson, and D. L. Purich, *J. Biol. Chem.*, **255**, 11853 (1980).
23) D. R. Marshak, T. J. Lukas, and D. M. Watterson, *Biochemistry*, **24**, 144 (1985).
24) J. Marcum, J. R. Dedman, B. R. Brinkley, and A. R. Means, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 3771 (1978).
25) S. A. Berkowita and J. Wolf, *J. Biol. Chem.*, **256**, 11216 (1981).

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The Effect of Di-(2-ethylhexyl)phthalate, a Chemical Leached from Blood Bags, on Platelet Adenosine Diphosphate Aggregability

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Recovery of adenosine diphosphate aggregability of stored platelets took place after incubation at 37 °C with Tyrode/bovine serum albumin solution as well as with fresh plasma. However, recovery with plasma from platelet concentrate (PC) stored in ordinary polyvinyl chloride bags was less, and the extent of the decrease depended upon the length of storage. The level of recovery with stored PC plasma, containing leached di-(2-ethylhexyl)phthalate (DEHP), was the same as that of fresh plasma to which DEHP had been added at the same concentration as found in the stored PC plasma. These results indicate that DEHP leached into the plasma of stored PC suppresses aggregability recovery. This suppression by DEHP is reversible.

Keywords——platelet; blood bag; DEHP; aggregability; polyvinyl chloride; storage; ADP

Introduction

Platelets in platelet concentrate (PC) stored at room temperature progressively lose their function. However, after transfusion of stored platelets to thrombocytopenic recipients, recovery of platelet glycogen and capacity for aggregation with adenosine diphosphate (ADP) occur within 24 h.[1] Recent studies showed that stored platelets, with decreased ability to function, recoverd platelet function *in vitro* after incubation at 37 °C with fresh plasma.[2,3] Although the recovery mechanism is still unclear, Watanabe *et al.* suggested that the change in calcium ion distribution plays a part.[4] They also suggested the possible existence of an unstable factor(s) since stored platelets did not regain aggregability as effectively after incubation with platelet-deprived plasma from stored PC as with fresh plasma. In this report we examine the relation between this *in vitro* aggregability recovery of stored platelets and the plasticizer di-(2-ethylhexyl) phthalate (DEHP) eluted from storage bags.

Methods

Preparation of Fresh Plasma and Stored PC Plasma——Separate pools from 5 bags of platelet-deprived fresh plasma (fresh plasma) and 5 bags of platelet-deprived plasma from stored PCs (stored PC plasma) were made after centrifugation at 16000*g* for 20 min.[3] The pH of the plasma was adjusted to 7.35. PCs were stored in polyvinyl chloride (PVC) bags (Teruflex, Terumo, Tokyo) or PVC bags after glow discharge treatment (PVC-G bags, TT 200C, Toray, Tokyo)[2] with horizontal agitation.[5]

Platelet Functional Recovery Experiments——Platelets were prepared from donor blood.[5] One milliliter of PC stored for 1 d in PVC bags with horizontal rotation (5 cm diameter, 30 rpm, at 22 °C) was mixed with 2 ml of fresh plasma, or 2 ml of stored PC plasma, or 2 ml of Tyrode solution containing bovine serum albumin (Tyrode/BSA),[6] or 2 ml of fresh plasma with various concentrations of DEHP (Wako, Osaka) in methanol. The mixtures were each incubated at 37 °C for 90 min. Before incubation, 0.1 μM prostaglandin E_1 (PGE_1, Nakarai, Kyoto) and 100 μg protein/ml apyrase, prepared from potato,[7] were added. In measuring the effects of DEHP during PC storage on platelet aggregability, 0.5 ml of PCs stored for 3 d in PVC bags or in PVC-G bags with horizontal rotation, were

mixed with 4.5 ml of fresh plasma and incubated. After incubation, the platelets were washed once,[6] and resuspended to $3 \times 10^5/\mu l$ in Tyrode/BSA with 2 μg protein/ml apyrase. Aggregation of platelets by 50 μM ADP (Grade I, Sigma, St. Louis, MO) with 0.2 mg/ml fibrinogen (Fraction L, Kabi Diagnostica, Stockholm) was measured 4 min after resuspension by a turbidimetric technique[8] using a four-channel aggregometer (Hema Tracer 1, model PAT-4A, Niko Bioscience, Tokyo). Aggregability of platelets before recovery was measured without incubation using washed platelets.

Measurement of DEHP Content in Plasma——DEHP was extracted from plasma with methanol and chloroform (1 : 2 mixture), and the extract was applied to the top of a silicic acid column, and eluted with *n*-hexane and ether (96 : 4). The DEHP fraction was determined by gas chromatography (model K-53, Hitachi, Tokyo).[9]

Results

Platelet functional recovery after incubation with fresh plasma (in terms of aggregability induced by ADP) increased from 13 ± 10% before incubation to 36 ± 13% after incubation ($n=12$). This level of recovery was also observed after incubation with Tyrode/BSA (34 ± 13%, $n=12$). The differences between fresh plasma and without incubation, and between Tyrode/BSA and without incubation are both significant as evaluated by the paired t test ($p<0.001$). However, the recovery of aggregability after incubation with stored PC plasma was suppressed (Fig. 1). The decrease of recovery depended on the period of storage of PC in PVC bags. The level of recovery on incubation with PVC-G PC plasma stored for 7 d was the same as that from fresh plasma. The difference between 7-d PVC and 7-d PVC-G was significant ($p<0.001$). PC storage conditions with both types of storage bags were similar except for the amount of DEHP leakage. The pH of PC after storage in both types of bags was similar (6.82 ± 0.28 with PVC, 6.83 ± 0.38 with PVC-G, 5 d, $n=8$).

The levels of the plasticizer DEHP in plasma leached during PC storage in PVC or PVC-G bags were measured. While that of PVC-G bags was low (0.12 mg/ml) after 7 d of storage, that of PVC bags was higher (0.35 mg/ml after 3 d, 0.58 mg/ml after 5 d and 0.75 mg/ml after 7 d). Accordingly, platelets were incubated with fresh plasma to which DEHP had been added (Fig. 2). Platelet functional recovery was suppressed depending on the concentration of added DEHP.

Aggregability and recovery after incubation with fresh plasma were observed using platelets stored for 3 d in PVC bags and PVC-G bags. Aggregability before incubation was 4 ± 2% with platelets stored in PVC bags and 10 ± 7% in PVC-G bags ($n=9$). The difference

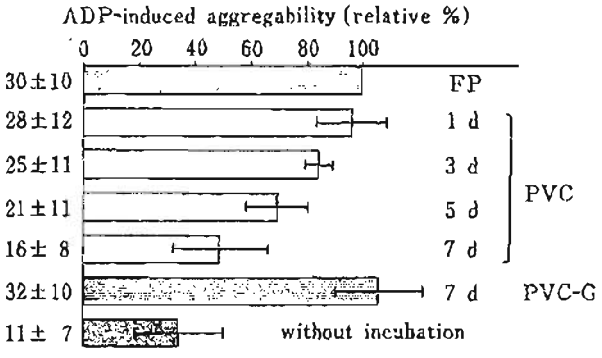

Fig. 1. Effect of PC Storage Period on Recovery of Platelet Aggregability

Platelets were incubated with fresh plasma (FP) or platelet-deprived plasma from PC stored in PVC bags or PVC-G bags. The number shown in the left side of each column is the absolute value of maximum aggregation (mean ± 1 S.D.; FP and without incubation, $n=13$; 1—5 d PVC, $n=4$; 7 d PVC or PVC-G, $n=9$).

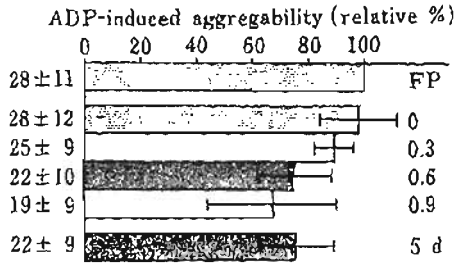

Fig. 2. DEHP Content and Recovery of Platelet Aggregability

Fresh plasma with added DEHP was prepared by adding 1/100 volume of DEHP in methanol to fresh plasma. The numbers in the right side of the columns are DEHP concentrations (final concentration, mg/ml; 0 mg/ml is methanol only). The number in the left side of each column is the absolute value of maximum aggregation (mean ± 1 S.D., $n=10$).

was significant as determined by the paired t test ($p<0.01$). Incubation with fresh plasma led to almost the same aggregability levels ($18\pm6\%$ with PVC, $20\pm6\%$ with PVC-G, $n=9$, $0.05<p<0.1$).

Discussion

It was shown many years ago that plasticizers, especially DEHP, are leached from blood bags on storage of blood components in PVC bags.[10,11] It has also been reported that DEHP has chronic and acute toxicity.[12] Despite these facts, PVC bags containing DEHP have been used, because DEHP is metabolized and excreted quickly,[13] and because its concentration in stored blood is low enough not to cause human toxicity. However, it can not be asserted even now that it is a completely safe practice. For stored platelets, Valeri *et al.* reported that platelet aggregability *in vitro*, or platelet survival and bleeding time after transfusion are not affected by DEHP.[14] As they probably did not consider prolonged storage at room temperature, only low concentrations of DEHP (0.05—0.15 mg/ml) were used in their studies on ADP aggregability. Ishikawa *et al.* reported that platelet ADP aggregability[2] and % hypotonic shock response[15] are lowered by addition of DEHP during storage. Recently, Rock *et al.* reported that DEHP inhibits platelet phospholipase A_2.[16] The results of our present study indicate that rather high concentrations of DEHP are leached during storage in PVC bags, and this does affect platelet functions.

In this work, platelet aggregability was measured with washed cells, and these were not affected by their own PC plasma. The level of recovery depended on the storage period of the PC from which the platelet-deprived PC plasma was obtained. After incubation with Tyrode/BSA, aggregability recovered to the same level as when fresh plasma was used. Therefore, suppressed recovery when using stored PC plasma does not depend on the inactivation of a special factor(s) in fresh plasma, but rather on some suppressing factor which appears in PC plasma during storage. This suppressing factor was found to be DEHP, because recovery suppression did not occur on incubation with plasma from PC stored in the PVC-G bags, and because the level of recovery using stored PC plasma was the same as with fresh plasma to which DEHP had been added at the same level as the measured value in the stored PC plasma.

Aggregability of platelets in both types of storage bags decreased with storage time, but that of platelets stored without DEHP, in PVC-G bags, was higher. Incubation with fresh plasma led to similar aggregability levels. These results indicate that the action of DEHP on platelets is reversible, and DEHP can be removed by replacement of the plasma with plasma containing no DEHP.

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References

1) S. Murphy and F. H. Gardner, *J. Clin. Invest.*, **50**, 370 (1971).
2) Y. Ishikawa, K. Honda, S. Sasakawa, K. Hatada and H. Kobayashi, *Vox Sang.*, **45**, 68 (1983).
3) T. Mazda, M. Bannai and S. Sasakawa, *J. Jpn. Soc. Blood Transfusion*, **29**, 331 (1983).
4) E. Watanabe, K. Tanaka and S. Sasakawa, *Thromb. Res.*, **32**, 537 (1983).
5) M. Bannai, T. Mazda and S. Sasakawa, *Transfusion*, **25**, 57 (1985).
6) C. Legrand, V. Dubernard and J. Caen, *Eur. J. Biochem.*, **142**, 465 (1984).
7) R. L. Kinlough-Rathbone, M. A. Packham and F. F. Mustard, "Methods in Hematology 8: Measurements of Platelet Function." ed. by L. A. Harker and T. S. Zimmerman, Churchill Livingstone, 1983, pp. 64—91.
8) G. V. R. Born, *Nature* (London), **194**, 927 (1962).
9) S. Sasakawa and Y. Mitomi, *Vox Sang.*, **34**, 81 (1978).

10) Y. L. Marcel and S. P. Noel, *Lancet*, i, 35 (1970).
11) R. J. Jaeger and R. J. Rubin, *Transfusion*, **13**, 107 (1973).
12) J. A. Thomas, T. D. Darby, R. F. Wallin, P. J. Garvin and L. Martis, *Toxicol. Appl. Pharmacol.*, **45**, 1 (1978).
13) R. J. Rubin and C. A. Shiffer, *Transfusion*, **16**, 330 (1976).
14) C. R. Valeri, T. J. Contreras, H. Feingold, R. H. Sheibley and R. J. Jaeger, *Environ. Health Perspect.*, Experimental Issue No. 3, 103 (1973).
15) Y. Ishikawa and S. Sasakawa, *Vox Sang.*, **47**, 330 (1984).
16) G. Rock, R. S. Labow and M. Tocchi, *Environ. Health Perspect.*, **65**, 309 (1986).

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Mutarotation of D-Glucose in Body Fluids and Perfused Rat Liver

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Mutarotation of D-glucose α- and β-anomers in some human body fluids, physiological salt solutions, and perfused rat liver was measured by the β-D-glucose oxidase-mutarotase method or by polarimetry. The mutarotational half-lives of α-D-glucose at 20 °C (37 °C) were as follows: whole blood, 9.0 min (2.3 min); serum, 10.6 min (2.5 min); saliva, 22.4 min (4.5 min); urine, 17.9 min (4.3 min); and distilled water, 41.0 min (9.8 min). It seems that the rapid mutarotation of D-glucose in blood may be due to the presence of mutarotase, phosphate ions, and bicarbonate ions. The mutarotation of α-D-glucose infused into isolated rat liver, warmed at 37 °C, was rapid ($t_{1/2}$ = 7.2 min), probably due to the presence of mutarotase in the liver, compared with that in the control system without liver ($t_{1/2}$ = 50.3 min). The anomeric composition of D-glucose released from the perfused rat liver was almost at equilibrium.

Keywords——α-D-glucose; β-D-glucose; mutarotase; D-glucose anomer mutarotation; blood; saliva; serum; urine; perfused liver

Introduction

D-Glucose in the crystalline state is well known to exist in either the α or β anomeric form. After either of these anomers is dissolved in aqueous solution, there is a mutarotation into an equilibrium state in which 63.5% is in the β form, 36.5% is in the α form, and 0.003% is in the so-called aldehyde form.[1,2] Many reports have recently appeared on the anomeric specificity of D-glucose metabolism[3,4] and D-glucose anomeric recognition[4-6] by tissues of higher animals.

The mutarotation of D-glucose anomers in pure water[1,7] and some salt solutions [8,9] has been studied by polarimetry. In body fluids, the mutarotation of the two anomers of D-glucose was supposed to be accelerated by the presence of mutarotase (aldose l-epimerase, EC 5.1.3.3), phosphate ions, *etc.* However, no report has heretofore been published on the mutarotation of D-glucose anomers in serum and blood. Thus we attempted to determine the mutarotational half-lives of D-glucose in normal human body fluids, such as blood, serum, saliva, and urine by our enzymatic method. We also determined by polarimetry the mutarotation of α-D-glucose in several physiological salt solutions which are frequently used in the physiological study of D-glucose anomers.

On the other hand, the mutarotation of D-glucose anomers in the organs has been studied by a few investigators. Hill *et al.* reported that among the organs rich in mutarotase activity, the liver of the anesthetized dog caused great changes in the relative D-glucose anomer content of the blood flowing through it.[10] Keston *et al.* reported that when α- or β-D-glucose was infused into the renal artery of the dog, the D-glucose appearing in urine due to the renal threshold being exceeded was not substantially mutarotated, but the D-glucose in the renal vein blood plasma had been subjected to catalyzed mutarotation.[11] However, their investigations were done under conditions influenced by blood, in which D-glucose anomers might have mutarotated very rapidly. Thus, we reevaluated the mutarotation of D-glucose

anomers in perfused rat liver by using a system with modified Ringer's solution to eliminate blood-related variables. The results are described in this paper.

Experimental

Materials——Normal human blood, serum, saliva, and urine samples were obtained from healthy adults. To prepare high-and low-molecular-weight fractions of human serum, normal human serum was fractionated by centrifugation at $600 \times g$ for 15 min using ultrafiltration membrane cones (Centriflo CF 25, Amicon Corporation, Lexington, U.S.A). The ultrafiltrate of serum contained low-molecular-weight substances (MW below 25000) including ions. The unfiltered substances (MW above 25000) were diluted with Tris–HCl buffer (20 mM, pH 7.4) to the initial volume of the serum, and were used as the high-molecular-weight fraction.

Water used in this experiment was distilled before use. The compositions of physiological buffers used in the experiment were as follows: Gamble's solution (119 mM NaCl, 2.5 mM $CaCl_2$, 0.8 mM $MgSO_4$, 50 mM $KHCO_3$, 21.0 mM Na_2HPO_4, pH 7.4); Ringer's solution (147 mM NaCl, 4.0 mM KCl, 2.3 mM $CaCl_2$, pH 7.5); Krebs–Ringer bicarbonate solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, pH 7.4); Krebs–Ringer phosphate solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 16 mM Na_2HPO_4, pH 7.4).

Improved Recrystallization Method of α- and β-D-Glucose[12]——α-D-Glucose: α-, β-, or equilibrated D-glucose (100 mg) was dissolved in 80% acetic acid (170 μl) and heated in a water bath at 90 °C until the crystals had dissolved completely. The solution was cooled to room temperature (20±2 °C) for 2—3 d. The crystals obtained were washed with cold ethyl alcohol (6 ml) and dried at 80 °C until the odor of acetic acid had disappeared. Yield, 63—70 mg, purity, over 98%.

β-D-Glucose: α-, β-, or equilibrated D-glucose (100 mg) was dissolved in anhydrous pyridine (900 μl) and heated in an oil bath at 100 °C until the crystals had dissolved completely. The solution was evaporated under reduced pressure and the resulting crystals were kept at room temperature (20±2 °C) for 2—3 d. The crystals were first washed with cold ethyl alcohol (2 ml), and then with cold ethyl ether (4 ml), and dried at 50 °C under reduced pressure until the odor of pyridine had disappeared. Yield, 80—90 mg, purity, over 98%.

The anomeric purities of α- and β-D-glucose were checked with a digital polarimeter (DIP 181, JASCO, Tokyo).

Determination of Mutarotational Half-Lives of α-D-Glucose in Buffers and Salt Solutions——The changes in mutarotation of α-D-glucose in various buffers and salt solutions were measured with a polarimeter. The half-lives of mutarotation of α-D-glucose in these solutions were estimated from semilogarithmic plots of the rotation by the method of Nelson and Beegle.[7]

We also measured the half-lives of mutarotation of β-D-glucose in some buffers, as well as of α-D-glucose, and similar data were obtained. Therefore, we measured the half-lives in body fluids and physiological salt solutions by using mainly α-D-glucose.

Determination of Mutarotational Half-Lives of α-D-Glucose in Body Fluids——For determination of mutarotational half-lives of α-D-glucose in blood, serum, saliva, and urine, the anomeric compositions of D-glucose in these body fluids were determined by our enzymatic method[13] with β-D-glucose oxidase (β-D-glucose: oxygen oxidoreductase, EC 1.1.3.4), mutarotase, and an oxygen analyzer (model 777, Beckman Instruments Inc., Fullerton, U.S.A.). α-D-Glucose (2 mg) was dissolved in the sample fluids (1 ml) and each mixture was incubated at 20 or 37 °C. Then a 15 μl aliquot was taken for analysis of the anomeric compositions of D-glucose at 5 min intervals until the anomers were almost fully equilibrated. In the case of blood, we used potassium ferricyanide to release oxygen from oxyhemoglobin in blood samples, and the anomers of D-glucose were determined by another of our enzymatic methods[14] with β-D-glucose oxidase and mutarotase.

After measurements of the anomeric compositions, the half-lives of mutarotation of α-D-glucose in these body fluids were calculated from semilogarithmic plots of the decrease of α-D-glucose.

Perfusion of Rat Liver——The liver perfusion methods reported by Sugano *et al.*[15] and us[6] were modified and used in this experiment. The isolated rat liver was perfused from a Teflon cannula inserted into the portal vein; the perfusion medium was a modified Ringer's solution: 154 mM NaCl, 1.8 mM $CaCl_2$, 2.7 mM $MgCl_2$, 2.0 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.2, saturated with 95% O_2+5% CO_2. The solutions of D-glucose anomers were cooled in the reservoir with ice in order to prevent spontaneous mutarotation as much as possible. The perfusion medium and D-glucose solution were warmed at 37 °C by passage through a stainless steel coil immersed in warm water just before their infusion into the liver. A peristaltic pump was used for perfusion, and the rate of flow was kept at about 37 ml/min. The mean oxygen consumption by a perfused rat liver was 0.93 mol O_2/min/g of wet liver. The bile production by the perfused rat liver was also measured and the mean flow rate was 16 μl/h/g of wet liver. For the determination of anomeric composition of D-glucose released from the perfused liver, perfusion was carried out in the flow-through mode. For study of the mutarotation of D-glucose anomers in the perfused liver, the recycle mode was used, in which the perfusate from the liver was returned to the initial reservoir cooled at about 4 °C with ice.

The liver was first perfused for 20 min with perfusion medium containing equilibrated D-glucose solution (120 mg/100 ml). Then, fresh α-D-glucose solution, fresh β-D-glucose solution (500 mg/100 ml) or the D-glucose-free perfusion medium, was introduced into the perfused liver by changing a three-way cock. Aliquots of the perfusate from the liver were collected at 2-min intervals for 8 min, and immediately frozen in a dry ice–acetone mixture. These samples were stocked at −70 °C for the determinations of anomeric composition and amount of D-glucose in the perfusate.

