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Porphyrins with Metal-Chelating Groups in the Peripheral Region. II.[1] *meso*-Tetra[2-(8-hydroxyquinolyl)]porphine

SETSURO SUGATA and YOSHIKAZU MATSUSHIMA*

Kyoritsu College of Pharmacy, Shibakoen 1-5-30, Minato-ku, Tokyo 105, Japan

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meso-Tetra[2-(8-hydroxyquinolyl)]porphine was synthesized by the Rothemund method as a model of porphyrin with metal chelating groups in the peripheral region. The compound was soluble in dimethylformamide (DMF) but practically insoluble in water. Addition of Cu(II) perchlorate to the DMF solution resulted in biphasic spectral changes, an instantaneous change and a subsequent slow change. The spectrum after the first step showed the formation of the free base porphyrin with metal-chelated 8-quinolinol moieties. The second step was observable in the presence of a large excess of Cu(II) and was ascribed to the formation of the Cu(II)-porphyrin with Cu(II)-chelated 8-quinolinol. Addition of perchloric acid to the solution of the metalloporphyrin gave rise to the spectrum assignable to the Cu(II)-porphyrin with unchelated 8-quinolinol.

Keywords——porphyrin; Cu(II)-porphyrin; synthetic porphyrin; tetraarylporphine; 8-quinolinol; metal chelation

It is well known that many natural and synthetic porphyrins accumulate *in vivo* in neoplastic tissues,[2] though the affinity for the tissue seems to be lost in metalloporphyrins.[3] Porphyrins with metal-chelating groups in the peripheral region can be obtained as the free base form of the porphyrin labeled with radioactive or nonradioactive metal ions at the periphery. The potential uses of such porphyrins in medical sciences and analytical chemistry are many.

In the previous paper,[1] we reported the synthesis of *meso*-tetra[5-(8-hydroxyquinolyl)]porphine as a model of such porphyrins. We have now prepared one of its isomers, *meso*-tetra[2-(8-hydroxyquinolyl)]porphine. The present paper describes the synthesis of

$PH_22(QOH)_4$: R = [2-(8-hydroxyquinolyl)]

$PH_25(QOH)_4$: R = [5-(8-hydroxyquinolyl)]

Fig. 1. Structures of *meso*-Tetra(8-hydroxyquinolyl)porphines

the new isomer, the chemical properties and chelation characteristics of the two isomers.

Results and Discussion

The Rothemund method,[4] which is a standard method for the preparation of *meso*-substituted porphyrins, consists of the direct condensation of pyrrole and a corresponding aldehyde in an appropriate solvent. *meso*-Tetra[2-(8-hydroxyquinolyl)]porphine ($PH_22(QOH)_4$) was prepared by the method in propionic acid. The yield (0.6%) was less than that (2%) of its 5-isomer, *meso*-tetra[5-(8-hydroxyquinolyl)]porphine ($PH_25(QOH)_4$).[1]

In this paper, the two porphyrins are represented by the symbols shown in the parentheses. PH_2 stands for the porphine moiety with two pyrrole hydrogens, and QOH for the quinolinol moiety. Three kinds of metal complexes are possible for these porphyrins. These are free base porphyrin with metal-chelated 8-quinolinol group ($PH_2(QOM)_4$), metalloporphyrin with unchelated 8-quinolinol group ($PM(QOH)_4$) and metalloporphyrin with metal-chelated 8-quinolinol group ($PM(QOM)_4$), where M indicates a metal ion.

The visible absorption spectrum of $PH_22(QOH)_4$ in dimethylformamide (DMF) is shown in Fig. 2. The spectrum is quite similar to the characteristic spectrum of free base porphyrins, with an intense band at the 400-nm region (Soret band) and four weak bands at the longer wavelength side.[5] The four weak bands are usually numbered I, II, III and IV starting from the low-energy end. The ratio of the intensity of band III to that of band II in $PH_22(QOH)_4$ was 1.08 in DMF and 1.09 in chloroform ($CHCl_3$). In the case of the 5-isomer, the ratio was 0.98 in DMF and 0.89 in $CHCl_3$. According to the conventional classification of the porphyrin spectra[5] the spectrum of $PH_22(QOH)_4$ can be classified as ethio-type, whereas that of the 5-isomer is phyllo-type.[1] This may be explained in terms of the greater electron-withdrawing character of the 8-quinolinol group in the 5-isomer.[6]

Addition of Cu(II) perchlorate to $PH_22(QOH)_4$ in DMF resulted in biphasic spectral changes, an instantaneous change and a subsequent slow change. The latter change was observable at room temperature only in the presence of a large excess of Cu(II) over the porphyrin. The spectra after the first step are shown in Fig. 2 and they indicate the formation of the free base porphyrin with Cu(II)-chelated 8-quinolinol ($PH_22(QOCu^+)_4$). The changes of the spectra with the concentration of Cu(II) show that the formation of $PH_22(QOCu^+)_4$ was incomplete at four-fold excess of Cu(II) over the porphyrin. It was also incomplete at six-fold excess, but was almost complete at eight fold excess. The unexpectedly low stability in Cu(II) chelation by the 8-quinolinol moiety is probably a result of the medium used. We have confirmed that the stability constant of the Cu(II) chelate of 8-quinolinol is much smaller in DMF ($\log K_1 = 1.62$) than in aqueous media.[7]

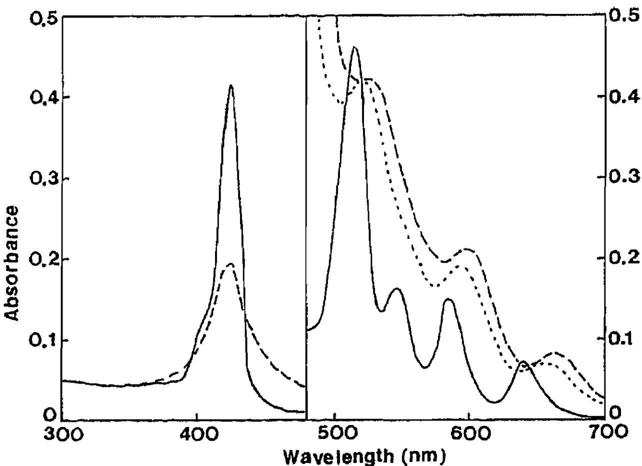

Fig. 2. Spectra of $PH_22(QOH)_4$ in DMF in the Presence and Absence of Cu(II)

Concentration of $PH_22(QOH)_4$: 1×10^{-6} M (300—480 nm) or 2×10^{-5} M (480—700 nm). Concentrations of Cu(II) were 0 (——), or 4-fold (------), or 8-fold (- - - -) excess over $PH_22(QOH)_4$.

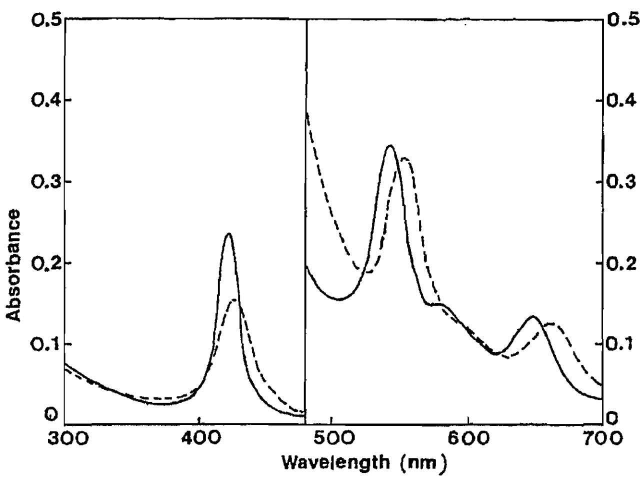

Fig. 3. Spectra of $PCu2(QOCu^+)_4$ and $PCu2(QOH)_4$ in DMF

$PCu2(QOCu^+)_4$, ----; $PCu2(QOH)_4$ (concentration of $HClO_4$: 15 mM), ——.
Concentration of the porphyrin: 1×10^{-6} M (300—480 nm) or 2×10^{-5} M (480—700 nm). Concentration of Cu(II): 2.6×10^{-5} M (300—480 nm) or 5.2×10^{-4} M (480—700 nm).

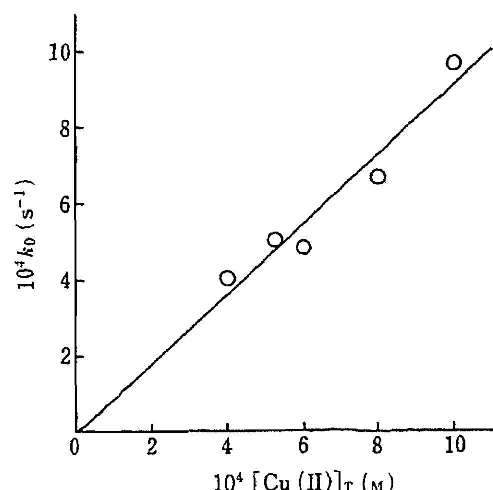

Fig. 4. Dependence of the Pseudo-First-Order Rate Constant, k_0, on the Total Concentration of Cu(II), $[Cu(II)]_T$

Concentration of the porphyrin: 2×10^{-5} M.

The spectrum after the completion of the second slow spectral change is shown in Fig. 3. The visible bands characteristic of the free base porphyrin were replaced by two bands. Since these bands are assigned as the α and β bands of metalloporphyrin,[5] the change should indicate the formation of $PCu2(QOCu^+)_4$. The kinetics of the second step in DMF were measured at 30°C by monitoring the absorption at 523 nm. The formation of the metalloporphyrin followed first-order kinetics in the presence of a large excess of Cu(II). The observed first-order rate constant, k_0, increased linearly with increase of the total concentration of Cu(II), $[Cu(II)]_T$, under the conditions used as shown in Fig. 4. The second-order rate constant, k, can be defined by the following equation:

$$d[PCu2(QOCu^+)_4]/dt = k_0[PH_22(QOCu^+)_4] = k[PH_22(QOCu^+)_4][Cu(II)]_T$$

The k value was calculated to be $0.92\,M^{-1}s^{-1}$, almost twice that for the 5-isomer ($0.41\,M^{-1}s^{-1}$).[1]

The spectrum of a DMF solution obtained by addition of $HClO_4$ to $PCu2(QOCu^+)_4$ is shown in Fig. 3. The concentration of $HClO_4$ was 15 mM. The spectrum can be ascribed to the species $PCu2(QOH)_4$. The two weak visible bands characteristic of the metalloporphyrin spectrum were blue-shifted. An increase of the absorbance of the Soret band with the demetallation of the quinolinol moiety was noted. A similar phenomenon was observed with the 5-isomer.

Infrared (IR) and mass spectra (MS) of $PH_22(QOH)_4$ confirmed its structure, as described in the experimental section. The compound and its 5-isomer were practically insoluble in water. The insolubility made it difficult to study the chelation phenomena in aqueous solutions of these bifunctional compounds. Studies of the biological and medical applications were also difficult. For further studies it is desirable to design water-soluble compounds with similar bifunctional nature.

Experimental

2-Formyl-8-quinolinol was prepared by the reported method.[8] A propionic acid solution (120 ml) of 2-formyl-8-

quinolinol (4.9 g, 28.3 mmol) and pyrrole (1.9 g, 28.3 mmol) was refluxed for 1.5 h. After removal of the solvent, the residue was washed with water, dried and extracted with $CHCl_3$. The extract was chromatographed on a silica gel column and 200 mg of a purple crystalline product was obtained from the 2% MeOH–$CHCl_3$ eluate. The product was dissolved in 66 ml of EtOH-free $CHCl_3$ and refluxed for 2 h. After addition of a dry benzene solution (1.3 ml) of 2,3-dichloro-5,6-dicyanobenzoquinone (40 mg), the mixture was refluxed for a further 2 h. Purification by silica gel chromatography afforded 35 mg of *meso*-tetra[2-(8-hydroxyquinolyl)]porphine ($PH_22(QOH)_4$) as purple crystals (yield, 0.6%). MS *m/z*: 882 (M^+), 738 ($M^+ - C_9H_6NO$). IR ν_{max}^{KBr} cm^{-1}: 3390 (QOH st.), 3290 (NH st.), 1586, 1560, 1497, 1455 (PH_2 C-H st.), 1427, 1309, 1231, 1185, 967 (PH_2 ring def.), 905, 788.

$PH_22(QOH)_4$ was very soluble in DMF, dimethylsulfoxide and 1 M HCl, soluble in $CHCl_3$, acetone, AcOEt and dioxane, slightly soluble in MeOH, EtOH, benzene, AcOH, ether and 1 M NaOH and practically insoluble in water.

A Shimadzu UV-240 spectrophotometer, a Hitachi EPI-G3 grating infrared spectrophotometer and a JEOL JMS-D100 mass spectrometer were used throughout the present study.

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Chemistry of 2-Methoxy-2,5-cyclohexadienone. V.[1] Photochemical Rearrangement and Oxidation of 2-Methoxy-4-methyl-4-phenyl-2-cyclohexenone and 2-Methoxy-4-methyl-4-phenyl-2,5-cyclohexadienone[2]

KATSUHIDE MATOBA,* HIROKO NODA, and TAKAO YAMAZAKI

Faculty of Pharmaceutical Sciences, Toyama Medical & Pharmaceutical University, Sugitani, Toyama 930-01, Japan

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A methanolic solution of 2-methoxy-4-methyl-4-phenyl-2-cyclohexenone (I) was irradiated with a 100 W mercury lamp to give 4-methyl-4-phenyl-2-cyclohexenone (III) and 1-methoxy-5-methyl-6-phenylbicyclo[3.1.0]hexan-2-one (IV). When a benzene solution of I was irradiated, only IV was obtained. On the other hand, 2-methoxy-4-methyl-4-phenyl-2,5-cyclohexadienone (II) gave 2,2-dimethoxy-6-methyl-6-phenylbicyclo[3.1.0]hexan-3-one (V) and 6,6-dimethoxy-5-methyl-5-phenyl-3-cyclohexenone (VI) on photoirradiation in methanol. In benzene, 6-methoxy-6-methyl-5-phenyl-2,4-cyclohexadienone (XI) was obtained.

Compound II was treated with *m*-chloroperbenzoic acid in a bilayer system of methylene chloride–aqueous disodium orthophosphate at room temperature to give 5,6-epoxy-6-methoxy-4-methyl-4-phenyl-2-cyclohexenone (XVI) and its stereoisomer (XVI′). 5,6-Epoxy-2-methoxy-4-methyl-4-phenyl-2-cyclohexenone (XVIII) was obtained by treatment of II with hydrogen peroxide in a basic medium. 2,3: 5,6-Diepoxy-6-methoxy-4-methyl-4-phenyl-6-hexanolide (XVII) rather than the expected diepoxide (XII) was obtained by the alternative oxidation of XVI or XVIII.

Keywords——photochemical rearrangement; epoxidation; 2,5-cyclohexadienone; 2-methoxy-2,5-cyclohexadienone; 2-methoxy-4-methyl-4-phenyl-2,5-cyclohexadienone; Baeyer–Villiger oxidation

The intermediacy of the 4,4-disubstituted 2-methoxy-2,5-cyclohexadienone structure, which occurs in natural alkaloids such as sinoactine, in the biosynthesis of sinomenine from reticuline, has prompted us to investigate chemical reactivity of this particular structure.[3] Previously, we have examined photochemical and oxidative reactions of a series of 4,4-disubstituted 2-methoxycyclohexenones and cyclohexadienones.[1,4] In this paper, we report the results obtained by photochemical and oxidative reactions of 2-methoxy-4-methyl-4-phenyl-2-cyclohexenone (I) and 2-methoxy-4-methyl-4-phenyl-2,5-cyclohexadienone (II) to clarify the effect of C_4-substituents on the reaction course.

First, we investigated the reactivity of the cyclohexenone (I) not only as a basic study but also for comparison with the reactivity of other cyclohexenones and cyclohexadienones. When a methanolic solution of I was irradiated with a high-pressure 100 W mercury lamp for 5 h, 4-methyl-4-phenyl-2-cyclohexenone (III), a demethoxylated compound, and 1-methoxy-5-methyl-6-phenylbicyclo[3.1.0]hexan-2-one (IV) were obtained in yields of 30.0 and 66.8%, respectively, together with a 2.9% recovery of I. The structure of III was suggested on the basis of the physical data and confirmed by comparison with an authentic sample prepared by the known method.[5] Compound IV exhibited in the nuclear magnetic resonance (NMR) spectrum a multiplet signal at δ 0.8—1.1 and a singlet peak at δ 2.80 due to the C_3-*endo*- and C_6-protons, respectively. The high-field shift of the C_3-*endo* proton could be explained in

terms of anisotropy induced by the cyclopropane ring system. The stereochemical structure of IV was deduced from the mechanism proposed in Chart 1, that is, the phenyl group migrates with retention of the configuration and is on the opposite side to the C_5-methyl group. On the other hand, a benzene solution of I was irradiated to give only IV in the yield of 44.6%. From these results, it would be suggested that methanol as a solvent might play a role in the reduction of I to III.

Then, we examined the photochemical behavior of the dienone, II. When a methanolic solution of II was irradiated with a high-pressure 100W mercury lamp for 30 min, two products, 2,2-dimethoxy-6-methyl-6-phenylbicyclo[3.1.0]hexan-3-one (V) and 6,6-dimethoxy-5-methyl-5-phenyl-3-cyclohexenone (VI), were obtained in 45.9 and 15.9% yields, respectively. Compound V exhibited an infrared (IR) absorption at 1760 cm^{-1} due to the carbonyl group and NMR signals at δ 2.36 and 2.80 (double doublet and broad doublet, respectively) due to the methylene protons at the C_4-position. Compound VI exhibited an absorption at 1730 cm^{-1} in the IR spectrum due to the unconjugated carbonyl group and NMR signals at δ 3.06, 3.29 due to the methylene protons and at δ 5.61, 5.77 due to two protons of the unconjugated double bond. The α-keto dimethylketal structures in these products (V and VI) were suggested by the formation of the adducts with *o*-phenylenediamine as shown in Chart 2. Although compound V readily reacted with this reagent in the presence of acid to give the corresponding adduct, 10-methyl-10-phenyl-2,3-dihydro-1,2-methano-1*H*-

Chart 1

Chart 2

Chart 3

cyclopenta[b]quinoxaline (VII), compound VI was found to be inert under the same conditions. However, 2-hydroxy-6-methyl-6-phenyl-2-cyclohexenone (IX), which was obtained by catalytic hydrogenation of VI followed by hydrolysis with 10% hydrochloric acid, gave an adduct, 1-methyl-1-phenyl-1,2,3,4-tetrahydrophenazine (X), with *o*-phenylenediamine.

Then, compound II was irradiated in benzene with a high-pressure 100 W mercury lamp in order to examine the solvent effect. The only product obtained (in poor yield) was 6-methoxy-6-methyl-5-phenyl-2,4-cyclohexadienone (XI), which exhibited a singlet at δ 1.72 due to the C_6-methyl proton in the NMR spectrum, supporting the structure of XI as shown.

A possible mechanism for the photochemical transformation of II to V, VI, and XI is shown in Chart 3, that is, it was considered that compound V was formed *via* the zwitterion (A), and VI and XI *via* bicyclo[3.1.0]hexenone (B) and dienolate (C) intermediates.[6] Thus, the photochemical products from I and II were characteristic ones compared with those obtained from 5,5-dimethyl or diphenyl analogues.[1,4c] In particular, the isolation of unconjugated ketone, VI, was surprising.

In view of the suggestion that the biosynthesis of acutumine possibly involves a diepoxide (see Chart 4),[3] studies on the oxidation of the cyclohexadienone (II) with hydrogen peroxide (HPO) or *m*-chloroperbenzoic acid (mCPBA) were carried out in order to obtain the diepoxide (XII). Prior to the study on the oxidation of cyclohexadienone (II), the oxidative behavior of the cyclohexenone (I) was examined to establish the optimal conditions, which might be applied to the preparation of XII, for the synthesis of a monoepoxide of I, 2,3-epoxy-2-methoxy-4-methyl-4-phenylcyclohexanone (XIII). When I was treated with HPO in trifluoroacetic acid (TFA) at 40 °C, a monomethyl ester of 3-methyl-3-phenyladipic acid (XIVa) was obtained in 97.1% yield. The structure of XIVa was suggested from the physical data and a consideration of its formation from I. Compound XIVa was treated with diazomethane to give the diester (XIVb) which was then hydrolyzed with sodium hydroxide to give the known 3-methyl-3-phenyladipic acid (XIVc).[7] When compound I was allowed to react with mCPBA at room temperature overnight, a lactone, 5,6-epoxy-6-methoxy-4-methyl-4-phenyl-6-hexanolide (XV), was obtained in 74.1% yield. The doublet signal at δ 4.95 ($J=2$ Hz) in the NMR spectrum of XV is attributable to the C_5-proton coupling through four σ bonds with one of the C_3-protons (*W* rule). From an examination of a molecular model, the C_5-proton must be quasi-equatorial. When compound I was then allowed to react with mCPBA in a bilayer system of methylene chloride–aqueous disodium orthophosphate at 4°C for 136.6 h, the epoxy ketone (XIII) and lactone (XV) were obtained in 8.6 and 64.3% yields, respectively, along with recovery of I (18.9%). Compound XIII exhibited a singlet signal at δ

2-methoxy-2,5-cyclohexadienone ----→ diepoxide ------→ acutumine

Chart 4

XII XIII XIV XV

XVI XVI′ XVII XVIII

a: R=Me, R′=H b: R=R′=Me c: R=R′=H

Chart 5

3.74 due to the C_3-proton. Thus, the expected monoepoxide, XIII, was obtained by the oxidation of the cyclohexenone (I) with mCPBA in a bilayer system at low temperature.

On the other hand, when II was treated with mCPBA in the bilayer system at 4°C, a large amount of the starting material was recovered. When this oxidation was carried out at room temperature, the expected epoxides, 5,6-epoxy-6-methoxy-4-methyl-4-phenyl-2-cyclohexenone (XVI) and its stereoisomer (XVI′), were obtained in yields of 43.5 and 5.9%, respectively, along with recovery of II (26.0%). In the NMR spectra of XVI and XVI′, the signals due to the C_5-protons appeared at δ 3.77 and 3.79, respectively, as a doublet ($J=4$ Hz) coupled with the C_3-protons. In the IR spectrum of XVI, a carbonyl band was observed at $1685\,\text{cm}^{-1}$. When XVI was treated with HPO in basic medium at room temperature, 2,3:5,6-diepoxy-6-methoxy-4-methyl-4-phenyl-6-hexanolide (XVII) was obtained in poor yield instead of the expected product, XII. In the NMR spectrum of XVII, signals due to the C_3-, C_2-, and C_5-protons were observed at δ 3.61 (d, $J=4$ Hz), 3.96 (d, $J=4$ Hz), and 5.02 (s), respectively. Thus the attempts to obtain XII *via* XVI were unsuccessful.

When, compound II was treated with HPO at room temperature, 5,6-epoxy-2-methoxy-4-methyl-4-phenyl-2-cyclohexenone (XVIII) was obtained in 52.6% yield accompanied with recovery of II (37.7%). Absorptions due to the carbonyl and enol ether at 1685 and $1620\,\text{cm}^{-1}$ in the IR spectrum of XVIII, and NMR signals due to two protons at the epoxy carbons and the C_3-proton at δ 3.54, 3.58, and 5.31 were observed. To convert XVIII into XII, XVIII was treated with mCPBA at 4°C in the bilayer system. Unfortunately the product (obtained in 46.8% yield) was XVII.

Thus all the attempts to obtain the corresponding diepoxides from 2-methoxy-2,5-cyclohexadienones were unsuccessful. Further study is necessary to obtain these diepoxides.

Experimental

All melting points (taken on a Kofler block) and boiling points are uncorrected. IR spectra were determined by using a JASCO A 102 diffraction grating spectrophotometer; absorption data are given in cm^{-1}. Ultraviolet (UV) spectra were obtained in MeOH with a Hitachi 220 spectrometer, and absorption maxima are given in nm. Gas chromatography (GC) was carried out on a Shimadzu GC-6AM instrument with a stainless steel column (3 mm × 2 m) packed with 5% SE-30. The N_2 gas flow was 40 ml/min. NMR spectra were recorded on a Varian EM-360, JEOL PMX-60, or Varian XL-200 spectrometer with tetramethylsilane as an internal standard. The chemical shifts and coupling constants (J) are given in δ and Hz, respectively. Mass spectra (MS) were measured with a JEOL D-200 or D-300 (70 eV, direct inlet system) spectrometer. All solvents were removed by evaporation under reduced pressure after drying the solution over anhyd. $MgSO_4$ or Na_2SO_4.

2-Methoxy-4-methyl-4-phenyl-2-cyclohexenone (I)[8)]——A methanolic solution of 2-phenylpropionaldehyde (8.04 g, 60 mmol), Triton B (40% in MeOH, 6.00 g, 14 mmol), and 1,4-dimethoxybutan-2-one (7.49 g, 60 mmol) was refluxed overnight under an Ar atmosphere. The reaction mixture was diluted with water and extracted with Et_2O. The organic layer was washed with brine and dried. The residue obtained after removal of the solvent was distilled to give a pale yellow oil. bp 148—150 °C (2 mmHg). Yield: 8.41 g (64.9%). IR (neat): $\nu_{C=O}$ 1690, $\nu_{C=C}$ 1620. UV λ_{max} (ε): 205 (9600), 258 (8200), 263 (sh. 8000). GC (200 °C): t_R 2.8 min. NMR ($CDCl_3$): 1.58 (3H, s, C_4-Me), 2.0—2.3 and 2.3—2.6 (each 2H, m, C_5- and C_6-H), 3.69 (3H, s, OMe), 5.80 (1H, s, C_3-H), 7.2—7.5 (5H, m, aromatic H). MS *m/z* (%): 216 (M^+, 91). 201 (M^+ − Me, 91), 173 (M^+ − CH_3CO, 100), 142 (*m/z* 173 − OMe, 50), 115 (54), 91 (44). *Anal.* Calcd for $C_{14}H_{16}O_2$: C, 77.75; H, 7.46. Found: C, 77.69; H, 7.19.

2-Methoxy-4-methyl-4-phenyl-2,5-cyclohexadienone (II)——a) With 2,3-Dichloro-5,6-dicyanobenzoquinone (DDQ): A solution of I (0.55 g, 2.6 mmol) and DDQ (96%, 0.73 g, 3.2 mmol) in dioxane (14.6 ml) was refluxed with stirring for 22 h, and then filtered. The filtrate was concentrated. The residue was rinsed with Et_2O and the Et_2O layer was washed with 10% NaOH and brine. The deep yellow oil obtained after removal of the solvent was distilled to give a colorless oil, bp 104—106 °C (0.5 mmHg). Yield: 0.43 g (78.8%). IR (neat): $\nu_{C=O}$ 1670, $\nu_{C=C}$ 1640, 1610. UV λ_{max} (ε): 212 (17500), 240 (9300), 318 (2200). GC (200 °C): t_R 3.7 min. NMR (CCl_4): 1.79 (3H, s, C_4-Me), 3.63 (3H, s, OMe), 5.75 (1H, d, J=3, C_3-H), 6.13 (1H, d, J=10, C_6-H), 6.80 (1H, dd, J=10, 3, C_5-H), 7.1—7.5 (5H, m, aromatic H). MS *m/z* (%): 214 (M^+, 100), 199 (M^+ − Me, 33), 171 (M^+ − CH_3CO, 33), 128 (24), 105 (13), 77 (14). *Anal.* Calcd for $C_{14}H_{14}O_2 + 1/5H_2O$: C, 77.12; H, 6.61. Found: C, 77.01; H, 6.58.

b) With SeO_2: A mixture of I (6.96 g, 32.2 mmol), SeO_2 (95%, 3.9 g every 13 h, total 31.2 g, 267 mmol), AcOH (41 ml), and anhyd. *tert*-amyl alcohol (138 ml) was refluxed with stirring and the reaction was monitored by GC during the course of 104 h. The residue obtained after evaporation of the filtrate was rinsed with CH_2Cl_2, and the organic layer was washed with 5% NaOH and brine. The oil obtained after removal of the solvent was distilled to give a mixture of I and II (3.28 g, 1 : 5). bp 144—162 °C (3 mmHg).

General Procedure for Photochemical Experiments——A methanolic or benzene solution of the starting material in a Pyrex vessel equipped with a thermometer, N_2-gas inlet system and a drying tube was well stirred and agitated for 1 h with N_2 which had been dried with anhyd. $CaCl_2$ and deoxygenated with a Badische Anilin und Soda Fabrik (BASF) catalyst. The solution was cooled and irradiated with a high-pressure mercury lamp. The irradiation was continued with monitoring of the reaction products by GC.

Irradiation of I in MeOH——A solution of I (121.8 mg, 0.56 mmol) in MeOH (240 ml) was irradiated with a high pressure 100 W mercury lamp at 5—20 °C for 5 h. After removal of MeOH, the residue (0.25 g) was fractionated through an SiO_2 column. I (3.6 mg, 2.9%), 4-methyl-4-phenyl-2-cyclohexenone (III, 31.4 mg, 30.0%), and 1-methoxy-5-methyl-6-phenylbicyclo[3.1.0]hexan-2-one (IV, 81.6 mg, 66.8%) were eluted successively with 3% AcOEt in hexane. III: bp < 120 °C (1 mmHg), GC (170 °C), t_R: 4.0 min. NMR ($CDCl_3$): 1.58 (3H, s, Me), 2.1—2.5 (each 2H, m, C_5- and C_6-H), 6.16 (1H, d, J=10, C_2-H), 6.97 (1H, d, J=10, C_3-H), 7.2—7.5 (5H, m, aromatic H). MS *m/z* (%): 186 (M^+, 100), 171 (M^+ − Me, 17), 158 (M^+ − CO, 48), 144 (M^+ − CH_2=C=O, 97), 129 (97), 115 (31). III was also prepared according to the known method[5)] from 2-phenylpropionaldehyde and methyl vinyl ketone; the product was identical with that obtained in this experiment. IV: bp < 120 °C (1 mmHg). IR (neat): $\nu_{C=O}$ 1720. GC (170 °C): t_R 4.5 min. NMR ($CDCl_3$): 0.8—1.1 (1H, m, C_3-*endo* H), 1.56 (3H, s, C_5-Me), 1.7—1.9 (1H, m), 1.9—2.1 (2H, m), 2.80 (1H, s, C_6-H), 3.71 (3H, s, OMe), 7.2—7.5 (5H, m, aromatic H). MS *m/z* (%): 216 (M^+, 100), 201 (M^+ − Me, 46), 184 (M^+ − MeOH, 16), 173 (M^+ − CH_3CO, 59), 142 (57), 128 (36), 114 (29), 90 (30). High-resolution MS Calcd for $C_{14}H_{16}O_2$: 216.1149. Found: 216.1149. *Anal.* Calcd for $C_{14}H_{16}O_2$: C, 77.75; H, 7.46. Found: C, 77.52; H, 7.30.

Irradiation of I in Benzene——A benzene solution (240 ml) of I (200.8 mg, 0.94 mmol) was irradiated with a 100 W mercury lamp at 7—23 °C for 10 h. GC (170 °C): t_R 4.5 min. Compound III could not be detected. An oily substance obtained after removal of the solvent was microdistilled to give a colorless oil. bp < 150 °C (3 mmHg). The NMR spectrum was superimposable on that of IV and exhibited no signal due to III. Yield: 89.5 mg (44.6%).

Irradiation of II in MeOH——A methanolic solution (240 ml) of II (196.4 mg, 0.92 mmol) was irradiated with a 100 W mercury lamp at 2—9 °C for 30 min. The residue (212.9 mg) obtained after removal of the solvent was fractionated through a medium-pressure SiO_2 column. 6,6-Dimethoxy-5-methyl-5-phenyl-3-cyclohexenone (VI) and 2,2-dimethoxy-6-methyl-6-phenylbicyclo[3.1.0]hexan-3-one (V) were eluted with 2% AcOEt–hexane and 3—5%

AcOEt–hexane, respectively. V: mp 91—94 °C (white prisms from hexane), yield 98.2 mg (43.5%). IR ($CHCl_3$): $\nu_{C=O}$ 1760, δ_{C-O} 1100. GC (170 °C): t_R 6.3 min. NMR ($CDCl_3$): 1.59 (3H, s, C_6-Me), 1.8—2.0 (2H, m, C_1- and C_5-H), 2.36 (1H, d, J=18, C_4-*endo* H), 2.80 (1H, dd, J=18, 5, C_4-*exo* H), 3.14 and 3.65 (each 3H, s, OMe), 7.2—7.4 (5H, m, aromatic H). MS *m/z* (%): 246 (M^+, 0.2), 218 (M^+ −CO, 100), 203 (M^+ −1−MeOH, 45), 187 (27), 171 (24), 155 (13), 143 (M^+ −1−$(MeO)_2C=C=O$, 48), 129 (85). *Anal.* Calcd for $C_{15}H_{18}O_3$: C, 73.14; H, 7.37. Found: C, 73.30; H, 7.40. VI: Oil, yield 42.6 mg (18.9%). IR ($CHCl_3$): $\nu_{C=O}$ 1730. UV, only end absorption. GC (200 °C): t_R 2.6 min. NMR ($CDCl_3$): 1.50 (3H, s, C_5-Me), 3.06 (1H, dm, J=20, C_2-H), 3.00 and 3.14 (each 3H, s, OMe), 3.29 (1H, dt, J=20, 4, C_2-H), 5.61 (1H, dm, J=10, C_4-H), 5.77 (1H, dt, J=10, 4, C_3-H), 7.2—7.6 (5H, m, aromatic H). MS *m/z* (%): 246 (M^+, 26), 218 (M^+ −CO, 43), 203 (M^+ −MeCO, 31), 143 (M^+ −$(MeO)_2C=C=O$, 29), 129 (100), 117 (33). High-resolution MS Calcd for $C_{15}H_{18}O_3$: 246.1255. Found: 246.1232.

10-Methyl-10-phenyl-2,3-dihydro-1,2-methano-1*H*-cyclopenta[*b*]quinoxaline (VII)——A solution of *o*-phenylenediamine (8.9 mg, 0.08 mmol) and V (19.5 mg, 0.08 mmol) in MeOH (5 ml) was added to an aqueous solution of AcONa·$3H_2O$ (13.0 mg, 0.10 mmol) and AcOH (5 drops). The mixture was stirred overnight at room temperature, and then diluted with water followed by extraction with CH_2Cl_2. The residue obtained after removal of the solvent was fractionated through an SiO_2 column. Compounds V and VII were successively eluted with CH_2Cl_2. The yields of V and VII were 10.0 mg (51.3%) and 4.7 mg (21.0%), respectively. VII: mp 147—150 °C (from hexane). NMR ($CDCl_3$): 1.56 (3H, s, Me), 2.40 (1H, dd, J=6, 6, C_2-H), 2.97 (1H, d, J=6, C_1-H), 3.06 (1H, d, J=20, C_3-H), 3.37 (1H, dd, J=20, 6, C_3-H), 6.9—7.2 (5H, m, C_{10}-phenyl-H), 7.5—7.7 (2H, m, C_6-, C_7-H), 7.77 (1H, dm, J=8, C_5-H), 8.02 (1H, dd, J=8, 1.5, C_8-H). MS *m/z* (%): 272 (M^+, 100), 257 (M^+ −Me, 79), 128 (26), 105 (28), 77 (12). High-resolution MS Calcd for $C_{19}H_{16}N_2$: 272.1313. Found: 272.1315.

2,2-Dimethoxy-3-methyl-3-phenylcyclohexanone (VIII)——A methanolic solution of VI (23.2 mg, 0.09 mmol) was shaken under an ordinary pressure of H_2 in the presence of PtO_2 (100 mg, 0.4 mmol) at room temperature for 3 h. The residue obtained after concentration of the filtrate was dissolved in CH_2Cl_2. Crude VIII obtained after removal of the organic solvent was purified through an SiO_2 column (CH_2Cl_2 as the eluent). VIII: mp 76—81 °C (from hexane). Yield: 14.7 mg (62.8%). IR ($CHCl_3$): $\nu_{C=O}$ 1720. NMR ($CDCl_3$): 1.36 (3H, s, C_3-Me), 1.5—1.8 and 1.8—2.1 (each 2H, m), 2.49 (1H, dm, J=12, C_6-H), 2.6—2.8 (1H, m, C_6-H), 2.81 and 3.11 (each 3H, s, OMe), 7.2—7.5 (3H, m, aromatic H), 7.64 (2H, dm, J=8, aromatic H). MS *m/z* (%): 248 (M^+, 5), 220 (M^+ −CO, 6), 214 (24), 199 (10), 131 (28), 117 (100), 101 (67), 91 (47), 57 (45). *Anal.* Calcd for $C_{15}H_{20}O_3$: C, 72.55; H, 8.12. Found: C, 72.82; H, 8.06.

2-Hydroxy-6-methyl-6-phenyl-2-cyclohexenone (IX)——Three drops of 10% HCl were added to an acetone solution of VIII (6.8 mg). The mixture was refluxed for 3 h on a water bath, then concentrated and extracted with CH_2Cl_2. The organic layer was washed with brine and concentrated to give crude IX (6.0 mg). GC (200 °C), t_R 2.5 min (single peak). NMR ($CDCl_3$): 1.20 (3H, s, Me), 1.8—2.5 (4H, m), 5.95 (1H, t, J=5, C_3-H), 7.1—7.4 (5H, m, aromatic H). MS *m/z* (%): 202 (M^+, 48), 187 (M^+ −Me, 26), 119 ($Ph\overset{+}{C}Me$, 26), 108 (M^+ −MeH−PhH, 100), 91 (38), 80 (56), 56 (60). High-resolution MS Calcd for $C_{13}H_{14}O_2$: 202.0993. Found: 202.1016.

1-Methyl-1-phenyl-1,2,3,4-tetrahydrophenazine (X)——An aqueous solution of AcONa·$3H_2O$ (2.5 mg, 0.018 mmol) was added to a mixture of IX (1.8 mg, 0.009 mmol) and *o*-phenylenediamine (0.9 mg, 0.08 mmol). After being stirred overnight, the mixture was concentrated. The residue was dissolved in CH_2Cl_2 and the organic layer was washed with brine and evaporated to give crude X (1.9 mg). MS *m/z* (%): 274 (M^+, 100), 259 (M^+ −Me, 55), 245 (18), 231 (33), 149 (77), 57 (80). High-resolution MS Calcd for $C_{19}H_{18}N_2$: 274.1469. Found: 274.1454.

Irradiation of II in Benzene——A benzene solution of II (208.4 mg, 0.97 mmol) was irradiated with a 100 W mercury lamp at 20 °C for 30 min. The residue obtained after removal of the solvent was fractionated through an SiO_2 column. 6-Methoxy-6-methyl-5-phenyl-2,4-cyclohexadienone (XI, 5.1 mg, 2.4%) and II (14.2 mg, 6.8%) were eluted with 2% AcOEt–hexane and 5% AcOEt-hexane, respectively. XI: GC (200 °C): t_R 3.2 min. NMR ($CDCl_3$): 1.72 (3H, s, Me), 3.66 (3H, s, OMe), 5.48 (1H, d, J=6, C_2-H), 5.82 (1H, d, J=10, C_4-H), 7.1—7.4 (6H, m, C_3- and aromatic H). MS *m/z* (%): 214 (M^+, 100), 199 (M^+ −Me, 16), 171 (39), 128 (34). High resolution MS Calcd for $C_{15}H_{18}O_3$: 246.1255. Found: 246.1232.

Treatment of I with HPO——HPO (31%, 0.27 ml, 2.37 mmol) was gradually added to a mixture of I (513 mg, 2.38 mmol) and TFA (1.95 ml) at room temperature with stirring (exothermic!). After being stirred at 40 °C for 3 h, the mixture was diluted with ice-water and extracted with CH_2Cl_2. The organic layer was washed with sat. $NaHCO_3$, 1% $NaHSO_3$, and brine. The residue (129 mg) obtained after removal of the solvent showed an NMR spectrum consistent with that of I. The basic layer was acidified with 5% HCl and extracted with AcOEt. The organic layer was dried and evaporated to give 4-methoxycarbonylmethyl-4-phenylpentanoic acid (XIVa, 431 mg, conversion yield was 97.1%). XIVa: IR (film): $\nu_{C=O}$ 1700—1740. NMR ($CDCl_3$): 1.48 (3H, s, CH_3), 2.0—2.3 (4H, m, 2×CH_2), 2.62 and 2.70 (each 1H, d, J=14, $\underline{CH_2}COOMe$), 3.53 (3H, s, OMe), 7.2—7.4 (5H, m, aromatic H). MS *m/z* (%): 250 (M^+, 0.6), 191 (M^+ −COOMe, 0.8), 84 (100). Compound XIVa was esterified with an excess of CH_2N_2 to give dimethyl 3-methyl-3-phenyladipate (XIVb) quantitatively. XIVb: bp < 120 °C (0.3 mmHg). IR (film): $\nu_{C=O}$ 1735. NMR ($CDCl_3$): 1.48 (3H, s, CH_3), 2.0—2.3 (4H, m, 2×CH_2), 2.61 and 2.71 (each 1H, d, J=14, C_2-H), 3.52 and 3.59 (each 3H, s, OMe), 7.1—7.4 (5H, m, aromatic H). MS *m/z* (%): 264 (M^+, 1.5), 177 ($Ph(Me)\overset{+}{C}$–CH_2COOMe, 100). *Anal.* Calcd for $C_{15}H_{20}O_4$: C, 68.16; H, 7.63. Found: C, 68.26; H, 7.68. NaOH (0.26 g) in aq. MeOH (1 ml) was added to a solution of

XIVa (412 mg) in MeOH (3 ml), and the mixture was refluxed for 1 h. The resulting mixture was diluted with ice-water, acidified with 5% HCl, and then extracted with AcOEt. The crystalline compound obtained after removal of the solvent was recrystallized from water to give 3-methyl-3-phenyladipic acid (XIVc). Yield: 362 mg (93.0%). mp 153—156 °C (Lit.[7] mp 153—155 °C).

Treatment of I with mCPBA——a) At Room Temperature in CH_2Cl_2: mCPBA (130 mg, 0.53 mmol) was added to a solution of I (99 mg, 0.46 mmol) under ice-cooling with stirring. After being stirred for 66.5 h at room temperature, the mixture was washed with sat. $NaHCO_3$ and brine. The residue obtained after removal of the solvent was purified through an SiO_2 column. 5,6-Epoxy-6-methoxy-4-methyl-4-phenyl-6-hexanolide (XV) was eluted with AcOEt. XV: bp < 200 °C (4 mmHg). 84.3 mg (74.1%). NMR ($CDCl_3$): 1.60 (3H, s, C_4-CH_3), 2.5—2.7 and 2.7—3.0 (each 2H, m, C_3- and C_2-H, respectively), 3.34 (3H, s, OMe), 4.95 (1H, d, $J=2$, C_5-H), 7.1—7.5 (5H, m, aromatic H). MS *m/z* (%): 248 (M^+, 2.4), 118 (Ph(Me)C=CH_2, 100). *Anal.* Calcd for $C_{14}H_{16}O_4+1/10\ H_2O$: C, 67.25; H, 6.49. Found: C, 67.26; H, 6.47.

b) At 4°C in a Bilayer System: mCPBA (70%, 138 mg, 0.56 mmol) was added to a solution of I (105 mg, 0.49 mmol) and $Na_2HPO_4 \cdot 12H_2O$ (0.97 g, 2.71 mmol) in CH_2Cl_2 (20 ml) and water (10 ml) under ice-cooling. The mixture was stirred at 4 °C for *ca.* 140 h and the organic layer was washed with sat. $NaHCO_3$ and brine. The residue obtained after removal of the solvent was fractionated through an SiO_2 column. 2,3-Epoxy-2-methoxy-4-methyl-4-phenylcyclohexanone (XIII, 9.7 mg, 8.6%), I (20.4 mg, 18.9%), and XV (77.6 mg, 64.3%) were eluted with 2% AcOEt–hexane, 5% AcOEt–hexane and AcOEt, successively. XIII: bp < 130 °C (3 mmHg). NMR ($CDCl_3$): 1.58 (3H, s, C_4-CH_3), 1.6—2.4 (4H, m, 2 × CH_2), 3.68 (3H, s, OMe), 3.74 (1H, s, C_3-H), 7.2—7.5 (5H, m, aromatic H). MS *m/z* (%): 232 (M^+, 9.8), 131 (Ph(Me)$\overset{+}{C}$–CH=CH_2, 67), 105 (Ph$\overset{+}{C}$HCH_3, 51), 91 ($PhCH_2^+$, 100).

5,6-Epoxy-6-methoxy-4-methyl-4-phenyl-2-cyclohexenone (XVI)——mCPBA (1.62 g, 6.6 mmol) was added to a solution of II (522 mg, 2.4 mmol) and $Na_2HPO_4 \cdot 12H_2O$ (5.17 g) in CH_2Cl_2 (76 ml) and water (53 ml) under ice-cooling. The mixture was stirred at room temperature for *ca.* 86 h. The organic layer was washed with 10% NaOH and brine, and then evaporated. The residue (613 mg) was fractionated through an SiO_2 column. Compound XVI and its isomer (XVI′) were successively eluted with 2% AcOEt–hexane. II (136 mg, 26.0%) was recovered from the fraction eluted with 20% AcOEt–hexane. XVI: 244 mg (43.5%). bp < 110 °C (0.5 mmHg). **IR** (film): $\nu_{C=O}$ 1685. NMR ($CDCl_3$): 1.72 (3H, s, C_4-CH_3), 3.59 (3H, s, OMe), 3.77 (1H, d, $J=4$, C_5-H), 6.05 (1H, d, $J=10$, C_2-H), 6.37 (1H, dd, $J=10$, 4, C_3-H), 7.3—7.5 (5H, m, aromatic H). MS *m/z* (%): 230 (M^+, 1.1), 215 (M^+-CH_3, 14), 201 (1.5), 187 (1.3), 171 (100). *Anal.* Calcd for $C_{14}H_{14}O_3$: C, 73.02; H, 6.13. Found: C, 73.11; H, 6.18. XVI′: 33 mg (5.9%). NMR ($CDCl_3$): 1.66 (3H, s, C_4-CH_3), 3.60 (3H, s, OMe), 3.79 (1H, d, $J=4$, C_5-H), 6.09 (1H, d, $J=10$, C_2-H), 6.62 (1H, dd, $J=10$, 4, C_3-H), 7.40 (5H, s, aromatic H). MS *m/z* (%): 230 (M^+, 1), 215 (M^+-CH_3, 28), 201 (1.7), 187 (1.7), 171 (100).

5,6-Epoxy-2-methoxy-4-methyl-4-phenyl-2-cyclohexenone (XVIII)——HPO (31%, 0.4 ml, 3.0 mmol) was added to a solution of II (214 mg, 1.0 mmol) in MeOH (2.8 ml). Then 6 N NaOH solution was added to the mixture (exothermic!). After being stirred at room temperature for 2.7 h, the mixture was diluted with water and extracted with CH_2Cl_2. The organic layer was washed with brine and evaporated. The residue (191 mg) was fractionated through an SiO_2 column. XVIII (121 mg, 52.6%) and II (81 mg, 37.7%) were eluted with 5% and 10% AcOEt–hexane, successively. XVIII: bp < 110 °C (0.4 mmHg). IR (film): $\nu_{C=O}$ 1685, $\nu_{C=C}$ 1620. NMR ($CDCl_3$): 1.78 (3H, s, C_4-CH_3), 3.5—3.7 (2H, m, C_5- and C_6-H), 3.60 (3H, s, OMe), 5.31 (1H, d, $J=4$, C_3-H), 7.2—7.6 (5H, m, aromatic H). MS *m/z* (%): 230 (M^+, 24), 215 (M^+-CH_3, 26), 202 (M^+-CO, 29), 187 (100), 171 (24). *Anal.* Calcd for $C_{14}H_{14}O_3$: C, 73.02; H, 6.13. Found: C, 72.91; H, 6.21.

2,3 : 5,6-Diepoxy-6-methoxy-4-methyl-4-phenyl-6-hexanolide (XVII)——a) From XVI by Oxidation with HPO: HPO (31%, 0.11 ml, 1.47 mmol) and 6 N NaOH (0.03 ml, 0.18 mmol) were added to a methanolic solution (1.1 ml) of XVI (71.4 mg, 0.31 mmol). The mixture was stirred at room temperature for 23.7 h and extracted with CH_2Cl_2. The organic layer was washed with brine and evaporated to give an oily residue (22.7 mg), which was fractionated through an SiO_2 column. XVII: bp < 110 °C (0.3 mmHg). NMR ($CDCl_3$): 1.91 (3H, s, C_4-CH_3), 3.52 (3H, s, OMe), 3.61 and 3.96 (each 1H, d, $J=4$, C_3- and C_2-H, respectively), 5.02 (1H, s, C_5-H), 7.2—7.4 (5H, m, aromatic H). MS *m/z* (%): 262 (M^+, 26), 145[Ph(Me)$\overset{+}{C}$–CH_2CHO, 100]), 117 (M^+-145, 51). *Anal.* Calcd for $C_{14}H_{14}O_5$: C, 64.11; H, 5.38. Found: C, 64.17; H, 5.44.

b) From XVIII by Oxidation of mCPBA: mCPBA (785 mg, 3.18 mmol) was added to a solution of XVIII (54.0 mg, 0.24 mmol) and Na_2HPO_4 (3.78 g, 10.6 mmol) in CH_2Cl_2 (7.3 ml) and water (5.2 ml) under ice-cooling. The mixture was stirred at 4 °C for 21.7 h. The crude product after the usual work-up was fractionated through an SiO_2 column. XVIII (14.8 mg, 21.8%) and XVII (28.8 mg, 46.8%) were successively eluted with benzene. The physical data of XVII were identical with those of XVII obtained in the above experiment a).

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Synthesis of Leukotriene B_3 Derivatives

Toshio Nakamura,* Mitsuo Namiki, and Keiichi Ono

Research Laboratories, Sumitomo Pharmaceuticals Co., Ltd., Konohana-ku, Osaka 554, Japan

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Four geometric isomers of a leukotriene B_3 derivative (**1b**, **2b**, **3b**, **4b**) and their nor derivatives (**1a**, **2a**, **3a**, **4a**) were synthesized selectively from a common starting material by the same methodology using the Wittig–Horner reaction. All optical isomers of all-*trans* nor leukotriene B_3 dimethylamide (**1a**), one of the eight compounds, were also synthesized.

Keywords——leukotriene B_4; leukotriene B_3 derivative; geometric isomer; hydrogenation; Wittig–Horner reaction; asymmetric reduction

Leukotriene B_4(LTB_4), a metabolite of arachidonic acid, has been reported to have various pharmacological actions such as chemotaxis, chemokinesis, aggregation and degranulation of leukocytes *in vitro*.[1] Therefore, it is thought to be a primary mediator in inflammatory and allergic states, and inhibitors of its actions are expected to have therapeutic potential. We planned to synthesize several LTB_4 derivatives for a study of the structure–activity relationships, focusing on the stereochemistry of the conjugated triene part.[2] The influence of the C-14,15 double bond was removed by saturation, and the carboxyl group was changed into an *N,N*-dimethylcarbamoyl group, because LTB_4 dimethylamide has a strong antagonistic activity.[3]

Though several synthetic studies on LTB_4 itself[4] have been reported since its discovery, we have devised a very useful method for constructing geometric isomers of LTB_3 derivatives. It is characterized by the combination of two Wittig reactions. In this synthesis, the butyne derivative (**5**) was used as the middle olefinic part and the other two olefinic parts were introduced by means of Wittig reaction with two phosphonate derivatives, as illustrated in Chart 1.

All-*trans* derivatives (**1a**, **1b**) were synthesized by Wittig reaction of the enal (**6**) obtained from **5**. *trans-cis-trans* derivatives (**2a**, **2b**) were synthesized by hydrogenation of the corresponding acetylenic intermediates, which were prepared in a similar manner starting from the ynal (**16**). *trans-trans-cis* and *cis-trans-trans* derivatives (**3a**, **3b**, **4a**, **4b**) were also synthesized by hydrogenation of the corresponding acetylenic intermediates, which were

leukotriene B_4(LTB_4)

Fig. 1

easily prepared by Wittig–Horner reaction in the presence of *N*-bromosuccinimide (NBS),[5] followed by dehydrobromination. The advantage of the present synthetic method is that many kinds of leukotriene B_3 (LTB_3) derivatives can be obtained easily from a common starting material by means of two Wittig reactions.

We were also interested in the structure–activity relationship with regard to the stereochemistry of the hydroxy groups. Thus, we synthesized all the optical isomers of all-*trans* nor LTB_3 dimethylamide (**1a**) by using this methodology.

(MeO)2P(O)–C(O)–(CH2)nCOOR; CHO–≡–CHO; (MeO)2P(O)–C(O)–octyl ⇒ 1a, b (all-*trans*); 2a, b (*trans*-*cis*-*trans*); 3a, b (*trans*-*trans*-*cis*); 4a, b (*cis*-*trans*-*trans*)

Chart 1

All-*trans* LTB_3 Derivatives (1a, 1b)

The key intermediates, the aldehydes (**7a**, **7b**), were synthesized in 3 steps starting from **6** in 42% and 52% overall yields, as follows. Wittig reaction of **6** obtained from **5**[6] with methyl 5-(triphenylphosphoranylidene) levulinate[7] or the sodium salt of dimethyl (5-methoxycarbonyl-2-oxopentyl)phosphonate afforded the corresponding dienones. These dienones were treated with *p*-toluenesulfonic acid and oxidized with active manganese (IV) oxide to give the aldehyde (**7a**, **7b**). Wittig-Horner reaction of **7a** or **7b** with the sodium salt of dimethyl (2-oxodecyl)phosphonate afforded the diones (**8a**, **8b**), which were reduced with $NaBH_4$ and treated with dimethylamine to give the all-*trans* LTB_3 derivatives (**1a**, 57% from **7a**; **1b**, 32% from **7b**). Each compound (**1a**, **1b**) was obtained as a mixture of two diastereomers which were separable by high performance liquid chromatography (HPLC).

5 → 6 → OHC–⋯–C(O)–(CH2)nCO2Me (7a (*n*=2), 7b (*n*=3)) → *n*-Oct–C(O)–⋯–C(O)–(CH2)nCO2Me (8a (*n*=2), 8b (*n*=3)) → *n*-Oct–CH(OH)–⋯–CH(OH)–(CH2)nCONMe2 (1a (*n*=2), 1b (*n*=3))

Chart 2

All optical isomers of **1a** were also synthesized in a similar manner *via* **12** and **14**, as follows. Oxidation of the alcohol (**9**) obtained from **5** with active manganese (IV) oxide afforded the *cis* enal (**10**), which was treated with the sodium salt of dimethyl (2-oxodecyl)phosphonate to give the dienone (**11**; 56% from **9**). Then, this dienone (**11**) was

reduced with some chiral reagents. *B*-3-Pinanyl-9-borabicyclo[3.3.1]nonane,[8a] Baker's yeast[8b] and lithium aluminum hydride decomposed by (−)-*N*-methylephedrine[8c] gave unsatisfactory results. However, with Noyori's reagent ((*R*)-lithium 1,1′-binaphthyl-2,2′-dioxy-ethoxyaluminum hydride ((*R*)-BINAL-H)),[9] the dienone (**11**) was reduced into (6*R*)-**12** (R=H; 49%, 80% ee). On the other hand, reduction of **11** with (*S*)-BINAL-H gave (6*S*)-**12** (R=H; 75%, 75% ee). Then, (6*R*)-**12** and (6*S*)-**12** (R=H) were acylated with (L)-menthoxyacetyl chloride to give (6*R*)-**12** and (6*S*)-**12**.

9 → 10 → 11 → (6*R*)-12, (6*S*)-12 (R=(L)-menthoxyacetyl) → (6*R*)-13, (6*S*)-13 → (11*R*)-14, (11*S*)-14 → 4 isomers of 1a

Chart 3

These absolute configurations were estimated on the basis of Noyori's general rule that (*R*)-alcohols are obtained by reduction of enones with (*R*)-BINAL-H, and that (*S*)-alcohols are obtained by reduction of them with (*S*)-BINAL-H.[9] These configurations were confirmed by comparison of the proton nuclear magnetic resonance (^{1}H-NMR) spectra of (6*R*)-**12** and (6*S*)-**12** (R=(*R*)-α-methoxy-α-trifluoromethylphenylacetyl (MTPAc)),[10] which were prepared from (6*R*)-**12** and (6*S*)-**12** (R=H) by acylation with (*R*)-α-methoxy-α-trifluoromethylphenylacetyl chloride (MTPAcCl). Namely, the olefinic protons of (6*R*)-**12** (R=MTPAc) are significantly shifted downfield in comparison with those of (6*S*)-**12** (R=MTPAc) as shown in Table I. This result suggested that (6*R*)-12 (R=MTPAc) has *R*

(6*R*)-12 : R=MTPAc (6*S*)-12 : R=MTPAc

Chart 4

Table I. ^{1}H-NMR Data for (6*R*)-12 and (6*S*)-12 (R=MTPAc)

	Chemical shifts (δ) (6*R*)-**12** (*X*)	(6*S*)-**12** (*Y*)	Coupling	δ (*X*−*Y*)
CH_3O	3.543	3.559	3H, q, *J*=1 Hz	−0.016
THP	4.627	4.624	1H, dd, *J*=7, 4 Hz	+0.003
2-H	5.70	5.66	1H, m	+0.03
3-H	6.107	6.061	1H, t, *J*=11 Hz	+0.046
4-H	6.636	6.542	1H, dd, *J*=15, 11 Hz	+0.094
5-H	5.657	5.608	1H, dd, *J*=15, 7 Hz	+0.049
6-H	5.526	5.525	1H, q, *J*=7 Hz	+0.001

THP: tetrahydropyranyl.

configuration at C-6, because this downfield shift may be caused by anisotropy of the phenyl group.

Compounds (6*R*)-**12** and (6*S*)-**12** (R = (L)-menthoxyacetyl) were also separated by chromatography on a LoBar column, as follows. The dienone (**11**) was reduced with $NaBH_4$ and acylated with (L)-menthoxyacetyl chloride to give a diastereomeric mixture of **12** quantitatively. This was chromatographed on a LoBar column to afford (6*R*)-**12** and (6*S*)-**12**, which showed the same ^{1}H-NMR and infrared (IR) spectra as (6*R*)-**12** and (6*S*)-**12** obtained by asymmetrical reduction.

n-Oct–CH(OR)–CH=CH–CH=CH–CH=CH–C(=O)–(CH2)3–CO2Me ⟶ n-Oct–CH(OR)–CH=CH–CH=CH–CH=CH–CH(MTPAcO)–(CH2)3–CO2Me ⟶ (4*R*, 11*R*)-1a or (4*S*, 11*R*)-1a

(11*R*)-14 : R=(L)-menthoxyacetyl

(4*R*, 11*R*)-15
(4*S*, 11*R*)-15

Chart 5

Then (6*R*)-**12** and (6*S*)-**12** (R = (L)-menthoxyacetyl) were deprotected with *p*-toluenesulfonic acid to give (6*R*)-**13** and (6*S*)-**13**, respectively. Oxidation of (6*R*)-**13** and (6*S*)-**13** with active manganese (IV) oxide followed by Wittig reaction with methyl 5-(triphenylphosphoranylidene)levulinate and isomerization with iodine gave the ester (11*R*)-**14** (11%) and (11*S*)-**14** (12%). Deprotection of each of (11*R*)-**14** and (11*S*)-**14** with K_2CO_3 in MeOH followed by reduction with $NaBH_4$, amidation with dimethylamine and separation by HPLC afforded two optically active all-*trans* nor LTB_3 derivatives ((4*R*,11*R*)-**1a**, (4*S*,11*R*)-**1a** and (4*R*,11*S*)-**1a**, (4*S*,11*S*)-**1a**), respectively.

The configurations at C-4 of (4*R*,11*R*)-**1a** and (4*S*,11*R*)-**1a** were confirmed as follows. Compounds (11*R*)-**14** was reduced with $NaBH_4$, acylated with MTPAcCl and separated into two diastereoisomers ((4*R*,11*R*)-**15** and (4*S*,11*R*)-**15**) by HPLC. Their configurations at C-4 were estimated by comparison of their ^{1}H-NMR spectra, in a manner similar to that described for the determination of the configurations of (6*R*)-**12** and (6*S*)-**12**. Namely, the olefinic protons of (4*R*,11*R*)-**15** are significantly shifted downfield in comparison with those of (4*S*,11*R*)-**15**. Deprotection of (4*R*,11*R*)-**15** and (4*S*,11*R*)-**15** with K_2CO_3 in MeOH followed by amidation with dimethylamine afforded (4*R*,11*R*)-**1a** and (4*S*,11*R*)-**1a**, respectively. On the other hand, (4*R*,11*S*)-**1a** and (4*S*,11*S*)-**1a** showed the same ^{1}H-NMR and IR spectra as (4*R*,11*R*)-**1a** and (4*S*,11*R*)-**1a**. Thus, the configurations at C-4 of (4*R*,11*S*)-**1a** and (4*S*,11*S*)-**1a** were determined by comparison with (4*R*,11*R*)-**1a** and (4*S*,11*R*)-**1a** in respect to optical rotations and retention times in HPLC.

trans-cis-trans LTB_3 Derivatives (2a, 2b)

The diolamides (**18a**, **18b**) were synthesized from **16**[11] by the same procedure as used in

HO–CH2–C≡C–CH2–OTHP (5) ⟶ OHC–C≡C–CH2–OTHP (16) ⟶ n-Oct–C(=O)–CH=CH–CH=CH–CH=CH–C(=O)–(CH2)nCO2Me

17a ($n=2$)
17b ($n=3$)

⟶ n-Oct–CH(OH)–CH=CH–C≡C–CH=CH–CH(OH)–(CH2)nCONMe2 ⟶ n-Oct–CH(OH)–CH=CH–CH=CH–CH=CH–CH(OH)–(CH2)nCONMe2

18a ($n=2$)
18b ($n=3$)

2a ($n=2$)
2b ($n=3$)

Chart 6

the syntheses of **1a** and **1b** in 33% and 23% yields respectively. Hydrogenation of **18a** or **18b** was carried out using Lindlar catalyst and quinoline[12] to give the *trans-cis-trans* LTB_3 derivatives (**2a**, **2b**), quantitatively. In this reaction, isomerization of the *cis* olefin in **2a** or **2b** did not occur.

trans-trans-cis LTB_3 Derivatives (3a, 3b)

Wittig–Horner reaction of **7a** or **7b** with the sodium salt of dimethyl (2-oxodecyl)phosphonate in the presence of NBS afforded the bromodiones (**19a**, **19b**),[5] which were reduced with $NaBH_4$ and treated with dimethylamine to give the diolamides (**20a**, **20b**). Dehydrobromination of **20a** or **20b** performed with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (toluene, 110 °C, 5 h) or KO^{t}Bu (dimethyl sulfoxide (DMSO), 10 °C, <10 s)[13] gave **21a** or **21b** (24% from **7a**, 35% from **7b**). We concluded that the geometry of the newly formed olefins in **20a** and **20b** is *trans*, as illustrated in Chart 7, because dehydrobromination of **20a** or **20b** with KO^{t}Bu was completed in 10 s at 10 °C.[13] Hydrogenation of **21a** and **21b** was carried out in the same manner as with the *trans-cis-trans* LTB_3 derivatives (**2a**, **2b**) to give the *trans-trans-cis* LTB_3 derivatives (**3a**, **3b**), quantitatively.

OHC–…–C(=O)(CH2)nCO2Me → n-Oct–C(=O)–C(Br)=…–C(=O)(CH2)nCO2Me

7a (n=2)
7b (n=3)

19a (n=2)
19b (n=3)

→ n-Oct–CH(OH)–C(Br)=…–CH(OH)(CH2)nCONMe2 →

20a (n=2)
20b (n=3)

n-Oct–CH(OH)–…–CH(OH)(CH2)nCONMe2 → n-Oct–CH(OH)–…–CH(OH)(CH2)nCONMe2

21a (n=2)
21b (n=3)

3a (n=2)
3b (n=3)

Chart 7

CHO / OTHP → n-Oct–C(=O)–…–CHO →

6

22

n-Oct–C(=O)–…–C(Br)–C(=O)(CH2)nCO2R → n-Oct–CH(OH)–…–C(Br)–CH(OH)(CH2)nCONMe2

23a : R=Et(n=2)
23b : R=Me(n=3)

24a (n=2)
24b (n=3)

→ n-Oct–CH(OH)–…–CH(OH)(CH2)nCONMe2 → n-Oct–CH(OH)–…–CH(OH)(CH2)nCONMe2

25a (n=2)
25b (n=3)

4a (n=2)
4b (n=3)

Chart 8

cis-trans-trans LTB_3 Derivatives (4a, 4b)

The *cis-trans-trans* LTB_3 derivatives (**4a**, **4b**) were obtained from **6** by the same procedure as shown in Chart 8 in 10% and 28% overall yields, respectively.

All of the LTB_3 derivatives prepared above have different retention times in HPLC. The geometry of their triene moiety was determined from the ^{1}H-NMR spectra. In the case of the series of *cis* derivatives (**2**, **3**, **4**), their geometries were confirmed by the coupling constants of their olefinic protons. In the case of all-*trans* derivatives, we could not assign their olefinic protons. Therefore, we determined their geometries from the coupling constants of the olefinic protons of the corresponding all-*trans* dione esters.

We have synthesized a series of LTB_3 derivatives with various α and ω chains by the method mentioned above. Some of them showed anti-LTB_4 activity or agonistic activity.[14] Further studies are planned on the structure–activity relationship.

Experimental

IR spectra were measured with a Hitachi model 260-10 spectrometer. ^{1}H-NMR spectra were measured on a Varian EM-390 (90 MHz) or a JEOL JNM-GX270 FT NMR spectrometer in $CDCl_3$ with Me_4Si as an internal standard. Mass spectra (MS) were taken on a JEOL JMS-D300 spectrometer as bis trimethylsilyl (TMS) ethers. Melting points were obtained on a Thomas Hoover capillary melting point apparatus and are uncorrected. Ultraviolet (UV) spectra were measured with a Hitachi 330 spectrometer in methanol. Optical rotations were measured on a JASCO DIP-181 digital polarimeter. HPLC was performed on a SUMIPAX OA-4200 column (Sumika Analysis Co. Ltd., 5 μm, 4 mm × 25 cm) (hexane : dichloroethane : ethanol = 25 : 5 : 1, 1 ml/min).

(5*E*,7*E*)-Methyl 8-Formyl-4-oxoocta-5,7-dienoate (7a)——Methyl 5-(triphenylphosphoranylidene)levulinate (35 g) was added to an *N*,*N*-dimethylformamide (DMF) solution (100 ml) of (2*E*)-4-tetrahydropyranoxy-2-butenal (**6**; 13 g), and the mixture was stirred at 50 °C for 3 h. The mixture was poured into water and extracted with AcOEt. The organic layer was washed, dried, concentrated, and chromatographed on silica gel (15—30% AcOEt/hexane (v/v)) to give a colorless oil (9 g).

p-Toluenesulfonic acid monohydrate (200 mg) was added to an MeOH solution (150 ml) of the above oil (9 g), and the mixture was stirred at 50°C for 1 h. The MeOH was evaporated off, and sat. $NaHCO_3$ aq. was added to the residue. Then the mixture was extracted with AcOEt. The extract was washed, dried, and concentrated to give a colorless oil.

Active manganese (IV) oxide (20 g) was added to a $CHCl_3$ solution (100 ml) of the above oil. The reaction mixture was stirred at room temperature for 10 h, then filtered on celite, concentrated and chromatographed on silica gel (30—50% AcOEt/hexane (v/v)) to give **7a** (6.4 g, 42%) as pale yellow plates. mp 86—86.5 °C (from CH_2Cl_2–hexane). ^{1}H-NMR ($CDCl_3$, 90 MHz) δ: 2.69 (2H, t, J=7 Hz), 2.98 (2H, t, J=7 Hz), 3.70 (3H, s), 6.47 (1H, dd, J=15, 8 Hz), 6.58 (1H, d, J=15 Hz), 7.18 (1H, dd, J=15, 11 Hz), 7.33 (1H, dd, J=15, 11 Hz), 9.69 (1H, d, J=8 Hz). IR γ_{max}^{film} cm^{-1}: 1730, 1695, 1680. *Anal.* Calcd for $C_{10}H_{12}O_4$: C, 61.22; H, 6.16. Found: C, 60.87; H, 6.03.

(6*E*,8*E*)-Methyl 9-Formyl-5-oxonona-6,8-dienoate (7b)——A tetrahydrofuran (THF) suspension (200 ml) of NaH (60% dispersion in mineral oil; 2.4 g) was treated with dimethyl (5-methoxycarbonyl-2-oxopentyl)phosphonate (16.4 g), and then a THF solution (20 ml) of **6** (11 g) was added at 0 °C. The reaction mixture was stirred at room temperature for 2 h, poured into water, and extracted with AcOEt. The extract was washed, dried, concentrated, and chromatographed on silica gel (15—30% AcOEt/hexane (v/v)) to give a colorless oil (10.2 g).

In a manner similar to that described for the synthesis of **7a**, **7b** (7.1 g, 52%) was obtained from the above oil as pale yellow plates. mp 67.5—68 °C (from AcOEt–hexane). ^{1}H-NMR ($CDCl_3$, 90 MHz) δ: 2.40 (2H, t, J=7 Hz), 2.73 (2H, t, J=7 Hz), 3.69 (3H, s), 6.5 (1H, m), 6.53 (1H, d, J=15 Hz), 7.20 (1H, dd, J=15, 11 Hz), 7.32 (1H, dd, J=15, 11 Hz), 9.76 (1H, d, J=8 Hz). IR γ_{max}^{Nujol} cm^{-1}: 1735, 1690, 1665. *Anal.* Calcd for $C_{11}H_{14}O_4$: C, 62.85; H, 6.71. Found: C, 62.86; H, 6.76.

(5*E*,7*E*,9*E*)-Methyl 4,11-Dioxononadeca-5,7,9-trienoate (8a)——Dimethyl (2-oxodecyl)phosphonate (700 mg) was added to a THF suspension (25 ml) of NaH (60% dispersion in mineral oil; 100 mg), and then a THF solution (10 ml) of **7a** (500 mg) was further added at 0 °C. The reaction mixture was stirred at room temperature for 2 h, poured into water, and extracted with AcOEt. The extract was washed, dried, concentrated, and chromatographed on silica gel (10—20% AcOEt/hexane (v/v)) to give **8a** (700 mg; 82%) as pale yellow needles. mp 108.5—109 °C (from AcOEt–hexane). ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7 Hz), 2.57 (2H, t, J=7 Hz), 2.66 (2H, t, J=7 Hz), 2.93 (2H, t, J=7 Hz), 3.69 (3H, s), 6.32 (1H, d, J=15 Hz), 6.33 (1H, d, J=15 Hz), 6.67 (1H, dd, J=15, 12 Hz), 6.70 (1H, dd, J=15, 12 Hz), 7.21 (1H, dd, J=15, 12 Hz), 7.24 (1H, dd, J=15, 12 Hz). IR γ_{max}^{Nujol} cm^{-1}: 1730, 1680. *Anal.* Calcd for $C_{20}H_{30}O_4$: C, 71.82; H, 9.04. Found: C, 71.89; H, 9.11.

(6*E*,8*E*,10*E*)-Methyl 5,12-Dioxoeicosa-6,8,10-trienoate (8b)——The dioxoester (**8b**) was prepared as pale yellow

needles, in 44% yield, from **7b** in a manner similar to that described for the synthesis of **8a**. mp 122—122.5 °C (from CH_2Cl_2–hexane). ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7 Hz), 1.96 (2H, qui, J=7 Hz), 2.39 (2H, t, J=7 Hz), 2.58 (2H, t, J=7 Hz), 2.66 (2H, t, J=7 Hz), 3.67 (3H, s), 6.27 (1H, d, J=15 Hz), 6.29 (1H, d, J=15 Hz), 6.60 (1H, dd, J=14, 11 Hz), 6.68 (1H, dd, J=14, 11 Hz), 7.1—7.4 (2H, m). IR γ_{max}^{KBr} cm^{-1}: 1730, 1680. *Anal.* Calcd for $C_{21}H_{32}O_4$: C, 72.38; H, 9.26. Found: C, 71.97; H, 9.42.

(5*E*,7*E*,9*E*)-*N*,*N*-Dimethyl-4,11-dihydroxynonadeca-5,7,9-trienamide (1a)——$NaBH_4$ (1 g) was added to an MeOH solution (300 ml) of **8a** (6.04 g) at 0 °C, and the mixture was stirred at 0 °C for 2 h. Then, Me_2NH (60 wt.% solution in water; 100 ml) was added. The resulting mixture was stirred at room temperature for 12 h, poured into water and extracted with AcOEt. The extract was washed, dried, concentrated, and chromatographed on silica gel (0—10% MeOH/AcOEt (v/v)) to give **1a** (4.3 g; 69%) as a colorless oil. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7 Hz), 2.48 (2H, t, J=6 Hz), 2.96 (3H, s), 3.02 (3H, s), 4.13 (1H, m), 4.26 (1H, m), 5.72 (2H, m), 6.20 (4H, m). IR γ_{max}^{film} cm^{-1}: 3400, 1630. HPLC: 22.4, 24.4 min. UV λ_{max}^{MeOH} nm: 258.5, 268.0, 279.0. MS m/z: 495 (M^+), 480, 405, 73. High-resolution MS m/z: M^+ Calcd for $C_{27}H_{53}NO_3Si_2$: 495.3563. Found: 495.3541.

(6*E*,8*E*,10*E*)-*N*,*N*-Dimethyl-5,12-dihydroxyeicosa-6,8,10-trienamide (1b)——The diolamide (**1b**) was prepared, in 72% yield, from **8b** in a manner similar to that described for the synthesis of **1a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7 Hz), 2.36 (2H, t, J=7 Hz), 2.94 (3H, s), 3.00 (3H, s), 4.1 (2H, m), 5.70 (2H, m), 6.20 (4H, m). IR γ_{max}^{film} cm^{-1}: 3400, 1630. HPLC: 22.8, 25.5 min. UV λ_{max}^{MeOH} nm: 259.0, 268.0, 279.0. MS m/z: 509 (M^+), 494, 419, 73. High-resolution MS m/z: M^+ Calcd for $C_{28}H_{55}NO_3Si_2$: 509.3719. Found: 509.3742.

(2*Z*)-4-Tetrahydropyranoxy-2-butenal (10)——The *cis* enal (**10**) was prepared, in 63% yield, from **9** in a manner similar to that described for the synthesis of **7a**. ^{1}H-NMR ($CDCl_3$, 90 MHz) δ: 4.5—4.7 (3H, m), 6.02 (1H, ddt, J=11, 7, 2 Hz), 6.65 (1H, dt, J=11, 5 Hz), 10.14 (1H, d, J=7 Hz).

(2*Z*,4*E*)-1-Tetrahydropyranoxy-6-oxo-2,4-tetradecadiene (11)——The ketone (**11**) was prepared, in 74% yield, from **10** in a manner similar to that described for the synthesis of **8a**. ^{1}H-NMR ($CDCl_3$, 90 MHz) δ: 0.88 (3H, t, J=7 Hz), 2.57 (2H, t, J=7 Hz), 4.42 (2H, m), 4.67 (1H, br s), 5.8—6.5 (2H, m), 6.19 (1H, d, J=16 Hz), 7.58 (1H, dd, J=16, 11 Hz).

Reduction of 11 with (*R*) or (*S*)-BINAL-H——EtOH (616 mg) in THF (7 ml) and (*R*)-binaphthol (3.83 g) in THF (21 ml) were successively added dropwise with stirring to an $LiAlH_4$ solution in THF (1.14 M; 11.7 ml) at 0 °C under an Ar atmosphere. After 1 h, **11** (0.82 g) in THF (3 ml) was added dropwise at −78 °C, and the whole was stirred for 4 h. The reaction mixture was decomposed with 1 N NaOHaq. and extracted with ether. The extract was washed, dried, concentrated, and chromatographed on silica gel (10—30% AcOEt/hexane (v/v)) to afford (6*R*)-**12** (R=H; 160 mg; 49%, 80% ee) and **11** (480 mg). In a similar manner, reduction of **11** with (*S*)-BINAL-H also afforded (6*S*)-**12** (R=H; 75%, 75% ee).

NEt_3 (0.36 ml), (L)-menthoxyacetyl chloride (240 mg) and *p*-dimethylaminopyridine (DMAP) (1 mg) were added to a THF solution (6.5 ml) of this alcohol ((6*R*)-**12** (R=H; 160 mg)) at 0 °C. The mixture was stirred at room temperature for 10 h. Ethanolamine (0.5 ml) was added, and the mixture was stirred for 30 min, then diluted with water, and extracted with AcOEt. The extract was washed, dried, concentrated, and chromatographed on silica gel (0—10% AcOEt/hexane (v/v)) to give (6*R*)-**12** (R=(L)-menthoxyacetyl; 260 mg) quantitatively. The alcohol ((6*S*)-**12** (R=H)) was also acylated quantitatively with (L)-menthoxyacetyl chloride in a similar manner. (6*R*)-**12**: ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.79 (3H, d, J=7 Hz), 0.9 (9H, m), 3.16 (1H, td, J=11, 4 Hz), 4.07 (1H, d, J=16 Hz), 4.12 (1H, d, J=16 Hz), 4.2—4.5 (2H, m), 4.63 (1H, br), 5.37 (1H, q, J=7 Hz), 5.6 (2H, m), 6.09 (1H, t, J=11 Hz), 6.54 (1H, dd, J=15, 11 Hz). IR γ_{max}^{film} cm^{-1}: 1760. $[\alpha]_D^{25}$ −50° (c=1.00, hexane). (6*S*)-**12**: ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.79 (3H, d, J=7 Hz), 0.9 (9H, m), 3.16 (1H, td, J=11, 4 Hz), 4.08 (1H, d, J=16 Hz), 4.10 (1H, d, J=16 Hz), 4.2—4.5 (2H, m), 4.63 (1H, br), 5.37 (1H, q, J=7 Hz), 5.6 (2H, m), 6.09 (1H, t, J=11 Hz), 6.54 (1H, dd, J=15, 11 Hz). IR γ_{max}^{film} cm^{-1}: 1760. $[\alpha]_D^{25}$ −45° (c=1.11, hexane).

(2*Z*,4*E*)-1-Tetrahydropyranoxy-6-((*R*)-α-methoxy-α-trifluoromethylphenylacetoxy)-2,4-tetradecadiene ((6*R*)-12 (R=MTPAc) and (6*S*)-12 (R=MTPAc))—— NEt_3 (0.4 ml), MTPAcCl (500 mg) and DMAP (1 mg) were added to a THF solution (10 ml) of (6*R*)-**12** (R=H, 127 mg) at 0 °C. The mixture was stirred at room temperature for 10 h. Ethanolamine (1 ml) was added, and the mixture was stirred for 30 min, then diluted with water, and extracted with AcOEt. The extract was washed, dried, concentrated, and chromatographed on silica gel (0—10% AcOEt/hexane (v/v)) to give (6*R*)-**12** (R=MTPAc; 160 mg; 77%) as a colorless oil. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7 Hz), 3.543 (3H, q, J=1 Hz), 4.1—4.45 (2H, m), 4.627 (1H, dd, J=7, 4 Hz), 5.526 (1H, q, J=7 Hz), 5.657 (1H, dd, J=15, 7 Hz), 5.70 (1H, m), 6.107 (1H, t, J=11 Hz), 6.636 (1H, dd, J=15, 11 Hz), 7.39 (3H, m), 7.52 (2H, m).

(6*S*)-**12** (R=MTPAc) was prepared, in 77% yield, from (6*S*)-**12** (R=H) in a similar manner. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7 Hz), 3.559 (3H, q, J=1 Hz), 4.1—4.4 (2H, m), 4.624 (1H, dd, J=7, 4 Hz), 5.525 (1H, q, J=7 Hz), 5.608 (1H, dd, J=15, 7 Hz), 5.66 (1H, m), 6.061 (1H, t, J=11 Hz), 6.542 (1H, dd, J=15, 11 Hz), 7.39 (3H, m), 7.50 (2H, m).

(2*Z*,4*E*)-1-Tetrahydropyranoxy-6-((L)-menthoxyacetoxy)-2,4-tetradecadiene (12)——$NaBH_4$ (500 mg) was added to an MeOH solution (100 ml) of **11** (5 g) at 0 °C, and the mixture was stirred at 0 °C for 2 h. The MeOH was removed *in vacuo*, and the mixture was diluted with water, and extracted with AcOEt. The extract was washed, dried, and concentrated to give (*dl*)-**12** (R=H). The alcohol ((*dl*)-**12** (R=H)) was acylated with (L)-menthoxyacetyl chloride

to give **12** (7.5 g; 92% from **11**), in a manner similar to that described for the reduction of **11** with (*R*)- or (*S*)-BINAL-H. ^{1}H-NMR ($CDCl_3$, 90 MHz) δ: 0.90 (12H, m), 3.16 (1H, td, J=11, 4 Hz), 4.10 (2H, br s), 4.3 (2H, br m), 4.62 (1H, br s), 5.3—5.7 (3H, m), 6.09 (1H, t, J=11 Hz), 6.54 (1H, dd, J=15, 11 Hz).

Separation into (6*R*)-12 and (6*S*)-12——Separation of the mixture of **12** was performed by chromatography on a LoBar column (Silica gel 60 (230—400 mesh; Merck), 8% Et_2O/hexane (v/v)).

(2*Z*,4*E*)-6-((L)-Menthoxyacetoxy)tetradeca-2,4-dien-1-ol ((6*R*)-13, (6*S*)-13)——The alcohol ((6*R*)-**13** or (6*S*)-**13**) was prepared quantitatively from (6*R*)-**12** or (6*S*)-**12**, respectively, in a manner similar to that described for the synthesis of **7a**. (6*R*)-**13**: ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.79 (3H, d, J=7 Hz), 0.9 (9H, m), 3.16 (1H, td, J=11, 4 Hz), 4.07 (1H, d, J=16 Hz), 4.12 (1H, d, J=16 Hz), 4.32 (2H, d, J=7 Hz), 5.37 (1H, q, J=7 Hz), 5.66 (2H, m), 6.05 (1H, t, J=11 Hz), 6.52 (1H, dd, J=15, 11 Hz). IR γ_{max}^{film} cm^{-1}: 3400, 1750. $[\alpha]_D^{25}$ $-58°$ (c=1.06, hexane). (6*S*)-**13**: ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.79 (3H, d, J=7 Hz), 0.9 (9H, m), 3.16 (1H, td, J=11, 4 Hz), 4.08 (1H, d, J=16 Hz), 4.11 (1H, d, J=16 Hz), 4.32 (2H, d, J=7 Hz), 5.36 (1H, q, J=7 Hz), 5.66 (2H, m), 6.04 (1H, t, J=11 Hz), 6.52 (1H, dd, J=15, 11 Hz). IR γ_{max}^{film} cm^{-1}: 3400, 1750. $[\alpha]_D^{25}$ $-56°$ (c=0.82, hexane).

(5*E*,7*E*,9*E*)-Methyl 11-((L))-Menthoxyacetoxy)-4-oxononadeca-5,7,9-trienoate ((11*R*)-14, (11*S*)-14)——A geometric mixture of the ester ((11*R*)-**14** or (11*S*)-**14**) was prepared in 11% or 12% yield from (6*R*)-**13** or (6*S*)-**13**, respectively, in a manner similar to that described for the synthesis of **7b**.

A CH_2Cl_2 solution (40 ml) of the crude ester ((11*R*)-**14**; 340 mg) was treated with iodine (10 mg). The reaction mixture was stirred at room temperature for 24 h, then washed with a saturated aq. solution of $Na_2S_2O_3$, dried and concentrated to give the ester ((11*R*)-**14**; 340 mg).

The ester ((11*S*)-**14**) was prepared quantitatively in a similar manner. (11*R*)-**14**: ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.79 (3H, d, J=7 Hz), 0.9 (9H, m), 2.65 (2H, t, J=7 Hz), 2.91 (2H, t, J=7 Hz), 3.16 (1H, td, J=11, 4 Hz), 3.69 (3H, s), 4.08 (1H, d, J=16 Hz), 4.14 (1H, d, J=16 Hz), 5.39 (1H, q, J=7 Hz), 5.83 (1H, dd, J=15, 7 Hz), 6.21 (1H, d, J=15 Hz), 6.2—6.4 (2H, m), 6.56 (1H, dd, J=15, 11 Hz), 7.21 (1H, dd, J=15, 11 Hz). IR γ_{max}^{film} cm^{-1}: 1750, 1740, 1660, 1600. $[\alpha]_D^{25}$ $-26°$ (c=0.29, hexane). (11*S*)-**14**: ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.79 (3H, d, J=7 Hz), 0.9 (9H, m), 2.65 (2H, t, J=7 Hz), 2.91 (2H, t, J=7 Hz), 3.16 (1H, td, J=11, 4 Hz), 3.69 (3H, s), 4.09 (1H, d, J=16 Hz), 4.12 (1H, d, J=16 Hz), 5.38 (1H, q, J=7 Hz), 5.82 (1H, dd, J=15, 7 Hz), 6.20 (1H, d, J=15 Hz), 6.2—6.4 (2H, m), 6.55 (1H, dd, J=15, 11 Hz), 7.21 (1H, dd, J=15, 11 Hz). IR γ_{max}^{film} cm^{-1}: 1750, 1740, 1665, 1605. $[\alpha]_D^{25}$ $-53°$ (c=0.40, hexane).

Optically Active (5*E*,7*E*,9*E*)-*N*,*N*-Dimethyl-4,11-dihydroxynonadeca-5,7,9-trienamide ((4*R*,11*R*)-1a, (4*S*,11*R*)-1a, (4*R*,11*S*)-1a, (4*S*,11*S*)-1a)——K_2CO_3 (35 mg) was added to an MeOH solution (2.5 ml) of (11*R*)-**14** (22 mg). The reaction mixture was stirred at room temperature for 30 min, then poured into water and extracted with Et_2O. The extract was washed and dried to give the alcohol. A mixture of **1a** was prepared from this alcohol in a manner similar to that described for the synthesis of **1a**. Separation of this mixture of **1a** by HPLC (SUMIPAX OA-4200, Sumica Analysis Co., Ltd., 5 μm, 8 mm × 25 cm) (hexane: dichloroethane : EtOH = 25 : 5 : 1) gave (4*R*,11*R*)-**1a** (0.51 mg, 4%) and (4*S*,11*R*)-**1a** (0.57 mg, 4%) in a manner similar to that employed in the case of **12**.

In a similar manner, (4*R*,11*S*)-**1a** (3.47 mg, 18%) and (4*S*,11*S*)-**1a** (2.40 mg, 12%) were prepared from (11*S*)-**14** (30 mg).

These optical isomers of **1a** showed the same ^{1}H-NMR and IR spectra as **1a**. (4*R*,11*R*)-**1a**: $[\alpha]_D^{25}$ $-5°$ (c=0.057, $CHCl_3$). HPLC: 22.4 min. (4*S*,11*R*)-**1a**: $[\alpha]_D^{25}$ $+4°$ (c=0.051, $CHCl_3$). HPLC: 24.4 min. (4*R*,11*S*)-**1a**: $[\alpha]_D^{25}$ $-8°$ (c=0.17, $CHCl_3$). HPLC: 24.4 min. (4*S*,11*S*)-**1a**: $[\alpha]_D^{25}$ $+12°$ (c=0.12, $CHCl_3$). HPLC: 22.4 min.

(5*E*,7*E*,9*E*)-Methyl 4-((*R*)-α-Methoxy-α-trifluoromethylphenylacetoxy)-11-((L)-menthoxyacetoxy)nonadeca-5,7,9-trienoate ((4*R*,11*R*)-15, (4*S*,11*R*)-15)——(4*R*,11*R*)-**15** and (4*S*,11*R*)-**15** were prepared, each in 25% yield, from (11*R*)-**14** in a manner similar to that described for the synthesis of **12**, followed by separation by HPLC (Hibar column (Merck) Si-60, 20% Et_2O/hexane (v/v)). (4*R*,11*R*)-**15**: ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.79 (3H, d, J=7 Hz), 0.90 (9H, m), 2.00 (2H, q-like, J=7 Hz), 2.24 (2H, t-like, J=7 Hz), 2.25 (2H, m), 3.16 (1H, td, J=11, 4 Hz), 3.524 (3H, q, J=1 Hz), 3.655 (3H, s), 4.062 (1H, d, J=16 Hz), 4.120 (1H, d, J=16 Hz), 5.359 (1H, q, J=7 Hz), 5.547 (1H, q, J=7 Hz), 5.622 (1H, dd, J=15, 7 Hz), 5.658 (1H, dd, J=15, 7 Hz), 6.15—6.40 (4H, m), 7.40 (3H, m), 7.50 (2H, m). (4*S*,11*R*)-**15**: ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.79 (3H, d, J=7 Hz), 0.9 (9H, m), 2.05 (2H, q-like, J=7 Hz), 2.30 (2H, m), 2.36 (2H, t-like, J=7 Hz), 3.18 (1H, td, J=11, 4 Hz), 3.544 (3H, q, J=1 Hz), 3.671 (3H, s), 4.065 (1H, d, J=16 Hz), 4.123 (1H, d, J=16 Hz), 5.357 (1H, q, J=7 Hz), 5.57 (2H, m), 5.655 (1H, dd, J=15, 7 Hz), 6.10—6.30 (4H, m), 7.40 (3H, m), 7.50 (2H, m).

(4*R*,11*R*) and (4*S*,11*R*)-(5*E*,7*E*,9*E*)-*N*,*N*-Dimethyl-4,11-dihydroxynonadeca-5,7,9-trienamide ((4*R*,11*R*)-1a, (4*S*,11*R*)-1a)——(4*R*,11*R*)-**1a** and (4*S*,11*R*)-**1a** were prepared, each in 85% yield from (4*R*,11*R*)-**15** and (4*S*,11*R*)-**15**, respectively, in a manner similar to that described for the synthesis of optically active **1a** ((4*R*,11*R*)-**1a**, (4*S*,11*R*)-**1a**, (4*R*,11*S*)-**1a**, (4*S*,11*S*)-**1a**). These optical isomers of **1a** showed the same ^{1}H-NMR and IR spectra as **1a**. (4*R*,11*R*)-**1a**: HPLC: 22.4 min. (4*S*,11*R*)-**1a**: HPLC: 24.4 min.

(5*E*,9*E*)-Methyl 4,11-Dioxononadeca-5,9-dien-7-ynoate (17a)——The dioxoester (**17a**) was prepared as pale yellow needles, in 39% yield, from **16** in a manner similar to that described for the syntheses of **7a** and **8a**. mp 80—81 °C (from CH_2Cl_2–hexane). ^{1}H-NMR ($CDCl_3$, 90 MHz) δ: 0.88 (3H, br t, J=7 Hz), 2.55 (2H, t, J=7 Hz), 2.66 (2H, t, J=7 Hz), 2.91 (2H, t, J=7 Hz), 3.68 (3H, s), 6.61 (1H, d, J=15 Hz), 6.62 (1H, d, J=15 Hz), 6.78 (2H, br d, J=15 Hz). IR γ_{max}^{Nujol} cm^{-1}: 1740, 1650. *Anal.* Calcd for $C_{20}H_{28}O_4$: C, 72.26; H, 8.49. Found: C, 72.20; H, 8.52.

(6*E*,10*E*)-Methyl 5,12-Dioxoeicosa-6,10-dien-8-ynoate (17*b*)——The dioxoester (**17b**) was prepared as pale yellow needles, in 45% yield, from **16** in a manner similar to that described for the syntheses of **7b** and **8b**. mp 74—75 °C (from CH_2Cl_2–hexane). ^{1}H-NMR ($CDCl_3$, 90 MHz) δ: 0.88 (3H, br t, J=7 Hz), 2.38 (2H, t, J=7 Hz), 2.56 (2H, t, J=7 Hz), 2.65 (2H, t, J=7 Hz), 3.69 (3H, s), 6.61 (2H, br d, J=15 Hz), 6.77 (2H, br d, J=15 Hz). IR γ_{max}^{Nujol} cm^{-1}: 1730, 1685. *Anal.* Calcd for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.75; H, 8.99.

(5*E*,9*E*)-*N*,*N*-Dimethyl-4,11-dihydroxynonadeca-5,9-dien-7-ynamide (18a)——The diolamide (**18a**) was prepared, in 84% yield, from **17a** in a manner similar to that described for the synthesis of **1a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7 Hz), 2.97 (3H, s), 3.03 (3H, s), 4.16 (1H, q, J=6 Hz), 4.30 (1H, br), 5.82 (1H, d, J=16 Hz), 5.92 (1H, d, J=16 Hz), 6.13 (1H, dd, J=16, 2.5 Hz), 6.15 (1H, d, J=16 Hz). IR γ_{max}^{film} cm^{-1}: 3400, 2200, 1630. HPLC: 23.1 min.

(6*E*,10*E*)-*N*,*N*-Dimethyl-5,12-dihydroxyeicosa-6,10-dien-8-ynamide (18b)——The diolamide (**18b**) was prepared, in 51% yield, from **17b** in a manner similar to that described for the synthesis of **1a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7 Hz), 2.34 (2H, t, J=7 Hz), 2.93 (3H, s), 3.00 (3H, s), 4.2 (2H, br), 5.81 (1H, d, J=15 Hz), 5.83 (1H, d, J=15 Hz), 6.14 (2H, dd, J=15, 6 Hz). IR γ_{max}^{film} cm^{-1}: 3400, 2200, 1630. HPLC: 24.9 min.

(5*E*,7*Z*,9*E*)-*N*,*N*-Dimethyl-4,11-dihydroxynonadeca-5,7,9-trienamide (2a)——Lindlar catalyst (2.2 mg) and quinoline (1.5 mg) were added to a CH_2Cl_2 solution (1 ml) of **18a** (10.8 mg). The mixture was stirred at room temperature for 2 h under a hydrogen atmosphere, then the catalyst was filtered off. The filtrate was concentrated and purified by HPLC (SUMIPAX OA-4200, Sumica Analysis Co., Ltd., 8 mm × 25 cm, hexane : dichloroethane : EtOH = 25 : 5 : 1) to give **2a** (10.8 mg). ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7 Hz), 2.49 (2H, t, J=6 Hz), 2.97 (3H, s), 3.02 (3H, s), 4.18 (1H, q, J=7 Hz), 4.31 (1H, br s), 5.72 (1H, dd, J=15, 6 Hz), 5.75 (1H, dd, J=15, 6 Hz), 5.96 (1H, dd, J=12, 10 Hz), 6.00 (1H, dd, J=12, 10 Hz), 6.70 (1H, dd, J=15, 10 Hz), 6.76 (1H, dd, J=15, 10 Hz). IR γ_{max}^{film} cm^{-1}: 3400, 1630. HPLC: 17.8, 18.2 min. UV λ_{max}^{MeOH} nm: 259.0, 268.0, 278.0. MS m/z: 495 (M^+), 480, 405, 73. High-resolution MS m/z: M^+ Calcd for $C_{27}H_{53}NO_3Si_2$: 495.3563. Found: 495.3589.

(6*E*,8*Z*,10*E*)-*N*,*N*-Dimethyl-5,12-dihydroxyeicosa-6,8,10-trienamide (2b)——The diolamide (**2b**) was prepared quantitatively from **18b** in a manner similar to that described for the synthesis of **2a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7 Hz), 2.36 (2H, t, J=7 Hz), 2.96 (3H, s), 3.01 (3H, s), 4.2 (2H, m), 5.73 (1H, dd, J=15, 5 Hz), 5.75 (1H, dd, J=15, 5 Hz), 5.96 (1H, dd, J=12, 8 Hz), 5.99 (1H, dd, J=12, 8 Hz), 6.69 (1H, dd, J=15, 8 Hz), 6.72 (1H, dd, J=15, 8 Hz). IR γ_{max}^{film} cm^{-1}: 3400, 1630. HPLC: 17.1, 18.5 min. UV λ_{max}^{MeOH} nm: 259.0, 268.0, 278.0. MS m/z: 509 (M^+), 494, 419, 73. High-resolution MS m/z: M^+ Calcd for $C_{28}H_{55}NO_3Si_2$: 509.3719. Found: 509.3712.

(5*E*,7*E*,9*Z*)-Methyl 10-Bromo-4,11-dioxononadeca-5,7,9-trienoate (19a)——Dimethyl (2-oxodecyl)phosphonate (0.3 g) and NBS (0.183 g) were added to a dimethoxyethane suspension (10 ml) of NaH (60% dispersion in oil; 41 mg) at 0 °C. The mixture was stirred for 1 h. Then **7a** (0.2 g) in dimethoxyethane (0.5 ml) was added, and the mixture was stirred for 1 h at room temperature, poured into water, and extracted with AcOEt. The extract was washed, dried, concentrated, and chromatographed on silica gel (10—30% AcOEt/hexane (v/v)) to give **19a** (200 mg, 48%) as a pale yellow solid. mp 85—86 °C (from AcOEt–hexane). ^{1}H-NMR ($CDCl_3$, 90 MHz) δ: 0.88 (3H, t, J=7 Hz), 2.67 (2H, d, J=7 Hz), 2.89 (2H, t, J=7 Hz), 2.96 (2H, t, J=7 Hz), 3.71 (3H, s), 6.37 (1H, d, J=15 Hz), 6.5—7.1 (2H, m), 7.32 (1H, dd, J=15, 10 Hz), 7.62 (1H, d, J=10 Hz). IR γ_{max}^{Nujol} cm^{-1}: 1730, 1680. *Anal.* Calcd for $C_{20}H_{29}BrO_4$: C, 58.12; H, 7.07; Br, 19.3. Found: C, 58.28; H, 7.11; Br, 18.7.

(6*E*,8*E*,10*Z*)-Methyl 11-Bromo-5,12-dioxoeicosa-6,8,10-trienoate (19b)——The bromodioxoester (**19b**) was prepared as a pale yellow solid, in 71% yield, from **7b** in a manner similar to that described for the synthesis of **19a**. mp 66—67 °C (from CH_2Cl_2–hexane). ^{1}H-NMR ($CDCl_3$, 90 MHz) δ: 0.88 (3H, t, J=7 Hz), 1.96 (2H, qui, J=7 Hz), 2.36 (2H, t, J=7 Hz), 2.66 (2H, t, J=7 Hz), 2.80 (2H, t, J=7 Hz), 3.65 (3H, s), 6.31 (1H, d, J=15 Hz), 6.6—7.1 (2H, m), 7.30 (1H, dd, J=15, 10 Hz), 7.62 (1H, d, J=10 Hz). IR γ_{max}^{Nujol} cm^{-1}: 1740, 1690, 1680. *Anal.* Calcd for $C_{21}H_{31}BrO_4$: C, 59.02; H, 7.31; Br, 18.7. Found: C, 59.16; H, 7.33; Br, 19.0.

(5*E*,7*E*,9*Z*)-*N*,*N*-Dimethyl-10-bromo-4,11-dihydroxynonadeca-5,7,9-trienamide (20a)——The bromodiolamide (**20a**) was prepared, in 78% yield, from **19a** in a manner similar to that described for the synthesis of **1a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7 Hz), 2.49 (2H, t, J=7 Hz), 2.97 (3H, s), 3.02 (3H, s), 4.13 (1H, br), 4.29 (1H, br), 5.82 (1H, dd, J=15, 6 Hz), 6.3—6.6 (4H, m).

(6*E*,8*E*,10*Z*)-*N*,*N*-Dimethyl-11-bromo-5,12-dihydroxyeicosa-6,8,10-trienamide (20b)——The bromodiolamide (**20b**) was prepared, in 72% yield, from **19b** in a manner similar to that described for the synthesis of **1a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7 Hz), 2.37 (1H, t, J=7 Hz), 2.95 (3H, s), 3.00 (3H, s), 4.13 (1H, m), 4.19 (1H, q, J=6 Hz), 5.83 (1H, dd, J=14, 6 Hz), 6.3—6.6 (4H, m).

(5*E*,7*E*)-*N*,*N*-Dimethyl-4,11-dihydroxynonadeca-5,7-dien-9-ynamide (21a)——A mixture of **20a** (500 mg) and DBU (1.3 g) in toluene (20 ml) was stirred for 10 h at 110 °C. The mixture was poured into water, extracted with AcOEt, washed, dried, concentrated and chromatographed on silica gel (0—10% MeOH/AcOEt (v/v)) to give **21a** (249 mg, 61%). ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7 Hz), 2.50 (2H, t, J=7 Hz), 2.98 (3H, s), 3.02 (3H, s), 4.30 (1H, br), 4.50 (1H, br t, J=6 Hz), 5.61 (1H, d, J=15 Hz), 5.80 (1H, dd, J=15, 6 Hz), 6.34 (1H, dd, J=15, 11 Hz), 6.58 (1H, dd, J=15, 11 Hz). IR γ_{max}^{film} cm^{-1}: 3400, 2210, 1630. HPLC: 27.9 min.

(6*E*,8*E*)-*N*,*N*-Dimethyl-5,12-dihydroxyeicosa-6,8-dien-10-ynamide (21b)——The acetylenic diolamide (**21b**) was prepared, in 64% yield, from **20b** in a manner similar to that described for the synthesis of **21a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7 Hz), 2.36 (2H, m), 2.95 (3H, s), 3.00 (3H, s), 4.18 (1H, q, J=6 Hz), 4.50 (1H, t,

$J=6$ Hz), 5.60 (1H, d, $J=15$ Hz), 5.80 (1H, dd, $J=15$, 6 Hz), 6.30 (1H, dd, $J=15$, 11 Hz), 6.55 (1H, dd, $J=15$, 11 Hz). IR γ_{max}^{film} cm^{-1}: 3400, 2200, 1630. HPLC: 28.8 min.

(5*E*,7*E*,9*Z*)-*N*,*N*-Dimethyl-4,11-dihydroxynonadeca-5,7,9-trienamide (3a)——The diolamide (**3a**) was prepared quantitatively from **21a** in a manner similar to that described for the synthesis of **2a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, $J=7$ Hz), 2.49 (2H, t, $J=6$ Hz), 2.97 (3H, s), 3.02 (3H, s), 4.27 (1H, br), 4.58 (1H, q, $J=7$ Hz), 5.41 (1H, dd, $J=11$, 8 Hz), 5.76 (1H, dd, $J=15$, 6 Hz), 6.08 (1H, t, $J=11$ Hz), 6.2—6.4 (2H, m), 6.49 (1H, dd, $J=15$, 11 Hz). IR γ_{max}^{film} cm^{-1}: 3400, 1630. UV λ_{max}^{MeOH} nm: 260.5, 270.0, 280.0. HPLC: 18.9, 22.7 min. MS m/z: 495 (M^+), 480, 405, 73. High-resolution MS m/z: M^+ Calcd for $C_{27}H_{53}NO_3Si_2$: 495.3563. Found: 495.3540.

(6*E*,8*E*,10*Z*)-*N*,*N*-Dimethyl-5,12-dihydroxyeicosa-6,8,10-trienamide (3b)——The diolamide (**3b**) was prepared quantitatively from **21b** in a manner similar to that described for the synthesis of **2a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, $J=7$ Hz), 2.16 (2H, br t, $J=6$ Hz), 2.97 (3H, s), 3.00 (3H, s), 4.18 (1H, q, $J=6$ Hz), 4.54 (1H, q, $J=7$ Hz), 5.41 (1H, dd, $J=11$, 8 Hz), 5.76 (1H, dd, $J=15$, 6 Hz), 6.07 (1H, t, $J=11$ Hz), 6.1—6.4 (2H, m), 6.48 (1H, dd, $J=12$, 11 Hz). IR γ_{max}^{film} cm^{-1}: 3400, 1630. UV λ_{max}^{MeOH} nm: 260.0, 270.0, 280.0. HPLC: 18.9, 24.3 min. MS m/z: 509 (M^+), 494, 419.73. High-resolution MS m/z: M^+ Calcd for $C_{28}H_{55}NO_3Si_2$: 509.3719. Found: 509.3704.

(2*E*,4*E*)-6-Oxotetradeca-2,4-dienal (22)——The aldehyde (**22**) was prepared, in 54% yield, from **6** in a manner similar to that described for the synthesis of **7b**. ^{1}H-NMR ($CDCl_3$, 90 MHz) δ: 0.88 (3H, br t, $J=7$ Hz), 2.63 (2H, t, $J=7$ Hz), 6.47 (1H, dd, $J=15$, 8 Hz), 6.56 (1H, d, $J=15$ Hz), 7.18 (1H, dd, $J=15$, 11 Hz), 7.33 (1H, dd, $J=15$, 11 Hz), 9.72 (1H, d, $J=8$ Hz). IR γ_{max}^{film} cm^{-1}: 1710.

(5*Z*,7*E*,9*E*)-Ethyl 5-Bromo-4,11-dioxononadeca-5,7,9-trienoate (23a)——The bromodioxoester (**23a**) was prepared as a pale yellow solid, in 53% yield, from **22** in a manner similar to that described for the synthesis of **19a**. mp 66—67 °C (from AcOEt–hexane). ^{1}H-NMR ($CDCl_3$, 90 MHz) δ: 0.86 (3H, br t, $J=7$ Hz), 2.56 (2H, t, $J=7$ Hz), 2.71 (2H, t, $J=7$ Hz), 3.13 (2H, t, $J=7$ Hz), 4.12 (2H, q, $J=7$ Hz), 6.32 (1H, d, $J=15$ Hz), 6.7—7.4 (3H, m), 7.60 (1H, d, $J=10$ Hz). IR γ_{max}^{Nujol} cm^{-1}: 1730, 1680. *Anal.* Calcd for $C_{21}H_{31}BrO_4$: C, 59.02; H, 7.31; Br, 18.7. Found: C, 59.23; H, 7.41; Br, 18.3.

(6*Z*,8*E*,10*E*)-Methyl 6-Bromo-5,12-dioxoeicosa-6,8,10-trienoate (23b)——The bromodioxoester (**23b**) was prepared as a pale yellow solid quantitatively from **22** in a manner similar to that described for the synthesis of **19a**. mp 66—67 °C (from CH_2Cl_2–hexane). ^{1}H-NMR ($CDCl_3$, 90 MHz) δ: 0.86 (3H, br t, $J=7$ Hz), 1.95 (2H, qui, $J=7$ Hz), 2.38 (2H, t, $J=7$ Hz), 2.58 (2H, t, $J=7$ Hz), 2.91 (2H, t, $J=7$ Hz), 3.68 (3H, s), 6.33 (1H, d, $J=15$ Hz), 6.7—7.4 (3H, m), 7.60 (1H, d, $J=10$ Hz). IR γ_{max}^{KBr} cm^{-1}: 1740, 1680. *Anal.* Calcd for $C_{21}H_{31}BrO_4$: C, 59.02; H, 7.31; Br, 18.7. Found: C, 59.22; H, 7.49; Br, 18.8.

(5*Z*,7*E*,9*E*)-*N*,*N*-Dimethyl-5-bromo-4,11-dihydroxynonadeca-5,7,9-trienamide (24a)——The bromodiolamide (**24a**) was prepared, in 58% yield, from **23a** in a manner similar to that described for the synthesis of **1a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, $J=7$ Hz), 2.50 (2H, t, $J=6$ Hz), 2.97 (3H, s), 3.02 (3H, s), 4.17 (2H, m), 4.36 (1H, m), 5.80 (1H, dd, $J=15$, 6 Hz), 6.2—6.6 (4H, m).

(6*Z*,8*E*,10*E*)-*N*,*N*-Dimethyl-6-bromo-5,12-dihydroxyeicosa-6,8,10-trienamide (24b)——The bromodiolamide (**24b**) was prepared, in 84% yield, from **23b** in a manner similar to that described for the synthesis of **1a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, $J=7$ Hz), 2.37 (2H, t, $J=6$ Hz), 2.95 (3H, s), 3.00 (3H, s), 4.18 (2H, m), 5.80 (1H, dd, $J=15$, 7 Hz), 6.2—6.6 (4H, m).

(7*E*,9*E*)-*N*,*N*-Dimethyl-4,11-dihydroxynonadeca-7,9-dien-5-ynamide (25a)——The acetylenic diolamide (**25a**) was prepared, in 54% yield, from **24a** in a manner similar to that described for the synthesis of **21a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, $J=7$ Hz), 2.51 (2H, m), 2.97 (3H, s), 3.05 (3H, s), 4.35 (1H, br), 4.68 (1H, br), 5.63 (1H, d, $J=15$ Hz), 5.79 (1H, dd, $J=15$, 7 Hz), 6.25 (1H, dd, $J=15$, 11 Hz), 6.56 (1H, dd, $J=15$, 11 Hz). IR γ_{max}^{film} cm^{-1}: 3400, 2220, 1630. HPLC: 28.1 min.

(8*E*,10*E*)-*N*,*N*-Dimethyl-5,12-dihydroxyeicosa-8,10-dien-6-ynamide (25b)——The acetylenic diolamide (**25b**) was prepared, in 61% yield, from **24b** in a manner similar to that described for the synthesis of **21a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, $J=7$ Hz), 2.40 (2H, m), 2.96 (3H, s), 3.01 (3H, s), 4.18 (1H, m), 4.53 (1H, m), 5.61 (1H, d, $J=15$ Hz), 5.79 (1H, dd, $J=15$, 6 Hz), 6.24 (1H, dd, $J=15$, 11 Hz), 6.55 (1H, dd, $J=15$, 11 Hz). IR γ_{max}^{film} cm^{-1}: 3400, 2220, 1630. HPLC: 29.5 min.

(5*Z*,7*E*,9*E*)-*N*,*N*-Dimethyl-4,11-dihydroxynonadeca-5,7,9-trienamide (4a)——The diolamide (**4a**) was prepared quantitatively from **25a** in a manner similar to that described for the synthesis of **2a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, $J=7$ Hz), 2.48 (2H, t, $J=6$ Hz), 2.97 (3H, s), 3.01 (3H, s), 4.16 (1H, q, $J=7$ Hz), 4.66 (1H, m), 5.47 (1H, dd, $J=11$, 9 Hz), 5.74 (1H, dd, $J=15$, 7 Hz), 6.06 (1H, t, $J=11$ Hz), 6.1—6.3 (2H, m), 6.51 (1H, dd, $J=15$, 11 Hz). IR γ_{max}^{film} cm^{-1}: 3400, 1630. UV λ_{max}^{MeOH} nm: 261.0, 270.0, 280.5. HPLC: 20.1, 23.5 min. MS m/z: 495 (M^+), 480, 405, 73. High-resolution MS m/z: M^+ Calcd for $C_{27}H_{53}NO_3Si_2$: 495.3563. Found: 495.3523.

(6*Z*,8*E*,10*E*)-*N*,*N*-Dimethyl-5,12-dihydroxyeicosa-6,8,10-trienamide (4b)——The diolamide (**4b**) was prepared quantitatively from **25b** in a manner similar to that described for the synthesis of **2a**. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, $J=7$ Hz), 2.36 (2H, t, $J=6$ Hz), 2.95 (3H, s), 3.01 (3H, s), 4.16 (1H, q, $J=6$ Hz), 4.58 (1H, m), 5.44 (1H, dd, $J=11$, 9 Hz), 5.74 (1H, dd, $J=15$, 7 Hz), 6.06 (1H, t, $J=11$ Hz), 6.1—6.3 (2H, m), 6.50 (1H, dd, $J=15$, 11 Hz). IR γ_{max}^{film} cm^{-1}: 3400, 1630. UV λ_{max}^{MeOH} nm: 260.5, 270.0, 280.0. HPLC: 20.6, 25.2 min. MS m/z: 509 (M^+), 494, 419, 73. High-resolution MS m/z: M^+ Calcd for $C_{28}H_{55}NO_3Si_2$: 509.3719. Found: 509.3692.

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Highly Regioselective Synthesis of Trisubstituted Pyrrolidines by 1,3-Cycloaddition

NOBUYUKI IMAI and KAZUO ACHIWA*

Shizuoka College of Pharmacy, 2-2-1 Oshika, Shizuoka 422, Japan

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It has been found that *N*-[α-(piperidinocarbonyl)benzylidene]trimethylsilylmethylamine reacts with unsymmetrical dipolarophiles to give 2,2,4- or 2,2,3-trisubstituted pyrrolidines in the presence of tetrabutylammonium fluoride or trifluoroacetic acid, respectively.

Keywords——1,3-cycloaddition; *N*-[α-(piperidinocarbonyl)benzylidene]trimethylsilylmethylamine; Schiff base; azomethine ylide; 2,2,4-trisubstituted pyrrolidine; 2,2,3-trisubstituted pyrrolidine

In recent studies on 1,3-dipolar cycloaddition of azomethine and thiocarbonyl ylides leading to pyrrolidine, 2,5-dihydropyrrole, and tetrahydrothiophene derivatives, we and other groups have reported a number of new methods[1,2] based on heterolysis of the silicon–carbon bond, explained their regio- and stereoselectivities in terms of frontier molecular orbital theory,[3,4] and described applications to the synthesis of alkaloids bearing a pyrrolidine nucleus.[5] In the previous communication, the regio- and stereoselectivities in the 1,3-cycloaddition of *N*-[α-(piperidinocarbonyl)benzylidene]trimethylsilylmethylamine (**1**) were clarified in connection with erythrinane alkaloid synthesis.[6] The details of these results are the subject of this paper.

The cycloaddition of the Schiff base **1** with olefinic and acetylenic unsymmetrical dipolarophiles in the presence of a catalytic amount of tetrabutylammonium fluoride (TBAF) was found to give 2,2,4-trisubstituted pyrrolidine derivatives *via* the intermediary carbanion (**2b**). On the other hand, the reaction catalyzed by trifluoroacetic acid (TFAA), trimethylsilyl trifluoromethanesulfonate (TMS-OTf) or trimethylsilyl chloride (TMS-Cl) was found to

$Me_3SiCH_2N=C(Ph)CON$ (**1**) — $Bu_4N^+F^-$ (cat.) → [**2a** ↔ **2b**] — $H_2C=CHX$ → 2,2,4-trisubstituted pyrrolidine

1 — CF_3CO_2H (cat.) or $Me_3SiOSO_2CF_3$ (cat.) → [**3**: R=H; **4**: R=$SiMe_3$] — $H_2C=CHX$ → 2,2,3-trisubstituted pyrrolidine

Chart 1

TABLE I. Solvent Effect[a] in the Presence of Several Catalysts

$$Me_3SiCH_2N{=}C(Ph)CON(C_5H_{10})\ (\mathbf{1}) + H_2C{=}CHCO_2Me \xrightarrow{\text{catalyst}} \mathbf{5a,b} \text{ or } \mathbf{5c,d}$$

Catalyst (mol%)	Solvent	Reaction conditions	Product	Conversion (%)[b]
$Bu_4N^+F^-$ (10)	DMF	r.t./47 h	**5a, b**	72
$Bu_4N^+F^-$ (10)	HMPA	r.t./40 h	**5a, b**	45
$Bu_4N^+F^-$ (10)	THF	r.t./20 h	**5a, b**	30
Me_3SiCl (20)	HMPA	r.t./25 h	**5c, d**	83
Me_3SiCl (20)	DMF	r.t./32 h	**5c, d**	30
Me_3SiCl (20)	THF	r.t./42 h	**5c, d**	20
Me_3SiCl (20)	Benzene	Reflux/20 h	**5c, d**	3
$Me_3SiOSO_2CF_3$ (20)	HMPA	60—65 °C/24 h	**5c, d**	90
CF_3CO_2H (20)	HMPA	r.t./20 h	**5c, d**	92

a) Molar ratio, **1** : $H_2C{=}CHCO_2Me$ = 1 : 1.2. *b*) Calculated from the disappearance of the Schiff base **1** as determined by GLC (10% SE-30 on Chromosorb-W). r.t. = room temperature.

proceed *via* the intermediary azomethine ylides (**3** or **4**) to give regioselectively 2,2,3-trisubstituted pyrrolidine derivatives as shown in Chart 1. It is noteworthy that these 1,3-cycloadditions of the Schiff base **1** gave products of different regioselectivity depending on the catalyst selected.

Preliminary experiments included the examination of solvent and catalyst effects using **1** as a substrate, methyl acrylate as an unsymmetrical dipolarophile, TBAF, TMS-OTf, TFAA or TMS-Cl as a catalyst and *N*,*N*-dimethylformamide (DMF), hexamethylphosphoramide (HMPA), tetrahydrofuran (THF) or benzene as a solvent. The results are summarized in Table I. The reaction of **1** with methyl acrylate in the presence of TBAF proceeded smoothly at room temperature for 47 h in DMF to give regio- and stereoselectively the corresponding 2,2,4-trisubstituted pyrrolidine (**5a**), along with a trace of by-product (**5b**). The Schiff base **1** reacted with methyl acrylate in the presence of TMS-OTf (at 60—65 °C for 24 h in HMPA), TFAA (at room temperature for 20 h in HMPA) or TMS-Cl (at room temperature for 25 h in HMPA) to give regio- and stereoselectively the corresponding 2,2,3-trisubstituted pyrrolidine (**5c**) along with a trace of by-product (**5d**).

The 1,3-cycloaddition of the Schiff base **1** with unsymmetrical dipolarophiles in the presence of TBAF (at room temperature for 6 h in DMF), TFAA (at room temperature for 7 h in HMPA) or TMS-OTf (at 50—60 °C for 3 h in HMPA) also proceeded smoothly to give the corresponding pyrrolidine derivatives (**5**—**9**). The results of these extensive experiments are summarized in Table II. In all cases, the products were regioselectively obtained and purified by bulb-to-bulb distillation and/or subsequent recrystallization from diisopropyl ether (IPE). In addition, the reaction of **1** with methyl acrylate or 2-cyclopentene-1-one proceeded stereoselectively because of the orbital interaction between the ester group and the phenyl group and/or the steric hindrance. The structures of these products were determined on the basis of proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13-nuclear magnetic resonance (^{13}C-NMR) spectra, and other spectral and physical data summarized in Tables IV and V.

The structures of **5a**—**d**, the four products isolated from the cycloaddition with methyl acrylate, were unequivocally determined as follows. The position and stereochemistry of the

TABLE II. Reaction of 1 with Unsymmetrical Dipolarophiles[a]

Entry	Dipolarophile Catalyst	Product[b]		Yield (%)	Regioselectivity (%) Stereoselectivity (%)
1	$H_2C=CHCO_2Me$ $Bu_4N^+F^-$ [c]	5a (97)	5b (trace)	56	97 ≈100
		5c (2)	5d (1)		
2	$H_2C=CHCO_2Me$ CF_3CO_2H [d]	5c (96)	5d (4)	88	≈100 96
3	$H_2C=CHCO_2Me$ $Me_3SiOSO_2CF_3$ [e]	5a (4) 5c (91)	5b (2) 5d (3)	94	94 97
4	$H_2C=CHCN$ $Bu_4N^+F^-$ [c]	6a (5)	6b (4)	55	≈100 56
5	$H_2C=CHCN$ CF_3CO_2H [d]	6c (3)	6d (1)	86	≈100 74
6	$H_2C=CHCOMe$ $Bu_4N^+F^-$ [c]	7a (3)	7b (2)	59	100 60
7	$HC\equiv CCO_2Me$ $Bu_4N^+F^-$ [e]	8		43	100 —
8	(cyclopentenone) $Bu_4N^+F^-$ [e]	9		58	100 100

a) All reactions were carried out with the Schiff base 1 (5 mmol), a dipolarophile (7.5 mmol), and a catalyst (0.75 mmol) in 20 ml of solvent. b) Ratio of the products is given in parentheses. c) Reaction conditions: solvent, DMF; temp., r.t.; time, 6 h. d) Reaction conditions: solvent, HMPA; temp., r.t.; time, 7 h. e) Reaction conditions: solvent, HMPA; temp., 50—60 °C; time, 3 h.

methoxycarbonyl group on the newly formed pyrrolidine ring of 5c were assigned to be at the C-3 carbon and *cis* to the 2-phenyl group on the basis of the ^{1}H-NMR spectrum, which showed a shifted signal (3.06 ppm) owing to the shielding effect of the 2-phenyl group.[7] The

Chart 2

structures of **5a**, **5b**, and **5d** were then assigned from the following experimental results. The catalytic hydrogenation of **8** afforded two stereoisomers (**5a** and **5b**) with the ratio of 1/2. Compound **8** was estimated to have the methoxycarbonyl group at the 4-position because the methylene protons at C_5 of **8** showed ABX coupling at 4.05 ppm (dd, $J=2.2$, 8.2 Hz) in the ^{1}H-NMR spectrum. The two hydrogenation products (**5a** and **5b**) were concluded to have 4-methoxycarbonyl groups because both of the stereoisomers were different from **5c**, and thus **8** was also determined to have the 4-methoxycarbonyl group. The major hydrogenation product **5b**, which has the 4-methoxycarbonyl group oriented *trans* to the 2-phenyl group, might be obtained by the attack of protons on the less sterically hindered side (phenyl group side). Therefore, the minor product **5a** has the 4-methoxycarbonyl group oriented *cis* to the 2-phenyl group, and the product **5d** has the 3-methoxycarbonyl group oriented *trans* to the 2-phenyl group. Furthermore, the regio- and stereochemistry of **6a** and **6b** were determined by converting the mixture of **6a** and **6b** with MeOH–HCl into **5a** and **5b**. The structures of **6c** and **6d** were determined on the basis of the fact that the mixture of **6c** and **6d** gave a signal pattern

Chart 3

Table III. Reaction of 1 with Symmetrical Dipolarophiles[a)]

Entry	Dipolarophile	Catalyst	Product (molar proportion) 10a	10b	10c	10d	11	Total yield (%)	Regioselectivity (%) / Stereoselectivity (%)
9	$MeO_2C(H)C=C(H)CO_2Me$ (trans)	$Bu_4N^+F^-$ [b)]	3	—	1	—	96	57	>96 / —
10	$MeO_2C(H)C=C(H)CO_2Me$ (trans)	$Me_3SiOSO_2CF_3$ [c)]	90	10	—	—	—	94	— / 90
11	$MeO_2C(H)C=C(H)CO_2Me$ (cis)	$Bu_4N^+F^-$ [b)]	10	—	4	—	86	77	>86 / —
12	$MeO_2C(H)C=C(H)CO_2Me$ (cis)	$Me_3SiOSO_2CF_3$ [c)]	6	—	90	4	—	69	— / 90

a) All reactions were carried out with the Schiff base 1 (5 mmol), a dipolarophile (7.5 mmol), and a catalyst (0.75 mmol) in 20 ml of solvent. b) Reaction conditions: solvent, DMF; temp., r.t.; time, 6 h. c) Reaction conditions: solvent, HMPA; temp., 50—60 °C; time, 3 h.

Table IV. ^{1}H- and ^{13}C-NMR Spectral Data (**5**—**10**)

Compound No.	^{1}H-NMR ($CDCl_3$, J=Hz) δ	^{13}C-NMR ($CDCl_3$) δ
5a	0.91—1.68 (6H, m, $CONCH_2(C\underline{H}_2)_3$), 2.29—3.66 (9H, 3-$CH_2$, 4-CH, 5-$CH_2$, $CON(CH_2)_2$), 2.74 (1H, s, NH), 3.67 (3H, s, OCH_3), 6.94—7.60 (5H, m, C_6H_5)	24.4 (t, $CONCH_2CH_2\underline{C}H_2$), 25.4 (t, $CONCH_2\underline{C}H_2\times 2$), 41.3 (t, 3-C), 44.9 (d, 4-C), 46.0 (br, $CON\underline{C}H_2\times 2$), 50.2 (t, 5-C), 51.8 (q, OCH_3), 73.5 (s, 2-C), 124.8, 127.1, 128.7, 143.4 (d, d, d, s, C_6H_5), 171.8 (s, CON), 175.0 (s, CO_2)
5b	0.70—1.61 (6H, m, $CONCH_2(C\underline{H}_2)_3$), 2.47—2.81 (3H, m, NH, 3-CH_2), 2.81—3.68 (7H, m, 4-CH, 5-CH_2, $CON(CH_2)_2$), 3.69 (3H, s, OCH_3), 7.27—7.32 (5H, m, C_6H_5)	24.4 (t, $CONCH_2CH_2\underline{C}H_2$), 25.4 (t, $CONCH_2\underline{C}H_2\times 2$), 41.2 (t, 3-C), 43.9 (d, 4-C), 45.6 (br, $CON\underline{C}H_2\times 2$), 50.1 (t, 5-C), 51.8 (q, OCH_3), 72.8 (s, 2-C), 125.1, 127.0, 128.7, 143.9 (d, d, d, s, C_6H_5), 171.8 (s, CON), 174.5 (s, CO_2)
5c	0.92—1.69 (6H, m, $CONCH_2(C\underline{H}_2)_3$), 1.91—2.28 (2H, m, 4-$CH_2$), 2.68—3.10 (2H, m, 5-$CH_2$), 3.06 (3H, s, OCH_3), 3.12—3.67 (5H, m, NH, $CON(CH_2)_2$), 4.32 (1H, dd, J=4.6, 7.6, 3-CH), 7.29 (5H, s, C_6H_5)	24.5 (t, $CONCH_2CH_2\underline{C}H_2$), 25.6 (t, $CONCH_2\underline{C}H_2\times 2$), 30.4 (t, 4-C), 45.6 (br, $CON\underline{C}H_2\times 2$), 46.4 (t, 5-C), 50.8 (q, OCH_3), 53.5 (d, 3-C), 78.6 (s, 2-C), 125.6, 127.3, 128.0, 138.3 (d, d, d, s, C_6H_5), 169.5 (s, CON), 175.9 (s, CO_2)
5d	0.84—1.53 (6H, m, $CONCH_2(C\underline{H}_2)_3$), 1.53—1.82 (2H, m, 4-$CH_2$), 2.66—2.95 (2H, m, 5-$CH_2$), 2.95—3.50 (6H, m, NH, 3-CH, $CON(CH_2)_2$), 3.74 (3H, s, OCH_3), 7.23—7.66 (5H, m, C_6H_5)	24.5 (t, $CONCH_2CH_2\underline{C}H_2$), 25.5 (t, $CONCH_2\underline{C}H_2\times 2$), 27.6 (t, 4-C), 44.5 (br, $CON\underline{C}H_2\times 2$), 46.5 (t, 5-C), 51.6 (q, OCH_3), 55.8 (d, 3-C), 77.3 (s, 2-C), 125.8, 127.2, 128.4, 141.2 (d, d, d, s, C_6H_5), 169.9 (s, CON), 176.5 (s, CO_2)
6a[a]	0.87—1.46 (12H, m, $CONCH_2(C\underline{H}_2)_3\times 2$), 2.17 (2H, dd, J=8.5, 13.4, NH$\times$2), 2.49—3.86 (18H, m, 3-$CH_2\times 2$, 4-CH$\times$2, 5-$CH_2\times 2$, $CON(CH_2)_2\times 2$), 7.31 (5H, s, C_6H_5 of **6a**), 7.34 (5H, s, C_6H_5 of **6b**)	24.3 (t, $CONCH_2CH_2\underline{C}H_2$), 25.3 (t, $CONCH_2\underline{C}H_2\times 2$), 28.1 (d, 4-C), 43.5 (t, 3-C), 45.8 (br, $CON\underline{C}H_2\times 2$), 51.1 (t, 5-C), 73.4 (s, 2-C), 121.3 (s, C$\equiv$N), 124.5, 127.4, 129.0, 142.7 (d, d, d, s, C_6H_5), 170.7 (s, CON)
6b[a]		24.3 (t, $CONCH_2CH_2\underline{C}H_2$), 25.3 (t, $CONCH_2\underline{C}H_2\times 2$), 29.1 (d, 4-C), 42.5 (t, 3-C), 45.8 (br, $CON\underline{C}H_2\times 2$), 51.1 (t, 5-C), 72.7 (s, 2-C), 120.8 (s, C$\equiv$N), 124.8, 127.4, 129.0, 142.4 (d, d, d, s, C_6H_5), 170.7 (s, CON)
6c[a]	1.16—1.72 (12H, m, $CONCH_2(C\underline{H}_2)_3\times 2$), 1.81—2.36 (6H, m, NH$\times$2, 4-$CH_2\times 2$), 2.99—3.81 (12H, m, 5-$CH_2\times 2$, $CON(CH_2)_2\times 2$), 4.21 (2H, t, J=7.2, 3-CH$\times$2), 7.29—7.66 (10H, m, $C_6H_5\times 2$)	24.3 (t, $CONCH_2CH_2\underline{C}H_2$), 25.4 (t, $CONCH_2\underline{C}H_2\times 2$), 30.7 (t, 4-C), 41.3 (d, 3-C), 45.4 (br, $CON\underline{C}H_2\times 2$), 46.1 (t, 5-C), 76.6 (s, 2-C), 120.5 (s, C$\equiv$N), 128.1, 125.6, 128.6, 139.1 (d, d, d, s, C_6H_5), 169.1 (s, CON)
6d[a]		24.3 (t, $CONCH_2CH_2\underline{C}H_2$), 25.4 (t, $CONCH_2\underline{C}H_2\times 2$), 29.1 (t, 4-C), 43.1 (d, 3-C), 45.3 (t, 5-C), 45.4 (br, $CON\underline{C}H_2\times 2$), 77.2 (s, 2-C), 121.0 (s, C$\equiv$N), 125.1, 128.3, 128.8, 139.6 (d, d, d, s, C_6H_5), 168.5 (s, CON)
7a[a]	0.60—1.65 (12H, m, $CONCH_2(C\underline{H}_2)_3\times 2$), 2.17 (3H, s, CCH_3 of **7a**), 2.19 (3H, s, CCH_3 of **7b**), 2.21—3.52 (20H, m, NH$\times$2, 3-$CH_2\times 2$, 4-CH$\times$2, 5-$CH_2\times 2$, $CON(CH_2)_2\times 2$), 7.33 (10H, br, $C_6H_5\times 2$)	24.4 (t, $CONCH_2CH_2\underline{C}H_2$), 25.4 (t, $CONCH_2\underline{C}H_2\times 2$), 28.9 (q, $C\underline{C}H_3$), 40.7 (t, 3-C), 45.7 (br, $CON\underline{C}H_2\times 2$), 49.0 (t, 5-C), 52.1 (d, 4-C), 73.0 (s, 2-C), 124.7, 127.1, 128.7, 144.0 (d, d, d, s, C_6H_5), 171.8 (s, CON), 208.6 (s, $\underline{C}CH_3$)
7b[a]		24.4 (t, $CONCH_2CH_2\underline{C}H_2$), 25.4 (t, $CONCH_2\underline{C}H_2\times 2$), 29.3 (q, $C\underline{C}H_3$), 40.9 (t, 3-C), 45.7 (br, $CON\underline{C}H_2\times 2$), 49.1 (t, 5-C), 52.9 (d, 4-C), 73.7 (s, 2-C), 124.9, 126.9, 128.7, 143.3 (d, d, d, s, C_6H_5), 171.8 (s, CON), 208.1 (s, $\underline{C}CH_3$)
8	0.87—1.63 (6H, m, $CONCH_2(C\underline{H}_2)_3$), 3.10—3.75 (5H, m, NH, $CON(CH_2)_2$), 3.76 (3H, s, OCH_3), 4.05 (2H, dd, J=2.2, 8.2, 5-CH_2), 7.01 (1H, t, J=2.2, 3-CH), 7.30 (5H, s, C_6H_5)	24.3 (t, $CONCH_2CH_2\underline{C}H_2$), 25.4 (t, $CONCH_2\underline{C}H_2\times 2$), 45.8 (br, $CON\underline{C}H_2\times 2$), 51.6 (q, OCH_3), 52.7 (t, 5-C), 80.2 (s, 2-C), 125.0, 127.6, 129.1, 135.7 (d, d, d, s, C_6H_5), 141.1 (d, 3-C), 143.0 (s, 4-C), 163.9 (s, CON), 170.1 (s, CO_2)

TABLE IV. (continued)

Compound No.	^{1}H-NMR ($CDCl_3$, J=Hz) δ	^{13}C-NMR ($CDCl_3$) δ
9	0.70—1.60 (8H, m, $CONCH_2(C\underline{H}_2)_3$, $COCH_2C\underline{H}_2$), 1.92—2.27 (3H, m, 3-CH, 5-CH_2), 2.71—3.76 (7H, m, NH, $CON(CH_2)_2$, $COCH_2$), 4.07 (1H, quintet, J=10.6, 4-CH), 7.34 (5H, s, C_6H_5)	23.8 (t, $COCH_2\underline{C}H_2$), 24.5 (t, $CONCH_2CH_2\underline{C}H_2$), 25.6 (t, $CONCH_2\underline{C}H_2\times 2$), 39.4 (t, $CO\underline{C}H_2$), 45.9 (t, $CON\underline{C}H_2\times 2$), 48.9 (t, 5-C), 49.4 (d, 3-C), 52.3 (d, 4-C), 77.3 (s, 2-C), 125.5, 127.2, 128.5, 139.7 (d, d, d, s, C_6H_5), 171.0 (s, CON), 220.6 (s, C=O)
10a	0.95—1.68 (6H, m, $CONCH_2(C\underline{H}_2)_3$), 2.68 (1H, br, NH), 3.07 (3H, s, OCH_3), 3.10—3.71 (7H, m, 4-CH, 5-CH_2, $CON(CH_2)_2$), 3.74 (3H, s, OCH_3), 4.73 (1H, d, J=2.7, 3-CH), 7.27 (5H, s, C_6H_5)	24.5 (t, $CONCH_2CH_2\underline{C}H_2$), 25.5 (t, $CONCH_2\underline{C}H_2\times 2$), 46.3 (br, $CON\underline{C}H_2\times 2$), 48.5 (d, 4-C), 48.7 (t, 5-C), 51.3 (q, OCH_3), 52.0 (q, OCH_3), 57.1 (d, 3-C), 78.5 (s, 2-C), 125.8, 127.5, 128.1, 137.9 (d, d, d, s, C_6H_5), 169.0 (s, CON), 172.8 (s, CO_2), 174.5 (s, CO_2)
10b	0.87—1.53 (6H, m, $CONCH_2(C\underline{H}_2)_3$), 3.13—3.37 (8H, m, NH, 4-CH, 5-CH_2, $CON(CH_2)_2$), 3.40 (3H, s, OCH_3), 3.71 (3H, s, OCH_3), 3.79 (1H, d, J=4.2, 3-CH), 7.29—7.75 (5H, m, C_6H_5)	24.4 (t, $CONCH_2CH_2\underline{C}H_2$), 25.5 (t, $CONCH_2\underline{C}H_2\times 2$), 45.5 (br, $CON\underline{C}H_2\times 2$), 47.2 (d, 4-C), 49.2 (t, 5-C), 51.8 (q, OCH_3), 51.9 (q, OCH_3), 59.4 (d, 3-C), 79.0 (s, 2-C), 126.4, 127.5, 128.3, 139.7 (d, d, d, s, C_6H_5), 169.6 (s, CON), 173.2 (s, CO_2), 173.9 (s, CO_2)
10c	0.86—1.62 (6H, m, $CONCH_2(C\underline{H}_2)_3$), 3.05 (3H, s, OCH_3), 3.16—3.87 (8H, m, NH, 4-CH, 5-CH_2, $CON(CH_2)_2$), 3.61 (3H, s, OCH_3), 4.51 (1H, d, J=7.1, 3-CH), 7.28 (5H, s, C_6H_5)	24.4 (t, $CONCH_2CH_2\underline{C}H_2$), 25.6 (t, $CONCH_2\underline{C}H_2\times 2$), 46.5 (br, $CON\underline{C}H_2\times 2$), 47.8 (t, 5-C), 48.9 (d, 4-C), 51.0 (q, OCH_3), 51.7 (q, OCH_3), 56.7 (d, 3-C), 78.7 (s, 2-C), 125.5, 127.5, 128.2, 137.2 (d, d, d, s, C_6H_5), 169.0 (s, CON), 172.8 (s, CO_2), 173.6 (s, CO_2)
10d	0.82—1.56 (6H, m, $CONCH_2(C\underline{H}_2)_3$), 2.70—3.83 (9H, m, NH, 3-CH, 4-CH, 5-CH_2, $CON(CH_2)_2$), 3.62 (3H, s, OCH_3), 3.75 (3H, s, OCH_3), 7.21—7.63 (5H, m, C_6H_5)	24.4 (t, $CONCH_2CH_2\underline{C}H_2$), 25.4 (t, $CONCH_2\underline{C}H_2\times 2$), 45.3 (t, 5-C), 47.0 (br, $CON\underline{C}H_2\times 2$), 48.8 (d, 4-C), 51.9 (q, $OCH_3\times 2$), 58.4 (d, 3-C), 80.2 (s, 2-C), 125.6, 127.7, 128.7, 140.3 (d, d, d, s, C_6H_5), 169.1 (s, CON), 172.0 (s, CO_2), 173.9 (s, CO_2)
11	0.83—1.78 (6H, m, $CONCH_2(C\underline{H}_2)_3$), 2.62—2.89 (2H, m, $COCH_2$), 3.12—3.41 (3H, m, CH, C=NCH_2), 3.41—3.77 (4H, m, $CON(CH_2)_2$), 3.65 (3H, s, OCH_3), 3.71 (3H, s, OCH_3), 7.32—7.89 (5H, m, C_6H_5)	24.3 (t, $CONCH_2CH_2\underline{C}H_2$), 25.6, 26.2 (t, t, $CONCH_2\underline{C}H_2\times 2$), 33.8 (t, $CO\underline{C}H_2$), 41.4 (t, $CON\underline{C}H_2$), 42.5 (d, CH), 46.7 (t, $CON\underline{C}H_2$), 51.5 (q, OCH_3), 51.8 (q, OCH_3), 54.3 (t, C=$N\underline{C}H_2$), 127.1, 128.5, 131.0, 134.6 (d, d, d, s, C_6H_5), 164.6, 164.9 (s, s, C=N, CON), 172.2 (s, CO_2), 173.5 (s, CO_2)

a) The NMR spectra of these compounds were obtained with the mixture of two stereoisomers in each case.

similar to that of **6a** and **6b** in the ^{13}C-NMR spectrum. Compounds **7a** and **7b** were considered to be 4-substituted pyrrolidine derivatives because the signals of the methyl protons of **7a** and **7b** were observed at 2.17 and 2.19 ppm respectively, uninfluenced by the phenyl group, in the ^{1}H-NMR spectra. The structure of **9** was confirmed by the ^{1}H-NMR signal of the C-4 proton at 4.07 ppm as a quintet, indicating the presence of three adjacent protons.

Next, the cycloaddition of **1** with symmetrical dipolarophiles such as dimethyl fumarate and dimethyl maleate was carried out. The results are collected in Table III. The reactions catalyzed by TMS-OTf gave the cyclized products **10a—d**, probably *via* the azomethine ylide (**4**). In the presence of TBAF, the reactions with dimethyl fumarate and dimethyl maleate regioselectively yielded the uncyclized product **11** as a main product *via* the carbanion **2a**, along with 4% and 14% yields of the cyclized products (**10a** and **10c**, respectively). The spectral and physical data are given in Tables IV and V. These results indicate that the carbanion **2b** plays an important role in the formation of **5a**, **6a, b**, **7a, b**, **8**, **9**, and **10a, c**, whereas **2a** gave the uncyclized product **11**. This distinct selectivity may be rationalized in terms of steric hindrance in the formation of the C_2–C_3 bond and the relative stability of both anions, **2a** and **2b**.

Table V. Pyrrolidine and 2,5-Dihydropyrrole Derivatives (**5**—**10**)

Compound No.	mp (°C)[a] bp (°C (mmHg))[b]	Appearance	Formula	Analysis (%) Calcd (Found) C	H	N	IR (liq. or KBr) cm^{-1}
5a	112—113	Prisms	$C_{18}H_{24}N_2O_3$	68.33	7.65	8.85	3380 1733 1620
				(68.12	7.64	8.84)	(NH) (CO_2) (CON)
5b	108—110	Prisms	$C_{18}H_{24}N_2O_3$	68.33	7.65	8.85	3320 1734 1628
				(67.90	7.61	8.77)	(NH) (CO_2) (CON)
5c	98—99	Prisms	$C_{18}H_{24}N_2O_3$	68.33	7.65	8.85	3350 1732 1620
				(68.46	7.66	8.95)	(NH) (CO_2) (CON)
5d	109—110	Prisms	$C_{18}H_{24}N_2O_3$	68.33	7.65	8.85	3352 1723 1624
				(68.32	7.60	8.74)	(NH) (CO_2) (CON)
6a, b[c]	140—148	Needles	$C_{17}H_{21}N_3O$	72.05	7.47	14.83	3380 2240 1613
				(71.79	7.34	14.74)	(NH) (C≡N) (CON)
6c, d[c]	220 (0.2)	Oil	$C_{17}H_{21}N_3O$	72.05	7.47	14.83	3305 2245 1622
				(72.13	7.31	14.95)	(NH) (C≡N) (CON)
7a, b[c]	240 (0.2)	Oil	$C_{18}H_{24}N_2O_2$	71.97	8.05	9.33	3316 1715 1620
				(72.25	8.08	9.18)	(NH) (CO) (CON)
8	116—117	Needles	$C_{18}H_{22}N_2O_3$	68.77	7.05	8.91	3370 1714 1624
				(68.85	7.09	8.83)	(NH) (CO_2) (CON, C=C)
9	174—175	Prisms	$C_{19}H_{24}N_2O_2$	73.04	7.74	8.97	3309 1738 1620
				(73.17	7.85	8.77)	(NH) (CO) (CON)
10a	158—159	Needles	$C_{20}H_{26}N_2O_5$	64.15	7.00	7.48	3315 1733 1724 1632
				(63.88	6.96	7.49)	(NH) (CO_2) (CO_2) (CON)
10b	131—132	Needles	$C_{20}H_{26}N_2O_5$	64.15	7.00	7.48	3370 1738 1625
				(63.88	7.00	7.48)	(NH) (CO_2) (CON)
10c	135—136	Needles	$C_{20}H_{26}N_2O_5$	64.15	7.00	7.48	3330 1730 1630
				(64.14	6.98	7.42)	(NH) (CO_2) (CON)
10d	198—199	Needles	$C_{20}H_{26}N_2O_5$	64.15	7.00	7.48	3360 1738 1625
				(64.00	6.97	7.44)	(NH) (CO_2) (CON)
11	240 (0.2)	Oil	$C_{20}H_{26}N_2O_5$	64.15	7.00	7.48	1744 1644 1633
				(64.31	7.01	7.40)	(CO_2) (N=C) (CON)

a) These compounds were recrystallized from diisopropyl ether (IPE). *b*) The boiling points refer the bath temperature in a "Kugelrohr" short-path apparatus. *c*) The physical and spectral data of these compounds were obtained with the mixture of two stereoisomers in each case.

$Me_3SiCH_2N=CHPh$ **12**

$Bu_4N^+F^-$ (cat.)

$Me_3SiOSO_2CF_3$ (cat.)

13a, **13b**, **16**

$H_2C=CHCO_2Me$

H_2O

PhCOCl

14 (45% yield)

15 (6% yield)

17a,b (83% yield)

Chart 4

The generality of the regioselectivities in TBAF- and TMS-OTf-catalyzed 1,3-cycloaddition of **1** is supported by the observations that *N*-(benzylidene)trimethylsilylmethylamine (**12**) reacted with methyl acrylate in the presence of TMS-OTf to give the 2,3-disubstituted pyrrolidine (**17a, b**) regioselectively *via* the azomethine ylide (**16**),[1c,m] and in the presence of TBAF to give the uncyclized products (**14** and **15**) after benzoylation, apparently *via* the intermediary carbanions (**13a** and **13b**, respectively), as shown in Chart 4.

It should be noted that this TFAA or TMS-OTf-catalyzed 1,3-cycloaddition proceeds with high regioselectivity to give 2,2,3-trisubstituted pyrrolidine derivatives, which form the skeletons of some biologically active natural products, especially erythrinane alkaloids, and that TBAF-promoted 1,3-cycloaddition gives regioselectively 2,2,4-trisubstituted derivatives.

Experimental

All melting and boiling points are uncorrected. Infrared (IR) spectra were measured with a Hitachi EPI-G2 infrared spectrometer. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM 90Q spectrometer (90 MHz) and all chemical shifts are given downfield from tetramethylsilane (TMS). Mass spectral (MS) data were recorded on a Hitachi RMS-4 MS spectrometer.

1-(Phenylglyoxyloyl)piperidine (17)[8]——A solution of ethyl phenylglyoxylate (26.7 g, 150 mmol) and piperidine (12.8 g, 150 mmol) was heated in an oil bath at 90—95 °C for 42 h with stirring. After cooling, the reaction mixture was solidified by trituration with hexane (80 ml) and recrystallized from hexane to give the pure product (17) in 79% yield. 17: mp 107—108 °C. IR ν_{max}^{KBr} cm^{-1}: 1674 (C=O), 1642 (CON). ^{1}H-NMR ($CDCl_3$) δ: 1.31—1.84 (6H, m, $NCH_2C\underline{H}_2CH_2C\underline{H}_2CH_2$), 3.01—3.84 (4H, m, $N(CH_2)_2$), 7.11—8.02 (5H, m, C_6H_5).

***N*-[α-(Piperidinocarbonyl)benzylidene]trimethylsilylmethylamine (1)**——In a 100 ml round-bottomed flask, fitted with a Clarke–Rahrs column with a water separator and surmounted by a reflux condenser, were placed 1-(phenylglyoxyloyl)piperidine (3.2 g, 14.7 mmol), trimethylsilylmethylamine[1c] (2.1 g, 20.0 mmol), and a catalytic amount of aluminum chloride in benzene (50 ml). The whole was vigorously refluxed in an oil bath at 110 °C. After being stirred for 48 h, the reaction mixture was evaporated and the residual oil was subjected to silica gel column chromatography (SGCC) using IPE–benzene (1 : 1) as an eluent to give the pure product 1 in an excellent yield. 1: Oil. Yield 99%. bp 240—250 °C (0.2 mmHg) (by bulb-to-bulb distillation). IR ν_{max}^{neat} cm^{-1}: 1640 (C=N), 1620 (C=O). ^{1}H-NMR ($CDCl_3$) δ: 0.13 (9H, s, $Si(CH_3)_3$), 1.28—1.75 (6H, m, $NCH_2C\underline{H}_2C\underline{H}_2C\underline{H}_2$), 3.16—3.41 (4H, m, $N(CH_2)_2$), 3.61—3.79 (2H, m, $SiCH_2N$), 7.32—7.76 (5H, m, C_6H_5). ^{13}C-NMR ($CDCl_3$) δ: −2.2 (q, $Si(CH_3)_3$), 24.3, 25.6, 26.3 (t, t, t, $NCH_2\underline{C}H_2CH_2\underline{C}H_2$), 41.3, 46.7 (t, t, $N(CH_2)_2$), 48.8 (t, $SiCH_2N$), 126.6, 128.5, 129.9, 135.7 (d, d, d, s, C_6H_5), 160.3 (s, C=N), 165.4 (s, C=O).

Solvent and Catalyst Effect on 1,3-Cycloaddition of 1 with Methyl Acrylate——General Procedure: TBAF, TMS-Cl, TFAA or TMS-OTf was added to a solution of the Schiff base 1 (1.51 g, 5 mmol) and methyl acrylate (0.52 g, 6 mmol) in 20 ml of the solvent indicated in Table I. The whole was stirred under a nitrogen atmosphere under the reaction conditions indicated in Table I. In the cases of DMF and HMPA, the reaction mixture was diluted with benzene (80 ml), and washed with sat. aq. NaCl–10% aq. $KHCO_3$ (150 ml + 10 ml) and sat. aq. NaCl (160 ml). In the case of benzene, the solution was diluted with benzene (60 ml) and washed as above. In the case of THF, the solvent was evaporated off and the residue was treated as above. The benzene layer was then separated, dried over $MgSO_4$, and submitted to gas liquid chromatographic (GLC) analysis (10% SE-30 on Chromosorb-W, 1 m). The solvent and catalyst efficiency in these reactions was assessed in terms of the conversion percentage, which was calculated on the basis of the disappearance of the starting material (1) determined by GLC analysis. The data are listed in Table I.

Pyrrolidines (5—10) and Michael Adduct (11)——General Procedure: A 1 mol/l solution of TBAF, TFAA or TMS-OTf in dichloromethane (0.75 mmol) was added at room temperature to a solution of 1 (1.51 g, 5 mmol) and an olefinic or acetylenic dipolarophile (7.5 mmol) in 20 ml of DMF (in the case of TBAF) or HMPA (in the cases of TFAA and TMS-OTf). The whole was stirred under a nitrogen atmosphere at room temperature for 6 h, at room temperature for 7 h or at 50—60 °C for 3 h in the case of TBAF, TFAA or TMS-OTf, respectively. Work-up of the reaction mixture was carried out by treatment with benzene (80 ml), sat. aq. NaCl (150 ml) and 10% aq. $KHCO_3$ (10 ml). The benzene layer was separated, dried over $MgSO_4$ and evaporated.

Purification of the products and isolation of the isomers from the residue were carried out as follows. In entries 1—3 and 10, 12, isolation of the main product was carried out by recrystallization from IPE, and then the filtrate after recrystallization was submitted to thin layer chromatography (TLC) on silica gel using ether (entries 1—3) or IPE (entries 10, 12) as an eluent to give very minor products in pure form in each case. In entry 4, the residue was purified by recrystallization from IPE after bulb-to-bulb distillation to give a mixture of **6a** and **6b** in 55% total yield. In entries 5 and 6, the residue was purified by bulb-to-bulb distillation to give a mixture of **6c** and **6d**, and **7a** and **7b**, respectively. In entries 7 and 8, purification of **8** and **9** from the residue was carried out by recrystallization from IPE.

In entries 9 and 11, the residue was purified by bulb-to-bulb distillation to give the main uncyclized product (**11**) along with very minor products (**10a** and **10c**).

These results are collected in Tables II and III in the cases of the cycloaddition of **1** with unsymmetrical and symmetrical dipolarophiles, respectively. The ratios of the isomers, shown in Tables II and III, were calculated on the basis of GLC or ^{1}H-NMR spectra of the crude products. The spectral and physical data are summarized in Tables IV and V.

Catalytic Hydrogenation of 8. Synthesis of 5a and 5b——A solution of **8** (100 mg, 0.32 mmol) and 10% palladium-on-charcoal (50 mg) in methanol (20 ml) was stirred at room temperature for 16 h under a hydrogen atmosphere. After removal of the catalyst by filtration and subsequent evaporation of the solvent, the residue was subjected to TLC on silica gel using ether as an eluent to give **5a** (48 mg, 45% yield) and **5b** (15 mg, 14% yield), and then each of **5a** and **5b** was purified by recrystallization from IPE. The product ratio (**5a**/**5b**) was found to be 2/1 by ^{1}H-NMR spectral analysis of the crude product.

Esterification of 6a, b——A solution of **6a, b** (180 mg, 0.64 mmol) and 3 N HCl/MeOH (10 ml) was heated in an oil bath at 40 °C for 26 h with stirring. After evaporation of the methanol, the residue was extracted with benzene (50 ml), washed with sat. aq. NaCl–10% aq. $KHCO_3$ (150 ml + 10 ml) and sat. aq. NaCl (150 ml), and dried over $MgSO_4$. The benzene solution was evaporated to give the crude products **5a** and **5b** in 74% total yield. ^{1}H-NMR spectral data of the crude products indicate that the ratio of the isomers **5a** and **5b** is 5:4.

Reaction of the Schiff Base 11 with Methyl Acrylate in the Presence of TBAF——This reaction was carried out using **11** (0.96 g, 5 mmol), methyl acrylate (0.65 g, 7.5 mmol), and a catalytic amount of TBAF (0.75 mmol) in a manner similar to that described for pyrrolidines (**5**—**10**). After evaporation of the benzene, benzoyl chloride (0.84 g, 6 mmol) was added dropwise to a solution of the residual oil in pyridine (5 ml) under ice-cooling. The mixture was stirred for 1.5 h under a nitrogen atmosphere and diluted with dichloromethane. The solution was washed with 15% aq. HCl, sat. aq. NaCl–10% aq. $KHCO_3$, and sat. aq. NaCl, then dried over $MgSO_4$. After evaporation of the dichloromethane and purification of the residue by SGCC, two amide derivatives (**14** (in 45% yield) and **15** (in 6% yield)) were obtained. **14**: bp 143—144 °C (0.2 mmHg). MS *m/z*: 221 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 1.84—2.06 (2H, m, $CH_2\underline{CH_2}CH_2$), 2.35 (2H, dd, J=7.6, 15.3 Hz, $\underline{CH_2}CO_2$), 3.44 (2H, J=7.1, 13.9 Hz, NCH_2), 3.62 (3H, s, OCH_3), 6.73 (1H, s, NH), 7.12—7.90 (5H, m, C_6H_5). ^{13}C-NMR ($CDCl_3$) δ: 24.7 (t, $CH_2\underline{C}H_2CH_2$), 31.7 (t, $CH_2\underline{C}H_2CO_2$), 39.6 (t, $N\underline{C}H_2$), 51.6 (q, OCH_3), 127.0 128.4, 131.3, 134.6 (d, d, d, s, C_6H_5), 167.7 (s, NHCO), 174.0 (s, CO_2). *Anal.* Calcd for $C_{12}H_{15}NO_3$: C, 65.14; H, 6.83; N, 6.33. Found: C, 65.03; H, 6.71; N, 5.98. **15**: mp 117—118 °C. MS *m/z*: 297 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 1.95—2.65 (5H, m, CH_2CH_2CH), 3.54 (3H, s, OCH_3), 5.13 (1H, d, J=6.6 Hz, NH), 6.86—7.88 (10H, m, $2\times C_6H_5$). ^{13}C-NMR ($CDCl_3$) δ: 31.0, 31.2 (t, t, CH_2CH_2), 51.7 (q, OCH_3), 53.8 (d, CH), 126.6, 127.1, 127.5, 128.5, 128.8, 131.4, 134.6, 141.9 (d, d, d, d, d, d, s, s, $2\times C_6H_5$), 166.9 (s, NHCO), 174.2 (s, CO_2). *Anal.* Calcd for $C_{18}H_{19}NO_3$: C, 72.70; H, 6.44; N, 4.71. Found: C, 72.55; H, 6.41; N, 4.71.

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Nitration of 3-Acylindoles in the Presence of Metal MeCN Solvates and Synthesis of the Antibiotic Alkaloid Chuangxinmycin

MASAYUKI MURASE, TAKESHI KOIKE, YUKARI MORIYA, and SEISHO TOBINAGA*

Showa College of Pharmaceutical Sciences, Tsurumaki, Setagaya-ku, Tokyo 154, Japan

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Nitration of 3-acylindoles in the presence of MeCN solvates of Cu^{2+}, Al^{3+}, and Fe^{2+} salts yielded 3-nitroindole, 4-nitro- and 6-nitro-3-acylindoles, of which 3-acetyl-4-nitroindole was subsequently transformed into dehydrochuangxinmycin (**7**), the dehydro derivative of the antibiotic alkaloid chuangxinmycin (**2**).

Keywords——nitration; 3-acylindole; metal MeCN solvate; 3-acetyl-4-nitroindole; synthesis; chuangxinmycin

Considerable interest has been focused on the direct C-4 substitution reaction of indoles in connection with the synthesis of C-4-substituted indole alkaloids, including the ergot family alkaloids (*e.g.* lysergic acid (**1**)) and chuangxinmycin (**2**). Although several direct C-4 substitution reactions of indole[1-4] have been developed, direct nitration or halogenation at C-4 of indoles has not been achieved to date. For example, nitration of 3-formyl- or 3-acetylindole in acidic media gave only a few percent of the 4-nitro derivative.[5,6] This paper deals with nitration of 3-acylindoles in the presence of metal acetonitrile (AN) solvates, $Cu(AN)_6(ClO_4)_2$, $Al(AN)_6(ClO_4)_3$, and $Fe(AN)_6(BF_4)_2$, to give the 4-nitro derivatives in significant yields. We also describe the subsequent transformation of the 3-acetyl-4-nitroindole (**3**) to dehydrochuangxinmycin (**7**), the dehydro derivative of the unique antibiotic alkaloid chuangxinmycin (**2**).

1 2

Chart 1

Nitration of 3-Acylindoles in the Presence of Metal MeCN Solvates

The solvates in AN, $Cu(AN)_6(ClO_4)_2$, $Al(AN)_6(ClO_4)_3$, and $Fe(AN)_6(BF_4)_2$, were prepared from the corresponding metal salt hydrates, $Cu(ClO_4)_2 \cdot 6H_2O$, $Al(ClO_4)_3 \cdot 8H_2O$, and $Fe(BF_4)_2 \cdot 6H_2O$, in AN by the addition of 6 or 8 mol of Ac_2O to remove water.[7] Nitrations of 3-acetylindole and 3-formylindole (3-indolecarbaldehyde) were carried out with 99% HNO_3 in the presence of the metal solvates in AN. The results are shown in Table I.

On the other hand, nitration of 3-acylindoles in the presence of Lewis acids, namely, BF_3, BCl_3, $AlCl_3$, *etc.*, in AN, glyme or CH_2Cl_2 afforded only the 6-nitro derivatives as the main

TABLE I. Nitration of 3-Acylindoles in the Presence of Metal MeCN Solvates

Entry	Substituent	Catalyst	Position of nitration, yield (%)				Time (h)	Temp. (°C)
			3-	4-	6-	Recover		
1	3-Ac	A	38.4	20.5	25.4	—	1	15
2	3-Ac	B	16.1	12.3	24.3	7.2	2	13
3	3-Ac	C	28.2	20.8	31.9	—	3.5	15
4	3-Formyl	A	21.7	19.1	19.3	3.4	19	13
5	3-Formyl	B	9.6	15.5	22.1	18.4	4	5
6	3-Formyl	C	21.9	5.9	21.8	29.8	24	15

Metal complex: A, $Cu(AN)_6(ClO_4)_2$; B, $Al(AN)_8(ClO_4)_3$; C, $Fe(AN)_6(BF_4)_2$.

metal solvate

complex A

HNO_3

complex B

Chart 2

product. The formation of significant amounts of 4-nitro derivatives and 3-nitroindole in the nitration of 3-acylindoles in the presence of the metal AN solvates may depend on the involvement of complexes such as A and B, whose existence is supported by the color changes from light blue (complex A) to deep green (complex B) during the course of the reaction and by the solubility changes of 3-acylindoles (3-acylindoles are less soluble in AN without the metal solvates).

A New Synthesis of Methyl Dehydrochuangxinmycin

Chuangxinmycin (a new kind of mycin) (**2**) is an antibiotic alkaloid having a unique indole skeleton, isolated from the microorganism *Actinoplanes tsinanensis* n. sp. in China. This compound is known to be active *in vitro* against a number of gram-positive and gram-negative bacteria and to be active *in vivo* in mice against *Escherichia coli* and *Shigella dysenteria* infections. Preliminary clinical results have shown that chuangxinmycin is effective in the treatment of septicemia and urinary and biliary infections caused by *E. coli*.[8,9] The structure of **2** was confirmed by X-ray crystallography[10] and syntheses.[11,12] We synthesized dehydrochuangxinmycin (**7**) from 3-acetyl-4-nitroindole (**3**) prepared by means of the above reaction.

The 4-amino derivative **4** prepared by reduction of **3** by catalytic hydrogenation or with $TiCl_3$, was transformed to the 4-iodo compound **5**[13] through the diazonium salt. Novel displacement of iodine with thioacetate to give the methyl thioacetate **6** was performed by treatment of the iodide with methyl thioglycolate in pyridine–methanol in 95% yield.

Chart 3

Treatment of the thioacetate **6** with ammonium acetate in AcOH afforded dehydrochuangxinmycin (**7**) in 93% yield. All physical data of the product **7** were identical with those given previously.[12] Thus, the formal synthesis of chuangxinmycin (**2**) by a new route was attained.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded with a Hitachi 260-10 spectrometer, proton nuclear magnetic resonance (^{1}H-NMR) spectra with a JEOL JNM-FX 100 or JEOL JNM-GX 270 spectrometer (with tetramethylsilane as an internal standard in $CDCl_3$, CD_3COCD_3 and dimethyl sulfoxide (DMSO)-d_6 solution) and mass spectra (MS) with a JEOL JMS-d 300 spectrometer. Wako Silica Gel C-200 (200 mesh) and Merck Kieselgel 60 F_{254} were used for column chromatography and thin layer chromatography (TLC), respectively.

Nitrations of 3-Acetylindole in the Presence of Metal AN Solvates——Method A: The solvate, $Cu(AN)_6(ClO_4)_2$, was prepared by the addition of 7.14 g (70 mmol) of Ac_2O to a solution of 3.70 g (10 mmol) of $Cu(ClO_4)_2 \cdot 6H_2O$ in AN (29 ml) at room temperature, and the mixture was stirred for 30 min. Then 1.59 g (10 mmol) of acetylindole was added and the mixture was stirred at room temperature for 30 min. At that time, the color of the solution changed from light blue to dark green.To the above mixture, 756 mg of HNO_3 (99%) in AN (1 ml) was added very slowly at 15 °C with stirring and the whole was stirred at the same temperature for 1 h. The precipitates (3-acetyl-6-nitroindole) were separated by filtration. The filtrate was poured into water and extracted with AcOEt. The organic layer was washed with sat. $NaHCO_3$ and brine. The AcOEt layer was dried and concentrated under vacuum. The residue was subjected to dry silica gel column chromatography. The first eluate with AcOEt–hexane (1 : 1) gave 623 mg (38.4%) of 3-nitroindole as light yellow needles (AcOEt–benzene), mp 213—214 °C. NMR (CD_3COCD_3) δ: 7.32—7.42 (2H, m, aromatic H), 7.59—7.65 (1H, m, aromatic H), 8.16—8.23 (1H, m, aromatic H), 8.49 (1H, s, C-2 H), and 11.54 (1H, br s, NH).[5a] The second eluate with the same solvent was combined with the previous precipitates and recrystallized from acetone to give 519 mg (25.4%) of 3-acetyl-6-nitroindole as brown crystals, mp 340—342 °C. NMR (CD_3COCD_3) δ: 2.53 (3H, s, $COCH_3$), 8.115 (1H, dd, J=9.28, 1.95 Hz, C-5 H), 8.43—8.58 (3H, m, aromatic H), 11.53 (1H, br s, NH).[5a] The third eluate afforded 420 mg (20.5%) of 3-acetyl-4-nitroindole as yellow needles (AcOEt), mp 229—230 °C. NMR (CD_3COCD_3) δ: 2.49 (3H, s, $COCH_3$), 7.39 (1H, t, J=7.81 Hz, C-6 H), 7.605 (1H, dd, J=7.8, 1 Hz, C-7 H), 7.85 (1H, dd, J=7.8, 1 Hz, C-5 H), 8.43 (1H, d, J=2.9 Hz, C-2 H), 11.53 (1H, br s, NH).[5a]

Method B: The solvate, $Al(AN)_6(ClO_4)_3$, was prepared with 4.69 g (10 mmol) of $Al(ClO_4)_3 \cdot 8H_2O$ and 9.18 g (90 mmol) of Ac_2O in 29 ml of AN. Nitration of 1.59 g (10 mmol) of 3-acetylindole was carried out with 756 mg of 99% HNO_3 in AN (1 ml) at 13 °C for 2 h. The reaction mixture was worked up as described in method A, and the residue obtained was purified according to method A to give 261 mg (16.1%) of 3-nitroindole, 114 mg (7.2%) of 3-acetylindole, 496 mg (24.3%) of 3-acetyl-6-nitroindole, and 252 mg (12.3%) of 3-acetyl-4-nitroindole.

Method C: The solvate, $Fe(AN)_6(BF_4)_2$, was prepared with 3.38 g (10 mmol) of $Fe(BF_4)_2 \cdot 6H_2O$ and 7.14 g (70 mmol) of Ac_2O in 29 ml of AN. Reaction of 1.59 g of 3-acetylindole in the presence of the solvate with 756 mg of 99% HNO_3 in AN (1 ml) was carried out at 15 °C for 3.5 h. The mixture was worked up as described in method A, and the residue obtained was purified according to method A to give 475 mg (28.2%) of 3-nitroindole, 652 mg (31.6%) of 3-acetyl-6-nitroindole, and 426 mg (20.8%) of 3-acetyl-4-nitroindole.

Nitration of 3-Indolecarbaldehyde in the Presence of Metal AN Solvates——Method A: 3-Indolecarbaldehyde

(1.45 g, 10 mmol) was added to a solution of the solvate, $Cu(AN)_6(ClO_4)_2$ [prepared from 3.70 g (10 mmol) of $Cu(ClO_4)_2 \cdot 6H_2O$ and 7.14 g (70 mmol) of Ac_2O], in 29 ml of AN as described for the reaction of 3-acetylindole above, and the mixture was stirred at room temperature for 30 min. Then 756 mg of 99% HNO_3 in AN (1 ml) was added very slowly at 13 °C and the whole was stirred at room temperature for 19 h. The reaction mixture was poured into water and extracted with AcOEt. The AcOEt layer was washed with sat. $NaHCO_3$ and brine. The organic layer was dried and concentrated under a vacuum. The residue was subjected to dry silica gel chromatography. The first eluate with AcOEt–hexane (1.5 : 2) gave 352 mg (21.7%) of 3-nitroindole as light yellow needles (AcOEt–benzene). The second eluate gave 49 mg (3.4%) of 3-indolecarbaldehyde. The third eluate with the same solvent afforded 368 mg (19.3%) of 6-nitro-3-indolecarbaldehyde as yellow crystals (acetone), mp 302—304 °C (dec.). NMR (DMSO-d_6) δ: 8.09 (1H, dd, J=8.79, 2 Hz, C-5 H), 8.25 (1H, d, J=8.79 Hz, C-4 H), 8.42 (1H, d, J=2 Hz, C-7 H), 8.65 (1H, s, C-2 H), 10.03 (1H, s, CHO), 12.65 (1H, brs, NH).[5b)] The 4th eluate with the same solvent yielded 364 mg (19.1%) of 4-nitro-3-indolecarbaldehyde as orange needles (AcOEt–hexane), mp 190—192 °C. NMR (CD_3COCD_3) δ: 7.49 (1H, t, J=8.0 Hz, C-6 H), 7.96 (1H, dd, J=8.0, 0.7 Hz, C-7 H), 8.00 (1H, dd, J=8.0, 0.7 Hz, C-5 H), 8.43 (1H, s, C-2 H), 10.29 (1H, s, CHO), 11.84 (1H, s, NH).[5b)]

Method B: The solvate, $Al(AN)_6(ClO_4)_3$, was prepared from 4.69 g (10 mmol) of $Al(ClO_4)_3 \cdot 8H_2O$ and 9.18 g (90 mmol) of Ac_2O in 29 ml of AN. Reaction of 1.45 g (10 mmol) of 3-indolecarbaldehyde in the presence of the solvate was carried out with 756 mg of 99% HNO_3 in AN (1 ml) at 5 °C for 4 h. The reaction mixture was worked up as described in method A and the residue obtained was purified according to method A to give 156 mg (9.6%) of 3-nitroindole, 267 mg (18.4%) of 3-indolecarbaldehyde, 420 mg (22.1%) of 6-nitro-3-indolecarbaldehyde, and 295 mg (15.5%) of 4-nitro-3-indolecarbaldehyde.

Method C: The solvate, $Fe(AN)_6(BF_4)_2$, was prepared with 3.38 g (10 mmol) of $Fe(BF_4)_2 \cdot 6H_2O$ and 7.14 g (70 mmol) of Ac_2O in AN (29 ml). Nitration of 1.45 g (10 mmol) of 3-indolecarbaldehyde with 765 mg of 99% HNO_3 in AN (1 ml) in the presence of the solvate was carried out at 15 °C for 24 h. The reaction mixture was worked up as described in method A, and purified according to method A to give 355 mg (21.9%) of 3-nitroindole, 430 mg (29.8%) of 3-indolecarbaldehyde, 415 mg (21.8%) of 6-nitro-3-indolecarbaldehyde, and 113 mg (5.9%) of 4-nitro-3-indolecarbaldehyde.

3-Acetyl-4-aminoindole (4)——A 31.8 ml portion of $TiCl_3$ solution (17—19%) was added at once to a suspension of 1.17 g of 3-acetyl-4-nitroindole (3) in MeOH (8 ml) and the mixture was stirred at room temperature for 1.5 h. The crystalline precipitates were separated by filtration, and washed with 23% HCl. The precipitates were dissolved in hot water, and then the solution was basified with sat. $NaHCO_3$. The basic solution was extracted with AcOEt. The organic layer was dried and concentrated. The residue was recrystallized from MeOH to give 980 mg (91.0%) of 4 as yellow crystals, mp 232—234 °C (dec.). IR (Nujol) cm^{-1}: 3425, 3280, 1590. NMR (CD_3COCD_3) δ: 2.48 (3H, s, $-COCH_3$), 5.6 (2H, br, NH_2), 6.32 (1H, dd, J=7.8, 1 Hz, C-5 H or C-7 H), 6.62 (1H, dd, J=8.3, 1 Hz, C-5 H or C-7 H), 6.92 (1H, dd, J=8.3, 7.8 Hz, C-6 H), 8.09 (1H, d, J=3.4 Hz, C-2 H), 10.8 (1H, br s, indole NH). MS m/z: Calcd for $C_{10}H_{10}N_2O$ (M^+): 174.0793. Found: 174.0811.

3-Acetyl-4-iodoindole (5)——A solution of 369 mg of $NaNO_2$ in water (9 ml) was added slowly to a solution of 422.8 mg of 4 in 2 N HCl with stirring at 0 °C. To the above diazonium salt solution, a solution of 25 g KI in water (18 ml) was added with stirring at 0 °C, and the whole was stirred at room temperature for 1 h and then at 85 °C for 10 min. After cooling, the mixture was extracted with AcOEt. The organic layer was washed with 5% $Na_2S_2O_3$ and brine. The AcOEt layer was dried and concentrated. The residue was subjected to dry silica gel chromatography, and the eluate with AcOEt–hexane (1 : 1.5) gave 505 mg (73%) of 5 as colorless needles (MeOH), mp 215—218 °C (dec.) (lit. mp 204—206 °C (dec.)).[13)] IR (Nujol) cm^{-1}: 3140, 1640. NMR (CD_3COCD_3) δ: 2.50 (3H, s, $COCH_3$), 6.95 (1H, dd, J=8.3, 7.32 Hz, C-6 H), 7.56 (1H, dd, J=8.3, 1 Hz, C-5 H or C-7 H), 7.76 (1H, dd, J=7.32, 1 Hz, C-5 H or C-7 H), 8.23 (1H, d, J=2.93 Hz, C-2 H), 11.1 (1H, brs, NH). MS m/z: Calcd for $C_{10}H_8INO$ (M^+): 284.9646. Found: 284.9645. *Anal.* Calcd for $C_{10}H_8INO$: C, 42.13; H, 2.83; N, 4.91. Found: C, 42.20; H, 2.93; N, 4.62.

Methyl [(3-Acetyl-4-indolyl)thio]acetate (6)——A solution of 28.5 mg of 5, 34 mg of methyl thioglycolate and 26.1 mg of pyridine in MeOH (1 ml) was heated at 90 °C under a nitrogen atmosphere for 48 h. Water was added to the mixture, and the whole was extracted with AcOEt. The organic layer was washed with dil. HCl, sat. $NaHCO_3$, and brine. The AcOEt layer was dried and concentrated. The residue was recrystallized from benzene to give 26 mg (95%) of 6 as colorless needles, mp 140—141.5 °C. NMR ($CDCl_3$) δ: 2.48 (3H, s, $COCH_3$), 3.72 (3H, s, CO_2CH_3), 3.78 (2H, s, $-SCH_2-$), 7.08—7.19 (3H, m, aromatic H), 7.69 (1H, d, C-2 H), 9.47 (1H, brs, NH). Ethyl [(3-acetyl-4-indolyl)thio]acetate was prepared by similar reaction of 5 and ethyl thioglycolate: mp 140—141 °C (MeOH). IR (Nujol) cm^{-1}: 3160, 1719, 1619. NMR (CD_3COCD_3) δ: 1.18 (3H, t, J=7.1 Hz, $COCH_2\underline{CH_3}$), 2.49 (3H, s, $COCH_3$), 3.74 (2H, s, $-SCH_2-$), 4.11 (2H, q, J=7.1 Hz, $CO\underline{CH_2}CH_3$), 7.14 (1H, dd, J=7, 2 Hz, C-5 H or C-7 H), 7.18 (1H, t, J=7 Hz, C-6 H), 7.32 (1H, dd, J=7, 2 Hz, C-5 H or C-7 H), 8.20 (1H, d, J=3.2 Hz, C-2 H), 11.05 (1H, br s, NH). MS m/z: Calcd for $C_{14}H_{15}NO_3S$ (M^+): 277.077. Found: 277.0763.

Dehydrochuangxinmycin Methyl Ester (7)——A mixture of 108 mg of 6, 144 mg of ammonium acetate monohydrate, and 277 mg of AcOH in benzene (8 ml) was heated at 110 °C for 15 h under a nitrogen atmosphere. Water was added to the mixture, and the whole was extracted with AcOEt. The organic layer was washed with sat. $NaHCO_3$ and brine. The AcOEt layer was dried and concentrated. The residue was recrystallized from benzene to

give 93 mg (93%) of methyl dehydrochuangxinmycin (7) as yellow needles, mp 167—168 °C.[12a] NMR ($CDCl_3$) δ: 2.34 (3H, s, CH_3), 3.81 (3H, s, CO_2CH_3), 6.51—6.94 (4H, m, aromatic H), 7.91 (1H, brs, NH). *Anal.* Calcd for $C_{13}H_{11}NO_2S$: C, 63.67; H, 4.52; N, 5.71. Found: C, 63.63; H, 4.68; N, 5.44. Ethyl dehydrochuangxinmycin was prepared by similar reaction of ethyl [(3-acetyl-4-indolyl)thio]acetate: mp 183—185 °C (MeOH). IR (Nujol) cm^{-1}: 3250, 1640. NMR (CD_3COCD_3) δ: 1.31 (3H, t, J=7 Hz, $CO_2CH_2\underline{CH_3}$), 2.32 (3H, s, CH_3), 4.22 (2H, q, J=7 Hz, $CO_2\underline{CH_2}CH_3$), 6.46 (1H, dd, J=7, 1.1 Hz, C-8 H or C-10 H), 6.81—6.91 (2H, m, aromatic H), 7.25 (1H, d, J=2.5 Hz, C-2 H), 10.32 (1H, brs, NH). *Anal.* Calcd for $C_{14}H_{13}NO_2S$: C, 64.84; H, 5.05; N, 5.40. Found: C, 64.64; H, 5.16: N, 5.19.

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Steric and Electronic Effects of Methyl Substitutents at the 2- and 6-Positions on *N*-Benzyl-1,4-dihydronicotinamide

JUN TAKEDA, SHIGERU OHTA, and MASAAKI HIROBE*

Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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N-Benzyl-1,4-dihydronicotinamides having 2-methyl, 6-methyl and 2,6-dimethyl substituents were prepared and their reactivities toward an activated carbonyl compound (hexachloroacetone) were investigated. The reaction rates were especially enhanced for the 2,6-dimethyl derivative, with the order of the rates being 2,6-dimethyl > 2-methyl > 6-methyl. The steric and electronic effects of the methyl group(s) are discussed based on the ultraviolet spectra and redox potential data.

Keywords——steric effect; electronic effect; methyl substituent; dihydronicotinamide; NADH model

Introduction

Extensive chemical studies of the nicotinamide adenine dinucleotide (phosphate)/reduced nicotinamide adenine dinucleotide (phosphate) $(NAD(P)^+/NAD(P)H)$-dependent dehydrogenation/hydrogenation reaction have been carried out in attempts to understand the mechanism and stereochemistry of the hydride (H^-) equivalent transfer process.[1-3] Most model studies have been carried out using simple reduced nicotinamide adenine dinucleotide (NADH) analogues such as *N*-benzyl-1,4-dihydronicotinamide (BzNAH) and 2,6-dimethyl-3,5-dicarboethoxy-1,4-dihydropyridine (Hantzsch ester). However, large differences in the reducing abilities exsist between these models and the enzymatic system. The reducing ability of the model systems can be accelerated by several factors, such as metal ion catalysis[4] and general acid catalysis[5] for substrate activation. Neighboring-group effects of carboxylate[6] and intramolecular interaction of bisdihydronicotinamides[7] are also known to activate dihydronicotinamide by electrostatic interaction. In this paper, we report the reducing ability of the 2- and/or 6-methyl-substituted *N*-benzyl-1,4-dihydronicotinamide, which is expected to throw light on the steric and electron-donating effects of the methyl group.[8]

	R_1	R_2	R_3	
1	H	H	benzyl	BzNAH
2	CH_3	H	benzyl	6-MeBzNAH
3	H	CH_3	benzyl	2-MeBzNAH
4	CH_3	CH_3	benzyl	2,6-diMeBzNAH
5	H	H	propyl	PrNAH

Chart 1

Results and Discussion

Nicotinamidinium bromides were prepared from the corresponding nicotinamide[9] and benzyl bromide; the ease of benzylation was in the order of nicotinamide > 6-methyl- > 2-methyl- > 2,6-dimethylnicotinamide. Subsequent sodium dithionite reduction gave the di-

TABLE I. Summary of UV Absorption Maxima, Redox Potentials and Second-Order Rate Constants for the Reduction of Hexachloroacetone with NADH Models[a)]

	λ_{max}, nm (ε, $M^{-1}cm^{-1}$)[a)]	k_2, $M^{-1}s^{-1}$[b)]	k_{rel}[c)]	$E°$, V vs. NHE[d)]
BzNAH	348 (6400)	0.311 ± 0.011	1	−0.361
6-MeBzNAH	343 (6100)	0.783 ± 0.023	2.5	−0.390
2-MeBzNAH	339 (3600)	1.82 ± 0.08	5.9	−0.418
2,6-DiMeBzNAH	329 (3400)	16.9 ± 0.5	54	−0.458

a) At 30 °C in CH_3CN. b) Mean ± standard deviation. c) k_{rel} values were represented by the relative rate based on the k_2 value of BzNAH. d) Redox potentials were determined by the direct hydride exchange method using ^{1}H-NMR in CD_3CN–$CDCl_3$. The known redox potential of BzNAH was used as the standard.

Chart 2

hydronicotinamides as white or yellow solids. The proton nuclear magnetic resonance (^{1}H-NMR) and ultraviolet (UV) spectra were consistent with the 1,4-dihydropyridine structure and the formation of the 1,2-dihydro isomer or hydration products was ruled out.[10)] The UV spectrum of each of the three 1,4-dihydronicotinamides showed that the introduction of methyl substituent(s) induces hypso- and hypochromic effects in the long-wavelength dihydropyridine transition. These effects increase in the order of *N*-benzyl-2,6-dimethyl-1,4-dihydronicotinamide (2,6-DiMeBzNAH (**4**)) > *N*-benzyl-2-methyl-1,4-dihydronicotinamide (2-MeBzNAH (**3**)) > *N*-benzyl-6-methyl-1,4-dihydronicotinamide (6-MeBzNAH (**2**)) > *N*-benzyl-1,4-dihydronicotinamide (BzNAH, (**1**)) (see Table I). This phenomenon would be caused by distortion of the planar dihydronicotinamide chromophore.[11)] Similar results have been observed in bridged and sterically hindered dihydropyridines.[12)] Determination of the redox potentials of **2**—**4** is also important to characterize the effects of the ring methyl groups. The two-electron redox potential (standard midpoint potential, $E°$, shown in Chart 2) was determined by the direct hydride exchange method.[13)] The $E°$ values for **2**—**4** listed in Table I indicated that $E°$ of 2,6-DiMeBzNAH is more negative than that of BzNAH by 97 mV. The order of the negative shift of $E°$ is the same as that of the hypsochromic shift of λ_{max}.

The effect of methyl groups was also observed in the reactivity toward an activated carbonyl compound. The reduction of hexachloroacetone[14)] was performed at 30 °C in acetonitrile under pseudo-first-order conditions ($[Cl_3CC=OCCl_3] \gg [PyH_2] = 0.5$—$1.0 \times 10^{-4}$ M), and the progress of the reaction was followed spectrophotometrically by monitoring the disappearance of the absorption band of dihydronicotinamide at 330—350 nm (Chart 3). All experiments gave good first-order plots over three half-lives. Figure 1 shows plots of the pseudo-first-order rate constants (k_{obsd}) against the concentration of hexachloroacetone. The observed linear dependence proves the reaction to be first-order in hexachloroacetone. The second-order-rate constants for the NADH model compounds are listed in Table I. The reaction products were identified from their ^{1}H-NMR spectra as oxidized nicotinamidinium cation and hexachloroisopropanol.

Consideration of the Table I data reveals that 2,6-DiMeBzNAH is the most reactive compound and the order of the rate of hexachloroacetone reduction shows the same tendency

Chart 3

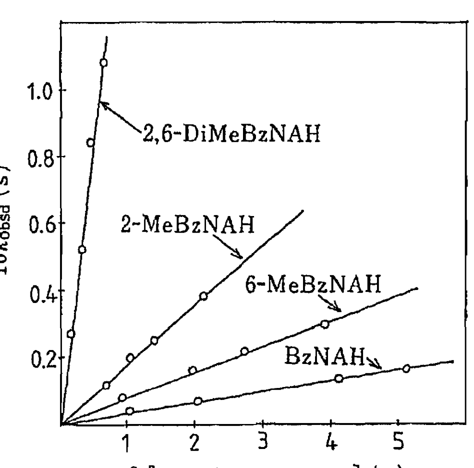

Fig. 1. Dependence of Pseudo-First-Order Rate Constant (k_{obsd}) upon Hexachloroacetone Concentration in CH_3CN at 30 °C

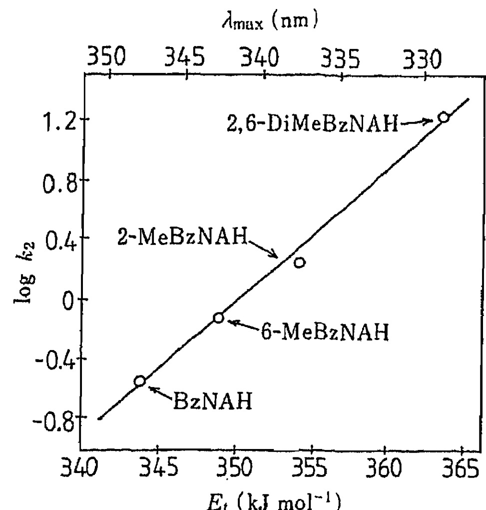

Fig. 2. Plot of the log of the Second-Order Rate Constants (log k_2) for the Reduction of Hexachloroacetone with NADH Models *vs.* the Transient Energy (E_t) of the NADH Models

E_t were calculated from the following relationship, $E_t = hcN_L/\lambda_{max}$.

as the λ_{max} and $E°$ data. The second-order rate constants of three methyl-substituted 1,4-dihydronicotinamides show different electronic and steric effects. The rate constant for the reference compound BzNAH is 0.311 $M^{-1}\,s^{-1}$, in agreement with the literature value.[14] The relatively small rate enhancement (0.783 $M^{-1}\,s^{-1}$) that was observed for 6-MeBzNAH results from the electron-donating effect rather than the steric effect of the 6-methyl group. This value of 0.783 $M^{-1}\,s^{-1}$ is comparable to that for *N*-propyl-1,4-dihydronicotinamide (PrNAH) (k_2 = 0.764 $M^{-1}\,s^{-1}$). In contrast to the 6-methyl group, however, the 2-methyl group of 2-MeBzNAH strongly affects the reducing ability, and increases the rate by *ca.* 2.4-fold. This, in turn, suggests that the 2-methyl group of 2-MeBzNAH affects the reducing ability not only electronically but also sterically. Most surprising is the high rate acceleration for 2,6-DiMeBzNAH (53-fold compared with BzNAH). The high reducing ability of 2,6-DiMeBzNAH can not be explained simply in terms of the electron-donating nature of two independent methyl groups. If the two methyl groups produced a simple electron-donating effect, a k_{rel} value of 15 (=5.9×2.5) for BzNAH would be expected. That is, the two methyl groups at the 2- and 6-position would cooperatively promote the reducing ability. Because λ_{max} is an index of ring distortion, we tried to correlate the λ_{max} values with the second-order rate constants.[15] The logarithm of these constants (log k_2) *vs.* the transition energy plot (Fig. 2) was found to be linear (γ=0.997) and could be expressed by Eq. 1.

$$\log k_2 = 0.0875\,E_t - 30.6 \qquad (1)$$

These results clearly show that steric effects control the hydride-donating ability. There

(i)

planary distortion

(ii)

Fig. 3. Proposed Conformations for the Distortion of Dihydronicotinamide Ring System

(i) pseudo boat form. (ii) rotation of carbamoyl group.

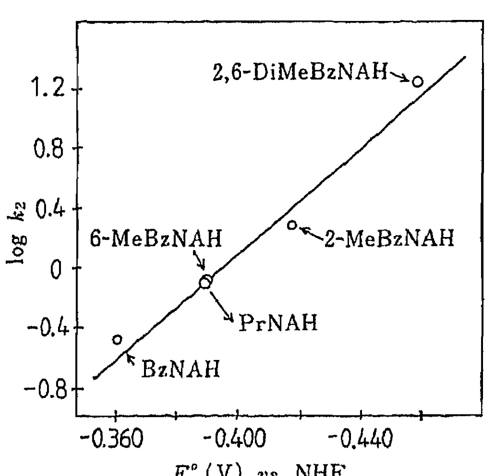

Fig. 4. Correlation of the log the Second-Order Rate Constant ($\log k_2$) *vs.* the Redox Potential for the Dihydronicotinamide (E°)

are two possible explanations for the steric effect of the methyl group. (i) The interaction of the 1-benzyl group with the 2- and/or 6-methyl group(s) causes distortion of the planar 1,4-dihydropyridine ring.[16] This would suggest that the (pseudo)axial C(4)H is more rapidly donated to the substrate by π—σ orbital interaction through hyperconjugation. (ii) The interactions of the 6-, 1- and 2-substituents with the 3-carbamoyl group lead to out-of-plane rotation of the 3-carbamoyl group. The rotation of the 3-carbamoyl group increases the hydride-donating ability because it decreases the conjugation of dihydropyridine and the electron-withdrawing carbamoyl group by a resonance inhibition effect. In the case of 2,6-DiMeBzNAH, this phenomenon is increased not only by the 2-methyl group but also by the 6-methyl group due to the buttressing effect. These steric effects are summarized in Fig. 3. Buck and Donkersloot[17] proposed that stereo- and regio-selective hydride transfer of NADH in the enzymatic system would be due to rotation of the carbamoyl group with respect to the plane of the dihydropyridine ring on the basis of quantum chemical calculation. Verhoeven *et al.*[12a] suggested that the rotation is most likely to be relevant to the catalytic activity of the enzyme. Our results also indicate that distortion of the 1,4-dihydronicotinamide structure produces high hydride-donating ability.

The effect of methyl substituents on the redox potential was also considered. In Fig. 4, the logarithm of k_2 is plotted against E° of dihydronicotinamides (**1**—**4**) and PrNAH. The plot was found to be linear ($\gamma = 0.988$) with a slope of $17.6\,V^{-1}$ and could be expressed by Eq. 2.

$$\log k_2 = -17.6 E^{\circ} - 6.95 \quad (2)$$

The acceleration of the rate by introduction of the methyl group(s) may be attributed mainly to the intramolecular steric interaction which causes distortion of the ring, as mentioned above. The negative shift of the redox potential results directly from the distortion.

In conclusion, the present study clearly shows that the hydride-donating ability of 1,4-dihydronicotinamide is increased by the 2- and 6-methyl groups due to steric as well as electronic effects. We suggest that distortion of the dihydronicotinamide ring system and rotation of the 3-carbamoyl group are also important in the enzymatic system. Such distortions of NAD(P)H in the enzyme may be caused by a suitable binding pocket for NAD(P)H and by hydrogen bonding.

Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H-NMR spectra were taken on a Hitachi R-24B (60 MHz) or a JNM FX-100 (100 MHz) pulse Fourier-transform spectrometer with tetramethylsilane as an internal standard. Mass spectra (MS) were obtained on a JEOL DX-300 mass spectrometer operating in the EI mode at 70 eV. UV-visible spectra were recorded on a Hitachi 557 double-beam spectrophotometer.

Materials——6-Methylnicotinamide,[9a,b] 2-methylnicotinamide[9c,d] and 2,6-dimethylnicotinamide[9e] were synthesized according to the cited methods. *N*-Benzyl-1,4-dihydronicotinamide was synthesized as described by Mauzerall and Westheimer.[18] *N*-Propyl-1,4-dihydronicotinamide was synthesized as described by Suelter and Metzler.[19] Perchlorate salts of *N*-benzylnicotinamides were prepared by addition of sodium perchlorate to an aqueous solution of *N*-benzylnicotinamidinium halide.

General Procedure for the Preparation of *N*-Benzylnicotinamidinium Bromides——The appropriate nicotinamide (5 mmol) was suspended in benzyl bromide (3—6 ml) and heated at 120 °C for 1—12 h. After the reaction mixture had cooled, ether was added. The white precipitate was collected and washed with ether. Recrystallization from ethanol gave colorless crystals.

N-Benzyl-6-methylnicotinamidinium Bromide: 74% yield. mp 175—177 °C. ^{1}H-NMR (D_2O) δ: 2.91 (3H, s, $-CH_3$), 5.92 (2H, s, $-CH_2-$), 7.44 (5H, m, Ar H), 8.11 (1H, d, $J=8.4$ Hz, 5-H), 8.80 (1H, dd, $J=8.4, 2.1$ Hz, 4-H), 9.23 (1H, d, $J=2.1$ Hz, 2-H). *Anal.* Calcd for $C_{14}H_{15}BrN_2O \cdot 0.75H_2O$: C, 52.43; H, 5.19; N, 8.74. Found: C, 52.26; H, 4.91; N, 8.54.

N-Benzyl-2-methylnicotinamidinium Bromide: 80% yield. mp 203—205 °C. ^{1}H-NMR (D_2O) δ: 2.88 (3H, s, $-CH_3$), 5.92 (2H, s, $-CH_2-$), 7.46 (5H, m, Ar H), 8.04 (1H, dd, $J=8.2, 6.4$ Hz, 5-H), 8.68 (1H, dd, $J=8.2, 1.4$ Hz, 4-H), 8.95 (1H, dd, $J=6.4, 1.4$ Hz, 6-H). *Anal.* Calcd for $C_{14}H_{15}BrN_2O$: C, 54.74; H, 4.92; N, 9.12. Found: C, 54.89; H, 4.95; N, 8.93.

N-Benzyl-2,6-dimethylnicotinamidinium Bromide: 84% yield. mp 241—243 °C (dec.). ^{1}H-NMR (D_2O) δ: 2.98 (6H, s, $-CH_3$), 6.08 (2H, s, $-CH_2-$), 7.55 (5H, m, Ar H), 7.87 (1H, d, $J=8.4$ Hz, 5-H), 8.63 (1H, d, $J=8.4$ Hz, 4-H). *Anal.* Calcd for $C_{15}H_{17}BrN_2O$: C, 56.09; H, 5.33; N, 8.72. Found: C, 55.89; H, 5.33; N, 8.56.

General Procedure for the Preparation of *N*-Benzyl-1,4-dihydronicotinamides——Sodium dithionite (1.0 g, 5.7 mmol) and sodium carbonate (0.5 g, 4.7 mmol) were added dropwise over 10 min to an Ar-purged solution of *N*-benzylnicotinamidinium bromide (2 mmol in 10 ml of water), and the mixture was stirred for 30 min at room temperature under an Ar atmosphere. A yellow or pale yellow precipitate resulted. Water (10 ml) was added to the mixture, and the whole was extracted with dichloromethane (20 ml × 2). The extract was dried over anhydrous sodium sulfate. Recrystallization from ethanol–water at 35 °C gave pale yellow or colorless crystals. For the preparation of 2,6-DiMeBzNADH, a larger excess of sodium dithionite was used and the precipitate formed was collected by filtration, washed with water and recrystallized as above. UV data are given in Table I.

6-MeBzNAH: 50% yield. mp 113—115 °C (dec.), pale yellow micro crystals. ^{1}H-NMR (CD_3CN) δ: 1.77 (3H, s, $-CH_3$), 3.16 (2H, br, 4-H_2), 4.57 (3H, m, $-CH_2-$ and 5-H), 5.63 (2H, brs, NH_2), 7.05 (1H, s, 2-H), 7.38 (5H, s, Ar H). MS *m/z*: 228 (M^+). *Anal.* Calcd for $C_{14}H_{16}N_2O$: C, 73.66; H, 7.06; N, 12.27. Found: C, 73.72; H, 7.13; N, 12.22.

2-MeBzNAH: 53% yield. mp 103—106 °C (dec.), colorless micro crystals. ^{1}H-NMR (CD_3CN) δ: 2.18 (3H, s, $-CH_3$), 3.17 (2H, d, $J=3.2$ Hz, 4-H_2), 4.56 (2H, s, $-CH_2-$), 4.74 (1H, dt, $J=7.8, 3.2$ Hz, 5-H), 5.62 (2H, brs, NH_2),

5.97 (1H, d, J=7.8 Hz, 6-H), 7.40 (5H, s, Ar H). MS m/z: 228 (M^+). *Anal.* Calcd for $C_{14}H_{16}N_2O$: C, 73.66; H, 7.06; N, 12.27. Found: C, 73.53; H, 7.14; N, 12.03.

2,6-DiMeBzNAH: 26% yield. mp 129—131 °C (sec.), colorless micro crystals. ^{1}H-NMR (CD_3CN) δ: 1.80 (3H, s, 6-CH_3), 2.20 (3H, s, 2-CH_3), 3.11 (2H, br, 4-H_2), 4.73 (3H, m, 5-H and –CH_2–), 5.68 (2H, br s, NH_2), 7.39 (5H, m, Ar H). MS m/z: 242 (M^+). *Anal.* Calcd for $C_{15}H_8N_2O$: C, 74.35; H, 7.49; N, 11.56. Found: C, 74.05; H, 7.49; N, 11.29.

Kinetic Study——The rates of reaction were determined by measuring the decrease of the absorption maximum for BzNAH and 6-MeBzNAH, and the absorption at 350 nm for 2-MeBzNAH and 2,6-DiMeBzNAH in modified Thunberg cuvettes. In a typical experiment, hexachloroacetone stock solution (1 ml, $>3\times10^{-3}$ M in acetonitrile) was placed in the upper arm of the cuvette and dihydronicotinamide stock solution (2 ml, 3×10^{-4} M in acetonitrile) was placed in the bottom part of the cuvette. After the cuvette had equilibrated at 30 °C, the reaction was initiated by mixing the contents of the cuvette. All kinetic studies were carried out at 30±0.2 °C in a thermostated cell compartment of the spectrophotometer. Control experiments indicated that the rate of decomposition of dihydronicotinamide was negligible.

Product Analysis——The products of reaction of dihydronicotinamide and hexachloroacetone were determined by ^{1}H-NMR spectroscopy. As a standard procedure, excess hexachloroacetone was added to a solution of dihydronicotinamide (0.1 mmol) in CD_3CN (0.5 ml) in an NMR tube. The reaction mixture was allowed to stand at room temperature. After 10 min, the ^{1}H-NMR signals of dihydronicotinamide completely disappeared and signals attributable to the nicotinamidinium cation and hexachloroisopropanol (4.8 ppm) appeared. Integration of the signals indicated that the reactions proceeded stoichiometrically.

Determination of Redox Potential——Two-electron redox potentials of dihydronicotinamide were determined by almost the same method as reported.[13] As a standard procedure, a nicotinamidinium perchlorate ($Py'H^+$) and the dihydronicotinamide (PyH_2) derived from a different nicotinamidinium cation were dissolved in CD_3CN–$CDCl_3$ (1 : 1, v/v, 0.5 ml) and allowed to stand at 25 °C (Eq. 3).

$$PyH_2 + Py'H^+ \underset{}{\overset{K_{eq}}{\rightleftarrows}} PyH^+ + Py'H_2 \tag{3}$$

The progress of the reaction was monitored by ^{1}H-NMR spectroscopy. In general, the reaction reached equilibrium within 2 h. The equilibrium constant (K_{eq}) was calculated from the results of peak integration of each species. Given the $E^\circ(Py'H_2)$ value for $Py'H_2$ (BzNAH $E^\circ=-0.361$ V; PrNAH, $E^\circ=-0.387$ V *vs.* NHE),[13,15,20] the two-electron redox potential for dihydronicotinamide (E°) could be obtained from Eq. 4, where F is the Faraday constant and n, the number of electrons transferred, is two in this case.

$$E^\circ(PyH_2) = E^\circ(Py'H_2) + (RT/nF)\ln K_{eq} \tag{4}$$

To ensure accuracy in the experiment, the forward and backward reactions were examined and cross checks were made as described by Kellogg and Piepers, and Ostovie *et al.*[13]

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(i).[2] When no steric hindrance exists, the dipolar structure (ii) is stabilized by hyperconjugation and the electron-donating nature of the methyl group leads to a hyperchromic shift of λ_{max}. The observed hypsochromic shift clearly indicates that the excited state can be preferentially unstabilized by the sterically hindered methyl group(s).

Chart 4

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Inhibitors of the Arachidonate Cascade from *Allium chinense* and Their Effect on *in Vitro* Platelet Aggregation

YUKIHIRO GODA, MASAAKI SHIBUYA and USHIO SANKAWA*

Faculty of Pharmaceutical Science, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan

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N-(*p-trans*-Coumaroyl)- and *N*-(*p-cis*-coumaroyl)tyramines (**1**, **2**), lunularic acid (**3**) and *p*-coumaric acid (**4**), which are inhibitors of prostaglandin (PG) and thromboxane synthetases, were isolated from the bulb of *Allium chinense* G. DON, a Chinese medicinal drug (Japanese name "薤白"). Stilbene derivatives structurally related to lunularic acid (**3**) were tested for inhibitory effect on prostaglandin synthetase. The compounds that inhibited PG biosynthesis also showed significant inhibitory effects on platelet aggregation induced by arachidonic acid and collagen.

Keywords——*Allium chinense*; phenolic amide; *N*-(*p-trans*-coumaroyl)tyramine; *N*-(*p-cis*-coumaroyl)tyramine; stilbene; lunularic acid; *p*-coumaric acid; prostaglandin synthetase inhibitor; thromboxane synthetase inhibitor; platelet aggregation inhibitor

During the course of our attempts to find biologically active principles of crude drugs used in traditional medicine, a hot aqueous extract of the bulb of *Allium chinense* G. DON (Japanese name"薤白") was found to inhibit prostaglandin synthetase (PG-ase) by 33% at a concentration of 0.75 mg/ml.[1] In Chinese medicine, the bulb of *A. chinense* is used to treat stenocardia, heart asthma and so-called stagnant blood.[2] On the other hand, it has been reported that oral administration of juice or oil of plants belonging to *Allium* species prevents hyperlipemia,[3] atherosclerosis[4] and myocardial infarction,[5] and decreases platelet aggregation[6] in man and animals. Furthermore, inhibitory effects of the extract of *Allium* plants on platelet aggregation were demonstrated *in vivo* studies.[7] In recent years the balance between thromboxane (TX) A_2 and prostaglandin (PG) I_2 (prostacyclin) has been recognized to play a very important role in the regulation of blood flow. These facts suggest that the effects of *Allium* plants may be attributable to the inhibition of PG and TX biosynthesis. Ariga *et al.* reported the separation and identification of several sulfides as inhibitors of platelet aggregation from garlic (*A. sativum*) oil and proposed that the anti-aggregatory mechanism is based on the inhibition of PG biosynthesis.[7c] However, little is known about biologically active constituents of *Allium* plants except for sulfides.[7d,f,8] In this paper, we describe the separation and identification of phenolic compounds contained in *A. chinense* and the inhibition of PG and TX biosynthesis by these compounds.

Experimental

All melting points were determined on a Yanagimoto melting point apparatus and are uncorrected. Ultraviolet (UV) spectra were taken on a Hitachi spectrophotometer, model 100-60. Infrared (IR) spectra were recorded on a JASCO DS-701G spectrometer. Mass spectra (MS) were measured on a JEOL JMS-DX-300 spectrometer. Proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra were determined on a JEOL FX-100 spectrometer with tetramethylsilane as an internal standard. Gas chromatography-mass spectra (GC-MS) were obtained with a Shimadzu LKB GC-MS 9000B combined with a GC-MSpac 300 computer system and a Shimadzu chromatopac 4B integrator.

Materials——All materials, unless otherwise specified, were of reagent grade or the highest grade available from usual commercial sources.

Assay of PG-ase——The method was described in a previous paper (radioisotope method).[1]

Preparation of Partially Purified PG Endoperoxide Synthetase——Sheep vesicular gland microsomes were prepared according to the procedure described by Miyamoto *et al.*[9] with some modifications. Frozen vesicular glands (50 g) stored at $-80\,^{\circ}C$ were thawed at $4\,^{\circ}C$. Extraneous tissues were removed and the vesicular glands were homogenized in a Waring blender twice for 1 min each with 50 ml of buffer I (20 mM potassium phosphate buffer, pH 7.4). The homogenate was further homogenized with a Waring blender twice for 1 min each with 75 ml of buffer I. The crude homogenate was centrifuged at $6000 \times g$ for 10 min and the supernatant was filtered through cotton gauze. The filtrate was centrifuged at $100000 \times g$ for 90 min. The precipitate was homogenized in a Potter homogenizer with 60 ml of buffer I and further centrifuged at $100000 \times g$ for 90 min. The precipitate was rehomogenized in a Potter homogenizer with 60 ml of buffer I and further centrifuged at $100000 \times g$ for 90 min. The precipitate was rehomogenized in a Potter homogenizer with 20 ml of buffer I (microsomal fraction, 322 mg of protein in 24 ml). The microsomal fraction was further purified according to the method described by Mizuno *et al.*[10] The microsomal fraction was mixed with 20 ml of buffer II (20 mM potassium phosphate buffer, pH 7.4, containing 2% Tween 20 and 40% glycerol), and stirred for 30 min in an ice bath, followed by centrifugation at $100000 \times g$ for 90 min. The supernatant solution (solubilized enzyme, 169 mg of protein in 43 ml) was applied to a diethylaminoethyl (DEAE)-cellulose column previously equilibrated with buffer III (20 mM potassium phosphate buffer, pH 7.4 containing 0.2% Tween 20 and 20% glycerol). The column was then washed with buffer III and the eluate was concentrated to 1/10 volume (partially purified enzyme, 41.8 mg of protein in 6.8 ml) by ultra-filtration with a Diaflo membrane XM-50 and stored at $-80\,^{\circ}C$. All manipulations described above were performed at $4\,^{\circ}C$. The partially purified enzyme was purified 5.74-fold from the microsomal fraction. The recovery of enzyme activity was 75%.

Preparation of [1-^{14}C]PGH_2——[1-^{14}C]PGH_2 was prepared from [1-^{14}C]arachidonic acid (58 mCi/mmol) obtained from Amersham with the partially purified enzyme prepared from sheep seminal vesicles, according to the method described by Ogino *et al.*[11] with some modifications. The reaction mixture (1 ml) containing [1-^{14}C]-arachidonic acid (361 μM, 20 μCi), hemoglobin (2 μM), tryptophan (10 mM), Tris-HCl buffer (100 mM, pH 8.0) and the partially purified enzyme (*ca.* 0.6 mg) was incubated at $24\,^{\circ}C$ for 90 s. The reaction was terminated by the addition of 0.2 M citric acid (250 μl). The acidified reaction mixture was extracted three times with 6 ml of ether precooled to $-20\,^{\circ}C$ and the ether extract was passed through an anhydrous $NaSO_4$ column (16×80 mm) at $4\,^{\circ}C$. The obtained eluate was evaporated in an ice bath with a vacuum pump and the residue was extracted twice with 0.4 ml of dry acetone. The acetone solution was applied to a precooled thin layer chromatography (TLC) plate (Merck Kieselgel 60 F_{254} with a concentration zone). A standard solution of PGB_2 was also spotted on the same plate as a reference compound. The plate was developed 15 cm with a solvent mixture of ether–petroleum ether–acetic acid (85 : 15 : 0.1) at $-20\,^{\circ}C$. The PGH_2 zone just above the PGB_2 zone was visualized under UV light, scraped off at $4\,^{\circ}C$ and extracted three times with 5 ml of dry ether–methanol (9 : 1) precooled to $-20\,^{\circ}C$. The eluate was evaporated in an ice bath with a vacuum pump. The residue was dissolved in a small volume of dry acetone and stored at $-80\,^{\circ}C$ as [1-^{14}C]PGH_2 solution. By this method more than 95% chromatographically pure [1-^{14}C]PGH_2 was obtained in a yield of 30 to 45%.

Assay of Thromboxane Synthetase (TX-ase)——Human platelet microsomes were purchased from Ran Biochem. The assay mixture (100 μl) contained [1-^{14}C]PGH_2 (5 μM, 7.5×10^4 dpm), human platelet microsomes (0.5 mg), potassium phosphate buffer (0.1 M, pH 7.5) and test samples which were solubilized with polyvinylpyrrolidone K 30 (0.5 mg). Polyvinylpyrrolidone K 30 itself had no effect on the reaction at this concentration. The reaction was carried out at $24\,^{\circ}C$ for 3 min and terminated by the addition of 0.3 ml of a mixture of ethyl acetate–methanol–1 M citric acid (30 : 4 : 1) precooled to $-80\,^{\circ}C$. Then *ca.* 0.5 g of anhydrous Na_2SO_4 was added to the reaction mixture. A 75 μl aliquot of the organic phase was removed and applied to a precooled TLC plate (Merck Kieselgel 60 F_{254} with a concentration zone) in a cold room maintained at $4\,^{\circ}C$. Standard solutions of TXB_2, PGE_2 and $PGF_{2\alpha}$ were spotted on the same zone and the TLC plate was developed with a solvent mixture of chloroform–ethyl acetate–methanol–acetic acid–water (70 : 30 : 8 : 1 : 0.5). The TXB_2 zone (between the $PGF_{2\alpha}$ and PGE_2 zones) was visualized with iodine vapor, and scraped off the plate. Radioactivity of TXB_2 was measured as a parameter of TX-ase activity with a scintillation counter (Aloka LSC-670) in toluene scintillation cocktail.

Isolation of Active Principles from *Allium chinense*——Dried bulbs (10 g) of *A. chinense* G. DON (Liliaceae) purchased from Uchida Pharmacy for Oriental Medicine (Tokyo) were successively extracted with hexane, chloroform, methanol and water. The inhibitory activities of these extracts were 76, 96, 25 and 0% against PG-ase at 0.75 mg/ml, respectively, and 56, 77, 0 and 0% against TX-ase at 1 mg/ml, respectively.

In a large-scale extraction, dried bulbs (15 kg) were successively extracted with hexane, chloroform and methanol. The methanol extract was further fractionated into ethyl acetate- and water-soluble portions. The ethyl acetate-soluble portion was combined with the chloroform extract.

After separation by repeated column chromatography, the hexane extract (10.3 g) gave diallyldisulfide and a mixture of fatty acids. An authentic sample of diallyldisulfide was kindly supplied by Dr. T. Fuwa (Wakunaga Pharmaceutical Co.). A GC-MS analysis of the mixture of fatty acids showed that it contained palmitic acid, oleic

acid, linoleic acid, myristic acid, pentadecanoic acid, hexadecenoic acid, heptadecanoic acid, heptadecenoic acid and stearic acid.

The combined chloroform and ethyl acetate extract (14.2 g) was applied to an oxalate-treated silica gel column and eluted with benzene and acetone to afford two fractions active against PG-ase. These fractions were separated and further purified by repeated chromatography on silica gel, Sephadex LH-20 and Lobar RP-8, and finally by high-performance liquid column chromatography (HPLC) followed by recrystallization, to give five phenolic compounds.

N-(*p-trans*-Coumaroyl)tyramine (**1**) was obtained as a colorless powder and was identified by direct comparison with an authentic sample, which was kindly supplied by Dr. C.-F. Tseng of the University of Tokyo.[12]

N-(*p-cis*-Coumaroyl)tyramine (**2**) was obtained as a colorless oil and was identified from its spectral data as follows. High-resolution MS: $C_{17}H_{17}N_1O_3$ (M^+ *m/z*: 283.1172, Calcd: 283.1206). MS *m/z* (rel. int. %): 283 (M^+, 3), 164 (21), 147 (29), 120 (27), 119 (22), 118 (33), 107 (100), 91 (23), 77 (38). ^{1}H-NMR (methanol-d_4) γ: 2.87 (2H, t, J=8.0 Hz), 3.57 (2H, t, J=8.0 Hz), 5.98 (1H, d, J=12.5 Hz), 6.80 (1H, d, J=12.5 Hz), 6.88 (2H, d, J=8.7 Hz), 6.91 (2H, d, J=8.9 Hz), 7.21 (2H, d, J=8.7 Hz), 7.56 (2H, d, J=8.9 Hz).

Lunularic acid (**3**) was obtained as colorless needles from aqueous methanol (mp 195—197 °C) and was identified by comparing its spectral data with the reported values.[13] UV λ_{max}^{MeOH} nm: 225, 246, 280, 287, 308. IR ν_{max}^{KBr} cm^{-1}: 3550 (OH), 1650 (COOH), 1602 (arom.), 1515 (arom.). High-resolution MS: $C_{15}H_{14}O_4$ (M^+ *m/z*: 258.0883, Calcd: 258.0890). MS *m/z* (rel. int. %): 258 (M^+, 6), 134 (3), 108 (9), 107 (100), 105 (5). ^{1}H-NMR (acetone-d_6) δ: 2.75—3.38 (4H, m), 6.44—6.75 (4H, m), 7.19 (2H, d, J=8.5 Hz), 7.72 (1H, dd, J=7.5, 8.5 Hz).

Both *p*-coumaric acid (**4**) and *p*-hydroxybenzoic acid (**5**) were obtained as colorless powders and were identified by direct comparison with authentic samples, which were purchased from Tokyo-Kasei Co. (Tokyo) and Wako Co. (Tokyo), respectively.

Stilbene Derivatives——Rhapontigenin (**10**), piceatannol (**11**), rhaponticin (**12**) and piceatannol glucoside (**13**) were kindly supplied by Prof. I. Nishioka (University of Kyushu). Other dihydrostilbene and stilbene derivatives except for lunularic acid (**3**) were kind gifts of Prof. M. Yamato (University of Okayama).

Derivatives of Cinnamic and Benzoic Acids——3-Hydroxydihydrocinnamic acid was prepared by catalytic reduction of *m*-coumaric acid. Other cinnamic acid and benzoic acid derivatives were purchased from Tokyo-Kasei Co. (Tokyo) and Wako Co. (Tokyo). Their inhibitory effects against TX-ase at a concentration of 2 mM are as follows: *p*-coumaric acid (**4**), 31%; *m*-coumaric acid, 39%; *o*-coumaric acid, 18%; caffeic acid (**15**), 40%. 3-Hydroxydihydrocinnamic acid, *p*-hydroxybenzoic acid, *m*-hydroxybenzoic acid, *o*-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid showed no effect at this concentration.

Assay of Platelet Aggregation——Blood was collected from the main leg artery of a male albino rabbit and prevented from coagulating by adding 1 volume of 3.8% sodium citrate to 9 volumes of blood. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 200 × *g* for 10 min. The precipitate was further centrifuged at 2000 × *g* for 15 min to afford platelet-poor plasma (PPP). The PRP was diluted with PPP to the concentration of 2.2×10^5 platelets/μl.

Aggregation experiments were performed in an aggregometer (Paiton Aggregation Module 600B and Lumiaggregation Module 1010). For each assay, rabbit PRP (400 μl) was preincubated with test samples (50 μl, 5% ethanol and 50 mM Tris–HCl buffer solution) at 37 °C for 2 min, before the addition of the aggregating agents (50 μl).

Results and Discussion

In a preliminary solvent extraction experiment, it was found that inhibitory activity against PG-ase was mainly present in the hexane and chloroform extracts. The extracts also significantly inhibited TX-ase, though their inhibitory activities were much less than those against PG-ase. We therefore separated active compounds mainly by following the inhibitory activity against PG-ase.

The bulbs of *A. chinense* were extracted with hexane, chloroform and methanol successively on a large scale (15 kg). The ethyl acetate-soluble portion of the methanol extract was combined with the chloroform extract, since it contained virtually the same compounds as the chloroform extract. After chromatographic separation, the hexane extract gave diallyldisulfide as a PG-ase inhibitor and a mixture of fatty acids (mainly palmitic acid, oleic acid and linoleic acid) as inhibitors of both enzymes. These compounds were identified by direct comparison with an authentic sample and by GC-MS, respectively.

In order to isolate more polar active principles, the combined chloroform and ethyl acetate extract was separated on an oxalate treated silica gel column to give two active

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fractions showing inhibitory activity against PG-ase. The more polar of the two was further separated and purified by repeated column chromatography and HPLC (following the inhibitory activity towards PG-ase) to give two compounds, *N*-(*p-trans*-coumaroyl)tyramine (**1**) and *N*-(*p-cis*-coumaroyl)tyramine (**2**) as active principles. The less polar of the two was recrystallized from aqueous methanol to yield another compound, lunularic acid (**3**), as an active principle. The mother liquid was also found to inhibit TX-ase rather than PG-ase. Therefore, we further separated the mother liquid by repeated column chromatography and HPLC (following the inhibitory activity towards TX-ase) and obtained two compounds, *p*-coumaric acid (**4**) and *p*-hydroxybenzoic acid (**5**).

Compounds **1**, **4** and **5** were identified as *N*-(*p-trans*-coumaroyl)tyramine (**1**), *p*-coumaric acid (**4**) and *p*-hydroxybenzoic acid (**5**), respectively, by direct comparison with authentic samples. Compound **2** gave spectral data very similar to those of **1** except for the ^{1}H-NMR signals assignable to vinylic protons at δ 5.98 (J=12.5 Hz) and δ 6.80 (J=12.5 Hz) instead of δ 6.47 (J=15.6 Hz) and δ 7.46 (J=15.6 Hz) in **1**. These high-field-shifted signals of vinylic protons with smaller coupling constants than those of **1** indicated that **2** was the *cis*-isomer, *N*-(*p-cis*-coumaroyl)tyramine. Although the *trans*-isomer (**1**) has been isolated from several plants,[14] this is the first time that **2** has been isolated from a natural source. Compound **3** was identified as lunularic acid (**3**) by comparing its spectral data with those reported.[13] Its ^{13}C-NMR data (Table I) were consistent with the structure **3**. Lunularic acid (**3**) is a well-known common endogenous growth inhibitor of liverworts,[15] but the isolation of this compound from higher plants has not previously been reported.

The 50% inhibitory concentration (IC_{50}) values of **1** and **3** against PG-ase were 280 and 45 μM, respectively. Compound **2** showed 56% inhibition against PG-ase at 100 μM, but its IC_{50} value was not determined because of its low yield. Compound **1**—**3** did not inhibit TX biosynthesis at the concentration where more than 90% of PG biosynthesis was inhibited. Though **4** showed an IC_{50} value of 5 mM against TX-ase, it activated PG-ase in the concentration range of 0.1—0.5 mM. In contrast, **5** had no effect on either of the enzymes. Structural requirements for the inhibition of PG biosynthesis by the amide derivatives have been reported in a separate paper.[12]

Lunularic acid (**3**) is a dihydrostilbene and the biological activities of stilbene derivatives

TABLE I. ^{13}C Chemical Shifts of Lunularic Acid

Carbon No.	3	Carbon No.	3
1	146.0 s	1′	133.4 s
2	113.0 s	2′	129.8 d
3	163.3 s	3′	115.7 d
4	116.0 d	4′	156.0 s
5	134.6 d	5′	115.7 d
6	122.9 d	6′	129.8 d
COOH	173.2 s	α	38.2 t[a]
		β	39.4 t[a]

δ values in acetone-d_6. The multiplicities of carbon signals are indicated as s, d and t. *a*) The assignments may be reversed.

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1 2 3

4 : R=H
15 : R=OH

5

6 : R_1=OH, R_2=OMe
7 : R_1=OMe, R_2=OH

8 : R_1=COOH, R_2=OH, R_3=OMe
9 : R_1=H, R_2=OMe, R_3=OH

10 : R_1=OH, R_2=OMe 12 : R_1=O-glu, R_2=OMe
11 : R_1=OH, R_2=OH 13 : R_1=O-glu, R_2=OH

14

Table II. Inhibitory Effects of Stilbene Derivatives on PG Biosynthesis

Compound	Inhibition % at 100 μM	IC_{50} (μM)
Lunularic acid (**3**)	64	45
3-Hydroxy-4-methoxydihydrostilbene (**6**)	99	0.14
4-Hydroxy-3-methoxydihydrostilbene (**7**)	98	0.27
2-(3-Hydroxy-4-methoxystyryl)benzoic acid (**8**)	97	38
4-Hydroxy-3-methoxystilbene (**9**)	99	1.0
Rhapontigenin (**10**)	93	17
Piceatannol (**11**)	90	24
Rhaponticin (**12**)	0	—[a]
Piceatannol glucoside (**13**)	0	—[a]
Monomethylphyllodulcin (**14**)	0	—[a]

a) 0% inhibition at 1.0 mM.

are of interest, since some stilbene derivatives have been isolated from several medicinal plants used to treat stagnant blood.[16] We therefore investigated the inhibitory effects of various stilbene derivatives (**6**—**14**) on PG biosynthesis (Table II). All derivatives having no substituents on either aromatic ring (**6**, **7** and **9**) strongly inhibited PG-ase. On the other hand, *O*-glucosides [rhaponticin (**12**) and piceantannol glucoside (**13**)], and a cyclic derivative [monomethylphyllodulcin (**14**)] did not inhibit PG-ase. It has been noted that potent phenolic inhibitors of PG-ase commonly possess a hydrophobic group in addition to a phenolic hydroxyl group.[17] The results of this study are consistent with the proposed structure–activity relationships.

Since *p*-coumaric acid (**4**) and *p*-hydroxybenzoic acid (**5**) were obtained from the fraction exhibiting an inhibitory effect against TX-ase, benzoic and cinnamic acid derivatives were tested for TX-ase inhibition. Cinnamic acid derivatives including *p*-coumaric and caffeic acids (**4**, **15**) significantly inhibited TX-ase at a concentration of 2 mM, whereas corresponding

TABLE III. Inhibitory Effects on Rabbit Platelet Aggregation Induced by ADP, Arachidonic Acid and Collagen

Test material	ADP (10 μM)	Arachidonic acid (128 μM)	Collagen (20 μg/ml)	IC_{50} values against PG-ase[a] (μM)
p-Coumaroyltyramine (**1**)	>1000	150	350	280
Lunularic acid (**3**)	>1000	320	390	45
Rhapontigenin (**10**)	>1000	20	200	17
p-Coumaric acid (**4**)	>1000	1000	1000	5.6 mM (TX-ase[b])
Caffeic acid (**15**)	>1000	280	>1000	40% inh. at 2 mM against TX-ase

Each figure indicates the concentration (μM) of test material which gave the same degree of inhibitory effect as the positive control. Positive control: ADP (10 μM)+adenosine (3.75 μM); arachidonic acid (128 μM)+aspirin (5.6 μM); collagen (20 μg/ml)+aspirin (111 μM). *a*) Prostaglandin synthetase. *b*) Thromboxane synthetase.

benzoic and dihydrocinnamic acid had no effects.

