Studies on Taxane Synthesis. III. Stereocontrolled Synthesis of a Twelve-Membered Lactam Sulfide as a Precursor of 4,8,11,11-Tetramethyl-3-oxobicyclo[5.3.1]undec-8-ene[1)]

Yasuo OHTSUKA* and Takeshi OISHI*

RIKEN (The Institute of Physical and Chemical Research) Hirosawa, Wako-shi, Saitama 351-01, Japan. *Received October 8, 1990*

Stereocontrolled synthesis of two twelve-membered lactam sulfides 37 and 38 as precursors of 8,11,11-trimethyl-3-oxobicyclo[5.3.1]undec-8-enes constituting the A and B rings of taxane-diterpenes was achieved. The key step involves a Diels–Alder reaction of maleic anhydride and the *E*-diene 18 for introduction of the requisite *cis*-arrangement of substitutions at the C-1 and C-7 positions. The resulting adduct was converted exclusively into the lactone 21b in five steps: 1) hydrolysis, 2) iodo-lactonization, 3) BH_3 reduction, 4) Zn reduction, 5) methylation. The benzyl group of 26 could be selectively removed by heating with Raney Ni (W-2) in EtOH in nearly quantitative yield without hydrogenation of the double bond.

Keywords taxane-type diterpene; stereocontrolled synthesis; Diels–Alder reaction; iodo-lactonization; anhydride; $NaBH_4$ reduction; BH_3 reduction; Raney Ni; selective debenzylation; twelve-membered lactam sulfide

A number of synthetic approaches to taxanes have already been reported[2)] and, recently, Holton and co-workers reported the conversion of (−)-patchoulene oxide into the enantiomer of (−)-taxusin (**1**).[3)] We reported previously syntheses of the bicyclo[5.3.1]undecanes **2**, **3** and **4** constituting the A and B rings of taxane-type diterpenes (*e.g.* **5**)[1)] from α- or β-ionone using a general method for constructing medium-sized ring ketones developed in this laboratory.[4)] Among them, the eight-membered ring ketone **4** was considered to be a potential intermediate for formation of the tricyclo[9.3.1.$0^{3,8}$]pentadecane **6**. However, 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) oxidation of the cyclohexenone derivative to the corresponding dienone and a Michael addition of nitromethane to the above dienone involved in the previous synthesis of **4** from α-ionone required 4 and 36d, respectively, and thus development of a more efficient strategy was desirable. The present paper deals with the synthesis of **4** *via* the mesyloxy acid **7** through a small number of steps.

The stereochemistry of two substituents at the C-1 and C-7 positions[5)] in the precursors **9** or **11** should be *cis* for eight-membered B ring formation. The requisite *cis*-

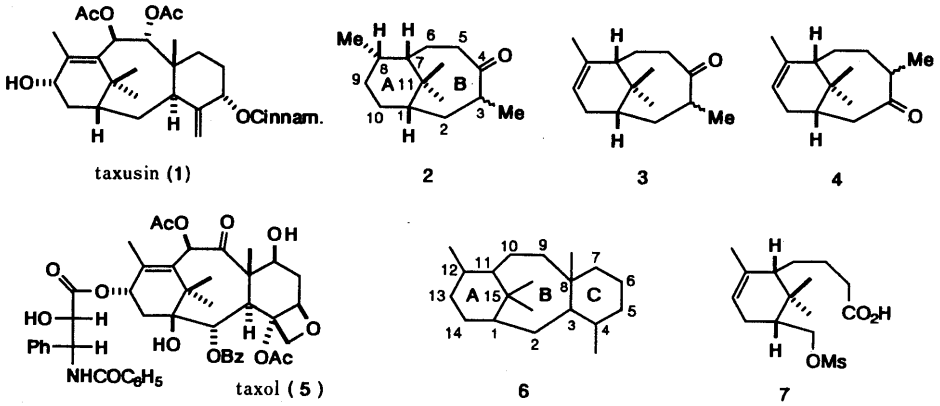


Fig. 1

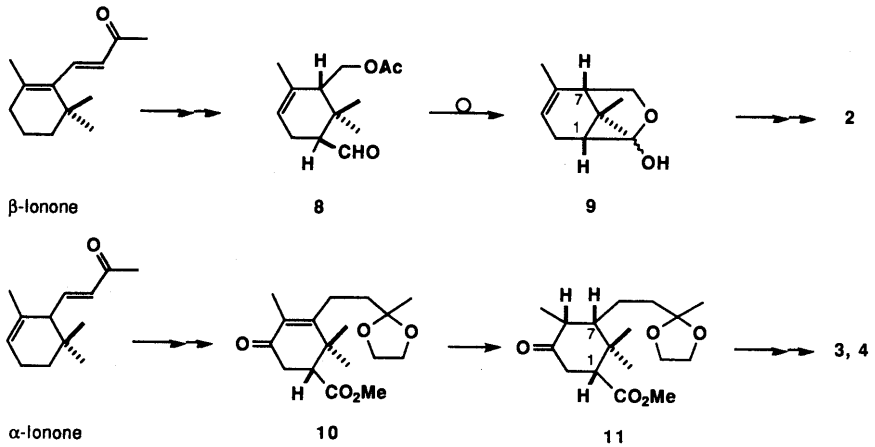


Fig. 2

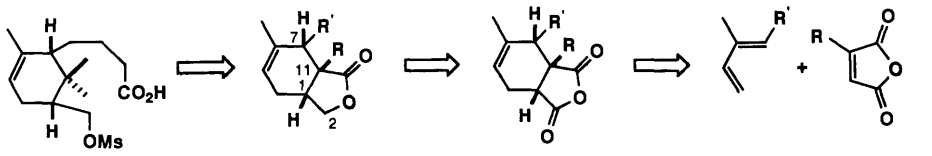


Fig. 3

arrangement of these substituents was effected in the previous synthesis by methanolysis of the acetoxy aldehyde **8** (epimerization at the C-1 position and concomitant formation of the cyclic hemiacetal **9**) or high pressure catalytic hydrogenation of the α,β-unsaturated ketone **10** leading to the ketone **11**, as shown in Fig. 2.[1]

In the present paper, the stereocontrolled synthesis of **7** (or its equivalent) was undertaken by utilizing a Diels–Alder reaction. Our retrosynthesis of **7** is shown in Fig. 3. Since it is known that the intermolecular Diels–Alder reaction generally produces the *endo*-adduct, reaction of the *E*-diene **14** and the anhydride **15** is expected to produce the 1,11,7-all-*cis*-anhydride **13**. The requisite lactone **12b** appears to be obtainable by either introduction of a methyl group into **12a**, which would be obtained from the anhydride **13a**, or selective reduction of the anhydride moiety in **13b**. There should be no difficulty in converting **12b** into **7** containing three substituents of the desired stereochemistry. Based on the above consideration, a Diels–Alder reaction with the *E*-diene **14** and the anhydride **15a**, **b** was carried out.

Diels–Alder Reaction of the *E*-Diene 14 and the Anhydride 15 The *E*-diene **18** corresponding to **14** was prepared from 1,5-pentanediol (**16a**) through 6 steps. Benzyl 5-hydroxypentyl ether (**16b**) obtained by benzylation of **16a** was treated with pyridinium chlorochromate (PCC) and the resulting aldehyde was condensed with (carbethoxyethylidene)triphenylphosphorane to give the α,β-unsaturated ester **17b** in 73% yield. The corresponding alcohol **17a** has been obtained directly from 2-hydroxypyran by the reaction with the same phosphorane but the yield was only 55%.[6] Attempted benzylation of **17a** under the usual conditions (NaH–$PhCH_2Br$–tetrahydrofuran (THF), 0—25 °C) did not produce **17b** but gave only unidentified products. The ester **17b** was subjected to $LiAlH_4$ reduction, PCC oxidation and the Wittig reaction successively to give the *E*-diene **18** in 72% yield.

Treatment of the diene **18** with maleic anhydride (**15a**) in toluene at 100 °C afforded the anhydride **19a** in 83% yield as a single product. Sodium borohydride reduction of **19a** produced a mixture of the desired lactone **21a** and the isomeric lactone **22a** (**21a** : **22a** = 1 : 2) in THF (0 °C).[7] Only **22a** was obtained when *N*,*N*-dimethylformamide (DMF)–toluene was used as the solvent at room temperature. The lactone **21a** could be exclusively prepared from **19a** *via* iodo-lactonization. Alkaline hydrolysis of **19a** with an aqueous $NaHCO_3$ solution followed by iodine (I_2–KI) treatment afforded the iodo lactone **20a**, whose carboxylic acid moiety was reduced with $BH_3 \cdot SMe_2$ complex in $B(OMe)_3$–THF at room temperature. The crude alcohol thus obtained was treated with Zn in refluxing acetic acid to give the desired lactone **21a** in 60—65% overall yield. In this transformation, reduction of **20a** with $BH_3 \cdot SMe_2$ complex afforded a mixture of the alcohol and a small amount of the corresponding aldehyde, which remained unchanged by Zn reduction. The overall yield of **21a** was improved to 78% when $NaBH_4$ (EtOH) was added prior to Zn reduction. Exclusive formation of **21a** from **19a** was in accord with our expectation that the iodo lactone **20a** (**20**-A) would be much more stable than the sterically congested isomeric lactone **27**-A. Introduction of a methyl group into **21a** (lithium diisopropylamide (LDA)–MeI) gave the lactone **21b** in 94% yield.

An alternative preparation of **21b** from citraconic anhydride (**15b**) was also examined. Reaction of **15b** with **18** in toluene (100 °C) provided a 1 : 1 mixture of adducts **19b** and **19c** in 85% yield, but the mixture was inseparable by silica gel (SiO_2) chromatography. When the mixture was treated with $NaBH_4$ in THF at 0 °C, the more hindered carbonyl group in the anhydride[7] was reduced to yield a mixture of γ-lactones **22b** (41% yield) and **23** (43% yield), which could be separated by careful column chromatography on SiO_2. Attempted cleavage of the lactone ring in **22b** with EtSH–$AlCl_3$ to prepare the alcohol **25** *via* the sulfide **24** led only to debenzylation[8] at 0 °C and decomposition at room temperature. Iodo-lactonization of a mixture of **19b**, **c** followed by selective reduction of the liberated carboxylic acid in the same way as described for **19a** produced the lactones **21b** (46% yield) and **23** (14% yield). Formation of **22b** was not detected in this case, either. The yield of **23**, which is expected to be derived from **19c**, was low, which might be ascribed to the sterically congested nature of the intermediary iodo lactone **20**-C (**20c**). In this route, however, separation of the desired lactone **21b** from the isomer **23** is rather difficult and the maximum yield of **21b** is 50% since a 1 : 1 mixture of anhydrides (**19b**, **c**) is used. Thus, the former route *via* the iodo lactone **20a** and the lactone **21a** was adopted for the synthesis of **21b**.

The structures of these lactones **21b**, **22b** and **23** were confirmed by ^{1}H-nuclear magnetic resonance (^{1}H-NMR) analysis (spin-decoupling method) and by conversion of **21b** into the twelve-membered lactam sulfide **37**.

Conversion of Lactone 21b into the Twelve-Membered Lactam Sulfides 37 and 38 The lactone **21b** was readily converted into the acetate **26** by successive treatment with diisobutylaluminum hydride in toluene (−78 °C), hydrazine–NaOH in diethylene glycol and acetic anhydride in pyridine (95% overall yield *via* **25**).

Treatment of **26** under the Pd–C (EtOH) catalyzed reduction conditions, in the expectation of selective debenzylation, gave only the dihydro alcohol **27** in nearly quantitative yield. Attempted debenzylation of **26** with Me_2S–$BF_3 \cdot Et_2O$ (CH_2Cl_2, 0 °C—room temperature)[9] or EtSH–$AlCl_3$ (CH_2Cl_2, 0 °C)[8] also failed. This difficulty

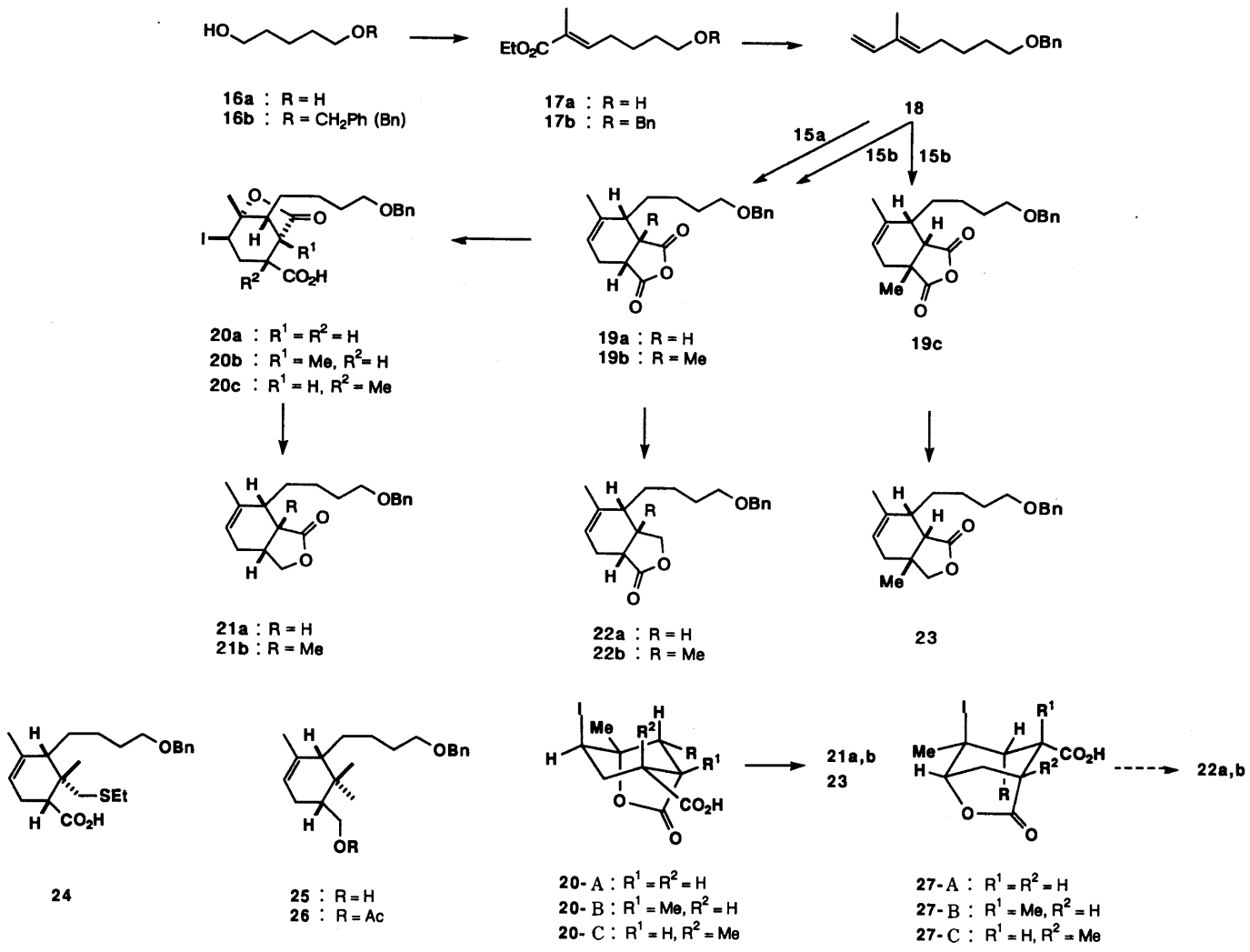


Fig. 4

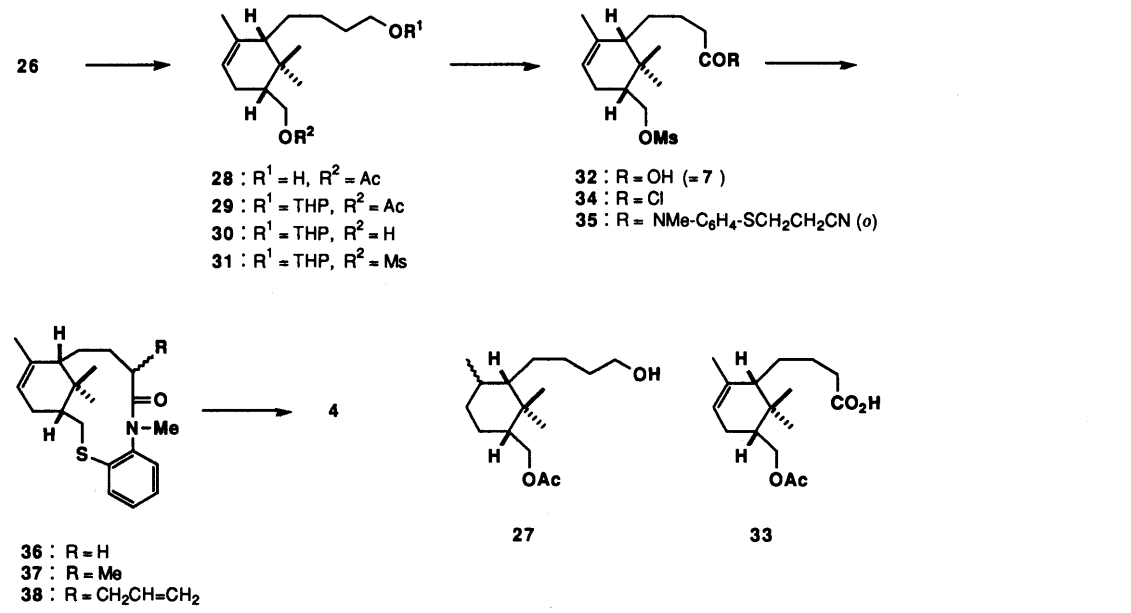


Fig. 5

could be overcome by the use of Raney Ni.[10] When a mixture of **26** with Raney Ni (W-2) in EtOH was refluxed for 7 min under nitrogen, the desired alcohol **28** was obtained in nearly quantitative yield. When heating was prolonged to 1 h, hydrogenation of the double bond took place, producing the dihydro alcohol **27** in about 15% yield

in addition to **28** (80% yield). The alcohol **28** was converted into the acid **32**, the precursor of **4**, in four steps: 1) masking of the primary alcohol with THP, 2) $LiAlH_4$ reduction, 3) mesylation, 4) Jones oxidation (94% overall yield *via* **29**, **30** and **31**). The acid **32** could also be prepared by Jones oxidation of **28** followed by hydrolysis and mesylation, but the yield was 61% from **28** *via* **33**.

The acid **32** was led to the lactam sulfide **36** in essentially the same manner as in the preparation of **4**.[1] 2-Cyanoethyl 2-*N*-methylaminophenyl sulfide was acylated with the acid chloride **34**, prepared from **32** with oxalyl chloride to give the amide **35** in 89% yield. This product was subjected to cyclization at high dilution with anhydrous K_2CO_3 (dried over P_2O_5 at 130 °C *in vacuo*)–$NaBH_4$ in DMF at 130—135 °C to afford the lactam sulfide **36** in 61% yield. No formation of the corresponding dimer was detected. Methylation of **36** with LDA and MeI yielded **37** as a sole product (90% yield), whose physical data (infrared (IR), thin layer chromatography (TLC), ^{1}H-NMR) were consistent with those of the less polar lactam sulfide A among the two isomeric lactam sulfides derived from α-ionone.[1] Treatment of **36** with LDA–allyl bromide also afforded a single isomer **38** in 94% yield, although the stereochemistry of the allyl group remained unknown.

Experimental

Melting point is uncorrected. ^{1}H-NMR spectra were taken on a JEOL FX-60 or GX-400 instrument in $CDCl_3$ solution with Me_4Si as an internal standard. A JEOL FX-60 instrument was routinely used. IR spectra were measured in CCl_4 solution with a JASCO A-3 spectrometer. Mass spectra (MS) were obtained with a Hitachi RMU-6M mass spectrometer and high-resolution MS were recorded on a Hitachi M-80 GC-MS instrument.

Ethyl (2*E*)-7-Benzyloxy-2-methylheptenoate (17b) A solution of 1,5-pentanediol monobenzyl ether (**16b**, 21.60 g, 111.3 mmol) in CH_2Cl_2 (110 ml) was added to a vigorously stirred suspension of PCC (49 g) in CH_2Cl_2 (330 ml) and the mixture was stirred at room temperature. After completion of the reaction (0.5—1.5 h), the mixture was diluted with Et_2O (1.7 l), dried ($MgSO_4$) and filtered through a column packed with Florisil. The filtrate was concentrated to afford the crude aldehyde (19.17 g). ^{1}H-NMR δ: 3.48 (2H, t, J=5.8 Hz), 4.49 (2H, s), 7.31 (5H, s), 9.74 (1H, t, J=1.7 Hz).

A mixture of the aldehyde (19.17 g) obtained above and (carbethoxypropylidene)triphenylphosphorane (54.3 g) in toluene (150 ml) was stirred at 100 °C (bath temperature) for 10 h under nitrogen. After removal of the solvent, the residue was extracted with hexane–Et_2O (4 : 1, 500 ml) and the extract was evaporated to give an oil, chromatography of which on SiO_2 afforded **17b** (22.51 g, 73.3% yield from **16b**) as a colorless oil from the hexane–AcOEt (19 : 1) eluate. ^{1}H-NMR δ: 1.28 (3H, t, J=7.1 Hz), 1.82 (3H, d, J=1.3 Hz), 3.48 (2H, t, J=6 Hz), 4.18 (2H, q, J=7.1 Hz), 4.50 (2H, s), 6.75 (1H, tq, J=1.4, 6.3 Hz), 7.32 (5H, s).

(3*E*)-8-Benzyloxy-3-methyl-1,3-octadiene (18) A solution of **17b** (3.59 g, 13 mmol) in Et_2O (26 ml) was added dropwise to a stirred suspension of $LiAlH_4$ (740 mg) in Et_2O (74 ml) over 10 min on an ice bath and the mixture was stirred for 10 min. Excess of $Na_2SO_4 \cdot 10H_2O$ was added carefully. The mixture was dried ($MgSO_4$) and filtered (Celite), and the filtrate was concentrated to give the allyl alcohol (3.30 g), which was rather unstable and was used immediately for the next oxidation reaction without further purification. ^{1}H-NMR δ: 1.64 (3H, d, J=1.2 Hz), 3.47 (2H, t, J=6.1 Hz), 3.98 (2H, br), 4.50 (2H, s), 7.32 (5H, s).

The allyl alcohol (3.30 g) obrained above was treated with PCC (4.30 g) in CH_2Cl_2 in the same way as for the oxidation of **16b** to give the unsaturated aldehyde (2.86 g). ^{1}H-NMR δ: 1.73 (3H, d, J=1.2 Hz), 3.49 (2H, t, J=5.8 Hz), 4.50 (2H, s), 6.47 (1H, tq, J=1.2, 7.3 Hz), 7.32 (5H, s), 9.38 (1H, s).

A solution of *n*-butyl lithium in hexane (17.6 mmol) was added dropwise to a stirred suspension of methyltriphenylphosphonium iodide (7.20 g, 17.8 mmol) in THF (72 ml) at −5—0 °C and the orange mixture was stirred for 30 min at room temperature under argon. A solution of the allyl aldehyde (2.86 g) obtained above in THF (40 ml) was added slowly to the stirred solution of the above ylide in THF over 10 min at −13—−15 °C. After 5 min, the mixture was stirred at room temperature for 30 min and the reaction was quenched with saturated aqueous NH_4Cl solution at −5 °C. An ethereal extract of the mixture was washed with brine, dried ($MgSO_4$) and evaporated to dryness. The residue was chromatographed on SiO_2 (hexane–AcOEt (19 : 1)) to afford **18** as a cololess oil (21.6 g, 72.2% from the unsaturated ester). ^{1}H-NMR δ: 1.72 (3H, br), 3.47 (2H, t, J=6.1 Hz), 4.49 (2H, s), 4.8—5.25 (2H, m), 5.48 (1H, br d, J=7.3 Hz), 6.36 (1H, br dd, J=10.5, 17.4 Hz), 7.32 (5H, s).

Diels–Alder Reaction of 18 and the Anhydrides 15a and 15b 1) **15a** (770 mg) was added to a solution of **18** (1.45 g, 6.28 mmol) and hydroquinone (70 mg) in toluene (16 ml) under argon and the container was sealed with a rubber cap. The mixture was stirred at 100 °C (bath temperature) for 15—17 h and then concentrated under reduced pressure to a quarter of the initial volume. The mixture was chromatographed on SiO_2 (hexane–AcOEt (17 : 3)) to afford 3α-(4-benzyloxybutyl)-4-methyl-4-cyclohexene-1α,2α-dicarboxylic anhydride (**19a**, 1.70 g, 82.6% yield from **18**) as a colorless oil. IR: 1845, 1775 cm^{-1}. ^{1}H-NMR δ: 1.77 (3H, br d), 3.2—3.65 (4H, m), 4.50 (2H, s), 5.5—5.8 (1H, m), 7.32 (5H, s). MS *m/z*: 328 (M^+), 237 ($M^+ - 91$). High-resolution MS Calcd for $C_{20}H_{24}O_4$ (M^+) *m/z*: 328.167. Found *m/z*: 328.165.

2) A solution of **18** (460 mg, 2.00 mmol), citraconic anhydride (**15b**, 280 mg, 2.50 mmol) and hydroquinone (46 mg) in toluene (5 ml) was stirred at 100 °C for 67 h in a sealed tube. After removal of the solvent, SiO_2 chromatography (hexane–AcOEt (4 : 1)) of the residue afforded a mixture of 3α-(4-benzyloxybutyl)-1,4-(**19c**) and -2,4-dimethyl-4-cyclohexene-1α,2α-dicarboxylic anhydrides (**19b**) in a ratio of 1 : 1 (582 mg, 85.1% from **18**) as a colorless oil. IR: 1840, 1775 cm^{-1}. ^{1}H-NMR δ: 1.38 and 1.41 (3H each, s), 1.75 (3H, br), 1.82 (3H, d, J=1.8 Hz), 4.47 and 4.51 (2H each, s), 5.4—5.8 (2H, m), 7.31 and 7.32 (5H each, s).

7α-(4-Benzyloxybutyl)-6-methyl-3aβ,4,7β,7aβ-tetrahydrophthalide (21a) *via* the Iodo Lactone 20a A suspension of the anhydride **19a** (3.10 g, 9.45 mmol) in 0.5 M $NaHCO_3$ aqueous solution (57 ml) was boiled for 30 min and allowed to cool to room temperature. To this mixture, $NaHCO_3$ (800 mg) was added and then a solution of I_2 (4.80 g, 2 eq) and KI (9.42 g, 6 eq) in water (57 ml) was added with stirring over 30 min. The reaction mixture was stirred for 3.5 h at room temperature and extracted with $CHCl_3$. The extract was washed with aqueous $Na_2S_2O_3$ solution and brine, dried ($MgSO_4$) and evaporated to give the crude iodo lactone **20a** (4.52 g). IR: 1780, 1715 cm^{-1}.

A solution of **20a** (4.52 g) obtained above in THF–$B(OMe)_3$ (4 : 1, 45 ml) was treated with borane–methylsulfide complex ($BH_3 \cdot Me_2S$, 1.50 ml, *ca.* 2.4 eq) at room temperature for 3 h. An excess of MeOH was added carefully to the ice cooled mixture and the solvent was removed *in vacuo*. The resulting oil was dissolved in MeOH and the solution was evaporated to dryness. The residue was treated with $NaBH_4$ (150 mg) in EtOH (90 ml) for 10 min at room temperature in order to reduce an aldehyde produced to a minor extent. After addition of AcOH (1 ml), the solvent was removed *in vacuo*. A mixture of the residue, IR: 3500, 1780 cm^{-1}, and Zn powder (9.50 g) in AcOH (190 ml) was refluxed for 1 h, cooled to room temperature and filtered. The filtrate was concentrated *in vacuo*. Column chromatography on SiO_2 of the residue afforded **21a** (2.31 g, 77.7% yield from **19a**) as a colorless oil using hexane–AcOEt (4 : 1) as an eluent. IR: 1770 cm^{-1}. ^{1}H-NMR δ: 1.77 (3H, br), 3.3—3.65 (2H, m), 3.87 (1H, dd, J=4.4, 9.0 Hz), *ca.* 4.39 (1H, dd, J=*ca.* 7.3, 9.0 Hz), 4.50 (2H, s), 5.35—5.75 (1H, m), 7.32 (5H, s). MS *m/z*: 314 (M^+), 223 ($M^+ - 91$). High-resolution MS Calcd for $C_{20}H_{26}O_3$ (M^+) *m/z*: 314.188. Found *m/z*: 314.184.

7α-(4-Benzyloxybutyl)-6,7aβ-dimethyl-3aβ,4,7β,7aβ-tetrahydrophthalide (21b) A solution of **21a** (2.31 g, 7.34 mmol) in THF (14.5 ml) was added dropwise to a solution of LDA in THF prepared from iso-Pr_2NH (3.20 ml, 22.86 mmol), a hexane solution of *n*-BuLi (20.56 mmol) and THF (14.5 ml) (−15 °C, 15 min) on a dry ice-acetone bath under Ar. The mixture was stirred for 30 min at −15 °C and then cooled on a dry ice–acetone bath. Methyl iodide (4.4 ml) was added dropwise to the stirred mixture, which was stirred at −78 °C for 30 min and at −15 °C for 30 min. The reaction was quenched with saturated NH_4Cl aqueous solution, and the mixture was extracted with $CHCl_3$. The extract was washed with brine, dried ($MgSO_4$) and evaporated to dryness. The resulting oil was chromatographed on SiO_2 (hexane–AcOEt (17 : 3)) to afford **21b** (2.27 g, 94.0%) as a colorless oil. IR: 1770 cm^{-1}. ^{1}H-NMR (400 MHz, decoupling) δ: 1.22 (3H, s), 1.83 (3H, d, J=1.4 Hz), 1.93—2.00 (1H, m, 4β-H), 2.07 (1H, dd, J=2.7, 10.0 Hz, 7β-H), 2.35—2.45 (1H, m, 4α-H), 2.4—2.5 (1H, m, 3aβ-H), 3.4—3.7 (2H, m), 3.81 (1H, dd, J=9.0, 9.3 Hz, 3-H), 4.49 (1H, dd, J=9.0, 9.3 Hz, 3-H), 4.48 (2H, s), *ca.* 5.5 (1H, m, 5-H), 7.2—7.4 (5H, m). MS *m/z*: 328 (M^+), 310 ($M^+ - 18$). High-resolution MS Calcd for $C_{21}H_{28}O_3$ (M^+) *m/z*: 328.204. Found *m/z*: 328.203.

$NaBH_4$ Reduction of the Anhydrides 19 1) The anhydride **19a** (33 mg, 0.1 mmol) was treated with an excess of $NaBH_4$ in toluene–DMF (2:1, 1.5 ml) for 17 h at room temperature. The mixture was acidified with diluted HCl and extracted with Et_2O. The extract was washed with water and dried ($MgSO_4$). Removal of the solvent gave an oil (32 mg), whose TLC and ^{1}H-NMR spectrum showed that the product consisted of a mixture of diacid and γ-lactone. Column chromatography (SiO_2) of the residual oil afforded 4α-(4-benzyloxybutyl)-5-methyl-3aβ,4β,7,7aβ-tetrahydrophthalide (**22a**), as a colorless oil (7.5 mg, 23.4%) from the hexane–AcOEt (4:1) eluate. IR: 1775 cm^{-1}. ^{1}H-NMR δ: 1.73 (3H, br), 3.48 (2H, t, J=5.2 Hz), 3.96 (1H, dd, J=6.0, 9.0 Hz), *ca.* 4.30 (1H, dd, J= *ca.* 7.9, 9.0 Hz), 4.50 (2H, s), 5.4—5.75 (1H, m), 7.32 (5H, s). MS *m/z*: 314 (M^+), 223 (M^+-91). High-resolution MS Calcd for $C_{20}H_{26}O_3$ (M^+) *m/z*: 314.188. Found *m/z*: 314.186.

2) The anhydride **19a** (33 mg, 0.1 mmol) was treated with an excess of $NaBH_4$ in THF (1 ml) for 1 h on an ice bath. The mixture was acidified with concentrated HCl (2 drops) and extracted with $CHCl_3$. The extract was filtered through a short column packed with SiO_2 and the filtrate was evaporated to dryness to give an oil (29 mg), whose ^{1}H-NMR and TLC showed that it was a mixture of **21a** and **22a** in a ratio of about 1:2.

3) A mixture of **19b** and **19c** (1:1, 292 mg, 0.855 mmol) was treated with $NaBH_4$ (50 mg) in THF (5 ml) at 0 °C for 30 min and at room temperature for 1.5 h. After addition of 6 N HCl (1 ml) on an ice both, the reaction mixture was stirred for 10 min at room temperature, diluted with water and extracted with $CHCl_3$. The extract was washed with brine, dried ($MgSO_4$) and evaporated to dryness. The resulting oil was chromatographed on SiO_2 using hexane–AcOEt (17:3) as an eluent to afford successively 7α-(4-benzyloxybutyl)-3aβ,6-dimethyl-3aβ,4,7β,7aβ-tetrahydrophthalide (**23**) (120 mg, 42.8%) as a less polar colorless oil and 4α-(4-benzyloxybutyl)-3aβ,5-dimethyl-3aβ,4β,7,7aβ-tetrahydrophthalide (**22b**) (114 mg, 40.5%) as a more polar colorless oil. (The less polar **23**) IR: 1775 cm^{-1}. ^{1}H-NMR (400 MHz, decoupling) δ: 1.16 (3H, s, 3aβ-Me), 1.73 (3H, br, 6-Me), *ca.* 1.87 and *ca.* 2.19 (1H each, m, 4-H_2), *ca.* 2.32 (1H, m, 7β-H), 2.41 (1H, d, J=5.6 Hz, 7aβ-H), 3.45—3.55 (2H, m), 3.87 and 3.91 (1H each, d, J=8.5 Hz, 3-H_2), 4.51 (2H, s), 5.4—5.5 (1H, m, 5-H), 7.34 (3H, s), 7.35 (2H, s). MS *m/z*: 328 (M^+), 237 (M^+-91). High-resolution MS Calcd for $C_{21}H_{28}O_3$ (M^+) *m/z*: 328.204. Found *m/z*: 328.204. (The more polar **22b**) IR: 1775 cm^{-1}. ^{1}H-NMR (400 MHz, decoupling) δ: 1.16 (3H, s, 3aβ-Me), 1.78 (3H, br, 5-Me), 2.35 (1H, dd, J=5.1, 9.0 Hz, 7aβ-H), 2.4—2.45 (2H, m, 7-H_2), 3.4—3.47 (2H, m), 4.00 and 4.25 (1H each, d, J=9.4 Hz, 3-H_2), 4.49 (2H, s), *ca.* 5.41 (1H, m, 6-H), *ca.* 7.33 (5H, m). MS *m/z*: 328 (M^+), 237 (M^+-91). High-resolution MS Calcd for $C_{21}H_{28}O_3$ (M^+) *m/z*: 328.204. Found *m/z*: 328.202.

Conversion of a Mixture of 19c and 19b into 23 and 21b *via* the Iodo Lactones A mixture of **19c** and **19b** (1:1, 342 mg, 1 mmol) obtained from **18** and citraconic anhydride (**15b**) was treated successively with 0.5 M $NaHCO_3$ aqueous solution (6 ml) and a solution of I_2 (508 mg, 2 eq)–KI (996 mg, 6 eq) in water (6 ml) in the same way as described for the preparation of **21a**. Work-up of the reaction mixture afforded a mixture of iodo lactones (506 mg) as a colorless oil. IR: 3500, 1775, 1720 (sh) cm^{-1}. ^{1}H-NMR δ: 1.26 and 1.71 (3H each, s), 1.59 (6H, s), 3.25—3.65 (4H, m), 4.3—4.5 (2H, m, –CH–I), 4.50 and 4.52 (2H each, s), 7.32 and 7.33 (5H each, s).

A part (352 mg) of the above mixture of iodo lactones was dissolved in THF–$B(OMe)_3$ (4:1, 3.5 ml) and the solution was treated with $BH_3 \cdot Me_2S$ (0.12 ml) for 13 h at room temperature. An excess of MeOH was added carefully and the solvent was removed to give an oily mixture of lactones (342 mg). IR: 3630, 3400, 1775 cm^{-1}.

A mixture of the resulting lactones and Zn powder (700 mg) in AcOH (17 ml) was refluxed for 2.5 h with vigorous stirring, filtered through Celite and concentrated under reduced pressure. Column chromatography (SiO_2) of the residue afforded successively **23** (38 mg, 16.6% from the mixture of anhydrides) and **21b** (92 mg, 40.2% from the mixture of anhydrides) from the hexane–AcOEt (17:3) eluate and the unidentified lactone (55.5 mg) as colorless oil from the hexane–AcOEt (2:1) eluate. IR: 3600, 1780 cm^{-1}. ^{1}H-NMR δ: 1.13 (3H, s), 1.70 (3H, br), 3.35—3.7 (2H, m), 3.8—4.3 (1H, m), 4.51 (2H, s), 7.32 (5H, s).

6α-(4-Benzyloxybutyl)-4α-hydroxymethyl-1,5,5-trimethylcyclohexene (25) and Its Acetate (26) A solution of diisobutylaluminum hydride in toluene (1 M solution, 7 ml) was added dropwise to a stirred solution of **21b** (1.54 g, 4.69 mmol) in toluene (47 ml) on a dry ice–acetone bath under Ar, and the mixture was stirred for 10 min. After addition of saturated NH_4Cl aqueous solution (15 ml), the mixture was stirred at room temperature for 10 min and then 5% H_2SO_4 (15 ml) was added on an ice bath. The mixture was stirred for 10 min, diluted with water and extracted with Et_2O–AcOEt. The extract was washed with $NaHCO_3$ aqueous solution and brine, and dried ($MgSO_4$). Removal of the solvent gave the cyclic hemiacetal (1.62 g) as a colorless oil, which was used for the next reduction without further purification.

A mixture of the cyclic hemiacetal (1.62 g) obtained above, 80% hydrazine hydrate (3.1 ml) and NaOH (1.84 g) in diethylene glycol (23 ml) was heated at 110°C (bath temperature) for 1 h with vigorous stirring under N_2. Then, the temperature was raised gradually to 210 °C (bath temperature) during 1—1.5 h with removal of water and excess hydrazine, and maintained for 2.5 h at 210 °C. After cooling, the mixture was diluted with water and extracted with Et_2O. The extract was washed with water, dried ($MgSO_4$) and evaporated to dryness to afford an oil (1.60 g), which was chromatographed on SiO_2 (hexane–AcOEt (4:1)) to provide the alcohol **25** (1.37 g, 92.1%) as a colorless oil. IR: 3620 cm^{-1}. ^{1}H-NMR δ: 0.74 and 0.97 (3H each, s), 1.70 (3H, br), 3.40 (1H, dd, J=8.1, 10.5 Hz), 3.3—3.65 (3H, m), 3.83 (1H, dd, J=4.1, 10.5 Hz), 4.50 (2H, s), 5.2—5.5 (1H, m), 7.32 (5H, s). MS *m/z*: 316 (M^+), 298 (M^+-18). High-resolution MS Calcd for $C_{21}H_{32}O_2$ (M^+) *m/z*: 316.240. Found *m/z*: 316.239.

The alcohol **25** (1.30 g, 4.13 mmol) was treated with Ac_2O (14 ml) and pyridine (28 ml) for 2 h at room temperature and the solvent was removed under reduced pressure. Column chromatography (SiO_2, hexane–AcOEt (19:1)) of the residue afforded **26** (1.41 g, 95.4%) as a colorless oil. IR: 1735 cm^{-1}. ^{1}H-NMR δ: 0.76, 0.98 and 2.03 (3H each, s), 1.69 (3H, br), 3.2—3.65 (2H, m), 3.85 (1H, dd, J=8.1, 10.6 Hz), 4.25 (1H, dd, J=3.8, 10.6 Hz), 4.50 (2H, s), 5.15—5.55 (1H, m), 7.32 (5H, s). MS *m/z*: 298 (M^+-60). High-resolution MS Calcd for $C_{21}H_{30}O$ ($M^+-CH_3CO_2H$) *m/z*: 298.229. Found *m/z*: 298.226.

4α-Acetoxymethyl-6α-(4-hydroxybutyl)-1,5,5-trimethylcyclohexene (28) Commercially available Raney Ni W-2 (16 ml, Aldrich Chemical Co.) was washed by suspension in distilled water and decantation until the washings were neutral to litmus and then the washing process was repeated three times with 99.5% EtOH (20 ml). A mixture of **26** (5.17 g, 14.4 mmol) and Raney Ni obtained above in 99.5% EtOH (100 ml) was refluxed for 7 min with vigorous stirring under N_2, then cooled rapidly below 20 °C and filtered through Celite. The filtrate was concentrated and the residual oil was dissolved in AcOEt. The mixture was passed through a short column packed with SiO_2. Removal of the solvent gave **28** (3.90 g, 100.7%) as a colorless oil, which was used for the next reaction without purification. An analytical sample was obtained by SiO_2 column chromatography (hexane–AcOEt (3:1)). IR: 3620, 1735 cm^{-1}. ^{1}H-NMR δ: 0.77, 0.99 and 2.04 (3H each, s), 1.70 (3H, br), 3.4—3.9 (2H, m), *ca.* 3.85 (1H, dd, J=8.0, 10.7 Hz), 4.23 (1H, dd, J=3.9, 10.7 Hz), 5.15—5.45 (1H, m). MS *m/z*: 268 (M^+), 208 (M^+-60). High-resolution MS Calcd for $C_{16}H_{28}O_3$ (M^+) *m/z*: 268.204. Found *m/z*: 268.199.

4-(5α-Acetoxymethyl-2,6,6-trimethyl-2-cyclohexen-1α-yl)butyl Tetrahydropyranyl Ether (29) A mixture of crude **28** (3.41 g) obtained by debenzylation of **26** (4.56 g, 12.73 mmol), dihydropyran (3 ml) and pyridinium *p*-toluenesulfonate (315 mg) in CH_2Cl_2 (50 ml) was stirred at room temperature for 1 h and diluted with Et_2O. The solution was washed with aqueous Na_2CO_3 solution and brine, dried ($MgSO_4$), and concentrated. SiO_2 column chromatography (hexane–AcOEt (9:1)) of the resulting oil provided **29** (4.47 g, 99.7% from **26**) as a colorless oil. IR: 1735 cm^{-1}. ^{1}H-NMR δ: 0.77, 0.99 and 2.04 (3H each, s), 1.69 (3H, br), 3.15—4.1 (5H, m), 4.26 (1H, dd, J=3.7, 9.7 Hz), 4.57 (1H, br), 5.15—5.45 (1H, m). MS *m/z*: 352 (M^+), 292 (M^+-60). High-resolution MS Calcd for $C_{21}H_{36}O_4$ (M^+) *m/z*: 352.261. Found *m/z*: 352.259.

4-(5α-Methanesulfonyloxymethyl-2,6,6-trimethyl-2-cyclohexen-1α-yl)-butanoic Acid (32) *via* 30 and 31 The acetoxy ether **29** (4.42 g, 12.54 mmol) was treated with $LiAlH_4$ (940 mg) in Et_2O (80 ml) for 10 min at 0 °C. Usual work-up of the mixture provided **30** (3.88 g) as a colorless oil. IR: 3620 cm^{-1}. ^{1}H-NMR δ: 0.76 and 0.98 (3H each, s), 1.68 (3H, br), 3.1—4.0 (6H, m), 4.57 (1H, br), 5.15—5.45 (1H, m). MS *m/z*: 310 (M^+), 292 (M^+-18).

Methanesulfonyl chloride (4.8 ml) was added dropwise to a stirred solution of **30** (3.88 g) obtained above and Et_3N (10.9 ml) in CH_2Cl_2 (129 ml) at 0 °C for 1 h. Work-up of the mixture in the usual manner gave **31** (5.44 g) as an oil. ^{1}H-NMR δ: 0.79, 1.01 and 3.00 (3H each, s), 1.70 (3H, br).

Jones reagent (27 ml) was added dropwise to a stirred solution of crude **31** (5.44 g) obtained above in acetone (220 ml) at 0 °C and stirring was continued for 1 h at room temperature. Work-up of the mixture in the usual manner and subsequent SiO_2 column chromatography (hexane–AcOEt (3:1)) afforded **32** (3.75 g, 94.0% from **29**) as a colorless gum. IR: 1705, 1175 cm^{-1}. ^{1}H-NMR δ: 0.79, 1.01 and 3.00 (3H, each, s), 1.72 (3H, br), 4.00 (1H, dd, J=8.1, 9.3 Hz), 4.39 (1H, dd, J=3.5, 9.3 Hz),

5.2—5.5 (1H, m). MS *m/z*: 318 (M^+), 303 (M^+-15), 222 (M^+-96). High-resolution MS Calcd for $C_{15}H_{26}O_5S$ (M^+) *m/z*: 318.150. Found *m/z*: 318.148.

4-(5α-Acetoxymethyl-2,6,6-trimethyl-2-cyclohexen-1α-yl)-butanoic Acid (33) The crude acetoxy alcohol **28** (1.03 g) prepared from **26** (1.41 g, 3.94 mmol) was oxidized with Jones reagent (10 ml) in acetone (80 ml) at room temperature for 30 min. Usual work-up of the reaction mixture and subsequent SiO_2 column chromatography (hexane–AcOEt (7 : 3)) afforded **33** (924 mg, 83.2% from **26**) as a colorless oil. IR: 1735, 1705 cm^{-1}. ^{1}H-NMR δ: 0.77, 0.99 and 2.04 (3H each, s), 1.71 (3H, br), 3.84 (1H, dd, J=8.0, 10.7 Hz), 4.26 (1H, dd, J=3.8, 10.7 Hz), 5.2—5.5 (1H, m). MS *m/z*: 282 (M^+), 222 (M^+-60). High-resolution MS Calcd for $C_{16}H_{26}O_4$ (M^+) *m/z*: 282.183. Found *m/z*: 282.184.

Conversion of 33 into 32 A solution of **33** (663 mg, 2.35 mmol) in MeOH–H_2O (1 : 3, 12 ml) was stirred with NaOH (260 mg) at room temperature overnight. The mixture was acidified with concentrated HCl and then concentrated. The residue was suspended in toluene and the solvent was evaporated off. The resulting residue was treated with methanesulfonyl chloride (1 ml) in CH_2Cl_2 (24 ml) in the presence of Et_3N (2.8 ml) at 0 °C for 1 h. The mixture was poured into ice, acidified, and extracted with AcOEt. The extract was washed with water, dried ($MgSO_4$), and concentrated under reduced pressure affording an oil. Column chromatography (SiO_2) (hexane–AcOEt (3 : 1)) gave **32** (547 mg, 73.2%) as a colorless gum.

***N*-Methyl-2'-[(2-cyanoethyl)thio]-4-(5α-methanesulfonyloxymethyl-2,6,6-trimethyl-2-cyclohexen-1α-yl)butananilide (35)** A solution of **32** (1.45 g, 4.60 mmol) and oxalyl chloride (2.3 ml) in benzene (23 ml) was stirred at room temperature for 1 h and then at 60 °C for 1 h. Removal of the solvent afforded the corresponding acid chloride **34** as a colorless oil, which was dissolved in THF (46 ml). The solution was added dropwise to a stirred mixture of 2-cyanoethyl (2-methylamino)phenyl sulfide (1.75 g) and anhydrous K_2CO_3 (2.52 g) in THF (90 ml) at 0 °C under Ar and stirring was continued for 30 min. The mixture was diluted with water and extracted with AcOEt. The extract was washed with brine, dried ($MgSO_4$), and concentrated. Column chromatography (SiO_2, hexane–AcOEt (1 : 1)) of the resulting oil gave **35** (2.00 g, 89.2%) as a pale yellow caramel. IR ($CHCl_3$): 2240, 1650 (sh), 1645 cm^{-1}. ^{1}H-NMR δ: 0.76, 0.94, 2.99 and 3.18 (3H each, s), 1.66 (3H, br), 2.5—2.85 (2H, m), 3.0—3.4 (2H, m), 3.7—4.6 (2H, m), 5.1—5.4 (1H, m), 7.0—7.5 (2H, m). MS (FD-MS) *m/z*: 492 (M^+).

3,9-Dimethyl-8β,12β-dimethylmethano-14-thia-3-aza-1,2-benzo-1,9-cyclotetradecadien-4-one (36) Anhydrous K_2CO_3 (4.40 g) and $NaBH_4$ (1.20 g) were dried over P_2O_5 at 130 °C for 2 h under reduced pressure and then DMF (318 ml) was added. To the stirred mixture, a solution of **35** (3.13 g, 6.36 mmol) in DMF (40 ml) was added over 48 h at 130 °C under Ar, and after complete addition stirring was continued for 5 h. The mixture was neutralized with diluted HCl, concentrated under reduced pressure, diluted with water and extracted with Et_2O–AcOEt (1 : 1). The extract was washed with aqueous Na_2CO_3 solution and brine, dried ($MgSO_4$), and concentrated. Column chromatography (SiO_2, hexane–AcOEt (3 : 1)) of the resulting residue afforded crystalline **36** (1.32 g, 60.6%), which was recrystallized from $CHCl_3$–hexane to give colorless prisms, mp 140—142 °C. IR: 1650 cm^{-1}. ^{1}H-NMR δ: 0.90, 1.35 and 3.24 (3H, each, s), 1.54 (3H, br), 2.75 (1H, dd, J=11.0, 12.9 Hz), 3.42 (1H, dd, J=6.7, 12.9 Hz), 4.85—5.1 (1H, m), 6.95—7.7 (4H, m). MS *m/z*: 343 (M^+), 328 (M^+-15). High-resolution MS Calcd for $C_{21}H_{29}NOS$ (M^+) *m/z*: 343.197. Found *m/z*: 343.194. *Anal.* Calcd for $C_{21}H_{29}NOS$: C, 73.42; H, 8.51; N, 4.08; S, 9.33. Found: C, 73.53; H, 8.52; N, 4.13; S, 9.32.

3,5ξ,9-Trimethyl-8β,12β-dimethylmethano-14-thia-3-aza-1,2-benzo-1,9-cyclotetradecadien-4-one (37) A solution of *n*-BuLi in hexane (1.55 M, 2.50 ml) was added dropwise to a stirred solution of **36** (686 mg, 2.00 mmol) and diisopropylamine (0.70 ml, 5 mmol) in THF (20 ml) at −78 °C under Ar, and after 20 min methyl iodide (0.8 ml) was added. Stirring was continued for 30 min at −78 °C and for 30 min at −10 °C. The reaction was quenched with saturated aqueous NH_4Cl solution and the mixture was extracted with $CHCl_3$. The extract was dried ($MgSO_4$) and the solvent was removed. Column chromatography on SiO_2 (hexane–AcOEt (9 : 1)) of the crude product afforded **37** (645 mg, 90.3%) as a colorless gum, whose IR and ^{1}H-NMR spectra and *Rf* values of TLC were identical with those of the less polar lactam sulfide A prepared from α-ionone.[1)]

5ξ-Allyl-3,9-dimethyl-8β,12β-dimethylmethano-14-thia-3-aza-1,2-benzo-1,9-cyclotetradecadien-4-one (38) According to the above procedure, the anion prepared from **36** (1.03 g, 3.00 mmol) and LDA (4.6 mmol) was treated with allyl bromide (1.30 ml). The crude product was chromatographed (SiO_2, hexane–AcOEt (9 : 1)) to give the allyl lactam sulfide **38** (1.07 g, 93.5%) as a pale yellow gum. IR: 1650 cm^{-1}. ^{1}H-NMR δ: 0.90, 1.41 and 3.23 (3H each, s), 1.54 (3H, br), 2.66 (1H, t, J=12.7 Hz), 3.58 (1H, dd, J=4.7, 12.7 Hz), 4.85—5.4 (3H, m), 5.4—6.0 (1H, m), 7.0—7.6 (4H, m). MS *m/z*: 383 (M^+), 368 (M^+-15). High-resolution MS Calcd for $C_{24}H_{33}NOS$ (M^+) *m/z*: 383.228. Found *m/z*: 383.229.

References and Notes

1) Studies on Taxane Synthesis. II: Y. Ohtsuka and T. Oishi, *Chem. Pharm. Bull.*, **36**, 4722 (1988).
2) Y. Horiguchi, T. Furukawa and I. Kuwajima, *J. Am. Chem. Soc.*, **111**, 8277 (1989); Over 40 papers had been published prior to 1988. See references cited in our previous paper; Y. Ohtsuka and T. Oishi, *Chem. Pharm. Bull.*, **36**, 4711 (1988).
3) R. A. Holton, R. R. Juo, H. B. Kim, A. D. Williams, S. Harusawa, R. E. Lowenthal and S. Yogai, *J. Am. Chem. Soc.*, **110**, 6558 (1988).
4) Y. Ohtsuka and T. Oishi, *Tetrahedron Lett.*, **1979**, 4487; *idem, Chem. Pharm. Bull.*, **31**, 443, 454 (1983).
5) In order to avoid confusion, the numbering for the bicyclo-[5.3.1]undecene to be synthesized as the final compound has been used for the cyclohexane derivatives in this paper.
6) L. D. Bergelson, E. V. Dyatlovitskaya and M. M. Shemyakin, *Izv. Akad. Nauk SSSR*, **1963**, 388; (*Chem. Abstr.*, **58**, 13783*h* (1963)).
7) D. M. Bailey and R. I. Johnson, *J. Org. Chem.*, **35**, 3574 (1970). *Cf.* J. J. Bloomfield and S. L. Lee, *ibid.*, **32**, 3919 (1967).
8) M. Node, K. Nishide, M. Sai, K. Ichikawa, K. Fuji and E. Fujita, *Chem. Lett.*, **1979**, 97; M. Node, K. Nishide, K. Fuji and E. Fujita, *J. Org. Chem.*, **45**, 4257 (1980).
9) K. Fuji, K. Ichikawa, M. Node and E. Fujita, *ibid.*, **44**, 1661 (1979); K. Fuji, T. Kawabata and E. Fujita, *Chem. Pharm. Bull.*, **28**, 3662 (1980).
10) Y. Oikawa, T. Tanaka, K. Horita and O. Yonemitsu, *Tetrahedron Lett.*, **25**, 5397 (1984).

Synthesis of *Erythrina* and Related Alkaloids. XXIV.[1] Total Synthesis of Erysotrine from 1,7-Cycloerythrinan Derivatives by the Use of a New 1,2-Carbonyl Transposition Method

Yoshisuke TSUDA,*[,a] Shinzo HOSOI,[a] Akira NAKAI,[a] Yuki SAKAI,[a] Tomoko ABE,[a] Yukari ISHI,[a] Fumiyuki KIUCHI,[a] and Takehiro SANO[b]

Faculty of Pharmaceutical Sciences Kanazawa University,[a] 13-1 Takara-machi, Kanazawa 920, Japan and Showa College of Pharmaceutical Sciences,[b] Higashi-tamagawagakuen 3-chome, Machida-shi, Tokyo 194, Japan. *Received October 15, 1990*

Treatment of 2,8-dioxo-1,7-cycloerythrinans with phenylselenenyl chloride in the presence of $BF_3 \cdot Et_2O$ as a catalyst gave 3-chloro-3-phenylselenenyl derivatives through the 3-phenylselenenyl derivative, which changed into the Δ^3-3-phenylselenenyl derivative on further reaction. Both the 3-phenylselenenyl and 3-chloro-3-phenylselenenyl derivatives gave the 3,3-dimethoxy derivative on treatment with mercury(II) perchlorate (MPC) in methanol, thus providing a new method for introduction of a masked carbonyl group at the α-position to the original carbonyl group. Thus, the reaction of 1 with phenylselenenyl chloride under acidic conditions followed by MPC treatment in methanol and borohydride reduction gave the 2α-hydroxy-3,3-dimethoxy derivative in 57% yield. This was converted to the conjugated ketone in four steps (72%). The carbomethoxy group of this compound was removed by the $CaCl_2$–dimethyl sulfoxide–3-ethylpentane-3-thiol method to give the enone 4 (70—80%) which isomerized to the conjugated ketone 25c (100%). This was converted to a natural *Erythrina* alkaloid, (±)-erysotramidine (5), in four steps (31%), and thence to (±)-erysotrine (6).

Keywords *Erythrina* alkaloid; erysotrine; total synthesis; 1,7-cycloerythrinan; phenylselenenylation; mercury(II) perchlorate; α,α-dimethoxylation; 1,2-carbonyl transposition; decarbomethoxylation; calcium chloride–dimethyl sulfoxide

The 6β-ethoxycarbonyl-2,8-dioxo-1,7-cyclo-*cis*-erythrinan (**1**) is a potential synthetic intermediate of natural erythrinan alkaloids, since it is easily preparable from homoveratrylamine and ethyl 5,5-ethylenedioxy-2-oxocyclohexanecarboxylate (seven steps with 40% overall yield) through intramolecular cyclization of an *N*-acyliminium compound of *N*-arylethylhydroindole type.[1,2] and it is appropriately protected at C-1 for further manipulation of rings A and B to synthesize the natural alkaloids.[1] most of which possess an oxygenated function at C-3. This paper describes transformation of **1** to natural erythrinan alkaloids of alkenoid and dienoid type, presenting a total synthesis of (±)-erysotrine (**6**).[3]

The procedure is schematically shown in Chart 1. This transformation requires the following three steps: 1) transposition of the oxygenated function from C-2 to C-3, 2) removal of the extra COOEt group, and 3) requisite alignment of the functional groups at rings A and B. These are treated in the above order.

Results and Discussion

Firstly, we attempted to introduce a methoxy group at C-3 by the action of mercury(II) ion in methanol on the 3-phenylselenenyl derivative **7**, because a soft–soft interaction of Hg and Se atoms might assist the expected substitution. However, the reaction of **7** with mercury(II) perchlorate (MPC) in methanol unexpectedly resulted in the introduction of two methoxy groups; this result instead opened up a new 1,2-carbonyl transposition method.

Introduction of a Carbonyl Function at C-3 *via* Phenylselenenylation (1→2) Treatment of **1a** with phenylselenyl chloride (PhSeCl) in tetrahydrofuran (THF) in the presence of $BF_3 \cdot Et_2O$ as a catalyst gave four products A—D, depending on the amount of reagent and the reaction conditions (Fig. 1). Compound A was formed initially and obtained as a major product by a short reaction (30 min) with 1 mol of PhSeCl. With an excess of the reagent under reflux for a longer time, compound D was obtained as a major product. Compound B and C were proved to be intermediary products. Isolation of each compound and

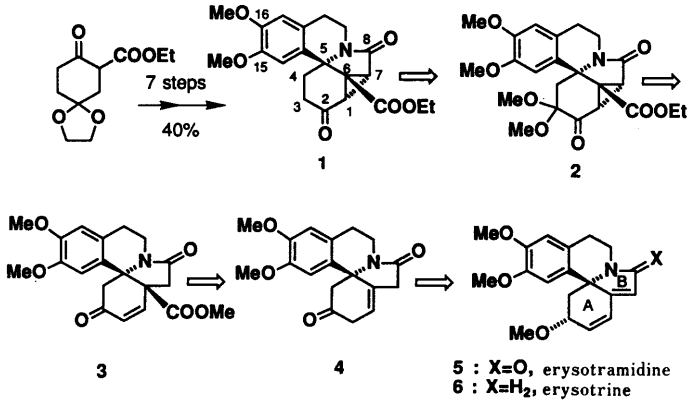


Chart 1. Synthetic Strategy for Erysotrine (**6**)

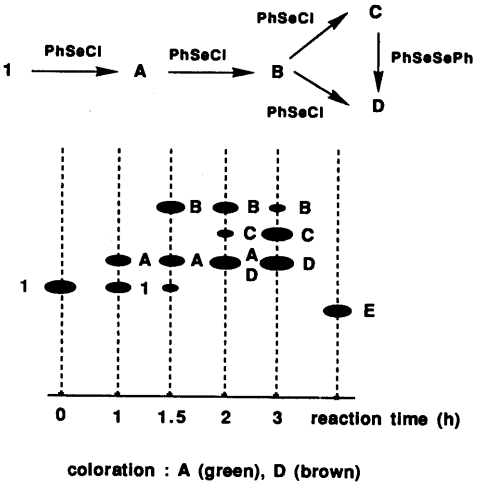


Fig. 1. TLC of the Reaction Mixture of **1** and PhSeCl

Reaction conditions, see text. Solvent, benzene : acetone = 4 : 1.

treatment of them with PhSeCl clarified that the reaction proceeds in the sequence A→B→C, and that the path C→D is catalyzed by $(PhSe)_2$ (see also below).

Chromatographic separation of the product over silica gel often gave a fifth compound E, which was not present in the reaction mixture, suggesting that some of the above four compounds changed into E on chromatography.

Compound A is the monophenylselenenyl derivative **7a**, which can also be prepared on treatment of **1a** with *n*-BuLi and diphenyl diselenide, though the yield was lower. The structure was proved by the desorption chemical ionization mass spectrum (DCI-MS), by other spectral data, and by conversion to the unsaturated ketone **8a** on treatment with hydrogen peroxide. On treatment with MPC in methanol, **7a** gave in high yield the α,α-dimethoxyketone **9a** (=**2**), whose structure was proved by analysis of the ^{1}H- and ^{13}C-nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra (for details of this new oxidative methoxylation reaction, see also below).

When compound A was adsorbed on silica gel and exposed to air, it gradually changed into the diketone **10a** which was identical with compound E, indicating that compound E was produced from compound A presumably by air oxidation as shown in Chart 3. On treatment with sodium sulfide, compound A regenerated compound **1a**, which was clearly the product due to the substitution reaction on the Se atom by sulfide anion.

Compound B had the formula $C_{27}H_{26}ClNO_6Se$ as evidenced from DCI-MS, and gave the chloro olefin **12a** and the dimethoxyketone **9a** on treatment with hydrogen peroxide in methanol. When dissolved in methanol, compound B rapidly changed into the methoxy derivative **13a** which gradually afforded the dimethoxyketone **9a** on prolonged standing in the same solvent. The dimethoxyketone **9a** was also obtained directly from compound B on treatment with MPC in methanol. These findings suggested the structure of compound B as the 3-chloro-3-phenylselenenyl derivative **11a** (for the stereochemistry, see below). On treatment with PhSeCl in THF, compound B gave compounds C and D.

Compound C had the same molecular formula as that of compound B (by DCI-MS), and gave the chloro olefin **12a** on treatment with hydrogen peroxide suggesting that it is a stereoisomer of compound B. In contrast to B, compound C was stable to methanol at room temperature, but changed into the dimethoxyketone **9a** and the diketone **10a** on heating in methanol or on treatment with a catalytic amount of TsOH in methanol at room temperature. The dimethoxyketone **9a** was obtained quantitatively on treatment of compound C with MPC in methanol. Compound C gave compound D and the chloro olefin **12a** on treatment with $(PhSe)_2$.

Compound D had the formula $C_{27}H_{25}NO_6Se$ and exhibited an olefinic proton signal (δ 6.36, s) in the ^{1}H-NMR spectrum. It was a conjugated ketone as evidenced from the infrared (IR) absorption band at 1689 cm^{-1}. It also showed the absorptions of a five-membered lactam (1710

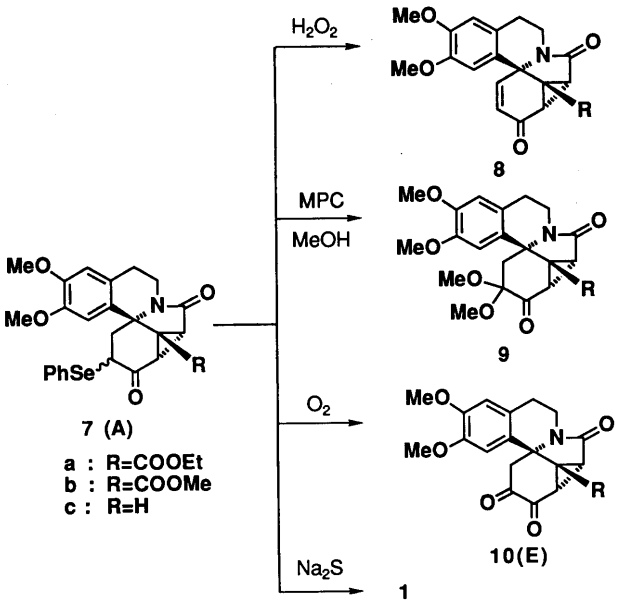


Chart 2

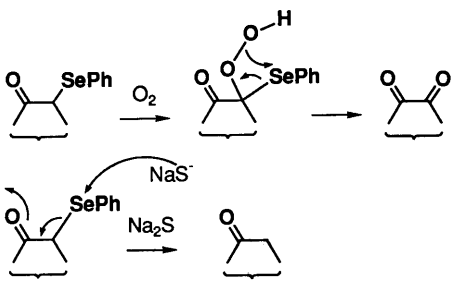


Chart 3

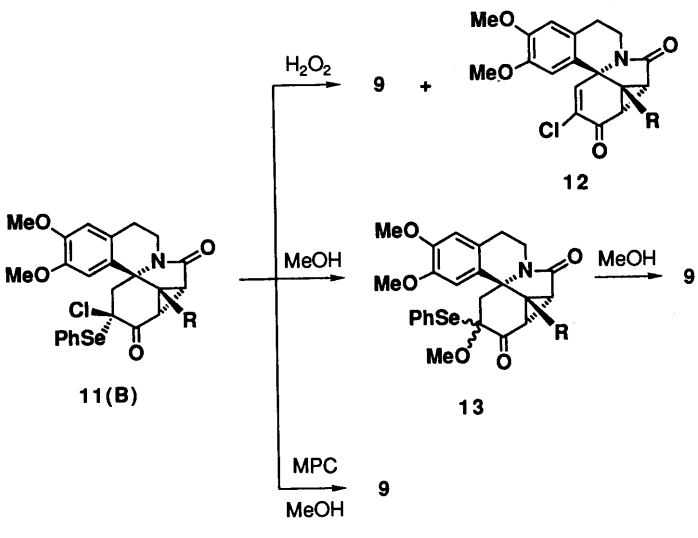


Chart 4

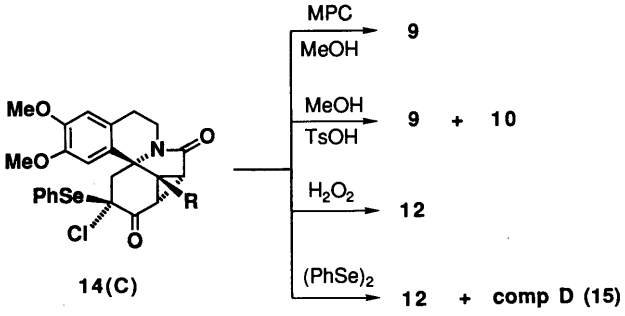


Chart 5

cm^{-1}) and an ester (1730 cm^{-1}) group. All of those data and the ^{13}C-NMR spectrum indicated the structure **15a** for compound D.

When compound D was treated with sodium sulfide in methanol, it changed into the methoxyketone **17a** which, on heating with 2.5% HCl, gave the conjugated ketone **8a**, identical with the compound obtained from compound A, together with the hydroxyketone **18a**, thus supporting the structure **15a**. The formation of **17a** from **15a** can be rationalized in terms of Michael addition of methanol followed by the substitution reaction of NaS^- anion on the Se atom in the resulting **16a** (see Chart 3). Attempts at chromatographic isolation of **16a** after the reaction of **15a** with NaOMe in methanol failed, the isolated product being a methoxy-diketone **19**, which is apparently the product of air oxidation of **16**, as discussed above, thus proving the intermediary formation of **16**. In contrast to A, B, and C, compound D was inert to MPC in methanol: it only formed an insoluble complex which regenerated compound D on decomposition with sodium sulfide.

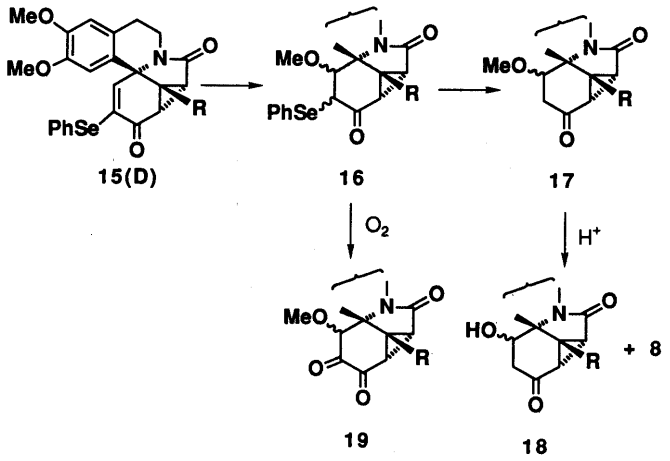


Chart 6

The above reaction with PhSeCl is explained as shown in Chart 7. The initially formed phenylselenenyl derivative **7** (A) is converted to compound B by one of the three possible paths (i—iii in Chart 8) where the second molecule of PhSeCl is reduced to $PhSe^-$. The kinetically formed compound B equilibrates with the thermodynamically more stable isomer C, probably through a Pummerer type intermediate **20**, which may isomerize to **15** (D) by deprotonation. Based on the above argument, we consider the stereochemistries of compounds B and C as the PhSe-inside (**11**) and PhSe-outside (**14**) configurations, respectively, since introduction of Cl^- (or Cl^+) from the β-face of the cycloerythrinan is favorable due to steric hindrance. Thus, in the kinetically produced compound (B), the PhSe group is at the more compressed position (PhSe-inside) and Cl is at the less hindered position (outside), *i.e.*, the structure **11**. On the contrary, the bulkier PhSe group is at the less compressed position (PhSe-outside) in the thermodynamically produced compound C, *i.e.*, the structure **14**. The reactions A→B→C are mediated by PhSeCl as suggested above, but compound C was stable to PhSeCl, implying that the reverse reaction **14**→**20** under catalysis of PhSeCl is too slow to be observed. Both compounds B and C changed, though slowly, into compound D on reaction with $(PhSe)_2$. The presence of $(PhSe)_2$ (or its equivalent) in the reaction mixture is conceivable, if we accept one of the three paths in Chart 8 for formation of compound B from compound A, producing $PhSe^-$, which will couple with PhSeCl to give $(PhSe)_2$.

TABLE I. ^{13}C-NMR Data for 1,7-Cycloerythrinan Derivatives (in Chloroform-*d*)

Carbon	1a	1c	7a	11a	14a	15a	15b	9a	9b	9c
1	35.1	30.3	34.0	32.5	30.0	39.1	38.9	32.2	31.9	30.1
2	200.7	203.0	198.1	190.7	186.8	186.1	185.9	195.3	195.2	197.2
3	34.9	34.7	44.7	69.3	70.9	140.4	140.4	97.2	97.2	97.2
4	28.7	27.5	28.4	28.3	28.2	147.5	147.5	28.3	28.3	27.4
5	61.9	59.1	62.8	61.8	62.1	61.3	61.3	61.9	61.5	59.0
6	46.5	28.8	45.4	43.1	41.2	48.0	47.7	42.7	42.3	26.2
7	33.6	33.6	32.9	30.3	27.9	34.1	33.9	31.7	31.4	27.2
8	167.1	168.8	167.5	168.3	168.8	166.6	167.0	167.6	167.6	169.1
10	36.9	36.2	44.1	53.5	52.9	28.5	28.6	45.4	45.4	45.4
11	35.2	33.6	34.4	35.7	35.6	36.3	36.2	34.9	34.9	33.8
12	127.5	129.8	127.5	127.4	127.3	127.0	126.8	127.4	127.4	130.0
13	126.5	125.3	126.5	126.6	126.3	126.5	126.6	127.3	127.3	126.2
14	108.9	107.3	108.9	109.6	109.7	108.7	108.6	109.8	109.6	107.7
15	147.6	148.0	147.6	147.5	147.4	148.1	148.1	147.5	147.6	146.7
16	148.5	148.2	148.5	148.8	148.8	148.6	148.6	148.6	148.7	148.2
17	112.3	112.0	112.1	112.1	112.3	111.7	111.8	112.1	112.2	112.1
OMe	55.9	55.7	55.8	56.0	55.9	55.9	55.8	56.1	56.1	56.0
	56.0	56.0	56.0	55.8	56.2	56.0	56.1	55.9	55.8	56.3
								49.9	49.9	49.7
								49.8	49.8	49.8
COOEt	167.1		166.8	166.8	167.0	165.3		167.1		
	61.9		62.0	62.1	62.1	62.0		61.9		
	13.6		13.7	13.7	13.9	13.5		13.7		
COOMe							167.0		167.6	
							52.8		61.9	
SePh s			126.6	125.9	127.8	126.0	126.0			
SePh d			128.6	129.1	129.2	129.3	129.8			
SePh d			135.3	137.8	137.5	136.2	136.2			
SePh d			131.5	130.0	130.7	129.9	129.9			

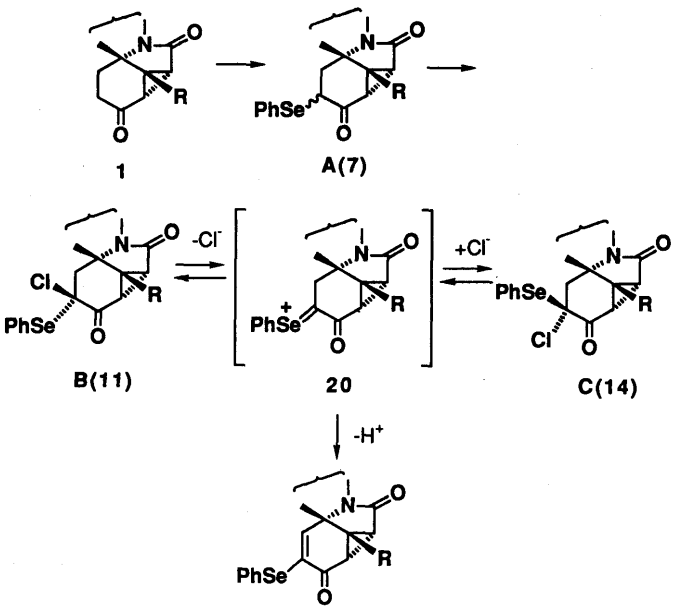


Chart 7

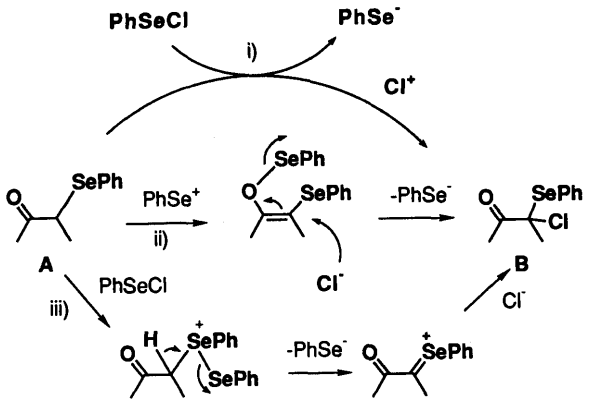


Chart 8

The 6-methoxycarbonyl derivative **1b** gave similar results, the products being **7b** and **11b** when a restricted amount of reagent was used, and **15b** with an excess of the reagent.

Phenylselenenylation of cycloalkanones under acidic conditions has so far been reported to give only the monophenylselenenyl derivatives.[4] Our present investigation has shown, for the first time, that the reaction gives not only the monophenylselenenyl derivative but also α-chloro-α-phenylselenenyl derivatives,[5] and sometimes the unsaturated phenylselenenyl derivative when an excess of the reagent is used. The similar phenylselenenylation of *O*-methylestrone[6] supports these findings.

As indicated above, all phenylselenenylated products (except compound D) gave the α,α-dimethoxyketone **9** when treated with MPC in methanol. Conversion of compound B or C to **9** by this reaction seems reasonable. However, the reaction of an α-phenylselenenyl ketone (A) to give the dimethoxyketone **9** by the action of MPC in methanol needs to be explained, because the reaction is accompanied with oxidation of the substrate. We consider that the reaction proceeds as shown in Chart 9, where Hg^{2+} mediates the methoxylation. This oxidative methoxylation is also

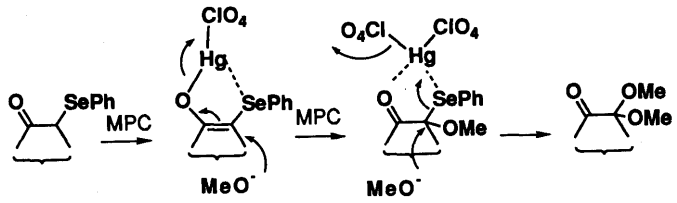


Chart 9. Oxidative Methoxylation of α-Phenylselenenyl Ketones with MPC

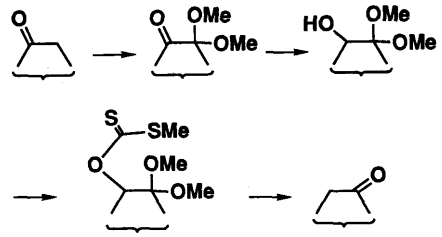


Chart 10. 1,2-Carbonyl Transposition

mediated by other metal cations such as Tl^{3+}. Thus, the reaction of either **7** or **11** (or **14**) with thallium(III) trinitrate (TTN) in MeOH gave the same α,α-dimethoxyketone **9**.[7] Interestingly, the reaction of **7** to **9** was more rapidly mediated by TTN, while that of **11** (or **14**) to **9** was more rapidly mediated by MPC. This metal effect will be discussed in a future communication.

The cycloerythrinan derivative **1a** was thus converted to the α,α-dimethoxyketone **9a** by one-pot reaction in a yield of *ca.* 60%: phenylselenenylation with PhSeCl in THF catalyzed by $BF_3 \cdot Et_2O$ and treatment of the resulting phenylselenenylated mixture with MPC in MeOH. The methyl ester **1b** also gave the dimethoxyketone **9b** in good yield. The 1,7-cycloerythrinan **1c** similarly gave the dimethoxylated product **9c** in 68% yield by one-pot treatment.

Removal of the C-2 Oxygenated Function (2→3) In the above section, we presented an efficient method of dimethoxylation at the α-position to a carbonyl group. Removal of the original carbonyl function from the resulting α,α-dimethoxyketone (followed by acid hydrolysis) completes a 1,2-carbonyl transposition procedure.[8] This can be done by reduction of the ketone to the alcohol followed by radical cleavage of its dithiocarbonate with tributyltin hydride (Chart 10). An example of the overall procedure of this 1,2-carbonyl transposition method is a successful conversion of estrone to isoestrone.[6] Here we deal with compounds **9a** (=**2**), **9b**, and **9c**.

Sodium borohydride reduction of **9a** gave the 2α-alcohol **21a** as a single product,[1] which was converted to the dithiocarbonate **22a**. Compound **22a** was smoothly reduced by tributyltin hydride, with concomitant ring opening of the conjugated cyclopropane ring, to give **23a**, which on acid treatment furnished the conjugated ketone **25a**.

For removal of the COOEt group at the next stage of the synthesis (see below), it was necessary to exchange the COOEt to COOMe. This transformation should be done at the stage of the 1,7-cyclo derivatives, since an alkoxycarbonyl group on the cyclopropane ring is easily hydrolyzable, or otherwise, it is highly resistant to hydrolysis or transesterification.[1] Thus, treatment of **21a** with NaOMe

in methanol resulted in the requisite transesterification in high yield to give **21b**.

This was converted to the dithiocarbonate **22b**, then treated with tributyltin hydride to yield **23b**, which, on mild acid hydrolysis, gave the required conjugated ketone **25b** (=**3**). Similarly the 6βH-derivative **9c**, by the above four successive reactions, gave the conjugated ketone **25c** in 66% yield.

Decarbomethoxylation from Vinylogous β-Keto Methyl Esters (3→4) Previously we presented a useful decarbalkoxylation method of β-keto esters ($MgCl_2$–dimethyl sulfoxide (DMSO) combination).[2,9] This method was also applicable to vinylogous β-keto esters, particularly for methyl esters.

The ethyl ester **25a** resisted decarbethoxylation even at 170 °C, but the methyl ester **25b** was smoothly decarbomethoxylated on heating with $MgCl_2$ in DMSO (containing EtSH) at 140 °C to give, in 55% yield, the enones **4** and **25c** in a 1 : 1 ratio. The difference of reactivity of ethyl *vs.* methyl ester is obviously due to the steric hindrance at the position where Cl^- attacks.

The structures of the products were proved spectroscopically and by chemical correlations. Treatment of **4** with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in benzene quantitatively isomerized it to a conjugated enone which was identical with **25c** obtained from 6βH-1,7-cycloerythrinan (see above), thus proving the stereochemistry at C-6. Those facts suggested that the enone **4** had been initially produced from the intermediary diene-enolate **26** by kinetic trapping of proton, which then migrated under the decarbomethoxylation conditions to give **25c**.

We found that $CaCl_2$ instead of $MgCl_2$ is more effective to remove the ester group from vinylogous β-keto methyl esters, in terms of both the yield and selectivity.[10] It must be emphasized that the reaction with the $CaCl_2$–DMSO combination always gives the kinetic product, the nonconjugated enone, predominantly. Thus, heating of **25b** (=**3**) with $CaCl_3$–DMSO (EtSH) at 140 °C gave the enones **4** and **25c**, in 74—83% yield, in a ratio of 5 : 1—4 : 3.

Several other points should be mentioned: the decarbomethoxylation of vinylogous β-keto esters is often accompanied with a side reaction, Michael addition of the thiol

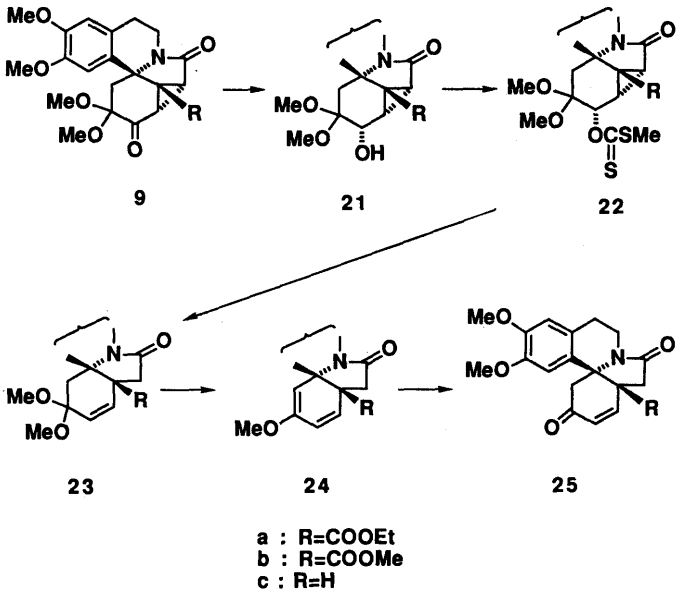


Chart 11

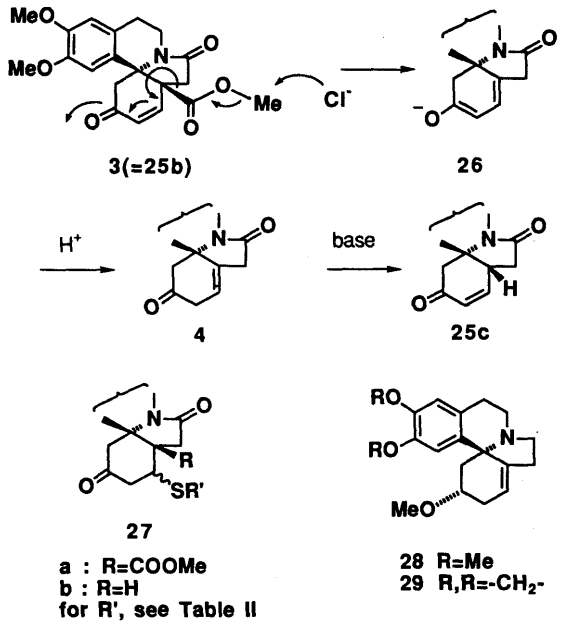


Chart 12

TABLE II. Decarbomethoxylation of **25b** by the $CaCl_2$–DMSO Method[a]

Additive R'SH	**25b** (recovery)	Product (yield, %)				
		4	**25c**	Adduct	**27a**	**27b**
$PhCH_2SH$	48	34	5		12	1 (R'=$PhCH_2$)
PhSH	16	34	18		15	18 (R'=Ph)
EtSH	13	43	31		7	6 (R'=Et)
tert-BuSH	12	63	24		0	0
tert-HepSH	9	80	11		0	0

a) Reaction conditions: see Experimental.

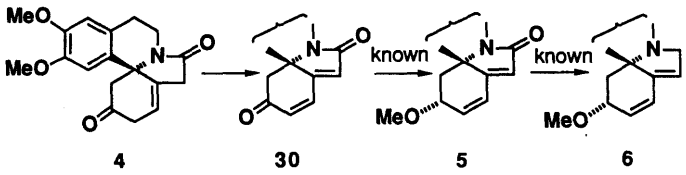


Chart 13

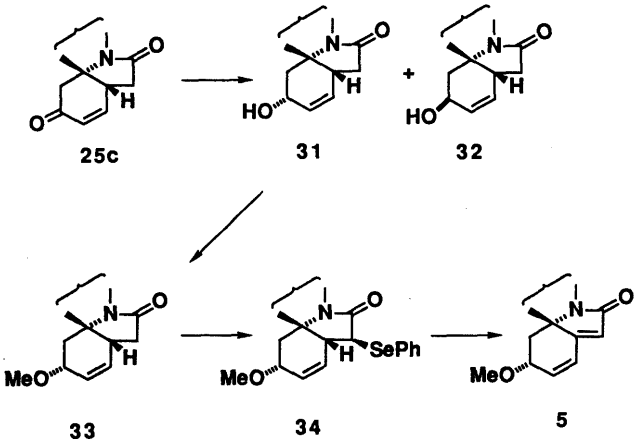


Chart 14

group to the conjugated ketone, forming **27**. The formation of **27a** prevents further decarbomethoxylation. This side reaction is particularly evident when EtSH or PhSH is used as an antioxidant[2] and can not readily be avoided by regulation of the amount of thiol. However, it was well avoided by the use of a bulky thiol such as 2-methylpropane-2-thiol or 3-ethylpentane-3-thiol, which are weak Michael addends. Thus, $CaCl_2$–DMSO (3-ethylpentane-3-thiol) was the best combination to prepare the nonconjugated ketones by decarbomethoxylation of vinylogous β-keto methyl esters. By this modified method, the methyl ester **25b** was converted into the enone **4** in an average yield of 70—80%. Enones of the structure **4** are potential intermediates in the preparation of alkenol type erythrinan alkaloids such as dihydroerysotrine **28** and erythramine (**29**).

Other applications of this decarbomethoxylation method to various vinylogous β-keto esters will be reported in a separate paper.

Total Synthesis of Erysotrine (4→5→6) Oxidation of the nonconjugated ketone **4** with dichlorodicyanobenzoquinone (DDQ) in benzene gave the dienone **30**, though in low yield (29%).[11] The yield of **30** was improved to 39% on treatment of **4** with pyridinium hydrobromide perbromide in chloroform followed by dehydrobromination with DBU.

Since the dienone **30** has already been converted into the dienoid type natural alkaloids erysotramidine (**5**) and erysotrine (**6**) by stereoselective reduction, methylation, and reductive removal of the lactam group,[12] the synthesis of the dienone **30** constitutes a total synthesis of (±)-erysotrine (**6**) in a formal sense.

A more efficient total synthesis of **5** and **6** was achieved from the conjugated enone **25c** as follows. The enone **25c** was reduced stereoselectively to the 3α-alcohol **31** with $NaBH_4$–$CeCl_3$ in methanol (**31**/**32** ratio was 3/1).[13] The alcohol **31** was methylated with iodomethane in the presence of a phase-transfer catalyst to give **33**. Phenylselenenylation of **33** with $(PhSe)_2$ and lithium diisopropylamide (LDA) followed by oxidative elimination of the phenylselenenyl group gave, in 95% yield, the dienoid lactam **5**, (±)-erysotramidine. Reduction of **5** with $LiAlH_4$ and $AlCl_3$[12] gave (±)-erysotrine (**6**). The identities of these products were confirmed by direct comparisons with the samples reported previously.[12] Thus, the total synthesis of erysotrine was accomplished.

Several new synthetic methods developed in the course of this work (presented in this and preceding several papers) are general ones and applicable not only to the synthesis of erythrinan alkaloids but also to the synthesis of other natural products and to their transformations.

Experimental

General Unless otherwise stated, the following procedures were adopted. Melting points were determined on a Yanaco micro hot stage melting point apparatus and are uncorrected. IR spectra were taken in KBr disks on a Jasco IR-G spectrometer, and the data are given in cm^{-1}. ^{1}H-NMR spectra were taken with a JEOL FX-100 (100 MHz) spectrometer in chloroform-*d* solution with tetramethylsilane as an internal standard, and the chemical shifts are given in δ values (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad). Mass spectra (MS) and high resolution mass spectra (HRMS) were taken with a Hitachi M-80 machine and M^+ and/or major peaks are indicated as *m/z*. DCI-MS were taken on a Shimadzu LKB-9000A spectrometer with ammonia or isobutane as a carrier gas. Column chromatography was performed on Wakogel C-200 (silica gel). For thin layer chromatography (TLC), Merck precoated plates GF_{254} were used and spots were monitored by ultraviolet (UV) (254 nm), then developed by spraying 1% $Ce(SO_4)_2$ in 10% H_2SO_4 and heating the plates at 100 °C until coloration took place. All organic extracts were washed with brine and dried over anhydrous sodium sulfate before concentration. Identities were confirmed by mixed melting point determination (for crystalline compounds) and also comparisons of TLC behavior and ^{1}H-NMR and IR spectra. MPC used was a trihydrate, $Hg(ClO_4)_2 \cdot 3H_2O$.

Phenylselenenylation of the 6β-Ethoxycarbonyl-2,8-dioxo-1,7-cycloerythrinan (1a) (1) Formation of Compound A: A mixture of **1a** (1.02 g), PhSeCl (504 mg, 1.0 eq), and $BF_3 \cdot Et_2O$ (10 drops) in THF (40 ml) was refluxed under an Ar atmosphere for 30 min. The cooled mixture was poured into $CHCl_3$ and the combined organic layer was washed with aqueous $NaHCO_3$ and concentrated. Chromatography of the residue gave compound A (**7a**, 412 mg, 28%), the starting material (**1a**, 560 mg), and the diketone E (**10a**, 260 mg, 25%).

7a (Compound A): Pale yellow oil. ^{1}H-NMR: 7.52 (2H, m, SePh), 7.24 (3H, m, SePh), 6.75, 6.45 (each 1H, s, ArH), 3.80 (6H, s, 2×OMe), 1.00 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$). DCI-MS (ammonia): 559 $[(M+NH_4)^+$ for $C_{27}H_{27}NO_6{}^{80}Se]$.

10a (Compound E): Yellow crystals (from CH_2Cl_2–$CHCl_3$), mp 252—253 °C. IR: 1725, 1715, 1690. ^{1}H-NMR: 7.04, 6.58 (each 1H, s, ArH), 3.86 (6H, s, 2×OMe), 1.13 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$). MS: 399 (M^+). *Anal.* Calcd for $C_{21}H_{21}NO_7$: C, 63.15; H, 5.30; N, 3.51. Found: C, 62.97; H, 5.35; N, 3.46.

(2) Formation of Compound B: A mixture of **1a** (1.005 g), PhSeCl (591 mg, 1.2 eq), and $BF_3 \cdot Et_2O$ (8 drops) in THF (50 ml) was treated as above for 2 h, then PhSeCl (200 mg, 0.4 eq) was added, and the reaction was continued for a further 1.5 h. Work-up of the mixture and chromatography of the product gave compound B (**11a**, 310 mg, 21%) from the benzene–acetone (6:1) eluate, and a mixture of compounds A (**7a**) and D (**15a**) (592 mg), and compound E (**10a**, 239 mg, 23%) from the benzene–acetone (2:1) eluate.

11a (Compound B): Pale yellow prisms (from benzene–hexane), mp 159—161 °C. IR: 1740, 1710, 1695. ^{1}H-NMR: 7.66 (2H, m, SePh), 7.32 (3H, m, SePh), 6.97, 6.56 (each 1H, s, ArH), 3.84, 3.83 (each 3H, s, OMe), 1.04 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$). DCI-MS (ammonia): 593 $[(M+NH_4)^+$ for $C_{27}H_{26}{}^{35}ClNO_6{}^{80}Se]$.

(3) Formation of Compound C: Compound **1a** (503 mg), PhSeCl (517 mg, 2.1 eq), and $BF_3 \cdot Et_2O$ (5 drops) in THF (20 ml) were heated as above. Portions of PhSeCl (259 mg, 1 eq, and 510 mg, 2 eq) were added to the mixture after 1 h and after a further 2 h, and the reflux was continued for a total of 11 h. Work-up as above and chromatography of the product gave compound C (**14a**, 436 mg, 58%) and compound D (**15a**, 276 mg, 39%) from the benzene–EtOAc (4:1) eluate.

14a (Compound C): Pale yellow prisms (from benzene–ether), mp 121—122 °C. IR: 1725, 1720, 1710. ^{1}H-NMR: 7.60 (2H, m, SePh), 7.38 (3H, m, SePh), 7.18, 6.56 (each 1H, s, ArH), 3.88, 3.86 (each 3H, s, OMe), 1.20 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$). DCI-MS (ammonia): 593 $[(M+NH_4)^+$ for $C_{27}H_{26}{}^{35}ClNO_6{}^{80}Se]$.

(4) Formation of Compound D: The reaction of **1a** (1.0 g) in THF (40 ml) in the presence of $BF_3 \cdot Et_2O$ (11 drops) as a catalyst for 11 h with occasional additions of PheSeCl (1.02, 0.85, and 0.571 g, total 4.8 eq) gave compound C (**14a**, 375 mg, 25%) and compound D (**15a**, 866 mg, 62%).

15a (Compound D): Yellow prisms (from EtOAc–ether), mp 224—225 °C. IR: 1725, 1705, 1695. ^{1}H-NMR: 7.52 (2H, m, SePh), 7.28 (3H, m, SePh), 6.54, 6.44 (each 1H, s, ArH), 6.36 (1H, s, H-4), 3.84, 3.76 (each 3H, s, OMe), 0.90 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$). DCI-MS (ammonia): 557 $[(M+NH_4)^+$ for $C_{27}H_{25}NO_6{}^{80}Se]$.

Treatment of Compound A (7a) with PhSeCl Compound A (**7a**, 92 mg) in THF (7 ml) containing $BF_3 \cdot Et_2O$ (3 drops) was treated with PhSeCl as follows under an Ar atmosphere with monitoring by TLC: i) 33 mg (1 eq) of PhSeCl and reflux for 2 h, and ii) further addition of 66 mg of PhSeCl (2 eq) and reflux for 8 h, iii) then for a further 5 h, and iv) the resulting mixture was heated with $(PhSe)_2$ (20 mg) for 2 h. The following compounds were formed: i) compound B, ii) compounds B and C, and iii) compounds B, C, and D. The major product in iv) was compound D.

Treatment of Compound B (11a) with PhSeCl Compound B (**11a**, 10 mg) was heated with PhSeCl (10 mg, 3 eq) and $BF_3 \cdot Et_2O$ (1 drop) in THF (2 ml) at 80 °C. After 1 h (i), $BF_3 \cdot Et_2O$ (1 drop) was added, and the reaction was monitored at 1 h (ii), 3 h (iii), and 5 h (iv), then 15 mg of PhSeCl was added and the mixture was heated for a further 3 h (v). The results were as follows: (i) compound B (unchanged), (ii) compound B and compound C, (iii) compounds B, C, and D, (iv) compounds B and C < compound D, and (v) compounds B and C ≪ compound D.

Treatment of Compound C (14a) with PhSeCl or $(PhSe)_2$ (1) Compound **14a** (5 mg) was heated with PhSeCl (10 mg, 6 eq) and $BF_3 \cdot Et_2O$ (1 drop) in THF (1.5 ml) at 80 °C for 5.5 h (the TLC pattern was unchanged). $(PhSe)_2$ (15 mg, 5.4 eq) was added to this mixture and the whole was heated for 3.5 h (i), then 15 mg (5.4 eq) of $(PhSe)_2$ was added and the mixture was heated for a further 1 h (ii). The TLC of (i) showed compounds C and D, and that of (ii) almost entirely D. The ^{1}H-NMR spectrum of the products in (ii) indicated the formation of compound D with a small amount of the chloro olefin **12a** and compound C.

(2) Compound **14a** (5 mg) and $(PhSe)_2$ (15 mg) in THF (2 ml) were heated in a sealed tube at 80 °C for 1 h. The TLC pattern and ^{1}H-NMR spectrum of the product (3 mg, isolated by chromatography) showed that it was a mixture of compound D **15a**, the chloro olefin **12a**, and the starting material **14a**.

15,16-Dimethoxy-6β-methoxycarbonyl-2,8-dioxo-1,7-cycloerythrinan (1b) Compound **1a** (300 mg) in dry MeOH was treated with 1% NaOMe–MeOH (15 ml) for 2 h at room temperature. The mixture was neutralized with Amberlite IRC-50 (H^+) and concentrated to give the methyl ester **1b** (280 mg, 97%), as colorless prisms (from MeOH), mp 225—227 °C (previously reported as a gum).[1] The spectral data were identical with those reported.[1]

Phenylselenenylation of Compound 1b (1) A mixture of **1b** (505 mg), PhSeCl (310, 324, 156 mg, total 3.0 eq), and $BF_3 \cdot Et_2O$ (15 drops) in THF (35 ml) was heated under reflux for a total of 9 h. Work-up of the mixture as described above gave compound **7b** (370 mg, 52%) and the starting material **1b** (150 mg, 30%).

7b: Gum. ^{1}H-NMR: 7.58 (2H, m, PhSe), 7.32 (3H, m, PhSe), 6.82, 6.54 (each 1H, s, ArH), 3.84 (6H, s, 2 × OMe), 3.52 (3H, s, COOMe). DCI-MS (isobutane): 528 (MH^+ for $C_{26}H_{25}NO_6{}^{80}Se$).

(2) A mixture of **1b** (502 mg), PhSeCl (543, 397, and 270 mg, total 4.5 eq), and $BF_3 \cdot Et_2O$ (5 drops) in THF (35 ml) was heated under reflux for total 5.5 h. Work-up of the mixture gave **11b** (318 mg, 42%) and **15b** (295 mg, 42%).

11b: Pale yellow prisms (from MeOH), mp 158—161 °C. ^{1}H-NMR: 7.67 (2H, m, PhSe), 7.35 (3H, m, PhSe), 6.96, 6.56 (each 1H, s, ArH), 3.86, 3.85, 3.59 (each 3H, s, OMe). DCI-MS (isobutane): 562 (MH^+ for $C_{26}H_{24}{}^{35}ClNO_6{}^{80}Se$).

15b: Yellow prisms (from AcOEt–ether), mp 106—107 °C. ^{1}H-NMR: 7.50 (2H, m, PhSe), 7.26 (3H, m, PhSe), 6.52, 6.44 (each 1H, s, ArH), 6.36 (1H, s, H-4), 3.84, 3.78, 3.42 (each 3H, s, OMe). DCI-MS (isobutane): 526 (MH^+ for $C_{26}H_{23}NO_6{}^{80}Se$).

Air Oxidation of Compound A (7a) Compound A (**7a**) in benzene was adsorbed on SiO_2 and kept at room temperature. After 1 d, TLC showed the formation of compound E, and after 10 d, elution of the column with benzene–acetone gave compound E (**10a**) exclusively.

Reaction of Compound A (7a) with MPC (1) A solution of **7a** (100 mg) and MPC (380 mg) in MeOH (10 ml) was stirred for 3 h at room temperature. The mixture was treated with dilute Na_2S solution until the solution became faintly alkaline, and then it was filtered. The filtrate was extracted with $CHCl_3$. The product obtained from the extract was purified by chromatography to give the dimethoxyketone **9a** (69 mg, 84%) as a colorless gum.

(2) A solution of **1a** (100 mg) and PhSeCl (100 mg) in THF (15 ml) was stirred for 22 h at room temperature, then refluxed for 5 h to give a mixture of **7a** and **11a** (on TLC). After evaporation of the solvent, the residue was dissolved in MeOH (10 ml) and treated with MPC (490 mg, 4 eq) for 17 h at room temperature. The mixture was diluted with water and extracted with $CHCl_3$. Concentration of the dried extract and chromatography of the residue gave **9a** (66 mg, 57%) as an oil from the benzene–AcOEt (4 : 1) eluate. IR: 1730, 1715, 1700. ^{1}H-NMR: 7.00, 6.50 (each 1H, s, ArH), 3.98 (2H, q, J = 7 Hz, $COOCH_2CH_3$), 3.82, 3.22 (each 6H, s, OMe), 1.07 (3H, t, J = 7 Hz, $COOCH_2CH_3$). MS: 445 (M^+). HRMS: Calcd for $C_{23}H_{27}NO_8$: 445.2171. Found: 445.2156.

Reaction of Compound A (7a) with Na_2S Compound **7a** (50 mg) in MeOH (5 ml) was stirred with an excess of Na_2S solution for 2 h at room temperature. Extraction of the mixture with $CHCl_3$ and chromatography of the product gave compound **1a** (20 mg) and compound **7a** (5 mg).

Reaction of Compound A (7a) with H_2O_2 A solution of **7a** (40 mg) and 30% H_2O_2 (5 drops) in MeOH (15 ml) was stirred for 15 min at room temperature. Chromatography of the product gave the conjugated ketone **8a** (28 mg, 100%) as colorless prisms (from MeOH), mp 189—192 °C. IR: 1730, 1720, 1700. ^{1}H-NMR: 7.08 (1H, d, J = 10 Hz, H-4), 6.65, 6.59 (each 1H, s, ArH), 6.24 (1H, dd, J = 10, 1 Hz, H-3), 3.90, 3.83 (each 3H, s, OMe), 0.91 (3H, t, J = 7 Hz, $COOCH_2CH_3$). *Anal.* Calcd for $C_{21}H_{21}NO_6 \cdot H_2O$: C, 62.84; H, 5.73; N, 3.49. Found: C, 63.23; H, 5.52; N, 3.42.

Reaction of Compound B with MeOH (1) Compound **11a** (11 mg) in MeOH (10 ml) was stirred at room temperature. After 30 min, the mixture showed almost a single spot corresponding to the methoxy compound **13a**. However, after evaporation of the solvent the dimethoxyketone **9a** was obtained.

(2) Compound **11b** (100 mg) in MeOH was stirred for 1.4 h at room temperature. Careful evaporation of the solvent gave the methoxy compound **13b** as a gum. ^{1}H-NMR: 7.41 (2H, m, PhSe), 7.27 (3H, m, PhSe), 6.96, 6.52 (each 1H, s, ArH), 3.82, 3.80, 3.60, 3.50 (each 3H, s, OMe). DCI-MS (isobutane): 558 (MH^+ for $C_{27}H_{27}NO_7{}^{80}Se$).

On further stirring of **13b** in MeOH, it changed into the dimethoxyketone **9b** (gum). ^{1}H-NMR: 6.95, 6.47 (each 1H, s, ArH), 3.80 (6H), 3.55 (3H), 3.20 (6H) (each s, 5 × OMe). MS: 431 (M^+).

Reaction of Compound B (11a) with H_2O_2 Compound **11a** (50 mg) and 30% H_2O_2 (10 drops) in MeOH (15 ml) were stirred for 50 min at room temperature. The mixture was diluted with $CHCl_3$, washed with aqueous $NaHCO_3$, and concentrated. Chromatography of the residue gave the chloro olefin **12a** (5 mg, 17%) and the dimethoxyketone **9a** (21 mg, 55%).

12a: Colorless prisms (from AcOEt–MeOH), mp 209—210 °C. IR: 1725, 1710, 1700. ^{1}H-NMR: 7.23 (1H, s, H-4), 6.67, 6.60 (each 1H, s, ArH), 3.89, 3.84 (each 3H, s, OMe), 0.91 (3H, t, J = 7 Hz, $COOCH_2CH_3$). MS: 417, 419 (M^+, 3 : 1). HRMS: Calcd for $C_{21}H_{20}{}^{35}ClNO_6$: 417.0974. Found: 417.1008.

Reaction of Compound B (11a) with MPC A solution of **11a** (19 mg) and MPC (40 mg, 2.5 eq) in MeOH–$CHCl_3$ (3 : 1, 8 ml) was stirred for 40 min at room temperature, then diluted with $CHCl_3$, and washed with aqueous $NaHCO_3$. The organic layer was washed with brine, dried, and concentrated. Chromatography of the residue gave the dimethoxyketone **9a** (12 mg, 83%) from the benzene–acetone (4 : 1) eluate.

Reaction of Compound C (14a) with MeOH (1) Compound **14a** (10 mg) in MeOH (2 ml) was heated under reflux for 8 h under an Ar atmosphere. TLC of the mixture showed the presence of the dimethoxyketone **9a** and the diketone **10a**.

(2) Compound **14a** (100 mg) in MeOH (40 ml) was heated under reflux with a catalytic amount of *p*-TsOH for 30 min, then stirred for 4 h at room temperature. Evaporation of the solvent and usual work-up of the residue gave the dimethoxyketone **9a** (30 mg, 39%) and the diketone **10a** (40 mg, 58%).

Reaction of Compound C (14a) with H_2O_2 A solution of **14a** (5 mg), 30% H_2O_2 (1 drop) and pyridine (1 drop) in CH_2Cl_2 was stirred for 30 min at room temperature. Chromatography of the product gave the chloro olefin **12a** (2.4 mg, 80%).

Reaction of Compound C (14a) with MPC A solution of **14a** (10 mg) and MPC (20 mg, 2.0 eq) in MeOH (2 ml) was stirred for 20 min at room temperature and worked up as above. Chromatography of the product gave the dimethoxyketone **9a** (6 mg, 77%).

Reaction of Compound D (15a) with Na_2S and MeOH An excess of Na_2S was added to a stirred solution of compound **15a** (145 mg) in MeOH (36 ml) and the mixture was stirred for 5 min, then neutralized with 1 N HCl and extracted with $CHCl_3$. Purification of the extract by preparative TLC gave the methoxyketone **17a** (93 mg, 86%), as colorless plates (from MeOH), mp 215—219 °C. IR: 1720, 1710, 1690. ^{1}H-NMR: 7.26, 6.62 (each 1H, s, ArH), 3.89, 3.83, 3.36 (each 3H, s, OMe), 0.95 (3H, t, J = 7 Hz, $COOCH_2CH_3$). MS: 415 (M^+). *Anal.* Calcd for $C_{22}H_{25}NO_7$: C, 63.60; H, 6.07; N, 3.37. Found: C, 63.48; H, 5.99; N, 3.45.

Reaction of Compound D (15a) with NaOMe A 5% NaOMe–MeOH solution (10 drops) was added to a stirred solution of **15a** (50 mg) in MeOH (25 ml) and the mixture was stirred for 1 h at room temperature, then diluted with water, and extracted with $CHCl_3$. Chromatography of the extract gave **19a** (30 mg, 78%) as a yellow oil from the benzene–acetone (4 : 1) eluate. IR ($CHCl_3$): 1730, 1720, 1695. ^{1}H-NMR: 7.28, 6.62 (each 1H, s, ArH), 3.88, 3.85, 3.45 (each 3H, s, OMe), 1.02 (3H, t, J = 7 Hz, $COOCH_2CH_3$). MS: 429 (M^+).

Acid Treatment of 17a Compound **17a** (11 mg) in 5% HCl–THF (1 : 1, 1 ml) was heated at 80 °C for 1 h. Addition of water and extraction with $CHCl_3$ gave 9 mg of the product, which was judged to be a mixture of the conjugated ketone **8a**, the hydroxyketone **18a**, and the starting material **17a** on the basis of TLC behavior and the ^{1}H-NMR spectrum.

Phenylselenenylation of 15,16-Dimethoxy-2,8-dioxo-1,7-cycloerythrinan (1c) (1) The cycloerythrinan **1c** (130 mg) was treated with 0.1 M *n*-BuLi (0.4 ml) and $(PhSe)_2$ (194 mg) in benzene (10 ml) in the presence of 12-crown-4 (8 drops) to give the phenylselenenyl derivative **7c** (103 mg, 53%) as colorless needles (from MeOH), mp 211—213 °C. IR: 1690. ^{1}H-NMR: 7.55 (2H, m, PhSe), 7.30 (3H, m, PhSe), 6.64, 6.56 (each 1H,

s, ArH), 4.16 (2H, m, H-3 and one of H-10), 3.89, 3.85 (each 3H, s, OMe). MS: 469 (M^+ for $C_{24}H_{23}NO_4{}^{80}Se$), 312 (base peak).

(2) Compound **1c** in THF (or CH_2Cl_2) was treated with PhSeCl (2 eq-excess) under reflux for 4 h. The product showed two spots on TLC: one corresponded to **7c** and the other (upper one) was supposed to be **11c**. This material was used for the next step without separation (see below).

The Conjugated Ketone 8c Compound **7c** (25 mg) in CH_2Cl_2 (2.5 ml) was treated with 15% H_2O_2–MeOH (2 drops) at 0 °C for 15 min. Work-up of the mixture gave the conjugated ketone **8c** (12 mg, 72%) as colorless prisms (from MeOH), mp 169—172 °C. IR: 1690. ^{1}H-NMR: 7.90 (1H, d, J=10 Hz, H-4), 6.53 (2H, s, ArH), 6.08 (1H, d, J=10 Hz, H-3), 4.17 (1H, m, one of H-10), 3.82 (6H, s, 2×OMe). MS: 311 (M^+), 257 (M^+-54). *Anal.* Calcd for $C_{18}H_{17}NO_4$: C, 69.44; H, 5.50; N, 4.50. Found: C, 69.62; H, 5.44; N, 4.28.

The Dimethoxyketone 9c (1) By MPC: Compound **7c** (20 mg) in MeOH (5.5 ml) was treated with MPC (79 mg, 4.0 eq) to give **9c** (16 mg, 100%) as colorless needles (from MeOH), mp 209—212 °C. IR: 1720, 1695. ^{1}H-NMR: 6.63, 6.45 (each 1H, s, ArH), 3.85, 3.80, 3.20, 3.17 (each 3H, s, OMe), 2.35 (2H, s, H-4). MS: 373 (M^+). *Anal.* Calcd for $C_{20}H_{23}NO_6$: C, 64.33; H, 6.21; N, 3.75. Found: C, 64.23; H, 6.19; N, 3.66.

(2) By TTN: Compound **7c** (22 mg) in MeOH (5 ml) was treated with TTN (82 mg, 4 eq) for 3 h at room temperature to give the dimethoxyketone **9c** (13 mg, 86%).

(3) One Pot Transformation of **1c** to **9c**: A solution of the cycloerythrinan **1c** (16 mg) and PhSeCl (19.5 mg, 2 eq) in THF (2 ml) was stirred at room temperature for 48 h. The solvent was evaporated off and the residue in MeOH (4 ml) was treated with MPC (150 mg, 6 eq) at room temperature for 1 h. Work-up of the mixture as described above gave the dimethoxyketone **9c** (13 mg, 68%).

The 2,3,8-Trioxo Derivative 10c The dimethoxyketone **9c** (19 mg) in acetone (3.5 ml) was heated with 5% HCl (3.0 ml) at 70 °C for 2 h. The product was taken up into $CHCl_3$ and worked up as usual to give the trioxo derivative **10c** (16 mg, 96%) as yellow crystals, mp 256—259 °C. IR: 1720, 1700. ^{1}H-NMR: 6.78, 6.63 (each 1H, s, ArH), 4.14 (1H, m, one of H-10), 3.93, 3.89 (each 3H, s, OMe). MS: 327 (M^+), 257 (M^+-70). *Anal.* Calcd for $C_{18}H_{17}NO_5$: C, 66.05; H, 5.24; N, 4.28. Found: C, 66.12; H, 5.20; N, 4.43.

The Dimethoxy-alcohol 21a The dimethoxyketone **9a** (120 mg) in MeOH (10 ml) was reduced with $NaBH_4$ (15 mg) to give the alcohol **21a** (100 mg, 83%) as colorless needles (from CH_2Cl_2–hexane), mp 189—191 °C. IR: 3320, 1715, 1675. ^{1}H-NMR: 6.68, 6.45 (each 1H, s, ArH), 4.52 (1H, br s, CHOH), 3.63 (6H), 3.33 (3H), 3.20 (3H) (each s, OMe), 0.88 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$). MS: 447 (M^+). *Anal.* Calcd for $C_{23}H_{29}NO_8$: C, 61.73; H, 6.53; N, 3.13. Found: C, 61.77; H, 6.41; N, 3.01.

On acetylation with pyridine–Ac_2O, **21a** gave the acetate as colorless needles (from MeOH), mp 230—232 °C. IR: 1745, 1730, 1690. ^{1}H-NMR: 6.83, 6.38 (each 1H, s, ArH), 5.35 (1H, m, CHOAc), 3.78 (6H), 3.35 (3H), 3.13 (3H) (each s, OMe), 0.97 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$). MS: 489 (M^+).

One-Pot Transformation of 1a to the Dimethoxy-alcohol 21a Compound **1a** (500 mg), PhSeCl (375 mg, 1.5 eq), and $BF_3\cdot Et_2O$ (4 drops) in THF (25 ml) were heated under reflux for 3 h under an Ar atmosphere, then concentrated *in vacuo*. The residue was dissolved in MeOH (60 ml), MPC (1.8 g) was added at 0 °C, and the mixture was stirred for 1.5 h at room temperature. After further addition of MPC (0.6 g) and stirring overnight, the mixture was separated by filtration into the precipitate and the filtrate. The filtrate was treated with Na_2S until the solution became faintly alkaline and the resultant black precipitate was removed by filtration with the aid of Celite. The filtrate was diluted with water and extracted with $CHCl_3$. Chromatography of the product from the $CHCl_3$ extract gave crude **9a** (508 mg) from the benzene–acetone (4:1) eluate; this product was dissolved in MeOH (40 ml) and reduced with $NaBH_4$ (44 mg) for 1.5 h at 0 °C. The mixture was acidified with 1 N HCl to pH 4 and extracted with CH_2Cl_2. Concentration of the dried extract gave **21a**, which was crystallized from MeOH–Et_2O (281 mg, 48%). The first precipitate gave **17a** (46 mg, 9%) on decomposition by treatment with Na_2S in MeOH.

Transesterification of 21a to the Methyl Ester 21b Compound **21a** (305 mg) was dissolved in dry MeOH (15 ml) and treated with 10% NaOMe–MeOH (15 ml) for 3 h at room temperature. The separated crystals were collected by filtration to give **21b** (184 mg). The filtrate was neutralized with Amberlite IRC-50 (H^+) and concentrated to give a residue which was crystallized from MeOH to give a further crop of **21b** (89 mg). Total yield: 273 mg (92%).

21b: Colorless prisms (from MeOH), mp 211—213 °C. IR: 1725, 1670, ^{1}H-NMR: 6.78, 6.56 (each 1H, s, ArH), 4.56 (1H, br d, J=6 Hz, H-2), 3.86, 3.84, 3.40, 3.38, 3.24 (each 3H, s, OMe). MS: 433 (M^+). *Anal.* Calcd for $C_{22}H_{27}NO_8\cdot 1/2H_2O$: C, 59.73; H, 6.33; N, 3.17. Found: C, 59.99; H, 6.15; N, 3.13.

The Dimethoxy-alcohol 21c The dimethoxyketone **9c** (100 mg) in MeOH (5 ml) was reduced with $NaBH_4$ (30 mg) for 2 h at room temperature to give the alcohol **21c** (97 mg, 96%) as colorless needles (from benzene), mp 122—124 °C. IR: 3400, 1658. ^{1}H-NMR: 6.67, 6.55 (each 1H, s, ArH), 4.50 (1H, d, J=11 Hz, H-2), 3.90, 3.86, 3.33, 3.26 (each 3H, s, OMe). MS: 375 (M^+), 258 (base peak). *Anal.* Calcd for $C_{20}H_{25}NO_6$: C, 63.98; H, 6.71; N, 3.73. Found: C, 63.70; H, 6.81; N, 3.79.

The Dithiocarbonate 22b A mixture of the alcohol **21b** (500 mg), NaH (60% oil dispersion, 291 mg), and a catalytic amount of imidazole (20 mg) in THF (250 ml) was heated under reflux for 4 h under an Ar atmosphere. Carbon disulfide (3 ml) and iodomethane (3 ml) were added successively, and the mixture was heated for a further 40 min, then concentrated to *ca.* 1/3 volume. The mixture was brought to pH 8 by addition of AcOH and water, and extracted with $CHCl_3$. Concentration of the extract and chromatography of the residue gave, from the $CHCl_3$–AcOEt (5:1) eluate, **22b** (490 mg, 81%) as colorless needles (from MeOH), mp 205—206 °C. IR: 1730, 1690. ^{1}H-NMR: 7.00, 6.54 (each 1H, s, ArH), 6.18 (1H, d, J=6 Hz, H-2), 3.87, 3.86, 3.46, 3.44, 3.18 (each 3H, s, OMe), 2.54 (3H, s, SMe). MS: 523 (M^+). *Anal.* Calcd for $C_{24}H_{29}NO_8S_2$: C, 55.05; H, 5.58; N, 2.67. Found: C, 54.76; H, 5.59; N, 2.65.

This product was hydrolyzed by 3% HCl to the keto-dithiocarbonate (gum). IR ($CHCl_3$): 1733, 1725, 1715. ^{1}H-NMR: 6.87, 6.58 (each 1H, s, ArH), 6.70 (1H, d, J=7 Hz, H-2), 3.86 (6H), 3.60 (3H) (each s, OMe), 2.56 (3H, s, SMe). MS: 477 (M^+).

15,16-Dimethoxy-6β-methoxycarbonyl-3,8-dioxo-Δ^1-erythrinan (25b=3) The dithiocarbonate **22b** (203 mg) in toluene (200 ml) was added dropwise to a hot stirred solution of tributyltin hydride (Bu_3SnH, 0.5 ml, 5 eq) and azobisisobutyronitrile (AIBN) (catalytic amount) in toluene (50 ml) under an Ar atmosphere, and the mixture was refluxed for a further 4 h. The cooled mixture was poured onto a silica gel column and the column was washed with benzene to remove tin compounds. Elution of the column with $CHCl_3$ and $CHCl_3$–AcOEt (1:1) gave **23b** (204 mg) as a gum, which was used for the next step without further purification. An analytical sample was crystallized from ether as colorless prisms, mp 194—196 °C. IR: 1740, 1695. ^{1}H-NMR: 6.66, 6.20 (each 1H, s, ArH), 6.59, 6.01 (each 1H, d, J=10 Hz, H-1 and H-2), 3.50, 3.36, 2.96, 2.94, 2.80 (each 3H, s, OMe). *Anal.* Calcd for $C_{22}H_{27}NO_7$: C, 63.30; H, 6.52; N, 3.36. Found: C, 63.30; H, 6.70; N, 3.18.

Compound **23b** (204 mg) was hydrolyzed with 2% HCl (10 ml)–acetone (40 ml) at 50 °C for 1 h to give the conjugated ketone **25b** (=**3**) (139 mg, 97% from **22b**), as colorless prisms (from ether), mp 206—207 °C. IR: 1730, 1700, 1680. ^{1}H-NMR: 7.24, 6.38 (each 1H, d, J=12 Hz, H-1 and H-2), 6.54, 6.48 (each 1H, s, ArH), 3.82, 3.68, 3.26 (each 3H, s, OMe). MS: 371 (M^+). *Anal.* Calcd for $C_{20}H_{21}NO_6$: C, 64.68; H, 5.70; N, 3.77. Found: C, 64.57; H, 5.76; N, 3.90.

Transformation of 21a to 6β-Ethoxycarbonyl-15,16-Dimethoxy-3,8-dioxo-Δ^1-erythrinan (25a) The ethyl ester **21a** (39 mg) was similarly converted to the dithiocarbonate **22a** (36 mg, 77%), colorless needles (from MeOH), mp 217—218 °C. IR: 1735, 1690. ^{1}H-NMR: 7.00, 6.53 (each 1H, s, ArH), 6.18 (1H, m, H-2), 3.86 (6H), 3.44 (3H), 3.20 (3H) (each s, OMe), 1.00 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$). MS: 537 (M^+). This was treated with Bu_3SnH to convert it into the olefin **23a** (18.6 mg, 96%), colorless prisms (from MeOH), mp 182—183 °C. IR: 1730, 1690. ^{1}H-NMR: 6.82, 6.48 (each 1H, s, ArH), 6.69, 6.13 (each 1H, d, J=10 Hz, H-1 and H-2), 3.82, 3.76, 3.48, 3.20 (each 3H, s, OMe), 0.86 (3H, t, J=8 Hz, $COOCH_2C\underline{H}_3$). MS: 431 (M^+). In the $CDCl_3$ solution, **23a** changed gradually into **24a**. ^{1}H-NMR: 6.82, 6.48 (each 1H, s, ArH), 6.33 (1H, d, J=10 Hz, H-1), 5.95 (1H, dd, J=10, 2 Hz, H-2), 4.72 (1H, d, J=2 Hz, H-4), 3.82, 3.76, 3.54 (each 3H, s, OMe), 0.86 (3H, t, J=8 Hz, $COOCH_2C\underline{H}_3$). This was hydrolyzed as above to the conjugated ketone **25a** (96% from **23a**), colorless prisms (from ether), mp 150—151 °C. ^{1}H-NMR: 7.26 (1H, d, J=12 Hz, H-1), 6.54, 6.50 (each 1H, s, ArH), 6.38 (1H, d, J=12 Hz, H-2), 3.82, 3.68 (each 3H, s, OMe), 0.84 (3H, t, J=8 Hz, $COOCH_2C\underline{H}_3$). MS: 385 (M^+). *Anal.* Calcd for $C_{21}H_{23}NO_6$: C, 65.44; H, 6.02; N, 3.63. Found: C, 65.61; H, 5.89; N, 3.62.

Transformation of 21c to 15,16-Dimethoxy-3,8-dioxo-Δ^1-6βH-erythrinan (25c) The alcohol **21c** (390 mg) was converted to the dithiocarbonate **22c** as described above. This was heated with AIBN (15 mg) and Bu_3SnH (2.5 ml) in toluene (50 ml) at 90 °C for 1 h under an Ar atmosphere.

Chromatography of the product gave **23c** as an oil, which was dissolved in acetone (25 ml) and treated with 2% HCl (25 ml) for 10 min at room temperature to give **25c** (220 mg, 68% from **21c**) as colorless needles (from CH_2Cl_2–MeOH), mp 176—178 °C. IR: 1690, 1675. ^{1}H-NMR: 7.00 (1H, dd, J=10.5, 4.5 Hz, H-1), 6.61, 6.55 (each 1H, s, ArH), 6.26 (1H, dd, J=10.5, 1.5 Hz, H-2), 3.82, 3.72 (each 3H, s, OMe). MS: 313 (M^+). *Anal.* Calcd for $C_{18}H_{19}NO_4$: C, 68.99; H, 6.11; N, 4.47. Found: C, 69.18; H, 6.29; N, 4.52.

Decarbomethoxylation of the Vinylogous β-Keto Ester (25b) (1) Effect of Additives (RSH): A mixture of **25b** (10 mg), $CaCl_2$ (24 mg, 8 eq) and RSH (1 drop) in DMSO (1 ml) was heated in a sealed tube at 140 °C for 1—3 h. The mixture was diluted with water, acidified with HCl, and extracted with $CHCl_3$. The product was separated by preparative TLC ($CHCl_3$–acetone = 10 : 1) to give the results summarized in Table II.

(2) $CaCl_2$–DMSO–*tert*-HepSH Method: A mixture of **25b** (238 mg), $CaCl_2$ (572 mg, 8 eq), and 3-ethylpentane-3-thiol (1.15 ml) in DMSO (23 ml) was heated at 140 °C for 3.3 h. After work-up as above, the product was chromatographed to give, from the benzene–acetone (5 : 1) eluate, the enone **4** (136 mg, 68%) as colorless needles (from MeOH), mp 180—184 °C. IR: 1710, 1680. ^{1}H-NMR: 6.56, 6.30 (each 1H, s, ArH), 6.06 (1H, br s, H-1), 3.82, 3.74 (each 3H, s, OMe). MS: 313 (M^+). *Anal.* Calcd for $C_{18}H_{19}NO_4$: C, 68.99; H, 6.11; N, 4.47. Found: C, 68.86; H, 6.18; N, 4.51.

(3) $MgCl_2$–DMSO–EtSH Method: A mixture of **25b** (100 mg), $MgCl_2$ (210 mg, 8 eq) and EtSH (1 ml) in DMSO (10 ml) was heated at 140 °C for 2 h and worked up as above to give **4** (24 mg, 29%) and the isomeric enone **25c** (22 mg, 26%).

The EtSH Adducts Two EtSH adducts, **27a** and **27b**, were obtained in the reaction of **25b** (160 mg) with $CaCl_2$–DMSO–EtSH at 140 °C for 2 h together with **4** (44 mg, 32%) and the starting material **25b** (53 mg). **27a** (R = Et): yield, 33 mg (17%). Colorless gum. ^{1}H-NMR: 6.52, 6.47 (each 1H, s, ArH), 3.84 (6H), 3.24 (3H) (each s, OMe), 2.72 (2H, q, J=7.5 Hz, $SC\underline{H}_2CH_3$), 1.28 (3H, t, J=7.5 Hz, $SCH_2C\underline{H}_3$). MS: 433 (M^+). **27b** (R = Et): yield, 1 mg (0.5%). Colorless gum. IR ($CHCl_3$): 1720, 1690. ^{1}H-NMR: 6.60, 6.54 (each 1H, s, ArH), 3.86, 3.85 (each 3H, s, OMe), 2.67 (2H, q, J=7.5 Hz, $SC\underline{H}_2CH_3$), 1.34 (3H, t, J=7.5 Hz, $SCH_2C\underline{H}_3$). MS: 375 (M^+).

Isomerization of the Enone 4 to the Conjugated Ketone 25c A mixture of the enone **4** (10 mg) and DBU (5 drops) in benzene (1 ml) was heated in a sealed tube at 100 °C for 1.5 h. The mixture was washed with 2% HCl and concentrated to give the conjugated ketone **25c** (10 mg, 100%).

The Dienone 30 (1) A mixture of the enone **4** (57 mg) and DDQ (288 mg, 7 eq) in dioxane (4 ml) was heated in a sealed tube at 110 °C for 5 h. The mixture was taken up into $CHCl_3$, washed with 1 N NaOH and concentrated. Chromatography of the residue gave, from the $CHCl_3$–acetone (5 : 1) eluate, the dienone **30** (16 mg, 29%) and a trienone (8 mg, 15%).[11]

(2) A mixture of the enone **4** (21 mg) and pyridinium hydrobromide perbromide (32 mg, 1.5 eq) in $CHCl_3$ (2.5 ml) was stirred for 1.5 h at room temperature. Addition of water, acidification with HCl, and extraction with $CHCl_3$ of the mixture gave, after concentration, a gummy residue, which was heated with DBU (60 mg) in benzene (4 ml) under reflux for 1 h under an Ar atmosphere. The cooled mixture was washed with HCl and concentrated. Chromatography of the product gave the dienone **30** (8 mg, 39%) as colorless prisms (from MeOH–ether), mp 192—194 °C. This product was identical with the authentic specimen described in a previous paper.[12]

Reduction of 25c The conjugated ketone **25c** (220 mg) and $CeCl_3 \cdot 7H_2O$ (1.4 g, 5.4 eq) in MeOH (10 ml) were stirred at 0 °C for 10 min, then $NaBH_4$ (378 mg) was added and the mixture was stirred for a further 1.5 h at 0 °C. The precipitated crystals (**31**, 135 mg) was collected by filtration and the filtrate, after addition of water, was extracted with $CHCl_3$. Medium pressure liquid chromatography (MPLC) of the residue from the $CHCl_3$ extract gave **32** (46 mg) and **31** (9.5 mg). Total yield: **32** (46 mg, 21%), **31** (144.5 mg, 65%).

31 (The 3α-Alcohol): Colorless needles (from MeOH), mp 285.5—287 °C. IR: 3246, 1658. ^{1}H-NMR (400 MHz, $CDCl_3$–CD_3OD): 6.66 (2H, s, ArH), 6.05 (2H, s, H-1, 2), 4.17 (1H, ddd, J=10, 5, 1 Hz, H-3), 3.99 (1H, ddd, J=13, 7, 6 Hz, H-10), 3.87, 3.78 (each 3H, s, OMe), 2.72 (1H, dd, J=16.5, 9.5 Hz, H-7), 2.40 (2H, m, H-4, 7), 1.69 (1H, dd, J=12, 10 Hz, H-4). MS: 315 (M^+), 284 (base peak). *Anal.* Calcd for $C_{18}H_{21}NO_4$: C, 68.55; H, 6.71; N, 4.44. Found: C, 68.39; H, 6.58; N, 4.28.

32 (The 3β-Alcohol): Colorless gum. IR ($CHCl_3$): 3420, 1680. ^{1}H-NMR (400 MHz): 6.69, 6.56 (each 1H, s, ArH), 5.95 (1H, dd, J=10, 1 Hz, H-2), 5.76 (1H, ddd, J=10, 4, 2 Hz, H-1), 4.29 (1H, m, H-3), 4.20 (1H, ddd, J=13.5, 7.5, 2 Hz, H-10), 3.86, 3.85 (each 3H, s, OMe), 3.26 (1H, ddd, J=13.5, 11.5, 5.5 Hz, H-10), 3.18 (1H, ddd, J=9.5, 4, 3 Hz, H-6), 3.07 (1H, ddd, J=16.5, 11.5, 5.5 Hz, H-11), 2.61 (1H, ddd, J=16.5, 5.5, 2 Hz, H-11), 2.55 (1H, dd, J=17, 9.5 Hz, H-7), 2.47 (1H, ddd, J=14, 9.5, 5 Hz, H-4e), 2.17 (1H, dd, J=17, 3 Hz, H-7), 1.89 (1H, dd, J=14, 9.5 Hz, H-4a). MS: 315 (M^+), 284 (base peak). *Anal.* Calcd for $C_{18}H_{21}NO_4$: C, 68.55; H, 6.71; N, 4.44. Found: C, 68.53; H, 6.80; N, 4.59.

3α,15,16-Trimethoxy-8-oxo-Δ^1-*cis*-erythrinan (33) A mixture of **31** (200 mg), NaH (60% oil dispersion, 508 mg), and a catalytic amount of imidazole in dioxane (50 ml) was heated under reflux for 1 h, then n-Bu_4NHSO_4 (215 mg) and iodomethane (3 ml) were added and the mixture was stirred for 3 h at 70 °C. The cooled mixture was acidified with 2% HCl, and extracted with $CHCl_3$. Concentration of the extract and chromatography of the residue gave **33** (190 mg, 91%) as colorless prisms (from MeOH–ether), mp 149—151 °C. IR: 1675. ^{1}H-NMR: 6.66 (2H, s, ArH), 6.09 (2H, s, H-1 and H-2), 3.87, 3.79, 3.33 (each 3H, s, OMe). MS: 329 (M^+). *Anal.* Calcd for $C_{19}H_{23}NO_4$: C, 69.28; H, 7.04; N, 4.25. Found: C, 69.02; H, 6.95; N, 4.32.