Results and Discussion

Mutarotation of α-D-Glucose in Body Fluids and Physiological Solutions

The mutarotational half-life of α-D-glucose in purified water at 20 or 37 °C was quite slow (41.0 or 9.8 min, respectively), and these values agree fairly well with those of previous reports (45 min at 20 °C,[8,9] 10 min at 37 °C[7]). The half-life of α-D-glucose in normal whole blood was 2.3 min at 37 °C. This value in blood is very low in comparison with the value (9.8 min) in water, as shown in Table I.

Bailey *et al.*[16] reported that the mutarotational half-life of α-D-glucose under physiological conditions was 7.1 min at 37 °C, although the conditions used for measurement of mutarotation were not described in detail. However, our results indicate that the mutarotational half-lives of α-D-glucose in normal human whole blood and serum are very small, 2.3 and 2.5 min respectively, at 37 °C, as shown in Table I.

The mutarotation of α-D-glucose in normal human saliva progressed slowly, and the half-lives at 20 and 37 °C were 22.4 and 4.5 min, respectively (Table I). Those in urine at 20 and

TABLE I. Mutarotational Half-Lives ($t_{1/2}$) of α-D-Glucose in Normal Human Body Fluids

Body fluid	$t_{1/2}$ (min)	
	20 °C	37 °C
Distilled water	41.0 ± 3.2[a]	9.8 ± 0.3[a]
Whole blood	9.0 ± 1.3[a]	2.3 ± 0.5[a]
Serum	10.6 ± 0.8[a]	2.5 ± 0.3[a]
Saliva	22.4 ± 8.2[a]	4.5 ± 0.2[a]
Urine	17.9 ± 1.7[a]	4.3 ± 0.5[b]

a) Values are means ± S.D. for 10 subjects. *b*) Values are means ± S.D. for 5 subjects. Final concentration of α-D-glucose: 2 mg/ml.

TABLE II. Mutarotational Half-Lives ($t_{1/2}$) of α-D-Glucose in Various Buffers and Physiological Salt Solutions

Buffer	$t_{1/2}$ (min)	
	20 °C	37 °C
20 mM EDTA buffer, pH 6.0	40.4	9.0
20 mM Tris–HCl buffer, pH 7.4	36.9	8.0
20 mM Phosphate buffer, pH 6.0	26.1	6.0
20 mM Phosphate buffer pH 7.4	10.5	2.5
Gamble's solution (pH 7.4)	21.2	4.0
Ringer's solution (pH 7.5)	41.3	10.0
Krebs–Ringer bicarbonate solution (pH 7.4)	20.6	2.8
Krebs–Ringer phosphate solution (pH 7.4)	12.9	2.5

Values are means for 3 experiments. Final concentration of α-D-glucose: 2 mg/ml. EDTA = ethylenediaminetetraacetic acid.

37 °C were 17.9 and 4.3 min.

We also investigated the mutarotation of α-D-glucose in 20 mM Tris–HCl buffer (pH 7.4) containing human serum albumin (4.2 g/100 ml) or human serum γ-globulin (4.7 g/100 ml), and these mutarotational half-lives were determined to be 7.0 min at 37 °C. The half-lives of mutarotation of α-D-glucose in these protein solutions were larger than those in blood and serum. The half-life of mutarotation of α-D-glucose in the low-molecular-weight fraction of serum (2.4 min) was almost the same as those in blood and serum, and that in the high-molecular fraction of serum (MW above 25000) was 3.7 min. These results indicate that the rapid spontaneous mutarotation of α-D-glucose in blood and serum is mainly caused by the low-molecular-weight compounds (MW below 25000), including ions, in it.

Table II shows the mutarotational half-lives of α-D-glucose in various buffers which are frequently used in biochemical studies. As shown in Table II, phosphate buffer (pH 6.0, 7.4) caused rapid mutarotation of α-D-glucose as compared with Tris–HCl buffer (pH 7.4) at the same molar concentration, and the half-life (6.0 min) of the mutarotation in phosphate buffer (pH 7.4) was consistent with that given in a previous report.[8]

We also investigated the mutarotation of α-D-glucose in various salt solutions (10 mM) at 37 °C. The chlorides of K^+, Na^+, and Mg^{2+} dissolved in 20 mM Tris–HCl buffer (pH 7.4) shortened the mutarotational half-life of α-D-glucose in comparison with that (8.0 min) in only 20 mM Tris–HCl buffer (pH 7.4) as follows: KCl, 6.5 min; NaCl, 7.0 min; and $MgCl_2$, 6.0 min. Calcium chloride, however, did not alter the half-life of the mutarotation. To compare the effects of anions on the mutarotation, we also measured the half-lives of mutarotation in the presence of potassium salts (20 mM) of HPO_4^{2-}, HCO_3^-, and SO_4^{2-}. The values were calculated to be 3.0, 6.0, and 8.0 min, respectively.

The half-life of mutarotation of D-glucose in Gamble's solution at 37 °C was calculated as 4.0 min, as shown in Table II. In Krebs–Ringer bicarbonate and Krebs–Ringer phosphate solutions, the α-D-glucose mutarotation was as rapid as that in the serum. However, the mutarotational half-life (10.0 min) of α-D-glucose in Ringer's solution at 37 °C was almost the same as that in distilled water.

Since human serum contains 1.35 mM phosphate ion and 25 mM bicarbonate ion, which accelerate the mutarotation of D-glucose,[8,9] the rapid mutarotation of α-D-glucose observed in blood and serum is undoubtedly due to the presence of phosphate and bicarbonate ions in them.

Mutarotation of α, β-D-Glucose in Perfused Rat Liver

Prior to the perfusion experiments with isolated rat liver, the mutarotation of D-glucose anomers in the perfusion medium at −70 °C and at 37 °C was measured. There was no mutarotation of α- or β-D-glucose at −70 °C over at least a 10 d period. Thus, the perfusates from the liver were frozen in a dry ice–acetone mixture and stocked at −70 °C prior to measurement. The mutarotational half-life of α-D-glucose in the perfusion medium at 37 °C

TABLE III. Mutarotational Half-Lives of D-Glucose Anomers in the Perfusion System at 37 °C

	$t_{1/2}$ (min)	
	α-D-Glucose	β-D-Glucose
Without liver	50.3	51.4
With liver[a]	7.2±0.1	7.6±0.4

a) Values are means±S.D. for 3 experiments.

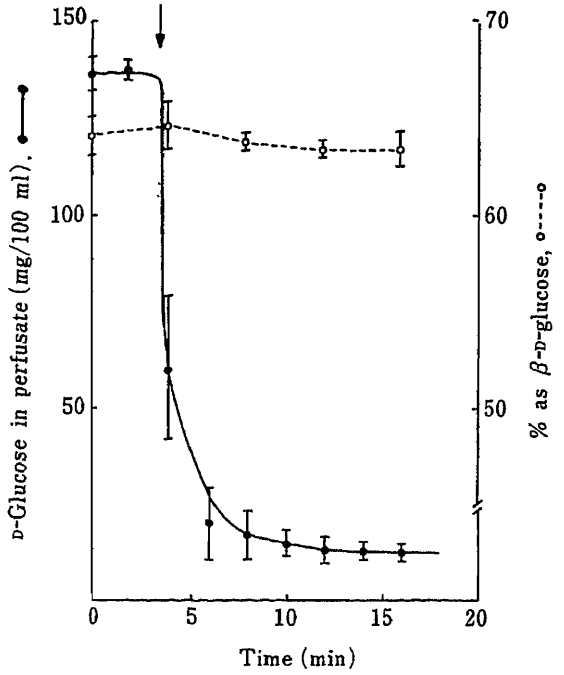

Fig. 1. The Concentration and the Anomeric Composition of D-Glucose Released from Perfused Rat Liver

Isolated rat liver (12.5±0.9 g, mean weight±S.D.) was perfused with modified Ringer's solution containing 120 mg/100 ml of D-glucose for about 20 min. Then the perfusion medium was replaced by the same medium without D-glucose. An arrow indicates the time of the medium change. The concentration (●) and the percent of D-glucose as β-D-glucose (○) in the perfusate were measured as described in Materials and Methods. Results are given as the means (±S.D.) of five experiments.

was calculated to be 9.2±0.7 min (mean±S.D., $n=5$). In the perfusion system in which the liver was replaced by a silicone tube (0.96 ml, mean volume of the vascular system of rat liver as determined by the method of Marklund[17]), a slow mutarotation of α- or β-D-glucose was observed at 37 °C: the half-lives of mutarotation of α- and β-D-glucose were calculated to be 50.3 min (α-D-glucose) and 51.4 min (β-D-glucose).

In contrast with the above results, the mutarotation of infused α- and β-D-glucose during perfusion with rat liver was considerably more rapid, as shown in Table III, with the half-lives being 7.2 min for α-D-glucose and 7.6 min for β-D-glucose. These values were about 7 times lower than those in the perfusion system using the silicone tube as described above. Since rat liver contains a large amount of mutarotase,[18] both α- and β-anomers of D-glucose entering the liver cells might be rapidly mutarotated by this enzyme.

The liver was first perfused with the medium containing D-glucose for 20 min; then perfusion was continued with medium lacking D-glucose. Within 8 min of the medium change, the concentration of D-glucose in the perfusate decreased from 135 mg/100 ml to 12 mg/100 ml (Fig. 1). The release of D-glucose from the perfused liver continued at least for 12 min. The anomeric composition of β-D-glucose in the perfusates from 4 to 12 min was about 63%, the equilibrium state. These phenomena can be explained by the presence of mutarotase in the liver and (or) the rapid spontaneous mutarotation of α- and β-D-glucose 6-phosphate as reported previously.[19] The result suggests that D-glucose released from the liver, probably by glycogenolysis and gluconeogenesis, is almost fully equilibrated.

Recently, the physiological roles of α- and β-D-glucose and the aldehyde form have become more clearly understood; *e.g.*, α-D-glucose is used as the signal for D-glucose sensing cells (Langerhans α-, β-cells and taste bud cells for sweetness of D-glucose), while β-D-glucose is used for energy production in many cells and organs in higher animals. Hence, the study of mutarotation of D-glucose anomers in body fluids, physiological salt solutions, and in organs is quite important for elucidation of the biochemical roles[4] of D-glucose anomers and the aldehyde form in higher animals.

References

1) W. Pigman and H. S. Isbell, "Advances in Carbohydrate Chemistry and Biochemistry," Vol. 23, ed. by M. L.

Wolfrom and R. S. Tipson, Academic Press, New York, 1968, pp. 11—57.
2) S. J. Angyal, "Advances in Carbohydrate Chemistry and Biochemistry," Vol. 42, ed. by R. S. Tipson and D. Horton, Academic Press, New York, 1984, pp. 15—68.
3) J. Okuda, *Trends Biochem. Sci.*, **3**, 161 (1978).
4) J. Okuda and I. Miwa, *J. Clin. Biochem. Nutr.*, **1**, 189 (1986).
5) T. Sakaguchi, T. Taguchi, and J. Okuda, *Biochem. Int.*, **7**, 299 (1983).
6) A. Niijima, A. Fukuda, T. Taguchi, and J. Okuda, *Am. J. Physiol.*, **244**, R611 (1983).
7) J. M. Nelson and F. M. Beegle, *J. Am. Chem. Soc.*, **41**, 559 (1919).
8) H. Murschhauser, *Biochem. Z.*, **110**, 181 (1920).
9) H. Murschhauser, *Biochem. Z.*, **136**, 66 (1923).
10) J. B. Hill, D. S. Cowart, T. U. Biber, and W. D. Huffines, *Biochem. Med.*, **1**, 62 (1967).
11) A. S. Keston, R. Brandt, and I. M. Barash, *Biochem. Biophys. Res. Commun.*, **46**, 610 (1972).
12) I. Miwa, J. Okuda, H. Niki, and A. Niki, *J. Biochem.* (Tokyo), **78**, 1109 (1975).
13) J. Okuda and I. Miwa, "Methods in Biochemical Analysis," Vol. 21, ed. by D. Glick, Interscience Pub., New York, 1973, p. 155.
14) J. Okuda and G. Okuda, *Biochem. Med.*, **7**, 257 (1973).
15) T. Sugano, K. Suda, M. Shimada, and N. Oshino, *J. Biochem.* (Tokyo), **83**, 995 (1978).
16) J. M. Bailey, P. H. Fishman, and P. G. Pentchev, *J. Biol. Chem.*, **243**, 4827 (1968).
17) S. Marklund, *Clin. Chim. Acta*, **92**, 229 (1979).
18) J. M. Bailey, P. G. Pentchev, and J. Woo, *Biochim. Biophys. Acta*, **94**, 124 (1965).
19) J. M. Bailey, P. H. Fishman, and P. G. Pentchev, *Biochemistry*, **9**, 1189 (1970).

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Degradation of Bromazepam by the Intestinal Microflora

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Degradation of four benzodiazepines, *i.e.* bromazepam, diazepam, desmethyldiazepam, and 7-bromo-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2(1*H*)-one, in a broth containing human feces was studied. Degradation of bromazepam was also investigated in a fecal medium, a homogenate of the liver and a homogenate of intestinal epithelial cells of rat. More than 80% of bromazepam, which contains a pyridine ring in its structure, was degraded in the broth containing 8% human feces under both anaerobic and aerobic conditions. The major degradation product of bromazepam was identified as 2-(2-amino-5-bromobenzoyl)pyridine by means of thin layer chromatography and gas chromatography-mass spectra analyses. About 70, 90, and 90% of bromazepam remained in the fecal medium, homogenate of the liver and homogenate of intestinal epithelial cells of rat, respectively, after incubation for 5 h at 37 °C.

Therefore, degradation of bromazepam by the intestinal microflora may be considered to be one of the major factors influencing the bioavailability after oral administration to man.

Keywords——bromazepam; degradation; fecal flora; gut flora; intestinal microflora; rat

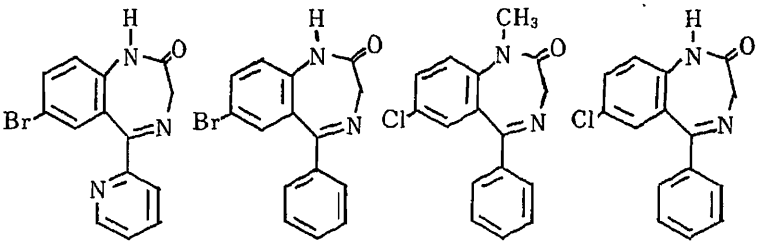
Recently, degradation of imipramine,[1] digoxin,[2,3] and nitroglycerin[4] by the intestinal microflora has been investigated in order to explain their low extents of bioavailability after oral administration to man. In the case of sulfinpyrazone,[5] the distal gut with its microflora is the principal and possibly the only site of reduction of sulfinpyrazone to its active form in humans, and metabolism of the drug by the gut flora is essential to its pharmacological response. Bromazepam, one of the 1,4-benzodiazepine antianxiety agents, has also been reported to exhibit low bioavailability after oral administration. Kasama *et al.*[6] reported that the area under the plasma concentration–time curve (*AUC*) value after rectal administration of 3 mg of bromazepam was equivalent to that after oral administration of 5 mg of bromazepam. The present study was undertaken to investigate the possible degradation of bromazepam in fresh human feces.

Experimental

Materials——Bromazepam was a gift from Eisai Co., Tokyo, and diazepam, desmethyldiazepam, and 7-bromo-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2(1*H*)-one (BDPB) were gifts from Nippon Roche Co., Tokyo. An incubation medium (shown in Table I) was purchased from Nissui Seiyaku, Tokyo. Chemicals used were of reagent grade, and were purchased from Wako Pure Chemical Industries, Osaka.

Degradation by the Intestinal Microflora——About 100 ml of the medium containing 8% (w/v) fresh feces, obtained from normal subjects who had not taken any antibiotic prior to the experiment, was centrifuged at 2000 rpm for 5 min. An 800-μl portion of bromazepam solution in dimethylformamide (DMF) (8 mg/ml) was added to 80 ml of the supernatant of the fecal medium and the mixture was incubated at 37 °C under both anaerobic (Gaspak®,BBL Microbiology Systems, Cackeysville, Md, U.S.A.) and aerobic conditions. A 2-ml portion of the fecal medium was collected at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 h and stored at −70 °C until analysis. Degradation of drugs in the sterilized 8% fecal medium was examined as a control. Degradation of drugs in a rat fecal medium was also studied similarly.

Determination of Undegraded Drugs——A sample (2 ml) was mixed with 3 ml of 0.2 M borate buffer, pH 8.0, and 5 ml of toluene containing prazepam as an internal standard. After being shaken for 10 min, the mixture was



bromazepam | 7-bromo-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2(1*H*)-one (BDPB) | diazepam | desmethyldiazepam

Chart 1. Structures of the Four Benzodiazepines

TABLE I. Components of the Medium (GAM Broth 'Nissui')[a]

Peptone	10.0	Potassium phosphate, monobasic	2.5
Soya peptone	3.0	Glucose	3.0
Proteose peptone	10.0	Sodium chloride	3.0
Digested blood powder	13.5	Soluble starch	5.0
Yeast extract	5.0	L-Cysteine HCl	0.3
Beef extract	2.2	Sodium thioglycolate	0.3
Liver extract powder	1.2		

a) g/l (total, 59.0 g).

centrifuged at 3000 rpm for 20 min and the organic phase was then evaporated *in vacuo* at 60 °C. The residue was dissolved in 50 μl of methanol and a 10-μl aliquot of the resultant solution was injected into the high performance liquid chromatography (HPLC) system (LC-4A, Shimadzu Manufacturing Co., Kyoto) equipped with a ultraviolet (UV) detector set at 233 nm. A 150 mm column of Shim-pack CLC-ODS (particle size 5 μm, Shimadzu Manufacturing Co.) was employed with a mobile phase of methanol : water : ammonium hydroxide = 270 : 230 : 3 at the flow rate of 1.0 ml/min at a column temperature of 50 °C.

Decrease in bromazepam concentration with time was expressed as the ratio of peak area of bromazepam/peak area of the internal standard at the sampling time divided by that at time zero.

Identification of the Degradation Product——An extract from fecal culture medium after incubation was spotted on a silica gel plate (Kieselgel 60 F_{254}, Kanto Chemical Co., Tokyo), developed in acetone : chloroform = 10 : 90 and visualized under UV light. The extract was also examined by gas chromatography-mass spectrum (GC-MS) analysis (QP-1000 GC-MS system, Shimadzu Manufacturing Co.). The GC part of the GC-MS system was equipped with a 1-m glass column packed with 3% OV-17 on Chromosorb Q. The flow rate of helium was 50 ml/min. The temperatures of the injection port, column, transfer line to the MS system, and ion source of the MS system were 275, 250, 280, and 280 °C, respectively. MS were obtained in an electron impact (EI) mode at an electron energy of 70 eV.

Metabolism in Homogenates of Liver and Intestinal Epithelial Cells in Rats——An 8-ml portion of 0.1 M Tris HCl buffer solution, pH 7.4 was added to 2 g each of the excised liver mass and intestinal epithelial cells of rat. Saline was introduced into the artery of the excised liver of a rat to remove blood, and the tissue was homogenized (Physcotron, Nichi-on Co., Chiba). Everted rat intestine was scraped with a slide glass to collect epithelial cells. Both mixtures were homogenized and centrifuged at 3000 rpm for 10 min. A 50-μl portion of bromazepam solution (2 mg/ml) in ethanol was added to 0.95 ml of the supernatant, and then the mixture was incubated at 37 °C. A 50-μl aliquot was collected after incubation for 0, 1, 2, 3, 4, and 5 h, and 100 μl of acetonitrile containing clonazepam as an internal standard (50 μg/ml) was immediately added to deproteinize the sample. Each mixture was centrifuged at 7000 rpm for 2 min. A 2-μl portion of each supernatant was injected into the HPLC system.

The HPLC conditions were identical to those given earlier in the section on determination of undegraded drugs. Total amounts of protein in the supernatant of homogenates of the rat liver and intestinal epithelial cells in this study were determined by the Lowry method.

Results and Discussion

Figure 1 shows the degradation profiles of four benzodiazepines in the broth containing

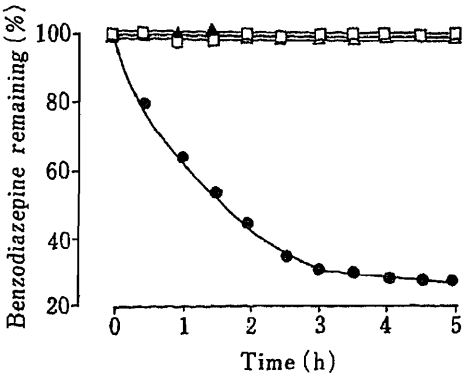


Fig. 1. Disappearance Patterns of Diazepam (□), Desmethyldiazepam (△), BDPB (▲) and Bromazepam (●) during Incubation with Human Fecal Flora

Average of three experiments.

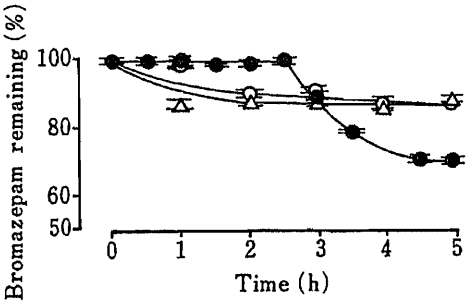


Fig. 2. Disappearance Patterns of Bromazepam during Incubation with a Homogenate of Liver (○), a Homogenate of Epithelial Cells of the Intestine (△), and Fecal Flora (●) in Rats

Average of three experiments.

8% human feces. Bromazepam was rapidly degraded in the broth at 37 °C, while the other three benzodiazepines were not degraded up to 5 h. About 80% of bromazepam was degraded within 5 h under both anaerobic and aerobic conditions, while it was not degraded in the sterilized fecal culture, indicating that degradation of bromazepam was not caused by the fecal contents themselves but by the gut microflora. The degradability of bromazepam may be related to the fact that this compound is the only one containing a pyridine ring, among those tested.