Next, we assayed three of the isolated compounds, [*N*-(*p*-*trans*-coumaroyl)tyramine (**1**), lunularic acid (**3**) and *p*-coumaric acid (**4**)] and two related compounds, [rhapontigenin (**10**) and caffeic acid (**15**)] for inhibitory effects on rabbit platelet aggregation induced by adenosine diphosphate (ADP), arachidonic acid and collagen. Rhapontigenin (**10**) showed the most potent inhibitory effect on PG-ase and strongly inhibited platelet aggregation induced by arachidonic acid and collagen (Table III). *p*-Coumaric acid (**4**) and caffeic acid (**15**) also showed significant inhibitory effects. In a previous paper we reported that hydroxycinnamic acids activated PG biosynthesis. Although the inhibitory effects of hydroxycinnamic acids are not strong, their inhibition of platelet aggregation is of interest from the mechanistic point of view.[12]

Excessive platelet aggregation is recognized to be a contributing factor to thrombosis and arteriosclerosis. Therefore the above compounds, isolated by following the inhibitory effect against PG or TX biosynthesis, probably play an important role in the therapeutic effects of *A. chinense*.

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Inhibitors of Prostaglandin Biosynthesis from *Mucuna birdwoodiana*

YUKIHIRO GODA, MASAAKI SHIBUYA and USHIO SANKAWA*

Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan

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Four phenolic compounds were isolated from the stalks of *Mucuna birdwoodiana* TUTCHER, a Chinese medicinal drug (Japanese name "鶏血藤") used to treat so-called stagnant blood. Of the four compounds 2,6-dimethoxyphenol and *N*-(*trans*-feruloyl)tyramine inhibited prostaglandin biosynthesis, and the former showed a potent inhibitory effect on *in vitro* platelet aggregation.

Keywords——*Mucuna birdwoodiana*; Leguminosae; stagnant blood; 2,6-dimethoxyphenol; phenolic amide; *N*-(*trans*-feruloyl)tyramine; prostaglandin biosynthesis inhibitor; platelet aggregation inhibitor

In the course of our studies to find inhibitors of prostaglandin (PG) biosynthesis from medicinal plants used as crude drugs in traditional medicine, extracts of several plants used to treat so-called stagnant blood were found to inhibit PG biosynthesis.[1] It has been recognized that PGs have very important roles in the regulation of blood flow. Therefore, the isolated inhibitors may be active principles of the medicinal plants. Previously, we reported the isolation of inhibitors of PG biosynthesis from *Dalbergia odorifera* (Japanese name "降真香")[2] and *Allium chinense* (Japanese name "薤白"),[3] which have also been used to treat stagnant blood. In this paper, we report on the inhibitors of PG biosynthesis isolated from the stalks of *Mucuna birdwoodiana* TUTCHER[4] (Japanese name "鶏血藤"). Its hot aqueous extract inhibited the PG biosynthesizing enzyme system (prostaglandin synthetase (PG-ase)) by 84% at a concentration of 0.75 mg/ml. This Chinese medicinal drug has been recognized as being effective to promote blood circulation or to relieve stasis, and is widely used to treat pain or numbness of the wrist, knees or other joints and irregular menstruation in present Chinese medicine.[5] However, no study on the biologically active components of this drug has been reported.

Experimental

All melting points were determined on a Yanagimoto melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a JASCO DS-701G spectrometer. Mass spectra (MS) were taken on a JEOL JMS-DX-300 spectrometer equipped with a JEOL JMA-200 computer. Proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra were measured on a JEOL FX-100 spectrometer with tetramethylsilane as an internal standard.

Assay of PG-ase——The previously reported radioisotope method was used.[1a)]

Isolation of Active Principles from *Mucuna birdwoodiana*——Dried stalks (4.7 kg) purchased at a Hong-Kong market were successively extracted with hexane, chloroform and methanol. The inhibition of PG biosynthesis by these extracts at a concentration of 0.5 mg/ml amounted to 93, 97 and 68%, respectively. The methanol extract was further partitioned with chloroform and 80% aqueous methanol, and the chloroform-soluble portion was combined with the chloroform extract. The combined chloroform extract (15.9 g) was applied to an oxalate-treated silica gel column and eluted with chloroform and methanol to give three active fractions, which were separated and purified by chromatography on silica gel, Sephadex LH-20 and Lobar RP-8, followed by recrystallization, to afford four phenolic compounds.

2,6-Dimethoxyphenol (1) was obtained as a colorless oil (12.9 mg) and gave the following spectral data. MS *m/z* (rel. int. %): 154 (M^+, 100), 137 (88), 109 (23), 81 (11), 63 (17). ^{1}H-NMR ($CDCl_3$) δ: 3.89 (6H, s), 6.39—6.90 (3H, AB_2 type coupling). ^{13}C-NMR ($CDCl_3$) δ: 56.1 (q, OMe), 104.7 (d, C-3 and C-5), 118.8 (d, C-4), 134.6 (s, C-1), 147.0 (s, C-

2 and C-6). Compound **1** was finally identified by direct comparison with an authentic sample purchased from Aldrich Co. (Milwaukee).

Syringic acid (**2**) and vanillic acid (**3**) were obtained as colorless powders (5.0 and 2.2 mg, respectively) and gave the following spectral data. **2**: MS *m/z* (rel. int. %): 198 (M^+, 100), 183 (32), 127 (17), 109 (14), 69 (19), 55 (16). ^{1}H-NMR (methanol-d_4) δ: 3.87 (6H, s), 7.33 (2H, s). **3**: MS *m/z* (rel. int. %): 168 (M^+, 100), 153 (67), 125 (19), 106 (17), 97 (25), 78 (34), 52 (17), 51 (20). ^{1}H-NMR (acetone-d_6) δ: 6.93 (1H, d, J=8.4 Hz), 7.57 (1H, d, J=1.0 Hz), 7.60 (1H, dd, J=8.4, 1.0 Hz). Compounds **2** and **3** were finally identified by direct comparison with authentic samples purchased from Tokyo-Kasei Co. (Tokyo) and Aldrich Co. (Milwaukee), respectively.

N-(*trans*-Feruloyl)tyramine (**4**) was obtained as colorless plates (mp 142—143 °C) from $CHCl_3$ and acetone and gave the following spectral data. IR ν_{max}^{KBr} cm^{-1}: 1650, 1580, 1510, 1445, 1360, 1275, 1120, 1030, 980. High-resolution MS: $C_{18}H_{19}NO_4$ (M^+ *m/z*: 313.1313, Calcd: 313.1313). MS *m/z* (rel. int. %): 313 (M^+, 16), 194 (33), 193 (55), 192 (52), 177 (100), 145 (24), 120 (31), 85 (16), 83 (25). ^{1}H-NMR (acetone-d_6) δ: 2.74 (2H, m), 3.49 (2H, m), 6.48 (1H, d, J=15.5 Hz), 6.75 (2H, d, J=8.6 Hz), 6.82 (1H, d, J=7.0 Hz), 7.02 (1H, d, J=2.5 Hz), 7.09 (2H, d, J=8.6 Hz), 7.14 (1H, dd, J=2.5, 7.0 Hz), 7.44 (1H, d, J=15.5 Hz), 8.03 (2H, s), 8.13 (1H, m). Compound **4** was finally identified by direct comparison with an authentic sample kindly supplied by Prof. S. Sakamura.

Assay of Platelet Aggregation——The assay was carried out as described in a previous paper.[3]

Results and Discussion

The stalks of *M. birdwoodiana* were successively extracted with hexane, chloroform and methanol. The hexane extract was found to contain fatty acids. Since the chloroform extract inhibited PG-ase by more than 90% at a concentration of 0.5 mg/ml, the chloroform-soluble portion of the methanol extract was combined with the chloroform extract and the inhibitory principles were separated from the combined chloroform extract.

The combined chloroform extract was separated first by oxalate-impregnated silica gel column chromatography to give three active fractions not containing fatty acids. The less polar fraction than fatty acids was further separated and purified with repeated column chromatography, guided by testing of the inhibitory effect, to give 2,6-dimethoxyphenol (**1**) as an active principle. On the other hand, the inhibitory effect of the more polar fraction than fatty acids was widely dispersed in the course of separation. Finally, we obtained syringic acid (**2**) and vanillic acid (**3**) from active fractions derived from the more polar fraction, but they did not inhibit PG-ase. The most polar fractions among the three active fractions was also separated by repeated column chromatography and purified by recrystallization to afford *N*-(*trans*-feruloyl)tyramine (**4**), as an active principle.

The 50% inhibitory concentration (IC_{50}) values of **1** and **4** were 12 and 210 μM, respectively. The process of PG biosynthesis as used in our studies involves three different reactions, cyclooxygenase, hydroperoxidase and PGE_2 synthetase. Ohki *et al.* showed that the hydroperoxidase reaction was stimulated by a variety of compounds, such as tryptophan, epinephrine, phenol and hydroquinone (tryptophan-like cofactors).[6] Furthermore, Baumann *et al.*[7] and Tseng *et al.*[8] reported that phenolic acids such as vanillic acid (**3**) and coumaric

OH, MeO, OMe — **1**; COOH, MeO, OMe, OH — **2**; COOH, MeO, OH — **3**

O, MeO, HO, C, N, H, OH — **4**

acids also acted as tryptophan-like cofactors. The previous observations and the results obtained in this study indicate that the Chinese medicinal drug "鶏血藤" contains inhibitors of PG biosynthesis (**1** and **4**) together with activators (**2** and **3**). *N*-(*trans*-Feruloyl)tyramine (**4**) has been isolated from various plants such as *Solanum melongena*,[9] *Capsicum annuum*,[10] *Tinospora tuberculata*[11] and *Ipomoea aquatica*.[8] Recently Okuyama *et al.* isolated **4** from *Allium chinense* as an inhibitor of human platelet aggregation.[12] Structure–activity relationships for the inhibition of PG biosynthesis by *N*-cinnamoyl-β-phenethylamine derivatives, including **4**, have been reported.[7]

Next, we assayed **1** for inhibitory effect on rabbit platelet aggregation. Adenosine (3.75 μM) and aspirin (11 and 111 μM) were used as positive controls for inhibition of platelet aggregation induced by adenosine diphosphate (ADP), arachidonic acid and collagen, respectively. The concentrations of **1** which gave the same degree of inhibitory effect as the positive controls were 0.45 μM in arachidonic acid-induced platelet aggregation and 32 μM in collagen-induced platelet aggregation. However, **1** did not show any effect on ADP-induced platelet aggregation even at a concentration of 1 mM. Among natural compounds isolated hitherto in our studies, **1** is the most potent inhibitor of platelet aggregation.

One of the contributing factors to blood stasis seems to be excessive platelet aggregation. Therefore, the PG biosynthesis inhibitors isolated from *M. birdwoodiana* may well be active principles in the therapeutic effect of the crude drug.

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Cyclobutyl Cation Rearrangements of 6-Protoilluden-8α-ol, 7-Protoilluden-6-ol and Related Compounds

NAOKO MORISAKI,*,[a] JUN FURUKAWA,[a] HISAYOSHI KOBAYASHI,[a]
SHIGEO IWASAKI,[a] SHIGEO NOZOE[b]
and SHIGENOBU OKUDA[a]

Institute of Applied Microbiology, The University of Tokyo,[a] Yayoi, Bunkyo-ku, Tokyo 113, Japan and Pharmaceutical Institute, Tohoku University,[b] Aobayama, Sendai 980, Japan

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6-Protoilluden-8α-ol (**15**) was formolyzed and then reduced with $LiAlH_4$ to give 7-protoilluden-6-ol (**16**) and its stereoisomer (**17**). 3-*epi*-6-Protoilluden-8α-ol (**19**) also gave the same products under the same conditions. These and other related reactions imply that the 7-protoilludene C-6 cation (**10**) is an important intermediate in the biosynthesis of illudane (**11**), marasmane (**12**) and illudalane (**13**) type sesquiterpenes.

Keywords——sesquiterpene; 6-protoilluden-8α-ol; 7-protoilluden-6-ol; biomimetic reaction; formolysis; cyclobutyl cation rearrangement; Basidiomycete; 7-protoilludene C-6 cation

Introduction

A group of sesquiterpenes with unique carbon skeletons, such as protoilludanes (*e.g.*, illudol (**1**)[1]), illudanes (*e.g.*, illudin-M (**2**)[1a,2] and -S (**3**)[1a,2,3]), marasmanes (*e.g.*, marasmic acid (**4**)[4]) and illudalanes (*e.g.*, fomajorin D (**5**)[5]), are produced by Basidiomycetes. These compounds[6] were postulated to be biosynthesized through 6-protoilludene (**7**)[7,8] as illustrated in Chart 1. In our previous work, the hydrocarbon **7** was shown to be a real biosynthetic precursor of illudin-M (**2**) and -S (**3**) by feeding *dl*-[13,13,13-$^{2}H_3$]-6-protoilludene to the illudin-producing fungus *Omphalotus olearius* (ATCC 11719).[9] The biosynthetic route from the hydrocarbon **7** to illudanes such as **2** and **3** as well as marasmanes and illudalanes may be as follows. The allylic carbonium ion **9** is generated from the hydrocarbon **7** by the introduction of a functional group such as a hydroxy group at C-8 (**8**) followed by solvolytic removal of the group. The cation **9** would easily isomerize to the 7-protoilludene C-6 cation **10**, and then this would undergo the cyclobutyl cation rearrangement to form illudane (**11**), marasmane (**12**) and illudalane (**13**) carbon skeletons. The aim of this study was to clarify the intermediary role of the 7-protoilludene C-6 cation **10** in the biosynthesis of **11**, **12** and **13**.

1 | **2** : R=H, **3** : R=OH | **4** | **5**

Fig. 1

farnesyl-PP → humulene or humulenium ion → 6 → 7 6-protoilludene

→ 8 → 9

11 illudane cation → illudanes ← 10 7-protoilludene cation → 13 illudalane cation → illudalanes

10 → 12 marasmane cation → marasmanes

Chart 1

This cation (**10**) was chemically generated from 6-protoilluden-8α-ol (**15**),[10] an equivalent of **8** (Chart 2). It was shown that cations **11** and **20** were formed by the cyclobutyl cation rearrangement of **10** and that the cations **10**, **11** and **20** were in equilibrium. The same equilibrium was attained by the solvolysis of 3-*epi*-6-protoilluden-8α-ol (**19**). The formation of the cation **13** from the cation **10** was also demonstrated (Chart 6). In connection with the results described above, the cyclobutyl cation rearrangement of related cations, such as **29**, **32**, **35**, **39** and **40** (Chart 5) and **47** (Chart 7), is discussed.[11,12]

Results

The allyl alcohol (**15**),[10] which was obtained by $LiAlH_4$(LAH) reduction of 6-protoilluden-8-one (**14**),[10] was treated with HCOOH(30%)-tetrahydrofuran (THF) (70%) overnight at room temperature, and the resulting formates were reduced with LAH. Two allyl alcohols **16** and **17** were obtained in a ratio of 4:5 (Chart 2). Their chromatographic properties (gas chromatography (GC) and thin-layer chromatography (TLC)) and proton nuclear magnetic resonance (^{1}H-NMR) spectra were similar. The ^{1}H-NMR spectrum of **16** showed four methyl signals at δ 0.96, 1.00, 1.21 and 1.74 and an olefinic proton signal at δ 5.27, and that of **17** showed four methyl signals at δ 0.97, 1.04, 1.15 and 1.80 and an olefinic proton signal at δ 5.66. The mass spectra (MS) of **16** and **17** were also very similar and showed the molecular ion peak (M^+) at *m/z* 220 and fragment ions at 205 ($M^+ - CH_3$), 192 ($M^+ - CH_2{=}CH_2$) and 177 ($M^+ - CH_3 - CH_2{=}CH_2$). Intense MS peaks at *m/z* 192 and 177 indicated the presence of a cyclobutanol moiety. The spectral and chromatographic data of **16**

Chart 2

coincided with those of 7-protoilluden-6-ol isolated from *Fomitopsis insularis*[7] and, therefore, **16** was determined to be *dl*-7-protoilluden-6-ol. From the almost identical MS and the similarity of ^{1}H-NMR signals between **16** and **17**, compound **17** was assumed to be a stereoisomer of **16**. The ring junction at C-3 and C-6 of **17** was supposed to be *cis* because the *cis* junction would be less strained than the *trans* junction. Moreover, the *cis* junction would be expected when formate ion attacks the cation **20** from the less hindered side. This was supported by the reported observation[13] that both *cis*- and *trans*-bicyclo[4.2.0]octan-1-ol esters gave only *cis*-bicyclo[4.2.0]octan-1-ol under solvolytic conditions. Thus, the *cis–syn–cis* structure would be the most plausible one for the major product **17**.

The structure assignment of **17** was supported by the following experiments. The *cis–syn* allyl alcohol **19**, which was obtained by LAH reduction of 3-*epi*-6-protoilluden-8-one (**18**),[14] was treated with HCOOH(30%)–THF(70%) and then reduced with LAH. The product was again a mixture of **16** and **17** in the same ratio (4:5) as that obtained from the *cis–anti* allyl alcohol **15**. The transformation of both **15** and **19** to the mixture of **16** and **17** can be well explained by the formation of cations **10** and **20** from **15** and **19**, respectively. The cations **10** and **20** could attain equilibrium through the illudane cation **11**. Attack of formate ion on the cations **10** and **20** at C-6 and LAH reduction of the formates would lead to **16** and **17** in a ratio of 4:5. Thus, compound **17** was assigned as the *cis–syn–cis* stereoisomer of **16**.[15] A similar epimerization between protoilludane type compounds **14** and **18** through an illudane C-3 cation **22** has also been observed under acidic conditions (CF_3COOH–$CHCl_3$ or $BF_3 \cdot Et_2O$–benzene) (Chart 3).[14]

21 22 23

Chart 3

24 26 27 25

R=3,5-dinitrobenzoyl

Chart 4

In the simpler model of bicyclo[4.2.0]octyl 1-derivatives, Wiberg *et al.* showed that solvolysis of *cis*- and *trans*-bicyclo[4.2.0]octyl 1-(3,5-dinitrobenzoates) (**24** and **25**) gave spiro[2.5]octan-4-ol (**26**) and *cis*-bicyclo[4.2.0]octan-1-ol (**27**) in a ratio of 2.5—2.6 : 1 (Chart 4).[13] However, in the case of the 7-protoilludene system, all the products were derived from cyclobutyl cations **10**, **20**, **21** and **23**, and no products from cyclopropylcarbinyl cations **11** and **22**, which correspond to **26**, could be identified.[16] A possible reason may be that the C-7(8) double bond of **10**, **20**, **21** and **23** stabilizes the carbonium cation at C-6. In addition, in the

28 → ($SOCl_2$) 29 → 30

31 → (TsCl pyridine) 32 → 33

34 → (H^+) 35 → 36

37 : 8α-OAc
38 : 8β-OAc
→ (MsCl)
39 : 8α-OAc
40 : 8β-OAc
→
41 : 8α-OAc
42 : 8β-OAc

Chart 5

case of formolysis of **15** and **19**, formate ion might not be able to attack C-3 of **11** because of the steric hindrance around this center. In the course of our chemical studies on protoilludanes, we obtained several illudane type compounds (**30**,[10] **33**,[8,17,18] **36**,[8] **41** and **42**[10,19]) from protoilludane C-6 cations (**29**, **32**, **35**, **39** and **40**), and all of them possessed a double bond at C-2(3) (Chart 5).

Since the formation of the cation **10** from the allyl alcohol **16** in acidic media was also expected, **16** was treated with formic acid. The allyl alcohol **16** was recovered unchanged after treatment with HCOOH(30%)–THF(70%) for 14 h at room temperature. Under more drastic conditions (99% HCOOH, 1 h at room temperature), the illudalane type compound **45** was obtained from **16** as a sole product after LAH reduction (Chart 6). Likewise the allyl alcohols **15**, **17** and **19** were also converted to **45** under the same conditions. These reactions could be explained by the formation of the illudalane cation **13** from intermediary cations **10**, **11** or **20**, followed by dehydrogenation to give cation **43**. The alcohol **45** has, indeed, been isolated from mycelium of *F. insularis* along with the alcohol **16**.[7]

Another illudalane type compound **48** was obtained from the epoxyketone **46**[20] by treatment with CF_3COOH in benzene. The structure of **48** was determined by ^{1}H-NMR and MS. A possible pathway leading to **48** is shown in Chart 7. The methyl ether **49** was obtained by Anchel and Morris.[2b]

15,16,17,19 —HCOOH→ [10 ⇄ 11 ⇄ 20] → [13] —(−2H)→ [43] → RO– 44 : R=HCO; 45 : R=H

Chart 6

46 —CF₃COOH→ [47] → 48 : R=CF₃CO; (49 : R=CH₃)

Chart 7

Discussion

Although no illudane type compound could be isolated, the results of solvolysis of the allyl alcohols **15** and **19** indicated the intervention of the illudane cation **11** (Chart 2). In the reaction of the allyl alcohols **15** and **16** to the illudalane **44**, rearrangement of the cation **10** into the illudalane cation **13** was also found (Chart 6). These facts suggest that, in enzymatic reactions also, 6-protoilludene (**7**) would undergo the cyclobutyl cation rearrangement to give illudane (**11**) and illudalane (**13**) cations through the cation **10**.

Under our solvolytic conditions [HCOOH(30%)–THF(70%) overnight at room temperature], when a double bond is present at C-7(8) (*e.g.*, in **10**), the protoilludane C-6 cation would be stabilized, and indeed the isolated products were *cis–anti–cis* protoilludane (**16**) and stereoisomeric *cis–syn–cis* protoilludane (**17**) type compounds. On the other hand, protoilludane C-6 cations without a double bond at C-7(8) gave illudane type compounds (**30**, **33**, **36**, **41** and **42**).

The rearrangements of cyclobutanones through cyclopropylcarbinyl cations under acidic conditions were studied by Katz and Dessan,[21] Erman *et al.*[22] and Ceccherelli *et al.*[23] They showed that two carbons on the cyclobutane ring were interchangeable during the rearrangement. The allyl alcohols **15** and **19**, and the enones **14** and **18** are vinylogs of cyclobutanol and cyclobutanone systems. Therefore, C-4 and C-5 of **14**, **15**, **18** and **19** might be transposed during the rearrangement (Chart 8 for **10** from **15** and a similar precess for **14**, **18** and **19**). However, this could not be clarified in this work. Concerning this point, Hanson *et al.*[24] concluded on the basis of their biosynthetic study that if protoilludane were the precursor of illudin-M (**2**) and -S (**3**), the C3–C4 bond of the protoilludane would migrate from the α-side to produce **2** and **3**.

Although conversion of the cation **10** to the marasmane cation **12** was expected, we could not obtain marasmanes as stable compounds. In order to stabilize the marasmane C-5 primary cation, some modification of the protoilludane C-6 cation, *e.g.*, **50** (R = electron-donating group), may be necessary (Chart 9). Expecting the formation of an illudane type compound, Matsumoto *et al.* formolyzed protoilludane C-7 cation (**6**) equivalents, but illudane type compounds were not obtained.[25]

Thus, our results indicate that the 7-protoilludene C-6 cation (**10**), formed from 6-protoilluden-8α-ol (**15**),[26] is the true intermediate in the rearrangement to illudanes (**11**) and illudalanes (**13**). The appropriately modified cation **10** may give marasmanes (**12**). In biological reactions, prior to the rearrangement of the cation **10**, some modification (*e.g.*, oxidation) might also occur and the introduced functionality could influence the course of the rearrangement.

10 ⇄ **11** ⇄ **10**

Chart 8

50 **51**

Chart 9

Experimental

^{1}H-NMR spectra were measured on a JEOL GX-400 FT-NMR spectrometer (400 MHz for ^{1}H) and chemical shifts were recorded in δ units relative to internal tetramethylsilane (TMS) ($\delta = 0$) in $CDCl_3$. Infrared (IR) spectra were measured on a JASCO DS-301 and MS on a Shimadzu-LKB 9000 at 70 eV.

7-Protoilluden-6-ol (16) and *cis–syn–cis* 7-Protoilluden-6-ol (17) from 6-Protoilluden-8α-ol (15)——The allyl alcohol **15**[10] (10 mg) was dissolved in 0.1 ml of HCOOH(30%)–THF(70%) and the solution was allowed to stand overnight at room temperature. After removal of the reagent and solvent with an N_2 stream and then with a rotary pump, the residue was dissolved in Et_2O and reduced with LAH. The products were analyzed by GC. Total yield was more than 95% and the ratio of **16** and **17** was 4 : 5. When the allyl alcohol **15** was treated with HCOOH(10%)–THF(90%), the reaction rate was very slow and **15** was recovered after 18 h at room temperature. Treatment of **15** with 99% HCOOH at room temperature gave only illudalane **44**, as described below. Compounds **16** and **17** were separated by TLC. The *Rf* values of **15**, **16** and **17** (precoated silica gel plate, benzene : acetone = 9 : 1) and relative

retention times (Rt_R) of **15** and **17** with respect to **16** (1.5% OV-17, 1.5 m, 150 °C, **16**, 3.0 min) were as follows.

	15	**16**	**17**	**19**
Rf	0.32	0.37	0.30	0.33
Rt_R	1.50	1.00	1.33	1.39

16: ^{1}H-NMR ($CDCl_3$) δ: 0.96 (3H, s, CH_3), 1.00 (3H, s, CH_3), 1.21 (3H, s, CH_3), 1.74 (3H, s, CH_3–C=C), 2.64 (1H, br s, H-9), 5.27 (1H, br s, H-8). MS *m/z* (110—220): 220 (M^+, 2.5%), 205 ($M^+ - 15$, 2.5%), 192 ($M^+ - 28$, 65%), 177 ($M^+ - 28 - 15$, 15%), 136 (55%), 135 (100%), 124 (88%), 121 (25%). **17**: ^{1}H-NMR ($CDCl_3$) δ: 0.97 (3H, s, CH_3) 1.04 (3H, s, CH_3), 1.15 (3H, s, CH_3), 1.8 (3H, s, CH_3–C=C), 2.66 (1H, br s, H-9), 5.66 (1H, s, H-8). MS *m/z* (110—220): 220 (M^+, 2%), 205 ($M^+ - 15$, 2%), 192 ($M^+ - 28$, 50%), 177 ($M^+ - 28 - 15$, 13%), 136 (48%), 135 (100%), 124 (88%), 121 (23%).

Reduction of 3-*epi*-6-Protoilluden-8-one (18) to 3-*epi*-6-Protoilluden-8α-ol (19)——The enone **18**[14] (74 mg) was reduced with LAH (13 mg)/Et_2O (1 ml). The allyl alcohol (**19**) was purified by silica gel column chromatography (56 mg, 75% yield). The *Rf* and Rt_R values of the allyl alcohol **19** were shown above. ^{1}H-NMR ($CDCl_3$) δ: 0.93 (3H, s, CH_3), 1.04 (3H, s, CH_3), 1.16 (3H, s, CH_3), 1.62 (3H, br s, CH_3–C=C), 2.42 (1H, br s, H-5), 2.65 (1H, br s, H-5), 4.43 (1H, br s, H-8). The stereochemistry of the hydroxy group of **19** was assigned as α from the negligibly small coupling constant between the protons on C-8 and C-9, which would not be expected for the β-isomer.

Conversion of 19 to the Allyl Alcohols 16 and 17——The allyl alcohol **19** (10 mg) was treated with 0.1 ml of HCOOH(30%)-THF(70%) and then with LAH as described for **15**. The product was again a mixture of **16** and **17** in the same yield and ratio as those from **15**.

Conversion of the Allyl Alcohols 15, 16, 17 and 19 to 5-(2-Hydroxyethyl)-2,2,4,6-tetramethylindan (45)——Each allyl alcohol (5 mg) was treated with 99% HCOOH (0.05 ml) for 1 h at room temperature. After HCOOH was removed with an N_2 stream, the product was reduced with LAH in Et_2O to give the alcohol **45**, quantitatively. The ^{1}H-NMR data, *Rf* on TLC and Rt_R on GC were in agreement with those reported for natural **45**.[7]

6-Trifluoroacetoxyethyl-2,2,5,7-tetramethylindan-4-ol (48) from 9-*epi*-6,7-Epoxyprotoilludan-8-one (46)——A solution of **46** (10 mg) in benzene (0.3 ml) was combined with a solution of CF_3COOH (80 mg) in benzene (0.3 ml) and the mixture was allowed to stand overnight at room temperature. The reagent and the solvent were removed with an N_2 stream. Compound **48** was purified by silica gel column chromatography (5 mg, 35% yield). IR ($CHCl_3$): 1782 cm^{-1} (CF_3COO). MS *m/z*: 330 (M^+), 315 ($M^+ - 15$), 216 ($M^+ - CF_3COOH$), 203 ($M^+ - CF_3COOCH_2$). ^{1}H-NMR ($CDCl_3$) δ: 1.18 (6H, s), 2.16 (3H, s), 2.23 (3H, s), 2.64 (2H, s), 2.69 (2H, s), 3.31 (2H, t, J=8 Hz), 4.38 (2H, t, J=8 Hz).

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52 **53** **54**

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20) When the crystalline *trans–anti* enone **52** was left to stand in air, it slowly changed to an oil, which contained **46** (after 2 weeks, **52**:**46** = 1:1). MS showed that compound **46** had one more oxygen atom than **52**. The structure of **46** was determined from the spectral data and its formation from **52** with H_2O_2/NaOH–MeOH. Though **46** was obtained as a single isomer, the configuration has not been established. The air oxidation of **52** to **46** was accelerated by heat. **46**: MS *m/z*: 234 (M^+), 219 (M^+-15), 201, 191 ($M^+-15-28$). ^{1}H-NMR ($CDCl_3$) δ: 0.98 (3H, s, CH_3), 1.02 (3H, s, CH_3), 1.25 (3H, s, CH_3), 1.35 (3H, s, CH_3), 2.6 (1H, H-2), 2.8 (1H, H-9). IR ($CHCl_3$): 1712 cm^{-1} (C=O): N. Morisaki, J. Furukawa, H. Kobayashi, S. Iwasaki, A. Itai, S. Nozoe and S. Okuda, *Chem. Pharm. Bull.*, **33**, 2783 (1985).
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Condensed Pyridazines. VI.[1] Reaction of 7-(Methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine with Carbanions and Enamines

ETSUO ŌISHI,* AKIYA YAMADA, EISAKU HAYASHI, and TAKEO HIGASHINO

Shizuoka College of Pharmacy, 2-2-1 Oshika, Shizuoka 422, Japan

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The reaction of 7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**1**) with active methylene compounds or ketones (**3a**—**g**) in the presence of sodium hydride gave the corresponding 7-substituted 1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazines (**4a**—**g**). A [4+2] cycloaddition occurred in the reaction with enamines or ynamines (**9a**—**d**) to give the corresponding arenotriazoles (**10a**—**d**). Similarly, the reaction of 7-chloro-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**2**) with **3** and **9** resulted in the formation of **4** and arenotriazoles (**12**), respectively.

Keywords——triazolopyridazine; carbanion; substitution; enamine; cycloaddition

In the previous paper,[1] we reported that 7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**1**) reacts with nucleophiles in three ways, *i.e.*, substitution, addition, and ring fission of the pyridazine portion of **1**, depending on the nature of the reagent. For example, the methylsulfonyl substituent of **1** was replaced by sodium methoxide, giving the 7-methoxytriazolopyridazine (A). Alkylmagnesium halides added to the 4,5-double bond of **1**,

Chart 1

giving the corresponding 4-alkylated dihydrotriazolopyridazines (B). The reaction of **1** with primary amines gave the ring fission products, such as the 4,5-disubstituted triazoles (C). As an extension of the above work, we examined the reactions of **1** with carbanions, enamines and ynamines, and found that cycloaddition took place in the reaction with enamines or ynamines. In the present paper, we describe the above reactions in detail, and compare the chemical properties of **1** with those of 7-chloro-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**2**).

The Reaction of 1 and 2 with Active Methylene Compounds or Ketones (3a—g) in the Presence of Sodium Hydride

Sources of carbanions used in this reaction were as follows; ethyl cyanoacetate (**3a**), ethyl acetoacetate (**3b**), malononitrile (**3c**), diethyl malonate (**3d**), phenylacetonitrile (**3e**), acetophenone (**3f**), and acetone (**3g**).

In boiling benzene or toluene, **1** did not react with **3a** in the presence of sodium hydride, resulting in complete recovery of **1**. However, when dioxane instead of benzene or toluene was used as a solvent, the reaction gave ethyl α-cyano-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]-pyridazine-7-acetate (**4a**) in good yield. Under the same conditions, the reactions of **1** with **3b—d** also proceeded smoothly to give the corresponding 7-substituted triazolopyridazines (**4b—d**). However, in the reaction of **1** with **3e**, the substitution did not take place, and 1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazin-7(6*H*)-one (**5**) instead of the expected α,1-diphenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine-7-acetonitrile (**4e**) was isolated. Although the yields were less than 10%, the reaction of **1** with ketones **3f** and **3g** similarly gave the corresponding 7-substituted triazolopyridazines **4f** and **4g**, respectively, together with **5**. The results are summarized in Chart 2.

1 —(R-CH$_2$-R′ **3**, NaH in dioxane)→ **4** (Ph, CH(R)(R′)) + **5** (Ph, O, NH)

3	R	R′	4	yield (%)	5 yield (%)
3a	CN	COOEt	4a	88	—
3b	Ac	COOEt	4b	29	—
3c	CN	CN	4c	38	—
3d	COOEt	COOEt	4d	73	—
3e	Ph	CN	4e	—	59
3f	H	COPh	4f	7	30
3g	H	Ac	4g	2	trace

Chart 2

The chlorine atom at the 7-position in **2** was similarly replaced by carbanions, generated from **3**, in boiling benzene, to give the corresponding 7-substituted triazolopyridazines (**4a**, **b**, **d—f**) as shown in Chart 3. However, **2** did not react with **3c** in boiling benzene, but in dimethylformamide (DMF), the substitution smoothly proceeded to give 1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine-7-malononitrile (**4c**) in good yield. Moreover, in benzene the reaction of **2** with **3g** did not give any reaction product, but that in tetrahydrofuran (THF) resulted in the formation of 1-(1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazin-7-yl)-2-propanone (**4g**) together with **5**.

The structures of **4a—g** and **5** were suggested by their elemental analyses and mass spectral (MS) data (Table I), and confirmed by their proton nuclear magnetic resonance (^{1}H-NMR) and infrared (IR) absorption spectral data (Table II).

Moreover, diethyl 1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine-7-malonate (**4d**) was con-

Ph Cl

2 —(3, NaH in benzene)→ 4 + 5

3	4	yield (%)	5 yield (%)
3a	4a	91	—
3b	4b	55	—
3c	4c[a]	90	—
3d	4d	61	—
3e	4e	19	—
3f	4f	23	—
3g	4g[b]	30	25

a) Reaction in DMF. *b*) Reaction in THF.

Chart 3

Table I. Melting Points, Elemental Analyses, and MS Data for **4**, **5**, **6**, and **7**

Compd.	mp (°C)	MS *m/z* M^+	Formula	Analysis (%) Calcd (Found)		
				C	H	N
4a	229—231[a,e]	308	$C_{15}H_{12}N_6O_2$	58.44	3.92	27.26
				(58.18	3.85	27.08)
4b	159—160[b,f]	325	$C_{16}H_{15}N_5O_3$	59.07	4.65	21.53
				(59.05	4.58	22.29)
4c	270—272[a,g] (dec.)	261	$C_{13}H_7N_7$	59.76	2.70	37.53
				(59.36	2.67	37.17)
4d	79—80[c]	355	$C_{17}H_{17}N_5O_4$	57.46	4.82	19.71
				(57.99	5.12	18.86)
4e	179—180[b,f]	312	$C_{18}H_{12}N_6$	69.22	3.87	26.91
				(69.42	3.91	26.46)
4f	214—216[c,f]		$C_{18}H_{13}N_5O$	68.56	4.16	22.21
				(68.28	4.07	22.25)
4g	180—183[c,f]		$C_{13}H_{11}N_5O$	61.65	4.38	27.66
				(61.39	4.32	27.32)
5	240—242[d,f]		$C_{10}H_7N_5O$	56.33	3.31	32.85
				(56.13	3.33	32.83)
6	180[d,e]	211	$C_{11}H_9N_5$	62.55	4.29	33.16
				(62.74	4.27	33.18)
7	190—193[d,e] (dec.)		$C_{17}H_{13}N_5O_2S$	58.11	3.73	19.93
				(58.02	3.69	19.70)

a) Yellow powder. *b*) Yellow prisms. *c*) Yellow needles. *d*) Colorless needles. *e*) Recrystallization from benzene–petr. ether. *f*) Recrystallization from MeOH. *g*) Recrystallization from dimethyl slufoxide.

verted to 7-methyl-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**6**) by alkaline hydrolysis, and the triazolopyridazinone 5 was synthesized by the reaction of **2** with sodium *p*-toluenesulfinate (**8**) in DMF, together with 1-phenyl-7-(*p*-tolylsulfonyl)-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (7).

The Reaction of 1 and 2 with Enamines and Ynamines

Enamines and ynamines used in this reaction were as follows: 1-methoxy-*N*,*N*-dimethylvinylamine (**9a**), 1-piperidinocyclopentene (**9b**), 1-piperidinocyclohexene (**9c**), and *N*,*N*-diethyl-1-propynylamine (**9d**).

When **1** was refluxed with **9a** in dioxane, 6-(*N*,*N*-dimethylamino)-7-(methylsulfonyl)-1-

Table II. IR and ^{1}H-NMR Spectral Data for 4, 5, 6, and 7

Compd.	IR ν_{max}^{KBr} cm^{-1}	^{1}H-NMR ($CDCl_3$) ppm
4a	2220 (CN) 1665 (CO)	14.73—14.35[a] (1H, brs, enolic H), 8.72 (1H, s, C^4-H), 7.72—7.25 (5H, m, C_6H_5), 4.25 (2H, q,[b] $OC\underline{H}_2CH_3$), 1.32 (3H, t,[b] $OCH_2C\underline{H}_3$)
4b	1660—1640 (CO)	12.96—12.85[a] (1H, brs, enolic H), 9.80 (1H, s, C^4-H), 7.67—7.06 (5H, m, C_6H_5), 4.05—3.10 (2H, m, $OC\underline{H}_2CH_3$), 2.01 (3H, s, $COCH_3$), 0.89 (3H, t,[b] $OCH_2C\underline{H}_3$)
4c[d]	3250—3180 (NH), 2230, 2220 (CN)	9.01 (1H, s, C^4-H), 7.59 (5H, brs, C_6H_5), 5.10—3.90 (1H, brs, enolic H)
4d	1775, 1735 (CO)	9.76 (1H, s, C^4-H), 7.75—7.03 (5H, m, C_6H_5), 4.97 (1H, s, $CH(CO)_2$), 4.06 (4H, q,[c] $2\times(OC\underline{H}_2CH_3)$, 1.15 (6H, t,[c] $2\times(OCH_2C\underline{H}_3)_2$)
4e	2250 (CN)	9.86 (1H, s, C^4-H), 7.75—6.46 (10H, m, C_6H_5), 5.63 (1H, s, NC-CH-Ph)
4f	1630 (CO)	15.30—15.02[a] (1H, brs, enolic H), 8.56 (1H, s, C^4-H), 7.90—7.28 (10H, m, $2\times C_6H_5$), 5.67 (1H, =CH-CO)
4g	1630 (CO)	15.20—14.47[a] (1H, brs, enolic H), 8.47 (1H, s, C^4-H), 7.70—7.36 (5H, m, C_6H_5), 4.91 (1H, s, =CH-CO), 1.93 (3H, s, $COCH_3$)
5[d]	3200—3050 (NH), 1690 (CO)	13.32—13.00[a] (1H, brs, OH or NH), 8.72 (1H, s, C^4-H), 8.02—7.21 (5H, m, C_6H_5)
6		9.68 (1H, s, C^4-H), 7.83—7.15 (5H, m, C_6H_5), 2.61 (3H, s, CH_3)
7	1330, 1165 (SO_2)	9.89 (1H, s, C^4-H), 8.18—7.04 (9H, m, C_6H_5 and C_6H_4), 2.44 (3H, s, $C\underline{H}_3$-C_6H_4)

a) Exchangeable with CD_3OD. *b*) $J=8$ Hz. *c*) $J=7$ Hz. *d*) NMR in $(CD_3)_2SO$.

Chart 4

phenyl-1*H*-benzotriazole (**10a**) was obtained in 9% yield. The enamine **9a**, which is an electron-rich dienophile,[2] adds selectively to C^4/C^7 of the triazolopyridazine nucleus, and there is a strong preference for the nucleophilic carbon atom of **9a** to attack the electron-deficient C^4 of the triazolopyridazine **1**. Loss of nitrogen from the initial [4+2] cycloadduct (D) followed by elimination of methanol gives the benzotriazole **10a** by way of an intermediate (E).

A similar [4+2] cycloaddition was found to proceed in the reaction of **1** with **9b** and **9c**, resulting in the formation of 8-(methylsulfonyl)-1-phenyl-1,5,6,7-tetrahydroindeno[5,6-*d*]-triazole (**10b**) and 9-(methylsulfonyl)-1-phenyl-5,6,7,8-tetrahydro-1*H*-naphtho[2,3-*d*]triazole (**10c**) in 47 and 45% yields, respectively. In the case of the reaction of **1** with **9d**, 6-(*N*,*N*-diethylamino)-5-methyl-7-(methylsulfonyl)-1-phenyl-1*H*-benzotriazole (**10d**) was obtained in 56% yield together with 8-(*N*,*N*-diethylamino)-4,7-dihydro-9-methyl-7-(methylsulfonyl)-1-phenyl-1*H*-4,7-etheno-1,2,3-triazolo[4,5-*d*]pyridazine (**11d**), corresponding to the initial [4+2] cycloadduct D, in 21% yield.

A similar [4+2] cycloaddition was found to occur in the reaction between **2** and **9**. Thus, the reaction of **2** with **9b** gave 8-chloro-1,4a,5,6,7,7a-hexahydro-1-phenyl-7a-piperidinoindeno[5,6-*d*]triazole (**12b**), corresponding to the intermediate E in Chart 5, in 51% yield. When **12b** was heated with concentrated hydrochloric acid in dioxane, **12b** was aromatized to 8-chloro-1-phenyl-1,5,6,7-tetrahydroindeno[5,6-*d*]triazole (**13b**) in 57% yield with loss of piperidine. The reaction of **2** with **9c** gave 9-chloro-1-phenyl-5,6,7,8-tetrahydro-1*H*-naphtho[2,3-*d*]triazole (**12c**) in 30% yield together with 1-phenyl-7-piperidino-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**14**)[1] in 22% yield. The tetrahydronaphthotriazole **12c** corresponds to **13b**, and **14** originates from the nucleophilic substitution of the chlorine atom of **2** with piperidine, which is eliminated from the initial [4+2] cycloadduct. However, in the case of the reaction of **2** with **9a** and **9d**, no reaction took place, resulting in the recovery of **2**.

2 —9b→ **12b** —conc. HCl / in dioxane→ **13b**

2 —9c→ **12c** + **14**

Chart 5

The structures of **10**, **11d**, **12**, and **13b** were suggested by the elemental analysis and confirmed by analyses of the IR, ^{1}H-NMR, and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectral data, as shown in Tables III, IV, and V. However, the stereochemistry of the ethenotriazolopyridazine **11d** and the hexahydroindenotriazole **12b** has not been determined yet.

The experimental results may be summarized as follows. i) On the reaction of **1** and **2** with carbanions generated from active methylene compounds or ketones (**3a**—**g**), both undergo nucleophilic substitution rather than addition of carbanions to the 4,5-double bond,

TABLE III. Yields, Melting Points, Elemental Analyses and MS Data for **10**, **11**, and **13**

Compd.	Yield (%)	mp (°C)	MS m/z M^+	Formula	Analysis (%) Calcd (Found)		
					C	H	N
10a	9	192—193[a,d]	316	$C_{15}H_{16}N_4O_2S$	56.95	5.10	17.71
					(57.02	5.16	17.61)
10b	47	211—213[b,d]	313	$C_{16}H_{15}N_3O_2S$	61.32	4.82	13.41
					(61.10	4.78	13.37)
10c	45	223—224[c,d]	327	$C_{17}H_{17}N_3O_2S$	62.37	5.23	12.83
					(62.46	5.27	12.70)
10d	56	149—150[b,d]	358	$C_{18}H_{22}N_4O_2S$	60.31	6.19	15.63
					(60.56	6.24	15.51)
11d	21	155—157[a,d]	386	$C_{18}H_{22}N_6O_2S$	55.94	5.74	21.75
					(56.31	5.75	21.48)
12b	51	195—197[b,d]	354	$C_{20}H_{23}ClN_4$	67.69	6.53	15.79
		(dec.)	356 (M+2)		(67.90	6.53	15.95)
12c	30	155—156[a,e]	283	$C_{16}H_{14}ClN_3$	67.72	4.97	14.81
			285 (M+2)		(67.85	4.98	14.87)
13b	57	138—139[c,d]	269	$C_{15}H_{12}ClN_3$	66.79	4.48	15.58
			271 (M+2)		(66.42	4.53	15.31)

a) Yellow prisms. *b*) White needles. *c*) Colorless plates. *d*) Recrystallization from MeOH. *e*) Recrystallization from petr. ether–benzene.

TABLE IV. IR and ^{1}H-NMR Spectral Datt for **10**, **11**, **12**, and **13**

Compd.	IR ν_{max}^{KBr} cm^{-1}	^{1}H-NMR ($CDCl_3$) ppm
10a	1295, 1130 (SO_2)	8.18 (1H, d,[b] C^4-H), 7.75—7.40 (5H, m, C_6H_5), 7.31 (1H, d,[b] C^5-H), 3.14 (3H, s, SO_2CH_3), 3.08 (6H, s, $N(CH_3)_2$)
10b	1315, 1140 (SO_2)	8.15 (1H, s, C^4-H), 7.74—7.25 (5H, m, C_6H_5), 3.45 (2H, t,[a] $C\underline{H}_2CH_2$), 3.10 (2H, t, [a] $C\underline{H}_2CH_2$), 2.88 (3H, s, SO_2CH_3), 2.19 (2H, quint,[a] $CH_2C\underline{H}_2CH_2$)
10c	1310, 1140 (SO_2)	8.10 (1H, s, C^4-H), 7.83—7.05 (5H, m, C_6H_5), 3.62—2.58 (4H, m, $2\times CH_2$), 3.00 (3H, s, SO_2CH_3), 2.10—1.45 (4H, m, $2\times CH_2$)
10d	1290, 1115 (SO_2)	8.05 (1H, s, C^4-H), 7.05 (5H, s, C_6H_5), 3.41 (4H, q,[a] $N(CH_2)_2$), 3.12 (3H, s, SO_2CH_3), 2.51 (3H, s, CH_3), 1.14 (6H, t,[a] $2\times CH_2C\underline{H}_3$)
11d	1320, 1140 (SO_2)	7.53 (5H, s, C_6H_5), 6.70 (1H, q,[c] C^4-H), 3.24 (4H, q,[a] $N(CH_2)_2$), 2.96 (3H, s, SO_2CH_3), 2.12 (3H, d,[c] CH_3), 1.05 (6H, t,[a] $2\times CH_2C\underline{H}_3$)
12b		7.45 (5H, s, C_6H_5), 4.03 (1H, d,[d] C^4-H), 3.59—1.30 (17H, m, $(CH_2)_8$ and CH)
12c		7.74 (1H, s, C^4-H), 7.52 (5H, s, C_6H_5), 3.22—2.68 (4H, m, C^5H_2 and C^8H_2), 2.13—1.58 (4H, m, C^6H_2 and C^7H_2)
13b		7.77 (1H, s, C^4-H), 7.53 (5H, s, C_6H_5), 3.06 (4H, q,[a] C^5H_2 and C^7H_2), 2.17 (2H, quint,[a] C^6H_2)

a) J=7 Hz. *b*) J=9 Hz. *c*) J=2 Hz. *d*) J=14.4 Hz.

resulting in the formation of 7-substituted triazolopyridazines (**4a**—**g**). ii) Electron-deficient **1** and **2** both react with electron-rich dienophiles, *i.e.*, enamines or ynamines (**9a**—**d**), to afford the products (**10**, **11d**, and **12**) of [4+2] cycloaddition rather than the products of nucleophilic substitution. This cycloaddition reaction of **1** and **2** with **9** is the first such finding in the condensed pyridazine ring system.

TABLE V. ^{13}C-NMR Spectral Data for **10**, **11**, **12**, and **13**

Compd.	^{13}C-NMR ($CDCl_3$) ppm
10a	154.90 (s), 143.41 (s), 140.44 (s), 131.82 (s), 129.00 (d, Ph), 128.89 (d, Ph), 125.75[a] (d, C^4), 124.78 (d, Ph), 118.11 (d, C^5), 116.54 (s), 45.95 (q, $N(CH_3)_2$), 44.70 (q, SO_2CH_3)
10b	149.70 (s), 147.96 (s), 143.79 (s), 139.46 (s), 134.85 (s), 129.93 (d, Ph), 128.73 (d, Ph), 127.59 (d, Ph), 120.93 (s), 120.66 (d, C^4), 44.43 (q, SO_2CH_3), 34.19 (t, C^7), 31.86 (t, C^5), 26.33 (t, C^6)
10c	146.23 (s), 143.74 (s), 140.76 (s), 137.02 (s), 130.58 (s), 129.28 (d, Ph), 128.79 (d, Ph), 126.24 (d, Ph), 124.83[a] (d, C^4), 44.70 (q, SO_2CH_3), 29.96 (t, C^8), 28.12 (t, C^5), 21.78 (t, C^7), 21.08 (t, C^6)
10d	151.38 (s), 144.88 (s), 140.55 (s), 136.10 (s), 130.58 (s), 128.89 (d, Ph), 128.73 (d, Ph), 126.89[a] (d, C^4), 125.75 (d, Ph), 124.72 (s), 48.55 (t, $N(CH_2)_2$), 45.57 (q, SO_2CH_3), 20.97 (q, CH_3), 13.17 (q, $N(CH_2\underline{C}H_3)_2$)
11d	151.06 (s), 150.73 (s), 150.03 (s), 139.57 (s), 136.91 (s), 129.93 (d, Ph), 129.71 (d, Ph), 123.10 (d, Ph), 122.28[a] (d, C^4), 42.10 (t, $N(CH_2)_2$), 41.29 (q, SO_2CH_3), 20.91 (q, CH_3), 13.06 (q, $2\times CH_3$)
12b	150.68 (s), 146.18 (s), 136.48 (s), 132.31 (s), 129.22 (d, Ph), 128.41 (d, Ph), 126.19 (d, Ph), 108.31 (s), 65.13[a] (d, C^4), 50.71 (t, $N(CH_2)_2$), 48.33, 33.65, 30.45 (each t, $-(CH_2)_3-$), 26.77, 24.76 (each t, $-(CH_2)_3-$)
12c	145.42 (s), 136.86 (s), 136.48 (s), 136.05 (s), 129.49 (d, Ph), 128.57 (d, Ph), 127.49 (d, Ph), 117.30 (d, C^4), 115.13 (s), 30.61, 27.52 (each t, C^8 and C^5), 22.81, 22.49 (each t, C^7 and C^6)
13b	147.64 (s), 144.88 (s), 142.93 (s), 136.59[b] (s), 129.55 (d, Ph), 128.57 (d, Ph), 127.33 (d, Ph), 119.42 (s), 112.86 (d, C^4), 111.45 (s), 33.16, 31.97 (each t, C^7 and C^5), 25.63 (t, C^6)

a) Assignment based on selective decoupling. *b*) Multiplicities in the off-resonance spectrum.

Experimental

All melting points are uncorrected. IR spectra were recorded on a Jasco A-102 diffraction grating IR spectrometer. ^1H-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 Hz. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, sept = septet, m = multiplet, and br s = broad singlet. MS were recorded on a JEOL JMS D-100 mass spectrometer. Samples were vaporized in a direct inlet system. Column chromatography was carried out on SiO_2 (Wakogel C-200; 200 mesh).

Reaction of 7-(Methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (1) with Active Methylene Compounds or Ketones (3a—g) in the Presence of NaH——A mixture of **1** (100 mg, 0.36 mmol), **3** (0.55 mmol), and 60% NaH (in oil, 35 mg, 0.88 mmol) in dioxane (1 ml) was refluxed for 1 h. After cooling, the reaction mixture was poured into H_2O (5 ml), neutralized with AcOH, and extracted with $CHCl_3$. The $CHCl_3$ extract was dried over Na_2SO_4, and chromatographed on a column of SiO_2 with $CHCl_3$. The first fraction gave 7-substituted 1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**4**), which was purified by recrystallization from an appropriate solvent (Table I). The second fraction gave 1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazin-7(6*H*)-one (**5**), which was purified by recrystallization from MeOH.

Reaction of 7-Chloro-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (2) with 3a—g in the Presence of NaH——i) A mixture of **2** (100 mg, 0.43 mmol), **3** (0.65 mmol), and 60% NaH (in oil, 35 mg, 0.88 mmol) in benzene (2 ml) was refluxed for 2 h. The reaction mixture was poured into H_2O (5 ml), neutralized with AcOH, and extracted with $CHCl_3$. The extract was dried over Na_2SO_4, and chromatographed on a column of SiO_2 with $CHCl_3$. The first fraction gave **4**.

ii) A mixture of **2** (100 mg, 0.43 mmol), **3c** (43 mg, 0.65 mmol), and 60% NaH (in oil, 35 mg, 0.88 mmol) in DMF (2 ml) was stirred for 1 h at room temperature. The reaction mixture was poured into H_2O (5 ml), and neutralized with AcOH. The separated crystals were collected by suction, washed with H_2O then MeOH, and recrystallized from dimethyl sulfoxide to give **4c**.

iii) A mixture of **2** (100 mg, 0.43 mmol), **3g** (38 mg, 0.65 mmol), and 60% NaH (in oil, 35 mg, 0.88 mmol) in THF (1 ml) was refluxed for 1 h. Work-up of the reaction mixture as described for reaction i) gave **4g** and **5**.

7-Methyl-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (6)——A 50% NaOH solution (0.2 ml) was added to a solution of **4d** (149 mg, 0.42 mmol) in MeOH (2 ml), and the mixture was refluxed for 1 h. After removal of the solvent, the residue was neutralized with diluted AcOH, and extracted with $CHCl_3$. The extract was washed with H_2O, dried over Na_2SO_4, and chromatographed on a column of SiO_2 with $CHCl_3$. The first fraction gave **6** in 86% yield.

Reaction of 2 with Sodium *p*-Toluenesulfinate (8)——A mixture of **2** (0.1 g) and the dihydrate of **8** (0.18 g) in DMF (2 ml) was heated at 90 °C for 1.5 h. After cooling, the reaction mixture was poured into H_2O (10 ml), and

extracted with $CHCl_3$. The extract was washed with saturated aqueous NaCl then H_2O, dried over Na_2SO_4, and chromatographed on a column of SiO_2 with $CHCl_3$. The first fraction gave 1-phenyl-7-(*p*-tolylsulfonyl)-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (7) in 49% yield (74 mg). The second fraction gave **5** in 30% yield (28 mg).

Reaction of 1 with Enamines or Ynamines (9a—d)——A solution of **1** (200 mg, 0.73 mmol) and **9** (1.46 mmol) in dioxane (2 ml) was refluxed for 4 h. After cooling, the reaction mixture was poured into H_2O (5 ml), neutralized with AcOH, and extracted with $CHCl_3$. The extract was dried over Na_2SO_4, and chromatographed on a column of SiO_2 with $CHCl_3$. The first fraction gave arenotriazole (**10**), which was purified by recrystallization from an appropriate solvent (Table III). In the reaction with **9d**, the second fraction gave 8-(*N*,*N*-diethylamino)-4,7-dihydro-9-methyl-7-(methylsulfonyl)-1-phenyl-1*H*-4,7-etheno-1,2,3-triazolo[4,5-*d*]pyridazine (**11d**) as a by-product.

Reaction of 2 with 9b——A solution of **2** (100 mg, 0.43 mmol) and **9b** (0.5 ml) was heated at 140 °C for 4 h. After cooling, the reaction mixture was poured into H_2O (5 ml), acidified with AcOH, and extracted with $CHCl_3$. The extract was dried over Na_2SO_4, and chromatographed on a column of SiO_2 with $CHCl_3$. The first fraction gave 8-chloro-1,4a,5,6,7,7a-hexahydro-1-phenyl-7a-piperidinoindeno[5,6-*d*]triazole (**12b**).

Reaction of 2 with 9c——A solution of **2** (100 mg, 0.43 mmol) and **9c** (0.5 ml) was heated at 140 °C for 4 h. Work-up of the reaction mixture as described for the reaction of **2** with **9b** gave 9-chloro-1-phenyl-5,6,7,8-tetrahydro-1*H*-naphtho[2,3-*d*]triazole (**12c**) from the first fraction and 1-phenyl-7-piperidino-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**14**, 22%) from the second fraction.

8-Chloro-1-phenyl-1,5,6,7-tetrahydroindeno[5,6-*d*]triazole (13b)——Concentrated HCl (0.5 ml) was added to a solution of **12b** (100 mg, 0.28 mmol) in dioxane (2 ml), and the mixture was refluxed for 8 h. After cooling, the reaction mixture was poured into H_2O (2 ml), neutralized with 2 N NaOH, and extracted with $CHCl_3$. The extract was dried over Na_2SO_4 and chromatographed on a column of SiO_2 with $CHCl_3$. The first fraction gave **13b**.

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The [4+2] Cycloaddition of 1,6-Dihydro-2-dimethylamino-4,6,6-trimethylpyrimidine with Acetylenic Compounds

CHOJI KASHIMA,* MASAO SHIMIZU, and YOSHIMORI OMOTE

Department of Chemistry, University of Tsukuba, Sakura-mura, Niihari-gun, Ibaraki 305, Japan

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1,6-Dihydro-2-dimethylamino-4,6,6-trimethylpyrimidine reacted with acetylenic compounds containing electron-withdrawing groups to yield 2-dimethylaminopyridine derivatives. When monosubstituted acetylenes were used, 4-unsubstituted pyridines were selectively obtained. The results of MINDO/3 calculations are consistent with this regioselectivity.

Keywords——1,6-dihydropyrimidine; [4+2] cycloaddition; acetylenic compound; pyridine derivative; MINDO/3 calculation

Dihydropyrimidines are of interest as aza-analogs of dihydropyridines which are the active moieties of nicotinamide adenine dinucleotide phosphate (NAD(P)H). Furthermore, dihydropyrimidines have various reactive sites in their rings such as the C=N and the C=C double bonds and enamines. In particular, 1,2- and 1,6-dihydropyrimidines contain the conjugated aza-diene moieties in their ring structures. A recent review[1] has dealt with the chemistry of dihydropyrimidines, *i.e.*, tautomerism, rearrangement (di-π-methane photochemical and thermal), and other reactions. We have reported nucleophilic reactions of dihydropyrimidines using metal hydride complexes[2] and on [2+2] cycloaddition with ketenes.[3] The reactions with sodium borohydride were also reported by Cho *et al.*[4]

However, the reactions of dihydropyrimidines as conjugated aza-dienes have not been explored. It is known that pyrimidine compounds which have electron-withdrawing or -donating substituents react with some dienophiles to yield pyridine derivatives.[5] In this paper, we describe the [4+2] cycloaddition reaction of 1,6-dihydropyrimidines as aza-dienes with dienophiles such as acetylenic compounds.

Results and Discussion

When 1,6-dihydro-4,6,6-trimethyl-2-dimethylaminopyrimidine (**1**) was treated with a small excess of dimethyl acetylenedicarboxylate (**2a**) in benzene at room temperature for 15 h, two products were isolated from the reaction mixture. The main product showed the following spectral data: in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum, signals at δ 2.42 (3H, s), 3.01 (6H, s), 3.86 (6H, s), and 6.75 ppm (1H, s) were attributable to methyl, N-Me_2, methyl esters, and an olefinic proton. The spectrum indicated loss of the N-1 nitrogen and the C-6 carbon moiety from the dihydropyrimidine ring and introduction of two methoxycarbonyl groups. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum was also consistent with this. From these results, the product was determined to be dimethyl 6-methyl-2-dimethylaminopyridine-3,4-dicarboxylate (**3a**), and this conclusion was also supported by the microanalytical data. The minor product (13% yield) was concluded to be dimethyl aminofumarate (**4**) from the spectral data (see Chart 1).

Chart 1

The reaction mechanism is speculated to be as follows. The [4+2] cycloaddition of 1,6-dihydropyrimidine with dimethyl acetylenedicarboxylate (**2a**) takes place across the C-2 and the C-5 carbons to afford the [4+2] adduct as an intermediate, though it was not detected. Retro-cycloaddition also takes place with loss of 2-iminopropane to afford the pyridine derivative (**3a**) through aromatization. Dimethyl aminofumarate is produced from the reaction of excess dimethyl acetylenedicarboxylate with ammonia, which is generated by the hydrolysis of 2-iminopropane. Although dihydropyrimidine (**1**) exists as a mixture of 1,6- and 4,5-dihydro forms in nonpolar solvents,[6] the cycloadduct derived from the 4,5-dihydropyrimidine isomer (**1′**) was not obtained.

A similar reaction of 2-dimethylaminodihydropyrimidine using the unsymmetrical dienophiles was attempted. Since 1-butyn-3-one is volatile and difficult to purify, 1-butyn-3-ol was oxidized by chromium trioxide in dichloromethane without isolation. When 1-butyn-3-one generated from 10 eq of 1-butyn-3-ol was treated with 1,6-dihydropyrimidine (**1**) in dichloromethane, two products, (**3e**) $C_{10}H_{14}N_2O$ and (**5e**) $C_{14}H_{18}N_2O_2$, were obtained in 37% and 10% yields, respectively. Compound **3e** was determined to be 3-acetyl-6-methyl-2-dimethylaminopyridine from the ^{1}H-NMR spectral data as compared with those of **3a**. The position of the acetyl group in **3e** was determined from the coupling constant (8 Hz) between the two protons on the pyridine ring, which was larger than would be expected for two bond separation (Chart 1). Compound **5e** was speculated to be 5-(3-oxo-1-butenyl)-3-acetyl-6-methyl-2-dimethylaminopyridine from the coupling constant of the two *trans* olefinic protons (16 Hz), which showed the two protons were not in the pyridine ring. Furthermore, the isomer, 4-acetyl-6-methyl-2-dimethylaminopyridine, was not detected.

To confirm this regioselective reaction, the LUMO and HOMO coefficients of the aza-diene and dienophile were calculated by MINDO/3. In general [4+2] cycloadditions, a σ-bond is formed between atoms on dienes having higher HOMO coefficient and those on dienophiles having higher LUMO coefficient. In the diene moiety of dihydropyrimidine, the coefficient of the C-5 carbon is higher than that of the C-2 carbon (Chart 2), and in 1-butyn-3-one the C-1 carbon has a higher coefficient than the C-2 carbon. Actually, the σ-bonds were formed between the C-5 carbon on the diene and the C-1 carbon on the dienophile, and between the C-2 carbon on the diene and the C-2 carbon of the dienophile, to yield the 3-acetylpyridine derivative (**3e**).

HOMO LUMO

0.1966 0.0614 0.0559 0.2830 0.0619 0.0112

Chart 2

TABLE I. Reaction of 1,6-Dihydropyrimidine (1) with Acetylenic Compounds, 2

2	R^1	R^2	Method[a]	Product No.	Yield (%)
a	CO_2Me	CO_2Me	A	**3a**	61
b	COMe	COMe	A	**3b**	23
c	CO_2Me	H	A	**3c**	27
d	$COPr^n$	H	A	**3d**	23
e	COMe	H	B	**3e**	37[b]

a) See the experimental section. *b*) **5e** was obtained in 10% yield.

To investigate the mechanism of the formation of **5e**, the reaction of the pyridine derivative (**3e**) with 1-butyn-3-one generated from the oxidation of 1-butyn-3-ol was carried out. However, the Michael addition of the pyridine ring to the ynone moiety did not occur, and the starting material (**3e**) was recovered. From this result, it was concluded that **5e** was formed simultaneously with **3e**.

Similar treatment of 1,6-dihydro-4,6,6-trimethyl-2-dimethylaminopyrimidine with various acetylenic compounds was carried out, and the results are summarized in Table I. The reactions of **2b**, **2c**, and **2d** were very complicated; the yields of **3** were low and **4** was not isolated. Although the reactions with electron-withdrawing dienophiles proceeded, neutral dienophiles such as styrene and diphenylacetylene did not react with dihydropyrimidines.

In conclusion, 1,6-dihydro-4,6,6-trimethyl-2-dimethylaminopyrimidine reacted with electron-poor acetylenic compounds to yield pyridine derivatives. Thus, ring transformation from pyrimidine to pyridine was accomplished by [4+2] cycloaddition.

Experimental

Melting points, determined on a Yanagimoto micro melting point apparatus, are uncorrected. Infrared (IR) spectra were measured on a Jasco IRA-1 infrared spectrophotometer. ^1H-NMR spectra were recorded on Hitachi R-24 (60 MHz) and JEOL-100 (100 MHz) spectrometers with tetramethylsilane as an internal standard. ^{13}C-NMR spectra were obtained on a JEOL-100 spectrometer with tetramethylsilane as an internal standard.

General Procedure for the Reaction with Acetylenic Compounds——Method A: An acetylenic compound (1.2 mmol) was added to a solution of dihydropyrimidine (1 mmol) in benzene (20 ml), and the mixture was stirred for 15 h at room temperature. After removal of the solvent, the reaction mixture was evaporated and the residue was purified by chromatography on silica gel with benzene–ethyl acetate (5 : 1, v/v) or hexane–ethyl acetate (5 : 1, v/v) as eluate.

Method B: A solution of chromium trioxide in diluted sulfuric acid (H_2O: 10 ml, H_2SO_4: 2 ml) was added dropwise to a solution of acetylene alcohol (*ca.* 50% in water, 5 mmol) in diluted sulfuric acid (H_2O: 10 ml, H_2SO_4: 3 ml) on an ice-bath. The mixture was stirred for 3 h at 2—10 °C, then dichloromethane (20 ml) was added. The organic layer was dried over anhydrous magnesium sulfate. The acetylene compound in dichloromethane was added to a solution of dihydropyrimidine (1 mmol) in dichloromethane (20 ml) and the mixture was stirred for 15 h at room temperature. The reaction mixture was evaporated and the residue was purified by chromatography on silica gel with benzene–ethyl acetate (5 : 1, v/v) or hexane–ethyl acetate (5 : 1, v/v) as the eluate.

Dimethyl 6-Methyl-2-dimethylaminopyridine-3,4-dicarboxylate (3a)——mp 83.5—84 °C (from hexane). IR (KBr): 1720, 1560 cm^{-1}. ^1H-NMR ($CDCl_3$, 100 MHz) δ: 2.42 (3H, s), 3.01 (6H, s), 3.86 (6H, s), 6.75 (1H, s). ^{13}C-

NMR ($CDCl_3$) δ: 24.4 (q), 40.2 (q), 52.4 (q), 52.5 (q), 110.5 (d), 140.3 (s), 157.2 (s), 158.4 (s), 167.0 (s), 168.6 (s). *Anal.* Calcd for $C_{12}H_{16}N_2O_4$: C, 57.13; H, 6.39; N, 11.10. Found: C, 57.15; H, 6.44; N, 11.08.

3,4-Diacetyl-6-methyl-2-dimethylaminopyridine (3b)——mp 71—72 °C (from hexane). IR ($CHCl_3$): 1700 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 2.46 (3H, s), 2.48 (3H, s), 2.93 (6H, s), 6.55 (1H, s). *Anal.* Calcd for $C_{12}H_{16}N_2O_2$: C, 65.43; H, 7.32; N, 12.71. Found: C, 65.50; H, 7.40; N, 12.61.

Methyl 6-Methyl-2-dimethylaminopyridine-3-carboxylate (3c)——IR ($CHCl_3$): 1700 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 2.40 (3H, s), 2.83 (6H, s), 3.83 (3H, s), 6.43 (1H, d, J=8.0 Hz), 7.78 (1H, d, J=8.0 Hz). Picrate of (3c): mp 131—132 °C (from ethanol). *Anal.* Calcd for $C_{16}H_{17}N_5O_9$: C, 45.39; H, 4.04; N, 16.54. Found: C, 45.33; H, 4.06; N, 16.63.

3-Butyryl-6-methyl-2-dimethylaminopyridine (3d)——bp 39 °C/10^{-3} mmHg. IR ($CHCl_3$): 1660 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 0.94 (3H, t, J=7.0 Hz), 1.68 (2H, hex, J=7.0 Hz), 2.39 (3H, s), 2.89 (3H, t, J=7.0 Hz), 2.92 (6H, s), 6.46 (1H, d, J=8.0 Hz), 7.56 (1H, d, J=8.0 Hz). *Anal.* Calcd for $C_{12}H_{18}N_2O$: C, 69.86; H, 8.79; N, 13.57. Found: C, 69.83; H, 8.85; N, 13.20.

3-Acetyl-6-methyl-2-dimethylaminopyridine (3e)——IR ($CHCl_3$): 1660 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 2.42 (3H, s), 2.49 (3H, s), 2.97 (6H, s), 6.57 (1H, d, J=8.0 Hz), 7.73 (1H, d, J=8.0 Hz). Picrate of **3e**: mp 114—115 °C (from ethanol). *Anal.* Calcd for $C_{16}H_{17}N_5O_8$: C, 47.17; H, 4.20; N, 17.19. Found: C, 47.20; H, 4.26; N, 17.07.

3-Acetyl-6-methyl-2-dimethylamino-5-(3-oxo-1-butenyl)pyridine (5e)——mp 152—153 °C (from hexane). IR ($CHCl_3$): 1660 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 100 MHz) δ: 2.36 (3H, s), 2.54 (3H, s), 2.56 (3H, s), 3.04 (6H, s), 6.53 (1H, d, J=16 Hz), 7.71 (1H, d, J=16 Hz), 8.00 (1H, s). ^{13}C-NMR ($CDCl_3$) δ: 22.7 (q), 27.8 (q), 28.1 (q), 41.0 (q), 117.0 (s), 118.2 (s), 124.5 (d), 136.7 (d), 138.9 (d), 157.9 (d), 159.4 (s), 197.6 (s), 198.3 (s). *Anal.* Calcd for $C_{14}H_{18}N_2O_2$: C, 68.26; H, 7.36; N, 11.37. Found: C, 68.26; H, 7.43; N, 11.32.

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New Methods and Reagents in Organic Synthesis. 69.[1] A New Synthesis of α-Amino Acid and Peptide Amides of Aromatic Amines Using a Modified Curtius Reaction with Diphenyl Phosphorazidate

TAKAYUKI SHIOIRI,* MITSUO MURATA,[2] and YASUMASA HAMADA

Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467, Japan

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As a basic study on the efficient synthesis of chromogenic and fluorogenic peptidase substrates, we investigated possible general methods for the synthesis of α-amino acid and peptide amides of aromatic amines. Preparation of Boc–L-Leu–*p*NA (**1**) as a model compound was attempted by (1) the coupling of Boc–L-Leu–OH with *p*-nitroaniline, (2) the reaction of Boc–L-Leu–OH with *p*-nitrophenyl isocyanate, and (3) the reaction of Boc–L-Leu–OH with the product formed from *p*-nitrobenzoic acid through the modified Curtius reaction with diphenyl phosphorazidate ($(C_6H_5O)_2P(O)N_3$) in a one-pot process. The third method was found to be a general and efficient method for the preparation of α-amino acid amides of aromatic amines. Application of this method to the preparation of a peptidase substrate, Bz–L-Ile–L-Glu(OMe)–Gly–L-Arg–*p*NA (**2**), has also been successfully achieved.

Keywords——α-amino acid arylamide; peptide arylamide; chromogenic peptidase substrate; fluorogenic peptidase substrate; diphenyl phosphorazidate; Curtius reaction; amide bond formation; amino group activation

Some α-amino acid and peptide amides of chromogenic and fluorogenic aromatic amines are good artificial substrates of various peptidases and are used clinically for the sensitive determination of these enzymes in body fluids. Preparation of these peptidase substrates usually starts by condensation of aromatic amines with N-protected α-amino acids, which generally results in low yields since aromatic amines have lower nucleophilicity.

As a basic study on the efficient synthesis of chromogenic and fluorogenic peptidase substrates, we have investigated the preparation of Boc–L-Leu–*p*NA (**1**),[3] which serves as a substrate for leucine aminopeptidase after deblocking of its Boc group, from Boc–L-Leu–OH by (1) coupling with *p*-nitroaniline, (2) reaction with *p*-nitrophenyl isocyanate, and (3) reaction with the product, *p*-nitrophenyl isocyanate, formed from *p*-nitrobenzoic acid through the modified Curtius reaction with diphenyl phosphorazidate (DPPA, $(C_6H_5O)_2P(O)N_3$) in a one-pot process. The third method using the modified Curtius reaction[4] was the most efficient and appears to be a general method for the preparation of various α-amino acid amides of aromatic amines. Application of this method to the preparation of a peptidase substrate, Bz–L-Ile–L-Glu(OMe)–Gly–L-Arg–*p*NA (**2**) has also been successfully achieved.

We first surveyed the coupling of Boc–L-Leu–OH with *p*-nitroaniline using the various coupling methods usually employed for peptide synthesis. As summarized in Table I, the efficiency of the coupling was moderate or poor. These results were not unexpected, since aromatic amines containing electron-withdrawing functions such as *p*-nitroaniline are usually poor nucleophiles.

The coupling methods examined in Table I are based on the activation of the carboxyl group of Boc–L-Leu–OH. We next turned our attention to the coupling methods based on the

TABLE I. Coupling of Boc–L-Leu–OH with p-Nitroaniline

$$\text{Boc–L-Leu–OH} + H_2N\text{–}C_6H_4\text{–}NO_2 \xrightarrow{\text{tetrahydrofuran}} \text{Boc–L-Leu–}p\text{NA (1)}$$

Run	Coupling method	Yield (%)
1	DPPA–TEA[a]	—
2	DEPC–TEA[a]	—
3	DCCD	22
4	DCCD (2.2 eq)[b]	43
5	DCCD–HOBt	—
6	DCCD–HONB	—
7	iso-BuOCOCl–N-methylmorpholine	25
8	EtOCOCl–N-methylmorpholine[c]	52
9	EEDQ	39
10	$POCl_3$–TEA–DMAP	3

a) DMF was used instead of tetrahydrofuran. *b*) p-Nitroaniline (5.8 eq) was used. *c*) p-Nitroaniline (5 eq) was used.

activation of the amino group of p-nitroaniline. The most well-known method to activate the amino function is conversion of the amino groups to the isocyanates.[5] Nishi and co-workers reported[6] a convenient preparation of Z–L-Arg(NO_2)–pNA by condensation of Z–L-Arg(NO_2)–OH with p-nitrophenyl isocyanate in hexamethylphosphoric triamide in the presence of TEA. Application of this method to the condensation of Boc–L-Leu–OH with p-nitrophenyl isocyanate analogously proceeded to give Boc–L-Leu–pNA (**1**) in 57% yield accompanied with the hydantoin (**3**) in 14% yield, as shown in Chart 1. The hydantoin (**3**) is presumably formed by the attack of the nitrogen atom of Boc–L-Leu–OH on the carbonyl carbon of p-nitrophenyl isocyanate, followed by cyclization as depicted in **4** (X = H or p-O_2N–C_6H_4–NHCO). Addition of DMAP[7] to the above reaction raised the yield of **1** to 70%, but the formation of the by-product **3** also increased to 22%.

Boc–L-Leu–OH + O_2N–C_6H_4–NCO ⟶ Boc–L-Leu–pNA (1) + 3

Boc, O, N, O, NO₂ — **3**

Boc, O⁻, N, C, N, NO₂, XO, C, O — **4**

Chart 1

Although the efficiency of the reaction was better than that of the coupling of Boc–L-Leu–OH with p-nitroaniline, commercially available p-nitrophenyl isocyanate is sometimes impure because of gradual deterioration during storage and should be purified by recrystallization from carbon tetrachloride before use. Furthermore, preparation of p-nitrophenyl isocyanate from p-nitroaniline requires the use of highly toxic phosgene,[6] and p-nitrophenyl isocyanate thus prepared should not be kept for a long period.

We have already reported a modified Curtius reaction using DPPA.[4] We presumed that p-nitrobenzoic acid would easily undergo the modified Curtius reaction to give p-nitrophenyl isocyanate, which would react with Boc–L-Leu–OH to give Boc–L-Leu–pNA (**1**). It should be

TABLE II. Preparation of Amino Acid Amides of Aromatic Amines Using the Modified Curtius Reaction with DPPA

$$ArCO_2H \xrightarrow[\text{2) Boc-L-Ama-OH, reflux 1—6 h in } ClCH_2CH_2Cl]{\text{1) DPPA, TEA, r.t. 2 h, reflux 15 min}} \text{Boc-L-Ama-NH-Ar}$$

Starting material		Reaction time for step 2 (h)	Product		Yield (%)
Boc-L-Ama-OH	$ArCO_2H$		Boc-L-Ama-NH-Ar	No.	
Boc-L-Leu-OH	$O_2N-C_6H_4-CO_2H$	1	Boc-L-Leu-*p*NA	**1**	95
Boc-L-Leu-OH	2-naphthoic acid ($C_{10}H_7-CO_2H$)	6	Boc-L-Leu-NH-(2-naphthyl)	**5**	76
Boc-L-Leu-OH	$Me_2N-C_6H_4-N=N-C_6H_4-CO_2H$	4	Boc-L-Leu-NH-C_6H_4-N=N-C_6H_4-NMe_2	**6**	53
Boc-L-Leu-OH	anthraquinone-2-carboxylic acid	4	Boc-L-Leu-NH-(anthraquinon-2-yl)	**7**	92
Boc-L-Leu-OH	quinoline-4-carboxylic acid N-oxide	1	Boc-L-Leu-NH-(quinolin-4-yl N→O)	**8**	54
Boc-L-Leu-OH	2-phenylquinoline-4-carboxylic acid N-oxide	3	Boc-L-Leu-NH-(2-phenylquinolin-4-yl N→O)	**9**	30[a]
Boc-L-Lys(Z)-OH	$O_2N-C_6H_4-CO_2H$	4	Boc-L-Lys(Z)-*p*NA	**11**	76
Boc-L-Arg(Mts)-OH	$O_2N-C_6H_4-CO_2H$	5	Boc-L-Arg(Mts)-*p*NA	**12**	54

r.t., room temperature.

a) Boc-L-Leu-NH-(2-phenylquinolin-4-yl) (**10**) was obtained in 29% yield.

possible to conduct the overall reaction in a one-pot procedure. After some trials to find the most suitable reaction conditions, we found that *p*-nitrophenyl isocyanate was very easily formed from *p*-nitrobenzoic acid and DPPA in 1,2-dichloroethane, and then reacted with Boc-L-Leu-OH under reflux in the same flask, giving a 95% yield of Boc-L-Leu-*p*NA (**1**). Not even a trace of **3** could be detected when *p*-nitrophenyl isocyanate was directly used. This may be due to the different reaction conditions, such as reaction solvents and temperatures. Two-fold excesses of *p*-nitrobenzoic acid, DPPA, and TEA afforded the best result.

The method has been shown to have generality, and Boc-amino acids readily condensed with aromatic isocyanates derived from aromatic carboxylic acids and DPPA in the presence of TEA in a one-pot procedure, giving Boc-amino acid amides of aromatic amines in preparatively satisfactory yields. The results are summarized in Table II. It is noteworthy that the *β*-naphthylamide (**5**) is also conveniently prepared by this method starting from *β*-

naphthoic acid, since it avoids the use of β-naphthylamine, a well-known bladder carcinogen.

As an extension of this new preparation of α-amino acid amides of aromatic amines, we prepared Bz–L-Ile–L-Glu(OMe)–Gly–L-Arg–*p*NA (**2**), which is a substrate for factor Xa,[8] as shown in Chart 2. H–Gly–OBu^t was quantitatively condensed with Boc–L-Glu(OMe)–OH by the DPPA[4a,9] or DEPC[9,10] method. After brief treatment with hydrogen chloride in ethyl acetate, the resulting H–L-Glu(OMe)–Gly–OBu^t·HCl was coupled with Z–L-Ile–OH by the same coupling methods to give Z–L-Ile–L-Glu(OMe)–Gly–OBu^t. Catalytic removal of the Z-group over palladium–carbon followed by treatment with benzoyl chloride afforded Bz–L-Ile–L-Glu(OMe)–Gly–OBu^t, which, after treatment with trifluoroacetic acid, was condensed with H–L-Arg(Mts)–*p*NA·HCl prepared from its Boc derivative **12**, giving Bz–L-Ile–L-Glu(OMe)–Gly–L-Arg(Mts)–*p*NA. Deblocking of the Mts group was achieved with methanesulfonic acid–anisole.[11] Treatment of the crude product with TEA followed by chromatographic purification afforded Bz–L-Ile–L-Glu(OMe)–Gly–L-Arg–*p*NA (**2**).

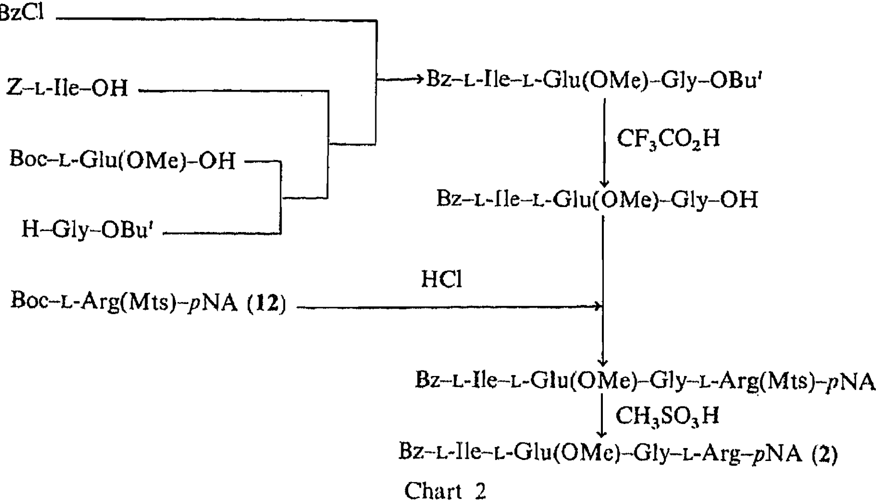

Chart 2

Preparation of α-amino acid amides of aromatic amines is now easily conducted as a one-pot procedure by the modified Curtius reaction of aromatic carboxylic acids with DPPA followed by reaction with N-protected α-amino acids. The method should be useful not only for the preparation and exploitation of chromogenic and fluorogenic substrates of peptidases, but also as a general method for the synthesis of various amides.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were measured with a JASCO IRA-2 spectrometer in Nujol mulls. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL JNM PMX-60 or JNM MH-100 spectrometer with tetramethylsilane as an internal standard in deuterochloroform unless otherwise stated. Optical rotations were measured with a Union Giken PM-201 or a JASCO DIP-140 automatic polarimeter. Silica gel (70—230 mesh ASTM, Merck Art. 7734) was used for column chromatography. Preparative layer chromatography was carried out on silica gel plate (2 mm thickness, Merck Art. 5717).

Condensation of Boc–L-Leu–OH with *p*-Nitroaniline——Reactions in runs 1—8 in Table I were carried out as described in our previous paper.[9] Reactions in runs 9 and 10 were carried out as described in the literature.[12,13]

Reaction of Boc–L-Leu–OH with *p*-Nitrophenyl Isocyanate——TEA (1.01 g, 10 mmol) in hexamethylphosphoric triamide (5 ml) was added to Boc–L-Leu–OH (2.31 g, 10 mmol) in hexamethylphosphoric triamide (20 ml) with ice-cooling under nitrogen, then *p*-nitrophenyl isocyanate (3.28 g, 20 mmol, recrystallized from carbon tetrachloride) was added in small portions. The mixture was stirred with ice-cooling for 1 h, then at room temperature for 16 h. Ethyl acetate–benzene (4 : 1, 200 ml) was added, and the mixture was successively washed with 10% aqueous citric acid, saturated aqueous sodium chloride, saturated aqueous sodium bicarbonate, water, and saturated aqueous sodium chloride. After being dried over sodium sulfate, the mixture was concentrated *in vacuo* to give the residue, which was

purified by column chromatography on silica gel (100 g) with hexane–diethyl ether (3 : 1) to give fractions 1 and 2.

Fraction 1, Boc–L-Leu–*p*NA (**1**), mp 91—96 °C (diethyl ether–hexane), $[\alpha]_D^{20}$ −30.4° (c=1, MeOH). IR ν_{max} cm^{-1}: 3280, 1670, 855. NMR δ: 0.8—2 (18H, m), 4.2—4.6 (1H, m), 5.66 (1H, br d, J=8 Hz), 7.60 (2H, d, J=9 Hz), 8.00 (2H, d, J=9 Hz), 9.76 (1H, s). *Anal.* Calcd for $C_{17}H_{25}N_3O_5$: C, 58.10; H, 7.19; N, 11.96. Found: C, 58.16; H, 7.23; N, 12.04.

Fraction 2, **3**, mp 99—101 °C (diethyl ether–hexane). IR ν_{max} cm^{-1}: 1810, 1740, 845. NMR δ: 0.8—2.2 (18H, m), 4.64 (1H, m), 7.68 (2H, d, J=9 Hz), 8.12 (2H, d, J=9 Hz). *Anal.* Calcd for $C_{18}H_{23}N_3O_6$: C, 57.28; H, 6.15; N, 11.14. Found: C, 57.47; H, 6.12; N, 11.43.

When DMAP was used, DMAP (0.1 eq) was added before the addition of TEA in the above experiment. Reaction and work-up as above afforded Boc–L-Leu–*p*NA (**1**) in 70% yield and **3** in 22% yield.

General Procedure for the Preparation of Boc-α-Amino Acid Amides of Aromatic Amines Using the Modified Curtius Reaction with DPPA——A mixture of an aromatic carboxylic acid (2 mmol), DPPA (550 mg, 2 mmol), and TEA (202 mg, 2 mmol) in 1,2-dichloroethane (2.5 ml) was stirred at room temperature for 2 h under nitrogen and refluxed for 15 min. Boc-α-amino acid (1 mmol) in 1,2-dichloroethane (2.5 ml) was added, and the mixture was refluxed for 1—6 h (see Table II). After concentration *in vacuo*, ethyl acetate–benzene (4 : 1, 100 ml) was added, and the mixture was successively washed with 100 ml each of 10% aqueous citric acid, water, saturated aqueous sodium bicarbonate, water, and saturated aqueous sodium chloride. It was then dried over sodium sulfate, filtered, and concentrated *in vacuo*. The residue was treated with ethyl acetate (100 ml) (diethyl ether was used in run 1 of Table II). The insoluble material was filtered off and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with the eluent described below, followed by recrystallization.

Boc–L-Leu–*p*NA (**1**): Eluent, diethyl ether–hexane (1 : 2); identical with the sample prepared as above.

Amide **5**: Eluent, ethyl acetate–hexane (1 : 3). IR ν_{max} cm^{-1}: 3280, 1665, 1590, 1530, 855, 815, 745. NMR δ: 1.00 (6H, d, J=5 Hz), 1.44 (9H, s), 1.76 (3H, m), 4.32—4.76 (1H, m), 5.72 (1H, d, J=8 Hz), 7.18—7.92 (6H, m), 8.20 (1H, s), 9.44 (1H, s). The amide **6** (89 mg) thus obtained was dissolved in 10% hydrogen chloride in ethyl acetate (10 ml), and the mixture was stirred at room temperature for 2 h. The solvent was removed *in vacuo* to give crystals, which were washed with ethyl acetate to give the hydrochloride of H–L-Leu–β-naphthylamide (57 mg, 78%). Recrystallization from methanol–ethyl acetate afforded colorless needles, mp 242—242.5 °C (dec.), $[\alpha]_D^{20}$ +92.8° (c=1, H_2O) (lit.[14] mp 241—242 °C (dec.), $[\alpha]_D^{20}$ +84.1° (c=1.73, H_2O)). IR ν_{max} cm^{-1}: 3280, 1695, 1610, 1585, 1560, 1510, 860, 820, 745. NMR δ (DMSO-d_6): 0.97 (m) and 1.73 (m) (9H), 4.17 (1H, m), 7.17—8.10 (m), 8.33 (s), and 8.57 (b) (10H), 11.23 (1H, s). *Anal.* Calcd for $C_{16}H_{21}ClN_2O$: C, 65.62; H, 7.24; N, 9.57. Found: C, 65.48; H, 7.35; N, 9.29.

Amide **6**: Eluent, diethyl ether–hexane (1 : 1); mp 190—193 °C (ethyl acetate–hexane); $[\alpha]_D^{20}$ −53.2° (c=0.2, MeOH). IR ν_{max} cm^{-1}: 3320, 1725, 1685, 1665, 1605, 1515, 1155, 1140. NMR δ: 0.98 (6H, d, J=6 Hz), 1.44 (9H, s), 1.74 (3H, m), 4.42 (1H, m), 5.52 (1H, d, J=8 Hz), 6.74 (2H, m), 7.80 (6H, m), 9.10 (1H, s). *Anal.* Calcd for $C_{25}H_{35}N_5O_3$: C, 66.20; H, 7.78; N, 15.44. Found: C, 66.48; H, 7.73; N, 15.51.

Amide **7**: Eluent, ethyl acetate–hexane (1 : 3); mp 126—128 °C (ethyl acetate–hexane); $[\alpha]_D^{20}$ −32.8° (c=1, MeOH). IR ν_{max} cm^{-1}: 3290, 1670, 1590, 1530, 1330. NMR δ: 1.02 (6H, t, J=6 Hz), 1.52 (9H, s), 1.80 (3H, m), 4.54 (1H, m), 5.72 (1H, m), 7.74 (2H, m), 7.88—8.26 (4H, m), 8.30 (1H, s), 9.72 (1H, s). *Anal.* Calcd for $C_{25}H_{28}N_2O_5$: C, 68.79; H, 6.47; N, 6.42. Found: C, 68.46; H, 6.18; N, 6.29.

Amide **8**; Eluent, chloroform–methanol (30 : 1); mp 163—166 °C (ethyl acetate); $[\alpha]_D^{20}$ −26.3° (c=1, MeOH). IR ν_{max} cm^{-1}: 3260, 1710, 1665, 1620, 1555, 1505, 1295, 1280, 1255, 1210. NMR δ: 0.7—2.1 (18H, m), 4.45 (1H, m), 5.89 (1H, br), 7.2—8.9 (6H, m), 9.92 (1H, br). *Anal.* Calcd for $C_{20}H_{27}N_3O_4$: C, 64.32; H, 7.29; N, 11.25. Found: C, 64.15; H, 7.11; N, 11.20.

Amides **9** and **10**: The crude product was purified by column chromatography on silica gel with ethyl acetate–hexane (2 : 1), followed by preparative layer chromatography with ethyl acetate, giving the amides **9** (*Rf* 0.37) and **10** (*Rf* 0.62). Amide **9**, mp 177—179 °C (ethyl acetate–hexane), $[\alpha]_D^{20}$ −48.5° (c=1, MeOH). IR ν_{max} cm^{-1}: 3320, 1715, 1690, 1615, 1550, 1240, 1175. NMR δ: 0.95 (4H, d, J=6 Hz), 1.2—2.1 (18H, m), 4.38 (1H, m), 5.73 (1H, d, J=8 Hz), 7.03—8.10 (8H, m), 8.25 (1H, s), 8.87 (1H, d, J=8 Hz), 9.77 (1H, s). *Anal.* Calcd for $C_{26}H_{31}N_3O_4$: C, 69.46; H, 6.95; N, 9.35. Found: C, 69.39; H, 6.91; N, 9.27. Amide **10**: IR ν_{max} cm^{-1}: 3260, 1675, 1510, 1240, 755. NMR δ: 0.97 (d, J=4 Hz) and 1.2—2.15 (m, 18H), 4.48 (1H, m), 5.27 (1H, br), 6.93—8.25 (10H, m), 9.30 (1H, br).

Boc–L-Lys(Z)–*p*NA (**11**): Eluent, ethyl acetate–hexane (1 : 2); mp 113—114 °C (ethyl acetate–hexane), $[\alpha]_D^{20}$ −8.3° (c=1, MeOH) (lit.[15] $[\alpha]_D^{25}$ −9.0° (c=0.9, MeOH)). IR ν_{max} cm^{-1}: 3300, 1675, 1615, 1595, 1550, 1500, 1340, 1250, 1160, 1110, 850, 750. NMR δ: 0.72—2.16 (1H, m), 3.20 (2H, m), 4.44 (1H, m), 5.13 (2H, s), 5.64 (1H, m), 7.36 (5H, s), 7.68 (2H, d, J=9 Hz), 8.12 (2H, d, J=9 Hz), 9.58 (1H, s). *Anal.* Calcd for $C_{25}H_{32}N_4O_7$: C, 59.99; H, 6.44; N, 11.19. Found: C, 59.55; H, 6.42; N, 11.67.

Boc–L-Arg(Mts)–*p*NA (**12**): Eluent, ethyl acetate–hexane (3 : 2); an amorphous solid; $[\alpha]_D^{20}$ +3.9° (c=1, MeOH). IR ν_{max} cm^{-1}: 3270, 1680, 1590, 1540, 850. NMR δ: 1.40 (9H, s), 1.70 and 1.88 (4H, m), 2.23 (3H, s), 2.62 (6H, s), 3.23 (2H, m), 4.50 (1H, m), 5.68 (1H, d, J=8 Hz), 6.38 (2H, s), 6.88 (2H, s), 7.24 (1H, s), 7.74 (2H, d, J=9 Hz), 8.08 (2H, d, J=9 Hz), 9.58 (1H, s). *Anal.* Calcd for $C_{26}H_{36}N_6O_7S$: C, 54.14; H, 6.30; N, 14.58. Found: C, 54.27; H, 6.42; N, 14.26.

General Procedure for the Formation of Peptide Bonds with DPPA or DEPC——DPPA or DEPC (1.1 eq) in DMF was added to an ice-cooled mixture of the amine and the carboxyl components in DMF, followed by the

addition of TEA in DMF, as described in our previous paper.[9] The mixture was stirred with ice-cooling for 2 h, then at room temperature overnight. The mixture was diluted with ethyl acetate–benzene (4 : 1, ten times the quantity of DMF), and washed successively with 10% aqueous citric acid, water, saturated aqueous sodium bicarbonate, water, and saturated aqueous sodium chloride. The organic layer was dried over sodium sulfate, and concentrated *in vacuo*. The crude residue was purified by column chromatography and/or recrystallizations.

Boc–L-Glu(OMe)–Gly–OBu^t——(i) With DEPC: The reaction was carried out according to the general procedure using Boc–L-Glu(OMe)–OH (1.20 g, 4.6 mmol), H–Gly–OBu^t (503 mg, 3.8 mmol), DEPC (810 mg, 5.0 mmol), and TEA (470 mg, 4.6 mmol) in DMF (12 ml). Work-up as usual quantitatively gave the dipeptide, mp 47—49.5 °C (diethyl ether–hexane), $[\alpha]_D^{23}$ −10.3° (c = 1, AcOEt). IR ν_{max} cm^{-1}: 3320, 1740, 1675, 1520. NMR δ: 1.44 (9H, s), 1.46 (9H, s), 1.6—2.6 (4H, m), 3.70 (3H, s), 3.92 (2H, d, J = 7 Hz), 4.12 (6H, m), 5.56 (1H, br), 7.00 (1H, br). *Anal.* Calcd for $C_{17}H_{30}N_2O_7$: C, 54.52; H, 8.09; N, 7.48. Found: C, 54.27; H, 8.14; N, 7.51.

(ii) With DPPA: Analogously obtained in quantitative yield.

Z–L-Ile–L-Glu(OMe)–Gly–OBu^t——(i) With DEPC: A mixture of Boc–L-Glu(OMe)–Gly–OBu^t (370 mg, 1 mmol) and 10% hydrogen chloride in ethyl acetate (10 ml) was stirred at room temperature for 20 min. The solvent was removed *in vacuo*, ethyl acetate (10 ml) was added to the residue, and the solvent was removed *in vacuo*. This work-up was carried out twice to give the hydrochloride. The reaction of the hydrochloride with Z–L-Ile–OH (530 mg, 2 mmol) and DEPC (340 mg, 2.1 mmol) in the presence of TEA (310 mg, 3 mmol) was carried out in DMF (5 ml) as described above. Column chromatographic purification with ethyl acetate–hexane (1 : 1) gave a white powder (228 mg, 44%), mp 129—133 °C. IR ν_{max} cm^{-1}: 3280, 1735, 1690, 1630, 700. NMR δ: 0.52—2.26 (22H, m), 3.63 (3H, s), 3.72—4.26 (3H, m), 4.68 (1H, br), 5.08 (2H, s), 5.81 (1H, br), 7.32 (7H, s).

(ii) With DPPA: Analogously obtained in 40% yield.

Bz–L-Ile–L-Glu(OMe)–Gly–OBu^t——Z–L-Ile–L-Glu(OMe)–Gly–OBu^t (1 g) in methanol (50 ml) was hydrogenated over 5% Pd–C (0.2 g) at room temperature for 5 h. Concentration of the solvent *in vacuo* afforded a colorless oil (655 mg, 87%), which was dissolved in tetrahydrofuran (7 ml). Benzoyl chloride (351 mg, 2.5 mmol) and TEA (253 mg, 2.5 mmol) were added dropwise, and the mixture was stirred with ice-cooling for 1 h, then at room temperature for 2 h. Work-up as in the general procedure for peptide bond formation gave colorless needles (613 mg, 74%), mp 199—202 °C (ethyl acetate), $[\alpha]_D^{20}$ −37.4° (c = 1, MeOH). IR ν_{max} cm^{-1}: 3260, 1735, 1675, 1620, 690. NMR δ: 0.68—2.62 (2H, m), 3.4—4.2 (7H, m), 4.78 (2H, m), 7.8—8.0 (6H, m). *Anal.* Calcd for $C_{25}H_{37}N_3O_7$: C, 61.08; H, 7.59; N, 8.55. Found: C, 61.24; H, 7.72; N, 8.71.

Bz–L-Ile–L-Glu(OMe)–Gly–OH——Trifluoroacetic acid (1.5 ml) was added to a stirred suspension of Bz–L-Ile–L-Glu(OMe)–Gly–OBu^t (220 mg, 0.45 mmol) in dichloromethane (1 ml). The mixture was stirred at room temperature for 2 h, and concentrated *in vacuo*. Benzene (2 ml) was added to the residue, and then removed *in vacuo*. This work-up was repeated three times to give a colorless foam (190 mg, 99%), which was directly used for the condensation with the Arg derivative.

H–L-Arg(Mts)–*p*NA·HCl——The amide **12** (576 mg, 1 mmol) was dissolved in 10% hydrogen chloride in ethyl acetate (10 ml), and the mixture was stirred for 17 h. The precipitated crystals were filtered off, and washed with ethyl acetate to give the hydrochloride (499 mg, 98%).

Bz–L-Ile–L-Glu(OMe)–Gly–L-Arg(Mts)–*p*NA——(i) With DEPC: Bz–L-Ile–L-Glu(OMe)–Gly–OH (190 mg, 0.45 mmol) was coupled with H–L-Arg(Mts)–*p*NA·HCl (461 mg, 0.90 mmol) using DEPC (88 mg, 0.54 mmol) and TEA (137 mg, 1.35 mmol) in DMF (5 ml) according to the general procedure. Purification of the crude product was done by column chromatography with ethyl acetate–tetrahydrofuran (4 : 1), giving a colorless powder (361 mg, 58.5%), mp 203—207 °C, $[\alpha]_D^{20}$ +4.4° (c = 0.22, DMF). IR ν_{max} cm^{-1}: 3400, 1740, 1630, 1550, 1510, 1340, 1110, 850. *Anal.* Calcd for $C_{42}H_{55}N_9O_{11}S \cdot H_2O$: C, 55.31; H, 6.30; N, 13.82. Found: C, 55.79; H, 6.09; N, 13.52.

(ii) With DPPA: Analogously obtained in 56% yield.

Bz–L-Ile–L-Glu(OMe)–Gly–L-Arg–*p*NA (2)——The above Mts–*p*NA (50 mg, 0.055 mmol) was added to a stirred mixture of methanesulfonic acid (0.5 ml) and anisole (0.1 ml). The whole was stirred at room temperature for 1 h, then diethyl ether (6 ml) was added. The precipitated oil was washed with diethyl ether and dissolved in a small quantity of methanol. TEA (780 mg, 7.7 mmol) was added, and the mixture was stirred for 0.5 h. After removal of the solvent *in vacuo*, the residue was chromatographed on silica gel (5 g) with chloroform–methanol (10 : 3). The crude product was further subjected to preparative layer chromatography with chloroform–methanol (5 : 2) to give a pale yellow powder (28 mg, 70%), which was identical with an authentic sample on thin layer chromatography using various solvent systems. The product was further passed through a Sephadex LH-20 column with methanol, hydrolyzed with 6 N hydrochloric acid and subjected to amino acid analysis: Ile, 0.91; Glu, 1.01; Gly, 1.01; Arg, 1.0.

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Acid-Induced Rearrangement of Triterpenoid Hydrocarbons Belonging to the Hopane and Migrated Hopane Series

HIROYUKI AGETA,* KENJI SHIOJIMA and YÔKO ARAI

Shôwa College of Pharmaceutical Sciences, 5–1–8 Tsurumaki, Setagaya-ku, Tokyo 154, Japan

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The acid-catalyzed rearrangement of triterpenoid monoenes belonging to the hopane and migrated hopane series with sulfuric acid and boron trifluoride etherate was investigated. By selecting the reaction conditions, a variety of the monoenes of these series, including three new compounds, 9*β*H-fern-7-ene (**3c**), 8*β*H-fern-9(11)-ene (**4b**), and adian-5(10)-ene (**5b**), were obtained. For comparison, oleanenes and migrated oleanenes were also subjected to the same reaction.

Keywords——acid-induced rearrangement; triterpenoid hydrocarbon; hopane, migrated hopane; 9*β*H-fern-7-ene; 8*β*H-fern-9(11)-ene; adian-5(10)-ene; sulfuric acid; boron trifluoride

Introduction

The acid catalyzed rearrangement of triterpenoid monoenes is one of their most interesting features. Analyses of the reaction products have been used to establish the structures of the carbon skeletons of the so-called migrated triterpenoids, such as friedelin,[1] glutinone (alnusenone),[2] multiflorenol, [3] bauerenol,[4] butyrospermol,[5] and various kinds of triterpenoid hydrocarbons of the migrated hopane series.[6,7] On the other hand, systematic investigations of the reaction products were also reported for friedel-3-ene with mineral[8,9] and Lewis acid.[10,11] In addition, experiments have been conducted on 3,4-epoxides of friedelane,[12] shionane,[13] and *D*:*A*-friedolupane[14] with boron trifluoride etherate. The present paper deals with a mineral acid (H_2SO_4)- and a Lewis acid (BF_3-etherate)-catalyzed rearrangement of triterpenoid monoenes having hopane and migrated hopane (neohopane, fernane, adianane and filicane) skeletons under various conditions (concentration of acid, temperature and reaction time). For comparison, some oleanenes and migrated oleanenes were also treated under similar conditions.

Results

Rearrangements of Hopenes with Sulfuric Acid

A similar reaction has been reported for zeorin[15] and hydroxyhopanone.[16] The reaction products of hop-22(29)-ene (**1a**)[17,18] and hop-21-ene (**1b**)[17] under various conditions are shown in Table I. Treatment of **1a** under mild conditions (1 N, 20 °C, 12 h) gave hop-17(21)-ene (**1c**)[17] in more than 90% yield with a small amount of neohop-13(18)-ene (**2b**).[17] With 2 N sulfuric acid, **1a** afforded **1c** and **2b** in various ratios according to the reaction temperature. Interestingly, the temperature giving the highest yield of **2b** was found to be 30—40 °C. With 4 N sulfuric acid at 20 °C for 96 h, **1a** gave **2b** in 95% yield.

The reaction products from **1b** were almost the same as those of **1a** under similar conditions. On the other hand, the reaction products mentioned above, **1c** or **2b**, needed more

TABLE I. Rearrangement Products from Hopenes with H_2SO_4

Starting materials	Conditions			% yields	
				1c	2b
1a	1 N	20 °C	12 h	94	6
	2 N	20 °C	12 h	45	55
			96 h	28	72
		30 °C	12 h	5	92
		40 °C	12 h	7	90
		50 °C	12 h	25	74
	4 N	20 °C	12 h	53	47
			36 h	25	75
			72 h	14	86
			96 h	5	95
1b	1 N	20 °C	24 h	95	5
	2 N	20 °C	24 h	60	40
		40 °C	12 h	13	87
	4 N	20 °C	24 h	30	70
1c	1 N	20 °C	12 h	91	8
	2 N	20 °C	12 h	66	31
		50 °C	12 h	69	25
	4 N	20 °C	12 h	19	79
2b	1 N	20 °C	12 h		100
	2 N	20 °C	12 h		100
		50 °C	12 h	23	75
	4 N	20 °C	12 h		100

TABLE II. Rearrangement Products from Hopenes with BF_3-Etherate

Starting materials	Conditions			% yields		
				1b	1c	2b
1a	(In ether)					
	10%	20 °C	12 h		100	
	20%	20 °C	12 h		100	
	(In AcOH–ether 1 : 1)					
	10%	20 °C	24 h		100	
	20%	20 °C	24 h		100	
			48 h		90	10
			336 h		40	60
1b	(In ether)					
	10%	20 °C	24 h	65	35	
			48 h	35	65	
			72 h		100	
	20%	20 °C	24 h	2	98	
			48 h		100	
	(In AcOH–ether 1 : 1)					
	10%	20 °C	24 h		100	
			48 h		95	5
			72 h		90	10
			336 h		40	60

drastic conditions as shown in Table I. In conclusion, the products formed from **1a**, **b**, **c** and **2b** were found to be mixtures of **1c** and **2b**, and no other hydrocarbon was found. In addition, the fact that the ratios of reaction products **1c** and **2b** formed from **1a** or **2a** with 2 N sulfuric acid, were different, suggests that they were not simply an equilibrium mixture.

Rearrangements of Hopenes with BF_3-Etherate

Next, **1a** and **1b** were treated with boron trifluoride-etherate in ether and in ether–acetic acid (1 : 1). The reaction products under various conditions are summarized in Table II. In ether solution, both **1a** and **1b** gave **1c** as the sole product, and **1a** was found to be more reactive than **1b**. In ether–acetic acid solution, the formation of **2b** was also observed. Thus, boron trifluoride-etherate in ether was a very good reagent to produce **1c** from **1a** or **1b**.

Rearrangement of Migrated Hopenes with Sulfuric Acid

Treatment of filic-3-ene (**6**)[7] with sulfuric acid in acetic acid–benzene under very mild conditions (1/4 N, 20 °C, 12 h) gave adian-5-ene (**5a**)[7] and adian-5(10)-ene (**5b**)[7] together with unchanged starting material. Under more severe condition (1/2 N, 20 °C, 12 h) the products were **5a**, **b** and fern-8-ene (**3b**).[6] Moreover, with 1 N sulfuric acid (20 °C, 12 h) **3b** was formed in a very pure state from **6**, while with 2 N and 4 N sulfuric acid at the same temperature, **6** gave almost the same equilibrium mixture of **3b** and a new compound, 8βH-fern-9(11)-ene (**4b**), in the ratio of 2 : 1. At a higher temperature (2 N, 40 °C, 12 h) **6** gave **4b**, **3b**, **2b** and **1c** with a second new compound, 9βH-fern-7-ene (**3c**). At 50 °C with 2 N sulfuric acid, **6** gave a mixture of **4b**, **3b**, **2b** and **1c**. Thus, treatment of a migrated hopene (**6**), yielded various kinds of compounds, **5a**, **b**, **4b**, **3b**, **c**, **2b** and **1c**, according to the concentration of acid used and the reaction temperatures.

TABLE III. Rearrangement Products from Migrated Hopenes with H_2SO_4

Starting materials	Conditions			% yields								
				6	5a	5b	4a	4b	3b	3c	2b	1c
6	1/4 N	20 °C	12 h	34	14	52						
	1/2 N	20 °C	12 h		10	50			40			
	1 N	20 °C	12 h						100			
		40 °C	12 h					35	56		6	1
	2 N	20 °C	12 h					33	66			
		40 °C	12 h					25	47	4	18	2
		50 °C	12 h					15	30		45	10
	4 N	20 °C	12 h					33	66			
5a	1 N	20 °C	12 h					33	66			
	2 N	20 °C	12 h					33	66			
	4 N	20 °C	12 h					33	66			
4a	1 N	20 °C	12 h				5		95			
	2 N	20 °C	12 h					33	66			
		30 °C	12 h					9	90			
		40 °C	12 h					6	37	10	33	3
		50 °C	12 h								74	25
3a	1 N	20 °C	12 h					33	65			
	2 N	20 °C	12 h					33	66			
	4 N	20 °C	12 h					32	63			
3b	1 N	20 °C	12 h						100			
	2 N	20 °C	12 h						100			
		50 °C	12 h					18	36		36	9
	4 N	20 °C	12 h					33	66			
3c	2 N	20 °C	12 h					33	66			
4b	2 N	20 °C	12 h					33	66			
2a	1 N	20 °C	12 h								99	
	2 N	20 °C	12 h								94	5

Other natural compounds, adian-5-ene (**5a**),[7] fern-9(11)-ene (**4a**),[6] fern-7-ene (**3a**)[7] and neohop-12-ene (**2a**)[19] were also subjected to rearrangement reactions. Compound **5a** or **3a** gave the equilibrium mixture of **4b** and **3b** under the conditions used. Compound **4a** furnished **3b** under mild conditions (1 N, 20 °C, 12 h) and the mixture of **4b** and **3b** with stronger acid (2 N, 20 or 30 °C, 12 h). However, at a higher temperature (2 N, 40 °C, 12 h), **4a** gave **4b**, **3b**, **c**, **2b** and **1c** as in the case of **6**. At 50 °C with 2 N sulfuric acid, **4a** gave a mixture of **2b** and **1c**, as in the case of **1a** or **1b** (Table I). One of the reaction products (also found in some ferns), **3b**, seemed to be rather resistant to the acid, but at 2 N, 50 °C, or 4 N, 20 °C the products were very similar to those formed from **6** under the same conditions. The other reaction product, **3c** or **4b**, yielded the mixture of **4b** and **3b** under the usual conditions.

Finally, **2a** was found to be rather unstable to acid, giving **2b** exclusively under mild conditions. It was also found that **2a** changes readily to **2b** during chromatography on neutral alumina.

Structures of New Reaction Products, 3c, 4b, and 5b

Compound **3c**, mp 195—197 °C, was shown to have the molecular formula $C_{30}H_{50}$ by mass (MS) and elemental analysis. The MS fragments of **3c** were similar to those of **3a**, but their relative intensities were remarkably different. The ^{1}H-chemical shifts of eight methyls and one olefinic proton of **3c** were assigned, as shown in Table V, while those of three methyl signals (C-25, C-26 and C-27) were found to be different from those of the corresponding

Table IV. Physical Constants and Mass Fragments of the Compounds

	mp (°C)	$[\alpha]_D$	Rt_R	Mass fragments (70/eV) m/z (rel. int.)
Hop-22(29)-ene (**1a**)	211—212	+60.2	2.61	410 (30), 395 (9), 367 (3), 299 (8), 218 (13), 205 (14), 204 (15), 203 (12), 191 (100), 189 (91)
Hop-21-ene (**1b**)	194—195	+29.8	2.67	410 (50), 395 (13), 367 (34), 341 (52), 231 (28), 218 (12), 205 (19), 203 (18), 191 (100), 189 (89), 161 (74)
Hop-17(21)-ene (**1c**)	188—189	+50.0	1.67	410 (59), 395 (17), 367 (100), 231 (80), 203 (16), 191 (71), 189 (45), 175 (45), 161 (75), 136 (95), 135 (100)
Neohop-12-ene (**2a**)	210—211	+41.6	2.25	410 (7), 395 (2), 367 (1), 218 (100), 203 (33), 191 (28), 175 (48)
Neohop-13(18)-ene (**2b**)	200—201	+2.9	1.90	410 (20), 395 (4), 367 (3), 229 (23), 218 (58), 206 (27), 205 (61), 204 (36), 203 (24), 191 (100), 189 (27), 175 (25)
Fern-7-ene (**3a**)	212—214	−27.8	2.26	410 (29), 395 (81), 271 (14), 257 (22), 243 (100), 231 (19), 205 (11), 203 (9), 191 (11), 189 (9)
Fern-8-ene (**3b**)	190—191	+25.3	1.91	410 (31), 395 (95), 271 (3), 257 (17), 243 (100), 231 (17), 205 (5), 203 (5), 191 (5), 189 (5)
9βH-Fern-7-ene (**3c**)	195—197	−148.0	1.98	410 (98), 395 (100), 286 (35), 271 (28), 257 (27), 243 (78), 231 (30), 205 (60), 203 (41), 191 (21), 189 (23)
7-Oxofern-8-ene (**3d**)	185—186	+26.0	2.90	424 (100), 409 (90), 381 (16), 354 (6), 271 (29), 257 (53), 245 (61), 201 (8), 189 (10)
Fern-9(11)-ene (**4a**)	170—171	−18.1	2.00	410 (32), 395 (86), 271 (4), 257 (20), 243 (100), 231 (16), 205 (8), 203 (7), 191 (9)
8βH-Fern-9(11)-ene (**4b**)	197—198	−104.4	2.05	410 (38), 395 (92), 271 (6), 257 (22), 243 (100), 231 (26), 205 (13), 203 (10), 191 (10), 189 (9)
Ferna-7,9(11)-diene (**4c**)	201—202	−189.5	1.87	408 (100), 393 (27), 365 (5), 323 (7), 255 (84), 243 (14), 241 (19), 229 (14), 215 (11), 199 (9), 187 (11)
12-Oxofern-9(11)-ene (**4d**)	222—223	−31.0	3.57	424 (95), 409 (100), 381 (7), 339 (71), 271 (66), 257 (14), 231 (8), 218 (16), 201 (11), 191 (10), 135 (95)
12-Oxo-8βH-fern-9(11)-ene (**4e**)	188—190	−168.0	3.60	424 (65), 409 (95), 381 (10), 339 (70), 271 (69), 257 (16), 231 (5), 218 (6), 201 (9), 135 (100)
Adian-5-ene (**5a**)	194—195	+51.9	2.15	410 (10), 395 (8), 367 (2), 274 (100), 259 (67), 231 (18), 205 (14), 191 (9), 189 (13)
Adian-5(10)-ene (**5b**)	208—209	−54.7	2.00	410 (100), 395 (65), 325 (5), 257 (8), 243 (11), 231 (12), 205 (49), 189 (31), 175 (41)
Adiana-1(10),5-diene (**5c**)	209—210	+105.0	2.11	408 (68), 393 (16), 365 (6), 255 (9), 241 (8), 229 (16), 203 (100), 190 (95), 187 (39), 173 (46)
Filic-3-ene (**6**)	232—234	+50.0	2.80	410 (94), 395 (47), 367 (10), 340 (9), 325 (33), 287 (23), 274 (21), 259 (19)
Olean-18-ene (**11**)	174—175	+6.2	1.57	410 (20), 395 (19), 233 (11), 229 (6), 218 (24), 204 (100), 191 (44), 189 (70), 177 (70)
Olean-12-ene (**12a**)	162—164	+96.2	1.57	410 (6), 395 (3), 257 (4), 218 (100), 203 (33), 191 (16), 189 (11)
Olean-13(18)-ene (**12b**)	192—193	−53.4	1.57	410 (18), 395 (10), 257 (4), 218 (100), 205 (27), 204 (31), 191 (29), 189 (24)
18αH-Olean-12-ene (**12c**)	205—206	+37.0	1.57	410 (10), 395 (17), 257 (10), 243 (5), 231 (6), 218 (100), 203 (39), 191 (26), 189 (12)
Multiflor-7-ene (**13a**)	146—147	−20.0	1.87	410 (9), 395 (14), 271 (7), 257 (9), 243 (47), 231 (46), 218 (19), 206 (26), 205 (55), 204 (100), 191 (24), 189 (12)
Multiflor-8-ene (**13b**)	188—189	+58.0	1.63	410 (16), 395 (20), 257 (9), 243 (100), 231 (72), 218 (20), 206 (36), 205 (53), 191 (35)
Multiflor-9(11)-ene (**14a**)	163—166	−2.0	1.65	410 (18), 395 (20), 257 (12), 243 (50), 231 (40), 218 (100), 206 (40), 205 (38), 203 (28), 191 (75)
Glutin-5-ene (**15a**)	182—184	+57.4	1.80	410 (16), 395 (10), 294 (100), 259 (86), 245 (10), 231 (8), 218 (10), 205 (47), 189 (20)
Glutin-5(10)-ene (**15b**)	231—233	−44.0	1.67	410 (69), 395 (38), 243 (7), 231 (18), 205 (100), 189 (66)
Friedel-3-ene (**16**)	272—273	−18.0	2.29	410 (43), 395 (30), 287 (12), 274 (20), 257 (24), 231 (20), 218 (100), 205 (59), 191 (41), 189 (39)

Table V. ¹H-Chemical Shifts (δ) in $CDCl_3$ Solution (JEOL FX 100)

	Methyl signals								Olefinic proton attached to C[ ]
	C-23	C-24	C-25	C-26	C-27	C-28	C-29	C-30	
1a	0.846	0.994	0.818	0.964	0.948	0.728	—	1.790	[29] 4.78 dd (0.9, 0.9)
1b	0.850	0.796	0.818	0.970	0.970	0.587	1.571	1.728	—
1c	0.846	0.794	0.836	0.938	1.044	0.846	0.917 d	0.978 d (6.9)	—
2a	0.858	0.816	0.878	0.738	1.126	0.760	0.847 d	0.936 d (6.4)	[12] 5.06 ddd (2.4, 2.4, 4.5)
2b	0.858	0.794	0.824	0.858	1.100	0.794	0.888 d	0.935 d (6.4)	—
3a	0.844	0.878	0.742	0.996	0.906	0.742	0.830 d	0.898 d (6.6)	[7] 5.35 ddd (3.2, 3.2, 3.7)
3b	0.876	0.828	0.946	0.946	0.770	0.770	0.826 d	0.888 d (6.2)	—
3c	0.888	0.878	0.888	1.096	0.706	0.782	0.832 d	0.902 d (6.6)	[7] 5.24 m ($W_{1/2h}$ 9.7)
3d	0.912	0.872	0.980	1.134	0.774	0.764	0.826 d	0.888 d (6.2)	—
4a	0.858	0.892	1.054	0.736	0.824	0.760	0.830 d	0.890 d (6.4)	[11] 5.29 ddd (3.0, 3.4, 4.0)
4b	0.898	0.830	1.020	0.898	0.830	0.762	0.830 d	0.898 d (6.7)	[11] 5.13 dd (3.6, 3.6)
4c	0.858	0.912	0.912	0.912	0.705	0.759	0.830 d	0.899 d (6.9)	[7] 5.40 m ($W_{1/2h}$ 10.0)
									[11] 5.15 br dd (4.0, 4.0)
4d	0.880	0.916	1.104	0.784	1.202	0.822	0.830 d	0.890 d (6.5)	[11] 5.64 d (2.7)
4e	0.872	0.966	1.100	0.872	1.236	0.798	0.840 d	0.904 d (6.5)	[11] 5.49 s
5a	1.002	1.058	0.836	1.002	0.924	0.782	0.824 d	0.894 d (6.4)	[5] 5.51 ddd (2.5, 2.5, 4.9)
5b	0.906	0.944	0.944	0.944	0.944	0.798	0.822 d	0.888 d (6.6)	—
5c	0.910	1.080	0.910	0.996	0.910	0.774	0.826 d	0.890 d (6.3)	[1] 5.39 dd (3.5, 3.5)
									[6] 5.57 br dd (3.5, 3.5)
6	1.574	0.988	0.900	0.920	0.920	0.782	0.824 d	0.888 d (6.4)	[3] 5.15 m ($W_{1/2h}$ 8.0)
11	0.846	0.802	0.876	1.078	0.746	1.020	0.938	0.938	[18] 4.86 d (1.5)
12a	0.872	0.822	0.932	0.968	1.144	0.834	0.872	0.872	[12] 5.19 dd (3.4, 3.4)
12b	0.856	0.796	0.856	0.856	1.164	1.008	0.702	0.954	—
12c	0.868	0.816	0.956	0.988	1.154	0.616	0.944	0.894	[12] 5.13 ddd (1.5, 3.6, 4.0)
13a	0.854	0.888	0.740	1.074	1.098	1.058	0.970	0.970	[7] 5.47 ddd (3.0, 3.0, 3.4)
13b	0.878	0.834	0.954	1.056	1.000	1.072	0.968	0.954	—
14a	0.848	0.898	1.054	0.784	0.898	1.054	0.976	0.976	[11] 5.30 ddd (2.5, 2.5, 5.0)
15a	1.002	1.062	0.792	1.002	1.084	1.160	0.988	0.952	[5] 5.22 ddd (2.0, 2.5, 5.0)
15b	0.914	0.946	0.946	1.008	1.008	1.184	0.958	1.007	—
16	1.578	0.998	0.860	0.998	0.998	1.174	0.946	0.998	[3] 5.16 m ($W_{1/2h}$ 6.5)

Assignments were confirmed by the $CDCl_3$–C_6D_6 solvent shift method. All signals, unless otherwise stated, are singlets. Coupling constants are shown in parentheses.

signals of **3a**. These observations suggested **3c** to be 9βH-fern-7-ene, a 9H-isomer of **3a**. To confirm the structure of **3c**, 7-oxofern-8-ene (**3d**), prepared from **3b** by CrO_3 oxidation, was reduced by the Wolff–Kishner method, to give two isomeric hydrocarbons, one of which was proved to be identical with **3a** and the other with **3c** [mp, relative retention time (Rt_R), and infrared (IR) and proton nuclear magnetic resonance (¹H-NMR) spectra].

Compound **4b**, mp 197—198 °C, was also indicated to have the molecular formula $C_{30}H_{50}$ by MS and elemental analysis. The fact that the fragmentation pattern of **4b** (Table IV) was indistinguishable from that of **4a**, suggested that **4b** is an isomer of **4a**. The ¹H-chemical shifts of four singlet methyl signals (C-23, C-24, C-27, and C-28) of **4b** were very similar to those of **4a**, while the other two (C-25 and C-26) were extraordinarily different. This observation was explained by considering the structures of **4a** to be 8αH-fern-9(11)-ene (ring B boat), and **4b** to be 8βH-fern-9(11)-ene (ring B chair), because the anisotropic effects of the 9(11)-double bond on the C-25 and C-26 methyls are different. Oxidation of **4b** with SeO_2 gave ferna-7,9(11)-diene (**4c**), which was identified by direct comparison with an authentic sample.[6] Meanwhile, treatment of **4b** with CrO_3 afforded 8βH-fern-9(11)-en-12-one (**4e**), confirming the structure of **4b**. The ¹H-chemical shifts of methyl groups of **4e** and its isomer

4d[6] were assigned, as shown in Table V; one methyl signal (C-26) of **4e** was observed 0.09 ppm to lower fiels than that of **4d**. Moreover, the olefinic proton signal of **4e** was observed as a singlet, while that of **4d** was a doublet (J=2.7 Hz). These observations clearly demonstrated that **4e** has the 8βH structure and **4d** 8αH, because the bond angle of 8βH–11H had been estimated as 50° and that of 8αH–11H as 90°.[20]

Finally, compound **5b**, mp 208—209 °C, was also shown to have the molecular formula $C_{30}H_{50}$ by high-resolution MS spectrum. The fragmentation pattern of **5b** (Table IV) indicated the compound to have a double bond in ring A or B. The ^{1}H-NMR spectrum (Table V) indicated the double bond to be tetrasubstituted and the chemical shifts of six singlet and two doublet methyl signals were reasonably assigned when **5b** was considered to be adian-5(10)-ene.[7] This identification was confirmed by direct comparison (mp, Rt_R, and IR and ^{1}H-NMR spectra) with a sample derived from **5a** *via* adian-1(10),5-diene (**5c**).[7]

Rearrangement of Migrated Hopenes with BF_3-Etherate

The reactions of filic-3-ene (**6**)[7] with boron trifluoride-etherate in ether solution were much slower than those with sulfuric acid, and a considerable amount of the starting material remained unreacted after 12 h, as shown in Table VI. Although the products were generally a mixture of adian-5-ene (**5a**), adian-5(10)-ene (**5b**) and fern-7-ene (**3a**), the formation of **3a** and the absence of fern-8-ene (**3b**) were in contrast to the case with sulfuric acid. Treatment of **6** in ether-acetic acid (1 : 1) with BF_3-etherate (20%, 20 °C, 1 h) gave fern-9(11)-ene (**4a**) and fern-8-ene (**3b**). In both benzene and chloroform solutions, the products from **6** were neohop-13(18)-ene (**2b**) and hop-17(21)-ene (**1c**), together with a large amount of oily mixtures. Fern-9(11)-ene (**4a**) was less reactive and no rearrangement had occurred under any of the conditions

TABLE VI. Rearrangement Products from Migrated Hopenes with BF_3-Etherate

Starting materials	Conditions	% yields							
		6	**5a**	**5b**	**4a**	**3a**	**3b**	**2b**	**1c**
6	(In ether)								
	10% 20 °C 12 h	98	1	1		1			
	20% 20 °C 12 h	91	1	4		4			
	20 °C 168 h	18	12	58		12			
	20% 27 °C 12 h	69	5	13		13			
	30% 20 °C 12 h	44	9	33		14			
	40% 20 °C 12 h	11	12	69		8			
	(In AcOH–ether 1 : 1)								
	10% 20 °C 12 h	26	8	48		4	14		
	20% 20 °C 12 h	18	14	16	24	1	16		
	(In benzene)[a]								
	10% 20 °C 12 h							10	17
	20% 20 °C 10 min							24	35
	30 min							24	18
	(In chloroform)[a]								
	10% 20 °C 12 h							14	19
	20% 20 °C 12 h							15	11
4a	(In ether)								
	10% 20 °C 12 h				100				
	20% 20 °C 12 h				100				
	(In AcOH–ether 1 : 1)								
	10% 20 °C 12 h				100				
	20% 20 °C 12 h				100				

a) Oily by-products of lower molecular weight (GC-MS) were also formed.

mentioned above.

Rearrangements of Oleanenes and Migrated Oleanenes with Sulfuric Acid

The reaction products formed from olean-18-ene (**11**), olean-12-ene (**12a**), friedel-3-ene (**16**), multiflor-9(11)-ene (**14a**) and multiflor-7-ene (**13a**)[21] with sulfuric acid under various conditions are summarized in Table VII. Compounds **11** and **12a** gave mixtures of **12a**, olean-13(18)-ene (**12b**), 18αH-olean-12-ene (**12c**) and **11**. The formation of the former three was already known,[22] but that of the last compound was found for the first time here. Compound **12a** was found to be stable to acid, as compared with **2a**. Compound **16** afforded glutin-5(10)-ene (**15b**) first under mild conditions, and the **12a** in high yield. Compounds **14a** and **13a** also gave **12a** as the main product. These observations indicate that the formation of **12a**, **b**, **c** does

Table VII. Rearrangement Products from Oleanenes and Migrated Oleanenes with H_2SO_4

Starting materials	Conditions			% yields						
				16	**15b**	**13b**	**12a**	**12b**	**12c**	**11**
11	1 N	20 °C	12 h				5	78	12	5
12a	1 N	20 °C	12 h					100		
	2 N	20 °C	12 h				100			
		40 °C	12 h			20	40	20	20	
	4 N	20 °C	12 h				20	40	20	20
16	1/2 N	0 °C	72 h	40	60					
	2 N	0 °C	12 h		20		80			
		20 °C	12 h				80	5	5	10
14a	1/2 N	20 °C	12 h			10	90			
13a	2 N	20 °C	12 h				90	5		5

Table VIII. Rearrangement Products from Friedelene with BF_3-Etherate

Starting materials	Conditions			% yields						
				16	**15a**	**15b**	**12a**	**12b**	**12c**	**11**
16	(In ether)									
	10%	20 °C	12 h	96	1	2	1			
	20%	20 °C	12 h	80	4	6	5			
			168 h	22	8	30	40			
	20%	27 °C	12 h	79	5	10	7			
	30%	20 °C	12 h	37	13	24	25			
	40%	20 °C	12 h	40	12	25	23			
	(In AcOH–ether 1 : 1)									
	10%	20 °C	12 h	42	7	41	10			
	20%	20 °C	12 h	22	5	45	28			
	(In benzene)[a]									
	10%	20 °C	12 h				13	30	13	13
	20%	20 °C	10 min				14	28	14	14
			30 min				13	30	13	13
	(In chloroform)[a]									
	10%	20 °C	12 h	5			8	63	8	16
	20%	20 °C	12 h				9	50	9	19

a) Oily by-products of lower molecular weight (GC-MS) were also formed.

not represent an equilibrium mixture as reported earlier.[22)]

Rearrangements of Friedel-3-ene (16) with BF_3-Etherate

The products formned from **16** with boron trifluoride-etherate under various conditions are shown in Table VIII. In ether or acetic acid–ether solution, the reactions were rather slow, and the products were always **15a**, **b** and **12a**, together with the starting material. Thus, the formation of multiflorane derivative was not observed, in contrast to the case of the migrated

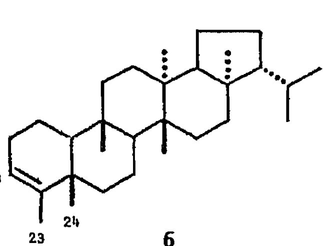
1a : 22(29)-ene
1b : 21-ene
1c : 17(21)-ene

2a : 12-ene
2b : 13(18)-ene

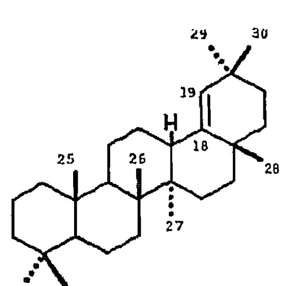
3a : 9αH 7-ene
3b : 8-ene
3c : 9βH 7-ene
3d : 8-ene, 7-oxo

4a : 8αH 9(11)-ene
4b : 8βH 9(11)-ene
4c : 7,9(11)-diene
4d : 8αH 9(11)-ene, 12-oxo
4e : 8βH 9(11)-ene, 12-oxo

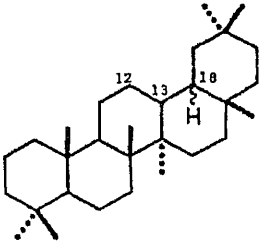
5a : 5-ene
5b : 5(10)-ene
5c : 1(10),5-diene



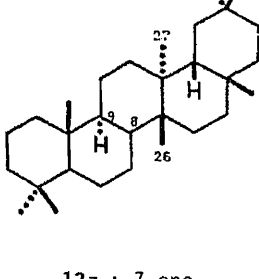
6



11



12a : 18βH 12-ene
12b : 13(18)-ene
12c : 18αH 12-ene



13a : 7-ene
13b : 8-ene

14a

15a 5-ene
15b 5(10)-ene

16

Chart 1

hopane (**6**). On the other hand, in benzene or chloroform solution, the reactions proceeded much more quickly and gave mixtures of four kinds of oleanane derivatives, **12a**, **b**, **c** and **11**. This situation was rather similar to the results with **16** in sulfuric acid, although the main product was **12b** with BF_3 and **12a** with acid.

Discussion

The acid-induced rearrangement of monoenes of the hopane and oleanane series with sulfuric acid and boron trifluoride-etherate in ether are summarized in Charts 2 and 3. The rearrangement of hopenes (**1a** and **1b**) proceeds in a biogenetical direction (**1a**→**1b**→**1c**→**2b**) to give mixtures of **1c** and **2b**, while that of the migrated hopenes (**6**, **5a**, **4a**, **3a** and **2a**) proceeds in the opposite direction to biogenesis [**2b**(**2a**)→**3a**→**3b**→**4a**→**5b**(**5a**)→**6**] to afford similar mixtures of **1c** and **2b**. This situation is also similar in the oleanane series, but the final products are **11** and **12a**, **b**, **c**. The only exception was the formation of **13b** from **12a** (Table VII).

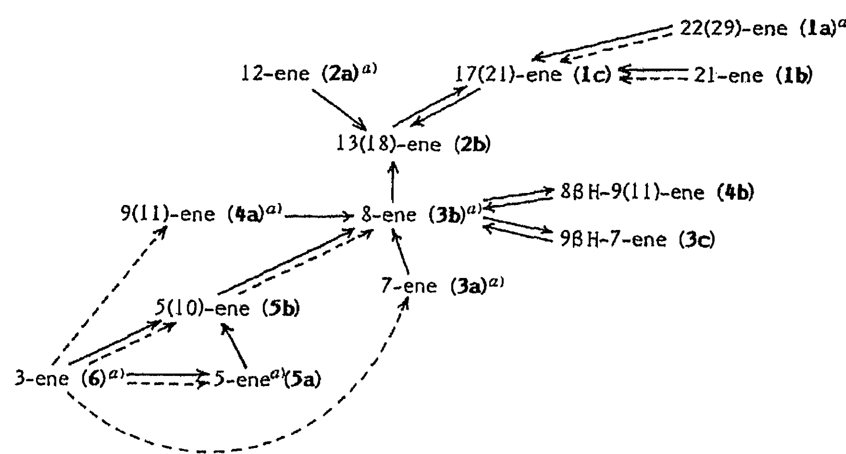


Chart 2. Direction of Rearrangement in the Hopane Series (——→H_2SO_4, ---→BF_3)

a) Naturally occurring compounds.

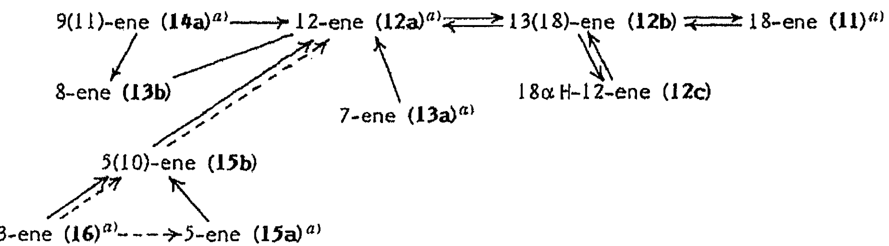


Chart 3. Direction of Rearrangement in the Oleanane Series (——→H_2SO_4, ---→BF_3)

a) Naturally occurring compounds.

The reaction products with sulfuric acid and with boron trifluoride-etherate are rather different. In the former case the products are generally the stable tetrasubstituted monoenes (**1c**, **2b**, **3b** and **5b**) except for **5a** and the two new products (**3c** and **4b**), and most of them do not occur naturally, while in the latter case some trisubstituted monoenes (**3a**, **4a** and **5a**) are formed and they are identical with natural products.

When the reactions of the hopenes and the migrated hopenes are compared with those of the oleanenes and the migrated oleanenes, the latter compounds are much more reactive than the former. The reaction of the 3-ene (**6**) gave various kinds of fernene derivatives (**3b**, **c** and **4b** with acid; **3a**, **b** and **4a** with BF_3) and no neohop-12-ene (**2a**), while that of **16** gave olean-12-ene (**12a**) and no multiflorane derivatives with sulfuric acid as well as with BF_3-etherate. It is noteworthy that, in the hopane series, fernene derivatives are widely distributed among fern plants, while in the oleanane series, olean-12-ene derivatives are the most common natural products.

The treatment of filic-3-ene (**6**) (or other migrated hopenes) with sulfuric acid and boron trifluoride-etherate can afford almost all kinds of known hopenes and migrated hopenes, such as **5a**, **b**, **4a**, **b**, **3a**, **b**, **c**, **2b** and **1c**, according to the conditions used. In many cases, the optimum conditions to produce a desired compound in high yield were found, as summarized in the Tables.

Experimental

Melting points were measured with a Yanagimoto microapparatus and are corrected. The $[\alpha]_D$s were observed in $CHCl_3$ solution ($c=0.3$—0.6) at 22—24 °C. ^1H-NMR spectra were taken at 100 MHz by the FT method with tetramethylsilane as an internal standard. MS spectra were recorded (direct inlet) at 70/eV and the relative intensities of peaks are reported with reference to the most intense peak higher than m/z 100. Gas liquid chromatography (GLC) was performed on a 1 m glass column containing Chromosorb G AW DMCS with 1.4% SE-30 at 260 °C under N_2. Cholestane was used as an internal reference (its retention time was set at about 3.5 min), and the Rt_R values of compounds are given (Table IV).

The starting materials were obtained as described below. Physical constants including MS fragments of the starting materials and the reaction products are shown in Table IV, and ^1H-NMR data in Table V.

Hop-22(29)-ene (1a) and Hop-21-ene (1b)——Hydroxyhopanone[23] obtained from Dammar (Gum Copal C2) was reduced by the Wolff–Kishner–Barton method to give hydroxyhopane, mp 256—258 °C, 1.0 g of which was dehydrated by boiling with Ac_2O (30 ml) and anhyd. K_2CO_3 (3.0 g) for 1 h. The products were separated into **1a** (0.63 g) and **1b** (0.27 g) by chromatography on 20% $AgNO_3$-impregnated silica gel, and the pure products were obtained after recrystallization from Me_2CO.

Neohop-12-ene (2a), Fern-7-ene (3a), Fern-8-ene (3b), Adian-5-ene (5a), and Filic-3-ene (6)——An n-hexane extract of the dried leaves (1.3 kg) of *Adiantum monochlamys* EATON (Pteridaceae),[7,19] collected in August, was chromatographed on silica gel and 20% $AgNO_3$-silica gel. The corresponding fractions were recrystallized from Me_2CO. **2a** 90 mg, **3a** 450 mg, **3b** 470 mg, **5a** 470 mg, **6** 310 mg.

Fern-9(11)-ene (4a)——The dried leaflets (1.2 kg) of *Dryopteris crassirhizoma* NAKAI (Aspidiaceae),[6] collected in August, were treated as described above. **4a** 410 mg.

Olean-18-ene (11), Olean-12-ene (12a), Multiflor-7-ene (13a), Multiflor-9(11)-ene (14a), and Friedel-3-ene (16)——Specimens obtained from the rhizomes of *Polypodium niponicum* METT.[21] were used.

General Procedure of Reaction with Sulfuric Acid——The starting material was dissolved in C_6H_6 and added to H_2SO_4 in AcOH at below the reaction temperature. The amount of the materials used was 1—4 mmol.

	Conc. H_2SO_4 (ml)	AcOH (ml)	C_6H_6 (ml)
1/4 N	0.07	8.00	1.03
1/2 N	0.13	8.00	1.87
1 N	0.27	8.00	1.73
2 N	0.53	7.00	2.47
4 N	1.06	7.00	1.94

The solution was allowed to react in a isothermal apparatus (NKS LP-45-2, ±0.5 °C) (10—50 °C) or in a Coolnics Mixer (0 °C) under a nitrogen atmosphere, then added to ice-water, and extracted with n-C_6H_{14}. The extract was washed with aqueous Na_2CO_3 and water, dried and evaporated to dryness. The residue was checked by GLC, gas chromatography-mass spectrometry (GC-MS) and ^1H-NMR spectroscopy to analyze the components qualitatively and quantitatively. In many cases, the residue was chromatographed on neutral alumina and on 20% $AgNO_3$-silica

gel to separate individual components. The products were recrystallized from Me_2CO and identified by comparison of melting points, Rt_R, and IR and ^{1}H-NMR spectra with those of authentic samples.

General Procedure of Reaction with BF_3-Etherate——The starting material (1—4 mmol) was dissolved in fresh BF_3-etherate solution (v/v%) and treated in the same way as above.

9βH-Fern-7-ene (3c)——Fern-9(11)-ene (**4a**) (120 mg) was treated with 2 N H_2SO_4 solution (20 ml) at 40 °C for 12 h. The oily products were separated by repeated chromatographies on alumina (1 kg) followed by 20% $AgNO_3$-silica gel (40 g), with n-C_6H_{14} eluent, to give **3b** 44 mg, **2b** 40 mg, **4b** 8 mg, **3c** 12 mg, and **1c** 4 mg in order of elution. **3c**, plates, mp 195—197 °C, was obtained by recrystallization from Me_2CO. IR ν_{max}^{KBr} cm^{-1}: 820 (CH=C). *Anal.* Calcd for $C_{30}H_{50}$: C, 87.73; H, 12.27. Found: C, 87.60; H, 12.42.

7-Oxofern-8-ene (3d)——A solution of 1 g of fern-8-ene (**3b**) (1 g) in AcOH (80 ml), C_6H_6 (60 ml), and $CHCl_3$ (15 ml) was treated with CrO_3 (0.4 g) in AcOH (50 ml) at room temperature overnight. The oily products were extracted with Et_2O and chromatographed on silica gel (100 g). The amount of the starting material recovered was 400 mg, and the fraction eluted with n-C_6H_{14}–C_6H_6 was purified by preparative thin layer chromatography [silica gel, n-C_6H_{14}–EtOAc (9:1)] followed by recrystallization from MeOH to give **3d**, mp 185—186 °C. IR ν_{max}^{KBr} cm^{-1}: 1645. UV λ_{max}^{EtOH} nm (ε): 257 (10700). MS m/z. Calcd for $C_{30}H_{48}O$: 424.3704 (M^+). Found: 424.3687.

Wolff–Kishner Reduction of 3d——A solution of 10 mg of **3d** in diethylene glycol was treated with Na (50 mg) and anhydrous hydrazine (2.5 ml) at 210 °C for 15 h. The oily hydrocarbons (7 mg) obtained by Florisil chromatography were found to be a mixture of **3a** and **3c** (1 : 4) by GLC and IR and ^{1}H-NMR spectral examination.

8βH-Fern-9(11)-ene (4b)——Fern-9(11)-ene (**4a**) (500 mg) was treated with 1 N H_2SO_4 solution (200 ml) at 20 °C for 12 h in a nitrogen atmosphere. The oily products were separated by chromatography on alumina (Woelm grade I, 800 g, n-C_6H_{14}) to give **3b** (320 mg), and **4b** (130 mg), the latter of which was recrystallized from Me_2CO to give plates, mp 197—198 °C. IR ν_{max}^{KBr} cm^{-1}: 815 (CH=C). *Anal.* Calcd for $C_{30}H_{50}$: C, 87.73; H, 12.27. Found C, 87.79; H, 12.27.

Ferna-7,9(11)-diene (4c)——A solution of 10 mg of 8βH-fern-9(11)-ene (**4b**) in AcOH (10 ml) was treated with SeO_2 at 95 °C for 1.5 h. An n-C_6H_{14} solution of the product was passed through a silica gel (5 g) column followed by recrystallization from Me_2CO to give **4c**, plates, mp 201—202 °C. IR ν_{max}^{KBr} cm^{-1}: 3030, 1633, 1614, 822, 817, 795 (CH=C–C=CH). UV λ_{max}^{EtOH} nm (ε): 232 (12900), 239 (14900), 248 (9800).

12-Oxo-8βH-fern-9(11)-ene (4e)——Compound **4b** (50 mg) was oxidized with CrO_3 (30 mg) in a AcOH (20 ml) solution under reflux for 1 h. The product was chromatographed on a silica gel (5 g) column with n-C_6H_{14}–C_6H_6 to give 32 mg of **4e**, which was recrystallized from Me_2CO to give plates, mp 188—190 °C. IR ν_{max}^{KBr} cm^{-1}: 1670 (CO), 1620, 860 (CH=C). UV λ_{max}^{EtOH} nm (ε): 241 (10600). MS m/z. Calcd for $C_{30}H_{48}O$: 424.3704 (M^+). Found: 424.3708.

Adian-5(10)-ene (5b)——Filic-3-ene (**6**) (60 mg) was treated with BF_3-etherate (4 ml), Et_2O (18 ml), and AcOH (18 ml) at 20 °C for 12 h. The oily products were separated by repeated chromatography on 20% $AgNO_3$-silica gel to give the following fractions, in order of elution (n-C_6H_{14}): **3b** 10 mg, **5b** 10 mg, **4a** 14 mg, **3a** trace, **5a** 8 mg, **6** 12 mg. Pure **5b**, mp 208—209 °C, needles, was obtained by recrystallization from Me_2CO. MS m/z. Calcd for $C_{30}H_{50}$: 410.3912. Found: 410.3901.

Adiana-1(10),5-diene (5c)——A solution of 250 mg of adian-5-ene (**5a**) in C_6H_6 (10 ml) and AcOH (40 ml) was heated with SeO_2 (270 mg) in a water bath for 1 h. The product was extracted with ether and passed through a silica gel (20 g, contained a small amount of Ag powder) column in n-C_6H_{14} to give 220 mg of **5c**, which was recrystallized from Me_2CO, mp 209—210 °C. IR ν_{max}^{KBr} cm^{-1}: 821, 815. UV λ_{max}^{EtOH} nm (ε): 232 (14800), 240 (16000), 248 (11000).

Catalytic Hydrogenation of 5c——A solution of 100 mg of **5c** in EtOAc (8 ml) and AcOH (2 ml) was hydrogenated with PtO_2 for 4 h. The crystalline products were separated by chromatography on 20% $AgNO_3$-silica gel (10 g) to give 5 mg of **5b** and 75 mg of **5a**.

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Studies on the Chemical Constituents of Rutaceous Plants. LXIV.[1] Structural Establishment of Oxyterihanine, a Phenolic Benzo[*c*]phenanthridone Alkaloid. Syntheses of Phenolic Benzo[*c*]phenanthridine Alkaloids, Terihanine and Isoterihanine, and Related Compounds

HISASHI ISHII,* IH-SHENG CHEN,[2] SATOSHI UEKI, MISAKO AKAIKE, and TSUTOMU ISHIKAWA

Faculty of Pharmaceutical Sciences, Chiba University, 1-33, Yayoi-cho, Chiba 260, Japan

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The structure of oxyterihanine (**2c**), a new phenolic benzo[*c*]phenanthridone alkaloid which was isolated from Formosan *Xanthoxylum nitidum* (ROXB.) D. C. (*Fagara nitida* ROXB.), was established as 8-hydroxy-9-methoxy-5-methyl-2,3-methylenedioxybenzo[*c*]phenanthridin-6(5*H*)-one by direct comparison with a synthetic specimen, which was derived from 2-(4-isopropoxy-3-methoxyphenyl)-6,7-methylenedioxy-3,4-dihydronaphthalen-1(2*H*)-one (**8a**) through the synthetic sequence shown in Charts 3 and 4.

9-Hydroxy-8-methoxy-5-methyl-2,3-methylenedioxybenzo[*c*]phenanthridin-6(5*H*)-one [oxyisoterihanine (**2d**)] and two phenolic quaternary benzo[*c*]phenanthridine alkaloids, terihanine (**1c**) and isoterihanine (**1d**), were also synthesized.

Keywords——phenolic benzo[*c*]phenanthridone alkaloid synthesis; oxyterihanine; structural establishment; oxyisoterihanine; phenolic quaternary benzo[*c*]phenanthridine alkaloid synthesis; terihanine; isoterihanine

It is well known that fully aromatized quaternary benzo[*c*]phenanthridine alkaloids[3] occur naturally in Rutaceous and Papaveraceous plants. These alkaloids, especially nitidine[4] (**1a**) and fagaronine[5] (**1b**), have attracted the interest of a number of chemists because of their antileukemic activities.

In the course of studies on the chemical constituents of Rutaceous plants, we[6] obtained a minute amount of a new phenolic benzo[*c*]phenanthridone alkaloid, designated as oxyterihanine (**2c**), from Formosan *Xanthoxylum nitidum* (ROXB.) D. C. (*Fagara nitida*

1 **2**

a: $R_1+R_2=CH_2$, $R_3=R_4=Me$
b: $R_1=H$, $R_2=R_3=R_4=Me$
c: $R_1+R_2=CH_2$, $R_3=H$, $R_4=Me$
d: $R_1+R_2=CH_2$, $R_3=Me$, $R_4=H$
e: $R_1=H$, $R_2=Me$, $R_3+R_4=CH_2$
f: $R_1=Me$, $R_2=H$, $R_3+R_4=CH_2$
g: $R_1+R_2=CH_2$, $R_3={}^iPr$, $R_4=Me$
h: $R_1+R_2=CH_2$, $R_3=Me$, $R_4={}^iPr$
i: $R_1={}^iPr$, $R_2=R_3=R_4=Me$

^{i}Pr: isopropyl

Chart 1

ROXB.) (Japanese name: teriha-zansho). Oxyterihanine[6] (**2c**) gave the molecular formula $C_{20}H_{15}NO_5$ and contains a hydroxy [infrared (IR) absorption: $3100\,cm^{-1}$], amide (IR: $1630\,cm^{-1}$), *N*-methyl and methoxy [proton nuclear magnetic resonance (^{1}H-NMR) spectrum: δ 3.98 and 4.11 (each 3H, s)], and methylenedioxy [^{1}H-NMR: δ 6.11 (2H, s)] moieties. Further, the signals due to aromatic protons were observed as four 1H singlets at δ 7.20, 7.61, 7.63, and 7.82 and two 1H doublets ($J=8.6$ Hz) at δ 7.60 and 8.02. These spectral features left only four possible structures (**2c**—**f**) for oxyterihanine. However, we could not obtain more information on its structure because of the small amount of the alkaloid isolated. Consideration of a statistical survey of the structures of naturally occurring benzo-[*c*]phenanthridine alkaloids suggested that oxyterihanine would have a methylenedioxy group at the C_2–C_3 position, and so we planned to synthesize 8-hydroxy-9-methoxy- and 9-hydroxy-8-methoxy-2,3-methylenedioxybenzo[*c*]phenanthridin-6(5*H*)-ones [oxyterihanine (**2c**) and oxyisoterihanine (**2d**)] for direct comparison with naturally occurring oxyterihanine. In addition, we also aimed at synthesizing the corresponding quaternary benzo[*c*]-phenanthridine alkaloids (**1c** and **1d**), because it is generally believed that quaternary alkaloids are the precursors of oxybases in plant bodies, although these phenolic quaternary alkaloids (**1c** and **1d**) have not so far been isolated from natural sources.

3 → → 4 —(H_2, Pd–C / AcOH)→ 5 —($POCl_3$ / $CHCl_3$)→ 6 → → 1

Chart 2

In the previous papers,[7] we have established a generally applicable and efficient synthetic sequence for the nonphenolic benzo[*c*]phenanthridines from chalcone derivatives (**3**) *via* 2-aryl-1-tetralone intermediates (**6**). (Chart 2) However, the synthetic sequence could not be directly applied to the syntheses of phenolic alkaloids, because it involved the steps of hydrogenolysis of 2,4-diaryl-4-oxobutyric acids (**4**) to 2,4-diarylbutyric acids (**5**) and of intramolecular acylation of the resulting butyric acids (**5**) with phosphorus oxychloride in chloroform to 2-aryl-1-tetralones (**6**), and these steps cause cleavage of common phenol-protecting groups, such as benzyl and methoxymethyl groups.

Recently, we[8] succeeded in the synthesis of fagaronine (**1b**) by using an isopropoxy group as a phenol-protecting group. Thus, we tried to prepare oxyterihanine (**2c**) and oxyisoterihanine (**2d**) by a similar approach. Unfortunately, the step of the intramolecular acylation of 2-(4-isopropoxy-3-methoxyphenyl)-4-(3,4-methylenedioxyphenyl)butyric acid (the isopropoxy methylene acid-A) (**7a**) to the corresponding tetralone (the isopropoxy tetralone-A) (**8a**) with phosphorus oxychloride in chloroform gave the desired nonphenolic isopropoxy tetralone-A (**8a**) together with a mixture of four phenolic products in 41.6 and 19.5% yields, respectively. Later, the conditions of the intramolecular acylation were modified

Chart 3

(phosphorus oxychloride in acetonitrile in the presence of potassium carbonate) and we succeeded in preparing the desired isopropoxy tetralone-A[1] (**8a**) and -B[1] (**8b**) without formation of the phenolic derivatives.

According to the reported synthetic sequence,[1,7,8] both the tetralones (**8a** and **8b**) were transformed into 2-(4-isopropoxy-3-methoxyphenyl)- and 2-(3-isopropoxy-4-methoxyphenyl)-6,7-methylenedioxy-1-(*N*-methylformamido) naphthalenes (the isopropoxy aromatized formamide-A and -B) (**11a** and **11b**), synthetic key intermediates for the desired oxybases (**2c** and **2d**) and the quaternary alkaloids (**1c** and **1d**).

Treatment of the isopropoxy tetralone-A (**8a**) with methylamine gas in the presence of titanium tetrachloride followed by reduction with sodium borohydride afforded the desired nonphenolic 1,2,3,4-tetrahydro-1-naphthylamine (the isopropoxy amine-A) (**9a**) along with the phenolic product (the hydroxy amine-C) (**9c**) in 85 and 0.79% yields, respectively. In the ^{1}H-NMR spectrum, the former (**9a**) shows a 1H doublet (J=3.5 Hz) at δ 3.56 and the latter (**9c**) at δ 3.55. These signals can be assigned to the C_1-proton in each case. In the previous study,[9] we prepared the related *cis*- and *trans*-formamides (**12** and **13**) and confirmed that the coupling constant between the C_1- and C_2-protons of the *cis*-isomer (**12**) is 3.4 Hz and that of the *trans*-isomer (**13**) is 9.6 Hz. These spectral data allow us to deduce that the above amines (**9a** and **9c**) have the *cis*-configuration.

On the other hand, the same treatment of the isopropoxy tetralone-B (**8b**) gave the nonphenolic isopropoxy amine-B (**9b**) as a sole product in 94.4% yield. It should be added here that, in the case of fagaronine[8] (**1b**), the corresponding isopropoxy amine derivative had also been obtained as a sole product.

Both the isopropoxy amine-A (**9a**) and -B (**9b**) were treated with freshly prepared chloral[1,7,8] to give 2-(4-isopropoxy-3-methoxyphenyl)- and 2-(3-isopropoxy-4-methoxyphenyl)-6,7-methylenedioxy-1-(*N*-methylformamido)-1,2,3,4-tetrahydronaphthalenes (the isopropoxy aliphatic formamide-A and -B) (**10a** and **10b**) in 89.9 and 83.0% yields, respectively. Dehydrogenation of these aliphatic formamides (**10a** and **10b**) with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) provided the desired aromatized formamides (**11a** and **11b**) in 89.9 and 82.6% yields, respectively.

11a → ($POCl_3$, MeCN) → **1g** → (KCN) → **14a**, **14b** → (NaH, O_2, HMPT) → **2g**

11b → ($POCl_3$, MeCN) → **1h** → (KCN) → **14a**, **14b** → (NaH, O_2, HMPT) → **2h**

14a : R_1=^{i}Pr, R_2=Me

14b : R_1=Me, R_2=^{i}Pr

11a, **11b** → (H_2SO_4/AcOH) → **11c**, **11d** → ($POCl_3$, MeCN) → **1c** (terihanine), **1d** (isoterihanine)

2g, **2h** → (H_2SO_4, AcOH) → **2c** (oxyterihanine), **2d** (oxyisoterihanine)

1c, **1d** → ($NaBH_4$) → **15c**, **15d**

15c : R_1=H, R_2=Me

15d : R_1=Me, R_2=H

Chart 4

Initially, we aimed at synthesizing the oxybases (**2c** and **2d**). The Bischler–Napieralski reaction of the above aromatized formamides (**11a** and **11b**) with phosphorus oxychloride in acetonitrile gave the respective 8-isopropoxy-9-methoxy- and 9-isopropoxy-8-methoxy-5-methyl-2,3-methylenedioxybenzo[*c*]phenanthridinium [isopropylterihanine (**1g**) and isopropylisoterihanine (**1h**)] chlorides quantitatively. These quaternary bases (**1g** and **1h**) were converted into the corresponding ψ-cyanides (**14a** and **14b**) by the usual procedure,[1,7,8] in 80.6 and 66.0% yields, respectively.

Air-oxidation of the carbanions prepared from the ψ-cyanides (**14a** and **14b**) furnished isopropyloxyterihanine (**2g**) and isopropyloxyisoterihanine (**2h**) in 80.6 and 92.5% yields, respectively.

In the case of oxyfagaronine[8] (**2b**), selective cleavage of the isopropoxy group of isopropyloxyfagaronine[8] (**2i**) took place smoothly under various acid conditions, because the reactivity of the isopropoxy group is quite distinct from that of the three methoxy groups present in isopropyloxyfagaronine (**2i**). However, since, in the cases of oxyterihanine (**2c**) and oxyisoterihanine (**2d**), a methylenedioxy group is more susceptible than a methoxy group under acidic conditions, we were obliged to examine the conditions for selective cleavage of the isopropoxy group of isopropyloxyterihanine (**2g**) and isopropyloxyisoterihanine (**2h**) in detail. Treatment of isopropyloxyterihanine (**2g**) with a 1.1% solution (v/v) of concentrated sulfuric acid in acetic acid under reflux for about 1 h gave the desired oxyterihanine (**2c**) in 82.2% yield. Since this material (**2c**) was identical with a sample of oxyterihanine isolated from a natural source, the structure of oxyterihanine was unambiguously established as the formula (**2c**). In addition, oxyisoterihanine (**2d**) was prepared from isopropyloxyisoterihanine (**2h**) by a similar procedure in 79.4% yield.

Finally, the phenolic quaternary alkaloids, terihanine (**1c**) and isoterihanine (**1d**), were prepared. Selective cleavage of the isopropoxy group of the aromatized formamides (**11a** and **11b**) gave the phenolic aromatized formamides (**11c** and **11d**) in 76.7 and 83.2% yields, respectively. The Bischler-Napieralski reaction of the resulting phenolic formamides (**11c** and **11d**) with phosphorus oxychloride in acetonitrile gave terihanine (**1c**) and isoterihanine (**1d**) chlorides in 87.6 and 99.9% yields, respectively.

The cytotoxic activities of these quaternary alkaloids (**1c** and **1d**) against the experimental tumor, P 388, together with those of other phenolic quaternary benzo[*c*]phenanthridines, will

be reported elsewhere.

Experimental

All melting points were measured on a micro melting point hot stage apparatus (Yanagimoto) and are uncorrected. IR spectra were recorded on a Hitachi 215 spectrometer in Nujol. ^{1}H-NMR spectra were recorded on a Hitachi R-24B spectrometer (60 MHz) in deuteriochloroform, unless otherwise stated, with tetramethylsilane as an internal reference. All NH and OH signals were confirmed by their disappearance after addition of deuterium oxide. Mass spectra (MS) were measured on a Hitachi RMU-6E spectrometer at 70 eV chamber voltage with a direct inlet system. For chromatography (column), silicic acid (100 mesh) (Mallinckrodt Chemical Works) and Silica gel 60 (70—230 mesh ASTM) (Merck) were used, while for thin layer chromatography (TLC) and preparative TLC, Silica gel GF_{254} (Merck) was used. Identification of products was done by IR and TLC comparisons and melting point determination. The abbreviations used are as follows: s, singlet; d, doublet; dd, double doublet; m, multiplet; br, broad; dif, diffused. All the quaternary bases were characterized as the ψ-cyanides (**14**), the oxybases (**2**), and/or the dihydrobases (**15**).

Oxyterihanine (2c)——As reported previously,[6] oxyterihanine was isolated in 0.00015% yield from the bark of *X. nitidum* (Roxb.) D.C. (*F. nitida* Roxb.) (Japanese name: teriha-zansho) along with twelve known alkaloids (nitidine, oxynitidine, 6-methoxy-5,6-dihydrochelerythrine, oxychelerythrine, norchelerythrine, chelerythrine, arnottianamide, liriodenine, bocconoline, decarine, integriamide, and isoarnottianamide), three known lignans (*l*-asarinin, *l*-sesamin, and *l*-syringaresinol), and two other known compounds (aesculetin dimethyl ether and β-sitosterol). Colorless prisms, mp > 300 °C. IR ν_{max}^{KBr} cm^{-1}: 3100, 1630. ^{1}H-NMR ($CDCl_3$–CD_3OD) (270 MHz) δ: 3.98 (3H, s, NMe or OMe), 4.11 (3H, s, OMe or NMe), 6.11 (2H, s, OCH_2O), 7.20 (1H, s, C_1-H), 7.60 (1H, d, J=8.6 Hz, C_{12}-H), 7.61 (1H, s, C_4- or C_{10}-H), 7.63 (1H, s, C_{10}- or C_4-H), 7.82 (1H, s, C_7-H), 8.02 (1H, d, J=8.6 Hz, C_{11}-H). High resolution MS *m/z*: Calcd $C_{20}H_{15}NO_5$: 349.0951. Found: 349.0962.

General Method for Preparation of *cis*-2-Aryl-*N*-methyl-1,2,3,4-tetrahydro-1-naphthylamine (the Amine) (9) from 2-Aryl-1-tetralone Derivative (the Tetralone) (8)——A solution of the tetralone (**8**) and $MeNH_2$ in dry $CHCl_3$ was gradually added to a solution of titanium tetrachloride ($TiCl_4$) in dry $CHCl_3$ under ice-cooling, and then the mixture was stirred at room temperature for several hours and refluxed for 30 min. After removal of the resulting precipitates by filtration, the filtrate was evaporated to dryness *in vacuo*. The residue (the ketimine) was dissolved in MeOH. The reaction mixture was treated with $NaBH_4$ at room temperature until the reaction was complete, then diluted with a large amount of H_2O and extracted with Et_2O. The ethereal solution was dried over K_2CO_3 and evaporated to dryness *in vacuo*. Recrystallization of the residue from an appropriate solvent gave the desired amine (**9**).

***cis*-*N*-Methyl-2-(4-isopropoxy-3-methoxyphenyl)-6,7-methylenedioxy-1,2,3,4-tetrahydro-1-naphthylamine (the Isopropoxy Amine-A) (9a)**——A solution of the isopropoxy tetralone-A[1] (**8a**) (14.2 g) and $MeNH_2$ (33 g) in dry $CHCl_3$ (280 ml) and a solution of $TiCl_4$ (4.5 ml) in dry $CHCl_3$ (140 ml) were mixed, stirred at room temperature for 2 h, and refluxed. A solution of the crude iminium salt in MeOH (430 ml) was treated with $NaBH_4$ (3.03 g) overnight. In this run, the ethereal extract of the crude product was washed with 5% NaOH aq. and extracted with 5% HCl aq. The hydrochloric acid solution was made alkaline with NH_4OH aq. and extracted with Et_2O again. The ethereal solution was treated according to the general method. Colorless prisms (12.6 g), mp 113—114 °C (MeOH or Et_2O). *Anal.* Calcd for $C_{22}H_{27}NO_4$: C, 71.52; H, 7.37; N, 3.79. Found: C, 71.57; H, 7.36; N, 3.81. ^{1}H-NMR δ: 1.19 (1H, s, NH), 1.35 (6H, d, J=6.0 Hz, $CH\underline{Me}_2$), 1.78—2.66 (2H, m, C_3-H_2), 2.19 (3H, s, NMe), 2.66—3.35 (3H, m, C_2-H and C_4-H_2), 3.56 (1H, d, J=3.5 Hz, C_1-H), 3.82 (3H, s, OMe), 4.48 (1H, septet, J=6.0 Hz, $OC\underline{H}Me_2$), 5.88 (2H, s, OCH_2O), 6.60 (1H, s, C_5-H), 6.72 (1H, s, ArH), 6.81 (3H, s, ArH).

***cis*-*N*-Methyl-2-(4-hydroxy-3-methoxyphenyl)-6,7-methylenedioxy-1,2,3,4-tetrahydro-1-naphthylamine (the Hydroxy Amine-C) (9c)**——The 5% NaOH aq. washings described above were made alkaline by addition of NH_4Cl and extracted with Et_2O. The ethereal solution was dried over $MgSO_4$ and evaporated to dryness *in vacuo*. Recrystallization of the residue from $CHCl_3$–MeOH gave colorless prisms (0.103 g), mp 175—177 °C. *Anal.* Calcd for $C_{19}H_{21}NO_4$: C, 69.70; H, 6.47; N, 4.28. Found: C, 69.69; H, 6.46; N, 4.25. ^{1}H-NMR δ: 1.75—2.62 (2H, m, C_3-H_2), 2.20 (3H, s, NMe), 2.68—3.32 (5H, m, C_2-H, C_4-H_2, OH, and NH), 3.55 (1H, d, J=3.5 Hz, C_1-H), 3.85 (3H, s, OMe), 5.89 (2H, s, OCH_2O), 6.62 (1H, s, C_5-H), 6.73 (1H, s, ArH), 6.79 (3H, s, ArH).

***cis*-*N*-Methyl-2-(3-isopropoxy-4-methoxyphenyl)-6,7-methylenedioxy-1,2,3,4-tetrahydro-1-naphthylamine (the Isopropoxy Amine-B) (9b)**——A solution of the isopropoxy tetralone-B[1] (**8b**) (4.43 g) and $MeNH_2$ (9 g) in dry $CHCl_3$ (88 ml) and a solution of $TiCl_4$ (1.4 ml) in dry $CHCl_3$ (44 ml) were mixed, stirred at room temperature for 2.5 h, and refluxed. A solution of the crude iminium salt in MeOH (130 ml) was treated with $NaBH_4$ (0.95 g) for 1.5 h to give a yellow oil (4.36 g). ^{1}H-NMR δ: 1.34 (6H, d, J=6.0 Hz, $CH\underline{Me}_2$), 1.39 (1H, s, NH), 1.80—2.60 (2H, m, C_3-H_2), 2.19 (3H, s, NMe), 2.60—3.30 (3H, m, C_2-H and C_4-H_2), 3.56 (1H, d, J=3.5 Hz, C_1-H), 3.83 (3H, s, OMe), 4.49 (1H, septet, J=6.0 Hz, $OC\underline{H}Me_2$), 5.58 (2H, s, OCH_2O), 6.61 (1H, s, C_5-H), 6.74 (1H, s, ArH), 6.83 (3H, s, ArH).

This material was characterized as the picrate. Yellow prisms, mp 172—173 °C ($CHCl_3$–MeOH). *Anal.* Calcd for $C_{22}H_{27}NO_4 \cdot C_6H_3N_3O_7$: C, 56.18; H, 5.05; N, 9.36. Found: C, 56.14; H, 5.04; N, 9.34.

General Method for Formylation of the Amine (9) [*cis*-2-Aryl-1-(*N*-methylformamido)-1,2,3,4-tetrahydronaphthalene (the Aliphatic Formamide) (10)]——A mixture of the amine (9) and freshly prepared chloral[10] in dry $CHCl_3$ was refluxed, then washed with H_2O, dried over K_2CO_3, and evaporated to dryness *in vacuo*. Recrystallization of the residue from an appropriate solvent gave the desired aliphatic formamide (10).

***cis*-2-(4-Isopropoxy-3-methoxyphenyl)-6,7-methylenedioxy-1-(*N*-methylformamido)-1,2,3,4-tetrahydronaphthalene (the Isopropoxy Aliphatic Formamide-A) (10a)**——A solution of the isopropoxy amine-A (**9a**) (12.0 g) and chloral (12.8 ml) in dry $CHCl_3$ (120 ml) was refluxed for 6 h. Column chromatography on silica gel was run with benzene–AcOEt (10:1, v/v). Colorless prisms[11] (11.6 g), mp 163—164 °C[12] (MeOH–Et_2O). *Anal.* Calcd for $C_{23}H_{27}NO_5$: C, 69.50; H, 6.85; N, 3.52. Found: C, 69.32; H, 6.78; N, 3.50. IR ν_{max} cm^{-1}: 1650. ^{1}H-NMR δ: 1.36 (6H, d, J=6.0 Hz, CH$\underline{Me}_2$), 1.87—2.36 (2H, m, C_3-H_2), 2.51 (3H, s, NMe), 2.75—3.50 (3H, m, C_2-H and C_4-H_2), 3.83 (3H, s, OMe), 4.50 (1H, septet, J=6.0 Hz, OC$\underline{H}$Me$_2$), 4.56 (1H, d, J=4.5 Hz, C_1-H), 5.92 (2H, s, OCH_2O), 6.51 (1H, s, C_5-H), 6.55—7.00 (4H, m, ArH), 7.62 (4/5H, s, NCHO), 7.75 (1/5H, s, NCHO).

***cis*-2-(3-Isopropoxy-4-methoxyphenyl)-6,7-methylenedioxy-1-(*N*-methylformamido)-1,2,3,4-tetrahydronaphthalene (the Isopropoxy Aliphatic Formamide-B) (10b)**——A solution of the isopropoxy amine-B (**9b**) (4.31 g) and chloral (4.6 ml) in dry $CHCl_3$ (43 ml) was refluxed for 6 h. Colorless prisms[11] (3.25 g), mp 131—135 °C (Et_2O). *Anal.* Calcd for $C_{23}H_{27}NO_5$: C, 69.50; H, 6.85; N, 3.52. Found: C, 69.55; H, 6.82; N, 3.50. IR ν_{max} cm^{-1}: 1670. ^{1}H-NMR δ: 1.34 (6H, d, J=6.0 Hz, CH$\underline{Me}_2$), 1.83—2.32 (2H, m, C_3-H_2), 2.50 (15/6H, s, NMe), 2.54 (3/6H, s, NMe), 2.70—3.50 (3H, m, C_2-H and C_4-H_2), 3.83 (3H, s, OMe), 4.49 (1H, septet, J=6.0 Hz, OC$\underline{H}$Me$_2$), 4.56 (1H, d, J=4.0 Hz, C_1-H), 5.94 (2H, s, OCH_2O), 6.50 (5/6H, s, C_5-H), 6.57—6.97 (25/6H, m, ArH), 7.62 (5/6H, s, NCHO), 7.76 (1/6H, s, NCHO).

Column chromatography of the crude material obtained from the mother liquor of recrystallization with benzene–AcOEt (5:1, v/v) gave an additional amount [0.60 g (total yield: 3.85 g)] of the pure product.

General Method for Dehydrogenation of the Aliphatic Formamide (10) [2-Aryl-1-(*N*-methylformamido)naphthalene (the Aromatized Formamide) (11)]——A solution of the aliphatic formamide (10) and DDQ[13] in dry benzene was refluxed. After removal of the precipitates by filtration, a large amount of 5% NaOH aq. was added to the filtrate and the mixture was extracted with $CHCl_3$. The chloroform solution was washed with 5% NaOH aq., dried over K_2CO_3, and then evaporated to dryness *in vacuo*. Recrystallization of the residue from an appropriate solvent gave the desired aromatized formamide (11).

2-(4-Isopropoxy-3-methoxyphenyl)-6,7-methylenedioxy-1-(*N*-methylformamido)naphthalene (the Isopropoxy Aromatized Formamide-A) (11a)——Refluxing of a solution of the isopropoxy aliphatic formamide-A (**10a**) (10.9 g) and DDQ (18.7 g) in dry benzene (494 ml) for 1 h gave colorless prisms[11] (8.63 g), mp 158—160 °C (softened at 140—142 °C) (benzene–hexane). *Anal.* Calcd for $C_{23}H_{23}NO_5$: C, 70.21; H, 5.89; N, 3.56. Found: C, 70.09; H, 5.87; N, 3.52. IR ν_{max} cm^{-1}: 1675. ^{1}H-NMR δ: 1.38 (6H, d, J=6.0 Hz, CH$\underline{Me}_2$), 2.90 (3/13H, s, NMe), 3.00 (36/13H, s, NMe), 3.83 (3H, s, OMe), 4.54 (1H, septet, J=6.0 Hz, OC$\underline{H}$Me$_2$), 6.06 (2H, s, OCH_2O), 6.76 (1H, dif d, J=8.0 Hz, ArH), 6.82 (1H, dif s, $C_{2'}$-H), 6.95 (1H, dif d, J=8.0 Hz, ArH), 7.07 (1H, s, C_5-H), 7.17 (1H, s, C_8-H), 7.33 (1H, d, J=9.0 Hz, C_4-H), 7.70 (1H, d, J=9.0 Hz, C_3-H), 8.13 (12/13H, s, NCHO), 8.34 (1/13H, s, NCHO).

The mother liquor of the above recrystallization was evaporated to dryness *in vacuo*. Purification of the residue by column chromatography on silica gel with benzene followed by AcOEt gave an additional amount of the above colorless prisms (1.08 g), which were recrystallized from benzene–hexane (total amount 9.71 g).

2-(3-Isopropoxy-4-methoxyphenyl)-6,7-methylenedioxy-1-(*N*-methylformamido)naphthalene (the Isopropoxy Aromatized Formamide-B) (11b)——Refluxing of a solution of the isopropoxy aliphatic formamide-B (**10b**) (3.67 g) and DDQ (6.29 g) in dry benzene (167 ml) for 70 min gave the crude product. Column chromatography of this with a mixed solvent [benzene–AcOEt, 5:1 (v/v)] gave colorless prisms[11] (3.00 g), mp 170—172 °C (benzene–hexane). *Anal.* Calcd for $C_{23}H_{23}NO_5$: C, 70.21; H, 5.89; N, 3.56. Found: C, 70.29; H, 5.92; N, 3.54. IR ν_{max} cm^{-1}: 1680. ^{1}H-NMR δ: 1.36 (6H, d, J=6.0 Hz, CH$\underline{Me}_2$), 2.93 (3/10H, s, NMe), 3.03 (27/10H, s, NMe), 3.88 (3H, s, OMe), 4.52 (1H, septet, J=6.0 Hz, OC$\underline{H}$Me$_2$), 6.07 (2H, s, OCH_2O), 6.87 (3H, dif s, ArH), 7.07 (1H, s, C_5-H), 7.18 (1H, s, C_8-H), 7.33 (1H, d, J=8.5 Hz, C_4-H), 7.72 (1H, d, J=8.5 Hz, C_3-H), 8.14 (9/10H, s, NCHO), 8.36 (1/10H, s, NCHO).

General Method for Bischler–Napieralski Reaction of the Aromatized Formamide (11) [the Quaternary Base (1)]——A mixture of the aromatized formamide (11) and $POCl_3$ in MeCN was heated. The solvent was distilled off under reduced pressure, then the residue was basified with 10% NaOH aq. and extracted with $CHCl_3$. The chloroform solution was dried over K_2CO_3 and evaporated to dryness *in vacuo*. A small amount of 10% HCl aq. was added to an ice-cooled solution of the residue in a minute amount of $CHCl_3$. The precipitates were collected by filtration and recrystallized from an appropriate solvent to give the desired quaternary base (1) as the chloride.

8-Isopropoxy-9-methoxy-5-methyl-2,3-methylenedioxybenzo[*c*]phenanthridinium (Isopropylterihanine) (1g) Chloride——A solution of the isopropoxy aromatized formamide-A (**11a**) (1.50 g) and $POCl_3$ (2.3 ml) in MeCN (68 ml) was heated at 60 °C for 100 min. Yellow needles (1.55 g), mp 228—230 °C (MeOH). ^{1}H-NMR (CF_3CO_2H) δ: 1.62 (6H, d, J=6.0 Hz, CH$\underline{Me}_2$), 4.37 (3H, s, OMe), 5.01 (3H, s, N^+Me), 5.16 (1H, septet, J=6.0 Hz, OC$\underline{H}$Me$_2$), 6.24 (2H, s, OCH_2O), 7.49 (1H, s, C_1-H), 7.76 (1H, s, C_7-H), 8.08 (1H, s, ArH), 8.16 (1H, d, J=9.0 Hz, C_{12}-H), 8.23 (1H, s, ArH), 8.51 (1H, d, J=9.0 Hz, C_{11}-H), 9.34 (1H, s, C_6-H).

Isopropylterihanine ψ-Cyanide (14a)——Potassium cyanide (0.305 g) was added to a solution of isopropylterihanine (**1g**) chloride (0.750 g) in MeOH aq. [MeOH–H_2O, 2:1 (v/v)] (245 ml) at 50 °C. The mixture was stirred at the

same temperature for 1.5 h. After addition of water, the reaction mixture was extracted with $CHCl_3$. The chloroform solution was dried over K_2CO_3 and evaporated to dryness *in vacuo*. Recrystallization of the residue from $CHCl_3$–MeOH gave colorless prisms (0.607 g), mp 211—217 °C (dec.) (softened at 190 °C). *Anal.* Calcd for $C_{24}H_{22}N_2O_4$: C, 71.62; H, 5.51; N, 6.96. Found: C, 71.33; H, 5.53; N, 6.82. ^{1}H-NMR δ: 1.42 (6H, d, J=6.0 Hz, CH$\underline{Me}_2$), 2.62 (3H, s, NMe), 3.97 (3H, s, OMe), 4.63 (1H, septet, J=6.0 Hz, OC$\underline{H}$Me$_2$), 5.07 (1H, s, C_6-H), 6.04 (2H, s, OCH_2O), 6.98 (1H, s, ArH), 7.10 (1H, s, ArH), 7.32 (1H, s, C_{10}-H), 7.50 (1H, d, J=9.0 Hz, C_{12}-H), 7.62 (1H, s, C_4-H), 7.70 (1H, d, J=9.0 Hz, C_{11}-H).

9-Isopropoxy-8-methoxy-5-methyl-2,3-methylenedioxybenzo[*c*]phenanthridinium (Isopropylisoterihanine) (1h) Chloride——A solution of the isopropoxy aromatized formamide-B (**11b**) (1.01 g) and $POCl_3$ (1.5 ml) in MeCN (45 ml) was refluxed for 1.5 h. Yellow needles (1.08 g), mp 290—299 °C[14] (MeOH–AcOEt). ^{1}H-NMR (CF_3CO_2H) δ: 1.66 (6H, d, J=6.0 Hz, CH$\underline{Me}_2$), 4.25 (3H, s, OMe), 5.00 (3H, s, N^+Me), 5.28 (1H, septet, J=6.0 Hz, OC$\underline{H}$Me$_2$), 6.25 (2H, s, OCH_2O), 7.52 (1H, s, C_1-H), 7.75 (1H, s, C_7-H), 8.09 (1H, s, ArH), 8.16 (1H, d, J=9.0 Hz, C_{12}-H), 8.23 (1H, s, ArH), 8.51 (1H, d, J=9.0 Hz, C_{11}-H), 9.30 (1H, br s, C_6-H).

Isopropylisoterihanine ψ-Cyanide (14b)——Potassium cyanide (0.406 g) was added to a solution of isopropylisoterihanine (**1h**) chloride (1.00 g) in H_2O (260 ml) at 50 °C. The mixture was stirred at the same temperature for 1.5 h. After cooling, the resulting precipitates were collected by filtration and washed with water followed by MeOH. Recrystallization of the precipitates from $CHCl_3$–MeOH gave colorless prisms (0.706 g), mp 208 °C (dec.) (softened at 187 °C). *Anal.* Calcd for $C_{24}H_{22}N_2O_4$: C, 71.62; H, 5.51; N, 6.96. Found: C, 71.57; H, 5.51; N, 6.74. ^{1}H-NMR δ: 1.41 (6H, d, J=6.0 Hz, CH$\underline{Me}_2$), 2.61 (3H, s, NMe), 3.92 (3H, s, OMe), 4.65 (1H, septet, J=6.0 Hz, OC$\underline{H}$Me$_2$), 5.07 (1H, s, C_6-H), 6.02 (2H, s, OCH_2O), 6.95 (1H, s, ArH), 7.09 (1H, s, ArH), 7.35 (1H, s, C_{10}-H), 7.50 (1H, d, J=8.0 Hz, C_{12}-H), 7.60 (1H, s, C_4-H), 7.65 (1H, d, J=8.0 Hz, C_{11}-H).

General Procedure for Synthesis of the Oxybase (2) by Air-Oxidation of the Carbanion Prepared from the ψ-Cyanide (14)——Sodium hydride[15] (2—8 mol) was added to a stirred solution of the ψ-cyanide (**14**) (1 mol) in hexamethylphosphoric triamide (HMPT) at room temperature. The reaction mixture was stirred at room temperature and then stirred by bubbling dry oxygen gas through the solution at room temperature for several hours. After addition of saturated brine, the reaction mixture was extracted with AcOEt. The organic layer was washed with saturated brine several times, dried over K_2CO_3, and evaporated to dryness *in vacuo*. Recrystallization of the residue from a suitable solvent gave the desired oxybase (**2**).

Isopropyloxyterihanine (2g)——A solution of isopropylterihanine ψ-cyanide (**14a**) (0.153 g) and NaH (0.035 g) in HMPT (8.7 ml) was stirred for 2 h. Colorless prisms (0.120 g), mp 234.5—236.5 °C ($CHCl_3$–MeOH). *Anal.* Calcd for $C_{23}H_{21}NO_5$: C, 70.57; H, 5.41; N, 3.58. Found: C, 70.35; H, 5.38; N, 3.51. IR ν_{max} cm^{-1}: 1645. ^{1}H-NMR δ: 1.47 (6H, d, J=6.0 Hz, CH$\underline{Me}_2$), 3.94 (3H, s, NMe or OMe), 4.05 (3H, s, OMe or NMe), 4.81 (1H, septet, J=6.0 Hz, OC$\underline{H}$Me$_2$), 6.07 (2H, s, OCH_2O), 7.11 (1H, s, C_1-H), 7.47 (1H, d, J=8.0 Hz, C_{12}-H), 7.54 (1H, s, C_4- or C_{10}-H), 7.56 (1H, s, C_{10}- or C_4-H), 7.92 (1H, s, C_7-H), 7.94 (1H, d, J=8.0 Hz, C_{11}-H).

Isopropyloxyisoterihanine (2h)——A solution of isopropylisoterihanine ψ-cyanide (**14b**) (0.607 g) and NaH (0.548 g) in HMPT (35 ml) was stirred for 5 h. Colorless needles (0.546 g), mp 262—264 °C ($CHCl_3$–MeOH). *Anal.* Calcd for $C_{23}H_{21}NO_5$: C, 70.57; H, 5.41; N, 3.58. Found: C, 70.50; H, 5.36; N, 3.54. IR ν_{max} cm^{-1}: 1635. ^{1}H-NMR δ: 1.49 (6H, d, J=6.0 Hz, CH$\underline{Me}_2$), 3.95 (3H, s, NMe or OMe), 4.02 (3H, s, OMe or NMe), 4.83 (1H, septet, J=6.0 Hz, OC$\underline{H}$Me$_2$), 6.07 (2H, s, OCH_2O), 7.13 (1H, s, C_1-H), 7.51 (1H, d, J=8.5 Hz, C_{12}-H), 7.60 (2H, s, C_4- and C_{10}-H), 7.91 (1H, s, C_7-H), 7.92 (1H, d, J=8.5 Hz, C_{11}-H).

General Procedure for Selective Cleavage of an Isopropoxy Derivative——A solution of an isopropoxy compound in AcOH containing conc. H_2SO_4 was heated at 100—120 °C under argon, if necessary. The mixture was poured into a large amount of H_2O. The crude product was obtained by filtration (method A) or by extraction with benzene (method B). In the case of the latter method, the benzene solution was extracted with 5% NaOH aq. The aqueous layer was acidified with 10% HCl and extracted with $CHCl_3$. The chloroform solution was dried over $MgSO_4$ and evaporated to dryness *in vacuo*. Recrystallization of the resulting crude material from an appropriate solvent gave the desired phenolic product.

Oxyterihanine (2c)——A solution of isopropyloxyterihanine (**2g**) (0.045 g) in AcOH (4.5 ml) containing conc. H_2SO_4 (0.05 ml) was refluxed for 55 min. The crude product was obtained through method B. Colorless prisms (0.033 g), mp > 300 °C ($CHCl_3$–MeOH). *Anal.* Calcd for $C_{20}H_{15}NO_5$: C, 68.76; H, 4.33; N, 4.01. Found: C, 68.47; H, 4.37; N, 3.98. IR ν_{max} cm^{-1}: 3120, 1635. ^{1}H-NMR ($CDCl_3$–CD_3OD) (270 MHz) δ: 3.98 (3H, s, NMe or OMe), 4.12 (3H, s, OMe or NMe), 6.11 (2H, s, OCH_2O), 7.21 (1H, s, C_1-H), 7.60 (1H, d, J=8.6 Hz, C_{12}-H), 7.63 (1H, s, C_4- or C_{10}-H), 7.64 (1H, s, C_{10}- or C_4-H), 7.82 (1H, s, C_7-H), 8.03 (1H, d, J=8.6 Hz, C_{11}-H).

This material was identical with a sample of oxyterihanine which was isolated from a natural source.

Oxyisoterihanine (2d)——A solution of isopropyloxyisoterihanine (**2h**) (0.079 g) in AcOH (7.8 ml) containing conc. H_2SO_4 (0.043 ml) was refluxed for 4.5 h. The crude product was obtained through method A. Colorless needles (0.056 g), mp > 300 °C ($CHCl_3$–MeOH). *Anal.* Calcd for $C_{20}H_{15}NO_5$: C, 68.76; H, 4.33; N, 4.01: Found: C, 68.89; H, 4.39; N, 3.98. IR ν_{max}^{KBr} cm^{-1}: 1635. ^{1}H-NMR ($CDCl_3$–CD_3OD) (270 MHz) δ: 3.98 (3H, s, NMe or OMe), 4.06 (3H, s, OMe or NMe), 6.11 (2H, s, OCH_2O), 7.19 (1H, s, C_1-H), 7.57 (1H, d, J=8.7 Hz, C_{12}-H), 7.64 (1H, s, C_4- or C_{10}-H), 7.71 (1H, s, C_{10}- or C_4-H), 7.89 (1H, s, C_7-H), 7.98 (1H, d, J=8.7 Hz, C_{11}-H).

2-(4-Hydroxy-3-methoxyphenyl)-6,7-methylenedioxy-1-(*N*-methylformamido)naphthalene (the Hydroxy Aromatized Formamide-C) (11c)——A solution of the isopropoxy aromatized formamide-A (**11a**) (2.00 g) in AcOH (194 ml) containing conc. H_2SO_4 (6 ml) was heated at 100 °C for 1 h. The crude product was obtained through method B. Colorless prisms[11] (1.37 g), mp 248—252 °C ($CHCl_3$–MeOH). *Anal.* Calcd for $C_{20}H_{17}NO_5$: C, 68.37; H, 4.88; N, 3.99. Found: C, 68.29; H, 5.00; N, 3.97. IR ν_{max} cm^{-1}: 3385, 1660. ^{1}H-NMR (270 MHz) δ: 2.92 (3/8H, s, NMe), 3.02 (21/8H, s, NMe), 3.89 (3H, s, OMe), 5.67 (1H, s, OH), 6.09 (2H, s, OCH_2O), 6.78 (1H, d, J=2.0 Hz, $C_{2'}$-H), 6.82 (1H, dd, J=8.0, 2.0 Hz, $C_{6'}$-H), 6.98 (1H, d, J=8.0 Hz, $C_{5'}$-H), 7.08 (1H, s, C_5-H), 7.19 (1H, s, C_8-H), 7.37 (1H, d, J=8.2 Hz, C_4-H), 7.72 (1H, d, J=8.2 Hz, C_3-H), 8.17 (7/8H, s, NCHO), 8.38 (1/8H, s, NCHO).

2-(3-Hydroxy-4-methoxyphenyl)-6,7-methylenedioxy-1-(*N*-methylformamido)naphthalene (the Hydroxy Aromatized Formamide-D) (11d)——A solution of the isopropoxy aromatized formamide-B (**11b**) (1.00 g) in AcOH (97 ml) containing conc. H_2SO_4 (3 ml) was heated at 100 °C for 70 min. The crude product was obtained through method B. Colorless prisms[11] (0.734 g), mp 259—260 °C ($CHCl_3$–hexane). *Anal.* Calcd for $C_{20}H_{17}NO_5$: C, 68.37; H, 4.88; N, 3.99. Found: C, 68.11; H, 4.90; N, 3.94. IR ν_{max} cm^{-1}: 3375, 1670. ^{1}H-NMR (270 Hz) δ: 3.05 (3/11H, s, NMe), 3.06 (30/11H, s, NMe), 3.93 (3H, s, OMe), 5.67 (1H, s, OH), 6.09 (2H, s, OCH_2O), 6.76 (1H, dd, J=8.3, 2.3 Hz, $C_{6'}$-H), 6.88 (1H, d, J=2.3 Hz, $C_{2'}$-H), 6.89 (1H, d, J=8.3 Hz, $C_{5'}$-H), 7.07 (1H, s, C_5-H), 7.19 (1H, s, C_8-H), 7.34 (1H, d, J=8.4 Hz, C_4-H), 7.71 (1H, d, J=8.4 Hz, C_3-H), 8.10 (10/11H, s, NCHO), 8.37 (1/11H, s, NCHO).

Terihanine (1c) Chloride——The general procedure for the Bischler–Napieralski reaction described above was carried out on a solution of the hydroxy aromatized formamide-C (**11c**) (1.50 g) and $POCl_3$ (2.7 ml) in MeCN (420 ml). Heating was done at 80 °C for 70 min. Yellow prisms (1.25 g), mp 280 °C[16] (MeOH). IR ν_{max}^{KBr} cm^{-1}: 3450. ^{1}H-NMR (CF_3CO_2D) (270 MHz) δ: 4.40 (3H, s, OMe), 5.00 (3H, s, N^+Me), 6.27 (2H, s, OCH_2O), 7.54 (1H, s, C_1- or C_7-H), 7.86 (1H, s, C_7- or C_1-H), 8.10 (1H, s, C_4- or C_{10}-H), 8.21 (1H, d, J=9.2 Hz, C_{12}-H), 8.23 (1H, s, C_{10}- or C_4-H), 8.54 (1H, d, J=9.2 Hz, C_{11}-H), 9.32 (1H, s, C_6-H).

Dihydroterihanine (15c)——Sodium borohydride (0.092 g) was added portionwise to a stirred solution of terihanine (**1c**) chloride (0.201 g) in MeOH (1.5 l) at room temperature. After being stirred at room temperature for a further 40 min, the mixture was evaporated to dryness *in vacuo*. The residue was suspended in 5% HCl aq. and the suspension was extracted with $CHCl_3$. The chloroform solution was dried over $MgSO_4$ and evaporated to dryness *in vacuo*. Recrystallization of the residue from $CHCl_3$–MeOH gave colorless prisms (0.118 g), mp 189—192 °C. *Anal.* Calcd for $C_{20}H_{17}NO_4$: C, 71.63; H, 5.11; N, 4.18. Found: C, 71.48; H, 5.13; N, 4.15. ^{1}H-NMR (270 MHz) δ: 2.59 (3H, s, NMe), 4.00 (3H, s, OMe), 4.10 (2H, s, C_6-H_2), 5.68 (1H, s, OH), 6.04 (2H, s, OCH_2O), 6.85 (1H, s, C_7-H), 7.11 (1H, s, C_1-H), 7.28 (1H, s, C_{10}-H), 7.48 (1H, d, J=8.4 Hz, C_{12}-H), 7.66 (1H, s, C_4-H), 7.67 (1H, d, J=8.4 Hz, C_{11}-H).

Isoterihanine (1d) Chloride——The general procedure for the Bischer–Napieralski reaction described above was carried out on a solution of the hydroxy aromatized formamide-D (**11d**) (3.01 g) and $POCl_3$ (5.3 ml) in MeCN (300 ml). Heating was done at 60 °C for 2.5 h. Yellow prisms (2.86 g), mp 243—247 °C (MeOH–AcOEt): IR ν_{max} cm^{-1}: 3375. ^{1}H-NMR (CF_3CO_2D) (270 MHz) δ: 4.26 (3H, s, OMe), 4.99 (3H, s, N^+Me), 6.26 (2H, s, OCH_2O), 7.53 (1H, s, C_1- or C_7-H), 7.72 (1H, s, C_7- or C_1-H), 8.10 (1H, s, C_4- or C_{10}-H), 8.18 (1H, d, J=9.2 Hz, C_{12}-H), 8.31 (1H, s, C_{10}- or C_4-H), 8.49 (1H, d, J=9.2 Hz, C_{11}-H), 9.31 (1H, s, C_6-H).

Dihydroisoterihanine (15d)——A solution of isoterihanine (**1d**) chloride (0.401 g) in MeOH (800 ml) was treated with $NaBH_4$ (0.157 g) by the same procedure as used for the synthesis of dihydroterihanine (**15c**) from terihanine (**1c**) chloride. Colorless prisms (from $CHCl_3$–MeOH) (0.240 g), mp 235—242 °C.[17] *Anal.* Calcd for $C_{20}H_{17}NO_4$: C, 71.63; H, 5.11; N, 4.18. Found: C, 71.21; H, 5.12; N, 4.12. IR ν_{max} cm^{-1}: 3490. ^{1}H-NMR (270 MHz) δ: 2.60 (3H, s, NMe), 3.95 (3H, s, OMe), 4.12 (2H, s, C_6-H_2), 5.60 (1H, br s, OH), 6.04 (2H, s, OCH_2O), 6.77 (1H, s, C_7-H), 7.11 (1H, s, C_1-H), 7.38 (1H, s, C_{10}-H), 7.48 (1H, d, J=8.6 Hz, C_{12}-H), 7.65 (1H, s, C_4-H), 7.65 (1H, d, J=8.6 Hz, C_{11}-H).

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11) This compound shows a complex pattern in the ^{1}H-NMR spectrum and gave two spots on TLC [benzene–AcOEt (1 : 1, v/v)], indicating that it exists as a relatively stable mixture of rotational isomers with respect to the *N*-formyl group.

12) This material melted at 143—145 °C and then solidified again.

13) Commercial DDQ was freshly recrystallized from $CHCl_3$–benzene before use.

14) This material melted at 242—244 °C and then solidified again.

15) A commercially available 52.9% suspension of NaH in mineral oil was used without any treatment.

16) This material melted at 240—245 °C and then solidified again.

17) This material melted at 218—222 °C and then solidified again.

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Chemical Synthesis of Deoxyribonucleotide with a 5'-Phosphoryl Group on a Polystyrene Polymer Support by the Phosphotriester Method

TOSHIKI TANAKA,* YASUKI YAMADA and MORIO IKEHARA

Faculty of Pharmaceutical Sciences, Osaka University, 1–6 Yamadaoka, Suita, Osaka 565, Japan

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Highly lipophilic amines [trityloxyethylamine (TEAm) and *N*-trityloxyethylaniline (TEAn)] were introduced onto a 5'-phosphoryl group and the stability of the phosphoramidate linkage to acid was studied at the diester level. It was found that the phosphoramidate linkage using TEAn was cleaved by 80% aqueous acetic acid within 1 h at room temperature. However, it required 2 h to remove TEAm from the phosphate. By using the TEAn group, 5'-phosphorylated pentadeca-deoxyribonucleotide pTCCAGGGTCTGGTAC was synthesized on a polystyrene support by the phosphotriester method. After partial deblocking, the pentadecamer with the TEAn group could be easily isolated on a reversed-phase column due to the high lipophilicity of the TEAn group.

The chemically synthesized 5'-phosphorylated pentadecamer was successfully joined by using deoxyribonucleic acid ligase in the presence of a template.

Keywords——oligodeoxyribonucleotide synthesis; phosphotriester method; 5'-phosphoryl group; polymer support; DNA ligase

Oligodeoxyribonucleotides, which can be synthesized rapidly on a polymer support by means of either the phosphotriester or phosphite-triester approach, are indispensable tools for biological studies. Since chemically synthesized oligonucleotide usually has a hydroxyl group at the 5'- end, it must be phosphorylated with T_4-polynucleotide kinase and adenosine triphosphate (ATP). Though several synthetic methods for 5'-phosphorylated oligonucleotide has been reported by other workers,[1] the methods usually involve a solution phase synthetic technique. In order to prepare 5'-phosphoryl oligonucleotide easily and rapidly, polymer-supported oligonucleotide synthesis seems preferable. Though some work on solid-phase synthesis was reported,[2] the need for rapid purification was not considered. By using a lipophilic trityl group for the protection of the 5'-hydroxyl group, the 5'-protected oligonucleotide could be purified easily on a reversed-phase column.[3] In this paper, we wish to describe lipophilic protecting groups for the protection of the 5'-phosphate of nucleosides and the synthesis of 5'-phosphoryl oligodeoxyribonucleotides on a polystyrene support by the phosphotriester approach.

A 5'-phosphorylated oligodeoxyribonucleotide can be used directly for the gene construction[4] of a protein or the synthesis of mutated forms[5] of the deoxyribonucleic acid (DNA). It can be attached to biotin[6] or ethylenediaminetetracetic acid (EDTA)[7] through an aminoethylene linker for use as a hybridization probe or in DNA strand cleavage studies. It should be also possible to prepare 5'-phosphoryl oligonucleotides on a large scale for conformational studies.[8]

Results and Discussion

For the preparation of trityloxyethylamine (TEAm) (**5**),[9] *N*-2-hydroxyethylphthalimide

Chart 1

(1) was first treated with 1.3 eq of trityl chloride (TrCl) in CH_2Cl_2 and diisopropylethylamine was added as a catalyst. Next, 33 eq of hydrazine hydrate was added. After the reaction, the product was separated on a silica gel column, to give **5** in 82% yield. To prepare *N*-trityloxyethylaniline (TEAn) (**6**),[10] *N*-hydroxylethylaniline (**4**) was reacted with 1.6 eq of TrCl in CH_2Cl_2 and diisopropylethylamine was again used as the catalyst. Following the usual reaction, **6** was obtained in 94% yield after separation on a silica gel column.

The preparation of nucleotide-5′-phosphoramidate derivatives by using **5** and **6** was done as follows. The 3′-*O*-benzoylthymidine-5′-*o*-chlorophenylphosphate (**8a**) (which was prepared from 3′-*O*-benzoylthymidine by the phosphorylation with *o*-chlorophenylphosphoroditriazolide followed by hydrolysis with H_2O), was first activated with 1,3,5-triisopropylbenzenesulfonyl chloride (TPSCl) and 1-methylimidazole (1-MeIm)[11] for 10 min at room temperature. Then 2 eq of lipophilic amine **5** or **6** was added to the mixture. The condensation reaction was very rapid (within 10 min) at room temperature. After separation on a silica gel column, the phosphoramidate derivative (**9a**) or (**10a**) was obtained as a solid in a yield of 58% or 45%, respectively, after precipitation into *n*-hexane from a solution in CH_2Cl_2. To find suitable deblocking conditions, **9a** or **10a** was treated with conc. NH_4OH at 50 °C for 5 h. It was found that the 3′-benzoyl and *o*-chlorophenyl protecting groups were removed from **9a**. In the case of **10a** only the benzoyl group was removed and the *o*-chlorophenyl group was completely stable. Though the *o*-chlorophenyl group was stable on treatment with 0.5 M N^1,N^1,N^3,N^3-tetramethylammonium *syn*-pyridine-2-aldoximate (TMG-PAO) in dioxane–pyridine–H_2O (4:2:1, v/v) at room temperature overnight, it was removed by the treatment with 0.5 M TMG-PAO at 55 °C overnight. This treatment did not cleave internucleotidic linkages.[12] After purification on a reversed-phase column, phosphoramidate derivatives which had only the TEAm or TEAn protecting group were treated with 80% aqueous acetic acid. It was observed that the TEAn group was smoothly removed with 80% acetic acid at room temperature within 1 h to give pT. However, the TEAm group was only partially removed by the same treatment. It required more than 2 h for complete deprotection.

Compound **9a** or **10a** was then treated with 2 N NaOH for 10 min at 0 °C for selective removal of the 3′-benzoyl group. After this treatment **11a** or **12a** was obtained in a yield of 75% or 72%, respectively, after isolation on a silica gel column. The free 3′-hydroxyl group of **11a** or **12a** was then phosphorylated with *o*-chlorophenylphosphoroditriazolide followed by hydrolysis with H_2O in quantitative yield. By the same procedure, phosphoroamidate derivatives of other nucleosides **13b—d** and **14b—d** were prepared from **7b—d**.

1) ArO-P(O)(triazolyl)$_2$ 2) H_2O → 8; 5 or 6, TPSCl, 1-MeIm → TrO(CH$_2$)$_2$N(R)-P(O)(OAr)-O-nucleoside-OBz

7a : B=T
7b : B=bzA
7c : B=bzC
7d : B=ibG

8

9 : R=H
10 : R=cyclohexyl

1) 2 N NaOH 2) 2 N HCl → TrO(CH$_2$)$_2$N(R)-P(O)(OAr)-O-nucleoside-OH; 1) ArO-P(O)(triazolyl)$_2$ 2) H_2O → TrO(CH$_2$)$_2$N(R)-P(O)(OAr)-O-nucleoside-O-P(O)(OAr)-O$^-$

11 : R=H
12 : R=cyclohexyl

13 : R=H
14 : R=cyclohexyl

Ar: *o*-chlorophenyl TPSCl: 1,3,5-triisopropylbenzenesulfonyl chloride
1-MeIm: 1-methylimidazole Bz: benzoyl

Chart 2

By using **13a** and **14a**, a 5′-*O*-phosphorylated dimer, pTpC, was prepared: 5 eq of **13a** or **14a** was condensed with the 5′-hydroxyl group of *N*-4-benzoyldeoxycytidine bound to a polystyrene support with 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT) at room temperature for 40 min. After treatment with 0.5 M TMG-PAO at either room temperature or 55 °C overnight followed by conc. NH_4OH at 55 °C for 6 h, the dimer which still retained the TEAm or TEAn group was separated on a reversed phase (C_{18}) column with a linear gradient of CH_3CN (10%→50%) in 50 mM triethylammonium acetate (TEAA) (pH 7). The product was eluted with 30% CH_3CN as a single peak, then treated with 80% acetic acid at room temperature for 2 h or 1 h, respectively. This reaction gave unprotected dimer pTpC which was hydrolyzed to give pT and pC in equal amounts by venom phosphodiesterase. However, as it is known that deoxyadenosine is sensitive to acid treatment, rather drastic acid treatment should be avoided. In fact, when (TEAm) pTpA, prepared by the same procedure, was treated with 80% aqueous acetic acid for 3 h, some depurination (7%) was observed. It seems that the TEAn group is superior for the synthesis of 5′-phosphorylated oligonucleotide. However, the TEAm group can be used for the synthesis of nucleoside 5′-di or triphosphate.[5]

The usefulness of this method was demonstrated by applying it to the synthesis of a longer chain oligonucleotide, the pentadecamer pTCCAGGGTCTGGTAC, using **14a**. Starting with 3 μmol of *N*-benzoyldeoxycytidine bound to polystyrene, six protected dimers TA, GG, CT, GT, GG, CA, one monomer C[3,13] and **14a** were condensed successively with MSNT according to the reaction cycle shown in Table I. After the reaction, the resin was treated successively with 0.5 M TMG-PAO at 60 °C overnight and conc. NH_4OH at 60 °C overnight to remove the base and phosphate protecting groups and to release the nucleotidic compounds from the resin. The pentadecamer which still possessed a lipophilic TEAm group was separated on a reversed-phase (C_{18}) silica gel column with a linear gradient of CH_3CN in 50 mM TEAA, pH 7. As shown in Fig. 1a, it was eluted slowly (with 25% CH_3CN) and could be separated easily from truncated short oligonucleotides and the other reagents, which were eluted at the front. To remove the triethylammonium acetate salt used as a buffer in the column eluant from the pentadecamer, the residue, after evaporation of the volatile organic

TABLE I. One Cycle of Operation

	Operation	Solution	Volume
1)	Wash	CH_2Cl_2	1 ml (3 times)
2)	Detritylation	3% Cl_3CCOOH in CH_2Cl_2	1 ml for 30 s (4 times)
3)	Wash	CH_2Cl_2–pyridine (9:1, v/v)	1 ml (3 times)
4)	Wash	Pyridine	1 ml (3 times)
5)	Coevaporation	Nucleotide+pyridine	0.2 ml
6)	Condensation	MSNT+pyridine	0.4 ml (40 min)
7)	Wash	Pyridine	1 ml
8)	Capping	1 M Ac_2O, 50 mM DMAP in pyridine	1 ml (1 min)
9)	Wash	Pyridine	1 ml

MSNT = 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole. DMAP = 4-dimethylaminopyridine.

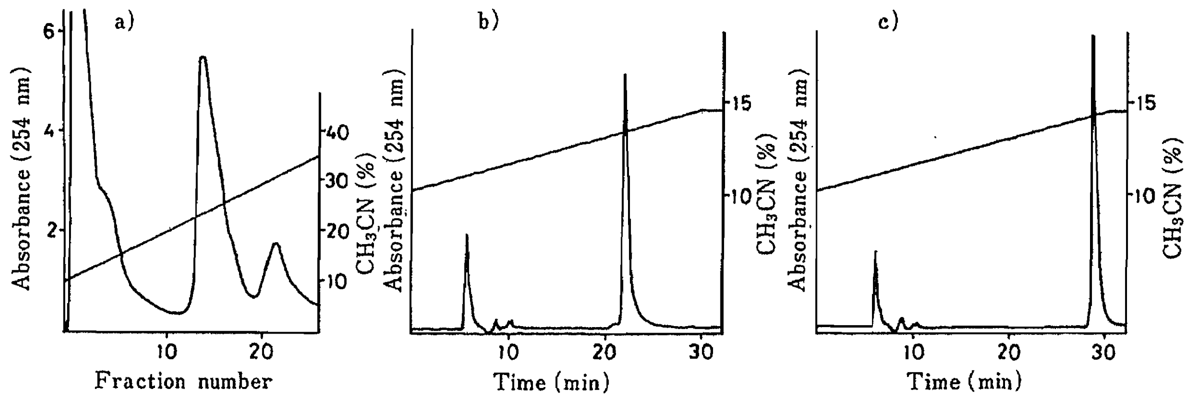

Fig. 1a. Isolation of d-(TEAn)-pTCCAGGGTCTGGTAC on a Reversed-Phase (C-18) Silica Gel Column (0.7 × 8 cm) Using a Linear Gradient of Acetonitrile (10—35%) in 50 mM Triethylammonium Acetate (Total, 100 ml)

Fig. 1b, c. Reversed-Phase HPLC Analysis of b) d-pTCCAGGGTCTGGTAC and c) d-TCCAGGGTCTGGTAC Using a Linear Gradient of Acetonitrile in 0.1 M Triethylammonium Acetate at a Flow Rate of 0.7 ml/min

solvent, was applied to a Sep-Pak C_{18}. After washing of the column with H_2O to remove the salt, the product was eluted with 80% acetic acid and the TEAn group was removed by keeping the eluate at room temperature for 1 h. Completely unprotected pentadecamer was purified by reversed-phase high pressure liquid chromatography) (HPLC) (Fig. 1b). To compare the retention times of the pentadecamer with and without phosphate at the 5′-end, the pentadecamer with the same sequence but no phosphate at the 5′-end was prepared by the same procedure. It is apparent that the 5′-phosphorylated pentadecamer was eluted faster than the non-phosphorylated one (Fig. 1b, c). The existence of the 5′-phosphate on the pentadecamer was analyzed as follows. At first, the purified pentadecamer was found to be unphosphorylated by [γ-^{32}P]ATP and polynucleotide kinase (Fig. 2, lane 1). However, after treatment with bacterial alkaline phosphatase to remove the 5′-phosphate, it could be phosphorylated as in the case of the non-phosphorylated pentadecamer (Fig. 2, lane 2, 3). The labeled pentadecamer was next subjected to mobility shift analysis to confirm its nucleotide sequence.[14]

In order to determine whether synthetic 5′-phosphorylated pentadecamer could be a substrate for the T_4-DNA ligase reaction, [γ-^{32}P]CCTCCTACCGTTGAAC (Fig. 3, I) and CAGACCCTGGAGTTCAACGG (Fig. 3, III) were prepared as an acceptor and the template, respectively. They were mixed with pTCCAGGGTCTGGTAC, annealed and then

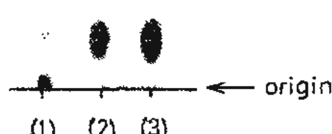

Fig. 2. Analysis by Homochromatography

Lane 1, 5′-*O*-phosphorylated pentadecamer was labeled with [γ-^{32}P]ATP and polynucleotide kinase; lane 2, 5′-*O*-phosphorylated pentadecamer was treated with bacterial alkaline phosphatase then labeled; lane 3, non-phosphorylated pentadecamer was labeled.

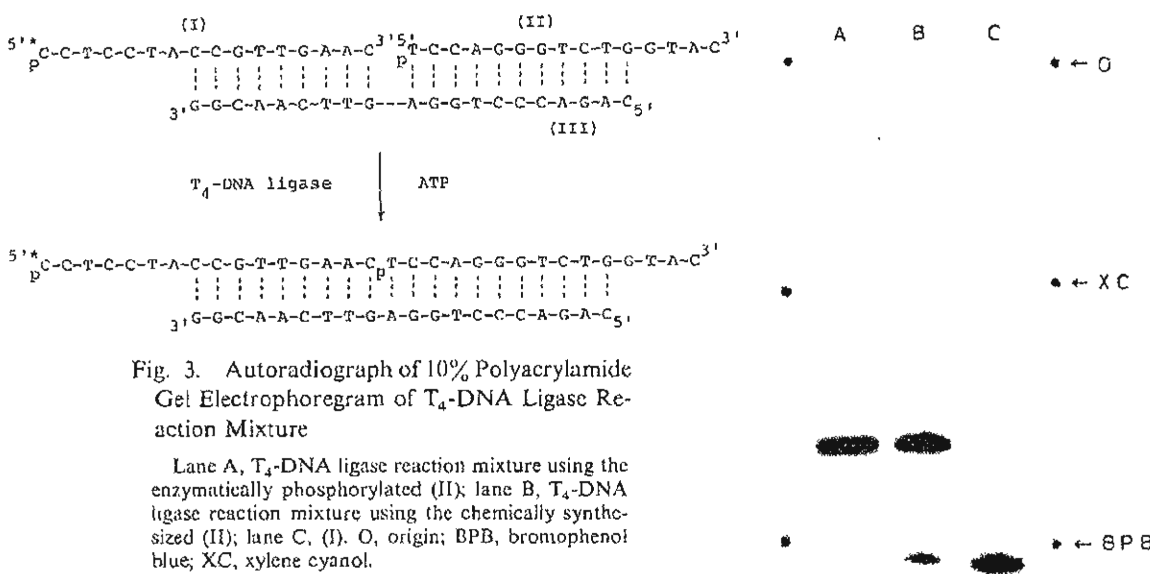

Fig. 3. Autoradiograph of 10% Polyacrylamide Gel Electrophoregram of T_4-DNA Ligase Reaction Mixture

Lane A, T_4-DNA ligase reaction mixture using the enzymatically phosphorylated (II); lane B, T_4-DNA ligase reaction mixture using the chemically synthesized (II); lane C, (I). O, origin; BPB, bromophenol blue; XC, xylene cyanol.

treated with DNA ligase at 20 °C for 9 h. To compare the regular DNA-ligase reaction, pentadecamer with the same sequence but no phosphate at the 5′-end was phosphorylated with ATP and polynucleotide kinase. It was also annealed with the acceptor and template,

and then treated with DNA ligase. The mixture was analyzed by 10% polyacrylamide gel electrophoresis. As shown in Fig. 3, very little difference was observed in the two autoradiograms (lanes A and B). A new product which migrated in the position of 30-mer was seen in both cases.

In this work, we prepared two lipophilic amines, TEAn and TEAm, for the protection of the phosphoryl group. Though both groups were successfully introduced onto the 5'-phosphoryl group of nucleoside by TPSCl and 1-MeIm, it was found that the TEAn group was more suitable for this purpose owing to its instability to acid and higher lipophilicity. We also achieved the synthesis and purification of a 5'-phosphorylated pentadecamer by the phosphotriester method on a polystyrene support.

Materials and Methods

Thin-layer chromatography (TLC) was performed on plates of Kieselgel $60F_{254}$ (Merck). Column chromatography was performed on Kieselgel 60 (Merck). Reversed-phase chromatography was performed on alkylated silica gel (C_{18}, 55—105 μ, Waters). Sep-Pak C-18 was purchased from Waters. HPLC was performed on an Altex 322MP chromatography system. Reversed-phase HPLC was carried out on Nucleosil C-18 (5 μ) packed into a stainless steel column (i.d. 0.6 × 20 cm) under a pressure of 500 kg/cm^2.
500 kg/cm^2.

Venom phosphodiesterase was purchased from Boehringer Mannheim. T_4-polynucleotide kinase and T_4-DNA ligase were purchased from Takara Shuzo Co. Ltd.

Synthesis of Trityloxyethylamine (TEAm) (5)——Trityl chloride (1.8 g, 6.5 mmol) was added to a solution of *N*-hydroxyethylphthalimide (955 mg, 5 mmol) in dry CH_2Cl_2 (20 ml) and diisopropylethylamine (1.4 ml, 8 mmol). After 2 h, the completion of the reaction being confirmed by TLC (CH_2Cl_2 : MeOH = 10 : 1, *Rf* 0.35→0.81), hydrazine hydrate (8 ml, 165 mmol) was added to the mixture. Then methanol was added until the mixture became a homogeneous solution. After 1 h, TLC showed that the reaction was completed (CH_2Cl_2 : MeOH = 10 : 1, *Rf* 0.81→0.24). The mixture was diluted with CH_2Cl_2 (30 ml), washed with 0.1 M triethylammonium bicarbonate (TEAB) (pH 7.5) (30 ml × 4) and evaporated. The residue was applied to a silica gel column (i.d. 3.5 × 7 cm) and eluted with an increasing MeOH concentration in CH_2Cl_2. The fractions containing **5** were pooled and evaporated. The residue was dried *in vacuo* to give a white precipitate. Yield, 1.25 g (4.12 mmol, 82%).

Synthesis of *N*-Trityloxyethylaniline (TEAn) (6)——Trityl chloride (2.2 g, 8 mmol) was added to a solution of β-anilinoethanol (0.63 ml, 5 mmol) in dry CH_2Cl_2 (20 ml) and diisopropylethylamine (1.7 ml, 10 mmol). The mixture was stirred for 2 h at room temperature, and the completion of the reaction was confirmed by TLC (CH_2Cl_2, *Rf* 0→0.81). The mixture was diluted with CH_2Cl_2 (20 ml), washed with 0.1 M TEAB (20 ml × 3) and evaporated. The residue was separated on a silica gel column (i.d. 3.5 × 4 cm). The fractions containing **6** were pooled and evaporated. Yield, 1.78 g (4.7 mmol, 94%).

Synthesis of 9 and 10——*o*-Chlorophenylphosphorodichloridate (0.11 ml, 0.65 mmol) was added dropwise to a solution 1,2,4-triazole (114 mg, 1.65 mmol) in dry CH_2Cl_2 (5 ml) and pyridine (0.5 ml) at 0 °C, and the mixture was stirred for 15 min, 3'-*O*-benzoylthymidine (**7a**) which had previously been co-evaporated with pyridine and then dissolved in CH_2Cl_2–pyridine (5 ml–0.5 ml) was added. After 15 min, a 0.1 M TEAB solution was added to the mixture and the phosphorylated product (**8**) was extracted with CH_2Cl_2 (20 ml). The organic layer was washed with 0.1 M TEAB (5 ml × 3) and evaporated. The residue was co-evaporated with pyridine to remove a trace of water and treated with TPSCl (606 mg, 2 mmol) and 1-methylimidazole (0.28 ml, 3.5 mmol) in CH_2Cl_2 (5 ml) for 10 min. Then TEAm (303 mg, 1 mmol), previously co-evaporated with pyridine and dissolved in CH_2Cl_2 (5 ml), was added. The mixture was stirred for another 30 min. After confirmation of completion of the reaction by TLC (CH_2Cl_2 : MeOH = 10 : 1, *Rf* 0→0.6), the mixture was dropped into *n*-hexane (100 ml) with stirring and kept in a freezer overnight. The supernatant was removed. The oily residue was applied to a silica gel column (i.d. 3 × 4.5 cm) and eluted with an increasing MeOH concentration in CH_2Cl_2. The fractions containing **9** were pooled and evaporated. The residue was taken up in CH_2Cl_2 (3 ml) and dropped into *n*-hexane (50 ml) with stirring. The precipitate was collected and dried *in vacuo*. Yield, 235 mg (0.29 mmol, 58%).

By the same procedure, **9b—d** were obtained in yields of 70%, 83% and 61%, respectively. For the synthesis of compound **10a—d**, TEAn instead of TEAm was used for the condensation reaction. The yields of **10a—d** were 45%, 75%, 45% and 47%, respectively.

Synthesis of 11a, b and 12a——Compound **9** (B = T, 164 mg, 0.2 mmol) was dissolved in MeOH (6 ml). Then 2 N NaOH solution (1 ml) was added with stirring at 0 °C. After 10 min, the completion of the reaction was confirmed by TLC (CH_2Cl_2 : MeOH = 10 : 1, *Rf* 0.65→0.52). The mixture was neutralized by adding 2 N HCl solution, then diluted with CH_2Cl_2 (10 ml), and the organic layer was washed with 0.1 M TEAB (10 ml × 3). The organic layer was evaporated, and the residue was applied to a silica gel column (i.d. 3 × 4 cm). Elution was carried out with an

increasing MeOH concentration in CH_2Cl_2. The fractions containing **11** were pooled and evaporated. The residue was taken up in CH_2Cl_2 (2 ml) and dropped into *n*-hexane (40 ml) with stirring. The precipitate was collected and dried *in vacuo*. Yield, 108 mg (0.15 mmol, 75%). By the same procedure, **11b** and **12a** were obtained in 70% and 88% yields, respectively, from **9b** or **10a**.

Synthesis of 13a, b and 14a——*o*-Chlorophenylphosphorodichloridate (0.05 ml, 0.30 mmol) was added dropwise to a solution of 1, 2, 4-triazole (23 mg, 0.33 mmol) in a mixture of CH_2Cl_2 (5 ml) and pyridine (0.5 ml) with stirring at 0 °C. After 15 min, **11a** (72 mg, 0.1 mmol), which had previously been co-evaporated with pyridine and then dissolved in CH_2Cl_2 (5 ml), was added. After another 20 min, 0.1 M TEAB (10 ml) was added and the product was extracted with CH_2Cl_2 (10 ml). The organic layer was washed with 0.1 M TEAB (10 ml × 4) and evaporated. The residue was co-evaporated with pyridine, and dissolved in CH_2Cl_2 (1 ml), and this solution was dropped into *n*-hexane (50 ml) with stirring. The precipitate was collected and dried *in vacuo*. Yield, 87 mg, 86%. By the same procedure, **13b** and **14a** were obtained in 92% yield in each case.