(±)-Erysotramidine (8-Oxoerysotrine, 5) Compound **33** (86 mg) in THF (6 ml) was treated with LDA (2.4 eq) in THF (2 ml) at −78 °C for 30 min under an Ar atmosphere, then $(PhSe)_2$ (180 mg) was added and the mixture was stirred for 20 min. The mixture was acidified with 1 N HCl and extracted with CH_2Cl_2. Concentration of the extract gave a gum, which was dissolved in MeOH–water and treated with $NaIO_4$ (560 mg) for 30 min at room temperature. Purification of the product by chromatography gave the dienoid lactam **5** (81 mg, 95%) as a gum, which was identical with (±)-erysotramidine.[12]

(±)-Erysotrine (6) Reduction of **5** in a manner previously reported[12] gave (±)-erysotrine (**6**).

Acknowledgement The authors thank Prof. Makoto Suzuki, Meijo University, for measurement of desorption chemical ionization mass spectra, and Mr. Natsuo Okada for his assistance in some of the experiments.

References and Notes

1) Intramolecular Cyclization Approach. (3). Part XXIII (2): Y. Tsuda, Y. Sakai, A. Nakai, T. Ohshima, S. Hosoi, K. Isobe, and T. Sano, *Chem. Pharm. Bull.*, **38**, 2136 (1990).
2) Y. Tsuda, Y. Sakai, A. Nakai, M. Kaneko, Y. Ishiguro, K. Isobe, J. Taga, and T. Sano, *Chem. Pharm. Bull.*, **38**, 1462 (1990).
3) Preliminary communication: Y. Tsuda, S. Hosoi, A. Nakai, T. Ohshima, Y. Sakai, and F. Kiuchi, *J. Chem. Soc., Chem. Commun.*, **1984**, 1216.
4) For example, see K. B. Sharpless, R. F. Lauer, and A. Y. Teranishi, *J. Am. Chem. Soc.*, **95**, 6137 (1973).
5) Such α-chloro-α-phenylselenenyl ketones have previously been prepared by the action of PhSeCl on diazoketones: D. J. Buckley, S. Kulkowit, and A. McKervey, *J. Chem. Soc., Chem. Commun.*, **1980**, 506.
6) Y. Tsuda and S. Hosoi, *Chem. Pharm. Bull.*, **33**, 1745 (1985).
7) An analogous reaction of phenylsulfenylketones was reported: Y. Nagao, K. Kaneko, and E. Fujita, *Tetrahedron Lett.*, **1978**, 4115.
8) For a review of 1,2-carbonyl transposition, see V. V. Kane, V. Singh, A. Martin, and D. L. Doyle, *Tetrahedron*, **39**, 345 (1983).
9) Y. Tsuda and Y. Sakai, *Synthesis*, **1981**, 118.
10) See also a footnote in Y. Tsuda, S. Hosoi, T. Ohshima, S. Kaneuchi, M. Murata, F. Kiuchi, J. Toda, and T. Sano, *Chem. Pharm. Bull.*, **33**, 3574 (1985).
11) For DDQ oxidation of erythrinan enones, see Y. Tsuda, F. Kiuchi, S. Hosoi, T. Sano, J. Toda, and R. Yamamoto, *Heterocycles*, **22**, 2255 (1984).
12) *a*) T. Sano, J. Toda, and Y. Tsuda, *Heterocycles*, **18**, 229 (1982); *b*) T. Sano, J. Toda, K. Kashiwaba, T. Ohshima, and Y. Tsuda, *Chem. Pharm. Bull.*, **35**, 479 (1987).
13) See also Y. Tsuda, S. Hosoi, and M. Murata, *Heterocycles*, **30**, 311 (1990).

Studies on Angiotensin Converting Enzyme Inhibitors. V.[1] The Diastereoselective Synthesis of 2-Oxoimidazolidine Derivatives[2]

Hitoshi KUBOTA,[a] Ken-ichi NUNAMI,[a] Masafumi YAMAGISHI,[b] Shigeru NISHIMOTO,[b] and Kimiaki HAYASHI*,[a]

Research Laboratory of Applied Biochemistry[a] and Pharmaceutical Technics Division,[b] Tanabe Seiyaku Co., Ltd., 16–89 Kashima-3-chome, Yodogawa-ku, Osaka 532, Japan. *Received October 15, 1990*

A diastereoselective synthesis of imidapril (1), which is under clinical study as an antihypertensive drug based on its angiotensin converting enzyme (ACE)-inhibitory activity, was established. *N*-Alkylation of (2*S*)-2-amino-4-phenylbutyric acid ester (12) with 3-((2*R*)-2-methane or toluenesulfonyloxypropionyl)-2-oxoimidazolidine derivative (11) diastereoselectively proceeded in an *SN*2 fashion to afford *tert*-butyl (4*S*)-3-[(2*S*)-2-[*N*-[(1*S*)-1-ethoxycarbonyl)-3-phenylpropyl]amino]propionyl]-1-methyl-2-oxoimidazolidine-4-carboxylate (13), a precursor of 1. Alternatively, benzyl (2*S*)-2-[*N*-[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propionate (15), which is the key building block of 13, was synthesized by the same strategy. This procedure was also applied to the synthesis of enalapril.

Keywords ACE inhibitor; imidapril; enalapril; racemization; diastereoselective synthesis

Since the discovery that angiotensin converting enzyme (ACE) inhibitors can be effective antihypertensive agents for the treatment of essential and renal hypertension, several synthetic inhibitors have been reported.[3] In our series of synthetic studies on ACE inhibitors, we previously reported that the 2-oxoimidazolidine derivative (**1**), which has (*S,S,S*)-configuration,[4] possesses potent and long-lasting ACE-inhibitory and antihypertensive activities.[1] It was designated as imidapril[5] and has been undergoing clinical trials to evaluate its efficacy in the treatment of hypertension.

In the previous paper,[1] we retro-synthetically divided **1** into three units (A, B and C). As a starting material for the A-unit, (4*S*)-2-oxoimidazolidine-4-carboxylic acid (**2**), which was readily prepared from L-asparagine according to a known procedure,[6] was used. To obtain the C-unit, (2*S*)-2-amino-4-phenylbutyric acid (**6**) was prepared by enantioselective deacylation of the corresponding *N*-acetyl-D,L-amino acid using aminoacylase. We have recently established a more advantageous synthetic procedure for **6** by enzymatic transamination of 2-oxo-4-phenylbutyric acid (**5**) by the use of *Paracoccus denitrificans* with L-aspartic acid as an amino group donor.[7] Meanwhile, 2-bromopropionic acid was adopted as a source of the B-unit. However, *N*-alkylation of the optically active amino acid ester (**12**) with a bromo compound (**7** or **8**) resulted in the formation of diastereomeric mixtures ((*S,S,S*)- and (*S,R,S*)-**13**, or (*S,S*)- and (*R,S*)-**15**), even when optically pure (*S,R*)-**7** or (*R*)-**8** was used. Therefore, an efficient method for stereoselective introduction of the B-unit was required as the key point in the synthesis of (*S,S,S*)-**1**. Now, we wish to report a diastereoselective preparation of (*S,S,S*)-**1** that should be suitable for industrial-scale synthesis.

First, in order to elucidate the nature of the *N*-alkylation reaction, a mixture of (*S,R*)-**7**, **12** and potassium carbonate (K_2CO_3) in hexamethylphosphoramide (HMPA) was stirred at room temperature and the reaction process was investigated periodically by high-performance liquid chromatography (HPLC). Figure 1 shows that (*S,R*)-**7** completely racemized within 5 min under the reaction conditions used, giving a mixture of (*S,R*)-**7** and (*S,S*)-**7**, while the *SN*2-type reaction proceeded much more slowly. This result prompted us to develop a new optically active B-synthon which would hardly racemize under such reaction conditions.

Recently, Iwasaki *et al.*[8] reported the diastereoselective synthesis of ACE inhibitors in which optically active ethyl 2-bromo-4-phenylbutyrate was coupled with dipeptide esters in the presence of a weak base such as ammonium

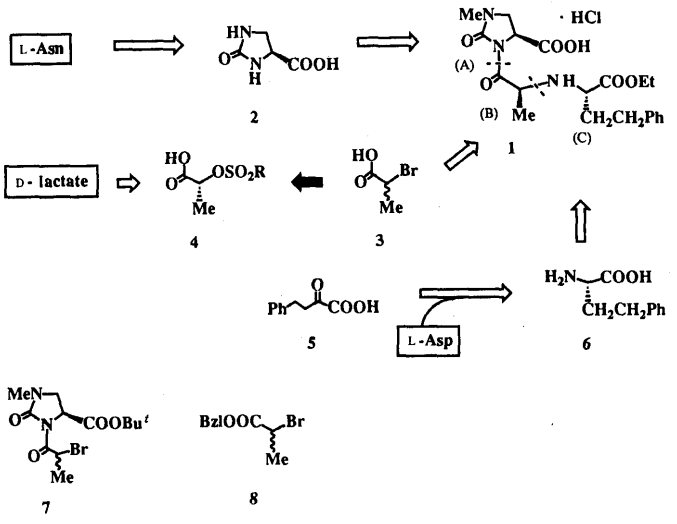


Chart 1

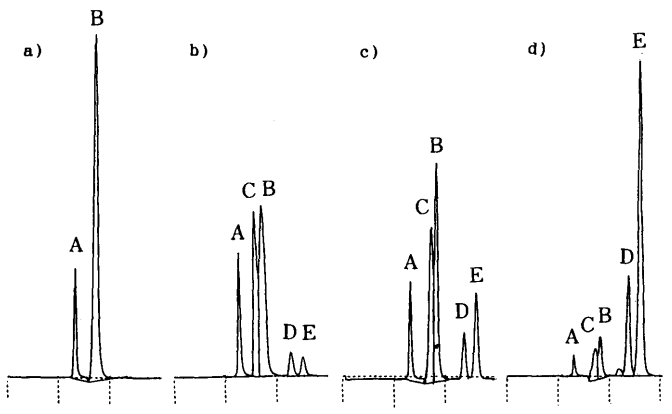


Fig. 1. HPLC Analysis of the Reaction Mixture of (*S,R*)-**7** with **12** in the Presence of K_2CO_3

a) initial; b) 5 min; c) 1 h; d) 24 h. A, **12**; B, (*S,R*)-**7**; C, (*S,S*)-**7**; D, (*S,S,S*)-**13**; E, (*S,R,S*)-**13**.

carbonate by an S_N2 process. However, a small amount of the undesired diastereoisomer was still formed, since the racemization of the bromo compound was not suppressed completely. On the other hand, Effenberger *et al.*[9] prepared several optically active *N*-substituted amino acid esters from 2-trifluoromethanesulfonyloxyalkanoic acid esters and amines. As the reaction proceeded with complete inversion of triflate, this method was supposed to be useful for the diastereoselective synthesis of (*S*,*S*,*S*)-**1**. However, triflates are too unstable and expensive to be used on an industrial scale.

Taking the above information into consideration, we chose (2*R*)-2-(4-toluenesulfonyloxy)- or (2*R*)-2-methanesulfonyloxypropionic acid (**4**) as the B-unit synthon. The optically pure tosylate or mesylate derivative (**11**) was synthesized as shown in Chart 2. Namely, the carboxylic acid (**4**) was prepared from D-lactate (**9**) according to the known procedure.[10] Compound **4** was converted to the acid chloride and condensed with a 2-oxoimidazolidine derivative (**10**) in the presence of potassium *tert*-butoxide (*tert*-BuOK) in tetrahydrofuran (THF), affording (*S*,*R*)-**11** in a good yield.

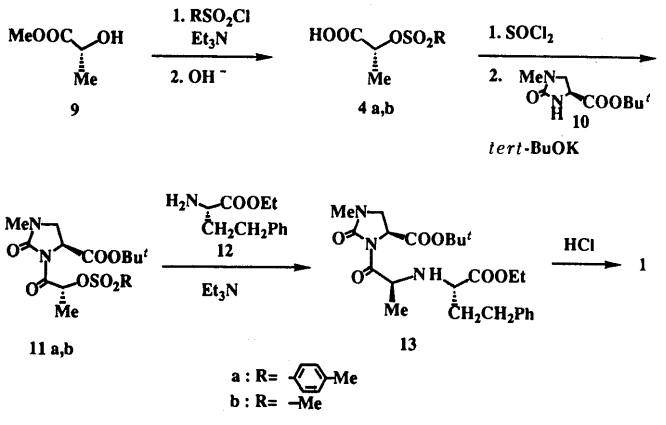


Chart 2

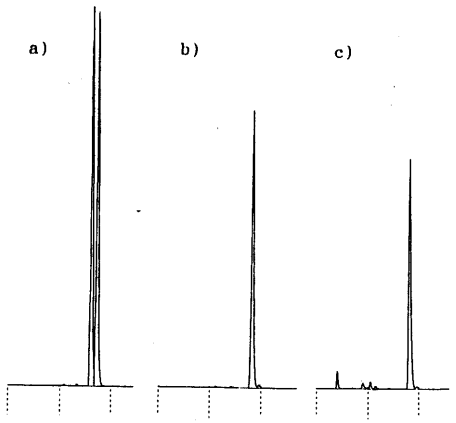


Fig. 2. HPLC Analysis of **11a** in Base-Solvent Systems

a) (*S*,*R*)-**11a**+(*S*,*S*)-**11a**; b) (*S*,*R*)-**11a**–K_2CO_3–HMPA, 25 °C, 24 h; c) (*S*,*R*)-**11a**–Et_3N–DMSO, 70 °C, 24 h.

TABLE I

$$(S,R)\text{-}\mathbf{11a}+(S)\text{-}\mathbf{12} \xrightarrow[\text{DMSO}]{\text{base}} (S,S,S)\text{-}\mathbf{13}$$

Run	**12** (eq)[a]	Base (eq)[a]	Temp. (°C)	Time (h)	Yield (%)[b]
1	1.0	K_2CO_3 (2.0)	60	5	27
2	1.0	K_2CO_3 (2.0)	60	24	39
3	1.0	Et_3N (1.5)	80	30	61
4	1.5	Et_3N (1.5)	80	24	85
5	1.5	Et_3N (2.0)	80	24	80

a) Molar equivalents to **11a**. *b*) Isolation yield.

The racemization tendency of (*S*,*R*)-**11** in several base-solvent systems was investigated by HPLC in the same manner as described in the case of the bromo compound ((*S*,*R*)-**7**) (Fig. 2). No racemization of (*S*,*R*)-**11a** was observed when K_2CO_3 in HMPA at 25°C or heating in the presence of triethylamine (Et_3N) in dimethyl sulfoxide (DMSO) was used. This result showed that the sulfonate derivative (**11**) was clearly less sensitive to racemization under weakly basic conditions than the bromide (**7**).

Next, the reaction conditions for *N*-alkylation of **12** with (*S*,*R*)-**11a** were optimized. The results are summarized in Table I. When the reaction was carried out in the presence of K_2CO_3 at room temperature, only the starting materials were recovered. Under heating, the reaction was accompanied with the decomposition of **11a**, resulting in a low yield of expected diester derivative (**13**). Interestingly, the employment of Et_3N instead of K_2CO_3 drastically changed the products. Thus, the reaction smoothly proceeded in the presence of 1.5 eq of Et_3N under heating at 80 °C for 24 h to afford the desired (*S*,*S*,*S*)-**13** exclusively in 85% yield (Run 4). The reaction of the mesylate derivative (**11b**) with **12** under the same reaction conditions also afforded (*S*,*S*,*S*)-**13**. Removal of the *tert*-butyl group of **13** by treatment with anhydrous hydrogen chloride gave the target compound (**1**).

Next, an alternative method for the preparation of **1** by using optically active tosylate was established in a similar manner (Chart 3). Namely, benzyl (2*R*)-2-(4-toluenesulfonyloxy)propionate (**14**) was submitted to the substitution reaction with **12** in the presence of Et_3N in DMSO to afford

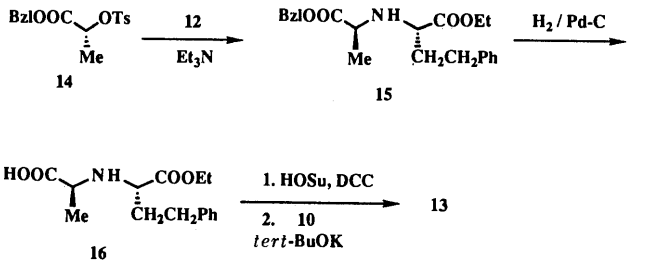


Chart 3

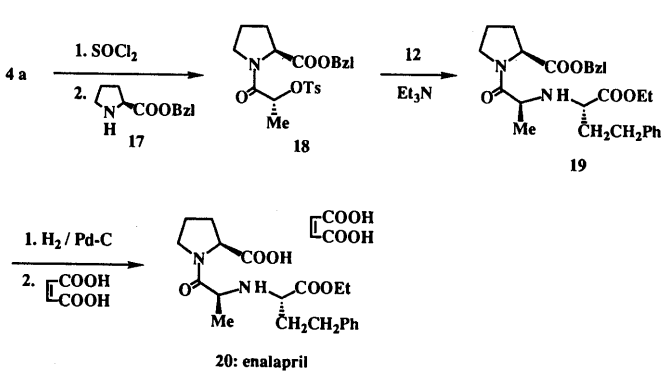


Chart 4

a single diastereoisomer, (*S,S*)-**15**, in 75% yield. Compound **15** was converted to (*S,S,S*)-**13** according to the procedure reported by us.[1)]

Furthermore, this method was extended to the synthesis of enalapril (**20**)[3c)] (Chart 4). The tosylate derivative (**18**), which was obtained from L-proline benzyl ester (**17**), was coupled with **12** under the same reaction conditions, predominantly giving (*S,S,S*)-**19** in 76% yield. The removal of the benzyl group of **19** by hydrogenolysis afforded enalapril.

As described above, we have established practical methods to synthesize the 2-oxoimidazolidine derivative (**1**) having (*S,S,S*) configuration diastereoselectively. Moreover, we have demonstrated that these methods can also be applied to the synthesis of other ACE inhibitors.

Experimental

Melting points (mp) are uncorrected. Infrared (IR) spectra were obtained on a Shimadzu IR-420 spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Hitachi R-40 or a Bruker AC-200 instrument, using tetramethylsilane as an internal standard. Mass spectra (MS) were taken on a Hitachi M-60 mass spectrometer. Specific rotations were measured with a Perkin-Elmer 243 polarimeter. HPLC was done on a Shimadzu LC-6A instrument equipped with an ultraviolet detector (SPD-6A, Shimadzu) and a computing integrator (CR-4A, Shimadzu). For silica gel column chromatography, Kieselgel 60 (0.063—0.20 mm, E. Merk) was employed.

HPLC Analysis of the Reaction Mixture of (*S,R*)-7 A mixture of (*S,R*)-**7** (335 mg, 1 mmol), **12** (207 mg, 1 mmol) and K_2CO_3 (138 mg, 1 mmol) in HMPA (1 ml) was stirred at 25 °C. At appropriate time intervals, a portion of the reaction mixture was taken and added to a mixture of Et_2O and H_2O, and the organic layer was analyzed by HPLC. Column, Nucleosil $5C_{18}$ 4.6 × 150 mm (Chemco); eluent, 0.05 N KH_2PO_4–CH_3CN (30 : 70); flow rate, 0.34 ml/min; column temperature, 40 °C; detection, 220 nm.

HPLC Analysis of 11a in a Base-Solvent System A mixture of (*S,R*)-**11** (86 mg, 0.2 mmol) and K_2CO_3 (28 mg, 0.2 mmol) in HMPA (0.2 ml), or (*S,R*)-**11** (200 mg, 0.46 mmol) and Et_3N (94 mg, 0.93 mmol) in DMSO (0.2 ml) was stirred at 25 or 70 °C for 24 h. The reaction mixture was partitioned with Et_2O and H_2O, and the organic layer was analyzed by HPLC. Column, Nucleosil $5C_{18}$ 4.6 × 150 mm (Chemco); eluent, 65% CH_3CN; flow rate, 0.56 ml/min; column temperature, 40 °C; detection, 220 nm.

(2*R*)-2-(4-Toluenesulfonyloxy)propionic Acid (4a) *p*-Toluenesulfonyl chloride (28.7 g, 0.15 mol) was added portionwise to a mixture of **9** (15.6 g, 0.15 mol) and Et_3N (15.2 g, 0.15 mol) in CH_2Cl_2 (100 ml) at 0—5 °C. After being stirred at room temperature overnight, the reaction mixture was washed successively with cold water, 10% HCl, saturated aqueous $NaHCO_3$, and brine, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was purified by column chromatography on silica gel with hexane–AcOEt (2 : 1) to give methyl (2*R*)-2-(4-toluenesulfonyloxy)propionate (25.5 g, 66%) as a colorless oil. A solution of the above product (12.9 g, 0.05 mol) in 10% NaOH (40 ml) was stirred at 5—10 °C for 15 min and then at room temperature for 45 min. The reaction mixture was washed with CH_2Cl_2, acidified with 1 N HCl, and extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The resulting residue was triturated with hexane to give crude crystals (11.3 g, 93%). Recrystallization from hexane–Et_2O gave pure **4a** as colorless needles. mp 106—108 °C. $[\alpha]_D^{25}$ +47.7° (*c* = 5.1, $CHCl_3$). IR (Nujol): 1730, 1595 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.53 (3H, d, *J* = 7 Hz), 2.45 (3H, s), 4.97 (1H, q, *J* = 7 Hz), 7.35 (2H, d, *J* = 9 Hz), 7.81 (2H, d, *J* = 9 Hz), 9.62 (1H, s). MS *m/z*: 244 (M^+). *Anal.* Calcd for $C_{10}H_{12}O_5S$: C, 49.17; H, 4.95; S, 13.13. Found: C, 49.12; H, 4.77; S, 13.27.

(2*R*)-2-Methanesulfonyloxypropionic Acid (4b) By the same procedure as described for the preparation of **4a**, **4b** was obtained in 63% yield as colorless needles. mp 71—74 °C. $[\alpha]_D^{25}$ +58.9° (*c* = 2, $CHCl_3$). IR (Nujol): 1715 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.66 (3H, d, *J* = 7 Hz), 3.13 (3H, s), 5.16 (1H, q, *J* = 7 Hz), 9.72 (1H, s). MS *m/z*: 123 ($M^+ - CO_2H$). *Anal.* Calcd for $C_4H_8O_5S$: C, 28.57; H, 4.79; S, 19.06. Found: C, 28.60; H, 4.63; S, 19.34.

***tert*-Butyl (4*S*)-1-Methyl-3-[(2*R*)-2-(4-toluenesulfonyloxy)propionyl]-2-oxoimidazolidine-4-carboxylate (11a)** Thionyl chloride (1.12 g, 9.4 mmol) was added to a solution of **4a** (1.15 g, 4.7 mmol) in $CHCl_3$ (5 ml), and the mixture was heated under reflux for 3 h, then concentrated *in vacuo*. The residue was taken up in $CHCl_3$ and the solution was reconcentrated to afford crude (2*R*)-2-(4-toluenesulfonyloxy)propionyl chloride as a syrup. Potassium *tert*-butoxide (607 mg, 5.4 mmol) was added to a solution of **10** (1.08 g, 5.4 mmol) in THF (12 ml) at −50 °C. The mixture was stirred at the same temperature for 20 min, then a solution of the above acid chloride in THF (2 ml) was added. Stirring was continued at −30 °C for 20 min, and a mixed solution of AcOEt (6 ml), AcOH (320 mg), and brine (6 ml) was added to the reaction mixture. The organic layer was separated and washed successively with 5% aqueous K_2CO_3 and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel with $CHCl_3$–AcOEt (2 : 1) and the product was crystallized from hexane to give **11a** (1.48 g, 74%) as colorless needles. mp 78—80 °C. $[\alpha]_D^{25}$ −3.0° (*c* = 1, $CHCl_3$). IR (Nujol): 1750, 1735, 1690 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.46 (9H, s), 1.47 (3H, d, *J* = 7 Hz), 2.41 (3H, s), 2.87 (3H, s), 3.31 (1H, dd, *J* = 4, 9 Hz), 3.70 (1H, t, *J* = 9 Hz), 4.50 (1H, dd, *J* = 4, 9 Hz), 6.26 (1H, q, *J* = 7 Hz), 7.29 (2H, d, *J* = 7 Hz), 7.80 (2H, d, *J* = 7 Hz). *Anal.* Calcd for $C_{19}H_{26}N_2O_7S$: C, 53.51; H, 6.14; N, 6.57; S, 7.52. Found: C, 53.37; H, 6.09; N, 6.50; S, 7.44.

***tert*-Butyl (4*S*)-1-Methyl-3-[(2*R*)-2-methanesulfonyloxypropionyl]-2-oxoimidazolidine-4-carboxylate (11b)** By the same procedure as described for the preparation of **11a**, **4b** (2.50 g, 14.9 mmol) was converted to **11b** (3.95 g, 76%) as colorless needles. mp 97—100 °C. $[\alpha]_D^{25}$ −3.2° (*c* = 2, $CHCl_3$). IR (Nujol): 1740, 1700 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.46 (9H, s), 1.61 (3H, d, *J* = 7 Hz), 2.89 (3H, s), 3.02 (3H, s), 3.35 (1H, dd, *J* = 4, 10 Hz), 3.73 (1H, t, *J* = 10 Hz), 4.58 (1H, dd, *J* = 4, 10 Hz), 6.35 (1H, q, *J* = 7 Hz). *Anal.* Calcd for $C_{13}H_{22}N_2O_7S$: C, 44.56; H, 6.33; N, 8.00; S, 9.15. Found: C, 44.55; H, 6.31; N, 7.91; S, 8.82.

***tert*-Butyl (4*S*)-3-[(2*S*)-2-[*N*-[(1*S*)-1-(Ethoxycarbonyl)-3-phenylpropyl]amino]propionyl]-1-methyl-2-oxoimidazolidine-4-carboxylate (13)** A mixture of **11a** (1.00 g, 2.3 mmol), **12** (0.73 g, 3.5 mmol), and Et_3N (0.36 g, 3.5 mmol) in DMSO (1 ml) was heated under stirring at 80 °C for 24 h. After cooling, the mixture was diluted with AcOEt (10 ml) and brine (3 ml). The separated organic layer was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with $CHCl_3$–AcOEt (2 : 1) to give **13** (0.92 g, 85%) as a syrup. The product and maleic acid (0.23 g, 2.0 mmol) were dissolved in AcOEt (2 ml) by heating on a water bath. Diisopropyl ether (iso-Pr_2O) (2 ml) was added to the above solution and the mixture was allowed to stand at room temperature for 3 h. The resulting crystals were collected by filtration and washed with AcOEt–iso-Pr_2O (1 : 1) to afford **13** hydrogen maleate as colorless needles (1.09 g, 95%). mp 122—124 °C. $[\alpha]_D^{25}$ −58.2° (*c* = 1, EtOH). IR (Nujol): 3600, 3500, 1740, 1690 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.32 (3H, t, *J* = 7 Hz), 1.47 (9H, s), 1.55 (3H, d, *J* = 6.5 Hz), 2.10—2.45 (2H, m), 2.70—2.93 (2H, m), 2.90 (3H, s), 3.36 (1H, dd, *J* = 4, 9.5 Hz), 3.67—3.95 (2H, m), 4.28 (2H, q, *J* = 7 Hz), 4.68 (1H, dd, *J* = 4, 9.5 Hz), 5.25 (1H, q, *J* = 6.5 Hz), 6.30 (2H, s), 7.15—7.40 (5H, m), 9.12 (3H, br). MS *m/z*: 461 (M^+).

By the same procedure as described above, **11b** (1.0 g, 2.9 mmol) was coupled with **12** (1.04 g, 4.3 mmol) to afford **13** (1.14 g, 86%).

(4*S*)-3-[(2*S*)-2-[*N*-[(1*S*)-1-(Ethoxycarbonyl)-3-phenylpropyl]amino]propionyl]-1-methyl-2-oxoimidazolidine-4-carboxylic Acid Hydrochloride (1) The diester **13** as the hydrogen maleate (28.9 g, 50 mmol) was suspended in H_2O, basified with K_2CO_3 and extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, and evaporated to dryness under reduced pressure. The residue was dissolved in 15% HCl–dioxane solution (140 ml), and the mixture was stirred at room temperature overnight. The crystalline precipitates were collected by filtration and washed with iso-Pr_2O to afford **1** (19.2 g, 90%) as colorless needles. mp 214—216 °C (dec.). $[\alpha]_D^{20}$ −64.1° (*c* = 0.5, EtOH). IR (Nujol): 1735, 1690 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.26 (3H, t, *J* = 7 Hz), 1.57 (3H, d, *J* = 6.5 Hz), 2.04—2.35 (2H, m), 2.45—2.80 (2H, m), 2.81 (3H, s), 3.45 (1H, dd, *J* = 4, 9.5 Hz), 3.83 (1H, t, *J* = 9.5 Hz), 4.02 (1H, t, *J* = 6.5 Hz), 4.24 (2H, q, *J* = 7 Hz), 4.82 (1H, dd, *J* = 4, 9.5 Hz), 5.23 (1H, q, *J* = 6.5 Hz), 7.20—7.40 (5H, m). MS *m/z*: 405 (M^+).

Benzyl (2*S*)-2-[*N*-[(1*S*)-1-(Ethoxycarbonyl)-3-phenylpropyl]amino]propionate (15) A mixture of **14** (1.00 g, 3.0 mmol), **12** (0.62 g, 3.0 mmol), and Et_3N (0.45 g, 4.5 mmol), in DMSO (1 ml) was heated under stirring at 65 °C for 24 h. After cooling, the reaction mixture was diluted with water and extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with toluene–AcOEt (10 : 1) to give **15** (0.83 g, 75%) as a colorless oil. IR (film): 3300, 1740 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.25 (3H, t, *J* = 7 Hz), 1.33 (3H, d, *J* = 6.5 Hz), 1.72—2.10 (2H, m), 1.83 (1H, s), 2.68 (2H, t, *J* = 7 Hz), 3.33 (1H, t, *J* = 6.5 Hz), 3.44 (1H,

q, J=6.5 Hz), 4.15 (2H, q, J=7 Hz), 5.12 (2H, s), 7.10—7.40 (10H, m). Compound **15** hydrogen maleate was obtained as colorless needles by treatment with maleic acid in AcOEt–iso-Pr_2O. mp 133—134 °C. $[\alpha]_D^{24}$ +1.1° (c=1, MeOH).

***N*-[(2*R*)-2-(4-Toluenesulfonyloxy)propionyl]-L-proline Benzylester (18)** Thionyl chloride (11.91 g, 0.1 mol) was added to a solution of **4a** (4.89 g, 20 mmol) in $CHCl_3$ (20 ml), and the mixture was heated under reflux for 1 h, then concentrated *in vacuo*. The residue was taken up in $CHCl_3$ and the solution was reconcentrated. The residue was dissolved in $CHCl_3$ (50 ml) and the solution obtained was added to a mixture of **17** (4.11 g, 20 mmol) and Et_3N (3.04 g, 30 mmol) in $CHCl_3$ (100 ml) at 0 °C. After being stirred at room temperature for 30 min, the mixture was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with $CHCl_3$–AcOEt (4 : 1) to give **18** (7.08 g, 82%) as a colorless syrup. IR (film): 1750, 1670, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.45 (3H, d, J=7 Hz), 1.80—2.30 (2H, m), 2.43 (3H, s), 3.30—3.80 (2H, m), 4.35—4.50 (1H, m), 5.00—5.20 (3H, m), 7.20—7.45 (7H, m), 7.79 (2H, d, J=8 Hz).

***N*-[(1*S*)-1-(Ethoxycarbonyl)-3-phenylpropyl]-L-alanyl-L-proline Benzylester (19)** A mixture of **18** (2.16 g, 5 mmol), **12** (1.55 g, 7.5 mmol), and Et_3N (1.01 g, 10 mmol) in DMSO (2 ml) was heated under stirring at 75 °C for 8 h. After cooling, the reaction mixture was diluted with water and extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with $CHCl_3$–AcOEt (2 : 1) to give **19** (1.78 g, 76%) as a colorless syrup. IR (film): 1740, 1650 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.15—1.35 (6H, m), 1.80—2.30 (6H, m), 2.55—2.80 (2H, m), 3.24 (1H, t, J=6.5 Hz), 3.40—3.65 (3H, m), 4.17 (2H, q, J=7 Hz), 4.50—4.65 (1H, m), 5.09 (1H, d, J=12 Hz), 5.20 (1H, d, J=12 Hz), 7.10—7.30 (5H, m), 7.34 (5H, s).

***N*-[(1*S*)-1-(Ethoxycarbonyl)-3-phenylpropyl]-L-alanyl-L-ploline Hydrogen Maleate (20)** Compound **19** (1.15 g, 2.5 mmol) dissolved in EtOH (20 ml) was subjected to hydrogenolysis in the presence of 10% palladium on carbon (0.2 g) at room temperature under atmospheric pressure for 2 h. After removal of the catalyst, the filtrate was concentrated *in vacuo*. The residue and maleic acid (0.29 g, 2.5 mmol) were dissolved in AcOEt (5 ml) by heating. The mixture was allowed to stand at room temperature for 3 h. The resulting crystals were collected by filtration, washed with AcOEt, and recrystallized from acetonitrile to give **20** (1.10 g, 91%) as colorless needles. mp 148—150 °C. $[\alpha]_D^{25}$ −42.2° (c=1, MeOH). IR (Nujol): 1750, 1730, 1650 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.24 (3H, t, J=7 Hz), 1.34 (3H, d, J=7 Hz), 1.80—2.35 (6H, m), 2.45—2.85 (2H, m), 3.30—4.35 (7H, m), 7.15—7.40 (5H, m). *Anal.* Calcd for $C_{24}H_{32}N_2O_9$: C, 58.53; H, 6.55; N, 5.69. Found: C, 58.25; H, 6.42; N, 5.59.

References and Notes

1) Part IV: K. Hayashi, K. Nunami, J. Kato, N. Yoneda, M. Kubo, T. Ochiai, and R. Ishida, *J. Med. Chem.*, **32**, 289 (1989).
2) A part of this work was presented at the Meeting of the Kinki Branch of the Pharmaceutical Society of Japan, Osaka, Nov. 1989.
3) *a*) M. A. Ondetti, B. Rubin, and D. W. Cushman, *Science*, **196**, 441 (1977); *b*) D. W. Cushman, H. S. Cheung, E. F. Sabo, and M. A. Ondetti, *Biochemistry*, **16**, 5484 (1977); *c*) A. A. Patchett, E. Harris, E. W. Tristram, M. J. Wyvratt, M. T. Wu, D. Taub, E. R. Peterson, T. J. Ikeler, J. ten Broeke, L. G. Payne, D. L. Ondeyka, E. D. Thorsett, W. J. Greenlee, N. S. Lohr, R. D. Hoffsommer, H. Joshua, W. V. Ruyle, J. W. Rothrock, S. D. Aster, A. L. Maycock, F. M. Robinson, R. Hirschmann, C. S. Sweet, E. H. Ulm, D. M. Gross, T. C. Vassil, and C. A. Stone, *Nature* (London), **288**, 280 (1980); *d*) E. W. Petrillo and M. A. Ondetti, *Med. Res. Rev.*, **2**, 1 (1982); *e*) M. J. Wyvratt and A. A. Patchett, *ibid.*, **5**, 483 (1985); *f*) J. B. Kostis and E. A. DeFelke (eds.), "Angiotensin Converting Enzyme Inhibitors," Alan R. Liss, Inc., New York, 1987; *g*) H. Gavras, *Circulation*, **81**, 381 (1990).
4) In this paper, the absolute configurations of asymmetric carbons in compounds are represented in parentheses in accordance with the order in the nomenclature: (4*S*)-3-[(2*S*)-2-[*N*-[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propionyl]-1-methyl-2-oxoimidazolidine-4-carboxylic acid hydrochloride and its (4*S*),(2*R*),(1*S*)-diastereoisomer are (*S*,*S*,*S*)-**1** and (*S*,*R*,*S*)-**1**, respectively.
5) Recommended International Nonproprietary Name (r-INN).
6) T. Shiba, A. Koda, S. Kusumoto, and T. Kaneko, *Bull. Chem. Soc. Jpn.*, **41**, 2748 (1968).
7) M. Senuma, K. Nakamichi, K. Nabe, S. Nishimoto, and T. Tosa, *Appl. Biochem. Biotechnol.*, **22**, 141 (1989).
8) G. Iwasaki, R. Kimura, N. Numao, and K. Kondo, *Chem. Pharm. Bull.*, **37**, 280 (1989).
9) F. Effenberger, U. Burkard, and J. Willfahrt, *Angew. Chem., Int. Ed. Engl.*, **22**, 65 (1983).
10) Daicel Chemical Industries Ltd., Japan Kokai Tokkyo Koho, Japan. Patent 61-210049 [*Chem. Abstr.*, **106**, 66922*r* (1987)].

Structure of Kifunensine, a New Immunomodulator Isolated from an Actinomycete[1)]

Hiroshi KAYAKIRI,[a] Shigehiro TAKASE,[a] Toshihiro SHIBATA,[a] Masashi HASHIMOTO,*[,a] Toshiji TADA,[b] and Shigetaka KODA[b]

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd.,[a] 5-2-3 Tokodai, Tsukuba, Ibaraki 300-26, Japan and Analytical Research Laboratories, Fujisawa Pharmaceutical Co., Ltd.,[b] 2-1-6 Kashima, Yodogawa-ku, Osaka 532, Japan. *Received October 17, 1990*

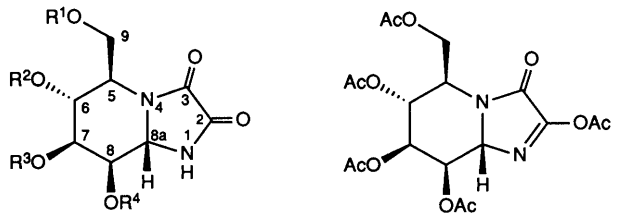
The structure of kifunensine, a new immunomodulator produced by a strain of *Kitasatosporia*, has been established as 1 on the basis of chemical and physicochemical evidence and X-ray crystallographic analysis. Kifunensine is unique both in its novel structure, containing a 4,5-dioxoimidazolidine ring included in the bicyclic framework, and in its potent immunomodulating activity. It is a representative of a new class of 1,5-iminopyranoses.

Keywords immunomodulator; α-mannosidase inhibitor; *Kitasatosporia kifunense*; polyhydroxylated piperidine; 4,5-dioxoimidazolidine; kifunensine; X-ray analysis

As part of a continuing program to screen for immunologically active compounds of microbial origin, a search was undertaken for substances having activity to restore mitogenic responses depressed by immunosuppressive factors of tumors. Kifunensine was isolated as such an immunoregulatory substance from an actinomycete, *Kitasatosporia kifunense* No. 9482.[2)] This compound was also found to possess an inhibitory activity against α-mannosidase.[1)] In this paper we report the structural elucidation of this natural product.

Kifunensine (**1**) was isolated as colorless prisms, mp > 280 °C, $[\alpha]_D$ +58.0° (c=0.1, H_2O). The molecular formula ($C_8H_{12}N_2O_6$) of **1** was established by elemental analysis and fast atom bombardment mass spectra (FAB-MS). The infrared (IR) spectrum showed absorption bands ascribable to hydroxy groups (3330, 3240, 3195 cm^{-1}) and carbonyl functions (1740, 1727, 1710 cm^{-1}).

The carbon nuclear magnetic resonance (^{13}C-NMR) spectrum (Table I) showed eight carbon signals, of which two were observed in the sp^2 region (δ 164.2 (s), 162.8 (s)) and the remainder (six carbons) in the sp^3 region (δ 62.7 (t), 73.9 (d), 73.8 (d), 71.8 (d), 66.0 (d), 61.3 (d)), being assigned to two carbonyls (C-2 (or C-3), C-3 (or C-2)), one methylene (C-9), and five methines (C-8, C-7, C-6, C-8a, C-5). The chemical shifts of the C-9 methylene and three of the five methines (C-8, C-7, C-6), which all resonated at relatively low field, suggest that they bear hydroxy groups.



kifunensine(1): R^1=R^2=R^3=R^4=H

3: R^1,R^2=R^3,R^4=>CMe$_2$

2

Chart 1

Acetylation of **1** with Ac_2O in pyridine gave the pentaacetate **2**, whose proton nuclear magnetic resonance (^{1}H-NMR) spectrum (Table II) showed five acetyl methyl signals at δ 2.60, 2.23, 2.10, 2.05, and 2.04. The relatively low chemical shift of one of these acetyl groups (δ 2.60) suggests this acetyl group to be an enol acetate. In the ultraviolet (UV) spectrum of **2**, a strong absorption band was observed at 232 nm (ε = 10000), revealing that **2** has an α,β-unsaturated carbonyl function and accordingly the original compound **1** has an enolizable α,β-dicarbonyl system.

The ^{1}H-NMR spectrum of **1** (Table II) showed methylene signals at δ 4.02 (dd, J = 12, 9.5 Hz, H_e) and 3.86 (dd, J = 12, 4.5 Hz, H-f) and methine signals at δ 3.72 (dd, J = 9, 3 Hz, H_g), 4.11 (dd, J = 3.5, 3 Hz, H_d), 4.20 (dd, J = 3.5, 1 Hz, H_c), 4.41 (ddd, J = 9.5, 4.5, 1 Hz, H_b), and 5.12 (d, J = 9 Hz, H_a). An analysis of these signals in conjunction with a ^{1}H–^{1}H shift correlation spectroscopy (^{1}H–^{1}H COSY) experiment clarified the H–H relationships as shown in Fig. 1. A two-dimensional incredible natural abundance double quantum transfer (2D INADEQUATE) experiment[3)] (D_2O–NaOD) on **1** revealed C–C couplings between the

TABLE I. ^{13}C-NMR (100 MHz) Chemical Shifts (in ppm) for **1** and **2**[a)]

C	1[b)]	2[c)]
2	164.2 (s)[d)]	156.1 (s)[e)]
3	162.8 (s)[d)]	156.0 (s)[e)]
5	61.3 (d)	52.7 (d)
6	71.8 (d)	66.9 (d)
7	73.8 (d)	68.5 (d)
8	73.9 (d)	71.0 (d)
8a	66.0 (d)	62.9 (d)
9	62.7 (t)	59.7 (t)

a) Abbreviations given in parentheses denote signals observed in the off-resonance experiments. *b*) D_2O. *c*) $CDCl_3$. *d, e*) Assignments may be interchanged in each column.

TABLE II. ^{1}H-NMR (400 MHz) Chemical Shifts (in ppm), Multiplicities, and Coupling Constants (in Hz, in Parentheses) for **1**, **2**, and **3**

H	1[a)]	2[b)]	3[b)]
1-H			9.00 br s
5-H	4.41 ddd (9.5, 4.5, 1)	4.83 ddt (10, 5, 1.5)	3.59 ddd (11, 10, 5)
6-H	4.20 dd (3.5, 1)	5.08 dd (4, 1.5)	4.23 dd (11, 8)
7-H	4.11 dd (3.5, 3)	5.33 ddd (4, 3, 1.5)	4.36 dd (8, 8)
8-H	3.72 dd (9, 3)	5.02 dd (9, 3)	4.05 dd (8, 8)
8a-H	5.12 d (9)	5.91 d (9)	4.91 d (8)
9-H_a	4.02 dd (12, 9.5)	4.60 dd (12, 10)	3.80 dd (12, 10)
9-H_b	3.86 dd (12, 4.5)	4.29 dd (12, 5)	4.67 dd (12, 5)
2-OAc, 3H s		2.60	
6,7,8,9-O-Ac, each 3H s		2.23, 2.10, 2.05, 2.04	
7,8;6,9-Acetonides, each 3H s			1.57, 1.55, 1.48, 1.36

a) D_2O. *b*) $CDCl_3$.

carbons bonded directly to each other, disclosing the sequence of these carbons as shown in partial structures A and B (Fig. 2).

A reasonable connection of these partial structures A and B to partial structure C was obtained by analysis of the ^{1}H-NMR spectrum of the diacetonide **3**, which was prepared by treatment of **1** with 2,2-dimethoxypropane in the presence of TsOH. The spectrum showed methylene and methine proton signals at δ 4.67 (dd, J=12, 5 Hz, 9-H$_a$), 3.80 (dd, J=12, 10 Hz, 9-H$_b$), 4.05 (dd, J=8, 8 Hz, 8-H), 4.36 (dd, J=8, 8 Hz, 7-H), 4.23 (dd, J=11, 8 Hz, 6-H), 4.91 (d, J=8 Hz, 8a-H), and 3.59 (ddd, J=11, 10, 5 Hz, 5-H) (Table II). The spectrum further showed an exchangeable amide proton at δ 9.00 (br s, 1-H), between which and the C-8a proton a cross-peak was observed in the ^{1}H–^{1}H COSY spectrum ($CDCl_3$). These data indicated the bonding of the amide N to C-8a and thereby led to partial structure C. The two acetonide bonds are postulated to be between C-9 and C-6 and between C-7 and C-8 on the assumption that they are five- or six-membered rings. A reasonable cyclization of this partial structure through the remaining tertiary nitrogen atom (N-4) finally leads to structure **3** for the diacetonide and the structure for kifunensine is hence deduced to be **1**.

The relative stereochemistry of **1** was presumed on the following grounds (Fig. 3). In the ^{1}H-NMR spectrum of **1**, nuclear Overhauser effect (NOE) was observed between 8a-H and 9-H$_a$, suggesting that 8a-H and the C9-hydroxymethyl group are 1,3-diaxial and hence that the piperidine ring of **1** takes a chair form. Supposing this is correct, the fact that the vicinal coupling constant of 8-H and 8a-H was large (J=9 Hz) suggests these protons to be in *trans* diaxial relationship. Since the coupling constant between 7-H and 8-H was small (J=3 Hz), 7-H was assigned to be equatorial. Regarding the relative configuration of the remaining C-6, an NOE experiment was undertaken on the diacetonide **3**. In this experiment, NOE was observed between 6-H and 8a-H in **3**, suggesting that the piperidine ring takes a boat form in which these protons exist on the same side of the piperidine ring. In the ^{1}H-NMR spectrum of **3**, the coupling constants between the two vicinal protons are all relatively large (5-H and 6-H, 11 Hz; 6-H and 7-H, 8 Hz; 7-H and 8-H, 8 Hz; 8-H and 8a-H, 8 Hz). All these protons are therefore presumed to be quasi-axial, in agreement with the conclusion derived from the above discussion. The relative stereochemistry of kifunensine has thus been deduced to be as shown in **1**.

The presumed structure of **1** was finally established by X-ray crystallographic analysis using crystals of kifunensine itself obtained from water. A perspective drawing of the

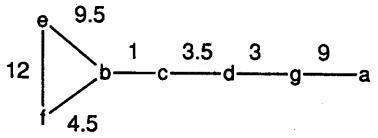


Fig. 1. ^{1}H–^{1}H Relationships and Coupling Constants (in Hz) in **1**

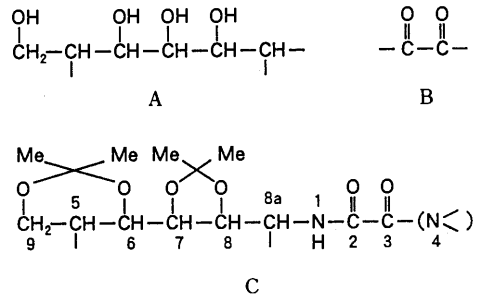


Fig. 2. Partial Structures of **1** and **3**

TABLE III. Atomic Coordinates and Thermal Parameters with e.s.d.'s (Å^2) in Parentheses

Atom	x	y	z	B_{eq}/B_{iso}
N1	0.2443 (3)	0.4465 (5)	0.0351 (3)	1.7
C2	0.4163 (4)	0.4671 (5)	0.0705 (4)	1.5
C3	0.4770 (4)	0.3539 (5)	0.2195 (3)	1.4
N4	0.3370 (3)	0.2615 (4)	0.2497 (3)	1.3
C5	0.3288 (4)	0.1240 (5)	0.3752 (3)	1.3
C6	0.1833 (4)	0.1949 (5)	0.4536 (3)	1.5
C7	0.0146 (4)	0.2328 (6)	0.3387 (3)	1.4
C8	0.0425 (4)	0.3824 (6)	0.2144 (3)	1.5
C8a	0.1817 (4)	0.3016 (5)	0.1338 (3)	1.4
C9	0.3135 (4)	−0.0944 (5)	0.3193 (4)	1.6
O10	0.5114 (4)	0.5533 (5)	−0.0012 (3)	2.3
O11	0.6243 (3)	0.3511 (5)	0.2926 (3)	2.1
O12	0.4676 (3)	−0.1562 (4)	0.2728 (3)	2.0
O13	0.2282 (3)	0.3805 (5)	0.5318 (3)	2.1
O14	−0.0493 (3)	0.0483 (4)	0.2684 (3)	1.8
O15	−0.1085 (3)	0.4057 (5)	0.0984 (3)	2.6
H1	0.173 (7)	0.513 (10)	−0.048 (6)	3.5
H5	0.446 (6)	0.131 (9)	0.456 (6)	2.0
H6	0.166 (6)	0.084 (9)	0.532 (6)	1.8
H7	−0.074 (6)	0.294 (9)	0.398 (6)	2.1
H8	0.076 (7)	0.526 (10)	0.268 (6)	2.8
H8a	0.131 (6)	0.174 (9)	0.072 (6)	2.0
H9a	0.207 (6)	−0.112 (10)	0.226 (5)	2.3
H9b	0.291 (6)	−0.191 (9)	0.407 (6)	2.4
H12	0.473 (7)	−0.085 (11)	0.182 (6)	3.6
H13	0.353 (7)	0.380 (13)	0.600 (6)	4.1
H14	−0.115 (8)	−0.006 (11)	0.338 (7)	4.1
H15	−0.204 (7)	0.401 (11)	0.147 (6)	3.7

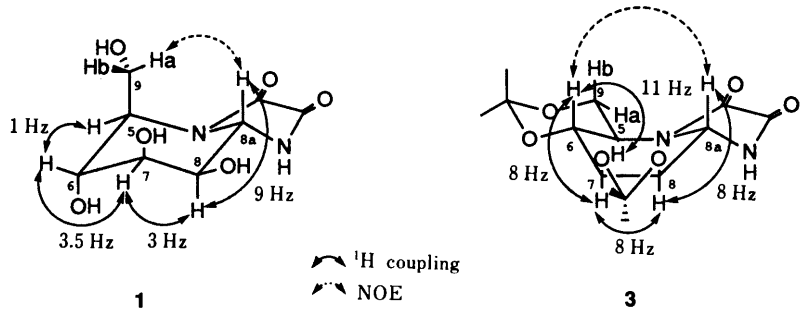


Fig. 3. ^{1}H-NMR Coupling Constants and NOE in **1** and **3**

TABLE IV. Bond Lengths (Å) and Angles (°) with Their e.s.d.'s in Parentheses

Bond length (Å)			
N1–C2	1.344 (5)	N1–C8a	1.458 (5)
N1–H1	0.95 (7)	C2–C3	1.520 (5)
C2–O10	1.226 (5)	C3–N4	1.343 (4)
C3–O11	1.219 (5)	N4–C5	1.457 (4)
N4–C8a	1.466 (5)	C5–C6	1.539 (5)
C5–C9	1.529 (5)	C5–H5	1.06 (6)
C6–C7	1.535 (5)	C6–O13	1.425 (5)
C6–H6	1.05 (6)	C7–C8	1.538 (5)
C7–O14	1.421 (5)	C7–H7	1.04 (6)
C8–C8a	1.531 (5)	C8–O15	1.426 (5)
C8–H8	1.087 (7)	C8a–H8a	1.05 (6)
C9–O12	1.428 (5)	C9–H9a	1.07 (7)
C9–H9b	1.05 (6)	O12–H12	0.95 (7)
O13–H13	1.05 (9)	O14–H14	0.96 (7)
O15–H15	0.94 (8)		
Bond angle (°)			
C2–N1–C8a	112.2 (3)	C2–N1–H1	124 (4)
C8a–N1–H1	124 (4)	N1–C2–C3	106.5 (3)
N1–C2–O10	128.8 (4)	C3–C2–O10	124.6 (3)
C2–C3–N4	105.8 (3)	C2–C3–O11	125.3 (3)
N4–C3–O11	128.9 (3)	C3–N4–C5	127.4 (3)
C3–N4–C8a	112.8 (3)	C5–N4–C8a	119.7 (3)
N4–C5–C6	108.2 (3)	N4–C5–C9	110.8 (3)
N4–C5–H5	109 (3)	C6–C5–C9	114.9 (3)
C6–C5–H5	108 (3)	C9–C5–H5	105 (3)
C5–C6–C7	112.2 (3)	C5–C6–O13	110.5 (3)
C5–C6–H6	108 (3)	C7–C6–O13	106.5 (3)
C7–C6–H6	111 (3)	O13–C6–H6	109 (3)
C6–C7–C8	110.8 (3)	C6–C7–O14	109.9 (3)
C6–C7–H7	108 (3)	C8–C7–O14	109.1 (3)
C8–C7–H7	109 (3)	O14–C7–H7	110 (3)
C7–C8–C8a	109.6 (3)	C7–C8–O15	111.9 (3)
C7–C8–H8	108 (3)	C8a–C8–O15	106.0 (3)
C8a–C8–H8	113 (3)	O15–C8–H8	109 (3)
N1–C8a–N4	101.9 (3)	N1–C8a–C8	114.8 (3)
N1–C8a–H8a	111 (3)	N4–C8a–C8	108.4 (3)
N4–C8a–H8a	114 (3)	C8–C8a–H8a	107 (3)
C5–C9–O12	110.6 (3)	C5–C9–H9a	111 (4)
C5–C9–H9b	111 (3)	O12–C9–H9a	110 (4)
O12–C9–H9b	109 (3)	H9a–C9–H9b	106 (5)
C9–O12–H12	107 (4)	C6–O13–H13	113 (5)
C7–O14–H14	103 (4)	C8–O15–H15	107 (5)

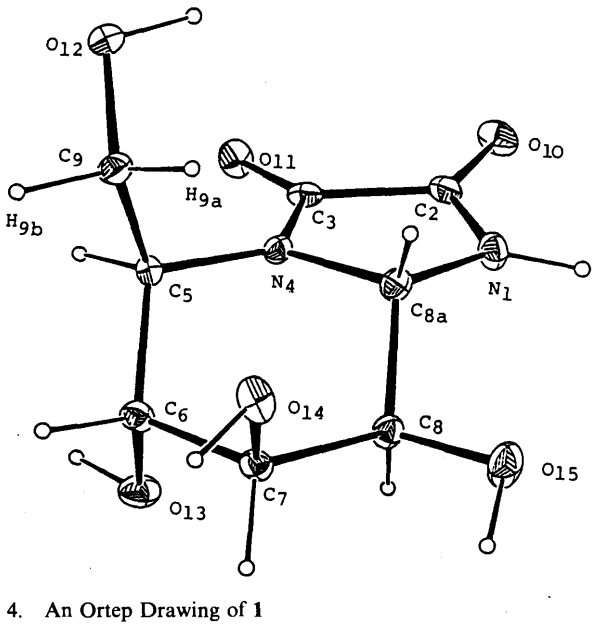


Fig. 4. An Ortep Drawing of **1**

structure of **1** is given in Fig. 3. Kifunensine was thus determined to have the structure **1** (relative stereochemistry). The absolute stereochemistry was presumed, by considering the fact that **1** showed inhibitory activity against α-mannosidase, to be the same as that of D-mannose and this was finally confirmed by a synthesis of **1** from D-mannosamine.[4]

The structural study described above thus revealed that kifunensine has a unique bicyclic structure **1** corresponding to a cyclic oxamide derivative of 1-amino-substituted mannojirimycin.[5,6] Synthetic studies of kifunensine and related compounds will be reported in subsequent papers in this series.

Experimental

General Procedures The melting points were determined in open capillary tubes with a Thomas-Hoover melting point apparatus and are uncorrected. The elemental analyses were performed using a Yanaco MT-3 CHN CORDER. The optical rotations were measured with a JASCO DIP-140 digital polarimeter. The IR spectra were taken on a JASCO A-102 infrared spectrophotometer. The UV spectra were taken on a Hitachi 220A spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra were taken on a Bruker AM 200 (200 MHz for ^{1}H-NMR) or Bruker AM 400 (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR) NMR spectrometer with tetramethylsilane (TMS) or sodium 3-(trimethylsilyl)propionate-d_4 (TSP-d_4) as an internal standard, and chemical shifts were recorded in δ values. Multiplicities of ^{13}C-NMR signals were determined by the distortionless enhancement by polarization transfer (DEPT) method. Pulse programs of the standard Bruker software library were used for 2D experiments. The FAB-MS were taken on a VG Analytical ZAB-SE mass spectrometer. Column chromatography was run on Merck Silica gel 60 (70—230 mesh). Thin layer chromatography (TLC) was performed on glass plates precoated with Silica gel 60F_{254} (Merck).

Kifunensine (1) Colorless prisms, mp>280 °C (H_2O), $[\alpha]_D$ +58.0° (c=0.1, H_2O). *Anal.* Calcd for $C_8H_{12}N_2O_6$: C, 41.38; H, 5.21; N, 12.06. Found: C, 40.94; H, 5.07; N, 11.78. IR (KBr): 3405, 3330, 3240, 3195, 2920, 1740, 1727, 1710, 1452, 1100, 1070, 1052, 1010 cm^{-1}. UV $\lambda_{max}^{H_2O}$ nm (ε): 226 (230). ^{1}H- and ^{13}C-NMR: see Tables I and II. FAB-MS m/z: 233 $(M+H)^+$.

Kifunensine Pentaacetate (2) Acetic anhydride (10 ml) was added to a suspension of **1** (1.73 g) in pyridine (20 ml) and the mixture was stirred at room temperature for 20 h. The reaction mixture was evaporated *in vacuo* and the residue was dissolved in AcOEt and washed with 1N aqueous HCl, water, saturated aqueous $NaHCO_3$, and water. The organic layer was dried over $MgSO_4$, evaporated *in vacuo*, and purified by column chromatography (SiO_2 30 g, CH_2Cl_2–MeOH) to furnish **2** (2.92 g, 84%). **2**, amorphous solid, $[\alpha]_D$ −35.3° (c=0.6, $CHCl_3$). *Anal.* Calcd for $C_{18}H_{22}N_2O_{11}$: C, 48.87; H, 5.01; N, 6.33. Found: C, 48.61; H, 4.92; N, 6.20. IR ($CHCl_3$): 1778, 1750, 1432, 1368, 1250, 1210, 1142, 1058 cm^{-1}. UV λ_{max}^{MeOH} nm (ε): 232 (10000). ^{1}H- and ^{13}C-NMR: see Tables I and II. FAB-MS m/z: 433 $(M+H)^+$.

Kifunensine Diacetonide (3) A suspension of **1** (200 mg) in *N,N*-dimethylformamide (DMF) (5.0 ml) was treated with 2,2-dimethoxypropane (3.2 ml) and TsOH·H_2O (20 mg) at 60 °C for 12 h under an N_2 atmosphere. After neutralization with saturated aqueous $NaHCO_3$, the solvent was removed under reduced pressure and the residue was extracted with MeOH (15 ml). The extract was evaporated *in vacuo* and purified by column chromatography (SiO_2 10 g, $CHCl_3$–AcOEt) to afford the diacetonide **3** (223 mg, 83%). **3**, colorless fine crystals, mp 275—278 °C (dec., *n*-hexane–AcOEt), $[\alpha]_D$ −66.6° (c=0.5, MeOH). *Anal.* Calcd for $C_{14}H_{20}N_2O_6$: C, 53.84; H, 6.45; N, 8.97. Found: C, 53.87; H, 6.77; N, 8.99. IR ($CHCl_3$): 3415, 2993, 1754, 1419, 1375, 1100, 1074 cm^{-1}. ^{1}H-NMR: see Table II. FAB-MS m/z: 313 $(M+H)^+$.

X-Ray Analysis of 1 The crystals were recrystallized from water: $C_8H_{12}N_2O_6$, prisms, monoclinic, space group $P2_1$, a=7.934(2), b=6.634(1), c=8.933(3) Å, β=101.59(3)°, V=460.6(2) Å^3, Z=2, D_x=1.68 g/cm^3, μ=0.93 cm^{-1}. The X-ray diffraction intensity data from a selected crystal (0.15×0.05×0.05 mm) were obtained on a Rigaku AFC-5R diffractometer equipped with a rotating anode X-ray generator (50 KV–180 mA), using graphite-monochromated Mo$K\alpha$ radiation (λ=0.71069 Å). A total of 1461 independent reflections with $2\theta<60°$ were collected in the ω–2θ scaling mode. The structure was solved by the direct

method using MULTAN 84 (Main *et al.*, 1984). The H atoms were located from difference Fourier syntheses. The refinement was carried out by the block-diagonal least-squares method with anisotropic thermal parameters for non H atoms and with isotropic thermal parameters for H atoms. The R factor was reduced to 0.047 using 1273 reflections with $F_o \geqq 3\sigma(F_o)$. The atomic parameters, bond lengths and bond angles are given in Tables III and IV.

References and Notes

1) A preliminary report of this work: H. Kayakiri, S. Takase, T. Shibata, M. Okamoto, H. Terano, M. Hashimoto, T. Tada, and S. Koda, *J. Org. Chem.*, **54**, 4015 (1989).
2) M. Iwami, O. Nakayama, H. Terano, M. Kohsaka, H. Aoki, and H. Imanaka, *J. Antibiot.*, **40**, 612 (1987). Kifunensine was tentatively designated FR900494 in that paper.
3) D. L. Turner, *J. Magn. Reson.*, **49**, 175 (1982).
4) H. Kayakiri, C. Kasahara, T. Oku, and M. Hashimoto, *Tetrahedron Lett.*, **31**, 225 (1990).
5) Mannojirimycin (nojirimycin B): T. Niwa, T. Tsuruoka, H. Goi, Y. Kodama, J. Itoh, S. Inoue, Y. Yamada, T. Niida, M. Nobe, and Y. Ogawa, *J. Antibiot.*, **37**, 1579 (1984).
6) For a review on 1,5-iminopyranoses and 1,4-iminofuranoses, see G. W. J. Fleet, *Spec. Publ. Royal Chem. Soc.*, **65**, 149 (1988).

Chemical Study on *Haematoxylon campechianum*: a Sweet Principle and New Dibenz[*b*,*d*]oxocin Derivatives

Hitoshi MASUDA,[a] Kazuhiro OHTANI,[a] Kenji MIZUTANI,[b] Susumu OGAWA,[b] Ryoji KASAI*,[a] and Osamu TANAKA[a]

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine,[a] Kasumi, Minami-ku, Hiroshima 734, Japan and Department of Biology and Chemistry, Maruzen Kasei Co., Ltd.,[b] 14703–10 Mukaihigashi-cho, Onomichi 722, Japan. *Received October 17, 1990*

The sweet principle of an extract of heartwood of *Haematoxylon campechianum* was identified as hematoxylin (1), the well-known staining reagent already isolated from this plant. In addition to 1, two new dibenz[*b*,*d*]oxocin derivatives (2 and 3) were obtained. The structure of 2, named hematoxylol A, was elucidated as 3,4,10,11-tetrahydroxy-7,8-dihydro-6*H*-dibenz[*b*,*d*]oxocin-7-one. The latter compound (3) was purified as a tetramethyl ether (3′) named tetra-*O*-methylhematoxylol B and its structure was assigned as 7-hydroxy-3,4,10,11-tetramethoxy-7,8-dihydro-6*H*-dibenz[*b*,*d*]oxocin-7-methanol.

Keywords *Haematoxylon campechianum*; Leguminosae; logwood; sweet principle; hematoxylin; hematoxylol A; tetra-*O*-methylhematoxylol B; dibenz[*b*,*d*]oxocin

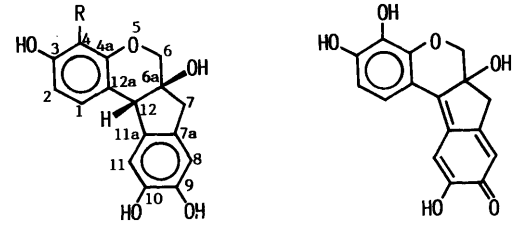
Haematoxylon campechianum L. (Leguminosae) is a well-known tree named "logwood," growing in Mexico, the West Indies and South America. Heartwood of this tree is a source of mordant dye. It is also known that extract of the heartwood tastes sweet, and it has been used as a sweetener. The present paper reports the identification of a sweet principle of this tree. Isolation and structure elucidation of two new dibenz[*b*,*d*]oxocin derivatives are also described.

The dried and powdered heartwood was extracted with 50% methanol and the sweet extract was chromatographed on highly porous synthetic polymer with water, 30% ethanol, 50% ethanol, ethanol and finally acetone. Of these eluates, the 30% ethanolic eluate tasted sweet. This eluate was chromatographed on silica gel, affording three fractions, tentatively designated as frs. 1—3. Rechromatography of the sweet fraction, fr. 2, on silica gel yielded a sweet crystalline compound (**1**). The bitter fraction, fr. 1, afforded a crystalline compound (**2**) on silica gel chromatography followed by high performance liquid chromatography (HPLC). The tasteless fraction, fr. 3, was subjected to chromatography on silica gel followed by HPLC to give a substance (**3**) which was further purified as its methyl ether, affording the pure compound (**3′**).

By inspection of the nuclear magnetic resonance (NMR) spectrum and fast-atom bombardment mass spectrum (FAB-MS), the sweet principle (**1**) was identified as hematoxylin,[1] which has already been isolated from this plant. Hematoxylin is a well-known staining reagent in biology, but this is the first report of the sweetness of this compound. Hematoxylin is readily oxidized to give a red pigment named hematein (**4**), which was found to be tasteless. Brazilin (**5**),[1] a congener of **1**, has been isolated from *Caesalpinia echinata* (Leguminosae), but it is tasteless. This indicates an important role of the 4-hydroxyl group of this skeleton for sweetness.

The elemental analysis of compound **2** coupled with the positive FAB-MS ($[M+H]^+$: *m/z* 289) led to the molecular formula $C_{15}H_{12}O_6$. The infrared (IR) spectrum (KBr) of **2** showed a carbonyl absorption band at 1700 cm^{-1}. The ^{1}H-NMR spectrum of **2** showed two methylene signals at δ 3.30 and 4.40, each as a 2H singlet. The ^{13}C-NMR spectrum of **2** (Table I) exhibited two methylene and one carbonyl carbon signals. The ^{13}C–^{1}H correlation spectroscopy (^{13}C–^{1}H COSY) spectrum established the correlation of these methylene carbon and proton signals. The ^{13}C–^{1}H long range COSY spectrum indicated the presence of long range coupling between the carbonyl carbon signal and the two methylene proton signals. Based on these results, a partial structure A (Chart 2) was proposed for **2**.

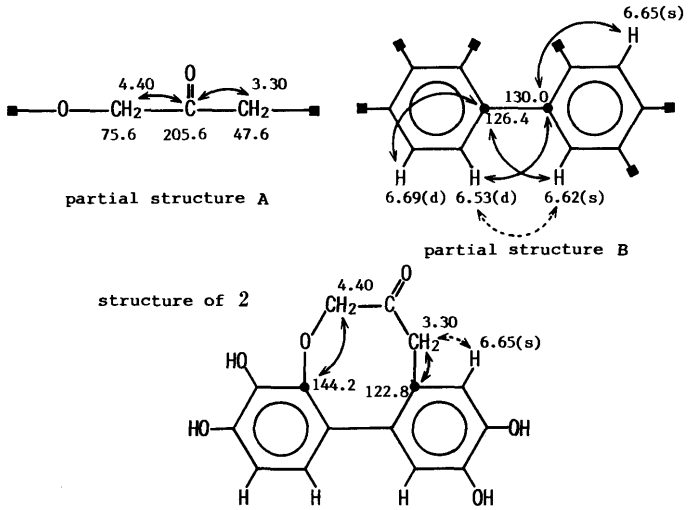
The ^{1}H-NMR spectrum of **2** showed a pair of *ortho*-located aromatic proton signals at δ 6.53 (1H, d, J=8.3 Hz), 6.69 (1H, d, J=8.3 Hz) and aromatic proton signals having



hematoxylin (**1**): R=OH
sweet
brazilin (**5**): R=H
tasteless

hematein (**4**)
tasteless

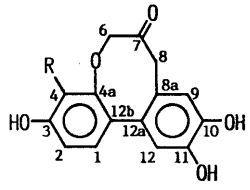
Chart 1



^{1}H- and ^{13}C-chemical shifts, long-range C–H correlation (⟷) and NOE observed in the NOE difference spectrum (←→) in DMSO-d_6 at 50 °C

Chart 2

TABLE I. ^{13}C-NMR Chemical Shifts of **2** and **6** in Acetone-d_6

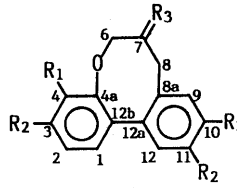


hematoxylol A (**2**): R=OH
protosappanin A (**6**): R=H

	2[a]	**6**
C-1	120.1 (117.8)	131.1
C-2	113.1 (111.8)	113.5
C-3	147.4 (146.0)	159.2
C-4	139.2 (137.7)	109.2
C-4a	145.7 (144.2)	159.8
C-6	77.2 (75.6)	78.9
C-7	206.4 (205.6)	206.0
C-8	49.1 (47.6)	49.4
C-8a	125.3 (122.8)	125.1
C-9	117.7 (116.5)	117.5[b]
C-10	145.5 (143.9)	145.4[c]
C-11	145.7 (144.1)	145.7[c]
C-12	117.7 (116.4)	117.7[b]
C-12a	132.4 (130.0)	131.9
C-12b	128.7 (126.4)	127.2

a) Values measured in DMSO-d_6 at 50°C in parentheses. *b, c*) These assignments may be interchanged.

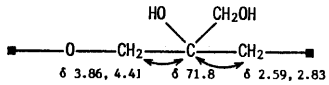
TABLE II. ^{13}C-NMR Chemical Shifts of **3′**, **7** and **9** in $CDCl_3$



3′: $R_1=R_2=OMe$, $R_3=$ 
7: $R_1=R_2=OMe$, $R_3=O$
8: $R_1=H$, $R_2=OH$, $R_3=$ 
9: $R_1=H$, $R_2=OMe$, $R_3=$ 

	3′	**7**	**9**
C-1	123.9	123.2	131.4
C-2	108.3	108.9	110.1
C-3	153.3	153.6	158.5
C-4	141.6	142.0	106.5
C-4a	152.0	149.7	160.5
C-6	80.8	76.9	78.6
C-7	71.8	205.8	74.2
C-8	38.9	48.9	38.9
C-8a	126.5	124.5	126.1[b]
C-9	115.4	112.9	114.9
C-10	148.1[a]	148.1[a]	148.1[c]
C-11	148.2[a]	148.5[a]	147.9[c]
C-12	111.3	112.5	113.3
C-12a	131.3	130.6	131.5
C-12b	128.3	128.6	124.9[b]
7-CH_2OH	67.1	—	67.2
-OCH_3	56.1	56.1	55.3
	56.2	56.1	56.0
	56.2	56.1	56.1
	60.7	60.9	—

a—*c*) These assignments may be interchanged in each column. *, *S* chirality.



(each 1H, d, J=12.6 Hz) (each 1H, d, J=13.9 Hz)
Long-range C–H correlation (measured in $CDCl_3$).

no vicinal proton at δ 6.62 (1H, s) and 6.65 (1H, s). The ^{13}C-NMR spectrum of **2** (Table I) exhibited twelve aromatic carbon signals and the distortionless enhancement by polarization transfer (DEPT) experiment disclosed that the aromatic carbon signals at δ 111.8, 116.4, 116.5 and 117.8 (in dimethyl sulfoxide-d_6 (DMSO-d_6)) are due to carbons bearing a proton and others are due to carbons without any proton. The ^{1}H–^{1}H COSY and ^{13}C–^{1}H COSY spectra furnished the correlations of these proton and carbon signals. The ^{13}C–^{1}H long range COSY spectrum revealed the presence of long range coupling between the following carbon and proton signals: a carbon signal at δ 126.4 with proton signals at δ 6.69 and 6.62; a carbon signal at δ 130.0 with proton signals at δ 6.53 and 6.65. In the nuclear Overhauser effect (NOE) differential spectrum, NOE was observed between the proton signals at δ 6.53 and 6.62. These observations led to a biphenyl-type partial structure B as illustrated in Chart 2.

The connection of the two partial structures A and B was established as follows. In the ^{13}C–^{1}H long range COSY spectrum, long range coupling was observed between a methylene proton signal at δ 3.30 and an aromatic carbon signal at δ 122.8 and also between a methylene proton signal at δ 4.40 and an aromatic carbon signal at δ 144.2. The NOE differential spectrum demonstrated the presence of NOE between a methylene proton signal at δ 3.30 and an aromatic proton signal at δ 6.65. Compound **2** was thus formulated as 3,4,10,11-tetrahydroxy-7,8-dihydro-6*H*-dibenz[*b,d*]oxocin-7-one (Chart 2). Isolation of a dibenz[*b,d*]oxocin derivative was first reported by Nagai *et al.*,[2] who isolated a compound named protosappanin A (**6**) from *Caesalpinia sappan* L. (Leguminosae) and elucidated the structure (see Table I). The structure of **2** (=4-hydroxyprotosappanin A) was further supported by comparison of the carbon signals in acetone-d_6 with those of **6** in the same solvent (Table I) by considering the hydroxylation shift of phenolic compounds. This is the second example of the isolation of compounds of this type. The name hematoxylol A is proposed for **2**.

The molecular formula of the optically active compound **3′** was determined by high resolution negative FAB-MS as $C_{20}H_{24}O_7$. The IR spectrum of **3′** indicated the absence of any carbonyl group and the ^{1}H-NMR spectrum of **3′** in $CDCl_3$ showed signals due to four methoxyl groups at δ 3.88, 3.90, 3.91 and 3.93 (each 3H, s). For ^{13}C-NMR comparison, a tetramethyl ether (**7**) was prepared from **2** after ketalization of the carbonyl group to protect it against methylation with diazomethane. The carbon signals of **7** due to C-1, 2, 3, 4, 10, 11, 12, 12a and 12b were observed at similar positions to those of **3′** (Table II), indicating the presence of the same biphenyl moiety as that of **7** in **3′**. This was supported by the aromatic proton signals of **3′** at δ 6.79 (1H, d, J=8.6 Hz), 7.01 (1H, d, J=8.6 Hz), 6.81 (1H, s) and 6.83 (1H, s) in $CDCl_3$. The ^{13}C-NMR spectrum of **3′** in $CDCl_3$ exhibited two signals due to a O–$\underline{C}H_2$–C group at δ 67.1 and 80.8, a signal due to a C–$\underline{C}H_2$–C group at δ 38.9 and a signal due to a tetra-substituted carbon having an oxygen function at δ 71.8 (Table II). The ^{1}H-NMR spectrum of **3′** showed signals due to two O–CH_2 groups as two pairs of doublets at δ 3.86, 4.41 (each 1H, d, J=12.6 Hz) and 3.46, 3.56 (each 1H, d, J=11.8 Hz), together with signals due to a phenyl-CH_2–C group at δ 2.59, 2.83 (each 1H, d, J=13.9 Hz). The

^{13}C–^{1}H COSY and ^{13}C–^{1}H long range COSY spectra of **3'** demonstrated the long range coupling of the tetra-substituted carbon signal with the methylene proton signals at δ 2.59, 2.83, 3.86 and 4.41 (Table II). It follows that **3'** can be formulated as 7-hydroxy-3,4,10,11-tetramethoxy-7,8-dihydro-6*H*-dibenz[*b*,*d*]oxocin-7-methanol (Table II). Since the ^{1}H-NMR spectrum of **3** showed no methoxy proton signal, the name tetra-*O*-methylhematoxylol B is proposed for **3'**. The absolute configuration at C-7 of **3'** has not been assigned as yet. Nagai and Nagumo[3] isolated 3,7,10,11-tetrahydroxy-7,8-dihydro-6*H*-dibenz-[*b*,*d*]oxocin-7-methanol, named protosappanin B (**8**), from *Caesalpinia sappan* L. Subsequently, Saitoh *et al.*[4] isolated 10-*O*-methylprotosappanin B from the same plant. A comparison of the ^{13}C-NMR spectrum of **3'** with that of the trimethyl ether (**9**) of protosappanin B[3] supported the formulation of **3'**. The biogenetic correlation of **1**, protosappanins and related compounds with chalcone derivatives has been proposed.[3,5–7]

Experimental

Melting points were measured on a micro hot-stage and are uncorrected. NMR spectra were recorded on a JEOL JNM-GX 400 spectrometer in DMSO-d_6 at 50°C unless otherwise stated. FAB-MS were taken on a JEOL JMS-SX 102 spectrometer. Conditions of HPLC: column, TSKgel ODS-120T (21.5 mm i.d. × 30 cm); flow rate of mobile phase, 6 ml/min; detection, UV 254 nm.

Extraction and Separation The powdered heartwood (125 g) collected in Mexico was extracted with hot MeOH. An aqueous suspension of the extract was chromatographed on Diaion HP-20 and eluted with H_2O, 30% EtOH, 50% EtOH, EtOH and acetone, successively. The 30% EtOH eluate, which tastes sweet, was chromatographed on silica gel. Elution with $CHCl_3$–MeOH–H_2O (40 : 10 : 1) provided three fractions, frs. 1—3.

The sweet fraction, fr. 2, was subjected to repeated chromatography on silica gel with C_6H_6–EtOAc–MeOH (5 : 10 : 1) followed by recrystallization from H_2O in a dark-colored flask under Ar gas to afford **1** (80 mg). **1**: sweet yellow prisms, mp 148—151 °C, $[\alpha]_D^{18}$ +90.3° (c = 1.04, MeOH). FAB-MS m/z: 331 $[M+Na]^+$. ^{1}H-NMR δ: 2.73, 2.88 (each 1H, d, J = 15.6 Hz, 7-H_2), 3.59, 3.90 (each 1H, d, J = 11.0 Hz, 6-H_2), 3.86 (1H, s, 12-H), 5.28 (1H, s, 6a-OH), 6.42 (1H, d, J = 8.2 Hz, 1-H), 6.54 (1H, s, 11-H), 6.62 (1H, d, J = 8.2 Hz, 2-H), 6.63 (1H, s, 8-H), 8.18 (1H, s, OH), 8.59 (3H, s, OH). ^{13}C-NMR δ: 41.9 (C-7), 49.8 (C-12), 69.7 (C-6), 76.3 (C-6a), 108.6 (C-2), 111.6 and 111.9 (C-8 or -11), 115.2 (C-12a), 119.2 (C-1), 130.0 (C-7a), 133.1 (C-4), 135.6 (C-11a), 142.4, 143.8, 144.0, 144.2 (C-3, -4a, -9 or -10).

The bitter fraction, fr. 1, was subjected to chromatography on silica gel with C_6H_6–acetone (2 : 1) followed by HPLC on an ODS column (mobile phase: 30% MeOH) to give **2** (115 mg). **2**: colorless needles from MeOH–H_2O, mp 274—277 °C, optically inactive. UV λ_{max}^{MeOH} nm (log ε): 243 (3.49), 293 (3.26). *Anal.* Calcd for $C_{15}H_{12}O_6 \cdot H_2O$: C, 58.82; H, 4.61. Found: C, 58.74; H, 4.59.

A solution of **2** (30 mg), $(CH_2)_2(OH)_2$ (1 ml) and p-CH_3–C_6H_4–SO_3H (10 mg) in THF (3 ml) was heated at 100 °C for 2 h. The mixture was neutralized with Amberlite MB-3 and concentrated to dryness. A solution of the residue in MeOH was repeatedly treated with a solution of CH_2N_2 in ether and the product was purified by chromatography on silica gel with C_6H_6–acetone (3 : 1) to give an ethylene ketal methyl ether. A solution of this compound and p-CH_3–C_6H_4–SO_3H (10 mg) in $CHCl_3$ (3 ml) containing a few drops of 95% EtOH (pH 3.0) was heated at 100 °C for 6 h. The reaction mixture was treated with Amberlite MB-3 and concentrated to dryness to give **7**, which was subjected to ^{13}C-NMR measurement.

The tasteless fraction, fr. 3, was chromatographed on silica gel with C_6H_6–EtOAc–MeOH (5 : 30 : 1) followed by HPLC on an octadecyl silica (ODS) column (mobile phase: 15% MeCN) to give a major fraction (168 mg). A solution of this fraction (100 mg) in MeOH (10 ml) was treated repeatedly with an ether solution of CH_2N_2. The product was purified by HPLC (mobile phase: 60% MeOH) to give **3'** (60 mg). **3'**: a white powder, $[\alpha]_D^{18}$ −1.7° (c = 1.67, $CHCl_3$). High-resolution FAB-MS m/z: Calcd for $C_{20}H_{24}O_7$ +H: 377.1600. Found: 377.1607 $(M+H)^+$.

Acknowledgment We are grateful to Professor M. Nagai, Hoshi University, Tokyo for valuable advice.

References

1) W. H. Perkin and R. Robinson, *J. Chem. Soc.*, **91**, 1073 (1907); R. Robinson, *Bull. Soc. Chim. Fr.*, **1985**, 125; J. C. Craig, A. R. Naik, R. Partt and E. Johnson, *J. Org. Chem.*, **30**, 1573 (1973).
2) M. Nagai, S. Nagumo, S. Lee, I. Eguchi and K. Kawai, *Chem. Pharm. Bull.*, **34**, 1 (1986).
3) M. Nagai and S. Nagumo, *Heterocycles*, **24**, 601 (1986); M. Nagai and S. Nagumo, *Chem. Pharm. Bull.*, **38**, 1490 (1990).
4) M. Namikoshi, H. Nakata and T. Saitoh, *Chem. Pharm. Bull.*, **35**, 3615 (1987).
5) M. Nagai and S. Nagumo, *Chem. Pharm. Bull.*, **35**, 3002 (1987).
6) K. Miyahara, T. Kawasaki, J. Kinjo, T. Shimokawa, J. Yamahara, M. Yamasaki, K. Harano and T. Nohara, *Chem. Pharm. Bull.*, **34**, 4166 (1986).
7) T. Saitoh, S. Sakashita, H. Nakata, T. Shimokawa, J. Kinjo, J. Yamahara, M. Yamasaki and T. Nohara, *Chem. Pharm. Bull.*, **34**, 2506 (1986).

Synthesis of a New Cerebroside from a *Chondropsis* sp. Sponge[1,2]

Masanori HONDA, Yoshitaka UEDA, Shigeo SUGIYAMA, and Tetsuya KOMORI*

Faculty of Pharmaceutical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan. *Received October 29, 1990*

A cerebroside, 1-*O*-(β-D-galactopyranosyloxy)-(2*S*,3*S*,4*R*,6*E*)-2-[(*R*)-2-hydroxytetracosanoylamino]-17-methyl-6-octadecene-3,4-diol (2), was asymmetrically synthesized from isobutyraldehyde. On the basis of a comparison of the physical data, the absolute structure of a new cerebroside 1b from a *Chondropsis* sp. sponge is thought to be the same as that of 2.

Keywords glycosphingolipid; asymmetric synthesis; absolute stereochemistry; phytosphingosine; galactosyl ceramide; hystidine decarboxylase; hypotensive activity; (*R*)-benzyl-2,3-epoxypropyl ether; sponge; *Chondropsis* sp.

Recently, a mixture of new galactosyl ceramides (**1a** and **1b**) was separated from a sea sponge, *Chondropsis* sp., and was shown to inhibit hystidine decarboxylase as well as to have hypotensive activity in anesthetized rats.[2] The mixture was characterized by acid hydrolysis, ozonolysis, and gas chromatography-mass spectrometry (GC-MS) analysis and found to be composed of D-galactose, α-hydroxytetracosanoic acid, and C_{17}- and C_{18}-phytosphingosines bearing a *trans*-double bond and an iso-type terminal in the long chain, as illustrated in Chart 1. However, neither the determination of the absolute stereochemistry nor the isolation of the pure cerebrosides **1a** and **1b** was achieved.

As a part of our studies on glycosphingolipids from starfishes, we were primarily interested in the absolute structure and biological activities of the new cerebrosides **1a** and **1b**. While the syntheses of many kinds of ceramides and cerebrosides have been achieved,[3] there seems to be no documentation of the synthesis of a phytosphingosine-type cerebroside possessing a hydroxy fatty acid, except for one example by our group.[4] For the synthesis of the new cerebrosides **1a** and **1b**, we focused on the synthesis of the ceramide, (2*S*,3*S*,4*R*,6*E*)-2-[(*R*)-2-hydroxytetracosanoylamino]-17-methyl-6-octadecene-1,3,4-triol (**2**), which contains the most common configurations among naturally occurring phytosphingosines and α-hydroxy fatty acids. The design of the synthesis of **2** is retrosynthetically outlined in Chart 1. The chiralities on the phytosphingosine were successively introduced into the C-4, C-3, and C-2 positions by utilizing the chiral synthon (*R*)-benzyl-2,3-epoxypropyl ether (**7**), asymmetric epoxidation, and amination with a regioselective epoxide opening, leading to **5**. (*R*)-2-Hydroxytetracosanoic acid (**4**) has been prepared by our group.[4a] The acid **4** and the phytosphingosine **5** were converted into the cerebroside **2** after glycosidation of the ceramide **3** with D-galactose.

Isobutyraldehyde (**9**) was used for the construction of the iso-type terminal of a phytosphingosine, (2*S*,3*S*,4*R*,6*E*)-2-amino-17-methyl-6-octadecene-1,3,4-triol (**5**). A Wittig reaction of **9** with α,α-dibromomethylenetriphenylphosphorane[5] gave an olefinic dibromide **10** in an 88% yield. The dibromide **10** was treated first with *n*-butyllithium and then bromooctane to afford an alkyne **11** in a 70% yield. Hexamethylphosphoric triamide (HMPA) and three equivalents of *n*-butyllithium are necessary for this alkylation. The migration of the internal triple bond of **11** with potassium aminopropylamide (KAPA)[6] resulted in the alkyne **8**, of which the terminals were an iso-type moiety and a triple bond. The alkyne **8** was treated with *n*-butyllithium and then (*R*)-benzyl-2,3-epoxypropyl ether (**7**)[7] to produce a chiral alcohol, which was protected as the methoxymethoxy (MOM) ether (**12**) with chloromethyl methyl ether (MOMCl) and *N*,*N*-diisopropylethylamine in a 64% yield from **8**. Debenzylation and the reduction of the MOM ether **12** with lithium in ethylamine and *tert*-

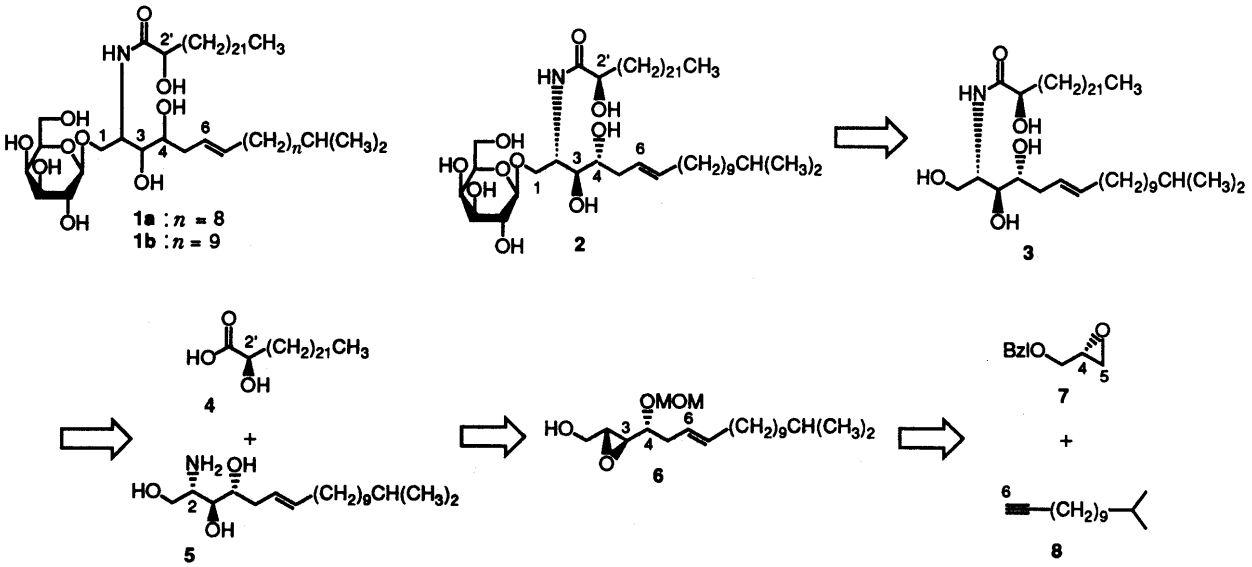


Chart 1

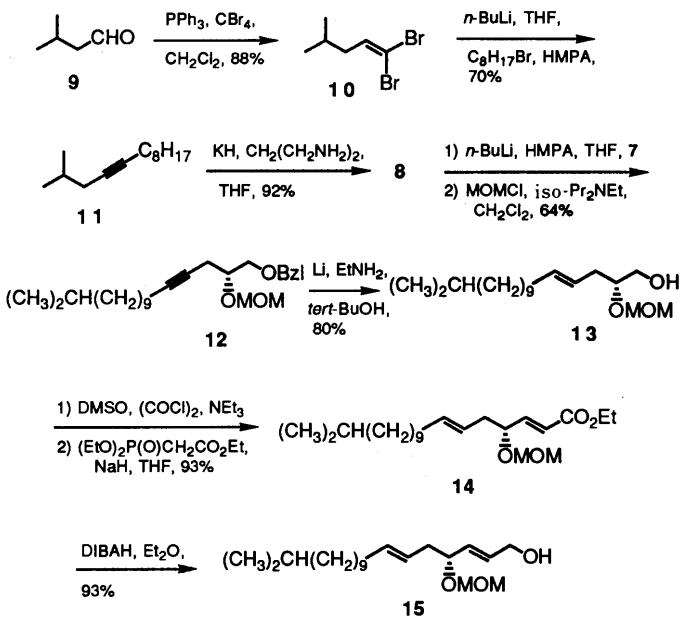


Chart 2

butanol gave an alcohol (**13**) bearing a *trans*-double bond in an 80% yield. Swern oxidation[8] of **13** followed by Horner–Emmons reaction afforded a *trans*-α,β-unsaturated ester **14** in a 93% yield. The ester **14** was reduced with diisobutylaluminum hydride (DIBAH) to give the (4*R*)-allylic alcohol **15** in a 93% yield.

Asymmetric epoxidation[9] of **15**, using (−)-diisopropyl tartrate (DIPT) as a chiral auxiliary, gave a mixture of the (2*R*,3*R*,4*R*)-epoxy alcohol **6** and the (2*S*,3*S*,4*R*)-epoxy alcohol **16** in a ratio of 3 : 1.[10] The formation of the diastereomeric epoxy alcohols was confirmed by a comparison of the epoxy alcohol prepared by epoxidation of **15** with vanadyl acetylacetonate [$VO(acac)_2$] and *tert*-butyl hydroperoxide (TBHP).[11] The configurations of **6** and **16** were determined by examination of the phytosphingosine tetraacetates **23** and **26**, as described later. The mixture of **6** and **16** was converted into the urethanes **17** and **18**, which were subjected to an intramolecular base-catalyzed epoxide opening with sodium hydride in tetrahydrofuran (THF)[12] to give *N*-benzyloxazolidinones **19** and **20** in 63 and 23% yields from **6** and **16**, respectively, after separation by silica gel chromatography.

Debenzylation of the *N*-benzyloxazolidinone **19** afforded an oxazoline **21** which was subjected to alkaline hydrolysis and cleavage of the MOM protective group to produce the phytosphingosine **5**. Acetylation of **5** to **23** and subsequent hydrogenation gave the phytosphingosine tetraacetate **25**. A comparison of the ^1H- and ^{13}C-nuclear magnetic resonance (NMR) data of **25** with those of authentic (2*S*,3*S*,4*R*)-phytosphingosine tetraacetate given in the literature[4b] revealed that the absolute structure of phytosphingosine **5** was (2*S*,3*S*,4*R*)-2-amino-17-methyl-6-octadecene-1,3,4-triol. The isomeric *N*-benzyloxazolidinone **20** was also converted sequentially into **22**, **24**, and **26** in a similar manner to that described above. The configurations of **24** were 2*R*, 3*R*, and 4*R* on the basis of a comparison of the ^1H- and ^{13}C-NMR data on **26** with those of authentic phytosphingosine tetraacetate, (2*R*,3*R*,4*R*)-2-acetamido-1,3,4-triacetoxyhexadecane.[4b]

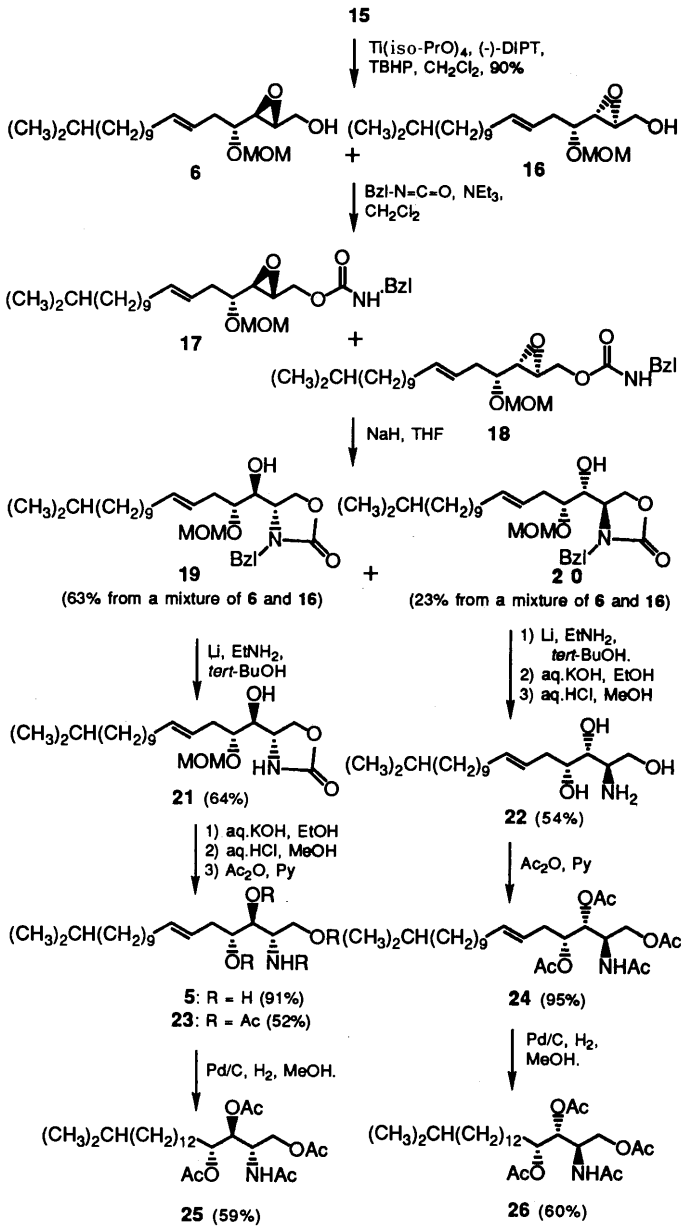


Chart 3

The amidation of **5** and (*R*)-2-(methoxymethoxy)tetracosanoic acid (**27**) was carried out with dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT) in THF[13] to give **28** in a 76% yield. The MOM protective group of **28** was removed with 1,2-ethanedithiol and boron trifluoride etherate[14] to give the ceramide **3** in a 76% yield. At this point, we could introduce all chiralities, the *trans*-double bond, and the iso-type terminal of the ceramide, requisite for synthesis of the cerebroside **2**. The secondary hydroxy groups of **3** were protected as benzoyl esters (except for the primary hydroxy group) by successive tritylation, benzoylation, and detritylation to afford **31** in an 84% yield. Glycosidation of **31** and a D-galactosyl imidate **32** in the presence of boron trifluoride etherate and 4 Å molecular sieves in dichloromethane[15] gave **33** together with **34**, which was also formed in the glycosidation of a pentaacetylglucosyl imidate.[3b,4b] Finally, deacylation of **33** with a methanolic potassium carbonate solution gave the D-galactosyl ceramide **2**. All of the NMR data of **2**

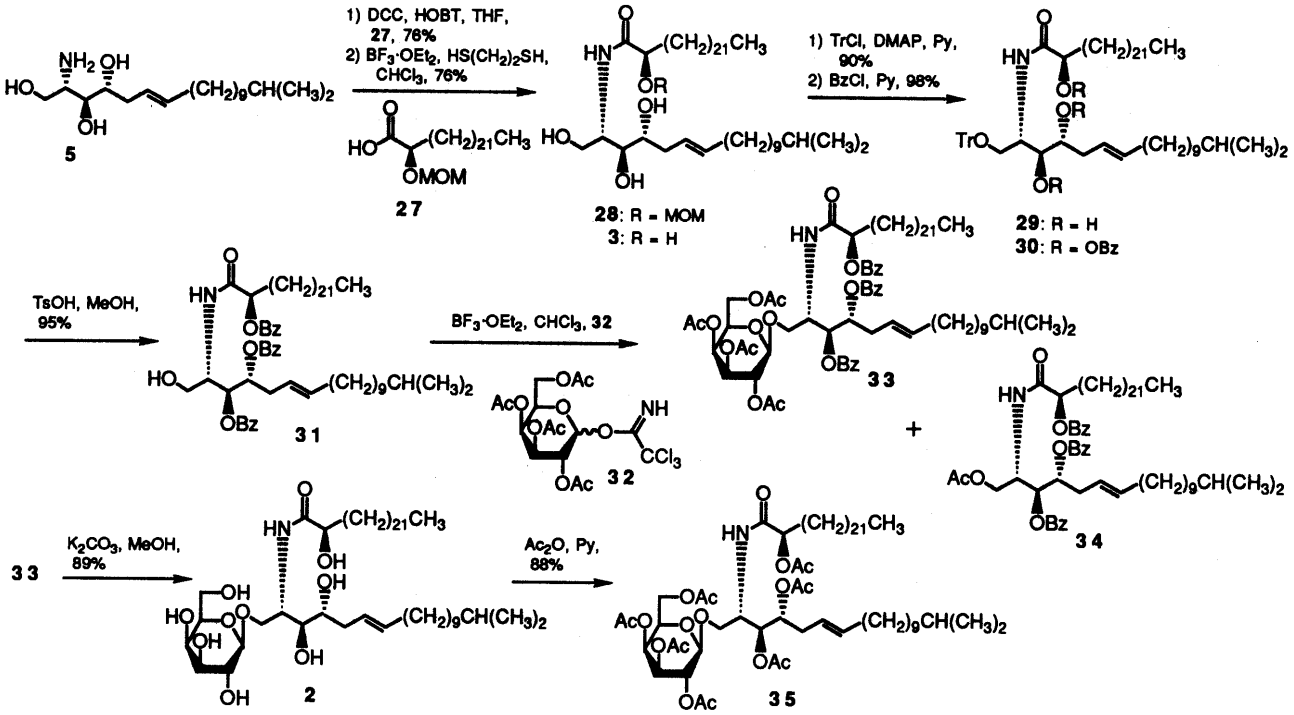


Chart 4

were in good agreement with those of the mixture of **1a** and **1b**.[2] Consequently, the first asymmetric synthesis of the new cerebroside **1b** with hystidine decarboxylase-inhibitory activity has been accomplished from isobutyraldehyde (**9**) in 22 steps. The cerebroside **2** was converted into a heptaacatate **35** in the usual manner. The ^{1}H- and ^{13}C-NMR spectra of **35** were also in good agreement with those of the mixture of acetyl derivatives of **1a** and **1b**.[2]

On the basis of the above results, the absolute structure of **1b** is thought to be 1-*O*-(β-D-galactopyranosyloxy)-(2*S*,3*S*,4*R*,6*E*)-2-[(*R*)-2-hydroxytetracosanoylamino]-17-methyl-6-octadecene-3,4-diol (**2**) and the other component of the mixture, **1a**, which has the same absolute stereochemistry is considered to consist of a nor-phytosphingosine residue with the same hydroxytetracosanoyl group.

Experimental

Melting points were determined with a Yanaco micro melting point apparatus (MP-3). Optical rotations: Jasco DIP-360 polarimeter. Infrared (IR) spectra: Jasco IR-810 spectrometer. ^{1}H-NMR spectra: 90 MHz, JEOL FX-90Q spectrometer; 270 MHz, JEOL GX-270 spectrometer; 400 MHz, JEOL GX-400 spectrometer. ^{13}C-NMR spectra: 67.8 MHz, JEOL GX-270 spectrometer; 100 MHz, JEOL GX-400 spectrometer. NMR spectra were obtained by using $CDCl_3$, C_6D_6, or pyridine-d_5 (C_5D_5N) as the solvent and tetramethylsilane as an internal standard. Field desorption mass spectra (FD-MS) and fast atom bombardment MS (FAB-MS): JEOL JMS-DX300/JMA-3500 data system. Electron impact MS (EI-MS) and high-resolution EI-MS: JEOL JMS-DX300/JMA-3100 data system. Silica gel column chromatography: Kieselgel 60 (70—230 mesh, No. 7734, Merck) if not otherwise specified, or Silica gel BW-300 (200—400 mesh, Fuji Davison Co., Ltd.). All solvents were distilled before use. Anhydrous THF and ether were distilled from sodium and benzophenone. Other anhydrous solvents were distilled from calcium hydride and stored over activated 4-Å molecular sieve pellets. Reactions were carried out under argon, as required.

1,1-Dibromo-4-methyl-1-pentene (10) Carbon tetrabromide (12.4 g, 37.4 mmol) was added at 0 °C to a solution of triphenylphosphine (19.6 g, 74.6 mmol) in CH_2Cl_2 (37 ml). The reaction mixture was stirred at room temperature for 1 h and isobutyraldehyde **9** (2.0 ml, 18.7 mmol) was added at 0 °C to the vigorously stirred mixture. Stirring was continued at room temperature for 1 h, then the mixture was diluted with hexane (400 ml) and the resulting precipitate was filtered off through a cotton plug. The filtrate was concentrated under atmospheric pressure and distilled to give **10** (3.98 g, 16.4 mmol, 88%) as a colorless oil, bp 96 °C (46 Torr). IR (neat): 2950 (C–H), 1615 (C=C), 1383 and 1365 [CH–$(CH_3)_2$] cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 6.40 (1H, t, *J*=7.1 Hz, 2-H), 2.00 (2H, m, 3-H), 1.73 (1H, m, 4-H), 0.93 (6H, d, *J*=6.3 Hz, 5-H and $CHCH_3$). *Anal.* Calcd for $C_6H_{10}Br_2$: C, 29.79; H, 4.17. Found: C, 29.63; H, 3.97.

2-Methyl-4-tridecyne (11) *n*-Butyllithium (73.2 ml, 110 mmol, 1.5 M in hexane) was added at −78 °C to a solution of **10** (8.57 g, 35.4 mmol) in THF (71 ml). The reaction mixture was allowed to warm to room temperature over 1.5 h and then cooled to −78 °C. A solution of 1-bromononane (6.44 ml, 37.2 mmol) in HMPA (71 ml) was added at −78 °C to the mixture. After being stirred at −78 °C for 30 min, the mixture was allowed to warm to room temperature over 1.5 h, diluted with hexane, and washed with water. The aqueous layer was extracted with hexane. The combined hexane layer was dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was distilled to give **11** (4.85 g, 70% yield) as a colorless oil, bp 125 °C (17 Torr). IR (neat): 2955 (C–H), 2930, 2850, 1383 and 1365 [CH–$(CH_3)_2$] cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 2.08 (4H, m, 3-H and 6-H), 1.38 (12H, m), 0.96 (6H, d, *J*=6.4 Hz, 1-H and $CHCH_3$). EI-MS *m/z* (%): 194 (M^+, 2), 151 ($M^+-C_3H_7$, 5), 81 ($M^+-C_8H_{17}$, 100). *Anal.* Calcd for $C_{14}H_{26}$: C, 86.51; H, 13.48. Found: C, 86.55; H, 13.48.

12-Methyl-1-tridecyne (8) 1,3-Diaminopropane (31 ml) was added to KH (2.24 g, 55.9 mmol; 20—25% wt. KH dispersion in mineral oil was washed 3 times with hexane and dried) under stirring and cooling in an ice bath. The reaction mixture was stirred at room temperature for 1 h and **11** (5.97 g, 30.7 mmol) was added dropwise to the vigorously stirred mixture. Stirring was continued at room temperature for 5 min, then the reaction mixture was quenched with water and extracted with hexane. The extracts were washed consecutively with water, 10% aqueous HCl, and water, dried (Na_2SO_4), filtered, and concentrated under atmospheric pressure. The residue was distilled to give **8** (5.50 g, 92% yield) as a colorless oil, bp 85 °C (1 Torr). IR (neat): 3315 (≡C–H), 2955 (C–H), 2930, 2855, 2120 (C≡C), 1383 and 1365 [CH–$(CH_3)_2$] cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 2.18 (2H, dd, *J*=7.0, 2.6 Hz, 3-H), 1.93 (1H, t, *J*=2.6, 1-H), 1.27 (10H, m), 0.86 (6H, d, *J*=6.6 Hz, 13-H and $CHCH_3$). EI-MS *m/z* (%): 194 (M^+, 1), 179 (M^+-CH_3, 3), 151 ($M^+-C_3H_7$, 5), 109 ($M^+-C_6H_{13}$, 100). *Anal.* Calcd for $C_{14}H_{26}$: C, 86.51; H, 13.48. Found: C, 86.48; H, 13.30.

(2*R*)-1-Benzyloxy-2-(methoxymethoxy)-15-methyl-4-hexadecyne (12) *n*-Butyllithium (5.37 ml, 8.06 mmol, 1.5 M hexane solution) was added at −25 °C to a solution of **8** (2.0 ml, 8.06 mmol) in THF (8 ml) and the

mixture was allowed to warm to room temperature over 10 min. The epoxide **7** (1.48 ml, 8.07 mmol) and HMPA (16 ml) were added at −25 °C to the mixture. After being stirred at room temperature for 30 min, the reaction mixture was diluted with hexane–Et_2O (2 : 1), washed with water, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane–AcOEt, 8 : 2) to give a mixture (2.55 g) of the desired alcohol and a trace of unreacted **7**. Chloromethyl methyl ether (812 μl, 10.7 mmol) and *N,N*-diisopropylethylamine (2.11 ml, 12.1 mmol) were added to the mixture (2.55 g). After being stirred at room temperature for 20 h, the reaction mixture was concentrated *in vacuo* and chromatographed on silica gel (hexane–AcOEt, 85 : 15) to give **12** (2.16 g, 64% yield from **8**) as a colorless oil. $[\alpha]_D^{26}$ −5.68° (*c* = 2.66, $CHCl_3$). IR (neat): 2920 (C–H), 2930, 2855, 735 and 790 (Ar–H) cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 7.32 (5H, s, aromatic H), 4.74 (2H, s, OCH_2O), 4.57 (2H, s, benzylic H), 3.85 (1H, m, 2-H), 3.62 (2H, dd, *J* = 4.9, 1.1 Hz, 1-H), 3.38 (3H, s, OCH_3), 2.48 (2H, m, 3-H), 2.11 (2H, br s, 6-H), 1.25 (m), 0.86 (6H, d, *J* = 6.1 Hz, 16-H and $CHC\underline{H}_3$). EI-MS *m/z* (%): 402 (M^+, 4), 357 ($M^+ - CH_2OCH_3$, 93), 207 ($M^+ - C_{11}H_{15}O_3$, 100). *Anal.* Calcd for $C_{26}H_{42}O_3$: C, 77.56; H, 10.51. Found: C, 77.32; H, 10.35.

(2*R*)-2-(Methoxymethoxy)-15-methyl-4-hexadecen-1-ol (13) Liquid $EtNH_2$ (50 ml) was added at 0 °C to a solution of **12** (2.05 g, 5.09 mmol) in *tert*-BuOH (10 ml), then several pieces of lithium (300 mg) were added at −20 °C. The blue-colored mixture was stirred at −20 °C for 10 min and quenched with ammonium chloride. The unreacted lithium was removed, then the mixture was concentrated to half the original volume, diluted with ether, washed with water, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane–AcOEt, 7 : 3) to give **13** (1.28 g, 80% yield) as a colorless oil. $[\alpha]_D^{26}$ −20.0° (*c* = 2.72, $CHCl_3$). IR (neat): 3440 (O–H), 2920 (C–H), 2855, 1381 and 1362 [CH–$(CH_3)_2$] cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 4.71 (2H, s, OCH_2O), 3.54 (3H, m, 1-H and 2-H), 3.42 (3H, s, OCH_3), 2.82 (1H, m, OH), 2.22 (2H, m, 3-H), 1.99 (2H, m, 6-H), 1.25 (m), 0.86 (6H, d, *J* = 6.1 Hz, 16-H and $CHC\underline{H}_3$). FD-MS *m/z* (%): 315 (M^+ + H, 100), 105 (50). *Anal.* Calcd for $C_{19}H_{38}O_3$: C, 72.56; H, 12.18. Found: C, 72.27; H, 12.23.

Ethyl (2*E*,4*R*,6*E*)-4-(Methoxymethoxy)-17-methyl-2,6-octadecadien-1-ate (14) Dimethyl sulfoxide (DMSO) (549 μl, 7.74 mmol) was added at −60 °C to a solution of oxalyl chloride (506 μl, 5.80 mmol) in CH_2Cl_2 (9.67 ml). The mixture was stirred at −60 °C for 2 min and a solution of **13** (1.21 g, 3.87 mmol) in CH_2Cl_2 (3.87 ml) was added. After being stirred at −50 to −60 °C for 15 min, the mixture was treated with triethylamine (3.24 ml, 23.2 mmol), stirred at −60 °C for 10 min, and then allowed to warm to room temperature over 1 h. The reaction mixture was diluted with ether, washed consecutively with 5% aqueous H_3PO_4, water, and aqueous saturated $NaHCO_3$, dried ($MgSO_4$), filtered, and concentrated *in vacuo*. The residue was used for the next reaction without further purification. NaH (242 mg, 6.05 mmol, 60% wt. dispersion in mineral oil) was added to a solution of triethyl phosphonoacetate (1.26 ml, 6.35 mmol) in THF (8 ml) under cooling with a water bath. A solution of the residue in THF (8 ml) was added to the mixture. After being stirred at room temperature for 20 min, the mixture was diluted with ether, washed with water, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The resulting residue was chromatographed on silica gel (hexane–AcOEt, 8 : 2) to give **14** (1.38 g, 93% yield) as a colorless oil. $[\alpha]_D^{27}$ +16.8° (*c* = 5.66, $CHCl_3$). IR (neat): 2920 (C–H), 2850, 1722 (C=O), 1566 (C=C) cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 6.83 (1H, dd, *J* = 15.6, 6.0 Hz, 3-H), 5.97 (1H, dd, *J* = 15.8, 1.1 Hz, 2-H), 5.51 (1H, d, *J* = 5.5 Hz, 6-H), 5.39 (1H, d, *J* = 5.3 Hz, 7-H), 4.62 (2H, s, OCH_2O), 4.20 (2H, q, *J* = 7.1 Hz, $OC\underline{H}_2CH_3$), 3.37 (3H, s, OCH_3), 2.31 (2H, m, 5-H), 1.99 (2H, m, 8-H), 1.26 (m), 0.86 (6H, d, *J* = 6.1 Hz, 18-H and $CHC\underline{H}_3$). FD-MS *m/z* (%): 382 (M^+, 30), 173 (100). *Anal.* Calcd for $C_{23}H_{42}O_4$: C, 72.20; H, 11.06. Found: C, 71.97; H, 11.06.

(2*E*,4*R*,6*E*)-4-(Methoxymethoxy)-17-methyl-2,6-octadecadien-1-ol (15) A DIBAH solution (12.7 ml, 12.7 mmol, 1.0 M in hexane) was added at −25 °C to a solution of **14** (1.22 g, 3.20 mmol) in ether (12.8 mmol). The reaction mixture was allowed to warm to room temperature and stirred at ambient temperature for 12 h. After dropwise addition of a 5% aqueous H_3PO_4 solution, the mixture was extracted with ether. The extracts were acidified with aqueous 5% H_3PO_4, washed with brine and aqueous saturated $NaHCO_3$, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was chromatographed on silica gel (BW-300, hexane–AcOEt, 7 : 3) to give **15** (1.01 g, 93% yield) as a colorless oil. $[\alpha]_D^{27}$ +30.5° (*c* = 0.78, $CHCl_3$). IR (neat): 3420 (O–H), 2930 (C–H), 2855 cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 5.71 (2H, m, 2-H and 3-H), 5.46 (2H, m, 6-H and 7-H), 4.69 (1H, d, *J* = 6.8 Hz, OCH_aO), 4.53 (1H, d, *J* = 6.6 Hz, OCH_bO), 3.35 (3H, s, OCH_3), 2.26 (2H, m, 5-H), 1.99 (2H, m, 8-H), 1.25 (m), 0.85 (6H, d, *J* = 6.1 Hz, 18-H and $CHC\underline{H}_3$). FD-MS *m/z* (%): 341 (M^+ + H, 2), 131 (100). *Anal.* Calcd for $C_{21}H_{40}O_3$: C, 74.07; H, 11.84. Found: C, 73.89; H, 11.87.

(2*R*,3*R*,4*R*,6*E*)-2,3-Epoxy-4-(methoxymethoxy)-17-methyl-6-octadecen-1-ol (6) and Its (2*S*,3*S*,4*R*,6*E*) Isomer (16) A mixture of titanium tetraisopropoxide (325 μl, 1.09 mmol) and powdered 4-Å molecular sieves (400 mg) in CH_2Cl_2 was cooled to −30 °C. (−)-Diisopropyl tartrate (290 μl, 1.37 mmol) and a *tert*-butyl hydroperoxide solution (1.68 ml, 4.10 mmol, 2.44 M in CH_2Cl_2) were added dropwise at −30 °C, sequentially. The reaction mixture was stirred at −25 °C for 30 min and a solution of **15** (931 mg, 2.74 mmol) in CH_2Cl_2 (11 ml) was added dropwise. The resulting mixture was stored at −20 °C for 15 h in a refrigerator and quenched with water (6.5 ml). The mixture was allowed to warm to room temperature over 1 h while being stirred vigorously, and filtered through a pad of Celite 545. The filtrate was treated with an aqueous 1 N KOH solution (5.5 ml) saturated with NaCl, stirred vigorously for 30 min, and filtered through a pad of Celite 545. The filtrate was extracted with $CHCl_3$. The extracts were washed with water, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane–AcOEt, 7 : 3) to give a mixture of **6** and **16** (854 mg, 88% yield) as a colorless oil. $[\alpha]_D^{27}$ +4.65° (*c* = 2.69, $CHCl_3$). IR (neat): 3440 (O–H), 2930 (C–H), 2855 cm^{-1}. FD-MS *m/z* (%): 357 (M^+ + H, 66), 147 (100). *Anal.* Calcd for $C_{21}H_{40}O_4$: C, 70.74; H, 11.31. Found: C, 70.56; H, 11.41.

(2*R*,3*R*,4*R*,6*E*)-1-[(Benzylcarbamoyl)oxy]-2,3-epoxy-4-(methoxymethoxy)-17-methyl-6-octadecene (17) and Its (2*S*,3*S*,4*R*,6*E*) Isomer (18) Triethylamine (962 μl, 6.90 mmol) and benzyl isocyanate (426 μl, 3.45 mmol) were added sequentially to a solution of a mixture of **6** and **16** (820 mg, 2.30 mmol) in CH_2Cl_2 (9.2 ml). The whole was stirred at room temperature for 15 h, then treated with water (83 μl, 4.6 mmol) and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane–AcOEt, 3 : 1) to give a mixture of **17** and **18** (1.09 g, 97% yield) as a colorless oil. $[\alpha]_D^{27}$ +13.0° (*c* = 2.66, $CHCl_3$). IR (CCl_4): 2930 (C–H), 2855, 1735 (C=O) cm^{-1}. FAB-MS *m/z* (%): 490 (M^+ + H, 25), 91 ($PhCH_2^+$, 100). *Anal.* Calcd for $C_{29}H_{47}NO_5$: C, 71.13; H, 9.67; N, 2.86. Found: C, 71.39; H, 9.69; N, 2.83.

(4*S*,1′*S*,2′*R*,4′*E*)-3-Benzyl-4-[1′-hydroxy-2′-(methoxymethoxy)-15′-methyl-4′-hexadecenyl]-2-oxazolidinone (19) and Its (4*R*,1′*R*,2′*R*,4′*E*) Isomer (20) A solution of a mixture of **17** and **18** (1.03 g, 2.11 mmol) in THF (42 ml) was added to NaH (146 mg, 3.65 mmol, 60% wt. oil suspension), and the mixture was stirred at 50 °C for 1 h. Then the reaction was quenched with 5% aqueous H_3PO_4 and the mixture was extracted with $CHCl_3$. The extracts were washed with aqueous saturated $NaHCO_3$, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane–AcOEt, 63 : 35—6 : 4) to give **19** (683 mg, 66% yield) and **20** (249 mg, 24% yield).

19: Viscous colorless oil. $[\alpha]_D^{27}$ −19.3° (*c* = 2.02, $CHCl_3$). IR (CCl_4): 3380 (O–H), 2930 (C–H), 2855, 1738 (C=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.32 (5H, m, aromatic H), 5.40 (1H, dt, *J* = 14.6, 7.1 Hz, 4′-H), 5.22 (1H, dt, *J* = 14.8, 7.3 Hz, 5′-H), 4.70 (1H, d, *J* = 15.5 Hz, benzylic H_a), 4.57 (1H, d, *J* = 6.4 Hz, OCH_aO), 4.53 (1H, d, *J* = 6.6 Hz, OCH_bO), 4.47 (1H, dd, *J* = 8.9, 7.4 Hz, 5-H_a), 4.25 (1H, d, *J* = 15.2 Hz, benzylic H_b), 4.23 (1H, t, *J* = 8.9 Hz, 5-H_b), 3.90 (1H, dd, *J* = 7.5, 1.5 Hz, 4-H), 3.81 (1H, m, 1′-H), 3.57 (1H, dd, *J* = 5.8, 5.8 Hz, 2′-H), 3.29 (3H, s, OCH_3), 2.87 (1H, d, *J* = 5.3 Hz, OH), 2.12 (2H, m, 3′-H), 1.95 (2H, m, 6′-H), 1.51 (1H, dqq, *J* = 6.5, 6.5, 6.5 Hz, 15′-H), 1.26 (m), 0.86 (6H, d, *J* = 6.6 Hz, 16′-H and $CHC\underline{H}_3$). FD-MS *m/z* (%): 490 (M^+ + H, 98), 489 (M^+, 100). *Anal.* Calcd for $C_{29}H_{47}NO_5$: C, 71.13; H, 9.67; N, 2.86. Found: C, 71.01; H, 9.75; N, 2.71.

20: Viscous colorless oil. $[\alpha]_D^{27}$ +5.99° (*c* = 1.74, $CHCl_3$). IR (CCl_4): 3410 (O–H), 2930 (C–H), 2855, 1740 (C=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.32 (5H, m, aromatic H), 5.40 (1H, dt, *J* = 14.6, 7.1 Hz, 4′-H), 5.20 (1H, dt, *J* = 14.7, 7.4 Hz, 5′-H), 4.80 (1H, d, *J* = 15.2 Hz, benzylic H_a), 4.64 (1H, d, *J* = 6.9 Hz, OCH_aO), 4.60 (1H, d, *J* = 6.8 Hz, OCH_bO), 4.48 (1H, dd, *J* = 8.7, 6.8 Hz, 5-H_a), 4.21 (1H, t, *J* = 8.8 Hz, 5-H_b), 4.18 (1H, d, *J* = 15.7 Hz, benzylic H_b), 3.87 (1H, m, 1′-H), 3.71 (1H, dd, *J* = 7.1, 1.7 Hz, 4-H), 3.34 (3H, s, OCH_3), 3.15 (1H, d, *J* = 3.6 Hz, OH), 2.25 (2H, m, 3′-H), 2.11 (2H, m, 6′-H), 1.51 (1H, dqq, *J* = 6.6, 6.6, 6.6 Hz, 15′-H), 1.26 (m), 0.86 (6H, d, *J* = 6.6 Hz, 16′-H and $CHC\underline{H}_3$). FD-MS *m/z* (%): 490 (M^+ + H, 93), 489 (M^+, 100). *Anal.* Calcd for $C_{29}H_{47}NO_5$: C, 71.13; H, 9.67; N, 2.86. Found: C, 71.01; H, 9.61; N, 2.84.

(4*S*,1′*S*,2′*R*,4′*E*)-4-[1′-Hydroxy-2′-(methoxymethoxy)-15′-methyl-4′-hexadecenyl]-2-oxazolidinone (21) Liquid $EtNH_2$ (30 ml) was added at 0 °C to a solution of **19** (631 mg, 5.09 mmol) in *tert*-BuOH (6.3 ml). Several pieces of lithium (100 mg) were added at −78 °C to the mixture. The blue-colored mixture was stirred at −78 °C for 10 min and the reaction

was quenched with ammonium chloride. After removal of the unreacted lithium, the mixture was concentrated to half of the original volume, diluted with ether, washed with brine, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane–AcOEt, 3:7) to give **21** (356 mg, 69% yield) as a colorless oil. $[\alpha]_D^{27}$ −26.6° (c=2.08, $CHCl_3$). IR (CCl_4): 3400 and 3280 (O–H and N–H), 2930 (C–H), 2850, 1752 (C=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 6.37 (1H, s, NH), 5.54 (1H, dt, J=14.4, 7.0 Hz, 4'-H), 5.39 (1H, dt, J=14.7, 7.2 Hz, 5'-H), 4.63 (2H, s, OCH_2O), 4.52 (1H, dd, J=8.9, 6.3 Hz, 5H$_a$), 4.41 (1H, t, J=8.7 Hz, 5-H$_b$), 4.08 (1H, m, 4-H), 3.68 (1H, t, J=4.6 Hz, 1'-H), 3.61 (1H, dt, J=3.1, 3.1 Hz, 2'-H), 3.37 (3H, s, OCH_3), 2.31 (2H, m, 3'-H), 2.00 (2H, m, 6'-H), 1.51 (1H, dqq, J=6.6, 6.6, 6.6 Hz, 15'-H), 1.26 (m), 0.86 (6H, d, J=6.6 Hz, 16'-H and CHC$\underline{H}_3$). FD-MS m/z (%): 400 (M^++H, 100). *Anal.* Calcd for $C_{22}H_{41}NO_5$: C, 66.13; H, 10.34; N, 3.51. Found: C, 65.91; H, 10.43; N, 3.50.

(2*S*,3*S*,4*R*,6*E*)-2-Amino-17-methyl-6-octadecene-1,3,4-triol (5) An aqueous 2 N KOH solution (3.52 ml, 7.04 mmol) was added to a solution of **21** (282 mg, 70.6 μmol) in EtOH (3.5 ml). The reaction mixture was heated for 5 h at reflux, cooled to room temperature, and extracted with Et_2O. The extracts were dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The residue (268 mg) was dissolved in 2 N HCl in MeOH (2 ml). The resulting mixture was heated for 5 h at reflux and alkalinized with a 0.1 M solution of K_2CO_3 in MeOH. The precipitate was filtered off through a cotton plug. The filtrate was concentrated by blowing a stream of nitrogen gas over it. The residue was chromatographed on silica gel (BW-300, $CHCl_3$–MeOH–H_2O, 7:3:0.5) to give crude **5** (515 mg), which was passed through a Sephadex LH-20 column (MeOH as the eluent). The obtained **5** (212 mg, 91% yield) was a viscous colorless oil. ^{1}H-NMR (270 MHz, C_5D_5N) δ: 5.91 (1H, dt, J=14.8, 7.3 Hz, 6-H), 5.67 (1H, dt, J=14.6, 7.1 Hz, 7-H), 4.78 (1H, dd, J=8.7, 3.3 Hz, 3-H), 4.72 (2H, m, 1-H), 4.27 (1H, dt, J=8.4, 2.8 Hz, 4-H), 3.05 (1H, m, 5-H$_a$), 2.61 (1H, quint., J=7.3 Hz, 5-H$_b$), 2.00 (2H, q, J=6.6 Hz, 8-H), 1.24 (m), 0.87 (6H, d, J=6.6 Hz, 18-H and CHC$\underline{H}_3$). ^{13}C-NMR (67.8 MHz, C_5D_5N) δ: 133.2 (d, (C7)), 127.4 (d, (C6)), 73.4 (d, (C3 and C4)), 60.0 (t, (C1)), 56.7 (d, (C2)), 39.2 (t), 38.6 (t, (C5)), 33.1 (t, (C8)), 30.2 (t), 30.0 (t), 29.9 (t), 29.8 (t), 29.6 (t), 28.2 (d, (C17)), 27.7 (t), 22.8 (q, (C18 and CH$\underline{C}H_3$)). FD-MS m/z (%): 330 (M^++H, 100). This product was used in the next reaction without further purification.

(2*S*,3*S*,4*R*,6*E*)-2-Acetamido-17-methyl-1,3,4-triacetoxy-6-octadecene (23) Acetic anhydride (147 μl, 1.56 mmol) and pyridine (127 μl, 1.57 mmol) were added to **5** (34.3 mg, 104 μmol). The reaction mixture was stirred at 50 °C for 1 h and at room temperature for 15 h, then concentrated by blowing nitrogen gas over it. The residue was chromatographed on silica gel (BW-300, hexane–AcOEt, 2:3) to give **23** (26.8 mg, 52% yield) as a colorless viscous oil. $[\alpha]_D^{26}$ +9.76° (c=1.24, $CHCl_3$). IR (CCl_4): 3370 (N–H), 2930 (C–H), 2855, 1755 (OC=O), 1690 (NC=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 6.06 (1H, d, J=9.4 Hz, NH), 5.49 (1H, dt, J=14.7, 7.2 Hz, 6-H), 5.28 (1H, dt, J=14.7, 7.3 Hz, 7-H), 5.09 (1H, dd, J=7.6, 3.4 Hz, 3-H), 4.96 (1H, dt, J=9.1, 3.8 Hz, 4-H), 4.51 (1H, m, 2-H), 4.26 (1H, dd, J=11.7, 5.0 Hz, 1-H$_a$), 4.01 (1H, dd, J=11.6, 3.4 Hz, 1-H$_b$), 2.43 (1H, m, 5-H$_a$), 2.28 (1H, m, 5-H$_b$), 2.09 (3H, s, CH_3CO), 2.05 (3H, s, CH_3CO), 2.04 (3H, s, CH_3CO), 2.02 (3H, s, CH_3CO), 1.96 (2H, m, 8-H), 1.51 (1H, dqq, J=6.6, 6.6, 6.6 Hz, 17-H), 1.25 (m), 0.86 (6H, d, J=6.6 Hz, 18-H and CHC$\underline{H}_3$). ^{13}C-NMR (67.8 MHz, $CDCl_3$) δ: 170.9 (s, $\underline{C}OCH_3$), 170.2 (s, $\underline{C}OCH_3$), 169.7 (s, $\underline{C}OCH_3$), 134.7 (d, (C6)), 124.0 (d, (C7)), 72.5 (d, (C4)), 72.2 (d, (C3)), 62.8 (t, (C1)), 47.7 (d, (C2)), 39.1 (t), 32.6 (t), 32.1 (t, (C5)), 30.0 (t), 29.7 (t), 29.6 (t), 29.5 (t), 29.4 (t), 29.2 (t), 28.0 (d, (C17)), 27.4 (t), 23.3 (q, $\underline{C}OCH_3$), 22.7 (q, (C18 and CH$\underline{C}H_3$)), 21.0 (q, $\underline{C}OCH_3$), 20.8 (q, $\underline{C}OCH_3$). FD-MS m/z (%): 498 (M^++H, 100). *Anal.* Calcd for $C_{27}H_{47}NO_7$: C, 65.16; H, 9.52; N, 2.81. Found: C, 64.83; H, 9.40; N, 2.77.

(2*S*,3*S*,4*R*)-2-Acetamido-17-methyl-1,3,4-triacetoxyoctadecane (25) A suspension of **23** (23.8 mg, 47.8 μmol) and 10% palladium on carbon (22.4 mg) in MeOH (2.4 ml) was stirred for 7 h under a hydrogen atmosphere. The mixture was filtered through a pad of Celite 545 and concentrated *in vacuo*. The residue was chromatographed on silica gel (BW-300, hexane–AcOEt, 2:3) to give **25** (14.2 mg, 59% yield) as a colorless oil. $[\alpha]_D^{26}$ +26.1° (c=0.71, $CHCl_3$). IR (CCl_4): 3350 (N–H), 2930 (C–H), 2855, 1750 (OC=O), 1690 (NC=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 6.00 (1H, d, J=9.4 Hz, NH), 5.11 (1H, dd, J=8.3, 3.1 Hz, 3-H), 4.93 (1H, dt, J=9.3, 3.5 Hz, 4-H), 4.47 (1H, m, 2-H), 4.29 (1H, dd, J=11.6, 4.9 Hz, 1-H$_a$), 4.00 (1H, dd, J=11.6, 3.1 Hz, 1-H$_b$), 2.08 (3H, s, CH_3CO), 2.05 (6H, s, CH_3CO × 2), 2.03 (3H, s, CH_3CO), 1.51 (1H, dqq, J=6.6, 6.6, 6.6 Hz, 17-H), 1.25 (m), 0.86 (6H, d, J=6.6 Hz, 18-H and CHC$\underline{H}_3$). ^{13}C-NMR (67.8 MHz, $CDCl_3$) δ: 171.2 (s, $\underline{C}OCH_3$), 170.9 (s, $\underline{C}OCH_3$), 170.1 (s, $\underline{C}OCH_3$), 169.7 (s, $\underline{C}OCH_3$), 73.0 (d, (C3)), 72.0 (d, (C4)), 62.9 (t, (C1)), 47.6 (d, (C2)), 39.1 (t), 30.0 (t), 29.7 (t), 29.6 (t), 29.5 (t), 29.3 (t), 28.2 (d), 28.0 (d, (C17)), 27.4 (t), 25.5 (d), 23.3 (q, $\underline{C}OCH_3$), 22.7 (q, (C18 and CH$\underline{C}H_3$)), 21.0 (q, $\underline{C}OCH_3$), 20.7 (q, $\underline{C}OCH_3$). FD-MS m/z (%): 500 (M^++H, 100). *Anal.* Calcd for $C_{27}H_{49}NO_7$: C, 64.90; H, 9.88; N, 2.80. Found: C, 64.51; H, 9.77; N, 2.71.