Disappearance profiles of bromazepam in homogenates of the liver, intestinal epithelial cells, and the fecal flora of rats are shown in Fig. 2. Total amounts of protein in the supernatant of homogenates of the liver and intestinal epithelial cells of rats were 29.4 and 22.5 mg/ml, respectively. Percentages of degradation of bromazepam by the liver and intestinal epithelial cells in rats were about 10% each, while that by rat feces was about 30% in 5 h. Degradation of bromazepam in homogenates of fecal cultures in rats was observed from 3 h after the initiation of incubation, presumably reflecting the time required for the growth of bacteria in a rat microfloral culture.

Kasama *et al.*[6] observed lower bioavailability of bromazepam after oral administration than after rectal administration. Fukushima *et al.*[7] reported that the plasma concentrations of bromazepam after rectal administration were higher than those after oral administration to 12 healthy volunteers. The *AUC* after rectal administration of bromazepam was about twice that after oral administration. Kasama *et al.*[6] also reported on the pharmacokinetics after oral administration of 5 mg of bromazepam and rectal administration of 3 mg of bromazepam to 7 healthy volunteers. The $AUC_{0-\infty}$ values after rectal administration of 3 mg were equivalent to those after oral administration of 5 mg, suggesting 60% relative bioavailability of bromazepam after oral administration of bromazepam with respect to bioavailability after rectal administration. Thus, one of the major reasons why low bioavailability was observed after oral administration of bromazepam is considered to be degradation of the drug by the intestinal microflora.

On a thin layer chromatogram, the *Rf* values for bromazepam and its degradation product were 0.04 and 0.64, respectively, and no other spot was detected by this method. A major metabolite of bromazepam separated by TLC was identical with authentic 2-(2-amino-5-bromobenzoyl) pyridine on GC-MS analysis, as shown in Fig. 3. Thus, the degradation of bromazepam in intestinal microflora may be represented by the scheme shown in Chart 2.

Reversible hydrolytic reactions of 1,4-benzodiazepines in acidic solutions at body

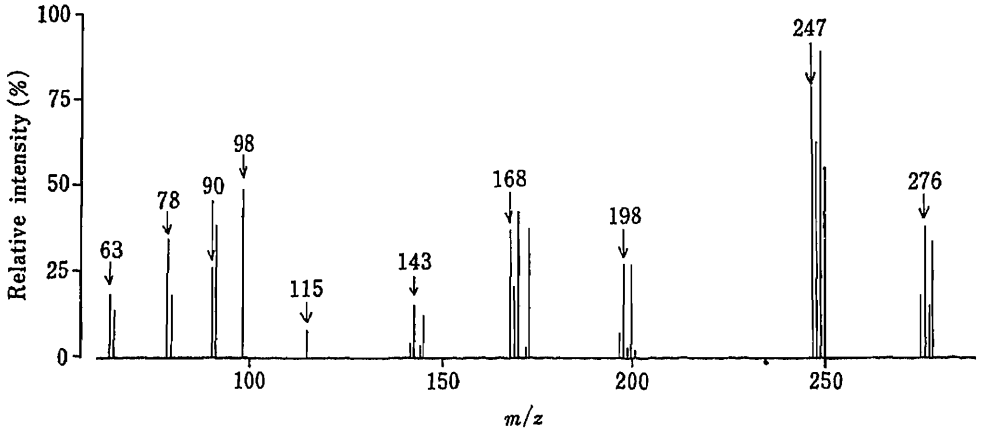


Fig. 3. Mass Spectrum of a Degradation Product of Bromazepam Formed by Incubation in a Human Fecal Medium

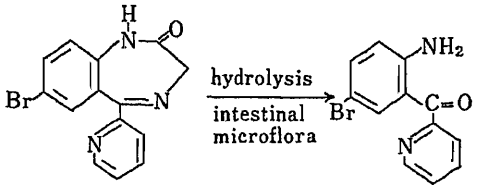


Chart 2. Hypothetical Scheme for the Degradation

temperature have been studied.[8-10] After oral administration of 1,4-benzodiazepines, the open-ring compounds would be produced from the parent closed-ring compounds. When the open-ring compounds enter the intestine, they are expected to revert to the parent 1,4-benzodiazepines due to the higher pH value of the medium. Therefore, these reversible reactions of most 1,4-benzodiazepines may not influence their oral bioavailability.[11] However, bromazepam was slowly but irreversibly degraded in the acidic media at 37 °C and decomposed by a two-step sequential reaction to 2-(2-amino-5-bromobenzoyl) pyridine and glycine.[12] Thus, the reason why lower bioavailability of bromazepam was obtained after oral administration than after rectal administration may be considered to be the degradation of bromazepam both by acid in the stomach and by intestinal microflora.

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References

1) A. W. Clark, R. T. Clinton, J. K. Baker, and C. D. Hufford, *J. Pharm. Sci.*, **72**, 1288 (1983).
2) Lindenbaum, D. G. Rund, V. P. Butler, Jr., D. Tse.-Eng, and J. R. Saha, *N. Engl. J. Med.*, **305**, 789 (1981).
3) J. O. Magunusson, B. Bergdabl, C. Bogentoft, S. Gustafsson, and U. E. Jonsson, *Eur. J. Clin. Pharmacol.*, **27**, 197 (1984).
4) M. S. A. Shamat and A. H. Beckett, *J. Pharm. Pharmacol.*, Suppl., 71P (1983).
5) H. A. Strong, J. Oates, J. Sembi, A. G. Renwick, and C. F. Georgel, *J. Pharmacol. Exp. Ther.*, **230**, 726 (1984).
6) T. Kasama, M. Hiura, M. Nakai, K. Sasaki, Y. Shinozaki, and H. Mizuta, *Kiso To Rinsho*, **17**, 1049 (1983).
7) K. Fukushima, T. Satou, T. Kasama, M. Hiura, M. Nakai, and Y. Shinozaki, *Masui*, **31**, 512 (1982).
8) M. Nakano, N. Inotsume, N. Kohri, and T. Arita, *Int. J. Pharmaceut.*, **3**, 195 (1979).
9) N. Inotsume and M. Nakano, *Chem. Pharm. Bull.*, **28**, 2536 (1980).
10) N. Inotsume and M. Nakano, *Int. J. Pharmaceut.*, **6**, 147 (1980).
11) N. Inotsume, J. Fujii, and M. Nakano, *Chem. Pharm. Bull.*, **34**, 937 (1986).
12) S. Inui, M. Yamamoto, H. Nakae, and S. Asada, *Yakuzaigaku*, **42**, 71 (1982).

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Rheological Studies on a Suspension of Water-Insoluble Glucan Produced by *Streptococcus mutans*

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Rheological properties of a suspensions of the water-insoluble glucan produced by *Streptococcus mutans* were investigated. Flow curves were obtained by using a rotational viscometer. The curve showed non-Newtonian and shear thinning behavior, and can be described by a power-law equation. A yield point was also observed but thioxotropic behavior was not seen. The rheological properties, *e.g.*, yield point and apparent viscosity, depended markedly on the concentration in the range of 6 to 10% glucan. However, these values of the suspension containing 10% glucan were little affected by temperature in the range of 10 to 50 °C and were not affected by pH in the range of 5 to 9.

Keywords——rheological property; water-insoluble glucan; *Streptococcus mutans*; flow curve; suspension; non-Newtonian behavior; shear-thinning behavior; power-law equation

We have reported on the pharmaceutical availability and physicochemical properties of the water-insoluble glucan produced by *Streptococcus mutans*.[1-4]

A directly compressed tablet containing lactose with the glucan at low concentration immediately disintegrated as a result of hydration and swelling of the glucan.[1] On the other hand, the tablet containing the glucan at high concentration showed a longer disintegration time.[2] The prolongation of the disintegration of the tablet containing the glucan at high concentration was supposed to be due to inhibition of permeation of the disintegration medium by the gel-like barrier of hydrated glucan. Therefore, we were interested in the physicochemical properties of the glucan gel. It has been reported that some polymers that form a gel at high concentration are useful as suspending agents at lower concentration.[5] Therefore, in the present study, in order to investigate the applicability of the glucan suspension as a suspending agent, we studied the rheological properties of suspensions containing the glucan at relatively low concentrations.

Experimental

Materials——Water-insoluble glucan was obtained by the same method as described in the previous paper.[3] All other chemicals used here were of analytical-reagent grade.

Apparatus——All the rheological data were obtained using a rotational viscometer, Haake Rotovisco RV 12, equipped with an M 150 or M 500 measuring head, PG 142 programmer and MV II sensor system (Haake Mess-Technik GmbH and Co.). The MV II sensor system had the following specifications: rotor radius, 18.4 mm; rotor length, 60 mm; gap width, 2.6 mm. An X-Y recorder, model RW-11 (Rikadenki Kogyo Co., Ltd., Tokyo, Japan), and circulator, model TE-105 (Toyo Seisakusho Co., Tokyo, Japan), were employed.

Preparation of Glucan Suspension——Glucan suspensions of various concentrations were prepared by mixing the glucan and water with a mortar and pestle, and left to stand overnight at 4 °C. All suspensions were prepared in terms of w/w percent. The pH was adjusted with diluted hydrochloric acid and sodium hydroxide solution.

Rheological Measurement——Glucan suspensions were introduced into the cup of the MV sensor system. Prior to measurement, the cup filled with the sample was incubated at the selected temperature for 10 min. Then, the sensor system was fitted to the measuring head equipped with a temperature controller. Rheological measurement was started after equilibration for 10 min. The range of the rheogram was 0—256 rpm, *i.e.*, 0—230.4 s^{-1}, and the program was 256 rpm·min^{-1}. Therefore, the overall rheogram was completed within 2 min. The shear stress was determined from the rheograms. When the M 150 and M 500 measuring heads were used, the full-scale values of shear stress were 113 and 376 Pa, respectively. The apparent viscosity at each shear rate was calculated from shear rate and shear stress by using the following equation:

$$\text{apparent viscosity} = \frac{\text{shear stress}}{\text{shear rate}} \times 1000 \text{ (cP)}$$

Results and Discussion

Description of Flow Curve

A typical flow curve obtained at 20 °C with a 10% glucan suspension and a plot of the data in terms of shear rate *versus* apparent viscosity are shown in Fig. 1. The flow curve shows that the ratio of shear stress to shear rate, *i.e.*, the viscosity, decreases continuously with increasing shear rate. Therefore, the glucan suspension is indicated to be of non-Newtonian and shear-thinning type. A yield point was observed, but thixotropic behavior was not seen. As already suggested in connection with the flow characteristics of polymers, *e.g.*, cellulose gum,[6] the shear-thinning behavior observed in this case should be due to unidirectional laminar motion of the polymer(glucan) molecules with shear.

Then, logarithmic values of shear rate as a function of the logarithmic shear stress were plotted. As shown in Fig. 2, these plots showed good linearity ($r=0.995$). Therefore, the flow curves can be described by a power-law equation[7]:

$$D = KS^n$$

$$\log D = \log K + n \log S$$

with $K = 10^{-7.45}$ and $n = 4.39$

where D is shear rate, and S is shear stress. The exponent n is an index of the deviation from Newtonian flow behavior. When n equals 1, K indicates the reciprocal viscosity and the flow is Newtonian. On the other hand, when n is greater than 1 the flow is pseudoplastic, and when n is less than 1 the flow is dilatant. In this case, n is much greater than 1, so the glucan

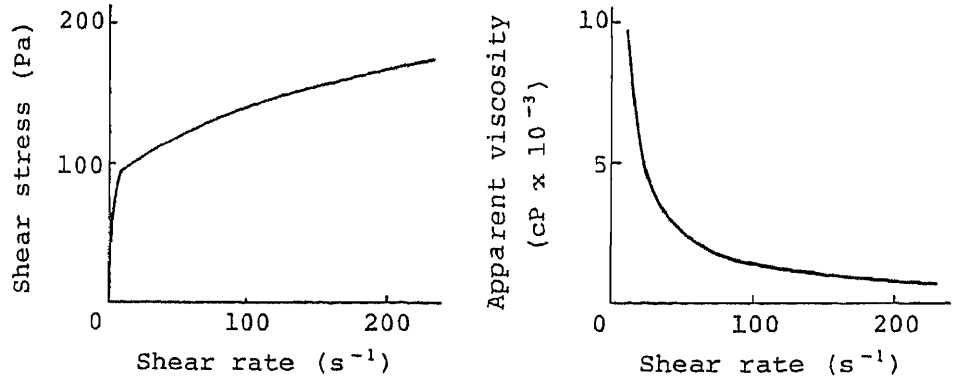

Fig. 1. Typical Flow Curves of Glucan Suspension

The concentration of glucan was 10% and the temperature was 20 °C.

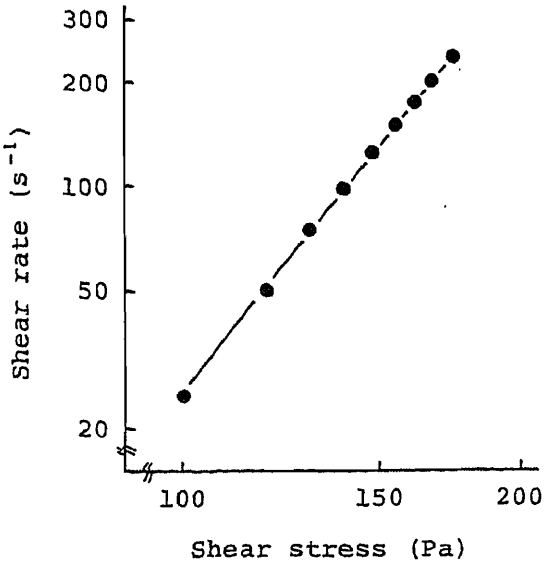

Fig. 2. Typical Flow Curve Plotted in Logarithmic Coordinates

The concentration of glucan was 10% and the temperature was 20 °C.

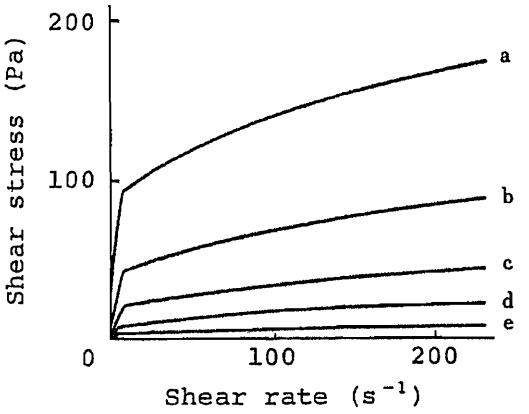

Fig. 3. Concentration Dependency of the Flow Curve

The concentrations of glucan were 10 (a), 9 (b), 8 (c), 7 (d) and 6% (e). The temperature was 20 °C.

TABLE I. Effect of Glucan Concentration on the Rheological Characteristics

Concentration of glucan (%)	Yield point (Pa)	Apparent viscosity at $100\,s^{-1}$ (cP)
10	92.0	1399
9	41.2	671
8	18.9	328
7	7.9	150
6	2.4	61

TABLE II. Effect of Temperature on the Rheological Characteristics

Temperature (°C)	Yield point (Pa)	Apparent viscosity at $100\,s^{-1}$ (cP)
10	93.0	1522
20	92.0	1399
30	90.5	1316
40	87.2	1203
50	82.0	1097

suspension is considered to show marked pseudoplasticity.

Concentration Dependency of Rheological Properties

The glucan suspensions were prepared at concentrations of 6, 7, 8, 9 and 10%. The effect of the glucan concentration on the flow curve is shown in Fig. 3. The apparent viscosity at $100\,s^{-1}$ and the yield point determined from the rheograms are summarized in Table I. The apparent viscosity and yield point both decreased markedly with decreasing concentration of glucan. However, the flow characteristic, *i.e.*, pseudoplasticity with a yield point, was found to be retained.

Effect of Temperature on Rheological Properties

The effects of temperature in the range of 10 to 50 °C on the yield point and apparent viscosity at $100\,s^{-1}$ are summarized in Table II. Although the rheological properties were affected by temperature, the extent of the effect was slight.

Effect of pH on Rheological Properties

The flow curves of the glucan suspensions (10%) were obtained at various pH values. No changes were observed in the rheological properties at pH 5.0 to 9.0. It has been shown that this water-insoluble glucan consists of α-1,3- and α-1,6-linked glucose,[3,8] *i.e.*, this polymer

dose not have ionic residues. Therefore, the flow characteristics of glucan may not be much influenced by the pH of the suspending medium.

It has already been proposed that a pseudoplastic vehicle is better as a suspending agent than a Newtonian vehicle,[9] and some pseudoplastic vehicles have been reported to be useful as suspending agents.[5,10] Moreover, pH, one of the problems associated with the formulation of a suspension, need not be taken into consideration in this case. Therefore, the application of glucan suspension as a suspending agent might be useful.

References

1) K. Masumoto, N. Nambu, A. Yoshida, S. Hayashi, and T. Nagai, *Chem. Pharm. Bull.*, **31**, 209 (1983).
2) K. Masumoto, K. Matsumoto, A. Yoshida, S. Hayashi, N. Nambu, and T. Nagai, *Chem. Pharm. Bull.*, **32**, 1055 (1984); *idem*, *ibid.*, **32**, 3720 (1984).
3) K. Masumoto, K. Yamashita, A. Yoshida, S. Hayashi, Y. Machida, and T. Nagai, *Chem. Pharm. Bull.*, **35**, 3813 (1987).
4) K. Masumoto, A. Yoshida, S. Hayashi, Y. Machida, and T. Nagai, *Chem. Pharm. Bull.*, **35**, 3940 (1987).
5) S. P. Kabre, H. G. DeKay, and G. S. Banker, *J. Pharm. Sci.*, **53**, 492 (1964); *idem*, *ibid.*, **53**, 495 (1964).
6) C. V. Walker and J. I. Wells, *Int. J. Pharmaceut.*, **11**, 309 (1982).
7) A. Osol (ed.), "Remington's Pharmaceutical Sciences," 16th ed., Mack Publishing Company, Easton, Pennsylvania, 1980, Part 2.
8) S. Ebisu and A. Misaki, *Carbohydr. Res.*, **38**, 374 (1974).
9) J. C. Samyn, *J. Pharm. Sci.*, **50**, 517 (1961).
10) W. D. Walkling and K. F. Shangraw, *J. Pharm. Sci.*, **57**, 1927 (1968).

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Preparation and Evaluation of Sustained Release Tablets Prepared with α-Starch[1)]

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A compressed tablet prepared from α-starch adsorbed water from the release medium and formed a gel, starting from the tablet surface. Theophylline was released slowly through the gelled layer. Release rates of theophylline from tablets prepared with α-starches from different sources were different. Release rates of theophylline from the tablets were faster in an acidic medium than in a neutral medium. The release rate of theophylline was faster in a medium containing α-amylase than in a medium without the enzyme.

Salivary levels were lower but were sustained for longer following oral administration of an α-starch tablet than those after administration of a rapidly dissolving tablet.

Keywords——α-starch; gel; sustained release; α-amylase; theophylline; salivary level

Introduction

Examination of natural gel-forming materials as matrices for the preparation of sustained release tablets is important from the viewpoint of employing economical and safe materials for designing pharmaceutical products. We have already reported on the possible use of hydroxypropylcellulose,[2)] seaweed extracts and seed gums[3)] to prepare sustained release theophylline tablets. Daly *et al.*[4)] reported on the release characteristics of a model drug from Synchron®, a specially treated form of hydroxypropylmethylcellulose.

Although natural starch is not soluble in water, it becomes water-soluble when it is heated in water and then quickly spray-dried or drum-dried. This pregelatinized starch is called α-starch. In the present study, we examined the gel-forming properties of α-starches to assess the suitability of the starches for the preparation of sustained-release tablets.

Experimental

Materials——α-Starches were generously supplied by Matsuya Kagaku, Itami, Hyogo Prefecture, while potato starch and low-substituted hydroxypropylcellulose were supplied by Hoei Yakko, Osaka and Shin-etsu Chemicals, Tokyo, respectively. Theophylline (anhydrous), 8-chlorotheophylline, and α-amylase were purchased from Wako Pure Chemical Ind., Osaka, while Neo-Amylase Test was purchased from Daiichi Chemicals, Tokyo. All other chemicals used for the preparation of release media and the mobile phase and for extraction were of analytical grade.

Preparation of Tablets——Tablets, 13 mm in diameter, were prepared by compressing directly 500 (or 400) mg of a 1 : 1 mixture of theophylline and each α-starch at 200 kg/cm^2 as reported earlier.[3)] Rapidly dissolving tablets were prepared by compressing directly a 1 : 1 mixture of theophylline and low-substituted hydroxypropylcellulose. Completion of dissolution of theophylline from rapidly dissolving tablet in about 10 min has already been shown.[5)]

Release Studies——Procedures reported earlier[3)] were employed except that JP X disintegration test medium No. 2 containing α-amylase at 5 μg/ml was used in some release studies. α-Amylase activity was determined by employing the Neo-Amylase Test, based on the blue-starch method. Average α-amylase activity was 1694 IU/l, which is close to the median α-amylase activity in pancreatic juice.

Measurement of Salivary Levels in Human Volunteers——Four healthy volunteers, one male and three females, of 23—47 years of age participated in the *in vivo* study. To eliminate ingestion of theophylline from other sources, the subjects were told to abstain from any drinks containing xanthines.