Synthesis of Dinucleotide (pTpC)——Compound **13a** (15 mg, 15 μmol) or **14a** (16 mg, 15 μmol) was mixed with *N*-4-benzoyldeoxycytidine bound to a polystyrene resin (17 mg, 3 μmol) and co-evaporated with pyridine (0.5 ml). The mixture was treated with MSNT (20 mg, 68 μmol) in pyridine (0.4 ml) at room temperature. After 40 min, the resin was washed successively with pyridine (2 ml × 2), CH_2Cl_2 (2 ml × 3) and ether (2 ml × 2), and dried. The resin was treated with 0.5 M TMG-PAO in dioxane–pyridine–water (5 : 4 : 1 v/v, 1 ml) at room temperature for 12 h (when **13a** was used) or at 60 °C for 12 h (when **14a** was used). After evaporation of the volatile materials, the product was treated with a mixture of conc. NH_4OH (2 ml) and pyridine (0.5 ml) at 60 °C for 5 h. The mixture was centrifuged and the resin was washed with 50% aqueous pyridine (1 ml). The combined solution was washed with ethyl acetate (6 ml × 3) and evaporated. The residue was dissolved in 0.1 M TEAA (pH 7, 0.2 ml) and applied to a reversed-phase (C_{18}) silica gel column (0.7 × 5 cm). Elution was carried out with a linear gradient formed from 50 ml of 10% acetonitrile in 0.1 M TEAA (mixing vessel) and 50 ml of 50% acetonitrile in 0.1 M TEAA (reservoir). The fractions containing the desired product (31 A_{267}) were concentrated to a small volume. The residue was dissolved in H_2O (2 ml) and applied to a Sep-Pak C-18 column previously washed successively with H_2O (10 ml), 60% aqueous acetonitrile (10 ml) and H_2O (20 ml). The column was washed with H_2O (20 ml), then the product was eluted with 80% aqueous acetic acid (6 ml), and the eluate was kept at room temperature for 1 h. The mixture was diluted with H_2O (3 ml), washed with ether (6 ml × 3) and evaporated. An aliquot of the sample (2.5 A_{267} units), after isolation by the paper electrophoresis, was treated with a bacterial alkaline phosphatase (69 units/ml) (3 μl) in 5 mM TEAB (100 μl) at 37 °C for 4 h. The mixture was separated by paper electrophoresis after heating at 100 °C for 5 min in the presence of 40 mM nitrilotriacetic acid (NTA) (13 μl). The de-phosphorylated dimer (TpC) was then treated with snake venom phosphorodiesterase [(1 mg/ml) (3 μl)] in 0.2 M TEAB at 37 °C for 4 h. The mixture was analyzed by paper electrophoresis to separate T (0.96 A_{267} at pH 7.5) and pC (1.30 A_{280} at pH 2). The ratio found was 1.03 : 1.

Synthesis of Oligonucleotides——The target 5′-*O*-phosphorylated oligonucleotide was synthesized according to the reaction cycle shown in Table I. Starting from *N*-4-benzoyldeoxycytidine bound to a polystyrene support (17 mg, 3 μmol), the protected dimer (20 mg, 15 μmol) was condensed with MSNT (20 mg, 67 μmol) in pyridine (400 μl). At the last coupling, **14a** (16 mg, 15 μmol) was used for condensation. After the reaction, the procedures used for deprotection and isolation were similar to those described for the synthesis of the dimer. The loaded polystyrene support was treated with 0.5 M TMG-PAO (1 ml) at 60 °C overnight and with conc. NH_4OH (2.5 ml). Then the mixture was separated on a reversed-phase (C_{18}) silica gel column to obtain the pentadecamer with a TEAn group at the 5′-end (65 A_{267}) (Fig. 1a). After the removal of the salt and treatment with 80% AcOH, the unprotected pentadecamer was further purified by reversed-phase HPLC (Fig. 1b). Other oligonucleotides with hydroxyl group at the 5′-end (CCTCCTACCGTTGAAC, TCCAGGGTCTGGTAC, CAGACCCTGGAGTTCAACGG) were prepared by the reported procedure.[3]

Kinase Reaction of Oligonucleotide——The synthetic 5′-*O*-phosphorylpentadecamer pTCCAGGGTCTGGTAC (0.02 A_{260} unit, 150 pmol), the de-phosphorylated pentadecamer (0.02 A_{260} unit, 150 μmol) obtained by treatment with bacterial alkaline phosphatase (0.04 unit) in 0.1 M TEAB (5 μl) at 55 °C for 1.5 h, followed by heating at 100 °C for 5 min in the presence of NTA, and the pentadecamer with the same sequence but no phosphoryl group at the 5′-end (0.02 A_{260} unit, 150 pmol) were treated with [γ-^{32}P]ATP (4000 cpm/pmol, 83 μM, 0.5 μl) and T_4-polynucleotide kinase (1 unit/μl, 1 μl) in 10 μl of 50 mM Tris–HCl (pH 9.6), 1 mM $MgCl_2$, 2 mM spermine, 10 mM dithiothreitol and 0.1 M KCl at 37 °C for 1 h. An aliquot (about one-tenth) was analyzed by homochromatography in Homomix I.[9a]

DNA Ligase Reaction——By means of the procedure mentioned above, the hexadecamer CCTCCTACCGTTGAAC was labeled with [γ-^{32}P]ATP and the pentadecamer TCCAGGGTCTGGTAC was phosphorylated at the 5′-end with cold ATP by using T_4-polynucleotide kinase. The labeled hexadecamer (0.05 A_{260} unit, 0.33 nmol), d-CAGACCCTGGAGTTCAACGG (0.05 A_{260} unit, 0.33 nmol) as a template were mixed with either the synthetic 5′-*O*-phosphorylated pentadecamer (0.05 A_{260} unit, 0.33 nmol) or enzymatically phosphorylated pentadecamer (0.05 A_{260} unit, 0.33 nmol) in 27 μl of 66 mM Tris–HCl pH 7.6, 6.6 mM $MgCl_2$, and 500 μM ATP. The mixture was heated to 75 °C for 10 min and then cooled slowly to 20 °C over a period of 1 h. Then 1.5 μl of 0.2 M β-mercaptoethanol and 1.5 μl of T_4-DNA ligase (350 units/μl, 1.5 μl) were added to the mixture. This mixture was then incubated at 20 °C for 9 h. An aliquot (5 μl) of the mixture was dissolved in 8 M urea, 10 mM EDTA and 40 mM Tris–borate

(pH 8.4) containing 0.04% (w/v) marker dye (3 μl) and loaded onto a 10% polyacrylamide gel for electrophoresis at 400 V. The result is shown in Fig. 3.

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Synthesis of Sceletium and Amaryllidaceae Alkaloids, (±)-Mesembrine and (±)-Dihydromaritidine, (±)-Epidihydromaritidine, (±)-Elwesine, and (±)-Epielwesine[1]

OSAMU HOSHINO,* SHOHEI SAWAKI, NAOMI SHIMAMURA, AKIRA ONODERA, and BUNSUKE UMEZAWA*

Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12, Ichigaya Funagawara-machi, Shinjuku-ku, Tokyo 162, Japan

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The alkylation of 2-arylcyclohexanones (**12b** and **6**) with alkyl halides or acrylonitrile and 50% aq. sodium hydroxide in the presence of a phase-transfer catalyst (18-crown-6) was found to give readily 2-alkyl-2-arylcyclohexanones (**14** and **16**) in fair to good yields. Furthermore, 2-allyl-2-aryl-5,5-ethylenedioxycyclohexanone (**16a** or **16b**) was coverted into (±)-mesembrine (**1**), (±)-dihydromaritidine (**4a**), and (±)-epidihydromaritidine (**5a**) or (±)-elwesine (dihydrocrinine) (**4b**) and (±)-epielwesine (**5b**), respectively, *via* 5,5-ethylenedioxy-2-formylmethyl-2-(3′,4′-dimethoxy- or 3′,4′-methylenedioxyphenyl)cyclohexyl acetate (**20a** or **20b**).

Keywords——alkylation; 18-crown-6; 2-aryl-5,5-ethylenedioxycyclohexanone; 2-allyl-2-aryl-5,5-ethylenedioxycyclohexanone; (±)-mesembrine; (±)-dihydromaritidine; (±)-elwesine

In the past decade, considerable efforts to synthesize a Sceletium alkaloid,[2] mesembrine (**1**), have been made by organic chemists,[3,4] because it is of interest pharmacologically, and is structurally a close relative of Amaryllidaceae alkaloids[5] such as (+)-maritidine (**2**) and (−)-crinine (**3**). In the course of our studies[6] on the synthesis of Amaryllidaceae alkaloids, we found that alkylation in the presence of a phase-transfer catalyst[7] gave a useful key compound for the synthesis of (±)-**1**. The present paper is concerned with the effective alkylation and synthesis of (±)-**1**, (±)-dihydromaritidine (**4a**),[3a,f] (±)-elwesine (dihydrocrinine) (**4b**),[8a,b] and related alkaloids (**5a**[3a] and **5b**[8]).

The desired 2-arylcyclohexanone (**6a**) was prepared in a reasonable yield as described below. Condensation of homoveratraldehyde[9] with methyl vinyl ketone *via* the pyrrolidine enamine and subsequent hydrolysis with 10% hydrochloric acid (HCl) produced mainly a keto-aldehyde (**7a**). Refluxing of the crude product with acetic acid gave a cyclized product (**8a**), ketalization of which afforded 4,4-ethylenedioxy-1-(3′,4′-dimethoxyphenyl)cyclohexene (**9a**) in 23.5% overall yield from homoveratraldehyde. A considerable improvement of the overall yield (46%) was achieved when homoveratraldehyde was treated with pyrrolidine in

1 2 3 4: R^1=OH, R^2=H; 5: R^1=H, R^2=OH 6

a: R=CH_3 b: R+R=CH_2

Chart 1

Chart 2

the presence of potassium carbonate[10] in tetrahydrofuran (THF) at room temperature. Epoxidation of **9a** with *m*-chloroperbenzoic acid (MCPBA) in ether gave an epoxide (**10**) in 91% yield. Rearrangement of **10** in boiling benzene in the presence of lithium perchlorate and tri-*n*-butylphosphine oxide[11] gave a mixture of the expected cyclohexanone (**6a**) and an allylic alcohol (**11**) in 40—70% and 20—23% yields, respectively. A high yield (80%) of **6a**, however, was attained by hydroboration–oxidation of **9a** followed by oxidation with Collins oxidant.[12]

The available data[13] on the alkylation of 2-arylcyclohexanones (**12a** and **12c**) indicate that a prolonged reflux in strongly basic solutions is required. In addition, Yamada *et al.*[14] have observed that 5,5-ethylenedioxy-2-methyl-2-phenylcyclohexanone undergoes fission to an enone (**13**) by Grignard reagent, alkyllithium or lithium diisopropylamide even at very low temperature. From these findings, a prolonged exposure of **6a** to strongly basic media was expected to cause undesirable fission of the ethylenedioxy grouping, lowering the yield of the expected 2-alkylated product.

The above serious problem might be solved if alkylation could readily take place at a benzylic position in 2-arylcyclohexanone. Therefore, the alkylation of 2-(3′,4′-methylenedioxyphenyl)cyclohexanone (**12b**)[6a] in the presence of a phase-transfer catalyst was explored, because **12c** is resistant to alkylation with ordinary bases such as sodium hydride or potassium *tert*-butoxide.[13b] Namely, alkylation of **12b** in a mixture of 50% aq. sodium hydroxide (NaOH) and benzene with allyl bromide in the presence of 18-crown-6 at 75 °C gave 2-allyl-2-(3′,4′-methylenedioxyphenyl)cyclohexanone (**14a**) in 96% yield; the structure of the product was confirmed on the basis of the spectral data [proton nuclear magnetic resonance (^{1}H-NMR) and mass spectrum (MS)]. Similarly, reaction with acrylonitrile at room temperature afforded a 2-cyanoethylcyclohexanone (**14b**) in 40% yield. The former yield is comparable to that of the alkylation of **12a** reported by Shamma and Rodriguez.[13a] Furthermore, ethylation with ethyl iodide gave **14c** in 46% yield. With chloroacetonitrile, however, two diastereomeric glycidonitriles (**15**) were formed. Methylation with methyl iodide afforded **14d**, when dimethylformamide (DMF)[15] was used in place of benzene as the solvent. Thus, it was proved that the alkylation in the presence of 18-crown-6 readily gave 2-alkylated products.

With the above results in mind, alkylation of **6a** with allyl bromide in the presence of 18-crown-6 at 75 °C was carried out to give a 1.5 : 1 mixture of 2-allyl-5,5-ethylenedioxy-2-(3′,4′-

12a : R=CH_3, R^1=H
12b : R+R=CH_2, R^1=H
12c : R+R=CH_2, R^1=CH_3

13

14a : R=$CH_2CH=CH_2$
14b : R=CH_2CH_2CN
14c : R=C_2H_5
14d : R=CH_3

15

18 16 17
a : R=CH_3 b : R+R=CH_2

Chart 3

Table I. Reaction Conditions and Yields of **16a** and **17a**

Reaction temperature (°C)	Reaction time (h)	Yields (%)	
		16a	**17a**
75	1.5	25	16.5
25	1.0	62.5	10
25	0.5	69.5	3

dimethoxyphenyl)cyclohexanone (**16a**)[16] and an undesired fission product (**17a**). However, a high ratio (23:1) in favor of the former was obtained by lowering the reaction temperature and shortening the reaction time. The results are summarized in Table I.

In contrast with **12b**, alkylation of **6a** with methyl or ethyl iodide failed, while **18** was formed by cyanoethylation under conditions similar to those noted above.

Since the key compound (**16a**) was available through alkylation in the presence of 18-crown-6, its conversion into (±)-**1** was carried out. Oxidation of **16a** with osmium tetroxide (OsO_4)–sodium metaperiodate ($NaIO_4$)[17] gave an aldehyde (**19**) in 62% yield, reductive amination of which with methylamine hydrochloride ($CH_3NH_2 \cdot HCl$) and sodium cyanoborohydride ($NaBH_3CN$)[18] followed by acid treatment afforded (±)-**1** in 15% yield. A better result was obtained as follows. Successive treatment of **16a** with sodium borohydride ($NaBH_4$), acetic anhydride, and OsO_4–$NaIO_4$ gave an acetoxy-aldehyde (**20a**), the ^{1}H-NMR spectrum of which showed the signal of one proton at the 1-position as a triplet ($J=7$ Hz) at δ 5.22.[19] From a consideration of the spectrum in conjunction with a Dreiding model, it was deduced that the formylmethyl and an acetoxyl groups in **20a** were *trans*-oriented, with the cyclohexane ring in a twist boat form due to 1,3-diaxial interaction of the acetoxyl and ethylenedioxy groups. This inference is supported by the presumption that both alkylation and $NaBH_4$ reduction would take place from the opposite side to the axially oriented C–O bond of the ethylenedioxy group.

Thus, the reductive amination of **20a** followed by boiling with 10% HCl gave (±)-**1** in

Chart 4

48% yield. The spectral and physical data were identical with those of the (±)-alkaloid in the literature.[20]

The above results suggested that **16** could also be transformed to (±)-dihydromaritidine (**4a**), (±)-epidihydromaritidine (**5a**), (±)-elwesine (**4b**), and (±)-epielwesine (**5b**).

Reductive amination of **19** or **20a** in the presence of ammonium acetate and subsequent acid treatment failed to give the desired amine (**21a**). Instead, a tertiary amine (**22**) [MS *m/z*: 791 (M^+)] was obtained in the latter case. Since **21a**[20b,21] was not obtained conveniently, reductive amination of **20a** in the presence of benzylamine ($C_6H_5CH_2NH_2$) was explored to give an *N*-benzylamine (**23a**)[3a] in 59% yield. On the other hand, when the Schiff base derived from **20a** and $C_6H_5CH_2NH_2$ was first reduced with $NaBH_4$ and then treated with 10% HCl, the yield of **23a** was increased to 75%.

Although a formal synthesis of the alkaloids (**4a** and **5a**) was achieved at that stage,[3a] we tried to develop our own route to the final products. Lithium aluminum hydride ($LiAlH_4$) reduction of **23a** gave a pair of epimeric alcohols (**24a** and **25a**) in 25.6% and 42.6% yields, respectively, acetylation of which gave the corresponding acetates (**26a** and **27a**). Reductive debenzylation of the acetates (**26a** and **27a**) with hydrogen over palladium on active carbon followed by Pictet–Spengler reaction afforded in moderate yields (±)-**4a** and (±)-**5a**, respectively, the spectral and physical data of which were coincident with those of the (±)-alkaloids in the literature.[3a]

Quite analogously, homopiperonal[9] was transformed to **23b**, $LiAlH_4$ reduction of which afforded a pair of epimeric alcohols (**24b** and **25b**) in a product ratio of 1 : 1. Thus, (±)-**4b** and (±)-**5b** were synthesized *via* the acetates (**26b** and **27b**). Except for a discrepancy in the melting point of the former, the spectral data given here agreed well with those of authentic samples.[22]

In conclusion, the alkylation of **6a** and **6b** in the presence of 18-crown-6 proceeded readily to give in moderate yields **16a** and **16b**, which were converted into several Sceletium and Amaryllidaceae alkaloids (**1**, **4**, and **5**).

Experimental

Melting points were measured on a Büchi melting point apparatus and all melting and boiling points are

uncorrected. Infrared (IR) spectra were run on a Hitachi 215 or 225 infrared spectrometer in $CHCl_3$ solution, unless otherwise noted. ^{1}H-NMR spectra were taken with a Hitachi 24B (60 MHz) or JEOL JNM-FX-100 (100 MHz) spectrometer in $CDCl_3$ solution using $(CH_3)_4Si$ as an internal standard. MS were measured with a Hitachi RMU-7M mass spectrometer (70 eV). Column chromatography was performed on silica gel (Kanto Chemical Co., Ltd.) and preparative thin layer chromatography (TLC) on Kieselgel HF_{254} (Merck).

4,4-Ethylenedioxy-1-(3′,4′-dimethoxyphenyl)cyclohexene (9a)——a) A mixture of homoveratraldehyde[9] (27.5 g) and pyrrolidine (16.2 ml) in benzene (140 ml) was refluxed for 2.25 h under a condenser equipped with a water separator. Methyl vinyl ketone (MVK) (15.5 ml) was added to a stirred dry benzene (60 ml) solution of the reddish-brown oil obtained on concentration of the reaction mixture *in vacuo*, and the whole was stirred for 20 h at room temperature under Ar. Then 15% HCl (33 ml) was added dropwise under ice-cooling, and the reaction mixture was stirred for 20 h at room temperature and for 1 h under heating at 110 °C (bath temperature). After cooling of the reaction mixture, H_2O was added and the product was taken up in ether. Usual work-up of the ether extract gave a reddish oil (22.3 g) containing mainly **7a** [^{1}H-NMR (60 MHz) δ: 2.05, 9.40]. A solution of the crude oil (22.3 g) in AcOH (50 ml) was refluxed for 4.5 h, then allowed to cool. H_2O was added to the reaction mixture, which was basified with K_2CO_3 (powder) and extracted with ether. Usual work-up of the ether extract gave a reddish-brown oil (15.7 g) containing mainly **8a**, ketalization [ethylene glycol (4.8 g), *p*-TsOH (70 mg), and dry benzene (120 ml)] of which (15.7 g) and usual work-up, followed by column chromatography (elution with benzene–MeOH (100 : 1)), gave **9a** (9.9 g, 23.5% from the aldehyde), mp 77.5—78 °C (hexane–ether). IR (KBr): 840 cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.80, 3.82 (each 3H, s, $OCH_3 \times 2$), 3.95 (4H, s, OCH_2CH_2O), 5.87 (1H, m, 2-H), 6.70—6.95 (3H, m, ArH). *Anal.* Calcd for $C_{16}H_{20}O_4$: C, 69.54; H, 7.30. Found: C, 69.30; H, 7.33.

b) Pyrrolidine (7.2 ml) was added dropwise to an ice-cooled, stirred mixture of homoveratraldehyde (6.53 g) and dried K_2CO_3 (1.88 g) in dry THF (45 ml)[10] over a period of 1 h under Ar and stirring was continued for 3 h at room temperature. Removal of K_2CO_3 by filtration and concentration of the filtrate *in vacuo* gave a red oil, which was dissolved in dry benzene (15 ml). A solution of MVK (3.8 ml) in dry benzene (5 ml) was added to the stirred benzene solution at room temperature under Ar and the whole was stirred for 22 h at the same temperature. Then 15% HCl (8 ml) was added to the reaction mixture under ice-cooling. The whole was stirred for 1 h at room temperature and refluxed for 0.5 h under Ar. Usual work-up gave a reddish-yellow oil (8.8 g) containing mainly **7a**. A solution of the crude oil (8.8 g) in AcOH (50 ml) was refluxed for 8 h under Ar to give an oil (7.9 g) containing mainly **8a**, ketalization [ethylene glycol (2.9 g), *p*-TsOH (43 mg), and dry benzene (70 ml)] of which, followed by one recrystallization from hexane–ether, furnished **9a** (4.59 g, 46% from the aldehyde), mp 69—73 °C. The spectral data were identical with those of the sample obtained in a).

4,4-Ethylenedioxy-1-(3′,4′-dimethoxyphenyl)cyclohexene Oxide (10)——A solution of MCPBA (440 mg) in dry ether (20 ml) was added dropwise to a stirred solution of **9a** (500 mg) in dry ether (15 ml) at room temperature and the whole was stirred for 7 h at the same temperature. Usual work-up of the reaction mixture gave, after recrystallization from ether, pale yellow crystals (**10**) (483 mg, 91%), mp 108—111 °C. IR (KBr): 1255, 800 cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.08 (1H, br s, 2-H), 3.85 (6H, s, $OCH_3 \times 2$), 3.93 (4H, m, OCH_2CH_2O), 6.76—7.03 (3H, m, ArH). *Anal.* Calcd for $C_{16}H_{20}O_5$: C, 65.74; H, 6.90. Found: C, 65.72; H, 6.92.

5,5-Ethylenedioxy-2-(3′,4′-dimethoxyphenyl)cyclohexanone (6a)——a) From **10**: A solution of **10** (250 mg) in benzene (6 ml) was added dropwise to a boiling mixture of $LiClO_4$ (140 mg) and *n*-Bu_3PO (187 mg) in benzene (6 ml),[11] and the whole was refluxed for 2 h. Benzene was added to the reaction mixture and a yellow oil was obtained on usual work-up. Column chromatography of the oil gave crystals (**6a**) (167 mg, 66.8%) (elution with benzene) and a colorless oil (**11**) (58 mg, 23.2%). **6a**: mp 94—95.5 °C (MeOH, rapid cooling). IR: 1710 (C=O) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 2.05—2.20 (4H, m, 3- and 4-H), 2.70 (2H, s, 6-H), 3.35—3.65 (1H, m, 2-H), 3.81 (6H, s, $OCH_3 \times 2$), 3.95 (4H, s, OCH_2CH_2O), 6.60—6.85 (3H, m, ArH). MS *m/z*: 292 (M^+). *Anal.* Calcd for $C_{16}H_{20}O_5$: C, 65.74; H, 6.90. Found: C, 65.75; H, 6.85. **11**: IR: 3500 (OH) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 3.85 (6H, s, $OCH_3 \times 2$), 4.00 (4H, m, OCH_2CH_2O), 4.65 (1H, m, 1-H), 5.90 (1H, t, J=4 Hz, 3-H), 6.80—7.20 (3H, m, ArH).

b) From **9a**: A 1 M BH_3-THF solution (9 ml) was added dropwise to an ice-cooled, stirred solution of **9a** (828 mg) in dry THF (18 ml) under Ar and stirring was continued for 3 h at room temperature. H_2O (0.5 ml), 3 N NaOH (1.5 ml) and 30% H_2O_2 (1.5 ml) were added to the reaction mixture under ice-cooling and the whole was stirred for 3 h at room temperature. The solvent was removed *in vacuo* to leave a residue, which was taken up in benzene. Usual work-up of the organic extract gave 5,5-ethylenedioxy-2-(3′,4′-dimethoxyphenyl)cyclohexanol as colorless crystals (774 mg, 88%), mp 118—118.5 °C (ether). IR (KBr): 3380, 3340 (OH) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.86, 3.88 (each 3H, s, $OCH_3 \times 2$), 3.98 (4H, s, OCH_2CH_2O), 6.68—6.92 (3H, m, ArH). *Anal.* Calcd for $C_{16}H_{22}O_5$: C, 65.29; H, 7.53. Found: C, 65.23; H, 7.60. A solution of the alcohol (588 mg) in CH_2Cl_2 (40 ml) was added dropwise to Collins reagent [CrO_3 (1.2 g), dry pyridine (2 ml) and CH_2Cl_2 (40 ml)] under stirring, and stirring was continued for 0.5 h at room temperature. Usual work-up of the reaction mixture gave **6a** as colorless crystals (532 mg, 91%), mp 116—116.5 °C (ether). *Anal.* Calcd for $C_{16}H_{20}O_5$: C, 65.74; H, 6.90. Found: C, 65.92; H, 6.88. The spectral data were identical with those of the sample obtained in a).

General Procedure for Alkylation of 12b——A 50% aq. NaOH solution was added to a stirred solution of **12b**[6a)] and 18-crown-6 in benzene (2—3 ml). A solution of alkyl halide or acrylonitrile in benzene (2 ml) was added, and the

whole was stirred at an appropriate temperature. Saturated aq. NH_4Cl was added to the water-cooled reaction mixture and the product was taken up in ether. Usual work-up of the organic extract gave an oil, which was subjected to column chromatography or preparative TLC.

a) With Allyl Bromide: **12b** (436 mg), 18-crown-6 (26.5 mg), 50% aq. NaOH (3 ml), and allyl bromide (291 mg) were used. The whole was stirred at 75 °C for 1.25 h. Column chromatography of the oily product (588 mg) (elution with benzene–hexane (10:1)) gave crystalline 2-allyl-2-(3',4'-methylenedioxyphenyl)cyclohexanone (**14a**) (498 mg, 96%), mp 35—41 °C, which was distilled at 140 °C (0.02 mmHg) (bath temperature) to give colorless prisms, mp 46—47 °C. IR: 1710 (C=O), 1640 (C=C) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 4.60—5.70 (3H, m, $CH=CH_2$), 5.90 (2H, s, OCH_2O), 6.40—6.85 (3H, m, ArH). High-resolution MS Calcd for $C_{16}H_{18}O_3$ (M^+) *m/z*: 258.1254. Found: 258.1250.

b) With Acrylonitrile: **12b** (218 mg), 18-crown-6 (14 mg), 50% aq. NaOH (2 ml), and acrylonitrile (64 mg) were used. The whole was stirred for 0.5 h at room temperature. Usual work-up and column chromatography of the resulting oil (290 mg) (elution with benzene–EtOAc (20:1)) gave 2-cyanoethyl-2-(3',4'-methylenedioxyphenyl)-cyclohexanone (**14b**) as an oil (164 mg, 40%); bp 170 °C (0.05 mmHg) (bath temperature). IR (film): 2250 (C≡N), 1710 (C=O) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 5.90 (2H, s, OCH_2O), 6.35—6.85 (3H, m, ArH). High-resolution MS Calcd for $C_{16}H_{17}O_3$ (M^+) *m/z*: 271. 1206. Found: 271.1205.

c) With Ethyl Iodide: **12b** (218 mg), 18-crown-6 (14 mg), 50% aq. NaOH (2 ml), and EtI (312 mg) were used. The reaction mixture was stirred for 1 h at room temperature. Usual work-up and preparative TLC (developing solvent; benzene–EtOAc (10:1)) gave an ethyl enol ether as an oil (6 mg) (less polar), 2-ethyl-2-(3',4'-methylenedioxyphenyl)cyclohexanone (**14c**) as an oil (more polar) (113 mg, 46%), and **12b** (20 mg) (the most polar), respectively. Ethyl enol ether: IR: 1045 cm^{-1}. ^{1}H-NMR (60 MHz) δ: 1.10 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 3.60 (2H, q, J=7 Hz, $=COC\underline{H}_2CH_3$), 5.85 (2H, s, OCH_2O), 6.55—6.95 (3H, m, ArH). **14c**: IR: 1710 (C=O) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 0.61 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 5.95 (2H, s, OCH_2O), 6.45—6.92 (3H, m, ArH). High-resolution MS Calcd for $C_{15}H_{18}O_3$ (M^+) *m/z*: 246.1255. Found: 246.1271.

d) With Chloroacetonitrile: **12b** (412 mg), 18-crown-6 (28 mg), 50% aq. NaOH (2 ml), and $ClCH_2CN$ (340 mg) were used. The whole was stirred for 1 h at room temperature. Usual work-up and column chromatography of the resulting oil (410 mg) (elution with benzene–hexane (5:1) gave two diasteromeric glycidonitriles (**15**). A less polar **15** (oil, 150 mg, 31%), bp 155 °C (0.05 mmHg) (bath temperature). IR (film): 2250 (C≡N) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 2.67 (1H, s, $\overset{O}{C-C}\underline{H}CN$), 5.85 (2H, s, OCH_2O), 6.40—6.75 (3H, m, ArH). High-resolution MS Calcd for $C_{15}H_{15}NO_3$ (M^+) *m/z*: 257.1050. Found: 257.1030. A more polar **15** (oil, 30 mg, 6.2%). IR (film): 2250 (C≡N) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 3.10 (1H, s, $\overset{O}{C-C}\underline{H}CN$), 5.85 (2H, s, OCH_2O), 6.55—6.90 (3H, m, ArH). MS *m/z*: 257 (M^+). Their stereochemistry was not determined.

e) With Methyl Iodide: **12b** (218 mg), 18-crown-6 (28 mg), 50% aq. NaOH (2 ml), MeI (284 mg), and DMF (4 ml) in place of benzene were used. The whole was stirred for 0.5 h at room temperature. Usual work-up and column chromatography of the resulting oil (235 mg) (elution with benzene–hexane (10:1)) gave 2-methyl-2-(3',4'-methylenedioxyphenyl)cyclohexanone (**14d**) as an oil (170 mg, 73.5%). IR: 1710 (C=O) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 1.23 (3H, s, CH_3), 5.93 (2H, s, OCH_2O), 6.50—6.90 (3H, m, ArH). MS *m/z*: 232 (M^+). 2,4-Dinitrophenylhydrazone, mp 175.5—176.5 °C (MeOH). *Anal.* Calcd for $C_{20}H_{20}N_4O_6$: C, 58.25; H, 4.89; N, 13.58. Found: C, 58.49; H, 4.74; N, 13.35.

Alkylation of 6a——The alkylation was carried out in a manner similar to that noted for **12b**. The results are shown in Table I.

i) With Allyl Bromide: **6a** (292 mg), 18-crown-6 (14 mg), 50% aq. NaOH (2 ml), allyl bromide (146 mg), and benzene (4 ml) were used. The whole was stirred for 1.5 h at 75 °C. Usual work-up and column chromatography of the resulting oil (266 mg) gave 2-allyl-5,5-ethylenedioxy-2-(3',4'-dimethoxyphenyl)cyclohexanone (**16a**) as an oil (elution with benzene–EtOAc (10:1)) (83 mg, 25%) and an enone (**17a**) as an oil (61 mg, 16.5%) (elution with benzene–EtOAc (8:1)). **16a**: IR: 1710 (C=O) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.82, 3.85 (each 3H, s, $OCH_3\times2$), 3.90 (4H, s, OCH_2CH_2O), 4.75—5.73 (3H, m, $CH=CH_2$), 6.58—6.95 (3H, m, ArH). MS *m/z*: 332 (M^+), 291 [M^+-41 ($CH_2CH=CH_2$)]. **17a**: IR (film): 1650 (α,β-unsaturated C=O), 1615 (C=C) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.84 (6H, s, $OCH_3\times2$), 4.75—6.20 (7H, m, $CH=CH_2\times2$ and 6-H), 6.79—6.82 (3H, m, ArH). MS *m/z*: 372 (M^+).

ii) With Acrylonitrile: **6a** (292 mg), 18-crown-6 (14 mg), 50% aq. NaOH (2 ml), acrylonitrile (64 mg), and benzene (3 ml) were used. The whole was stirred for 1 h at room temperature. Usual work-up and column chromatography of the resulting oil (240 mg) (elution with benzene–EtOAc (10:1)) gave 2-cyanoethyl-5,5-ethylenedioxy-2-(3',4'-dimethoxyphenyl)cyclohexanone (**18**) as colorless prisms (80 mg, 23%), mp 114—114.5 °C (ether). IR (KBr): 2250 (CN), 1715 (C=O) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.84, 3.88 (each 3H, s, $OCH_3\times2$), 3.90—4.00 (4H, m, OCH_2CH_2O), 6.50—6.96 (3H, m, ArH). MS *m/z*: 345 (M^+). *Anal.* Calcd for $C_{19}H_{23}NO_3$: C, 66.07; H, 6.71; N, 4.06. Found: C, 66.10; H, 6.67; N, 4.06.

5,5-Ethylenedioxy-2-formylmelthyl-2-(3',4'-dimethoxyphenyl)cyclohexanone (19)——OsO_4 (32 mg) was added to a dioxane (9.5 ml) solution of **16a** (400 mg) in a flask wrapped with aluminum foil, and the whole was stirred for 15 min at room temperature. After addition of H_2O (2.5 ml), a solution of $NaIO_4$ (678 mg) in H_2O (5.2 ml) was added

to the stirred mixture over a period of 1 h and stirring was continued for 6 h at room temperature. The reaction mixture was filtered through a celite bed and the bed was well rinsed with ether. The combined filtrate and ether solution were washed with 0.5% aq. Na_2S (100 ml) and then H_2O. Usual work-up gave a pale yellow oil (288 mg), which was subjected to column chromatography. Elution with benzene–EtOAc (10 : 1) gave **19** as an oil (249 mg, 62%). IR: 1720, 1715 (C=O) cm^{-1}. ^1H-NMR (60 MHz) δ: 3.82, 3.85 (each 3H, s, $OCH_3\times2$), 3.92 (4H, s, OCH_2CH_2O), 6.68 (1H, br s, ArH), 6.82 (2H, br s, ArH), 9.55 (1H, t, J=3 Hz, CHO). High-resolution MS Calcd for $C_{18}H_{20}O_6$ (M^+) m/z: 334.1414. Found: 334.1388.

(1*R,2*S**)-5,5-Ethylenedioxy-2-formylmethyl-2-(3',4'-dimethoxyphenyl)cyclohexyl Acetate (20a)**——$NaBH_4$ (58 mg) was added to a stirred solution of **16a** (200 mg) in MeOH (10 ml) and stirring was continued for 3 h at room temperature. The solvent was removed *in vacuo* and the product was taken up in benzene. Usual work-up of the benzene layer gave 2-allyl-5,5-ethylenedioxy-2-(3',4'-dimethoxyphenyl)cyclohexanol as an oil (194 mg, 97%). IR (film): 3500 (OH) cm^{-1}. ^1H-NMR (60 MHz) δ: 2.38 (2H, br d, J=7 Hz, $C\underline{H}_2CH=$), 3.78 (6H, s, $OCH_3\times2$), 3.90 (4H, s, OCH_2CH_2O), 3.77—4.20 (1H, m, 1-H), 4.60—5.33 (3H, m, $CH=CH_2$), 6.50—6.85 (3H, m, ArH). MS m/z: 334 (M^+). Acetylation of the oil (194 mg) gave (1*R**,2*S**)-2-allyl-5,5-ethylenedioxy-2-(3',4'-dimethoxyphenyl)cyclohexyl acetate (216 mg, 99%), mp 85.5—86 °C (ether). IR (KBr): 1730 (OC=O) cm^{-1}. ^1H-NMR (100 MHz) δ: 1.95 (3H, s, $OCOCH_3$), 2.40 (2H, d, J=7 Hz, $C\underline{H}_2CH=$), 3.85 (6H, s, $OCH_3\times2$), 3.90 (4H, s, O-CH_2CH_2O), 4.65—5.40 (4H, m, $CH=CH_2$ and 1-H), 6.75—7.05 (3H, m, ArH). MS m/z: 376 (M^+). *Anal.* Calcd for $C_{21}H_{28}O_6$: C, 67.00; H, 7.50. Found: C, 67.07; H, 7.49. Reaction of the acetate (194 mg) with OsO_4 (13 mg) and $NaIO_4$ (291 mg) in dioxane (4 ml)–H_2O (4 ml) was carried out by the procedure noted above, giving **20a** (oil, 147 mg, 75.6%). IR (film): 1750 (OC=O), 1720 (CHO) cm^{-1}. ^1H-NMR (100 MHz) δ: 2.02 (3H, s, $OCOCH_3$), 2.60 (2H, d, J=3 Hz, $C\underline{H}_2CHO$), 3.88 (6H, s, $OCH_3\times2$), 3.90 (4H, m, OCH_2CH_2O), 5.22[19] (1H, t, J=7 Hz, 1-H), 6.68—7.10 (3H, m, ArH), 9.28[19] (1H, t, J=3 Hz, $CH_2C\underline{H}O$). MS m/z: 378 (M^+).

(±)-Mesembrine (1)——a) From **19**: A mixture of **19** (76 mg), $NaBH_3CN$ (46 mg), and $CH_3NH_2\cdot HCl$ (18.5 mg) in anhydrous MeOH (2 ml) was stirred for 5 d at room temperature, 10% HCl (1.5 ml) was added, and the whole was refluxed for 0.5 h. The mixture was evaporated to half the original volume *in vacuo* and the ice-cooled mixture was basified with 10% aq. NaOH. Extraction of the product with $CHCl_3$ and usual work-up of the $CHCl_3$ extract gave a red oil (68 mg), purification of which by preparative TLC (developing solvent; $CHCl_3$–MeOH (20 : 1)) yielded (±)-**1** (oil, 10 mg, 15%). The spectral data were identical with those of the sample obtained in b).

b) From **20a**: A mixture of **20a** (100 mg), $NaBH_3CN$ (20 mg), and $CH_3NH_2\cdot HCl$ (106 mg) in anhydrous MeOH (3 ml) was stirred for 3 d at room temperature. Acid treatment as noted above and usual work-up gave an oil (77 mg), column chromatography of which (elution with $CHCl_3$) gave (±)-**1** (oil, 37 mg, 48%). IR: 1720 (C=O) cm^{-1}. ^1H-NMR (100 MHz) δ: 2.32 (3H, s, NCH_3), 3.87, 3.89 (each 3H, s, $OCH_3\times2$), 6.88 (3H, m, ArH). Picrate: mp 173—175 °C (dec.) (EtOH–EtOAc) (lit.[20a] 171.5—172.5 °C). (±)-**1**·HCl, mp 176—178 °C (dec.) (iso-PrOH) [lit.[20b] 181 °C (dec.)]. The spectral and physical data were coincident with those of the (±)-alkaloid in the literature.[20]

Reductive Amination of 20a with Ammonium Acetate——A mixture of **20a** (126 mg), $NaBH_3CN$ (15 mg), and NH_4OAc (257 mg) in anhydrous MeOH (1 ml) was stirred for 90 h at room temperature and the whole was refluxed for 0.5 h. Work-up as above and column chromatography of the resulting oil (91 mg) (elution with $CHCl_3$–MeOH (100 : 1—50 : 1)) gave a pale yellow amorphous amine (**22**) (55 mg, 21%). IR 1680 (C=CHC=O) cm^{-1}. ^1H-NMR (100 MHz) δ: 3.83, 3.87 (each 9H, s, $OCH_3\times6$), 6.13 (3H, d, J=11 Hz, olefinic H), 6.60—6.90 (9H, m, ArH), 7.04 (3H, d, J=11 Hz, olefinic H). MS m/z: 791 (M^+).

(3a*S,7a*S**)-1-Benzyl-3a,4,5,6,7,7a-hexahydro-3a-(3',4'-dimethoxyphenyl)-6-oxoindoline (23a)**——a) By Reductive Amination: **23a** (oil, 570 mg, 59%) was obtained by treatment of **20a** (1.0 g) with $NaBH_3CN$ (300 mg) and $C_6H_5CH_2NH_2\cdot HCl$ (5.6 g) in anhydrous MeOH (23 ml) for 3 d at room temperature according to the above procedure. Usual work-up and column chromatography gave an oil (4.9 g) (elution with benzene–EtOAc (10 : 1—5 : 1)). **23a**: IR: 1720 (C=O) cm^{-1}. ^1H-NMR (100 MHz) δ: 3.15, 4.10 (2H, ABq, J=13 Hz, $C\underline{H}_2C_6H_5$), 3.89 (6H, s, $OCH_3\times2$), 6.74—7.02 (3H, m, ArH). High-resolution MS Calcd for $C_{23}H_{27}NO_3$ (M^+) m/z: 365.1988. Found: 365.1968.

b) *Via* Schiff Base: A mixture of **20a** (1.6 g), $C_6H_5CH_2NH_2$ (1.1 g), and dried K_2CO_3 (584 mg) in anhydrous MeOH (60 ml) was stirred for 1 h at room temperature. $NaBH_4$ (406 mg) was added to the water-cooled, stirred reaction mixture and stirring was continued for 2 h at room temperature. The reaction mixture was acidified with 10% HCl under ice-cooling and the whole was refluxed for 0.5 h. After evaporation to half the initial volume *in vacuo*, the remainder was basified with K_2CO_3 (powder) under ice-cooling. The product was taken up in $CHCl_3$ and purification as above gave **23a** (oil, 1.16 g, 75%), spectral data of which were identical with those of the sample obtained in a).

(3a*S,6*R**,7a*S**)- and (3a*S**,6*S**,7a*S**)-1-Benzyl-3a,4,5,6,7,7a-hexahydro-6-hydroxy-3a-(3',4'-dimethoxyphenyl)indolines (24a and 25a) and Their Acetates (26a and 27a)**——$LiAlH_4$ (53 mg) was added to a water-cooled, stirred solution of **23a** (500 mg) in dry ether (5 ml) and the whole was stirred for 0.5 h at room temperature. Excess $LiAlH_4$ was decomposed with H_2O (0.5 ml) under ice-cooling and then a precipitate was filtered off through a celite bed, which was well rinsed with $CHCl_3$. Usual work-up of the combined filtrate and the $CHCl_3$ layers and subsequent column chromatography gave **25a** as a colorless oil (213 mg, 42.6%) (elution with $CHCl_3$–acetone (10 : 1)) and **24a** as a colorless oil (128 mg, 25.6%) (elution with $CHCl_3$–acetone (5 : 1)). **25a**: IR (CCl_4): 3310 (OH) cm^{-1}. ^1H-NMR

(100 MHz) δ: 3.20, 4.45 (2H, ABq, J=12.5 Hz, $NC\underline{H}_2C_6H_5$), 3.87, 3.90 (each 3H, s, $OCH_3\times2$), 4.02 (1H, m, $W_{1/2}$=9 Hz, 6-H), 6.70—7.00 (3H, m, ArH), 7.33 (5H, s, C_6H_5). **25a**·HCl: mp 228—230 °C (iso-PrOH) (lit.[3a] 215—230 °C). Acetate (**27a**) of **25a**: mp 104—105 °C (MeOH). IR (KBr): 1720 (OC=O) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 2.00 (3H, s, $OCOCH_3$), 3.26, 4.02 (2H, ABq, J=13 Hz, $NC\underline{H}_2$-C_6H_5), 3.87 (6H, s, $OCH_3\times2$), 4.87 (1H, m, $W_{1/2}$=13 Hz, 6-H), 6.80—6.96 (3H, m, ArH), 7.17—7.45 (5H, m, C_6H_5). *Anal.* Calcd for $C_{25}H_{31}NO_4$: C, 73.32; H, 7.63; N, 3.42. Found: C, 73.60; H, 7.68; N, 3.30. **24a**: IR (CCl_4): 3600, 3400 (OH) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.22, 4.22 (2H, ABq, J=13 Hz, $NC\underline{H}_2C_6H_5$), 3.88, 3.90 (each 3H, s, $OCH_3\times2$), 4.18 (1H, m, $W_{1/2}$=25 Hz, 6-H), 6.76—7.00 (3H, m, ArH), 7.20—7.40 (5H, m, C_6H_5). Acetate (**26a**) of **24a** (Oil): IR: 1720 (OC=O) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 1.98 (3H, s, $OCOCH_3$), 3.84, 3.87 (each 3H, s, $OCH_3\times2$), 5.25 (1H, m, $W_{1/2}$=18 Hz, 6-H), 6.80—7.00 (3H, m, ArH), 7.20—7.50 (5H, m, C_6H_5). Spectral and physical data of **24a** and **25a** were coincident with those in the literature.[3a]

(±)-Dihydromaritidine (4a) and (±)-Epidihydromaritididine (5a)——(±)-**4a**: A mixture of **26a** (110 mg), 2% aq. $PdCl_2$ (0.4 ml) and active carbon (64 mg) in MeOH (6 ml) was shaken for 2 h in an atmosphere of H_2 at room temperature. Work-up as usual gave a yellow oil (83 mg, 96%). IR: 1725 (OC=O) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 1.98 (3H, s, $OCOCH_3$), 3.85, 3.88 (6H, each s, $OCH_3\times2$), 5.10 (1H, m, $W_{1/2}$=18 Hz, 6-H), 6.75—7.00 (3H, m, ArH). A mixture of the crude oil, and 37% HCHO (0.83 ml) in anhydrous MeOH (0.72 ml) was stirred for 10 min at room temperature. The reaction mixture was diluted with 10% HCl (30 ml) and the whole was stirred for 19 h at room temperature. Work-up as usual and purification by preparative TLC (developing solvent; $CHCl_3$–MeOH (5 : 1)) gave (±)-**4a** (48.5 mg, 63%), mp 229.5—232 °C (MeOH) (lit.[3a] 230—233 °C). IR (KBr): 3200 (OH) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.78, 4.38 (2H, ABq, J=17 Hz, 6-H_2), 3.83, 3.87 (each 3H, s, $OCH_3\times2$), 6.48, 6.75 (each 1H, s, ArH). MS m/z: 289 (M^+). The spectral and physical data were identical with those of the (±)-alkaloid in the literature.[3a]

(±)-**5a**: Treatment of **27a** (470 mg) as noted above gave (±)-**5a** (245 mg, 73%), mp 192—194 °C (MeOH) (lit.[3a] 190—194 °C) [IR (KBr): 3130 (OH) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.63, 4.40 (2H, ABq, J=16.5 Hz, 6-H_2), 3.83, 3.87 (each 3H, s, $OCH_3\times2$), 6.51, 6.72 (each 1H, s, ArH). MS m/z: 289 (M^+)] *via* a debenzylated amine (oil) [IR: 1730 (OC=O) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 2.08 (3H, s, $OCOCH_3$), 3.85, 3.87 (each 3H, s, $OCH_3\times2$), 5.00 (1H, m, $W_{1/2}$=10 Hz, 6-H), 6.77—7.00 (3H, m, ArH)]. The spectral and physical data were identical with those of the (±)-alkaloid in the literature.[3a]

2-Allyl-5,5-ethylenedioxy-2-(3′,4′-methylenedioxyphenyl)cyclohexanone (16b)——Starting from the crude homopiperonal[9] (3.05 g) in the same manner as noted for **9a**, **9b** was obtained (1.99 g, 35% from the aldehyde). **9b**: mp 81—82 °C (MeOH–H_2O). ^{1}H-NMR (100 MHz) δ: 4.00 (4H, s, OCH_2CH_2O), 5.88 (1H, m, 1-H), 5.93 (2H, s, OCH_2O), 6.66—6.92 (3H, m, ArH). *Anal.* Calcd for $C_{15}H_{16}O_4$: C, 69.21; H, 6.20. Found: C, 69.50; H, 6.16. Hydroboration of **9b** (1.3 g) in THF (30 ml) with 1 M BH_3–THF (10 ml) (3 h; room temperature) followed by oxidation with 3 N NaOH (2.5 ml) and 30% H_2O_2 (2.5 ml) (3h; room temperature) gave 5,5-ethylenedioxy-2-(3′,4′-methylenedioxyphenyl)cyclohexanol (0.95 g, 68%), mp 118—119 °C (MeOH–H_2O). IR: 3570 (OH) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 3.45—3.80 (1H, m, 1-H), 3.85 (4H, s, OCH_2CH_2O), 5.80 (2H, s, OCH_2O), 6.50—6.75 (3H, m, ArH). *Anal.* Calcd for $C_{15}H_{18}O_5$: C, 64.73; H, 6.52. Found: C, 64.88; H, 6.48. Oxidation of the above cyclohexanol (0.6 g) with Collins reagent [CrO_3 (1.3 g) and dry pyridine (2.1 g) in CH_2Cl_2 (40 ml)] as noted for **6a** gave 5,5-ethylenedioxy-2-(3′,4′-methylenedioxyphenyl)cyclohexanone (**6b**) (0.53 g, 89%), mp 128.5—130 °C (MeOH). IR (KBr): 1710 (C=O) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 2.73 (2H, s, 6-H_2), 4.00 (4H, s, OCH_2CH_2O), 5.94 (2H, s, OCH_2O), 6.50—6.84 (3H, m, ArH). *Anal.* Calcd for $C_{15}H_{16}O_5$: C, 65.21; H, 5.84. Found: C, 65.37; H, 5.84.

Allylation of **6b** (500 mg) with allyl bromide (265 mg), 50% aq. NaOH (3 ml), and 18-crown-6 (24 mg) in benzene (8 ml) (0.5 h; room temperature) as noted for **16a** and subsequent column chromatography gave **16b** (351 mg, 61%) (elution with benzene–EtOAc (30 : 1—20 : 1) and **17b** (18 mg, 3%) (elution with benzene–EtOAc (20 : 1)). **16b**: mp 109—109.5 °C (ether–hexane). IR (KBr): 1710 (C=O) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.88—4.00 (4H, m, OCH_2CH_2O) 4.79—5.04 (2H, m, $CH=C\underline{H}_2$), 5.24—5.68 (1H, m, $C\underline{H}=CH_2$), 5.97 (2H, s, OCH_2O), 6.52—6.87 (3H, m, ArH). MS m/z: 275 (M^+). *Anal.* Calcd for $C_{18}H_{20}O_5$: C, 68.34; H, 6.37. Found: C, 68.26; H, 6.32. **17b** (Oil): IR: 1650 (α,β-unsaturated C=O) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.68 (2H, t, J=6 Hz, $OCH_2C\underline{H}_2OCH_2CH$=), 3.84—4.08 (4H, m, $OC\underline{H}_2CH_2OC\underline{H}_2CH$=), 4.85—6.10 (7H, m, $CH=CH_2\times2$ and 6-H), 6.70—6.82 (3H, m, ArH). MS m/z: 356 (M^+).

(3a*S,7a*S**)-1-Benzyl-3a,4,5,6,7,7a-hexahydro-3a-(3′,4′-methylenedioxyphenyl)-6-oxoindoline (23b)**——A solution of **16b** (323 mg) in MeOH (22 ml) was reduced with $NaBH_4$ (95 mg) for 2 h at room temperature. Usual work-up gave 2-allyl-5,5-ethylenedioxy-2-(3′,4′-methylenedioxyphenyl)cyclohexanol (255 mg, 79%), mp 124—124.5 °C (MeOH). IR (KBr): 3500 (OH) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.98 (4H, s, OCH_2CH_2O), 4.08 (1H, m, $W_{1/2}$=12 Hz, 1-H), 4.80—5.54 (3H, m, $CH=CH_2$), 5.93 (2H, s, OCH_2O), 6.80 (2H, br s, ArH). *Anal.* Calcd for $C_{18}H_{22}O_5$: C, 67.91; H, 6.97. Found: C, 67.60; H, 6.91. Oxidation, as noted for **20a**, of (1*R**,2*S**)-2-allyl-5,5-ethylenedioxy-2-(3′,4′-methylenedioxyphenyl)cyclohexyl acetate (oil) (256 mg) [IR: 1730 (OC=O) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 2.00 (3H, s, $OCOCH_3$), 2.37 (2H, d, J=7 Hz, -$C\underline{H}_2CH$=), 3.91 (4H, s, OCH_2H_2O), 5.60 (4H, m, $CH=CH_2$ and 1-H), 5.92 (2H, s, OCH_2O), 6.60—7.10 (3H, m, ArH)], derived from the above cyclohexanol, with OsO_4 (20 mg)–$NaIO_4$ (440 mg) in H_2O (8 ml) and dioxane (9 ml) gave **20b** (oil) (225 mg, 87.4%). IR: 1735 (OC=O), 1720 (CHO) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 2.00 (3H, s, $OCOCH_3$), 2.53 (2H, d, J=3 Hz, $C\underline{H}_2CHO$), 3.90 (4H, s, OCH_2CH_2O), 5.13 (1H, t, J=7 Hz, 1-H), 5.92 (2H, s, OCH_2O), 6.51—7.11 (3H, m, ArH), 9.25 (1H, t, J=3 Hz, $CH_2C\underline{H}O$).

Treatment of the crude **20b** (225 mg) with $C_6H_5CH_2NH_2$ (166 mg) and dried K_2CO_3 (86 mg) in anhydrous MeOH (9 ml) (1 h; room temperature) followed by reduction with $NaBH_4$ (59 mg) (1.5 h; room temperature) gave an oil, which was refluxed with 10% HCl (6.1 ml) for 0.5 h. Work-up of the reaction mixture as noted for (±)-**1** gave an oil (206 mg), which was subjected to column chromatography. Elution with benzene–EtOAc (10 : 1) afforded **23b** (114 mg, 52.8%), mp 96—97.5 °C (benzene-hexane) (lit.[8a] 98.5—99 °C). IR (KBr): 1720 (C=O) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.12, 4.08 (2H, ABq, J = 13 Hz, $NC\underline{H}_2C_6H_5$), 5.96 (2H, s, OCH_2O), 6.68—6.96 (3H, m, ArH), 7.17 (5H, m, C_6H_5). The spectral and physical data were identical with those in the literature.[8a]

(3aS*,6R*,7aS*)- and (3aS*,6S*,7aS*)-1-Benzyl-3a,4,5,6,7,7a-hexahydro-6-hydroxy-3a-(3′,4′-methylenedioxyphenyl)indoline (24b and 25b) and Their Acetates (26b and 27b)——A solution of **23b** (440 mg) in dry ether (26 ml) was treated with $LiAlH_4$ (44 mg) for 0.5 h at room temperature. Work-up as noted for **24a** gave an oil (425 mg), separation of which by preparative TLC (developing solvent; $CHCl_3$–ether (1 : 1)) gave **24b** (more polar part) (161 mg, 40%) and **25b** (less polar part) (163 mg, 40%). **24b**: mp 132.5—133 °C (ether) (lit.[8a] 135.5—136 °C). IR: 3600 (OH) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.21, 4.20 (2H, ABq, J = 13 Hz, $NC\underline{H}_2C_6H_5$), 4.17 (1H, m, $W_{1/2}$ = 18 Hz, 6-H), 5.94 (2H, s, OCH_2O), 6.64—6.94 (3H, m, ArH), 7.32 (5H, brs, C_6H_5). Acetate (**26b**) of **24b**: mp 110—111 °C (MeOH). IR: 1730 (OC=O) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 1.97 (3H, s, $OCOCH_3$), 3.10, 4.20 (2H, ABq, J = 13 Hz, $NC\underline{H}_2C_6H_5$), 5.04—5.44 (1H, m, 6-H), 5.94 (2H, s, OCH_2O), 6.60—6.95 (3H, m, ArH), 7.15—7.45 (5H, m, C_6H_5). *Anal.* Calcd for $C_{21}H_{27}NO_4$: C, 73.26; H, 6.92; N, 3.56. Found: C, 73.51; H, 7.00; N, 3.34. **25b** (Oil): IR: 3275 (OH) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.19, 4.44 (2H, ABq, J = 13 Hz, $NC\underline{H}_2C_6H_5$), 4.00 (1H, m, $W_{1/2}$ = 8 Hz, 6-H), 5.94 (2H, s, OCH_2O), 6.68—6.94 (3H, m, ArH), 7.32 (5H, brs, C_6H_5). Picrate, mp 224—226 °C (dec.) (lit.[8a] 229—231 °C). Acetate (**27b**) (oil) of **25b**: IR: 1730 (OC=O) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 2.00 (3H, s, $OCOCH_3$), 3.25, 4.05 (2H, ABq, J = 13 Hz, $NC\underline{H}_2C_6H_5$), 4.87 (1H, m, $W_{1/2}$ = 12 Hz, 6-H), 5.94 (2H, s, OCH_2O), 6.60—6.95 (3H, m, ArH), 7.30 (5H, brs, C_6H_5). Spectral data of **24b** and **25b** were identical with those in the literature.[8a]

(±)-Elwesine (4b) and (±)-Epielwesine (5b)——The procedure was similar to that noted for **4a**.

(±)-4b: **26b** (74 mg), 2% aq. $PdCl_2$ (0.26 ml), active carbon (44 mg), and MeOH (4 ml) were used. Usual work-up gave (3aS*,6R*,7aS*)-6-acetoxy-3a,4,5,6,7,7a-hexahydro-3a-(3′,4′-methylenedioxyphenyl)indoline as an oil (56 mg). IR: 1725 (OC=O) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 2.00 (3H, s, $OCOCH_3$), 5.05 (1H, m, $W_{1/2}$ = 18 Hz, 6-H), 5.90 (2H, s, OCH_2O), 6.75—6.95 (3H, m, ArH). The Pictet–Spengler reaction of the oil (56 mg) gave (±)-**4b** (40 mg, 77%), mp 238—239 °C (MeOH) (lit.[8a] 216—220 °C). IR (KBr): 3350 (OH) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.74, 4.36 (2H, ABq, J = 17 Hz, 6-H_2), 5.90 (2H, s, O–CH_2O), 6.64, 6.75 (each 1H, s, ArH). *Anal.* Calcd for $C_{16}H_{19}NO_3$: C, 70.31; H, 7.01; N, 5.13. Found: C, 70.45; H, 7.09; N, 4.90. The spectral data were identical with those of an authentic sample.[22]

(±)-5b: **27b** (156 mg), 2% aq. $PdCl_2$ (0.56 ml), active carbon (95 mg), and MeOH (13 ml) were used. Usual work-up gave (3aS*,6S*,7aS*)-6-acetoxy-3a,4,5,6,7,7a-hexahydro-3a-(3′,4′-methylenedioxyphenyl)indoline as an oil (102 mg). IR: 1735 (OC=O) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 2.12 (3H, s, $OCOCH_3$), 5.02 (1H, m, $W_{1/2}$ = 10 Hz, 6-H), 5.96 (2H, s, OCH_2O), 6.70—6.95 (3H, m, ArH). The Pictet–Spengler reaction of the oil (102 mg) have (±)-**5b** (80 mg, 74%), mp 181.5—182 °C (benzene–hexane) (lit.[8a] 184—188 °C). IR (KBr): 3150 (OH) cm^{-1}. ^{1}H-NMR (100 MHz) δ: 3.74, 4.34 (2H, ABq, J = 16.5 Hz, 6-H_2), 5.90 (2H, s, OCH_2O), 6.46, 6.70 (each 1H, s, ArH). MS m/z: 273 (M^+). The spectral data were identical with those of an authentic sample.[22]

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16) **16a** was formed in 34.5% or 60% yield by a similar reaction in CH_2Cl_2 in place of benzene or in 40% aq. KOH in place of 50% aq. NaOH. On the other hand, the reaction in the presence of NaH or *tert*-BuOK in THF afforded **16a** in 23% or 18% yield.
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Cardenolide Monoglycosides from the Leaves of *Cerbera odollam* and *Cerbera manghas* (*Cerbera*. III)[1]

TATSUO YAMAUCHI,*,[a] FUMIKO ABE,[a] and ALFRED S.C. WAN[b]

Faculty of Pharmaceutical Sciences, Fukuoka University,[a] 8–19–1 Nanakuma, Jonan-ku, Fukuoka 814–01, Japan and Department of Pharmacy, Faculty of Science, National University of Singapore,[b] 10 Kent Ridge Crescent, Singapore 0511

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Six minor monosides (L-thevetosides of oleagenin and of 8β-hydroxy-17β- and 17α-digitoxigenin, L-acofriosides of 17β- and 17α-digitoxigenin and of tanghinigenin) were isolated from the air-dried leaves of *Cerbera odollam* GAERTN. and *C. manghas* L., along with the major monosides, 17β- and 17α-neriifolin and 17β- and 17α-deacetyltanghinin.

Keywords——*Cerbera odollam*; *Cerbera manghas*; Apocynaceae; cardenolide; oleagenin α-L-thevetoside; neriifolin; deacetyltanghinin; solanoside; 8β-hydroxydigitoxigenin α-L-thevetoside

Genus *Cerbera* belongs to Apocynaceae and is distributed widely in the coastal areas of South-East Asia and the Indian Ocean. The cardenolides from the seeds of *Cerbera* have been investigated by many groups.[2] The Ryukyu Islands are the northern border of the habitat of *C. manghas* L., and previously, we reported on the cardenolide glycosides from the seeds, bark and leaves of *C. manghas* collected on Okinawa Island.[3] This paper deals with the isolation and structure determinations of six minor cardenolide monosides as well as the isolation of four known monosides from the air-dried leaves of *C. odollam* GAERTN. collected in Singapore and *C. manghas* L. collected in Taiwan.

The air-dried leaves of *C. odollam* and *C. manghas* were percolated with MeOH. The benzene and $CHCl_3$ extractives of the MeOH percolate were fractionated by ordinary and reversed-phase column chromatographies, and by high-performance liquid chromatography (HPLC), and six minor monosides were isolated along with the four major monosides, neriiforlin (I), 17α-neriifolin (II), deacetyltanghinin (III), and 17α-deacetyltanghinin (IV). The minor monosides were tentatively designated as substances V—X.

I: 17β; R_1=OH, R_2=H
II: 17α; R_1=OH, R_2=H
VIII: 17β; R_1=H, R_2=OH
IX: 17α; R_1=H, R_2=OH

III: 17β; R_1=OH, R_2=H
IV: 17α; R_1=OH, R_2=H
X: 17β; R_1=H, R_2=OH

V

VI: 17β
VII: 17α

Chart 1

TABLE I. ^{1}H Chemical Shifts of the Monosides, δ (ppm) from TMS in Pyridine-d_5 (J/Hz Parentheses)

	I	II	V	VI	VII	VIII	IX	X
H-18, 19	0.83	0.99	0.71	1.23	1.34	0.89	0.93	1.05
	1.02	1.04	0.90	1.32	1.41	1.04	1.20	1.06
H-21	5.03	4.99	4.73	5.00	4.81	5.04	4.82	5.00
	5.31	5.20	4.83	5.27	4.95	5.33	4.95	5.20
	(dd, 18, 1)	(dd, 18, 1)	(dd, 18, 1)	(dd, 18, 2)	(dd, 18, 1)	(dd, 18, 2)	(dd, 18, 2)	(dd, 18, 1)
H-22	6.13	6.13	5.88	6.10	6.11	6.14	6.13	6.14
	(br s)	(br s)	(br s)	(br s)	(br s)	(br s)	(br s)	(br s)
H-3α	4.18	4.11	4.14	4.20	4.21	4.19	4.21	4.13
	(br s)	(br s)	(br s)	(br s)	(br s)	(br s)	(br s)	(br s)
H-17	2.80	2.81	2.98	2.80	3.34	2.81	3.43	2.83
	(dd, 9, 6)	(dd, 9, 5)	(br d, 7)	(dd, 10, 5)	(t, 9)	(dd, 9, 6)	(t, 9)	(dd, 9, 5)
H-1′	5.24	5.22	5.23	5.27	5.28	5.42	5.42	5.40
	(d, 4)	(d, 4)	(d, 3)	(d, 4)	(d, 3)	(d, 2)	(d, 2)	(d, 1)
H-2′	4.08	4.08	4.06	4.08	4.09	4.57	4.57	4.57
	(dd, 4, 9)	(dd, 4, 9)	(dd, 3, 9)	(dd, 4, 9)	(dd, 3, 9)	(br s)	(dd, 2, 3)	(br s)
H-3′	4.00	4.02	3.99	4.02	4.06	3.96	3.95	3.94
	(t, 9)	(t, 9)	(t, 9)	(t, 9)	(t, 9)	(dd, 3, 8)	(dd, 3, 9)	(dd, 3, 9)
H-4′	3.66	3.66	3.66	3.67	3.67	4.28	4.30	4.25
	(t, 9)	(t, 9)	(t, 9)	(t, 9)	(t, 9)	(dd, 8, 9)	(t, 9)	(t, 9)
H-5′	4.31	4.29	4.29	4.34	4.35	4.28	4.29	4.25
	(m)	(m)	(m)	(m)	(m)	(m)	(m)	(m)
H-6′	1.61	1.62	1.61	1.63	1.63	1.66	1.66	1.66
	(d, 6)	(d, 6)	(d, 6)	(d, 6)	(d, 6)	(d, 6)	(d, 6)	(d, 6)
3′-OMe	3.84	3.83	3.83	3.84	3.84	3.56	3.55	3.56
Others		3.39						
		(d, 6)						
		(H-7α)						

Substance V, mp 213—217 °C, showed the molecular formula $C_{30}H_{44}O_8$, on the basis of the fast atom bombardment (FAB)-mass spectrum (MS) (m/z: 555.297, $M^+ + Na$). In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of V, the characteristic methylene protons at C-21 and the olefinic proton at C-22 were observed. In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum, however, no C-14 carbinol carbon peak was observed at δ 83—85. Since the carbon signals ascribable to L-thevetose were seen, the aglycone seemed to be $C_{23}H_{32}O_4$, which is consistent with a cardenolide having one hydroxyl group (δ 73.3) and one carbonyl (δ 221.2) group. The hydroxyl group was assignable to C-3. On thin layer chromatography (TLC), V showed blue staining, characteristic of oleagenin, on treatment of the plate by spraying dil. H_2SO_4 and heating. In fact, the carbon signals due to the aglycone moiety coincided with those of oleaside B (oleagenin β-D-digitaloside), which has been isolated from the leaves of some cultivars of *Nerium oleander*.[4] Substance V was therefore considered to be the L-thevetoside of oleagenin (=3β-hydroxy-15(14→8)-*abeo*-5β-(8*R*)-14-oxo-card-20(22)-enolide). On hydrolysis of V with HCl in acetone, the identities of the aglycone (mp 285—291 °C) and thevetose were confirmed by direct comparisons with authentic samples. Substance V was named cerleaside A.

The glycosidic linkage of L-thevetose to digitoxigenin was formerly assigned to be β, based on the assignment by Voigtländer *et al.*,[5] since the coupling constant of the anomeric proton in I was observed to be 7 Hz in $CDCl_3$, whereas that of cerberin (=2′-*O*-acetylneriifolin) was 4 Hz.[3] In this study, however, all anomeric proton peaks of L-thevetosides showed couplings with 3—4 Hz in pyridine-d_5, and $J_{C1'-H1'}$ of I was observed to

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

	I	II	V	VI	VII	VIII	IX	X
C-1	30.3	32.0[a]	30.3[a]	30.5	30.6	30.1	30.2	32.1[a]
C-2	26.9[a]	27.3	26.8[b]	27.4[a]	27.2	26.9[a]	27.2[a]	27.3
C-3	73.7	73.0	73.3[c]	73.7	73.7	72.2[b]	72.4	71.4
C-4	31.0	32.9[a]	29.1[a]	32.6	32.6	31.0	31.0	32.5[a]
C-5	36.8	34.2	37.0	36.5	36.6	37.1	37.2	34.5
C-6	27.1[a]	28.2	27.0[b]	28.4	28.0	27.2[a]	26.9[a]	28.3
C-7	21.5[b]	50.9	24.4	23.3	23.2	22.0[c]	21.6	51.0
C-8	41.9	64.4	47.4[d]	76.7	76.9	41.9	41.7	64.4
C-9	35.8	31.9	45.9	37.4	37.5	35.8	36.0	31.9
C-10	35.5	33.8	37.5	35.7	35.6	35.5	35.5	33.9
C-11	21.9[b]	20.9	21.2	18.3	17.4	21.6[c]	20.7	20.9
C-12	39.8	40.8	43.9[e]	40.7	31.9	39.9	31.7	40.8
C-13	50.1	52.6	48.8[d]	50.8	50.5	50.1	49.4	52.3
C-14	84.6	81.7	221.2	85.9	86.2	84.6	85.2	81.7
C-15	33.2	35.2	42.5[e]	35.2	34.0	33.2	31.0	35.3
C-16	27.1[a]	28.7	32.2	27.3[a]	25.3	26.9[a]	24.9	28.7
C-17	51.5	50.9	52.8	52.2	49.4	51.5	48.9	51.0
C-18	16.2	17.3	23.3	18.6[b]	20.2	16.2	18.6	17.3
C-19	23.8	24.4	26.2	26.2	26.1	24.0	24.0	24.5
C-20	175.9	175.0	171.7	175.7	172.1	175.9	172.8	175.0
C-21	73.6	73.6	73.3	73.6	74.2	73.7	74.1	73.7
C-22	117.6	117.7	116.2	117.7	117.1	117.6	116.6	117.7
C-23	174.4	174.3	173.7	174.4	172.1	174.4	174.1	174.3
C-1′	98.8	98.9	98.9	98.9	98.9	99.7	99.7	99.6
C-2′	73.4	73.3	73.4[c]	73.4	73.4	70.0	70.0	69.9
C-3′	85.4	85.3	85.3	85.4	85.4	82.8	82.8	82.8
C-4′	76.6	76.5	76.5	76.6	76.6	72.4[b]	72.3	72.4
C-5′	68.9	69.0	68.9	69.0	69.0	68.7	68.8	68.7
C-6′	18.5	18.5	18.5	18.5[b]	18.5	18.6	18.6	18.6
-OMe	60.5	60.5	60.5	60.5	60.5	57.0	57.0	57.1

a—e) Signal assignments marked *a—e)* in each column may be reversed.

be 164.8 Hz (α-L-rhamnoside, 164—168 Hz; β-L-rhamnoside, 152—158 Hz).[6] Therefore, L-thevetosides in this plant are considered to retain the α-linkage.

Substance VI also seemed to be an α-L-thevetoside of a cardenolide, based on the ^1H- and ^{13}C-NMR spectra. The $M^+ + Na$ peak was observed at *m/z*: 573.306, indicating the molecular formula to be $C_{30}H_{46}O_9$, so that the aglycone bears an additional hydroxyl group, besides the C-3 and C-14 carbinols. In the ^{13}C-NMR spectrum of VI, signals of two tertiary carbinol carbons were observed at δ 85.9 and 76.7, of which the former was assigned to C-14. The other was ascribable to C-8, since other tertiary carbons at C-5, C-9, and C-17 were assignable on the basis that their chemical shifts are close to those of I, and also, the C-7, C-9, and C-14 signals were shifted downfield by 1.3—1.8 ppm in comparison with those of I. On acetylation, VI afforded a diacetate (VI-1), which is consistent with the structure having two tertiary hydroxyl groups. When VI was treated with $SOCl_2$ in pyridine, the product (VI-2) showed the molecular peak at *m/z*: 619 ($C_{30}H_{44}O_{10}S + Na$) and large downfield shifts of the C-8 (+16.4 ppm) and C-14 (+19.5 ppm) signals in the ^{13}C-NMR spectrum, indicating cyclosulfite formation at C-8 and C-14 as previously found in a derivative of affinoside A,[7] and thus, the 8β,14β-glycol structure was established. Substance VI was therefore 8β-hydroxydigitoxigenin α-L-thevetoside, and was named cerdollaside.

The ^1H-NMR spectrum of VII showed the H-17β signal at δ 3.34, suggesting VII to be a

TABLE III. Yields of Monosides from the Air-Dried Leaves (mg/kg)[a]

	I	II	III	IV	V	VI	VII	VIII	IX	X
C. odollam	38	50	33	13	83	3[b]	7[b]	5[b]	7	+[c]
C. manghas	49	190	21	66	6	2[b]	+[c]	2	3	1

a) Obtained as crystals unless otherwise mentioned. *b*) Obtained as a homogeneous solid. *c*) Not isolated but detectable by HPLC.

17α-cardenolide. In the ^{13}C-NMR spectrum, signals due to C-12, C-15, C-16, and C-17 were shifted upfield and that of C-18 was shifted downfield, in comparison with those of VI, as observed in uzarigenin and 17α-uzarigenin.[8] The peaks due to the sugar moiety were in good agreement with those of α-L-thevetosides such as I—VI, and FAB-MS also afforded the same molecular formula, $C_{30}H_{46}O_9$ as that of VI. Substance VII was thus considered to be 17α-cerdollaside.

Substances VIII and IX also formed a pair as regards the stereochemistry at C-17, and VIII was 17β-cardenolide. The aglycone of VIII was assigned as digitoxigenin, based on the ^{1}H- and ^{13}C-NMR spectra. In the sugar moiety, all the proton signals and their coupling constants were assignable by COSY, and the component sugar was considered to be L-acofriose (=3-*O*-methyl-L-rhamnose). On hydrolysis of VIII, the component sugar showed, after methylation with MeOH–HCl, the same behavior on TLC and gas liquid chromatography (GLC) as authentic methyl 3-*O*-methyl-α-L-rhamnoside. Substance VIII was therefore identified as digitoxigenin α-L-acofrioside, which was formerly reported as solanoside by Kaufmann *et al.*[9] and IX was 17α-solanoside.

Substance X was obtained from *C. manghas* in a small amount. The structure was considered to be the α-L-acofrioside of tanghinigenin, based on the ^{1}H- and ^{13}C-NMR spectra and molecular peak at *m/z*: 571.288 ($C_{30}H_{44}O_9$+Na).

In a comparison of the monosides obtained from the air-dried leaves of *C. odollam* and *C. manghas*, the yields of the major glycosides (I—IV) showed similar patterns and II was obtained in high yield (Table III). It should be noted that V showed the highest yield in *C. odollam*.

In this study, five new minor cardenolide glycosides (V, VI, VII, IX and X) were isolated along with four major glycosides, I—IV, and one minor glycoside, VIII. Since adynerigenin was smoothly converted into oleagenin on reaction with BF_3, we previously suggested that an 8,14β-epoxy-type cardenolide such as adynerigenin is possibly the biogenetic precursor of the oleagenin framework in *Nerium oleander*. In *Cerbera*, however, no 8,14-epoxycardenolide has been found. The immediate precursor of oleagenin glycosides in this plant remains to be investigated. Although 8β,14β-dihydroxysteroids are known among pregnanes and bufadienolides, VI and VII are the first cardenolides having a 8β,14β-glycol moiety. The polar cardenolide glycosides from the BuOH extractives in the air-dried leaves as well as those from the fresh leaves are under investigation.

Experimental

Melting points were measured on a Kofler block and are uncorrected. The samples for ^{1}H- or ^{13}C-NMR spectroscopy were dissolved in pyridine-d_5 and the spectra were measured with a JEOL GX-400. Chemical shifts are given in δ values, referred to internal tetramethylsilane (TMS), and the following abbreviations are used: s=singlet, brs=broad singlet, d=doublet, dd=doublet of doublets, t=triplet, m=multiplet. FAB-MS were recorded on a JEOL D-300-FD spectrometer. Optical rotations were measured with a JASCO DIP-360. HPLC was run on a Waters model ALC 200 equipped with radial pack C_{18} column. For GLC, a Shimadzu GC 8A apparatus was used. For silica gel chromatography and TLC, the following solvent systems were employed: solv. 1, benzene–acetone (5:1—2:1);

solv. 2, $CHCl_3$–MeOH–H_2O (7:1:1—7:3:1, bottom layer); solv. 3, EtOAc–MeOH–H_2O (6:1:5—4:1:3). As spray reagents, a) a 1:1 mixture of 2% 3,5-dinitrobenzoic acid in MeOH and 2 N NaOH (Kedde's reagent), and b) 5% H_2SO_4 were used. When b) was applied, the plate was heated until the spots appeared.

Isolation of Cardenolide Monosides from the Air-Dried Leaves——From *C. odollam* L.: The leaves, collected in July 21, 1986 on the West Coast area of Singapore (*ca.* 2.4 kg), were air-dried in the shade for 1 month. The powdered leaves (400 g) were percolated with MeOH. The MeOH percolate was concentrated *in vacuo* to *ca.* 1 l, and diluted with 1 l of H_2O. After filtration, the filtrate was partitioned with benzene and then with $CHCl_3$. The benzene and $CHCl_3$ layers were combined and chromatographed on a silica gel column with solv. 2 (7:1:1—7:2:2—7:2:1). The fractions containing the monosides were further chromatographed on an ODS-column (Fuji-gel, RQ-1, 30% MeCN), and then on silica gel columns with solv. 1 (5:1—3:1) and with solv. 3 (6:1:5—4:1:5). The minor monosides were further subjected to HPLC with 40% MeCN to obtain pure monosides (homogeneous on TLC and HPLC), which were crystallized from EtOAc–hexane or MeOH. The yields are presented in Table III.

From *C. manghas*: The leaves were harvested at the beginning of March, 1986 at Taipei, Taiwan, and air-dried in the shade for 1 month. The powdered leaves (2.7 kg) were percolated with MeOH, and worked up as in the case of *C. odollam*. The yields of the monosides are presented in Table III.

Major Monosides——Neriifolin (I) (mp 230—234 °C, $J_{C_{1'}-H_{1'}}=168.4$ Hz), 17α-neriifolin (II) (mp 238 °C), deacetyltanghinin (III) (mp 232—233 °C), and 17α-deacetyltanghinin (IV) (mp 222—225 °C) were identified by comparison with authentic samples obtained from *C. manghas* in previous work.[3]

Cerleaside A (V)——Colorless needles from MeOH, mp 213—217 °C, $[\alpha]_D^{27}$ −22.3° (c=0.13, MeOH). FAB-MS *m/z*: 555.297 (M^++Na, Calcd for $C_{30}H_{44}O_8$+Na: 555.293). The diacetate of V was obtained as prisms by usual acetylation with Ac_2O and pyridine, followed by crystallization from MeOH, mp 244—245 °C. FAB-MS *m/z*: 639 ($C_{34}H_{48}O_{10}$+Na). Substance V (3 mg) was dissolved in 1 ml of acetone containing 0.01 ml of conc. HCl, allowed to stand for 10 d, and then diluted with MeOH. The solution was deacidified with IRA-410 and the solvent was evaporated off *in vacuo*. The residue was again dissolved in H_2O and extracted with $CHCl_3$. The $CHCl_3$ layer, was evaporated to yield a product, which was crystallized from MeOH to give prisms, mp 285—291 °C. On admixture with the authentic oleagenin, no melting point depression was observed. The H_2O layer was concentrated *in vacuo*, and the residue was examined on TLC in parallel with authentic L-thevetose. The two samples showed the same *Rf* values (solv. 2, 7:3:1).

Cerdollaside (VI)——A solid, $[\alpha]_D^{29}$ −17.7° (c=0.61, MeOH). FAB-MS *m/z*: 573.306 (M^++Na, Calcd for $C_{30}H_{46}O_9$+Na: 573.304). On acetylation with Ac_2O and pyridine at room temperature, VI-diacetate was obtained as a solid (VI-1). FAB-MS *m/z*: 657 ($C_{34}H_{50}O_{11}$+Na). ^{1}H-NMR δ: 1.25, 1.43 (3H, each, s, H-18, H-19), 2.81 (1H, dd, J=9, 4 Hz, H-17), 4.06 (1H, br s, H-3α), 5.03, 5.30 (1H each, dd, J=18, 2 Hz, H-21α, β), 6.11 (1H, br s, H-22), 5.42 (1H, d, J=4 Hz, H-1′), 5.10 (1H, dd, J=4, 9 Hz, H-2′), 4.09 (1H, t, J=9 Hz, H-3′), 5.15 (1H, t, J=9 Hz, H-4′), 4.19 (1H, m, H-5′), 1.29 (3H, d, J=6 Hz, H-6′), 3.53 (3H, s, 3′-OMe), 2.151, 2.153 (3H each, s, -OAc), 5.58 (1H, s, -OH). A solution of VI (8 mg) in pyridine (0.5 ml) was treated with $SOCl_2$ (0.05 ml), and the mixture was stirred at 0 °C for 2 h. The mixture was then diluted with ice-H_2O, and extracted with BuOH. The BuOH extractives were purified on a silica gel column (solv. 2, 7:1:3) to afford the product as a homogeneous solid, which was crystallized from MeOH to give prisms (VI-2), mp 195—205 °C. FAB-MS *m/z*: 619 ($C_{30}H_{44}O_{10}S$+Na), negative FAB-MS *m/z*: 595 ($C_{30}H_{44}O_{10}S$-1). ^{13}C-NMR δ: 93.1 (C-8), 40.3 (C-9), 36.6 (C-10), 16.8 (C-11), 41.0 (C-12), 49.3 (C-13), 105.4 (C-14), 33.4 (C-15), 29.9 (C-16), 51.5 (C-17), 21.2 (C-18), 25.5 (C-19), 171.7 (C-20, 23). Other peaks showed the same chemical shifts as those of VI.

17α-Cerdollaside (VII)——Colorless prisms from EtOAc–hexane, mp 198—200 °C, $[\alpha]_D^{27}$ −16.8° (c=0.22, MeOH). FAB-MS *m/z*: 573.306 (M^++Na, Calcd for $C_{30}H_{46}O_9$+Na: 573.304).

Solanoside (VIII)[8]——Colorless prisms from EtOAc–hexane, mp 198—200 °C, $[\alpha]_D^{27}$ −51.6° (c=0.16, MeOH). FAB-MS *m/z*: 557.308 (M^++Na, Calcd for $C_{30}H_{46}O_8$+Na: 557.309). On hydrolysis of VIII (3 mg) with acetone–HCl (1 ml–0.05 ml) at room temperature for 7 d, Δ^{14}-anhydrodigitoxigenin was observed on TLC (solv. 1, 2:1, solv. 3, 9:1:0.5). The H_2O layer was deacidified with IRA-410 and the solution was concentrated *in vacuo*. The residue was refluxed with 0.5 ml of 0.5 N HCl in MeOH for 1 h. The mixture was deacidified and the MeOH was evaporated off *in vacuo*. The residue was examined by TLC (solv. 2, 7:3:1) and GLC (column: PEG-20M, N_2: 0.8 kg/cm^2, 170 °C) in parallel with methyl 2-*O*-methyl-, 3-*O*-methyl-, and 4-*O*-methyl-α-L-rhamnoside. The behavior of the sample from VIII was in good agreement with that of methyl 3-*O*-methyl-α-L-rhamnoside (t_R; 26.0 min).

17α-Solanoside (IX)——Colorless prisms from EtOAc–hexane, mp 247—248 °C, $[\alpha]_D^{27}$ −30.4° (c=0.14, MeOH). FAB-MS *m/z*: 557.310 (M^++Na, Calcd for $C_{30}H_{46}O_8$+Na: 557.309). Hydrolysis of IX (3 mg) was carried out in the same manner as with VIII, and Δ^{14}-anhydro-17α-digitoxigenin and methyl 3-*O*-methyl-α-L-rhamnoside were observed on TLC and GLC.

Tanghinigenin α-L-Acofrioside (X)——A solid, $[\alpha]_D^{28}$ −15.0° (c=0.04, MeOH). FAB-MS *m/z*: 571.288 (M^++Na, Calcd for $C_{30}H_{44}O_9$+Na: 571.288).

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Synthesis of (±)-*endo*- and (±)-*exo*-Brevicomin *via* Ene Reaction of the Pummerer Rearrangement Product Derived from 4-Chlorophenyl Methyl Sulfoxide

HIROYUKI ISHIBASHI,* CHIAKI UEHARA, HAJIME KOMATSU, and MASAZUMI IKEDA

Kyoto Pharmaceutical University, Misasagi, Yamashina, Kyoto 607, Japan

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(±)-*endo*- (**4**) and (±)-*exo*-Brevicomin (**5**) were synthesized by employing the ene-type reaction of 4-chlorophenylthiomethyl trifluoroacetate (**2**) as the key step. The ene product **7** was converted, *via* two steps, into the ketosulfone **9**, whose epoxidation followed by acid hydrolysis afforded the cyclic acetal as a mixture, **12a** and **12b** (89 : 11). Recrystallization of the mixture followed by desulfurization yielded (±)-*endo*-brevicomin (**4**). On the other hand, successive treatment of **9** with osmium tetroxide, hydrochloric acid, and Raney nickel afforded a mixture of (±)-*exo*- (**5**) and (±)-*endo*-brevicomin (**4**) (89 : 11).

Keywords——(±)-*endo*-brevicomin; (±)-*exo*-brevicomin; Pummerer rearrangement; ene reaction; Wacker oxidation; desulfurization; Raney nickel

In previous papers,[1] we reported that the Pummerer rearrangement product **2** derived from 4-chlorophenyl methyl sulfoxide (**1**) and trifluoroacetic anhydride reacts with terminal olefins in trifluoroacetic acid to give the ene-type reaction products **3**. Here we wish to describe an application of this method to the synthesis of (±)-*endo*- (**4**) and (±)-*exo*-brevicomin (**5**). (+)-*exo*-Brevicomin is a component of the aggregation pheromone of the western pine beetle.[2] Although (+)-*endo*-brevicomin is inactive in relation to the western pine beetle, this compound inhibits the olfactory response of flying male and female southern pine

Cl–C₆H₄–S(O)CH₃ (1) —[$(CF_3CO)_2O$, CH_2Cl_2]→ Cl–C₆H₄–SCH_2OCOCF_3 (2) —[R–CH=CH₂, CF_3COOH]→ [intermediate] → R–CH=CH–CH₂CH₂–S–C₆H₄–Cl (3)

4 5

Chart 1

beetles to the female-produced pheromone, frontalin.[3]

The common key intermediate **9** for our syntheses of both stereoisomers **4** and **5** was prepared as follows. On treatment of the Pummerer rearrangement product **2**[1] with 2/3 eq of octa-1,7-diene (**6**) in trifluoroacetic acid at 0 °C for 1 h, the ene product **7** ($E:Z=89:11$, *vide infra*) was obtained in 50% yield (based on **6**). Structure determination of **7** was based on microanalysis and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. The signal due to the methylene protons α to the arylthio group appears as a triplet ($J=7$ Hz) at δ 2.87 and those due to the olefinic protons as multiplets at δ 4.7—5.2 (2H) and at δ 5.3—6.1 (3H). Oxidation of **7** with 2 eq of *m*-chloroperbenzoic acid (MCPBA) gave, in 67% yield, the sulfone **8**, which was then subjected to the Wacker oxidation to afford the keto-sulfone **9** in 67% yield.

Ar=4-ClC$_6$H$_4$

Chart 2

The transformation of **9** to (±)-*endo*-brevicomin (**4**) was achieved as follows. Oxidation of **9** with 1 eq of MCPBA gave, in an almost quantitative yield, the epoxide **10**, which, without further purification, was then treated with sulfuric acid in aqueous acetone to give, *via* the keto-diol **11**, a mixture of the *endo*- and *exo*-isomers of the cyclic acetal, **12a** and **12b**, in 94% combined yield after chromatography (silica gel). The ratio of the isomers **12a** and **12b** was estimated to be 89 : 11 from the integrated intensities of the signals due to the methyl protons at δ 1.37 (for **12a**) and 1.33 (for **12b**) (300 MHz ^{1}H-NMR). This, in turn, suggests the E/Z ratio of **7** to be 89 : 11. This mixture crystallized on standing, and one recrystallization from hexane–ethyl acetate (3 : 1) gave the pure *endo*-isomer **12a** (mp 121—122 °C) in 53% yield. Desulfurization of **12a** with Raney nickel in boiling ethanol afforded (±)-*endo*-brevicomin (**4**)[4] in 69% yield.

The intermediary epoxy-ketone **10** could alternatively be prepared from the sulfide **7** *via* two steps, but with less satisfactory results. Thus, on treatment of **7** with 3 eq of MCPBA, the epoxy-sulfone **13** was obtained in 32% yield.[5] The Wacker oxidation of **13** afforded the epoxy-ketone **10**, which was then treated with hydrochloric acid to give a mixture of **12a** and **12b** (89:11 by 300 MHz ^{1}H-NMR) in 54% yield (based on **13**).

3 eq MCPBA; 13; $PdCl_2$-CuCl-O_2, H_2O-DMF

[10 —HCl→ 11] → 12a + 12b

Chart 3

On the other hand, treatment of the keto-sulfone **9** with osmium tetroxide in pyridine followed by acid treatment of the resultant keto-diol **14** gave a mixture of the *exo*- and *endo*-isomers of the cyclic acetal, **12b** and **12a** (89:11 by 300 MHz ^{1}H-NMR), in 72% combined yield (based on **9**) as an oily substance. Desulfurization of this mixture with Raney nickel gave a mixture of (±)-*exo*- (**5**)[6] and (±)-*endo*-brevicomin (**4**) [89:11 by ^{1}H-NMR spectroscopy and gas liquid chromatography (GLC)] in 43% combined yield (in another run for preparation of **5** and **4**, the ratio of **5** and **4** was 88:12). No attempt was made to separate these isomers owing to the small quantity of the mixture thus obtained.

9 —OsO_4, C_5H_5N→ [14] —HCl→

12b + 12a —Raney Ni→ 5 + 4

Chart 4

Experimental

Melting points are uncorrected. Infrared (IR) spectra were recorded with a JASCO IRA-1 spectrophotometer. ^{1}H-NMR spectra were determined with a JEOL JNM-PMX 60 (60 MHz) spectrometer or a Varian XL-300 (300 MHz) spectrometer using tetramethylsilane as an internal standard. Low- and high-resolution mass spectra (MS) were obtained with a Hitachi M-80 instrument at 20 eV. GLC was carried out on a Shimadzu GC-5APTF gas chromatograph using a SCOT glass capillary column (20 m) coated with Silicone OV-17 at 60 °C. Column chromatography was performed on Silica gel 60 PF_{254} (Merck) under pressure.

9-(4-Chlorophenylthio)nona-1,6-diene (7)——Trifluoroacetic anhydride (1.2 g, 5.7 mmol) was added to a solution of 4-chlorophenyl methyl sulfoxide (**1**) (500 mg, 2.86 mmol) in methylene dichloride (4 ml) at 0 °C and the mixture was stirred at room temperature for 2.5 h. The solvent was removed by evaporation below 40 °C and a solution of octa-1,7-diene (**6**) (219 mg, 1.97 mmol) in trifluoroacetic acid (2 ml) was added to the residue containing the trifluoroacetate **2** at 0 °C, then the mixture was stirred at the same temperature for 1 h. Methylene dichloride (20 ml) was added to the reaction mixture and the organic phase was washed with saturated $NaHCO_3$ solution to remove trifluoroacetic acid, then dried ($MgSO_4$). The solvent was evaporated off and the residue was chromatographed on silica gel (hexane) to give the ene product **7** (260 mg, 50%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1635, 1430, 1090, 965, 910. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.1—2.6 [8H, m, $CH_2C{=}C(CH_2)_3C{=}C$], 2.87 (2H, t, J=7 Hz, SCH_2), 4.7—5.2 (2H, m, $=CH_2$), 5.3—6.1 (3H, m,

$-C\underline{H}=C\underline{H}-$, $C\underline{H}=CH_2$), 7.16 (4H, s, aromatic protons). *Anal.* Calcd for $C_{15}H_{19}ClS$: C, 67.52; H, 7.18. Found: C, 67.22; H, 7.10.

9-(4-Chlorophenylsulfonyl)nona-1,6-diene (8)——MCPBA (80%) (2.03 g, 9.4 mmol) was added to a solution of the sulfide 7 (1.26 g, 4.7 mmol) in methylene dichloride (60 ml) in small portions at 0 °C and the mixture was stirred at the same temperature for 10 h. The reaction mixture was washed with saturated $NaHCO_3$ solution to remove *m*-chlorobenzoic acid and dried ($MgSO_4$). The solvent was removed by evaporation and the residue was chromatographed on silica gel (hexane–ethyl acetate, 6 : 1) to give the sulfone **8** (941 mg, 67%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1640, 1305, 1140, 1085, 970, 910. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.1—2.7 [8H, m, $CH_2C=C(CH_2)_3C=C$], 2.9—3.3 (2H, m, SO_2CH_2), 4.7—5.2 (5H, m, olefinic protons), 7.52 (2H, d, $J=9$ Hz, aromatic protons), 7.88 (2H, d, $J=9$ Hz, aromatic protons). *Anal.* Calcd for $C_{15}H_{19}ClO_2S$: C, 60.29; H, 6.41. Found: C, 59.92; H, 6.33.

9-(4-Chlorophenylsulfonyl)non-6-en-2-one (9)——Cuprous chloride (521 mg, 5 mmol) and palladium chloride (177 mg, 1 mmol) were suspended in dimethylformamide (DMF) (5 ml) and water (0.6 ml), and the mixture was stirred vigorously at room temperature under an oxygen atmosphere until absorption of oxygen ceased. Then a solution of the sulfone **8** (1.5 g, 5 mmol) in DMF (1 ml) was added and the mixture was stirred vigorously at room temperature for 24 h under an oxygen atmosphere. The reaction mixture was poured into cold 3 N HCl (10 ml) and extracted with methylene dichloride. The extract was dried ($MgSO_4$), the solvent was removed by evaporation, and the residue was chromatographed on silica gel (hexane–ethyl acetate, 3 : 2) to give the keto-sulfone **9** (1.06 g, 67%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1710, 1310, 1125, 1090, 970. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.2—2.3 (6H, m, $CH_2C=CCH_2CH_2$), 2.09 (3H, s, COMe), 2.36 (2H, t, $J=7$ Hz, $COCH_2$), 2.9—3.3 (2H, m, SO_2CH_2), 5.2—5.5 (2H, m, olefinic protons), 7.43 (2H, d, $J=9$ Hz, aromatic protons), 7.75 (2H, d, $J=9$ Hz, aromotic protons). *Anal.* Calcd for $C_{15}H_{19}ClO_3S$: C, 57.23; H, 6.08. Found: C, 56.68; H, 6.01.

7-*endo*-[(4-Chlorophenylsulfonyl)ethyl]-5-methyl-6,8-dioxabicyclo[3.2.1]octane (12a)——MCPBA (80%) (370 mg, 1.72 mmol) was added to a solution of **9** (366 mg, 1.16 mmol) in chloroform (3 ml) at 0 °C and the mixture was stirred at room temperature for 15 h. The reaction mixture was washed successively with 10% NaOH solution and brine, then dried ($MgSO_4$). The solvent was removed by evaporation and the residue, which contained the epoxide **10**, was dissolved in acetone (10 ml) and water (1.4 ml). Two drops of concentrated sulfuric acid were added and the mixture was refluxed for 1.5 h. The reaction mixture was poured into water (20 ml) and extracted with chloroform. The extract was dried ($MgSO_4$), the solvent was removed by evaporation, and the residue was chromatographed on silica gel (chloroform) to give a mixture of **12a** and **12b** (365 mg, 94%), which solidified on standing. This mixture was recrystallized from hexane–ethyl acetate (3 : 1) to give pure **12a** (204 mg, 53%): mp 121—122 °C. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1585, 1320, 1150, 1090. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.37 (3H, s, Me), 1.5—1.9 (6H, m, H-2, 3, 4), 1.92—2.05 [1H, m, one of $CH_2C(7)$], 2.08—2.23 [1H, m, one of $CH_2C(7)$], 3.13 (1H, ddd, $J=14.0$, 11.5, 5.0 Hz, one of SO_2CH_2), 3.40 (1H, ddd, $J=14.0$, 11.7, 4.9 Hz, one of SO_2CH_2), 3.99 (1H, dt, $J=10.0$, 4.0 Hz, H-7), 4.21 (1H, br s, H-1), 7.56 (2H, d, $J=8.8$ Hz, aromatic protons), 7.87 (2H, d, $J=8.8$ Hz, aromatic protons). *Anal.* Calcd for $C_{15}H_{19}ClO_4S$: C, 54.46; H, 5.79. Found: C, 54.34; H, 5.84.

(±)-*endo*-Brevicomin (4)——A solution of **12a** (175 mg, 0.53 mmol) in ethanol (4 ml) containing Raney nickel (W-2) (*ca.* 7 g) was heated under reflux for 3.5 h. The Raney nickel was removed by filtration and the filtrate was concentrated at atmospheric pressure to give **4** (57 mg, 69%) as an oil [pure by GLC (N_2: 40 ml/min, t_R: 4.19 min)], whose ^{1}H-NMR spectrum ($CDCl_3$, 300 MHz) was virtually identical with that depicted ($CDCl_3$, 400 MHz) in the literature.[4d] ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 0.99 (3H, t, $J=7.2$ Hz, $CH_2C\underline{H}_3$), 1.44 (3H, s, Me), 1.5—2.0 (8H, m, H-2, 3, 4, and $C\underline{H}_2CH_3$), 3.99 (1H, dt, $J=4.2$, 7.3 Hz, H-7), 4.21 (1H, br s, H-1).

9-(4-Chlorophenylsulfonyl)-6,7-epoxynon-1-ene (13)——MCPBA (80%) (2.33 g, 10.82 mmol) was added to a solution of **7** (976 mg, 3.66 mmol) in methylene dichloride (40 ml) in small portions at 0 °C, and the mixture was stirred at room temperature for 15 h. The reaction mixture was washed successively with saturated $NaHCO_3$ solution and brine, then dried ($MgSO_4$). The solvent was evaporated off and the residue was chromatographed on silica gel (hexane–ethyl acetate, 2 : 1) to give **13** (364 mg, 32%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1640, 1580, 1320, 1150, 1090. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.2—2.3 (8H, m), 2.5—2.9 (2H, m, H-6, H-7), 3.20 (2H, t, $J=7.5$ Hz, SO_2CH_2), 4.8—5.2 (2H, m, $C=CH_2$), 5.4—6.1 (1H, m, $C\underline{H}=CH_2$), 7.45 (2H, d, $J=9$ Hz, aromatic protons), 7.80 (2H, d, $J=9$ Hz, aromatic protons). *Anal.* Calcd for $C_{15}H_{19}ClO_3S$: C, 57.23; H, 6.08. Found: C, 57.22; H, 6.12.

Transformation of 13 to 12a, b——By the same procedure as that described for the preparation of **9**, the epoxy-sulfone **13** (109 mg, 0.35 mmol) was subjected to the Wacker oxidation with cuprous chloride (36 mg, 0.35 mmol) and palladium chloride (12 mg, 0.068 mmol) under oxygen in DMF (0.85 ml) and water (0.05 ml). After completion of the reaction, the reaction mixture was poured into 3 N HCl (3 ml) and the whole was stirred at room temperature for 15 min. The reaction mixture was extracted with methylene dichloride. The extract was dried ($MgSO_4$), the solvent was evaporated off, and the residue was chromatographed on silica gel (hexane–ethyl acetate, 3 : 1) to give a mixture of **12a** and **12b** (62 mg, 54%), whose product ratio (89 : 11) proved to be identical with that of the mixture **12a, b** obtained from **9** (300 MHz ^{1}H-NMR).

7-*exo*-[(4-Chlorophenylsulfonyl)ethyl]-5-methyl-6,8-dioxabicyclo[3.2.1]octane (12b)——Osmium tetroxide (163 mg, 0.64 mmol) was added to a solution of **9** (201 mg, 0.64 mmol) in pyridine (2.5 ml) at room temperature and the mixture was stirred at the same temperature for 24 h. A solution of $NaHSO_3$ (295 mg) in water (5 ml) and pyridine

(3.3 ml) was added and the mixture was stirred at room temperature for 25 min, then extracted with chloroform. The solvent was evaporated off, the residue, which contained the diol **14**, was dissolved in 10% HCl (10 ml), and the solution was stirred at room temperature for 30 min. The reaction mixture was extracted with methylene dichloride. The extract was dried ($MgSO_4$), the solvent was evaporated off, and the residue was chromatographed on silica gel (hexane–ethyl acetate, 3 : 1) to give **12b** containing 11% of **12a** (total 152 mg, 72% based on **9**) as an oil. **12b**: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.33 (3H, s, Me), 1.45—2.0 [8H, m, H-2, 3, 4 and $CH_2C(7)$], 3.08—3.32 (2H, m, SO_2CH_2, 4.07—4.13 (2H, m, H-1, H-7), 7.55 (2H, d, J=8.7 Hz, aromatic protons), 7.85 (2H, d, J=8.7 Hz, aromatic protons).

(±)-*exo*-Brevicomin (5)——A solution of **12b** (containing 11% of **12a**) (152 mg, 0.46 mmol) in ethanol (5 ml) was heated under reflux in the presence of Raney nickel (W-2) (*ca.* 7 g) for 10 h. The Raney nickel was removed by filtration and the filtrate was concentrated at atmospheric pressure to give (±)-*exo*-brevicomin (**5**) [containing 11% (another run for preparation of **5** and **4**: 12%) of (±)-*endo*-brevicomin (**4**) as determined by ^{1}H-NMR and GLC (N_2, 40 ml/min; t_R for *exo*-isomer **5**, 3.38 min; for *endo*-isomer **4**, 4.19 min)] (31 mg, 43%). This mixture was not further purified owing to the small quantity obtained. ^{1}H-NMR ($CDCl_3$, 300 MHz, the signals for **5** only) δ: 0.91 (3H, t, J= 7.4 Hz, $CH_2C\underline{H}_3$), 1.4—1.65 (6H, m), 1.42 (3H, s, Me), 1.75—2.0 (2H, m), 3.93 (1H, t, J=6.5 Hz, H-7), 4.14 (1H, br, H-1). MS m/z: 156 (5, M^+), 127 (6), 114 (66), 99 (11), 98 (30), 86 (25), 85 (58), 68 (25), 43 (100%). These data (^{1}H-NMR and MS) were in accord with those reported for pure *exo*-brevicomin (**5**).[4d] Exact MS m/z: Calcd for $C_9H_{16}O_2$: 156.1148. Found: 156.1135.

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5) In this instance a small amount of the starting sulfide **7** was recovered. The use of MCPBA in more than the stoichiometric amount gave a complex mixture of products. A similar result was obtained by oxidation of **8** with 1 eq of MCPBA, leading to **13** (29%).
6) For other recent syntheses of *exo*-brevicomin, see (optically active form) *a*) K. Mori, *Tetrahedron*, **30**, 4223 (1974); *b*) M. Larcheveque and J. Lalande, *J. Chem. Soc., Chem. Commun.*, **1985**, 83; (racemic form) *c*) U. Kufner and R. R. Schmidt, *Synthesis*, **1985**, 1060; and 4*d*, *e*).

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Quinolizidines. XX.[1] Racemic and Chiral Syntheses of the *Alangium* Alkaloids 9-Demethylprotoemetinol and 10-Demethylprotoemetinol

TOZO FUJII,* MASASHI OHBA, ESAHAK ALI, HITOSHI SUZUKI, and JUN SAKAGUCHI

Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi, Kanazawa 920, Japan

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The synthesis of (±)-9-demethylprotoemetinol [(±)-**3d**] was accomplished by $LiAlH_4$ reduction of the tricyclic ester (±)-**5** and subsequent debenzylation of the resulting tricyclic alcohol (±)-**10**. Acetylation of (±)-**3d** with acetic anhydride and pyridine gave the diacetate (±)-**11**. The same sequence of reactions starting with (−)-**5** afforded (−)-9-demethylprotoemetinol [(−)-**3d**] and the diacetate (−)-**11** through (−)-**10**. Parallel synthetic routes starting with the isomeric tricyclic esters (±)-**9** and (−)-**9** produced (±)- and (−)-10-demethylprotoemetinols [(±)-**4d** and (−)-**4d**] and the corresponding diacetates [(±)-**13** and (−)-**13**] through (±)-**12** and (−)-**12**, respectively. The correctness of the structure and absolute stereochemistry of an *Alangium* alkaloid inferred to be 10-demethylprotoemetinol was confirmed by a direct comparison of its diacetate with synthetic (−)-**13**.

Keywords——*Alangium lamarckii* alkaloid; demethylprotoemetinol; racemic synthesis; chiral synthesis; lithium aluminum hydride ester reduction; benzyl ether hydrogenolysis; acetic anhydride–pyridine *O*-acetylation

In previous papers,[2,3] we have classified a number of benzo[*a*]quinolizidine alkaloids found in *Alangium lamarckii* THW. (Alangiaceae)[4] into four groups (types **1**—**4**) (R = CH_2OH, CO_2H, or a heterocyclic ring)[5] according to their substitution patterns in the aromatic ring A. Prior to the present study, the isolation of the **1**-type alkaloids psychotrine (**1a**),[6-8] cephaeline (**1b**),[6-9] and tubulosine (**1c**),[8-12] accompanied with their demethylated bases (types **3** and **4**) such as 9-demethylpsychotrine (**3a**),[8,13,14] demethylcephaeline (**3b** or **4b**),[8,15] and 10-demethyltubulosine (**4c**),[12,13,16] from the same plant suggested the possibility of co-occurrence of the 9-demethylated (**3d**) and/or 10-demethylated (**4d**) bases of pro-

toemetinol (dihydroprotoemetine) (**1d**), which had also been encountered[9,17] in *A. lamarckii*. This suggestion seemed to be very probable because ankorine (**2d**),[18] the **2**-type alkaloid at the protoemetinol level ($R=CH_2OH$), had already been found[17,19] to occur in the same plant. If the two demethylprotoemetinols were available in advance by synthesis, searching for them as natural products would be greatly facilitated. Our previous work has shown that the racemic syntheses of all the alkaloids of **1**—**4**-types are possible through the "lactim ether route" or "3-acetylpyridine route" and the chiral syntheses, through the "cincholoipon-incorporating route" or "lactim ether route".[20] We therefore undertook the racemic and chiral syntheses of 9-demethylprotoemetinol (**3d**) and 10-demethylprotoemetinol (**4d**), based on these unified synthetic strategies.[21]

The first target selected for synthesis was (±)-9-demethylprotoemetinol [(±)-**3d**], which would be accessible from the known tricyclic ester (±)-**5**, a common key intermediate utilized in our previous syntheses of (±)-9-demethylpsychotrine [(±)-**3a**][22] and (±)-9-demethyltubulosine [(±)-**3c**].[23] The starting tricycle (±)-**5** was available from the lactim ether (±)-**6** by the previously reported 6-step synthesis[22] or from 3-acetylpyridine (**7**) by the recently reported multistep syntheses.[24] Reduction of (±)-**5** with $LiAlH_4$ in ether gave the tricyclic alcohol (±)-**10** in 98% yield. On debenzylation using hydrogen and Pd–C catalyst, (±)-**10** produced the first target (±)-**3d** in 93% yield. The diacetate (±)-**11** was prepared from (±)-**3d**

Chart 1

in 93% yield by acetylation with acetic anhydride and pyridine.

Our second target for synthesis was (−)-9-demethylprotoemetinol [(−)-**3d**], and the same sequence of reactions starting with (−)-**5** afforded (−)-**10**, (−)-**3d**, and (−)-**11** in excellent yields. The starting material [(−)-**5**], a common key intermediate in our recent unified syntheses of (+)-9-demethylpsychotrine [(+)-**3a**],[14] (−)-9-demethylcephaeline [(−)-**3b**],[15] and (−)-9-demethyltubulosine [(−)-**3c**],[1] was prepared from cincholoipon ethyl ester [(+)-**8**][25] according to the previously reported 10-step procedure.[14]

For the syntheses of (±)- and (−)-10-demethylprotoemetinols, the third and fourth targets, synthetic routes starting with the known tricyclic esters (±)-**9** and (−)-**9** and parallel to those employed for the above 9-demethyl series were separately followed. All reactions proceeded smoothly and compounds **12**, **4d**, and **13**, in both the (±) and (−) forms, were obtained in high yields. The racemic starting material [(±)-**9**], a common key intermediate used by us in the syntheses of (±)-10-demethylpsychotrine [(±)-**4a**][22] and (±)-10-demethyltubulosine [(±)-**4c**],[16] was prepared from (±)-**6** by the previously reported 6-step synthesis[22] or from **7** according to the "3-acetylpyridine method".[24c] The chiral starting material [(−)-**9**], a key intermediate used also in our recent synthesis of (−)-10-demethylcephaeline [(−)-**4b**],[15] was obtained from (+)-**8**[25] as described previously.[2]

In the meantime, Pakrashi's group isolated two new alkaloids from the seeds of *A. lamarckii* and, on the basis of spectral and chemical evidence, inferred them to be 9-demethylprotoemetinol (**3d**) and 10-demethylprotoemetinol (**4d**).[26] We thus tried to confirm the structures of these compounds by direct comparisons with our authentic samples. It was found that the infrared (IR), nuclear magnetic resonance (NMR), and mass spectra and chromatographic behavior of the diacetate of the second alkaloid were identical with those of synthetic (±)-10-demethylprotoemetinol diacetate [(±)-**13**] [hence with those of synthetic (−)-**13**]. The chiral identity of the diacetate of the natural base with synthetic (−)-**13** was shown by the same sign of their specific rotations, establishing that the second alkaloid was actually (−)-10-demethylprotoemetinol [(−)-**4d**]. On the other hand, a direct comparison of the other alkaloid, inferred to be 9-demethylprotoemetinol (**3d**), with synthetic (−)-**3d** at the diacetate or the original level was not possible on account of paucity of the natural base, thus leaving its chemistry incomplete.

In conclusion, the above results not only represent an extension of the scope of our unified synthetic strategies for benzo[*a*]quinolizidine-type *Alangium* alkaloids but also have unequivocally established the structure and absolute stereochemistry of the *Alangium* alkaloid (−)-10-demethylprotoemetinol [(−)-**4d**]. Interestingly, among the many benzo[*a*]-quinolizidine-type *Alangium* alkaloids hitherto known, the tricyclic alcohol level is the only one that embraces all the **1**—**4**-types of alkaloids: protoemetinol (**1d**), ankorine (**2d**), 9-demethylprotoemetinol (**3d**), and 10-demethylprotoemetinol (**4d**). Though the very limited availability of the third of the four alkaloids at this moment precludes us from providing conclusive evidence to support the correctness of its structure, it is hoped that the knowledge obtained on the synthetic (±)-**3d** and (−)-**3d** or (±)-**11** and (−)-**11** will be of great help toward further isolation of this base from natural sources.

Experimental

General Notes——All melting points were determined by using a Yamato MP-1 capillary melting point apparatus and are corrected. See refs. 3 and 18*b* for details of instrumentation and measurements. Microanalyses were performed by Mr. Y. Itatani and his associates at Kanazawa University. The following abbreviations are used: br = broad, m = multiplet, s = singlet, sh = shoulder, t = triplet.

(±)-9-Benzyloxy-3α-ethyl-1,3,4,6,7,11bα-hexahydro-10-methoxy-2*H*-benzo[*a*]quinolizine-2β-ethanol [(±)-10]——To a stirred, ice-cooled suspension of $LiAlH_4$ (304 mg, 8 mmol) in dry ether (40 ml) was added dropwise a solution of (±)-**5**[22,24] (1.75 g, 4 mmol) in dry ether (40 ml) over a period of 15 min. After the mixture had been stirred

at room temperature for 1 h, H_2O (0.3 ml), 10% aqueous NaOH (0.3 ml), and H_2O (0.7 ml) were successively added under ice-cooling. The insoluble material that resulted was filtered off and washed with three 15-ml portions of $CHCl_3$. The filtrate and washings were combined, dried over anhydrous K_2CO_3, and concentrated *in vacuo* to leave (±)-**10** (1.55 g, 98%) as a brown oil. The oil was crystallized from hexane–AcOEt (3 : 1, v/v) to afford an analytical sample as colorless pillars, mp 111.5—112 °C; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3640 (OH), 2820, 2760 (*trans*-quinolizidine ring);[27] ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, t, J=6.5 Hz, $CCH_2\underline{Me}$), 1.78 (1H, s, OH), 3.78 (2H, br, $C\underline{H}_2OH$), 3.86 (3H, s, OMe), 5.10 (2H, s, $OC\underline{H}_2Ph$), 6.60 (1H, s, $H_{(8)}$ or $H_{(11)}$), 6.71 (1H, s, $H_{(11)}$ or $H_{(8)}$), 7.2—7.5 (5H, m, Ph). *Anal.* Calcd for $C_{25}H_{33}NO_3$: C, 75.92; H, 8.41; N, 3.54. Found: C, 75.81; H, 8.65; N, 3.49.

(2*R*,3*R*,11b*S*)-9-Benzyloxy-3-ethyl-1,3,4,6,7,11b-hexahydro-10-methoxy-2*H*-benzo[*a*]quinolizine-2-ethanol [(−)-10]——This was prepared in 93% yield from (−)-**5**[14] (1.53 g, 3.5 mmol) by reduction with $LiAlH_4$ (266 mg, 7 mmol) in dry ether (70 ml) in a manner similar to that described above for (±)-**10**. Purification by means of recrystallization from hexane–AcOEt (3 : 1, v/v) gave (−)-**10** as colorless needles, mp 103—104 °C; $[\alpha]_D^{25}$ −35.0° (c=0.50, EtOH). *Anal.* Calcd for $C_{25}H_{33}NO_3$: C, 75.92; H, 8.41; N, 3.54. Found: C, 75.91; H, 8.46; N, 3.80. The IR ($CHCl_3$) and ^{1}H-NMR ($CDCl_3$) spectra and thin-layer chromatographic (TLC) mobility of this sample were identical with those of the racemic modification [(±)-**10**] described above.

(±)-10-Benzyloxy-3α-ethyl-1,3,4,6,7,11bα-hexahydro-9-methoxy-2*H*-benzo[*a*]quinolizine-2β-ethanol [(±)-12]——To a chilled (0 °C), stirred solution of (±)-**9**[22,24c] (662 mg, 1.51 mmol) in dry ether (30 ml) was added $LiAlH_4$ (86 mg, 2.3 mmol) in small portions. The reaction mixture was stirred at room temperature for 1 h and then heated under reflux for 10 min. After successive additions of H_2O (2 drops) and anhydrous Na_2SO_4 at 0 °C, the resulting solid was filtered off and washed with $CHCl_3$. The filtrate and washings were combined and dried over anhydrous Na_2SO_4. Removal of the solvent *in vacuo* yielded a colorless oil, which was crystallized from hexane containing a small amount of EtOH to give (±)-**12** (542 mg, 91%) as colorless prisms. Recrystallization from the same solvent afforded an analytical sample, mp 98.5—99.5 °C; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3640 (OH), 2815, 2760 (*trans*-quinolizidine ring);[27] ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, t, J=6.5 Hz, $CCH_2\underline{Me}$), 1.43 (1H, s, OH), 3.69 (2H, br, $C\underline{H}_2OH$), 3.84 (3H, s, OMe), 5.10 (2H, s, $OC\underline{H}_2Ph$), 6.59 (1H, s, $H_{(8)}$ or $H_{(11)}$), 6.69 (1H, s, $H_{(11)}$ or $H_{(8)}$), 7.2—7.5 (5H, m, Ph). *Anal.* Calcd for $C_{25}H_{33}NO_3$: C, 75.92; H, 8.41; N, 3.54. Found: C, 76.01; H, 8.63; N, 3.64.

(2*R*,3*R*,11b*S*)-10-Benzyloxy-3-ethyl-1,3,4,6,7,11b-hexahydro-9-methoxy-2*H*-benzo[*a*]quinolizine-2-ethanol [(−)-12]——According to the procedure described above for (±)-**10**, except that the reaction time was prolonged to 2 h, (−)-**9**[2] (1.09 g, 2.5 mmol) was reduced with $LiAlH_4$ (190 mg, 5 mmol) in dry ether (60 ml) to give (−)-**12** (910 mg, 92%). Recrystallization of crude (−)-**12** from hexane–AcOEt (3 : 1, v/v) furnished an analytical sample as almost colorless prisms, mp 85—86 °C; $[\alpha]_D^{16}$ −50.6° (c=0.50, EtOH). *Anal.* Calcd for $C_{25}H_{33}NO_3$: C, 75.92; H, 8.41; N, 3.54. Found: C, 76.14; H, 8.44; N, 3.66. The IR ($CHCl_3$) and ^{1}H-NMR ($CDCl_3$) spectra and TLC mobility of this sample were identical with those of the racemic modification [(±)-**12**] described above.

(±)-3α-Ethyl-1,3,4,6,7,11bα-hexahydro-9-hydroxy-10-methoxy-2*H*-benzo[*a*]quinolizine-2β-ethanol [(±)-9-Demethylprotoemetinol] [(±)-3d]——A solution of (±)-**10** (1.19 g, 3 mmol) in EtOH (40 ml) was hydrogenated over 10% Pd–C (400 mg) at atmospheric pressure and 30 °C for 1 h. Removal of the catalyst by filtration and concentration of the filtrate under reduced pressure gave (±)-**3d** (852 mg, 93%) as a pale brown solid, mp 166.5—168 °C. Recrystallization of the solid from acetone yielded an analytical sample as colorless prisms, mp 171—171.5 °C; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3630, 3560 (OH's), 2810, 2750 (*trans*-quinolizidine ring);[27] ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, t, J=6.5 Hz, $CCH_2\underline{Me}$), 3.77 (2H, m, $C\underline{H}_2OH$), 3.85 (3H, s, OMe), 6.63 and 6.66 (1H each, s, aromatic protons). *Anal.* Calcd for $C_{18}H_{27}NO_3$: C, 70.79; H, 8.91; N, 4.59. Found: C, 70.66; H, 9.11; N, 4.75.

(2*R*,3*R*,11b*S*)-3-Ethyl-1,3,4,6,7,11b-hexahydro-9-hydroxy-10-methoxy-2*H*-benzo[*a*]quinolizine-2-ethanol [(−)-9-Demethylprotoemetinol] [(−)-3d]——This was obtained in 97% yield from (−)-**10** (990 mg, 2.5 mmol) by catalytic hydrogenolysis [10% Pd–C (400 mg), EtOH (40 ml), 1 atm, 24 °C, 1 h] similar to that described above for (±)-**3d**. Purification by means of recrystallization from acetone gave (−)-**3d** as slightly yellowish prisms, mp 157—158.5 °C; $[\alpha]_D^{25}$ −61.0° (c=0.50, EtOH); MS m/z (relative intensity): 306 (12), 305 (M^+) (66), 304 (100), 260 (11), 233 (10), 232 (49), 191 (47), 178 (12), 177 (50), 176 (13); UV λ_{max} (EtOH) 225 nm (sh) (log ε 3.87), 285 (3.60), 288 (3.60); λ_{max} (0.1 N aq. NaOH) 242 (3.91), 299 (3.70); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3630, 3560, 3005, 2930, 2810, 2750, 1508, 1467, 1448, 1361, 1336, 1254, 1148, 1133, 1034, 1018; ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, t, J=6.5 Hz, $CCH_2\underline{Me}$), 1.0—3.2 (unresolved m), 3.74 (2H, m, $C\underline{H}_2OH$), 3.84 (3H, s, OMe), 6.60 and 6.65 (1H each, s, aromatic protons). *Anal.* Calcd for $C_{18}H_{27}NO_3$: C, 70.79; H, 8.91; N, 4.59. Found: C, 70.59; H, 8.91; N, 4.56. The IR and ^{1}H-NMR spectra and TLC mobility of this sample were identical with those of the racemic sample [(±)-**3d**] described above.

(±)-3α-Ethyl-1,3,4,6,7,11bα-hexahydro-10-hydroxy-9-methoxy-2*H*-benzo[*a*]quinolizine-2β-ethanol [(±)-10-Demethylprotoemetinol] [(±)-4d]——Catalytic hydrogenolysis of (±)-**12** was carried out as described above for (±)-**3d** except that the reaction time was extended to 2 h. The crude product (96% yield) that resulted was recrystallized from hexane–$CHCl_3$ to provide (±)-**4d** as colorless prisms, mp 151—152 °C; MS m/z (relative intensity): 306 (11), 305 (M^+) (62), 304 (100), 290 (13), 260 (12), 248 (12), 233 (11), 232 (56), 191 (46), 178 (13), 177 (62), 176 (11); UV λ_{max} (EtOH) 225 nm (sh) (log ε 3.87), 285 (sh) (3.61), 288 (3.61); λ_{max} (0.1 N aq. NaOH) 243 (3.87), 300 (3.73); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3635, 3560 (OH's), 2810, 2755 (*trans*-quinolizidine ring);[27] ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, J=6.5 Hz, $CCH_2\underline{Me}$), 3.70 (2H, m, $C\underline{H}_2OH$), 3.81 (3H, s, OMe), 6.53 and 6.74 (1H each, s, aromatic protons). *Anal.* Calcd for

$C_{18}H_{27}NO_3$: C, 70.79; H, 8.91; N, 4.59. Found: C, 70.79; H, 8.83; N, 4.63.

(2*R*,3*R*,11b*S*)-3-Ethyl-1,3,4,6,7,11b-hexahydro-10-hydroxy-9-methoxy-2*H*-benzo[*a*]quinolizine-2-ethanol [(−)-10-Demethylprotoemetinol] [(−)-4d]——Catalytic hydrogenolysis of (−)-**12** (870 mg, 2.2 mmol) was effected [10% Pd–C (350 mg), EtOH (40 ml), 1 atm, 17 °C, 2 h] as described above for (±)-**3d**. The crude glassy material (656 mg, 98%) that resulted was purified on an alumina column [$CHCl_3$–EtOH (20 : 1, v/v)] to give (−)-**4d** as a pale yellow glass, $[\alpha]_D^{17}$ −35.2° (*c*=0.50, EtOH); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3635, 3560, 3005, 2930, 2810, 2755, 1508, 1465, 1448, 1370, 1266, 1147, 1131, 1032, 1018; ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, *J*=6.5 Hz, $CCH_2\underline{Me}$), 1.0—3.2 (unresolved m), 3.68 (2H, m, $C\underline{H}_2OH$), 3.81 (3H, s, OMe), 6.53 and 6.73 (1H each, s, aromatic protons). Apart from the chiroptical property, this sample was identical with (±)-**4d** by direct comparison of the mass, UV (EtOH or 0.1 N aq. NaOH), IR ($CHCl_3$), and ^{1}H-NMR ($CDCl_3$) spectra and TLC mobility.

(±)-9-Acetyloxy-3α-ethyl-1,3,4,6,7,11bα-hexahydro-10-methoxy-2*H*-benzo[*a*]quinolizine-2β-ethanol Acetic Ester [(±)-11]——A stirred mixture of (±)-**3d** (92 mg, 0.3 mmol), pyridine (0.5 ml), and acetic anhydride (0.3 ml) was heated at 60 °C for 30 min. After cooling, the reaction mixture was concentrated *in vacuo*. The resulting oil was purified by column chromatography [silica gel, AcOEt–$CHCl_3$ (2 : 1, v/v)] to afford (±)-**11** (109 mg, 93%) as a yellow oil; MS *m/z* (relative intensity): 390 (14), 389 (M^+) (66), 388 (84), 347 (13), 346 (54), 332 (13), 330 (29), 300 (15), 274 (41), 260 (15), 233 (47), 232 (33), 191 (21), 178 (15), 177 (100); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2810, 2760 (*trans*-quinolizidine ring),[27] 1754, 1730 (ester CO's); ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, t, *J*=6.5 Hz, $CCH_2\underline{Me}$), 2.06 (3H, s, $CH_2OCO\underline{Me}$), 2.29 (3H, s, $ArOCO\underline{Me}$), 3.81 (3H, s, OMe), 4.19 (2H, t, *J*=6.5 Hz, $C\underline{H}_2OAc$), 6.76 (2H, s, aromatic protons).

(2*R*,3*R*,11b*S*)-9-Acetyloxy-3-ethyl-1,3,4,6,7,11b-hexahydro-10-methoxy-2*H*-benzo[*a*]quinolizine-2-ethanol Acetic Ester [(−)-11]——Acetylation of (−)-**3d** (92 mg, 0.3 mmol) was carried out [pyridine (0.5 ml), Ac_2O (0.3 ml), 60 °C, 30 min] as described above for (±)-**11**, giving (−)-**11** (110 mg, 94%) as an unstable yellow oil, $[\alpha]_D^{25}$ −34.3° (*c*=0.40, $CHCl_3$); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2950, 2810, 2760, 1754, 1730, 1508, 1466, 1366, 1136, 1030; ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, t, *J*=6.5 Hz, $CCH_2\underline{Me}$), 1.0—3.2 (unresolved m), 2.06 (3H, s, $CH_2OCO\underline{Me}$), 2.29 (3H, s, $ArOCO\underline{Me}$), 3.81 (3H, s, OMe), 4.18 (2H, t, *J*=6.5 Hz, $C\underline{H}_2OAc$), 6.75 (2H, s, aromatic protons). The mass, IR, and ^{1}H-NMR spectra of this specimen were superimposable on those of the racemic modification [(±)-**11**] described above.

(±)-10-Acetyloxy-3α-ethyl-1,3,4,6,7,11bα-hexahydro-9-methoxy-2*H*-benzo[*a*]quinolizine-2β-ethanol Acetic Ester [(±)-13]——Acetylation of (±)-**4d** was effected as described above for (±)-**11**, and the resulting crude oil was crystallized from hexane to afford (±)-**13** in 85% yield. Recrystallization from hexane–$CHCl_3$ yielded an analytical sample as colorless prisms, mp 97—98 °C; MS *m/z* (relative intensity): 390 (15), 389 (M^+) (69), 388 (100), 346 (21), 332 (17), 330 (15), 302 (11), 300 (13), 275 (15), 274 (64), 260 (10), 234 (12), 233 (61), 178 (12), 177 (84); UV λ_{max} (EtOH) 222 nm (sh) (log ε 4.00), 277 (3.43), 281 (3.44), 286 (3.42); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2815, 2760 (*trans*-quinolizidine ring),[27] 1755, 1730 (ester CO's); ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, t, *J*=6.5 Hz, $CCH_2\underline{Me}$), 2.06 (3H, s, $CH_2OCO\underline{Me}$), 2.31 (3H, s, $ArOCO\underline{Me}$), 3.79 (3H, s, OMe), 4.17 (2H, t, *J*=6.4 Hz, $C\underline{H}_2OAc$), 6.66 and 6.86 (1H each, s, aromatic protons). *Anal.* Calcd for $C_{22}H_{31}NO_5$: C, 67.84; H, 8.02; N, 3.60. Found: C, 68.11; H, 8.05; N, 3.66. The mass, UV, IR, and ^{1}H-NMR spectra and TLC mobility of this sample were identical with those of the diacetate derived from natural 10-demethylprotoemetinol[26] as well as with those of synthetic (−)-**13** described below.

(2*R*,3*R*,11b*S*)-10-Acetyloxy-3-ethyl-1,3,4,6,7,11b-hexahydro-9-methoxy-2*H*-benzo[*a*]quinolizine-2-ethanol Acetic Ester [(−)-13]——Treatment of (−)-**4d** (92 mg, 0.3 mmol) with acetic anhydride (0.3 ml) and pyridine (0.5 ml) in a manner similar to that described above for (±)-**11** and purification of the crude oily product by column chromatography [silica gel, $CHCl_3$–EtOH (20 : 1, v/v)] gave (−)-**13** (102 mg, 87%) as a pale orange oil, $[\alpha]_D^{25}$ −29.7° (*c*=0.38, $CHCl_3$); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3015, 2950, 2815, 2760, 1755, 1730, 1508, 1465, 1366, 1145, 1030; ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, t, *J*=6.5 Hz, $CCH_2\underline{Me}$), 1.0—3.2 (unresolved m), 2.06 (3H, s, $CH_2OCO\underline{Me}$), 2.31 (3H, s, $ArOCO\underline{Me}$), 3.79 (3H, s, OMe), 4.17 (2H, t, *J*=6.2 Hz, $C\underline{H}_2OAc$), 6.65 and 6.86 (1H each, s, aromatic protons). This sample was identical [by comparison of mass, IR ($CHCl_3$), and ^{1}H-NMR ($CDCl_3$) spectra, TLC behavior, and specific rotation] with the diacetate [lit.[26] $[\alpha]_D$ −15.8° ($CHCl_3$)] of an *Alangium* alkaloid inferred to be 10-demethylprotoemetinol.[26]

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Homoisoflavonoids and Related Compounds. II.[1] Isolation and Absolute Configurations of 3,4-Dihydroxylated Homoisoflavans and Brazilins from *Caesalpinia sappan* L.

MICHIO NAMIKOSHI,* HIROYUKI NAKATA, HIROYUKI YAMADA, MINAKO NAGAI and TAMOTSU SAITOH

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan

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Three new phenolic compounds, 3'-*O*-methylsappanol, 3'-*O*-methylepisappanol and 3'-*O*-methylbrazilin, were isolated from Sappan Lignum, the dried heartwood of *Caesalpinia sappan*. The absolute configurations of sappanol, episappanol, 3'-deoxysappanol, 3'-*O*-methylsappanol, 3'-*O*-methylepisappanol, brazilin and 3'-*O*-methylbrazilin were determined by means of Horeau's partial resolution method and chemical correlations.

Sappanol, episappanol, 3'-deoxysappanol, 3'-*O*-methylsappanol and 3'-*O*-methylepisappanol form a novel class of homoisoflavonoids.

Keywords——*Caesalpinia sappan*; heartwood; Sappan Lignum; Leguminosae; homoisoflavonoid; 3,4-dihydroxylated homoisoflavan; brazilin; 3'-*O*-methylbrazilin; absolute configuration

A number of homoisoflavonoids[2,3] have been isolated from several genera in Liliaceae[3,4] and Caesalpinioideae (Legumiosae).[1,5,6] The structural features of homoisoflavonoids can generally be classified into three types, that is, eucomin type (3-benzylidenechroman-4-one), dihydroeucomin type (3-benzylchroman-4-one) and eucomol type (3-hydroxy-3-benzylchroman-4-one). Geometrical (*Z*-isomer) and position ($\Delta^{2(3)}$) isomers[4c] of eucomin-type compounds have also been isolated as natural products. Some unusual compounds were also isolated together with homoisoflavanones. One such group is scillascillin type compounds, which have a characteristic 3-spirocyclobutene ring system. Another example is comosin,[4a] whose 9-carbon is not benzylic, and which is a 3-acetyloxymethyl-3-phenylchroman-4-one. All these compounds have a carbonyl function at the C-4 position. Other novel compounds, reported by Camarda *et al.*,[4b] have a homoisoflavan structure, in which the 4-carbon is a methylene instead of the carbonyl in homoisoflavanones.

In the course of our studies on homoisoflavonoids and related compounds, we have been investigating the phenolic components of Sappan Lignum, the dried heartwood of *Caesalpinia sappan* L. (Leguminosae), and reported the isolation and structural determination of various homoisoflavonoidal components.[1,5] Among these compounds, newly named sappanol (1) and episappanol (2) form a novel class of homoisoflavonoids, which have a 3,4-dihydroxyhomoisoflavan structure. In the previous paper,[1] we reported the isolation and structural assignment of three homoisoflavonoids from the same source. One of these compounds, newly named 3'-deoxysappanol (3), is the third example of 3,4-dihydroxyhomoisoflavans.

Chemical constituents of Sappan Lignum have also been studied by two other groups. Nagai *et al.* reported the isolation of sappanchalcone[7] and dibenzoxocins[8] (protosappanins A, B and C) in a study on the compounds having a sleeping-time-prolonging effect in mice. Nohara and his co-workers isolated some phenolic components from this source in the course of their systematic screening for antihypercholesteremic activity,[5,9] and reported that

benzofran compounds were responsible for the effect.[9a] They determined the relative configuration of caesalpin J by means of X-ray crystallographic study.[9b]

Thus, Sappan Lignum contains various structural types of phenolic components, that is dibenzoxocins, homoisoflavonoids, brazilin and so on. Brazilin (**14**),[10] a well known main component of this plant, is interesting from the viewpoint of its characteristic skeleton and pharmacological activities.[11,12] Sappan Lignum has been used as an emmenagogue, hemostatic and antiinflammatory agent, and as a medical treatment for contusion and thrombosis prescribed in traditional oriental medicine.[11,13] An extract of Sappan Lignum has a suppressing effect on the central nervous system and has antimicrobial activities against *Staphylococcus*, *Diplococcus*, *Corynebacterium*, *Shigella baydii*, *etc.*[13]

We are interested in structural and biogenetic aspects of these compounds, as well as in the structure–activity relationships. In the present paper we deal with the isolation from Sappan Lignum and the absolute stereochemistries of three new components, 3′-*O*-methylsappanol (4), 3′-*O*-methylepisappanol (**5**) and 3′-*O*-methylbrazilin (**15**), and the

1: $R_1=R_3=H$, $R_2=OH$
3: $R_1=R_2=R_3=H$
4: $R_1=R_3=H$, $R_2=OMe$
6: $R_1=R_3=Me$, $R_2=OMe$
8: $R_1=R_3=Me$, $R_2=H$
26: $R_1=R_3=CH_2Ph$, $R_2=OMe$
28: $R_1=Me$, $R_2=OCH_2Ph$, $R_3=CH_2Ph$
38: $R_1=R_3=CH_2Ph$, $R_2=OCH_2Ph$

2: $R_1=R_2=R_3=H$
5: $R_1=R_3=H$, $R_2=Me$
7: $R_1=R_2=R_3=Me$
27: $R_1=R_3=CH_2Ph$, $R_2=Me$
29: $R_1=Me$, $R_2=R_3=CH_2Ph$
39: $R_1=R_2=R_3=CH_2Ph$

9

10: $R_1=R_3=H$, $R_2=OH$
11: $R_1=R_3=H$, $R_2=OMe$
12: $R_1=R_2=R_3=H$
13: $R_1=R_3=Me$, $R_2=OMe$
30: $R_1=R_3=CH_2Ph$, $R_2=OMe$
31: $R_1=Me$, $R_2=OCH_2Ph$, $R_3=CH_2Ph$
32: $R_1=Me$, $R_2=OH$, $R_3=H$

14: $R_1=R_2=R_3=R_4=H$
15: $R_1=R_3=R_4=H$, $R_2=Me$
16: $R_1=R_2=R_3=Me$, $R_4=H$
33: $R_1=Me$, $R_2=R_3=R_4=H$
40: $R_1=R_2=R_3=H$, $R_4=OH$

Chart 1

elucidation of the absolute stereochemistries of sappanol (1), episappanol (2), 3′-deoxysappanol (3) and brazilin (14).

Absolute Stereochemistries of Sappanol (1), Episappanol (2) and 3′-Deoxysappanol (3)

Sappanol (1) and episappanol (2) were obtained as a mixture of epimers at the C-4 position.[5] The ratio of **1** and **2** in the mixture varied between 2:1 and 3:2 as estimated from the proton nuclear magnetic resonance (^{1}H-NMR) spectra. Compounds **1** and **2** were not chromatographically separable from each other, but could be separated as their methyl derivatives (**6** and **7**), obtained by methylation with dimethyl sulfate. Compounds **6** and **7** were isolated from each other by silica gel column chromatography (hexane–acetone (4:1)). Compound **6** has a specific rotation of +27.6° (c=3.91, $CHCl_3$), and **7** has −13.6° (c=1.43, $CHCl_3$). The ^{1}H-NMR data for **6** and **7** are summarized in Table I. Since **6** and **7** gave a trimethyl ether of brazilin (**16**) upon acid catalyzed ring closure, the difference in the structure of these compounds is the stereochemistry at the C-4 position. On treatment with dry acetone in the presence of an acid catalyst, **6** afforded **13** within 10 h, but about 50% of **7** remained unchanged even after 24 h. In addition, **6** was readily oxidized to form **9** within 20 min upon HIO_4 treatment in methanol, while **7** was not (a half of the starting material remained even after 2 h). These facts suggested that the relative stereochemistries at C-3 and C-4 of **6** and **7** are *cis* and *trans*, respectively.

The absolute configurations at C-4 position of **6** and **7** were determined by Horeau's partial resolution method.[14] Compound **6** was treated with two equivalents of racemic 2-phenylbutanoic anhydride in pyridine, and the residual acid was levorotatory. The absolute stereochemistry at C-4 of **6** is thus (*S*) according to Horeau's rule.[14] Compound **6** is therefore (3*R*,4*S*)-3,4-dihydroxy-3-(3,4-dimethoxybenzyl)-7-methoxychroman. Consequently, sappanol (**1**) is (3*R*,4*S*)-3-(3,4-dihydroxybenzyl)-3,4,7-trihydroxychroman, as shown in Chart 1.

Similarly, **7** was treated with racemic 2-phenylbutanoic anhydride and the obtained acid was dextrorotatory. Therefore, the absolute stereochemistry at the C-4 position of **7** is (*R*), and **7** is (3*R*,4*R*)-3,4-dihydroxy-3-(3,4-dimethoxybenzyl)-7-methoxychroman. Accordingly, episappanol (**2**) is (3*R*,4*R*)-3-(3,4-dihydroxybenzyl)-3,4,7-trihydroxychroman (Chart 1).

^{1}H-NMR data for sappanol (**1**) and episappanol (**2**), summarized in Table I, were obtained with synthetic (±)-**1** and (±)-**2** as described later.

3′-Deoxysappanol (**3**) was obtained as a single epimer,[1] and afforded the dimethyl derivative (**8**) upon treatment with dimethyl sulfate. The circular dichroism (CD) curve of **8** showed a negative Cotton effect (Fig. 1). A negative Cotton effect was observed for **6**, while a positive Cotton effect was found for **7** (Fig. 1). Consequently the stereochemical correspondence of **6** and **8** was confirmed. Accordingly, 3′-deoxysappanol (**3**) is (3*R*,4*S*)-3-(4-hydroxybenzyl)-3,4,7-trihydroxychroman, as shown in Chart 1.

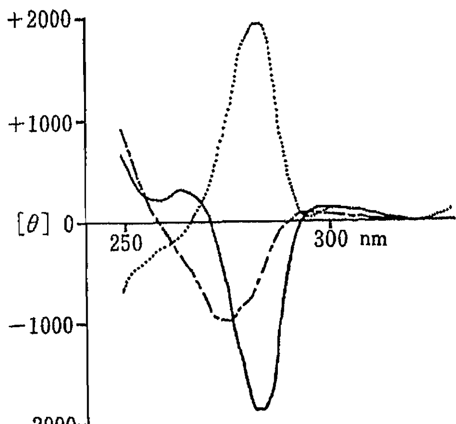

Fig. 1. CD Curves of Compounds **6** (——), **7** (------) and **8** (—·—)

TABLE I. ^{1}H-NMR Data for Compounds **1**, **2**, **4**, **5**, **6** and **7**

	1 (CD$_3$OD)$^{a)}$	**2** (CD$_3$OD)$^{a)}$	**4** (CD$_3$OD)$^{a)}$	**5** (CD$_3$OD)$^{a)}$	**6** (CDCl$_3$)	**7** (CDCl$_3$)
H-2	3.67 dd J=10.6, 1.2$^{b)}$	3.74 dd J=11.2, 1.6	3.66 dd J=10.5, 1.3	3.75 dd J=11.2, 1.6	c)	c)
	3.88 d J=10.6	4.01 d J=11.2	3.89 d J=10.5	4.02 d J=11.2	c)	4.07 d J=11.5
H-4	4.21 br s	4.19 br s	4.19 br s	4.18 br s	4.26 br s	4.30 br s
H-5	7.09 d J=8.2	7.07 d J=8.2	7.10 d J=8.2	7.08 d J=8.2	7.20 d J=8.6	7.18 d J=8.6
H-6	6.41 dd J=8.2, 2.3	6.37 dd J=8.2, 2.4	6.42 dd J=8.2, 2.2	6.37 dd J=8.2, 2.4	6.53 dd J=8.6, 2.4	6.50 dd J=8.6, 2.4
H-8	6.26 d J=2.3	6.23 d J=2.4	6.27 d J=2.2	6.24 d J=2.4	6.40 d J=2.4	6.39 d J=2.4
H-9	2.57 d J=14.0 $^{d)}$	2.67 d J=14.4	2.65 d J=14.0 $^{d)}$	2.72 d J=14.0	2.76 s	2.72 d J=14.0
	2.63 d J=14.0	2.84 d J=14.4	2.69 d J=14.0	2.91 d J=14.0		3.00 d J=14.0
H-2′	6.69 d J=2.0	6.81 d J=1.9	6.77 d J=2.0	6.94 d J=1.8	6.62—6.78 3H m	6.76—7.00 3H m
H-5′	6.66 d J=8.0	6.70 d J=8.0	6.69 d J=8.0	6.73 d J=8.0		
H-6′	6.50 dd J=8.0, 2.0	6.65 dd J=8.0, 1.9	6.60 dd J=8.0, 2.0	6.76 dd J=8.0, 1.8		
			3.81 s (3′-OMe)	3.85 s (3′-OMe)	3.76, 3.83, 3.83 3s (OMe)	3.74, 3.84, 3.86 3s (OMe)

a) Measured at 400 MHz. *b*) Coupling constants (*J*) are given in Hz. *c*) Overlapped with methyl signals. *d*) These signals were observed as a singlet at 100 MHz.

TABLE II. ^{1}H-NMR Data for Compounds **10**, **11**, **12**, **13** and **32**

	10 (Acetone-d_6)$^{a)}$	**11** (Acetone-d_6)$^{a)}$	**32** (Acetone-d_6)	**12** (Acetone-d_6)	**13** (CDCl$_3$)
H-2	3.61 d J=10.5$^{c)}$	3.63 d J=10.5	3.59 d J=10.5	3.59 d J=10.5	b)
	3.78 dd J=10.5, 1.3	3.79 dd J=10.5, 1.3	3.79 dd J=10.5, 1.3	3.78 dd J=10.5, 1.3	
H-4	4.61 br s	4.62 br s	4.62 br s	4.62 br s	4.58 br s
H-5	7.12 d J=8.5	7.16 d J=8.5	7.16 d J=8.5	7.15 d J=8.5	7.22 d J=8.5
H-6	6.46 dd J=8.5, 2.2	6.52 dd J=8.5, 2.2	6.52 dd J=8.5, 2.2	6.51 dd J=8.5, 2.2	6.58 dd J=8.5, 2.2
H-8	6.33 d J=2.2	6.39 d J=2.2	6.41 d J=2.2	6.39 d J=2.2	6.48 d J=2.2
H-9	2.70 d J=14.5 $^{d)}$	2.78 d J=14.4 $^{d)}$	2.76 s	2.79 s	2.77 d J=14.5
	2.76 d J=14.5	2.85 d J=14.4			2.95 d J=14.5
H-2′	6.66 d J=2.0	6.81 d J=2.0	6.77 d J=2.0	7.04 d J=8.8	
H-3′	—	—	—	6.75 d J=8.8	— 6.68—6.86 3H m
H-5′	6.67 d J=8.0	6.75 d J=8.0	6.80 d J=8.2	6.75 d J=8.8	
H-6′	6.48 dd J=8.0, 2.0	6.63 dd J=8.0, 2.0	6.61 dd J=8.2, 2.0	7.04 d J=8.8	
		3.80 s (3′-OMe)	3.78 s (4′-OMe)		3.76, 3.84 3s (OMe)
C(CH$_3$)$_2$	1.24, 1.35	1.25, 1.33	1.29, 1.33	1.26, 1.33	1.26, 1.41

a) Measured at 400 MHz. *b*) Overlapped with methyl signals. *c*) Coupling constants (*J*) are given in Hz. *d*) These signals were observed as a singlet at 100 MHz.

Absolute Stereochemistry of Brazilin (14)

The relative stereochemistry at C-3 and C-4 of brazilin (**14**) has been established by synthetic, spectral and chemical evidence; the compound has a *cis* C/D ring junction.[10a,15] Compounds **1** and **2** were readily cyclized to form brazilin (**14**) on treatment with a catalytic amount of acid. Compounds **6** and **7** were also transformed by similar acid treatment into the trimethyl derivative of brazilin (**16**) which was identical with the compound obtained from brazilin (**14**) upon methylation with dimethyl sulfate. Consequently, the absolute stereochemistry at the C-3 and C-4 positions of brazilin (**14**) must be (3*S*,4*R*), *i.e.*, brazilin is (6a*S*,11b*R*)-3,6a,9,10-tetrahydroxy-6a,11b-dihydro-7*H*-indeno[2,1-*c*]chromene, as shown in Chart 1.

Isolation, Synthesis and Absolute Stereochemistries of 3′-*O*-Methylsappanol (4) and 3′-*O*-Methylepisappanol (5)

3′-*O*-Methylsappanol (4) and 3′-*O*-methylepisappanol (5) were obtained as a mixture of two epimers at the C-4 position as in the case of sappanol (**1**) and episappanol (**2**). The ^{1}H-NMR spectrum of the mixture of **4** and **5** resembled that of the mixture of **1** and **2**, except for two three-proton singlets at δ 3.81 and 3.85, due to the *O*-methyl groups at C-3′ of **4** and **5**, respectively. The ratio of **4** and **5** in the mixture was about 2 : 1. When the separation was performed with the intact compounds, a very small amount of the mixture of **4** and **5** was obtained. Satisfactory amounts were obtained in the form of the isopropylidene derivative (**11**) separated after treatment of the methanolic extract with dry acetone in the presence of an acid catalyst. In a similar way, compound **10**, the isopropylidene derivative of sappanol (**1**) was obtained in greatly improved yield compared with the case of the separation with intact compounds. Compound **12** was also isolated as the isopropylidene derivative of 3′-deoxysappanol (3). As the mixture of 3′-*O*-methylsappanol (4) and 3′-*O*-methylepisappanol (5) was not separable and the yields were very low, the structural elucidation was accomplished by utilizing the isopropylidene derivative (**11**).

The ^{1}H-NMR spectrum of **11** was similar to that of **10**, except for the phenolic methoxy signal (3H, s) at δ 3.80 (summarized in Table II). Compound **11** gave a dimethyl derivative (**13**) on methylation with dimethyl sulfate, and its spectral properties coincided with those of **13** derived from **10**. Therefore, **11** was deduced to be a monomethyl ether of **10**. The fragment ion peaks derived from the A-ring of **10** and **11** were the same (*m/z* 163 and 164), but a fragment ion peak derived from the B-ring of **11** (at *m/z* 137) appeared at a position 14 mass units higher than the corresponding peak of **10** (at *m/z* 123). These facts suggested that the *O*-

Chart 2

methyl group is attached to either C-3′ or C-4′ in the B-ring of **11**.

A nuclear Overhauser effect (NOE) enhancement was observed for a proton signal at δ 6.81 (H-2′) upon irradiation of the methoxy proton signal at δ 3.80 in the ^{1}H-NMR spectrum of **11**. Therefore, the *O*-methyl group could be attached to the C-3′ position. The structure of **11** was further confirmed by the synthesis of (±)-**11** and (±)-**32**, which has an *O*-methyl group at the C-4′ position.

Vanillin (**18**) reacted with 7-hydroxychroman-4-one[16] (**17**) in dry ethanol saturated with dry HCl gas,[17a] to afford a benzylidenechromanone (**20**). It was transformed into its benzyl ether (**21**) with benzyl chloride in K_2CO_3/DMF. Compound **21**, on epoxidation with alkaline hydrogen peroxide in acetone–methanol,[17a,18] gave (±)-**24**, which was reduced to a mixture of (±)-**26** and (±)-**27** (about 9:1 mixture) with $LiALH_4$ in tetrahydrofuran (THF).[19] The mixture of (±)-**26** and (±)-**27** was treated with dry acetone in the presence of Amberlyst 15 without separation, and yielded the isopropylidene derivative (±)-**30** and a small amount of unchanged (±)-**27**. Compound (±)-**30**, on hydrogenation with palladium-on-charcoal (Pd–C) catalyst in acetone–methanol, afforded (±)-**11**. The physicochemical properties of (±)-**11** coincided with those of **11** isolated from the acetone-treated methanolic extract. Compound (±)-**32** was synthesized from isovanillin (**19**) and **17** in a manner similar to that used for the synthesis of (±)-**11**. Compound (±)-**32** was not distinguishable chromatographically from **11**, but the spectral data of (±)-**32** showed the distinct differences from those of **11**. The ^{1}H-NMR data for (±)-**32** are summarized in Table II together with those for **11**.

On the basis of these results, the position of the *O*-methyl group in **11**, and also in **4** and **5** was determined to be C-3′ of the B-ring.

Compound **13** derived from **11** showed the same levorotatory character as **13** derived from **10**. Consequently, the absolute stereochemistries at C-3 and C-4 of **11** are (3*R*,4*S*), as in **10**. 3′-*O*-Methylsappanol (**4**) and 3′-*O*-methylepisappanol (**5**) are therefore (3*R*,4*S*)-3-(4-hydroxy-3-methoxybenzyl)-3,4,7-trihydroxychroman and (3*R*,4*R*)-3-(4-hydroxy-3-methoxybenzyl)-3,4,7-trihydroxychroman, respectively (Chart 1).

Isolation, Synthesis and Absolute Stereochemistry of 3′-*O*-Methylbrazilin (15)

3′-*O*-Methylbrazilin (**15**) was obtained by repeated column chromatography (silica gel and LH-20) and preparative thin layer chromatography (prep. TLC) from the fractions remaining after separation of the previously reported compounds.[1,5] 3′-*O*-Methylbrazilin (**15**) has the molecular formula $C_{17}H_{16}O_5$ (high-resolution mass spectrum). The ^{1}H-NMR spectrum of 3′-*O*-methylbrazilin (**15**) was similar to that of brazilin (**14**) (summarized in Table III), but it showed the signals due to H-2′ and H-5′ together at δ 6.79, and also a phenolic methoxy signal (3H, s) at δ 3.79. The presence of one *O*-methyl group is consistent with the observation of a 14 mass units increase of the M^+ ion in the mass spectrum of 3′-*O*-methylbrazilin (**15**) compared with brazilin (**14**). The lower field shift (0.14 ppm) of the proton signal due to H-5′ in the ^{1}H-NMR spectrum of **15** suggested that a methoxy group is attached to C-3′ of the B-ring. The structure of **15** was determined on the basis of the following chemical correlations.

3′-*O*-Methylbrazilin (**15**) was readily synthesized from 3′-*O*-methylsappanol (**4**) and 3′-*O*-methylepisappanol (**5**), and also from **11** upon heating in methanol in the presence of an acid. Compound (±)-**33** (4′-*O*-methylbrazilin) was also synthesized from (±)-**32** under similar condition for the comparison of the spectral data. The ^{1}H-NMR spectrum of (±)-**33** was different from that of 3′-*O*-methylbrazilin (**15**) (data are summarized in Table III). The location of the methoxy group was thus determined to be at C-3′ of the B-ring.

3′-*O*-Methylbrazilin (**15**) showed a specific rotation of +113.2° (methanol), and the compound derived from **11** showed the same dextrorotatory character. Moreover, **15**, on methylation with dimethyl sulfate, afforded **16**, whose optical rotatory character was the same

TABLE III. ^{1}H-NMR Data for Compounds **14**, **15**, **16** and **33**

	14 (Acetone-d_6)	**15** (Acetone-d_6)	**16** ($CDCl_3$)	**33** (Acetone-d_6)
H-2	3.72 d $J=11.5$[a]	3.74 d $J=11.5$	[b]	3.77 d $J=11.5$
	3.96 dd $J=11.5, 2.0$	3.97 dd $J=11.5, 2.0$	3.98 dd $J=12.0, 2.0$	4.01 d $J=11.5$
H-4	4.01 d $J=2.0$	4.02 br s	4.04 br s	4.06 br s
H-5	7.21 d $J=8.6$	7.23 d $J=8.6$	7.24 d $J=8.8$	7.30 d $J=8.6$
H-6	6.50 dd $J=8.6, 2.4$	6.51 dd $J=8.6, 2.4$	6.58 dd $J=8.8, 2.5$	6.53 dd $J=8.6, 2.4$
H-8	6.32 d $J=2.4$	6.31 d $J=2.4$	6.41 d $J=2.5$	6.35 d $J=2.4$
H-9	2.80 d $J=16.0$	2.88 d $J=16.0$	2.81 d $J=16.0$	2.88 d $J=16.0$
	3.06 d $J=16.0$	3.10 d $J=16.0$	3.20 d $J=16.0$	3.10 d $J=16.0$
H-2′	6.79 s	6.79 s	6.76 s	6.90 s
H-5′	6.65 s	6.79 s	6.69 s	6.69 s
		3.79 s (3′-OMe)	3.72 s, 3.79 2s (OMe)	3.79 s (4′-OMe)

a) Coupling constants (J) are given in Hz. *b*) Overlapped with methyl signals.

as that of **16** derived from brazilin (**14**). Therefore, the absolute stereochemistries at C-3 and C-4 of 3′-*O*-methylbrazilin (**15**) are (3*S*,4*R*), *i.e.*, **15** is (6a*S*,11b*R*)-9-methoxy-3,6a,10-trihydroxy-6a,11b-dihydro-7*H*-indeno[2,1-*c*]chromene, as shown in Chart 1.

The stereochemical correspondence of brazilin (**14**) and haematoxylin[10b] (**40**), a constituent of *Haematoxylon campechianum*, was established in an optical rotatory dispersion (ORD) experiment carried out on their peracetates.[10a] The great similarity of the ORD curves of the two acetates revealed the stereochemistry at C-3 and C-4 of haematoxylin (**40**) to be (3*S*,4*R*), as shown in Chart 1 ((6a*S*,11b*R*)-3,4,6a,9,10-pentahydroxy-6a,11b-dihydro-7*H*-indeno[2,1-*c*]chromene). Studies on the absolute stereochemistries of the other components obtained from Sappan Lignum are in progress.

In the preceding paper,[1] we reported the isolation of three flavonoidal components, quercetin, rhamnetin and ombuin, from this source. The contents of these flavonoids are significantly less than those of homoisoflavonoidal components. Therefore, methylation at the C-2′ position of a chalcone to form a 2′-methoxychalcone is preferred to isomerization of the chalcone into a flavonoidal skeleton in the heartwood of this plant. 2′-Methoxychalcone is thought to be important in the biogenetic transformation into the homoisoflavonoidal skeleton, based on an isotope labeling study reported by Dewick.[20] He also proposed the biogenetic route to brazilin (**14**) from 2′-methoxychalcone via homoisoflavonoids;[20] this was supported recently by the isolation of homoisoflavanones and 3,4-dihydroxyhomoisoflavans.[5] Among these biogenetic transformations, the hydrogenation of a carbonyl group at the C-4 position to a hydroxyl group, that is, the formation of sappanol (**1**) and/or episappanol (**2**), will be a key step for brazilin biosynthesis.[5,20] Hydrogenation at the C-4 carbonyl group has never been recognized in homoisoflavonoids isolated from other sources. Sappanol (**1**) and episappanol (**2**) are readily transformed into brazilin (**14**) in the presence of a catalytic amount of acids or bases, and upon heating even under neutral conditions.[5] 3′-*O*-Methylsappanol (**4**) and 3′-*O*-methylepisappanol (**5**) afforded 3′-*O*-methylbrazilin (**15**) under similar conditions. However 3′-deoxysappanol (**3**) was not transformed into a brazilin-type compound by similar treatments. This can be attributed to the lack of an oxygen function at the C-3′ position, and 3′-deoxysappanol (**3**) should not biogenetically transform into a brazilin-type compound on the basis of this result.[3,20] When a methanol solution of 3′-deoxysappanol (**3**) was refluxed in the presence of an acid catalyst, two methylated compounds were formed, which were found to be 3′-deoxy-4-*O*-

methylsappanol and its C-4 epimer.[21] A similar phenomenon was observed with sappanol (**1**) and episappanol (**2**), which both gave a mixture of two epimers at the C-4 position of 4-*O*-methylated sappanol together with brazilin (**14**) upon heating in methanol.[5] 4-*O*-Methylsappanol and 4-*O*-methylepisappanol were also obtained from the methanolic extract of Sappan Lignum (compounds **5** and **6** in ref. 5), and were proposed to be activated precursors for brazilin biosynthesis, like sappanol (**1**) and episappanol (**2**).[3,20,22] Studies on the details of biosynthesis of brazilin and related compounds originating from homoisoflavonoids have been started in our laboratory.

From this series of studies, it has been shown that there are three substitution patterns of oxygen functions on aromatic rings in homoisoflavonoidal components of this plant material. One group consists of 3′,4′,7-trihydroxy compounds,[5] and the second consists of 4′,7-dihydroxy compounds,[1] which could be synthesized biogenetically from the corresponding methylated chalcones.[1,5] The third group consists of 4′,7-dihydroxy-3′-methoxy compounds as reported in this paper. The isolation of the biosynthetic precursors of 3′-*O*-methylsappanol (**4**) and 3′-*O*-methylepisappanol (**5**) is in progress.

The spectral data for sappanol (**1**), episappanol (**2**), 3′-*O*-methylsappanol (**4**) and 3′-*O*-methylepisappanol (**5**) were obtained by utilizing the synthetic compounds. The epimers **1** and **2**, and **4** and **5** could not be separated from each other, as already mentioned. The methyl ethers and the benzyl ethers of these compounds were separable. The mixtures of benzyl ethers (±)-**26** and (±)-**27**, and (±)-**38** and (±)-**39** were each subjected to column chromatography on silica gel with a hexane–acetone mixture (4:1). The hydrogenation of (±)-**26**, (±)-**27**, (±)-**37** and (±)-**38** in acetone–methanol in the presence of Pd–C catalyst afforded the free hydroxyl compounds (±)-**4** and (±)-**5**, and (±)-**1** and (±)-**2**, respectively. (±)-Sappanol (**1**) and (±)-3′-*O*-methylsappanol (**4**) gave a trimethyl ether (±)-**6** upon methylation with dimethyl sulfate, and on similar methylation, (±)-episappanol (**2**) and (±)-3′-*O*-methylepisappanol (**5**) yielded (±)-**7**. The synthesis of other components of Sappan Lignum will be reported elsewhere.

Experimental

All melting points were taken on a Yanagimoto micro melting point determination apparatus and are not corrected. Specific rotations were measured with a JASCO DIP-181 digital polarimeter. Circular dichroism (CD) spectra were observed on a JASCO J-20A automatic recording spectropolarimeter, equipped with a JASCO J-DPY data processor. ^{1}H-NMR spectra were recorded at 100 MHz on a Varian XL-100 instrument and at 400 MHz on a JEOL JNM-GX 400 spectrometer. Chemical shifts are reported on the δ scale (ppm) relative to tetramethylsilane (TMS) as an internal standard and coupling constants (J) are given in Hz. Abbreviations used are as follows: s = singlet, d = doublet, dd = doublet of doublets, m = multiplet, br = broad. Electron impact mass spectra (EI-MS) were measured with a JEOL JMS-D 300 at 70 eV. Ultraviolet (UV) spectra were obtained with a Shimadzu UV-240 spectrometer. TLC was performed on precoated Silica gel 60 F_{254} plates (0.25 mm thickness, Merck), and the spots were visualized by UV irradiation (254 nm) and by spraying 10% H_2SO_4 followed by heating.

Extraction and Isolation——Sappan Lignum, the dried heartwood of *Caesalpinia sappan* L. (500 g), purchased in Tokyo, was extracted 3 times with methanol at room temperature for 3 d. The methanolic extract (48.9 g) was repeatedly subjected to column chromatography and prep. TLC as described in the preceding papers.[1,5] Compounds **4** and **5**, and **15** were obtained by further separation from the fractions remaining after isolation of the previously reported compounds,[1,5] giving a mixture of **4** and **5** (1.2 mg), $[\alpha]_D^{25}$ +2.6° (c = 0.39, MeOH), in a ratio of about 2:1 estimated from the ^{1}H-NMR spectrum, and 5.6 mg of **15**.

Well-dried methanolic extract (52.6 g) was dissolved in dry acetone (300 ml), and 2 ml of Amberlyst 15 was added. The mixture was stirred at room temperature for 24 h, then the catalyst was filtered off, and the filtrate was evaporated to dryness. Compounds **10**, **11** and **12** were detectable on TLC ($CHCl_3$–MeOH (9:1), benzene–acetone (4:1), hexane–acetone (3:2)) of the crude reaction mixture. Repeated column chromatography (silica gel and LH-20) and prep. TLC of the crude product gave **10** (782 mg), **11** (26 mg) and **12** (254 mg).

Compound 10——$[\alpha]_D^{25}$ −24.7° (c = 1.50, MeOH). UV λ_{max}^{MeOH} nm (log ε): 283 (3.79), 278 (3.76). EI-MS m/z: 344.1257 (M^+, Calcd for $C_{19}H_{20}O_6$: 344.1257), 269, 220, 164, 163, 123, 107. ^{1}H-NMR data are summarized in Table II.

From the Mixture of Sappanol (**1**) and Episappanol (**2**): The mixture of **1** and **2** (38 mg) in dry acetone (20 ml) was stirred at room temperature for 24 h in the presence of Amberlyst 15 (0.2 ml). The mixture was filtered, and the filtrate was evaporated to dryness. The residue was purified by prep. TLC (benzene–acetone (7:3)) to afford **10** (36 mg). The physical and spectral properties coincided with those of an authentic sample of **10**.

Compound 11——$[\alpha]_D^{25}$ $-24.0°$ (c=1.04, MeOH). UV λ_{max}^{MeOH} nm (log ε): 283 (3.69), 278 (3.71), 225 (4.10). EI-MS m/z: 358.1420 (M^+, Calcd for $C_{20}H_{22}O_6$: 358.1415), 283, 220, 164, 163, 137, 122, 107. ^{1}H-NMR data are summarized in Table II. A difference NOE spectrum was measured on a 400 MHz ^{1}H-NMR spectrometer. Irradiating at δ 3.80 (3′-OMe) [one of geminal methylene signals due to H-2 (δ 3.79) overlapped] caused NOE enhancements at δ 6.81 (H-2′) and at δ 3.63 (the other geminal methylene signal due to H-2).

Compound 12——$[\alpha]_D^{25}$ $-21.1°$ (c=3.56, MeOH). UV λ_{max}^{MeOH} nm (log ε): 283 (3.58), 278 (3.63), 224 (4.20). EI-MS m/z: 328.1311 (M^+, Calcd for $C_{19}H_{20}O_5$: 328.1311), 164, 163, 107, 77. ^{1}H-NMR data are summarized in Table II.

Methylation of the Mixture of Sappanol (1) and Episappanol (2) (Compounds 6 and 7)——Dimethyl sulfate (180 mg) and anhydrous K_2CO_3 (3 g) were added to a solution of the mixture of **1** and **2** (138 mg) in dry acetone (50 ml). The mixture was refluxed for 3 h, then filtered, and the filtrate was evaporated to dryness. The residue was applied to a silica gel column and eluted with hexane–acetone (9:1) to afford **6** (83 mg) and **7** (45 mg).

Compound **6** was obtained as colorless needles. mp 108—109 °C. $[\alpha]_D^{25}$ $+27.6°$ (c=3.90, $CHCl_3$). EI-MS m/z: 346 (M^+), 328, 262, 261, 194, 193, 153, 152, 151, 121, 86, 84. CD (c=0.00026 mol/l, $CHCl_3$) $[\theta]^{25}$ (nm): +583 (250), +202 (257), +291 (264), −1830 (282), −1830 (284), +132 (300). ^{1}H-NMR data are summarized in Table I.

Compound **7** was obtained as colorless prisms. mp 95—96 °C. $[\alpha]_D^{25}$ $-13.7°$ (c=1.41, $CHCl_3$). EI-MS m/z: 346 (M^+), 344, 272, 271, 257, 194, 193, 152, 151. CD (c=0.00025 mol/l, $CHCl_3$) $[\theta]^{25}$ (nm): −629 (250), +1920 (281), +1930 (283), +117 (302). ^{1}H-NMR data are summarized in Table I.

Compound 13——From Compound **10**: Compound **10** (186 mg) was methylated as described above. The crude product was purified on a silica gel column (benzene–acetone (98:2)) to yield **13** (203 mg). $[\alpha]_D^{25}$ $-29.6°$ (c=4.36, $CHCl_3$). EI-MS m/z: 386 (M^+), 311, 234, 177, 151, 121. ^{1}H-NMR data are summarized in Table II.

From Compound **11**: Compound **11** (6 mg), on methylation in a similar manner, gave **13** (5 mg). The physicochemical properties of this compound coincided with those of **13** derived from **10**.

From Compound **6**: A solution of **6** (12 mg) in dry acetone (10 ml) was stirred at room temperature for 10 h in the presence of Amberlyst 15 (0.2 ml). The reaction mixture was filtered, and the filtrate was evaporated to dryness. The residue was purified by prep. TLC (benzene–acetone (30:1)) to yield pure **13** (13 mg). The physical and spectral properties coincided with those of **13** derived from **10**.

From Compound **7**: Compound **7** (16 mg) was treated as described above. After 24 h, about 50% of unchanged **7** was detected on TLC. The product was separated to afford **13** (8 mg) and **7** (7 mg). The physicochemical properties of **13** coincided with those of **13** derived from **10**, **11** and **6**.

Compound **13** gave a mixture of **6**, **7** and **16** (small amount) upon heating at 60 °C for 5 h in 80% AcOH.

The HIO_4 Oxidation of Compound 6——A solution of HIO_4 in H_2O (10 mg, 1 ml) was added to a solution of **6** (11 mg) in methanol (9 ml) with stirring. After 20 min, H_2O (50 ml) was added, and the mixture was extracted with EtOAc (50 ml × 3). The EtOAc extract was washed twice with H_2O (60 ml) and dried over Na_2SO_4. After evaporation of the solvents, the residue was purified by prep. TLC (hexane–acetone (1:1)) to afford **9** (9 mg), EI-MS m/z: 344 (M^+), 326, 193, 178, 175, 165, 164, 162, 152, 151, 107. ^{1}H-NMR ($CDCl_3$) δ: 3.79 (2H, s, H-9), 3.83 and 3.84 (3H and 6H, each s, OMe × 3), 4.68 (2H, s, H-2), 6.14 (1H, d, J=2.2 Hz, H-8), 6.58 (1H, dd, J=8.8, 2.2 Hz, H-6), 6.68—6.88 (3H, m, H-2′, 5′ and 6′), 7.82 (1H, d, J=8.8 Hz, H-5), 10.38 (1H, s, H-4).

The HIO_4 Oxidation of Compound 7——Compound **7** (10 mg) was treated with HIO_4 (10 mg) as described above. About 50% of starting material remained even after stirring for 2 h. The residue, obtained after EtOAc extraction, was purified by prep. TLC (hexane–acetone (1:1)) to give **9** (4 mg), whose physicochemical properties coincided with those of **9** derived from **6**, and recovered **7** (3 mg).

Compound 8——Compound **3** (10 mg) was methylated as described for **6** and **7**. The product was purified by prep. TLC (hexane–acetone (3:2)) to afford **8** (9 mg) as colorless needles, mp 154—156 °C. $[\alpha]_D^{25}$ $+31.0°$ (c=0.29, $CHCl_3$). EI-MS m/z: 316 (M^+), 177, 164, 153, 122, 121. CD (c=0.00027 mol/l, $CHCl_3$) $[\theta]^{25}$ (nm): +677 (250), −1000 (275), −818 (280), +104 (295). ^{1}H-NMR ($CDCl_3$) δ: 2.78 (2H, s, H-9), 3.74 (1H, dd, J=10.5, 1.3 Hz, H-2), 3.77 (3H, s, OMe), 3.79 (3H, s, OMe), 3.93 (1H, d, J=10.5 Hz, H-2), 4.35 (1H, br s, H-4), 6.41 (1H, d, J=2.2 Hz, H-8), 6.56 (1H, dd, J=8.2, 2.2 Hz, H-6), 6.84 (2H, d, J=8.8 Hz, H-3′ and 5′), 7.15 (2H, d, J=8.8 Hz, H-2′ and 6′). 7.24 (1H, d, J=8.2 Hz, H-5).

Brazilin (14)——From the Mixture of Sappanol (**1**) and Episappanol (**2**): A solution of the mixture of **1** and **2** in methanol (26 mg, 10 ml) and conc. HCl (0.1 ml) was refluxed for 1 h. The mixture was evaporated to dryness and the residue was purified by prep. TLC ($CHCl_3$–MeOH (9:1)) to afford **14** (18 mg), $[\alpha]_D^{25}$ $+125.6°$ (c=0.61, MeOH).

From Compound **10**: Compound **10** (42 mg) was treated in a similar manner to yield dextrorotatory brazilin (**14**) (21 mg).

The physicochemical properties of **14** coincided with those of the natural product. ^{1}H-NMR data are summarized in Table III.

3′-*O*-Methylbrazilin (15)——$[\alpha]_D^{25}$ $+113.2°$ (c=0.21, MeOH). UV λ_{max}^{MeOH} nm (log ε): 288 (3.82), 282 (sh). EI-MS

m/z: 300.0994 (M^+, Calcd for $C_{17}H_{16}O_5$: 300.0995), 283, 282, 281, 267, 243. ^{1}H-NMR data are summarized in Table III.

From Compound **11**: Compound **11** (12 mg) was treated as described for **14** to yield **15** (7 mg). The physical and spectral properties coincided with those of **15**.

Compound 16——From Brazilin (**14**): Brazilin (**14**) (270 mg) was methylated as described for **6** and **7**, to afford **16** (246 mg) as colorless needles from benzene, mp 136—137 °C. $[\alpha]_D^{25}$ +127.4° (c=0.51, $CHCl_3$). EI-MS *m/z*: 328 (M^+), 310, 309, 297, 279, 271, 204, 155, 151, 121. ^{1}H-NMR data are summarized in Table III.

From 3'-*O*-Methylbrazilin (**15**): Compound **15** (5 mg) on similar methylation afforded **16** (4 mg). The physical and spectral properties coincided with those of **16** derived from **14**.

From Compound **6**: A mixture of **6** (18 mg), methanol (9 ml) and conc. HCl (1 ml) was refluxed for 2 h. The solution was evaporated to dryness and the residue was purified by prep. TLC to yield **16** (12 mg).

From Compound **7**: Compound **7** (22 mg) was treated in a similar manner to give **16** (15 mg).

From Compound **13**: Similar treatment of **13** (10 mg) afforded **16** (6 mg).

Determination of the Absolute Configurations by Horeau's Partial Resolution Method[14]——Compound **6**: Racemic 2-phenylbutanoic anhydride (22.6 mg) was added to a solution of **6** (12.6 mg) in pyridine (1 ml). The mixture was allowed to stand at room temperature for 20 h, then 0.5 ml of water was added and the mixture was stirred for 1 h. After addition of further water (3 ml), the solution was extracted with benzene (3 ml × 3), and the benzene extract was extracted with 5% Na_2CO_3 aq. (3 ml × 3). The free acid (10.4 mg) was obtained after acidification of the alkaline extract and extraction with benzene (4 ml × 3). The residual acid had a specific rotation of −12.0° (c=0.42, benzene).

Compound **7**: Compound **7** (14.7 mg) was similarly reacted with racemic 2-phenylbutanoic anhydride (26.3 mg) in pyridine (1 ml), and worked up in a similar manner to afford the free acid (16.1 mg), which had a specific rotation of +6.2° (c=0.64, benzene).

Compounds 20, 22 and 35——A mixture of 7-hydroxychroman-4-one[16] (**17**) (10 mmol) and the aldehyde (11 mmol) in dry EtOH (25 ml) was saturated with dry HCl gas under cooling and then stirred at room temperature. After 20 h, the mixture was poured into water (100 ml), and 1 N NaOH aq. (20 ml) was added. The mixture was stirred at room temperature for 5 h, and the precipitate was filtered off, washed sufficiently with H_2O and dried over P_2O_5.

Compound **20** (2.86 g) was obtained by the reaction of **17** (1.64 g) and vanillin (**18**) (1.67 g). EI-MS *m/z*: 298 (M^+), 297, 162, 147, 137, 119, 108, 102, 91, 89, 80, 77, 69, 65, 63. ^{1}H-NMR (CD_3OD) δ: 3.89 (3H, s, 3'-OMe), 5.36 (2H, d, J=1.8 Hz, H-2), 6.29 (1H, d, J=2.2 Hz, H-8), 6.51 (1H, dd, J=8.5, 2.2 Hz, H-6), 6.80—7.00 (3H, m, H-2', 5' and 6'), 7.70 (1H, t, J=1.8 Hz, H-9), 7.80 (1H, d, J=8.5 Hz, H-5).

Compound **22** (2.88 g) was obtained by the reaction of **17** (1.64 g) and isovanillin (**19**) (1.67 g). EI-MS *m/z*: 298 (M^+), 297, 273, 181, 167, 161, 151, 147, 137, 136, 119, 108, 91. ^{1}H-NMR (CD_3OD) δ: 3.92 (3H, s, 4'-OMe), 5.36 (2H, d, J=1.8 Hz, H-2), 6.32 (1H, d, J=2.2 Hz, H-8), 6.53 (1H, dd, J=8.5, 2.2 Hz, H-6), 6.85 (1H, d, J=2.0 Hz, H-2'), 6.87 (1H, dd, J=8.8, 2.0 Hz, H-6'), 7.02 (1H, d, J=8.8 Hz, H-5'), 7.68 (1H, t, J=1.8 Hz, H-9), 7.81 (1H, d, J=8.5 Hz, H-5).

Compound **35** (2.70 g) was obtained by the reaction of **17** (1.64 g) and 3,4-dihydroxybenzaldehyde (**34**) (1.52 g). EI-MS *m/z*: 284 (M^+), 272, 268, 255, 237, 175. ^{1}H-NMR (CD_3OD) δ: 5.39 (2H, d, J=1.8 Hz, H-2), 6.34 (1H, d, J=2.2 Hz, H-8), 6.55 (1H, dd, J=8.9, 2.2 Hz, H-6), 6.60—7.00 (3H, m, H-2', 5' and 6'), 7.68 (1H, br s, H-9), 7.83 (1H, d, J=8.9 Hz, H-5). These data are identical with those of the natural product.[5]

These products each showed a single spot on TLC ($CHCl_3$–MeOH (9:1), benzene–acetone (3:2), hexane–acetone (1:1)), and the following benzylation was performed without separation.

Compounds 21, 23 and 36——A mixture of compound **20** (602 mg), benzyl chloride (280 mg) and anhydrous K_2CO_3 (3 g) in dry dimethylformamide (DMF) (30 ml) was heated at 100 °C for 2 h with stirring. The mixture was cooled and poured into H_2O, and the precipitate was filtered off, washed with H_2O and digested with methanol to afford **21** (867 mg). EI-MS *m/z*: 478 (M^+), 388, 387, 92, 91, 65. ^{1}H-NMR ($CDCl_3$) δ: 3.90 (3H, s, 3'-OMe), 5.09 and 5.20 (each 2H, s, $OC\underline{H_2}Ph$ × 2), 5.34 (2H, d, J=1.8 Hz, H-2), 6.47 (1H, d, J=2.2 Hz, H-8), 6.69 (1H, dd, J=8.5, 2.2 Hz, H-6), 6.78—7.00 (3H, m, H-2', 5' and 6'), 7.28—7.50 (10H, m, $OCH_2\underline{Ph}$ × 2), 7.77 (1H, t, J=1.8 Hz, H-9), 7.96 (1H, d, J=8.5 Hz, H-5).

Compound **23** (613 mg) was obtained from **22** (422 mg) by similar treatment. EI-MS *m/z*: 478 (M^+), 388, 387, 92, 91, 65. ^{1}H-NMR ($CDCl_3$) δ: 3.94 (3H, s, 4'-OMe), 5.10 (2H, s, $OC\underline{H_2}Ph$), 5.17 (2H, d, J=1.8 Hz, H-2), 5.18 (2H, s, $OC\underline{H_2}Ph$), 6.46 (1H, d, J=2.2 Hz, H-8), 6.69 (1H, dd, J=8.5, 2.2 Hz, H-6), 6.76—7.02 (3H, m, H-2', 5' and 6'), 7.28—7.74 (10H, m, $OCH_2\underline{Ph}$ × 2), 7.71 (1H, t, J=1.8 Hz, H-9), 7.95 (1H, d, J=8.5 Hz, H-5).

Compound **36** (1.62 g) was obtained from **35** (850 mg) by similar benzylation. EI-MS *m/z*: 554 (M^+), 464, 463, 91. ^{1}H-NMR ($CDCl_3$) δ: 5.10 and 5.20 (2H and 4H, each s, $OC\underline{H_2}Ph$ × 3), 5.22 (2h, s, H-2), 6.47 (1H, d, J=2.2 Hz, H-8), 6.70 (1H, dd, J=8.8, 2.2 Hz, H-6), 6.78—6.92 (2H, m, H-2' and 6'), 6.99 (1H, d, J=9.0 Hz, H-5'), 7.26—7.58 (15H, m, $OCH_2\underline{Ph}$ × 3), 7.72 (1H, br s, H-9), 7.97 (1H, d, J=8.8 Hz, H-5).

These products were used for the following epoxidation without purification.

Compounds (±)-24 and (±)-25——Hydrogen peroxide (1 ml) and 2 N NaOH aq. (1.2 ml) were added to a solution of **21** (520 mg) in acetone (40 ml) and methanol (10 ml). The mixture was stirred at room temperature for 24 h, then 25 ml of water was added and the organic solvents were evaporated off. The precipitate was filtered off and washed

with H_2O and methanol successively to yield (±)-**24** (516 mg). EI-MS *m/z*: 494 (M^+), 404, 403, 227, 92, 91. ^{1}H-NMR ($CDCl_3$) δ: 3.88 (3H, s, 3′-OMe), 4.14 (1H, d, *J*=12.5 Hz, H-2), 4.52 (1H, d, *J*=12.5 Hz, H-2), 4.52 (1H, s, H-9), 5.09 and 5.16 (each 2H, s, $OCH_2Ph\times2$), 6.45 (1H, d, *J*=2.2 Hz, H-8), 6.72 (1H, dd, *J*=8.8, 2.2 Hz, H-6), 6.80—7.00 (3H, m, H-2′,5′ and 6′), 7.28—7.52 (10H, m, $OCH_2Ph\times2$), 7.91 (1H, d, *J*=8.8 Hz, H-5).

Compound (±)-**25** (438 mg) was obtained from **23** (450 mg) on similar epoxidation. EI-MS *m/z*: 494 (M^+), 404, 403, 387, 227 177, 92, 91, 65. ^{1}H-NMR ($CDCl_3$) δ: 3.90 (3H, s, 4′-OMe), 3.94 (1H, d, *J*=12.5 Hz, H-2), 4.38 (1H, d, *J*=12.5 Hz, H-2), 4.46 (1H, s, H-9), 5.08 and 5.16 (each 2H, 2s, $OCH_2Ph\times2$), 6.43 (1H, d, *J*=2.2 Hz, H-8), 6.70 (1H. dd, *J*=8.8, 2.2 Hz, H-6), 6.78—6.96 (3H, m, H-2′, 5′ and 6′), 7.26—7.74 (10H, m, $OCH_2Ph\times2$), 7.90 (1H, d, *J*=8.8 Hz, H-5).

These compounds each showed a single spot on TLC (benzene–acetone (50 : 1), $CHCl_3$, hexane–acetone (9 : 1)).

Compound (±)-37——Compound **36** (1.22 g) in dioxane (120 ml) was treated with alkaline hydrogen peroxide as described for (±)-**24** to afford (±)-**37** (1.08 g). EI-MS *m/z*: 570 (M^+), 479, 318, 268, 254, 228, 181, 92, 91. ^{1}H-NMR ($CDCl_3$) δ: 3.95 (1H, d, *J*=12.5 Hz, H-2), 4.37 (1H, d, *J*=12.5 Hz, H-2), 4.44 (1H, s, H-9), 5.07 and 5.15 (2H and 4H, each s, $OCH_2Ph\times3$), 6.42 (1H, d, *J*=2.2 Hz, H-8), 6.68 (1H, dd, *J*=8.8, 2.2 Hz, H-6), 6.84—7.00 (3H, m, H-2′, 5′ and 6′), 7.20—7.56 (15H, m, $OCH_2Ph\times3$), 7.78 (1H, d, *J*=8.8 Hz, H-5).

The product showed a single spot on TLC, and was used for the following reduction without separation.

Compounds (±)-26 and (±)-27——A solution of (±)-**24** in THF (496 mg, 15 ml) was added dropwise to a solution of $LiAlH_4$ (36 mg) in THF (10 ml) with stirring at 0 °C. The mixture was stirred at room temperature for 2 h, then 100 ml of 1 N HCl aq. was added and the products were extracted with CH_2Cl_2 (100 ml × 3). The organic extract was washed twice with H_2O (each 100 ml), dried over Na_2SO_4 and evaporated to afford a mixture of (±)-**26** and (±)-**27**. The ratio of (±)-**26** and (±)-**27** was about 9 : 1,[19] as estimated from the ^{1}H-NMR spectrum and TLC behavior (benzene–acetone (9 : 1), benzene–EtOAc (7 : 1)).

The mixture of (±)-**26** and (±)-**27** was chromatographed on silica gel with benzene–acetone (9 : 1) to yield the pure compounds (±)-**26**, EI-MS *m/z*: 498 (M^+), 480, 270, 254, 253, 229, 228, 227, 137, 92, 91, ^{1}H-NMR ($CDCl_3$) δ: 2.75 (2H, s, H-9), 3.74 (1H, br d, *J*=10.5 Hz, H-2), 3.84 (3H, s, 3′-OMe), 3.92 (1H, d, *J*=10.5 Hz, H-2), 4.33 (1H, br s, H-4), 5.02 and 5.11 (each 2H, s, $OCH_2Ph\times2$), 6.48 (1H, d, *J*=2.2 Hz, H-8), 6.61 (1H, dd, *J*=8.5, 2.2 Hz, H-6), 6.65 (1H, dd, *J*=8.0, 2.0 Hz, H-6′), 6.78 (1H, d, *J*=2.0 Hz, H-2′), 6.82 (1H, d, *J*=8.0 Hz, H-5′), 7.21 (1H, d, *J*=8.5 Hz, H-5), 7.20—7.54 (10H, m, $OCH_2Ph\times2$), and (±)-**27**, EI-MS *m/z*: 498 (M^+), 271, 270, 254, 253, 227, 180, 179, 137, 92, 91, ^{1}H-NMR ($CDCl_3$) δ: 2.73 (1H, d, *J*=14.0 Hz, H-9), 2.99 (1H, d, *J*=14.0 Hz, H-9), 3.82 (1H, br d, *J*=11.5 Hz, H-2), 3.88 (3H, s, 3′-OMe), 4.09 (1H, d, *J*=11.5 Hz, H-2), 4.27 (1H, br s, H-4), 5.00 and 5.12 (each 2H, s, $OCH_2Ph\times2$), 6.48 (1H, d, *J*=2.4 Hz, H-8), 6.58 (1H, dd, *J*=8.5, 2.4 Hz, H-6), 6.76—7.00 (3H, m, H-2′, 5′ and 6′), 7.14 (1H, d, *J*=8.5 Hz, H-5), 7.20—7.54 (10H, m, $OCH_2Ph\times2$).

Compound (±)-30——The mixture of (±)-**26** and (±)-**27** (168 mg) in dry acetone (30 ml) was stirred for 24 h in the presence of Amberlyst 15 (0.5 ml). The reaction mixture was filtered, and the filtrate was evaporated to dryness. The residue was chromatographed on silica gel (eluted with benzene–EtOAc (99 : 1)) to afford (±)-**30** (146 mg). EI-MS *m/z*: 538 (M^+), 301, 300, 254, 253, 228, 227, 137, 92, 91. ^{1}H-NMR ($CDCl_3$) δ: 1.22 and 1.39 (each 3H, 2s, $C(CH_3)_2$), 2.75 (1H, d, *J*=14.5 Hz, H-9), 2.92 (1H, d, *J*=14.5 Hz, H-9), 3.69 (1H, d, *J*=10.5 Hz, H-2), 3.82 (3H, s, 3′-OMe), 3.85 (1H, br d, *J*=10.5 Hz, H-2), 4.54 (1H, br s, H-4), 5.04 and 5.11 (each 2H, s, $OCH_2Ph\times2$), 6.53 (1H, d, *J*=2.2 Hz, H-8), 6.62 (1H, dd, *J*=8.0, 2.0 Hz, H-6′), 6.64 (1H, dd, *J*=8.2, 2.2 Hz, H-6), 6.76 (1H, d, *J*=2.0 Hz, H-2′), 6.79 (1H, d, *J*=8.0 Hz, H-5′), 7.21 (1H, d, *J*=8.2 Hz, H-5), 7.20—7.54 (10H, m, $OCH_2Ph\times2$). A small amount of unchanged (±)-**27** was also obtained after the elution of (±)-**30** (eluted with a 9 : 1 mixture of the same solvents).

Compound (±)-11——Compound (±)-**30** (62 mg) was hydrogenated in acetone–methanol (4 : 1, 24 ml) under a hydrogen atmosphere in the presence of 5% Pd–C catalyst (50 mg). After 2 h, the mixture was filtered, and the filtrate was evaporated to dryness. The residue was chromatographed on silica gel (eluted with benzene–acetone (9 : 1)) to give (±)-**11** (32 mg). The spectral properties of (±)-**11** coincided with those of (−)-**11**.

Compound (±)-32——Compound (±)-**25** (402 mg) was treated with $LiAlH_4$ (32 mg) in THF as described for (±)-**26** and (±)-**27** to give a mixture of (±)-**28** and (±)-**29** (294 mg), which was transformed into its isopropylidene derivative (±)-**31** as described for (±)-**30** without separation. Compound (±)-**31** (85 mg) was hydrogenated as described for (±)-**11**, yielding (±)-**32** (48 mg). EI-MS *m/z*: 358 (M^+), 283, 220, 181, 176, 164, 163, 147, 138, 137, 135, 122, 107, 94. ^{1}H-NMR data are summarized in Table II.

Compounds (±)-38 and (±)-39——Compound (±)-**37** (980 mg) was treated with $LiAlH_4$ (75 mg) as described for (±)-**26** and (±)-**27** to give a mixture of (±)-**38** and (±)-**39** (768 mg), which was chromatographed on silica gel (eluted with benzene–EtOAc (92 : 8)) to afford (±)-**38**, EI-MS *m/z*: 556 (M^+-H_2O), 304, 303, 254, 211, 182, 181, 151, 123, 92, 91 ^{1}H-NMR ($CDCl_3$) δ: 2.68 (2H, s, H-9), 3.62 (1H, br d, *J*=10.5 Hz, H-2), 3.79 (1H, d, *J*=10.5 Hz, H-2), 4.24 (1H, br s, H-4), 5.00 and 5.11 (2H and 4H, each s, $OCH_2Ph\times3$), 6.46 (1H, d, *J*=2.2 Hz, H-8), 6.59 (1H, dd, *J*=8.5, 2.2 Hz, H-6), 6.67 (1H, dd, *J*=8.0, 2.0 Hz, H-6′), 6.80 (1H, d, *J*=2.0 Hz, H-2′), 6.85 (1H, d, *J*=8.0 Hz, H-5′), 7.15 (1H, d, *J*=8.5 Hz, H-5), 7.20—7.54 (15H, m, $OCH_2Ph\times3$), and (±)-**39**, EI-MS *m/z*: 574 (M^+), 556, 312, 303, 254, 181, 152, 151, 91, ^{1}H-NMR ($CDCl_3$) δ: 2.64 (1H, d, *J*=14.0 Hz, H-9), 2.92 (1H, d, *J*=14.0 Hz, H-9), 3.74 (1H, br d, *J*=11.5 Hz, H-2), 4.00 (1H, d, *J*=11.5 Hz, H-2), 4.08 (1H, br s, H-4), 4.98, 5.12 and 5.16 (each 2H, s, $OCH_2Ph\times3$), 6.46 (1H, d, *J*=2.4 Hz, H-8), 6.54 (1H, dd, *J*=8.5, 2.4 Hz, H-6), 6.68—7.00 (3H, m, H-2′, 5′ and 6′), 7.05

(1H, d, J=8.5 Hz, H-5), 7.20—7.54 (15H, m, $OCH_2\underline{Ph}\times3$).

(±)-Sappanol (1)——Compound (±)-**38** (126 mg) was hydrogenated as described for (±)-**11** to give (±)-**1** (52 mg). UV λ_{max}^{MeOH} nm (log ε): 284 (3.73), 279 (3.73). EI-MS m/z: 286 (M^+-H_2O), 285, 269, 268, 267, 123, 111. ^{1}H-NMR data are summarized in Table I.

(±)-Episappanol (2)——Compound (±)-**39** (86 mg) was hydrogenated as described for (±)-**11** to afford (±)-**2** (32 mg). UV λ_{max}^{MeOH} nm (log ε): 284 (3.83), 280 (3.83). EI-MS m/z: 286 (M^+-H_2O), 269, 268, 267, 229, 164, 163, 123, 111, 91. ^{1}H-NMR data are summarized in Table I.

(±)-3′-*O*-Methylsappanol (4)——Compound (±)-**26** (72 mg) was similarly hydrogenated to yield (±)-**4** (36 mg). UV λ_{max}^{MeOH} nm (log ε): 284 (3.67), 279 (3.89), 224 (sh). EI-MS m/z: 318 (M^+), 300, 180, 164, 163, 138, 137, 122, 107, 91. ^{1}H-NMR data are summarized in Table I.

(±)-3′-*O*-Methylepisappanol (5)——Hydrogenation of (±)-**27** (58 mg) gave (±)-**5** (28 mg). UV λ_{max}^{MeOH} nm (log ε): 285 (3.80), 279 (3.84), 224 (4.23). EI-MS m/z: 318 (M^+), 300, 180, 164, 163, 147, 139, 138, 137, 128, 127, 107. ^{1}H-NMR data are summarized in Table I.

Compound (±)-6——A mixture of (±)-**1** (18 mg), dimethyl sulfate (29 mg) and anhydrous K_2CO_3 (0.4 g) in dry acetone (15 ml) was refluxed for 3 h. The reaction mixture was filtered, and the filtrate was evaporated. The residue was purified by prep. TLC (hexane–acetone (3:2)) to afford (±)-**6** (12 mg).

Compound (±)-**4** (10 mg) gave (±)-**6** (6 mg) on similar methylation. The spectral properties of (±)-**6** coincided with those of (+)-**6**.

Compound (±)-7——Compound (±)-**2** (16 mg) and (±)-**5** (13 mg) were each methylated as described for (±)-**6** to give (±)-**7** (9 mg and 5 mg, respectively). The spectral properties of (±)-**7** coincided with those of (−)-**7**.

Compound (±)-33 (4′-*O*-Methylbrazilin)——A mixture of (±)-**32** (86 mg), conc. HCl (0.2 ml) and methanol (20 ml) was refluxed for 1 h. The solution was evaporated to dryness, and the residue was purified on a silica gel column (eluted with benzene–acetone (7:3)) to give (±)-**33** (48 mg). EI-MS m/z: 300 (M^+), 285, 283, 282, 281, 243. ^{1}H-NMR data are summarized in Table III.

Acid Treatment of (±)-Sappanol (1) and (±)-Episappanol (2) [(±)-Brazilin (14)]——A mixture of (±)-**1** (38 mg), methanol (10 ml) and conc. HCl (0.1 ml) was refluxed for 1 h. The solution was evaporated to dryness, and the residue was purified by prep. TLC ($CHCl_3$–MeOH (9:1)) to afford 23 mg of (±)-brazilin (**14**).

(±)-Episappanol (2) (12 mg) was treated in a similar manner to yield (±)-**14** (6 mg). The spectral properties of (±)-**14** coincided with those of (+)-**14**.

Acid Treatment of (±)-3′-*O*-Methylsappanol (4) and (±)-3′-*O*-Methylepisappanol (5) [(±)-3′-*O*-Methylbrazilin (15)]——A solution of (±)-**4** (19 mg) in methanol (10 ml) and conc. HCl (0.1 ml) was refluxed for 1 h, then the solvent was evaporated off. The residue was purified by prep. TLC (benzene–acetone (7:3)) to gave 10 mg of (±)-3′-*O*-methylbrazilin (**15**).

Similarly, (±)-**5** (11 mg) was treated with conc. HCl/methanol to give (±)-**15** (6 mg). The spectral properties of (±)-**15** coincided with those of (+)-**15**.

Acid Treatment of 3′-Deoxysappanol (3) and Compound 12——Compound **12** (36 mg) in 60% AcOH was stirred at 50 °C for 5 h. The solution consisted of an about 4:1 mixture of two compounds (**3** and its epimer at the C-4 position). When the mixture was heated at 100 °C for 3 h, the solution contained an about 1:1 mixture of **3** and its epimer. Similar treatment of **3** resulted in an identical mixture of these compounds.

Compound **3** and its epimer were separable on TLC (benzene–acetone (7:3), hexane–acetone (1:1)). The Rf values of these compounds obtained with the different solvent mixtures were reversed. The compound eluted from the lower spot on prep. TLC (benzene–acetone (7:3)) was identified as **3** and the compound eluted from the upper spot proved to be the C-4 epimer of **3**, having the following physical and chemical properties: $[\alpha]_D^{25}$ −77.5° (c=0.42, MeOH). EI-MS m/z: 288 (M^+), 270, 165, 164, 163, 151, 108, 107, 77. ^{1}H-NMR (CD_3OD) δ: 2.71 (1H, d, J=14.4 Hz, H-9), 2.93 (1H, d, J=14.4 Hz, H-9), 3.74 (1H, dd, J=11.2, 1.6 Hz, H-2), 4.02 (1H, d, J=11.2 Hz, H-2), 4.20 (1H, br s, H-4), 6.24 (1H, d, J=2.5 Hz, H-8), 6.36 (1H, dd, J=8.2, 2.5 Hz, H-6), 6.72 (2H, d, J=8.8 Hz, H-3′ and 5′), 7.08 (1H, d, J=8.2 Hz, H-5), 7.17 (2H, d, J=8.8 Hz, H-2′ and 6′).

When **3** and **12** were each treated under conditions similar to those used for acid treatment of (±)-**1** (reflux in methanol with conc. HCl), the reaction solution contained mainly two components (two spots on TLC (benzene–acetone (4:1))). These compounds were found to be the 4-*O*-methyl derivative of **3** and its C-4 epimer.[21]

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Benzylpiperazine Derivatives. I.[1] Syntheses and Biological Activities of 1-(2,3,4-Trimethoxybenzyl)piperazine Derivatives

HIROSHI OHTAKA,* MUTSUO MIYAKE, TOSHIRO KANAZAWA, KEIZO ITO and GORO TSUKAMOTO

Pharmaceuticals Research Center, Kanebo Ltd., 5-90, Tomobuchi-cho 1-chome, Miyakojima-ku, Osaka 534, Japan

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A series of 1-(2,3,4-trimethoxybenzyl)piperazine (trimetazidine) derivatives were prepared and tested for cerebral vasodilating activity. Almost all the compounds possess stronger activity than trimetazidine and among them, the γ-amino tertiary alcohols **6e—j** exhibit potent cerebral vasodilating activities which are superior to those of cinnarizine and papaverine. Moreover, these compounds show a selective vasodilating effect on vertebral arteries.

Keywords——piperazine; trimetazidine; Mannich reaction; Grignard reaction; cerebral vasodilator

1-(2,3,4-Trimethoxybenzyl)piperazine dihydrochloride (trimetazidine dihydrochloride; 1) is one of a few monosubstituted piperazines used clinically, and there are some patents concerning 4-substituted derivatives of this coronary vasodilator.[2] It is of interest to note that trimetazidine was reported to be distributed in the brain as well as in the heart,[3] and to relax dog basilar arteries more effectively than coronary arteries after contraction with prostaglandin $F_{2\alpha}$.[4] Trimetazidine seems to be more hydrophilic than cinnarizine (CNZ, **2**), a well known cerebral vasodilator, and therefore we thought it pertinent to prepare a group of lipophilic trimetazidine derivatives, with the aim of finding a new cerebral vasodilator. The structure of the trimetazidine derivatives was designed taking the structure of **2** into consideration. This paper describes the synthesis of a group of trimetazidine derivatives (**3**—**6**), as well as the cerebral vasodilating activities of the compounds.

MeO MeO MeO N NH·2 HCl

1

N N

2

Chart 1

Most of the trimetazidine derivatives listed in Table I were prepared by the methods shown in Chart 2. Thus, acetophenone was subjected to the Mannich reaction with trimetazidine dihydrochloride and paraformaldehyde in refluxing ethanol, and the product, 1-(2-benzoylethyl)-4-(2,3,4-trimethoxybenzyl)piperazine (**3a**), was precipitated as its dihydrochloride from the cooled reaction mixture. As for the substituent on the phenyl ring, we applied Topliss's method,[5] which was a practical application of the Hansch theory,[6] to obtain the highly active compound, and synthesized a further four compounds, the 4-methyl,

4-methoxy, 4-chloro and 3,4-dichloro derivatives, **3b**—**e**. The resulting Mannich bases **3a**—**e** were reduced with sodium borohydride in methanol to give the γ-amino secondary alcohols **4a**—**e**. The alcohols were dehydrated to provide the cinnamylpiperazines **5a**—**e** by treatment with 50% phosphoric acid solution. Some cinnamylpiperazines (**5a**, **e**, **f**) were prepared by the reaction of trimetazidine with substituted cinnamyl chlorides[7] in the presence of triethylamine as shown in Chart 2.

MeO MeO MeO NH N 1 — X-C6H4-COCH3, HCHO → 3 (NaBH4/MeOH) → 4 (H+) → 5; 1 — X-C6H4-CH=CH-CH2Cl, Et3N → 5

Chart 2

From the biological test results obtained with the above trimetazidine derivatives in dogs, it was found that two of the γ-amino secondary alcohols, **4d**, **e**, induced a considerable increase in vertebral blood flow, indicative of cerebral vasodilating action. The benzoylethyl derivatives (**3**) and the cinnamyl derivatives (**5**) were less potent than cinnarizine and the effects of the substituents on the potency were less and unclear in these derivatives. These results suggest that the benzylic hydroxy group is a prerequisite for high cerebral vasodilating activity, and in a series of γ-amino secondary alcohols the activity depends positively on the lipophilicity (π) of the substituent. To clarify this point we synthesized some further γ-amino secondary alcohols **4f**—**i** by the method described above. The nitro derivative, **4f**, was practically insoluble in many solvents and could not be recrystallized or tested. The results for the other compounds supported the above hypothesis.

Thus the γ-amino tertiary alcohols **6a**—**n** (Table II) were synthesized by the methods shown in Chart 3 in order to obtain more lipophilic compounds.

One method was to convert the Mannich base **3c** into the γ-amino tertiary alcohol **6c** by means of the Grignard reaction. This method required a large excess (about 10-fold excess) of methylmagnesium iodide, otherwise a mixture of the γ-amino tertiary alcohol **6c** and the starting Mannich base **3c** was obtained. The naphthyl derivative, **6f**, was also prepared by this method.

As the other method, substituted ω-chloropropiophenones **7a**—**e**[8] were subjected to the Grignard reaction and the resulting tertiary alcohols **8a**—**l** were allowed to react with trimetazidine to give the γ-amino tertiary alcohols **6a**, **b**, **d**, **e**, **g**—**n**. In this method, equimolar Grignard reagent was sufficient.

Topliss's method was applied to introduce the alkyl group at the benzylic position, but

TABLE I. Trimetazidine Derivatives (**3**, **4** and **5**)

Compd. No.	X	Yield[a] (%)	mp (°C)	Recrystn. solvent	Formula	Analysis (%) Calcd (Found) C	H	N	Potency[b]
3a	H	40	201—203 (dec.)	MeOH	$C_{23}H_{30}N_2O_4 \cdot 2HCl$	58.60 (58.56	6.84 6.83	5.94 5.90)	0.49
3b	4-Me	32	207—211 (dec.)	MeOH	$C_{24}H_{32}N_2O_4 \cdot 2HCl$	59.38 (59.08	7.06 7.04	5.77 5.67)	0.37
3c	4-OMe	54	210—214 (dec.)	MeOH	$C_{24}H_{32}N_2O_5 \cdot 2HCl \cdot 0.25H_2O$	56.97 (56.88	6.87 6.76	5.54 5.57)	0.50
3d	4-Cl	38	214—216 (dec.)	MeOH	$C_{23}H_{29}ClN_2O_4 \cdot 2HCl$	54.61 (54.26	6.18 6.13	5.54 5.47)	0.46
3e	3,4-Cl_2	35	205—209 (dec.)	MeOH	$C_{23}H_{38}Cl_2N_2O_4 \cdot 2HCl$	51.13 (50.97	5.60 5.67	5.18 5.08)	0.48
4a	H	76	214—217	MeOH	$C_{23}H_{32}N_2O_4 \cdot 2HCl$	58.35 (58.16	7.24 7.39	5.92 5.87)	0.28
4b	4-Me	82	225—228 (dec.)	MeOH	$C_{24}H_{34}N_2O_4 \cdot 2HCl$	59.14 (58.90	7.44 7.48	5.75 5.76)	0.44
4c	4-OMe	53	227—229 (dec.)	MeOH	$C_{24}H_{34}N_2O_5 \cdot 2HCl$	57.26 (57.54	7.21 7.27	5.56 5.39)	N.T.
4d	4-Cl	56	204—206 (dec.)	MeOH	$C_{23}H_{31}ClN_2O_4 \cdot 2HCl$	54.40 (54.16	6.55 6.46	5.52 5.42)	0.61
4e	3,4-Cl_2	81	228—232 (dec.)	MeOH	$C_{23}H_{30}Cl_2N_2O_4 \cdot 2HCl$	50.94 (50.88	5.95 5.96	5.16 5.24)	0.79
4f	4-NO_2	89	250—254 (dec.)	—	$C_{23}H_{31}N_3O_6 \cdot 2HCl$	53.29 (53.55	6.42 6.58	8.11 8.18)	N.T.
4g	4-NHAc	62	200—203 (dec.)	MeOH–EtOH	$C_{25}H_{35}N_3O_5 \cdot 2HCl$	56.60 (56.46	7.03 7.14	7.92 7.91)	Inact.
4h	3,4-Me_2	28	228—230 (dec.)	EtOH	$C_{25}H_{36}N_2O_4 \cdot 2HCl$	59.88 (59.72	7.64 7.54	5.59 5.62)	0.63
4i	3,4-$(CH)_4$	43	223—227 (dec.)	MeOH–EtOH	$C_{27}H_{34}N_2O_4 \cdot 2HCl$	61.95 (61.81	6.93 7.14	5.35 5.38)	0.89
5a	H[c]	26[d]	205—208	EtOH	$C_{23}H_{30}N_2O_3 \cdot 2HCl$	60.66 (60.47	7.08 7.16	6.15 6.19)	0.44
5b	4-Me	16	235—238 (dec.)	CH_3CN	$C_{24}H_{32}N_2O_3 \cdot 2HCl \cdot 0.25H_2O$	60.82 (60.88	7.34 7.41	5.91 6.11)	0.17
5c	4-OMe	14	229—232 (dec.)	EtOH	$C_{24}H_{32}N_2O_4 \cdot 2HCl$	59.38 (59.19	7.06 6.91	5.77 5.70)	N.T.
5d	4-Cl	13	249—253 (dec.)	EtOH	$C_{23}H_{29}ClN_2O_3 \cdot 2HCl \cdot 0.75H_2O$	54.88 (54.86	6.51 6.21	5.57 5.55)	N.T.
5e	3,4-Cl_2	10[d]	240—243 (dec.)	CH_3CN	$C_{23}H_{28}Cl_2N_2O_3 \cdot 2HCl$	52.69 (52.45	5.77 5.88	5.34 5.31)	0.43
5f	2,4-Cl_2	29[d]	177—179 (dec.)	CH_3CN	$C_{23}H_{28}Cl_2N_2O_3 \cdot 2(C_4H_4O_4)$[e]	54.47 (54.43	5.31 5.28	4.10 4.09)	0.45

a) Yields are based on the preceding isolated intermediates. *b*) The potency is expressed as the ratio of cerebral vasodilating activity to that of papaverine taken as 1. The potency of trimetazidine is 0.06 and that of cinnarizine is 0.71. N.T.=not tested. Inact.=inactive. *c*) See refs. 2*f* and 2*g*. *d*) See experimental section. *e*) Maleate.

the attempt failed, because *sec*-alkylmagnesium halides (such as isopropylmagnesium bromide) did not afford the corresponding tertiary alcohols. The γ-chloro tertiary alcohols (**8**) were purified by column chromatography and yields and nuclear magnetic resonance (NMR) data are given in Table III.

Most of the γ-amino tertiary alcohols (**6**) were stable, but **6c** was dehydrated in the salt-forming step with hydrochloric acid to give **9**.

Table II. γ-Amino Tertiary Alcohols (**6**)

Compd. No.	R	X	Yield[a] (%)	mp (°C)	Recrystn. solvent	Formula	Analysis (%) Calcd (Found)			Potency[b]
							C	H	N	
6a	CH_3	H	20	214—215 (dec.)	CH_3CN–EtOH	$C_{24}H_{34}N_2O_4 \cdot 2HCl \cdot 0.25H_2O$	58.59 (58.48	7.48 7.53	5.69 5.71)	0.88
6b	CH_3	4-Me	19	165—171 (dec.)	EtOH	$C_{25}H_{36}N_2O_4 \cdot 2(C_4H_4O_4)$[c]	59.99 (59.82	6.71 6.58	4.24 4.19)	0.92
6c	CH_3	4-OMe	38	169—172 (dec.)	EtOH	$C_{25}H_{36}N_2O_5 \cdot 2(C_4H_4O_4) \cdot 0.5H_2O$	57.80 (57.80	6.61 6.61	4.09 4.12)	0.69
6d	CH_3	4-Cl	31	168—169 (dec.)	MeOH–EtOH	$C_{24}H_{33}ClN_2O_4 \cdot 2(C_4H_4O_4)$	56.43 (56.63	6.07 6.37	4.11 4.14)	0.94
6e	CH_3	3,4-Cl_2	57	220—224 (dec.)	MeOH–EtOH	$C_{24}H_{32}Cl_2N_2O_4 \cdot 2HCl$	51.81 (51.53	6.16 6.34	5.04 5.01)	1.36
6f	CH_3	3,4-$(CH)_4$	14	203—205 (dec.)	iso-PrOH	$C_{28}H_{36}N_2O_4 \cdot 2HCl \cdot H_2O$	60.53 (60.66	7.26 7.05	5.04 5.16)	1.17
6g	CH_3	2,4-Cl_2	14	231—233 (dec.)	EtOH	$C_{24}H_{32}Cl_2N_2O_4 \cdot 2HCl$	51.81 (51.71	6.16 6.20	5.04 5.04)	1.46
6h	C_2H_5	3,4-Cl_2	44	229—230 (dec.)	MeOH	$C_{25}H_{34}Cl_2N_2O_4 \cdot 2HCl$	52.64 (52.39	6.36 6.37	4.90 4.91)	1.77
6i	C_2H_5	2,4-Cl_2	55	232—237 (dec.)	EtOH	$C_{25}H_{34}Cl_2N_2O_4 \cdot 2HCl$	52.64 (52.48	6.36 6.45	4.90 4.93)	1.64
6j	n-C_3H_7	3,4-Cl_2	49	224—226 (dec.)	EtOH–Et_2O	$C_{26}H_{36}Cl_2N_2O_4 \cdot 2HCl \cdot 0.5H_2O$	52.62 (52.78	6.62 6.60	4.72 4.86)	1.36
6k	n-C_4H_9	3,4-Cl_2	40	210—214 (dec.)	EtOH–Et_2O	$C_{27}H_{38}Cl_2N_2O_4 \cdot 2HCl \cdot H_2O$	52.61 (52.45	6.87 6.88	4.54 4.53)	0.81
6l	n-C_5H_{11}	3,4-Cl_2	11	212—215 (dec.)	EtOH–Et_2O	$C_{28}H_{40}Cl_2N_2O_4 \cdot 2HCl \cdot 1.5H_2O$	52.59 (52.66	7.09 6.88	4.38 4.52)	N.T.
6m	n-C_6H_{13}	3,4-Cl_2	16	205—208 (dec.)	EtOH–Et_2O	$C_{29}H_{42}Cl_2N_2O_4 \cdot 2HCl \cdot 1.5H_2O$	53.30 (53.41	7.24 7.25	4.29 4.33)	Inact.
6n	$CH_2C_6H_5$	3,4-Cl_2	29	223—226 (dec.)	MeOH	$C_{30}H_{36}Cl_2N_2O_4 \cdot 2HCl \cdot 0.5H_2O$	56.17 (56.07	6.13 6.03	4.37 4.26)	0.58

a, *b* and *c*) See footnotes *a*), *b*) and *c*), respectively, of Table I.

TABLE III. γ-Chloro Tertiary Alcohols **8**

Compd. No.	R	X	Yield (%)	^{1}H-NMR ($CDCl_3$)
8a[a]	CH_3	H	91	1.53 (3H, s), 2.00 (1H, s), 2.23 (2H, t, J=8 Hz), 3.00—3.80 (2H, m), 7.0—7.6 (5H, m)
8b	CH_3	4-CH_3	90	1.52 (3H, s), 2.00 (1H, s), 2.27 (3H, s), 2.18 (2H, t, J=8 Hz), 3.00—3.70 (2H, m), 6.80—7.40 (4H, m)
8c	CH_3	4-Cl	95	1.53 (3H, s), 2.20 (1H, s), 2.20 (2H, t, J=8 Hz), 3.00—3.70 (2H, m), 7.17 (4H, s)
8d	CH_3	3,4-Cl_2	93	1.55 (3H, s), 2.14 (1H, s), 2.23 (2H, t, J=8 Hz), 3.00—3.70 (2H, m), 7.00—7.60 (3H, m)
8e	C_2H_5	3,4-Cl_2	98	0.74 (3H, t, J=7 Hz), 1.82 (2H, q, J=7 Hz), 1.97 (1H, s), 2.23 (2H, t, J=7 Hz), 3.00—3.70 (2H, m), 6.80—7.60 (3H, m)
8f	n-C_3H_7	3,4-Cl_2	32	0.50—2.00 (7H, m), 2.00 (1H, s), 2.23 (2H, t, J=7 Hz), 2.90—3.70 (2H, m), 6.90—7.60 (3H, m)
8g	n-C_4H_9	3,4-Cl_2	32	0.50—2.00 (9H, m), 2.00 (1H, s), 2.23 (2H, t, J=7 Hz), 6.95—7.60 (3H, m)
8h	n-C_5H_{11}	3,4-Cl_2	48	0.50—2.00 (11H, m), 2.10 (1H, s), 2.20 (2H, t, J=7 Hz), 2.90—3.70 (2H, m), 6.90—7.50 (3H, m)
8i	n-C_6H_{13}	3,4-Cl_2	47	0.50—2.00 (13H, m), 2.00 (1H, s), 2.20 (2H, t, J=7 Hz), 2.90—3.70 (2H, m), 6.90—7.50 (3H, m)
8j	$CH_2C_6H_5$	3,4-Cl_2	93	2.10 (1H, s), 2.00—2.40 (2H, m), 2.80—3.70 (4H, m), 6.70—7.50 (8H, m)
8k	CH_3	2,4-Cl_2	99	1.68 (3H, s), 2.50 (1H, s), 2.00—3.70 (4H, m), 7.00—7.80 (3H, m)
8l	C_2H_5	2,4-Cl_2	36	0.70 (3H, t, J=7 Hz), 2.33 (1H, s), 1.10—3.60 (6H, m), 6.90—7.70 (3H, m)

a) J. W. Baker, *J. Chem. Soc.*, **1948**, 89.

3 —RMgX→ 6

7 —RMgX→ 8 —1/Et_3N→ 6

Chart 3

9 (·2HCl)

Chart 4

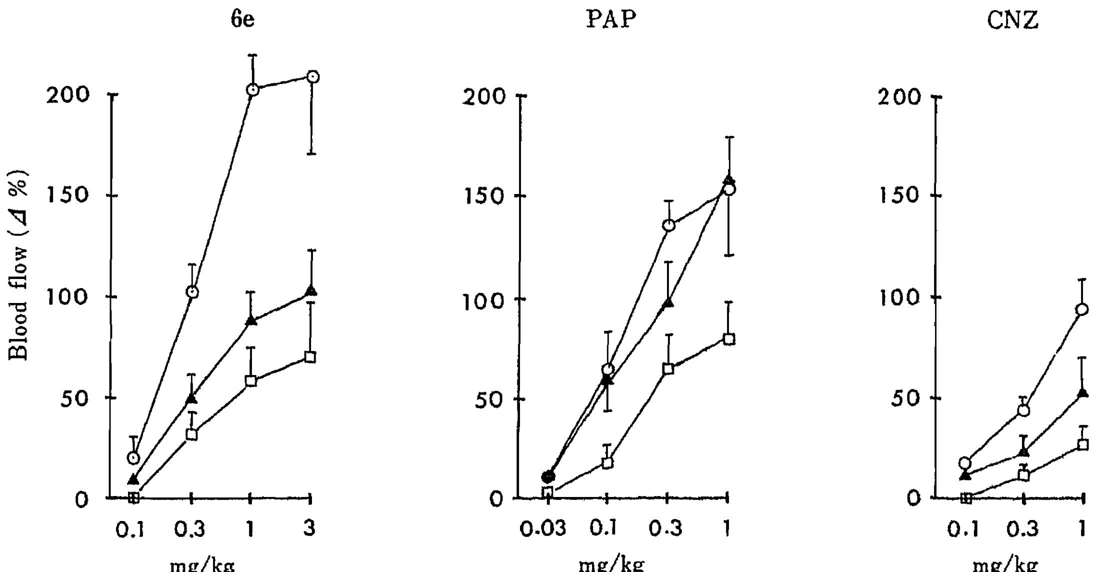

Fig. 1. Effect of Cerebral Vasodilators on Vertebral (○—○), Common Carotid (▲—▲) and Femoral (□—□) Blood Flows in Anesthetized Dog

Drugs were intravenously injected. CNZ, cinnarizine; PAP, papaverine. Means ± standard errors of 5 experiments.

Biological Testing

Most of the compounds **3a**—**6n** listed above were tested for cerebral vasodilating activity. The cerebral vasodilating activity was evaluated in dogs anesthetized with pentobarbital. The potency is expressed in terms of the ratio of the maximum change of blood flow in vertebral arteries after intravenous administration of the test compound (1 mg/kg) to that of papaverine (1 mg/kg). The results are summarized in Tables I and II. Almost all the compounds prepared possess stronger activity than trimetazidine. Potent cerebral vasodilating activity was observed in six compounds **6e**—**j**, which were superior in activity to cinnarizine and papaverine.

Moreover, in order to study the effects of these compounds on various arteries, **6e** was selected as a representative and its effect was compared with those of cinnarizine and papaverine. Figure 1 shows that **6e** exhibits a selective effect on vertebral arteries.

Experimental

Melting points were determined on a Yamato capillary melting point apparatus, model MP-21, and are uncorrected. ^{1}H-NMR spectra were determined on a Hitachi R-24A NMR spectrometer using 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. Trimetazidine dihydrochloride (**1**) was prepared according to the reported method.[9]

Preparation of the Mannich Bases 3a—e

1-(2-Benzoylethyl)-4-(2,3,4-trimethoxybenzyl)piperazine Dihydrochloride (3a)——A mixture of **1** (10 g), paraformaldehyde (4 g), acetophenone (7 g) and EtOH (100 ml) was refluxed for 6 h. After the mixture had cooled to room temperature, the precipitated solid was filtered off. Recrystallization of the solid from MeOH yielded 5.6 g (yield, 40%) of **3a** as colorless needles, mp 201—203 °C (dec.).

Compounds **3b**—**e** were obtained in the same manner as described for **3a**. The yields, melting points and elementary analytical data are given in Table I.

Preparation of the γ-Amino Secondary Alcohols 4a—i

1-[3-(3,4-Dichlorophenyl)-3-hydroxypropyl]-4-(2,3,4-trimethoxybenzyl)piperazine Dihydrochloride (4e)——A mixture of **3e** (11.6 g), 10% NaOH (50 ml) and AcOEt (100 ml) was stirred at room temperature. The organic layer was separated, washed with water and dried over Na_2SO_4. After removal of the solvent by evaporation,

the residue was suspended in MeOH (100 ml), and $NaBH_4$ (0.41 g) was added portionwise to the mixture. After being stirred for 30 min at room temperature, the mixture was poured into water and extracted with Et_2O (100 ml). The Et_2O layer was washed with water and dried over Na_2SO_4. HCl gas was bubbled into the solution and the precipitated solid was filtered off. Recrystallization of the solid from MeOH yielded 9.45 g (yield, 81%) of **4e** as colorless fine needles, mp 228—232 °C (dec.).

Compounds **4a**—**d** were obtained in the same manner as described for **4e**. The yields, melting points and elementary analytical data are given in Table I.

1-[3-Hydroxy-3-(2-naphthyl)propyl]-4-(2,3,4-trimethoxybenzyl)piperazine Dihydrochloride (4i)——A mixture of **1** (17 g), paraformaldehyde (6 g), 2-acetonaphthone (8.5 g) and CH_3NO_2 (200 ml) was refluxed for 4.5 h. After the mixture had cooled to room temperature, 10% NaOH (100 ml) was added and the organic layer was separated, washed with water and dried over Na_2SO_4. Removal of the solvent by evaporation gave crude 1-[2-(2-naphthoyl)ethyl]-4-(2,3,4-trimethoxybenzyl)piperazine. $NaBH_4$ (0.25 g) was added portionwise to the mixture of the Mannich base (3 g) and MeOH (30 ml). After being stirred for 2 h, the mixture was poured into water and extracted with AcOEt. The organic layer was washed with water and dried over $MgSO_4$. HCl–MeOH (5 ml) was added to the solution and the precipitated solid was filtered off. Recrystallization of the solid from MeOH–EtOH (1 : 1) yielded 1.34 g (yield, 43%) of **4i** as pale yellow fine prisms, mp 223—227 °C (dec.).

Compounds **4f**—**h** were obtained in the same manner as described for **4i**, though, **4f** could not be recrystallized because of its insolubility. The yields, melting points and elementary analytical data are given in Table I.

Preparation of the Cinnamylpiperazines 5a—f

1-(3,4-Dichlorocinnamyl)-4-(2,3,4-trimethoxybenzyl)piperazine Dihydrochloride (5e)——A mixture of 4e (4.0 g), phosphoric acid (15 ml) and water (15 ml) was stirred at 90 °C for 7 h. After the mixture had cooled to room temperature, the mixture was poured into water and the solution was made basic by the addition of 10% NaOH and extracted with Et_2O. The Et_2O layer was washed with water. The dried extract was concentrated and the residue was chromatographed on a silica gel column with Et_2O. After concentration of the eluate, HCl–EtOH was added and precipitated solid was filtered off. Recrystallization of the solid from CH_3CN yielded 0.39 g (yield, 10%) of **5e** as colorless fine needles, mp 240—243 °C (dec.).

Compounds **5a**—**d** were obtained in the same manner as described for **5e**. The yields, melting points and elementary analytical data are given in Table I.

1-(2,4-Dichlorocinnamyl)-4-(2,3,4-trimethoxybenzyl)piperazine Dihydrochloride (5f)——A mixture of **1** (3.39 g), 2,4-dichlorocinnamyl chloride[7] (2.22 g), benzene (60 ml) and triethylamine (4 ml) was refluxed for 3 h. After the mixture had cooled to room temperature, water (50 ml) was added and the organic layer was separated, washed with water, dried over Na_2SO_4 and concentrated. The residue was diluted with MeOH (10 ml) and a MeOH solution of maleic acid (2.5 g) was added. The precipitated solid was filtered off and recrystallized from CH_3CN to yield 2 g (yield, 29%) of **5f**, mp 177—179 °C (dec.).