(2*R*,3*R*,4*R*,6*E*)-2-Amino-17-methyl-6-octadecene-1,3,4-triol (22) According to the procedures described for the preparation of **5** from **19**, compound **20** (332 mg) was converted into **22** (120 mg, 54% yield) as a colorless viscous oil. ^{1}H-NMR (270 MHz, C_5D_5N) δ: 5.64 (1H, dt, J=14.5, 7.1 Hz, 7-H), 2.78 (2H, t, J=6.7 Hz, 5-H), 2.00 (2H, q, J=6.8 Hz, 8-H), 1.49 (1H, dqq, J=6.6, 6.6, 6.6 Hz, 17-H), 1.25 (m), 0.87 (6H, d, J=6.6 Hz, 18-H and CHC$\underline{H}_3$). FD-MS m/z (%): 330 (M^++H, 100). This product was used in the next reaction without further purification.

(2*R*,3*R*,4*R*,6*E*)-2-Acetamido-17-methyl-1,3,4-triacetoxy-6-octadecene (24) According to the procedures described for the preparation of **23**, compound **22** (49.1 mg) was converted into **24** (70.1 mg, 95% yield) as a colorless oil. $[\alpha]_D^{27}$ −2.14° (c=1.00, $CHCl_3$). IR (CCl_4): 3320 (N–H), 2930 (C–H), 2855, 1745 (OC=O), 1695 (NC=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 5.73 (1H, d, J=9.9 Hz, NH), 5.48 (1H, dt, J=14.6, 7.1 Hz, 6-H), 5.27 (1H, dt, J=14.9, 7.4 Hz, 7-H), 4.54 (1H, m, 2-H), 4.22 (1H, dd, J=11.7, 4.5 Hz, 1-H$_a$), 3.96 (1H, dd, J=11.7, 3.3 Hz, 1-H$_b$), 2.13 (3H, s, CH_3CO), 2.08 (3H, s, CH_3CO), 2.07 (3H, s, CH_3CO), 1.98 (3H, s, CH_3CO), 1.51 (1H, dqq, J=6.6, 6.6, 6.6 Hz, 17-H), 1.25 (m), 0.86 (6H, d, J=6.6 Hz, 18-H and CHC$\underline{H}_3$). ^{13}C-NMR (67.8 MHz, $CDCl_3$) δ: 170.8 (s, $\underline{C}OCH_3$), 170.4 (s, $\underline{C}OCH_3$), 170.3 (s, $\underline{C}OCH_3$), 169.6 (s, $\underline{C}OCH_3$), 135.3 (d, (C6)), 123.3 (d, (C7)), 71.6 (d, (C4)), 70.9 (d, (C3)), 63.1 (t, (C1)), 47.5 (d, (C2)), 39.1 (t), 34.4 (t, (C5)), 32.6 (t), 29.9 (t), 29.7 (t), 29.5 (t), 29.4 (t), 29.2 (t), 28.0 (d, (C17)), 27.4 (q, $\underline{C}OCH_3$), 23.3 (q, $\underline{C}OCH_3$), 22.7 (q, (C18 and CH$\underline{C}H_3$)), 21.0 (q, $\underline{C}OCH_3$), 20.8 (q, $\underline{C}OCH_3$). FD-MS m/z (%): 498 (M^++H, 100). *Anal.* Calcd for $C_{27}H_{47}NO_7$: C, 65.16; H, 9.52; N, 2.81. Found: C, 64.97; H, 9.43; N, 2.75.

(2*R*,3*R*,4*R*)-2-Acetamido-17-methyl-1,3,4-triacetoxyoctadecane (26) According to the procedures described for the preparation of **25**, compound **24** (36.3 mg) was converted into **26** (21.7 mg, 60% yield) as a colorless powder. mp 77 °C. $[\alpha]_D^{27}$ −3.09° (c=1.09, $CHCl_3$). IR (CCl_4): 3380 (N–H), 2920 (C–H), 2850, 1745 (OC=O), 1690 (NC=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 5.79 (1H, d, J=9.7 Hz, NH), 5.09 (2H, m, 3-H and 4-H), 4.53 (1H, m, 2-H), 4.23 (1H, dd, J=11.7, 4.6 Hz, 1-H$_a$), 3.97 (1H, dd, J=11.7, 3.3 Hz, 1-H$_b$), 2.13 (3H, s, CH_3CO), 2.09 (6H, s, CH_3CO × 2), 2.07 (3H, s, CH_3CO), 1.98 (3H, s, CH_3CO), 1.51 (1H, dqq, J=6.6, 6.6, 6.6 Hz, 17-H), 1.24 (m), 0.86 (6H, d, J=6.6 Hz, 18-H and CHC$\underline{H}_3$). ^{1}H-NMR (400 MHz, C_6D_6) δ: 5.30 (1H, d, J=9.9 Hz, NH), 5.23 (1H, ddd, J=8.1, 5.4, 2.6 Hz, 4-H), 4.80 (1H, ddt, J=9.6, 5.1, 3.2 Hz, 3-H), 4.25 (1H, dd, J=11.7, 5.1 Hz, 1-H$_a$), 3.90 (1H, dd, J=11.7, 3.2 Hz, 1-H$_b$), 1.99 (3H, s, CH_3CO), 1.78 (6H, s, CH_3CO × 2), 1.73 (3H, s, CH_3CO), 1.66 (3H, s, CH_3CO), 1.31 (m), 0.91 (6H, d, J=6.6 Hz, 18-H and CHC$\underline{H}_3$). ^{13}C-NMR (67.8 MHz, $CDCl_3$) δ: 170.7 (s, $\underline{C}OCH_3$), 170.6 (s, $\underline{C}OCH_3$), 170.3 (s, $\underline{C}OCH_3$), 169.6 (s, $\underline{C}OCH_3$), 71.9 (d, (C3)), 71.2 (d, (C4)), 63.2 (t, (C1)), 47.5 (d, (C2)), 39.1 (t), 30.9 (t), 30.0 (t), 29.7 (t), 29.6 (t), 29.5 (t), 29.4 (t), 28.0 (d, (C17)), 27.4 (t), 25.2 (d), 23.3 (q, $\underline{C}OCH_3$), 22.7 (q, (C18 and CH$\underline{C}H_3$)), 21.0 (q, $\underline{C}OCH_3$), 20.75 (q, $\underline{C}OCH_3$), 20.70 (q, $\underline{C}OCH_3$). ^{13}C-NMR (100 MHz, C_6D_6) δ: 170.4 (s, $\underline{C}OCH_3$), 170.2 (s, $\underline{C}OCH_3$), 169.1 (s, $\underline{C}OCH_3$), 71.9 (d, (C3)), 70.8 (d, (C4)), 63.6 (t, (C1)), 47.5 (d, (C2)), 39.5 (t), 31.6 (t), 30.4 (t), 30.2 (t), 30.1 (t), 30.0 (t), 27.9 (t), 25.9 (d, (C17)), 22.9 (q, (C18, $\underline{C}OCH_3$, and CH$\underline{C}H_3$)), 20.9 (q, $\underline{C}OCH_3$), 20.4 (q, $\underline{C}OCH_3$), 20.2 (q, $\underline{C}OCH_3$). FD-MS m/z (%): 500 (M^++H, 100). *Anal.* Calcd for $C_{27}H_{49}NO_7$: C, 64.90; H, 9.88; N, 2.80. Found: C, 64.79; H, 9.83; N, 2.77.

(2*S*,3*S*,4*R*,6*E*)-2-[(*R*)-2-(Methoxymethoxy)tetracosanoylamino]-17-methyl-6-octadecene-1,3,4-triol (28) A solution of DCC (44.8 mg, 217 μmol) in THF (1.5 ml) was added to a mixture of **5** (47.7 mg, 145 μmol), **27** (62.2 mg, 145 μmol), and HOBT (33.3 mg, 217 μmol). The reaction mixture was stirred at room temperature for 2 d and filtered through a cotton plug. The filtrate was concentrated and chromatographed on silica gel (BW-300, $CHCl_3$–acetone–water, 85:15:0.3) gave **28** (80.9 mg, 76% yield) as a colorless powder. mp 67.5 °C. $[\alpha]_D^{27}$ +9.08° (c=1.96, $CHCl_3$). IR (CCl_4): 3420 (O–H and N–H), 2930 (C–H), 2850, 1655 (NC=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.41 (1H, d, J=7.6 Hz, NH), 5.60 (1H, dt, J=14.6, 7.1 Hz, 6-H), 5.41 (1H, dt, J=15.0, 7.4 Hz, 7-H), 4.69 (2H, s, OCH_2O), 4.17 (1H, m, 2-H), 4.07 (1H, t, J=5.6 Hz, 2'-H), 3.94 (1H, dd, J=11.9, 2.3 Hz, 1-H$_a$), 3.74 (1H, dd, J=11.9, 4.9 Hz, 1-H$_b$), 3.56 (2H, m, 3-H and 4-H), 3.41 (3H, s, CH_3O), 2.56 (1H, m, 5-H$_a$), 2.17 (1H, quint., J=7.5 Hz, 5-H$_b$), 2.03 (2H, q, J=6.8 Hz, 8-H), 1.77 (2H, m, 3'-H), 1.51 (1H, dqq, J=6.6, 6.6, 6.6 Hz, 17-H), 1.25 (m), 0.864 (3H, t, J=4.1 Hz,

24'-H), 0.860 (6H, d, J=6.6 Hz, 18-H and $CHC\underline{H}_3$). FAB-MS m/z (%): 740 (M^+, 14), 708 (M^+-CH_3OH, 21). *Anal.* Calcd for $C_{45}H_{89}NO_6$: C, 73.02; H, 12.12; N, 1.89. Found: C, 72.77; H, 12.02; N, 1.88.

(2*S*,3*S*,4*R*,6*E*)-2-[(*R*)-2-Hydroxytetracosanoylamino]-17-methyl-6-octadecene-1,3,4-triol (3) $BF_3\cdot OEt_2$ (38.2 μl, 311 μmol) was added to a solution of **28** (76.7 mg, 104 μmol) in 1,2-ethanedithiol (1 ml) and $CHCl_3$ (1 ml), and the mixture was stirred at room temperature for 30 min. The reaction was quenched with triethylamine (144 μl, 1.0 mmol), and the mixture was concentrated by blowing a stream of nitrogen gas over it. The residue was chromatographed on silica gel (BW-300, $CHCl_3$–acetone–water, 65:35:0.7) to give **3** (54.5 mg, 76% yield) as a colorless powder. mp 125—126 °C. $[\alpha]_D^{27}$ +2.89° (c=1.06, $CHCl_3$). IR (CCl_4): 3360 (O–H and N–H), 2925 (C–H), 2850, 1642 (NC=O) cm^{-1}. ^{1}H-NMR (270 MHz, C_5D_5N) δ: 8.59 (1H, d, J=8.9 Hz, NH), 5.99 (1H, dt, J=14.8, 7.3 Hz, 6-H), 5.74 (1H, dt, J=14.7, 7.2 Hz, 7-H), 5.13 (1H, m, 2-H), 4.62 (1H, dd, J=7.6, 3.6 Hz, 2'-H), 4.51 (1H, dd, J=10.6, 4.76 Hz, 1-H_a), 3.07 (1H, m, 5-H_a), 2.72 (1H, quint., J=7.1 Hz, 5-H_b), 2.23 (1H, m, 3'-H_a), 1.50 (1H, dqq, J=6.6, 6.6, 6.6 Hz, 17-H), 1.32 (m), 1.26 (m), 0.88 (3H, t, J=6.6 Hz, 24'-H), 0.86 (6H, d, J=6.6 Hz, 18-H and $CHC\underline{H}_3$). ^{13}C-NMR (67.8 MHz, C_5D_5N) δ: 175.3 (s, CO), 132.8 (d, (C7)), 128.3 (d, (C6)), 76.3 (d, (C3)), 73.3 (d, (C4)), 42.5 (d, (C2')), 62.0 (t, (C1)), 52.9 (d, (C2)), 39.3 (t), 37.7 (t), 33.2 (t, (C8)), 32.1 (t), 30.2 (t), 30.0 (t), 29.9 (t), 29.6 (t), 28.2 (t), 27.7 (d, (C17)), 25.8 (t), 22.9 (t), 22.8 (q, (C18 and $CHC\underline{H}_3$)), 14.3 (q, (C24')). FD-MS m/z (%): 696 (M^++H, 100), 678 (M^+-H_2O, 42). *Anal.* Calcd for $C_{43}H_{85}NO_5$: C, 74.19; H, 12.31; N, 2.01. Found: C, 74.11; H, 12.23; N, 2.05.

(2*S*,3*S*,4*R*,6*E*)-2-[(*R*)-2-Hydroxytetracosanoylamino]-17-methyl-1-(triphenylmethyloxy)-6-octadecene-3,4-diol (29) Compound **3** (18.6 mg, 26.7 μmol), trityl chloride (73.2 mg, 263 μmol), and dimethylaminopyridine (DMAP) (21.4 mg, 175 μmol) were dissolved in pyridine (534 μl). After being stirred at 60 °C for 4 h, the reaction mixture was diluted with $CHCl_3$, washed with water, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$–MeOH–H_2O, 6:4:0.8) to give **29** (15.8 mg, 90% yield) as a colorless viscous oil. $[\alpha]_D^{25}$ +16.0° (c=0.725, $CHCl_3$). IR (CCl_4): 3410 (O–H and N–H), 3060 and 3030 (Ar–H), 2930 (C–H), 2855, 1660 (NC=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.41 (6H, m, aromatic H), 7.27 (9H, m, aromatic H), 7.12 (1H, d, J=8.6 Hz, NH), 5.53 (1H, dt, J=14.6, 7.1 Hz, 6-H), 5.34 (1H, dt, J=14.6, 7.3 Hz, 7-H), 4.25 (1H, m, 2-H), 4.07 (1H, t, J=5.6 Hz, 2'-H), 4.00 (1H, m, 2'-H), 3.62 (1H, m, 3-H), 3.52 (1H, dd, J=11.9, 2.3 Hz, 1-H_a), 3.43 (1H, dd, J=9.9, 4.0 Hz, 1-H_b), 3.26 (1H, m, 4-H), 2.47 (1H, m, 5-H_a), 2.02 (2H, m, 8-H), 1.26 (m), 0.86 (6H, d, J=6.6 Hz, 18-H and $CHC\underline{H}_3$). Negative FAB-MS m/z (%): 936 (M^+−H, 4), 694 (M^+-CPh_3, 19), 424 (41), 153 (100). *Anal.* Calcd for $C_{62}H_{99}NO_5$: C, 79.35; H, 10.63; N, 1.49. Found: C, 79.38; H, 10.57; N, 1.45.

(2*S*,3*S*,4*R*,6*E*)-2-[(*R*)-2-Benzoyloxytetracosanoylamino]-3,4-dibenzoyloxy-17-methyl-1-(triphenylmethyloxy)-6-octadecene (30) Benzoyl chloride (95.0 μl, 820 μmol) was added to a solution of **29** (44.2 mg, 47.1 μmol) and DMAP (99.8 mg, 820 μmol) in pyridine (1.6 ml). After being stirred at 70—75 °C for 15 h, the reaction mixture was diluted with water and extracted with $CHCl_3$. The extracts were washed with water, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane–AcOEt, 3:1) to give **30** (58.0 mg, 98% yield) as a colorless viscous oil. $[\alpha]_D^{29}$ +30.9° (c=2.43, $CHCl_3$). IR (CCl_4): 3060 and 3030 (Ar–H), 2920 (C–H), 2850, 1725 (OC=O), 1682 (NC=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 8.05 (2H, d, J=7.6 Hz, aromatic H), 7.96 (2H, d, J=7.6 Hz, aromatic H), 7.86 (2H, d, J=7.6 Hz, aromatic H), 7.52 (3H, m, aromatic H), 7.39 (4H, t, J=7.6 Hz, aromatic H), 7.27 (2H, t, J=7.8 Hz, aromatic H), 7.12 (6H, m, aromatic H), 7.00 (9H, m, aromatic H), 5.85 (1H, dd, J=8.2, 3.3 Hz, 3-H), 5.57 (1H, t, J=5.8 Hz, 2'-H), 5.53 (1H, m, 6-H), 5.39 (1H, m, 4-H), 5.34 (1H, m, 7-H), 4.64 (1H, m, 2-H), 3.24 (d, J=3.3 Hz, 2H, 1-H), 2.66 (m, 1H, 5-H_a), 2.49 (1H, m, 5-H_b), 2.06 (2H, m, 3'-H), 1.87 (2H, m, 8-H), 1.26 (m), 0.88 (3H, t, J=6.3 Hz, $CH_2C\underline{H}_3$), 0.85 (6H, d, J=6.6 Hz, 18-H and $CHC\underline{H}_3$). Negative FAB-MS m/z (%): 1248 (M^+−H, 1.2), 486 (1.1). *Anal.* Calcd for $C_{83}H_{111}NO_8$: C, 79.70; H, 8.94; N, 1.12. Found: C, 79.83; H, 8.94; N, 1.12.

(2*S*,3*S*,4*R*,6*E*)-2-[(*R*)-2-Benzoyloxytetracosanoylamino]-3,4-dibenzoyloxy-17-methyl-6-octadecen-1-ol (31) A solution of $TsOH\cdot H_2O$ (17.7 mg, 93 μmol) in $CHCl_3$ (1.25 ml) and MeOH (0.6 ml) was added to **30** (58.0 mg, 46.4 μmol). After being stirred at room temperature for 8 h, the mixture was concentrated to 1/4 of the original volume by blowing a stream of nitrogen gas over it, and the concentrate was chromatographed on silica gel (hexane–AcOEt, 7:3) to give **31** (44.3 mg, 95% yield) as a colorless viscous oil. $[\alpha]_D^{26}$ +43.7° (c=1.88, $CHCl_3$). IR (CCl_4): 3520 and 3420 (O–H and N–H), 3060 and 3030 (Ar–H), 2930 (C–H), 2850, 1725 (OC=O), 1685 (NC=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 8.14 (2H, d, J=7.9 Hz, aromatic H), 8.01 (2H, d, J=8.2 Hz, aromatic H), 7.96 (2H, d, J=8.4 Hz, aromatic H), 7.45 (9H, m, aromatic H), 7.13 (1H, d, J=9.2 Hz, NH), 5.65—5.35 (5H, m, 3-H, 4-H, 6-H, 7-H, and 2'-H), 4.44 (1H, m, 2-H), 3.64 (2H, brs, 1-H), 2.78 (1H, m, 5-H_a), 2.65 (1H, m, 5-H_b), 2.07 (2H, m), 2.92 (2H, m), 1.26 (m), 0.88 (3H, t, J=6.6 Hz, $CH_2C\underline{H}_3$), 0.86 (6H, d, J=6.6 Hz, 18-H and $CHC\underline{H}_3$). FAB-MS m/z (%): 1008 (M^++H, 11), 990 (M^+−OH, 16), 886 (M^+−PhCOO, 100). *Anal.* Calcd for $C_{64}H_{97}NO_8$: C, 76.22; H, 9.70; N, 1.39. Found: C, 75.97; H, 9.67; N, 1.42.

1-*O*-(2,3,4,6-Tetra-*O*-acetoxy-β-D-galactopyranosyloxy)-(2*S*,3*S*,4*R*,6*E*)-2-[(*R*)-2-benzoyloxytetracosanoylamino]-3,4-dibenzoyloxy-17-methyl-6-octadecene (33) and (2*S*,3*S*,4*R*,6*E*)-1-Acetoxy-2-[(*R*)-2-benzoyloxytetracosanoylamino]-3,4-dibenzyloxy-17-methyl-6-octadecane (34) A suspension of **31** (15.9 mg, 15.8 μmol), **32** (25.3 mg, 53.1 μmol), and 4 Å molecular sieves (38.9 mg) in CH_2Cl_2 (315 μl) was stirred at room temperature for 1 h and boron trifluoride etherate (5.82 μl, 47.3 μmol) was added at −25 °C. After being stirred at −25 °C for 1 h, the reaction mixture was allowed to warm to room temperature over 1 h and stirred at ambient temperature for 1 h. The mixture was diluted with $CHCl_3$, washed with aqueous saturated $NaHCO_3$, dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane–AcOEt, 8:2—7:3) to give **33** (15.7 mg, 74% yield) and **34** (4.2 mg, 25% yield).

33: $[\alpha]_D^{27}$ +24° (c=0.82, $CHCl_3$). IR (CCl_4): 2930 (C–H), 2850, 1755 and 1725 (OC=O), 1685 (NC=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 8.16 (2H, dd, J=7.8, 1.7 Hz, aromatic H), 8.00 (2H, dd, J=5.9, 1.3 Hz, aromatic H), 7.97 (2H, dd, J=9.8, 1.8 Hz, aromatic H), 7.60 (3H, m, aromatic H), 7.45 (6H, m, aromatic H), 6.07 (1H, d, J=8.9 Hz, NH), 5.60 (1H, dd, J=7.89, 4.0 Hz, 6-H), 4.63 (1H, m, 2-H), 4.38 (1H, d, J=7.9 Hz, 1''-H), 2.07 (3H, s, Ac), 1.96 (3H, s, Ac), 1.91 (3H, s, Ac), 1.71 (3H, s, Ac), 1.25 (m), 0.88 (3H, t, J=6.8 Hz, $CH_2C\underline{H}_3$), 0.85 (6H, d, J=6.6 Hz, 18-H and $CHC\underline{H}_3$). FAB-MS m/z (%): 1338 (M^++H, 2.0), 1216 (M^+−PhCOO, 3.5), 990 (M^+−tetraacetylgalactose unit, 8.4). This product was used in the next reaction without further purification.

34: $[\alpha]_D^{25}$ +17° (c=0.21, $CHCl_3$). IR (CCl_4): 2925 (C–H), 2850, 1730 (OC=O), 1690 (NC=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 8.10 (2H, dd, J=8.3, 1.3 Hz, aromatic H), 8.00 (2H, dd, J=8.4, 1.5 Hz, aromatic H), 7.96 (2H, dd, J=8.9, 1.5 Hz, aromatic H), 7.59 (3H, m, aromatic H), 7.43 (6H, m, aromatic H), 6.92 (1H, d, J=9.2 Hz, NH), 5.68—5.30 (5H, m, 3-H, 4-H, 6-H, 7-H, and 2'-H), 4.77 (1H, m, 2-H), 4.24 (1H, dd, J=11.7, 5.1 Hz, 1-H_a), 4.16 (1H, dd, J=11.7, 4.1 Hz, 1-H_b), 2.06 (2H, m), 1.88 (2H, m), 1.70 (3H, s, Ac), 1.25 (m), 0.87 (3H, t, J=7.3 Hz, $CH_2C\underline{H}_3$), 0.86 (6H, d, J=6.6 Hz, 18-H and $CHC\underline{H}_3$). FD-MS m/z (%): 1050 (M^++H, 100). *Anal.* Calcd for $C_{66}H_{99}NO_9$: C, 75.46; H, 9.50; N, 1.33. Found: C, 75.27; H, 9.46; N, 1.29.

1-*O*-(β-D-Galactopyranosyloxy)-(2*S*,3*S*,4*R*,6*E*)-2-[(*R*)-2-hydroxytetracosanoylamino]-17-methyl-6-octadecene-3,4-diol (2) A 0.1 M solution of K_2CO_3 in MeOH (1.2 ml) was added to a solution of **33** (15.7 mg) in MeOH (0.6 ml). After being stirred at room temperature for 30 min, the reaction mixture was concentrated by blowing a stream of nitrogen gas over it, and the residue was chromatographed on silica gel (BW-300, $CHCl_3$–MeOH–H_2O, 85:15:1.5) to give **2** (8.7 mg, 89% yield) as colorless crystals. mp 218—220 °C. $[\alpha]_D^{27}$ +18° (c=0.34, $CHCl_3$–MeOH, 2:1). IR (KBr): 3400 (O–H), 2920 (C–H), 2850 (C–H), 1645 (NC=O), 1540, 1075, 720 cm^{-1}. ^{1}H-NMR (270 MHz, C_5D_5N) δ: 8.57 (1H, d, J=9.6 Hz, NH), 5.99 (1H, dt, J=14.6, 7.2 Hz, 6-H), 5.74 (1H, dt, J=14.5, 7.1 Hz, 7-H), 5.29 (1H, m, 2-H), 4.89 (1H, d, J=7.9 Hz), 4.78 (1H, dd, J=10.6, 6.6 Hz), 4.13 (3H, dd, J=9.2, 3.3 Hz), 4.02 (1H, t, J=5.9 Hz), 3.04 (1H, dd, J=12.5, 6.6 Hz, 5-H_a), 2.70 (1H, quint., J=7.2 Hz, 5-H_b), 1.50 (1H, dqq, J=6.5, 6.6, 6.6 Hz, 17-H), 1.33 (m), 1.27 (m), 0.879 (6H, d, J=6.6 Hz, 18-H and $CHC\underline{H}_3$), 0.877 (3H, t, J=6.9 Hz, 24'-H). ^{13}C-NMR (67.8 MHz, C_5D_5N) δ: 175.7 (s), 132.7 (d), 128.3 (d), 106.3 (d), 77.1 (d), 75.3 (d), 75.2 (d), 72.8 (d), 72.6 (d), 72.4 (d), 70.6 (t), 70.2 (d), 62.4 (t), 51.6 (d), 39.3 (t), 37.5 (t), 35.6 (t), 33.3 (t), 32.1 (t), 30.3 (t), 30.1 (t), 29.9 (t), 29.73 (t), 29.65 (t), 29.6 (t), 28.2 (d), 27.7 (t), 25.9 (t), 22.9 (t), 22.8 (q), 14.3 (q). FD-MS m/z (%): 858 (M^++H, 100), 840 (M^+−OH, 27). *Anal.* Calcd for $C_{64}H_{97}NO_8\cdot 0.5H_2O$: C, 67.86; H, 11.16; N, 1.61. Found: C, 67.79; H, 10.97; N, 1.60. [Spectral data of the mixture of **1a** and **1b**.[2)] IR (KBr): 3361, 1076, and 1047 (O–H), 1656 and 1544 (NC=O) cm^{-1}. ^{1}H-NMR (270 MHz, C_5D_5N) δ: 5.94 (dt, J=15.4, 8.1 Hz), 5.71 (dt, J=15.4, 8.1 Hz), 4.0—4.7 (sugar protons), 4.87 (d, J=7.5 Hz, anomeric proton), 1.25 (long chain methylene protons), 0.85 (6H, d, J=7.3 Hz, secondary methyls), 0.85 (3H, t, J=7.0 Hz, primary methyl). ^{13}C-NMR (67 MHz, C_5D_5N) δ: 175.61 (carbonyl), 133.67 and 126.60 (olefinic carbons)]. The ^{1}H-NMR spectrum of **2** was superimposable on that of the mixture of **1a** and **1b**

provided by Dr. M. Nakagawa.

1-*O*-(2,3,4,6-Tetra-*O*-acetoxy-β-D-galactopyranosyloxy)-(2*S*,3*S*,4*R*,6*E*)-2-[(*R*)-2-acetoxytetracosanoylamino]-3,4-diacetoxy-17-methyl-6-octadecene (35) Acetic anhydride (175 μl) and pyridine (151 μl) were added to **2** (8.0 mg). After being stirred at 50 °C for 3 h, the reaction mixture was concentrated by blowing a stream of nitrogen gas over it and the residue was chromatographed on silica gel (BW-300, hexane–AcOEt, 6 : 4) to give **35** (9.5 mg, 88% yield) as a colorless viscous oil. $[\alpha]_D^{26}$ +2.8° (c = 0.44, $CHCl_3$). IR (CCl_4): 2925 (C–H), 2850 (C–H), 1755 (OC=O), 1685 (NC=O) cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 6.78 (1H, d, J = 8.9 Hz, NH), 5.48 (1H, dt, J = 14.6, 7.2 Hz, 6-H), 5.37 (1H, d, J = 2.8 Hz, 4″-H), 5.26 (1H, dt, J = 14.7, 7.3 Hz, 7-H), 5.19—5.08 (2H, m, 3-H, and 2′-H), 5.00 (1H, dd, J = 10.6, 3.3 Hz, 3″-H), 4.92 (1H, dt, J = 9.1, 3.7 Hz, 4-H), 4.45 (1H, d, J = 7.6 Hz, 1″-H), 4.33 (1H, m, 2-H), 4.13 (2H, d, J = 6.6 Hz, 6″-H), 3.90 (1H, dd, J = 7.6, 6.6 Hz, 5″-H), 3.86 (1H, dd, J = 10.9, 3.6 Hz, 1-H_a), 3.68 (1H, dd, J = 10.9, 3.6 Hz, 1-H_b), 2.23 (3H, s, CH_3CO), 2.15 (3H, s, CH_3CO), 2.10 (3H, s, CH_3CO), 2.05 (3H, s, CH_3CO), 2.04 (3H, s, CH_3CO), 1.97 (3H, s, CH_3CO), 1.25 (m), 0.88 (3H, t, J = 6.6 Hz, CH_2CH_3), 0.86 (6H, d, J = 6.6 Hz, 18-H and $CHCH_3$). ^{13}C-NMR (67.8 MHz, $CDCl_3$) δ: 170.7 (s), 170.3 (s), 170.2 (s), 170.1 (s), 170.05 (s), 169.99 (s), 169.8 (s), 169.4 (s), 134.6 (d), 124.0 (d), 100.7 (d), 73.9 (d), 72.6 (d), 72.0 (d), 70.8 (d), 68.6 (d), 67.0 (d), 66.2 (t), 61.1 (t), 48.1 (d), 39.1 (t), 32.6 (t), 32.1 (t), 31.9 (t), 31.8 (t), 30.0 (t), 29.7 (t), 29.6 (t), 29.5 (t), 29.4 (t), 29.3 (t), 29.2 (t), 28.0 (d), 27.4 (t), 24.9 (t), 22.7 (q), 20.99 (q), 20.95 (q), 20.7 (q), 20.6 (q), 20.5 (q), 14.1 (q). FAB-MS m/z (%): 1152 (M^++H, 1.2), 1092 (M^+−AcOH, 1.5), 804 (M^+−tetraacetylgalactose unit, 8.9). *Anal.* Calcd for $C_{64}H_{97}NO_8$: C, 65.65; H, 9.53; N, 1.22. Found: C, 65.38; H, 9.50; N, 1.24. [NMR data of the mixture of acetates of **1a** and **1b**.[16] ^{1}H-NMR (360 MHz, $CDCl_3$) δ: 4.44 (1H, d, J = 7.5 Hz, 1″-H), 3.86 (1H, dd, J = 10.8, 3.0 Hz, 1-H_a), 3.67 (1H, dd, J = 10.8, 3.5 Hz, 1-H_b). ^{13}C-NMR (67 MHz, $CDCl_3$) δ: 134.3 (d), 123.9 (d), 100.6 (d), 73.9 (d), 72.6 (d), 72.1 (d), 70.9 (d), 68.7 (d), 67.0 (d), 66.3 (t), 61.1 (t), 48.2 (d), 39.2 (t), 28.2 (d), 25.1 (t), 22.8 (q), 14.3 (q)]. The ^{13}C- and ^{1}H-NMR spectral data of **35** were superimposable on those of the mixture of acetates of **1a** and **1b** provided by Dr. M. Nakagawa.

Acknowledgements We are grateful to Dr. M. Endo and Dr. M. Nakagawa of Suntory Institute for Biological Research for providing the NMR data on the cerebrosides. Thanks are also due to Mr. A. Tanaka, Miss Y. Soeda, and Mr. R. Isobe of the Faculty of Pharmaceutical Sciences, Kyushu University, for recording NMR and mass spectra. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 01771919) from the Ministry of Education, Science, and Culture of Japan.

References and Notes

1) Some of these data were presented at the 31st Symposium on the Chemistry of Natural Products, Nagoya, Japan, Oct. 1989, Symposium Papers, p. 22.
2) M. Endo, M. Nakagawa, Y. Hamamoto, and M. Ishihama, *Pure Appl. Chem.*, **58**, 387 (1986).
3) *a*) K. Mori and T. Kinsho, *Justus Liebigs Ann. Chem.*, **1988**, 807; *b*) T. Bär and R. R. Schmidt, *ibid.*, **1990**, 669; *c*) S. Kodato, M. Nakagawa, K. Nakayama, and T. Hino, *Tetrahedron*, **45**, 7247 (1989); *d*) S. Kodato, M. Nakagawa, and T. Hino, *ibid.*, **45**, 7263 (1989); *e*) M. Nakagawa, S. Kodato, K. Nakayama, and T. Hino, *Tetrahedron Lett.*, **28**, 6281 (1987); *f*) K. Mori and Y. Funami, *Tetrahedron*, **41**, 2379 (1985).
4) *a*) S. Sugiyama, M. Honda, and T. Komori, *Justus Liebigs Ann. Chem.*, **1990**, 1069; *b*) *Idem*, *ibid.*, **1988**, 619.
5) E. J. Corey and P. L. Fuchs, *Tetrahedron Lett.*, **1972**, 3769.
6) C. A. Brown and A. Yamashita, *J. Am. Chem. Soc.*, **97**, 891 (1975).
7) J. Jurczak, S. Pikul, and T. Bauer, *Tetrahedron*, **42**, 447 (1986).
8) A. J. Mancuso, S. L. Huang, and D. Swern, *J. Org. Chem.*, **43**, 2480 (1978).
9) *a*) R. M. Hanson and K. B. Sharpless, *J. Org. Chem.*, **51**, 1922 (1986); *b*) T. Katsuki and K. B. Sharpless, *J. Am. Chem. Soc.*, **102**, 5974 (1980).
10) J. M. Finan and Y. Kishi, *Tetrahedron Lett.*, **23**, 2719 (1982).
11) S. Tanaka, H. Yamamoto, H. Nozaki, K. B. Sharpless, R. C. Michaelson, and J. D. Cutting, *J. Am. Chem. Soc.*, **96**, 5254 (1974).
12) W. R. Roush and M. A. Adam, *J. Org. Chem.*, **50**, 3752 (1985).
13) *a*) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970); *b*) *Idem*, *ibid.*, **103**, 2024 (1970); *c*) *Idem*, *ibid.*, **103**, 2034 (1970).
14) M. Node, H. Hori, and E. Fujita, *J. Chem. Soc., Perkin Trans. 1*, **1976**, 2237.
15) R. R. Schmidt, *Angew. Chem. Int. Ed. Engl.*, **25**, 212 (1986).
16) Unpublished data of M. Endo, M. Nakagawa, Y. Hamamoto, and M. Ishihama.[2])

Synthesis of Kifunensine, an Immunomodulating Substance Isolated from a Microbial Source[1)]

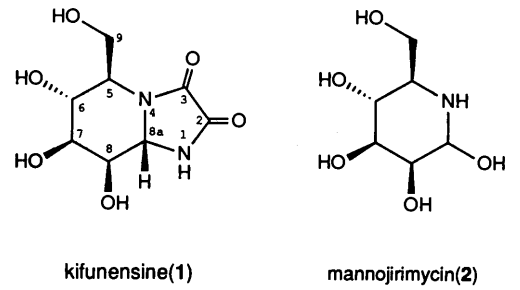
Hiroshi KAYAKIRI, Chiyoshi KASAHARA, Katsuya NAKAMURA, Teruo OKU,* and Masashi HASHIMOTO

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-26, Japan. *Received October 30, 1990*

Kifunensine (1), a novel immunomodulator isolated from an actinomycete, was enantiospecifically synthesized from D-mannosamine *via* a double cyclization of the oxamide-aldehyde precursor with ammonia as a key step. The absolute stereochemistry of natural kifunensine was confirmed to be the D form.

Keywords kifunensine; D-mannosamine; enantiospecific synthesis; double cyclization; polyhydroxylated piperidine; 4,5-dioxoimidazolidine; immunomodulator; α-mannosidase inhibitor

In the preceding papers[2)] we reported the structure of kifunensine (**1**) isolated from *Kitasatosporia kifunense* no. 9482 as a new immunomodulator with α-mannosidase-inhibitory activity.[3)] It induces the expression of Ia antigen on mouse peritoneal macrophages[4)] and restores, in mouse spleen cells, the immune response depressed by immunosuppressive factors in the tumor-bearing mouse serum. Kifunensine has a unique basic framework, an octahydro-2,3-dioxoimidazo[1,2-*a*]pyridine ring system, which to our knowledge has not previously been found in nature, and corresponds structurally to a cyclic oxamide derivative of 1-amino-substituted mannojirimycin[5,6)] (Chart 1).

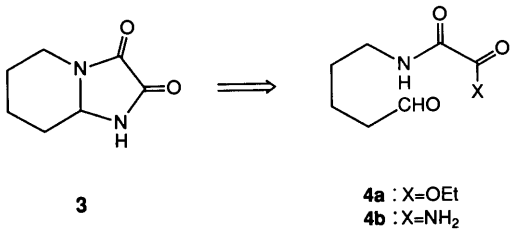


kifunensine(**1**) mannojirimycin(**2**)

Chart 1

This novel structure of **1** and its interesting biological activity prompted us to establish an efficient route for the synthesis of this natural product. Herein we report a highly stereo-controlled synthesis of kifunensine from D-mannosamine, *via* a double cyclization of the oxamide-aldehyde precursor with ammonia as a key step.

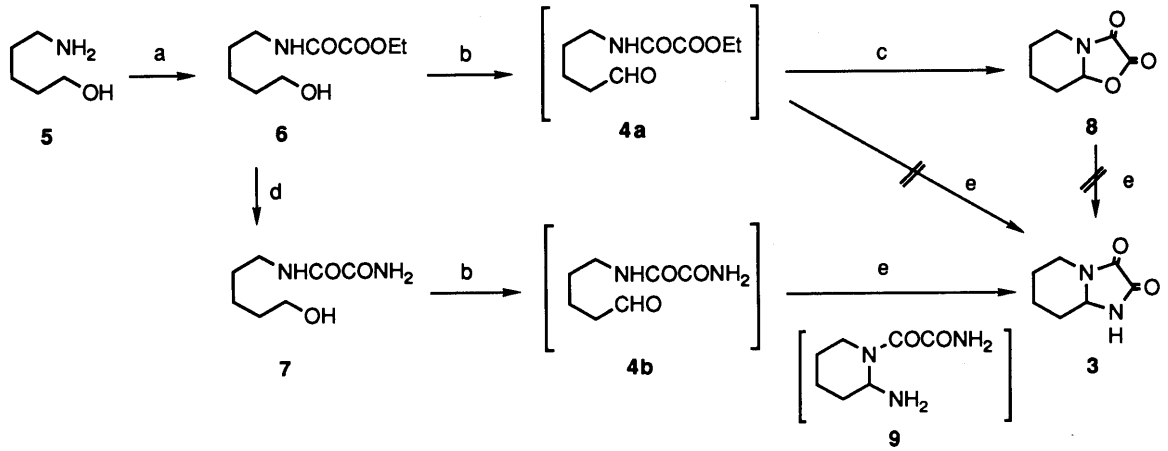
The main problem to be solved for the synthesis of this substance was the construction of the bicyclic framework. In order to find a solution to this problem, we initially investigated in a model study preparation of the simplest octahydro-2,3-dioxoimidazo[1,2-*a*]pyridine system **3**, consisting of the basic framework of **1**. We envisioned that this bicyclic structure would be constructed by a double cyclization of oxalylamino-aldehyde precursor **4** with ammonia (Chart 2). At first we chose the ethoxy group as the leaving group X and prepared the precursor **4a** from 1-aminopentanol (**5**) as follows (Chart 3).



3 **4a** : X=OEt
4b : X=NH_2

Chart 2

Selective N-acylation of **5** was achieved by silylation with bistrimethylsilylacetamide (BSA) followed by acylation with ethyl oxalyl chloride and by subsequent acidic desilylation to give the alcohol **6** in 95% yield. Collins oxidation of **6** afforded the required aldehyde **4a**, which was directly used without further purification because of its instability during chromatography on silica gel or Florisil. We found that by heating in toluene, this aldehyde was transformed into the 4,5-dioxooxazolidine **8**, which corresponds to the 1-oxa

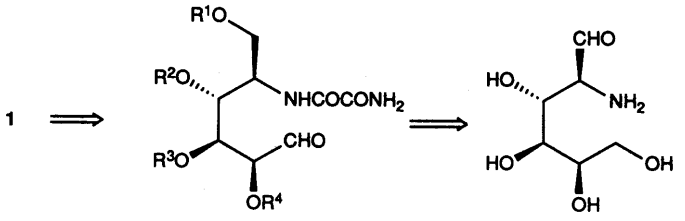


a: 1) BSA, THF; 2) ClCOCOOEt; 3) 1 N aq. AcOH b: $CrO_3 \cdot 2Py$, CH_2Cl_2 c: reflux in toluene d: 2.4 N NH_3–MeOH e: 6 N NH_3–MeOH.

Chart 3

derivative of **3**, probably *via* an intramolecular double cyclization, in 30% yield from **6**. Encouraged by this result, we examined treatment of **4a** and **8** with ammonia in MeOH. In both cases, however, an unknown material was mainly produced and only trace amounts of **3** were detected on thin layer chromatography (TLC). It was supposed that polymerization might occur much more quickly than the desired cyclization. We then attempted a double cyclization of the oxamide-aldehyde precursor **4b** whose oxalyl group might be much less reactive than that of **4a**. Compound **4b**, prepared from **6** by ammonolysis to **7** (quantitative yield) followed by Collins oxidation, was also unstable, and was used directly for the next reaction without further purification. After several attempts, we found that the desired cyclization took place in **4b** to afford **3** in 48% yield from **7** on treatment with 6 N ammonia–MeOH at room temperature for 48 h. Since this cyclization did not occur in the case of treatment with tertiary amines such as Et_3N and diisopropylethylamine, it was presumed that **3** arose *via* the intermediary amine **9**.

With these results in hand, we devised a synthetic route for kifunensine. Though the absolute stereochemistry of **1** was unknown, it was presumed to be the D form because **1** showed α-mannosidase-inhibitory activity. In our strategy, the piperidine portion of **1** was retrosynthetically related to D-mannosamine (**11**), which could be converted into the precursor **10** for **1** *via* interchange of its C-1 aldehyde and C-6 hydroxymethyl groups: reduction of C-1 to hydroxymethyl and oxidation of C-6 to aldehyde (Chart 4). This starting material could provide four of the five asymmetric centers in **1**. For protection of the four hydroxyl functions in **10**, we chose the acetonide groups in the expectation that the cyclization would proceed stereoselectively as a result of restricting the flexibility of the molecule.

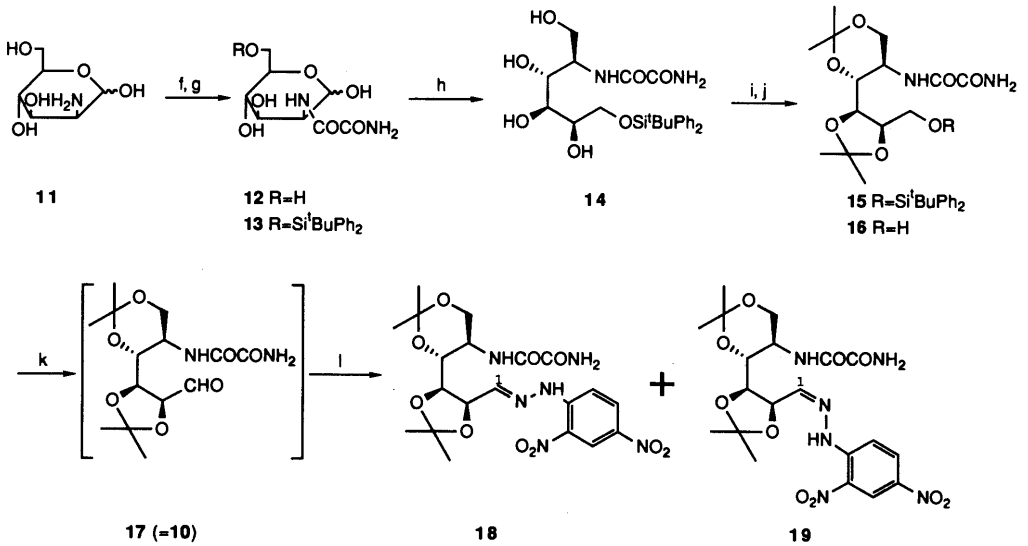


10 **11**

Chart 4

The requisite intermediate **17** (**10**) was prepared from D-mannosamine (**11**) as follows (Chart 5). Selective N-acylation of **11** with oxamic acid, using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT) in dimethylformamide (DMF), and subsequent silylation of the primary alcohol gave, *via* **12**, an anomeric mixture (*ca.* 7:3) of **13** in 66% yield from **11**. Compound **13** was subjected to $NaBH_4$ reduction to furnish the tetrol **14** in 92% yield. Diacetonization of the four hydroxyl groups in **14** was achieved successfully with acetone–$BF_3 \cdot Et_2O$ to give the diacetonide **15** in 86% yield, and this was desilylated with *n*-Bu_4NF in tetrahydrofuran (THF) to afford the alcohol **16** quantitatively. Collins oxidation of **16** provided the desired oxamide-aldehyde precursor **17** as a crude oil, which was directly subjected to the key cyclization reaction because of its instability. The structure of this key intermediate was supported by infrared (IR, $CHCl_3$, 1720 cm^{-1}) and proton nuclear magnetic resonance (^{1}H-NMR, $CDCl_3$, δ 9.60, 1H s) data, and confirmed by derivatization to the dinitrophenylhydrazone derivatives, **18** (*anti*) and **19** (*syn*). The geometries of these hydrazones were presumed on the basis of comparison of the chemical shifts of the C-1 protons,[7] δ 8.05 for **18** and δ 6.96 for **19**, in their ^{1}H-NMR spectra.

The key double cyclization was carried out by treating **17** with 6 N NH_3–MeOH at room temperature for 6 h to afford the objective kifunensine diacetonide **20** in 76% yield from **16** along with its **8a**-epimer **21** (4.0% yield) (Chart 6). This diacetonide **20** was identical with an authentic sample derived from the natural product. The remarkable stereoselectivity might be explained by the relative ther-



11 **12** R=H, **13** R=Si^tBuPh_2 **14** **15** R=Si^tBuPh_2, **16** R=H

17 (=**10**) **18** **19**

Si^tBuPh_2 = *tert*-butyldiphenylsilyl

f: $H_2NCOCOOH$, DCC, HOBT, DMF g: $^tBuPh_2SiCl$, imidazole, DMF, 0 °C h: $NaBH_4$, MeOH i: acetone, $BF_3 \cdot OEt_2$, −20 °C j: *n*-Bu_4NF, THF, −20 °C k: $CrO_3 \cdot 2Py$, CH_2Cl_2 l: 2,4-DNP, H_3PO_4, EtOH.

Chart 5

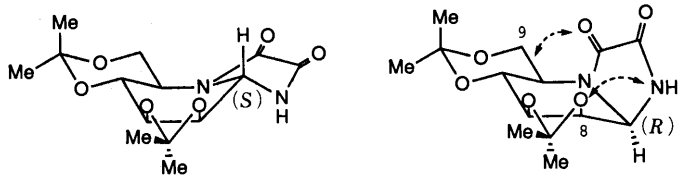
modynamic stability of the desired (**8a**-*S*)epimer **20** and its (**8a**-*R*)epimer **21**. In our study using molecular models, it seemed that **20** is much more stable than **21** because, in the latter compound, the dioxoimidazolidine ring is hindered by the methylene (C-9) or/and the oxygen on C-8 (Fig. 1). Probably the direction of ring closure was regulated by this difference of thermodynamic stability between **20** and **21**.

All other cyclization methods examined under alkaline conditions (NaH/THF, NaOMe/MeOH, 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU)/MeOH, *etc.*), acidic conditions (camphorsulfonic acid/THF, $BF_3 \cdot Et_2O$/THF, *etc.*), and other conditions (heating, pyridinium *p*-toluenesulfonate/CH_2Cl_2, trimethylsilyl chloride–pyridine/THF, *etc.*) were unsuccessful. On the other hand, treatment of **17** with saturated aqueous NH_4HCO_3 or $(NH_4)_2CO_3$ afforded the desired cyclization product **20** in a stereoselective manner, but in lower yield. On the basis of these results, we speculated that this reaction proceeded through the intermediacy of the amine **B**, formed by a condensation of the aldehyde **A** with ammonia (Chart 7). This speculation is supported by the fact that similar treatment of the oxamide-aldehyde **17** with 30% $MeNH_2$–MeOH in a similar manner afforded ^1N-methylkifunensine diacetonide **22**, identical with an authentic sample derived from the natural product, in 81% yield from the alcohol **16**. In this cyclization, the (**8a**-*R*)epimer was not obtained.

Removal of the acetonide protecting groups in **20** with aqueous trifluoroacetic acid (TFA) furnished kifunensine (**1**) which was identical with an authentic sample, confirming the absolute stereochemistry of **1** to be the D form, as presumed. Similar treatment of **21** and **22** also afforded **8a**-*epi*-kifunensine (**23**) and ^1N-methylkifunensine (**24**), respectively.

The basic framework **3**, its 1-oxa derivative **8**, ^1N-methyl derivative **24** and **8a**-epimer **23** did not inhibit α-mannosidase and had no effect on Ia antigen expression. These facts might suggest that the hydroxyl groups,amide NH and the stereochemistry of kifunensine (**1**) are important for its biological activities.[8]

In conclusion, we have developed a double cyclization method to construct the octahydro-2,3-dioxoimidazo[1,2-*a*]pyridine ring system and by adopting it as the key step, we have established an efficient route for the synthesis of kifunensine (**1**). This synthetic route is capable of providing sufficient amounts for detailed biological evaluation and may also be applicable to the preparation of analogous compounds.



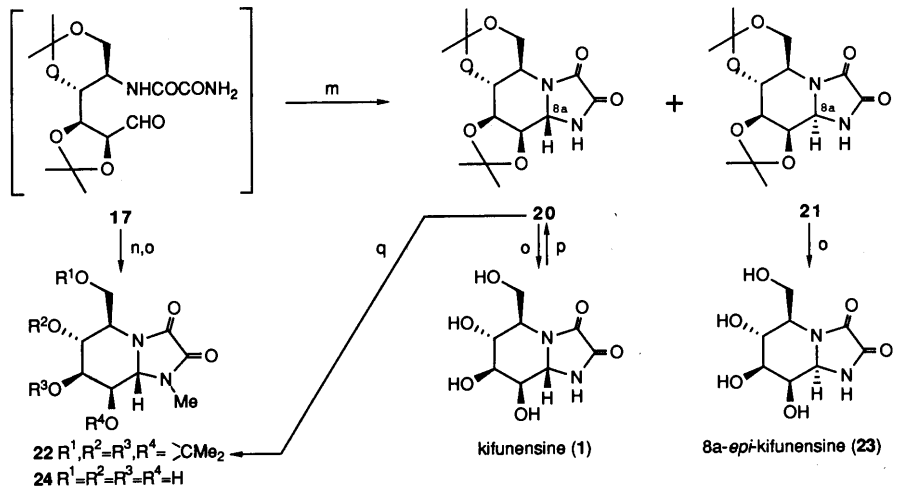
20 (8a-S) **21** (8a-*R*)

Fig. 1

Experimental

The instruments used to obtain physical data and the experimental conditions for chromatography were the same as described in our preceding paper.[2b]

Ethyl *N*-(5-Hydroxypentyl)oxamate (6) BSA (20 ml) was added dropwise to a stirred anhydrous solution of 5-amino-1-pentanol (**5**, 2.06 g) in freshly distilled THF (100 ml) at room temperature over a period of 20 min under an N_2 atmosphere and the mixture was stirred for 1 h. The



m: 6 N NH_3–MeOH n: 30% $MeNH_2$–MeOH o: 75% aq. TFA p: 2,2-dimethoxypropane, TsOH, DMF, 60 °C q: MeI, K_2CO_3, acetone, reflux.

Chart 6

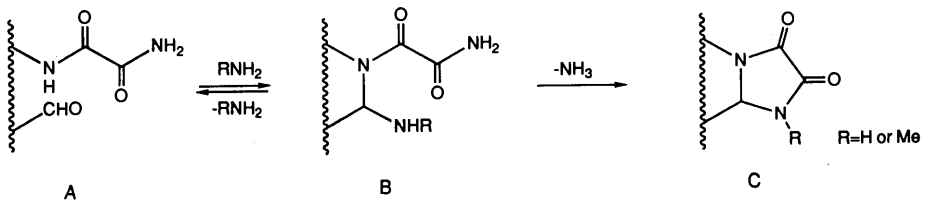


Chart 7

reaction mixture was cooled in an ice-water bath and a solution of ethyl oxalyl chloride (2.5 ml) in freshly distilled THF (7.5 ml) was added dropwise at 7—14 °C over a period of 5 min. The mixture was stirred for 1 h, then 1 N aqueous AcOH (20 ml) was added dropwise at 6—14 °C over a period of 5 min. The mixture was washed with brine and the aqueous layer was extracted with AcOEt twice. The combined organic layer was washed with saturated aqueous $NaHCO_3$, dried over $MgSO_4$, and evaporated *in vacuo* to give a pale yellow oil (4.15 g), which was purified by column chromatography (SiO_2 200 g, CH_2Cl_2 : EtOH = 20 : 1) to afford **6** (3.85 g, 95%). **6**: a colorless oil. IR (neat): 3300 (br), 2940, 1732, 1682, 1532 cm^{-1}. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 7.20 (1H, br s), 4.36 (2H, q, J = 7 Hz), 3.68 (2H, t, J = 6 Hz), 3.37 (2H, q, J = 7 Hz), 1.70—1.40 (6H, m), 1.40 (3H, t, J = 7 Hz), Fast atom bombardment mass spectra (FAB-MS) m/z: 204 $(M+H)^+$. High-resolution FAB-MS Calcd for $C_9H_{18}NO_4$ $(M+H)^+$: 204.124. Found: 204.123.

***N*-(5-Hydroxypentyl)oxamide (7)** An anhydrous solution of **6** (1.50 g) in MeOH (10 ml) was treated with 6 N NH_3–MeOH (5 ml) at room temperature for 10 min under an N_2 atmosphere. Removal of the solvent under reduced pressure afforded **7** (1.28 g, quant.). **7**: colorless fine crystals, mp 168—170 °C (MeOH). *Anal.* Calcd for $C_7H_{14}N_2O_3$: C, 48.26; H, 8.10; N, 16.08. Found: C, 47.97; H, 7.85; N, 16.12. IR (Nujol): 3380, 3305, 1652, 1540 cm^{-1}. ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 8.66 (1H, t, J = 6 Hz, D_2O-exchangeable), 8.03, 7.75 (each 1H, s, D_2O-exchangeable), 4.36 (1H, t, J = 5 Hz, D_2O-exchangeable), 3.37 (2H, q, J = 5 Hz), 3.16 (2H, q, J = 6 Hz), 1.55—1.15 (6H, m). FAB-MS m/z: 175 $(M+H)^+$.

Octahydro-2,3-dioxoimidazo[1,2-*a*]pyridine (3) A stirred anhydrous solution of pyridine (1.0 ml) in CH_2Cl_2 (25 ml) was treated with CrO_3 (615 mg) at room temperature under an N_2 atmosphere. The mixture was stirred for 15 min, then a suspension of **7** (100 mg) in anhydrous pyridine (3.5 ml) was added and the mixture was stirred for an additional 30 min followed by vacuum filtration through cellulose powder. The insoluble material was washed with CH_2Cl_2 (25 ml). The filtrate and washings were combined and evaporated *in vacuo* to give *N*-(4-formylbutyl)oxamide (**4b**, 825 mg) as a crude oil. ^{1}H-NMR (DMSO-d_6) δ: 9.68, 1H, br s. This crude aldehyde was directly treated with 6 N NH_3–MeOH (5.0 ml) for 48 h at room temperature under an N_2 atmosphere. After vacuum filtration through cellulose powder, the filtrate was evaporated *in vacuo* and the residue was purified by preparative TLC (CH_2Cl_2 : MeOH = 9 : 1) to furnish **3** (42 mg, 48% from **7**). **3**: colorless fine crystals, mp 164—165 °C (AcOEt). *Anal.* Calcd for $C_7H_{10}N_2O_2$: C, 54.54; H, 6.54; N, 18.17. Found: C, 54.25; H, 6.33; N, 17.90. IR (Nujol): 3220, 1748, 1718, 1698 cm^{-1}. ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 9.92 (1H, br s, D_2O-exchangeable), 4.72 (1H, dd, J = 10, 4 Hz), 4.06 (1H, dd, J = 12, 5 Hz), 2.91 (1H, td, J = 12, 4 Hz), 2.20—1.00 (6H, m). FAB-MS m/z: 155 $(M+H)^+$.

Octahydro-2,3-dioxooxazo[3,2-*a*]pyridine (8) A stirred anhydrous solution of pyridine (5.0 ml) in CH_2Cl_2 (125 ml) was treated with CrO_3 (3.08 g) at room temperature under an N_2 atmosphere. The mixture was stirred for 15 min, then a solution of **6** (613 mg) in anhydrous CH_2Cl_2 (5 ml) was added and the whole was stirred for an additional 30 min. The reaction mixture was diluted with Et_2O (125 ml), filtered through cellulose powder, and evaporated *in vacuo* to give a residue, which was extracted with Et_2O (100 ml). The extract was evaporated *in vacuo* to give ethyl *N*-(4-formylbutyl)oxamate (**4a**, 601 mg) as a crude oil. ^{1}H-NMR ($CDCl_3$) δ: 9.81 (1H, t, J = 2 Hz). This crude aldehyde was directly heated under reflux in anhydrous toluene (12 ml) for 1 h under an N_2 atmosphere. The reaction mixture was evaporated *in vacuo* and the residue was purified by column chromatography (SiO_2 10 g, CH_2Cl_2 : MeOH = 30 : 1) to afford **8** (140 mg, 30% from **6**). **8**: colorless fine crystals, mp 72—73 °C (isopropyl ether). *Anal.* Calcd for $C_7H_9NO_3$: C, 54.19; H, 5.85; N, 9.03. Found: C, 54.08; H, 5.79; N, 9.00. IR ($CHCl_3$): 2950, 2930, 1818, 1732 cm^{-1}. ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 5.51 (1H, dd, J = 10, 5 Hz), 4.06 (1H, ddd, J = 12, 4, 2 Hz), 3.01 (1H, td, J = 12, 4 Hz), 2.23 (1H, m), 1.97—1.22 (5H, m). FAB-MS m/z: 156 $(M+H)^+$.

6-*O*-*tert*-Butyldiphenylsilyl-2-deoxy-2-oxamoylamino-D-mannose (13) DCC (4.95 g), HOBT (3.24 g) and Et_3N (2.8 ml) were added to a stirred ice-cold suspension of D-mannosamine hydrochloride (**11**, 4.31 g) and oxamic acid (2.14 g) in DMF (40 ml). The mixture was stirred at room temperature for 15 h under an N_2 atmosphere, then DCC (1.24 g) and oxamic acid (534 mg) were added and the whole was stirred for an additional 5 h. The insoluble material was removed by vacuum filtration and washed with water (200 ml). The filtrate and washings were combined, washed five times with CH_2Cl_2 and evaporated *in vacuo* to give 2-deoxy-2-oxamoylamino-D-mannose (**12**) as a crude white amorphous solid (9.53 g), which was directly subjected to silylation without further purification. *tert*-Butyldiphenylsilyl chloride (7.8 ml) was added dropwise to an ice-cold solution of this residue (9.51 g) and imidazole (2.04 g) in DMF (80 ml). The mixture was stirred in an ice-water bath for 3 h under an N_2 atmosphere, then *tert*-butyldiphenylsilyl chloride (2.6 ml) and imidazole (1.36 g) were added and the whole was sirred for an additional 3 h. The reaction mixture was poured into water (400 ml) and extracted with AcOEt (300 ml). The organic layer was washed with 1 N aqueous HCl 3 times, brine, saturated aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$, and evaporated *in vacuo*. The residue was purified by column chromatography (SiO_2 300 g, CH_2Cl_2 : MeOH = 40 : 1—15 : 1) to afford **13** (6.47 g, 66% from **11**). **13**: amorphous solid, $[\alpha]_D$ +12.9° (c = 0.6, MeOH). *Anal.* Calcd for $C_{24}H_{32}N_2O_7Si$: C, 59.00; H, 6.60; N, 5.73. Found: C, 58.72; H, 6.62; N, 5.72. IR ($CHCl_3$): 3580, 3500, 3450, 3390, 2930, 1680, 1530, 1110 cm^{-1}. ^{1}H-NMR (200 MHz, DMSO-d_6–D_2O) δ: 7.78—7.60 (4H, m), 7.52—7.36 (6H, m), 5.08 (0.7H, br s), 4.86 (0.3H, br s), 4.18—3.22 (6H, m), 1.01 (9H, s). FAB-MS m/z: 511 $(M+Na)^+$.

6-*O*-*tert*-Butyldiphenylsilyl-2-deoxy-2-oxamoylamino-D-mannitol (14) $NaBH_4$ (2.13 g) was added to a stirred ice-cold solution of **16** (5.51 g) in MeOH (120 ml) and the mixture was stirred at room temperature for 30 min. After an acidic treatment with 0.1 N HCl (500 ml) in an ice-water bath, the reaction mixture was extracted with AcOEt twice. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated *in vacuo* to give **14** (5.12 g, 92%). **14**: colorless fine crystals, mp 174—175 °C (AcOEt), $[\alpha]_D$ −16.9° (c = 0.7, MeOH). *Anal.* Calcd for $C_{24}H_{34}N_2O_7Si$: C, 58.75; H, 6.98; N, 5.71. Found: C, 58.42; H, 7.01; N, 5.67. IR (Nujol): 3380, 3315, 1660, 1537, 1112 cm^{-1}. ^{1}H-NMR (200 MHz, DMSO-d_6), δ: 8.33 (1H, d, J = 7 Hz, D_2O-exchangeable), 8.08 (1H, br s, D_2O-exchangeable), 7.86 (1H, br s, D_2O-exchangeable), 7.75—7.60 (4H, m), 7.50—7.35 (6H, m), 4.77 (1H, t, J = 4 Hz, D_2O-exchangeable), 4.68 (1H, d, J = 4 Hz, D_2O-exchangeable), 4.51 (1H, d, J = 6 Hz, D_2O-exchangeable), 4.29 (1H, d, J = 6 Hz, D_2O-exchangeable), 3.96—3.30 (8H, m), 0.99 (9H, s). FAB-MS m/z: 513 $(M+Na)^+$, 491 $(M+H)^+$.

6-*O*-*tert*-Butyldiphenylsilyl-2-deoxy-1,3 : 4,5-di-*O*-isopropylidene-2-oxamoylamino-D-mannitol (15) $BF_3 \cdot OEt_2$ (1.40 ml) was added dropwise to a stirred solution of **14** (3.80 g) in acetone (70 ml) at −25 °C under an N_2 atmosphere. After being stirred for 4 h at −25——18 °C, the reaction mixture was poured into stirred, ice-cold saturated aqueous $NaHCO_3$ and extracted twice with AcOEt. The extracts were combined, washed with brine, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was purified by column chromatography (SiO_2 150 g, CH_2Cl_2 : AcOEt = 1 : 1, then CH_2Cl_2 : MeOH = 8 : 1) to afford **15** (3.82 g, 86%). **15**: amorphous solid, $[\alpha]_D$ −39.0° (c = 0.6, MeOH). *Anal.* Calcd for $C_{30}H_{42}N_2O_7Si$: C, 63.13; H, 7.42; N, 4.91. Found: C, 62.87; H, 7.51; N, 4.78. IR ($CHCl_3$): 3520, 3400, 2945, 1692, 1530, 1381, 1116 cm^{-1}. ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 8.60 (1H, d, J = 7 Hz, D_2O-exchangeable), 8.10 (1H, br s, D_2O-exchangeable), 7.82 (1H, br s, D_2O-exchangeable), 7.74—7.58 (4H, m), 7.55—7.35 (6H, m), 4.42 (1H, td, J = 6, 2 Hz), 4.10—3.20 (7H, m), 1.41, 1.28, 1.08, 1.03 (each 3H, s), 1.02 (9H, s). FAB-MS m/z: 593 $(M+Na)^+$, 571 $(M+H)^+$.

2-Deoxy-1,3 : 4,5-di-*O*-isopropylidene-2-oxamoylamino-D-mannitol (16) A solution of *n*-$Bu_4NF \cdot 3H_2O$ (789 mg) in THF (15 ml) was added dropwise to a stirred solution of **15** (571 mg) in THF (10 ml) at −17——14 °C over a period of 20 min. The mixture was stirred at −17——21 °C for 1.5 h, then the solvent was removed under reduced pressure. The residue was purified by column chromatography (SiO_2 28 g, CH_2Cl_2 : AcOEt : MeOH = 50 : 50 : 2) to furnish the primary alcohol **16** (332 mg, quant.). **16**, amorphous solid, $[\alpha]_D$ −77.5° (c = 0.5, MeOH). *Anal.* Calcd for $C_{14}H_{24}N_2O_7$: C, 50.59; H, 7.28; N, 8.43. Found: C, 50.87; H, 7.08; N, 8.10. IR ($CHCl_3$): 3525, 3475, 3402, 3325, 3002, 1687, 1532, 1381, 1162 cm^{-1}. ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 8.68 (1H, d, J = 7 Hz, D_2O-exchangeable), 8.12 (1H, br s), 7.83 (1H, br s), 4.81 (1H, t, J = 4 Hz), 4.28—3.80 (4H, m), 3.78—3.50 (4H, m), 1.44, 1.40, 1.28, 1.24 (each 3H, s). FAB-MS m/z: 355 $(M+Na)^+$, 333 $(M+H)^+$.

5-Deoxy-2,3 : 4,6-di-*O*-isopropylidene-5-oxamoylamino-D-mannose (17) A stirred anhydrous solution of pyridine (3.6 ml) in CH_2Cl_2 (90 ml) was treated with CrO_3 (2.22 g) at room temperature under an N_2 atmosphere. The mixture was stirred for 15 min, then a solution of **16** (730 mg) in anhydrous CH_2Cl_2 (90 ml) was added dropwise over a period of 5 min. The mixture was stirred for an additional 30 min, diluted with Et_2O (200 ml) and filtered through cellulose powder. Removal of the solvent under reduced pressure gave a residue, which was extracted with Et_2O (250 ml). The combined extracts were evaporated *in vacuo* to afford the aldehyde **17** (846 mg) as a crude oil. (IR ($CHCl_3$): 1720 cm^{-1}. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 9.60 (1H, s)).

2,4-Dinitrophenylhydrazones of 17 (18, 19) 2,4-Dinitrophenylhydrazine (90 mg) and H_3PO_4 (0.02 ml) were added to a stirred solution of **17** (43 mg)

in EtOH (1 ml). After being stirred for 1 h, the reaction mixture was diluted with CH_2Cl_2 (5 ml) and washed with saturated aqueous $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2–MeOH (4 : 1) 3 times. The organic layers were combined, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was purified by preparative TLC (CH_2Cl_2 : MeOH = 20 : 1) to afford the *anti*-isomer **18** (32 mg, 41% from **16**) and the *syn*-isomer **19** (16 mg, 21% from **16**). **18**: yellow amorphous solid, $[\alpha]_D$ +185.5° (c = 0.5, $CHCl_3$). IR ($CHCl_3$): 3520, 3400, 3310, 1692, 1618, 1596, 1338 cm^{-1}. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 8.86 (1H, d, J = 1 Hz), 8.72 (1H, d, J = 10 Hz, D_2O-exchangeable), 8.39 (1H, dd, J = 10, 1 Hz), 8.09 (1H, br s, D_2O-exchangeable), 8.05 (1H, d, J = 8 Hz), 7.90 (1H, d, J = 10 Hz), 7.81 (1H, br s, D_2O-exchangeable), 4.83 (1H, t, J = 8 Hz), 4.32 (1H, d, J = 8 Hz), 4.07 (1H, d, J = 11 Hz), 3.97 (1H, m), 3.66 (1H, t, J = 11 Hz), 3.58 (1H, dd, J = 11, 7 Hz), 1.49 (3H, s), 1.35 (6H, s), 1.32 (3H, s). FAB-MS m/z: 533 $(M+Na)^+$. High-resolution FAB-MS Calcd for $C_{20}H_{26}N_6NaO_{10}$ $(M+Na)^+$: 533.161. Found: 533.159. **19**: yellow amorphous solid, $[\alpha]_D$ +175.2° (c = 0.5, $CHCl_3$). IR ($CHCl_3$): 3520, 3400, 3280, 1690, 1618, 1592, 1338 cm^{-1}. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 8.90 (1H, d, J = 1 Hz), 8.68 (1H, d, J = 10 Hz, D_2O-exchangeable), 8.42 (1H, dd, J = 10, 1 Hz), 8.11 (1H, br s, D_2O-exchangeable), 7.96 (1H, d, J = 10 Hz), 7.83 (1H, br s, D_2O-exchangeable), 6.96 (1H, d, J = 3 Hz), 5.30 (1H, dd, J = 7, 3 Hz), 4.48 (1H, d, J = 7 Hz), 4.02 (1H, d, J = 10 Hz), 3.97 (1H, m), 3.66 (1H, t, J = 11 Hz), 3.60 (1H, dd, J = 11, 6 Hz), 1.67, 1.37, 1.20, 0.96 (each 3H, s). FAB-MS m/z: 533 $(M+Na)^+$. High-resolution FAB-MS Calcd for $C_{20}H_{26}N_6NaO_{10}$ $(M+Na)^+$: 533.161. Found: 533.161.

Double Cyclization of 17 The crude aldehyde **17** (460 mg), obtained as described above, was treated with 6 N NH_3–MeOH (20 ml) at room temperature for 20 h under an N_2 atmosphere. After removal of the solvent under reduced pressure, the residue was purified by column chromatography (SiO_2 25 g, CH_2Cl_2 : MeOH = 50 : 1) to afford kifunensine diacetonide **20** (284 mg, 76% from **16**) along with its **8a**-epimer **21** (15 mg, 4.0% from **16**). Recrystallization of **20** from *n*-hexane–AcOEt provided colorless fine crystals. This product was identical with an authentic sample derived from the natural product as judged from mixed melting-point determination and direct TLC comparison (CH_2Cl_2 : MeOH = 9 : 1, Rf = 0.55; AcOEt, Rf = 0.50), $[\alpha]_D$, IR ($CHCl_3$), and ^{1}H-NMR ($CDCl_3$).[2] **21**: colorless fine crystals, mp >280 °C (MeOH), $[\alpha]_D$ −15.8° (c = 0.1, DMSO). *Anal.* Calcd for $C_{14}H_{20}N_2O_6 \cdot H_2O$: C, 50.90; H, 6.71; N, 8.48. Found: C, 50.92; H, 6.43; N, 8.45. IR (Nujol): 3130, 1740, 1722, 1708, 1220, 1200, 1160, 1024 cm^{-1}. ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 10.03 (1H, s, D_2O-exchangeable), 5.25 (1H, d, J = 3 Hz), 4.70 (1H, t, J = 11 Hz), 4.38 (1H, dd, J = 4, 3 Hz), 4.29 (1H, dd, J = 11, 5 Hz), 4.15 (1H, dd, J = 8, 4 Hz), 3.78 (1H, dd, J = 10, 8 Hz), 3.25 (1H, td, J = 11, 5 Hz), FAB-MS m/z: 335 $(M+Na)^+$, 313 $(M+H)^+$.

Kifunensine (1) Compound **20** (219 mg) was treated with 75% aqueous TFA (5.0 ml) at room temperature for 5 h. The precipitate was collected by vacuum filtration and washed with water (2.0 ml) to give **1** as a white powder (134 mg, 82%). Recrystallization from water furnished colorless prisms. This product was identical with an authentic sample as judged from mixed melting-point determination and direct TLC comparison ($CHCl_3$: MeOH : H_2O = 6 : 4 : 1, Rf = 0.31; isopropyl alcohol : H_2O = 9 : 1, Rf = 0.37), $[\alpha]_D$, IR (KBr), and ^{1}H-NMR (400 MHz, D_2O).[2]

8a-*epi*-Kifunensine (23) Compound **21** (10 mg) was treated with 75% aqueous TFA (1 ml) at room temperature for 3 h. After removal of the solvent under reduced pressure, the residue was purified by preparative TLC ($CHCl_3$: MeOH : H_2O = 6 : 4 : 1) to afford **8a**-*epi*-kifunensine (**23**, 7 mg, 94%). **23**: amorphous solid, $[\alpha]_D$ −38.7° (c = 0.1, H_2O). IR (KBr): 3350, 3290, 3190, 1720, 1700, 1680, 1400, 1095, 1080, 1056, 1036 cm^{-1}. ^{1}H-NMR (400 MHz, D_2O) δ: 5.16 (1H, d, J = 2 Hz), 4.38 (1H, dd, J = 13, 2 Hz), 4.30 (1H, t, J = 2 Hz), 4.27 (1H, dd, J = 13, 5 Hz), 3.87—3.83 (2H, m), 3.45 (1H, ddd, J = 8, 5, 2 Hz). FAB-MS m/z: 255 $(M+Na)^+$. High-resolution FAB-MS Calcd for $C_8H_{12}N_2NaO_6$ $(M+Na)^+$: 255.059. Found: 255.058.

^{1}*N*-Methylkifunensine Diacetonide (22) Compound **17** (130 mg) was treated with 30% $MeNH_2$–MeOH (5 ml) at room temperature for 1 h under an N_2 atmosphere. The reaction mixture was evaporated *in vacuo* and the residue was purified by column chromatography (SiO_2 7 g, CH_2Cl_2 : MeOH = 200 : 1—50 : 1) to afford **22** (88 mg, 80% from **16**). This compound was also prepared from the natural product (**1**) *via* the diacetonide **20** as follows. A mixture of **20** (100 mg), MeI (114 mg), K_2CO_3 (45 mg), and acetone (5 ml) was heated under reflux for 1.5 h. The reaction mixture was cooled to room temperature, filtered, and evaporated *in vacuo*. The residue was purified by preparative TLC to give authentic **22** (65 mg, 62%). Synthetic **22** was identical with this authentic sample as judged from mixed melting-point determination and direct TLC comparison (CH_2Cl_2 : MeOH = 9 : 1, Rf = 0.68; AcOEt, Rf = 0.57) and ^{1}H-NMR (200 MHz, $CDCl_3$). **22**: colorless fine crystals, mp 245—246 °C (AcOEt), $[\alpha]_D$ −64.8° (c = 0.5, MeOH). *Anal.* Calcd for $C_{15}H_{22}N_2O_6$: C, 55.21; H, 6.79; N, 8.58. Found: C, 55.21; H, 6.75; N, 8.67. IR ($CHCl_3$): 2990, 1752, 1420, 1377, 1090, 1068 cm^{-1}. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 4.73 (1H, dd, J = 10, 4 Hz), 4.70 (1H, d, J = 8 Hz), 4.38 (1H, t, J = 8 Hz), 4.19 (1H, dd, J = 10, 8 Hz), 4.02 (1H, t, J = 8 Hz), 3.77 (1H, t, J = 10 Hz), 3.59 (1H, td, J = 10, 4 Hz), 3.19, 1.59, 1.57, 1.50, 1.39 (each 3H, s). FAB-MS m/z: 327 $(M+H)^+$.

^{1}*N*-Methylkifunensine (24) Compound **22** (65 mg) was treated with 75% aqueous TFA (2 ml) in the same way as described for deprotection of **20** to afford **24** (41 mg, 84%). **24**: colorless fine crystals, mp 283—285 °C (dec., MeOH), $[\alpha]_D$ +66.0° (c = 0.4, H_2O). *Anal.* Calcd for $C_9H_{14}N_2O_6$: C, 43.90; H, 5.73; N, 11.38. Found: C, 43.68; H, 5.68; N, 11.25. IR (KBr): 3405, 3310, 3190, 2910, 1720, 1706, 1692, 1430, 1232, 1110, 1061, 1050, 1000 cm^{-1}. ^{1}H-NMR (200 MHz, D_2O) δ: 5.08 (1H, d, J = 10 Hz), 4.43 (1H, dd, J = 10, 4 Hz), 4.18 (1H, d, J = 3 Hz), 4.07 (1H, t, J = 3 Hz), 3.98 (1H, d, J = 10 Hz), 3.92—3.78 (2H, m), 3.30 (3H, s). FAB—MS m/z: 247 $(M+H)^+$.

Acknowledgement We are grateful to Dr. T. Kaizu and Dr. M. Okamoto (Fujisawa Pharmaceutical Co., Ltd.) for the biological assays.

References and Notes

1) A preliminary report of this work: H. Kayakiri, C. Kasahara, T. Oku, and M. Hashimoto, *Tetrahedron Lett.*, **31**, 225 (1990).
2) *a*) H. Kayakiri, S. Takase, T. Shibata, M. Okamoto, H. Terano, M. Hashimoto, T. Tada, and S. Koda, *J. Org. Chem.*, **54**, 4015 (1989); *b*) *Idem*, *Chem. Pharm. Bull.*, **39**, 1378 (1991).
3) M. Iwami, O. Nakayama, H. Terano, M. Kohsaka, H. Aoki, and H. Imanaka, *J. Antibiot.*, **40**, 612 (1987).
4) EC_{70} = 0.02 μg/ml: S. Izumi, T. Kaizu, M. Yamashita, M. Okamoto, and M. Okuhara, "Novel Microbial Products for Medicine and Agriculture," ed. by A. L. Demain, G. A. Somkuti, J. C. Hunter-Cevera, and H. W. Rossmoore, Elsevier Science Publishers, Amsterdam, in press.
5) Mannojirimycin (nojirimycin B): T. Niwa, T. Tsuruoka, H. Goi, Y. Kodama, J. Itoh, S. Inoue, Y. Yamada, T. Niida, M. Nobe, and Y. Ogawa, *J. Antibiot.*, **37**, 1579 (1984).
6) For a review of 1,5-iminopyranoses and 1,4-iminofuranoses, see G. W. J. Fleet, *Spec. Publ. Royal Chem. Soc.*, **65**, 149 (1988).
7) G. J. Karabatsos, R. A. Taller, and F. M. Vane, *J. Am. Chem. Soc.*, **85**, 2326 (1963).
8) Compounds **3**, **8**, **23**, **24** and kifunensine (**1**) did not inhibit α-glucosidase (rabbit intestine).

Synthesis of 8-*epi*-Kifunensine[1)]

Hiroshi KAYAKIRI, Teruo OKU,* and Masashi HASHIMOTO

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5–2–3 Tokodai, Tsukuba, Ibaraki 300-26, Japan. *Received November 5, 1990*

A synthesis of 8-*epi*-kifunensine (2) in optically active form was achieved starting from D-glucose *via* a modified double cyclization of the oxamide-hemiacetal precursor 9 with 2,4-dimethoxybenzylamine as a key step.

Keywords 8-*epi*-kifunensine; polyhydroxylated piperidine; 4,5-dioxoimidazolidine; D-glucose; X-ray analysis

Kifunensine (**1**) is a novel immunomodulator isolated from an actinomycete. This natural product is a representative of a new class of polyhydroxylated piperidines, having a novel structure, which corresponds to a cyclic oxamide derivative of 1-amino-substituted mannojirimycin (**3**),[2)] and interesting biological activities.[3)] These unique features of **1** prompted us to investigate the synthesis of new compounds belonging to this class. Herein we report a synthesis of 8-*epi*-kifunensine (**2**), which is similarly related to nojirimycin (**4**)[4)] (Chart 1).

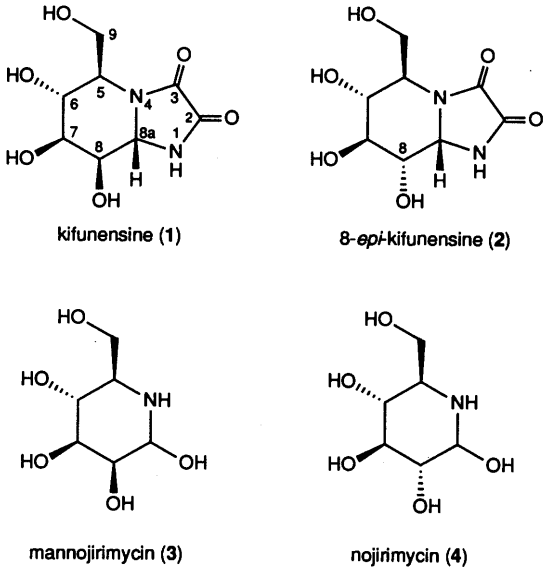


Chart 1

In the previous paper[5)] we demonstrated that construction of the basic framework of kifunensine, the octahydro-2,3-dioxoimidazo[1,2-*a*]pyridine ring system, could be achieved *via* a double cyclization of the oxamide-aldehyde precursor with ammonia (**5**→**6**). We established a synthesis of kifunensine in optically active form by a route involving this cyclization reaction (**7**→**8**) as the key step. We anticipated that 8-*epi*-kifunensine could be synthesized *via* a similar cyclization process. For this synthesis of **2**, we chose the non-protected oxamide-hemiacetal precursor (**9**) as the key intermediate (Chart 2).

The requisite intermediate **9** was prepared as follows (Chart 3). A mixture of 5-amino-5-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose (**12**) and 5-amino-5-deoxy-1,2-*O*-isopropylidene-β-L-idofuranose (**13**) (about 5 : 2, as judged from the proton nuclear magnetic resonance (^{1}H-NMR) spectrum) was prepared from 1,2-*O*-isopropylidene-α-D-glucofuranose (**11**) in 65% overall yield according to the method reported in the literature.[6)] It was found to be difficult to separate these epimers and accordingly we examined the feasibility of separating them as the N-acyl derivatives. Thus, N-acylation of the mixture with carbobenzyloxy chloride followed by silica gel column chromatography gave the (5*R*)-*N*-carbobenzyloxy (Cbz) derivative **14** in 28% yield along with its (5*S*)-epimer **15** (11% yield). Hydrogenolysis of the *N*-Cbz derivatives on 10% Pd–C afforded the desired (5*R*)-amine **12** in quantitative yield from **14** and the (5*S*)-amine **13** in 95% yield from **15**. Compounds **12**,[7)] **13**,[8)] and **14**[9)] were identified by comparison of their physical data with the reported values. N-Selective acylation of **12** was achieved by silylation

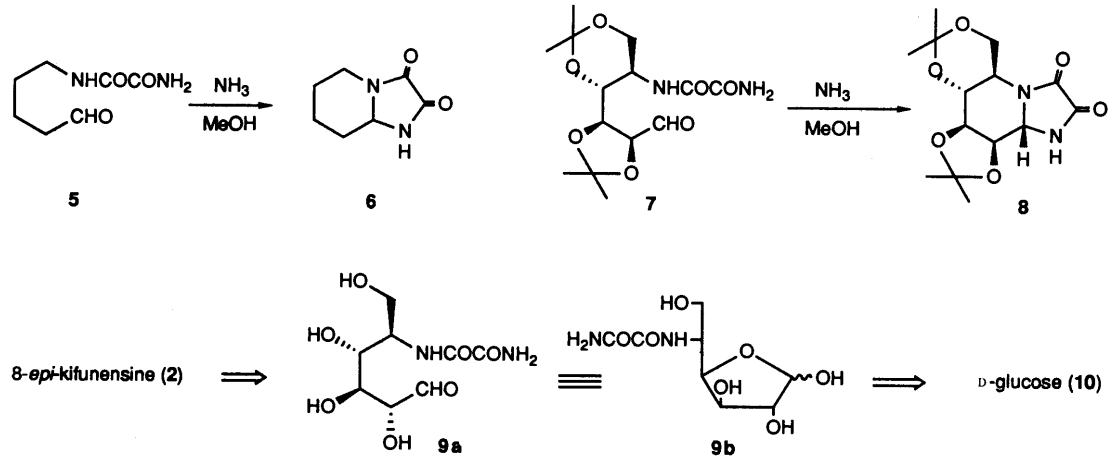
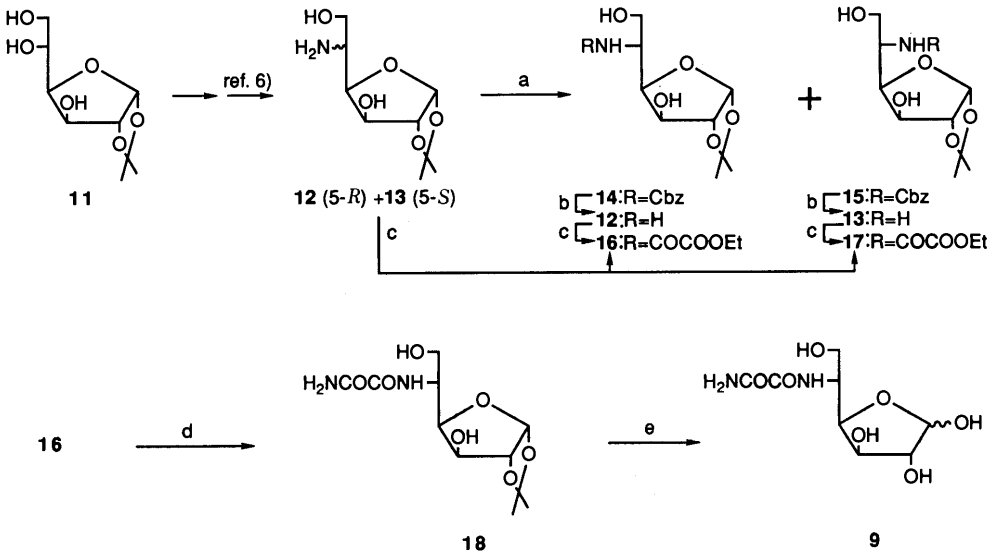
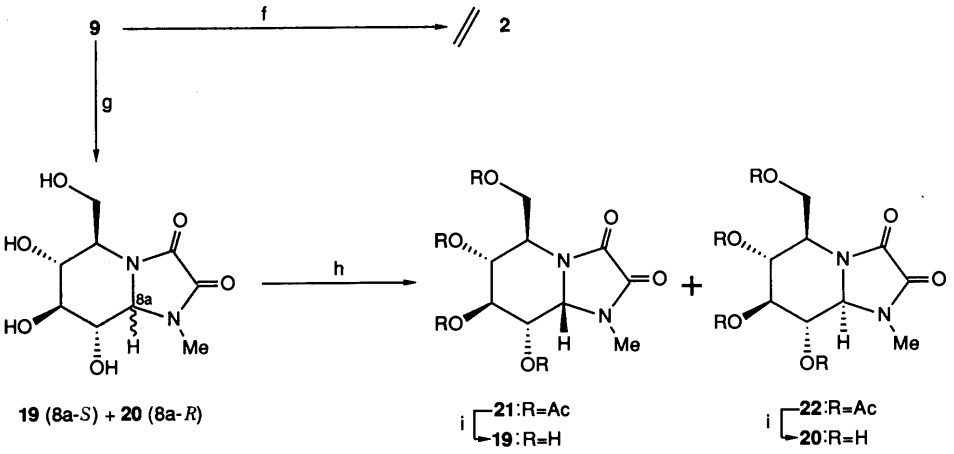


Chart 2



(a) CbzCl, NaOH, dioxane-H_2O,0°C (b) H_2, 10% Pd-C, MeOH (c) i) BSA, THF; ii) EtOOCCOCl, 0°C; iii) 1 N aq. AcOH, 0°C (d) 6 N NH_3-MeOH (e) 75% aq. TFA, 0°C

Chart 3

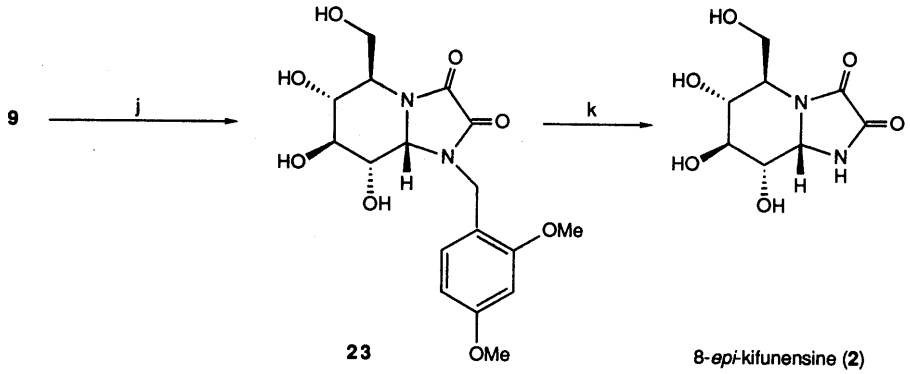


(f) 6 N NH_3-MeOH (g) 40% aq. $MeNH_2$ (h) Ac_2O, Py (i) NaOH, MeOH-H_2O

Chart 4

with bis(trimethylsilyl)acetamide (BSA) followed by acylation with ethyl oxalyl chloride and subsequent desilylation with 1 N aqueous AcOH to afford the (5*R*)-*N*-acyl derivative **16** in 98% yield from **12**. The (5*S*)-isomer **17** was also prepared in the same way in 91% yield from **13**. This procedure could be applied directly to the mixture of **12** and **13** to afford **16** in 31% yield and **17** in 13% yield; both products were identical with the respective samples prepared from pure **12** and **13**. Compound **16** thus obtained was treated with 6N NH_3 in MeOH to give the oxamide **18** in quantitative yield, and this was deprotected with 75% aqueous trifluoroacetic acid (TFA) to afford the key intermediate **9** as an about 1 : 1 mixture of α and β anomers in 77% yield.

Pursuing the initial plan, we examined a double cyclization of the oxamide-hemiacetal **9** with 6 N NH_3 in MeOH in the same manner as used for our synthesis of kifunensine.[5] The desired cyclization, however, did not occur at all and the reactant **9** decomposed slowly. Although we examined various modified conditions (*e.g.*, 28% aqueous NH_3, at room temperature or 100 °C in a sealed tube; saturated aqueous NH_4HCO_3, at room temperature; liquid NH_3, at −78 °C—reflux), all of them failed to afford the cyclization product. As compared with **7** in the synthesis of kifunensine, there are two major differences in **9** in this case. The aldehyde group in **9** is masked as a hemiacetal, and accordingly the distance between the oxamide and aldehyde moieties is much larger than that in **7**. We therefore speculated that more nucleophilic amines are required for formation of stable addition products which might undergo the desired reaction. When **9** was treated with 40% aqueous $MeNH_2$ at room temperature for 12 h,



(j) 2,4-dimethoxybenzylamine, MeOH (k) $K_2S_2O_8$, Na_2HPO_4, CH_3CN-H_2O, reflux

Chart 5

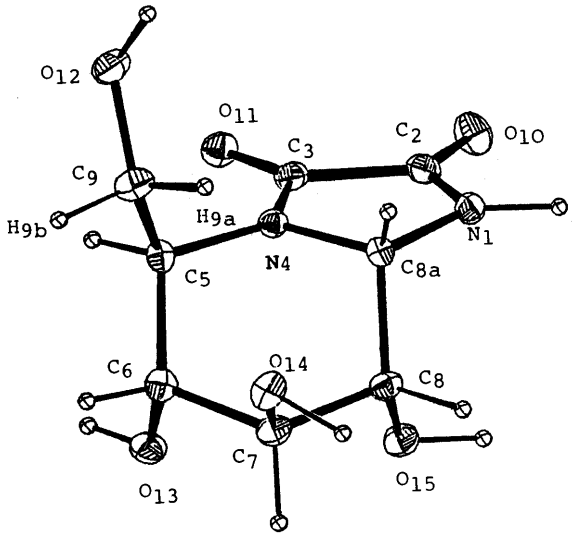


Fig. 1

the cyclization reaction proceeded as expected to give a mixture of N^1-methyl-8-*epi*-kifunensine (**19**) and its 8a-epimer **20** which could not be separated on thin layer chromatography (TLC). The mixture was acylated with acetic anhydride to furnish, after silica gel column chromatography, N^1-methyl-8-*epi*-kifunensine tetraacetate (**21**) in 33% yield from **9** along with its 8a-epimer **22** (13% yield). The stereochemistry at C-8a was assigned on the basis of the $J_{8,8a}$ values of 2 Hz (8, 8a-*cis*) for **21** and 9 Hz (8, 8a-*trans*) for **22** in the ^{1}H-NMR spectra. Alkaline hydrolysis of these compounds with NaOH in aqueous MeOH afforded N^1-methyl-8-*epi*-kifunensine (**19**) in 96% yield from **21** and its 8a-epimer **20** in 98% yield from **22**.

With these results in hand, we devised for the synthesis of **2** a two-step procedure consisting of a double cyclization of **9** with an appropriate benzylamine and subsequent removal of the benzyl group. Investigations revealed that treatment of **9** with 2,4-dimethoxybenzylamine (1.5 eq) in MeOH achieved the desired double cyclization to afford, after cation exchange resin column chromatography followed by preparative TLC, N^1-(2,4-dimethoxybenzyl)-8-*epi*-kifunensine (**23**) in 25% yield as a single diastereomer. The configuration of C-8a was presumed to be *S* (the same as that of kifunensine) on the basis of ^{1}H-NMR evidence ($J_{8,8a}$ = 1 Hz, (8, 8a-*cis*)). The low yield might be due to a steric factor. The stereoselectivity may be attributable to the bulkiness of 2,4-dimethoxybenzylamine. Finally, oxidative removal of the benzyl group in **23** with $K_2S_2O_8$ furnished 8-*epi*-kifunensine (**2**) in 94% yield. The configuration was assigned as *S* based on the fact that nuclear Overhauser effect (NOE) was observed between 8a-H (δ 5.45, d, J = 2 Hz) and 9-Ha (δ 3.99, dd, J = 12, 9 Hz) in the ^{1}H-NMR spectrum (400 MHz, D_2O) of **2**. Furthermore the structure of 8-*epi*-kifunensine was confirmed by X-ray crystallographic analysis using crystals obtained from water (Fig. 1).[10]

We have thus completed the synthesis of 8-*epi*-kifunensine (**2**) by adopting the modified double cyclization approach. As this procedure could be applied to a non-protected oxamide-hemiacetal precursor, this work suggests a wider usefulness of the double cyclization method for synthesis of this class of compounds.

Experimental

The instruments used to obtain physical data and the experimental conditions for chromatography were the same as described in our preceding paper.[11]

5-Benzyloxycarbonylamino-5-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose (14) and 5-Benzyloxycarbonylamino-5-deoxy-1,2-*O*-isopropylidene-β-L-idofuranose (15) A mixture of 5-amino-5-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose (**12**) and 5-amino-5-deoxy-1,2-*O*-isopropylidene-β-L-idofuranose (**13**) was prepared from 1,2-*O*-isopropylidene-α-D-glucofuranose (**11**) in 65% overall yield by a four step procedure reported by Tsuda *et al.*[6] To a stirred ice-cold solution of this mixture (523 mg) in 1,4-dioxane (10 ml) and water (10 ml), a solution of carbobenzyloxy chloride (706 mg) in 1,4-dioxane (5 ml) and 1 N aqueous NaOH (6 ml) were added over a period of 5 min. After being stirred at room temperature for 1 h, the mixture was extracted with CH_2Cl_2 three times. The organic layers were combined, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was purified by column chromatography (SiO_2 44 g, *n*-hexane : AcOEt = 3 : 1—3 : 2) to afford **14** (240 mg, 28%) and **15** (93 mg, 11%). **14**, a colorless oil, $[\alpha]_D$ +32.2° (c = 1.0, $CHCl_3$). IR ($CHCl_3$): 3430, 2995, 2940, 1694, 1512, 1390, 1380, 1076 cm^{-1} (lit.,[9] oil, $[\alpha]_D$ +25.5° (c = 1). IR ($CHCl_3$): 3430, 2995, 2940, 1695, 1510, 1390, 1380, 1080 cm^{-1}). **15**, colorless fine crystals, mp 140—141 °C (AcOEt), $[\alpha]_D$ −24.5° (c = 0.5, $CHCl_3$), *Anal.* Calcd for $C_{17}H_{23}NO_7$: C, 57.78; H, 6.56; N, 3.96. Found: C, 57.78; H, 6.43; N, 3.96. IR ($CHCl_3$): 3440, 1710, 1515, 1384, 1376, 1074 cm^{-1}. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 7.36 (5H, s), 5.92 (1H, d, J = 4 Hz), 5.35 (1H, d, J = 6 Hz), 5.12 (1H, d, J = 12 Hz), 5.07 (1H, d, J = 12 Hz), 4.52 (1H, d, J = 4 Hz), 4.26 (1H, d, J = 3 Hz), 4.20 (1H, dd, J = 6, 3 Hz), 4.06 (1H, m), 3.89 (1H, dd, J = 12, 4 Hz), 3.69 (1H, dd, J = 12, 6 Hz), 1.48, 1.30 (each 3H, s). Fast-atom bombardment mass spectra (FAB-MS) m/z: 354 $(M+H)^+$.

5-Amino-5-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose (12) A mixture of **14** (220 mg), 10% Pd–C (44 mg), and MeOH (5 ml) was stirred under hydrogen (3 atm) at room temperature for 3 h. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo*. The residue was partitioned into a mixture of CH_2Cl_2–H_2O. The organic layer was extracted twice with H_2O. The aqueous layers were combined, filtered, and lyophilized to give **12** (136 mg, quant.). **12**, colorless needles, mp 125—126 °C (EtOH–Et_2O), $[\alpha]_D$ −13.0° (c=1.0, MeOH) (lit.,[7] mp 125—126 °C (EtOH–Et_2O), $[\alpha]_D$ −13.8° (c=4, MeOH)).

5-Amino-5-deoxy-1,2-*O*-isopropylidene-β-L-idofuranose (13) Compound **15** (34 mg) was hydrogenated in the same way as described for the deprotection of **14** to afford **13** (20 mg, 95%). **13**, colorless leaflets, mp 183—185 °C (EtOH–Et_2O), $[\alpha]_D$ −3.3° (c=1.0, MeOH) (lit.,[8] mp 184—185 °C (EtOH–Et_2O), $[\alpha]_D$ −3.7° (c=1.1, MeOH)).

5-Deoxy-5-(2-etoxyethanedioyl)amino-1,2-*O*-isopropylidene-α-D-glucofuranose (16) BSA (0.3 ml) was added to a stirred anhydrous solution of **12** (52 mg) in tetrahydrofuran (THF) (2 ml) at room temperature under an N_2 atmosphere, and the mixture was stirred for 2 h. Then a 1 M solution (0.24 ml) of ethyl oxalyl chloride in THF was added dropwise under ice-cooling. At the same temperature the mixture was stirred for 2 h and treated with 1 N aqueous AcOH (0.5 ml) for 20 min. The reaction mixture was poured into brine and extracted with AcOEt three times. The organic layers were combined, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was purified by preparative TLC (CH_2Cl_2 : EtOH = 9 : 1) to afford **16** (74 mg, 98%). **16**, colorless fine crystals, mp 164—165 °C (Et_2O), $[\alpha]_D$ +35.1° (c=0.5, $CHCl_3$). *Anal.* Calcd for $C_{13}H_{21}NO_8$: C, 48.90; H, 6.63; N, 4.39. Found: C, 48.87; H, 6.54; N, 4.34. IR ($CHCl_3$): 3390, 1739, 1690, 1377, 1075, 1015 cm^{-1}. ^{1}H-NMR (200 MHz, CD_3OD) δ: 5.89 (1H, d, J=4 Hz), 4.50 (1H, d, J=4 Hz), 4.40—4.22 (4H, m), 4.16 (1H, br s), 3.84—3.68 (2H, m), 1.47 (3H, s), 1.37 (3H, t, J=7 Hz), 1.31 (3H, s). FAB-MS m/z: 342 $(M+Na)^+$, 320 $(M+H)^+$.

5-Deoxy-5-(2-ethoxyethanedioyl)amino-1,2-*O*-isopropylidene-β-L-idofuranose (17) In the same way as described for **16**, compound **13** (90 mg) was acylated with BSA (0.5 ml) and ethyl oxalyl chloride (1 M solution in THF, 0.42 ml) followed by desilylation with 1 N aqueous AcOH (0.9 ml) to afford **17** (120 mg, 91%). **17**, amorphous solid, $[\alpha]_D$ −24.4° (c=0.5, $CHCl_3$). *Anal.* Calcd for $C_{13}H_{21}NO_8$: C, 48.90; H, 6.63; N, 4.39. Found: C, 48.62; H, 6.55; N, 4.38. IR ($CHCl_3$): 3405, 1736, 1695, 1376, 1073, 1014 cm^{-1}. ^{1}H-NMR (200 MHz, CD_3OD) δ: 5.87 (1H, d, J=4 Hz), 4.51 (1H, d, J=4 Hz), 4.40—4.26 (4H, m), 4.12 (1H, m), 3.75—3.65 (2H, m), 1.48 (3H, s), 1.37 (3H, t, J=7 Hz), 1.30 (3H, s). FAB-MS m/z: 342 $(M+Na)^+$, 320 $(M+H)^+$.

Acylation of a Mixture of 12 and 13 In the same way as described for **16**, a mixture (55 mg) of **12** and **13**, which was used for the synthesis of **14** and **15**, was acylated with BSA (0.4 ml) and ethyl oxalyl chloride (1 M solution in THF, 0.26 ml) followed by desilylation with 1 N aqueous AcOH (0.5 ml) to afford **16** (25 mg, 31%) and **17** (10 mg, 13%).

5-Deoxy-1,2-*O*-isopropylidene-5-oxamoylamino-α-D-glucofuranose (18) An anhydrous solution of **16** (638 mg) in MeOH (9 ml) was treated with 6 N NH_3 in MeOH (6 ml) at room temperature for 20 min under an N_2 atmosphere. Removal of the solvent under reduced pressure afforded the oxamide **18** (582 mg, quant.). **18**, amorphous solid, $[\alpha]_D$ +26.8°C (c=1.1, MeOH). *Anal.* Calcd for $C_{11}H_{18}N_2O_7$: C, 45.52; H, 6.25; N, 9.65. Found: C, 45.32; H, 6.11; N, 9.36. IR (KBr): 3350, 2905, 1650, 1527, 1370, 1060, 1006 cm^{-1}. ^{1}H-NMR (200 MHz, CD_3OD) δ: 5.89 (1H, d, J=4 Hz), 4.50 (1H, d, J=4 Hz), 4.29—4.08 (3H, m), 3.85—3.70 (2H, m), 1.48, 1.30 (each 3H, s). FAB-MS m/z: 313 $(M+Na)^+$, 291 $(M+H)^+$.

5-Deoxy-5-oxamoylamino-D-glucose (9) Compound **18** (4.00 g) was treated with 75% aqueous TFA (80 ml) under ice-cooling for 6 h. Removal of the solvent under reduced pressure gave a colorless viscous oil which was crystallized from CH_2Cl_2–MeOH–H_2O (130 : 30 : 2) to afford **9** (2.65 g, 77%). **9**, colorless fine crystals, mp 100—102 °C (CH_2Cl_2 : MeOH : H_2O = 130 : 30 : 2), $[\alpha]_D$ −0.2° (c=0.5, H_2O). *Anal.* Calcd for $C_8H_{14}N_2O_7 \cdot 1/3H_2O$: C, 37.50; H, 5.77; N, 10.93. Found: C, 37.60; H, 5.55; N, 11.02. IR(KBr): 3320, 2910, 1648, 1532, 1392, 1010 cm^{-1}. ^{1}H-NMR (200 MHz, D_2O) δ: 5.39 (0.5H, d, J=4 Hz), 5.10 (0.5H, s), 4.30—3.95 (4H, m), 3.85—3.56 (2H, m). FAB-MS m/z: 251 $(M+H)^+$.

8-*epi*-N^1-Methylkifunensine Tetraacetate (21) and Its 8a-Epimer 22 Compound **9** (1.00 g) was treated with 40% aqueous $MeNH_2$ (50 ml) at room temperature for 12 h and then the mixture was evaporated *in vacuo*. The residue was dissolved in anhydrous pyridine (50 ml) and the solution was treated with acetic anhydride (25 ml) at room temperature for 12 h. After concentration *in vacuo*, the residue was dissolved in AcOEt and washed with 1 N aqueous HCl. The aqueous layer was extracted with CH_2Cl_2–MeOH (4 : 1) three times. The organic layers were combined, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was purified by column chromatography (SiO_2 150 g, CH_2Cl_2 : MeOH = 100 : 1) to afford **21** (552 mg, 33%) and **22** (221 mg, 13%). **21**, colorless fine crystals, mp 228—229 °C (*n*-hexane–AcOEt), $[\alpha]_D$ −24.5° (c=0.4, $CHCl_3$). *Anal.* Calcd for $C_{17}H_{22}N_2O_{10}$: C, 49.28; H, 5.35; N, 6.76. Found: C, 49.20; H, 5.38; N, 6.75. IR ($CHCl_3$): 1750, 1430, 1367, 1220 cm^{-1}. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 5.33—5.22 (2H, m), 5.19 (1H, d, J=2 Hz), 4.92 (1H, m), 4.78 (1H, dd, J=10, 5 Hz), 4.49 (1H, dd, J=11, 10 Hz), 4.18 (1H, dd, J=11, 5 Hz), 3.04, 2.21, 2.08 (each 3H, s), 2.05 (6H, s). FAB-MS m/z: 437 $(M+Na)^+$, 415 $(M+H)^+$. **22**, colorless fine crystals, mp > 280 °C (CH_2Cl_2–MeOH), $[\alpha]_D$ −36.9° (c=0.1, $CHCl_3$). *Anal.* Calcd for $C_{17}H_{22}N_2O_{10}$: C, 49.28; H, 5.35; N, 6.76. Found: C, 48.98; H, 5.37; N, 6.77. IR ($CHCl_3$): 1762, 1748, 1724, 1710, 1438, 1367, 1260 cm^{-1}. ^{1}H-NMR (200 MHz, $CDCl_3$: CD_3OD = 5 : 1) δ: 5.37—5.07 (3H, m), 4.88 (1H, d, J=9 Hz), 4.82—4.69 (2H, m), 3.90 (1H, m), 3.09, 2.17, 2.10, 2.09, 2.03 (each 3H, s). FAB-MS m/z: 437 $(M+Na)^+$, 415 $(M+H)^+$.

8-*epi*-N^1-Methylkifunensine (19) A solution of **21** (112 mg) in MeOH (1 l ml) was treated with 1 N aqueous NaOH (1.7 ml) at room temperature and the mixture was stirred for 1.5 h. After neutralization of the mixture with cation exchange resin Dowex 50W × 8 (H^+ form), the solvent was removed under reduced pressure to afford **19** (64 mg, 96%). **19**, colorless fine crystals, mp 259—260 °C (MeOH), $[\alpha]_D$ −67.6° (c=0.5, H_2O). *Anal.* Calcd for $C_9H_{14}N_2O_6$: C, 43.90; H, 5.73; N, 11.38. Found: C, 43.64; H, 5.61; N, 11.27. IR (KBr): 3330, 2905, 1717, 1698, 1437, 1229, 1065, 1005 cm^{-1}. ^{1}H-NMR (400 MHz, D_2O) δ: 5.38 (1H, d, J=2 Hz), 4.50 (1H, dd, J=9, 5 Hz), 4.37 (1H, dd, J=2, 3 Hz), 4.23 (1H, t, J=3 Hz), 4.08 (1H, d, J=3 Hz), 3.98 (1H, dd, J=12, 9 Hz), 3.88 (1H, dd, J=12, 5 Hz), 3.15 (3H, s). FAB-MS m/z: 247 $(M+H)^+$.

8a-Epimer of 19 (20) Compound **22** (100 mg) was treated with 1 N aqueous NaOH (1.5 ml) in MeOH (10 ml) in the same way as described for **19** to afford **20** (58 mg, 98%). **20**, colorless fine crystals, mp 230—231 °C (MeOH), $[\alpha]_D$ −61.0° (c=0.5, H_2O). *Anal.* Calcd for $C_9H_{14}N_2O_6$: C, 43.90; H, 5.73; N, 11.38. Found: C, 43.74; H, 5.65; N, 11.28. IR(KBr): 3310, 2910, 1715, 1681, 1425, 1219, 1080, 1060 cm^{-1}. ^{1}H-NMR (400 MHz, D_2O): δ: 4.78 (1H, d, J=9 Hz), 4.39 (1H, dd, J=13, 2 Hz), 4.27 (1H, dd, J=13, 5 Hz), 3.69—3.46 (4H, m), 3.29 (3H, s). FAB-MS m/z: 247 $(M+H)^+$.

N^1-(2,4-Dimethoxybenzyl)-8-*epi*-kifunensine (23) Compound **9** (50 mg) was treated with 2,4-dimethoxybenzylamine (50 mg) in MeOH (2 ml) at room temperature for 3 d under an N_2 atmosphere. The reaction mixture was subjected to cation exchange resin column chromatography (Dowex

TABLE I. Atomic Coordinates and Thermal Parameters with e.s.d.'s (Å^2) in Parentheses

Atom	x	y	z	B_{eq}/B_{iso}
N1	0.1083 (3)	0.5547 (5)	0.8437 (7)	1.8
C2	0.1870 (4)	0.5946 (6)	0.7558 (9)	2.1
C3	0.2241 (4)	0.7293 (6)	0.8603 (8)	2.0
N4	0.1606 (3)	0.7578 (5)	1.0010 (7)	1.6
C5	0.1605 (4)	0.8831 (6)	1.1329 (8)	1.8
C6	0.0739 (4)	0.9742 (6)	1.0982 (8)	1.9
C7	−0.0150 (4)	0.8812 (6)	1.0772 (8)	1.9
C8	−0.0055 (3)	0.7490 (6)	0.9431 (8)	1.9
C8a	0.0827 (4)	0.6580 (5)	0.9955 (9)	1.8
C9	0.1700 (4)	0.8312 (7)	1.3431 (9)	2.4
O10	0.2250 (3)	0.5392 (4)	0.6152 (6)	2.8
O11	0.2937 (2)	0.7981 (4)	0.8218 (6)	2.3
O12	0.2645 (3)	0.7755 (4)	1.3646 (6)	2.6
O13	0.0823 (3)	1.0575 (4)	0.9247 (5)	2.2
O14	−0.0358 (3)	0.8301 (4)	1.2704 (6)	2.3
O15	−0.0002 (3)	0.8001 (4)	0.7482 (6)	2.1
H1	0.072 (5)	0.461 (8)	0.804 (11)	4.8
H5	0.217 (4)	0.952 (7)	1.107 (8)	2.2
H6	0.068 (5)	1.047 (8)	1.222 (11)	4.7
H7	−0.068 (5)	0.948 (7)	1.009 (10)	4.2
H8	−0.069 (5)	0.677 (7)	0.958 (10)	4.8
H8a	0.070 (5)	0.600 (8)	1.134 (10)	4.2
H9a	0.123 (4)	0.750 (7)	1.368 (9)	3.2
H9b	0.157 (4)	0.922 (7)	1.441 (9)	3.1
H12	0.261 (6)	0.687 (7)	1.448 (11)	5.5
H13	0.137 (4)	1.113 (8)	0.903 (10)	3.8
H14	−0.103 (5)	0.778 (8)	1.253 (11)	4.7
H15	−0.025 (5)	0.719 (8)	0.681 (11)	5.1

TABLE II. Bond Lengths (Å) and Angles (°) with Their e.s.d.'s in Parentheses

Bond length (Å)			
N1–C2	1.337 (7)	N1–C8a	1.457 (7)
N1–H1	1.033 (74)	C2–C3	1.518 (8)
C2–O10	1.226 (7)	C3–N4	1.363 (7)
C3–O11	1.210 (7)	N4–C5	1.458 (7)
N4–C8a	1.442 (8)	C5–C6	1.516 (8)
C5–C9	1.538 (8)	C5–H5	1.044 (62)
C6–C7	1.539 (8)	C6–O13	1.427 (7)
C6–H6	1.084 (75)	C7–C8	1.524 (8)
C7–O14	1.450 (7)	C7–H7	1.082 (71)
C8–C8a	1.558 (8)	C8–O15	1.432 (7)
C8–H8	1.130 (78)	C8a–H8a	1.108 (74)
C9–O12	1.460 (7)	C9–H9a	1.016 (67)
C9–H9b	1.084 (67)	O12–H12	0.993 (82)
O13–H13	0.954 (74)	O14–H14	1.083 (78)
O15–H15	0.945 (77)		
Bond angle (°)			
C2–N1–C8a	111.7 (5)	C2–N1–H1	121.8 (41)
C8a–N1–H1	126.5 (41)	N1–C2–C3	107.4 (5)
N1–C2–O10	129.1 (6)	C3–C2–O10	123.5 (5)
C2–C3–N4	105.0 (5)	C2–C3–O11	127.0 (5)
N4–C3–O11	128.0 (5)	C3–N4–C5	126.7 (5)
C3–N4–C8a	112.6 (5)	C5–N4–C8a	120.3 (5)
N4–C5–C6	109.0 (4)	N4–C5–C9	110.9 (5)
N4–C5–H5	111.0 (34)	C6–C5–C9	113.0 (5)
C6–C5–H5	106.6 (34)	C9–C5–H5	106.2 (34)
C5–C6–C7	113.6 (5)	C5–C6–O13	110.7 (4)
C5–C6–H6	105.5 (40)	C7–C6–O13	106.3 (4)
C7–C6–H6	110.3 (40)	O13–C6–H6	110.5 (40)
C6–C7–C8	114.5 (5)	C6–C7–O14	105.1 (4)
C6–C7–H7	108.9 (38)	C8–C7–O14	109.3 (4)
C8–C7–H7	103.8 (38)	O14–C7–H7	115.6 (38)
C7–C8–C8a	110.3 (5)	C7–C8–O15	109.1 (5)
C7–C8–H8	109.1 (40)	C8a–C8–O15	110.4 (5)
C8a–C8–H8	109.4 (40)	O15–C8–H8	108.6 (40)
N1–C8a–N4	103.1 (5)	N1–C8a–C8	112.2 (5)
N1–C8a–H8a	111.2 (38)	N4–C8a–C8	108.0 (5)
N4–C8a–H8a	113.7 (38)	C8–C8a–H8a	108.5 (38)
C5–C9–O12	106.6 (5)	C5–C9–H9a	108.9 (38)
C5–C9–H9b	110.1 (36)	O12–C9–H9a	110.6 (38)
O12–C9–H9b	111.0 (36)	H9a–C9–H9b	109.4 (52)
C9–O12–H12	107.1 (48)	C6–O13–H13	119.2 (44)
C7–O14–H14	102.9 (42)	C8–O15–H15	101.3

50W × 8, H^+ form, 1 ml) and eluted with MeOH. The fractions containing the desired product were combined and evaporated *in vacuo*. The residue was purified by preparative TLC (CH_2Cl_2 : MeOH : H_2O = 130 : 30 : 2) to afford **23** (19 mg, 25%). **23**, amorphous solid, $[\alpha]_D$ +50.4° (c = 0.5, MeOH). IR (KBr): 3350, 2915, 1705, 1666, 1604, 1585, 1500, 1422, 1200, 1015 cm^{-1}. ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 7.18 (1H, d, J=9 Hz), 6.59 (1H, d, J=2 Hz), 6.52 (1H, dd, J=9, 2 Hz), 5.70 (1H, d, J=5 Hz, D_2O-exchangeable), 5.34 (1H, d, J=6 Hz, D_2O-exchangeable), 5.18 (1H, d, J=8 Hz, D_2O-exchangeable), 4.90 (1H, d, J=6Hz, D_2O-exchangeable), 4.88 (1H, d, J=1 Hz). 4.77 (1H, d, J=15 Hz), 4.20 (1H, t, J=6 Hz), 4.12 (1H, d, J=15 Hz), 4.03 (1H, dd, J=3, 1 Hz), 3.90 (1H, t, J=3 Hz), 3.80, 3.77 (each 3H, s), 3.85—3.70 (1H), 3.60 (1H, dd, J=11, 6 Hz), 3.49 (1H, dd, J=11, 6 Hz). FAB-MS m/z: 405 $(M+Na)^+$, 383 $(M+H)^+$. High-resolution FAB-MS Calcd for $C_{17}H_{23}N_2O_8$ $(M+H)^+$: 383.145. Found: 383.145.

8-*epi*-Kifunensine (2) A mixture of compound **23** (155 mg), $K_2S_2O_8$ (438 mg), Na_2HPO_4 (217 mg), and 40% aqueous CH_3CN (15 ml) was heated under reflux for 45 min. The reaction mixture was cooled in an ice-water bath and its pH value was adjusted to 4 with saturated aqueous $NaHCO_3$. Silica gel (1.0 g) was added, and the solvent was removed under reduced pressure. The residue was subjected to column chromatography (SiO_2 7.5 g, CH_2Cl_2 : MeOH : H_2O = 130 : 30 : 2) to afford **2** (88 mg, 94%). **2**, colorless prisms, mp 272—274 °C (dec., H_2O), $[\alpha]_D$ −54.6° (c = 0.1, H_2O). *Anal.* Calcd for $C_8H_{12}N_2O_6$: C, 41.38; H, 5.21; N, 12.06. Found: C, 41.08; H, 5.20; N, 11.98. IR (KBr): 3300, 3200, 3090, 2950, 2910, 1695, 1434, 1395, 1351, 1335, 1045, 1009 cm^{-1}. ^{1}H-NMR (400 MHz, D_2O) δ: 5.45 (1H, d, J=2 Hz), 4.47 (1H, m), 4.21—4.17 (2H, m), 4.04 (1H, br s), 3.99 (1H, dd, J=12, 9 Hz), 3.88 (1H, dd, J=12, 5 Hz). FAB-MS m/z: 255 $(M+Na)^+$, 233 $(M+H)^+$.

X-Ray Analysis of 2 The crystals were recrystallized from water: $C_8H_{12}N_2O_6$, prisms, orthorhombic, space group $P2_12_12_1$, a = 14.407(1), b = 9.070(1), c = 6.938(1) Å, V = 906.6 (2) Å^3, Z = 4, D_x = 1.701 g/cm^3, μ = 12.91 cm^{-1}. The X-ray diffraction intensity data from a selected crystal (0.35 × 0.30 × 0.25 mm) were obtained on a Rigaku AFC-5 diffractometer equipped with a rotating anode X-ray generator (50 KV-180 mA), using graphite-monochromated Cu K_α radiation (λ = 1.54178 Å). A total of 925 independent reflections with $2\theta < 130°$ were collected in the $2\theta/\omega$ scanning mode. The structure was solved by the direct method using MULTAN 84. Hydrogen atoms were located from the difference Fourier synthesis. The refinement was carried out by the block-diagonal least-squares method with anisotropic thermal parameters for non H atoms and with isotropic thermal parameters for H atoms. The R factor was reduced to 0.061 using 908 reflections with $F_O > 3\sigma$ (F_O). The atomic parameters, bond lengths and angles are given in Tables I and II.

Acknowledgement We thank Dr. T. Tada of Analytical Research Laboratories for X-ray crystal analysis of 8-*epi*-kifunensine.

References and Notes

1) A preliminary report of this work: H. Kayakiri, T. Oku, and M. Hashimoto, *Chem. Pharm. Bull.*, **38**, 293 (1990).
2) Mannojirimycin (nojirimycin B): T. Niwa, T. Tsuruoka, H. Goi, Y. Kodama, J. Itoh, S. Inoue, Y. Yamada, T. Niida, M. Nobe, and Y. Ogawa, *J. Antibiot.*, **37**, 1579 (1984).
3) M. Iwami, O. Nakayama, H. Terano, M. Kohsaka, H. Aoki, and H. Imanaka, *J. Antibiot.*, **40**, 612 (1987). Kifunensine was tentatively designated FR 900494 in that paper.
4) *a*) S. Inouye, T. Tsuruoka, T. Ito, and T. Niida, *Tetrahedron*, **23**, 2125 (1968); *b*) For a review on 1,5-iminopyranoses and 1,4-iminofuranoses, see G. W. J. Fleet, *Spec. Publ. Royal Chem. Soc.*, **1988**, No. 65, 149.
5) H. Kayakiri, C. Kasahara, T. Oku, and M. Hashimoto, *Tetrahedron Lett.*, **31**, 225 (1990).
6) Y. Tsuda, Y. Okuno, and K. Kanemitsu, *Heterocycles*, **27**, 63 (1988).
7) U. G. Nayak and R. I. Whistler, *J. Org. Chem.*, **33**, 3582 (1968).
8) H. Saeki and E. Ohki, *Chem. Pharm. Bull.*, **16**, 2471 (1968).
9) A. Vasella and R. Voeffray, *Helv. Chim. Acta*, **65**, 1134 (1982).
10) 8-*epi*-Kifunensine (**2**) did not inhibit α-mannosidase and it had no effect on the expression of Ia antigen on mouse peritoneal macrophages.
11) H. Kayakiri, S. Takase, T. Shibata, M. Okamoto, H. Terano, M. Hashimoto, T. Tada, and S. Koda, *Chem. Pharm. Bull.*, **39**, 1378 (1991).

Synthesis and Reactions of 3,7-Cycloerythrinans: Skeletal Rearrangement of 2,7,8-Trioxoerythrinans to 4-Oxo-4*H*-pyrido[2,1-*a*]isoquinolines[1)]

Yoshisuke TSUDA,*[,a] Yuki SAKAI, [a] Takehiro SANO,[b] and Jun TODA[b]

Faculty of Pharmaceutical Sciences, Kanazawa University,[a] 13-1 Takara-machi, Kanazawa 920, Japan and Showa College of Pharmaceutical Sciences,[b] Higashi-tamagawagakuen 3-chome, Machida-shi, Tokyo 194, Japan. *Received November 5, 1990*

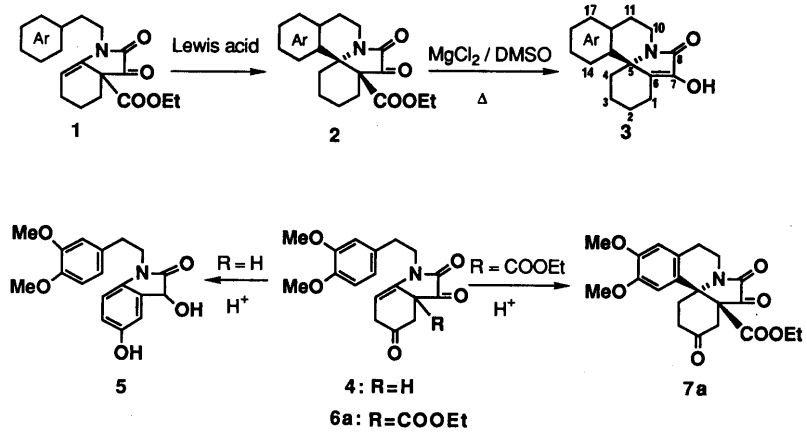
Treatment of 2,7,8-trioxoerythrinan derivatives with anhydrous phosphoric acid or Lewis acids gave 3,7-cycloerythrinan derivatives *via* an intramolecular aldol condensation. The structures of the products were established by spectroscopic and chemical means. Further treatment of the 3,7-cycloerythrinans with anhydrous phosphoric acid resulted in skeletal rearrangement to yield 6,7-dihydro-4-oxo-4*H*-pyrido[2,1-*a*]isoquinolines, whose structures were also determined mainly by spectroscopic means.

Keywords *Erythrina* alkaloid; 2,7,8-trioxoerythrinan; 3,7-cycloerythrinan; intramolecular aldol reaction; 4-oxo-4*H*-pyrido[2,1-*a*]isoquinoline; anhydrous phosphoric acid; Lewis acid; skeletal rearrangement

In a previous paper[2)] we described high yield cyclization of 1-β-arylethyl-3a-ethoxycarbonyl-2,3,3a,4,5,6-hexahydro-1*H*-indole-2,3-diones **1** to 6-ethoxycarbonyl-7,8-dioxoerythrinans **2**. The ethoxycarbonyl group in the product can be smoothly removed by heating with $MgCl_2$ in dimethyl sulfoxide (DMSO) [or in hexamethylphosphoric triamide (HMPA)]. One of the particular advantages in the use of the 3a-ethoxycarbonyl derivative is in the synthesis of oxoerythrinans such as **7a**, because attempted cyclization of the 3a-H derivative **4** to the erythrinan was reported to fail, giving instead the hydroxyisatin **5**.[3)] On the other hand, cyclization of **6a** proceeded smoothly to give **7a**, but this product was sometimes accompanied with several by-products which apparently were the products of over-reaction.[2)] This paper deals with structural elucidations of these by-products and describes a new skeletal rearrangement of 2,7,8-trioxoerythrinans to pyrido[2,1-*a*]isoquinolines.[4)]

Results and Discussion

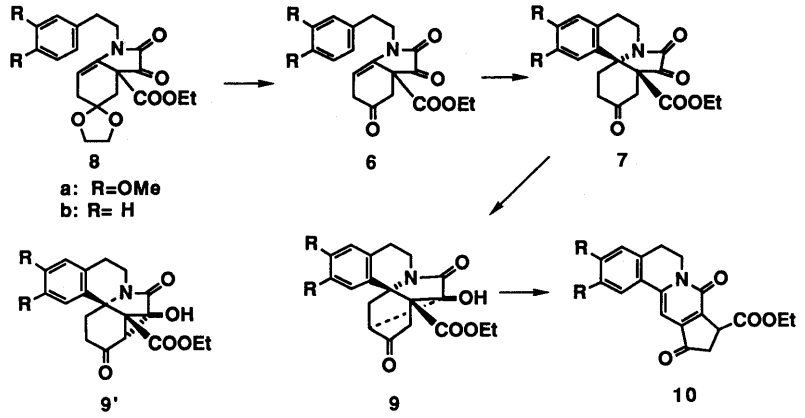
Formation of 3,7-Cycloerythrinans from 2,7,8-Trioxoerythrinans Treatment of **8a** with anhydrous phosphoric acid at room temperature for 1.5 h resulted only in cleavage of the ethylene acetal group to give **6a**. Further treatment of



1 2 3

5 4: R=H 6a: R=COOEt 7a

Chart 1



8 a: R=OMe b: R= H 6 7

9' 9 10

Chart 2

6a with the same reagent at 70 °C for 3.5 h produced the trioxoerythrinan **7a** and the 3,7-cycloerythrinan **9a**, while on treatment at 100 °C, **9a** and a new yellow compound **10a** were produced.

Compound **7a** was identical with that reported previously.[2] Compound **9a** showed infrared (IR) absorptions at 1760, 1725, 1695 and 3400 cm^{-1}, indicative of five membered ketone, ester, lactam, and hydroxyl moieties, respectively, and showed the presence of three quaternary carbons (δ 65.9, 68.6, 88.2, each s) and one tertiary carbon (δ 54.2, d) in the ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectrum.

Treatment of **7a** with $BF_3 \cdot Et_2O$ in methylene chloride or with an excess of anhydrous phosphoric acid gave **9a** in 55% or 70% yield. On the other hand, heating of **9a** in 80% AcOH for 1.5 h produced **7a**, though in low yield. All of these results indicate that **7a** and **9a** are interconvertible and **9a** is an intramolecular aldol product of **7a**, *i.e.*, the 3,7-cyclo (**9a**) or 1,7-cyclo (**9'**) derivative.

The structure **9a** was verified by the following chemical correlations. Reduction of **9a** with Zn–AcOH gave a mixture of two stereoisomeric alcohols, **11** and **12**, which on Jones oxidation gave **7a**. Reduction of **9a** with $NaBH_4$ in ethanol gave a 1 : 1 mixture of stereoisomeric alcohols, **13** and **14**, which, without separation, was mesylated and treated with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in benzene to give the olefin **17** and the mesylate **16**. The olefin **17** was identical with the Diels–Alder adduct of the dioxopyrroline **18** to butadiene.[5] Since **17** is the product of concerted fragmentation from the β-*O*-mesylate **15**, the structure of **9a** was thus established. The *O*-mesylate **16** must therefore be of α configuration. Treatment of **16** with lithium iodide in dimethylformamide (DMF) followed by heating of the resulting iodide **19** with DBU in benzene afforded the olefin **17** as expected. Reduction of **9a** with LiAlH(*O*-*tert*-Bu)$_3$ gave a single stereoisomer **14**.

The above results show that the cyclization of **8** with phosphoric acid proceeds firstly with hydrolytic cleavage of the ethylene acetal group, followed by cyclization to **7**, which is further converted to **9** by an intramolecular aldol reaction.

Treatment of **8a** with Lewis acids gave somewhat different results. Lewis acids were found to catalyze the cyclization of **8a** prior to hydrolysis of the ethylene acetal group. Although silver perchlorate did not affect **8a** at room temperature, it gave three products, **20**, **7a**, and **9a**, on heating at 90 °C for 1 h, in the yields of 25, 32, and 25%. Treatment of **8a** with $BF_3 \cdot Et_2O$ in methylene chloride produced, at room temperature for 19 h, **20**, **7a**, **9a**, and **21** in the yields of 35, 23, 4, and 4%, and gave **20**, **7a**, and **9a** in a ratio of 1 : 1 : 1 on reflux for 40 min. Compound **20** was identical with that previously reported[2] and gave **7a** on hydrolysis with 80% AcOH. The structure of **21** was assigned on the basis of spectral data and confirmed by acid hydrolysis to **9a**. The formation of this compound can be rationalized by considering the intermediacy of the enolate **22**, which might be produced by the action of a Lewis acid on **20**.

3,7-Cycloerythrinans from Photochemical Routes The formation of 3,7-cycloerythrinans by intramolecular aldol reaction of 2,7,8-trioxoerythrinans was also observed in photochemical approaches to erythrinans. The photoadduct **23** of an activated butadiene to **18**, upon thermolysis followed by treatment with potassium fluoride, gave the 3,7-cycloerythrinan derivative **25**.[6] Obviously, the intermediate enolate anion underwent an intramolecular aldol reaction to yield **25**. Further examples and details of this photochemical approach will be given in a separate paper.

Rearrangement of 3,7-Cycloerythrinans to Pyrido[2,1-*a*]-isoquinolines In the above section we reported that, when **8a** was treated with anhydrous phosphoric acid at 100 °C for 3 h, a new yellow compound **10a** was produced together with **9a**. Compound **10a** was obtained in better yield on treatment of **9a** with hot phosphoric acid or on heating with polyphosphoric acid (PPA) at 70—100 °C.