After overnight fasting, a single 200-mg dose of a rapidly dissolving tablet or a potato α-starch tablet was administered with 200 ml of water. The mouth was quickly rinsed with water to remove any theophylline adhering to the oral mucosa.

Saliva samples were collected at appropriate intervals up to 50 h. A small amount of citric acid, a salivary flow stimulant, was put on the tongue and held in the mouth for 1 min, then about 2-ml of saliva was collected in a test tube and kept frozen until analysis. No food was taken for 4 h post-dose but food and drink were taken *ad lib.* after 4 h. A crossover design was used and a minimum interval of 1 week was allowed between trials.

Analysis of Theophylline——Amounts of theophylline released into release media were determined with a spectrophotometer. Theophylline in saliva was analyzed by using reverse-phase high-performance liquid chromatography[6] employing 8-chlorotheophylline as an internal standard.

Pharmacokinetic Analysis——Moment analysis[7] was employed to calculate the area under the salivary concentration curve ($AUC_{0-\infty}$) and the mean residence time (MRT).

Results and Discussion

Behavior in Aqueous Media

Natural starch such as potato starch was not soluble in water and a compressed tablet prepared from natural starch disintegrated when it was placed in water. When a compressed tablet prepared from α-starch such as potato α-starch was placed in water, it did not disintegrate but it adsorbed water to form a gel starting from the tablet surface.

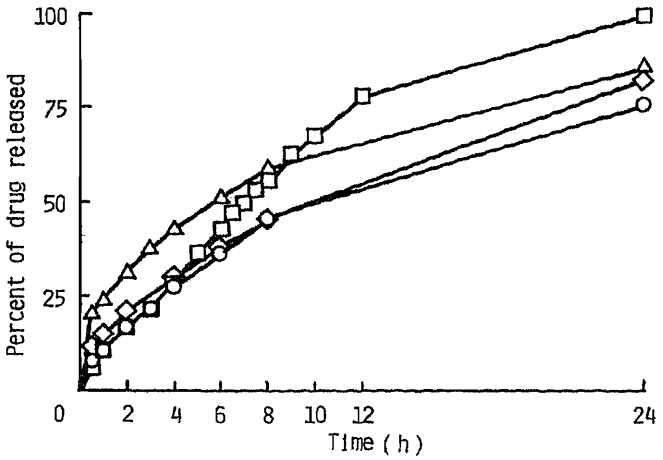

Fig. 1. Release Profiles of Theophylline from α-Starch Tablets

◇, corn; ○, potato; □, waxy corn; △, wheat. Medium No. 1. $n=3$.

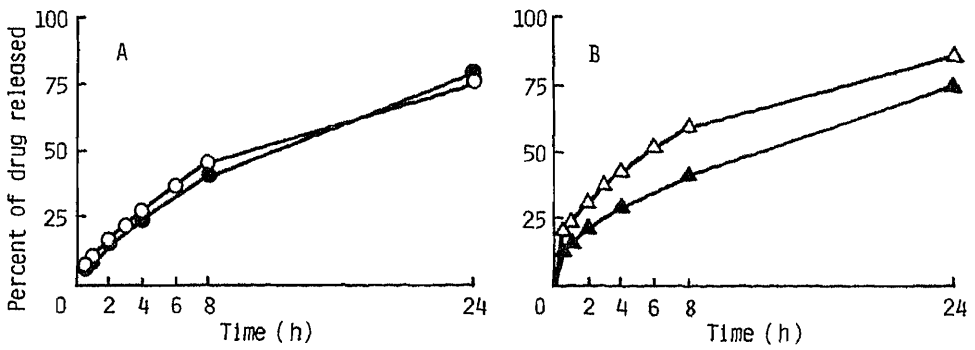

Fig. 2. Effect of pH Value of Release Media on Release of Theophylline from α-Starch Tablet

A (Potato α-starch): ○, medium No. 1; ●, medium No. 2. $n=3$. B (Wheat α-starch): △, medium No. 1; ▲, medium No. 2. $n=3$.

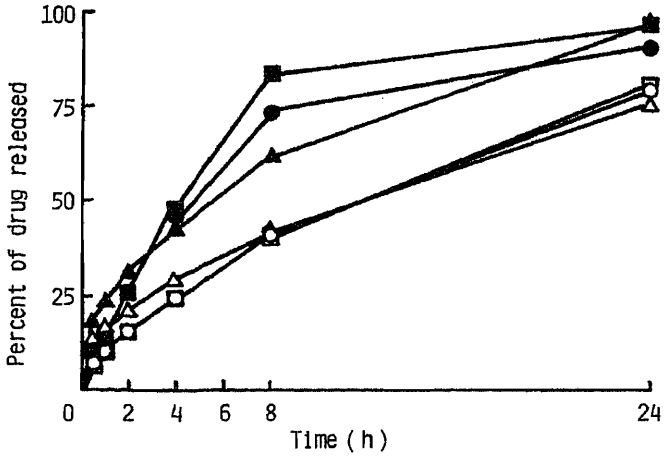

Fig. 3. Effect of α-Amylase on Release of Theophylline from Three α-Starch Tablets

○, potato without α-amylase; ●, potato with α-amylase; □, waxy corn without α-amylase; ■, waxy corn with α-amylase; △, wheat without α-amylase; ▲, wheat with α-amylase. Medium No. 2. $n=3$.

Effect of Source of Starch

Release profiles of theophylline in JP disintegration medium No. 1 from tablets prepared from four α-starches from different sources are shown in Fig. 1. The drug was released fastest from the tablet prepared from α-starch of wheat origin while it was released most slowly from the tablets prepared from α-starch of potato or corn origin. These difference may be attributed to differences in hydration (*i.e.* gelation) rates due to differences in starch structure and contents of amylose and amylopectin.

Effects of Release Media

The effects of the media on the release profiles of theophylline from tablets prepared with α-starches of potato and wheat origin are shown in Fig. 2A and 2B, respectively. Rates of release tended to be faster in the acidic medium (medium No. 1, pH 1.2) than in the neutral medium (medium No. 2, pH 6.8). The effect of medium pH was more pronounced with α-starch of wheat origin than with that of potato origin. A significant effect of medium pH was also observed in α-starch of waxy corn origin (not shown). The difference may be attributed to differences in the susceptibility of the starch molecules to acid hydrolysis.

Effect of α-Amylase

The effect of α-amylase in the release media on the release patterns of theophylline from tablets is shown in Fig. 3. In tablets prepared from three types of α-starches, the drug release in media containing α-amylase was faster than that in media without the enzyme. The increase in release rates may be attributed to the destruction of starch matrices caused by hydrolysis of starch molecules with α-amylase.

***In Vivo* Study**

Based on the effects of gastrointestinal pH and a digestive enzyme on the release patterns of theophylline from compressed tablets, potato α-starch was selected for the *in vivo* study. Salivary levels of theophylline after oral administration of a rapidly dissolving tablet or a sustained-release matrix tablet prepared with α-starch of potato origin are shown in Fig. 4. Salivary levels rose quickly to reach the maximum concentration at 1.4 h following administration of the rapidly dissolving tablet, whereas they rose slowly to reach the maximum concentration at 5 h following administration of the sustained release tablet.

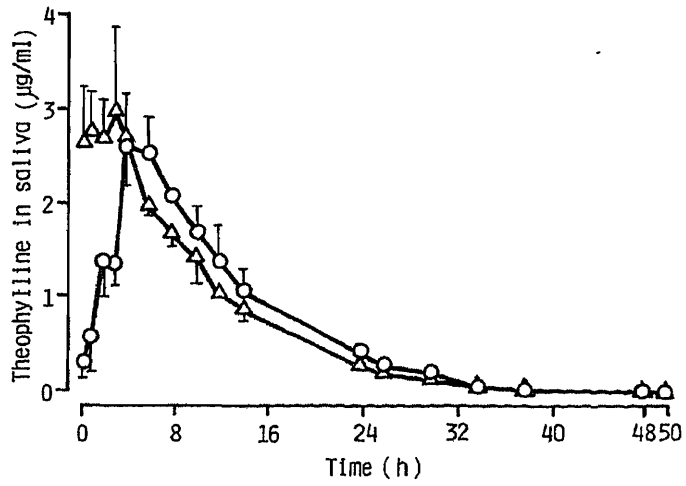

Fig. 4. Salivary Levels of Theophylline after Oral Administration of Potato α-Starch Tablet and Rapidly Dissolving Tablet in Volunteers

△, rapidly dissolving tablet; ○, potato α-starch tablet. Mean±S.E.M. $n=4$.

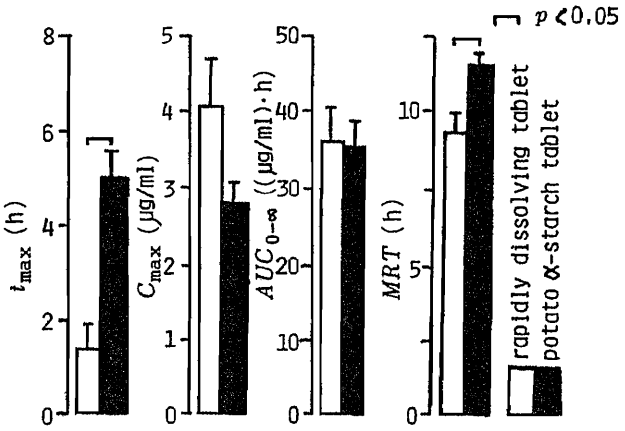

Fig. 5. Comparison of Bioavailability Parameters between Potato α-Starch Tablet and Rapidly Dissolving Tablet

Figure 5 shows bioavailability parameters for two theophylline preparations. Although t_{max} and MRT values were significantly different among the two, $AUC_{0-\infty}$ and C_{max} values were not significantly different. This observation indicates that theophylline was absorbed to a similar extent despite the difference in release rates from the tablets and resultant difference in absorption patterns.

Conclusion

The present results suggest that

1) Compressed α-starch tablets form a gel by adsorbing water from aqueous media.
2) Some α-starches are more susceptible to acid hydrolysis than others.
3) All α-starches are susceptible to enzymatic hydrolysis with α-amylase but the degree of susceptibility is variable.
4) α-Starches can be used as a matrix material for sustained-release tablets.

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References and Notes

1) Presented at the 105th Annual Meeting of the Pharmaceutical Society of Japan, April 1985 in Kanazawa.
2) M. Nakano, N. Ohmori, A. Ogata, K. Sugimoto, Y. Tobino, R. Iwaoku, and K. Juni, *J. Pharm. Sci.*, **72**, 378 (1983).
3) M. Nakano and A. Ogata, *Chem. Pharm. Bull.*, **32**, 782 (1984).
4) P. B. Daly, S. S. Davis, M. Frier, J. Hardy, J. Kennerley, and C. G. Wilson, *J. Pharm. Pharmacol.*, **33**, 47P (1981).
5) N. Nakazono, N. Inotsume, Y. Tobino, R. Iwaoku, and M. Nakano, *Jpn. J. Clin. Pharmacol. Ther.*, **16**, 401 (1985).
6) M. Nakano, Y. Nakamura, K. Juni, and T. Tomitsuka, *J. Pharmacobio-Dyn.*, **3**, 702 (1980).
7) K. Yamaoka, T. Nakagawa, and T. Uno, *J. Pharmacokinet. Biopharm.*, **6**, 547 (1978).

Communications to the Editor

[Chem. Pharm. Bull. 35(10)4351—4354(1987)]

NEW REDUCTION OF SULFOXIDES AND SULFILIMINES TO SULFIDES WITH ZINC/1,4-DIBROMOBUTANE. A PROPOSAL FOR A RADICAL-FORMING ELECTRON TRANSFER PROCESS[1)]

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Based upon ^{13}C NMR spectroscopic observations of the deuteriorated methylcyclopropane and 1-butene, a bromoalkyl radical process has been considered in the reduction of sulfoxides and sulfilimines to sulfides by zinc/1,4-dibromobutane.

KEYWORDS——sulfide; reduction; sulfoxide; sulfilimine; zinc; 1,4-dibromobutane; single electron transfer; ^{13}C NMR; deuterio-methylcyclopropane; deuterio-butene-1

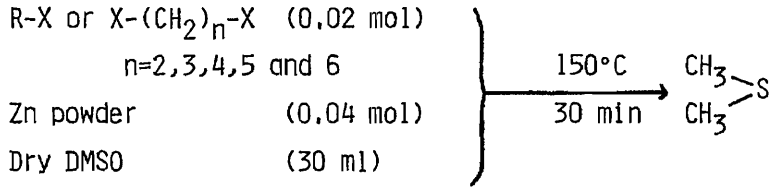
Reduction of sulfoxides and sulfilimines to sulfides is still a fascinating field in organic synthesis because of their abilities in various transformations, such as alkylation and/or condensation, and numerous methods have been exploited as of now.[2)] However, only few radical-mediated reductions of these compounds have been known so far.[3)] Therefore, as part of our ongoing program in organosulfur chemistry we wish to present in this communication our unique methods concerning this subject.

Dimethyl sulfoxide(DMSO) was found to react surprisingly with 1,4-dibromobutane at 150°C for 30 min in the presence of zinc metal(2 eq) to give dimethyl sulfide(DMS) in almost quantitative yield and reduction does not occur at all in the absence of zinc metal or 1,4-dibromobutane. Therefore, we have tried this reaction by using other kinds of metals such as tin, cobalt, nickel and iron in order to examine the effects of metals. As a result, DMS was obtained in the order of Sn(80%)>>> Co≃Ni(~10%)≥ Fe(~5%). Among the metals so far as concerned, zinc proved to be by far the best. In addition, the results are illustrated as follows(Chart 1) when applied to alkane dihalides and alkyl halides, in which 1,4-dibromobutane being the most efficient agent.

In an effort to determine whether this reducing reagent system is rather specific for dimethyl sulfoxide, we have, next, performed a similar procedure, developing to a wide range of the succeeding aliphatic and aromatic sulfoxides and sulfilimines and the results obtained are shown in the Table.

As for the mechanism of this hard-to-believe transformation, the following have been rationalized. A twenty percent yield of n-butanal was produced along

Chart 1. Effects of Various Alkyl Halides and Alkane Dihalides



$$Br\text{-}(CH_2)_4\text{-}Br > Br\text{-}(CH_2)_5\text{-}Br \gg Br\text{-}(CH_2)_6\text{-}Br \simeq Br\text{-}(CH_2)_3\text{-}Br \gg$$

quant. 70% 30%

$$n\text{-}C_5H_{11}\text{-}Br,\ n\text{-}C_6H_{13}\text{-}Br \simeq n\text{-}C_5H_{11}\text{-}I,\ n\text{-}C_7H_{15}\text{-}I \gg Cl\text{-}(CH_2)_4\text{-}Cl,\ n\text{-}C_7H_{15}\text{-}Cl$$

5-10% 0%(almost inactive) based on (di)halides

Table. Reduction of Sulfoxides and Sulfilimines

R	R'	Sulfide yield(%)[a] based on dibromide from sulfoxide (R-SO-R')	from sulfilimine [R-S(NSO_2-C_6H_4-CH_3-p)-R']
CH_3CH_2	CH_3CH_2	90[b]	31
CH_2=$CHCH_2$	CH_3	15[b]	
-$(CH_2)_4$-		93[b]	20
C_6H_5	CH_3	60	15
C_6H_5	C_6H_5	65	45
Thianthrene		Quant.	40

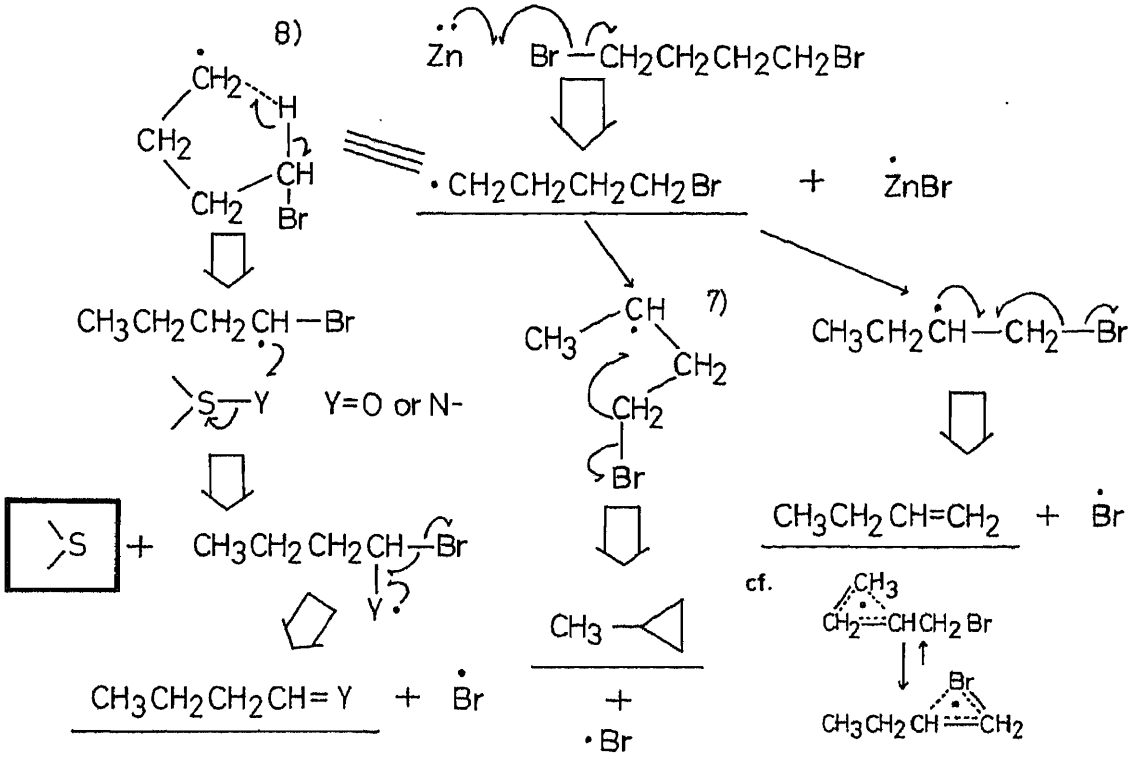
a) Unless otherwise indicated, reduction was carried out at 150°C/60 min for sulfoxide and at 150°C/120 min for sulfilimine.
b) Reaction was performed at 80°C/30 min with activated zinc powder by lithium metal reduction.

with the expected sulfide (40%) and the sulfoxide recovered (50%) after 30-min-heating at 150°C of a mixture of diphenyl sulfoxide, 1,4-dibromobutane and zinc powder, which suggests the oxosulfonium intermediates[4] just like those in the Kornblum oxidation,[5] while heating of the last two reagents gave a considerable amount (50-60%) of methylcyclopropane (bp 4-5°C/760 Torr), 10-20% of 1-butene (bp -6.3°C/760 Torr) and n-butyl bromide trapped by a Dry ice/acetone bath. This might indicate a carbene generation. However, the Kornblum oxidation has been completely cancelled as none of the sulfides were given from a reaction mixture of zinc and the oxosulfonium salts prepared separately according to a literature method,[4] and just the starting salts were recovered instead. The other prospect could be seemingly eliminated owing to no detectable carbene-adducts.

Careful inspection of the reaction of zinc and 1,1,4,4-tetradeuterio-1,4-di-

bromobutane by ^{13}C NMR spectra[6] revealed that there must be present *four deuterium atoms* in each of the products, *i.e.*, α,α,2,2-tetradeuteriomethylcyclopropane and 1,1,4,4-tetradeuterio-1-butene. At this stage it seems reasonable to propose that the reduction of sulfoxides and sulfilimines to sulfides by using the zinc dust/1,4-dibromobutane system involves the generation of free radical intermediates arising from a single electron transfer process according to the results described above and the mechanism could be finally settled as follows(Chart 2).

Chart 2. Reaction Pathway for Reduction of Sulfoxides and Sulfilimines by Employing Zinc Dust/1,4-Dibromobutane



In conclusion, we have advocated the reduction of sulfoxides and sulfilimines by using the zinc dust/1,4-dibromobutane system *via* a single electron transfer mechanism. The advantages of this procedure lie in its simplicity, its low cost and the possibility of its being carried out under neutral conditions when applied to the compounds bearing acid- and/or base-sensitive functionalities, although the yields leave much to be desired, especially in the case of sulfilimine derivatives.

REFERENCES AND NOTES

1) Organosulfur Chemistry IV. For Part III, see K. Nagasawa, A. Yoneta, T. Umezawa and K. Ito, Heterocycles, in press. A part of this work was presented at the 1984 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii, December, 1984. Abstract No. 10-O-115.

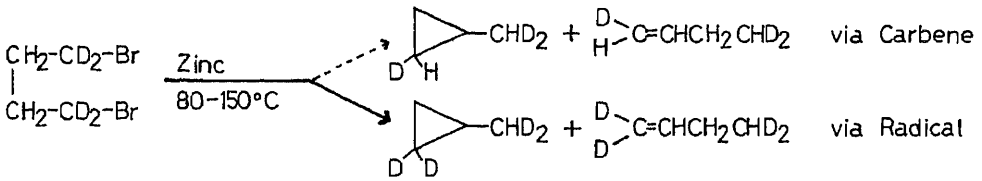
2) For sulfoxides, a) J. Drabowicz, T. Numata and S. Oae, Org. Prep. Proc. Int., 9, 63(1977); b) H.S. Dilanjan Soysa and W.P. Weber, Tetrahedron Lett., 1969 (1978); c) C. Tenca, A. Dossena, R. Marchelli and G. Casnati, Synthesis, 141 (1981); d) J.R. Babu and M.V. Bhatt, Tetrahedron Lett., 27, 1073(1986); e) Y. Guindon, J.G. Atkinson and H.E. Morton, J. Org. Chem., 49, 4538(1984) and references cited therein. For sulfilimines, T.L. Gilchrist and C.J. Moody, Chem. Rev., 77, 409(1977).