Compounds **5a** and **5e** were prepared in the same manner as described for **5f**. The yields were 30% and 29%, respectively.

Preparation of the γ-Amino Tertiary Alcohols 6a—n

1-[3-Hydroxy-3-(4-methoxyphenyl)butyl]-4-(2,3,4-trimethoxybenzyl)piperazine Dimaleate (6c)——A small portion of a solution of methyl iodide (33 g) in dry Et_2O (100 ml) was added dropwise to magnesium turnings (5.7 g) under a nitrogen atmosphere. After the spontaneous reaction had begun, residual methyl iodide solution was added at a rate sufficient to maintain gentle reflux. When the addition was complete, the mixture was stirred for 1 h at room temperature then cooled to ice-bath temperature. The free base of **3c** (10 g) in dry benzene (50 ml) was added dropwise, and the reaction mixture was stirred for 1 h. After the usual work-up, the product was chromatographed on silica gel with $CHCl_3$–MeOH (20 : 1). Concentration of the eluate gave 7.2 g of the free base of **6c** (yield, 70%) as a colorless oil.

HCl–MeOH (25 ml) was added to this oil (6 g) in EtOH (25 ml) and the precipitated solid was filtered off. The TLC and NMR analysis revealed that this solid was a mixture of **6c** and **9**. It was refluxed for 1 h in HCl–MeOH, then the solid was filtered off and recrystallized from water to yield 1.6 g (total yield, 24%) of **9**, mp 222—224 °C (dec.). NMR (D_2O–DMSO-d_6-CD_3OD): 2.0 (3H, s, allylic methyl). *Anal.* Calcd for $C_{25}H_{34}N_2O_4 \cdot 2HCl \cdot 0.5H_2O$: C, 59.05; H, 7.33. N, 5.51. Found: C, 58.90; H, 7.44; N, 5.54.

Maleic acid (0.62 g) in EtOH (10 ml) was added to a solution of the oil obtained by the Grignard reaction described above (1.2 g) in EtOH (10 ml) and the precipitated solid was filtered off. The solid was recrystallized from EtOH to give 1.0 g (total yield, 38%) of **6c**, mp 169—172 °C (dec.).

1-[3-(2,4-Dichlorophenyl)-3-hydroxypentyl]-4-(2,3,4-trimethoxybenzyl)piperazine Dihydrochloride (6i)——A small portion of a solution of ethyl iodide (24 g) in dry Et_2O (100 ml) was added dropwise to magnesium turnings (2.6 g) under a nitrogen atmosphere. After the spontaneous reaction had begun, residual ethyl iodide solution was added at a rate sufficient to maintain gentle reflux. When the addition was complete, the mixture was stirred for 1 h at room temperature and then cooled to ice-bath temperature. Then **7e**[8c] (24.5 g) in dry benzene (100 ml) was added dropwise and the reaction mixture was stirred for 1 h. After the usual work-up, the product was chromatographed on silica gel with *n*-hexane–AcOEt (10 : 1). Concentration of the eluate gave 9.6 g of **8l** (yield, 36%) as a pale brown oil. A

mixture of **8l** (9.5 g), **1** (11 g), triethylamine (11 g) and xylene (150 ml) was refluxed overnight. The cooled mixture was washed with water and dried over $MgSO_4$. After removal of the solvent by evaporation, conc. HCl (6 ml) and EtOH (60 ml) were added to the residue. The precipitated solid was filtered off and recrystallization from EtOH yielded 10 g (yield, 55%) of **6i** as colorless crystals, mp 232—237 °C (dec.).

Compounds **6a, b, d, e, g—n** were obtained in the same manner as described for **6i**. The yields, melting points and elementary analytical data are given in Table II.

Biological Testing Method——The cerebral blood flow-increasing activity was measured by using the amount of vertebral blood flow as an index.[10] 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.

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Benzylpiperazine Derivatives. II.[1] Syntheses and Cerebral Vasodilating Activities of 1-[(3-Alkyl-3-hydroxy-3-phenyl)propyl]-4-benzylpiperazine Derivatives

HIROSHI OHTAKA,* YOSHIAKI FUJIMOTO, KENJI YOSHIDA, TOSHIRO KANAZAWA, KEIZO ITO and GORO TSUKAMOTO

Pharmaceuticals Research Center, Kanebo Ltd., 5–90, Tomobuchi-cho 1-chome, Miyakojima-ku, Osaka 534, Japan

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Analogs of 1-[(3-alkyl-3-hydroxy-3-phenyl)propyl]-4-(2,3,4-trimethoxybenzyl)piperazine (**1**) were prepared and tested for cerebral vasodilating activity. It was found that the potency depends positively on the number of methoxyl groups on the benzyl moiety, and the homopiperazine analogs seem to be equipotent to the corresponding piperazines.

From the standpoints of potency and ease of synthesis, **8k** was selected for further study. Further analogs, which have various substituents in place of the benzyl moiety of **8k**, were prepared and tested for cerebral vasodilating activity. These analogs were less potent than **8k**. It was suggested that the benzyl moiety of **8k** plays an important role in the high cerebral vasodilating activity.

Keywords——piperazine; homopiperazine; benzylpiperazine; acylation; alkylation; Grignard reaction; cerebral vasodilator

A previous report from our laboratory described the syntheses and biological properties of 1-[(3-alkyl-3-hydroxy-3-phenyl)propyl]-4-(2,3,4-trimethoxybenzyl)piperazine derivatives (1) as a novel class of compounds with interesting cerebral vasodilating activity.[1] Encouraged by these results, we undertook further studies to prepare other analogs of **1**.

MeO MeO MeO N N R HO X

2 HCl

1: R = Me or Et
X = 2,4- or 3,4-Cl_2

Chart 1

2

3

4

Chart 2

Results and Discussion

Analogs of 1

Several compounds, which have various substitution patterns of methoxyl groups on the phenyl moiety, are used clinically as vasodilators; these include dilazep (**2**), verapamil (**3**) and buflomedil (**4**). Therefore, the number and the locations of methoxyl groups of **1** were varied in order to clarify the role of the substituents. In addition, the piperazine moiety of **1** was changed to homopiperazine.

The compounds were prepared by methods A and B as shown in Chart 3. Thus, in method A, 3-alkyl-3-(3,4-dichlorophenyl)-3-hydroxypropyl chloride (**7**),[1] was allowed to react with 1-(substituted benzyl)piperazine (**5**) to give 1-[3-alkyl-3-(3,4-dichlorophenyl)-3-hydroxypropyl]-4-(substituted benzyl)piperazines (**8**) (Table I).

Chart 3

From the data in Table I, it is likely that the greater the number of methoxyl group in the benzyl moiety, the stronger the cerebral vasodilating activity. A similar situation was reported for the negative inotropic action of verapamil derivatives.[2]

Since the 3,4,5-trimethoxyphenyl moiety is often present in pharmaceuticals,[3] the effect of an alkyl group (R) at the benzylic position on the potency was examined in the 1-(3,4,5-trimethoxybenzyl)piperazine series in order to compare the results with those in the 1-(2,3,4-trimethoxybenzyl)piperazine series reported previously.

The compounds were prepared by method B as shown in Chart 3. 1-(3,4,5-Trimethoxybenzyl)piperazine (**5h**) was condensed with ω,3,4-trichloropropiophenone (**6a**)[4] to give benzoylethylpiperazine (**9a**), and **9b** was obtained in a similar manner. In these cases the yields were nearly quantitative. In our previous study, benzoylethylpiperazines similar to **9** were prepared by the Mannich reaction of 1-(substituted benzyl)piperazine, acetophenone and paraformaldehyde, but the yields were poor compared to that of the present method. As **9** is the key intermediate in this method, the present method was preferred. Next, **9** was subjected to the Grignard reaction. In this step about a 10-fold excess of Grignard reagent was used, as before (Table II).

The ethyl residue showed the most potent activity (Table II), and the effects of the alkyl group on the potency in the 1-(3,4,5-trimethoxybenzyl)piperazine series seem to be the same

TABLE I. 1-Benzyl-4-(3-hydroxy-3-phenyl)propylpiperazine Salts (**8**) Prepared by Method A

Compd. No.	$(OMe)_n$	R	Yield (%)	mp (°C)	Recrystn. solvent	Formula	Analysis (%) Calcd (Found) C	H	N	Potency[a]
8a	2-OMe	Me	16	232—235	MeOH	$C_{22}H_{28}Cl_2N_2O_2 \cdot 2HCl$	53.24	6.09	5.64	0.48
				(dec.)			(53.37	6.05	5.63)	
8b	3-OMe	Me	24	220—222	MeOH	$C_{22}H_{28}Cl_2N_2O_2 \cdot 2HCl$	53.24	6.09	5.64	N.T.
				(dec.)			(53.24	6.12	5.75)	
8c	4-OMe	Me	26	246—247	EtOH	$C_{22}H_{28}Cl_2N_2O_2 \cdot 2HCl$	53.24	6.09	5.64	0.56
				(dec.)			(53.05	6.18	5.62)	
8d	2,3-$(OMe)_2$	Me	15	224—226	MeOH	$C_{23}H_{30}Cl_2N_2O_3 \cdot 2HCl \cdot H_2O$	50.75	6.30	5.15	0.68
				(dec.)			(50.77	6.24	5.08)	
8e	2,4-$(OMe)_2$	Me	32	222—224	EtOH	$C_{23}H_{30}Cl_2N_2O_3 \cdot 2HCl$	52.49	6.13	5.32	1.33
				(dec.)			(52.22	6.13	5.31)	
8f	3,4-$(OMe)_2$	Me	26	190—191	MeOH-H_2O	$C_{23}H_{30}Cl_2N_2O_3 \cdot 2MA$[b]	54.31	5.59	4.09	0.94
				(dec.)			(54.18	5.57	4.04)	
8g	3,5-$(OMe)_2$	Me	17	222—223	EtOH	$C_{23}H_{30}Cl_2N_2O_3 \cdot 2HCl$	52.49	6.13	5.32	0.40
				(dec.)			(52.47	6.16	5.33)	
8h	3,4,5-$(OMe)_3$	Me	21	189—191	MeOH-H_2O	$C_{24}H_{32}Cl_2N_2O_4 \cdot 2MA$	53.71	5.63	3.91	1.43
				(dec.)			(53.61	5.55	3.86)	
8i	2,4,6-$(OMe)_3$	Me	7	210—211	EtOH	$C_{24}H_{32}Cl_2N_2O_4 \cdot 2HCl \cdot H_2O$	50.19	6.32	4.88	1.24
				(dec.)			(50.16	6.30	4.84)	
8j[c]	3,4,5-$(OMe)_3$	Me	21	223—225	EtOH	$C_{24}H_{32}Cl_2N_2O_4 \cdot 2HCl \cdot 0.5H_2O$	50.99	6.24	4.96	1.40
				(dec.)			(50.99	6.43	5.00)	
8k	3,4,5-$(OMe)_3$	Et	24	184—186	EtOH	$C_{25}H_{34}Cl_2N_2O_4 \cdot 2MA$	54.33	5.80	3.84	1.46
							(54.09	5.71	4.01)	
8l	3,5-$(OMe)_2$	Et	18	233—234	EtOH	$C_{24}H_{32}Cl_2N_2O_3 \cdot 2HCl$	53.35	6.34	5.18	1.36
				(dec.)			(53.36	6.39	5.08)	
8m	4-OMe	Et	18	255—256	EtOH	$C_{23}H_{30}Cl_2N_2O_2 \cdot 2HCl$	54.13	6.32	5.49	0.83
				(dec.)			(54.25	6.47	5.44)	

a) The potency is expressed as the ratio of cerebral vasodilating activity to that of papaverine taken as 1. N.T. = not tested. *b*) Maleate. *c*) 2,4-Dichloro derivative of **8h**.

TABLE II. 1-Benzyl-4-(3-hydroxy-3-phenyl)propylpiperazine Salts (8) Prepared by Method B

Compd. No.	R	X	Yield (%)	mp (°C)	Recrystn. solvent	Formula	Analysis (%) Calcd (Found)			Potency[a)]
							C	H	N	
8n	C_2H_5	2,4-Cl_2	21	236—240 (dec.)	EtOH	$C_{25}H_{34}Cl_2N_2O_4\cdot 2HCl\cdot 0.5H_2O$	51.83 (52.10	6.44 6.63	4.84 4.88)	1.34
8o	n-C_3H_7	3,4-Cl_2	17	178—181 (dec.)	EtOH	$C_{26}H_{36}Cl_2N_2O_4\cdot 2MA$[b)]	54.92 (54.71	5.96 5.91	3.77 3.76)	1.29
8p	n-C_3H_7	2,4-Cl_2	22	225—227 (dec.)	EtOH	$C_{26}H_{36}Cl_2N_2O_4\cdot 2HCl$	53.44 (53.19	6.55 6.62	4.79 4.77)	1.20
8q	n-C_4H_9	3,4-Cl_2	24	182—185 (dec.)	EtOH	$C_{27}H_{38}Cl_2N_2O_4\cdot 2MA$	55.48 (55.35	6.12 6.14	3.70 3.72)	0.36
8r	n-C_4H_9	2,4-Cl_2	29	221—222 (dec.)	iso-PrOH	$C_{27}H_{38}Cl_2N_2O_4\cdot 2HCl\cdot 0.25H_2O$	53.79 (53.79	6.77 6.81	4.65 4.64)	0.70
8s	n-C_5H_{11}	3,4-Cl_2	30	183—185 (dec.)	MeOH	$C_{28}H_{40}Cl_2N_2O_4\cdot 2MA$	56.03 (55.70	6.27 6.21	3.63 3.57)	Inact.
8t	n-C_5H_{11}	2,4-Cl_2	19	212—215 (dec.)	iso-PrOH	$C_{28}H_{40}Cl_2N_2O_4\cdot 2HCl\cdot H_2O$	53.34 (53.34	7.03 7.03	4.44 4.48)	Inact.
8u	n-C_6H_{13}	3,4-Cl_2	24	181—183 (dec.)	MeOH	$C_{29}H_{42}Cl_2N_2O_4\cdot 2MA$	56.56 (56.47	6.41 6.59	3.57 3.54)	Inact.
8v	n-C_6H_{13}	2,4-Cl_2	35	206—209 (dec.)	iso-PrOH	$C_{29}H_{42}Cl_2N_2O_4\cdot 2HCl\cdot H_2O$	54.04 (53.90	7.19 7.17	4.35 4.34)	Inact.

a, b) See footnotes *a*) and *b*), respectively, of Table I.

TABLE III. 1-Benzyl-4-(3-hydroxy-3-phenyl)propylhomopiperazine Salts (8) Prepared by Method A

Compd. No.	$(OMe)_n$	R	Yield (%)	mp (°C)	Recrystn. solvent	Formula	Analysis (%) Calcd (Found) C	H	N	Potency[a]
8w	3,4,5-$(OMe)_3$	H	42	157—160	EtOH	$C_{24}H_{32}Cl_2N_2O_4 \cdot$ 2MA[b]	53.71 (53.56	5.63 5.59	3.91 3.89)	0.80
8x	2,3,4-$(OMe)_3$	Me	10	201—203 (dec.)	MeOH	$C_{25}H_{34}Cl_2N_2O_4 \cdot$ 2OX[c]$\cdot 0.5H_2O$	50.74 (50.63	5.73 5.84	4.08 4.15)	1.36
8y	2,3,4-$(OMe)_3$	Et	7	179—182 (dec.)	iso-PrOH	$C_{26}H_{36}Cl_2N_2O_4 \cdot$ 2OX	52.10 (52.03	5.83 5.95	4.05 4.18)	1.78
8z	3,4,5-$(OMe)_3$	Et	27	168—170	MeOH	$C_{26}H_{36}Cl_2N_2O_4 \cdot$ $2MA \cdot H_2O$	53.62 (53.88	6.09 5.91	3.68 3.67)	1.69

a, b) See footnotes *a*) and *b*), respectively, of Table I. *c*) Oxalate.

as in the 1-(2,3,4-trimethoxybenzyl)piperazine series reported previously.

Homopiperazine analogs were prepared by method A (Table III). The homopiperazines seem to be equipotent to the corresponding piperazines, but they are expensive and difficult to purify. From the standpoints of potency and ease of synthesis, **8k** was selected for further study.

Modification of the Benzyl Moiety

As mentioned above, the highest activity is observed when R is an ethyl group and the activity seems to depend positively on the number of methoxyl groups on the benzyl moiety. However, the role of the trimethoxybenzyl moiety itself in the activity remained unclear. Therefore, we synthesized compounds with various substituents in place of the benzyl moiety of **8k**, and tested their cerebral vasodilating activities.

The compounds were prepared by the methods shown in Charts 4—6. In method C, 1-[3-(3,4-dichlorophenyl)-3-hydroxypentyl]piperazine (**10**) was used as the key intermediate and various derivatives were prepared by acylation or alkylation of this compound. Preparation of **10** was as follows; 3-(3,4-dichlorophenyl)-3-hydroxypentyl chloride (**7b**)[1] was allowed to react with piperazine hexahydrate (2 eq) to give **10** along with the 1,4-disubstituted piperazine **11**. Acylation or alkylation of **10** was done by usual procedures (Table IV).

Chart 4

10 → alkylation → 12

acylation → 13

method C

Chart 5

R—N NH → 7b/Et_3N → 12

($R=CH_2CH_2OH$) acylation → 14

method D

Chart 6

In method D, monosubstituted piperazines were allowed to react with **7b** to give 1,4-disubstituted piperazines **12c**—**i**. Monosubstituted piperazines were commercially available except for 1-(3,4,5-trimethoxyphenyl)piperazine and 1-[α-(2-pyridyl)benzyl]piperazine. The former was prepared according to the literature[5] and the latter was prepared by the reaction of α-(2-pyridyl)benzyl chloride[6] with piperazine. The hydroxyethyl derivative **12i** was further acylated to give **14a, b** (Table V).

From the results listed in Tables IV and V, the following structure–activity relationships can be deduced. In general, as a substituent in place of the benzyl moiety of **8k**, sterically small substituents (**10**,**12d**) appear to favor high activity and compounds containing bulky derivatives (**11**, **12c**, **12g** and **14b**) are inactive. However, bulky 3,4,5-trimethoxyphenylalkyl derivatives, especially **8k**, show exceptionally high activity. Acyl derivatives are almost inactive. It seems clear that the trimethoxybenzyl moiety of **8k** plays an important role in the cerebral vasodilating activity.

From the beginning of this series of studies, we have worked on the assumption that the 3-alkyl-3-hydroxy-3-phenylpropyl moiety of **1** plays a similar role in cerebral vasodilating activity to the cinnamyl group of cinnarizine, and the former results in higher activity. However, the results for **12c** and **12g** indicate that a 3-alkyl-3-hydroxy-3-phenylpropyl moiety can not directly replace the cinnamyl group without considerable or complete loss of activity; the two groups are not bioisosteric in our case. The mechanisms of cerebral vasodilating

TABLE IV. 1-Substituted 4-[3-(3,4-Dichlorophenyl)-3-hydroxypentyl]piperazines Prepared by Method C

Compd. No.	R	Yield (%)	mp (°C)	Recrystn. solvent	Formula	Analysis (%) Calcd (Found)			Potency[a]
						C	H	N	
10	H	44	227—229 (dec.)	MeOH	$C_{15}H_{22}Cl_2N_2O \cdot 2HCl$	46.17 (45.80	6.20 6.35	7.18 7.32)	1.03
11	A[b]	2	206—208	AcOEt	$C_{26}H_{34}Cl_4N_2O_2$	56.95 (57.17	6.25 6.33	5.11 5.24)	Inact.
12a	iso-Pr	46	249—251 (dec.)	MeOH–EtOH	$C_{18}H_{28}Cl_2N_2O \cdot 2HCl \cdot 0.5H_2O$	48.99 (49.05	7.08 6.92	6.35 6.36)	Inact.
12b	TMP–CH_2CH_2[c]	8	240—242 (dec.)	EtOH	$C_{26}H_{36}Cl_2N_2O_4 \cdot 2HCl$	53.44 (53.49	6.55 6.57	4.79 4.68)	1.00
13a	MeCO	30	182—186	MeCOMe	$C_{17}H_{24}Cl_2N_2O_2 \cdot HCl \cdot 0.25H_2O$	51.01 (50.96	6.42 6.37	7.00 6.85)	Inact.
13b	TMP–CO	45	159—162	EtOH	$C_{25}H_{32}Cl_2N_2O_5 \cdot HCl$	54.80 (54.51	6.07 5.95	5.11 5.08)	0.53
13c	TMP–CH_2CO	17	158—160	AcOEt	$C_{26}H_{34}Cl_2N_2O_5$	59.43 (59.64	6.52 6.57	5.33 5.27)	Inact.
13d	TMP–CH=CHCO	39	205—210 (dec.)	MeOH–AcOEt	$C_{27}H_{34}Cl_2N_2O_5 \cdot HCl \cdot 0.5H_2O$	55.63 (55.88	6.22 6.14	4.81 5.03)	Inact.

a) See footnote *a*) of Table I. *b*) A: 3-(3,4-dichlorophenyl)-3-hydroxypentyl. *c*) TMP: 3,4,5-trimethoxyphenyl.

TABLE V. 1-Substituted 4-[3-(3,4-Dichlorophenyl)-3-hydroxypentyl]piperazines Prepared by Method D

Compd. No.	R	Yield (%)	mp (°C)	Recrystn. solvent	Formula	Analysis (%) Calcd (Found)			Potency[a]
						C	H	N	
12c	$(Ph)_2CH$	34	209—212 (dec.)	MeOH–Et_2O	$C_{28}H_{32}Cl_2N_2O \cdot 2HCl$	60.44 (60.21	6.16 6.03	5.03 5.01)	Inact.
12d	Me	23	230—234 (dec.)	EtOH	$C_{16}H_{24}Cl_2N_2O$	47.54 (47.32	6.48 6.57	6.93 6.98)	0.82
12e	Ph	19	216—220 (dec.)	MeOH	$C_{21}H_{26}Cl_2N_2O \cdot 2HCl$	54.09 (54.24	6.05 6.09	6.01 6.02)	N.T.
12f	TMP[b]	8	178—179 (dec.)	EtOH	$C_{24}H_{32}Cl_2N_2O_4 \cdot HCl \cdot 1.5H_2O$	52.71 (52.83	6.63 6.57	5.12 5.08)	0.20
12g	$(Ph\text{-}4F)_2CH$[c]	18	150—151	EtOH	$C_{28}H_{30}Cl_2F_2N_2O$	64.74 (65.23	5.82 5.99	5.39 5.41)	Inact.
12h	Ph(2-Py)CH[d]	45	161—163 (dec.)	CH_3CN	$C_{27}H_{31}Cl_2N_3O \cdot 4OX$	49.77 (49.66	4.65 4.62	4.98 4.88)	0.62
12i	$HOCH_2CH_2$	14	230—233 (dec.)	MeOH	$C_{17}H_{26}Cl_2N_2O_2 \cdot 2HCl$	47.02 (47.12	6.50 6.44	6.45 6.69)	0.72
14a	$AcOCH_2CH_2$	16	169—171 (dec.)	EtOH	$C_{19}H_{28}Cl_2N_2O_3 \cdot 2MA$[f]	51.03 (51.07	5.71 5.77	4.41 4.35)	0.22
14b	TMP–$COOCH_2CH_2$	16	162—167	EtOH	$C_{27}H_{36}Cl_2N_2O_6 \cdot 2MA$	53.37 (53.30	5.63 5.49	3.56 3.47)	Inact.

a, b) See footnotes *a*) and *c*), respectively, of Table IV. *c*) Ph-4F: 4-fluorophenyl. *d*) 2-Py: 2-pyridyl. *e*) Oxalate. *f*) Maleate.

action of **1** and cinnarizine are likely to be different.

Experimental

Melting points were determined on a Yamato capillary melting point apparatus, model MP-21, and are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were determined on a Hitachi R-24A NMR spectrometer using tetramethylsilane (TMS) as an internal standard. Silica gel 60 F_{254} (Merck) thin layer chromatography plates were used for thin layer chromatography. For column chromatography, Silica gel 60 (Merck) and alumina N, Akt. I (Woelm) were used. An Aldrich Kugelrohr apparatus was used for bulb-to-bulb distillation.

General Method for Benzyl(homo)piperazines——a) By Leuckart-Wallach Reaction[7a]: 3,4,5-Trimethoxybenzaldehyde (49.1 g) and anhydrous piperazine (43 g) were heated in an oil bath at 90—100 °C. Formic acid (45 ml) was added dropwise to the molten mixture, and the whole was stirred for 5 h, then 20% NaOH aq. was added and the reaction mixture was refluxed for 2 h. After the mixture had cooled to room temperature, the product was extracted with benzene (200 ml x 2). The benzene layer was washed with sat. NaCl aq. and concentrated under reduced pressure. The residue was distilled by Kugelrohr apparatus to give a clear oil, which solidified on standing. HCl–MeOH was added to a methanol solution of the oil, and the precipitated solid was filtered off and recrystallized from EtOH. **5h**: mp 210—211 °C (dec.).

Compounds **5a**—**i** were obtained in the same manner as described for **5h**. The yields, melting points and elementary analytical data are given in Table VI. The homopiperazine analogs **5j**, **k** failed to crystallize, so they were used in the next step without further purification.

b) By the Method of Craig and Young[7f]: 3,4,5-Trimethoxybenzyl chloride[8] (43.4 g) in EtOH (400 ml) was added

TABLE VI. Substituted Benzyl(homo)piperazine Dihydrochlorides 5

(MeO)$_n$-C$_6$H$_4$-CH$_2$-N(ring)NH, (CH$_2$)$_m$

Compd. No.	(OMe)$_n$	m	Yield (%)	mp (°C)	Recrystn. solvent	Formula	Analysis (%) Calcd (Found) C	H	N
5a	2-OMe[a]	2	35	207—209	EtOH	$C_{12}H_{18}N_2O\cdot$ 2HCl	51.62 (51.62	7.22 7.26	10.03 10.02)
5b	3-OMe[b]	2	35	216—218	EtOH	$C_{12}H_{18}N_2O\cdot$ 2HCl	51.62 (51.63	7.22 7.21	10.03 10.05)
5c	4-OMe[c]	2	27	253—255 (dec.)	EtOH	$C_{12}H_{18}N_2O\cdot$ $2HCl\cdot H_2O$	48.49 (48.33	7.46 7.48	9.42 9.64)
5d	2,3-(OMe)$_2$	2	32	215—218 (dec.)	EtOH	$C_{13}H_{20}N_2O_2\cdot$ 2HCl	50.49 (50.45	7.17 7.23	9.59 9.39)
5e	2,4-(OMe)$_2$	2	34	187—188 (dec.)	EtOH	$C_{13}H_{20}N_2O_2\cdot$ $2HCl\cdot 0.25H_2O$	49.76 (49.72	7.23 7.18	8.93 9.05)
5f	3,4-(OMe)$_2$[d]	2	34	228—229 (dec.)	MeOH–Et$_2$O	$C_{13}H_{20}N_2O_2\cdot$ $2HCl\cdot 0.5H_2O$	49.06 (48.79	7.29 7.31	8.80 8.95)
5g	3,5-(OMe)$_2$	2	35	220—221 (dec.)	EtOH–Et$_2$O	$C_{13}H_{20}N_2O_2\cdot$ $2HCl\cdot 0.25H_2O$	49.76 (49.70	7.23 7.19	8.93 8.90)
5h	3,4,5-(OMe)$_3$[e]	2	47[f]	210—211 (dec.)	EtOH	$C_{14}H_{22}N_2O_3\cdot$ 2HCl	49.56 (49.34	7.13 7.26	8.26 8.12)
5i	2,4,6-(OMe)$_3$	2	36	218—224 (dec.)	MeOH–Et$_2$O	$C_{14}H_{22}N_2O_3\cdot$ 2HCl	49.56 (49.61	7.13 7.14	8.26 8.24)
5j	2,3,4-(OMe)$_3$	3	50			[g]			
5k	3,4,5-(OMe)$_3$	3	43			[g]			

a) For free base and maleate, see ref. 7*b*). *b*) For free base and maleate, see ref. 7*c*). *c*) Lit.[7d] mp 261—263 °C. *d*) Lit.[7e] mp 222—226 °C. For free base, see J. R. Boissier, R. Ratouis and C. Dumont, *J. Med. Chem.*, **6**, 541 (1963). *e*) For free base, see H. G. Morren, Belg. Patent 560330 (1958) [*Chem. Abstr.*, **53**, 16169*c* (1959)]. *f*) See experimental section. *g*) Used in the next step without purification.

dropwise to a mixture of piperazine·$6H_2O$ (38.8 g) and piperazine·2HCl·H_2O (35.4 g) in EtOH (200 ml) at 60 °C. After being stirred for 2 h at 60 °C, the mixture was allowed to cool. The precipitated solid was filtered off and the solution was concentrated under reduced pressure. Then 1.5 N HCl (200 ml) and benzene (200 ml) were added to the residue, and the water layer was separated, made basic with 20% NaOH and extracted with benzene (200 ml × 2) by the salting-out technique. Concentration of the benzene layer gave free **5h** (38.5 g).

1-[3-(3,4-Dichlorophenyl)-3-hydroxypentyl]-4-(3,4,5-trimethoxybenzyl)piperazine Dimaleate (8k)——Method A: A mixture of **5h** (1.7 g), 3-(3,4-dichlorophenyl)-3-hydroxypentyl chloride (1.27 g), triethylamine (2.2 g) and xylene (30 ml) was refluxed for 9 h. The cooled mixture was washed with water and dried over $MgSO_4$. After removal of the solvent by evaporation, maleic acid (0.8 g) and MeOH (30 ml) were added to the residue. The precipitated solid was filtered off and recrystallized from MeOH–water. **8k**: 0.75 g (yield, 21%), mp 184—186 °C (dec.).

Compounds **8a—m** and **8w—z** were obtained in the same manner as described for **8k**. The yields, melting points and elementary analytical data are given in Tables I and III.

1-(3,4-Dichlorobenzoylethyl)-4-(3,4,5-trimethoxybenzyl)piperazine Dihydrochloride (9a)——A mixture of **5h** (3.0 g), ω,3,4-trichloropropiophenone (2.1 g), triethylamine (3.1 g) and xylene (50 ml) was refluxed for 9 h. The cooled mixture was washed with water and dried over $MgSO_4$. Removal of the solvent by evaporation gave free **9a** (3.5 g, yield, 85%) as a pale brown oil. The oil, which solidified on standing, was used in the next step without further purification. An analytical sample was prepared as follows; HCl–MeOH was added to an ethanol solution of the oil to give a precipitated solid, which was filtered off and recrystallized from MeOH. **9a**: mp 211—215 °C (dec.). *Anal.* Calcd for $C_{23}H_{28}Cl_2N_2O_4$·2HCl: C, 51.13; H, 5.60; N, 5.18. Found: C, 51.00; H, 5.55; N, 5.07.

9b was obtained in the same manner as described for **9a**. Crude free base of **9b** (15.4 g) was obtained from **5h** (10.6 g) as a pale brown oil and used in the next step without further purification. **9b**: mp 206—207 °C (dec.). *Anal.* Calcd for $C_{23}H_{28}Cl_2N_2O_4$·2HCl: C, 51.13; H, 5.60; N, 5.18. Found: C, 51.13; H, 5.58; N, 5.17.

1-[3-(2,4-Dichlorophenyl)-3-hydroxypentyl]-4-(3,4,5-trimethoxybenzyl)piperazine Dihydrochloride (8n)—— Method B: A small portion of a solution of ethyl iodide (68.9 g) in dry Et_2O (100 ml) was added dropwise to magnesium turnings (10.7 g) under a nitrogen atmosphere. After the spontaneous reaction had begun, residual ethyl iodide solution was added at a rate sufficient to maintain gentle reflux. When the addition was complete, the mixture was stirred for 1 h at room temperature then cooled in an ice-bath. The free base of **9b** (15.8 g) in dry benzene (200 ml) was added dropwise, and the reaction mixture was stirred for 2 h. After the usual work-up, the product was chromatographed on silica gel with AcOEt. Concentration of the eluate gave 10.1 g of the free base of **8n** as a brown oil. HCl–MeOH was added to a methanol solution of the oil and the resulting precipitate was filtered off and recrystallized from EtOH. **8n**: 7.8 g (yield, 40%), mp 236—240 °C (dec.).

Compounds **8o—v** were obtained in the same manner as described for **8n**. The yields, melting points and elementary analytical data are given in Table II.

1-[3-(3,4-Dichlorophenyl)-3-hydroxypentyl]piperazine (10) and 1,4-Bis[3-(3,4-dichlorophenyl)-3-hydroxypentyl]-piperazine (11)——Piperazine hexahydrate (77.7 g), **7b** (53.5 g) and xylene (500 ml) were refluxed for 5 h. The cooled mixture was washed with 10% NaOH then water and dried over $MgSO_4$. After removal of the solvent by evaporation, the oily residue was chromatographed on alumina. Concentration of the AcOEt eluate gave 1.7 g of **11**. An analytical sample was recrystallized from AcOEt. Elution with AcOEt–MeOH (2 : 1) gave 28 g of **10** as an oil. To obtain an analytical sample, the free base was converted to the dihydrochloride and recrystallized from MeOH. The yields, melting points and elementary analysis data are given in Table IV.

1-[3-(3,4-Dichlorophenyl)-3-hydroxypentyl]-4-(3,4,5-trimethoxyphenethyl)piperazine Dihydrochloride (12b)—— Method C: A mixture of 3,4,5-trimethoxyphenethyl chloride[9] (1.7 g), **10** (2.3 g), triethylamine (0.8 g) and xylene (50 ml) was refluxed for 30 h. The cooled mixture was washed with water and extracted with 3 N HCl. The aqueous layer was made basic with 1 N NaOH then extracted with benzene. The benzene layer was dried and concentrated. The residual brown oil was chromatographed on silica gel. Elution with $CHCl_3$–MeOH (20 : 1) gave the free base of **12b** (0.7 g), which was converted to the dihydrochloride and recrystallized from EtOH.

Compound **12a** was obtained in a similar manner. The yields, melting points and elementary analytical data are given in Table IV.

1-[3-(3,4-Dichlorophenyl)-3-hydroxypentyl]-4-(3,4,5-trimethoxycinnamoyl)piperazine Hydrochloride (13d)—— Method C: 3,4,5-Trimethoxycinnamic acid (1.5 g) and **10** (2.0 g) were dissolved in AcOEt (50 ml) and the mixture was cooled in an ice-bath. Then dicyclohexylcarbodiimide (DCC) (1.3 g) was added and the reaction mixture was kept in an ice box overnight. The precipitated solid was filtered off and 3 N HCl was added to the filtrate. Deposited crude **13d** was collected and recrystallized from AcOEt–MeOH.

Compounds **13a—c** were obtained in a similar manner. The yields, melting points and elementary analytical data are given in Table IV.

1-[3-(3,4-Dichlorophenyl)-3-hydroxypentyl]-4-[α-(2-pyridyl)benzyl]piperazine Tetraoxalate (12h)——A solution of piperazine (26.6 g) and α-(2-pyridyl)benzyl chloride[6] (6.3 g) in xylene (250 ml) was refluxed overnight. The mixture was washed with water and sat. NaCl, then dried over $MgSO_4$. Evaporation of the solvent gave crude 1-[α-(2-pyridyl)benzyl]piperazine (3.5 g). To obtain an analytical sample, the crude product was converted to the hydrochloride and recrystallized from EtOH. mp 245—247 °C (dec.). *Anal.* Calcd for $C_{16}H_{19}N_3$·HCl·$0.25H_2O$: C,

65.29; H, 6.94; N, 14.28. Found: C, 65.36; H, 7.00; N, 14.29.

A solution of the crude product obtained above (2.0 g), **7b** (1.9 g) and triethylamine (1.45 g) in xylene (100 ml) was refluxed overnight, then washed with water followed by sat. NaCl, and dried over $MgSO_4$. After removal of the solvent, the product was chromatographed on silica gel. Elution with AcOEt gave the free base of **12h** (1.2 g), which was converted to the tetraoxalate and recrystallized from CH_3CN.

1-[3-(3,4-Dichlorophenyl)-3-hydroxypentyl]-4-(2-hydroxyethyl)piperazine Dihydrochloride (12i)——Method D: A solution of triethylamine (1.6 g), 1-piperazineethanol (2 g) and **7b** (4.3 g) in xylene (40 ml) was refluxed for 4.5 h. The mixture was washed with water and dried. The solvent was evaporated off and the residue was diluted with EtOH (20 ml). Then HCl–MeOH was added and the precipitated solid was filtered off. Recrystallization from MeOH gave **12i** (0.94 g).

Compounds **12c**—**g** were obtained in a similar manner. The yields, melting points and elementary analytical data are given in Table V.

1-(2-Acetoxyethyl)-4-[3-(3,4-dichlorophenyl)-3-hydroxypentyl]piperazine Dimaleate (14a)——Triethylamine (1.7 g) and **12i** (2.1 g) were dissolved in CH_2Cl_2 and the mixture was cooled in an ice-bath. Acetyl bromide (0.9 g) in CH_2Cl_2 (10 ml) was added dropwise and the mixture was stirred for 1.5 h, washed with water, and dried over $MgSO_4$. The solvent was evaporated off and the residue was diluted with EtOH (10 ml). Maleic acid (1.21 g) in EtOH (25 ml) was added and the precipitated solid was collected. Recrystallization from EtOH gave **14a** (2.4 g).

Compound **14b** was obtained in a similar manner. The yields, melting points and elementary analytical data are given in Table V.

Biological Testing Method——The cerebral blood flow-increasing activity was measured by using the amount of vertebral blood flow as an index.[10] The potency of the test compounds was evaluated as described previously.[1]

References and Notes

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Benzylpiperazine Derivatives. III.[1] Quantitative Structure–Cerebral Vasodilating Activity Relationships of 1-Benzyl-4-(3-hydroxy-3-phenylpentyl)piperazine Derivatives

HIROSHI OHTAKA* and GORO TSUKAMOTO

Pharmaceuticals Research Center, Kanebo Ltd., 5–90, Tomobuchi-cho 1-chome, Miyakojima-ku, Osaka 534, Japan

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Quantitative structure–cerebral vasodilating activity relationships of 1-benzyl-4-(3-hydroxy-3-phenylpropyl)piperazines were examined. The analyses indicate that the potency depends on the number of methoxyl groups on the benzyl moiety, and the local lipophilicity around the asymmetric carbon atom is also of importance.

This work reveals that even initial screening data at a single dose can be analyzed quantitatively.

Keywords——quantitative structure–activity relationship analysis; regression analysis; indicator variable; benzylpiperazine; cerebral vasodilating activity

In previous studies we have synthesized 1-benzyl-4-(3-hydroxy-3-phenylpropyl)-piperazines (I) and examined their cerebral vasodilating activities.[1b,2] Among these compounds, 1-[3-(3,4-dichlorophenyl)-3-hydroxypentyl]-4-(3,4,5-trimethoxybenzyl)piperazine (**28**) was found to be more potent than cinnarizine or papaverine, and was selected for further study. Some chemical modifications of these derivatives revealed preliminary structure–activity relationships. The lipophilicity of the substituent X appears to be important with respect to the activity and some other factors are also expected to influence the activity. These results prompted us to attempt a quantitative structure–activity relationship (QSAR) 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 compounds reported previously[1b,2] were expressed in terms of the ratio of the maximum change of blood flow in vertebral arteries after intravenous administration of the test compound to that in the case of papaverine. For either the extrathermodynamic or the *de novo* method, the potency of compounds should be properly expressed on a molar basis. In this case, all compounds were administered at the same dose and the dose–response curves of the four most potent derivatives (**14**, **28**, **38** and **39**) had been determined (Fig. 1). The log(dose)–response plots of these analogs gave parallel lines (Fig. 1). 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 of the four compounds (= 0.8). This assumption may be expressed in equation form:

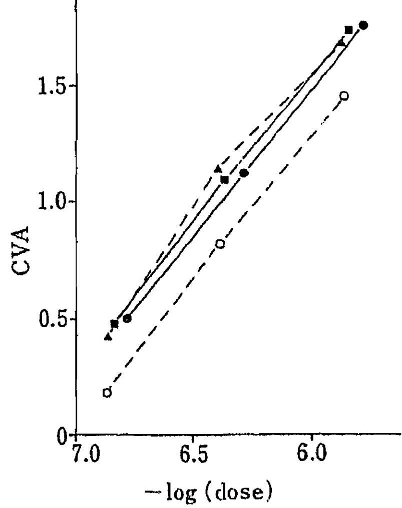

Fig. 1. log(dose)–Response Curves of Compounds **14** (—●—), **28** (---○---), **38** (—■--) and **39** (---▲--)

$$\log(\text{dose}) = a_x + b \text{ response}_x \quad (1)$$

in which x refers to the analog x, and b is the slope of the log(dose)–response curve (=0.8). 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 potency (log RP) was then estimated by using Eq. 2.[3]

$$\log \text{RP} = -\log(\text{dose}) + 0.8\text{CVA} \quad (2)$$

Regression Analysis——A regression analysis was carried out to investigate the relationships between the various parameters of the substituents (X, Y and R) in 1-benzyl-4-(3-hydroxy-3-phenylpropyl)piperazines (I) and the cerebral vasodilating activities. All the calculations were performed on a TRS-80 microcomputer system. The BASIC program for multiple regression analysis was written by us, based on a program given in the literature.[4]

Parameters——The classical physicochemical parameters for 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.[5] The indicator variables, I_1, I_2 and I_3 for the number of *ortho-*, *meta-* and *para-*methoxyl groups on the benzyl moiety, respectively, were also used.

Results and Discussion

In the first attempt, a series of 2,3,4-trimethoxybenzylpiperazine derivatives (**1**—**18**) were subjected to the analysis. In our previous paper,[2] we assumed that the cerebral vasodilating activity depends positively on the lipophilicity of the substituent X when R is H (**1**—**6**). This situation is reflected by Eq. 3.

$$\log \text{RP} = 0.392(\pm 0.128)\pi_X + 5.873 \quad (3)$$
$$n=6, \quad r=0.973, \quad s=0.051, \quad F=72.17$$

In Eq. 3 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. When R is Me (**7**—**13**), a similar equation was obtained.

$$\log \text{RP} = 0.310(\pm 0.119)\pi_X + 6.390 \quad (4)$$
$$n=7, \quad r=0.948, \quad s=0.069, \quad F=44.73$$

Addition of an electronic factor and/or steric factor to Eq. 3 or Eq. 4 did not give a significantly better result. Thus, it appears that the effect of the lipophilicity of the substituent X on the potency is predominantly important, as expected from the qualitative study. Next, all 2,3,4-trimethoxybenzylpiperazine derivatives (**1**—**18**) were considered, and Eq. 5 was

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obtained as the best equation.

$$\log RP = -0.654(\pm 0.100)\pi_R^2 + 1.328(\pm 0.201)\pi_R + 0.361(\pm 0.087)\pi_X + 5.884 \quad (5)$$

$$n=18, \quad r=0.979, \quad s=0.079, \quad F=105.70$$

TABLE I. Structural Features and Cerebral Vasodilating Activities of 1-Benzyl-4-(3-hydroxy-3-phenylpropyl)piperazines

Compd. No.	Y	R	X	I_1	I_2	I_3	I_4[a]	π_R[b]	π_X[c]	log RP[d]	
										Obs.	Calcd[e]
1	2,3,4-Tri-OMe	H	H	1	1	1	3	0.00	0.00	5.90	5.89
2	2,3,4-Tri-OMe	H	4-Me	1	1	1	3	0.00	0.56	6.04	6.09
3	2,3,4-Tri-OMe	H	4-Cl	1	1	1	3	0.00	0.71	6.19	6.14
4	2,3,4-Tri-OMe	H	3,4-Di-Cl	1	1	1	3	0.00	1.25	6.37	6.32
5	2,3,4-Tri-OMe	H	3,4-Di-Me	1	1	1	3	0.00	0.99	6.20	6.23
6	2,3,4-Tri-OMe	H	3,4-CH_4[f]	1	1	1	3	0.00	1.32	6.43	6.35
7	2,3,4-Tri-OMe	Me	H	1	1	1	3	0.50	0.00	6.40	6.40
8	2,3,4-Tri-OMe	Me	4-Me	1	1	1	3	0.50	0.56	6.56	6.60
9	2,3,4-Tri-OMe	Me	4-OMe	1	1	1	3	0.50	−0.02	6.39	6.40
10	2,3,4-Tri-OMe	Me	4-Cl	1	1	1	3	0.50	0.71	6.59	6.65
11	2,3,4-Tri-OMe	Me	3,4-Di-Cl	1	1	1	3	0.50	1.25	6.83	6.83
12	2,3,4-Tri-OMe	Me	3,4-CH_4	1	1	1	3	0.50	1.32	6.68	6.85
13	2,3,4-Tri-OMe	Me	2,4-Di-Cl	1	1	1	3	0.50	1.42	6.91	6.89
14	2,3,4-Tri-OMe	Et	3,4-Di-Cl	1	1	1	3	1.00	1.25	7.17	7.00
15	2,3,4-Tri-OMe	Et	2,4-Di-Cl	1	1	1	3	1.00	1.42	7.07	7.06
16	2,3,4-Tri-OMe	*n*-Pr	3,4-Di-Cl	1	1	1	3	1.50	1.25	6.86	6.82
17	2,3,4-Tri-OMe	*n*-Bu	3,4-Di-Cl	1	1	1	3	2.00	1.25	6.44	6.30
18	2,3,4-Tri-OMe	Bz[g]	3,4-Di-Cl	1	1	1	3	2.01	1.25	6.27	6.29
19	3,4,5-Tri-OMe	Me	3,4-Di-Cl	0	2	1	3	0.50	1.25	7.00	6.83
20	2,4,6-Tri-OMe	Me	3,4-Di-Cl	2	0	1	3	0.50	1.25	6.75	6.83
21	2,4-Di-OMe	Me	3,4-Di-Cl	1	0	1	2	0.50	1.25	6.79	6.54
22	3,4-Di-OMe	Me	3,4-Di-Cl	0	1	1	2	0.50	1.25	6.59	6.54
23	3,5-Di-OMe	Me	3,4-Di-Cl	0	2	0	2	0.50	1.25	6.04	6.34
24	2,3-Di-OMe	Me	3,4-Di-Cl	1	1	0	2	0.50	1.25	6.28	6.34
25	2-OMe	Me	3,4-Di-Cl	1	0	0	1	0.50	1.25	6.08	6.05
26	4-OMe	Me	3,4-Di-Cl	0	0	1	1	0.50	1.25	6.14	6.25
27	3,4,5-Tri-OMe	Me	2,4-Di-Cl	0	2	1	3	0.50	1.42	6.87	6.89
28	3,4,5-Tri-OMe	Et	3,4-Di-Cl	0	2	1	3	1.00	1.25	7.03	7.00
29	3,4,5-Tri-OMe	Et	2,4-Di-Cl	0	2	1	3	1.00	1.42	6.83	7.06
30	3,4,5-Tri-OMe	*n*-Pr	3,4-Di-Cl	0	2	1	3	1.50	1.25	6.90	6.82
31	3,4,5-Tri-OMe	*n*-Pr	2,4-Di-Cl	0	2	1	3	1.50	1.42	6.73	6.88
32	3,4,5-Tri-OMe	*n*-Bu	3,4-Di-Cl	0	2	1	3	2.00	1.25	6.17	6.30
33	3,4,5-Tri-OMe	*n*-Bu	2,4-Di-Cl	0	2	1	3	2.00	1.42	6.34	6.36
34	3,5-Di-OMe	Et	3,4-Di-Cl	0	2	0	2	1.00	1.25	6.82	6.50
35	4-OMe	Et	3,4-Di-Cl	0	0	1	1	1.00	1.25	6.37	6.42
36[h]	3,4,5-Tri-OMe	H	3,4-Di-Cl	0	2	1	3	0.00	1.25	6.50	6.32
37[h]	2,3,4-Tri-OMe	Me	3,4-Di-Cl	1	1	1	3	0.50	1.25	6.93	6.83
38[h]	2,3,4-Tri-OMe	Et	3,4-Di-Cl	1	1	1	3	1.00	1.25	7.26	7.00
39[h]	3,4,5-Tri-OMe	Et	3,4-Di-Cl	0	2	1	3	1.00	1.25	7.23	7.00

a) I_1, I_2, I_3 and I_4 stand for the number of *ortho*-, *meta*-, *para*- and total methoxyl groups, respectively. *b*) Calcd from ref. 7*b*. *c*) From ref. 7*a*. *d*) See the text. *e*) From Eq. 11. *f*) β-Naphthyl moiety. *g*) Benzyl residue. *h*) Homopiperazine derivatives.

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Equation 5 represents a highly significant correlation, the F-value being significant at the 99.5% level ($F3,14(0.005)=6.68$). In the case that R is H ($\pi_R=0$) or R is Me ($\pi_R=0.5$), Eq. 5 becomes essentially identical to Eq. 3 or Eq. 4, respectively. The optimum lipophilicity of the substituent R is calculated to be 1.01 from Eq. 5 which indicates that R = Et is the best. Many kinds of drug activity have been found to depend upon lipophilicity, which is one of the most fundamental characteristics of drug structure controlling biological activity. In the present case, Eq. 5 indicates that the local lipophilicity around the asymmetric carbon atom is of importance. This may be the case because intravenous administration of a drug does not involve an absorption process or first-pass effect, so that the interaction of a drug with the active site is the critical step. Total lipophilicity of the molecule and steric effects of the substituent (R) are less important, as shown in Fqs. 6—8.

$$\log RP = -0.227(\pm 0.123)(\pi_R+\pi_X)^2 + 0.976(\pm 0.436)(\pi_R+\pi_X)+5.753 \quad (6)$$
$$n=18,\ r=0.812,\ s=0.217,\ F=14.57$$

$$\log RP = -0.125(\pm 0.063)L_R^2+1.059(\pm 0.486)L_R + 0.304(\pm 0.206)\pi_X+4.297 \quad (7)$$
$$n=18,\ r=0.872,\ s=0.188,\ F=14.83$$

$$\log RP = -0.003(\pm 0.001)MR_R^2+0.096(\pm 0.035)MR_R + 0.331(\pm 0.179)\pi_X+5.886 \quad (8)$$
$$n=18,\ r=0.906,\ s=0.163,\ F=21.31$$

In Eq. 7 L is the Verloop's steric parameter.[6]

In the second stage of the analysis, nine compounds (**11**, **19**—**26**), which have different substitution patterns on the benzyl moiety, were considered. The analysis using classical parameter combinations was unsuccessful, but the utilization of indicator variables (I_1-I_3) gave a significant result.

$$\log RP = 0.301(\pm 0.276)I_1+0.272(\pm 0.239)I_2 + 0.641(\pm 0.329)I_3+5.661 \quad (9)$$
$$n=9,\ r=0.926,\ s=0.176,\ F=10.00$$

Equation 9 suggests that the potency depends essentially on the number of methoxyl groups, and the contribution of the *para*-methoxyl group is the largest. This situation is formulated in Eq. 10.

$$\log RP = 0.282(\pm 0.198)I_4+0.362(\pm 0.310)I_3 + 5.664 \quad (10)$$
$$n=9,\ r=0.924,\ s=0.162,\ F=17.59$$

In Eq. 10, I_4 stands for the number of methoxyl groups on the benzyl moiety and I_3 is the indicator variable for the presence (1) or absence (0) of *para*-methoxyl group.

Next, total 35 compounds were subjected to the analysis using parameters π_R, π_X, I_3 and I_4 to obtain Eq. 11.

$$\log RP = -0.686(\pm 0.129)\pi_R^2+1.362(\pm 0.274)\pi_R + 0.342(\pm 0.128)\pi_X+0.288(\pm 0.091)I_4 + 0.207(\pm 0.171)I_3+4.822 \quad (11)$$
$$n=35,\ r=0.934,\ s=0.132,\ F=39.70\ [F(5,29,0.005)=4.26]$$

This equation supports the above QSAR (Eqs. 3—5 and 10).

Equations 9, 10 and 11 indicate the additive nature of the effect of methoxyl groups on potency. The lipophilicity of 1,2,3-trimethoxybenzene is known to be extremely low compared

TABLE II. Correlation Matrix for Variables Used in Eq. 11

	π_R^2	π_R	π_X	I_3	I_4
π_R^2	1.000				
π_R	0.954	1.000			
π_X	0.311	0.388	1.000		
I_3	0.142	0.079	−0.135	1.000	
I_4	0.193	0.102	−0.191	0.540	1.000

to the calculated value based on the additivity.[5] Therefore the lipophilicity of the methoxyl groups on the benzyl moiety does not seem to play an important role. Moreover, a good agreement of the calculated potencies of homopiperazine derivatives using Eq. 11 with the observed ones[1b] seems to indicate that the difference of lipophilicity between piperazine and homopiperazine has little effect on the potency. These results also support the hypothesis that the total lipophilicity of the molecule does not play an important role. The physicochemical meaning of the indicator variables (I_1-I_4) is unclear. From Eq. 11, the most potent substitution pattern is suggested to be 2,3,4,5,6-pentamethoxy, though this is not practicable.

The potency depends positively on the lipophilicity of the substituent X, and therefore the introduction of a more lipophilic substituent or substituent combination for group X would make the compound more active.

This work indicates that even the initial screening data at a single dose can be analyzed quantitatively, and this QSAR analysis is helpful for the rational design of analogs to be synthesized.

References and Notes

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Studies on α_2-Plasmin Inhibitor Fragment T-11. I.[1] Synthesis of the Protected Hexadecapeptide Ester Corresponding to Positions 11 through 26 of α_2-Plasmin Inhibitor Fragment T-11

TAKASHI SASAKI* and SUMIRO ISODA

Research Institute, Daiichi Seiyaku Co., Ltd., 16-13, Kitakasai 1-chome, Edogawa-ku, Tokyo 134, Japan

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The protected hexadecapeptide ester corresponding to positions 11 through 26 of human α_2-plasmin inhibitor fragment T-11, which consists of 26 amino acids and binds to the plasmin(ogen) lysine-binding site(s), was synthesized by assembling five peptide fragments by the azide method or the dicyclohexylcarbodiimide-*N*-hydroxybenzotriazole method.

Keywords——α_2-plasmin inhibitor; α_2-plasmin inhibitor fragment synthesis; protected hexadecapeptide ester; peptide synthesis; fragment condensation

α_2-Plasmin inhibitor is physiologically the most important inhibitor of plasmin in blood plasma. This inhibitor is a single-chain glycoprotein with a molecular weight of 67000. It contains about 500 amino acid residues and 12% carbohydrates.[2] This protein inhibits plasmin very quickly, and its inhibitory reaction involves two steps: the first step is reversible binding to the lysine-binding site(s) of plasmin, and the second is irreversible binding to the active site of plasmin. The high reaction rate of this inhibition is due to the first step of interaction between α_2-plasmin inhibitor and plasmin.[3]

In the previous paper,[4] we reported that the human α_2-plasmin inhibitor fragment named T-11 showed high binding activity to the lysine binding site(s) of plasmin with a dissociation constant of 5.5 μM. This indicated that T-11 contains the binding site of α_2-plasmin inhibitor to the lysine-binding site(s) of plasmin. The peptide was shown to have 26 amino acid residues, and its amino acid sequence determined by the Edman method is illustrated in Fig. 1.[4]

```
 1             5                  10                 15
H-Gly-Asp-Lys-Leu-Phe-Gly-Pro-Asp-Leu-Lys-Leu-Val-Pro-Pro-Met-Glu-Glu-Asp-Tyr-
 20              25
Pro-Gln-Phe-Gly-Ser-Pro-Lys-OH
```

Fig. 1. Amino Acid Sequence of α_2-Plasmin Inhibitor Fragment, T-11

Only 300 μg of this peptide was obtained from 10 mg of human α_2-plasmin inhibitor.[4] It was necessary to obtain a larger amount of this fragment for further studies on the interaction between α_2-plasmin inhibitor and plasmin. Thus, we wished to obtain the peptide in quantity by chemical synthesis.

We have synthesized the hexacosapeptide corresponding to the entire amino acid sequence of T-11 and made a preliminary examination of the physiological properties of this synthetic peptide and some of its intermediates. In a series of studies described in this and the following papers, we intend to give a detailed account of our synthetic studies on the inhibitor

fragment, which may cast some light on the structure–activity relationship of this important plasma protein inhibitor fragment.

This paper describes the synthesis of the protected C-terminal hexadecapeptide (positions 11—26 in T-11) as a starting material for the synthesis of T-11. A conventional solution method was used for the synthesis of T-11 and its fragments in order to obtain sufficient amounts of these peptides and to ensure their purity. As α-amino protecting groups, the TFA-labile Boc group[5] was chosen for temporary protection, and the α-amino group of the Ser derivative was protected with the Z(OMe) group.[6] TFMSA–thioanisole–TFA was used as the final deprotecting reagent.[7] Amino acid derivatives with side-chain protecting groups, *i.e.*, Asp(OBzl), Glu(OBzl) and Lys(Z), easily removable by the above mixed reagent and capable of surviving under the restricted TFA treatment conditions were employed. In order to prevent partial oxidation of the Met residue during the various steps involved, the Met residue was protected as Met(O)[8] and deprotected with dithiothreitol at the final stage.[9]

The scheme employed in the synthesis of the hexadecapeptide ester is illustrated in Fig. 2. We used the azide method[10] and the DCC–HOBT method[11] as fragment condensing techniques. As building blocks for the construction of the C-terminal portion of T-11, five relatively small peptide fragments were synthesized, namely, Z(OMe)–Ser–Pro–Lys(Z)–OBzl (I-1) (positions 24—26), Boc–Gln–Phe–Gly–$NHNH_2$ (I-2) (positions 21—23), Boc–Asp(OBzl)–Tyr–Pro–OH (I-3) (positions 18—20), Boc–Met(O)–Glu(OBzl)–OH (I-4) (positions 15, 16), and Boc–Leu–Val–Pro–Pro–$NHNH_2$ (I-5) (positions 11—14).

The protected C-terminal tripeptide ester (I-1) was synthesized from H–Lys(Z)–OBzl[12] by stepwise chain elongation with Boc–Pro–OH and Z(OMe)–Ser–$NHNH_2$ by the DCC[13] and azide procedures, respectively. The reason for the usage of Z(OMe)–Ser–$NHNH_2$ instead of Boc–Ser–$NHNH_2$ was that the resulting tripeptide is easily crystallized, whereas the Boc derivative is not.

For the synthesis of (I-2), Boc–Phe–Gly–OMe was prepared by the DCC–HOBT procedure. The resulting dipeptide ester, after treatment with TFA, was condensed with Boc–Gln–OH by the *p*-nitrophenyl ester procedure.[14] Boc–Gln–Phe–Gly–OMe was then converted to the corresponding hydrazide.

The azide condensation of Boc–Tyr–$NHNH_2$ and H–Pro–OH afforded Boc–Tyr–Pro–OH, which was treated with TFA and condensed with Boc–Asp(OBzl)–OH by the *p*-nitrophenyl ester procedure in the presence of *N*-methylmorpholine to give the protected tripep-

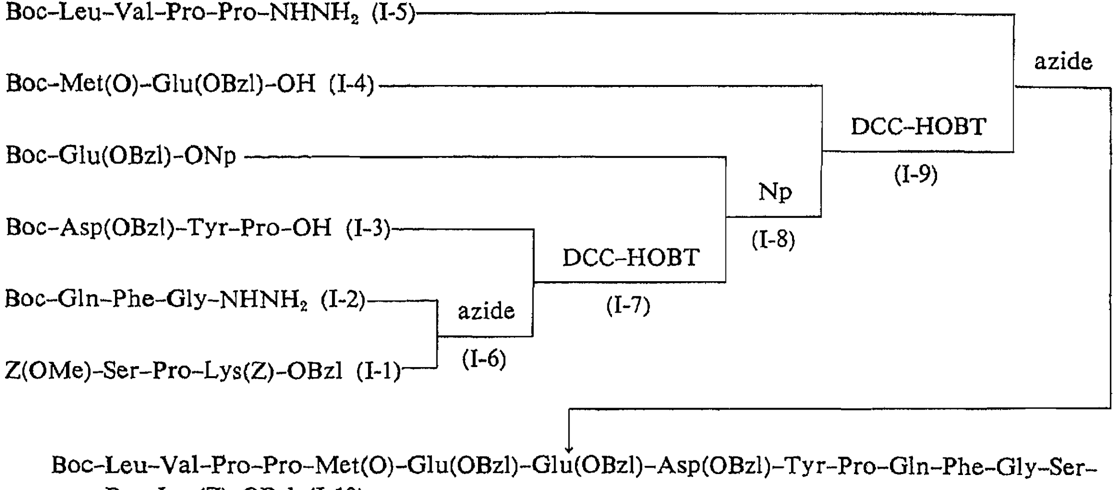

Fig. 2. Synthetic Route to the Protected Hexadecapeptide Ester (I-10) Corresponding to Positions 11 to 26 of T-11

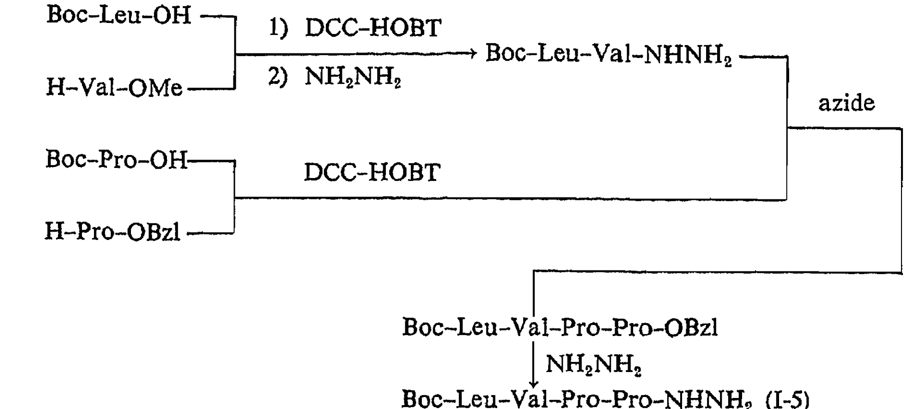

Fig. 3. Synthetic Scheme for the Protected Tetrapeptide Hydrazide (I-5)

TABLE I. Amino Acid Ratios in 6 N HCl Hydrolysates of the Intermediates of Synthetic T-11

	I-6	I-7	I-8	I-9	I-10
Asp		1.03 (1)	1.00 (1)	1.07 (1)	1.09 (1)
Ser	0.81 (1)	0.87 (1)	0.87 (1)	0.81 (1)	0.77 (1)
Glu	0.96 (1)	0.96 (1)	1.90 (2)	3.44 (3)	3.30 (3)
Pro	0.89 (1)	1.86 (2)	1.91 (2)	2.03 (2)	4.22 (4)
Gly	0.96 (1)	1.20 (1)	0.95 (1)	1.21 (1)	1.29 (1)
Val					1.24 (1)
Met+Met(O)				1.01 (1)	0.95 (1)
Leu					1.35 (1)
Tyr		0.86 (1)	0.97 (1)	0.77 (1)	0.71 (1)
Phe	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Lys	0.89 (1)	0.95 (1)	1.06 (1)	0.89 (1)	0.92 (1)
Rec. (%)	89	91	86	91	89

tide (I-3).

The oily compound Boc–Met–Glu(OBzl)–OH, which was prepared by condensation of Boc–Met–OH and H–Glu(OBzl)–OH by the trichlorophenyl ester procedure,[15] was oxidized with sodium periodate[8] to give the easily crystallized compound (I-4).

The protected tetrapeptide hydrazide (I-5) was synthesized as illustrated in Fig. 3. The azide procedure was used to condense the TFA–treated sample of Boc–Pro–Pro–OBzl (obtained by the DCC–HOBT technique) and Boc–Leu–Val–$NHNH_2$, which was prepared from Boc–Leu–Val–OMe[16] by hydrazine hydrate treatment. The resulting tetrapeptide ester, Boc–Leu–Val–Pro–Pro–OBzl, was converted to the corresponding hydrazide (I-5) in the usual manner.

As shown in Fig. 2, two fragments, Boc–Gln–Phe–Gly–$NHNH_2$ (I-2) and Boc–Leu–Val–Pro–Pro–$NHNH_2$ (I-5), were assembled by the azide procedure to avoid racemization.[17] The other two fragments, Boc–Met(O)–Glu(OBzl)–OH (I-4) and Boc–Asp(OBzl)–Tyr–Pro–OH (I-3), were condensed by the DCC–HOBT method to minimize racemization. Only one amino acid residue, Boc–Glu(OBzl)–OH (position 17 in T-11), was introduced by the *p*-nitrophenyl ester procedure. All of the protected peptide esters, (I-7) to (I-10) except for (I-6), obtained in the chain elongation steps were readily soluble in ethyl acetate. Therefore, purification of (I-7) to (I-10) was performed by washing of the ethyl acetate extract with diluted citric acid and sodium bicarbonate solution, followed by precipitation. The less soluble

compound (I-6) was washed batchwise.

The purity of these synthetic protected peptides was assessed by thin-layer chromatography, elemental analysis and amino acid analysis (Table I) of 6 N HCl hydrolysates.[18] The well characterized protected hexadecapeptide thus synthesized served as an amino component for the total synthesis of T-11, which will be described in the following paper.[19]

Experimental

All melting points are uncorrected. Thin-layer chromatography was performed on silica gel plates (Kiesel gel $60F_{254}$, Merck) and *Rf* values refer to the following solvent systems: Rf^1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf^2 $CHCl_3$–MeOH–AcOH (9:1:0.5). Optical rotations were determined with a JASCO DIP 140 digital polarimeter. Acid hydrolyses were performed in 6 N HCl at 110 °C in evacuated tubes for 24 h. The amino acid compositions of acid hydrolysates were determined with a Hitachi amino acid analyzer 835 and are not corrected for amino acid destruction.

Boc–Pro–Lys(Z)–OBzl——DCC (11.24 g) was added to a solution of Boc–Pro–OH (10.81 g) and H–Lys(Z)–OBzl (prepared from 27.13 g of the tosylate with 6.93 ml of Et_3N) in DMF (90 ml) and the mixture was stirred at room temperature overnight. After filtration, the filtrate was concentrated *in vacuo* and the residue was dissolved in ether. The ether phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, and dried over Na_2SO_4. Evaporation of the solvent gave an oily residue; yield 24.20 g (85%), Rf^1 0.87.

Z(OMe)–Ser–Pro–Lys(Z)–OBzl (I-1)——Boc–Pro–Lys(Z)–OBzl (8.45 g) was treated with TFA (15 ml) with ice-cooling for 45 min. The excess TFA was removed by evaporation *in vacuo* and the residue was washed twice with *n*-hexane by decantation. This oily residue was then dissolved in DMF (20 ml) and neutralized with Et_3N. The azide (prepared from 4.64 g of Z(OMe)–Ser–$NHNH_2$ with 12.71 ml of 3.87 N HCl–DMF, 3.31 ml of isoamyl nitrite and 8.89 ml of Et_3N) in DMF (15 ml) was added to the N^{α}-deprotected peptide solution and the mixture was stirred at 4 °C for 48 h. The solvent was removed by evaporation and the residue was dissolved in AcOEt. The AcOEt phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and then evaporated. The residue was triturated with IPE and a white powder was obtained after recrystallization from MeOH and ether; yield 5.78 g (54%), mp 78—80 °C, $[\alpha]_D^{20}$ −49.4° (*c* = 1.1, MeOH), Rf^1 0.58. *Anal.* Calcd for $C_{38}H_{46}N_4O_{10}\cdot 0.5H_2O$: C, 62.71; H, 6.51; N, 7.70. Found: C, 62.81; H, 6.36; N, 7.94.

Boc–Phe–Gly–OMe——DCC (6.13 g) and HOBT·H_2O (4.59 g) were combined with a solution of Boc–Phe–OH (7.96 g) in THF (50 ml) and H–Gly–OMe (prepared from 4.24 g of the hydrochloride with 4.57 ml of Et_3N) in $CHCl_3$ (50 ml). The solution was stirred at room temperature overnight. After filtration, the solvent was removed by evaporation and the residue was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, and dried over Na_2SO_4. After evaporation of the AcOEt, the residue was triturated with *n*-hexane. White crystals were obtained after recrystallization from ether and *n*-hexane; yield 9.54 g (95%), mp 69—71 °C, $[\alpha]_D^{24}$ −4.2° (*c* = 1.0, MeOH), Rf^1 0.86. *Anal.* Calcd for $C_{17}H_{24}N_2O_5$: C, 60.70; H, 7.19; N, 8.33. Found: C, 61.09; H, 7.13; N, 8.67.

Boc–Gln–Phe–Gly–OMe——Boc–Phe–Gly–OMe (6.12 g) was treated with TFA (15 ml) at 0 °C for 45 min. The excess TFA was removed by evaporation *in vacuo* and the residue was washed with *n*-hexane twice, dissolved with DMF (30 ml), and neutralized with Et_3N. Boc–Gln–ONp (6.69 g) and Et_3N (2.52 ml) were combined with the above solution and the mixture was stirred at room temperature overnight, then evaporated. The residue was triturated with ether and 5% citric acid. The resulting solid was washed batchwise with 5% citric acid, $NaHCO_3$ and H_2O, followed by recrystallization from MeOH–THF and IPE; yield 6.83 g (81%), mp 164—165 °C, $[\alpha]_D^{24}$ −30.4° (*c* = 1.0, MeOH), Rf^1 0.66. *Anal.* Calcd for $C_{22}H_{32}N_4O_7$: C, 56.88; H, 6.94; N, 12.06. Found: C, 56.66; H, 6.77; N, 12.14.

Boc–Gln–Phe–Gly–$NHNH_2$ (I-2)——Hydrazine hydrate (80%, 8.93 ml) was added to a solution of Boc–Gln–Phe–Gly–OMe (6.83 g) in MeOH–dioxane (15 ml–15 ml), and the mixture was left to stand overnight at room temperature. The resulting crystalline mass was collected by filtration and recrystallized from MeOH–dioxane and ether; yield 4.89 g (72%), mp 174—176 °C, $[\alpha]_D^{25}$ −39.0° (*c* = 1.0, DMF), Rf^1 0.41. *Anal.* Calcd for $C_{21}H_{32}N_6O_6\cdot 0.5H_2O$: C, 53.26; H, 7.02; N, 17.75. Found: C, 53.31; H, 6.83; N, 17.59.

Boc–Tyr–Pro–OH——A solution of proline (4.14 g) in H_2O–DMSO (5 ml–45 ml) containing Et_3N (9.15 ml) was added to the azide (prepared from 8.86 g of Boc–Tyr–$NHNH_2$ with 18.6 ml of 3.87 N HCl–DMF, 4.85 ml of isoamyl nitrite and 9.98 ml of Et_3N) in DMF (10 ml). The mixture was stirred at 4 °C for 48 h, then evaporated, and the residue was dissolved in 5% $NaHCO_3$. This solution was washed with AcOEt, then the aqueous phase was acidified with citric acid and the resulting precipitate was extracted with AcOEt. The AcOEt phase was washed with H_2O, dried over Na_2SO_4 and then evaporated. IPE was added to the residue and the resulting powder was recrystallized from MeOH and IPE; yield 7.63 g (67%), mp 114—116 °C, $[\alpha]_D^{24}$ −26.6° (*c* = 1.0, MeOH), Rf^1 0.40. *Anal.* Calcd for $C_{19}H_{26}N_2O_4\cdot 0.25H_2O$: C, 59.95; H, 7.24; N, 7.32. Found: C, 59.70; H, 7.07; N, 7.07.

Boc–Asp(OBzl)–Tyr–Pro–OH (I-3)——The protected dipeptide (6.50 g) mentioned above was treated with TFA (20 ml) in an ice-bath for 1 h, then dry IPE and *n*-hexane were added to the solution. The resulting powder was dried

over KOH pellets *in vacuo* for 1 h and then dissolved in DMF (50 ml) and neutralized with Et_3N. To this solution, Boc–Asp(OBzl)–ONp (7.64 g) and *N*-methylmorpholine (1.89 ml) were added and the mixture was stirred at room temperature overnight. The solvent was removed by evaporation under reduced pressure and the residue was dissolved in 5% Na_2CO_3. This solution was washed with AcOEt, then acidified with citric acid and the precipitate was extracted with AcOEt. The organic extract was washed with 5% citric acid and H_2O, dried over Na_2SO_4 and then evaporated. The residue was triturated with IPE and the resulting powder was recrystallized from MeOH–AcOEt and IPE; yield 8.56 g (85%), mp 93—96 °C, $[\alpha]_D^{24}$ −43.2° (c=1.0, MeOH), Rf^1 0.65. *Anal.* Calcd for $C_{30}H_{37}N_3O_9$: C, 61.74; H, 6.39; N, 7.20. Found: C, 61.48; H, 6.43; N, 6.98.

Boc–Met(O)–Glu(OBzl)–OH (I-4)——H–Glu(OBzl)–OH (5.69 g) in DMF–DMSO (20 ml–20 ml) and Et_3N (3.33 ml) were added to a solution of Boc–Met–OTcp (8.58 g) in THF (40 ml) containing *N*-methylmorpholine (2.20 ml), and the mixture was stirred at room temperature for 18 h. Evaporation of the solvent gave a residue which was dissolved in 5% Na_2CO_3. The aqueous phase was washed with AcOEt and acidified with citric acid, and the precipitate was extracted with AcOEt. The organic phase was washed with H_2O, dried over Na_2SO_4 and then evaporated. The resulting oily residue was dissolved in MeOH (75 ml) followed by the addition of sodium periodate (4.49 g) in H_2O (90 ml). The mixture was stirred at room temperature for 2 h, and the precipitated white mass was filtered off. The filtrate was evaporated and the residue was extracted with AcOEt. After being washed with H_2O and dried over Na_2SO_4, the extract was evaporated and IPE was added to the residue. The resulting white mass was recrystallized from AcOEt–MeOH and IPE; yield 8.15 g (84%), mp 93—95 °C, $[\alpha]_D^{24}$ −5.2° (c=1.0, MeOH), Rf^1 0.41. *Anal.* Calcd for $C_{22}H_{32}N_2O_8S$: C, 54.53; H, 6.66; N, 5.78. Found: C, 54.56; H, 6.58; N, 5.73.

Boc–Pro–Pro–OBzl——DCC (6.13 g) and HOBT·H_2O (1.0 g) were added to a solution of Boc–Pro–OH (6.49 g) in DMF (30 ml) and the mixture was stirred for 15 min. Then H–Pro–OBzl (prepared from 8.70 g of the hydrochloride with 4.99 ml of Et_3N) in $CHCl_3$ (25 ml) was added and the whole was stirred at room temperature for 18 h, and filtered. The filtrate was evaporated and the residue was extracted with AcOEt. After washing in the usual manner, the organic phase was dried over Na_2SO_4 and the oily residue was obtained by evaporation; yield 12.0 g (99%), Rf^1 0.81.

Boc–Leu–Val–OMe——DCC (3.57 g) and HOBT·H_2O (2.43 g) were added to a solution of Boc–Leu–OH·H_2O (4.10 g) in DMF (10 ml). The mixture was stirred for 15 min, then H–Val–OMe (prepared from 2.91 g of the hydrochloride with 2.42 ml of Et_3N) in $CHCl_3$ (10 ml) was added. The reaction mixture was stirred at room temperature for 18 h. Work-up as described for Boc–Phe–Gly–OMe afforded a white mass, which was recrystallized from AcOEt and *n*-hexane; yield 4.19 g (77%), mp 139—141 °C (Lit.[15] 143—144 °C), $[\alpha]_D^{24}$ −41.1° (c=1.1, MeOH) (Lit.[15] $[\alpha]_D^{25}$ −44.0° (c=2.4, MeOH), Rf^1 0.85. *Anal.* Calcd for $C_{17}H_{32}N_2O_5$: C, 59.28; H, 9.36; N, 8.13. Found: C, 59.09; H, 9.25; N, 8.16.

Boc–Leu–Val–NHNH$_2$——Hydrazine hydrate (80%, 7.39 ml) was added to a solution of Boc–Leu–Val–OMe (4.19 g) in MeOH (15 ml), and the mixture was stirred overnight. The solvent was removed by evaporation *in vacuo*, and the residue was triturated with H_2O. The resulting mass was reprecipitated from MeOH and H_2O; yield 3.07 g (74%), mp 143—145 °C, $[\alpha]_D^{24}$ −52.4° (c=1.0, MeOH), Rf^1 0.61. *Anal.* Calcd for $C_{16}H_{32}N_4O_4$: C, 55.79; H, 9.36; N, 16.27. Found: C, 55.74; H, 9.27; N, 16.01.

Boc–Leu–Val–Pro–Pro–NHNH$_2$ (I-5)——Boc–Pro–Pro–OBzl (3.78 g) was treated with TFA (15 ml) in the usual manner, and dry ether and *n*-hexane were added and decanted off twice. The resulting residue was dissolved in DMF (10 ml) and neutralized with Et_3N. To this ice-chilled solution, the azide (prepared from 3.07 g of Boc–Leu–Val–NHNH$_2$ with 6.10 ml of 3.87 N HCl–DMF, 1.58 ml isoamyl nitrite and 4.33 ml of Et_3N) in DMF (10 ml) was added. The mixture was stirred at 4 °C for 48 h, and then evaporated. The residue was extracted with AcOEt. The AcOEt phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and then evaporated. The resulting residue was dissolved in MeOH (15 ml) and 80% hydrazine hydrate (4.57 ml) was added to the solution. After standing overnight, the resulting gelatinous mass was collected by filtration and precipitated from MeOH with IPE; yield 3.36 g (80%), mp 118—121 °C, $[\alpha]_D^{24}$ −155.8° (c=1.0, MeOH), Rf^1 0.64. *Anal.* Calcd for $C_{26}H_{46}N_6O_6 \cdot H_2O$: C, 56.09; H, 8.69; N, 15.10. Found: C, 56.31; H, 8.56; N, 15.02.

Boc–Gln–Phe–Gly–Ser–Pro–Lys(Z)–OBzl (I-6)——Z(OMe)–Ser–Pro–Lys(Z)–OBzl (5.78 g) was treated with TFA (20 ml)–anisole (2.0 ml) in an ice-bath for 1 h and then the excess TFA was removed by evaporation. IPE and *n*-hexane were added to the residue. The resulting powder was dried over KOH pellets *in vacuo* for 1 h, dissolved in DMF (15 ml) and neutralized with Et_3N. To this ice-chilled solution, the azide (prepared from 4.48 g of Boc–Gln–Phe–Gly–NHNH$_2$ with 7.48 ml of 3.87 N HCl–DMF, 1.95 ml of isoamyl nitrite and 5.12 ml of Et_3N) in DMF–DMSO (10 ml–15 ml) was added. The mixture was stirred at 4 °C for 48 h, then evaporated to give a residue, which was treated with ether and H_2O. The resulting powder was washed batchwise with 5% citric acid, 5% $NaHCO_3$ and H_2O and precipitated from MeOH–THF with ether; yield 7.05 g (89%), mp 149—151 °C, $[\alpha]_D^{25}$ −37.5° (c=1.0, DMF), Rf^1 0.47. *Anal.* Calcd for $C_{50}H_{66}N_8O_{13} \cdot H_2O$; C, 59.74; H, 6.82; N, 11.15. Found: C, 59.87; H, 6.69; N, 11.59.

Boc–Asp(OBzl)–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–Lys(Z)–OBzl (I-7)——The above protected hexapeptide ester (I-6) (2.41 g) was treated with TFA (10 ml) as stated above and dry ether was added to the residue. The resulting powder was dried over KOH pellets *in vacuo* for 1 h, then dissolved in DMF (10 ml) and neutralized with Et_3N. DCC (0.90 g) and HOBT·H_2O (0.62 g) were added to a solution of Boc–Asp(OBzl)–Tyr–Pro–OH (2.14 g) in DMF (10 ml),

and the reaction mixture was stirred for 15 min. Then the solution containing the N^{α}-deprotected hexapeptide ester was added and the whole was stirred at room temperature for 18 h. After work-up as described for Boc–Phe–Gly–OMe, the product was precipitated from AcOEt with ether; yield 3.24 g (92%), mp 117—119 °C, $[\alpha]_D^{25}$ −52.8° (c=1.1, DMF), Rf^1 0.60. *Anal.* Calcd for $C_{75}H_{93}N_{11}O_{19}$: C, 60.15; H, 6.60; N, 10.29. Found: C, 60.01; H, 6.34; N, 10.45.

Boc–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–Lys(Z)–OBzl (I-8)——The above protected nonapeptide ester (I-7) (3.24 g) was treated with TFA (15 ml) in the usual manner, and the N^{α}-deprotected peptide, isolated as stated above, was dissolved in DMF (15 ml) and neutralized with Et_3N. To this ice-chilled solution, Boc–Glu(OBzl)–ONp (1.53 g) and N-methylmorpholine (0.25 ml) were added, and the mixture was stirred at room temperature for 18 h. After usual work-up as stated above, the residue was treated with ether and the resulting powder was collected by filtration. The product was further purified by silica-gel column chromatography (SiO_2 150 g, 4×21.5 cm) using MeOH–$CHCl_3$–acetic acid (85:10:5) as an eluent. The desired product was finally precipitated from AcOEt with ether; yield 1.70 g (46%), mp 115—118 °C, $[\alpha]_D^{25}$ −52.9° (c=1.1, DMF), Rf^1 0.67. *Anal.* Calcd for $C_{87}H_{106}N_{12}O_{22}\cdot 2H_2O$: C, 61.18; H, 6.49; N, 9.84. Found: C, 61.14; H, 6.28; N, 9.77.

Boc–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–Lys(Z)–OBzl (I-9)——The above protected decapeptide ester (I-8) (1.67 g) was treated with TFA (5 ml) in the usual manner and dry ether was added to the residue. The resulting powder was collected by filtration and dried over KOH pellets *in vacuo* for 1 h, then dissolved in DMF (7 ml), and neutralized with Et_3N. To the N^{α}-deprotected peptide solution, Boc–Met(O)–Glu(OBzl)–OH (0.63 g) in THF (3 ml), DCC (0.32 g) and HOBT·H_2O (0.15 g) were added, and the mixture was stirred at room temperature for 18 h. DCurea was removed by filtration and the filtrate was evaporated *in vacuo*. The residue was extracted with AcOEt, and the organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, then dried over Na_2SO_4. Evaporation and trituration with IPE gave a white powder, which was precipitated from MeOH with ether; yield 1.97 g (97%), mp 105—108 °C, $[\alpha]_D^{25}$ −42.0° (c=1.1, DMF), Rf^1 0.60. *Anal.* Calcd for $C_{104}H_{128}N_{14}O_{27}S\cdot 3H_2O$: C, 59.70; H, 6.46; N, 9.37. Found: C, 59.92; H, 6.33; N, 9.20.

Boc–Leu–Val–Pro–Pro–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–Lys(Z)–OBzl (I-10)——The above protected dodecapeptide ester (I-9) (1.86 g) was treated with TFA (6 ml) in the usual manner, and the deprotected peptide, isolated as stated above, was dissolved in DMF (3 ml) and neutralized with Et_3N. To this ice-chilled solution, the azide (prepared from 0.79 g of Boc–Leu–Val–Pro–Pro–$NHNH_2$ with 0.99 ml of 3.87 N HCl–DMF, 0.26 ml of isoamyl nitrite and 0.66 ml of Et_3N) in DMF (3 ml) was added. The mixture was stirred at 4 °C for 48 h. After usual work-up as stated above, the residue was treated with ether and the resulting white mass was precipitated from MeOH–AcOEt with ether; yield 2.07 g (93%), mp 123—126 °C, $[\alpha]_D^{25}$ −63.1° (c=1.1, DMF), Rf^1 0.70, Rf^2 0.30. *Anal.* Calcd for $C_{125}H_{162}N_{18}O_{31}S\cdot H_2O$: C, 59.65; H, 6.81; N, 10.02. Found: C, 59.70; H, 6.74; N, 10.05.

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References and Notes

1) Amino acid and peptide derivatives mentioned in this paper are of the L-configuration. The following abbreviations were used: Z=benzyloxycarbonyl, Z(OMe)=p-methoxybenzyloxycarbonyl, Bzl=benzyl, Boc=*tert*-butoxycarbonyl, Np=p-nitrophenyl, Tcp=2,4,5-trichlorophenyl, DCC=dicyclohexylcarbodiimide, DCurea=dicyclohexylurea, DMF=dimethylformamide, DMSO=dimethylsulfoxide, AcOEt=ethyl acetate, HOBT=N-hydroxybenzotriazole, IPE=isopropyl ether, MeOH=methanol, THF=tetrahydrofuran, Et_3N=triethylamine, TFA=trifluoroacetic acid, TFMSA=trifluoromethanesulfonic acid.
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Studies on α_2-Plasmin Inhibitor Fragment T-11. II.[1,2] Synthesis of the Entire Amino Acid Sequence of α_2-Plasmin Inhibitor Fragment T-11

TAKASHI SASAKI* and SUMIRO ISODA

Research Institute, Daiichi Seiyaku Co., Ltd., 16-13 Kitakasai 1-chome, Edogawa-ku, Tokyo 134, Japan

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The hexacosapeptide corresponding to the entire amino acid sequence of human α_2-plasmin inhibitor fragment T-11 was synthesized by a conventional solution method. Three newly synthesized fragments were combined successively with the protected C-terminal hexadecapeptide ester previously obtained by using the dicyclohexylcarbodiimide-*N*-hydroxybenzotriazole and azide procedures to afford the protected hexacosapeptide ester. The trifluoromethanesulfonic acid–thioanisole–trifluoroacetic acid procedure was employed to remove all protecting groups of the protected peptide ester at the final stage. The dissociation constant for the interaction between the synthetic α_2-plasmin inhibitor fragment T-11 and plasmin was equal to that of the native T-11 from human α_2-plasmin inhibitor.

Keywords——α_2-plasmin inhibitor; α_2-plasmin inhibitor fragment synthesis; peptide synthesis; thioanisole–mediated TFMSA–TFA deprotection; dissociation constnat

As described in the preceding paper,[1] the human α_2-plasmin inhibitor fragment named T-11 was found to show high binding activity to plasmin.[3] To clarify the structure–activity relationship of this important plasma protein inhibitor fragment, we undertook the synthesis of this fragment and its intermediates by a conventional solution method. In this paper, we describe the synthesis of the hexacosapeptide that covers the entire amino acid sequence of T-11 by further chain elongation with the three newly synthesized fragments of the intermediate, hexadecapeptide ester, whose synthesis has already been reported,[1] followed by the thioanisole–mediated deprotection of all protecting groups with TFMSA–TFA[4a,b] and purification steps.

The strategy employed here for the synthesis was essentially the same as that described in the preceding paper.[1] Amino acid derivatives having side chain-protecting groups removable by the above deprotecting reagents at the final step were employed, *i.e.*, Asp(OBzl), Lys(Z) and Glu(OBzl). The Met residue was protected as its sulfoxide as previously described.[1]

The first fragment, Boc–Asp(OBzl)–Leu–Lys(Z)–OH (I-11), was prepared stepwise starting with the condensation of H–Lys(Z)–OH and Boc–Leu–OH by means of the pentachlorophenyl ester procedure.[5] Next, the *p*-nitrophenyl ester procedure[6] was used for the combination of Boc–Asp(OBzl)–OH with a TFA–treated sample of the above dipeptide, Boc–Leu–Lys(Z)–OH.

The fragment, Boc–Leu–Phe–Gly–Pro–$NHNH_2$ (I-12), was prepared from the dipeptides Boc–Gly–Pro–OBzl and Boc–Leu–Phe–$NHNH_2$. The DCC method[7] was useful for condensation of Boc–Gly–OH and H–Pro–OBzl to give the above dipeptide. Boc–Leu–OH and H–Phe–OMe were combined by the DCC–HOBT method[8] and the resulting dipeptide ester was converted to the corresponding hydrazide with hydrazine hydrate. A TFA–treated sample of Boc–Gly–Pro–OBzl was condensed with Boc–Leu–Phe–$NHNH_2$, *via* the azide,[9] and the

resulting tetrapeptide ester, Boc–Leu–Phe–Gly–Pro–OBzl, was then converted to the corresponding hydrazide in the usual manner.

The N-terminal fragment of T-11, Boc–Gly–Asp(OBzl)–Lys(Z)–OH (I-13), was also prepared in a stepwise manner starting with H–Lys(Z)–OH, which was successively condensed with Boc–Asp(OBzl)–OH and Boc–Gly–OH by the *p*-nitrophenyl ester procedure.

Subsequent fragment condensation was carried out as follows. The fragment Boc–Asp(OBzl)–Leu–Lys(Z)–OH (I-11) was condensed with a TFA–treated sample of the protected hexadecapeptide ester (I-10)[1] by the DCC–HOBT method to give the nonadecapeptide ester (I-14). Then, Boc–Leu–Phe–Gly–Pro–$NHNH_2$ (I-12) was coupled with a TFA–treated sample of I-14 by the azide procedure. The DCC–HOBT method was used for the final condensation of Boc–Gly–Asp(OBzl)–Lys(Z)–OH (I-13) and a TFA–treated sample of I-15 as indicated in Fig. 1.

After completion of the coupling reactions had been confirmed by the ninhydrin test, each product was purified by batchwise washing with 5% citric acid and 5% $NaHCO_3$, followed by precipitation.

The purity of these intermediates was checked by TLC, elemental analysis and amino acid analysis. The amino acid ratios of the acid hydrolysates of these intermediates are shown in Table I.

The deprotection procedure and the subsequent purification methods are shown in Fig. 2. The protected hexacosapeptide ester (I-16) thus obtained was treated with 1 M TFMSA in

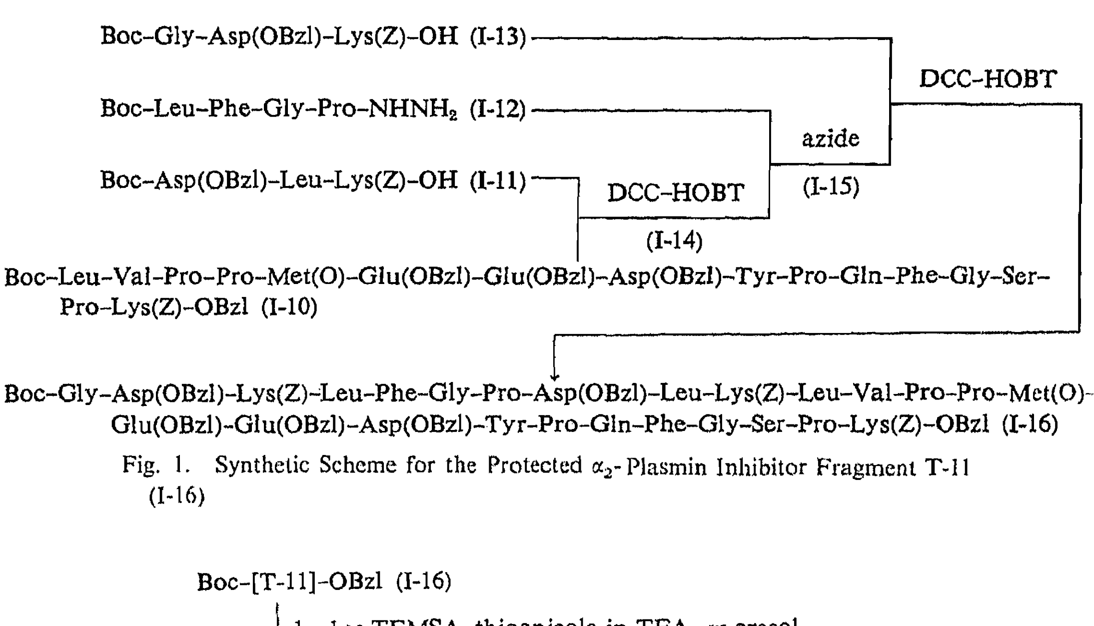

Fig. 1. Synthetic Scheme for the Protected α_2-Plasmin Inhibitor Fragment T-11 (I-16)

Boc-[T-11]-OBzl (I-16)

1. 1 M TFMSA-thioanisole in TFA, *m*-cresol
2. Amberlite IRA-410 (acetate form)
3. treatment with 5% NH_4OH
4. reduction with dithiothreitol

crude T-11

5. Sephadex G-25 (1 N AcOH)
6. DEAE-cellulose (gradient elution with NH_4HCO_3)

purified T-11

Fig. 2. Deprotection of (I-16) and Purification of T-11

TABLE I. Amino Acid Ratios in 6 N HCl Hydrolysates and Aminopeptidase M Digests of Synthetic T-11 and Its Intermediates

	I-14[a)]	I-15[a)]	I-16[a)]	T-11[a)]	T-11[b)]
Asp	2.28 (2)	1.85 (2)	3.01 (3)	3.01 (3)	2.84 (3)
Ser	0.82 (1)	0.68 (1)	0.73 (1)	0.85 (1)	0.92 (1)
Glu	3.16 (3)	2.72 (3)	2.98 (3)	3.01 (3)	2.04 (2)
Gln					1.02 (1)
Pro	4.24 (4)	5.17 (5)	4.55 (5)	5.21 (5)	5.31 (5)
Gly	1.01 (1)	1.95 (2)	3.16 (3)	3.01 (3)	3.33 (3)
Val	1.13 (1)	1.12 (1)	1.23 (1)	1.02 (1)	1.05 (1)
Met	0.56 (1)	0.58 (1)	0.84 (1)	0.79 (1)	0.92 (1)
Leu	2.18 (2)	3.04 (3)	3.40 (3)	3.11 (3)	3.09 (3)
Tyr	0.86 (1)	0.69 (1)	0.77 (1)	0.92 (1)	1.09 (1)
Phe	1.00 (1)	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)
Lys	2.02 (2)	1.97 (2)	3.13 (3)	2.85 (3)	2.98 (3)
Rec. (%)	92	90	89	91	81

a) A 6 N HCl hydrolysate. *b*) Aminopeptidase M digest.

TFA in the presence of thioanisole[4a)] and *m*-cresol[4b)] in an ice-bath for 1 h to remove all protecting groups employed. After precipitation of the peptide with ether, the deprotected peptide was converted to the corresponding acetate by treatment with Amberlite IRA-410 (acetate form). The product was next treated with diluted ammonia in order to reverse the possible N→O shift at the Ser residue.[10)] To ensure the complete reduction of the Met(O) residue, the deprotected peptide was incubated with dithiothreitol,[11)] and then subjected to gel-filtration on Sephadex G-25. The main peak portions were collected and lyophilized. The lyophilized product was further purified by ion-exchange chromatography on DEAE-cellulose. In elution with a gradient up to 0.20 M NH_4HCO_3, minor peaks were detected before the main peak (Fig. 3). The main product thus obtained exhibited a single peak on HPLC (Fig. 4) and a single spot on TLC in two different solvent systems. Its purity was further confirmed by elemental analysis and amino acid analysis after acid hydrolysis and enzymatic digestion.

The dissociation constant for the interaction between our synthetic peptide and plasmin measured by the method of Wiman *et al.*[12)] was determined to be 4—7 μM, which was the same as that reported for the native T-11 (5.5 μM).[3)]

Experimental

Melting points are uncorrected. Optical rotations were determined with a JASCO DIP 140 digital polarimeter. Acid hydrolysis was performed in 6 N HCl at 110 °C for 24 h in evacuated tubes. The amino acid compositions of acid hydrolysates were determined with a Hitachi amino acid analyzer 835 and are not corrected for amino acid destruction. TLC was performed on silica-gel plates (Kiesel gel 60 F_{254}, Merck) and *Rf* values refer to the following solvent systems: Rf^1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf^2 *n*-butanol–pyridine–acetic acid–H_2O (4:1:1:2), Rf^3 *n*-butanol–pyridine–H_2O (1:1:1).

Analytical HPLC was conducted on a JASCO TWINCLE apparatus equipped with a Cosmosil $5C_{18}$-P (5 μ, Nakarai Chem. Co.) column (4.6 × 150 mm) by linear gradient elution with acetonitrile (10% to 50%, 15 min) in 0.05% TFA at a flow rate of 1.0 ml/min with monitoring at 230 nm. Aminopeptidase M (Lot. 012583-1) was purchased from Pierce Chemical Co., Ltd.

Boc–Leu–Lys(Z)–OH——Boc–Leu–OPcp (3.88 g) was added to a suspension of H–Lys(Z)–OH (2.59 g) and Et_3N (2.47 ml) in DMSO–DMF (10 ml–15 ml) and the mixture was stirred at room temperature for 48 h. The solvent was removed by evaporation and the residue was dissolved in 5% $NaHCO_3$. This solution was washed with AcOEt, then acidified with citric acid and the precipitate was extracted with AcOEt. The AcOEt layer was washed with 5%

citric acid and H_2O, dried over Na_2SO_4 and then evaporated. The resulting oily residue was applied to a silica-gel column(65 g, 2.5 x 24 cm) and eluted with $CHCl_3$. The purified dipeptide was treated with IPE and recrystallized from AcOEt and IPE; yield 2.80 g (72%), mp 72—74 °C, $[\alpha]_D^{25}$ −11.9° (*c*=1.1, MeOH), *Rf*¹ 0.51. *Anal.* Calcd for $C_{25}H_{39}N_3O_7 \cdot 0.5H_2O$: C, 59.74; H, 8.02; N, 8.36. Found: C, 60.18; H, 7.82; N, 8.33.

Boc–Asp(OBzl)–Leu–Lys(Z)–OH (I-11)——Boc–Leu–Lys(Z)–OH (2.47 g) was treated with TFA (10 ml) in the usual manner and the resulting oily product was dissolved in THF (20 ml) and neutralized with Et_3N. To this solution, Boc–Asp(OBzl)–ONp (2.29 g) and Et_3N (1.43 ml) were added, and the mixture was stirred at room temperature overnight. The solvent was removed by evaporation under reduced pressure. The residue was dissolved in AcOEt and the AcOEt layer was washed with 5% citric acid and H_2O, dried over Na_2SO_4 and evaporated. The resulting residue was crystallized from *n*-hexane and recrystallized from AcOEt and IPE; yield 2.56 g (71%), mp 98—100 °C, $[\alpha]_D^{24}$ −25.0° (*c*=1.1, MeOH), *Rf*¹ 0.78. *Anal.* Calcd for $C_{36}H_{50}N_4O_{10}$: C, 61.87; H, 7.21; N, 8.02. Found: C, 61.68; H, 7.18; N, 8.09.

Boc–Gly–Pro–OBzl——DCC (3.21 g) was added to a mixture of Boc–Gly–OH (2.51 g) and H–Pro–OBzl (prepared from 3.80 g of the hydrochloride with 2.18 ml of Et_3N) in THF–$CHCl_3$ (10 ml–5 ml), and the solution was stirred at room temperature for 18 h, then filtered. The filtrate was evaporated, and the residue was dissolved in AcOEt. This solution was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4, and evaporated to give an oily residue; yield 4.98 g (96%), *Rf*¹ 0.67.

Boc–Leu–Phe–OMe——DCC (4.49 g) and HOBT·H_2O (3.06 g) were added to a solution of Boc–Leu–OH·H_2O (5.17 g) in dioxane–THF (10 ml–20 ml). After stirring of the mixture for 15 min, H–Phe–OMe (prepared from 4.31 g of the hydrochloride with 2.79 ml of Et_3N) in $CHCl_3$–dioxane (20 ml–10 ml) was added, and the whole was stirred at room temperature for 18 h, then filtered. The filtrate was evaporated, and the residue was extracted with AcOEt. The AcOEt layer was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and then evaporated. The resulting residue was triturated with IPE and *n*-hexane and recrystallized from ether and IPE–*n*-hexane; yield 7.61 g (97%), mp 86—87 °C (Lit.[13] 78—79 °C), $[\alpha]_D^{25}$ −20.7° (*c*=1.2, MeOH) (Lit.[13] $[\alpha]_D^{25}$ −27.6° (*c*=1.0, MeOH), *Rf*¹ 0.83. *Anal.* Calcd for $C_{21}H_{32}N_2O_5$: C, 64.24; H, 8.22; N, 7.14. Found: C, 64.19; H, 8.25; N, 7.08.

Boc–Leu–Phe–NHNH₂——Hydrazine hydrate (80%, 11.70 ml) was added to a solution of Boc–Leu–Phe–OMe (7.55 g) in MeOH (50 ml), and the gelatinous mass that formed after standing overnight was collected by filtration and recrystallized from MeOH and IPE; yield 6.95 g (92%), mp 165—166 °C, $[\alpha]_D^{25}$ −36.0° (*c*=1.0, MeOH), *Rf*¹ 0.80. *Anal.* Calcd for $C_{20}H_{32}N_4O_4$: C, 61.20; H, 8.22; N, 14.28. Found: C, 61.06; H, 8.08; N, 14.17.

Boc–Leu–Phe–Gly–Pro–OBzl——Boc–Gly–Pro–OBzl (2.62 g) was treated with TFA (5 ml) in the usual manner, and the excess TFA was removed by evaporation. The residue was decanted with IPE and *n*-hexane, and the residue thus obtained was dissolved in DMF (10 ml) and neutralized with Et_3N. This solution was combined with the azide (prepared from 2.35 g of Boc–Leu–Phe–$NHNH_2$ with 3.72 ml of 3.87 N HCl–DMF, 0.97 ml of isoamyl nitrite and 2.83 ml of Et_3N) in DMF (10 ml). The mixture was stirred at 4 °C for 48 h, then evaporated *in vacuo*, and the residue was extracted with AcOEt. The extract was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and evaporated. The residue was triturated with IPE and *n*-hexane, followed by recrystallization from ether and IPE–*n*-hexane; yield 3.40 g (91%), mp 78—80 °C, $[\alpha]_D^{25}$ −84.4° (*c*=1.0, MeOH), *Rf*¹ 0.90. *Anal.* Calcd for $C_{34}H_{46}N_4O_7 \cdot 0.5H_2O$: C, 64.64; H, 7.50; N, 8.87. Found: C, 64.82; H, 7.30; N, 8.85.

Boc–Leu–Phe–Gly–Pro–NHNH₂ (I-12)——Hydrazine hydrate (80%, 3.84 ml) was added to a solution of the above protected tetrapeptide ester (3.94 g) dissolved in MeOH (40 ml). The mixture was left to stand at room temperature for 18 h, then the solvent was removed by evaporation. The residue was dissolved in AcOEt, and the organic phase was washed with NaCl–H_2O, dried over Na_2SO_4 and then evaporated. The residue was triturated with IPE, and the mass obtained was recrystallized from MeOH and IPE; yield 3.04 g (88%), mp 113—115 °C, $[\alpha]_D^{25}$ −80.5° (*c*=1.1, MeOH), *Rf*¹ 0.48. *Anal.* Calcd for $C_{27}H_{42}N_6O_6 \cdot 0.5H_2O$: C, 58.36; H, 7.80; N, 15.13. Found: C, 58.36; H, 7.80; N, 15.19.

Boc–Asp(OBzl)–Lys(Z)–OH——Boc–Asp(OBzl)–ONp (8.89 g) was added to a suspension of H–Lys(Z)–OH (6.92 g) in DMF (70 ml) containing Et_3N (3.63 ml) and *N*-methylmorpholine (2.20 ml), and the mixture was stirred at room temperature for 48 h. Work-up as described for Boc–Leu–Lys(Z)–OH afforded an oily product; yield 10.23 g (87%), *Rf*¹ 0.54.

Boc–Gly–Asp(OBzl)–Lys(Z)–OH (I-13)——Boc–Asp(OBzl)–Lys(Z)–OH (5.65 g) was treated with TFA (15 ml) in an ice-bath for 45 min, then the excess TFA was removed by evaporation. IPE was added to the residue. The resulting powder was dried over KOH pellets *in vacuo* for 1 h and then dissolved in THF (20 ml) followed by neutralization with Et_3N. To this solution, Boc–Gly–ONp (3.27 g) and Et_3N (1.33 ml) were added, and the mixture was stirred at room temperature for 48 h. After usual work-up as described above, the residue was triturated with ether and the gelatinous mass obtained was precipitated from AcOEt–MeOH with ether–IPE; yield 3.32 g (54%), mp 81—83 °C, $[\alpha]_D^{25}$ −5.6° (*c*=1.0, MeOH), *Rf*¹ 0.43. *Anal.* Calcd for $C_{32}H_{42}N_4O_{10}$: C, 59.80; H, 6.59; N, 8.72. Found: C, 59.95; H, 6.62; N, 9.01.

Boc–Asp(OBzl)–Leu–Lys(Z)–Leu–Val–Pro–Pro–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–Lys(Z)–OBzl (I-14)——The protected hexadecapeptide ester (I-10) (2.07 g) was treated with TFA (10 ml) in the usual manner, and the resulting dried powder was dissolved in DMF (10 ml) and neutralized with Et_3N.

DCC (0.28 g) and HOBT·H_2O (0.13 g) were added to a solution of Boc–Asp(OBzl)–Leu–Lys(Z)–OH (I-11) (0.83 g) in DMF (5 ml). The above N^{α}-deprotected peptide solution was added to this mixture. The reaction mixture was stirred at room temperature for 18 h, then DCurea was filtered off and the filtrate was concentrated under reduced pressure. The residue was treated with 5% citric acid and ether, and the resulting powder was collected and washed batchwise with 5% citric acid, 5% $NaHCO_3$ and H_2O, followed by precipitation from MeOH with AcOEt; yield 2.35 g (92%), mp 139—141 °C, $[\alpha]_D^{25}$ −58.9° (c=1.1, DMF), Rf^1 0.64. *Anal.* Calcd for $C_{156}H_{202}N_{22}O_{38}S \cdot 3H_2O$: C, 60.84; H, 6.81; N, 10.01. Found: C, 60.78; H, 6.72; N, 10.02.

Boc–Leu–Phe–Gly–Pro–Asp(OBzl)–Leu–Lys(Z)–Leu–Val–Pro–Pro–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–Lys(Z)–OBzl (I-15)——The above nonadecapeptide ester (I-14) (1.70 g) was treated with TFA (10 ml) as stated above and the excess TFA was removed by evaporation. The residue was treated with ether, and the resulting powder was dried over KOH pellets *in vacuo* and then dissolved in DMF (5 ml). After neutralization with Et_3N, the azide (prepared from 0.46 g of Boc–Leu–Phe–Gly–Pro–$NHNH_2$ with 0.65 ml of 3.87 N HCl–DMF, 0.17 ml of isoamyl nitrite and 0.43 ml of Et_3N) in DMF (5 ml) was combined with the above N^{α}-deprotected peptide solution and the mixture was stirred at 4 °C for 48 h. After work-up as described above, the product was precipitated from MeOH with ether; yield 1.86 g (96%), mp 133—136 °C, $[\alpha]_D^{25}$ −62.7° (c=1.1, DMF), Rf^1 0.66. *Anal.* Calcd for $C_{178}H_{232}N_{26}O_{42}S \cdot 4H_2O$: C, 60.87; H, 6.89; N, 10.37. Found: C, 60.64; H, 6.69; N, 10.44.

Boc–Gly–Asp(OBzl)–Lys(Z)–Leu–Phe–Gly–Pro–Asp(OBzl)–Leu–Lys(Z)–Leu–Val–Pro–Pro–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–Lys(Z)–OBzl (I-16)——The above compound (I-15) (1.40 g) was treated with TFA (10 ml) as stated above, and ether was added. The resulting powder was dissolved in DMF (7 ml) and neutralized with Et_3N. This solution was added to a mixture of Boc–Gly–Asp(OBzl)–Lys(Z)–OH (0.39 g), DCC (0.15 g) and HOBT·H_2O (0.10 g) in DMF (3 ml) and the mixture was stirred at room temperature for 18 h, then filtered. The filtrate was evaporated, and the residue was treated with ether and H_2O. The resulting powder was washed batchwise as stated above and precipitated from MeOH–THF with ether; yield 1.41 g (88%), mp 198—202 °C, $[\alpha]_D^{25}$ −58.6° (c=1.0, DMF), Rf^1 0.64. *Anal.* Calcd for $C_{205}H_{264}N_{30}O_{49}S \cdot 4H_2O$: C, 60.99; H, 6.79; N, 10.41. Found: C, 60.77; H, 6.63; N, 10.40.

H–Gly–Asp–Lys–Leu–Phe–Gly–Pro–Asp–Leu–Lys–Leu–Val–Pro–Pro–Met–Glu–Glu–Asp–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–Lys–OH (T-11)——The above protected hexacosapeptide ester (I-16) (200 mg) was treated with 1 M TFMSA–thioanisole in TFA (3.97 ml) in the presence of *m*-cresol (0.53 ml) in an ice-bath for 1 h, then the solvent was removed by evaporation and dry ether was added to the residue. The resulting powder was collected by filtration, and dried over KOH pellets *in vacuo* for 30 min. This treatment was repeated twice to ensure complete deprotection. The deprotected peptide thus obtained was dissolved in H_2O (4 ml), and treated with Amberlite IRA-410 (acetate form) for 30 min with stirring in an ice-bath. The mixture was filtered, and the pH of the filtrate was adjusted to 8.0 with 5% NH_4OH and after 30 min to 4.0 with AcOH in an ice-bath. The solution was lyophilized to give a hygroscopic powder, which was incubated with dithiothreitol (200 mg) in H_2O (5 ml) in an atmosphere of nitrogen at 37 °C for 19 h. The solution was applied to a Sephadex G-25 column (3.3 × 133 cm) and eluted with 1 N AcOH at a flow rate of 71.2 ml/h. The UV absorption at 275 nm was determined in each fraction (10.4 ml). The fractions corresponding to the front main peak (tube Nos. 53—66) were collected and the solvent was removed by lyophilization to give a white powder; yield 114 mg (78%).

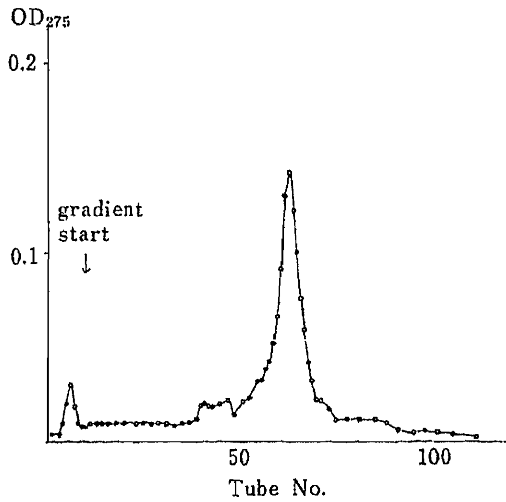

Fig. 3. Purification of Crude Synthetic T-11 by Ion-Exchange Chromatography on DEAE-Cellulose

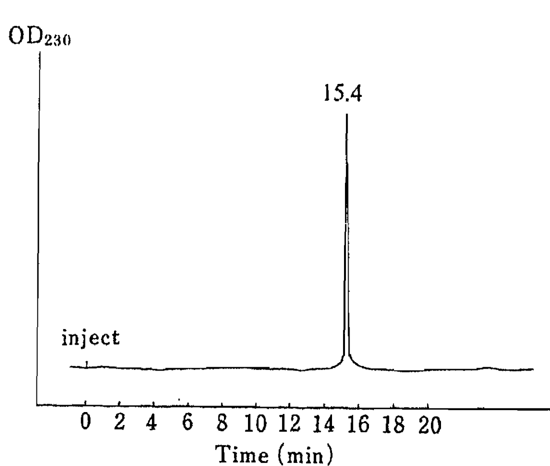

Fig. 4. HPLC of Synthetic T-11

The crude product was dissolved in H_2O (3 ml), and the solution was applied to a column of DEAE–cellulose (1.5 × 34.5 cm), which was first washed with the starting buffer 0.01 M NH_4HCO_3 (pH 8.1) and then eluted with a linear gradient formed from the starting buffer (500 ml) and 0.20 M NH_4HCO_3 (500 ml) at a flow rate of 41 ml/h. The UV absorption at 275 nm in each fraction (10.2 ml) was determined (Fig. 3). The fractions corresponding to the main peak (tube Nos. 59—70) were combined and the solvent and ammonium salt were removed by lyophilization. After the addition of 1 N AcOH to the residue, the solvent was removed by lyophilization to give a fluffy powder; yield 52 mg (36%), $[\alpha]_D^{25}$ −76.0° (c=0.2, 0.1 N AcOH), Rf^3 0.12, Rf^4 0.70. The synthetic peptide exhibited a single peak at a retention time of 15.4 min on HPLC with a Cosmosil $5C_{18}$-P column (4.6 × 150 mm) (Fig. 4). Amino acid ratios in a 6 N HCl hydrolysate and an aminopeptidase M digest of the synthetic peptide are shown in Table I. *Anal.* Calcd for $C_{134}H_{202}N_{30}O_{40}S \cdot 4AcOH \cdot 15.5H_2O$: C, 49.80; H, 7.33; N, 12.27. Found: C, 49.97; H, 7.52; N, 12.53.

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References and Notes

1) Part I: T. Sasaki and S. Isoda, *Chem. Pharm. Bull.*, **35**, 2797 (1987).
2) Amino acid and peptide derivatives mentioned in this paper are of the L-configuration. The following abbreviations were used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Bzl = benzyl, Boc = *tert*-butoxycarbonyl, Np = *p*-nitrophenyl, Pcp = pentachlorophenyl, DCC = dicyclohexylcarbodiimide, DCurea = dicyclohexylurea, DEAE = diethylaminoethyl, DMSO = dimethylsulfoxide, AcOEt = ethyl acetate, DMF = dimethylformamide, HPLC = high performance liquid chromatography, HOBT = *N*-hydroxybenzotriazole, IPE = isopropyl ether, MeOH = methanol, THF = tetrahydrofuran, TLC = thin-layer chromatography, Et_3N = triethylamine, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, UV = ultraviolet.
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Studies on α_2-Plasmin Inhibitor Fragment T-11. III.[1a,b,2] Structure–Activity Relationships among the Fragments of T-11, the Plasminogen Binding Site(s) of Human α_2-Plasmin Inhibitor

TAKASHI SASAKI,* NOBUTOSHI SUGIYAMA, MASAHIRO IWAMOTO and SUMIRO ISODA

Research Institute, Daiichi Seiyaku Co., Ltd., 16-13 Kitakasai 1-chome, Edogawa-ku, Tokyo 134, Japan

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Twelve shortened fragments of peptide T-11, which is a part of human α_2-plasmin inhibitor and contains its plasmin(ogen)-binding site(s) were synthesized by a conventional solution method. The dissociation constants for the interaction between these synthetic fragments and plasmin were determined. Among these fragments, the octadecapeptide containing C-terminal lysine of T-11, which consists of 26 amino acids, was found to be the smallest active fragment of T-11. The N-terminal and the central portion fragments of T-11 possessed no binding activity. The heptadecapeptide which has the same sequence as the octadecapeptide but lacks the C-terminal lysine showed no binding activity. The hexadecapeptide containing the C-terminal lysine but lacking the 10th lysine in T-11 scarcely exhibited the activity. Thus, the lysine residues at positions 10 and 26 in T-11 must be important for the activity.

Keywords——α_2-plasmin inhibitor; α_2-plasmin inhibitor fragment; peptide synthesis; thioanisole-mediated TFMSA deprotection; dissociation constant; structure–activity relationship

α_2-Plasmin inhibitor is the most important inhibitor of plasmin in mammalian blood. Peptide T-11, which consists of 26 amino acid residues, was isolated from human α_2-plasmin inhibitor and shown to be the plasmin(ogen)-binding site(s) of the inhibitor.[3] A study on the structure activity relationship of T-11 has been carried out. This paper deals with the synthesis and activity of many shortened fragments of T-11.

Previously we demonstrated that peptide T-11 binds to the plasmin(ogen) lysine-binding site(s).[3] ω-Amino acids, for example, ε-aminocaproic acid[4] and *trans*-4-aminomethylcyclohexanecarboxylic acid (*t*-AMCHA),[5] also bind to plasmin(ogen). Amino and carboxyl groups of these ω-amino acids are reported to be essential for their binding activity to the lysine-binding site(s).[6] The fragment T-11 and *t*-AMCHA have been shown to be competitive inhibitors of binding to the lysine-binding site(s).[7] Thus, at least one pair of acidic and basic amino acids among three lysines and two glutamic acids or three aspartic acids in this peptide T-11 seems to be essential for binding to the same site(s) as that of ω-amino acids. To identify the pair of amino acids, twelve shortened fragments of T-11 lacking those basic and/or acidic amino acid residues were synthesized and their activities were determined.

In order to obtain fragments II, III, V and VI of T-11, deprotection of all protecting groups of their intermediates (I-15, I-14, I-10 and I-9), which were obtained in the course of the total synthesis of T-11,[1a,b] was carried out, and the deprotected products were purified. The protected N-terminal fragments (XII-4 and XIII-1), central fragments (X-7 and XI-2) and C-terminal fragments (IV-1 and VII-2) were newly synthesized and deprotected, follow-

ed by purification to give the peptides (XII, XIII, X, XI, IV and VII, respectively). Fragments VIII and IX, lacking C-terminal lysine were prepared from III and IV, respectively, by carboxypeptidase B[8] digestion.

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1     5        10        15        20        25                    Kd (μM)
G-D-K-L-F-G-P-D-L-K-L-V-P-P-M-E-E-D-Y-P-Q-F-G-S-P-K   (I) (T-11)   4—7
      L.............................................K   (II)         18
              D.....................................K   (III)        13
                L...................................K   (IV)         35
                    L...............................K   (V)         250
                            M.......................K   (VI)        250
                                          F.........K   (VII)       700
              D...................................P     (VIII)       —
                L.................................P     (IX)         —
              Ac-L....................P-NH2             (X)          —
            Ac-D......................P-NH2             (XI)         —
      L.................P-NH2                           (XII)        —
G.......................P-NH2                           (XIII)       —
                                          Ac-Lys-OH                 800
                                  ε-aminocaproic acid           100—200
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Fig. 1. Amino Acid Sequences and Dissociation Constants (K_d) of the Synthesized Fragments of T-11

The standard IUPAC one-letter codes[9] for amino acid residues are used.

The strategy for the synthesis of the peptides was essentially the same as the method previously described.[1a,b] Asp(OBzl), Glu(OBzl) and Lys(Z) were employed as side chain-protected amino acid derivatives so that deprotection by thioanisole–mediated TFMSA treatment in TFA[10] could be performed at the final stage. The TFA–labile Boc-group[11] was employed as a temporary protector of α-amino groups.

The protected octadecapeptide ester (IV-1) was synthesized by coupling of Boc–Leu–Lys(Z)–OH[1b] with a TFA–treated sample of Boc–Leu–Val–Pro–Pro–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–Lys(Z)–OBzl (I-10)[1a] by the DCC–HOBT procedure[12] and purified by batchwise washing with dilute acid and base, followed by precipitation.

The protected hexapeptide ester (I-6)[1a] was treated with TFA and hydrogenated with Pd catalyst. After purification, three spots were detected on TLC. Therefore the shortened peptide fragment VII lacking the N-terminal Gln was synthesized. Fragment VII was prepared by condensation of a TFA–treated sample of Z(OMe)–Ser–Pro–Lys(Z)–OBzl[1a] and Boc–Phe–Gly–$NHNH_2$ obtained from Boc–Phe–Gly–OMe[1a] using the azide procedure,[13] followed by deprotection with TFA and hydrogenation with a Pd catalyst (Fig. 2).

As described before, fragments VIII and IX were prepared by the removal of the C-terminal lysine residue of III and IV, respectively, with carboxypeptidase B.[8] As the second amino acid from the C-terminal of III or IV was proline, the enzyme reaction stopped at this residue, releasing only lysine. Preparative HPLC was used for purification of VIII and IX.

Fragment X-7 was synthesized starting with Boc–Pro–NH_2 as shown in Fig. 3. The Tyr residue was introduced into a TFA–treated sample of the starting material by the azide procedure and the Asp(OBzl) and the Glu(OBzl) residues were introduced stepwise by the *p*-nitrophenyl ester procedure.[14] The resulting protected tetrapeptide amide, Boc–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–NH_2 (X-4), was exposed to TFA and condensed with Boc–Met(O)–Glu(OBzl)–OH (I-4)[1a] by the DCC–HOBT procedure. Then Boc–Leu–Val–Pro–Pro–$NHNH_2$ (I-5)[1a] was condensed with a TFA–deprotected sample of the above hexapeptide

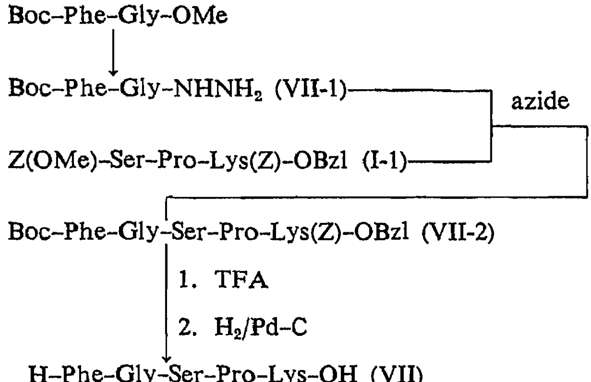

Fig. 2. Synthetic Scheme for the Pentapeptide (VII)

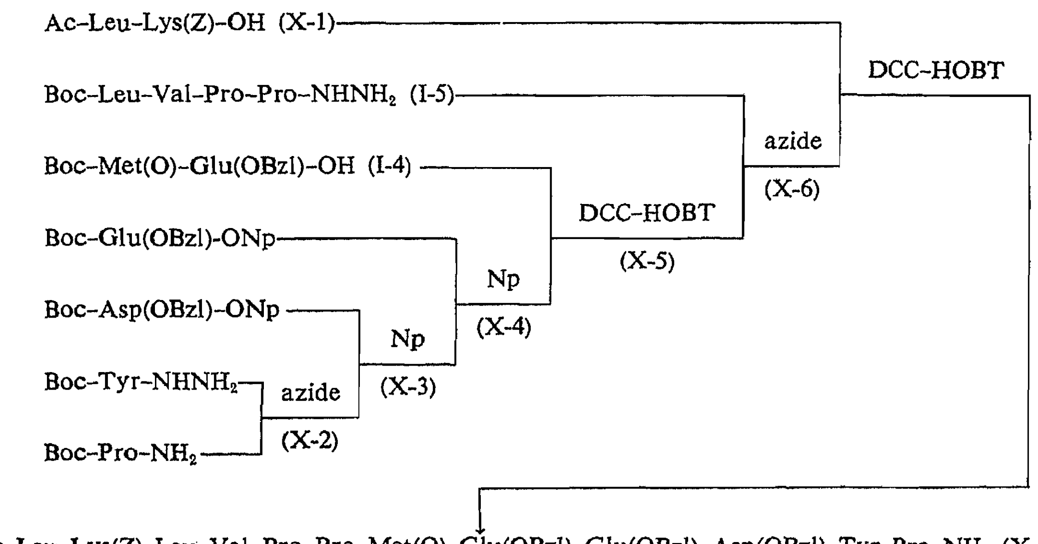

Fig. 3. Synthetic Scheme for the Protected Dodecapeptide Amide (X-7)

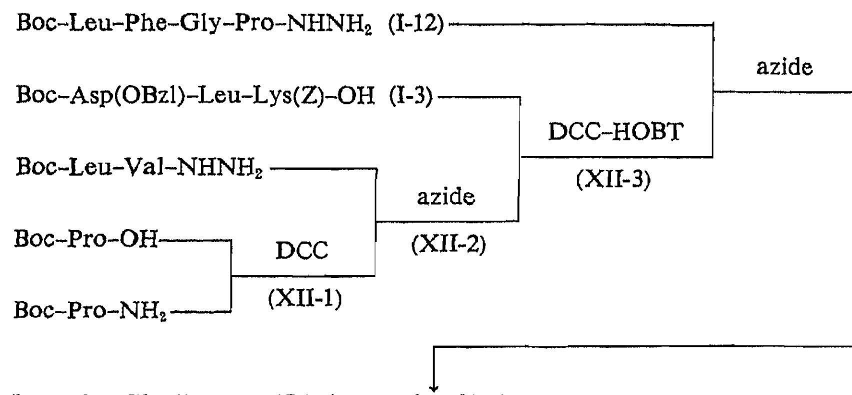

Fig. 4. Synthetic Scheme for the Protected Undecapeptide Amide (XII-4)

amide by the azide procedure. Finally, Ac–Leu–Lys(Z)–OH, which was prepared by the acetylation of a TFA-treated sample of Boc–Leu–Lys(Z)–OH[1b] with AcONp, was combined with the N^{α}-deprotected decapeptide amide.

Fragment XI-2 was prepared as follows. The above decapeptide amide (X-6) was treated with TFA and condensed by the DCC–HOBT method with Ac–Asp(OBzl)–Leu–Lys(Z)–OH, which was obtained by acetylation in the same manner as described for Ac–Leu–Lys(Z)–OH, after TFA treatment of Boc–Asp(OBzl)–Leu–Lys(Z)–OH (I-11).[1b]

Fragment XII-4 was synthesized as shown in Fig. 4. Boc–Pro–Pro–NH_2 (XII-1), which was synthesized by the DCC condensation of Boc–Pro–OH with a TFA-treated sample of Boc–Pro–NH_2, was exposed to TFA and coupled with Boc–Leu–Val–$NHNH_2$[1b] by the azide procedure. After removal of the Boc group of the resulting tetrapeptide amide (XII-2), Boc–Asp(OBzl)–Leu–Lys(Z)–OH (I-3)[1b] was condensed by the DCC–HOBT procedure. The azide procedure was employed to prepare the protected fragment (XII-4) for the condensation of Boc–Leu–Phe–Gly–Pro–$NHNH_2$ (I-12)[1b] and a TFA-treated sample of the heptapeptide amide (XII-3) obtained above.

The condensation of a TFA-treated sample of fragment XII-4 and Boc–Gly–Asp(OBzl)–Lys(Z)–OH (I-13)[1b] by the DCC–HOBT technique afforded fragment XIII-1.

The deprotection and subsequent purification of these fragments were carried out by essentially the same procedures as described for the synthesis of T-11.[1b] The product was purified by ion-exchange chromatography on DEAE–cellulose or CM–cellulose. If necessary, to remove small amounts of impurities, preparative reversed-phase HPLC on a Senshupack N5C_{18} column was carried out for further purification.

Each peptide thus obtained exhibited a single peak on HPLC and a sharp single spot on TLC. Its purity was further confirmed by amino acid analysis after acid hydrolysis and elemental analysis. The yield and the deprotection and purification procedures used for these peptides are shown in Table I, and the analytical data are given in Tables II and III.

The dissociation constants for the complexes between human plasmin and the T-11 fragments obtained were determined as the peptide concentrations that caused a 50% decrease of the apparent rate constant, according to the method of Wiman *et al.*[15] The values of the dissociation constants (K_d) determined for the peptides are summarized in Fig. 1. The K_d values for fragments II, III and IV were determined to be 18, 13 and 35 μM, respectively, and were of the same order as that of the fragment I (T-11).[1b,3] On the other hand, the values for fragments V, VI and VII were estimated to be 250, 250 and 700 μM, respectively, indicating that these fragments have weak binding activities similar to those of ε-aminocaproic acid and Ac–Lys–OH used as the controls. The N-terminal and central fragments, X to XIII, and fragments VIII and IX, lacking the C-terminal lysine, had no ability to bind to plasmin. Among these T-11 fragments prepared, fragment IV was the smallest one that exhibited binding activity to plasmin.

T-11 or its fragment III competed with *t*-AMCHA for binding to the lysine-binding site(s) of plasmin.[7] Therefore, some of the ε-amino and ω-carboxyl groups of T-11 might be involved in binding to the lysine-binding site(s) by the same mechanism as in the case of ω-amino acids, *e.g.*, ε-aminocaproic acid and *t*-AMCHA. Thus at least one pair of basic and acidic amino acid residues in T-11 seems to be essential for the binding.

Concerning basic amino acids, T-11 contains three lysines at positions 3, 10 and 26. The binding activity of fragment V, which contains only one lysine at position 26 in T-11, was greatly decreased, and its dissociation constant value was almost identical to that of the control as shown in Fig. 1. Fragments VIII and IX, lacking the C-terminal lysine at position 26, had no activity. However, fragment IV, which contains two lysines at positions 10 and 26 and consists of 18 amino acids, retained the binding activity. Thus, fragment IV might be the smallest active fragment of T-11, suggesting that the two lysines at positions 10 and 26 in T-11

are essential for the activity. Previously, we showed that the T-11 derivative amidinated at positions 3 and 10, but intact at position 26, lost its binding activity completely.[3)] Therefore, the 10th lysine in T-11 might play an important role in the binding.

In relation to the acidic amino acids in T-11, fragment IV, which contains two glutamic acids at positions 16 and 17 and aspartic acid at position 18, retained the activity. However, fragments V and VI containing these acidic amino acids and fragment VII containing no acidic amino acid showed very small binding activity similar to that of the controls. Further study will be necessary to clarify which acidic amino acid residues and which pair of basic and acidic amino acid residues in T-11 are important for binding activity to plasmin.

Experimental

Melting points are uncorrected. Optical rotations were determined with a JASCO DIP 140 digital polarimeter. The amino acid compositions of 6 N HCl hydrolysates were determined with a Hitachi amino acid analyzer 835 and are not corrected for amino acid destruction. TLC was performed on silica gel plates (Kiesel gel 60 F_{254}, Merck) and *Rf* values refer to the following solvent systems: Rf^1 $CHCl_3$–MeOH–H_2O (8 : 3 : 1), Rf^2 $CHCl_3$–MeOH–acetic acid (85 : 10 : 5), Rf^3 *n*-butanol–acetic acid–pyridine–H_2O (4 : 1 : 1 : 2), Rf^4 *n*-butanol–pyridine–H_2O (1 : 1 : 1).

Analytical HPLC was conducted with a JASCO TWINCLE apparatus equipped with a Cosmosil $5C_{18}$-P (5 μ, Nakarai Chem. Co.) column (4.6 × 150 mm) by linear gradient elution with acetonitrile (10% to 50%; 15 min) in 0.05% TFA at a flow rate of 1.0 ml/min with monitoring at 230 nm. Preparative HPLC was conducted with the same apparatus but with a Senshupack $N5C_{18}$ column (8 × 250 mm), using the same linear gradient system as that used for analytical HPLC at a flow rate of 2.0 ml/min with monitoring at 275 nm.

Carboxypeptidase B (Lot J1K698) was obtained from Worthington Biochemical Corp.

The final deprotection and purification procedures that were used for the synthesis of fragment III are described in detail as a representative example.

Boc–Leu–Lys(Z)–Leu–Val–Pro–Pro–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–Lys(Z)–OBzl (IV-1)——The hexadecapeptide ester (I-10)[1a)] (0.49 g) was treated with TFA (3 ml) at 0 °C for 1 h. The excess TFA was removed by evaporation *in vacuo*, and dry ether was added to the residue. The resulting powder was dried over KOH pellets *in vacuo* for 1 h and then dissolved in DMF (3 ml) and neutralized with Et_3N. DCC (62 mg) and HOBT·H_2O (34 mg) were added to a solution of Boc–Leu–Lys(Z)–OH[1b)] (0.23 g) in DMF (2 ml), then the above N^α-deprotected peptide solution was added to this mixture. The whole was stirred for 18 h, then filtered. The filtrate was evaporated, and the residue was treated with ether. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O and precipitated from MeOH with ether; yield 0.53 g (94%), mp 128—130 °C, $[\alpha]_D^{28}$ −72.4° (*c* = 1.0, MeOH), Rf^1 0.72. Amino acid ratios in 6 N HCl hydrolysate: Asp 0.95, Ser 0.83, Glu 2.76, Pro 4.15, Gly 0.98, Val 1.06, Met 0.63, Leu 2.23, Tyr 0.80, Phe 1.00, Lys 1.83 (recovery 91%). *Anal.* Calcd for $C_{145}H_{191}N_{21}O_{35}S \cdot 4H_2O$: C, 60.21; H, 6.94; N, 10.17. Found: C, 60.33; H, 6.77; N, 10.17.

Boc–Phe–Gly–NHNH$_2$ (VII-1)——Hydrazine hydrate (80%, 8.38 ml) was added to a solution of Boc–Phe–Gly–OMe[1a)] (4.65 g) in MeOH (30 ml). After standing overnight at room temperature, the mixture was concentrated *in vacuo* at room temperature and the residue was extracted with AcOEt. The AcOEt layer was washed with NaCl-saturated H_2O, dried over Na_2SO_4 and evaporated. The resulting product was recrystallized from MeOH and ether; yield 3.70 g (80%), mp 131—132 °C, $[\alpha]_D^{28}$ −7.6° (*c* = 1.1, MeOH), Rf^1 0.67. *Anal.* Calcd for $C_{16}H_{24}N_4O_4$: C, 57.13; H, 7.19; N, 16.66. Found: C, 57.46; H, 7.17; N, 16.60.

Boc–Phe–Gly–Ser–Pro–Lys(Z)–OBzl (VII-2)——Z(OMe)–Ser–Pro–Lys(Z)–OBzl[1a)] (1.08 g) was treated with TFA (6 ml) and anisole (0.81 ml) at 0 °C for 1 h. TFA was removed *in vacuo* and ether was added to the residue. The resulting powder was dried over KOH pellets, dissolved in DMF (5 ml) and neutralized with Et_3N. To this ice-chilled solution, the azide (prepared from 0.61 g of Boc–Phe–Gly–NHNH$_2$ with 0.61 ml of 7.62 N HCl–DMF, 0.31 ml of isoamyl nitrite and 0.86 ml of Et_3N) in DMF (1 ml) was added. The mixture was stirred at 4 °C for 48 h, then evaporated, and the residue was dissolved in AcOEt. The AcOEt phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O and then dried over Na_2SO_4. Evaporation gave the desired product, which was crystallized with ether and recrystallized from MeOH and ether; yield 1.03 g (80%), mp 119—121 °C, $[\alpha]_D^{28}$ −53.8° (*c* = 1.0, MeOH), Rf^1 0.77. *Anal.* Calcd for $C_{45}H_{58}N_6O_{11} \cdot 0.5H_2O$: C, 62.27; H, 6.85; N, 9.68. Found: C, 62.26; H, 6.75; N, 9.89.

H–Phe–Gly–Ser–Pro–Lys–OH (VII)——Compound VII-2 (400 mg) was treated with TFA (2 ml) at 0 °C for 1 h, then evaporated and ether was added to the residue. The resulting powder was dissolved in MeOH (5 ml) and a few drops of acetic acid and 5% palladium carbon (200 mg) were added. After hydrogenation, the catalyst was removed by filtration, and the filtrate was evaporated *in vacuo*. Then 1 N acetic acid was added to the residue, and the solvent was removed by lyophilization. The lyophilized product was treated with ether to give a white powder; yield 240 mg (96%), $[\alpha]_D^{28}$ −47.1° (*c* = 0.62, 1 N AcOH), Rf^3 0.21, Rf^4 0.51. Amino acid ratios in a 6 N HCl hydrolysate: Ser 0.89, Pro 1.17, Gly 1.00, Phe 1.00, Lys 0.94 (average recovery 90%). *Anal.* Calcd for $C_{25}H_{38}N_6O_7 \cdot 2AcOH \cdot 3H_2O$: C, 49.14;

H, 7.40; N, 11.86. Found: C, 48.85; H, 7.18; N, 12.13.

H–Asp–Leu–Lys–Leu–Val–Pro–Pro–Met–Glu–Glu–Asp–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–OH (VIII)——Peptide III (40.15 mg) was dissolved in 0.2 M NH_4HCO_3 (4.0 ml; pH 7.9). Carboxypeptidase B (1.60 mg) was added to the peptide solution and incubation was carried out at 37 °C for 3 d. The reaction mixture was subjected directly to preparative HPLC on a Senshupack $N5C_{18}$ column (8 x 250 mm). The main peak portions were collected and lyophilized. The lyophilized product was dissolved in 1 N AcOH and the solvent was removed by lyophilization to give a white fluffy powder; yield 25.3 mg (67%), $[\alpha]_D^{27}$ −145.7° (*c*=0.07, 1 N AcOH), Rf^4 0.77. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.95, Ser 0.83, Glu 2.76, Pro 4.15, Gly 0.98, Val 1.06, Met 0.63, Leu 2.23, Tyr 0.80, Phe 1.00, Lys 0.83 (average recovery 95%). *Anal.* Calcd for $C_{94}H_{140}N_{20}O_{30}S \cdot 2AcOH \cdot 18H_2O$: C, 46.95; H, 7.40; N, 11.18. Found: C, 47.32; H, 7.58; N, 11.03.

H–Leu–Lys–Leu–Val–Pro–Pro–Met–Glu–Glu–Asp–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–OH (IX)——This was obtained in the same manner as described for fragment VIII. Thus, fragment IV (16.0 mg) dissolved in 0.2 M NH_4HCO_3 (1.60 ml; pH 7.9) was treated with carboxypeptidase B (1.07 mg) and incubated at 37 °C for 2 d. Purification of the mixture by preparative HPLC as described above, followed by lyophilization, gave a white fluffy powder; yield 8.48 mg (57%), $[\alpha]_D^{27}$ −80.0° (*c*=0.12, 1 N AcOH), Rf^4 0.77. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.06, Ser 0.90, Glu 2.96, Pro 3.95, Gly 1.01, Val 0.99, Met 0.97, Leu 1.91, Tyr 1.00, Phe 1.00, Lys 0.91 (average recovery 95%). *Anal.* Calcd for $C_{90}H_{135}N_{19}O_{27}S \cdot 2AcOH \cdot 16.5H_2O$: C, 47.74; H, 7.50; N, 11.26. Found: C, 47.42; H, 7.38; N, 11.22.

Ac–Leu–Lys(Z)–OH (X-1)——Boc–Leu–Lys(z)–OH[1b] (2.13 g) was treated with TFA (6 ml) in the usual manner. IPE was added to the residue. The resulting powder was collected by filtration, dissolved in DMF (10 ml) and neutralized with Et_3N. AcONp (0.97 g) and Et_3N (0.62 ml) were added to this solution, followed by overnight stirring at room temperature. The solvent was removed by evaporation, and the residue was dissolved in AcOEt. This solution was washed with 5% citric acid and H_2O, dried over Na_2SO_4 and evaporated. Addition of IPE to the residue gave a powder, which was recrystallized from MeOH and IPE; yield 1.12 g (56%), mp 132—134 °C, $[\alpha]_D^{26}$ −15.3° (*c*=1.1, MeOH), Rf^2 0.56. *Anal.* Calcd for $C_{22}H_{33}N_3O_6$: C, 60.67; H, 7.64; N, 9.65. Found: C, 60.50; H, 7.54; N, 9.67.

Boc–Tyr–Pro–NH_2 (X-2)——Boc–Pro–NH_2 (5.22 g) was treated with TFA (15 ml) in the usual manner. The resulting powder was dissolved in DMF (10 ml) and neutralized with Et_3N. To this solution, the azide (prepared from 6.58 g of Boc–Tyr–$NHNH_2$ with 7.61 ml of 7.62 N HCl–DMF, 4.20 ml of isoamyl nitrite and 11.13 ml of Et_3N) in DMF (10 ml) was added, and the mixture was stirred at 4 °C for 48 h. After evaporation of the solvent, the residue was dissolved in AcOEt. This solution was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4, and evaporated. Treatment of the residue with IPE gave white crystals, which were recrystallized from MeOH and ether; yield 5.25 g (62%), mp 105—108 °C, $[\alpha]_D^{28}$ −27.8° (*c*=1.0, MeOH), Rf^1 0.57. *Anal.* Calcd for $C_{19}H_{27}N_3O_5$: C, 60.46; H, 7.21; N, 11.13. Found: C, 60.56; H, 7.35; N, 11.43.

Boc–Asp(OBzl)–Tyr–Pro–NH_2 (X-3)——Compound X-2 (5.14 g) was treated with TFA (20 ml) in an ice-bath for 1h. A white powder that formed on addition of ether was collected by filtration, dried over KOH pellets *in vacuo*, dissolved in DMF (20 ml), and neutralized with *N*-methylmorpholine. Boc–Asp(OBzl)–ONp (6.35 g) and *N*-methylmorpholine (1.20 ml) were added to the solution and the mixture was stirred at room temperature overnight. Removal of the solvent by evaporation gave a residue, which was dissolved in AcOEt. This solution was washed as usual with acid and base, dried over Na_2SO_4, and evaporated to give a residue, which was used for the next step without further purification; yield 5.23 g (66%), Rf^1 0.60.

Boc–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–NH_2 (X-4)——Compound X-3 (2.00 g) was treated with TFA (8 ml) in the usual manner, and coupled with Boc–Glu(OBzl)–ONp (1.73 g) as described above. The residue was further purified by silica-gel column chromatography (150 g, 4.5 x 23 cm) with $CHCl_3$–MeOH (20 : 1) as an eluent to afford the desired product after recrystallization from MeOH and ether; yield 1.33 g (48%), mp 88—90 °C, $[\alpha]_D^{26}$ −34.0° (*c*=1.1, MeOH), Rf^1 0.60. *Anal.* Calcd for $C_{42}H_{51}N_5O_{11}$: C, 62.91; H, 6.41; N, 8.73. Found: C, 63.03; H, 6.65; N, 8.46.

Boc–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–NH_2 (X-5)——The above tetrapeptide amide (1.27 g) was treated with TFA (5 ml) as usual. The residue obtained by evaporation of the TFA was dissolved in DMF (6 ml) and neutralized with Et_3N. DCC (0.37 g) and HOBT·H_2O (0.27 g) were added to a solution of Boc–Met(O)–Glu(OBzl)–OH[1a] (0.81 g), and the N^{α}-deprotected peptide solution was added to this mixture. The reaction mixture was stirred at room temperature overnight. After filtration to remove DCurea, the filtrate was evaporated, and the residue was dissolved in *n*-butanol. This solution was washed with the usual acid and base, dried over $MgSO_4$ and evaporated. The residue was treated with ether and precipitated from MeOH with ether; yield 1.81 g (98%), mp 103—106 °C, $[\alpha]_D^{26}$ −30.5° (*c*=1.1, MeOH), Rf^1 0.60. *Anal.* Calcd for $C_{59}H_{73}N_7O_{16}S \cdot 2H_2O$: C, 58.84; H, 6.44; N, 8.14. Found: C, 58.78; H, 6.28; N, 8.20.

Boc–Leu–Val–Pro–Pro–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp–(OBzl)–Tyr–Pro–NH_2 (X-6)——The above hexapeptide amide (1.79 g) was treated with TFA (8 ml) as usual, and coupled with the azide (prepared from 0.94 g of Boc–Leu–Val–Pro–Pro–$NHNH_2$ with 0.55 ml of 7.62 N HCl–DMF, 0.28 ml of isoamyl nitrite and 0.79 ml of Et_3N) in DMF (3 ml). Work-up as described for X-2 gave a white powder, which was precipitated from MeOH–AcOEt with ether; yield 1.81 g (76%), mp 119—121 °C, $[\alpha]_D^{26}$ −77.6° (*c*=1.1, MeOH), Rf^1 0.62. *Anal.* Calcd for $C_{80}H_{107}N_{11}O_{20}S \cdot 4H_2O$: C, 58.34; H, 7.04; N, 9.36. Found: C, 58.37; H, 6.84; N, 9.43.

Ac–Leu–Lys(Z)–Leu–Val–Pro–Pro–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–NH_2 (X-7)——The above decapeptide amide (0.60 g) was treated with TFA (3 ml) in the usual manner. The TFA salt was dissolved in DMF (3 ml), neutralized with Et_3N and treated with a solution of DCC (0.15 g), HOBT·H_2O (0.10 g) and Ac–Leu–Lys(Z)–OH (X-1) (0.20 g) in DMF (2 ml). The mixture was stirred at room temperature overnight, then filtered. The filtrate was concentrated *in vacuo* and the residue was treated with H_2O and ether. The resulting powder was washed batchwise with acid and base as usual, followed by precipitation from MeOH with ether; yield 0.66 g (87%), mp 152—155 °C, $[\alpha]_D^{26}$ −70.4° (c=1.1, MeOH), Rf^1 0.67. Amino acid ratios in a 6 N HCl hydrolysate: Asp 0.93, Glu 1.86, Pro 2.94, Val 1.00, Met 0.55, Leu 2.09, Tyr 0.72, Lys 1.09 (average recovery 92%). *Anal.* Calcd for $C_{97}H_{130}N_{14}O_{23}S \cdot 2H_2O$: C, 60.42; H, 7.01; N, 10.17. Found: C, 60.45; H, 7.03; N, 9.78.

Ac–Asp(OBzl)–Leu–Lys(Z)–OH (XI-1)——This compound was prepared from Boc–Asp(OBzl)–Leu–Lys(Z)–OH (I-11)[1b] (0.40 g) in the same manner as described for the synthesis of Ac–Leu–Lys(Z)–OH (X-1). TFA treatment of I-11 and coupling with AcONp (0.13 g) gave the product, which was recrystallized from MeOH and IPE; yield 0.34 g (89%), mp 123—127 °C, $[\alpha]_D^{26}$ −25.6° (c=1.1, MeOH), Rf^2 0.62. *Anal.* Calcd for $C_{33}H_{44}N_4O_9$: C, 61.86; H, 6.92; N, 8.75. Found: C, 61.89; H, 6.84; N, 8.86.

Ac–Asp(OBzl)–Leu–Lys(Z)–Leu–Val–Pro–Pro–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–NH_2 (XI-2)——This compound was prepared by the condensation of a TFA (3 ml)-treated sample of X-6 and XI-1 (0.29 g) with DCC (0.10 g) and HOBT·H_2O (0.07 g) in DMF (3 ml) as usual. After work-up as described for X-7, the resulting powder was precipitated from MeOH and ether; yield 0.79 g (98%), mp 166—169 °C, $[\alpha]_D^{26}$ −79.2° (c=0.5, MeOH), Rf^1 0.66. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.82, Glu 1.86, Pro 2.95, Val 1.00, Met 0.60, Leu 2.03, Tyr 0.76, Lys 1.11 (average recovery 91%). *Anal.* Calcd for $C_{108}H_{141}N_{15}O_{26}S \cdot 3H_2O$: C, 60.29; H, 6.89; N, 9.77. Found: C, 59.73; H, 6.41; N, 9.76.

Boc–Pro–Pro–NH_2 (XII-1)——In the usual manner, DCC (2.14 g) and HOBT·H_2O (1.0 g) were added to a solution of a TFA (8 ml)-treated sample of Boc–Pro–NH_2 (2.28 g) and Boc–Pro–OH (2.05 g) in THF (10 ml). After usual work-up, a low-melting mass was obtained; yield 1.88 g (57%), Rf^1 0.76.

Boc–Leu–Val–Pro–Pro–NH_2 (XII-2)——A TFA (8 ml) treated sample of XII-1 (1.88 g) was condensed with the azide (prepared from 2.25 g of Boc–Leu–Val–$NHNH_2$ with 4.45 ml of 3.87 N HCl–DMF, 1.16 ml of isoamyl nitrite and 3.23 ml of Et_3N) in DMF (10 ml). After usual work-up, the residue was treated with ether and IPE to afford a white powder, which was recrystallized from ether and IPE–n-hexane; yield 1.12 g (40%), mp 110—114 °C, $[\alpha]_D^{26}$ −104.3° (c=1.2, MeOH), Rf^1 0.71. *Anal.* Calcd for $C_{26}H_{45}N_5O_6$: C, 59.63; H, 8.66; N, 13.38. Found: C, 59.56; H, 8.45; N, 13.12.

Boc–Asp(OBzl)–Leu–Lys(Z)–Leu–Val–Pro–Pro–NH_2 (XII-3)——The above protected tetrapeptide amide (XII-2) (1.13 g) was treated with TFA (5 ml). The resulting TFA salt was neutralized and condensed with Boc–Asp(OBzl)–Leu–Lys(Z)–OH (I-11)[1b] (1.64 g) with DCC (0.49 g) and HOBT·H_2O (0.36 g). After usual work-up, ether was added to the residue and the product was precipitated from MeOH–THF with ether; yield 1.72 g (72%), mp 140—142 °C, $[\alpha]_D^{26}$ −52.2° (c=1.1, DMF), Rf^1 0.63. *Anal.* Calcd for $C_{57}H_{85}N_9O_{13}$: C, 60.99; H, 7.81; N, 11.23. Found: C, 60.98; H, 7.72; N, 11.06.

Boc–Leu–Phe–Gly–Pro–Asp(OBzl)–Leu–Lys(Z)–Leu–Val–Pro–Pro–NH_2 (XII-4)——A TFA (8 ml)-treated sample of XII-3 (1.61 g) was condensed with the azide (prepared from 0.96 g of Boc–Leu–Phe–Gly–Pro–$NHNH_2$ with 1.36 ml of 3.87 N HCl–DMF, 0.29 ml of isoamyl nitrite and 0.93 ml of Et_3N) in DMF (4 ml). After usual work-up, treatment of the residue with IPE gave a fine powder, which was precipitated from MeOH–THF with IPE; yield 2.00 g (90%), mp 146—148 °C, $[\alpha]_D^{26}$ −54.5° (c=1.2, DMF), Rf^1 0.58. Amino acid ratios in a 6 N HCl hydrolysate: Asp 0.98, Pro 2.59, Gly 0.99, Val 0.85, Leu 2.81, Phe 1.00, Lys 1.03 (average recovery 92%). *Anal.* Calcd for $C_{79}H_{115}N_{13}O_{17} \cdot 1.5H_2O$: C, 61.38; H, 7.69; N, 11.78. Found: C, 61.31; H, 7.61; N, 11.57.

Boc–Gly–Asp(OBzl)–Lys(Z)–Leu–Phe–Gly–Pro–Asp(OBzl)–Leu–Lys(Z)–Leu–Val–Pro–Pro–NH_2 (XIII-1)——This compound was prepared by the condensation of a TFA (5 ml)-treated sample of XII-4 (0.50 g) and Boc–Gly–Asp(OBzl)–Lys(Z)–OH (I-13)[1b] (0.32 g) with DCC (0.12 g) and HOBT·H_2O (0.08 g) in DMF (3 ml). After usual work-up, the resulting powder was washed batchwise with the usual acid and base and precipitated from MeOH–THF with ether; yield 0.62 g (92%), mp 201—205 °C, $[\alpha]_D^{26}$ −54.7° (c=0.6, DMF), Rf^1 0.69. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.99, Pro 2.50, Gly 2.03, Val 0.93, Leu 3.20, Phe 1.00, Lys 2.02 (average recovery 92%). *Anal.* Calcd for $C_{106}H_{147}N_{17}O_{24} \cdot 4H_2O$: C, 60.18; H, 7.39; N, 11.26. Found: C, 60.32; H, 7.09; N, 11.01.

H–Asp–Leu–Lys–Leu–Val–Pro–Pro–Met–Glu–Glu–Asp–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–Lys–OH (III)——The protected nonadecapeptide ester (I-14)[1b] (817 mg) was treated with 1 M TFMSA–thioanisole in TFA (15.88 ml) in the presence of m-cresol (2.27 ml) in an ice-bath for 30 min, and then at room temperature for 1 5.h. The solvent was removed by evaporation *in vacuo* and then dry ether was added to the residue. The resulting powder was collected by filtration, washed with ether, and dried over KOH pellets *in vacuo* for 1 h. The deprotected crude peptide was dissolved in H_2O (8 ml) containing a few drops of acetic acid, and treated with Amberlite IRA-410 (acetate form) for 30 min in an ice-bath. After filtration, the filtrate was adjusted to pH 10.0 with 5% NH_4OH, stirred for 30 min in an ice-bath,[16] then readjusted to pH 4.0 with 1 N acetic acid and lyophilized to give a hygroscopic powder. This was dissolved in 1 N acetic acid (3 ml). The solution was applied to a Sephadex G-25 column (3.3 × 136 cm), which was eluted with 1 N acetic acid at a flow rate of 81.2 ml/h. The UV absorption at 275 nm was

TABLE I. Deprotection, Purification Methods and Physical Properties of the Peptides

	II	IV	V	VI	X	XI	XII	XIII
Protected peptide (mg)	120	280	200	200	200	200	200	200
Deprotection method[a]	A	A	A	A	A	A	C	C
Purification method[b]	B	B	A	A	C	C	D	D
Yield (mg)	16.8	31.2	28.8	30.0	38.0	23.0	40.0	25.0
(%)	(18%)	(15%)	(19%)	(21%)	(25%)	(14%)	(23%)	(16%)
Rf	Rf^4 0.74	Rf^4 0.70	Rf^3 0.33	Rf^3 0.32	Rf^3 0.31	Rf^3 0.21	Rf^3 0.42	Rf^3 0.11
$[\alpha]_D^{25}$ in 1 N AcOH	−91.4°	−122.0°	−107.2°	−78.8°	−137.9°	−148.0°	−141.4°	−136.9°
	c=0.1	c=0.1	c=0.3	c=0.2	c=0.2	c=0.2	c=0.1	c=0.1
Retention time on HPLC[c] (min)	18.5 (1)	13.0 (2)	16.0 (3)	19.5 (1)	23.0 (4)	24.0 (4)	15.7 (3)	16.0 (3)

a) Deprotection method A: 1 M TFMSA–thioanisole in TFA with *m*-cresol. Method B: 1) TFA, 2) H_2/Pd–C in MeOH. Method C: 1 M TFMSA–thioanisole in TFA. *b*) Purification method A: 1) Amberlite IRA 410 (acetate form), 2) 5% NH_4OH, 3) Sephadex G-25, 4) 2-mercaptoethanol, 5) DEAE-cellulose. Method B: 1—5) are the same procedures as described above in A, 6) reversed-phase HPLC. Method C: the same procedures as in B, but step 2) is omitted. Method D: 1) Amberlite IRA 410 (acetate form), 2) Sephadex G-25, 3) CM-cellulose, 4) reversed-phase HPLC. *c*) HPLC solvent system A: 0.05% TFA–H_2O. System B: 0.05% TFA–CH_3CN. (1) B: 10%—50%/30 min (1.0 ml/min). (2) B: 10%—50%/15 min (1.0 ml/min). (3) B: 10%—50%/20 min (1.0 ml/min). (4) B: 0%—50%/35 min (1.0 ml/min).

TABLE II. Elemental Analysis of the Peptides

	Formula	Analysis (%) Calcd (Found)		
		C	H	N
II	$C_{122}H_{182}N_{26}O_{35}S \cdot 3AcOH \cdot 24H_2O$	47.78 (47.96)	7.58 (7.83)	11.32 (11.03)
IV	$C_{96}H_{147}N_{21}O_{28}S \cdot 3AcOH \cdot 19H_2O$	47.16 (47.32)	7.64 (7.48)	11.32 (11.02)
V	$C_{84}H_{124}N_{18}O_{26}S \cdot 2AcOH \cdot 18H_2O$	46.39 (46.68)	7.43 (7.35)	11.07 (10.78)
VI	$C_{63}H_{90}N_{14}O_{22}S \cdot 2AcOH \cdot 15.5H_2O$	44.05 (43.94)	7.12 (7.21)	10.73 (10.58)
X	$C_{68}H_{106}N_{14}O_{20}S \cdot 2AcOH \cdot 20H_2O$	44.30 (43.66)	7.95 (8.01)	10.05 (10.23)
XI	$C_{72}H_{111}N_{15}O_{23}S \cdot 2AcOH \cdot 19H_2O$	44.54 (44.48)	7.72 (7.85)	10.25 (10.52)
XII	$C_{59}H_{95}N_{13}O_{13} \cdot 2AcOH \cdot 14.5H_2O$	48.02 (47.98)	8.44 (8.23)	11.56 (11.62)
XIII	$C_{71}H_{115}N_{17}O_{18} \cdot AcOH \cdot 17H_2O$	46.68 (46.81)	8.19 (8.25)	12.02 (11.82)

TABLE III. Amino Acid Analysis of the Peptides (6 N HCl Hydrolysis)

	II	IV	V	VI	X	XI	XII	XIII
Asp	2.07 (2)	1.01 (1)	0.95 (1)	1.06 (1)	1.02 (1)	2.14 (2)	1.03 (1)	1.87 (2)
Ser	0.84 (1)	0.91 (1)	0.63 (1)	0.78 (1)				
Glu	3.15 (3)	2.92 (3)	2.67 (3)	3.01 (3)	1.95 (2)	2.13 (2)		
Pro	5.30 (5)	4.01 (4)	3.78 (4)	2.00 (2)	3.24 (3)	3.15 (3)	2.79 (3)	2.80 (3)
Gly	1.95 (2)	0.96 (1)	1.00 (1)	1.03 (1)			1.06 (1)	1.92 (2)
Val	1.10 (1)	0.99 (1)	0.99 (1)		1.08 (1)	1.14 (1)	1.00 (1)	1.00 (1)
Met	1.08 (1)	0.96 (1)	0.85 (1)	0.98 (1)	1.00 (1)	1.02 (1)		
Leu	3.30 (3)	2.06 (2)	1.22 (1)		2.08 (2)	2.11 (2)	3.13 (3)	2.80 (3)
Tyr	0.88 (1)	0.98 (1)	0.76 (1)	0.96 (1)	1.04 (1)	1.07 (1)		
Phe	2.00 (2)	1.00 (1)	1.00 (1)	1.00 (1)			1.07 (1)	1.00 (1)
Lys	2.10 (2)	1.96 (2)	0.94 (1)	1.06 (1)	1.00 (1)	1.00 (1)	0.99 (1)	1.80 (2)
Rec. (%)	90	91	90	93	92	90	93	91

determined for each fraction (10 ml). The fractions corresponding to the front main peak (tube Nos. 66—84) were combined and the solvent was removed by lyophilization to give a white fluffy powder.

The above product was dissolved in H_2O (10 ml), then incubated with 2-mercaptoethanol (0.70 ml) under an N_2 atmosphere at 70 °C for 18 h.[17] H_2O was added to this mixture, and the solvent was lyophilized. The crude product was dissolved in H_2O (4 ml), and the solution was applied to a column of DEAE-cellulose (2.5 × 40 cm), which was eluted first with 0.02 M NH_4HCO_3, and then with a linear gradient formed from 0.02 M NH_4HCO_3 (800 ml) and 0.25 M NH_4HCO_3 (800 ml) at a flow rate of 79.2 ml/h. The UV absorption in each fraction (11.3 ml) was determined. The fractions corresponding to the main peak (tube Nos. 97—110) were combined and the solvent and ammonium salt were removed by repeated lyophilization. The powder obtained was dissolved in 1 N acetic acid and lyophilized to give the acetate as a white fluffy powder; yield 114 mg (19.3%), $[\alpha]_D^{25}$ −109.6° (c=0.3, 1 N AcOH), Rf^4 0.77. The synthetic peptide (III) thus purified exhibited a single peak on HPLC at a retention time of 14.9 min on a Cosmosil $5C_{18}$-P column (4.6 × 150 mm). Amino acid ratios in a 6 N HCl hydrolysate: Asp 2.04 (2), Ser 0.77 (1), Glu 2.88 (3), Pro 3.68 (4), Gly 1.02 (1), Val 1.05 (1), Met 0.78 (1), Leu 2.05 (2), Tyr 0.86 (1), Phe 1.00 (1), Lys 2.01 (2) (recovery 91%). *Anal.* Calcd for $C_{100}H_{152}N_{22}O_{31}S \cdot 3AcOH \cdot 19H_2O$: C, 46.93; H, 7.51; N, 11.36. Found: 47.01; H, 7.59; N, 11.13.

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Synthesis and Pharmacological Activities of 3-Phenyl-2-(1-piperazinyl)quinolines and Related Compounds

KATSUHIKO HINO,* YASUTAKA NAGAI and HITOSHI UNO

Research Laboratories, Dainippon Pharmaceutical Co., Ltd., 33–94, Enoki-cho, Suita, Osaka 564, Japan

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Various 3-phenyl-2-piperazinylquinolines and their 3-methyl analogs were synthesized and the effects of these compounds on the central nervous system were evaluated pharmacologically in mice. Some compounds having a 3-phenyl group showed potent antagonism of maximal electroshock seizures, but the 3-methyl analogs had no significant activity in any of the tests.

Keywords——3-phenyl-2-piperazinylquinoline; 3-methyl-2-piperazinylquinoline; anticonvulsant activity; anti-maximal electroshock seizure activity; central nervous system activity; structure–activity relationship; phosphorus pentachloride

It has been reported that 2-(1-piperazinyl)quinoline (I) (quipazine) acts on the central nervous system (CNS), showing some activity in common with tricyclic antidepressants.[1] We have reported the potential antidepressant activity of 4-phenyl-2-piperazinylquinolines (II)[2] in mice. Among these compounds, 2-(4-ethyl-1-piperazinyl)-4-phenylquinoline dihydrochloride (IIa, AD-1308) was examined by Karasawa *et al.* of this laboratory, in order to establish the mechanism of its antidepressant action compared with that of tricyclic antidepressants.[3] Recently the effect on the antidepressant activity caused by replacing the piperazinyl group in IIa with either NH, NCH_3, O or S having an open chain (dialkylamino)alkyl group has been examined, and it was confirmed that the 2-piperazinyl moiety caused the optimal antagonistic effect on reserpine-induced hypothermia.[4]

We were interested in synthesizing 3-phenyl-2-piperazinylquinolines (III, R^2 = Ph), which have a phenyl group at the 3 position instead of the 4 position, in the hope of finding compounds having a new profile of activity, and also determining the structure–activity relationships in this class of compounds acting on the CNS. Such compounds have never previously been reported. This paper deals with the synthesis of the above compounds and their 3-methyl analogs (III, $R^2=CH_3$), and the results of primary evaluation of their pharmacological activity on the CNS.

Catalytic hydrogenation of (*E*)-*o*-nitro-α-phenylcinnamic acid (1)[5] over Pd–C afforded

I II III

IIa : R=Et

Chart 1

3,4-dihydro-3-phenylcarbostyril (**3a**) in good yield. The reaction of **3a** with bromine afforded the 6-bromo derivative (**3b**) accompanied with a trace amount of 6-bromocarbostyril (**4b**). The Schmidt reaction of 2-methylbenzocyclopentan-1-one (**5**),[6] on the other hand, gave 3,4-dihydro-3-methylcarbostyril (**6a**) although in rather low yield. The reaction of **6a** with bromine gave the 6-bromo derivative (**6b**).

Treatment of the dihydrocarbostyrils (**3a**, **b** and **6a**, **b**) with phosphorus pentachloride (PCl_5) afforded a mixture of three products, 3-chloro-3,4-dihydrocarbostyrils (**7a**, **b** and **8a**, **b**), carbostyrils (**4a**, **b** and **9a**, **b**) and 2-chloroquinolines (**10a**, **b** and **11a**, **b**), respectively. 3-Phenylcarbostyril (**4a**) was identical with the compound obtained by the cyclization of **2**, which was prepared by the selective hydrogenation of the nitro group of **1**, with acetic anhydride and concentrated sulfuric acid.[7] The structures of these products were determined on the basis of spectroscopic and chemical evidence. On treatment with potassium carbonate (K_2CO_3), the α-chlorolactams (**7a** and **8b**) lost hydrogen chloride with ease to give the carbostyrils (**4a** and **9b**). The conversion of **4a** into **10a** was incomplete on treatment with PCl_5 and phosphorus oxychloride ($POCl_3$), but with the Vilsmeier reagent, this conversion proceeded in an excellent yield. On the other hand, the treatment of the 3-methyl analogs (**9a** and **9b**) with $POCl_3$ readily gave the 2-chloro derivatives (**11a** and **11b**). The chlorides (**10a**, **b**

1 —(H_2, Pd-C)→ 2 —(H_2, Pd-C)→ 3a —(Br_2)→ 3b + 4b

2 —(Ac_2O–H_2SO_4)→ 4a

5 —(NaN_3, PPA)→ 6a : R^1=H; 6b : R^1=Br

3a, 3b, 6a, 6b —(PCl_5)→ 7a, 7b, 8a, 8b + 4a, 4b, 9a, 9b + 10a, 10b, 11a, 11b

3a : R^1=H, R^2=Ph
3b : R^1=Br, R^2=Ph
6a : R^1=H, R^2=CH_3
6b : R^1=Br, R^2=CH_3

7a : R^1=H, R^2=Ph
7b : R^1=Br, R^2=Ph
8a : R^1=H, R^2=CH_3
8b : R^1=Br, R^2=CH_3

4a : R^1=H, R^2=Ph
4b : R^1=Br, R^2=Ph
9a : R^1=H, R^2=CH_3
9b : R^1=Br, R^2=CH_3

10a : R^1=H, R^2=Ph
10b : R^1=Br, R^2=Ph
11a : R^1=H, R^2=CH_3
11b : R^1=Br, R^2=CH_3

10a,b / 11a,b —(HN⟨⟩NR^3)→

12a–d : R^1=H, R^2=Ph
12e,f : R^1=Br, R^2=Ph
13a–c : R^1=H, R^2=CH_3
13d,e : R^1=Br, R^2=CH_3

Chart 2

TABLE I. Physicochemical Properties and Analytical Data of Intermediates, **3b**, **4b**, **6b**, **7a**, **7b**, **8a**, **8b**, **9b**, **10b**, and **11b**

Compd. No.	mp (°C)	Recryst. solvent[a]	Formula	Analysis (%) Calcd (Found)				
				C	H	Br	Cl	N
3b	175	C–E	$C_{15}H_{12}BrNO$	59.62	4.00	26.45		4.64
				(59.70	3.89	26.57		4.60)
4b	258	C–E	$C_{15}H_{10}BrNO$	60.02	3.36	26.62		4.67
				(60.27	3.21	26.48		4.69)
7a	188—189	C–Al	$C_{15}H_{12}ClNO$	69.90	4.69		13.76	5.44
				(69.66	4.44		13.59	5.23)
7b	190	E–H	$C_{15}H_{11}BrClNO$	53.52	3.29	23.74	10.53	4.16
				(53.47	3.09	23.71	10.77	4.10)
10b	144—145	E–H	$C_{15}H_{9}BrClN$	56.55	2.85	25.08	11.13	4.40
				(56.78	2.73	25.18	11.18	4.47)
6b	157—159	C–E	$C_{10}H_{10}BrNO$	50.02	4.20	33.28		5.83
				(49.86	4.20	33.11		5.73)
8a	175—177	Ac–H	$C_{10}H_{10}ClNO$	61.37	5.15		18.12	7.16
				(61.19	5.24		18.16	7.09)
8b	194—195	C–H	$C_{10}H_{9}BrClNO$	43.74	3.30	29.11	12.91	5.10
				(43.98	3.12	29.10	12.95	5.01)
9b	248—251	C–H	$C_{10}H_{8}BrNO$	50.45	3.39	33.57		5.88
				(50.32	3.23	33.34		5.81)
11b	146	Ac–H	$C_{10}H_{7}BrClNO$	46.82	2.75	31.15	13.82	5.46
				(46.79	2.68	31.18	13.84	5.54)

a) Recrystallization solvents used were as follows: Ac, acetone; Al, EtOH; C, $CHCl_3$; E, Et_2O; H, hexane.

and **11a**, **b**) were allowed to react with various piperazines to give the desired 2-piperazinyl derivatives (**12a**—**f** and **13a**—**e**). The results are summarized in Tables I and II.

Pharmacology

The compounds listed in Table II were examined for CNS activities in mice at 100 mg/kg *p.o.* in a set of primary tests and the results are summarized in Table III in comparison with those for IIa (which has potent antidepressant activity) and quipazine (I). Compounds **12b**—**d**, **f** having a 3-phenyl group, but not **12a** or **12e**, exhibited potent anticonvulsant activity as measured in terms of protection against maximal electroshock seizure (MES), and the potency was comparable to that of the antiepileptic drug, carbamazepine. Neither antagonistic effect against reserpine-induced hypothermia nor neuroleptic-like properties were shown by **12a**—**f**. This is in contrast to the activity profile of the 4-phenyl isomers.[2] Some of the compounds (**12a**—**d**) showed a mild antagonizing effect on tremor induced by tremorine, comparable to that of IIa or quipazine. The 3-methyl derivatives (**13a**—**e**) showed weak or no activities in the above tests. Weak activity was observed only in the anti-reserpine (**13a**, **b**) and anti-MES (**13d**, **e**) tests.

From these results, all the compounds examined in this study proved to have different CNS pharmacological activity profiles from those of the potentially antidepressive 4-phenyl derivatives and quipazine. It appears that the phenyl group at the 4 position but not the 3 position in the quinoline nucleus, in combination with the 2-piperazinyl moiety, is essential for potent antidepressant activity, and that the aromatic group conjugating with the quinoline nucleus plays an important role in modifying the biological response. Structural modification of biologically active compounds by introducing such a group seems to be a useful strategy for finding compounds with different activity profiles.

TABLE II. Physicochemical Properties and Analytical Data of 2-Piperazinylquinolines (12 and 13)

Compd. No.	R^1	R^2	R^3	mp (°C) Recryst. solvent[a]	Yield (%)	Formula	Analysis (%) Calcd (Found)				
							C	H	Br	Cl	N
12a	H	Ph	H	184—186	83	$C_{19}H_{19}N_3\cdot$	68.13	5.72			10.36
				Al		$C_4H_4O_4$[b]	(68.37	5.93			10.34)
12b	H	Ph	CH_3	258—260	76	$C_{20}H_{21}N_3\cdot$	60.91	6.39		17.98	10.66
				Al–Ac		$2HCl\cdot H_2O$	(60.70	6.26		17.70	10.69)
12c	H	Ph	C_2H_5	240—242	72	$C_{21}H_{23}N_3\cdot$	61.76	6.67		17.56	10.29
				Al–Ac		$2HCl\cdot H_2O$	(61.63	6.68		17.78	10.21)
12d	H	Ph	CH_2CH_2OH	184—189	75	$C_{21}H_{23}N_3O\cdot$	59.43	6.41		16.71	9.90
				Al		$2HCl\cdot H_2O$	(59.34	6.31		16.55	9.82)
12e	Br	Ph	CH_3	147	87	$C_{20}H_{20}BrN_3$	62.83	5.27	20.95		10.99
				E–H			(63.08	5.31	20.90		10.80)
12f	Br	Ph	CH_2CH_2OH	250—255	80	$C_{21}H_{22}BrN_3O\cdot$	50.12	5.21	15.88	14.09	8.35
				Al		$2HCl\cdot H_2O$	(49.98	5.37	15.71	13.95	8.32)
13a	H	CH_3	CH_3	241—243	67	$C_{15}H_{19}N_3\cdot$	50.14	7.29		19.74	11.69
				Al		$2HCl\cdot 5/2\,H_2O$	(50.37	6.99		19.51	11.77)
13b	H	CH_3	C_2H_5	220—225	40	$C_{16}H_{21}N_3\cdot$	49.11	7.73		18.12	10.74
				Al		$2HCl\cdot 7/2\,H_2O$	(49.01	7.76		17.89	10.77)
13c	H	CH_3	CH_2CH_2OH	225—229	55	$C_{16}H_{21}N_3O\cdot$	55.82	6.73		20.60	12.20
				Al		$2HCl$	(55.92	6.91		20.41	12.17)
13d	Br	CH_3	CH_3	97—98	88	$C_{15}H_{18}BrN_3$	56.26	5.66	24.96		13.12
				E–H			(56.27	5.55	24.86		13.04)
13e	Br	CH_3	CH_2CH_2OH	103	87	$C_{16}H_{20}BrN_3O$	54.86	5.76	22.82		12.00
				E			(54.99	5.74	22.76		11.91)

a) For abbreviations of recrystallization solvents, see footnote *a*) in Table I. *b*) Maleate.

TABLE III. Results of Primary Tests at 100 mg/kg, *p.o.* in Mice

Compd. No.	Anti-MES activity[a]	Anti-reserpine activity (%)	Anti-tremorine activity (%)
12a	0/5	−14	20
12b	4/5	−21	47
12c	5/5	−21	33
12d	5/5	−9	33
12e	1/5	−40	0
12f	5/5	−6	0
13a	0/5	21	0
13b	0/5	19	0
13c	0/5	7	0
13d	1/5	−7	0
13e	2/5	−3	0
Carbamazepine	5/5		
Quipazine (I)		46	40
IIa	0/5	85	53

a) Number of positive effects/number of mice tested.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Proton magnetic resonance (^{1}H-NMR) spectra were taken on a Varian HA-100 or Varian A-60 spectrometer using

tetramethylsilane as an internal standard. Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. The chemical shifts are given as δ (ppm). Mass spectra (MS) were taken on a JEOL JMS-D300 spectrometer and infrared (IR) spectra were recorded on a Hitachi EPI-S2 spectrometer in KBr disks. Organic extracts were dried over magnesium sulfate.

3,4-Dihydro-3-phenylcarbostyril (3a)——Compound **1**[5] (6.4 g) was hydrogenated with 5% Pd-C (0.5 g) in MeOH at room temperature under atmospheric pressure. The theoretical amount of hydrogen (4 mol eq) was absorbed during about 6 h. The catalyst and the solvent were removed, and the residue was recrystallized to give **3a** (4.5 g, 85%), mp 170—171 °C ($CHCl_3$-EtOH) (lit. mp 170—172 °C).[8] IR: 1680 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.25 (2H, d, J=7 Hz, 4-H_2), 3.86 (1H, dd, J=7, 7 Hz, 3-H), 6.6—7.3 (4H, m, -C_6H_4-), 7.26 (5H, s, C_6H_5), 8.60 (1H, NH).

3-Phenylcarbostyril (4a)——a) Compound **1**[5] (26.9 g) was hydrogenated with 5% Pd-C (2 g) in MeOH at room temperature under atmospheric pressure. The theoretical amount of hydrogen (3 mol eq) was absorbed during about 40 min. The catalyst and the solvent were removed to give crude **2** as a crystalline residue. MS m/z: 239 (M^+). Without further purification, this crude product was treated with acetic anhydride and concentrated sulfuric acid as reported[7] to give **4a** (16 g, 73%), mp 235 °C ($CHCl_3$-EtOH) (lit. mp 228 °C).[7] ^{1}H-NMR (DMSO-d_6) δ: 8.09 (1H, s, 4-H), 7.0—7.9 (9H, m, Ar-H), 11.97 (1H, NH).

b) A mixture of **7a** (77 mg), K_2CO_3 (46 mg, 1.1 mol eq) and MeOH (10 ml) was refluxed for 30 min. After being cooled, the reaction mixture was diluted with water and the resulting precipitates were collected and recrystallized to give **4a** (62 mg, 94%).

The Reaction of 3,4-Dihydro-3-phenylcarbostyril (3a) with Bromine——A mixture of **3a** (2.23 g), $CHCl_3$ (30 ml) and Br_2 (1.92 g, 1.2 mol eq) was refluxed for 6 h. The reaction mixture was concentrated *in vacuo* and the residue was chromatographed on silica gel with $CHCl_3$. The first eluate afforded **3b** (2.68 g, 89%) after recrystallization. IR: 1690 (C=O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.18 (2H, d, J=8 Hz, 4-H_2), 3.83 (1H, t, J=8 Hz, 3-H), 6.86 (1H, d, J=9 Hz, 8-H), 7.26 (5H, s, C_6H_5), 7.35 (1H, m, 7-H), 7.41 (1H, d, J=2 Hz, 5-H), 10.46 (1H, NH). The second eluate gave **4b** (155 mg, 5%) after recrystallization. ^{1}H-NMR (DMSO-d_6) δ: 7.2—7.9 (7H, m, C_6H_5, 7-H and 8-H), 7.97 (1H, d, J=2 Hz, 5-H), 8.07 (1H, s, 4-H), 12.08 (1H, NH).

3,4-Dihydro-3-methylcarbostyril (6a)——NaN_3 (17.2 g, 1.1 mol eq) was added portionwise to a mixture of **5**[6] (35 g) and polyphosphoric acid (370 g) at 65—70 °C, and the reaction mixture was stirred for 3 h at the same temperature. After being cooled, the reaction mixture was poured into ice and water, and extracted with AcOEt. The extracts were washed with water and dried, and the solvent was evaporated off. The residue was recrystallized to give **6a** (13.7 g, 32%), mp 129—130 °C (acetone–hexane) (lit. mp 129—130 °C).[9] IR: 1665 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.29 (3H, d, J=6.5 Hz, CH_3), 2.4—3.2 (3H, m, 3-H and 4-H_2), 6.7—7.3 (4H, m, Ar-H), 9.10 (1H, NH).

6-Bromo-3,4-dihydro-3-methylcarbostyril (6b)——A mixture of **6a** (1.61 g), $CHCl_3$ (30 ml) and Br_2 (1.92 g, 1.2 mol eq) was refluxed for 4 h. The reaction mixture was concentrated *in vacuo* and the residue was recrystallized to give **6b** (2.14 g, 89%). IR: 1670 (C=O) cm^{-1}.

The Reaction of 3,4-Dihydro-3-phenylcarbostyril (3a) with PCl_5——A mixture of **3a** (1.12 g), PCl_5 (2.5 g, 2.5 mol eq) and benzene (3 ml) was refluxed for 4 h. After being cooled, the reaction mixture was diluted with water and extracted with $CHCl_3$. The extracts were washed with water and dried, and the solvent was evaporated off. The residue was chromatographed on silica gel with $CHCl_3$ to give firstly **10a** (0.48 g, 40%) as an oil. MS m/z: 239 (M^+) (lit. bp 175 °C/1.1 mm).[10] The second fraction gave **7a** (80 mg, 6%) after recrystallization. MS m/z: 257 (M^+). IR: 1680 (C=O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.52 and 3.98 (1H each, both d, J=16 Hz, 4-H_2), 6.7—7.7 (9H, m, Ar-H), 10.70 (1H, NH). The third fraction afforded **4a** (0.38 g, 41%) after recrystallization.

The Reaction of 6-Bromo-3,4-dihydro-3-phenylcarbostyril (3b) with PCl_5——Compound **3b** (2.41 g) was treated in the same manner as described for the reaction of **3a** to give the following three compounds. **10b** (0.13 g, 5%): MS m/z: 317 (M^+). **7b** (0.62 g, 23%): MS m/z: 335 (M^+). IR: 1690 (C=O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.53 and 4.07 (1H each, both d, J=17 Hz, 4-H_2), 6.87 (1H, d, J=8.5 Hz, 8-H), 7.2—7.8 (7H, m, C_6H_5, 5-H and 7-H), 10.90 (1H, NH). **4b** (1.27 g, 53%).

The Reaction of 3,4-Dihydro-3-methylcarbostyril (6a) with PCl_5——A mixture of **6a** (3.2 g), PCl_5 (10.4 g, 2.5 mol eq) and benzene (5 ml) was refluxed for 3 h. After being cooled, the reaction mixture was diluted with water and extracted with AcOEt. The extracts were washed with water and dried, and the solvent was evaporated off. The residue was chromatographed on silica gel. The eluate with benzene gave **11a** (3.2 g, 90%) after recrystallization. mp 83 °C (ether–hexane) (lit. mp 83—84 °C).[11] ^{1}H-NMR ($CDCl_3$) δ: 2.50 (3H, d, J=1 Hz, CH_3), 7.4—7.9 (4H, m, benzene ring H), 7.92 (1H, q, J=1 Hz, 4-H). The eluate with benzene–$CHCl_3$ (1:1) gave **8a** (0.21 g, 5%) after recrystallization. MS m/z: 195 (M^+). IR: 1690 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.91 (3H, s, CH_3), 3.29 (2H, s, 4-H_2), 6.8—7.4 (4H, m, Ar-H), 9.25 (1H, NH). The eluate with $CHCl_3$ gave **9a** (50 mg, 2%) after recrystallization. mp 240—242 °C ($CHCl_3$–hexane) (lit. mp 238—240 °C).[11] IR: 1650 (C=O) cm^{-1}.

The Reaction of 6-Bromo-3,4-dihydro-3-methylcarbostyril (6b) with PCl_5——Compound **6b** (4.1 g) was treated in the same manner as described for the reaction of **6a** to give the following three compounds. **11b** (1.88 g, 43%): MS m/z: 255 (M^+). **8b** (1.65 g, 35%): MS m/z: 273 (M^+). IR 1680 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.79 (3H, s, CH_3), 3.37 (2H, br s, 4-H_2), 6.90 (1H, d, J=9 Hz, 8-H), 7.41 (1H, q, J=9, 2 Hz, 7-H), 7.48 (1H, d, J=2 Hz, 5-H), 10.05 (1H,

NH). **9b** (0.19 g, 5%). IR: 1665 (C=O) cm^{-1}.

6-Bromo-3-methylcarbostyril (9b)——A mixture of **8b** (1.37 g), K_2CO_3 (0.76 g) and MeOH (30 ml) was refluxed for 15 min. After being cooled, the reaction mixture was diluted with water and the resulting precipitates were collected and recrystallized to give **9b** (1.15 g, 97%).

2-Chloro-3-phenylquinoline (10a)——a) A mixture of **4a** (2.21 g), $CHCl_3$ (40 ml), $SOCl_2$ (6 ml) and dimethylformamide (DMF) (1.3 ml) was refluxed for 1 h and concentrated *in vacuo*. The residue was diluted with water and extracted with ether. The extracts were washed with water, dried and evaporated to give **10a** (2.27 g, 95%) as a residual oil.

b) A mixture of **4a** (4.42 g), PCl_5 (20.8 g) and $POCl_3$ (20 ml) was refluxed for 18 h. After being cooled, the reaction mixture was poured into ice and water, and extracted with $CHCl_3$. The residue was recrystallized to give the starting **4a** (2.8 g) and the mother liquor was chromatographed on silica gel with $CHCl_3$ to give **10a** (1.2 g, 25%) as an oil.

6-Bromo-2-chloro-3-phenylquinoline (10b)——A mixture of **4b** (60 mg), $CHCl_3$ (5 ml), $SOCl_2$ (0.1 ml) and DMF (0.1 ml) was refluxed for 1 h and concentrated *in vacuo*. The residue was diluted with water and extracted with AcOEt. The extracts were washed with water and dried, and the solvent was evaporated off. The residue was recrystallized to give **10b** (56 mg, 88%).

2-Chloro-3-methylquinoline (11a)——A mixture of **9a** (160 mg) and $POCl_3$ (5 ml) was refluxed for 2.5 h and concentrated *in vacuo*. The residue was dissolved in ether and the solution was washed with water, dried and concentrated, then the residue was recrystallized to give **11a** (150 mg, 85%).

6-Bromo-2-chloro-3-methylquinoline (11b)——Compound **9b** (1.15 g) was treated in the same manner as described for **11a** to give **11b** (1.17 g, 95%) after recrystallization.

3-Phenyl-2-piperazinylquinolines (12a—f) and 3-Methyl-2-piperazinylquinolines (13a—e)——General Procedure: A mixture of the 2-chloro derivatives (**10a, b** and **11a, b**) and 3 mol eq of the piperazine was stirred for 4—12 h at 130 °C. The reaction mixture was dissolved in a mixture of ether and water, and the organic layer was washed with water and dried, then the solvent was evaporated off. The residue was recrystallized from an appropriate solvent directly or after the conversion into a salt. The results are summarized in Table II.

Pharmacological Methods——Male Std-ddY strain mice (Shizuoka Lab. Animal Center, SLAC, Shizuoka, Japan), weighing 20—25 g, were employed in the experiments. Test compounds were dissolved or suspended in 0.5% aqueous tragacanth and orally administered to a group of five mice.

Antagonistic Effect on Maximal Electroshock Seizure: This experiment was carried out according to the method of Masuda *et al.*[12] Test compounds were evaluated for ability to prevent the hind-limb extensor component of maximal electroshock seizure induced by applying a current of 25 mA at 60 Hz for 0.2 s, delivered through corneal electrodes 2 h after dosing.

Antagonistic Effect on Hypothermia Induced by Reserpine: This experiment was carried out according to the method described in ref. 2. The inhibitory effect was calculated as the average value (percentage), as compared with the control.

Antagonistic Effect on Tremor Induced by Tremorine: This experiment was carried out according to the method described in ref. 2. The inhibitory effect was calculated as a percentage from the scores compared with those of the control mice.

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Synthesis of 2-Phenylthiazolidine Derivatives as Cardiotonic Agents. IV.[1) Modification of the Phenylpiperazino Moiety of 2-(Phenylpiperazino-alkoxyphenyl)thiazolidine-3-carbothioamides and the Corresponding Carboxamides

HIROYUKI NATE,[a] AKISHIGE WATANABE,[a] KENJI MATSUKI,[a] ISAO INOUE,[a] HISAO OHTSUKA,[a] YASUO SEKINE,[a] KUNIYUKI ODA,[a] YASUSHI HONMA,[a] AKIHIKO ISHIDA,[a] TAKESHI KANNO,[a] MIKIHIKO KONDA,[a] HIDEO NAKAI,*[,a] HIROSHI WADA,[a] MIKIO TAKEDA,[a] HIDEO YABANA,[b] and TAKU NAGAO[b]

Organic Chemistry Research Laboratory[a] and Biological Research Laboratory,[b] Tanabe Seiyaku Co., Ltd., 2-2-50, Kawagishi, Toda-shi, Saitama 335, Japan

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Examination of the structure–activity relationships of 2-(phenylpiperazinoalkoxyphenyl)-thiazolidine-3-carbothioamides and the corresponding carboxamides (**1**) as new cardiotonic agents was extended by the chemical modification of the phenylpiperazino moiety. The 4-phenylpiperidine (**13**), 4-phenyltetrahydropyridines (**17**), and related derivatives were prepared from the chlorides (**10**) through several intermediates (**12**, **14**, and **16**) and tested for cardiotonic activity. Generally, both the 4-phenylpiperidine (**13**) and 4-phenyltetrahydropyridine (**17**) derivatives exhibited potent positive inotropic activity comparable to that of **1**. The *N*-phenylpiperidines (**9**) and amide derivatives (**22**, **25**, and **28**) exhibited no significant positive inotropy. This is also the case for the phenylpropylamines (**29**) and the ethylenediamines (**30**), which are pseudo-ring analogues of **1** with respect to the piperazine moiety. The activity of the homopiperazine derivative (**23**) was approximately one-thirtieth of that of the corresponding piperazine derivative (**1**). Thus, the presence of the six-membered, basic azacycloalkane ring (piperidine or piperazine) with a 4-phenyl group at the end of the alkoxy side chain appears to be essential for the appearance of potent positive inotropic activity in this series of compounds.

Keywords——2-(phenylpiperidinoalkoxyphenyl)thiazolidine-3-carbothioamide; 2-(phenyl-piperidinoalkoxyphenyl)thiazolidine-3-carboxamide; 2-(tetrahydropyridylalkoxyphenyl)thiazolidine-3-carbothioamide; 2-(tetrahydropyridylalkoxyphenyl)thiazolidine-3-carboxamide; 2-(phenyl-homopiperazinoalkoxyphenyl)thiazolidine-3-carboxamide; 2-phenylthiazolidine; structure–activity relationship; positive inotropic activity; cardiotonic agent

The synthesis and cardiotonic activity of novel 2-phenylthiazolidine-3-carboxamides or carbothioamides (**1**) were described in our previous papers.[1-3] Many of these derivatives exhibited potent and long-lasting positive inotropic activity without producing significant effects on heart rate or blood pressure in anesthetized dogs. In those studies, the structure–activity relationships (SAR) of **1** were examined by varying the structural parameters such as substituents (R^1—R^3, X), length of the alkyl chain (n), position of the alkoxyl group, and the chirality at C_2 in the thiazolidine ring. Our continued interest in the SAR of **1** as a new type of cardiotonic agent led to further examination of the effects of modifying the phenylpiperazino moiety. This paper describes the synthesis and cardiotonic activity of the *N*-phenylpiperidine (**9**) and 4-phenylpiperidine (**13** and **17**) congeners, in which one of the two nitrogen atoms of the piperazine was replaced by carbon. Several amide derivatives (**22**, **25**, and **28**), pseudo-ring analogues (**29** and **30**), and the homopiperazine derivative (**23**) were also synthesized and

I

Chart 1

tested for cardiotonic activity.

Chemistry

The *N*-phenylpiperidine congeners (**9**) were synthesized through the sequence of reactions outlined in Chart 2. Wittig reaction of 1-phenyl-4-piperidone (**2**)[4] with methoxymethylenetriphenylphosphorane[5] followed by acidic hydrolysis gave the aldehyde (**3**) in 47% yield. The same procedure effected the homologation of **3** to **4** in 54% yield. Sodium borohydride ($NaBH_4$) reduction of **4** readily gave the alcohol (**5a**). Wittig reaction of **3** with formylmethylenetriphenylphosphorane[6] gave the α,β-unsaturated aldehyde (**6**) in 50% yield. Catalytic hydrogenation and subsequent $NaBH_4$ reduction of **6** gave the alcohol (**5b**) in 60% yield. Tosylation of **5a, b** followed by condensation with salicylaldehyde gave the substituted benzaldehydes (**8a, b**). Reaction of **8a, b** with cysteamine and then with methyl isothiocyanate (MeNCS) readily gave the thiazolidine-3-carbothioamides (**9a, b**).

The 4-phenylpiperidine derivatives (**13**), other carba analogues of phenylpiperazine, were synthesized by the two routes shown in Chart 3. Since this type of compounds exhibited potent cardiotonic activity (see below), a number of derivatives having various substituents were synthesized. Condensation of the chlorides (**10**)[2] with various 4-phenylpiperidines (**11**)[7] followed by acidic hydrolysis gave the substituted benzaldehydes (**12**) listed in Table I. Treatment of **12** with cysteamine and then with methyl isocyanate (MeNCO) or MeNCS gave the thiazolidine-3-carboxamides or 3-carbothioamides (**13**) (method A). Alternatively, condensation of the thiazolidines (**14**)[2] with **11** also afforded **13** (method B). The physical properties of **13** are summarized in Table II. The 4-phenyltetrahydropyridine derivatives (**17**) were also

a : n = 2
b : n = 3

Chart 2

Table I. 2-Piperidinoalkoxybenzaldehydes (12 and 16)

Compd. No.	n	R	Yield (%)	mp (°C) (Recryst. solvent)[a]	^{1}H-NMR δ ($CDCl_3$, J=Hz)
12a	2	H	62.6	59—62 (A)	1.55—2.60 (7H, m), 2.89—3.35 (4H, m), 4.24 (2H, t, J=6), 6.85—7.95 (9H, m), 10.49 (1H, s)
12b	3	H	58.0	—[b]	1.53—2.69 (11H, m), 2.99—3.17 (2H, m), 4.16 (2H, t, J=6), 6.88—7.92 (9H, m), 10.53 (1H, s)
12c	2	3-F	65.9	—[b]	1.60—3.30 (11H, m), 4.27 (2H, t, J=6), 6.70—7.90 (8H, m), 10.56 (1H, s)
12d	3	3-F	63.3	—[b]	1.60—3.30 (13H, m), 4.17 (2H, t, J=6), 6.70—7.90 (8H, m), 10.45 (1H, s)
12e	2	3-Me	41.3	—[b]	1.50—2.70 (7H, m), 2.30 (3H, s), 2.70—3.30 (4H, m), 4.20 (2H, t, J=6), 6.70—7.90 (8H, m), 10.56 (1H, s)
12f	2	4-OMe	59.1	79—82[c] (B)	1.50—2.70 (7H, m), 2.70—3.30 (4H, m), 3.79 (3H, s), 4.28 (2H, t, J=6), 6.70—7.90 (8H, m), 10.56 (1H, s)
12g	3	4-OMe	71.9	52—56[d] (B)	1.50—2.80 (11H, m), 2.80—3.30 (2H, m), 3.76 (3H, s), 4.15 (2H, t, J=6), 6.70—8.00 (8H, m), 10.49 (1H, s)
16a	2	H	21.0	—[b]	2.45—3.10 (6H, m), 3.20—3.45 (2H, m), 4.20 (2H, t, J=6), 6.10 (1H, m), 6.70—7.90 (9H, m), 10.50 (1H, s)
16b	3	H	43.6	—[b]	1.77—2.31 (2H, m), 2.55—2.78 (6H, m), 3.13—3.18 (2H, m), 4.14 (2H, t, J=6), 6.10 (1H, m), 6.80—7.85 (9H, m), 10.46 (1H, s)
16c	2	3-F	61.4	—[b]	2.35—2.75 (2H, m), 2.80—3.10 (2H, m), 3.01 (2H, t, J=5.5), 3.25—3.50 (2H, m), 4.30 (2H, t, J=5.5), 6.12 (1H, m), 6.70—7.95 (8H, m), 10.51 (1H, s)
16d	3	3-F	63.7	—[b]	1.90—2.35 (2H, m), 2.40—3.00 (6H, m), 3.10—3.40 (2H, m), 4.18 (2H, t, J=6.5), 6.10 (1H, m), 6.70—7.90 (8H, m), 10.48 (1H, s)
16e	2	3-Me	40.5	—[b]	2.30—3.20 (6H, m), 2.38 (3H, s), 3.20—3.50 (2H, m), 4.30 (2H, t, J=6), 6.10 (1H, m), 6.70—7.90 (8H, m), 10.50 (1H, s)
16f	2	4-OMe	43.0	124—126[e] (B)	2.35—2.70 (2H, m), 2.70—3.20 (4H, m), 3.20—3.45 (2H, m), 3.79 (3H, s), 4.31 (2H, t, J=6), 5.97 (1H, m), 6.75—7.95 (8H, m), 10.50 (1H, s)
16g	3	4-OMe	39.1	90—93[f] (B)	1.80—2.30 (2H, m), 2.30—2.90 (6H, m), 3.00—3.30 (2H, m), 3.80 (3H, s), 4.20 (2H, t, J=6), 5.99 (1H, m), 6.70—7.90 (8H, m), 10.50 (1H, s)

a) A = Et_2O–hexane, B = AcOEt–hexane. *b*) Obtained as an oil. *c*) *Anal.* Calcd for $C_{21}H_{25}NO_3$: C, 74.31; H, 7.42; N, 4.13. Found: C, 74.15; H, 7.55; N, 4.03. *d*) *Anal.* Calcd for $C_{22}H_{27}NO_3$: C, 74.76; H, 7.70; N, 3.96. Found: C, 74.59; H, 7.78; N, 3.91. *e*) *Anal.* Calcd for $C_{21}H_{23}NO_3$: C, 74.75; H, 6.87; N, 4.15. Found: C, 74.73; H, 6.81; N, 4.12. *f*) *Anal.* Calcd for $C_{22}H_{25}NO_3$: C, 75.19; H, 7.17; N, 3.99. Found: C, 74.98; H, 7.28; N, 3.91.

synthesized in a similar manner *via* the aldehydes (**16**, Table I) or from **14**, and are listed in Table III.

The effect of conversion of the basic nitrogen in the piperazine moiety to an amido group was examined next. The amide derivatives (**22**, **25**, and **28**) were, therefore, synthesized by the usual method as outlined in Chart 4. Reaction of *N*-phenylethylenediamine (**18**) with benzyloxycarbonyl chloride (CbzCl) followed by treatment with bromoacetyl bromide and cyclization with potassium carbonate in dimethylformamide (DMF) gave the *N*-Cbz-piperazinone (**19**). Reductive removal of the Cbz group by the use of ammonium formate and palladium on carbon in methanol[8] gave the *N*-formyl derivative (**20**) and the desired

1) HN 11 2) H^+

1) HS~NH_2 2) MeNCX method A

10 12 13

1) HN 15 2) H^+

11 method B

method A

15 method B

16 17 14

Chart 3

1) CbzCl 2) $BrCH_2COBr$ 3) K_2CO_3−DMF

Pd-C/HCO_2NH_4 MeOH

18 19

20 : R=CHO
21 : R=H
Cbz=$PhCH_2OCO$

21

22a : n = 2
22b : n = 3

14a : n = 2
14b : n = 3

23

1) PhCON NH 2) H^+

10 : n = 3 24 25

1) $SOCl_2$ 2) PhN NH

26 27 28

Chart 4

Chart 5

piperazinone (21) in 75.4 and 22.9% yields, respectively. Acidic hydrolysis of **20** readily gave **21**. Condensation of the thiazolidines (**14a, b**, X = S) with **21** gave the piperazinone derivatives (**22a, b**). Reaction of **10** ($n = 3$)[2] with *N*-benzoylpiperazine[9] followed by acidic hydrolysis gave the aldehyde (**24**). The phenoxyacetic acid derivative (**26**)[3] was condensed with phenylpiperazine *via* the acid chloride to give the aldehyde (**27**). The benzaldehydes (**24** and **27**) were converted to the thiazolidine-3-carbothioamides (**25** and **28**), respectively. The homopiperazine derivative (**23**) was prepared by the reaction of **14a** (X = O) with *N*-phenylhomopiperazine.[10]

Finally, the straight chain derivatives (**29** and **30**), which may be looked upon as the pseudo-ring analogues of **13** and **1** with respect to the piperidine and piperazine moieties, were synthesized (Chart 5). Formylation of *N*-phenylethylenediamine (**18**) with ethyl formate followed by methylation with methyl iodide gave the formyl derivative (**31**) in 34% yield. Lithium aluminum hydride reduction of **31** readily gave the diamine (**32**). Condensation of **14a, b** (X = S) with *N*-methyl-3-phenylpropylamine[11] and **32** gave the thiazolidines (**29a, b** and **30a, b**), respectively.

Pharmacology and Structure–Activity Relationships

The positive inotropic activity of the piperidines (**13**), 4-phenyltetrahydropyridines (**17**), and related derivatives prepared in this study was determined by measuring the increase in the maximum derivative of left ventricular pressure (LVdP/dt_{max}) after i.v. administration to anesthetized dogs by the method reported previously.[12] The results for **13** and **17** are included in Tables II and III together with comparative data for amrinone.

Generally, both the 4-phenylpiperidine (**13**) and 4-phenyltetrahydropyridine (**17**) derivatives exhibited positive inotropic activity comparable to that of the 4-phenylpiperazine derivatives (**1**).[2] As for the positional isomers with respect to the piperidinoalkoxy group, *ortho* isomers were invariably more potent than the corresponding *para* isomers in both the 4-phenylpiperidine (**13**) and 4-phenyltetrahydropyridine (**17**) series. The piperidinoethoxy derivatives ($n = 2$) always exhibited more pronounced activity than the corresponding propoxy derivatives ($n = 3$) in both series. These SAR are in good accordance with the

TABLE II. 2-(Piperidinoalkoxyphenyl)thiazolidines (13)

Compd. No.	Position[a]	n	R	X	Yield (%)	Method[b]	Salt[c]	mp (dec.) (°C) (Recryst. solvent)[d]	Formula and Analysis (%) Calcd (Found) C H N	Myocardial contractility Anesthetized dog[e] Dose mg/kg i.v.	LVdP/dt_{max} Δ%	Duration min
13a	2	2	H	S	89.3	A	Ox	178—180 (A)	$C_{26}H_{33}N_3O_5S_2$ 58.73 6.26 7.90 (58.56 6.49 7.94)	0.1	30	43
13b	2	3	H	S	51.0	A	HCl	109—129 (B)	$C_{25}H_{34}ClN_3OS_2 \cdot H_2O$ 58.86 7.11 8.24 (58.82 7.04 8.08)	0.3	20	24
13c	2	2	H	O	84.0	B	Ox	168—169 (C)	$C_{26}H_{33}N_3O_6S$ 60.56 6.45 8.15 (60.51 6.43 8.09)	0.1	45	23
13d	2	3	H	O	67.2	B	Ox	113—117 (D)	$C_{27}H_{35}N_3O_6S \cdot 1/2H_2O$ 60.20 6.74 7.80 (60.56 6.61 7.94)	1	33	17
13e	2	2	3-F	S	71.9	A	Fum	138—139 (A)	$C_{28}H_{34}FN_3O_5S_2$ 58.42 5.95 7.30 (58.51 6.02 7.18)	0.03	22	25
13f	2	3	3-F	S	90.0	A	Ox	189—191 (C)	$C_{27}H_{34}FN_3O_5S_2$ 57.53 6.08 7.45 (57.49 6.07 7.46)	1	−11	—
13g	2	2	3-F	O	78.0	B	Ox	168—170 (C)	$C_{26}H_{32}FN_3O_6S$ 58.52 6.04 7.87 (58.37 5.98 7.75)	0.01	28	5

13h	2	3	3-F	O	42.2	B	Fum	145—146 (C)	$C_{29}H_{36}FN_3O_6S$ 60.72 6.32 7.32 (60.95 6.20 7.08)	0.1	36	19
13i	2	2	3-Me	S	79.3	A	Ox	152—153 (C)	$C_{27}H_{35}N_3O_5S_2 \cdot H_2O$ 57.53 6.61 7.45 (57.76 6.31 7.42)	0.3	26	18
13j	2	2	3-Me	O	72.0	B	Ox	163—164 (C)	$C_{27}H_{35}N_3O_6S$ 61.23 6.66 7.93 (61.47 6.78 8.07)	0.01	32	5
13k	2	2	3-OMe	S	63.5	B	Ox	154—156 (C)	$C_{27}H_{35}N_3O_6S_2$ 57.73 6.28 7.48 (57.53 6.38 7.60)	0.01	43	10
13l	2	2	3-OMe	O	85.2	B	Ox	142—145 (C)	$C_{27}H_{35}N_3O_7S$ 59.43 6.47 7.70 (59.53 6.48 7.86)	0.01	42	5
13m	2	2	4-OMe	S	76.7	A	Ox	185—187 (A)	$C_{27}H_{35}N_3O_6S_2$ 57.73 6.28 7.48 (57.63 6.24 7.49)	1	38	30
13n	2	3	4-OMe	S	85.0	A	Ox	188—189 (A)	$C_{28}H_{37}N_3O_6S_2$ 58.41 6.48 7.30 (58.44 6.46 7.31)	1	7	15
13o	4	2	H	S	55.4	B	Ox	104—105 (C)	$C_{26}H_{33}N_3O_5S_2$ 58.73 6.26 7.90 (58.58 6.31 7.95)	1	18	27
13p	4	3	H	S	59.8	B	Fum	176—177 (A)	$C_{29}H_{37}N_3O_5S_2$ 60.92 6.52 7.35 (60.69 6.56 7.38)	0.3	18	5
13q	4	2	H	O	98.0	B	Ox	*f*)	$C_{26}H_{33}N_3O_6S$ 60.56 6.45 8.15 (60.36 6.42 8.18)	0.3	27	23
13r	4	3	H	O	70.6	B	Ox	78—80 (E)	$C_{27}H_{35}N_3O_6S$ 61.23 6.66 7.93 (61.03 6.65 7.90)	1	8	20
Amrinone										0.3	25	25

a) The position of the alkoxyl group. *b*) See Experimental. *c*) Ox = oxalate, Fum = fumarate. *d*) A = MeOH–Et_2O, B = iso-PrOH–iso-Pr_2O, C = Me_2CO, D = Me_2CO–EtOH–Et_2O, E = Me_2CO–MeOH–hexane. *e*) For methodology, see reference 12. *f*) Amorphous powder. The spectral data of the free base (oil) were similar to those of **13a** and compatible with the assigned structure.

TABLE III. 2-(Tetrahydropyridylalkoxyphenyl)thiazolidines (**17**)

Compd. No.	Position[a]	n	R	X	Yield (%)	Method[b]	Salt[c]	mp (dec.) (°C) (Recryst. solvent)[d]	Formula and Analysis (%) Calcd (Found)				Myocardial contractility Anesthetized dog[e]		
									Formula	C	H	N	Dose mg/kg i.v.	LVdP/dt_{max} Δ%	Duration min
17a	2	2	H	S	21.0	A	HCl	210—212 (A)	$C_{24}H_{30}ClN_3OS_2$	60.54 (60.29)	6.35 (6.45)	8.83 (8.74)	0.03	19	58
17b	2	3	H	S	20.3	A	HCl	207—210 (A)	$C_{25}H_{32}ClN_3OS_2 \cdot 1/2H_2O$	60.16 (60.44)	6.66 (6.76)	8.42 (8.18)	0.03	34	30
17c	2	2	H	O	61.0	B	Ox	183—184 (D)	$C_{26}H_{31}N_3O_6S$	60.80 (60.96)	6.08 (5.92)	8.18 (8.06)	0.03	31	32
17d	2	3	H	O	64.0	B	Ox	112—115 (E)	$C_{27}H_{33}N_3O_6S$	61.46 (61.25)	6.30 (6.33)	7.96 (7.76)	0.1	30	57
17e	2	2	3-F	S	59.5	A	Ox	158—159 (B)	$C_{26}H_{30}FN_3O_5S_2$	57.02 (57.16)	5.52 (5.67)	7.67 (7.75)	0.03	21	20
17f	2	3	3-F	S	54.8	A	Ox	201—202 (D)	$C_{27}H_{32}FN_3O_5S_2$	57.74 (57.63)	5.74 (5.72)	7.48 (7.46)	0.3	35	24
17g	2	2	3-F	O	58.0	B	Ox	179—181 (D)	$C_{26}H_{30}FN_3O_6S$	58.74 (58.78)	5.69 (5.67)	7.90 (7.75)	0.03	24	22

17h	2	3	3-F	O	34.8	B	Ox	139—142 (D)	$C_{27}H_{32}FN_3O_6S \cdot 1/2\,H_2O$ 58.47 6.00 7.58 (58.60 5.76 7.74)	1	−16	—
17i	2	2	3-Me	S	78.8	A	Ox	156—158 (D)	$C_{27}H_{33}N_3O_5S_2 \cdot H_2O$ 57.73 6.28 7.48 (57.40 5.91 7.72)	0.3	39	32
17j	2	3	3-Me	S	49.1	B	Ox	162—165 (G)	$C_{28}H_{35}N_3O_5S_2$ 60.30 6.33 7.54 (60.44 6.33 7.62)	0.1	27	22
17k	2	2	3-Me	O	49.0	B	Ox	175—176 (D)	$C_{27}H_{33}N_3O_6S$ 61.46 6.30 7.96 (61.28 6.26 7.74)	0.3	47	25
17l	2	2	3-OMe	S	35.3	B	Ox	151—152 (F)	$C_{27}H_{33}N_3O_6S_2$ 57.94 5.94 7.51 (58.04 6.05 7.49)	0.03	30	35
17m	2	2	3-OMe	O	58.1	B	Ox	177—178 (E)	$C_{27}H_{33}N_3O_7S$ 59.65 6.12 7.73 (59.81 6.14 7.78)	0.1	29	22
17n	2	2	4-OMe	S	88.1	A	Ox	144—146 (B)	$C_{27}H_{33}N_3O_6S_2$ 57.94 5.94 7.51 (57.80 5.93 7.61)	0.3	27	20
17o	2	3	4-OMe	S	89.8	A	Ox	188—190 (C)	$C_{28}H_{35}N_3O_6S_2$ 58.62 6.15 7.32 (58.53 6.10 7.40)	1	12	25
17p	4	2	H	S	48.4	B	Ox	116—120 (D)	$C_{26}H_{31}N_3O_5S_2 \cdot 1/3\,H_2O$ 58.30 5.96 7.84 (58.29 5.93 7.75)	0.03	−4	—
17q	4	3	H	S	53.8	B	Fum	167—169 (H)	$C_{29}H_{35}N_3O_5S_2$ 61.14 6.19 7.38 (61.03 6.21 7.41)	0.3	19	12
17r	4	2	H	O	84.0	B	Ox	120—121 (F)	$C_{26}H_{31}N_3O_6S \cdot 1/2\,C_2H_5OH$ 60.43 6.39 7.83 (60.71 6.58 7.70)	1	22	15
17s	4	3	H	O	51.0	B	Ox	115—116 (F)	$C_{27}H_{33}N_3O_6S$ 61.46 6.30 7.96 (61.26 6.37 7.93)	0.1	−9	—

a—c) See footnotes *a—c* in Table II, respectively. *d*) A = MeOH–EtOH–Et_2O, B = MeOH–Et_2O, C = aq. MeOH, D = Me_2CO, E = EtOH–Et_2O, F = EtOH, G = Me_2CO–EtOH–Et_2O, and H = Me_2CO–MeOH–Et_2O. *e*) See footnote *e* in Table II.

TABLE IV. 2-(Aminoalkoxyphenyl)thiazolidines

Compd.	X	R	Dose mg/kg i.v.	Myocardial contractility Anesthetized dog[a] LVdP/dt_{max} Δ%	Duration min
9a	S	-(CH$_2$)$_2$-(piperidin-4-yl)N-Ph	1	0	0
9b	S	-(CH$_2$)$_3$-(piperidin-4-yl)N-Ph	1	0	0
22a	S	-(CH$_2$)$_2$N(oxopiperazine)N-Ph	1	9	20
22b	S	-(CH$_2$)$_3$N(oxopiperazine)N-Ph	1	25	7
23	O	-(CH$_2$)$_2$N(homopiperazine)N-Ph	0.1	28	21
25	S	-(CH$_2$)$_3$N(piperazine)N-C(=O)-Ph	1	−5	—
28	S	-CH$_2$C(=O)-N(piperazine)N-Ph	1	−4	—
29a	S	-(CH$_2$)$_2$N(Me)-(CH$_2$)$_3$-Ph	1	−8	—
29b	S	-(CH$_2$)$_3$N(Me)-(CH$_2$)$_3$-Ph	1	−21	—
30a	S	-(CH$_2$)$_2$N(Me)-(CH$_2$)$_2$N(Me)-Ph	1	27	24
30b	S	-(CH$_2$)$_3$N(Me)-(CH$_2$)$_2$N(Me)-Ph	1	14	25

a) See footnote *e* in Table II.

previous observation in the phenylpiperazine series (1).[2] Decrease in activity, previously seen with the phenylpiperazine series on converting the carboxamido group to the carbothioamido group, was not apparent in this series of compounds. Thus, no significant change in activity was noted between the carbothioamides (**13a** and **17a**) and the corresponding carboxamides (**13c** and **17c**). There is little uniformity in the effect of the substituents (R) on the benzene ring of **13** and **17**. In the piperidine series (**13**), a marked enhancement of activity was observed on fluoro substitution (**13a** *vs.* **13e** and **13c** *vs.* **13g**) in accordance with the previous experience in the piperazine series.[2] This favorable effect, however, was not apparent in the 4-phenyltetrahydropyridine series (**17**). Generally, 4-phenyltetrahydropyridine derivatives exhibited more potent positive inotropy than the corresponding piperidine derivatives. Compounds **17a**—**d** were thus from three to ten times more potent than **13a**—**d**.

None of the *N*-phenylpiperidines (**9**), amide derivatives (**22**, **25**, and **28**), and pseudo-ring derivatives (**29** and **30**) exhibited significant positive inotropy even at 1 mg/kg. The homopipe-

razine derivative (**23**) produced a 28% increase in LVdP/dt_{max} at 0.1 mg/kg (Table IV). Thus, the activity of **23** is approximately one-thirtieth of that of the corresponding piperazine derivative (**1**, $R^1=R^2=H$, $R^3=Me$, X=O, *ortho* isomer), which exhibited a 30% increase in LVdP/dt_{max} at 0.003 mg/kg.[2]

In summary, the presence of the six-membered, basic azacycloalkane ring (piperidine or piperazine) with a 4-phenyl group as an amino function at the end of the alkoxy side chain appears to be essential for the appearance of potent positive inotropic activity in this series of compounds.

Further studies on the synthesis and SAR of 2-phenylthiazolidine-3-carbothioamides and the corresponding carboxamides as new cardiotonic agents are being continued.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded on a Hitachi IR-215 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken at 60 MHz on a JEOL PMX-60 spectrometer with tetramethylsilane (TMS) as an internal reference. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. Mass spectra (MS) were measured with a Hitachi RMU-6M instrument.

1-Phenyl-4-piperidinecarbaldehyde (3)——A solution of lithium diisopropylamide [prepared from diisopropylamine (6.92 g), BuLi (43.8 ml of 15% hexane solution), and tetrahydrofuran (THF) (50 ml)] was added to a stirred suspension of methoxymethyltriphenylphosphonium chloride[5] (23.5 g, 0.064 mol) in THF (150 ml) at −20 °C. The mixture was stirred at −20 °C for 30 min, then a solution of **2**[4] (9.35 g, 0.0534 mol) was added to the mixture at −40 °C, and the whole was stirred at room temperature overnight. The mixture was diluted with H_2O and extracted with AcOEt. The organic layer was dried and evaporated. The residue was purified by SiO_2 chromatography (C_6H_6) to give 6 g of an oil. A mixture of this oil, 10% HCl (20 ml), and THF (40 ml) was stirred at room temperature overnight, made basic with K_2CO_3, and extracted with AcOEt. The extracts were washed with H_2O, dried, and evaporated. The residue was purified by column chromatography (SiO_2, AcOEt–hexane (1 : 4)) to give 4.7 g (46.7% from **2**) of **3** as an oil. IR ν_{max}^{liquid} cm^{-1}: 1720, 1600, 1500. MS *m/z*: 189 (M$^+$), 161, 160, 132. ^{1}H-NMR ($CDCl_3$) δ: 1.70—2.10 (4H, m), 2.10—2.55 (1H, m), 2.55—3.15 (2H, m), 3.52 (1H, t, J=4 Hz), 3.74 (1H, t, J=4 Hz), 6.85—7.40 (5H, m), 9.70 (1H, s).

1-Phenyl-4-piperidineacetaldehyde (4)——This compound was obtained from **3** by the same procedure as described above in 54.3% yield as an oil. IR ν_{max}^{liquid} cm^{-1}: 1720. MS *m/z*: 203 (M$^+$), 175, 174. ^{1}H-NMR ($CDCl_3$) δ: 1.05—2.10 (5H, m), 2.30—3.10 (4H, m), 3.70 (2H, br d, J=12 Hz), 6.65—7.10 (5H, m), 9.80 (1H, t, J=2 Hz).

1-Phenyl-4-piperidineethanol (5a)——A mixture of **4** (2.9 g, 0.0143 mol), $NaBH_4$ (2.2 g, 0.0582 mol), and EtOH (30 ml) was stirred at room temperature for 1 h. The usual work-up and purification by SiO_2 chromatography (C_6H_6–AcOEt (4 : 1)) gave **5a** (2.6 g, 88.7%) as an oil. IR ν_{max}^{liquid} cm^{-1}: 3300, 1590. MS *m/z*: 205 (M$^+$), 204, 174. ^{1}H-NMR ($CDCl_3$) δ: 1.00—2.10 (8H, m), 2.70 (2H, br t, J=11 Hz), 3.50—4.00 (4H, m), 6.75—7.45 (5H, m).

3-(1-Phenyl-4-piperidyl)-2-propenal (6)——A solution of **3** (3 g, 0.0159 mol) and formylmethylenetriphenylphosphorane[6] (4.83 g, 0.0159 mol) in benzene (40 ml) was refluxed overnight. Evaporation of the mixture and purification of the residue by SiO_2 chromatography (C_6H_6–AcOEt (9 : 1)) gave 1.7 g (50%) of **6** as an oil. IR ν_{max}^{liquid} cm^{-1}: 1680. MS *m/z*: 215 (M$^+$), 158. ^{1}H-NMR ($CDCl_3$) δ: 1.40—2.10 (4H, m), 2.55—3.10 (3H, m), 3.40—4.00 (2H, m), 6.12 (1H, dd, J=7 Hz and 2 Hz), 6.60—7.60 (6H, m).

1-Phenyl-4-piperidinepropanol (5b)——A mixture of **6** (1.7 g, 0.0079 mol), 10% Pd–C (0.5 g), and EtOH (30 ml) was hydrogenated at room temperature and ordinary pressure. The catalyst was filtered off, and $NaBH_4$ (1.2 g, 0.0317 mol) was added to the filtrate. After being stirred at room temperature for 1 h, the mixture was concentrated, diluted with H_2O, and extracted with AcOEt. The extracts were washed with H_2O, dried, and evaporated. Chromatographic purification of the residue (SiO_2, C_6H_6–AcOEt (4 : 1)) gave 1.04 g (60%) of **5b** as a wax. IR ν_{max}^{Nujol} cm^{-1}: 3280, 1590. MS *m/z*: 220, 219 (M$^+$), 218, 174, 132. ^{1}H-NMR ($CDCl_3$) δ: 1.05—2.10 (10H, m), 2.70 (2H, br t, J=12 Hz), 3.45—3.90 (4H, m), 6.60—7.40 (5H, m).

1-Phenyl-4-piperidineethanol *O*-Tosylate (7a)——A mixture of **5a** (2.6 g, 0.0127 mol), *p*-toluenesulfonyl chloride (2.9 g, 0.0152 mol), pyridine (4 ml), and CH_2Cl_2 (40 ml) was stirred at room temperature overnight. The mixture was worked up in the usual manner and purified by chromatography (SiO_2, C_6H_6–AcOEt (9 : 1)) to give 2.3 g (50%) of **7a** as an oil. IR ν_{max}^{liquid} cm^{-1}: 1600, 1360. MS *m/z*: 359 (M$^+$), 204, 188. ^{1}H-NMR ($CDCl_3$) δ: 1.00—1.95 (7H, m), 2.00—3.00 (5H, m), 3.65 (2H, br d, J=11 Hz), 4.10 (2H, t, J=6 Hz), 7.80 (2H, d, J=8 Hz).

1-Phenyl-4-piperidinepropanol *O*-Tosylate (7b)——The carbinol (**5b**) was tosylated in the same manner as described above to give 61.5% yield of **7b**, mp 78—80 °C (from AcOEt–hexane). *Anal.* Calcd for $C_{21}H_{27}NO_3S$: C, 67.53; H, 7.29; N, 3.75; S, 8.58. Found: C, 67.59; H, 7.24; N, 3.60; S, 8.38. IR ν_{max}^{Nujol} cm^{-1}: 1590, 1380. MS *m/z*: 373

(M^+), 202. ^{1}H-NMR ($CDCl_3$) δ: 1.00—2.20 (9H, m), 2.20—3.15 (5H, m), 3.70 (2H, brd, J=11 Hz), 4.06 (2H, t, J=6 Hz), 6.60—7.50 (7H, m), 7.80 (2H, d, J=8 Hz).

2-(2-(1-Phenyl-4-piperidyl)ethoxy)benzaldehyde (8a)——A mixture of **7a** (2.3 g, 0.0064 mol), salicylaldehyde (0.78 g, 0.0064 mol), K_2CO_3 (0.81 g, 0.0064 mol), and DMF (40 ml) was heated at 50 °C overnight. The mixture was concentrated, diluted with H_2O, and extracted with AcOEt. The extracts were washed with H_2O, dried, and evaporated. The residue was purified by chromatography (SiO_2, hexane–AcOEt (4:1)) to give 1.41 g (71.2%) of **8a**, mp 46—49 °C (from AcOEt–hexane). *Anal.* Calcd for $C_{20}H_{23}NO_2$: C, 77.64; H, 7.49; N, 4.53. Found: C, 77.51; H, 7.38; N, 4.49. IR ν_{max}^{Nujol} cm^{-1}: 1680. MS m/z: 309 (M^+), 188, 158. ^{1}H-NMR ($CDCl_3$) δ: 1.20—2.30 (7H, m), 2.30—3.10 (2H, m), 3.50—3.90 (2H, m), 4.16 (2H, t, J=6 Hz), 6.60—8.00 (9H, m), 10.50 (1H, s).