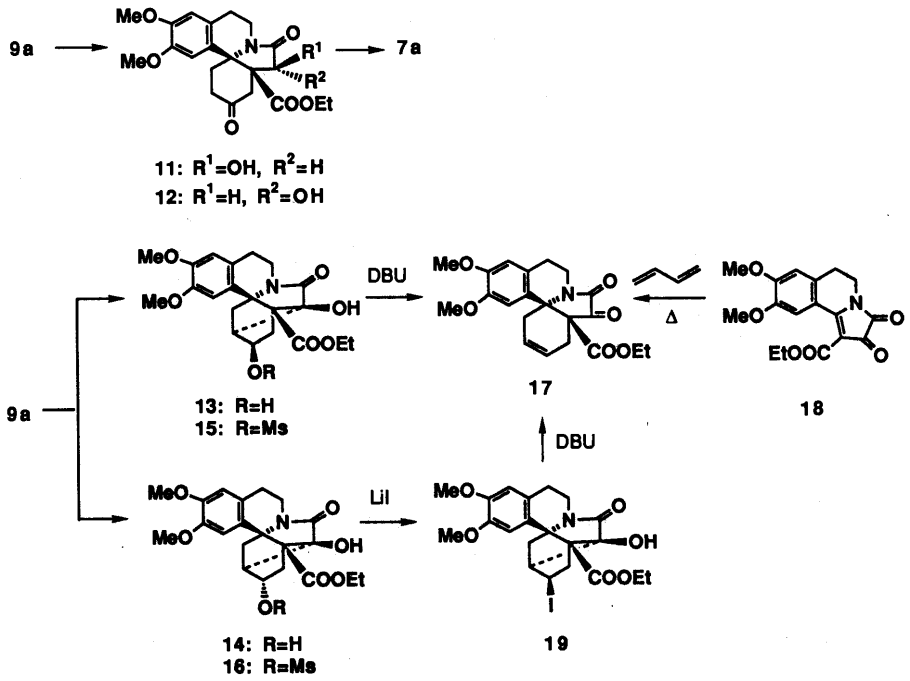


Chart 3

8a	Lewis acids	7a	+ 9a	+ 20	+ 21
	$AgClO_4$, 90°C	32%	25%	25%	---
	BF_3·Et_2O, r.t.	23%	4%	35%	4%

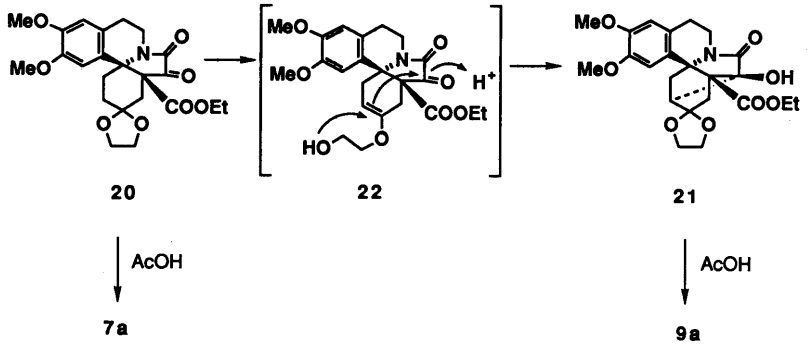


Chart 4

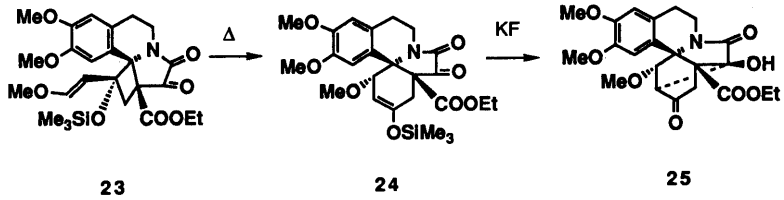


Chart 5

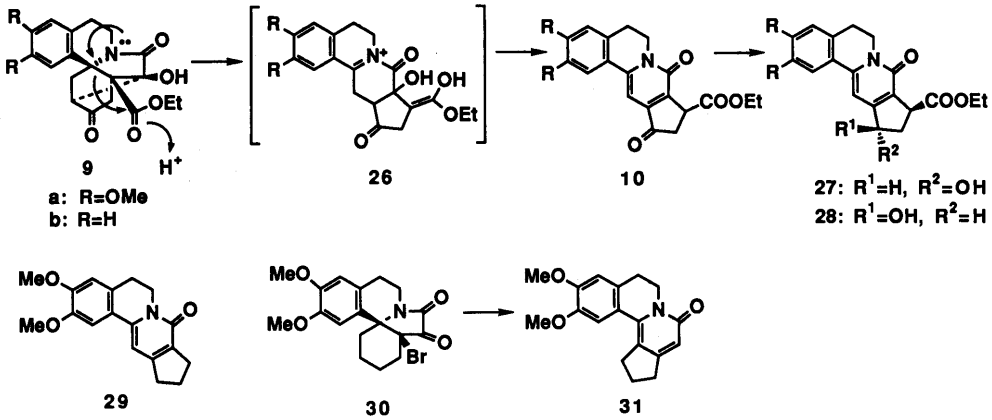


Chart 6

Compound **10a**, mp 182—183 °C, had the formula $C_{21}H_{21}NO_6$ and showed ultraviolet (UV) absorptions at 286 (ε 15600), 303 sh (ε 13900) and 380 nm (ε 15000), indicating that it has an elongated conjugated system. In the ^{13}C-NMR spectrum, it exhibited signals due to ten aromatic carbons, seven of which were quaternary (δ 151.4, 148.7, 145.5, 144.7, 141.5, 128.9, 121.4) and three tertiary (δ 110.3, 108.2, 94.0), which corresponded to the singlet signals at δ 7.21, 6.76, and 6.82 in the ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrum. In the IR spectrum, **10a** showed absorptions at 1720 and 1653 cm^{-1}. The ^{1}H-NMR chemical shifts of the three aromatic protons and the IR absorption at 1653 cm^{-1} are in agreement with the data for the pyrido-isoquinoline **29** reported by Mondon *et al.*[7] Reduction of **10a** with $NaBH_4$ gave two alcohols, **27** and **28**. The UV spectrum [λ_{max} nm (ε): 269 (9000), 348 (24800)] of the major product (stereochemistry was not determined). mp 199—201 °C, was again in good agreement with that of **29** [λ_{max} nm (ε): 267(6760), 343 (18200)].[7] We therefore consider that the product is the 6,7-dihydro-4-oxo-4*H*-pyrido[2,1-*a*]isoquinoline (**10a**), which was produced from the 3,7-cycloerythrinan **9a** through acid-catalyzed fragmentation as illustrated in Chart 6.

The non-methoxy derivative **8b** similarly gave the 3,7-cycloerythrinan **9b** and the pyrido-isoquinoline **10b** on heating with phosphoric acid, thus revealing that this rearrangement is a general one for 3,7-cycloerythrinans. In support of this consideration, compound **2**, which can not form an intramolecular aldol, was recovered unchanged on heating with PPA at 100—110 °C. Mondon[8] reported a pyrido-isoquinoline **31** (which is structurally isomeric to **29**) as one of the products of Wolff–Kishner reduction of the

bromo-ketone **30**. Apparently our pyrido-isoquinoline **10** is different from **31**-type product in substitution pattern and formation mechanism.

The pyrido[2,1-*a*]isoquinoline structure of the type **29** has been found in natural alkaloids such as alamarine,[9] so the above described transformation should provide a synthetic route to these alkaloids.

Experimental

General Unless otherwise stated, the following procedures were adopted. Melting points were determined on a Yanaco micro hot stage melting point apparatus and are uncorrected. IR spectra were taken in KBr disks, recorded on a Jasco IR-G spectrometer, and the data are given in cm^{-1}. UV spectra were taken in ethanol solution and λ_{max} values are given in nm (ε). ^{1}H-NMR spectra were taken with a JNM PMX-60 (60 MHz) or a JEOL FX-100 (100 MHz) spectrometer in chloroform-*d* solution with tetramethylsilane as an internal standard, and the chemical shifts are given in δ values (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad). High resolution mass spectra (HR-MS) were taken with a Hitachi M-80 spectrometer. Column chromatography was performed on Wakogel C-200 (silica gel). For thin layer chromatography (TLC), Merck precoated plates GF_{254} were used and spots were monitored by UV (254 nm), then developed by spraying 1% $Ce(SO_4)_2$ in 10% H_2SO_4 and heating the plates at 100 °C until coloration took place. All organic extracts were washed with brine and water, dried over anhydrous sodium sulfate, and concentrated. Identities were confirmed by mixed melting point determination (for crystalline compounds) and also by comparisons of TLC behavior and ^{1}H-NMR and IR spectra.

Anhydrous H_3PO_4 was prepared from P_2O_5 (7 g) and 80% H_3PO_4 (15 g), and PPA was prepared from 80% H_3PO_4 (33 g) and P_2O_5 (25 g), by heating each mixture at 110 °C for 6 h.

Treatment of Dioxopyrroline 8a with Phosphoric Acid 1) Compound **8a**[2] (0.31 g) was stirred with an excess of anhydrous H_3PO_4 for 1.5 h at room temperature. The mixture was decomposed with ice-water and extracted with $CHCl_3$. Chromatography of the product gave **6a** (0.22 g, 80%) and the ketol **9a** (0.03 g, 11%) from the $CHCl_3$–AcOEt eluate. Compound **6a** was obtained as a yellow gum. ^{1}H-NMR: 6.70 (3H, s, ArH), 5.28 (1H, t, J=4.7 Hz, =CH), 4.09 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 3.81, 3.78 (each 3H, s, OMe), 1.10 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$). HR-MS: Calcd for $C_{21}H_{23}NO_7$ (M^+) m/z: 401.1473. Found: 401.1422.

2) Crude **8a** prepared from 13 g of ethyl 5,5-ethylenedioxy-2-oxocyclohexanecarboxylate by the reported method,[2] was heated with anhydrous H_3PO_4 (80 ml) at 70 °C for 3.5 h. The mixture was decomposed with ice-water and the separated precipitate was collected by filtration, and washed with hot benzene to give the 2,7,8-trioxo derivative **7a** (5.7 g, 25%), as colorless prisms from $CHCl_3$, mp 282—283 °C. This product was identical with **7a** previously reported.[2]

Concentration of the hot benzene washings gave a solid which was recrystallized from MeOH then from benzene to give the ketol **9a** (12.6 g, 53%) as colorless prisms, mp 279—282 °C (dec.). IR: 3400, 1760, 1725, 1695. ^{1}H-NMR: 6.75, 6.67 (each 1H, s, ArH), 4.01 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 3.89, 3.88 (each 3H, s, OMe), 0.94 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$). ^{13}C-NMR: 207.1 s, 169.1 s, 168.2 s, 149.2 s, 148.4 s, 128.1 s, 122.8 s, 112.1 d, 108.7 d, 88.2 s, 68.6 s, 65.9 s, 61.3 t, 56.5 q, 56.0 q, 54.2 d, 39.2 t, 36.7 t, 34.9 t, 28.7 t, 13.8 q. *Anal.* Calcd for $C_{21}H_{23}NO_7$: C, 62.83; H, 6.02; N, 3.63. Found: C, 62.54; H, 5.75; N, 3.37.

3) Compound **8a** (2.6 g) was heated with an excess of anhydrous H_3PO_4 at 100 °C for 3 h and worked up as described in 1). Chromatography of the product gave the pyridone **10a** (0.55 g, 25%) from the benzene–AcOEt eluate and the ketol **9a** (0.5 g, 21%) from the $CHCl_3$–MeOH eluate. Compound **10a** formed yellow prisms from ether, mp 182—183 °C. UV: 286 (15600), 303 sh (13900), 380 (15000). IR: 1720, 1653, 1605. ^{1}H-NMR: 7.21, 6.82, 6.76 (each 1H, s, ArH), 4.26 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 3.95 (6H, s, OMe×2), 1.32 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$). ^{13}C-NMR: 204.7 s, 172.3 s, 160.7 s, 151.4 s, 148.7 s, 145.5 s, 144.7 s, 141.5 s, 128.9 s, 121.4 s, 110.3 d, 108.2 d, 94.0 d, 61.6 t, 56.3 q, 56.1 q, 41.2 d, 41.0 t, 39.9 t, 27.5 t, 14.1 q. *Anal.* Calcd for $C_{21}H_{21}NO_6$: C, 65.78; H, 5.52; N, 3.65. Found: C, 65.70; H, 5.59; N, 3.20.

Treatment of Dioxopyrroline 8a with Lewis Acids 1) Compound **8a** (79 mg) and $AgClO_4$ (*ca.* 50 mg) in dry benzene (10 ml) were heated at 90 °C for 1 h. The mixture was decomposed with water and extracted with $CHCl_3$. The product was triturated with MeOH to give the trioxo derivative **7a** (25 mg, 32%) as insoluble crystals. Chromatography of the mother liquor gave **20** (20 mg, 25%) and **9a** (20 mg, 25%). Compound **20** formed colorless needles from ether, mp 185—186 °C, and was identical with the compound reported previously.[2]

2) Compound **8a** (475 mg) and $BF_3 \cdot Et_2O$ (420 mg, 3 mol eq) in CH_2Cl_2 (15 ml) were stirred for 20 h at room temperature. Work-up of the mixture as described above gave **7a** (110 mg, 23%). Chromatography of the mother liquor gave **20** (164 mg, 35%) from the $CHCl_3$–AcOEt (5 : 1) eluate, and **9a** (20 mg, 4%) and **21** (22 mg, 4%) from the $CHCl_3$–AcOEt (1 : 1) eluate; the latter compounds were separated by preparative TLC. Compound **21** formed colorless needles from ether, mp 176—177 °C. IR: 3300, 1730, 1708. ^{1}H-NMR: 6.86, 6.63 (each 1H, s, ArH), 3.91, 3.87 (each 3H, s, OMe), 4.2—3.8 (overlapped m, $COOC\underline{H}_2CH_3$, $OC\underline{H}_2C\underline{H}_2O$), 0.95 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$). ^{13}C-NMR: 169.4 s, 168.8 s, 148.8 s, 148.0 s, 128.3 s, 123.6 s, 116.3 s, 111.9 d, 109.2 d, 90.3 s, 71.3 s, 65.5 s, 65.2 t, 64.5 t, 60.8 t, 56.3 q, 55.9 q, 46.9 d, 38.4 t, 37.2 t, 34.6 t, 28.7 t, 13.9 q. *Anal.* Calcd for $C_{23}H_{27}NO_8$: C, 62.01; H, 6.11; N, 3.14. Found: C, 62.11; H, 5.98; N, 3.20.

3) Compound **8a** in CH_2Cl_2 was heated with 3 mol eq of $BF_3 \cdot Et_2O$ under reflux for 40 min to give a mixture of **20**, **7a**, and **9a** in a ratio of *ca.* 1 : 1 : 1 (TLC detection).

Deacetalization of 21 Compound **21** (400 mg) in 80% AcOH (10 ml) was heated under reflux for 1.5 h and the solvent was evaporated off to leave a mixture of **9a** and **7a** (4 : 1, 337 mg), which were separated by fractional crystallizations and chromatography.

The Ketol 9a from the Trioxo Derivative 7a 1) Compound **7a** (3.8 g) and $BF_3 \cdot Et_2O$ (20.5 g, 15 mol eq) in CH_2Cl_2 (200 ml) were stirred overnight at room temperature. The mixture was washed with saturated $NaHCO_3$ solution and concentrated to *ca.* 10 ml. The separated starting material **7a** was removed by filtration. The mother liquor was treated with hot benzene to obtain a fruther crop of **7a**. Chromatography of the mother liquor gave a further crop of **7a** (total recovery, 2.08 g, 54.7%) together with **9a** (1.3 g, 34.2%).

2) Compound **7a** (6.1 g) was heated with a mixture of anhydrous H_3PO_4 (20 ml) and 80% H_3PO_4 (10 ml) at 50—60 °C for 2 h. The reaction mixture was decomposed with ice-water and extracted with $CHCl_3$. Concentration of the extract and treatment of the residue with MeOH recovered **7a** (1.85 g, 30%). Chromatography of the mother liquor gave the ketol **9a** (4.3 g, 70%).

The 6,7-Dihydro-9,10-dimethoxy-4-oxo-4*H*-pyrido[2,1-*a*]isoquinoline 10a from the Ketol 9a The ketol **9a** (100 mg) was heated with an excess of PPA at 100 °C for 1 h. The mixture was decomposed and extracted with $CHCl_3$. Chromatography of the product gave the pyridone **10a** (57 mg, 60%).

Zn–AcOH Reduction of the Ketol 9a Zn powder (3 g) was added in portions to a stirred and refluxed solution of the ketol **9a** (200 mg) in AcOH (15 ml). After being refluxed for 2 h, the mixture was filtered, and the filtrate was diluted with water and extracted with $CHCl_3$. Chromatography of the extract gave a mixture of **11** and **12** (180 mg) from the $CHCl_3$–MeOH (30 : 1) eluate. Although this mixture gave a single spot on TLC, the ^{1}H-NMR spectrum showed two triplets of $COOCH_2C\underline{H}_3$ at δ 0.80 and 0.75, of which the former was identical with that of **11**[2] as judged from a detailed inspection of the spectrum.

This mixture (50 mg) in acetone (15 ml) was oxidized with Jones reagent at 0 °C and worked up as usual to give the trioxo derivative **7a** (40 mg).

The Olefin 17 from the Ketol 9a The ketol **9a** (5 g) and $NaBH_4$ (200 mg) in EtOH–tetrahydrofuran (THF) (1 : 1, 300 ml) were stirred at 0 °C for 1 h. After evaporation of the solvent, the mixture was extracted with $CHCl_3$ to give a mixture of **13** and **14** [^{1}H-NMR: ArH δ 7.03 (0.5H), 6.72 (0.5H), 6.53 (1H)]. Although these products could be partially separated by fractional crystallizations after conversion to the tosylates (2β-*O*-tosylate: colorless needles from EtOH, mp 133—142 °C, and 2α-*O*-tosylate: colorless prisms from EtOH, mp 102—112 °C), they were used without separation for the next step.

The mixture of **13** and **14** was mesylated with methanesulfonyl chloride (4.2 g) and pyridine (50 ml) for 1 h at room temperature, then the mixture was poured into water and extracted with $CHCl_3$ to yield the mesylates, **15** and **16**.

The mixture of mesylates was dissolved in benzene (100 ml) and treated with DBU (5 g) at 80 °C for 15 min. The cooled mixture was diluted with CH_2Cl_2 (300 ml), washed with 1 N HCl and water, dried, and concentrated. The residue was treated with benzene to give the 2α-*O*-mesylate **16** (2.8 g, 46.7% from **8a**) as an insoluble mass, which was recrystallized from acetone as colorless prisms, mp 192.5—193.5 °C. IR: 1720, 1700. ^{1}H-NMR: 6.70, 6.62 (each 1H, s, ArH), 5.19 (1H, d, J=7.5 Hz, CHOMs), 3.90, 3.87 (each 3H, s, OMe), 3.12 (3H, s, Ms), 0.91 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$).

Anal. Calcd for $C_{22}H_{27}NO_9S$: C, 54.88; H, 5.65; N, 2.91. Found: C, 54.72; H, 5.88; N, 2.80.

The mother liquor was chromatographed to give the olefin **17** (1.7 g, 34% from **9a**), as pale yellow prisms from MeOH, mp 176—180 °C. This product was identical with **17** previously reported.[5]

Reduction of the Ketol 9a with LiAlH(*O*-*tert*-Bu)$_3$ Compound **9a** (0.5 g) and LiAlH(*O*-*tert*-Bu)$_3$ (1 g) in THF (30 ml) were stirred for 1 h at room temperature. After decomposition of excess reagent with a small amount of water, the mixture was filtered and washed thoroughly with CH_2Cl_2. The combined filtrate and washings were concentrated and the residue was chromatographed to give the starting material **9a** (0.1 g) from the $CHCl_3$–AcOEt (3 : 1) eluate and the 2α-alcohol **14** (0.4 g), as a gum, from the $CHCl_3$–AcOEt (1 : 1) eluate. Mesylation of this in a usual way gave the 2α-*O*-mesylate **16**, mp 192—193 °C.

Reduction of the Ketol 9a with Zn(BH$_4$)$_2$ Reduction of **9a** with zinc borohydride in THF gave a 1 : 1 mixture of **13** and **14**.

Conversion of the 2α-*O*-Mesylate 16 to the Olefin 17 Compound **16** (1.15 g) and LiI (2 g) in DMF (50 ml) were stirred and heated at 130 °C for 5 h. After addition of water, the mixture was extracted with $CHCl_3$. The extract was washed with dilute $Na_2S_2O_3$ solution and water, dried, and concentrated. The residue in benzene (30 ml) was heated with DBU (5 g) at 90 °C for 1 h and worked up as usual. Chromatography of the product gave the olefin **17** (420 mg, 45.6%) from the benzene–$CHCl_3$ (1 : 1) eluate and the starting material **16** (340 mg) from the $CHCl_3$–AcOEt (2 : 1) eluate.

Hydride Reduction of the Pyridone 10a The pyridone **10a** (100 mg) and $NaBH_4$ (10 mg) in THF–EtOH (1 : 1, 20 ml) were stirred at 0 °C for 1 h. After addition of water, the product was taken into $CHCl_3$. Concentration of the extract gave a mixture of two alcohols (**27** and **28**), which were separated by chromatography. The major product obtained from the $CHCl_3$–AcOEt (1 : 1) eluate crystallized in colorless plates from benzene, mp 199—201 °C. UV: 269 (9000), 348 (24800). IR: 1720, 1608, 1595. ^{1}H-NMR: 7.21, 6.80, 6.73 (each 1H, s, ArH), 4.95—5.15 (1H, m, C$\underline{H}$OH), 4.25 (2H, q, J=7 Hz, COOC$\underline{H}_2$CH$_3$), 3.95, 3.94, (each 3H, s, OMe), 3.57 (1H, d, J=11 Hz, OH, disappeared on addition of D_2O), 1.34 (3H, t, J=7 Hz, COOCH$_2$C$\underline{H}_3$). *Anal.* Calcd for $C_{21}H_{23}NO_6$: C, 65.44; H, 6.02; N, 3.63. Found: C, 65.66, H, 6.04; N, 3.57.

Elution of the column with AcOEt gave a mixture of the major and minor products (41 mg).

The 6,7-Dihydro-4-oxo-4*H*-pyrido[2,1-*a*]isoquinoline 10b from 8b Compound **8b** was prepared from phenylethylamine by condensation with 5,5-ethylenedioxy-2-oxocyclohexanecarboxylate followed by oxalylation in the manner described previously.[2] Crude **8b** (2.38 g) thus obtained was heated with a large excess of PPA at 110 °C for 4 h. After decomposition with water, the mixture was extracted with $CHCl_3$. Chromatography of the product gave the pyridone **10b** (0.26 g, 11.2%) and the ketol **9b** (0.17 g, 7%).

The pyridone **10b** formed yellow prisms from ether, mp 159—161 °C. IR: 1725, 1650. ^{1}H-NMR: 7.1—7.9 (4H, m, ArH), 6.89 (1H, s, ArH), 4.23 (2H, q, J=7 Hz, COOC$\underline{H}_2$CH$_3$), 1.30 (3H, t, J=7 Hz, COOCH$_2$C$\underline{H}_3$). *Anal.* Calcd for $C_{19}H_{17}NO_4$: C, 70.57; H, 5.30; N, 4.33. Found: C, 70.28; H, 5.10; N, 4.43.

The ketol **9b** formed colorless prisms from MeOH–ether, mp 174—176 °C. IR: 3200, 1765, 1700. ^{1}H-NMR; 7.1—7.5 (4H, m, ArH), 0.87 (3H, t, J=7 Hz, COOCH$_2$C$\underline{H}_3$). ^{13}C-NMR: 206.5 s, 169.0 s, 168.4 s, 135.4 s, 129.5 d, 128.4 d, 127.3 d, 125.5 d, 88.4 s, 68.7 s, 65.9 s, 61.4 t, 54.0 d, 39.5 t, 36.6 t, 34.8 t, 29.2 t, 13.7 q. *Anal.* Calcd for $C_{19}H_{19}NO_5$: C, 66.85; H, 5.61; N, 4.10. Found: C, 66.58; H, 5.58; N, 3.98.

Acknowledgement The authors thank Miss S. Koike for her technical assistance in some experiments.

References and Notes

1) Synthesis of *Erythrina* and Related Alkaloids. XXV. Part XXIV: Y. Tsuda, S. Hosoi, A. Nakai, Y. Sakai, T. Abe, Y. Ishi, F. Kiuchi, and T. Sano, *Chem. Pharm. Bull.*, **39**, 1365 (1991).
2) Y. Tsuda, Y. Sakai, A. Nakai, M. Kaneko, Y. Ishiguro, K. Isobe, J. Taga, and T. Sano, *Chem. Pharm. Bull.*, **38**, 1462 (1990).
3) A. Mondon, K. F. Hansen, K. Boehme, H. P. Faro, H. J. Nestler, H. G. Vilhuber, and K. Boettcher, *Chem. Ber.*, **103**, 615 (1970).
4) Preliminary communication: Y. Tsuda, Y. Sakai, and T. Sano, *Heterocycles*, **15**, 1097 (1981).
5) T. Sano, J. Toda, K. Kashiwaba, T. Ohshima, and Y. Tsuda, *Chem. Pharm. Bull.*, **35**, 479 (1987).
6) Details will be published in a forthcoming paper.
7) A. Mondon, E. Oelrich, and R. Schickfluss, *Chem. Ber.*, **105**, 2036 (1972).
8) A. Mondon, *Chem. Ber.*, **104**, 270 (1971).
9) S. C. Pakrashi, B. Achari, E. Ali, P. P. G. Dastidar, and R. R. Sinha, *Tetrahedron Lett.*, **1980**, 2667.

Synthesis and Absolute Configuration of Wybutine, the Fluorescent Minor Base from Phenylalanine Transfer Ribonucleic Acids

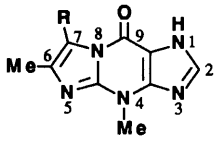
Taisuke ITAYA,* Akemi MIZUTANI, and Takehiko IIDA

Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi, Kanazawa 920, Japan. *Received November 17, 1990*

The phosphonium chloride 6 having an optically active amino acid moiety was synthesized from (*S*)-serine benzyl ester tosylate (2b) through a six-step route. The utility of 6 as a reagent for the Wittig reaction was exemplified in the olefination with benzaldehyde, affording the (*E*)-β,γ-unsaturated amino acid derivative 11 as a sole geometrical isomer. This new method of amino acid homologation was successfully employed for the first chiral synthesis of wybutine (1c), the minor base isolated from yeast phenylalanine transfer ribonucleic acids: the Wittig reaction between 6 and the tricyclic aldehyde 16 followed successively by methylation and catalytic reduction afforded 1c. Comparison of wybutine with synthetic 1c has unequivocally established that wybutine has an *S* configuration.

Keywords wybutine; Wittig reaction; β,γ-unsaturated amino acid; chiral synthesis; amino acid homologation; phenylalanine transfer ribonucleic acid; hypermodified base; stereoselective olefination; alanine synthon; absolute configuration

The base sequences of more than 450 transfer ribonucleic acids (tRNAs) have been determined[1] since the publication of the pioneering work by Holley *et al.* on yeast alanine tRNA in 1965.[2] The most prominent feature of tRNAs is a frequent occurrence of modification at the base moiety, and more than 50 modified nucleosides or bases from tRNAs have been characterized.[1,3] RajBhandary *et al.* determined the base sequence of yeast phenylalanine tRNA ($tRNA^{Phe}$)[4] and discovered a fluorescent component at the next position to the 3'-end of the anticodon.[5] The chromophore was isolated as the nucleoside, wybutosine,[5,6] whose *N*-glycosidic bond was shown to be unusually susceptible to acidic hydrolysis by Thiebe and Zachau.[6] Thus the fluorescent base, wybutine, was selectively obtained by mild acid treatment of the tRNA,[6] and the structure **1c** was assigned to this base by Nakanishi's and Zachau's groups without determining the stereochemistry.[7] One of the fluorescent bases isolated from rat and calf liver $tRNAs^{Phe}$ was also reported to be wybutine.[8] The congeners **1a, b, d—f** have subsequently been isolated from $tRNAs^{Phe}$ of various eukaryotic species and unfractionated tRNAs of archaebacteria.[9] The chemical structures of the members of the family **1** are quite unique because they embody not only a 3-methylguanine skeleton but also a condensed tricycle: no other 3-methylguanine derivative has been reported to occur naturally and no condensed tricyclic base other than **1** has been found in nucleic acids. Nakanishi *et al.* achieved a synthesis of the racemic modification of **1c** by cyclocondensation of 3-methylguanine with (±)-5-bromo-2-[(methoxycarbonyl)amino]-6-oxoheptanoate, ascertaining the correctness of the two-dimensional structure of wybutine.[10] They also reported that the reaction of 7-benzyl-3-methylguanine with the bromoketone followed by catalytic hydrogenolysis gave a better result.[11] The absolute configuration of wybutine was reported to be *S* by comparison of the circular dichroism (CD) spectrum of *N*-(methoxycarbonyl)glutamic acid dimethyl ester, obtained from a degradation product of wybutine, with that of an authentic sample derived from (*S*)-glutamic acid.[10] Unfortunately, the dimethyl ester obtained from wybutine was not fully characterized and there is an inconsistency in this report.[12] Since wybutine is available in only a minute quantity from $tRNA^{Phe}$, we planned a synthesis of optically active **1c** for unambiguous determination of the absolute configuration of wybutine. This paper reports the first chiral synthesis of **1c**.[13]



1

a: R = H

b: R = Me

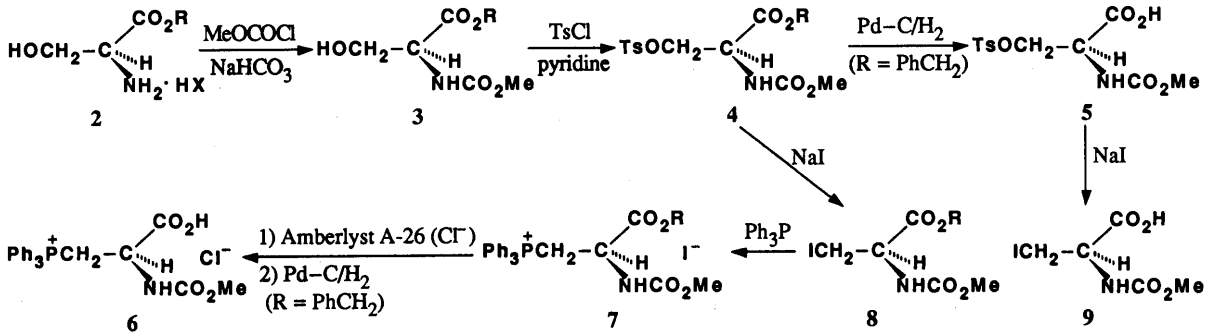
c: R = $CH_2CH_2C(H)(NHCO_2Me)CO_2Me$ (γ β α)

d: R = $CH_2CH(OH)CH(NH_2)CO_2H$

e: R = $CH_2CH(OH)CH(NHCO_2Me)CO_2Me$

f: R = $CH_2CH(O_2H)CH(NHCO_2Me)CO_2Me$

In preceding papers, we described some model experiments for construction of a side chain at the 7-position of 1-benzylwye, and found that the Wittig reaction on 1-benzyl-7-formylwye (**16**) was most promising.[14] Along this line, the phosphonium reagent **7a** was required for the preparation of **18**, which appeared to be a good intermediate for the synthesis of not only **1c** but also its congeners **1d—f**. We first attempted to synthesize **7a** (TsO^- for I^-) by metathesis of the tosylate **4a**, which is easily accessible from (*S*)-serine methyl ester hydrochloride (**2a**: X = Cl) according to the procedure reported for the *N*-benzyloxycarbonyl analog.[15] The reaction of **4a** with triphenylphosphine in *N*,*N*-dimethylformamide (DMF), however, took place only sluggishly at 40 °C and that at a more elevated temperature failed to afford the pure phosphonium salt. We then converted **4a** into the iodide **8a** by following the general method.[16] The reaction of **8a** with triphenylphosphine in DMF at 50 °C gave a mixture, whose proton nuclear magnetic resonance (^{1}H-NMR) spectrum indicated the presence of **7a** and methyltriphenylphosphonium iodide. When the reaction was conducted in toluene under reflux, 2-[(methoxycarbonyl)amino]-2-propenoic acid methyl ester,[17] a product formed *via* β-elimination,[18] was also obtained. Although the product obtained in the reaction at 80 °C was still contaminated with methyltriphenylphosphonium iodide, prolonged reaction at 50 °C gave pure **7a** in good yield. Nevertheless, treatment of **7a** with *n*-butyllithium in tetrahydrofuran (THF) at −78 °C followed



a: R = Me
b: R = $PhCH_2$

Chart 1

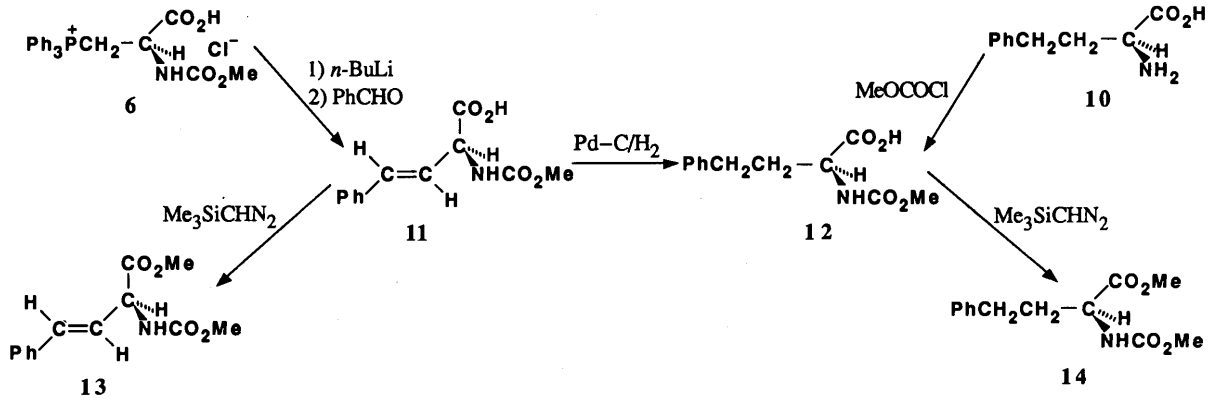
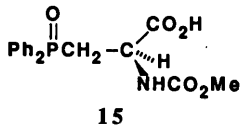


Chart 2

by addition of 1-benzyl-7-formylwye (**16**) resulted in the β-elimination in preference to the Wittig reaction. We considered that the corresponding phosphonium salt **6** with a free carboxy group might not undergo the β-elimination in view of a successful precedent with (2-carboxyethyl)-triphenylphosphonium chloride.[19] An attempt to obtain **6** by treatment of **7a** with sodium hydroxide in aqueous methanol again resulted in the β-elimination. We then prepared **7b** through the iodide **8b**, which was synthesized from (*S*)-serine benzyl ester *p*-toluenesulfonate (**2b**, X=TsO),[20] in a manner similar to that described for **7a**. Although direct hydrogenolysis of **7b** was not achieved, conversion of the iodide **7b** into the chloride followed by hydrogenolysis over Pd–C afforded **6** in 59% overall yield based on (*S*)-serine. We failed in an alternative synthesis of **6** (I^- for Cl^-) by the reaction of triphenylphosphine with (*R*)-3-iodo-*N*-(methoxycarbonyl)alanine (**9**), which was obtained by hydrogenolysis of **4b** followed by metathesis with sodium iodide, because of its poor reactivity.

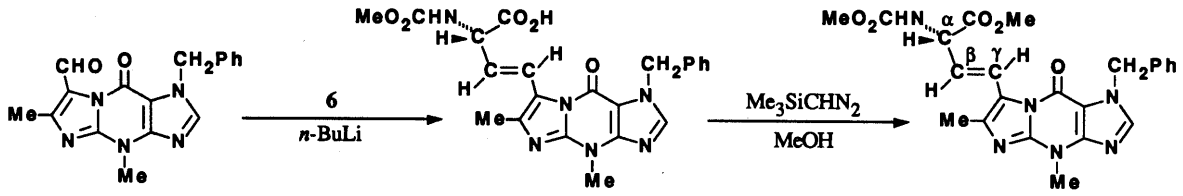
In order to examine the behavior of **6** in the Wittig reaction, we first performed the reaction of **6** with benzaldehyde using 3 molar eq of *n*-butyllithium in a mixed solvent of THF and hexamethylphosphoric triamide (HMPA), obtaining [*S*-(*E*)]-2-(methoxycarbonyl)amino-4-phenyl-3-butenoic acid (**11**) (isolated as the methyl ester **13** in 28% yield). Even the use of 2 molar eq of the base afforded **11**, but in somewhat lower yield. An *E* configuration of **11** was assignable on the basis of the coupling constant (16 Hz) observed for the olefinic protons. The formation of the (*Z*)-isomer of **11** was not observed by ^{1}H-NMR



15

spectroscopy, although nonstabilized triphenylphosphorus ylides often produce (*Z*)-alkenes preferentially.[21] The observed stereoselectivity may be interpreted by analogy with the preponderance of (*E*)-alkenes reported for similar reactions of nonstabilized ylides bearing an oxido, carboxylato, or amido anion,[21b] but nevertheless, such a marked preference for the (*E*)-isomer in the reaction of **6** is noteworthy. A major side product of this reaction was the phosphine oxide **15**. On hydrogenation over Pd–C **11** afforded (*S*)-2-[(methoxycarbonyl)amino]-4-phenylbutanoic acid (**12**) in 27% overall yield. The specific rotation of this sample was identical with that of an authentic specimen of **12** [$[\alpha]_{365}^{25}$ +30.2° (MeOH)] derived from (*S*)-homophenylalanine (**10**).[22] We can not, however, say whether the configuration of the chiral center was completely retained throughout these transformations, since we can not rule out the possibility that **12** thus obtained from **6** was contaminated by a trace of **15** having a large specific rotation [$[\alpha]_{365}^{24}$ +98.5° (MeOH)] because of the difficulty of purification. Compound **12** was then converted into the methyl ester **14**, from which the methyl ester of **15** was easily removable by chromatography. The specific rotation of **14** thus obtained was 92% of that of an authentic sample derived from **10** *via* **12**.[23]

The next step toward access to wybutine should be

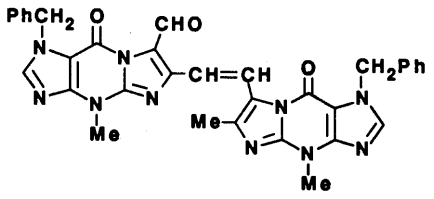


16 17 18

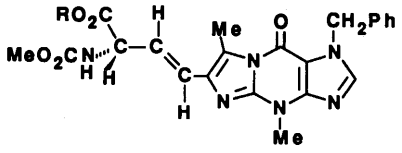
Chart 3

utilization of the aldehyde **16** as a substrate of the present method of constructing optically active β,γ-unsaturated amino acid derivatives. However, the reaction between **16** and the phosphorane generated from **6** gave poorer results than the case of benzaldehyde and proved to depend markedly on subtle changes of the reaction conditions. The best result was obtained when **6** was dried over molecular sieves in a mixture of THF and HMPA, then treated with 3 molar eq of *n*-butyllithium at $-78\,^{\circ}C$, and the resulting phosphorane was then allowed to react with **16** at $-78\,^{\circ}C$ to $-18\,^{\circ}C$. Flash chromatography[24] of the crude product afforded unchanged **16** (28% recovery), a mixture of products, whose ^{1}H-NMR spectrum suggested that the main component was **19**, and a polar fraction containing the desired acid **17**. Compound **19** should be a product formed through vinylogous aldol condensation. For obtaining **17**, it was indispensable to dry the solution of the phosphonium salt **6** over molecular sieves before use; otherwise no **17** was formed, but **15** was produced in 83% yield. When the temperature of the Wittig reaction was raised to room temperature at the final stage, a small amount of the rearranged product **20a** was suggested to be formed. Although we obtained its methyl ester **20b** as a mixture with the methyl ester of **15**, we could not purify it because of the instability of this type of compounds.[14b] Compound **20b**[25] was also obtained as a yellow oil in 3% yield by the Wittig reaction using sodium (methylsulfinyl)methanide in dimethyl sulfoxide at room temperature followed by methylation. We previously reported on this type of rearrangement of a model compound under similar conditions.[14b] Analogous transformations through cleavage of a pyrimidone ring followed by recyclization have been recorded.[26]

Compound **17**, which was contaminated with other polar products, was isolated as the methyl ester **18** by flash chromatography after treatment with trimethylsilyldiazomethane[27] in 16% yield.[28] In this case again, no (*Z*)-isomer of **18** was detected by ^{1}H-NMR spectroscopy. It should be noted that the β,γ-unsaturated amino acid ester **18** underwent racemization when the methylated mixture was allowed to stand at room temperature without acidification. This was probably caused by contaminating bases such as **21**.[29] Indeed, **16**, which should be formed through cyclization of **21**, was detected by means of ^{1}H-NMR spectroscopy in the methylated mixture. The ^{1}H-NMR spectrum of **18** is in accord with that of a model compound, (*E*)-1-benzyl-7-(3-methyl-1-butenyl)wye (**22**),[14b] except for the C(γ)-olefinic proton, which is more deshielded by 0.5 ppm than the corresponding one [C(1′)-H] of **22**. The ultraviolet (UV) spectrum of **18** [$\lambda_{max}^{95\%\ EtOH}$ 232 nm (ε 21200), 260 (21600), 294 (13300), 322 (sh) (7400)], however, exhibited a marked difference from that of **22** [$\lambda_{max}^{95\%\ EtOH}$

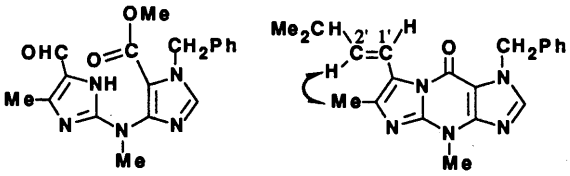


19



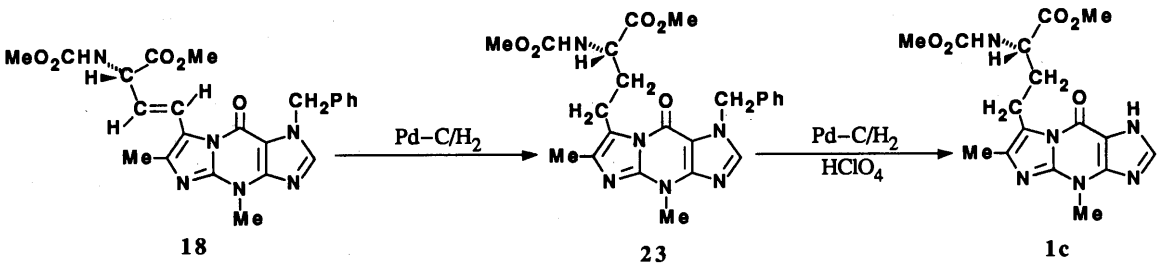
20

a: R = H
b: R = Me



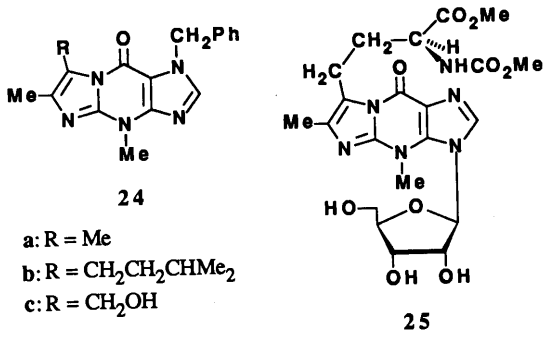
21 22

254 nm (ε 25500), 282 (sh) (8200), 324 (5200).[14b] This should stem from the different extent in conjugation of the exocyclic double bond with the tricycle, because the UV spectrum of compound (±)-**23**[30] with a saturated side chain was practically identical with the spectra of the model compounds **24**.[14] A consideration of a space-filling molecular model for **22** suggested that the exocyclic double bond should be arranged as the s-*cis* form so that the maximum conjugation is attained. This was supported by the nuclear Overhauser effect (NOE)[31]: when the C(6)-Me resonance was irradiated, the enhancement obtained for C(2′)-H was 22%, whereas that for C(1′)-H was only 3%. With **18** also, saturation of the C(6)-Me resonance gave 28% and 2% enhancements of the intensities of the C(β)-H and C(γ)-H signals, respectively, indicating that there was no substantial difference in conformation between **22** and **18**. It is unlikely that the inductive effect of the functional groups of **18** alone brings about the UV spectral difference. We might suppose that the exocyclic double bond in **18** is forced slightly out of the plane of the heterocycle owing to an intramolecular hydrogen bonding between the amino hydrogen at the side chain and the carbonyl oxygen at the 9-position to cause some decrease in the conjugation. However, we found no evidence for such hydrogen bonding in the stretching absorption band due to the carbonyl at the 9-position. A π-stacking interaction between the phenyl



18 23 1c

Chart 4



24

a: R = Me
b: R = $CH_2CH_2CHMe_2$
c: R = CH_2OH

25

of the 1-benzyl group and the carbamate of the side chain might be another factor in the supposed deviation of the coplanarity. In such a defined conformer, the C(γ)-H resonance observed at unexpectedly low magnetic field may be interpreted as a result of the anisotropic effect of the carbamate group.[32]

Now that a key intermediate **18** had become available, saturation of the side chain and removal of the benzyl group were necessary as the next steps for access to **1c**. Although debenzylation of (±)-**23** had been accomplished by Nakanishi's group by hydrogenolysis over Pd–C in 2-propanol in the presence of acetic acid and hydrochloric acid with special care,[11] we had smoothly removed the benzyl group from 1-benzyl-7-methylwye (**24a**) by hydrogenolysis over Pd–C in methanol in the presence of aqueous perchloric acid.[14a] However, we previously experienced the formation of a by-product when 1-benzyl-7-(hydroxymethyl)wye (**24c**) was subjected to hydrogenolysis under similar conditions, probably owing to acid-catalyzed generation of the stabilized carbocation.[14a] To avoid the predictable formation of such a carbocation we first saturated the side chain of **18** over 10% Pd–C in the absence of acid. The reduction was continued by addition of perchloric acid to afford **1c** as the monohydrate [$[\alpha]_D^{23}$ −45° (MeOH)] in 75% yield. The UV, mass (MS), and ^{1}H-NMR spectra of **1c** thus obtained were identical with those of (±)-**1c**[10,33] confirming the correctness of the structure in a two-dimensional sense. We have already assigned the 1,4-dihydro structure [N(1)-H tautomer] to **1a** rather than the alternative 3,4-dihydro structure [N(3)-H tautomer] on the basis of UV spectral comparison with model compounds.[34] This assignment has been supported by recent fluorescence studies on **1a** and related compounds.[35] The UV spectrum of **1c** determined in 95% aqueous ethanol resembles that of (±)-1-benzylwybutine [(±)-**23**] rather than that of 3-β-D-ribofuranosylwybutine (**25**),[36] suggesting that **1c** also exists as the 1,4-dihydro structure [N(1)-H tautomer].

The identity of wybutine had been established by comparison of the MS[7a] and ^{1}H-NMR spectra[7,37] with those of (±)-**1c**.[10] Further evidence in support of the identity was provided by direct comparison of wybutine[38] with the present sample of synthetic **1c** by means of high-performance liquid chromatography (HPLC). Although comparison of the CD spectrum of wybutine[39] with that of synthetic **1c** enabled us to assign an *S* configuration to wybutine, the intensity of the Cotton effect at 235 nm reported for wybutine was only a half of that of the present sample of **1c**. Direct comparison of the CD spectra of natural[38] and synthetic **1c** revealed that they were superimposable except for the larger intensity (1.2 times) of the latter. The same intensity relationship was also recognized in the UV spectra of natural and synthetic **1c**; the low potency of the natural sample was probably due to purification difficulties because of the minute amount available, as had already been noted.[10] Although these results suggested that synthetic **1c** was equivalent to the natural one in optical purity, both samples were ultimately demonstrated to be enantiomerically pure by means of HPLC using a chiral column.

In conclusion, we have established a new method for the synthesis of optically active β,γ-unsaturated α-amino acid derivatives by use of the phosphonium chloride **6** as a synthetic equivalent for the nucleophilic alanine synthon.[40] This method enabled us to perform the first chiral synthesis of **1c** and to assign an *S* configuration to wybutine.

Experimental

General Notes All melting points were taken on a Yamato MP-1 capillary melting point apparatus and are corrected. Spectra reported herein were recorded on a Hitachi 320 UV spectrophotometer, a JASCO J-500C spectropolarimeter equipped with a JASCO DP-500N data processor, a Hitachi M-80 mass spectrometer, and a JEOL JNM-FX-100 NMR spectrometer at 25 °C with tetramethylsilane as an internal standard. Optical rotations were measured with a JASCO DIP-181 polarimeter using a 1-dm sample tube. The liquid chromatographic system was a Waters model 204 ALC which included a 6000A pump, a U6K injector, and a model 440 absorbance detector operating at 254 nm. Microanalyses were determined by Mr. Y. Itatani and his associates at Kanazawa University. Pre-coated silica gel plates (0.25 mm) with a fluorescent indicator (Merck) were used for analytical thin-layer chromatography (TLC). Flash chromatography was performed on silica gel according to the reported procedure.[24] The following abbreviations are used: br = broad, d = doublet, dd = doublet-of-doublets, ddd = doublet-of-doublets-of-doublets, dt = doublet-of-triplets, m = multiplet, q = quartet, s = singlet, sh = shoulder, t = triplet.

(*S*)-*N*-(Methoxycarbonyl)serine Methyl Ester (3a) Methyl chloroformate (11.3 g, 120 mmol) was added to a pre-cooled (10 °C) solution of (*S*)-serine methyl ester hydrochloride (**2a**: X = Cl) (15.56 g, 100 mmol) in water (250 ml) in the presence of sodium bicarbonate (25 g) over a period of 5 min with vigorous stirring on a magnetic stirrer. The mixture was stirred at room temperature for a further 5 min, brought to pH 6 with 10% hydrochloric acid, and concentrated *in vacuo* to *ca.* 70 ml. The resulting solution was extracted with dichloromethane (8 × 50 ml). The

combined organic layers were dried over magnesium sulfate and then concentrated *in vacuo* to leave a colorless heavy oil (16.53 g, 93%), $[\alpha]_D^{19}$ −21.6° (*c* = 1.00, MeOH); ^{1}H-NMR ($CDCl_3$) δ: 2.30 (1H, s, OH), 3.71 and 3.79 (3H each, s, two Me's), 3.91 and 3.99 (1H each, dd, *J* = 10.5, 3.5 Hz, CH_2), 4.42 (1H, m, CH), 5.74 (1H, d, *J* = 7.5 Hz, NH).

(*S*)-*N*-(Methoxycarbonyl)serine Benzyl Ester (3b) (*S*)-Serine benzyl ester *p*-toluenesulfonate (**2b**: X = TsO), which was prepared from (*S*)-serine (25.27 g, 240 mmol) according to the reported procedure,[20] was dissolved in water (600 ml). Sodium bicarbonate (60.0 g, 714 mmol) and methyl chloroformate (22.3 ml, 288 mmol) were successively added to this solution under cooling in ice water over a period of 10 min with vigorous stirring. Stirring was continued for a further 1.5 h at room temperature. The resulting oily precipitate crystallized on being kept in a refrigerator. The crystals were collected by filtration and dried to give a first crop of **3b** (43.45 g), mp 39—44 °C. A second crop [3.27 g; the total yield was 76% based on (*S*)-serine] was obtained by extraction of the mother liquor with dichloromethane (3 × 200 ml) followed successively by drying over magnesium sulfate and concentration *in vacuo*. Recrystallization from 50% (v/v) aqueous methanol followed by drying over phosphorus pentoxide at 2 mmHg and room temperature for 18 h gave an analytical sample as colorless pillars, mp 42—44 °C; $[\alpha]_D^{13}$ −17.8° (*c* = 1.05, MeOH); ^{1}H-NMR ($CDCl_3$) δ: 1.77 (br, 1/5H_2O), 2.38 (1H, br, OH), 3.69 (3H, s, Me), 3.92 and 4.01 (1H each, dd, *J* = 12, 3 Hz, CH_2CH), 4.46 (1H, m, CH), 5.22 (2H, s, PhCH_2), 5.71 (1H, brd, *J* = 9 Hz, NH), 7.35 (5H, s, Ph). *Anal.* Calcd for $C_{12}H_{15}NO_5 \cdot 1/5H_2O$: C, 56.11; H, 6.04; N, 5.45. Found: C, 56.23; H, 6.01; N, 5.55.

(*S*)-*N*-(Methoxycarbonyl)-*O*-(*p*-toluenesulfonyl)serine Methyl Ester (4a) *p*-Toluenesulfonyl chloride (13.38 g, 70.1 mmol) was added to a solution of **3a** (10.34 g, 58.4 mmol) in dry pyridine (29 ml) at −10 °C over a period of 20 min with stirring. The mixture was stirred for a further 3.5 h at −10 °C then poured onto crushed ice (170 ml). The solid that separated was collected by filtration, washed with cold water (50 ml), and dried to give **4a** (16.03 g, 83%), mp 83—85 °C. Recrystallization from ethanol afforded an analytical sample as colorless prisms, mp 87—88 °C; $[\alpha]_D^{15}$ +6.6° (*c* = 1.04, MeOH); ^{1}H-NMR ($CDCl_3$) δ: 2.46 (3H, s, CMe), 3.65 and 3.72 (3H each, s, two OMe's), 4.32 (1H, dd, *J* = 10, 3.5 Hz) and 4.41 (1H, dd, *J* = 10, 3 Hz) (CH_2), 4.53 (1H, ddd, *J* = 7, 3.5, 3 Hz, CH), 5.47 (1H, d, *J* = 7 Hz, NH), 7.36 (2H, d, *J* = 8 Hz, phenyl protons *meta* to the sulfonyl group), 7.76 (2H, d, *J* = 8 Hz, phenyl protons *ortho* to the sulfonyl group). *Anal.* Calcd for $C_{13}H_{17}NO_7S$: C, 47.12; H, 5.17; N, 4.23. Found: C, 47.27; H, 5.20; N, 4.33.

(*S*)-*N*-(Methoxycarbonyl)-*O*-(*p*-toluenesulfonyl)serine Benzyl Ester (4b) A solution of **3b** · 1/5H_2O (24.83 g, 96.7 mmol) in dry pyridine (120 ml) was cooled to −10 °C and *p*-toluenesulfonyl chloride (32 g, 170 mmol) was added over a period of 20 min. The resulting mixture was stirred at −10 ± 5 °C for 3 h and then poured onto crushed ice (*ca.* 500 ml). The resulting solid was collected by filtration, washed with water (200 ml), and dried to give a slightly brown solid (32.08 g, 81%), mp 75—83 °C. Recrystallization from ethanol (30 ml) gave colorless needles (25.80 g, 65%), mp 89—90.5 °C. Further recrystallization from ethanol gave an analytical sample with unchanged melting point, $[\alpha]_D^{16}$ +2.7° (*c* = 1.08, MeOH); MS *m/z*: 407 (M^+); ^{1}H-NMR ($CDCl_3$) δ: 2.43 (3H, s, CMe), 3.64 (3H, s, OMe), 4.33 and 4.40 (1H each, dd, *J* = 9, 3 Hz, CH_2CH), 4.55 (1H, ddd, *J* = 9, 3, 3 Hz, CH), 5.10 and 5.19 (1H each, d, *J* = 12 Hz, PhCH_2), 5.53 (1H, d, *J* = 9 Hz, NH), 7.31 (2H, d, *J* = 10 Hz, phenyl protons *meta* to the sulfonyl group), 7.34 (5H, s, PhCH_2), 7.71 (2H, d, *J* = 10 Hz, phenyl protons *ortho* to the sulfonyl group). *Anal.* Calcd for $C_{19}H_{21}NO_7S$: C, 56.01; H, 5.20; N, 3.44. Found: C, 55.99; H, 5.25; N, 3.23.

(*S*)-*N*-(Methoxycarbonyl)-*O*-(*p*-toluenesulfonyl)serine (5) A solution of **4b** (8.15 g, 20 mmol) in ethanol (280 ml) was hydrogenated over 10% Pd–C (0.82 g) at atmospheric pressure and room temperature for 35 min. The catalyst was filtered off and the filtrate was concentrated *in vacuo* to leave **5** (6.29 g, 99%), mp 127—131 °C. Recrystallization from ethanol gave an analytical sample as colorless pillars, mp 137—139 °C; $[\alpha]_D^{28}$ +19.4° (*c* = 0.500, MeOH); ^{1}H-NMR [$(CD_3)_2SO$] δ: 2.43 (3H, s, CMe), 3.50 (3H, s, OMe), 4.06—4.42 (3H, m, CH_2 CH), 7.49 (2H, d, *J* = 8 Hz, phenyl protons *meta* to the sulfonyl group), 7.60 (1H, d, *J* = 8 Hz, NH), 7.77 (2H, d, *J* = 8 Hz, phenyl protons *ortho* to the sulfonyl group), 13.07 (1H, br, CO_2H). *Anal.* Calcd for $C_{12}H_{15}NO_7S$: C, 45.42; H, 4.76; N, 4.41. Found: C, 45.37; H, 4.71; N, 4.47.

(*R*)-3-Iodo-2-[(methoxycarbonyl)amino]propanoic Acid (9) A solution of sodium iodide (0.68 g, 4.54 mmol) in dry acetone (4 ml) was added to a solution of **5** (1.07 g, 3.37 mmol) in dry acetone (7 ml) and the whole was stirred at 25 °C for 55 h. The resulting precipitate was filtered off and the filtrate was concentrated *in vacuo*. The residue was dissolved in chloroform (30 ml) and the solution was washed with saturated aqueous sodium chloride (2 × 20 ml), dried over magnesium sulfate, and concentrated *in vacuo* to leave a slightly yellow solid (0.706 g, 77%). Recrystallization from benzene gave an analytical sample as colorless prisms, mp 125—127 °C; $[\alpha]_D^{28}$ +22.7° (*c* = 1.00, MeOH); ^{1}H-NMR [$(CD_3)_2SO$] δ: 3.33 (1H, dd, *J* = 10, 8.5 Hz) and 3.54 (1H, dd, *J* = 10, 4.5 Hz) (CH_2), 3.57 (3H, s, Me), 4.19 (1H, ddd, *J* = 8.5, 8.5, 4.5 Hz, CH), 7.55 (1H, d, *J* = 8.5 Hz, NH). *Anal.* Calcd for $C_5H_8INO_4$: C, 22.00; H, 2.95; N, 5.13. Found: C, 22.21; H, 2.93; N, 5.38.

(*R*)-3-Iodo-2-[(methoxycarbonyl)amino]propanoic Acid Methyl Ester (8a) A solution of sodium iodide (16.4 g, 110 mmol) in dry acetone (125 ml) was added to a solution of **4a** (18.22 g, 55 mmol) in dry acetone (25 ml) and the whole was stirred at 25 °C for 5 d. The precipitate that separated was filtered off and washed with acetone. The combined filtrate and washings were concentrated *in vacuo* to leave a solid residue. This was partitioned between chloroform (40 ml) and water (20 ml). The organic layer was washed with water (20 ml), dried over magnesium sulfate, and concentrated *in vacuo* to leave a slightly yellow solid, which was recrystallized from ethanol (20 ml) to afford colorless needles (13.91 g, 88%), mp 105—106 °C. Further recrystallization from ethanol gave an analytical sample with unchanged melting point, $[\alpha]_D^{13}$ +0.5° (*c* = 1.13, MeOH); $[\alpha]_{365}^{13}$ +3.5° (*c* = 1.13, MeOH); ^{1}H-NMR ($CDCl_3$) δ: 3.59 (2H, d, *J* = 4 Hz, CH_2), 3.72 and 3.81 (3H each, s, two OMe's), 4.56 (1H, dt, *J* = 7, 4 Hz, CH), 5.52 (1H, br, NH). *Anal.* Calcd for $C_6H_{10}INO_4$: C, 25.11; H, 3.51; N, 4.88. Found: C, 25.12; H, 3.51; N, 4.72.

(*R*)-3-Iodo-2-[(methoxycarbonyl)amino]propanoic Acid Benzyl Ester (8b) A solution of sodium iodide (12.62 g, 84.2 mmol) in dry acetone (50 ml) was added to a solution of **4b** (25.80 g, 63.3 mmol) in dry acetone (30 ml) and the whole was stirred at room temperature for 3 d. The precipitate that separated was filtered off and washed with dry acetone (3 × 10 ml). The filtrate and the washings were combined and concentrated *in vacuo* to leave a slightly brown oil. This was partitioned between chloroform (100 ml) and water (100 ml). The organic layer was washed with water (2 × 100 ml), dried over magnesium sulfate, and concentrated *in vacuo* to leave a slightly brown solid (22.94 g, 100%), mp 59—61 °C. Recrystallization from methanol gave an analytical sample as colorless plates, mp 60—62 °C; $[\alpha]_D^{17}$ −18.6° (*c* = 1.21, MeOH); ^{1}H-NMR ($CDCl_3$) δ: 3.58 (2H, m, ICH_2), 3.70 (3H, s, Me), 4.57 (1H, m, CH), 5.22 (2H, s, Ph CH_2), 5.55 (1H, br, NH), 7.37 (5H, s, Ph). *Anal.* Calcd for $C_{12}H_{14}INO_4$: C, 39.69; H, 3.89; N, 3.86. Found: C, 39.99; H, 3.97; N, 3.78.

(*R*)-[2-(Methoxycarbonyl)-2-[(methoxycarbonyl)amino]ethyl]triphenylphosphonium Iodide (7a) A solution of **8a** (5.74 g, 20 mmol) and triphenylphosphine (5.77 g, 22 mmol) in dry toluene (100 ml) was kept at 50 °C for 34 d. The precipitate that separated was collected by filtration, washed with a little toluene, and dried to afford **7a** (8.39 g, 76%) as colorless prisms, mp 147.5—153 °C (dec.); $[\alpha]_D^{18}$ +5.81° (*c* = 3.46, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 3.40 and 3.73 (3H each, s, two Me's), 3.80—4.14 (1H, m, P^+CH), 4.65—5.25 (2H, m, P^+CHCH), 7.33 (1H, d, *J* = 8 Hz, NH), 7.61—8.00 (15H, m, Ph_3).

(*R*)-[2-(Benzyloxycarbonyl)-2-[(methoxycarbonyl)amino]ethyl]triphenylphosphonium Iodide (7b) A solution of **8b** (19.90 g, 54.8 mmol) and triphenylphopsphine (15.81 g, 60.3 mmol) in dry toluene (100 ml) was kept at 50 °C for 5 d. The resulting precipitate was collected by filtration, washed with toluene (40 ml), and dried to afford colorless plates, 21.57 g, mp 149—151 °C; $[\alpha]_D^{18}$ +9.5° (*c* = 2.6, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 3.38 (3H, s, Me), 3.77—4.12 (1H, m, P^+CH), 4.79—5.11 (2H, m, P^+CHCH), 5.16 (2H, s, PhCH_2), 7.32 (5H, s, overlapped with a broad 1H signal due to NH, PhCH_2), 7.90—7.98 (15H, m, Ph_3). The combined filtrate and washings were concentrated *in vacuo* to *ca.* 20 ml and kept at 50 °C for a further 5 d. The precipitate that separated was filtered off, washed with toluene (50 ml), and dried to give a second crop of **7b** (11.80 g) having the same melting point, infrared (IR) spectrum, and specific rotation as those of the first crop described above. The total yield was 97%.

(*R*)-[2-Carboxy-2-[(methoxycarbonyl)amino]ethyl]triphenylphosphonium Chloride (6) A solution of **7b** (24.70 g, 39.5 mmol) in 50% (v/v) aqueous ethanol (720 ml) was passed through a column of Amberlyst A-26 (Cl^-) (330 ml) and the column was eluted with the aqueous ethanol (700 ml). The combined eluate was concentrated *in vacuo* to leave a slightly brown glass (22.80 g). This was dissolved in ethanol (290 ml) and the whole was hydrogenated over 10% Pd–C (13 g) at atmospheric pressure and room temperature for 21 h. The catalyst was filtered off and washed with ethanol (700 ml). The filtrate and the washings were combined and concentrated *in vacuo*. The residue was dried by coevaporation, after dissolving it in a mixture of dry chloroform and dry benzene (1 : 1, v/v), *in vacuo* three times. It was further dried over phosphorus pentoxide at

2 mmHg and 80 °C for 21 h to afford **6** (17.20 g, 98%) as a colorless glass, $[\alpha]_D^{24}$ +52° (c=0.50, $CHCl_3$); ^{1}H-NMR [$(CD_3)_2SO$] δ: 3.30 (3H, s, Me), 3.70—4.50 (3H br, CH_2CH), 7.50 (1H, br, NH), 7.60—8.00 (15H, m, Ph_3). This sample was of *ca.* 95% purity: the ^{1}H-NMR spectrum indicated that it was contaminated by a small amount of benzene and a trace of ethanol.

(*S*)-2-[(Methoxycarbonyl)amino]-4-phenylbutanoic Acid (12) i) From (*S*)-Homophenylalanine (**10**): Methyl chloroformate (0.04 ml, 0.48 mmol) was added to an ice-cooled mixture of (*S*)-2-amino-4-phenylbutanoic acid (**10**)[22] (54 mg, 0.30 mmol) and sodium carbonate (95 mg, 0.90 mmol) in water (6 ml). The whole was stirred at 0 °C for 10 min and then at room temperature for 2 h. The resulting mixture was brought to pH 1 by addition of 10% hydrochloric acid and extracted with dichloromethane (5 × 6 ml). The organic layers were dried over magnesium sulfate and concentrated *in vacuo* to leave **12** (71 mg, 100%), mp 101—103 °C. Recrystallization from benzene gave an analytical sample of colorless plates, mp 103—105 °C; $[\alpha]_{365}^{25}$ +30.2° (c=0.540, MeOH); ^{1}H-NMR ($CDCl_3$) δ: 2.11 [2H, m, C(3)-H_2], 2.73 [2H, dd, J=8 Hz each, C(4)-H_2], 3.71 (3H, s, Me), 4.40 (1H, m, CH), 5.24 (1H, br, NH), 5.48 (1H, br, CO_2H), 7.24 (5H, m, Ph). *Anal.* Calcd for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.90. Found: C, 60.57; H, 6.43; N, 5.85.

ii) By the Hydrogenation of **11**: A solution of **6** (*ca.* 1.1 mmol as estimated by ^{1}H-NMR spectroscopy) in THF–HMPA (7 : 3, v/v) (31.5 ml), which had been dried over molecular sieves 4A (2.5 g) at 30 °C for 18 h and filtered in the same way as described for the preparation of **18** (see below), was chilled in a dry ice–acetone bath and *n*-butyllithium (1.55 M solution in hexane, 1.94 ml, 3.01 mmol) was added with stirring under argon over a period of 20 min. Benzaldehyde (0.090 ml, 0.88 mmol) was added to the stirred mixture at −78 °C and the whole was allowed to warm to 0 °C over a period of 17 min. The mixture was neutralized with 10% aqueous phosphoric acid and then concentrated *in vacuo* using a mechanical pump to remove HMPA. The residue was partitioned between saturated aqueous sodium bicarbonate (5 ml) and chloroform (5 ml). The organic layer was extracted with saturated aqueous sodium bicarbonate (5 ml). The combined aqueous layers were brought to pH 2 by addition of 10% hydrochloric acid and then extracted with ether (3 × 10 ml). The ethereal extracts were dried over magnesium sulfate and concentrated *in vacuo* to leave crude **11** as a colorless solid (98 mg). This was dissolved in methanol (8 ml) and hydrogenated over 10% Pd–C (100 mg) at atmospheric pressure and room temperature for 1 h. The catalyst was filtered off and washed with hot methanol (50 ml). The combined filtrate and washings were concentrated *in vacuo* to leave a colorless glass (87 mg), whose ^{1}H-NMR spectrum ($CDCl_3$) showed that this product was a mixture of **12** and **15** (molar ratio, 4 : 1). This was dissolved in ether (23 ml) and insoluble **15** was filtered off. Recrystallization of crude **15** from ethyl acetate afforded colorless needles, mp 115—125 °C (dec.), $[\alpha]_{365}^{24}$ +98.5° (c=0.466, MeOH); ^{1}H-NMR ($CDCl_3$) δ: 1.26 (3/5H, t, J=7 Hz, $AcOCH_2\underline{Me}$), 2.04 (3/5H, s, $\underline{Ac}OEt$), 2.82—3.32 (2H, m, $C\underline{H}_2CH$), 3.44 (3H, s, CO_2Me), 4.12 (2/5H, q, J=7 Hz, $AcOC\underline{H}_2Me$), 4.38—4.80 (1H, m, $CH_2C\underline{H}$), 5.98 (1H, d, J=5.5 Hz, NH), 7.34—8.01 (11H, m, Ph_2 and CO_2H). *Anal.* Calcd for $C_{17}H_{18}NO_5P \cdot 1/5CH_3CO_2C_2H_5$: C, 58.59; H, 5.41; N, 3.84. Found: C, 58.23; H, 5.41; N, 3.78. Drying at 50 °C and 2 mmHg for 38 h did not remove the ethyl acetate.

The ethereal solution was concentrated and the solid residue was suspended in water (8 ml). The mixture was extracted with ether (5 × 8 ml) after it had been brought to pH 1—2 with 10% hydrochloric acid. The ethereal solution was dried over magnesium sulfate and concentrated *in vacuo* to leave **12** (57 mg, 27%), $[\alpha]_{365}^{18}$ +29.3° (c=0.747, MeOH). Recrystallization from benzene gave colorless plates, mp 102—105 °C; $[\alpha]_{365}^{24}$ +29.9° (c=0.538, MeOH). This sample was identical (IR spectrum and TLC) with the authentic sample described above.

[*S*-(*E*)]-2-[(Methoxycarbonyl)amino]-4-phenyl-3-butenoic Acid Methyl Ester (13) Crude **11** prepared from benzaldehyde (0.254 ml, 2.5 mmol) in a manner similar to that described for **12** under item (ii), was dissolved in a mixture of benzene (7 ml) and methanol (2 ml) and trimethylsilyldiazomethane (*ca.* 10% in hexane)[27] (2 ml) was added. Acetic acid was added to the solution after a delay of 1 min to acidify it. Removal of the solvents by evaporation and purification by flash chromatography [hexane–ethyl acetate (3 : 1, v/v)] afforded **13** (177 mg, 28%) as a colorless oil, $[\alpha]_D^{26}$ +92° (c=0.20, MeOH); ^{1}H-NMR ($CDCl_3$) δ: 3.72 and 3.79 (3H each, s, two Me's), 5.06 (1H, m, =CHC$\underline{H}$), 5.48 (1H, br d, J=8 Hz, NH), 6.20 (1H, dd, J=6, 16 Hz, =C$\underline{H}$CH), 6.68 (1H, dd, J=1.2, 16 Hz, PhC$\underline{H}$=CH), 7.33 (5H, m, Ph).

(*S*)-2-[(Methoxycarbonyl)amino]-4-phenylbutanoic Acid Methyl Ester (14) i) From **10** through **12**: A solution of trimethylsilyldiazomethane in hexane (*ca.* 10%) (0.8 ml) was added to a solution of **12** (52 mg, 0.22 mmol) in a mixture of methanol (0.4 ml) and benzene (1.4 ml). The resulting solution was concentrated *in vacuo* and the residue was purified by flash chromatography [hexane–ethyl acetate (3 : 1, v/v)] to afford **14** as a colorless oil (51 mg, 93%), $[\alpha]_D^{29}$ −8.6° (c=2.88, MeOH); $[\alpha]_{365}^{31}$ −14.1° (c=2.88, MeOH); ^{1}H-NMR ($CDCl_3$) δ: 1.76—2.36 (2H, m, $CH_2C\underline{H}_2CH$), 2.68 (2H, m, $PhC\underline{H}_2$), 3.70 and 3.72 (3H each, s, two Me's), 4.38 (1H, m, CH), 5.23 (1H, d, J=8 Hz, NH), 7.24 (5H, m, Ph).

ii) From **11** through **12**: Crude **11**, which was obtained from benzaldehyde (0.276 ml, 2.71 mmol) according to the method described above under item (ii) for the preparation of **12**, was hydrogenated over 10% Pd–C in methanol (35 ml) at room temperature for 1 h. The catalyst was removed by filtration and washed with hot methanol. The combined filtrate and washings were concentrated *in vacuo* and the residue was dissolved in a mixture of methanol (1.6 ml) and benzene (5.6 ml). Trimethylsilyldiazomethane (10% in hexane) (2 ml) was added to this solution and the mixture was concentrated *in vacuo*. The oily residue was purified by flash chromatography [hexane–ethyl acetate (2 : 1, v/v)] to afford **14** (100 mg, 15%), $[\alpha]_{365}^{28}$ −13.0° (c=3.07, MeOH). This was identical (IR and ^{1}H-NMR spectra) with **14** prepared by method (i).

[*S*-(*E*)]-4-[1-Benzyl-4,9-dihydro-4,6-dimethyl-9-oxo-1*H*-imidazo-[1,2-*a*]purin-7-yl]-2-[(methoxycarbonyl)amino]-3-butenoic Acid Methyl Ester (18) The phosphonium chloride **6** (3.3 mmol) was gently stirred with molecular sieves 4A (8.40 g) in a mixture of dry THF (74 ml) and dry HMPA (31 ml) under argon at 30 °C for 23 h. The supernatant of the mixture (96 ml) was withdrawn with a syringe and transferred to a reaction vessel through a funnel with a fritted disk under argon. The solution was cooled to −78 °C and *n*-butyllithium (1.55 M solution in hexane, 5.70 ml, 8.84 mmol) was added over a period of 1 h with stirring, and then **16** (977 mg, 3.04 mmol) was added under argon. The whole was stirred for 1 h and then allowed to warm to −18 °C over a period of 6 h. The resulting mixture was neutralized with 10% aqueous phosphoric acid and concentrated *in vacuo* to a small volume. After addition of water (40 ml), the mixture was brought to pH 3 by addition of 10% aqueous phosphoric acid and extracted with chloroform (4 × 30 ml). The organic layers were combined, dried over magnesium sulfate overnight, and concentrated *in vacuo* to leave a brown oil. This was subjected to flash chromatography [column diameter, 50 mm; ethyl acetate–ethanol (10 : 1, v/v)] to afford unchanged **16** (254 mg, 28%). Further elution of the column with chloroform–methanol (10 : 1, v/v) gave a yellow solid (115 mg), whose ^{1}H-NMR spectrum suggested that it was **19** contaminated with small amounts of some components having similar structures, ^{1}H-NMR [$(CD_3)_2SO$] δ: 1.99 (s, CMe), 3.75 and 3.77 (s each, two NMe's), 5.65 (br, two $PhC\underline{H}_2$'s), 7.34 (s overlapped with m, two Ph's and olefinic protons), 8.45 and 8.52 (s each, two heterocyclic protons), 10.54 (s, CHO). Attempts to purify this compound failed. The column was finally eluted with methanol to afford a yellow solid (958 mg) containing **17**. This was dissolved in a mixture of methanol (6 ml) and benzene (21 ml). Trimethylsilyldiazomethane (1.5 M solution in ether, 2.10 ml, 3.2 mmol) was added to the solution and then acetic acid (0.6 ml) was added after a delay of 1 min. The resulting mixture was concentrated *in vacuo* and purified by flash chromatography [column diameter, 30 mm; ethyl acetate–ethanol (10 : 1, v/v)] to afford **18** (221 mg, 16%) as a slightly yellow solid. Recrystallization from methanol gave slightly yellow needles (158 mg, 11%), mp 179—181 °C; $[\alpha]_D^{19}$ +56.9° (c=0.181, MeOH). Further recrystallization from methanol afforded an analytical sample with unchanged melting point, $[\alpha]_D^{20}$ +58.0° (c=0.207, MeOH); UV: given in the text; MS m/z: 464 (M^+), 405 ($M^+ - CO_2Me$), 373 ($M^+ - PhCH_2$); ^{1}H-NMR ($CDCl_3$) δ: 2.38 [3H, br s, C(6)-Me], 3.72 and 3.81 (3H each, s, two OMe's), 3.89 (3H, s, NMe), 5.08 [1H, m, C(α)-H], 5.53 (1H, br, NH), 5.58 (2H, s, $PhC\underline{H}_2$), 5.77 [1H, dd, J=6.5, 16 Hz, =C(β)-H], 7.35 (5H, s, Ph), 7.64 [1H, s, C(2)-H], 7.67 [1H, ddq, J=16, 1.5, 0.5 Hz, =C(γ)-H]. *Anal.* Calcd for $C_{23}H_{24}N_6O_5$: C, 59.48; H, 5.21; N, 18.09. Found: C, 59.42; H, 5.01; N, 18.38.

(*S*)-4,9-Dihydro-α-[(methoxycarbonyl)amino]-4,6-dimethyl-9-oxo-1*H*-imidazo[1,2-*a*]purine-7-butanoic Acid Methyl Ester (1c) A solution of **18** (144 mg, 0.31 mmol) in methanol (110 ml) was hydrogenated over 10% Pd–C (150 mg) at *ca.* 60 °C for 1 h. After confirmation of the consumption of **18** by TLC [ethyl acetate–ethanol (10 : 1, v/v)], 70% aqueous perchloric acid (0.07 ml) and further 10% Pd–C (312 mg) were added. The reduction was continued for a further 24 h at the same temperature. The catalyst was filtered off and the filtrate was neutralized with saturated aqueous sodium bicarbonate. The mixture was then concentrated *in vacuo* and the residue was treated with water (1 ml). The resulting precipitate was collected by filtration after storage of the mixture in a refrigerator overnight, washed with water (2 ml), and dried. It was dissolved in hot methanol (3 ml) and

insoluble material was removed by filtration while the solution was hot. The solution was concentrated *in vacuo* to give **1c** (79 mg), mp 179—184 °C (dec.). The catalyst was extracted with methanol using a Soxhlet extractor. The methanolic solution was concentrated *in vacuo* and the residue was purified by layer chromatography on silica gel (0.5 mm) [ethyl acetate–ethanol (10 : 1, v/v)] to afford a second crop of **1c** (12 mg; the total yield was 75% as the monohydrate). Recrystallization from methanol gave colorless needles, mp 208—211 °C (dec.). This sample was dried over phosphorus pentoxide at 2 mmHg and 80 °C for 8 h and then exposed to air until constant weight was reached to give an analytical sample as the monohydrate with unchanged melting point, $[\alpha]_D^{23}$ −45° (c=0.130, MeOH); CD ($c=2.80\times10^{-5}$ M, 10% aqueous MeOH) $[\theta]^{13}$ (nm): −13800 (232) (neg. max.), −4300 (260) (neg. max.); UV $\lambda_{max}^{95\%\ EtOH}$ 236 nm (ε 32000), 260 (sh) (5300), 309 (5300); $\lambda_{max}^{10\%\ MeOH}$ 236 (34500), 261 (6100), 313 (5500); MS m/z (relative intensity): 376 (M^+) (8), 344 (5), 242 (1), 230 (8), 216 (100); ^{1}H-NMR ($CDCl_3$) δ: 2.17 [2H, m, C(β)-H_2], 2.27 [3H, s, C(6)-Me], 3.21 [2H, m, C(γ)-H_2], 3.70 (6H, s, two OMe's), 3.97 (3H, s, NMe), 4.35 [1H, m, C(α)-H], 5.85 [1H, br, C(α)-NH], 7.96 [1H, s, C(2)-H], 11.07 [1H, br, N(1)-H]; [$(CD_3)_2SO$] δ: 1.96 [2H, m, C(β)-H_2], 2.09 [3H, s, C(6)-Me], 3.06 [2H, m, C(γ)-H_2], 3.56 and 3.58 (3H each, s, two OMe's), 3.75 (3H, s, NMe), 3.96 [1H, m, C(α)-H], 7.64 [1H, d, J=8 Hz, C(α)-NH], 8.16 [1H, d, J=1 Hz, C(2)-H], 13.55 [1H, br, N(1)-H]. *Anal.* Calcd for $C_{16}H_{20}N_6O_5\cdot H_2O$: C, 48.73; H, 5.62; N, 21.31. Found: C, 48.81; H, 5.76; N, 21.51. HPLC analyses were performed on pre-packed columns of 4 mm inner diameter and 250 mm length at the flow rate of 0.5 ml per min at room temperature (20 °C). HPLC [column, solvent (ratio by volume), retention time (min)]: Merck LiChrosorb Si-60 (5 μm), chloroform–methanol (95 : 5), 16.4; 1,2-dichloroethane–ethanol (88 : 12), 22.8; Merck LiChrosorb RP-18 (7μm), acetonitrile–water (20 : 80), 30.8; methanol–water (50 : 50), 18.0. The chiral column used was Sumichiral OA-4600 (5 μm) supplied by Sumika Chemical Analysis Service, Ltd. When eluted with hexane–1,2-dichloroethane–methanol–trifluoroacetic acid (50 : 45 : 5 : 0.2), (±)-**1c**[33] was cleanly resolved to the (*R*)-isomer (retention time, 32.4 min) and the (*S*)-isomer (34.8 min). Analysis of the present sample of **1c** under these conditions indicated that it was free from its antipode.

For comparison of **1c** (39 μg) with wybutine from natural sources (36 μg),[38] both samples were dried over phosphorus pentoxide at 2 mmHg and 50 °C for 6 h and then exposed to air overnight, and separately dissolved in 10% aqueous methanol (4 ml). The UV and CD spectra were taken using these solutions; the results are given in the text. The samples were recovered by evaporation of the solvent and the residual solids were analyzed by HPLC. These behaved in an identical manner under the five different chromatographic conditions defined above.

Acknowledgment This work was supported by a Grant-in-Aid for Scientific Research (No. 59570890) from the Ministry of Education, Science and Culture, Japan, and a grant from the Japan Research Foundation for Optically Active Compounds. We wish to express our gratitude to Professor H. G. Zachau, the University of Munich, for an invaluable sample of wybutine from natural sources, and to Professor K. Nakanishi, Columbia University, for a private communication.

References and Notes

1) M. Sprinzl, T. Hartmann, J. Weber, J. Blank, and R. Zeidler, *Nucleic Acids Res.*, **17**, r1 (1989).
2) R. W. Holley, J. Apgar, G. A. Everett, J. T. Madison, M. Marquisee, S. H. Merrill, J. R. Penswick, A. Zamir, *Science*, **147**, 1462 (1965).
3) For recent reviews, see *a*) T. Itaya, *Yuki Gosei Kagaku Kyokai Shi*, **45**, 431 (1987); *b*) G. R. Björk and J. Kohli, *J. Chromatogr. Libr.*, **45B** (Chromatogr. Modif. Nucleosides, Pt. B), B13 (1990).
4) U. L. RajBhandary, S. H. Chang, A. Stuart, R. D. Faulkner, R. M. Hoskinson, and H. G. Khorana, *Proc. Natl. Acad. Sci. U.S.A.*, **57**, 751 (1967).
5) U. L. RajBhandary, R. D. Faulkner, and A. Stuart, *J. Biol. Chem.*, **243**, 575 (1968).
6) R. Thiebe and H. G. Zachau, *Eur. J. Biochem.*, **5**, 546 (1968).
7) *a*) K. Nakanishi, N. Furutachi, M. Funamizu, D. Grunberger, and I. B. Weinstein, *J. Am. Chem. Soc.*, **92**, 7617 (1970); *b*) R. Thiebe, H. G. Zachau, L. Baczynskyj, K. Biemann, and J. Sonnenbichler, *Biochim. Biophys. Acta*, **240**, 163 (1971).
8) *a*) K. Nakanishi, S. Blobstein, M. Funamizu, N. Furutachi, G. Van Lear, D. Grunberger, K. W. Lanks, and I. B. Weinstein, *Nature, New Biol.*, **234**, 107 (1971); *b*) S. H. Blobstein, D. Grunberger, I. B. Weinstein, and K. Nakanishi, *Biochemistry*, **12**, 188 (1973).
9) References cited in ref. 14*a*.
10) M. Funamizu, A. Terahara, A. M. Feinberg, and K. Nakanishi, *J. Am. Chem. Soc.*, **93**, 6706 (1971).
11) C. R. Frihart, A. M. Feinberg, and K. Nakanishi, *J. Org. Chem.*, **43**, 1644 (1978).
12) According to a private communication from Professor K. Nakanishi, he believes that the CD values reported for (*S*)-*N*-(methoxycarbonyl)glutamic acid dimethyl ester derived from (*S*)-glutamic acid[10] should be read as Δε −0.14 (232 nm) and +0.76 (206).
13) A preliminary communication of this work has been published: T. Itaya and A. Mizutani, *Tetrahedron Lett.*, **26**, 347 (1985).
14) *a*) T. Itaya, A. Mizutani, M. Takeda, and C. Shioyama, *Chem. Pharm. Bull.*, **37**, 284 (1989); *b*) T. Itaya, A. Mizutani, and N. Watanabe, *ibid.*, **37**, 1221 (1989).
15) D. Theodoropoulos, I. L. Schwartz, and R. Walter, *Biochemistry*, **6**, 3927 (1967).
16) M. L. P. Monsigny, D. Delay, and M. Vaculik, *Carbohydr. Res.*, **59**, 589 (1977).
17) ^{1}H-NMR ($CDCl_3$) δ: 3.75 and 3.84 (3H each, s, two Me's), 5.78 (1H, d, J=1.5 Hz) and 6.23 (1H, s) (=CH_2), 7.19 (1H, br, NH). This compound has appeared in the literature without a description of its characteristics or the method of preparation: H. Horikawa, T. Nishitani, T. Iwasaki, Y. Mushika, I. Inoue, and M. Miyoshi, *Tetrahedron Lett.*, **21**, 4101 (1980). See also, M. J. Mullins and P. J. Brondsema, U. S. Patent 4734453 (1988) [*Chem. Abstr.*, **109**, 129814*s* (1988)].
18) *a*) P. F. Alewood, J. W. Perich, and R. B. Johns, *Aust. J. Chem.*, **37**, 429 (1984); *b*) J. A. Bajgrowicz, A. El Hallaoui, R. Jacquier, Ch. Pigière, Ph. Viallefont, *Tetrahedron Lett.*, **25**, 2759 (1984).
19) H. S. Corey, Jr., J. R. D. McCormick, and W. E. Swensen, *J. Am. Chem. Soc.*, **86**, 1884 (1964).
20) G. Fölsch, *Acta Chem. Scand.*, **13**, 1407 (1959).
21) For reviews of the Wittig reaction, see *a*) I. Gosney and A. G. Rowley, "Organophosphorus Reagents in Organic Synthesis," ed. by J. I. G. Cadogan, Academic Press, New York, 1979 pp. 17—153; *b*) B. E. Maryanoff and A. B. Reitz, *Chem. Rev.*, **89**, 863 (1989).
22) A kind gift from Dr. N. Takamura, Tanabe Seiyaku Co., Ltd.
23) Further investigation on the stereochemistry of the reaction sequence will be reported elsewhere.
24) W. C. Still, M. Kahn, and A. Mitra, *J. Org. Chem.*, **43**, 2923 (1978).
25) ^{1}H-NMR for **20b** ($CDCl_3$) δ: 2.74 [3H, s, C(7)-Me], 3.71 and 3.79 (3H each, s, two OMe's), 3.87 [3H, s, NMe], 5.08 (1H, br, =CHC$\underline{H}$), 5.40 (1H, br, NH), 5.57 (2H, s, PhC$\underline{H}_2$), 6.47 (1H, dd, J=6, 15 Hz, =C$\underline{H}$CH), 6.65 [1H, d, J=15 Hz, C(6)-CH=], 7.35 (5H, s, Ph), 7.62 [1H, s, C(2)-H].
26) B. Bhat, K. A. Cruickshank, and N. J. Leonard, *J. Org. Chem.*, **54**, 2028 (1989) and references cited therein.
27) *a*) N. Hashimoto, T. Aoyama, and T. Shioiri, *Chem. Pharm. Bull.*, **29**, 1475 (1981); *b*) S. Mori, I. Sakai, T. Aoyama, and T. Shioiri, *ibid.*, **30**, 3380 (1982).
28) This value of the yield was our best result. In many runs, we obtained **18** in 10% yield or so in spite of employing the same procedure.
29) The parent carboxylic acid of **21** has been reported.[14b]
30) Prepared according to the reported procedure[11] in 6% yield, mp 176—177 °C; UV $\lambda_{max}^{95\%\ EtOH}$ 240 nm (ε 31400), 259 (sh) (6600), 319 (5400); MS m/z: 466 (M^+); ^{1}H-NMR ($CDCl_3$) δ: 2.12 [2H, m, C(β)-H_2], 2.23 [3H, s, C(6)-Me], 3.15 [2H, m, C(γ)-H_2], 3.68 and 3.72 (3H each, s, two OMe's), 3.88 (3H, s, NMe), 4.32 [1H, m, C(α)-H], 5.59 (2H, s, PhC$\underline{H}_2$), 6.05 (1H, d, J=8 Hz, NH), 7.37 (5H, s, Ph), 7.65 [1H, s, C(2)-H]; [$(CD_3)_2SO$] δ: 1.90 [2H, m, C(β)-H_2], 2.08 [3H, s, C(6)-Me], 3.04 [2H, m, C(γ)-H_2], 3.56 and 3.58 (3H each, s, two OMe's), 3.73 (3H, s, NMe), 3.88 [1H, m, C(α)-H], 5.58 (2H, s, PhC$\underline{H}_2$), 7.34 (5H, s, Ph), 7.68 (1H, d, J=8 Hz, NH), 8.37 [1H, s, C(2)-H].
31) D. Neuhaus and M. Williamson, "The Nuclear Overhauser Effect in Structural and Conformational Analysis," VCH, New York, 1989.
32) L. M. Jackman and S. Sternhell, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry," 2nd ed., Pergamon Press, Oxford, 1969, pp. 88—92.
33) Obtained by hydrogenolysis of (±)-**23** over 10% Pd–C in methanol in the presence of perchloric acid in 89% yield, mp 214—215 °C (dec.) (lit.[10] mp 204—206 °C).
34) T. Itaya, H. Matsumoto, T. Watanabe, and T. Harada, *Chem. Pharm. Bull.*, **33**, 2339 (1985).
35) T. M. Nordlund, R. Rigler, C. Glemarec, J.-C. Wu, H. Bazin, G.

Remaud, and J. Chattopadhyaya, *Nucleosides Nucleotides*, **7**, 805 (1988).
36) T. Itaya, M. Shimomichi, and M. Ozasa, *Tetrahedron Lett.*, **29**, 4129 (1988).
37) The solvent, which is not specified in the literature,[7a] should be $CDCl_3$ according to a private communication from the senior author.
38) A very generous sample (colorless needles) was provided by Professor H. G. Zachau.
39) A. M. Feinberg, K. Nakanishi, J. Barciszewski, A. J. Rafalski, H. Augustyniak, and M. Wiewiórowski, *J. Am. Chem. Soc.*, **96**, 7797 (1974).
40) For other methods of homologation of amino acids using synthetic equivalents for the nucleophilic alanine synthon, see *a*) N. A. Sasaki, C. Hashimoto, and P. Potier, *Tetrahedron Lett.*, **28**, 6069 (1987); *b*) J. E. Baldwin, M. G. Moloney, and M. North, *J. Chem. Soc., Perkin Trans. 1*, **1989**, 833; *c*) R. F. W. Jackson, K. James, M. J. Wythes, and A. Wood, *J. Chem. Soc., Chem. Commun.*, **1989**, 644; *d*) R. F. W. Jackson, M. J. Wythes, and A. Wood, *Tetrahedron Lett.*, **30**, 5941 (1989).

Pharmacological Activities of the Prenylcoumarins, Developed from Folk Usage as a Medicine of *Peucedanum japonicum* THUNB.[1)]

Naoki TAKEUCHI,[a] Toshio KASAMA,[b] Yoko AIDA,[b] Junji OKI,[a] Izumi MARUYAMA,[a] Kiyoshi WATANABE,[b] and Seisho TOBINAGA*,[a]

Showa College of Pharmaceutical Sciences,[a] Machida, Tokyo 194, Japan and Research laboratory of Biological Sciences, Kodama Ltd.,[b] Matsudo, Chiba 271, Japan. *Received October 8, 1990*

In connection with the chemical structure of coumarin 1 (a mixture of acetylangeloylkhellactone and acetyltigloylkhellactone), a compound isolated from *Peucedanum japonicum* THUNB., we synthesized eight coumarin compounds (3—10) and performed pharmacological studies on these nine compounds, as well as on another coumarin, praeruptorin A (=Pd-Ia) (2), a compound isolated from *Peucedanum praeruptorum* DUNN. We studied the effects of compounds 1—5 on isolated smooth muscle and of compounds 1—10 on the cardiovascular system. These compounds showed dose-related antagonistic effects on histamine- and Ca^{2+}-induced contractions in smooth muscle and the potencies were in the order 2>1>seselin (3)>xanthyletin (4)=2,2,10-trimethyl-2*H*,8*H*-benzo[1,2-*b*:3,4-b']dipyran-8-one (5). All the compounds except 7-geranyloxy-4-methylcoumarin (10) produced a dose-related increase in vertebral, carotid and femoral blood flow. Compounds 1, 5, and 4-methyl-7-(3-methyl-2-butenyloxy)coumarin (8) caused an increase in blood pressure, but 3 and 4 caused a slight decrease. Compounds 2, 3, 4, 5, and 8 increased heart rate. Jatamansinone (6) and jatamansinol (7) caused only slight changes in blood pressure. All the compounds except 10 increased heart rate. Compound 1 also increased blood flow in the cerebral cortex. Thus, compound 1 was confirmed to have an inhibitory effect on contraction in isolated smooth muscle and an action increasing arterial blood flow. Among the compounds tested in this study, 3, as well as 6 and 7 synthesized on the basis of 3, showed actions similar to those of Ca^{2+} blockers and some compounds had papaverine-like activities. These results suggest that the chemical moiety of compound 3 may be the basis for the pharmacological activities of *Peucedanum japonicum* THUNB.

Keywords *Peucedanum japonicum*; *Peucedanum praeruptorum*; coumarin; seselin; synthesis; smooth muscule contraction; arterial blood flow; calcium ion blocker

The leaves and roots of *Peucedanum japonicum* THUNB. have been used as a wholesome vegetable (folk name: chyoumeigusa) and a folk medicine for the treatment of coughs, respectively, in the Ryukyu Islands.[2)] The plant, called chyoumeigusa (grass for longevity), is indispensable in dishes served in ceremonies such as shirayoi (ceremony for naming a newborn) and yahnuyoi (celebration of the completion of a new house) in the Yaeyama Islands.[3)] Coumarin **1** (a mixture of acetylangeloylkhellactone and acetyltigloylkhellactone) is reported to be one of the principal constituents of this plant.[4)] On the other hand, there is a plant called zenko (*Peucedanum praeruptorium* DUNN.) a relative of chyomeigusa, among Chinese medicines. It is reported that zenko contains coumarins which have concompetitive anticholinergic, antihistaminic, and Ca^{2+} blocking actions and that praeruptorin A (Pd-Ia; **2**) is the most active compound among them.[5)]

We are interested in the relationship between the folk usage of chyomeigusa as a medicine and the pharmacological activities of coumarin **2**, and compared the pharmacological

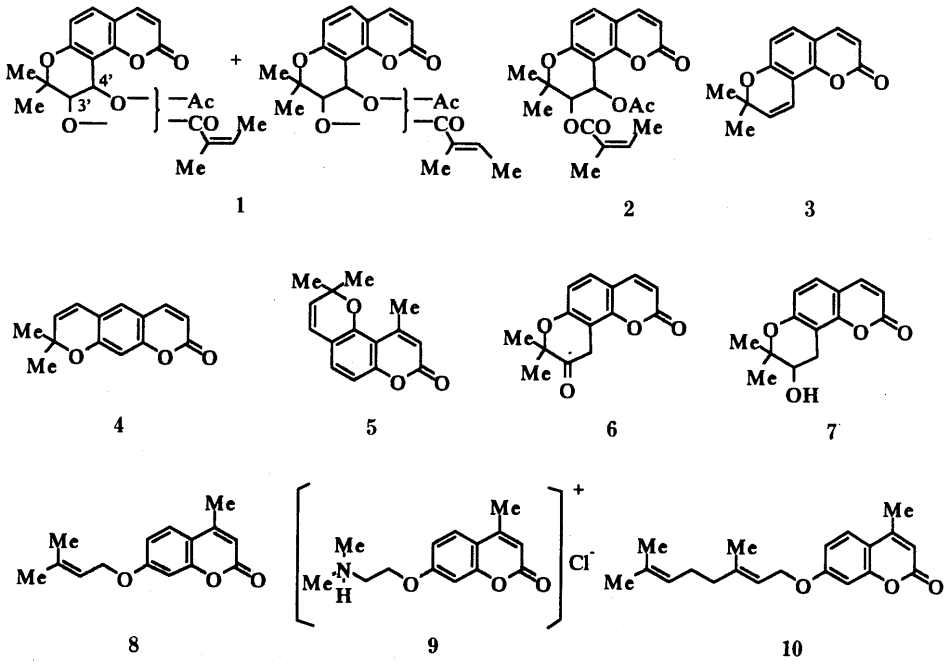


Chart 1

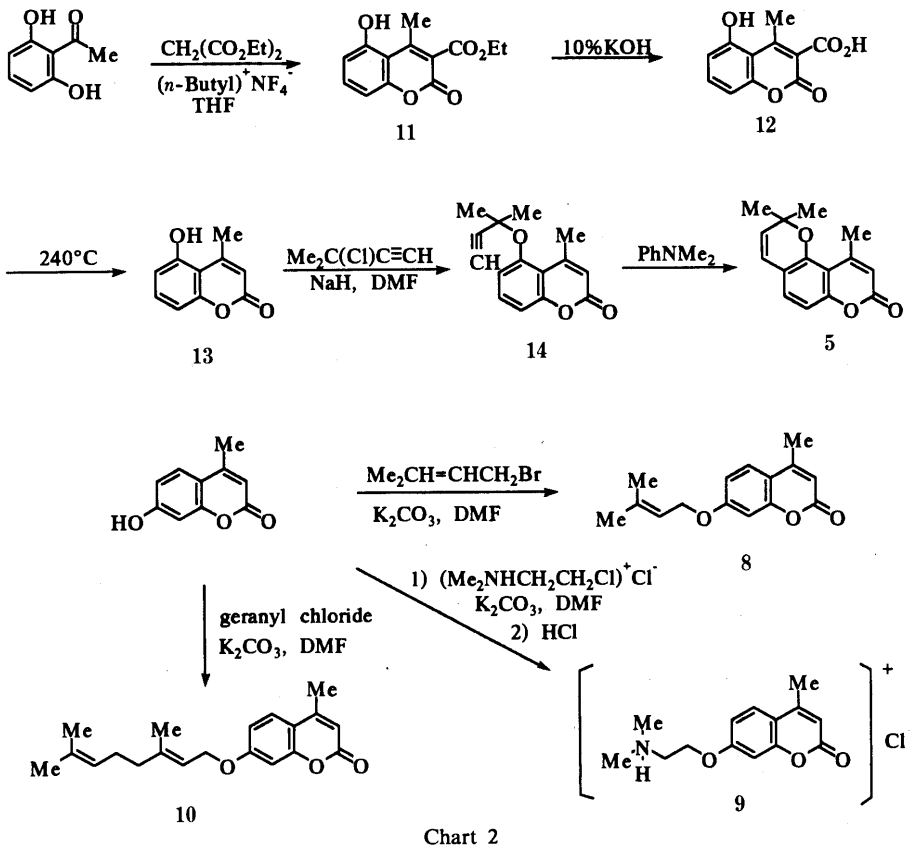


Chart 2

activities of coumarin **1** with those of coumarin **2**. Further, in order to ivestigate the structural requirement for the activities of the two compounds, we synthesized eight related compounds, seselin (**3**),[6] xanthyletin (**4**),[6] 2,2,10-trimethyl-2*H*,8*H*-benzo[1,2-*b*:3,4-*b'*]dipyran-8-one (**5**), jatamansinone (**6**),[4,7] jatamansinol (**7**),[4,7] 4-methyl-7-(3-methyl-2-butenyloxy)coumarin (**8**), 7-(2-dimethylaminoethoxy)-4-methylcoumarin hydrochloride (**9**), and 7-geranyloxy-4-methylcoumarin (**10**) and examined their pharmacological activities. We performed the following pharmacological studies: (1) experiments with isolated smooth muscle; histamine- and Ca^{2+}-induced contraction in guinea pig ileum and (2) experiments with the car diovascular system; blood flow, blood pressure, and heart rate in dogs and cerebral blood flow in rabbits.

Materials Coumarins **1** and **2** were isolated from the ether extracts of the roots of *Peucedanum japonicum* THUNB.[4] and the methanol extracts of the roots of *Peucedanum praeruptorum* DUNN.[5] Seselin (**3**) and xanthyletin (**4**) were synthesized from umbelliferone.[6] Coumarin **5** was synthesized from 2,6-dihydroxyacetophenone as shown in Chart 2. That is, the reaction of 2,6-dihydroxyacetophenone with diethyl malonate in the presence of tetrabutylammonium fluoride gave the condensation product **11** (82.1% yield) which was transformed to the acid **12** by hydrolysis with 10% KOH in 88.8% yield. The resulting acid was converted to **13** by decarboxylation at 240 °C in 90.7% yield. Coumarin **13** and 3-chloro-3-methylbutyne[8] were condensed in the presence of NaH in *N*,*N*-dimethylformamide (DMF) to give the product **14** in 35.6% yield. The conversion of **14** to **5** was established by heating with *N*,*N*-dimethylaniline in 82.0% yield. Jatamansinone (**6**) and jatamansinol (**7**) were synthesized from seselin (**3**) by the method of Bohlman and Hata.[4,7] Coumarins **8**, **9**, and **10** were synthesized by the reactions of 8-hydroxy-4-methylcoumarin with 4-bromo-2-methyl-2-butene, 2-chloroethyldimethylammonium chloride, and geranyl chloride in the presence of K_2CO_3 in DMF in yields of 61.0, 21.0, and 89.0%, respectively.

Agents Coumarins **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, and **10** were dissolved in ethanol and diluted with water or saline for *in vitro* or *in vivo* studies and suspended in 0.5% (w/v) carboxymethyl cellulose (CMC) for *in vivo* study. Compound **9** was dissolved in water. Other agents used were histamine dihydrochloride (histamine; Wako Pure Chemical Industries, Ltd.), $CaCl_2$ (Ca^{2+}; Wako Pure Chemical Industries, Ltd.), KCl (K^+; Wako Pure Chemical Industries, Ltd.), diphenhydramine hydrochloride (diphenhydramine; Wako Pure Chemical Industries, Ltd.), sodium pentobarbital (Tokyo Kasei Co., Ltd.), *d*-tubocurarine chloride (Wako Pure Chemical Industries, Ltd.), and diltiazem (Tanabe Seiyaku Co., Ltd.; extracted from the tablets in our laboratory).

Animals Male Hartley strain guinea pigs weighing about 200 g (Japan SLC, Inc.), male albino rabbits weighing about 2.5 kg (Japan Laboratory Animal Inc.), and adult mongrel dogs weighing 9.0 to 16 kg (Takeda Kaseijho Co., Ltd.) were acclimatized for more than one week after purchase. The animals that had been in good health during the acclimatization period were selected for use in this study. They were housed in rooms controlled to maintain temperature and relative humidity at 22 ± 3 °C and 55 ± 10%, respectively, with air change 10 to 15 times/h and 12 h (07:00—19:00) of artificial light.

Methods (1) Experiments with Isolated Smooth Muscle (i) Effects of Coumarins on Histamine-Induced Contraction

in Isolated Guinea Pig Ileum: The guinea pigs were sacrificed by a sharp blow to the head and the ileum removed. The preparation was suspended in a 10 ml organ bath of Tyrode's solution maintained at 26 °C and bubbled with 95% O_2 and 5% CO_2. Contractile responses were measured with an isotonic transducer (ME-4012, Medical Electrics Co. and TD1125—DT1125, Nihon Koden Corporation) and the resting tension was set at 0.5 g. Compounds were applied at 5 min before the addition of the agonist.

(ii) Effects of Coumarins on Ca^{2+}-Induced Contraction in Isolated Guinea Pig Ileum: The preparation suspended as described in (i) was washed with Ca-free Locke-Ringer's solution added with 1 mM ethylenediaminetetraacetic acid (EDTA) for 40 to 60 min. Then, the preparation was placed in Ca-free Locke-Ringer's solution. After an addition of 75 mM K^+ (final concentration; 80 mM K^+) to the solution, Ca^{2+} was cumulatively added to induce contraction of the ileum. Compounds were applied at 5 min before the addition of 75 mM K^+.

(2) Experiments with Cardiovascular System Adult mongrel dogs were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). The trachea was cannulated for artificial respiration (Harberd). Blood pressure in the left femoral artery and heart rate were routinely measured by means of a pressure transducer (MPU-0.5, Nihon Koden Corporation) and a cardiotachograph (AT-601G, Nihon Koden Corporation), respectively. Mean blood flow was measured with an electromagnetic flow meter (FM-27, Nihon Koden Corporation) in three blood vessels: right carotid, right vertebral, and right femoral arteries. Compounds were given in the cannulated left femoral vein.

(3) Statistical Method Student's *t*-test was used for statistical analysis.

Results and Discussion

(1) Effects of Coumarins on Isolated Smooth Muscle (i) Effects of Coumarins on Histamine-Induced Contraction: Compounds **2** at 10^{-6} g/ml or more, **1**, **3**, and **5** at 10^{-5} g/ml or more, and **4** at 3×10^{-5} g/ml or more caused a rightward shift of the dose–response curve for histamine with a reduction of the maximal responses (Fig. 1). The vehicle (ethanol: 0.01—0.5% (v/v)) showed no effect on the histamine-induced contraction. Table I summarized the pD'_2 values of drugs and the antagonistic potencies were in the order **2**>**1**>**3**≧**5**≧**4**. Diphenhydramine, a histamine antagonist, caused a parallel shift of the dose–response curve without a reduction of the maximal response.

(ii) Effects of Coumarins on Ca^{2+}-Induced Contraction: Compounds **1** and **2** at 10^{-6} g/ml and **3** at 3×10^{-6} g/ml produced a slight shift of the dose–response curve for Ca^{2+} to the right (Fig. 2). However, **1** and **2** at 3×10^{-6} g/ml or more and **3** at 10^{-5} g/ml or more further shifted the curves to the right with the concomitant decrease in the maximum responses. Compounds **4** and **5** at 10^{-5} or more and 3×10^{-5} g/ml or more, respectively, showed a noncompetitive antagonistic tendency of the Ca^{2+}-induced contraction. The vehicle showed no effect on this contraction. Table I summarized the pD'_2 values of drugs and the Ca^{2+}-antagonistic potencies were in the order **2**>**1**>**3**≧**4**=**5**.

Table I. Antagonistic Activity of Drugs on Histamine- and Ca^{2+}-Induced Contraction in the Isolated Guinea Pig Ileum

Drugs	Antagonistic activity (pD'_2; mean ± S.E.)			
	n	Histamine	*n*	Ca^{2+}
1	6	5.10±0.10	5	4.73±0.10
2	6	5.47±0.13	6	5.26±0.14
3	5	4.52±0.18	6	4.55±0.17
4	5	4.37±0.05	6	4.00±0.12
5	5	4.38±0.21	5	4.00±0.13
Diphenhydramine	6	9.31±0.05[a)]		—
Diltiazem		—	6	7.75±0.06[a)]

a) pA_2.

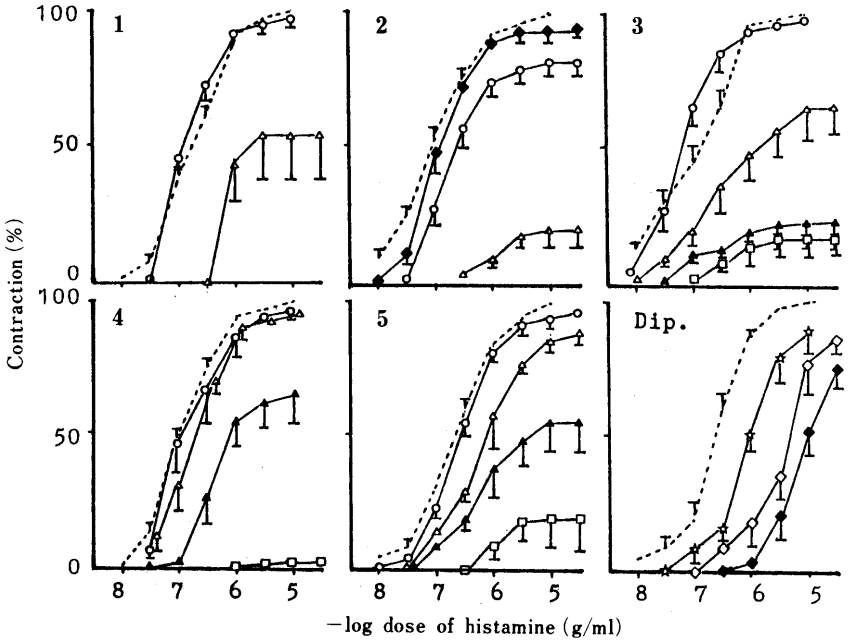


Fig. 1. Effects of **1**, **2**, **3**, **4**, **5** and Diphenhydramine (Dip.) on Histamine-Induced Contraction in the Isolated Guinea Pig Ileum

---, control; —☆—, 10^{-9}; —◇—, 10^{-8}; —◆—, 10^{-7}; —○—, 10^{-6}; —△—, 10^{-5}; —▲—, 3×10^{-5}; —□—, 10^{-4} g/ml. Each point represents the mean ± S.E. of 5 to 6 experiments.

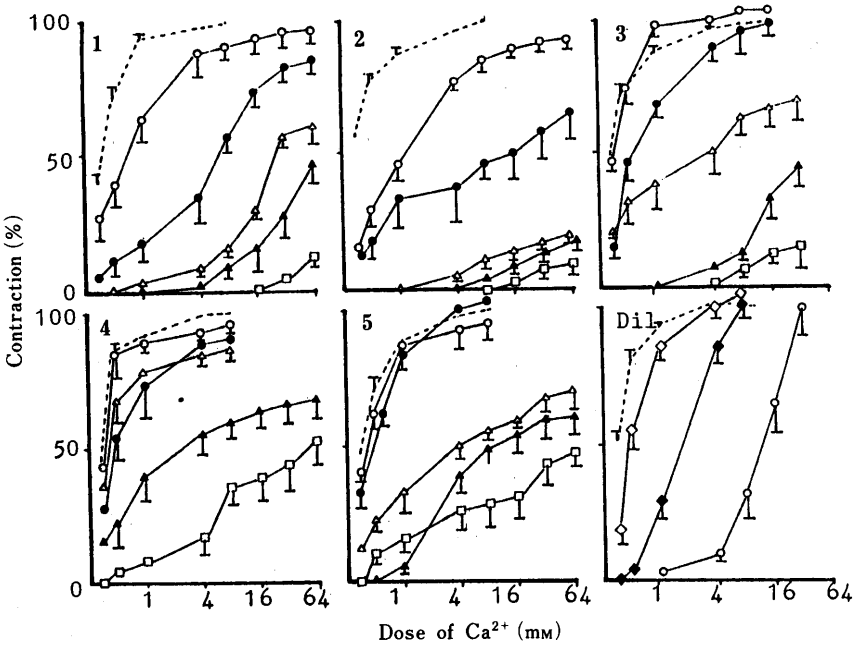


Fig. 2. Effects of **1**, **2**, **3**, **4**, **5** and Diltiazem (Dil.) on Ca^{2+}-Induced Contraction in the Isolated Guinea Pig Ileum

---, control; —◇—, 10^{-8}; —◆—, 10^{-7}; —○—, 10^{-6}; —●—, 3×10^{-6}; —△—, 10^{-5}; —▲—, 3×10^{-5}; —□—, 10^{-4} g/ml. Each point represents the mean ± S.E. of 5 to 6 experiments.

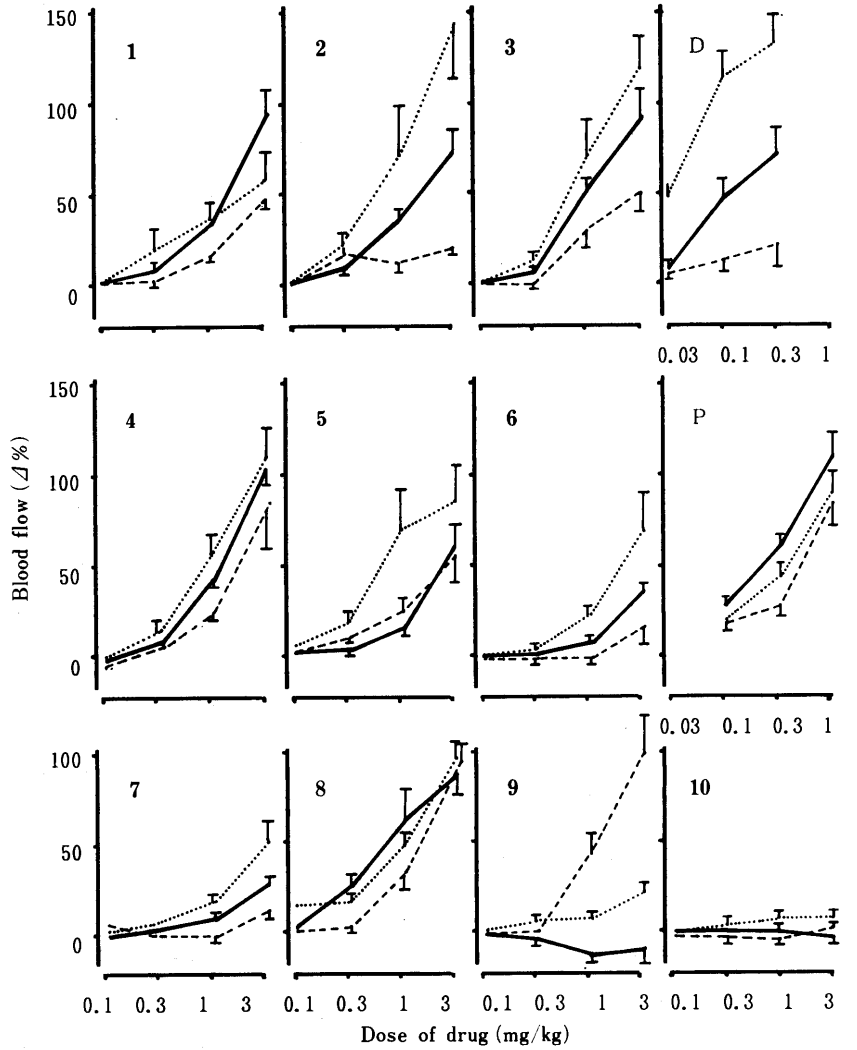


Fig. 3. Effects of Coumarins (**1**—**10**) and Other Drugs on Carotid, Vertebral and Femoral Arterial Blood Flows in Anesthetized Dogs

D, diltiazem; P, papaverine; ——, carotid; ------, vertebral; ——, femoral artery. Each point represents the mean ± S.E. of 5 to 7 experiments.

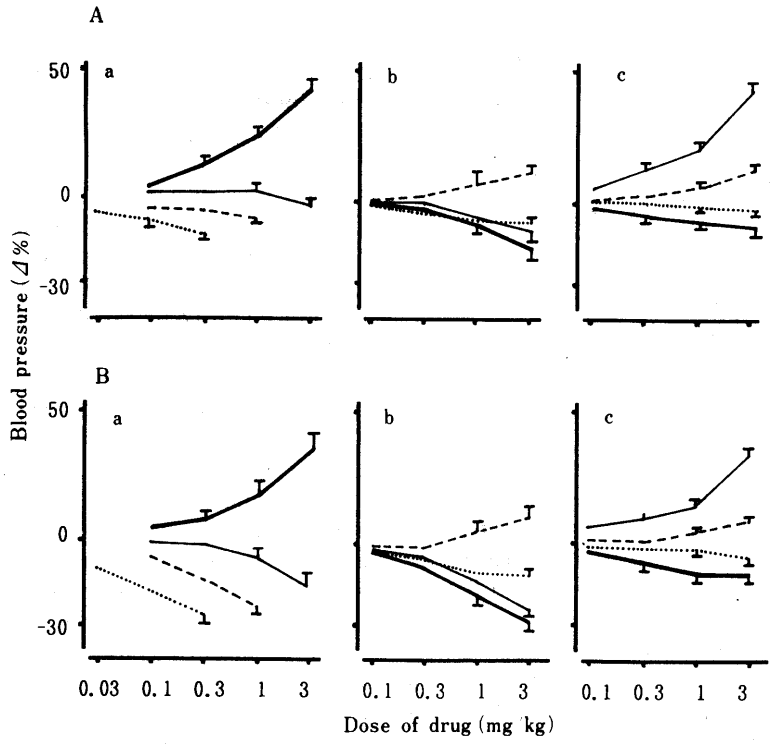


Fig. 4. Effects of Coumarins (**1—10**) and Other Drugs on Blood Pressure in Anesthetized Dogs

D, diltiazem; P, papaverine. Each point represents the mean ± S.E. of 5 to 7 experiments. A: Systolic blood pressure. a ——, **1**; ——, **2**; – – –, P; ------, D. b ——, **3**; ——, **4**; – – –, **5**; ------, **6**. c ——, **7**; ——, **8**; – – –, **9**; ------, **10**. B: Diastolic blood pressure.

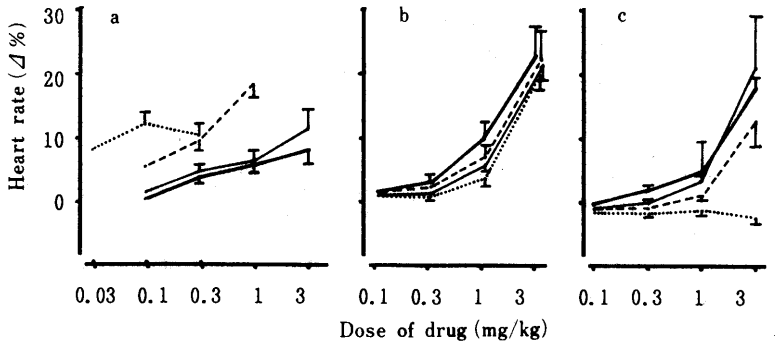


Fig. 5. Effects of Coumarins (**1—10**) and Other Drugs on Heart Rate in Anesthetized Dogs

D, diltiazem; P, papaverine; Each point represents the mean ± S.E. of 5 to 7 experiments. a ——, **1**; ——, **2**; – – –, P; ------, D. b ——, **3**; ——, **4**; – – –, **5**; ------, **6**. c ——, **7**; ——, **8**; – – –, **9**; ------, **10**.

Diltiazem, a Ca^{2+} blocker, caused a parallel shift of the dose–response curve.

(2) Effects of Coumarins on Cardiovascular System Since compounds **1—5** showed inhibitory effects on histamine- and Ca^{2+}-induced contractions, compounds **6—10** in addition to **1—5** were studied for their effects on blood flow, blood pressure, and heart rate. As values (mean ± S.E.) before treatment with the test compounds, blood flow was 34.4 ± 1.4 ml/min for vertebral artery, 140.0 ± 3.9 ml/min for carotid artery and 60.4 ± 2.2 ml/min for femoral artery, and systolic blood pressure was 193.0 ± 1.3 mmHg, diastolic blood pressure was 125.3 ± 1.2 mmHg and heart rate was 168.4 ± 1.7 beat/min. All the compounds except **10** produced dose-related effects on these cardiovascular parameters (Figs. 3—5). Compounds **1—9** increased blood flow. The percent increase in carotid and vertebral blood flow was larger than that in femoral blood flow at 1.0 mg/kg or more of **1**. Compounds **2** and **3** at 0.3 mg/kg or more and at 1.0 mg/kg or more, respectively, produced a marked increase in blood flow; vertebral blood flow showed the largest increase, followed in order by carotid and femoral blood flow; however, the data of vertebral blood flow for **2** showed no significant difference because of their large variances. Compounds **4** and **8** increased the three arterial blood flows almost to the same extent. For compound **5** the vertebral artery showed the most pronounced reaction, followed by the carotid and femoral arteries. Compounds **6** and **7** produced an effect similar to, but generally lower than, that of **3**. Compound **9** markedly increased femoral arterial blood flow. Compound **10** did not increase blood flow in any of the three arteries. Diltiazem at 0.03 mg/kg or more caused an increase in flood flow: vertebral blood flow showed the largest increase, followed in order by carotid and femoral blood flow. Papaverine at 0.1 mg/kg or more increased the three arterial blood flows almost to the same extent. Thus,

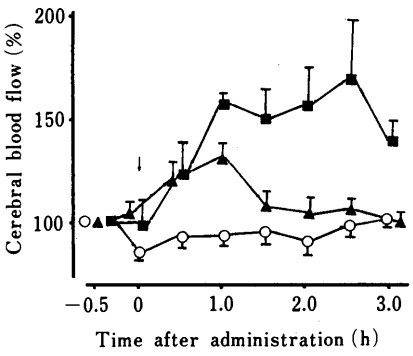


Fig. 6. Effects of 1 on Cerebral Blood Flows in Rabbits

—○—, control; —▲—, 30; —■—, 100 mg/kg. ↓, administration. Each point represents the mean ± S.E. of 5 to 6 experiments.

the potencies of the coumarins in increasing blood flow at the doses of 1.0 mg/kg or more were in the order of **2**≧**3**>**4**≧**8**>**1**>**5**>**6**>**7**>**9** for the vertebral artery, **1**=**3**=**4**=**8**>**2**>**5**>**6**>**7** for the carotid artery, and **9**≧**8**>**4**>**5**>**1**≧**3**>**2**>**6**>**7** for the femoral artery.

Compounds **1**, **5**, and **8** produced a dose-related increase in systolic and diastolic blood pressure. Compounds **2**, **3**, **4**, **6**, and **7** lowered blood pressure. Compounds **9** and **10** had no effect on blood pressure. These results are correlated with those of effects on smooth muscle contraction and blood pressure except for compound **1**. Compound **1** produced a marked increase in blood pressure although it inhibited smooth muscle contraction and increased blood flow fairly strongly. All the compounds except **10** produced an increase in heart rate. Diltiazem at 0.03 mg/kg and more and papaverine at 0.1 mg/kg and more produced a dose-related decrease in mean blood pressure and an increase in heart rate.

Since several coumarins showed inhibitory effects on contraction in guinea pig ileum and produced increases in some arterial blood flows in dogs as described above, the effect of coumarin **1**, the main compound, on cerebral blood flow was studied in rabbits. Cerebral blood flow was determined by the method of hydrogen clearance using an inserted platinum electrode in rabbits which received inhalation of hydrogen gas for 2 min under paralyzed conditions with *d*-tubocurarine.[9,10] Compound **1** suspended in 0.5% CMC was given intraduodenally and as values (mean ± S.E.) before treatment with the compounds, blood flow was 35.0 ± 5.8 ml/min. The compound at 30 mg/kg produced an increase in blood flow of the cerebral cortex after 30 min, with a peak increase appearing at 1 h after administration (Fig. 6). At 100 mg/kg, it produced a significant increase similar to that at 30 mg/kg, and the effect lasted until about 3 h after administration.

From the results of the present studies, we conclude that (1) the coumarins, particularly compounds **1**—**5**, showed noncompetitive antagonistic effects on histamine- and Ca^{2+}-induced contractions in smooth muscle and the potencies were in the order of **2**>**1**>**3**≧**4**=**5**, (2) compounds **2** and **3** caused a transient decrease in blood pressure and increases in blood flow in three arteries; their effects on vertebral and carotid arteries were more marked than that on the femoral artery, suggesting their actions to be similar to those of diltiazem,[11-14] a Ca^{2+} blocker, which strongly inhibits Ca^{2+}-induced contraction by the selective inhibition of calcium flux through smooth muscle membrane; and compounds **6** and **7**, synthesized on the basis of **3**, showed similar effects, (3) compound **4** increased the blood flow of the three arteries to almost the same extent, suggesting its action to be similar to that of papaverine,[15-17] which is said to inhibit non-selectively smooth muscle contraction through inhibitory effects on calcium flux and phosphodiesterase. Compound **8** receiving cleavage of the pyran ring of **4** showed a similar effect; and compound **9** in which a nitrogen atom was introduced into **8** markedly increased femoral arterial blood flow, (4) compound **1** increased blood flow of the cerebral cortex; this suggests the possibility for this compound to be used as a drug for improvement of cerebral circulation, and (5) thus, the action of compound **1** to blood pressure is different from that of **2** or **3**, though **1** has a Ca^{2+} blocking action as part of its pharmacological actions. Therefore, compound **1** is considered to be different from diltiazem in its mode of action. There is the possibility that compound **1** is mixed with another compound that is different from compound **2** at 3′ and 4′ in its chemical structure. In this study, diltiazem was used as a control Ca^{2+} blocking drug. For elucidation the mechanism of action of compound **1**, it is considered necessary to further study the comparison with dihydropyridine and verapamil Ca^{2+} blockers and to perform more detailed studies on the action to the cardiovascular system. The results of the pharmacological studies of coumarins **1**—**10** may account for the folk usage of *Peucedanum japonicum* THUNB.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded with a Hitachi 260-10 spectrometer, nuclear magnetic resonance (NMR) spectra with a Varian T-60 or a JEOL JNM-FX 100 spectrometer with tetramethylsilane as an internal standard and mass spectra (MS) with a JEOL JMS-D 300 spectrometer. Mallinckrodt silica gel (100 mesh) and Merck kieselgel G nach Stahl were used for column chromatography and thin layer chromatography (TLC), respectively.

3-Ethoxycarbonyl-5-hydroxy-4-methylcoumarin (11) Diethyl malonate (10.4 g) was added to a solution of 1.0 M tetrabutylammonium fluoride in tetrahydrofuran (THF; 19.5 ml), and the whole was concentrated under a vacuum at 40 °C. 2,6-Dihydroxyacetophenone (5 g) was added to a solution of the residue in dry toluene (150 ml), then the mixture was refluxed overnight and concentrated under a vacuum. The residue was poured into ice-water and extracted with chloroform. The organic layer was washed with dilute HCl and water, then dried and concentrated. The residue was subjected to silica gel chromatography. The chloroform eluate gave 6.7 g (82.1%) of **11** as colorless crystals (methanol), mp 191—193 °C. IR (KBr) cm^{-1}: 3310, 1730, 1685, 1600, 1500. NMR ($CDCl_3$) δ: 1.33 (3H, t, J=8 Hz, –Me), 2.60 (3H, s, –Me), 4.30 (2H, q, J=8 Hz, $-CH_2-$), 6.50—6.73 (2H, m, aromatic H), 7.06 (1H, t, J=8 Hz, aromatic H). MS m/z Calcd for $C_{13}H_{12}O_5$ (M^+): 248.0684. Found: 248.0684.

3-Carboxy-5-hydroxy-4-methylcoumarin (12) 10% KOH (104 ml) was added to a solution of **11** (3.47 g) in ethanol (104 ml), and the whole was refluxed for 3 h. The reaction mixture was poured into ice–water, then acidified with conc. HCl and extracted with ethyl acetate. The organic layer was washed with water, then dried and concentrated. The residue was recrystallized from methanol–benzene to yield 2.4 g (88.8%) of **12** as straw crystals, mp 229—230.5 °C. IR (KBr) cm^{-1}: 3300, 1700, 1650, 1600, 1500. NMR ($CDCl_3$ + DMSO-d_6) δ: 2.68 (3H, s, –Me), 5.43 (2H, br, –OH and $-CO_2H$), 6.58 (1H, dd, J=8, 2 Hz, aromatic H), 6.66 (1H, J=8, 2 Hz, aromatic H), 7.16 (1H, t, J=8 Hz, aromatic H).

5-Hydroxy-4-methylcoumarin (13) Compound **12** (4.25 g) was heated at 240 °C for 4 min and allowed to stand at room temperature. The resulted solid was recrystallized from ethyl acetate to give 3.09 g (90.7%) of **13** as light brown crystals, mp 266—268 °C. IR (KBr) cm^{-1}: 3150, 1680, 1610, 1500. NMR ($CDCl_3$–DMSO-d_6) δ: 2.56 (3H, d, J=1 Hz, –Me), 3.18 (1H, br, –OH), 5.86 (1H, q, J=1 Hz, olefinic H), 6.48—6.66 (2H, m, aromatic

H), 7.08 (1H, dd, $J=8, 7$ Hz). MS m/z Calcd for $C_{10}H_8O_3$ (M^+): 176.0474. Found: 176.0477.

4-Methyl-5-(2-methyl-3-butyn-2-yloxy)coumarin (14) Sodium hydride (55%, 2.05 g) was added to a solution of **13** (3.0 g) in DMF (103 ml) with stirring at room temperature under a nitrogen atmosphere, and the whole was stirred for awhile at room temperature. A solution of 3-chloro-3-methylbutyne[8] (4.1 g) in DMF (1 ml) was added and the mixture was stirred for 2 h under the same conditions. The reaction mixture was poured into ice-water, acidified with conc. HCl, and extracted with ethyl acetate. The organic layer was washed with water, then dried and concentrated. The residue was subjected to silica gel chromatography. The benzene eluate gave 1.47 g (35.6%) of **14** as yellow crystals (ether–hexane), mp 102—103 °C. IR (KBr) cm^{-1}: 3220, 1700, 1680, 1590. NMR ($CDCl_3$) δ: 1.73 (6H, s, –Me), 2.53 (3H, s, –Me), 2.62 (1H, s, acetylenic H), 5.96 (1H, m, olefinic H), 6.70—6.85 (1H, m, aromatic H), 7.18—7.30 (2H, m, aromatic H). MS m/z Calcd for $C_{15}H_{14}O_3$ (M^+): 242.0943. Found: 242.0945.

2,2,10-Trimethyl-2*H*,8*H*-benzo[1,2-*b*:3,4-*b'*]dipyran-8-one (5) A solution of **14** (1.0 g) in *N*,*N*-dimethylaniline (40 ml) was refluxed for 40 min. The reaction mixture was poured into ice-water, acidified with 10% HCl, and then extracted with ethyl acetate. The organic layer was washed with 10% HCl, 5% K_2CO_3, and brine, then dried and concentrated. The residue was recrystallized from ether–hexane to give 0.82 g (82.0%) of **5** as yellow crystals, mp 115—117 °C. IR (Nujol) cm^{-1}: 1723, 1685, 1645, 1620, 1590, 1578. NMR ($CDCl_3$) δ: 1.47 (6H, s, –Me), 2.53 (3H, d, $J=1$ Hz, –Me), 5.45 (1H, d, $J=10$ Hz, olefinic H), 5.93 (1H, q, $J=1$ Hz, olefinic H), 6.16 (1H, $J=10$ Hz, olefinic H), 6.63 (1H, d, $J=8$ Hz, aromatic H), 6.94 (1H, d, $J=8$ Hz, aromatic H). MS m/z Calcd for $C_{15}H_{14}O_3$ (M^+): 242.0943. Found: 242.0954.

4-Methyl-7-(3-methyl-2-butenyloxy)coumarin (8) 4-Bromo-2-methyl-2-butene (1 ml) and anhydrous K_2CO_3 (400 mg) were added to a solution of 7-hydroxy-4-methylcoumarin (100 mg) in DMF (3 ml), and the whole was stirred at room temperature. The reaction mixture was poured into ice-water and extracted with ether. The organic layer was washed with water, the dried and concentrated. The residue was subjected to silica gel chromatography. The chloroform eluate gave 81.1 mg (61.0%) of **8** as light yellow prisms (ether–hexane), mp 84—86 °C. IR (Nujol) cm^{-1}: 1718, 1675, 1606, 1550, 1500. NMR ($CDCl_3$) δ: 1.78 (6H, s, –Me), 2.40 (3H, s, –Me), 4.58 (2H, d, $J=8$ Hz, $-CH_2-$), 5.48 (1H, t, $J=8$ Hz, olefinic H), 6.16 (1H, s, olefinic H), 6.77—6.96 (2H, m, aromatic H), 7.50 (1H, d, $J=9$ Hz, aromatic H). MS m/z Calcd for $C_{15}H_{16}O_3$ (M^+): 244.1100. Found: 244.1105.