3) a) S. Oae and S. Kawamura, Bull. Chem. Soc. Jpn., 36, 163(1963); b) S. Oae, K. Tsujihara and N. Furukawa, ibid., 45, 2856(1972); c) S. Kozuka, S. Furumai, T. Akasaka and S. Oae, Chem. Ind.(London), 496(1974); d) For a single electron transfer in Organic Chemistry, see W. Kaim, Acc. Chem. Res., 18, 160(1985) and A. Pross, ibid., 18, 212(1985).

4) D.H.R. Barton, B.J. Garner and R.H. Wightman, J. Chem. Soc., 1964, 1855.

5) N. Kornblum, J.W. Powers, G.J. Anderson, W.J. Jones, H.O. Larson, O. Levand and W.M. Weaver, J. Am. Chem. Soc., 79, 6562(1957).

6) a) W.G. Schneider and H. Spiesecke, J. Chem. Phys., 35, 731(1961); b) Y.K. Grishin, N.M. Sergeyev and Y.A. Ustynyuk, Mol. Phys., 22, 711(1971); c) J.B. Stothers, C.T. Tan, A. Nickon, F. Huang, R. Sridhar and R. Weglein, J. Am. Chem. Soc., 94, 8581(1972); d) K. Nagasawa and T. Kana-iwa, Chem. Pharm. Bull., 30, 4189(1982). Formation of the corresponding sulfides and/or the accompanied products described in this communication was effectively suppressed when reaction had been carried out in the presence of 2,4,6-tri t-butylphenol or galvinoxyl, which indicating largely a radical process.



In case of α,α,2,2-tetradeuteriomethylcyclopropane, when recorded in the complete proton noise decoupling mode at 25.0 MHz, 20°C, the last two of three original carbon-13 sharp singlets(in ppm) at 5.11(>CH-), 5.69($-CH_2-$), 19.49(CH_3-) were found to be replaced by the characteristic, very weak and low-intensity quintets(upfield shift, 0.70 and 0.64 ppm, respectively) with a 1:4:6:4:1 ratio of each peak area, in which $^1J_{C-D}$ was confirmed to be 23.53 Hz. The two newly appeared singlets(in ppm) at 4.76 and 5.46, which indicated upfield shift again by 0.35&0.23 ppm, should be the corresponding methine and another non-deuterium-bearing methylene carbon of cyclopropane ring, respectively. All of these observations are quite consistent with the isotopic effects expected for two deuterium atoms in the literature. Likewise, for 1,1,4,4-tetradeuterio-1-butene a reasonable interpretation could be performed.

7) A.S. Gordon and J.R. McNesby, J. Chem. Phys., 31, 853(1959).

8) A.S. Gordon and J.R. McNesby, J. Chem. Phys., 33, 1882(1960).

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Communications to the Editor

[Chem. Pharm. Bull. 35(10)4355—4358(1987)]

DETERMINATION OF THE STEREOSTRUCTURE OF THE δ-LACTONES OF 5,7-DIHYDROXY-2,3-UNSATURATED ACIDS BY ^{1}H NMR SPECTROSCOPY

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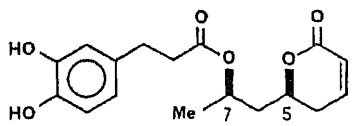
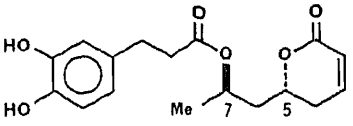
The stereostructures of the syn- and anti-δ-lactones of 5,7-dihydroxy-2,3-unsaturated acids were assigned by a structure-specific ^{1}H NMR splitting pattern of the C-4 protons of the corresponding acetate.

KEYWORDS —— stereostructure determination; δ-lactone of 5,7-dihydroxy 2,3-unsaturated acid; syn-, anti-isomer; ^{1}H NMR; structure-specific splitting pattern

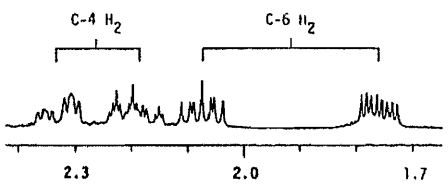
Recently, the optically active δ-lactones, 5,7-syn-1 and 5,7-anti-isomer 2, were synthesized by a strategy for 1,3-polyol synthesis.[1] The ^{1}H NMR spectrum of the former proved to be identical with that of (-)-tarchonanthuslactone. Thus, the relative and absolute configurations of the natural lactone were established as shown in structure 1. During the comparison studies using the ^{1}H NMR (400 MHz) technique, we found that the splitting pattern of the C-4 and C-6 protons of 5,7-syn-1 was clearly different from that of 5,7-anti-2 (Fig. 1).



5,7-syn-1



5,7-anti-2



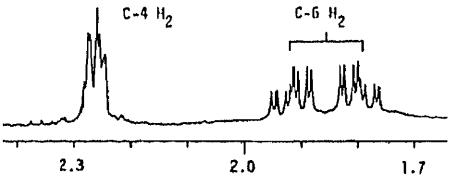


Fig. 1. ^{1}H NMR Signals of the C-4 and C-6 Protons of 1 and 2 (400 MHz)

To examine whether a similar spectral pattern difference is observed in other isomer pairs of this type, we synthesized several stereochemically well-defined C-7-mono- and C-7,9-diacetates (3~10) by an authenticated method.[2,3] On comparison of their ^{1}H NMR spectra, all of the 5,7-syn- and anti-isomers examined proved to have characteristic splitting patterns similar to 1 and 2, respectively.[4]

The ^{1}H NMR spectra (400 MHz) of the C-4 protons of the synthetic δ-lactones are shown in Fig 2. Two protons at C-4 in the 5,7-syn-lactones appeared separately at δ 2.29~2.33 and 2.45~2.53 with each dddd splitting pattern, while the protons of 5,7-anti-lactones appeared overlapped at ca. δ 2.35.[5] These results show that the pattern strongly depends on the relative configuration at C-5 and C-7 but is not affected by the configuration at the other positions. As for the C-6 protons, the spectral characteristic of 1 and 2 holds well for 3, 4 and for 5, 6, respectively. However, the signals of these protons in the diacetoxy lactones (7~10) overlapped those of other protons (acetyl and/or methylene protons). So the use of the spectral pattern of C-6 protons for structural study is rather limited.

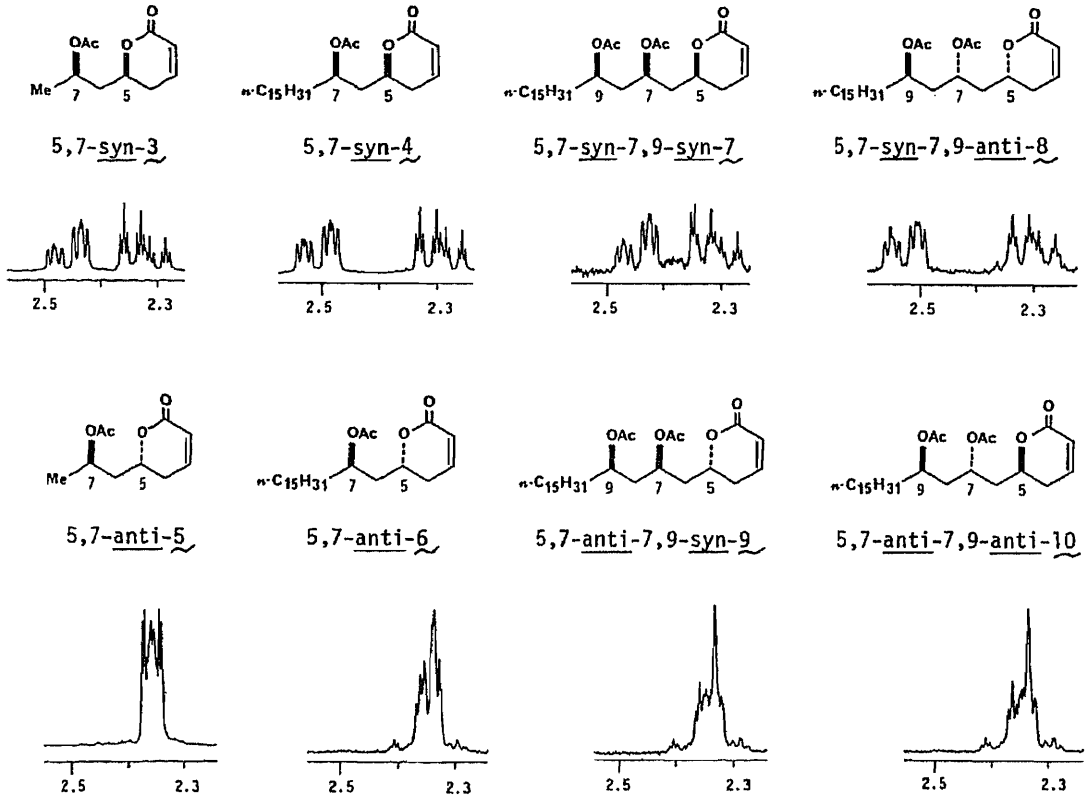


Fig. 2. ^{1}H NMR Signals of the C-4 Protons of 3~10 (400 MHz)

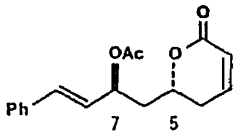
The chemical shifts of the acetyl groups of the above stereoisomers are shown in Table I. It is noteworthy that signals of the acetyl groups of 5,7-syn-isomers 3, 4 appeared at a slightly lower field than those of anti-isomers 5, 6, respectively. The same trend appeared in the chemical shifts of 7,9-syn- and anti-isomer pairs (7/8 and 9/10).

Clearly the present findings serve as a very simple and reliable method for determining the relative configuration at C-5 and C-7 of the naturally occurring α,β-unsaturated δ-lactones having a functionalized side chain of unknown configuration. The method's applicability was confirmed in the following cases. The absolute stereostructures of (+)-cryptocaryalactone and ACRL Toxin I was

Table I. Chemical Shifts (400 MHz, δ, ppm) of Acetyl Groups of Compounds 3~10

5,7-syn-3	2.053	5,7-syn-7,9-syn-7	2.061, 2.067
5,7-anti-5	2.039	5,7-syn-7,9-anti-8	2.031, 2.059
5,7-syn-4	2.068	5,7-anti-7,9-syn-9	2.053, 2.066
5,7-anti-6	2.049	5,7-anti-7,9-anti-10	2.036, 2.038

recently determined to be 5,7-anti-11 and 10,12-syn-12, respectively, by physicochemical methods.[6a,7a] The reported NMR data of the C-4 protons of anti-acetate 11[6b,8] and the C-13 protons of syn-acetate 13[7b] show the characteristic patterns for 5,7-anti- and 10,12-syn-compounds, respectively. Thus, the above spectral trends for determining the relative configurations were found to hold well even for compounds having double bonds on the side chain and/or a methoxy group on the lactone ring.



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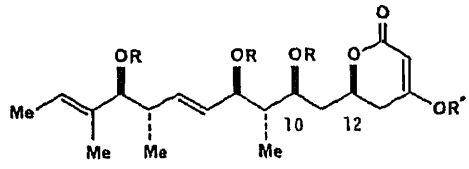
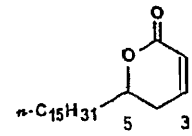


12: R=R'=H
13: R=Ac, R'=Me



14

The spectral pattern and the chemical shift of the C-4 protons of 14 having no oxygen function at the C-7 position are similar to those of 5,7-anti-isomers (5, 6, 9, and 10). This shows that the acyloxy group at the C-7 position in these isomers should be located far from the C-4 protons in the preferred conformation, while in the 5,7-syn-isomers (3, 4, 7, and 8), 4α(equatorial)-H is apparently shifted to a lower field influenced by the near-by acyloxy group at the C-7 position. The structure of the preferred conformers should serve to rationalize the observed structure-specific spectral pattern at the C-4 and C-6 protons. Examination of the preferred conformation of these isomers is in progress.

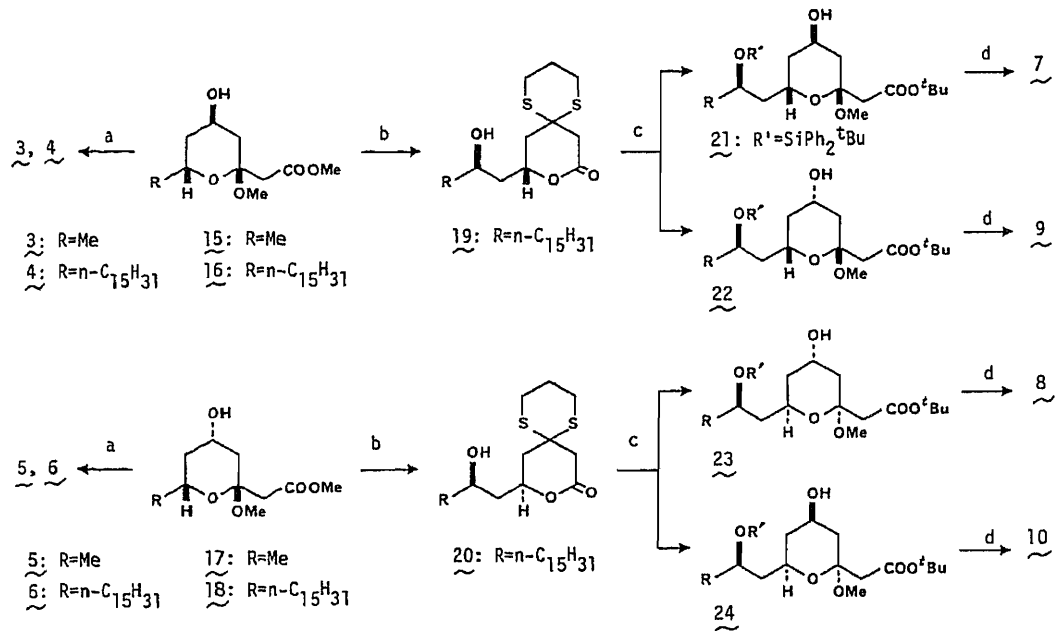
Other special cases where the present findings are effectively used for elucidation of stereostructure of natural lactones will be reported elsewhere.[1] Furthermore, based on the above structure-specific ^{1}H NMR spectra, development of a general method for determination of the stereostructure of the 1,3-polyol system is now being investigated in our laboratory.

ACKNOWLEDGEMENT The authors are grateful to Dr. Y. Kono (RIKEN) for providing 400 MHz ^{1}H NMR spectra of ACRL Toxin I (12) and 13. This work was supported in part by a Grant-in-Aid for Special Research from the Ministry of Education, Science, and Culture "Chemical Syntheses for Elucidation of Biological Functions".

REFERENCES AND NOTES

1) T. Nakata, N. Hata, K. Iida, and T. Oishi, Tetrahedron Lett., in press.
2) a) T. Nakata, S. Takao, M. Fukui, T. Tanaka, and T. Oishi, Tetrahedron Lett., 24, 3873 (1983); b) T. Nakata, S. Nagao, and T. Oishi, Tetrahedron Lett., 26, 75 (1985).
3) The stereoisomers (3~10) were synthesized based on the synthetic method for

1,3-syn- and anti-polyol[2] as shown below. Alcohols 16 and 18 were prepared from cetyl alcohol. See, reference 1.



a) 1N HCl/THF; $NaBH_4$/MeOH/THF; CSA/PhH; Ac_2O/DMAP/Py/CH_2Cl_2; DBU/PhH, b) $HS(CH_2)_3SH$/$BF_3 \cdot Et_2O$/CH_2Cl_2, c) t-$BuPh_2SiCl$/imidazole/DMF; LDA/MeCOO-t-Bu/THF; $CH(OMe)_3$/CSA/MeOH/CH_2Cl_2; NBS/$AgNO_3$/Na_2CO_3/aq MeCN; n-Bu_4NBH_4/aq THF, d) 1N HCl/THF; $NaBH_4$/MeOH/THF; CSA/PhH; n-Bu_4NF/THF; Ac_2O/DMAP/Py/CH_2Cl_2; DBU/PhH.

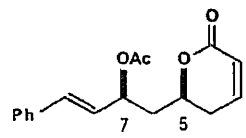
4) In the corresponding C-7 hydroxy compounds, there was no spectral pattern difference between the syn- and anti-isomers.

5) In the case of 5,7-syn-3, signals centered at δ 2.33 and at δ 2.46 were assigned as C-4 axial and equatorial protons, respectively, since the coupling constant between the C-4 and C-5 protons in the former was found to be 12 Hz and that in the latter, 6 Hz by an extensive decoupling experiment. 5,7-syn-3: δ 2.33 (dddd, $J_{4\alpha,4\beta}$=18 Hz, $J_{4\beta,5\alpha}$=12 Hz, $J_{2,4\beta}=J_{3,4\beta}$=2 Hz; 4β-H), 2.46 (dddd, $J_{4\alpha,4\beta}$= 18 Hz, $J_{4\alpha,5\alpha}$=6 Hz, $J_{2,4\alpha}$=4 Hz, $J_{3,4\alpha}$=1 Hz; 4α-H).

6) a) H. H. Meyer, Liebigs Ann. Chem., 1984, 977; b) T. R. Govindachari, P. C. Parthasarathy, and J. D. Modi, Indian J. Chem., 10, 149 (1972).

7) a) Y. Kono, J. M. Gardner, K. Kobayashi, Y. Suzuki, S. Takeuchi, and T. Sakurai, Phytochemistry, 25, 69 (1986); b) J. M. Gardner, Y. Kono, J. H. Tatum, Y. Suzuki, and S. Takeuchi, Phytochemistry, 24, 2861 (1985).

8) Both anti-acetate 11 and syn-acetate 25 were synthesized for comparison in this laboratory. The ^1H NMR (400 MHz) data of synthetic 11 were identical with those of natural 11. The C-4 protons of syn-25 exhibit a typical splitting pattern for 5,7-syn-compounds.



25

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Communications to the Editor

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THE STRUCTURES OF DATURAMETELIN A AND B[1)]

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Two withanolide glucosides, daturametelin A and B, were obtained as major ingredients from the methanol extract of the whole plants of Datura metel L. Their structures were determined by the spectroscopic means.

KEYWORDS —— Solanaceae; Datura metel; withanolide glucoside; daturametelin A; daturametelin B; withametelin

In the course of our studies on the constituents of Solanaceous plants, we found the withanolide-related glucosides, tubocapside A and B,[2)] in the mature berries of Tubocapsicum anomalum Makino. It is known[3)] that withanolides are included also in Datura genus as well as Withania and Physalis. Recently, Hikino et al. isolated a withanolide, named withametelin (1),[4)] from the petroleum-ether extract of the leaves of Datura metel. Its structure is characteristically oxygenated at C-21 and cyclized between C_{21}-OH and C_{24}. We have now independently obtained two major withanolide glucosides from the methanol extract of the same origin. This paper describes with their structures.

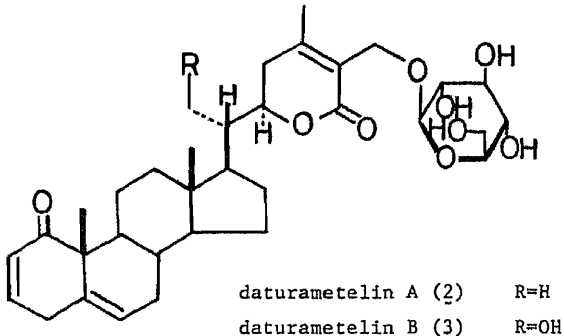
Two new withanolide glucosides, designated daturametelin A (2) and B (3), along with 1 have been isolated in yields of 0.05% and 0.25%, respectively, from the fresh whole plants harvested in June.