2-(3-(1-Phenyl-4-piperidyl)propoxy)benzaldehyde (8b)——Condensation of **7b** with salicylaldehyde in the same manner as described for **7a** gave 93.8% yield of **8b**, mp 65—67 °C (from AcOEt–hexane). *Anal.* Calcd for $C_{21}H_{25}NO_2$: C, 77.99; H, 7.79; N, 4.33. Found: C, 78.05; H, 7.75; N, 4.27. IR ν_{max}^{Nujol} cm^{-1}: 1680. MS m/z: 323 (M^+), 203, 158. ^{1}H-NMR ($CDCl_3$) δ: 1.00—2.30 (9H, m), 2.45—3.10 (2H, m), 3.70 (2H, brd, J=12 Hz), 4.10 (2H, t, J=6 Hz), 6.65—7.95 (9H, m), 10.48 (1H, s).

***N*-Methyl-2-(2-(2-(1-phenyl-4-piperidyl)ethoxy)phenyl)thiazolidine-3-carbothioamide (9a)**——A mixture of **8a** (1.4 g, 0.00453 mol), cysteamine hydrochloride (0.57 g, 0.00498 mol), NaOH (0.21 g, 0.0052 mol), and EtOH (40 ml) was stirred at room temperature for 2 h. MeNCS (0.46 g, 0.00634 mol) was added, and the whole was stirred at room temperature overnight and then refluxed for 1 h. The reaction mixture was concentrated, diluted with H_2O, and extracted with AcOEt. Evaporation of the dried extracts gave, after recrystallization from AcOEt–hexane, 1.55 g (77.6%) of **9a**, mp 151—152 °C. IR ν_{max}^{Nujol} cm^{-1}: 3420, 1590. MS m/z: 441 (M^+), 367, 335, 290, 188, 185. ^{1}H-NMR ($CDCl_3$) δ: 1.10—2.30 (7H, m), 2.40—3.30 (7H, m), 3.70 (2H, brd, J=12 Hz), 4.00—4.80 (4H, m), 5.40 (1H, brs), 6.34 (1H, s), 6.60—7.60 (9H, m). The fumarate was recrystallized from Me_2CO–hexane and had mp 152—153 °C. *Anal.* Calcd for $C_{28}H_{35}N_3O_5S_2$: C, 60.30; H, 6.33; N, 7.53; S, 11.50. Found: C, 60.47; H, 6.32; N, 7.77; S, 11.45.

***N*-Methyl-2-(2-(3-(1-phenyl-4-piperidyl)propoxy)phenyl)thiazolidine-3-carbothioamide (9b)**——Treatment of **8b** with cysteamine and then with MeNCS in the same manner as described above gave 50.6% yield of **9b**, mp 126—130 °C (from AcOEt–hexane). The fumarate was recrystallized from Me_2CO–hexane and had mp 129—131 °C (dec.). *Anal.* Calcd for $C_{29}H_{37}N_3O_5S_2$: C, 60.92; H, 6.52; N, 7.35; S, 11.22. Found: C, 60.81; H, 6.46; N, 7.38; S, 11.37.

2-(2-(4-Phenyl-1-piperidyl)ethoxy)benzaldehyde (12a)——A mixture of **10** (n=2)[2] (2.84 g, 0.0124 mol), 4-phenylpiperidine (**11**, R=H, 2 g, 0.0124 mol), K_2CO_3 (1.89 g, 0.0137 mol), and DMF (20 ml) was heated at 80 °C for 22 h. The mixture was diluted with H_2O and extracted with AcOEt. The extracts were washed with H_2O, dried, and evaporated. The residue was purified by chromatography (SiO_2, C_6H_6–AcOEt (3:2)) to give 2.74 g of an oil. A mixture of this oil, 10% HCl (20 ml), and MeOH (20 ml) was heated at 70 °C for 20 min. The mixture was diluted with H_2O, made basic with 10% aq. NaOH, and extracted with benzene. The extracts were washed with H_2O, dried, and evaporated. Recrystallization of the residue from Et_2O–hexane gave 1.54 g (62.6%) of **12a**, mp 59—62 °C. IR ν_{max}^{Nujol} cm^{-1}: 1680. MS m/z: 309 (M^+), 174. *Anal.* Calcd for $C_{20}H_{33}NO_2$: C, 77.64; H, 7.49; N, 4.53. Found: C, 77.52; H, 7.54; N, 4.41. The substituted benzaldehydes (**12b—g**) were prepared in a similar manner and their physical properties are summarized in Table I.

***N*-Methyl-2-(2-(2-(4-phenyl-1-piperidyl)ethoxy)phenyl)thiazolidine-3-carbothioamide (13a) (Method A)**——A mixture of **12a** (1.5 g, 0.00485 mol), cysteamine hydrochloride (0.61 g, 0.00534 mol), NaOH (0.22 g, 0.00558 mol), and EtOH (30 ml) was refluxed for 1 h. MeNCS (0.57 g, 0.00776 mol) was then added to the mixture, and the whole was refluxed for 1.5 h. The mixture was evaporated, diluted with H_2O, and extracted with AcOEt. The extracts were washed with H_2O, dried, and evaporated. Recrystallization of the residue from aq. EtOH gave 1.91 g (89.3%) of **13a**, mp 141—142 °C. IR ν_{max}^{Nujol} cm^{-1}: 3220, 1600. MS m/z: 441 (M^+), 368, 308. ^{1}H-NMR ($CDCl_3$) δ: 1.60—2.10 (4H, m), 2.10—2.60 (3H, m), 2.80—3.40 (10H, m), 4.10—4.80 (4H, m), 5.64 (1H, br), 6.35 (1H, s), 6.75—7.60 (9H, m). Analytical data are included in Table II. The thiazolidine-3-carbothioamides (**13**) prepared by method A are listed in Table II.

***N*-Methyl-2-(4-(3-(4-phenyl-1-piperidyl)propoxy)phenyl)thiazolidine-3-carboxamide (13r) (Method B)**——A mixture of 1.89 g (0.006 mol) of **14** (X=O, *para*-substituted, n=3),[2] NaI (0.9 g, 0.006 mol), K_2CO_3 (0.83 g, 0.006 mol), 4-phenylpiperidine (**11**, R=H, 0.97 g, 0.006 mol), and DMF (20 ml) was heated at 80 °C overnight. The usual work-up gave 1.86 g (70.6%) of **13r**, mp 129—131 °C (from AcOEt–Me_2CO–hexane). IR ν_{max}^{Nujol} cm^{-1}: 3380, 1620. MS m/z: 439 (M^+), 382, 381, 322, 174. ^{1}H-NMR ($CDCl_3$) δ: 1.50—2.35 (9H, m), 2.35—2.90 (6H, m), 2.90—3.30 (4H, m), 3.60—4.50 (5H, m), 6.33 (1H, s), 6.90 (2H, d, J=9 Hz), 7.25 (5H, s), 7.23 (2H, d, J=9 Hz). *Anal.* Calcd for $C_{25}H_{33}N_3O_2S$: C, 68.30; H, 7.57; N, 9.56; S, 7.29. Found: C, 68.57; H, 7.62; N, 9.67; S, 7.13. Analytical data are included in Table II. Other 4-phenylpiperidine derivatives (13) prepared by method B are listed in Table II.

2-(1,2,5,6-Tetrahydro-4-phenyl-1-pyridyl)alkoxybenzaldehydes (16)——These compounds were prepared by the condensation of **10** with **15**[13] in the same manner as described for the piperidine derivative (**12**) and are listed in Table I.

2-((1,2,5,6-Tetrahydro-4-phenyl-1-pyridyl)alkoxy)phenyl-*N*-methylthiazolidine-3-carboxamides or -carbothioamides (17)——These compounds were prepared from the aldehyde (**16**) by method A or from **14** by method B in the same manner as described for the piperidine derivative (**13**). Their physical properties are summarized in Table

III.

4-Benzyloxycarbonyl-1-phenyl-2-piperazinone (19)——*N*-Phenylethylenediamine (**18**) (8 g, 0.0587 mol) was allowed to react with CbzCl (10 g, 0.0587 mol) and NEt_3 (6.54 g, 0.0645 mol) in THF (150 ml) in the usual manner to give 15.8 g of the *N*-Cbz derivative. Schotten–Baumann reaction of this material with bromoacetyl bromide (12.2 g, 0.06 mol) and 10% aq. NaOH (30 ml) in AcOEt (150 ml) gave 22.4 g of the bromoacetyl derivative as an oil. A mixture of this oil, K_2CO_3 (25 g), and DMF (120 ml) was stirred at room temperature for 2 d. An insoluble material was filtered off, and the filtrate was evaporated, diluted with H_2O, and extracted with AcOEt. Evaporation of the extracts and purification of the residue by chromatography (SiO_2, C_6H_6–AcOEt (5 : 1)) gave 12.7 g (79.7% from **18**) of **19**, mp 87—88 °C (from Et_2O–hexane). IR ν_{max}^{Nujol} cm^{-1}: 1700, 1665, 1600, 1500. MS *m/z*: 310 (M^+), 219, 196, 191, 175, 91. ^1H-NMR ($CDCl_3$) δ: 3.60—4.00 (4H, m), 4.36 (2H, s), 5.21 (2H, s), 7.20—7.60 (10H, m). *Anal.* Calcd for $C_{18}H_{18}N_2O_3$: C, 69.66; H, 5.85; N, 9.03. Found: C, 69.87; H, 5.80; N, 8.89.

1-Phenyl-2-piperazinone (21)——A mixture of **19** (12.3 g, 0.0396 mol), HCO_2NH_4 (5 g, 0.0792 mol), 10% Pd–C (2.4 g), and MeOH (150 ml) was stirred at room temperature for 10 min and evaporated. The residue was chromatographed over SiO_2 and eluted with $CHCl_3$–EtOH (20 : 1). The first eluate gave 6.1 g (75.4%) of the *N*-formyl derivative (**20**), mp 97—99 °C (from AcOEt–hexane). IR ν_{max}^{Nujol} cm^{-1}: 1650, 1595, 1495, 750, 690. MS *m/z*: 204 (M^+), 160, 106, 77. ^1H-NMR ($CDCl_3$) δ: 3.60—4.00 (4H, m), 4.18 (1H, s), 4.16 (1H, s), 7.10—7.60 (5H, m), 8.11 (1H, s). *Anal.* Calcd for $C_{11}H_{12}N_2O_2$: C, 64.69; H, 5.92; N, 13.72. Found: C, 64.72; H, 5.88; N, 13.58. The second eluate gave, after recrystallization from Et_2O–hexane, 1.6 g (22.9%) of **21**, mp 93—94 °C. IR ν_{max}^{Nujol} cm^{-1}: 3250, 1640, 1585, 1490, 760, 690. MS *m/z*: 176 (M^+), 148, 106, 77. ^1H-NMR ($CDCl_3$) δ: 1.88 (1H, s), 3.09—3.25 (2H, m), 3.65 (2H, s), 3.58—3.74 (2H, m), 7.16—7.52 (5H, m). *Anal.* Calcd for $C_{10}H_{12}N_2O$: C, 68.16; H, 6.86; N, 15.90. Found: C, 68.35; H, 6.80; N, 15.85. Refluxing of the *N*-formyl derivative (**20**) with conc. HCl in MeOH gave **21** in 92.8% yield.

***N*-Methyl-2-(2-(2-(3-oxo-4-phenylpiperazino)ethoxy)phenyl)thiazolidine-3-carbothioamide (22a)**——A mixture of **14a** (X=S)[2] (2.5 g, 0.00789 mol), **21**·HCl (1.75 g, 0.00789 mol), K_2CO_3 (2.18 g, 0.0158 mol), NaI (1.18 g, 0.00789 mol), and DMF (20 ml) was heated at 80 °C overnight. The mixture was worked up in the usual manner and purified by chromatography (SiO_2, $CHCl_3$–AcOEt–Me_2CO (5 : 4 : 1)). Recrystallization from $CHCl_3$–MeOH–hexane gave 0.82 g (22.8%) of **22a**, mp 207—209 °C. IR ν_{max}^{Nujol} cm^{-1}: 3300, 1640. MS *m/z*: 456 (M^+), 383, 306, 203, 202, 200. ^1H-NMR ($CDCl_3$–DMSO-d_6) δ: 2.80—3.30 (9H, m), 3.40 (2H, s), 3.71 (2H, brt, J=7 Hz), 3.95—4.00 (5H, m), 6.80—7.50 (9H, m). *Anal.* Calcd for $C_{23}H_{28}N_4O_2S_2$: C, 60.50; H, 6.18; N, 12.27; S, 14.04. Found: C, 60.29; H, 6.09; N, 12.43; S, 14.28. The oxalate was recrystallized from $CHCl_3$–MeOH–hexane and had mp 125—127 °C (dec.). *Anal.* Calcd for $C_{25}H_{30}N_4O_6S_2$: C, 54.93; H, 5.53; N, 10.25; S, 11.73. Found: C, 54.93; H, 5.66; N, 10.35; S, 11.35. The propoxy analogue (**22b**) was similarly obtained by the condensation of **14b** (X=S) with **21** in 38.3% yield. mp 190—195 °C (from $CHCl_3$–AcOEt–hexane). The oxalate was recrystallized from MeOH and had mp 178—179.5 °C (dec.). *Anal.* Calcd for $C_{26}H_{32}N_4O_6S_2 \cdot 0.25H_2O$: C, 55.25; H, 5.80; N, 9.91; S, 11.34. Found: C, 55.28; H, 5.91; N, 9.90; S, 11.38.

2-(2-(2-(Hexahydro-4-phenyl-1,4-diazepin-1-yl)ethoxy)phenyl)-*N*-methylthiazolidine-3-carboxamide (23)——A mixture of **14** (X=O, n=2, 1.65 g, 0.0055 mol), hexahydro-4-phenyl-1,4-diazepine[10] (0.97 g, 0.0055 mol), K_2CO_3 (0.76 g, 0.0055 mol), NaI (0.82 g, 0.0055 mol), and DMF (20 ml) was allowed to react in the same manner as described above. Chromatographic purification (SiO_2, AcOEt–MeOH (30 : 1)) gave 1.84 g (76%) of **23**, mp 117—120 °C (from AcOEt–hexane). IR ν_{max}^{Nujol} cm^{-1}: 3325, 1630, 1595, 760. MS *m/z*: 440 (M^+), 425, 382, 203, 189. ^1H-NMR ($CDCl_3$) δ: 1.80—2.20 (2H, m), 2.60—3.30 (11H, m), 3.40—3.80 (4H, m), 3.80—4.60 (5H, m), 6.20 (1H, s), 6.50—7.40 (9H, m). The oxalate was recrystallized from Me_2CO–Et_2O and had mp 168—170 °C (dec.). *Anal.* Calcd for $C_{26}H_{34}N_4O_6S$: C, 58.85; H, 6.46; N, 10.56; S, 6.04. Found: C, 59.12; H, 6.55; N, 10.64; S, 5.97.

2-(3-(4-Benzoylpiperazino)propoxy)benzaldehyde (24)——A mixture of *N*-benzoylpiperazine hydrochloride[9] (2.3 g, 0.01 mol), **10**[2] (n=3; 2.43 g, 0.01 mol), K_2CO_3 (4 g, 0.029 mol), and DMF (30 ml) was heated at 80—100 °C overnight. The usual work-up and chromatographic purification (SiO_2, $CHCl_3$–acetone (4 : 1)) gave 2.29 g (64.9%) of **24** as an oil. IR ν_{max}^{liquid} cm^{-1}: 1685, 1635, 1600. MS *m/z*: 352 (M^+), 218, 203, 148, 105. ^1H-NMR ($CDCl_3$) δ: 1.93—2.25 (2H, m), 2.40—2.93 (6H, m), 3.30—3.80 (4H, m), 4.10 (2H, t, J=6 Hz), 6.93—8.26 (4H, m), 7.40 (5H, s).

2-(2-(3-(4-Benzoylpiperazino)propoxy)phenyl)-*N*-methylthiazolidine-3-carbothioamide (25)——The usual treatment of **24** with cysteamine and MeNCS gave **25** in 88.8% yield as an oil. IR ν_{max}^{Nujol} cm^{-1}: 3300, 1620. MS *m/z*: 484 (M^+), 411, 351, 229, 203, 201. ^1H-NMR ($CDCl_3$) δ: 1.90—2.12 (2H, m), 2.99 (3H, d, J=4.6 Hz), 3.40—3.80 (4H, m), 5.44—5.52 (12H, m), 6.32 (1H, s), 6.82—7.18 (4H, m), 7.36 (5H, m). The hydrochloride was recrystallized from EtOH and had mp 206—208 °C (dec.). *Anal.* Calcd for $C_{25}H_{33}ClN_4O_2S_2$: C, 57.62; H, 6.38; Cl, 6.80; N, 10.75; S, 12.31. Found: C, 57.48; H, 6.41; Cl, 6.82; N, 10.66; S, 12.18.

2-(((4-Phenylpiperazino)carbonyl)methoxy)benzaldehyde (27)——A mixture of the carboxylic acid (**26**)[3] (2.8 g, 0.016 mol), $SOCl_2$ (3 ml), and benzene (20 ml) was refluxed for 2 h and evaporated. The residual acid chloride was allowed to react with phenylpiperazine (2.64 g, 0.0165 mol), 10% aq. NaOH (20 ml), and CH_2Cl_2 (40 ml) with vigorous stirring. The mixture was evaporated, and the residue was dissolved in 10% aq. HCl (10 ml) and MeOH (30 ml). The mixture was evaporated, diluted with H_2O, made basic with 10% aq. NaOH, and extracted with AcOEt. The extracts were washed with H_2O, dried, and evaporated. Chromatographic purification of the residue gave 2.9 g (59.7%) of **27**, mp 117—118 °C (from AcOEt–hexane). IR ν_{max}^{Nujol} cm^{-1}: 1670, 1655, 1590, 760. ^1H-NMR ($CDCl_3$) δ:

3.06—3.22 (4H, m), 3.68—3.84 (4H, m), 4.86 (2H, s), 6.75—7.92 (9H, m), 10.48 (1H, s). *Anal.* Calcd for $C_{19}H_{20}N_2O_3$: C, 70.35; H, 6.21; N, 8.64. Found: C, 70.31; H, 6.19; N, 8.60.

***N*-Methyl-2-(2-(((4-phenylpiperazino)carbonyl)methoxy)phenyl)thiazolidine-3-carbothioamide (28)**——Reaction of **27** with cysteamine and MeNCS in the usual manner gave 92.1% yield of **28**, mp 188—189 °C (from $CHCl_3$–EtOH). IR ν_{max}^{Nujol} cm^{-1}: 3280, 1630, 1595, 750. MS *m/z*: 382, 220, 202, 160, 132 (M^+ was not seen). ^{1}H-NMR ($CDCl_3$) δ: 2.98 (3H, d, J=4.5 Hz), 4.89 and 5.04 (2H, ABq, J=15 Hz), 6.63 (1H, s), 6.74—7.40 (9H, m). *Anal.* Calcd for $C_{23}H_{28}N_4O_2S_2$: C, 60.50; H, 6.18; N, 12.27; S, 14.04. Found: C, 60.51; H, 6.15; N, 12.36; S, 13.76.

***N*-Methyl-2-(2-(2-(*N*-methyl-3-phenylpropylamino) ethoxy) phenyl) thiazolidine-3-carbothioamide (29a)**——Condensation of **14a** (X=S) with *N*-methyl-3-phenylpropylamine[11] by the usual method gave **29a** in 84% yield as an oil. IR ν_{max}^{liquid} cm^{-1}: 3400, 3300, 1590. MS *m/z*: 429 (M^+), 356, 295, 162, 160, 91, 71, 58. ^{1}H-NMR ($CDCl_3$) δ: 1.50—2.10 (2H, m), 2.20—3.20 (14H, m), 3.90—4.90 (4H, m), 5.40—5.80 (1H, m), 6.25 (1H, s), 6.70—7.50 (9H, m). The oxalate, mp 171—172 °C (dec.), was recrystallized from EtOH. *Anal.* Calcd for $C_{25}H_{33}N_3O_5S$: C, 57.78; H, 6.40; N, 8.09; S, 12.34. Found: C, 57.47; H, 6.35; N, 8.33; S, 12.30.

The propoxy analogue (**29b**) was similarly prepared from **14b** (X=S) in 82% yield. The oxalate, mp 152—153 °C (dec.), was recrystallized from EtOH. *Anal.* Calcd for $C_{26}H_{35}N_3O_5S$: C, 58.51; H, 6.61; N, 7.87; S, 12.01. Found: C, 58.57; H, 6.50; N, 8.07; S, 12.14.

***N*-(2-(Methylphenylamino)ethyl)formamide (31)**——A mixture of *N*-phenylethylenediamine (**18**) (4.09 g, 0.03 mol) and HCO_2Et (50 ml) was refluxed for 1.5 h and evaporated. Chromatography (SiO_2, $CHCl_3$–AcOEt (30:1)) of the residue gave 4.79 g of the *N*-formate as an oil. A mixture of this oil (3.8 g, 0.0231 mol), MeI (4.68 g, 0.033 mol), K_2CO_3 (3.83 g, 0.028 mol), and DMF (20 ml) was heated at 40—50 °C for 4 h. The mixture was diluted with AcOEt and washed with sat. NaCl. The organic layer was dried and evaporated. The residue was purified by chromatography (SiO_2, $CHCl_3$–AcOEt (10:1)) to give 1.83 g (34.2% from **18**) of **31** as an oil. IR ν_{max}^{liquid} cm^{-1}: 3350, 3270, 1660, 1590, 750, 690. MS *m/z*: 178 (M^+), 120. ^{1}H-NMR ($CDCl_3$) δ: 2.92 (3H, s), 3.30—3.80 (4H, m), 5.70—6.20 (1H, m), 6.60—7.00 (3H, m), 7.10—7.50 (2H, m), 8.13 (1H, br s).

***N,N'*-Dimethyl-*N*-phenylethylenediamine (32)**——A mixture of **31** (1.92 g, 0.0108 mol), lithium aluminum hydride (0.61 g, 0.0161 mol), and THF (40 ml) was refluxed for 4 h. The usual work-up and chromatographic purification (SiO_2, $CHCl_3$–MeOH (10:1)) gave 1.74 g (98%) of **32** as an oil. IR ν_{max}^{liquid} cm^{-1}: 3300, 1600, 750. MS *m/z*: 164 (M^+), 120. ^{1}H-NMR ($CDCl_3$) δ: 2.45 (3H, s), 2.79 (2H, t, J=6 Hz), 2.94 (3H, s), 3.45 (2H, t, J=6.4 Hz), 6.5—7.5 (5H, m).

***N*-Methyl-2-(2-(2-(methyl(2-(methylphenylamino)ethyl)amino)ethoxy)phenyl)thiazolidine-3-carbothioamide (30a)**——Condensation of **14a** (X=S) and **32** in the usual manner gave **30a** in 64% yield as an oil. IR ν_{max}^{liquid} cm^{-1}: 3400, 3300, 1590. MS *m/z*: 444 (M^+), 371, 324, 251, 133, 120, 73, 70. ^{1}H-NMR ($CDCl_3$) δ: 2.43 (3H, s), 2.95 (3H, s), 2.00—3.20 (9H, m), 3.30—3.70 (2H, m), 3.80—4.90 (4H, m), 5.40—5.70 (1H, m), 6.30 (1H, s), 6.50—7.50 (9H, m). The oxalate, mp 181—182 °C (dec.), was recrystallized from EtOH. *Anal.* Calcd for $C_{25}H_{34}N_4O_5S_2$: C, 56.16; H, 6.41; N, 10.48; S, 11.99. Found: C, 56.17; H, 6.34; N, 10.60; S, 11.75.

The propoxy analogue (**30b**) was similarly obtained by the condensation of **14b** (X=S) and **32** in 78% yield. The oxalate was recrystallized from EtOH and had mp 167—168 °C (dec.). *Anal.* Calcd for $C_{26}H_{36}N_4O_5S_2$: C, 56.91; H, 6.61; N, 10.21; S, 11.69. Found: C, 57.11; H, 6.60; N, 10.42; S, 11.97.

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Synthesis of 1,2,3,4-Tetrahydro-β-carboline Derivatives as Hepatoprotective Agents. I. Dithiocarbamates of Several α-Amino Acids

YUTAKA SAIGA,[a] IKUO IIJIMA,*[,a] AKIHIKO ISHIDA,[a] TOSHIKAZU MIYAGISHIMA,[a] TOKURO OH-ISHI,[a] MAMORU MATSUMOTO,[b] and YUZO MATSUOKA[b]

Organic Chemistry Research Laboratory[a] and Safety Research Laboratory,[b] Tanabe Seiyaku Co., Ltd., 2-2-50, Kawagishi, Toda-shi, Saitama 335, Japan

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A series of dithiocarbamates of α-amino acids was synthesized and tested for hepatoprotective activity against CCl_4-induced liver damage in mice. Reaction of the amino acids (**2**, **4**, **6** and **8**) with carbon disulfide followed by methylation gave the corresponding dithiocarbamates. Among the compounds synthesized, the β-carboline derivative (**9a**) exhibited remarkable hepatoprotective activity. The oxa analogues (**9b**—**d**) with respect to the dithiocarbamate group of **9a** were also prepared. The activity of **9a**—**d** decreased in the following order: dithiocarbamate (**9a**)>*S*-methyl thiocarbamate (**9b**)>*O*-methyl thiocarbamate (**9c**)>carbamate (**9d**). The structure–activity relationships are discussed.

Keywords——dithiocarbamate; α-amino acid; hepatoprotective activity; tetrahydro-β-carboline-3-carboxylic acid; tetrahydro-β-carboline; structure–activity relationship; carbon tetrachloride-induced liver damage

In recent years, the role of reactive oxygen species such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide, and hydroxy radical ($\dot{O}H$) in biochemical reactions has received a great deal of attention, together with the effect of the enzymatic defense system (*e.g.* superoxide dismutase, *etc.*).[1] The finely tuned balance between reactions of reactive oxygen species and the enzymatic defense system can, on occasion, be upset, with pathological consequences.[2] The presence of reactive oxygen species in amounts greater than normal thus causes cytotoxicity, mainly through oxidation of unsaturated fatty acids in the cellular membrane to lipid peroxides. Consequently, reactive oxygen species have been assumed to cause senescence and various diseases such as hepatitis, cataract, arteriosclerosis, and cancer.[2] The relationship with hepatitis appears to be of particular interest. Acute hepatitis in experimental animal models has been induced most commonly by administration of carbon tetrachloride (CCl_4). The oxidation of CCl_4 to $\cdot OOCCl_3$ radical by superoxide[3] is thought to cause hepatic damage in animals. Thus, compounds with radical scavenging activity may be of use as hepatoprotective agents.

Several types of thioamides have been reported to be readily desulfurized to the carbonyl compounds by superoxide.[4] Significant reactivity in several radical reactions such as peroxide decomposition and radical copolymerization has been reported for thioethers, among which 1,1-bis(ethylthio)ethene displayed potent reactivity.[5] This probably accounts[6] for part of the hepatoprotective activity of malotilate (**1**).[7] These observations indicate that thioamides and thioethers can act as radical scavengers. We are interested in the hepatoprotective activity of compounds with a dithiocarbamate group, which may exhibit radical scavenging activity in view of the structural similarity to thioamides and thioethers.

This paper describes the synthesis of dithiocarbamates of several α-amino acids and their hepatoprotective activities against acutely CCl_4-induced liver damage in mice.

Chemistry

Reaction of pipecolic acid (**2**), 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**4**),[8] and 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**8**)[9,10] with CS_2 in the presence of NaOH followed by alkylation with methyl iodide readily gave the corresponding dithiocarbamates (**3**, **5**, and **9a**). Since the β-carboline derivative (**9a**) exhibited potent hepatoprotective activity (see below), its ring-fissioned derivatives (**7a**, **b**) were similarly synthesized from **6a**, **b**. To examine the role of the dithiocarbamate group in **9a** in the manifestation of hepatoprotective activity, the oxa analogues (**9b**—**d**) were synthesized. The methylthiocarbonyl derivative (**9b**) was obtained by the reaction of **8** with carbon oxysulfide (COS),[11] followed by methylation. The reaction of **8** with *O*-methyl chlorothioformate[12] gave the methoxythiocarbonyl derivative (**9c**) in 35% yield. The carbamate (**9d**) was readily obtained by the reaction of **8** with methyl chloroformate.

malotilate **1**   **2**   **3**   **4**   **5**

6a : R=H
6b : R=Me

7a : R=H
7b : R=Me

8

9a : X=S, Y=S
9b : X=O, Y=S
9c : X=S, Y=O
9d : X=O, Y=O

Chart 1

Pharmacology and Structure–Activity Relationships

The dithiocarbamates and related compounds prepared in this study were tested for hepatoprotective activity against CCl_4-induced liver damage in mice after oral administration. The results were evaluated according to the criteria defined in Table I, which includes the combined effects both on the increase in relative liver weight (RLW) and on the appearance of liver by macroscopic observation (MO) [see Experimental]. The results are summarized in Table II together with comparative data for malotilate.

Of the five dithiocarbamates prepared in this study, the β-carboline derivative (**9a**) exhibited remarkable hepatoprotective activity. It was significantly effective (AA) even at 10 mg/kg orally. At this dose, malotilate exhibited C rank of activity. The tryptophan derivatives (**7a**, **b**), the ring-fissioned derivatives of **9a**, were ineffective. The importance of the β-carboline skeleton was further demonstrated by the much reduced activity of **3** and **5**. The effect of the dithiocarbamate group of **9a** was examined next by replacement of the two sulfur atoms by oxygen one by one (**9b**—**d**). The hepatoprotective activity of these oxa analogues decreased in the following order: **9a** (AA) > **9b** (A) > **9c** (B) > **9d** (D), indicating the importance of the presence of the SMe group. This is probably related to the significant reactivity of thioethers in radical reactions.[5]

TABLE I. Criteria of Hepatoprotective Effect

MO of the liver	Supressive % of the increase in RLW[a] >20%	>−20% to <20%	<−20%
Grade I	AA	C	D
Grade II	A	C	D
Grade III	B	D	D

AA = significantly effective; A, B and C = effective; D = ineffective. *a*) Relative liver weight.
Grade I = almost the same as normal control group; Grade II = intermediate between normal and CCl_4-treated control groups; Grade III = almost the same as CCl_4-treated control group.

TABLE II. Hepatoprotective Activity of Dithiocarbamates

Compd. No.	Hepatoprotective activity[a] 100 mg/kg *p.o.*	10 mg/kg *p.o.*
3	D	
5	B	
7a	D	
7b	D	
9a	AA	AA
9b	A	
9c	B	
9d	D	
Malotilate	AA	C

a) For methodology and criteria, see Table I and Experimental.

Further studies on a series of dithiocarbamates of various β-carbolines as new hepatoprotective agents are in progress and will be the subject of a forthcoming paper.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded on a Hitachi IR-215 spectrometer. Proton nuclear magnetic resonance (^1H-NMR) spectra were taken on a JEOL PMX-60 instrument. Chemical shifts are given as δ values from tetramethylsilane as an internal standard. The following abbreviation is used: s = singlet. Mass spectra (MS) were measured with a Hitachi RMU-6M instrument.

1-[(Methylthio)thiocarbonyl]pipecolic Acid (3)——A mixture of pipecolic acid (2) (2.58 g, 20 mmol), NaOH (1.65 g, 40 mmol), and CS_2 (1.33 ml, 22 mmol) in dimethyl sulfoxide (DMSO) (20 ml)–H_2O (2 ml) was stirred at room temperature for 45 min, and then methyl iodide (1.5 ml, 24 mmol) was added. After being stirred for 3 h, the mixture was poured into ice-water, acidified with 10% HCl, and extracted with AcOEt. The extract was washed with sat. NaCl, dried, and concentrated to leave a crystalline solid. Recrystallization from EtOH–hexane gave 3 (4.0 g, 91%), mp 130—131 °C. IR ν_{max}^{Nujol} cm^{-1}: 1705. MS *m/z*: 219 (M^+), 171 (M^+ − MeSH). NMR ($CDCl_3$) δ: 2.66 (3H, s). *Anal.* Calcd for $C_8H_{13}NO_2S_2$: C, 43.81; H, 5.97; N, 6.39; S, 29.24. Found: C, 43.89; H, 6.04; N, 6.35; S, 29.14. The following compounds were prepared in a similar manner.

1,2,3,4-Tetrahydro-2-[(methylthio)thiocarbonyl]isoquinoline-3-carboxylic Acid (5)——mp 141—142 °C (EtOH), 77%. IR ν_{max}^{Nujol} cm^{-1}: 1710. MS *m/z*: 267 (M^+), 219 (M^+ − MeSH). NMR ($CDCl_3$) δ: 2.69 (3H, s). *Anal.* Calcd for $C_{12}H_{13}NO_2S_2$: C, 53.91; H, 4.90; N, 5.24; S, 23.98. Found: C, 53.71; H, 4.88; N, 5.17; S, 24.01.

N^α-[(Methylthio)thiocarbonyl]tryptophan (7a)——A pale yellow oil (purified by column chromatography on silica gel (benzene–AcOEt = 4 : 6)) 35.7%. IR ν_{max}^{liquid} cm^{-1}: 1720. MS *m/z*: 294 (M^+), 246 (M^+ − MeSH). NMR ($CDCl_3$) δ: 2.49 (3H, s).

N^α-Methyl-N^α-[(methylthio)thiocarbonyl]tryptophan (7b)——A powder (purified by column chromatography on silica gel (hexane–AcOEt = 1 : 1)), 60%. IR ν_{max}^{Nujol} cm^{-1}: 1710. MS *m/z*: 308 (M^+), 260 (M^+ − MeSH). NMR ($CDCl_3$) δ: 2.63 (3H, s), 3.18 (3H, s). *Anal.* Calcd for $C_{14}H_{16}N_2O_2S_2$: C, 54.52; H, 5.23; N, 9.08; S, 20.79. Found: C, 54.69; H, 5.44; N, 9.21; S, 20.73.

1,2,3,4-Tetrahydro-2-[(methylthio)thiocarbonyl]-β-carboline-3-carboxylic Acid (9a)——mp 160—161 °C (dec.) (aq. EtOH), 59%. IR ν_{max}^{Nujol} cm^{-1}: 1710. MS *m/z*: 306 (M^+), 258 (M^+ − MeSH). NMR ($CDCl_3$–DMSO-d_6) δ: 2.68 (3H, s). *Anal.* Calcd for $C_{14}H_{14}N_2O_2S_2 \cdot C_2H_5OH$: C, 54.52; H, 5.72; N, 7.95; S, 18.19. Found: C, 54.47; H, 5.64; N, 7.94; S, 18.02.

1,2,3,4-Tetrahydro-2-[(methylthio)carbonyl]-β-carboline-3-carboxylic Acid (9b)——Carbon oxysulfide (1.35 g, 22.5 mmol) in dioxane (39 ml) was added to a stirred solution of 8 (3.24 g, 15 mmol) and triethylamine (4.22 g, 42 mmol) in MeOH (40 ml)–H_2O (20 ml), and the mixture was stirred at room temperature for 50 min. Methyl iodide (0.97 ml, 15.5 mmol) was added to the mixture, and the whole was stirred at room temperature for 1.5 h. The reaction mixture was concentrated *in vacuo*, and the residue was acidified with 5% HCl and extracted with AcOEt. The extract was washed with water and dried over Na_2SO_4. After removal of the solvent, the residual solid was recrystallized from ether to give **9b** (1.40 g, 32.2%). mp 195—197 °C. IR ν_{max}^{Nujol} cm^{-1}: 1720. MS *m/z*: 290 (M^+), 242 (M^+ − MeSH). NMR ($CDCl_3$–DMSO-d_6) δ: 2.37 (3H, s). *Anal.* Calcd for $C_{14}H_{14}N_2O_3S \cdot 0.5C_4H_{10}O$: C, 58.69; H, 5.85; N, 8.55; S,

9.79. Found: C, 58.67; H, 6.00; N, 8.42; S, 9.86.

1,2,3,4-Tetrahydro-2-[(methoxy)thiocarbonyl]-β-carboline-3-carboxylic Acid (9c)——An ethereal solution of *O*-methyl chlorothioformate [prepared from NaOMe (0.54 g, 10 mmol) and thiophosgene (0.76 ml, 10 mmol) in ether (20 ml)] and 2 N NaOH (5 ml) were added alternately to a stirred solution of **8** (1.51 g, 7 mmol) and 2 N NaOH (3.5 ml) in H_2O (20 ml) under ice-cooling, and the mixture was stirred at room temperature overnight. The ethereal layer was separated, and the aqueous layer was made acidic with 10% HCl and extracted with AcOEt. The extract was washed with water, dried, and evaporated. The residue was purified by chromatography on silica gel using $CHCl_3$–MeOH–AcOH (98 : 1 : 1) to afford a solid, which was crystallized from aq. EtOH to give **9c** (0.82 g, 35%), mp 125—128 °C. IR ν_{max}^{Nujol} cm^{-1}: 1705. MS *m/z*: 290 (M^+), 258 ($M^+ - MeOH$). NMR ($CDCl_3$) δ: 4.09 and 4.12 (1.4H and 1.6H, a pair of singlets, OCH_3, collapsed to a singlet (4.11) on heating at 90 °C). *Anal.* Calcd for $C_{14}H_{14}N_2O_3S \cdot C_2H_5OH$: C, 57.13; H, 5.99; N, 8.33; S, 9.53. Found: C, 56.83; H, 5.88; N, 8.28; S, 9.83.

1,2,3,4-Tetrahydro-2-methoxycarbonyl-β-carboline-3-carboxylic Acid (9d)——This compound was prepared from **8** and methyl chloroformate in a manner similar to that described for **9c**. mp 203—204 °C (dec.) (aq. EtOH), 43%. IR ν_{max}^{Nujol} cm^{-1}: 1715. MS *m/z*: 274 (M^+), 256, 143. NMR (DMSO-d_6) δ: 3.72 (3H, s). *Anal.* Calcd for $C_{14}H_{14}N_2O_4 \cdot H_2O$: C, 57.53; H, 5.52; N, 9.58. Found: C, 57.28; H, 5.41; N, 9.63.

Determination of Hepatoprotective Activity——Groups of 3 ddY male mice, weighing 25—30 g, were used. Test compounds were orally administered to mice at a dose of 100 mg/kg twice, 3 h before and after oral administration of 50 μl/kg of CCl_4. The animals were fasted after the first administration of test compounds. Following measurement of the body weight of each animal 24 h after administration of CCl_4, the whole liver was extirpated, weighed and macroscopically observed. The hepatoprotective effect of each test compound against CCl_4-induced liver damage was evaluated according to the criteria shown in Table I, in which suppression (%) of the increase in RLW, liver weight/100 g body wt., was calculated from the following equation and MO (color tone and external appearance) of the liver were ranked in the following 3 grades in comparison with those in normal and CCl_4-treated control groups: suppression (%) of the increase in RLW=

$$\left(1-\frac{\begin{matrix}\text{mean RLW of}\\ \text{test compound group}\end{matrix}-\begin{matrix}\text{mean RLW of}\\ \text{normal control group}\end{matrix}}{\begin{matrix}\text{mean RLW of}\\ CCl_4\text{-treated control group}\end{matrix}-\begin{matrix}\text{mean RLW of}\\ \text{normal control group}\end{matrix}}\right)\times 100$$

and MO grades were as described in Table I.

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Mutagenic Principles in Sinomeni Caulis *et* Rhizoma. I. The Structure of a Mutagenic Alkaloid, *N*-Demethyl-*N*-formyldehydronuciferine, in the Neutral Fraction of the Methanol Extract

TOMIO NOZAKA,*[,a] ISAO MORIMOTO,[a] MASAZO ISHINO,[a] TOMOAKI OKITSU,[a] HIDEAKI KONDOH,[b] KAZUAKI KYOGOKU,[b] YOKO SUGAWARA,[c] and HITOSHI IWASAKI[c]

Saitama Institute of Public Health,[a] 639–1 Kamiokubo, Urawa-shi, Saitama 338, Japan, Research Laboratory, Taisho Pharmaceutical Co., Ltd.,[b] 1–403 Yoshino-cho, Omiya-shi, Saitama 330, Japan, and RIKEN (The Institute of Physical and Chemical Research),[c] Wako-shi, Saitama 351–01, Japan

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A mutagenic principle in the neutral fraction of the methanol extract from Sinomeni Caulis *et* Rhizoma (Menispermaceae) was isolated by conventional techniques, and identified as a new dehydroaporphine alkaloid, *N*-demethyl-*N*-formyldehydronuciferine, on the basis of mass, ultraviolet, proton and carbon-13 nuclear magnetic resonance spectra, and X-ray analysis. *N*-Demethyl-*N*-formyldehydronuciferine was mutagenic to *Salmonella typhimurium* TA98 and TA100.

Keywords——mutagenicity; Sinomeni Caulis *et* Rhizoma; *N*-demethyl-*N*-formyldehydronuciferine; ^{1}H-NMR; ^{13}C-NMR; X-ray analysis

Ames and his co-workers have devised a simple short-term bioassay for detecting chemical carcinogens as mutagens.[1] The test has been widely used to detect mutagens among man-made and naturally-occurring compounds in the environment.[2] We have used Ames' test for assessing the potential health hazards of crude drugs, and reported the results of screening tests for mutagenicity in extracts of crude drugs.[3] Xanthone derivatives were characterized as mutagenic components in Gentianae Radix and Swertiae Herba, which are widely used in Japan as medicines.[4]

As a continuation of that work, we have tried to identify the mutagenic factors of Sinomeni Caulis *et* Rhizoma and have isolated a mutagenic principle from the neutral fraction of the methanol extract of the drug. This paper deals with the structural elucidation of the mutagen, as well as its specific activities and dose–effect curves in Ames' test. Sinomeni Caulis *et* Rhizoma originates from the root, root-stalk, and stem of *Sinomenium acutum* REHDER *et* WILSON (Menispermaceae; Japanese name, Oh-tsuzurafuji), and sinomenine, disinomenine, sinactine, tuduranine, acutumine, acutumidine, and magnoflorine have been reported as alkaloidal components.[5]

I

Fig. 1. Structure of *N*-Demethyl-*N*-formyldehydronuciferine (I)

The methanol extract of Sinomeni Caulis *et* Rhizoma was fractionated into the acidic, basic, and neutral fractions, and the mutagenicity of the fractions was examined.[6] A mutagen in the neutral fraction was separated and purified as described in Experimental (Ames' test was emloyed to monitor the progress of purification), and was identified as *N*-demethyl-*N*-formyldehydronuciferine (Fig. 1).

The mutagen was crystallized from methanol to give colorless needles, mp 140.5 °C. The mass spectrum (MS) showed a molecular ion (M^+) at *m/z* 307, which corresponded to $C_{19}H_{17}NO_3$, and chemical ionization MS showed the $(M+H)^+$ ion at *m/z* 308. The molecular formula was consistent with the result of elemental analysis. The ultraviolet (UV) absorption spectrum exhibited maxima at 205, 253, 317, 348, and 367 nm, suggesting that the mutagen might be a dehydroaporphine alkaloid.[7] The infrared (IR) absorption spectrum showed the

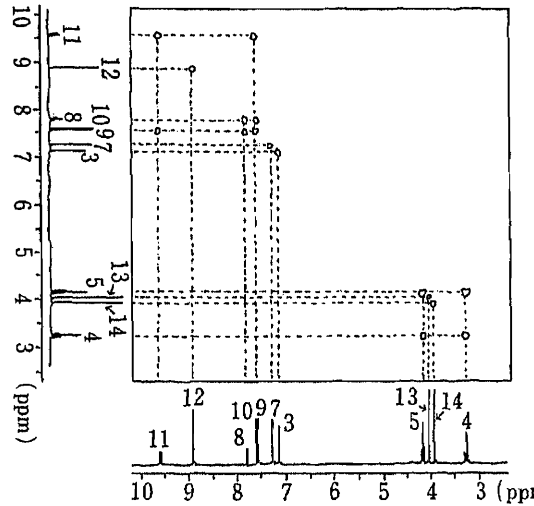

Fig. 2. ^1H-^1H Shift Correlation Two-Dimensional NMR Spectrum of I

TABLE 1. Assignments for ^1H-NMR and ^{13}C-NMR Signals

Carbon or proton	Chemical shifts (δ ppm, *J*=Hz)	
	^{13}C-NMR ($CDCl_3$)	^1H-NMR ($CDCl_3$)
1	146.0 (s)	—
2	151.8 (s)	—
3	112.9 (d, *J*=155)	7.13 (1H, s)
4	30.5 (t, *J*=130)	3.23 (2H, t, *J*=5.9)
5	38.5 (t, *J*=143)	4.15 (2H, t, *J*=5.8)
7	112.1 (d, *J*=159)	7.26 (1H, s)
8	127.8 (d)	7.78 (1H)
9	127.3 (d)	} 7.58 (2H)
10	126.2 (d)	
11	127.9 (d)	9.59 (1H)
12	160.8 (d, *J*=197)	8.90 (1H, s)
13	56.5 (q, *J*=144)	4.03 (3H, s)
14	59.9 (q, *J*=144)	3.93 (3H, s)
3a	128.2 or 128.4 (s)	—
6a	128.2 or 128.4 (s)	—
7a	132.4 (s)	—
11a	133.0 (s)	—
11b	125.7 (s)	—
11c	118.9 (s)	—

presence of a carbonyl group (1680 cm^{-1}). Its proton nuclear magnetic resonance (^{1}H-NMR) spectrum exhibited two methoxyl singlets at δ 3.93 and 4.03, two methylene signals at δ 3.23 and 4.15, and two aromatic proton singlets at δ 7.13 and 7.26. It also exhibited four aromatic proton signals showing complicated couplings at δ 7.58 (2H), 7.78 (1H), and 9.59 (1H) on the D ring of the dehydroaporphine alkaloid. The last signal is a characteristic downfield C-11 proton signal of the dehydroaporphine alkaloids.[7] The signal at δ 8.90 (1H, singlet) indicated the presence of an *N*-formyl group. The signals of the protons were assigned by measurements of the ^{1}H–^{1}H shift correlation spectrum (Fig. 2) and the nuclear Overhauser effect (NOE) difference spectra.

In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) studies, the proton noise-decoupled (PND) ^{13}C-NMR spectrum of the compound showed 19 resolved carbon signals. The signals of the carbon atoms were assigned on the basis of selective proton decoupling (SPD) and long-range selective proton decoupling (LSPD) experiments. All these ^{1}H-NMR and ^{13}C-NMR data were consistent with the structure (I) and the signals were assigned as shown in Table I.

The structure of the mutagen was finally confirmed by X-ray analysis. The structure was solved by the direct method using MULTAN 78,[8] and refined by the block-diagonal least-squares method. The final positional parameters are listed in Table II and an ORTEP drawing[9] of the molecule with the atomic numbering scheme is shown in Fig. 3.

Several alkaloids having an *N*-formyl group such as *N*-formylcorydamine, iwamide, and arnottianamide have been isolated from plant sources.[10] *N*-Demethyl-*N*-formyldehydro-nuciferine is a new *N*-formylaporphine alkaloid[11] from Sinomeni Caulis *et* Rhizoma.

TABLE II. Atomic Positional Parameters ($\times 10^4$) of Nonhydrogen Atoms and Equivalent Temperature Factors (Å^2)[a]

Atom	x	y	z	B_{eq}
N	1365 (2)	8222 (2)	3934 (2)	3.4
C(1)	−57 (2)	3163 (2)	1013 (3)	3.2
C(2)	1428 (2)	3675 (2)	663 (3)	3.4
C(3)	2513 (2)	5164 (2)	1246 (3)	3.6
C(4)	3319 (2)	7773 (2)	2751 (3)	3.9
C(5)	3003 (2)	8623 (2)	4279 (3)	3.8
C(7)	−1155 (2)	6274 (2)	3943 (3)	3.9
C(8)	−3796 (3)	4349 (3)	3797 (4)	5.0
C(9)	−4999 (3)	2927 (3)	3206 (4)	5.0
C(10)	−4776 (3)	1872 (2)	2122 (3)	4.3
C(11)	−3357 (2)	2225 (2)	1690 (3)	3.6
C(12)	969 (3)	9249 (2)	3958 (3)	4.3
C(13)	3182 (3)	3105 (3)	−759 (4)	5.0
C(14)	−848 (4)	914 (3)	1445 (4)	5.8
C(3a)	2131 (2)	6158 (2)	2163 (3)	3.1
C(6a)	232 (2)	6712 (2)	3475 (2)	3.0
C(7a)	−2334 (2)	4757 (2)	3355 (3)	3.6
C(11a)	−2073 (2)	3675 (2)	2312 (2)	3.0
C(11b)	−513 (2)	4135 (2)	1939 (2)	2.8
C(11c)	631 (2)	5665 (2)	2517 (2)	2.7
O(1)	1896 (2)	10565 (2)	4446 (3)	6.1
O(2)	1711 (2)	2620 (2)	−276 (2)	4.6
O(3)	−1068 (2)	1669 (1)	377 (2)	4.1

a) e.s.d.'s in parentheses. Equivalent temperature factors are defined by the expression $B_{eq} = 4/3 \sum_i \sum_j a_i a_j B_{ij}$, where a_i's are cell constants.

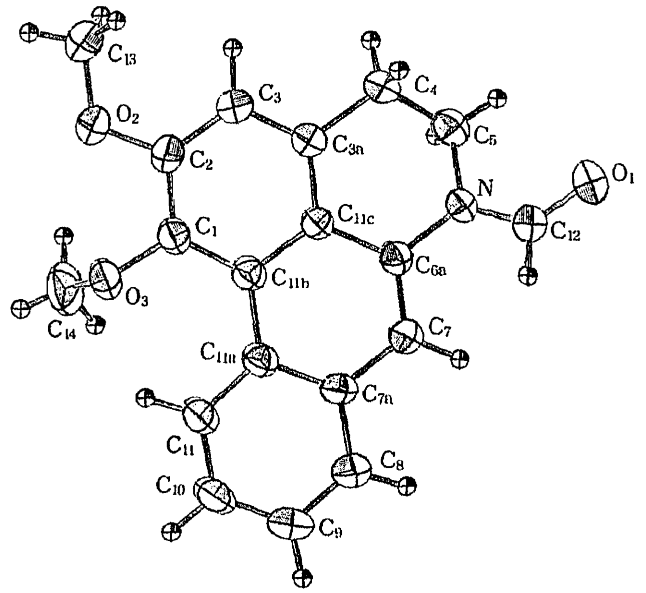

Fig. 3. ORTEP Drawing of I

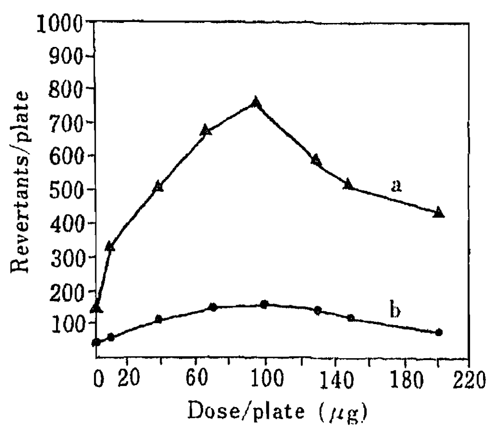

Fig. 4. Dose–Effect Curves of I

a, TA100 (with S9 mixture); b, TA98 (with S9 mixture). (Mutagenicity was assayed by the preincubation method. Each point is the average of 3 plates. Spontaneous revertants have not been subtracted.)

The dose–effect curves of *N*-demethyl-*N*-formyldehydronuciferine are shown in Fig. 4. A linear relationship was observed in the range of doses of 0 to 100 μg. At over 100 μg, toxicity was observed. The specific activities were 2 and 19 revertants per μg on *Salmonella typhimurium* TA98 and TA100, respectively, after treatment with liver homogenate (9000 *g* supernatant) (S9 mixture).

In addition to the neutral fraction, the acidic and basic fractions of the methanol extract from Sinomeni Caulis *et* Rhizoma showed mutagenicity in Ames' test. In thin-layer chromatographic analysis of the acidic and basic fractions, *N*-demethyl-*N*-formyldehydronuciferine was not detected, suggesting the presence of other kinds of mutagen in the acidic and basic fractions. Further work to characterize them is in progress.

Experimental

Melting points were determined on a Yazawa BY-2 apparatus and are uncorrected. The UV spectrum was recorded on a Shimadzu UV-240 UV-visible recording photometer. The IR spectrum was measured in a KBr disk with a Shimadzu IR spectrophotometer, model IR 435. ^{1}H-NMR and ^{13}C-NMR spectra were measured in $CDCl_3$ with tetramethylsilane (TMS) as an internal reference using a JEOL TNM-GX 400 instrument. Chemical shifts (δ) are expressed in ppm downfield from TMS, and coupling constants (*J*) in Hz. Abbreviations: s = singlet, d = doublet, t = triplet, q = quartet. Elementary analysis was carried out on a Perkin-Elmer 240 elementary analyzer. The mass spectrum (MS) was recorded on a Shimadzu LKB-9000 GC-MS high-resolution spectrometer. Wakogel B-5FM was used for thin layer chromatography (TLC) and preparative TLC, and Wakogel C-200 for column chromatography. Spots on the TLC plate were visualized under UV irradiation or by heating the plate at 105 °C after spraying it with 10% H_2SO_4.

Mutagenicity Assay——The preincubation method[12] (modified Ames' test) was used with *Salmonella typhimurium* strains TA98 and TA100 with or without S9 mixture to evaluate mutagenicity. S9 was prepared from the liver of male Sprague-Dawley rats pretreated with phenobarbital and β-naphthoflavone. The S9 mixture was prepared according to the reported method[3,4] Benzo[*a*]pyrene and furylfuramide (trade name AF-2) were used as positive controls.

Isolation of *N*-Demethyl-*N*-formyldehydronuciferine——Sinomeni Caulis *et* Rhizoma (from plants cultivated in Shikoku) was purchased from Kinokuniya Kanyakkyoku Ltd. (Japan). Dried, ground material (500 g) was extracted with MeOH (1 l) at room temperature for 2 d. The MeOH extract (16.8 g) was fractionated into acidic, basic, and neutral fractions with 1 N HCl, 1 N NaOH, ammonia water, and $CHCl_3$. The neutral fraction (1.1 g) was fractionated by preparative TLC ($CHCl_3$: ethyl acetate = 9 : 1) to give the crude mutagenic fraction (10 mg). This fraction was further fractionated sequentially by silica gel (100 g) column chromatography with $CHCl_3$–ethyl acetate (9 : 1, 500 ml)

and Sephadex LH-20 column (2 cm × 30 cm) chromatography with MeOH to give *N*-demethyl-*N*-formyldehydronuciferine (2 mg). All fractions were screened for mutagenicity, and the mutagenic activity of the neutral fraction was followed.

X-Ray Analysis——A single crystal, 0.65 × 0.6 × 0.04 mm, was used for intensity data measurement. The cell dimensions and intensity data were collected on a Rigaku AFC-4 automated four-circle diffractometer with graphite-monochromated MoK_α radiation. The crystal data are as follows: triclinic, $P\bar{1}$, $a=9.948(4)$, $b=10.970(4)$, $c=8.285(3)$ Å, $\alpha=105.34(4)$, $\beta=93.37(4)$, $\gamma=118.06(3)$°, $V=751.4(4)$ Å^3, $Z=2$, and $D_x=1.358$ Mg m^{-3}. A total of 4661 independent reflections were measured through the range of $0<2\theta\leqq 60$° in the ω–2θ scan mode, and 2833 reflections of $|F_o|\geqq 3\sigma(F_o)$ were used for structure determination. The data were corrected for Lorentz and polarization factors, but not for absorption (μ(MoK_α) = 0.086 mm^{-1}). A difference Fourier map revealed all the hydrogen atom positions. Inclusion of H atoms with isotropic temperature factors gave a final R value of 0.064 for 2833 independent reflections. The weighting scheme for the final refinement was: $w=0.5$ for $|F_o|<3.0$, $w=1.0$ for $3.0\leqq|F_o|<8.0$, $w=(5.0/|F_o|)^2$ for $8.0\leqq|F_o|$ and $R_w=0.081$. The maximum and minimum peaks in the final difference Fourier map were 0.25 and −0.34 e/Å^3, respectively. Calculations were performed on a FACOM M-380 computer at RIKEN using the UNICS III program system.[13]

***N*-Demethyl-*N*-formyldehydronuciferine**——Colorless needles from MeOH, mp 140.5 °C. UV λ_{max}^{MeOH} nm: 205, 253, 317, 348, 367. IR ν_{max}^{KBr} cm^{-1}: 1680 (C=O). MS *m/z* 307 (M^+), CI-MS (reagent gas, isobutane) *m/z* 308 ($(M+H)^+$). *Anal.* Calcd for $C_{19}H_{17}NO_3$: C, 74.25; H, 5.57; N, 4.55. Found: C, 74.09; H, 5.73; N, 4.68. ^{1}H-NMR ($CDCl_3$) δ, ^{13}C-NMR ($CDCl_3$) δ: Table I.

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Novel Guaiane- and Secoguaiane-Type Sesquiterpenes from *Alpinia japonica* (THUNB.) MIQ.

HIDEJI ITOKAWA,*,[a] HIROSHI MORITA,[a] KENJI OSAWA,[a] KINZO WATANABE[a] and YOICHI IITAKA[b]

Tokyo College of Pharmacy,[a] 1432-1, Horinouchi, Hachioji, Tokyo 192-03, Japan and Faculty of Pharmaceutical Sciences, The University of Tokyo,[b] Hongo, Bunkyo-ku, Tokyo 113, Japan

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Three novel guaiane-type sesquiterpenes, hanalpinone (I), isohanalpinone (II), and alpinenone (III), and two novel secoguaiane-type sesquiterpenes, alpinolide peroxide (IV) and 6-hydroxyalpinolide (V) have been isolated from the rhizomes of *Alpinia japonica*. Their structures were determined by spectroscopic methods, chemical conversions, and X-ray analysis. Their biosynthetic relationships with previously identified sesquiterpenes from *Alpinia japonica* are discussed.

Keywords——*Alpinia japonica*; Zingiberaceae; sesquiterpene; guaiane; secoguaiane; peroxide; X-ray analysis; ^{13}C-NMR; biosynthesis

In our previous papers,[1-4] a number of endesmanes, agarofurans, eremophilanes, guaianes, and secoguaianes isolated from *Alpinia japonica* were reported. These sesquiterpenes included novel sesquiterpenes which possess a hydroperoxide or a cyclic peroxide moiety. Biogenetic considerations indicated a possible biogenetic pathway[2] for eudesmanes, agarofurans, and eremophilanes, while the chemical conversions of hanalpinol (VI) with a guaiane skeleton into furopelargones (VII and VIII) and alpinolide (IX) with a secoguaiane skeleton seemed to suggest a possible biogenetic pathway for secoguaianes.[3,4]

Further examination of the constituents of the rhizomes of *Alpinia japonica* resulted in the isolation and structure determination of five new guaianes and secoguaianes, named hanalpinone (I), isohanalpinone (II), alpinenone (III), alpinolide peroxide (IV), and 6-hydroxyalpinolide (V). Furthermore, biogenetic considerations based on the structural relationships led to a proposed biogenetic pathway for these compounds.

Fresh rhizomes of *A. japonica* were extracted with methanol. The extraction was carried out under an argon atmosphere at 0 °C to avoid autooxidation by atmospheric oxygen. The methanolic extract was shaken with *n*-hexane and chromatographic purification of the *n*-hexane-soluble fraction gave the new guaianes (I—III) and secoguaianes (IV and V).

Hanalpinone (I) and isohanalpinone (II), which appeared to be new peroxide sesquiter-

I II III IV V

Chart 1

penes because of their positive color reaction for peroxide,[5] were both isolated as colorless needles having the same molecular formula, $C_{15}H_{22}O_3$. The physical properties and spectral data of I were identical with those recently published.[3] Compound II (mp 90.0—91.5 °C) was found to be a diastereomer of I as shown below. The infrared (IR) (1675 cm^{-1}) and nuclear magnetic resonance (NMR) spectra (Tables I and II) showed that II possesses a guaiane skeleton like that of I. As in the case of the reaction from hanalpinol (VI) to furopelargone B (VII),[3] acid treatment of the product X which was obtained by reduction of II with sodium borohydride gave VII, whose spectral data and specific rotation were identical with those of natural furopelargone B[6] (Chart 2). Therefore, the absolute configurations of C-1, -4 and -5 were determined to be as shown in Chart 2. The configuration of the peroxide moiety was determined from the NMR spectra. In the ^{1}H-NMR spectra of I and II, only the H-6 and H-14 signals showed appreciable differences. The lack of vicinal coupling between H-5 and H-6 of II indicates that the dihedral angle between them is *ca.* 90° and the chemical shift (1.14 ppm) of H-14 indicates that the methyl group on C-4 is shielded by the lone pair of the peroxide oxygen. These NMR data suggested that the configuration of the cyclic peroxide is α. Nuclear Overhauser effect (NOE) was observed between H-11 and H-14 of I and VI (8.0 and 6.3%, respectively), suggesting that the molecules of I and VI take a basket conformation. On the other hand, no NOE was observed between the corresponding hydrogens of II.

Chart 2

Chart 3

8.0% NOE I | non NOE II | 10.0% NOE III | 6.3% NOE VI

Chart 4

TABLE I. ^{1}H-NMR Data for I—VI (400 MHz in $CDCl_3$)

Proton No.	I	II	III	VI
1	2.69, dt		2.90, dt	2.66, ddd
5	3.05, dt		2.80, t	2.79, dt
6	4.73, dd	4.68, d		4.38, dd
8	6.31, br s	6.09, t	5.93, s	5.61, ddd
9				4.16 dd
11	2.58, sept	2.61, sept	2.93, sept	2.23, sept
12	1.20, d	1.12, d	1.18, d	1.08, d
13	1.23, d	1.16, d	1.25, d	1.10, d
14	0.96, d	1.14, d	1.11, d	1.06, d
15	1.43, s	1.34, s	1.48, s	1.35, s

Coupling constants in Hz. I: 1,2=10.0; 1,2=4.0; 1,5=10.0; 4,14=7.0; 4,5=10.0; 5,6=7.0; 6,8=1.0; 11,12=7.0; 11,13=7.0. II: 4,14=6.6; 6,8=1.2; 8,11=1.2; 11,12=7.0; 11,13=7.0. III: 1,2=9.0; 1,2=2.4; 1,5=9.0; 4,14=7.3; 4,5=9.0; 11,12=7.0; 11,13=7.0. VI: 1,2=11.7; 1,2=5.5; 1,5=8.0; 4,14=7.0; 4,5=8.0; 5,6=4.9; 6,8=1.5; 8,9=5.3; 8,11=1.3; 9,OH=12.0; 11,12=7.0; 11,13=7.0.

TABLE II. ^{13}C-NMR Data for I—VI (100 MHz in $CDCl_3$)

No.	I	II	III	VI
1	41.7	38.9	52.4	44.7
2	32.9	31.9	33.0	33.0
3	26.8	25.4	24.6	25.4
4	37.1	38.6	36.3	37.3
5	43.7	47.0	62.8	46.4
6	81.8	77.6	105.5	80.3
7	166.6	165.0	176.7	152.4
8	128.7	127.8	123.3	124.6
9	201.3	205.1	199.1	73.1
10	88.5	88.3	88.0	83.3
11	36.4	35.8	30.0	32.6
12	20.0	20.1	20.6	20.3
13	22.4	22.4	20.8	23.1
14	15.6	14.9	14.4	15.2
15	22.5	20.6	25.6	25.4

On reaction with BF_3–Et_2O, hanalpinone (I) gave alpinolide (IX),[4] which had previously been isolated from *Alpinia japonica*. The reaction steps involved are shown in Chart 3.

Alpinenone (III) was obtained as colorless needles (mp 140.0—142.0 °C) having the molecular formula $C_{15}H_{22}O_3$, based on elemental analysis. The IR (3610 and 1685 cm^{-1}),

ultraviolet (UV) (244 nm, ε 8500), and ^{1}H-NMR spectra (Table I) suggested the presence of a hydroxyl group, an α,β-unsaturated carbonyl group, and an isopropyl group attached to the β-carbon of the α,β-unsaturated carbonyl group. Further inspection of the spectral data of III indicated that III is a similar guaiane-type sesquiterpene to I and II, but as it gave a negative color reaction for peroxides and as the ^{13}C-NMR spectrum showed signals at δ 88.0 (s) and 105.5 (s), III was considered to have a cyclic hemiketal linkage between C-6 and C-10 in place of a peroxide. 10.0% NOE between H-11 and H-14 of III was observed, suggesting that it has a basket-like structure similar to those of I and VI. Therefore the configuration of the cyclic hemiketal linkage was determined to be β. Reductive cleavage of III with lithium aluminum hydride gave XI, and oxidative ring cleavage of the seven-membered carbocycle of XI with pyridinium chlorochromate (PCC) gave VII (Chart 5). Therefore the absolute structure of III was determined to be as shown in Chart 1.

III —LiAlH4→ XI —PCC→ [ ] → VII

Chart 5

Alpinolide peroxide (IV), a novel secoguaiane-type sesquiterpene with a cyclic peroxide linkage, was obtained as colorless needles (mp 128.0—130.0 °C) having the molecular formula $C_{15}H_{22}O_5$, based on high-resolution mass spectrum (MS). The IR (1780 cm^{-1}) and ^{1}H-NMR (δ 5.95, 1H) spectra suggested the presence of an α,β-unsaturated butenolide as in IX. However, the lack of a methyl ketone group and a lactonic methine proton in the IR spectrum and ^{1}H-NMR spectrum, respectively, the presence of a hydroxyl group (3610 cm^{-1}) and carbon atoms giving signals at δ 100.8 and 109.8 ppm, and the positive peroxide color reaction suggested the presence of a peroxide linkage between C-6 and C-10. This assumption was confirmed by the fact that chemical oxidation of furopelargone B (VII) with *m*-chloroperbenzoic acid gave XII, autooxidation of which with oxygen gave IV (Chart 6). Therefore the absolute configurations of C-1, -4 and -5 were determined to be the same as in VII. The absolute configurations of the remaining two asymmetric carbons, C-6 and C-10, were determined to be "*S*" by X-ray analysis (Fig. 1).

VII —m-CPBA→ XII —O2→ IV

Chart 6

6-Hydroxyalpinolide (V) was obtained as a colorless oil having the molecular formula $C_{15}H_{22}O_4$, based on high-resolution MS. The IR (CCl_4) spectrum showed typical absorption bands of α,β-unsaturated butenolide (1750 and 1770 cm^{-1}) and a methyl ketone group (1713 cm^{-1}). By comparison of the spectroscopic data with those of IX, V was deduced to be IX with a hydroxyl group at C-6, because there was no lactonic methine proton signal in the

Fig. 1. Molecular Structure Drawn by the PLUTO[9] Program

^{1}H-NMR spectrum and a hydroxyl absorption band (3600 cm^{-1}) was observed in the IR spectrum. The location of the hydroxyl group at C-6 was finally confirmed by methylation of V with diazomethane to give XIII (Chart 7). The absolute structure was determined by chemical conversion of IV into V: reduction of IV with triphenylphosphine gave an epimeric mixture of XIV at C-10, followed by acid-catalyzed cleavage of the hemiketal linkage to give V (Chart 7). It is considered that the hemiketals (XIV) were not the epimeric mixture at C-6 because V remained unaltered on exposure to the same conditions. Therefore, the absolute configuration of C-6 was determined to be "*S*," as in compound IV. The absolute structure of V was determined to be as shown in Chart 1. It is considered that the hemiketals (XIV) were produced when C-1 had α-configuration, while V was isomerized by acid catalysis to β-configuration. This assumption was supported by a comparison of the C-2 and C-3 chemical shifts in a series of similar compounds (Table III). The carbon signal of C-2 appears at lower field than 34.0 ppm when C-1 takes β-configuration, and at higher field in the case of α-configuration. Furthermore, the borderline in the case of the C-3 chemical shift seems to be in the vicinity of 26.0 ppm. The configuration of the methyl ketone group can also be determined from the chemical shift values.

IV —PPh3→ XIV —silica gel→ [ ] → V —CH2N2→ XIII

Chart 7

The biogenetic pathway proposed in Chart 8 is based on the chemical conversions discussed above. Furopelargones B and A (VII and VIII) are considered to be derived from hanalpinol (VI) or its analog containing a hydroxyl group at C-9 through a cleavage of the C-9–C10 bond of its guaiane skeleton. Alpinolide (IX) is considered to be derived from

Chart 8. Proposed Biogenetic Pathway of Guaiane- and Secoguaiane-Type Sesquiterpenes Obtained from *Alpinia japonica*

Table III. ^{13}C-NMR Data for IV, V, VII, VIII, XII, XIII, XV and IX (100 MHz in $CDCl_3$)

No.	IV	V	VII	VIII	XII	XIII	XV	IX
1	45.1	55.0	57.8	55.5	56.9	58.6	54.5	51.2
2	31.0	34.5	31.9	34.2	31.7	34.6	34.5	34.1
3	25.9	29.1	24.7	28.0	25.5	29.4	29.2	26.7
4	37.6	35.1	40.3	39.3	38.5	37.8	38.0	35.1
5	39.6	53.2	43.8	42.1	42.9	55.4	39.9	43.4
6	109.8	109.9	147.3	149.4	146.6	209.2	146.5	83.3
7	170.6	170.9	128.4	127.1	121.1	167.5	120.0	173.3
8	119.6	116.2	108.0	108.6	31.6	114.8	32.4	114.6
9	174.5	177.5	140.9	140.2	175.8	166.2	174.8	178.8
10	100.8	213.9	208.1	209.8	207.7	209.5	209.1	210.2
11	27.6	27.6	24.5	24.3	25.2	31.7	25.0	28.1
12	21.6	21.3	23.6	23.9	21.5	20.9	21.8	20.2
13	21.7	21.3	24.4	24.1	22.2	21.2	22.2	22.1
14	15.7	22.7	16.1	16.3	16.1	20.5	16.1	16.2
15	24.2	29.1	28.6	29.2	29.2	29.2	29.2	29.9
COOMe						51.6		

XV: Epimer of XII at the methyl ketone group obtained by oxidation of VIII with *m*-CPBA. The measurements were made on a Brucker AM400 spectrometer in $CDCl_3$ with tetramethylsilane as an internal reference, and the chemical shifts are expressed in ppm.

hanalpinone (I) and isohanalpinone (II) through a C9–C10 bond cleavage similar to that involved in the above conversion. However, the lactone ring of alpinolide (IX) may be biosynthesized by oxidation of the furan ring of furopelargone A (VIII). Alpinolide peroxide (IV) and 6-hydroxyalpinolide (V) are considered to be biosynthesized by partial oxidation of

TABLE IV. Atomic Coordinates and Equivalent Isotropic Temperature Factors

No.	Atom	x 10^4	y 10^4	z 10^4	B_{eq} (Å^2)
1	C1	4026 (6)	−960 (0)	1049 (13)	3.7 (0.1)
2	C2	3045 (6)	−719 (5)	1641 (14)	4.4 (0.1)
3	C3	2575 (7)	−152 (6)	−147 (15)	5.1 (0.2)
4	C4	3361 (7)	−62 (5)	−1620 (13)	4.5 (0.1)
5	C5	4346 (6)	−295 (5)	−223 (13)	3.7 (0.1)
6	C6	4908 (6)	296 (4)	1331 (13)	3.6 (0.1)
7	C7	5491 (7)	882 (5)	321 (14)	4.3 (0.1)
8	C8	5163 (8)	1544 (5)	849 (16)	5.1 (0.2)
9	C9	4372 (8)	1452 (5)	2074 (15)	4.9 (0.2)
10	C10	4839 (6)	−1213 (4)	2859 (13)	3.8 (0.1)
11	C11	3288 (8)	679 (7)	−2857 (15)	6.1 (0.2)
12	C12	4455 (8)	−1724 (5)	4516 (16)	5.3 (0.2)
13	C13	6320 (8)	689 (6)	−804 (18)	5.8 (0.2)
14	C14	7280 (9)	596 (8)	677 (22)	8.3 (0.3)
15	C15	6453 (9)	1294 (8)	−2548 (18)	7.2 (0.2)
16	O1	5584 (5)	−1525 (4)	1936 (9)	4.9 (0.1)
17	O2	5203 (5)	−580 (3)	4218 (8)	4.2 (0.1)
18	O3	5659 (5)	−37 (3)	2928 (9)	4.3 (0.1)
19	O4	4238 (5)	708 (3)	2389 (9)	4.5 (0.1)
20	O5	3877 (6)	1899 (4)	2856 (12)	6.9 (0.1)

No.	Atom	x 10^3	y 10^3	z 10^3	B_{eq} (Å^2)
21	HC1	384 (6)	−144 (5)	−7 (13)	5. (2.)
22	HC2	315 (7)	−42 (6)	317 (15)	7. (2.)
23	H'C2	253 (6)	−116 (5)	178 (13)	5. (2.)
24	HC3	236 (7)	38 (5)	56 (15)	6. (2.)
25	H'C3	186 (7)	−38 (5)	−94 (14)	6. (2.)
26	HC4	323 (6)	−49 (5)	−289 (14)	6. (2.)
27	HC5	491 (6)	−45 (5)	−115 (12)	4. (2.)
28	HC8	545 (7)	208 (5)	46 (15)	7. (3.)
29	HC11	384 (7)	71 (6)	−376 (16)	8. (3.)
30	H'C11	332 (6)	115 (5)	−181 (13)	5. (2.)
31	H''C11	252 (6)	73 (5)	−390 (13)	5. (2.)
32	HC12	505 (8)	−195 (6)	572 (15)	7. (3.)
33	H'C12	406 (6)	−222 (5)	375 (13)	6. (2.)
34	H''C12	384 (6)	−145 (6)	518 (14)	6. (2.)
35	HC13	622 (8)	21 (7)	−156 (19)	10. (3.)
36	HC14	790 (7)	47 (6)	−14 (15)	7. (3.)
37	H'C14	719 (8)	13 (6)	191 (17)	8. (3.)
38	H''C14	748 (8)	112 (7)	159 (18)	11. (4.)
39	HC15	715 (6)	118 (5)	−331 (13)	6. (2.)
40	H'C15	668 (7)	185 (5)	−170 (14)	6. (2.)
41	H''C15	582 (7)	140 (6)	−368 (16)	8. (3.)
42	HO1	601 (7)	−184 (5)	300 (14)	6. (2.)

Equivalent positions: x y z
$-x$ $1/2+y$ $-z$.

furopelargones, or 6-hydroxyalpinolide (V) may also be derived by way of alpinolide peroxide. Alpinenone (III) may be derived from hanalpinone (I) by formation of a hemiketal after the cleavage of the peroxide.

These sesquiterpenes obtained from *Alpinia japonica*, in particular, I, II, IV and VI, which contains a cyclic peroxide in the molecule, are unique and are also of interest from the

viewpoint of phytophysiology.

Experimental

All melting points were recorded on a Yanagimoto micro melting point apparatus and are uncorrected. Spectral data were obtained on the following instruments; optical rotation on a JASCO DIP-4, IR on a JASCO A-302, UV on a Hitachi 557, NMR on a Brucker AM400, and MS on a Hitachi M-80. High-performance liquid chromatography (HPLC) was carried out on a CIG column system (Kusano Scientific Co., Tokyo) with Iatrobeads (60 μ silica gel, IATRON Co., Tokyo) as the stationary phase.

Extraction and Isolation——Fresh rhizomes (70.0 kg) of *Alpinia japonica* were extracted twice with methanol under an argon atmosphere. The methanolic extract was partitioned with *n*-hexane, and the *n*-hexane layer was concentrated to give a yellow oil (65.0 g). The residue was subjected to column chromatography on silica gel with an *n*-hexane–ethyl acetate gradient system. Repeated HPLC and $AgNO_3$–HPLC of each fraction using a benzene–ethyl acetate system, a benzene–chloroform–acetonitrile system, an *n*-hexane–ethyl acetate system, and an *n*-hexane–chloroform–acetonitrile system gave I (200 mg), II (200 mg), III (2.0 g), IV (200 mg), and V (300 mg). Compounds I—IV were recrystallized from benzene.

Compound I (Hanalpinone): Colorless needles, mp 80.5—82.0 °C, $[\alpha]_D$ +149.5 ° (c=0.20, EtOH). MS *m/z* (%): 250 (M^+, 56, Calcd for $C_{15}H_{22}O_3$, 250.1566; Found 250.1547), 235 (5), 232 (4), 217 (6), 207 (29), 192 (12), 189 (18), 179 (50), 166 (33), 165 (45), 153 (32), 147 (24), 139 (38), 126 (100), 125 (37), 111 (42), 97 (58), 81 (71). IR (KBr) cm^{-1}: 2960, 2875, 1665, 1465, 1380, 1240, 980, 885. UV (EtOH) nm (ε): 237 (9330), 345 (107).

Compound II (Isohanalpinone): Colorless needles, mp 90.0—91.5 °C, $[\alpha]_D$ −61.0 ° (c=0.25, EtOH). MS *m/z* (%): 250 (M^+, 10, Calcd for $C_{15}H_{22}O_3$, 250.1567; Found 250.1554), 235 (1), 232 (0.8), 217 (1.5), 207 (5), 189 (4), 179 (6), 168 (13), 153 (9), 126 (100), 125 (15), 111 (19), 95 (16), 81 (25). IR (KBr) cm^{-1}: 2950, 2870, 1675, 1630, 1450, 1375, 1275, 980, 875. UV (EtOH) nm (ε): 236 (8880), 339 (113).

Compound III (Alpinenone): Colorless needles, mp 140.0—142.0 °C, $[\alpha]_D$ +322.7 ° (c=0.30, $CHCl_3$). *Anal.* Calcd for $C_{15}H_{22}O_3$: C, 71.97; H, 8.86. Found: C, 71.88; H, 8.93. MS *m/z* (%): 250 (M^+, 40), 235 (4), 232 (6), 217 (15), 207 (100), 189 (26), 179 (68), 165 (57), 153 (40), 147 (32), 139 (44), 125 (32), 109 (42), 97 (82), 81 (63), 69 (49), 67 (47), 55 (48), 53 (51). IR (CCl_4) cm^{-1}: 3600, 3400, 2970, 2900, 2840, 1685, 1620, 1465, 1450, 1385, 1375, 1340, 1265, 1240, 1185, 1165, 1145, 1090, 1000, 965, 920, 885. UV (EtOH) nm (ε): 202 (3300), 244 (8500), 348 (310).

Compound IV (Alpinolide Peroxide): Colorless needles, mp 128.0—130.0 °C, $[\alpha]_D$ +366.3 ° (c=0.08, $CHCl_3$). MS *m/z* (%): 264 (M^+ − 18, 1, Calcd for $C_{15}H_{20}O_4$, 264.1360; Found 264.1371), 249 (80), 206 (30), 165 (13), 124 (20), 81 (70), 67 (100). IR (CCl_4) cm^{-1}: 3610, 2970, 2880, 1780, 1640, 1470, 1390, 1375, 1230, 1160, 1100, 1045, 1010, 980, 960, 920, 910, 870, 680. ^{1}H-NMR ($CDCl_3$) δ ppm: 0.83 (3H, d, J=7.0 Hz), 1.19 (3H, d, J=7.0 Hz), 1.27 (3H, d, J= 7.0 Hz), 1.40 (3H, s), 5.95 (1H, d, J=1.5 Hz). UV (EtOH) nm (ε): 216 (8020), 290 (330).

Compound V (6-Hydroxyalpinolide): A colorless oil, $[\alpha]_D$ −26.2 ° (c=0.63, $CHCl_3$). MS *m/z* (%): 266 (M^+, 5, Calcd for $C_{15}H_{22}O_4$, 266.1517; Found 266.1525), 249 (15), 206 (30), 142 (60), 126 (90), 111 (100), 81 (55), 71 (45). IR (CCl_4) cm^{-1}: 3600, 3380, 2970, 2880, 1770, 1750, 1713, 1640, 1460, 1380, 1360, 1260, 1165, 1095, 930, 865. UV (EtOH) nm (ε): 212 (9800). ^{1}H-NMR ($CDCl_3$) δ ppm: 0.92 (3H, d, J=7.0 Hz), 1.22 (3H, d, J=7.0 Hz), 1.29 (3H, d, J= 7.0 Hz), 2.30 (3H, s), 3.37 (1H, m), 5.59 (1H, br s), 5.77 (1H, s).

Oxidation of VI with Pyridinium Chlorochromate to Give I——VI (64 mg) was treated with pyridinium chlorochromate (87 mg) in methylene chloride (2 ml) for 3 h at room temperature. Then, dry ether (20 ml) was added. The reaction mixture was filtered and the filtrate was evaporated. Purification by HPLC (*n*-hexane : ethyl acetate = 19 : 1) and recrystallization from *n*-pentane gave I (48 mg) as colorless needles.

Reduction of II with Sodium Borohydride to Give X——A methanol solution of II (25 mg) was treated with an excess of sodium borohydride. After work-up in the usual way, the product was subjected to HPLC (*n*-hexane : ethyl acetate = 4 : 1) to give X (22 mg) as colorless needles; $[\alpha]_D$ −21.0 ° (c=0.10, $CHCl_3$), mp 67.0 °C. MS *m/z* (%): 252 (M^+, 54), 235 (100), 201 (40), 175 (54). IR (CCl_4) cm^{-1}: 3630, 3530, 2960, 2880, 1465, 1385, 1380, 1000, 970. ^{1}H-NMR ($CDCl_3$) δ ppm: 1.06 (6H, d, J=7.0 Hz), 1.11 (3H, d, J=6.7 Hz), 1.30 (3H, s), 2.37 (1H, sept. d, J=7.0, 1.2 Hz), 2.73 (1H, ddd, J=6.6, 9.5, 9.5 Hz), 4.17 (1H, d, J=4.5 Hz), 4.40 (1H, d, J=1.1 Hz), 5.55 (1H, ddd, J=1.1, 1.2, 4.5 Hz).

Conversion of X to VII——X (20 mg) was stirred with *p*-toluenesulfonic acid (50 mg) in benzene (2 ml) for 12 h at room temperature, then the reaction mixture was filtered. The filtrate was washed with saturated sodium bicarbonate solution and brine, dried over magnesium sulfate and evaporated. The product was purified by HPLC (*n*-hexane : ethyl acetate = 4 : 1) to give VII (10 mg) whose spectral data (including specific rotation) were identical with those of natural furopelargone B.

Reduction of III with Lithium Aluminium Hydride to Give XI——An ether solution of III (100 mg) was treated with lithium aluminum hydride (500 mg) in dry ether under a nitrogen atmosphere for 3 h at room temperature. After work-up in the usual way, the product was subjected to HPLC (*n*-hexane : ethyl acetate = 1 : 1) to give XI as a colorless oil (80 mg). MS *m/z* (%): 236 (M^+ − 18, 10), 218 (10), 193 (52), 175 (80), 123 (100), 95 (86), 81 (66). IR ($CHCl_3$) cm^{-1}: 3610, 3400, 2960, 2880, 1600, 1465, 1380, 1050, 1020, 900. ^{1}H-NMR ($CDCl_3$) δ ppm: 1.03 (3H, d, J=

6.9 Hz), 1.04 (3H, d, J=6.7 Hz), 1.12 (3H, d, J=6.9 Hz), 1.38 (3H, s), 4.03 (1H, br d, J=6.3 Hz), 4.24 (1H, br s), 5.73 (1H, br d, J=5.1 Hz).

Oxidative Conversion of XI with PCC to Give VII——XI (20 mg) was treated with PCC (50 mg) in methylene chloride (2 ml) for 15 min at room temperature. Then, dry ether (20 ml) was added. The reaction mixture was filtered, and the filtrate was evaporated. Purification by HPLC (n-hexane : benzene : ethyl acetate = 10 : 10 : 1) gave VII (5 mg) whose spectral data (including specific rotation) were identical with those of natural furopelargone B.

Conversion of I with Boron Trifluoride Etherate to Give IX——I (35 mg) was treated with two drops of boron trifluoride etherate in benzene (2 ml) for 11 h at room temperature. Then, the solvent was evaporated off and the residue was subjected to HPLC (n-hexane : ethyl acetate = 7 : 3) to give colorless needles (12 mg), whose spectral data (including specific rotation) were identical with those of natural alpinolide.

Oxidation of VII with *m*-Chloroperbenzoic Acid to Give XII——A solution of VII (20 mg) and m-chloroperbenzoic acid (15 mg) in benzene (2 ml) was kept for 2 d at room temperature. Then it was washed with 10% $Na_2S_2O_3$, saturated $NaHCO_3$ solution, and brine, dried, and evaporated. The residue was subjected to HPLC (benzene : ethyl acetate = 9 : 1) to give VIII as a colorless oil (8 mg) and XII as colorless needles (11 mg); $[\alpha]_D$ +15.9°

TABLE V. Temperature Factors

No.	Atom	U_{11}	U_{22}	U_{33}	U_{12}	U_{13}	U_{23}
		$U(ij)$'s are multiplied by 10^3					
1	C1	54 (5)	40 (4)	45 (4)	0 (4)	6 (4)	−4 (4)
2	C2	58 (5)	55 (5)	53 (5)	0 (4)	10 (4)	4 (4)
3	C3	53 (5)	82 (7)	59 (5)	15 (5)	7 (4)	6 (5)
4	C4	62 (5)	65 (6)	43 (4)	15 (5)	6 (4)	−3 (4)
5	C5	61 (5)	42 (4)	41 (4)	5 (4)	15 (4)	−2 (3)
6	C6	61 (5)	36 (4)	44 (4)	8 (4)	16 (4)	−2 (3)
7	C7	71 (6)	43 (4)	50 (5)	1 (4)	18 (4)	1 (4)
8	C8	92 (7)	47 (5)	63 (6)	−2 (5)	34 (5)	1 (4)
9	C9	94 (7)	43 (5)	53 (5)	5 (5)	24 (5)	−2 (4)
10	C10	58 (5)	33 (4)	52 (5)	1 (4)	7 (4)	−1 (4)
11	C11	92 (8)	91 (8)	49 (5)	30 (7)	18 (5)	22 (6)
12	C12	87 (7)	46 (5)	66 (6)	−6 (5)	12 (5)	14 (5)
13	C13	97 (8)	48 (5)	87 (7)	−8 (5)	44 (6)	−4 (5)
14	C14	87 (8)	118 (11)	116 (10)	28 (8)	37 (8)	53 (9)
15	C15	103 (9)	104 (9)	70 (7)	−7 (8)	27 (6)	17 (7)
16	O1	73 (4)	57 (4)	53 (3)	20 (3)	6 (3)	1 (3)
17	O2	80 (4)	41 (3)	38 (3)	−10 (3)	6 (3)	4 (2)
18	O3	73 (4)	44 (3)	46 (3)	−13 (3)	3 (3)	3 (3)
19	O4	85 (4)	41 (3)	49 (3)	6 (3)	28 (3)	−5 (3)
20	O5	139 (7)	54 (4)	82 (5)	25 (4)	54 (5)	−12 (4)

No.	Atom	$U(ij)$'s are multiplied by 10^2 U_{11}	No.	Atom	$U(ij)$'s are multiplied by 10^2 U_{11}
21	HC1	6 (3)	32	HC12	9 (3)
22	HC2	8 (3)	33	H′C12	7 (3)
23	H′C2	6 (2)	34	H″C12	7 (3)
24	HC3	8 (3)	35	HC13	13 (4)
25	H′C3	8 (3)	36	HC14	9 (3)
26	HC4	7 (3)	37	H′C14	10 (4)
27	HC5	6 (2)	38	H″C14	14 (5)
28	HC8	9 (3)	39	HC15	7 (3)
29	HC11	10 (3)	40	H′C15	8 (3)
30	H′C11	7 (3)	41	H″C15	10 (3)
31	H″C11	7 (3)	42	HO1	8 (3)

Temperature factor T is in the form of

$$T=\exp(-2\pi^2(U_{11}H^2A^{*2}+U_{22}K^2B^{*2}+U_{33}L^2C^{*2}+2U_{12}HKA^*B^*+2U_{13}HLA^*C^*+2U_{23}KLB^*C^*)).$$

TABLE VI. Bond Lengths (Å)

	Length (S.T.D.)		Length (S.T.D.)
C1–C2	1.527 (12)	C7–C8	1.336 (13)
C1–C5	1.545 (10)	C7–C13	1.486 (15)
C1–C10	1.510 (10)	C8–C9	1.449 (16)
C2–C3	1.563 (13)	C9–O4	1.370 (11)
C3–C4	1.553 (14)	C9–O5	1.212 (13)
C4–C5	1.532 (11)	C10–C12	1.548 (13)
C4–C11	1.534 (15)	C10–O1	1.385 (11)
C5–C6	1.549 (11)	C10–O2	1.453 (9)
C6–C7	1.528 (12)	C13–C14	1.479 (16)
C6–O3	1.431 (9)	C13–C15	1.575 (17)
C6–O4	1.435 (11)	O2–O3	1.477 (8)

S.T.D.: The standard deviation.

TABLE VII. Bond Angles (°)

	Angle (S.T.D.)		Angle (S.T.D.)
C2–C1–C5	104.6 (6)	C8–C7–C13	130.4 (9)
C2–C1–C10	118.1 (6)	C6–C7–C13	122.8 (8)
C5–C1–C10	112.2 (6)	C9–C8–C7	110.5 (9)
C3–C2–C1	106.3 (7)	O4–C9–C8	108.7 (8)
C4–C3–C2	104.6 (7)	O4–C9–O5	119.2 (9)
C5–C4–C3	105.8 (7)	C8–C9–O5	132.0 (9)
C5–C4–C11	119.7 (8)	C12–C10–C1	112.6 (7)
C3–C4–C11	113.7 (8)	C12–C10–O1	114.1 (7)
C6–C5–C1	111.3 (6)	C12–C10–O2	101.3 (6)
C6–C5–C4	117.4 (7)	C1–C10–O1	108.2 (6)
C1–C5–C4	101.6 (6)	C1–C10–O2	109.5 (6)
C7–C6–C5	116.9 (7)	O1–C10–O2	111.0 (6)
C7–C6–O3	102.1 (6)	C14–C13–C7	113.9 (10)
C7–C6–O4	105.4 (6)	C14–C13–C15	108.0 (10)
C5–C6–O3	111.5 (6)	C7–C13–C15	111.4 (9)
C5–C6–O4	110.7 (6)	O3–O2–C10	109.5 (5)
O3–C6–O4	109.6 (6)	C6–O3–O2	109.5 (5)
C8–C7–C6	106.5 (8)	C6–O4–C9	108.8 (6)

(c=0.11, $CHCl_3$), mp 52.0—54.0 °C. MS m/z (%): 250 (M^+, 60), 235 (70), 207 (90), 179 (40), 165 (95), 153 (53), 126 (43), 109 (35), 97 (72), 81 (55), 69 (100). IR (CCl_4) cm^{-1}: 2960, 2880, 1805, 1710, 1470, 1360, 1220, 1170, 1150, 1030, 975, 890. ^1H-NMR ($CDCl_3$) δ ppm: 0.93 (3H, d, J=6.8 Hz), 1.05 (3H, d, J=6.9 Hz), 1.07 (3H, d, J=6.8 Hz), 2.11 (3H, s), 2.73 (1H, sept, J=6.8 Hz), 3.05 (2H, br s), 3.32 (1H, dd, J=6.9, 6.9 Hz).

Autooxidation of XII with Oxygen to Give IV——A solution of XII (10 mg) in benzene (3 ml) was stirred for a week at room temperature under an O_2 atmosphere. Then the solvent was evaporated off to give IV as colorless needles (9 mg). The spectral data (including specific rotation) were identical with those of natural alpinolide peroxide.

Conversion of V with Diazomethane to Give XIII——A solution of V (20 mg) and diazomethane in ether was kept for 30 min at room temperature and then evaporated. Purification by HPLC (n-hexane : ethyl acetate = 4 : 1) gave XIII as a colorless oil (14 mg). MS m/z (%): 280 (M^+, 0.5), 263 (2), 249 (6), 237 (2), 209 (20), 155 (100). IR (CCl_4) cm^{-1}: 2960, 2930, 2880, 1735, 1715, 1690, 1635, 1460, 1435, 1370, 1315, 1240, 1220, 1175, 1045, 1015, 920, 870, 680. UV (EtOH) nm (ε): 217 (6700). ^1H-NMR ($CDCl_3$) δ ppm: 1.09 (3H, d, J=6.7 Hz), 1.12 (3H, d, J=6.8 Hz), 1.13 (3H, d, J=6.8 Hz), 2.21 (3H, s), 5.70 (1H, d, J=1.4 Hz).

Reduction of IV with Triphenylphosphine to Give XIV——A solution of IV (20 mg), triphenylphosphine (30 mg) and p-toluenesulfonic acid (2 mg) in ether (5 ml) was stirred for 2 d at room temperature. The solvent was removed by evaporation and the residue was subjected to HPLC (n-hexane : ethyl acetate = 3 : 2) to give XIV as colorless needles (15 mg). This product was an epimeric mixture (2 : 1). IR (CCl_4) cm^{-1}: 3610, 2960, 2880, 1775, 1645, 1480, 1385, 1310,

1240, 1220, 1170, 1100, 1080, 1060, 1040, 950, 915, 890, 865, 850. ^{1}H-NMR ($CDCl_3$) δ ppm: main product, 0.92 (3H, d, $J=7.2$ Hz), 1.22 (3H, d, $J=6.8$ Hz), 1.26 (3H, d, $J=6.8$ Hz), 1.53 (3H, s), 5.88 (1H, d, $J=1.0$ Hz); minor product, 0.91 (3H, d, $J=7.2$ Hz), 1.23 (3H, d, $J=6.8$ Hz), 1.25 (3H, d, $J=6.8$ Hz), 1.59 (3H, s), 5.83 (1H, d, $J=0.5$ Hz).

Reaction of XIV with Silica Gel to Give V——A solution of XIV (10 mg) in benzene (3 ml) was stirred with silica gel for 12 h at room temperature, then the silica gel was removed by filtration and the filtrate was evaporated to give a colorless oil, V (7 mg) whose spectral data (including specific rotation) were identical with those of natural 6-hydroxyalpinolide.

X-Ray Analysis of IV——Crystals were grown in *n*-hexane solution as transparent thick plates. The X-ray diffraction intensities of 3366 reflections were measured as above the $2\sigma(I)$ level out of 3656 in the 2θ range of 6° through 156°, using graphite-monochromated CuK_α radiation. The crystal structure was determined by the direct method using the MULTAN program[7] and refined by the block-diagonal-matrix least-squares method[8] to an R value of 0.089. Crystal data are: alpinolide peroxide, $C_{15}H_{22}O_5$, $M_r=282$. Monoclinic, space group $P2_1$, $Z=4$. Lattice constants, $a=13.761(7)$, $b=17.974((9)$, $c=6.259(4)$ Å, $\beta=100.47(5)$°, $V=1522$ Å^3. $D_{calc}=1.232$ gcm^{-3}. Hydrogen atoms were located on the difference electron-density map and their atomic parameters including isotropic temperature factors were refined. Two crystallographically independent molecules have almost the same structure and Fig. 1 shows one of them.

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Novel Sesquiterpenes from *Alpinia intermedia* GAGNEP.

HIDEJI ITOKAWA,*,[a] HIROSHI MORITA,[a] TAKESHI KOBAYASHI[a] KINZO WATANABE,[a] and YOICHI IITAKA[b]

Tokyo College of Pharmacy,[a] Horinouchi 1432-1, Hachioji, Tokyo 192-03, Japan and Faculty of Pharmaceutical Science,[b] University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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Three novel sesquiterpene peroxides, hanalpinol peroxide (I), isohanalpinol (II), and aokumanol (III), a new secoguaiane-type sesquiterpene, epialpinolide (IV), and a new elemophilane-type sesquiterpene, $\Delta^{11(12)}$-eremophilen-10β-ol (V), have been isolated from the rhizomes of *Alpinia intermedia* together with eight known sesquiterpenes, hanalpinol (VI), hanalpinone (VII), isohanalpinone (VIII), alpinenone (IX), furopelargone B (X), furopelargone A (XI), intermedeol (XII) and β-selinene (XIII). The structures of I—V were determined by spectroscopic methods, chemical conversion, and X-ray analysis. Their biosynthetic relationships are discussed.

From the point of view of chemotaxonomy, it is suggested that *Alpinia intermedia* and *Alpinia japonica* are closely related plants.

Keywords——*Alpinia intermedia*; Zingiberaceae; sesquiterpene; guaiane; cyclic peroxide; X-ray analysis; eremophilane; secoguaiane; chemotaxonomy; biosynthesis

In our continuing work on the constituents of *Alpinia* spp. (Zingiberaceae),[1] we have investigated *Alpinia intermedia*. Seeds of *A. intermedia* have been used as an aromatic stomachic in the same way as Amomi Semen.[2] In our previous studies, many novel sesquiterpene peroxides[3] have been isolated from *Alpinia japonica*, which belongs to the same genus. This time, separation of the *n*-hexane- and chloroform-soluble fractions obtained from the rhizomes of *A. intermedia* permitted us to isolate five new sesquiterpenes (I—V) together with eight known sesquiterpenes (VI—XIII). This paper describes the structure determination of the new sesquiterpenes and discusses the structural relationships between them. The sesquiterpenes obtained from *A. intermedia* are compared with those from *A. japonica*.

Compounds I—III were found to be sesquiterpene peroxides because they were positive to the color reactions for peroxides.[4]

Compound I, named hanalpinol peroxide, was obtained as a colorless oil having the molecular formula $C_{15}H_{24}O_4$, based on chemical ionization mass spectrometry (MS) and high-resolution MS. The proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) data are shown in Tables I and II. All the spectroscopic data of I were similar to those of hanalpinol (VI).[3a] The stereochemistry of the cyclic peroxide was suggested to be β, as in the case of VI, from the coupling constant (5.3 Hz) between H-5 and H-6. The infrared (IR) absorption band at 3560 cm^{-1} showed the presence of either a hydroxyl group or a hydroperoxide with intramolecular hydrogen bonding. However, as I gave a stronger peroxide reaction than VI, the four oxygen atoms of I were assumed to belong to two peroxides, one being a cyclic peroxide and the other a hydroperoxide. This assumption was proved to be correct: reduction of I with triphenylphosphine gave a product which was identical with natural hanalpinol (VI). Therefore, the absolute structure of I was determined to be as shown in Chart 1. The structure and this conversion of I into VI may suggest that I is a biogenetic precursor of VI.

I II III IV V VI VII VIII IX X XI XII XIII XIV XV $\Delta^{9(10)}$-eremophilen-11-ol alpinolide

Chart 1

Compound II, named isohanalpinol, was obtained as colorless needles (mp 67 °C). The molecular formula, $C_{15}H_{24}O_3$ was established by high-resolution MS. The IR spectrum ($3620\,cm^{-1}$) and the positive color reactions for peroxides showed that the three oxygen atoms belonged to a hydroxyl group and a peroxide. The ^{13}C-NMR spectrum (Table II) suggested the presence of three oxygen-linked carbons, one being hydroxyl group-linked and two being cyclic peroxide-linked (δ 76.6, 77.7 and 82.3). The ^{1}H-NMR spectrum (Table I) showed signals ascribable to four methyl groups, that is, two belonging to an isopropyl group attached to an olefin (δ 1.06, 6H), one doublet methyl group (δ 1.11) and one methyl group attached to an oxygen atom (δ 1.30). Compound II was shown to possess the same plane structure as VI. Furthermore, the stereochemistry of the peroxide was deduced to be α, as in isohanalpinone (VIII),[3c] because of the lack of vicinal coupling between H-5 and H-6. For confirmation of the structure, chemical conversion of VIII into II was carried out: VIII was reduced with sodium borohydride to give two products which were epimers of a hydroxyl moiety. Compound II was identical with the main product. The configuration of the hydroxyl group of II was determined to be β, because the minor product (XIV) showed the presence of intramolecular hydrogen bonding in the IR spectrum.

Compound III, named aokumanol, was obtained as colorless needles (mp 118.0—119.0 °C) having the molecular formula $C_{15}H_{24}O_3$, based on high-resolution MS. The ^{13}C-NMR spectrum showed three signals of carbon atoms bearing an oxygen function (δ 79.3, 79.8 and 80.3). Its color reaction for peroxides was positive and the IR spectrum ($3620\,cm^{-1}$) suggested the presence of a hydroxyl group. Therefore the three oxygen atoms were revealed to belong to a peroxide and a hydroxyl group. The double irradiation experiment in ^{1}H-NMR and the proton correlation spectrum showed two olefinic protons (δ 5.59 and 5.71) with the coupling constant of 10.7 Hz. Hence, the double bond in III was concluded to be *cis*. Further,

TABLE I. ^{1}H-NMR Data for I—IV (400 MHz in $CDCl_3$)

Proton	I	II	III	IV
1	2.67, ddd	2.73, ddd	2.45, ddd	3.06, dt
4	2.20, m		2.17, m	2.27, m
5	2.85, dt		2.26, t	2.40, dt
6	4.44, dd	4.40, d	4.21, d	5.59, dd
8	5.75, dt	5.55, dt	5.59, dd	5.76, t
9	4.63, dd	4.17, d	5.71, d	
11	2.31, sept. d	2.37, sept. d	2.36, sept.	2.54, sept. d
12	1.12, d	1.06, d	1.01, d	1.17, d
13	1.16, d		1.03, d	1.27, d
14	1.08, d	1.11, d	1.02, d	0.94, d
15	1.46, s	1.30, s	1.23, s	2.23, s
-OOH	8.82, br s			

Coupling constants in Hz. I: 1,2=4.6; 1,2=11.5; 1,5=9.0; 4,5=9.0; 4,14=7.2; 5,6=5.3; 6,8=1.3; 8,9=4.6; 8,11=1.3; 9,OOH=1.3; 11,12=6.7; 11,13=6.7. II: 1,2=6.5; 1,2=9.5; 1,5=8.0; 4,5=8.0; 4,14=7.6; 6,8=1.2; 8,9=10.7; 8,11=1.2; 11,12=6.9; 11,13=6.9. III: 1,2=6.5; 1,2=9.5; 1,5=8.0; 4,5=8.0; 4,14=7.6; 6,8=1.8; 8,9=10.7; 11,12=6.9; 11,13=6.9. IV: 1,2=9.4; 1,2=9.4; 1,5=7.0; 4,5=7.0; 4,14=7.0; 5,6=4.7; 6,8=1.5; 8,11=1.5; 11,12=7.0; 11,13=7.0.

TABLE II. ^{13}C-NMR Data for I—IV (100 MHz in $CDCl_3$)

No.	I	II	III	IV
1	44.5 (d)	38.4 (d)	39.8 (d)	55.4 (d)
2	33.2 (t)	32.5 (t)	33.6 (t)	32.3 (t)
3	26.2 (t)	26.2 (t)	24.9 (t)	27.3 (t)
4	37.5 (d)	37.1 (d)	37.9 (d)	37.7 (d)
5	45.9 (d)	44.9 (d)	44.5 (d)	46.0 (d)
6	85.3 (d)[a]	77.7 (d)[a]	79.3 (d)	82.6 (d)
7	154.5 (s)	150.0 (s)	79.8 (s)[a]	173.4 (s)
8	120.9 (d)	124.4 (d)	135.4 (d)[b]	114.3 (d)
9	80.6 (d)[a]	76.6 (d)[a]	136.4 (d)[b]	180.7 (s)
10	84.7 (s)	82.3 (s)	80.3 (s)[a]	210.5 (s)
11	33.9 (d)	34.8 (d)	34.0 (d)	28.3 (d)
12	20.4 (q)	20.9 (q)	16.3 (q)	20.5 (q)
13	23.2 (q)	21.0 (q)	16.8 (q)	22.3 (q)
14	15.3 (q)	15.4 (q)	15.1 (q)	16.1 (q)
15	25.7 (q)	24.3 (q)	24.8 (q)	30.0 (q)

a, b) Tentative.

the signal at δ 5.59 showed a long-range coupling (J=1.8 Hz) with the proton (δ 4.21) linked to an oxygen atom-bearing carbon. The signal at δ 4.21 can not be that of the proton linked to the hydroxyl group-bearing carbon but must be that of the proton linked to a cyclic peroxide-bearing carbon because its chemical shift did not change after the addition of trichloroacetyl isocyanate (TIA)[5]: the hydroxyl group must be tertiary. The spectral investigation and comparison of the IR and NMR data of III with those of known sesquiterpene peroxides (Tables I and II) suggested that III has a guaiane skeleton. The stereochemistry of the cyclic peroxide was considered to be α because of the lack of vicinal coupling between H-5 and H-6. However, to elucidate the whole structure including stereochemical configuration, X-ray analysis of III was undertaken.

Aokumanol (III) was crystallized from *n*-hexane–ethyl acetate solutions as colorless fine

needles elongated along the c axis. The lattice constants and intensity data were measured on a Philips PW1100 diffractometer using graphite-monochromated CuK_α radiation. The size of the crystal used was about 0.02 × 0.1 × 0.15 mm. Crystal data are: orthorhombic, space group $P2_12_12_1$, $Z=4$, $D_{calc}=1.161$ g cm^{-3}, $a=14.608(8)$, $b=17.592(8)$, $c=5.617(4)$ Å, $U=1443$ Å^3. Intensities of 1330 reflections were measured as above the 2σ (I) level out of 1755 in the 2θ range of 6° through 178°. The crystal was deteriorated by X-ray irradiation. The intensities of the three standard reflections, 902, 080 and 0$\bar{3}$4, were monitored every 2 h and it was found that the extents of decrease of the intensities during the 2 h intervals were 6.1, 20.5 and 4.5%, respectively, throughout the measurement. The intensities were, therefore, corrected under the simple assumption that they decreased uniformly with respect to time and the direction of the Bragg reflection. The crystal structure was determined by the direct method and refined by the block-diagonal-matrix least-squares method. The R value could not be reduced beyond 0.147. The final difference electron-density map showed 8 small peaks having peak height greater than 0.35 e/Å^3, with the maximum value 0.5 e/Å^3. Some of them were assigned as hydrogen atoms. Presumably errors arising from the crystal deterioration and inadequate correction of the intensities prevented further refinement. The molecular structure having the *cis*-linked guaiane skeleton, the bond lengths and the bond angles are shown in Fig. 1 and Tables V and VI. No abnormal features were found in the structure. Hydrogen atoms were located by calculation assuming sp^3 or sp^2 configuration about the carbon atoms and their atomic parameters were refined by the least-squares method. The relative configuration of the cyclic peroxide was concluded to be α estimated from the lack of vicinal coupling in the ^{1}H-NMR spectrum.

Compound IV, a colorless oil, was a secoguaiane-type sesquiterpene named epialpinolide. The molecular formula, $C_{15}H_{22}O_3$, was established by high-resolution MS. The IR, ultraviolet (UV) and ^{1}H-NMR spectra indicated the presence of an unsaturated butenolide (1760 and 1630 cm^{-1}; 214 nm, ε 10900) and a methyl ketone group (1700 cm^{-1}; δ 2.23, 3H, s). The above spectral data, ^{13}C-NMR spectrum (Table II) and the coupling relationships observed in a detailed proton decoupling experiment were in reasonable agreement with those of alpinolide,[6] which has been isolated from *Alpinia japonica*. The whole structure including the absolute configuration was determined by chemically converting IV into alpinolide by treatment with p-toluenesulfonic acid; the spectral data and specific rotation of the product were identical with those of natural alpinolide. Consequently, compound IV was identified as an epimer of alpinolide and the stereochemistry of the methyl ketone group of IV should be α. This was supported by a proton nuclear Overhauser effect (NOE) experiment, which showed an NOE of 4.1% between H-1 and H-5 in IV. On the other hand, no NOE was observed between the corresponding protons in alpinolide.

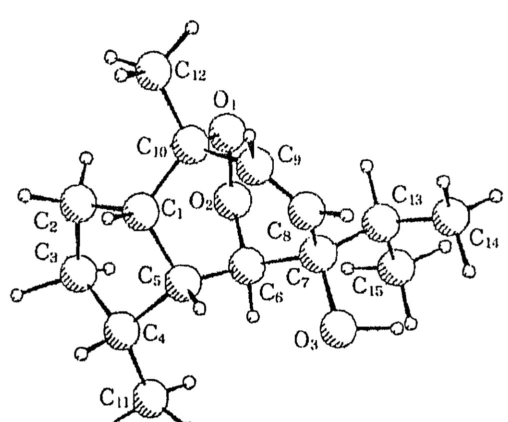

Fig. 1. Molecular Structure Drawn by the PLUTO[11] Program

TABLE III. Atomic Coordinates and Equivalent Isotropic Temperature Factors

No.	Atom	x 10^4	y 10^4	z 10^4	B_{eq} (Å^2)
1	C1	8563 (10)	3987 (7)	3997 (23)	3.9 (0.2)
2	C2	9134 (11)	4530 (10)	5694 (22)	5.4 (0.3)
3	C3	10146 (12)	4363 (13)	5214 (23)	7.1 (0.3)
4	C4	10137 (10)	3964 (9)	2659 (22)	4.8 (0.2)
5	C5	9248 (9)	3440 (7)	2733 (17)	3.2 (0.2)
6	C6	9292 (9)	2705 (7)	3945 (15)	3.4 (0.2)
7	C7	8648 (9)	2077 (8)	3053 (19)	3.8 (0.2)
8	C8	7723 (9)	2382 (8)	2431 (21)	3.8 (0.2)
9	C9	7358 (9)	2978 (9)	3279 (23)	4.4 (0.2)
10	C10	7798 (10)	3542 (8)	5219 (27)	4.6 (0.2)
11	C11	11021 (11)	3510 (11)	2081 (29)	6.4 (0.3)
12	C12	7099 (11)	4032 (12)	6513 (30)	7.5 (0.4)
13	C13	8577 (10)	1364 (8)	4577 (21)	4.4 (0.2)
14	C14	7987 (12)	738 (10)	3548 (29)	5.9 (0.3)
15	C15	9535 (11)	1017 (11)	5205 (26)	6.0 (0.3)
16	O1	8197 (6)	3026 (7)	7043 (12)	5.0 (0.2)
17	O2	9162 (6)	2809 (6)	6578 (11)	4.3 (0.2)
18	O3	9097 (7)	1835 (5)	817 (12)	4.4 (0.1)

No.	Atom	x 10^3	y 10^3	z 10^3	B_{eq} (Å^2)
19	HC1	824 (9)	445 (7)	265 (28)	7. (4.)
20	HC2	896 (8)	515 (6)	534 (24)	5. (3.)
21	H'C2	891 (12)	444 (10)	723 (41)	13. (6.)
22	HC3	1041 (10)	396 (9)	629 (32)	9. (5.)
23	H'C3	1059 (7)	496 (6)	514 (21)	3. (3.)
24	HC4	1007 (8)	446 (6)	122 (21)	4. (3.)
25	HC5	904 (11)	334 (9)	102 (34)	11. (5.)
26	HC6	1002 (7)	252 (5)	358 (19)	3. (2.)
27	HC8	737 (10)	204 (8)	144 (30)	9. (5.)
28	HC9	670 (7)	316 (6)	284 (23)	4. (3.)
29	HC11	1098 (13)	313 (10)	73 (40)	13. (6.)
30	H'C11	1163 (10)	394 (8)	206 (33)	9. (5.)
31	H''C11	1112 (11)	303 (10)	329 (37)	11. (5.)
32	HC12	743 (10)	441 (8)	788 (31)	9. (5.)
33	H'C12	678 (10)	448 (8)	529 (29)	8. (4.)
34	H''C12	657 (9)	369 (7)	719 (28)	7. (4.)
35	HC13	824 (12)	153 (9)	626 (35)	11. (6.)
36	HC14	827 (11)	54 (9)	185 (34)	11. (5.)
37	H'C14	797 (9)	22 (7)	468 (27)	7. (4.)
38	H''C14	730 (9)	94 (7)	325 (27)	6. (4.)
39	HC15	994 (9)	145 (7)	608 (24)	6. (3.)
40	H'C15	946 (8)	47 (7)	624 (24)	5. (3.)
41	H''C15	991 (12)	83 (10)	364 (36)	13. (6.)
42	HO3	898 (7)	122 (6)	79 (20)	3. (2.)

Equivalent positions: x, y, z; $1/2-x$, $-y$, $1/2+z$; $1/2+x$, $1/2-y$, $-z$; $-x$, $1/2+y$, $1/2-z$.

Compound V, a colorless oil having the molecular formula $C_{15}H_{26}O$, was an eremophilane-type sesquiterpene. ^{1}H-NMR (δ 1.73, 3H, br s; 4.68, 4.70, each 1H, br s) and IR (3080 and 890 cm^{-1}) spectra indicated the presence of an isopropenyl group. The ^{1}H-NMR

Table IV. Temperature Factors

No.	Atom	U_{11}	U_{22}	U_{33}	U_{12}	U_{13}	U_{23}
		$U\ (ij)$'s are multiplied by 10^3					
1	C1	73 (9)	42 (6)	33 (7)	−2 (7)	−6 (7)	4 (6)
2	C2	79 (10)	102 (12)	23 (6)	−6 (10)	−9 (8)	−26 (8)
3	C3	84 (12)	169 (18)	18 (7)	−22 (13)	−8 (8)	−33 (10)
4	C4	71 (9)	96 (11)	14 (6)	−5 (9)	−4 (6)	−16 (7)
5	C5	61 (7)	53 (7)	5 (4)	5 (7)	−2 (6)	3 (5)
6	C6	60 (7)	76 (8)	−6 (4)	0 (7)	5 (5)	−9 (5)
7	C7	58 (7)	74 (9)	12 (5)	7 (7)	9 (6)	−21 (6)
8	C8	58 (8)	69 (8)	17 (5)	8 (7)	−8 (6)	−4 (6)
9	C9	56 (8)	81 (10)	32 (7)	−7 (8)	−15 (7)	10 (7)
10	C10	69 (9)	47 (7)	58 (9)	14 (7)	−7 (8)	−9 (8)
11	C11	61 (9)	138 (15)	46 (8)	−13 (11)	5 (8)	−12 (11)
12	C12	67 (11)	167 (20)	51 (10)	45 (12)	7 (9)	−23 (12)
13	C13	83 (10)	67 (9)	18 (6)	14 (8)	22 (7)	10 (6)
14	C14	93 (12)	81 (11)	48 (9)	−25 (10)	16 (9)	−11 (9)
15	C15	74 (10)	123 (14)	31 (7)	14 (10)	6 (8)	9 (10)
16	O1	67 (6)	123 (8)	−1 (3)	21 (6)	0 (4)	10 (5)
17	O2	59 (5)	120 (8)	−14 (3)	9 (6)	−9 (3)	−1 (4)
18	O3	82 (6)	79 (6)	6 (3)	2 (6)	7 (4)	−12 (4)

No.	Atom	$U\ (ij)$'s are multiplied by 10^2 U_{11}	No.	Atom	$U\ (ij)$'s are multiplied by 10^2 U_{11}
19	HC1	9 (5)	31	H″C11	14 (7)
20	HC2	6 (4)	32	HC12	11 (6)
21	H′C2	16 (8)	33	H′C12	10 (5)
22	HC3	11 (6)	34	H″C12	9 (5)
23	H′C3	4 (3)	35	HC13	14 (7)
24	HC4	5 (4)	36	HC14	13 (7)
25	HC5	14 (7)	37	H′C14	9 (5)
26	HC6	4 (3)	38	H″C14	8 (5)
27	HC8	11 (6)	39	HC15	7 (4)
28	HC9	5 (4)	40	H′C15	6 (4)
29	HC11	17 (8)	41	H″C15	16 (8)
30	H′C11	11 (6)	42	HO3	4 (3)

Temperature factor T is in the form of

$$T=\exp(-2\pi^2(U_{11}H^2A^{*2}+U_{22}K^2B^{*2}+U_{33}L^2C^{*2}+2U_{12}HKA^*B^*+2U_{13}HLA^*C^*+2U_{23}KLB^*C^*)).$$

spectrum indicated the presence of a singlet methyl (δ 0.84) and a doublet methyl (δ 0.80) groups. A tertiary hydroxyl group was indicated by the IR (3630 cm^{-1}) and ^{13}C-NMR (δ 73.2, s) spectra. These spectral data suggest that compound V is of eudesmane or eremophilane type, possessing a tertiary hydroxyl group. Dehydration of V gave XV which was also produced by dehydration of $\Delta^{9(10)}$-eremophilen-11-ol[7] isolated from *Alpinia japonica*. Consequently, V was concluded to have the eremophilane skeleton and a hydroxyl group at C-10. The configuration of the hydroxyl group was deduced from the pyridine-induced solvent effect[8] in the ^{1}H-NMR spectrum. In the ^{1}H-NMR spectrum (pyridine-d_5), the singlet methyl group signal showed a downfield shift (−0.22 ppm) owing to the vicinal deshielding effect. Therefore the hydroxyl group at C-10 and the methyl group at C-5 must be syn. That is, the configuration of the hydroxyl group must be β. Compound V was determined to be $\Delta^{11(12)}$-eremophilen-10β-ol.

TABLE V. Bond Lengths (Å)

	Length (S.T.D.)		Length (S.T.D.)
C1–C2	1.585 (20)	C7–C8	1.495 (18)
C1–C5	1.559 (18)	C7–C13	1.522 (18)
C1–C10	1.528 (20)	C7–O3	1.479 (14)
C2–C3	1.531 (24)	C8–C9	1.269 (20)
C3–C4	1.598 (20)	C9–C10	1.608 (20)
C4–C5	1.593 (20)	C10–C12	1.521 (23)
C4–C11	1.552 (23)	C10–O1	1.487 (17)
C5–C6	1.464 (17)	C13–C14	1.513 (22)
C6–C7	1.535 (18)	C13–C15	1.566 (22)
C6–O2	1.502 (11)	O1–O2	1.485 (13)

S.T.D.: The standard deviation.

TABLE VI. Bond Angles (°)

	Angle (S.T.D.)		Angle (S.T.D.)
C2–C1–C5	108.0 (11)	C6–C7–C13	116.8 (10)
C2–C1–C10	115.0 (11)	C6–C7–O3	102.3 (9)
C5–C1–C10	110.9 (11)	C13–C7–O3	105.7 (10)
C3–C2–C1	106.6 (12)	C9–C8–C7	126.1 (12)
C4–C3–C2	103.6 (13)	C10–C9–C8	126.6 (13)
C5–C4–C3	103.7 (11)	C12–C10–C1	114.5 (13)
C5–C4–C11	112.7 (12)	C12–C10–C9	114.0 (12)
C3–C4–C11	114.0 (13)	C12–C10–O1	106.2 (12)
C6–C5–C1	111.2 (10)	C1–C10–C9	107.7 (11)
C6–C5–C4	119.2 (10)	C1–C10–O1	109.6 (11)
C1–C5–C4	100.2 (10)	C9–C10–O1	104.3 (10)
C7–C6–C5	117.2 (10)	C14–C13–C7	115.1 (12)
C7–C6–O2	109.4 (9)	C14–C13–C15	108.2 (12)
C5–C6–O2	110.2 (9)	C7–C13–C15	112.8 (11)
C8–C7–C6	111.8 (10)	O2–O1–C10	114.1 (9)
C8–C7–C13	111.5 (11)	C6–O2–O1	109.0 (8)
C8–C7–O3	107.8 (10)		

Chart 2. Proposed Biogenetic Pathway of I, II, III and VI

Eight known sesquiterpenes, hanalpinol (VI), hanalpinone (VII), isohanalpinone (VIII), alpinenone (IX), furopelargone B (X), furopelargone A (XI), intermedeol (XII),[9] and β-selinene (XIII)[10] were also isolated. The structures of I—V are very similar to those of the above known sesquiterpenes VI—XI which have already been isolated from *Alpinia japonica*.[3] From the chemotaxonomic viewpoint, it is suggested that *Alpinia intermedia* and *Alpinia japonica* are closely related plants.

Furthermore, from the biogenetic point of view, I was considered to be an immediate biogenetic precursor of VI, and these sesquiterpene peroxides were assumed to be derived from similar compounds which possess a hydroperoxide moiety at C-9. We propose that these compounds are derived from compound XVI, which has two double bonds at C-6 and C-9, by successive oxidations with oxygen as shown in Chart 2.

Experimental

All melting points were recorded on a Yanagimoto micro melting point apparatus, and are uncorrected. Spectral data were obtained on the following instruments; optical rotation on a JASCO DIP-4, IR on a JASCO A-302, UV on a Hitachi 557, NMR on a Brucker AM400, and MS on a Hitachi M-80. High-performance liquid chromatography (HPLC) was carried out on a CIG column system (Kusano Scientific Co., Tokyo) with Iatrobeads (60 μ silica gel, IATRON Co., Tokyo) as the stationary phase.

Extraction and Isolation——Fresh rhizomes (14.0 kg) of *Alpinia intermedia* collected at Nomosaki, Nagasaki, in August 1984 were extracted twice with methanol under an argon atmosphere. The methanol extract was treated with *n*-hexane, and the *n*-hexane layer was concentrated to give a yellow oil (18.5 g). Then the methanol layer was treated with chloroform, and the chloroform layer was concentrated to give a brown oil (24.0 g). The *n*-hexane and chloroform extracts were each subjected to column chromatography on silica gel with a linear *n*-hexane–ethyl acetate gradient system and a linear chloroform–methanol gradient system, respectively. Repeated HPLC and $AgNO_3$–HPLC of each fraction using an *n*-hexane–ethyl acetate system, an *n*-hexane–chloroform–acetonitrile system, a benzene–ethyl acetate system, and a benzene–chloroform–acetonitrile system gave I (100 mg), II (100 mg), III (50 mg), IV (100 mg), V (100 mg), VI (500 mg), VII (300 mg), VIII (200 mg), IX (100 mg), X (1.5 g), XI (200 mg), XII (100 mg), and XIII (500 mg).

Compound I (Hanalpinol Peroxide): A colorless oil, $[\alpha]_D$ +135.0° (c=0.50, $CHCl_3$), CI-MS m/z (%): 269 (M^++1, 1); EI-MS m/z (%) 250 (M^+ − 18, 5, Calcd for $C_{15}H_{22}O_3$, 250.1559; Found 250.1569), 219 (5), 191 (10), 147 (20), 95 (60), 81 (100), 71 (65). IR (CCl_4) cm^{-1}: 3560, 3480, 2970, 2880, 1465, 1390, 1380, 970, 885.

Compound II (Isohanalpinol): Colorless needles, mp 67.0 °C, $[\alpha]_D$ −21.0° (c=0.10, $CHCl_3$). MS m/z (%): 252 (M^+, 54, Calcd for $C_{15}H_{24}O_3$, 252.1724; Found 252.1694), 235 (100), 201 (40), 175 (54), 149 (12), 95 (12). IR (CCl_4) cm^{-1}: 3620, 2960, 2940, 2880, 1460, 1385, 1005.

Compound III (Aokumanol): Colorless needles, mp 118.0—119.0 °C, $[\alpha]_D$ +6.7° (c=0.06, $CHCl_3$). MS m/z (%): 252 (M^+, 1, Calcd for $C_{15}H_{24}O_3$, 252.1724; Found 252.1740), 234 (10), 191 (15), 163 (18), 141 (23), 123 (100), 81 (37), 71 (32), 55 (35). IR (CCl_4) cm^{-1}: 3620, 2960, 2880, 1465, 1455, 1380, 1370, 1135, 985, 925.

Compound IV (Epialpinolide): A colorless oil, $[\alpha]_D$ −7.1° (c=0.07, $CHCl_3$). MS m/z (%): 250 (M^+, 100, Calcd for $C_{15}H_{22}O_3$, 250.1567; Found 250.1549), 207 (37), 179 (62), 165 (55), 139 (42), 126 (86), 96 (49), 81 (54), 69 (48). IR (CCl_4) cm^{-1}: 2970, 2880, 1760, 1700, 1630, 1470, 1385, 1360, 1325, 1265, 1190, 1175, 1160, 1045, 1005, 970, 915, 865. UV (EtOH) nm (ε): 214 (10900).

Compound V ($\Delta^{11(12)}$-Eremophilen-10β-ol): A colorless oil, $[\alpha]_D$ +29.2° (c=0.12, $CHCl_3$). MS m/z (%): 222 (M^+, 6, Calcd for $C_{15}H_{26}O$, 222.1981; Found 222.1971), 204 (60), 189 (44), 161 (35), 126 (44), 123 (44), 111 (69), 109 (69), 81 (74), 69 (64), 67 (58), 55 (100). IR (CCl_4) cm^{-1}: 3630, 3080, 2930, 2870, 1645, 1450, 1380, 1010, 960, 890. ^{1}H-NMR ($CDCl_3$) δ ppm: 0.80 (3H, d, J=6.8 Hz), 0.84 (3H, s), 1.73 (3H, br s), 4.68 (1H, br s), 4.70 (1H, br s). ^{13}C-NMR ($CDCl_3$) δ ppm: 15.4 (q), 15.4 (q), 21.0 (q), 23.0 (t), 26.8 (t), 30.2 (t), 32.9 (t), 33.1 (d), 36.6 (t), 36.8 (t), 38.8 (d), 41.2 (s), 73.2 (s), 108.2 (t), 150.5 (s).

Compounds VI—XI: Compounds VI—XI were identical with those which have been isolated from *Alpinia japonica* and reported in our previous paper.

Compound XII (Intermedeol): Colorless needles, mp 46.0—47.0 °C, $[\alpha]_D$ +7.1° (c=0.17, $CHCl_3$). MS m/z (%): 222 (M^+, 4), 204 (90), 189 (70), 161 (50), 135 (40), 123 (51), 109 (58), 95 (62), 81 (100), 71 (82), 67 (60), 55 (60). IR (CCl_4) cm^{-1}: 3625, 3085, 2940, 1640, 1450, 1385, 1220, 1170, 1090, 1065, 910, 890. ^{1}H-NMR (CCl_4) δ ppm: 0.92 (3H, s), 1.02 (3H, s), 1.73 (3H, s), 4.86 (2H, br s).

Compound XIII (β-Selinene): A colorless oil, $[\alpha]_D$ +41.9° (c=0.09, $CHCl_3$). MS m/z (%): 204 (65, M^+), 189 (37), 175 (20), 161 (53), 147 (39), 133 (42), 122 (70), 105 (100). IR (neat) cm^{-1}: 3050, 2910, 1640, 1440, 1380, 885. ^{1}H-NMR ($CDCl_3$) δ ppm: 0.72 (3H, s), 1.76 (3H, s), 4.44 (1H, br s), 4.73 (3H, br s). ^{13}C-NMR ($CDCl_3$) δ ppm: 16.4 (q),

21.0 (q), 23.5 (t), 26.9 (t), 29.5 (t), 36.0 (s), 36.9 (t), 41.2 (t), 42.0 (t), 45.9 (d), 49.9 (d), 105.4 (t), 108.2 (t), 150.9 (s), 151.1 (s).

Reduction of I with Triphenylphosphine to Give VI——An ether solution (2 ml) of I (15 mg) was treated with triphenylphosphine (25 mg) for a few minutes at room temperature, then evaporated. The residue was subjected to HPLC (*n*-hexane : chloroform : acetonitrile = 7 : 2.6 : 0.5) to give VI as a colorless oil (13 mg), whose spectral data (including specific rotation) were identical with those of natural hanalpinol.

Reduction of VIII with Sodium Borohydride to Give II——A methanol solution of VIII (25 mg) was treated with an excess of sodium borohydride. After work-up in the usual way, the product was subjected to HPLC (*n*-hexane : ethyl acetate = 4 : 1) to give II as colorless needles (22.0 mg) whose spectral data (including specific rotation) were identical with those of natural isohanalpinol, and XIV as a colorless oil (1.2 mg). ^{1}H-NMR ($CDCl_3$) of XIV δ ppm: 1.05 (6H, d, J=7.0 Hz), 1.17 (3H, d, J=7.0 Hz), 1.24 (3H, s), 3.90 (1H, dd, J=4.5, 11.5 Hz), 4.41 (1H, br s), 5.52 (1H, ddd, J=1.5, 1.5, 4.5 Hz). IR (CCl_4) of XIV cm^{-1}: 3580, 2960, 2880, 1460, 1385, 1370, 1010.

Conversion of IV to Alpinolide——IV (10 mg) was stirred with *p*-toluenesulfonic acid (20 mg) in benzene (2 ml) for a day at room temperature. Then the reaction mixture was filtered, and the filtrate was washed with saturated sodium bicarbonate solution and brine, dried over magnesium sulfate, and evaporated. The product was purified by HPLC (benzene : chloroform : acetonitrile = 5 : 3 : 1) to give alpinolide (7 mg), whose spectral data (including specific rotation) were identical with those of natural alpinolide.

Dehydration of V to Give XV——Thionyl chloride (0.2 ml) was added to a cooled solution of V (30 mg) in pyridine (2 ml) and the reaction mixture was left for 1.5 h at 0 °C. The mixture was poured into ice-water and extracted with ether, and the ether layer was washed with saturated sodium bicarbonate solution and saturated sodium chloride solution, then dried (magnesium sulfate) and evaporated, leaving a colorless oil (20 mg). This was purified by HPLC (*n*-hexane) to give XV as a colorless oil (10 mg) whose spectral data (including specific rotation) were identical with those of $\Delta^{9(10),11(12)}$-eremophilene.

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Studies on Sesquiterpene Glycosides from *Sonchus oleraceus* L.

TOSHIO MIYASE* and SEIGO FUKUSHIMA

Shizuoka College of Pharmacy, 2-2-1, Oshika, Shizuoka 422, Japan

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Four new sesquiterpene glycosides, sonchusides A (**6**), B (**7**), C (**8**) and D (**9**), have been isolated from *Sonchus oleraceus* L., together with five known glycosides, glucozaluzanin C (**1**), macrocliniside A (**2**), crepidiaside A (**3**) and picrisides B (**4**) and C (**5**). The structures of the new compounds were established on the basis of chemical and spectral data.

Keywords——*Sonchus oleraceus*; Compositae; sesquiterpene glycoside; sonchuside

As a part of our studies on sesquiterpene glycosides from Compositae plants, we now report the isolation from methanolic extract of *Sonchus oleraceus* L. and the structure elucidation of four new sesquiterpene lactone glycosides, sonchusides A—D, together with five known glycosides, glucozaluzanin C (**1**), macrocliniside A (**2**), crepidiaside A (**3**) and picrisides B (**4**) and C (**5**). The structures of these compounds were determined on the basis of some chemical transformations and spectroscopic studies.

Three guaianolide-type sesquiterpene glycosides, glucozaluzanin C (**1**), macrocliniside A (**2**) and crepidiaside A (**3**), were identified by direct comparison [proton nuclear magnetic resonance (^{1}H-NMR), carbon-13 nuclear magnetic resonance (^{13}C-NMR) and infrared (IR) spectra] with authentic samples.[1,2] Two germacranolide-type sesquiterpene glycosides, picrisides B (**4**) and C (**5**), were also identified by direct comparison (^{1}H-NMR, ^{13}C-NMR, IR) with authentic samples.[3]

Sonchuside A (**6**), $C_{21}H_{32}O_8 \cdot 1/2H_2O$, $[\alpha]_D$ +29.1°, was obtained as an amorphous powder. The IR spectrum suggested the presence of hydroxyl groups (3450 cm^{-1}), γ-lactone ring (1770 cm^{-1}) and double bonds (1665, 1640 cm^{-1}). The ^{1}H-NMR spectrum was similar to that of **5** except for the appearance of a methyl signal at δ 1.27 (3H, d, J=7 Hz) instead of two doublet signals due to exomethylene protons. A comparison of the ^{13}C-NMR spectrum of **6** with that of **5** showed a downfield shift of 8.2 ppm in the C-12 signal (lactone carbonyl), suggesting that **6** is an 11,13-dihydro derivative of **5**. Acid hydrolysis of **6** afforded glucose and enzymatic hydrolysis afforded an aglycone (**6a**), whose mass spectrum (MS) showed a molecular ion peak at m/z 250, in agreement with the molecular formula $C_{15}H_{22}O_3$. In the ^{1}H-NMR spectrum of **6a**, the methyl signal showed an upfield shift of 0.20 ppm in benzene-d_6 relative to chloroform-d_1 solution, suggesting that the configuration of the C-13 methyl group at the γ-lactone is α.[4] Thus, the structure of **6** was established by comparison (IR, ^{1}H-NMR, ^{13}C-NMR) with the $NaBH_4$ reduction product of **5**.

Sonchuside B (**7**), $C_{30}H_{38}O_{11} \cdot 1/2H_2O$, $[\alpha]_D$ +72.0°, was obtained as an amorphous powder. The IR spectrum suggested the presence of hydroxyl groups (3450 cm^{-1}), ester group (1730 cm^{-1}) and double bonds (1660, 1615 cm^{-1}). The ultraviolet (UV) spectrum showed absorption maxima at 275 and 282 nm. The ^{1}H-NMR spectrum was similar to that of **5** except for the appearance of signals due to a p-methoxyphenylacetate moiety [δ 3.67 (3H, s), 3.76 (2H, s), 7.00 (2H, d, J=9 Hz), 7.34 (2H, d, J=9 Hz)]. In the ^{13}C-NMR spectrum, the C-7 signal (δ 46.9) was shifted upfield by 3.2 ppm and the C-8 signal (δ 34.0) was shifted downfield

by 5.6 ppm compared with those of **5**, suggesting that C-9 has an O-function. Saponification afforded *p*-methoxyphenylacetic acid and enzymatic hydrolysis afforded the aglycone (**7a**), $C_{24}H_{28}O_6$, as an amorphous powder. In the ^{13}C-NMR spectrum of **7a**, the C-3 signal (δ 77.7) was shifted upfield by 5.2 ppm, and the C-2 (δ 35.7) and C-4 (δ 145.0) signals were shifted downfield by 3.0 and 2.8 ppm, respectively, but the C-8, C-9 and C-10 signals showed almost the same chemical shifts in comparison with those of **7**. Thus, *p*-methoxyphenylacetic acid was attached to C-9. In the ^{1}H-NMR spectrum of **7a**, a nuclear Overhauser effect (NOE) was observed in the signal of H-6 [δ 3.94 (1H, t, J=9 Hz)] (12%) on irradiating the vinyl methyl signal (δ 1.41) due to H_3-15, but no effect was observed in the H-1 signal on irradiating H_3-14. The stereochemistry of the hydroxyl group at C-3 was concluded to be β from the coupling constants of H-3 [δ 3.75 (dd, J=10, 7 Hz)] compared with those of 3β-hydroxygermacranolides (dd, J=10, 5.5—7 Hz)[5] and 3α-hydroxygermacranolide (t, J=3 Hz).[6] The stereochemistry of the ester side chain at C-9 was also concluded to be β from the coupling constants of H-9 [δ 5.01 (dd, J=10, 3 Hz)], suggesting an antiperiplanar orientation of H-9 and H-8.[7,8a] In the ^{13}C-NMR spectrum of **7a**, the chemical shifts of C-3 (δ 77.7) and C-9 (δ 81.2) were very similar to those of 3β-hydroxy- (δ 75.4—76.7)[8] and 9β-acetoxygermacranolides (δ 80.7—81.0),[9] respectively. From these data the structure of sonchuside B was concluded to be **7**.

Sonchuside C (**8**), $C_{21}H_{32}O_8 \cdot 1/2H_2O$, $[\alpha]_D$ $-12.0°$, was obtained as an amorphous powder. The ^{1}H-NMR spectrum exhibited a tertiary methyl signal at δ 1.13 (s), a doublet methyl signal at δ 1.17 (J=7 Hz), a vinyl methyl signal at δ 1.90 (br s), a proton signal due to H-6 at δ 4.60 (br d, J=10 Hz) and an anomeric proton signal at δ 4.90 (d, J=7 Hz). In the ^{13}C-NMR spectrum, twenty-one signals, including six signals due to a glucopyranosyl moiety, were observed. A quaternary carbon signal was observed at δ 41.6, suggesting that this compound is a eudesmanolide-type sesquiterpene,[10] and two olefinic carbon signals were observed at δ 125.0 and 130.5 (each s in off-resonance) which were assigned to C-5 and C-4, respectively. Acid hydrolysis afforded glucose as the sugar moiety and enzymatic hydrolysis afforded an aglycone (**8a**), $C_{15}H_{22}O_3$, mp 173—175 °C. The ^{1}H-NMR spectrum of **8a** exhibited a carbinyl proton signal at δ 3.52 (br t, J=7 Hz, $W_{1/2}$=17 Hz) and a proton signal due to H-6 at δ 4.59 (br d, J=10 Hz) besides three methyl signals. The doublet methyl signal due to H_3-13 was shifted upfield in benzene-d_6 by 0.26 ppm, suggesting that this methyl group has pseudo-equatorial (α) orientation. The circular dichroism (CD) spectrum of the *p*-nitrobenzoate of **8a** showed a negative Cotton effect $[\theta]_{262}$ -5797 suggesting that the hydroxyl

TABLE I. ^{1}H-NMR Chemical Shifts and Coupling Constants

Proton No.	6	7	8	9
Aglycone moiety				
5		4.90 (1H, br d, J=10 Hz)		
6		4.80 (1H, t, J=10 Hz)	4.60 (1H, br d, J=10 Hz)	
13a	1.27 (3H, d, J=7 Hz)	5.58 (1H, d, J=3.0 Hz)	1.17 (3H, d, J=7 Hz)	5.31 (1H, d, J=3.0 Hz)
13b		6.36 (1H, d, J=3.4 Hz)		6.12 (1H, d, J=3.3 Hz)
14	1.37 (3H, br s)	1.48 (3H, br s)	1.13 (3H, s)	0.87 (3H, s)
15	1.96 (3H, br s)	2.00 (3H, br s)	1.90 (3H, br s)	
Anomeric	4.84 (1H, d, J=8 Hz)	4.84 (1H, d, J=8 Hz)	4.90 (1H, d, J=7 Hz)	4.89 (1H, d, J=8 Hz)
Ester moiety				
β		3.76 (2H, s)		
2, 6		7.34 (2H, d, J=9 Hz)		
3, 5		7.00 (2H, d, J=9 Hz)		
OMe		3.67 (3H, s)		

Run at 89.55 MHz in pyridine-d_5 solution.

TABLE II. ^{13}C-NMR Chemical Shifts

Carbon No.	**6**	**7**	**8**	**9**
Aglycone moiety				
1	125.3	126.5	24.3[d)]	83.8
2	33.6	32.7	33.5[e)]	28.3
3	83.5	82.9[a)]	82.8[f)]	36.4
4	140.8	139.8	130.5	144.2
5	127.4	128.8	125.0	53.5
6	80.7	81.4[a)]	83.5[f)]	79.8
7	54.4	46.9	53.2	49.8
8	28.6	34.0	23.6[d)]	21.5
9	41.3	80.7[a)]	38.7[e)]	33.8
10	137.8	135.8	41.6	42.9
11	42.3	142.2	41.0	140.5
12	178.5	169.7	178.5	170.8
13	13.5	119.9	12.5	116.3
14	16.3	11.4[b)]	19.7[g)]	12.8
15	12.3	12.4[b)]	19.5[g)]	110.0
Sugar moiety				
1	102.8	102.6	102.0	102.3
2	75.3	75.1	75.0	75.2
3	78.5	78.4[c)]	78.5[h)]	78.7[i)]
4	72.0	71.8	72.0	72.1
5	78.5	78.3[c)]	78.0[h)]	78.5[i)]
6	63.0	62.8	63.1	63.3
Ester moiety				
α		170.7		
β		40.8		
1		126.9		
2, 6		130.8		
3, 5		114.5		
4		159.2		
OMe		55.2		

Run at 22.5 MHz in pyridine-d_5 solution. *a*—*i*) Assignments may be interchanged in each column.

group at C-3 is β oriented (from the allyl benzoate rule).[11] If the assumption is made that the absolute configuration of the C-7 side chain is as shown (as in all other known sesquiterpene lactones of authenticated stereochemistry), the structure of sonchuside C can be concluded to be **8**.

Sonchuside D (**9**), $C_{21}H_{30}O_8 \cdot H_2O$, $[\alpha]_D$ +64.3°, was obtained as an amorphous powder. The ^{1}H-NMR spectrum exhibited a tertiary methyl signal at δ 0.87 (s), an anomeric proton signal at δ 4.89 (d, J=8 Hz) and two olefinic proton signals at δ 5.31 (d, J=3.0 Hz) and 6.12 (d, J=3.3 Hz), which are characteristic of an exocyclic-α-methylene-γ-lactone. Enzymatic hydrolysis afforded the aglycone (**9a**). The MS of **9a** showed a molecular ion peak at m/z 248 in agreement with the molecular formula $C_{15}H_{20}O_3$. The ^{1}H-NMR spectrum of **9a** exhibited a tertiary methyl signal at δ 0.92 (s), a carbinyl proton signal at δ 3.54 (dd, J=11, 5 Hz), a proton signal due to H-6 at δ 4.04 (t, J=11 Hz), exomethylene signals at δ 4.88 (br s) and 5.00 (br s), and two olefinic proton signals at δ 5.43 (d, J=3.0 Hz) and 6.11 (d, J=3.1 Hz). From these data, **9a** was assumed to be reynosin and this was confirmed by direct comparison (^{1}H-NMR, MS) with an authentic sample.[12] Therefore the structure of sonchuside D was decided to be **9**.

1 : R=H
2 : R=OH

3

4

5 : R=Glc, X=CH_2
6 : R=Glc, X=CHCH_3
6a : R=H, X=CHCH_3

7 : R=Glc
7a : R=H

8 : R=Glc
8a : R=H

9 : R=Glc
9a : R=H

Chart 1

Experimental

Melting points were taken on a Yanaco MP-500 micro melting point apparatus and are uncorrected. Optical rotations were determined with a JASCO DIP-140 digital polarimeter. IR spectra were run on a JASCO A-201 IR spectrometer and UV spectrum on a Shimadzu UV-360 recording spectrometer. MS were measured on a JEOL JMS-D 100 mass spectrometer. CD spectra were recorded on a JASCO J-20A spectropolarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL FX-90Q NMR spectrometer (89.55 and 22.5 MHz, respectively). Chemical shifts are given on the δ scale with tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; m, multiplet; br, broad). Gas chromatography (GC) was done on a Hitachi K53 gas chromatograph. High-performance liquid chromatography (HPLC) was done on a Kyowa Seimitsu model K880 instrument.

Isolation——Air-dried whole plants of *S. oleraceus* (6 kg) were extracted twice with methanol under reflux. The extract was concentrated under reduced pressure and the residue was partitioned between hexane–benzene (1 : 1) and methanol–water (4 : 1). The lipophilic layer was concentrated to give a dark green residue (210 g). The hypophilic layer was concentrated and the residue was partitioned between *n*-butanol and water. The *n*-butanol layer was concentrated to dryness *in vacuo*. After repeated chromatography of the *n*-butanol layer (80 g) on silica gel with a chloroform–methanol system and HPLC with a water–acetonitrile system, nine sesquiterpene glycosides were isolated.

Glucozaluzanin C (1)——Amorphous powder (7 mg). IR ν_{max}^{KBr} cm^{-1}: 3450, 1765, 1660, 1635. ^{1}H-NMR (pyridine-d_5) δ: 5.38 (1H, d, J=3.1 Hz, H-13a), 5.53, 5.85 (each 1H, br s, H_2-15), 6.22 (1H, d, J=3.3 Hz, H-13b). ^{13}C-NMR (pyridine-d_5) δ: 30.7 (C-8), 34.1 (C-9), 38.2 (C-2), 44.9, 45.4 (C-1/C-7), 50.4 (C-5), 63.1 (C-6′), 72.0 (C-4′), 75.3 (C-2′), 78.3, 78.6 (C-3′/C-5′), 80.6 (C-3), 83.6 (C-6), 104.1 (C-1′), 112.5 (C-15), 114.0 (C-14), 119.2 (C-13), 141.1 (C-11), 148.9 (C-10), 150.5 (C-4), 169.9 (C-12).

Macrocliniside A (2)——Amorphous powder (45 mg). IR ν_{max}^{KBr} cm^{-1}: 3450, 1760, 1665, 1640. ^{1}H-NMR (pyridine-d_5) δ: 5.03 (1H, d, J=7 Hz, H-1′), 5.15 (2H, s, H_2-14), 5.42 (1H, d, J=3.1 Hz, H-13a), 5.53, 5.92 (each 1H, br s, H_2-14), 6.23 (1H, d, J=3.6 Hz, H-13b). ^{13}C-NMR (pyridine-d_5) δ: 37.0 (C-7), 37.5 (C-2), 39.8 (C-8), 41.6 (C-1), 49.5 (C-5), 63.1 (C-6′), 72.0 (C-4′), 72.3 (C-9), 75.3 (C-2′), 78.2, 78.6 (C-3′/C-5′), 80.8 (C-3), 84.4 (C-6), 104.4 (C-1′), 111.1 (C-14), 112.2 (C-15), 118.9 (C-13), 141.2 (C-11), 150.9 (C-4), 153.3 (C-10), 170.1 (C-12).

Crepidiaside A (3)——Amorphous powder (23 mg). IR ν_{max}^{KBr} cm^{-1}: 3450, 1770, 1680, 1620, 1080, 1030. ^{1}H-NMR (pyridine-d_5) δ: 2.46 (3H, s, H_3-14), 4.93 (1H, d, J=7 Hz, H-1′), 5.00, 5.20 (each 1H, br d, J=17 Hz, H_2-15), 5.38 (1H, d, J=3.2 Hz, H-13a), 6.18 (1H, d, J=3.5 Hz, H-13b), 6.93 (1H, br s, H-3). ^{13}C-NMR (pyridine-d_5) δ: 21.5 (C-14), 24.4 (C-8), 37.1 (C-9), 50.1 (C-5), 52.5 (C-7), 62.7 (C-6′), 68.7 (C-15), 71.5 (C-4′), 75.1 (C-2′), 78.2, 78.4 (C-3′/C-5′), 84.0 (C-6), 104.1 (C-1′), 118.2 (C-13), 131.7 (C-1), 134.4 (C-3), 139.6 (C-11), 152.5 (C-10), 169.1 (C-4, C-12), 194.9 (C-2).

Picriside B (4)——Amorphous powder (7 mg). IR ν_{max}^{KBr} cm^{-1}: 3450, 1765, 1665, 1635, 1550, 1450, 1230, 1080, 960. ^{1}H-NMR (pyridine-d_5) δ: 1.37 (3H, br s, H_3-14), 5.49 (1H, d, J=3.1 Hz, H-13a), 6.31 (1H, d, J=3.6 Hz, H-13b).

^{13}C-NMR (pyridine-d_5) δ: 16.2 (C-14), 27.1, 27.8 (C-2/C-8), 35.9 (C-3), 41.1 (C-9), 50.8 (C-7), 63.0 (C-6'), 67.7 (C-15), 71.8 (C-4'), 75.1 (C-2'), 78.5, 78.6 (C-3'/C-5'), 80.3 (C-6), 105.2 (C-1'), 119.0 (C-13), 126.8 (C-1), 130.1 (C-5), 137.5 (C-10), 141.0, 141.1 (C-4/C-11), 170.2 (C-12).

Picriside C (5)——Amorphous powder (10 mg). IR ν_{max}^{KBr} cm^{-1}: 3450, 1765, 1665, 1550, 1450, 1410, 1295, 1250, 1150, 1080, 970, 940. ^{1}H-NMR (pyridine-d_5) δ: 1.36 (3H, br s, H$_3$-14), 1.97 (3H, br s, H$_3$-15), 5.55 (1H, d, J=3.1 Hz, H-13a), 6.38 (1H, d, J=3.4 Hz, H-13b). ^{13}C-NMR (pyridine-d_5) δ: 12.5 (C-15), 16.3 (C-14), 28.4 (C-8), 33.6 (C-2), 41.2 (C-9), 50.1 (C-7), 63.0 (C-6'), 71.8 (C-4'), 75.3 (C-2'), 78.5, 78.6 (C-3'/C-5'), 81.3 (C-6), 83.3 (C-3), 102.8 (C-1'), 119.5 (C-13), 125.3 (C-1), 127.1 (C-5), 137.8 (C-10), 140.9 (C-4), 142.0 (C-11), 170.3 (C-12).

Sonchuside A (6)——Amorphous powder (15 mg), $[\alpha]_D^{25}$ +29.1° (c=0.55, methanol). *Anal.* Calcd for $C_{21}H_{32}O_8 \cdot 1/2H_2O$: C, 59.84; H, 7.89. Found: C, 59.54; H, 7.72. IR ν_{max}^{KBr} cm^{-1}: 3450, 1770, 1665, 1640, 1160, 1080, 1040, 1020, 965. ^{1}H- and ^{13}C-NMR: Tables I and II.

Sonchuside B (7)——Amorphous powder (48 mg), $[\alpha]_D^{25}$ +72.0° (c=0.93, methanol). *Anal.* Calcd for $C_{30}H_{38}O_{11} \cdot 1/2H_2O$: C, 61.74; H, 6.74. Found: C, 61.57; H, 6.65. IR ν_{max}^{KBr} cm^{-1}: 3450, 1770, 1730, 1660, 1615, 1515, 1250, 1150, 1070, 1025, 965. UV λ_{max}^{MeOH} nm (log ε): 275 (3.37), 282 (3.31). CD (c=0.36, methanol) [θ] (nm): −6218 (258). ^{1}H- and ^{13}C-NMR: Tables I and II.

Sonchuside C (8)——Amorphous powder (35 mg), $[\alpha]_D^{25}$ −12.0° (c=0.50, methanol). *Anal.* Calcd for $C_{21}H_{32}O_8 \cdot 1/2H_2O$: C, 59.84; H, 7.89. Found: C, 59.71; H, 7.74. IR ν_{max}^{KBr} cm^{-1}: 3450, 1780, 1640, 1460, 1380, 1235, 1170, 1080, 1030, 985, 905. ^{1}H- and ^{13}C-NMR: Tables I and II.

Sonchuside D (9)——Amorphous powder (7 mg), $[\alpha]_D^{25}$ +64.3° (c=0.14, methanol). *Anal.* Calcd for $C_{21}H_{30}O_8 \cdot H_2O$: C, 58.87; H, 7.53. Found: C, 58.60; H, 7.31. IR ν_{max}^{KBr} cm^{-1}: 3430, 1765, 1660, 1630, 1415, 1385, 1255, 1125, 1075. CD (c=0.14, methanol) [θ] (nm): −2548 (253). ^{1}H- and ^{13}C-NMR: Tables I and II.

Sodium Borohydride Reduction of Picriside C (5)——A solution of picriside C (5) (15 mg) in methanol (2 ml) was treated at 0 °C with sodium borohydride (20 mg) and the mixture was stirred for 20 min. The reaction mixture was acidified with acetic acid, diluted with water and then passed through an Amberlite XAD-2 column. The column was washed with water and the reaction product was eluted with methanol. Purification by HPLC (Develosil ODS-10) with water–acetonitrile (78 : 22) as the eluent provided **6** (4 mg) as an amorphous powder. The IR, ^{1}H- and ^{13}C-NMR spectra were identical to those of sonchuside A.

Enzymatic Hydrolysis of Sonchuside A (6)——A solution of sonchuside A (6) (10 mg) in water (1 ml) was treated with crude hesperidinase (10 mg) at 38 °C for 1 h. The reaction mixture was diluted with water and then passed through an Amberlite XAD-2 column. The column was washed with water and the reaction product was eluted with methanol. Purification by HPLC (Develosil ODS-7) with water–acetonitrile (55 : 45) as the eluent provided the aglycone **6a** (1.5 mg) as an amorphous powder. MS m/z: 250 (M$^+$, 32), 125 (37), 109 (39), 98 (42), 95 (55), 93 (45), 81 (100). ^{1}H-NMR (C_6D_6) δ: 1.06 (3H, d, J=7 Hz, H$_3$-13), 1.10 (3H, br s, H$_3$-14), 1.52 (3H, d, J=1 Hz, H$_3$-15), 3.85 (1H, dd, J=10, 7 Hz, H-3), 4.05 (1H, dd, J=10, 8 Hz, H-6), 4.34 (1H, br d, J=10 Hz, H-5), 4.50 (1H, m, H-1). ^{1}H-NMR ($CDCl_3$) δ: 1.26 (3H, d, J=7 Hz, H$_3$-13), 1.45 (3H, br s, H$_3$-14), 1.72 (3H, br s, H$_3$-15), 4.25 (1H, dd, J=10, 7 Hz, H-3).

Enzymatic Hydrolysis of Sonchuside B (7)——Sonchuside B (7) (12 mg) was hydrolyzed in the same way as **6** to give the aglycone, $C_{24}H_{28}O_6$, M$^+$ 412.1866 (Calcd for $C_{24}H_{28}O_6$ 412.1889) (**7a**) (5 mg) as an amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3450, 1770, 1730, 1615, 1518, 1250, 1135, 970. MS m/z: 412 (M$^+$, 5), 206 (5), 166 (5), 148 (6), 121 (100), 91 (15), 84 (61). ^{1}H-NMR (C_6D_6) δ: 1.17 (3H, d, J=1 Hz, H$_3$-14), 1.41 (3H, d, J=1 Hz, H$_3$-15), 3.29 (3H, s, OMe), 3.38 (2H, s, H$_2$-β), 3.75 (1H, dd, J=10, 7 Hz, H-3), 3.94 (1H, t, J=9 Hz, H-6), 4.14 (1H, br d, J=10 Hz, H-5), 4.77 (1H, br dd, J=11, 6 Hz, H-1), 4.97 (1H, d, J=3.1 Hz, H-13a), 5.01 (1H, dd, J=10, 3 Hz, H-9), 6.18 (1H, d, J=3.5 Hz, H-13b), ^{13}C-NMR (pyridine-d_5) δ: 11.6, 12.5 (C-14/C-15), 34.0 (C-8), 35.7 (C-2), 41.0 (C-β), 47.1 (C-7), 55.3 (OMe), 77.7 (C-3), 81.2, 81.5 (C-6/C-9), 114.5 (C-3, C-5 of ester), 119.8 (C-13), 127.1 (C-1 of ester), 130.0 (C-1, C-5), 131.0 (C-2, C-6 of ester), 136.2 (C-10), 140.2 (C-11), 145.0 (C-4), 159.5 (C-4 of ester), 170.0, 170.8 (C-12/C-α).

Saponification of Sonchuside B (7)——A solution of sonchuside B (7) (*ca.* 0.1 mg) in aqueous 2% NaOH solution (2 drops) was stirred for 1 h at room temperature under a nitrogen atmosphere. The solution was acidified with 2 N HCl and extracted with n-butanol. The extract was concentrated to dryness and the residue was refluxed with acetyl chloride–methanol (1 : 20) for 20 min. The reaction mixture was concentrated to give methyl p-methoxyphenylacetate, which was detected by HPLC. Conditions: column, TSK GEL LS-410AK, 4 mm × 30 cm; flow rate, 1.3 ml/min; detector, UV 245 nm; solvent, water–methanol (1 : 1); t_R 4.7 min.

Enzymatic Hydrolysis of Sonchuside C (8)——Sonchuside C (8) (15 mg) was hydrolyzed in the same way as **6**, and the product was purified by HPLC [YMC Pack A-212, water–acetonitrile (55 : 45)]. The aglycone of **8**, **8a**, was obtained as colorless needles (chloroform–hexane) (5 mg), mp 173—175 °C, as $C_{15}H_{22}O_3$, M$^+$ 250.1552 (Calcd for $C_{15}H_{22}O_3$ 250.1570). IR ν_{max}^{KBr} cm^{-1}: 3500, 1755, 1665, 1145, 1020, 980. MS m/z: 250 (M$^+$, 28), 235 (M$^+$ −CH$_3$, 8), 232 (M$^+$ −H$_2$O, 25), 217 (19), 206 (100), 193 (64), 165 (67). ^{1}H-NMR ($CDCl_3$) δ: 1.11 (3H, s, H$_3$-14), 1.23 (3H, d, J=7 Hz, H$_3$-13), 1.85 (3H, br s, H$_3$-15), 3.52 (1H, br t, J=7 Hz, $W_{1/2}$=17 Hz, H-3), 4.59 (1H, br d, J=10 Hz, H-6). ^{1}H-NMR (C_6D_6) δ: 0.94 (3H, s, H$_3$-14), 0.97 (3H, d, J=7 Hz, H$_3$-13), 1.95 (3H, d, J=1 Hz, H$_3$-15), 3.20 (1H, dd, J=10, 5 Hz, $W_{1/2}$=17 Hz, H-3), 4.11 (1H, br d, J=10 Hz, H-6). ^{13}C-NMR ($CDCl_3$) δ: 12.3 (C-13), 18.4, 19.7 (C-14/C-15), 24.4, 27.0 (C-1/C-7), 32.2, 38.3 (C-2/C-9), 41.1 (C-11), 41.8 (C-1), 52.8 (C-7), 77.6 (C-3), 82.9 (C-6), 125.9 (C-5), 128.8

(C-4), 178.7 (C-12). CD (*p*-nitrobenzoate) (c=0.0483, methanol) [θ] (nm): −5797 (262).

Enzymatic Hydrolysis of Sonchuside D (9)——Sonchuside D (**9**) (1.5 mg) was hydrolyzed in the same way as **6**, and the product was purified by HPLC [Develosil ODS-7, water–acetonitrile (6:4)]. **9a** was obtained as an amorphous powder (0.2 mg). ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, s, H_3-14), 3.54 (1H, dd, J=11, 5 Hz, H-1), 4.04 (1H, t, J=11 Hz, H-6), 4.88, 5.00 (each 1H, br s, H_2-15), 5.43 (1H, d, J=3.0 Hz, H-13a), 6.11 (1H, d, J=3.1 Hz, H-13b). MS *m/z*: 248 (M^+, 3), 130 ($M^+ - H_2O$, 52), 163 (52), 83 (81), 42 (100).

Acid Hydrolysis of Sonchusides A (6), B (7), C (8) and D (9)——A solution of a glycoside (*ca.*0.1 mg) in 10% H_2SO_4 (2 drops) were heated in a boiling water bath for 30 min. The solution was passed through an Amberlite IRA-45 column and concentrated to give a residue, which was reduced with $NaBH_4$ (*ca.* 1 mg) for 1 h at room temperature. The reaction mixture was passed through an Amberlite IR-120 column and the eluate was concentrated to dryness. Boric acid was removed by co-distillation with methanol and the residue was acetylated with acetic anhydride and pyridine (1 drop each) at 100 °C for 1 h. The reagents were evaporated off *in vacuo*. From each glycoside, glucitol acetate was detected by GC. Conditions: column, 1.5% OV-17, 3 mm × 1 m; column temperature, 215 °C; carrier gas,. N_2; t_R 6.0 min.

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Circular Dichroism Studies on the Ellagitannins–Nucleic Acids Interaction

NOBUKO KAKIUCHI,[a] XUAN WANG,[a] MASAO HATTORI,[a]
TAKUO OKUDA[b] and TSUNEO NAMBA*,[a]

Research Institute for Wakan-Yaku (Oriental Medicines), Toyama Medical and Pharmaceutical University,[a] 2630 Sugitani, Toyama 930–01, Japan and Faculty of Pharmaceutical Sciences, Okayama University,[b] 1-1-1 Tsushima-naka, Okayama 700, Japan

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The interaction of homopolynucleotides complexes and ellagitannins was studied by circular dichroism (CD) spectroscopy. The interaction difference CD spectra of mixtures of $(rC)_n(dG)_n$ and dimeric ellagitannins showed extremely large positive peaks especially around 250 nm, suggesting a strong interaction. The mixtures with $(rA)_n(dT)_n$ also had large difference CD spectra, although the interaction showed no apparent contribution to the stabilities of the nucleotides complexes.

Keywords——ellagitannin; dimeric ellagitannin; homopolynucleotide complex; nucleotide–tannin interaction; CD; thermal denaturation temperature

Previously, we reported that some hydrolyzable tannins, especially dimeric ellagitannins, had strong inhibitory activity on reverse transcriptase isolated from a ribonucleic acid (RNA) tumor virus.[1] We also found a relationship between inhibitory activity and structures of tannins dimeric tannins inhibited the enzyme reaction in the presence of either $(rA)_n \cdot (dT)_{12-18}$ or $(rC)_n \cdot (dG)_{12-18}$ as a template-primer, whereas a lesser inhibitory activity of monomeric ellagitannins and galloylglucoses was observed in the $(rC)_n \cdot (dG)_{12-18}$-directed reaction. To elucidate the mechanism of this nucleic acids-specific inhibition of the reverse transcriptase reaction by tannins, we studied the properties of the interaction of various tannins and nucleic acids.

Materials and Methods

Instruments——Ultraviolet (UV) absorption spectra were recorded on a Shimadzu UV-210 spectrophotometer. Circular dichroism (CD) spectra were taken with a JASCO J-500 equipped with a JASCO DP-500 data processor. Temperature was measured with a microprobe of DIGIMULTI D611 thermistor (Takara Thermistor Co., Ltd., Yokohama). The cuvette holder was thermostated by a Thermosupplier EZL-80 (Taiyo Co., Ltd.). All UV and CD measurements were made in 0.01 M phosphate buffer (pH 7.0) containing 0.1 M NaCl.

Nucleic Acids——Polynucleotides, $(rA)_n$, $(dA)_n$, $(rC)_n$, $(dG)_n$, $(dT)_n$ and $(rU)_n$, and double strand complexes, $(rA)_n \cdot (rU)_n$, $(dA)_n \cdot (dT)_n$ and $(dC)_n \cdot (rG)_n$ were purchased from PL Biochemicals. These polynucleic acids were serially dialyzed against 0.5 M NaCl–0.01 M Tris–HCl (pH 7.0), 0.1 M NaCl–0.01 M Tris–HCl, then 0.01 M Tris–HCl. $(rA)_n \cdot (dT)_n$, $(dA)_n \cdot (rU)_n$ and $(rC)_n \cdot (dG)_n$ were made by mixing the indicated components in equimolar quantity. The complexes were checked by thermal denaturation temperatures (T_ms) and CD spectra. Before use, nucleic acids were heated for 5 min at 50—60 °C, and left for 30 min at room temperature. The following molecular extinction coefficients were used: $(rA)_n \cdot (rU)_n$: 7.0×10^3 (257 nm), $(rA)_n \cdot (dT)_n$: 6.9×10^3 (257 nm), $(dA)_n \cdot (dT)_n$: 6.0×10^3 (259 nm), $(dA)_n \cdot (rU)_n$: 6.5×10^3 (257 nm) and $(rC)_n \cdot (dG)_n$: 7.4×10^3 (261 nm).[2,3]

Tannins——Tannins were isolated from the following plants as previously reported; agrimoniin was from *Agrimonia pilosa* LEDEB.,[4] coriariin A was from *Coriaria japonica* A. GRAY,[5] cornusiin A was from *Cornus officinalis* SIEB. *et* ZUCC.,[6] gemin A was from *Geum japonica* THUNB.,[7] geraniin from *Geranium thunbergii* SIEB. *et* ZUCC.,[8] granatin B from *Punica granatum* L.,[9] nobotanin A was from *Tibouchina semidecandra* COGN.,[10] pedunculagin from

Psidium guajava L.,[11] and rugosin D was from *Rosa rugosa* THUNB.[12]

Measurement of Difference CD Spectra——Solutions of tannins and nucleic acids were separately taken in a cuvette having a quartz partition half way along with the light path, and CD spectra were measured before and after mixing. The difference in CD spectra between these two measurements was obtained by microcomputer processing.

Abbreviations——$(rA)_n \cdot (dT)_{12-18}$: complex of poly(riboadenylic acid) and oligo(deoxyribothymidylic acid).
$(rC)_n \cdot (dT)_{12-18}$: complex of poly(ribocytidylic acid) and oligo(deoxyguanylic acid).
$(rA)_n$: poly(riboadenylic acid).
$(dA)_n$: poly(deoxyriboadenylic acid).
$(rC)_n$: poly(ribocytidylic acid).
$(dG)_n$: poly(deoxyriboguanylic acid).
$(dT)_n$: poly(deoxyribothymidylic acid).
$(rU)_n$: poly(ribouridylic acid).
$(rA)_n \cdot (rU)_n$: complex of poly(riboadenylic acid) and poly(ribouridylic acid).
$(rA)_n \cdot (dU)_n$: complex of poly(riboadenylic acid) and poly(deoxyribouridylic acid).
$(dA)_n \cdot (dT)_n$: complex of poly(deoxyriboadenylic acid) and poly(deoxyribothymidylic acid).
$(rA)_n \cdot (dT)_n$: complex of poly(riboadenylic acid) and poly(deoxyribothymidylic acid).
$(rC)_n \cdot (dG)_n$: complex of poly(ribocytidylic acid) and poly(deoxyguanylic acid).

Results and Discussion

CD spectra of the tannins studied are presented in Fig. 1. Seven tannins out of nine, agrimoniin, coriariin A, cornusiin A, gemin A, nobotanin A, pedunculagin and rugosin D, show characteristic positive Cotton effects around 240 nm due to the (*S*)-hexahydroxydiphenoyl (HHDP) group as previously reported.[13] Geraniin and granatin B contain an (*R*)- and (*S*)-dehydrohexahydroxydiphenoyl (DHHDP) group, respectively, and showed opposite profiles to each other.

The interaction of polynucleotides and tannins was directly monitored by measuring the difference in the CD spectra between before and after mixing of two components. When $(rC)_n \cdot (dG)_n$ and agrimoniin were mixed at the same concentration (1×10^{-5} M), the CD spectrum was changed as shown in Fig. 2a. Small red-shifts of the two Cotton effects centered at 244 and 274 nm together with the increase of their amplitude resulted in a large positive Cotton band at around 250 nm and a negative one at around 275 nm in the interaction difference CD spectrum. Although the CD spectra of tannin–nucleotide mixtures reflect the overall conformational changes of the two components, the shift of the Cotton effects and enhancement of the amplitudes of the 1 : 1 mixture mainly originated from the tannin

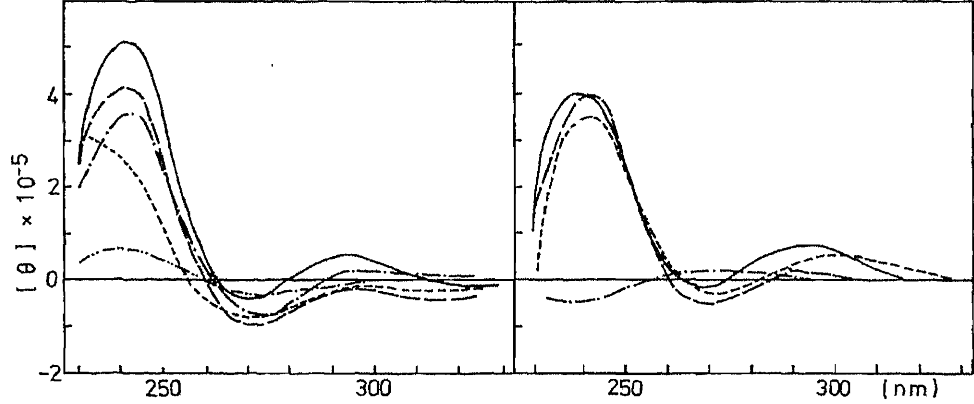

Fig. 1. CD Spectra of Tannins Measured in 0.1 M NaCl, Potassium Phosphate Buffer (pH 7.0) and 1% Dimethylsulfoxide (DMSO)

Left: ——, gemin A; – – –, nobotanin A; ------, cornusiin A; –·–·–, pedunculagin; –··–··–, granatin B.
Right: ——, rugosin D; – – –, agrimoniin; ------, coriariin A; –·–·–, geraniin.

components because of large amplitudes (order of 10^5) of tannin signals compared to those of nucleotide signals (order of 10^4) at wavelengths shorter than 260 nm. Since the CD bands of this region were generated from the chirality in the HHDP group, this change could be explained in terms of optically active transitions in HHDP, probably a decrease of the torsion angle between the two phenyl rings of HHDP around the conjugation bond in a way similar to that suggested by Mislow *et al.*[14] The flatter conformation may be favorable for interaction with polynucleotide helices since less steric hindrance would be expected. On the other hand, a 10:1 mixture of $(rA)_n \cdot (dT)_n$ and agrimoniin showed a CD spectrum with diminished amplitude at longer wavelengths where the CD bands of the nucleotide complex were mainly observed (Fig. 2b), reflecting some conformational alterations of the nucleotide component, such as destacking and conformational transition.[15]

The mixtures of $(rC)_n \cdot (dG)_n$ and the other tannins, except geraniin, showed difference CD spectra with a positive and a negative Cotton effect at around 250 and 275 nm, respectively, and their magnitudes are summarized in Table I. The difference CD spectra for dimeric ellagitannins had relatively large Cotton bands at around 250 nm, as well as 275 nm, in comparison with those of monomeric ellagitannins.

The difference CD spectra of $(rA)_n \cdot (dT)_n$–tannin (1:1) mixtures are presented in Table

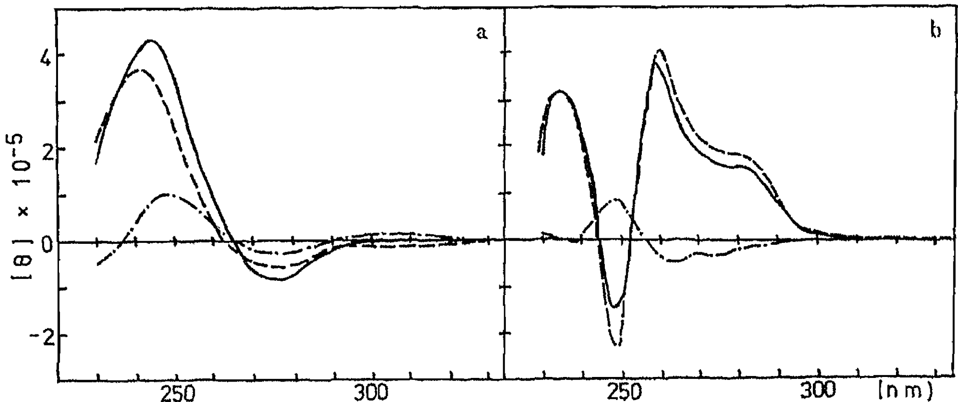

Fig. 2. CD Spectra of Mixtures of Agrimoniin (1×10^{-5} M) and (a) $(rC)_n \cdot (dG)_n$ (1×10^{-5} M), or (b) $(rA)_n \cdot (dT)_n$ (1×10^{-4} M)

-·-·-, before mixing; ——, after mixing and - - - -, difference. Same experimental conditions as in Fig. 1.
Molecular ellipticities were calculated on the basis of the agrimoniin concentration.

TABLE I. Interaction Difference CD Spectra of $(rC)_n \cdot (dG)_n$ with Tannins at 0.1 M NaCl, 0.01 M Potassium Phosphate Buffer (pH 7.0) and 1% DMSO

Ellagitannin	λ_1	$\theta_1 \times 10^{-4}$	λ_2	$\theta_2 \times 10^{-4}$
Dimeric				
Agrimoniin	276	−3.0	250	9.7
Coriariin A	276	−4.6	248	13.2
Cornusiin A	278	−2.8	250	8.6
Gemin A	278	−7.6	250	12.7
Nobotanin A	280	−4.1	250	12.7
Rugosin D	280	−6.8	250	8.1
Monomeric				
Geraniin	274	1.8	250	−2.0
Granatin B	272	−2.1	250	4.1
Pedunculagin	282	−4.3	252	4.6

Concentrations of $(rC)_n \cdot (dG)_n$ and tannins were 1×10^{-5} M.

TABLE II. Interaction Difference CD Spectra of $(rA)_n \cdot (dT)_n$ with Tannins

Ellagitannin	λ_1	$\theta_1 \times 10^{-4}$	λ_2	$\theta_2 \times 10^{-4}$
Dimeric				
Agrimoniin	270	−0.59	240	4.67
Coriariin A	258	−6.54	248	3.27
Cornusiin A	278	−0.49	244	0.88
Gemin A	266	−0.64	238	3.29
Rugosin D	256	2.16	238	5.12
Monomeric				
Geraniin	268	0.49	246	−0.98
Granatin B	266	1.64	240	−1.17
Pedunculagin	268	0.69	242	1.71

Same experimental conditions as in Table I.

TABLE III. Interaction CD Spectra of Nobotanin A–Nucleic Acids (1 : 1) Mixtures

Polynucleotide	$\theta \times 10^{-4}$ (nm)	
$(rA)_n \cdot (dT)_n$	−3.25 (240)	
$(rC)_n \cdot (dG)_n$	12.7 (250)	−4.7 (280)
$(dA)_n \cdot (dT)_n$	0.21 (260)	
$(rA)_n \cdot (rU)_n$	0.98 (240)	
$(dA)_n \cdot (rU)_n$	−1.5 (244)	
$(dG)_n$	11.0 (250)	3.5 (280)
$(rA)_n$	5.2 (246)	−1.5 (270)
$(rC)_n$	0.2 (250)	
$(dA)_n$	0.3 (246)	
$(dT)_n$	0.7 (246)	

Same conditions as in Table I.

TABLE IV. T_m Values (°C) of $(rA)_n \cdot (dT)_n$ in the Presence of Tannins

Compound	T_m (°C)
—	63.8
Agrimoniin	62.8
Coriariin A	64.1
Cornusiin A	63.8
Gemin A	64.1
Geraniin	63.0
Granatin B	63.9
Nobotanin A	62.8
Rugosin D	63.9

0.1 M NaCl, 0.01 M phosphate buffer (pH 7.0) and 1% DMSO.

II, and similar results were obtained in this case, suggesting the stronger binding of dimeric ellagitannins. These findings accord with the results on inhibitory potency against reverse transcriptase, and larger molecules with polyphenol groups are presumed to interact more tightly with nucleotides.

The interaction of a dimeric ellagitannin, namely nobotanin A, and various polynucleotide duplexes was also studied by CD spectrometry (Table III). The differences in the CD spectra between the solution before and after mixing of $(rC)_n \cdot (dG)_n$ or $(rA)_n \cdot (dT)_n$ were considerable. The purine homopolymer components of these two duplexes, $(dG)_n$ and $(rA)_n$, showed large difference CD spectra, whereas those of the pyrimidine homopolymer components, $(rC)_n$ and $(dT)_n$, were almost negligible.

Table IV presents the denaturation temperatures of $(rA)_n \cdot (dT)_n$ in the presence or absence of various tannins. Although the stability of these duplexes was not remarkably influenced by the presence of tannins, a slight decrease of Tm of $(rA)_n \cdot (dT)_n$ in the presence of either agrimoniin or nobotanin A was observed.

Figure III shows the changes in the CD spectrum of $(rA)_n \cdot (dT)_n$ at increasing concentration of agrimoniin. The binding curve monitored in terms of the CD band at 275 nm (Fig. 3 insert) shows a break point at about $r = 0.1$, suggesting that the interaction of tannin and nucleotide complex reached an equilibrium state at approximately 1 : 10 molar ratio.

In a comparison of either $(rA)_n \cdot (dT)_n$ with $(dA)_n \cdot (dT)_n$ or $(rA)_n \cdot (rU)_n$ with $(dA)_n \cdot (rU)_n$, larger interaction difference CD were found in deoxyribonucleic acid (DNA)–RNA hetero-

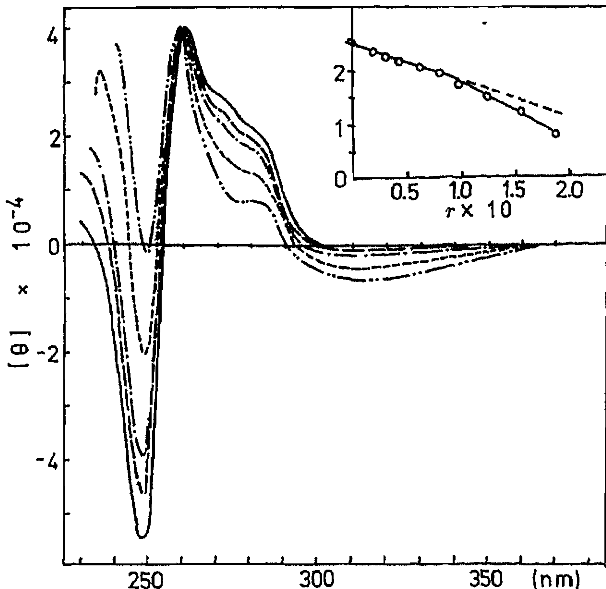

Fig. 3. Titration of $(rA)_n \cdot (dT)_n$ with Agrimoniin

——, $r=0$; ----, $r=0.03$, ---·-, $r=0.06$; ······, $r=0.125$; -··-··-, $r=0.19$ (r = molar ratio of agrimoniin/nucleotide-P).
Insert: Ellipticities at 275 nm *versus* r.

duplexes, $(rA)_n \cdot (dT)_n$ or $(dA)_n \cdot (rU)_n$. These duplexes were thermally less stable and more flexible than the corresponding ribo-, or deoxyribo-duplexes.[3] This property is appropriate for an anti-reverse transcriptase agent because the enzyme works on DNA–RNA duplexes in infected cells.

Tannins can bind to single strand nucleotides, $(dG)_n$ and $(rA)_n$. This suggests that tannins interact with nucleotides by hydrogen bonding between hydroxy groups of tannins and functional groups of the nucleotide sugar–phosphate backbone. Dimeric ellagitannins, bulky molecules with larger numbers of hydroxy groups, are unlikely to intercalate between the base pairs. Therefore, the mode of interaction with double-stranded nucleic acids may be similar.

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Studies on Crude Drugs Effective on Visceral Larva Migrans. I. Identification of Larvicidal Principles in Betel Nuts

FUMIYUKI KIUCHI,[a] NAOSHI MIYASHITA,[a] YOSHISUKE TSUDA,*[,a] KAORU KONDO,[b] and HIROYUKI YOSHIMURA[b]

Faculty of Pharmaceutical Sciences, Kanazawa University,[a] and School of Medicine, Kanazawa University,[b] 13–1 Takara-machi, Kanazawa 920, Japan

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Larvicidal principles obtained from the 50% aqueous acetone extract of betel nuts (*Areca catechu*) were revealed to be a mixture of fatty acids. They were identified by GC-MS analysis as lauric, myristic, palmitic, and oleic acids. Determination of the larvicidal activities of fatty acids and aliphatic alcohols with various alkyl chain lengths, and of analogous compounds with various functional groups, suggested that the balance between hydrophilicity and hydrophobicity is important for the larvicidal activity.

Keywords——anthelmintic; *Areca catechu*; fatty acid; tannin; aliphatic alcohol; betel chewing; *Toxocara canis*; larva; larvicide

There are many anthelmintics effective against parasites living in the gastrointestinal tract.[1] However, very few are known to be effective against parasitic diseases caused by nematodes parasitizing tissues. This presents a problem, since reports on the occurrence of visceral larva migrans, in which pathogenic larvae of a nematode migrate through blood vessels to the liver, lung, eyes, brain or muscles, and cause persistent hypereosinophilia together with hepatomegaly or pneumonitis,[2] have been increasing in recent years. In most cases, the pathogenic helminth was derived from dogs or cats kept as pets. No anthelmintic is known to be certainly effective against this disease. With the aim of finding a new anthelmintic effective against parasites living in tissues, we started *in vitro* screening tests of medicinal drugs used in traditional medicines as larvicides against the larva of dog round worm, *Toxocara canis*, which is a common pathogenic parasite in visceral larva migrans. This larva is useful for bioassay because it is highly resistant to drugs and can be kept in synthetic medium for several months.[3] In this report, we describe the identification of larvicidal principles from betel nuts, edible endosperm of the palm, *Areca catechu* L. (檳榔子). In South East Asia, many people chew this nut with betel leaves as a luxury. The anthelmintic effect of this nut is interesting from the viewpoint of prevention of parasitic diseases.

Materials and Methods

Melting points were taken on a Yanagimoto micro hot-stage melting point apparatus, and are uncorrected. Infrared (IR) spectra were taken on a JASCO A-202 spectrometer and are given in cm^{-1}. Proton nuclear magnetic resonance (^1H-NMR) spectra were measured in $CDCl_3$ solution with tetramethylsilane as an internal standard on a JEOL FX-100 spectrometer. Mass spectra (MS) and gas liquid chromatography-mass spectra (GC-MS) were taken on a Hitachi M-80 machine. Gas liquid chromatography (GLC) analyses were performed on a Shimadzu GC4CM-PF instrument with an FID detector using a 1.5% OV-1 glass column. Fuji-Davison BW-820 MH (silica gel) was used for column chromatography. For thin layer chromatography (TLC), Macherey-Nagel SIL G-25 UV_{254} plates were used and spots were observed by spraying 1% ceric sulfate in 10% H_2SO_4 followed by heating until coloration appeared.

Chemicals——All chemicals were purchased from Nakarai Chemicals Ltd. unless otherwise mentioned. 1-

Decanamide[4] and 1-decanonitrile[5] were prepared according to the cited methods. Decylamine was prepared by the reduction of decanamide with $NaBH_4$ in the presence of ethanedithiol.[6]

5-Nonyltetrazole[7]——1-Decanonitrile (940 mg), NaN_3 (1.78 g), and NH_4Cl (490 mg) were dissolved in dimethylformamide (DMF, 10 ml) and kept at 140 °C for 12 h. The reaction mixture was diluted with ethyl acetate (AcOEt), washed with 1 N HCl, then extracted with 5% $NaHCO_3$. The aqueous layer was acidified with HCl and extracted with AcOEt. The extract was washed with brine, dried over Na_2SO_4, and concentrated. The residue was recrystallized from hexane to give colorless platelets (437 mg, 36%), mp 57—58 °C. *Anal.* Calcd for $C_{10}H_{20}N_4$: C, 61.18; H, 10.27; N, 28.55. Found: C, 61.22; H, 10.37; N, 28.45.

Test Solution——Test samples were dissolved in 0.7% saline or, in the case of samples not readily soluble in water, dissolved in dimethylsulfoxide (DMSO) and diluted with saline. DMSO showed no effect on the assay up to the concentration of 2% (v/v).

Assay Method——The second stage larvae of dog round worm (*Toxocara canis*) were collected by the method previously described[8] and kept in Eagle-MEN medium (Nissui Pharmaceutical Co.) at 37 °C. For one assay, 20 larvae were incubated with the test solution in a Corning cell well at 37 °C and the behavior of the larvae was observed under a microscope at 1, 3, 6, and 24 h after the start of incubation. All assays were done in duplicate. The effect of each test material was assessed according to the state of the larvae based on the criteria listed in Table I. The relative movability (RM) value was calculated from Eqs. 1 and 2, and used as a measure of the anthelmintic activity of the test material. Minimal lethal concentration (MLC) was determined as the lowest concentration with an RM value of 0 after 24 h of incubation.

Extraction and Separation——Cut betel nuts (250 g) were extracted with three 500 ml portions of 50% aqueous acetone under reflux for 3 h. After removal of the acetone under reduced pressure, the residual aqueous suspension was extracted with AcOEt to give 7.08 g of AcOEt extract (fr. B) and a residual fraction (fr. A). Fr. B, after concentration, was extracted with 100 ml of hot hexane (fr. C) and the residual fraction (fr. D) was further partitioned with $CHCl_3 : MeOH : H_2O = 3:3:2$ to give frs. E (aqueous layer) and F (organic layer). The yield and larvicidal activity of each fraction are shown in Chart 2.

Identification of Larvicidal Principles——Fr. F (110 mg) was applied to a silica gel column and eluted with AcOEt (fr. G) and MeOH (fr. H) successively. A part of fr. G (10 mg), which showed larvicidal activity, was dissolved in MeOH and methylated with ethereal diazomethane. GLC and GC-MS analyses showed the presence of lauric, myristic, palmitic, and oleic acids. GC-MS conditions: column, 1.5% OV-1, 1 m; column temperature, 100→220 °C, 5 °C/min; flow rate, 40 ml He/min; injector temperature, 235 °C. Peak 1 (methyl laurate), t_R: 5.3 min. MS *m/z* (%): 214 (M^+, 2), 183 (2), 171 (3), 143 (3), 129 (2), 87 (18), 74 (100). Peak 2 (methyl myristate), t_R: 10.5 min. MS *m/z* (%): 242 (M^+, 3), 211 (2), 199 (3), 143 (4), 129 (2), 87 (20), 74 (100). Peak 3 (methyl palmitate), t_R: 15.6 min. MS *m/z* (%): 270 (M^+, 4), 239 (2), 227 (3), 149 (2), 143 (4), 135 (2), 87 (17), 74 (100). Peak 4 (methyl oleate), t_R: 19.4 min. MS *m/z* (%): 296 (M^+, 1), 294 (1), 264 (2), 222 (1), 180 (1), 123 (2), 110 (2), 96 (4), 81 (100).

Results and Discussion

Extraction of Larvicidal Principles from Betel Nuts

In the screening test for larvicidal activity of crude drugs used for the treatment of parasitic diseases in traditional medicines, a hot water extract of betel nuts showed only weak anthelmintic activity (RM = 52, 10 mg/ml, 24 h incubation).[9] However, the 50% aqueous acetone extract showed strong larvicidal activity with a "burst" effect on dog round worm

TABLE I. Criteria for Evaluating Effect on Larvae

State	Score (n)
Moving with whole body	3
Moving with only a part of body in the observation period	2
Immobile but not dead	1
Dead	0

$$\text{movability index (MI)} = \frac{\sum nN_n}{\sum N_n} \quad (1)$$

N_n: number of larvae with the score of n

$$\text{relative movability (RM)} = \frac{\text{MI}_{\text{sample}}}{\text{MI}_{\text{control}}} \times 100 \quad (2)$$

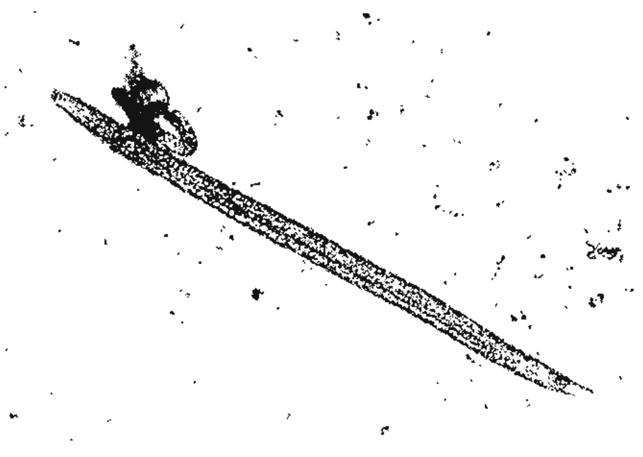

Fig. 1. The "Burst" of a Larva of *T. canis* (*ca.* 400 μm Length) Caused by the 50% Aqueous Acetone Extract of *A. catechu*

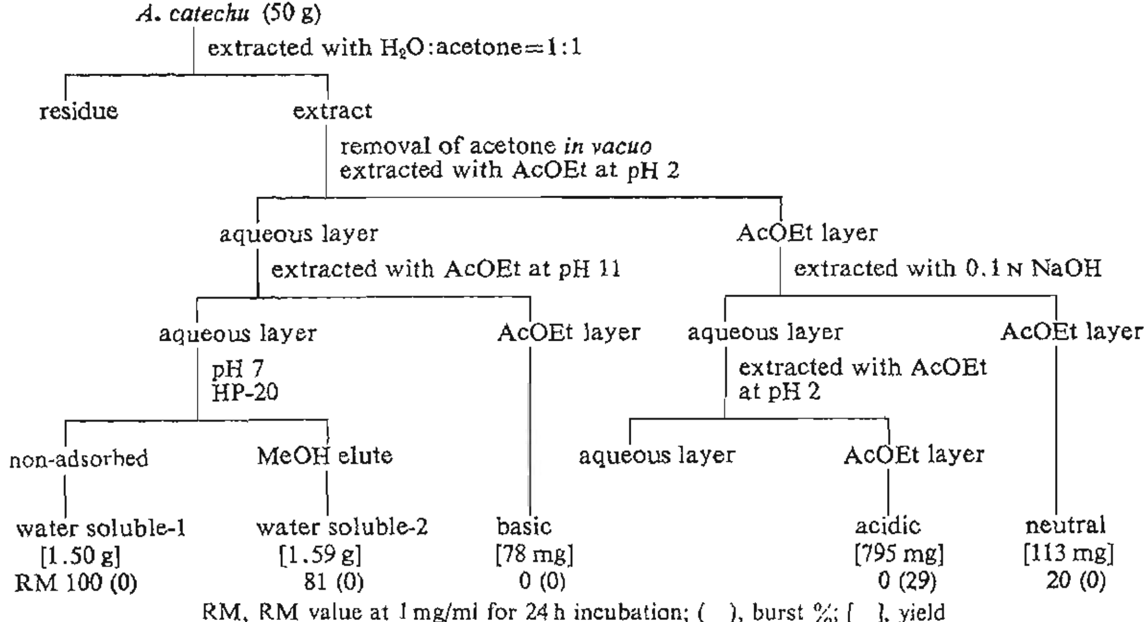

Chart 1. Preliminary Test

larvae (Fig. 1). In a preliminary experiment to identify the larvicidal principles, the 50% acetone extract was fractionated into acidic, basic, neutral, and water-soluble fractions in the usual manner. The water-soluble fraction was passed through a Diaion HP-20 column and eluted with MeOH to give the non-adsorbed and adsorbed fractions (Chart 1). Among these five fractions, the basic and acidic fractions showed larvicidal activity at a concentration of 1 mg/ml, but the "burst" was observed only in the acidic fraction.

As regards the anthelmintic principle in the basic fraction, arecoline is known to be one of the major constituents of betel nuts.[10] In fact, the larvicidal activity of arecoline was RM=2 (at 24 h incubation) at a concentration of 0.5 mg/ml, though its hydrobromide showed a paralyzing effect with only a very weak killing activity (RM=34) on larvae even at a concentration of 1 mg/ml. However, the quantity of the basic fraction was too small to be responsible for the larvicidal activity of betel nuts. Therefore, based on the quantities of the fractions, we concluded that the major constituent(s) responsible for the larvicidal activity of betel nuts is present in the acidic fraction.

Identification of Larvicidal Principles

To identify the larvicidal principles in the acidic fraction, 50% aqueous acetone extract of

betel nuts was fractionated into eight fractions as shown in Chart 2 (see Materials and Methods). Fr. B showed larvicidal and "burst" activity, and was further divided into frs. C and D. Both fractions showed larvicidal activity, whereas only the latter showed "burst" activity. Next, fr. D was fractionated into lipophilic and hydrophilic fractions (frs. F and E). At this stage, only fr. F showed larvicidal activity and fr. E did not show killing activity at a concentration of 1 mg/ml. The "burst" activity seemed to have disappeared. However, when the same amounts of frs. E and F were combined, the "burst" activity was restored. This result suggested that there were at least two substances taking part in the larvicidal and "burst" effects, *i.e.* compound(s) with only larvicidal activity and compound(s) without larvicidal activity but causing a "burst" in combination with the larvicidal compound(s). The fraction causing the "burst" (fr. E) was rich in tannins. Therefore, some tannins purified from *A. catechu*[11] were assayed for the "burst" effect and some of them were revealed to cause the "burst" in combination with capric (decanoic) acid. Details of the "burst" effect of tannins will be reported elsewhere.

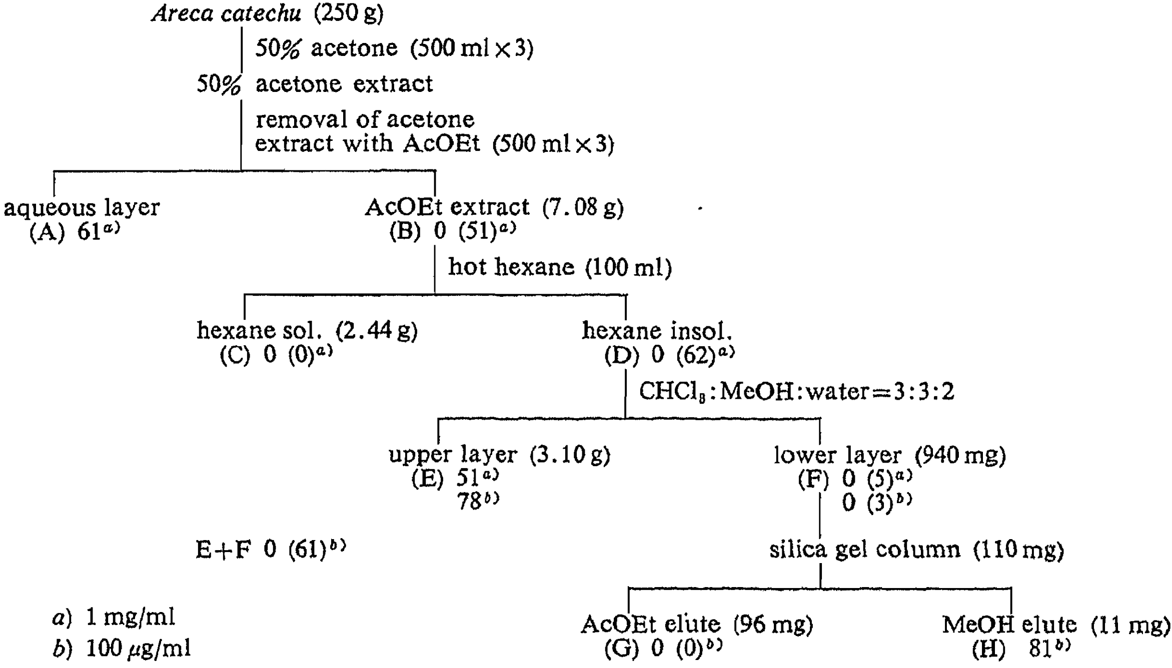

Chart 2. Purification of Larvicidal Principles of *A. catechu*

For the identification of the constituents responsible for the larvicidal activity, fr. F was subjected to silica gel column chromatography, and the activity was found to be eluted by AcOEt (fr. G). From the ^{1}H-NMR and MS, the major constituent of this fraction was suggested to be a mixture of fatty acids, so this fraction was treated with diazomethane and subjected to GLC and GC-MS analyses (Fig. 2). GC-MS analysis revealed that this fraction consisted of lauric, myristic, palmitic, and oleic acids in the ratio shown in Fig. 2. The other active fraction (fr. C) was also treated with diazomethane and analyzed by GLC (Fig. 2), and the constituents were found to be very similar to those of fr. G.

Larvicidal Activity of Fatty Acids and Related Compounds

The major constituents of the larvicidal fractions in betel nuts were found to be fatty acids, so the MLC of straight chain fatty acids of various chain lengths were determined. Interestingly, as shown in Fig. 3, the larvicidal activity of fatty acids depended markedly on the chain length. Fatty acids shorter than heptanoic acid were inactive up to the concentration of

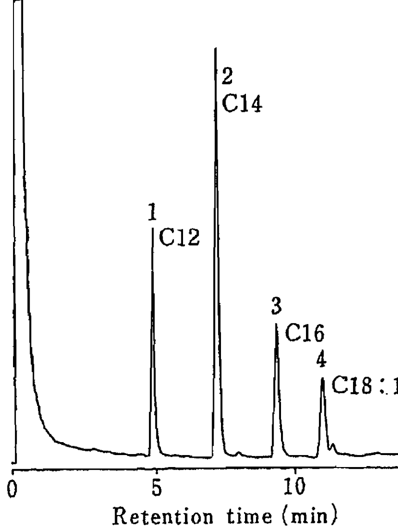

Fig. 2. GLC Analyses of the Active Fractions after Treatment with Diazomethane

Column, 1.5% OV-1, 1 m; column temp., 100→220 °C (10 °C/min); injector temp., 250 °C; flow rate, 60 ml N_2/min.

Peak	Retention time (min)	Relative intensity (%) Fr. C	Fr. G	Identification
1	4.9	25	29	Methyl laurate
2	7.2	45	37	Methyl myristate
3	9.4	16	29	Methyl palmitate
4	11.1	14	24	Methyl oleate

1 mM. Between C_8 and C_{12}, larvicidal activity increased gradually as the alkyl chain length increased, and the strongest activity was obtained with lauric (dodecanoic) acid (MLC 150 μM). However, the activity decreased rapidly above C_{13}. Hexadecanoic and octadecanoic acids were inactive, though they are hardly soluble in water and had to be assayed in suspension. Such a "cut-off" of activity has been observed in several biological activities of analogous compounds.[12] The phenonenon is related to the partition coefficient of the compounds and can be explained by models in which the concentration of the compound at the active site is governed by the partition coefficient of the compound.[13]

Next, straight chain aliphatic alcohols, which have a neutral functional group at the end of the alkyl chain, were tested for larvicidal activity (Fig. 3). An analogous relationship between chain length and MLC was obtained, but in this case, tetradecyl alcohol showed the strongest activity (MLC 20 μM, about one-tenth of that of dodecanoic acid). A similar action curve was reported for the antimicrobial activity of aliphatic alcohols against *Clostridium botulinum*[14] and *Streptococcus mutans*.[15] However, to our knowledge, this is the first report on the anthelmintic activity of fatty acids and aliphatic alcohols against larva of dog round worm.

Since fatty acids and alcohols with long alkyl chains showed strong larvicidal activity, some analogous compounds were also tested for larvicidal activity (Table II). Methyl decanoate and decanonitrile, which have relatively hydrophobic groups at the end of the alkyl chain, were inactive. On the other hand, decylamine, and nonyltetrazole, with hydrophilic groups, were as larvicidal as the acid and the alcohol. The sodium salt of decanoic acid was inactive. Therefore, some balance between the hydrophobicity (length of hydrophobic alkyl chain) and the hydrophilicity of the functional group seems to be essential for the larvicidal activity.

In the South Asia, betel chewing is a common habit. Betel nuts contain large amounts of

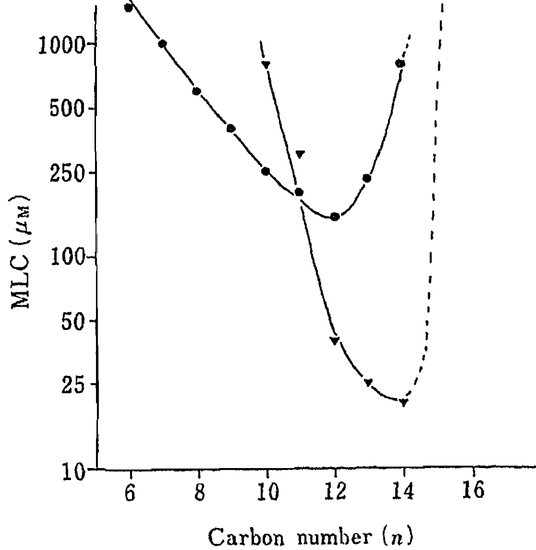

Fig. 3. Minimal Lethal Concentration of Fatty Acids and Aliphatic Alcohols

—●—, $CH_3(CH_2)_{n-2}COOH$;
—▼—, $CH_3(CH_2)_{n-1}OH$.

TABLE II. Larvicidal Activity of Decanoic Acid Analogues (RM Value)

Sample	Conc.	1 h	3 h	6 h	24 h
Decanoic acid	500 μM	0	0	0	0
	250 μM	71	9	1	0
	125 μM	96	94	84	82
Sodium decanoate	1 mM	99	99	81	78
Methyl decanoate	1 mM	94	100	95	87
Decanonitrile	1 mM	85	102	102	100
Decanamide	1 mM	101	101	102	61
Decylamine	500 μM	34	0	0	0
	320 μM	33	54	74	72
Decyl alcohol	1 mM	35	0	0	0
Nonyltetrazole	500 μM	40	0	0	0
	250 μM	53	7	0	0
	125 μM	95	81	59	10

fatty acids and tannins.[10] In our *in vitro* assay system, the combination of these compounds showed strong larvicidal activity against larvae of dog round worm. These facts suggested that the widespread habit of betel chewing in the South East Asia may be effective in the prevention of parasitic diseases.

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Selective Measurement of Hepatic Triglyceride Lipase and Lipoprotein Lipase in Rat Postheparin Plasma with Pentachlorophenol and Dichlorodiphenyltrichloroethane

HIROYUKI KANEKI, MIZUO NAKAUCHI and MITSUYA TANAKA*

Department of Hygienic Chemistry, School of Pharmaceutical Science, Toho University, Miyama 2-2-1, Funabashi-shi, Chiba 274, Japan

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Postheparin plasma lipolytic activity consists of hepatic triglyceride lipase (H-TGL) and lipoprotein lipase (LPL) activities. The effect of chlorinated pesticides, pentachlorophenol (PCP) and 1,1,1-trichloro-2,2-bis-(4-chlorophenyl)ethane (DDT), on these lipase activities in rat postheparin plasma was examined. PCP completely inactivated H-TGL but did not change LPL activity. On the other hand, DDT completely inactivated LPL but did not change H-TGL activity. By utilizing these specific inhibitions, a procedure for selective measurement of H-TGL and LPL in postheparin plasma of young and old rats, with and without diabetes, was developed. The values of H-TGL and LPL activity obtained by this method were in good agreement with those obtained by immunochemical methods.

Keywords——selective lipase measurement; lipoprotein lipase; hepatic triglyceride lipase; rat; postheparin plasma; pentachlorophenol; DDT; immunochemical lipase assay; lipase; inhibition

The hydrolysis of plasma lipoprotein is mediated by two different lipases, hepatic triglyceride lipase (H-TGL) and (extrahepatic) lipoprotein lipase (LPL), which are released into the blood stream upon intravenous administration of heparin.[1] Measurement of the two lipase activities appears to be important in determining the possible pathogenesis of lipid disorders.[2] H-TGL activity is often quite low in patients with uremia,[2a] estrogen treatment,[2b] or parenchymal liver disease.[2c] The activity of LPL is decreased in patients with exogenous familial hypertriglyceridemia.[2b]

Several methods have been described for the selective determination of H-TGL and LPL activities in postheparin plasma.[3] Krauss *et al.* described a method utilizing the inhibition of LPL activity by protamine sulfate or sodium chloride in rat[3a] and human[3b] postheparin plasma. This method, however, involves incomplete inactivation of LPL and incomplete survival of H-TGL.[3a] Baginsky and Brown described a method utilizing the specific inhibition of H-TGL activity by sodium dodecyl sulfate.[3c] An immunochemical method based on the use of an antibody for H-TGL[3d] or LPL[3e] and a column method based on affinity chromatography on heparin-Sepharose[3f] permit specific measurement of H-TGL and LPL in postheparin plasma. However, specific antisera for the immunochemical method are not readily available and the column method is laborious and involves a significant loss of lipase activities during chromatography.[3c]

This paper shows that the use of pentachlorophenol (PCP) and 1,1,1-trichloro-2,2-bis-(4-chlorophenyl)ethane (DDT) as selective inhibitors of H-TGL and LPL, respectively, in normal and diabetic rat postheparin plasma provides a rapid method that is comparable to the immunochemical method in specificity.

Materials and Methods

Materials——Male Wistar rats weighing between 150 and 520 g and Japanese albino rabbits weighing *ca.* 2.5 kg were purchased from Nippon Biosupp. Center Co., Ltd. and fasted for 18 h prior to the experiments. Diabetic rats were prepared by the intravenous administration of 65 mg of streptozotocin (Sigma Chemical Co., Ltd.) per kg body weight by the method of Junod *et al.*[4] Diabetes was determined by the measurement of rat serum glucose with *o*-toluidine.[4] Essentially fatty acid-free bovine serum albumin and heparin sodium salt from porcine intestinal mucosa were from Sigma Chemical Co., Ltd. NEFA-test Wako, which is a diagnostic kit for the colorimetric determination of free fatty acid in serum, and chlorinated pesticides, DDT and PCP, were purchased from Wako Pure Chemical Industries Co., Ltd. Heparin-Sepharose CL-6B was purchased from Pharmacia Fine Chemicals Co., Ltd. Intralipid (10% soybean oil emulsion containing 1.2% egg phosphatidylcholine and 2.5% glycerol) was a product from Kabi-Vitrum AB Co., Ltd. All the other materials used were special-grade reagents purchased from Nakarai Chemicals Co., Ltd.

Preparation of Rat Postheparin Plasma——Rat postheparin plasma samples were obtained exactly 10 min after the intravenous administration of 10 units of heparin sodium salt per 100 g of body weight under ether anesthesia according to the method of Murase *et al.*[5] The lipase activity in plasma was then assayed immediately. One milliliter of rat plasma contained *ca.* 60 mg of protein, 1.82—2.86 units of H-TGL and 0.97—1.63 units of LPL.

Preparation of H-TGL and LPL——Separation of H-TGL and LPL was performed by the method of Murase *et al.*[5] Rat postheparin plasma was applied to a column of heparin-Sepharose CL-6B equilibrated with 5 mM sodium barbital-HCl buffer (pH 7.4) containing 0.4 M sodium chloride. H-TGL was eluted with the same buffer containing 0.75 M sodium chloride and LPL was eluted with the same buffer containing 1.5 M sodium chloride. H-TGL (6.62 units per mg protein) and LPL (6.63 units per mg protein) fractions were desalted by ultrafiltration, lyophilized and stored at −30 °C.

Preparation of Antibodies to H-TGL and LPL——Antibody to H-TGL or LPL was prepared by the method described by Murase *et al.*[5] for rat anti-H-TGL serum. The emulsion (1.5 ml) containing 1—2 mg of partially purified H-TGL or LPL and Freund's complete adjuvant (Difco Co., Ltd.) was injected subcutaneously into rabbits. After two weeks, and then at two-week intervals, the same procedure was repeated with each animal. The titer was checked 10 d after each injection by precipitation of H-TGL or LPL. One unit of partially purified H-TGL was completely inhibited by the addition of 2.7 mg of anti-H-TGL serum, whereas the activity of partially purified LPL was unaffected by the addition of anti-H-TGL serum up to 16.5 mg. One unit of partially purified LPL was completely inhibited by the addition of 4.0 mg of anti-LPL serum, whereas the activity of partially purified H-TGL was unaffected by the addition of anti-LPL serum up to 21 mg. Antisera were lyophilized and stored at −30 °C.

Assay of Lipase Activity——Measurement of lipase activities was performed according to the method of Bolzano *et al.*[6] Five milliliters of intralipid was preincubated with 5 ml of rat "preheparin" serum at 37 °C for 30 min. This mixture was incubated with 10 ml of 0.3 M Tris-HCl buffer (pH 8.0) and 10 ml of 20% bovine serum albumin at 37 °C for 10 min to make an activated substrate. The enzyme reaction was initiated by the addition of 0.2 ml of enzyme solution to 0.5 ml of the activated substrate at 37 °C at pH 8.0. The liberated free fatty acid was colorimetrically measured at 480 nm by using NEFA-test Wako, essentially according to Duncombe.[7] Time courses were completely linear under the experimental conditions. The units of enzyme activity refer to micromols of free fatty acid liberated per h under the above experimental condition.

Selective Measurement with Pesticide——One-hundredth milliliter of 0.8 mM pesticide acetone solution was added to a mixture of rat postheparin plasma (0.4 ml) and 0.15 M Tris-HCl buffer (0.4 ml, pH 8.0). After preincubation at 37 °C for 1 h, the mixture was centrifuged and the supernatant was assayed for lipase activity. A control experiment was carried out in exactly the same way except that acetone was used instead of pesticide acetone solution. The addition of acetone did not affect the enzyme activity within the limit of experimental error.

Selective Measurement with Antibody——An antiserum for H-TGL (4.0 mg protein, 0.2 ml) or LPL (6.0 mg protein, 0.2 ml) was added to a mixture of rat postheparin plasma (0.4 ml) and 0.3 M Tris-HCl buffer (0.2 ml, pH 8.0). After incubation at 4 °C for 1 h, the mixture was centrifuged and the supernatant was assayed for lipase activity. A control experiment was carried out in exactly the same way except that the normal serum was used instead of the antiserum.

Results

Effect of Anti-H-TGL or Anti-LPL Serum on Lipase Activity

Subcutaneous injection of partially purified H-TGL or LPL protein into rabbits resulted in the production of antiserum against H-TGL or LPL as described in Materials and Methods. The specificity of antisera against H-TGL and LPL in postheparin plasma was examined according to the method described by Baginsky and Brown[3c] for an antisera–

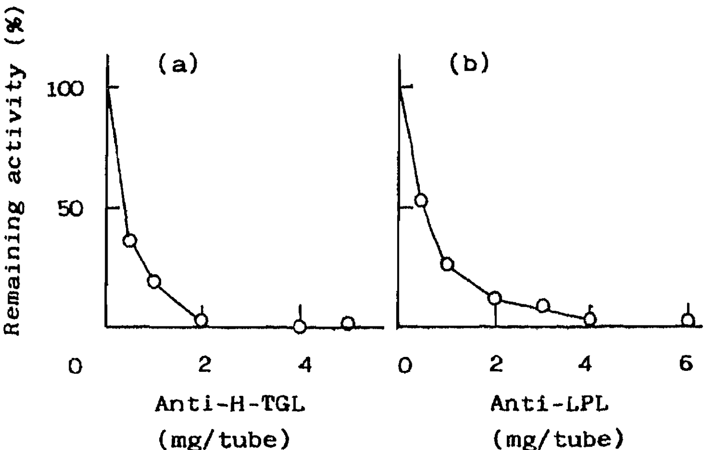

Fig. 1. Effect of Anti-H-TGL or Anti-LPL Serum on the Lipase Activities in Postheparin Plasma

(a) Postheparin plasma (0.4 ml) was preincubated with anti-LPL (6.0 mg) to remove LPL activity. The preincubated mixture was centrifuged and the supernatant, in which only H-TGL activity remained, was preincubated with various amounts of anti-H-TGL. After centrifugation, the remaining activity in the supernatant was measured.

(b) Postheparin plasma (0.4 ml) was preincubated with anti-H-TGL (4.0 mg) to remove H-TGL activity. The preincubated mixture was centrifuged and the supernatant, in which only LPL activity remained, was preincubated with various amounts of anti-LPL. After centrifugation, the remaining activity in the supernatant was measured.

sodium dodecyl sulfate system in human postheparin plasma.

As shown in Fig. 1a, the lipase (H-TGL) activity after removal of LPL from postheparin plasma with anti-LPL serum decreased with increasing amount of anti-H-TGL serum added and was completely suppressed by the addition of 2 mg of anti-H-TGL per tube. Similarly, as shown in Fig. 1b, the lipase (LPL) activity after removal of H-TGL with anti-H-TGL serum decreased with increasing amount of anti-LPL serum added and was completely suppressed by the addition of 4 mg of anti-LPL serum per tube.

Effect of Pesticides on the Lipase Activities

The effect of the concentration of PCP or DDT on the activities of partially purified H-TGL and LPL from rat postheparin plasma was examined, with various times of preincubation of H-TGL or LPL with pesticide.

As shown in Fig. 2a, the activity of H-TGL decreased with increasing concentration of PCP and the degree of decline of activity was found to be dependent on the time of preincubation with PCP. The concentrations of PCP required for complete inhibition were 0.6 μM when preincubated for 60 min, 0.9 μM for 30 min and 5 μM for 10 min. The result of preincubation for 120 min was essentially the same as that of preincubation for 60 min (data not shown). In contrast with H-TGL activity, LPL activity was not changed by the preincubation with PCP for 60 min. No effect of PCP on LPL activity was observed on prolonged preincubation even for 180 min (data not shown).

As shown in Fig. 2b, the activity of LPL decreased with increasing concentration of DDT. The inhibition was dependent on the time of preincubation with DDT. The concentrations of DDT required for complete inhibition were 0.4 μM when preincubated for 60 min and 5 μM for 30 min. The result of preincubation for 120 min was essentially the same as that of preincubation for 60 min (data not shown). Preincubation for 10 min, however, did not cause complete inhibition. On the other hand, H-TGL activity was not changed by prolonged preincubation with 10 μM DDT even for 180 min (data not shown).

The specificity of PCP or DDT against H-TGL and LPL activities in postheparin plasma

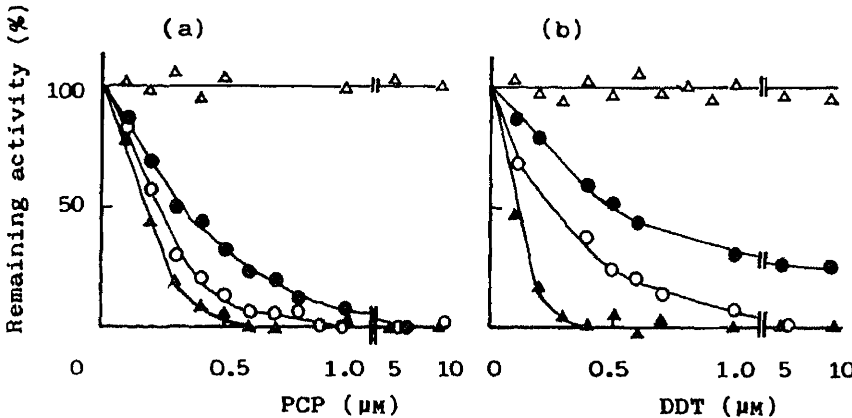

Fig. 2. Effect of Concentration of PCP or DDT and the Time of Preincubation on the Activities of Partially Purified H-TGL and LPL

(a) Preincubation of lipase at various concentrations of PCP for a given time. H-TGL activity (1.88 units): ●, 10 min; ○, 30 min; ▲, 60 min. LPL activity (1.28 units): △, 60 min.
(b) Preincubation of lipase at various concentrations of DDT for a given time. LPL activity: ●, 10 min; ○, 30 min; ▲, 60 min. H-TGL activity: △, 60 min.

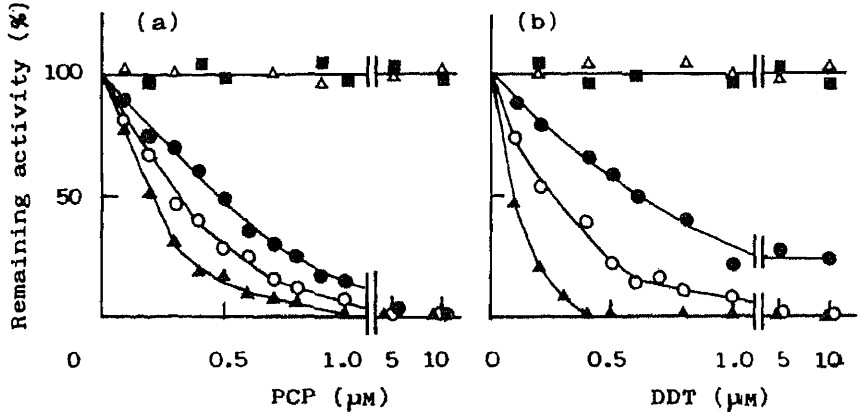

Fig. 3. Effect of PCP and DDT on the Lipase Activities in Postheparin Plasma

Postheparin plasma (0.4 ml) was preincubated with anti-LPL (6.0 mg) to remove LPL activity or with anti-H-TGL (4.0 mg) to remove H-TGL activity. The preincubated mixture was centrifuged.
(a) The supernatant, in which only H-TGL activity remained, was preincubated at various concentrations of PCP for a given time: ●, 10 min; ○, 30 min; ▲, 60 min. The supernatant, in which only LPL activity remained, was preincubated at various concentrations of PCP for a given time: △, 60 min; ■, 120 min.
(b) The supernatant, in which only LPL activity remained, was preincubated at various concentrations of DDT for a given time: ●, 10 min; ○, 30 min; ▲, 60 min. The supernatant, in which only H-TGL activity remained, was preincubated at various concentrations of DDT for a given time: △, 60 min; ■, 120 min.

was confirmed according to the method of Baginsky and Brown.[3c)]

As shown in Fig. 3a, the lipase activity after removal of LPL from postheparin plasma with anti-LPL serum was completely inhibited by PCP. The required concentrations of PCP were 1.0 μM when preincubated for 60 min, 5.0 μM for 30 min and 5.0 μM for 10 min. Complete inhibition of H-TGL in postheparin plasma required a higher concentration of PCP than in the case of partially purified H-TGL. On the other hand, the lipase activity after removal of H-TGL from postheparin plasma with anti-H-TGL serum was not changed by prolonged preincubation with PCP even for 120 min.

As shown in Fig. 3b, the lipase activity after removal of H-TGL from postheparin plasma

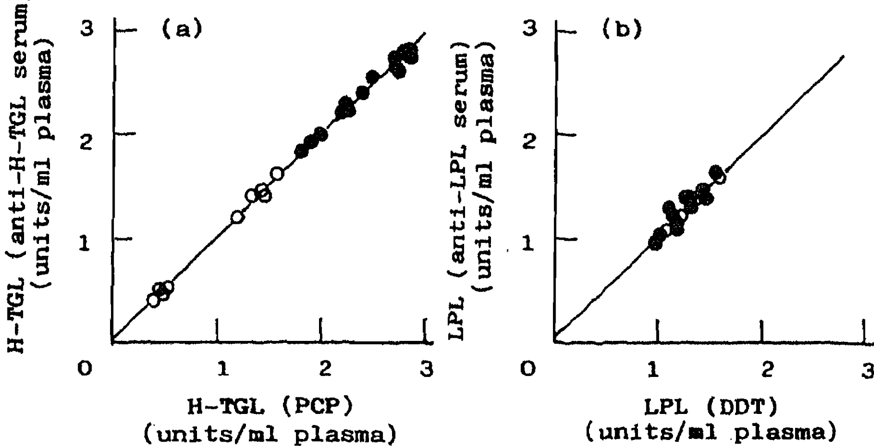

Fig. 4. Correlation of Lipase Activities in Rat Postheparin Plasma Determined by the Selective Inhibition Method with Pesticide and by the Immunochemical Method with Antiserum

(a) H-TGL activity. (b) LPL activity. ○, diabetes; ●, normal.

TABLE I. Comparison of the Lipase Activities in Postheparin Plasma of Normal and Diabetic Rats Determined by the Selective Inhibition Method with Pesticide and by the Immunochemical Method with Antiserum

Subject[a]	Age (weeks)	Serum[b] glucose (mg/dl)	Total activity (units/ml)	H-TGL activity (units/ml)		LPL activity (units/ml)	
				PCP	Anti-H-TGL	DDT	Anti-LPL
1	5	248	3.88	1.82	1.84	—	—
2	5	240	3.55	1.91	1.93	—	—
3	5	217	4.09	2.00	2.00	—	—
4	8	197	3.96	2.49	2.57	1.11	1.32
5	8	220	4.02	2.72	2.76	1.05	1.07
6	8	235	3.50	2.20	2.22	1.20	1.14
7	8	185	3.40	2.23	2.30	1.29	1.41
8	8	215	3.80	2.26	2.26	1.34	1.29
9	10	228	3.50	2.20	2.22	1.20	1.22
10	10	253	3.72	2.40	2.41	1.34	1.42
11	10	227	4.20	2.73	2.65	1.46	1.44
12	10	264	4.20	2.80	2.80	1.47	1.40
13	10	199	4.39	2.86	2.77	1.58	1.63
14	13	230	3.43	2.40	2.41	0.98	0.97
15	13	222	3.86	2.73	2.64	1.20	1.13
16	13	209	4.19	2.80	2.80	1.34	1.29
17	13	219	4.30	2.86	2.77	1.47	1.40
18	13	188	4.26	—	—	1.46	1.44
19	13	268	4.58	—	—	1.58	1.63
20	10	722	2.35	1.20	1.22	1.10	1.08
21	10	684	2.40	1.34	1.42	1.10	1.08
22	10	589	2.71	1.46	1.44	1.23	1.22
23	10	643	2.80	1.47	1.40	1.39	1.40
24	10	698	3.07	1.58	1.63	1.60	1.61
25	13	716	2.04	0.42	0.42	—	—
26	13	634	2.02	0.49	0.46	—	—
27	13	758	2.22	0.48	0.51	—	—
28	13	589	2.15	0.54	0.53	—	—

a) No. 1—19, normal rats; No. 20—28, streptozotocin-diabetic rats. *b*) Mean plasma glucose levels in normal and diabetic rats were 224±23.5 and 670±59.9 mg/dl, respectively.

with anti-H-TGL serum was completely inhibited by DDT. The required concentrations of DDT were 0.4 μM when preincubated for 60 min and 5 μM for 30 min. Preincubation for 10 min, however, did not cause complete inhibition. The lipase activity after removal of LPL from postheparin plasma with anti-LPL serum was not changed by prolonged preincubation with DDT even for 120 min.