7-(2-Dimethylaminoethoxy)-4-methylcoumarin Hydrochloride (9) 2-Dimethylaminoethylchloride hydrochloride (288 mg) and anhydrous K_2CO_3 (414 mg) were added to a solution of 7-hydroxy-4-methylcoumarin (178 mg) in DMF (3 ml) and the whole was stirred overnight at room temperature. The reaction mixture was poured into ice-water and extracted with ethyl acetate. The organic layer was washed with water, then dried and concentrated. The residue was recrystallized from ether–hexane to give 100 mg of 2-dimethylaminoethoxy-4-methylcoumarin as colorless prisms, mp 65—67 °C. IR (Nujol) cm^{-1}: 1725, 1615, 1605, 1560, 1510. NMR ($CDCl_3$) δ: 2.36 (9H, s, –Me), 2.77 (2H, t, $J=10$ Hz, $-CH_2-$), 4.14 (2H, t, $J=10$ Hz, $-CH_2-$), 6.12 (1H, s, olefinic H), 6.82—6.99 (2H, m, aromatic H), 7.42 (1H, d, $J=13$ Hz, aromatic H). MS m/z Calcd for $C_{14}H_{17}NO_3$ (M^+): 247.1209. Found: 247.1213. To a solution of 2-methylaminoethoxy-4-methylcoumarin (100 mg), conc. HCl (3 gtt) was added and the whole was stirred at room temperature for 1 h. The reaction mixture was concentrated under a vacuum. The residue was recrystallized from ether to give 120 mg (41.9%) of **9** as colorless prisms, mp 224—225 °C.

7-Geranyloxy-4-methylcoumarin (10) Geranyl chloride (5.16 g) and anhydrous K_2CO_3 (5 g) were added to a solution of 7-hydroxy-4-methylcoumarin (1.1 g) and the whole was stirred overnight at room temperature. The reaction mixture was poured into ice-water and extracted with ethyl acetate. The organic layer was washed with water, then dried and concentrated. The residue was subjected to silica gel chromatography to yield 5.6 g (89.0%) of **10** from benzene eluate as a colorless oil. IR (Nujol) cm^{-1}: 1715, 1605, 1565, 1505. NMR ($CDCl_3$) δ: 1.62 (3H, s, –Me), 1.68 (3H, s, –Me), 1.78 (3H, s, –Me), 2.14 (4H, m, $-CH_2CH_2-$), 2.41 (3H, s, –Me), 4.61 (2H, d, $J=11$ Hz, $-CH_2-$), 5.05 (1H, m, olefinic H), 5.45 (1H, m, olefinic H), 6.13 (1H, s, olefinic H), 6.81—6.96 (2H, m, aromatic H), 7.51 (1H, d, $J=13$ Hz, aromatic H). MS m/z Calcd for $C_{20}H_{24}O_3$ (M^+): 312.1815. Found: 312.1825.

References

1) A part of this work was presented on Proceeding of the 5th Symposium on the Development and Application of Naturally Occuring Drug Materials, Hiroshima, 1985; N. Takeuchi, I. Maruyama, J. Ohki, S. Tobinaga, T. Kasama, Y. Aida, K. Watanabe, M. Koizumi, and K. Mayuzumi, *J. Pharmacobio-Dyn.*, **8**, s-53 (1985).
2) T. Amano, "Ryukyuretto Syokubutu Hougensyu (The Botanical Dialects in the Ryukyus)," Shinseitosho Shuppan, Naha, 1987.
3) J. Ueseto "Taketomizimashi (The Documents of Taketomi Island)," Houseidaigaku Shuppankyoku, Tokyo, 1987.
4) K. Hata, M. Kozawa, Y. Ikeshiro, and K. Y. Yen, *Yakugaku Zasshi*, **88**, 513 (1968).
5) Z. X. Chen, B. S. Huang, Q. L. Shi, and G. F. Zeng, *Acta Pharm. Sin.*, **14**, 486 (1979); T. Okuyama and S. Shibata, *Planta Medica*, **42**, 89 (1981); T. Kozawa, K. Sakai, M. Uchida, T. Okuyama, and S. Shibata, *J. Pharm. Pharmacol.*, **33**, 317 (1981); T. Suzuki, Y. Kobayashi, M. Uchida, I. Sakakibara, T. Okuyama, and S. Shibata, *J. Pharmacobio-Dyn.*, **8**, 257 (1985).
6) E. Spath and R. Hillel, *Chem. Ber.*, **72**, 2093 (1939); J. Hlubucek, E. Ritchie, and W. C. Taylor, *Aust. J. Chem.*, **24**, 2347 (1971).
7) F. Bohlmann and H. Franke, *Chem. Ber.*, **104**, 3229 (1971).
8) G. F. Hennion and A. P. Boisselle, *J. Org. Chem.*, **26**, 725 (1961).
9) Y. Shinohara, J. S. Meyer, A. Kitahara, M. Toyoda, and T. Ryu, *Circulation Research*, **25**, 735 (1969).
10) J. H. Halsey, N. F. Capra, and R. S. McFarland, *Stroke*, **8**, 351 (1977).
11) T. Nagao, M. Sato, H. Nakajima, and A. Kiyomoto, *Jpn. J. Pharmacol.*, **22**, 1 (2972).
12) A. Fleckenstein, *Ann. Rev. Pharmacol. Toxycol.*, **17**, 149 (1977).
13) Y. Ito, H. Kuriyama, and H. Suzuki, *Br. J. Pharmacol.*, **64**, 503 (1978).
14) R. A. Craven, S. F. Flaim, and R. Zelis, *Clin. Res.*, **27**, 230 (1979).
15) G. Poch and W. R. Kukovetz, *Life Sci.*, **10**, 133 (1971).
16) I. Takayanagi, M. Uchida, N. Inatomi, A. Tomiyama, and K. Takagi, *Jpn. J. Pharmacol.*, **22**, 869 (1972).
17) R. Anderson, *Acta Physiol. Scand.*, **87**, 348 (1973).

2-Oxo-1,3-dioxoles as Specific Substrates for Measurement of Arylesterase Activity

Hiroshi KAWAI,* Fumio SAKAMOTO, Masahiro TAGUCHI, Mikiya KITAMURA, Mikio SOTOMURA and Goro TSUKAMOTO

Pharmaceuticals Research Center, Kanebo Co., Ltd., Tomobuchicho 1-5-90, Miyakojimaku, Osaka 534, Japan. *Received October 29, 1990*

Various 4-arylthiomethyl-2-oxo-1,3-dioxole derivatives IIIa—o were synthesized. Their hydrolysis rates by arylesterase (EC 3.1.1.2) and cholinesterase (EC 3.1.1.8) in human serum were evaluated. Some of them were not hydrolyzed by cholinesterase, but were hydrolyzed easily by arylesterase.

Among the substrates, sodium 4-((5-methyl-2-oxo-1,3-dioxol-4-yl)methylthio)benzenesulfonate (IIIg) was selected for its substrate reactivity toward arylesterase and its good water solubility. In addition, neither aliesterase (EC 3.1.1.1), acetylesterase (EC 3.1.1.6) nor cholesterol esterase (EC 3.1.1.13) hydrolyzed the compound. IIIg is thus concluded to be a specific substrate for arylesterase.

Our assay system for serum arylesterase using IIIg can be readily applied to an automatic analyzer in the diagnosis of liver cirrhosis.

Keywords human serum; arylesterase activity; specific substrate; cirrhosis diagnosis; 4-arylthiomethyl-2-oxo-1,3-dioxole

Augustinsson *et al.* studied the types of esterase present in vertebrate plasma, and found three to be present: aliesterase (carboxylic-ester hydrolase, EC 3.1.1.1, AliE), arylesterase (aryl-ester hydrolase, EC 3.1.1.2, ArE), and cholinesterase (acyl-choline acyl-hydrolase, EC 3.1.1.8, ChE). ArE and ChE are present in mammalian plasma.[1] Serum ArE activity, as determined by phenyl acetate[2,3] or β-naphthyl acetate[4] as a substrate, has been reported to decrease in patients with liver disease, particularly cirrhosis. However, these substrates for the assay of ArE activity are hydrolyzed by ChE as well as ArE.[5,6] Their lack of specificity makes necessary the separation of ArE from ChE by electrophoresis,[7] chromatography or other troublesome methods prior to accurate measurement of ArE activity.

In the studies of enzymatic hydrolysis of a series of ampicillin prodrugs, it was suggested that lenampicillin, (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl ester of ampicillin,[8] is hydrolyzed to ampicillin by ArE, and ArE recognizes the (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl group as a substrate.

5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB: Ellman's reagent) reacts rapidly with a thiol and the resulting product (5-thio-2-nitrobenzoic acid, TNB) is yellow in color (Fig. 1).[9] If a thiol is released following the hydrolysis of an ArE-specific substrate, it should be possible to readily assay ArE activity by colorimetric determination using an automatic analyzer in the diagnosis of cirrhosis. Therefore, we combined a benzenethiol with the (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl group, regarded as the recognition domain of ArE.

The present study is conducted to examine the synthesis, properties, structures and reactivity toward the ArE relationship of several (2-oxo-1,3-dioxol-4-yl)methyl sulfide derivatives, and to choose substrate for the determination of serum ArE activity.

Chemistry 4-Halomethyl-2-oxo-1,3-dioxole derivatives[8] I were allowed to react with thiols II in the presence of triethylamine in dichloromethane at room temperature to afford sulfides III (Fig. 1). Sulfides IIIa—f and IIIj—o were isolated upon silica-gel column chromatography. Sulfides IIIg and IIIi were obtained by salting-out techniques with NaCl. Sulfide IIIh was obtained upon ion-exchange column chromatography using a Na-form resin.

Enzymatic Hydrolysis As human plasma contains ArE and ChE, the hydrolysis rates of sulfides IIIa—o by these were measured (Table I). When the 5-substituent (R) was fixed on a methyl group, the hydrolysis rates of substrate III by ArE varied with the phenyl substituent (X), being fast when X was an electron withdrawing group. The relationship between the rate by ArE (R_{ArE}) and Hammett's σ-value of X (m- and p-position) is as follows:

$$\log R_{ArE} = 0.255\sigma - 0.491 \quad (n=8,\ r=0.752).$$

Substrates IIIa—o were scarcely hydrolyzed by ChE.

Among the substrates examined for enzymatic hydrolysis (Table I), sodium 4-((5-methyl-2-oxo-1,3-dioxol-4-yl)methylthio)benzenesulfonate (IIIg) was further studied for clinical application described below, because it was easy to handle owing to its high solubility in water (about 110 mg/ml). Other compounds except for IIIh and IIIi were

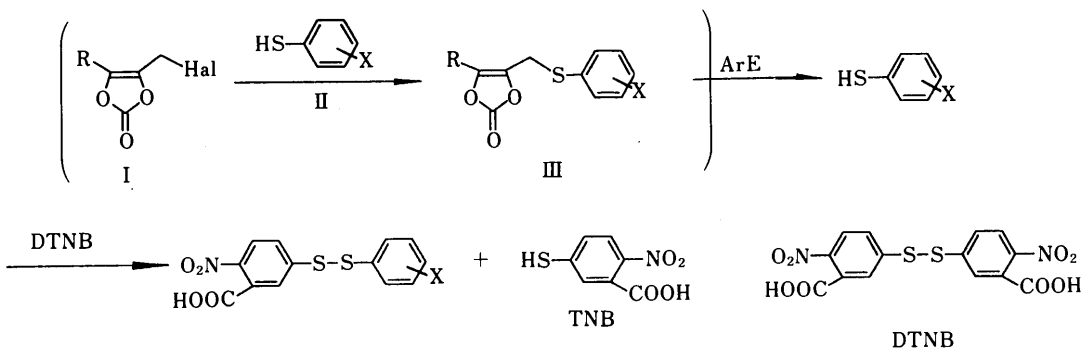


Fig. 1. The Synthetic Method and Principle of the Assay System

TABLE I. Hydrolysis Rates of Compound IIIa—o by ArE and ChE

Compound No.	R	X	Formula	Analysis (%) Calcd (Found) C	H	N	σ-Value of X	Hydrolysis rate[a] ArE	ChE
IIIa	CH_3	H	$C_{11}H_{10}O_3S$	59.44 (59.31	4.54 4.47	)	0.00	0.385	$<10^{-3}$
IIIb	CH_3	*p*-F	$C_{11}H_9FO_3S$	54.99 (54.78	3.78 3.79	)	0.06	0.387	$<10^{-3}$
IIIc	CH_3	*p*-Cl	$C_{11}H_9ClO_3S$	51.47 (51.57	3.53 3.44	)	0.23	0.423	$<10^{-3}$
IIId	CH_3	*p*-NO_2	$C_{11}H_9NO_5S$	49.44 (49.31	3.39 3.40	5.24 5.25)	0.78	0.437	$<10^{-3}$
IIIe	CH_3	*p*-CH_3	$C_{12}H_{12}O_3S$	61.00 (60.93	5.12 5.00	)	−0.17	0.223	$<10^{-3}$
IIIf	CH_3	*p*-OCH_3	$C_{12}H_{12}O_4S$	57.13 (57.01	4.80 4.84	)	−0.27	0.247	$<10^{-3}$
IIIg	CH_3	*p*-SO_3Na	$C_{11}H_9NaO_6S_2 \cdot 1/2H_2O$ (39.64	3.02 2.96	)	0.09	0.368	$<10^{-3}$
IIIh	CH_3	*m*-SO_3Na	$C_{11}H_9NaO_6S_2 \cdot 1/2H_2O$ (39.91	3.02 2.98	)	0.05	0.335	$<10^{-3}$
IIIi	CH_3	*o*-SO_3Na	$C_{11}H_9NaO_6S_2 \cdot 1/2H_2O$ (39.69	3.02 3.09	)		0.115	$<10^{-3}$
IIIj	CH_3	*p*-Cl, *m*-CO_2H	$C_{12}H_9ClO_5S$ (48.09	3.02 2.84	)		0.111	$<10^{-3}$
IIIk	H	H	$C_{10}H_8O_3S$ (57.54	3.87 3.82	)		0.280	$<10^{-3}$
IIIl	H	*p*-F	$C_{10}H_7FO_3S$ (52.96	3.12 2.96	)		0.258	$<10^{-3}$
IIIm	H	*p*-NO_2	$C_{10}H_7NO_5S$ (47.26	2.79 2.82	5.53 5.57)		0.349	$<10^{-3}$
IIIn	C_6H_5	H	$C_{16}H_{12}O_3S$ (67.79	4.25 4.17	)		0.024	$<10^{-3}$
IIIo	C_6H_5	*p*-NO_2	$C_{16}H_{11}NO_5S$ (58.36	3.37 3.17	4.25 4.38)		0.012	$<10^{-3}$

a) Hydrolysis rate of each compound was expressed as μmol of compoud hydrolyzed/min per U esterase preparation.[6]

insoluble in water.

Substrate Reactivity The substrate reactivity of compound IIIg for several esterases was compared with that of reported substrates, phenyl acetate and β-naphthyl acetate (Table II). Of the 3 substrates tested for enzymatic hydrolysis, IIIg was not hydrolyzed by ChE, but was easily by ArE. In addition, it was not hydrolyzed by AliE, acetylesterase (EC 3.1.1.6, AcE) or cholesterol esterase (EC 3.1.1.13, CE). IIIg was thus concluded to be a specific substrate for ArE. Its hydrolysis by ArE may possibly be correlated with the fact that aliphatic esters such as isopropenyl acetate and vinyl acetate are hydrolyzed by ArE.[10] Indeed, these esters have a double bond in the alcohol moiety nearest to the ester linkage, as shown in Fig. 2, which is essential for ArE hydrolysis.

Phenyl acetate[2,3] and β-naphthyl acetate[4] were hydrolyzed by ChE, AliE, AcE, and CE, as well as by ArE.

TABLE II. Substrate Reactivity for Several Types of Esterase

Substrate	Conc. (mM)	Hydrolysis rate (μmol/min per U) AliE	ArE	AcE	ChE	CE
Compd. IIIg	3.2	$<10^{-3}$	0.368	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
Phenyl acetate	4.0	0.098	1.000	0.001	0.028	0.031
β-Naphthyl acetate	0.49	2.66	0.190	0.354	0.049	1.94

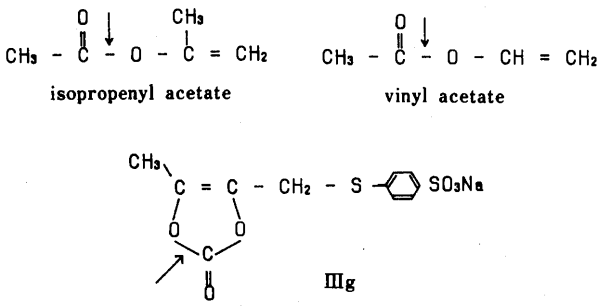


Fig. 2. Aliphatic Esters Hydrolyzed by ArE
Arrows show positions of the ester linkage

Conclusions

For the diagnosis of acute hepatitis, there are excellent biochemical parameters such as glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT), which can be easily measured and are widely used. However, only a few reliable biochemical parameters which indicate liver cirrhosis are presently available. The activity of serum ArE has been shown to decrease in patients with certain liver diseases, particularly cirrhosis.[2-4] In the determination of ArE activity, the main problems related to the substrate are: (a) low specificity to ArE[5] and (b) poor solubility in water.

To solve these problems, IIIg was selected as a substrate for the determination of ArE activity. It was found possible to assay ArE activity without disturbing other esterases by using the new specific substrate for ArE. The assay is easy

to conduct using an automatic analyzer.[11] Activity can thus be easily measured, as can other biochemical parameters.

In a preliminary clinical study,[11] the activity of ArE, as calculated by this assay, markedly decreased in patients with cirrhosis. Particularly, the activities of severely decompensated cirrhosis patients were lower than those of compensated ones. Therefore, further clinical studies on the positive correlation between serum ArE activity and the severity of chronic liver disease are now being conducted.

Experimental

Enzymatic Hydrolysis ArE was purified from human serum by precipitation with ammonium sulfate and chromatography on Affi-Gel Blue (Bio-Rad Laboratories, U.S.A.) and on DEAE-Toyopearl (Tosoh, Japan) at pH 7.4.[6] The purified preparation had a specific activity of 34.6 μmol phenyl acetate hydrolyzed/min per mg protein by the method of Junge and Klees,[12] and was confirmed to be free from ChE by electrophoresis. ChE, AliE and CE were obtained from Boehringer Mannheim GmbH (FRG). AcE was obtained from Sigma chemical company (U.S.A.). The hydrolysis rates of sulfides IIIa—o were assayed using DTNB as the coloring reagent by modification[6,11,13] of Ellman's method[9] according to the principle shown in Fig. 1. The summary of the procedure was as follows: 100 μl of 10 U/ml esterase preparation was added to 100 μl of the color reagent (3.2 mM DTNB solution), and 800 μl of substrate solution (0.4 mM, pH 6.5) was added and reacted at 25 °C. An increase in absorbance at 412 nm/min was measured, and the hydrolysis rate (μmol/min per U) was calculated. The methods used for other substrates were; phenyl acetate using the method of Junge and Klees,[12] and β-naphthyl acetate according to Burlina and Galzigna.[14]

Chemistry Melting points were determined on a Yamato capillary melting point apparatus, Model MP-21, and were uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were determined at 60 MHz on a Hitachi R-24B spectrometer using tetramethylsilane as an internal reference. Infrared (IR) spectra were recorded with a Hitachi IR 270-50 infrared spectrophotometer. Compounds IIIa—o were analyzed for C, H, and N, and the analytical values were within $\pm$0.4% of the theoretical values.

5-Methyl-4-phenylthiomethyl-1,3-dioxol-2-one (IIIa) Benzenethiol (1.5 g, 13.6 mmol) was added to a solution of 4-chloromethyl-5-methyl-1,3-dioxol-2-one (2.0 g, 13.5 mmol) in 40 ml of dichloromethane. Then, triethylamine (1.4 g, 13.8 mmol) was added slowly, and the mixture was stirred at room temperature for 30 min. The reaction mixture was evaporated *in vacuo*, and the residue was dissolved in ethyl acetate. The insoluble materials were filtered off, and the filtrate was evaporated *in vacuo*. The residue was chromatographed on a column of Silica gel 60 (230—400 mesh, E. Merck) with chloroform-*n*-hexane (1 : 2) as an eluent under medium pressure. The desired fraction was evaporated *in vacuo*, and the residue was subjected to bulb-to-bulb distillation (about 180 °C/0.1 mmHg) to give IIIa (2.3 g, 76%) as a colorless liquid, bp 185 °C (0.1 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 1.7 (s, 3H, CH_3), 3.7 (s, 2H, CH_2), 7.2—7.7 (m, 5H, aromatic protons). IR ($CDCl_3$): 1819 (C=O), 1733 (C=C) cm^{-1}.

4-(4-Fluorophenyl)thiomethyl-5-methyl-1,3-dioxol-2-one (IIIb) By a procedure similar to that described for IIIa, 4-fluorobenzenethiol (1.8 g, 14.0 mmol) and 4-chloromethyl-5-methyl-1,3-dioxol-2-one (2.0 g, 13.5 mmol) gave IIIb (1.8 g, 55%) as pale yellow crystals after recrystallization from a mixture of ethyl acetate and *n*-hexane, mp 60—62 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.7 (s, 3H, CH_3), 3.7 (s, 2H, CH_2), 6.9—7.7 (m, 4H, aromatic protons). IR (KBr): 1811 (C=O), 1734 (C=C) cm^{-1}.

4-(4-Chlorophenyl)thiomethyl-5-methyl-1,3-dioxol-2-one (IIIc) By a procedure similar to that described for IIIa, 4-chlorobenzenethiol (2.0 g, 13.8 mmol) and 4-chloromethyl-5-methyl-1,3-dioxol-2-one (2.0 g, 13.5 mmol) gave IIIc (2.9 g, 83%) as colorless crystals after recrystallization from a mixture of ethyl acetate and *n*-hexane, mp 54—57 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.8 (s, 3H, CH_3), 3.7 (s, 2H, CH_2), 7.2—7.4 (m, 4H, aromatic protons). IR (KBr): 1814, 1804 (C=O), 1730 (C=C) cm^{-1}.

5-Methyl-4-(4-nitrophenyl)thiomethyl-1,3-dioxol-2-one (IIId) By a procedure similar to that described for IIIa, 4-nitrobenzenethiol (2.1 g, 13.5 mmol) and 4-chloromethyl-5-methyl-1,3-dioxol-2-one (2.0 g, 13.5 mmol) gave IIId (2.4 g, 66%) as yellow crystals after recrystallization from ethyl acetate, mp 80—83 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.1 (s, 3H, CH_3), 4.0 (s, 2H, CH_2), 7.3—7.6 (m, 2H, aromatic protons), 8.0—8.6 (m, 2H, aromatic protons). IR (KBr): 1846, 1818 (C=O), 1733 (C=C) cm^{-1}.

5-Methyl-4-(4-methylphenyl)thiomethyl-1,3-dioxol-2-one (IIIe) By a procedure similar to that described for IIIa, 4-methylbenzenethiol (1.7 g, 13.7 mmol) and 4-chloromethyl-5-methyl-1,3-dioxol-2-one (2.0 g, 13.5 mmol) gave IIIe (2.2 g, 69%) as yellow crystals after recrystallization from a mixture of ethyl acetate and *n*-hexane, mp 57—59 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.7 (s, 3H, CH_3), 2.3 (s, 3H, $PhCH_3$), 3.6 (s, 2H, CH_2), 7.0—7.5 (m, 4H, aromatic protons). IR (KBr): 1808 (C=O), 1734 (C=C) cm^{-1}.

4-(4-Methoxyphenyl)thiomethyl-5-methyl-1,3-dioxol-2-one (IIIf) By a procedure similar to that described for IIIa, 4-methoxybenzenethiol (1.9 g, 13.6 mmol) and 4-chloromethyl-5-methyl-1,3-dioxol-2-one (2.0 g, 13.5 mmol) gave IIIf (2.3 g, 67%) as pale yellow crystals after recrystallization from a mixture of ethyl acetate and *n*-hexane, mp 59—61 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.6 (s, 3H, CH_3), 3.6 (s, 2H, CH_2), 3.8 (s, 3H, OCH_3), 6.7—7.0 (m, 2H, aromatic protons), 7.2—7.5 (m, 2H, aromatic protons). IR ($CDCl_3$): 1819 (C=O), 1733 (C=C) cm^{-1}.

Sodium 4-((5-Methyl-2-oxo-1,3-dioxol-4-yl)methylthio)benzenesulfonate (IIIg) To a solution of a mixture of 4-chloromethyl-5-methyl-1,3-dioxol-2-one (0.4 g, 2.7 mmol) and 4-mercaptobenzenesulfonic acid (0.5 g, 2.6 mmol) in 20 ml of dichloromethane was slowly added triethylamine (0.5 g, 4.9 mmol). After being stirred at room temperature for 3 h, the reaction mixture was concentrated *in vacuo*, and the residue was dissolved in water (50 ml) and washed with ethyl acetate. The aqueous layer was chromatographed on an ion-exchange column (20 ml of Dowex 50W-X2, 100—200 mesh, H-form) with water as an eluent. The acidic fraction was collected and concentrated *in vacuo* to a volume of about 15 ml, then saturated with NaCl. The resulting precipitate was collected and recrystallized from ethanol to give IIIg (0.51 g, 58%) as colorless crystals, mp 260 °C (dec.). ^{1}H-NMR (DMSO-d_6) δ: 1.9 (s, 3H, CH_3), 4.1 (s, 2H, CH_2), 7.2—7.7 (m, 4H, aromatic protons). IR (KBr): 1815 (C=O), 1732 (C=C) cm^{-1}.

4-Mercaptobenzenesulfonic acid was prepared by modification of the reported method[15] as follows. 4-Aminobenzenesulfonic acid (86.6 g, 0.5 mol) was dissolved in a solution of sodium hydroxide (20 g, 0.5 mol) in 500 ml of water, then 100 ml of conc. hydrochloric acid was added. To the resulting suspension was gradually added a solution of sodium nitrite (34.5 g, 0.5 mol) in 100 ml of water at 1—3 °C, and the resulting mixture was poured into a solution of sodium disulfide, prepared by co-melting sulfur (16 g, 0.5 mol) and sodium sulfide nonahydrate (120.1 g, 0.5 mol) at 65 °C, in 500 ml of water. After being warmed until the evolution of gas ceased, the mixture was returned to room temperature, and the insoluble materials were filtered off. A solution of aniline (93.4 g) and conc. hydrochloric acid (84 ml) in 200 ml of water was added to the filtrate. The resulting precipitate was collected by filtration, recrystallized from water, and washed with ethanol and *n*-hexane to give dianilinium 4,4'-dithiobis(benzenesulfonate) (58.7 g, 41%) as colorless crystals. Then, this salt (6.0 g, 10.6 mmol) was subjected to an ion-exchange column chromatography (28 ml of Dowex 50W-X2, 100—200 mesh, H-form) with methanol–water (2 : 1) as an eluent. An acidic fraction was collected and evaporated *in vacuo*. The residue was dissolved in methanol (100 ml) and to the resulting solution were added triphenylphosphine (6.1 g, 23.3 mmol) and about 1 ml of water. After being stirred overnight at room temperature, the mixture was evaporated *in vacuo* and the residue was dissolved in 60 ml of dichloromethane, then the products were extracted with water. The extract was washed with dichloromethane and evaporated *in vacuo*. The residue was dissolved in dioxane, and insoluble materials were filtered off. The filtrate was evaporated *in vacuo* and the resulting syrup was crystallized from benzene to give 4-mercaptobenzenesulfonic acid (1.6 g, 39% yield from dianilinium salt) as pale yellow crystals (mp 99—101 °C).

Sodium 3-((5-Methyl-2-oxo-1,3-dioxol-4-yl)methylthio)benzenesulfonate (IIIh) By a procedure similar to that described for 4-mercaptobenzenesulfonic acid, 3-aminobenzenesulfonic acid (17.3 g, 0.1 mol) gave dianilinium 3,3'-dithiobis(benzenesulfonate) (9.8 g, 34%) as colorless crystals, and from this salt (5.0 g, 8.9 mmol) 3-mercaptobenzenesulfonic acid was obtained as a pale yellowish-brown syrup (2.4 g, 70%) and was subjected to the following reaction. To a solution of 3-mercaptobenzenesulfonic acid (2.2 g, 11.6 mmol) and triethylamine (2.4 g, 23.7 mmol) in dichloromethane (20 ml) was added a solution of 4-chloromethyl-5-methyl-1,3-dioxol-2-one (1.9 g, 12.8 mmol) in 4 ml of dichloromethane. After being stirred at room temperature for 2.5 h, the reaction mixture was evaporated *in vacuo*, the residue was dissolved in water (60 ml), and the solution was washed with ethyl acetate. The aqueous layer was chromatographed on an ion-exchange column (30 ml of Dowex 50W-X2, 100—200 mesh, Na-form) with water as an eluent. The desired fraction (about 140 ml) was collected, and thallium acetate (20 g) was added to this fraction. After being allowed to stand overnight at 4 °C, the insoluble materials were

filtered off, and the filtrate was concentrated to a volume of about 14 ml. The precipitated solid was collected by filtration, dissolved in water (120 ml), and concentrated *in vacuo* to afford crystals. This operation was repeated once more to give 2 g of colorless crystals, and they were chromatographed on an ion-exchange column (8 ml of Dowex 50W-X2, 100—200 mesh, Na-form) with water as an eluent. The desired fraction was evaporated *in vacuo*, and the residue was recrystallized from ethanol to give IIIh (1.1 g, 28%) as colorless crystals, mp 190 °C (dec.). ^{1}H-NMR (DMSO-d_6) δ: 1.9 (s, 3H, CH_3), 4.1 (s, 2H, CH_2), 7.2—7.7 (m, 4H, aromatic protons). IR (KBr): 1811, 1795 (C=O), 1736 (C=C) cm^{-1}.

Sodium 2-((5-Methyl-2-oxo-1,3-dioxol-4-yl)methylthio)benzenesulfonate (IIIi) To a solution of 4-chloromethyl-5-methyl-1,3-dioxol-2-one (0.36 g, 2.4 mmol) and triethylammonium 2-mercaptobenzenesulfonate (0.63 g, 2.2 mmol) in 10 ml of dichloromethane was added triethylamine (0.22 g, 2.2 mmol). After being stirred at room temperature for 2 h, the reaction mixture was worked up by a procedure similar to that described for IIIg to give IIIi (0.36 g, 50%) as colorless crystals, mp 110 °C (dec.). ^{1}H-NMR (DMSO-d_6) δ: 1.8 (s, 3H, CH_3), 4.1 (s, 2H, CH_2), 7.1—8.0 (m, 4H, aromatic protons). IR (KBr): 1826 (C=O), 1730 (C=C) cm^{-1}.

Triethylammonium 2-mercaptobenzenesulfonate was prepared by modification of the reported method[15] as follows. 2-Aminobenzenesulfonic acid (17.3 g, 0.1 mol) was dissolved in a solution of sodium hydroxide (4.0 g, 0.1 mol) in 100 ml of water, then 20 ml of conc. hydrochloric acid was added. To the resulting suspension was gradually added a solution of sodium nitrite (6.9 g, 0.1 mol) in 20 ml of water at 1—3 °C, and the resulting mixture was poured into a solution of sodium disulfide, prepared by co-melting sulfur (3.2 g, 0.1 mol) and sodium sulfide nonahydrate (24.0 g, 0.1 mol) at 65 °C, in 100 ml of water. After being warmed until the evolution of gas ceased, the mixture was returned to room temperature and the insoluble materials were filtered off. The filtrate was evaporated *in vacuo*, and the residue was dissolved in 200 ml of methanol. The insoluble materials were filtered off and the filtrate was evaporated *in vacuo*. The residue was dissolved in 120 ml of water, and 30 ml of acetic acid and 20 g of zinc dust were added. After being warmed for 40 min, the insoluble materials were filtered off and the products were extracted with aniline–benzene (1:1). The extract was evaporated *in vacuo* and the resulting residue was stirred in 150 ml of benzene to give powder. The powder was recrystallized from methanol to give anilinium 2-mercaptobenzenesulfonate (5.39 g, 19%) as colorless crystals. Then, this salt (2.0 g, 7.1 mmol) was subjected to ion-exchange column chromatography (30 ml of Dowex 50W-X2, 100—200 mesh, H-form) with methanol–water (4:1) as an eluent. An acidic fraction was collected and evaporated *in vacuo*. The residue was dissolved in 50 ml of isopropanol and evaporated *in vacuo* to give 1.1 g of syrup. This syrup was dissolved in 4 ml of isopropanol following the addition of triethylamine (0.58 g, 5.7 mmol) to give triethylammonium 2-mercaptobenzenesulfonate (0.84 g, 40%) as colorless crystals (mp 89—90 °C).

2-Chloro-5-((5-methyl-2-oxo-1,3-dioxol-4-yl)methylthio)benzoic Acid (IIIj) By a procedure similar to that described for IIIa, 2-chloro-5-mercaptobenzoic acid (5.1 g, 27.0 mmol) and 4-chloromethyl-5-methyl-1,3-dioxol-2-one (4.0 g, 26.9 mmol) gave IIIj (4.8 g, 59%) as colorless crystals after recrystallization from ethanol, mp 162—164 °C. ^{1}H-NMR (DMSO-d_6) δ: 1.9 (s, 3H, CH_3), 4.2 (s, 2H, CH_2), 7.4—7.9 (m, 3H, aromatic protons). IR (KBr): 1814, 1800 (C=O), 1738 (C=C) cm^{-1}.

4-Phenylthiomethyl-1,3-dioxol-2-one (IIIk) By a procedure similar to that described for IIIa, benzenethiol (0.7 g, 6.4 mmol) and 4-bromomethyl-1,3-dioxol-2-one[8] (1.0 g, 5.6 mmol) gave IIIk (0.7 g, 60%) as a colorless oil after bulb-to-bulb distillation (about 170 °C/0.1 mmHg), bp 175 °C (0.1 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 3.8 (d, 2H, CH_2), 6.7 (t, 1H, CH), 7.2—7.7 (m, 5H, aromatic protons). IR ($CDCl_3$): 1849, 1817 (C=O) cm^{-1}.

4-(4-Fluorophenyl)thiomethyl-1,3-dioxol-2-one (IIIl) By a procedure similar to that described for IIIa, 4-fluorobenzenethiol (0.8 g, 6.2 mmol) and 4-bromomethyl-1,3-dioxol-2-one (1.0 g, 5.6 mmol) gave IIIl (0.52 g, 41%) as pale yellow crystals after recrystallization from a mixture of ethyl acetate and *n*-hexane, mp 57—59 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.7 (d, 2H, CH_2), 6.7 (t, 1H, CH), 6.9—7.6 (m, 4H, aromatic protons). IR ($CDCl_3$): 1849, 1819 (C=O) cm^{-1}.

4-(4-Nitrophenyl)thiomethyl-1,3-dioxol-2-one (IIIm) By a procedure similar to that described for IIIa, 4-nitrobenzenethiol (0.9 g, 5.8 mmol) and 4-bromomethyl-1,3-dioxol-2-one (1.0 g, 5.6 mmol) gave IIIm (0.5 g, 37%) as yellow crystals after recrystallization from a mixture of chloroform and *n*-hexane. mp 109—112 °C. ^{1}H-NMR ($CDCl_3$) δ: 4.0 (d, 2H, CH_2), 6.9 (t, 1H, CH), 7.2—7.6 (2H, aromatic protons), 8.0—8.5 (2H, aromatic protons). IR (KBr): 1870, 1838, 1806 (C=O), 1786 (C=C) cm^{-1}.

5-Phenyl-4-phenylthiomethyl-1,3-dioxol-2-one (IIIn) By a procedure similar to that described for IIIa, benzenethiol (0.09 g, 0.82 mmol) and 4-bromomethyl-5-phenyl-1,3-dioxol-2-one[8] (0.20 g, 0.78 mol) gave IIIn (0.20 g, 85%) as colorless crystals after recrystallization from a mixture of chloroform and *n*-hexane, mp 87—89 °C. ^{1}H-NMR ($CDCl_3$) δ: 4.0 (s, 2H, CH_2), 7.1—7.7 (m, 10H, aromatic protons). IR (KBr): 1818 (C=O) cm^{-1}.

4-(4-Nitrophenylthio)methyl-5-phenyl-1,3-dioxol-2-one (IIIo) By a procedure similar to that described for IIIa, 4-nitrobenzenethiol (0.12 g, 0.77 mmol) and 4-bromomethyl-5-phenyl-1,3-dioxol-2-one[8] (0.20 g, 0.78 mmol) gave IIIo (0.22 g, 86%) as pale yellow crystals after recrystallization from a mixture of chloroform and *n*-hexane, mp 137—140 °C. ^{1}H-NMR ($CDCl_3$) δ: 4.2 (s, 2H, CH_2), 7.2—7.6 (m, 7H, aromatic protons), 7.9—8.2 (m, 2H, aromatic protons). IR (KBr): 1826 (C=O) cm^{-1}.

References

1) K.-B. Augustinsson, *Ann. N.Y. Acad. Sci.*, **94**, 844 (1961).
2) Y. Takahashi, I. Aoyama, F. Ito and Y. Yamamura, *Clin. Chim. Acta*, **18**, 21 (1967)
3) K. Lorentz, B. Flatter and E. Augustin, *Clin. Chem.*, **25**, 1714 (1979).
4) A. Burlina, E. Michielin and L. Galzigna, *Europ. J. Clin. Invest.*, **7**, 17 (1977).
5) K.-B. Augustinsson and G. Ekedahl, *Acta Chem. Scand.*, **16**, 240 (1962).
6) H. Tanaka, M. Kitamura and Y. Inoue, *Clin. Chim. Acta*, **170**, 105 (1987).
7) G. C. Secchi and N. Dioguardi, *Enzymol. Biol. Clin.*, **5**, 29 (1965).
8) F. Sakamoto, S. Ikeda and G. Tsukamoto, *Chem. Pharm. Bull.*, **32**, 2241 (1984).
9) G. L. Ellman, K. D. Courtney, V. Andres and R. M. Featherstone, *Biochem. Pharmacol.*, **7**, 88 (1961).
10) K.-B. Augustinsson, *J. Histochem. Cytochem.*, **12**, 744 (1964).
11) H. Kawai, F. Sakamoto and Y. Inoue, *Clin. Chim. Acta*, **188**, 177 (1990).
12) W. Junge and H. Klees, "Methods of Enzymatic Analysis," 3rd ed., Vol. 4, ed. by J. Bergmeyer and M. Grassl, Verlag Chemie, Weinheim, 1984, p. 8.
13) H. Tanaka, M. Kitamura, F. Sakamoto, M. Taguchi and M. Sotomura, U.S. Patent 4777267 (1988) [*Chem. Abstr.*, **108**, 5996*n* (1988)].
14) A. Burlina and L. Galzigna, *Clin. Chim. Acta*, **39**, 255 (1972).
15) A. E. Kretov, A. S. Bespalyi and N. N. Politun, *J. Gen. Chem. USSR.*, **34**, 2078 (1964).

Quantitative Structure–Activity Relationship Analysis of Phencyclidine Derivatives. I

Yoshiharu INAMI, Tatsuya TOMITA, and Yukimasa TERADA*

Meijo University, Faculty of Pharmacy, Tenpaku, Nagoya 468, Japan. *Received November 13, 1990*

Quantitative structure–activity relationship analysis has been accomplished on 24 derivatives of phencyclidine (PCP). By analysis with conceivable parameters effecting the variation of activity, it was shown that a compound with a smaller dipole moment, larger hydrophobicity, and a smaller principal moment of inertia is a stronger ligand of the receptor.

By further examination of this fact, the direction of the dipole vector of the ligand molecule was demonstrated to be important for the activity. Consequently, an equation, which sufficiently explains the variation of the activity, was derived using the difference in direction of the dipole vector as the only parameter. This is the first quantitative analysis explaining the variation of activity of PCP derivatives.

Keywords phencyclidine; QSAR; regression analysis; AM1 method; dipole moment; PCP derivative; physicochemical property

Although phencyclidine [PCP, 1-(1-phenylcyclohexyl)-piperidine (**1**)] was developed as an excellent anesthetic, its clinical use was discontinued because of psychomotor side effects during convalescence. However, since the undesirable symptoms induced by PCP are restorable by ordinary drugs for schizophrenia, an antagonist of PCP is expected to be a psychopathic new remedy.[1]

Many PCP analogs have been synthesized and studied concerning their biological activities in connection with their structural features.[2–4] However, no quantitative analysis has been done to explain the reason for the variation of activity of PCP derivatives. Therefore, it was deemed worthwhile for precise understanding of the mechanism of drug action to express the structural effect numerically.

We analyzed the quantitative structure–activity relationships (QSAR) of PCP derivatives which revealed a good correlation between the direction of the dipole vector of the ligand molecule and the binding affinity for the receptor.

Calculation Method

Biological Activity Data The published data concerning 24 PCP derivatives, which were synthesized and measured biological activities by Kamenka *et al.*, were used.[2,3] The pattern of chemical substitution and the biological activity of each compound are shown in Table I. The activity is expressed as the logarithm of the reciprocal concentration of a compound required to substitute 50% of [^{3}H]-PCP bound to its receptor, and a larger number means the higher binding ability of the compound.

Physicochemical Parameters In general QSAR analysis, the pharmacological property of a compound is expressed by the sum of substituent effects on a basic structure. However, the PCP derivatives discussed in this paper have too many substitution sites compared to the variety of substituents; therefore, parameters expressing the property of the whole molecule were devised as described below and were used instead of the usual substituent constants.

Dipole moment, ionization potential and the net atomic charge of the nitrogen atom of the piperidine ring were estimated by molecular orbital calculations and were regarded as the parameters depicting the electronic properties. Furthermore, molecular van der Waals (VDW) volume and VDW surface area were calculated on the basis of the structure optimized by the molecular orbital calculations and used as the steric parameters. The calculations of the volume and the surface area were carried out using the MOLSV program by Smith.[5]

Moment of inertia and length of the principal ellipsoidal axis were adopted as the structure related parameters. The principal moments of inertia were determined by diagonalization of a matrix constructed by three-dimensional coordinates and weights of the constituting atoms in a molecule. Then, the molecule was rotated to coincide these principal moments of inertia with *z*-, *y*-, and *x*-axes in the order of magnitude. Finally, the length of the principal ellipsoidal axis was calculated by the projection of the VDW surface onto these three axes.[6]

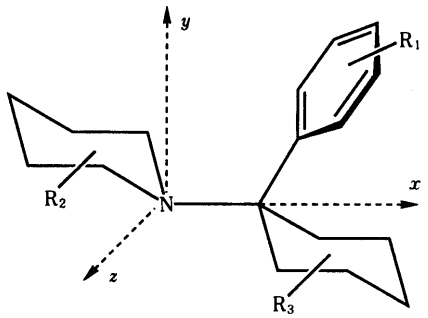


Fig. 1. The Active Conformation of PCP Derivatives

It is known that hydrophobicity plays an important role in the action of drugs, and log *P*, the logarithm of the partition coefficient between *n*-octanol and water, is often used in QSAR analysis. We used log *P* estimated by the fragment method of Ghose *et al.*[7] In this method, a molecule is divided into fragments according to atom types, and the molecular log *P* is calculated by the sum of the fragment contribution.

All physicochemical parameters considered in the QSAR analysis are summarized in Table II.

Molecular Orbital Calculations The AM1 method, one of the semi-empirical molecular orbital methods developed by Dewar *et al.*,[8] was employed. We assumed all compounds are in the bioactive conformation[3,9,10] regardless of the stability,[11] and the starting structure (Fig. 1) was constructed using standard geometries with Dreiding models. This was followed by calculations in which all flexibility, except for the planarity of the phenyl ring, was optimized.

Statistical Calculations Multiple regression analysis using the physicochemical parameters in Table II as the explanatory variables and the biological activity as the criterion variable was executed. Selection of the explanatory variables was achieved by the stepwise method on the basis of the variance ratio (*F*), and the best correlated equation was searched. A program package for statistic analyses by Tanaka *et al.*[12] was used for multiple regression analysis.

The molecular orbital calculations were executed on a HITAC M-680H at the Computer Center of the Institute for Molecular Science. All other calculations were carried out on a personal computer NEC PC-9801.

Results and Discussion

Analysis by Physicochemical Parameters Equation 1, shown below, was obtained by multiple regression analysis of 24 PCP derivatives,

$$\log(1/K_d) = -0.322DM + 0.604LOGP - 0.102MIZ + 6.150 \quad (1)$$
$$(\pm 0.219) \quad (\pm 0.403) \quad (\pm 0.074) \quad (\pm 2.193)$$
$$(n=24,\ r=0.808,\ s=0.548,\ F=12.556)$$

TABLE I. Parameter Values and Observed and Calculated $\log(1/K_d)$ of PCP Derivatives

No.	R_1	R_2	R_3[a]	$\log(1/K_d)$[b]	*DM*	*LOGP*	*MIZ*	$\log(1/K_d)$[c]	DV_{angle}	$\log(1/K_d)$[d]
1	H	H	H	6.602	0.949	4.528	17.122	6.841	0.56	6.608
2	*o*-OH	H	H	6.125	2.138	4.224	17.301	6.257	0.71	6.425
3	*o*-OMe	H	H	6.301	2.199	4.438	18.167	6.279	0.94	6.144
4	*m*-OH	H	H	7.523	0.383	4.224	19.353	6.613	0.00	7.992
5	*m*-OMe	H	H	7.046	0.555	4.438	21.196	6.500	0.27	6.962
6	*m*-NO_2	H	H	4.939	4.919	4.579	25.051	4.791	1.46	5.509
7	*p*-OH	H	H	4.699	1.178	4.224	20.959	6.194	1.82	5.070
8	*p*-OMe	H	H	5.921	1.582	4.438	24.977	5.785	1.48	5.485
9	*p*-NO_2	H	H	4.602	4.797	4.579	28.779	4.445	2.58	4.142
10	*m,p*-$(OMe)_2$	H	H	5.022	0.715	4.295	26.970	5.774	2.20	4.606
11	H	3′-Me	H	6.796	0.946	4.877	19.382	6.824	0.58	6.584
12	H	4′-Me	H	6.398	0.933	4.877	21.312	6.631	0.56	6.608
13	H	4′-OH	H	5.658	1.810	3.129	21.553	5.268	1.36	5.631
14	H	3′,4′-Dehydro	H	7.097	0.760	4.257	17.096	6.741	0.55	6.620
15	H	H	*trans*-2-Me	6.921	0.795	4.877	18.526	6.841	0.54	6.633
16	H	H	*trans*-2-OMe	6.081	1.754	3.562	20.523	5.574	1.13	5.912
17	H	H	*trans*-3-Me	6.222	0.938	4.877	19.233	6.709	0.57	6.596
18	H	H	*trans*-3-OH	5.658	1.550	3.129	19.372	6.709	0.90	6.193
19	H	H	*trans*-3-OMe	5.523	1.180	3.562	22.424	5.200	0.94	6.144
20	H	H	*cis*-4-Me	6.886	0.965	4.877	20.451	5.542	0.56	6.608
21	H	H	*cis*-4-OH	4.770	2.321	3.129	20.613	6.370	1.80	5.094
22	H	H	*cis*-4-OMe	5.398	0.648	3.562	25.133	5.644	1.25	5.766
23	*m*-OMe	H	*cis*-4-Me	7.237	0.547	4.787	24.568	6.959	0.27	6.962
24	*p*-OMe	H	*cis*-4-Me	5.658	1.590	4.788	28.417	5.653	1.47	5.947

a) *cis* and *trans* denote the relation between the phenyl ring and R_3. *b*) Taken from reference 2. *c*) Calculated by Eq. 1. *d*) Calculated by Eq. 2.

TABLE II. Physicochemical Parameters Used in the Derivation of Eq. 1

Electronic parameter[a]	
Dipole moment (D)	*DM*
x-Component of dipole vector (D)	*DVX*
y-Component of dipole vector (D)	*DVY*
z-Component of dipole vector (D)	*DVZ*
Ionization potential (eV)	*IP*
HOMO energy (eV)	E_{HOMO}
LUMO energy (eV)	E_{LUMO}
Charge of piperidine nitrogen (e)	*QN*
Steric parameter[b]	
VDW volume of the molecule ($Å^3$)	*VOL*
VDW surface area of the molecule ($Å^2$)	*SA*
Structure related parameter[b]	
x-Component of moment of inertia (dalton·$Å^2$)	*MIX*
y-Component of moment of inertia (dalton·$Å^2$)	*MIY*
z-Component of moment of inertia (dalton·$Å^2$)	*MIZ*
Length of principal ellipsoidal *x*-axis (Å)	*PEX*
Length of principal ellipsoidal *y*-axis (Å)	*PEY*
Length of principal ellipsoidal *z*-axis (Å)	*PEZ*
Hydrophobicity parameter[c]	
Calculated log *P*	*LOGP*

a) Calculated by AM1 method.[8] *b*) Calculated following the description of Chuman[6] based on the molecular structure resulting from AM1 optimization. *c*) Estimated by the fragment method of Ghose.[7]

where n is the number of compounds, r is the correlation coefficient, s is the standard deviation, F is the variance ratio, and the figures in parentheses are the 95% confidence intervals. Both equations in this paper represent the best correlation, and no problematic collinearity in the squared cross-correlation matrix of the parameters was recognized. The parameter values, together with the calculated values of $\log(1/K_d)$, are listed in Table I.

Equation 1 predicts that a compound with smaller dipole moment, considerable hydrophobicity, and a smaller first component of moment of inertia would have a stronger affinity for the receptor. The negative coefficient of a dipole moment is consistent with the low activity of the nitro derivatives which have a large dipole moment. The positive sign of the partition coefficient may be a reflection of the general high activity of methyl derivatives. However, the activity of the polar compounds varies remarkably.

Although *ca.* 65% of the variation of the activity was explained by Eq. 1, it is not a sufficient result as not so many samples were taken into consideration. Since Eq. 1 was the best equation derived using the parameters in Table II, another possibility was explored to parameterize unexplained molecular properties.

Analysis with the Direction of the Dipole Vector The size of the dipole moment (*DM*) was significant in Eq. 1. Being a vector quantity, the dipole moment includes both the size and the direction. Similarly, *LOGP* can be considered the expression of a balance between the electrostatic property and the steric extent of the molecule. Moreover, *MIZ* is an expression of a type of molecular dimension. The significance of these three terms, therefore, suggests that some kind of molecular balance or some unknown directionality is important for the effective binding of the PCP derivatives. In fact, the activity of PCP derivatives varies remarkably when a substituent exists on different positions. Hence, it is interesting to parameterize such directional differences.

The dipole vector was calculated by the AM1 method fixing all of the compounds in the biologically active conformation. As shown by a few examples (Fig. 2), the dipole vector of each compound is oriented differently. Accordingly, the correlation of the direction of the dipole vector with the activity was inspected. The vector of the most potent compound (*m*-OH-PCP, **4**) was tentatively chosen as the standard axis.

The angle between the standard axis and the dipole vector of a trial molecule measured in radian was denoted by DV_{angle} whose values are shown in Table I. A QSAR

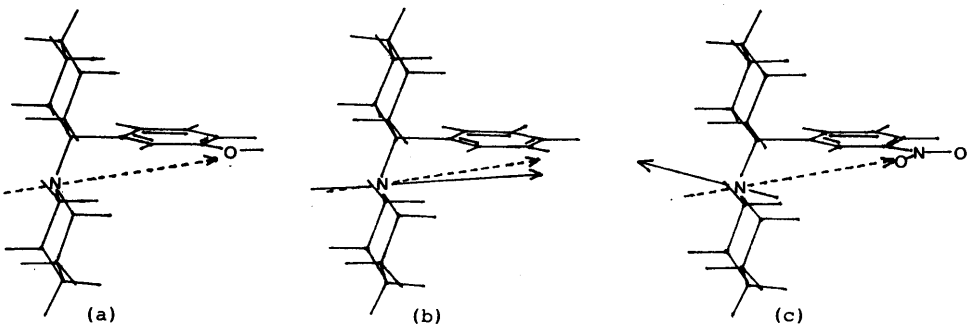


Fig. 2. A Few Examples of Dipole Vector of PCP Derivatives (a) *m*-OH-PCP (**4**), (b) PCP (**1**), and (c) *m*-NO_2-PCP (**6**)
The dipole vector of *m*-OH-PCP (**4**) was chosen as the standard axis and is shown by the dotted arrow.

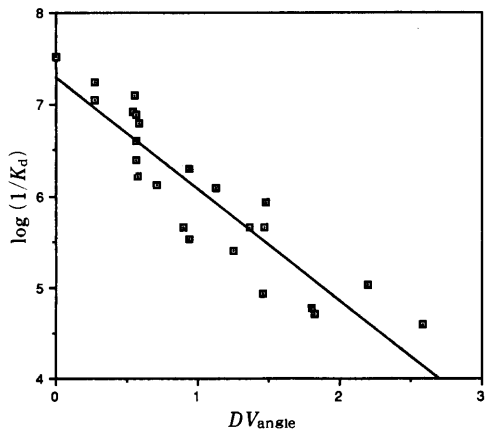


Fig. 3. Linear Relation between Observed $\log(1/K_d)$ and DV_{angle}

analysis with DV_{angle} as the only parameter gave a well-correlated equation.

$$\log(1/K_d) = -1.221 DV_{angle} + 7.292 \quad (2)$$
$$(\pm 0.240) \qquad (\pm 0.290)$$
$$(n=24,\ r=0.913,\ s=0.361,\ F=109.756)$$

No further improvement was obtained by the addition of the size of the dipole moment (DM) to the analysis. In Fig. 3 the observed value of $\log(1/K_d)$ is plotted against DV_{angle}. It is obvious that the compounds with a dipole vector similarly oriented to the standard axis exhibit a stronger affinity for the PCP receptor. This would be interpreted as follows.

Manallack and coworkers proposed a receptor model for PCP-like drugs belonging to several chemical groups.[9] At the same time, they pointed out that a phenyl ring and a basic nitrogen are common elements of the PCP-like drugs and that these groups anchor the drugs in the receptor site. Carroll *et al.*[10] and Kamenka and Geneste[3] suggested that the biologically active conformation of PCP is the one with an axial phenyl and an equatorial piperidine group as shown in Fig. 1. Consequently, all PCP derivatives discussed here must bind to the same site of the receptor in the same direction.

When a ligand enters into the electronic environment of the receptor cavity, a compound with an electronic field as complementary as possible to the receptor is favorable. In other words, the strongest interaction occurs when the charge density around a molecule is identical, but in the opposite sign, with that of the receptor cavity. Furthermore, the dipole moment is referred to as the outcome of the intramolecular electrostatic distribution, and it effects the electrostatic circumstance around the molecule. Therefore, it is conceivable that strong ligands have a similar electrostatic character.[13]

We revealed that the binding affinity of PCP derivatives is strongly related to the direction of the dipole vector, and as far as we know, the present analysis provides the first quantitative results revealing the source of the variation in biological activity of PCP derivatives.

Acknowledgement The authors are indebted to Professor T. Nabeshima (Nagoya University) for drawing their attention to this problem.

References

1) V. H. Maddox, "PCP (Phencyclidine): Historical and Current Perspectives," ed. by E. F. Domino, NPP Books, Ann Arbor, 1981, Chapter 1.
2) J. P. Vincent, B. Kartalovski, P. Geneste, J.-M. Kamenka, and M. Lazdunski, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4678 (1979); J. Vignon, J. P. Vincent, J. N. Bidard, J.-M. Kamenka, P. Geneste, S. Monier, and M. Lazdunski, *Eur. J. Pharmacol.*, **81**, 5531 (1982); J.-M. Kamenka and P. Geneste, "PCP (phencyclidine): Historical and Current Perspectives," ed. by E. F. Domino, NPP Books, Ann Arbor, 1981, pp. 47—82.
3) J.-M. Kamenka and P. Geneste, "Phencyclidine and Related Arylcyclohexylamines: Present and Future Applications," ed. by J.-M. Kamenka, E. F. Domino, and P. Geneste, NPP Books, Ann Arbor, 1983, Chapter 1.
4) V. H. Maddox, E. F. Godefroi, and R. F. Parcell, *J. Med. Chem.*, **8**, 230 (1964); A. Kalir, H. Edery, Z. Pelah, D. Balderman, and G. Porath, *ibid.*, **12**, 473 (1969); H. E. Shannon, Phencyclidine and Related Arylcyclohexylamines: Present and Furture Applications, ed. by J.-M. Kamenka, E. F. Domino, and P. Geneste, NPP Books, Ann Arbor, 1983, pp. 311—335; E. F. Domino, W. Rocki, E. J. Cone, R. L. McQuinn, and H. E. Shannon, *J. Pharmacol. Exp. Ther.*, **228**, 147 (1984).
5) G. M. Smith, MOLSV program (QCPE #509), A modified version for the PC-9801 personal computer by T. Nagao (Hakodate Technical College) was used.
6) K. Chuman and A. Tomonaga, "Structure–Activity Relationships Quantitative Approaches II; The Applications to Drug Design and Mode-of-Action Studies," ed. by The Conversazione of Structure–Activity Relationship, Nankodo, Tokyo, 1982, Chapter 12; R. C. Glen and V. S. Rose, *J. Mol. Graph.*, **5**, 79 (1987).
7) A. K. Ghose and G. M. Crippen, *J. Comp. Chem.*, **7**, 565 (1986).
8) M. J. S. Dewar, E. G. Zoebisch, E. F. Heali, and J. J. Stewart, *J. Am. Chem. Soc.*, **107**, 3902 (1985). AM1 method included in MOPAC program version 3, library program of Computer Center of Institute for Molecular Science (Okazaki), was used.
9) D. T. Manallack, M. G. Wong, M. Costa, P. R. Andrews, and P. M. Beart, *Mol. Pharmacol.*, **34**, 863 (1988).
10) F. I. Carroll, G. A. Brine, K. G. Boldt, S. W. Mascarella, C. G.

Moreland, S. J. Burgess, and E. O. Stejskal, "Sigma and Phencyclidine-Like Compounds as Molecular Probes in Biology," ed. by E. F. Domino and J.-M. Kamenka, NPP Books, Ann Arbor, 1988, pp. 91—106.

11) Each compound discussed in this paper is in the global minimum conformation or within 1.5 kcal mol^{-1} of it. The binding conformation should not necessarily be the global minimum, because even if the binding conformation is unstable, stabilization by intermolecular interaction with the receptor can be compensated for the energy for unfavorable conformational change. (C. Tosi, R. Fusco, and L. Caccianotti, *J. Mol. Struct.* (THEOCHEM), **183**, 361 (1989)).

12) Y. Tanaka, T. Tarumi, and K. Wakimoto, "Handbook for Personal Computer-Aided Statistical Analysis," Vol. II, Kyoritsu Shuppan, Tokyo, 1984, pp. 1—48.

13) Binding activity of PCP derivatives was correlated to the spatial distribution of electrostatic potential. (Y. Terada, Y. Inami, and T. Tomita, "NMDA Receptor Related Agents: Biochemistry, Pharmacology and Behavior," ed. by T. Kameyama, T. Nabeshima, and E. F. Domino, NPP Books, Ann Arbor, 1991, Chapter 12).

Antiulcer Agents. II. Synthesis and Gastric Acid Antisecretory Activity of *N*-[3-{3-(Piperidinomethyl)phenoxy}propyl]-4-(1-methyl-1*H*-tetrazol-5-ylthio)butanamide and Related Compounds

Ikuo UEDA,*[a] Katsuyuki ISHII,[a] Katsuo SINOZAKI,[b] Masao SEIKI[b] and Minoru HATANAKA[a]

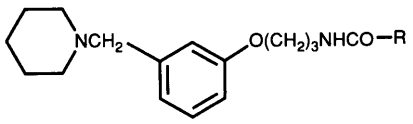
The Institute of Scientific and Industrial Research, Osaka University,[a] Ibaraki, Osaka 567, Japan and Central Research Laboratories, Zeria Pharmaceutical Co., Ltd.,[b] 2512-1, Oshikiri, Konan-machi, Osato-gun, Saitama 360-01, Japan. *Received November 19, 1990*

***N*-[3-{3-(Piperidinomethyl)phenoxy}propyl]butanamides having a 1-methyl-1*H*-tetrazol-5-ylthio moiety as a pharmacophore and related compounds were prepared and tested for their antisecretory activity against histamine-induced gastric acid secretion in conscious rats with gastric fistulas. Most of the compounds showed antisecretory activity. Among them, *N*-[3-{3-(piperidinomethyl)phenoxy}propyl]-4-(1-methyl-1*H*-tetrazol-5-ylthio)butanamide (5f) was found to possess the most potent activity, and a possibility of isosteric replacement of the methoxycarbonyl group with 1-methyl-1*H*-tetrazol-5-yl group was indicated. The structure–activity relationships are also discussed.**

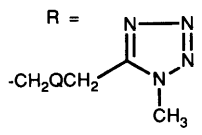
Keywords gastric acid antisecretory activity; antiulcer activity; *N*-[3-{3-(piperidinomethyl)phenoxy}propyl]-4-(1-methyl-1*H*-tetrazol-5-ylthio)butanamide; 5-mercapto-1-methyl-1*H*-tetrazole; structure–activity relationship; histamine H_2-receptor antagonist

In the previous paper,[1] we reported that *N*-[3-{3-(piperidinomethyl)phenoxy}propyl]-2-(2-hydroxyethylthio)-acetamide (**1**) showed potential gastric acid antisecretory and gastrointestinal cytoprotective activities. Compounds **2** and **3**, which are analogues of **1**, were also found to possess significant gastric acid antisecretory activity.[1,2] It has been suggested that successive bioisosteric replacement of key positions of a biologically important molecule results in an improvement of its biological effects.[3]

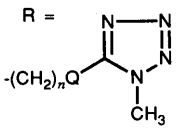
Structual Alteration of Compounds 2 and 3 The similarity between the spatial and acidic properties of the tetrazole ring and the carboxylic acid group has prompted a search for tetrazoles with pharmacological application.[3,4] We are now estimating the possibility of bioisosteric replacement of the methoxycarbonyl ($-COOCH_3$) group with 1-methyl-1*H*-tetrazol-5-yl group. Structural alteration of **2** was tried on the basis of bioisosterism principles. Isosteric replacement of the $-COOC_2H_5$ group by a 1-methyl-1*H*-tetrazol-5-yl group brought compound **4**. As the $-CH_2SCH_2-$ ($-CH_2SO_2CH_2-$) group may be substituted for the $-CH_2CH_2S-$ ($-CH_2CH_2SO_2-$) group with similar electronic structures, **4** will be changeable into compounds **5d** and **5e**. And it has been shown that the $-NHCOCH_2S-$ group was effective in imparting the desired level of gastric acid antisecretory activity.[1] Thus, *N*-[3-{3-(piperidinomethyl)phenoxy}propyl]-2-(1-methyl-1*H*-tetrazol-5-ylthio)-acetamide (**5a**) was the first target compound in this series. Secondly, the replacement of the methoxycarbonyl group of **3** with a 1-methyl-1*H*-tetrazol-5-yl group led to *N*-[3-{3-(piperidinomethyl)phenoxy}propyl]-2-(1-methyl-1*H*-tetrazol-5-yl)acetamide (**5k**). Both compounds **5a** and **5k** exhibited significant antisecretory activity against histamine-induced gastric acid secretion in conscious rats with gastric fistulas. The inhibition percent upon intraduodenal administration of the dose of 30 mg/kg was 66.9% for **5a** and 77.4% for **5k**, respectively. These results led us to prepare compounds illustrated in a general formula **5** having a 1-methyl-1*H*-tetrazol-5-yl moiety as its pharmacophore (Chart 2). One of these compounds, *N*-[3-{3-(piperidinomethyl)phenoxy}propyl]-4-(1-methyl-1*H*-tetrazol-5-ylthio)-butanamide (**5f**), in terms of its ED_{50} value, was shown to be 1.5 times more potent than **1**. In this paper, we describe the synthesis, histamine-induced gastric acid antisecretory activity of **5f** and related compounds, and determination of structure–activity relationships with gastric acid antisecretory activity data.



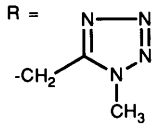
1 : R = $-CH_2SCH_2CH_2OH$ (79.5)
2 : R = $-CH_2SO_2CH_2COOC_2H_5$ (39.7)
3 : R = $-CH_2COOCH_3$ (46.9)



4 : Q=S or SO_2



5a : n=1, Q=S
5d : n=2, Q=S
5e : n=2, Q=SO_2



5k

( ) : %inhibition of gastric acid secretion in conscious rats with gastric fistulas at the dose of 30 mg/kg (i.d.)

Chart 1

Synthesis Compounds illustrated in a general formula **5** were prepared according to methods A—E (Chart 2). In this series, most compounds were prepared by the reaction of 3-{3-(piperidino(or pyrrolidino)methyl)phenoxy}propanamine [**6**[5] (or **7**[6])] with an ester derivative (**12**) in good yields (method A). Compound **5d** was synthesized by treatment of **6** with 3-(1-methyl-1*H*-tetrazol-5-ylthio)propionyl chloride (method B). *N*-[3-{3-(piperidinomethyl)phenoxy}-propyl]-2-(1-methyl-1*H*-tetrazol-5-ylthio)acetamide (**5a**) was prepared by the reaction of *N*-[3-{3-(piperidinomethyl)phenoxy}propyl]-2-chloroacetamide[1] (**8**) with 5-mercapto-1-methyl-1*H*-tetrazole (TzSH) in the presence of a base in a good yield (method C). Variation of the piperidino group was conveniently achieved by the use of *N*-[3-(3-chloromethylphenoxy)propyl]-4-(1-methyl-1*H*-tetrazol-5-ylthio)butanamide (**10**) as a starting material. Compound **10**, which was prepared by the reaction of *N*-[3-(3-hydroxymethylphenoxy)propyl]-4-(1-methyl-1*H*-

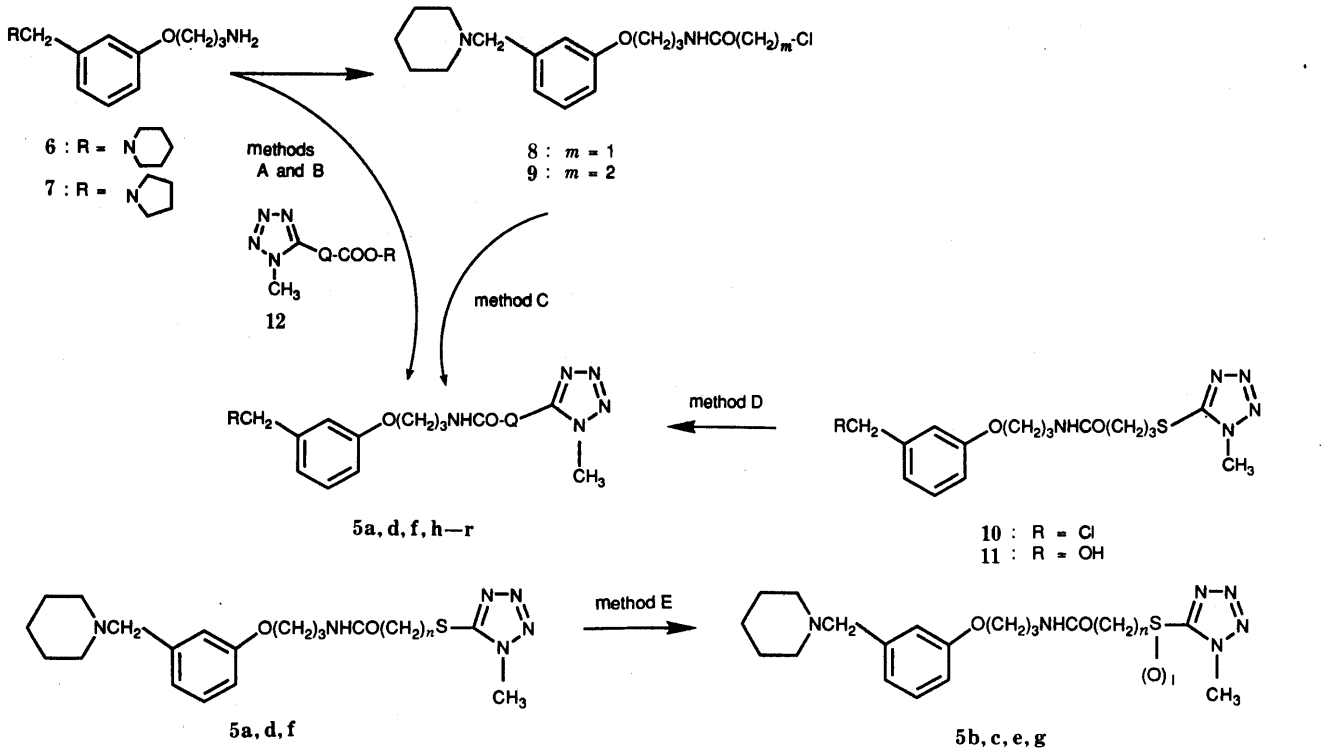
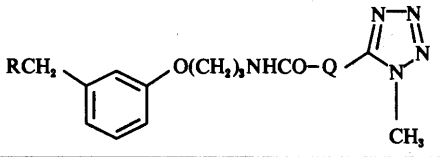


Chart 2

TABLE I. *N*-[3-{3-(Piperidinomethyl)phenoxy}propyl]-4-(1-methyl-1*H*-tetrazol-5-ylthio)butanamide and Related Compounds



Compd. No.	R[a]	Q	Method	Yield (%)	mp °C [Recryst. solv.]	Formula	Analysis (%) Calcd (Found) C	H	N	Activity[c] (%)
5a	P	CH_2S–[b]	A, C	88	90.0—90.5 [IPE]	$C_{19}H_{28}N_6O_2S$	56.41 (56.60	6.98 6.75	20.77 20.81)	66.9
5b	P	CH_2SO_2–[b]	E	58	Oil	$C_{19}H_{28}N_6O_4S$	436.1893 (436.1909)[g]			19.6
5c	P	CH_2SO–[b]	E	63	Oil	$C_{19}H_{28}N_6O_3S$	420.1944 (420.1953)[g]			51.6
5d	P	CH_2CH_2S–[b]	B	70	Oil	$C_{20}H_{30}N_6O_2S$	418.2149 (418.2178)[g]			63.1
5e	P	$CH_2CH_2SO_2$–[b]	E	81	Oil	$C_{20}H_{30}N_6O_4S$	450.2049 (450.2037)[g]			35.5
5f	P	$CH_2CH_2CH_2S$–[b]	A	94	87.0—88.0 [EtOH–Et_2O]	$C_{21}H_{32}N_6O_2S\cdot HCl$	53.78 (53.81	7.09 7.21	17.94 18.06)	86.1 (9.5)[d]
5g	P	$CH_2CH_2CH_2SO_2$–[b]	A, E	34	76.5—77.5 [IPE]	$C_{21}H_{32}N_6O_4S$	54.29 (54.53	6.94 6.79	18.09 18.12)	50.4
5h	P	$CH_2SCH_2CH_2S$–[b]	A	61	Oil	$C_{21}H_{32}N_6O_2S_2$	464.2026 (464.2030)[g]			68.9
5i	P	$CH_2OCH_2CH_2S$–[b]	A	75	Oil	$C_{21}H_{32}N_6O_3S$	448.2257 (448.2243)[g]			13.9
5j	P	$CH_2SCH_2CH_2$–[b]	A	60	Oil	$C_{21}H_{32}N_6O_2S$	432.2306 (432.2272)[g]			18.4
5k	P	CH_2	A	63	95.0—95.9 [C_6H_6–hexane]	$C_{19}H_{28}N_6O_2$	61.27 (61.37	7.58 7.39	22.56 22.45)	77.4
5l[e]	P	CH_2	A	56	126—127 (dec.) [EtOH–Et_2O]	$C_{19}H_{28}N_6O_2\cdot HCl \cdot 1/3H_2O$	55.00 (55.15	7.21 7.39	20.25 19.95)	19.5
5m	P	—[f]	A	84	Oil	$C_{18}H_{26}N_6O_2$	358.2117 (358.2119)[g]			−23.9
5n[e]	P	—[f]	A	76	Oil	$C_{18}H_{26}N_6O_2$	358.2117 (358.2101)[g]			17.7
5o	Py	$CH_2CH_2CH_2S$–[b]	B, D	86	Oil	$C_{20}H_{30}N_6O_2S$	418.2149 (418.2138)[g]			66.7
5p	PyOH	$CH_2CH_2CH_2S$–[b]	D	86	Oil	$C_{20}H_{30}N_6O_3S$	434.2100 (434.2106)[g]			41.8
5q	DM	$CH_2CH_2CH_2S$–[b]	D	82	Oil	$C_{18}H_{28}N_6O_2S$	392.1993 (392.1996)[g]			61.1
5r	HE	$CH_2CH_2CH_2S$–[b]	D	88	Oil	$C_{19}H_{30}N_6O_3S$	422.2100 (422.2126)[g]			−2.4
1[h]										79.5 (12.9)[d]
Ranitidine[h]										72.1 (17.7)[d]

a) P: piperidino, Py: pyrrolidino, PyOH: 3-hydroxypyrrolidino, DM: *N*,*N*-dimethylamino, HE: *N*-2-hydroxyethyl-*N*-methylamino. *b*) Binding site to tetrazole ring. *c*) % inhibition at the dose of 30 mg/kg (i.d.) in rats. Each value represents the mean of three rats. *d*) ED_{50} value; mg/kg. *e*) 1*H*-2-Methyltetrazole analogue. *f*) Direct binding to tetrazole ring. *g*) High-resolution MS analysis. *h*) See ref. 1.

tetrazol-5-ylthio)butanamide (**11**) with thionyl chloride, was treated with various amines to give the corresponding derivatives (**5o—r**) (method D). Sulfinyl compound (**5c**) and sulfonyl compounds (**5b**, **5e** and **5g**) were synthesized from methane sulfonates of corresponding thio derivatives (**5a**, **5d** and **5f**) and *m*-chloroperbenzoic acid (*m*-CPBA) in CH_2Cl_2 (method E). Derivatives of **5** prepared in this series are summarized with their antisecretory activity in Table I. Infrared (IR) and proton nuclear magnetic resonance (^{1}H-NMR) spectral data for **5** are shown in Table II (Experimental section).

In the course of synthesizing **5**, some interesting reactions were observed. When methyl 2-(1-methyl-1*H*-tetrazol-5-ylsulfonyl)acetate (**12b**) was allowed to react with **6** in refluxing toluene for 5 h, *N*-(1-methyl-1*H*-tetrazol-5-yl)-*N*-[3-{3-(piperidinomethyl)phenoxy}propyl]acetamide (**13**) was obtained in a 68% yield. The structure of **13** was confirmed by spectral data and high-resolution mass spectral analysis and conversion of **13** into 5-[*N*-[3-{3-(piperidinomethyl)phenoxy}propyl]]amino-1-methyl-1*H*-tetrazole[7] (**16**). In order to estimate the reaction pathway to **13** in this reaction, both reactions of 5-methoxycarbonyl-1methyl-1*H*-tetrazole[8] (**14**) with **6**, and 5-methylsulfonyl-1-methyl-1*H*-tetrazole[8] (**15**) with **6** were carried out as model reactions. When **14** was allowed to react with **6** for 1 h at 120 °C, 3-{3-(piperidinomethyl)phenoxy}-*N*-(1-methyl-1*H*-tetrazol-5-ylcarbonyl)propanamine (**5m**) was obtained in an 84% yield. On the other hand, the reaction of **15** with **6** for 48 h in refluxing toluene gave **16** in a 22% yield. These results suggest that, in the reaction of **12b** having two reaction sites of $-COOCH_3$ and CH_3SO_2-Tz in the molecule with **6**, amidation proceeds preferentially, compared with replacement of the CH_3SO_2- group. Additionally, treatment of **5b** in refluxing toluene for 13 h gave compound **13** in a 73% yield. These observations suggest that first **12b** reacts with **6**, yielding amide **5b**, followed by the Smiles rearrangement of **5b** to give **13**. Studies on the Smiles rearrangement of **5b** will be reported elsewhere.[9] The reaction of respective ethyl 3-(1-methyl-1*H*-tetrazol-5-ylthio)-propionate (**12c**) and its sulfonyl derivative (**12d**) with **6** gave the same product of ethyl 3-[*N*-[3-{3-(piperidinomethyl)phenoxy}propyl]]aminopropionate (**17**) in good yields without giving corresponding amides (**5d** and **5e**). Compound **17** was also obtained in a 58% yield by the reaction of **6** with ethyl acrylate in toluene at 70 °C. When *N*-[3-{3-(piperidinomethyl)phenoxy}propyl]-3-chloropropanamide (**9**) was allowed to react with TzSH in the presence of NaH, *N*-[3-{3-(piperidinomethyl)phenoxy}propyl]-acrylamide (**18**) was obtained in an 88% yield as a main product without yielding amide **5d**. These findings indicate that **17** was produced by the reaction of **6** with ethyl acrylate, which was produced from **12c** and **12d**, respectively.

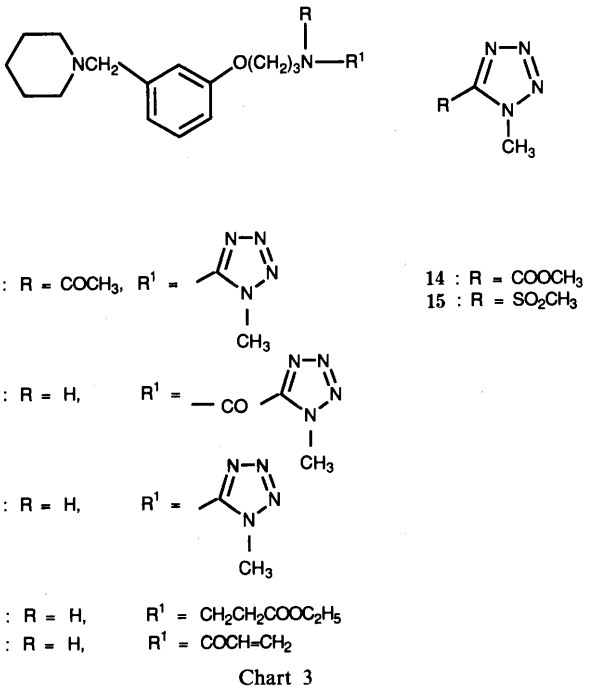


Chart 3

Results and Discussion

The compounds were evaluated for their antisecretory activity against histamine-induced gastric acid secretion in conscious rats with gastric fistulas as a primary screen. In this test, the compounds were administered intraduodenally (i.d.) at a dose of 30 mg/kg, and the reduction in acid output was measured at 1-h intervals for 5 h after administration of the test compound. The gastric acid antisecretory activity determined for the compounds in the rat model is described in Table I. The compounds having a 1-methyl-1*H*-tetrazol-5-yl moiety exhibited significant antisecretory activity. Among these compounds, **5f** was the most active compound. The potency of **5f**, in terms of ED_{50} value, was superior to that of **1** and the reference compound, ranitidine; Each ED_{50} value was 9.5 mg/kg for **5f**, 12.9 mg/kg for **1** and 17.7 mg/kg for ranitidine, respectively.

Structure–Activity Relationships Gastric acid antisecretory activity data described in Table I suggested the following structure–activity relationships.

Extension of the alkylene side chain (Q) in a function of –HNCO-Q-Tz, in which Tz is tetrazole ring, was examined. The function of $-CH_2S-$ in $-HNCOCH_2S-$ of **5a** was homologated by increasing (**5d** and **5f**) the number of methylene groups and by incorporation of $-CH_2SCH_2CH_2S-$ (**5h**) and $-CH_2OCH_2CH_2S-$ (**5i**) instead of $-CH_2S-$. In this case, **5d** showed a tendency to reduce antisecretory activity, compared to **5a**. Compound **5f** was the most active, and **5h** retained its antisecretory activity. As observed in **5i**, isosteric replacement of one of the sulfur atoms in **5h** by an oxygen atom reduced the activity. Compound **5j**, an isomer of **5f**, exhibited diminished antisecretory activity compared to **5f**. The order of activity was **5f**≫**5h**>**5a**, **5d**≫**5j**≒**5i**. These results suggest that the distance of about 8 Å between the –HNCO– and Tz ring brings a maximum potency. As observed in compounds **5k—n**, the shorter distance between –NHCO– group and Tz ring gave variable effects on antisecretory activity. Compound **5k** exhibited an enhanced antisecretory activity relative to that of **1**, while compound **5m** showed a tendency to increase acid secretion. It was difficult to rationalize the pattern of structure–activity relationships within the shorter distance between the –NHCO– and Tz ring.

Oxidation of the sulfur atom in **5a** gave sulfonyl derivative (**5b**) and sulfinyl derivative (**5c**), both of which exhibited reduced antisecretory activity. Compound **5e**, prepared by oxidation of **5d**, was also less active than **5d**. The increase

of hydrophilicity by oxidation of the sulfur atom may be a cause of the reduction in activity. A similar tendency has been observed among **1** and related compounds.[1] Regarding the position of the methyl group of the Tz ring, the data indicates that the 1-methyl derivative (**5k**) is superior to the 2-methyl derivative (**5l**) in antisecretory activity.

At present, it is difficult to discuss structure–toxicity relationships, because there is not enough data on these relationships. As is seen in certain cases,[10,11] toxicity may be reduced by increasing hydrophilicity suitably. Then, we attempted to change the piperidino group on the benzene ring to pyrrolidino, 3-hydroxypyrrolidino, *N*,*N*-dimethylamino and *N*-2-hydroxyethyl-*N*-methylamino groups. The pyrrolidino compound (**5o**) retained antisecretory activity and 3-hydroxypyrrolidino compound (**5p**) was less active than **5o**. The activity of the *N*,*N*-dimethylamino compound (**5q**) was comparable to that of **5o**, but the *N*-2-hydroxyethyl-*N*-methylamino compound (**5r**) was devoid of antisecretory activity.

In conclusion, we were able to show that isosteric replacement of the methoxycarbonyl group with a 1-methyl-1*H*-tetrazol-5-yl group achieved an improvement in biological effect. Since an increase of hydrophilicity resulted in reducing antisecretory activity, toxicological study of compounds **5o**, **5p**, **5q** and **5r** was not carried out.

Experimental

Melting points were measured in a Gallenkamp melting point apparatus and are uncorrected. IR spectra were recorded on a Hitachi 260-10 Model infrared spectrophotometer and ^{1}H-NMR spectra were measured on Hitachi R-90H (90 MHz) and Bruker AM 360 (360 MHz) spectrometers with tetramethylsilane as an internal standard. Chemical shifts are given as δ values (ppm); s, singlet; d, doublet; t, triplet; q, quartet; qt, quintet; br, broad; m, multiplet. All spectra were consistent with the assigned structures. Mass spectra (MS) were obtained on a JMS-DX 300 spectrometer. Combustion analyses were performed on a Perkin–Elmer Model 240C elemental analyzer and high-resolution MS analyses were used for oily products.

Solvents used were dried over molecular sieves 4A overnight. 3-Hydroxypyrrolidine, 3-hydroxymethylphenol and 3-chloropropanamine were commercially available. Compounds **14** and **15** were prepared according to the procedure described in the literature.[8]

Typical procedures for the preparation of **5** are shown:

Method A ***N*-[3-{3-(Piperidinomethyl)phenoxy}propyl]-4-(1-methyl-1*H*-tetrazol-5-ylthio)butanamide (5f)** Amine **6**[5] (2.40 g, 9.7 mmol) and **12e** (3.15 g, 14.6 mmol) were added to a solution of $NaOCH_3$ in MeOH (sodium; 222 mg, dry MeOH; 40 ml). The mixture was refluxed for 5 h with stirring. After removal of the solvent, the residue obtained was treated with 3 N HCl and washed with Et_2O, and then made alkaline with 3 N NaOH and extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with brine, dried over $MgSO_4$ and evaporated *in vacuo* to give an oil. The oil was purified by column chromatography on silica gel with a (10 : 1) mixture of $CHCl_3$ and MeOH to give pure **5f** as an oil. Compounds **5a** and **5g**—**n** were prepared by a procedure similar to that used for **5f**.

Method B ***N*-[3-{3-Piperidinomethyl)phenoxy}propyl]-3-(1-methyl-1*H*-tetrazol-5-ylthio)propanamide (5d)** 3-(1-Methyl-1*H*-tetrazol-5-ylthio)propionyl chloride, prepared from 3-(1-methyl-1*H*-tetrazol-5-ylthio)propionic acid[12] (1.05 g, 5.6 mmol) and thionyl chloride (3 ml, 41 mmol), was added to a solution of **6** (1.24 g, 5 mmol) and pyridine (600 mg, 7.6 mmol) in CH_3CN (15 ml). The resulting mixture was stirred for 3 h at room temperature. After removal of the solvent, the obtained residue dissolved in CH_2Cl_2 was washed with saturated aqueous $NaHCO_3$ and then extracted with 1 N HCl. The aqueous layer was made alkaline with 4 N NaOH and extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with brine, dried over $MgSO_4$ and evaporated *in vacuo* to give an oil. The oil was purified by column chromatograpy on silica gel with a (14 : 1) mixture of $CHCl_3$ and MeOH to give pure **5d**. Compound **5o** was prepared by a procedure similar to that used for **5d**.

Method C ***N*-[3-{3-(Piperidinomethyl)phenoxy}propyl]-2-(1-methyl-1*H*-tetrazol-5-ylthio)acetamide (5a)** Compound **8**[1] (1.31 g, 4 mmol) and TzSH (581 mg, 5 mmol) were added to a solution of KOH (85% purity; 396 mg, 6 mmol) in MeOH (15 ml). The resulting mixture was stirred for 3 h at room temperature. After removal of the solvent, the obtained residue dissolved in CH_2Cl_2 was extracted with 1 N HCl. The aqueous layer was made alkaline with $NaHCO_3$. The oil obtained was extracted with C_6H_6, and the C_6H_6 layer was washed with brine, dried over $MgSO_4$ and evaporated *in vacuo* to give crude crystals. The crystals were recrystallized from IPE to give pure **5a**.

Method D ***N*-[3-[3-{(3-Hydroxy)pyrrolidinomethyl}phenoxy]propyl]-4-(1-methyl-1*H*-tetrazol-5-ylthio)butanamide (5p)** Chloro derivative (**10**), prepared from **11** (547 mg, 1.5 mmol) and thionyl chloride (196 mg, 1.6 mmol) in CH_2Cl_2, was added to a mixture of 3-hydroxypyrrolidine (191 mg, 2.1 mmol) and K_2CO_3 (416 mg, 3 mmol) in acetone (10 ml). The resulting mixture was refluxed for 3 h with stirring and filtered for the removal of inorganic materials. The filtrate and washing were combined and evaporated *in vacuo* to give an oil. The oil in CH_2Cl_2 was extracted with 1 N HCl, and the acidic aqueous layer was made alkaline with 3 N NaOH and extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with brine, dried over $MgSO_4$ and evaporated *in vacuo* to give an oil. The oil was purified by column chromatography on silica gel with a (10 : 1) mixture of $CHCl_3$ and MeOH to give pure **5p** as an oil. Compounds **5o**, **5q** and **5r** were prepared by a procedure similar to that used for **5p**.

***N*-[3-{(3-Hydroxymethyl)phenoxy}propyl]-4-(1-methyl-1*H*-tetrazol-5-ylthio)butanamide (11)** *N*-(3-Chloropropyl)-4-(1-methyl-1*H*-tetrazol-5-ylthio)butanamide (**19**) was prepared from 4-(1-methyl-1*H*-tetrazol-5-ylthio)butyryl chloride[12] and 3-chloropropanamine under a Schotten–Baumann reaction condition. Oily material **19**: ^{1}H-NMR ($CDCl_3$) δ: 1.86—2.58 (6H, m), 3.29—3.71 (6H, m), 3.92 (3H, s), 6.21—6.74 (1H, br s). IR ν (neat): 3300, 1650, 1540 cm^{-1}.

A mixture of **19** (278 mg, 1 mmol) with sodium (3-hydroxymethyl) phenolate, prepared from 3-hydroxymethylphenol (125 mg, 1 mmol) and NaOH (40 mg, 1 mmol) in a mixed solvent of dimethyl sulfoxide (DMSO) (3 ml) and C_6H_6 (2 ml) was heated for 3 h at 150 °C with stirring. The reaction mixture dissolved in CH_2Cl_2 was washed with H_2O, followed by 3 N HCl and brine. After removal of the solvent, the residue obtained was purified by column chromatography on silica gel with a (15 : 1) mixture of $CHCl_3$ and MeOH to give pure **11** (311 mg) as a viscous oil in an 85% yield. ^{1}H-NMR ($CDCl_3$) δ: 1.80—2.42 (6H, m), 2.49—3.68 (1H, br), 3.16—3.54 (4H, m), 3.85 (3H, s), 3.99 (2H, t, J = 6.0 Hz), 4.61 (2H, s), 6.48—7.39 (5H, m). IR ν (neat): 1655, 1440, 1260 cm^{-1}.

Method E ***N*-[3-{3-(Piperidinomethyl)phenoxy}propyl]-2-(1-methyl-1*H*-tetrazol-5-ylsulfonyl)acetamide (5b)** *m*-CPBA (80% purity; 1.19 g, 5.5 mmol) was added to a mixture of **5a** (1.03 g, 2.5 mmol) and methane sulfonic acid (244 mg, 2.5 mmol) in CH_2Cl_2 (20 ml). The mixture was stirred for 18 h at room temperature and then washed with 10% aqueous $Na_2S_2O_3$, followed by saturated aqueous $NaHCO_3$ and brine, and dried over $MgSO_4$ and evaporated *in vacuo* to give an oil. The oil was purified by column chromatography on silica gel with a (6 : 1) mixture of $CHCl_3$ and MeOH to give pure **5b** as an oil. Compound **5e** was prepared from **5d** by a procedure similar to that used for **5b**.

***N*-[3-{3-(Piperidinomethyl)phenoxy}propyl]-2-(1-methyl-1*H*-tetrazol-5-ylsulfinyl)acetamide (5c)** **5c** was prepared from **5a** (505 mg, 1.3 mmol), methane sulfonic acid (120 mg, 1.3 mmol) and *m*-CPBA (80% purity; 297 mg, 1.42 mmol) at −10 °C. Work-up by a procedure similar to that used for **5b** gave **5c**. IR and ^{1}H-NMR spectral data for **5** are shown in Table II.

***N*-(1-Methyl-1*H*-tetrazol-5-yl)-*N*-[3-{3-(piperidinomethyl)phenoxy}-propyl]acetamide (13)** 1. A solution of **6** (2.0 g, 8.0 mmol) and **12b** (1.9 g, 9.5 mmol) in toluene (40 ml) was refluxed for 5 h with stirring. After removal of the solvent, the residue obtained was purified by column chromatography on silica gel with a (10 : 1) mixture of $CHCl_3$ and MeOH to give pure **13** (2.0 g) as an oil in a 68% yield. High-resolution MS: 372.2247 (Calcd for $C_{19}H_{28}N_6O_2$: 372.2273). IR ν (neat): 1690 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.51—1.55 (2H, m, 4-CH_2 of piperidine), 1.62—1.69 (4H, m, 3,5-CH_2 of piperidine), 2.21 (2H, m, OCH_2–$\underline{CH_2}$–CH_2N), 2.41—2.49 (7H, m, $COCH_3$ and 2,6-CH_2 of piperidine), 3.52 (2H, s, $ArCH_2O$), 3.92—4.03 (5H, br s, CH_3 and CH_2N), 4.10 (2H, t, J = 6.0 Hz, OCH_2), 6.79—7.31 (4H, m, ArH).

2. A solution of **5b** (500 mg, 1.1 mmol) in toluene (50 ml) was refluxed for 13 h with stirring. Work-up by a procedure similar to that described above gave **13** (310 mg) as an oil in a 73% yield. The structure of the product was assigned on the basis of IR, ^{1}H-NMR and MS spectral data.

5-[*N*-[3-{3-(Piperidinomethyl)phenoxy}propyl]]amino-1-methyl-1*H*-tetrazole (16) A aqueous solution of **13** (500 mg) in 1 N HCl (25 ml) was

TABLE II. IR and ^{1}H-NMR Spectral Data for **5**

Compd. No.	IR[c] cm^{-1}	^{1}H-NMR ($CDCl_3$) δ (ppm)
5a[a]	k: 3300, 1640, 1565, 1265	1.39—1.47 (2H, m), 1.53—1.62 (4H, m), 1.98 (2, qt, J=6.0 Hz), 2.33—2.41 (4H, m), 3.43 (2H, s), 3.47 (2H, q, J=6.0, 6.5 Hz), 3.90 (3H, s), 3.93 (2H, s), 3.98 (2H, t, J=6.0 Hz), 6.71—7.23 (5H, m)
5b[a]	c: 1690, 1355, 1260	1.40—1.78 (6H, m), 2.01—2.27 (2H, m), 2.31—2.65 (4H, m), 3.38—3.67 (4H, m), 3.97 (3H, s), 4.05 (2H, t, J=6.0 Hz), 4.32 (2H, s), 6.63—7.58 (5H, m)
5c[a]	n: 1660, 1445, 1255, 1065	1.38—1.74 (6H, m), 1.74—2.13 (2H, m), 2.23—2.57 (4H, m), 3.27—3.73 (2H, m), 3.48 (2H, s), 3.99 (2H, t, J=6.0 Hz), 4.26 (3H, s), 4.37 (2H, dd, J=4.0 Hz), 6.62—7.53 (5H, m)
5d[a]	c: 3440, 1665, 1520, 1260	1.39—1.50 (2H, m), 1.54—1.66 (4H, m), 1.99 (2H, qt, J=6.3 Hz), 2.37—2.49 (4H, m), 2.79 (2H, t, J=6.5 Hz), 3.47 (2H, q, J=6.3 Hz), 3.48 (2H, s), 3.60 (2H, t, J=6.5 Hz), 3.86 (3H, s), 4.02 (2H, t, J=6.3 Hz), 6.47 (1H, br s), 6.64—7.21 (4H, m)
5e[b]	c: 3420, 1660, 1340, 1260, 1135	1.21—2.44 (12H, m), 2.84—3.35 (6H, m), 3.80—4.15 (4H, m), 4.34 (3H, s), 6.68—7.31 (4H, m), 8.01—8.22 (1H, br)
5f[a]	n: 3300, 1650, 1440, 1260	1.38—1.48 (2H, m), 1.52—1.63 (4H, m), 2.02 (2H, qt, J=6.0 Hz), 2.17 (2H, qt, J=7.0 Hz), 2.32—2.44 (6H, m), 3.38 (2H, t, J=7.0 Hz), 3.45 (2H, s), 3.48 (2H, q, J=6.0 Hz), 3.90 (3H, s), 4.04 (2H, t, J=6.0 Hz), 6.38—7.22 (5H, m)
5g[a]	k: 3320, 1640, 1580, 1345	1.43—1.53 (2H, m), 1.61—1.70 (4H, m), 1.99 (2H, qt, J=6.0 Hz), 2.25 (2H, qt, J=7.0 Hz), 2.44 (2H, t, J=7.0 Hz), 2.48—2.56 (4H, m), 3.45 (2H, q, J=6.0 Hz), 3.57 (2H, s), 3.78 (2H, t, J=7.0 Hz), 4.06 (2H, t, J=6.0 Hz), 4.34 (3H, s), 6.43 (1H, br s), 6.78—7.23 (4H, m)
5h[b]	n: 3290, 1645, 1445, 1365	1.37—1.66 (6H, m), 2.02 (2H, qt, J=6.0 Hz), 2.26—2.48 (4H, m), 3.10 (2H, t, J=7.0 Hz), 3.30 (2H, s), 3.44 (2H, s), 3.52 (2H, q, J=6.0 Hz), 3.85 (3H, s), 4.07 (2H, t, J=6.0 Hz), 4.49 (2H, t, J=7.0 Hz), 6.68—7.33 (5H, m)
5i[b]	n: 3400, 1665, 1275, 1255, 1110	1.29—1.72 (6H, m), 2.03 (2H, qt, J=6.0 Hz), 2.26—2.48 (4H, m), 3.43 (2H, s), 3.55 (4H, t, J=6.0 Hz), 3.88 (3H, s), 3.90—4.17 (4H, m), 4.00 (2H, s), 6.65—7.28 (5H, m)
5j[b]	n: 3320, 1650, 1445, 1255	1.33—1.74 (6H, m), 2.02 (2H, qt, J=6.0 Hz), 2.27—2.53 (4H, m), 3.08 (4H, br s), 3.23 (2H, s), 3.47 (2H, s), 3.51 (2H, q, J=6.0 Hz), 3.98 (3H, s), 4.07 (2H, t, J=6.0 Hz), 6.71—7.34 (5H, m)
5k[b]	c: 3320, 1685, 1475, 1265	1.38—1.72 (6H, m), 2.03 (2H, qt, J=6.0 Hz), 2.22—2.46 (4H, m), 3.43 (2H, s), 3.46 (2H, q, J=6.0 Hz), 3.88 (2H, s), 3.99 (2H, t, J=6.0 Hz), 4.07 (3H, s), 6.64—7.59 (5H, m)
5l[b]	n: 3290, 1655, 1485, 1260	1.38—1.69 (6H, m), 1.99 (2H, qt, J=6.0 Hz), 2.23—2.48 (4H, m), 3.43 (2H, s), 3.49 (2H, q, J=6.0 Hz), 3.87 (2H, s), 4.02 (2H, t, J=6.0 Hz), 4.28 (3H, s), 6.67—7.31 (5H, m)
5m[b]	n: 3290, 1685, 1550, 1445, 1255	1.33—1.71 (6H, m), 2.12 (2H, qt, J=6.0 Hz), 2.29—2.47 (4H, m), 3.43 (2H, s), 3.71 (2H, q, J=6.0 Hz), 4.11 (2H, t, J=6.0 Hz), 4.36 (3H, s), 6.70—7.32 (4H, m), 7.89—8.18 (1H, br)
5n[b]	n: 3320, 1680, 1545, 1440, 1250	1.31—1.73 (6H, m), 2.13 (2H, qt, J=6.0 Hz), 2.23—2.48 (4H, m), 3.44 (2H, s), 3.74 (2H, q, J=6.0 Hz), 4.13 (2H, t, J=6.0 Hz), 4.42 (3H, s), 6.70—7.30 (4H, m), 7.52—7.83 (1H, br)
5o[b]	c: 3450, 1665, 1515, 1450, 1265	1.67—2.73 (14H, m), 3.29—3.63 (6H, m), 3.89 (3H, s), 4.05 (2H, t, J=6.0 Hz), 6.38—6.61 (1H, br), 6.69—7.32 (4H, m)
5p[b]	c: 3450, 1660, 1450, 1265	1.92—2.63 (12H, m), 2.99 (1H, br), 3.27—3.59 (6H, m), 3.88 (3H, s), 4.03 (2H, t, J=6.0 Hz), 4.19—4.42 (1H, m), 6.54—7.34 (5H, m)
5q[b]	n: 3300, 1645, 1445, 1265	1.84—2.48 (6H, m), 2.23 (6H, s), 3.25—3.67 (6H, m), 3.89 (3H, s), 4.05 (2H, t, J=6.0 Hz), 6.18—6.42 (1H, br), 6.63—7.38 (4H, m)
5r[b]	n: 3280, 1645, 1445, 1260	1.94—2.64 (12H, m), 3.29—3.68 (8H, m), 3.89 (3H, s), 4.04 (2H, t, J=6.0 Hz), 6.38—6.57 (1H, br), 6.67—7.29 (4H, m)

a) 360 MHz. *b*) 90 MHz. *c*) c: $CHCl_3$, k: KBr, n: neat.

stirred at room temperature for 30 min. The resulting mixture was made alkaline with 10% NaOH, and extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with H_2O and dried over $MgSO_4$. After removal of the solvent, the crystalline materials obtained were recrystallized from a mixture of C_6H_6 and hexane to give pure **16** (440 mg) in a quantitative yield. mp 124.5 °C. *Anal.* Calcd for $C_{17}H_{26}N_6O$: C, 61.79; H, 7.93; N, 25.44. Found: C, 61.96; H, 8.03; N, 25.17. IR ν (KBr): 3280 (NH), 1630 (C=N) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.42—1.46 (2H, m, 4-CH_2 of piperidine), 1.53—1.59 (4H, m, 3,5-CH_2 of piperidine), 2.16 (2H, qt, J=6.0 Hz, OCH_2-$\underline{CH_2}$-CH_2N), 2.31—2.41 (4H, m, 2,6-CH_2 of piperidine), 3.42 (2H, s, $ArCH_2$), 3.67 (2H, q, J=6.0 Hz, CH_2NH), 3.73 (3H, s, CH_3), 4.09 (2H, t, J=6.0 Hz, OCH_2), 5.47 (1H, t, J=6.0 Hz, NH), 6.70—7.20 (4H, m, ArH).

Ethyl 3-[*N*-[3-{3-(Piperidinomethyl)phenoxy}propyl]aminopropionate (17) 1. A mixture of **6** (418 mg, 1.7 mmol) and ester **12c** (410 mg, 1.9 mmol) in toluene (5 ml) was stirred for 4 h in refluxing toluene. The resulting mixture was extracted twice with 3 N HCl. The acidic aqueous layer was made alkaline with 4 N NaOH and extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with H_2O, followed by brine, dried over $MgSO_4$ and evaporated *in vacuo* to give an oil. The oil was purified by column chromatography on silica gel with a (10 : 1) mixture of $CHCl_3$ and MeOH to give pure **17** (223 mg) as an oil in a 38% yield. High-resolution MS: 348.2420 (Calcd for $C_{20}H_{32}N_2O_3$: 348.2413). MS m/z 348 (M^+). IR ν (neat): 2925, 1725 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.25 (3H, t, J=7.0 Hz), 1.41—1.44 (2H, m), 1.54—1.63 (4H, m), 1.96 (2H, q, J=6.7, 6.0 Hz), 2.36—2.42 (4H, m), 2.52 (2H, t, J=6.4 Hz), 2.82 (2H, t, J=6.7 Hz), 2.91 (2H, t, J=6.4 Hz), 3.46 (2H, s), 4.03 (2H, t, J=6.0 Hz), 4.13 (2H, q, J=7.0 Hz), 6.74—7.23 (4H, m).

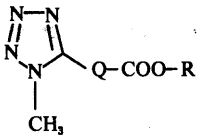
2. A mixture of **6** (1.03 g, 4.1 mmol), ester **12c** (1.80 g, 8.3 mmol) and C_2H_5ONa [Na (95 mg)] in dry EtOH (20 ml) was stirred for 1 h at room temperature. After removal of the solvent, the residue obtained was dissolved in 3 N HCl and extracted with CH_2Cl_2. Work-up in a procedure similar to procedure 1 gave **17** (608 mg) in a 42% yield.

3. The reaction of **6** (703 mg, 2.8 mmol) with ester **12d** (781 mg, 3.1 mmol) in toluene (10 ml) according to procedure 1 gave **17** (271 mg) in a 27% yield.

4. A mixture of **6** (146 mg, 0.59 mmol) and ethyl acrylate (60 mg, 0.61 mmol) in toluene (5 ml) was stirred for 10 h at 70 °C. Work-up in procedure 1 gave **17** (119 mg) in a 58% yield. Compound **17**, prepared by procedures 2—4, was assinged on the basis of IR, ^{1}H-NMR and MS spectral data, compared with those of **17** prepared by procedure 1.

***N*-[3-{3-(Piperidinomethyl)phenoxy}propyl]acrylamide (18)** A mixture of TzSH (581 mg, 5 mmol) and 60% NaH (203 mg, 5 mmol) in dry dimethylformamide (DMF) (10 ml) was stirred for 10 min at room temperature. A mixture of **9** (1.52 g, 4.5 mmol) and KI (747 mg, 4.5 mmol) in DMF (10 ml) was added dropwise to the above mixture at room temperature. The resulting mixture was stirred for 5 h at 70 °C and evaporated *in vacuo* to give an oil. The oil dissolved in C_6H_6 was extracted with 1 N HCl. The acidic aqueous layer was made alkaline with 4 N NaOH and extracted C_6H_6. The C_6H_6 layer was washed with brine and H_2O, dried over $MgSO_4$ and evaporated *in vacuo* to crude oil **18**, which was purified by column chromatography on silica gel with a (5 : 1) mixture of $CHCl_3$ and MeOH to give pure **18** (1.21 g) as an oil in an 88% yield. High-resolution MS: 302.2009 (Calcd for $C_{18}H_{26}N_2O_2$: 302.1994). MS m/z (M^+) 302. IR ν (neat): 3270, 2420, 1650, 1440 cm^{-1}. ^{1}H-NMR

TABLE III. 1-Methyl-1*H*-tetrazolylalkanoic Acid Ester (**12**)



Compd. No.	R	Q	Formula	Ref.
12a	CH_3	CH_2S–[a]	$C_5H_8N_4O_2S$	2
12b	CH_3	CH_2SO_2–[a]	$C_5H_8N_4O_4S$	2
12c	C_2H_5	CH_2CH_2S–[a]	$C_7H_{12}N_4O_2S$	12
12d	C_2H_5	$CH_2CH_2SO_2$–[a]	$C_7H_{12}N_4O_4S$	This work
12e	CH_3	$CH_2CH_2CH_2S$–[a]	$C_7H_{12}N_4O_2S$	12
12f	CH_3	$CH_2CH_2CH_2SO_2$–[a]	$C_7H_{12}N_4O_4S$	This work
12g	CH_3	CH_2	$C_5H_8N_4O_2$	13
12h[b]	CH_3	CH_2	$C_5H_8N_4O_2$	13
12i	CH_3	$CH_2SCH_2CH_2S$–[a]	$C_7H_{12}N_4O_2S_2$	This work
12j	CH_3	$CH_2OCH_2CH_2S$–[a]	$C_7H_{12}N_4O_3S$	This work
12k	CH_3	$CH_2SCH_2CH_2$–[a]	$C_7H_{12}N_4O_2S$	12
12l	C_2H_5	—[c]	$C_5H_8N_4O_2$	8
12m[b]	C_2H_5	—[c]	$C_5H_8N_4O_2$	8

a) Binding site to tetrazole ring. *b*) 1*H*-2-Methyltetrazole analogue. *c*) Direct binding to tetrazole ring.

($CDCl_3$) δ: 1.43—1.46 (2H, m), 1.55—1.60 (4H, m), 2.05 (2H, dd, J=6.0 Hz), 2.38 (4H, br s), 3.44 (2H, s), 3.54 (1H, t, J=6.0 Hz), 3.56 (1H, t, J=6.0 Hz), 4.07 (2H, t, J=6.0 Hz), 5.63 (1H, dd, J=10.2 Hz), 6.10 (1H, dd, J=10.2 Hz), 6.06—6.13 (1H, br s), 6.26 (1H, dd, J=1.8, 17.2 Hz), 6.77—7.21 (4H, m).

Preparation of 1-Methyl-1*H*-tetrazolylalkanoic Acid Esters 12 Methyl 4-(1-Methyl-1*H*-tetrazol-5-ylsulfonyl)butyrate (12f) A solution of **12e** (2.17 g, 0.01 mol) and *m*-CPBA (4.33 g, 0.02 mol) in C_6H_6 (40 ml) was stirred for 1 h at 70 °C. The reaction mixture was allowed to stand overnight at room temperature, yielding crystalline materials. After removal of the crystalline materials by filtration, the C_6H_6 layer was washed with 10% $Na_2S_2O_3$, followed by saturated aqueous $NaHCO_3$ and brine and dried over $MgSO_4$. After removal of the solvent, the residue obtained was purified by column chromatography with a (10 : 1) mixture of $CHCl_3$ and MeOH to give pure **12f** (2.36 g) in a 95% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.26 (2H, q, J=7.0 Hz), 2.58 (2H, t, J=7.0 Hz), 3.70 (3H, s), 3.78 (2H, t, J=7.0 Hz), 4.36 (3H, s). IR ν (neat): 1730, 1340, 1145 cm^{-1}.

Ethyl 3-(1-Methyl-1*H*-tetrazol-5-ylsulfonyl)propionate (12d) 12d was prepared in a 65% yield from **12c** and *m*-CPBA by a procedure similar to that used for **12f**.

Methyl 2-[2-(1-Methyl-1*H*-tetrazol-5-ylthio)ethoxy]acetate (12j) A mixture of 5-(2-hydroxyethylthio)-1-methyl-1*H*-tetrazole[12] (2.23 g, 0.014 mol) and NaH (60% purity; 561 mg, 0.014 mol) in dry DMF (20 ml) was stirred for 10 min at room temperature. Then, methyl 2-bromoacetate (2.30 g, 0.015 mol) was added to the reaction mixture. The resulting mixture was stirred for 2 h at room temperature and poured into ice-water. The mixture was made acidic with 1 N HCl, and extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with brine and evaporated to give **12j** (1.94 g) as an oil in a 60% yield. ^{1}H-NMR ($CDCl_3$) δ: 3.57 (2H, t, J=5.5 Hz), 3.75 (3H, s), 3.93 (2H, t, J=5.5 Hz), 3.94 (3H, s), 4.13 (2H, s). IR ν (neat) 1750, 1275, 1210, 1130 cm^{-1}.

Methyl 2-[2-(1-Methyl-1*H*-tetrazol-5-ylthio)ethylthio]acetate (12i) 12i was prepared from 5-(2-chloroethylthio)-1-methyl-1*H*-tetrazole[12] (100 mg, 0.56 mmol) and methyl thioglycolate (73 mg, 0.68 mmol) by a procedure similar to that used for **12j** in a 78% yield. ^{1}H-NMR ($CDCl_3$) δ: 3.18 (2H, t, J=7.0 Hz), 3.34 (2H, s), 3.76 (3H, s), 3.90 (3H, s), 4.54 (2H, t, J=7.0 Hz). IR ν (neat): 1735, 1365, 1300 cm^{-1}.

Methyl 2-(1-methyl-1*H*-tetrazol-5-ylthio)acetate (**12a**)[2] and its sulfonyl (**12b**),[2] ethyl 3-(1-methyl-1*H*-tetrazol-5-ylthio)propionate (**12c**),[12] 4-(1-methyl-1*H*-tetrazol-5-ylthio)butyrate (**12e**),[12] 1-methyl- and 2-methyl-5-methoxycarbonylmethyl-1*H*-tetrazoles (**12g** and **12h**),[13] 2-[2-(1-methyl-1*H*-tetrazol-5-ylethyl)thio]acetate (**12k**)[12] and 1-methyl- and 2-methyl-5-ethoxycarbonyl-1*H*-tetrazoles (**12l** and **12m**)[8] were prepared by procedures descibed in the literature, respectively. The structure of **12** is shown in Table III.

Biological Method Gastric acid antisecretory activity in rats was tested by the reported method.[1]

Acknowledgements We would like to express our thanks to the Material Analytical Center of ISIR, Osaka University, for spectral measurement and microanalyses.

References

1) Part I: I. Ueda, K. Ishii, K. Shinozaki, M. Seiki, H. Arai and M. Hatanaka, *Chem. Pharm. Bull.*, **38**, 3035 (1990).
2) K. Shinozaki, K. Ishii and I. Ueda, Japan. Patent 63-165823 (1988) [*Chem. Abstr.*, **113**, 132008*v* (1990)].
3) C. W. Thornber, *Chem. Soc. Rev.*, **8**, 563 (1979).
4) P. F. Juby and T. W. Hudyma, *J. Med. Chem.*, **12**, 396, (1969).
5) J. W. Clitherow, J. Bradshaw, J. W. Mackinnon, B. J. Price, M. Martin-Smith and D. B. Judd, DE 2917026 (1980) [*Chem. Abstr.*, **92**, 181197*n* (1980)].
6) M. Martin-Smith, B. J. Price, J. W. Clitherow and J. Bradshaw, DE 2821410 (1979) [*Chem. Abstr.*, **90**, 121258*g* (1979)].
7) I. Ueda, M. Nagano and A. Akabane, Jpn. Kokai Tokkyo Koho JP 58 90569 (1983) [*Chem. Abstr.*, **99**, 175769 (1983)]; L. Carey, B. J. Price, J. W. Clitherow, J. Bradshaw and M. Martin-Smith, Brit. UK Patent Appl. GB 2082584 (1980) [*Chem. Abstr.*, **97**, 55813*a* (1982)].
8) R. J. Spear, *Aust. J. Chem.*, **37**, 2453 (1984).
9) K. Ishii, M. Hatanaka and I. Ueda, submitted for publication in *Bull. Chem. Soc. Jpn.*
10) T. Takashima, Y. Kadoh and S. Kumada, *Arzneim.-Forsch.*, (*Drug. Res.*) **22**, 711 (1973).
11) S. D. Nelson, Alteration of drug metabolism through structural modification in "Design of Biopharmaceutical Properties through Prodrugs and Analogs," ed. by E. B. Roche, American Pharmaceutical Association, Washington DC, 1977, pp. 316—343.
12) M. Uchida, M. Komatsu, S. Morita, T. Kanbe and K. Nakagawa, *Chem. Pharm. Bull.*, **37**, 322 (1989).
13) R. Raap and J. Howard, *Can. J. Chem.*, **47**, 813 (1969).

A New Group of Antibiotics, Hydroxamic Acid Antimycotic Antibiotics. IV.[1] Structures of Enactins Ia, Ib_1, Ib_2 and Va

Katsumi YAMAMOTO,[a] Yoshikazu SHIINOKI,[a] Jun FURUKAWA[b] and Shoshiro NAKAMURA*,[a]

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine,[a] 1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan and Institute of Applied Microbiology, University of Tokyo,[b] 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan. *Received November 26, 1990*

Structures of enactins Ia, Ib_1, Ib_2 and Va were determined by spectroscopic studies using their bis-2,4-dinitrophenyl derivatives. Both enactins and neoenactins contain L-serine to form the hydroxamic acid structure. Their physico-chemical and biological properties are closely related. The structures of neoenactin congeners have been previously reported and enactins Ia and Va are proved to be 19-hydroxyneoenactin B_2 and 14-dihydroneoenactin M_1, respectively. Both enactins Ib_1 and Ib_2 are determined to be 19-hydroxyneoenactin B_1 and considered to be diastereoisomers at the 19-position, though their steric structures at this position remain to be determined.

Keywords enactin; neoenactin; hydroxamic acid; antimycotic antibiotics; potentiator; *Streptomyces roseoviridis*

Enactins (ENs) produced by *Streptomyces roseoviridis*[1,2] and neoenactins (NEs) produced by *Streptoverticillium olivoreticuli*[3,4] are antimycotic antibiotics discovered as potentiators for polyene antifungal antibiotics (PAA) by our simple and multipurpose screening method, taking advantage of the ability of sterols to reverse the fungicidal activity of PAA.[5] Both ENs and NEs contain L-serine to form the hydroxamic acid structure (see Fig. 1) and have quite similar physico-chemical and biological properties, though NEs are more hydrophobic and more active against fungi and yeasts. Thus, we have proposed the group name "hydroxamic acid antimycotic antibiotics (HAAA)" for ENs, NEs and other related antibiotics.[6,7]

As reported in earlier, we have isolated several EN congeners called EN-Ia, -Ib, -IIc and -Va as their free bases by repetitive reverse-phase high performance liquid chromatography (HPLC) and revealed their physico-chemical and biological properties.[1]

In this paper, we will describe the separation of EN-Ib into EN-Ib_1 and -Ib_2 by HPLC as their bis-1,2-dinitrophenyl (DNP) derivatives and the structures of EN-Ia, -Ib_1, -Ib_2 and -Va elucidated by studying proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra and MS data of their DNP derivatives.

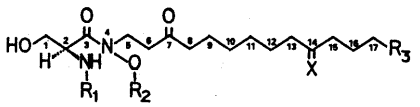
The molecular formulae of EN-Ia ($C_{20}H_{38}N_2O_6$), -Ib_1 ($C_{20}H_{38}N_2O_6$), -Ib_2 ($C_{20}H_{38}N_2O_6$) and -Va ($C_{19}H_{38}N_2O_5$) were determined by the fast atom bombardment mass spectra (FAB-MS) data and ^{1}H- and ^{13}C-NMR spectra of their DNP derivatives.

The structures of NEs were previously elucidated as

TABLE I. ^{1}H-NMR Data[a] ($CDCl_3$) of DNP Derivatives of Enactin-Ia, -Ib_1 and -Ib_2

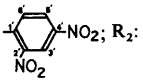
Assignment (Position)	Chemical shift (δ, ppm)				
	DNP-EN-Ia (27 °C)	DNP-NE-B_2	DNP-EN-Ib_1 (27 °C)	DNP-EN-Ib_2 (27 °C)	DNP-NE-B_1
$CHCH_3$ (20, 21)			0.88 (3H, d, J=6.9, H-21)	0.87 (3H, d, J=6.9, H-21)	0.85
			1.13 (3H, d, J=6.5, H-20)	1.12 (3H, d, J=6.2, H-20)	
CH_2CH_3 (20)					0.85
$C(CH_3)_2$ (20, 21)	1.12 (6H, s)				
$CH(CH_3)_2$ (20, 21)		0.75			
CH_2 (18, 19)		1.16 (H-18)			1.12 (H-19)
CH_2 and CH (10, 11, 17, 18, 19)	1.27 (7H, m, H-10, 11, 17, 18)	1.27 (H-10, 11, 17, 19)	1.26 (7H, m, H-10, 11, 17, 18)	1.26 (7H, m, H-10, 11, 17, 18)	1.28 (H-10, 11, 17, 18)
CH_2CH_2CO (9, 12, 16)	1.54 (6H, m)	1.54	1.54 (6H, m)	1.54 (6H, m)	1.54
CH_2CO (8, 13, 15)	2.42 (6H, m)	2.40	2.41 (6H, m, H-8, 13, 15)	2.41 (6H, m, H-8, 13, 15)	2.40
NCH_2CH_2CO (6)	2.84 (2H, t, J=5.2)	2.84	2.84 (2H, t, J=5.0)	2.84 (2H, t, J=5.5)	2.84
CHOH (19)			3.75 (1H, dq, J=4.2, 6.5)	3.63 (1H, dq, J=6.2, 6.2)	
NCH_2CH_2CO (5)	4.01 (1H, dt, J=14.5, 5.2)	4.01	4.02 (1H, dt, J=14.5, 5.0)	4.01 (1H, dt, J=15.0, 5.5)	4.02
	4.28 (1H, dt, J=14.5, 5.2)	4.28	4.27 (1H, dt, J=14.5, 5.0)	4.28 (1H, dt, J=15.0, 5.5)	4.26
$HOCH_2$ (1)	4.09 (1H, ddd, J=4.5, 4.5, 11.0)	4.09	4.10 (1H, ddd, J=5.2, 5.2, 11.5)	4.10 (1H, ddd, J=5.2, 5.2, 11.5)	4.09
	4.17 (1H, ddd, J=4.5, 4.5, 11.0)	4.18	4.17 (1H, ddd, J=5.2, 5.2, 11.5)	4.16 (1H, ddd, J=5.2, 5.2, 11.5)	4.16
NHCHCO (2)	4.86 (1H, br, m)	4.86	4.85 (1H, br, m)	4.86 (1H, br, m)	4.85
H-Ar (6′)	6.88 (1H, d, J=9.0)	6.88	6.88 (1H, d, J=9.2)	6.88 (1H, d, J=9.0)	6.85
H-Ar (6″)	7.60 (1H, d, J=9.0)	7.61	7.60 (1H, d, J=9.2)	7.61 (1H, d, J=9.0)	7.57
H-Ar (5′)	8.27 (1H, dd, J=2.7, 9.0)	8.26	8.27 (1H, dd, J=2.6, 9.2)	8.26 (1H, dd, J=2.6, 9.0)	8.25
H-Ar (5″)	8.52 (1H, dd, J=2.7, 9.0)	8.52	8.52 (1H, dd, J=2.7, 9.2)	8.52 (1h, dd, J=2.6, 9.0)	8.50
H-Ar (3′)	9.12 (1H, d, J=2.7)	9.11	9.12 (1H, d, J=2.6)	9.12 (1H, d, J=2.6)	9.12
H-Ar (3″)	8.93 (1H, d, J=2.7)	8.95	8.94 (1H, d, J=2.7)	8.95 (1H, d, J=2.6)	8.95
–CNHAr	9.17[b] (1H, d, J=8)	9.18[b]	9.17[b] (1H, d, J=8)	9.17[b] (1H, d, J=8)	9.16[b]

a) ^{1}H-NMR spectrum was recorded on a JEOL GX 400 and JEOL GX 500 spectrometer. Tetramethylsilane (TMS (0 ppm)) was used as an internal standard. Number of protons, multiplicity, coupling constants in Hz and position of protons where necessary are indicated in parenthesis. *b*) Temperature dependent.



	R_1	R_2	X	R_3
EN-Ia	H	H	=O	
EN-Ib_1	H	H	=O	
EN-Ib_2	H	H	=O	
EN-Va	H	H		
NE-A	H	H	=O	
NE-M_1	H	H	=O	
NE-M_2	H	H		
NE-B_1	H	H	=O	
NE-B_2	H	H	=O	
NE-NL_1	H	H	=O	
NE-NL_2	H	H	=O	

Fig. 1. Structures of Enactin, Neoenactin and Their DNP Derivatives[a)]

a) DNP derivatives, R_1:  R_2:

shown in Fig. 1 by studying ^{1}H- and ^{13}C-NMR spectra and MS data of their DNP derivatives.[4,8)] Thus, EN-Ia, -Ib and -Va were converted to DNP derivatives to elucidate their structures as done for NEs.

In the ^{1}H-NMR spectrum of DNP-Ib, unexplainable proton signals were observed at δ 3.63 (approximately 0.5H, dq) and δ 3.75 (approximately 0.5H, dq) and the two protons were not coupled to each other. Thus, DNP-Ib was suggested to be composed of almost equal amounts of the two closely related isomers and further separated by normal phase HPLC to the isomers designated DNP-EN-Ib_1 and -Ib_2.

^{1}H-NMR data of DNP-EN-Ia, -Ib_1 and -Ib_2 are listed in Table I together with those of DNP-NE-B_2 and -B_4[c)] and the structures of EN congeners are shown in Fig. 1 together with those of NE-congeners. EN-Ia, -Ib_1 and -Ib_2 have the same molecular formula ($C_{20}H_{38}N_2O_6$) containing one more extra oxygen atom than NE-B_1, -B_2 and -NL_2 ($C_{20}H_{38}N_2O_5$). Since EN-Ia and NE-B_2 have geminal methyl groups, EN-Ia is presumed to be a hydroxyl derivative of NE-B_2. In the ^{1}H-NMR data, the geminal methyl doublets at δ 0.75 (6H, J=6.5, H-20, -21) of DNP-NE-B_2 appear as the singlets at δ 1.12 (6H, H-20, -21) in DNP-EN-Ia by substitution of the hydroxyl group at 19-position as seen in Table I. Further, the presence of a hydroxyl group at 19-position in EN-Ia is advocated by the low field shift of methylene protons at δ 1.16 (2H, m, H-18) of DNP-NE-B_2 to δ 1.27 in DNP-EN-Ia. Other ^{1}H-NMR data of DNP-EN-Ia and DNP-NE-B_2 are substantially identical to each other from H-1 through H-17. Thus, the structure of EN-Ia is elucidated to be 19-hydroxy-NE-B_2.

NE-B_1 has the ante-iso type methyl groups and the triplet at δ 0.85 (3H, J=6.5) and the doublet at δ 0.85 (3H, J=6.5) in ^{1}H-NMR data of DNP-NE-B_1 are assigned to H-20 and -21, respectively, as seen in Table I.[4c)] EN-Ib_1 also has the ante-iso type methyl groups and is considered to be a hydroxyl derivative of NE-B_1. The triplet at δ 0.85 of DNP-NE-B_1 apppears as the doublet at δ 1.13 (3H, J=6.5)

TABLE II. ^{1}H-NMR Data[a)] ($CDCl_3$) of DNP Derivatives of Enactin-Va

Assignment (Position)	Chemical shift (δ, ppm)	
	DNP-EN-Va (50 °C)	DNP-NE-M_2
$CH_2\underline{C}H_3$ (20)		0.87
$CH(\underline{C}H_3)_2$ (19,20)	0.89 (6H, d, J=6.8)	
CH_2 and CH (10, 11, 12, 13, 15, 17, 18, 19)	1.20—1.43 (15H, m, H-10, 11, 12, 13, 15, 16, 17, 18)	1.21—1.46 (H-10, 11, 12, 13, 15, 16, 17, 18, 19)
$C\underline{H}_2CH_2CO$ (9)	1.55 (2H, m)	1.54
CH_2CO (8)	2.43 (2H, t, J=7.4)	2.42
$NCH_2C\underline{H}_2CO$ (6)	2.84 (2H, t, J=5.9)	2.83
$C\underline{H}OH$ (14)	3.59 (1H, br)	3.58
$NC\underline{H}_2CH_2CO$ (5)	4.04 (1H, dt, J=14.5, 5.9)	4.00
	4.26 (1H, dt, J=14.5, 5.9)	4.27
$HOC\underline{H}_2$ (1)	4.09 (1H, ddd, J=5.2, 5.2, 11.5)	4.07
	4.13 (1H, ddd, J=5.2, 5.2, 11.5)	4.14
$NHC\underline{H}CO$ (2)	4.87 (1H, br, m)	4.84
$\underline{H}$-Ar (6′)	6.90 (1H, d, J=9.1)	6.87
$\underline{H}$-Ar (6″)	7.58 (1H, d, J=9.1)	7.59
$\underline{H}$-Ar (5′)	8.26 (1H, dd, J=2.8, 9.1)	8.26
$\underline{H}$-Ar (5″)	8.50 (1H, dd, J=2.8, 9.1)	8.51
$\underline{H}$-Ar (3′)	9.09 (1H, d, J=2.8)	9.11
$\underline{H}$-Ar (3″)	8.92 (1H, d, J=2.8)	8.94
$-CN\underline{H}Ar$	9.08[b)] (1H, d, J=8)	9.15[b)]

a) ^{1}H-NMR spectrum was recorded on a JEOL GX 400 and JEOL GX 500 spectrometer. TMS (0 ppm) was used as an internal standard. Number of protons, multiplicity, coupling constants in Hz and position of protons where necessary are indicated in parenthesis. *b)* Temperature dependent.

in DNP-EN-Ib_1 by substitution of the hydroxyl group at β-position. The other methyl doublet at δ 0.85 (3H, J=6.5) in DNP-NE-B_1 is shifted to δ 0.88 (3H, d, J=6.9) in DNP-EN-Ib_1. Further, the methylene multiplet at δ 1.12 (2H, H-19) of DNP-NE-B_1 is shifted to lower field (δ 3.75, dq, J=4.2, 6.5) by the hydroxyl group at 19-position in DNP-EN-Ib_1. ^{1}H-NMR data of DNP-EN-Ib_1 from H-1 through H-17 are almost identical with those of DNP-NE-B_1. Thus, the structure of EN-$Ib_1$is elucidated to be 19-hydroxy-NE-B_1. EN-Ib_1 and -Ib_2 are closely related isomers and ^{1}H-NMR data of the two DNP derivatives are essentially the same except that the double quartet at δ 3.75 (1H, J=4.2, 6.5) in DNP-EN-Ib_1 is shifted to δ 3.63 (1H, dq, J=6.2, 6.2) in DNP-EN-Ib_2. Consequently, EN-Ib_2 is postulated to be the diastereoisomer of EN-Ib_1 at 19-position, though the steric configuration at this position remains to be solved.

EN-Va has the same molecular formula as NE-M_2 ($C_{19}H_{38}N_2O_5$). ^{1}H-NMR data of DNP-EN-Va and their assignments are listed in Table II together with those of DNP-NE-M_2. The existence of geminal methyl groups in DNP-EN-Va is shown by the methyl doublets at δ 0.89 (6H, J=6.8, H-19, -20) instead of the methyl triplet at δ 0.87 (3H, J=6.8, H-20) for DNP-NE-M_2. Further, fifteen methylene and methine protons exist as multiplets at δ 1.20—1.43 in ^{1}H-NMR data of DNP-EN-Va. On the other hand, eighteen methylene protons are observed in the same region in DNP-NE-M_2. Essentially no other difference is observed in ^{1}H-NMR data of both DNP derivatives from H-1 through H-18, as seen in Table II. Thus, the structure of EN-Va is determined to be 14-dihydro-NE-M_1 (=14-dihydrolipoxamycin).

^{13}C-NMR data of DNP-EN-Ia, -Ib_1 and -Ib_2 and their assignments are listed in Table III together with those of

TABLE III. ^{13}C-NMR Data[a] ($CDCl_3$) of DNP Derivatives of Enactin-Ia, -Ib_1 and -Ib_2

Position	Chemical shift (δ, ppm)						
	DNP-EN-Ia		DNP-NE-B_2	DNP-EN-Ib_1	DNP-EN-Ib_2		DNP-NE-B_1
	Exptl.	Calcd	Exptl.	Exptl.	Exptl.	Calcd	Exptl.
C-1	62.11		62.11	62.10	62.11		62.11
C-2	56.26		56.17	56.25	56.26		56.19
C-3	172.5		172.5	172.5	172.5		172.5
C-5	43.5		43.5	43.5	43.5		43.5
C-6	38.82		38.76	38.82	38.82		38.76
C-7	208.33		208.30	208.32	208.35		208.34
C-8	42.86[b]		42.86[b]	42.84[b]	42.84[b]		42.86[b]
C-9	23.39[c]		23.36[c]	23.36[c]	23.37[c]		23.38[c]
C-10	28.69[d]		28.72	28.67[d]	28.67[d]		28.73
C-11	28.67[d]		28.72	28.66[d]	28.65[d]		28.73
C-12	23.21[c]		23.20[c]	23.19[c]	23.20[c]		23.20[c]
C-13	42.57[b]		42.53[b]	42.60[b]	42.60[b]		42.53[b]
C-14	211.73		211.99	211.84	211.93		212.02
C-15	42.76		42.96	42.99	42.92		42.23
C-16	24.30		24.14	21.52	21.01		21.47
C-17	23.94	22.0	27.01	32.18	32.00	31.1	36.13
C-18	43.57	46.7	38.72	39.54	40.03	42.3	34.27
C-19	70.98	68.3	27.83	70.88	71.63	70.3	29.30
C-20	29.29	30.6	22.56	20.10	19.86	19.3	11.31
C-21	29.29	30.6	22.56	14.06	14.87	14.1	19.06
C-1′	146.67[e]		146.61[d]	146.66[e]	146.67[e]		146.62[d]
C-2′	131.62[e]		131.59[d]	131.61[e]	131.62[e]		131.59[d]
C-3′	124.25		124.25	124.25	124.25		124.23
C-4′	137.19[e]		137.16[d]	137.17[e]	137.18[e]		137.15[d]
C-5′	130.50		130.49	130.49	130.50		130.48
C-6′	113.99		113.93	114.27	114.00		113.94
C-1″	154.87		154.84	154.87	154.88		154.85
C-2″	137.34[e]		137.31[d]	137.17[e]	137.34[e]		137.29[d]
C-3″	122.84		122.83	122.83	122.84		122.81
C-4″	143.06[e]		143.03[d]	143.05[e]	143.06[e]		143.03[d]
C-5″	129.90		129.89	129.90	129.90		129.89
C-6″	115.43		115.37	115.42	115.43		115.40

a) ^{13}C-NMR spectra were recorded on a JEOL GX 400 and JEOL GX 500 spectrometer. TMS (0 ppm) was used as an internal standard. *b*—*e*) Values with identical superscript within a column may be interchanged.