Daturametelin A (2), $C_{34}H_{48}O_9$, an amorphous powder, $[\alpha]_D$ -22.4° (MeOH), showed absorption bands of strong hydroxyls(3500 cm^{-1}) and carbonyl groups (1710cm^{-1}). The respective signals on the ^{1}H-NMR spectrum of the acetate (4), $C_{42}H_{56}O_{13}$, an amorphous powder, $[\alpha]_D$ +5.0° ($CHCl_3$), could be assigned by comparing with those of 1 and withafelin A diacetate (5)[5)]. They suggested the existence of the following functional groups; two angular methyls (each 3H, s, δ 0.73, 1.24, 18- and 19-Me) and one secondary methyl (3H, d, J=7.0 Hz, δ 1.02), an 1-one-2-ene system (1H, dd, J=2.2, 9.9 Hz, δ 5.88, 2-H; 1H, ddd, J=2.6, 4.8, 9.9 Hz, δ 6.79, 3-H), a Δ^5 system (1H, d, J=5.9 Hz, δ 5.57, 6-H), and a hydroxymethyl (2H, ABq, J=10.0 Hz, δ 4.58, 4.47, 27-H_2), a vinyl methyl (3H, br s, δ 2.05, 28-Me) and a proton adjacent to the oxygen atom (1H, dt, J=3.3, 13.2 Hz, δ 4.41, 22-H) on the δ-lactone ring, together with the 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl moiety (1H, d, J=8.1 Hz, δ 4.65, H-1'; 1H, dd, J=8.1, 9.5 Hz, δ 4.96, H-2'; 1H, t, J=9.5 Hz, δ 5.21, H-3'; 1H, t, J=9.5 Hz, δ 5.08, H-4'; 1H, ddd, J=2.4, 4.5, 9.5 Hz, δ 3.69, H-5'; 2H, dd, J=4.6, 12.3 Hz, δ 4.24 and dd, J=2.4, 12.3 Hz, δ 4.16, 6'-H_2). The ^{13}C-NMR spectrum of 2 supported the above assignments and suggested the presence of the hydroxymethyl group at C-25. The chemical shifts at C-1-19, except for C-17 in the ^{13}C-NMR in 2,

Table I. ^{13}C-NMR Spectrum Data for 1 - 3, 5 and 7

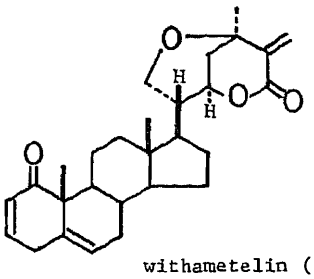
	1	2	3	5	7
C-1	204.5 (s)	203.9 (s)	203.9 (s)	201.1 (s)	214.7 (s)
2	128.0 (d)	127.7 (d)	127.9 (d)	133.9 (d)	38.4 (t)
3	145.0 (d)	145.8 (d)	145.9 (d)	139.8 (d)	27.4 (t)
4	33.4 (t)	33.4 (t)	33.6 (t)	72.2 (d)	31.2 (t)
5	135.9 (s)	136.2 (s)	136.3 (s)	61.0 (s)	141.4 (s)
6	124.5 (d)	124.6 (d)	124.8 (d)	60.2 (d)	121.8 (d)
7	30.7 (t)	30.8 (t)	31.1 (t)	31.1 (t)	32.2 (t)
8	33.2 (d)	33.1 (d)	33.4 (d)	29.6 (d)	32.0 (d)
9	42.8 (d)	43.3 (d)	43.5 (d)	44.1 (d)	45.5 (d)
10	50.4 (s)	50.6 (s)	50.8 (s)	48.1 (s)	53.9 (s)
11	23.6 (t)	23.8 (t)	24.1 (t)	21.3 (t)	22.7 (t)
12	39.6 (t)	39.8 (t)	39.3 (t)	39.2 (t)	39.1 (t)
13	42.5 (s)	42.5 (s)	42.6 (s)	42.6 (s)	42.5 (s)
14	56.0 (d)	56.2 (d)	56.3 (d)	56.1 (d)	56.3 (d)
15	24.0 (t)	24.2 (t)	24.5 (t)	24.2 (t)	24.3 (t)
16	26.5 (t)	26.9 (t)	27.1 (t)	27.3 (t)	26.3 (t)
17	47.6 (d)	51.9 (d)	47.2 (d)	51.9 (d)	46.6 (d)
18	12.7 (q)	11.7 (q)	12.4 (q)	11.5 (q)	12.6 (q)
19	18.9 (q)	18.8 (q)	19.0 (q)	15.7 (q)	19.4 (q)
20	39.9 (d)	38.9 (d)	46.0 (d)	38.8 (d)	42.6 (d)
21	60.4 (t)	13.3 (q)	58.9 (t)	13.3 (q)	60.0 (t)
22	75.8 (d)	78.2 (d)	77.9 (d)	78.2 (d)	78.2 (d)
23	33.2 (t)	29.9 (t)	33.3 (t)	30.1 (t)	31.0 (t)
24	69.2 (s)	157.0 (s)	157.9 (s)	157.1 (s)	150.3 (s)
25	138.9 (s)	122.8 (s)	123.1 (s)	121.9 (s)	122.8 (s)
26	165.2 (s)	166.0 (s)	166.2 (s)	165.3 (s)	166.8 (s)
27	129.7 (t)	63.2 (t)	63.4 (t)	58.0 (t)	12.3 (q)
28	25.6 (q)	20.4 (q)	20.5 (q)	20.6 (q)	20.6 (q)
1'		104.6 (d)	104.6 (d)		
2'		75.0 (d)	75.0 (d)		
3'		78.3 (d)	78.4 (d)		
4'		71.5 (d)	71.4 (d)		
5'		78.3 (d)	78.4 (d)		
6'		62.6 (t)	62.8 (t)		

Solv. 1, 5 and 7 : in $CDCl_3$; 2 and 3 : in pyridine d_5

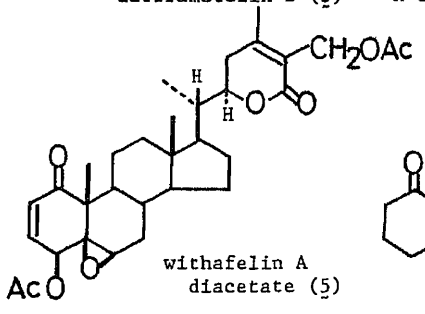


daturametelin A (2) R=H

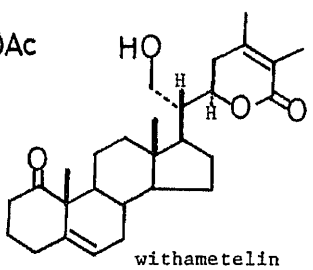
daturametelin B (3) R=OH



withametelin (1)



withafelin A diacetate (5)



withametelin H_2-minor P (7)

were consistent with those of 1, and at C-20-28, except the C-27 in 2 also showed good coincidence with those of 5 as listed in Table I. Signals at C-17 and -27 shifted down-field owing to structural difference of the C_{17}-side chain and glycosidation shifts,[6] respectively. Therefore, the location of the hydroxymethyl group could be corroborated and the glycosidic linkage should attach to this hydroxyl group. Thus, the structure of 2 is as shown in the formula.

Daturametelin B (3), $C_{34}H_{48}O_{10}$, an amorphous powder, $[\alpha]_D$ +1.4° (MeOH), showed ^{1}H- and ^{13}C-NMR (Table I) spectra similar to 2. However, the C-21 methyl proton signal in 2 was absent. Instead the compound had new methylene protons adjacent to the oxygen atom in the ^{1}H-NMR spectrum[7] of the acetate (6) of 3, with colorless needles, mp 147-148°, $[\alpha]_D$ +29.8° ($CHCl_3$). The signals of the ^{13}C-NMR spectrum of 3 were superimposable on those of 2 except for C-17, 20, 21 and 23, whose chemical shifts were almost identical with those of the whithametelin H_2-minor product (7)[4] derived from 1 by Hikino et al. Since the ^{13}C-NMR signals ascribable to C-24-28 in 3 coincided with those of 2, the sugar residue should also be linked to the C-27 hydroxyl group. Therefore, the structure of 3 is represented as shown in the formula.

We observed that 3 changed into 1 during column chromatographic separation using silicagel (solv. $CHCl_3$-MeOH-H_2O=7:3:0.2 system). Aside from this, the silica gel treatment (stirring at r.t. overnight) of 3 in $CHCl_3$-MeOH afforded predominently a product identical with 1 along with D-glucose. Consequently, withametelin (1) might be an artifact derived from the major component, daturametelin B (3), during the separation procedure. The two glucosides of 2 and 3 obtained here were the first examples of a glycosyl residue in the δ-lactone part of withanolide, and the latter (3) appeared to be a key compound to produce a hexacyclic withanolide, withametelin (1).

REFERENCES AND NOTES

1) Part 9 in the series of "Studies on the Constituents of Solanaceous Plants", Part 8; K.Yoshida, S.Yahara, R.Saijo, K.Murakami, T.Tomimatsu and T.Nohara, Chem. Pharm. Bull., 35, 1645 (1987).

2) K.Yoshida, F.Yokote, S.Yahara, T.Nohara, R.Saijo, K.Murakami and T.Tomimatsu, Abstract Papers of 28th Symposium on the Chemistry of Natural Products, p. 235 (Sendai, 1986).

3) E.Glotter, I.Kirson, D.Lavie and A.Abraham, "Bioorganic Chemistry," E.E. van Tamelen Ed., Academic Press, New York, 1978, vol. II, pp. 57-95; I.Kirson and E.Glotter, J. Nat. Products, 44, 633 (1981).

4) Y.Oshima, A.Bagchi and H.Hikino, Tetrahedron Lett., 28, 2025 (1987).

5) H.E.Gottlieb and I.Kirson, Org. Magn. Resn., 16, 20 (1981).

6) R.Kasai, M.Suzuo, J.Asakawa and O.Tanaka, Tetrahedron Lett., 1977, 175; K.Tori, S.Seo, Y.Yoshimula, H.Arita and T.Tomita, Tetrahedron Lett., 1977, 179.

7) ^{1}H-NMR spectrum of 6 (in $CDCl_3$) δ (ppm) : 6.77 (1H, ddd, J=2.3, 5.1 and 10.0 Hz, 3-H), 5.87 (1H, dd, J=2.0, 10.0 Hz, 2-H), 5.57 (1H, d, J=5.9 Hz, 6-H), 5.20 (1H, t, J=9.5 Hz, 3'-H), 5.07 (1H, t, J=9.8 Hz, 4'-H), 4.94 (1H, dd, J=9.5, 8.1 Hz, 2'-H), 4.64 (1H, d, J=8.1 Hz, 1'-H), 4.59(1H, d, J=10.8 Hz, 27-H), 4.46 (2H, br d, J=10.8 Hz, 27-H' and 22-H), 4.36 (1H, d, J=10.3 Hz, 21-H), 4.23 (1H, dd, J=4.4, 10.3 Hz, 21-H'), 4.20 (2H, dt, J=2.6, 12.5 Hz, 6'-H), 3.69 (1H, dt, J=9.5, 2.6 Hz, 5'-H), 3.29 (1H, br d, J=21.6 Hz, 4-H), 2.83 (1H, dd, J=5.1, 21.6 Hz, 4-H').

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Communications to the Editor

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26,27-DIETHYL-1α,25-DIHYDROXYVITAMIN D_3 AND 24,24-DIFLUORO-24-HOMO-1α,25-DIHYDROXYVITAMIN D_3: HIGHLY POTENT INDUCER FOR DIFFERENTIATION OF HUMAN LEUKEMIA CELLS HL-60

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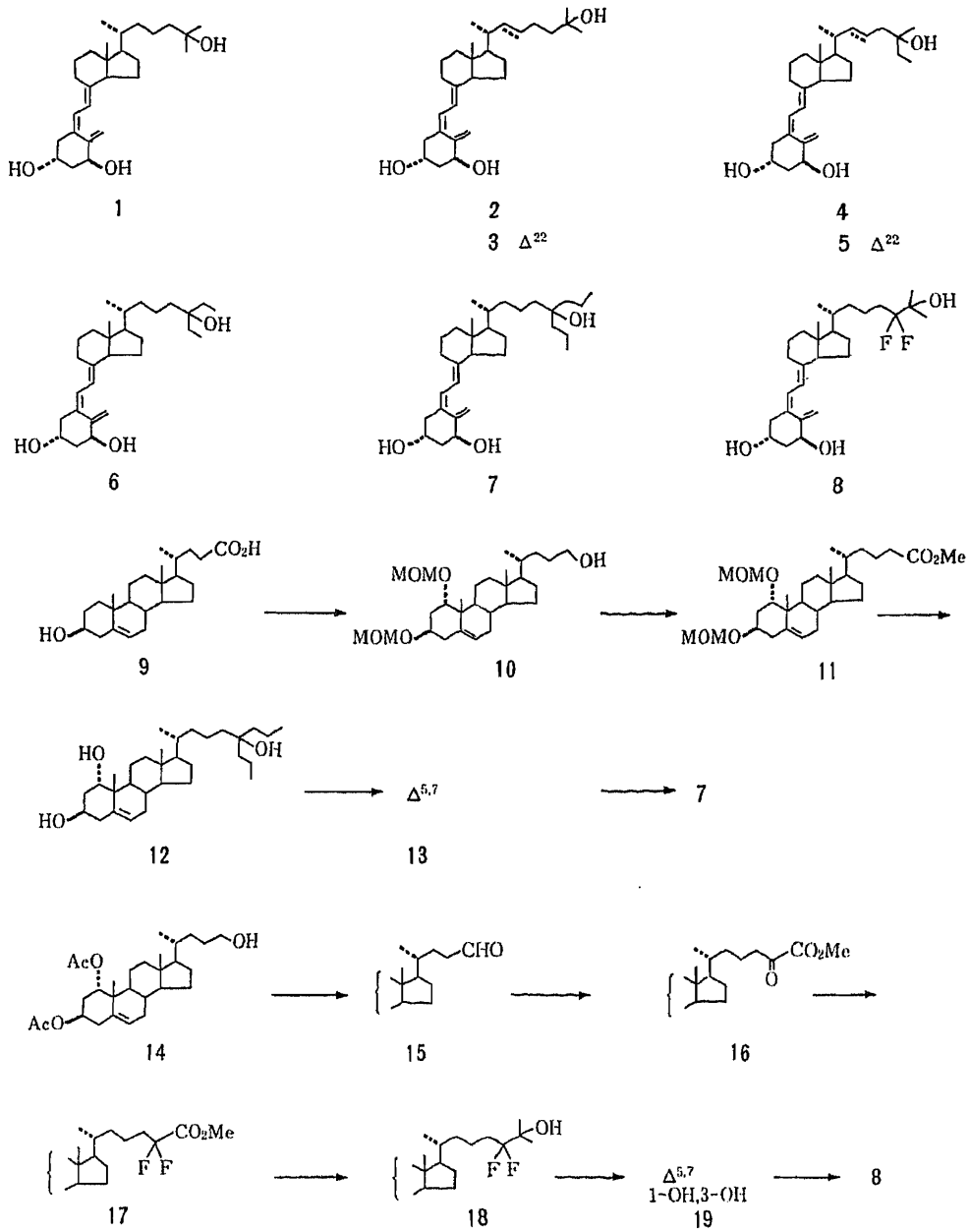
26,27-Diethyl-1α,25-dihydroxyvitamin D_3 (7) and 24,24-difluoro-24-homo-1α,25-dihydroxyvitamin D_3 (8) were synthesized. They had almost no vitamin D activity but were more active than 1α,25-dihydroxyvitamin D_3 (1) in tests for induction of cell differentiation.

KEYWORDS——vitamin D_3 analog; 1α,25-dihydroxyvitamin D_3; 26,27-diethyl-1α,25-dihydroxyvitamin D_3; 24,24-difluoro-24-homo-1α,25-dihydroxyvitamin D_3; cell differentiation; HL-60 cell

Since the discovery of 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) (1), the hormonal form of vitamin D_3, many analogs have been synthesized in order to obtain higher activity and to separate various biological activities.[2] The target organs for 1,25-$(OH)_2D_3$ (1) have been understood as intestine, bone and kidney. Additional target organs, though previously unrecognized, have been recently identified including brain, stomach, skin, pituitary, parathyroid, pancreas, etc.[3] A specific receptor for 1,25-$(OH)_2D_3$ (1) was also demonstrated in a number of tumor cells. 1,25-$(OH)_2D_3$ (1) also strongly induces cell differentiation in myeloid leukemia cells.[4] By testing many analogs of 1,25-$(OH)_2D_3$ (1) for activities, parallel activities were observed usually for the binding affinity to the receptor and for the induction of differentiation.[4] It seems likely that 1,25-$(OH)_2D_3$ (1) would induce severe hypercalcemia at the concentration required to induce myeloid differentiation *in vivo*. Therefore, separation of these activities is now greatly desired in the search for useful therapeutic agents.

Several compounds have a strong effect on cell differentiation, but weak or almost no calcium regulating effect. They are 1α,25,26-trihydroxy-Δ^{22}-vitamin D_3,[5] 19-nor-10-oxo-25-hydroxyvitamin D_3,[6] 26,26,26,27,27,27-hexafluoro-1α-hydroxyvitamin D_3,[7] and 20-oxa and 22-oxa vitamin D_3 analogs.[8]

During the course of our structure determination of a uniquely rearranged metabolite of 24-epi-1α,25-dihydroxyvitamin D_2, 24-homo- (2) and 26-homo-1α,25-



dihydroxyvitamin D_3 (4) and their Δ^{22} analogs (3 and 5) were synthesized.[9] These analogs were about ten-fold more potent than 1,25-$(OH)_2D_3$ (1) in inducing differentiation of HL-60 cells in vitro, but in the mobilization of bone calcium, the 24-homo analogs were significantly less active than 1,25-$(OH)_2D_3$ (1), whereas the 26-homo analogs were more active.[10]

In order to obtain further information on the structural requirement for cell differentiation and therapeutic possibility, 26,27-diethyl-1α,25-dihydroxyvitamin D_3 (7) and 24,24-difluoro-24-homo-1α,25-dihydroxyvitamin D_3 (8) were synthesized. Here we describe the synthesis of the compounds (7) and (8) and the results of their preliminary biological tests.

The diethyl analog (7) was synthesized from cholenic acid (9). Cholenic acid (9) was converted to the 24-ol (10) by the same reaction sequence as in our previous reports.[9,11] The 24-alcohol (10) was converted to the 25-methyl ester (11) *via* 24-cyanide (TsCl in pyridine, NaCN in DMSO at 70-80°C, KOH in aqueous EtOH at reflux, excess CH_2N_2) [oil, ν_{max} (neat) 1730 cm^{-1} (carbonyl)] in 54% yield. Grignard reaction of (11) with *n*-propylmagnesium bromide and subsequent acid hydrolysis gave the triol (12) [mp 76-79°C, MS *m/z*, 474 (M^+)] in 66% yield. After acetylation of (12), it was transformed into vitamin D_3 form by the standard method as follows. Bromination at 7-position with *N*-bromosuccinimide in refluxing CCl_4 and dehydrobromination with tetra-*n*-butylammonium fluoride gave a mixture of 4,6-diene and 5,7-diene. Saponification with 5% KOH-MeOH and subsequent preparative TLC afforded the pure 5,7-diene (13) λ_{max}(EtOH); 294, 282, and 271 nm, in 19% yield. Irradiation of the 5,7-diene (13) with a medium pressure mercury lamp through a Vycor filter in benzene-ethanol (2:1) at 0°C for 4 min and thermal isomerization of previtamin under reflux condition in the same solvent, after purification by preparative TLC and HPLC, gave the D_3 analog (7) in 16% yield.

The difluoro analog (8) was synthesized from the known alcohol (14).[12] The alcohol (14) was oxidized to the 24-aldehyde (15) by Swern oxidation. Wittig reaction of (14) with α-tetrahydropyranyloxyphosphonoacetate[13] and subsequent acid hydrolysis gave the keto-ester (16) [oil, δ($CDCl_3$) 2.74 (2H, t, *J*=6.6Hz, 24-H_2)] in 61% yield. Introduction of fluorine atoms into (16) was performed by treatment with DAST[14] in CH_2Cl_2 at r.t. to give the difluoro-ester (17) [oil, ν_{max} ($CHCl_3$) 1210 (F-C-F), 1720 (carbonyl) cm^{-1})] in 69% yield. Grignard reaction of (17) with methylmagnesium bromide followed by acetylation gave the 24-F_2-24-homo-25-ol (18) [oil, δ($CDCl_3$) 0.65 (3H, s, 18-H_3), 1.05 (3H, s, 19-H_3), 1.30 (6H, s, 26- and 27-H_3), 2.02 and 2.05 (6H, each s, acetyl), 4.91 (1H, m, 3-H), 5.08 (1H, m, 1-H), 5.54 (1H, m, 6-H)] in 60% yield. Transformation of (18) into vitamin D_3 form was accomplished by the standard method described above.

The synthetic analogs (7) and (8) had the following spectral data: (7); UV (EtOH) λ_{max}: 265 nm, λ_{min}: 228 nm, MS *m/z*, 472 (M^+), 454, 436, 410, 393, 269, 251, 152, 134. (8); UV (EtOH) λ_{max}: 265 nm, λ_{min}: 228 nm, MS *m/z*, 466 (M^+), 448, 430, 415, 407, 287, 269, 251, 134, 43.

26,27-Dimethyl-1α,25-dihydroxyvitamin D_3 (6) is slightly less active than 1,25-$(OH)_2D_3$ (1) in increasing serum calcium concentration.[11] The effects of this compound on the cell growth and differentiation of human promyecytic leukemia cells (HL-60 cells) *in vitro*[15] was about 2.5 times stronger than 1,25-$(OH)_2D_3$ (1); IC_{50} (concentrations required to inhibit cell growth by 50%) values: 10 nM for (6), and 25 nM for (1).[16]

Surprisingly, both the 26,27-diethyl analog (7) and the 24,24-F_2-homo analog (8) had almost no vitamin D activity, but induced differentiation of HL-60 cells into monocyte-macrophage. Furthermore, these compounds were at least 10 fold more active than 1,25-$(OH)_2D_3$ (1) for cell growth inhibition of HL-60 cells. The IC_{50} values for (7) and (8) were 2 nM and 2-3 nM, respectively, but 25 nM for (1).

It can be concluded that for the vitamin D activity, (intestinal calcium absorption, bone calcium mobilization), the distance between the two hydroxyl groups at the 1- and 25-positions may be critical. Furthermore, the alkyl

substituents at the 25-position should be methyl or ethyl groups. Thus, if the 25-position is substituted by fluorine atoms, the vitamin D activity is extinguished as shown in the 24,24-F_2-24-homo analog (8) and 25-fluoro-1-hydroxyvitamin D_3.[17] On the other hand, the lengthening the side chain by one carbon significantly increases the activity against HL-60 cells. The lengthening at the 26 and 27-positions also causes potent activity for leukemia cells.