Preincubation of postheparin plasma with a mixture of PCP and DDT (each 10 μM) for 60 min produced complete inactivation (data not shown).

From these results, it was concluded that preincubation with 10 μM PCP or DDT for 60 min is satisfactory for selective activity measurement.

Correlation of Lipase Activities Found by the Present Method and the Immunochemical Method

H-TGL and LPL activities in postheparin plasma of normal and diabetic rats were measured both by the present selective inhibition method and by the immunochemical method.

As shown in Table I, the H-TGL activity determined by the immunochemical method using anti-H-TGL serum and the activity determined by the present method using PCP were in good agreement for each rat. As shown in Fig. 4a, a good correlation was observed (correlation coefficient, 0.998; slope, 0.987). The H-TGL activity of diabetic rats was quite low, in agreement with the report by Nakai *et al.*[8]

As also shown in Table I, the LPL activity determined by the immunochemical method using anti-LPL serum and the activity determined by the present method using DDT were in good agreement for each rat. As shown in Fig. 4b, a good correlation was observed (correlation coefficient, 0.933; slope, 0.966).

Discussion

We had found that lipase activities from various mammalian liver acetone–ether powders were completely resistant to DDT but were strongly inhibited by PCP, in contrast to lipoprotein lipase activities from *Pseudomonas* and *Alcaligenes* that were completely inhibited by DDT but were unaffected by PCP (unpublished data). These results suggested the possibility of selective measurement of H-TGL and LPL in postheparin plasma by using PCP or DDT as a selective inhibitor, although the LPL preparations used were not mammalian enzymes. In the present study, the selective measurement of H-TGL and LPL by using pesticide was found to be successful with rat postheparin plasma. H-TGL activity from rat postheparin plasma was completely inhibited by PCP but was unaffected by DDT. LPL activity was completely inhibited by DDT but not by PCP.

The values of H-TGL and LPL activity in rat postheparin plasma obtained by using PCP or DDT were in good agreement with those obtained by the immunochemical method. The method described herein should allow the rapid determination of H-TGL or LPL activity in postheparin plasma of mammals other than the rat.

The enzyme activity was not recovered from an incubated mixture of H-TGL and PCP by gel filtration and dialysis, suggesting very tight or irreversible binding of the pesticide to lipase. The results of a kinetic study were not sufficient to determine the mode of inhibition with intralipid as a substrate. In a separate experiment using a triolein emulsion as a substrate, Lineweaver–Burk plots ($1/v-1/s$ plots) of the inhibition of H-TGL by PCP suggested non-competitive inhibition. A competitive inhibitor that binds irreversibly or very tightly to the enzyme would also give Lineweaver–Burk plots indicating apparent non-competitive inhibition, however.

Tsujita *et al.*[9] reported the isolation of electrophoretically homogeneous H-TGL from rat postheparin plasma, and found the molecular weight of the lipase to be 65000. Values for the

concentration of enzyme used in the experiments depicted in Figs. 2a and 3a are not far from 0.1 μM as estimated roughly on the basis of their experimental results. It is noteworthy that the inhibition of H-TGL occurred at very low concentrations of PCP, comparable to that of enzyme, as shown in Figs. 2a and 3a. This indicates that PCP binds to H-TGL very tightly and specifically even in the presence of the bulk of plasma proteins and that Lineweaver–Burk plots or Dixon plots ($1/v-[I]$ plots) to determine the mode of inhibition cannot be used here because the concentration of free inhibitor would be greatly affected by the formation of the enzyme–inhibitor complex.

The inhibition was strongly dependent on the preincubation time, and equilibrium was obtained after preincubation for 60 min. It is known that co-lipase binds tightly to porcine pancreatic lipase and then activates the lipase very slowly with a conformational change of the lipase.[10]

At present, the mode of inhibition is not known but it is likely that PCP binds to a site other than the active site and gradually inactivates the enzyme to form a very tightly bound inactive inhibitor–enzyme complex with a conformational change. DDT seems to inhibit LPL in a similar manner.

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Studies on Synthesis of 3-*O*-Alkyl-D-glucose and 3-*O*-Alkyl-D-allose Derivatives and Their Biological Activities

TETSURO IKEKAWA,*[,a] KAZUHIKO IRINODA,[a,1)] KOICHI SAZE,[a] TATSUHIKO KATORI,[b] HIDEAKI MATSUDA,[b] MASANORI OHKAWA,[c] and MARTIN KOSIK[d]

National Cancer Center Research Institute,[a] Tsukiji 5-1-1, Chuo-ku, Tokyo 104, Japan, Central Research Laboratories, SS Pharmaceutical Co., Ltd.,[b] Nampeidai 1143, Narita, Chiba 286, Japan, Faculty of Education, Kanazawa University,[c] Marunouchi 1-1, Kanazawa, Ishikawa 920, Japan, and Slovak Polytechnical University,[d] Janska 1, Bratislava, Czechoslovakia

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Twenty two 3-*O*-alkyl derivatives of D-glucose and D-allose, four 3-*O*-alkenyl derivatives of D-glucose having an end methylene group, and four 3-*O*-ω-hydroxyalkyl- or -methoxyalkyl derivatives of D-glucose were synthesized. Their cytotoxicity *in vitro* against the cultured leukemia L-5178Y cell line, antimicrobial activity and plant growth-inhibitory effect were determined.

Keywords——*O*-alkyl glucose; *O*-alkyl allose; antitumor activity; cytotoxicity; plant growth-inhibitory activity

We have studied the antitumor activity of polysaccharides isolated from *Basidiomycetes*[2)] and found the antitumor activity of polysaccharides to be associated mainly with glucans. In order to elucidate the relationship between the structure and the activity, we investigated the antitumor activity of oligosaccharides composed of D-glucose and monosaccharide derivatives.[3-5)] It was concluded from the studies on disaccharides that the *in vitro* cytotoxicity of sucrose derivatives was related to the values of hydrophile–lipophile-balance (HLB). Among trehalose-6,6′-diester derivatives the lauroyl ester had the highest activity.[3,5)] Concerning monosaccharide derivatives, we reported previously on the antitumor activity of 1-*O*-, 3-*O*- and 6-*O*-acyl-D-glucopyranoses and determined their IC_{50} values by *in vitro* bioassay using a cultured L-5178Y cell line.[4)]

In this study, we report on syntheses of 3-*O*-alkyl-D-glucose and -D-allose derivatives

RX
H⁺
3-*O*-alkyl-D-glucose
oxidation
reduction
R′X
H⁺
3-*O*-alkyl-D-allose

Chart 1. Synthesis of 3-*O*-Alkyl-D-glucose and -D-allose Derivatives

having substituents of various chain-length, and on the investigation of their *in vitro* cytotoxicity, antimicrobial activity and plant growth-inhibitory activity. Syntheses of 3-*O*-alkyl-D-glucose and -D-allose derivatives were carried out by the method shown in Chart 1. Among the compounds synthesized in this study, 3-*O*-allyl- and 3-*O*-lauryl-D-glucose and 3-*O*-alkyl-D-glucoses having an alkyl chain shorter than 4 carbon atoms, as well as 3-*O*-methyl-D-allose, have been previously prepared, but the antitumor activities have not been reported.

Materials and Methods

Physicochemical Properties——Melting points are not corrected. Measurement of infrared (IR) spectra was performed in KBr tablets with a Jasco A-102 infrared spectrophotometer. Nuclear magnetic resonance (NMR) spectra for solutions in dimethyl sulfoxide (DMSO)-d_6 or CD_3OD were measured using a Hitachi R-24B high resolution NMR spectrometer.

Preparation of 3-*O*-Alkyl-D-glucose Derivatives——DMSO (15 ml) containing 20 mmol of sodium hydride was slowly added, with stirring and cooling, to a solution of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (10 mmol) in 15 ml of DMSO. With continued cooling, the mixture was stirred for 30 min and then 20 mmol of alkyl halide (RX) was added dropwise. The reaction mixture was stirred at room temperature for 2 h, the resulting solution was poured into ice water, and the product was extracted with ether. The ethereal extract was concentrated and the crude product was purified by silica gel column chromatography using chloroform as the eluant. A solution of the resulting 3-*O*-alkyl-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (1.0 g) in water (10 ml) was refluxed in the presence of Amberlite CG-120 (H^+ type, 1.0 g) for 4—20 h. After cooling, the crude product was extracted with ether and purified by silica gel column chromatography using chloroform–methanol (10:1) as the eluant, and the 3-*O*-alkyl-D-glucopyranose was recrystallized from ethanol. Yields: $R=C_{11}H_{23}$, 28.1%; $R=C_{12}H_{25}$, 22.1%; $R=C_{13}H_{27}$, 29.2% from 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose. Melting points of the D-glucose series are: $R=CH_3$, 166—168 °C; C_2H_5, 143—145 °C; C_3H_7, 130—131 °C; C_4H_9, 139—143 °C; C_6H_{13}, 122—124 °C; C_8H_{17}, 128—130 °C; $C_{10}H_{21}$, 141—143 °C; $C_{11}H_{23}$, 137—139 °C; $C_{12}H_{25}$, 139—141 °C; $C_{13}H_{27}$, 138—140 °C; $C_{14}H_{29}$, 136—138 °C; $C_{16}H_{33}$, 134—135 °C; $C_{18}H_{37}$, 125—127 °C; $C_{20}H_{41}$, 110—113 °C; $C_{22}H_{45}$, 105—108 °C; $CH_2=CH-CH_2$, 131—132 °C; $CH_2=CH-(CH_2)_8$, 113—115 °C; $CH_2=CH-(CH_2)_9$, 134—136 °C; $CH_2=CH-(CH_2)_{10}$, 128—130 °C; $HO(CH_2)_{10}$, 108—110 °C; $HO(CH_2)_{16}$, 110—112 °C; $CH_3O(CH_2)_{10}$, 71—73 °C; $CH_3O(CH_2)_{12}$, 92—94 °C.

3-*O*-Alkyl-D-allose Derivatives[6]——1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose (10 mmol) was dissolved in 40 ml of dichloromethane and oxidized with pyridinium chlorochromate[7] (40 mmol) in the presence of 3 Å molecular sieves (10 g). The resulting mixture was extracted with dichloromethane and, after evaporation of the solvent, the 3-keto compound thus obtained was dissolved in 15 ml of ethanol–water (3:7), and reduced with sodium borohydride (0.22 g). After extraction with dichloromethane and crystallization from cyclohexane, 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose (1.35 g) was obtained. 1,2:5,6-Di-*O*-isopropylidene-α-D-allofuranose (1.30 g) was dissolved in DMSO (20 ml) and 60% sodium hydride (0.40 g) was added. The mixture was stirred at room temperature for 1 h. Then, in the case of the lauryl derivative, lauryl bromide (1.37 g) was added under continuous stirring. Stirring was continued for 2 h at room temperature, then the resulting solution was poured into ice water and extracted with ether. The ethereal solution was concentrated and 3-*O*-lauryl-1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose (1.70 g, 79%) was obtained. The foregoing compound (0.50 g) was suspended in water (15 ml), Amberlite CG-120 (H^+ type, 0.50 g) was added and the mixture was refluxed for 17 h. After cooling, the product was extracted with ether, and the ethereal phase was concentrated. The crude product was purified by elution from a silica gel column with chloroform–methanol (10:1) to give 3-*O*-lauryl-D-allopyranose (0.15 g) as colorless crystals. Yields: $R=C_{11}H_{23}$, 26.1%; $R=C_{12}H_{25}$, 29.2%; $R=C_{13}H_{27}$, 40.5% from 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose. Melting points of the D-allose series are: $R=CH_3$, 112—114 °C; C_6H_{13}, 52—54 °C; $C_{10}H_{21}$, 67—69 °C; $C_{11}H_{23}$, 69—70 °C; $C_{12}H_{25}$, 67—69 °C; $C_{13}H_{27}$, 67—68 °C; $C_{18}H_{37}$, 61—63 °C.

***In Vitro* Cytotoxic Activity**[3c]——The leukemia L-5178Y cell line maintained at this institute was cultured in RPMI-1640 medium supplemented with 10% calf serum, and the test sample was added at the desired concentration. The cells (1.0×10^5 cells/ml) were cultured in sealed tubes at 37 °C in a 5% CO_2 atmosphere in the presence or absence of the test sample. After a cultivation period of 48 h the cell growth-inhibitory effect was determined from the ratio of cell numbers counted visually with the aid of a microscope. The 50% inhibition concentration (IC_{50}) value was determined by a probit diagraming analysis.

Antimicrobial Activity——The first screening was performed by a paper disc assay using bacteria and fungi, that is, *Bacillus subtilis* ATCC 6633, *Escherichia coli* 0—1 and *Trichophyton mentagrophytes* QM 248. Active compounds selected by the first screening were subjected to a dilution assay and the minimum inhibition concentration (MIC) was determined by MIC determination assay, according to the procedure established by the Japanese Society of Chemotherapy.[8] The microorganisms used in this study were 8 gram-positive, 9 gram-negative bacteria and 20 fungi.

***In Vivo* Antitumor Activity**——Leukemia P-388 maintained in CDF_1 mice by weekly passage was used. P-388

(1.0×10^5 cells/mouse) was intraperitoneally (i.p.) inoculated into female CDF_1 mice, and the test sample was dissolved or suspended in an adequate vehicle, and administered i.p. once daily from day 1 to day 5. The increase in life-span was determined from the mean survival time by comparison with that of the control animals. The test samples were dissolved in 0.9% aqueous NaCl solution in the case of compounds containing less than 14 carbons, in 5% DMSO–saline for those containing 16—22 carbons, and in 30% DMSO–0.2% Tween 80–saline for the allyl derivatives.

***Avena* Coleoptile Straight Growth Test**——The method used in this study was identical with that reported in the previous paper.[9] Oat seeds (*Avena sativa* L.) were cultivated at 25 °C in darkness for 3 d after seeding, in the presence or absence of 3-indolylacetic acid (IAA). The test sample was dissolved in 0.1% ethanol solution. A section (6 mm long) was cut from the coleoptile about 2—3 mm below the tip, placed in a test tube containing 1 ml each of 10 mM KH_2PO_4–Na_2HPO_4 buffer solution (pH 5.2), 3 ppm IAA solution and 167 ppm test solution, and incubated at 25 °C for 18 h. For each sample 11 sections were used. After 18 h of incubation the section length was measured, and the growth was calculated as follows: Growth (%)$=\Delta T/\Delta C \times 100$, where ΔT is the average length (mm) after the incubation of the test group minus the initial length (6 mm), and ΔC is the corresponding value for the control group.

Results and Discussion

As reported in our previous paper,[4] monosaccharide esters did not show high *in vitro* cytotoxic activity. The *in vitro* cytotoxicity of ester derivatives of disaccharides, for example, sucrose monoester or trehalose diester derivatives,[5] was also not very high; at best, it was

TABLE I. Cell Growth-Inhibitory Effect on the Cultured L-5178Y Cell Line of Positional Isomers of *O*-Alkyl Monosaccharides

Compd.	IC_{50} (mcg/ml)
1-*O*-Lauryl-D-glucoside	21.8
3-*O*-Lauryl-D-glucose	0.6
6-*O*-Lauryl-D-glucose	31.0
6-*O*-Lauryl-D-galactose	100

TABLE II. Cell Growth-Inhibitory Effect on the Cultured L5178Y Cell Line of 3-*O*-Alkyl-D-glucose and -D-allose Derivatives

Compd.	R	IC_{50} (mcg/ml)	Compd.	R	IC_{50} (mcg/ml)
GC-1	CH_3-	7.0	GC-A3	$CH_2=CH-CH_2-$	100
GC-2	C_2H_5-	14.5	GC-A10	$CH_2=CH-(CH_2)_8-$	9.0
GC-3	C_3H_7-	20.0	GC-A11	$CH_2=CH-(CH_2)_9-$	1.3
GC-4	C_4H_9-	100	GC-A12	$CH_2=CH-(CH_2)_{10}-$	20.0
GC-6	$C_6H_{13}-$	4.4	GC-B10	$HO(CH_2)_{10}-$	100
GC-8	$C_8H_{17}-$	3.8	GC-B16	$HO(CH_2)_{16}-$	100
GC-10	$C_{10}H_{21}-$	6.5	GC-C10	$CH_3O(CH_2)_{10}-$	49
GC-11	$C_{11}H_{23}-$	7.6	GC-C12	$CH_3O(CH_2)_{12}-$	57
GC-12	$C_{12}H_{25}-$	0.6			
GC-13	$C_{13}H_{27}-$	5.0	AC-1	CH_3-	100
GC-14	$C_{14}H_{29}-$	4.4	AC-6	$C_6H_{13}-$	100
GC-16	$C_{16}H_{33}-$	1.6	AC-10	$C_{10}H_{21}-$	29
GC-18	$C_{18}H_{37}-$	1.1	AC-11	$C_{11}H_{23}-$	33
GC-20	$C_{20}H_{41}-$	3.4	AC-12	$C_{12}H_{25}-$	68
GC-22	$C_{22}H_{45}-$	100	AC-13	$C_{13}H_{27}-$	10.7
			AC-18	$C_{18}H_{37}-$	10.5

GC, 3-*O*-alkyl-D-glucose; GC-A, 3-*O*-ω-methylenealkyl-D-glucose; GC-B, 3-*O*-ω-hydroxyalkyl-D-glucose; GC-C, 3-*O*-ω-methoxyalkyl-D-glucose; AC, 3-*O*-alkyl-D-allose. Inoculum size, 1.0×10^5 cells/ml. Cell counting was performed 48 h after the inoculation.

50 mcg/ml (IC_{50}). Some structural relation to *in vitro* cytotoxicity[3,5] could, however, be found.

In this work we studied the biological activities of *O*-alkyl derivatives of D-glucose and D-allose, and the relationship between the structure and *in vitro* cytotoxicity. We also examined the difference in cytotoxic activity of the positional isomers of the *O*-alkyl derivatives of D-glucose. As shown in Table I, it was found that 3-*O*-alkyl-D-glucoses show high inhibitory activity on tumor cell growth, but the 1- or 6-*O*-alkyl derivatives do not. Therefore, we synthesized 3-*O*-alkyl derivatives of D-glucopyranose and D-allopyranose having various chain-length substituents, and found that it was essential for the carbon chain-length to be over 8 and below 20 in order for the derivatives to manifest cytotoxic activity. As shown in Table II, the IC_{50} values of compounds with carbon chain-lengths of C-8 to C-22 were less than 10 mcg/ml. The 3-*O*-lauryl derivative of D-glucose had the highest activity (IC_{50} below 1 mcg/ml). Among the tested alkenyl derivatives having an ω-methylene group the 11-carbon compound (GC-A11) showed a highly promising activity. Among the 6,6′-diester derivatives of, α,α-trehalose, the lauroyl ester showed the highest activity,[5] and in the D-glucose ester series the lauroyl derivative also showed the highest *in vitro* cytotoxicity[4] and plant growth-inhibitory effect.[9] Although it is not our intention to discuss here the reason why the lauryl or lauroyl derivatives of carbohydrates show such high biological activities, it may be suggested that the 12-carbon chain constitutes an important factor in some biological systems. As 3-*O*-lauryl-D-glucose showed the highest activity among the compounds synthesized in this study, we also tested by *in vivo* bioassay the antitumor activity of this and other structurally similar

TABLE III. Antitumor Activity of 3-*O*-Alkyl-D-glucose Derivatives

Compd.	Dose mg/kg × d	Mean survival (d)	ILS (%)	Result
Control		8.92		
GC-8	30 × 5	10.83	21	++
GC-10	30 × 5	10.83	16	++
Control		8.70		
GC-11	30 × 5	10.17	17	++
GC-12	30 × 5	10.67	23	++
GC-13	30 × 5	9.00	3	±

Tumor: P-388, 1.0×10^5 cells/mouse, i.p. Vehicle: 0.9% NaCl aq. solution. Animal: female BDF_1 mouse. Route: i.p. ILS: increase in life-span.

TABLE IV. Plant Growth-Inhibitory Activity on *Avena* Coleoptile Sections[a] of 3-*O*-Alkyl-D-glucose and -D-allose Derivatives

Compd.	Plant growth[b] (%)
GC-3	95.1 ± 4.9
GC-10	82.9 ± 4.9
GC-12	97.6 ± 2.4
GC-A3	97.6 ± 7.2
GC-A10	91.0 ± 3.0
GC-A11	46.3 ± 2.4
GC-A12	48.8 ± 7.2
AC-10	97.0 ± 3.0
AC-11	93.9 ± 0.0

a) 167 ppm. *b*) Mean ± S.E.

TABLE V. Spectral Data for 3-O-Alkyl-D-glucose and -D-allose Derivatives

Compd.	IR λ_{max}^{KBr} cm^{-1}	NMR[a] (δ ppm)
GC-1	2970, 1030	3.45 (s, 3H), 2.80—5.20 (m, 11H)
GC-2	2920, 1030	1.11 (t, 3H), 3.5 (q, 2H), 2.75—5.10 (m, 11H)
GC-3	2920, 1035	0.87 (t, 3H), 1.20—1.95 (m, 2H), 2.70—5.15 (m, 13H)
GC-4	2930, 1035	0.90 (t, 3H), 1.10—1.80 (m, 4H), 2.70—5.15 (m, 13H)
GC-6	2930, 1035	0.87 (t, 3H), 1.31 (br, 8H), 2.70—5.30 (m, 13H)
GC-8	2920, 1030	0.87 (t, 3H), 1.24 (br, 12H), 2.85—4.95 (m, 13H)
GC-10	2930, 1030	0.88 (t, 3H), 1.24 (br, 16H), 2.75—5.10 (m, 13H)
GC-11	2930, 1035	0.86 (t, 3H), 1.23 (br, 18H), 2.80—4.95 (m, 13H)
GC-12	2920, 1030	0.86 (t, 3H), 1.24 (br, 20H), 2.75—5.00 (m, 13H)
GC-13	2920, 1035	0.87 (t, 3H), 1.23 (br, 22H), 2.70—4.95 (m, 13H)
GC-14	2920, 1030	0.88 (t, 3H), 1.23 (br, 24H), 2.80—4.93 (m, 13H)
GC-16	2920, 1030	0.87 (t, 3H), 1.23 (br, 28H), 2.85—5.00 (m, 13H)
GC-18	2920, 1030	0.90 (t, 3H), 1.23 (br, 32H), 2.90—4.96 (m, 13H)
GC-20	2920, 1030	0.87 (t, 3H), 1.23 (br, 36H), 2.85—4.98 (m, 13H)
GC-22	2920, 1030	0.85 (t, 3H), 1.21 (br, 40H), 2.80—4.90 (m, 13H)
GC-A3	2920, 1030	2.70—5.50 (m, 15H), 5.60—6.30 (m, 1H)
GC-A10	2925, 1030	1.30 (br, 12H), 1.79—2.30 (m, 2H), 2.80—5.20 (m, 15H), 5.20—6.10 (m, 1H)
GC-A11	2925, 1035	1.25 (br, 14H), 1.74—2.20 (m, 2H), 2.80—5.20 (m, 15H), 5.20—6.10 (m, 1H)
GC-A12	2925, 1030	1.25 (br, 16H), 1.72—2.20 (m, 2H), 2.80—5.20 (m, 15H), 5.20—6.10 (m, 1H)
GC-B10	2935, 1035	1.25 (br, 16H), 2.70—5.20 (m, 16H)
GC-B16	2935, 1040	1.23 (br, 28H), 2.70—5.20 (m, 16H)
GC-C10	2930, 1035	1.27 (br, 16H), 3.21 (s, 3H), 2.70—5.20 (m, 15H)
GC-C12	2935, 1035	1.23 (br, 20H), 3.20 (s, 3H), 2.70—5.10 (m, 15H)
AC-1	2950, 1040	3.53 (s, 3H), 3.10—4.20 (m, 6H), 4.70—5.20 (m, 1H)
AC-6	2950, 1040	0.90 (t, 3H), 1.34 (br, 8H), 3.10—4.18 (m, 8H), 4.60—5.20 (m, 1H)
AC-10	2935, 1035	0.90 (t, 3H), 1.28 (br, 16H), 3.10—4.30 (m, 8H), 4.50—5.10 (m, 1H)
AC-11	2940, 1035	0.88 (t, 3H), 1.28 (br, 18H), 3.10—4.20 (m, 8H), 4.50—5.10 (m, 1H)
AC-12	2945, 1035	0.88 (t, 3H), 1.28 (br, 18H), 3.10—4.20 (m, 8H), 4.65—5.10 (m, 1H)
AC-13	2930, 1030	0.88 (t, 3H), 1.24 (br, 22H), 3.10—4.20 (m, 8H), 4.60—5.10 (m, 1H)
AC-18	2940, 1030	0.88 (t, 3H), 1.24 (br, 32H), 3.10—4.20 (m, 8H), 4.60—5.10 (m, 1H)

a) Glucose derivatives measured in DMSO-d_6; allose derivatives measured in CD_3OD.

compounds. The results correlate farily well with those of *in vitro* bioassay. Next, the antimicrobial activity of monosaccharide derivatives synthesized in this study was examined. The assay was done by a disc method using bacteria and fungi. Three compounds, namely 3-*O*-tridecyl-(GC-13), 3-*O*-myristyl-D-glucose (GC-14) and 3-*O*-lauryl-D-allose (AC-12), were selected by the preliminary disc assay. It is interesting to note that the alkyl chain-length in all these three compounds is close to 12. However, not all of the alkyl compounds tested showed high antimicrobial activity, and MIC was over 12.5 mcg/ml.

Plant growth-inhibition studies by the *Avena* coleoptile straight growth test[9] were also performed with these alkyl monosaccharides. In sugar ester derivatives, the chain-length and location of the acyl group are critical for the plant growth-inhibitory activity.[9] More specifically, only 1-*O*-lauroyl-D-glucose had a high plant growth-inhibitory activity. 3-*O*-Alkyl-D-glucose derivatives were less active. Among them, two 3-*O*-alkenyl derivatives having an ω-methylene group showed more than 50% plant growth-inhibitory activity, and the other compounds tested in this study were not active. As regards antitumor activity, the result of *in vitro* bioassay is rather well correlated with the *in vivo* data, but it is of interest that the inhibitory effects against animal tumor cells were quite different from those against plant growth or cell growth.

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Effect of Chronic Treatment with Saikosaponin d and Dexamethasone on Responsiveness of Pituitary and Adrenal Cortex and Adrenal Weight in Rats

SUSUMU HIAI* and HIROOMI YOKOYAMA

Research Institute for WAKAN-YAKU, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan

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Basal and maximum corticosterone secretions of the whole pituitary–adrenal cortex system or the adrenal cortex were determined in rats treated with saikosaponin d (saiko-d) from *Bupleurum falcatum* L. alone or with dexamethasone for 5 or 9 d. In rats treated with 0.5, 1.0 or 2.5 mg/kg/d of saiko-d, or saiko-d and 0.025 mg/kg/d of dexamethasone for 5 d, both basal and acutely stimulated evening levels of plasma corticosterone on day 5 were very slightly below normal; the stimulated level due to saiko-d was significantly higher than the basal level, but was not significantly lower than the normal and adrenocorticotropin (ACTH)-stimulated maximum level. In rats chronically treated with saiko-d (2.5 mg/kg/d) alone, or with saiko-d and a high dose of dexamethasone (0.05 mg/kg/d) for 9 d, the stimulated level of corticosterone was nearly equal to the basal level, and saiko-d administered with dexamethasone reduced the adrenal weight on day 9. These chronic effects of saiko-d on the adrenal activity and weight were fully effective in the early phase, but were less effective and then suppressive in the late phase, especially at a high dose of saiko-d or on co-administration of saiko-d and dexamethasone.

Keywords——saikosaponin d; dexamethasone; basal adrenal cortex responsiveness; maximum hypothalamic–pituitary system responsiveness; plasma basal corticosterone; plasma maximum corticosterone; adrenal growth; thymus growth; plasma glucose

In acute experiments, saikosaponins a and d increased plasma levels of adrenocorticotropin (ACTH) and corticosterone,[1] and a high dose of saikosaponins a and d antagonized the dexamethasone-induced blocking of corticosterone (and ACTH) secretion.[2] In a chronic experiment[3] saikosaponin d induced adrenal hypertrophy and thymus atrophy both in normal and dexamethasone-treated rats, while these effects were not found in hypophysectomized rats.[3] This suggests that saikosaponin acted on the adrenals *via* the pituitary; the adrenals hypertrophied due to saikosaponin and the hypothalamic–pituitary system were active, and a large amount of corticosterone secreted from the hypertrophied adrenals resulted in atrophy of the thymus. However, in the chronic experiments, the adrenal weight and the basal level of corticosterone on the final day had been determined, but not the stimulated level. Yamamoto *et al.*[4] reported that saikosaponin d administered repeatedly had antigranulomatous action, but it did not increase the adrenal weight or plasma 11-OH corticosteroid. Abe *et al.*[5] showed that repeated administration of saikosaponin d potentiated the antigranulomatous action of dexamethasone, while it was slightly hypertrophic to the adrenals in normal rats, but it was rather atrophic in the dexamethasone-treated rats. The present study was undertaken to examine the basal and maximum corticosterone secretions of the whole pituitary–adrenal cortex system and the adrenal cortex as well as the adrenal and thymus growth in rats chronically treated with saikosaponin d.

Materials and Methods

Male Wistar rats initially weighing 80—95 g were used. They were fed on laboratory chow (CE-2, CLEA Japan Inc., Tokyo) and tap water *ad libitum*, and maintained with artificial light (light phase: 0700 to 1900 h) at 22 °C for more than 7 or 11 d. In order to minimize stress-stimulated corticosterone release, they were "gentled" by daily handling and weighing, twice a day in the morning and evening. A test substance or 5 ml/kg of saline (pyrogen-free saline or 2.5% ethanol–saline) was injected intraperitoneally once a day between 1800 and 1900 h (considering the circadian adrenalcortical rhythm of the nocturnal animal) for 5 or 9 consecutive days. In the morning or evening 15 or 24 h after the final treatment, the rats were decapitated with a guillotine. Trunk blood was collected into a chilled heparinized tube to obtain plasma. Immediately after this, the adrenal glands and thymus were rapidly removed, put into ice-cold saline, and weighed after the removal of adipose tissues in cold saline followed by blotting on a filter paper.

Saikosaponin d from Bupleuri Radix was kindly supplied by Drs. K. Takeda and K. Sakurai of Shionogi Research Laboratories, Shionogi and Co., Ltd., Osaka. The ED_{50} value of corticosterone secretion-inducing activity of saikosaponin d was 0.33 mg/kg.[6] Saikosaponin d and dexamethasone were dissolved in 2.5% ethanol-containing pyrogen-free saline just before use. Cortrosyn z from N. V. Organon, Netherlands, was diluted with saline and used as corticotropin (1 mg of cortrosyn z has an activity of 40 U). Histamine dihydrochloride (Nakarai Chemicals, Kyoto) was used as saline solution.

Plasma corticosterone was determined by the competitive protein binding method of Murphy[7] as described in a previous paper.[8] Addition of dexamethasone to the assay system did not affect the determination. Plasma glucose was determined by the glucose oxidase method.

Results

Time Course of Effect of Saikosaponin d on the Adrenal Weight, and Basal and Stimulated Levels of Corticosterone and Glucose

As shown in Table I, in rats treated daily with saikosaponin d, adrenal weight was not significantly changed during day 2 to day 5, but adrenal weight seemed to increase on day 3, and to decrease on days 4 and 5. Thymus growth for 3 d was small but significant in these

TABLE I. Time Course of Effect of Saikosaponin d on the Adrenal and Thymus Weight and Basal and Stimulated Levels of Plasma Corticosterone or Glucose

Acute treatment	Days of treatment with saikosaponin d (2.5 mg/kg/d)			
	2	3	4	5
Adrenal weight (mg/100 g of initial body weight)[a]				
Unstimulated[b]	21.2±1.0 (7)[c]	22.3±1.0 (6)	20.9±0.6 (6)	19.3±0.9 (6)
Stimulated[d]	21.1±0.9 (7)	21.8±1.2 (6)	20.3±0.9 (6)	23.0±0.9 (6)
Thymus weight (mg/100 g of initial body weight)				
Unstimulated	359±19 (7)	371±24 (6)	370±19 (6)	401±21 (6)
Stimulated	350±21 (7)	394±17 (6)	385±16 (6)	424±12 (6)[e]
Plasma corticosterone (μg/100 ml)				
Unstimulated	17.5±2.6 (7)	19.0±3.3 (6)	23.8±3.5 (6)	21.3±2.7 (6)
Stimulated	33.0±2.7 (7)	36.6±4.5 (6)	33.9±6.4 (6)	34.6±6.1 (6)
p	<0.01	<0.02	NS	<0.1
Plasma glucose (mg/100 ml)				
Unstimulated	144±2 (7)	138±6 (6)	146±2 (6)	134±3 (6)[e]
Stimulated	144±3 (7)	139±2 (6)	139±4 (6)	143±2 (6)
p	NS	NS	NS	<0.05

a) Adrenal and thymus weight was determined after a 30-min saline or saikosaponin d treatment 24 h after the final treatment. *b*) A 30-min treatment with 5 ml/kg of saline 24 h after the final treatment. *c*) Figures are mean±S.E., and figures in parentheses are number of rats. *d*) A 30-min treatment with 2.5 mg/kg of saikosaponin d 24 h after the final treatment. *e*) $p<0.05$ *vs.* rats treated twice with saikosaponin.

young adult rats treated with saikosaponin d. The basal evening level of corticosterone seemed to increase and then to decrease, and the peak value was 23.8±3.5 μg/100 ml on day 4 (Table I). A significant acute increase in plasma corticosterone due to a 30-min treatment with saikosaponin was found on days 2 and 3, but not on days 4 and 5. This indicates that the pituitary–adrenal cortex system was active through the experimental period, but higher responsiveness of the system was found in the rather early phase.

Transient hyperglycemia due to acute saikosaponin treatment[1] was not found in rats treated with 2.5 mg/kg/d of saikosaponin for 2, 3, 4 or 5 d. This result clearly shows that corticosterone secretion-inducing activity of saikosaponin is dissociated from its hyperglycemic activity in rats previously treated with saikosaponin, and the former activity did not depend on the latter in saikosaponin d-treated rats, as in normal rats.[1]

Time Course of Effect of Saikosaponin on Adrenal Growth and Activity in the Recovery Phase from Dexamethasone Suppression

The chronic effect of saikosaponin d on the pituitary–adrenal cortex system was determined in the recovery phase from dexamethasone-induced suppression. The relative adrenal weight did not increase in control rats treated for one day with saline (17.5±1.3 and 17.5±0.5 mg/100 g of body weight, Table II). The effect of dexamethasone on the adrenal weight remained for 2 d after the 5th dexamethasone treatment. In control rats treated with saline for 3 or 5 d, however, the adrenal weight gradually recovered (Table II). Administration of a low dose of saikosaponin d (1.0 mg/kg/d) accelerated the recovery of the adrenal weight by one day ($p<0.02$). The relative thymus weight significantly increased in control rats treated with saline for 3 or 5 d but not one day. In rats treated with saikosaponin for 3 and 5 d,

Table II. Time Course of Effect of Saikosaponin d (Sd) on Adrenal and Thymus Growth and Stimulated Levels of Plasma Corticosterone and Glucose in Previously Dexamethasone-Treated Rats in the Recovery Phase

Treatment[a] (mg/kg/d)	Days of treatment with saline or saikosaponin d			
	0	1	3	5
Adrenal weight (mg/100 g of initial body weight)[b]				
Saline	17.5±1.3	17.5±0.5	21.7±1.8[c]	22.3±0.8[d]
+Sd 1.0	—	20.6±0.9[e]	22.0±1.1[d]	24.1±0.8[e]
p	—	<0.02	NS	NS
Thymus weight (mg/100 g of initial body weight)				
Saline	284±18	313±17	431±30[e]	476±5[f]
+Sd 1.0	—	319±9	369±9[e]	422±25[e]
p	—	NS	<0.1	<0.1
Plasma corticosterone (μg/100 ml)				
Saline	16.9±4.8	33.8±1.8[e]	38.3±1.5[e]	35.3±1.2[e]
+Sd 1.0	—	19.5±7.5	35.1±0.9[e]	24.9±6.6
p	—	<0.1	<0.1	NS
Plasma glucose (mg/100 ml)				
Saline	263±32	291±15	295±7	272±15
+Sd 1.0	—	181±14[d]	177±6[d]	171±10[d]
p	—	<0.001	<0.001	<0.001

a) Seven groups of 6 rats were treated with 0.025 mg/kg/d of dexamethasone every evening for 5 d. Six groups of rats were treated with 5 ml/kg/d of saline or 1.0 mg/kg/d of saikosaponin d for 1, 3 or 5 d 24 h after the 5th treatment with dexamethasone. All these rats were stimulated with 2.5 mg/kg of saikosaponin d for 30 min at 15 h after final treatment with dexamethasone, saline or saikosaponin d. b) Body weight in the evening of day 0 of the 1st dexamethasone treatment. c—f) $p<0.1$, $p<0.05$, $p<0.01$, $p<0.001$ vs. rats on day 0 of recovery phase.

significant growth of the thymus was also found, but saikosaponin suppressed the recovery on days 3 and 5 ($p<0.1$).

In rats treated with dexamethasone for 5 d the stimulated morning level of corticosterone (16.9±4.9 μg/100 ml) was higher than the normal basal morning level (0.5—1.5 μg/100 ml) 15 h after the 5th treatment, but it was low and one-half of the normal morning maximum level. In rats treated with saline for 1, 3 or 5 d, the activity of the pituitary–adrenal cortex system recovered and reached normal maximum activity, whereas treatment with a low dose of saikosaponin delayed the recovery in the early phase (day 1), and become suppressive again in the late phase (day 5, Table II). This may suggest that remaining dexamethasone and saikosaponin-induced corticosterone additively suppressed the hypothalamic–pituitary system in the early phase, and corticosterone alone suppressed the system in the late phase.

The stimulated level of plasma glucose in control rats did not change in the wash-out period. Hyperglycemia due to acute saikosaponin was abolished by a single previous treatment with 1.0 mg/kg of saikosaponin d 15 h after the dexamethasone treatment (Table II).

Effect of a Low Dose of Saikosaponin d on Basal and Stimulated Levels of Plasma Corticosterone and Glucose Determined in the Morning and Evening

Wet weights of the adrenals and thymus were determined in the morning or evening 15 or 24 h after the 5th treatment with a lower dose of saikosaponin d (0.5 mg/kg/d). As shown in Table IIIa, chronic treatment with saikosaponin d tended to increase the relative morning

TABLE IIIa. Effect of Saikosaponin d (Sd) on Adrenal and Thymus Weights

Treatment[a] (mg/kg/d × 5 d)	Weight (mg/100 g of body weight)[b]			
	Adrenal (%)	p	Thymus (%)	p
Saline-M[c]	19.7±0.8 (100)	—	463±30 (100)	—
+Sd 0.5-M[c]	20.7±0.7 (105)	NS	409±23 (88)	NS
Saline-E[d]	18.1±1.2 (100)	—	429±32 (100)	—
+Sd 0.5-E[d]	19.5±1.1 (108)	NS	385±25 (90)	NS

a) Eight groups of 6 rats were treated with saline or saikosaponin d every evening. *b*) Body weight was determined in the evening on day 0. Wet weight was determined after a 30-min treatment with saline at 15 or 24 h after the 5th treatment. *c*) Adrenal and thymus weights were determined 15 h after the 5th treatment. *d*) Adrenal and thymus weights were determined at 24 h after the 5th treatment.

TABLE IIIb. Effect of Saikosaponin d (Sd) on Evening (E) and Morning (M) Levels of Plasma Corticosterone and Glucose

Treatment (mg/kg/d × 5 d)	Corticosterone (μg/100 ml)			Glucose (mg/100 ml)		
	Basal[a]	Stimulated[b] (%)	p	Basal	Stimulated (%)	p
Saline-M	0.7±0.3	34.7±6.9 (100)	<0.001	143±2	192±14 (100)	<0.001
+Sd 0.5-M	1.6±0.6	27.7±6.7 (80)	<0.01	139±2	164±13 (85)	NS
p	NS	NS		NS	NS	
Saline-E	18.2±3.1	39.4±4.1 (100)	<0.01	148±3	215±20 (100)	<0.01
+Sd 0.5-E	16.2±3.2	42.7±2.4 (108)	<0.001	149±4	175±5 (81)	<0.01
p	NS	NS		NS	<0.1	

a) A 30-min treatment with 5 ml/kg of saline 15 or 24 h after the 5th treatment. *b*) A 30 min treatment with 2.5 mg/kg of saikosaponin d 15 or 24 h after the 5th treatment.

weight of the adrenals, and tended to decrease the relative morning weight of the thymus. The results of evening determination of wet weight were similar to those of the morning determination (Table IIIa).

The basal morning level of plasma corticosterone was not affected by treatment with saikosaponin d for 5 d (Table IIIb). Increase in plasma corticosterone due to acute treatment with saikosaponin d was found as usual, and the increased level was not affected by chronic treatment. The basal evening level of corticosterone was 10 to 20 times the morning level (Table IIIb). Chronic treatment with saikosaponin d did not affect the basal or stimulated evening level of plasma corticosterone. This suggests that the 5-d treatment with the low dose of saikosaponin d did not affect the circadian rhythm of plasma corticosterone or the maximum activity of the pituitary–adrenocortical system 15 or 24 h after the final treatment.

Chronic treatment with a low dose of saikosaponin d (0.5 mg/kg/d) for 5 d did not affect the basal morning or evening level of plasma glucose. In rats treated with saikosaponin d, hyperglycemia due to acute treatment with saikosaponin d was rather weak in the evening, and it was not significant in the morning (Table IIIb).

Effect of Higher Doses of Saikosaponin d on the Activity of the Pituitary–Adrenal Cortex System in Normal or Dexamethasone-Treated Rats

Administration of 1.0 mg/kg/d of saikosaponin d for 5 d induced a small increase in adrenal weight, as did that of 0.5 mg/kg/d of saikosaponin. Chronic treatment with dexamethasone (0.025 mg/kg/d) induced a significant decrease in adrenal weight, which was completely overcome by co-administration of the lower dose of saikosaponin (Table IVa). A 5-d treatment with 2.5 mg/kg/d of saikosaponin induced marked adrenal hypertrophy (123%), whereas in rats treated with dexamethasone the effect of the dose of saikosaponin was not statistically significant, though it appeared to be slightly atrophic, and additive to the atrophic effect of dexamethasone (from 95% to 91%). A 9-d treatment with 2.5 mg/kg of saikosaponin induced an increase in adrenal weight (113%, $p<0.1$), which was smaller than that due to 5-d treatment. In rats treated with a high dose of dexamethasone (0.05 mg/kg/d) the effect of this dose of saikosaponin was not statistically significant, though it appeared to be rather atrophic, and additive to the atrophic effect of dexamethasone (from 91% to 85%) as in the case of the 5-d treatment (Table IVa).

As shown in Table IVa, saikosaponin decreased the thymus weight dose-dependently. In dexamethasone-treated rats, the effect of co-administered saikosaponin on the thymus also was dose-dependent, and additive to that of dexamethasone (Table IVa).

The effect of administration of 1.0 mg/kg/d of saikosaponin d for 5 d, and 2.5 mg/kg/d for 5 and 9 d on the basal evening level of plasma corticosterone was not statistically significant on day 5 or 9, though there seemed to be some decrease. Dexamethasone (0.025 or 0.05 mg/kg/d) tended to decrease the basal level, and co-administered saikosaponin tended to decrease the basal level further (Table IVb). The acute increase in plasma corticosterone due to saikosaponin on the final day was statistically significant in rats treated with saikosaponin or dexamethasone alone or both for 5 d, as well as in normal rats. The pituitary and the adrenal cortex were fully active on the final day in rats treated with saikosaponin d for 5 d (126%, 96%), whereas in rats treated with dexamethasone alone or dexamethasone and saikosaponin they were not fully active (61%, 64%, 59%). In rats treated with saikosaponin or dexamethasone alone or both for 9 d, statistically significant stimulation due to acute treatment was not found on day 9 (49%, 57%, 32%). The 9-d treatment with 2.5 mg/kg/d of saikosaponin may be an "over-dose" which resulted in atrophy of both adrenals (123% to 113%) and thymus (86% to 83%) in the late phase due to "over-production" of glucocorticoid from the hypertrophied adrenals on day 5 in the early phase (Table IVa).

The basal level of glucose was not affected by chronic treatment with saikosaponin

TABLE IVa. Effect of Saikosaponin d (Sd) on Adrenal and Thymus Weight in Chronically Saline- or Dexamethasone (Dex)-Treated Rats

Treatment[a] (mg/kg/d)	Weight (mg/100 g of initial body weight)[b]			
	Adrenal (%)	p	Thymus (%)	p
Saline × 5 d	20.2 ± 0.9 (100)	—	463 ± 17 (100)	—
+Sd 1.0 × 5 d	21.3 ± 1.3 (105)	NS	441 ± 22 (95)	NS
Dex 0.025 × 5 d	16.5 ± 1.2 (82)	<0.05	338 ± 14 (73)	<0.001
+Sd 1.0 × 5 d	19.7 ± 1.0 (98)	NS	303 ± 20 (66)	<0.001
Saline × 5 d	23.2 ± 0.2 (100)	—	468 ± 28 (100)	—
+Sd 2.5 × 5 d (7)	28.6 ± 1.6 (123)	<0.02	403 ± 19 (86)	<0.1
Dex 0.025 × 5 d	22.0 ± 1.3 (95)	NS	327 ± 12 (70)	<0.001
+Sd 2.5 × 5 d	21.1 ± 0.8 (91)	<0.05	250 ± 9 (53)	<0.001
Saline × 9 d	20.7 ± 0.7 (100)	—	486 ± 26 (100)	—
+Sd 2.5 × 9 d	23.4 ± 1.0 (113)	<0.1	405 ± 28 (83)	<0.1
Dex 0.05 × 9 d	18.8 ± 1.1 (91)	NS	298 ± 25 (61)	<0.001
+Sd 2.5 × 9 d	17.5 ± 0.4 (85)	<0.01	222 ± 14 (46)	<0.001

a) The number of rats was 6, except in one case (indicated by 7 in parentheses). *b*) Adrenal and thymus weights were determined after a 30-min treatment with saline 24 h after the final daily treatment. Initial body weight was determined in the evening of day 0.

TABLE IVb. Effect of Saikosaponin d (Sd) on Plasma Levels of Corticosterone and Glucose in Normal or Dexamethasone (Dex)-Treated Rats

Treatment (mg/kg/d)	Corticosterone (μg/100 ml)			Glucose (mg/100 ml)		
	Basal[a]	Stimulated[b] (%)	p	Basal	Stimulated (%)	p
Saline × 5 d	23.1 ± 3.2	33.2 ± 1.9 (100)	<0.05	149 ± 3	226 ± 12 (100)	<0.001
+Sd 1.0 × 5 d	15.4 ± 1.9	41.8 ± 1.2 (126)	<0.001	145 ± 6	162 ± 5 (72)	NS
Dex 0.025 × 5 d	12.1 ± 1.5	17.7 ± 2.9 (53)	NS	142 ± 4	214 ± 25 (95)	<0.01
+Sd 1.0 × 5 d	14.1 ± 1.4	20.1 ± 2.1 (61)	<0.05	147 ± 5	148 ± 2 (65)	NS
Saline × 5 d	17.6 ± 0.7	40.1 ± 1.5 (100)	<0.001	149 ± 4	230 ± 12 (100)	<0.001
+Sd 2.5 × 5 d	19.2 ± 1.7[c]	38.5 ± 6.3 (96)[d]	<0.01	139 ± 3	146 ± 7 (64)	NS
Dex 0.025 × 5 d	15.3 ± 2.4	25.7 ± 2.0 (64)	<0.01	143 ± 6	264 ± 7 (115)	<0.001
+Sd 2.5 × 5 d	11.8 ± 1.8	23.8 ± 2.0 (59)	<0.01	138 ± 6	149 ± 3 (65)	NS
Saline × 9 d	25.8 ± 4.5	37.8 ± 1.6 (100)	<0.05	129 ± 3	194 ± 9 (100)	<0.001
+Sd 2.5 × 9 d	19.2 ± 3.2	18.6 ± 2.3 (49)	NS	151 ± 4	155 ± 3 (80)	NS
Dex 0.05 × 9 d	15.4 ± 2.6	21.5 ± 2.3 (57)	NS	153 ± 4	219 ± 22 (113)	<0.02
+Sd 2.5 × 9 d	12.0 ± 1.2	12.2 ± 1.2 (32)	NS	141 ± 3	154 ± 2 (79)	<0.01

a) Effect of a 30-min treatment with 5 ml/kg of saline on basal levels of corticosterone and glucose 24 h after the final treatment. The number of rats was 6, except that 7 were used for *c*). *b*) Effect of a 30-min treatment with 2.5 mg/kg of saikosaponin d on stimulated levels 24 h after the final treatment. The number of rats was 6, except that 5 were used for *d*).

and/or dexamethasone. However, transient hyperglycemia due to acute treatment with saikosaponin was abolished in rats previously treated with saikosaponin (1.0 or 2.5 mg/kg) alone or with dexamethasone, whereas saikosaponin d increased the stimulated level of corticosterone as usual (Table IVb).

Effect of Chronic Treatment with Saikosaponin and Dexamethasone on the Activities of the Pituitary and the Adrenal Cortex

Saikosaponin directly acted on the hypothalamic–pituitary system, and secretion of ACTH and corticosterone was accompanied with transient hyperglycemia, which was not

blocked by the H-1 receptor antagonist diphenhydramine.[1] Histamine also directly acted on the system, and secretion of corticosterone was accompanied with hyperglycemia, which was completely blocked by pretreatment with diphenhydramine.[1] ACTH directly acts on the adrenal cortex, but saikosaponin[2] and histamine[9] do not.

In rats chronically treated with saikosaponin, the activity of the whole pituitary–adrenal cortex system due to acute treatment with saikosaponin or histamine, and the activity of the adrenal cortex due to ACTH were determined (Table V). Chronic treatment with saikosaponin did not significantly affect the basal evening level of plasma corticosterone on day 5, which was rather low. The stimulated level of corticosterone due to acute saikosaponin was higher than the basal level, but the increase was not significant, whereas the adrenal weight was significantly increased 18.5±0.9 to 23.4±0.7 mg/100 g of initial body weight ($p<0.01$), and the thymus weight was decreased 493±25 to 426±20 mg/100 g of initial body weight ($p<0.1$). The stimulated level of corticosterone due to histamine was significantly higher than the basal value, but was rather low. A submaximum dose of ACTH induced a significant increase in plasma corticosterone in saikosaponin-treated rate ($p<0.001$), whereas these doses of ACTH induced a small increase in saline-treated rats. This shows that in rats chronically treated with saikosaponin, the adrenal cortex was fully active, but the capability of the whole pituitary–adrenal cortex system or the hypothalamic–pituitary system was lower than that of the hypertrophied adrenal cortex, and it limited that of the adrenal cortex on day 5.

In rats co-administered dexamethasone and saikosaponin or saline, plasma corticosterone was significantly increased by acute treatment with saikosaponin, histamine or ACTH (Table V). However, the degree of increase in rats previously treated with saikosaponin was lower than that in rats not given saikosaponin, or equal to it (Table V). This shows that the adrenal cortex was active, but saikosaponin-induced corticosterone and exogenous dexamethasone cooperatively suppressed the adrenal cortex as well as the hypothalamic–pituitary system in the late phase, and that the capability of the adrenal cortex was almost

TABLE V. Chronic Effect of Saikosaponin d (Sd) on the Activity of the Whole Pituitary–Adrenal Cortex System and the Adrenal Cortex in Normal and Dexamethasone (Dex)-Treated Rats

Repeated treatment (mg/kg/d × 4 d)	Acute treatment[a]			
	Saline[b] (5 ml/kg)	Sd[b] (2.5 mg/kg)	Histamine[b] (5.0 mg/kg)	ACTH[b] (0.035 mg/kg)
Plsama corticosterone (μg/100 ml)				
Saline	22.3±2.5	37.2±5.3[d]	34.2±2.2[e]	28.6±2.6
+Sd 2.5	15.5±2.0	28.3±6.9	31.6±2.6[f]	35.1±2.0[f]
p	<0.1	NS	NS	<0.1
Dex 0.025	14.8±0.8	28.0±1.2[f]	24.7±2.8[e]	26.0±2.9[e]
+Sd 1.0	15.5±1.1	21.1±1.6[d]	20.8±2.8	24.5±2.0[e]
p	NS	<0.01	NS	NS
Plasma glucose (mg/100 ml)				
Saline	136±5	195±17[e]	177±9[e]	144±3
+Sd 2.5	137±4	136±4	182±4[f]	127±4[e]
p	NS	<0.01	NS	<0.01
Dex 0.025	153±4	244±19[f]	214±16[e]	153±8
+Sd 1.0	158±3	164±7	197±19[c]	129±9[d]
p	NS	<0.01	NS	<0.1

a) The number of rats was 6 in each group. *b*) A 30-min treatment 24 h after the 5th treatment. *c*—*f*) $p<0.1$, $p<0.05$, $p<0.01$, $p<0.001$ *vs.* basal level.

equal to that of the whole pituitary–adrenal cortex system or the hypothalamic–pituitary system on the final day.

In saikosaponin-treated rats with or without dexamethasone, acute saikosaponin treatment induced an increase in plasma corticosterone, but it did not induce hyperglycemia, whereas histamine did both (Table V). This may suggest that disappearance of saikosaponin-induced hyperglycemia was not due to loss of saikosaponin (*e.g.* by rapidly metabolic degradation) or glucose stock, and arose from an acceleration of glucose consumption and/or inhibition of glucose formation. In control rats, ACTH treatment did not affect plasma glucose, but in previously saikosaponin-treated rats it decreased the plasma glucose level ($p<0.1$, $p<0.01$). This result may also suggest that previous treatment with saikosaponin accelerated process(es) of glucose disappearance, and/or inhibited that (those) of glucose accumulation.

Discussion

In rats treated with 2.5 mg/kg/d of saikosaponin d for 5 d, saikosaponin d significantly increased the relative weight of the adrenals and decreased the thymus weight, whereas saikosaponin did not significantly affect basal and maximum levels of plasma corticosterone on day 5 (Table IVa and b). In rats treated with the same dose of saikosaponin for 2, 3, 4 or 5 d, basal and maximum levels of corticosterone were almost equal to normal levels, but saikosaponin tended to decrease them 24 h after the final treatment, especially on day 4 or 5 (Table I). However, in rats treated with the same doses of saikosaponin, the capability of the hypertrophied adrenal cortex for corticosterone secretion was shown to be higher than that of the whole pituitary–adrenal cortex system on day 5 (Table V). Therefore, it was clear that the hypertrophied adrenals retained maximum capacity of corticosterone secretion even on day 5. These results suggest that daily administration of saikosaponin induced daily extra secretion of ACTH at the maximum level in the early phase, and that the daily increase in ACTH due to saikosaponin induced extra increase in adrenal weight and extra secretion of corticosterone, which resulted in atrophy of the thymus.

Recently, ACTH has been shown to be derived from a precursor, proopiomelanocortin, which contains lipotropin and N-termianl glycopeptide.[10] The N-terminal peptides of proopiomelanocortin (N-POC) (1—28) and (2—59), were active in causing adrenal growth and deoxyribonucleic acid (DNA) synthesis,[11] while synthetic ACTH inhibited compensatory adrenal growth.[12] Therefore, saikosaponin might concomitantly release ACTH and the N-terminal peptides from the corticotrophs, and then saikosaponin may induce adrenocortical hyperplasia as well as hypertrophy.

In rats treated with 2.5 mg/kg/d of saikosaponin, the 5-d treatment with saikosaponin induced a 23% increase in adrenal weight, but a 9-d treatment give only a 13% increase (Table IVa). Maximum activity of the pituitary–adrenal system on day 5 (96%) decreased to 49% on day 9. Nine administrations of saikosaponin might represent an "over-dosage." The 6th to 9th treatments might induce ACTH and corticosterone secretion as the 1st to 5th did, but in less effectively. "Over-secretion" of ACTH due to saikosaponin in the early phase might induce "over-production" of corticosterone, which would result in suppression of the pituitary by negative feedback action of glucocorticoid. The possible suppression of the pituitary or decrease in ACTH secretion might reduce adrenal growth and adrenalcortical activity in the late phase. The present results support the antigranulomatous action of saikosaponin d,[4] and the potentiating action of saikosaponin d on antigranulomatous action of dexamethasone,[5] and may be compatible with the lack of effect of saikosaponin d on the basal level of plasma 11-OH corticosteroid level[4] and the adrenal weight.[4,5]

In dexamethasone-treated rats (0.025 mg/kg/d), the basal level of corticosterone was

slightly decreased and the stimulated level was significantly decreased, but the latter was significantly higher than the former (Tables IVb and V). In the rats, the corticosterone secretion-inducing activity of the pituitary–adrenal system was almost equal to that of the adrenal cortex (Table V). Co-administration of 1.0 or 2.5 mg/kg/d of saikosaponin and dexamethasone for 5 d affected neither the basal nor the stimulated level of corticosterone (Tables IVb and V). Therefore, these results showed that in rats chronically treated with dexamethasone, co-administration of saikosaponin could induce a significant increase in corticosterone secretion, but even the high dose of saikosaponin could not completely antagonize the suppression by 0.025 mg/kg/d of dexamethasone, while in the acute experiment, the dose of saikosaponin completely released the suppression by 0.25 mg/kg of dexamethasone of corticosterone secretion.[2] These results suggested that in the acute experiment, saikosaponin could competitively antagonize dexamethasone in processes of ACTH secretion,[13] but in the chronic experiment saikosaponin could not antagonize dexamethasone in processes of messenger ribonucleic acid (mRNA) synthesis of ACTH precursor.[14,15] The main site of action of saikosaponin might be similar to but distinct from that of dexamethasone. This remains to be elucidated.

In rats treated with 0.05 mg/kg/d of dexamethasone for 9 d, the stimulated level of plasma corticosterone was higher than the basal level, but the difference was not statistically significant. In rats treated with saikosaponin and dexamethasone for 9 d, the stimulated level of corticosterone was lower than the basal level in dexamethasone-treated rats. These result shows that in rats treated with 0.05 mg/kg of dexamethasone for 9 d, stimulation due to saikosaponin did not occur on day 9, while in rats treated with 0.025 mg/kg of dexamethasone for 5 d daily stimulation of the pituitary due to saikosaponin was still effective on day 5. Therefore, in order to obtain the maximum efficacy it is necessary to carefully select the dose of saikosaponin, as well as duration and dosage schedule, especially in relation to the dose and class of glucocorticoid.[16] However, it seems clear that chronic treatment with saikosaponin was effective to stimulate or to maintain activity and growth of the adrenal cortex, and that saikosaponin itself did not have any direct suppressive action on the hypothalamic–pituitary system and thymus.[3] Therefore, saikosaponin is suggested to be a useful drug for reducing the dose of glucocorticoid and preventing glucocorticoid-induced adrenal insufficiency in therapy, because it appears to be a corticotropin releasing factor (CRF)-like, ACTH-like and corticosterone-like substance, which has no direct suppressive action on the hypothalamic–pituitary system, and does not directly affect the hormone action of ACTH and glucocorticoids.

Administration of saikosaponin d induced a transient hyperglycemia and an increase in plasma ACTH and corticosterone which occurred independently but concomitantly.[1] In the present chronic experiment, it was found that the hyperglycemic response disappeared in rats treated previously with saikosaponin d, while the increase in plasma corticosterone remained. In the saikosaponin d-treated rats, histamine normally induced marked hyperglycemia, but ACTH induced hypoglycemia. The saikosaponin d-induced phenomena *in vivo*, therefore, may represent a kind of refractoriness or heterologous desensitization. Detailed studies on the phenomenon are in progress.

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Improvement in Percutaneous Absorption of Prednisolone by β- and γ-Cyclodextrin Complexations

KANETO UEKAMA,* KAZUHIKO ARIMORI, ATSUSHI SAKAI, KATSUHIRO MASAKI, TETSUMI IRIE, and MASAKI OTAGIRI

Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1, Oe-honmachi, Kumamoto 862, Japan

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In vitro release characteristics of prednisolone (PD) and its β- and γ-cyclodextrin (β- and γ-CyD) complexes were investigated by using an ointment release simulator with artificial double-layer membranes. The release of PD from hydrophilic ointment was significantly improved by β- and γ-CyD complexations. Permeation and uptake studies indicated that the enhanced release of PD from the ointment may be mainly due to the faster dissolution of PD in the base and the lower binding affinity of PD to the ointment base as a result of the CyD complexations. The percutaneous absorption of PD from hydrophilic ointment after application to the rabbit skin was also increased by CyD complexations. The *in vitro* and *in vivo* data suggest that CyDs can improve the topical bioavailability of PD.

Keywords——prednisolone; β-cyclodextrin; γ-cyclodextrin; inclusion complex; membrane permeation; ointment release; percutaneous absorption; rabbit; hydrophilic ointment

Introduction

Since the introduction of topical corticosteroid formulations, their use has become widespread because of the potent antiinflammatory activity of these drugs. The activities of topical corticosteroids are expected to depend upon the physicochemical properties of the drugs, such as solubility and partition coefficient.[1] Moreover, it was found that the release of drugs, including steroid hormones, from topical dosage forms is affected by the composition of the vehicle and the thermodynamic activity of the drugs in the vehicle.[2] We have previously reported that the release rate of betamethasone from gel and hydrophilic ointments was significantly improved by inclusion complexation with β- and γ-cyclodextrins (β- and γ-CyDs).[3] It was also shown that the apparent rates of dissolution and membrane permeation of prednisolone (PD) were greatly improved by β- and γ-CyD complexations, resulting in increased serum levels of the drug following oral and rectal administrations.[4] Thus, the present paper deals with the effects of β- and γ-CyDs on the release rate of PD from hydrophilic ointment *in vitro*, in comparison with *in vivo* data.

Experimental

Materials——PD was donated by Nakarai Chemicals Ltd. (Kyoto, Japan), and recrystallized from ethanol–water. β- and γ-CyDs were purchased from Nihon Shokuhin Kako Co., Ltd. (Tokyo, Japan), and recrystallized from water. All other materials and solvents were of analytical reagent grade. Deionized and double-distilled water was used throughout the study. The solid complexes of PD with β- and γ-CyDs in the molar ratio of 1:2 and 2:3 were prepared in the same manner as previously described.[5]

Ointment Release Studies——Hydrophilic ointment base was prepared according to JPX. After being passed through a 100 mesh sieve, CyD complexes were kneaded thoroughly with the base, and the content of PD was adjusted to 1.0% in the base. By means of polarized microscopic observations (× 1000), fine solid particles of PD or

its CyD complexes were found to be distributed in both the inner and outer phases of o/w type ointment. The release of PD from ointment bases containing PD or its complexes was determined by using an ointment release apparatus (Sartorius Co., Ltd., Göttingen, FRG) with artificial double-layer membranes, as previously reported.[3] The temperature of the release phase (100 ml of saline) was held at 34 °C. At appropriate intervals, 1 ml samples were removed from the release phase, and mixed with 5 ml of methylene chloride. After centrifugation (2000 rpm, 10 min), the organic phase (3 ml) was transferred to a 15-ml glass-stoppered centrifuge tube and evaporated to dryness at 40 °C. The extract was reconstituted in 100 μl of methanol and then 10 μl of the solution was injected into a high-performance liquid chromatography (HPLC) equipped with a LiChrosorb RP-18 column (10 μm in 4.6 i.d. × 250 mm, Merck) operating at a flow rate of 1.0 ml/min, with methanol–acetonitrile–water mixture (3 : 3 : 4) as a mobile phase. The eluate was monitored spectrophotometrically at 248 nm by measuring the peak height in comparison with those of known amounts of internal standard (cortisone acetate).

Membrane Permeation Studies——The permeation behavior of PD through the double-layer membranes was examined by using the permeation cell apparatus described previously.[6] The artificial double-layer membranes used were the same as those in the ointment release experiments. The sample powder (100 mg) of PD or an equivalent amount of β- or γ-CyD complex was placed in 50 ml of saline solution in a donor cell. The solution in the permeation cell was stirred with a magnetic bar at 91 rpm at 34 °C. At appropriate intervals, 1 ml samples were pipetted from the receptor solution and extracted with 4 ml of chloroform. After centrifugation (2000 rpm, 10 min), the organic phase (3 ml) was transferred to a new tube, and the solvent was evaporated off on a water bath at 40 °C under reduced pressure. The residue was dissolved in 100 μl of methanol and assayed for PD by HPLC, as described above. Corrections were made for the cumulative dilution caused by replacement of samples with equal volumes of the original medium.

Uptake by Ointment Base——The general procedure is essentially the same as that of Nakano and Patel.[7] The ointment base was packed in one compartment cell and a 100 ml portion of 4.7×10^{-4} M PD solution in the absence or presence of 9.4×10^{-4} M β-CyD or 7.1×10^{-4} M γ-CyD was placed in the release compartment. The decrease in PD content of the release solution was determined by HPLC, as described above.

***In Vivo* Studies**——Five rabbits weighing 2.7—3.0 kg were used at intervals between applications of more than two weeks. The hair was removed with electric hair clippers from the intended dosing region of the back, 24 h prior to application of the ointment. The ointment (50 mg) containing PD or its CyD complexes were spread uniformly over the surface of six sheets of thin plastic films (2×2 cm^2), and the sheets were applied to the shaved surface of the dorsal skin of rabbits. To ensure close contact between the ointment and the skin, the films were covered with adhesive tape. The ointment samples were recovered periodically from the dorsal skin by wiping with absorbent cotton. The ointment samples and the absorbent cotton were then transferred to a new tube and dissolved with 10 ml of methanol with sonication for 30 min. After centrifugation (2000 rpm, 10 min), the supernatant was discarded and a 10 μl aliquot of the aqueous layer was assayed by HPLC as described above. In the pretreatment experiment, the ointment containing β-CyD alone was applied on the rabbit skin. After the removal of that ointment, the ointment containing PD was then applied, as described above.

Results and Discussion

Drug Release from Ointment Base

The release behavior of the β- and γ-CyD complexes from the hydrophilic ointment base was compared with that of PD alone. Figure 1 shows the amount of PD released from hydrophilic ointments containing PD or its complexes as a function of the square root of time. It is evident that the release rate of PD was significantly increased by complexation, particularly with β-CyD. The linearity of the plots, except for the initial delay in the case of the β-CyD complex, may indicate that release of PD is diffusion-controlled.[8] However, there was no detectable amount of CyDs in the release phase under these experimental conditions. This suggests that only the free form of the drug can penetrate into the release phase from the ointment base through the artificial membranes.

To gain insight into the mechanism of enhanced drug release due to β- and γ-CyD complexations, uptake and membrane permeation studies were carried out. The drug uptake from saline solution through a cellophane membrane into the hydrophilic ointment was measured to evaluate conveniently the relative affinities of the drug and its complexes for the base in a manner similar to that reported recently.[9] As shown in Fig. 2, the uptake of PD from the complexes into the base was fairly slow compared with that of the drug itself. Figure 3 shows the permeation profiles of PD through the artificial double-layer membranes

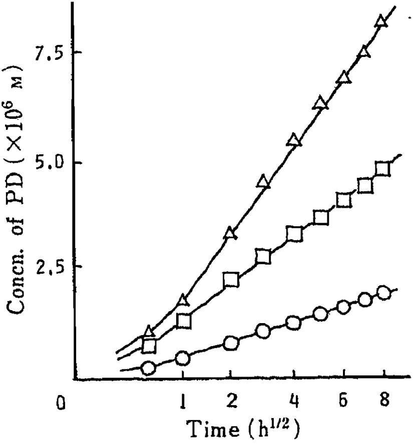

Fig. 1. Release Profiles of PD from Hydrophilic Ointment Containing PD or Its CyD Complexes in Normal Saline Solution at 34 °C

○, PD alone; △, β-CyD complex; □, γ-CyD complex.

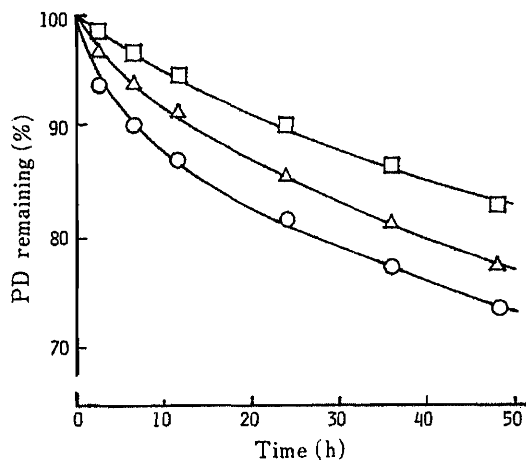

Fig. 2. Uptake of PD or Its CyD Complexes into Hydrophilic Ointment Base at 34 °C

○, PD alone; △, β-CyD complex; □, γ-CyD complex.

following the dissolution from PD or its complexes in the donor cell. The rapid dissolution of β- and γ-CyD complexes resulted in an increase in the net amount of PD permeating into the receptor cell. The bulky and hydrophilic complexes seem to have poorer permeability because the permeation through the double-layer membrane is mainly pore size- and partition-controlled.[10,11] In fact, the increase in the permeation rate of the complexes was small compared with that expected from the dissolution profiles.[4] Therefore, the rapid dissolution of the complexes may overcome the negative effect of the poor permeability, resulting in a net increase in drug permeation.

These results indicate that the enhancement of drug release may be mainly ascribable to the decrease in binding affinity of PD to the inner phase of the o/w type ointment together with the increase in the dissolution rate of PD in the outer phase of the ointment, owing to the hydrophilic CyD complex formation.

In this regard, it is anticipated that PD in CyD complexes may be displaced by some components of the ointment base. Microscopic observation revealed that small amounts of PD crystallites were present in the ointment containing CyD complexes. However, it seems likely that the extent of displacement is not significant because of the relatively large stability constant of PD–CyD complexes (3600 M^{-1} for β-CyD complex and 3240 M^{-1} for γ-CyD complex), as reported previously.[5] In fact, it is apparent from Fig. 1 that β-CyD complex (having a larger stability constant) is much more effective in enhancing the drug release than γ-CyD complex.

***In Vivo* Studies**

From the *in vitro* observations, it was suggested that β- and γ-CyDs may be useful to improve the topical bioavailability of PD from ointment preparations. Therefore, as a preliminary study on percutaneous absorption, the decrease in PD concentration in the ointment base was measured after application of the ointment to the rabbit skin. Figure 4 shows the time course of the residual amount of PD in the ointment base after the application of hydrophilic ointment containing PD or its complexes in the dorsal region of rabbits. As shown in Fig. 4, application of both CyD complexes resulted in extensive elimination of PD from the ointment base as compared with the drug alone. The average amounts of PD eliminated from the ointment base at 24 h after application of β- and γ-CyD complexes to

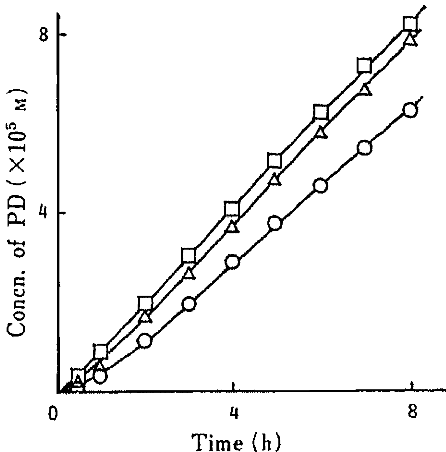

Fig. 3. Permeation Profiles of PD from Suspensions of PD Powder or Its CyD Complexes through Artificial Double-Layer Membranes in Saline Solution at 34 °C

○, PD alone; △, β-CyD complex; □, γ-CyD complex.

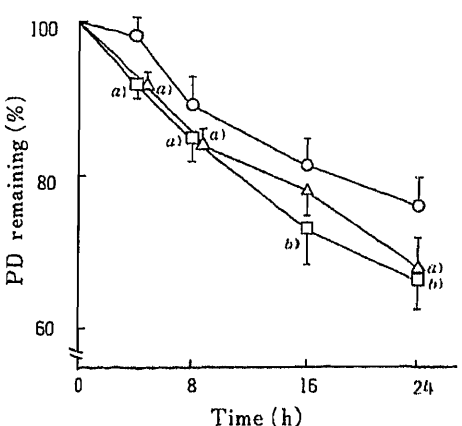

Fig. 4. Elimination Profiles of PD from Ointment Base after Application of the Hydrophilic Ointment Containing PD or Its CyD Complexes in Rabbits

○, PD alone; △, β-CyD complex; □, γ-CyD complex. Each value is the mean ± S.E. of 5 rabbits. *a*) $p<0.05$, *b*) $p<0.01$ in (△, □) *versus* (○).

rabbits were 31.5% and 33.4% of the dose, respectively, while that in the case of the drug alone was 23.2%. Although little difference was found between β- and γ-CyD complexes, the degree of elimination of the drug *in vivo* was well correlated with that in the *in vitro* release studies. Interestingly, it was observed that pretreatment of rabbit skin with CyDs did not significantly alter the elimination rate of PD from the ointment base. Therefore, the superior percutaneous absorption expected for CyD complexes may be mainly owing to the faster dissolution and the lower binding affinity of the complex to the ointment bases rather than to direct interaction of CyD with the skin. However, recent studies have demonstrated that hydrophobic derivatives of CyDs such as dimethyl-β-CyD significantly enhanced the percutaneous absorption of drugs owing to the extraction of membrane components, which may result in modification of the skin barrier.[12,13] In this regard, detailed data on the PD–dimethyl-β-CyD system will be reported elsewhere.

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Influence of Diethyl Maleate-Induced Loss of Thiols on Cefmetazole Uptake into Isolated Epithelial Cells and on Cefmetazole Absorption from Ileal Loop of Rats

TOSHIAKI NISHIHATA,* TAKUMI SUZUKA, ATSUSHI FURUYA, MASARU YAMAZAKI, and AKIRA KAMADA

Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565, Japan

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Uptake of cefmetazole into isolated rat intestinal epithelial cells increased with increasing nonprotein thiol loss induced by diethyl maleate (DEM) at concentrations up to 1 mM. However, 5 mM DEM decreased cefmetazole uptake, accompanied with a loss of protein thiol, despite the loss of nonprotein thiol. The coaddition of calmodulin inhibitors such as trifluoperazine (TFPZ) and chlorpromazine (CPZ) with 5 mM DEM restored cefmetazole uptake and inhibited protein thiol loss. The administration of DEM at 0.75 mM into the lumen of a rat ileal loop increased cefmetazole absorption along with loss of nonprotein thiol in the tissue. However, significant protein thiol loss occurred on administration of 7.5 mM DEM, and cefmetazole absorption was decreased. The appearance of cefmetazole in plasma after an injection of cefmetazole into connective tissue of the ileal loop was also inhibited by preadministration of 7.5 mM DEM into the loop. Coadministration of calmodulin inhibitors at a concentration of less than 100 μM with 7.5 mM DEM inhibited protein thiol loss but not nonprotein thiol loss. Coadministration of calmodulin inhibitors with 7.5 mM DEM increased cefmetazole absorption from the ileal lumen, and restored cefmetazole appearance in the plasma after injection into connective tissue. Thus, nonprotein thiol loss in cells and tissue of the intestine increased cefmetazole absorption from the mucosal lumen into the blood circulation, but protein thiol loss inhibited it.

Keywords——intestinal absorption; ileal loop; isolated epithelial cell; connective tissue; cefmetazole; diethyl maleate; calmodulin inhibitor; nonprotein thiol; protein thiol

It has been demonstrated that significant nonprotein thiol loss induced by a sulfhydryl depleter is accompanied with ulceration in the stomach[1] and with a decrease of viability of hepatocytes.[2] Further, it has been reported that the decrease of hepatocyte viability might be related to protein thiol loss and/or to an increase of cytosolic free Ca^{2+} concentration rather than to nonprotein thiol loss,[3,4] though a significant nonprotein thiol loss induced protein thiol loss by oxidation and also increased cytosolic free Ca^{2+} by inhibiting microsomal Ca^{2+} sequestration.[5] Thus, it is considered that both nonprotein and protein thiols play important roles in maintaining cell integrity. It has also been reported[6] that glutathione (a major endogenous nonprotein thiol) mediated the active transport of L-amino acid in the small intestine. However, it is not clear how both thiols in the intestine affect the permeability of the intestinal mucosal membrane (and thus the passive transport of compounds).

It is known that passive transport of hydrophilic compounds through living intestinal mucosal membrane is poor, though the cell membrane permeability to hydrophilic compounds increases after death.[7] It has been reported[8] that rat intestinal absorption of cefmetazole, though poor, occurred predominantly through the tight junctional area of intestinal epithelium. We have recently reported that nonprotein thiol loss in intestinal tissue induced by diethyl maleate (DEM) is accompanied with an increase of intestinal permeability to cefmetazole, a hydrophilic drug,[9,10] but we did not measure protein thiol levels or cell

viability in the intestine in the presence of DEM. Since significant nonprotein thiol loss in the intestine may cause protein thiol loss and cell death, it is of importance to investigate whether the increase of intestinal cefmetazole transport by DEM observed in the previous studies[9,10] is related to nonprotein thiol loss, protein thiol loss or cell death.

In the present report, we investigated the effect of DEM on both nonprotein and protein thiols in isolated rat intestinal epithelial cells and in rat ileal tissue. Further, changes of cefmetazole uptake into the cells and of cefmetazole absorption from the ileal loop were studied concomitantly.

Experimental

Materials——DEM was obtained from Sigma Inc. (St. Louis, U.S.A.). Sodium cefmetazole was supplied by Sankyo Co., Ltd. (Tokyo, Japan). Trifluoperazine hydrochloride (TFPZ), trifluoperazine sulfoxide (TFPZ-sulfoxide), chlorpromazine hydrochloride (CPZ), chlorpromazine sulfoxide (CPZ-sulfoxide) were supplied by Yoshitomi Pharmaceutical Industry (Osaka, Japan). *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) was obtained from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Other reagents used were of analytical grade.

Animals——Wistar male rats, 180 to 200 g, were fasted for 16 h prior to experiments, but water was given freely.

Isolation and Incubation of Small Intestinal Epithelial Cells——Isolation of rat small intestinal epithelial cells was performed by the method of Levine and Weintraub.[11] Briefly, the small intestine of a rat was excised, and immediately placed in a beaker of physiological saline at 4 °C. A segment of the intestine was everted over a glass spiral and was immersed in saline, in a cylinder. The cylinder was vibrated to obtain a suspension of isolated epithelial cells. After centrifugation of the fluid at $500 \times g$ for 5 min, cells were collected and resuspended in Krebs–Henseleit buffer (pH 7.4), which was saturated with O_2 and CO_2 gases (95% : 5%). Incubation of this cell suspension in the present study was performed at 30 °C (this was because cell viability decreased on prolonged incubation at 37 °C). Although isolated intestinal epithelial cells included both columner tip cells and rounded crypt cell in approximately equal numbers, the present study was performed without distinction of cell types. In the present method, in which medium without albumin and trypsin was used, some aggregation of cells was observed. However, it is considered that addition of albumin influences the concentration of free additives, and the addition of trypsin decreased the cell viability rapidly. Thus, the present study was performed in medium without albumin and trypsin despite some aggregation of isolated cells.

The cell viability was assayed by the trypan blue exclusion method.[11,12] Briefly, after incubation of cells in the presence of DEM, trypan blue (0.4% in saline) was added directly to the cell suspension at a final concentration of 0.01%. Within 10 min the numbers of cells that excluded the dye were counted, using an inverted microscope at $\times 200$ magnification. There is some controversy concerning the determination of cell viability by the trypan blue exclusion method, because of its low sensitivity. However, since this method can be performed rapidly and is widely accepted, cell viability was determined by this method in the present study. Thus, the cell viability determined in this study represents the ability of cell to exclude trypan blue.

To investigate the effect of DEM on nonprotein thiol and protein thiol in cells, cells were collected by rapid centrifugation after incubation in the medium containing DEM. The cells were resuspended in saline and homogenized.

Uptake of Cefmetazole into Isolated Cells——At 5 min after the start of incubation of the cells in the medium containing DEM, cefmetazole (200 mM in the buffer) was added in the cell suspension at a final concentration of 4 mM. After further incubation for 2 min, the cells were separated from the medium containing cefmetazole by rapid centrifugation through a suspension of Percoll® (final density: 1.06 g/ml) in saline solution.[3] After homogenation of the cell suspension, cefmetazole was assayed by the method described below.

Ileal Loop Study——A rat was anesthetized with sodium pentobarbital (30 mg/kg, i.p.), and kept on a hot plate at 38 °C. Cefmetazole absorption from the ileal loop was measured by the method described previously.[13] Briefly, after middle abdominal incision, a 10 cm ileal segment (proximal to the large intestine) was ligated at both ends with thread. Then 0.5 ml of saline containing 6 mg of sodium cefmetazole was administered at 30 min after an administration of 1.5 ml of saline containing DEM into the lumen of the ileal loop. Blood samples were collected from the jugular vein at designated time intervals for 3 h to obtain plasma by centrifugation.

In a separate study, after administration of 1.5 ml of saline containing DEM into the loop, a segment of ligated loop was removed at 0.5, 1, 2, 3 and 5 h, and homogenized in saline to assay both nonprotein and protein thiols.

In another series of experiments, 20 μl of saline containing 0.5 mg of sodium cefmetazole was injected into connective tissue of the ileal loop at 30 min after administration of 1.5 ml of saline containing DEM into the lumen of the ileal loop, and then blood samples were collected from the jugular vein at 15, 30 and 60 min to obtain plasma.

Assay Procedures——Assays of nonprotein and protein thiols were performed by the method of Di Monte *et al.*[14] Briefly, trichloroacetic acid was added to the homogenate at a final concentration of 3%. After centrifugation,

acid-soluble thiol in the supernatant was designated as nonprotein thiol, and acid-precipitated thiol in sediment was designated as protein thiol. Next, 4 ml of 0.5 M Tris–HCl buffer (pH 7.4) containing 100 μM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) was added to 0.5 ml of the supernatant and to the sediment to assay thiol content by the colorimetric method at 420 nm. Protein content in the homogenate was determined by the method of Lowry *et al.*[15] Assay of cefmetazole was carried out by high-performance liquid chromatography as described previously.[16]

Statistical Analyses——Statistical analyses were performed by means of Student's *t*-test.

Results and Discussion

Uptake of Cefmetazole into Isolated Intestinal Epithelial Cells

The viability of the isolated cells decreased with increase of DEM concentration in the medium (Table I). Although DEM over 5 mM caused a significant decrease of cell viability at 45 min after the incubation, the use of 5 mM DEM was required to investigate the effect of

TABLE I. Effect of DEM on Viability of Isolated Small Intestinal Epithelial Cells

Additive DEM Concn. of DEM (mM)	Viability (%) Time after incubation (min)		Additive	Viability (%) Coadministration of calmodulin inhibitors[a]	
	20	45		With 0.5 mM DEM	With 5 mM DEM
No additive	88.2 ± 3.6	85.3 ± 2.9	50 μM TFPZ	89.2 ± 5.9	82.1 ± 3.6
0.025	86.2 ± 4.7	89.0 ± 7.3	50 μM TFPZ-sulfoxide	85.1 ± 3.9	86.4 ± 4.9
0.10	84.6 ± 2.7	86.1 ± 3.1	50 μM CPZ	84.6 ± 4.1	88.2 ± 5.1
0.50	87.4 ± 3.6	84.7 ± 3.8	50 μM CPZ-sulfoxide	89.3 ± 6.2	87.3 ± 3.9
1.0	86.2 ± 2.8	81.6 ± 3.9	50 μM W-7	85.7 ± 4.6	84.2 ± 4.3
5.0	86.8 ± 5.0	64.3 ± 9.4[c]			
10.0	75.1 ± 6.1	32.6 ± 18.3[d]			
15.0	61.4 ± 10.9[d]	24.6 ± 16.8[d]			
50.0[b]	5.1 ± 2.4[d]	2.7 ± 1.9[d]			

Each value represents the mean ± S.D. ($n=4$ to 6). *a*) Incubation time: 20 min. *b*) DEM was in suspension, because the solubility of DEM was only about 19 mM in the medium at 30 C. *c*) $p<0.05$ *versus* no additive. *d*) $p<0.01$ *versus* no additive.

TABLE II. Effect of DEM on Thiols[a] and on Cefmetazole Uptake[b] in the Isolated Cells

Concn. of DEM (mM)	Nonprotein thiol (μmol/g-protein)		Protein thiol (μmol/g-protein)		Cefmetazole uptake (nmol/g-protein)
	Time after incubation (min)				
	5	20	5	20	2
0	14.2 ± 2.1	12.6 ± 2.1	29.7 ± 1.4	31.9 ± 2.1	0.37 ± 0.06
0.025	11.4 ± 1.6	9.7 ± 2.1	32.4 ± 1.9	30.2 ± 2.7	0.40 ± 0.09
0.10	9.2 ± 1.9[d]	7.8 ± 1.4[d]	33.2 ± 3.1	29.1 ± 1.9	0.56 ± 0.07[e]
0.50	6.9 ± 3.2[d]	6.1 ± 1.7[d]	32.6 ± 2.9	29.8 ± 4.1	0.62 ± 0.11[d]
1.0	6.2 ± 1.4[d]	5.8 ± 2.6[d]	33.2 ± 4.6	27.5 ± 1.0	0.70 ± 0.13[d]
5.0	4.3 ± 1.7[d]	3.8 ± 1.1[d]	20.6 ± 4.0[d]	18.2 ± 3.7[d]	0.22 ± 0.04[e]
10.0	3.2 ± 0.7[d]	No study	19.4 ± 2.5[d]	No study	1.52 ± 0.26[c,d]

Each value represents the mean ± S.D. ($n=3$ to 5). *a*) Thiols in isolated intestinal epithelial cells were measured at 5 or 20 min after the start of incubation with DEM. *b*) Cefmetazole uptake was measured at 2 min after addition of cefmetazole (4 mM) to cell suspension which had been incubated with DEM for 5 min. Before the incubation nonprotein thiol was 13.9 ± 1.7 μmol/g-protein and protein thiol was 31.2 ± 2.4 μmol/g-protein. *c*) Cells were preincubated with 10 mM DEM for 45 min before addition of cefmetazole in order to investigate the effect of cell death on cefmetazole uptake (Table I). *d*) $p<0.01$ *versus* no additive. *e*) $p<0.05$ *versus* no additive.

DEM-induced protein thiol loss (Table II) on cefmetazole uptake in living cells. Since cell viability was more than 80% at 20 min after the start of incubation even in the presence of 5 mM DEM (Table I), an uptake study was performed within 20 min after addition of DEM.

The addition of 0.1, 0.5 or 1.0 mM DEM in the medium caused rapid nonprotein thiol loss, but did not affect protein thiol levels in the isolated cells (Table II). The addition of DEM at 5 mM or more caused loss of both nonprotein and protein thiols within 5 min. Thus, the uptake of cefmetazole into isolated cells was examined by the addition of cefmetazole to the cell suspension after incubation with DEM for 5 min, as described in the experimental section. The cefmetazole uptake at 2 min after addition increased greatly with increase of DEM concentration up to 1 mM in the medium (Table II). A large loss of nonprotein thiol in the cells seems to result in a large uptake of cefmetazole into the cells. However, preincubation with 5 mM DEM decreased cefmetazole uptake. Thse observations may indicate that nonprotein thiol loss increases the cefmetazole uptake into the isolated cells, but protein thiol loss decreases it (even in the case of nonprotein thiol loss) in isolated, living intestinal epithelial cells.

It has been reported that a significant nonprotein thiol loss caused by various toxic agents leads to an alteration of intracellular Ca^{2+} homeostasis in hepatocytes.[3,4,14] It was also suggested that protein thiol loss might cause an increase of cytosolic free Ca^{2+} through an inhibition of Ca^{2+}-adenosine triphosphatase (ATPase) activity. We have further reported that a change of intestinal mucosal permeability was observed along with Ca^{2+} release from intestinal mucosa after nonprotein thiol loss, in the presence of 1 mM DEM in the Ca^{2+}- and Mg^{2+}-free mucosal medium of an *in vitro* everted intestinal sac.[8] Thus, it is suggested that the change of cell membrane permeability of cells in the presence of DEM is related to a change of intracellular Ca^{2+} homeostasis, as a secondary effect after nonprotein thiol loss and/or protein thiol loss. Therefore, it is of interest to investigate the effect of calmodulin inhibitors on the cell membrane permeability of the isolated cells.

The effect of calmodulin inhibitors was examined in the concentration range of less than 100 μM, since they are known to inhibit calmodulin-dependent enzyme activity in this concentration range.[17] The coaddition of each calmodulin inhibitor at 50 μM with DEM at

TABLE III. Effect of Calmodulin Inhibitors on Thiols[a] and on Cefmetazole Uptake[b] in Isolated Intestinal Epithelial Cells

Additive	Nonprotein thiol (μmol/g-protein)	Protein thiol (μmol/g-protein)	Cefmetazole uptake (nmol/g-protein)
In the presence of 0.5 mM DEM			
50 μM TFPZ	7.2±1.9	32.4±2.9	0.68±0.14
50 μM TFPZ-sulfoxide	6.1±2.7	33.1±4.3	0.62±0.04
50 μM CPZ	7.4±3.0	32.6±1.0	0.69±0.07
50 μM CPZ-sulfoxide	5.9±3.1	33.4±5.1	0.66±0.15
50 μM W-7	6.2±0.5	32.6±3.9	0.71±0.11
In the absence of DEM			
50 μM TFPZ	14.6±2.7	30.2±2.2	0.36±0.07
50 μM TFPZ-sulfoxide	12.9±1.4	35.2±5.6	0.42±0.05
50 μM CPZ	13.2±0.6	29.6±1.3	0.33±0.11
50 μM CPZ-sulfoxide	12.5±2.7	33.0±3.8	0.35±0.07
50 μM W-7	13.9±0.6	32.7±4.2	0.39±0.02

Each value represents the mean±S.D. (n=3 to 4). *a*) Thiols were determined at 5 min after the incubation. *b*) Cefmetazole uptake was determined at 2 min after addition of cefmetazole (4 mM) to cell suspension which had been incubated with calmodulin inhibitor and DEM for 5 min.

0.5 or 5 mM in the medium did not affect cell viability (Table I). The addition of each calmodulin inhibitor at 50 μM to the cell suspension also did not affect the cefmetazole uptake in the absence or presence of 0.5 mM DEM (Table III; 50 μM calmodulin inhibitor was used because it was sufficient to inhibit the effect of 5 mM DEM, as described below). The inhibitors did not affect nonprotein or protein thiols in the presence of 0.5 mM DEM.

TFPZ, CPZ and W-7 inhibited protein thiol loss induced by 5 mM DEM (Fig. 1A) in spite of having no effect on nonprotein thiol loss (data not shown), and also increased cefmetazole uptake in comparison with that in the absence of DEM (Fig. 1B). However, TFPZ-sulfoxide and CPZ-sulfoxide even at 100 μM did not inhibit the protein thiol loss, and did not increase cefmetazole uptake (Fig. 1); it has been reported that TFPZ-sulfoxide and CPZ-sulfoxide did not inhibit calmodulin-dependent enzyme activity at 100 μM.[17]

The above observations seem to indicate that nonprotein thiol loss in living epithelial cells is involved in the increase of cell membrane permeability to cefmetazole when protein thiol does not decrease, *i.e.*, when DEM at a concentration of less than 1 mM was present in the medium or a calmodulin inhibitor was present with 5 mM DEM. However, protein thiol loss even in the case of nonprotein thiol loss decreased the permeability of the cell membrane to cefmetazole. The concentration dependency of the inhibitory effect of calmodulin inhibitors on the 5 mM DEM-induced protein thiol loss was similar to that of their inhibitory effect on calmodulin-dependent cap formation of lymphocyte.[17] Thus, it is considered that protein thiol loss induced by 5 mM DEM occurs by a calmodulin-dependent mechanism, and the loss is probably induced by an increase of cytosolic free Ca^{2+} in the presence of 5 mM DEM, *i.e.*, protein thiol loss may arise from the association of calmodulin with Ca^{2+}, which dissociates the cytoskeleton related calmodulin-binding protein from the cytoskeleton, as suggested in the cap formation of lymphocytes.[17,18] Further, it is qualitatively considered that protein thiol loss is related directly to the decrease of cell membrane permeability to cefmetazole. Thus, the decrease of cell membrane permeability by protein thiol loss may be due to conformational change of the cytoskeleton, because a conformation change of a structural protein is often controlled by formation or dissociation of disulfide bridge(s).

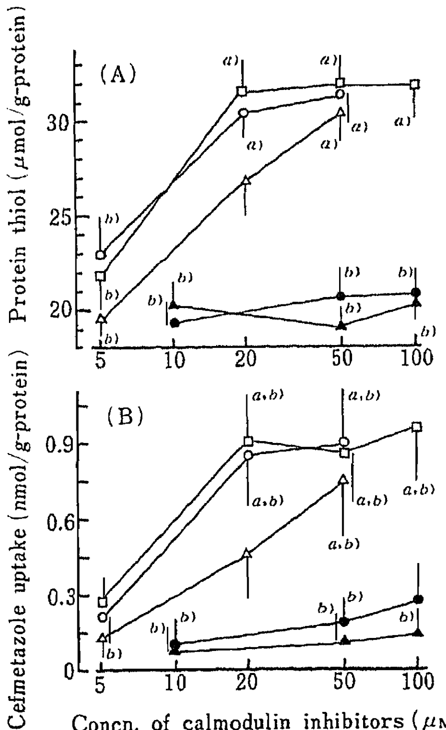

Fig. 1. Effect of Calmodulin Inhibitors on Protein Thiol in Isolated Intestinal Epithelial Cells (A) and on the Cefmetazole Uptake (B) in the Presence of 5 mM DEM in the Medium

Calmodulin inhibitors added in the medium were as follows: ○, TFPZ; ●, TFPZ-sulfoxide; △, CPZ; ▲, CPZ-sulfoxide; □, W-7. Each value represents the mean ± S.D. (n = 3 to 4). *a*) $p<0.01$ *versus* 5 mM DEM alone in Table II; *b*) $p<0.01$ *versus* no addition of DEM in Table II.

We did not investigate in detail whether nonprotein thiol loss is related directly or indirectly to the increase of cell membrane permeability to cefmetazole, though it has been suggested previously[9] that Ca^{2+} release from the mucosal membrane after nonprotein thiol loss induces an increase of the mucosal permeability. It has also been reported that calcium ionophore A23187, which releases Ca^{2+} from the extramitochondrial Ca^{2+} pool, increased the cell membrane permeability of hepatocytes,[19] and that nonprotein thiol loss caused a depletion of the extramitochondrial Ca^{2+} pool.[2] Since nonprotein thiol loss is known to result in a lipid oxidation in the cell membrane,[20] possible perturbation of the lipid layer in the membrane by nonprotein thiol loss may be involved in the increase of cell membrane permeability. However, it has been reported[9] that the presence of cysteamine, which is an exogenous nonprotein thiol but is not a reductant, inhibited the effect of DEM in increasing cefmetaxole transport in an everted rat intestinal sac. Thus, it is qualitatively considered that the increase of the epithelial cell membrane permeability along with nonprotein thiol loss induced by DEM occurs predominantly as a result of the effect of DEM in depleting the extramitochondrial Ca^{2+} pool. It is also considered that the change of permeability of the cell membrane is induced by lipid oxidation when the cells are kept in a state of nonprotein thiol loss for a long period. However, the effect of protein thiol loss in suppressing cell membrane permeability to cefmetazole seems to overcome the effect of nonprotein thiol loss in increasing the permeability.

Under condition causing the death of significant numbers of epithelial cells (incubation with 10 mM DEM for 45 min), cefmetazole uptake into the cells increased significantly (Table II). In our previous study[10] using rat colonic loop, 50 mM DEM (DEM did not dissolve completely in the administered solution) caused a significant increase of cefmetazole absorption. We also found in the present study that 50 mM DEM caused rapid death of isolated epithelial cells (Table I). Thus, the significant increase of cefmetazole absorption in the previous study might be due to significant cell death following administration of 50 mM DEM.

Cefmetazole Absorption from Rat Ileal Loop

To investigate the effect of DEM on cefmetazole absorption from rat ileal loop, a solution containing 0.75 or 7.5 mM DEM was administered into the loop. These concentrations were chosen because 0.75 mM DEM caused a rapid nonprotein thiol loss in the loop tissue without protein thiol loss, but 7.5 mM DEM caused a rapid loss of both nonprotein and protein thiols within 30 min (Fig. 2A, B). Cefmetazole was administered at 30 min after administration of DEM, as described in the experimental section, and cefmetazole absorption was compared with plasma cefmetazole concentration (Fig. 2C, D).

Plasma cefmetazole concentration was increased by the preadministration of 0.75 mM DEM, but decreased by that of 7.5 mM DEM (Fig. 2C). When TFPZ, CPZ or W-7 was coadministered with 0.75 mM DEM, they did not affect the area under the plasma cefmetazole concentration curve (*AUC*), which was increased by 0.75 mM DEM (Table IV). We have reported that calmodulin inhibitors such as TFPZ did not have a marked effect on *in vivo* intestinal absorption of cefmetazole, after administration in solution at physiological ionic strength.[21] In the present study, 50 μM TFPZ, CPZ or W-7 in the absence of DEM also did not increase the *AUC*, or increased it only slightly (Table IV). However, when they were coadministered at more than 20 μM with 7.5 mM DEM, they increased the 7.5 mM DEM-suppressed *AUC* (Table IV and Fig. 3B), and also inhibited the protein thiol loss in the tissue (Fig. 3A). We have recently found that 2,4-dinitrophenol (DNP) suppressed intestinal cefmetazole absorption along with protein thiol loss in tissue in spite of there being no change of nonprotein thiol, and coadministration of calmodulin inhibitors with DNP restored the intestinal cefmetazole absorption along with a restoration of the protein thiol loss in tissue

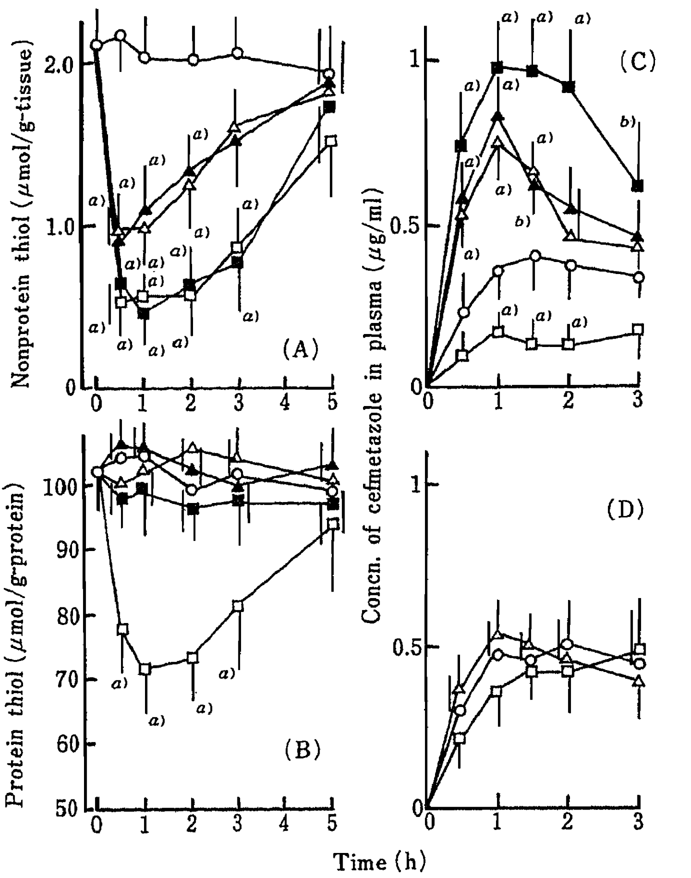

Fig. 2. Effect of DEM on Nonprotein Thiol (A) and on Protein Thiol (B) in Ileal Tissue of the Loop after an Administration of DEM

In (C) and (D), the effect of preadministration of DEM is shown on plasma cefmetazole concentrations after cefmetazole administration (at a dose of 6 mg per rat) at 30 min (C) and 5 h (D) after administration of DEM. The concentration of DEM in the administered solution was as follows: ○, saline alone; △, 0.75 mM DEM; ▲, 0.75 mM DEM and 50 μM TFPZ; □, 7.5 mM DEM; and ■, 7.5 mM DEM and 50 μM TFPZ. Each value represents the mean ± S.D. ($n=4$ to 5). *a*) $p<0.01$ *versus* absence of DEM; *b*) $p<0.05$ *versus* absence of DEM.

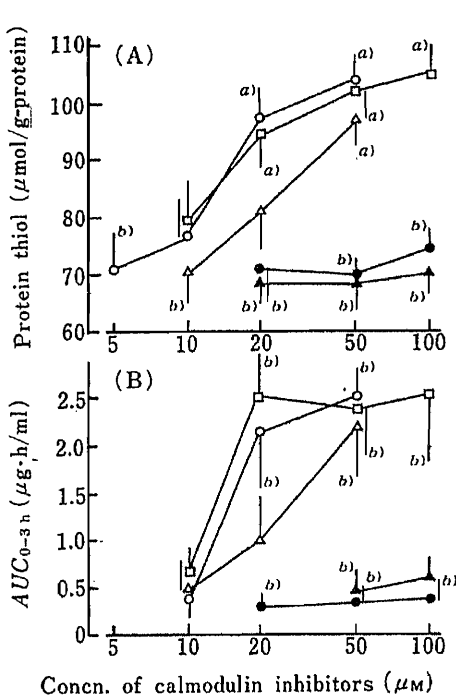

Fig. 3. Effect of Calmodulin Inhibitors in Administered Solution Containing 7.5 mM DEM on Protein Thiol (A) and on *AUC* of Plasma Cefmetazole Concentration (B)

Protein thiol was measured at 30 min after administration of DEM. *AUC* was determined for 3 h after administration of cefmetazole (6 mg per rat) into the lumen of the ileal loop at 30 min after the administration of 7.5 mM DEM. Calmodulin inhibitors added to the administered solution were as follows: ○, TFPZ; ●, TFPZ-sulfoxide; △, CPZ; ▲, CPZ-sulfoxide; □, W-7. Each value represents the mean ± S.D. ($n=3$ to 4). *a*) $p<0.01$ *versus* 7.5 mM DEM alone (Table IV); *b*) $p<0.05$ *versus* no additive (Table IV).

(presented at the annual meeting of the Pharmaceutical Society of Japan, Chiba city, April 1986). Thus, it is suggested that protein thiol loss in intestinal tissue suppresses intestinal cefmetazole absorption.

Both protein and nonprotein thiols recovered at 5 h after administration of 7.5 mM DEM (Fig. 2A, B). The plasma cefmetazole concentrations following administration of cefmetazole at 5 h after administration of 7.5 mM DEM were similar to those following administration of cefmetazole without preadministration of DEM (Fig. 2D). Thus, the change of cefmetazole transport induced by nonprotein and protein thiol loss after administration of 7.5 mM DEM was reversible.

When cefmetazole was injected into connective tissue of the ileal loop at 30 min after administration of DEM into the lumen of the ileal loop, 0.75 mM DEM did not affect the appearance of cefmetazole in plasma, but 7.5 mM DEM delayed the appearance of cefmetazole (Table V). Coadministration of 50 μM TFPZ or W-7 restored the appearance of

Table IV. Effect of Calmodulin Inhibitors on Thiol[a] in Ileal Tissue and on AUC[b] after Administration of Cefmetazole (6 mg per Rat) into the Lumen of the Ileal Loop

Additive	Nonprotein thiol (μmol/g-tissue)	Protein thiol (μmol/g-protein)	AUC (μg·h/ml)
In the presence of 0.75 mM DEM in administered solution			
None	0.98±0.21[c]	103.6±10.1	1.42±0.14[c]
50 μM TFPZ	0.90±0.17[c]	98.2±14.6	1.79±0.31[c]
50 μM TFPZ-sulfoxide	1.02±0.14[c]	106.8±7.4	1.40±0.27[c]
50 μM CPZ	0.87±0.21[c]	112.9±21.7	1.69±0.43[c]
50 μM CPZ-sulfoxide	1.24±0.16[c]	109.4±19.6	1.51±0.29[c]
50 μM W-7	1.18±0.29[c]	117.5±10.2	1.82±0.23[c]
In the absence of DEM in administered solution			
None	2.06±0.37	102.7±16.1	0.98±0.16
50 μM TFPZ	2.27±0.38	128.3±29.1	1.21±0.12
50 μM TFPZ-sulfoxide	1.91±0.13	92.4±11.6	0.81±0.26
50 μM CPZ	2.32±0.42	118.5±19.2	1.07±0.09
50 μM CPZ-sulfoxide	2.04±0.19	97.3±11.0	1.04±0.13
50 μM W-7	1.91±0.24	126.2±29.4	1.19±0.11
In the presence of 7.5 mM DEM in administered solution			
None	0.51±0.27[c]	78.7±6.9[c]	0.47±0.18[c]

Each value represents the mean±S.D. ($n=4$ to 5). *a*) Thiols in ileal tissue were measured at 30 min after administration of calmodulin inhibitor with DEM. *b*) AUC calculated from plasma cefmetazole concentrations up to 3 h after the administration of cefmetazole. Cefmetazole was administered at 30 min after administration of calmodulin with DEM. *c*) $p<0.05$ *versus* in the absence of DEM.

Table V. Appearance of Cefmetazole in Plasma after an Injection of Cefmetazole (0.5 mg in a Rat) into Connective Tissue of the Ileal Loop at 30 min after Administration of Saline Containing DEM and Calmodulin Inhibitors into the Lumen of the Ileal Loop

Calmodulin inhibitor in administered solution	Plasma cefmetazole concentration (μg/ml) Time after incubation (min)		
	15	30	60
Absence of DEM			
None	3.52±0.53	3.17±0.42	2.43±0.46
50 μM TFPZ	3.16±0.69	2.94±0.25	2.21±0.51
50 μM TFPZ-sulfoxide	3.91±0.20	3.02±0.55	2.19±0.18
50 μM W-7	4.01±0.52	2.71±0.62	2.04±0.30
Presence of 0.75 mM DEM			
None	3.71±0.56	3.10±0.63	1.97±0.41
50 μM TFPZ	3.27±0.46	2.96±0.42	2.26±0.53
50 μM TFPZ-sulfoxide	3.94±0.63	3.36±0.27	2.12±0.31
50 μM W-7	3.88±0.71	2.64±0.61	2.23±0.29
Presence of 7.5 mM DEM			
None	1.62±0.31[a]	1.58±0.26[a]	1.49±0.57
50 μM TFPZ	3.74±0.42[b]	2.82±0.31[b]	2.53±0.49
50 μM TFPZ-sulfoxide	1.46±0.47[a]	1.52±0.31[a]	1.41±0.28
50 μM W-7	3.41±0.29[b]	2.93±0.25[b]	2.17±0.41
Cefmetazole was injected at 5 h after administration of 7.5 mM DEM			
None	3.10±0.73[c]	2.93±0.41[c]	2.06±0.52

Each value represents the mean±S.D. ($n=3$). *a*) $p<0.01$ *versus* no DEM (none in (1)). *b*) $p<0.01$ *versus* 0.75 mM DEM alone (none in (3)). *c*) $p<0.01$ *versus* none in (3).

cefmetazole in plasma. The injection of cefmetazole 5 h after administration of 7.5 mM DEM also restored the appearance of cefmetazole. Thus, it is considered that nonprotein thiol loss does not affect diffusion of cefmetazole through the connective tissue into the blood, but protein thiol loss suppressed it. However, it is not clear at present whether protein thiol loss inhibits diffusion of cefmetazole through connective tissue or suppresses the permeability of the capillary wall.

Since TFPZ, CPZ and W-7 at a concentration of less than 100 μM inhibited protein thiol loss in ileal tissue induced by 7.5 mM DEM, in spite of having no inhibitory effect on nonprotein thiol loss, protein thiol loss in the tissue may also occur by a calmodulin-dependent mechanism.

From the findings obtained in the present study, we may qualitatively conclude that the increase of intestinal cefmetazole absorption by 0.75 mM DEM seems to occur predominantly as a result of nonprotein thiol loss in the epithelial cells, which increases the transport of cefmetazole through the mucosal membrane. In the present study, we did not investigate the effect of DEM on the permeability at the tight junctional area of the epithelium, in spite of the report[8] that intestinal cefmetazole absorption, though poor, occurs predominantly *via* the paracellular route. Thus, it is not clear whether the increase of cefmetazole absorption occurs only through the increase of cefmetazole uptake into intestinal epithelial cells. On the other hand, the decrease of intestinal cefmetazole absorption by 7.5 mM DEM seems to occur as a result of protein thiol loss in intestinal tissue, including epithelial cells, which suppresses both the uptake of cefmetazole into the epithelial cells and the transport into the blood after uptake from the mucosal membrane. However, it is also not clear whether protein thiol loss affects the permeability of the tight junctional region where cefmetazole transport occurs predominantly under normal conditions. Calmodulin inhibitors inhibited the effect of 7.5 mM DEM, probably by restoration of protein thiol in the ileum.

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Infusion Rate-Dependent Positive Inotropic Action of Ouabain in Rabbits[1)]

HIDEYOSHI HARASHIMA,*[a] YUICHI SUGIYAMA,[a] YASUFUMI SAWADA,[a] KOKI SHIGENOBU,[b] YUTAKA KASUYA,[b] TATSUJI IGA[a] and MANABU HANANO[a]

Department of Pharmaceutics[a] and Department of Chemical Pharmacology,[b] Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

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The effect of the infusion rate on the relationship between plasma concentration (Cp) and positive inotropic action (PIA) of ouabain was studied in rabbits. The maximum of the first derivative of the left ventricular pressure (dP/dt_{max}) was used as an index of PIA. The Cp of ouabain was measured by counting the radioactivity of ^{3}H-ouabain, a nonmetabolizable cardiac glycoside. ^{3}H-Ouabain was infused *via* the femoral vein at 2.7, 13 and 38 nmol/min/kg, and the time courses of Cp and dP/dt_{max} were measured simultaneously. Non-linear initial volume of distribution was suggested from the different slopes of the Cp curves normalized by infusion rate during the initial 5 min. The relationship between Cp and PIA depended remarkably on the infusion rate. The values of Cp which produced the maximum PIA at the low, medium and high infusion rates were 136±18, 700±210 and 2300±545 nM (mean±S.E.M.), respectively. The total amounts of infused ouabain until the maximum PIA at the low, medium and high infusion rates were 81±6.8, 130±6.5 and 152±7.2 nmol/kg (mean±S.E.M.), respectively. It was clear that ouabain does not exhibit its action in the central compartment, although it is considered to be a fast-acting cardiac glycoside. It is suggested that a slow step exists in the appearance of PIA of ouabain in rabbits.

Keywords——ouabain; positive inotropic action; rabbit; infusion rate dependency; pharmacodynamics; pharmacokinetics; non-linear distribution volume

Introduction

Cardiac glycosides are used most frequently to increase the adequacy of the circulation in patients with congestive heart failure. This effect is owing to their direct action to increase the force of myocardial contraction, *i.e.*, a positive inotropic action (PIA).[2)] At present, it seems reasonable to assume that the following sequence of events occurs.[3)] Cardiac glycosides bind to a specific site on the membrane Na^+, K^+-adenosine triphosphatase (ATPase) and decrease the active extrusion of intracellular Na^+. The consequent increase in the intracellular Na^+ decreases the exchange of the extracellular Na^+ for the intracellular Ca^{2+}. The elevated concentration of intracellular Ca^{2+} might increase contractility by several mechanisms.

Both in man and dog, a good correlation was found between the plasma concentration (Cp) and PIA in the elimination phase of digoxin and digitoxin.[4—6)] Reuning *et al.*[7)] and Kramer *et al.*[8)] reported a pharmacokinetic analysis of the time course of PIA in man based on the amounts of digoxin in the deep compartment, which was calculated from Cp–time curve after i.v. administration.

In the present study, we examined the effect of the infusion rate on the relationship between PIA and Cp of ouabain, which is known to be a fast-acting, nonmetabolizable cardiac glycoside.[9)]

Experimental

Materials——^{3}H-Ouabain was obtained from New England Nuclear Corp., Boston, MA. The specific activity

given by the manufacturer was 20 Ci/mmol. The radioactive compound was confirmed to be at least 98% pure by thin-layer chromatography (TLC) with chloroform–methanol–water (65 : 30 : 5). Unlabelled ouabain was purchased from Merck, Darmstadt, F.R.G. All other chemicals were commercial products of analytical grade.

Animal Experiments——Male, Japanese White rabbits (Nihon Ikagaku Dobutsu, Tokyo, Japan) weighing 2.5—3.5 kg were used. The rabbits were anesthetized with ethyl carbamate (urethan; 600 mg/kg) and α-chloralose (60 m/kg) intraperitoneally. Body temperature was kept at 37 °C using a heat lamp. The femoral vein and artery were cannulated with polyethylene tubing (PE-50) for drug administration and blood sampling, respectively. The throat was opened and a tracheal cannula was introduced for spontaneous respiration. A rigid catheter (i.d. 1.5 mm) was introduced into the left ventricle through the carotid artery. Systolic and diastolic ventricular pressures were recorded with a Gould Stathum transducer (P23 i.d., Gould Inc., Oxnard, CA). The left ventricular pressure was damped by using a hemodynamic damping device (CorrecTORR; Norton, Akron, OH), to obtain an appropriate frequency response.[10] From the ventricular pressure (P), the first derivative of $P(\mathrm{d}P/\mathrm{d}t)$ was obtained by electronic differentiation. The maximum of $\mathrm{d}P/\mathrm{d}t$ was used as an index of PIA ($\mathrm{d}P/\mathrm{d}t_{max}$). The lead II electrocardiogram (ECG) was also recorded continuously. Unlabelled ouabain was dissolved in saline and was mixed with radioactive glycoside in the range of 0.8—2.5 μCi/ml. Approximately 30 min after surgery, ouabain solution was infused intravenously through the femoral vein at 2.7, 13 and 38 nmol/min/kg (flow rates: 35—45 μl/min/kg) using an infusion pump (Harvard Apparatus, model 975E, South Natick, MA). Blood samples (approximately 1 ml) were taken into a heparinized syringe. For the control study, physiological saline without ouabain was infused.

Determination of Radioactivity——The purity of ^{3}H-ouabain in the plasma sample at 35 min was examined by TLC as mentioned above; more than 93% was intact ouabain and no metabolite was found. Thus, we determined directly the Cp of ouabain by measuring the radioactivity in plasma. A 500 μl aliquot of plasma was mixed with 10 ml of Biofluor, high-efficiency emulsifier cocktail (New England Nuclear Corp., Boston, MA), and the radioactivity was determined in a liquid scintillation spectrometer (Packard Instruments Corp., Downers Grove, IL). Quenching was determined using automatic external standardization.

Data Analysis——The index of the PIA, I_t, was calculated as follows:

$$I_t = \frac{E_t - E_0}{E_{max} - E_0}$$

where E_0 and E_t are the values of $\mathrm{d}P/\mathrm{d}t_{max}$ at the base line and at time t, respectively, and E_{max} is the maximal value of $\mathrm{d}P/\mathrm{d}t_{max}$. E_t increased with time and reached a maximum then decreased due to toxicity in each experiment. Therefore, E_{max} represents the maximum $\mathrm{d}P/\mathrm{d}t_{max}$ just before the appearance of toxicity.

Results

Time Course of Cp of Ouabain

Ouabain was administered intravenously by constant infusion at one of three rates, *i.e.*, 2.7, 13 and 38 nmol/min/kg. Time courses of ouabain Cp from the initiation of the infusion

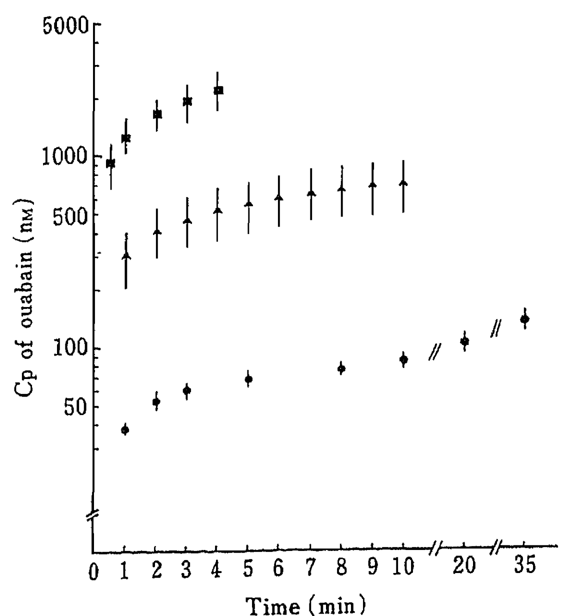

Fig. 1. Time Courses of Cp of Ouabain at Three Infusion Rates

(●) 2.7, (▲) 13, and (■) 38 nmol/min/kg. Each point and vertical bar represents the mean and S.E.M. of 4 or 5 experiments.

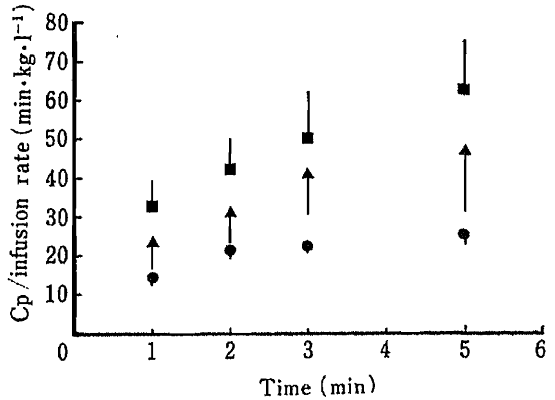

Fig. 2. Time Courses of Ouabain Cp Normalized with Respect to the Infusion Rate

Cp of ouabain was divided by its infusion rate. Symbols are the same as those in Fig. 1.

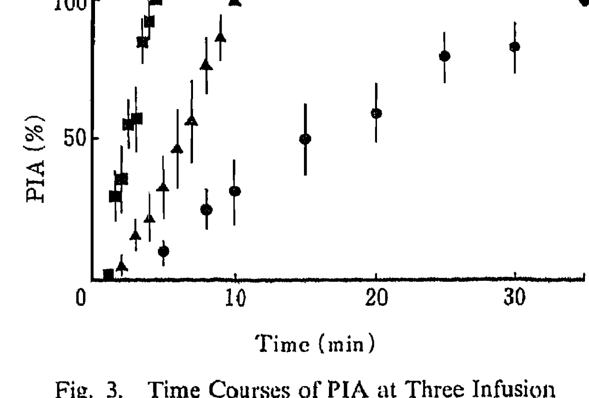

Fig. 3. Time Courses of PIA at Three Infusion Rates

The change of dP/dt_{max} was expressed as a percentage of the maximum value according to the equation. Each point and vertical bar represents the mean and S.E.M. Symbols are the same as those in Fig. 1.

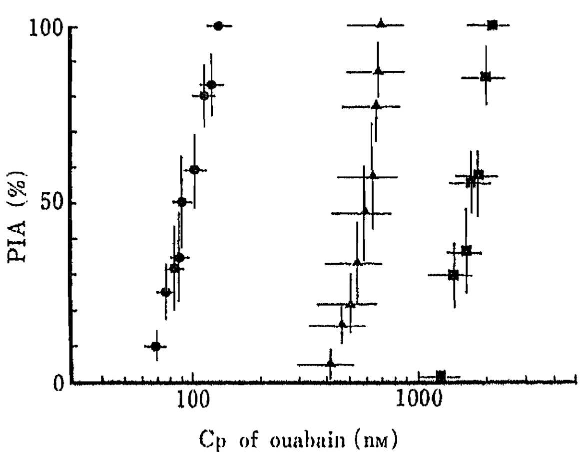

Fig. 4. Relationship between Cp and PIA of Ouabain

Each point and vertical bar represents the mean and S.E.M. Symbols are the same as those in Fig. 1.

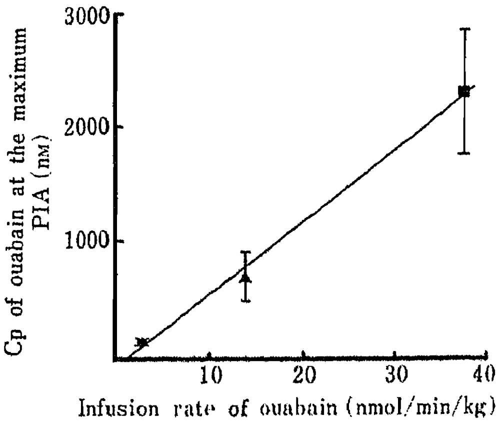

Fig. 5. Effect of the Infusion Rate of Ouabain on Cp Which Produced the Maximum PIA (EC_{max})

Each point and vertical bar represents the mean and S.E.M. Symbols are the same as those in Fig. 1. The solid line represents the linear regression line, $EC_{max}=61.8 \cdot IR-60.0$, where IR is the infusion rate (nmol/min/kg). The regression coefficient (r) is 0.999 and is statistically significant ($p<0.05$).

until the PIA reached the maximum value are shown in Fig. 1. The first rapid distribution phase finished within 5 min, and a rather slow phase followed. Each time course of Cp of ouabain was fitted to the two-compartment open model and the calculated pharmacokinetic parameters were obtained (not shown); however, it is not necessarily valid to compare them directly in order to evaluate the infusion rate dependency of the pharmacokinetic parameters, because of the different periods of data collection. Thus, each time course for the initial 5 min was normalized with respect to the infusion rate (Fig. 2). The higher the infusion rate, the higher the normalized Cp. Infusion rate dependency was clearly observed in Cp during the initial 5 min.

Time Courses of PIA of Ouabain

The time courses of PIA at the three infusion rates are shown in Fig. 3. The maximum effects were observed at 32.5 ± 2.5 min after initiation of the infusion for the low infusion rate, at 9.3 ± 0.5 min for the medium rate and at 4.0 ± 0.2 min for the high rate (mean ± S.E.M.; $n=$ 4 or 5). The values of Cp of ouabain which produced the maximum PIA at the low, medium and high infusion rates were 135 ± 18, 700 ± 210 and 2300 ± 545 nM, respectively and the total amounts of infused ouabain were 81 ± 6.8, 130 ± 6.5 and 152 ± 7.2 nmol/kg, respectively. The values of E_0 were 8695 ± 521, 7199 ± 956 and 7293 ± 1080 mmHg/s at the low, medium and high infusion rates, respectively. There is no significant difference among them. The maximum values of dP/dt_{max} for the low, medium and high infusion rates were 32.8 ± 5.0, 51.2 ± 22.8 and 44.4 ± 8.3%, respectively, and again there is no significant difference among them. In the control study, E_t decreased by 0.5, 14.2 and 10.6% at 10, 20 and 40 min after the initiation of infusion, respectively. However, correction for this was not performed in this study.

Relationship between Cp and PIA of Ouabain

From the time courses of Cp and PIA, the relationship between Cp and PIA was plotted as shown in Fig. 4. Remarkable infusion rate dependency is apparent in this relationship. The concentration-response curve shifted to the right with increase of the infusion rate. Then, the relationship between infusion rate and effective concentration (EC_{max}), the Cp which produced the maximum PIA, was plotted to examine the effect of the infusion rate on PIA; the result is shown in Fig. 5. There was a good linear relation between infusion rate and EC_{max}.

Discussion

The present study was focused on the distribution phase within 1 h after i.v. administration of ouabain, and the sampling times differed at the three infusion rates. It is not reasonable to discuss the non-linearity based on the pharmacokinetic parameters obtained from data sampled at different periods. Thus, Cp of ouabain was normalized with respect to the infusion rate, and a remarkable infusion rate dependency was observed (Fig. 2). Since the inverse of the initial slope represents the central volume of distribution,[11] this infusion rate dependency may result from a difference in the central volume of distribution, depending on the infusion rate. Further study is needed to confirm the non-linear pharmacokinetics of ouabain.

In clinical studies, evidence of saturable binding of digoxin to skeletal muscle was reported.[12] In addition, an increase in the volume of distribution of the peripheral compartment was reported for digoxin in hyperthyroidism.[13,14] These studies suggested that the cause of the increased volume of distribution might be an increase in the amount of Na^+, K^+-ATPase, because Lindsay and Parker[15] demonstrated that both digoxin and thyroxine treatment increased tissue Na^+, K^+-ATPase activity in rats. Lüllmann *et al.*[16] reported that the binding of ouabain in isolated papillary muscle of the guinea pig was saturable. Thus, it is suggested that the binding of ouabain to Na^+, K^+-ATPase may contribute to the distribution of ouabain in the body.

In this study, we used the maximum value of the first derivative of the left ventricular pressure (dP/dt_{max}) as a sensitive and direct index of PIA, although it is difficult to apply dP/dt_{max} to small animals.[17] Kramer *et al.*[8] used the electromechanical systole corrected for the heart rate (QS_2I) as an index of PIA in man. The merit of thier method is the applicability to small animals, although it is an indirect method.[18] There are some reports on the effect of infusion rate on the toxicity and inotropic action[19,20]; it was shown that the lethal dose of ouabain changed depending on the infusion rate. As to toxicity, all guinea pigs producing arrhythmias died as a result of their ventricular arrhythmias, independently of the adminis-

tered dose of ouabain.[21] In this study, the maximum effect was not altered significantly at the various infusion rates. Therefore, we expressed PIA in terms of I_t which is normalized with respect to E_{max}.

A remarkable infusion rate dependency was demonstrated in the relationship between Cp and PIA (Fig. 4) and in the relationship between infusion rate and Cp at the maximum PIA (Fig. 5), although ouabain is known to be a fast-acting cardiac glycoside. This suggests that the effective compartment exists kinetically not in the plasma compartment but in the peripheral compartment. Kramer *et al.*[8] reported that PIA of digoxin after i.v. administration to man could be explained in terms of the amount in the deep compartment. It is known that ouabain binds to Na^+, K^+-ATPase from the extracellular side[22] and this step is considered to be the first step of PIA.[23] Thus, this remarkable infusion rate-dependent PIA of ouabain (Fig. 4) may be explained by the slow binding process of ouabain with Na^+, K^+-ATPase or by a slow step after the occupation of Na^+, K^+-ATPase. In fact, extensive studies have been done on the binding of cardiac glycosides to Na^+, K^+-ATPase[24] and the slow binding process of ouabain to Na^+, K^+-ATPase might well be one reason for the infusion rate dependency.[25]

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Percutaneous Absorption of Indomethacin from Mixtures of Fatty Alcohol and Propylene Glycol (FAPG Bases) through Rat Skin: Effects of Fatty Acid Added to FAPG Base[1]

FUSAO KAIHO,* HIDEAKI NOMURA, ERI MAKABE, and YURIKO KATO

Faculty of Pharmaceutical Sciences, Science University of Tokyo, Ichigaya Funagawara-machi, Shinjuku-ku, Tokyo 162, Japan

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The effects of two kinds of fatty acids (stearic acid and palmitic acid) added to mixture of fatty alcohol and propylene glycol (FAPG base) on the percutaneous absorption of indomethacin (ID) were investigated by using the normal abdominal skin of rats *in vivo*. The percutaneous absorption of propylene glycol (PG) from FAPG base was simultaneously examined.

The percutaneous absorption profiles of ID from No. 1 (consisting of stearyl alcohol and 75% PG) and from No. 2 (consisting of stearyl alcohol, stearic acid and 75% PG) bases were almost the same. On the other hand, the absorption of ID from No. 3 base (consisting of stearyl alcohol, palmitic acid and 75% PG) was significantly higher than those from Nos. 1 and 2 bases. Furthermore, PG was readily absorbed through the rat skin from Nos. 1, 2 and 3 FAPG bases and its percutaneous absorption profiles were similar to those of ID.

In order to establish the reason for the differences in percutaneous absorption of ID from the three bases, the apparent partition ratio of ID between water and vehicle was determined as a parameter of the affinity of ID for the vehicle. There was no difference in apparent partition ratio between Nos. 1 and 2 bases, while that of No. 3 base was the highest among the vehicles tested. The release of ID from Nos. 1, 2 and 3 bases *in vitro* was also studied by using a silicone rubber membrane. The results of the *in vivo* test were broadly consistent with those of the *in vitro* tests.

Keywords——fatty alcohol; stearyl alcohol; fatty acid; stearic acid; palmitic acid; propylene glycol; FAPG base; percutaneous absorption; indomethacin, propylene glycol blood concentration

The topical vehicle consisting of a mixture of fatty alcohol (FA), propylene glycol (PG) and other excipients, which is usually abbreviated as FAPG, was invented and put into practical use by Katz and Neiman.[2] It has been used as a topical vehicle for various corticosteroids. However, FAPG base has been little used as a topical vehicle for other drugs. In the previous paper, we reported that indomethacin (ID) was readily absorbed through the depilated abdominal skin of rats from FAPG base,[3] and we suggested that FAPG base could be a useful vehicle for percutaneous drug administration in topical use.

On the other hand, we further observed that PG separated from the vehicle (bleeding) during storage. However, Katz and Neiman described in a patent how such bleeding could be prevented by adding a suitable coupling agent such as saturated fatty acids, amides of fatty acids and esters of fatty acids.[2] Among these coupling agents, saturated fatty acid has been used as an additive for dosage forms.[4] However, it appears that little or no attention has been paid to the effect of fatty acid added to the vehicle on the percutaneous absorption of a drug.

In the present study, we investigated the effects of two saturated fatty acids (stearic acid and palmitic acid) added to FAPG on the percutaneous absorption of ID from FAPG base using normal abdominal skin of rats *in vivo*, and simultaneously examined the percutaneous absorption of PG from FAPG base.

Experimental

Materials——Stearyl alcohol, palmitic acid, stearic acid and PG were purchased from Tokyo Kasei Co., Ltd. ID was supplied by Toho Pharmaceutical Co., Ltd. Flufenamic acid was purchased from Aldrich Chemical Company. All the solvents used in this experiment were of reagent grade from Kanto Chemical Co., Ltd. The silicon rubber membrane (Silastics® 5001) was purchased from Dow Corning Co., Ltd.

Preparation of FAPG Bases——FAPG bases containing 1% ID were prepared according to the formulae in Table I. The stearyl alcohol and fatty acid were heated at 75°C, then ID dissolved in PG, previously heated to the same temperature, was added. The mixture was stirred until it congealed.

***In Vitro* Experiment**——The release of ID from FAPG base was determined by using diffusion cells as shown in Fig. 1. One side of the cell was filled with FAPG base containing ID and this side (donor phase) was separated by the silicon rubber membrane from the other side (receiver), which was filled with pH 7.4 phosphate buffer. The cells were immersed in a constant-temperature bath (34 °C). An aliquot (0.5 ml) of receiver fluid was withdrawn for analysis at appropriate times and the concentration of ID released was determined by high-performance liquid chromatography (HPLC).

Measurement of Viscosity——The Rheomat 30 cone-and-plate viscometer (Contraves Co., Ltd.) was used to determine the viscosity of FAPG bases at 34 °C (average skin temperature[5]). The maximum shear rate was 42.1 s^{-1} with a 30 s sweep time. Apparent viscosity was obtained from the shear stress at the maximum shear rate.

***In Vivo* Experiment**——Male Wistar rats weighing between 230 and 250 g were used. The hair of the abdominal region was carefully removed with electric hair clippers and an electric razor without breaking the skin, one day before the experiments. The rat was fixed on its back, and 2 g of vehicle was spread on the skin (18 cm^2). Blood samples (0.6 ml) were withdrawn from the jugular vein into a syringe at predetermined intervals, and were centrifuged at 3000 rpm for 10 min. The resulting plasma samples were individually subjected to ID and PG content measurement by HPLC and gas chromatography (GC).

Assay of ID——In the *in vivo* and *in vitro* experiments, the HPLC method was applied for measurement of ID. A plasma sample (0.2 ml) or an aliquot (0.5 ml) of receiver fluid in the release test was placed in a test tube (5 mm i.d. × 50 mm) and 0.5 ml of internal standard (flufenamic acid, 10 μg/ml) acetonitrile solution was added. After being shaken for 5 min, the mixture was centrifuged at 3000 rpm for 10 min. The upper layer was transferred to another test tube, and was concentrated to about one-twentieth of its original volume under a stream of nitrogen. A 30 μl aliquot of this solution was injected into the HPLC apparatus (Nippon Seimitsu Kagaku Co., Ltd.). The conditions for analysis were as follows: column, SSC-ODS-262 (Senshu Scientific Co., Ltd.); mobile phase, 0.01 M sodium acetate buffer (pH 3.2)–methanol (3 : 7, v/v); flow rate, 2.0 ml/min; detector, ultraviolet (UV) (260 nm); range, 0.16. The signal from the detector was fed into an integrator (Chromatopack E-1A, Shimadzu Seisakusho Co., Ltd.).

Assay of PG——The analysis of PG in plasma was carried out according to the method of Yu and Sawchuk with some modifications.[6] A 7 ml aliquot of solvent mixture (anhydrous ether : *tert*-butyl alcohol, 9 : 1) and 0.1 ml of

TABLE I. Formulae of FAPG Bases

Composition[a]	Base No.		
	1	2	3
Stearyl alcohol	25	20	20
Stearic acid	—	5	—
Palmitic acid	—	—	5
Propylene glycol	75	75	75

a) In grams.

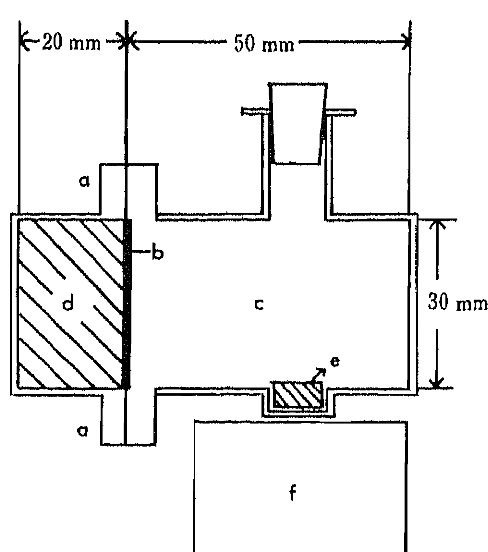

Fig. 1. Diagrammatic Section of the Apparatus Used to Study Drug Release from FAPG Bases

a, stopper; b, membrane; c, receiver fluid; d, vehicle; e, stirring bar; f, magnetic stirrer.

internal standard (2,3-butanediol, 99.5 μg/ml) aqueous solution were added to 0.1 ml of plasma sample in a centrifuge tube. After being shaken for 5 min, the mixture was centrifuged at 3000 rpm for 10 min. The upper layer was transferred to another tube, and evaporated at 20 °C for about 20 min. The volume left in the tube should be about 15—20 μl, and 7—10 μl of the residue was injected into the GC column.

A Shimadzu GC-5A gas chromatograph (Shimadzu Seisakusho Co., Ltd.) equipped with a flame ionization detector was used under the following conditions: column, 3 mm × 2 m glass tube packed with 10% PEG/Chromosorb DEGS (80—100 mesh, Nippon Chromato Kogyo Co., Ltd.); column temperature, 110 °C; injection port temperature, 275 °C; detector block temperature, 275 °C; carrier gas, 99.99% nitrogen gas (80 ml/min).

Measurement of Partition Ratio——FAPG base (12 g) and water (12 ml) containing 90 mg of ID were placed in a glass-stoppered test tube and shaken in a water bath at 60 °C for 2 h. Then, the tube was removed and left at room temperature in a vertical position for 1 h to allow separation of the two phases and solidification of the fatty alcohol and/or fatty acid phase. The amount of ID in the water phase was determined with a UV spectrophotometer (Hitachi 220A, Hitachi Seisakusho Co., Ltd.).

PG passed into the water phase from the FAPG base at the time of measurement of the partition ratio. Thus, if this measurement is carried out below about 60 °C, accurate results can not be obtained because of precipitation of fatty alcohol and/or fatty acid in the water phase after transfer of PG from the FAPG base. Accordingly, the measurement was carried out at 60 °C in this study in order to maintain the molten state of fatty alcohol and/or fatty acid.

The partition ratio (w/o) was calculated by comparison of the amount of ID in the water phase (w) with that in the fatty alcohol and/or fatty acid phase (o).

Results

Percutaneous Absorption of ID

The percutaneous absorption profiles of ID after topical application of Nos. 1, 2 and 3 bases to the normal abdominal skin of rats are shown in Fig. 2. There was no significant difference in absorption patterns or mean plasma concentrations of ID between No. 1 (consisting of stearyl alcohol and PG) and No. 2 (consisting of stearyl alcohol, stearic acid and PG) bases for 8 h after application. On the other hand, the percutaneous absorption rate of ID from No. 3 base (consisting of stearyl alcohol, palmitic acid and PG) after application was the fastest among the vehicles tested in this study. Furthermore, plasma ID concentration at 8 h after application of No. 3 base was about twice that of Nos. 1 and 2 bases. Mean plasma concentrations of ID from No. 3 base at 4, 6 and 8 h were significantly different from those of Nos. 1 and 2 bases ($p<0.05$).

In the previous paper,[3] we reported that ID from various FAPG bases was readily absorbed through the depilated abdominal skin of rats, prepared by pretreatment with depilatory cream (containing calcium thioglycolate). The mean plasma concentration of ID obtained after application of the vehicle, corresponding to the No. 1 base in this study, was

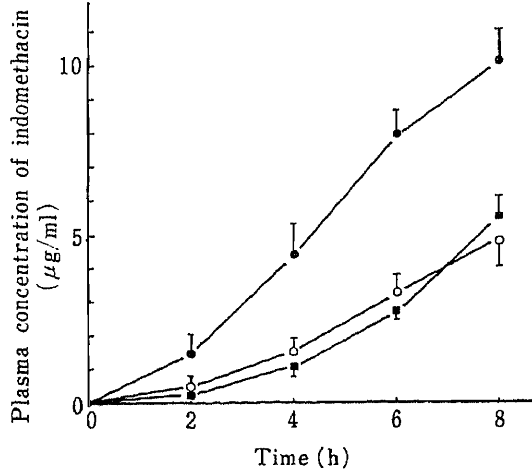

Fig. 2. Effects of Fatty Acids Added to FAPG Base on Percutaneous Absorption of Indomethacin in Rats

■, No. 1 base; ○, No. 2 base; ●, No. 3 base. Each value represents the mean ± S.E. of five experiments.

about 10 μg/ml at 2 h. On the other hand, the mean plasma concentration of ID obtained after application of No. 1 base to normal abdominal skin of rats was less than 1 μg/ml at 2 h in this study. These results suggest that a significant barrier of the normal skin was removed from the stratum corneum by pretreatment with the depilatory cream, so that the drug was able to penetrate more easily through this pretreated skin in comparison with normal skin.

Percutaneous Absorption of PG

Figure 3 shows the PG plasma concentration–time curves after application of Nos. 1, 2 and 3 bases. There was no significant difference in mean plasma concentration of PG between Nos. 1 and 2 bases in the period up to 8 h after application. On the other hand, the plasma concentration of PG from No. 3 base was significantly higher than that of Nos. 1 and 2 bases at 4, 6 and 8 h after application ($p<0.05$), though no difference was seen in mean plasma concentration of PG among Nos. 1, 2 and 3 bases at 2 h.

Release of ID from Vehicles

The release profiles of ID through the silicon rubber membrane used as a model membrane are shown in Fig. 4, and the release rate constant ($\mu g \cdot cm^{-2} \cdot t^{-1/2}$) calculated from Higuchi's equation is shown in Table II.[7]

No difference was detected in the amount of ID released or in the release patterns among Nos. 1, 2 and 3 bases up to 60 min. Subsequently, the amount of ID released from No. 3 base gradually increased in comparison with those from Nos. 1 and 2 bases. The ID release rate

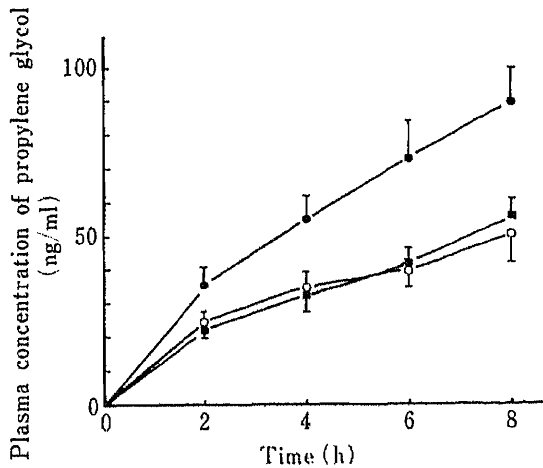

Fig. 3. Effect of Fatty Acids Added to FAPG Base on Percutaneous Absorption of Propylene Glycol in Rats

■, No. 1 base; ○, No. 2 base; ●, No. 3 base. Each value represents the mean ± S.E. of five experiments.

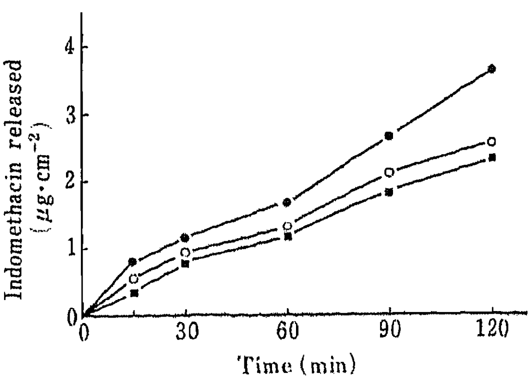

Fig. 4. Release of Indomethacin from FAPG Bases at 34 °C

■, No. 1 base; ○, No. 2 base; ●, No. 3 base. Each value represents the mean of four experiments.

TABLE II. Release Rate Constants of Indomethacin from FAPG Bases

	Base No.		
	1	2	3
Release rate constant ($\mu g \cdot cm^{-2} \cdot t^{-1/2}$)	0.27±0.036	0.29±0.039	0.39±0.033

Each value represents the mean ± S.D. of four experiments and was determined by using Higuchi's equation.

constant of No. 3 base was the highest among the three vehicles, though there was no difference in release rate constant of ID between Nos. 1 and 2 bases.

Viscosity of Vehicles

The apparent viscosity of Nos. 1, 2 and 3 bases measured using the rheometer is shown in Table III. There was no significant difference in viscosity or percutaneous absorption of ID between Nos. 1 and 2 bases. On the other hand, the apparent viscosity of No. 3 base was about 1.6 times those of Nos. 1 and 2 bases.

Bleeding tended to take place in No. 1 base (without fatty acid) but not in Nos. 2 and 3 bases. Furthermore, it was observed that addition of fatty acid to FAPG base resulted in a fine-grained vehicle.

Discussion

PG has been used as a food additive and as a solvent for some drugs. It is generally considered that PG enhances the absorption of drugs through the skin.[8] In addition, it has been confirmed that PG passed readily through excised human or animal skin from a vehicle containing PG *in vitro*.[9] Turi *et al.* has also reported that PG penetrated the skin rather easily *in vitro*, and further that it decreased the diffusional resistance of the skin, *i.e.*, the barrier function of skin.[10]

However, little attention has been paid to the percutaneous absorption of PG from a vehicle *in vivo*. In this study, we attempted to investigate whether or not PG is absorbed through normal rat skin after application of Nos. 1, 2 and 3 FAPG bases *in vivo*.

It became clear that PG was readily absorbed through the rat skin from FAPG bases and passed directly into the systemic circulation (Fig. 3), though the blood concentration of PG absorbed through the skin were fairly low as compared with that of ID. Nevertheless, the mean plasma concentration–time profiles of PG obtained from the three bases were similar to those of ID as shown in Figs. 2 and 3. It can be presumed from these results that the differences in the release of PG from the vehicle influence the percutaneous absorption of ID, and also that PG and ID penetrate together through the skin.

It was reported that the percutaneous absorption of a drug from a vehicle containing PG after application involves two processes; one is drug release from the vehicle in a dissolved state in PG, and the other is subsequent drug absorption through the skin barrier together with PG.[11,12] In this study, it was observed that the skin section after the application of FAPG base was altered, losing its suppleness. This may be due to the effect of absorption of PG through the skin, and may be responsible for facilitating the absorption of a drug through the skin.

The following equation was reported to describe the process of drug penetration by Higuchi.[13]

TABLE III. Viscosity of FAPG Bases at 34 °C

	Base No.		
	1	2	3
Viscosity (P)	3118	3415	5553

Each value represents the mean of three experiments.

TABLE IV. Apparent Partition Ratio of Indomethacin between Water and FAPG Base (Water/Base)

	Base No.		
	1	2	3
Partition ratio $\times 10^{-2}$	6.35	6.52	7.47

Each value represents the mean of three experiments.

$$J=K\cdot C\cdot D\cdot A/T \qquad (1)$$

where J is the mean flux of a drug through the skin barrier, K is the effective skin–vehicle drug partition coefficient, C is the drug concentration dissolved in the vehicle, D is the drug diffusivity through the barrier, A is the surface area of application of the vehicle, and T is the effective barrier thickness. Furthermore, Eq. 1 can be expressed as follows for various vehicle compositions;

$$J=a\cdot D\cdot A/r\cdot T \qquad (2)$$

where a is the thermodynamic activity of the drug in the vehicle and r is the activity coefficient of a drug in the skin barrier. The value of r is usually taken to be constant, so changes of the flux (J) are attributed to alteration in the activity of a drug in the vehicle. Since PG as well as ID is absorbed through the skin from Nos. 1, 2 and 3 bases, it may be strictly unreasonable to regard the value of r obtained from these bases as constant. However, the amount of PG absorbed through the skin from the vehicle was fairly low compared with that of ID, and thus we assumed that the effect of PG on the value of r was negligible, and further that the value obtained after the application of Nos. 1, 2 and 3 bases could be taken to be constant.

For absorption of a drug through the skin from the vehicle, the drug must first diffuse out of the vehicle to the skin surface. Then, if the drug has a low affinity for the vehicle, it will be readily released. Further, it is clear that the affinity of a drug for the vehicle is reduced as the thermodynamic activity of a drug in the vehicle is increased.

In the present study, in order to estimate the degree of affinity of ID for the vehicle, the apparent partition ratio of ID between water and vehicle was determined. The results are shown in Table IV. There was no difference in apparent partition ratio between Nos. 1 and 2 bases, while that of No. 3 base showed the highest value among the vehicles. These results suggest that the affinity of ID for No. 3 base is the lowest among three bases tested in this study. Since PG passed into the water phase from FAPG base during the experiment, the apparent partition ratio obtained reflects the affinity of ID for fatty alcohol and/or fatty acid.

As shown in Fig. 2, ID was readily absorbed through the skin from No. 3 base compared with Nos. 1 and 2 bases. These results appear to be due to the differences of partition ratio, that is affinity, of the vehicles. Furthermore, since the partition ratios of Nos. 1 and 2 bases were almost the same, it is not surprising that there was no significant difference of percutaneous absorption of ID between the two bases. It can be considered from the above results that the release properties of PG from the vehicle and the affinity of ID for the vehicle both influence the percutaneous absorption of ID from Nos. 1, 2 and 3 bases. Furthermore, it can be assumed that the permeation of ID itself through the skin is not the rate-determining step for percutaneous absorption of ID from FAPG base.

The silicon rubber membrane is a partition membrane and is known to be permeable to nonionic drug molecules. Thus, it can be used as a model membrane to examine the drug release from the various vehicles *in vitro*.[14-16] In the present study, we used the silicon rubber membrane to study the release of ID from Nos. 1, 2 and 3 bases *in vitro*. The release rate constant of ID from No. 3 base was relatively high compared with those of Nos. 1 and 2 bases, which were almost the same. These results are similar to those of the *in vivo* experiment and are consistent with the apparent partition ratios. It seems reasonable to assume that the results obtained with Nos. 1, 2 and 3 bases *in vitro* experiments using the silicon rubber membrane approximately reflect the absorption of ID through the skin *in vivo*. The silicon rubber membrane seems to be useful for comparing the early release patterns of ID from the vehicles.

The percutaneous absorption of a drug from the vehicle may increase with decrease in the viscosity of the vehicle. However, in the previous paper, we found that the percutaneous

absorption of ID from FAPG bases containing different kinds of fatty alcohols in a given volume of PG through the depilated skin of rats was not affected by the viscosity.[3] In this study, it was similarly observed that the ease of percutaneous absorption of ID through the normal skin of rats was independent of the viscosity of Nos. 1, 2 and 3 bases.

It has been empirically proven that bleeding can be prevented, and furthermore, FAPG base itself was stabilized by the addition of the fatty acid. It may be presumed that these findings can be attributed to formation of mixed crystals in the FAPG base. Further experiments on these points are being carried out.

It is not yet evident whether or not the fatty acid itself has an enhancing effect on ID absorption through the skin. However, it is considered that fatty acid could be useful as an additive for FAPG base from a practical viewpoint, *e.g.*, in improving the storage properties.

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Application of Synthetic Liposomes Based on Acyl Amino Acids or Acyl Peptides as Drug Carriers. I. Their Preparation and Transport of Glutathione into the Liver

HIROSHI KIWADA,*[,1] MASAMI AKIMOTO, MICHIYO ARAKI, MITSUKO TSUJI and YURIKO KATO

Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12, Ichigaya Funagawara-machi, Shinjuku-ku, Tokyo 162, Japan

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Palmitoyl amino acids and palmitoyl glutathione were synthesized. Liposome-like vesicles based on these compounds were prepared and their barrier functions were examined. These vesicles showed encapsulation efficiencies for aqueous solute comparable to that of conventional phosphatidylcholine liposomes (PC-liposomes). They were also stable in fresh rat plasma at 37 °C.

The biological behavior (blood clearance, urinary excretion and tissue distribution) of the vesicles based on palmitoyl serine (PSer-liposomes) or palmitoyl glutathione (PGSH-liposomes) was examined after intravenous injection in rats. The synthetic liposomes were cleared very rapidly from the blood compared with PC-liposomes. PSer-Liposomes showed a large amount of urinary excretion of aqueous marker ([^{3}H]inulin), suggesting that the mechanisms of vesicle degradation *in vivo* and *in vitro* are different. These synthetic liposomes showed low affinity to the spleen and high affinity to the liver in the tissue distribution study, and thus they may be expected to be a useful drug carrier which is targetable to the liver.

A suppressing effect of PGSH-liposomes on the increase of plasma glutamate oxaloacetate transaminase (GOT) induced by a high dose of acetaminophen in mice was observed, and transport of glutathione into the liver cells apparently occurred. The suppressing effect was greater than that of free glutathione or PC-liposomes containing free glutathione. However, the effect was not observed in the case of PGSH-liposomes without phosphatidylcholine, which appears to have an important role in the liposome-cell interaction.

Keywords——liposome; drug carrier; palmitoyl serine; palmitoyl glutathione; stability; tissue distribution; targeting; acetaminophen; liver necrosis

Introduction

In a previous report,[2] it was shown that synthetic alkyl glycosides formed lamellar vesicles like phosphatidylcholine vesicles (liposomes), and the preparation and physical properties of these vesicles were described. The targeting efficiency of the vesicles based on palmitoyl galactoside to the liver was also reported.[3] Application of synthetic acyl amino acids and acyl peptides was attempted in the present study, and the preparation of the vesicles and glutathione-transporting ability of the acyl glutathione vesicles are described here.

Yatvin *et al.*[4] reported pH-sensitive liposomes composed of synthetic palmitoyl homocysteine. This is an example of application of an acyl amino acid as a liposomal component. If acyl amino acids form lamellar liposome-like vesicles, as alkyl glycosides do, they could be useful for the development of effective drug carriers or functional liposomes, because amino acids have various functional groups and it should be possible to carry out chemical modifications on the liposomal surface. Neuman and Ringsdorf[5] prepared liposomes composed of amino acids coupled with a long hydrocarbon chain. They attempted to stabilize the liposomes by the formation of amide bonds on the surface of the liposomes.

In this report, preparation of liposomes based on *N*-palmitoyl amino acids and the encapsulation capacities of the liposomes were investigated, and the stability in plasma and disposition *in vivo* of palmitoyl serine liposomes were examined as an example.

Many applications of lipophilized peptides which are biologically active, such as muramyl dipeptide (MDP), have recently been attempted.[6] Liposomes injected *in vivo* were phagocytosed by macrophages and the peptide then activated the macrophages. We focussed on glutathione, which has an important role in detoxication in the liver.[7] Glutathione is not readily taken up by the liver in free form,[8] but esters of glutathione[9] are taken up more effectively. Anderson *et al.*[10] studied the uptake of glutathione monoethyl ester by tissues and its conversion to glutathione. On the other hand, it is known that substantial fractions of intravenously injected liposomes are taken up by the liver,[11] probably mainly by the Kupffer cells. However, small liposomes such as small unilamellar vesicles (SUV) may pass through the fenestration of the liver sinusoids and could reach the parenchymal cells.[12,13] Malnoe *et al.*[14] studied the effect of liposomal entrapment on the protective action of glutathione against acetaminophen-induced liver necrosis. Wendel *et al.* reported[15] the transport of glutathione by liposomes encapsulating glutathione in the aqueous phase, but a part of the aqueous contents was apparently released very rapidly in the blood.[16,17] Further, the multilamellar vesicles (MLV) that they used may not be able to be taken up by the parenchymal cells, because they cannot pass through the fenestration and thus cannot reach the cells.[12,13] The use of small liposomes might not improve the situation, because their capacity for glutathione transport would be small.

In this study, we attempted to transport glutathione into the hepatocytes by using small vesicles composed of acyl glutathione. The effectiveness of transport was assessed in terms of the suppressing effect on the increase of plasma glutamate oxaloacetate transaminase (GOT) induced by a high dose of acetaminophen.[15] The stability in plasma and disposition *in vivo* of the palmitoyl glutathione liposomes were also examined.

Experimental

Materials——Palmitic acid, L-serine and L-lysine were purchased from Nakarai Chem. Ltd. (Kyoto). Glycine, L-cystine and L-glutamic acid were from Kanto Chem. Co., (Tokyo). *N*-Hydroxysuccinimide and dicyclohexylcarbodiimide were of special grade for peptide synthesis, and were purchased from Nakarai Chem. Ltd. 5(6)-Carboxyfluorescein (Kodak, Rochester, N.Y.) was used without purification. Hydrogenated egg-phosphatidylcholine and glutathione were gifts from Nippon Fine Chem. Co., (Osaka) and Yamanouchi Pharm. Co., (Tokyo), respectively. All other chemicals and reagents were as described in the previous papers.[2,3]

Synthesis of *N*-Palmitoyl Amino Acids——*N*-Hydroxysuccinimide ester of palmitic acid (PS) was prepared and crystallized (yield 67.5%, mp 90 °C) as described by Lapidot *et al.*[18] This active ester was reacted with amino acids (L-serine, L-lysine, L-cystine, L-glutamic acid and glycine) in the manner described by Lapidot *et al.*[18] Yield (Y) and melting point (mp) of obtained palmitoyl amino acids were as follows: palmitoyl glycine (PGly, Y 27.3%, mp 120 °C); palmitoyl serine (PSer, Y 52.3%, mp 95—97 °C); dipalmitoyl cystine (PCys–CysP, Y 64.0%, mp 105 °C); dipalmitoyl lysine (PlysP, Y 59.2%, mp 101—103 °C); palmitoyl glutamic acid (PGlu, Y 15.8%, mp 110—111 °C).

Synthesis of *N*-Palmitoyl Glutathione——Glutathione (GSH) was oxidized by air in alkaline solution ($NaHCO_3$) at 40 °C by the method of Aoyagi *et al.*[19] Oxidized glutathione (GSSG) was coupled with palmitic acid in the same manner as used for amino acids, as described above. Oxidized palmitoyl glutathione (PGSSGP) was reduced with $NaBH_4$. The reaction mixture was adjusted to pH 1 with 10% HCl, then condensated. Water was added to the residue and the mixture was filtered. The product was recrystallized from MeOH–H_2O several times. Palmitoyl glutathione (PGSH) was obtained in 49.8% yield (mp 170—171 °C). These compounds was checked by infrared (IR) spectrometry, mass spectrometry and elemental analysis. The results of elemental analyses are shown in Table I.

Preparation of Liposomes——Liposomes were prepared by the conventional lipid-film-hydration method.[2] In the case of lipid with low solubility in $CHCl_3$, MeOH was used as the solvent. The lipid composition of the liposomes was palmitoyl residue of palmitoyl amino acid or palmitoyl glutathione and cholesterol (CH) in a molar ratio of 2 : 1 and the lipid concentration was 60 μmol/ml as total lipids. Liposomes used for biological experiments were sonicated with a bath-type sonicator (Tocho IUC-2811, Tokyo) for 2 h at 0 °C after preparation as above. [^{14}C]Sucrose and 5(6)-carboxyfluorescein (CF) were used as aqueous markers in the encapsulation study and stability study,

Table I. Elemental Analyses of Synthetic Palmitoyl Compounds

Compounds	Found (%)			Calc (%)		
	C	H	N	C	H	N
PSer·H_2O	63.15	10.91	3.94	63.16	10.80	3.88
PGly	68.75	11.40	4.50	69.01	11.18	4.47
PGlu	60.59	9.41	3.32	60.50	9.35	3.64
PCys-CysP	63.26	10.19	3.77	63.69	10.06	3.91
PLysP	72.04	11.89	4.58	73.31	11.90	4.50
PGSH	56.78	8.85	7.01	56.83	8.37	7.65

respectively. The fluorescent marker (CF) provides an immediate and easily measurable index of membrane permeability and has been widely used for stability studies.[20] Unencapsulated marker was removed by dialysis in cellulose tubing. [^{3}H]Inulin was used as a marker in the *in vivo* behavior study, and dialysis was carried out in a flow-type dialysis cell with a polycarbonate membrane (Nucleopore Co., CA) having a pore size of 0.05 μm as presented in the previous paper.[3] Inulin is considered to be a suitable liposomal marker for *in vivo* disposition studies, because it is recognized to be biologically inactive and excreted rapidly and completely.[17]

Stability in Plasma——A 0.1 ml aliquot of the liposome (sonicated) suspension containing 100 mM CF was incubated with 0.9 ml of fresh rat plasma at 37 °C. After appropriate time intervals, a 10 μl aliquot of the incubation mixture was transfered into a tube containing 5.0 ml of cold phosphate-buffered saline (PBS). A 1 ml aliquot of the diluted mixture was treated with Triton X-100. The latency of the liposomes was calculated from the fluorescence intensities with and without Triton X-100 treatment.

Blood Concentration and Urinary Excretion——Male albino Wistar rats (body weight; 250±20 g) were cannulated in the femoral vein, femoral artery and bladder, and treated as described in the previous paper.[17] A 0.5 ml aliquot of the liposome suspension (30 μmol total lipids) was injected through the cannula inserted into the femoral vein. At appropriate times after injection, arterial blood and urine samples were collected and the radioactivity of [^{3}H]inulin was counted as described in a previous paper.[17]

Tissue Distribution——Immediately after the last sampling of blood and urine (4 h after injection), the animal was sacrificed and the liver, lungs, spleen and kidneys were isolated. The remaining radioactivity of [^{3}H]inulin in each organ was counted as described in a previous paper.[21]

Transport of Glutathione into the Liver——Transport of glutathione into the liver was examined in terms of the suppressing effect on the increase of plasma GOT induced by a high dose of acetaminophen.[15] Male ddY mice (body weight; 25—30 g) were given acetaminophen intraperitoneally at the dose of 500 mg/kg in dimethylsulfoxide solution (250 mg/ml). More acetaminophen (same dose) was injected at 1.5 h after the first injection in order to confirm the increase of plasma GOT. Various preparations of sonicated liposomes were injected intravenously at 30 min before the first injection of acetaminophen. The animal was sacrificed by puncturing the jugular vein under light anesthesia with ether at 24 h after the second acetaminophen injection. The blood was sampled rapidly with a heparinized pipet. The activity of plasma GOT was determined by the method of Reitman and Frankel[22] and expressed in terms of the absorbance of 2,4-dinitrophenylhydrazone at the wavelength of 505 nm.

Results and Discussion

Characterization and Encapsulation Capacity of Liposomes

Optical micrographs of palmitoyl serine liposomes (PSer-liposomes) and palmitoyl glutathione liposomes (PGSH-liposomes) are shown in Fig. 1. Spherical liposomal structures were observed. Liposomes composed with other palmitoyl amino acids showed similar spherical structure.

The values of encapsulation ratio of aqueous [^{14}C]sucrose solution in the liposomes calculated from the radioactivity of the suspension after and before dialysis are shown in Table II. Liposomes based on palmitoyl amino acid or palmitoyl glutathione showed an encapsulation capacity for aqueous marker that was comparable to that of phosphatidylcholine liposomes (PC-liposomes) with cholesterol. On the other hand, they showed no encapsulation capacity without cholesterol. This result indicates that these compounds form a lamellar structure encapsulating an aqueous phase, like liposomes, when cholesterol is

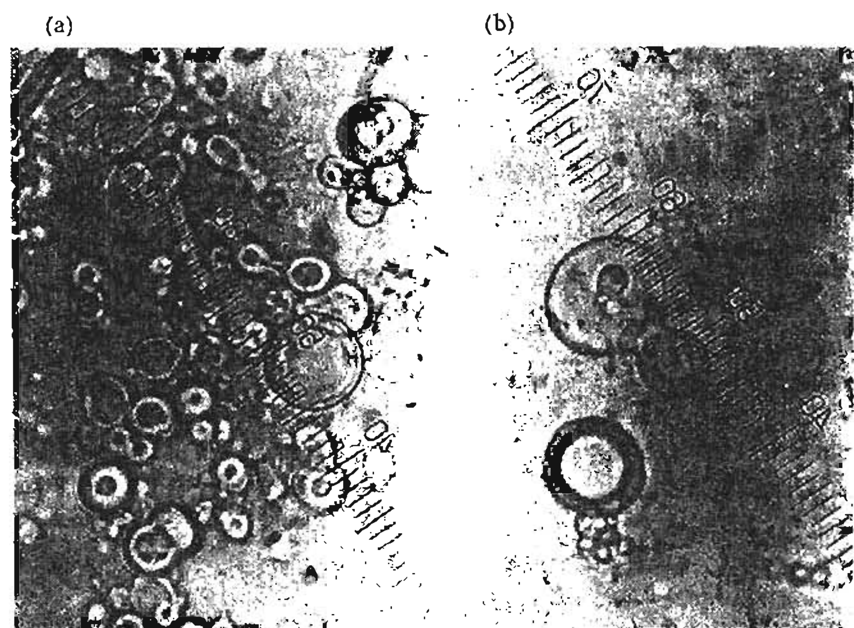

Fig. 1. Optical Micrographs of PSer-Liposomes (a) and PGSH-Liposomes (b)

Liposomes were prepared by the lipid-film-hydration method and were composed of palmitoyl compound (as palmitoyl residue) and cholesterol in a molar ratio of 2 : 1 (1 div. = 1.67 μm).

TABLE II. Encapsulation Ratios of [^{14}C]Sucrose Entrapped in Liposomes Based on Synthetic Palmitoyl Amino Acids and Palmitoyl Peptides

Base	Encapsulation ratio (%)	
	With CH	Without CH
PC	17.4 ± 2.4	—
PSer	13.0 ± 1.8	0.02
PGly	18.8 ± 1.5	0.03
PCys-CysP	13.3 ± 2.4	0.03
PLysP	15.0 ± 3.4	0.05
PGlu	18.8 ± 1.3	0.02
PGSH	14.9 ± 2.9	0.04
PGSSGP	12.8 ± 2.6	0.18

Lipid composition was base (as palmitoyl residue) and cholesterol (CH) in a molar ratio of 2 : 1. Values are expressed as means ± S.D. of three experiments.

present. However, they do not form such a structure without cholesterol. Therefore, the lamellar structure was suggested to be stably constructed with alternate hydrophobic anchors of palmitoyl chain and cholesterol in the lipid layer.

In the case of sonicated liposomes, their encapsulation ratios were about 1%, comparable to that of sonicated PC-liposomes. Very small particles were observed by optical microscopy. The palmitoyl compounds seem to form very small liposomes and retain their barrier function even after sonication. Small liposomes are advantageous for interaction with the hepatocytes because they are able to pass through the fenestration of the liver sinusoids,[12,13] and therefore the sonicated liposomes were used in the following experiments.

Stability in Plasma

It is known that liposomes are destroyed in the blood and release the entrapped aqueous

contents.[16,17] For the application of the vesicles described here as drug carriers *in vivo*, they must be stable in plasma. The stabilities of the sonicated PSer-liposomes and PGSH-liposomes in fresh plasma were examined and compared with that of PC-liposomes. The results are shown in Fig. 2 as the time course of latency during incubation with fresh rat plasma at 37 °C. PC-Liposomes showed gradual degradation followed by a plateau latency of 85%. On the other hand, PSer-liposomes were very stable and showed little leakage during incubation for 2 h. They showed a leakage of about 6% even at the first sampling time (1.5 min), but this is considered to be due to the adsorbed dye on the liposome surface or very rapid leakage because of temperature shock at the initiation of the incubation, or some other such mechanisms. PGSH-Liposomes showed a degradation profile similar to that of PC-liposomes and they are considered to have comparable stability to PC-liposomes in plasma.

Clearance from the Blood

Clearance of the sonicated liposomes from the blood after intravenous injection is shown in Fig. 3. PC-Liposomes were retained in the blood for a long time, and radioactivity amounting to about 2.4% of injected dose was observed in 1 ml of the blood even at 4 h after the injection. This value corresponds to about 50% of dose in whole blood (blood volume was estimated as 8% of body weight). On the other hand, PSer-liposomes and PGSH-liposomes were cleared rapidly and little radioactivity was observed in the blood even at 1 h after injection. In particular, PSer-liposomes were cleared very rapidly and only 1.9%/ml of the injected radioactivity was observed at 3 min after the injection. It is known that the clearance of liposomes is affected by particle size.[17] The size of particles used in this experiment were not determined, but there should not be much difference between PC- and palmitoyl-compound-liposomes because they were prepared by the same procedure and the particle sizes were confirmed to be very small by optical microscopy. Therefore, it is reasonable to consider that the clearance mechanisms of the PC-liposomes and synthetic liposomes presented here may be different.

Excretion of an Aqueous Marker in Urine

Cumulative radioactivity excreted in urine after intravenous injection of the liposomes is presented in Fig. 4 as a percentage of the injected dose. The initial increasing phase in the

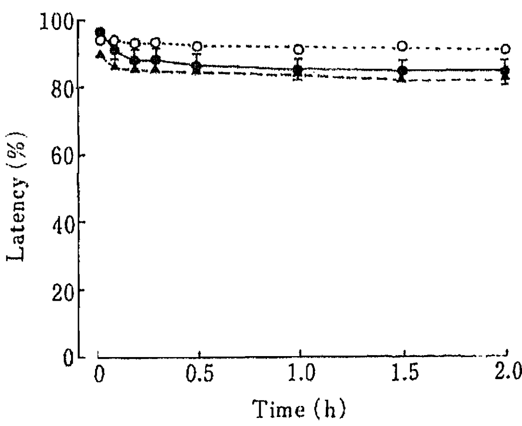

Fig. 2. Stability of PC-Liposomes (—●—), PSer-Liposomes (---○---) and PGSH-Liposomes (--▲--) in Plasma

Liposomes were prepared by the sonication procedure and their lipid compositions were the same as those in Fig. 1. Latency was calculated from the fluorescence intensity of CF lost from the liposomes during incubation with fresh rat plasma at 37 °C.

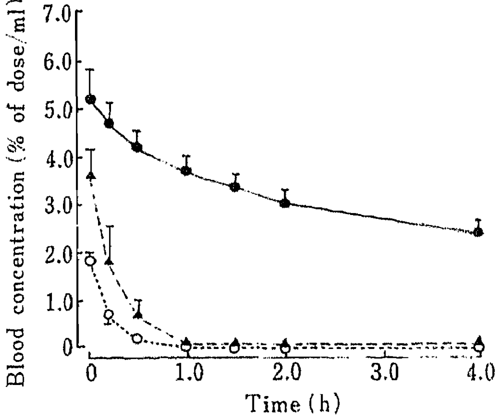

Fig. 3. Time Courses of Blood Levels of [^{3}H]-Inulin Entrapped in Liposomes after Intravenous Injection into Rats

Liposome preparations and symbols are the same as those in Fig. 2. Blood concentration was determined based on the radioactivity of [^{3}H]inulin used as an aqueous space marker. The dose was 30 μmol/head as total lipids.

cumulative excretion curve may represent the rapid leakage of the aqueous marker, [^{3}H]inulin, from the liposomes in the circulation as described in the previous paper.[17] PSer-Liposomes showed a large excretion in the initial phase, even though the *in vitro* stability study in fresh plasma showed that the liposomes were very stable, as mentioned previously. This result suggests the possibility that the *in vivo* stability in the early stage after the injection is affected by some other factor(s) than those existing in plasma, *e.g.* turbulence of the blood flow, interaction with cells in the blood or tissues, *etc.* PC-Liposomes and PGSH-liposomes seemed to be resistant to these factor(s) and showed lower urinary excretion.

Tissue Distributions

Remaining radioactivity in each organ at 4 h after injection is listed as a percentage of the injected dose in Table III. It is known that intravenously injected liposomes accumulate in the liver and spleen.[11] In the present experiment, 23.6% of dose was found in the liver and 8.7% in the spleen after injection of PC-liposomes. Little radioactivity was found in the kidneys and lungs (less than 1%). In the case of PSer-liposomes, 30.1% and 1.8% of the dose were found in the liver and spleen, respectively. Radioactivities in the kidneys and lungs were very low (<0.4%). The most remarkable difference between PC-liposomes and PSer-liposomes was in the accumulation in the spleen. PSer-Liposomes showed less accumulation than PC-liposomes (about one-fifth of that of PC-liposomes). The tissue distribution pattern of PGSH-liposomes was very similar to that of PSer-liposomes except for the liver. PGSH-liposomes accumulated in the liver more markedly than PSer-liposomes (67.4% of dose).

The results obtained in this experiment indicated that the liposomes based on palmitoyl amino acid or palmitoyl peptide are selectively accumulated in the liver, not in the spleen, and their blood concentration is very low after intravenous administration. Though PSer-liposomes are less stable in the circulation and their aqueous contents are rapidly excreted in the urine, the synthetic liposomes described here may be useful for targeting to the liver. It is

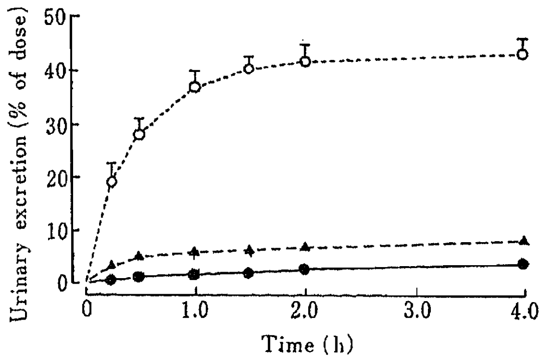

Fig. 4. Cumulative Excretion of [^{3}H]Inulin in Urine after Intravenous Injection of [^{3}H]Inulin Entrapped in Liposomes into Rats

Liposome preparation, symbols and method of determination are the same as those in Fig. 3.

TABLE III. Tissue Distribution of [^{3}H]Inulin Entrapped in Liposomes after Intravenous Injection into Rats at the Dose of 30 μmol/head

Organ	Distribution (% of dose)		
	PGSH	PSer	PC
Liver	67.42±2.47	30.14±2.65	23.57±4.73
Kidney	0.33±0.08	0.36±0.04	0.58±0.15
Lung	0.31±0.07	0.37±0.04	0.76±0.25
Spleen	1.91±0.31	1.83±0.48	8.71±3.07

Values are expressed as mean±S.D. of four experiments.

TABLE IV. Effects of Glutathione in Various Dosage Forms against Plasma GOT Induction by Acetaminophen in Mice

Dosage form	Dose of GSH (mg/kg)	Plasma GOT (Absorbance)
PBS (Control)	—	0.892±0.061
Free GSH	81.8	0.729±0.211
PC-liposomes	81.8	0.724±0.156[a]
PGSH-liposomes/PC	81.8	0.496±0.072[b]
PGSH-liposomes/CH	81.8	0.751±0.248

a) Significantly different from PBS ($p<0.05$). *b*) Significantly different from PBS ($p<0.01$). Values are expressed as means±S.D. of six or seven experiments. The dose of palmitoyl glutathione was expressed as free glutathione. The dose of acetaminophen was 500 mg/kg×2.

not yet clear how the properties (stability and *in vivo* behavior) of the liposomes described above are related to the species of amino acid or the length of amino acid sequence. This is currently under study.

Transport of Glutathione into the Liver

PGSH-Liposomes accumulate in the liver after intravenous injection as described above. The effectiveness of the liposomes as transporters of glutathione to prevent liver necrosis was examined in termes of their suppressing effect on the increase of plasma GOT induced by a high dose of acetaminophen. The results are expressed as absorbance determined by the method of Reitman and Frankel,[22] as shown in Table IV.

PGSH-Liposomes containing phosphatidylcholine in an equimolar ratio showed remarkable suppression of the increase of plasma GOT ($p<0.01$). PC-Liposomes containing free glutathione in the aqueous phase were also slightly effective ($p<0.05$). However, PGSH-liposomes without phosphatidylcholine were not effective ($p>0.05$). Though it is not clear whether the accumulated PGSH-liposomes in the liver are taken up by the Kupffer cells or parenchymal cells, the results obtained in this experiment suggest that glutathione was transferred into the parenchymal cells. Uptake of small liposomes by the parenchymal cells has been reported,[12,13] so the sonicated PGSH-liposomes may be directly taken up by the cells. It is uncertain whether the suppressing effect observed in this study is attributable to acylated glutathione or hydrated free glutathione. However, Anderson *et al.*[10] reported the conversion of the glutathione ester to the free form in the cells, so free glutathione might be the effective agent in this case. Phosphatidylcholine seems to be indispensable for the effectiveness of glutathione or palmitoyl glutathione in liposomal form and it may play an important role in the uptake of the liposomes. A study of the liposome-cell interaction should yield interesting results.

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Glassy State of Pharmaceuticals. II.[1] Bioinequivalence of Glassy and Crystalline Indomethacin[2]

EIHEI FUKUOKA,* MIDORI MAKITA and SHIGEO YAMAMURA

School of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama Funabashi, Chiba 274, Japan

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Bioinequivalence of glassy and crystalline indomethacin was investigated in rabbits. *In vivo* absorption studies were carried out by determining the plasma levels of indomethacin following oral and rectal administrations in rabbits. The *AUC* (area under the concentration-time curve) and the maximum plasma level following the administration of the glassy preparation were larger than those in the case of the crystalline preparation. The amounts of indomethacin absorbed were calculated by the deconvolution method. It was shown that the amounts absorbed following oral and rectal administrations of the glassy preparation were larger than those in the case of the crystalline preparation. These results indicated that the bioavailability of the glassy indomethacin was better than that of the crystalline indomethacin.

Keywords——glassy state; indomethacin; bioavailability; stability; X-ray analysis; deconvolution

In the previous paper,[1] the glassy state of indomethacin was confirmed by detection of the jump of heat capacity and the anomalous endothermic peak in the differential scanning calorimeter (DSC) curve, and the relaxation process below the glass transition temperature (T_g) was traced in terms of the area under the anomalous endothermic peak in the DSC curve. It was also reported that the rate of dissolution of glassy indomethacin was far greater than that of crystalline indomethacin. Further, the rate of crystallization of pulverized glassy indomethacin followed first-order kinetics and the degree of crystallization reached a maximum of 60% after 2 months.

It was expected that the glassy indomethacin would show better bioavailability than crystalline indomethacin because the dissolution rate of the glass is faster than that of the crystals as described in the previous paper.[1] In the present paper, *in vivo* absorption studies of glassy and crystalline indomethacin were carried out in rabbits in order to compare the bioavailability of the two forms.

Experimental

Materials——Indomethacin (Sigma Chemical Co., Ltd.) was of reagent grade. Other materials were of special reagent grade except sodium bicarbonate and lactose, which were of JP XI grade. Glassy indomethacin was prepared by cooling the melt as reported in the previous paper.[1] Physical mixtures of the glass and the crystals with lactose were prepared by mixing the two ingredients as follows: 200—250 mesh (63—74 μm) fraction of glassy or crystalline indomethacin was collected by using standard sieves after pulverizing the drug in an agate mortar. Then, each powder was mixed with lactose (200—250 mesh) in 1 : 4 weight ratio by using a spatula.

X-Ray Diffraction——A Rigaku Denki Geigerflex instrument was used for the present investigation. The X-ray source was Cu-K_α with an Ni filter (voltage 35 kV, current 10 mA), and a scintillation counter was used as a detector.

DSC——DSC curves were measured with a DSC-2 (Perkin-Elmer). The measurement conditions were the same as those reported in the previous paper.[1]

Infrared (IR) Absorption Spectra——IR spectra were measured on a model 295 infrared spectrophotometer (Hitachi). The measurements were made by the KBr disc method.

Thin Layer Chromatography (TLC)——The conditions of TLC were the same as those reported in the previous paper[1] and the spots were visualized under ultraviolet (UV) light.

Dissolution Studies——The dissolution behavior of glassy and crystalline indomethacin was studied by the beaker method with 100 ml of 0.02 M phosphate buffer (pH 7.2) maintained at 37 °C as a solvent. The samples were prepared as reported in the previous paper.[1] Concentration of indomethacin in the solvent was determined spectrophotometrically at 317 nm with a Hitachi 340 spectrophotometer.

Wettability Test——The penetration rate of liquid into glassy and crystalline indomethacin powder (200—250 mesh) beds and into the glassy and crystalline preparations was measured by the powder method. Distilled water and distilled water saturated with lactose were used as penetrating liquids.

Animal Studies——Male rabbits (2.5—3.0 kg) were used after fasting for about 18 h.

i) Intravenous Administration: Indomethacin was dissolved in water containing sodium bicarbonate (weight ratio of indomethacin and sodium bicarbonate, 5 : 2) and indomethancin solutions of 7.5, 15, and 30 mg/ml were used as sample solutions.

Three dosage levels of indomethacin (5, 10, and 20 mg/kg body weight) were administered intravenously. Blood samples were taken from an ear vein at appropriate intervals into a heparinized syringe.

ii) Oral and Rectal Administrations: A two-way cross-over design was employed for the oral and rectal administrations. A dose of 20 mg/kg body weight as indomethacin was enclosed in a No. 0 gelatin capsule, and then administered orally or rectally. Blood samples were taken from an ear vein at appropriate intervals into a heparinized syringe.

Determination of the Plasma Levels of Indomethacin——Plasma level of indomethacin was determined according to Nogami's modification[3] of Hucker's fluorescence method.[4] Fluorescence intensity was measured with a Hitachi MPF-4 spectrofluorometer (activation maximum, 295 nm; fluorescence maximum, 375 nm uncorrected).

Calculation of Amount Absorbed——The amount of indomethacin absorbed following oral or rectal administration was calculated by the least-squares deconvolution method developed by Pedersen.[5]

Results and Discussion

Preparation and Characterization of Glassy and Crystalline Indomethacin

Glassy indomethacin was prepared by cooling the melt, and the glassy state was confirmed by detection of the glass transition temperature in the DSC curve and a halo in the X-ray diffraction pattern.[1] Crystalline indomethacin used was γ-type crystals; it was characterized by measurements of the X-ray diffraction pattern, melting point (by DSC) and IR spectrum.[6] Decomposition products of glassy and crystalline indomethacin were not detectable by TLC.

Dissolution Rates of Glassy and Crystalline Indomethacin

Figure 1 shows the dissolution profiles of glassy and crystalline indomethacin in

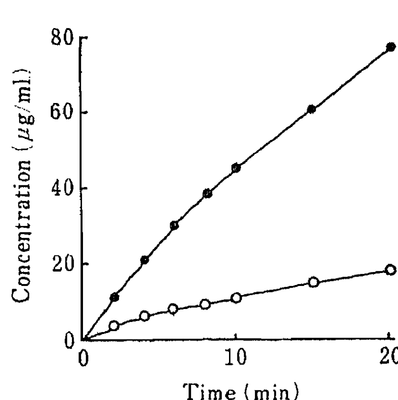


Fig. 1. Dissolution Profiles of Glassy and Crystalline (γ-Type) Indomethacin Determined by the Beaker Method in Phosphate Buffer (pH 7.2)

●, glassy indomethacin; ○, crystalline indomethacin.

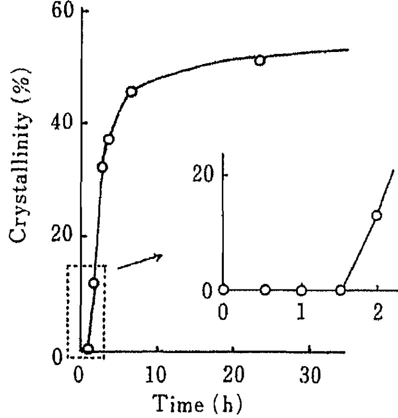


Fig. 2. The Rate of Crystallization of Pulverized Glassy Indomethacin in Water

phosphate buffer (pH 7.2). Glassy indomethacin dissolved about 4 times faster than the crystalline material.

Crystallization of Glassy Indomethacin in Water

If crystallization of the glass occurred during dissolution, the dissolution and subsequent absorption of the glass might be affected. Thus, crystallization of the glass in water was investigated.

After being pulverized and mounted on a glass plate for X-ray diffraction measurement, glassy indomethacin was immersed in distilled water for a given period of time and then dried under vacuum in a desiccator containing P_2O_5. The X-ray diffraction pattern was measured immediately after the sample was dried. The crystallinity of the sample was determined by Hermans' method.[7] The crystallization process of the sample immersed in water is shown in Fig. 2 as a plot of crystallinity against time.

After standing for 1.5 h in water, the glass began to crystallize and then crystallization proceeded rapidly. It took 10 h to reach maximum crystallization and at this stage indomethacin had 50% crystallinity. It was considered that if glassy indomethacin was dissolved and absorbed rapidly after administration, *in vivo* absorption might not be influenced by the crystallization of the glass.

Some Physicochemical Properties of Sample Powders

Wettability of the Sample Powders——The *in vivo* absorption rate of the drug after the administration of powder dosage forms is influenced in the first place by the wettability of the sample. Thus, the wettability of powder beds of glassy and crystalline indomethacin was examined. Figure 3 shows the penetration of water into the powder beds in terms of a plot of the square of the penetration length against time. Water did not penetrate into the glassy indomethacin powder bed, and crystalline indomethacin was hardly wetted. Thus, the contact angle as a main factor governing the wettability was measured according to the method reported in the previous paper[8] and values as large as 95° (glassy indomethacin) and 84° (crystalline indomethacin) were obtained. Improvement of the wettability of glassy and

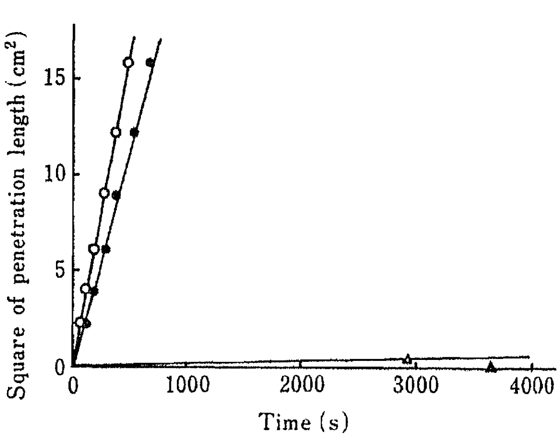


Fig. 3. Penetration of Liquid into the Powder Beds

●, mixture of glassy indomethacin and lactose (the penetrating liquid was distilled water saturated with lactose); ○, mixture of crystalline indomethacin and lactose (the penetrating liquid was distilled water saturated with lactose); ▲, glassy indomethacin (the penetrating liquid was distilled water); △, crystalline indomethacin (the penetrating liquid was distilled water).

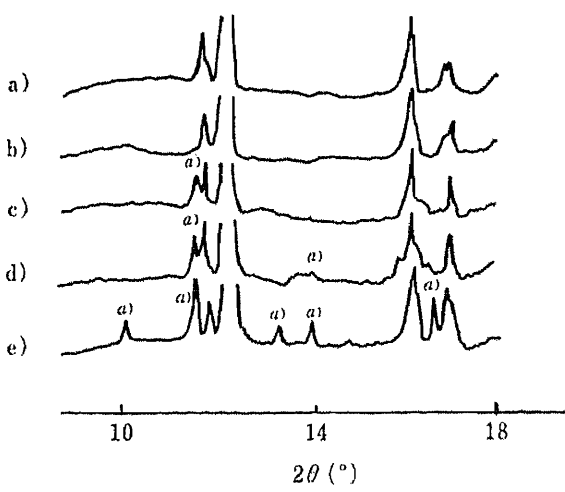


Fig. 4. Crystallization of Glassy Indomethacin Mixed with Lactose at Room Temperature

a) immediately after mixing; b) after standing for 5 h; c) after standing for 30 h; d) after standing for 56 h; e) after standing for 180 h.

a) Indicates diffraction peaks of indomethacin crystals.

crystalline indomethacin was attempted by mixing them with lactose. As shown in Fig. 3, the wettabilities were improved and a linear relationship was found between the square of the penetration length and time for the two preparations.

Crystallization of Glassy Indomethacin at Room Temperature——The crystallization of the glass at room temperature was followed by the X-ray diffraction method. Since indomethacin gave a very stable glass, devitrification did not occur for several years in the laboratory at room temperature. It was considered, however, that crystallization of the glass in the preparation might be induced by pulverizing and by mixing the sample with lactose.

Figure 4 shows the X-ray diffraction patterns of the glassy preparation after standing for a given period of time at room temperature. Only diffraction peaks due to crystalline lactose were observed in the X-ray diffraction pattern immediately after mixing the glass with lactose. After the sample had been left for 30 h, slight diffraction due to indomethacin crystals was observed in the vicinity of 12° (2θ). Subsequently, several diffraction peaks due to indomethacin crystals appeared and the intensity of the peaks increased gradually as crystallization proceeded.

These results indicated that the crystallization of the glass was scarcely induced by pulverizing or by mixing the sample with lactose in the initial stage. Therefore, the following experiments were run within 1 h after pulverization of the glass.

In Vivo Absorption Studies

Figure 5 shows the mean plasma levels of indomethacin following bolus intravenous administration of indomethacin solution. Each curve could be adequately approximated by a biexponential equation (Eq. 1), and the pharmacokinetic paramaters obtained are shown in

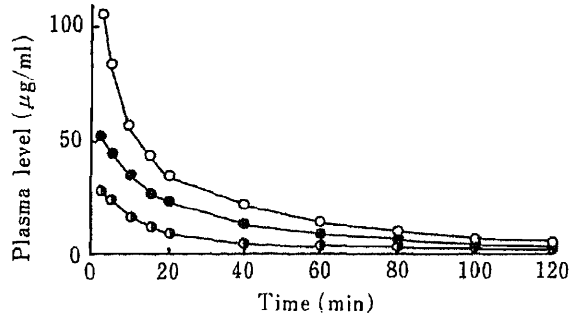


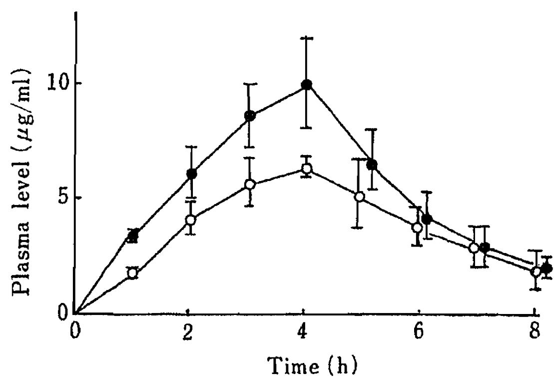
Fig. 5. Mean Plasma Levels Following Intravenous Administration of Indomethacin Solution

○, 20 mg/kg ($n=3$); ●, 10 mg/kg ($n=4$); ◑, 5 mg/kg ($n=3$).

Table I. Pharmacokinetic Parameters Obtained from the Plasma Levels Following Intravenous Administration

Parameter	Dose		
	5 mg/kg	10 mg/kg	20 mg/kg
a_1 (μg/ml)	27.31±3.65	37.68±6.92	93.77±13.25
b_1 (/min)	0.1222±0.0056	0.1034±0.0048	0.1335±0.0104
a_2 (μg/ml)	9.59±2.87	23.97±4.50	39.27±3.18
b_2 (/min)	0.0132±0.0008	0.0146±0.0024	0.0154±0.0005
k_{12} (/min)	0.0578±0.0055	0.0365±0.0034	0.0581±0.0048
k_{21} (/min)	0.0421±0.0101	0.0504±0.0107	0.0521±0.0077
k_{10} (/min)	0.0423±0.0072	0.0311±0.0053	0.0407±0.0016
V_1 (ml/kg)	136±8	163±9	153±15
V_2 (ml/kg)	176±31	131±37	173±18
$AUC_{0\rightarrow\infty}$ (μg/ml min)	939±148	2057±214	3261±282

Each value represents the mean±S.E. of 3—4 rabbits.



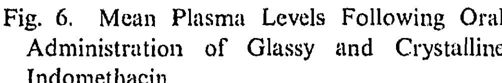


Fig. 6. Mean Plasma Levels Following Oral Administration of Glassy and Crystalline Indomethacin

●, glassy indomethacin; ○, crystalline indomethacin. Vertical lines represent the S.E. of the mean for six experiments.

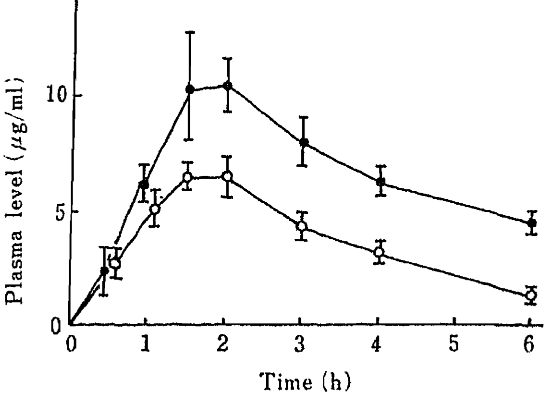


Fig. 7. Mean Plasma Levels Following Rectal Administration of Glassy and Crystalline Indomethacin

●, glassy indomethacin; ○, crystalline indomethacin. Vertical lines represent the S.E. of the mean for six experiments.

Table I.

$$c^*(t)=a_1\exp(b_1 t)+a_2\exp(b_2 t) \qquad (1)$$

where $c^*(t)$ is plasma level time course of indomethacin and a_1, a_2, b_1 and b_2 are constants.

Figures 6 and 7 show the plasma levels following oral and rectal administration of the glass and crystal preparations. Maximum plasma level and area under the concentration–time curve (AUC) following administration of the glass were significantly larger than those of the crystals ($p<0.05$). These results indicate that glassy and crystalline indomethacin are bioinequivalent.

Calculation of Absorption Rate and Amount Absorbed

Absorption rate and the amount absorbed were calculated by the least-squares deconvolution method developed by Pedersen.[5] The theory of this method is as follows. The response, $c^*(t)$, (plasma level time course of indomethacin following intravenous administration) to unit impulse input is expressed by Eq. 1. The rate of input (absorption rate), $f(t)$, is approximated to a polynomial function and expressed as Eq. 2.

$$f(t)=\sum_{i=0}^{N} x_i t^i \qquad (2)$$

where x_i are the polynomial coefficients and the highest degree of the polynomial function (N) is the number of data points minus one. The response, $c(t)$, (plasma level time course of indomethacin following oral or rectal administration) to an arbitrary input can be expressed by Eq. 3. Substituting Eq. 3 into Eqs. 1 and 2 yields Eq. 4.

$$c(t)=\frac{1}{q^*}\int_0^t f(t-u)c^*(u)du \qquad (3)$$

$$c(t)=\frac{1}{q^*}\int_0^t \sum_{i=0}^{N} x_i(t-u)^i[a_1\exp(b_1 u)+a_2\exp(b_2 u)]du \qquad (4)$$

where q^* is an intravenous bolus dose. When Eq. 4 is integrated and rearranged, Eq. 5 is obtained.

$$c(t) = \sum_{i=0}^{N} x_i \theta_i(t) \tag{5}$$

where,

$$\theta_i(t) = \frac{1}{q^*} i! \sum_{j=1}^{2} \frac{a_j}{b_j^{i+1}} \left[\exp(b_j t) - \sum_{k=0}^{i} \frac{(b_j t)^k}{k!} \right]$$

The polynomial coefficients x_i of the input function were calculated by multiple linear regression analysis and the cumulative amount of input (amount absorbed) was calculated from the integrated form of $f(t)$. Figures 8 and 9 show the cumulative amount of input following oral and rectal administrations of glassy and crystalline preparations, respectively. In both cases, the amounts of input following the administration of the glassy preparation were larger than those of the crystalline preparation.

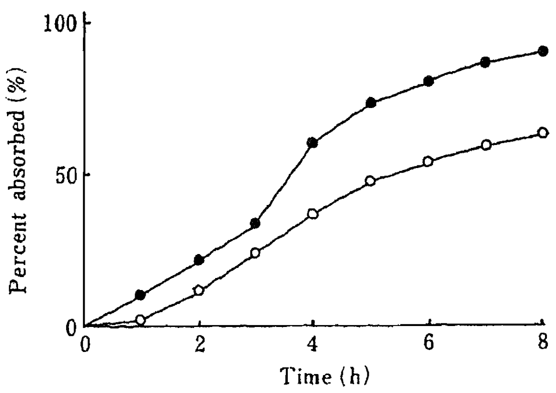


Fig. 8. Percent Absorbed Following Oral Administration of Glassy and Crystalline Indomethacin Calculated by the Deconvolution Method

●, glassy indomethacin; ○, crystalline indomethacin.

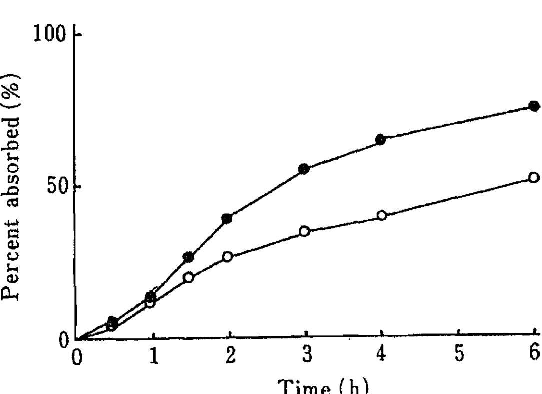


Fig. 9. Percent Absorbed Following Rectal Administration of Glassy and Crystalline Indomethacin Calculated by the Deconvolution Method

●, glassy indomethacin; ○, crystalline indomethacin.

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Coating of Pharmaceutical Powders by Fluidized Bed Process. I. Aqueous Enteric Coating with Methacrylic Acid–Ethylacrylate Copolymer and the Dissolution Behavior of Products

YOSHINOBU FUKUMORI,*,[a] TOMOAKI FUKUDA,[a] YOSHINOBU HANYU,[a] YOSHIKAZU TAKEUCHI[a] and YOSHIFUMI OSAKO[b]

Faculty of Pharmaceutical Sciences, Kobe-Gakuin University,[a] Arise, Igawadani-cho, Nishi-ku, Kobe 673, Japan and Fuji Paudal,[b] Chuo 2-2-30, Jyoto-ku, Osaka 536, Japan

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Phenacetin and lactose powders, as models of slightly and highly water-soluble drugs, respectively, were coated by means of the Wurster process.

Phenacetin (80—250 mesh) was a fragile crystalline material and hence fluidization had to be moderate. As a result, the agglomeration of some particles or the adhesion of small particles to large particles could not be avoided. However, the particles coated with triacetin(plasticizer)-containing lacquer (60% as dry lacquer relative to the core material) released only 6.0% of phenacetin in JP XI disintegration medium No. 1. This result suggested that slight agglomeration did not prevent the encapsulation. When a polyethyleneglycol 6000 and polysorbate 80 mixture was used as a plasticizer in the coating of phenacetin, the membrane became more permeable than when triacetin was used.

With lactose (100 mesh), which is a comparatively hard crystalline material, discretely encapsulated particles could easily be produced. The lactose particles coated with triacetin- and with polyethyleneglycol 6000-polysorbate 80-containing lacquer exhibited identical dissolution properties. Their dissolution profiles were characterized by a lag time, which increased with the amount of coating applied, followed by a rapid release. This lag time was the period needed for lactose crystals to be dissolved in rapidly taken up water. The inward flow of water should prevent lactose from diffusing out during the lag time. The high solubility and high permeation rate of lactose and the large specific surface area of particles produced by the Wurster process presumably account for the rapid release after the lag time. These dissolution profiles of coated lactose particles suggested that they should be useful as a model to study oral drug delivery systems with various lag times of dissolution.

Keywords—enteric coating; powder; fluidized bed; dissolution; lactose; phenacetin; microencapsulation; Wurster process; methacrylic acid–ethylacrylate copolymer

The fluidized bed coating process is already a widely used technique for tablets and granules which have a large particle size. Recently, dosage forms composed of coated small particles have been proposed to be useful because of their gastric emptying characteristics, rapid response to the medium and so on.[1] However, small particles sprayed in the conventional fluidized bed tend to be agglomerated. Thus, the Wurster process has been proposed for powder coating,[2] since the rapidly recycled particle flow is expected to prevent agglomeration as a result of the high-speed particle motion. However, it is still not clear what conditions of spraying and fluidizing and what properties of powders result in the particles with desired properties.

A serious problem in powder coating is that a large amount of coating material may be needed to encapsulate fine powders with a large specific surface area, whereas 20—30% coating is usually sufficient for tablets and granules. This may make the practical application

of powder coating too time-consuming and expensive.

Preliminary experiments to obtain some fundamental data on the powder coating were planned. An aqueous enteric coating was applied by the Wurster process with a methacrylic acid–ethylacrylate (1 : 1) copolymer dispersion, a typical aqueous coating material, and the dissolution behaviour of the products was evaluated in an aqueous acidic medium.

Experimental

Materials——As core materials, lactose (DMV 100 M) and phenacetin (JPX grade, Hoei Yakko) were used. They were regarded as models of hydrophilic and hydrophobic drugs, respectively. Purchased phenacetin powder with a large particle size was forced through an 80 mesh sieve and particles under 250 mesh were removed by the use of an air-jet sieve (Alpine). An aqueous dispersion (30% dry lacquer) of methacrylic acid–ethylacrylate copolymer, (MA–EA, Eudragit L30D-55, Röhm Pharma), was used as the coating material, and triacetin (TA) or polyethyleneglycol 6000–polysorbate 80 (PEG–PS, Nakarai Chemicals) was used as the plasticizer. Talc (JP XI grade, Maruishi) was used as purchased.

Coating Apparatus——A Glatt GPCG-1 apparatus was used.

Dissolution——Dissolution tests were performed on an NTR 5S3 dissolution apparatus (Toyama Sangyo) by the JP XI paddle method at 200 rpm and 37 °C. The dissolution medium was JP XI disintegration medium No. 1 (pH 1.2). The sample weight was 150 mg as core material. An aliquot of 1 ml was tken through a 0.45 μm filter (Ekicrodisc 3, Gelman Sciences Japan, Ltd.) every 30 min. The phenacetin concentration in the sample was determined by measuring the absorbance at 245 nm on a Shimazu UV 190 spectrophotometer. The lactose concentration was detected by the phenol–sulfuric acid method.

Sieve Analysis——Particle size distribution was determined by sieve analysis with a row-tap shaker (Iida Seisakusho Co., Ltd.). The shaking time was 10 min and the charged weight was 100 g.

Scanning Electron Microscopy (SEM)——SEM was performed on Hitachi S430.

Polarizing Microscopy——An Olympus POM polarizing microscope was used with a heating stage (MHS, Union Optical).

Results

Coating of Phenacetin and Its Dissolution

Phenacetin crystals were used as a model of a fragile core material,[3] and as a model hydrophobic drug. The operating conditions and the composition of the spray dispersion are shown in Tables I and II, respectively. Until the coating reached 20% as MA–EA dry lacquer, the fluidizing conditions had to be moderate in order to avoid fracture of the crystals. During that stage, the powder was, therefore, under conditions where agglomeration could easily occur. To avoid agglomeration, talc was conventionally added to the spray dispersion (Table II) and, in addition, 70 or 150 g of talc powder was gradually inserted into the coating chamber in coating with TA or with PEG–PS, respectively. The larger amount of talc added in

TABLE I. Operating Conditions in Coating Phenacetin Powder

	TA		PEG–PS
Core material		Phenacetin	
Mesh size		80—250	
Charged weight (g)		500	
Plasticizer	TA		PEG–PS
Inlet air temperature (°C)		60	
Material temperature (°C)	33→36		33→36
Outlet air temperature (°C)	31→32		31→33
Air flow rate (m^3/min)	0.75→0.9		0.75→1.25
Spray rate (ml/min)		6.9	
Spray pressure (atm)	1.5→2.0		1.5→2.6
Diameter of spray nozzle (mm)		0.8	
Drying conditions		60 °C, 60 min	

TABLE II. Composition of Spray Dispersion

MA–EA dispersion	1000 g		1000 g
TA	30 g	PEG 6000	30 g
		Polysorbate 80	15 g
Talc	90 g		90 g
Water	Added		Added
Total	2400 ml		2400 ml
Total solid	420 g (84%)		435 g (87%)
MA–EA dry lacquer	60%		60%

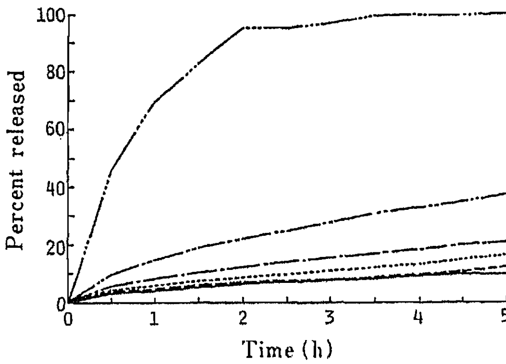
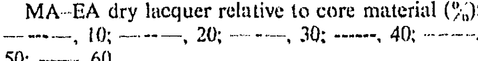


Fig. 1. Dissolution of Phenacetin Coated with TA-Containing Lacquer in JP XI Disintegration Medium No. 1 (pH 1.2)



MA–EA dry lacquer relative to core material (%): ——, 10; ——, 20; ——, 30; ------, 40; ——, 50; ——, 60.

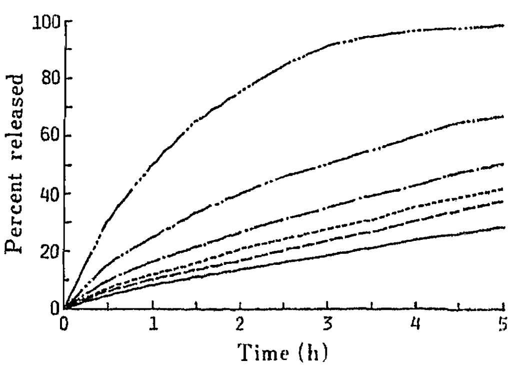


Fig. 2. Dissolution of Phenacetin Coated with PEG–PS Containing Lacquer in JP XI Disintegration Medium No. 1 (pH 1.2)

MA–EA dry lacquer relative to core material (%): ——, 10; ——, 20; ——, 30; ------, 40; - - - -, 50; ——, 60.

coating with PEG–PS was due to the more wettable character of the sprayed powder. At 20% coating, where the coated particles seemed not to be fractured any more, the conditions were altered as shown by the arrows in Table I.

In this study, the inlet air temperature was kept at 60 °C. The inlet air rate and the spray pressure were set so that particles would not be fractured. The spray rate was also adjusted so that the air-suspended particles could be slightly wetted in the upper part of chamber and the fluidized bed at the bottom could be dry.

The dissolution curves for the product coated with TA are shown in Fig. 1. The coating with 20% MA–EA dry lacquer relative to phenacetin provided a fairly good barrier against phenacetin release. The release at 2 h was 6.0% at 60% coating. This approaches a practical level for enteric coating. Figure 1 also suggests that sustained-release products may be produced by less than 20% coating.

The dissolution curves for the product coated with PEG–PS are shown in Fig. 2. The membrane with PEG–PS was a less effective barrier against phenacetin diffusion than the membrane containing TA. As a result, a larger amount of coating material would be needed for enteric coating.

Coating of Lactose and Its Dissolution

Lactose was used as a model water-soluble, hydrophilic drug. The lactose crystal is comparatively hard and can not be fractured easily.[3] Hence, lactose powder could be fluidized under more severe conditions (Table III) than phenacetin (Table I), so that the particles were not agglomerated.

The dissolution curves for the lactose powder coated with TA are shown in Fig. 3. When

TABLE III. Operating Conditions in Coating Lactose Powder

Core material	Lactose
Mesh size	100
Charged weight (g)	500
Plasticizer	TA or PEG–PS
Inlet air temperature (°C)	60
Material temperature (°C)	40
Outlet air temperature (°C)	35
Air flow rate (m^3/min)	1.7
Spray rate (ml/min)	6.4
Spray pressure (atm)	2.5
Diameter of spray nozzle (mm)	0.8
Drying conditions	60 °C, 60 min

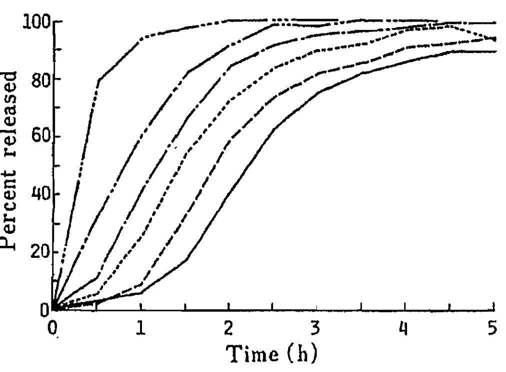


Fig. 3. Dissolution of Lactose Coated with TA-Containing Lacquer in JP XI Disintegration Medium No. 1 (pH 1.2)

TA dry lacquer relative to core material (%): —·—·—, 10; — — —, 20; —·—, 30; ------, 40; - - - -, 50; ——, 60.

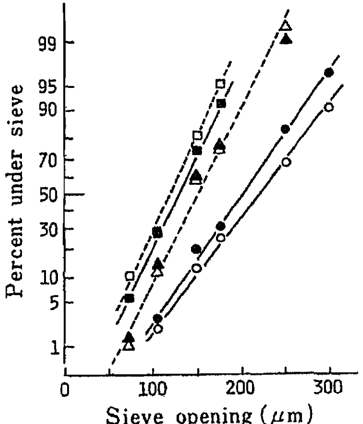


Fig. 4. Particle Size Distributions of Uncoated and Coated Powders on a Normal Probability Scale as Determined by Sieve Analysis

Symbols and 50% diameters (μm):

	Uncoated		Coated			
Core material	Lactose	Phenacetin	Lactose		Phenacetin	
Plasticizer	—	—	PEG–PS	TA	PEG–PS	TA
50% diameter	120	128	150	150	200	223
Symbol	□	■	▲	△	●	○

the plasticizer was changed to PEG–PS, the dissolution curves (not shown here) were almost identical with those in Fig. 3. Figure 3 shows that the dissolution of lactose had a lag time above 30% coating and became very rapid after the lag time. The rate at the rapid dissolution stage changed only moderately above 20% coating, suggesting that the particles were almost wholly encapsulated at 20% coating.

Particle Size Distribution

The particle size distributions are shown in Fig. 4 for the raw powders and the products. The coated phenacetin powder had a remarkably increased particle size (see the legend to Fig. 4). This was because phenacetin was fluidized under relatively mild conditions, and hence

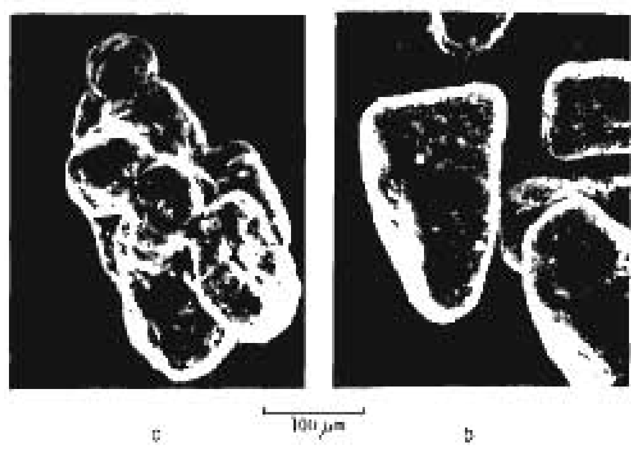


Fig. 5. Photographs of Coated Particles (×300)

Core material and plasticizer: a, phenacetin–TA; b, lactose–TA.

small particles could adhere to large particles and even large particles could be agglomerated to some extent. The photograph in Fig. 5a shows this feature of the particles.

On the other hand, the particle size of lactose powder was not much enlarged by coating, presumably because of the more severe fluidizing conditions used in coating lactose powder (Table III). The powder visualized by SEM consisted of discrete particles, as shown in Fig. 5b. The increase of the 50% diameter by 30 μm was clearly due to the thickness of the formed membrane.

Discussion

Particle Flow in the Wurster Chamber

In the Wurster chamber (Fig. 6), particles are forced into a cylindrical partition by air flow and sprayed there. The particles thereafter fall with the recycling air flow in the upper part of chamber. The particles deposited outside the partition are fluidized by distributed air and again drawn into the partition.[2]

To produce discretely coated particles without agglomeration, it is desirable for particles to be sprayed and dried without flocculation. Even if particles were sprayed into flocs in the partition or in the upper part of chamber, they had to be separated into discrete particles at latest until they were again forced into the partition. Although the lactose particles were kept separate under the severe fluidizing conditions, the phenacetin particles gradually agglomerated because of their more moderate motion (Tables I and III).

To obtain a homogenously coated product, it is desirable for all particles to have the same recycling time. For a powder with a wide particle size distribution, however, this is difficult in principle. In particular, small particles easily adhere to the wall surface and the filter through electrostatic attraction or moisture on the particle surfaces. In addition, small particles may sometimes be ejected to the filter. In the runs with lactose and phenacetin reported here, the small particles were carried by adhering temporarily to the slightly wet large particles.

In the case of phenacetin, which is composed of fragile crystalline particles, like many other pharmaceutical powders, relatively moderate conditions were employed at the initial stage of coating to avoid fracture. Sufficiently coated crystals are, however, not so easily fractured as the uncoated crystals, and thus conditions were made more severe after the initial stage to avoid agglomeration of the coated particles.

Talc powder added to avoid excessive agglomeration at the initial stage of phenacetin coating could also prevent the adhesion of small particles to the wall, especially to the upper part of the chamber. Since the talc powder adhering to phenacetin particles acted as an anti-

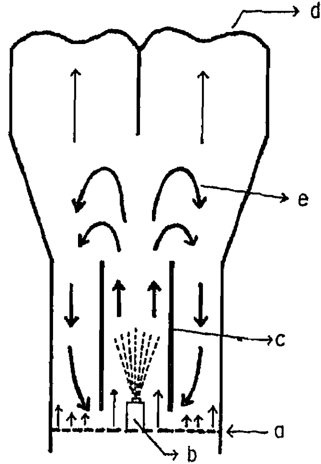


Fig. 6. Schematic Diagram of the Wurster Coating Chamber

a, air distributor; b, spray; c, cylindrical partition; d, bag filter; e, particle and air flow.

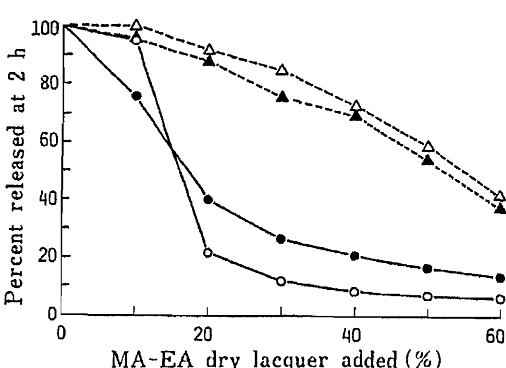


Fig. 7. Release after 2 h from Coated Particles in JP XI Disintegration Medium No. 1 (pH 1.2)

Core material and plasticizer: ○, phenacetin-TA; ●, phenacetin-PEG-PS; △, lactose-TA; ▲, lactose-PEG-PS.

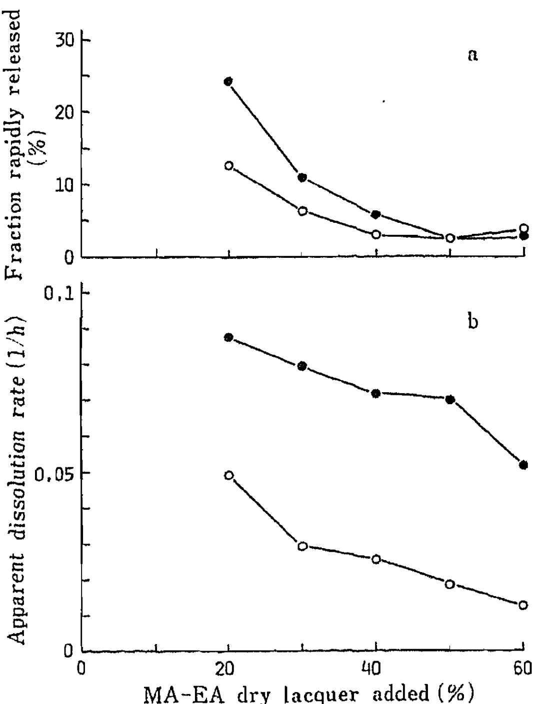


Fig. 8. Characteristic Parameters of Dissolution of Phenacetin

a. Fraction of particles which exhibited rapid dissolution. b. Apparent dissolution rate. Plasticizer used in coating: ○, TA; ●, PEG-PS.

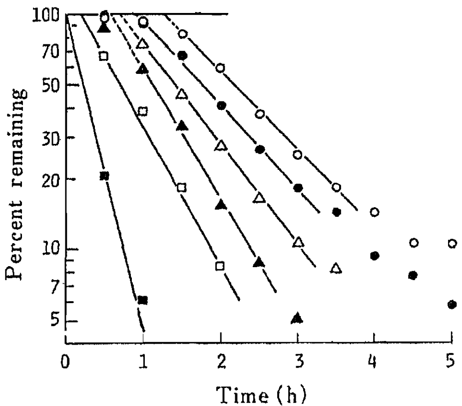


Fig. 9. Semi-log Plots of Percent of Lactose Remaining

MA-EA dry lacquer relative to core material (%): ■, 10; □, 20; ▲, 30; △, 40; ●, 50; ○, 60.

agglomerant, the coating could be performed under wet conditions. As a result, the temporary capture of small particles by wet large particles was accelerated and effectively prevented particle adhesion to the wall. Since particle adhesion to the wall would result in insufficiently coated particles, the addition of talc was critical for successful coating of phenacetin. Because excessive talc addition might prevent a compact membrane being formed, it was limited to

20% coating. This approach should be generally useful for coating of fragile particles.

Characteristics of Dissolution

Since a lag time of 2 h is required in drug release from enteric-coated pharmaceuticals to prevent drug release in gastric juice, the amount of drug released in 2 h is plotted in Fig. 7 against MA–EA dry lacquer added. For phenacetin, the amount released in 2 h rapidly decreased at the beginning, especially in the case of TA. However, the change becomes moderate above 20% coating. For lactose, neither of the membranes sufficiently restrained the drug release at the coating level studied here.

The dissolution rate of phenacetin became almost constant after the initial rapid release except for 10% coating (Figs. 1 and 2). Hence, the slope of the linear regression line for the data at 2.5—5 h was regarded as the apparent dissolution rate. The intercept of the linear portion on the ordinate could be regarded as representing the fraction of insufficiently coated particles. The results are plotted against MA–EA dry lacquer added (Fig. 8).

For phenacetin, the particles coated with PEG–PS have higher permeability above 20% coating than those coated with TA (Fig. 8). Figure 8 also shows that the fraction of initial rapid release is nearly 50% of total 2 h release. These results suggest that for a moderately soluble material such as phenacetin, an impermeable, homogeneously coated membrane has to be produced.

For lactose, the lag time and the rate at the stage of rapid release were characteristic parameters. Semi-log plots of the percent remaining (R) are shown in Fig. 9. The slope and the intercept with $R=100$ (%) of the linear regions of the plots gave the desired parameters. The results are plotted in Fig. 10 against MA–EA dry lacquer added.

For lactose, a water-soluble drug model, the dissolution rate was high even at 60% coating (Fig. 10). The MA–EA membrane does not seem to provide a simple diffusion barrier which is sufficient to act as an enteric coating for water-soluble core materials. On the other hand, the increase in lag time is nearly linear above 10%. This suggests that the prolongation

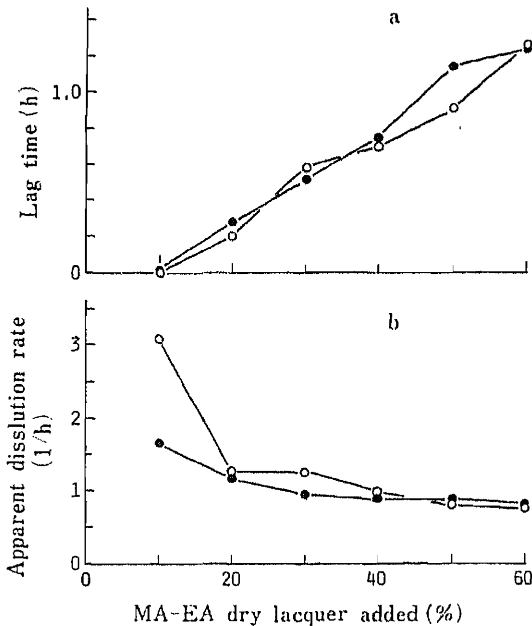


Fig. 10. Characteristic Parameters of Dissolution of Lactose

a. Lag time. b. Apparent dissolution rate. Plasticizer used in coating: ○, TA; ●, PEG–PS.