TABLE IV. ^{13}C-NMR Data[a] ($CDCl_3$) of DNP Derivative of Enactin-Va

Position	Chemical shift (δ, ppm)		
	DNP-EN-Va		DNP-NE-M_2
	Exptl.	Calcd	Exptl.
C-1	62.31		62.11
C-2	56.28		56.16
C-3	172.5		172.5
C-5	43.76		43.5
C-6	38.80		38.78
C-7	208.15		208.37
C-8	43.01		42.96
C-9	23.47		23.36
C-10	28.97[b]		28.88[b]
C-11	29.25[b]		29.17[b]
C-12	25.34		25.63[c]
C-13	37.93[c]		37.24[d]
C-14	72.09		72.05
C-15	37.35[c]		37.59[d]
C-16	23.47	23.3	25.26[c]
C-17	39.10	39.4	29.35[b]
C-18	27.98	27.8	31.83
C-19	22.59	21.6	22.60
C-20	22.59	21.6	14.07
C-1′	146.66[d]		146.61[e]
C-2′	131.84[d]		131.60[e]
C-3′	124.18		124.25
C-4′	137.44[d]		137.22[e]
C-5′	130.46		130.49
C-6′	113.96		113.90
C-1″	154.84		154.79
C-2″	137.44[d]		137.22[e]
C-3″	122.74		122.83
C-4″	143.22[d]		143.04[e]
C-5″	129.77		128.88
C-6″	115.41		115.33

a) ^{13}C-NMR spectra were recorded on a JEOL GX 400 and JEOL GX 500 spectrometer. TMS (0 ppm) was used as an internal standard. *b*—*e*) Values with identical superscript within a column may be interchanged.

DNP-NE-B_2 and DNP-NE-B_1.[4c] Chemical shifts from C-1 through C-16 of DNP-EN-Ia are essentially the same as those of DNP-NE-B_2, while low field shifts (α and β effects of hydroxyl group) of C-19 ($\Delta\delta_C$ 43.15 ppm), C-18 ($\Delta\delta_C$ 4.85 ppm) and C-20 ($\Delta\delta_C$ 6.73 ppm) and a high field shift (γ effect of hydroxyl group) of C-17 ($\Delta\delta_C$ 3.07 ppm) were observed on going from DNP-NE-B_2 to DNP-EN-Ia. Expected chemical shifts from C-17 through C-21 of DNP-19-hydroxy-NE-B_2 (=DNP-EN-Ia) were calculated using parameters of substituent effects[9] on ^{13}C-chemical shifts and experimental values of DNP-NE-B_2.[4c] Experimental chemical shifts from C-17 through C-21 of DNP-EN-Ia are in good agreement with those calculated for DNP-19-hydroxy-NE-B_2, as seen in Table III.

Further, ^{13}C-NMR data of DNP-EN-Ib_1 and -Ib_2 indicate that both compounds have the same structure as DNP-EN-B_1[4c] from C-1 through C-16. Experimental values from C-17 through C-21 of ^{13}C chemical shifts of DNP-EN-Ib_1 and -Ib_2 well agree with the anticipated data for DNP-19-hydroxy-NE-B_1, as seen in the same table.

^{13}C-NMR data of DNP-EN-Va and those calculated for DNP-14-dihydro-NE-M_1 are shown in Table IV in comparison with those of DNP-NE-M_2.[4c] DNP-EN-Va and DNP-NE-M_2 have the same molecular formula and DNP-EN-Va was shown to have the same structure as DNP-NE-M_2 from C-1 through C-15 by their essentially common chemical shifts. The existence of an iso type structure in DNP-EN-Va is suggested by chemical shifts at δ 22.59 (C-19, 20) and δ 27.98 (C-18), which well agree with those calculated for DNP-14-dihydro-NE-M_1 and those of DNP-NE-B_2. Thus, the EN-Va was confirmed to be 14-dihydro-NE-M_1, a positional isomer of NE-M_2.

Experimental

All chemicals and solvents used in this experiment were of reagent grade.

Isolation of Enactins Ia, Ib and Va EN-Ia, -Ib and -Va were isolated from the cultured filtrate of *Streptomyces roseoviridis*, as reported previously.[1]

Preparation of Bis-2,4-dinitrophenyl Enactins Ia, Ib and Va 0.1 ml of 2,4-dinitrofluorobenzene solution (210 mg/ml) in dry MeOH was added to 0.5 ml of dry MeOH solution of EN-Ia, -Ib or -Va (24 mg/ml). Approximately 1 ml of 1% triethylamine in dry MeOH was added dropwise to the mixture and stirred for 2 h. The reaction mixture was evaporated *in vacuo* to give yellow oil. The residual oil was purified by silica gel column chromatography using $CHCl_3$–MeOH (30:1 for DNP-EN-Ia and -Ib, 20:1 for DNP-EN-Va) as a developing solvent. The main yellow eluate was evaporated *in vacuo* to give DNP-EN-Ia, -Ib or -Va (10—12 mg) in 60% yield. DNP-EN-Va thus obtained was recrystallized from $CHCl_3$ to give yellow needles, mp 145 °C. DNP-EN-Ia and -Ib were further purified by reverse phase HPLC on an ODS column (YMC-Pack S-343 I-15, 20 × 250 mm, Yamamura Chem. Lab. Co., Ltd.) using CH_3CN–H_2O (3:2) as a mobile phase. The flow rate was maintained at 9.0 ml/min and the eluate was monitored by a ultraviolet (UV) detector at 340 nm. The retention times of DNP-EN-Ia and -Ib were 25.1 and 32.2 min, respectively. After removing the solvent of eluates, DNP-EN-Ia or -Ib was extracted with EtOAc. Each EtOAc extract was dried over Na_2SO_4 anhydride,

filtered and evaporated *in vacuo* to given yellow oil.

Separation of Bis-2,4-dinitrophenyl Enactins Ib_1 and Ib_2 DNP-EN-Ib was further separated into DNP-EN-Ib_1 and -Ib_2 by HPLC on a Radial pak nova pak silica cartridge (8 × 100 mm, Waters Assoc., U.S.A.) using *n*-hexane–tetrahydrofuran (13 : 7) as a mobile phase. The flow rate was maintained at 2.5 ml/min and the eluate was monitored by a UV detector at 340 nm. The retention times of DNP-EN-Ib_1 and -Ib_2 were 24.3 and 25.1 min, respectively. Both DNP-EN-Ib_1 and -Ib_2 were separated by repeated HPLC and recovered by evaporation of the solvent *in vacuo*.

MS Data of Enactins Ia, Ib and Va and Their DNP Derivatives MS were measured with a JEOL HX-110 mass spectrometer.

(i) EN-Ia: High resolution FAB-MS *m/z* Calcd for $C_{20}H_{39}N_2O_6^+$ $(M+H)^+$: 403.2808. Found: 403.2810.

(ii) EN-Ib: FAB-MS *m/z*: 403 $(M+H)^+$.

(iii) EN-Va: FAB-MS *m/z*: 375 $(M+H)^+$. Electron impact MS *m/z*: 271 $(M-H_2O-C_6H_{13})^+$, 253 $(M-H_2O-C_6H_{13}-H_2O)^+$.

(iv) DNP-EN-Ib_1: High resolution FAB-MS *m/z* Calcd for $C_{32}H_{43}N_6O_{14}^+$ $(M+H)^+$: 735.2837. Found: 735.2883.

(v) DNP-NE-Ib_2: High resolution FAB-MS *m/z* Calcd for $C_{32}H_{43}N_6O_{14}^+$ $(M+H)^+$: 735.2837. Found: 735.2848.

(iv) DNP-EN-Va: High resolution FAB-MS *m/z* Calcd for $C_{31}H_{43}N_6O_{13}^+$ $(M+H)^+$: 707.2888. Found: 707.2899.

References

1) K. Yamamoto, Y. Shiinoki, M. Nishio, Y. Matsuda, Y. Inouye and S. Nakamura, *J. Antibiot.*, **43**, 1012 (1990).
2) T. Otani, S. Arai, K. Sakano, Y. Kawakami, K. Ishimaru, H. Kondo and S. Nakamura, *J. Antibiot.*, **30**, 182 (1977).
3) *a*) H. Kondo, H. Sumomogi, T. Otani and S. Nakamura, *J. Antibiot.*, **32**, 13 (1979); *b*) T. Otani, K. Ishimaru, Y. Kawakami, H. Yoshiyama, H. Kondo and S. Nakamura, *Jpn. J. Antibiot.*, **32**, 720 (1979).
4) *a*) M. Nishio, N. Yasuda and S. Nakamura, *J. Antibiot.*, **36**, 1399 (1983); *b*) S. K. Roy, S. Nakamura, J. Furukawa and S. Okuda, *ibid.*, **39**, 717 (1986); *c*) S. K. Roy, Y. Inouye, S. Nakamura, J. Furukawa and S. Okuda, *ibid.*, **40**, 266 (1987).
5) H. Fukuda, Y. Kawakami and S. Nakamura, *Chem. Pharm. Bull.*, **21**, 2057 (1973).
6) *a*) H. A. Whaley, O. K. Sebek and C. Lewis, *Antimicrob. Agents Chemother.*, **1970**, 455; *b*) H. A. Whaley, *J. Am. Chem. Soc.*, **93**, 3767 (1971); *c*) H. Kasakabe, H. Sugawara, T. Mizuno and S. Suzuki, *J. Antibiot.*, **25**, 541 (1972).
7) Y. Inouye, H. Okada and S. Nakamura, *Ann. N.Y. Acad. Sci.*, **544**, 180 (1988).
8) *a*) H. Okada, K. Yamamoto, S. Tsutano and S. Nakamura, *J. Antibiot.*, **41**, 869 (1988); *b*) H. Okada, K. Yamamoto, S. Tsutano, Y. Inouye, S. Nakamura and J. Furukawa, *ibid.*, **42**, 276 (1989).
9) F. W. Wehrli and T. Wirthlin (ed.), "Interpretation of Carbon-13 NMR Spectra," Heydon, London, 1976.

Studies on Antidiabetic Agents. X.[1] Synthesis and Biological Activities of Pioglitazone and Related Compounds

Yu MOMOSE, Kanji MEGURO, Hitoshi IKEDA, Chitoshi HATANAKA, Satoru OI, and Takashi SOHDA*

Research and Development Division, Takeda Chemical Industries, Ltd., 17-85, Jusohommachi 2-chome, Yodogawa-ku, Osaka 532, Japan.
Received December 17, 1990

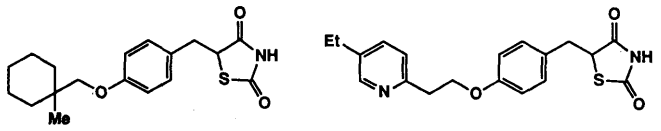
Various analogues of a new antidiabetic agent, pioglitazone (AD-4833, U-72107), were synthesized in order to study in more detail the structure–activity relationships of this class of drug. 5-(4-Pyridylalkylthiobenzyl)-2,4-thiazolidinediones (I), thia-analogues of pioglitazone, were prepared *via* Meerwein arylation of the alkylthioanilines (IV). 5-(4-Pyridylalkoxybenzylidene)-2,4-thiazolidinediones (IIa) and related heterocyclic analogues (IIb) were synthesized by Knoevenagel condensation of the aldehydes (VIII) with the corresponding azolidinones. Compounds I and II were evaluated for hypoglycemic and hypolipidemic activity in genetically obese and diabetic yellow KK (KKA^y) mice. Several 5-[4-[2-(2-pyridyl)ethoxy]-benzylidene]-2,4-thiazolidinediones (IIa) were equipotent to pioglitazone. However, the thia-analogues (I) and the benzylideneheterocycles (IIb) had decreased activity. Catalytic hydrogenation of the 5-benzylidene analogue (14) was found to be a convenient new synthetic method for pioglitazone. The configuration of 14 is also discussed.

Keywords 5-benzyl-2,4-thiazolidinedione; 5-benzylidene-2,4-thiazolidinedione; hypoglycemic activity; hypolipidemic activity; pioglitazone; Knoevenagel condensation

In the course of our study aimed at developing a new oral agent for the treatment of non-insulin dependent diabetes mellitus (NIDDM), we found that a *p*-alkoxybenzyl-substituted thiazolidinedione derivative, ciglitazone,[2] showed hypoglycemic and hypolipidemic activity[3] in insulin-resistant animal models such as KKA^y mice[4] and Wistar fatty rats.[5] Further evaluation of this series of compounds led to the finding that the introduction of a 2-(2-pyridylethoxy) group as a *p*-alkoxy substituent remarkably potentiates the pharmacological effects. Among the compounds synthesized, pioglitazone[6] (AD-4833, U-72107), 5-[4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione, was selected as a candidate for further development. Pioglitazone is expected to effectively ameliorate the abnormal glucose and lipid metabolism associated with NIDDM or obesity and is currently under clinical evaluation.[6] Recently, several other studies based on ciglitazone as a prototype have been reported,[7] and drug development along this line is becoming one of the major concerns in the field of diabetes.

In this paper we describe the further modification of 5-(4-pyridylalkoxybenzyl)-2,4-thiazolidinediones such as replacement of the *p*-alkoxybenzyl portion with a *p*-alkoxybenzylidene or a *p*-alkylthiobenzyl moiety and replacement of the thiazolidinedione ring with other acidic heterocycles. In addition, an alternative method for the synthesis of pioglitazone involving catalytic hydrogenation of the 5-benzylidene-2,4-thiazolidinedione prepared by Knoevenagel condensation of the corresponding aldehyde with 2,4-thiazolidinedione is described. The stereochemistry of the benzylidene intermediate is also discussed.

Chemistry The 5-(4-alkylthiobenzyl)-2,4-thiazolidinediones (Ia: X=S) were synthesized as shown in Chart 2 from the 4-alkylthioanilines (IV) by a procedure similar to that previously described for the synthesis of their oxa-analogues (Ib: X=O).[2,6a] The requisite anilines (IV) were prepared by coupling the vinylarenes (III)[8] with 4-aminothiophenol and subjected to the Meerwein arylation. Radical coupling of the diazonium bromide of IV with methyl acrylate in the presence of cuprous oxide provided the α-bromopropionates (V). Subsequent cyclization of the intermediate (V) with thiourea followed by acid hydrolysis afforded the desired thiazolidinediones (Ia).



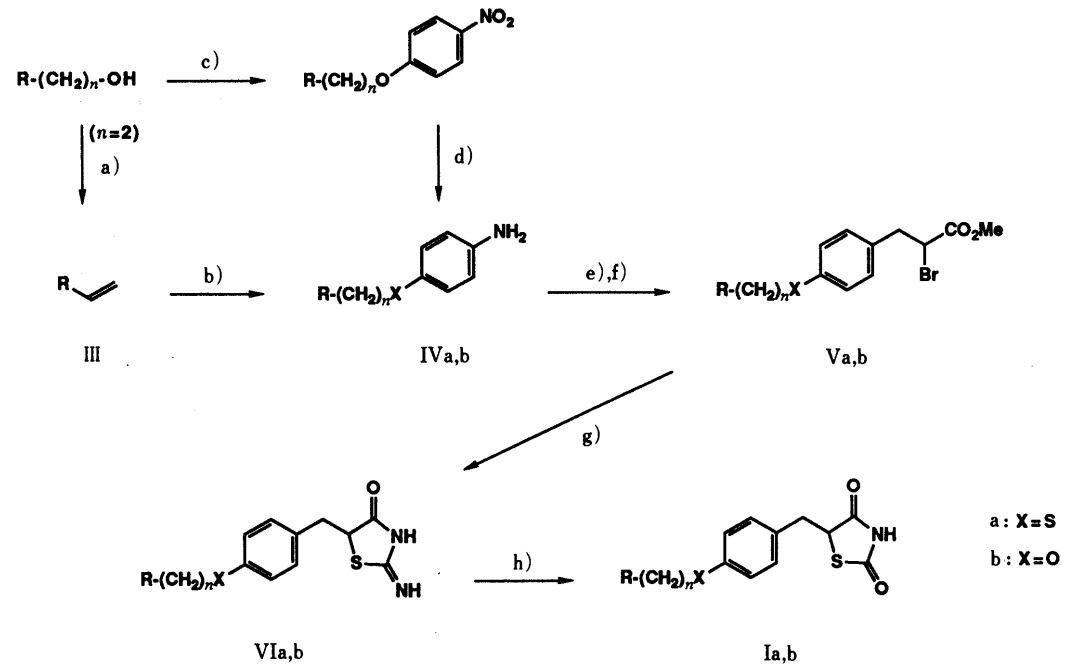
ciglitazone pioglitazone(AD-4833, U-72107)

Chart 1

The 5-benzylidene-2,4-thiazolidinediones (IIa) and their heterocyclic analogues (IIb) were synthesized by Knoevenagel condensation of the benzaldehydes (VIII) with various five-membered acidic heterocycles as shown in Chart 3. Although piperidine was generally used as the base in this condensation, pyrrolidine was more effective for the synthesis of benzylidenehydantoin (**19**). The requisite aldehydes (VIII) were obtained by a base mediate coupling of the pyridylethanols and 4-fluorobenzonitrile followed by treatment with Raney Ni alloy in aqueous formic acid.[9] An alternative and more efficient one-pot route to VIII was tosylation of the 2-pyridylethanols and subsequent coupling with 4-hydroxybenzaldehyde in the presence of benzyltributylammonium chloride as a phase transfer catalyst.[10]

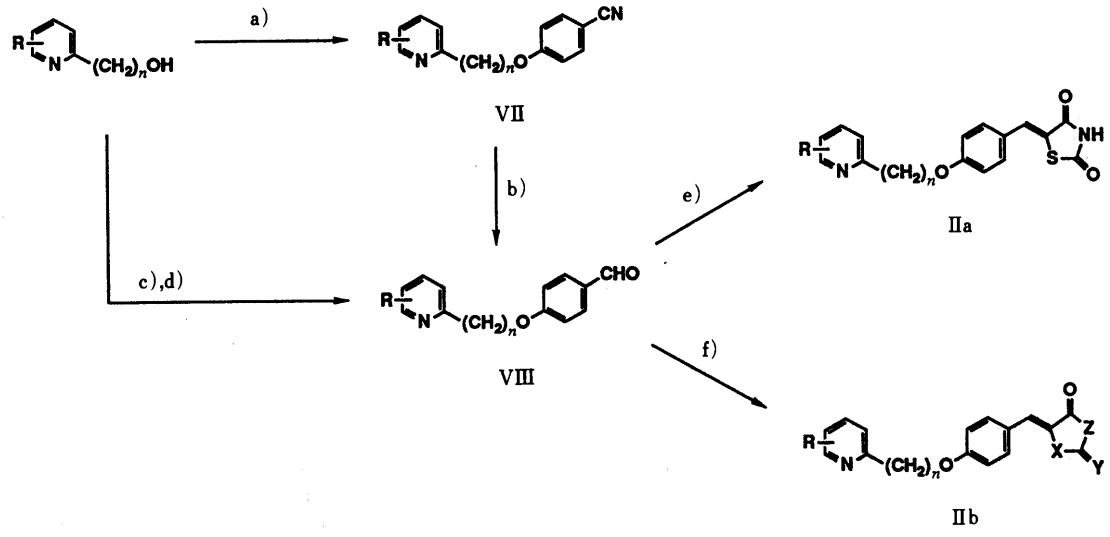
Catalytic hydrogenation of 5-[4-[2-(5-ethyl-2-pyridyl)-ethoxy]-benzylidene]-2,4-thiazolidinedione (**14**) gave pioglitazone in good yield (Chart 4).[10] This procedure, in combination with Knoevenagel condensation, provides a convenient method for large-scale synthesis of pioglitazone.

The configuration (*E* or *Z*) of the key intermediate (**14**) was determined as follows. Irradiation of **14** with a high pressure mercury-vapor lamp in acetonitrile gave an equilibrium mixture of **14** and **15** (ratio, *ca.* 3:2). The stereoisomer (**15**) was isolated using silica gel column chromatography. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **15** was very similar to that of **14** except for the signal due to the methine proton (H_a) in the benzylidene group. As shown in Chart 4, the methine proton of the starting **14** appeared at a lower field than that of the



a) KOH b) 4-aminothiophenol c) NaH, 4-fluoronitrobenzene d) H_2/Pd-C e) $NaNO_2$, aq.HBr f) $CH_2=CHCO_2Me$, Cu_2O g)$(H_2N)_2CS$, NaOAc h) aq.HCl

Chart 2



a) NaH, 4-fluorobenzonitrile b) Raney Ni, aq.HCO_2H c) TsCl, $PhCH_2NBu_3Cl$, aq.NaOH d) 4-hydroxybenzaldehyde, $PhCH_2NBu_3Cl$, aq. NaOH e) 2,4-thiazolidinedione,base f) heterocycles, base

Chart 3

photoisomerized compound (**15**). This suggests that the proton in **14** is on the same side as the carbonyl group at the 4-position of the thiazolidinedione ring while that in **15** is on the opposite side. In addition, Pascual and co-workers reported the prospective method to calculate the chemical shifts of the protons on variously substituted olefins.[11] The calculated values of the methine protons of *Z*-**14** and *E*-**15** by this method were δ 7.90 and δ 7.42, respectively, in good accordance with the observed values. Thus the configurations *Z*-**14** and *E*-**15** were determined.

Biological Methods The biological activities of the compounds prepared were tested using genetically obese and diabetic KKA^y mice[4] (8—11 weeks old). After being fed a laboratory chow (CE-2, Clea Japan Inc., Tokyo, Japan) for 3 d, the mice were divided into experimental groups of five mice each according to their blood glucose levels. The test compounds were given as a dietary admixture at 0.005% or 0.01% concentration in the CE-2 powdered diet. The mice were fed the experimental diet and water *ad libitum* for 4 d. Blood samples were taken from the orbital vein. Blood glucose was determined using the glucose oxidase method[12] and plasma triglyceride using

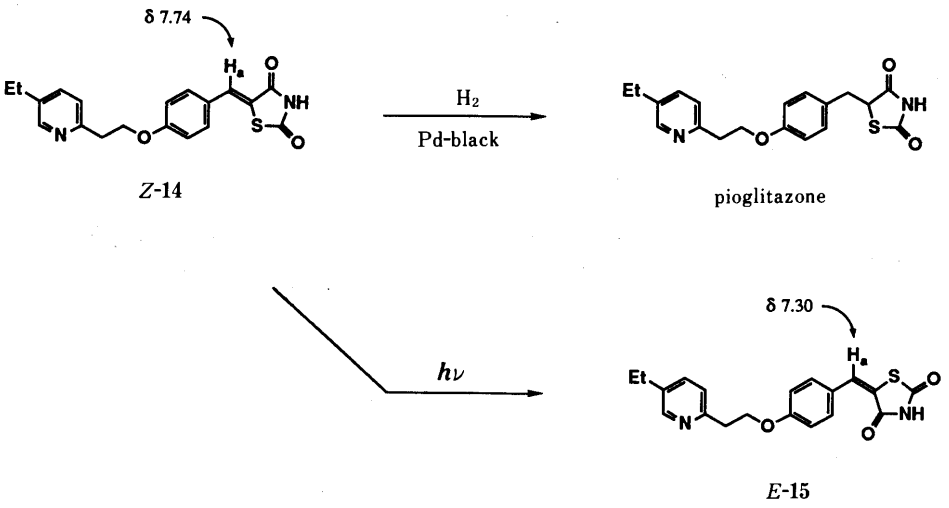
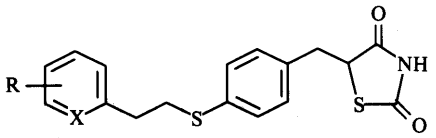


Chart 4

TABLE I. Physical and Biological Properties of 5-[4-(2-Arylethylthio)benzyl]-2,4-thiazolidinediones (I)



No.	R	X	Yield[a] (%)	mp (°C)	Recrystn. solvent	Formula[b]	Hypoglycemic activity[c]	Plasma triglyceride lowering activity[c]
1	H	CH	69	285—289[g]	EtOH	$C_{18}H_{16}NO_2S_2Na$	11	13
2	H	N	83	162—164	$CHCl_3$–EtOH	$C_{17}H_{16}N_2O_2S_2$	0	20[d]
3	3-Me	N	63	151—152	$CHCl_3$–EtOH	$C_{18}H_{18}N_2O_2S_2$	16	12
4	5-Me	N	79	154—155	CH_2Cl_2–EtOH	$C_{18}H_{18}N_2O_2S_2$	18	7
5	6-Me	N	95	146—147	EtOH	$C_{18}H_{18}N_2O_2S_2$	4	5
6	4,6-Me_2	N	84	162—163	CH_2Cl_2–EtOH	$C_{19}H_{21}N_2O_2S_2$	20[d]	9
7	5-Et	N	75	118—118.5	$CHCl_3$–EtOH	$C_{19}H_{20}N_2O_2S_2$ ·1/3EtOH[h]	14	8
	Pioglitazone						54[f]	47[e]

a) Yield from the corresponding 5-[4-(2-arylethylthio)benzyl]-2-imino-4-thiazolidinone (VI). *b*) All compounds were analyzed for C, H, and N; analytical results obtained for these elements were within ±0.4% of calculated values. *c*) Maximum reduction in blood glucose and plasma triglyceride levels at the dosage of 0.01% diet were calculated as percentage reduction with respect to the control value. *d*) $p<0.05$. *e*) $p<0.02$. *f*) $p<0.001$. *g*) Decomposition. *h*) EtOH solvate.

a commercially available assay kit (Cleantech TG-S, Iatron Laboratories Inc., Tokyo, Japan). The maximum decreases in blood glucose and plasma triglyceride levels were calculated as percentage change from the control value.

Results and Discussion

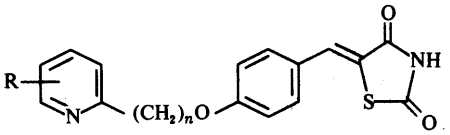
The structures and the biological data on the compounds prepared are shown in Tables I, II and III. The 5-(4-alkylthiobenzyl)-2,4-thiazolidinediones (**1**—**7**) were inactive or less potent than pioglitazone as shown in Table I. For example, compound **7**, the thia-analogue of pioglitazone, was inactive which indicates that the oxygen atom at the 4-position of the 5-benzyl moiety plays an important role in the pharmacological activity. Among the benzylidene-type analogues (II), the 5-[4-(2-pyridylalkoxy)-benzylidene]-2,4-thiazolidinediones (**8**, **9**, **11**, and **14**) had potent hypoglycemic and hypolipidemic activities (Table II) that were comparable to those of pioglitazone. However, 2-(4-pyridyl)ethoxy derivative (**16**) was inactive. The distance between the benzene ring and the pyridine ring appeared to influence the activity (**14** > **13**). Introduction of alkyl substituent(s) to the pyridine ring did not potentiate the activities. These structure–activity relationships are similar to those of the previously reported 5-(4-alkoxybenzyl)-2,4-thiazolidinediones.[2,6a] No significant difference was observed between the activities of compound **14** and the stereoisomer (**15**). However, replacement of the 2,4-thiazolidinedione in **11** with other heterocycles, such as a rhodanine, hydantoin, thiohydantoin, and 2-thioxo-5-thiazolidinone, resulted in a complete loss of activity as seen in compounds (**17**—**21**) (Table III).

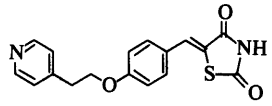
The above mentioned results indicate that the 2,4-thiazolidinedione moiety and the 4-oxybenzyl group are essential for the compound to exert favorable hypoglycemic and hypolipidemic activity. It also appeared that the methine portion is effective as a linker between the benzene and the thiazolidinedione rings, and its configuration does not affect the activity.

Conclusion

We synthesized 5-(4-arylalkylthiobenzyl)-2,4-thiazolidinediones (I) and 5-(4-pyridylalkoxybenzylidene)-substituted heterocycles (II) and evaluated them for hypoglycemic and hypolipidemic activity. Among them, several 5-[4-(2-

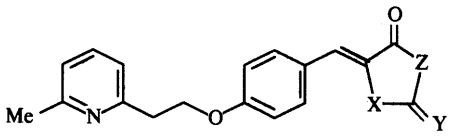
TABLE II. Physical and Biological Properties of 5-[4-[2-(2-Pyridyl)ethoxy]benzylidene]-2,4-thiazolidinediones (IIa)



No.	R	n	Yield[a] (%)	mp (°C)	Recrystn. solvent	Formula[b]	Hypoglycemic activity[c]	Plasma triglyceride lowering activity[c]
8	H	2	73	212—213	DMF–H_2O	$C_{17}H_{14}N_2O_3S$	44[e]	31[d]
9	3-Me	2	65	204—205	DMF–H_2O	$C_{18}H_{16}N_2O_3S$	48[e]	26[d]
10	5-Me	2	79	201—202	DMF–H_2O	$C_{18}H_{16}N_2O_3S$	31[d]	37
11	6-Me	2	78	181—182	MeOH	$C_{18}H_{16}N_2O_3S$	41[f]	41[d]
12	4,6-Me_2	2	45	181—182	EtOH	$C_{19}H_{18}N_2O_3S$	14	6
13	5-Et	1	71	223—224	DMF–H_2O	$C_{18}H_{16}N_2O_3S$	12	6
14	5-Et	2	64	165.5—167	AcOEt	$C_{19}H_{18}N_2O_3S$	38[d]	24[d]
15	5-Et(*E*)[h]	2		181—182	CH_2Cl_2–EtOH	$C_{19}H_{18}N_2O_3S$	47[f]	58[f]
16			83	219—220	DMF–H_2O	$C_{18}H_{16}N_2O_3S$	−1	8
	Pioglitazone						54[g]	47[e]

a) Yield from the corresponding benzaldehydes (VIII). *b*) All compounds were analyzed for C, H, and N; analytical results obtained for these elements were within ±0.4% of calculated values. *c*) Maximum reduction in blood glucose and plasma triglyceride levels at the dosage of 0.01% diet were calculated as percentage reduction with respect to the control value. *d*) $p<0.05$. *e*) $p<0.02$. *f*) $p<0.01$. *g*) $p<0.001$. *h*) *E*-Isomer of **14**.

TABLE III. Physical and Biological Properties of the Benzylidene-Type Analogues (IIb)



No.	X	Y	Z	Yield[a] (%)	mp (°C)	Recrystn. solvent	Formula[b]	Hypoglycemic activity[c]	Plasma triglyceride lowering activity[c]
11[e]	S	O	NH					31[d]	16
17	S	S	NH	89	197—198	$CHCl_3$–EtOH	$C_{18}H_{16}N_2O_2S_2$	16	4
18	S	S	NCH_2CO_2H	75	206—207	$CHCl_3$–MeOH	$C_{20}H_{18}N_2O_4S_2$	−20	−10
19	NH	O	NH	67	195—196	CH_2Cl–EtOH	$C_{18}H_{17}N_3O_3$	7	−15
20	NH	S	NH	85	183—185	Acetone–hexane	$C_{18}H_{17}N_3O_2S$	−2	5
21	NH	S	S	50	173—175	Acetone–hexane	$C_{18}H_{16}N_2O_2S_2$	0	21

a) Yield from the corresponding benzaldehyde. *b*) All compounds were analyzed for C, H, and N; analytical results obtained for these elements were within ±0.4% of calculated values. *c*) Maximum reduction in blood glucose and plasma triglyceride levels at the dosage of 0.005% diet were calculated as percentage reduction with respect to the control value. *d*) $p<0.05$. *e*) See Table II.

pyridylethoxy)benzylidene]-2,4-thiazolidinediones (IIa) were equipotent to pioglitazone, indicating that the *p*-alkoxybenzyl or *p*-alkoxybenzylidene moiety is essential for activity. We also arrived at an alternative method for the synthesis of pioglitazone using catalytic hydrogenation of the 5-benzylidene-2,4-thiazolidinedione derivative (**14**) prepared by Knoevenagel condensation of the corresponding aldehyde and 2,4-thiazolidinedione. In addition, we determined that the benzylidene intermediate (**14**) was *Z*-form.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Infrared (IR) spectra were taken on a Hitachi IR-260-10 or a Jasco IR-810 spectrophotometer. NMR spectra were recorded on a Varian EM-390 or a Varian Gemini-200 spectrometer in $CDCl_3$ unless otherwise noted. Chemical shifts are given in ppm with tetramethylsilane as the internal standard, and the following abbreviations are used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, dd=doublet of doublets, td=triplet of doublets, br=broad.

5-[4-(2-Arylthioethyl)benzyl]-2,4-thiazolidinediones (I) A typical example to illustrate the general procedure is given below.

5-[4-[2-(2-Pyridyl)ethylthio]benzyl]-2,4-thiazolidinedione (2): A mixture of 2-imino-5-[4-[2-(2-pyridyl)ethylthio]benzyl]-4-thiazolidinone (5.00 g), 2 N HCl (40 ml), and EtOH (40 ml) was refluxed for 16 h. After removal of EtOH, the reaction mixture was neutralized with aq. $NaHCO_3$ to give **2** as crystals. Recrystallization from $CHCl_3$–EtOH gave colorless prisms (4.14 g, 83%), mp 162—164 °C. IR ν_{max}^{KBr} cm^{-1}: 3450, 1700, 1600. ^{1}H-NMR (DMSO-d_6) δ: 2.9—3.55 (6H, m), 4.88 (1H, dd, J=9, 4.5 Hz), 7.1—7.4 (6H, m), 7.68 (1H, td, J=8, 2 Hz), 8.49 (1H, dd, J=6, 2 Hz), 12.0 (1H, br s). *Anal.* Calcd for $C_{17}H_{16}N_2O_2S_2$: C, 59.28; H, 4.68; N, 8.13. Found: C, 59.17; H, 4.68; N, 7.86.

The other compounds (I) listed in Table I were prepared similarly.

5-Benzylidene-2,4-thiazolidinediones (IIa) A typical example to illustrate the general procedure is given below.

5-[4-[2-(6-Methyl-2-pyridyl)ethoxy]benzylidene]-2,4-thiazolidinedione (11): A mixture of 4-[2-(6-methyl-2-pyridyl)ethoxy]benzaldehyde (1.21 g), 2,4-thiazolidinedione (0.59 g), piperidine (0.33 g), and EtOH (50 ml) was refluxed for 16 h. The reaction mixture was poured into H_2O and acidified with AcOH to give **11** as crystals (1.34 g, 78%). Recrystallization from MeOH gave colorless prisms, mp 181—182 °C. IR ν_{max}^{KBr} cm^{-1}: 1730, 1695, 1685. ^{1}H-NMR (DMSO-d_6) δ: 2.43 (3H, s), 3.13 (2H, t, J=6.5 Hz), 4.40 (2H, t, J=6.5 Hz), 6.95—7.7 (7H, m), 7.71 (1H, s). *Anal.* Calcd for $C_{18}H_{16}N_2O_3S$: C, 63.51; H, 4.74; N, 8.23. Found: C, 63.40; H, 4.84; N, 8.30.

The other 5-benzylidene-2,4-thiazolidinediones (IIa) listed in Table II were prepared similarly.

5-[4-[2-(6-Methyl-2-pyridyl)ethoxy]benzylidene]rhodanine (17) A mixture of 4-[2-(6-methyl-2-pyridyl)ethoxyl]benzaldehyde (1.21 g), rhodanine (0.67 g), piperidine (0.39 g), and EtOH (50 ml) was refluxed for 40 min. The reaction mixture was poured into H_2O and acidified with AcOH to give **17** as crystals (1.60 g, 89%). Recrystallization from $CHCl_3$–EtOH gave pale yellow prisms, mp 197—198 °C. IR ν_{max}^{KBr} cm^{-1}: 3450, 1700. ^{1}H-NMR (DMSO-d_6) δ: 2.44 (3H, s), 3.15 (2H, t, J=6.5 Hz), 4.42 (2H, t, J=6.5 Hz), 6.95—7.7 (7H, m), 7.56 (1H, s). *Anal.* Calcd for $C_{18}H_{16}N_2O_2S_2$: C, 60.65; H, 4.52; N, 7.86. Found: C, 60.69; H, 4.72; N, 8.12.

5-[4-[2-(6-Methyl-2-pyridyl)ethoxy]benzylidene]rhodanine-3-acetic Acid (18) A mixture of 4-[2-(6-methyl-2-pyridyl)ethoxy]benzaldehyde (1.21 g), rhodanine-3-acetic acid (0.96 g), piperidine (0.85 g), and EtOH (50 ml) was refluxed for 1 h. The reaction mixture was poured into H_2O and acidified with AcOH to give **18** as crystals (1.57 g, 75%). Recrystallization from $CHCl_3$–MeOH gave pale yellow prisms, mp 206—207 °C. IR ν_{max}^{KBr} cm^{-1}: 3450, 1705. ^{1}H-NMR (DMSO-d_6) δ: 2.42 (3H, s), 3.14 (3H, t, J=6.5 Hz), 4.43 (2H, t, J=6.5 Hz), 4.71 (2H, s), 7.0—7.25 (4H, m), 7.5—7.7 (3H, m), 7.81 (1H, s). *Anal.* Calcd for $C_{20}H_{18}N_2O_4S_2$: C, 57.96; H, 4.38; N, 6.76. Found: C, 57.68; H, 4.25; N, 6.61.

5-[4-[2-(6-Methyl-2-pyridyl)ethoxy]benzylidene]hydantoin (19) A mixture of 4-[2-(6-methyl-2-pyridyl)ethoxy]benzaldehyde (1.00 g), hydantoin (0.42 g), pyrrolidine (0.15 g), and EtOH (50 ml) was refluxed for 24 h. The reaction mixture was poured into H_2O to give **19** as crystals (0.90 g, 67%). Recrystallization from CH_2Cl_2–EtOH gave colorless prisms, mp 195—196 °C. IR ν_{max}^{KBr} cm^{-1}: 3230, 1740, 1725, 1660, 1605. ^{1}H-NMR (DMSO-d_6) δ: 2.43 (3H, s), 3.10 (2H, t, J=6.5 Hz), 4.38 (2H, t, J=6.5 Hz), 6.34 (1H, s), 6.85—7.7 (7H, m), 10.37 (1H, br s), 11.09 (1H, br s). *Anal.* Calcd for $C_{18}H_{17}N_3O_3$: C, 66.86; H, 5.30; N, 13.00. Found: C, 67.03; H, 5.22; N, 13.30.

5-[4-[2-(6-Methyl-2-pyridyl)ethoxy]benzyl]-2-thiohydantoin (20) A mixture of 4-[2-(6-methyl-2-pyridyl)ethoxy]benzaldehyde (0.70 g), 2-thiohydantoin (0.35 g), piperidine (0.20 g), and EtOH (30 ml) was refluxed for 1.5 h. The reaction mixture was poured into H_2O to give **20** as crystals (0.84 g, 85%). Recrystallization from acetone–hexane gave pale yellow prisms, mp 183—185 °C. IR ν_{max}^{KBr} cm^{-1}: 3450, 1730, 1660, 1600. ^{1}H-NMR (DMSO-d_6) δ: 2.42 (3H, s), 3.12 (2H, t, J=6.5 Hz), 4.39 (2H, t, J=6.5 Hz), 6.43 (1H, s), 6.8—7.2 (4H, m), 7.5—7.8 (3H, m), 12.00 (1H, br s), 12.25 (1H, br s). *Anal.* Calcd for $C_{18}H_{17}N_3O_2S$: C, 63.70; H, 5.05; N, 12.38. Found: C, 63.68; H, 4.96; N, 12.20.

4-[4-[2-(6-Methyl-2-pyridyl)ethoxy]benzylidene]-2-thioxo-5-thiazolidinone (21) A mixture of 4-[2-(6-methyl-2-pyridyl)ethoxy]benzaldehyde (3.75 g), 2-thioxo-5-thiazolidinone (2.09 g), piperidine (0.85 g), and AcOH (100 ml) was refluxed for 2 h. After removal of the solvent, the residue was treated with H_2O to give **21** as crystals (2.80 g, 50%). Recrystallization from acetone–hexane gave yellow prisms, mp 173—175 °C. IR ν_{max}^{KBr} cm^{-1}: 3450, 1705. ^{1}H-NMR (DMSO-d_6) δ: 2.46 (3H, s), 3.23 (2H, t, J=7 Hz), 3.75 (2H, t, J=7 Hz), 6.79 (2H, d, J=9 Hz), 6.96 (1H, s), 7.09 (2H, d, J=7.5 Hz), 7.60 (1H, d, J=7.5 Hz), 8.11 (2H, d, J=9 Hz), 10.34 (1H, br s). *Anal.* Calcd for $C_{18}H_{16}N_2O_2S_2$: C, 60.65; H, 4.52; N, 7.86. Found: C, 60.93; H, 4.52; N, 7.87.

4-(2-Arylethylthio)anilines (IV) A typical example to illustrate the general procedure is given below.

4-[2-(2-Pyridyl)ethylthio]aniline: A mixture of 2-vinylpyridine (4.32 g) and 4-aminothiophenol (4.63 g) was stirred at room temperature for 30 min. The reaction mixture was purified by column chromatography using silica gel and CH_2Cl_2 as the eluent to give an oil (7.96 g, 93%). IR ν_{max}^{neat} cm^{-1}: 3340, 3225, 1620. ^{1}H-NMR δ: 2.49 (3H, s), 2.8—3.3 (4H, m), 3.68 (2H, br s), 6.57 (2H, d, J=8.5 Hz), 7.24 (2H, d, J=8.5 Hz), 7.0—7.35 (2H, m), 7.56 (1H, td, J=7.5, 2 Hz), 8.51 (1H, dd, J=6, 2 Hz).

The other compounds (IV) listed in Table IV were prepared similarly.

5-[4-(2-Arylethylthio)benzyl]-2-imino-4-thiazolidinone (VI) A typical example to illustrate the general procedure is given below.

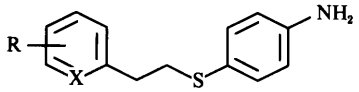
2-Imino-5-[4-[2-(2-pyridyl)ethylthio]benzyl]-4-thiazolidinone: A solution of $NaNO_2$ (2.31 g) in H_2O (10 ml) was added to a stirred and ice-cooled mixture of 4-[2-(2-pyridyl)ethylthio]aniline (7.00 g), 47% HBr (14.0 ml), and MeOH–acetone (1 : 1, v/v, 100 ml). The mixture was stirred at 5 °C for 30 min, and then methyl acrylate (15.5 g) was added to the mixture. The temperature was raised to 35 °C and Cu_2O (0.3 g) was added to the mixture in small portions with vigorous stirring. After N_2 gas evolution had ceased, the reaction mixture was concentrated *in vacuo*, diluted with H_2O, neutralized with conc. NH_4OH, and extracted with AcOEt. The extract was washed with H_2O, dried over $MgSO_4$, and concentrated *in vacuo* to give crude methyl 2-bromo-3-[4-[2-(2-pyridyl)-ethylthio]phenyl]propionate as an oil. A mixture of the oil, thiourea (1.98 g), sodium acetate (2.13 g), and EtOH (50 ml) was refluxed for 2 h. The reaction mixture was poured into H_2O to give crystals which were collected by filtration and washed with Et_2O. Yield: 5.40 g (52%). Recrystallization from EtOH gave colorless prisms, mp 189—190 °C. IR ν_{max}^{KBr} cm^{-1}: 3400, 1700, 1620. ^{1}H-NMR (DMSO-d_6) δ: 2.88 (1H, dd, J=14, 9.5 Hz), 3.00 (2H, t, J=7.5 Hz), 3.2—3.5 (3H, m), 4.57 (1H, dd, J=9.5, 4 Hz), 7.15—7.3 (6H, m), 7.70 (1H, td, J=7.5, 2 Hz), 8.50 (1H, br d, J=5 Hz), 8.69 (1H, br s), 8.92 (1H, br s). *Anal.* Calcd for $C_{17}H_{17}N_3OS_2$: C, 59.45; H, 4.99; N, 12.23. Found: C, 59.60; H, 5.07; N, 11.86.

The other compounds (VI) listed in Table V were prepared similarly.

4-[2-(2-Pyridyl)ethoxy]benzonitriles (VII) A typical example to illustrate the general procedure is given below.

4-[2-(6-Methyl-2-pyridyl)ethoxy]benzonitrile: Sodium hydride (60% in oil, 29.0 g) was added gradually to a stirred and ice-cold solution of 6-methyl-2-pyridylethanol (97.2 g) and 4-fluorobenzonitrile (85.8 g) in tetrahydrofuran (THF 600 ml). After stirring for 2 h, the reaction mixture

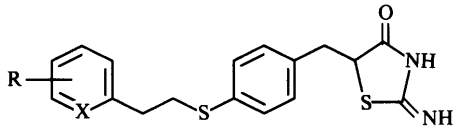
TABLE IV. 4-(2-Arylethylthio)anilines (IV)



R	X	Yield (%)[a]	Formula[b]
H	CH	91	$C_{14}H_{15}NS$
3-Me	N	90	$C_{14}H_{16}N_2S$
5-Me	N	76	$C_{14}H_{16}N_2S$
6-Me	N	96	$C_{14}H_{16}N_2S$
4,6-Me_2	N	66	$C_{15}H_{18}N_2S$
5-Et	N	95	$C_{15}H_{18}N_2S$

a) Yield from the corresponding vinylarenes (III). *b*) Oily compounds were used for the next reaction after silica gel column chromatography.

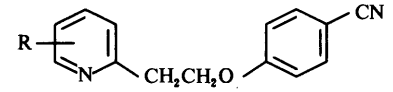
TABLE V. 5-[4-(2-Arylethylthio)benzyl]-2-imino-4-thiazolidinones (VI)



R	X	Yield (%)[a]	mp (°C)	Recrystn. solvent	Formula[b]
H	CH	50	Oil[c]	—	$C_{18}H_{18}N_2OS_2$
3-Me	N	58	207—208	$CHCl_3$–MeOH	$C_{18}H_{19}N_3OS_2$
5-Me	N	46	203—204	$CHCl_3$–EtOH	$C_{18}H_{19}N_3OS_2$
6-Me	N	34	169.5—171	Acetone–hexane	$C_{18}H_{19}N_3OS_2$
4,6-Me_2	N	28	Oil[c]	—	$C_{19}H_{21}N_3OS_2$
5-Et	N	59	185—187	EtOH	$C_{19}H_{21}N_3OS_2$

a) Yield from the corresponding anilines (IV). *b*) See foot note *b* of Table I. *c*) See footnote of *b* of Table IV.

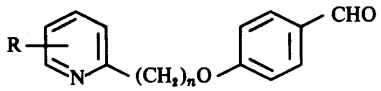
TABLE VI. 4-[2-(2-Pyridyl)ethoxy]benzonitriles (VII)

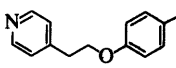


R	Yield (%)[a]	mp (°C)	Recrystn. solvent	Formula[b]
H	36	Oil[c]	—	$C_{14}H_{12}N_2O$
3-Me	49	98—99	AcOEt–hexane	$C_{15}H_{14}N_2O$
5-Me	53	74—75	AcOEt–hexane	$C_{15}H_{14}N_2O$
4,6-Me_2	29	87—88	AcOEt–hexane	$C_{16}H_{16}N_2O$
5-Et	67	Oil[c]	—	$C_{16}H_{16}N_2O$

a) Yield from the corresponding 2-pyridylethanols. *b*) See footnote *b* of Table I. *c*) See footnote *b* of Table IV.

TABLE VII. 4-[2-(2-Pyridyl)alkoxy]benzaldehydes (VIII)



R	n	Yield (%)[a]	mp (°C)	Recrystn. solvent	Formula[b]
H	2	80	Oil[c]	—	$C_{14}H_{13}NO_2$
3-Me	2	78	69—70	Et_2O–hexane	$C_{15}H_{15}NO_2$
5-Me	2	83	73—74	AcOEt–hexane	$C_{15}H_{15}NO_2$
4,6-Me_2	2	73	69—70	Et_2O–hexane	$C_{16}H_{17}NO_2$
5-Et	1	75[d]	52—53	Et_2O–hexane	$C_{15}H_{15}NO_2$
5-Et	2	70	Oil[c]	—	$C_{16}H_{17}NO_2$
		82	Oil[c]	—	$C_{15}H_{15}NO_2$

a) Yield from the corresponding 4-(2-pyridylethoxy)benzonitriles (VII). *b*) See footnote *b* of Table I. *c*) See footnote *b* of Table IV. *d*) Prepared by alkylation of 4-hydroxybenzaldehyde with 2-chloromethyl-5-ethylpyridine.

was poured into H_2O and extracted with Et_2O. The extract was washed with H_2O, dried over $MgSO_4$, and concentrated *in vacuo*. The residual crystals were recrystallized from hexane to give colorless prisms (85.9 g, 50%), mp 66—67 °C. IR ν_{max}^{KBr} cm^{-1}: 2215, 1600. ^{1}H-NMR δ: 2.52 (3H, s), 3.22 (2H, t, J=6.5 Hz), 4.39 (2H, t, J=6.5 Hz), 6.8—7.65 (7H, m). *Anal.* Calcd for $C_{15}H_{14}N_2O$: C, 75.61; H, 5.92; N, 11.76. Found: C, 75.62; H, 6.01; N, 11.73.

The other compounds (VII) listed in Table VI were prepared similarly.

4-[2-(2-Pyridyl)ethoxy]benzaldehydes (VIII) A typical example to illustrate the general procedure is given below.

4-[2-(6-Methyl-2-pyridyl)ethoxy]benzaldehyde: A mixture of 4-[2-(6-methyl-2-pyridyl)ethoxy]benzonitrile (9.62 g), Raney Ni alloy (10.0g), and 75% HCO_2H (150 ml) was refluxed for 1 h. After removal of the alloy by filtration, the filtrate was diluted with H_2O, made alkaline with 4 N KOH, and extracted with Et_2O. The extract was washed with H_2O, dried over $MgSO_4$, and concentrated *in vacuo*. The residual crystals were recrystallized from Et_2O–hexane to give colorless prisms (6.20 g, 64%), mp 53—54 °C. IR ν_{max}^{KBr} cm^{-1}: 1695, 1600. ^{1}H-NMR δ: 2.52 (3H, s), 3.23 (2H, t, J=6.5 Hz), 4.42 (2H, t, J=6.5 Hz), 6.8—7.2 (4H, m), 7.49 (1H, t, J=7.5 Hz), 7.78 (2H, d, J=9 Hz), 9.87 (1H, s). *Anal.* Calcd for $C_{15}H_{15}NO_2$: C, 74.67; H, 6.27; N, 5.80. Found: C, 74.74; H, 6.16; N, 5.65.

The other compounds (VIII) listed in Table VII were prepared similarly.

One-Pot Synthesis of 4-[2-(5-Ethyl-2-pyridyl)ethoxy]benzaldehyde: A mixture of 2-(5-ethyl-2-pyridyl)ethanol (15.0 g), benzyltributylammonium chloride (50%, 6.0 g), *p*-toluenesulfonyl chloride (23.0 g), and CH_2Cl_2 (100 ml) was added to a solution of NaOH (5.0 g) In H_2O (30 ml). After stirring at room temperature for 2 h, a mixture of 4-hydroxybenzaldehyde (12.0 g), NaOH (8.0 g), and H_2O (100 ml) was added to the mixture, and then the resultant mixture was stirred at 40—50 °C for 12 h. The organic layer was removed, dried over $MgSO_4$, and concentrated *in vacuo* to give a crude oil, which was purified by column chromatography using SiO_2 to yield the title compound (15.8 g, 62%).

Catalytic Hydrogenation of 14 to Pioglitazone Pd-black (0.2 g) was added to a solution of 5-[4-[2-(5-ethyl-2-pyridyl)ethoxy]benzylidene]-2,4-thiazolidinedione (**14**, 1.0 g) in dimethylformamide (DMF) (20 ml), and the resultant mixture was hydrogenated under 50 kg/cm^2 at 50 °C for 5 h. After removal of the catalyst by filtration, the filtrate was concentrated *in vacuo*. The residue was dissolved in 6 N HCl and neutralized with aq. $NaHCO_3$ to give crystals of pioglitazone (650 mg, 65%). *Anal.* Calcd for $C_{19}H_{20}N_2O_3S$: C, 64.02; H, 5.66; N, 7.86. Found: C, 63.73; H, 5.65; N, 7.84.

(*E*)-5-[4-[2-(5-Ethyl-2-pyridyl)ethoxy]benzylidene]-2,4-thiazolidinedione (15) A solution of **14** (800 mg) in acetonitrile (1.2 l) was irradiated for 45 min in a quartz tube with a 400W high pressure mercury-vapor lamp with continuous bubbling of N_2 gas. After removal of the solvent, the residue was purified by column chromatography and recrystallized from CH_2Cl_2–EtOH to yield pure **15** as colorless needles, mp 181—182 °C. IR ν_{max}^{KBr} cm^{-1}: 3420, 1710, 1690, 1605. ^{1}H-NMR (DMSO-d_6) δ: 1.18 (3H, t, J=7.5 Hz), 2.59 (2H, q, J=7.5 Hz), 3.17 (2H, t, J=6.5 Hz), 4.41 (2H, t, J=6.5 Hz), 6.98 (2H, d, J=9 Hz), 7.29 (1H, d, J=8 Hz), 7.30 (1H, s), 7.58 (1H, dd, J=8, 2 Hz), 8.02 (2H, d, J=9 Hz), 8.38 (1H, d, J=2 Hz), 12.24 (1H, br s). *Anal.* Calcd for $C_{19}H_{18}N_2O_3S$: C, 64.39; H, 5.12; N, 7.90. Found: C, 64.06; H, 5.15; N, 7.93.

Acknowledgement The authors are grateful to Drs. H. Iwatsuka, T. Fujita, and Y. Kawamatsu for encouragement throughout this work.

References and Notes

1) Part IX: H. Tawada, Y. Sugiyama, H. Ikeda, Y. Yamamoto, and K. Meguro, *Chem. Pharm. Bull.*, **38**, 1238 (1990).
2) T. Sohda, K. Mizuno, E. Imamiya, Y. Sugiyama, T. Fujita, and Y. Kawamatsu, *Chem. Pharm. Bull.*, **30**, 3580 (1982).
3) T. Fujita, Y. Sugiyama, S. Taketomi, T. Sohda, Y. Kawamatsu, H. Iwatsuka, and Z. Suzuoki, *Diabetes*, **32**, 804 (1983).
4) H. Iwatsuka, A. Shino, and Z. Suzuoki, *Endocrinol. Jpn.*, **10**, 611 (1974).
5) H. Ikeda, A. Shino, T. Matsuo, H. Iwatsuka, and Z. Suzuoki, *Diabetes*, **30**, 1045 (1981).
6) *a*) T. Sohda, Y. Momose, K. Meguro, Y. Kawamatsu, Y. Sugiyama, and H. Ikeda, *Arzneim.-Forsch.*, **40**, 37 (1990); *b*) Y. Sugiyama, S. Taketomi, Y, Shimura, T. Sohda, K. Meguro, and T. Fujita, *ibid.*, **40**, 253 (1990); *c*) Y. Sugiyama, Y. Shimura, and H. Ikeda, *ibid.*, **40**, 436 (1990).
7) Y. Aizawa, T. Kanai, K. Hasegawa, T. Yamaguchi, Y. Iizuka, T. Iwaoka, and T. Yoshioka, *J. Med. Chem.*, **33**, 1491 (1990); D. A. Clark, S. W. Goldstein, B. Hulin, R. A. Volkmann, G. F. Holland, J. F. Eggler, M. R. Johnson, M. N. Krupp, R. W. Stevenson, D. K. Kreutter, E. M. Gibbs, and N. J. Hutston, Abstract of Papers, 199th National Meeting of the American Chemical Society, Boston, April 1990, MEDI 70; A. Zask, I. Jirkovsky, J. W. Nowicki, and M. L. McCaleb, *J. Med. Chem.*, **33**, 1418 (1990).
8) The vinylarene (III) were commercially available or were prepared by standard methods. For representative examples, see: F. Melichar, *Chem. Ber.*, **88**, 1208 (1955); R. L. Frank, J. R. Blegen, R. J. Dearborn, R. L. Myers, and F. E. Woodward, *J. Am. Chem. Soc.*, **68**, 1368 (1946); F. Bohlmann, E. Winterfeldt, P. Studt, H. Laurent, G. Boroschewski, and K. M. Kleine, *Chem. Ber.*, **94**, 3151 (1961).
9) T. Van Es and B. Staskun, *J. Chem. Soc.*, **1965**, 5775.
10) K. Meguro, T. Fujita, C. Hatanaka, and S. Oi, Japan. Patent 139182 (1988) [*Chem. Abstr.*, **109**, 6504*h* (1988)].
11) C. Pascual, J. Meier, and W. Simon, *Helv. Chim. Acta*, **49**, 164 (1966).
12) W. Werner, H. G. Rey, and H. Wielinger, *Fresenius' Z. Anal. Chem.*, **252**, 224 (1970).

Synthesis and Antitumor Activity of 20(*S*)-Camptothecin Derivatives: Carbamate-Linked, Water-Soluble Derivatives of 7-Ethyl-10-hydroxycamptothecin[1)]

Seigo SAWADA,*[,a] Satoru OKAJIMA,[a] Ritsuo AIYAMA,[a] Ken-ichiro NOKATA,[a] Tomio FURUTA,[a] Teruo YOKOKURA,[a] Eiichi SUGINO,[b] Kentaro YAMAGUCHI[c] and Tadashi MIYASAKA[c]

Yakult Institute for Microbiological Research,[a] Yaho 1796, Kunitachi-shi, Tokyo 186, Japan. Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University,[b] Fukuyama-shi, Fukuyama, Japan and School of Pharmaceutical Sciences, Showa University,[c] Hatanodai, 1-5-8, Shinagawa-ku, Tokyo 142, Japan. *Received December 20, 1990*

Novel 36 derivatives (6), bonding the phenolic hydroxyl group of 7-ethyl-10-hydroxycamptothecin (4) with diamines through a monocarbamate linkage, were synthesized and their antitumor activity was evaluated *in vivo*. The derivatives were soluble in water as their HCl salts with the E lactone ring intact and exhibited significant antitumor activity. One of the derivatives, 6-27 showed excellent activity against L1210 leukemia and other murine tumors. The structure of its hydrochloride trihydrate (CPT-11) was determined by spectroscopic and crystallographic methods.

Keywords 20(*S*)-camptothecin; 7-ethyl-10-hydroxycamptothecin; carbamate; CPT-11; X-ray crystallography; antitumor activity

20(*S*)-Camptothecin (**1**, Chart 1) is an alkaloid which was first isolated from *Camptotheca acuminata* (Nyssaceae) by Wall and his co-workers in 1966.[2)] It has attracted much attention because of its significant inhibitory activity towards L1210 leukemia in mice and Walker 256 sarcoma in rats. The clinical trials of this alkaloid were carried out using its water soluble sodium salt, in which the E-lactone ring was cleaved by sodium hydroxide, but its severe toxicity to bone marrow and bladder ruled out the salt for the cancer treatment.[3)]

Camptothecin (lactone) administered in suspension exhibited a satisfactory high cytotoxic activity towards L1210 leukemia *in vivo* whereas the salt in aqueous solution has rather low activity. The lactone moiety of the alkaloid including the 20-hydroxyl group was said to be the responsible group for the activity,[3)] and the salt was assumed to lactonize *in vivo* although detailed behavior of the salt was not yet clear.

We describe here the synthesis of derivatives of 7-ethyl-10-hydroxycamptothecin (**4**), whose 10-hydroxyl group is connected with a hydrophilic functional side chain through a carbamate linkage, and the evaluation of their antitumor activity. We already reported the significant antitumor activity of the compound obtained herein, **6-27** (its hydrochloride trihydrate; CPT-11), toward various tomor systems[4)] and CPT-11 is now under phase II of clinical trials in Japan.[5)]

Chemistry

We reported the synthesis and antitumor activity of various 7-*C*-substituted and the A-ring modified derivatives starting from 20(*S*)-camptothecin.[6)] Compound **4**, which has a phenolic hydroxyl group as a foothold for hydrophilic modifications, was selected as the starting material because of its good therapeutic index [TI: optimal dose/minimum effective dose, **4**: TI=50, **1**: TI=3.1, L1210 in mice]. Compound **4** is also a potent inhibitor of deoxyribonucleic acid (DNA) topoisomerase I.[7c)]

Synthesis of **4** was depicted in Chart 1. Hydrogen peroxide was added dropwise to an ice-cold solution of **1** in sulfuric acid in the presence of ferrous sulfate and freshly distilled propionaldehyde. From the reaction mixture **2** was obtained in a good yield. Compound **2** was converted into its *N*-oxide (**3**). The *N*-oxide was then dissolved in dioxane containing equimolar amounts of sulfuric acid, and the mixture was irradiated with a high pressure Hg lamp (450 W) through a Pyrex filter to afford the 10-hydroxyl compound (**4**) in 30—35% overall yields.[1)]

Since the introduction of a bulky substituent either in the A or in the B-ring of camptothecin weakened or completely vanished its activity, a hydrophilic side chain is bound to the phenolic hydroxyl group of **4** through such linkages as organic or inorganic esters, glycosides, which seemed to be susceptible to enzymatical hydrolysis *in vivo*. We at first attempted to synthesize the derivatives having an amino acid group as the side chain, but these carboxyester derivatives decomposed to the starting phenol during purification. We selected more stable carbamate linkage, which was hydrolyzable *in vivo*.[8)] Furthermore, the fact[9)] that the linkage is selectively hydrolyzed in some tumor cells attracted our interest for a homing device.

A series of carbamate derivatives (**6**) were prepared either by the reaction of chlorocarbonyl derivatives of diamines with **4** in pyridine, or by the reaction of diamines with chlorocarbonyloxy compound **5**, which was obtained in a good yield by the treatment of **4** with phosgen in the presence of triethylamine (Chart 1 and Table I). In this case, the phenol **4** was yielded in addition to **6**. The secondary–secondary or secondary–tertiary diamines used in this work were mostly commercially available, but acyclic diamines and 4-dialkylaminopiperidines were prepared according to the reported methods.[10)] The yields were fairly low in the reaction of **4** with isocyanates and the reactions of **5** with primary–secondary or tertiary diamines and amino acid esters, since the products seemed to be hydrolyzed *via* a deprotonation–elimination mechanism in the basic reaction media.

CPT-11, hydrochloride trihydrate of **6-27**, was chosen for the determination of its structure. CPT-11 was obtained as slightly pale yellow needles or crystalline powder by crystallization from water and the compound has three molecules of water of crystallization suggested by its elemental analysis. The crystals were stable to heat (100 °C for a week) but some degradation was observed by exposure to visible light. The salt dissolved in water into a clear acidic solution (pH 4 in 2% solution) in which the compound was

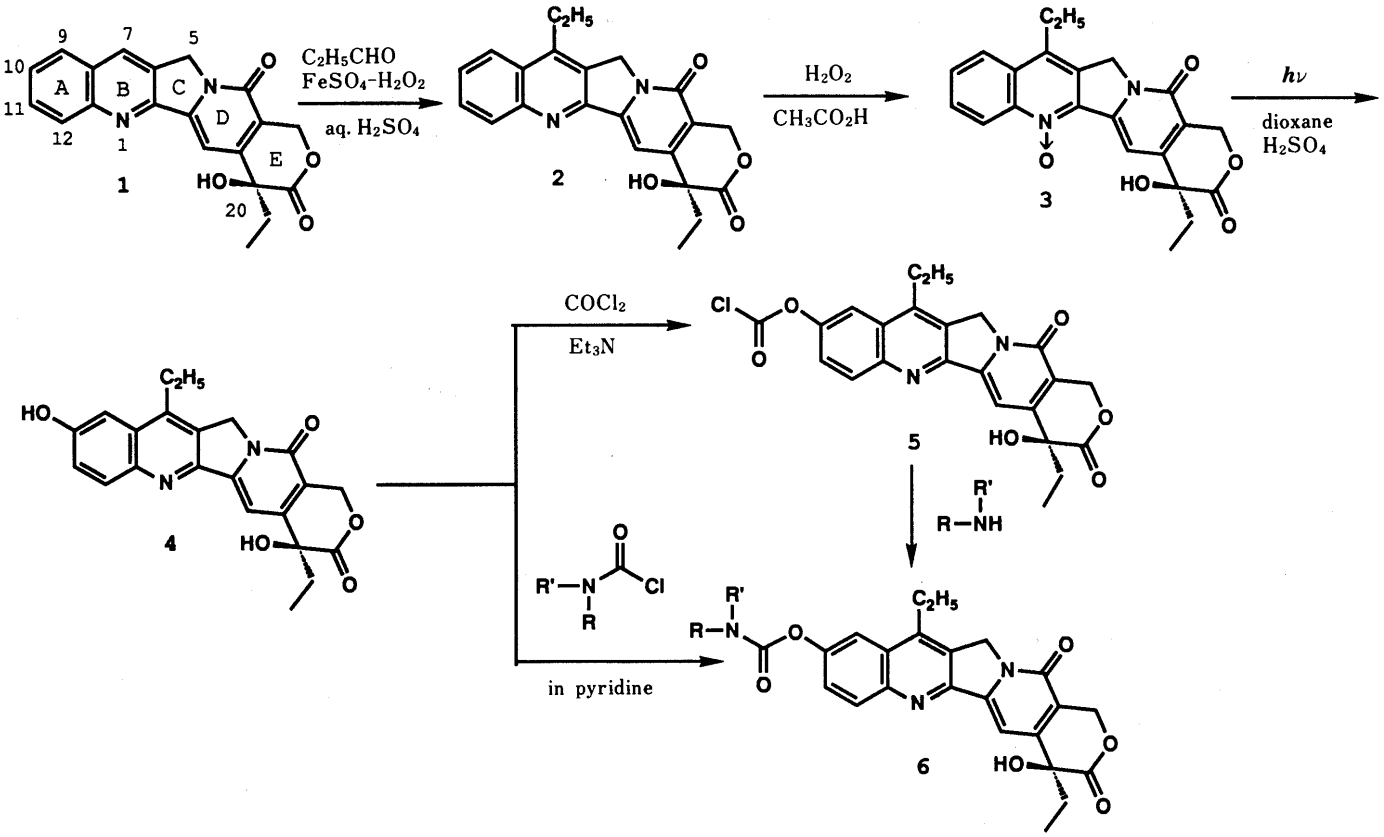


Chart 1

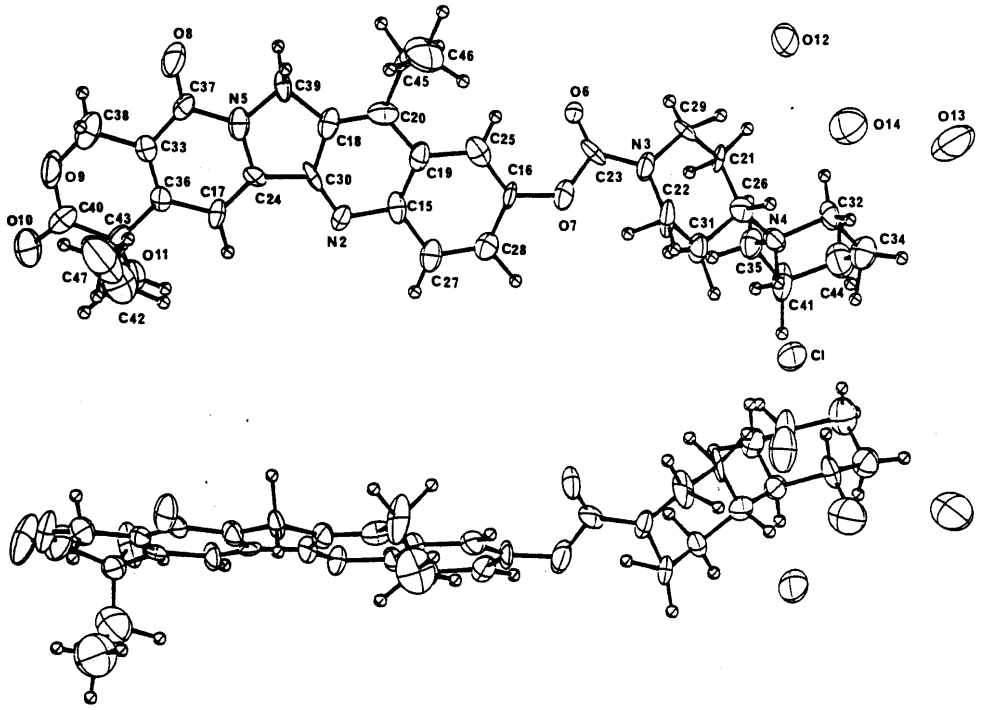


Fig. 1. ORTEP Drawings of CPT-11 from a Different View

stable (100 °C for 16 h) under the interception of light. The full assignments of proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy by homo and hetero correlation spectroscopy (COSY) and correlation spectroscopy *via* long range coupling (COLOC) methods, and also other spectroscopic data, were met in Experimental.

The proposed structure was confirmed by the single crystal X-ray crystallographic study. The observed cell parameters for a crystal of CPT-11 ($0.35 \times 0.05 \times 0.20$ mm) recrystallized from aqueous acetonitrile were as follows: $C_{33}H_{38}N_4O_6 \cdot HCl \cdot 3H_2O$, $M_w = 677.19$, orthorhombic,

TABLE I. Antitumor Activity of the Camptothecin Derivatives on L1210 Leukemia in CDF_1 Mice

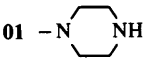
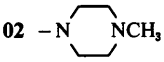
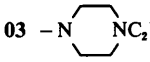
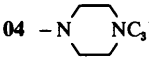
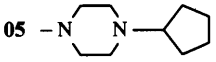
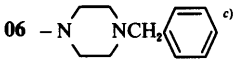
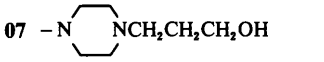
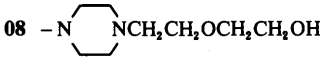
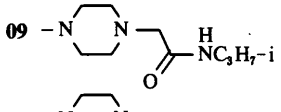
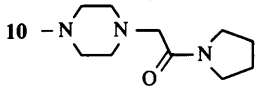
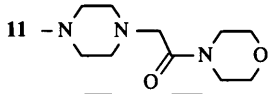
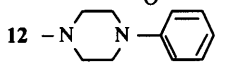
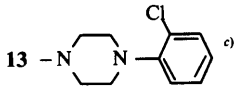
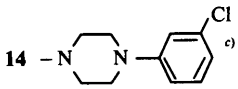
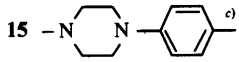
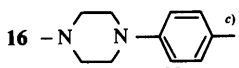
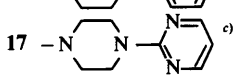
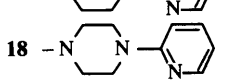
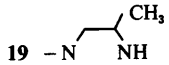
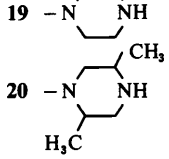
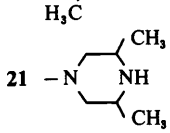
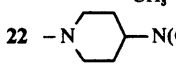
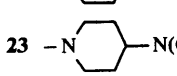
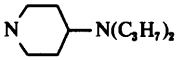
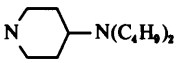
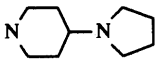
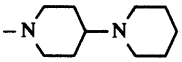
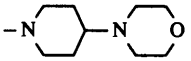
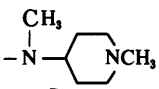
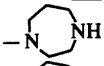
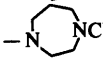
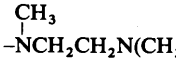
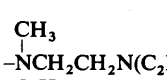
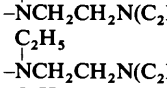
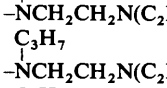
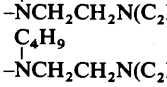
Compd. 6 Diamine side chain	Survival time of control animals (d, mean ± S.D.)	T/C%[a] Total dose (mg/kg)								
		1.56	3.12	6.25	12.5	25	50	100	200	400
01 	7.0±0.0	—[b]	111	124	131	167	210	226	352	552 (5/6)[d]
02 	7.0±0.0	114	121	119	129	133	176	217	—	—
03 	7.0±0.0	114	114	121	121	138	188	238	293	—
04 	7.0±0.0	119	121	124	126	124	136	186	205	—
05 	7.2±0.4	—	—	—	—	127	134	167	211	—
06 	6.7±0.5	128	137	172	201	287	520 (4/6)	122	60	—
07 	7.0±0.0	—	114	119	119	127	137	164	197	269
08 	7.0±0.0	—	—	—	—	126	126	138	143	—
09 	7.0±0.0	—	112	119	119	148	148	279	571 (6/6)	129
10 	7.3±0.5	116	119	123	123	129	139	167	434 (3/6)	162
11 	7.0±0.0	114	114	124	126	124	129	131	148	229
12 	6.3±0.5	119	122	135	148	153	161	169	183	—
13 	7.2±0.4	—	—	—	—	380 (3/6)	556 (6/6)	286 (2/6)	88	—
14 	7.2±0.4	—	—	—	—	130	150	183	245	—
15 	7.0±0.0	—	—	—	—	213	299 (1/6)	500 (5/6)	104	—
16 	7.0±0.0	—	—	—	—	114	129	152	193	—
17 	7.5±0.5	—	—	—	—	307 (2/6)	387 (3/6)	347 (3/6)	107	—
18 	7.2±0.4	—	—	—	—	169	167	206	396 (2/6)	—
19 	7.0±0.0	—	—	—	—	140	167	198	302	—
20 	7.0±0.0	—	—	—	—	114	212	140	171	—
21 	7.0±0.0	—	—	—	—	124	133	143	193	—
22 	7.3±0.5	112	114	119	137	180	192	237	297	352 (1/6)
23 	7.0±0.0	—	—	—	—	143	205	250	302	—

TABLE I. (continued)

Compd. 6	Diamine side chain	Survival time of control animals (d, mean ± S.D.)	T/C%[a] Total dose (mg/kg) 1.56	3.12	6.25	12.5	25	50	100	200	400
24		7.0±0.0	—	—	—	—	150	205	245	312 (1/6)	—
25		7.0±0.0	—	—	—	—	154	207	229	340 (1/6)	—
26		7.3±0.5	112	121	148	153	199	224	274	463 (4/6)	495 (4/6)
27		7.0±0.0	—	119	129	167	226	274	571 (6/6)	571 (6/6)	124
28		8.0±0.0	104	113	119	117	125	160	192	338 (2/6)	81
29		6.3±0.5	100	98	127	130	130	132	148	178	—
30		7.0±0.0	—	—	—	—	117	114	119	126	—
31		7.0±0.0	—	—	—	—	129	155	198	298 (1/6)	—
32		7.0±0.0	124	129	124	133	145	226	310 (1/6)	495 (5/6)	90
33		7.0±0.0	—	—	—	—	114	157	183	236	—
34		7.3±0.5	—	—	110	110	121	142	176	201	—
35		7.5±0.5	109	118	118	116	113	127	153	180	—
36		7.5±0.5	—	—	102	104	104	116	127	144	—

a) T/C% = (mean survival time of the tested animals)/(mean survival time of the control animals) × 100. b) Not tested. c) Administered in suspension. d) Number of cured mice/number of mice tested.

$P2_12_12_1$, $a=12.154$ (5), $b=42.847$ (5), $c=6.422$ (5) (Å), $V=3344(3)$ Å^3, $Z=4$, $D_x=1.345$ Mgm^{-3}, $\lambda(\mathrm{Cu}K\alpha_1)=1.54050$ Å, $\mu=1.521$ mm^{-1}, $F(000)=1440$, $T=295$ K. The structures were solved by the direct method, and refined by full-matrix least-squares calculations assuming anisotropic temperature factors for nonhydrogen atoms and isotropic ones for hydrogen atoms. Final $R=0.083$, $R_w=0.080$ for 1724 reflections above $3\sigma(F)$. Atomic coordinates and isotopic thermal parameters are shown in Table IV.

As illustrated in Fig. 1, the structure of the compound consists of a 7-ethylcamptothecin skeleton with a piperidinopiperidine side chain and the presence of a chlorine atom and three oxygen atoms demonstrated the hydrochloride trihydrate structure. The side chain has an intertwisted structure and the E ring lactone moiety constitutes a *quasi* chair form and the ethyl group at C_{20} installs in *quasi* axial and the free hydroxyl group is equatorial, of which conformation was similar to that of 20-*O*-iodoacetylcamptothecin.[11]

The importance of the 20(*S*)-geometry of camptotheca alkaloids for antitumor activity has been demonstrated in comparison with its antipode in the animal tumor system.[12] We examined the optical purity of compound **6-27** by NMR spectrum using a shift reagent, {tris-[3-(heptafluoropropylhydroxymethylene)-(+)-camphorate]-europium (III)}. The hydrogen at the 9-position of compound **6-27** synthesized starting from 20(*S*)-camptothecin, has one doublet at δ 7.78, whereas the racemic **6-27**, synthesized according to Wall's report,[13] showed two doublets at δ 7.78 and δ 7.80 ppm arising from *S* and *R* form, respectively. A chiral stationary phase high performance liquid chromatography (HPLC) separation of CPT-11 also indicates that there was no detectable *R*-form.

Results and Discussion

The activity of novel 36 derivatives synthesized in this work was estimated toward L1210 leukemia in mice (Table I). The test was carried out using a clear aqueous solution of their HCl salt, but the compounds **6-06**, **13**—**17**, and **18** were administered in suspension because of the low solubility of the HCl salts of these compounds. The HCl salt of **6-23** gave compound **4** as a precipitate in the aqueous solution. All the water soluble derivatives exhibited the activity [$T/C\% > 125$ (the percentage of the mean survival time of the treated and that of the untreated group)]. Cured mice were observed in 9 derivatives, **6-01**, **09**, **10**, **22**, **24**—**27**, and **31** at a total dose of 100 to 400 mg/kg.

TABLE II. Antitumor Activity of the Selected Derivatives against Acites Tumors

Tumor system	Total dose (mg/kg)	T/C%[a]		
		6-01	6-09	6-27
P338 leukemia	3.12	141	111	144
i.p.–i.p.	6.25	156	115	165
q4d d 1—9	12.5	173	126	194
	25.0	187	146	235
	50.0	211	246	281
	100.0	265	393 (4/6)[b]	343 (1/6)
	200.0	404 (4/6)	404 (4/6)	428 (3/6)
	400.0	444 (6/6)	74	333 (4/6)
Survival time of control animals (d, 9.0±0.0)				
L1210 leukemia	6.25	103	98	98
i.p.–*p.o.*	12.5	98	100	103
q4d d 1—9	25.0	98	100	105
	50.0	98	114	119
	100.0	98	132	151
	200.0	98	160	199
	400.0	103	192	240
Survival time of control animals (d, 7.3±0.0)				
B16 melanoma	3.12	104	107	99
i.p.–i.p.	6.25	104	101	109
qd d 1—17	12.5	109	107	111
	25.0	107	106	117
	50.0	121	103	138
	100.0	139	113	155
	200.0	153	144	158
	400.0	179	171	215 (2/6)
Survival time of control animals (d, 22.5±1.5)				
B16 melanoma	12.5	107	111	109
i.p.–i.v.	25.0	112	111	112
q4d d 1—17	50.0	115	117	121
	100.0	121	118	123
	200.0	121	118	132
	400.0	125	117	143
Survival time of control animals (d, 22.5±1.5)				

a) $T/C\% = (\text{mean survival time of the tested animals})/(\text{mean survival time of the control animals}) \times 100$. *b*) Number of cured mice/number of mice treated.

TABLE III. Antitumor Activity of the Selected Derivatives against Solid Tumors

Tumor system	Total dose (mg/kg)	I.R.%[a]		
		6-01	6-09	6-27
Sarcoma 180	50.0	27.8	38.5	59.0[b,c]
s.c.–i.v.	100.0	40.0	59.0	74.4[d]
q4d d 1—9	200.0	72.2[d]	69.2[c]	92.3[d]
	400.0	94.8[d]	89.7[d]	—[e]
			(3/10)[f]	
Tumor weight of control animals (g, 1.15±0.7)				
Sarcoma 180	50.0	7.9	11.4	14.3
s.c.–*p.o.*	100.0	0	72.9[d]	59.8[c]
q4d d 1—9	200.0	29.3	69.3[d]	80.0[d]
	400.0	26.4	77.9[d]	90.7[d]
Tumor weight of control animals (g, 1.40±0.82)				
Meth A	50.0	3.8	45.1	51.0[d]
s.c.–i.v.	100.0	31.3	49.1[d]	74.0[d]
q4d d 1—9	200.0	51.8[d]	53.9[d]	90.3[d]
	400.0	86.3[d]	67.5[d]	98.5[d]
Tumor weight of control animals (g, 2.06±0.8)				
Meth A	50.0	24.2	38.2[c]	37.6[c]
s.c.–i.v.	100.0	27.9	33.9[c]	46.1[d]
q4d d 1—9	200.0	26.1	52.7[d]	73.3[d]
	400.0	36.4	60.6[d]	90.3[d]
	600.0	—	—	94.5[d]
	800.0	—	—	97.3[d]
	1000.0	—	—	99.2[d]
				(6/10)
Tumor weight of control animals (g, 1.65±0.4)				

a) Inhibition rate (I.R.)% =

$$\left(1 - \frac{\text{mean tumor weight of tested animals}}{\text{mean tumor weight of control animals}}\right) \times 100 .$$

b) Statistical significance of difference from control, *c*) $p < 0.01$, *d*) $p < 0.001$ (Student's *t* test). *e*) Not tested. *f*) Number of tumor-free mice/number of mice tested.

We selected three water-soluble derivatives, **6-01**, **09**, and **27** for further evaluation of their activity of several tumor systems (Tables II and III). The compounds showed good activity in these tumor systems, but compounds **6-01** and **09** were inactive by oral administrations (*p.o.*) and on B16 melanoma by intravenous (i.v.) administration. Compound **6-27** had satisfactory activity in all systems examined.

The highlighting of the carbamate linkage is susceptible to hydrolysis mainly in serum and liver, in this case, that is, CPT-11 was gradually converted into compound **4**. On the administration of CPT-11 in mice, a rather higher concentration of **4** is sustained for a longer term than by the administration of the sodium salt of **4** in serum and some tissues.[14] The compound derived the excellent activity because *Camptotheca* alkaloids were said to exert the activity time-dependently.[7b]

Among the 36 derivatives, there were great differences in their activity depending on the structure of the side chains which held the diamine-monocarbamate moiety. The diamine side chain furnishes the derivatives with a more lipophilic property and also endows a specific structure as the substrate for the hydrolyzing enzyme. Characterization of the enzyme is now being undergone. We intend to measure the hydrolysis profile of the enzyme on derivatives synthesized herein to understand the structure–activity relationships.

In conclusion, we synthesized here the E ring intact water-soluble derivatives of compound **4** by connecting with diamines through carbamate linkage and evaluated the compound in mice tumor screens. CPT-11, hydrochloride trihydrate of **6-27**, was found as a potential nominee for a new anticancer agent.

A synthesis of other types of water-soluble derivatives of compound **4** on this line is now under progress. Synthesis and activity of sulfates, phosphates and glycosides of **4** will be published elsewhere.

Experimental

Melting points (with decomposition) were uncorrected. Ultraviolet (UV) spectra were taken with a Shimadzu UV-240 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL GX-400 (400 MHz) spectrometer with tetramethylsilane as an internal standard. Infrared (IR) spectra were recorded on a Shimadzu IR 435 and FTIR-5000 (Japan Spectroscopic Co., Ltd.). Mass spectra (MS) were measured with a Hitachi M-80B Mass Spectra. Optical rotation was taken with a DIP-360 Digital Polarimeter (Japan Spectroscopic Co., Ltd.) with a Thermostat Model RCS 6-D (Messengeraete-Werk Lauda, West Germany). X-Ray crystallography was conducted with a Rigaku AFC-5 Diffractometer.

7-Ethylcamptothecin (2) To a suspension of **1** (1.00 g, 2.9 mmol) in water (100 ml) containing $FeSO_4 \cdot 7H_2O$ (300 mg, 1.1 mmol) and propionaldehyde (2 ml), conc. H_2SO_4 (11 ml) was dropwise added in an ice bath. To the mixture 30% H_2O_2 (720 mg, 6.4 mmol) was added with stirring. The stirring was continued for 3 h at room temperature. The mixture was diluted with H_2O and the suspension was extracted with $CHCl_3$ (100 ml × 3). The extracts were evaporated and the residue was

passed through silica gel column chromatography with 2% MeOH–$CHCl_3$. **2**: 0.84 g, 77% yield as pale yellow needles, mp 258—261 °C [EtOH]. IR (KBr) ν: 1750, 1650, 1595 cm^{-1}. ^{1}H-NMR ($CDCl_3$–DMSO-d_6) δ: 0.97 (3H, t, J=7 Hz), 1.39 (3H, t, J=7 Hz), 1.91 (2H, q, J=7 Hz), 3.21 (2H, q, J=7 Hz), 521 (2H, s), 5.24, 5.57 (two 1H's, d, J=16 Hz), 7.49 (1H, s), 7.44—8.21 (4H, m). MS m/z: 376.1399 [M^+]. *Anal.* Calcd for $C_{22}H_{20}N_2O_4$: C, 70.20; H, 5.36; N, 7.44. Found: C, 70.37; H, 5.35; N, 7.39.

7-Ethylcamptothecin 1-Oxide (3) A solution of **2** (3.00 g, 8.0 mmol) and 30% H_2O_2 (50 ml) in acetic acid (800 ml) was heated at 70—80 °C for 3.5 h. The mixture was condensed to about one third at 45—55 °C and the residue was poured into ice-water (3 l). The precipitated material in the solution was collected by suction. The filtered material was purified by recrystallization. **3**: 2.4 g, 78% yield as yellow-orange needles, mp 255 °C [$CHCl_3$–n-C_6H_{14}]. ^{1}H-NMR (DMSO-d_6) δ: 0.87 (3H, t, J=7 Hz), 1.28 (3H, t, J=7 Hz), 1.84 (2H, q, J=7 Hz), 3.10 (2H, q, J=7 Hz), 5.26 (2H, s), 5.36 (2H, s), 6.24 (1H, s), 7.80 (3H, m), 8.19 (1H, s), 8.35 (1H, m). MS m/z: 392 [M^+] for $C_{22}H_{20}N_2O_5$=392.

7-Ethyl-10-hydroxycamptothecin (4) A solution of **3** (1.00 g, 2.6 mmol) and 1 N H_2SO_4 (2.6 ml) in dioxane (1 l) was degassed by bubbling N_2 for 20 min. The mixture was irradiated by a high pressure Hg lamp (450 W, Usio UM-452) with a Pyrex filter for 30 min under stirring. The reaction mixture was evaporated to dryness and the residue was dissolved in 10% MeOH–$CHCl_3$ (50 ml). The solution was washed with water (500 ml) and the insoluble material of both phases was collected on a Celite pad by suction and the Celite pad was eluted with 10% MeOH–$CHCl_3$ (200 ml × 3). The eluents were condensed and the residue was washed with MeOH and then recrystallized. **4**: 0.5 g, 49% yield as pale yellow needles, mp 216 °C [EtOH]. IR (KBr) ν: 3600, 3400, 3220, 2970, 1760, 1650, 1590, 1570, 1510 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.98 (3H, t, J=7 Hz), 1.38 (3H, t, J=7 Hz), 1.90 (2H, q, J=7 Hz), 3.08 (2H, q, J=7 Hz), 5.17 (2H, s), 5.23, 5.54 (two 1H's d, J=16 Hz), 7.34—7.39 (3H, m), 7.92 (1H, d, J=9 Hz). MS m/z: 392 [M^+]. *Anal.* Calcd for $C_{22}H_{20}N_2O_5 \cdot H_2O$: C; 64.38, H; 5.40, N; 6.83. Found: C; 64.24, H; 5.31, N; 6.68.

General Procedure for the Preparation of Carbamate Linked Derivatives (6) by the Reaction of 4 with Chlorocarbonyldiamines To a benzene solution of diamine (60 g), phosgen dimer (50 ml) in benzene was dropwise added with stirring under N_2 atmosphere below 10 °C. The stirring was continued for an additional hour at an ambient temperature. The precipitates were collected on a filter paper by suction. The collected material was dissolved in CH_2Cl_2 and the solution was washed with 7% $NaHCO_3$, dried over $MgSO_4$, filtered, and then evaporated to dryness *in vacuo*. The residue was passed through a silica gel column with CH_2Cl_2–acetone (500 : 1).

The solution of compound **4** and chlorocarbonyl diamine (1.1 eq) in pyridine was stirred for 15 h at an ambient temperature. The mixture was evaporated to dryness *in vacuo*. The residue was dissolved in CH_2Cl_2 and the solution was shaken with 7% $NaHCO_3$, the organic layer was dried over $MgSO_4$, filtered, and condensed *in vacuo*. The residue was purified through a silica gel column with MeOH–$CHCl_3$ (1 : 20).

Compounds **6-09**, **10**, **11**, **23**—**27**, and **28** were obtained by this method.

General Procedure for the Preparation of 6 by the Reaction of 7-Ethyl-10-chlorocarbonyloxycamptothecin (5) with Diamines: **4** (5.00 g, 12.8 mmol) was dissolved in dioxane (10 l) containing triethylamine (50 ml), and phosgen [generated from phosgen dimer (3.75 ml) on active charcoal] was passed through the mixture with stirring at room temperature. The stirring was continued for an additional hour. The solution was filtered by suction and the filtrate was condensed to dryness *in vacuo*. The residue was triturated with acetone and collected on a filter paper to afford **5** (5.2 g, 90.0% yield) as colorless powder.

5 (3.0 g) was dissolved in a mixture of CH_2Cl_2–MeOH (500 ml–150 ml) containing pyridine (15 ml), and then diamine (2.0 eq) in CH_2Cl_2 was added dropwise to the mixture with stirring. The mixture was stirred for 15 h at room temperature and was condensed to dryness *in vacuo*. The residue was dissolved in CH_2Cl_2 and the solution was washed with 7% $NaHCO_3$ and then the organic layer was dried over $MgSO_4$, filtered, and evaporated *in vacuo*. The residue was purified through silica gel column chromatography with 4% MeOH–CH_2Cl_2.

Carbamate linked derivatives (**6**) other than described above were obtained by this method.

6-01: 25.5% yield as pale yellow needles, mp 228—230 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3430, 2960, 1745, 1718, 1660, 1590, 1413 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.90 (3H, t, J=7 Hz), 1.32 (3H, t, J=7 Hz), 1.97 (2H, q, J=7 Hz), 3.04—3.65 (10H, m), 5.32 (2H, s), 5.44 (2H, s), 6.50 (1H, s), 7.34 (1H, s), 7.66 (1H, dd, J=2, 8 Hz), 7.97 (1H, d, J=2 Hz), 8.16 (1H, d, J=8 Hz). *Anal.* Calcd for $C_{27}H_{28}N_4O_6 \cdot H_2O$: C, 62.05; H, 5.79; N, 10.72. Found: C, 62.05; H, 5.42; N, 10.96.

6-02: 54.2% yield as pale yellow needles, mp 236—239 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3430, 2970, 1743, 1715, 1655, 1598, 1459 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.88 (3H, t, J=7 Hz), 1.29 (3H, t, J=7 Hz), 1.87 (2H, q, J=7 Hz), 2.25 (3H, s), 3.18 (2H, q, J=7 Hz), 3,49—3.64 (8H, m), 5.31 (2H, s), 5.43 (2H, s), 6.50 (1H, s), 7.31 (1H, s), 7.64 (1H, dd, J=2, 9 Hz), 7.97 (1H, d, J=2 Hz), 8.15 (1H, d, J=9 Hz). MS m/z 518 [M^+]. *Anal.* Calcd for $C_{30}H_{34}N_4O_7 \cdot 1/2H_2O$: C, 63.75; H, 5.92; N, 10.62. Found: C, 63.87; H, 5.74; N, 10.71.

6-03: 75.3% yield as pale yellow needles, mp 200—203 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3430, 2960, 1742, 1720, 1655, 1597, 1412 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.90 (3H, t, J=7 Hz), 1.06 (3H, t, J=7 Hz), 1.32 (3H, t, J=7 Hz), 1.90 (2H, q, J=7 Hz), 2.42 (2H, q, J=7 Hz), 2.18—3.17 (10 H, m), 5.32 (2H, s), 5.44 (2H, s), 6.48 (1H, s), 7.35 (1H, s), 7.66 (1H, dd, J=2, 8 Hz), 7.99 (1H, d, J=2 Hz), 8.18 (1H, d, J=8 Hz). MS m/z: 532 [M^+] for $C_{29}H_{32}N_4O_6$=532.

6-04: 34.7% yield as pale yellow needles, mp 210—213 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3440, 2960, 1750, 1720, 1655, 1598, 1412 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.93 (3H, t, J=7 Hz), 1.02 (3H, t, J=7 Hz), 1.38 (3H, t, J=7 Hz), 1.50—1.60 (2H, m), 1.87 (2H, q, J=7 Hz), 2.51 (2H, q, J=7 Hz), 3.11 (2H, q, J=7 Hz), 3.50—3.90 (8H, m), 5.16 (2H, s), 5.23 (1H, d, J=16 Hz), 5.47 (1H, d, J=16 Hz), 7.45 (1H, dd, J=2, 8 Hz), 7.50 (1H, s), 7.70 (1H, d, J=2 Hz), 8.07 (1H, d, J=8 Hz). MS m/z: 546 [M^+] for $C_{30}H_{34}N_4O_6$=546.

6-05: 29% yield as pale yellow needles, mp 226—230.5 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr)ν: 3400, 2950, 1750, 1715, 1650, 1600, 1450 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.41 (3H, t, J=7 Hz), 1.40—2.20 (9H, br), 1.80—2.20 (2H, m), 2.50—2.70 (4H, br m), 3.16 (2H, q, J=7 Hz), 3.60—3.90 (4H, br m), 5.26 (2H, s), 5.31 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 7.60 (1H, dd, J=2, 8 Hz), 7.64 (1H, s), 7.85 (1H, d, J=2 Hz), 8.22 (1H, d, J=8 Hz).

6-06: 81.8% yield as pale yellow needles, mp 160—162 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3440, 2940, 1745, 1720, 1655, 1600, 1415 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.29 (3H, t, J=7 H), 1.87 (2H, q, J=7 Hz), 3.19 (2H, q, J=7 Hz), 3.56 (2H, s), 3.50—3.70 (8H, m), 5.32 (2H, s), 5.43 (2H, s), 6.50 (1H, s), 7.32 (1H, s), 7.34 (5H, br s), 7.45 (1H, dd, J=2, 8 Hz), 7.97 (1H, d, J=2 Hz), 8.16 (1H, d, J=8 Hz). *Anal.* Calcd for $C_{34}H_{34}N_4O_6 \cdot 1/2H_2O$: C, 66.65; H, 5.92; N,9.14.

6-07: 48.5% yield as pale yellow powder, mp 228—230 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3300, 2940, 1745, 1709, 1655, 1592, 1412 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.30 (3H, t, J=7 Hz), 1.63 (2H, m), 1.88 (2H, q, J=7 Hz), 3.20—3.65 (14H, m), 5.32 (2H, s), 5.43 (2H, s), 6.51 (1H, s), 7.32 (1H, s), 7.65 (1H, dd, J=2, 8 Hz), 7.98 (1H, d, J=2 Hz), 8.17 (1H, d, J=8 Hz).

6-08: 23% yield as pale yellow powder, mp 198—200 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3390, 2960, 1745, 1715, 1655, 1595, 1455 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.41 (3H, t, J=7 Hz), 1.80—2.00 (2H, m), 2.64 (4H, t, J=4 Hz), 2.68 (2H, t, J=4 Hz), 3.16 (2H, q, J=7 Hz), 3.63 (2H, t, J=3 Hz), 3.70 (2H, t, J=5.5 Hz), 3.73 (6H, m), 5.25 (2H, s), 5.29 (1H, d, J=16 Hz), 5.74 (2H, d, J=16 Hz), 7.58 (1H, dd, J=2, 8 Hz), 7.62 (1H, s), 7.85 (1H, d, J=2 Hz), 8.21 (1H, d, J=8 Hz).

6-09: 75.9% yield as pale yellow needles, mp 237—240 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3420, 3340, 2960, 1750, 1720, 1655, 1595 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.09 (6H, d, J=6 Hz), 1.30 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 2.60 (4H, br s), 3.40—3.70 (4H, m), 3.70—4.00 (1H, m), 5.32 (2H, s), 5.43 (2H, s), 6.50 (1H, s), 7.32 (1H, s), 7.65 (1H, dd, J=2, 8 Hz), 7.98 (1H, d, J=2 Hz), 8.16 (1H, d, J=8 Hz). *Anal.* Calcd for $C_{32}H_{37}N_5O_7 \cdot H_2O$: C, 61.88; H, 6.33; N, 11.28. Found: C, 61.89; H, 6.33; N, 11.28.

6-10: 78.5% yield as pale yellow needles, mp 165.5—166.5 °C [EtOH]. IR (KBr) ν: 3395, 2955, 1745, 1715, 1655, 1615, 1450 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, t, J=7 Hz), 1.40 (3H, t, J=7 Hz), 1.80—1.95 (2H, m), 1.95—2.05 (4H, m), 2.65—2.80 (4H, m), 3.16 (2H, q, J=7 Hz), 3.23 (2H, s), 3.50 (4H, dd, J=7, 14 Hz), 3.65—3.85 (4H, m), 5.25 (2H, s), 5.30 (1H, d, J=16 Hz), 5.74 (1H, d, J=16 Hz), 7.58 (1H, dd, J=2, 9 Hz), 7.65 (1H, s), 7.84 (1H, d, J=2 Hz), 8.21 (1H, d, J=9 Hz). *Anal.* Calcd for $C_{33}H_{37}N_5O_8 \cdot H_2O$: C, 62.55; H, 6.20; N, 11.05. Found: C, 62.45; H, 6.05; N, 11.12.

6-11: 62.7% yield and pale yellow needles, mp 205.5—208 °C [EtOH]. IR (KBr) ν: 3400, 2960, 1745, 1710, 1650, 1595, 1455 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.40 (3H, t, J=7 Hz), 1.61 (2H, m), 1.80—2.00 (2H, m), 1.90—2.05 (2H, m), 2.46 (1H, m), 2.61 (4H, m), 2.96 (1H, br m), 3.13 (1H, m), 3.16 (2H, q, J=7 Hz), 3.76 (4H, t, J=3.5 Hz), 3.85 (1H, br s), 4.23 (1H, d, J=13 Hz), 4.42 (1H, d, J=13 Hz), 5.26 (2H, s), 5.31 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 7.58 (1H, dd, J=2,

9 Hz), 7.65 (1H, s), 7.83 (1H, d, J=2 Hz), 8.22 (1H, d, J=9 Hz). *Anal.* Calcd for $C_{33}H_{37}N_5O_8 \cdot 1/2H_2O$: C, 61.86; H, 5.98; N, 10.93. Found: C, 62.06; H, 5.78; N, 10.94.

6-12: 38.0% yield as pale yellow needles, mp 202—204 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3400, 2960, 1745, 1715, 1655, 1600, 1510, 1450 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.42 (3H, t, J=7 Hz), 1.80—2.00 (2H, m), 3.10—3.30 (4H, m), 3.17 (2H, q, J=7 Hz), 3.80—4.00 (4H, m), 5.27 (2H, s), 5.31 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 7.04—7.45 (5H, br s), 7.62 (1H, dd, J=2, 8 Hz), 7.65 (1H, s), 7.91 (1H, d, J=2 Hz), 8.21 (1H, d, J=8 Hz). MS m/z: 580 [M$^+$] for $C_{33}H_{32}N_4O_6$=580.

6-13: 36% yield as pale yellow powder, mp 241—242 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3400, 2960, 1743, 1715, 1655, 1600, 1510, 1450 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.42 (3H, t, J=7 Hz), 1.80—2.00 (2H, m), 3.10—3.30 (4H, m), 3.16 (2H, q, J=7 Hz), 3.80—4.00 (4H, m), 5.27 (2H, s), 5.31 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 7.04—7.42 (4H, m), 7.62 (1H, dd, J=2, 8 Hz), 7.65 (1H, s), 7.88 (1H, d, J=2 Hz), 8.24 (1H, d, J=8 Hz).

6-14: 33% yield as pale yellow powder, mp 159—163 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3400, 2960, 1750, 1720, 1655, 1590, 1455 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.41 (3H, t, J=7 Hz), 1.80—2.00 (2H, m), 3.16 (2H, q, J=7 Hz), 3.30 (4H, m), 3.70—3.90 (4H, m), 5.24 (2H, s), 5.29 (1H, d, J=16 Hz), 5.72 (1H, d, J=16 Hz), 6.83—7.20 (4H, m), 7.59 (1H, dd, J=2, 8 Hz), 7.62 (1H, s), 7.87 (1H, d, J=2 Hz), 8.22 (1H, d, J=8 Hz).

6-15: 31% yield as pale yellow powder, mp 179—180.5 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3410, 2970, 1740, 1715, 1660, 1595, 1490 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.41 (3H, t, J=7 Hz), 1.80—2.00 (2H, m), 3.17 (2H, q, J=7 Hz), 3.20—3.30 (4H, m), 3.70—4.00 (4H, m), 5.26 (1H, s), 5.31 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 6.90 (2H, d, J=9 Hz), 7.26 (2H, d, J=9 Hz), 7.61 (1H, dd, J=2, 8 Hz), 7.87 (1H, d, J=2 Hz), 8.24 (1H, d, J=8 Hz).

6-16: 63.3% yield as pale yellow needles, mp 156—158 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3440, 2970, 1745, 1720, 1658, 1600, 1515, 1415 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.30 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 3.14 (6H, br s), 5.26 (1H, s), 5.44 (2H, s), 6.50 (1H, s), 6.91 (4H, m), 7.32 (1H, s), 7.69 (1H, dd, J=2, 8 Hz), 8.01 (1H, d, J=2 Hz), 8.18 (1H, d, J=8 Hz).

6-17: 38% yield as pale yellow needles, mp 230.5—232.5 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3400, 2960, 1745, 1720, 1655, 1585, 1540, 1490 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, t, J=7 Hz), 1.41 (3H, t, J=7 Hz), 1.80—2.00 (2H, m), 3.17 (2H, q, J=7 Hz), 5.26 (1H, s), 5.31 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 6.58 (1H, t, J=5 Hz), 7.62 (1H, dd, J=2, 8 Hz), 7.65 (1H, s), 7.87 (1H, d, J=2 Hz), 8.23 (1H, d, J=8 Hz), 8.37 (2H, d, J=5 Hz). MS m/z: 581 [M$^+$] for $C_{32}H_{31}N_5O_6$=581.

6-18: 34% yield as pale yellow needles, mp 229—230.5 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3400, 2960, 1745, 1720, 1655, 1590, 1475 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.41 (3H, t, J=7 Hz), 1.80—2.00 (2H, m), 3.17 (2H, q, J=7 Hz), 3.60—4.00 (8H, m), 5.26 (1H, s), 5.31 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 6.70—6.72 (2H, m), 7.55—7.61 (2H, m), 7.61 (1H, dd, J=2, 8 Hz), 7.65 (1H, s), 7.87 (1H, d, J=2 Hz), 8.24 (1H, d, J=8 Hz).

6-19: 36% yield as pale yellow powder, mp 220.5—221.5 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3400, 2960, 1740, 1710, 1660, 1590, 1510 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.14 (two 1.5H, s), 1.41 (3H, t, J=7 Hz), 1.80—2.20 (2H, br m), 2.69—2.93 (2H, br m), 3.09—4.16 (4H, br m), 3.16 (2H, q, J=7 Hz), 5.24 (2H, s), 5.29 (1H, d, J=16 Hz), 5.73 (1H, d, J=16 Hz), 7.58 (1H, dd, J=2, 8 Hz), 7.62 (1H, s), 7.85 (1H, d, J=2 Hz), 8.21 (1H, d, J=8 Hz). MS m/z: 518 [M$^+$] for $C_{28}H_{30}N_4O_6$=518.

6-20: 32% yield as pale yellow powder, mp 215.5—217 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3400, 2960, 1740, 1710, 1660, 1590, 1450 cm^{-1}. ^{1}H-NMR ($CDCl_3$)δ: 1.03 (3H, t, J=7 Hz), 1.32 (3H, d, J=7 Hz), 1.41 (3H, t, J=4 Hz), 1.42 (3H, d, J=7 Hz), 1.80—2.20 (2H, br m), 2.64 (1H, m), 3.17 (2H, q, J=7 Hz), 3.27 (1H, m), 3.37 (1H, dd, J=2, 14 Hz), 3.52 (1H, dd, J=2, 14 Hz), 3.79 (1H, m), 4.35 (1H, m), 5.26 (2H, s), 5.31 (1H, d, J=16 Hz), 5.74 (1H, d, J=16 Hz), 7.58 (1H, dd, J=2, 8 Hz), 7.66 (1H, s), 7.84 (1H, d, J=2 Hz), 8.23 (1H, d, J=8 Hz). MS m/z: 532 [M$^+$] for $C_{29}H_{32}N_4O_6$=532.

6-21: 23% yield as pale yellow powder, mp 216—227 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3400, 2980, 1750, 1720, 1660, 1610, 1450 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.14 (3H, d, J=7 Hz), 1.16 (3H, d, J=7 Hz), 1.41 (3H, t, J=7 Hz), 1,80—2.00 (2H, br m), 2.52 (1H, t, J=12 Hz), 2.69 (1H, t, J=12 Hz), 3.16 (2H, q, J=7 Hz), 4.15 (2H, d, J=12 Hz), 4.23 (2H, d, J=12 Hz), 5.26 (2H, s), 5.31 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 7.59 (1H, dd, J=2, 8 Hz), 7.64 (1H, s), 7.85 (1H, d, J=2 Hz), 8.22 (1H, d, J=8 Hz).

6-22: 36% yield as pale yellow needles, mp 235—237 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3370, 2955, 1745, 1700, 1655, 1595, 1410 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.41 (3H, t, J=7 Hz), 1.50—2.00 (4H, m), 1.80—2.00 (2H, m), 2.34 (6H, s), 2.40 (1H, m), 2.34 (1H, br t, J=12 Hz), 3.11 (1H, br t, J=12 Hz), 3.16 (2H, q, J=7 Hz), 3.82 (1H, br s), 4.32 (1H, br), 4.41 (1H, br), 5.26 (2H, s), 5.31 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 7.59 (1H, dd, J=2, 9 Hz), 7.64 (1H, s), 7.84 (1H, d, J=2 Hz), 8.22 (1H, d, J=9 Hz).

6-23: 45% yield as pale yellow needles, mp 236—238 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3385, 2955, 1745, 1710, 1655, 1595, 1410 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.11 (6H, t, J=7 Hz), 1.41 (3H, t, J=7 Hz), 1.50—2.00 (4H, m), 1.80—2.00 (2H, m), 2.66 (4H, q, J=7 Hz), 2.82 (1H, m), 2.91 (1H, br t, J=12 Hz), 3.07 (1H, br t, J=12 Hz), 3.16 (2H, q, J=7 Hz), 3.80 (1H, br s), 4.36 (1H, br), 4.45 (1H, br), 5.26 (2H, s), 5.31 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 7.59 (1H, dd, J=2, 9 Hz), 7.64 (1H, s), 7.84 (1H, d, J=2 Hz), 8.22 (1H, d, J=9 Hz).

6-24: 48% yield as pale yellow needles, mp 196—199 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3395, 2945, 1745, 1715, 1655, 1600, 1455 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.90 (6H, t, J=7 Hz), 1.04 (3H, t, J=7 Hz), 1.41 (3H, t, J=7 Hz), 1.46 (4H, sex, J=7 Hz), 1.50—2.00 (4H, m), 1.80—2.00 (2H, m), 2.45 (4H, t, J=7 Hz), 2.73 (1H, m), 2.88 (1H, br t, J=12 Hz), 3.04 (1H, br t J=12 Hz), 3.16 (2H, q, J=7 Hz), 3.86 (1H, br s), 4.35 (1H, br), 4.45 (1H, br), 5.25 (2H, s), 5.31 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 7.59 (1H, dd, J=2, 9 Hz), 7.65 (1H, s), 7.84 (1H, d, J=2 Hz), 8.22 (1H, d, J=9 Hz).

6-25: 52% yield as pale yellow needles, mp 203.5—206.5 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3390, 2940, 1750, 1715, 1665, 1610, 1455 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.93 (6H, t, J=7 Hz), 1.04 (3H, t, J=7 Hz), 1.33 (4H, sex, J=7 Hz), 1.35—1.50 (4H, m), 1.41 (3H, t, J=7 Hz), 1.16 (4H, sex, J=7 Hz), 1.50—2.00 (4H, m), 1.80—2.00 (2H, m), 2.48 (4H, t, J=7 Hz), 2.71 (1H, m), 2.88 (1H, br t, J=13 Hz), 3.04 (1H, br t, J=13 Hz), 3.16 (2H, q, J=7 Hz), 3.85 (1H, br s), 4.35 (1H, br), 4.44 (1H, br), 5.26 (2H, s), 5.31 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 7.59 (1H, dd, J=2, 9 Hz), 7.65 (1H, s), 7.84 (1H, d, J=2 Hz), 8.22 (1H, d, J=9 Hz).

6-26: 58.2% yield as pale yellow powder, mp 219—223 °C [n-$C_{16}H_{14}$–$CHCl_3$]. IR (KBr) ν: 3400, 2955, 1745, 1715, 1655, 1600, 1450 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.30 (3H, t, J=7 Hz), 1.40 (3H, t, J=7 Hz), 1.66 (2H, br t, J=7 Hz), 1.84 (4H, br s), 1.80—2.00 (2H, m), 1.95—2.20 (2H, br s), 2.30 (1H, br), 2.64 (4H, br s), 3.04 (1H, br), 3.15 (2H, q, J=7 Hz), 3.18 (1H, br), 3.95 (1H, br s), 4.22 (1H, br d, J=14 Hz), 4.33 (1H, br d, J=14 Hz), 5.25 (2H, s), 5.30 (1H, d, J=16 Hz), 5.74 (1H, d, J=16 Hz), 7.57 (1H, dd, J=2, 9 Hz), 7.64 (1H, s), 7.82 (1H, d, J=2 Hz), 8.22 (1H, d, J=9 Hz).

6-27: 79.8% yield as pale yellow powder, mp 222—223 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3420, 2930, 1745, 1715, 1655, 1600, 1412 cm^{-1} ^{1}H-NMR (DMSO-d_6) δ: 0.90 (3H, t, J=7 Hz), 1.28 (3H, t, J=8 Hz), 1.38 (2H, br), 1.49 (6H, br), 1.78 (2H, br d), 1.85 (1H, q, J=7 Hz), 1.90 (1H, q, J=7 Hz), 2.46 (5H, br), 2.89 (1H, br t), 3.05 (1H, br t), 3.14 (2H, q, J=8 Hz), 4.31 (1H, br d), 4.24 (1H, br d), 5.24 (2H, s), 5.42 (2H, s), 6.51 (1H, s), 7.29 (1H, s), 7.60 (1H, dd, J=3, 9 Hz), 7.90 (1H, d, J=3 Hz), 8.09 (1H, d, J=9 Hz). *Anal.* Calcd for $C_{33}H_{38}N_4O_6$: C, 61.88; H, 6.39; N, 9.38. Found: C, 65.28; H, 6.39; N, 9.38.

6-28: 52.4% yield as pale yellow powder, mp 221.5—223 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3400, 2950, 1745, 1710, 1655, 1595, 1410 cm^{-1} ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.40 (3H, t, J=7 Hz), 1.61 (2H, br t, J=4 Hz), 1.80—2.00 (2H, m), 1.90—2.05 (2H, m), 2.46 (1H, m), 2.61 (4H, t, J=4 Hz), 2.96 (1H, br), 3.13 (1H, br), 3.16 (2H, q, J=7 Hz), 3.76 (4H, t, J=4 Hz), 3.85 (1H, br s), 4.32 (1H, d, J=12 Hz), 4.42 (1H, d, J=4 Hz), 5.26 (2H, s), 5.31 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 7.58 (1H, dd, J=2, 9 Hz), 7.65 (1H, s), 7.83 (1H, d, J=2 Hz), 8.22 (1H, d, J=9 Hz).

6-29: 60.8% yield as pale yellow powder, mp 159—162 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3420, 2940, 1745, 1720, 1656, 1600, 1405 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, t, J=7 Hz), 1.41 (3H, t, J=7 Hz), 1.08—2.15 (6H, m), 2.04 (3H, s), 3.00—3.20 (6H, m), 4.12 (2H, q, J=7 Hz), 5.25 (2H, s), 5.32 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 7.59 (1H, dd, J=2, 8 Hz), 7.65 (1H, s), 7.85 (1H, d, J=2 Hz), 8.22 (1H, d, J=8 Hz).

6-30: 31% yield as pale yellow powder, mp 222—223.5 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3400, 2970, 1750, 1700, 1660, 1590, 1460 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, t, J=7 Hz), 1.43 (3H, t, J=7 Hz), 1.80—2.00 (4H, m), 2.98—3.00 (2H, m), 3.04—3.10 (2H, m), 3.16 (2H, q, J=7 Hz), 3.64 (2H, t, J=4.5 Hz), 3.69 (2H, t, J=4.5 Hz), 5.24 (2H, s), 5.29 (1H, d, J=16 Hz), 5.74 (1H, d, J=16 Hz), 7.59 (1H, dd, J=2, 8 Hz), 7.62 (1H, s), 7.78 (1H, d, J=2 Hz), 8.21 (1H, d, J=8 Hz).

6-31: 39% yield as pale yellow powder, mp 220—222.5 °C [n-C_6H_{14}–

$CHCl_3$]. IR (KBr) ν: 3400, 1750, 1710, 1660, 1590, 1460 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.41 (3H, t, J=7 Hz), 1.80—2.00 (4H, m), 2.42, 2.44 (two 1.5H, s), 2.66—2.77 (4H, m), 3.14 (2H, q, J=7 Hz), 3.68—3.81 (4H, m), 5.24 (2H, s), 5.29 (1H, d, J=16 Hz), 5.73 (1H, d, J=16 Hz), 7.59 (1H, dd, J=2, 8 Hz), 7.62 (1H, s), 7.86 (1H, d, J=2 Hz), 8.21 (1H, d, J=8 Hz). MS m/z: 532 [M^+] for $C_{29}H_{32}N_4O_6$=532.

6-32: 48.9% yield as pale yellow powder, mp 202—203.1 °C [n-C_6H_{14}–$CHCl_3$]. ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, t, J=7 Hz), 1.39 (3H, t, J=7 Hz), 1.84 (2H, q, J=7 Hz), 2.36 (6H, br s), 2.64 (2H, t, J=7 Hz), 3.09 (1.5H, s), 3.22 (1.5H, s), 3.16 (2H, q, J=7 Hz), 3.58 (2H, t, J=7 Hz), 5.24 (2H, s), 5.27 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 7.26 (1H, s), 7.41 (1H, dd, J=2, 9 Hz), 7.62 (1H, d, J=2 Hz), 8.09 (1H, d, J=9 Hz).

6-33: 28% yield as pale yellow needles, mp 172—175 °C [n-C_6H_{14}–$CHCl_3$]. IR(KBr)ν: 3400, 2970, 1745, 1715, 1660, 1610, 1460 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.07 (6H, t, J=7 Hz), 1.40 (3H, t, J=7 Hz), 1.80—2.00 (2H, m), 2.60 (4H, q, J=7 Hz), 2.74 (2H, m), 2.74 (2H, m), 3.09 (1.5H, br s), 3.21 (1.5H, br s), 3.15 (2H, q, J=7 Hz), 5.29 (1H, d, J=16 Hz), 5.73 (1H, d, J=16 Hz), 7.59 (1H, dd, J=2, 8 Hz), 7.62 (1H, s), 7.85 (1H, d, J=2 Hz), 8.20 (1H, d, J=8 Hz). MS m/z: 548 [M^+] for $C_{30}H_{36}N_4O_6$=548.

6-34: 17% yield as pale yellow powder, mp 166—169 °C [n-C_6H_{14}–$CHCl_3$]. IR (KBr) ν: 3400, 2965, 1750, 1715, 1650, 1610, 1460 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.07 (3H, t, J=7 Hz), 1.08 (3H, t, J=7 Hz), 1.26 (3H, t, J=7 Hz), 1.34 (3H, t, J=7 Hz), 1.41 (3H, t, J=7 Hz), 1.80—2.00 (2H, m), 2.61 (4H, m), 2.70—2.80 (2H, m), 3.16 (2H, q, J=7 Hz), 3.40—3.60 (2H, m), 5.26 (2H, s), 5.31 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 7.60 (1H, dd, J=2, 8 Hz), 7.64 (1H, s), 7.83 (1H, d, J=2 Hz), 8.22 (1H, d, J=8 Hz).

6-35: 6% yield as pale yellow syrup. IR ($CHCl_3$) ν:3400, 2955, 1800, 1730, 1650, 1550, 1510, 1460 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.98 (3H, t, J=7 Hz), 1.00—1.10 (6H, m), 1.04 (3H, t, J=7 Hz), 1.42 (3H, t, J=7 Hz), 1.80—2.00 (2H, m), 3.15 (2H, q, J=7 Hz), 3.40—3.50 (4H, m), 3.50—3.70 (4H, m), 3.87 (2H, t, J=7 Hz), 4.10 (2H, t, J=7 Hz), 5.24 (2H, s), 5.29 (1H, d, J=16 Hz), 5.73 (1H, d, J=16 Hz), 7.58 (1H, dd, J=2, 8 Hz), 7.62 (1H, s), 7.84 (1H, d, J=2 Hz), 8.20 (1H, d, J=8 Hz).

6-36: 8% yield as pale yellow syrup. IR ($CHCl_3$) ν: 3370, 2950, 1745, 1715, 1655, 1460 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.80—4.40 (23H, m), 0.85—1.15 (5H, m), 1.80—2.00 (2H, m), 5.28 (2H, s), 5.35 (1H, d, J=16 Hz), 5.69 (1H, d, J=16 Hz), 7.69 (1H, dd, J=2, 8 Hz), 8.12 (1H, d, J=2 Hz), 8.13 (1H, s), 8.55 (1H, d, J=8 Hz).

Antitumor Activity of Derivatives 6 on L1210 Leukemia, Test Method (Table I) L1210 leukemia cells (10^5) were implanted intraperitoneally (i.p.) in 7 week old CDF_1 female mice on day 0, and 6 mice were used at the each dose. The samples were administered i.p. on days 1, 5, and 9. The control mice were injected with saline. Cured mice were calculated on day 40.

Antitumor Activity of the Selected Derivatives against Acites Tumors, Test Method (Table II) An aliquot of each suspension (P338, 1.7×10^6 cell/ml, L1210, 3×10^5 cell/ml; B16, 2×10^6 cell/ml) was implanted i.p. in 7 week old female mice (CDF_1 for P388 and L1210; C57BL/6 for B16) on day 0. The sample were administered i.p., *p.o.*, and i.v., and the control mice were injected with saline. Cured mice were observed on day 40 for P388 and L1210, day 60 for B16.

Antitumor Activity of the Selected Derivatives against Solid Tumors, Test Method A 0.1 ml aliquot of each tumor suspension (S180, 1×10^6 cells/ml; Meth A, 1×10^5 cells/ml) was implanted subcutaneously in 7 week old female mice (ICR for S180 and BALB/c for Meth A) on day 0. The samples were administered i.v. or *p.o.* on days indicated in Table III. Inhibition rates and cured mice were calculated on day 21.

CPT-11 (+)-(4*S*)-4,11-Diethyl-4-hydroxy-9-[(4-piperidinopiperidino)-carbonyloxy]-1*H*-pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-3,14-(4*H*,12*H*)-dione hydrochloride trihydrate

a) Preparation: To a suspension of **6-27** (1000 g) in distilled water (10.7 l), 5 N HCl (0.4 l) was added and the mixture was warmed at 70 °C. To the solution activated carbon (200 g) was added and the mixture was stirred for 30 min at 70 °C. After cooling the mixture to an ambient temperature, the mixture was stirred with AcOEt (3.7 l). The mixture was filtered through a Celite pad by suction and the aqueous layer was separated and passed through a membrane filter (0.22 μm) and then the filtrate was condensed to about 9 l *in vacuo*. To the solution, 5 N HCl (75 ml) was added and the solution was stirred for 40 h at an ambient temperature. The precipitate was collected and the material was dried *in vacuo*. The dried material was placed in a 75% relative humidity chamber for 70 h.

b) Structural Determination of CPT-11: Slightly pale yellow needles, mp 256.5 °C [H_2O]. UV (EtOH) λ nm (ε): 221 (53800), 254 (36600), 359 (26200), 372 (25300). $[\alpha]_D^{20}$ +67.7° (c=1, water). IR (KBr) ν: 3376 (O–H), 2630, 2546 (NH^+), 1748 (lactone carbonyl), 1688 (carbamate carbonyl), 1663 (pyridone carbonyl), 1613 (quinoline C=N), 1568 (C=C) cm^{-1}. ^{1}H-NMR (DMSO-d_6, 90 °C) δ: 0.92 (3H, t, J=7 Hz, C_{20}-$CH_2C\underline{H}_3$), 1.33 (3H, t, J=8 Hz, C_7-$CH_2C\underline{H}_3$), 1.47 (1H, q-like, $C_{4''}$-H_{ax}), 1.73 (1H, d-like, $C_{4''}$-H_{eq}), 1.85 (2H, overlap, $C_{3''}$-H_{ax} and $C_{5''}$-H_{ax}), 1.89 (2H, overlap, $C_{3'}$-H_{ax} and $C_{5'}$-H_{ax}), 1.94 (2H, q, J=7 Hz, C_{20}-$C\underline{H}_2$), 2.01 (2H, q-like, $C_{3''}$-H_{eq} and $C_{5''}$-H_{eq}), 2.28 (2H, d-like, $C_{3'}$-H_{eq} and $C_{5'}$-H_{eq}), 2.95 (2H, q-like, $C_{2''}$-H_{ax} and $C_{6''}$-H_{ax}), 3.08 (2H, t-like, $C_{2'}$-H_{ax} and $C_{6'}$-H_{ax}), 3.17 (2H, q, J=8 Hz, C_7-$C\underline{H}_2$), 3.42 (3H, br, $C_{4''}$-H, $C_{2''}$-H_{eq} and $C_{6''}$-H_{eq}), 4.31 (2H, d-like, $C_{2'}$-H_{eq} and $C_{6'}$-H_{eq}), 5.27 (2H, s, C_5-H_2), 5.36 (1H, d, J=17 Hz, C_{17}-H_{ax}), 5.45 (1H, d, J=17 Hz, C_{17}-H_{eq}), 7.34 (1H, s, C_{14}-H), 7.65 (1H, dd, J=3, 9 Hz, C_{11}-H), 7.95 (1H, dd, J=3 Hz, C_9-H), 8.13 (1H, d, J=9 Hz, C_{12}-H), 11.10 (1H, br s, N^+-H). ^{13}C-NMR (DMSO-d_6, 90 °C) δ: 7.6 (q, C_{20}-$CH_2\underline{C}H_3$), 13.5 (q, C_7-$CH_2\underline{C}H_3$), 21.7 (t, $C_{4''}$), 22.2 (t, C_7-$\underline{C}H_2CH_3$), 22.4 (t, $C_{3''}$ and $C_{5''}$), 25.7 (t, $C_{3'}$ and $C_{5'}$), 31.0 (t, C_{20}-$\underline{C}H_2CH_3$), 42.9 (t, $C_{2'}$ and $C_{6'}$), 49.2 (t, $C_{2''}$ and $C_{6''}$), 49.4 (t, C_5), 62.2 (d, $C_{4'}$), 65.5 (t, C_{17}), 72.4 (s, C_{20}), 96.6 (d, C_{14}), 114.6 (d, C_9), 119.0 (s, C_{16}), 125.5 (d, C_{11}), 127.1 (s, C_8), 128.2 (s, C_6), 131.0 (d, C_{12}), 145.2 (s, C_7), 146.0 (s, C_3), 146.5 (s, C_{13}), 150.0 (s, C_{10} and/or C_{15}), 150.1 (s, C_{15} and/or C_{10}), 151.8 (s, C_2), 152.6 (s, carbamate carbonyl), 156.9 (s, pyridone carbonyl), 172.1 (s, lactone carbonyl). MS m/z: 586.2786 for

TABLE IV. Fractional Atomic Coordinates and Isotopic Thermal Parameters of CPT-11

Atom	X	Y	Z	B_{eq}
Cl(1)	0.5106 (3)	0.2653 (1)	0.1492 (7)	5.89 (16)
N(2)	0.8520 (10)	0.3943 (2)	1.6368 (20)	3.16 (38)
N(3)	0.3955 (11)	0.3497 (3)	0.8631 (21)	3.61 (41)
N(4)	0.3296 (10)	0.2694 (3)	0.4931 (21)	3.06 (38)
N(5)	0.8362 (11)	0.4438 (2)	2.0741 (21)	3.26 (40)
O(6)	0.3906 (8)	0.3653 (3)	1.2029 (19)	3.88 (35)
O(7)	0.5485 (9)	0.3680 (3)	1.0176 (18)	4.68 (41)
O(8)	0.8177 (11)	0.4750 (2)	2.3576 (22)	4.88 (39)
O(9)	1.1280 (12)	0.4494 (3)	2.5755 (19)	5.62 (46)
O(10)	1.2702 (14)	0.4176 (4)	2.5581 (23)	8.15 (61)
O(11)	1.1818 (11)	0.3897 (3)	2.2213 (27)	6.01 (50)
O(12)	0.0185 (10)	0.3480 (3)	0.7072 (22)	6.19 (44)
O(13)	−0.0168 (14)	0.3044 (4)	0.0060 (23)	8.84 (61)
O(14)	0.0864 (12)	0.3329 (3)	0.3136 (26)	7.14 (50)
C(15)	0.7699 (14)	0.3896 (3)	1.4874 (26)	2.85 (46)
C(16)	0.6155 (13)	0.3757 (4)	1.1831 (26)	3.34 (52)
C(17)	0.9987 (16)	0.4144 (4)	2.0119 (25)	3.13 (49)
C(18)	0.7248 (14)	0.4300 (3)	1.8046 (26)	3.11 (47)
C(19)	0.6650 (15)	0.4041 (3)	1.4965 (26)	3.15 (48)
C(20)	0.6437 (13)	0.4248 (3)	1.6609 (30)	3.26 (46)
C(21)	0.2739 (14)	0.3074 (4)	0.7722 (28)	3.44 (51)
C(22)	0.4609 (16)	0.3468 (5)	0.6659 (31)	4.58 (59)
C(23)	0.4360 (12)	0.3614 (4)	1.0427 (34)	3.16 (45)
C(24)	0.8965 (12)	0.4230 (3)	1.9507 (23)	2.22 (44)
C(25)	0.5882 (14)	0.3963 (4)	1.3366 (32)	3.64 (53)
C(26)	0.3353 (13)	0.3027 (3)	0.5768 (26)	2.71 (46)
C(27)	0.8019 (13)	0.3675 (3)	1.3221 (29)	3.36 (48)
C(28)	0.7214 (13)	0.3613 (4)	1.1794 (28)	3.27 (48)
C(29)	0.2832 (12)	0.3414 (5)	0.8468 (40)	4.88 (66)
C(30)	0.8248 (12)	0.4139 (4)	1.7788 (27)	3.06 (50)
C(31)	0.4560 (15)	0.3113 (4)	0.6021 (28)	3.57 (53)
C(32)	0.2202 (14)	0.2637 (4)	0.3780 (32)	3.47 (52)
C(33)	0.9850 (13)	0.4478 (3)	2.3102 (25)	2.59 (45)
C(34)	0.2285 (17)	0.2333 (4)	0.2647 (31)	4.15 (57)
C(35)	0.3462 (16)	0.2453 (4)	0.6450 (30)	3.60 (53)
C(36)	1.0438 (12)	0.4287 (4)	2.1931 (24)	2.57 (44)
C(37)	0.8765 (14)	0.4571 (3)	2.2595 (26)	2.72 (48)
C(38)	1.0280 (18)	0.4630 (4)	2.5046 (32)	4.58 (65)
C(39)	0.7252 (13)	0.4503 (3)	1.9949 (29)	3.00 (47)
C(40)	1.1890 (19)	0.4283 (5)	2.4758 (31)	5.10 (69)
C(41)	0.3588 (20)	0.2133 (5)	0.5444 (37)	5.29 (72)
C(42)	1.2396 (24)	0.4355 (8)	2.1002 (53)	7.45 (89)
C(43)	1.1622 (16)	0.4224 (4)	2.2500 (28)	4.05 (56)
C(44)	0.2505 (21)	0.2061 (5)	0.4178 (42)	5.49 (80)
C(45)	0.5337 (21)	0.4417 (6)	1.6702 (39)	6.38 (81)
C(46)	0.5267 (22)	0.4707 (6)	1.5210 (49)	7.16 (95)
C(47)	1.2396 (31)	0.4677 (12)	2.1100 (72)	10.99 (99)

$C_{33}H_{38}N_4O_6$=586.2784. *Anal.* Calcd for $C_{33}H_{38}N_4O_6 \cdot HCl \cdot 3H_2O$, C; 58.53, H; 6.70, N; 8.27, Cl; 5.23. Found: C, 58.59, H; 6.43, N; 8.30, Cl; 5.34.

c) Determination of Optical Purity: 1) ^{1}H-NMR: **6-27** or (*RS*)-**6-27** (10 mg) was dissolved in $CDCl_3$ (5 ml), and the shift reagent (13.6 mg) was dissolved in $CDCl_3$ (2 ml). To the solution of **6-27** or (*RS*)-**6-27** (0.5 ml), the shift reagent solution (0.1 ml) was added.

2) Liquic Chromatography; CPT-11 or (*RS*)-CPT-11 (10 mg) was dissolved in $CHCl_3$–EtOH (4 : 1, 50 ml) and 20 μl of the solutions were used for HPLC operation with a UV deterctor (wave length: 254 nm) and CHIRALCEL OD column (4.6 mm × 25 cm, Daicel Chem. Ind. Co., Osaka Japan) at 40 °C. A mixture of *n*-C_6H_{14}–EtOH (1 : 1) containing 0.2 (v/v)% of diethylamine was used as an eluent at 1 ml/min. (*RS*)-CPT-11 revealed two peaks at 11.8 min and 16.7 min, whereas, CPT-11 prepared by the method described above showed one peak at 16.7 min.