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REFERENCES AND NOTES

1) Present address: Iwaki Meisei University, Iwaki New-Town, Iwaki, Fukushima 970, Japan.
2) N. Ikekawa, Medicinal Research Reviews, 7, 333 (1987).
3) A. W. Norman, J. Roth, and L. Orch, Endoc. Rev., 3, 331 (1982).
4) T. Suda, C. Miura, E. Abe, and T. Kuroki, "Bone and Mineral Research," Ed. W. A. Peck, Elsevier Science Publisher B. V., 4, p. 1 (1986).
5) P. M. Wovkulich, A. D. Batcho, E. G. Baggiolini, A. Boris, G. Truitt, and M. R. Uskokovic, "Vitamin D, A Chemical, Biological and Clinical Update," Ed. A. W. Norman et al., de Gruynter, p. 755 (1985).
6) T. K. Gray, D. S. Millington, D. A. Maltby, M. E. Williams, M. S. Cohen, and R. C. Dodd, Proc. Natl. Acad. Sic. U.S.A., 82, 8218 (1985).
7) M. Inaba, K. Yukioka, Y. Nishizawa, S. Okuno, S. Otani, S. Morisawa, H. F. DeLuca, and H. Morii, "Calcium Regulation and Bone Metabolism: Basic and Clinical Aspects," Ed. D. V. Cohn et al., Elsevier Science Publisher B. V., 9, p. 523 (1987).
8) a) N. Kubodera, K. Miyamoto, K. Ochi, and I. Matsunaga, Chem. Pharm. Bull., 54, 2286 (1986); b) E. Maruyama, K. Miyamoto, N. Kubodera, T. Mori, and I. Matsunaga, Chem. Pharm. Bull., 34, 4410 (1986).
9) H. Sai, S. Takatsuto, N. Ikekawa, Y. Tanaka, C. Smith, and H. F. DeLuca, Chem. Pharm. Bull., 32, 3866 (1984).
10) V. K. Osterm, Y. Tanaka, J. Prahl, H. F. DeLuca, and N. Ikekawa, Proc. Natl. Acad. Sci. U.S.A., 84, 2610 (1987).
11) Although we previously reported that compound (6) is a highly active analog of 1,25-$(OH)_2$ D_3 (1) (H. Sai, S. Takatsuto, N. Hara, and N. Ikekawa, Chem. Pharm. Bull., 33, 878 (1985)), the subsequent study indicated that this is not the case. This observation is being under investigated.
12) Y. Kobayashi, T. Taguchi, S. Mitsuhashi, T. Eguchi, E. Ohshima, and N. Ikekawa, Chem. Pharm. Bull., 30, 4297 (1982).
13) E. Nakamura, Tetrahedron Lett., 1981, 663.
14) W. J. Middleton and E. M. Bingham, J. Org. Chem., 45, 2883 (1983).
15) A. Honda, I. Morita, S-I. Murota, and Y. Mori, Biochim. Biophys. Acta, 877, 423 (1986).
16) N. Ikekawa, S. Takatsuto, H. Sai, N. Hara, A. Honda, Y. Mori, S. Higuchi, M. Muramatsu, S. Otomo, and H. Aihara, "Calcium Regulation and Bone Metabolism: Basic and Clinical Aspects," Ed. D. V. Cohn et al., Elsevier Science Publisher B. V., 9, p. 598 (1987).
17) a) J. L. Napoli, M. A. Fivizzani, H. K. Schnoes, and H. F. DeLuca, Biochemistry, 17, 2387 (1978); b) J. L. Napoli, H. A. Hamstra, H. K. Schnoes, H. F. DeLuca, and P. H. Stern, Steroids, 32, 2322 (1978).

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A NEW BROMINATED NAPHTHOQUINONE FROM *DIOSPYROS MARITIMA* BLUME

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From the fresh fruit of *Diospyros maritima* Blume a new brominated naphthoquinone, 3-bromoplumbagin (1), was isolated, besides three known naphthoquinones, 3-chloroplumbagin (2), plumbagin (3), and droserone (4). The structure of the new compound was established by spectral analysis and synthesis.

KEYWORDS —— 3-bromoplumbagin; 3-chloroplumbagin; plumbagin; droserone; naphthoquinone; brominated naphthoquinone; *Diospyros maritima*; Ebenaceae

Diospyros maritima Blume (Japanese name; ryukyugaki)(Ebenaceae) is a shrub growing in south-east Asia. Its fruit is 2-3 cm in diameter in spring. The isolation of plumbagin, elliptinone, maritinone, isoshinanolone, scopoletin, lupeol, betulin and betulinic acid from the bark and roots has been reported.[1,2] Here we report a new brominated naphthoquinone from the fruit of the same plant.

The ethanol extract of the fresh fruit afforded, after column chromatographic separation, four naphthoquinones 1, 2, 3, and 4.

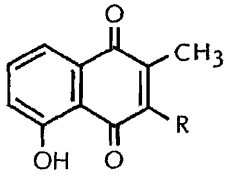
The quinone (3), orange needles of mp 75-76°C from hexane, which is the major constituent is plumbagin,[3-5] UV $\lambda_{max}^{CHCl_3}$ nm(log ε): 252sh(4.04), 267(4.10), 422 (3.60); IR ν_{max}^{KBr} cm^{-1}: 1665, 1645; ^{1}H-NMR δ($CDCl_3$): 2.13(3H, d, J=1.5 Hz, quinonoid CH_3), 6.67(1H, q, J=1.5 Hz, quinonoid H), 7.0-7.6(3H, m, Ar H), 11.83(1H, s, bonded OH).

The quinone (4), orange needles of mp 180-182°C from acetone-pet. ether, is droserone,[4,5] UV $\lambda_{max}^{CHCl_3}$ nm(log ε): 244(4.05), 286(4.13), 417(3.74); IR ν_{max}^{KBr} cm^{-1}: 3330, 1624; ^{1}H-NMR δ($CDCl_3$): 2.10(3H, s, quinonoid CH_3), 7.0-7.9(3H, m, Ar H), 7.25(1H, s, quinonoid OH), 11.10(1H, s, bonded OH). This is the first report of the isolation of droserone from Ebenaceae.

The quinone (2), orange leaflets of mp 123-125°C from hexane, Beilstein test (+), is 3-chloroplumbagin, MS *m/z*(%): 224(M^++2, 39), 222(M^+, 100), 187(M^+-Cl, 84); UV $\lambda_{max}^{CHCl_3}$ nm(log ε): 246(3.79), 280(4.09), 429(3.59); IR ν_{max}^{KBr} cm^{-1}: 1630, 1655; ^{1}H-NMR δ($CDCl_3$): 2.32(3H, s, quinonoid CH_3), 7.1-7.7(3H, m, Ar H), 11.71(1H, s, bonded OH). 3-Chloroplumbagin is quite rare.[4,7] So far it has only been isolated from the roots of *Plumbago zeylanica*[3] and from *Drosera intermedia* and *D. anglica*.[6]

The quinone (1), orange needles of mp 121-122°C from hexane, Beilstein test (+), has a molecular formula $C_{11}H_7O_3Br$ (M^+ 266, M^++2 268. Anal. Calcd: C, 49.47; H, 2.64. Found: C, 49.59; H, 2.54). The IR (ν_{max}^{KBr} cm^{-1}: 1655, 1631, 1593) and UV

[$\lambda_{max}^{CHCl_3}$ nm(log ε): 241(3.89), 287(4.13), 429(3.69)] spectra showed the characteristics of juglone derivatives. The MS spectrum exhibited a molecular ion peak at *m/z* 266 with a satellite peak at *m/z* 268. The relative intensities of M^++2 to M^+ as well as those of the M^+-CO peak (*m/z* 238) to its satellite peak (*m/z* 240) were 97%, which indicates the presence of one bromine atom in 1. The ^{1}H-NMR spectrum (in $CDCl_3$) revealed the presence of one hydrogen bonded hydroxyl [δ 11.77(1H, s)], one quinonoid methyl [δ 2.36(3H, s)] and three aromatic protons [δ 7.1-7.7(3H, m)] in 1. Since the absence of the allylic coupling in the quinonoid methyl group indicates that the 3-position is occupied by a substituent, the new quinone (1) must be 3-bromoplumbagin. The alternative 3-methyljuglone structure was excluded on biogenetical grounds. The final assignment of the structure has been accomplished by the following synthesis. The bromination of plumbagin (3) with bromine in tetrachlorocarbon afforded a dibromide (5), colorless plates of mp 92-93°C from benzene, which is assumed to be the racemate of *trans*-2,3-dibromide, MS *m/z*(%): 350(M^++4, 15), 348(M^++2, 31), 346(M^+, 17); IR ν_{max}^{KBr} cm^{-1}: 1700, 1650; ^{1}H-NMR δ ($CDCl_3$): 2.18(3H, s, CH_3), 5.01(1H, s, C_3-H), 7.2-7.8(3H, m, Ar H), 11.30(1H, s, bonded OH). The dehydrobromination of the dibromide (5) with sodium acetate in acetic acid afforded 3-bromoplumbagin, mp 121-122°C, and it was identical with the natural product. The isolation of brominated compounds from non-marine sources is extremely rare[7] and 1 is the first example of a brominated naphthoquinone isolated as a plant constituent.



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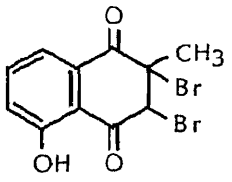
3 R = H 4 R = OH



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REFERENCES AND NOTES

1) Th. M. Meijer, Rec. Trav. Chim. Pays-Bas, 66, 193 (1947).
2) M. Tezuka, C. Takahashi, M. Kuroyanagi, M. Satake, K. Yoshihira, and S. Natori, Phytochemistry, 12, 175 (1973).
3) G. S. Sidhu and A. V. B. Sankaram, Tetrahedron Lett., 1971, 2385.
4) R. H. Thomson, "Naturally Occurring Quinones," 2nd ed., Academic Press Inc., London, 1971.
5) J. R. Cannon, V. Lojanapiwatna, C. L. Raston, W. Sinchai, and A. H. White, Aust. J. Chem., 33, 1073 (1980).
6) G. Bendz and G. Lindberg, Acta Chem. Scand., 22, 2722 (1968).
7) J. F. Suida and J. F. DeBernardis, Lloydia, 36, 107 (1973).

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Communications to the Editor

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AN EFFICIENT 3(C)-ACYLATION OF TETRAMIC ACIDS INVOLVING ACYL MIGRATION OF 4(O)-ACYLATES

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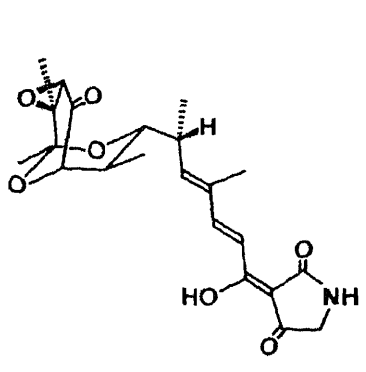
3(C)-Acylation of 3-unsubstituted tetramic acids with carboxylic acid has been achieved by two sequential reactions in one flask: (1) 4(O)-acylation in the presence of DCC and a catalytic amount of DMAP, and (2) 4(O)→3(C) acyl-migration promoted by adding Et_3N.

KEYWORDS ——— tetramic acid; acylation; carboxylic acid; phosphonoacetic acid; acyl-migration

In connection with the total syntheses of triandamycin A (1), ikarugamycin (2), and their congeners,[1] there have been reported various methods for synthesizing the 3-acyltetramic acid structure. Tetramic acids (pyrrolidine-2,4-diones) can be directly 3(C)-acylated in moderate to good yields with carboxylic chloride in the presence of Lewis acid (BF_3-Et_2O or $TiCl_4$, heating)[2]. But this method is apparently not suitable for those carboxylic acid which are sensitive to harsh reaction conditions. Base-induced acylation, on the other hand, has been reported to result in predominant formation of 4-O-acyltetramic acid.[3] For the construction of the tetramic acid units in 1 and 2, it has been introduced in a novel method[4] that employs Emmons-Horner reaction of the (phosphonoacetyl)tetramic acid 3.[5] We report herein a new and efficient method for the direct 3(C)-acylation of 5 which involves an acyl migration of the kinetic O-acylate induced by 4-N,N-dimethylaminopyridine (DMAP).



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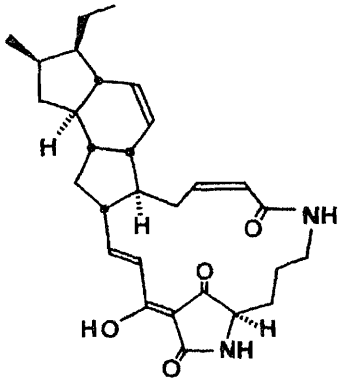

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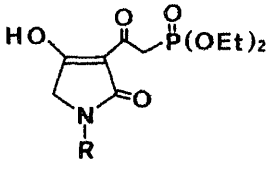
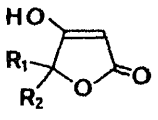
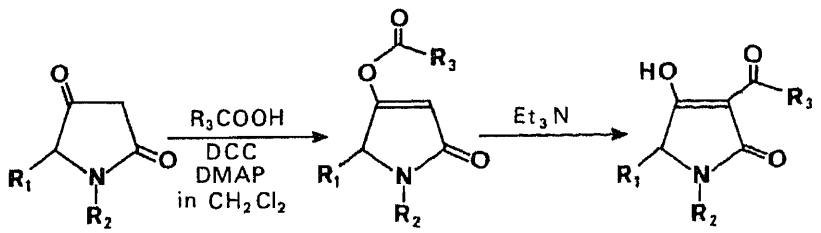


3 (R=H, $ArCH_2$)



4

We recently demonstrated[6] that tetronic acid (4) can be nicely acylated with carboxylic acid at the 3-position in the presence of dicyclohexylcarbodiimide (DCC), DMAP (0.3 eq) and triethylamine (1.1 eq) in CH_2Cl_2. The reaction proceeds via kinetic O-acyl intermediate. This prompted us to investigate whether the same experimental protocol can be utilized for 3(C)-acylation of tetramic acids which are less acidic (pKa 6.4-7.1).[7] A preliminary experiment using 5-benzyltetramic acid (5a) and hexanoic acid resulted in formation of a complex mixture of products, from which the desired 5-benzyl-3-hexanoyltetramic acid (7a)(R= n-C_5H_{11}) was isolated in low yield (15-20%) after chromatography. A serious side reaction observed here was N-acylation and a double-bond migration in the O-acylate 6a to the 5(exo) position.[8] Fortunately, however, the yield of the acyltetramic acid could be greatly improved up to 95% by conducting the reaction in a stepwise manner in one flask: (1) formation of kinetic O-acylate mediated with 1.1 eq DCC and ca. 0.1 eq DMAP and (2) subsequent slow O(4)→C(3) acyl migration induced by addition of 1.1 eq triethylamine. This technique, which can be easily controlled by thin layer chromatography, was equally effective for 3-alkanoylation of both N-substituted and unsubstituted tetramic acids (5a-d)[9] (Table I).[10] 2-Butenoylation and 2,4-hexadienoylation should be carried out at low temperatures (0° to 8°C) to minimize side reactions,[11] giving modest yields (37 - 50%) of the corresponding 3-acyltetramic acids.



	R_1	R_2
a	$C_6H_5CH_2$	H
b	$(CH_3)_2CH$	H
c	$(CH_3)_2CH$	CH_3
d	H	$C_6H_5CH_2$

Table I. 3-Acyltetramic Acids[17]

	R_3	% yield[a]	mp/bp(°C)		R_3	% yield[a]	mp/bp(°C)
7a	n-C_5H_{11}	95	126-8	7c	n-C_5H_{11}	89	oil
	$C_6H_5CH_2$	83	143-6		$(EtO)_2P(O)CH_2$	(ca.80)	oil
	$(CH_3)_2CH$	58	125-8				
	$CH_3CH=CH$	50	198-203	7d	n-C_5H_{11}	72	57
	$CH_3CH=CH-CH=CH$	50	201-3		$CH_3CH=CH$	37	81-3
	$(EtO)_2P(O)CH_2$	84	oil		$CH_3CH=CH-CH=CH$	49	100-2
					$(EtO)_2P(O)CH_2$	84(96)	oil
7b	n-C_5H_{11}	88	85-95/0.04				
	cyclo-C_3H_5	87	129-131				
	$C_6H_5CH_2$	86	68-73				
	$CH_3CH=CH-CH=CH$	50	153-7				
	$(EtO)_2P(O)CH_2$	73(97)	105-8				

a) Isolated yield by distillation or silica gel chromatography. Yield determined by proton NMR (270 MHz) spectral analysis of the crude product is indicated in parenthesis.

Since acylation with the conjugated acids was less efficient than alkanoylation, we turned our attention to (diethoxyphosphinyl)acetylation in the light of the literature precedent that the compound 3 can serve as an excellent precursor of enoyl and dienoyl tetramic acids.[4] The reaction of $(EtO)_2P(O)CH_2COOH$[12,13] with N-benzyltetramic acid (**5d**) occurred smoothly to give **7d** [R_3 = $(EtO)_2P(O)CH_2CO$] in 96% yield after an acid-base extraction (0.15 M Na_3PO_4/HCl) of the reaction mixture. The (phosphinyl)acetylation of **5a-c** was also achieved in high yields (Table I).

In conclusion, we could have introduced an efficient method for 3(C)-acylation of tetramic acids that can be performed under weakly basic conditions. It is also significant that the present method provides a ready access to 3-(diethoxyphosphinyl)acetyl tetramic acids which are useful intermediates in the synthesis of a variety of 3-alkenoyl derivatives.

A typical experimental procedure: A stirred mixture of **5a** (80 mg, 0.42 mmol) and DMAP (10 mg, 0.082 mmol) in dry dichloromethane (3 ml) at 0°C (an ice-water bath) was treated sequentially with hexanoic acid (58 µl, 0.46 mmol) and DCC (105 mg, 0.50 mmol). The cooling bath was removed after 10 min, and stirring of the mixture at room temperature was continued for 1.5 h to complete formation of O-acylate **6a** (R_3 = C_5H_{11}) [essentially one spot on a silica gel plate, R_f = 0.58 ($CHCl_3$/MeOH = 19:1)]. The resulting suspension (dicyclohexylurea) was cooled to 0°C again before adding triethylamine (71 µl, 0.50 mmol) and, after 10 min, it was stirred at room temperature for 4.5 h. The reaction mixture was filtered with suction, and the filtrate was washed with 10% HCl and brine. The organic layer was dried over anhydrous $MgSO_4$ and concentrated with a rotary evaporator. The solid residue was subjected to column chromatography (KH_2PO_4-treated silica gel,[15] 8 g; solvent, benzene/AcOEt = 4:1) to give **7a** (R_3 = C_5H_{11}) (115 mg, 95%) as a white solid, which was homogeneous on KH_2PO_4-impregnated silica gel thin-layer,[16] R_f = 0.42 (benzne/AcOEt = 4:1). An analytical sample was obtained by recrystallization from diisopropyl ether, mp 126-128°C.

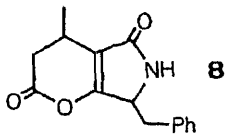
REFERENCES AND NOTES

1) Synthetic study on 3-acyltetramic acid antibiotics: a) R. E. Ireland and R. B. Wardle, J. Org. Chem., 52, 1780(1987); b) R. K. Boeckman, Jr., J. C. Potenza, and E. J. Enholm, ibid., 52, 469(1987); c) C. Neukom, D. P. Richardson, J. H. Myerson, and P. A. Bartlett, J. Am. Chem. Soc., 108, 5559(1986); d) R. K. Boeckman, Jr., J. E. Starrett, Jr., D. G. Nickell, and P.-E. Sum, J. Am. Chem. Soc., 108, 5549(1986); e) R. K. Boeckman, Jr. and R. B. Perni, J. Org. Chem., 51, 5486(1986); f) P. DeShong, S. Ramesh, V. Elango, and J. J. Perez, J. Am. Chem. Soc., 107, 5219(1985); g) R. H. Schlessinger, G. R. Bebernitz, and P. Lin, J. Am. Chem. Soc., 107, 1777(1985).

2) R. C. F. Jones and G. E. Peterson, Tetrahedron Lett., 24, 4757(1983); R. C. F. Jones and S. Sumaria, ibid., 3173(1978).

3) S. Toda, S. Nakagawa, T. Naito, and H. Kawaguchi, J. Antibiot., 33, 173(1980); V. J. Lee, A. R. Branfman, T. R. Herrin, and K. L. Rinehart, Jr., J. Am. Chem. Soc, 100, 4225(1978); J. L. van der Baan, J. W. F. K. Barnick, and F. Bickelhaupt, Tetrahedron, 34, 223(1978).

4) R. H. Schlessinger and G. R. Bebernitz, J. Org. Chem., 50, 1344(1985); R. K. Boeckman, Jr. and A. J. Thomas, ibid., 47, 2823(1982).
5) Other synthetic methods of 3-acyltetramic acid structure: R. C. F. Jones and A. D. Bates, Tetrahedron Lett., 28, 1565(1987); R. C. F. Jones and G. E. Peterson, ibid., 24, 4751(1983); P. DeShong, N. E. Lowmaster, and O. Bavalt, J. Org. Chem., 48, 1149(1983).
6) K. Nomura, K. Hori, M. Arai, and E. Yoshii, Chem. Pharm. Bull., 34, 5188(1986).
7) T. P. C. Mulholland, R. Foster, and D. B. Haydock, J. Chem. Soc. Perkin Trans. 1, 2121(1972).
8) R. C. F. Jones and G. E. Peterson, Tetrahedron Lett., 24, 4755(1983).
9) Compounds 5a-c are racemic.
10) O-Acylate intermediates (6) can be isolated in excellent yields.
11) In the 2-butenoylation of 5a, a bicyclic compound 8 was isolated, presumably formed by an internal Michael reaction of 6a.