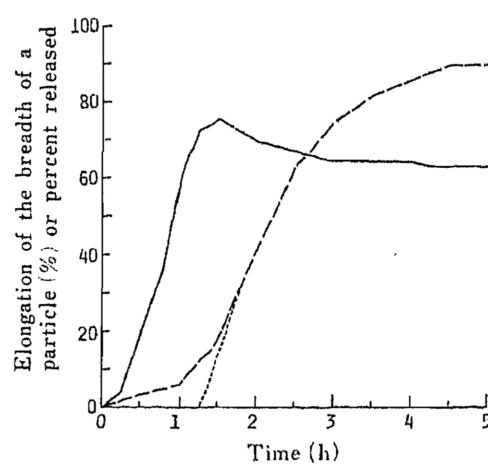


Fig. 11. Expansion of Lactose Particle 60% Coated with TA-Containing Lacquer in JP XI Disintegration Medium No. 1 (pH 1.2) at 37 °C and the Corresponding Dissolution Curve

——, particle expansion; – – – –, dissolution.

of lag time is desirable for enteric coating of water-soluble drugs.

Dissolution of Water-Soluble Drug from Capsules

In this study, the dissolution profiles of lactose and phenacetin used as core materials were very different, even though the same coating material was used (Figs. 1 and 3). The main difference was in the lag time of dissolution in the case of lactose. It seemed to result only from the large difference in solubility. However, Koida *et al.*[4] reported that ethylcellulose microcapsules of core materials with various solubilities comparable to that of lactose exhibited no lag time in the dissolution.

The membrane on lactose particles did not seem to be ruptured by stirring during the dissolution tests, when particles were filtered off and observed under an optical microscope. This indicates that the rapid dissolution after the lag time did not result from macroscopic rupture of the membrane.

When coated particles on the heated stage of a polarizing microscope were immersed in acidic medium and observed at 37 °C, the lactose particles exhibited a marked increase in their breadth, whereas only a slight increase (less than 10%) of their length was observed. An example of such an expansion is shown in Fig. 11 with the corresponding dissolution curve. At the maximum of expansion, which coincided well with the lag time of dissolution, the dissolving core crystals just disappeared. These results suggested that the lag time in the dissolution of lactose was due to the rapid water intake, resulting from the high solubility of lactose, and the restraint of lactose diffusion by this inward water flow.

Kawashima *et al.*[5] reported that encapsulated theophylline particles showed a lag time in dissolution. Their dissolution profiles showed less restraint at the beginning of dissolution and thereafter a slower rate of release, compared to the case of lactose reported here. The lag time in their dissolution profiles seemed to result from the swelling of the thick membrane, different from the mechanism of the time lag of dissolution reported here.

The dissolution profiles of coated lactose suggested that hydrophilic drugs coated as fine capsules by the Wurster method and orally administered may be delivered to any desired position in the gastro-intestinal tract and rapidly released there, if a pH-independently insolule membrane with the same water- and drug-permeation characteristics as MA–EA membrane is used. The averaging effect in gastro-intestinal transit of such small particles should provide good availability of drugs delivered in this way. The present model system should be useful in providing a basis for the design of membranes with such release patterns.

Conclusion

The success of powder coating is limited by many kinds of powder properties such as particle density, crystal hardness, wettability, electrostatic charging, adhesion, particle size distribution and so on. However, even for such a fragile powder as phenacetin, a successful procedure could be found in this study. Talc powder prevented the adhesion of small particles to the wall and also prevented excessive agglomeration. The adhesion of small particles to large particles and the agglomeration of some large particles seemed not to present a serious problem. These results have important implications for the practical coating of various pharmaceutical powders.

Dissolution through the MA–EA membrane was markedly affected by the properties of the plasticizer and core material. The amount of coating materials and the kind of plasticizer have to be determined according to the required characteristics of products. In the enteric coating of a hydrophobic substance such as phenacetin, a membrane with low permeability has to be designed. In such a case, triacetin can be effectively used as a plasticizer. For a highly water-soluble substance such as lactose, a membrane with a large lag time has to be designed

for enteric coating purposes. For sustained release, a 20% coating may be sufficient, though more than 60% coating seems to be needed for enteric coating at the present stage of study. The present model system should be useful in developing oral drug delivery system with a controlled lag time of dissolution.

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Possible Eye-Irritant Test Using Polysaccharide-Coated Liposomes as a Corneal Epithelium Model

JUNZO SUNAMOTO,*,[a] KIYOSHI IWAMOTO,[a,1)] GENJI IMOKAWA,[b] and SHUICHI TSUCHIYA[b]

Laboratory of Artificial Cell Technology, Department of Industrial Chemistry, Faculty of Engineering, Nagasaki University,[a] Nagasaki 852 and Department of Dermatology, Tochigi Research Laboratories, Kao Corporation,[b] 2606, Akabane, Ichikai-machi, Haga, Tochigi 321-34, Japan

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An alternative nonanimal test to the Draize test to evaluate the eye irritancy of surfactants has been newly developed. The physicochemical lysis of liposomal membranes by various surfactants, such as sodium dodecyl sulfate (SDS), dodecyldimethylbenzylammonium chloride (BTC), tetradecyldimethylbenzylammonium chloride (Sanisol M-100), benzyldimethyl-2-(2-*p*-1,1,3,3-tetramethylbutylphenoxy)ethoxyethylammonium chloride (Hyamine 1622), dodecanoyldiethanolamide (LDE), and polyoxyethylene ($n=21$)-sorbitan monostearate (Tween 80), was correlated with the results of the Draize test with the same surfactants. Egg phosphatidylcholine small unilamellar vesicles (SUVs) coated with *O*-palmitoylamylopectinsulfonic acid were employed as a model of the human corneal epithelium. The lysis of liposomes with the surfactants was monitored quantitatively by following the release of carboxyfluorescein (CF) from the interior water phase of the liposomes suspended in an aqueous medium containing almost the same salt composition as that in the human tear film. The order of efficiencies of these surfactants in perturbing the liposomal membrane was Sanisol M-100>Hyamine 1622>BTC>(Tween 80>)LDE>SDS. With the exception of Tween 80, whose critical micelle concentration (cmc) is relatively low (9.6×10^{-6} M) compared with those of the other surfactants (approximately 5—7×10^{-4} M), a reliable correlation was found between the results of animal and nonanimal tests.

Keywords——liposome; Draize test; eye irritancy; corneal epithelium

At the present time the Draize test is the best established to evaluate the eye irritancy of surfactants or detergents.[2-6)] However, application of the Draize test still presents several problems. More specifically, this test requires skilful techniques of objective judgement and a large number of animals must be used. Not unexpectedly, animal rights activists object to this procedure. Consequently, a simpler and more reliable *in vitro* test has been sought to replace the animal test. Recently, several research groups have developed a nonanimal test to evaluate the eye irritancy of cosmetics ingredients.[4,5)] Borenfreund and Shopsis have reported that both the uptake of radioactive uridine by cells and the morphological change of cells observed by light microscopy in a mammalian cell culture are closely correlated to the results of the Draize test.[4)] They employed either a mouse fibroblast cell culture (3T3) or a human liver cancer cell line (Hep G_2) for the test. Further, Kemp has developed a new methodology to evaluate the toxicity of surfactants to mouse fibroblast cells from the coloration of the cultured cells by fluorescein diacetate and ethidium bromide, arising from the damage to the cells caused by the surfactants.[5)]

Liposomes have gained wide acceptance in chemotherapy and immunotherapy as targetable drug carriers.[7-11)] Moreover, liposomes (artificial cells) may also be available as a tool to investigate the pharmacological activity and/or the toxicity of physiologically active

substances at the cell membrane level. Very recently, Sunamoto and his coworkers have established several methodologies to mimic and evaluate cell damage with a variety of membrane-permeable drugs, by using a liposomal system. For example, spermicidal activity of several nonionic surfactants has been investigated by the technique of surfactant-induced release of carboxyfluorescein (CF) from liposomes.[12,13] Furthermore, the antihypertensive activity of ethyl decaprenoate has been shown to be correlated with the drug-induced fusion between egg phosphatidylcholine liposomes[14] and the cosolubilization of cholesterol in gallstones was rationalized on the basis of the physicochemical damage to cholesterol-containing liposomes caused by bile salts.[15]

In this paper, we suggest a new methodology, employing a liposomal system, for the preliminary evaluation of the eye irritancy of surfactants. The procedure is a relatively simple, reliable, and inexpensive one for the preliminary screening of potential irritants.

Experimental

Materials——Egg phosphatidylcholine (PC) was isolated and purified from fresh egg yolk according to the method described previously.[16,17] Sodium dodecyl sulfate (SDS, Wako Pure Chemical Industries, Ltd., Osaka) was recrystallized from ether–ethanol (5:2, by vol.). Other surfactants, dodecyldimethylbenzylammonium chloride (BTC), tetradecyldimethylbenzylammonium chloride (Sanisol M-100), benzyldimethyl-2-(2-*p*-1,1,3,3-tetramethylbutylphenoxy)ethoxyethylammonium chloride (Hyamine 1622), dodecanoyldiethanolamide (LDE), and polyoxyethylene ($n=21$)-sorbitan monostearate (Tween 80) were all chromatographically pure and were used without further purification. Their chemical structures are given in Table I along with their critical micelle concentrations (cmc). This value, for most surfactants, is very sensitive to pH, temperature, salt composition, and ionic strength. Thus, any attempt to correlate surfactant structure with micellar or other properties must be made using cmc values obtained under identical conditions. We have therefore measured the cmc values of the surfactants, except Tween 80 and LDE, in 20 mM $KHCO_3$ (pH 8.4) containing 149 mM NaCl at 37.0 °C, since this salt composition is comparable to that of the human tear film.[18] In the measurements of the cmc, pinacyanol chloride[19] (for SDS) and eosin Y[20] (for

Table I. Structures and cmc Values of Surfactants Employed in This Work

Surfactant	Structure	cmc (M)[a] (at 37.0 °C)
SDS	$C_{12}H_{25}OSO_3^- Na^+$	6.4×10^{-4}
BTC	$C_{12}H_{25}N^+(CH_3)_2CH_2C_6H_5 \cdot Cl^-$	6.0×10^{-4}
Sanisol M-100	$C_{14}H_{29}N^+(CH_3)_2CH_2C_6H_5 \cdot Cl^-$	4.7×10^{-4}
Hyamine 1622	$(CH_3)_3CCH_2C(CH_3)_2C_6H_4OC_2H_4OC_2H_4N^+(CH_3)_2CH_2C_6H_5 \cdot Cl$	7.0×10^{-4}
LDE	$C_{12}H_{25}CON(C_2H_4OH)_2$	—[b]
Tween 80	(sorbitan ring) $CH_2OCOC_{18}H_{35}$; HO; $O-(CH_2CH_2O)_{21}-H$	9.6 × 10[illegible] [b,c]

a) In 20 mM $KHCO_3$ containing 149 mM NaCl at 37.0 °C. *b*) It was impossible to determine the cmc because of the unusual behavior in solution. *c*) At 25.0 °C, from ref. 29.

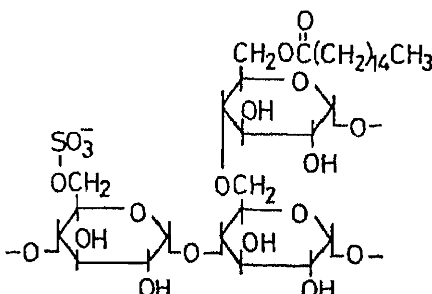

Fig. 1. Structure of *O*-Palmitoylamylopectin-112-(1.6)-sulfate(2.1), Employed in This Work Instead of Mucin

other cationic surfactants) were used as the dye probes.

O-Palmitoylamylopectin-112-(1.6)-sulfate(2.1) (Fig. 1) was prepared by the same method as described elsewhere.[21] The number 112 means that the average molecular weight of the polysaccharide is 112000. The degree of substitution of palmitoyl residues per 100 glucose units in amylopectin was determined by proton nuclear magnetic resonance (^{1}H-NMR) to be 1.6, while that of the sulfate moiety was determined by elemental analysis to be 2.1.

CF was purchased from Eastman Kodak, Rochester, N.Y. Other reagents were commercial products of analytical grade and were used without further purification.

Preparation of Liposomes——Conventional small unilamellar vesicles (SUVs) and amylopectin derivative-coated SUV containing 200 mM CF in the interior water phase were prepared in 20 mM $KHCO_3$ (pH 8.4) by essentially the same method as described before.[22,23] A CF-loaded SUV suspension (4.0 ml), prepared from 7.3 mg of egg PC, was mixed with 1.0 ml of 20 mM $KHCO_3$ containing 10.0 mg of *O*-palmitoylamylopectin-112-(1.6)-sulfate(2.1) and 149 mM NaCl. After being stirred for 1 h at 20.0 °C, the resulting mixed suspension was applied to a Sepharose 4B gel-chromatographic column (i.d. 1.8 × 41 cm) pre-equilibrated with the same buffered solution, by which uncapsulated CF was eliminated. The liposome concentration was determined as inorganic phosphate according to Allen's procedure.

CF-Release from Liposomes Perturbed by Eye Irritant Surfactants——Fluorescence measurements were run on a Hitachi 650-10S fluorospectrophotometer equipped with a thermoregulated cell compartment. When CF is encapsulated in liposomes at a concentration above 200 mM, its fluorescence is almost completely suppressed by the concentration quenching mechanism, but it fluoresces strongly upon liberation from the interior of the liposomes to the bulk aqueous phase.[25,26] A CF-loaded SUV suspension in 20 mM $KHCO_3$ (pH 8.4) containing 149 mM NaCl was placed in a thermoregulated cuvette cell and preincubated for 10 min at 37.0 °C. The total volume in the cuvette cell was kept constant at 3.0 ml ([egg PC] = 3.0×10^{-4} M). During the preincubation, no spontaneous release of CF was observed. CF-release from the liposomes was induced by the addition of an appropriate amount of surfactant (1.0—100.0 µl) in the same buffered solution and was monitored by following the increase in the fluorescence intensity at 520 nm on excitation at 470 nm. The final intensity at infinite time, corresponding to the total amount of CF released, I_∞, was determined by complete destruction of the liposomes by adding 100.0 µl of an aqueous Triton X-100 solution (10% v/v). The percent CF-release was calculated by means of the following equation: % CF-release $= ((I_t - I_0)/(I_\infty - I_0)) \times 100$, where I_0 is the fluorescence intensity before the addition of an aqueous surfactant solution and I_t is that at time t after the addition of the surfactant solution to the liposomal suspension.

Cytotoxicity of the Surfactants Evaluated from the Plating Efficiency of Human Epithelium——With the same surfactants as those used in the liposomal system, cytotoxicity against human epithelium was investigated by the colony-forming method in cell culture.

A 5 ml aliquot of surfactant solution (10^{-4}, 10^{-5}, or 10^{-6} M) in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS) was placed in a plastic dish (i.d. 60 mm) and preincubated at 37.0 °C in a 5.0% CO_2-air incubator. At the same time, human epithelium (JTC-17 (XX male cells)) in the logarithmic growth phase was treated with 0.25% aqueous trypsin solution to obtain a cell suspension. After counting the number of cells (stained with trypan blue) on a hemocytometer (Burker-Turk) and adjusting the count to 10^3 cells ml^{-1}, 0.1 ml of cell suspension (10^2 cells) was added to the preincubated surfactant-containing MEM.

After incubation for 14 d, the dishes were washed once with Hank's phosphate-buffered saline (PBS), then treated with a neutral formalin solution for 30 min, and 5.0 ml of crystal violet staining solution was added. The plating efficiency (%) was obtained by dividing the number of colonies by the initial number of cells (10^2).

The Draize Test——The technique established by Draize and Kellery,[2] was employed using female Japanese albino rabbits weighing 2.5—3.0 kg. Ten rabbits were divided into two equal groups. Each surfactant solution was tested, at low (2.5 mM) and high (250.0 mM) concentrations by instilling 0.1 ml of the sample into the conjunctival sac of the right eye of each of the five rabbits in the set. The left hand eye remained untreated, in each case, to serve as the standard for comparison. At 24 and 168 h after the instillation, ocular changes about the cornea, iris, and conjunctiva were scored according to the standard method. In addition, in all cases, one drop of 2% aqueous fluorescein solution was instilled after 168 h to check corneal epithelium abruption.

Results and Discussion

The Draize test has been widely adopted for testing the eye-irritating activity of, for example, the ingredients of cosmetics. In this test, lesions on the cornea, iris and palpebral and bulbar conjunctiva are scored independently.[2] In the scoring system, approximately 80 percent of the score is given to the cornea and iris, because the structure of the eye, and especially the cornea, is crucial for vision. In fact, after instillation of benzalkonium chloride or SDS solution into a rabbit's eye, opacity of the cornea and desquamation of the corneal epithelium have been observed.[27]

Corneal epithelium is covered by the tear film, which is constructed of a teary coacervate with a thickness of 6—10 μm and a superficial lipid monolayer with the thickness of 10—40 Å.[28] The teary coacervate containing mucin is further divided into the three phases of adsorbed mucin layer, mucin semigel layer, and middle aqueous layer.[28-30] After consideration of these structural characteristics of the tear film, we designed a system to mimic the interaction between eye-irritant surfactants and the eye. First, a lipid monolayer was developed over an appropriate aqueous buffered solution, and we tried to observe the physicochemical perturbation of the lipid monolayer by careful instillation of the surfactant solution onto the lipid monolayer. However, we could not obtain reliable and reproducible results by this method. Then, we considered that the first step in eye irritation is the direct interaction between the cell membrane of the corneal epithelium, which consists mostly of PC, and the surfactant. In order to monitor the interaction, therefore, we designed a liposomal system sensitive to physicochemical perturbation by surface-active agents.[12-15] In addition, we noted the important role of mucin in the tear film,[29,30] especially at the surface of the corneal epithelium. Thus, the anionic polysaccharide, amylopectin sulfate, was added as a model of the mucin in the tear film. This technique has been developed independently by ourselves to mimic the morphology of the cell wall of plant cells or gram-positive bacteria.[21-23]

Surfactant-Induced CF-Release from Polysaccharide-Coated SUV

Figure 2 shows the percent CF-release from the polysaccharide coated liposomes at 5 min after the addition of the surfactant solution to the liposome suspension, as a function of the surfactant concentration. Under the conditions used, the surfactants, except Tween 80, induced the release of CF from the liposomes at a relatively low concentration of surfactant which was below the cmc (Table I). This result means that, under our experimental conditions, the CF-release induced by these surfactants is brought about by permeation of monodispersed surfactant molecules into the liposomal bilayer, not by solubilization of liposomes by surfactant micelles.

The use of a surfactant solution more concentrated than the cmc is not considered to be feasible in the animal test, because the solubilization of membrane lipids by micelles will lead to complete lysis of the cells and to loss of vision. This does not correspond to simple eye irritancy. Even if one were to add a surfactant at a concentration higher than the cmc, most of the surfactant molecules should be absorbed by the superficial lipid layer of the tear film and it is assumed that the surfactant concentration would be lower at the corneal epithelium level. Therefore, eye irritancy with surfactants, at the level of the corneal epithelium, should always involve concentrations below the cmc.

With Tween 80, irritancy was not found because the cmc of Tween 80 is relatively low (9.6×10^{-6} M at 25.0 °C[30]) and the surfactant is in the micellar phase under the conditions

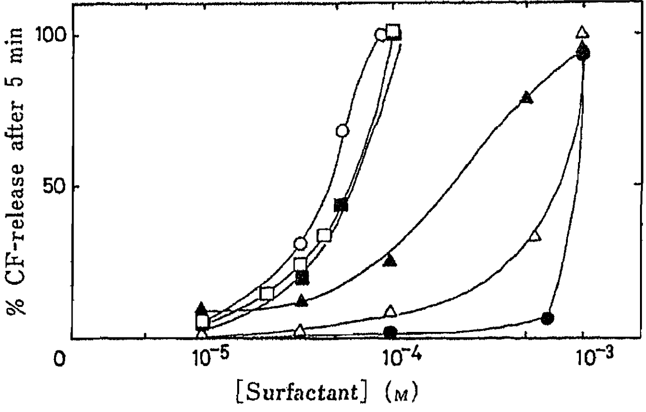

Fig. 2. Surfactant-Induced Release of CF from the Polysaccharide-Coated SUV of Egg Phosphatidylcholine as a Function of Surfactant Concentration

The extent of CF release (%) was determined at 5 min after injecting a surfactant solution into the liposomal suspension: —○—, Sanisol M-100; —■—, BTC; —□—, Hyamine 1622; —▲—, Tween 80; —△—, LDE; and —●—, SDS.

employed in this work. Consequently, the mechanism of lysis of liposomes by Tween 80 must be different from that with the other surfactants and, most probably, is a micellar solubilization mechanism.[13] This conclusion is also supported by the profile of CF-release *vs.* surfactant concentration, which is rather different from those of the other surfactants (Fig. 2). Although LDE is relatively commonly used as an ingredient of shampoo, the solution behavior of the surfactant is not fully understood and the cmc could not be determined. However, the behavior of LDE in the liposomal lysis was completely different from that of Tween 80.

From the data shown in Fig. 2, the order of effectiveness for the surfactants to induce lysis of the liposomes is: Sanisol M-100 > Hyamine 1622 > BTC > (Tween 80>) LDE > SDS. This order does not, of course, correlate with that of their cmc values. The effective concentration of surfactant needed to induce 50% release of CF from the liposomes 5 min after injection is defined as $R_{CF(50)}$ and data for three different liposomal systems are summarized in Table II.

TABLE II. Effective Concentrations ($R_{CF(50)}$) of Surfactants Needed to Induce 50% Release of CF from Three Kinds of Liposomes at 37.0 °C

Surfactant	$R_{CF(50)}$ (M)		
	Liposome A	Liposome B	Liposome C
Sanisol M-100	5.3×10^{-5}	4.7×10^{-5}	4.7×10^{-5}
BTC	6.2×10^{-5}	7.6×10^{-5}	6.0×10^{-5}
Hyamine 1622	6.4×10^{-5}	8.3×10^{-5}	5.8×10^{-5}
Tween 80	23×10^{-5}	49×10^{-5}	21×10^{-5}
LDE	30×10^{-5}	69×10^{-5}	74×10^{-5}
SDS	94×10^{-5}	96×10^{-5}	91×10^{-5}

Liposome A: conventional liposomes without added cholesterol or polysaccharide coat. Liposome B: liposomes with 25 mol% cholesterol content, but without polysaccharide coat. Liposome C: liposomes without added cholesterol, but coated with the polysaccharide derivative.

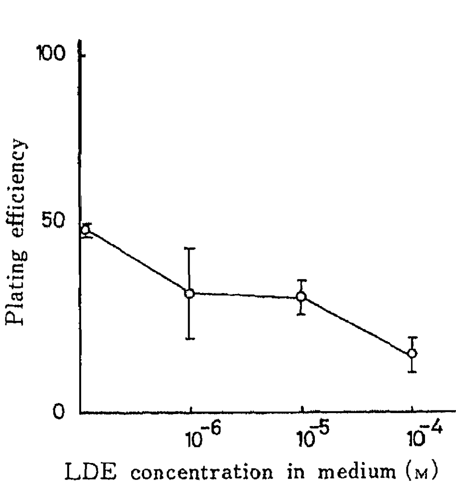

Fig. 3. Plating Efficiency of JTC-17 (XX Male Cells) in the Presence of LDE, within the Surfactant Concentration Range of 10^{-6} to 10^{-4} M, at 37.0 °C

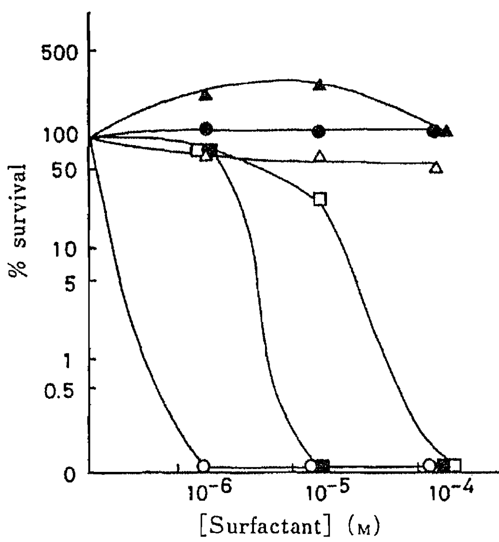

Fig. 4. Survival of JTC-17 Cells as a Function of Surfactant Concentration

—○—, Sanisol M-100; —■—, BTC; —□—, Hyamine 1622; —▲—, Tween 80; —△—, LDE; and —●—, SDS (for details, see the text).

Compared with the values of $R_{CF(50)}$ for the conventional SUV without cholesterol or a polysaccharide coat, those for the SUV with 25 mol% cholesterol were higher. This increase means that the addition of cholesterol decreases the membrane fluidity[13,20] leading to a decrease in the permeability of the lipid bilayer to the surfactants.[13] Conversely, when the SUV were coated with the amylopectin sulfate derivative, the $R_{CF(50)}$-values decreased except in the cases of SDS and LDE. This decrease indicates that the electrostatic repulsive force between the anionic species of the surfactant and the polysaccharide on the liposomal surface may not necessarily be a major factor but that the hydrophobic interaction may be more important.[15] On the other hand, in the case of cationic surfactants, the electrostatic attractive force between the species of opposite charge might assist the hydrophobic interaction.

Cytotoxicity of the Surfactants against Intact Cells

A typical example of the plating efficiency with LDE, as a function of the surfactant concentration, is given in Fig. 3. The plating efficiency decreased as the surfactant concentration increased and no colony formation was observed at a concentration of 10^{-4} M. Tween 80 appears to be grossly different from the other five surfactants, and showed an abnormal effect on the plating efficiency: namely, at the surfactant concentrations of 10^{-6} and 10^{-5} M, the plating efficiency increased. In the case of Sanisol M-100, no colony formation was observed at all even at 10^{-6} M. Conversely, SDS did not show any cytotoxicity over the concentration range investigated (10^{-6}—10^{-4} M).

In order to examine in more detail the cytotoxicity of the surfactants to the cells, JTC-17, the percent survival of the cells was calculated by means of the following equation:

$$\text{survival\%} = \frac{\text{plating efficiency of the treated cells}}{\text{plating efficiency of the control}} \times 100$$

From the curves shown in Fig. 4, it seems that the order of cytotoxicity of the surfactants investigated was Sanisol M-100 > BTC > Hyamine 1622 > LDE > SDS ≫ Tween 80. Except for the order of BTC and Hyamine 1622 and the anomalous behavior of Tween 80, the order of cytotoxicity was identical to that for liposomal membrane damage with these surfactants.

The Draize Test

Figure 5 shows the results of the Draize test for all six surfactants at 24 and 168 h after the instillation of aqueous surfactant solution at concentrations of 25 mM (below the cmc except for Tween 80) and 250 mM (above the cmc for all the surfactants). At 24 h after instillation, BTC, Sanisol M-100, and Hyamine 1622 showed eye irritancy in the rabbit's eye even at 25 mM surfactant concentration, while SDS and LDE showed almost no irritancy at 25 mM but a significant irritacny at 250 mM. Tween 80 did not show any significant irritancy, even at 250 mM.

The order of the eye irritancy in the Draize test was Sanisol M-100 > Hyamine 1622 > BTC > LDE > SDS ≫ Tween 80, and this sequence is almost identical with that found in the liposomal system, the only exception being Tween 80 (*vide supra*). The present results are consistent with the previous data reported by Matsuura and his coworkers.[27] They showed that the irritancy of cationic surfactants in rabbits' and guinea-pigs' eyes is relatively high and that of nonionic surfactants is low.[27]

Figure 6 shows the relationship between percent CF-release from the polysaccharide-coated liposomes and both the score for the cornea and the total score in the Draize test at 24 h after instillation of surfactants into a rabbit's eye. Clearly, from Fig. 6, there exists a close correlation between the animal and nonanimal test results, Tween 80 being the only exception. Although the data are not shown, we also investigated the relationship for conventional liposomes which contained either no cholesterol or 25 mol% cholesterol without a polysac-

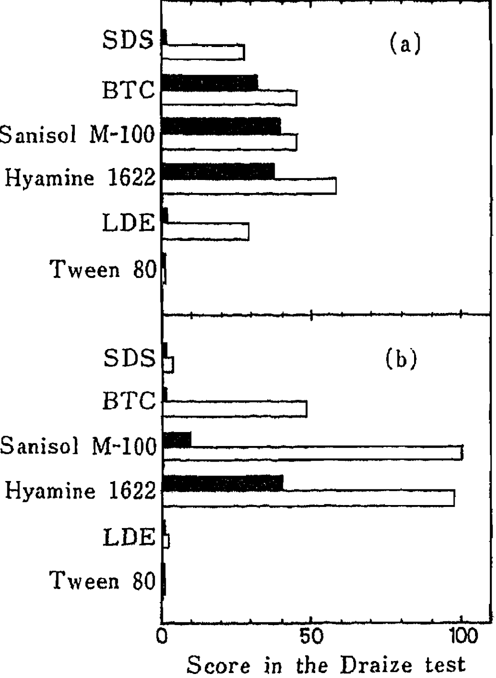

Fig. 5. Total Score in the Draize Test at 24 h (a) and 168 h (b) after the Instillation of Aqueous Surfactant Solution of 25 mM (■) and 250 mM (□) into a Rabbit's Eye

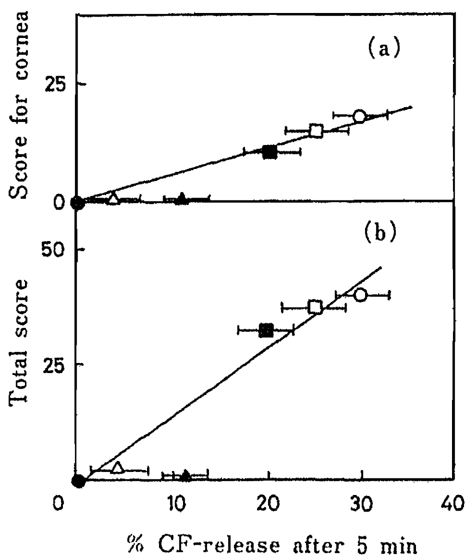

Fig. 6. Correlation between the Percent CF-Release from Polysaccharide-Coated Liposomes Perturbed by the Surfactants and the Scores in the Draize Test

—○—, Sanisol M-100; —□—, Hyamine 1622; —■—, BTC; —△—, LDE; —●—, SDS; and —▲—, Tween 80. (a) Score for cornea. (b) Total score.

charide coat. However, we did not obtain a reliable correlation between the results of the two tests. This finding suggests that the surface structure of the liposome, including its hydrophobicity and surface charge, is important in mimicking the microenvironment around the corneal epithelium.

One of the present authors (G.I.) has previously investigated the biological properties of several ionic and nonionic surfactants with particularly attention to skin irritancy,[31,32] and found that cationic surfactants show a relatively strong labilizing effect compared with anionic ones and that the liposome-labilizing effect is due to an increase in the permeability of the liposomal membrane. The effect is due to the absorption of monomeric surfactant rather than solubilization of membrane lipids in surfactant micelles.[31,32] These previous findings are consistent with the present results.

The results obtained in this work suggest that the use of liposomes, which may be regarded as artificial cells, is a potentially valuable approach for evaluating the pharmacological activity of drugs at the cell membrane level.

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Biphasic Binding of ^{125}I-Iodocyanopindolol to β-Adrenergic Receptors in Rat Cerebral Cortical Membranes. I. Assessment by the Use of Agonists

HIROSHI TSUCHIHASHI* and TAKAFUMI NAGATOMO

Department of Pharmacology, Niigata College of Pharmacy, 5-13-2 Kamishinei-cho, Niigata 950-21, Japan

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When saturation binding studies of β-adrenoceptors in rat brain with ^{125}I-iodocyanopindolol (^{125}I-ICYP) were carried out by means of radioligand binding assay, the Scatchard plots showed a biphasic character. The addition of guanosine triphosphate (GTP, 3×10^{-4} M) had no effect on the biphasic character. The inhibition constants of various ligands for the high- and low-affinity sites of ^{125}I-ICYP binding were determined from the biphasic Scatchard plots. Competition by the agonists at the high-affinity sites was in the order of *l*-isoproterenol > *l*-epinephrine > *l*-norepinephrine, whereas at the low-affinity sites, the order was *l*-isoproterenol > *l*-epinephrine = *l*-norepinephrine. The specificities of the agonists for the high- and low-affinity sites were high = low (*l*-isoproterenol), high > low (*l*-epinephrine), or high < low (*l*-norepinephrine). These results suggest that two distinct types of β-adrenoceptor sites can be differentiated in rat cerebral cortical membranes.

Keywords——β-adrenoceptor; rat brain; Scatchard analysis; two-site model

There is increasing evidence that data obtained by the radioligand binding assay method are useful to evaluate the pharmacological potencies of chemicals in relation to various receptors.[1-3] We reported that the potencies of β-blocking action of newly synthesized chemicals could be assessed from K_i values obtained from displacement experiments using radioligand binding assay.[4] In these experiments, the Scatchard plots of the saturation data were mainly uniphasic. Many other investigators[3] have also reported that Scatchard plots obtained with ^{125}I-iodocyanopindolol (^{125}I-ICYP) as the radioligand were uniphasic in character. However, Hoyer *et al.*[5] found that the Scatchard plots were biphasic when the binding study was carried out with (+)-, (±)-, and (−)-^{125}I-ICYP as the ligands using guinea-pig left ventricle membranes. Thus, the present study was designed to examine whether or not the Scatchard plots of ^{125}I-ICYP binding to β-adrenoceptors in rat brain are biphasic.

Experimental

(−)-^{125}I-ICYP (2200 Ci/mmol) was purchased from New England Nuclear Corp. *l*-Isoproterenol (from Nakarai Chemicals Ltd.), *l*-epinephrine and *l*-norepinephrine (from Sigma Chem. Comp.) were used.

The membrane-enriched fraction from cerebral cortex was prepared by using the method described previously.[6] The membrane-enriched fraction was frozen in liquid nitrogen, stored at −80 °C and diluted to appropriate concentrations immediately before use. Protein concentrations were determined by the method of Lowry *et al.*[7] using bovine serum albumin as the standard.

The β-adrenoceptor binding assay was carried out in duplicate with ^{125}I-ICYP in the presence (non-specific) and absence (total) of 10 μM *l*-propranolol. In brief, 0.25 ml of membrane suspension (0.1 mg of protein) was incubated for 60 min at 23 °C with various concentrations (0.015—1.0 nM) of ^{125}I-ICYP in a total volume of 0.5 ml containing 60 mM Tris-HCl and 20 mM $MgCl_2$ (pH 7.2). At the end of the incubation period, the incubation mixture was immediately filtered through a Whatman GF/C glassfiber filter according to an improved method.[8] The radioactivity of the filter in a glass tube was counted with an auto well gamma counter (Aloka, ARC-500). The difference in mean

values between total and non-specific binding was taken as the specific binding.

All kinetic analyses were carried out on an NEC PC-9801F computer by iterative non-linear regression, based on the theory of Munson and Rodbard.[9] The goodness-of-fit was evaluated with a model having only one receptor subtype and a model having two receptor subtypes by Scatchard analysis on the basis of the mass-action law.[10] The saturation binding curves can be described as follows:

(I) One receptor site model

$$\frac{B}{F}=\frac{1}{K_{d1}}(R_1-B)$$

(II) Two receptor site model

$$\frac{B}{F}=\frac{1}{2}\left[\frac{R_1-B}{K_{d1}}+\frac{R_2-B}{K_{d2}}+\sqrt{\left(\frac{R_1-B}{K_{d1}}-\frac{R_2-B}{K_{d2}}\right)^2+4\frac{R_1\cdot R_2}{K_{d1}\cdot K_{d2}}}\right]$$

where K_{d1} and K_{d2} are dissociation constants between a radioligand and receptors 1 and 2, and R_1 and R_2 are the total concentrations of receptors 1 and 2. For the purpose of this report, R_1 and R_2 are expressed as percentages of the total concentration of the binding sites R_1+R_2.

The Scatchard plots based on the one site model were done by the least-squares fit method. In the two site model, the Scatchard plots were arbitrarily divided, and the first half of the data points were assigned to the high-affinity site and the second half to the low-affinity site. The plot obtained could be displayed on the computer, and four initial parameter values (K_{d1}, K_{d2}, R_1 and R_2) were obtained. Iterative non-linear regression analysis was continued until the difference between the initial parameter values and the estimated parameter values in a run was below 10^{-6}. In many cases this condition was achieved but up to 100 iterations were required in some cases; when it had not been achieved after 100 iterations, calculation was stopped and the best parameter values were chosen.

The applicability of the model was checked by applying Akaike's information criterion (AIC)[11]:

$$AIC=n\cdot\ln SS+2m$$

where SS is the sum of squares, and n and m are sets of data and parameters. The model which gives a lower AIC value has a better goodness-of-fit.

The computer programs were made available by Mr. S. Nagatsuka (Daiichi Pure Chem. Co., Ltd.) and partially modified in our laboratory. In order to quantify the mode of saturation, Hill numbers for ^{125}I-ICYP binding to the membranes were determined by means of the Hill plot.[12] The Hill coefficients of models I (one receptor site model) and II (two receptor site model) are 1 and below one, respectively.

In order to determine the inhibition constant (K_i) of a cold ligand, the Scatchard plot was separated into two affinity components (high- and low-affinity sites) and each component was compared with the control values. The K_i values were calculated by using the following equations:

(a) competitive inhibition

$$1/K_{d'}=1/K_d\times K_i/(K_i+[\mathrm{I}])$$

(b) non-competitive inhibition

$$B_{max'}=B_{max}\times K_i/(K_i+[\mathrm{I}])$$

where K_d and $K_{d'}$ are the dissociation constants in the absence (control) and presence of the cold ligand, B_{max} and $B_{max'}$ are the receptor capacities for the radioligand in the absence (control) and presence of the cold ligand and [I] is the concentration of cold ligand.

The significance of differences was evaluated by Student's t-test.

Results

Preliminary experiments were performed in order to standardize the β-adrenergic receptor binding assays with the membrane preparation. The specific binding of ^{125}I-ICYP to the membrane fraction was linear with respect to protein concentration below 0.15 mg per incubation when 0.05 nM ^{125}I-ICYP was used. Total and specific bindings at 23 °C were rapid, reaching the steady state within 10 min when 0.05 nM ^{125}I-ICYP was used. Binding in the presence of 10 μM l-propranolol (non-specific binding) using the same concentration of ^{125}I-ICYP reached equilibrium within 10 min and remained constant thereafter.

Figure 1 shows the result of the ^{125}I-ICYP saturation experiment with β-adrenoceptors in the cerebral cortical membranes. The Scatchard plots were all biphasic, and the biphasic

TABLE I. Effects of GTP on β-Adrenoceptor Binding

	High-affinity site		Low-affinity site		High : low	Hill coefficient
	K_d (pM)	B_{max} (fmol/mg protein)	K_d (pM)	B_{max} (fmol/mg protein)		
Control (4)	27.10±11.83	25.53±5.00	534.99±118.31	161.15±25.98	13.7 : 86.3	0.76±0.05
GTP 3×10^{-4}(4)	36.95± 4.77	29.05±1.20	455.00± 69.10	140.78±10.46	17.1 : 82.9	0.79±0.08

Each value in parenthesis is the number of experiments. Data are the mean values±S.E.

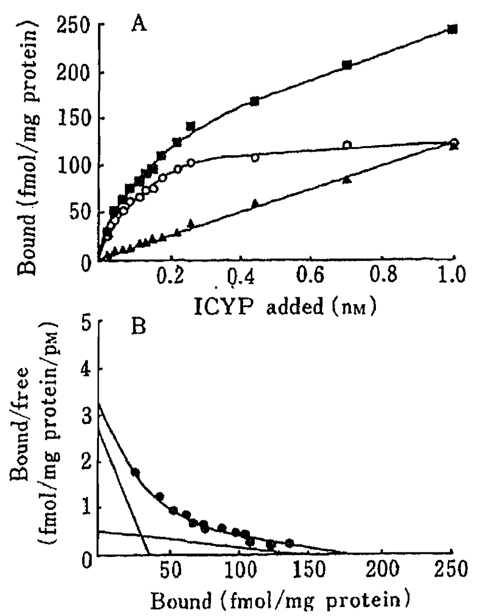

Fig. 1. The Results of Saturation Experiments (A) and Scatchard Plots (B) of ^{125}I-ICYP Binding to the Rat Brain

Specific binding is defined as the difference between total binding and the binding in the presence of 10 μM *l*-propranolol at ^{125}I-ICYP concentrations between 0.01 and 1.0 nM. The values of K_d and B_{max} with ^{125}I-ICYP binding were as follows: 13.13 pM and 36.29 fmol/mg protein (high-affinity site); 301.89 pM and 147.73 fmol/mg protein (low-affinity site). The Hill coefficient of the ^{125}I-ICYP binding was 0.66. The data shown are those from a single experiment performed in duplicate. The points show total (■), specific (○ or ●), and non-specific (▲) binding.

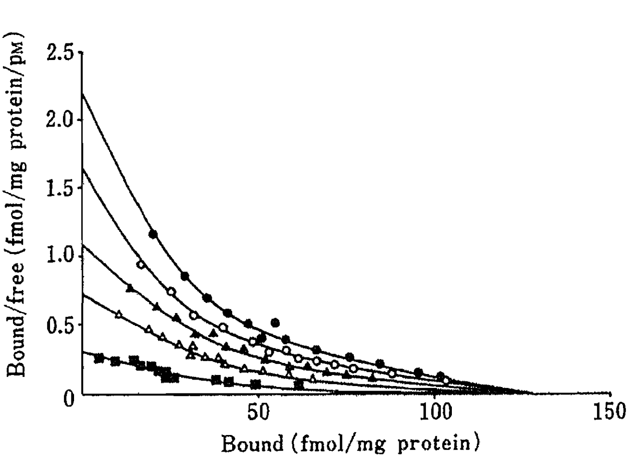

Fig. 2. Inhibition of ^{125}I-ICYP Binding to Rat Brain Membrane by *l*-Isoproterenol

Membranes with ^{125}I-ICYP (0.01—1.0 nM) in the absence or presence of various concentrations of *l*-isoproterenol: absence (●); 1×10^{-7} M (○); 1×10^{-6} (▲); 1×10^{-5} (△); and 1×10^{-4} M isoproterenol (■). Values are those from a single experiment performed in duplicate.

character was not affected by the addition of guanosine triphosphate (GTP) (3×10^{-4} M) (Table I). These results suggest the existence of high- and low-affinity sites for ^{125}I-ICYP binding to the β-adrenoceptors. In order to characterize the high- and low-affinity sites inhibition studies with various agonists were carried out.

Figure 2 shows the Scatchard plots in the presence or absence (control) of various concentrations of *l*-isoproterenol. *l*-Isoproterenol increased the K_d values of both sites. Thus, the inhibition by *l*-isoproterenol was competitive in this study, and the K_i values were calculated (Tables II and III). Similar experiments were also carried out with other agonists, *l*-epinephrine and *l*-norepinephrine (Tables II and III). The plots of $-\log K_i$ *vs.* $-\log[\mathrm{I}]$ thus

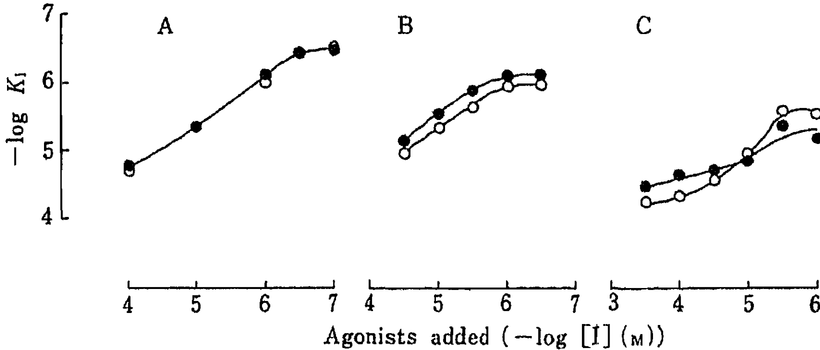

Fig. 3. Plots of $-\log K_i$ *vs.* $-\log[I]$ of *l*-Isoproterenol (A), *l*-Epinephrine (B), and *l*-Norepinephrine (C)

The K_i values for high- (●) or low-affinity sites (○) in the presence of the agonists were plotted. Each K_i value was calculated based on the equation of competitive inhibition. The data shown are those from a single experiment performed in duplicate.

TABLE II. Effects of Agonists on β-Adrenoceptor Binding

	Drugs added (M)	High-affinity site			Low-affinity site		
		K_d (pM)	B_{max} (fmol/mg protein)	K_i (μM)	K_d (pM)	B_{max} (fmol/mg protein)	K_i (μM)
l-Isoproterenol							
Control	—	15.93	30.60		339.56	101.51	
	1×10^{-7}	20.70	30.02	0.33	451.83	102.19	0.30
	1×10^{-6}	35.99	35.98	0.79	686.36	93.02	0.98
	1×10^{-5}	60.99	34.89	4.54	1054.51	91.01	4.75
	1×10^{-4}	112.04	30.30	16.57	2054.51	98.18	19.80
Control	—	13.13	36.29		301.89	147.73	
	3×10^{-7}	23.74	30.57	0.37	547.99	140.90	0.37
l-Epinephrine							
Control	—	26.95	25.16		369.21	133.90	
	1×10^{-6}	64.24	31.70	0.72	700.79	147.89	1.11
	3×10^{-6}	88.85	29.77	1.31	843.00	144.62	2.34
	1×10^{-5}	120.05	25.21	2.89	1200.02	145.11	4.44
	3×10^{-5}	140.08	25.37	7.15	1400.13	145.23	10.74
Control	—	13.13	36.29		301.89	147.73	
	3×10^{-7}	18.05	33.22	0.80	388.14	145.12	1.05
l-Norepinephrine							
Control	—	16.76	17.11		393.64	110.86	
	1×10^{-5}	28.27	16.97	14.56	741.15	102.65	11.33
	3×10^{-5}	41.89	14.35	20.01	841.15	103.62	26.39
	1×10^{-4}	89.37	17.28	23.08	1311.30	102.94	42.90
	3×10^{-4}	165.54	18.41	33.79	2489.18	108.21	56.35
Control	—	13.13	36.29		301.89	147.73	
	1×10^{-6}	15.15	29.64	6.50	398.49	155.61	3.13
	3×10^{-6}	22.83	36.35	4.06	649.26	156.40	2.61

The data shown are those from a single experiment performed in duplicate. The K_i values were calculated based on the equation of competitive inhibition.

obtained are shown in Fig. 3. The K_i values of the agonists would be independent of concentration if the agonists each possessed a single K_i value for both high- and low-affinity sites. However, the K_i values of the agonists were not constant, thereby indicating the existence of both high and low K_i values (against both high and low affinity sites). These results can be

TABLE III. Effects of Agonists on β-Adrenoceptor Binding

	Drugs added (M)	High-affinity site		Low-affinity site		High:low ratio	Hill coefficient
		K_d (pM)	B_{max} (fmol/mg protein)	K_d (pM)	B_{max} (fmol/mg protein)		
Control	— (4)	7.54±2.51	19.57±5.59	266.75±19.64	125.34±10.59	13.5:86.5	0.76±0.03
l-Isoproterenol	3×10^{-7} (3)	17.58±6.81	16.01±7.75	561.03±73.89	123.59± 9.24	11.5:88.5	0.81±0.03
l-Epinephrine	3×10^{-7} (3)	8.96±3.19	18.57±7.34	311.74±11.25	129.63± 8.59	12.5:87.5	0.83±0.02
l-Norepinephrine	1×10^{-6} (3)	8.59±3.37	17.24±6.21	477.70±93.81	145.72± 7.66	10.6:89.4	0.78±0.02

Each value in parenthesis is the number of experiments. Data are the mean values±S.E.

TABLE IV. Inhibition Constants (K_i) of Agonists in β-Adrenoceptor Binding

	Drugs added (M)	K_i (nM) High-affinity site	K_i (nM) Low-affinity site	Low K_i/high K_i
l-Isoproterenol	3×10^{-7} (3)	268.92± 52.02	249.65± 65.97	0.94±0.06
l-Epinephrine	3×10^{-7} (3)	571.20± 92.91	2255.33±547.55	4.78±2.02
l-Norepinephrine	1×10^{-6} (3)	6637.26±557.62	2626.93±771.74	0.37±0.14

Each value in parenthesis is the number of experiments. Data are the mean values±S.E.

explained in terms of two affinity states of the receptors for the agonists. Thus, all the high-affinity-state receptors were converted in the presence of GTP into low-affinity-state recetors.[1] In fact, it is known that the high-affinity-state receptors for the agonists are identical with the physiological receptors for the agonists.[13] Therefore, as we wished to examine the potency of agonists in terms of effect on ^{125}I-ICYP binding, concentrations of agonists which showed low K_i values were chosen. Thus, the concentrations of *l*-isoproterenol, *l*-epinephrine, and *l*-norepinephrine were set at 3×10^{-7}, 3×10^{-7}, and 1×10^{-6} M, respectively.

Table IV summarizes the K_i values of the agonists for ^{125}I-ICYP binding to rat cerebral cortical membranes. Agonists competed with ^{125}I-ICYP binding to the high- and low-affinity sites in the following order of potency: *l*-isoproterenol > *l*-epinephrine > *l*-norepinephrine and *l*-isoproterenol > *l*-epinephrine = *l*-norepinephrine.

Discussion

Although many investigators have found that Scatchard plots of ^{125}I-ICYP binding as a radioligand in various tissues[3] were uniphasic in character, we obtained biphasic plots in this work. The results may be summarized as follows. (1) The Scatchard plots of ^{125}I-ICYP binding to rat brain membranes were all biphasic and the Hill coefficients were below one (Table III and Fig. 1). (2) The K_d and B_{max} values were determined by the use of selective radioligands for β-adrenoceptors, such as ^{3}H-dihydroalprenolol (^{3}H-DHA) and ^{125}I-ICYP. If both radioligands bind to the same sites, the B_{max} values for the two radioligands should be identical. When the saturation experiment showed in Fig. 1 was carried out with ^{3}H-DHA as the radioligand, the Scatchard plot was uniphasic and the B_{max} and K_d values were 170.33 fmol/mg protein and 20.41 nM, respectively (data not shown). Thus, the B_{max} value obtained by ^{3}H-DHA binding was identical with that obtained by a two receptor site model analysis of

^{125}I-ICYP binding, suggesting that the Scatchard plots of ^{125}I-ICYP binding can be well described by the two receptor site model. (3) If ICYP behaves as partial agonist, there is the possibility that the Scatchard plot would be biphasic. In fact, although ICYP behaved as partial agonist in the case of lipolytic activity on rat fat cells,[14] the addition of GTP (3×10^{-4} M) did not have any effect in this study (Table I). These results are consistent with a report[5] that competition experiments with pindolol or ICYP for ($\pm$)-^{125}I-ICYP binding did not show any shift of the competition curve to the right upon addition of guanyl-5'-yl-imidodiphosphate. These results suggested that the radioligand, ^{125}I-ICYP, did not behave as a partial agonist in the radioligand binding assay. (4) The agonists *l*-isoproterenol, *l*-epinephrine, and *l*-norepinephrine possessed specific patterns of affinity for the high- and low-affinity sites in the ^{125}I-ICYP binding. If the saturation curves conformed to the one receptor model, the K_i values for the high-affinity sites of the agonists would be identical with those for the low-affinity sites in the biphasic Scatchard plots, whereas in fact the respective K_i values of *l*-norepinephrine and *l*-epinephrine for the high- and low-affinity sites in the biphasic Scatchard plots were extremely different. Thus, the ratios of the low K_i/high K_i values of *l*-norepinephrine and *l*-epinephrine were 0.37 and 4.78, respectively (Table IV).

The K_d and B_{max} values of the high-affinity sites of the control in the absence of the agonists varied widely as compared with those of the low-affinity sites (Tables I, II and III). The variable character of the values of high-affinity sites may be based on the number of data points taken because fewer data points were used in the regression for the high-affinity sites than for the low-affinity sites (Fig. 1). Thus, more data points at low concentrations of ^{125}I-ICYP may be required.

The plots of $-\log K_i$ *vs.* $-\log[I]$ for the agonists used in the present study showed that the agonists may be possessed four K_i values (two each for the high- and low-affinity sites), because the K_i values of the agonists were concentration-dependent, which would not be the case if a single class of binding sites existed for both high- and low-affinity sites. These results can be explained in terms of two affinity states of receptors for the agonists. The high-affinity-state receptors are converted into low-affinity-state receptors in the presence of guanine nucleotides (GTP).[1] In fact, it is known that the high-affinity-state receptors for the agonists are identical with the physiological receptors for the agonists.[13] In displacement experiments, other investigators[1] found that the displacement curves with agonists to ^{3}H-DHA binding were shifted to the right and steepened in the presence of guanine nucleotides (GTP), indicating that the guanine nucleotides, which are required for hormonal activation of adenyl cyclase, mediate the transition of the high-affinity-state receptors to low-affinity-state receptors in the binding of the agonists to β-adrenoceptors. In fact, Scatchard plots with the agonists, *l*-isoproterenol (3×10^{-7} M), *l*-epinephrine (3×10^{-7} M), and *l*-norepinephrine (1×10^{-6} M), in the presence of GTP (3×10^{-4} M) showed variable K_i values which approached the K_i values obtained at high concentration of the agonists (data not shown). Thus, the results suggested that the Scatchard plots possessed high- and low-affinity sites and these two affinity sites may be have additional two different state receptors like so-called high- and low-affinity-state receptors for the agonists.

Partial selectivity of ^{125}I-ICYP to β_2-adrenoceptor subtypes colud be seen in the Scatchard plots in the presence and absence of competing agonists. Pharmacologically, the subtype specificity of drugs or tissues can be assessed by analyzing the relative potency of the agonists, isoproterenol, epinephrine, and norepinephrine.[1] Thus, the β_1-adrenoceptor potency was in the order of isoproterenol > epinephrine = norepinephrine, whereas the β_2-adrenoceptor potency was in the order of isoproterenol > epinephrine > norepinephrine. In the present study, the agonists competing with ^{125}I-ICYP for the high- and low-affinity binding sites may correspond to β_2- and β_1-adrenoceptor subtypes, respectively. It is well known that the specificity of the agonists for β_1- and β_2-adrenoceptors is $\beta_2 = \beta_1$ (*l*-

isoproterenol), $\beta_2 > \beta_1$ (*l*-epinephrine), and $\beta_2 < \beta_1$-adrenoceptors (*l*-norepinephrine). In the present study, the specificity of the agonists for the high- and low-affinity sites was high = low (*l*-isoproterenol), high > low (*l*-epinephrine), and high < low affinity sites (*l*-norepinephrine). These results imply that the high-affinity site in ^{125}I-ICYP binding may be the β_2-adrenoceptor binding site and the low-affinity site may be the β_1-adrenoceptor binding site. Further investigations by the use of selective antagonists for β_1- or β_2-adrenoceptors and examination of the characteristics of the displacement curves with selective antagonists as reported by Minneman *et al.*[2b] seem to be desirable.

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Pharmacological Studies of Furo[3,2-*b*]indole Derivatives. III. Correlation between Analgesic Effect and Effect on Central Nervous System of *N*-(3-Piperidinopropyl)-4-methyl-6-trifluoromethyl Furo[3,2-*b*]indole-2-carboxamide (FI-302) in Mice

FUSAO AMANUMA*,[a] and TSUTOMU KAMEYAMA[b]

Department of Pharmacology, Research Center, Taisho Pharmaceutical Co., Ltd.,[a] Ohmiya 330, Japan and Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Meijo University,[b] Nagoya 468, Japan

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N-(3-Piperidinopropyl)-4-methyl-6-trifluoromethyl furo[3,2-*b*]indole-2-carboxamide (FI-302) is a new non-steroidal anti-inflammatory compound. The analgesic effect of FI-302 and its effect on the central nervous system (CNS) were compared with those of aspirin, chlorpromazine and chlordiazepoxide in mice subjected to various experimental conditions. FI-302 showed an analgesic effect with all the methods used, but aspirin, chlorpromazine and chlordiazepoxide showed analgesic effects only with the acetic acid-induced writhing method. Moreover, FI-302 inhibited spontaneous motor activity and fighting behavior induced by isolation and foot-shock, but had no effect on the hexobarbital-induced sleeping time. Aspirin had no effect on the CNS, but chlorpromazine and chlordiazepoxide showed inhibitory effects with all the methods used. Fighting behavior is related to emotional response, and pain is associated with emotional responses such as distress and anxiety. Thus, it is suggested that FI-302 is a new analgesic compound which has an inhibitory effect on the emotional response in the CNS.

Keywords——*N*-(3-piperidinopropyl)-4-methyl-6-trifluoromethyl furo[3,2-*b*]indole-2-carboxamide; analgesic effect; central nervous system; anti-aggressive effect

Introduction

N-(3-Piperidinopropyl)-4-methyl-6-trifluoromethyl furo[3,2-*b*]indole-2-carboxamide (FI-302) is a new non-steroidal anti-inflammatory compound. Previously, we reported that FI-302 has analgesic effects which may be produced by both peripheral and central mechanisms, though it is a non-narcotic compound.[1,2]

In general, non-steroidal anti-inflammatory drugs (NSAID) have little or no effect on the central nervous system (CNS). In the present study, we compared the analgesic effects of FI-302 with those of aspirin, chlorpromazine and chlordiazepoxide, and examined its effects on the CNS.

Materials and Methods

Figure 1 shows the chemical structure of FI-302. It is a white powder melting at 155—156 °C, and is insoluble in water but soluble in organic solvents such as ethanol and acetone.

CH_3 / CF_3 / N / O / $CONHCH_2CH_2CH_2N$

Fig. 1. Chemical Structure of FI-302

As reference drugs, aspirin (Nihon Kayaku), chlorpromazine hydrochloride (Aldrich) and chlordiazepoxide (Roche) were used. These drugs were suspended or dissolved in 0.4% sodium carboxymethylcellulose solution.

Analgesic Effects

Acetic Acid-Induced Writhing——Groups of 10 male ddY mice each weighing 19 to 24 g were used. The drugs were administered orally 30 min before intraperitoneal injection (10 ml/kg) of 0.7% acetic acid solution. The number of writhes was counted for each mouse during a period of 10 to 20 min after the acetic acid injection.

The percent inhibition was calculated by comparison with the number of writhes in a control group.

Tail Pressure——Groups of 10 male ddY mice each weighing 20 to 25 g were used. A pressure stimulus was applied to the base of the mouse tail. The drugs were administered orally to mice showing a pain threshold within 40 to 70 mmHg, and the pain threshold was measured every 30 min for 120 min.

Animals in which the post-drug pain threshold was raised above 50% compared with the pre-drug threshold were regarded as positive for analgesic activity.

Hot Plate——Groups of 10 male ddY mice each weighing 20 to 25 g were used. The drugs were administered orally to mice showing a pain response (paw licking or jumping) time of 4 to 6 s, and the pain response time was measured every 30 min for 120 min.

Animals in which the post-drug pain response time was prolonged by more than 50% compared with the pre-drug time were regarded as positive for analgesic activity.

D'Amour and Smith Method——Groups of 10 male ddY mice each weighing 20 to 25 g were used. The drugs were administered orally to mice showing a pain response (tail flick) time of within 3 to 5 s, and the pain response time was measured every 30 min for 120 min.

Animals in which the post-drug pain response time was prolonged by more than 50% compared with the pre-drug time were regarded as positive for analgesic activity.

Effect on Hexobarbital-Induced Sleeping Time

Groups of 10 male ddY mice each weighing 19 to 24 g were used. The drugs were administered orally 30 min before intraperitoneal injection of hexobarbital-Na 100 mg/kg. The sleeping time (from loss to recovery of righting reflex) was measured for each mouse.

Animals for which the sleeping time was prolonged by more than 2 times compared with the mean sleeping time in a control group were regarded as positive for potentiation of hexobarbital-induced sleeping time.

Effect on Spontaneous Motor Activity (Wheel Cage Method)

Groups of 10 male ddY mice each weighing 19 to 24 g were used. The drugs were administered orally to mice for which the number of revolutions was 400 to 600 per 30 min before the day of the experiment, and the number of revolutions was measured for each mouse every 15 min for 90 min.

The percent inhibition was calculated from the number of revolutions for 90 min compared with that in a control group.

Effect on Fighting Behavior

Isolated Mice——Male ddY mice each weighing 19 to 24 g were used and isolated in single cages for more than 4 weeks. A mouse was placed in the home cage of another mouse and the pair of mice showing fighting behavior within 30 s were selected. Groups of 6 to 8 pairs were used. The drugs were administered orally and, 30 min after dosing, the fighting behavior by the same pairs was observed for 3 min.

Pairs that showed no fighting behavior during these 3 min were regarded as positive for anti-aggressive activity.

Foot-Shock[3]——Male ddY mice each weighing 20 to 24 g were used. The fighting behavior was produced by a foot-shock (40 V direct current, 8 ms, 2 Hz) for 2 min and pairs of mice showing fighting behavior more than 6 times were selected. Groups of 6 to 8 pairs were used. The drugs were administered orally and 30 min after dosing, the fighting behavior was observed for 2 min.

Pairs that exhibited fighting behavior less than one-half as many times as before administration were regarded as positive for anti-aggressive activity.

Muscle-Relaxing Effect (Traction Test)

Groups of 10 male ddY mice each weighing 19 to 23 g were used. The drugs were administered orally, and mice were suspended by the fore-limbs from a wire (1 mm diameter) fixed 30 cm above the experimental table every 30 min for 120 min.

Animals that did not hang one hind-limb on the wire during the following 10 s were regarded as positive for muscle relaxation.

Results

The ED_{50} value and 95% confidence limits were calculated according to the method of Litchfield and Wilcoxon.[4]

Analgesic Effect

Acetic Acid-Induced Writhing——FI-302 showed an analgesic effect at doses of more

Table I. Analgesic Effects of FI-302, Aspirin, Chlorpromazine and Chlordiazepoxide as Determined by the Acetic Acid-Induced Writhing Method in Mice

Drug	ED_{50} in mg/kg, *p.o.*
FI-302	26.5 (16.0— 43.8)
Aspirin	265.0 (152.4—460.7)
Chlorpromazine	3.2 (1.9— 5.3)
Chlordiazepoxide	17.0 (9.5— 30.3)

Table II. Analgesic Effects of FI-302, Aspirin, Chlorpromazine and Chlordiazepoxide as Determined by the Tail Pressure Method in Mice

Drug	ED_{50} in mg/kg, *p.o.*
FI-302	31.0 (19.4—49.4)
Aspirin	>400
Chlorpromazine	>20
Chlordiazepoxide	>30

Table III. Analgesic Effects of FI-302, Aspirin, Chlorpromazine and Chlordiazepoxide as Determined by the Hot Plate Method in Mice

Drug	ED_{50} in mg/kg, *p.o.*
FI-302	55.0 (36.6—82.7)
Aspirin	>400
Chlorpromazine	>20
Chlordiazepoxide	>30

Table IV. Analgesic Effects of FI-302, Aspirin, Chlorpromazine and Chlordiazepoxide as Determined by the D'Amour and Smith Method in Mice

Drug	ED_{50} in mg/kg, *p.o.*
FI-302	47.0 (34.6—63.8)
Aspirin	>400
Chlorpromazine	>20
Chlordiazepoxide	>30

than 10 mg/kg and the ED_{50} value was 26.5 mg/kg. Aspirin, chlorpromazine and chlordiazepoxide also showed analgesic effects and their ED_{50} values were 265.0, 3.2 and 17.0 mg/kg, respectively (Table I).

Tail Pressure——FI-302 showed an analgesic effect at doses of more than 15 mg/kg and the ED_{50} value was 31.0 mg/kg. However, aspirin chlorpromazine and chlordiazepoxide showed no such effect at doses of 400, 20 and 30 mg/kg, respectively (Table II).

Hot Plate——FI-302 showed an analgesic effect at doses of more than 25 mg/kg and the ED_{50} value was 55.0 mg/kg. However, aspirin chlorpromazine and chlordiazepoxide showed no such effect at doses of 400, 20 and 30 mg/kg, respectively (Table III).

D'Amour and Smith Method——FI-302 showed an analgesic effect at doses of more than 25 mg/kg and the ED_{50} value was 47.0 mg/kg. However, aspirin chlorpromazine and chlordiazepoxide showed no such effect at doses of 400, 20 and 30 mg/kg, respectively (Table IV).

Effect on Hexobarbital-Induced Sleeping Time

FI-302 and aspirin showed no effect at doses of 100 and 400 mg/kg, respectively. On the other hand, chlorpromazine and chlordiazepoxide were found to prolong the sleeping time and their ED_{50} values were 3.1 and 9.4 mg/kg, respectively (Table V).

Effect on Spontaneous Motor Activity

FI-302 showed inhibitory activity at doses of more than 10 mg/kg and the ED_{50} value was 26.5 mg/kg. Aspirin showed slight promoting activity at a dose of 400 mg/kg, but chlorpromazine and chlordiazepoxide showed inhibitory activity and their ED_{50} values were 6.2 and 31.0 mg/kg, respectively (Table VI).

Effect on Fighting Behavior

Isolated Mice——FI-302 showed inhibitory activity at doses of more than 5 mg/kg and the ED_{50} value was 14.0 mg/kg. Aspirin showed no effect at a dose of 400 mg/kg, but chlorpromazine and chlordiazepoxide showed inhibitory activity and their ED_{50} values were

Table V. Effects of FI-302, Aspirin, Chlorpromazine and Chlordiazepoxide on Hexobarbital-Induced Sleeping Time in Mice

Drug	ED_{50} in mg/kg, *p.o.*
FI-302	>100
Aspirin	>400
Chlorpromazine	3.1 (2.2— 4.4)
Chlordiazepoxide	9.4 (7.3—12.1)

Table VI. Effects of FI-302, Aspirin, Chlorpromazine and Chlordiazepoxide on Spontaneous Motor Activity in Mice

Drug	ED_{50} in mg/kg, *p.o.*
FI-302	26.5 (15.0—46.8)
Aspirin	>400
Chlorpromazine	6.2 (4.3— 9.0)
Chlordiazepoxide	31.0 (15.5—62.1)

Table VII. Effects of FI-302, Aspirin, Chlorpromazine and Chlordiazepoxide on Fighting Behavior in Isolated Mice

Drug	ED_{50} in mg/kg, *p.o.*
FI-302	14.5 (10.7—19.6)
Aspirin	>400
Chlorpromazine	5.4 (4.1— 7.1)
Chlordiazepoxide	42.0 (35.2—50.1)

Table VIII. Effects of FI-302, Aspirin, Chlorpromazine and Chlordiazepoxide on Foot-Shock-Induced Fighting Behavior in Mice

Drug	ED_{50} in mg/kg, *p.o.*
FI-302	25.0 (16.7— 37.3)
Aspirin	>400
Chlorpromazine	36.0 (12.0—108.4)
Chlordiazepoxide	38.0 (30.8— 46.8)

Table IX. Muscle-Relaxing Effects of FI-302, Aspirin, Chlorpromazine and Chlordiazepoxide in Mice

Drug	ED_{50} in mg/kg, *p.o.*
FI-302	>100
Aspirin	>400
Chlorpromazine	15.2 (10.7—21.7)
Chlordiazepoxide	28.0 (18.0—43.6)

5.4 and 42.0 mg/kg, respectively (Table VII).

Foot-Shock——FI-302 showed inhibitory activity at doses of more than 10 mg/kg and the ED_{50} value was 25.0 mg/kg. Aspirin showed no effect at a dose of 400 mg/kg, but chlorpromazine and chlordiazepoxide showed inhibitory activity and their ED_{50} values were 36.0 and 38 mg/kg, respectively (Table VIII).

Muscle-Relaxing Effect (Traction Test)

FI-302 showed no effect at a dose of 100 mg/kg. Aspirin also showed no effect at a dose of 400 mg/kg. However, chlorpromazine exhibited a muscle-relaxing effect at doses of more than 10 and the ED_{50} value was 15.2 mg/kg. Chlordiazepoxide also exhibited a muscle-relaxing effect at doses of more than 20 mg/kg and the ED_{50} value was 28.0 mg/kg (Table IX).

Discussion

FI-302 is a new non-steroidal anti-inflammatory compound. In our previous paper, we reported that FI-302 has analgesic effects which may be produced by both peripheral and central mechanisms, though it is a non-narcotic compound.[1,2] In this study, we demonstrated that FI-302 has analgesic effects when tested by various methods and also has an inhibitory effect on the CNS.

FI-302 showed analgesic effects when assessed by the acetic acid-induced writhing, tail pressure, hot plate and D'Amour and Smith methods. On the other hand, aspirin, chlorpromazine and chlordiazepoxide showed an analgesic effect with only the acetic acid-induced writhing method. The methods used in this study for evaluation of the analgesic activity are widely used, but the acetic acid-induced writhing method has the disadvantage that, although mild analgesic drugs are detected, many types of drugs which are not generally classified as analgesic drugs are also detected as being positive.[5] As FI-302 showed an analgesic effect with all methods, its inhibitory effect determined by the acetic acid-induced writhing method is considered to be due to its analgesic effect. Aspirin is an acidic NSAID and, in general, acidic NSAID produce their analgesic effects by inhibiting prostaglandin biosynthesis in inflamed tissue.[6,7] Therefore, the acidic NSAID show an analgesic effect on writhing induced by chemicals, such as acetic acid and acetylcholine,[8] but have little or no effect on animals subjected to the tail pressure, hot plate and D'Amour and Smith assessment methods. Chlorpromazine and chlordiazepoxide are psychotropic drugs, but they also inhibited acetic acid-induced writhing. However, since these drugs did not show any analgesic effect with the other methods at high doses, but caused muscle relaxation, the inhibitory effect of these drugs on acetic acid-induced writhing is considered to be due to their inhibitory effect on the CNS, and not to analgesic action.

From this study, FI-302 appears to have an inhibitory effect on the CNS. FI-302 inhibited spontaneous motor activity and fighting behavior caused by isolation and foot-shock at analgesic dose levels, whereas it had no effect on hexobarbital-induced sleeping time or muscle relaxation. We reported that FI-302 has an antipyretic effect, without affecting normal body temperature.[1] Moreove, FI-302 had no effect on the conditioned avoidance response and did not cause catalepsy (data not shown). On the other hand, aspirin did not have any effect but chlorpromazine and chlordiazepoxide had an inhibitory effect and also a muscle-relaxing effect in all the methods used. Chlorpromazine inhibits the conditioned avoidance response, has hypothermic activity and causes catalepsy. Chlordiazepoxide shows various inhibitory effects on the CNS at the doses that cause muscle relaxation. Therefore, although FI-302 has an inhibitory effect on the CNS, it is clear that the spectrum of the inhibitory effects of FI-302 on the CNS differs from those of chlorpromazine and chlordiazepoxide.

Fighting behavior is related to emotional response. We previously reported that FI-302 has an inhibitory effect on vocalization response, which is an emotional parameter associated with pain.[2] Therefore, FI-302 at analgesic dose levels is considered to show an inhibitory effect on emotional response. As NSAIDs generally have little or no effect on the CNS, this inhibitory effect of FI-302 may be a side effect. However, the findings that FI-302 inhibits emotional responses without affecting the hexobarbital-induced sleeping time suggests that the agent is a promising new analgesic compound.

In conclusion, although the mechanism of action of FI-302 on the central pain pathway and the CNS is still unknown, FI-302 is a new type of analgesic compound which has an inhibitory effect on the emotional response in the CNS.

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Characterization of ^{3}H-Dihydroalprenolol Binding to β-Adrenergic Receptors of Rat Brain: Two Binding Sites of Racemic Propranolol in Displacement Experiments

HIROSHI TSUCHIHASHI* and TAKAFUMI NAGATOMO

Department of Pharmacology, Niigata College of Pharmacy, 5-13-2 Kamisinei-cho, Niigata 950-21, Japan

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The binding affinities of racemic derivatives of propranolol to β-adrenoceptors were examined by means of radioligand binding assay to determine whether racemic propranolol shows biphasic binding to β-adrenoceptors in a displacement experiment. Rat brain was used and the β-adrenoceptor binding assay was carried out with ^{3}H-dihydroalprenolol as the radioligand. A higher affinity of *l*-propranolol than of *d*-propranolol was observed and *d*- and *l*-propranolol each gave a uniphasic curve. However, *dl*-propranolol showed a biphasic curve. Furthermore, a mixture of *d*- and *l*-propranolol also showed a biphasic curve. These results imply that β-adrenoceptors of rat brain may bind the *d*- and *l*-isomers at distinct sites.

Keywords——β-adrenoceptor; rat brain; propranolol; displacement experiment; two-site model

It is well known that *l*- and *d*-propranolol have different pharmacological effects, and *d*-propranolol has 1/100 to 1/20 of the potency of *l*-propranolol in blocking β-adrenergic receptors.[1] Bylund and Snyder reported different affinities of *l*- and *d*-propranolol in a radioligand binding assay using rat brain membrane with ^{3}H-dihydroalprenolol (^{3}H-DHA) as the radioligand,[2] and the same result was obtained in our laboratory using neuraminidase-treated membrane from rat heart.[3] On the other hand, the binding of ligands such as ^{3}H-DHA to the receptors has all the characteristics of specificity, stereospecificity, saturability and reversibility that one would expect of binding to physiological receptors.[4] An obvious outgrowth of radioligand-binding techniques was the development of methods for determining β-adrenoceptor subtypes (β_1- and β_2-receptor) in tissues containing either homogeneous or heterogeneous populations of β-adrenergic receptors.[4,5] These techniques using the competition curves of β-adrenergic subtype-selective antagonists with non-subtype-selective radioligands such as ^{3}H-DHA gave competition curves that were biphasic in character,[6] whereas the curves obtained with non-subtype-selective antagonists (optical isomers or racemates) in competition with non-subtype-selective radioligands were mainly uniphasic[5b,7] or slightly biphasic[7] in character. To analyze such complex competition curves, a mathematical method of analysis was applied to quantitate the relative proportions of the two receptor subtypes and the affinity of the competitor for each receptor. Thus, the object of this experiment was to examine whether the competition curves of *dl*-propranolol in the radioligand binding method using rat brain membrane with ^{3}H-DHA as the radioligand are uniphasic or not.

Experimental

(−)-^{3}H-DHA (104.8 Ci/mmol) was purchased from New England Nuclear Corp., and *l*-, *d*-, and *dl*-propranolol were kindly donated by ICI Pharmaceuticals.

The membrane-enriched fraction from the rat brain was prepared by using the method previously described.[8] In brief, after the removal of the rat brain, cerebral cortex was minced and homogenized with a glass homogenizer. The homogenate was filtered through 4 layers of gauze, and the filtrate was centrifuged at 40000*g* for 30 min. The resultant pellets were rinsed at once with 75 mM Tris–HCl, 25 mM $MgCl_2$, pH 7.2, and homogenized with a glass homogenizer using 20 ml of the same buffer. These membrane-enriched fractions were frozen in liquid nitrogen, stored at −80 °C, and, immediately before use, further diluted to the appropriate concentrations as indicated below in the text. During 2 months' storage there was no observable decrease in antagonist binding. Protein concentrations were determined by the method of Lowry *et al.*[9] using bovine serum albumin as the standard.

The β-adrenoceptor binding assay was carried out in duplicate with ^{3}H-DHA. For ^{3}H-DHA binding, 0.25 ml of membrane suspension (0.25 mg of protein) was incubated with shaking for 30 min at 23 °C with 1.2 nM ^{3}H-DHA and with various concentrations of antagonist in a total volume of 0.5 ml containing 60 mM Tris–HCl and 20 mM $MgCl_2$ (pH 7.2). At the end of the incubation period, the incubation medium was immediately filtered through a GF/C glass fiber filter using the improved method.[10] The filter was added to 5 ml of Tt 76 scintillation fluid. The difference in mean values between total and non-specific binding determined in the presence of 10 μM *l*-propranolol was taken as the specific binding. The specific binding with ^{3}H-DHA routinely amounted to approx. 80% of the total binding.

All kinetic analyses were carried out on an NEC PC-9801F computer system that performs iterative non-linear regression. This program fits the binding data to equations based on the law of mass action for one or more classes of binding sites and determines whether the fit for the two-site model is statistically better than that for the one-site model by using the theory of Munson and Rodbard.[11] Using the weighted sum of squares principle, the goodness of fit was evaluated between either a model having only one receptor subtype and a model having two receptor subtypes in the displacement analysis, or between a model having one receptor site and a model having two receptor sites in Scatchard analysis. In brief, both saturation binding curves and competition curves are analyzed according to a general model for the interactions of several ligands with several classes of sites on the basis of the mass-action law.[12] The one receptor site model and two site model were examined by means of Scatchard plots of saturation binding data obtained as described previously.[13] The models of the competition curves were as follows:

(I) One receptor–one ligand model

$$\frac{B_1}{B_0}=\frac{1}{1+\dfrac{x}{\mathrm{IC}_{50}}}$$

(II) One receptor–two ligands model

$$\frac{B_1}{B_0}=\frac{1}{1+\dfrac{F_a}{\mathrm{IC}_{50a}}+\dfrac{F_b}{\mathrm{IC}_{50b}}}$$

(III) Two receptors–one ligand model

$$\frac{B_1}{B_0}=\frac{z}{1+\dfrac{x}{\mathrm{IC}_{50c}}}+\frac{1-z}{1+\dfrac{x}{\mathrm{IC}_{50d}}}$$

where either B_1 or B_0 is the concentration of the radioligand bound with or without the cold ligand, x is the cold ligand concentration, IC_{50} is an apparent inhibition constant (concentration of the cold ligand which inhibits the radioligand binding by 50%), F_a and F_b are the concentrations of ligands a and b for a receptor, IC_{50a} and IC_{50b} are the apparent inhibition constants of ligands a and b, IC_{50c} and IC_{50d} are the apparent inhibition constants of a cold ligand for receptors c and d, and z is the proportion of the ligand bound with receptor c.

In these non-linear regression analyses, the parameter fitting method, termination of iteration and justification of the models were carried out by the methods described previously.[13]

These programs were donated by Mr. S. Nagatsuka (Daiichi Pure Chem. Co., Ltd.) and partially modified at our laboratory. As the value of inhibition constant (IC_{50}) in displacement analysis was obtained as the concentration of antagonist which inhibits ^{3}H-DHA binding by 50%, the values of inhibition constants (K_i) were calculated by applying the equation of Cheng and Prusoff.[14] In order to quantitate the mode of saturation, Hill numbers of ^{3}H-DHA binding to the membranes were determined by means of the Hill plot,[15] and the slope factors of competition curves were calculated.[16] The slope factors of the models of competition curves, I (one receptor–one ligand model), II (one receptor–two ligands model), and III (two receptors–one ligand model), are 1, 1, and below one, respectively.

Results

The yield of membrane protein obtained by the present method from cerebral cortex was

73.76±2.36 mg per g wet weight ($n=7$). Figure 1 shows the Scatchard plots of specific ^{3}H-DHA bindings to membranes. Values of the equilibrium dissociation constant (K_d) and the maximum binding capacity (B_{max}) of ^{3}H-DHA were found from Scatchard analysis to be 20.58±2.16 nM, and 170.83±13.76 fmol/mg protein ($n=4$). The Scatchard plots for ^{3}H-DHA binding were uniphasic and the Hill coefficients were 1.00±0.02 ($n=4$).

In order to study the effect of high-affinity racemic antagonists on competition curves, we constructed curves using as the antagonist (a) *dl*-propranolol, (b) a mixture of *l*-propranolol and *d*-propranolol (molar ratio = 1 : 2), and (c) *l*- : *d*-propranolol = 2 : 1. When *dl*-propranolol was used as the competitor (Fig. 2), the competition curves using ^{3}H-DHA as a radioligand were biphasic and the slope factor was 0.71 (Table I). Furthermore, when mixtures of *l*- and *d*-propranolol were used as competitors (Fig. 2), the competition curves were biphasic and the slope factors were below one (Table I). In contrast, when either *d*- or *l*-propranolol was the competitor (Fig. 2), the respective competition curves were uniphasic and the slope factors were about one (Table I).

Table I summarizes the K_i values for propranolol derivatives in displacement assays using ^{3}H-DHA as a radioligand. The K_i values of *l*-propranolol on the ^{3}H-DHA bindings indicated an affinity about 100 times greater than that of *d*-propranolol. The K_i values of high-affinity sites of *dl*-propranolol and the mixtures were identical with those of *l*-propranolol, and those of low-affinity sites were identical with those of *d*-propranolol. Furthermore, the percentage ratios of high- to low-affinity sites of *dl*-propranolol, and those of mixtures of

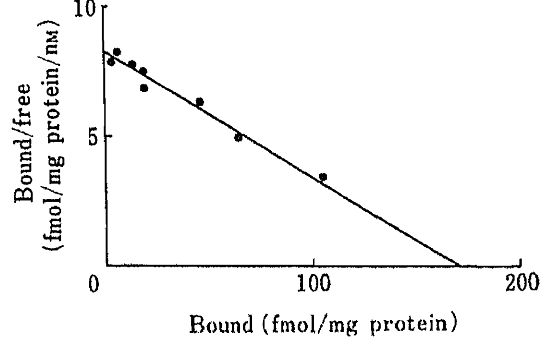

Fig. 1. Scatchard Plots of ^{3}H-DHA Bindings in Rat Brain

Specific ^{3}H-DHA binding was measured as described in Experimental in the concentration range between 0.1 and 10 nM ^{3}H-DHA. The values of K_d and B_{max} for ^{3}H-DHA binding were 20.41 nM and 170.33 fmol/mg protein. The data shown are those from a single experiment performed in duplicate.

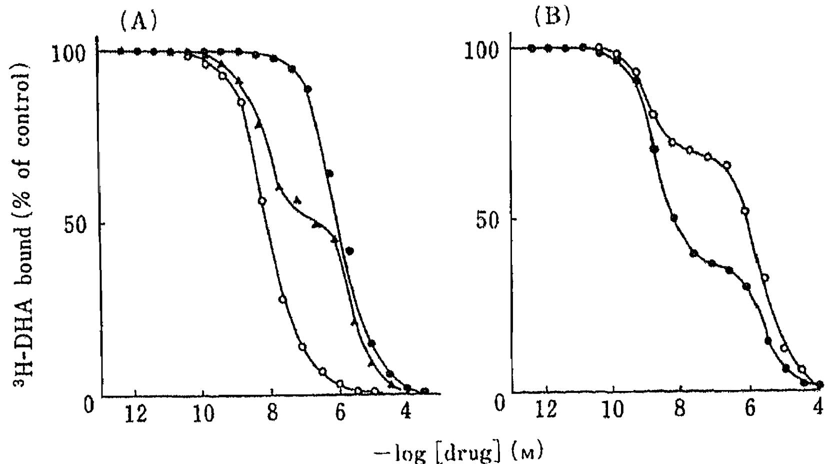

Fig. 2. Inhibition of ^{3}H-DHA Binding by *d*- (●), *dl*- (▲), and *l*-Propranolol (○) (A) and Mixtures of *d*- : *l*-Propranolol, 1 : 2 (●) and 2 : 1 (○) (B)

Membranes were incubated with ^{3}H-DHA (1.2 nM) in the presence of various concentrations of the antagonists. Values are means of three experiments, each performed in duplicate.

TABLE I. Inhibition Constants of Racemic Propranolol Derivatives for ^{3}H-DHA Binding

Drug	n	High-affinity site		Low-affinity site		Slope factor
		K_i (nM)	%	K_i (μM)	%	
l-Propranolol	3	16.73±14.39	100			1.00±0.02
d-Propranolol	3			1.24±0.88	100	0.96±0.02
dl-Propranolol	3	13.32± 9.92	49.4± 3.6	6.67±2.14	50.6± 3.6	0.71±0.03
$d:l$=1:2	3	6.68± 4.32	64.6± 5.0	2.61±1.02	35.4± 5.0	0.70±0.01
$d:l$=2:1	3	1.58± 0.52	33.7±10.1	1.12±0.86	66.3±10.1	0.71±0.04

Each value in parenthesis is the number of experiments. Data are the mean values±S.E. In order to facilitate the understanding of this table, l- and d-propranolol are located the columns under high- and low-affinity sites, respectively. The proportional percentages (%) are calculated by using the equation based on two receptors-one ligand model.

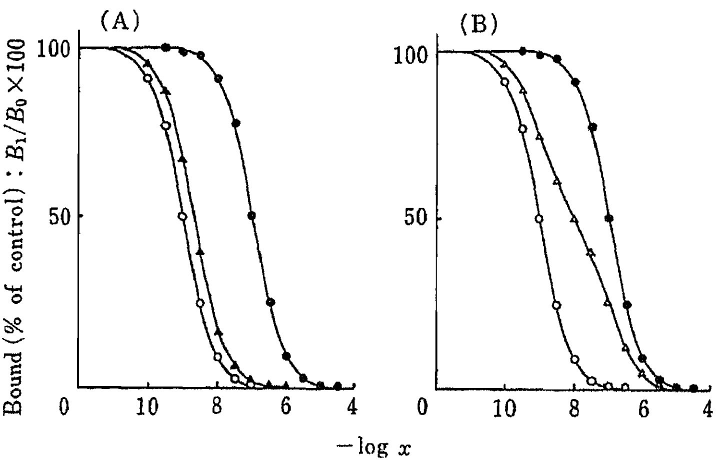

Fig. 3. The Expected Competition Curves Obtained by Computerized Analysis

Part A shows theoretical curves based on the models of one receptor–one ligand (model I) (○ and ●), one receptor–two ligands (model II) (▲), and part B shows curves based on one receptor-one ligand (model I) (○ and ●), and two receptors-one ligand (model III) (△). Each line represents either one or two components. A single component has an IC_{50} of 1 nM (○) or 100 nM (●) and a proportional percent of 100. In the case of two components, (▲) and (△), one component has an IC_{50} of 1 nM and a proportional percent of 50 and the other component has an IC_{50} of 100 nM and a proportional percent 50. The slope factors of models I, II, and III are 1.00, 1.00, and 0.73, respectively.

d:l=2:1 and d:l=1:2 in ^{3}H-DHA binding were 49.4:50.6, 33.7:66.3, and 64.6:35.4, respectively.

Figure 3 shows the expected competition curves with computer curve fitting according to models I (one receptor–one ligand model), II (one receptor–two ligands model), and III (two receptors–one ligand model). The competition curve of a mixture of two non-selective antagonists such as racemic propranolol would be expected to be consistent with the model of one receptor–two ligands (model II), whereas the competition curves of dl-propranolol and the mixtures of l- and d-propranolol found in this study were biphasic and fitted the model of two receptors–one ligand (model III).

Discussion

Among the isomers of propranolol, the d-isomer has less than about one-hundredth of the potency of the l-isomer. These results are consistent with the physiological function of both isomers[1] and the results of Bylund and Snyder,[2] as propranolol possesses only one

asymmetric carbon atom. Thus, the difference between the *d*- and *l*-isomers in propranolol lies in the situation of the hydroxyl group linked to the asymmetric carbon atom. These results suggest that the β-adrenergic receptor recognized the hydroxyl group and the binding sites is specific for the *l*-isomer of propranolol.

Our present data suggest the possibility that the racemic compound may show a biphasic drug–receptor interaction in ligand binding assay using ^{3}H-DHA. This possibility is supported by the following results. (1) The competition curves of *dl*-propranolol with this ligand were biphasic (slope factor = 0.71) in character, with a percentage ratio of high- to low-affinity sites of 49.4 : 50.6. (2) The competition curves of the mixtures of *d*- and *l*-propranolol were also biphasic and the slope factors were below one, whereas those of *d*- or *l*-propranolol alone were uniphasic and the slope factors were about one. (3) The percentage ratios of high- to low-affinity sites of the mixtures as competitors were 33.7 : 66.3 for the mixture of *l*- : *d*-propranolol (1 : 2) and 64.6 : 35.4 for the mixture of *l*- : *d*-propranolol (2 : 1). (4) Furthermore, the K_i values of the high-affinity sites of *dl*-propranolol and the mixtures as competitors to this ligand binding were identical with those of *l*-propranolol, and those of the low-affinity sites were identical with those of *d*-propranolol.

It is known that the slope factors of non-selective antagonists (both optical isomers and racemates) are unity,[5b,7] though there are some exceptions.[7] In (−)-^{3}H-DHA binding to frog erythrocyte membranes, the slope factors of (−)- and (±)-carazolol as competitors were over one but that of (+)-carazolol was one, and those of (−)- and (±)-carazolol on (−)-^{3}H-carazolol binding were also one while that of (+)-carazolol was below one.[7a] Furthermore, the slope factor of (+)-propranolol was below one on ^{3}H-DHA binding using bovine teat muscle membranes.[7b] These results can be explained by the law of mass action, which indicates that when the competitor has very much higher or lower affinity than the radioligand, the slope factor of the competition curve is greater or lower than one.[7a] In the present study, the slope factors of *d*- and *l*-propranolol were one, thus suggesting that the situation described above does not apply to propranolol derivatives and ^{3}H-DHA as a radioligand.

The kinetics of racemic compounds are complex, and biphasic dissociations have only been described for racemic antagonist radioligands such as (±)-^{3}H-carazolol,[17] (±)-^{125}I-iodohydroxybenzylpindolol,[17] and (±)-^{125}I-iodocyanopindolol (^{125}I-ICYP),[18] and pure enantiomers such as (−)-^{3}H-DHA and (−)-^{125}I-ICYP showed only monophasic dissociation curves.[18] These results suggested that the biphasic dissociation kinetics of racemic compounds arise from the presence of the *d*- and *l*-isomers.[17,18]

The Scatchard plots with ^{3}H-DHA were uniphasic (Fig. 1). Many other investigators[4,5] have reported that Scatchard plots obtained with this ligand were uniphasic in character, though there were some exceptions.[19] The plots using human lymphocyte membrane were biphasic because of non-specific binding by 10^{-5} M *dl*-propranolol, whereas at 10^{-6} M *l*-propranolol the plot would have been uniphasic.[19a] Furthermore, the plots using human frontal cortex were biphasic.[19b] These experiments[19] suggested that non-specific binding to biological membranes was partially due to partitioning of the ligand into these membranes. The addition of propranolol, which also partitions into the membranes, could alter the membrane to such an extent that the ligand partition coefficient decreases, thereby causing inhibition of this binding. When the displacement experiments were carried out under conditions which allow non-specific binding, the competition curves were biphasic. As in our study, they showed that the more hydrophilic agonist, *l*-isoproterenolol (10^{-3} M), which has a much lower partition coefficient than the above-mentioned antagonists, or *dl*-propranolol (10^{-4} M) had no significant non-specific binding with the membrane (data not shown).

In conclusion, we consider that the results of competition experiments with racemic compounds in β-adrenoceptor binding assay using ^{3}H-DHA are consistent with the two

receptors-one ligand model, and that the β-adrenoceptor may bind *d*- and *l*-isomers at distinct sites.

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Metabolism of Thiabendazole and Teratogenic Potential of Its Metabolites in Pregnant Mice

TOSHIE TSUCHIYA,* AKIRA TANAKA, MASAMICHI FUKUOKA, MICHIO SATO, and TSUTOMU YAMAHA

Division of Medical Chemistry, National Institute of Hygienic Sciences, Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158, Japan

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In connection with the teratogenic activity of thiabendazole (TBZ), the metabolic fate of ^{14}C-TBZ in pregnant mice was studied by administration of the drug in olive oil and gum arabic as vehicles. Absorption of TBZ in the olive oil group occurred more rapidly than in the gum arabic group, and the maximum blood level in the former group was 7-fold higher than that in the latter group. 5-Hydroxylated TBZ (5-HY TBZ), its glucuronide and its sulfate were identified as urinary and fecal metabolites. A very small quantity of *N*-methyl TBZ was also identified for the first time in the urine and feces. The percentages of TBZ, 5-HY TBZ, and the glucuronide and sulfate of 5-HY TBZ in the urine were 12—15, 22—24, 28—29 and 30—31%, respectively. About 97% of the dose was excreted into the urine (60—62%) and the feces (34—37%) within 7 d in each group. In a limb bud cell culture system for the assay of teratogenic potential, the concentrations of TBZ, 5-HY TBZ and *N*-methyl TBZ necessary to reduce the amounts of cartilage proteoglycan by 50% were estimated to be about 0.09, 0.09 and 0.24 mM, respectively. However, the concentrations of 5-HY TBZ glucuronide and sulfate were over 2 mM. Consequently, teratogenesis caused by TBZ appeared to be induced by the high levels of direct-acting teratogens such as TBZ and 5-HY TBZ when olive oil was used as a vehicle.

Keywords——teratogenicity; teratogenic potential; limb bud cell; thiabendazole; 5-hydroxylated thiabendazole; *N*-methyl thiabendazole; metabolism; pregnant mouse

Thiabendazole (TBZ), a broad-spectrum anthelmintic affecting gastrointestinal parasites of domestic animals, has been shown to be rapidly absorbed, metabolized, and excreted in these species.[1,2] Tocco *et al.* reported[3] that an average of 85% of the dose appeared in the urine (61%) and feces (24%) in 24 h, and most of the drug was excreted by the kidney within 48 h as 5-hydroxylated TBZ (5-HY TBA) or its glucuronide and sulfate in rats.

Ogata *et al.* found[4] that TBZ was teratogenic in mice when olive oil was used as a vehicle, but not when administered in gum arabic. Compared to other teratogens, TBZ's selective toxicity in the embryo, particularly for the developing limbs, and its relative lack of toxicity in the adult were striking.

In relation to the mouse limb malformations caused by TBZ, we tested the teratogenicity of TBZ in a mouse limb bud culture system.[5] TBZ interfered with the development of the explants under our experimental conditions, and it was concluded that TBZ had a direct effect on mouse limb development. Further, we also examined the possible association between TBZ-induced teratogenicity and adenosine triphosphate (ATP) decrease.[6]

In this paper, we deal with the metabolic fate of ^{14}C-TBZ given in different vehicles (olive oil and 0.5% gum arabic solution) to pregnant mice. In addition, the teratogenic potentials of TBZ and its metabolites were compared by the use of an embryonic limb bud cell culture system.

Experimental

Spectrometry——Carbon-13 nuclear magnetic resonance (^{13}C-NMR) and proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL JNM-FX 200 Fourier-transform NMR spectrophotometer at 50 and 200 MHz, respectively, at 27 °C. Samples were prepared in ^{2}H-methanol, unless otherwise specified. ^{13}C- and ^{1}H-chemical shifts were measured relative to Me_4Si.

Chemicals——TBZ and 4-methoxy-*o*-phenylenediamine were obtained from Merck Japan Ltd. and Aldrich Chemical Co., Milwaukee, Wisc., U.S.A., respectively. ^{14}C-Aniline hydrochloride (612 mCi/mmol) was purchased from CEA, Saclay, France. All other chemicals were analytical-grade products available commercially.

Synthesis of Labeled TBZ——Synthesis of ^{14}C-TBZ was carried out by a modification of the method of Grenda *et al.*[7] ^{14}C-Aniline hydrochloride (1 mCi) was diluted with 164 mg of aniline hydrochloride as a carrier and dissolved in 4 ml of water. The aqueous solution was alkalized with 0.35 ml of 5 N NaOH containing 0.1 ml of aniline, and extracted three times with 2.5 ml of dichloromethane. After evaporation of the solvent, 107 mg of diluted labeled aniline was obtained. Aluminum chloride powder (320 mg) was added rapidly to a stirred solution of 265 mg of 4-cyanothiazole[8] and 204 mg of the diluted aniline in 1 ml of *sym*-tetrachloroethane. After addition of 0.1 ml of tetrachloroethane, the mixture was heated for 20 min under reflux. The mixture was cooled to room temperature and after addition of 4.5 ml of 5 N NaOH, extracted with a total of 10 ml of dichloromethane. The extract was dried over anhydrous potassium carbonate overnight and filtered. Dried hydrogen chloride gas was introduced into the filtrate, giving a precipitate, which was recrystallized from a mixture of ethanol and ethyl acetate (1 : 3) to provide 275 mg of the pure amidine. The compound was dissolved in 4 ml of 50% methanol and chlorinated with 0.8 ml of commercially available sodium hypochlorite solution (effective chlorine: 10%). The N-chlorinated amidine was cyclized into ^{14}C-TBZ by refluxing for 20 min in a saturated sodium carbonate solution. In total, 219 mg of ^{14}C-TBZ (0.95 μCi/mg) was obtained. The radiochemical yield was 21%, based on labeled aniline hydrochloride, and the chemical yield was 86% based on the amidine hydrochloride. The radiochemical purity of ^{14}C-TBZ was found to be more than 98% by thin-layer chromatography (TLC). The following *Rf* values were obtained: 0.93 in BuOH–AcOH–H_2O (4 : 1 : 5), 0.61 in hexane–acetone (1 : 9) and 0.18 in acetonitrile–benzene (7 : 13).

Animals——JcL/ICR mice were obtained from Nihon CLEA Co. After quarantine for acclimatization to the laboratory environment, the animals were housed in cages with food (diet CE-2) and water *ad lib.* in an air-conditioned room maintained at 25 ± 1 °C.

Two female mice were mated with a male overnight. When a vaginal plug was observed next morning, that day was termed day 0 of pregnancy.

Administration of TBZ——The labeled TBZ was suitably-diluted with unlabeled TBZ. ^{14}C-TBZ (40 mg) was suspended in 0.3 ml of olive oil or of 0.5% gum arabic solution. A dose of 1300 mg/kg (16—33 μCi/kg) of ^{14}C-TBZ was orally administered to pregnant mice (30—35 g) after fasting overnight on day 11 of pregnancy. This dose was chosen as having the greatest teratogenic potential in mice according to the results of Ogata *et al.*[4]

On the other hand, unlabeled TBZ was repeatedly administered to pregnant mice once a day for 5 d at a dose of 1300 mg/kg/d for isolation of urinary and fecal metabolites.

Collection of Radioactive Samples——Three or five animals given ^{14}C-TBZ were killed by cervical dislocation at various time intervals for 3 d. The organs and tissues (0.1—0.5 g) were dissolved in 1 ml of Soluene 100 (Packard Instrument Co., Inc.). Urine samples were collected in vessels containing 20 ml of distilled water, every 24 h for 7 d. Feces were collected every 24 h for 7 d, dried, weighed and ground. Blood samples were drawn from the tail artery without killing the animals.

Radioactivity Measurement——The radioactivities were measured with an Aloka LSC-751 scintillation spectrometer, model LSC-673, according to the method of Sato *et al.*[9]

Detection, Isolation and Identification of the Urinary and Fecal Metabolites——Paper Chromatography (PC): A radioactive urine sample was chromatographed on a paper 20 cm in length and 2 cm in width (Toyo Roshi, No. 51A) in *n*-butanol–acetic acid–H_2O (4 : 1 : 1). Spots of the metabolites were detected by two methods as follows: 1) an autoradiogram was prepared by placing the paper on Xomat TL film (XTL-5, Kodak) and 2) the paper was cut lengthwise into 2 cm strips, and the radioactivity on each strip was determined.

TLC: Urinary samples were chromatographed on preparative thin-layer plates (Kiesel gel 60F_{254} 200 × 200 × 2 mm, Merck) using the following solvent systems: A) *n*-butanol–acetic acid–H_2O (4 : 1 : 1), B) chloroform–methanol (100 : 1) and C) benzene–dioxane–acetic acid (30 : 5 : 1). Fecal samples were chromatographed on thin-layer plates (Spot film, Tokyo Kasei) using the following solvent systems: D) dichlorobenzene and E) chloroform–methanol (9 : 1).

High-Pressure Liquid Chromatography (HPLC): The non-radioactive urine samples were combined and the combined urine (102 ml) was centrifuged at 3000 rpm for 15 min. The supernatant was, after being adjusted to pH 7.0 with 0.1 N NaOH or 0.1 N HCl solution, chromatographed on an Amberlite XAD-2 resin column (18 g, 20 × 2.5 cm). The column was eluted with 100 ml of water followed by 300 ml of methanol. The methanol eluate was concentrated to dryness. The residue was dissolved in methanol at the concentration of 10 mg/ml, and analyzed on a JASCO high-pressure liquid chromatograph at 298 nm using a μBondapak C_{18} column (30 cm × 3.9 mm i.d.). The column was

eluted with 75% aqueous methanol at a flow rate of 0.3 ml/min.

Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS): The above methanol extract of the urine sample was analyzed on a Shimadzu GC-7A unit with dual flame ionization detectors or FPD using a 2—3 m × 0.3 cm glass column packed with 3% OV-1 on 60 to 80 mesh Chromosorb W. Operating conditions were as follows: column temperature, 220 °C; injector temperature, 240 °C; carrier gas (nitrogen) flow rate, 30 ml/min. The above methanol extract was also analyzed on a JEOL JMS-DX 300 wtih a JMA DA 5000 system. The GC column was a 2% OV-1 column (2 m × 3 mm) or a 100% methyl silicone megabore column (5 m).

Enzymatic Hydrolysis of Sulfate and Glucuronide——Equal volumes of bacterial β-glucuronidase solution (Sigma, Type VII, 130 units/ml in 0.1 M sodium phosphate buffer, pH 7.0) and the urinary sample solution were mixed, filter-sterilized, and incubated at 37 °C for 24 h by the modified procedure of Durston and Ames.[10] The urinary sample solution was adjustd to pH 5.0 with 1 M sodium acetate and 0.2 M acetate buffer (pH 5.0). One drop of toluene was added and then the mixture was incubated with sulfatase (arylsulfatase, Sigma Type IV, 6.8 U/ml) in the presence of 50 mM saccharo-1,4-lactone (Sigma) at 37 °C for 24 h.

Acid Hydrolysis of Conjugates I and II——The conjugates I and II (about 10 mg) isolated by TLC were each heated with 1 drop of conc. HCl and water (0.5 ml) on a steam bath for 90 min. Each reaction mixture was extracted with benzene (5 ml) to give aqueous and benzene layers. The benzene layer was proved to contain B (5-HY TBZ) in both cases. The aqueous layer from conjugate I gave a white precipitate of $BaSO_4$ on addition of $BaCl_2$ solution, and that from conjugate II gave a spot identical with glucuronic acid on a thin-layer plate with solvent system A (Rf= 0.21) and with ethanol (Rf=0.15).

Calculations——The half-life of the blood radioactivity data was calculated by least-squares regression analysis using the method of residuals.[11] The area under the blood concentration–time curve from zero to 4 d and the mean residence time of urinary or fecal radioactivity were calculated from the experimental data by means of the trapezoidal rule, using the program of Yamaoka and Tanigawara.[11]

Synthesis of 5-Methoxy-2-(4-thiazolyl)benzimidazole (5-Methoxy TBZ)——A mixture of 876 mg of 4-methoxy-*o*-phenylenediamine and 567 mg of 4-cyanothiazole in a sealed glass tube was heated for 2 h at 200 °C in an oil bath. After 90 mg of sublimed 4-cyanothiazole had been recovered, the residue was dissolved in conc. HCl, followed by addition of a further 15 ml of conc. HCl, and the solution was filtered. The filtrate was alkalized with conc. ammonium hydroxide to afford a precipitate, which was dissolved in hot methanol. The solution was filtered. Concentration of the methanol-soluble fraction gave 256 mg of a colored compound. The compound was dissolved in methanol (8 ml) and passed through an alumina column (aluminum oxide, grade 1, Merck, 14 g: 1.4 × 9.0 cm). The column was eluted with methanol (12 ml). The methanol eluates were combined, concentrated *in vacuo* and subjected to preparative TLC (silica gel, 200 × 200 × 2.5 mm) in benzene–methyl acetate (4 : 1). The band below Rf=0.1 was repeatedly developed in the same solvent system and eluted with methanol. The methanol eluate was concentrated to give the desired 5-methoxy TBZ (20 mg). The ^{1}H-NMR spectrum showed signals at 3.80 (3H, MeO), 6.88 (1H, dd, J=8.79, 1.7 Hz), 7.09 (1H, br s), 7.49 (1H, d, J=8.79 Hz), 8.21 (1H, s), 9.04 (1H, s) ppm; ^{13}C-NMR at 158.3 (5-C), 155.7 (2′-C), 148.0 (2-C and 4′-C), 139.6 (8-C), 135.1 (9-C), 119.1 (5-C), 117.2 (4′-C), 113.9 (7-C), 98.3 (6-C), 56.2 (5-MeO) ppm.

Synthesis of *N*-Methyl TBZ——TBZ (10 mg) was methylated with diazomethane in ether (10 ml). The product was identified as *N*-methyl TBZ by GC-MS.[12]

Assay of Cartilage Proteoglycan in a Mouse Limb Bud Cell System——The assay procedure was performed according to the method of Hassell and Horigan.[13] The limb buds were isolated from pregnant mice and dissected from the day 10 mouse embryos. The limb buds were dissociated in a solution of 0.1% trypsin and 0.1% ethylenediaminetetraacetic acid (EDTA) in calcium-magnesium-free saline to obtain a single cell suspension. Aliquots of 20 μl of this cell suspension were dropped in the center of the wells of 24-wells Costar dishes (5 × 10^5 cells/well). The dishes were placed in an humidified CO_2 incubator at 37 °C for 2 h to allow the cells to attach and then CMRL medium containing 10% fetal bovine serum was added (0.5 ml) to each well. Compounds to be tested were dissolved in the medium. Medium containing various concentrations of the compounds was changed on days 1, 3 and 5. The accumulation of sulfated proteoglycans, a measure of the extent of cartilage differentiation, was determined by staining with 0.5% alcian blue on day 6. The bound dye was extracted from the cultures with 0.3 ml of 4 M guanidine HCl, and the absorbance of the extracted solution was measured with a spectrophotometer.

Results

Recovery of Administered Radioactivity

Daily excretion was compared between the two groups (Fig. 1). Radioactivity in the 24-h urine in the olive oil group was two-fold higher than in the gum arabic group (p<0.05). However, the urinary radioactivity in the gum arabic group was almost constant for 3 d, then gradually decreased (Fig. 1). Fecal radioactivity in the olive oil group was 10% of the dose in

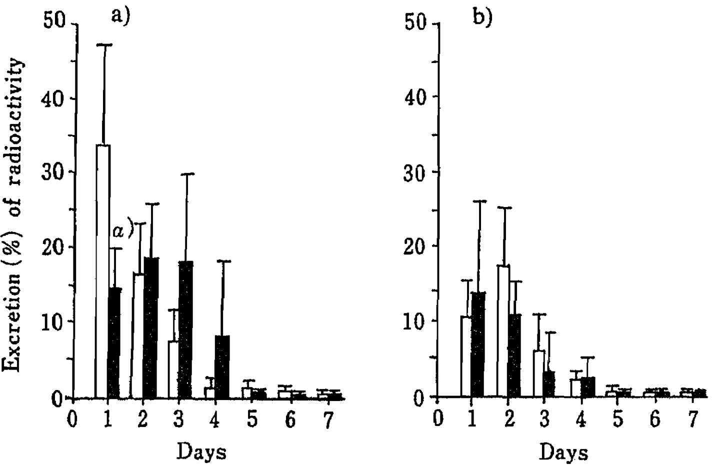

Fig. 1. Daily Excretion (%) of Radioactivity in the Urine (a) or Feces (b) after an Oral Administration of ^{14}C-TBZ to Pregnant Mice

Bars indicate S.D. Olive oil group, □; gum arabic group, ■. Significant difference between olive oil group and gum arabic group: *a*) $p<0.05$.

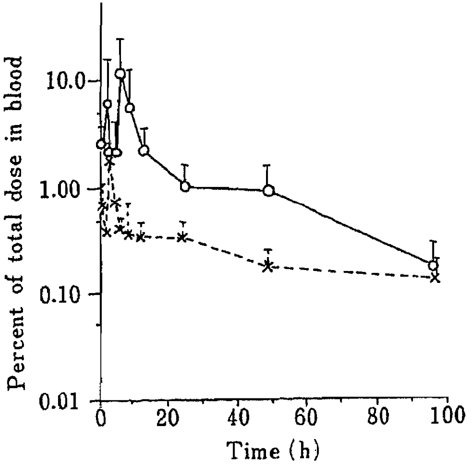

Fig. 2. Percentage of Total TBZ Dose in Blood *vs.* Time

^{14}C-TBZ suspended in olive oil (○) or gum arabic solution (×) was administered to groups of 3 mice. Blood samples were drawn from the upper tail artery of the mice. Radioactivities were measured as described in Experimental. Bars indicate S.D.

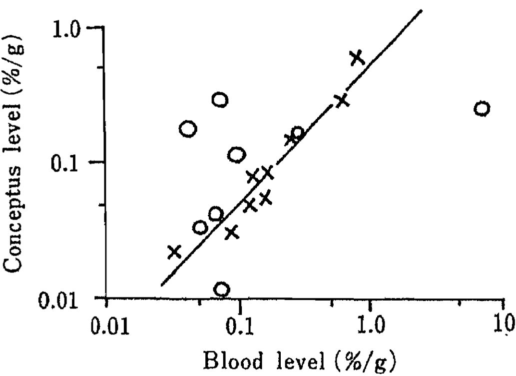

Fig. 3. Relationship of Blood Level to Conceptus Level

The samples were obtained at 6, 12, 24 h after the oral administration of ^{14}C-TBZ suspended in olive oil (○) or gum arabic solution (×).

the first day, increased to 17—18% in the second day, and then decreased (Fig. 1). In the gum arabic group, the fecal label gradually decreased with time (Fig. 1).

Recovered radioactivity in the olive oil group was higher up to day 2, but lower between day 3 and day 4 than in the gum arabic group. Pregnant mice of both groups excreted cumulatively *ca.* 97% of the dose in their urine (60—62%) and feces (34—37%) within 7 d.

Mean residence times (MRT)[11] of the urinary radioactivity were smaller in the olive oil group (29.84±5.88 h) than in the gum arabic group (44.06±12.31 h) ($p<0.1$), while the MRT of the fecal label was similar between the olive oil group (36.95±8.06 h) and the gum arabic group (36.84±11.36 h).

Blood Levels

Figure 2 shows the time courses of blood levels. Total dose (%) in the blood of three mice

was compared between the two groups. Absorption in the olive oil group occurred more rapidly than that in the gum arabic group. Two peaks of the blood levels were observed in the olive oil group. The maximum level in the olive oil group was reached at 6 h after dosing of TBZ, and was 7-fold higher than that in the gum arabic group, which reached the maximum at 3 h. Further, blood levels in the olive oil group remained higher than those in the gum arabic group for 96 h.

In the period from 6 to 96 h in the olive oil group, two phases in the clearance of ^{14}C-TBZ were clearly discernible, with half-lives[14] of 1.10 h (first) and 23.9 h (second). The blood levels followed the equation: $Y=26.6\exp(-0.631t)+2.27\exp(-0.029t)$ (Y = total radioactivity in blood, t = hour). In the case of the gum arabic group, the half-lives were 0.65 h (first) and 63 h (second) and the blood levels followed the equation $Y=17.5\exp(-1.059t)+0.34\exp(-0.011t)$. The areas under the blood curves of total radioactivity[15] were higher in the olive oil group (52.27±35.66% h/g) than in the gum arabic group (9.39±2.39% h/g).

Tissue Distribution

Table I shows that the radioactivity (%/g) in organs and tissues in the olive oil group was higher at 6 h than in the gum arabic group, except for the intestine. However, at 12 h, the radioactivity in the gum arabic group was higher than in the olive oil group.

As for the conceptus level, the concentration in the olive oil group was around 0.1 (%/g) or more for the first 24 h, but in the gum arabic group, the maximum conceptus level was higher. In Fig. 2, blood levels are shown successively with the same mice. Accordingly, individual differences are much more significant in the data of Table I than in Fig. 2.

Figure 3 shows the relationship of blood level to the conceptus level. In the gum arabic group, the greater the blood level, the more the conceptus level was increased. On the other hand, there was no correlation between the blood and conceptus levels in the olive oil group.

Table II shows the relationship of the blood levels to the organ and tissue levels ($Y=AX^B$; X = blood level, Y = organ and tissue level), within 24 h after the oral administration of ^{14}C-TBZ. In the gum arabic group, the levels of the organs and tissues examined were closely correlated with the blood levels (adipose, $p<0.05$; the others, $p<0.01$), but the correlation was poor in the olive oil group (Table II). It was clarified from the values of A and B in the equation that the radioactivity in the kidney was inclined to be highest among the organs and

TABLE I. Radioactivity Levels (%/g) in Organs and Tissues after a Single Oral Dose of ^{14}C-Thiabendazole (TBZ) to Pregnant Mice

	Gum arabic group				Olive oil group			
(h)	6	12	24	72	6	12	24	72
Brain	0.07	0.29	0.04	0.01[b]	0.14	0.18	0.02	0.02[b]
Heart	0.10	0.45	0.08	0.03	0.34	0.13	0.04	0.03
Lung.	0.12	0.79	0.10	0.04	0.39	0.19	0.07	0.17
Liver	0.24[b]	1.45	0.22	0.07	0.72[b]	0.27	0.14	0.06
Kidney	0.21[a]	3.57	0.34	0.04	0.74[a]	0.33	0.13	0.07
Spleen	0.10	0.74	0.08	0.02	0.68	0.19	0.13	0.34
Stomach	1.84	7.00	1.94	0.03	7.23	3.88	1.26	0.04
Intestine	3.71	17.3[a]	1.13	0.06	1.91	2.03[a]	0.27	0.02
Conceptus	0.06	0.32	0.04	0.01	0.17	0.09	0.17	0.01
Muscle	0.08	0.97	0.05	0.04	0.29	0.11	0.03	0.04
Blood	0.11	0.55	0.12	0.05	3.33	0.05	1.73	0.07
Adipose	0.16	0.67[a]	0.07	0.04	2.16	0.25[a]	0.05	0.07

The data show the mean of 3 mice for 6, 12 and 24 h, but 4 mice for 72 h. Significance of the difference between the olive oil group and the gum arabic group: *a*) $p<0.01$, *b*) $p<0.02$.

TABLE II. Correlation of Blood Level to Organ and Tissue Levels
$Y=AX^B$ (X, blood level; Y, organ and tissue levels)

Vehicle	Gum arabic			Olive oil		
	A	*B*	*r*	*A*	*B*	*r*
Brain	0.472	0.985	0.944[a]	0.291	0.552	0.471
Heart	0.740	0.967	0.977[a]	0.662	0.619	0.456
Lung	1.428	1.153	0.965[a]	1.443	0.775	0.710
Liver	2.807	1.153	0.992[a]	2.129	0.776	0.603
Kidney	4.614	1.329	0.931[a]	1.700	0.627	0.553
Spleen	1.266	1.204	0.964[a]	0.414	0.384	0.281
Muscle	1.085	1.154	0.826[a]	0.427	0.547	0.356
Adipose	0.726	0.759	0.703[b]	2.115	0.868	0.443
Conceptus	0.525	1.056	0.970[a]	0.212	0.393	0.217

a) $p<0.01$, *b*) $p<0.05$.

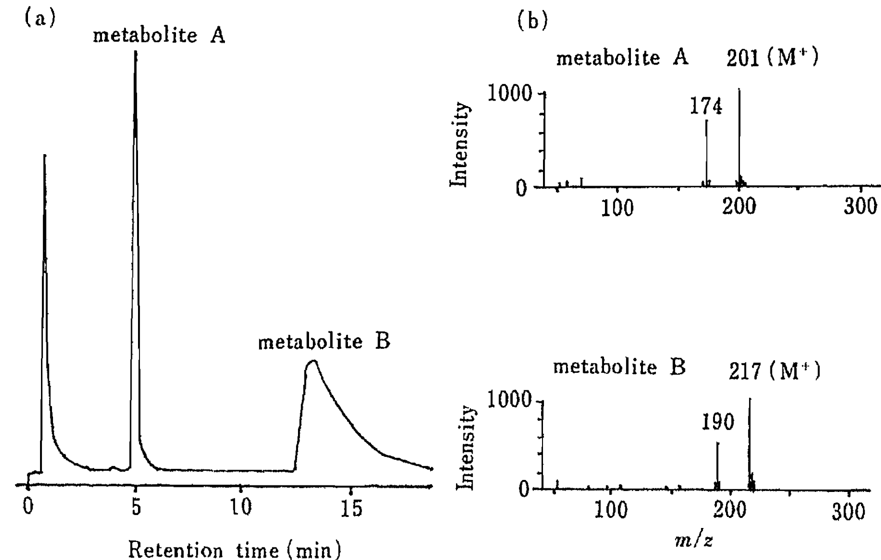

Fig. 4. Gas Chromatogram (a) and Mass Spectra (b) of Urinary Metabolites of TBZ

Urinary samples were obtained from the mice given TBZ suspended in olive oil. The preparation and analysis of the samples were carried out according to the method described in Experimental.

tissues in the gum arabic group (Table II). When olive oil was used as a vehicle, tissue radioactivities were inclined to persist independently of the blood level. The tissue radioactivities were dependent on the blood level when gum arabic solution was used.

Identification of the Metabolites

TLC of urine samples prepared from pregnant mice on day 6 to day 10 gave four metabolites, A, B and a mixture of conjugates, in solvent system A. Metabolite A in Fig. 4a was identified as TBZ by GC-MS (Fig. 4b), and metabolite B in Fig. 4a was identified as 5-HY TBZ (Fig. 4b), because the mass spectrum of the methylated metabolite B was identical with that of authentic 5-methoxy TBZ. The mass spectrum of 5-methoxy TBZ showed the molecular ion at *m/z* 231 (base peak), and fragment ions appeared at *m/z* 216 $(M-CH_3)^+$ and at *m/z* 188.

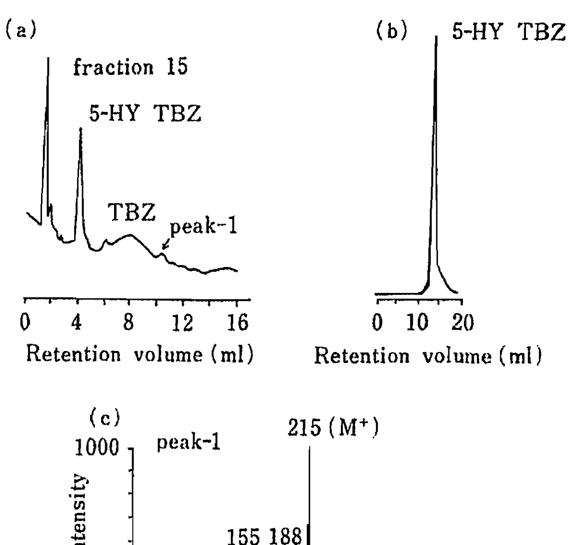

Fig. 5. High-Performance Liquid Chromatogram of Urinary Sample (a) and Isolated 5-HY TBZ (b) and Mass Spectrum of *N*-Methyl TBZ (c)

Urinary samples were obtained from mice given TBZ suspended in olive oil. The preparation and analysis were described in Experimental.

TABLE III. Effects of Thiabendazole (TBZ) and Its Metabolites on the Amounts of the Cartilage Proteoglycan in the Limb Bud Cell Culture System

Compound	TP_{50}[a] (μg/ml)	TP_{50} (mM)
TBZ	18	0.090
5-HY TBZ	19	0.088
5-HY TBZ glucuronide	1000	2.54
5-HY TBZ sulfate	1000	3.37
N-Methyl TBZ	52	0.242

The assay of cartilage proteoglycan in the cell system was carried out as described in Experimental. TP_{50}'s of cytosine arabinoside and caffeine were 0.0045 and 3.76 mM, respectively. *a*) TP_{50} is the dose of a compound needed to reduce alcian blue staining by 50%.

The mixture of the conjugates was rechromatographed on a thin-layer plate using the same solvent system to afford two metabolites, conjugates I and II. Conjugate I showed a white color with metaperiodate–benzidine reagent[16] on a thin-layer plate but the other did not. The *Rf* values of metabolites A and B, and conjugates I and II were 0.50, 0.40, 0.25 and 0.06, respectively. Conjugates I and II were hydrolyzed to give metabolite B with sulfatase and β-glucuronidase, respectively.

The structure of metabolite B was confirmed to be 5-HY TBZ by its ^{13}C-NMR spectrum, which showed signals at 155.8 (2′-1), 155.5 (5-C), 148.2 (2-C or 5′-C), 148.0 (2-C or 5′-C), 139.8 (8-C), 135.1 (9-C), 119.1 (4′-C), 117.1 (4-C), 114.1 (7-C), and 100.4 (6-C) ppm.

The urinary samples were fractionated by HPLC. Fraction-15 was analyzed by HPLC and GC-MS (Fig. 5a and c). Peak-1 was identified as *N*-methyl TBZ by GC-MS in comparison with an authentic sample. The identities of TBZ and 5-HY TBZ were also confirmed by comparison with authentic samples, and the latter was isolated from the urinary samples by HPLC on a semi-preparative μBondapak C_{18} column (Fig. 5b), and used in the limb bud cell assay.

Autoradiography of thin-layer chromatograms of the fecal metabolites showed the existence of TBZ, 5-HY TBZ, *N*-methyl TBZ and more polar metabolites than 5-HY TBZ. The *Rf* values of TBZ, 5-HY TBZ and *N*-methyl TBZ were 0.52, 0.45 and 0.80, respectively, in solvent system E.

Paper chromatography of the 24-h urine indicated that nearly all of the radioactivity was present in metabolites of TBZ. The percentages of TBZ, 5-HY TBZ, and the glucuronide and sulfate of 5-HY TBZ with respect to the total radioactivities on the paper were 12—15, 22—24, 28—29 and 30—31%, respectively, in 24-h urine of each group. The *Rf* values of TBZ, 5-HY TBZ, 5-HY TBZ sulfate and 5-HY TBZ glucuronide were 0.79, 0.60, 0.43 and 0.23, respectively, in solvent system A.

Assay of Effect of TBZ Metabolites on the Amount of Cartilage Proteoglycan in a Mouse Limb Bud Cell Culture

Teratogenic potentials of the metabolites were estimated and compared as shown in Table III. The data showed that the TP_{50}'s of TBZ, 5-HY TBZ and *N*-methyl TBZ are about 90, 88 and 242 μM, respectively. However, the conjugated metabolites such as 5-HY TBZ glucuronide and 5-HY TBZ sulfate were very weakly inhibitory on chondrogenesis. The TP_{50}'s of these conjugates were over 2 mM (Table III).

Discussion

Our studies showed that, in mouse, 97% of the dose was recovered in the urine (60%) and feces (37%) within one week after the oral administration of labeled TBZ in olive oil. It was clear that the excretion rates in 24-h urine after the dose of ^{14}C-TBZ were higher in the olive oil group than in the gum arabic group (Fig. 1), suggesting that the absorption of ^{14}C-TBZ at early times was higher in the olive oil group than in the gum arabic group.

The biphasic absorption was clearly observed in the olive oil group, judging from the time course of blood levels. A biphasic decay was also observed in the two groups. It was apparent that the half-life of the total radioactivity of the first component was longer in the olive oil group than in the gum arabic group (Fig. 2). Further, the values of the area under the blood curves of total radioactivities varied with the two vehicles, and the ratio of the value of the olive oil group to that of the gum arabic group was *ca.* 5.6 (Fig. 2).

Our results showed that radioactivity in the olive oil group tended to be retained in the conceptus, although the blood levels were decreased (Fig. 3). There was a high correlation between blood level and tissue/organ level in the gum arabic group but not in the olive oil group (Table II). However, the reason for this is unclear at present.

From the parameter values in Table II, the radioactivity of ^{14}C-TBZ in the gum arabic group tended to be distributed in the kidney, liver, lung, muscle in decreasing order. It was found that the distribution was lower in the conceptus and brain than in other tissues/organs. Toda *et al.*[17] also pointed out that the total concentration of TBZ and its metabolites was higher in the kidney than in the liver, but the concentration of TBZ itself was higher in the liver than in the kidney.

The main metabolic pathways of TBZ in mice were similar to those in rats[3] as indicated in Chart 1. However, a very small quantity (below 0.1% of the dose) of *N*-methyl TBZ was detected for the first time in urine and feces.

N-methyl TBZ

TBZ

5-HY TBZ

5-HY TBZ glucuronide

5-HY TBZ sulfate

Chart 1. Metabolic Pathways of TBZ in Mice

The ratios of the free forms such as TBZ and 5-HY TBZ were increased in the mouse urine compared with those in the rat urine. The increase in the unconjugated forms might be due to the difference of the doses between the mice (1300 mg/kg) and rats (25 mg/kg).[3]

The present paper shows that 5-HY TBZ also has an inhibitory action on chondrogenesis, but the conjugates have no inhibitory action below 1 mM. *N*-Methyl TBZ showed a lower inhibition than TBZ and 5-HY TBZ. Pratt and Willis pointed out that the high concentration (>1 mM) in their assay system appeared to have little relevance to animal or human teratogenesis, and presumably represented non-specific effects on the cell, due to the altered osmolarity.[18]

Yoneyama *et al.* reported[19] that the radioactivity in the fetus and placenta was 2—3 fold higher in mice treated with ^{14}C-TBZ suspended in olive oil than in those given TBZ suspended in gum arabic solution.

We consider that teratogenesis was induced by administration of TBZ suspended in olive oil owing to the high concentrations of direct-acting teratogens, such as TBZ and 5-HY TBZ, but not by TBZ suspended in gum arabic solution because of the low concentrations of these free compounds. Studies on the mechanisms of the inhibition of proteoglycan synthesis by TBZ *in vivo* are in progress.

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Chemical Conversion of L-α,ω-Diamino Acids to L-ω-Carbamoyl-α-amino Acids by Ruthenium Tetroxide Oxidation[1]

SHIGEYUKI YOSHIFUJI,* KEN-ICHI TANAKA and YOSHIHIRO NITTA

School of Pharmacy, Hokuriku University, Kanagawa-machi, Kanazawa 920-11, Japan

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Ruthenium tetroxide oxidation of *N,C*-protected derivatives of L-2,4-diaminobutyric acid, L-ornithine and L-lysine was carried out under two-phase conditions and the corresponding imides, formed through the oxidation of the methylene adjacent to the ω-amino group, were obtained in good yields. Removal of the protecting groups from the products gave L-asparagine, L-glutamine and L-2-aminoadipic acid 6-amide, respectively. Thus, the first chemical conversion of L-α,ω-diamino acids into the corresponding L-ω-carbamoyl-α-amino acids without racemization has been established. L-2-Aminoadipic acid 6-amide was further converted to L-2-aminoadipic acid by acid hydrolysis. This represents a convenient method for the synthesis of L-2-aminoadipic acid starting from L-lysine.

Keywords——oxidation; ruthenium tetroxide oxidation; L-α-amino acid synthesis; *N*-protecting group; ruthenium tetroxide; L-α,ω-diamino acid; L-ω-carbamoyl-α-amino acid; L-2-aminoadipic acid

Although a wide range of methods is available for the synthesis of alkylamides, there is no general method for the direct oxidation of primary and secondary alkylamines to the corresponding amides, *i.e.*, constructing a carbonyl function at the methylene next to the amine nitrogen. With existing methods, these amines undergo more or less oxidative degradation.[2] One possible approach may be by oxidation of *N*-protected amines derived from primary and secondary alkylamines, followed by deprotection of the resulting *N*-protected amides. A suitable oxidant for this purpose might be ruthenium tetroxide (RuO_4), a multi-purpose oxidant which oxidizes various types of organic compounds.[3] In the oxidation of nitrogen-containing compounds, application of RuO_4 has been limited to the oxidative transformation of some cyclic amines into the corresponding lactams or cyclic imides.[4] On the other hand, this oxidant had been claimed not to be applicable to straight-chain alkylamines.[5] However, we recently developed a new synthetic route to imides from alkylamines,[6] involving an effective RuO_4 oxidation of *N*-acyl derivatives of alkylamines. We thought that it might be possible to extend this methodology to the synthesis of amides by protection of the amino function with urethane-type protecting groups which would be easily removable after the oxidation. With this idea in mind, we have examined RuO_4 oxidation of L-α-amino acids possessing side chain alkylamines which should be oxidized to the corresponding amides, and have succeeded in the first chemical conversion of L-2,4-diaminobutyric acid (**1**), L-ornithine (**2**), and L-lysine (**3**) to L-asparagine (**10**), L-glutamine (**11**), and L-2-aminoadipic acid 6-amide (**12**), respectively, as shown in Chart 1. In this paper, we wish to report the details of this conversion.[1]

As urethane type protecting groups, *tert*-butoxycarbonyl (Boc) and trichloroethoxycarbonyl (Troc) groups were chosen in this work. These groups are widely used for protection of amines[7] and were confirmed by us to be stable to RuO_4.[8] The starting materials for the RuO_4 oxidation, protected L-α,ω-diamino acid esters (**4**, **5**, **6**), were prepared from L-amino acids (**1**,

NH_2–CH_2–$(CH_2)_n$–$H_2NCHCOOH$ (1, 2, 3) ⟶ R^1–NH–CH_2–$(CH_2)_n$–R^1–$HNCHCOOR^2$ (4, 5, 6) —RuO_4⟶ R^1–NH–C=O–$(CH_2)_n$–R^1–$HNCHCOOR^2$ (7, 8, 9) ⟶ NH_2–C=O–$(CH_2)_n$–$H_2NCHCOOH$ (10, 11, 12)

1 ($n=1$): L-2,4-diaminobutyric acid
2 ($n=2$): L-ornithine
3 ($n=3$): L-lysine
R^1, R^2 = protecting groups (see Table I)

10 ($n=1$): L-asparagine
11 ($n=2$): L-glutamine
12 ($n=3$): L-2-aminoadipic acid 6-amide

Chart 1

2, 3) by *N*-acylation with *tert*-butyl *S*-4,6-dimethylpyrimid-2-ylthiocarbonate (Boc-S reagent)[9] or trichloroethyl chloroformate, followed by esterification with diazomethane or with *tert*-butyl alcohol or 4-nitrobenzyl alcohol in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine.[10]

Although two oxidation sites are available in the molecules of these substrates (**4**—**6**) having two amino groups, only the methylene carbon adjacent to the ω-amino group is expected to be susceptible to RuO_4, based on the common feature of the RuO_4 oxidation of *N*-acylated cyclic amines[4] and cyclic ethers (to the corresponding lactones)[11] that RuO_4 preferentially oxidizes a secondary carbon rather than a tertiary one. The RuO_4 oxidation of these *N*,*C*-protected amino acids was carried out at room temperature according to our standard procedure[8] using a catalytic amount of ruthenium dioxide (RuO_2) hydrate and an excess of 10% aqueous sodium metaperiodate ($NaIO_4$) in a two-phase system of ethyl acetate (AcOEt)–water. Progress of the reaction was monitored by observing the disappearance of the starting materials by thin layer chromatography (TLC). After the reaction had gone to completion, the oxidation products were isolated from the organic phase and purified by silica gel column chromatography to afford the desired imides (**7**—**9**), which were characterized on the basis of their analytical and spectral properties. Carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were most effective for detection of the imide carbonyl functions produced by the oxidation; the signals appeared in the narrow range of 173—174 ppm downfield from tetramethylsilane (TMS). As expected, the RuO_4 oxidation occurred regioselectively at the terminal carbon without exception, and the α-carbon remained intact. The results are summarized in Table I. It may be seen that the *tert*-butoxy group in both the urethane and ester functions shortened the reaction time.

The target compounds (**7**—**9**) obtained by the above oxidation are novel L-ω-carbamoyl-α-amino acid derivatives having urethane-type protecting groups on the amide nitrogen. These protecting groups are generally difficult to introduce directly into the amide function of such L-amino acids as L-asparagine or L-glutamine.[12] These products, therefore, should be useful for peptide synthesis by the DCC or mixed anhydride method without nitrile formation by dehydration of the unprotected amide group.[13]

In the above oxidation of the *N*-Boc series (**5a**—**c**, **6a**—**c**) of L-ornithine and L-lysine, the concomitant formation of small amounts of the cyclization products (**13a**—**c**, **14a**—**c**, Table I) was observed. Their structures were determined by spectroscopic analyses to be the pyrrolidine (**13a**—**c**) and piperidine (**14a**—**c**) derivatives, respectively, as shown in Chart 2. Formation of these compounds, which are presumably diastereomeric mixtures, can be well explained by an intramolecular cyclization[14] of *N*-acyliminium ions (**15**) generated from the starting materials at the first stage of the oxidation.

Next, representative *N*,*C*-protected L-ω-carbamoyl-α-amino acids (**7a**, **8a**, **9a**) were converted to free amino acids. The Boc group attached to the carbamoyl group could be

TABLE I. Oxidation of *N*,*C*-Protected Diamino Acids with RuO_4

Substrate	*n*	R^1	R^2	Reaction time (h)	Product yield (%)	
4a	1	Boc	NBzl	10	**7a**: 84	
4b			Me	10	**7b**: 79	
5a	2	Boc	*tert*-Bu	6	**8a**: 54	**13a**: 12
5b			NBzl	24	**8b**: 73	**13b**: 8
5c			Me	5	**8c**: 72	**13c**: 10
5d		Troc	NBzl	96	**8d**: 72	
5e			Me	120	**8e**: 72	
6a	3	Boc	*tert*-Bu	6	**9a**: 85	**14a**: 8
6b			NBzl	8	**9b**: 84	**14b**: 10
6c			Me	5	**9c**: 79	**14c**: 6
6d		Troc	*tert*-Bu	35	**9d**: 83	
6e			NBzl	50	**9e**: 79	
6f			Me	120	**9f**: 81	

Boc = *tert*-butoxycarbonyl; Troc = trichloroethoxycarbonyl; NBzl = *p*-nitrobenzyl.

R^1–$HNCH_2(CH_2)_nCH(NH$–$R^1)COOR^2$ —RuO_4→ [R^1–$H\overset{+}{N}$=$CH(CH_2)_nCH(NH$–$R^1)COOR^2$] —RuO_4→ R^1–$HNCO(CH_2)_nCH(NH$–$R^1)COOR^2$

5a–c (*n*=2)
6a–c (*n*=3)

8a–c (*n*=2)
9a–c (*n*=3)

15 ↓ cyclization

R^1–HN–(ring: $(CH_2)_{n-1}$, N–R^1)–$COOR^2$

13a–c (*n*=2)
14a–c (*n*=3)

R^1, R^2 = protecting groups (see Table I)

Chart 2

removed under mild conditions by the use of the common procedure for removal of the group from *N*-Boc amines.[7] Thus, **7a** was hydrogenated in ethanol (Pd–C/H_2, room temperature) to give N^{α},$N^{c\omega}$-diBoc-L-asparagine, which was treated with trifluoroacetic acid (TFA) at room temperature for 10 min to furnish L-asparagine (**10**) in 73% yield from **7a**. On treatment with TFA (room temperature, 10 min), **8a** gave directly L-glutamine (**11**) in 79% yield. In the same manner, L-2-aminoadipic acid 6-amide (**12**) was obtained from **9a** in 82% yield. Structures and optical rotations of **10** and **11** were in good agreement with those of authentic L-asparagine and L-glutamine, respectively. L-2-Aminoadipic acid 6-amide (**12**) was further hydrolyzed in refluxing 6 N hydrochloric acid to afford in 83% yield an amino dicarboxylic acid, which was proved to be identical with authentic L-2-aminoadipic acid. Thus, the first chemical conversion of L-α,ω-diamino acids to L-ω-carbamoyl-α-amino acids without racemization has been accomplished. We have also concomitantly developed an excellent method for high-yielding synthesis of L-2-aminoadipic acid, which has previously been prepared in low overall yields by multi-step conversions from L-aspartic acid[15] and L-lysine.[16]

The present conversion provides an efficient and general synthetic route to optically active L-α-amino acids bearing a protected (7—9 type) or unprotected carbamoyl group from the corresponding L-α,ω-diamino acids. Introduction of an urethane-type protecting group at an amide function should be especially useful in organic synthesis of nitrogen-containing compounds, including amino acids and peptides.[17] Further investigation is in progress to develop a new general synthesis of amides from alkylamines by RuO_4 oxidation.

Experimental

Melting points were taken on a Yanagimoto melting point apparatus. All melting points are uncorrected. Infrared (IR) spectra were recorded in $CHCl_3$ solution on a JASCO IRA-2 or a Hitachi 270-30 spectrometer. Mass spectra (MS) were measured on a JEOL JMS D-300 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra in $CDCl_3$ were obtained at 23 °C using TMS as an internal standard on a JEOL JNM-MH-100 spectrometer. ^{13}C-NMR spectra were measured in $CDCl_3$ on a JEOL JNM-FX-100 spectrometer. Optical rotations were measured in $CHCl_3$ (c = 1.0) on a JASCO DIP-4 spectrometer.

The organic solutions obtained after extraction were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Column chromatographic purification or separation of the products was done on Merck Silica gel 60 (70—230 mesh) using AcOEt–hexane as the eluent.

Starting Materials for the RuO_4 Oxidation——Protected L-α,ω-diamino acids (4—6) were prepared from commercial L-amino acids[18] by acylation with Troc-Cl under basic conditions (40% aqueous EtOH, K_2CO_3, 0—10 °C) or with Boc-S reagent,[9] followed by esterification with diazomethane (MeOH–ether) or with *tert*-BuOH or *p*-nitrobenzyl alcohol in the presence of DCC and 4-dimethylaminopyridine.[10] The crude materials were purified by column chromatography, and then recrystallized in the case of the solid substrates. These compounds were characterized as follows.

$N^α$,$N^γ$-DiBoc-L-2,4-diaminobutyric Acid *p*-Nitrobenzyl Ester (4a)——Recrystallized from isopropyl ether as colorless needles, mp 98—100 °C. $[\alpha]_D^{26}$ −8.0°. MS *m/z*: 453 (M^+). IR ν_{max} cm^{-1}: 3440 (NH), 1742, 1712 (C=O). ^{1}H-NMR δ: 1.44 (18H, s, $(CH_3)_3$ × 2), 1.64—2.28 (2H, m, C_3-H_2), 2.92—3.52 (2H, m, C_4-H_2), 4.28—4.56 (1H, m, C_2-H), 5.04 (1H, br s, C_4-NH), 5.26 (2H, s, $C\underline{H}_2$Ar), 5.28 (1H, br d, J = 10 Hz, C_2-NH), 7.70, 8.21 (2H × 2, each d, J = 8 Hz, aromatic protons). *Anal.* Calcd for $C_{21}H_{31}N_3O_8$: C, 55.62; H, 6.89; N, 9.27. Found: C, 55.79; H, 6.94; N, 9.35.

$N^α$,$N^γ$-DiBoc-L-2,4-diaminobutyric Acid Methyl Ester (4b)——A colorless oil. $[\alpha]_D^{26}$ −8.8°. MS *m/z*: 332 (M^+). IR ν_{max} cm^{-1}: 3440 (NH), 1740, 1710 (C=O). ^{1}H-NMR δ: 1.32—2.24 (2H, m, C_3-H_2), 1.44 (18H, s, $(CH_3)_3$ × 2), 2.84—3.52 (2H, m, C_4-H), 3.74 (3H, s, CO_2CH_3), 4.16—4.28 (1H, m, C_2-H), 5.09 (1H, br s, C_4-NH), 5.27 (1H, br d, J = 10 Hz, C_2-NH). *Anal.* Calcd for $C_{15}H_{28}N_2O_6$: C, 54.20; H, 8.49; N, 8.43. Found: C, 54.01; H, 8.32; N, 8.59.

Boc–Orn(Boc)–O*tert*-Bu (5a)——A colorless oil. $[\alpha]_D^{25}$ +3.7°. MS *m/z*: 388 (M^+). IR ν_{max} cm^{-1}: 3440 (NH), 1710 (C=O). ^{1}H-NMR δ: 1.08—2.11 (4H, m, C_3-H_2, C_4-H_2), 1.46, 1.55 (27H, each s, $(CH_3)_3$ × 3), 3.35—3.69 (2H, m, C_5-H_2), 4.04—4.42 (1H, m, C_2-H), 4.65 (1H, br s, C_5-NH), 5.54 (1H, br d, J = 10 Hz, C_2-NH). *Anal.* Calcd for $C_{19}H_{36}N_2O_6$: C, 58.74; H, 9.34; N, 7.21. Found: C, 58.90; H, 9.41; N, 7.10.

Boc–Orn(Boc)–ONBzl (5b)——Recrystallized from AcOEt–petroleum ether as colorless needles, mp 129—130 °C. $[\alpha]_D^{25}$ +2.5°. MS *m/z*: 467 (M^+). IR ν_{max} cm^{-1}: 3450 (NH), 1742, 1710 (C=O). ^{1}H-NMR δ: 1.11—2.07 (4H, m, C_3-H_2, C_4-H_2), 1.42 (18H, s, $(CH_3)_3$ × 2), 2.97—3.25 (2H, m, C_5-H_2), 4.21—4.49 (1H, m, C_2-H), 4.71 (1H, br s, C_5-NH), 5.18 (1H, br d, J = 10 Hz, C_2-NH), 5.28 (2H, s, $C\underline{H}_2$Ar), 7.52, 8.24 (2H × 2, each d, J = 8 Hz, aromatic protons). *Anal.* Calcd for $C_{22}H_{33}N_3O_8$: C, 56.52; H, 7.11; N, 8.99. Found: C, 56.45; H, 7.01; N, 8.92.

Boc–Orn(Boc)–OMe (5c)——A colorless oil. $[\alpha]_D^{25}$ +13.2°. MS *m/z*: 346 (M^+). IR ν_{max} cm^{-1}: 3450 (NH), 1740, 1710 (C=O). ^{1}H-NMR δ: 1.29—1.89 (4H, m, C_3-H_2, C_4-H_2), 1.44 (18H, s, $(CH_3)_3$ × 2), 3.00—3.27 (2H, m, C_5-H_2), 3.75 (3H, s, CO_2CH_3), 4.17—4.41 (1H, m, C_2-H), 4.71 (1H, brs, C_5-NH), 5.16 (1H, br d, J = 10 Hz, C_2-NH). *Anal.* Calcd for $C_{16}H_{30}N_2O_6$: C, 55.47; H, 8.73; N, 8.09. Found: C, 55.40; H, 8.65; N, 8.11.

Troc–Orn(Troc)–ONBzl (5d)——A colorless oil. $[\alpha]_D^{25}$ +1.2°. MS *m/z*: 618, 620, 622 (M^+). IR ν_{max} cm^{-1}: 3450 (NH), 1740 (C=O). ^{1}H-NMR δ: 1.49—2.09 (4H, m, C_3-H_2, C_4-H_2), 3.16—3.44 (2H, m, C_5-H_2), 4.33—4.64 (1H, m, C_2-H), 4.75 (4H, s, CH_2CCl_3 × 2), 5.31 (2H, s, $C\underline{H}_2$Ar), 5.37 (1H, brs, C_5-NH), 5.87 (1H, br d, J = 10 Hz, C_2-NH), 7.54, 8.26 (2H × 2, each d, J = 8 Hz, aromatic protons). *Anal.* Calcd for $C_{18}H_{19}Cl_6N_3O_8$: C, 34.98; H, 3.10; N, 6.80. Found: C, 35.01; H, 3.18; N, 6.72.

Troc–Orn(Troc)–OMe (5e)——A colorless oil. $[\alpha]_D^{25}$ +5.9°. MS *m/z*: 496, 498, 500 (M^+). IR ν_{max} cm^{-1}: 3450 (NH), 1738 (C=O). ^{1}H-NMR δ: 1.52—2.11 (4H, m, C_3-H_2, C_4-H_2), 3.15—3.41 (2H, m, C_5-H_2), 3.78 (3H, s, CO_2CH_3), 4.27—4.55 (1H, m, C_2-H), 4.73, 4.75 (2H × 2, each s, CH_2CCl_3 × 2), 5.31 (1H, brs, C_5-H), 5.82 (1H, br d, J = 10 Hz, C_2-NH). *Anal.* Calcd for $C_{12}H_{16}Cl_6N_2O_6$: C, 29.00; H, 3.24; N, 5.64. Found: C, 28.84; H, 3.52; N, 5.50.

Boc–Lys(Boc)–O*tert*-Bu (6a)——Recrystallized from AcOEt–isopropyl ether as colorless needles, mp 94—96 °C. $[\alpha]_D^{25}$ +2.6°. MS *m/z*: 402 (M^+), 301 (M^+ − Boc). IR ν_{max} cm^{-1}: 3440 (NH), 1710 (C=O). ^{1}H-NMR δ: 1.00—2.09 (6H, m, C_3-H_2, C_4-H_2, C_5-H_2), 1.46 (27H, s, $(CH_3)_3$ × 3), 2.93—3.25 (2H, m, C_6-H_2), 3.97—4.33 (1H, m, C_2-H),

4.65 (1H, br s, C_6-NH), 5.10 (1H, br d, $J=10$ Hz, C_2-NH). *Anal.* Calcd for $C_{20}H_{38}N_2O_6$: C, 59.68; H, 9.51; N, 6.96. Found: C, 59.78; H, 9.60; N, 6.80.

Boc–Lys(Boc)–ONBzl (6b)——Recrystallized from AcOEt–petroleum ether as colorless needles, mp 95—96 °C. $[\alpha]_D^{25}$ +1.8°. MS *m/z*: 481 (M^+). IR ν_{max} cm^{-1}: 3450 (NH), 1742, 1710 (C=O). ^{1}H-NMR δ: 1.07—1.94 (6H, m, C_3-H_2, C_4-H_2, C_5-H_2), 1.45 (18H, s, $(CH_3)_3 \times 2$), 2.95—3.23 (2H, m, C_6-H_2), 4.18—4.46 (1H, m, C_2-H), 4.58—4.82 (1H, br s, C_6-NH), 5.24 (1H, br d, $J=10$ Hz, C_2-NH), 5.27 (2H, s, $C\underline{H}_2$Ar), 7.52, 8.22 (2H × 2, each d, $J=8$ Hz, aromatic protons). *Anal.* Calcd for $C_{23}H_{35}N_3O_8$: C, 57.37; H, 7.33; N, 8.73. Found: C, 57.45; H, 7.30; N, 8.61.

Boc–Lys(Boc)–OMe (6c)——Recrystallized from isopropyl ether as colorless needles, mp 82—83 °C. $[\alpha]_D^{25}$ +7.2°. MS *m/z*: 360 (M^+). IR ν_{max} cm^{-1}: 3460 (NH), 1740, 1710 (C=O). ^{1}H-NMR δ: 1.08—1.95 (6H, m, C_3-H_2, C_4-H_2, C_5-H_2), 1.45 (18H, s, $(CH_3)_3 \times 2$), 2.95—3.23 (2H, m, C_6-H_2), 3.74 (3H, s, CO_2CH_3), 4.11—4.40 (1H, m, C_2-H), 4.70 (1H, br s, C_6-NH), 5.18 (1H, br d, $J=10$ Hz, C_2-NH). *Anal.* Calcd for $C_{17}H_{32}N_2O_6$: C, 56.65; H, 8.95; N, 7.77. Found: C, 56.23; H, 8.74; N, 7.70.

Troc–Lys(Troc)–O*tert*-Bu (6d)——A colorless oil. $[\alpha]_D^{25}$ +3.2°. MS *m/z*: 553, 555, 557 (M^+). IR ν_{max} cm^{-1}: 3430 (NH), 1730 (C=O). ^{1}H-NMR δ: 1.24—2.17 (6H, m, C_3-H_2, C_4-H_2, C_5-H_2), 1.50 (9H, s, $(CH_3)_3$), 3.13—3.45 (2H, m, C_6-H_2), 4.16—4.42 (1H, m, C_2-H), 4.78 (4H, s, $CH_2CCl_3 \times 2$), 5.24 (1H, br s, C_6-NH), 5.84 (1H, br d, $J=10$ Hz, C_2-NH). *Anal.* Calcd for $C_{16}H_{24}Cl_6N_2O_6$: C, 34.75; H, 4.37; N, 5.06. Found: C, 34.48; H, 4.29; N, 5.11.

Troc–Lys(Troc)–ONBzl (6e)——A colorless oil. $[\alpha]_D^{25}$ +1.5°. MS *m/z*: 632, 634, 636 (M^+). IR ν_{max} cm^{-1}: 3450 (NH), 1740 (C=O). ^{1}H-NMR δ: 1.18—2.26 (6H, m, C_3-H_2, C_4-H_2, C_5-H_2), 3.06—3.42 (2H, m, C_6-H_2), 4.28—4.54 (1H, m, C_2-H), 4.73 (4H, s, $CH_2CCl_3 \times 2$), 5.28 (2H, s, $C\underline{H}_2$Ar), 5.42 (1H, br s, C_6-NH), 5.98 (1H, br d, $J=10$ Hz, C_2-NH), 7.52, 8.22 (2H × 2, each d, $J=8$ Hz, aromatic protons). *Anal.* Calcd for $C_{19}H_{21}Cl_6N_3O_8$: C, 36.10; H, 3.35; N, 6.65. Found: C, 36.31; H, 3.24; N, 6.68.

Troc–Lys(Troc)–OMe (6f)——A colorless oil. $[\alpha]_D^{25}$ +5.5°. MS *m/z*: 511, 513, 515 (M^+). IR ν_{max} cm^{-1}: 3450 (NH), 1738 (C=O). ^{1}H-NMR δ: 1.27—2.03 (6H, m, C_3-H_2, C_4-H_2, C_5-H_2), 3.11—3.39 (2H, m, C_6-H_2), 3.77 (3H, s, CO_2CH_3), 4.23—4.51 (1H, m, C_2-H), 4.75 (4H, s, $CH_2CCl_3 \times 2$), 5.25 (1H, br s, C_6-H), 5.78 (1H, br d, $J=10$ Hz, C_2-NH). *Anal.* Calcd for $C_{13}H_{18}Cl_6N_2O_6$: C, 30.56; H, 3.55; N, 5.48. Found: C, 30.28; H, 3.80; N, 5.29.

Standard Procedure for the RuO_4 Oxidation in a Two-Phase System——A solution of a substrate (3.0 mmol) to be oxidized in AcOEt (40 ml) was added to a mixture of RuO_2 hydrate [Aldrich Chemical Co.] (120 mg) and 10% aqueous $NaIO_4$ (120 ml). The mixture was vigorously stirred using a mechanical stirrer with a glass blade at room temperature in a sealed flask. When the starting material was no longer detectable on a TLC plate, the layers were separated. The aqueous layer was extracted with three 20-ml portions of AcOEt. The combined organic solution was treated with isopropyl alcohol (2 ml) for 2—3 h to decompose the RuO_4 oxidant and filtered. The filtrate was washed with H_2O (20 ml) and dried over anhydrous Na_2SO_4. The solution was filtered and evaporated *in vacuo* to leave a residue, which was purified by column chromatography. The cyclized products (**13**, **14**) produced in the oxidation of the Boc-ornithine and Boc-lysine series (**5a**—**c**, **6a**—**c**) were eluted faster than the desired imide products. The results are summarized in Table I. The characteristics of the products were as follows.

Boc–Asn(Boc)–ONBzl (7a)——Recrystallized from AcOEt–isopropyl ether as colorless prisms, mp 126—127 °C. $[\alpha]_D^{25}$ +22.4°. MS *m/z*: 467 (M^+). IR ν_{max} cm^{-1}: 3440, 3400 (NH), 1784, 1755, 1712 (C=O). ^{1}H-NMR δ: 1.45, 1.50 (total 18H, each s, $(CH_3)_3 \times 2$), 3.27 (1H, dd, $J=16$, 4 Hz, one proton of C_3-H_2), 3.60 (1H, dd, $J=16$, 4 Hz, one proton of C_3-H_2), 4.59—4.87 (1H, m, C_2-H), 5.29 (2H, s, $C\underline{H}_2$Ar), 5.66 (1H, br d, $J=10$ Hz, C_2-NH), 7.52, 8.23 (2H × 2, each d, $J=8$ Hz, aromatic protons), 7.86 (1H, br s, C_4-NH). ^{13}C-NMR δ: 39.01 (t, C_3), 49.71 (d, C_2), 150.53 (N^{ω}C=O), 155.76 (N^{α}C=O), 171.38 (C_1), 172.95 (C_4). *Anal.* Calcd for $C_{21}H_{29}N_3O_9$: C, 53.95; H, 6.25; N, 8.99. Found: C, 53.84; H, 6.34; N, 8.86.

Boc–Asn(Boc)–OMe (7b)——A colorless oil. $[\alpha]_D^{25}$ +26.5°. MS *m/z*: 346 (M^+). IR ν_{max} cm^{-1}: 3440, 3400 (NH), 1785, 1754, 1710 (C=O). ^{1}H-NMR δ: 1.46, 1.51 (18H, each s, $(CH_3)_3 \times 2$), 3.18 (1H, dd, $J=16$, 4 Hz, one proton of C_3-H_2), 3.50 (1H, dd, $J=16$, 4 Hz, one proton of C_3-H_2), 3.74 (3H, s, CO_2CH_3), 4.46—4.78 (1H, m, C_2-H), 5.61 (1H, br d, $J=10$ Hz, C_2-NH), 8.00 (1H, br s, C_4-NH). ^{13}C-NMR δ: 39.11 (t, C_3), 49.61 (d, C_2), 150.63 (N^{ω}C=O), 155.76 (N^{α}C=O), 172.12 (C_1), 172.95 (C_4). *Anal.* Calcd for $C_{15}H_{26}N_2O_7$: C, 52.01; H, 7.57; N, 8.09. Found: C, 52.28; H, 7.78; N, 8.24.

Boc–Gln(Boc)–O*tert*-Bu (8a)——A colorless oil. $[\alpha]_D^{25}$ +2.4°. MS *m/z*: 402 (M^+). IR ν_{max} cm^{-1}: 3420 (NH), 1780, 1700 (C=O). ^{1}H-NMR δ: 1.46, 1.48, 1.50 (27H, each s, $(CH_3)_3 \times 3$), 1.69—2.40 (2H, m, C_3-H_2), 2.83 (2H, t, $J=7$ Hz, C_4-H_2), 4.12—4.40 (1H, m, C_2-H), 5.30 (1H, br d, $J=10$ Hz, C_2-NH), 7.91 (1H, br s, C_5-NH). ^{13}C-NMR δ: 27.25 (t, C_3), 32.28 (t, C_4), 53.56 (d, C_2), 150.68 (N^{ω}C=O), 155.64 (N^{α}C=O), 171.62 (C_1), 174.02 (C_5). *Anal.* Calcd for $C_{19}H_{34}N_2O_7$: C, 56.70; H, 8.51; N, 6.96. Found: C, 56.69; H, 8.40; N, 7.14.

Boc–Gln(Boc)–ONBzl (8b)——Recrystallized from AcOEt–isopropyl ether as colorless needles, mp 105—107 °C. $[\alpha]_D^{25}$ +1.1°. MS *m/z*: 481 (M^+). IR ν_{max} cm^{-1}: 3440, 3410 (NH), 1790, 1750, 1710 (C=O). ^{1}H-NMR δ: 1.47, 1.53 (18H, each s, $(CH_3)_3 \times 2$), 1.93—2.34 (2H, m, C_3-H_2), 2.91 (2H, t, $J=7$ Hz, C_4-H_2), 4.30—4.54 (1H, m, C_2-H), 5.38 (2H, s, $C\underline{H}_2$Ar), 5.44 (1H, br d, $J=10$ Hz, C_2-NH), 7.69, 8.39 (2H × 2, each d, $J=8$ Hz, aromatic protons), 7.84 (1H, br s, C_5-NH). ^{13}C-NMR δ: 26.60 (t, C_3), 32.18 (t, C_4), 53.22 (d, C_2), 150.73 (N^{ω}C=O), 155.71 (N^{α}C=O), 172.36 (C_1), 174.02 (C_5). *Anal.* Calcd for $C_{22}H_{31}N_3O_9$: C, 54.88; H, 6.49; N, 8.73. Found: C, 55.03; H, 6.58; N, 8.55.

Boc–Gln(Boc)–OMe (8c)——A colorless oil. $[\alpha]_D^{25}$ +7.7°. MS *m/z*: 360 (M^+). IR ν_{max} cm^{-1}: 3450, 3420 (NH),

1790, 1750, 1710 (C=O). ^{1}H-NMR δ: 1.44, 1.49 (18H, each s, $(CH_3)_3\times 2$), 1.74—2.34 (2H, m, C_3-H_2), 2.81 (2H, t, J=7 Hz, C_4-H_2), 3.72 (3H, s, CO_2CH_3), 4.18—4.46 (1H, m, C_2-H), 5.30 (1H, br d, J=10 Hz, C_2-NH), 7.84 (1H, br s, C_5-NH). ^{13}C-NMR δ: 27.10 (t, C_3), 32.28 (t, C_4), 53.08 (d, C_2), 150.78 ($N^{ca}C$=O), 155.76 ($N^{\alpha}C$=O), 173.14 (C_1), 174.07 (C_5). *Anal.* Calcd for $C_{16}H_{28}N_2O_7$: C, 53.32; H, 7.83; N, 7.77. Found: C, 53.60; H, 7.98; N, 7.92.

Troc–Gln(Troc)–ONBzl (8d)——A colorless oil. $[\alpha]_D^{25}$ +1.6°. MS *m/z*: 632, 634, 636 (M^+). IR ν_{max} cm^{-1}: 3430, 3400 (NH), 1810, 1780, 1720 (C=O). ^{1}H-NMR δ: 1.96—2.44 (2H, m, C_3-H_2), 2.94 (2H, t, J=7 Hz, C_4-H_2), 4.40—4.74 (1H, m, C_2-H), 4.72, 4.77 (2H×2, each s, $CH_2CCl_3\times 2$), 5.30 (2H, s, $C\underline{H}_2Ar$), 5.94 (1H, br d, J=10 Hz, C_2-NH), 7.54, 8.23 (2H×2, each d, J=8 Hz, aromatic protons), 8.32 (1H, br s, C_5-NH). ^{13}C-NMR δ: 26.46 (t, C_3), 32.08 (t, C_4), 53.52 (d, C_2), 150.43 ($N^{ca}C$=O), 154.63 ($N^{\alpha}C$=O), 171.43 (C_1), 173.48 (C_5). *Anal.* Calcd for $C_{18}H_{17}Cl_6N_3O_9$: C, 34.21; H, 2.71; N, 6.65. Found: C, 34.42; H, 2.68; N, 6.52.

Troc–Gln(Troc)–OMe (8e)——A colorless oil. $[\alpha]_D^{25}$ +6.8°. MS *m/z*: 510, 512, 514 (M^+). IR ν_{max} cm^{-1}: 3430, 3400 (NH), 1810, 1780, 1732 (C=O). ^{1}H-NMR δ: 1.88—2.48 (2H, m, C_3-H_2), 2.92 (2H, t, J=7 Hz, C_4-H_2), 3.77 (3H, s, CO_2CH_3), 4.32—4.60 (1H, m, C_2-H), 4.70, 4.78 (2H×2, each s, $CH_2CCl_3\times 2$), 5.90 (1H, br d, J=10 Hz, C_2-NH), 8.34 (1H, br s, C_5-NH). ^{13}C-NMR δ: 26.71 (t, C_3), 32.23 (t, C_4), 53.42 (d, C_2), 150.53 ($N^{ca}C$=O), 154.69 ($N^{\alpha}C$=O), 172.31 (C_1), 173.77 (C_5). *Anal.* Calcd for $C_{12}H_{14}Cl_6N_2O_7$: C, 28.21; H, 2.76; N, 5.48. Found: C, 28.31; H, 2.96; N, 5.56.

N^{α},N^{ca}-DiBoc-L-2-aminoadipamic Acid *tert*-Butyl Ester (9a)——Recrystallized from isopropyl ether–pentane as colorless needles, mp 113—114 °C. $[\alpha]_D^{25}$ +1.0°. MS *m/z*: 416 (M^+). IR ν_{max} cm^{-1}: 3420 (NH), 1780, 1752, 1710 (C=O). ^{1}H-NMR δ: 1.33—1.97 (4H, m, C_3-H_2, C_4-H_2), 1.44, 1.48, 1.49 (27H, each s, $(CH_3)_3\times 3$), 2.72 (2H, t, J=7 Hz, C_5-H_2), 3.97—4.25 (1H, m, C_2-H), 5.16 (1H, br d, J=10 Hz, C_2-NH), 7.68 (1H, br s, C_6-NH). ^{13}C-NMR δ: 20.12 (t, C_4), 32.32 (t, C_3), 35.69 (t, C_5), 53.76 (d, C_2), 150.73 ($N^{ca}C$=O), 155.71 ($N^{\alpha}C$=O), 171.97 (C_1), 174.27 (C_6). *Anal.* Calcd for $C_{20}H_{36}N_2O_7$: C, 57.67; H, 8.71; N, 6.73. Found: C, 57.55; H, 8.70; N, 6.52.

N^{α},N^{ca}-DiBoc-L-2-aminoadipamic Acid *p*-Nitrobenzyl Ester (9b)——A colorless oil. $[\alpha]_D^{25}$ +3.8°. MS *m/z*: 495 (M^+). IR ν_{max} cm^{-1}: 3450, 3410 (NH), 1782, 1742, 1710 (C=O). ^{1}H-NMR δ: 1.60—1.96 (4H, m, C_3-H_2, C_4-H_2), 1.45, 1.50 (18H, each s, $(CH_3)_3\times 2$), 2.76 (2H, t, J=7 Hz, C_5-H_2), 4.16—4.48 (1H, m, C_2-H), 5.23 (1H, br d, J=10 Hz, C_2-NH), 5.30 (2H, s, $C\underline{H}_2Ar$), 7.58, 8.28 (2H×2, each d, J=8 Hz, aromatic protons), 7.62 (1H, br s, C_6-NH). ^{13}C-NMR δ: 20.07 (t, C_4), 31.69 (t, C_3), 35.35 (t, C_5), 53.47 (d, C_2), 150.78 ($N^{ca}C$=O), 155.71 ($N^{\alpha}C$=O), 172.60 (C_1), 174.22 (C_6). *Anal.* Calcd for $C_{23}H_{33}N_3O_9$: C, 57.75; H, 6.71; N, 8.48. Found: C, 55.88; H, 6.84; N, 8.55.

N^{α},N^{ca}-DiBoc-L-2-aminoadipamic Acid Methyl Ester (9c)——A colorless oil. $[\alpha]_D^{25}$ +9.6°. MS *m/z*: 374 (M^+). IR ν_{max} cm^{-1}: 3440, 3410 (NH), 1780, 1745, 1710 (C=O). ^{1}H-NMR δ: 1.45, 1.49 (18H, each s, $(CH_3)_3\times 2$), 1.58—2.18 (4H, m, C_3-H_2, C_4-H_2), 2.72 (2H, t, J=7 Hz, C_5-H_2), 3.72 (3H, s, CO_2CH_3), 4.10—4.38 (1H, m, C_2-H), 5.24 (1H, br d, J=10 Hz, C_2-NH), 7.91 (1H, br s, C_6-NH). ^{13}C-NMR δ: 20.12 (t, C_4), 32.03 (t, C_3), 35.60 (t, C_5), 53.32 (d, C_2), 150.83 ($N^{ca}C$=O), 155.76 ($N^{\alpha}C$=O), 173.44 (C_1), 174.41 (C_6). *Anal.* Calcd for $C_{17}H_{30}N_2O_7$: C, 54.53; H, 8.08; N, 7.48. Found: C, 54.43; H, 8.20; N, 7.51.

N^{α},N^{ca}-DiTroc-L-2-aminoadipamic Acid *tert*-Butyl Ester (9d)——A colorless oil. $[\alpha]_D^{25}$ +2.6°. MS *m/z*: 567, 569, 571 (M^+). IR ν_{max} cm^{-1}: 3440, 3400 (NH), 1800, 1770, 1727 (C=O). ^{1}H-NMR δ: 1.50 (9H, s, $(CH_3)_3$), 1.70—2.10 (4H, m, C_3-H_2, C_4-H_2), 2.90 (2H, t, J=7 Hz, C_5-H_2), 4.22—4.60 (1H, m, C_2-H), 4.82, 4.86 (2H×2, each s, $CH_2CCl_3\times 2$), 6.00 (1H, br d, J=10 Hz, C_2-NH), 8.46 (1H, br s, C_6-NH). ^{13}C-NMR δ: 19.87 (t, C_4), 31.40 (t, C_3), 35.40 (t, C_5), 53.95 (d, C_2), 150.53 ($N^{ca}C$=O), 154.54 ($N^{\alpha}C$=O), 170.70 (C_1), 173.77 (C_6). *Anal.* Calcd for $C_{16}H_{22}Cl_6N_2O_7$: C, 33.89; H, 3.91; N, 4.94. Found: C, 33.90; H, 3.85; N, 4.86.

N^{α},N^{ca}-DiTroc-L-2-aminoadipamic Acid *p*-Nitrobenzyl Ester (9e)——A colorless oil. $[\alpha]_D^{25}$ +3.2°. MS *m/z*: 646, 648, 650 (M^+). IR ν_{max} cm^{-1}: 3440, 3400 (NH), 1800, 1775, 1735 (C=O). ^{1}H-NMR δ: 1.64—2.08 (4H, m, C_3-H_2, C_4-H_2), 2.82 (2H, t, J=7 Hz, C_5-H_2), 4.36—4.60 (1H, m, C_2-H), 4.73, 4.78 (2H×2, each s, $CH_2CCl_3\times 2$), 5.29 (2H, s, $C\underline{H}_2Ar$), 5.91 (1H, br d, J=10 Hz, C_2-NH), 7.53, 8.23 (2H×2, each d, J=8 Hz, aromatic protons), 8.34 (1H, br s, C_6-NH). ^{13}C-NMR δ: 19.73 (t, C_4), 31.45 (t, C_3), 35.35 (t, C_5), 53.95 (d, C_2), 150.48 ($N^{ca}C$=O), 154.59 ($N^{\alpha}C$=O), 171.82 (C_1), 173.77 (C_6). *Anal.* Calcd for $C_{19}H_{19}Cl_6N_3O_9$: C, 35.32; H, 2.96; N, 6.50. Found: C, 35.44; H, 3.20; N, 6.49.

N^{α},N^{ca}-DiTroc-L-2-aminoadipamic Acid Methyl Ester (9f)——A colorless oil. $[\alpha]_D^{25}$ +8.3°. MS *m/z*: 525, 527, 529 (M^+). IR ν_{max} cm^{-1}: 3430, 3400 (NH), 1800, 1778, 1715 (C=O). ^{1}H-NMR δ: 1.64—2.12 (4H, m, C_3-H_2, C_4-H_2), 2.86 (2H, t, J=7 Hz, C_5-H_2), 3.83 (3H, s, CO_2CH_3), 4.36—4.63 (1H, m, C_2-H), 4.82, 4.86 (2H×2, each s, $CH_2CCl_3\times 2$), 5.94 (1H, br d, J=10 Hz, C_2-NH), 8.43 (1H, br s, C_6-NH). ^{13}C-NMR δ: 19.82 (t, C_4), 31.79 (t, C_3), 35.50 (t, C_5), 53.81 (d, C_2), 150.48 ($N^{ca}C$=O), 154.59 ($N^{\alpha}C$=O), 172.55 (C_1), 173.77 (C_6). *Anal.* Calcd for $C_{13}H_{16}Cl_6N_2O_7$: C, 29.74; H, 3.07; N, 5.34. Found: C, 29.94; H, 3.21; N, 5.41.

***N*-Boc-5-*tert*-butoxycarbonylaminopyrrolidine-2-carboxylic Acid *tert*-Butyl Ester (13a)**——A colorless oil. MS *m/z*: 386 (M^+). IR ν_{max} cm^{-1}: 3500 (NH), 1735, 1698 (C=O). ^{1}H-NMR δ: 1.44 (27H, s, $(CH_3)_3\times 3$), 1.58—2.50 (4H, m, C_3-H_2, C_4-H_2), 4.02—4.26 (1H, m, C_2-H), 4.82—5.06 (1H, m, C_5-H), 5.30—5.64 (1H, br s, NH). ^{13}C-NMR δ: 26.95 (t, C_3), 30.71, 32.47 (each t, C_4, diastereomeric), 60.01, 60.55 (each d, C_2, diastereomeric), 65.77, 66.65 (each d, C_5, diastereomeric). *Anal.* Calcd for $C_{19}H_{34}N_2O_6$: C, 59.05; H, 8.87, N, 7.25. Found: C, 59.21; H, 8.70; N, 7.18.

***N*-Boc-5-*tert*-butoxycarbonylaminopyrrolidine-2-carboxylic Acid *p*-Nitrobenzyl Ester (13b)**——A colorless oil. MS *m/z*: 465 (M^+). IR ν_{max} cm^{-1}: 3452 (NH), 1750, 1710, 1698 (C=O). ^{1}H-NMR δ: 1.43 (18H, s, $(CH_3)_3\times 2$), 1.75—

2.59 (4H, m, C_3-H_2, C_4-H_2), 4.28—4.51 (1H, m, C_2-H), 5.15—5.35 (3H, m, C_5-H, CH_2Ar), 5.40—5.72 (1H, br s, NH), 7.50, 7.51, 8.17, 8.18 (total 4H, each d, J=7 Hz, diastereomeric aromatic protons). ^{13}C-NMR δ: 26.95 (t, C_3), 30.81, 32.57 (each t, C_4, diastereomeric), 58.98, 59.72 (each d, C_2, diastereomeric), 65.67, 66.55 (each d, C_5, diastereomeric) *Anal.* Calcd for $C_{22}H_{31}N_3O_8$: C, 56.76; H, 6.71; N, 9.03. Found: C, 56.65; H, 6.82; N, 9.20.

N-Boc-5-*tert*-butoxycarbonylaminopyrrolidine-2-carboxylic Acid Methyl Ester (13c)——Recrystallized from hexane as colorless needles, mp 135—136 °C. MS *m/z*: 344 (M^+). IR ν_{max} cm^{-1}: 3370 (NH), 1740, 1720, 1694 (C=O). ^{1}H-NMR δ: 1.46 (18H, s, $(CH_3)_3 \times 2$), 1.72—2.44 (4H, m, C_3-H_2, C_4-H_2), 3.67 (3H, s, CO_2CH_3), 4.17—4.37 (1H, m, C_2-H), 4.68—4.92 (1H, m, C_5-H), 5.32—5.60 (1H, m, NH). ^{13}C-NMR δ: 26.95, 27.88 (each t, C_3, diastereomeric), 30.81, 32.42 (each t, C_4, diastereomeric), 58.94, 59.81 (each d, C_2, diastereomeric), 65.77, 66.70 (each d, C_5, diastereomeric). *Anal.* Calcd for $C_{16}H_{28}N_2O_6$: C, 55.80; H, 8.19; N, 8.13. Found: C, 55.93; H, 8.29; N, 8.20.

N-Boc-6-*tert*-butoxycarbonylaminopiperidine-2-carboxylic Acid *tert*-Butyl Ester (14a)——Recrystallized from petroleum ether–pentane as colorless needles, mp 133—134 °C. MS *m/z*: 400 (M^+). IR ν_{max} cm^{-1}: 3416 (NH), 1724, 1708, 1696 (C=O). ^{1}H-NMR δ: 1.46, 1.49 (27H, each s, $(CH_3)_3 \times 3$), 1.41—2.25 (6H, m, C_3-H_2, C_4-H_2, C_5-H_2), 4.49—4.66 (1H, m, C_2-H), 5.57—5.81 (1H, m, C_6-H), 6.63 (1H, br s, NH). ^{13}C-NMR δ: 14.75 (t, C_4), 26.76 (t, C_3), 30.85 (t, C_5), 54.74 (d, C_2), 58.74 (d, C_6). *Anal.* Calcd for $C_{20}H_{36}N_2O_6$: C, 59.98; H, 9.06; N, 6.99. Found: C, 59.87; H, 9.12; N, 7.09.

N-Boc-6-*tert*-butoxycarbonylaminopiperidine-2-carboxylic Acid *p*-Nitrobenzyl Ester (14b)——A colorless oil. MS *m/z*: 479 (M^+). IR ν_{max} cm^{-1}: 3400 (NH), 1738, 1710, 1695 (C=O). ^{1}H-NMR δ: 1.45 (18H, s, $(CH_3)_3 \times 2$), 1.45—2.32 (6H, m, C_3-H_2, C_4-H_2, C_5-H_2), 4.71—4.88 (1H, m, C_2-H), 5.31 (2H, s, CH_2Ar), 5.61—5.83 (1H, m, C_6-H), 6.53 (1H, br s, NH), 7.53, 8.20 (2H × 2, each d, J=7 Hz, aromatic protons). ^{13}C-NMR δ: 14.65 (t, C_4), 26.51 (t, C_3), 30.76 (t, C_5), 54.00 (d, C_2), 58.84 (d, C_6). *Anal.* Calcd for $C_{23}H_{33}N_3O_8$: C, 57.61; H, 6.94; N, 8.76. Found: C, 57.92; H, 6.72; N, 8.66.

N-Boc-6-*tert*-butoxycarbonylaminopiperidine-2-carboxylic Acid Methyl Ester (14c)——Recrystallized from petroleum ether–pentane as colorless needles, mp 102—103 °C. MS *m/z*: 358 (M^+). IR ν_{max} cm^{-1}: 3460 (NH), 1718, 1700 (C=O). ^{1}H-NMR δ: 1.45 (18H, s, $(CH_3)_3 \times 2$), 1.43—2.25 (6H, m, C_3-H_2, C_4-H_2, C_5-H_2), 3.75 (3H, s, CO_2CH_3), 4.61—4.77 (1H, m, C_2-H), 5.57—5.81 (1H, m, C_6-H), 6.63 (1H, m, NH). ^{13}C-NMR δ: 14.70 (t, C_4), 26.61 (t, C_3), 30.76 (t, C_5), 53.91 (d, C_2), 58.79 (d, C_6). *Anal.* Calcd for $C_{17}H_{30}N_2O_6$: C, 55.97; H, 8.44; N, 7.82. Found: C, 56.90; H, 8.18; N, 7.69.

L-Asparagine (10)——A solution of **7a** (470 mg, 1.0 mmol) in EtOH (20 ml) was hydrogenated over 10% Pd–C (0.20 g) at room temperature. The catalyst was removed by filtration, and the filtrate was concentrated *in vacuo* to give an oily residue, which was dissolved in 10% aqueous HCl (10 ml) under cooling, and then extracted with ether (20 ml × 3 times). The extracts were washed with H_2O, dried, and concentrated to leave Boc–Asn(Boc)–OH as a colorless solid (310 mg), which was dissolved in TFA (2 ml). The solution was stirred at room temperature for 10 min, and then concentrated *in vacuo*. The residue was dissolved in H_2O (10 ml) and passed through an Amberlite IRA-45 column to give **10**·H_2O (110 mg, 73% yield from **7a**) as a colorless powder; mp 230—233 °C (dec.). $[\alpha]_D^{25}$ −5.4° (c=2, H_2O). This product was identical with authentic L-asparagine monohydrate.[18]

L-Glutamine (11)——Compound **8a** (500 mg, 1.25 mmol) was treated with TFA (3 ml) at room temperature for 10 min. Excess TFA was removed *in vacuo* and the residue was dissolved in H_2O (10 ml). The solution was passed through an Amberlite IRA-45 column to give **11** (144 mg, 79%) as a colorless powder. Recrystallization of the product from 50% aqueous EtOH afforded colorless needles, mp 180—182 °C (dec.). $[\alpha]_D^{25}$ +6.2° (c=1, H_2O). This product was identical with authentic L-glutamine.[18]

L-2-Aminoadipamic Acid (12)——The foregoing product (**9a**) was treated with TFA as discribed for **11**, producing **12** in 82% yield as colorless solid, which was recrystallized from 70% aqueous EtOH to give pure **12** as colorless leaflets, mp 172.5—174 °C. $[\alpha]_D^{25}$ +1.5° (c=0.6, H_2O). IR ν_{max} cm^{-1}: 3420 (OH, NH_2), 1655, 1610 (C=O, COOH). ^{1}H-NMR (D_2O) δ: 1.47—2.05 (4H, m, C_3-H_2, C_4-H_2), 2.32 (2H, t, J=7 Hz, C_5-H_2), 3.71 (1H, t, J=7 Hz, C_2-H). ^{13}C-NMR (D_2O) δ: 23.53 (t, C_4), 32.47 (t, C_3), 37.06 (t, C_5), 57.18 (d, C_2), 177.29, 181.73 (C=O). *Anal.* Calcd for $C_6H_{12}N_2O_3$: C, 44.99; H, 7.55; N, 17.49. Found: C, 44.90; H, 7.55; N, 17.54.

L-2-Aminoadipic Acid——A solution of **12** (100 mg, 0.6 mmol) in 6 N HCl (5 ml) was refluxed for 5 h. The reaction solution was concentrated *in vacuo* to dryness and the residue was dissolved in H_2O (5 ml). The resulting solution was treated with powdered K_2CO_3 (0.6 mmol) under cooling. The mixture was allowed to stand at 5 °C for 24 h to leave L-2-aminoadipic acid as a colorless powder (82 mg, 83%), mp 200—203 °C (dec.). $[\alpha]_D^{23}$ +22.8° (c=1.0, 6 N HCl). This product was identical with authentic L-2-aminoadipic acid[18] (lit.[16] $[\alpha]_D^{25}$ +23.2° (c=2.0, 5 N HCl)).

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Notes

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Protosappanin C from Sappan Lignum and Absolute Configuration of Protosappanins

MASAHIRO NAGAI*,[a] and SEIJI NAGUMO[b]

Institute of Medicinal Chemistry[a] and Faculty of Pharmaceutical Sciences,[b] Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142, Japan

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A new dibenz[*b,d*]oxocin derivative, named protosappanin C (3), $C_{16}H_{14}O_6$, amorphous powder, $[\alpha]_D^{23}$ −39.8° (MeOH), was isolated from Sappan Lignum (the dried heart-wood of *Caesalpinia sappan* L. (Leguminosae)). It yielded sappanin (4) on alkaline fusion, an oxime (5), a dimethyl acetal (6) on treatment with *p*-toluenesulfonic acid in methanol, and protosappanin B (2) on reduction with sodium borohydride. The circular dichroism spectra of 3 and 6 indicated that their biphenyl system preferentially takes *S* configuration, from which *R* configuration was assigned to C-7 of 3. On the basis of the above chemical and spectroscopic evidence, the chemical structure of 3 was established as (7*R*)-7,8-dihydro-3,7,10,11-tetrahydroxy-7(6*H*)-dibenz[*b,d*]oxocincarbaldehyde. A possible route of biosynthesis of protosappanins from sappanchalcone is discussed.

Keywords——Leguminosae; *Caesalpinia sappan*; Sappan Lignum; dibenz[*b,d*]oxocin; biphenyl; protosappanin C; sappanin; stereochemistry

The methanol extract of Sappan Lignum, the dried heart-wood of *Caesalpinia sappan* L. (Leguminosae) has sleeping time-prolonging effect in mice. In the course of our studies on its chemical components responsible for the effect, we have reported on a chalcone (sappanchalcone)[1a] and two biphenyls (protosappanins A[1b] and B[1c]) with minor activities in relation to the effect of the methanol extract. The two biphenyls belong to a new class of natural products, the dibenz[*b, d*]oxocin group. The present paper deals with protosappanin C (3), another dibenz[*b,d*]oxocin which was inactive in respect of the sleeping time-prolonging effect at the dose of 160 mg/kg (i.p.).[2]

Protosappanin C (3), amorphous powder, $[\alpha]_D^{23}$ −39.8° (MeOH), showed the molecular ion peak at *m/z* 302.078 corresponding to $C_{16}H_{14}O_6$, two hydrogens less than the molecular formula of protosappanin B (2), in the high-resolution mass spectrum (MS). Hydroxyl (3330 cm^{-1}) and aromatic (1605 and 1495 cm^{-1}) absorption bands were observed in the infrared (IR) spectrum (KBr), which roughly resembled that of protosappanin B (2) except for a weak carbonyl absorption at 1720 cm^{-1}. The ultraviolet (UV) spectra of protosappanins A (1), B (2) and C (3) were superimposable on each other. In the MS of 3, prominent fragment peaks were observed at 230, 229 (ion a in Chart 1) and 213, of which the *m/z* 229 peak also appeared in the spectra of 1 and 2.

The proton and carbon-13-nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra of protosappanin C (3) were measured at 90 °C in dimethylsulfoxide (DMSO)-d_6 since indistinguishably split or broadened signals were obtained on measurement at room temperature. A carbonyl carbon resonating at δ_C 202.3 ppm (doublet) and one proton singlet at δ_H 9.67 ppm indicated the presence of an aldehyde group in protosappanin C (3), which was

confirmed by the formation of an oxime (5).[3)]

The above findings suggested that protosappanin C (3) is a dibenz[*b*,*d*]oxocin having an aldehyde group instead of the carbinol at C-7 of protosappanin B (2). In fact, alkaline fusion of protosappanin C yielded sappanin (4), as in the case of protosappanins A and B, and reduction of 3 with sodium borohydride provided an alcohol, $C_{16}H_{16}O_6$,[3)] $[\alpha]_D^{21}$ $-12.6°$ (MeOH). This alcohol was identical with protosappanin B (2) on the basis of the IR, NMR circular dichroism (CD) spectra, MS and thin layer chromatographic (TLC) behavior. Consequently the chemical structure of protosappanin C was established as 3 (Chart 1) except for its stereochemistry.

protosappanin A (1): R^1=H, R^2=O

protosappanin B (2): R^1=H, R^2=$\langle$CH₂OH / OH

2a: R^1=CH_3, R^2=$\langle$CH₂OH / OH

protosappanin C (3)

ion a

ion b

3′: R^1=R^2=R^3=H
3″: R^1=CH_3, R^2=R^3=H
6: R^1=R^2=CH_3, R^3=H
7: R^1=R^2=R^3=CH_3

Chart 1

Since protosappanin C (3) is an aldehyde, a strong carbonyl absorption should be observed in its IR spectrum (KBr). However the absorption was weak at 1720 cm^{-1}. We may explain this phenomenon as follows. Protosappanin C (3), obtained as a powder by concentration of a methanolic solution (see Experimental), would exist as a mixture of aldehyde (3), hydrate (3′) and hemiacetal (3″). The aldehyde (3) has a partial structure similar to those of aldoses of monosaccharides, which usually do not take an aldehyde form but exist almost exclusively in an intramolecular hemiacetal form, since the aldehyde function of 3 has electron-withdrawing atoms at the α- and β- positions, namely an α,β-dioxygenated aldehyde structure. It is well known that an aldehyde, its hydrate and its methyl hemiacetal are easily convertible to each other in the presence of water and methanol. On treatment with *p*-toluenesulfonic acid in methanol, 3 afforded a dimethyl acetal (6),[3)] which was successfully methylated with diazomethane to a trimethyl ether (7).[3)] In the MS, 6 and 7 showed a characteristic base peak at m/z 75 due to the ion b (Chart 1).[4)]

The stereochemistry of protosappanin B (2) was discussed in the previous paper and its absolute configuration at C-7 was tentatively assigned as *S*.[1c)] However, the assignment was not certain, because the negative Cotton effect due to the UV conjugation band of the biphenyl system was weak in the CD spectrum of 2. In the present study, the CD spectra of protosappanin C (3) and dimethyl acetal (6) were measured in methanol, resulting in the observation of strong Cotton effects (3, $[\theta]$ -20200 at 250 nm; 6, $[\theta]$ -22400 at 246 nm) (Fig. 1). Comparable magnitudes of the Cotton effects of 3 and 6 could be rationalized by the assumption of hemiacetal structure (3″) of 3 in methanol (*vide supra*). In the case of 6, there is a large difference of bulkiness between the two substituents at C-7, dimethoxymethyl and hydroxyl. The bulkier dimethoxymethyl group is likely to exist as an equatorial substituent at C-7 of a twist-boat-chair conformer[5)] of the dihydrodibenz[*b*,*d*]oxocin ring rather than as an axial one, which seems consistent with a greater population of *S* rotamer than of *R* rotamer of

the biphenyl system in the solution (Chart 1). In consequence protosappanins B (**2**) and C (**3**) are defined as (7*S*)-7,8-dihydro-3,7,10,11-tetrahydroxy-7(6*H*)-dibenz[*b*, *d*]oxocinmethanol and (7*R*)-7,8-dihydro-3,7,10,11-tetrahydroxy-7(6*H*)-dibenz[*b*, *d*]oxocincarbaldehyde, respectively.[6]

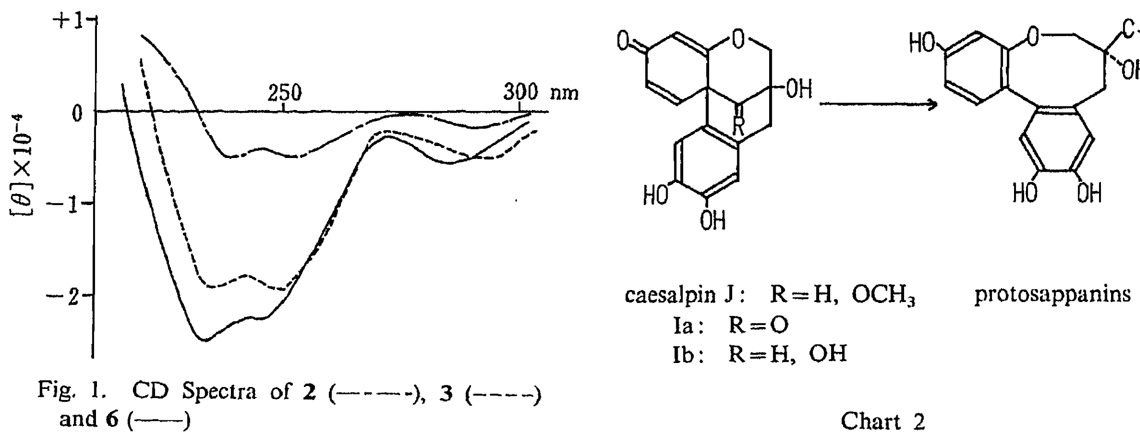

Fig. 1. CD Spectra of **2** (—·—·—), **3** (- - - -) and **6** (——)

Chart 2

Dewick has already discussed the possible biosynthesis of brazilin from a chalcone *via* a homoisoflavone[7] and we have also proposed a biogenetic relationship between sappanchalcone, brazilin and protosappanin A (**1**).[1b] Recently we isolated two other dibenzoxocins having an extra C_1 unit at C-7 of **1**, which led us to revise a part of the hypothetical biogenesis, namely the route to the dibenzoxocins, as follows (Chart 2). Nohara and his coworkers suggested a possible biosynthetic route to caesalpin J (Chart 2), a dienone compound, from a homoisoflavone.[8] A putative dienone (Ia or Ib), which has a 2-ene-1,5-dione or 5-hydroxy-2-en-1-one partial structure, may undergo retro-Claisen or retro-aldol condensation reaction to yield a dibenzoxocin. Protosappanin C (3) could thus be derived from Ib.

Experimental

Details of the instruments and TLC procedures used in this work were essentially the same as described in our previous paper (ref. 1*b*). CD spectra were measured with a JASCO J-40 apparatus in a 1 cm tube.

Extraction and Isolation——Sappan Lignum (500 g) was extracted five times with MeOH (1.5 l) for 2 h each under reflux. The total MeOH solution was concentrated under reduced pressure, affording the extract (62.5 g). A mixture of the extract and MeOH (150 ml) was stirred slowly for a while, and allowed to stand overnight. Crystalline insoluble matter (4 g) was filtered off and washed once with MeOH. The filtrate was concentrated to dryness, leaving a residue (58 g). A part of the residue (40 g) was chromatographed on silica gel and divided into the following eight fractions: fr. 1, 5.7 g; fr. 2, 4.6 g; fr. 3, 4.4 g; fr. 4, 2.8 g; fr. 5, 5.5 g; fr. 6, 4.1 g; fr. 7, 1.9 g; fr. 8, 6.9 g. Fractions 1—7 were eluted with $CHCl_3$–MeOH–H_2O (800 : 150 : 8) (solvent A) and fr. 8, with MeOH. Fraction 3 was rechromatographed on silica gel with benzene–EtOAc–MeOH (25 : 25 : 1), affording crude brazilin (3.2 g) and protosappanin C (**3**); **3** was repeatedly purified by chromatography on Sephadex LH-20 with MeOH as a solvent. Fraction 5 was rechromatographed on silica gel (eluent, solvent A) and successively on Sephadex LH-20. Elution with MeOH provided two compounds, protosappanin B (0.5 g) and an unknown compound (0.2 g). Protosappanin C (3), amorphous powder (0.3 g), $[\alpha]_D^{23}$ −39.8° (c=1.0, MeOH). MS m/z: 302 (M^+), 230, 229, 213, 185. High MS m/z: Calcd for $C_{16}H_{14}O_6$ (M^+), 302.079; $C_{13}H_9O_4$, 229.049; $C_9H_9O_6$, 213.041. Found: 302.078, 229.048, 213.041. IR ν_{max}^{KBr} cm^{-1}: 3330, 1720, 1605, 1495, 810. ^{1}H-NMR (DMSO-d_6, 90 °C) δ: 3.18 (2H, s, 8-H_2), 3.4—4.5 (2H, m, 6-H_2), 6.94 (1H, d, J=8.3, 1-H), 9.67 (1H, s, -CHO). ^{13}C-NMR (DMSO-d_6, 90 °C) δ: 39.4 (C-8, value on measurement in pyridine-d_5 at 90 °C), 72.4 (C-6), 75.1 (C-7), 106.7 (C-4), 110.4 (C-2), 116.4 (C-12), 118.6 (C-9), 121.2, 123.9 (C-8a and -12b), 130.2 (C-12a), 131.5 (C-1), 143.4, 143.7 (C-10 and -11), 157.2, 157.5 (C-3 and -4a), 202.3 (–CHO). UV λ_{max}^{EtOH} nm (log ε): 210 (end absorption, 4.60), 255 (4.09), 288 (3.89). CD (MeOH) $[\theta]^{23}$ (nm): −19400 (234), −20200 (250), −5400 (294).

Alkaline Fusion of Protosappanin C (3)——**3** (0.1 g) was fused with KOH (2 g) in the same way as in the case of **1** (ref. 1*b*). Chromatographic separation provided sappanin (**4**), 10 mg. **4**, colorless needles (MeOH–H_2O), mp 203.5—

204 °C. ^{1}H-NMR (DMSO-d_6): 6.23 (1H, dd, J=8.1, 2.3 Hz, 5-H), 6.34 (1H, d, J=2.3 Hz, 3-H), 6.67 (2H, br s, 5′-H and 6′-H), 6.91 (1H, br s, 2′-H), 6.93 (1H, d, J=8.1 Hz, 6-H). UV ν_{max}^{MeOH} nm (log ε): 210 (end absorption, 4.59), 257 (4.06), 292 (3.92).

Reduction of Protosappanin C (3)——Sodium borohydride (50 mg) was added to a solution of **3** (40 mg) in MeOH (13 ml) with stirring, and the mixture was allowed to stand for 2 h. After decomposition of the excess reagent, the reaction product was taken up in EtOAc, concentrated to dryness, and then applied to a column of silica gel. Elution with solvent A afforded an alcohol, $[\alpha]_D^{21}$ −12.6° (c=0.5, MeOH), which was identical with an authentic sample of **2** on the basis of TLC, IR, NMR, MS and CD comparisons.

Oxime (5)——A solution of **3** (102 mg) in MeOH (2 ml) was added to an aqueous solution (3 ml) of $NH_2OH \cdot HCl$ (CH_3COONa buffer). The solution was stirred for 2 h at 50 °C. After removal of the solvent, the residue was dissolved in EtOAc. The EtOAc solution was washed with H_2O, dried over anhydrous Na_2SO_4 and concentrated. The residue was passed through a column of silica gel (eluent, solvent A) and then a column of Sephadex LH-20 (eluent, MeOH), affording an oxime (**5**), 85 mg. **5**, amorphous powder, $[\alpha]_D^{18}$ −3° (c=0.8, MeOH). MS m/z: 317 (M^+), 272, 244, 230, 229, 213. NMR (DMSO-d_6, 90 °C) δ_C: 152.3 (–$\underline{C}$H=N–); δ_H: 7.39 (1H, s, –C$\underline{H}$=N–).

Dimethyl Acetal (6)——A solution of **3** (60 mg) and p-TsOH (5 mg) in MeOH (2 ml) was refluxed for 10 min. After removal of the solvent, the residue was dissolved in EtOAc. The EtOAc solution was washed with H_2O, dried over anhydrous Na_2SO_4, and concentrated. The residue was chromatographed over silica gel. Elution with solvent A–$CHCl_3$ (3:5) afforded **6** (38 mg). **6**, amorphous powder, $[\alpha]_D^{20}$ −31.6° (c=0.8, MeOH). High MS m/z: Calcd for $C_{18}H_{20}O_7$ (M^+) 348.121, $C_3H_7O_2$ 75.045. Found: 348.121, 75.044. ^{1}H-NMR (DMSO-d_6, 80 °C) δ: 3.43, 3.44 (each CH_3O–), 3.95 (2H, br s, 6-H_2), 4.10 (1H, s, –CH(O–)$_2$), 6.40 (1H, d, J=2.4 Hz, 4-H), 6.50 (1H, dd, J=2.4, 8.3 Hz, 2-H), 6.59 (1H, s, 9-H or 12-H), 6.67 (1H, s, 12-H or 9-H), 6.92 (1H, d, J=8.3 Hz, 1-H). ^{13}C-NMR (DMSO-d_6, 80 °C) δ: 39.0 (C-8, value on measurement in pyridine-d_5 at 90 °C), 57.2×2 (–OCH_3×2), 73.4 (C-7), 76.5 (C-6), 107.6 (C-4), 109.9 (–CH(O–)$_2$), 110.7 (C-2), 116.0 (C-12), 119.5 (C-9), 123.6, 126.1 (C-8a and -12b), 129.1 (C-12a), 130.7 (C-1), 143.4×2 (C-10 and -11), 157.7, 158.4 (C-3 and -4a). UV λ_{max}^{MeOH} nm (log ε): 210 (end absorption, 4.63), 255 (4.13), 287 (3.90). CD (MeOH) $[\theta]^{23}$ (nm): −24600 (233), −22400 (246), −5500 (285).

Trimethyl Ether (7)——An excess of diazomethane in ether was repeatedly added to a solution of **6** (30 mg) in MeOH (30 ml). The product was purified by chromatography over silica gel (eluent, benzene–EtOAc (4:1)). **7**, amorphous powder, $[\alpha]_D^{23}$ −76.0° (c=1.0, $CHCl_3$). MS m/z: 390 (M^+), 75. ^{1}H-NMR ($CDCl_3$) δ: 2.72 (2H, s, 8-H_2), 3.56 (6H, s, –OCH_3×2), 3.83, 3.89, 3.92 (each 3H, s, CH_3O–), 4.00, 4.34 (each 1H, d, J=12.2 Hz, 6-H_2), 4.24 (1H, –CH(O–)$_2$), 6.89 (1H, dd, J=2.7, 8.1 Hz, 2-H), 6.64 (1H, d, J=2.7 Hz, 4-H), 6.79 (1H, s, 9-H or 12-H), 6.81 (1H, s, 12-H or 9-H), 7.18 (1H, d, J=8.1 Hz, 1-H). ^{13}C-NMR ($CDCl_3$, r.t.) δ: 38.3 (C-8), 55.3 (CH_3O–), 55.9×2 (–OCH_3×2), 57.7×2 (–OCH_3×2), 73.8 (C-7), 77.6 (C-6), 106.6 (C-4), 109.2 (–CH(O–)$_2$), 110.0 (C-2), 113.1 (C-12), 115.3 (C-9), 125.1, 126.5 (C-8a and -12b), 131.5 (C-12a), 131.9 (C-1), 147.8 148.0 (C-10 and -11), 158.8, 160.4 (C-3 and -4a).

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Synthesis of 28-Norbrassinolides and Related 2-Deoxysteroids

SUGURU TAKATSUTO*,[a] and NOBUO IKEKAWA[b]

Department of Chemistry, Joetsu University of Education,[a] Joetsu, Niigata 943, Japan and Department of Chemistry, Tokyo Institute of Technology,[b] Ookayama, Meguro-ku, Tokyo 152, Japan

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(22*R*,23*R*)-28-Norbrassinolide (**1**), (22*S*,23*S*)-28-norbrassinolide (**24**), and two related 2-deoxysteroids (**7** and **8**) were synthesized from cholesta-5,22*E*-dien-3*β*-ol (**9**) and 3*β*-tetrahydropyranyloxydinorchol-5-en-22-al (**14**).

Keywords——28-norbrassinolide; 2-deoxysteroid; brassinolide; plant growth promoter

28-Norbrassinolide (**1**) and brassinone (**2**) are naturally-occurring C_{27}-steroids, and are plant growth promoters.[1] These steroids are structurally related to the hormonal steroids, brassinolide (**3**)[2] and castasterone (**4**),[3] respectively. Typhasterol (2-deoxycastasterone) (**5**) and teasterone (**6**), which might be biosynthetic precursors of castasterone (**4**) and brassinolide (**3**), have recently been isolated from several plant sources together with **3** and **4**.[4] Thus, the 2-deoxysteroids (**7** and **8**) corresponding to **1** and **2** might also be found. Accordingly, synthesis is desirable in order to obtain standard samples for identification purposes. In this paper, we report the synthesis of the 2-deoxysteroids **7** and **8** and our alternative synthesis[5a,b] of (22*R*,23*R*)- and (22*S*,23*S*)-28-norbrassinolide (**1** and **24**).

1 : R=H
3 : R=Me

2 : R=H
4 : R=Me

5 : R=Me
7 : R=H

6 : R=Me
8 : R=H

Fig. 1

The mesylate of the known cholesta-5,22*E*-dien-3β-ol (**9**) was solvolyzed[6] and then oxidized to give the cyclopropyl ketone **10** in 79% yield. Refluxing of **10** with 5 M aqueous sulfuric acid and acetic acid (AcOH), followed by saponification, gave the 3β-ol **11** (85% yield), which was then hydroxylated with a catalytic amount of osmium tetroxide (OsO_4) and an excess of *N*-methylmorpholine *N*-oxide (NMO).[7] Chromatographic separation of the resulting products gave the less polar triol **12** and the more polar triol **8** in 55 and 27% yields, respectively. Compound **8** was led to the known sulfonate **13a**[5b] by acetonide formation and mesylation, which determined the stereochemistry of **8** and **12** as (22*R*,23*R*) and (22*S*,23*S*), respectively.

The sulfonate **13a** was alternatively prepared from the known 22-aldehyde **14**[8] as follows. Coupling of **14** with the anion of 1,3-dithiane gave exclusively the (22*R*)-22-ol **15** in 85% yield.[5a] After exchange of the protecting group into a methoxymethyl group, the adduct **16** was dethioacetallized with red mercury(II) oxide and boron trifluoride etherate in aqueous

9 10 11

12 + 8 13a(22*R*, 23*R*) 13b(22*S*, 23*S*)

14 15 16

17 18 19

THP: tetrahydropyranyl

Fig. 2

13a (22*R*, 23*R*) → 20a (22*R*, 23*R*)
13b (22*S*, 23*S*) → 20b (22*S*, 23*S*) + 21

20 → 22 → 23 : R=Ac, 24 : R=H

21 → 7

Fig. 3

tetrahydrofuran (THF) to give the 23-aldehyde **17** in 90% yield. This was reacted with isobutylmagnesium bromide at $-78\,^{\circ}C$ to give stereoselectively the (22*R*,23*R*)-compound **18** in 82% yield, according to Cram's cyclic model.[9] Removal of the protecting group gave the known triol **19**[5a,b] in 87% yield. Conversion of **19** into the sulfonate **13a** has already been described in our previous paper.[5b]

With the desired intermediate **13a** in hand, transformation of **13a** into the 3α-triol **7**, brassinone (**2**), and 28-norbrassinolide (**1**) was carried out. Treatment of **13a** with lithium carbonate in refluxing dimethylformamide (DMF), followed by saponification gave the 2-ene **20a** and the 3α-ol **21** in 37 and 43% yields, respectively. Deprotection of **21** with 70% aqueous AcOH gave the 3α-triol **7** in 76% yield. Conversion of the 2-ene **20a** into brassinone (**2**) and 28-norbrassinolide (**1**) has already been reported in our previous papers.[5a,b]

For the synthesis of (22*S*,23*S*)-28-norbrassinolide (**24**), the sulfonate **13b**, derived from the triol **12**, was heated with lithium bromide in DMF. The 2-ene **20b** was obtained in 87% yield and it was hydroxylated with OsO_4, as described for **11**. Deprotection of the resulting product, followed by acetylation gave the tetraacetoxy-6-ketone **22**. Baeyer–Villiger oxidation[10] of **22** with trifluoroperacetic acid gave the 7-oxalactone **23** in 61% yield from **20b**. (22*S*,23*S*)-28-Norbassinolide (**24**) was obtained by saponification of **23**, followed by acidification.

Experimental

Melting points were determined on a hot-stage microscope and are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Hitachi R-24A (60 MHz) spectrometer in deuteriochloroform solution with tetramethylsilane as an internal standard, unless otherwise noted. Mass spectra (MS) were taken with a Shimadzu LKB-9000S mass spectrometer at 20 eV. Infrared (IR) spectra were taken with a Hitachi 260-10 spectrometer. Column chromatography and thin layer chromatography were carried out with Merck Kieselgel 60 (70—230 mesh) and precoated Merck Kieselgel 60 F_{254} plates (0.25 mm thickness), respectively. Work-up refers to dilution of the reaction mixture with water, extraction with the organic solvent indicated in parentheses, washing of the extract with water to neutrality, drying over anhydrous magnesium sulfate, filtration, and removal of the solvent under reduced pressure. The following abbreviations are used; EtOAc, ethyl aceate; MeOH, methanol; EtOH,

ethanol; ether, diethyl ether; $CHCl_3$, chloroform; CH_2Cl_2, dichloromethane; *p*-TsOH, *p*-toluenesulfonic acid; KOH, potassium hydroxide; $NaHCO_3$, sodium hydrogen carbonate.

3β-Hydroxy-5α-cholest-22*E*-en-6-one (11)——Cholesta-5,22*E*-dien-3β-ol (9)[5a] (2.0 g, 5.2 mmol) in pyridine (10 ml) was treated with mesyl chloride (1.0 ml) at room temperature for 1 h. Work-up (EtOAc) gave the corresponding mesylate, and a solution of this in acetone (100 ml) and water (10 ml) was treated with potassium bicarbonate (3.0 g) under reflux for 7 h. Removal of the acetone by distillation and work-up (ether) gave a crude product (1.9 g). This was dissolved in acetone (20 ml) and oxidized with Jones reagent (1.2 eq) at room temperature for 20 min. Work-up (ether) and chromatography on silica gel (2.5 cm i.d. × 20 cm) with benzene gave the cyclopropyl ketone **10** (1.56 g, 79%): mp 87—88 °C (EtOH). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1695. ^{1}H-NMR δ: 0.73 (3H, s, 18-H_3), 0.80 (3H, s, 19-H_3), 5.20 (2H, m, 22-H and 23-H). The product **10** (1.55 g, 4.06 mmol) in AcOH (10 ml) was treated with 5 M aqueous sulfuric acid (3 ml) under reflux for 1 h. Work-up (EtOAc) gave a crude product, which was then refluxed with 5% KOH/MeOH (5 ml) for 1 h. Work-up (ether) and chromatography on silica gel (2.5 cm i.d. × 15 cm) with benzene–EtOAc (10 : 1) gave the 3β-ol **11** (1.38 g, 85%): mp 163—164 °C (MeOH). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1710. ^{1}H-NMR δ: 0.68 (3H, s, 18-H_3), 0.75 (3H, s, 19-H_3), 0.85 (6H, d, J=6 Hz, 26-H_3 and 27-H_3), 0.98 (3H, d, J=6 Hz, 21-H_3), 3.45 (1H, m, 3-H), 5.20 (2H, m, 22-H and 23-H). *Anal.* Calcd for $C_{27}H_{44}O_2$: C, 80.94; H, 11.07. Found: C, 80.82; H, 11.21.

(22*R*,23*R*)- and (22*S*,23*S*)-3β,22,23-Trihydroxy-5α-cholestan-6-one (8 and 12)——Compound **11** (1.25 g, 3.38 mmol) in 95% aqueous THF (24 ml) was treated with OsO_4 (50 mg) and NMO (4.0 g) at room temperature for 65 h. Work-up (CH_2Cl_2) gave crude products, which were applied to a column of silica gel (2.5 cm i.d. × 21 cm). Elution with benzene–EtOAc (3 : 2) gave the less polar (22*S*,23*S*)-triol **11** (807 mg, 55%): mp 176—177 °C (EtOH). ^{1}H-NMR δ: 0.70 (3H, s, 18-H_3), 0.76 (3H, s, 19-H_3), 3.15—3.80 (3H, m, 3-H, 22-H, and 23-H). *Anal.* Calcd for $C_{27}H_{46}O_4$: C, 74.61; H, 10.67. Found: C, 74.79; H, 10.64.

Further elution with benzene–EtOAc (2 : 3) gave the more polar (22*R*,23*R*)-triol **8** (396 mg, 27%): mp 200—201 °C (EtOAc). ^{1}H-NMR δ: 0.69 (3H, s, 18-H_3), 0.76 (3H, s, 19-H_3), 3.10—3.80 (3H, m, 3-H, 22-H, and 23-H). *Anal.* Calcd for $C_{27}H_{46}O_4$: C, 74.61; H, 10.67. Found: C, 74.65; H, 10.77.

(22*R*,23*R*)-22,23-Isopropylidenedioxy-3β-methanesulfonyloxy-5α-cholestan-6-one (13a)——The (22*R*,23*R*)-triol **8** (350 mg, 0.806 mmol) in acetone (10 ml) was treated with *p*-TsOH (10 mg) at room temperature for 2 h. Work-up (ether) gave a crude product, and a solution of this in pyridine (4 ml) was treated with mesyl chloride (0.2 ml) at room temperature for 1 h. Work-up (EtOAc) gave the mesylate **13a** (445 mg). The spectral data of **13a** were identical with those of the reported compound.[5b]

(22*R*)-3β,22-Dimethoxymethoxynorchol-5-en-23-al 1,3-Propanedithioacetal (16)——A solution of *n*-butyl lithium in hexane (4.0 ml, 6.24 mmol) was added to a solution of 1,3-dithiane (720 mg, 6.0 mmol) in dry THF (5.0 ml) at 0 °C under argon. The mixture was stirred at room temperature for 1 h. Then, a solution of the 22-aldehyde **14**[8] (1.24 g, 3.0 mmol) in dry THF (10 ml) was added dropwise to the resulting anion solution at 0 °C. The whole was stirred at 0 °C for 30 min. Work-up (ether) and chromatography on silica gel (2.5 cm i.d. × 20 cm) with benzene–EtOAc (100 : 1) gave the 22-ol **15** (1.36 g, 85%): mp 190—193 °C (acetone). ^{1}H-NMR δ: 0.72 (3H, s, 18-H_3), 0.92 (3H, d, J=6 Hz, 21-H_3), 1.02 (3H, s, 19-H_3), 2.80 (4H, m, -CH_2S-), 5.32 (1H, m, 6-H). Compound **15** (1.3 g) in THF–MeOH (2 : 1, 30 ml) was treated with 2 M HCl (1.0 ml) at room temperature for 1 h. Work-up (ether) gave a crude product, and a solution of this in dioxane (10 ml) was treated with chloromethyl methyl ether (2 ml) and diethylcyclohexylamine (3 ml) at room temperature for 18 h. Work-up (EtOAc) and chromatography on silica gel (2.5 cm i.d. × 20 cm) with benzene–EtOAc (25 : 1) gave the product **16** (1.14 g, 90%): mp 106—108 °C (hexane). ^{1}H-NMR δ: 0.69 (3H, s, 18-H_3), 0.99 (3H, s, 19-H_3), 1.00 (3H, d, J=6 Hz, 21-H_3), 2.75 (4H, m, -CH_2S-), 3.34 (3H, s, -OCH_3), 3.40 (3H, s, -OCH_3), 3.62 (1H, d, J=8 Hz, 23-H), 4.20 (1H, d, J=8H, 22-H), 5.32 (1H, m, 6-H). *Anal.* Calcd for $C_{30}H_{50}O_4S_2$: C, 66.87; H, 9.35. Found: C, 67.16; H, 9.35.

(22*R*,23*R*)-3β,22,23-Trihydroxycholest-5-ene 3,22-Dimethoxymethyl Ether (18)——Red mercury (II) oxide (700 mg) and boron trifluoride etherate (0.4 ml) were added to 50% aqueous THF (10 ml) at room temperature. The mixture was stirred at room temperature for 10 min. A solution of the dithiane **16** (772 mg, 1.43 mmol) in THF (10 ml) was added and the whole was stirred at room temperature for 30 min. Then, sat. $NaHCO_3$ solution was added and the mixture was filtered through a pad of Hyflo Super Cel. The filtrate was extracted with EtOAc and concentrated to give the 23-aldehyde **17** (640 mg): amorphous solid. ^{1}H-NMR δ: 0.71 (3H, s, 18-H_3), 0.93 (3H, d, J=6 Hz, 21-H_3), 1.01 (3H, s, 19-H_3), 3.35 (3H, s, -OCH_3), 3.42 (3H, s, -OCH_3), 3.92 (1H, s, 22-H), 5.32 (1H, m, 6-H), 9.75 (1H, s, 23-H). The aldehyde **17** (320 mg, 0.717 mmol) in dry THF (5 ml) was treated with isobutylmagnesium bromide (1.0 mmol) in dry THF (5 ml) at −78 °C under argon for 1 h. Work-up (ether) and chromatography on silica gel (1.5 cm i.d. × 20 cm) with benzene–EtOAc (50 : 1) gave the 23-ol **18** (297 mg, 82%): mp 122—123 °C (MeOH). ^{1}H-NMR δ: 0.69 (3H, s, 18-H_3), 0.92 (6H, d, J=6 Hz, 26-H_3 and 27-H_3), 1.01 (3H, s, 19-H_3), 3.37 (3H, s, -OCH_3), 3.42 (3H, s, -OCH_3), 5.33 (1H, m, 6-H). *Anal.* Calcd for $C_{31}H_{54}O_5$: C, 73.47; H, 10.74. Found: C, 73.75; H, 10.69.

(22*R*,23*R*)-3β,22,23-Trihydroxycholest-5-ene (19)——The 23-ol **18** (280 mg, 0.556 mmol) was treated with acetic anhydride (1 ml) and pyridine (2 ml) at room temperature for 16 h. Work-up (EtOAc) gave a crude product, which was then treated with 6 M HCl (5 ml) in THF–MeOH (1 : 1, 20 ml) at 70 °C for 3 h. Then 30% KOH (5 ml) was added, and the whole was refluxed for 30 min. Work-up (ether) and chromatography on silica gel (2.5 cm i.d. × 15 cm) with benzene–EtOAc (20 : 1) gave the triol **19** (202 mg, 87%): mp 189—191 °C (acetone) (lit.,[5b] mp 187—191 °C), whose

spectral data were identical with the reported values.[5b)]

(22*R*,23*R*)-22,-23-Isopropylidenedioxy-5α-cholest-2-en-6-one (20a) and (22*R*,23*R*)-3α-Hydroxy-22,23-isopropylidenedioxy-5α-cholestan-6-one (21)——The mesylate **13a** (445 mg, 0.80 mmol) in DMF (8 ml) was treated with lithium carbonate (450 mg) under reflux for 1 h. Work-up (EtOAc) followed by saponification with 5% KOH/MeOH (10 ml) at room temperature for 1 h gave, after work-up (ether), crude products, which were applied to a column of silica gel (2.5 cm i.d. × 15 cm). Elution with benzene–EtOAc (50 : 1) gave the 2-ene **20a** (134 mg, 37%), mp 186—188 °C (MeOH) (lit.,[5b)] mp 186—188 °C), whose spectral data were identical with the reported values.[5b)]

Further elution with benzene–EtOAc (10 : 1) gave the 3α-ol **21** (165 mg, 43%): mp 222—223 °C (MeOH). ^{1}H-NMR δ: 0.69 (3H, s, 18-H_3), 0.75 (3H, s, 19-H_3), 1.40 (6H, s, acetonide), 2.70 (1H, m, 5-H), 3.45—3.80 (2H, m, 22-H and 23-H), 4.32 (1H, m, $W_{1/2}=7$ Hz, 3-H). *Anal.* Calcd for $C_{30}H_{50}O_4$: C, 75.90; H, 10.62. Found: C, 75.72; H, 10.55.

(22*S*,23*S*)-22,23-Isopropylidenedioxy-5α-cholest-2-en-6-one (20b)——The (22*S*,23*S*)-triol **12** (610 mg, 1.41 mmol) was converted into the mesylate **13b** (774 mg), as described for **13a**. A mixture of **13b** (774 mg), lithium bromide (500 mg), and DMF (10 ml) was refluxed for 1 h. Work-up (EtOAc) and chromatography on silica gel (2.5 cm i.d. × 16 cm) with benzene–EtOAc (50 : 1) gave the 2-ene **20b** (560 mg, 87%): mp 159—161 °C (MeOH). ^{1}H-NMR δ: 0.73 (6H, s × 2, 18-H_3 and 19-H_3), 0.96 (6H, d, $J=6.5$ Hz, 26-H_3 and 27-H_3), 1.03 (3H, d, $J=6$ Hz, 21-H_3), 1.37 and 1.38 (6H, s × 2, acetonide), 3.65—4.02 (2H, m, 22-H and 23-H), 5.40—5.80 (2H, m, 2-H and 3-H). MS *m/z* 456 (M^+), 441, 399, 381, 355, 312, 297, 271, 269, 255, 157, 128, 85, 43.

(22*R*,23*R*)-3α,22,23-Trihydroxy-5α-cholestan-6-one (7)——Compound **21** (150 mg, 0.317 mmol) was refluxed with 70% aqueous acetic acid (15 ml) for 4 h. Removal of the solvent under reduced pressure and chromatography on silica gel (1.5 cm i.d. × 15 cm) with $CHCl_3$–MeOH (15 : 1) gave the 3α-triol **7** (105 mg, 76%): mp 207—210 °C (EtOAc). ^{1}H-NMR δ: 0.68 (3H, s, 18-H_3), 0.72 (3H, s, 19-H_3), 2.68 (1H, m, 5-H), 3.10—3.77 (2H, m, 22-H and 23-H), 4.08 (1H, m, 3-H). *Anal.* Calcd for $C_{27}H_{46}O_4$: C, 74.61; H, 10.67. Found: C, 74.57; H, 10.71.

(22*S*,23*S*)-2α,3α,22,23-Tetraacetoxy-*B*-homo-7-oxa-5α-cholestan-6-one (23)——The 2-ene **20b** (550 mg, 1.21 mmol) in 95% aqueous THF (15 ml) was treated with OsO_4 (30 mg) and NMO (700 mg) at room temperature for 17 h. Work-up (CH_2Cl_2) gave a crude product, which was refluxed with 70% aqueous AcOH (30 ml) for 4 h. Removal of the solvent followed by acetylation with acetic anhydride (5 ml) and pyridine (10 ml) at 60 °C for 16 h gave, after work-up (EtOAc), a crude product, which was chromatographed on silica gel (2.5 cm i.d. × 23 cm) with benzene–EtOAc (10 : 1) to give the tetraacetoxy-6-ketone **22** (610 mg, 81%). ^{1}H-NMR δ: 0.62 (3H, s, 18-H_3), 1.92 (3H, s, acetyl), 1.99 (3H, s, acetyl), 2.02 (3H, s, acetyl), 2.06 (3H, s, acetyl), 4.50—5.25 (4H, m, 2-H, 3H, 22-H, and 23-H). This in CH_2Cl_2 (10 ml) was treated with two equivalents of trifluoroperacetic acid in the presence of disodium hydrogen phosphate (1.2 g) at 0 °C for 6 h. Work-up (EtOAc) and chromatography on silica gel (2.5 cm i.d. × 25 cm) with benzene–EtOAc (10 : 1) gave the 7-oxalactone **23** (468 mg, 75%): amorphous solid. ^{1}H-NMR δ: 0.65 (3H, s, 18-H_3), 1.92 (3H, s, acetyl), 1.97 (3H, s, acetyl), 2.05 (6H, s × 2, two acetyls), 2.90 (1H, dd, $J=13$, 6 Hz, 5-H), 4.02 (2H, m, 7-H_2), 4.60—4.95 (2H, m, 2-H and 22-H), 4.94—5.30 (2H, m, 3-H and 23-H).

(22*S*,23*S*)-2α,3α,22,23-Tetrahydroxy-*B*-homo-7-oxa-5α-cholestan-6-one (24)——Compound **23** (468 mg, 0.734 mmol) was refluxed with 5% KOH/MeOH (70 ml) for 1 h. The reaction mixture was cooled to 0 °C and acidified with conc. HCl. Work-up (EtOAc) and chromatography on silica gel (2.5 cm i.d. × 25 cm) with $CHCl_3$–MeOH (15 : 1) gave (22*S*,23*S*)-28-norbrassinolide (**24**) (301 mg, 88%): mp 130—133 °C (lit.,[5c)] mp 129.5—133.5 °C) (EtOH), whose spectral data were identical with the reported values.[5c)]

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Bitter Principles of *Ailanthus altissima* SWINGLE.[1] Studies on Oxidative Degradation of the A-Ring of Ailanthone Derivatives

MASAMI ISHIBASHI, TETSUO FURUNO, HÉLÈNE HIETTER,[2] TAKAHIKO TSUYUKI,* and TAKEYOSHI TAKAHASHI

Department of Chemistry, Faculty of Science, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

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12,20-Diacetoxy-11,16-dioxo-1,2-secopicrasa-3,12-diene-1,2-dioic acid and 12α-acetoxy-11β,20-epoxy-11α-methoxy-16-oxo-1,2-secopicrasa-3,13(21)-diene-1,2-dioic acid were prepared from ailanthone and their oxidation reactions with lead tetraacetate were investigated.

Keywords——quassinoid; norpicrasane; ailanthone derivative; oxidation; skeletal rearrangement

In our studies on the bitter principles of *Ailanthus altissima* SWINGLE, we have obtained three new compounds possessing modified picrasane skeletons, shinjudilactone,[3] shinjulacone C,[3] and shinjulactone B (**1**).[4] The structures of these new quassionoids have been determined by single crystal X-ray diffraction analyses, and chemical conversion of ailanthone (**2**)[5] into shinjudilactone[3] and shinjulactone C[6] has been reported. This paper describes some chemical findings obtained from oxidative degradation reactions of the A-ring of ailanthone derivatives aiming at a preparation of **1**.