References

1) A part of this study was presented: S. Sawada, K. Nokata, S. Okajima, T. Furuta, T. Yokokura, M. Mutai, E. Sugino and T. Miyasaka, Poster Abstractr. at the Japanese–United States Congress of Pharmaceutical Sciences, 1987, Honolulu, Hawaii, Dec. 1987, p. 166 and S. Sawada, K. Nokata and T. Miyasaka, p. 172.
2) M. E. Wall, M. C. Wani, C. E. Cook, K. H. Palmer and G. A. Sim, *J. Am. Chem. Soc.*, **88**, 3888 (1966).
3) Recent review on *camptotheca* alkaloids; J.-C. Cai and C. R. Hutchingson, "The Alkaloids," Vol. 21, ed. by A. Brossi, Academic Press, New York, 1983, p. 101 and references cited herein.
4) K. Nitta, T. Yokokura, S. Sawada, T. Kunimoto, T. Tanaka, N. Uehara, H. Baba, M. Takeuchi, T. Miyasaka and M. Mutai, ed, by J. Ishigami, "Recent Advances in Chemotherapy, Antitumor Section I, The Proceedings of the 14th International Congress of Chemother., Tokyo Univ. Press, Tokyo, 1985, p. 29.
5) *a*) Phase I trial of CPT-11: T. Taguchi, A. Wakui, H. Niitani, H. Furue, K. Ohta and T. Hattori, *Jpn. J. Cancer Chemother.*, **17**, 115 (1990); *b*) Phase II trial of CPT-11 (non-small cell lung cancer): M. Fukuoka, S. Negoro, H. Niitani and T. Taguchi, 1990 American Society of Clinical Oncology, Annual Meeting, Washington D. C., May 1990.
6) T. Miyasaka, S. Sawada and K. Nokata, *Heterocycles*, **16**, 1713, 1719 (1981).
7) *a*) Y.-H. Hsing, R. Hertbeg, S. Hecht and L.-F. Liu, *J. Biol. Chem.*, **27**, 14873, (1985); *b*) Y. Kawamoto, K. Yasuoka, K. Matsumoto and M. Tomikawa, Proceedings of the Japanese Cancer Association, 46th Annual Meeting, Tokyo, 1987, p. 408; *c*) T. Andoh, K. Ishii, Y. Suzuki, Y. Ikegami, Y. Kusunoki, Y. Takemoto and K. Okada, *Proc. Natl. Acad. Sci., U.S.A.*, **84**, 5565 (1987).
8) E. Heymann, "Metabolic Basis of Detoxication," ed. by W. B. Jakoby, J. R. Bend and J. Caldwell, Academic Press, New York, 1982, p. 229
9) A. A. Sinkula and S. H. Yalkowsky, *J. Pharm. Sci.*, **64**, 181 (1975) and references cited herein.
10) *a*) N. B. Chapmann, K. Clarke and K. Wilson, *J. Chem. Soc.*, **1963**, 2256; *b*) B. Hermans, P. van Daele, C. van de Westeringh, C. van der Eycken, J. Boey and P. A. J. Janssen, *J. Med. Chem.*, **9**, 49 (1966).
11) A. T. McPhail and G. A. Sim, *J. Chem. Soc.* (*B*), **1968**, 923.
12) A. Ejima, H. Terasawa, M. Sugimori and H. Tagawa, *J. Chem. Soc. Perkin Trans. 1*, **1990**, 27.
13) M. C. Wani, P. E. Ronman, J. T. Lindley and M. E. Wall, *J. Med. Chem.*, **23**, 554 (1980).
14) N. Kaneda, H. Nagata, T. Furuta and T. Yokokura, *Cancer Res.*, **50**, 1715 (1990).

Studies on the Constituents of Palmae Plants. V.[1a] Steroid Saponins and Flavonoids of Leaves of *Phoenix rupicola* T. ANDERSON, *P. loureirii* KUNTH, *P. reclinata* N. J. JACQUIN, and *Arecastrum romanzoffianum* BECCARI

Kotaro IDAKA, Yasuaki HIRAI, and Junzo SHOJI*

School of Pharmaceutical Sciences, Showa University, Hatanodai, Shinagawa-ku, Tokyo 142, Japan. *Received October 9, 1990*

Steroid saponins and flavonoids of leaves of *Phoenix rupicola* T. ANDERSON, *P. loureirii* KUNTH, *P. reclinata* N. J. JACQUIN, and *Arecastrum romanzoffianum* BECCARI have been investigated. Tricin 7-*O*-β-D-glucopyranoside (I), vitexin (II), methyl proto-Pb (VII), methyl proto-taccaoside (26-*O*-β-D-glucopyranosyl (25*R*)-22-*O*-methyl-furost-5-en-3β,26-diol 3-*O*-[α-L-rhamnopyranosyl-(1→2)][α-L-rhamnopyranosyl-(1→3)]-β-D-glucopyranoside, VI), and methyl proto-rupicolaside (26-*O*-β-D-glucopyranosyl (25*R*)-22-*O*-methy-furost-5-en-3β,26-diol 3-*O*-[β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)][α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside, VIII) from *P. rupicola*, II, glucoluteolin (III), orientin (IV), isoorientin (V), VII, VIII, and methyl proto-loureiroside (26-*O*-β-D-glucopyranosyl (25*R*)-22-*O*-methyl-furost-5-en-3β,26-diol 3-*O*-[β-D-glucopyranosyl-(1→5)-α-L-arabinofuranosyl-(1→4)][α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside, IX) from *P. loureirii*, VII, VIII and methyl proto-reclinatoside (26-*O*-β-D-glucopyranosyl (25*R*)-22-*O*-methyl-furost-5-en-3β,26-diol 3-*O*-[α-L-rhamnopyranosyl-(1→5)-α-L-arabinofuranosyl-(1→4)][α-L-rhamnopyranosyl(1→2)]-β-D-glucopyranoside, X) from *P. reclinata*, III and VII from *A. romanzoffianum* were isolated and identified. VI, VIII, IX and X were the first isolated and characterized from natural sources.

Keywords Palmae; *Phoenix rupicola*; *Phoenix loureirii*; *Phoenix reclinata*; *Arecastrum romanzoffianum*; flavonoid; steroid saponin; furostanol oligoside; proto-diosgenin; diosgenin

A series of phytochemical studies on plants of the Palmae family has been undertaken by us, and we previously reported the isolation and structure elucidation of flavonoids and steroid glycosides from Palmae plants, *Trachycarpus fortunei* (HOOK.) H. WENDL.,[1d] *T. wagnerianus* BECC.,[1b] *Rhapis exelsa* HENRY,[1c] *R. humilis* BL.,[1c] *Chamaerops humilis* L.,[1b] and *Sabal causiarum* BECC.[1a] The present paper is mainly concerned with studies on the constituents of *Phoenix rupicola* T. ANDERSON, *P. loureirii* KUNTH, *P. reclinata* N. J. JACQUIN, and *Arecastrum romanzoffianum* BECCARI.

The habitat of *P. rupicola* (Japanese name: iwayama-natsumeyashi) is Nepal and the northern region of India; that of *P. loureirii* (Japanese name: shinnohyashi) is India, Vietnam and Myanmar. Furthermore, the habitat of *P. reclinata* (Japanese name: senegaruyashi) is the southern region of Africa, and that of *A. romanzoffianum* (Japanese name: joōyashi) is Brazil. Most of these plants are cultivated in Japan for appreciative purposes.

The fresh leaves of *P. rupicola*, *P. loureirii*, *P. reclinata*, and *A. romanzoffianum* harvested from Chichi Island, Ogasawara Islands, Tokyo, in March 1984, were respectively chopped and extracted with methanol at room temperature. Each methanol extract was treated by the method described in the experimental section.

Five compounds, I, II, VI, VII and VIII, were separated from the methanol extract of *P. rupicola*. Based on the general properties, I, II and VII were deduced to be tricin 7-*O*-β-D-glucopyranoside,[2] vitexin[1c] and methyl proto-Pb,[1d] respectively, and they were characterized by comparing the thin layer chromatography (TLC) behavior and the infrared (IR) and nuclear magnetic resonance (NMR) spectra with those of respective authentic samples.

VI and VIII were positive in the Liebermann–Burchard reaction and in the Ehrlich reaction.[3] The IR spectra of VI and VIII show strong absorption bands due to hydroxyl groups. The ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectra of VI shows four anomeric carbon signals at δ 100.1, 102.4, 103.7 and 104.7, while that of VIII shows six anomeric carbon signals at δ 100.4, 102.0, 102.1, 102.3, 104.7 and 107.0. On enzymatic hydrolysis with almond emulsin, VI and VIII afford D-glucose and a prosapogenin (VIa from VI and VIIIa from VIII). VIa was identified as taccaoside[4] by comparing the TLC behavior and the IR and NMR spectra with an authentic sample. Accordingly, VI was deduced to be 26-*O*-β-D-glucopyranosyl (25*R*)-22-*O*-methyl-furost-5-en-3β,26-diol 3-*O*-[α-L-rhamnopyranosyl-(1→2)][α-L-rhamnopyranosyl-(1→3)]-β-D-glucopyranoside (methyl proto-taccaoside). The IR spectrum of VIIIa showed strong absorption bands due to hydroxyl and (25*R*)-spiroketal groups,[5] and, on acidic hydrolysis, VIIIa gave diosgenin, D-glucose and L-rhamnose. The absolute configuration of these sugars were determined using the method of Oshima *et al.*[6] The ^{13}C-NMR spectrum of VIIIa showed five anomeric carbon signals at δ 100.6, 102.2, 102.3, 102.5, 107.2 and three methyl signals at δ 18.7, 19.1, 19.2 corresponding to the C-6 methyl groups of three rhamnoses. Consequently, VIIIa was suggested to be a diosgenin pentaoside, which contains two moles of glucose and three moles of rhamnose. The $J_{C_1-H_1}$ coupling constants of the ^{13}C-NMR spectrum of each monosaccharide of VIII showed that the configurations of all D-glucoses were β (156 Hz (δ 100.4, 104.7), 158 Hz (δ 107.0)) and those of L-rhamnose were α (172 Hz (δ 102.0), 169 Hz (δ 102.1, 102.3)).

Methylation of VIIIa by Hakomori's method[7] afforded a per-*O*-methyl derivative (VIIIb), which was methanolyzed to afford diosgenin, methyl 3,6-di-*O*-methylglucopyranoside, methyl 2,3,4-tri-*O*-methylrhamnopyranoside, methyl 2,3,4,6-tetra-*O*-methylglucopyranoside, methyl 2,3-di-*O*-methylrhamnopyranoside and methyl 3,4-di-*O*-methylrhamnopyranoside. The partial methanolysis of VIIIb afforded the reaction mixture, which was methylated by Hakomori's method to give three products (VIIIc, d and

e). VIIIc was identified to be 3-*O*-methyldiosgenin by comparing the TLC behavior. Furthermore, VIIId and VIIIe were methanolyzed to afford diosgenin, methyl 3,4,6-tri-*O*-methylglucopyranoside and methyl 2,3,4-tri-*O*-methylrhamnopyranoside from the former, diosgenin, methyl 2,3,4,6-tetra-*O*-methylglucopyranoside, methyl 2,3,6-tri-*O*-methylglucopyranoside, methyl 2,3-di-*O*-methylrhamnopyranoside and methyl 3,4-di-*O*-methylrhamnopyranoside from the latter. To confirm the structure of the oligosaccharide moiety, VIIIa was hydrolyzed with pectinase to afford Pb (diosgenin 3-*O*-[α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)][α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside,[1d] VIIa).

Consequently, the structure of VIII was concluded to be 26-*O*-β-D-glucopyranosyl (25*R*)-22-*O*-methyl-furost-5-en-3β,26-diol 3-*O*-[β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)][α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside. Furthermore, the structure of VIIIa was also established to be diosgenin 3-*O*-[β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)][α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside.

This is the first report of the isolation of VI, VIII and VIIIa from natural sources, and we propose the names methyl prototaccaoside, methyl proto-rupicolaside and rupicolaside for these compounds.

Seven compounds, II, III, IV, V, VII, VIII and IX, were separated from the methanol extract of *P. loureirii*. Based on the physical data of these compounds, II, III, IV, V, VII and VIII were deduced to be vitexin, glucoluteolin,[1d] orientin,[8] isoorientin,[1c] methyl proto-Pb and methyl proto-rupicolaside, respectively, and they were characterized by comparing the TLC behavior, as well as the IR and NMR spectra, with those of respective authentic samples. IX was positive in the Liebermann–Burchard reaction and in the Ehrlich reaction. The IR spectrum of IX shows a strong absorption band due to hydroxyl groups. The ^{13}C-NMR spectra of IX shows five anomeric carbon signals at δ 100.5, 102.2, 105.0, 105.4 and 109.9. On enzymatic hydrolysis with emulsin, IX afforded D-glucose and a prosapogenin (IXa). The IR spectrum of IXa showed absorption bands due to hydroxyl and (25*R*)-spiroketal groups, and, on acidic hydrolysis, IXa gave diosgenin, D-glucose, L-arabinose and L-rhamnose. The ^{13}C-NMR spectrum of IXa showed four anomeric carbon signals at δ 100.2, 102.1, 105.4 and 109.7, and one methyl signal at δ 18.7 corresponding to the C-6 methyl group of the L-rhamnose. The ^{13}C-NMR signals of C_1, C_2 and C_3 of the arabinose in IXa appeared at δ 109.7, 83.1 and 78.4, which were lower than those of arabinopyranose (δ 104.5, 72.0 and 73.9).[9] Consequently, the arabinose moiety of IXa was suggested to be present in a furanose type. The configurations of D-glucoses of IX were deduced to be β, and that of L-rhamnose to be α based on the $J_{C_1-H_1}$ coupling constants, 160 Hz (δ 100.5, glc), 172 Hz (δ 102.2, rha), 157 Hz (δ 105.0, glc), and 158 Hz (δ 105.4, glc), but the configuration of L-arabinose could not be deduced based on the $J_{C_1-H_1}$ coupling constant. The configuration of L-arabinose was inferred as follows. It is known that the coupling pattern of α-arabinofuranoside shows a triplet-like or singlet-like signal, but that of β-arabinofuranoside shows a doublet-like signal (J=4—5 Hz).[10] The signal corresponding to the anomeric proton of arabinose of IX shows a singlet-like signal at δ 5.22. Consequently, the configuration of L-arabinofuranoside of IX was deduced to be α.

Methylation of IXa by Hakomori's method afforded a per-*O*-methyl derivative (IXb), which was methanolyzed to afford methyl 2,3,4,6-tetra-*O*-methylglucopyranoside, methyl 3,6-di-*O*-methylglucopyranoside, methyl 2,3,4-tri-*O*-methylrhamnopyranoside, and methyl 2,3-di-*O*-methylarabinofuranoside (or methyl 2,3-di-*O*-methylarabinopyranoside). Furthermore, the partial acidic hydrolysis of IXa afforded trillin[11] and prosapogenin A of dioscin,[1c] which were identified by comparing the TLC behavior and NMR spectra with those of respective authentic samples.

Finally, the structure of IX was concluded to be 26-*O*-β-D-glucopyranosyl (25*R*)-22-*O*-methyl-furost-5-en-3β,26-diol 3-*O*-[β-D-glucopyranosyl-(1→5)-α-L-arabinofuranosyl-(1→4)][α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside, while the structure of IXa was elucidated to be diosgenin 3-*O*-[β-D-glucopyranosyl-(1→5)-α-L-arabinofuranosyl-(1→4)][α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside. IX and IXa were the first isolated compounds from natural sources, and we propose the names methyl proto-loureiroside for the former, and loureiroside for the latter.

Three compounds, VII, VIII and X, were separated from the methanol extract of *P. reclinata* N. J. JACQUIN. Based on the general properties, VII and VIII were deduced to be methyl proto-Pb and methyl proto-rupicolaside, and they were characterized by comparing the TLC behavior, and the IR and NMR spectra with those of respective authentic samples.

X was positive in the Liebermann–Burchard reaction and in the Ehrlich reaction. The IR spectrum of X shows a strong absorption band due to hydroxyl groups. The ^{13}C-NMR spectra of X shows five anomeric carbon signals at δ 100.5, 101.8, 102.1, 104.9 and 110.0. On enzymatic hydrolysis with emulsin, X afforded D-glucose and a prosapogenin (Xa). The IR spectrum of Xa showed absorption bands due to hydroxyls and a (25*R*)-spiroketal group, and, on acidic hydrolysis, Xa gave diosgenin, D-glucose, L-arabinose and L-rhamnose. The ^{13}C-NMR spectrum of Xa showed four anomeric carbon signals at δ 100.4, 101.7, 102.2 and 110.0, two methyl signals at δ 18.5 and 18.6 corresponding to the C-6 methyl group of two L-rhamnoses, and C_1, C_2 and C_3 carbon signals corresponding to an arabinose moiety, which were identical with those of methyl proto-loureiroside (IX). Consequently, the arabinose of Xa should be present in α-furanoside structure. The configurations of two D-glucoses of X were deduced to be β and those of two L-rhamnoses to be α based on the $J_{C_1-H_1}$ coupling constants of ^{13}C-NMR spectrum of X, 157 Hz (δ 100.5, glc), 170 Hz (δ 101.8, 102.1, rha), and 156 Hz (δ 104.9, glc). The signal corresponding to the anomeric proton of the arabinose of X shows a singlet-like signal at δ 5.23. Consequently, the configuration of L-arabinofuranoside was deduced to be α.

Methylation of Xa by Hakomori's method afforded a per-*O*-methyl derivative (Xb), which was methanolyzed to afford methyl 3,6-di-*O*-methylglucopyranoside, methyl 2,3,4-tri-*O*-methylrhamnopyranoside, and methyl 2,3-di-*O*-methylarabinofuranoside (or methyl 2,3-di-*O*-methylarabinopyranoside). Furthermore, partial acidic hydrolysis

of Xa afforded diosgenin, trillin and prosapogenin A of dioscin, which were identified by comparing the TLC behavior and NMR spectra with those of respective authentic samples.

Consequently, the structure of X was concluded to be 26-*O*-*β*-D-glucopyranosyl (25*R*)-22-*O*-methyl-furost-5-en-3*β*,26-diol 3-*O*-[α-L-rhamnopyranosyl-(1→5)-α-L-arabinofuranosyl-(1→4)][α-L-rhamnopyranosyl-(1→2)]-*β*-D-

TABLE I. ^{13}C-NMR Chemical Shifts of the Sugar Moieties of VI, VIII, VIIIa, IX, IXa, X and Xa

		VI		VIII		VIIIa	IX		IXa	X		Xa
C_3 sugars			$J_{C_1-H_1}$		$J_{C_1-H_1}$			$J_{C_1-H_1}$			$J_{C_1-H_1}$	
glc	1	100.1	(156 Hz)	100.4	(156 Hz)	100.6	100.5	(160 Hz)	100.2	100.5	(157 Hz)	100.4
	2	78.5		81.0		81.3	78.9		78.6	79.0		79.0
	3	87.7		76.7		77.1	76.9		76.7	76.7		76.6
	4	70.3		78.4		78.7[a]	78.5[a]		78.3[a]	78.6		78.5
	5	77.8		77.6		77.9	77.9		77.8	77.7		77.7
	6	62.5		61.5		61.8	61.9		61.5	61.8		61.7
rha (→ ^{2}glc)	1	102.4	(169 Hz)	102.0[a]	(172 Hz)	102.2[b]	102.2	(172 Hz)	102.1	102.1[a]	(170 Hz)	102.2[a]
	2	72.4		72.3		72.6	72.6		72.4	72.3		72.3
	3	72.7		72.6		72.9	73.1		72.8	72.9[b]		72.8[b]
	4	73.8[a]		74.1		74.5[c]	74.5		74.2	74.3		74.3
	5	69.8		69.4		69.7	69.7		69.5	69.9		69.9
	6	18.5[b]		18.5[b]		19.2[d]	19.1		18.7	18.6[c]		18.6[c]
rha (→ ^{3}glc)	1	103.7	(167 Hz)									
	2	72.2										
	3	72.4										
	4	73.5[a]										
	5	70.6										
	6	18.3[b]										
rha (→ ^{4}glc)	1			102.3[a]	(169 Hz)	102.5[b]						
	2			69.9		70.3						
	3			72.4		72.8						
	4			78.0		77.9						
	5			68.3		68.6						
	6			18.5[b]		19.1[d]						
rha (→ ^{4}rha)	1			102.1[a]	(169 Hz)	102.3[b]						
	2			82.4		82.7						
	3			72.7		73.1						
	4			74.1		74.4[c]						
	5			73.1		73.4						
	6			18.2[b]		18.7						
glc (→ ^{2}rha)	1			107.0	(158 Hz)	107.2						
	2			75.6		75.9						
	3			78.0		78.5[a]						
	4			71.8		72.3						
	5			78.4		78.6[a]						
	6			62.9		63.3						
ara (→ ^{4}glc)	1						109.9	(173 Hz)	109.7	110.0	(172 Hz)	110.0
	2						83.4		83.1	83.3		83.2
	3						78.6[a]		78.4[a]	78.7		78.7
	4						84.3		84.5	83.6		83.6
	5						70.6		70.7	67.9		67.8
glc (→ ^{5}ara)	1						105.4[b]	(158 Hz)	105.4			
	2						75.5		75.0			
	3						78.1		77.8			
	4						72.1[c]		71.7			
	5						78.5[a]		78.2[a]			
	6						63.2[d]		62.7			
rha (→ ^{5}ara)	1									101.8[a]	(170 Hz)	101.7[a]
	2									72.1		72.1
	3									72.7[b]		72.7[b]
	4									74.0		74.0
	5									69.5		69.5
	6									18.5[c]		18.5[c]
C_{26} sugar												
glc	1	104.7	(157 Hz)	104.7	(156 Hz)		105.0[b]	(157 Hz)		104.9	(156 Hz)	
	2	75.1		75.0			75.5			75.3		
	3	78.1		78.0			78.2			78.4		
	4	71.9		71.8			72.3[c]			72.1		
	5	78.5		78.4			78.9			78.7		
	6	63.0		62.9			63.4[d]			63.2		

Chemical shifts were measured in pyridine-d_5 at 50 °C. *a—d*) Assignments may be interchangeable within the same column.

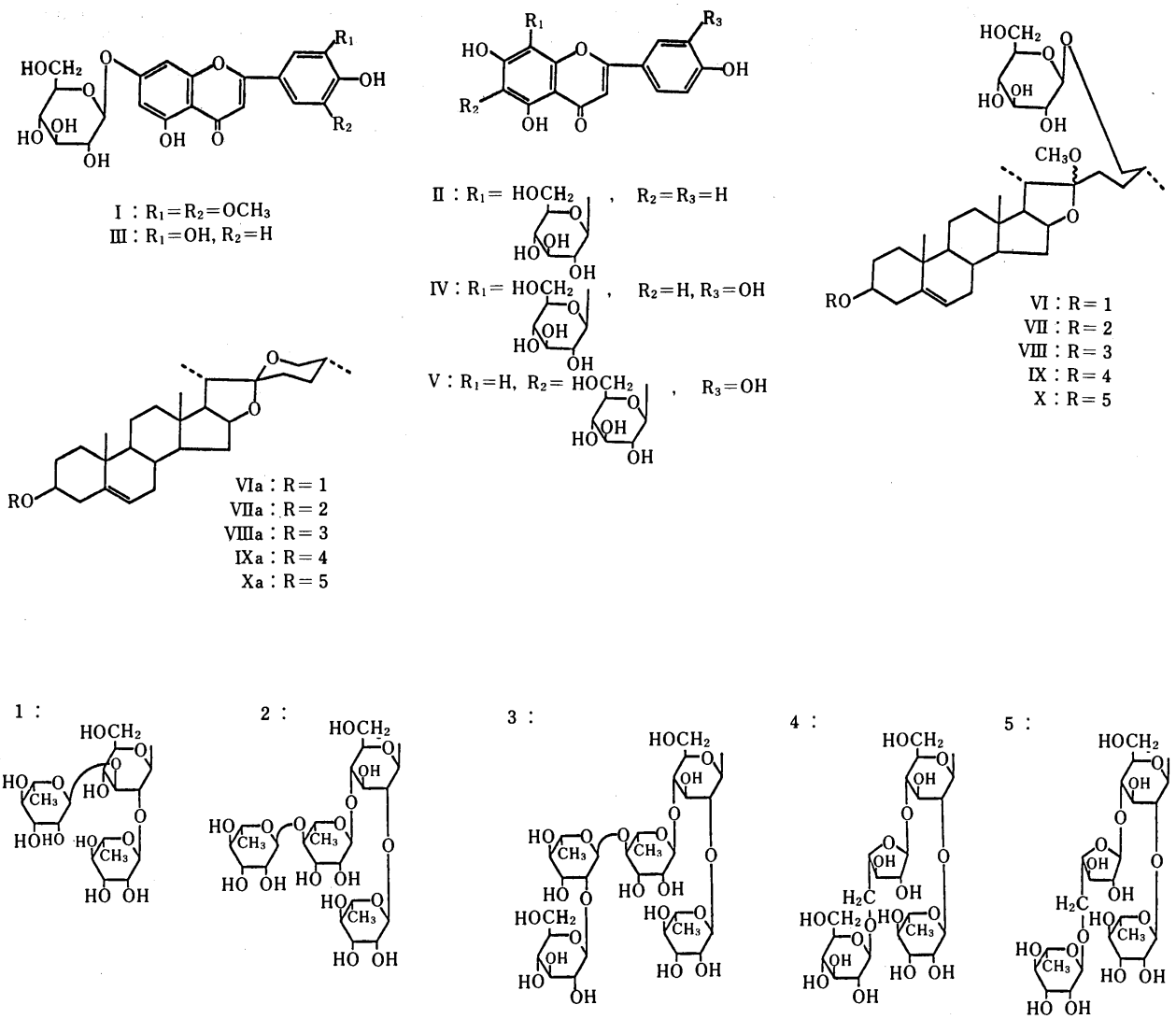


Chart 1

glucopyranoside, while the structure of Xa was established to be diosgenin 3-*O*-[*β*-D-glucopyranosyl-(1→5)-α-L-arabinofuranosyl-(1→4)][α-L-rhamnopyranosyl-(1→2)]-*β*-D-glucopyranoside. X and Xa were the first isolated compounds from natural sources, and we propose the names methyl proto-reclinatoside for the former, and reclinatoside for the latter.

Finally, glucoluteolin (III) and methyl proto-Pb (VII) were separated from the methanol extract of *A. romanzoffianum* BECCARI. These constituents were characterized by comparing the TLC behavior and the IR and NMR spectra with those of respective authentic samples.

In this paper, we described the isolation and structure elucidation of steroid glycosides and flavonoids of four Palmae plants. The new steroid glycosides, namely methyl proto-taccaoside (VI) from *P. rupicola*, methyl proto-rupicolaside (VIII) from *P. rupicola*, *P. loureirii* and *P. reclinata*, methyl proto-loureiroside (IX) from *P. loureilii*, methyl proto-reclinatoside (X) from *P. reclinata* were isolated and the chemical structures were elucidated. The isolation of IX and X, which have an arabinofuranoside in the oligosaccharide moiety of saponins from Palmae plants, were the first example. On the other hand, methyl proto-Pb, which is a known compound, was isolated from *P. rupicola*, *P. loureirii*, *P. reclinata* and *A. romanzoffianum*.

Williams[12] reported the distribution of flavonoids in Palmae plants by using TLC-methods. In this paper, the isolation and identification of flavonoids has been reported and tricin 7-*O*-*β*-D-glucoside and vitexin from *P. rupicola*, vitexin, glucoluteolin, orientin and isoorientin from *P. loureirii*, and glucoluteolin from *A. romanzoffianium* were confirmed, but no flavonoid has been isolated from *P. reclinata*. These results described above are very interesting from the standpoint of the biogenesis of steroid glycosides and flavonoids of Palmae plants.

Experimental

All melting points were determined on a Yanagimoto micro-melting point apparatus (hot-stage type) and are uncorrected. The optical rotations were measured with a JASCO DIP-140 polarimeter at room temperature. The IR spectra were recorded with a Hitachi EPI-2, and the NMR spectra were recorded with a JEOL GX-400 spectrometer (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR). Chemical shifts are given on a δ (ppm) scale with tetramethylsilane as an internal standard. Gas-liquid chromatography (GLC) was run on a Shimadzu GC-6A unit equipped with a flame ionization detector. Experimental conditions for sugars: (a) column, 5% SE-52 on Chromosorb W 3 mm × 2 m; column temp., 200 °C; injection temp., 220 °C; carrier gas N_2, 1.0 kg/cm^2 or (b) column, 5% SE-52

on Chromosorb W 3 mm × 2 m; column temp., 180 °C; injection temp., 200 °C; carrier gas N_2, 1.0 kg/cm^2; samples, trimethylsilyl (TMS) ether. Experimental conditions for *O*-methylated sugars: (c) column, 5% neopentylglycol succinate (NPGS) on Shimalite 3 mm × 2 m; column temp., 160 °C; injection temp., 180 °C; carrier gas N_2, 1.0 kg/cm^2 or (d) column, 5% NPGS on Shimalite 3 mm × 2 m; column temp., 150 °C; injection temp., 170 °C; carrier gas N_2, 1.0 kg/cm^2; samples, TMS ether. TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck) using the following solvents: a, hexane–acetone (3 : 1, v/v); b, hexane–acetone (4 : 1, v/v); c, $CHCl_3$–MeOH–H_2O (7 : 3 : 0.4, v/v). Detection was achieved by spraying with 10% H_2SO_4 followed by heating.

Extraction and Isolation of the Compounds from the Leaves The dried leaves of *P. rupicola* T. ANDERSON (3.2 kg), *P. loureirii* KUNTH. (6.4 kg), *P. reclinata* N. J. JACQUIN (1.4 kg) and *A. romanzoffianum* BECCARI (1.3 kg), harvested from the Tokyo-to Ogasawara Subtropical Agricultural Experiment Station in January 1985, were chopped and extracted with MeOH at room temperature.

a) *P. rupicola*: The MeOH extract was evaporated to dryness *in vacuo*. The residue (273 g) was suspended in water (500 ml) and partitioned with ether (200 ml × 3). The ether layer was concentrated *in vacuo* to afford an ether extract (61 g), and the aqueous layer was partitioned with BuOH saturated with water (250 ml × 3). The BuOH-soluble layer was concentrated under reduced pressure to afford the BuOH extract (67 g), and the aqueous layer was concentrated *in vacuo* to give the water extract (147 g). The BuOH extract was subjected to column chromatography on Sephadex LH-20 with MeOH to afford four fractions, fr. 1 (27.1 g), fr. 2 (22.8 g), fr. 3 (9.2 g), and fr. 4 (1.4 g). Fraction 1 was subjected to column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (13 : 7 : 1, v/v) to yield three fractions, namely VI containing fraction, VII containing fraction and VIII containing fraction. Each fraction was refluxed with MeOH for 1 h, and each solution was concentrated to 10 ml. AcOEt (300 ml) was added to the methanolic solution, and the precipitate was collected by filtration to afford VI (3.31 g), VII (0.20 g) and VIII (0.32 g), respectively. Fraction 2 was subjected to column chromatography on polyamide with 80% MeOH, to fractionate into two fractions, frs. A and B, and the former was subjected to column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (7 : 3 : 0.4, v/v) to afford I containing fraction and II containing fraction. Crude I and II were recrystallized from MeOH to afford I (0.06 g) and II (0.09 g), respectively.

b) *P. loureirii*: The MeOH extract (454 g) was treated by the same method as described above, and afforded the ether extract (40 g), the BuOH extract (93 g) and the water extract (320 g). The BuOH extract was subjected to column chromatography on Sephadex LH-20 with MeOH to afford four fractions, fr. 1 (49.7 g), fr. 2 (34.8 g), fr. 3 (6.7 g) and fr. 4 (1.3 g). Fraction 1 was treated by the same method as described above to afford VII (0.45 g), VIII (0.34 g) and IX (0.30 g). Fraction 2 was treated by the same method as described above to afford II (0.02 g), III (0.02 g), IV (0.15 g), and V (0.12 g), which were purified by recrystallization from MeOH.

c) *P. reclinata*: The MeOH extract (183 g) was treated by the same method as described above, and afforded the ether extract (30 g), the BuOH extract (31 g) and the water extract (115 g). The BuOH extract was subjected to column chromatography on Sephadex LH-20 with MeOH to afford three fractions, fr. 1 (14.4 g), fr. 2 (7.8 g) and fr. 3 (7.3 g). Fraction 1 was treated by the same method as described above to afford VII (0.16 g), VIII (0.68 g) and X (1.03 g).

d) *A. romanzoffianum*: The MeOH extract (189 g) was treated by the same method as described above, and afford the ether extract (58 g), the BuOH extract (29 g) and the water extract (101 g). The BuOH extract was subjected to column chromatography on Sephadex LH-20 with MeOH to afford four fractions, fr. 1 (1.0 g), fr. 2 (9.8 g), fr. 3 (17.2 g) and fr. 4 (0.9 g). Fraction 2 was treated by the same method as described above to afford VII (0.44 g). Furthermore, fr. 4 was subjected to column chromatography on Avicel with $CHCl_3$–MeOH–H_2O (7 : 3 : 1, v/v, lower phase) to afford III containing fraction. Crude III was recrystallized from MeOH to afford III (0.02 g).

Properties of I, II, III, IV, V, Methyl Proto-taccaoside (VI), VII, Methyl Proto-rupicolaside (VIII), Methyl Proto-loureiroside (IX) and Methyl Proto-reclinatoside (X) I (Tricin 7-*O*-β-D-glucopyranoside): Yellow needles from MeOH, mp 251—254 °C (dec.). $FeCl_3$, dark green; Mg–HCl, red. $[\alpha]_D$ −65.9° (*c* = 0.26, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3500—3300, 1660, 1605. UV λ_{max}^{MeOH} nm (log ε): 248 (4.25), 270 (4.19), 350 (4.34). $\lambda_{max}^{MeOH+AlCl_3}$ nm: 278, 302, 394. $\lambda_{max}^{MeOH+CH_3COONa}$ nm: 248, 270, 350. *Anal.* Calcd for $C_{23}H_{24}O_{12}$: C, 56.10; H, 4.91. Found: C, 56.11; H, 5.03. II (Vitexin): Yellow needles from MeOH, mp 259—263 °C (dec.). $FeCl_3$, dark green; Mg–HCl, red. $[\alpha]_D$ −18.9° (*c* = 0.23, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3400—3200, 1655, 1608. UV λ_{max}^{MeOH} nm (log ε): 323 (4.18), 362 (4.18). *Anal.* Calcd for $C_{21}H_{20}O_{10}\cdot 1/3H_2O$: C, 57.53; H, 4.75. Found: C, 57.52; H, 4.58. III (Glucoluteolin): Yellow needles from MeOH, mp 253—257 °C (dec.). $FeCl_3$, dark green; Mg–HCl, red. $[\alpha]_D$ −65.9° (*c* = 0.26, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3500—3300, 1660, 1605. UV λ_{max}^{MeOH} nm (log ε): 248 (4.25), 270 (4.19), 342 (4.20). *Anal.* Calcd for $C_{23}H_{24}O_{12}\cdot 2/3H_2O$: C, 54.78; H, 4.67. Found: C, 54.69; H, 4.67. IV (Orientin): A yellow powder from MeOH, (mp 209—213 °C (dec.)). $FeCl_3$, dark green; Mg–HCl, red. $[\alpha]_D$ −22.6° (*c* = 0.40, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3500—3300, 1650, 1620. UV λ_{max}^{MeOH} nm (log ε): 250 (4.21), 258 (4.19), 342 (4.27). *Anal.* Calcd for $C_{21}H_{20}O_{11}$: C, 56.25; H, 4.50. Found: C, 56.24; H, 4.63. V (Isoorientin); A yellow powder from MeOH, (mp 229—233 °C (dec.)). $FeCl_3$, dark green; Mg–HCl, red. $[\alpha]_D$ +19.8° (*c* = 0.24, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3500—3300, 1650, 1620. UV λ_{max}^{MeOH} nm (log ε): 250 (4.22), 260 (4.22), 342 (4.34). *Anal.* Calcd for $C_{21}H_{20}O_{10}\cdot 1/2H_2O$: C, 55.14; H, 4.62. Found: C, 55.04; H, 4.73. VI: A white powder from MeOH–AcOEt, (mp 180—183 °C (dec.)). Ehrlich reaction, positive. $[\alpha]_D$ −79.2° (*c* = 1.05, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3600—3200 (OH). ^{13}C-NMR δ: aglycone: 37.2 (C_1), 30.2 (C_2), 78.1 (C_3), 38.8 (C_4), 141.0 (C_5), 121.7 (C_6), 32.3 (C_7), 31.8 (C_8), 50.5 (C_9), 37.2 (C_{10}), 21.1 (C_{11}), 40.0 (C_{12}), 40.9 (C_{13}), 56.7 (C_{14}), 32.3 (C_{15}), 81.4 (C_{16}), 64.2 (C_{17}), 16.3 (C_{18}), 19.4 (C_{19}), 40.6 (C_{20}), 16.1 (C_{21}), 112.8 (C_{22}), 30.8 (C_{23}), 28.3 (C_{24}), 34.3 (C_{25}), 75.1 (C_{26}), 17.2 (C_{27}), 47.4 (OCH_3); sugar moiety: Table I. *Anal.* Calcd for $C_{52}H_{86}O_{22}\cdot 3/2H_2O$: C, 57.28; H, 8.23. Found: C, 57.09; H, 7.75. VII (Methyl proto-Pb): A white powder from MeOH–AcOEt, (mp 192—195 °C (dec.)). Ehrlich reaction, positive. $[\alpha]_D$ −91.8° (*c* = 0.91, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3600—3200 (OH). VIII: A white powder from MeOH–AcOEt, (mp 195—199 °C (dec.)). Ehrlich reaction, positive. $[\alpha]_D$ −82.6° (*c* = 0.71, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3600—3200 (OH). ^{13}C-NMR δ: each signal of the aglycone moiety of VIII was analogous to that of VI; sugar moiety: Table I. *Anal.* Calcd for $C_{64}H_{106}O_{31}\cdot 3/2H_2O$: C, 54.96; H, 7.86. Found: C, 55.08; H, 7.77. IX: A white powder from MeOH–AcOEt, (mp 183—186 °C (dec.)). Ehrlich reaction, positive. $[\alpha]_D$ −84.1° (*c* = 1.06, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3600—3200 (OH). ^{13}C-NMR δ: each signal of the aglycone moiety of IX was analogous to that of VI; sugar moiety: Table I. *Anal.* Calcd for $C_{57}H_{94}O_{27}\cdot 2H_2O$: C, 55.02; H, 7.70. Found: C, 55.11; H, 7.87. X: A white powder from MeOH–AcOEt, (mp 165—168 °C (dec.)). Ehrlich reaction, positive. $[\alpha]_D$ −44.2° (*c* = 1.05, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3600—3200 (OH). ^{13}C-NMR δ: each signal of the aglycone moiety of X was analogous to that of VI; sugar moiety: Table I. *Anal.* Calcd for $C_{57}H_{94}O_{26}\cdot 3H_2O$: C, 54.79; H, 8.07. Found: C, 54.79; H, 7.64.

Enzymatic Hydrolysis of VI, VIII, IX, and X An aqueous solution of each of VI (0.25 g), VIII (0.30 g), IX (0.25 g) and X (0.20 g, was incubated with almond emulsin (50 mg) at 37 °C for 8 h. The precipitate was collected by filtration, dried and recrystallized from MeOH. The aqueous filtrate was evaporated to dryness *in vacuo* and the residue was examined by GLC (condition a) t_R (min) 5.8, 7.9 (TMS-glucose). The hydrolysate of each compound was characterized as follows. Hydrolysate of VI (VIa, taccaoside[4]): The prosapogenin (0.08 g) was obtained as colorless needles from diluted MeOH, mp 262—264 °C (dec.), $[\alpha]_D$ −113.1° (*c* = 1.05, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3600—3200 (OH), 980, 920, 900, 860 (intensity 920 < 900, (25*R*)-spiroketal). *Anal.* Calcd for $C_{45}H_{72}O_{16}\cdot H_2O$: C, 60.92; H, 8.41. Found: C, 60.87; H, 8.29. Hydrolysate of VIII (rupicolaside, VIIIa): The prosapogenin (0.18 g) was obtained as a white powder from diluted MeOH, (mp 229—234 °C (dec.)), $[\alpha]_D$ −97.6° (*c* = 0.50, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3600—3200 (OH), 980, 920, 900, 860 (intensity 920 < 900, (25*R*)-spiroketal). ^{13}C-NMR δ: aglycone: 38.0 (C_1), 30.7 (C_2), 78.7 (C_3), 39.5 (C_4), 140.9 (C_5), 121.7 (C_6), 32.8 (C_7), 32.3 (C_8), 50.8 (C_9), 37.6 (C_{10}), 21.7 (C_{11}), 40.4 (C_{12}), 41.0 (C_{13}), 57.1 (C_{14}), 32.7 (C_{15}), 81.4 (C_{16}), 63.4 (C_{17}), 16.9 (C_{18}), 19.9 (C_{19}), 42.5 (C_{20}), 15.5 (C_{21}), 109.4 (C_{22}), 32.4 (C_{23}), 29.8 (C_{24}), 31.1 (C_{25}), 67.2 (C_{26}), 17.8 (C_{27}); sugar moiety: Table I. *Anal.* Calcd for $C_{57}H_{82}O_{25}\cdot 5/2H_2O$: C, 55.01; H, 7.83. Found: C, 55.89; H, 7.83. Hydrolysate of IX (loureiroside, IXa): The prosapogenin (0.12 g) was obtained as a white powder from diluted MeOH, (mp 272—276 °C (dec.)), $[\alpha]_D$ −107.0° (*c* = 0.38, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3600—3200 (OH), 980, 920, 900, 860 (intensity 920 < 900, (25*R*)-spiroketal). ^{13}C-NMR δ: each signal of the aglycone moiety of IXa was analogous to that of VIIIa; sugar moiety: Table I. *Anal.* Calcd for $C_{50}H_{80}O_{21}\cdot H_2O$: C, 58.25; H, 8.08. Found: C, 58.25; H, 8.00. Hydrolysate of X (reclinatoside, Xa): The prosapogenin (0.14 g) was obtained as colorless needles from diluted MeOH, mp 227—230 °C (dec.), $[\alpha]_D$ −111.3° (*c* = 0.55, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3600—3200 (OH), 980, 920, 900, 860 (intensity 920 < 900, (25*R*)-spiroketal). ^{13}C-NMR δ: each signal of the aglycone moiety of Xa was analogous to that of VIIIa; sugar moiety: Table I. *Anal.* Calcd for

$C_{50}H_{80}O_{20}\cdot 2H_2O$: C, 57.90; H, 8.16. Found: C, 57.61; H, 7.83.

Acidic Hydrolysis of VIIIa, IXa and Xa VIIIa (5 mg), IXa (20 mg) and Xa (50 mg) were separately hydrolyzed with 2 N HCl–50% dioxane (0.2 ml/mg prosapogenin) by refluxing for 2 h on a water bath. Each reaction mixture was cooled and neutralized with $NaHCO_3$. After filtration, each filtrate was evaporated to dryness and the residue was examined by TLC and GLC. TLC (solvent a), VIIIa, IXa and Xa: *Rf* 0.48 (diosgenin). GLC (condition a) VIIIa t_R (min) 2.0, 2.6 (TMS-rhamnose), 5.8, 7.9 (TMS-glucose). IXa and Xa GLC (condition b) t_R (min) 3.8, 4.2 (TMS-arabinose), 3.9, 4.5 (TMS-rhamnose), 11.3, 16.9 (TMS-glucose).

Determination of Absolute Configurations of Sugars by HPLC Each solution of VIIIa—Xa (1 mg) in 2 N HCl–50% dioxane (2 ml) was heated in a sealed tube for 3 h at 100 °C. The reaction mixture was diluted with water and evaporated to remove dioxane. The solution was neutralized with Amberlite IRA-93ZU (OH^- form) and passed through a SEP-PAK C_{18} cartridge to give a sugar fraction. Each component sugar in the solution was derivated to 1-(*N*-acetyl-L-α-methylbenzylamino)-1-deoxyalditol acetate using the method of Asada *et al.*[13] and analyzed by normal- and reversed-phase HPLC. Conditions of normal-phase HPLC: column, supermicro bead silica gel B-5, 5 μm (10 × 250 mm); solvent, hexane–EtOH (9 : 1); flow rate, 4 ml/min; detection, ultraviolet (UV) (230 nm). t_R (min) from VIIIa: L-rhamnose 22.8, D-glucose 37.7 (reference: D-rhamnose 20.5, L-glucose 36.0). Conditions of reversed-phase HPLC: column, Chromatorex-ODS DU0015MT (10 × 250 mm); solvent, 40% CH_3CN; flow rate, 3 ml/min; detection, UV (230 nm). t_R (min) L-rhamnose 41.3, D-glucose 36.6 (reference: D-rhamnose 41.3, L-glucose 34.0). From IXa and Xa: normal-phase HPLC t_R (min): L-arabinose 33.0, L-rhamnose 22.8, D-glucose 37.7 (reference: D-arabinose 28.9, D-rhamnose 20.5, L-glucose 36.0); reversed-phase HPLC t_R (min): L-arabinose 23.2, L-rhamnose 41.3, D-glucose 36.6 (reference: D-arabinose 23.6, D-rhamnose 41.3, L-glucose 34.0).

Methylation of VIIIa, IXa and Xa by Hakomori's Method According to Hakomori's method, NaH (200 mg) was defatted by anhydrous benzene followed by petroleum ether, then warmed with dimethylsulfoxide (DMSO, 8 ml) at 65 °C in an oil bath for 1 h with stirring under N_2 flow. A solution of VIIIa (100 mg) in a small amount of (DMSO 0.5 ml) was then added and the mixture was stirred for 1 h under an N_2 flow. CH_3I (10 ml) was then added and the reaction mixture was allowed to stand at room temperature for 1 h with stirring. After dilution with water (50 ml), the reaction mixture was extracted with $CHCl_3$ (10 ml × 3) and the organic layer was washed with water, dried and evaporated to dryness. The residue was chromatographed on silica gel with hexane–acetone (3 : 1, v/v) to afford a per-*O*-methylate of VIIIa (95 mg). Per-*O*-methylation of IXa (30 mg) and Xa (50 mg) by Hakomori's method was carried out, and 30 mg of per-*O*-methylate of IXa and 53 mg of per-*O*-methylate of Xa were obtained, respectively. Per-*O*- methylate of VIIIa (VIIIb): A white powder from aqueous MeOH, (mp 99—100 °C). IR ν_{max}^{Nujol} cm^{-1}: OH (nil). ^{1}H-NMR ($CDCl_3$) δ: 4.38, 4.49 (each 1H, d, J=7 Hz, C_1-H of glc × 2), 5.22 (2H, br s, C_1-H of rha × 2). *Anal.* Calcd for $C_{70}H_{118}O_{25}\cdot H_2O$: C, 61.03; H, 8.78. Found: C, 61.11; H, 8.75. Per-*O*-methylate of IXa (IXb): Colorless needles from aqueous MeOH, mp 131—132 °C. IR ν_{max}^{Nujol} cm^{-1}: OH (nil). ^{1}H-NMR ($CDCl_3$) δ: 4.26, 4.34 (each 1H, d, J=7 Hz, C_1-H of glc × 2), 5.16 (1H, d, J=1 Hz, C_1-H of rha), 5.22 (1H, br s, C_1-H of ara). *Anal.* Calcd for $C_{61}H_{102}O_{21}$: C, 62.06; H, 8.79. Found: C, 62.09; H, 8.75. Per-*O*-methylate of Xa (Xb): Colorless needles from aqueous MeOH, mp 118—121 °C. IR ν_{max}^{Nujol} cm^{-1}: OH (nil). ^{1}H-NMR ($CDCl_3$) δ: 4.35 (1H, d, J=7 Hz, C_1-H of glc), 4.90, 5.17 (each 1H, br s, C_1-H of rha × 2), 5.23 (1H, br s, C_1-H of ara).

Methanolysis of VIIIb, IXb and Xb VIIIb (5 mg), IXb (3 mg) and Xb (3 mg) were individually refluxed with methanolic 5% HCl (1 ml) for 3 h. After cooling, each reaction mixture was neutralized with Ag_2CO_3, and the resulting precipitate was filtered off. The filtrate was concentrated to dryness and the residue was examined by GLC (condition c or d). GLC, t_R (min) as TMS derivative: from VIIIb (condition c): 2.4 (methyl 2,3,4-tri-*O*-methylrhamnopyranoside), 2.5 (methyl 2,3-di-*O*-methylrhamnopyranoside), 1.4 (methyl 3,4-di-*O*-methylrhamnopyranoside), 4.9, 6.8 (methyl 2,3,4,6-tetra-*O*-methylglucopyranoside), 5.8, 6.8 (methyl 3,6-di-*O*-methylglucopyranoside). From IXb (condition d): 3.1, 4.8 (methyl 2,3,4-tri-*O*-methylrhamnopyranoside), 3.3, 4.1 (methyl 2,3-di-*O*-methylarabinofuranoside or methyl 2,3-di-*O*-methylarabinopyranoside), 7.0, 10.3 (methyl 2,3,4,6-tetra-*O*-methylglucopyranoside), 8.5, 10.0 (methyl 3,6-di-*O*-methylglucopyranoside). From Xb (condition d): 3.1, 4.8 (methyl 2,3,4-tri-*O*-methylrhamnopyranoside), 3.3, 4.1 (methyl 2,3-di-*O*-methylarabinofuranoside or methyl 2,3-di-*O*-methylarabinopyranoside), 8.5, 10.0 (methyl 3,6-di-*O*-methylglucopyranoside).

Partial Hydrolysis of IXa and Xa with 0.5 N HCl IXa (70 mg) was dissolved in 0.5 N HCl–50% dioxane (10 ml) and the solution was stirred for 24 h at room temperature. The reaction mixture was neutralized with $NaHCO_3$ and evaporated to dryness *in vacuo*. The residue was subjected to column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (8 : 2 : 0.2, v/v) to afford compound **1** (5 mg) and compound **2** (4 mg). These products were identified as trillin and prosapogenin A of dioscin by comparing TLC behavior and ^{13}C-NMR spectra. On the other hand, Xa (50 mg) was dissolved in 0.5 N HCl–50% MeOH (10 ml) and refluxed for 2 h on a water bath. After cooling, the reaction mixture was neutralized with $NaHCO_3$ and evaporated to dryness *in vacuo*. The residue was subjected to column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (17 : 3 : 1, v/v) to afford compound **1** (5 mg) and compound **2** (23 mg). These products were identified as trillin and prosapogenin A of dioscin by comparing TLC behavior and ^{13}C-NMR spectra.

Partial Methanolysis of VIIIb A solution of VIIIb (60 mg) in 10 ml of methanolic 0.25 N HCl was kept at 65 °C on a water bath. After cooling, the reaction mixture was neutralized with Ag_2CO_3 and filtered. The filtrate was evaporated to dryness *in vacuo*. The residue was methylated by Hakomori's method. The product was chromatographed on silica gel with hexane–acetone (4 : 1, v/v) to afford three compounds, VIIIc (7 mg), VIIId (7 mg) and VIIIe (6 mg). VIIIc: A colorless syrup (not crystallized). This compound was identified as 3-*O*-methyl diosgenin by comparing the TLC behavior (solvent b, *Rf* 0.50). VIIId: Colorless needles from MeOH, mp 167—168 °C. IR ν_{max}^{Nujol} cm^{-1}: OH (nil). ^{1}H-NMR ($CDCl_3$) δ: 4.36 (1H, d, J=7 Hz, C_1-H of glc), 5.24 (1H, br s, C_1-H of rha). VIIIe: Colorless needles from MeOH, mp 191—193 °C. IR ν_{max}^{Nujol} cm^{-1}: OH (nil). ^{1}H-NMR ($CDCl_3$) δ: 4.34, 4.49 (each 1H, d, J=7 Hz, C_1-H of glc × 2), 4.93, 5.22 (each 1H, br s, C_1-H of rha × 2).

Methanolysis of VIIId and VIIIe with Methanolic 5% HCl VIIId (3 mg) and VIIIe (3 mg) were individually refluxed with methanolic 5% HCl (1 ml) for 3 h. After cooling, each reaction mixture was neutralized with Ag_2CO_3 and the resulting precipitate was filtered off. The filtrate was concentrated to dryness and the residue was examined by GLC (condition d). t_R (min) from VIIId: 3.2 (methyl 2,3,4-tri-*O*-methylrhamnopyranoside), 7.5, 8.8 (methyl 3,4,6-tri-*O*-methylglucopyranoside). From VIIIe: 1.9 (methyl 3,4-di-*O*-methylrhamnopyranoside), 3.5 (methyl 2,3-di-*O*-methylrhamnopyranoside), 6.8, 10.3 (methyl 2,3,4,6-tetra-*O*-methylglucopyranoside), 7.5, 11.5 (methyl 2,3,6-tri-*O*-methylglucopyranoside).

Enzymatic Hydrolyses of VIIIa with Pectinase McIlvaine buffer (pH 4.0, 10 ml) and pectinase (20 mg, Sigma Co. Lot. No. 122F-422) were added to a solution of VIIIa (100 mg) in ethanol (1 ml) and the solution was left at 37 °C for 72 h. The precipitate was collected by filtration and subjected to column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (8 : 2 : 0.2, v/v) to afford a prosapogenin (11 mg). The prosapogenin was identified as Pb on the basis of comparisons of TLC behavior and ^{13}C-NMR spectra.

Acknowledgement The authors are grateful to Dr. S. Kawamata, Tokyo-to Ogasawara Subtropical Agricultural Experiment Station, Mr. T. Yasui, a teacher of Tokyo-to Ogasawara High School, and Mr. S. Isoda of the Medicinal Plant Garden of Showa University for providing the plant material. Thanks are also due to the staff of the Analytical Laboratory of this school for elemental analysis, IR and NMR spectral measurements.

References

1) *a*) K. Idaka, Y. Hirai, and J. Shoji, *Chem. Pharm. Bull.*, **36**, 1783 (1988); *b*) Y. Hirai, S. Sanada, Y. Ida, and J. Shoji, *ibid.*, **34**, 82 (1986); *c*) *Idem*, *ibid.*, **32**, 4003 (1984); *d*) *Idem*, *ibid.*, **32**, 295 (1984).
2) N. A. M. Saleh, B. A. Bohm, and J. R. Maze, *Phytochemistry*, **10**, 490 (1971).
3) S. Kiyosawa, M. Hutoh, T. Komori, T. Nohara, I. Hosokawa, and T. Kawasaki, *Chem. Pharm. Bull.*, **16**, 1162 (1968).
4) H. N. Pharn, A. N. Kelginbaev, M. B. Gorovits, and N. K. Abubakirov, *Khim. Prir. Soedin.*, **1980**, 352.
5) M. E. Wall, C. R. Eddy, M. L. McClennan, and M. E. Klumpp, *Anal. Chem.*, **24**, 1337 (1952).
6) R. Oshima, Y. Yamauchi, and J. Kumanotani, *Carbohydr. Res.*, **107**, 169 (1982).
7) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
8) D. K. Bhardwaj, M. S. Bisht, and C. K. Mehta, *Phytochemistry*, **19**, 2040 (1980).
9) E. Fujita, S. Johne, R. Kasai, M. Node, and O. Tanaka, "Progress

in the Chemistry of Natural Products," Vol. 46, Springer-Verlag, Wien, 1984, p. 26.
10) K. Mizutani, M. Nakamura, R. Kasai, O. Tanaka, and H. Matsuura, Abstracts of Papers, the 105th Annual Meeting of the Pharmaceutical Society of Japan, Kanazawa, April 1985, p. 513.
11) *a*) T. Tsukamoto, T. Kawasaki, and T. Yamauchi, *Chem. Pharm. Bull.*, **4**, 35 (1956); *b*) T. Kawasaki and T. Yamauchi, *ibid.*, **10**, 703 (1962).
12) C. A. Williams, J. B. Harbone, and H. T. Clifford, *Phytochemistry*, **12**, 2417 (1973).
13) Y. Asada, M. Ikeno, T. Ueoka, and T. Furuya, *Chem. Pharm. Bull.*, **37**, 2747 (1989).

Relative Inhibitory Activity of Berberine-Type Alkaloids against 12-*O*-Tetradecanoylphorbol-13-acetate-Induced Inflammation in Mice

Ken YASUKAWA,*[,a] Michio TAKIDO,[a] Tetsuro IKEKAWA,[b] Fumitake SHIMADA,[b] Mieko TAKEUCHI[c] and Shigeki NAKAGAWA[d]

College of Pharmacy, Nihon University,[a] Narashinodai, Funabashi-shi, Chiba 274, Japan, National Cancer Center Research Institute,[b] Tsukiji, Chuo-ku, Tokyo 104, Japan, Institute of Microbial Chemistry,[c] Kamiosaki, Shinagawa-ku, Tokyo 141, Japan and Department of Biochemistry, Nihon University School of Medicine,[d] Ohyaguchikami-machi, Itabashi-ku, Tokyo 173, Japan. *Received October 29, 1990*

Forty eight derivatives of berberine-type alkaloids were examined for their inhibition activity against the induction of edema on mouse ear by application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Berberine had an inhibitory effect against TPA-induced ear edema at a grade corresponding to those of quercetin, caffeine and cepharanthine. Berberine derivatives had stronger inhibitory activity than palmatine derivatives. 9-*N*,*N*-Diphenylcarbamoyl derivatives of both 9-demethylberberine and 9-demethylpalmatine had rather strong activity. These inhibitory activities are about ten times the activity of the respective mother compounds. Furthermore, 9-*N*-monophenylcarbamoyl derivatives and *N*,*N*-diphenylcarbamoyl chloride are found to have no effect.

Keywords anti-tumor promoter; 12-*O*-tetradecanoylphorbol-13-acetate (TPA); berberine-type alkaloids; anti-inflammation; berberine; isoquinoline alkaloid

Introduction

Since it is known that, in general, promoters of carcinogenesis also have potent irritant activity,[1] we studied the inhibitory effects of chemical components of higher plants against inflammation in mouse skin induced by a strong tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). We have reported the relative inhibitory activity of flavonoids,[2] sterols and triterpenes[3] on TPA-induced inflammation in mice, from results of screening tests for antitumor-promoting activity. These studies revealed a certain correlation between the inhibitory effect against the TPA-induced inflammation and the inhibition of TPA-induced tumor promotion in a two-stage carcinogenesis experiment.[2-5]

As berberine is a major compound present in Phellodendri Cortex (Obaku in Japanese) and Coptidis Rhizoma (Oren in Japanese), which are used for treating diarrhea and other gastrointestinal diseases in Japan and other Eastern countries, there are many reports of studies on its anti-inflammatory activity,[6] anti-hypertensive activity,[7] and anti-bacterial activity.[8] The anti-tumor activity[9,10] of berberine, and its inhibition of promotion in two-stage carcinogenesis[11] have also been reported. Cepharanthine, a dimer of benzylisoquinoline derivatives, is also known to inhibit promotion in two-stage carcinogenesis.[12] Berberine has almost the same level of inhibitory activity against TPA-induced inflammation as quercetin, which was reported by Sugimura *et al.* to have an inhibitory effect against promotion in two-stage carcinogenesis.[13,14]

In the present study, the inhibitory effect of 48 derivatives of berberine and palmatine on TPA-induced inflammation in mice was investigated. It was found that berberine derivatives had stronger inhibitory activity than palmatine derivatives. However, 9-*N*,*N*-diphenylcarbamoyl derivatives of both 9-demethylberberine and 9-demethylpalmatine had rather strong activity. Of interest may be that *N*-monophenylcarbamoyl derivatives had no influence on the substitution at 9-position of the compounds.

Materials and Methods

Chemicals TPA was purchased from Chemicals for Cancer Research Inc., Minnesota, U.S.A. Berberine chloride (**3**), berberine sulfate (**4**), *N*-phenylcarbamic acid methyl ester, *N*-phenylcarbamic acid ethyl ester, phenylcarbamide, *N*,*N*-dimethylcarbamoyl chloride, *N*,*N*-diethylcarbamoyl chloride, *N*,*N*-diphenylcarbamoyl chloride, acetone, chloroform and methanol were purchased from Tokyo Kasei Kogyo Co., Ltd., Japan. Palmatine chloride (**27**) was offered by Alps Pharmaceutical Industrial Co., Ltd., Japan. Other compounds were synthesized by one of the authors (F. Shimada): berberrubine (**1**),[15] berberrubine hydrochloride (**2**),[16] 9-ethyl, 9-demethylberberine chloride (**5**), 12-bromoberberrubine (**16**),[17] 9-*n*-propyl, 9-demethylberberine bromide (**6**),[18] 9-acetyl, 9-demethylberberine chloride (**7**), 9-pivaloyl, 9-demethylberberine chloride (**8**),[19] 9-cinnamoyl, 9-demethylberberine chloride (**9**),[20] 9-*N*-methylcarbamoyl, 9-demethylberberine chloride (**10**), 9-*N*,*N*-dimethylcarbamoyl, 9-demethylberberine chloride (**11**), 9-*N*,*N*-diethylcarbamoyl, 9-demethylberberine chloride (**12**), 9-*N*,*N*-diphenylcarbamoyl, 9-demethylberberine chloride (**13**), 9-*N*-methylcarbamoyl, 9-demethyl, 13-methylberberine chloride (**24**), 9-*N*-phenylcarbamoyl, 9-demethyl, 13-methylberberine chloride (**25**), 9-*N*-methylcarbamoyl, 9-demethylpalmatine chloride (**35**), 9-*N*,*N*-dimethylcarbamoyl, 9-demethylpalmatine chloride (**36**), 9-*N*,*N*-diphenylcarbamoyl, 9-demethylpalmatine chloride (**37**),[21] 8-methylberberine bromide (**14**), 8-*n*-propylberberine bromide (**15**),[22] 13-methylberberrubine hydrogensulfate (**17**), 13-ethylberberrubine (**18**), palmatrubine hydrochloride (**26**), 13-methylpalmatine chloride (**38**), 2,3,9,10-tetraethyl, 2,3,9,10-tetrademethylpalmatine bromide (**40**), 2,3,9,10-tetraallyl, 2,3,9,10-tetrademethylpalmatine bromide (**41**),[23] 13-methylberberine iodide (**19**), 13-ethylberberine iodide (**20**), 13-allylberberine bromide (**21**), 13-ethoxyberberine chloride (**22**), 13-methyl, 2,9-didemethylpalmatine chloride (**39**), 9-demethyl, 13-methyltetrahydroberberine (**45**),[24] 9-4'-methoxybenzoyl, 9-demethyl, 13-methylberberine chloride (**23**),[25] 9-acetyl, 9-demethylpalmatine chloride (**28**), 9-propionyl, 9-demethylpalmatine chloride (**29**), 9-*n*-butyroyl, 9-demethylpalmatine chloride (**30**), 9-palmitoyl, 9-demethylpalmatine chloride (**31**), 9-isovaleroyl, 9-demethylpalmatine chloride (**32**), 9-benzoyl, 9-demethylpalmatine chloride (**33**), 9-4'-chlorobenzoyl, 9-demethylpalmatine chloride (**34**),[26] tetrahydroberberine (**42**), 9-propyl, 9-demethyltetrahydroberberine hydrochloride (**43**), 9-acetyl, 9-demethyltetrahydroberberine hydrochloride (**44**),[27] *N*-methyl, 9-demethyltetrahydroberberine methosulfate (**46**), *N*-methyltetrahydroberberine chloride (**47**), *N*-ethyltetrahydroberberine chloride (**48**).[28]

Animals Female ICR mice were obtained from Shizuoka Laboratory Animal Center, Shizuoka, Japan, and housed in an air-conditioned room (22—23 °C) lit from 08:00 to 20:00. Food and water were available *ad libitum*.

Assay of TPA-Induced Inflammation TPA (1 μg) dissolved in acetone (20 μl) was applied to the right ear only of ICR mice by means of a micropipette. A volume of 10 μl was delivered to both the inner and outer surfaces of the ear. The sample or its vehicle, chloroform–methanol (1:1, 20 μl), as a control, was applied topically about 30 min before each TPA treatment. The edema was measured 8 h after TPA treatment. Application of the sample (2, 1, 0.5, 0.2, 0.1 or 0.05 mg/ear) completely inhibited TPA-induced inflammation and this inhibitory activity was reduced in a dose-dependent manner. For ear thickness determinations, a pocket

thickness gauge (Mitsutoyo Co., Ltd. Japan) with a range of 0—9 mm, graduated at 0.01-mm intervals and modified so that the contact surface area was increased, thus reducing the tension, was applied to the tip of the ear. Each value was the mean of individual determinations from 5 mice, and ED_{50} values were determined by method of probit-graphic interpolation for at least 4 dose levels.

Results

Berberine sulfate (**4**) had an inhibitory effect against TPA-induced inflammation in the ears of mice at a grade corresponding to those of quercetin, caffeine and cepharanthine (Table I). As shown in Tables II and III, berberine and palmatine derivatives were tested for their ability to reduce the intensity of TPA-induced ear edema.

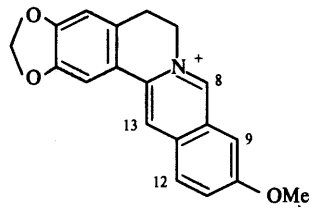
TABLE I. Inhibitory Effect of Anti-tumor Promoters on TPA-Induced Inflammation in Mice

Compound	ED_{50}	
	μmol/ear	mg/ear
Quercetin	6.6	2.0
Caffeine	8.8	1.7
Cepharanthine	0.9	0.5
Berberine sulfate	1.2	0.9

Compounds were applied 30 min before TPA: ear thickness was determined at 8 h after TPA treatment.

The inhibitory effect of berberine derivatives, berberine chloride (**3**), 9-acetyl, 9-demethylberberine chloride (**7**), 9-*N*,*N*-dimethylcarbamoyl, 9-demethylberberine chloride (**11**) and 9-*N*,*N*-diphenylcarbamoyl, 9-demethylberberine chloride (**13**), were found to be stronger than that of palmatine derivatives, palmatine chloride (**27**), 9-acetyl, 9-demethylpalmatine chloride (**28**), 9-*N*,*N*-dimethylcarbamoyl, 9-demethylpalmatine chloride (**36**) and 9-*N*,*N*-diphenylcarbamoyl, 9-demethylpalmatine chloride (**37**). Berberrubine hydrochloride (**2**), 12-bromoberberrubine (**16**) and 13-ethylberberrubine (**18**) were stronger than the mother compound, berberrubine (**1**). On the 8-position of berberine, 8-*n*-propyl berberine bromide (**15**) was stronger than 8-methylberberine bromide (**14**). On the 13-position of berberine, 13-ethoxycarbonylberberine chloride (**22**) and 13-methyl, 9-*N*,*N*-diphenylcarbamoyl, 9-demethylberberine chloride (**25**) were lower than these 8-hydro derivatives (**3**) and (**11**), respectively. When substituted with an *N*,*N*-diphenylcarbamoyl function at 9-position of both 9-demethylberberine and 9-demethylpalmatine, the compounds had about ten times the activity of the respective mother compound (Tables II and III). Of interest may be that *N*-monophenylcarbamoyl derivatives had no influence on the substitution at 9-position of the compounds. *N*-Phenylcarbamic acid derivatives and *N*,*N*-diphenylcarbamoyl chloride had no apparent effect (Table IV). Of the

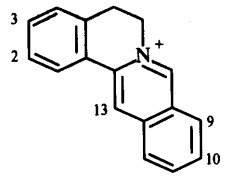
TABLE II. Inhibitory Effect of Berberine Derivatives on TPA-Induced Inflammation in Mice



No.	Substituent					ED_{50}	Inhib.[a]
	C_8	C_9	C_{12}	C_{13}	X	μmol/ear	(%)
1	H	O^-	H	H	—	6.7	34[d]
2	H	OH	H	H	Cl^-	1.3	52[c,e]
3	H	OMe	H	H	Cl^-	6.5	36[d]
4	H	OMe	H	H	SO_4^{2-}	1.2	53[e]
5	H	OEt	H	H	Cl^-	—	45[e]
6	H	OC_3H_7	H	H	Br^-	2.0	51[e]
7	H	OAc	H	H	Cl^-	—	44[b,e]
8	H	$OCOCMe_3$	H	H	Cl^-	3.1	84[b,e]
9	H	$OCOCHCH{=}CH{-}C_6H_5$	H	H	Cl^-	—	26[b]
10	H	OCO–NHMe	H	H	Cl^-	—	9
11	H	$OCO{-}NMe_2$	H	H	Cl^-	2.0	51[e]
12	H	$OCO{-}NEt_2$	H	H	Cl^-	1.0	79[e]
13	H	$OCO{-}N(C_6H_5)_2$	H	H	Cl^-	0.6	88[e]
14	Me	OMe	H	H	Br^-	—	16[c]
15	C_3H_7	OMe	H	H	Br^-	3.2	60[b,e]
16	H	O^-	Br	H	—	1.0	86[b,e]
17	H	OH	H	Me	HSO_4^-	—	76[b,e]
18	H	O^-	H	Et	—	2.5	69[b,e]
19	H	OMe	H	Me	I^-	—	19[c]
20	H	OMe	H	Et	I^-	—	18
21	H	OMe	H	$CH_2CH{=}CH_2$	Br^-	3.8	52[b,e]
22	H	OMe	H	CO–OEt	Cl^-	—	12
23	H	$OCO{-}C_6H_4$-4-OMe	H	Me	Cl^-	6.3	43[e]
24	H	OCO–NHMe	H	Me	Cl^-	—	22
25	H	$OCO{-}NH(C_6H_5)$	H	Me	Cl^-	—	32[d]

Compounds were applied 30 min before TPA: ear thickness was determined at 8 h after TPA treatment. *a*) Inhibition ratio at 1 mg/ear. *b*) Inhibition ratio at 2 mg/ear. *c*) Inhibition ratio at 0.5 mg/ear. *d*) $p<0.05$, *e*) $p<0.01$ by Student's *t* test as compared with the control group.

TABLE III. Inhibitory Effect of Palmatine Derivatives on TPA-Induced Inflammation in Mice



No.	Substituent						ED_{50}	Inhib.[a]
	C_2	C_3	C_9	C_{10}	C_{13}	X	μmol/ear	(%)
26	OMe	OMe	OH	OMe	H	Cl^-	—	22
27	OMe	OMe	OMe	OMe	H	Cl^-	—	29[b,d]
28	OMe	OMe	OAc	OMe	H	Cl^-	—	6
29	OMe	OMe	OCOEt	OMe	H	Cl^-	—	15
30	OMe	OMe	OCOBu	OMe	H	Cl^-	1.9	68[e]
31	OMe	OMe	$OCOC_{15}H_{31}$	OMe	H	Cl^-	1.7	49[e]
32	OMe	OMe	$OCOCH_2CHMe_2$	OMe	H	Cl^-	1.7	62[e]
33	OMe	OMe	OCO–C_6H_5	OMe	H	Cl^-	—	32[c,d]
34	OMe	OMe	OCO–C_6H_4-4-Cl	OMe	H	Cl^-	1.4	71[e]
35	OMe	OMe	OCO–NHMe	OMe	H	Cl^-	—	4
36	OMe	OMe	$OCO–NMe_2$	OMe	H	Cl^-	2.1	53[e]
37	OMe	OMe	$OCO–N(C_6H_5)_2$	OMe	H	Cl^-	1.0	87[e]
38	OMe	OMe	OMe	OMe	Me	Cl^-	—	19
39	OH	OMe	OH	OMe	Me	Cl^-	—	10
40	OEt	OEt	OEt	OEt	H	Br^-	2.0	81[b,e]
41	$OCH_2CH=CH_2$	$OCH_2CH=CH_2$	$OCH_2CH=CH_2$	$OCH_2CH=CH_2$	H	Br^-	1.5	77[e]

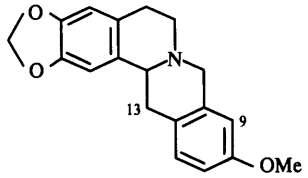
Compounds were applied 30 min before TPA: ear thickness was determined at 8 h after TPA treatment. *a*) Inhibition ratio at 1 mg/ear. *b*) Inhibition ratio at 2 mg/ear. *c*) Inhibition ratio at 0.5 mg/ear. *d*) $p<0.05$, *e*) $p<0.01$ by Student's *t* test as compared with the control group.

TABLE IV. Inhibitory Effect of Carbamoyl Derivatives on TPA-Induced Inflammation

Compound	Inhib.[a] (%)
N-Phenylcarbamic acid methyl ester	0
N-Phenylcarbamic acid ethyl ester	7
Phenylcarbamide	0
N,N-Dimethylcarbamoyl chloride	0
N,N-Diethylcarbamoyl chloride	0
N,N-Diphenylcarbamoyl chloride	0

Compounds were applied 30 min before TPA: ear thickness was determined at 8 h after TPA treatment. *a*) Inhibition ratio at 2 mg/ear.

TABLE V. Inhibitory Effect of Tetrahydroberberine Derivatives on TPA-Induced Inflammation



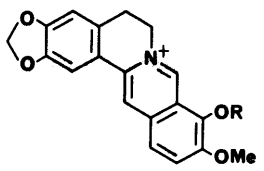
No.	Substituent				ED_{50}	Inhib.[a]
	C_9	C_{13}	N	X	μmol/ear	(%)
42	OMe	H	—	—	—	43[d]
43	OC_3H_7	H	—	HCl	—	39[d]
44	OAc	H	—	HCl	1.6	47[c,d]
45	OH	Me	—	—	—	15[c]
46	OH	—	Me	$MeSO_4^-$	—	27
47	OMe	—	Me	Cl^-	—	6
48	OMe	—	Et	Cl^-	—	22[b]

Compounds were applied 30 min before TPA: ear thickness was determined at 8 h after TPA treatment. *a*) Inhibition ratio at 1 mg/ear. *b*) Inhibition ratio at 2 mg/ear. *c*) Inhibition ratio at 0.5 mg/ear. *d*) $p<0.01$ by Student's *t* test as compared with the control group.

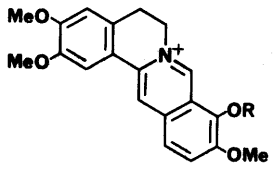
various tetrahydroberberines, that in which an acetyl function was substituted for the methyl group at the 9-position of tetrahydroberberine was proved to inhibit TPA-induced inflammation (Table V).

Discussion

It was demonstrated that berberine, a main alkaloid of Phellodendri Cortex and Coptidis Rhizoma, had an inhibitory effect against TPA-induced inflammation in the ears of mice at a level corresponding to those of quercetin,[13,14] caffeine[5] and cepharanthine[12] (Table I), which are known to have an anti-tumor promotion effect in two-stage carcinogenesis. In general, the inhibitory effect of berberine derivatives was found to be stronger than that of palmatine derivatives (Tables II and III). This finding suggested that the methylene-dioxy function at the 2,3-position of the alkaloids is more important for production of anti-inflammation activity against TPA-induced ear edema than in the dimethoxyl group. Substitution of the methyl group at the 9-position of both berberine and palmatine to other functions generally augmented the inhibitory activity. In particular, when substituted with an *N,N*-diphenylcarbamoyl function, the compounds had about ten times the activity of the respective mother compound (Fig. 1). However, substitution with *N*-methylcarbamoyl or *N*-phenylcarbamoyl groups did not produce the same augmentation as that by substitution with an *N,N*-diphenylcarbamoyl group. Furthermore, *N,N*-diphenylcarbamoyl chloride had no apparent effect (Table IV). Among the *N,N*-di-substituted carbamoyl derivatives, the diphenyl derivative had the highest activity, followed by the diethyl and dimethyl derivatives in that order. Therefore, it is suggested that introduction of lipophilic functions into the *N*-terminus of carbamoyl in these



R	ED_{50}
methyl	6.5 μM
N-methylcarbamoyl	-
N, *N*′-dimethylcarbamoyl	2.0 μM
N, *N*′-diethylcarbamoyl	1.0 μM
N, *N*′-diphenylcarbamoyl	0.6 μM



R	ED_{50}
methyl	-
N-methylcarbamoyl	-
N, *N*′-dimethylcarbamoyl	2.1 μM
N, *N*′-diphenylcarbamoyl	1.0 μM

Fig. 1. Relative Inhibitory Activities of Berberine and Palmatine Derivatives on TPA-Induced Inflammation in Mice

alkaloids augments their anti-tumor promotion activity. Of the various tetrahydroberberines, that in which an acetyl function was substituted for the methyl group at the 9-position of tetrahydroberberine was proved to inhibit TPA-induced inflammation (Table V).

Based on the studies described above, the following structure–activity relationship can be postulated:

1. Berberine derivatives had stronger inhibitory activity than palmatine derivatives.
2. The 9-*N*,*N*-diphenylcarbamoyl function was substituted for the methyl group at the 9-position of berberine and palmatine had about ten times the activity of the respective mother compounds. In addition, *N*,*N*-diphenylcarbamoyl chloride proved to have no effect.

As berberine and some of its derivatives are present in Chinese traditional crude drugs, which are used in combined Kampo prescriptions, it is of interest from the viewpoints of both Chinese traditional medicine and pharmacognostical science that such alkaloids have inhibitory activity against promotion of carcinogenesis.

Acknowledgement This study was supported in part by Grants-in-Aid for Scientific Research, Nihon University.

References

1) H. Fujiki, M. Mori, M. Nakayama, M. Tarada and T. Sugimura, *Biochem. Biophys. Res. Commun.*, **90**, 976 (1979).
2) K. Yasukawa, M. Takido, M. Takeuchi and S. Nakagawa, *Chem. Pharm. Bull.*, **37**, 1071 (1989).
3) K. Yasukawa, M. Takido, T. Matsumoto, M. Takeuchi and S. Nakagawa, *Oncology*, **48**, 72 (1991).
4) K. Yasukawa, M. Takido, M. Takeuchi, Y. Sato, K. Nitta and S. Nakagawa, *Chem. Pharm. Bull.*, **38**, 771 (1990); K. Yasukawa, M. Takido, M. Takeuchi and K. Nitta, "Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular and Medicinal Properties," ed. by V. Cody, E. Middleton, Jr., J. B. Harborne and A. Beretz, Alan R. Liss, Inc., New York, 1988, pp. 247—250.
5) K. Yasukawa, M. Takido, M. Takeuchi and S. Nakagawa, *Yakugaku Zasshi*, **108**, 794 (1988).
6) H. Fujimura, T. Sawada and M. Goto, *Yakugaku Zasshi*, **90**, 782 (1970).
7) S. Suzuki, *Tohoku J. Exptl. Med.*, **36**, 134 (1939).
8) A. H. Amin, T. V. Subbaiah and K. M. Abbasi, *Can. J. Microbiol.*, **15**, 1067 (1969).
9) A. Hoshi, T. Ikekawa, Y. Ikeda, S. Shirakawa, M. Iigo, K. Kuretani and F. Fukuoka, *Gann*, **67**, 321 (1976).
10) T. Ikekawa and Y. Ikeda, *J. Pharmacobio-Dyn.*, **5**, 469 (1982).
11) H. Nishino, K. Kitagawa, H. Fujiki and A. Iwashima, *Oncology*, **43**, 131 (1986).
12) K. Yasukawa, M. Takido, M. Takeuchi, M. Akasu and S. Nakagawa, *Nihon Univ. J. Med.*, **31**, 229 (1989).
13) R. Kato, T. Nakadate, S. Yamamoto and T. Sugimura, *Carcinogenesis*, **4**, 1301 (1983).
14) H. Nishino, A. Iwashima, H. Fujiki and T. Sugimura, *Gann*, **75**, 113 (1984).
15) E. Spath and G. Burger, *Ber.*, **59**, 1489 (1926).
16) Y. D. Sadykov, S. B. Davidyants, K. T. Poroshin and I. N. Grigina, *Dokl. Akad. Nauk. Tadzh.*, **10**, 29 (1967).
17) G. Frerichs and P. Stoepel, *Arch. Pharm.*, **251**, 321 (1913).
18) M. Ota, H. Tani and T. Teshigawara, Japan. Patent 21530 (1964) [*Chem. Abstr.*, **62**, 13194*d* (1964)].
19) M. Ota, H. Tani and T. Teshigawara, Japan. Patent 21529 (1964) [*Chem. Abstr.*, **62**, 13194*d* (1964)].
20) F. Shimada, T. Ikekawa, T. Endo, H. Kuroda, Y. Ikeda, K. Tachibana and Y. Okazaki, Ger. Patent 2342709 (1974) [*Chem. Abstr.*, **80**, 146399*k* (1974)].
21) T. Ikekawa, F. Shimada, Y. Okazaki, K. Tachibana and N. Aikawa, Ger. Patent 2403572 (1974) [*Chem. Abstr.*, **81**, 120848*b* (1974)].
22) C. Tani and K. Ishibashi, *Yakugaku Zasshi*, **74**, 317 (1954).
23) F. Shimada, T. Ikekawa, K. Tachibana, T. Endo, T. Kohno, H. Kuroda, Y. Ikeda, Y. Okazaki and Y. Sawa, Ger. Patent 2352632 (1974) [*Chem. Abstr.*, **81**, 91778*w* (1974)].
24) S. Naruto and H. Kaneko, *Yakugaku Zasshi*, **92**, 1017 (1972).
25) K. Tachibana, T. Ikekawa, F. Shimada and Y. Okazaki, Japan. Patent 135997 (1974) [*Chem. Abstr.*, **83**, 164407*c* (1975)].
26) F. Shimada, T. Ikekawa, S. Daibo, Y. Okazaki, K. Tachibana, T. Endo, T. Kohno, H. Kuroda and Y. Ikeda, Japan. Patent 72298 (1974) [*Chem. Abstr.*, **83**, 114716*g* (1975)].
27) C. Tani, K. Ishibashi and M. Wada, *Yakugaku Zasshi*, **74**, 315 (1954).
28) C. Tani, K. Ishibashi and K. Nakasato, *Yakugaku Zasshi*, **72**, 447 (1954).

Studies on Nepalese Crude Drugs. XIV.[1] New Flavonoids from the Root of *Scutellaria prostrata* JACQ. *ex* BENTH.[2]

Yuichi KIKUCHI, Yukinori MIYAICHI and Tsuyoshi TOMIMORI*

School of Pharmacy, Hokuriku University, 3 Ho, Kanagawa-machi, Kanazawa 920-11, Japan. *Received December 13, 1990*

Six flavonoids, named scutellaprostins A, B, C, D, E and F, were isolated from the root of *Scutellaria prostrata* JACQ. ex BENTH. (Labiatae). Their structures were established to be (2*R,3*R**)-6-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-2-hydroxymethyl-9-phenyl-2,3-dihydro-7*H*-1,4-dioxino[2,3-*h*]chromene-7-one (I), (2*R**,3*R**)-6-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-2-hydroxymethyl-9-(4-hydroxyphenyl)-2,3-dihydro-7*H*-1,4-dioxino[2,3-*h*]-chromene-7-one (II), (2*R**,3*R**)-6-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-2-hydroxymethyl-9-(3,4-dihydroxyphenyl)-2,3-dihydro-7*H*-1,4-dioxino-[2,3-*h*]chromene-7-one (III), (2*R**,3*R**)-5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-8-phenyl-2,3-dihydro-6*H*-1,4-dioxino[2,3-*g*]chromene-6-one (IV), (2*R**,3*R**)-5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-8-(4-hydroxyphenyl)-2,3-dihydro-6*H*-1,4-dioxino[2,3-*g*]chromene-6-one (V) and (2*R**,3*R**)-5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-8-(3,4-dihydroxyphenyl)-2,3-dihydro-6*H*-1,4-dioxino[2,3-*g*]chromene-6-one (VI), respectively, by chemical and spectral data. They were synthesized by oxidative coupling of the flavones and coniferyl alcohol in the presence of silver oxide.**

Keywords *Scutellaria prostrata*; Labiatae; flavonoid; flavone: scutellaprostin; structure elucidation; biomimetic synthesis

In the previous paper,[3] we reported the structural identification of nineteen flavonoids and five phenylethanoid glycosides which were isolated from the root of *Scutellaria prostrata* JACQ. ex BENTH. Further extensive fractionation of the extract of the root led to the isolation of six new flavonoids (I—VI), for which we now give the names, scutellaprostins A, B, C, D, E and F, respectively. The present paper deals with their structural determination.

Compounds I—VI gave dark green colorations with a dil. H_2SO_4 reagent on thin-layer chromatography (TLC), and showed positive color reactions to Mg–HCl. They had absorption bands assignable to hydroxyls, conjugated carbonyl groups and aromatic rings in infrared (IR) spectra. Circular dichroism (CD) spectra revealed them to be racemates.

Scutellaprostin A (I) was obtained as yellow needles, mp 221—222 °C (dec.), $C_{25}H_{20}O_8$. The ultraviolet (UV) spectrum of I was characteristic of the flavone series and showed a bathochromic shift by the addition of $AlCl_3$/HCl, indicating the presence of a chelated hydroxyl at the C-5 position.[4] The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of I showed the presence of one methoxyl (3H, δ 3.79), two hydroxyls (1H, δ 9.12; 1H, δ 5.09), one chelated hydroxyl (δ 12.25) and one C-3 proton (δ 7.10). In the aromatic region of the spectrum, there were nine proton signals, which appeared as a singlet (1H, δ 6.43) due to the A-ring proton, a double doublet (2H, δ 8.13, J=8.2 Hz and 1.7 Hz) and a multiplet (3H, δ 7.53—7.65) due to the non-substituted B-ring protons and an ABC system (H-2″,5″, and 6″ in Table I) suggesting the presence of a 1,3,4-trisubstituted aromatic ring. In the aliphatic region of the spectrum, the remaining four protons appeared as an AMXY system (H-7″,8″,9″a and 9″b in Table I), indicating the presence of a partial structure –CH–CH–CH_2– in I. The methylene proton signals (H-9″a and H-9″b) exhibited downfield shifts by 0.64 and 0.84 ppm when I was acetylated, demonstrating that the methylene bears a hydroxyl. Carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of I showed the presence of 25 carbon signals consisting of 21 sp^2 carbons and four sp^3 carbons bearing oxygen atoms. Among them, 15 sp^2 carbons and one sp^3 carbon are due to a flavone skeleton and a methoxyl group, respectively. I was, therefore, suggested to have a phenylpropane (C_6–C_3) moiety in the molecule. This was also supported by the mass spectrum (MS) of I, exhibiting fragment ion peaks at m/z 180 (F_1) and 137 (F_2). The formation of the former ion can be rationalized in terms of a retro-Diels–Alder cleavage in the dioxane ring. And the latter peak pointed to two oxygen functions in the phenyl ring of a phenylpropane unit to be allocated to a hydroxyl and a methoxyl. The MS of I also exhibited a fragment ion peak originating from the B-ring at m/z 105 (), which, in conjunction with the ^{1}H-NMR data, confirmed that no substituents attached to the B-ring.

From these results, I was considered to be a flavone bearing a C_6–C_3 unit, in which the two methine groups of the phenylpropane moiety should be connected with the two oxygens of the flavone molecule forming a benzodioxane bridge. The coupling constant between the H-7″ and H-8″ signals in the dioxane ring was 7.7 Hz, demonstrating that the two hydrogens are *trans*-oriented.

The characterization of flavone and phenylpropane moieties in I was performed chemically as follows. (1) On treatment with pyridine hydrobromide, I afforded a product found to be identical with norwogonin (5,7,8-trihydroxyflavone).[5] (2) I was treated with alkali to give a product, which was proved to be identical with coniferyl alcohol. This ring opening of the dioxane ring is probably initiated by ionization of the para (4″) phenolic group (Chart 1) since the corresponding 4″-*O*-methyl ether remains unchanged under the same condition.

Based on these findings, the structure of scutellaprostin A could be restricted to two alternative formulations, I or Ia. Distinction between the two formulae was obtained by selective heteronuclear decoupling experiments according to the strategy developed by Rey *et al.*[6] towards solving similar problems with regard to coumarinolignoids. In triacetate of I, when the C-7″ and C-8″ hydrogen signals (δ 5.36 and 4.84, respectively) were irradiated, the carbon

signals for C-7 (δ 146.5) and C-8 (δ 129.5), respectively, showed significant sharpening. This result could rule out the possibility of formula Ia.

The structure of scutellaprostin A could thus be formulated as I.

Next, I was synthesized by adoption of the biomimetic approach developed by Merlini *et al.*[7] for the synthesis of silybin/isosilybin regioisomers. Thus, oxidative coupling of norwogonin and coniferyl alcohol in the presence of Ag_2O afforded I in 45% yield, which was identical in all respects to the natural product. We could find no evidence for the formation of a regioisomer of type Ia or of 7″,8″-*cis*-isomer.

Scutellaprostin B (II) was obtained as yellow needles, mp 255—256 °C (dec.), $C_{25}H_{20}O_9$. The molecular formula suggested the presence of one more hydroxyl group than I. The structural similarity of I and II became evident from MS, ^{1}H- and ^{13}C-NMR spectral comparison. In the MS of II, the fragment ion peaks at *m/z* 180 (F_1) and 137 (F_2) suggested the presence of the same phenylpropane unit as I. The spectrum also showed the ion at *m/z* 121 (HO-C$_6$H$_4$-C≡O$^+$),which appeared at *m/z* 105 in I, indicating that the additional hydroxyl group is present in the B-ring. The ^{1}H-NMR spectrum of II showed the signals of an A_2X_2 type grouping due to the B-ring protons, indicating the presence of a hydroxyl at the C-4′ position. In the ^{13}C-NMR spectrum, the signal patterns of the A-, C-, D- and E-rings and of the B-ring were almost identical with those of I and isoscutellarein (5,7,8,4′-tetrahydroxyflavone),[8] respectively. From these findings, the structure of scutellaprostin B was shown to be II. Chemical evidence for supporting this structure was secured by the formation of coniferyl alcohol and isoscutellarein by the treatment of II with alkali and with pyridine hydrobromide, respectively. In addition, II was synthesized from isoscutellarein and coniferyl alcohol in the same manner as in the case of I.

Scutellaprostin C (III) was obtained as yellow needles, mp 250—251 °C (dec.), $C_{25}H_{20}O_{10}$. The molecular formula suggested the presence of one more hydroxyl group than II. The ^{1}H-NMR spectra of II and III were virtually superimposable except for the signal patterns of their B-ring protons, in which an A_2X_2-system in II was replaced by an ABC-system in III. In the ^{13}C-NMR spectrum of III, the signal patterns of the A-, C-, D- and E-rings and of the B-ring were almost superimposable on those of I and hypolaetin (5,7,8,3′,4′-pentahydroxyflavone),[9] respectively. Coniferyl alcohol and hypolaetin were obtained from III by the same degradation procedure as in the case of I.

Thus, the structure of scutellaprostin C was determined to be as represented by formula III. Compound III was synthesized from hypolaetin and coniferyl alcohol in the same manner as in the case of I.

Scutellaprostin D (IV) was obtained as yellow needles, mp 234—235 °C (dec.), $C_{25}H_{20}O_8$ having the same molecular formula as I. A diagnostic shift in the UV spectrum suggested the presence of a hydroxyl at the C-5 position.[4] The structural resemblance of IV and I became manifest from MS, ^{1}H- and ^{13}C-NMR spectral analysis. The MS of IV showed peaks at *m/z* 180 due to F_1 and at *m/z* 270 due to $C_{15}H_{10}O_5^+$, which indicated that IV has a trihydroxyflavone moiety. The ^{1}H-NMR spectrum of IV showed typical signals of a baicalein (5,6,7-trihydroxy-

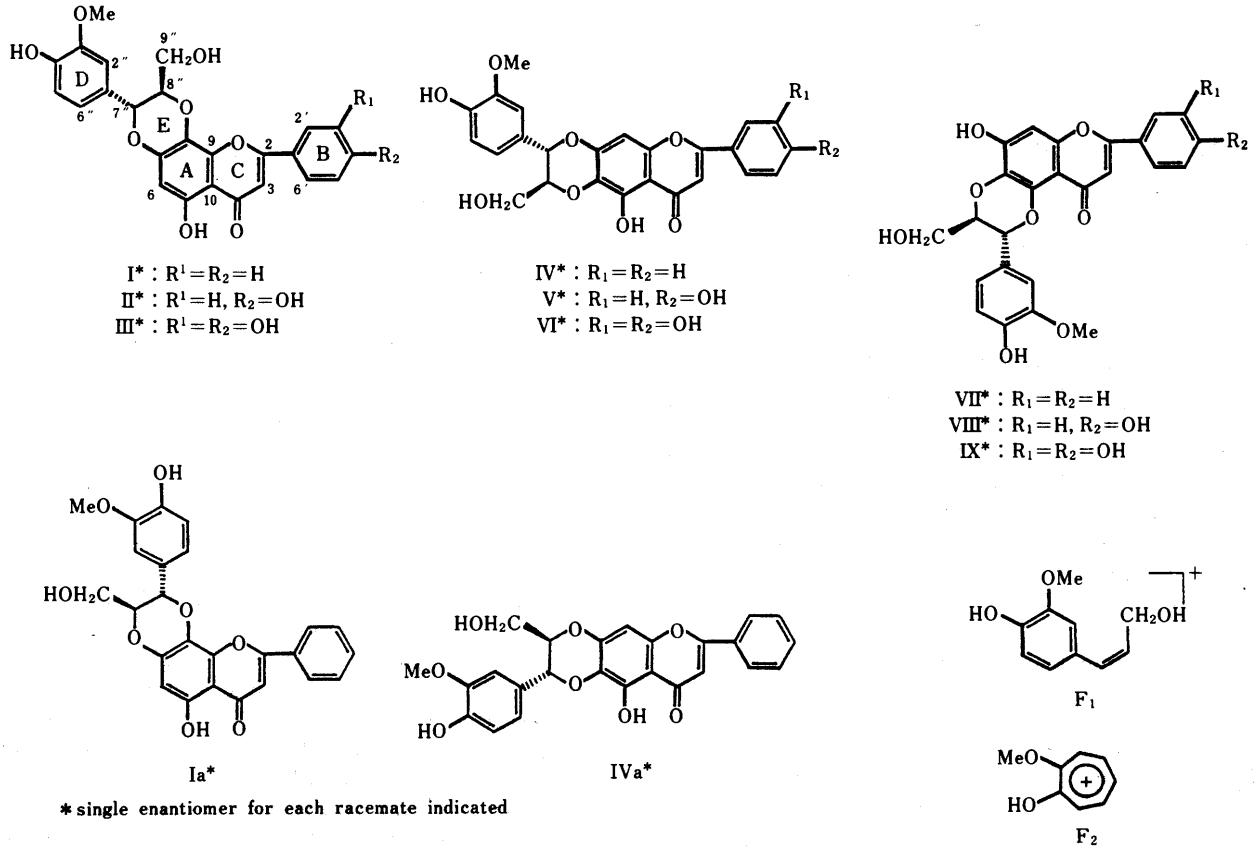


Fig. 1

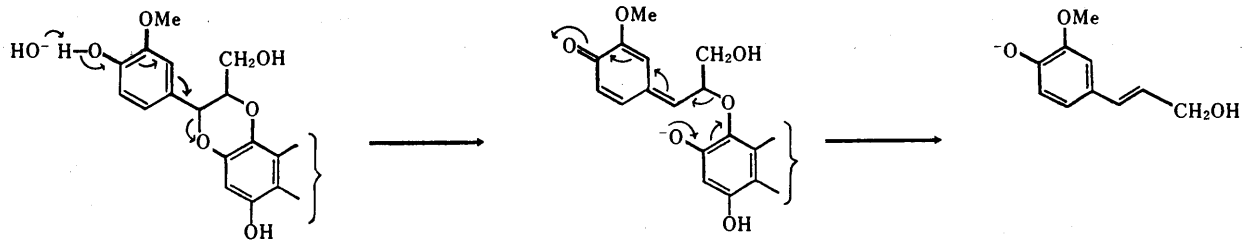


Chart 1

TABLE I. ^{1}H Chemical Shifts of Compounds I—IX, δ (ppm) in DMSO-d_6 (J/Hz in Parentheses)

H	I	II	III	IV	V	VI	VII	VIII	IX
3	7.10 s	6.84 s	6.77 s	7.00 s	6.80 s	6.68 s	6.67 s	6.48 s	6.36 s
6	6.43 s	6.36 s	6.38 s						
8				6.85 s	6.77 s	6.71 s	6.72 s	6.67 s	6.60 s
2′	8.13 br dd	7.96 d	7.45 d	8.09 br d	7.93 d	7.40 d	8.01 m	7.83 d	7.32 d
	(8.2, 1.7)	(8.8)	(2.2)	(6.7)	(8.8)	(2.2)		(8.8)	(2.2)
3′		6.94 d			6.93 d			6.89 d	
		(8.8)			(8.8)			(8.8)	
4′	7.53—7.65 m			7.53—7.62 m			7.54—7.61 m		
5′		6.94 d	6.91 d		6.93 d	6.89 d		6.89 d	6.85 d
		(8.8)	(8.2)		(8.8)	(8.1)		(8.8)	(8.4)
6′	8.13 br dd	7.96 d	7.48 dd	8.09 br d	7.93 d	7.41 dd	8.01 m	7.83 d	7.30 dd
	(8.2, 1.7)	(8.8)	(8.2, 2.2)	(6.7)	(8.8)	(8.1, 2.2)		(8.8)	(8.4, 2.2)
2″	7.06 d	7.05 d	7.05 d	7.05 d	7.04 d	7.04 d	7.09 d	7.06 d	7.07 d
	(1.8)	(1.7)	(1.8)	(1.8)	(1.8)	(1.7)	(1.8)	(1.8)	(1.8)
5″	6.83 d	6.83 d	6.82 d	6.84 d	6.82 d	6.83 d	6.83 d	6.81 d	6.81 d
	(8.4)	(8.2)	(8.0)	(8.2)	(8.1)	(8.1)	(8.2)	(8.1)	(8.1)
6″	6.90 dd	6.89 dd	6.89 dd	6.91 dd	6.89 dd	6.89 dd	6.91 dd	6.89 dd	6.89 dd
	(8.4, 1.8)	(8.2, 1.7)	(8.0, 1.8)	(8.1, 1.8)	(8.1, 1.8)	(8.1, 1.7)	(8.2, 1.8)	(8.1, 1.8)	(8.1, 1.8)
7″	5.16 d	5.12 d	5.15 d	5.13 d	5.11 d	5.08 d	5.02 d	4.97 d	4.97 d
	(7.7)	(7.9)	(7.7)	(8.1)	(7.7)	(8.1)	(7.4)	(7.0)	(7.3)
8″	4.31 ddd	4.26 ddd	4.30 ddd	4.23 ddd	4.21 ddd	4.20 br d	4.19 ddd	4.16 ddd	4.15 m
	(7.7, 4.4, 2.6)	(7.9, 4.9, 2.8)	(7.7, 4.8, 3.0)	(8.1, 4.1, 2.1)	(7.7, 4.1, 2.6)	(8.1)	(7.4, 5.0, 2.9)	(7.0, 5.0, 3.0)	
9″	3.39 ddd	3.45 ddd	3.44 ddd	3.39 ddd	3.37 br dd	3.36 br dd	3.48 dd	3.48 dd	3.44 m
	(12.5, 5.5, 4.4)	(12.1, 5.5, 2.8)	(13.2, 5.5, 4.8)	(12.4, 5.3, 2.1)	(12.5, 4.1)	(12.3, 3.9)	(12.1, 5.0)	(12.1, 5.0)	
	3.65 ddd	3.66 ddd	3.70 ddd	3.65 ddd	3.67 ddd	3.63 br d	3.57 dd	3.53 dd	3.53 br d
	(12.5, 5.5, 2.6)	(12.1, 5.5, 4.9)	(13.2, 5.5, 3.0)	(12.4, 5.3, 4.1)	(12.5, 2.6)	(12.3)	(12.1, 2.9)	(12.1, 3.0)	(12.3)
OMe	3.79 s	3.80 s	3.78 s	3.79 s	3.79 s	3.79 s	3.79 s	3.76 s	3.77 s
5-OH	12.25 s	12.38 s	12.43 s	12.94 s	13.09 s	13.12 s			
Ar-OH	9.20 s	9.16 s	9.21 s	9.18 s	9.16 s	9.20 br s	9.12 s	9.18 br s	9.20 br s
		10.36 s	9.44 s		10.33 s	9.45 br s	10.50 s	10.30 br s	9.45 br s
			10.02 s			9.95 br s		10.51 br s	10.00 br s
									10.50 br s
9″-OH	5.09 t	5.03 t	5.05 t	5.02 t	5.00 br s	5.03 br s	4.91 br s	4.95 br s	5.03 br s
	(5.5)	(5.5)	(5.5)	(5.3)					

flavone) substitution pattern (H-3,8,2′,3′,4′,5′ and 6′ in Table I). The spectrum also showed signals due to the same phenylpropane moiety as I (H-2″,5″,6″,7″,8″ and 9″ in Table I). Comparison of the ^{13}C-NMR spectrum of IV with that of I revealed that all of the carbon signals due to the phenylpropane moiety and the B- and C-rings of IV appeared at almost the same positions as in the spectrum of I. These findings suggest that IV is also a dioxane-type flavone composed of coniferyl alcohol and baicalein moieties.

IV, on treatment with alkali and with pyridine hydrobromide, produced conifery alcohol and baicalein, respectively. Based on these findings, the structure of scutellaprostin D was considered to be restricted to two alternative formulations IV or IVa. The discrimination between the two structures was performed by a selective heteronuclear decoupling experiment in the triacetate of IV. Irradiations of the C-7″ and C-8″ hydrogen signals result in considerable sharpening of the C-7 and C-6 signals, respectively.

Scutellaprostin D, hence, can be represented as IV. The coupling constant between H-7″ and H-8″ in IV was observed to be 8.1 Hz, demonstrating that the two hydrogens are *trans*-oriented.

IV was synthesized in a similar manner to I as follows. Oxidation of an equimolar mixture of baicalein and coniferyl alcohol in benzene/acetone with Ag_2O afforded a mixture of two compounds (IV and VII). Comparison of the major compound with natural IV established their identity. The second compound (VII) was obtained as colorless needles, mp 206—207 °C (dec.), $C_{25}H_{20}O_8$, having the same molecular formula as IV. The structural similarity of this compound and IV became evident from MS, ^{1}H- and ^{13}C-NMR spectral comparisons. Inspection of the UV spectrum using a diagnostic reagent[4] indicated the absence of a free hydroxyl at the C-5 position, which was also

TABLE II. ^{13}C Chemical Shifts of Compounds I—IX, δ (ppm) in DMSO-d_6

C	I	II	III	IV	V	VI	VII	VIII	IX
2	163.1	163.6	163.9	163.5	164.0	164.4	159.4	160.2	160.1
3	105.3	102.9	103.1	104.4	102.1	102.3	107.2	105.3	105.3
4	182.2	181.8	181.9	182.5	182.3	182.3	175.5	175.9	175.6
5	153.0	152.9	153.1	147.8	147.8	148.0	142.5	142.6	142.5
6	99.0	98.6	98.8	128.0	127.9	128.0	129.1	129.2	129.3
7	149.6	149.3	149.4	150.1	149.8	149.9	151.3	151.3	151.9
8	124.6	124.4	124.5	94.7	94.4	94.5	95.9	96.0	95.8
9	144.5	144.3	144.5	149.7	149.5	149.7	151.7	151.8	151.9
10	104.9	104.6	104.8	104.9	104.6	104.8	107.2	107.1	106.7
1′	130.5	121.0	121.4	130.6	121.1	121.5	131.1	121.7	122.0
2′	126.4	128.4	113.6	126.3	128.4	113.4	125.7	127.8	112.9
3′	129.2	115.9	145.7	129.0	115.8	145.8	128.9	115.9	145.7
4′	132.2	161.2	150.0	132.0	161.1	149.8	131.1	160.4	148.9
5′	129.2	115.9	116.0	129.0	115.8	116.0	128.9	115.9	115.9
6′	126.4	128.4	119.2	126.3	128.4	119.1	125.7	127.8	118.0
1″	126.6	126.7	126.8	126.6	126.6	126.8	127.2	127.3	127.3
2″	111.7	111.8	111.8	111.8	111.8	111.9	111.8	111.9	111.8
3″	147.6	147.5	147.6	147.5	147.5	147.7	147.5	147.7	147.6
4″	147.2	147.1	147.2	147.2	147.1	147.3	146.8	147.0	146.9
5″	115.3	115.3	115.4	115.3	115.3	115.4	115.2	115.4	115.3
6″	120.5	120.5	120.6	120.5	120.5	120.7	120.3	120.4	120.4
7″	77.1	77.0	77.0	76.9	76.9	77.1	75.6	75.8	75.7
8″	77.6	77.6	77.6	77.4	77.3	77.5	76.8	77.0	76.9
9″	60.0	60.0	60.1	59.8	59.8	60.0	60.1	60.3	60.2
OMe	55.7	55.7	55.7	55.6	55.6	55.7	55.6	55.8	55.7

confirmed by the ^1H-NMR spectrum showing the absence of a chelated hydroxyl proton. These findings indicated that the C_6–C_3 unit is attached to the baicalein unit at C-5 and C-6 through a dioxane bridge, and the structure of this compound is considered to be either VII or its regioisomer. The latter structure was ruled out by a selective heteronuclear decoupling experiment in a manner similar to I. The *trans* orientation of the phenyl and hydroxymethyl groups in the dioxane ring is apparent from the coupling constant of a benzylic methine proton (J=7.4 Hz). Thus, the structure of this compound was determined to be as represented by the formula VII.

Scutellaprostin E (V) was obtained as yellow needles, mp 240—241 °C (dec.), $C_{25}H_{20}O_9$. The ^1H-NMR spectrum of V was similar to that of IV except for the signals due to the B-ring protons, which appeared as an A_2X_2-system, indicating the presence of a hydroxyl at the C-4′ position. The ^{13}C-chemical shifts of the A-, C-, D- and E-rings of V are almost the same as those of IV, and the ^{13}C-chemical shift of the B-ring of V closely resembles that of scutellarein (5,6,7,4′-tetrahydroxyflavone).[8] Coniferyl alcohol and scutellarein were detected from V by the same degradation procedure as in the case of I. Scutellaprostin E was thus framed as V. Oxidation of scutellarein and coniferyl alcohol gave a mixture of two products, one of which (main product) was proven to be identical with scutellaprostin E by direct comparison. The second product was obtained as colorless needles, mp 235—236 °C, $C_{25}H_{20}O_9$, whose structure was determined to be VIII by spectroscopic means in the same way described for VII.

Scutellaprostin F (VI) was obtained as yellow needles, mp 204—205 °C (dec.), $C_{25}H_{20}O_{10}$. The molecular formula indicated the presence of one more hydroxyl than V. The ^{13}C-NMR spectrum of VI was closely related to that of V; in particular, the chemical shifts of the signals from the A-, C-, D- and E-rings of VI were almost identical with those of V. And the ^{13}C-chemical shift of the B-ring of VI was almost identical with that of 6-hydroxyluteolin (5,6,7,3′,4′-pentahydroxyflavone).[11] Hence, scutellaprostin F can be depicted as VI. The ^1H-NMR spectrum also supported this structure. Oxidation of 6-hydroxyluteolin and coniferyl alcohol gave VI as a main product and IX, colorless needles, mp 213—214 °C, $C_{25}H_{20}O_{10}$, as a minor product. Characterization of IX was performed by spectroscopic means in the same way described for VII.

Experimental

The instruments used to obtain physical data were the same as described in our previous paper.[3]

Isolation Fractions 16, 20 and 22, described in the previous paper,[3] were further examined. Fraction 16 was subjected to chromatography on silica gel [solvent: benzene–dioxane–AcOH (90 : 15 : 3)] to give I (120 mg) and IV (98 mg). Fraction 20 was chromatographed on silica gel [solvent: benzene–dioxane–AcOH (90 : 15 : 3)] to give II (85 mg) and V (68 mg). Fraction 22 was chromatographed on silica gel [solvent: benzene–dioxane–AcOH (90 : 20 : 4)] to give III (70 mg) and VI (59 mg).

Scutellaprostin A (I) Yellow needles (from MeOH), mp 221—222 °C (dec.). $[\alpha]_D^{20}$ ±0° (c=0.05, MeOH). *Anal.* Calcd for $C_{25}H_{20}O_8$: C, 66.96; H, 4.50. Found: C, 66.91; H, 4.53. Mg–HCl (+). UV λ_{max}^{MeOH} nm (log ε): 234 (4.24), 286 (4.45), 370 (3.40); $\lambda_{max}^{MeOH-NaOMe}$ nm (log ε): 253 (4.27), 289 (4.44), 394 (3.27); $\lambda_{max}^{MeOH-AlCl_3}$ nm (log ε): 221 (4.35), 236 (4.31), 290 (4.34), 310 (4.38), 346 sh (3.74), 428 (3.42); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm (log ε): 221 (4.36), 238 (4.29), 290 (4.33), 309 (4.38), 346 sh (3.63), 440 (3.44); $\lambda_{max}^{MeOH-NaOAc}$ nm (log ε): 285 (4.44), 360 (3.40). IR ν_{max}^{KBr} cm^{-1}: 3460 (OH), 1664 (conjugated CO), 1616 (arom. C=C). ^1H-NMR: Table I. ^{13}C-NMR: Table II. EI-MS m/z (%): 448 (M^+, 100), 270 ($C_{15}H_{10}O_5$, 89), 180 ($C_{10}H_{12}O_3$, 72), 168 ($C_7H_4O_5$, 40), 137 ($C_8H_9O_2$, 97), 124 ($C_7H_8O_2$, 54), 105 (C_7H_5O, 27).

Acetylation of I: A mixture of I, Ac_2O and pyridine was kept at 40 °C for 24 h. Usual work up followed by crystallization from $CHCl_3$–MeOH yielded a triacetate as colorless needles, mp 195—197 °C (dec.). *Anal.* Calcd for $C_{31}H_{26}O_{11}$: C, 64.80; H, 4.56. Found: C, 64.73; H, 4.61. UV λ_{max}^{MeOH} nm (log ε): 223 sh (4.52), 272 (4.54), 284 sh (4.36), 315 sh (4.05). IR ν_{max}^{KBr} cm^{-1}: 1773, 1753 (acetyl), 1650 (conjugated CO), 1617 (arom. C=C). ^1H-NMR (DMSO-d_6) δ: 6.89 (1H, s, H-3), 6.90 (1H, s, H-6), 8.06 (2H, br dd, J=7.2, 1.7 Hz, H-2′, 6′), 7.60 (3H, m, H-3′,4′,5′), 7.35 (1H, d, J=1.7 Hz, H-2″), 7.21 (1H, d, J=8.1 Hz, H-5″), 7.14 (1H, dd, J=8.1,

1.7 Hz, H-6″), 5.36 (1H, d, J=8.0 Hz, H-7″), 4.84 (1H, ddd, J=8.0, 5.4, 3.5 Hz, H-8″), 4.23 (1H, dd, J=12.5, 3.5 Hz, H-9″a), 4.29 (1H, dd, J=12.5, 5.4 Hz, H-9″b), 3.83 (3H, s, $-OCH_3$), 2.06, 2.29, 2.31 (each 3H, s, $-OCOCH_3$). ^{13}C-NMR (DMSO-d_6) δ: 160.6 (C-2), 107.4 (C-3), 175.3 (C-4), 141.5 (C-5), 108.4 (C-6), 146.5 (C-7), 129.5 (C-8), 145.9 (C-9), 111.1 (C-10), 130.5 (C-1′), 125.9 (C-2′, 6′), 129.0 (C-3′, 5′), 131.7 (C-4′), 133.7 (C-1″), 112.2 (C-2″), 151.0 (C-3″), 140.0 (C-4″), 123.0 (C-5″), 120.1 (C-6″), 76.4 (C-7″), 74.5 (C-8″), 61.7 (C-9″), 55.9 ($-OCH_3$), 20.2 ($-OCOC\underline{H}_3\times2$), 20.7 ($-OCOC\underline{H}_3$), 168.2, 169.0, 169.8 ($-O\underline{C}OCH_3$). EI-MS m/z (%): 574 (M^+, 2), 532 (100), 490 (6), 430 (3), 281 (23), 270 (10), 269 (26), 222 (55), 179 (23).

Partial Methylation of I: I was treated with CH_2N_2 as usual to afford a monomethyl ether as yellow needles (from MeOH), mp 234—244 °C (dec.). *Anal.* Calcd for $C_{26}H_{22}O_8$: C, 67.52; H, 4.80. Found: C, 67.49; H, 4.89. Mg–HCl (+). UV λ_{max}^{MeOH} nm (log ε): 230 (4.38), 284 (4.60), 357 (3.40); $\lambda_{max}^{MeOH-NaOMe}$ nm (log ε): 232 (4.46), 284 (4.54), 384 (3.18); $\lambda_{max}^{MeOH-AlCl_3}$ nm (log ε): 233 (4.44), 288 (4.46), 307 (4.51), 345 sh (3.78), 430 (3.47); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm (log ε): 233 (4.44), 288 (4.46), 307 (4.51), 345 sh (3.60), 426 (3.45); $\lambda_{max}^{MeOH-NaOAc}$ nm (log ε): 234 (4.61), 325 (3.55), 354 (3.42). IR ν_{max}^{KBr} cm^{-1}: 3460 (OH), 1664 (conjugated CO), 1616, 1590 (arom. C=C). ^{1}H-NMR (DMSO-d_6) δ: 7.07 (1H, s, H-3), 6.45 (1H, s, H-6), 8.11 (2H, br dd, J=8.4, 1.8 Hz, H-2′, 6′), 7.58—7.64 (3H, m, H-3′,4′,5′), 7.08 (1H, d, J=1.6 Hz, H-2″), 7.00 (1H, d, J=8.4 Hz, H-5″), 7.02 (1H, dd, J=8.4, 1.6 Hz, H-6″), 5.20 (1H, d, J=7.7 Hz, H-7″), 4.32 (1H, ddd, J=7.7, 4.7, 2.6 Hz, H-8″), 3.48 (1H, ddd, J=12.0, 5.5, 4.7 Hz, H-9″a), 3.68 (1H, ddd, J=12.0, 5.5, 2.6 Hz, H-9″b), 3.77 (6H, s, $-OCH_3\times2$), 5.13 (1H, t, J=5.5 Hz, –OH), 12.25 (1H, s, –OH). ^{13}C-NMR (DMSO-d_6) δ: 163.2 (C-2), 105.4 (C-3), 182.3 (C-4), 153.1 (C-5), 99.1 (C-6), 149.7 (C-7), 124.7 (C-8), 144.6 (C-9), 105.1 (C-10), 130.6 (C-1′), 126.5 (C-2′, 6′), 129.3 (C-3′, 5′), 132.3 (C-4′), 128.3 (C-1″), 111.3 (C-2″), 148.8 (C-3″), 149.4 (C-4″), 111.7 (C-5″), 120.4 (C-6″), 77.0 (C-7″), 77.7 (C-8″), 60.1 (C-9″), 55.6 ($-OCH_3$), 55.7 ($-OCH_3$). EI-MS m/z (%): 462 (M^+, 100), 194 ($C_{11}H_{14}O_3$, 33), 151 ($C_9H_{11}O_2$, 22).

Scutellaprostin B (II) Yellow needles (from MeOH), mp 255—256 °C (dec.). $[\alpha]_D^{20}\pm0°$ (c=0.05, MeOH). *Anal.* Calcd for $C_{25}H_{20}O_9$: C, 64.65; H, 4.34. Found: C, 64.60; H, 4.41. Mg–HCl (+). UV λ_{max}^{MeOH} nm (log ε): 229 (4.45), 285 (4.41), 303 sh (4.32), 310 sh (4.31), 332 sh (4.27); $\lambda_{max}^{MeOH-NaOMe}$ nm (log ε): 233 sh (4.49), 258 (4.49), 281 (4.51), 303 sh (4.31), 400 (4.54); $\lambda_{max}^{MeOH-AlCl_3}$ nm (log ε): 237 (4.46), 290 (4.36), 323 (4.34), 357 (4.35), 424 (3.84); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm (log ε): 236 (4.46), 290 (4.36), 322 (4.38), 353 (4.34), 422 (3.82); $\lambda_{max}^{MeOH-NaOAc}$ nm (log ε): 230 sh (4.49), 283 (4.39), 310 sh (4.23), 333 (4.17), 396 (4.11). IR ν_{max}^{KBr} cm^{-1}: 3457 (OH), 1667 (conjugated CO), 1612 (arom. C=C). ^{1}H-NMR: Table I. ^{13}C-NMR: Table II. EI-MS m/z (%): 464 (M^+, 26), 286 ($C_{15}H_{10}O_6$, 100), 180 ($C_{10}H_{12}O_3$, 55), 168 ($C_7H_4O_5$, 81), 137 ($C_8H_9O_2$, 90), 124 ($C_7H_8O_2$, 48), 121 ($C_7H_5O_2$, 20).

Acetylation of II: Ac_2O–pyridine treatment of II yielded a tetraacetate, which was recrystallized from $CHCl_3$–MeOH to obtain colorless needles, mp 197—198 °C (dec.). *Anal.* Calcd for $C_{33}H_{28}O_{13}$: C, 62.66; H, 4.46. Found: C, 62.60; H, 4.52. UV λ_{max}^{MeOH} nm (log ε): 224 sh (4.41), 273 (4.39), 284 sh (4.26), 321 sh (4.02). IR ν_{max}^{KBr} cm^{-1}: 1766, 1750 (acetyl), 1646 (conjugated CO), 1614, 1590 (arom C=C). ^{1}H-NMR (DMSO-d_6) δ: 6.89 (1H, s, H-3), 6.88 (1H, s, H-6), 8.08 (2H, d, J=8.8 Hz, H-2′, 6′), 7.35 (2H, d, J=8.8 Hz, H-3′, 5′), 7.35 (1H, br s, H-2″), 7.20 (1H, d, J=8.1 Hz, H-5″), 7.13 (1H, br d, J=8.1 Hz, H-6″), 5.35 (1H, d, J=7.7 Hz, H-7″), 4.82 (1H, ddd, J=7.7, 5.2, 3.3 Hz, H-8″), 4.22 (1H, dd, J=12.5, 3.3 Hz, H-9″a), 4.27 (1H, dd, J=12.5, 5.2 Hz, H-9″b), 3.81 (3H, s, $-OCH_3$), 2.05, 2.28, 2.29, 2.31 (each 3H, s, $-OCOCH_3$). ^{13}C-NMR (DMSO-d_6) δ: 160.0 (C-2), 107.5 (C-3), 175.4 (C-4), 141.6 (C-5), 108.6 (C-6), 146.6 (C-7), 129.6 (C-8), 146.0 (C-9), 111.1 (C-10), 128.2 (C-1′), 127.5 (C-2′, 6′), 122.7 (C-3′, 5′), 153.1 (C-4′), 133.8 (C-1″), 112.3 (C-2″), 151.2 (C-3″) 140.1 (C-4″), 123.2 (C-5″), 120.2 (C-6″), 76.5 (C-7″), 74.6 (C-8″), 61.8 (C-9″), 56.0 ($-OCH_3$), 20.4, ($-OCOC\underline{H}_3\times2$), 20.9 ($-OCOC\underline{H}_3\times2$), 168.4, 168.9, 169.2, 170.0 ($-O\underline{C}OCH_3$). EI-MS m/z (%): 590 (100), 548 (27), 488 (23), 328 (70), 286 (53), 222 (84), 179 (44).

Scutellaprostin C (III) Yellow needles (from MeOH), mp 150—251 °C (dec.). $[\alpha]_D^{20}\pm0°$ (c=0.05, MeOH). *Anal.* Calcd for $C_{25}H_{20}O_{10}$: C, 62.50; H, 4.20. Found: C, 62.51; H, 4.26. Mg–HCl (+). UV λ_{max}^{MeOH} nm (log ε): 230 sh (4.82), 283 (4.74), 304 sh (4.48), 348 (4.74); $\lambda_{max}^{MeOH-NaOMe}$ nm (log ε): 234 sh (4.75), 270 (4.81), 282 sh (4.77), 304 sh (4.47), 416 (4.75); $\lambda_{max}^{MeOH-AlCl_3}$ nm (log ε): 235 sh (4.87), 290 (4.77), 318 sh (4.40), 444 (4.75); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm (log ε): 235 sh (4.82), 290 (4.70), 317 sh (4.50), 366 (4.63); $\lambda_{max}^{MeOH-NaOAc}$ nm (log ε): 282 (4.73), 410 (4.52); $\lambda_{max}^{MeOH-NaOAc-H_3BO_3}$ nm (log ε): 270 sh (4.73), 283 sh (4.73), 307 (4.32), 384 (4.65). IR ν_{max}^{KBr} cm^{-1}: 3424 (OH), 1664 (conjugated CO), 1612 (arom. C=C). ^{1}H-NMR: Table I. ^{13}C-NMR: Table II. EI-MS m/z (%): 302 ($C_{15}H_{10}O_7$, 20), 180 ($C_{10}H_{12}O_3$, 45), 168 ($C_7H_4O_5$, 21), 137 ($C_8H_9O_2$, 100), 124 ($C_7H_8O_2$, 74). FAB-MS m/z (%): 481 (M^++1, 8), 154 (100).

Acetylation of III: Ac_2O–pyridine treatment of III yielded a pentaacetate as colorless needles (from $CHCl_3$–MeOH), mp 224—225 °C (dec.). *Anal.* Calcd for $C_{35}H_{30}O_{15}$: C, 60.87; H, 4.38. Found: C, 60.80; H, 4.41. UV λ_{max}^{MeOH} nm (log ε): 212 (4.46), 272 (4.39), 305 sh (4.12), 335 (3.95). IR ν_{max}^{KBr} cm^{-1}: 1758, 1732 (acetyl), 1646 (conjugated CO), 1616 (arom. C=C). ^{1}H-NMR (DMSO-d_6) δ: 6.91 (1H, s, H-3), 6.94 (1H, s, H-6), 7.96 (1H, d, J=1.5 Hz, H-2′), 7.53 (1H, d, J=8.7 Hz, H-5′), 8.02 (1H, dd, J=8.7, 1.5 Hz, H-6′), 7.34 (1H, br s, H-2″), 7.20 (1H, d, J=8.1 Hz, H-5″), 7.13 (1H, br d, J=8.1 Hz, H-6″), 5.35 (1H, d, J=7.7 Hz, H-7″), 4.83 (1H, ddd, J=7.7, 5.5, 3.0 Hz, H-8″), 4.21 (1H, dd, J=12.5, 5.5 Hz, H-9″a), 4.27 (1H, dd, J=12.5, 3.0 Hz, H-9″b), 3.81 (3H, s, $-OCH_3$), 2.05, 2.09, 2.28, 2.30, 2.33 (each 3H, s, $-OCOCH_3$). ^{13}C-NMR (DMSO-d_6) δ: 159.1 (C-2), 108.0 (C-3), 175.3 (C-4), 141.5 (C-5), 108.6 (C-6), 146.7 (C-7), 129.6 (C-8), 145.9 (C-9), 111.1 (C-10), 129.3 (C-1′), 121.5 (C-2′), 142.6 (C-3′), 144.7 (C-4′), 124.6 (C-5′), 124.5 (C-6′), 133.8 (C-1″), 112.3 (C-2″), 151.2 (C-3″), 140.0 (C-4″), 123.2 (C-5″), 120.2 (C-6″), 76.5 (C-7″), 74.8 (C-8″), 61.5 (C-9″), 56.0 ($-OCH_3$), 20.3 ($-OCOC\underline{H}_3\times2$), 20.4 ($-OCOC\underline{H}_3\times2$), 20.8 ($-OCOC\underline{H}_3$), 168.0, 168.1, 168.4, 169.1, 170.0 ($-O\underline{C}OCH_3$). EI-MS m/z (%): 648 (80), 606 (31), 588 (17), 546 (22), 504 (13), 386 (18), 344 (31), 222 (100), 179 (24).

Scutellaprostin D (IV) Yellow needles (from MeOH), mp 234—235 °C (dec.). $[\alpha]_D^{20}\pm0°$ (c=0.05, MeOH). *Anal.* Calcd for $C_{25}H_{20}O_8$: C, 66.96; H, 4.50. Found: C, 66.91; H, 4.59. Mg–HCl (+). UV λ_{max}^{MeOH} nm (log ε): 219 (4.59), 239 sh (4.31), 280 (4.49), 320 (4.27); $\lambda_{max}^{MeOH-NaOMe}$ nm (log ε): 233 sh (4.50), 256 (4.43), 295 (4.49), 400 (3.46); $\lambda_{max}^{MeOH-AlCl_3}$ nm (log ε): 223 (4.55), 287 (4.49), 297 sh (4.44), 348 (4.35), 402 sh (3.58); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm (log ε): 225 (4.56), 287 (4.47), 297 sh (4.43), 345 (4.33), 402 sh (3.58); $\lambda_{max}^{MeOH-NaOAc}$ nm (log ε): 280 (4.49), 321 (4.26). IR ν_{max}^{KBr} cm^{-1}: 3280 (OH), 1664 (conjugated CO), 1622, (arom. C=C). ^{1}H-NMR: Table I. ^{13}C-NMR: Table II. EI-MS m/z: (%): 448 (M^+, 59), 270 ($C_{15}H_{10}O_5$, 100), 180 ($C_{10}H_{12}O_3$, 23), 168 ($C_7H_4O_5$, 11), 137 ($C_8H_9O_2$, 40), 124 ($C_7H_8O_2$, 21).

Acetylation of IV: Ac_2O–pyridine treatment of IV yielded a triacetate as colorless needles (from $CHCl_3$–MeOH), mp 209—210 °C (dec.). *Anal.* Calcd for $C_{31}H_{26}O_{11}$: C, 64.80; H, 4.56. Found: C, 64.82; H, 4.59. UV λ_{max}^{MeOH} nm (log ε): 217 (4.68), 271 (4.36), 284 sh (4.17), 315 (4.36). IR ν_{max}^{KBr} cm^{-1}: 1770, 1752 (acetyl), 1640 (conjugated CO), 1620 (arom. C=C). ^{1}H-NMR (DMSO-d_6) δ: 6.80 (1H, s, H-3), 7.37 (1H, s, H-8), 8.05 (2H, br dd, J=7.6, 1.7 Hz, H-2′, 6′), 7.58 (3H, m, H-3′, 4′, 5′), 7.34 (1H, d, J=1.8 Hz, H-2″), 7.18 (1H, d, J=8.1 Hz, H-5″), 7.12 (1H, dd, J=8.1 1.8 H, H-6″), 5.36 (1H, d, J=7.6 Hz, H-7″), 4.76 (1H, ddd, J=7.6, 5.2, 4.0 Hz, H-8″), 4.11 (1H, dd, J=12.4, 5.2 Hz, H-9″a), 4.19 (1H, dd, J=12.4, 4.0 Hz, H-9″b), 3.81 (3H, s, $-OCH_3$), 2.02, 2.28, 2.36 (each 3H, s, $-OCOCH_3$). ^{13}C-NMR (DMSO-d_6) δ: 161.1 (C-2), 106.9 (C-3), 175.4 (C-4), 135.6 (C-5), 133.5 (C-6), 148.4 (C-7), 102.5 (C-8), 150.6 (C-9), 111.3 (C-10), 130.7 (C-1′), 126.0 (C-2′, 6′), 128.9 (C-3′, 5′), 131.6 (C-4′), 133.7 (C-1″), 112.2 (C-2″), 151.0 (C-3″), 139.9 (C-4″), 123.0 (C-5″), 120.1 (C-6″), 76.3 (C-7″), 74.3 (C-8″), 61.6 (C-9″), 55.8 ($-OCH_3$), 20.2 ($-OCOC\underline{H}_3\times2$), 20.4 ($-OCOC\underline{H}_3$), 168.1, 168.2, 169.7 ($-O\underline{C}OCH_3$). EI-MS m/z (%): 574 (M^+, 4), 532 (100), 490 (9), 430 (6), 281 (13), 270 (49), 222 (42), 179 (17).

Scutellaprostin E (V) Yellow needles (from MeOH), mp 240—241 °C (dec.). $[\alpha]_D^{20}\pm0°$ (c=0.05, MeOH). *Anal.* Calcd for $C_{25}H_{20}O_9$: C, 64.65; H, 4.34. Found: C, 64.69; H, 4.30. Mg–HCl (+). UV λ_{max}^{MeOH} nm (log ε): 220 (4.52), 236 sh (4.38), 340 (4.40); $\lambda_{max}^{MeOH-NaOMe}$ nm (log ε): 218 (4.61), 255 (4.37), 293 (4.30), 374 (4.39); $\lambda_{max}^{MeOH-AlCl_3}$ nm (log ε): 224 (4.51), 239 sh (4.41), 290 sh (4.24), 309 sh (4.28), 373 (4.45); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm (log ε): 224 (4.51), 238 sh (4.42), 292 sh (4.28), 307 sh (4.31), 364 (4.44); $\lambda_{max}^{MeOH-NaOAc}$ nm (log ε): 223 (4.59), 284 (4.27), 344 (4.29), 408 sh (4.06). IR ν_{max}^{KBr} cm^{-1}: 3440 (OH), 1664 (conjugated CO), 1610 (arom. C=C). ^{1}H-NMR: Table I. ^{13}C-NMR: Table II. EI-MS m/z (%): 464 (M^+, 26), 286 ($C_{15}H_{10}O_6$, 100), 180 ($C_{10}H_{12}O_3$, 55), 168 ($C_7H_4O_5$, 81), 137 ($C_8H_9O_2$, 90), 124 ($C_7H_8O_2$, 48), 121 (C_7H_5O, 20).

Acetylation of V: Ac_2O–pyridine treatment of V yielded a tetraacetate as colorless needles (from $CHCl_3$–MeOH), mp 199—200 °C (dec.). *Anal.* Calcd for $C_{33}H_{28}O_{13}$: C, 62.66; H, 4.46. Found: C, 62.59; H, 4.52. UV λ_{max}^{MeOH} nm (log ε): 219 (4.67), 275 sh (4.27), 292 sh (4.18), 326 (4.37). IR ν_{max}^{KBr} cm^{-1}: 1772, 1754 (acetyl), 1644 (conjugated CO), 1618 (arom. C=C). ^{1}H-NMR: (DMSO-d_6) δ: 6.79 (1H, s, H-3), 7.35 (1H, s, H-8), 8.08 (2H, d, J=8.8 Hz, H-2′, 6′), 7.35 (2H, d, J=8.8 Hz, H-3′, 5′), 7.35 (1H, d, J=1.8 Hz, H-2″), 7.17 (1H, d, J=8.1 Hz, H-5″), 7.11 (1H, dd, J=8.1, 1.8 Hz, H-6″), 5.35 (1H, d, J=7.7 Hz, H-7″), 4.74 (1H, ddd, J=7.7, 5.2, 4.1 Hz, H-8″), 4.08 (1H, dd, J=12.5, 5.2 Hz, H-9″a), 4.18 (1H, dd, J=12.5, 4.1 Hz, H-9″b), 3.80 (3H, s, $-OCH_3$), 2.00, 2.27, 2.31, 2.35 (each 3H, s, $-OCOCH_3$). ^{13}C-NMR (DMSO-d_6) δ: 160.5 (C-2), 106.9 (C-3), 175.5

(C-4), 135.7 (C-5), 133.6 (C-6),148.5 (C-7), 102.6 (C-8), 150.6 (C-9), 111.4 (C-10), 128.3 (C-1′), 127.6 (C-2′, 6′), 122.5 (C-3′, 5′), 152.9 (C-4′), 133.7 (C-1″), 112.2 (C-2″), 151.1 (C-3″), 140.0 (C-4″), 123.0 (C-5″), 120.1 (C-6″), 76.3 (C-7″), 74.4 (C-8″), 61.6 (C-9″), 55.9 (–OCH_3), 20.2, 20.3, 20.5, 20.8 (–OCOCH_3), 168.2, 168.3, 168.8, 169.8 (–OCOCH_3). EI-MS *m/z* (%): 632 (M^+, 2), 590 (100), 548 (16), 488 (11), 328 (52), 286 (28), 222 (67), 179 (33).