8

12) We prepared this compound by the following sequence.

$$BrCH_2COOH + (CH_3)_2C{=}C(OMe)OSiEt_3 \xrightarrow[98\%]{14)} BrCH_2COOSiEt_3 \xrightarrow[84\%]{(EtO)_3P,\ 95\text{-}100^{\circ}C}$$

$$(EtO)_2P(O)CH_2COOSiEt_3 \xrightarrow[62\%]{HF,\ MeCN} (EtO)_2P(O)CH_2COOH$$

13) M. P. Cooke, Jr. and K. P. Biciunas, Synthesis, 283(1981); J. P. Clayton, K. Luk, and N. H. Rogers, J. Chem. Soc. Perkin Trans. 1, 308(1979).
14) E. Yoshii and K. Takeda, Chem. Pharm. Bull., 31, 4586(1983).
15) W. W. Lee, A. P. Martinez, T. H. Smith, and D. W. Henry, J. Org. Chem., 41, 2296(1976).
16) J. St. Pyrek, O. Achmatowicz, Jr., and A. Zamojski, Tetrahedron, 33, 673(1977).
17) All compounds were characterized by ^{1}H-NMR and mass spectroscopy, and crystalline compounds gave satisfactory combustion analyses (C, H).

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Communications to the Editor

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SYNTHESIS OF 7-METHYL-3-β-D-RIBOFURANOSYLWYE, THE MOST PROBABLE STRUCTURE FOR THE FLUORESCENT NUCLEOSIDE ISOLATED FROM ARCHAEBACTERIAL TRANSFER RIBONUCLEIC ACIDS

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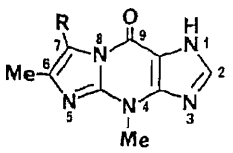
3-β-D-Ribofuranosylwye (**2a**) was converted into 7-methyl-3-β-D-ribofuranosylwye (**2b**) through the 2',3',5'-tri-*O*-acetate by formylation with $POCl_3$ and $HCONMe_2$ followed by catalytic hydrogenolysis.

KEYWORDS —— fluorescent nucleoside; tricyclic nucleoside; archaebacterium tRNA; Vilsmeier-Haack formylation; catalytic hydrogenolysis; glycosidic bond cleavage; acidic hydrolysis

Since the finding[1] of the fluorescent component at the position adjacent to the 3'-end of the anticodon of yeast phenylalanine transfer ribonucleic acid ($tRNA^{Phe}$), five tricyclic bases have been obtained from eukaryotic $tRNAs^{Phe}$ and their structures have been elucidated as **1a**,[2] **1c**,[3] **1d**,[4] **1e**,[5] and **1f**.[6,7] The parent nucleosides of **1a**[8] and **1c**[1,9] have been isolated from yeast $tRNAs^{Phe}$ and 3-β-D-ribofuranosylwye (**2a**), the most probable structure for the former, has been synthesized.[10] Recently, McCloskey and coworkers[11] found a new fluorescent component in unfractionated tRNAs of three extremely thermophilic archaebacteria and assigned 7-methylwye (**1b**) to the structure of the base by direct comparison with an authentic sample,[3c] which had been synthesized by us. Quite recently, Chattopadhyaya *et al.* reported the synthesis of 7-methyl-3-β-D-ribofuranosylwye (**2b**),[10e] the proposed structure[11] for the new fluorescent nucleoside. This paper presents an alternative synthesis of **2b**.

Probably, the most straightforward synthesis of **2b** is cyclocondensation of 3-methylguanosine[10a,b,c,12] with AcCHBrMe. This method, however, did not work effectively. We have reported the synthesis of **1b** by the Vilsmeier-Haack reaction of 1-benzylwye followed by reduction with $NaBH_4$ and hydrogenolysis over Pd–C.[3c] This procedure was now applied successfully to the nucleoside level as follows.

Acetylation of **2a** with Ac_2O and pyridine followed by treatment with a mixture of $POCl_3$ and $HCONMe_2$ at −25°C for 4 h gave the aldehyde **3** as a glass, ^{1}H-NMR ($CDCl_3$) δ: 10.83 (s, CHO), in 92% yield. Although reduction of **3** with $NaBH_4$ in tetra-



1

a: R = H wye

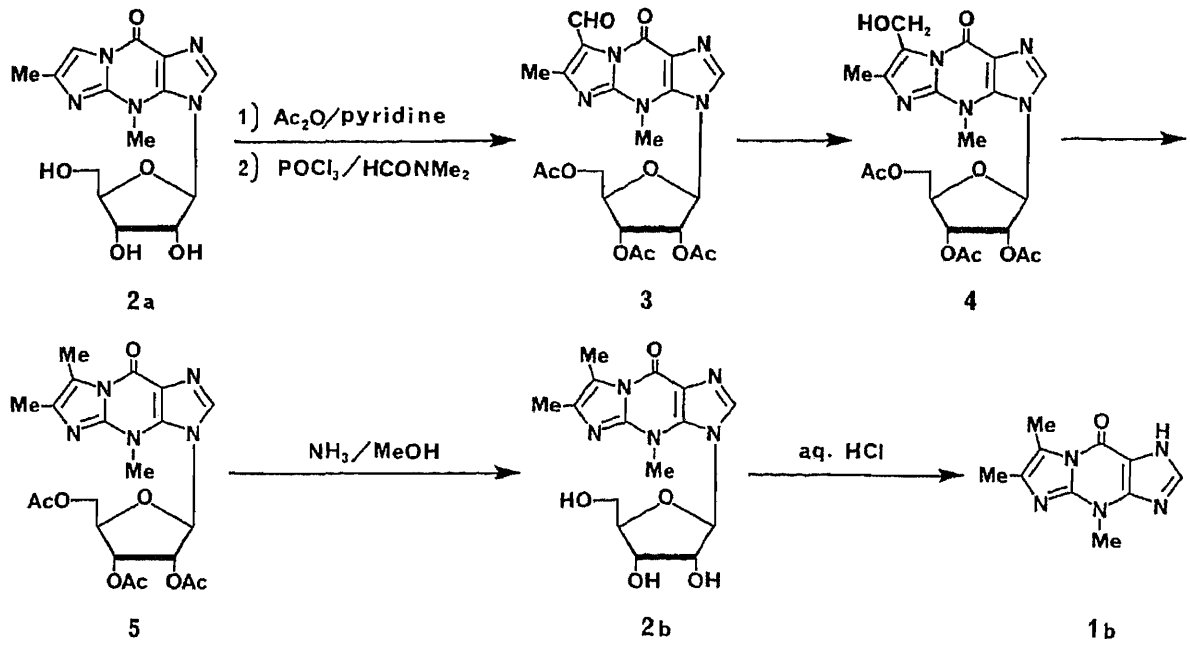
b: R = Me

c: R = CH_2CH_2–C(H, CO_2Me, $NHCO_2Me$)

d: R = $CH_2CH(OH)CHCO_2Me$ ($NHCO_2Me$)

e: R = $CH_2CH(O_2H)CHCO_2Me$ ($NHCO_2Me$)

f: R = $CH_2CH(OH)CHCO_2H$ (NH_2)



hydrofuran gave the alcohol **4**, the yield was poor, probably owing to partial migration of an acetyl group to the oxygen of the 7-hydroxymethyl group of **4**. Compound **3** was then directly submitted to catalytic reduction over Pd–C in EtOH to give **5** as a glass, ^{1}H-NMR ($CDCl_3$) δ: 2.11 (s, Ac), 2.15 (s, Ac), 2.18 [s and q, Ac and C(6)Me], 2.63 [q, J = 0.7 Hz, C(7)Me], 4.10 (s, NMe), 4.31 [d, J = 3 Hz, C(5')H_2], 4.49 [m, J = 3 and 4 Hz, C(4')H], 5.48 [d-d, J = 4 and 5 Hz, C(3')H], 5.85 [d-d, J = 5 and 6 Hz, C(2')H], 6.21 [d, J = 6 Hz, C(1')H], 7.66 [s, C(2)H]; CD (H_2O) $[\theta]^{20}_{232}$ +6700, in 32% yield. Deprotection of **5** was performed by treating with saturated methanolic NH_3 at 0°C for 5 h to afford **2b** as colorless needles (from H_2O), mp ca. 190°C (dec.); UV $\lambda^{95\%\ EtOH}_{max}$ 239 nm (ε 28800), 298 (5700); $\lambda^{H_2O}_{max}$ (pH 2) 233 (31300), 279 (10000); $\lambda^{H_2O}_{max}$ (pH 7) 241 (29800), 302 (5300); $\lambda^{H_2O}_{max}$ (pH 13) 241 (30800), 302 (5500); ^{1}H-NMR [$(CD_3)_2SO$] δ: 2.11 [q, J = 0.7 Hz, C(6)Me], 2.56 [q, J = 0.7 Hz, C(7)Me], 3.63 [m, C(5')H_2], 3.98 [m, C(4')H], 4.01 (s, NMe), 4.12 [m, C(3')H], 4.45 [m, C-(2')H], 5.11 (br, 5'-OH), 5.30 (d, J = 5 Hz, 3'-OH), 5.69 (d, J = 6 Hz, 2'-OH), 6.08 [d, J = 5 Hz, C(1')H], 8.17 [s, C(2)H]; CD (H_2O) $[\theta]^{20}_{244}$ −7300, in 72% yield. Compound **2b** underwent hydrolysis of the glycosidic bond at the rate (rate constant 4.7×10^{-1} min^{-1}, half-life 88 s) comparable to that (4.4×10^{-1} min^{-1})[10b,c] of **2a** in 0.1 N aq. HCl at 25°C to give **1b**[3c] in 86% yield, confirming the correctness of the structure **2b**.

Although the ultimate identification of the nucleoside from natural sources[11] was difficult owing to the extremely minute amounts available, it was identical with the synthetic one according to fast atom bombardment mass spectrometry and high performance liquid chromatography.[13,14] These results further support the proposal that the structure of the fluorescent nucleoside from the archaebacterial tRNAs is **2b**.[11]

It appears that analogues of **2b** oxidized at the 7-methyl group to a variable extent may be components of tRNAs of some species. The present synthesis has an advantage over the reported procedure[10e] in that it comprises a potential for synthesizing these nucleosides.

ACKNOWLEDGMENT The author is grateful to Professors J. A. McCloskey and T. Hashizume, University of Utah, for mass spectral and chromatographic comparison of **2b** with the natural nucleoside.

REFERENCES AND NOTES

1) U. L. RajBhandary, R. D. Faulkner, and A. Stuart, J. Biol. Chem., 243, 575 (1968).
2) a) H. Kasai, M. Goto, S. Takemura, T. Goto, and S. Matsuura, Tetrahedron Lett., 1971, 2725; b) H. Kasai, M. Goto, K. Ikeda, M. Zama, Y. Mizuno, S. Takemura, S. Matsuura, T. Sugimoto, and T. Goto, Biochemistry, 15, 898 (1976).
3) a) K. Nakanishi, N. Furutachi, M. Funamizu, D. Grunberger, and I. B. Weinstein, J. Am. Chem. Soc., 92, 7617 (1970); b) R. Thiebe, H. G. Zachau, L. Baczynskyj, K. Biemann, and J. Sonnenbichler, Biochim. Biophys. Acta, 240, 163 (1971); c) T. Itaya and A. Mizutani, Tetrahedron Lett., 26, 347 (1985).
4) H. Kasai, Z. Yamaizumi, Y. Kuchino, and S. Nishimura, Nucleic Acids Res., 6, 993 (1979).
5) a) K. Nakanishi, S. Blobstein, M. Funamizu, N. Furutachi, G. Van Lear, D. Grunberger, K. W. Lanks, and I. B. Weinstein, Nature, New Biol., 234, 107 (1971); b) S. H. Blobstein, D. Grunberger, I. B. Weinstein, and K. Nakanishi, Biochemistry, 12, 188 (1973); c) D. Yoshikami and E. B. Keller, ibid., 10, 2969 (1971); d) A. M. Feinberg, K. Nakanishi, J. Barciszewski, A. J. Rafalski, H. Augustyniak, and M. Wiewiórowski, J. Am. Chem. Soc., 96, 7797 (1974); e) A. Mochizuki, Y. Omata, and Y. Miyazawa, Bull. Chem. Soc. Jpn., 53, 813 (1980); f) M. Barciszewska, M. Kaminek, J. Barciszewski, and M. Wiewiórowski, Plant Sci. Lett., 20, 387 (1981).
6) Y. Kuchino, H. Kasai, Z. Yamaizumi, S. Nishimura, and E. Borek, Biochim. Biophys. Acta, 565, 215 (1979).
7) For a recent review, see T. Itaya, Yuki Gosei Kagaku Kyokai Shi, 45, 431 (1987).
8) S. Takemura, H. Kasai, and M. Goto, J. Biochem., 75, 1169 (1974).
9) a) R. Thiebe and H. G. Zachau, Eur. J. Biochem., 5, 546 (1968); b) S. H. Blobstein, R. Gebert, D. Grunberger, K. Nakanishi, and I. B. Weinstein, Arch. Biochem. Biophys., 167, 668 (1975).
10) a) S. Nakatsuka, T. Ohgi, and T. Goto, Tetrahedron Lett., 1978, 2579; b) T. Itaya, T. Watanabe, and H. Matsumoto, J. Chem. Soc., Chem. Commun., 1980, 1158; c) T. Itaya, H. Matsumoto, T. Watanabe, and T. Harada, Chem. Pharm. Bull., 33, 2339 (1985); d) B. Golankiewicz and W. Folkman, Nucleic Acids Res., 11, 5243 (1983); e) H. Bazin, X-X. Zhou, C. Glemarec, and J. Chattopadhyaya, Tetrahedron Lett., 28, 3275 (1987).
11) J. A. McCloskey, P. F. Crain, C. G. Edmonds, R. Gupta, T. Hashizume, D. W. Phillipson, and K. O. Stetter, Nucleic Acids Res., 15, 683 (1987).
12) T. Itaya and K. Ogawa, Tetrahedron Lett., 1978, 2907.
13) The comparison was done by J. A. McCloskey's group at the University of Utah.
14) Liquid chromatographic analysis was carried out according to reported procedure: M. Buck, M. Connick, and B. N. Ames, Anal. Biochem., 129, 1 (1983).

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Communications to the Editor

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A NEW MARINE EPOXY PROSTANOID WITH AN ANTIPROLIFERATIVE ACTIVITY FROM THE STOLONIFER CLAVULARIA VIRIDIS QUOY AND GAIMARD

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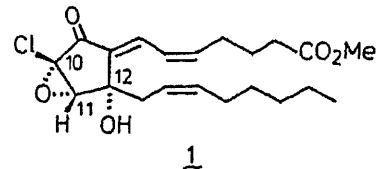
A new marine epoxy prostanoid 1 with an antiproliferative activity was isolated from the Japanese stolonifer Clavularia viridis Quoy and Gaimard. The structure of 1 was established on the basis of spectroscopy and chemical transformation.

KEYWORDS —— marine prostanoid; epoxy prostanoid; Clavularia viridis; stolonifer; antiproliferative activity; HL-60 leukemia cell

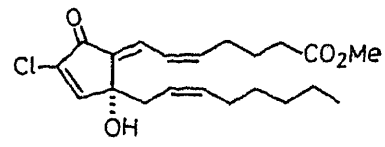
Recently octocoral-derived marine prostanoids, clavulones[1] (claviridenones)[2] and their halogenated congeners,[3] have received much interest owing to their unique structural features and great antitumor activities.[3a,c,d,4] During our continuing investigations on marine prostanoids from the Japanese stolonifer Clavularia viridis Quoy and Gaimard from which clavulones and their halogenated congeners were found, we have isolated a new antiproliferative prostanoid 1, which is the first encounter of a natural prostanoid having a 1,2-epoxide ring. This paper describes the isolation and structure of 1.

The freeze-dried organisms (1.2 kg) of Clavularia viridis[5] were extracted with hexane. The hexane extract (22.6 g) was repeatedly chromatographed on a silica gel column using a hexane-ethyl acetate mixture as an eluent. This gave the eluate containing the compound 1 and other halogenated prostanoids (chlorovulones,[3a] bromovulone I,[3c] and iodovulone I[3c]) prior to the elution of clavulones.[1] High pressure liquid chromatography [silica gel, hexane-ether (2:1)] of the eluate gave 1[6] [colorless oil, 10 mg, $C_{21}H_{29}ClO_5$, $[\alpha]_D$ -24.1° (c 0.44, $CHCl_3$)], iodovulone I, bromovulone I, and chlorovulones, in that order of increasing polarity.

The UV spectrum (EtOH) of 1 showed an absorption at 300 nm (ε 4300) due to the conjugated dienone system. But there was no absorption due to the conjugated cyclopentenone system which is common in clavulones and their congeners. The IR spectrum ($CHCl_3$) showed absorptions due to a hydroxy group (3580 cm^{-1}), an ester group (1740 cm^{-1}), and an α-chlorocyclopentanone moiety conjugated with the diene system (1740, 1625 cm^{-1}). The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of 1 showed the signals due to the above-mentioned conjugated dienone system [δ_{ppm} 6.13 (1H, tdd, J = 7.9, 10.9, 1.0



1



2

Hz), 6.82 (1H, tdd, J = 1.6, 10.9, 12.5 Hz), 7.54 (1H, d, J = 12.5 Hz)], a non-conjugated olefin [5.23 (1H, m), 5.60 (1H, m)], a methoxycarbonyl group [3.68 (3H, s)], a trisubstituted epoxide [3.96 (1H, s)], and a methyl group [0.88 (3H, t, J = 7.2 Hz)]. The ^{1}H-NMR spectrum of 1 is very similar to that of chlorovulone I (2),[3a] except for the appearance of the epoxy proton at 3.96 ppm instead of the olefinic proton signal at C-11 present in 2, suggesting that the compound 1 is 10,11-epoxy-chlorovulone I. In order to confirm this structure of 1, the following chemical transformation was performed. Epoxidation of chlorovulone I (2) with excess 5% aqueous sodium hypochlorite solution[7] in N,N-dimethylformamide at 0°C gave exclusively 1 in 96% yield. This transformation revealed the same α-orientation of the hydroxy group at C-12 in 1 as that in 2, whose absolute configuration at C-12 was already established.[3b] The cis relationship between the epoxide and the hydroxy group was elucidated by the observation of the intramolecular hydrogen bond due to the hydroxy group and the oxygen of the epoxide in high dilution IR measurement; IR (CCl_4, 2.7×10^{-3} M), 3580 cm^{-1}.

The compound 1 showed an antiproliferative activity against HL-60 leukemia cells (IC_{50} 0.04 μg/ml) in vitro, which is stronger than that of clavulone I.[3a,8] This provides an interesting problem regarding the inhibition mechanism[8] of leukemia cell growth by clavulones and their congeners. The antiproliferative activity of these prostanoids is assumed to be the effect of the cross-conjugated cyclopentenone system,[4] but 1 lacks this system.

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REFERENCES AND NOTES

1) H. Kikuchi, Y. Tsukitani, K. Iguchi, and Y. Yamada, Tetrahedron Lett., 23, 5171 (1982); H. Kikuchi, Y. Tsukitani, K. Iguchi, and Y. Yamada, ibid, 24, 1549 (1983); K. Iguchi, Y. Yamada, H. Kikuchi, and Y. Tsukitani, ibid, 24, 4433 (1983).

2) M. Kobayashi, T. Yasuzawa, M. Yoshihara, H. Akutsu, Y. Kyogoku, and I. Kitagawa, Tetrahedron Lett., 23, 5331 (1982); M. Kobayashi, T. Yasuzawa, M. Yoshihara, B. W. Son, Y. Kyogoku, and I. Kitagawa, Chem. Pharm. Bull., 31, 1440 (1983); I. Kitagawa, M. Kobayashi, T. Yasuzawa, B. W. Son, M. Yoshihara, and Y. Kyogoku, Tetrahedron, 41, 995 (1985).

3) a) K. Iguchi, S. Kaneta, K. Mori, Y. Yamada, A. Honda, and Y. Mori, Tetrahedron Lett., 26, 5787 (1985); b) H. Nagaoka, K. Iguchi, T. Miyakoshi, N. Yamada, and Y. Yamada, ibid, 27, 223 (1986); c) K. Iguchi, S. Kaneta, K. Mori, Y. Yamada, A. Honda, and Y. Mori, J. Chem. Soc., Chem. Commun., 1986, 981; d) B. J. Baker, R. K. Okuda, P. T. K. Yu, and P. J. Scheuer, J. Am. Chem. Soc., 107, 2976 (1985); e) H. Nagaoka, H. Miyaoka, T. Miyakoshi, and Y. Yamada, ibid, 108, 5019 (1986); f) M. Suzuki, Y. Morita, A. Yanagisawa, and R. Noyori, ibid, 108, 5021 (1986).

4) M. Fukushima and S. Narumiya, Japanese Journal of Inflammation, 4, 152 (1984).

5) The soft coral was collected at the coral reef of Ishigaki Island (Okinawa, Japan) in September, 1985.

6) 1: high resolution CIMS m/z 397.1815 (M + 1, calcd for $C_{21}H_{30}{}^{35}ClO_5$, 397.1780); ^{1}H-NMR (400 MHz, $CDCl_3$) δ_{ppm} 1.80 (2H, quint., J = 7.4 Hz), 1.99 (2H, br q, J = 7.7 Hz), 2.33 (2H, t, J = 7.3 Hz), 2.39 (2H, m), 2.72 (2H, br d, J = 7.3 Hz).

7) D. D. Keane, W. I. O'Sullivan, E. M. Philbin, R. M. Simons, and P. C. Teague, Tetrahedron, 27, 3535 (1971).

8) A. Honda, Y. Yamamoto, Y. Mori, Y. Yamada, and H. Kikuchi, Biochem. Biophys. Res. Commun., 130, 515 (1985).

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