Shinjulactone B (**1**) possesses a unique 1,2-seco-1-nor-6(5→10)*abeo*-picrasane skeleton, and Chart 1 showed a conceivable biogenetic process of **1** through a 1,2-diketone (A) and a dicarboxylic acid (B) from ailanthone (**2**).[4] On the basis of these considerations, an α-diketone (**3**), an equivalent of A, was prepared from ailanthone (**2**),[6] and treated with hydrogen peroxide in acetic acid.[7] A 1,2-seco-dicarboxylic acid (**4**) corresponding to B, was

(Z: oxidizing agent)

Chart 1

obtained together with an acid anhydride (**5**). On treatment with lead tetraacetate (4.5 eq), the 1,2-seco-dicarboxylic acid (**4**) suffered oxidative decarboxylation to give two spiro lactones (**6** and **7**) in 35 and 8% yields, respectively. The configurations at the C-5 position of **6** and **7** were determined to be 5*R* and 5*S*, respectively, from nuclear Overhauser effect (NOE) difference spectra obtained at 400 MHz. In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **6**, irradiation at δ 1.98 (C_4-Me) caused an increase in the area of the signals at δ 2.46 ($C_{6\beta}$-H) and δ 4.21 (C_{20}-H), while in that of **7**, saturation of the signal at δ 2.00 (C_4-Me) occasioned enhancement of the signal at δ 2.40 ($C_{6\alpha}$-H). The assignments of $C_{6\alpha}$-H and $C_{6\beta}$-H in the ^{1}H-NMR spectra of **6** and **7** were also based on the respective NOE measurements. When the resonance frequencies of C_{20}-H (δ 4.21 for **6** and δ 4.32 for **7**) were irradiated, signals at δ 2.46 for **6** and δ 2.83 for **7** showed increases in area, respectively.

Shinjulactone B (**1**) is considered to be formed biogenetically from the intermediate (B) by loss of carbon dioxide and two hydrogen atoms. On treatment with lead tetraacetate, however, the dicarboxylic acid (**4**) corresponding to B gave the spiro lactones (**6** and **7**) by losing carbon dioxide and four hydrogen atoms. This reaction comprises elimination of the carboxyl group at C-10 followed by not the bond migration from C-5 to C-10, but a deprotonation of a hydrogen atom at C-9, yielding a double bond between C-9 and C-10. The oxidative γ-lactonization took place concomitantly or successively to afford **6** and **7**. The preferential deprotonation could not be avoided even if the reaction was carried out using equimolar lead tetraacetate in various solvents. Previously, Robertson *et al.*[8] and Valenta *et al.*[9] reported the oxidation of deoxodicarboxylic acid monomethyl ester (**8**) with lead tetraacetate to afford an unsaturated keto ester (**9**). The susceptibility to deprotonation at C-9 in these reactions may be ascribed to a relatively high acidity of C_9-H, because of the presence of the carbonyl group at C-11. These results revealed that the presence of the carbonyl group at C-11 is undesirable for preferential bond migration.

3 4 5 6

7 8 9

Then we attempted to introduce a 9(11)-enol acetate into the picrasane skeleton. However, it has been shown that the carbonyl group at C-11 of quassinoids is generally subjected to severe steric hindrance, when a hydroxyl group or other functionality is present at C-1, and that the lactone carbonyl group at C-16 exhibits a reactivity nearly equal to that of an isolated ketone.[10] A monosilyl diacetate (**10**)[6] was treated with isopropenyl acetate in the presence of *p*-toluenesulfonic acid. The reaction product, however, was not the desired 9(11)-

enol acetate, but ailanthone triacetate (**11**), which corresponds to an exchange product of the protective group. This result was explainable in terms of the reasons mentioned above.

10 : R = Si(CH$_3$)$_2$C(CH$_3$)$_3$
11 : R = Ac
12 : R = Ac
18 : R = CH$_3$
13 : R = Ac
19 : R = CH$_3$
14 : R = Ac
20 : R = CH$_3$
15 : R = Ac
21 : R = CH$_3$
16 : R = Ac
17 : R = CH$_3$

Chart 2

Table I. Mass Spectral Data for 11β,20-Epoxypicrasane Derivatives

Compound	M^+	x	y	Other ions		
2	376 (100)	264 (14)	248 (58)	151 (92)	135 (31)	
12	432 (51)	278 (62)	262 (100)	390 (18)	373 (68)	248 (84)
13	430 (89)	276 (47)	260 (100)	388 (15)	371 (33)	245 (48)
14	464 (18)	310 (34)	294 (100)	405 (38)	276 (44)	216 (61)
15	446 (15)	292 (28)	276 (100)	387 (42)	343 (9)	217 (46)
16	418 (25)	264 (47)	248 (90)	375 (9)	359 (78)	233 (100)
17	390 (5)	264 (7)	248 (100)	375 (2)	359 (4)	233 (47)
18	404 (8)	278 (15)	262 (100)	386 (3)	373 (4)	135 (40)
19	402 (8)	276 (6)	260 (100)	217 (17)	135 (22)	
20	436 (0.3)	310 (1)	294 (13)	392 (5)	361 (3)	250 (100)
21	418 (0.8)	292 (4)	276 (100)	387 (2)	234 (29)	217 (21)

m/z (relative intensity, %).

12-*O*-Acetyl-11-*O*-methylailanthone (**12**)[11] was subjected to Jones oxidation to give an α-diketone, 12-*O*-acetyl-11β,20-epoxy-11α-*O*-methylpicrasa-3,13(21)-diene-1,2,16-trione (**13**), which was treated with hydrogen peroxide under the same conditions as those for **3** to afford

12α-acetoxy-11β,20-epoxy-11α-methoxy-16-oxo-1,2-secopicrasa-3,13(21)-diene-1,2-dioic acid (**14**) and its anhydide (**15**). On oxidation with lead tetraacetate in the presence of copper(II) acetate[12] in benzene solution, **14** afforded (in 26% yield) an oxidation product (**16**), which was shown to have the molecular formula, $C_{22}H_{26}O_8$, by high-resolution mass spectrometry (HR-MS), indicating that carbon dioxide and two hydrogen atoms had been removed from the dicarboxylic acid (**14**). The ultraviolet (UV) and infrared (IR) spectra of **16** showed the presence of an α,β-unsaturated δ-lactone grouping and the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum exhibited a singlet signal due to the lactone-terminal carbon at δ 81.7, which is assignable to C-10. The mass spectrum (MS) of **16** gave prominent peaks at *m/z* 264 and 248, which are ascribable to the fragment ions x and y in Chart 2, respectively. The fragment ion x or y is characteristic of 11β,20-epoxypicrasane derivatives (Table I).[13] However, **16** did not show a peak at *m/z* 321 ($[M-C_5H_5O_2]^+$) due to a loss of 3-methyl-2-butenolide, characteristic of shinjulactone B (**1**).[4] From these observations, the oxidation product (**16**) was determined to be 12α-acetoxy-11β,20-epoxy-11α-methoxy-1-oxapicrasa-3,13(21)-diene-2,16-dione. The configuration at C-10 was determined to be *S* from the NOE measurement[14] of the corresponding 12α-methoxy derivative (**17**).[15]

It is suggested that the precursor for the simultaneous bond migration with oxidative decarboxylation is likely to be a dicarboxylic acid with a 9(11)-en-12-one structure in the C-ring.

Experimental[16]

12,20-Diacetoxy-11,16-dioxo-1,2-secopicrasa-3,12-diene-1,2-dioic Acid (4) and Its Anhydride (5)——A 30% hydrogen peroxide solution (12 ml) was added to a solution of 12,20-diacetoxypicrasa-3,12-diene-1,2,11,16-tetrone (3; 266 mg)[6] in acetic acid (12 ml), and the reaction mixture was stirred at room temperature for 16 h. After addition of dichloromethane and 2 M hydrochloric acid (10 ml), the reaction product was extracted with dichloromethane and separated by silica-gel column chromatography to give a 1,2-seco-dicarboxylic acid (4; 185 mg) and an acid anhydride (5; 22 mg). 4: mp 183—186 °C ($CHCl_3$). IR (KBr): 3420, 1740, 1695, 1640, 1220 cm^{-1}. ^{1}H-NMR δ: 1.33, 1.90 (each 3H, s), 1.96 (3H, br s), 2.11, 2.24 (each 3H, s), 3.94 (1H, s), 4.30, 4.74 (each 1H, d, *J* = 12.5 Hz); 4.52 (1H, br s), 5.81 (1H, br s). MS *m/z*: 492 (M^+), 474, 432, 414 (base peak). HR-MS (M^+) Calcd for $C_{24}H_{28}O_{11}$: 492.1629. Found: *m/z* 492.1623. 5: mp 266—269 °C ($CHCl_3$). IR (KBr): 1750, 1740, 1720, 1690, 1225, 1040 cm^{-1}. UV $\lambda_{max}^{ethanol}$: 265 nm (ε 22800). ^{1}H-NMR δ: 1.46, 1.91 (each 3H, s), 1.96 (3H, br s), 2.11, 2.26 (each 3H, s), 3.80 (1H, s), 4.21, 4.62 (each 1H, d, *J* = 12.5 Hz), 4.55 (1H, t, *J* = 3 Hz), 6.10 (1H, br s). MS *m/z* (%): 474 (M^+), 432, 414 (base peak), 372, 315, 189, 60. HR-MS (M^+) Calcd for $C_{24}H_{26}O_{10}$: 474.1525. Found: *m/z* 474.1502.

On treatment with diazomethane, 4 afforded a dimethyl ester, mp 210—212 °C (EtOAc).

(5*R*)- and (5*S*)-12,20-Diacetoxy-11,16-dioxo-1,2-seco-1-norpicrasa-3,12-dien-2,5-olides (6 and 7)——The acid (4; 40 mg) in pyridine (0.5 ml) was added to a suspension of 90% lead tetraacetate (180 mg) in benzene (6 ml) and the reaction mixture was heated at 80 °C for 80 min. The usual work-up and separation by preparative thin layer chromatography (TLC) developed with 3% methanol–chloroform gave the (5*R*)-spiro lactone (6; 12.5 mg) and (5*S*)-isomer (7; 3 mg). 6: mp 233—236 °C (EtOH). IR (KBr): 1760, 1740, 1680, 1665, 1225 cm^{-1}. UV $\lambda_{max}^{ethanol}$ nm (ε): 259 (7400), 276 (8600). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.84, 1.97 (each 3H, s, 13-Me and 10-Me), 1.98 (3H, d, *J* = 1.5 Hz, 4-Me), 2.11, 2.29 (each 3H, s, AcO), 2.23 (1H, dd, *J* = 16.0, 3.4 Hz, 6α-H), 2.41 (1H, dd, *J* = 17.6, 13.7 Hz, 15α-H), 2.46 (1H, dd, *J* = 16.0, 3.1 Hz, 6β-H), 2.93 (1H, dd, *J* = 17.6, 4.6 Hz, 15β-H), 3.05 (1H, dd, *J* = 13.2, 4.6 Hz, 14-H), 4.21, 4.50 (each 1H, d, *J* = 12.3 Hz, 20-H), 4.83 (1H, dd, *J* = 3.4, 3.1 Hz, 7-H), 6.06 (1H, br s, 3-H). ^{13}C-NMR δ: 13.2 q, 15.4 q, 16.4 q, 20.1 q, 20.6 q, 33.0 t, 33.3 t, 38.9 d, 43.3 s, 63.8 t, 74.9 d, 85.1 s, 120.4 d, 130.2 s, 142.6 s, 144.2 s, 147.5 s, 166.5 s, 168.4 s, 168.5 s, 170.0 s, 171.2 s, 180.1 s. MS *m/z*: 444 (M^+), 402, 372, 371, 330 (base peak), 312, 239. HR-MS (M^+) Calcd for $C_{23}H_{24}O_9$: 444.1420. Found: *m/z* 444.1424. 7: mp 191—193 °C (EtOH). IR (KBr): 1750, 1665, 1640, 1220 cm^{-1}. UV $\lambda_{max}^{ethanol}$ nm (ε): 258 (6800), 277 (7400). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.94, 2.00 (each 3H, s, 13-Me and 10-Me), 2.00 (3H, d, *J* = 1.5 Hz, 4-Me), 2.11, 2.29 (each 3H, s, AcO), 2.40 (1H, dd, *J* = 15.6, 3.7 Hz, 6α-H), 2.42 (1H, dd, *J* = 17.7, 12.2 Hz, 15α-H), 2.83 (1H, dd, *J* = 15.6, 3.4 Hz, 6β-H), 2.87 (1H, dd, *J* = 12.2, 5.7 Hz, 14-H), 3.04 (1H, dd, *J* = 17.7, 5.7 Hz, 15β-H), 4.32, 4.51 (each d, *J* = 12.5 Hz, 20-H), 4.81 (1H, dd, *J* = 3.7, 3.4 Hz, 7-H), 5.97 (1H, br s, 3-H). ^{13}C-NMR δ: 14.4, 15.1, 16.4, 20.1, 20.6, 33.8, 34.2, 38.4, 42.3, 64.7, 69.3, 87.6, 119.1, 131.9, 142.6, 142.7, 149.8, 166.9, 168.4, 169.8, 170.9, 171.4, 187.5. MS *m/z* 444 (M^+), 402, 372, 371, 330 (base peak), 312, 239. HR-MS (M^+) Calcd for $C_{23}H_{24}O_9$: 444.1420. Found: *m/z* 444.1426.

12α-Acetoxy-11β,20-epoxy-11α-methoxypicrasa-3,13(21)-diene-1,2,16-trione (13)——12-*O*-Acetoxy-11-*O*-meth-

ylailanthone (**12**; 326 mg)[11] in acetone (30 ml) was treated with Jones reagent (in excess) at 0 °C for 20 min. The usual work-up and purification by silica-gel column chromatography gave the 12-acetoxy-11-methoxy-1,2-dione (**13**; 271 mg), mp 140—141 °C (Me_2CO–hexane). IR (KBr): 1740, 1680, 1235, 1205 cm^{-1}. ^{1}H-NMR δ: 1.56 (3H, s), 2.04 (3H, br s), 2.24, 3.27 (each 3H, s), 3.46 (1H, s), 3.60, 3.95 (each 1H, d, J=8 Hz), 4.52 (1H, t, J=2.5 Hz), 5.29, 5.43, 5.72 (each 1H, s), 6.26 (1H, br s). MS (Table I). HR-MS (M^+) Calcd for $C_{23}H_{26}O_8$: 430.1626. Found: m/z: 430.1614.

12α-Acetoxy-11β,20-epoxy-11α-methoxy-16-oxo-1,2-secopicrasa-3,13(21)-diene-1,2-dioic Acid (14) and Its Anhydride (15)——Hydrogen peroxide (30%; 15 ml) was added to the 12-acetoxy-11-methoxy-1,2-dione (**13**; 262 mg) in acetic acid (15 ml) and the mixture was stirred at room temperature for 25 h. The usual work-up and chromatographic separation on silica gel afforded the 12-acetoxy-11-methoxy-1,2-seco-dicarboxylic acid (**14**; 210 mg) and its anhydride (**15**; 26 mg). **14**: mp 285—290 °C (Me_2CO–Et_2O). IR (KBr): 3600—2700, 1730, 1710, 1635, 1240, 1020 cm^{-1}. ^{1}H-NMR δ: 1.57, 1.99, 2.14, 3.34 (each 3H, s), 3.58, 4.03 (each 1H, d, J=8.5 Hz), 3.76 (1H, s), 4.47 (1H, t, J=2.5 Hz), 5.26, 5.39, 5.66 (each 1H, s), 5.80 (1H, br s). MS (Table I). HR-MS (M^+) Calcd for $C_{23}H_{28}O_{10}$: 464.1680. Found: m/z 464.1675. **15**: Amorphous solid. IR (KBr): 1785, 1740, 1240, 1030 cm^{-1}. ^{1}H-NMR δ: 1.64 (3H, s), 1.97 (3H, br s), 2.16, 3.38 (each 3H, s), 3.51 (1H, s), 3.64, 4.00 (each 1H, d, J=8.5 Hz), 4.58 (1H, t, J=2.5 Hz), 5.34, 5.48, 5.71 (each 1H, s), 6.06 (1H, m). MS (Table I). HR-MS (M^+) Calcd for $C_{23}H_{26}O_9$: 446.1576. Found: m/z 446.1556.

12α-Acetoxy-11β,20-epoxy-11α-methoxy-1-oxapicrasa-3,13(21)-diene-2,16-dione (16)——The 12-acetoxy-11-methoxy-1,2-seco-dicarboxylic acid (**14**; 40 mg) was treated with 90% lead tetraacetate (158 mg) in the presence of copper(II) acetate monohydrate (12 mg) in benzene (10 ml) at reflux temperature for 9 h. Then 2 M hydrochloric acid was added to the cooled reaction mixture and the reaction product was extracted with dichloromethane. Chromatography on silica gel yielded the 12-acetoxy-11-methoxy-1-nor-δ-lactone (**16**; 9.5 mg), mp 112—115 °C (Me_2CO). IR (KBr): 1740, 1720, 1240, 1030 cm^{-1}. UV $\lambda_{max}^{ethanol}$: 215 nm (ε 7700). ^{1}H-NMR δ: 1.72 (3H, s), 1.95 (3H, br s), 2.12, 3.41 (each 3H, s), 3.62, 3.93 (each 1H, d, J=8.5 Hz), 4.49 (1H, t, J=3 Hz), 5.32, 5.50, 5.79 (each 1H, s), 5.91 (1H, m). ^{13}C-NMR δ: 15.8 q, 20.4 q, 21.4 q, 27.8 t, 33.8 t, 39.9 d, 45.3 d, 46.5 s, 46.8 d, 50.7 q, 71.3 d, 72.8 t, 77.0 d, 81.7 s, 110.0 s, 118.2 d, 123.7 t, 140.4 s, 155.8 s, 162.9 s, 168.3 s, 169.3 s. MS (Table I). HR-MS (M^+) Calcd for $C_{22}H_{26}O_8$: 418.1627. Found: m/z 418.1627.

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References and Notes

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14) On irradiation at δ 1.73 (C_{10}-Me), the signal at δ 3.92 due to one of the methylene protons at C-20 was enhanced, and irradiation at δ 3.92 caused an enhancement of the signal at δ 1.73.
15) 11β,20-Epoxy-11α,12α-dimethoxy-1-oxapicrasa-3,13(21)-diene-2,16-dione (**17**) was prepared from 11,12-dimethylailanthone (**18**) through **19** and **20** by using the same procedures as described for **16**. **17**: ^{1}H-NMR (270 MHz) δ: 1.73 (3H, s), 1.93 (3H, br s), 3.05 (1H, s), 3.30 and 3.46 (each 3H, s), 3.57 and 3.92 (each 1H, d, J=8.5 Hz), 4.45 (1H, t, J=3 Hz), 5.23 and 5.31 (each 1H, s), 5.89 (1H, m). HR-MS (M^+) Calcd for $C_{21}H_{26}O_7$: 390.1677. Found: m/z 390.1697. Mass spectral data for **17**—**21** are given in Table I.
16) General procedures are the same as those in ref. 10. ^{1}H-NMR spectra were measured for $CDCl_3$ solutions at 90 MHz unless otherwise stated. ^{13}C-NMR spectra were measured for $CDCl_3$ solutions at 22.5 MHz.

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Antitumor Principles from *Ginkgo biloba* L.

HIDEJI ITOKAWA,*[,a] NOBUO TOTSUKA,[a] KEISUKE NAKAHARA,[a] KOICHI TAKEYA,[a] JEAN-PIERRE LEPOITTEVIN,[b] and YOSHINORI ASAKAWA[b]

Tokyo College of Pharmacy,[a] Horinouchi 1432–1, Hachioji, Tokyo 192–03, Japan and Institute of Pharmacognosy, Tokushima Bunri University,[b] Yamashirocho, Tokushima 770, Japan

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Seven long-chain phenols were isolated from *Ginkgo biloba* L. Three of them, anacardic acid (Ib), bilobol (IIa), and cardanol (IIIa), showed antitumor activity against Sarcoma 180 ascites in mice. The antitumor effectiveness was rated (+ +) for Ib, (+ + +) for IIa, and (+ + +) for IIIa at 40 mg/kg/d by the total packed cell volume method.

Keywords——*Ginkgo biloba*; Ginkgoaceae; antitumor activity; Sarcoma 180 ascites; anacardic acid; bilobol; cardanol

Preliminary antitumor screening tests of crude drugs and collected plants[1,2] have been carried out by means of the total packed cell volume method[3] using Sarcoma 180 ascites in mice. In subsequent tests, the methanol extract from the sarcotesta of *Ginkgo biloba* showed remarkable activity. In this paper, we describe the isolation and the activity of the antitumor principles in *Ginkgo biloba* L.

When an aqueous solution of the methanolic extract prepared from the sarcotesta of *Ginkgo biloba* was partitioned successively with chloroform, ethyl acetate, and *n*-butanol as shown in Chart 1, the antitumor activity was concentrated in the chloroform extract. The extract was subjected to silica gel and/or alumina column chromatography, and fractions containing anacardic acid, bilobol, and cardanol were obtained. Each of them was subjected

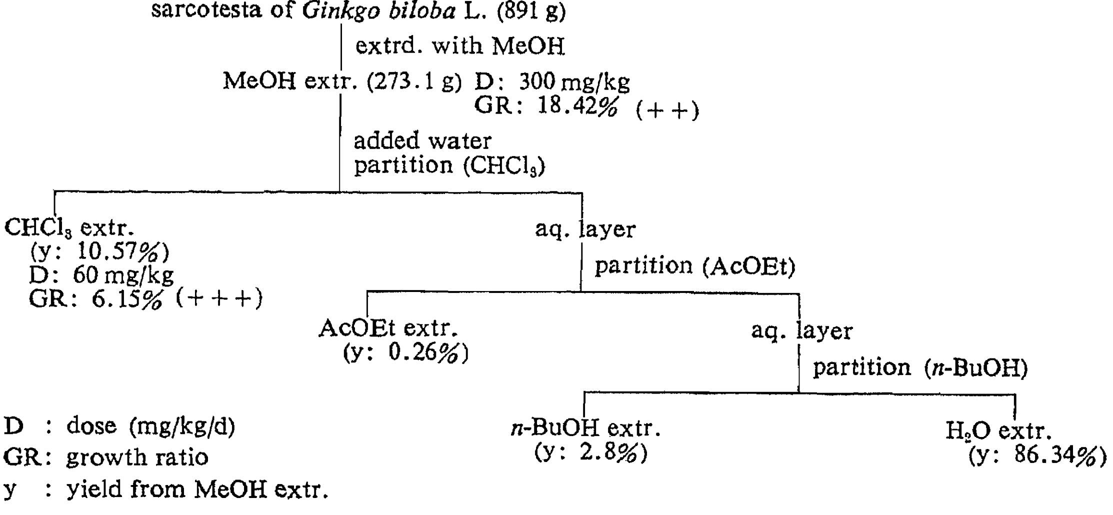

Chart 1

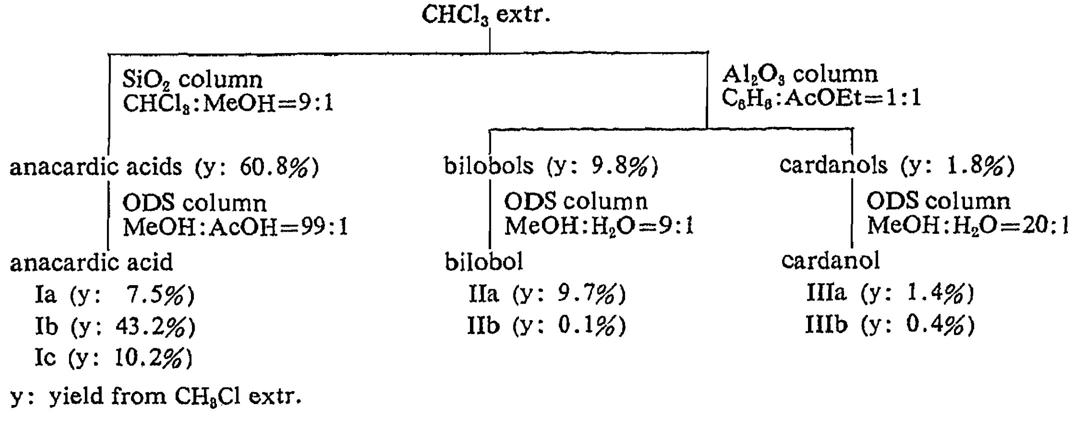

Chart 2

to octadecylsilyl (ODS) column chromatography as shown in Chart 2, yielding anacardic acid (Ia, b, c), bilobol (IIa, b), and cardanol (IIIa, b).

Compound Ib was isolated as a major antitumor principle from the chloroform extract. The spectral data led us to conclude that Ib is one of the anacardic acids[4] (Fig. 1). The length and degree of unsaturation of the side chain of Ib′, the methyl ester of Ib, were deduced from the molecular ion peak at m/z 360 in the mass spectrum (MS). The position of the double bond was determined to be $\Delta^8-15:1$ from the molecular ion peak at m/z 278 in the MS after ozonolysis of Ib′. In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum, carbons at allylic positions in the side chain of Ib were observed at δ 27.21 ppm. Therefore, the double bond was concluded to be *cis*.[5] The compounds Ia and Ic were similarly determined to be the anacardic acids with 13:0 and $\Delta^{10}-17:1$ side chains, respectively.

Compounds IIa and IIIa were isolated as minor antitumor principles. Compounds IIa, b and IIIa, b were determined to be bilobols and cardanols, respectively, by procedures similar to those used to identify anacardic acids. The side chain of IIb was $C_{17}H_{33}$, different from those of bilobols reported in the literature,[6,7] whose side chains were $C_{13}H_{27}$, $C_{15}H_{29}$, $C_{15}H_{27}$, and $C_{15}H_{25}$.

Compounds Ib, b′, IIa, a′, and IIIa, a′ were tested by means of the total packed cell volume method[3] using Sarcoma 180 ascites in mice. The results are shown in Table I. Compounds Ib, IIa, and IIIa showed potent activity, but Ib′, IIa′, and IIIa′ showed no activity.

On the other hand, antimicrobial activity of Ib has been reported,[7,8] and we found that IIa and IIIa also have weak antimicrobial activity (Table II). Thus, the antitumor activity against Sarcoma 180 ascites appears not to require the carboxyl group, whereas the antimicrobial activity does require it.

Ia:	$R^1=(CH_2)_{12}CH_3$,	$R^2=COOH$,	$R^3=OH$,	$R^4=H$
Ib:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$,	$R^2=COOH$,	$R^3=OH$,	$R^4=H$
Ic:	$R^1=(CH_2)_9CH=CH(CH_2)_5CH_3$,	$R^2=COOH$,	$R^3=OH$,	$R^4=H$
Ia′:	$R^1=(CH_2)_{12}CH_3$,	$R^2=COOCH_3$,	$R^3=OH$,	$R^4=H$
Ib′:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$,	$R^2=COOCH_3$,	$R^3=OH$,	$R^4=H$
Ic′:	$R^1=(CH_2)_9CH=CH(CH_2)_5CH_3$,	$R^2=COOCH_3$,	$R^3=OH$,	$R^4=H$
IIa:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$,	$R^2=H$,	$R^3=R^4=OH$	
IIb:	$R^1=(CH_2)_9CH=CH(CH_2)_5CH_3$,	$R^2=H$,	$R^3=R^4=OH$	
IIa′:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$,	$R^2=H$,	$R^3=R^4=OCOCH_3$	
IIIa:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$,	$R^2=R^4=H$,	$R^3=OH$	
IIIb:	$R^1=(CH_2)_9CH=CH(CH_2)_5CH_3$,	$R^2=R^4=H$,	$R^3=OH$	
IIIa′:	$R^1=(CH_2)_7CH=CH(CH_2)_5CH_3$,	$R^2=R^4=H$,	$R^3=OCOCH_3$	

Fig. 1. The Structures of Ia—IIIa′

TABLE I. Antitumor Activity on Sarcoma 180 Ascites in Mice

Compound	Dose (mg/kg)	GR (%)	Assessment
Ib	40	17.4	++
IIa	40	0.4	+++
IIIa	40	0.0	+++
Ib′	60	110.4	−
IIa′	40	81.9	−
IIIa′	40	105.7	−

TABLE II. Antimicrobial Activity (MIC, μg/ml)

Organisms	IIa (Bilobol)	IIIa (Cardanol)
S. aureus 209PJC-1	>100	50
S. aureus TERAJIMA	>100	50
S. aureus SMITH	>100	25
S. aureus 911-5	25	25
S. aureus E31080	>100	50
S. aureus JS1	25	25
E. faecalis E22018	25	25

MIC: minimum inhibitory concentration.

Experimental

Silica gel column chromatography was carried out on Wakogel C-200 (100—200 mesh). Alumina column chromatography was carried out on Merck Art. 1097 (Aluminiumoxide 90 standardisiert) (70—230 mesh). In general, silica gel and alumina for column chromatography were employed in amounts equivalent to 100 times the sample amount. For further purification, high-performance liquid chromatography (HPLC) was carried out on a CIG column system (Kusano Scientific Co., Tokyo) with IATROBEADS (60 μ silica gel, IATRON Co., Tokyo) as the stationary phase. Spectral data were obtained on the following instruments; ultraviolet spectrum (UV) on a Hitachi 557, infrared spectrum (IR) on a Jasco A-302, NMR on a Bruker AM400, MS on a Hitachi M-80, and optical rotation on a Jasco DIP-4.

Extraction and Isolation——The sarcotesta of *Ginkgo biloba* L. (891 g) was extracted with MeOH (3 l). The concentrated MeOH extract (273.1 g) was diluted with water and then shaken successively with chloroform, ethyl acetate, and *n*-butanol in a separatory funnel three times. The three portions of each organic solvent were combined and evaporated. The chloroform extract, which showed potent activity, was separated as shown in Chart 2.

Compound Ia: Colorless oil. MS *m/z* (%): 276 (28, $M^+ - CO_2$), 150 (2), 149 (3), 121 (10), 120 (4), 109 (8), 108 (100), 107 (26). IR (CCl_4) cm^{-1}: 3520, 3450, 3040, 2950, 2870, 1615, 1592, 1470, 1465, 1275, 1155. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.30 (20H, br), 1.60 (2H, quintet, 7.6), 2.96 (2H, t, 7.6), 6.75 (1H, dd, 7.4, 1.0), 6.85 (1H, dd, 7.4, 1.0), 7.32 (1H, t, 7.4), 11.33 (1H, s).

Compound Ia′: Colorless oil. MS *m/z* (%): 334 (50, M^+), 302 (10), 185 (10), 175 (20), 166 (55), 161 (50), 147 (100), 134 (70), 121 (20), 107 (35), 105 (57), 55 (40). IR (CCl_4) cm^{-1}: 3100, 3030, 2920, 2850, 1735, 1608, 1575, 1448, 1313, 1247, 1209, 1165, 1118. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.30 (20H, br), 1.53 (2H, quintet, 7.7), 2.88 (2H, t, 7.7), 3.96 (3H, s), 6.72 (1H, dd, 8.0, 1.0), 6.83 (1H, dd, 9.0, 1.0), 7.28 (1H, dd, 9.0, 8.0).

Compound Ib: Colorless powder, mp 40—41 °C. MS *m/z* (%): 302 (15, $M^+ - CO_2$), 276 (5), 147 (6), 133 (6), 121 (14), 120 (25), 108 (100), 107 (75). IR (CCl_4) cm^{-1}: 3520, 3440, 3030, 3000, 2920, 1675, 1645, 1606, 1450, 1380, 1300, 1245, 1207, 1175. UV λ_{max}^{EtOH} nm (ε): 240.0 (2050), 300.0 (1840). ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.30 (16H, br), 1.60 (2H, quintet, 7.6), 2.01 (4H, q, 6.0), 2.97 (2H, t, 7.6), 5.34 (2H, m), 6.75 (1H, dd, 7.4, 1.0), 6.85 (1H, dd, 7.4, 1.0), 7.32 (1H, t, 7.4), 11.32 (1H, s). ^{13}C-NMR ($CDCl_3$) δ: 14.05 (t), 22.65 (t), 27.21 (t), 29.25 (t), 29.32 (t), 29.38 (t), 29.49 (t), 29.58 (t), 29.61 (t), 29.76 (t), 31.79 (t), 31.94 (t), 36.42 (t), 110.50 (s), 115.90 (d), 122.72 (d), 129.78 (d), 129.90 (d), 135.36 (d), 147.79 (s), 163.58 (s), 176.41 (s).

Compound Ib′: Colorless oil. MS *m/z* (%): 360 (27, M^+), 328 (10), 310 (10), 175 (8), 166 (100), 161 (40), 147 (80), 134 (65), 121 (60), 107 (30), 105 (55), 55 (85). IR (CCl_4) cm^{-1}: 3100, 3030, 2920, 2850, 1735, 1665, 1608, 1575, 1448, 1313, 1247, 1209, 1165, 1118. UV λ_{max}^{EtOH} nm (ε): 217.5 (2610), 243.3 (2730), 283.0 (1410), 310.0 (1490). ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.1 Hz), 1.30 (16H, br), 1.53 (2H, quintet, 7.7), 2.03 (4H, q, 6.0), 2.88 (2H, t, 7.7), 3.96 (3H,

s), 5.35 (2H, m), 6.72 (1H, dd, 8.0, 1.0), 6.84 (1H, dd, 9.0, 1.0), 7.29 (1H, dd, 9.0, 8.0).

Compound Ic: Colorless powder, mp 45—46 °C. MS m/z (%): 330 (46, $M^+ - CO_2$), 304 (45), 234 (2), 175 (4), 149 (19), 147 (22), 133 (5), 121 (22), 120 (25), 108 (100), 107 (65). IR (CCl_4) cm^{-1}: 3520, 3440, 3050, 2980, 2840, 1615, 1598, 1590, 1490, 1450, 1275, 1152. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.30 (20H, br), 1.60 (2H, quintet, 7.6), 2.02 (2H, q, 6.0), 2.97 (2H, t, 7.6), 5.34 (2H, m), 6.75 (1H, dd, 7.4, 1.0), 6.85 (1H, dd, 7.4, 1.0), 7.32 (1H, t, 7.4), 11.33 (1H, s).

Compound Ic′: Colorless oil. MS m/z (%): 388 (30, M^+), 356 (20), 338 (15), 299 (8), 175 (10), 166 (75), 161 (35), 133 (65), 121 (15), 107 (35), 105 (40), 55 (100). IR (CCl_4) cm^{-1}: 3100, 3030, 2920, 2850, 1735, 1665, 1608, 1575, 1450, 1310, 1245, 1205, 1165, 1117. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.28 (20H, br), 1.53 (2H, quintet, 7.0), 2.02 (4H, q, 6.0), 2.88 (2H, t, 7.0), 3.97 (3H, s), 5.35 (2H, m), 6.72 (1H, dd, 8.0, 1.0), 6.83 (1H, dd, 9.0, 1.0), 7.28 (1H, dd, 9.0, 8.0).

Compound IIa: Colorless powder, mp 30—31 °C. MS m/z (%): 318 (33, M^+), 292 (4), 222 (14), 205 (10), 191 (13), 177 (9), 166 (23), 163 (22), 149 (18), 137 (100). IR (CCl_4) cm^{-1}: 3620, 3450, 3030, 1635, 1600, 1467, 1380, 1340, 1300, 1210, 1150, 1000. UV λ_{max}^{EtOH} nm (ε): 231.0 (1760), 274.5 (1260), 280.5 (1240). ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, J= 7.0 Hz), 1.30 (16H, br), 1.57 (2H, quintet, 7.5), 2.01 (4H, q, 6.5), 2.48 (2H, t, 7.5), 5.36 (2H, m), 6.17 (1H, t, 2.2), 6.24 (2H, d, 2.2). ^{13}C-NMR ($CDCl_3$) δ: 14.12 (q), 22.68 (t), 27.25 (t), 29.01 (t), 29.34 (t), 29.46 (t), 29.76 (t), 29.83 (t), 31.10 (t), 31.82 (t), 35.88 (t), 100.27 (d), 108.21 (d), 129.89 (d), 129.99 (d), 146.44 (s), 156.30 (s).

Compound IIa′: Colorless oil. MS m/z (%): 402 (14, M^+), 360 (12), 318 (28), 292 (6), 222 (8), 205 (5), 166 (8), 163 (7), 137 (11), 124 (100), 123 (24). IR (CCl_4) cm^{-1}: 3020, 2940, 2860, 1775, 1620, 1595, 1450, 1370, 1200, 1175, 1120, 1020. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, J=7.0 Hz), 1.29 (16H, br), 1.60 (2H, quintet, 7.5), 2.01 (4H, q, 6.0), 2.27 (3H, s), 2.60 (2H, t, 7.5), 5.34 (2H, m), 6.74 (1H, t, 2.1), 6.80 (2H, d, 2.1).

Compound IIb: Colorless powder, mp 35—36 °C. MS m/z (%): 346 (60, M^+), 320 (24), 250 (10), 205 (8), 191 (12), 177 (8), 166 (41), 163 (22), 149 (20), 137 (14), 124 (100). IR (CCl_4) cm^{-1}: 3620, 3400, 3030, 2930, 1635, 1600, 1470, 1145, 995. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.30 (20H, br), 1.57 (2H, quintet, 7.5), 2.01 (4H, q, 6.5), 2.48 (2H, t, 7.5), 5.36 (2H, m), 6.17 (1H, t, 2.2), 6.24 (2H, d, 2.2).

Compound IIIa: Colorless oil. MS m/z (%): 302 (25, M^+), 276 (8), 206 (2), 175 (3), 161 (5), 149 (6), 147 (13), 133 (13), 120 (46), 108 (100), 107 (72). IR (CCl_4) cm^{-1}: 3620, 3450, 3050, 1615, 1598, 1590, 1490, 1470, 1455, 1275, 1185, 1152. UV λ_{max}^{EtOH} nm (ε): 226.0 (1710), 273.0 (1500). ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.30 (16H, br), 1.57 (2H, quintet, 8.0), 2.01 (4H, q, 6.0), 2.52 (2H, t, 8.0), 5.35 (2H, m), 6.64 (1H, d, 7.7), 6.65 (1H, s), 6.73 (1H, d, 7.7), 7.11 (1H, t, 7.7). ^{13}C-NMR ($CDCl_3$) δ: 14.10 (q), 22.67 (t), 27.21 (t), 27.23 (t), 29.00 (t), 29.24 (t), 29.30 (t), 29.41 (t), 29.69 (t), 29.76 (t), 31.28 (t), 31.80 (t), 35.84 (t), 112.52 (d), 115.35 (d), 120.93 (d), 129.37 (d), 129.86 (d), 129.98 (d), 144.93 (s), 155.49 (d).

Compound IIIa′: Colorless oil. MS m/z (%): 344 (16, M^+), 302 (30), 276 (20), 147 (11), 133 (5), 120 (19), 108 (100), 107 (56). IR (CCl_4) cm^{-1}: 3020, 2940, 2860, 1770, 1610, 1590, 1490, 1370, 1260, 1210, 1145. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, 7.0), 1.28 (16H, br), 1.60 (2H, quintet, 8.0), 2.00 (4H, q, 6.0), 2.29 (3H, s), 2.60 (2H, t, 8.0), 5.34 (2H, m), 6.88 (1H, s), 6.89 (1H, d, 7.7), 7.03 (1H, d, 7.7), 7.27 (1H, t, 7.7).

Compound IIIb: Colorless oil. MS m/z (%): 330 (48, M^+), 304 (44), 234 (2), 175 (4), 149 (18), 147 (22), 133 (6), 121 (22), 120 (23), 108 (100). IR (CCl_4) cm^{-1}: 3620, 3450, 3050, 2940, 1615, 1598, 1490, 1470, 1455, 1275, 1185, 1152. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.0 Hz), 1.30 (20H, br), 1.57 (2H, quintet, 8.0), 2.02 (4H, q, 6.0), 2.53 (2H, t, 8.0), 5.35 (2H, m), 6.64 (1H, d, 7.7), 6.65 (1H, s), 6.73 (1H, d, 7.7), 7.11 (1H, t, 7.7).

Selective Methylation of Anacardic Acid——Selective methylation of the carboxyl group of anacardic acid was achieved by short treatment with CH_2N_2 in anhydrous ether. After removal of the solvent, methyl anacardic ester was obtained.

Ozonolysis of Long-Chain Phenols——A solution of 1—10 mg of an unsaturated long-chain phenol (Ib′, c′, IIa, b, IIIa, or IIIb) in 10 ml of MeOH was ozonized by bubbling ozone through the solution. After ozonolysis, the ozonide was reduced with 10 mg of zinc and 10 drops of acetic acid at 30 °C for 1 h, and neutralized with 1 N NaOH. The reaction mixture was partitioned between chloroform and water, then the organic layer was evaporated to give the corresponding aldehyde.

Assay of Activity Against Sarcoma 180 Ascites[9]——ICR male mice, 5 weeks old, supplied by Clea Japan Co., Ltd., were used in groups of 6 animals. Sarcoma 180 ascites, provided by the National Cancer Center Research Institute and maintained in successive generations by us, was implanted i.p. at 1×10^6 cells/body. Administration of a test drug was started at 1 d after the implantation and continued for 5 d by the i.p. route. The effectiveness was evaluated by means of the total packed cell volume method[3]: growth ratio (GR%) = (packed cell volume (PCV) of test groups/PCV of control groups) × 100; GR = 0—10% (+++), 11—47% (++), 41—65% (+), and over 66% (−).

Drug Treatment——A 0.5% solution of carboxymethylcellulose (CMC) in isotonic sodium chloride was used as a vehicle for the injection of test drugs. The dose ranges used for treatment are shown in Charts 1 and 2. Control group mice received equal volumes of normal saline containing 0.5% CMC. The results were evaluated according to the standard methods described above.

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Diterpene Glycosides from Leaves of Chinese *Rubus chingii* and Fruits of *R. suavissimus*, and Identification of the Source Plant of the Chinese Folk Medicine "Fu-pen-zi"

WEN-HUA CHOU,[a] TOSHIHIKO OINAKA,[b] FUJIKO KANAMARU,[b] KENJI MIZUTANI,[b] FENG-HUAI CHEN[a] and OSAMU TANAKA*,[b]

South China Institute of Botany, Academia Sinica,[a] Guangzhou, China and Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine,[b] Kasumi, Minami-ku, Hiroshima 734, Japan

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Five diterpene glycosides, goshonoside-F1, -F2, -F3, -F4 and -F5 (4—8), which are characteristic of the leaves of Japanese *R. chingii* (Gosho-ichigo), were isolated from the leaves of Chinese *R. chingii* (Zhan-yei fu-pen-zi). This proved the chemotaxonomical identity of the plants. Compound **8** was also detected in commercial Chinese folk medicine, Fu-pen-zi, indicating that the source plant of this drug is *R. chingii*. Rubusoside (**1**), which is the sweet diterpene glycoside of the leaves of *R. suavissimus* (Chinese name: Gan-yei xuen-gou-zi), was also identified in fruits of the plant.

Keywords——*Rubus chingii*; *Rubus suavissimus*; Rosaceae; Chinese folk medicine; fu-pen-zi; rubusoside; sweetener; goshonoside; diterpene glycoside; chemotaxonomy

A rosaceous plant, the leaves of which taste very sweet, grows wild in Guang-xi and Guang-dong, China. In our studies on Chinese sweet plants, the sweet principle, named rubusoside (**1**), was isolated from leaves of this plant cultivated in the Botanical Garden of the South China Institute of Botany, Guangzhou, Guang-dong.[1] Compound **1** was identified as the β-D-glucosyl ester of 13-*O*-β-D-glucosyl-steviol, which had already been derived from stevioside (**2**), the major sweet principle of the leaves of *Stevia rebaudiana* BERTONI (Compositae), by enzymic partial hydrolysis.[2,3] Another sweet glycoside named rebaudioside A (**3**) has been isolated from the leaves of *S. rebaudiana*, and is considered to taste better than **2**.[3] It is noteworthy that **1** is an important intermediate in the synthesis of **3** from **2**.[2,3] This is the first example of the isolation of a diterpene glycoside from rosaceous plants. Enzymic transglucosylation of **1** and the structure–sweetness relationship of bisglycosides of this type have been reported.[4]

In a previous report,[1] this sweet plant tentatively was assigned as *Rubus chingii* HU (Chinese name: Zhan-yei fu-pen-zi, 掌葉覆盆子) by Emeritus Professor H. Hara, University of Tokyo. Previously, Migo reported that Gosho-ichigo growing in Yamaguchi-ken, Kohchi-ken and Fukuoka-ken, Japan, is taxonomically identical with *R. chingii*[5] growing in An-hui, Jiang-su, Zhe-jiang, Jiang-xi and Fu-jien, China. Leaves of Gosho-ichigo were collected at Nodani, Yamaguchi-ken and Sameura, Kohchi-ken, and the close morphological similarity to the above Chinese sweet plant was confirmed. However, the leaves of Gosho-ichigo do not show any sweet taste. Further, **1** could not be obtained from leaves of Gosho-ichigo, but five new characteristic labdane-type diterpene glycosides named goshonosides-F1 through -F5 (**4**—**8**) were isolated.[6] Based on this chemical difference from *R. chingii*, the name *R.*

suavissimus S. LEE (Chinese name: Gan-yei xuen-gou-zi, 甘葉懸鈎子) has been proposed for the above sweet plant.[7]

Remarkable differences in chemical constituents have been occasionally found between Chinese and Japanese plants assigned the same botanical name. The present report deals with identification of glycosides of the leaves of Chinese *R. chingii*, Zhan-yei fu-pen-zi, which has remained to be investigated. Isolation of a diterpene glycoside from fruits of *R. suavissimus* is also reported. On the basis of the results, we consider that the source plant of commercial Chinese folk medicine, Fu-pen-zi (fruits of a kind of *Rubus* spp., 覆盆子), can be identified.

1: R = Glc
2: R = Glc^{2}—Glc
3: R = Glc^{2}—Glc, ^{3}—Glc

	R_1	R_2	R_3
4	-OH	-Glc	$-CH_2OH$
5	-OH	-H	$-CH_2O$-Glc
6	-H	-Glc	-COO-Glc
7	-H	-Glc	$-CH_2O$-Glc
8	-OH	-Glc	$-CH_2O$-Glc

Glc: β-D-glucopyranosyl

Chart 1

Leaves of Zhan-yei fu-pen-zi collected in Zhe-jian, China were extracted with methanol. An aqueous suspension of the methanolic extract was washed with ether and then the water-soluble portion was chromatographed on highly porous polymer to give a glycoside fraction, which was further rechromatographed, affording five glycosides. These were identified as **4**, **5**, **6**, **7** and **8**, which have already been isolated from leaves of Japanese *R. chingii*, Gosho-ichigo, in similar yields.[6] Of these glycosides, the major glycoside, **8**, was isolated from both plants in a significantly high yield (more than 5%). This result indicated that Japanese Gosho-ichigo is identical with Chinese Zhan-yei fu-pen-zi not only morphologically but also chemically, being different from the sweet plant, *R. suavissimus*.

Glycosides of the leaves of 39 *Rubus* species other than *R. suavissimus* and *R. chingii* have been examined.[8] In this study, glucosyl esters of 19α-hydroxyursolic acid derivatives were isolated from some of the species, while no diterpene glycosides such as **1** or goshonosides were isolated from any of the plants. This suggested a special chemotaxonomical position of both *R. chingii* and *R. suavissimus* in this genus.

It was reported that **1** could not be obtained from roots of *R. suavissimus*, but glucosyl esters of 19α-hydroxyursolic acid derivatives were isolated.[9] The chemical constituents of the fruits have not been investigated. Thus, fruits of *R. suavissimus* cultivated at the Botanical Garden of the South China Institute of Botany were extracted with methanol. The extract was separated by chromatography on highly porous polymer to give a glycoside fraction, which was further rechromatographed, affording **1** (yield: 0.018%), the characteristic sweet glycoside of the leaves of this plant.

Fu-pen-zi, a Chinese folk medicine for aged people, has been described as the fruits of Chinese *R. chingii*. However, in the Chinese literature, it is also stated that fruits of several other plants of *Rubus* spp. are sometimes also used as Fu-pen-zi.[10] Further, because of the close morphological similarity to *R. chingii*, fruits of *R. suavissimus* might be used as Fu-pen-zi. Commercial Fu-pen-zi obtained from a Hong Kong market was extracted with methanol. The methanolic extract was separated by chromatography on highly porous polymer to give a

glycoside fraction which was further rechromatographed, affording **8** (yield: 0.017%) which is a characteristic constituent of the leaves of Japanese and Chinese *R. chingii* and has not been detected in leaves of other *Rubus* species as yet. The present results thus allow us to identify the source plant of this crude drug as *R. chingii*, in agreement with the morphological study by Namba *et al.*[10] It is noteworthy that **1** and **8** can be used as a marker substances for the identification of the source plant of commercial *Rubus* fruits, Fu-pen-zi.

Experimental

High-performance liquid chromatography (HPLC) was carried out with a Toyo Soda CCPM pump. Each peak was monitored with a differential refractometer (Toyo Soda RI 8000) and an absorbance detector (Toyo Soda UV 8000, set at 210 nm). The plant materials of *R. chingii* and *R. suavissimus* used in the present study were collected and taxonomically identified by F. Chen of the South China Institute of Botany, and specimens are deposites in the Herbarium of this Institute.

Identification of Known Glycosides——Each known glycoside was identified by comparison of the optical rotation and proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra with those of a corresponding authentic specimen.

Isolation of Goshonosides from Leaves of Chinese *R. chingii*, Zhan-yei Fu-pen-zi——The leaves (100 g) collected in Zhe-jian Province were extracted with hot MeOH to give the MeOH extract (47 g) after concentration to dryness. A suspension of the MeOH extract in H_2O was washed with Et_2O and the H_2O layer was subjected to chromatography on highly porous polymer (Diaion HP-20, Mitsubishi Kasei Co., Ltd.). Elution was carried out with H_2O, 45% MeOH, 80% MeOH, MeOH and Me_2CO, successively. The eluate with 80% MeOH was rechromatographed on silica gel with EtOAc–EtOH–H_2O (gradient from 80 : 8 : 1 to 80 : 20 : 10) to give four fractions, Fr. 1—4. Fr. 2 was separated into two fractions, Fr. 2-1 and Fr. 2-2 by rechromatography on silica gel (solvent: EtOAc–EtOH–H_2O (80 : 8 : 1)). Fr. 1 and Fr. 2-1 were combined and further chromatographed on silanized silica gel (LiChroprep RP-8) (solvent: 60% MeOH) followed by HPLC on TSKgel ODS-120T (4 mm i.d. × 25 cm, Toyo Soda Co., Ltd.; mobile phase, 65% MeCN; flow rate, 0.6 ml/min) to give **4** as a white powder, $[\alpha]_D^{22}$ $-49.5°$ (c=0.74, MeOH), and **5** as a white powder, $[\alpha]_D^{20}$ $-28.2°$ (c=0.74, MeOH), in yields of 0.6 and 0.4%, respectively. Fr. 2-2 and Fr. 3 were combined and chromatographed on silica gel (solvent: EtOAc–EtOH–H_2O (90 : 10 : 1)), affording **6** as a white powder, $[\alpha]_D^{21}$ $-34.3°$ (c=0.70, MeOH) and **7** as a white powder, $[\alpha]_D^{22}$ $-37.0°$ (c=0.94, MeOH) in yields of 0.03 and 0.05%, respectively. Fr. 4 was chromatographed on silica gel and eluted with EtOAc–EtOH–H_2O (8 : 5 : 1) to give **8** as a white powder, $[\alpha]_D^{22}$ $-42.0°$ (c=1.0, MeOH) in a yield of 6.9%.

Isolation of 1 from Fruits of *R. suavissimus*——The fruits (100 g) collected in the Botanical Garden of the South China Institute of Botany, Academia Sinica, were extracted with hot MeOH to give the MeOH extract (34 g) after concentration to dryness. An aqueous suspension of the MeOH extract was chromatographed on Diaion HP-20. Elution was carried out with H_2O, 40% MeOH, 60% MeOH, 80% MeOH, MeOH and Me_2CO, successively. The 80% MeOH eluate was separated by chromatography on LiChroprep RP-8 (solvent: 65% MeOH) and then by HPLC on TSKgel ODS-120T (21.5 mm i.d. × 30 cm; mobile phase, 67% MeOH; flow rate, 6 ml/min), affording **1**, after recrystallization from MeOH, as colorless prisms, mp 178—181 °C, $[\alpha]_D^{25}$ $-39.4°$ (c=1.01, MeOH) in a yield of 0.018%.

Isolation of 8 from Commercial Fu-pen-zi——Fu-pen-zi (400 g) purchased in Hong Kong market was extracted with hot MeOH to give the MeOH extract (38 g) after concentration to dryness. An aqueous suspension of the MeOH extract was chromatographed on Diaion HP-20. Elution was carried out with H_2O, 40% MeOH, 80% MeOH, MeOH and Me_2CO, successively. The 80% MeOH eluate was subjected to chromatography on silica gel (solvent: EtOAc–EtOH–H_2O (8 : 2 : 1)) and then on LiChroprep RP-8 (solvent: 65% MeOH) followed by HPLC on TSKgel ODS-120A (21.5 mm i.d. × 30 cm, Toyo Soda Co., Ltd.; mobile phase, 67.5% MeOH; flow rate, 3 ml/min), affording **8** as a white powder, $[\alpha]_D^{20}$ $-40.3°$ (c=1.15, MeOH) in a yield of 0.017%.

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Chemotaxonomic Studies on the Genus *Citrus*. I. Distribution of Flavones in the Subgroup Microcarpa

MIZUO MIZUNO,*[a] MUNEKAZU IINUMA,[a] TOSHIYUKI TANAKA,[a] YOSHINOBU MATOBA,[a] YOSHIKO FUJII,[a] JIN MURATA,[b] HIROKO MURATA,[c] and MASAO IWAMASA[d]

Department of Pharmacognosy, Gifu Pharmaceutical University,[a] 5-6-1 Mitahora-higashi, Gifu 502, Japan, Botanical Gardens, Faculty of Science, The University of Tokyo,[b] 3-7-1 Hakusan, Bunkyo-ku, Tokyo 112, Japan, Faculty of Pharmaceutical Sciences, Setsunan University,[c] Hirakata, Osaka 571-01, Japan, and Department of Horticultural Science, Saga University,[d] Honjo 1, Saga 840, Japan

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The distribution of polyoxygenated flavones in the subgroup microcarpa of the genus *Citrus* was examined by the use of high-performance liquid chromatography. 3′,4′,5,6,7,8-Hexamethoxy-, 4′,5,6,7,8-pentamethoxy- and 3′,4′,5,6,7-pentamethoxyflavone were detected in all species of the subgroup and their contents in the fruit peels were quantitated.

Keywords——genus *Citrus*; subgroup microcarpa; flavone content; flavone distribution; chemotaxonomy

Systematic classification of the genus *Citrus* based on morphological characters (Engler 1896, Swingle 1943, Tanaka 1944 and 1961)[1] has been used for delimitation of *Citrus* species. With progressive hybridization and apomictic reproduction, however, it has become difficult to assess the relationship between different taxa only on the basis of morphology, because the characters are highly variable within the genus and extremes have been artificially chosen as cultivars. Therefore, chemotaxonomic studies on the genus *Citrus* are expected to provide important clues. In the present study, flavone contituents and their distribution in the species belonging to the group Citridora subgroup microcarpa (Tanaka's systematics),[2] *i.e.* *C. depressa* Hayata and its var. *kugani* TANAKA, *C. tachibana* TANAKA, *C. erythrosa* TANAKA, *C. kinokuni* TANAKA, and *C. leiocarpa* TANAKA, have been examined to obtain chemotaxonomic information. Seasonal fluctuation of flavones was also examined in *C. reticulata* BLANCO.

Results and Discussion

Isolation of Flavones from *C. depressa*

From the methanol extracts of dry fruit peels of *C. depressa*, five known polyoxygenated flavones were isolated by repeated chromatography on silica gel. The structures of the isolated flavones were determined by spectroscopic analysis as 4′,5,6,7,8-pentamethoxy- (ponkanetin; also commonly called tangeritin), 3′,4′,5,6,7,8-hexamethoxy- (nobiletin), 3′,4′,5,6,7-pentamethoxy- (sinensetin), 5-hydroxy-4′,6,7,8-tetramethoxy-, and 5-hydroxy-3′,4′,6,7,8-pentamethoxyflavone, which were confirmed by comparison with authentic samples isolated previously from *C. reticulata* BLANCO.[3]

Gas chromatography was found to be inadequate for separation of such polymethoxyflavones as ponkanetin, nobiletin, and sinensetin,[3] but high-performance liquid

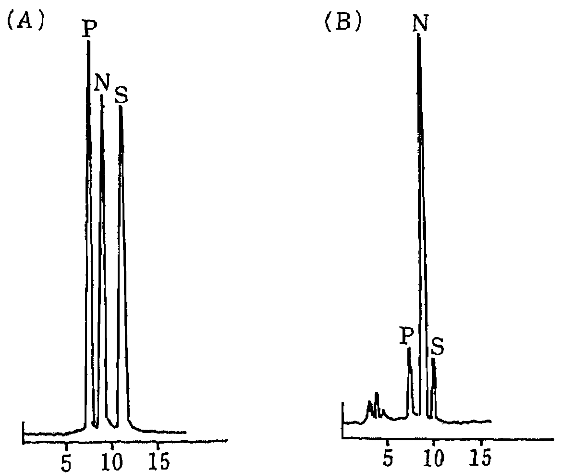

Fig. 1. Chromatograms of Ponkanetin (P), Nobiletin (N) and Sinensetin (S) [A] and a Crude Methanolic Extract of Fruit Peels of *Citrus reticulata* [B]

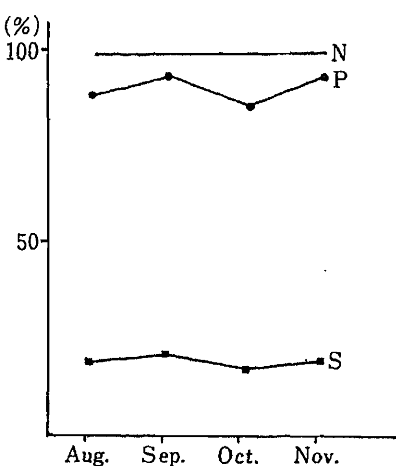

Fig. 2. Variations of Relative Contents of Nobiletin (N) (100%), Ponkanetin (P) and Sinensetin (S) from August (Young Fruit) to November (Fully Matured Fruit) in *Citrus reticulata*

TABLE I. Amounts of Flavones in Fruit Peels in Subgroup Microcarpa of the Genus *Citrus*

Sample number	Species	Common name	Location	Collected date	Ponkanetin	Nobiletin	Sinensetin
10	*C. tachibana*	Tachibana	Miyazaki	Dec. 12, '84	3.73[a] (69.3)	5.38 (100)	0.76 (14.0)
37	*C. tachibana*	Tachibana	Wakayama	Dec. 24, '84	1.98 (69.6)	2.59 (100)	0.42 (16.1)
90	*C. tachibana*	Tachibana	Wakayama	Jan. 8, '85	2.62 (73.8)	3.55 (100)	0.57 (15.9)
93	*C. tachibana*	Tachibana	Shizuoka	Nov. 29, '84	2.77 (63.8)	4.43 (100)	0.58 (13.3)
118	*C. erythrosa*	Kobeni-mikan	Wakayama	Jan. 10, '85	1.03 (59.5)	1.73 (100)	0.12 (6.99)
35	*C. kinokuni*	Hosotaka-kishu	Wakayama	Dec. 24, '84	0.91 (44.1)	2.07 (100)	0.23 (15.0)
36	*C. kinokuni*	Hira-kishu	Wakayama	Dec. 24, '84	1.45 (42.3)	3.43 (100)	0.51 (15.0)
47	*C. kinokuni*	Hira-kishu	Saga	Dec. 24, '84	1.23 (38.7)	3.18 (100)	0.37 (11.5)
48	*C. kinokuni*	Kishu	Saga	Dec. 24, '84	2.11 (48.7)	4.33 (100)	0.64 (14.9)
104	*C. depressa*	Hirami-lemon	Formosa	Jan. 11, '85	1.35 (48.2)	2.80 (100)	0.41 (20.0)
15	*C. depressa* var. *kugani*	Okinawa	Okinawa	Dec. 11, '85	0.97 (39.4)	2.46 (100)	0.49 (16.5)
30	*C. leiocarpa*	Surugayuko	Shizuoka	Dec. 11, '84	1.44 (56.9)	2.53 (100)	0.42 (16.5)
31	*C. leiocarpa*	Surugakohji, Kohji	Wakayama	Dec. 11, '84	0.79 (53.3)	1.49 (100)	0.27 (18.4)

a) mg/g.

chromatography (HPLC) on a polar stationary phase gave a satisfactory result, as shown in Fig. 1. The minor peaks that precede the main peak (Fig. 1B) are due to the 5-hydroxy derivatives of ponkanetin and nobiletin.

Seasonal Fluctuation of the Contents of Flavones in *C. reticulata*

To examine the variation of the contents of polymethoxyflavones in fruit peels of *C. reticulata* at different stages of fruit development, peels were collected at intervals of one month from August to November. Fruit peels of *C. reticulata* usually begin to change color from green to orange-yellow at the end of October, reaching a fully colored stage in December. As shown in Fig. 2, no significant variation in flavone contents was observed during the period of fruit development.

Distribution of Flavones in the Subgroup Microcarpa

The contents of nobiletin, ponkanetin, and sinensetin in five species belonging to the subgroup microcarpa were determined (Table I). These flavones were found to exist in all five species examined and their relative proportions were similar within the examined and their relative proportions were similar within the same species irrespective of the place of collection. The relative proportion of ponkanetin was higher in *C. tachibana*, *C. erythrosa* and *C. leiocarpa* than in *C. kinokuni* and *C. depressa*.

Experimental

Melting points were determined on a Büchi melting point apparatus, and are uncorrected. Ultraviolet (UV) spectra were recorded on a Hitachi 323 spectrometer and mass spectrum (MS) on a JEOL JMS-300 mass spectrometer at 70 eV. Proton nuclear magnetic resonance (^{1}H-NMR) measurements were taken on a Hitachi R-20B at 60 MHz; chemical shifts are given in δ value (ppm) with tetramethylsilane as an internal standard. The origin and data of collection of the materials are summarized in Table I.

Extraction and Isolation of Polymethoxyflavones from *C. depressa*——Dry peels (900 g) were extracted twice with MeOH on a water bath under reflux for 10 h. The combined MeOH extracts were concentrated under reduced pressure to give a brown oily residue (102 g). After addition of AcOEt and water, the residue was partitioned. AcOEt-soluble materials (35 g) were chromatographed on silica gel with AcOEt–C_6H_{14} = 1 : 1 as the solvent. Fractions 1—8 (each 100 ml) of the eluate were rechromatographed on silica gel with C_6H_6 containing acetone. The structures of the isolated polymethoxyflavones were determined by spectroscopic analysis.

4′,5,6,7,8-Pentamethoxyflavone (Ponkanetin or Tangeritin)——mp 153—154 °C (MeOH), colorless needles. ^{1}H-NMR (CCl_4) δ: 3.88, 3.90, 3.92, 3.98, 4.02 (3H, each s, OMe), 6.42 (1H, s, H-3), 6.99 (2H, d, J=9.0 Hz, H-3′, 5′), 7.88 (2H, d, J=9.0 Hz, H-2′, 6′). MS m/z (rel. int.): 372 [M$^+$] (72), 357 (100). UV λ_{max}^{MeOH} nm (log ε): 273 (4.19), 325 (4.32).

3′,4′,5,6,7,8-Hexamethoxyflavone (Nobiletin)——mp 134—135 °C (MeOH), colorless needles. ^{1}H-NMR (CCl_4) δ: 3.86, 3.89, 3.92, 3.98, 4.02 (3H, each s, OMe), 6.44 (1H, s, H-3), 6.90 (1H, d, J=9.0 Hz, H-5′), 7.38 (1H, d, J=2.0 Hz, H-2′), 7.51 (1H, dd, J=9.0, 2.0 Hz, H-6′). MS m/z (rel. int.): 402 [M$^+$] (63), 387 (100). UV λ_{max}^{MeOH} nm (log ε): 272 (4.08), 335 (4.26).

3′,4′,5,6,7-Pentamethoxyflavone (Sinensetin)——mp 175—177 °C (MeOH), colorless needles. ^{1}H-NMR ($CDCl_3$) δ: 3.92 (3H, s, OMe), 3.98 (6H, s, 2×OMe), 4.00, 4.02 (3H, each s, OMe), 6.62 (1H, s, H-3), 6.72 (1H, s, H-8), 7.00 (1H, d, J=9.0 Hz, H-5′), 7.37 (1H, d, J=2.0 Hz, H-2′), 7.57 (1H, dd, J=9.0, 2.0 Hz, H-6′). MS m/z (rel. int.): 372 [M$^+$] (57), 357 (100).

5-Hydroxy-4′,6,7,8-tetramethoxyflavone——mp 175—177 °C (MeOH), yellow needles. ^{1}H-NMR ($CDCl_3$) δ: 3.90 (3H, s, OMe), 3.96 (6H, s, 2×OMe), 4.08 (3H, s, OMe), 6.56 (1H, s, H-3), 7.01 (2H, d, J=9.0 Hz, H-3′, 5′), 7.92 (2H, d, J=9.0 Hz, H-2′, 6′). MS m/z (rel. int.): 358 [M$^+$] (47), 343 (100). UV λ_{max}^{MeOH} nm: 294, 328; $\lambda^{MeOH+AlCl_3}$: 290 sh, 312, 365.

5-Hydroxy-3′,4′,6,7,8-pentamethoxyflavone——mp 143—145 °C (MeOH), yellow needles. ^{1}H-NMR ($CDCl_3$) δ: 3.92 (12H, s, 4×OMe), 4.02 (3H, s, OMe), 6.46 (1H, s, H-3), 6.90 (1H, d, J=9.0 Hz, H-5′), 7.32 (1H, d, J=2.0 Hz, H-2′), 7.51 (1H, dd, J=9.0, 2.0 Hz, H-6′). MS m/z: 388 [M$^+$]. UV λ_{max}^{MeOH} nm: 254, 285, 342; $\lambda^{MeOH+AlCl_3}$: 265, 292, 304 sh, 376.

HPLC Equipment: Liquid chromatograph: Shimadzu LC-6A. Detector: Shimadzu UV spectrophotometric detector SPD 6A. Injector: Rheodyne 7215. Recorder: Shimadzu R-11. Conditions: column, Lichrosorb Si 60 (5 μm) (Merck) 250 mm × 4 i.d.; flow rate, 1.0 ml/min; solvent, C_6H_6–$(CH_3)_2CO$ = 3 : 1; detection, UV 350 nm; chart speed, 2 ml/min.

Procedure for Quantative Determination——About 200 mg of dry powdered peels was weighed accurately, placed

in a spitt tube (10 ml) and extracted with MeOH (5 ml) under ultrasonication in a water bath at 60 °C for 30 min. After filtration, the residue was washed with MeOH. The filtrate and washing were put in a 10 ml measuring flask and made up to 10 ml with MeOH. The solution (5 μl) was subjected to HPLC. The amounts of ponkanetin, nobiletin and sinensetin in the peels were calculated by the use of calibration curves prepared in advance.

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Preparation and Evaluation of Fatty Acid Esters of Fluorescent *p*-Substituted Phenols as Substrates for Measurement of Lipase Activity[1)]

SHUZO AKIYAMA,*,[a] TAKEHIDE OCHIAI,[a] SHIN'ICHI NAKATSUJI,[a] KENICHIRO NAKASHIMA,[a] and YOSUKE OHKURA[b]

Faculty of Pharmaceutical Sciences, Nagasaki University,[a] Bunkyo-machi, Nagasaki 852, Japan and Faculty of Pharmaceutical Sciences, Kyushu University,[b] 62 Maidashi, Higashiku, Fukuoka 812, Japan

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Several kinds of fatty acid esters of fluorescent *p*-substituted phenols (**1a**, **2a**, **3a**, and **4a**) were prepared. Their absorption and fluorescence spectral properties and their usefulness as substrates for the measurement of lipase activity were investigated. Among them, long alkyl chain esters were found to be suitable for fluorescent substrates, because their emission wavelengths were fairly different from those of the corresponding phenols. In particular, 4-(2-benzothiazolyl)phenyl myristate (**3e**), 4-(2-benzothiazolylvinyl)phenyl laurate (**4d**) and 4-(2-benzothiazolylvinyl)phenyl myristate (**4e**) were ascertained to be easily hydrolyzed by porcine pancreatic lipase.

Keywords——lipase; lipase activity; fluorescent *p*-substituted phenol; fluorogenic substrate; fluorometry

In the area of clinical testing, the development of simple, rapid, and sensitive assay methods for lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) activity is important for the diagnosis and follow-up of diseases of the pancreas.[2)] Therefore, many kinds of synthetic substrates for measurement of lipase activity have been reported.[3)] Recently, Orange I-laurate, a synthetic substrate, was found to be most rapidly hydrolyzed in a lipase catalyzed reaction and was applied to the colorimetric assay of lipase activity in blood.[3c)]

In this study, in order to improve in the sensitivity of the assay, various aliphatic carboxylic acid esters [**1**—**4** (R = acyl)] of fluorescent phenols [**1a**—**4a** (R = H)] were newly prepared as potential lipase substrates (Chart 1) and their fluorescence properties were examined. Among the twelve kinds of ester prepared, the laurate (**3d** and **4d**), the myristate (**3e** and **4e**), the palmitate (**3f**), and the stearate (**3g**) were found to be reasonably well hydrolyzed in the lipase-catalyzed reaction.

1: benzofuranyl–phenyl–OR
2: benzofuranyl–vinyl–phenyl–OR
3: benzothiazolyl–phenyl–OR
4: benzothiazolyl–vinyl–phenyl–OR

R	compound			
H	**1a**	**2a**	**3a**	**4a**
$COCH_3$	**1b**		**3b**	**4b**
$CO(CH_2)_2CH_3$			**3c**	
$CO(CH_2)_{10}CH_3$	**1d**	**2d**	**3d**	**4d**
$CO(CH_2)_{12}CH_3$			**3e**	**4e**
$CO(CH_2)_{14}CH_3$			**3f**	
$CO(CH_2)_{16}CH_3$			**3g**	

Chart 1

Experimental

Reagents and Materials——All chemicals were of reagent grade, unless otherwise noted. Porcine pancreatic lipase (100000 U/1.9 mg lyophilized powder) was obtained from Sigma Chemical Co., Ltd., U.S.A. Water was deionized and distilled.

Apparatus——Infrared (IR) spectra were measured in Nujol mull with a JASCO IRA2 grating infrared spectrophotometer, and ultraviolet (UV) and visible absorption spectra and absorbances were obtained with a Hitachi 210 spectrophotometer. Fluorescence spectra were measured with a Hitachi 650 10S fluorescence spectrophotometer and 10 x 10 mm quartz cells. A Toa HM 5A pH-meter was used for pH measurements. Melting points are uncorrected.

Preparation of Substrates[4]——4-(2-Benzofuranyl)phenyl Laurate (**1d**): Acetone (10 ml) solution of lauric anhydride (3.93 g) was added to a solution of 2-(4-hydroxyphenyl)benzofuran (**1a**)[5] (980 mg, 4.7 mmol) in a mixture of pyridine (2 ml) and ether (8 ml). The mixture was refluxed for 5 h and poured into a mixture of ice-water (50 ml). The deposited crystals were filtered off, washed with water, and dried *in vacuo*. Crude **1d** was recrystallized from ethanol; 1.59 g, 87%, mp 103—104 °C. *Anal.* Calcd for $C_{13}H_{32}O_3$: C, 79.59; H, 8.16. Found: C, 79.20; H, 8.15.

4-(2-Benzofuranyl)phenyl Acetate (**1b**): 91%, mp 140—141 °C. *Anal.* Calcd for $C_{16}H_{12}O_3$: C, 76.18; H, 4.80. Found: C, 76.16; H, 4.78.

4-(2-Benzofuranylvinyl)phenyl Laurate (**2d**): **2d** was prepared according to the above procedure (*cf.*, **1d**) in 96% yield from **2a** (mp 201—213 °C), which was obtained by a demethylation of the corresponding methylether (mp 140 °C) with sodium ethylthiolate in *N*,*N*-dimethylformamide (120 °C, 4 h, 54%); mp 122 °C. *Anal.* Calcd for $C_{28}H_{34}O_3$: C, 80.35; H, 8.19. Found: C, 80.30; H, 8.22.

4-(2-Benzothiazolyl)phenyl Myristate (**3e**): A solution of myristic acid (1.92 g, 8.4 mmol) and *N*,*N*′-carbodiimidazole (1.43 g, 8.8 mmol) in dry tetrahydrofuran (THF) (10 ml) was refluxed for 1 h in an atmosphere of Ar and then cooled to room temperature. Next, solution of 2-(4-hydroxyphenyl)benzothiazole (**3a**)[6] (2.00 g, 8.8 mmol) in dry THF (9 ml) containing sodium imidazolate (0.66 mmol) was added to the above solution, and the mixture was stirred overnight. The residue obtained by evaporating the solvent *in vacuo* was extracted with benzene (10 ml). The extract was percolated through a short column of alumina (10 g) and concentrated to give colorless crystals (**3e**, 1.72 g, 45%, mp 88—89 °C). *Anal.* Calcd for $C_{27}H_{35}NO_2S$: C, 74.14; H, 8.01; N, 3.20; S, 7.32. Found: C, 74.13; H, 8.18; N, 2.96; S, 7.18.

4-(2-Benzothiazolyl)phenyl Acetate (**3b**): 94%, mp 147 °C. *Anal.* Calcd for $C_{15}H_{11}NO_2S$: C, 66.91; H, 4.09; N, 5.20; S, 11.90. Found: C, 66.79; H, 4.13; N, 5.20; S, 11.90.

4-(2-Benzothiazolyl)phenyl Butyrate (**3c**): 81%, mp 144—145 °C. *Anal.* Calcd for $C_{17}H_{15}NO_2S$: C, 68.69; H, 5.05; N, 4.71; S, 10.77. Found: C, 68.68; H, 5.15; N, 4.11; S, 10.77.

4-(2-Benzothiazolyl)phenyl Laurate (**3d**): 44%, mp 83.5—84 °C. *Anal.* Calcd for $C_{25}H_{31}NO_2S$: C, 73.35; H, 7.58; N, 3.42; S, 7.82. Found: C, 73.60; H, 7.69; N, 3.17; S, 7.81.

4-(2-Benzothiazolyl)phenyl Palmitate (**3f**): 54%, mp 91—92 °C. *Anal.* Calcd for $C_{29}H_{39}NO_2S$: C, 74.84; H, 8.44; N, 2.51; S, 6.68. Found: C, 74.84; H, 8.39; N, 3.01; S, 6.88.

4-(2-Benzothiazolyl)phenyl Stearate (**3g**): 37%, mp 95—96 °C. *Anal.* Calcd for $C_{31}H_{43}NO_2S$: C, 75.41; H, 8.78; N, 2.84; S, 6.49. Found: C, 75.47; H, 8.94; N, 2.73; S, 6.57.

4-(2-Benzothiazolylvinyl)phenyl Acetate (**4b**): The compound was prepared according to the acid anhydride method; 87%, mp 142—143 °C. The starting material, 2-(4-hydroxystyryl)benzothiazole[7] (**4a**), was synthesized by demethylation of the corresponding methylether [**1** (R = Me)].[8]

4-(2-Benzothiazolylvinyl)phenyl Laurate (**4d**): 80%, mp 96—97 °C. *Anal.* Calcd for $C_{27}H_{33}NO_2S$: C, 74.44; H, 7.64; N, 3.33; S, 7.44. Found: C, 74.65; H, 7.67; N, 3.33; S, 7.44.

4-(2-Benzothiazolylvinyl)phenyl Myristate (**4e**): 86%, mp 95—97 °C. *Anal.* Calcd for $C_{29}H_{37}NO_2S$: C, 75.13; H, 8.05; N, 3.02. Found: C, 74.98; H, 8.42; N, 2.93.

Evaluation Procedure for Esters as Lipase Substrates——A solution of a synthetic ester (50 μl) in methyl cellosolve or dioxane was mixed with 40 mM sodium dodecyl sulfate (SDS) in 0.1 M barbital buffer (pH 8.0, 50 μl) and this mixture was further mixed with the same buffer (0.35 ml). After the solution had been sonicated for 1 min, it was mixed with a solution of lipase (1250 U/ml, 50 μl), and incubated at 37 °C for 30 min, then a 0.05 M $Na_2B_4O_7 \cdot 10H_2O$–Na_2CO_3 buffer (pH 10.0, 3.5 ml) was added. The resulting mixture was centrifuged at 2000 *g* for 10 min at 20 °C. The relative fluorescence intensity (RFI) of the solution was measured, *e.g.*, at 425 nm (excitation (Ex) at 360 nm) for **3a** or 520 nm (Ex at 398 nm) for **4a**. For the blank, 0.1 M barbital buffer (pH 8.0) was added instead of the indicated volume of lipase solution.

The relation between the concentration of the phenol (*e.g.*, **2a** or **4a**) and RFI was examined according to the standard procedure except that ester and lipase were replaced by the corresponding phenol and water, respectively. Linear relationships were obtained in the range of 4.88×10^{-8}—1.56×10^{-6} M (**3a**) and 4.88×10^{-8}—3.13×10^{-6} M (**4a**).

Results and Discussion

Preparation of the Fatty Acid Esters of Phenols (1a—4a)

The phenols (**1a**—**4a**) could be converted to various aliphatic carboxylic acid esters by the acid anhydride or *N*,*N*′-carbonyldiimidazole method. The esters prepared are shown in Table I with the correpsonding phenols.

Electronic Absorption and Fluorescence Spectral Properties of the Phenols and the Esters

As shown in Table I, it has become apparent that the vinylogues (**2a**, **4a**, and their esters) show red shifts (33—39 nm) relative to the corresponding compounds in the UV spectra.

Fluorescence spectra of the phenols and the esters were measured both in EtOH and in an alkaline ethanol solution (pH 10) (Table I). Generally, the emission maxima of the phenols and the esters in the alkaline solution were at longer wavelengths than in EtOH. For the use of these esters as synthetic substrates, it is required that the emission maxima of the phenols liberated in the enzyme reaction are well separated from those of the corresponding esters. Therefore, the esters (**1**—**4**, R = acyl) might be suitable as substrates for the assay of lipase activity by fluorometry.

Evaluation of Esters as Substrates for Measurement of Enzyme Activity

The suitability of these esters for use as substrates for the fluorometric assay of lipase activity was examined. As a representative, **3e** was used to establish the evaluation method, with porcine pancreatic lipase as the enzyme. The effect of buffers [0.1 M barbital (pH 6.0—8.2) and 0.1 M phosphate (pH 7.5—9.0)] was examined and the optimal pH was found to be near 8.0 in both cases. We used 0.1 M barbital buffer (pH 8.0) which showed a larger RFI than that of 0.1 M phosphate buffer. The effect of the incubation time on RFI was examined, and a linear relation was obtained in the range of 10—60 min at 37 °C. The effect of concentration of **3e** on lipase activity was examined and a maximum RFI was obtained at 60 μM. The apparent K_m value for **3e** obtained from Lineweaver–Burk plots was 2.27×10^{-5} M and thus the substrate concentration was set at 3.00×10^{-4} M. For six kinds of ester (**3d**—**g** and **4d**, **e**), K_m

TABLE I. Spectral Data for Phenols and Their Carboxylic Acid Esters

Compound	UV (nm) EtOH		Excitation Maximum (nm)	Emission Maximum (nm)	Excitation Maximum (nm)	Emission Maximum (nm)
	λ_{max}	log ε	(Ethanol solution)		(Alkaline solution)[a]	
1a	308	4.60	310	354	326	408
1b	304	4.54	313	353	326	407
1d	304	4.28	311	352	335	407
2a	343	4.50	343	405	360	470
2d	337	4.42	343	386	288	430
3a	319	4.39	326	376	360	425
3b	300	4.03	325	362	350	425
3c	301	4.31	325	365	350	425
3d	301	4.18	320	360	315	378
3e	301	3.81	314	362	330	380
3f	300	3.92	314	360	330	380
3g	301	3.82	315	365	290	370
4a	358	4.43	310	370	398	520
4b	335	4.62	330	407	290	355
4d	336	4.52	330	405	305	422
4e	338	4.15	332	407	330	414

a) EtOH: (0.05 M $Na_2B_4O_7 \cdot 10H_2O$–Na_2CO_3 (pH 10.0)) = 10 : 90 (v/v).

TABLE II. Relative Reaction Velocity, K_m Value, and V_{max} for Substrates in the Reaction of Lipase

Substrate	Relative reaction velocity[a] (M/min)	K_m (M)	V_{max} (M/min)	V_{max}/K_m (min^{-1})
3d	4.17×10^{-9}	1.79×10^{-5}	6.17×10^{-9}	3.45×10^{-4}
3e	5.33×10^{-9}	2.27×10^{-5}	2.46×10^{-9}	1.08×10^{-4}
3f	1.83×10^{-9}	7.25×10^{-6}	8.17×10^{-10}	1.13×10^{-4}
3g	6.17×10^{-10}	2.50×10^{-6}	3.34×10^{-10}	1.34×10^{-4}
4d	1.60×10^{-8}	4.55×10^{-5}	5.67×10^{-9}	1.25×10^{-4}
4e	9.67×10^{-9}	8.33×10^{-5}	3.27×10^{-9}	3.93×10^{-5}

a) Relative reaction velocity was estimated from the amount of phenol liberated according to the procedure described in Experimental. All substrate concentrations were fixed at 2.00×10^{-4} M.

values and relative reaction velocities at 2.00×10^{-4} M are summarized together with the maximum velocities (V_{max}) in Table II. Among the substrates, **3d**, **4d**, and **4e** showed larger V_{max} values than the others. The order of hydrolysis rates was as follows: **4d** > **4e** > **3e** > **3d** > **3f** > **3g**. From the above results, **3d**, **4d**, and **4e** seem to be preferable as substrates for lipase. Though V_{max}/K_m for **4e** was the smallest owing to its large K_m value, **4e** should be useful as a substrate because of its relatively large reaction velocity. The reactivity of lipase appears to be practically independent of the carbon chain length of the esters. A linear relationship between the amount of lipase and the amount of **3a** hydrolyzed was obtained in the range of 0—75 U/tube, and an amount of lipase of 62.5 U/tube was selected for the evaluation procedure. Fluorescence stability of **3a** produced in the enzyme reaction was also checked; the RFI was constant for at least 180 min.

In conclusion, it is apparent that the fluorogenic esters listed in Table II can be used as substrates for assay of lipase activity. However, before using these esters in this way, it is necessary to examine the effects of various other enzymes on them. The application of the study to practical samples is now under study.

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Determination of Dibucaine and Its Metabolites in Human Urine by High-Performance Liquid Chromatography with Fluorescence Detector

KAZUO IGARASHI,*[a] FUMIYO KASUYA,[a] MIYOSHI FUKUI,[a] and HISASHI NANJYOU[b]

Faculty of Pharmaceutical Sciences, Kobegakuin University,[a] Nishi, Kobe 673, Japan and Taisyou Hospital,[b] Taisyou, Osaka 551, Japan

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A high-performance liquid chromatographic (HPLC) method using a fluorescence detector is described for the simultaneous determination of dibucaine and its metabolites (M-4, M-8 and M-10) in human urine. Urine samples from obstetric patients were chromatographed in a reversed-phase system. When a ultraviolet detector (320 nm) was used, some interfering peaks appeared on the chromatogram, but this interference could be overcome by employing a fluorescence detector (Ex 330 nm and Em 440 nm) instead. The calibration curves were linear in the range of 0.05—5.0 μg/ml for all compounds and the detection limits of dibucaine and its metabolites were about 5 ng/ml in urine. The urinary excretion of dibucaine and its metabolites by obstetric patients infused with Percamine S® in the spinal cord were determined. The mean cumulative amounts of dibucaine, M-4, M-8 and M-10 excreted during 10 h after administration were 1.1, 10.5, 3.5 and 1.1% of the dose, respectively. The total urinary excretion was 16.2% of the dose. This method is sufficiently sensitive and specific to permit the determination of dibucaine and its metabolites in biological fluids.

Keywords——dibucaine; metabolite; human urine; spinal anesthesia; HPLC; fluorescence detector; Percamine S®; urinary excretion; metabolism

Dibucaine is a potent local anesthetic used for relief of pain or for spinal anesthesia. This drug has occasionally caused unsuspected death because it is highly toxic. From the viewpoint of clinical toxicology it would be useful to determine dibucaine concentration in the biological fluids and to elucidate the metabolism of dibucaine in order to minimize the risks.

The analysis of dibucaine in the biological fluids is complicated by the low dose levels employed. A specific and sensitive method for the analysis of dibucaine is therefore needed. Several methods for the micro-determination of dibucaine alone in the biological fluids by gas chromatography (GC) or gas chromatography–mass spectrometry (GC-MS) have been reported,[1-4] and the analysis of dibucaine and its metabolites by high performance liquid chromatography (HPLC) is also possible.[5,6]

In the previous study,[7] we showed that in humans dibucaine is metabolized to six basic metabolites, of which the 2-hydroxyethoxy (M-8), O-debutyl (M-4) and ω-1 hydroxy (M-10) compounds are the major ones (Fig. 1). The present work describes a sensitive and specific assay for the determination of dibucaine and its metabolites in urine by HPLC with a fluorescence detector, and its application to the analysis of urine from obstetric patients infused with Percamine S® in the spinal cord.

Experimental

Chemicals and Reagents——Dibucaine hydrochloride was purchased from Teikoku Chemical Industry Co., Ltd. (Osaka, Japan). Metabolites of dibucaine, O-debutyl (M-4), 2-hydroxyethoxy (M-8) and ω-1 hydroxy (M-10)

CONHCH₂CH₂N(R₁)₂ / N / OR₂

compound	R_1	R_2
dibucaine	$-C_2H_5$	$-C_4H_9$
M-4	$-C_2H_5$	-H
M-8	$-C_2H_5$	$-CH_2CH_2OH$
M-10	$-C_2H_5$	$-CH_2CH_2CH(OH)CH_3$
I.S.	$-CH_3$	$-C_4H_9$

Fig. 1. Chemical Structures of Dibucaine, Its Metabolites and the Internal Standard (I.S.)

compounds, and the internal standard (I.S.), 2-butoxy-*N*-(2-dimethylaminoethyl)cinchoninamide were prepared as described previously (Fig. 1).[4,7] All other solvents and reagents used were of analytical-reagent grade.

Apparatus and Chromatographic Conditions——A Shimadzu LC-3A high-performance liquid chromatograph was used. The ultraviolet (UV) detector (Shimadzu SPD-2A) was set at 320 nm. The excitation (Ex) and the emission (Em) wavelengths of the fluorescence spectromonitor (Shimadzu RF-530) were 330 and 440 nm, respectively. The column used was a Cosmosil® 5 C18 (15 cm × 4.6 mm i.d., Nakarai Chemical Co., Japan). The mobile phase was consisted of methanol–water (60 : 40, v/v) containing 30 mM triethylamine, the pH being adjusted to 7.5 with acetic acid. The mobile phase was passed through the column at a flow-rate of 1 ml/min.

Extraction Procedure——A urine sample (4 ml) was adjusted to pH 11 with conc. NH_4OH, and then 0.2 ml of the I.S. solution (20 μg/ml) and 5 ml of ethyl acetate were added. The mixture was vigorously shaken for 1 min and centrifuged. After phase separation, the organic layer was back-extracted by shaking with 2 ml of 0.5 N HCl. The aqueous layer was made alkaline with conc. NH_4OH and reextracted with 5 ml of dichloromethane. The organic layer was evaporated to dryness, and the residue was dissolved in 0.1 ml of the mobile phase. A 10 μl sample was injected into the HPLC column.

Clinical Study——Urine samples were taken from seven obstetric patients (25—33 years old) infused with 2 ml of Percamine S® (containing 0.3% dibucaine) in the spinal cord for the surgical treatment; urine was collected every 2 h up to 10 h after dosing. The urine samples were stored at −20 °C until analysis.

Results and Discussion

Typical chromatograms of urine samples from obstetric patients infused with Percamine S® in the spinal cord are shown in Fig. 2. Although they differed from sample to sample, some unidentified peaks were observed on the chromatogram when the UV detector (320 nm) was used and interfered with the simultaneous determination of dibucaine and its metabolites (Fig. 2A). These interfering peaks were not seen in the urine of healthy volunteers[6] or patients before surgical treatment. Therefore, they may have arisen from administered drug(s) other than dibucaine. In order to overcome this problem, we carried out the HPLC analysis with a fluorescence detector. The HPLC analysis of dibucaine using a fluorescence detector has been reported by Takeoka *et al.*[8] However, the method was directed to the analysis of dibucaine in injectable ampoules. We used the fluorescence detector for the simultaneous analysis of dibucaine and its metabolites. From the viewpoints of sensitivity and specificity of analysis, the detection wavelengths were set at Ex 330 nm and Em 440 nm. As shown in Fig. 2B, we were able to minimize the interference by exogenous substances in the urine samples under clinical conditions.

The peak height ratios of authentic samples of dibucaine and its metabolites with respect to the I.S. were linearly related ($r > 0.995$) with the concentrations in urine in the range from 0.05 μg/ml to 5.0 μg/ml. The calibration curves showed little day-to-day variability in slopes and intercepts (CVs below 5%). The minimum detectable amounts of dibucaine and its metabolites in urine were calculated to be about 5 ng/ml with a signal-to-noise ratio of 5 : 1. As compared with the UV detector, the use of the fluorescence detector offered a considerable sensitivity enhancement in the detection of dibucaine and its metabolites (10 times for M-4, 6 times for M-8, 4 times for M-10 and dibucaine).

The recoveries of known concentrations (0.25 and 2.5 μg/ml) of dibucaine and its

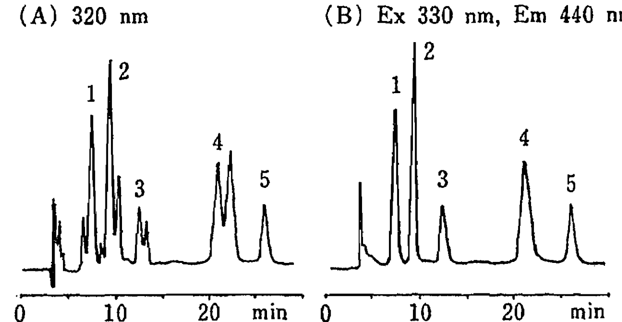

Fig. 2. Typical Chromatograms with (A) UV and (B) Fluorescence Detection of a Urine Sample from an Obstetric Patient Infused with Percamine S® in the Spinal Cord

1, M-4 (2.33 μg/ml); 2, M-8 (1.15 μg/ml); 3, M-10 (0.40 μg/ml); 4, I.S. and 5, dibucaine (0.81 μg/ml).

Table I. Reproducibility in the Determination of Dibucaine and Its Metabolites in Urine of Obstetric Patients

Patient	Metabolite	Mean concentration (μg/ml)	CV (%)
A	Dibucaine	0.12	7.3
	M-4	0.78	5.1
	M-8	0.53	5.4
	M-10	0.11	6.7
B	Dibucaine	0.51	5.9
	M-4	5.48	3.0
	M-8	1.84	2.8
	M-10	0.86	4.2

Experiments were performed with urine samples from the two patients infused 2 ml of Percamine S® in the spinal cord. Each value represents the mean of five determinations.
CV: coefficient of variation.

metabolites from blank human urine were determined by comparing the peak heights of the extracted compounds to those obtained from aqueous standard solutions injected directly. The mean recoveries of dibucaine, M-4, M-8 and M-10 were approximately 98%, 76%, 90% and 94%, respectively ($n=5$). The CV values were below about 5%. The recovery of M-4 was relatively poor, probably due to higher polarity.

The reproducibility of the method was also assessed by the repeated analysis of urine samples from obstetric patients infused with Percamine S® in the spinal cord. As shown in Table I, the CV values were satisfactory for all compounds (below 7%). Thus, the method was proved to be reproducible.

Using this method, we determined the urinary excretion of dibucaine and its metabolites by obstetric patients infused with Percamine S® in the spinal cord. In Table II the urinary excretion of dibucaine and its metabolites during 10 h after administration is presented. In the early period a small amount of dibucaine was observed (about 0.02 mg), and thereafter the amount was very small. On the other hand, metabolites were excreted at an approximately constant rate during 10 h after dosing. Ranges of cumulative excreted M-4 and M-8 in seven patients were 5.2—19.9% and 2.2—5.6% of the dose, respectively. Dibucaine and M-10 were excreted in the ranges of 0.3—3.2% and 0.6—1.7%, respectively. The range of total urinary excretion was 9.1—28.3%, with an average of 16.2% of the dose. These values are reasonably consistent with the findings in male healthy volunteers receiving dibucaine orally (10 mg); total excretion during 9 h after dosing, averaged 12.6% of the dose.[6] Fukui has also reported that only 1—2% of the dose was excreted as unchanged drug in the 0—12 h urine of patients infused with Percamine L® (containing 0.5% dibucaine) in the spinal cord.[9] From these results, we consider that dibucaine is rapidly and extensively metabolized in humans.

In conclusion, we have developed a sensitive and specific method to determine dibucaine and its metabolites in urine under clinical conditions.

TABLE II. Urinary Excretion of Dibucaine and Its Metabolites in Seven Obstetric Patients Infused with Percamine S® in Spinal Cord

Collection time (h)	Urinary excretion (mg)			
	M-4	M-8	M-10	Dibucaine
0— 2	0.041 (0.011—0.104)	0.011 (0.006—0.025)	0.007 (0.002—0.017)	0.018 (0.004—0.037)
2— 4	0.116 (0.042—0.361)	0.036 (0.015—0.114)	0.019 (0.009—0.051)	0.021 (0.004—0.070)
4— 6	0.109 (0.020—0.186)	0.042 (0.008—0.092)	0.015 (0.004—0.032)	0.013 (0.002—0.065)
6— 8	0.174 (0.047—0.460)	0.069 (0.022—0.072)	0.018 (0.007—0.033)	0.008 (0.001—0.025)
8—10	0.090 (0.016—0.180)	0.043 (0.007—0.075)	0.010 (0.001—0.021)	0.006 (0.001—0.027)
0—10	0.528 (0.260—0.997)	0.201 (0.125—0.321)	0.068 (0.035—0.104)	0.066 (0.015—0.197)

Mean values are given with ranges in parentheses ($n=7$).

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Colorimetric Assay of Diamine Oxidase Activity with Histamine as the Substrate

YUKARI YAMAGAMI,[a] TOSHIKO NAITO,[a] MIYUKI TAKAYANAGI,[b] SHOJI GOTO,[b] and TAMOTSU YASHIRO*,[a]

Faculty of Pharmaceutical Sciences, Nagoya City University,[a] 3-1, Tanabe-dori, Mizuho-ku, Nagoya 467, Japan and Aichi Prefecture Red Cross Blood Center,[b] 3-2-2, San-nomaru, Naka-ku, Nagoya 460, Japan

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A simple method for the determination of diamine oxidase (DAO) activity is described. The method is based on oxidation of histamine with DAO, formation of histamine-copper(II) chelated cation from the remaining histamine, and extraction of the ion associated with tetrabromophenolphthalein ethyl ester. A linear calibration curve between absorbance and DAO activity was obtained for DAO activity up to 250 mU/ml. The inter-assay coefficient of variation was 2.12% with a recovery of 101.7% at the DAO activity of 123.5 mU/ml. A close correlation was found between the results of this method and the 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) method.

Keywords——diamine oxidase; histaminase activity; colorimetry; solvent extraction; histamine-cupric ion-tetrabromophenolphthalein ethyl ester associate

Diamine oxidase [diamine: oxygen oxidoreductase (diaminating), EC 1.4.3.6] (DAO) is widely distributed in animals and microorganisms.[1] In animals, DAO is relatively abundant in the kidney,[2] and is also present in the plasma and other cells. DAO activity has been considered a useful parameter in clinical studies.[3]

DAO activity has been determined by the use of radioactive substances,[4,5] high-performance liquid chromatography[6,7] (HPLC), and colorimetric methods.[8-11] The first method is the most sensitive but required special equipment, and is not suitable for general use. The second method is time-consuming and troublesome. The third method is not as sensitive as the other methods, but is the simplest, and is suitable for routine use.

In the colorimetric method, the reactions are based on DAO oxidase activity towards several short-chain diamines,[11] such as ethylenediamine, putrescine (Pu), cadaverine (Ca), and histamine (Hi). The enzymatic activity of DAO towards Hi as a substrate is called histaminase activity[12] (Fig. 1). The activity of DAO may be measured in terms of such parameters as the amount of hydrogen peroxide, ammonia, or aldehydes produced in these reactions. Pu or Ca is commonly used to determine DAO activity, but Hi is not.

Recently, Sakai *et al.*[13] reported a method to determine Hi using solvent extraction and colorimetry as an associate with Cu(II) and tetrabromophenolphthalein ethyl ester (TBPE)

$H_2O + O_2$ — DAO → $H_2O_2 + NH_3$

HN N — $CH_2CH_2NH_2$ (Hi) → HN N — CH_2CHO (imidazoleacetaldehyde)

Fig. 1. Scheme of Oxidation of Hi by DAO

Fig. 2. Proposed Reaction Mechanism for Formation of Hi-Cu(II)-TBPE Associate

(Fig. 2). The precision of this method was better than that of other methods.[14,15] We therefore examined the application of this method to the determination of histaminase activity in various samples.

Experimental

Reagents, Solutions, and Apparatus——DAO and human serum albumin were obtained from Sigma Chemical Company (St. Louis). Bilirubin, Hi dihydrochloride, perchloric acid solution (PCA solution, 50%), potassium salt of tetrabromophenolphthalein ethyl ester (TBPE), cupric chloride, and 1,2-dichloroethane were purchased from Wako Pure Chemical (Osaka). Other solvents and inorganic chemicals were of reagent grade.

The substrate solution was prepared by dissolving 23 mg of Hi dihydrochloride in 5 ml of 0.1 M phosphate buffer (pH 6.5) and diluting it with additional buffer to obtain 0.5 mM Hi. Borate-phosphate buffer was prepared by mixing equal volumes of 0.3 M potassium dihydrogen orthophosphate and 0.1 M sodium tetraborate and adjusting the pH to 9.5 with 1 N sodium hydroxide or 1 N sulfuric acid. Standard solutions for preparing the calibration curve were prepared by dissolving DAO in 0.1 M phosphate buffer (pH 6.5) to yield DAO standards with activities of up to 250 mU/ml.

Absorbance was measured with a spectrophotometer (Specta-20, Beckman) in 10 × 2 mm cells.

Procedure——A mixture of 0.5 ml of 0.1 M phosphate buffer (pH 6.5), 0.6 ml of a substrate solution, and 0.4 ml of a sample (DAO activity up to 250 mU/ml) or a standard solution was incubated at 45 °C for 60 min, then 0.5 ml of 0.5 M PCA was added and the whole was centrifuged at 1500 × *g* for 5 min. A mixture of 0.1 ml of this deproteinized supernatant and 1.5 ml of 0.5 M potassium hydroxide was centrifuged at 1500 × *g* for 5 min. A mixture of 1.0 ml of the supernatant (free of PCA), 0.2 ml of 5 mM cupric chloride and 4.5 ml of borate-phosphate buffer (pH 9.5) was incubated at room temperature for 5 min. After the addition of 0.4 ml of a 4 mM TBPE ethanol solution and 2 ml of 1,2-dichloroethane, the whole was shaken vigorously for 10 min to extract the chromogen. The absorbance of the organic layer was measured at 515 nm.

One DAO unit was defined as the oxidation of 1.0 μM Hi under the proposed conditions (pH 6.5, 45 °C, and 60 min). For comparison, we also determined the DAO activity by the 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) method[10] (ABTS method) simultaneously.

Results

Effect of Reaction Conditions on Extent of Oxidation and Color Reaction

The optimal concentration of each reagent in the reaction mixture for the assay of DAO activity up to 250 mU/ml was first examined. The Hi solution was tested at concentrations between 0.025 and 2.5 mM. A concentration of 0.25 mM or more gave an optimum reaction. Therefore, we adopted the concentration of 0.5 mM. Optimum volumes of PCA solution and potassium hydroxide solution were 0.5 and 1.5 ml (tested in the ranges of 0—0.5 and 0—2.0 ml, respectively). The pH of the 0.1 M phosphate buffer that gave the optimum absorbance was 6.5 (tested between 5.7 and 8.0). The oxidation process proceeded smoothly and was the most effective at 45 °C, of the three incubation temperatures tested. It proceeded very slowly at 37 °C, and Hi was completely transformed at 60 °C after about 70 min of incubation. Hi was determined according to the method described by Sakai *et al.*[13]

Time Course of Oxidation Reaction

The oxidation reaction by which Hi was converted to imidazoleacetaldehyde by DAO

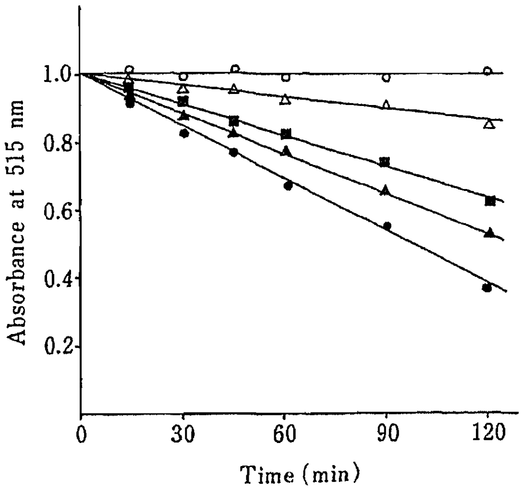

Fig. 3. Time Course of Oxidation Reaction at 45 °C

Diamine oxidase activities in samples: 0 (○), 49.4 (△), 123.5 (■), 172.9 (▲), and 247.0 (●) mU/ml.

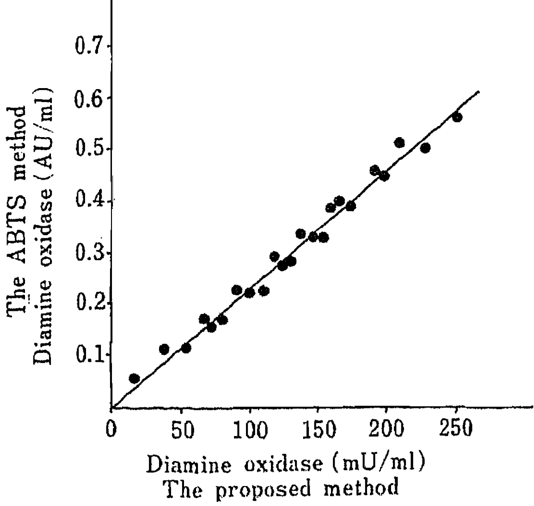

Fig. 4. Correlation between Results Obtained by the Proposed Method and the ABTS Method

One activity unit (AU) is defined as the amount of enzyme that catalyzes the oxidation of 1.0 μM putrescine per 90 min at 45 °C and pH 7.0.

proceeded in proportion to the reaction time up to at least 120 min (Fig. 3). A 60-min reaction time was considered to be maximum for routine use, so that the reaction was terminated by the addition of PCA solution after incubation for 60 min.

Effect of Plasma Components and Anticoagulants

We examined the interference by albumin and bilirubin as plasma components. When albumin at final concentrations of 15, 30, 60, and 90 mg/ml and bilirubin at final concentrations of 0.07, 0.15, 0.31, 0.62, 1.25, and 2.50 mg/ml were added to plasma, the DAO activities found by the present method were between 97.1 and 103.5% of that in their absence. Generally, in the determination of the activity of an oxidizing enzyme, such as DAO, anticoagulants are used as chelating reagents. Therefore, we studied the effects of sodium heparin, disodium ethylenediaminetetraacetate (EDTA-2Na), and sodium citrate at final concentrations of 18 IU/ml, 1.5 mg/ml, and 3.8 mg/ml, respectively. With DAO activities of 49.4, 123.5, and 197.6 mU/ml, the activities found under the standard conditions were between 94.3 and 99.1% at the values obtained without these anticoagulants. In this method, Cu was employed as a coloring reagent, and the normally used concentrations of the above anticoagulants in blood did not affect the result.

Accuracy

A linear relationship between the absorbance and the activity (mU/ml) of DAO was found up to 250 mU/ml. Ten human plasma samples obtained from normal subjects were tested, and the DAO activity was 1.2 to 7.7 mU/ml with a mean of 4.0 mU/ml.

The intra-assay precision was studied with plasma DAO activities of 74.1 and 172.9 mU/ml in 10 repreated tests, and the inter-assay precision was examined at a plasma DAO activity of 123.5 mU/ml in 5 repeated tests. The intra-assay precision, or coefficient of variation (C.V.), was 1.96%; the inter-assay C.V. was 2.12%.

Figure 4 compares the DAO activities in identical samples measured by the proposed method and the ABTS method. A good correlation ($n=23$, $r=1.007$) was observed, and the regression equation of the curve was $y=0.0022x$ (y, the ABTS method; x, the proposed method).

Discussion

DAO has oxidase activity towards several short-chain diamines[11] and Pu, Ca, or Hi is generally used as the substrate in DAO assay. The oxidation activity of DAO for Pu or Ca was higher than that for Hi,[16] but our method was nevertheless based on the measurement of histaminase activity, because solvent extraction of the Hi–Cu^{2+}–TBPE associate was convenient for evaluating remaining Hi.

In the present method, a good linear relationship was found between absorbance of residual Hi and reaction time (Fig. 3). This reaction was terminated after a 60 min incubation by the addition of PCA; that is, DAO and other proteins in the samples were denatured with PCA. Generally, determination of the DAO activity by colorimetric methods is performed with an oxidation time of 10—30 min, but in our method, the incubation time used was 60 min because a better sensitivity was obtained with the longer incubation time, making our proposed method as sensitive as other colorimetric methods. Although the present method required a longer time for measurement than other methods, it gives good reproducibility (C.V. =2.12%) as compared with other quick methods[6-9] (C.V. = 2.5%). The present method based on solvent extraction appears to be suitable for routine measurement of DAO activity.

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Nasal Absorption of Nifedipine from Gel Preparations in Rats

KAZUHIRO MORIMOTO,* HIROBUMI TABATA and KATSUAKI MORISAKA

Department of Pharmaceutical Sciences, Osaka University of Pharmaceutical Sciences, 2-10-65 Kawai, Matsubara City 580, Japan

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Nasal absorption of nifedipine from polyethylene glycol (PEG) 400, aqueous Carbopol gel and Carbopol–PEG gel was investigated with the aim of obtaining high bioavailability and prolonged action. Nasal administration of nifedipine in PEG resulted in rapid absorption and high maximum concentration (C_{max}). However, the elimination of nifedipine from plasma was very rapid. Nifedipine plasma concentration after nasal administration of nifedipine in aqueous Carbopol gel was very low. On the other hand, Carbopol–PEG gel (containing 50% w/v PEG) showed a relatively high nifedipine concentration and a prolonged action.

Keywords——gel preparation; polyethylene glycol 400; Carbopol 941; nifedipine; nasal administration; nasal absorption

Nifedipine, dimethyl 1,4-dihydro-2,6-dimethyl-4, (nitrophenyl)-3,5-pyridinedicarboxylate, is a highly active Ca^{2+}-channel blocker affecting the excitation–concentration coupling in smooth vascular muscle and myocardium, and is used in the treatment of angina pectoris and hypertension. However, nifedipine is a poorly water-soluble drug whose bioavailability is very low when it is administered in crystalline form,[1] and its biological half-life is relatively short.[2] Furthermore, recent studies have indicated that nifedipine shows a first-pass effect following oral administration.[3,4] Intranasal administration has been employed with drugs which are susceptible to a first-pass effect, such as propranolol[5] and peptides.[6,7] Recently, new powder dosage forms using water-soluble polymers have been developed for nasal administration.[8,9]

In the present study, nasal absorption of nifedipine from gel preparations made with polyethylene glycol (PEG) 400, aqueous Carbopol gel and aqueous Carbopol–PEG gel was investigated in order to obtain high bioavailability and prolonged action after intranasal administration.

Materials and Methods

Materials——Nifedipine was obtained from Bayer Yakuhin, Osaka, Japan, Carbopol 941 from B. F. Goodrich Chem. Co., Oh., U.S.A., and PEG 400 from Wako Pure Chem. Ind. Ltd., Osaka, Japan. The other reagents were of the best commercially available grade.

Methods——All experiments were carried out in a dark room in view of the high sensitivity of nifedipine to light.

Preparations——Carbopol gel was prepared with Carbopol 941 presoaked in distilled water at room temperature, and 10% NaOH solution was added to adjust the pH to 6.5 as previously described.[9] The concentrations of Carbopol 941 in the gel were 0.05% (w/v), 0.1% (w/v) and 0.5% (w/v). Carbopol–PEG gel was prepared by dissolution of PEG 400 at the concentration of 35% (w/v), 50% (w/v) or 70% (w/v) in Carbopol gel (0.05% w/v, pH 6.5). Nifedipine ($\sim 177\,\mu m$ particle size) was suspended or dissolved in the vehicles at the concentration of 100 mg/ml. The viscosities of the vehicles were measured with a cone and plate viscometer (Tokyo Keiki Co., Ltd., Tokyo, Japan) at 37 °C and at a shear rate of $38.4\,s^{-1}$. The viscosities and pH values are shown in Table I.

Administrations——Wistar-strain male rats, 200—250 g, were fasted for 20 h prior to the experiments but allowed free access to water, and anesthetized with Na-pentobarbital (50 mg/ml body weight) by intraperitoneal

TABLE I. Components and Viscosity of Vehicles

	PEG 400 (% w/v)	Carbopol 941 (% w/v)	Viscosity[a] cP	pH
PEG 400	100	—	51.2	—
Carbopol gel	—	0.05	256.0	6.0
Carbopol gel	—	0.1	366.1	6.0
Carbopol gel	—	0.5	1054.7	6.0
Carbopol–PEG gel (A)	35	0.05	215.0	6.5
(B)	50	0.05	100.1	7.0
(C)	70	0.05	58.2	7.2

a) The viscosities of vehicles were measured with a cone and plate viscometer at the shear rate of $38.4\,s^{-1}$ at 37 °C.

injection. The surgical operation was carried out as described by Hirai *et al.*[10] After an incision had been made in the neck, the trachea was cannulated with polyethylene tubing. Another similar cannula was inserted from the esophagus to the nasal cavity for the administration of nifedipine preparations. The nasopalatine was closed with an adhesive agent to prevent drainage of the drug from the nasal cavity to the mouth. The preparations were administered to the nasal cavity through the tube by means of a syringe at a volume of 1 ml/kg. In a comparative study, nifedipine, dissolved in a mixture of ethanol–PEG 400–water (15 : 15 : 17) was intravenously injected and nifedipine powder was administered to the duodenum through a polyethylene catheter to separate groups of rats. Blood samples (0.5 ml) were taken at appropriate times.

Analytical Methods——Plasma was separated by the centrifugation at 3000 rpm/min and nifedipine in plasma was assayed by the high-performance method reported by Pietta *et al.*[11]

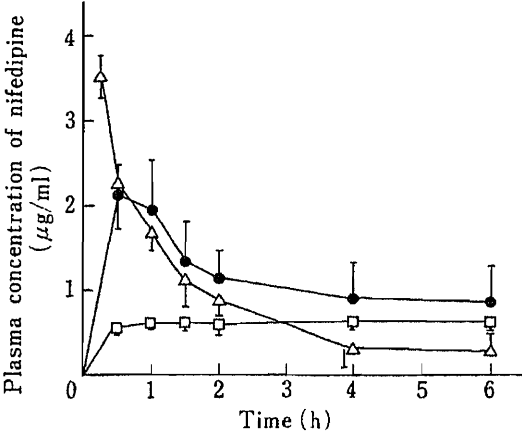
Results and Discussion

Figure 1 shows the plasma concentration of nifedipine after nasal administration of nifedipine in PEG 400, and after oral and intravenous administration of nifedipine in rats. Nasal administration of nifedipine in PEG 400 caused an early peak in the plasma level and a high maximum concentration compared with oral administration. However, the elimination of nifedipine from plasma was very rapid after the peak.

Figure 2 shows the plasma concentration of nifedipine after nasal administration of nifedipine in aqueous Carbopol gels at various concentrations of Carbopol in rats. Nifedipine plasma concentration profiles and peak plasma concentrations were similar among 0.05% (w/v), 0.1% (w/v) and 0.5% (w/v) Carbopol gels. The nifedipine concentrations in plasma were very low and sustained compared to that in the case of PEG 400.

Figure 3 shows the plasma concentration of nifedipine after nasal administration of nifedipine in Carbopol–PEG gels. Carbopol–PEG gels containing 35% (w/v) (A), 50% (w/v) (B) and 70% (w/v) (C) of PEG 400 in aqueous Carbopol gel (0.05% w/v, pH 6.5) were administered to rats. The nifedipine plasma profile after administration in Carbopol–PEG gel (C) was similar to that in the case of PEG 400. However, the nifedipine plasma concentration with Carbopol–PEG gel (C) was lower than that with PEG 400. The nifedipine plasma profile after administration of Carbopol–PEG gel (A) was similar to that with Carbopol gel. Carbopol–PEG gel (B) gave lower plasma concentrations as compared with PEG 400 and Carbopol–PEG gel (C) from 30 min to 6 h after administration, but the nifedipine plasma concentration was maintained near the maximum level from 4 to 6 h after administration.

Nasal administration of nifedipine in PEG 400, a water-soluble base resulted in rapid absorption and a high maximum plasma concentration (C_{max}), because the PEG 400 entrapped nifedipine and rapidly dissolved in the mucosal fluid. However, the elimination of nifedipine from plasma was very rapid after the C_{max}. This result was similar to that after rectal administration of nifedipine in a PEG suppository base.[2] On the other hand, the low



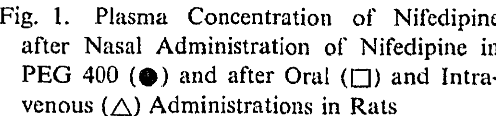


Fig. 1. Plasma Concentration of Nifedipine after Nasal Administration of Nifedipine in PEG 400 (●) and after Oral (□) and Intravenous (△) Administrations in Rats

The dose of nifedipine for nasal and oral administrations was 20 mg/kg and that for intravenous administration was 2 mg/kg. Each point is the mean ± S.E. of 4 animals.

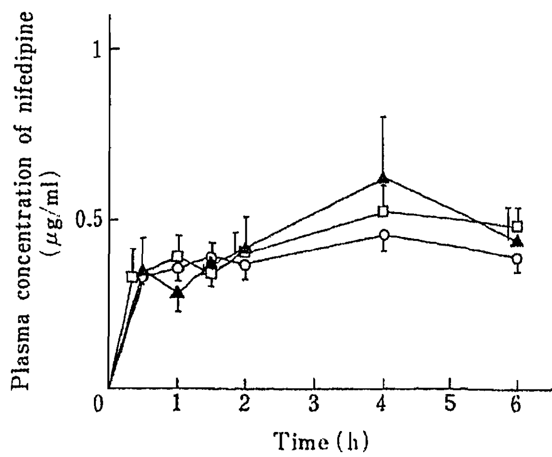


Fig. 2. Plasma Concentration of Nifedipine after Nasal Administration of Nifedipine in Aqueous Carbopol Gel in Rats

Concentration of Carbopol in gel: ○, 0.05% (w/v) Carbopol; ▲, 0.1% (w/v) Carbopol; □, 0.5% (w/v) Carbopol.

The dose of nifedipine for nasal administration was 20 mg/kg. Each point is the mean ± S.E. of 4 animals.

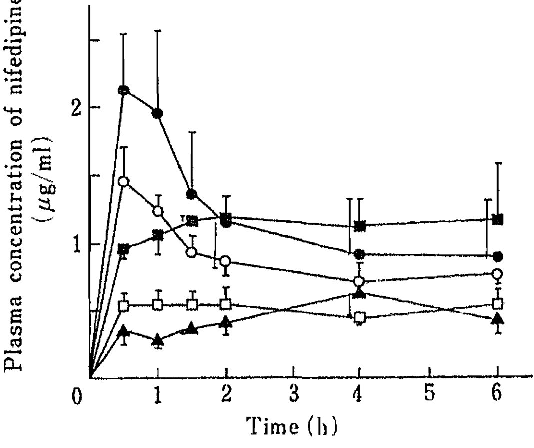


Fig. 3. Plasma Concentration of Nifedipine after Nasal Administration of Nifedipine in Carbopol–PEG Gel in Rats

□, Carbopol–PEG gel (containing 35% w/v PEG 400) (A); ■, Carbopol–PEG gel (containing 50% w/v PEG 400) (B); ○, Carbopol–PEG gel (containing 70% w/v PEG 400) (C); ●, PEG 400; △, Carbopol gel (0.1% w/v, pH 6.5).

The dose of nifedipine for nasal administration was 20 mg/kg. Each point is the mean ± S.E. of 4 animals.

nifedipine plasma concentration after nasal administration of nifedipine in aqueous Carbopol gel was caused by the poor solubility of nifedipine in aqueous gel. In the case of Carbopol–PEG gel, higher plasma concentrations of nifedipine were obtained with gel preparations containing PEG 400 at higher concentrations. Umeda *et al.* also reported that in a cellulose acetate phthalate–PEG matrix suppository, PEG enhanced the bioavailability of nifedipine.[12] The reason may be that PEG functions as a cosolvent of nifedipine, a poorly water-soluble drug in the aqueous gel.[13] Carbopol–PEG gel (containing 50% w/v PEG 400) gave an optimum nifedipine plasma concentration, which was well maintained.

In conclusion, nasal administration of nifedipine in PEG 400 resulted in rapid absorption and a high C_{max}. However, the elimination of nifedipine from plasma was very rapid after the C_{max}. Nasal administration of nifedipine in Carbopol–PEG gel (containing 50% w/v PEG) showed a relatively high plasma concentration and a prolonged action.

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Photo-Stabilization and Solubilization of an Aldose Reductase Inhibitor, (*E*)-3-Carboxymethyl-5-[(2*E*)-methyl-3-phenylpropenylidene]rhodanine (ONO-2235), by Human Serum Albumin

YUKIHISA KURONO,* AKIHIRO FURUKAWA, YOKO TAKESUE, FENGLONG LI, and KEN IKEDA

Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1, Tanabe-dori, Mizuho-ku, Nagoya 467, Japan

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Photo-stabilization and solubilization of an aldose reductase inhibitor, (*E*)-3-carboxymethyl-5-[(2*E*)-methyl-3-phenylpropenylidene]rhodanine (ONO-2235), by human serum albumin (HSA) were investigated in aqueous solution. ONO-2235 dissolved in pH 7.4 phosphate buffer was photo-stabilized by a factor of about 150 in the presence of a 5-fold excess of HSA per mole of the drug. The solubilities of ONO-2235 in the buffer and distilled water were increased about 17-fold and 57-fold, respectively, by the presence of 6×10^{-4} M or more HSA. The binding parameters for the interaction between ONO-2235 and HSA were determined from equilibrium dialysis data. These results indicate the usefulness of HSA as a drug additive.

Keywords——(*E*)-3-carboxymethyl-5-[(2*E*)-methyl-3-phenylpropenylidene]rhodanine (ONO-2235); human serum albumin; solubility; solubilization; photo-stability; stabilization; drug additive; binding; binding parameter; equilibrium dialysis

Human serum albumin (HSA) is a major protein component of human blood, and strongly binds numerous endogenous and exogenous compounds.[1-3] (*E*)-3-Carboxymethyl-5-[(2*E*)-methyl-3-phenylpropenylidene]rhodanine (ONO-2235) is an aldose reductase inhibitor and is expected to be used therapeutically in some diabetic complications.[4,5] However, ONO-2235 is photo-unstable and has low water solubility. As a part of our continuing studies on the utilization of HSA as a drug additive (*e.g.*, as a solubilizer and stabilizer),[6-8] we examined its effect on the properties of ONO-2235. In this paper, we report the photo-stabilization and solubilization of ONO-2235 by HSA, and also the binding parameters for the interaction between ONO-2235 and HSA.

Experimental

Materials——HSA (Sigma Chemical Co., lot 35F-9432) was used after purification by the method of Chen.[9] The concentration of HSA was determined by use of its molar absorptivity ($\varepsilon_{HSA} = 3.66 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$) at 278 nm, assuming a molecular weight of 69000.[10-12] ONO-2235 (lot J-3006) was supplied by Ono Pharmaceutical Co., Ltd. and was used without further purification. All other chemicals were purchased and were of reagent grade.

Analysis of ONO-2235——Since ONO-2235 exhibits absorption maxima at 400 nm ($\varepsilon_{ONO\text{-}2235} = 4.04 \times 10^4\ \text{M}^{-1}\text{cm}^{-1}$) and 294 nm ($\varepsilon'_{ONO\text{-}2235} = 1.13 \times 10^4\ \text{M}^{-1}\text{cm}^{-1}$) and the photo-decomposition products do not have absorbance at 400 nm, the absorbance at 400 nm was used for the quantitative analysis of ONO-2235 in the absence of HSA. In the presence of HSA, the absorbance at 433 nm was employed because the spectra of HSA-bound ONO-2235 and unbound ONO-2235 show an isosbestic point at 433 nm ($\varepsilon_{mixture} = 1.68 \times 10^4\ \text{M}^{-1}\text{cm}^{-1}$).

High-performance liquid chromatography (HPLC) was carried out for the separation of ONO-2235 and its photo-decomposition products. The apparatus (TRI ROTOR®, JASCO) was equipped with a Cosmosil $_5C_{18}$ column (Nakarai Kagaku Inc., 5 μm, 4.6 i.d. × 150 mm) and a variable-wavelength spectrophotometric detector (UVIDEC 100-II, JASCO), operated at 294 nm. The mobile phase was pH 7.4, 0.01 M phosphate buffer : acetonitrile (65 : 35, v/v)

and the flow rate was 1.0 ml/min. Peak area analyses were carried out with a C-R1B data processor (Shimadzu). The injection reproducibility was within $\pm 1\%$. Under these conditions the retention time, t_R, of ONO-2235 was about 7.5 min, and two other peaks due to the photo-decomposition products were observed at t_R's of about 5.5 and 6.5 min. The chemical structures of the products are unknown at present. The peak area of t_R 7.5 min was used for the quantitative analysis of ONO-2235.

Photo-Reaction——The photo-reaction was carried out in an irradiation apparatus (Riko, Type UVL-100P). The buffered ONO-2235 solution in the presence and absence of HSA was placed in the reaction vessel (made from quartz), and was photo-irradiated with a 100 W mercury vapor lamp (Riko, Type UVL-100H). At appropriate time intervals, samples were withdrawn and were analyzed by the ultraviolet spectrum (UV) method or HPLC method described above. The reaction temperature was 21—27 °C.

Solubility——ONO-2235 (10 mg) was placed in vials containing 3 ml of 0—9.40×10^{-4} M HSA solution prepared from the pH 7.4 buffer or distilled water. The vials were shaken for about 20 h in a thermostated room at 25 ± 1 °C. The samples were filtered with a Millipore filter (Millipore Corporation, 0.5 μm) and diluted with the pH 7.4 buffer. The concentrations of ONO-2235 were determined by the UV method.

Equilibrium Dialysis——Visking® cellulose tubing (Viskase Corporation) was used after pretreatment (it was boiled for 30 min twice in distilled water and in phosphate buffer).[12,13] The compartments for the dialysis contained 10 ml of 7.40×10^{-6} M—9.90×10^{-5} M HSA solution (inside) and 40 ml of 9.00×10^{-6} M—9.70×10^{-5} M ONO-2235 solution (outside). After 17—23 h under shaking in a room at 25 ± 1 °C, the concentration of ONO-2235 outside was determined by the UV method.

Results and Discussion

Photo-Stabilization

Figure 1 shows the first-order plot for the photo-decomposition of ONO-2235 in the presence and absence of HSA. All the plots are linear, and the pseudo first-order rate constant, k_{obs}, was determined according to Eq. 1.

$$\log(A_t/A_0) = -k_{obs} \cdot t/2.303$$

In Eq. 1, A_0 and A_t are the peak areas (or absorbances) at times 0 and t, respectively. The k_{obs} values in the absence of HSA, determined from the plots based on the UV method (●) and the HPLC method (○), were 2.45×10^{-3} s^{-1} (half life, $t_{1/2} = 2.83 \times 10^2$ s) and 2.33×10^{-3} s^{-1} ($t_{1/2} = 2.97 \times 10^2$ s), respectively. Both values are in good agreement with each other. In the presence of HSA the value of k_{obs} was 1.62×10^{-5} s^{-1} ($t_{1/2} = 4.28 \times 10^4$ s $= 11.9$ h), which is about 1/150 of that in the absence of HSA. It is apparent that the photo-stabilizing effect of HSA is very large.

Solubilization

Figure 2 shows the effect of HSA concentration on the solubility of ONO-2235. In both the buffer and distilled water, maximum solubility (about 3.80×10^{-3} M) was obtained at 6×10^{-4} M or more HSA concentration. In the absence of HSA the solubilities in the buffer and distilled water are about 2.25×10^{-4} M and 6.66×10^{-5} M, respectively. The differences in the values between the buffer and distilled water may result from the difference in the degree

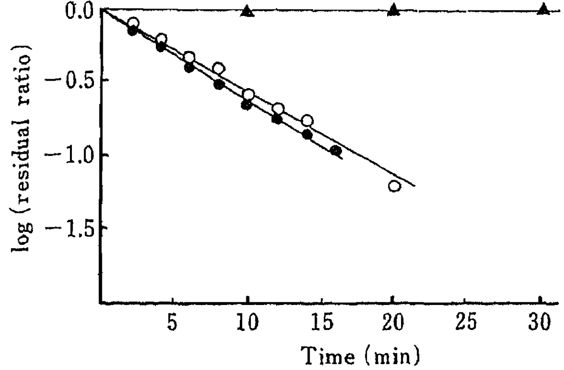

Fig. 1. First-Order Plots for the Photo-Decomposition of ONO-2235 Irradiated with a UVL-100H Lamp at 21—27 °C in 0.067 M Phosphate Buffer, pH 7.4 ($\mu = 0.2$ with NaCl)

○, 2.00×10^{-5} M ONO-2235 (determined by HPLC method); ●, 2.50×10^{-5} M ONO-2235 (determined by UV method); ▲, 2.50×10^{-5} M ONO-2235 + 1.25×10^{-4} M HSA (determined by the UV method).

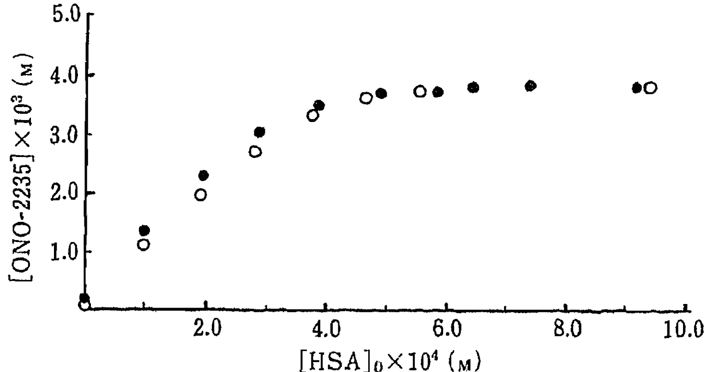

Fig. 2. Effects of HSA Concentration on the Solubility of ONO-2235 at 25 °C

●, 0.067 M phosphate buffer (pH 7.4); ○, distilled water.

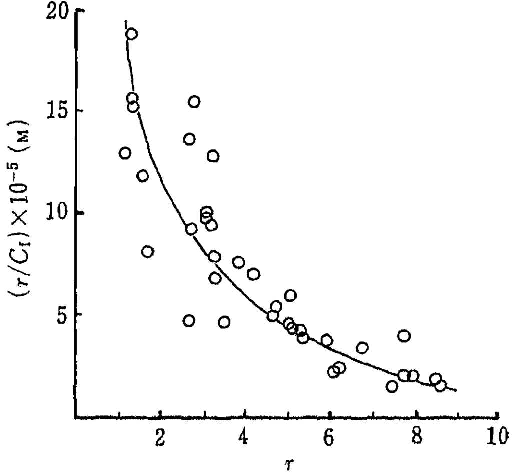

Fig. 3. Scatchard Plot for Binding of ONO-2235 to HSA Using Equilibrium Dialysis Data at 25 °C

The solid curve was calculated by using the obtained parameters.

of dissociation of the carboxyl group in the ONO-2235 molecule. The ratios of the maximum solubility to the solubilities of ONO-2235 in the absence of HSA are about 17 and 57 in the buffer and distilled water, respectively. The solubilizing effect of HSA is large.

Binding Parameters for Interaction between ONO-2235 and HSA

Since the binding of ONO-2235 to HSA is considered to be one of the mechanisms of the photo-stabilization and solubilization of ONO-2235 by HSA, equilibrium dialysis was carried out for the determination of the binding parameters. Figure 3 shows the Scatchard plot[12-15] for the equilibrium dialysis data, where r represents moles of bound ONO-2235 per mole of HSA and C_f is the unbound (free) concentration of ONO-2235. The curvature of the plot indicates the presence of more than two classes of binding sites. When two classes of binding sites on HSA are assumed, the following equation[12-15] can be used for the analysis.

$$r = n_1 K_1 C_f/(1 + k_1 C_f) + n_2 K_2 C_f/(1 + K_2 C_f) \qquad (2)$$

In Eq. 2, n and K represent the number of the binding sites and the association constant, respectively. Subscripts 1 and 2 indicate the high- and low-affinity binding sites, respectively. The binding parameters (n_i and K_i) were determined by applying non-linear least-squares analysis (MULTI[16]) to Eq. 2. The obtained values are: $n_1 = 2.12$, $K_1 = 1.12 \times 10^6\ \text{M}^{-1}$, $n_2 = 8.38$ and $K_2 = 5.41 \times 10^4\ \text{M}^{-1}$. These values indicate that there is a strong interaction between ONO-2235 and HSA.

In conclusion, the binding of ONO-2235 to HSA leads to the photo-stabilization and solubilization of ONO-2235, indicating the usefulness of HSA as a new drug additive.

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Preparation of Controlled-Release Granules of Sodium Diclofenac

YOSHIHIKO HIROTANI,*,[a] YUKIO ARAKAWA,[a] YOSHIMI MAEDA,[a] AKIRA YAMAJI,[a] AKIRA KAMADA,[b] and TOSHIAKI NISHIHATA[b]

Department of Pharmacy, Osaka University Hospital,[a] 1-50, Fukushima 1-chome, Fukushima-ku, Osaka 553, Japan and Faculty of Pharmaceutical Science, Osaka University,[b] 1-6 Yamadaoka, Suita, Osaka 565, Japan

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The release of sodium diclofenac from granules prepared with hydrogenated soya lecithin (lecithin) alone occurred slightly more slowly than that from a commercial tablet. The addition of cholesterol to the granules caused a significantly slower release as the cholesterol content in the granules was increased. The release of sodium diclofenac from granules containing cholesterol seems to occur by the leaching mechanism proposed by Higuchi.

Keywords——controlled release; granule; sodium diclofenac; lecithin; cholesterol; *in vitro* study

Although the intensity of a pharmacological effect is related to the drug concentration at the site of action, which is in turn generally related to the plasma drug concentration, an ideal situation is obtained when the concentration in the body is continuously maintained between the minimal effective and the maximal safe values. However, when the drug has a relatively short elimination half life, it is impossible to maintain the concentration within the therapeutic range without frequent dosing or the use of sustained-release formulations.

Among non-steroidal anti-inflammatory drugs, oral administration of sodium diclofenac in a commercial tablet caused a transient rapid increase of plasma diclofenac concentration, followed by relatively rapid elimination from the plasma in humans.[1] Recently we have reported[2,3] that addition of lecithin in a triglyceride suppository base gave sustained release of sodium diclofenac from the suppository, and that the administration of the suppository avoided a transient high plasma diclofenac concentration and gave a well maintained plasma diclofenac concentration in dogs and humans. Since lecithin is known to be hydrated[4] in spite of its poor solubility in water, it was suggested that lecithin in the suppository base regulated the infiltration of rectal fluid.[3] Thus, it is expected that a vehicle matrix prepared with lecithin can control the release of drugs contained in the matrix.

In the present study, we prepared granules of sodium diclofenac with hydrogenated soya lecithin, and investigated the release of sodium diclofenac from them.

Experimental

Materials——Sodium diclofenac was supplied by Ciba Geigy Japan (Takarazuka, Japan). Hydrogenated soya lecithin (lecithin), more than 95% hydrogenated, was supplied by Nikko Chemical (Tokyo, Japan). Cholesterol was obtained from Sigma Inc. (St. Louis, U.S.A.). Other reagents used were of analytical grade.

Preparation of Granules——Granules of sodium diclofenac were prepared as follows; the constituents are listed in Table I. Sodium diclofenac, lecithin and cholesterol (total amount, 1 g) were dissolved in 20 ml of chloroform–ethanol (50% : 50%), with warming if necessary. After complete dissolution, the solvents were evaporated off at 50 °C under reduced pressure for 5 h, followed by drying of the solid at room temperature for 24 h under reduced pressure. The solid was pulverized with a mortar and pestle, and then granules in the size range of 74 to 149 μm were collected.

Table I. Codes and Constituents of Granules of Sodium Diclofenac

Code	Content of each constituent (mg)			Recovery[a] of sodium diclofenac (mg/g)
	Sodium diclofenac	Lecithin	Cholesterol	
1	50	950	0	49.5±3.1
2	50	855	95	50.5±3.4
3	50	760	190	49.3±5.2
4	50	475	475	48.6±4.8
5	200	640	160	47.5±3.5
6	100	720	180	52.2±4.4
7	25	780	195	49.6±5.0

a) Recovery of sodium diclofenac was measured by the dissolution of granules in 1 N NaOH solution. Each value represents the mean±S.D. ($n=3$).

Granules prepared are listed in Table I.

Release Study——Release of sodium dichlofenac was examined by the rotating basket method in JP X at 100 rpm or at 150 rpm.[5] One gram of granules was employed for each study and 500 ml of JP X 2nd fluid (pH 6.8; 0.2 M KH_2PO_4–NaOH buffer) was used as the medium at 37 °C. The basket was covered with cotton gauze. After starting the experiment, 1 ml aliquots were collected through a Millipore filter (pore size of 0.45 μm) at designated time intervals for 24 h. The concentration of sodium diclofenac in each sample solution was measured by spectrophotometric method at the wavelength of 275 nm, or by high-performance liquid chromatography.[6]

Statistical Analysis——Statistical analyses were performed by using Student's t-test.

Results and Discussion

Granules of code-1 to code-4 with the same content of sodium diclofenac (50 mg/g) were examined, together with a commercial tablet containing 25 mg of sodium diclofenac. As shown in Fig. 1, release of diclofenac from granules of code-1 was only slightly slower than that from the commercial tablet. A greater content of cholesterol in the granules caused a slower release of diclofenac. Thus, it is considered that cholesterol delays the degradation of granules, or decreases the volume of medium infiltration into the matrix or the rate of its infiltration.

Release of a drug from granules often occurs as a result of degradation of the granules, as suggested by Hixson and Crowell.[7] In this case, drug release may be represented by Eq. 1 (the Hixson–Crowell equation),[7]

$$1-(W_t/W_0)^{1/3}=k_1 t \qquad (1)$$

where W_0 represents the total amount of drug in the granules, W_t represents the remaining amount of drug in the granules after time t (remaining amount was calculated from the measurement of the released amount in the present study), and k_1 is a constant for each type of granules. As shown in Fig. 2A, data analyzed according to Eq. 1 did not give a good straight line, especially at the early stage, except for code-1. Thus, release of diclofenac seems to occur through the mechanism of granule degradation only from granules of code-1. Since degradation of granules of code-2, code-3 and code-4 was not apparent to the naked eye at least up to 8 h, another mechanism may be involved in the release of diclofenac from these granules. Further, it was observed that the initial release of diclofenac was greater than expected. This initial burst of release of diclofenac may reflect rapid dissolution of diclofenac which was adsorbed on the granule surface (see below).

A possible mechanism of the drug release from granules of code-2 to -4 may be leaching, as proposed by Higuchi.[8] This is described by Eq. 2:

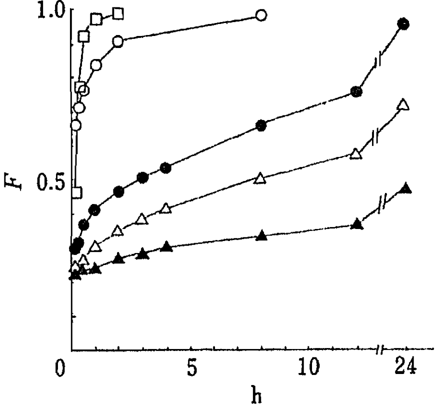

Fig. 1. Release of Sodium Diclofenac from Various Granules as a Function of Time

Release of sodium diclofenac is represented as the fraction of sodium diclofenac released, F. Granules used are symbolized as follows: ○, code-1; ●, code-2; △, code-3; and ▲, code-4. The symbol □ represents the release of sodium diclofenac from a commercial tablet containing 25 mg of sodium diclofenac. Rotation speed was 100 rpm. Data in this figure were obtained with one example of each formulation.

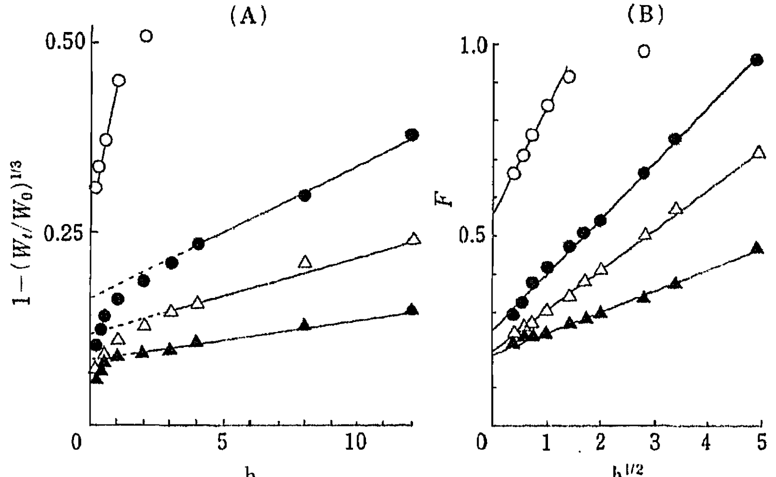

Fig. 2. Release Profiles of Sodium Diclofenac from Various Granules

(A) The profile according to Eq. 1 (Hixson–Crowell's equation); (B) The profile according to Eq. 2 (Higuchi's equation). Symbols were as described in Fig. 1.

$$Q=[DC_s\varepsilon(2A-\varepsilon C_s)t/\tau]^{1/2} \tag{2}$$

where Q represents the amount of drug released from unit surface area, D represents the diffusivity of the drug in the infiltration solvent, ε represents the porosity of granules, τ represents the toutuosity factor of granules, A represents the total amount of drug in unit volume and C_s is the solubility of the drug in the infiltration medium (C_s of sodium diclofenac in the solvent was 18 mM at 37 °C). The fraction, F, of diclofenac released from test granules can be represented by Eq. 3:

$$Q'=QS$$

$$F=Q'/AV_0=S[DC_s\varepsilon(2A-\varepsilon C_s)t/\tau]^{1/2}/AV_0 \tag{3}$$

where S represents the total surface area, V_0 represents the total volume of granules, and Q' represents the amount of diclofenac released from the granules tested.

As shown in Fig. 2B, a plot of F against the square root of time (h) gave a good straight line for each of the granules. These results suggest that release of diclofenac from granules occurs by leaching. The slope, k_2, of the line is represented by Eq. 4

$$k_2=S[DC_s\varepsilon(2A-\varepsilon C_s)/\tau]^{1/2}/AV_0 \tag{4}$$

Since the solubility of sodium diclofenac is low, it may be estimated that $2A$ is greater

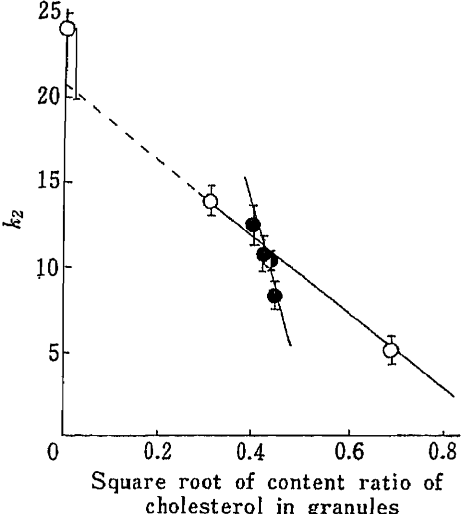

Fig. 3. Relationship between Estimated Release Rate of Sodium Diclofenac, k_2, and Square Root of Content Ratio (w/w) of Cholesterol in Granules

Open symbols represent code-1, code-2, code-3 and code-4 granules. Closed symbols represent code-3, code-5, code-6 and code-7 granules. Rotation speed was 100 rpm. Each value represents the mean ± S.D. ($n=3$). A straight line was obtained based on the open symbols excluding code-1 granules: $k_2=-22.8$ (content ratio of cholesterol)$^{1/2}$ ± 20.7 ($r=-0.998$). Another straight line was obtained based on the closed symbols: $k_2=-90.12$ (content ratio of cholesterol)$^{1/2}$ ± 48.4 ($r=-0.941$).

TABLE II. Comparison of k_2 Values[a] as a Parameter of Release from Various Granules, and the Effect of Rotation Speed

Code	k_2 at 100 rpm	r[b]	k_2 at 150 rpm	r[b]
1[c]	24.2 ± 4.2	>0.994	34.7 ± 3.9[d]	>0.989
2	13.9 ± 0.8	>0.994	14.6 ± 2.1	>0.991
3	10.4 ± 0.5	>0.990	11.2 ± 1.7	>0.992
4	5.1 ± 0.8	>0.991	5.7 ± 0.4	>0.994
5	12.5 ± 1.2	>0.997	Not determined	
6	10.8 ± 1.1	>0.991	Not determined	
7	8.1 ± 0.8	>0.993	Not determined	

a) The k_2 values were obtained from the slope of the straight line obtained when the fraction of sodium diclofenac released was plotted against the square root of time. *b*) Correlation coefficient of the straight line to obtain k_2 value. *c*) A straight line was obtained up to 1 h. *d*) $p<0.05$ *versus* value at 100 rpm. Each value represents the mean ± S.D. ($n=3$).

than εC_s. Equation 5 was obtained from Eq. 4

$$k_2=[S(2ADC_s)^{1/2}/AV_0](\varepsilon/\tau)^{1/2} \quad (5)$$

In Eq. 5, the change of k_2 is related to the value of ε/τ. Thus, the decrease of k_2 value with the increase of cholesterol content in the granules may indicate that cholesterol decreases the value of ε/τ in Eq. 5. When the k_2 values for granules of code-2 to -4 were plotted (closed circles in Fig. 3) against the square root of the content ratio of cholesterol, a good straight line was obtained. This result suggests that cholesterol in granules decreases the porosity of the granules and/or increases the tortuosity of the granules. Thus, release of sodium diclofenac from granules prepared with lecithin can be controlled easily by adjusting the cholesterol content.

Since sodium diclofenac dissolves in the infiltration medium, decrease of the content of sodium diclofenac may decrease the value of ε/τ in Eq. 5. To investigate the effect of content of sodium diclofenac, code-3, code-5, code-6 and code-7 granules were used. As shown in Table II, a smaller content of sodium diclofenac in granules resulted in a smaller k_2 value. However, since increase of the content of sodium diclofenac in the granules prepared in this study was accompanied with a decrease of cholesterol content, the k_2 value was also plotted against the square root of the content ratio of cholesterol. In this case, the value of slope obtained from

the apparent straight line (closed circles in Fig. 3) was significantly greater than that obtained from the open circle in Fig. 3. Thus, the content of sodium diclofenac seems to affect the value of ε/τ in Eq. 5.

When the speed of the rotating basket was changed, the release of diclofenac from granules of code-2 to code-4 remained unchanged between 100 and 150 rpm (Table II). However, the release from code-1 granules at 150 rpm was greater than that at 100 rpm (Table II). Thus, although the release from code-1 granules may occur predominantly by degradation of the granules, the release from code-2, -3 and -4 granules occurs predominantly by the leaching mechanism rather than by degradation.

As can be seen in Fig. 2B, each regression line gave an intercept with a positive value at zero time. In plots based on Higuchi's equation, the intercept often gives a negative value at zero time because there should be a lag time before the infiltration of solvent into the granules. Thus, the granules in the present study might have adsorbed more sodium diclofenac than expected on their surface during the process of granule preparation. Some sodium diclofenac dissolved in the organic solvent may have been adsorbed on the surface of solids which precipitated before the complete evaporation of the solvent.

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Effect of Solvents on the Permeation of Nicardipine Hydrochloride through the Hairless Rat Skin[1]

TOSHINOBU SEKI, KENJI SUGIBAYASHI and YASUNORI MORIMOTO*

Faculty of Pharmaceutical Sciences, Josai University, 1–1 Keyakidai, Sakado, Saitama 350–02, Japan

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The effect of several pure or mixed solvent systems on the skin permeation of nicardipine hydrochloride (NC), a potential calcium antagonist, was examined. The effect was evaluated by measuring the steady-state permeation rate through the excised hairless rat skin mounted in a 2-chamber diffusion cell and by measuring the solubilities of NC in these solvents at 37 °C. Although the solubility of NC in propylene glycol (PG) was higher than those in water, ethanol (EtOH) and methylethylketone (MEK), the skin permeation rate of NC from PG suspension was as low as that from aqueous suspension and lower than those from EtOH and MEK suspensions. The solubilities of NC in a binary EtOH–water solvent system at various ratios were higher than those in the pure solvents. Such cosolvency was also observed in the ternary EtOH–MEK–water solvent system. The relationship between the logarithm of the calculated maximum flux of NC from the binary and ternary solvent systems and solvent composition was similar to that between the solubility of NC and the solvent composition. The highest permeability was observed from the solvent system which gave the highest solubility. These results suggest that the use of mixed solvents is effective as a vehicle for enhancing the skin permeation of NC.

Keywords——skin permeation; nicardipine hydrochloride; calcium antagonist; cosolvency; hairless rat skin

Nicardipine hydrochloride (NC), a calcium antagonist in the 1,4-dihydropyridine class, is useful for the therapy of hypertension.[2] Although NC is rapidly absorbed following oral administration, its bioavailability is relatively low due to the first-pass metabolism in the liver, and its biological half-life is very short.[3] The skin is useful as an administration site to avoid such first-pass metabolism in the liver. In addition, the use of the skin as an administration site can help to maintain the plasma concentration of drugs and also to improve patient compliance.[4] Thus, it might be worthwhile to develop a transdermal therapeutic system (TTS) as a possible replacement for conventional dosage forms of NC such as oral tablet.

In the present study, we examined the effect of several pure or mixed solvent systems on the skin permeation of NC as the first step to develop a TTS. The effects were evaluated by measuring the steady-state permeation rate of NC through the excised hairless rate skin and by measuring the solubilities of NC in these solvents.

Experimental

Materials——NC was kindly supplied by Nissan Chemical Industries (Tokyo, Japan). Distilled water and reagent-grade ethanol (EtOH, Wako Pure Chemical Ind., Osaka, Japan), propylene glycol (PG, Wako Pure Chemical Ind.) and methylethylketone (MEK, Wako Pure Chemical Ind.) were used as solvents.

Preparation of Mixed (Binary or Ternary) Solvents——EtOH and MEK were mixed with water to give a total volume of 100 ml. The resulting solvent was designated as, for example, EtOH : MEK : $H_2O = 1:2:1$ ternary solvent system (after mixing 25 ml of EtOH, 50 ml of MEK and an appropriate volume of water).

Solubility Measurements——Excess NC was added to pure or mixed solvents. Each suspension was incubated in a water bath at 37 °C for 24 h. The concentration of NC in the supernatant was determined by using a high

performance liquid chromatography (HPLC) system (LC-6A, Shimadzu Seisakusho, Kyoto, Japan). The conditions were as follows: column, 4.6 mm × 250 mm stainless steel column packed with Nucleosil $5C_{18}$ (Nagel, Germany); mobile phase, methanol : 0.02 M KH_2PO_4 (3 : 1); detector, UV 240 nm.

***In Vitro* Skin Permeation Experiments**——The abdominal skin of WBN/kob hairless rat (180—200 g, Saitama Laboratory Animals, Sugito, Saitama, Japan) was excised and mounted in a 2-chamber diffusion cell.[5] NC was suspended in various pure solvents or dissolved (10 mg/ml) in various mixed solvents. The resulting suspension or solution was added to the donor-side half-cell. The same solvent (NC-free) was added to the receiver-side half-cell, in order to prevent the effect of solvent permeation from the donor to the receiver side or *vice versa* on the NC permeation through the skin. The cumulative amount of NC that permeated through the skin per unit area, Q, was determined by HPLC (conditions: same as above).

Theoretical

The steady-state permeation rate per unit area, F, which is the same as the differential of Q against time, t, is expressed by[6]:

$$F = \frac{dQ}{dt} = \frac{a_d}{\gamma_m} \frac{D}{L} \tag{1}$$

where a_d and γ_m are the thermodynamic activity of the drug in the donor compartment and the activity coefficient of the drug in the skin barrier, respectively, and D and L are the diffusion coefficient of the drug in the skin barrier and the thickness of the skin barrier, respectively. The activity, a_d, can be expressed by:

$$a_d = C_d \times \gamma_d \tag{2}$$

where C_d and γ_d are the concentration and activity coefficient of the drug in the donor compartment, respectively. When the drug is suspended in the donor compartment, Eqs. 1 and 2 can be expressed as follows:

$$F_{max} = \frac{a_{max}}{\gamma_m} \frac{D}{L} \tag{3}$$

$$a_{max} = C_s \times \gamma_{d,max} \tag{4}$$

where a_{max} and $\gamma_{d,max}$ are the activity and activity coefficient of the drug in the saturated solution, respectively, and C_s is the solubility of the drug. Assuming that $\gamma_d = \gamma_{d,max}$, the calculated maximum flux, F_{max}, is expressed by[7]:

$$F_{max} = F \times \frac{C_s}{C_d} \tag{5}$$

The effect of solvents on the skin permeation of NC from the binary and ternary solvent systems was evaluated in terms of the F_{max} value. In the case of permeation experiments using pure solvents on the donor side, the F value can be assumed to be F_{max}. On the other hand, with mixed solvents, F_{max} was calculated by using Eq. 5 from the observed F value. Comparison in terms of F_{max} was done to ensure equal thermodynamic activity of NC in different solvents.

Results and Discussion

Table I shows the solubilities of NC in pure solvents at 37 °C. The solubility of NC in PG was higher than those in water, EtOH and MEK.

Figure 1 shows the solubilities of NC in binary EtOH–water and ternary EtOH–MEK–water solvent systems. The solubilities of NC in several binary EtOH–water solvents were higher than those in both pure solvents; this effect is called cosolvency. The highest solubility

TABLE I. Solubility of NC at 37 °C

	Solubility at 37 °C (mg/ml)
Water[a]	8.71
EtOH	19.1
PG	48.5
MEK	2.75

a) The pH of NC suspension in water was 4.0.

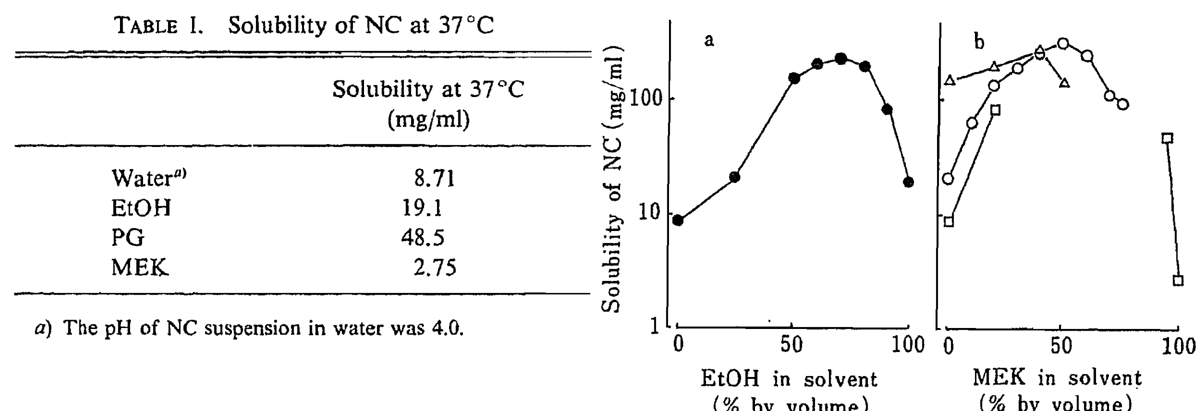

Fig. 1. Solubility of NC in Mixed Solvent Systems at 37 °C

a: EtOH–water binary system. b: EtOH–MEK–water ternary system. (○), EtOH 25% (v/v); (△), EtOH 50% (v/v); (□), without EtOH. The data are means of three experiments.

(228 mg/ml) in the binary EtOH–water solvent system was observed in EtOH : H_2O = 7 : 3 (70% (v/v) EtOH); it amounted to about 20 and 10 times those in water and EtOH, respectively. Cosolvency was also observed in the ternary EtOH–MEK–water solvent system. The highest solubility (316 mg/ml) was observed in the ternary EtOH : MEK : H_2O = 1 : 2 : 1 solvent system; it was about 1.5 times higher than the highest value for the binary EtOH–water system (EtOH : H_2O = 7 : 3).

Figure 2 shows the skin permeation profiles from NC suspensions in pure water, EtOH, PG and MEK. Although the solubility of NC in PG was higher than those in the other pure solvents, the skin permeation rate of NC from PG suspension was as low as that from aqueous suspension. Skin permeation rates of NC from EtOH and MEK suspensions were higher than those from aqueous and PG suspensions.

The reason why the permeation rate of NC from PG suspension is similar to that from aqueous suspension might be that the thermodynamic activity of NC in PG suspension is the same as that in the aqueous suspension for the donor compartment. The partition of drugs from the donor compartment containing highly drug-solubilizing solvents to the skin barrier phase is usually low.[8] If drugs were suspended in solvents and the solvents did not affect the skin barrier, the skin permeation rate of drugs should be constant independently of the kind of solvent. This conclusion is easily derived from Higuchi's theory[6] (Eq. 1).

On the other hand, the skin permeation rates of NC from EtOH and MEK suspensions were much higher than those in aqueous and PG suspensions. The rapid skin permeations from EtOH and MEK suspensions might be related to the changes of D and/or γ_m which are induced by the penetration of such solvents, since the thermodynamic activities of NC in these suspensions are the same. Although PG and water also affect such parameters, the changes of these parameters should be very low compared to those by EtOH and MEK in this experimental system.

Figure 3a and b shows the F_{max} values calculated from the observed F values in the experiments using NC solutions prepared from binary EtOH–water and ternary EtOH–MEK–water solvent systems, respectively. The relationship between the logarithm of the calculated maximum flux of NC from the binary and ternary solvent systems and the composition of the solvent was similar to that between the solubility of NC and the composition of the solvent. Within the F_{max} values calculated by using Eq. 5 from comparable F values, the highest value (4.6×10^{-1} μg/cm^2/s) was observed in the ternary

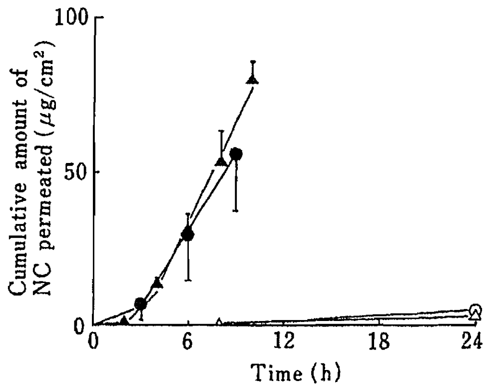

Fig. 2. Effect of Solvents on NC Permeation through the Hairless Rat Skin

(○), water; (●), EtOH; (△), PG; (▲), MEK. The data are means of three experiments and vertical bars show standard errors.

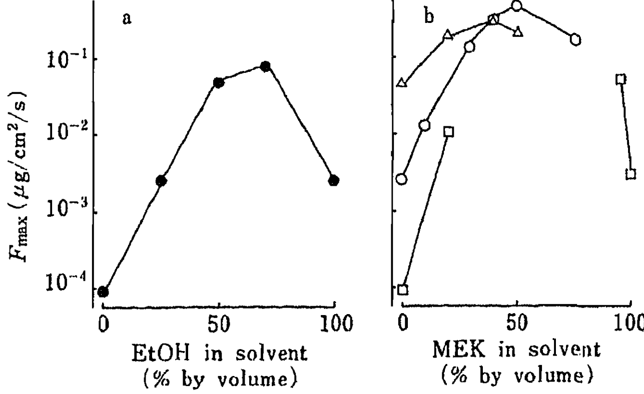

Fig. 3. Relationship between F_{max} Values and Compositions of Mixed Solvent Systems

a: EtOH–water binary system. b: EtOH–MEK–water ternary system. (○), EtOH 25% (v/v); (△), EtOH 50% (v/v); (□), without EtOH. The data are means of three experiments.

system of EtOH : MEK : H_2O = 1 : 2 : 1, which also gives the highest solubility. The F_{max} value was about 5000 times higher than that from pure water.

The marked difference of F_{max} values would result from changes of D and/or γ_m values. The changes of these parameters might be caused by solvent penetration into the skin barrier (stratum corneum). The penetration rates of EtOH, MEK and other solvents containing either one into the skin barrier might be very fast, so that these solvent systems would give high skin permeation of NC regardless of the thermodynamic activity of NC on the donor side.

These results suggest that mixed solvents containing EtOH and MEK might be effective as vehicles for topical application to enhance the skin permeation of NC. Transdermal absorption of drugs, therefore, might be enhanced by using various solvents which could not only freely solubilize the drugs but also change the values of D and γ_m in the skin barrier. Further experiments are under way in our laboratory using indomethacin,[9] an anti-inflammatory drug, and nicorandil,[10] a potent coronary vasodilator.

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Communications to the Editor

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FACILE PREPARATION OF γ-CHLORO-SUBSTITUTED ALLYLMETALLIC REAGENTS AND THEIR REACTIONS. STEREOSELECTIVE ACCESS TO CIS-VINYLOXIRANES

Akira Hosomi,*[,a] Shinya Kohra,[a] Yoshinori Tominaga,[a]
Masatomo Ando,[b] and Hideki Sakurai[b]
Faculty of Pharmaceutical Sciences, Nagasaki University,[a] Nagasaki 852, Japan and Department of Chemistry, Tohoku University,[b] Sendai 980, Japan

The title compounds are readily prepared from metallated allyl chloride and organotin chloride or organoaluminum chloride. After treatment with a base, they react with aldehydes in highly regio- and diastereo-selective modes, giving cis-vinyloxiranes selectively.

KEYWORDS —— lithiated allyl chloride; Z-γ-chloroallyltrimethyltin; γ-chloroallyldiethylaluminum; stereoselective synthesis; syn-chlorohydrin; cis-vinyloxirane

The heteroatom-substituted allylic carbanions are recent topics in organic synthesis.[1] Although sulfur is one of the most popular and important heteroatoms in α-carbanion chemistry, the use of halogen atoms for such purpose is considerably limited, presumably due to their thermal instability.[2] We report here that the synthesis of vinyl-substituted chlorohydrins and oxiranes, important in organic synthesis,[3] can be readily achieved in highly regio- and stereo-selective mode by in situ quenching of the lithiated allyl chloride[4] with chlorostannane and chloroaluminum compounds and successive reaction with aldehydes.

We have previously demonstrated that lithiated allyl chlorides react with chlorosilanes in situ to afford α-chloroallylsilanes exclusively.[5] In sharp contrast, treatment of a solution containing chlorotrimethylstannane and allyl chloride with lithium dicyclohexylamide cleanly provides Z-(3-chloro-2-propenyl)trimethylstannane (2a) in a single step. The 2a is easily isolated by distillation and stored.[6] The structure of 2a is clear from complete spectral analysis of the product, so the reaction is completely regio- and stereospecific.[7]

It is noteworthy that after treatment with an aqueous base, the 2a thus isolated reacts with aldehydes such as cyclohexanecarboxaldehyde very smoothly with the aid of boron trifluoride etherate[8] to give the corresponding cis-vinyloxiranes (6) via syn-chlorohydrins (5) in highly stereoselective mode. Without the base treatment, 5 can also be isolated from the reaction mixture,

R^1 Cl R_2NLi/L_nMX (3)   THF, -78°C L_nM R^1 Cl

1   2a, L_nM = Me_3Sn
b, = Et_2Al
c, = $(iso\text{-}PrO)_3Ti$

R^2CHO (4) R^1 HO H R^2 Cl H   + R^1 H OH R^2 Cl H

syn-5   anti-5

OH^-

R^1 R^2 O   + R^1 R^2 O

cis-6   trans-6

Chart 1[9)]

though with lower yield due to ready dehydrochlorination to **6** during isolation of 5 by TLC. The major results of our findings are illustrated in Table I along with the data of in-situ generated [chloroallyl]-aluminum and -titanium reagents of type **2**. (Chart 1)

Quite similarly but more importantly from the viewpoint of experimental simplicity in organic synthesis, the one-pot synthesis of vinyloxiranes can be attained by the reaction of lithiated allyl chloride with diethylaluminum chloride, aldehydes and a base, successively. The reaction proceeds with high stereoselectivity to afford the corresponding cis-6. Activation of the carbonyl groups is not necessary in this case. Apparently γ-chloroallylaluminum species is formed in the reaction mixture as the active species. It is interesting to note that lithium 2,2,6,6-tetramethylpiperidide (LiTMP) is one of the most effective proton-abstracting bases in this case.

In contrast, [chloroallyl]titanium (2c) is an unsatisfactory reagent for the stereoselective synthesis of vinyloxiranes, the selectivity being at most 2:1 with cis preference. Loss of the stereochemistry compared with reactions of **2b** might be due to relatively rapid isomerization from Z- to E-isomer of 2c during the reaction with carbonyl compounds, although further study is required before the mechanism rationale of these reactions are fully elucidated.

Regardless of the mechanistic differences between reactions of 2a and 2b where acyclic and cyclic transition states are considered, respectively,[1)] the results of the present work constitute an unprecedented and expedient route to synthetically useful cis-vinyloxiranes (**6**) by the simple manipulation from readily available starting materials under mild conditions.

Table I. Reactions of [Chloroallyl]organometallic Species with Aldehydes

$$CH_2{=}CR^1CH_2Cl\ (1) \xrightarrow[L_nMX\ (3)]{R_2NLi} \xrightarrow[OH^-]{R^2CHO\ (4)} CH_2{=}CR^1CH\underset{O}{\diagdown\diagup}CHR^2\ (6)$$

Entry	Allyl Chloride (1)	L_nMX (3)	Aldehyde (4)	Product (5 or 6),[a)] % Yield[b)]	(cis:trans)[c)]
1	$CH_2{=}CHCH_2Cl$ (1a)	Me_3SnCl (3a)	cyclo-$C_6H_{11}CHO$ (4a)	81	(>30:1)[d,e)]
2	1a	3a	Me_2CHCH_2CHO (4b)	83[g)]	(>30:1)[d,e,f)]
3	1a	3a	$CH_3(CH_2)_3CHO$ (4c)	75[g)]	(>30:1)[d,e,f)]
4	1a	3a	$Ph(CH_2)_2CHO$ (4d)	61	(>30:1)[d,e,f)]
5	1a	3a	4d	88	(>30:1)[d,e)]
6	1a	3a	PhCHO (4e)	59	(4.1:1)[d,f)]
7	1a	3a	4e	89	(4.1:1)[d)]
8	1a	Et_2AlCl (3b)	4a	83	(5.3:1)
9	1a	3b	4c	78	(9:1)
10	1a	3b	4d	86	(>30:1)[e)]
11	1a	3b	4e	77	(4.3:1)
12	1a	$(iso\text{-}PrO)_4Ti$ (3c)	4a	67	(0.9:1)
13	1a	3c	4d	58	(1.9:1)
14	1a	3c	4e	60	(1:1)
15	$CH_2{=}C(CH_3)CH_2Cl$ (1b)	3b	4a	67	(>30:1)[e)]
16	1b	3b	4d	77	(>30:1)[e)]

a) Isolation by TLC. b) Yield was not always optimized. c) Determined by ^1H-NMR and ^{13}C-NMR. d) Isolated **2a** was used. e) Within detection limit by NMR. f) Isolated as a chlorohydrin (5) without base treatment. Syn:anti ratio is given in parenthesis. g) Determined by GLC.

A typical procedure is as follows. A solution of lithium 2,2,6,6-tetramethylpiperidide, prepared from the corresponding piperidine (2.2 mmol) and n-butyllithium (2.1 mmol) in THF (5 ml), was added slowly to a mixture of allyl chloride (2.1 mmol) and diethylaluminum chloride (4.0 mmol) in THF at -78°C. The mixture was stirred for 1-1.5 h. Subsequently, without isolation of **2b**, an aldehyde (1.0 mmol) was added to the resulting reaction mixture at -78°C. The mixture was stirred for 5-7 h at -78°C-rt and hydrolyzed with excess aqueous potassium hydroxide at room temperature for 3 h. After work-up as usual, a vinyloxirane (**6**) was isolated by TLC. Allylation with **2a** was conducted according to the procedure shown in ref. 8.

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7) A solution of lithium dicyclohexylamide, prepared from dicyclohexylamine (12.7 g, 0.06 mol) and n-butyllithium (0.075 mol in hexane) in THF (40 ml), was added to a mixture of allyl chloride (5.7 g, 0.075 mol), chlorotrimethyltin (12.0 g, 0.06 mol) and THF (80 ml) at −78°C. The reaction mixture was stirred for 3 h and hydrolyzed with aq NH_4Cl. After work-up as usual, 2a (10.3 g, 0.043mol) was isolated by distillation in 72% yield. bp 56.6-58.0°C (15 mmHg). ^{1}H-NMR (CCl_4) δ: 0.17 (s, 9H), 1.92 (d, J=8.25Hz, 2H), 5.73 (d, J=7.25Hz, 1H), 5.99 (dt, J=7.25, 8.25Hz, 1H); ^{13}C-NMR ($CDCl_3$) δ: 9.47 (q), 12.40 (t), 112.47 (d), 130.49 (d). The yield depended on the proton-abstracting base employed. Similarly Z-ClCH=CHCH$_2$Sn(n-Bu)$_3$, bp 89°C (0.1 mmHg), and Z-BrCH=CHCH$_2$SnMe$_3$, bp 70-71°C (11 mmHg), were prepared from corresponding starting materials in 42% and 37% yield, respectively. Thus the present reaction provides one of the simplest procedure for stereoselective synthesis of γ-halogen-substituted allylstannanes. All new compounds obtained in this work gave satisfactory spectral data and elemental analysis.

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Communications to the Editor

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A PRACTICAL CONVERSION OF 4-PHENYL-2-ISOXAZOLINE-2-OXIDES INTO 3,3a,8,8a-TETRAHYDRO-2-OXO-2H-FURO[2,3-b]INDOLES VIA 1-OXIDO-3H-INDOLE-3-ACETATE AS A KEY INTERMEDIATE[1)]

Eisuke Kaji, Kiyobumi Takahashi, Miyako Kitazawa, and Shonosuke Zen*

School of Pharmaceutical Sciences, Kitasato University, Shirokane, Minato-ku, Tokyo 108, Japan

5-Substituted 1-oxido-3H-indole-3-acetate (2), a readily available ring-transformed product of 4-phenylisoxazoline-2-oxides (1), were converted into 3,3a,8,8a-tetrahydro-2-oxo-2H-furo[2,3-b]indoles (6) by a three-step procedure comprising O-tosylation, elimination, and reduction.

KEYWORDS ——isoxazoline-2-oxide; ring transformation; 1-oxido-3H-indole-3-acetate; oxaziridine; (E)-, (Z)-isomer; 2-oxo-2H-furo[2,3-b]-indole

Our previous papers[2,3)] have described a unique ring transformation of 3,5-bis(methoxycarbonyl)-4-phenyl-2-isoxazoline-2-oxides (1) into 2-methoxycarbonyl-1-oxido-3H-indole-3-acetate (2) and tricyclic benzofuro[3,3a-d]isoxazoles of type 7. Since 2 is multi-functionalized at the 1,2,3, and 5-positions of the indole ring, it can serve as an expedient building block for a functionalized indoline skeleton of some indole alkaloids.

We wish to report here a novel procedure for the synthesis of 5-substituted 3,3a,8,8a-tetrahydro-2-oxo-2H-furo[2,3-b]indoles of type 6. Such indolines as 2H-furo[2,3-b]indole and 2-oxo-2H-furo[2,3-b]indole systems occur naturally in the Calabar bean alkaloid physovenine[4)] and the akuammiline-group alkaloid lanciferine,[5)] respectively.

Although considerable attention has been focused on the synthesis of 3,3a, 8,8a-tetrahydro-2H-furo[2,3-b]indoles directed towards the total synthesis of physovenine,[6)] their 2-oxo analog has rarely been synthesized. The only reliable synthesis of the system to date was presented by Kametani et al.,[7)] who described the formation of 8-benzyl-3a-hydroxy-3,3a,8,8a-tetrahydro-2-oxo-2H-furo[2,3-b]-indole (8) and its 8a-methyl derivative (9) by photo-oxygenation of N-benzyl-indole-3-acetic acid.

Our approach to the ring system of 3,3a,8,8a-tetrahydro-2-oxo-2H-furo[2,3-b]-indole involves the ring transformation of isoxazoline-2-oxide (1)[8)] affording highly functionalized 1-oxido-3H-indole-3-acetate (2),[2)] which has been converted into 6 by a three-step procedure comprising O-tosylation, elimination, and reduction, as illustrated in Chart 1. This method signifies an expedient construction of 2-oxo-2H-furo[2,3-b]indoles from 3H-indole-1-oxido-3-acetates via bicyclic oxaziridine.

A typical procedure is exemplified by a series of the compounds **a** (R = H).[9] When 1-oxido-3*H*-indole-3-acetate (**2a**),[2] readily accessible by ring transformation of **1a**[8a] with $TiCl_4$ in CH_2Cl_2, was *O*-tosylated with a two-fold molar amount of tosyl chloride in pyridine at 0°C overnight, the corresponding 1'-*O*-tosylate[10] (**3a**) was obtained in 80% yield. A fused oxaziridine ring formation would be rationalized by the following facts: 1) Although mass spectra of both **2a**[11] and **3a**[10] showed that a characteristic fragment of m/z 261 resulted from $[M^+-OH]$ and $[M^+-TsOH]$, respectively, the regions lower than that in the two are quite different. 2) The UV spectrum of **3a** showed reasonable hypsochromic and hypochromic shifts at 266 nm (log ε 3.58) and 280 nm (log ε 3.59) compared with that of **2a** (282 nm, log ε 3.62). 3) **3a** did not form a 1,3-dipolar cycloadduct in general conditions (refluxed in toluene for 4 h) with dimethyl fumarate as a dipolarophile. 4) Lusinchi et al.[12] have also reported a similar conversion of nitrons into oxaziridines by treatment with tosyl chloride.

Subsequent treatment of **3a** with an equimolar amount of triethylamine (r. t., 4 h) readily gave the two possible geometric isomers (*E*)-**4a**[13] and (*Z*)-**4a**[14] in a quantitative yield. The two isomers were separated by silica-gel column elution with chloroform, whereupon the ratio of *E* : *Z* was determined as 77 : 23%. Other analogs of **4** are summarized in Table I, where (*E*)-isomers are preferred to (*Z*)-isomers in all of the cases studied. The assignment of the configuration of both

4 (*E*): R^1 = COOMe, R^2 = H
(*Z*): R^1 = H, R^2 = COOMe

8 : R = H, **9** : R = Me

a: R = H, **b**: 4,5,6,7-tetradeuterio deriv., **c**: R = Me, **d**: R = Cl, **e**: R = F
Ts = $SO_2C_6H_4CH_3(p)$, Bn = $CH_2C_6H_5$

Chart 1

isomers was unambiguously determined on the basis of ^{1}H-NMR spectra, where the H-4 of (E)-4 is strongly deshielded to a lower field (δ 7.8-8.0 ppm) by a magnetic anisotropy of 1'-ester carbonyl group, while the H-4 of (Z)-4 is exceptionally overlapped with other aromatic protons (H-5,6,7) at δ 7.0-7.7. Moreover, NOE experiments showed increases of 19.4% for the H-4→1'-ester Me and 22.6% for the H-1'→2-ester Me of (E)-4d, whilst 18.9% for the H-4→H-1' of (Z)-4d. This cogently reflects both stereochemical relationships.

The elimination process (3→4) may proceed mainly by cis- rather than trans-elimination, so that the 3,1'-erythro-configuration[2] of 2 should give (E)-olefin in preference to (Z)-olefin.

Finally, (E)- or (Z)-3-methyleneindolines (4a) were reduced catalytically in acidic media (PtO_2/AcOH) under 2 atm (202630 Pa) of hydrogen. 2-Oxo-2H-furo[2,3-b]indoles (6a)[15] was obtained in 58% or 55% yield from the respective (E)- or (Z)-4a. This conversion may be anticipated by an initial 2-indolinol (5) formation followed by cyclization. An NH signal (δ 7.05) was distinctly observed in the ^{1}H-NMR spectrum of the 4,5,6,7-tetradeuterio derivative (6b).

As mentioned above, our procedure should be of significance in the following ways: a facile procedure using neither expensive reagents nor drastic conditions; ready availability of various 5-substituted derivatives; applicability to the synthesis of the akuammiline-group alkaloid by the C-extension of the 8a-ester function of 6; usefulness of 2-oxo-2H-furo[2,3-b]indole as a synthetic precursor for the pyrrolo[2,3-b]indole derivative.[16]

Further investigations and the full account of the present work will be published in due course.

Table I. Yields (%) of the Products

Compd.	R	2	3	4	(E : Z)	6
a	H	85	80	99	(77 : 23)	58
b	D[a]	59	72	67	(93 : 7)	56
c	Me	69	60	86	(63 : 37)	44
d	Cl	69	44	98	(89 : 11)	45
e	F	76	74	97	(89 : 11)	50

a) 4,5,6,7-tetradeuterio derivative.

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10) Colorless prisms of mp 147-149°C (MeOH). $C_{20}H_{19}NO_8S$, MS m/z: 433 (M^+), 374 (M^+-CO_2Me), 261 (M^+-TsOH), 190 ($C_{10}H_8NO_3$), 91 (C_7H_7), 59 ($C_2H_3O_2$), 43 (C_2H_3O). UV λ_{max}^{MeOH} nm (log ε): 221 (4.15), 266 (3.58), 280 (3.59). ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 2.40 (3H, s, tosyl Me), 3.63 and 3.88 (each 3H, s, ester Me), 4.57 (1H, d, J=4.0 Hz, H-3), 4.89 (1H, d, J=4.0 Hz, H-1'), 6.9 (4H, m, H-4,5,6,7), 7.22 and 7.56 (each 2H, d, J=7.5 Hz, tosyl H).

11) MS m/z: 279 (M^+), 261 (M^+-OH), 190 ($C_{10}H_8NO_3$), 159 ($C_9H_5NO_2$), 131 (C_8H_5NO), 116 (C_8H_6N), 106 (C_7H_6O), 91 (C_7H_7). UV λ_{max}^{MeOH} nm (log ε): 282 (3.62).

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13) Yield: 76%. Yellowish needles of mp 90-91°C (MeOH). $C_{13}H_{11}NO_5$, MS m/z: 261 (M^+). UV λ_{max}^{MeOH} nm (log ε): 206 (4.28), 258 (3.51), 336 (3.91). ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 3.75 and 3.95 (each 3H, s, ester Me), 6.26 (1H, s, H-1'), 6.8-7.6 (3H, m, H-5,6,7), 8.01 (1H, dd, J=2.0 and 9.0 Hz, H-4).

14) Yield: 23%. Yellowish prisms of mp 84-85°C (MeOH-H_2O). $C_{13}H_{11}NO_5$, MS m/z: 261 (M^+). UV λ_{max}^{MeOH} nm (log ε): 208 (4.35), 258 (3.98), 338 (4.08). ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 3.72 and 3.91 (each 3H, s, ester Me), 6.22 (1H, s, H-1'), 7.1-7.7 (4H, m, H-4,5,6,7).

15) Colorless needles of mp 145-146°C ($CHCl_3$-hexane). $C_{12}H_{11}NO_4$, MS m/z: 233 (M^+). ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 2.56 (1H, dd, J=17.4 and 3.0 Hz, H-3), 3.00 (1H, dd, J=17.4 and 9.3 Hz, H-3), 3.86 (3H, s, ester Me), 4.41 (1H, dd, J=9.3 and 3.0 Hz, H-3a), 6.79-7.37 (4H, m, H-4,5,6,7), ca. 7.1 (1H, m, NH, disappeared on deuteration). ^{13}C-NMR (25 MHz, $CDCl_3$) δ: 36.9 (C-3), 45.1 (C-3a), 53.3 (ester Me), 99.3 (C-8a), 111.1, 122.8, 124.8, and 129.9 (C-4,5,6,7), 128.4 (C-3b), 157.5 (C-7a), 168.2 (ester C=O), 176.1 (C-2).

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USE OF THE (BUTYLTHIO)CARBONYL GROUP TO PROTECT URACIL AND GUANINE RESIDUES IN OLIGORIBONUCLEOTIDE SYNTHESIS

Masayo Fujii, Yuzoh Horinouchi, and Hiroshi Takaku*
Laboratory of Bioorganic Chemistry, Chiba Institute of Technology,
Tsudanuma, Narashino, Chiba 275

We describe the protection of the O^6-amide group of guanosine and the N^3-imide group of uridine with the (butylthio)carbonyl group. This group is introduced in good yields and is cleaved under mild conditions.

KEYWORDS——uracil residue protection; guanine residue protection; (butylthio)carbonyl group; oligoribonucleotide; phosphorylation; coupling reaction

The synthesis of oligoribonuclerotyides containing guanosine and uridine units has long been associated with low yields and obvious side reactions.[1) The source of the side reactions have been shown recently to be the reactivity of the O^6-position of guanosine and the N^3-position of uridine toward the condensing and phosphorylating agents commonly employed inoligoribonucleotide synthesis.[2) Several protecting groups have recently been proposedtoprevent the side reactions.[3) In preceding papers,[4,5) we have demonstrated the utility of guanosine and uridine residues. Here, we reportthe (butylthio)-carbonyl group[6) as a more sufficient protecting group to the amide functions of guanosine and uridine residues than several other protecting groups.[3,4,5) And we describe its application to the synshesis of the trimer AUG (14).

First, we examined the one-pot synthesis of N^3-protected uridine (2). Uridine (1) (4.88 g, 20 mmol) was treated with trimethylsilyl chloride (TMSCl) (25 ml, 200 mmol) in dry pyridine (100 ml) at 0°C; subsequently butyl chlorothioformate (BuSCOCl) (5.36 ml, 40 mmol) and diisorpopylethylamine (iPr_2NEt) (7.0 ml, 40 mmol) were added, and the reaction mixture was gradually warmed to room temperature. After the usual workup, silica gel column chromatography, using a mixture of CH_2Cl_2 and MeOH (95:5, v/v) eluants, afforded compound 2[7) (5.01 g, 70%). The silylated nucleoside 3 was prepared in 75% yield by the treatment of 2 with a slight excess of 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane ($TIPDSiCl_2$) in pyridine according to the procedure reported in the literature.[8) Compound 3 (5.6 g, 9.1 mmol) was treated with 2,3-hydropyran (10.8 ml, 118 mmol) in the presence of $TsOH.H_2O$ (1.82 g, 9.5 mmol) in dioxane (80 ml) at room temperature for 3 h to give 4, which was then desilylated to 5[9) in 70% (2.81 g) yield by 1M tri-1-butylammonium hydrogen fluoride (TBAHF). Treatment of 5 with DMTrCl in dry pyridine gave the expected 5'-tritylated product (6) in good yield. The tritylated (6) (1.0 mmol) thus obtained was treated with 5-chloro-8-quinolyl phosphate (1.1 mmol) and 8-quinolinesulfonyl chloride (QsCl) (2.0 mmol) in dry pyridine for 2 h to give the corresponding phosphodiester (7) in 92% yield.[10)

Table I. Stabilities of the Uridine and Guanosine Derivatives Under Various Conditions[a)]

Compound	A	B	C	D	E	F
2	stable	stable	stable	stable	97%	98%
10	stable	stable	stable	stable	86%	97%

a) (A) 3% Cl_3CCOOH/CH_3NO_2 -MeOH (9:1, v/v), room temperature, 1 h; (B) 80% AcOH, room temperature, 2 h; (C) Et_3N-CH_3CN (1:1, v/v), room temperature, 1 day; (D) pyridine-t-$BuNH_2$-H_2O (8:1:1, v/v), room temperature, 1 day; (E) 0.2 M NaOH-dioxane (1:1, v/v), room temperature, 2 h; (F) concentrated NH_4OH-MeOH (9:1, v/v), room temperature, 3 h.

Next, we examined the introduction of the (butylthio)carbonyl group onto O^6-position of guanosine as 3'-terminal nucleoside derivative. The 2',3',5'-O-N^2-protected guanosine derivative (8) (6.53 g, 7,7 mmol) was treated with BuSCOCl (3.2 ml, 23.1 mmol) in the presence of iPr_2NEt (4.0 ml, 23.1 mmol) in dry pyridine (38 ml) to afford the corresponding fully protected nucleoside (9), which was detritylated to 10[11)] in 67% (3.49 g) yield by 80% AcOH treatment. In order to determine whether the (butylthio)carbonyl group can be used in oligoribonucleotide synthesis, the corresponding nucleoside derivatives 2 and 10 were treated under the conditions as shown in Table I. The results indicate that the (butylthio)carbonyl group had the suitable properties for our oligoribonucleotide synthesis. In particular, compounds 2 and 10 were treated with a mixture of conc. ammonia and methanol (9:1, v/v) at room temperature for 3 h to afford uridine and guanosine without the formation of cytidine and 2,6-diamino-purine derivative (16).[12)] An additional advantage of the (butylthio)carbonyl group is that the derivatives can be easily detected as orange spots on TLC by spraying the samples with 1% aqueous silver nitrate solution. On the basis of the above facts, the utility of the (butylthio)-carbonyl group can be demonstrated in the synthesis of the trimer AUG (14). The triethylammonium salt of 7 (970 mg, 0.9 mmol) was treated with 10 (410 mg, 0.6 mmol) in the presence of QsCl (410 mg, 1.8 mmol) and N-methylimidazole (MeIm) (0.15 ml, 1.8 mmol) in dry pyridine (2 ml) for 2 h. After the usual workup, the resulting residue was applied to a column of silica gel and eluted with a

stepwise gradient of MeOH (0-5%) in CH_2Cl_2 to give the fully protected dimer (11) (815 mg, 82%). The DMTr group was removed from 11 (815 mg, 0.5 mmol) by treatment with 3% Cl_3CCOOH in a mixture of CH_3NO_2 and MeOH (95:5, v/v, 30 ml) at room temperature for 5 min. After the usual workup, silica gel column chromatography, using a mixture of CH_2Cl_2 and MeOH (95:5, v/v) eluants, afforded the detritylated dimer (12) (570 mg). A solution of both compounds 12 (570 mg, 0.4 mmol) and 13[13] (660 mg, 0.6 mmol) in dry pyridine (2 ml) was treated with QsCl (270 mg, 1.2 mmol) and MeIm (0.1 ml, 1.2 mmol) for 1 h. The fully protected AUG (14) was obtained in 80% (750 mg) yield after separation by silica gel column chromatography. 14 was deprotected as follows: 1) zinc acetate in pyridine-water (9:1, v/v) at room temperature for 24 h to remove the 5-chloro-8-quinolyl group; 2) conc. ammonia-pyridine (9:1, v/v) at 60°C for 6 h to remove the benzoyl, isobutyryl, and (butylthio)carbonyl groups; 3) 0.01 M HCl in dioxane-water (1:1, v/v) at room temperature for 2 days to remove the THP and DMTr groups. Thus the trimer 15 could be isolated in 52% yield by paper chromatography. It was degradated by snake venom phosphodiesterase to give A, pU, and pG in the ratio of 1.00:1.05:1.10. Further, the trimer 15 was completely degradated by nuclease P1. This hydrolysis indicated that AUG had a 3'-5' internucleotidic bonds.

In conclusion, introduction of the (butylthio)carbonyl group onto guanosine and uridine residues provides a promising method for synthesizing oligoribonucleotides and it is removed very easily under the usual conditions for removing N-acyl protecting groups.

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MARKED CHANGES IN THE CIRCULAR DICHROISM OF THE TRIPEPTIDE CATALYST RELATED TO ENHANCED ENANTIOSELECTIVE HYDROLYSIS IN MICELLAR SYSTEMS

Ryuichi Ueoka,[*,a] Yōko Matsumoto,[a] Katsuyuki Nagamine,[a]
Hidehisa Yasui,[a] Kumiko Harada,[b] and Atsushi Sugii[*,b]
Department of Industrial Chemistry, Faculty of Engineering,
Kumamoto Institute of Technology,[a] Ikeda, Kumamoto 860 and
Faculty of Pharmaceutical Sciences, Kumamoto University,[b]
Ohe-honmachi, Kumamoto 862, Japan

The enantioselectivity for the hydrolytic cleavage of p-nitrophenyl N-dodecanoyl-D(L)-phenylalaninates in the micellar systems was augmented by using the active tripeptide (Z-L-Phe-L-His-L-Leu), which showed an α-helix-like CD curve, combined with a marked change of the CD intensity of the tripeptide above the cmc.

KEYWORDS —— enantioselectivity; amino acid ester; peptide catalyst; hydrolysis; circular dichroism; micellar catalysis

The sequence of amino acids is fairly important in providing the rigid conformation of peptides and may also be closely related to the biological activity of peptides. With respect to the enhancement of the stereoselective hydrolytic cleavage of p-nitrophenyl esters derived from N-protected amino acids,[1] the importance of the amino acid sequence of peptide catalysts[2] has been emphasized. It is also important to control the reaction field by temperature regulation[3] or by changing the composition of vesicular and micellar surfactants.[4]

However, there have been only a few reports of the relation between stereoselectivity and the conformation of peptide catalysts for the enantioselective hydrolysis of amino acid esters,[5] though the helical structure of polypeptides and cyclopeptides has been discussed in detail on the basis of the circular dichroism (CD) experiments.[6]

In this paper, we report the dramatic changes of the CD curves and CD intensities of peptide catalysts in relation to the enhancement of enantioselective hydrolysis of p-nitrophenyl N-dodecanoyl-D(L)-phenylalaninates [C_{12}-D(L)-Phe-PNP] in the micellar systems.

We have examined the hydrolytic cleavage of long-chain enantiomers [C_{12}-D(L)-Phe-PNP] by peptide catalysts including the L-histidine part in the micellar systems (benzylhexadecyldimethylammonium chloride:CBzAC, hexadecyltrimethylammonium chloride:CTAC)[7] at room temperature (25 °C) and pH 7.6 in 0.08 M Tris-KCl buffer [3% (v/v) CH_3CN-H_2O]. The results are summarized in Table I. The noteworthy aspects are as follows: (a) The tripeptide Z-L-Phe-L-His-L-Leu and the dipeptide Z-L-Phe-L-His catalysts, having in common an L-Phe-L-His unit, greatly enhance

enantioselectivity (reflected in $k_{a,obsd}(L)/k_{a,obsd}(D)$)[8] compared with the other peptides (Z-L-His, Z-L-His-L-Phe, and Bz-Gly-L-His-L-Leu) in the presence of micelles (CBzAC and CTAC). (b) The CBzAC micelles having a benzyl group more greatly enhanced both the catalytic efficiency (reflected in $k_{a,obsd}$) and the enantioselectivity for the hydrolysis of C_{12}-D(L)-Phe-PNP when Z-L-Phe-L-His-L-Leu was used as a catalyst, and the highest enantiomer rate ratio (L/D = 35) was attained. This suggests that the L-Phe-L-His unit in Z-L-Phe-L-His-L-Leu and Z-L-Phe-L-His, which was attained directly to the Z-group, and the hydrophobicity in the C_{12}-L-Phe-PNP substrate with an L-Phe unit are of great importance in enhancing the rate and enantioselectivity in the micellar systems.

The CD spectra of L-histidine derivatives (Z-L-His, Z-L-His-L-Phe, Z-L-Phe-L-His, Bz-Gly-L-His-L-Leu, and Z-L-Phe-L-His-L-Leu) with CTAC micelles were measured as shown in Fig. 1.[9] It is noteworthy that the specific CD spectra at ca. 235 nm occured commonly in the active peptides (Z-L-Phe-L-His-L-Leu and Z-L-Phe-L-His) with CTAC micelles, which promoted the enantioselectivity for the hydrolysis of the long-chain enantiomers (C_{12}-D(L)-Phe-PNP). This implies that these specific CD patterns of Z-L-Phe-L-His-L-Leu and Z-L-Phe-L-His may support the rigid conformation of the common unit of L-Phe-L-His. The Phe, His, and Leu units in Z-L-Phe-L-His-L-Leu are well known for forming a stable α-helix structure. And interestingly, the CD curve of this tripeptide in the presence of micelles (CBzAC or CTAC) closely resembles those of α-helical peptides.[10]

The authors also examined the dependence of the CD intensity of the active tripeptide (Z-L-Phe-L-His-L-Leu) at ca. 235 nm on the concentration of CBzAC micelles and compared the kinetic results as shown in Fig. 2. The enantioselectivity for the hydrolysis of the long-chain enantiomers (C_{12}-D(L)-Phe-PNP) sharply increased at the cmc[7] of CBzAC and the high enantioselectivity remained almost constant above the cmc. It is of interest also (Fig. 2) that the same relationship exists between the CD intensity and the concentration of CBzAC, though the CD intensity was kept constant above the somewhat higher concentration than the cmc. These results suggest that the rigid conformation of Z-L-Phe-L-His-L-Leu (like an α-helical arrangement) should be stable in the hydrophobic micellar system and may be related to the enhancement of the enantioselective hydrolysis when above the cmc.

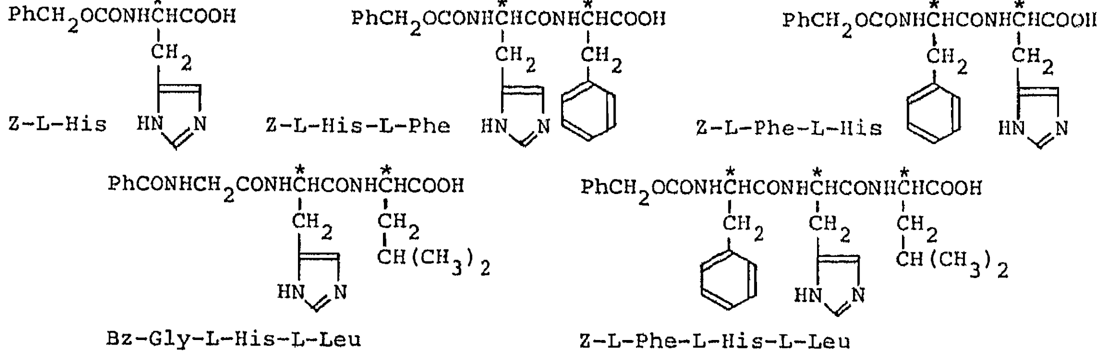

Table I. Rate Constants ($k_{a,obsd}$, $M^{-1}s^{-1}$) and Enantioselectivity ($k_{a,obsd}(L)/k_{a,obsd}(D)$) in the CBzAC or CTAC Micellar Catalytic System[a)]

Catalyst	C_{12}-L-Phe-PNP		C_{12}-D-Phe-PNP		L/D	
Z-L-His	28	(30)	7.0	(15)	4.0	(2.0)
Z-L-His-L-Phe	50	(30)	28	(26)	1.8	(1.2)
Z-L-Phe-L-His	2800	(1600)	190	(110)	15	(15)
Bz-Gly-L-His-L-Leu	9.5	(3.4)	3.9	(2.2)	2.4	(1.5)
Z-L-Phe-L-His-L-Leu	1900	(410)	55	(19)	<u>35</u>	(22)

Values in the parentheses were obtained in the CTAC micellar system.[11)]

a) pH 7.6, 25°C, 0.08 M Tris buffer (0.08 M KCl), 3% (v/v) CH_3CN-H_2O, [Sub] = 1×10^{-5} M, [Cat] = 1×10^{-4} M, [CBzAC] = 1×10^{-3} M, [CTAC] = 3×10^{-3} M.

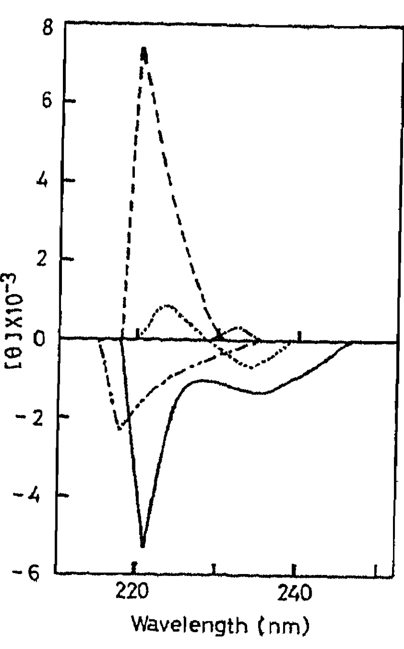

Fig. 1. CD Spectra of Peptide Catalysts with CTAC Micelles in CH_3OH-H_2O (5:95 v/v): —·—, Z-L-His; ---, Z-L-His-L-Phe; ·····, Z-L-Phe-L-His; —··—, Bz-Gly-L-His-L-Leu; ——, Z-L-Phe-L-His-L-Leu. [cat] = 2.5×10^{-4} M, [CTAC] = 3×10^{-3} M

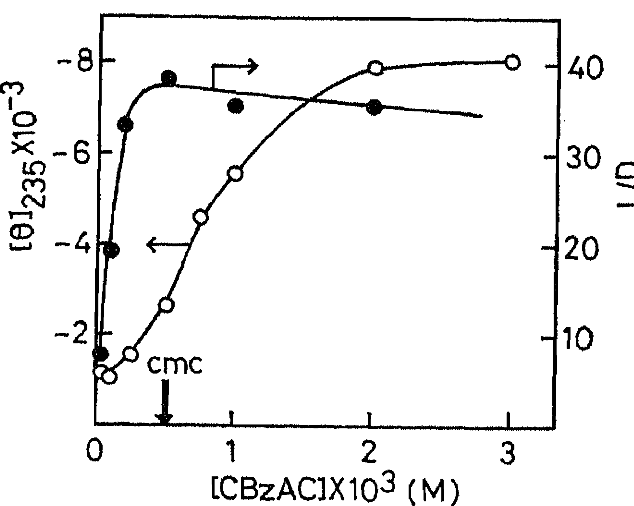

Fig. 2. Concentration Dependence of CBzAC Micelles on CD Intensity ([θ]) of Z-L-Phe-L-His-L-Leu and Enantioselectivity (L/D) for the Hydrolysis of C_{12}-D(L)-Phe-PNP Catalyzed by Z-L-Phe-L-His-L-Leu: ○, [θ] ([cat] = 2.5×10^{-4} M); ●, L/D ([cat] = 1×10^{-4} M).

In conclusion, it should be emphasized that the enantioselectivity for the hydrolysis of the long-chain enantiomers (C_{12}-D(L)-Phe-PNP) in the CBzAC or CTAC micellar system was augmented by using the active tripeptide (Z-L-Phe-L-His-L-Leu), which showed an α-helix-like CD curve. Also a drastic change of the CD intensity of the tripeptide above the cmc and amplified enantioselectivity (L/D = 38) was attained in the micelles of CBzAC having a benzyl head group.

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8) The second-order rate constants ($k_{a,obsd}$) were calculated from $(k_t - k_s)/[cat]_0$, where k_t and k_s denote the first-order rate constants with and without catalyst, respectively, and $[cat]_0$ stands for the initial concentration of the catalyst.
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Communications to the Editor

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PHOTOCHEMICAL SYNTHESIS OF ANTITUMOR PLATINUM PYRIMIDINE GREENS; POSSIBLE CONTRIBUTION OF SINGLET OXYGEN

Yohmei Okuno,[*,a] Takayuki Inoue,[b] Osamu Yonemitsu,[b] Takenori Tomohiro,[a] and Tarja Laitalainen[a,1)]
National Chemical Laboratory for Industry (NCLI),[a] Tsukuba 305, Japan and Faculty of Pharmaceutical Sciences, Hokkaido University,[b] Sapporo 060, Japan

A method is given for the selective synthesis of antitumor platinum greens in a reaction of the hydrolysis products of cis-diiododiammineplatinum (II) and nucleosides via visible-light induced oxidation. A role of singlet oxygen in the green formation is indicated by the sensitized reactions with methylene blue and rose bengal and by experimental quenching of singlet oxygen with 1,4-diaza-bicyclooctane (DABCO).

KEYWORDS —— platinum pyrimidine green; antitumor activity; photosensitized reaction; singlet oxygen; DABCO quenching

Much attention has been focused on the synthesis of potent anticancer platinum complexes and the mechanism of their biological actions. One promising way to reduce such strong side effects as nephrotoxicity and emesis, which occurs in the best known cis-dichlorodiammineplatinum (II), (cis-DDP), is, other than the methods of administration, to develope its oligomer chelates with pyrimidine derivatives.[2)]

Our recent findings confirm this.[3)] The complexes can be prepared by treating the diaquo derivative of cis-diiododiammineplatinum (II), (cis-DIDP),[4)] with pyrimidines under air. The reaction produces several kinds of characteristic colors including blue, green, violet and yellow. We have reported that the uridine green species had remarkably high anticancer activity against L1210 cells, employing SO_4^{2-} as a counter anion.[3)] Since then our efforts have been focused on developing selective methods for synthesizing the green complexes by hydrogen peroxide oxidation.[5,6,7)] Here, we report the first direct formation of Pt-greens by photo-sensitized reactions, which may involve a contribution of singlet oxygen.

When a mixture of hydrolysis products of cis-DIDP (0.3 mmol), uridine (0.3 mmol) and methylene blue[8)] (0.3 µmol) in 3 ml of water (pH 4.3) was irradiated with a 60 W tungsten bulb under air or oxygen atmosphere at 60°C,

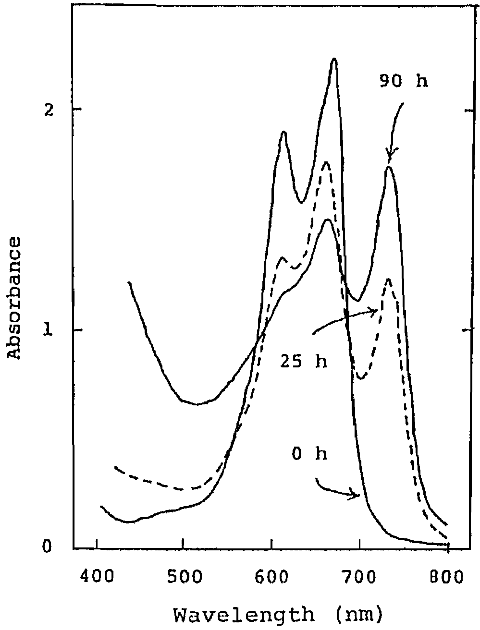

Fig.1. A Time Course of Visible Absorption Spectra in the Formation of Platinum Uridine Green Sensitized by Methylene Blue
(path length, 2 mm)

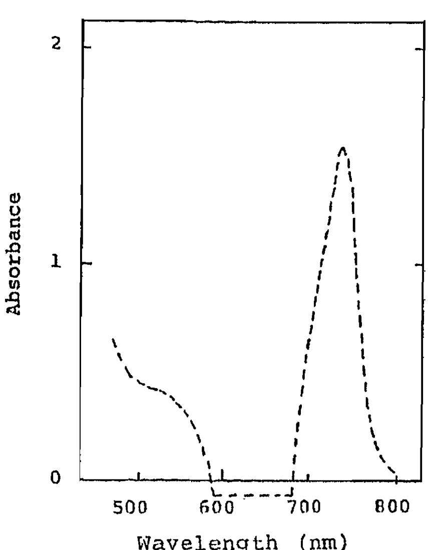

Fig.2. A Difference Spectrum after 90 h Illumination (Mb)
(path length, 2 mm)

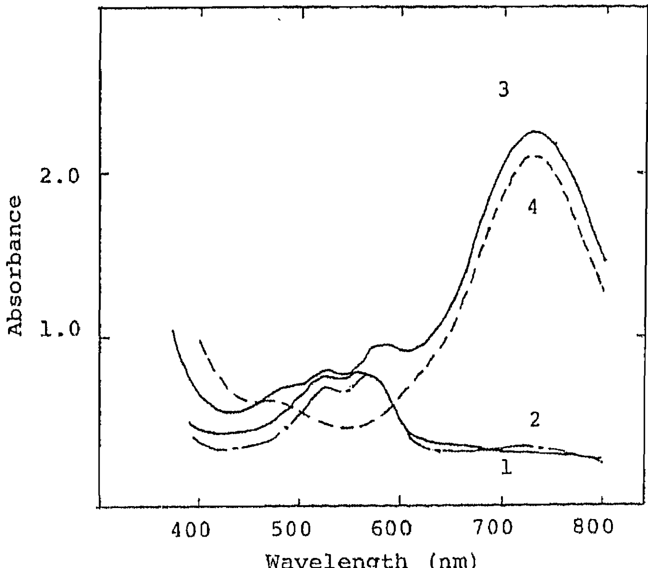

Fig.3. Photochemical Formation of Platinum Uridine Green Sensitized by Rose Bengal

1:(——) 0 h; 2:(—•—) After 3 h irradiation; 3:(——) After 21 h irradiation; 4:(----) Purified platinum green in water (arbitrary scale)

the absorption maxima at 730 nm, corresponding to typical green chelates in the visible region, increased with reaction time. An example is shown in Fig. 1. Though the absorption between 600 and 700 nm due to the sensitizer decreased to some extent (ca.20% after 1 day) during the reaction, the mixture turned green after 25 h. A difference spectrum after 90 h using the corresponding mixture kept in the dark as a reference clearly showed exclusive formation of the green complex, and there was no blue absorption maximum around 570 nm (Fig. 2). Gel filtration (Toyopearl HW-40) showed a single zone on the column, and mostly the same green substance was isolated[9] [Yield: 22.4 mg, mp>300°C, UV-VIS (water):λ max(ε) nm; 268(28,700), 730(1,140); Circular Dichroism (water): [θ](λ_{max} nm); 37,800(272), -37,800(219)].[3,10] There were no significant changes in absorption spectra in water solutions of the isolated complexes after 24 h in the presence or absence of the tungsten light under air.

The above observations indicate a contribution of singlet oxygen in the formation of the platinum greens, since it is widely accepted that reactions sensitized by methylene blue efficiently produce singlet oxygen (cf. quantum yield >0.23).[8]

When this photo-oxidation was carried out in D_2O, the rate of the green formation, which was monitored by visible spectra, was 27-29% higher than that in H_2O. The stability of singlet oxygen is known to increase ca.10 times more in D_2O[11] than in H_2O. This tends to substantiate our idea about singlet oxygen.

Further support has come from reactions employing rose bengal as a sensitizer, which gives singlet oxygen very efficiently (cf. quantum yield = 0.76).[8] A solution containing uridine (146.4 mg, 0.6 mmol), the diaquo-complex (6 ml, 0.6 mmol) and rose bengal (0.6 µmol) was illuminated at room temperature with stirring for 21 h at pH 5. The reaction proceeded very cleanly (see Fig. 3). Gel filtration (a single zone on the column) gave a platinum green. With further purification by reprecipitation from water-acetone, the yield was 74.4 mg.

Another source of supporting data utilizes quenching experiments. For example, using 1,4-diazabicyclooctane (DABCO, 0.3 mmol), known as an efficient quencher of singlet oxygen,[12,13,14] completely suppressed the green formation.

The present results are in sharp contrast with simple air oxidation in the dark which produces mostly blue materials.[3]

Though the experimental findings described here are still somewhat preliminary,[15] we have found novel photochemical methods for the synthesis of anticancer platinum greens. And it appears that singlet oxygen is responsible for selective synthesis of the platinum green complexes. Further work including biological activities against other malignant cells are in progress.

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EPOXIDATION CATALYZED BY Mn(III)TPPCl USING DIOXYGEN ACTIVATED BY A NOVEL SYSTEM CONTAINING N-HYDROXYPHTHALIMIDE AND STYRENE

Masaichiro Masui,* Keiichi Tsuchida, Yukio Kimata and Shigeko Ozaki

Faculty of Pharmaceutical Sciences, Osaka University,
1-6, Yamadaoka, Suita, Osaka-fu, 565 Japan

Olefins were epoxidated by molecular oxygen activated by a system composed of Mn(III)TPPCl,[1)] N-hydroxyphthalimide, pyridine and styrene.

KEYWORDS —— catalytic epoxidation; molecular oxygen; N-hydroxyphthalimide; Mn(III)TPPCl; styrene; cyclohexene

There have been several successful attemps to design a system producing cytochrome P-450 type dioxygen activation, in the presence of reducing agents such as borohydride, H_2-Pt and sodium ascorbate.[2)]

N-hydroxyphthalimide (NHPI) is known as a mediator in electrochemistry.[3)] We wish to report here the catalytic epoxidation of styrene by a novel system containing NHPI, O_2, pyridine and Mn(III)TPPCl as a catalyst. The catalytic epoxidatioin did not proceed when any of those components was omitted. Cyclohexene and stilbene were also epoxidated in the present system in the presence of styrene. This is a preliminary report of results of the catalytic epoxidation of olefins using the present system.

In a typical experiments (3 h, 30° C), a solution containing styrene (2.4 mmol), Mn(III)TPPCl (0.0028 mmol), pyridine (1.6 mmol), and NHPI (0.4 mmol) in 20 ml of acetonitrile consumed O_2 (0.57 mmol), and gave styrene oxide (0.11 mmol, 27% based on NHPI), benzaldehyde (0.04 mmol, 11%), a small amount of phenylacetaldehyde, and (II)[4)] (0.2 mmol, 50%) (see Scheme). The added NHPI was almost consumed. In a control experiment (2 h, 30°C) without Mn(III)TPPCl, the O_2 uptake was only 0.1 ml. Plots of O_2 uptake versus time showed sigmoid curves with a variety of induction times (from 5 to 120 min) characteristic of many catalytic autoxidation reactions. The plot for NHPI consumption showed a

reversel sigmoid curve. There was no induction period in the reaction initiated by adding phthalimide N-oxyl(PINO), which was separately prepared by an electrochemical method.[3] But when 2,2,6,6-tetramethylpiperidine-1-oxyl was added instead of PINO, the epoxidation reaction did not proceed. When a free radical trap, 4-butylcatechol, was added to the solution, neither consumption of NHPI nor production of styrene oxide occurred. Cyclohexene was also epoxidated in the present system, but the products from autoxidation, such as 2-cyclohexen-1-one and 2-cyclohexen-1-ol, were also produced in fair amounts. Products and their distribution from a reaction without styrene are similar to those from Mn(III)TPPCl- catalysed autoxidation,[5] but different from the reaction with oxygen activated by the present system.

Table. Products from Oxidation of Olefins Catalysed by Mn(III)TPPCl [a)]

Run	Conc. olefins (m M) (1)	Conc. olefins (m M) cyclohexene	Products and yields (%) [b,c)] (2)	PhCHO [d)]	cyclohexene oxide	cyclohexenone [d)]	cyclohexenol [d)]
1	120	0	27 (3860)	11	0	0	0
2	120	120	15	11	20	16	5
3	60	120	10	10	28	27	9
4	25	120	5	8	41	70	20
5	25	240	5	8	65 (9290)	130	44
6	0	120	0	0	13	180	30

a) Reaction conditions are shown in the text.
b) Yields based on NHPI.
c) Yields in parentheses are based on Mn(III)TPPCl.
d) See text.
(1) : $Ph-CH=CH_2$, (2) : $Ph-CH(O)CH_2$ (epoxide)

Yields of styrene oxide and cyclohexene oxide based on Mn(III)TPPCl were 3860%, and 9290%, respectively (runs 1 and 5, Table). These excellent turnovers indicate that Mn(III)TPPCl acts as a real catalyst for the epoxidation in the present system. The stereochemistry of the epoxidation in this system was investigated using trans and cis-stilbenes as the substrates under the same condition as run 5. The epoxidation of both trans- and cis-stilbenes were stereospecific,i.e., trans-stilbene and cis-stilbene yielded trans-stilbene

oxide (19%) and cis-stilbene oxide (25% together with a trace amount of the trans form) respectively, the same as the results with the system studied using NaClO, Mn(III)TPPCl and pyridine.[6] Attempts to get information about a direct electron transfer from NHPI to Mn(III)TPPCl by means of visible light absorption were not successful because the new absorption spectrum expected for Mn(II)TPPCl was developed at almost the same wavelength (430 nm) as the characteristic spectrum of Mn(II)TPPCl.[2,7] A similar absorption band was also developed on addition of either pyridine or NHPI to the acetonitrile solution of Mn(III)TPPCl.

High valent O=Mn(V)TPPCl is also difficult to observe in the present system,[8] because the high-valent oxo manganese complex is only attainable in our case in the presence of excess styrene, which must react with the oxo manganese complex instantaneously (Chart). Tentatively, the experimental results described above suggest the following reaction chart for the epoxidation.

NHPI —(Mn(III)TPPCl; pyridine, O_2)→ PINO —(O_2; $PhCH=CH_2$)→ (I),

Ph-CH($\cdot O_2$)CH_2-ON(CO)$_2$C$_6$H$_4$
(I)

(I) → Mn(III)TPPCl / (II) ← O=Mn(V)TPPCl
O=Mn(V)TPPCl + >C=C< → Mn(III)TPPCl + >C(O)C< (epoxide)

Ph-C(=O)CH_2-ON(CO)$_2$C$_6$H$_4$
(II)

Chart

The probable oxygen donor expressed as (I) might be the corresponding hydrogen peroxide formed by the reaction with NHPI or water contamination in acetonitrile.[10] Although the positive role of added pyridine has not been elucidated definitely, it seems to make the NHPI more susceptible to oxidation to PINO.[3] It is also likely to coordinate to manganese porphirin in order to effect the rate of the epoxidation.[6,11] Several other N-substituted hydroxamic acids also showed a similar effect on epoxidation as NHPI, but the reactivity was lower than NHPI.

Procedure for measuring O_2 uptake and products analysis

The container holding the solution except for pyridine was attached to a gas buret and an O_2 supply, and flushed with O_2 for 5 minutes. The uptake was

observed after adding pyridine dissolved in acetonitrile by means of a needle inserted in a serum stopper. Products were confirmed and determined by GLC (PEG 20M, 2 m, glass column) and consumptiom of NHPI was followed by HPLC. The cis/trans- epoxide ratio in the epoxidation of the cis- and trans- stilbene was determined by H^1-NMR using the crude products after passage of the reaction mixture through a silica gel column. The structure of (II) was confirmed by elemental analysis and spectra (IR, H^1-NMR).

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ACUTE INSECTICIDAL ACTIVITY OF QUASSIN AND ITS CONGENERS AGAINST THE AMERICAN COCKROACH

Myung Hwan Park,[a] Mitsuru Maeda,[b] Hajime Komura,*,[a]
Koji Nakanishi,[a] and Kyosuke Nomoto[a]
Suntory Institute for Bioorganic Research (SUNBOR),[a] and
Laboratories of Applied Microbiology, Research Center, Suntory Ltd,[b]
Wakayamadai, Mishima-gun, Osaka 618, Japan

Quassin and 8 congeners, **1-9**, were identified as active principles in *Picrasma quassioides* for the paralytic/acute insecticidal activity upon subcutaneous injection in the American cockroach. The activity was prolonged by applying the metabolic inhibitors for oxidation and esterification before injecting the quassinoids. The nature of the insecticidal activity seems to differ from that of other cytotoxic quassinoids, such as brusatol.

KEYWORDS——*Picrasma quassioides;* American cockroach (*Periplaneta americana*); quassinoid; paralytic/acute insecticidal activity; subcutaneous injection

Acute insecticidal activity upon subcutaneous injection in the American cockroach (*Periplaneta americana*) is an easy, therefore useful, preliminary bioassay for isolation and identification of the neurologically active substances.[1] Under the assay condition employed, regardless of the hydrophobic (or hydrophilic) nature of the substances tested, the neurologically active substances produce some *acute* effect(s), mostly acute insecticidal activity at high dosages and paralytic activity at lower dosages. This trend is quite different from *delayed* toxicity which is generally characteristic of cytotoxic substances. The acute insecticidal principles in *Chondria armata* were reexamined identifying a series of new congener amino acids[2] in addition to the major insecticidal principle domoic acid, which is well known as a neuromodulator.[3] A palytoxin analogue was also purified[4] from the same origin under the guidance of this bioassay method.

The root bark of *Picrasma quassioides* (Simaroubaceae) has been used as a stomachic, and it is classified as an insecticidal and anthelmintic material in China.[5] Quassia, the aqueous extract of the wood and bark of the tropical tree *Quassia amara* and the members of other Simaroubaceae family plants, is commonly used also against insects.[6] The strong *acute* insecticidal activity of the crude methanol extract of the *P. quassioides* root bark[7] stimulated our interest in relation to neurological

1 Quasin, R,R': =O
2 Neoquassin, R, R': H, OH

3 Picrasin B, R: H
6 Picrasin G, R: OH

5 Picrasin D, R, R': Me, Me
7 Nigakilactone B, R: H, R': Me
8 Nigakilactone C, R: Ac, R': Me

4 Picrasin C, R: H
9 14-Hydroxypicrasin C, R: OH

activity. The active principle was isolated using the acute insecticidal activity against American cockroaches with subcutaneous injection as a guide. Nine active substances were isolated after the solvent extraction and Diaion HP-20 column chromatography, followed by repeated preparative TLC on silica. Seven of the active principles were identified by their ^{1}H-NMR and CD spectra and other physical constants as quassin (1),[8,9] neoquassin (2),[9,10] picrasins C (4), D(5), and G(6),[11,12] and nigakilactones B (7)[9,13] and C(8).[9] The structure of a new active compound isolated in 0.001% yield, mp 245–247°, $C_{23}H_{34}O_8$ (by high resolution mass spectroscopy), was determined as 14-hydroxy picrasin C(9). This compound had a CD curve [$\Delta\varepsilon_{240}$(MeOH) -0.40, $\Delta\varepsilon_{265}$ -0.15, and $\Delta\varepsilon_{290}$ -0.23] and ^{1}H-NMR spectrum very similar to those of picrasin C except for some differences caused by substitution of 14-H(β) with a hydroxyl group. The configuration of the 14-OH was determined to be β because of an appreciable paramagnetic shift (0.39 ppm) observed on 7-H, which is in the 1,3-diaxial relationship to the 14-β position, with no changes in coupling constants. One remaining component was finally determined to be picrasin B (3)[11-14] by precise ^{1}H-NMR studies including n.O.e. experiments and X-ray crystallography.[15] However, contrary to the report, the CD data, $\Delta\varepsilon_{225}$ (MeOH) -0.79, $\Delta\varepsilon_{256}$ +2.05, and $\Delta\varepsilon_{326}$ -1.59, were not in agreement with any of the published values, $[\theta]_{341}$ -1,100,[11] and $[\theta]_{246}$ -16,400, $[\theta]_{300}$ +2,690 and $[\theta]_{341}$ -100.[12]

The specific paralytic/insecticidal activity of the major active components isolated from *P. quassioides* are summarized in the Table.[16] All of these compounds had strong acute paralytic activity at dosages as low as 25 μg/g of insect body weight, but after 24 h most of the insects recovered and the specific activity fell to 100 μg/g of insect body weight or more. When a higher dosage was applied, acute insecticidal activity resulted and there was no recovery. This trend in

Table. Paralytic/Insecticidal Activity of Quassinoids

Quassinoids	Metabolic inhibitors	Acute[a] (3 h)	24 h[a]	48 h[a]
Quassin(1)	—	25	>100	>100
	PB[b]	<12.5	25	50
	NIA[c]	<12.5	≤12.5	12.5
Neoquassin(2)	—	12.5-25	>100	>100
	PB	<12.5	12.5	50
	NIA	<12.5	12.5	12.5
Picrasin B(3)	—	25	>100	>100
	PB	12.5	25	25
	NIA	12.5	12.5	>100
Picrasin C(4)	—	25	>100	>100
	PB	12.5	>100	>100
	NIA	<12.5	50	>100
Picrasin D(5)	—	25	100	100
	PB	<12.5	25	100
	NIA	<12.5	<12.5	100
cf. Brusatol	—	N[d]	N[d]	D[e]
	PB	N	N	D
	NIA	N	N	A[f]

[a] Minimum effective dose (μg/g of insect body weight) to cause ataxia.
[b] Piperonyl buthoxide, 50 μg/g of insect body weight.
[c] Propargyl propyl benzenephosphonate, 50 μg /g of insect body weight.
[d] N: Not effective at 100 μg/g. [e] D: Dead at 50 μg/g. [f] A: Ataxia at 50 μg/g.

activity is very similar to that of domoic acid and its congeners, which possess neurologic activity, and quite different from that of brusatol[17,18] which is known to be cytotoxic. Brusatol showed no acute activity up to 24 h after injection even at the level of 100 μg/g. However, after 48 h, strong delayed insecticidal activity was observed at dosages less than 50 μg/g. Injection (50 μg/g of insect body weight)[19] of piperonyl buthoxide (PB)[20] or propargyl propyl benzenephosphonate (NIA 16388),[21] which are respectively inhibitors of oxidative detoxification and esterification, 1 h prior to the injection of the samples maintained the activity of most of the compounds at levels of 50 μg/g or less even after 48 h. This phenomenon probably results from the fact that the quassinoids are easily metabolized *in vivo* to non- or less-active substances mainly by two detoxification pathways, oxidation/hydroxylation of the carbon skeleton and esterification of the hydroxyl groups.

Quassinoids, the bitter principles of Simaroubaceae plants, are well studied because of their biological activities, such as cytotoxic/antitumor,[18b,22] antiviral,[23] antimalarial,[24] and insecticidal[25] activities. In order to maintain the cytotoxic activity, the oxygen bridging between C-30 (the methyl group on C-8) and C-12/13 is essential,[26] as, for example, in brusatol. However, the series of paralytic/acute insecticidal quassinoids **1** – **9** share no such oxygen-bridging structure, and it is difficult to rationalize the *acute* insecticidal activity in terms of cytotoxicity. It is noteworthy that quassia, which contains quassin and neoquassin as major quassinoid constituents,[27] was reported to be non-effective as an insecticide against the cockroach[6,28] under a different evaluation method focussed mainly on *delayed* toxicity. Thus, it would be plausible to expect, taking an earlier

preliminary study[29] into account, that these quassinoids have the activity because of their neuro-modulatory activity. Detailed mechanistic studies including structure-activity relationships are in progress.

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