Scutellaprostin F (VI) Yellow needles (from MeOH), mp 204—205 °C (dec.). $[\alpha]_D^{20} \pm 0°$ (*c* = 0.05, MeOH). *Anal.* Calcd for $C_{25}H_{20}O_{10}$: C, 62.50; H, 4.20. Found: C, 62.45; H, 4.29. Mg–HCl (+). UV λ_{max}^{MeOH} nm (log ε): 220 sh (4.61), 237 sh (4.40), 261 (4.12), 284 (4.28), 352 (4.39); $\lambda_{max}^{MeOH-NaOMe}$ nm (log ε): 258 (4.40), 294 (4.28), 394 (4.32); $\lambda_{max}^{MeOH-AlCl_3}$ nm (log ε): 224 (4.58), 285 (4.26), 311 sh (4.05), 433 (4.46); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm (log ε): 224 (4.56), 240 sh (4.36), 265 (4.05), 290 (4.24), 302 sh (4.20), 376 (4.40); $\lambda_{max}^{MeOH-NaOAc}$ nm (log ε): 282 (4.23), 354 (4.19), 414 (4.06); $\lambda_{max}^{MeOH-NaOAc-H_3BO_3}$ nm (log ε): 266 (4.27), 282 (4.23), 378 (4.37). IR ν_{max}^{KBr} cm^{-1}: 3428 (OH), 1664 (conjugated CO), 1610 (arom. C=C). ^{1}H-NMR: Table I. ^{13}C-NMR: Table II. EI-MS *m/z* (%): 480 (M^+, 3), 302 ($C_{15}H_{10}O_7$, 77), 180 ($C_{10}H_{12}O_3$, 63), 168 ($C_7H_4O_5$, 28), 137 ($C_8H_9O_2$, 100), 124 ($C_7H_8O_2$, 61).

Acetylation of VI: Ac_2O–pyridine treatment of VI yielded a pentaacetate as colorless needles (from $CHCl_3$–MeOH), mp 181—181 °C (dec.). *Anal.* Calcd for $C_{35}H_{30}O_{15}$: C, 60.87; H, 4.38. Found: C, 60.89; H, 4.40. UV λ_{max}^{MeOH} nm (log ε): 215 (4.79), 270 (4.43), 314 (4.44). IR ν_{max}^{KBr} cm^{-1}: 1772, 1742 (acetyl), 1644 (conjugated CO), 1616 (arom. C=C). ^{1}H-NMR (DMSO-d_6) δ: 6.84 (1H, s, H-3), 7.38 (1H, s, H-8), 8.01 (1H, d, *J* = 2.2 Hz, H-2′), 7.49 (1H, d, *J* = 9.2 Hz, H-5′), 8.01 (1H, dd, *J* = 9.2, 2.2 Hz, H-6′), 7.33 (1H, d, *J* = 1.8 Hz, H-2″), 7.17 (1H, d, *J* = 8.1 Hz, H-5″), 7.10 (1H, dd, *J* = 8.1, 1.8 Hz, H-6″), 5.36 (1H, d, *J* = 7.7 Hz, H-7″), 4.75 (1H, ddd, *J* = 7.7, 5.2, 3.7 Hz, H-8″), 4.07 (1H, dd, *J* = 12.4, 5.2 Hz, H-9″a), 4.17 (1H, dd, *J* = 12.4, 3.7 Hz, H-9″b), 3.79 (3H, s, –OCH_3), 2.00, 2.27, 2.32, 2.33, 2.35 (each 3H, s, –$OCOCH_3$). ^{13}C-NMR (DMSO-d_6) δ: 159.7 (C-2), 107.4 (C-3), 175.5 (C-4), 135.7 (C-5), 133.7 (C-6), 148.7 (C-7), 102.8 (C-8), 150.7 (C-9), 111.4 (C-10), 129.5 (C-1′), 121.7 (C-2′), 142.5 (C-3′), 144.6 (C-4′), 124.8 (C-5′), 124.5 (C-6′), 133.8 (C-1″), 112.2 (C-2″), 151.1 (C-3″), 140.0 (C-4″), 123.1 (C-5″), 120.2 (C-6″), 76.4 (C-7″), 74.4 (C-8″), 61.7 (C-9″), 56.0 (–OCH_3), 20.3 (–OCOCH_3 × 2), 20.4 (–OCOCH_3 × 2), 20.6 (–OCOCH_3), 168.0, 168.2, 168.3, 168.4, 169.9 (–OCOCH_3). EI-MS *m//z* (%): 690 (M^+, 7), 648 (33), 606 (22), 588 (52), 546 (100), 504 (48), 386 (35), 344 (26), 302 (29), 222 (28), 179 (21).

Treatment of I—VI with Pyridine Hydrobromide To freshly fused pyridine hydrobromide (450 mg) was added each of the compounds I—VI (15 mg), and the mixture was heated at 210—220 °C for 15 min. The reaction mixture was cooled and poured into ice-water. After extraction with AcOEt, the extract was washed with water and dried over anhydrous Na_2SO_4. The solution was evaporated to dryness and the residue was crystallized from MeOH to give the respective flavones, which were identified as norwogonin, isoscutellarein, hypolaetin, baicalein, scutellarein and 6-hydroxyluteolin, respectively, by direct comparisons with authentic specimens (TLC, UV, IR, ^{1}H-NMR, mixed fusion).

Treatment of I—VI with Alkali A solution of each of the compounds I–VI (10 mg) in 0.5 N KOH (3 ml) was heated at 75 °C for 2.5 h. The reaction mixture was acidified with diluted HCl and extracted with AcOEt. The organic layer was washed with water and dried over anhydrous Na_2SO_4. The solution was evaporated to dryness and the residue was chromatographed on silica gel using $CHCl_3$ to give colorless needles (3 mg) (from benzene–petr. ether), mp 75 °C; this product was shown to be identical with coniferyl alcohol by direct comparison (IR, ^{1}H-NMR, mixed fusion).

Oxidative Coupling of Norwogonin and Coniferyl Alcohol with Ag_2O (Formation of I) Norwogonin (500 mg) and coniferyl alcohol (320 mg) were dissolved in dry benzene–acetone (18 : 5) (200 ml). Ag_2O (925 mg) was added and the suspension was stirred at 55 °C. The reaction was monitored by TLC and interrupted when one of the starting materials was no longer detectable by TLC. The reaction mixture was then filtered and evaporated to dryness. The residue obtained was purified by column chromatography on a silica gel with $CHCl_3$–MeOH–H_2O (100 : 10 : 2) to give compound I (365 mg), which was identical to scutellaprostin A by direct comparison (TLC, $[\alpha]_D$, IR, ^{1}H- and ^{13}C-NMR).

Oxidative Coupling of Isoscutellarein and Coniferyl Alcohol with Ag_2O (Formation of II) A mixture of isoscutellarein (320 mg) and coniferyl alcohol (210 mg) in benzene–acetone (18 : 5) (460 ml) in the presence of Ag_2O (570 mg) as treated by a procedure similar to that described for the formation of I. The resulting product was purified by chromatography on a silica gel with $CHCl_3$–MeOH–H_2O (100 : 10 : 2) to give compound II (190 mg), which was identical to scutellaprostin B by direct comparison (TLC, $[\alpha]_D$, ^{1}H- and ^{13}C-NMR).

Oxidative Coupling of Hypolaetin and Coniferyl Alcohol with Ag_2O (Formation of III) A mixture of hypolaetin (500 mg) and coniferyl alcohol (300 mg) in benzene–acetone (18 : 5) (500 ml) in the presence of Ag_2O (800 mg) was treated by a procedure similar to that described for the formation of I. The resulting product was purified by chromatography on a silica gel with $CHCl_3$–MeOH–H_2O (100 : 10 : 2) to give compound III (290 mg), which was identical with scutellaprostin C by direct comparison (TLC, $[\alpha]_D$, ^{1}H- and ^{13}C-NMR).

Oxidative Coupling of Baicalein and Coniferyl Alcohol with Ag_2O (Formation of IV and VII) A mixture of baicalein (1 g) and coniferyl alcohol (670 mg) in benzene–acetone (18 : 5) (400 ml) in the presence of Ag_2O (1.8 g) was treated by a procedure similar to that described for the formation of I. The resulting product was chromatographed on silica gel with a gradient of $CHCl_3$–MeOH (100 : 0→100 : 20) as an eluent to give compounds IV (570 mg) and VII (145 mg). Compound IV was identical with scutellaprostin D by direct comparison (TLC, $[\alpha]_D$, ^{1}H- and ^{13}C-NMR).

Compound VII: Colorless needles (from MeOH), mp 206—207 °C (dec.). $[\alpha]_D^{25} \pm 0°$ (*c* = 0.05, MeOH). *Anal.* Calcd for $C_{25}H_{20}O_8$: C, 66.96; H, 4.50. Found: C, 66.92; H, 4.51. Mg–HCl (+). UV λ_{max}^{MeOH} nm (log ε): 215 (4.37), 237 (4.07), 274 (4.26), 314 (3.98); $\lambda_{max}^{MeOH-NaOMe}$ nm (log ε): 248 (4.25), 267 (4.29), 357 (3.73); $\lambda_{max}^{MeOH-AlCl_3}$ nm (log ε): 215 (4.36), 237 sh (4.05), 275 (4.23), 314 (3.95); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm (log ε): 215 (4.30), 238 (4.04), 277 (4.19), 320 (3.82); $\lambda_{max}^{MeOH-NaOAc}$ nm (log ε): 270 (4.15), 356 (3.58). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1638 (conjugated CO), 1588 (arom. C=C). ^{1}H-NMR: Table I. ^{13}C-NMR: Table II. EI-MS *m/z* (%): 448 (M^+, 52), 270 ($C_{15}H_{10}O_5$, 100), 180 ($C_{10}H_{12}O_3$, 23), 168 ($C_7H_4O_5$, 11), 137 ($C_8H_9O_2$, 40), 124 ($C_7H_8O_2$, 23).

Acetylation of VII: Ac_2O–pyridine treatment of VII yielded a triacetate as colorless needles (from $CHCl_3$–MeOH), mp 206—207 °C (dec.). *Anal.* Calcd for $C_{31}H_{26}O_{11}$: C, 64.80; H, 4.56. Found: C, 64.73; H, 4.60. UV λ_{max}^{MeOH} nm (log ε): 217 (4.44), 274 (4.49), 303 (4.12), 330 (3.85). IR ν_{max}^{KBr} cm^{-1}: 1774, 1750 (acetyl), 1648 (conjugated CO), 1616 (arom. C=C). ^{1}H-NMR (DMSO-d_6) δ: 6.84 (1H, s, H-3), 7.31 (1H, s, H-8), 8.06 (2H, m, H-2′, 6′), 7.54—7.61 (3H, m, H-3′, 4′, 5′), 7.33 (1H, d, *J* = 1.5 Hz, H-2″), 7.16 (1H, d, *J* = 8.1 Hz, H-5″), 7.10 (1H, dd, *J* = 8.1, 1.5 Hz, H-6″), 5.27 (1H, d, *J* = 7.0 Hz, H-7″), 4.65 (1H, ddd, *J* = 7.0, 5.5, 3.1 Hz, H-8″), 4.06 (1H, dd, *J* = 12.5, 5.5 Hz, H-9″a), 4.20 (1H, dd, *J* = 12.5, 3.1 Hz, H-9″b), 3.78 (3H, s, –OCH_3), 2.02, 2.27, 2.33 (each 3H, s, –$OCOCH_3$). ^{13}C-NMR (DMSO-d_6) δ: 160.6 (C-2), 107.6 (C-3), 175.8 (C-4), 142.7 (C-5), 131.9 (C-6), 143.1 (C-7), 104.8 (C-8), 150.6 (C-9), 112.2 (C-10), 130.7 (C-1′), 126.1 (C-2′, 6′), 129.1 (C-3′, 5′), 131.7 (C-4′), 134.3 (C-1″), 112.2 (C-2″), 151.0 (C-3″), 139.7 (C-4″), 123.0 (C-5″), 120.0 (C-6″), 75.5 (C-7″), 73.8 (C-8″), 61.6 (C-9″), 55.9 (–OCH_3), 20.2 (–OCOCH_3), 20.4 (–OCOCH_3 × 2), 167.7, 168.5, 170.0 (–OCOCH_3). EI-MS *m/z* (%): 574 (M^+, 4), 532 (100), 490 (9), 430 (6), 281 (13), 270 (49), 222 (42), 179 (17), 147 (8), 131 (13).

Oxidative Coupling of Scutellarein and Coniferyl Alcohol with Ag_2O (Formation of V and VIII) A mixture of scutellarein (235 mg) and coniferyl alcohol (148 mg) in benzene–acetone (18 : 5) (400 ml) in the presence of Ag_2O (400 mg) was treated by a procedure similar to that described for the formation I. The resulting product was chromatographed on silica gel with a gradient of $CHCl_3$–MeOH (100 : 0→100 : 20) as an eluent to give compounds V (109 mg) and VIII (32.8 mg). Compound V was identical to scutellaprostin E by direct comparison (TLC, $[\alpha]_D$, ^{1}H- and ^{13}C-NMR).

Compound VIII: Colorless needles (from MeOH), mp 235—236 °C (dec.). $[\alpha]_D^{25} \pm 0°$ (*c* = 0.05, MeOH), *Anal.* Calcd for $C_{25}H_{20}O_9$: C, 64.65; H, 4.34. Found: C, 64.58; H, 4.40. Mg–HCl (+). UV λ_{max}^{MeOH} nm (log ε): 219 (4.56), 278 (4.35), 328 (4.39); $\lambda_{max}^{MeOH-NaOMe}$ nm (log ε): 235 sh (4.49), 252 sh (4.38), 273 (4.27), 322 (4.19), 380 (4.51); $\lambda_{max}^{MeOH-AlCl_3}$ nm (log ε): 219 (4.56), 279 (4.32), 331 (4.36), 400 (3.62); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm (log ε): 220 sh (4.52), 281 (4.27), 305 sh (4.18), 347 sh (4.18), 393 (4.28); $\lambda_{max}^{MeOH-NaOAc}$ nm (log ε): 276 (4.44), 305 sh (4.11), 358 (4.27). IR ν_{max}^{KBr} cm^{-1}: 3300 (OH), 1634 (conjugated CO), 1608, 1576 (arom. C=C). ^{1}H-NMR: Table I. ^{13}C-NMR: Table II. EI-MS *m/z* (%): 464 (M^+, 15), 286 ($C_{15}H_{10}O_6$, 100), 180 ($C_{10}H_{12}O_3$, 23), 168 ($C_7H_4O_5$, 34), 137 ($C_8H_9O_2$, 34).

Acetylation of VIII: Ac_2O–pyridine treatment of VIII yielded a tetraacetate as colorless needles (from $CHCl_3$–MeOH), mp 213—214 °C (dec.). *Anal.* Calcd for $C_{33}H_{28}O_{13}$: C, 62.66; H, 4.46. Found: C, 62.68; H, 4.52. UV λ_{max}^{MeOH} nm (log ε): 217 sh (4.52), 277 (4.53), 305 (4.25), 335 (3.85). IR ν_{max}^{KBr} cm^{-1}: 1772, 1744 (acetyl), 1648 (conjugated CO), 1616 (arom. C=C). ^{1}H-NMR (DMSO-d_6) δ: 6.83 (1H, s, H-3), 7.31 (1H, s, H-8), 8.10 (2H, d, *J* = 8.8 Hz, H-2′, 6′), 7.33 (2H, d, *J* = 8.8 Hz, H-3′, 5′), 7.33 (1H, d, *J* = 1.8 Hz, H-2″), 7.16 (1H, d, *J* = 8.2 Hz, H-5″), 7.09 (1H, dd, *J* = 8.2, 1.8 Hz, H-6″), 5.27 (1H, d, *J* = 7.0 Hz, H-7″), 4.64 (1H, m,

H-8″), 4.20 (1H, dd, J=12.6, 3.4 Hz, H-9″a), 4.06 (1H, dd, J=12.6, 5.9 Hz, H-9″b), 3.77 (3H, s, $-OCH_3$), 2.01, 2.27, 2.30, 2.32 (each 3H, s, $-OCOCH_3$). ^{13}C-NMR (DMSO-d_6) δ: 160.0 (C-2), 107.6 (C-3), 175.9 (C-4), 142.7 (C-5), 132.0 (C-6), 143.2 (C-7), 104.9 (C-8), 150.7 (C-9), 112.2 (C-10), 128.3 (C-1′), 127.7 (C-2′, 6′), 122.7 (C-3′, 5′), 153.1 (C-4′), 134.4 (C-1″), 112.2 (C-2″), 151.1 (C-3″), 139.8 (C-4″), 123.1 (C-5″), 120.0 (C-6″), 75.6 (C-7″), 73.9 (C-8″), 61.7 (C-9″), 56.0 ($-OCH_3$), 20.3 ($-OC\underline{O}CH_3$), 20.5 ($-OCO\underline{C}H_3\times2$), 21.0 ($-OCO\underline{C}H_3$), 167.9, 168.6, 170.1 ($-O\underline{C}OCH_3$). EI-MS m/z (%): 632 (M^+, 27), 590 (14), 530 (13), 488 (12), 328 (70), 327 (100), 286 (49), 222 (53), 179 (22), 131 (24).

Oxidative Coupling of 6-Hydroxyluteolin and Coniferyl Alcohol with Ag_2O (Formation of VI and IX) A mixture of 6-hydroxyluteolin (1.9 g) and coniferyl alcohol (1.13 g) in benzene–acetone (18 : 5) (1900 ml) in the presence of Ag_2O (3.2 g) was treated by a procedure similar to that described for the formation of I. The resulting product was chromatographed on silica gel with a gradient of $CHCl_3$–MeOH (100 : 0→100 : 20) as an eluent to give compounds VI (650 mg) and IX (105 mg). Compound VI was identical to scutellaprostin F by direct comparison (TLC, $[\alpha]_D$, ^{1}H- and ^{13}C-NMR).

Compound IX: Colorless needles (from MeOH), mp 213—214 °C (dec.). $[\alpha]_D^{25}\pm0°$ (c=0.05, MeOH). *Anal.* Calcd for $C_{25}H_{20}O_{10}$: C, 62.50; H, 4.20. Found: C, 62.45; H, 4.29. Mg–HCl (+). UV λ_{max}^{MeOH} nm (log ε): 222 sh (4.60), 238 (4.35), 281 (4.24), 343 (4.35); $\lambda_{max}^{MeOH-NaOMe}$ nm (log ε): 259 sh (4.42), 294 sh (4.08), 334 (4.02), 394 (4.39); $\lambda_{max}^{MeOH-AlCl_3}$ nm (log ε): 221 sh (4.54), 239 sh (4.38), 279 (4.24), 432 (4.24); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm (log ε): 221 sh (4.52), 238 sh (4.34), 285 (4.21), 376 (4.21); $\lambda_{max}^{MeOH-NaOAc}$ nm (log ε): 277 (4.38), 323 sh (4.34), 376 (4.32); $\lambda_{max}^{MeOH-NaOAc-H_3BO_3}$ nm (log ε): 264 sh (4.31), 278 sh (4.29), 368 (4.34). IR ν_{max}^{KBr} cm^{-1}: 3460 (OH), 1636 (conjugated CO), 1614 (arom. C=C). ^{1}H-NMR: Table I. ^{13}C-NMR: Table II. EI-MS m/z (%): 302, ($C_{15}H_{10}O_7$, 100), 168 ($C_7H_4O_5$, 51), 137 ($C_8H_9O_2$, 13).

Acetylation of IX: Ac_2O–pyridine treatement of IX yielded a pentaacetate as colorless needles (from $CHCl_3$–MeOH), mp 213—215 °C. (dec.). *Anal.* Calcd for $C_{35}H_{30}O_{15}$: C, 60.87; H, 4.38. Found: C, 60.90; H, 4.43. UV λ_{max}^{MeOH} nm (log ε): 219 sh (4.59), 278 (4.63), 307 sh (4.26), 340 sh (3.94). IR ν_{max}^{KBr} cm^{-1}: 1773, 1749 (acetyl), 1650 (conjugated CO), 1617 (arom. C=C). ^{1}H-NMR (DMSO-d_6) δ: 6.87 (1H, s, H-3), 7.31 (1H, s, H-8), 8.03 (1H, br s, H-2′), 7.48 (1H, d, J=8.8 Hz, H-5′), 8.02 (1H, dd, J=8.8, 2.2 Hz, H-6′), 7.33 (1H, d, J=1.8 Hz, H-2″), 7.16 (1H, d, J=8.2 Hz, H-5″), 7.10 (1H, dd, J=8.2, 1.8 Hz, H-6″), 5.26 (1H, d, J=7.0 Hz, H-7″), 4.64 (1H, m, H-8″), 4.06 (1H, dd, J=12.6, 5.7 Hz, H-9″a), 4.20 (1H, dd, J=12.6, 3.3 Hz, H-9″b), 3.78 (3H, s, $-OCH_3$), 2.02, 2.28, (each 3H, s, $-OCOCH_3$) 2.32, (6H, s, $-OCOCH_3\times2$), 2.33 (3H, s, $-OCOCH_3$). ^{13}C-NMR (DMSO-d_6) δ: 159.0 (C-2), 108.0 (C-3), 175.7 (C-4), 142.7 (C-5), 132.0 (C-6), 143.1 (C-7), 104.8 (C-8), 150.6 (C-9), 112.2 (C-10), 129.1 (C-1′), 121.7 (C-2′), 142.5 (C-3′), 144.5 (C-4′), 124.6 (C-5′), 124.4 (C-6′), 134.2 (C-1″), 112.2 (C-2″), 151.0 (C-3″), 139.8 (C-4″), 123.0 (C-5″), 120.0 (C-6″), 75.6 (C-7″), 73.9 (C-8″), 61.6 (C-9″), 55.9 ($-OCH_3$), 20.2 ($-OCO\underline{C}H_3$), 20.3 ($-OCO\underline{C}H_3\times2$), 20.4 ($-OCO\underline{C}H_3\times2$), 167.7, 168.0, 168.2, 168.4, 170.0 ($-O\underline{C}OCH_3$). EI-MS m/z (%): 690 (M^+, 18), 648 (11), 588 (7), 546 (12), 504 (4), 427 (24), 385 (100), 343 (43), 302 (31), 222 (91), 179 (61).

Acknowledgements We are grateful to Mrs. R. Igarashi and Miss A. Nakashima of this university for elemental analysis and MS measurement.

References and Notes

1) Part XIII: Y. Kikuchi, Y. Miyaichi and T. Tomimori, *Chem. Pharm. Bull.*, **39**, 1051 (1991).
2) Presented at the 109th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1989 and the 36th Annual Meeting of the Japanese Society of Pharmacognosy, Kumamoto, October 1989.
3) Y. Kikuchi, Y. Miyaichi, Y. Yamaguchi, H. Kizu and T. Tomimori, *Chem. Pharm. Bull.*, **39**, 1047 (1991).
4) T. J. Mabry, K. R. Markham and M. B. Thomas, "The Systematic Identification of Flavonoids," Springer-Verlag, New York, 1970, Chapter V.
5) T. Tomimori, Y. Miyaichi, Y. Imoto, H. Kizu and Y. Tanabe, *Yakugaku Zasshi*, **103**, 607 (1983).
6) A. B. Ray, S. K. Chattopadhyay, S. Kumar, D. Konno, Y. Kiso and H. Hikino, *Tetrahedron*, **41**, 209 (1985).
7) L. Merlini, A. Zanarotti, A. Pelter, M. P. Rochefort and R. Hänsel, *J. Chem. Soc., Perkin Trans. 1*, **1980**, 775.
8) Y. Miyaichi, Y. Imoto, H. Saida and T. Tomimori, *Shoyakugaku Zasshi*, **42**, 216 (1988).
9) Synthesized from 5,8-dihydroxy-7,3′,4′-trimethoxyflavone[11] by demethylation with pyridine hydrobromide.
10) T. Tomimori, Y. Miyaichi, Y. Imoto and H. Kizu, *Yakugaku Zasshi*, **38**, 249 (1984).
11) Synthesized according to the literature [N. Morita, *Chem. Pharm. Bull.*, **8**, 66 (1960)].

Two New β-Hydroxychalcones from the Root Bark of *Pongamia pinnata*

Toshiyuki TANAKA, Munekazu IINUMA, Kaoru YUKI, Yuko FUJII and Mizuo MIZUNO*

Department of Pharmacognosy, Gifu Pharmaceutical University, 6–1 Mitahora-higashi 5-chome, Gifu 502, Japan. *Received December 25, 1990*

Two new β-hydroxychalcones named ponganones I and II were isolated from the root bark of *Pongamia pinnata* in Japan. The structures were characterized as 7-hydroxy-2',5'-dimethoxy-[6'',6''-dimethylpyrano(2'',3'' : 4',3')]chalcone for ponganone I, and 7-hydroxy-2',5'-dimethoxy-3,4-methylenedioxy-[6'',6''-dimethylpyrano(2'',3'' : 4',3')]chalcone for ponganone II, respectively, by means of spectroscopic analysis.

Keywords *Pongamia pinnata*; Leguminosae; β-hydroxychalcone; ponganone I; ponganone II

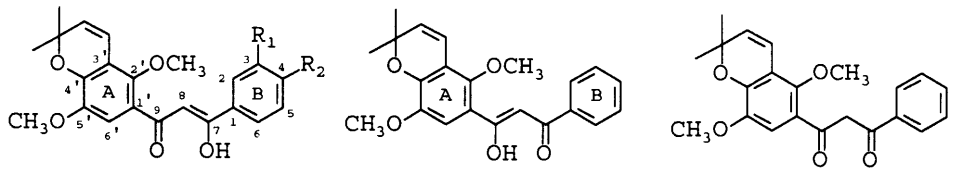
Introduction

The genus *Pongamia* (family: Leguminosae, subfamily: Papilionodae, tribe: Tephrosiae) consists of only one species, *P. pinnata*, which is widely distributed along Southeast Asia to the West Pacific and North Australia.[1] In Japan, the plant grows on the sandy coast along the Pacific Ocean and in limestone shrub forests.[2] From a botanical standpoint, the plant is classified in the same tribe as *Millettia*, and the flower of *Pongamia* is indistinguishable from that of *Millettia* (subgenus: Otosema, section: Fragilliflorae).[1]

All parts of the plant have been used as a crude drug for the remedy of tumors, piles, skin diseases, wounds, ulcers *etc.*,[3] especially in India. Previous phytochemical studies showed rich resources of flavonoid compounds such as flavones,[4,5] flavanones,[6,7] chalcones,[8] β-diketones and β-hydroxychalcones.[6,9,10] Re-examination of a dichloromethane soluble fraction of the root bark of *P. pinnata* in Japan revealed two new β-hydroxychalcones. In this paper, the isolation and structural determination of the two compounds is described.

Repeated chromatographies eluted with *n*-hexane–acetone mixtures, preparative thin layer chromatography (TLC) and recrystallization of the CH_2Cl_2 extract afforded two new hydroxychalcones, together with seven known compounds such as β-diketone or flavonol.

Compound **1**, ponganone I, mp 127—128 °C was obtained as yellow plates. By high-resolution mass spectrometry (HRMS), the empirical formula was determined to be $C_{22}H_{25}O_5$ (M^+ *m/z* 366.1490; Calcd 366.1467). The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** showed a strongly chelated hydroxyl group (δ 16.95) and an olefinic proton (δ 7.28) which correlated with a carbon at δ 97.0 in ^{1}H–^{13}C correlation spectroscopy (COSY). These results showed the presence of a partial structure such as –COCH=C(OH), which suggested that **1** was a hydroxylchalcone derivative in a *Z*-configuration.[11] The ^{1}H-NMR spectral data, furthermore, exhibited the presence of an unsubstituted benzene ring attributable to ring B [δ 7.50 (3H, m) and 7.97 (2H, m)], a dimethylpyrane ring [δ 1.50 (6H, Me×2), 5.72, 6.63 (1H, each d, J=10 Hz)], two methoxyl groups (δ 3.77 and 3.91), and an aromatic proton (δ 7.39). In the electron ionization (EI) MS, prominent fragment ions were observed at *m/z* 351, 335, 247, 231 and 105. A fragment at *m/z* 351 was regarded as an ion after elimination of a methyl group from the dimethylpyrane ring. The other fragments were also characterized as **1a—d** shown in Chart 2. Among them, **1a** ($M^+ - OMe$) intensively supported a methoxyl group at C-2 or C-2'. In the ^{13}C-NMR spectrum, two methoxyl carbon signals were observed at δ 56.4 and 62.8. The methoxyl proton at δ 3.91 correlated with the former carbon (56.4), and another such proton at δ 3.77 correlated with the latter (62.8) in the ^{1}H–^{13}C COSY. On the other hand, the methoxyl proton at δ 3.91 correlated with the aromatic proton at δ 7.39 in ^{1}H–^{1}H long range COSY, and the aromatic proton further correlated with a carbon at δ 112.2 assigned to the carbon bearing the aromatic proton itself in ^{1}H–^{13}C COSY. These spectral data showed that one of two methoxyl groups was surrounded by *ortho*-substituents, the other methoxyl group was located at an *ortho*-position to be unsubstituted. Accordingly, six possible partial structures (A—F) shown in Chart 3 were taken into consideration. Among the partial structures, C and F, the oxygenation pattern of which correspond to 6,8-dioxygenation of the flavone numbering system and are unprecedented as a natural product. In the ^{1}H–^{13}C long range, the olefinic proton at δ 7.28 assigned to a proton at C-8 correlated with two signals at δ 184.0 and 185.3 assigned to C-7 and C-9 in the β-hydroxylchalcone skeleton, the former of which further correlated with H-2 and H-6 of the unsubstituted ring of the ring B, and the latter of which (185.3) correlated with the aromatic proton at δ 7.39 (Chart 4). These findings suggested that the partial structure of **1** to be A or C and the other partial structures were deleted. Not only because of evidence that the aromatic proton correlated with carbons with an oxygen function such as the methoxyl group, and further correlated with a

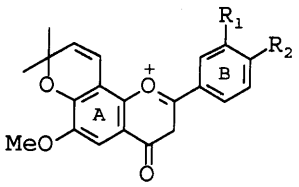


1 : R_1=R_2=H
2 : R_1+R_2=OCH_2O

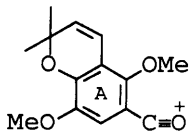
1′ 1″

Chart 1

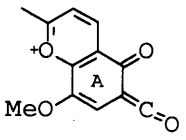
carbon at C-2, but also due to biosynthetic considerations, a partial structure of A is preferable to that of C. The alternative structure (**1'**) caused by tautomerism must be considered. Comparison between **1** and pongamol [7-hydroxy-2'-methoxy-furano(2",3" : 4',3')chalcone] (**3**),[11] the structure of which was determined by X-ray analysis,[12] showed that the ^{1}H- and ^{13}C-NMR signals due to ring B of **1** were completely identical to those of **3**, indicating that ponganone I was a 7-hydroxyl form. Consequently, the structure of ponganone I in a $CDCl_3$ solution was concluded to be 7-hydroxy-2',5'-dimethoxy-[6",6"-dimethylpyrano-(2",3" : 4',3')]chalcone. The β-diketone form shown as **1"**, however, occupies about 3% in the solution.



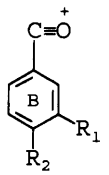
1a : $R_1=R_2=H$ *m/z* 335
2a : $R_1+R_2=OCH_2O$ *m/z* 379



1b *m/z* 247

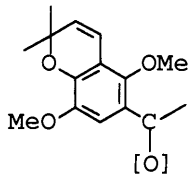


1c *m/z* 231

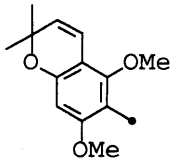


1d : $R_1=R_2=H$ *m/z* 105
2d : $R_1+R_2=OCH_2O$ *m/z* 149

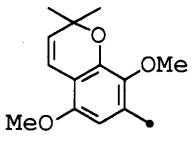
Chart 2



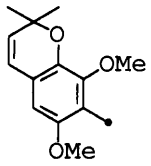
A



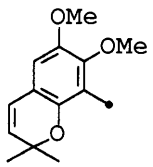
B



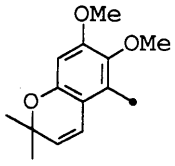
C



D



E



F

Chart 3

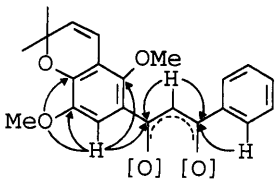


Chart 4

Compound **2**, ponganone II, mp 138—139 °C, was obtained as yellow needles. By HRMS, its empirical formula was determined as $C_{23}H_{22}O_7$ (M^+ *m/z* 410.1341; Calcd 410.1365). The signal pattern based on ring B was different from that of **1**, that is, an ABX system was observed. And a one-proton doublet at δ 7.00 ($J=8$ Hz) and 7.60 ($J=2$ Hz) and a one-proton double doublet at δ 7.71 ($J=8$ and 2 Hz) were assigned to H-5, H-6 and H-2, respectively. In addition to two methoxyl groups, the presence of a methylenedioxyl group (δ 6.11) was confirmed by the ^{1}H-NMR, and its group was located on ring B supported by the EIMS exhibiting a fragment ion (**2d**) at *m/z* 149 shown in Chart 2. The methylenedioxyl group was, then, at C-3 and C-4. Consequently, the structure of ponganone II was characterized as 7-hydroxy-2',5'-dimethoxy-3,4-methylenedioxy-[6",6"-dimethyl-pyrano(2",3" : 4',3')]chalcone. The β-diketone form of **2** corresponding to **1"** also existed in 4% in a solution.

The structures of known compounds were determined to be pongamol (**3**), ovalitenone [7-hydroxy-2'-methoxy-3,4-methylenedioxy-furano(2",3" : 4',5')chalcone] (**4**), and milletenone (7-hydroxy-2',4'-dimethoxy-3,4-methylenedioxy-chalcone) (**5**), fisetin tetramethyl ether (3,7,3',4'-tetramethoxyflavone) (**6**), demethylkanugin (3,7-dimethoxy-3',4'-methylenedioxyflavone) (**7**), karanjin [3-methoxy-furano(2",3",7,8)flavone] (**8**), pongapin [3-methoxy-3',4'-methylenedioxy-furano(2",3" : 7,8)flavone] (**9**), respectively, by means of spectroscopic analysis.

Other flavonoid compounds in the root bark are now being examined.

Experimental

Plant Materials Root bark of *Pongamia pinnata* was collected at Iriomote Island, Okinawa, in February, 1988. The voucher specimens have been deposited at the Herbarium in Gifu Pharmaceutical University.

Extraction and Isolation of Compounds 1—9 The dried root bark (300 g)

TABLE I. ^{13}C-NMR Spectral Data on Compounds **1** and **2**

Carbon No.	1	2
1	135.6 (s)	130.2 (s)
2	127.1 (d)	107.2 (d)
3	128.7 (d)	148.2 (s)
4	132.2 (d)	151.2 (s)
5	128.7 (d)	108.2 (d)
6	127.1 (d)	122.8 (d)
7	184.0 (s)	183.1 (s)
8	97.0 (d)	96.3 (d)
9	185.3 (s)	184.5 (s)
1'	120.7 (s)	120.7 (s)
2'	150.5 (s)	150.3 (s)
3'	116.0 (s)	116.0 (s)
4'	146.8 (s)	146.5 (s)
5'	145.3 (s)	145.2 (s)
6'	112.2 (d)	112.1 (d)
4"	116.8 (d)	116.8 (d)
5"	130.8 (d)	130.8 (d)
6"	77.3 (s)	77.2 (s)
6"-Me	28.0 (q)	28.0 (q)
2'-OMe	62.8 (q)	62.6 (q)
5'-OMe	56.4 (q)	56.4 (q)
OCH_2O		101.8 (t)

Measured in $CDCl_3$. All signals were assigned by the aid of ^{1}H–^{13}C, ^{1}H–^{13}C long range COSY and INEPT.

of *P. pinnata* was extracted with CH_2Cl_2, and acetone, successively. The CH_2Cl_2 extract was concentrated under reduced pressure. The residue was subjected to column chromatography on silica gel (*n*-hexane : acetone = 10 : 1→3 : 1) and purified by preparative TLC (cyclohexane–acetone–methylethylketone system) to give **1** ponganone I (110 mg), **2** ponganone II (150 mg), **3** pongamol (15 mg), **4** ovalitenone (5 mg), **5** milletenone (5 mg), **6** fisetin tetramethyl ether (25 mg), **7** demethylkanugin (200 mg), **8** karanjin (72 mg), and **9** pongapin (60 mg), respectively, in pure form.

Compound **1** (Ponganone I): mp 127—128 °C, yellow plates (MeOH). EIMS *m/z* (%): 366 [M^+] (29), 351 (78), 335 (100), 247 (9), 231 (22), 217 (9), 215 (10), 205 (9), 105 (51), 77 (29). UV λ_{max}^{MeOH} nm: 248, 290, 367. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.50 (6H, s, Me × 2), 3.77 (3H, s, 2′-OMe), 3.91 (3H, s, 5′-OMe), 5.72 (1H, d, J = 10 Hz, H-5″), 6.63 (1H, d, J = 10 Hz, H-4″), 7.28 (1H, s, H-8), 7.39 (1H, s, H-6′), 7.50 (3H, m, H-3, 4, 5), 7.97 (2H, m, H-2, 6), 16.95 (1H, s, C_7-OH), and 3.75, 3.87 (2′- and 5′-OMe), 4.67 ($COCH_2CO$) based on a β-diketone structure (**1′**). ^{13}C-NMR spectral data are listed in Table I.

Compound **2** (Ponganone II): mp 138—139 °C, yellow needles (MeOH). EIMS *m/z* (%): 410 [M^+] (29), 395 (69), 379 (100), 247 (13), 231 (21), 215 (13), 149 (53), 121 (13). UV λ_{max}^{MeOH} nm: 270 sh, 298, 366. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.56 (6H, s, Me × 2), 3.80 (3H, s, 2′-OMe), 4.01 (3H, s, 5′-OMe), 5.80 (1H, d, J = 10 Hz, H-5″), 6.11 (2H, s, OCH_2O), 6.67 (1H, d, J = 10 Hz, H-4″), 7.00 (1H, d, J = 8 Hz, H-5), 7.23 (1H, s, H-8), 7.50 (1H, s, H-6′), 7.60 (1H, d, J = 2 Hz, H-2), 7.71 (1H, dd, J = 8, 2 Hz, H-6), 17.20 (1H, s, C_7-OH), and 3.77, 3.99 (2′,5′-OMe) and 4.70 ($COCH_2CO$) based on a corresponding β-diketone structure. ^{13}C-NMR spectral data are shown in Table I.

References

1) R. Geesink, "Advances in Legume Systematics," ed. by R. M. Polhill and P. H. Raven, Royal Botanical Gardens, Kew, Richmond, 1981, p. 245.
2) H. Ohashi, "Wild Flowers of Japan, Woody Plants I," ed. by Y. Satake, H. Hara, S. Watari and T. Tominari, Heibonsha, Tokyo, 1989, p. 229.
3) B. S. Parmar, K. L. Sahrawat and S. K. Mukerjee, *J. Sci. India Res.*, **35**, 608 (1976).
4) P. Lakshmi, G. Srimannarayana and N. V. Subba Rao, *Indian J. Chem.*, **12**, 8 (1974).
5) S. B. Malik, P. Sharma and T. R. Seshadri, *Indian J. Chem.*, **15B**, 536 (1977).
6) S. K. Talapatra, A. M. Malik and B. Talapatra, *Phytochemistry*, **19**, 1199 (1980).
7) S. K. Talapatra, A. K. Malik and B. Talapatra, *Phytochemistry*, **21**, 761 (1982).
8) K. Subrehmanyan, J. M. Rao and K. V. J. Rao, *Indian J. Chem.*, **15B**, 12 (1977).
9) P. Sharma and M. R. Parthasarthy, *Indian J. Chem.*, **15B**, 866 (1977).
10) R. Gandhidasan, S. Neelakantan, P. V. Raman and S. Devaraj, *Phytochemistry*, **26**, 281 (1987).
11) F. Kikuchi, X. Chen and Y. Tsuda, *Chem. Pharm. Bull.*, **38**, 1862 (1990).
12) G. Venkataraman, E. V. Rao and C. Vilain, *J. Chem. Soc.*, **1987**, 2723.

A New Enzyme Immunoassay for a Solid Chinese Crude Drug, Pinellia Tuber

Hideaki TANIMORI,[a] Akira AKAHORI,[b] Shinsuke SONOI,[b] and Tsunehiro KITAGAWA*,[a]

Faculty of Pharmaceutical Sciences, Nagasaki University,[a] Bunkyo-machi 1–14, Nagasaki 852, Japan and Research Institute of Kotaroh Pharmaceutical Co., Ltd.,[b] Takatsuki 569, Japan. *Received December 12, 1990*

A new immunoassay for a solid Chinese crude drug was studied. An antiserum specific for Pinellia tuber was elicited in two rabbits. Using the antiserum and powdered Pinellia tuber-coated microtiter plate as the immunological reagents, and β-D-galactosidase-labeled goat anti-rabbit immunoglobulin G (IgG) as the tracer, a new enzyme immunoassay for a solid Pinellia tuber with a working range between 0.1 and 1000 μg/ml was developed. The assay was specific for a solid Pinellia tuber and showed low cross-reaction values on other Chinese crude drugs and the extract of Pinellia tuber. The specificity of the assay was compared with the selected antibody enzyme immunoassay (SAEIA) for the extract of Pinellia tuber recently developed. Both methods utilized the same immunological reagents such as the serum and the enzyme-labeled goat anti-rabbit IgG, and the only difference between them was the solid-phase antigen used. The assay results of several antigens determined by them were quite different, showing that selective measurements of different antigens, either solid or the extract of Pinellia tuber, were possible using the same antiserum, when the tracing reaction in the immunoassay was adequately selected.

Keywords competitive enzyme-linked immunosorbent assay; CELISA; selected antibody enzyme immunoassay; SAEIA; Chinese traditional medicine; Chinese crude drug; Pinellia tuber

Introduction

Immunological technology has been useful for various medical and biological studies. Most applications of the technology, however, have been limited to water-soluble antigens and solid materials have been outside the scope, though the recent developments in immunoassay methods have made it possible to use solid-phase antigens and antibodies.[1–4] Most Chinese traditional medicines consist of an extract of the mixture of solid Chinese crude drugs and any member of both the medicines and the drugs has been outside the scope of immunoassay.

In order to overcome this situation, we have been studying the development of a new assay system which is capable of being applied to both the medicines and the drugs. A part of the study has been reported in a preliminary communication of a new enzyme immunoassay method[5] for the extract of Pinellia tuber, in which detailed procedures for the assay were not reported. Further study was performed and a new immunoassay for a solid Pinellia tuber was developed.

In the present paper we also report on the characterization of the new assay by comparing it with the selected antibody enzyme immunoassay (SAEIA) for the extract of Pinellia tuber recently developed.[5]

Materials and Methods

Materials A microtiter plate with 96 wells (Immunoplate II) was bought from A/S Nunc, Denmark. β-D-Galactosidase (GAL)- labeled goat antibody specific for rabbit immunoglobulin G (IgG) was prepared according to the previous method.[6,7] Other chemicals used were of reagent grade.

Media Phosphate-buffered saline (PBS) (0.01 M phosphate buffer, pH 7.0, containing 0.1 M NaCl); coupling buffer (10 mM Tris–HCl buffer, pH 8.5, containing 10 mM NaCl and 10 mM NaN_3); buffer A (0.02 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin and 0.1% NaN_3); buffer B (0.06 M sodium phosphate buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid); washing buffer (buffer A containing 0.05% Tween 20).

Equipment The ELISA reader used was EAR 400AT. SLT Labinstruments, Austria.

Preparation of Immunogen Pinellia tuber was ground with a mortar and pestle and then filtrated with a bolter (mesh No. 48, 297 μm). A saline suspension of powdered Pinellia tuber was disrupted by Branson Sonifier (Branson Sonic Power Co., model 185E, U.S.A.) at 60 W for 3 min in an ice-water bath. The disrupted tuber was used as the immunogen and also for preparation of the solid-phase antigen.

Immunization One ml of a saline suspension of 1 mg of powdered Pinellia tuber was emulsified with an equal volume of Freund's complete adjuvant. Two Japanese white female rabbits were given multiple subcutaneous and intramuscular injections along both sides of their backs. A booster injection was given at biweekly intervals with half the dose of the first except that Freund's incomplete adjuvant was used instead of the complete type. The rabbit was bled from the ear vein two weeks after the final injection and the antiserum specific for Pinellia tuber (anti-PT) was stored at −30 °C until use.

Preparation of the Extract of Pinellia Tuber The powdered Pinellia tuber was extracted with a four times volume of water at 100 °C, and then the mixture was filtrated with cotton cloth. The filtrate and the residue were lyophilized, respectively. The extracts of Arisaema erubenscens, Pinellia cordata, Ginseng and Glycyrrhiza were also prepared in a similar way.

Preparation of the Solid-Phase Antigen The wells in a microtiter plate were coated by loading 200 μl of either a sonic disintegrated suspension of powdered Pinellia tuber (50 μg/ml) for a competitive enzyme-linked immunororbent assay (CELISA) system or a solution of the extract of Pinellia tuber (50 μg/ml) for a SAEIA system in a coupling buffer and being left at 25 °C for 1 h. After washing with 200 μl of buffer B each well was further incubated with 300 μl of buffer B at 25 °C for 1 in order to avoid non-specific adsorption. The microtiter plate was stored at 4 °C until use.

Immunoassay Procedure by CELISA Method The wells coated with powdered Pinellia tuber were incubated at 25 °C overnight with 100 μl of a variously concentrated suspension of powdered Pinellia tuber and 100 μl of 10000 times diluted anti-PT. After three washes with 200 μl of the washing buffer, each well was incubated with 200 μl of GAL-labeled goat anti-rabbit IgG antibody at 25 °C for 3 h. The wells were washed three times with 200 μl of buffer A, and the GAL activity bound to the well was assayed.

SAEIA for the extract of Pinellia tuber was performed by the method reported.[5]

Measurement of GAL Activity The amount of the bound enzyme conjugate to each well was measured by a modification of a published method.[5] Each well was incubated at 37 °C for a suitable period with 0.2 ml of 0.1% *o*-nitrophenyl-β-D-galactoside in buffer A as the substrate. The reaction was stopped by adding 25 μl of 1 M glycine–NaOH buffer, pH 10.6, and the resulting color intensity was spectrophotometrically measured at 414 nm using an ELISA reader (EAR 400AT).

Results

Antibody Response Anti-PT was produced in each of

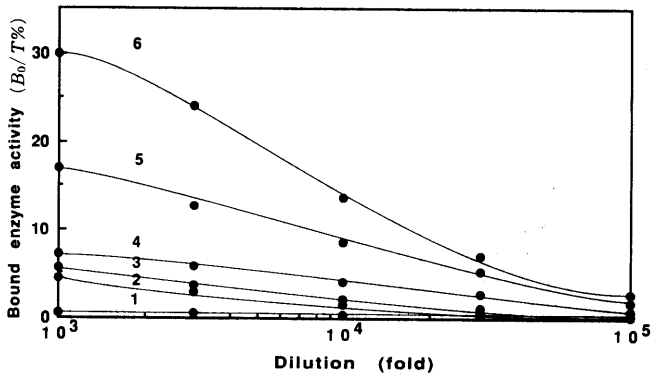


Fig. 1. Quantitative Estimation of the Titer of Anti-Pinellia Tuber Antiserum Samples

Sample 1 is bled before immunization. Samples 2, 3, 4, 5, and 6 are collected 2, 4, 6 and 8 weeks after the first injection. The samples were diluted with buffer B.

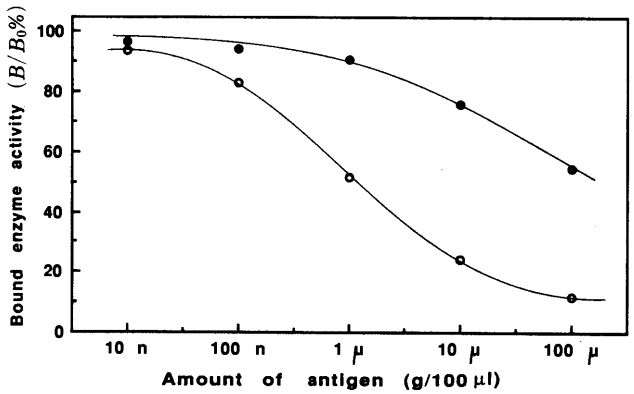


Fig. 2. Typical Dose–Response Curves of CELISA for Powdered Pinellia Tuber (Open Circles) and for the Extract of Pinellia Tuber (Closed Circles)

Bound enzyme activities measured at 414 nm were plotted against a log dose of the corresponding antigen.

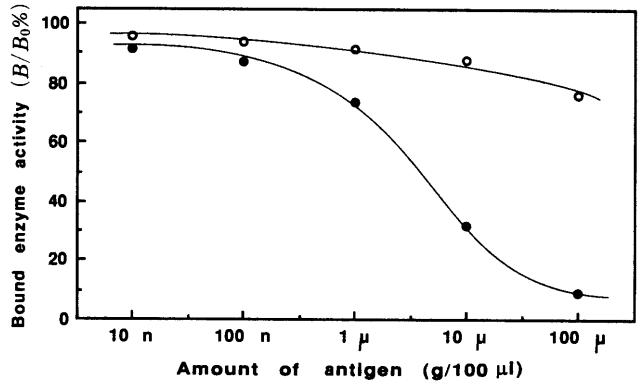


Fig. 3. Typical Dose–Response Curves of SAEIA for Powdered Pinellia Tuber (Open Circles) and for the Extract of Pinellia Tuber (Closed Circles)

Bound enzyme activities measured at 141 nm were plotted against a log dose of the corresponding antigen.

two rabbits immunized with a suspension of powdered Pinellia tuber fragments. The specific binding of the serum was detected by reacting variously diluted anti-PT serum with a solid-phase antigen, the immobilized Pinellia tuber fragments on the surface of wells of a microtiter plate, using the GAL-labeled antibody to anti-rabbit IgG as the tracer. Typical binding curves of anti-PT serum from bleeding of one of the two rabbits are shown in Fig. 1. The antibody

TABLE I. Intra- and Inter-assay Variations for the Solid Pinellia Tuber Measured by the CELISA Method and for the Extract of Pinellia Tuber Performed by the SAEIA Method

		Coefficient of variation	
		Levels of PT[a]	Concentration (μg/well)
CELISA	Intra-assay	Low (0.01)	6.7
		Moderate (1.0)	7.3
		High (100.0)	8.9
	Inter-assay	Low (0.01)	8.2±1.5
		Moderate (1.0)	6.5±0.6
		High (100.0)	10.5±1.5
SAEIA	Intra-assay	Low (0.01)	3.5
		Moderate (1.0)	3.6
		High (100.0)	5.7
	Inter-assay	Low (0.01)	4.4±0.8
		Moderate (1.0)	5.1±2.0
		High (100.0)	9.2±3.1

a) Pinellia tuber.

TABLE II. Cross-Reaction Values at B/B_0 50% of Several Chinese Crude Drugs and Their Extracts, Together with the Extracted Residue of Pinellia Tuber Using CELISA and SAEIA Methods

System C.T.M.[a]	CELISA		SAEIA	
	Whole[b]	Extract[c]	Whole	Extract
	Cross-reactivity (%)			
Pinellia tuber	100	0.75	0.37	100
Reside[d]	32.5	—	<0.01	—
Arisaema erubenscens	0.32	<0.01	<0.01	3.4
Pinellia cordata	0.06	<0.01	<0.01	<0.01
Glycyrrhiza	NT[e]	<0.01	NT	<0.01
Ginseng	<0.01	<0.01	<0.01	<0.01

a) Chinese traditional medicine. *b*) Whole of Chinese crude drug. *c*) The extract of Chinese crude drug. *d*) A dried residue prepared from 1 g of powdered Pinellia tuber after extraction with 4 ml of boiling water for 3 h. *e*) Not tested.

titer increased gradually and reached a maximum two weeks after the final booster. Judging from the binding value, a 10000 times diluted solution of the highest titered serum was chosen for the assay. Use of preimmunization serum showed little non-specific binding to the solid-phase Pinellia tuber.

CELISA for Pinellia Tuber After extensive trials, the optimall conditions of CELISA for powdered Pinellia tuber were established. Using the corresponding optimal conditions, the dose response curves of powdered Pinellia tuber and the extract of Pinellia tuber were measured by CELISA and the results were shown in Fig. 2. The dose response curves of SAEIA for the extract of Pinellia tuber and powdered Penellia tuber are shown in Fig. 3.

Assay Precision Intra- and inter-assay variations of the CELISA for the solid Pinellia tuber using the samples of high, moderate and low concentrations determined in multiple replicates are shown in Table I. The results are expressed as mean and standard deviation with the coefficient of variations for these samples. Similar experiments were performed with the SAEIA method for the

extract of Pinellia tuber and the results are also shown in Table I.

Specificity of the CELISA Method The specificities of the CELISA for solid Pinellia tuber was established by measuring Chinese crude drugs such as Arisaema erubenscens and Pinellia cordata, the same family members of Pinellia tuber, and Ginseng and Glycyrrhiza as well as their individual extract. Cross-reaction values were calculated from their dose response curves according to the method of Abraham[8] (Table II). For comparison, their cross-reaction values were also determined by the SAEIA for the extract of Pinellia tuber and the results are also summarized in Table II.

The CELISA clearly recognize the solid Pinellia tuber but not its aqueous extract. Chinese crude drugs, Ginseng and Glycyrrhiza showed extremely low cross-reactions. The values of Arisaema erubenscens and Pinellia cordata were also low (0.32% and $<0.06\%$, respectively) and of the extract of Pinellia tuber showed less than 0.75%. The SAEIA only recognized the extract of Pinellia tuber.

Discussion

A new immunoassay for a solid Pinellia tuber was studied. It was found that an antiserum specific for Pinellia tuber was elicited in two rabbits using powdered Pinellia tuber as the immunogen (Fig. 1). A new enzyme immunoassay of a CELISA type allowing the measurement of a solid Pinellia tuber has been developed using rabbit anti-PT antiserum, powdered Pinellia tuber-coated immunoplate as the solid-phase antigen, and GAL-labeled goat anti-rabbit IgG as a tracer. After extensive trials optimal conditions for the assay were established. The measuring range of the assay for the solid tuber was 0.1 to 1000 μg/ml.

This assay is specific for solid Pinellia tuber and similar Chinese crude drugs, Arisaema erubenscens and Pinellia cordata showed less than 0.32% cross-reaction values, and the different drugs such as Ginseng and Glycyrrhiza possessed values less than 0.01%. The value of the extract of Pinellia tuber was only 0.75%, and those of the extracts of other Chinese crude drugs were less than 0.01% (Table II).

As reported in the recent communication,[5] a specific assay method, SAEIA, for the extract of Pinellia tuber was developed. The working range and precision of the SAEIA and the CELISA methods for their corresponding antigens were compared and similar results were obtained (Figs. 2 and 3, Table I).

The specificities of both methods were compared using several solid Chinese crude drugs, such as Ginseng, Glycyrrhiza, Arisaema erubenscens, Pinellia cordata and Pinellia tuber, as well as the aqueous extract of each drug (Table II). The CELISA method clearly recognized solid Pinellia tuber while the SAEIA method showed less than a 0.37% cross-reaction. However, the CELISA showed a low cross-reaction value to its extract. Both methods used the same anti-PT serum and the tracer of GAL-labeled goat anti-rabbit IgG as the immunological reagents. The difference between the assay systems of the CELISA for a solid Pinellia tuber and of the SAEIA for the extract of Pinellia tuber is the solid-phase antigens used. Powdered Pinellia tuber was used for the antigen of the former method, while the extract of Pinellia tuber was used for the later. The assay results of SAEIA for the extract of Pinellia tuber and of CELISA for solid Pinellia tuber were completely different.

The solid-phase antigens for the CELISA and SAEIA were prepared from the same concentration of antigens, 10 μg/ml, of either powdered Pinellia tuber fragments or the extract of Pinellia tuber. The B_0 values of both assay methods, which represent amounts of bound rabbit IgG to the corresponding solid-phase antigens, against an aliquot of anti-PT were measured by using GAL-labeled goat anti-rabbit IgG as the tracer. The B_0 values of the CELISA and the SAEIA were quite different: the B_0 values for the CELISA were almost twice that of the SAEIA (data not shown). Judging from the evidence, it was assumed that anti-PT serum must consist of two groups of antibodies. The antibodies belonging to one group should be specific for the epitopes contained in the aqueous extract of Pinellia tuber. The antibodies participating in the other group would be specific for the epitopes contained in insoluble fragments of Pinellia tuber. It was also assumed that the total immunologically reactive amount of the former members may be smaller than that of the latter members calculating from the B_0 values.

The difference in the assay principles of these enzyme immunoassays (EIAs) are as follows: rabbit anti-PT serum should contain a certain population of various antibodies specific for the corresponding epitopes on the surface or the internal epitopes of Pinellia tuber. When the extract of Pinellia tuber was used as the solid-phase antigen, only the selected antibodies specific for the epitopes contained in the extract would bind to the solid-phase antigen, but other populations of antibodies specific for other epitopes of Pinellia tuber should not react. In fact, the amount of the extract of the tuber was successfully measured by following the competition of the selected antibodies against free (measuring) and the solid-phase antigens, using the SAEIA for the extract of the tuber.

When powdered fragments of Pinellia tuber were used as the solid-phase antigen, competition could be expected between the solid-phase antigen and the solid tuber against a limited amount of anit-PT antibodies. In fact, the antibodies specific for the epitopes contained in insoluble fragments of the tuber were assumed to play a predominant role in the immunological equilibrium of the CELISA, judging from the evidence that the dose response of CELISA was the most sensitive to the amount of solid Pinellia tuber. However, the CELISA possessed a low sensitivity to the extract of the tuber. Consequently, the dose response curve of a free antigen was quite different depending on the assay system used (Figs. 2 and 3, and Tables I and II).

In the classical radio- and enzyme-immunoassays, only the equilibrium state between the antibody and the antigen was followed by using a tracer to make a quantitative measurement of either antigen or antibody. In the present paper, we report interesting evidence caused by a new principle of the selection of a tracing reaction in immunoassay. Using the solid Pinellia tuber-loaded palate as the tracing antigen, a new method for the immunoassay of a solid Chinese crude drug was developed.

Since most solid materials have been outside the scope of immunoassay, this technology will be a useful means for various immunoassays of solid materials, though additional

detailed and systematic studies would be required for what epitopes were determined to get the quantitative measurement of a solid antigen.

As far as we know, this would be the first report in which completely different antigens were separately determined using the same antiserum.

References

1) A. P. Colwich and N. O. Kaplan (eds.), "Methods in Enzymology," Vol. 73, Academic Press, New York, 1982, pp. 1—691.
2) T. Chard, "An Introduction to Radioimmunoassay and Related Techniques," 3rd revised ed., Elsevier, Amsterdam, 1986, pp. 1—270.
3) H. V. Bergmyer (ed.), "Methods of Enzymatic Analysis," Vol. 12, VCH Publishers, Weinheim, 1986, pp. 1—490.
4) B. A. Morris, M. N. Clifford, and R. Jackman (eds.), "Immunoassay for Veterinary and Food Analysis-1," Elsevier Applied Science, London, 1987, pp. 1—373.
5) T. Kitagawa, H. Tanimori, Y. Kohno, A. Akahori, S. Sonoi, and S. Kondo, *Chem. Pharm. Bull.*, **37**, 1134 (1989).
6) H. Tanimori, F. Ishikawa, and T. Kitagawa, *J. Immunol. Methods*, **62**, 123 (1983).
7) H. Tanimori, S. Takaoka, N. Ishii, T. Koji, F. Ishikawa, and T. Kitagawa, *J. Immunol. Methods*, **83**, 327 (1985).
8) G. E. Abraham, *J. Clin. Endcrinol. Metab.*, **29**, 866 (1969).

New Polyphenolic 5′-Nucleotidase Inhibitors Isolated from the Wine Grape "Koshu" and Their Biological Effects

Toshikatsu TOUKAIRIN,[a] Keijiro UCHINO,*,[a] Masaya IWAMOTO,[a] Sachie MURAKAMI,[a] Takatoshi TATEBAYASHI,[a] Hiroshi OGAWARA,[b] and Yasuhiro TONOSAKI[a]

Central Laboratory, Nippon Flour Mills Co., Ltd.,[a] 2114–2, Nurumizu, Atsugi, Kanagawa 243, Japan and Department of Biochemistry, Meiji College of Pharmacy,[b] Nozawa-1, Setagaya-ku, Tokyo 154, Japan. *Received October 29, 1990*

New 5′-nucleotidase inhibitors designated as NPF-88BU-IA, NPF-88BU-IB, NPF-88BU-IIA and NPF-88BU-IIB, respectively, were isolated from the seeds and skin of the wine grape "Koshu". They were purified by solvent extraction, dialysis, and reversed-phase high performance liquid chromatography (HPLC). Their physico-chemical properties revealed these compounds to be polyphenolic substances. The average relative molecular masses of the four were estimated by gel permeation chromatography (GPC) analysis to be 7850, 5950, 11900, and 11300, respectively. They strongly inhibited 5′-nucleotidase activities from snake venom and rat liver membrane, and displayed significant therapeutic activity against Ehrlich ascites carcinoma. They also showed inhibitory effects on the growth of *Streptococcus mutans* MT8148(c), a primary cariogenic bacterium. Furthermore, these 5′-nucleotidase inhibitors inhibited the glucan formation from sucrose. These results suggest that the 5′-nucleotidase inhibitors can prevent the cause of *caries* of tooth.

Keywords 5′-nucleotidase inhibitor; polyphenol; tumor; *Streptococcus mutans*; grape; membrane

In the course of screening for new types of 5′-nucleotidase inhibitors, we found new compounds designated nucleoticidin,[1] melanocidin A, and melanocidin B[2] from microbial metabolites. These compounds were polysaccharides and showed antitumor activity.[3] We further screened 5′-nucleotidase inhibitors and isolated NPF-86IA, NPF-86IB, NPF-86IIA and NPF-86IIB from plant origin.[4] These inhibitors were polyphenolic substances and showed antitumor activity.[5] They also showed inhibitory effects on the growth of *Streptococcus mutans*, a primary cariogenic bacterium.[6] Here, we report other 5′-nucleotidase inhibitors designated NPF-88BU-IA, NPF-88BU-IB, NPF-88BU-IIA, and NPF-88BU-IIB, which were obtained from the seeds and skin of the wine grape "Koshu".

Materials and Methods

General Ultraviolet (UV) spectra were determined by a Shimadzu UV-160 spectrophotometer. Infrared (IR) spectra were obtained using a Jasco IR-810 spectrophotometer (KBr pellets).

Materials The seeds and the skin of the wine grape "Koshu" were kindly provided by Sainte Neige Wine Co., Ltd. (Yamanashi, Japan). ASF medium 104 was purchased from Ajinomoto Co., Inc. Cellulose tube (spectra/pore 6: molecular mass cut off: 1000 and 10000) was obtained from Spectral Medical Industries Inc. ICR mice, propagated at Japan SLC Inc. (Hamamatsu, Japan) were used.

Assay of 5′-Nucleotidase Inhibitors 5′-Nucleotidase and its inhibitory activities were determined by the method described previously.[1] One unit was defined as the reciprocal of the concentration, expressed as mg/ml, which showed 50% inhibitory activity.

High Performance Liquid Chromatography (HPLC) A Jasco Tri rotar-V system equipped with a model ML-611 auto injector (Jasco), Uvidec-100-VI detector (Jasco), and a Shodex RSpak DE-613 column (6 mm × 15 cm) (Showa Denko K.K.) was used with detection by UV at 280 nm. The flow rate was 1 ml/min. The mobile phase was 90% aqueous MeOH.

Gel Permeation Chromatography (GPC) New 5′-nucleotidase inhibitors were chromatographed on a Waters gel permeation chromatograph fitted with RI-8000 refractive index detector (Tosoh), and TSK-GEL ($G4000H_{XL}$, $G3000H_{XL}$, $G2000H_{XL}$) columns. The mobile phase was *N,N*-dimethylformamide at a flow rate of 0.5 ml/min.

Isolation Fresh skin and seeds (1 kg) of grape were defatted with hexane and dried at room temperature. The washed materials were extracted with ethyl acetate, and the residue was extracted with methanol under reflux. The methanol extracts were concentrated to dryness *in vacuo* below 40 °C. The residue was dissolved in a small volume of water and insoluble material was removed by filtration. The filtrate was dialyzed in cellulose tube (spectra/pore 6: molecular mass cut off: 1000) against distilled water for 6 d. The inner solution was further dialyzed in cellulose tube (spectra/pore 6: molecular mass cut off: 10000). The outer (NPF-88BU-I) and the inner (NPF-88BU-II) solutions were lyophilized separately; when subjected to HPLC, their chromatograms indicated that each consisted of two components (Fig. 1). Then, these components were isolated by repeated HPLC from NPF-88BU-I (185 mg): 17 mg of NPF-88BU-IA (6.7×10^2 units/mg) and 77 mg of NPF-88BU-IB (5.6×10^2 units/mg). In the same manner, 48 mg of NPF-88BU-IIA (10×10^2 units/mg) and 75 mg of NPF-88BU-IIB (8.3×10^2 units/mg) were obtained from NPF-88BU-II (200 mg).

Tumor Cells Line and Cell Culture Ehrlich ascites carcinoma was maintained by weekly intraperitoneal injection in ICR mice. For studies on the chemotherapeutic effects of 5′-nucleotidase inhibitors, Ehrlich carcinoma cells (1×10^5 cells) were inoculated intraperitoneally into 6-week old ICR male mice. The inhibitors were administered intraperitoneally from 24 h after the tumor inoculation. Each group consisted of 10 animals.

Ehrlich ascites carcinoma strain E cells were maintained on RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum under a humidified atmosphere of 5% CO_2 in air at 37 °C. The cells (4×10^4/ml) were planted on 60 mm dishes. After incubation for 24 h, the medium was replaced by fresh medium containing various concentrations of the 5′-nucleotidase inhibitors and cultured for 1, 2, 3 or 4 d. Cell numbers were counted with a Coulter counter model ZM (Coulter Electronics, Inc. U.S.A.).

Growth Inhibitory Activity The minimum inhibitory concentrations of

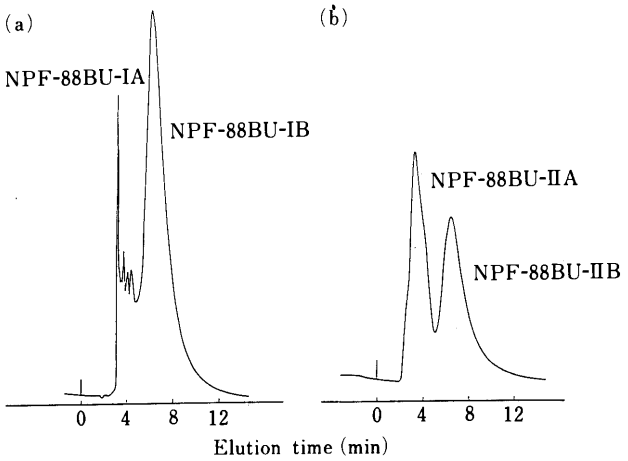


Fig. 1. HPLC Separation of NPF-88BU-I (a) and NPF-88BU-II (b)

Column, Shodex RSpak DE-613 (6 mm × 15 cm); mobile phase, 90% aqueous MeOH; flow rate, 1 ml/min; detection, UV280 nm.

the 5′-nucleotidase inhibitors for the bacterial strains used were determined by the method described previously.[6]

Glucan Synthesis by *Streptococcus mutans* The minimum inhibitory concentration of the water-insoluble glucan formation by *S. mutans* was determined by the method described previously.[6]

Results

Isolation Isolation and purification of NPF-88BU-IA, NPF-88BU-IB, NPF-88BU-IIA and NPF-88BU-IIB from the seeds and the skin of the wine grape "Koshu" were carried out as demonstrated in Fig. 2 and Table I. These inhibitors gave a single peak in HPLC, which coincided with the inhibitory activity against 5′-nucleotidase. Their physico-chemical properties are summarized in Table II. The compounds are weakly brown in color in neutral and acidic solutions, and reddish brown in alkaline solution. The elementary analysis showed the presence of carbon, hydrogen and oxygen but no nitrogen. The relative molecular masses of these inhibitors were calculated by analyzing with GPC on a column of TSK-GEL. When *N*,*N*-dimethylformamide was used as an eluant, only one major peak was observed in each of the inhibitors. The average relative molecular masses of NPF-88BU-IA, -88BU-IB, -88BU-IIA and -88BU-IIB were estimated to be 7850, 5950, 11900 and 11300, respectively, with polyethylene glycol as a standard. These compounds have a UV absorption maximum at about 280 nm, typical of polyphenol. $FeCl_3$ was positive in color reaction, but ninhidrin, *p*-anisidine-phthalic acid, aniline-diphenylamine and Dragendorff reactions were negative. All these compounds were soluble in water and methanol, and insoluble in other common organic solvents. Based on the above data, these inhibitors are concluded to be polyphenolic compounds. The IR absorption spectra of these compounds (Fig. 3) are similar and absorption at about 3410, 1600, 1520, 1440 and 1100 cm^{-1} confirmed the above conclusion. The detailed structure of these inhibitors has not been established because of their high molecular weight.

Table I. Purification of 5′-Nucleotidase Inhibitors

Purification step	Total weight (mg)	Total activity ($\times 10^5$ units)	Specific activity ($\times 10^2$ units/mg)	Yield (%)
Ethyl acetate extract	1050	0.2	0.2	
Methanol extract	2369	3.1	1.3	100
NPF-88BU-I	292	1.7	5.8	55
NPF-88BU-II	105	1.0	9.6	32

Table II. Physico-Chemical Properties of NPF-88BU-IA, NPF-88BU-IB NPF-88BU-IIA and NPF-88BU-IIB

	NPF-88BU-IA	NPF-88BU-IB	NPF-88BU-IIA	NPF-88BU-IIB
Molecular weight[a]	7850	5950	11900	11300
Elementary analysis (%) C:	49.63	55.66	52.75	54.24
H:	4.71	4.63	4.73	4.50
O:	35.09	39.75	39.12	38.83
N:	<0.1	<0.3	<0.3	<0.3
$UV_{max}(H_2O/nm)$	280	278	280	278

a) Standard compound: polyethylene glycol.

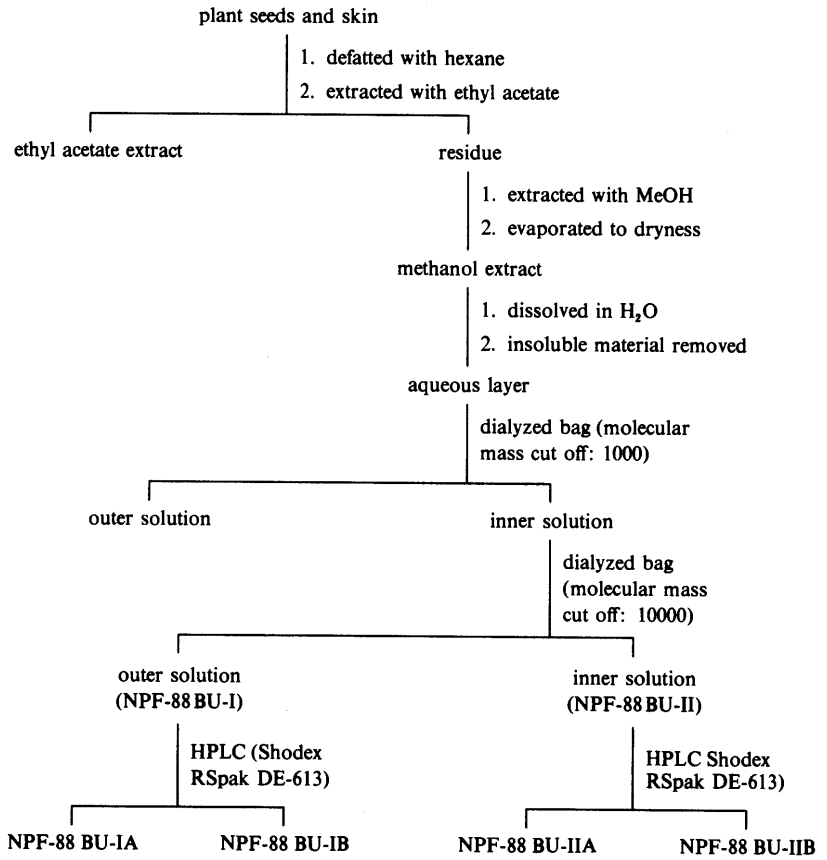


Fig. 2. Extraction Procedure for 5′-Nucleotidase Inhibitors from the Plant Seeds and Skin of the Wine Grape "Koshu"

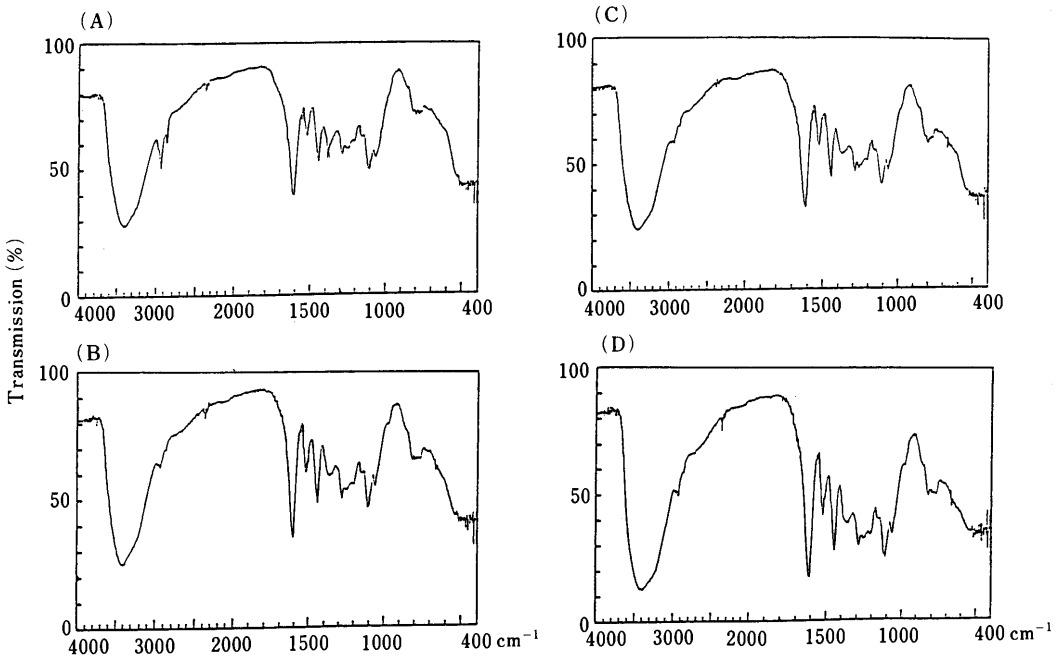


Fig. 3. IR Absorption Spectra of NPF-88BU-IA(A), NPF-88BU-IB (B), NPF-88BU-IIA (C) and NPF-88BU-IIB (D) in KBr Disk

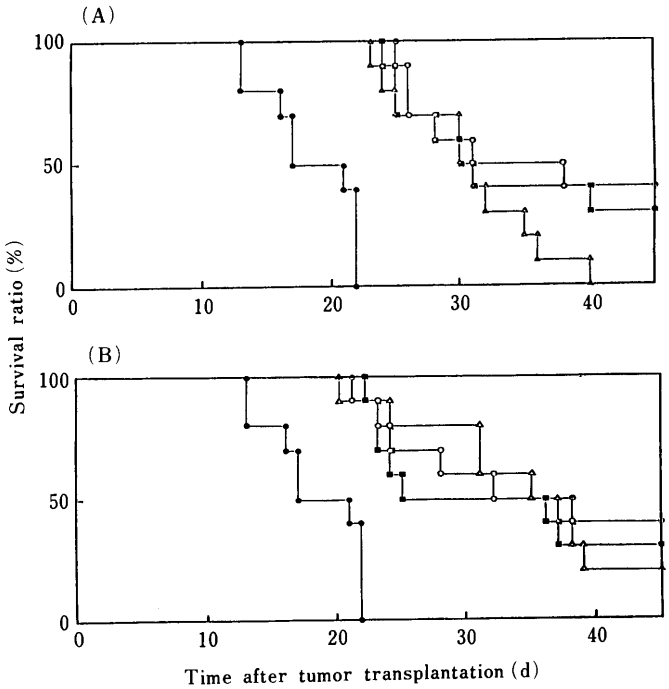


Fig. 4. Life Prolongation Effect of NPF-88BU-I (A) and NPF-88BU-II (B) on Ehrlich Ascites Tumor Bearing Mice

●, control; ○, (day 1—10) 10 mg/kg × 10, i.p.; △, (day 1—10) 20 mg/kg × 10, i.p.; ■, (day 1—10) 30 mg/kg × 10, i.p.

5′-Nucleotidase Inhibition NPF-88BU-I, -88BU-II, -88BU-IA, -88BU-IB, -88BU-IIA and -88BU-IIB inhibited 5′-nucleotidase activity from snake venom (*Crotalus atrox*, Sigma) (Table III). Catechin, a kind of polyphenol did not show any inhibitory activity on 5′-nucleotidase at 200 μg/ml. NPF-88BU-I and NPF-88BU-II also inhibited 5′-nucleotidase activity from rat liver membrane obtained by the procedure of Nakamura,[7] and their IC_{50} was determined to be 10 and 9.8 μg/ml, respectively.

Table III. Effects of NPF-88BU-IA, NPF-88BU-IB, NPF-88BU-IIA and NPF-88BU-IIB on 5′-Nucleotidase from Snake Venom

Compound	IC_{50} (μg/ml)
NPF-88BU-IA	1.5
NPF-88BU-IB	1.8
NPF-88BU-IIA	1.0
NPF-88BU-IIB	1.2
(+)-Catechin	>200

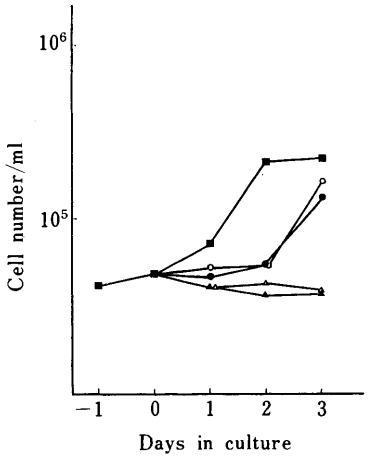


Fig. 5. Effects of NPF-88BU-I and NPF-88BU-II on Growth of Ehrlich Ascites Carcinoma Strain E Cells

■, control; ●, NPF-88BU-I 50 μg/ml; ▲, NPF-88BU-I 100 μg/ml; ○, NPF-88BU-II 50 μg/ml; △, NPF-88BU-II 100 μg/ml.

Anti-tumor Activity NPF-88BU-I and NPF-88BU-II were examined for their antitumor activity against Ehrlich ascites tumor in mice. As shown in Fig. 4, the survival period of mice was prolonged by administration of these inhibitors at a dose range of 10—30 mg/kg × 10 d.

The *in vitro* cytotoxic study using Ehrlich ascites carcinoma strain E cells revealed that neither NPF-88BU-I

TABLE IV. Minimum Growth Inhibitory Concentrations of NPF-88BU-I and NPF-88BU-II for Growth of Various Microorganisms (μg/ml)

Microorganism	NPF-88BU-I	NPF-88BU-II	(+)-Catechin
Streptococcus mutans MT8148 (c)	6.25	3.13	>200
Streptococcus mutans MT6715 (g)	200	200	>200
Streptococcus faecium ATCC 8043	12.5	12.5	—
Staphylococcus aureus ATCC 6538P	12.5	6.25	—
Bacillus subtilis ATCC 6633	50	50	—
Proteus mirabilis ATCC 9921	>200	>200	—
Proteus vulgaris ATCC 6380	>200	>200	—
Escherichia coli K-12 W3630	>200	>200	—
Candida albicans ATCC E10231	>200	>200	—
Saccharomyces cerevisiae ATCC 4226	>200	>200	—

—, not tested.

TABLE V. Minimum Inhibitory Concentration of the Water-Insoluble Glucan Formation by *Streptococcus mutans* (μg/ml)

Compound	*S. mutans* MT8148 (c)	*S. mutans* MT6715 (g)
NPF-88BU-I	100	200
NPF-88BU-II	100	200
(+)-Catechin	>400	>400

nor NPF-88BU-II showed severe cytotoxicity (Fig. 5).

Growth Inhibitory Activity The minimum inhibitory concentrations of NPF-88BU-I and NPF-88BU-II for the bacterial strains on ASF medium 104 supplemented with 1% glucose are shown in Table IV. The inhibitors demonstrated the growth inhibitory activity against *Streptococcus mutans* MT8148(c) and *S. mutans* MT6715(g) at the concentration of 3.13 and 200 μg/ml, respectively. These inhibitors also moderately inhibited the growth of other gram-positive bacteria, but did not inhibit the growth of gram-negative bacteria or fungi at 200 μg/ml.

Glucan Synthesis by *Streptococcus mutans* Inhibitory effect against glucan synthesis was examined in ASF medium 104 supplemented with 1% glucose and 5% sucrose. NPF-88BU-I and NPF-88BU-II moderately inhibited the formation of water-insolubel glucan by *S. mutans* at concentrations of 100 to 200 μg/ml (Table V).

Discussion

In the course of a survey of new types of 5′-nucleotidase inhibitors, we have screened various plant materials. The novel inhibitors NF-86I and NF-86II were isolated[4] from the seeds of *Areca catechu* L. and determined from their physico-chemical properties to be polyphenolic substances. In a previous paper,[6] we found that NF-86I amd NF-86II showed growth inhibition of *Streptococcus mutans*, and inhibited the synthesis of water-insoluble glucan from sucrose. This paper reports the isolation and characterization of other 5′-nucleotidase inhibitors, NPF-88BU-IA, -88BU-IB, -88BU-IIA and -88BU-IIB from the seeds and skin of the grape "Koshu". "Koshu", a species of grape produced in Japan, is cultivated for the production of wine. These inhibitors are polyphenolic compounds and strongly inhibit 5′-nucleotidase from snake venom and rat liver membrane.

As inhibitors of membrane-bound enzymes such as 5′-nucleotidase,[3,5] aminopeptidase and alkaline phosphatase[8] show antitumor and immunopotentiator activities, we examined the antitumor activity. The inhibitors displayed significant therapeutic activity by intraperitoneal administration against intraperitoneally implanted Ehrlich ascites carcinoma. The *in vitro* studies using Ehrlich ascites carcinoma strain E cells revealed that the inhibitors did not show severe cytotoxicity. It is considered that they showed antitumor activity against experimental murine tumors in association with modification of the immune system.

Next, we examined the growth inhibition of *Streptococcus mutans* and the inhibitory effect against water-insoluble glucan formation. The adherence of cariogenic *S. mutans* to the tooth surface may in part be mediated by insoluble glucan synthesized from sucrose. *Streptococcus mutans* MT8148(c) is mainly distributed in the oral cavity of human and the potential for glucan formation from sucrose by this bacterium is greater than *S. mutans* MT6715(g). The 5′-nucleotidase inhibitors isolated from the wine grape "Koshu" showed potent growth inhibitory activity toward *S. mutans* MT8148(c) at the concentration of 3.13 to 6.25 μg/ml, and inhibited the water-insoluble glucan formation by *S. mutans* MT8148(c) at the concentration of 100 μg/ml.

We recently isolated polyphenolic 5′-nucleotidase inhibitors from the seed of *Areca catechu* L.[4]; their physico-chemical properties are similar to those of inhibitors from "Koshu". Inhibitors from *Areca catechu* L. inhibited the growth of *S. mutans* MT8148(c) at the concentration of 6.25 to 12.5 μg/ml, and inhibited the water-insoluble glucan formation by *S. mutans* MT8148(c) at the concentration of 400 μg/ml.[6] The 5′-nucleotidase inhibitors isolated from the wine grape "Koshu" thus show more potent anti-plaque preventing agent than those from *Areca catechu* L.

The results obtained in the present study showed that the polyphenolic 5′-nucleotidase inhibitors isolated from "Koshu" wine grapes possessed an antitumor effect and an inhibitory effect against insoluble glucan formation by *S. mutants*.

References and Notes

1) H. Ogawara, K. Uchino, T. Akiyama and S. Watanabe, *J. Antibiot.*, **38**, 153 (1985).
2) H. Ogawara, K. Uchino, T. Akiyama and S. Watanabe, *J. Antibiot.*, **38**, 587 (1985).
3) K. Uchino, H. Ogawara, T. Akiyama, S. Watanabe, and A. Fukuchi, *J. Antibiot.*, **38**, 682 (1985).
4) K. Uchino, T. Matsuo, M. Iwamoto, Y. Tonosaki and A. Fukuchi, *Planta Medica*, **54**, 419 (1988).
5) M. Iwamoto, T. Matsuo, K. Uchino, Y. Tonosaki and A. Fukuchi, *Planta Medica*, **54**, 422 (1988).
6) M. Iwamoto, K. Uchino, T. Toukairin, K. Kawaguchi, T. Tatebayashi, H. Ogawara and Y. Tonosaki, *Chem. Pharm. Bull.*, **39**, 1323 (1991).
7) S. Nakamura, *Biochim. Biophys. Acta*, **426**, 339 (1976).
8) H. Umezawa, "Small Molecular Immunomodifiers of Microbial Origin," Jpn. Sci. Soc. Press, Pergamon Press, Tokyo, 1981.

Isolation and Physiological Activity of the Chitosan from Conidia and Mycelia of *Mycosphaerella pinodes*[1)]

Machiko MATSUBARA* and Hisatora KURODA

Kobe Women's College of Pharmacy, Motoyama-Kita-machi, Higashinada-ku, Kobe 658, Japan. *Received October 29, 1990*

Chitin- and chitosan-like substances were isolated from mycelia and conidia of *Mycosphaerella* (*M.*) *pinodes*, a plant pathogen of peas causing brown spots. The elicitor activities of these substances were compared with those of commercially available crustacean chitin and chitosan or several neutral polysaccharides. Only low activities were detected in the chitin-like substances of this microbe and in the authentic chitin commercially obtained. Although the activity was not detectable in chitosan oligomers whose degree of polymerization was less than 6, the activity which appeared in the chitosan hexamer and the octamer had almost the same activity as the commercially available chitosan. All of the authentic neutral linear polysaccharides tested were inactive. After having applied *M. pinodes* suspension on pea leaves, activities of both chitinase and chitosanase increased quickly. Fluorescein isothiocyanate (FITC)-labelled chitosan was treated with a crude extract of pea leaves, and FITC-oligomers were obtained.

Keywords *Mycosphaerella pinodes*; pisatin; phytoalexin; chitosan; chitin; fungi; elicitor activity

It is generally known that some plants synthesize antimicrobial substances as a defense mechanism against invasive microorganisms. These antimicrobial substances synthesized are called phytoalexins and their inducer is called an elicitor. When peas synthesize pisatin as a phytoalexin, it is reported that several substances secreted in the germination of fungal conidia,[2-4)] compounds found in culture filtrates of fungal mycelia[5,6)] and cell wall-polysaccharides of the mycelia[4,5)] work as elicitors. We found elicitor activity on a glycoprotein in a culture filtrate of the germinating *Mycosphaerella* (*M.*) *pinodes* conidia and determined its structure as a glycosyl chain.[1)] Others reported elicitor activity on a pectic polysaccharide[7)] derived from the decomposed cell wall of host plants, and chemically derived chitosan[7,8)] or chitosan oligomer[9,10)] from crustacean chitin. However, nobody has reported that chitosan or its related compounds, supposedly widespread in the cell wall of plant-pathogenic mold fungi, could work as an elicitor for the production of pisatin, which was one of the host-parasite interactions between peas and fungi. Therefore, our strategies for this investigation were (a) to isolate substances, which were referred to as chitin and chitosan, from the cell wall of mycelia or conidia of *M. pinodes*, a pathogen of peas causing brown spots, (b) to confirm whether they were real components of the cell wall, and (c) to compare their elicitor activities on pea leaves with that of crustacean chitin or chitosan commercially obtained.

Materials and Methods

Microorganism The strain of the fungus, *M. pinodes*, and the harvesting procedure of the conidia were described in the previous report.[1)] A conidia suspension (10 ml) was inoculated in 250 ml of a modified Czapek solution medium containing 30 g glucose, 5 g pepton, 2 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 6 mg thiamine mononitrate, 2 mg pyridoxin–HCl, 2 mg riboflavin and 3 mg calcium pantothenate in 1 l of distilled water. Cultivation was carried out for 5 d at 26 °C. For harvesting the mycelia, the culture broth was filtered through a nylon sieve and the residue was rinsed in running water for one day until the passed water turned sugar-free according to the phenol–sulfuric acid method. The mycelia thus obtained were lyophilized.

Preparation of Cell Wall The lyophilized mycelia (3 g) or conidia (5 g) of *M. pinodes* were suspended in 60 ml of a 1% NaCl solution and disrupted 5 times with a French press (1000—1200 kg/cm^2). The sample was rinsed once in about 4 l of a 1% NaCl solution and further in about 4 l of distilled water until the washings became sugar-free as detected by the phenol–sulfuric acid method. Complete removal of cell contents from the washed precipitate was verified by microscopic observation after staining with lactophenol cotton blue reagent. Fractions of the cell wall thus prepared were lyophilized.

Preparation of Fractions Refered to as Chitin and Chitosan We prepared 2 fractions corresponding to chitin and chitosan from the cell wall of mycelia or conidia using the method of Garcia[11)] *et al.*, as shown in Chart 1.

Chromatography These two fractions, corresponding to chitin and chitosan, were hydrolyzed in 6 N HCl in a sealed tube at 100 °C for 6 h. The lysates were analyzed by paper, thin layer and column chromatographies, as shown in i—iii). Pisatin in the lysate was quantified by high performance liquid chromatography (HPLC) under the condition described in iv). i) Paper chromatography (PPC): filter paper, Whatman No. 1; solvent system, water-saturated phenol; detection, ninhydrin. ii) Thin layer chromatography (TLC): plate, silica gel; solvent system, *n*-BuOH–AcOH–H_2O (25 : 6 : 25, v/v); detection, ninhydrin or 50% H_2SO_4. The plates were heated at 105 °C for 10 min or at 130 °C for 30 min after spraying with ninhydrin or 50% H_2SO_4. iii) Column chromatography: Glucosamine was eluted in one peak by using a Hitachi 835-30 amino acid analyzer under the following conditions: column, 2.6 × 250 mm; resin, Hitachi 2619; initial temperature, 30 °C; final temperature, 68 °C; initial buffer, 0.155 N lithium citrate (pH 3.0); final buffer, 1.20 N lithium citrate (pH 7.0); flow speed, 0.275 ml/min. iv) HPLC: The content of pisatin was determined with a Shimadzu LC-3A instrument; column, Lichrospher Si 100, 4 × 250 mm; temperature 30 °C; solvent systems, *n*-hexane–tetrahydrofuran (THF), 88-9; flow speed, 2 ml/min; detection, 307 nm.

X-Ray Diffraction and Infrared (IR) Absorption Analysis An IR absorption spectrum of the sample was analyzed in a KBr tablet. X-Ray diffraction analysis was carried out in an apparatus (Rigaku Denki, Japan) with CuK_α radiation filtered through iron under conditions of 45 kV and 7 or 160 mA.

Elicitor Activity We measured elicitor activities (μmol pisatin/cm^2 leaf of peas) under the condition shown in Chart 2.

Assay of Enzymatic Activities Pea leaves without epidermis on the reverse sides were put in a suspension containing *M. pinodes* at 26 °C for a certain time. Then, the leaves were rinsed in distilled water and the water drops on the leaves were wiped off. Other leaves were put in distilled water instead of the suspension as a control. The treated leaves (2 g) were ground with 1 ml of a buffer (described below) and 1 g of glass beads in a mortar cooled on an ice-cold water bath. A crude enzyme solution of the leaves was obtained by taking the supernatant after centrifugation of the homogenate at 700 rpm at 4 °C for 10 min. Protein concentration in the enzyme solution was determined using the Lowry method.[12)]

i) β-Glucanase: The buffer used for the extraction of enzymes was an 0.05 M acetate buffer (pH 5.25). The crude enzyme solution (0.5 ml) was reacted with 0.5 ml of a laminarin solution in a 0.05 M acetate buffer (5 mg/ml) at 37 °C for 1 h. The reaction was stopped by keeping the sample in boiling water for 2 min. The sample was centrifuged at 3000 rpm and the supernatant was collected for β-glucanase assay. β-Glucanase activity was determined by evaluating released glucose in the supernatant

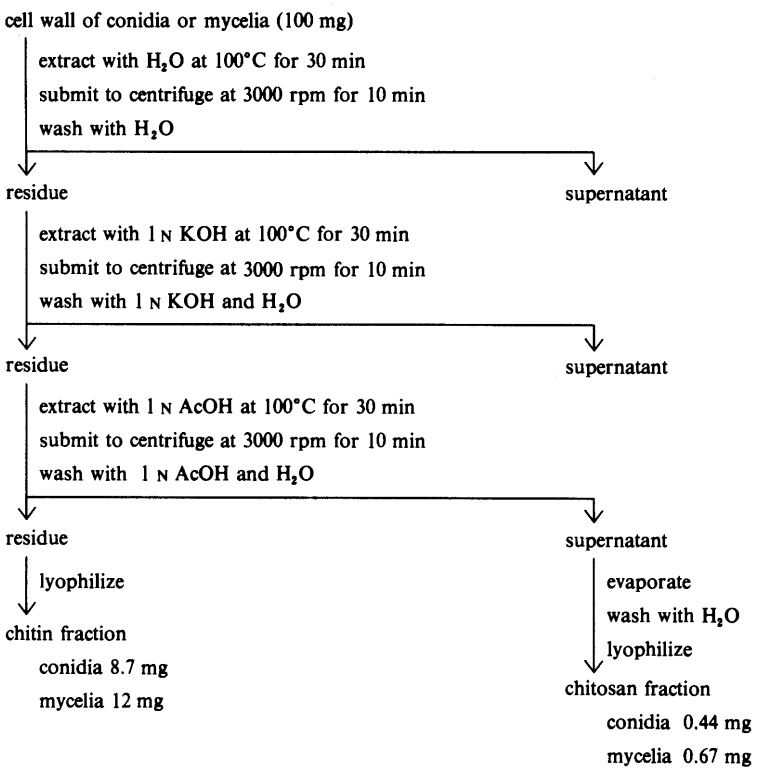


Chart 1. Fractions of Cell Wall from *M. pinodes*

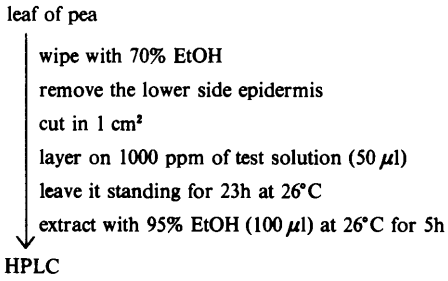


Chart 2. Method of Bioassay for Elicitor Activity

with a glucose oxidase–peroxidase method kit (Yatron), reading absorbances at 580 nm.

ii) Chitinase: The method of Boller[13] *et al.* was applied with some modification. The buffer used to extract enzymes was a 0.1 M citrate buffer (pH 5.0). The crude enzyme extract (0.3 ml) was mixed with 0.7 ml of a 0.6 μM sodium azide, and 2 mg of chitin was added and suspended. After keeping the mixture at 37 °C for 17 h, 0.1 ml of a 0.8 M sodium acetate buffer (pH 9.1) was added to stop the reaction. The sample was centrifuged at 2100 rpm (1000 × *g*) for 5 min and the supernatant was taken for *exo*- and *endo*-chitinase assay. We assayed the amount of released *N*-acetylglucosamine in the supernatant using the Reissig method by reading absorbances at 544 nm to determine *exo*-chitinase activity. Snail gut enzyme (40 μl, Helicase, obtained from *Lactobacillus bulgaricus* factor (IBF)) and 60 μl of a 1 M potassium phosphate buffer (pH 7.1) was added to 0.6 ml of the supernatant and kept at 37 °C for 2 h. After adding 0.1 ml of a 0.8 M sodium borate buffer (pH 9.1), the sample was centrifuged at 2100 rpm for 5 min. Released *N*-acetylglucosamine in the supernatant was assayed as described above.

iii) Chitosanase: The buffer used for extraction of the enzymes was a 0.1 M citrate buffer (pH 5.0). The crude enzyme solution (0.3 ml) was mixed with 0.7 ml of a buffer of 0.6 μM sodium nitrite in 40 mM citrate (pH 5.0) and 2 mg of chitosan. The mixture was reacted as described in ii). We assayed the amount of released glucosamine in the supernatant using the Brix method by reading absorbances at 530 nm to determine chitosanase activity.

Fluorescence Labelling of Chitosan Fluorescein isothiocyanate isomer I (FITC, 15 μg, Sigma) was dissolved in 50 μl of a 0.1 M carbonate buffer (pH 9.0) just before use. Chitosan (1 mg) was suspended in a 0.1 M carbonate buffer (pH 9.0) in a test tube and kept on an ice-cold water bath. Stirring slowly, 50 μl of a FITC solution was added to the suspension. The tube was sealed when the pH was stable for 15 min and stirred while cooling for 18 h in the dark. After the reaction, excess FITC was washed out with the 0.1 M carbonate buffer (pH 9.0) followed by hot ethanol, before lyophilizing the residue. The absorbance of 1500—1600 cm^{-1} (S=C<) in its IR spectrum revealed that only a small part of chitosan molecule was fluorescence-labelled.

Detection of Chitosan Oligomer The substrate, FITC-labelled chitosan, was reacted for 30 min as described in the measurement of activities of chitosanase. The reaction mixture was applied to a column with Sephadex G-50 (2 × 42 cm), and 5 ml-fractions were collected. Fluorescence intensity of the fractions was measured at 492 nm in excitation and at 520 nm in emission with the Shimadzu digital fluorometer RF-510.

Reduction of Chitosan Chitosan (2 mg) was reduced in 500 μl of distilled water and 500 μl of a 0.22 M $NaBH_4$ solution at 26 °C for 17 h according to Lehnhardt *et al.*[14] Excess $NaBH_4$ was removed by adding glacial acetic acid. The residue was rinsed in distilled water and lyophilized.

Determination of Molecular Weight Using dextran as the standard, the intrinsic viscosity of the sample in 50% H_2SO_4 was determined and the molecular weight was calculated by an equation of Staudinger–Sakurada–Houwink as follows: $[\eta]=KM^a$.

Reagents Chitosan dimer, tetramer and hexamer, Chitin and laminarin oligomer were commercially obtained from Seikagaku Kogyo. The heptamer was also prepared by Seikagaku Kogyo at our request. The octamer was kindly provided by Yaidzu Suisan Kagaku Kogyo.

Results and Discussion

Four fractions, which were estimated to be chitin or chitosan, were obtained from the cell walls of mycelia or conidia of *M. pinodes* with the method described in Chart 1. These fractions were hydrolysed in 6 N HCl at 100 °C for 6 h. The hydrolysates were analyzed by paper, thin layer and column chromatographies (Table I). Four hydrolysates from the fractions estimated to be chitin or chitosan from the mycelia or conidia were detected in one spot at an *Rf* value, or one peak at a retention time

TABLE I. *Rf* Values and Elution Time of Hydrolysate of Chitin and Chitosan Fractions

Fraction	*Rf* (PPC)	*Rf* (TLC)	Elution time by amino acid analyzer (min)
Chitin from conidia	0.54	0.12	60.34
Chitin from mycelia	0.54	0.12	60.69
Chitosan from conidia	0.53	0.12	60.52
Chitosan from mycelia	0.53	0.12	60.34
Authentic glucosamine	0.54	0.12	60.34

TABLE II. Elicitor Activities of Chitin and Chitosan Obtained from Conidia and Mycelia of *M. pinodes*

Compound	Accumulated pisatin (μmol/cm^2)
Chitin from crabs	0.123
Chitin-like substance from conidia	0.134
Chitin-like substance from mycelia	0.143
Chitosan from crabs	0.908
Chitosan-like substance from conidia	1.011
Chitosan-like substance from mycelia	1.224

corresponding to glucosamine. These results indicate that these four fractions mainly consist of *N*-acetylglucosamine or glucosamine. IR absorption analysis and X-ray diffraction analysis of these four fractions were carried out. Two chitin- and two chitosan-fractions from mycelia or conidia indicated similar spectra with authentic chitin and chitosan, respectively. These findings allow us to conclude that the cell walls of conidia or mycelia of *M. pinodes* contain chitin and chitosan, or their related compounds (chitin- or chitosan-like substances). The contents of the chitin-like substance in the cell walls of conidia and mycelia were 8.7 and 12%, respectively, whereas those of the chitosan-like substance were 0.44 and 0.67%, respectively. The optical rotation and the molecular weight of the chitin-like substance obtained from conidia or mycelia were $[\alpha]_D^{25}$ $-68.5°$ ($c=1$, 50% H_2SO_4) and 8.8×10^5, or $[\alpha]_D^{25}$ $-55.8°$ ($c=1$, 50% H_2SO_4) and 1.5×10^6. Those of the chitosan-like substance from conidia or mycelia were $[\alpha]_D^{25}$ $-56.0°$ ($c=1$, 1 N CH_3COOH) and 7.0×10^5, or $[\alpha]_D^{25}$ $-51.0°$ ($c=1$, 1 N CH_3COOH) and 1.1×10^6 respectively. Elicitor activities of these two chitin- or chitosan-like substances on pea leaves were determined and compared with a control (chitin or chitosan from crabs) as shown in Table II. The activity of the chitin-control was much less than that of the chitosan-control. The chitin- and chitosan-like substances indicated more elicitor activities than chitin- and chitosan-controls, respectively. Moreover, chitin- and chitosan-like substances in mycelia indicated more activities than those of conidia. The activities of chitosan-like substances, the two highest in six determinations, were about 6 times greater than of glycoprotein isolated from the culture filtrate of germinating conidia as reported previously.[1)]

It has been proven that chitin- and chitosan-like substances separated from this microbe, and some biological compounds such as β-glucan[15,16)] and glycoprotein, have elicitor activity. Accordingly, elicitor activities of other biological compounds, such as crustacean chitin, chitosan, some neutral polysaccharides and their derivatives and

TABLE III. Elicitor Activities of Carbohydrate

Compound	Accumulated pisatin (μmol/cm^2)
Chitosan	0.908
Chitosan octamer	0.832
Chitosan heptamer	0.720
Cnitosan hexamer	0.464
Chitosan tetramer	—
Chitosan dimer	—
Glucosamine	—
Reduced chitosan	0.944
FITC-chitosan	0.708
Chitin	0.123
Chitohexaose	0.085
Chitotetraose	—
Chitobiose	—
N-Acetylglucosamine	—
Glucan (from conidial cell content of *C. miyabeanus*)[14)]	0.195
Glucan (from conidial cell wall content of *C. miyabeanus*)[15)]	0.249
Glycoprotein (from conidia of *M. pinodes*)	0.152
Laminarin (β-1,3-glucan)	—
Laminariheptaose	—
Laminaripentaose	—
Laminaritriose	—
Laminaribiose	—
Cellulose (β-1,4-glucan)	—
Pustulan (β-1,6-glucan)	—
Dextran (α-1,6-glucan)	—
Agar	—
Mannan	—
Inulin (β-1,2-fructan)	—
Glucose	0.079

oligomers, were measured to compare with each other. The activity of glucose was also measured as a negative control. The results are shown in Table III, indicating an activity less than that of glucose as "—". The activity was detectable in chitosan oligomers or chitin oligomers when the degrees of polymerization of the oligomer were 6 or more. In chitosan oligomers, moreover, the more degrees of polymerization the oligomer had, the more activity was measured. Chitosan octamer, in particular, showed nearly the same high activity as chitosan. Similar results were obtained by Kendra[10)] on investigating the elicitor activity of a chitosan oligomer up to 7 degree of polymerization against pisatin. On the other hand, chitin, a derivative of chitosan whose amino groups were totally acetylated, showed much less activity than chitosan. Cellulose, a related compound to chitosan whose amino groups were totally substituted by hydroxy groups, had no detectable activity, although FITC–chitosan, whose amino groups partially remained, had a considerable activity. These results indicate that the amino groups of chitosan may play an important role in elicitor activity. No significant difference in activity was found between chitosan with or without reduced terminal glucosamine residue, and appreciable activity was found both in β-glucan, polysaccharide with side chains, obtained from the whole cell or cell wall of *C. miyabeanus*, and glycoprotein in the conidia of *M. pinodes*. However, no detectable activity was found in linear neutral polysaccharides tested or in laminarin oligomers, which may ensure that side chains are necessary for activity in neutral polysaccharides. Higher activity was obtained from

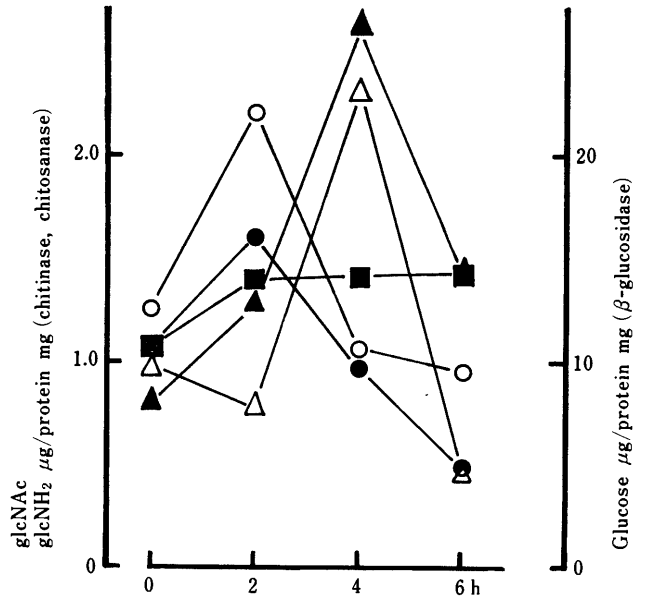


Fig. 1. Chitinase, Chitosanase and β-Glucanase Activities in Pea Leaves Kept in Contact with *M. pinodes*

▲, *exo*-chitinase; △, *endo*-chitinase; ●, *exo*-chitosanase; ○, *endo*-chitosanase; ■, β-glucanase.

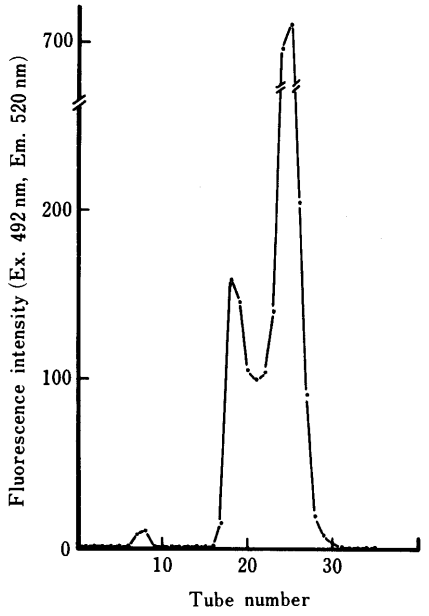


Fig. 2. Gel Filtration Pattern of Chitosanase-Cleaved FITC-Chitosan

chitosan-like substances and authentic chitosan than the others, so the mechanism of high elicitor activity was further investigated. Considering that solubilization of chitosan or chitin by enzymatic degradation may be needed for their acting as elicitors on pea leaves, the activity of enzymes in pea leaves was measured for 6 h as shown in Fig. 1. Time-courses of the activity of both *exo*- and *endo*-chitinase were almost identical. Chitosanase showed the same tendency. Both chitosanase activities increased just after application of *M. pinodes* on pea leaves with a peak after 2 h, then decreased. Both chitinase activities increased with a short time-lag with a peak after 4 h and then decreased, though the activity of glucanase was almost stable during the time period examined. Thus, the following mechanism was presumed. That is, the activity of chitosanase and chitinase increase after infection with *M. pinodes*, and these enzymes react with chitosan- and chitin-like substances, which are cell wall components of this microbe, decomposing them to smaller molecules and solubilizing them. Moreover, the activity of both chitosanases or both chitinases with chitosan- or chitin-like substances as a substrate about 5 times higher than the enzymes with authentic crustacean chitosan or chitin (data not shown). The difference between chitosan- or chitin-like substances and authentic chitosan or chitin in molecular weight or in the structure was implied by this result, as was the fact of the higher elicitor activity of the chitosan- or chitin-like substances than the authentic chitosan or chitin of crustacean (Table II). Next, FITC–chitosan as a substrate was reacted with a crude emzyme solution prepared from pea leaves at 37 °C for 30 min, followed by gel filtration through a column with Sephadex G-50 (2 × 42 cm) as shown in Fig. 2 to prove the decomposition of chitosan on pea leaves. Three peaks, a peak of the void volume, a bigger peak of the bed volume and a smaller peak corresponding to oligomer of FITC–glucosamine (about molecular weight 2500), were observed, indicating that the chitosan oligomer produced by endochitosanase was probably further decomposed by exochitosanase into smaller molecules.

Young *et al.*,[17,18] reported that chitosan increased the permeability of the cell membrane of a microbe, and Hadwiger *et al.*[8] reported that chitosan easily combined with microbial deoxyribonucleic acid (DNA). Further, phenylalanine ammonia-lyase (PAL)-enzyme and its (mRNA) accumulated in the cells of pea leaves after contact with an elicitor.[19] These results lead us to the idea that the solubilized chitosan-like substance originated in the fungal cell wall permeates into pea leaf cells to activate some genes directly. On the other hand, Yoshikawa *et al.*,[20] and Schmidt *et al.*[21] estimated a receptor, detecting a protein which combined with [^{14}C]micolaminaran and β-1,3-[^{3}H] glucan in the cell membrane fraction of cellular extract of soybeans. Considering these results, it is probable that chitosan specific receptors on the cell membrane of peas combine with chitosan first. Now, we are undertaking to detect protein in a membrane fraction of pea leaves, which combine with a chitosan-like substance according to the method described by Yoshikawa *et al.*

References

1) M. Matsubara and H. Kuroda, *Chem. Pharm. Bull.*, **35**, 249 (1987).
2) Y. Yamamoto, H. Oku, T. Shiraishi, S. Ouchi and K. Koshizawa, *J. Phytopathol.*, **117**, 136 (1986).
3) I. A. M. Cruickshank and M. M. Smith, *J. Phytopathol.*, **116**, 48 (1986).
4) P. Thanutong, H. Oku, T. Shiraishi and S. Ouchi, *Okayama Daigaku Nogakubu Gakujutsu Hokoku*, **59**, 1 (1982).
5) P. J. G. M. De Wit and P. H. M. Roseboom, *Physiol. Plant Pathol.*, **16**, 391 (1980).
6) D. L. Daniels and L. A. Hadwiger, *Physiol. Plant Pathol.*, **8**, 9 (1976).
7) M. W. Simmons, L. Hadwiger and C. A. Ryan, *Biochem. Biophys. Res. Commun.*, **110**, 194 (1983).
8) L. A. Hadwiger and J. M. Beckman, *Plant Physiol.*, **66**, 205 (1980).
9) M. W. Simmons, J. Donald, C. A. West, L. Hadwiger and C. A. Ryan, *Plant Physiol.*, **76**, 833 (1984).

10) D. F. Kendra and L. A. Hadwiger, *Exp. Mycol.*, **8**, 276 (1984).
11) S. B. Garcia and W. J. Nickerson, *Biochim. Biophys. Acta*, **58**, 102 (1962).
12) O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
13) T. Boller, A. Gehri, F. Mauch and V. Vögeli, *Planta*, **157**, 22 (1983).
14) W. F. Lehnhardt and P. J. Winzler, *J. Chromatogr.*, **34**, 471 (1968).
15) M. Matsubara and H. Kuroda, *Chem. Pharm. Bull.*, **31**, 2371 (1983).
16) M. Matsubara and H. Kuroda, *Chem. Pharm. Bull.*, **33**, 1175 (1985).
17) D. H. Young, H. Kohle and H. Kauss, *Plant Physiol.*, **70**, 1449 (1982).
18) D. H. Young and H. Kauss, *Plant Physiol.*, **73**, 698 (1983).
19) D. C. Loschke, L. A. Hadwiger and W. Wagoner, *Physiol. Plant Pathol.*, **23**, 163 (1983).
20) M. Yoshikawa, N. T. Keen and M. C. Wang, *Plant Physiol.*, **73**, 497 (1983).
21) W. E. Schmidt and J. Ebel, *Proc. Natl. Acad. Sci., U.S.A.*, **84**, 4117 (1987).

Vertebrate Collagenase Inhibitor. II.[1] Tetrapeptidyl Hydroxamic Acids

Shinjiro ODAKE,[a] Toru OKAYAMA,[a] Masami OBATA,[a] Tadanori MORIKAWA,*,[a] Shunji HATTORI,[b] Hisae HORI[b] and Yutaka NAGAI[b]

Research Institute, Fuji Chemical Industries, Ltd.,[a] 530, Chokeiji, Takaoka, Toyama 933 and Department of Tissue Physiology, Medical Research Institute, Tokyo Medical and Dental University,[b] Kanadasurugadai, Chiyoda-ku, Tokyo 101, Japan. *Received November 5, 1990*

To develop a potent and specific collagenase inhibitor, a series of tetrapeptidyl hydroxamic acids were synthesized, based on the previous findings with tripeptidyl derivatives (*Chem. Pharm. Bull.*, 38, 1007—1011, 1990). Among the series of tetrapeptidyl derivatives synthesized, R-Gly-Pro-Leu-Ala-NHOH and R-Gly-Pro-D-Leu-D-Ala-NHOH were found to be highly specific and potent inhibitors against vertebrate collagenase with an IC_{50} of 10^{-6} M order, where R stands for Boc or acyl group. Analysis of their structure-activity relationships showed a characteristic feature of the substrate-binding site of collagenase as follows: 1) the S_1 subsite forms a shallow hydrophobic pocket, although glycine residue corresponds to the subsite of the natural collagen substrate: 2) the S_2 subsite constitutes a bulky pocket with less requirement for hydrophobicity: 3) the S_3 subsite preferentially accommodates Pro residue: and 4) the accommodation of the P_4–P_1 subsites of peptidyl collagenase inhibitor to the S_4–S_1 subsites is required to form a tight binding of its hydroxamic acid moiety to the zinc ion at the catalytic site of the enzyme. The introduction of an enantiometric dipeptide unit, D-Leu-D-Ala, to the P_2–P_1 subsites demonstrated an increased binding capacity to the extended S_4–S_1 subsites of collagenase, thus providing proteinase-resistant inhibitor.

Keywords collagenase; hydroxamic acid; inhibitor; peptide synthesis; tetrapeptide

Introduction

The involvement of matrix metalloproteinases, that is, collagenase, gelatinase and stromelysin, in tissue degrading diseases such as rheumatoid arthritis and tumor metastasis has become evident recently.[2] The level of these proteinase activities is regulated in at least three different ways: 1) modulation of transcriptional levels of metalloproteinase genes by cytokines and glucocorticoids, 2) activation of the precursor forms of these enzymes which are latent and 3) inhibition of activated enzymes by tissue inhibitor of metalloproteinases or by synthetic inhibitors.[1,3]

In our previous paper,[1] we reported that tripeptidyl hydroxamic acid with the sequence of Z–Pro–Leu–Ala–NHOH showed a higher inhibitory activity against human skin fibroblast collagenase (IC_{50} value: 10^{-6} M order) than Z–Pro–Leu–Gly–NHOH (IC_{50} value: 10^{-5} M order), which was designed from the amino acid sequence of the collagenase cleavage site of type I collagen molecule and synthetic substrate (Fig. 1).[4,5]

These results strongly suggest that a potent peptidyl inhibitor for collagenase can be designed to recognize only Sn site of the enzyme molecule. This is quite a contrast to previous studies on collagenase inhibitors, which were mostly designed based on P'_n site of collagen cleavage site, –Gly–Ile(Leu)–, where collagenase was supposed to recognize Ile(Leu)residue (P'_1–S'_1 interaction)[6] (Fig. 1).

In this paper, we describe the synthesis of a series of tetrapeptidyl hydroxamic acids and assays of their inhibitory activity against vertebrate collagenase. The relationship between structure and activity of the inhibitors in terms of P*n*–S*n* interaction is also discussed.

Results and Discussion

Tetrapeptidyl hydroxamic acids were synthesized as shown in Chart 1. A Boc-derivative of amino acid was coupled with *O*-benzylhydroxylamine (NH_2-OBzl) by the dicyclohexylcarbodiimide (DCC) –*N*-hydroxybenzotriazole (HOBt) method[7] to obtain its fully protected hydroxamic

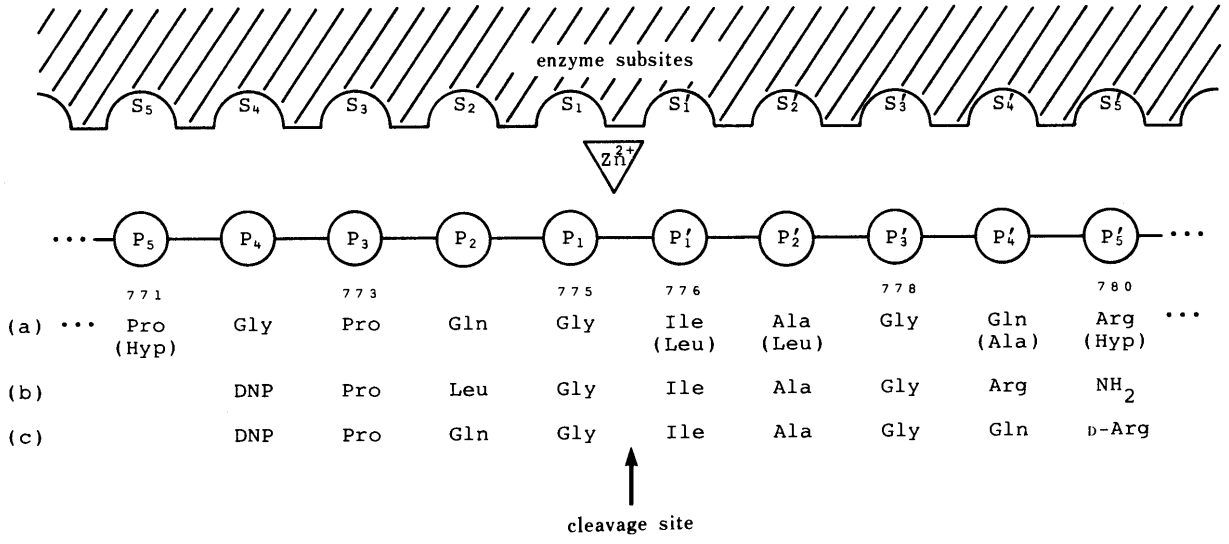


Fig. 1. Schematic Drawing Showing an Expected Interaction between Vertebrate Collagenase and the Cleavage Region of α1 and α2 (in Parentheses) Chains of Bovine Type I Collagen (a) and Synthetic Substrates (b and c) for Vertebrate Collagenase[5]

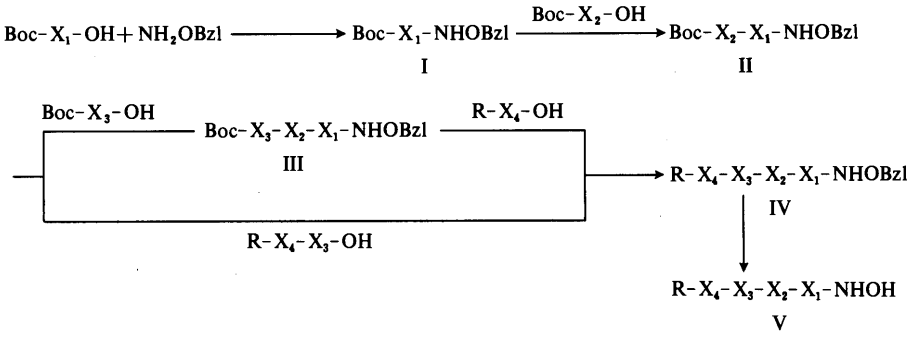


Chart 1. The Synthetic Route to Tetrapeptidyl Hydroxamic Acids

TABLE I. Inhibition (IC_{50}) of Vertebrate Collagenase and Other Enzymes by Hydroxamic Acid Derivatives of a Series of Oligopeptides

Compd. No.	Structure	Tadpole collagenase (M)	Bacterial collagenase (M)	Urease (M)	Thermolysin (M)
1	Boc–Leu–Gly–NHOH	6.8×10^{-4}	9.2×10^{-3}	1.0×10^{-4}	1.3×10^{-2}
2	Boc–Pro–Leu–Gly–NHOH	9.6×10^{-5}	2.0×10^{-2}	3.0×10^{-2}	3.2×10^{-2}
3	Boc–Gly–Pro–Leu–Gly–NHOH	3.3×10^{-5}	3.0×10^{-2}	5.5×10^{-2}	3.4×10^{-2}
4	Boc–Pro–Gly–Pro–Leu–Gly–NHOH	3.9×10^{-5}	1.6×10^{-2}	1.1×10^{-2}	1.6×10^{-2}
5	Boc–Gln–Gly–NHOH	4.1×10^{-4}	1.9×10^{-2}	9.6×10^{-3}	2.0×10^{-2}
6	Boc–Pro–Gln–Gly–NHOH	2.0×10^{-4}	7.0×10^{-2}	8.6×10^{-2}	3.3×10^{-2}
7	Boc–Gly–Pro–Gln–Gly–NHOH	5.6×10^{-5}	5.7×10^{-2}	7.5×10^{-2}	3.9×10^{-2}

acid derivative (I). Then, dipeptide derivative (II) was prepared from (I) by eliminating its Boc group, and coupling with a desired Boc–amino acid (Boc–X_2–OH; functional group(s) in its side chain was protected with Bzl or Z by the DCC–HOBt method). After synthesizing fully protected tri-(III) and then tetrapeptidyl hydroxamic acid (IV) as shown in Chart 1, its Bzl group was eliminated by treatment with hydrogen on Pd–C to give rise to tetrapeptidyl hydroxamic acid (V).

Enzyme inhibitory activities of a series of di- to tetrapeptidyl hydroxamic acids thus synthesized were assayed against tadpole collagenase[8] as reported,[9] and compared with those against clostridial collagenase (Sigma Chem. Co., St. Louis, MO), urease (jack bean, Toyobo Co., Osaka)[10] and thermolysin (Wako Pure Chemical Industries, Osaka)[11] to design vertebrate collagenase-specific inhibitors.

The relationships between primary structure and enzyme inhibitory activity as well as enzyme specificity of the peptidyl hydroxamic acids assayed are summarized as follows:

Peptide Chain Length and Enzyme Inhibitory Activity To obtain the size of peptide giving the maximum inhibitory activity against collagenase with high specificity, peptide length was elongated along the sequence of the P*n* subsite of the collagenase cleavage region of type I collagen and synthetic substrates (see Fig. 1). As shown in Table I, the extension to tetrapeptide covering P_4–P_1 subsites reached a maximum inhibitory activity against collagenase with high specificity, indicating that both P_3–S_3 and P_4–S_4 interactions are indispensable to give a tight binding of collagenase and inhibitor. Based on these findings, Boc–Gly–Pro–Leu–Gly–NHOH was selected as a leading compound in the following experiments. The kinetic analysis of this leading compound against tadpole collagenase by Lineweaver–Burk plot showed the mode of inhibition to be competitive with a K_i value of 7.7 μM ($K_m = 1.6\,\mu$M).

TABLE II. Inhibition (IC_{50}) of Vertebrate Collagenase and Other Enzymes by R–Gly–Pro–Leu–Gly–NHOH

Compd. No.	R	Tadpole collagenase (M)	Bacterial collagenase (M)	Urease (M)	Thermolysin (M)
3	Boc	3.3×10^{-5}	3.0×10^{-2}	5.5×10^{-2}	3.4×10^{-2}
8	H	1.1×10^{-4}	5.0×10^{-2}	1.8×10^{-3}	9.8×10^{-2}
9	CH_3	2.3×10^{-4}	5.4×10^{-2}	9.6×10^{-3}	1.5×10^{-1}
10	Ac	8.6×10^{-5}	3.2×10^{-2}	1.7×10^{-2}	2.9×10^{-1}
11	Bz	2.8×10^{-5}	7.0×10^{-3}	1.4×10^{-3}	3.9×10^{-2}
12	Boc(CH_3)	4.1×10^{-5}	3.1×10^{-2}	3.9×10^{-2}	3.6×10^{-2}

TABLE III. Inhibition (IC_{50}) of Vertebrate Collagenase and Other Enzymes by Boc–Gly–Pro–Leu–X_1–NHOH

Compd. No.	X_1	Tadpole collagenase (M)	Bacterial collagenase (M)	Urease (M)	Thermolysin (M)
3	Gly	3.3×10^{-5}	3.0×10^{-2}	5.5×10^{-2}	3.4×10^{-2}
25	β-Ala	5.2×10^{-3}	2.9×10^{-2}	1.3×10^{-3}	2.0×10^{-3}
26	GABA[a]	1.3×10^{-2}	7.8×10^{-3}	1.3×10^{-3}	1.3×10^{-2}
27	Ala	8.9×10^{-6}	4.9×10^{-2}	$>1.0\times10^{-1}$	3.6×10^{-2}
28	D-Ala	3.6×10^{-4}	7.2×10^{-3}	6.0×10^{-2}	3.7×10^{-2}
29	Val	9.8×10^{-6}	3.4×10^{-2}	$>1.0\times10^{-2}$	1.0×10^{-2}
30	Sar[b]	2.7×10^{-5}	2.1×10^{-3}	7.0×10^{-3}	1.5×10^{-2}

a) γ-Aminobutyric acid. *b*) Sarcosine.

Since the removal of Boc group from tetrapeptidyl derivative showed a marked decrease in collagenase inhibitory activity (compound **8**), and benzoylation of the unmasked amino group resulted in a full return of its inhibitory activity (compound **11**), S_5 subsite of the enzyme is apparently a bulky hydrophobic pocket (Table II). The finding that protection of the amino group with methyl- or

TABLE IV. Inhibition (IC_{50}) of Vertebrate Collagenase and Other Enzymes by Bz–Gly–Pro–X_2–X_1–NHOH

Compd. No.	X_2	X_1	Tadpole collagenase (M)	Bacterial collagenase (M)	Urease (M)	Thermolysin (M)
31	Leu	Gly	2.8×10^{-5}	7.0×10^{-3}	1.4×10^{-3}	3.9×10^{-2}
32	Leu	Ala	7.7×10^{-6}	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$
33	D-Leu	D-Ala	3.1×10^{-6}	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$
34	Leu	Leu	2.3×10^{-5}	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$
35	D-Leu	D-Leu	5.3×10^{-6}	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$
36	Leu	Sar	1.1×10^{-5}	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$	$>1.0\times10^{-3}$
37	D-Leu	Sar	1.2×10^{-5}	$>1.0\times10^{-3}$	$>1.0\times10^{-2}$	$>1.0\times10^{-2}$

acetyl-group showed only a partial recovery of collagenase inhibitory activity (compounds **9** and **10**) supports this hypothesis.

P_1–S_1 Interaction To detect the optimum P_1 subsite for tight interaction with enzyme S_1 subsite, X_1 residue in the sequence of Boc–Gly–Pro–Leu–X_1–NHOH was substituted with a variety of amino acids. As shown in Table III, substitution with sarcosine for Gly did not affect its collagenase inhibitory activity (compare compounds **3** and **30**). This suggests that the NH-moiety of Gly at P_1 subsite may not be significantly involved in the interaction with S_1 subsite. However, substitution with β-Ala (compound **25**) or GABA (compound **26**) showed a marked decrease in collagenase inhibitory activity, indicating that the presence of α-amino acid at P_1 subsite is essential for its hydroxamic acid moiety to access Zn ion at the catalytic site of the enzyme.

On the other hand, substitution with Ala (compound **27**) or Val (compound **29**) for Gly at X_1 residue caused an increase in the inhibitory activity against collagenase without impairing enzyme specificity. This strongly suggests that S_1 subsite constitutes a shallow hydrophobic pocket, since introduction of Leu to P_1 subsite resulted in the reduction of collagenase inhibitory activity (Table IV).

P_2–S_2 and P_3–S_3 Interactions Table V shows that S_2 subsite seems to be a bulky pocket but is less strict in the requirement for hydrophobicity, since X_2 residue corresponding to P_2 subsite can be substituted with D-Leu, Gln, Glu, Ile or even with Phe for Leu without impairing enzyme specificity. However, some charge effect seems to be involved in the interaction between P_2 subsite and S_2 subsite, since the reduction of collagenase inhibitory activity was observed with compound **23** containing Lys at P_2 subsite. The findings that substitution of X_2 residue with Gly or Ser resulted in a marked decrease in their collagenase inhibitory activity strongly suggest that tight interaction of P_2 subsite with bulky S_2 subsite is essential for the hydroxamic acid moiety to access Zn ion in the enzyme.

Of greatest importance in this study was the finding that S_3 subsite preferentially accommodates Pro residue as P_3 subsite. Neither Hyp nor D-Pro residue can be employed as a substitute for Pro (Table VI). Substitution with Ala residue showed a comparable inhibitory activity to that of the leading compound, but became less specific to collagenase. A marked decrease in collagenase inhibitory activity of Boc–Gly–Pro–Pro–Gly–NHOH (compound **24** in Table V) indicates that S_3–S_1 subsites require an extended structure of P_3–P_1 subsites to accommodate.

Configuration Requirement for P_2–P_1 Subsite Since collagenase inhibitors designed from the sequence of P*n*

TABLE V. Inhibition (IC_{50}) of Vertebrate Collagenase and Other Enzymes by Boc–Gly–Pro–X_2–Gly–NHOH

Compd. No.	X_2	Tadpole collagenase (M)	Bacterial collagenase (M)	Urease (M)	Thermolysin (M)
3	Leu	3.3×10^{-5}	3.0×10^{-2}	5.5×10^{-2}	3.4×10^{-2}
17	D-Leu	1.2×10^{-5}	5.7×10^{-2}	$>1.0\times10^{-2}$	2.4×10^{-3}
7	Gln	5.6×10^{-5}	5.7×10^{-2}	7.5×10^{-2}	3.9×10^{-2}
18	Glu	5.8×10^{-5}	8.0×10^{-3}	7.6×10^{-2}	1.5×10^{-2}
19	Gly	$>1.0\times10^{-3}$	$>1.0\times10^{-2}$	3.4×10^{-2}	4.8×10^{-3}
20	Ile	2.4×10^{-5}	6.9×10^{-3}	5.2×10^{-4}	6.8×10^{-3}
21	Phe	9.7×10^{-5}	1.1×10^{-2}	1.4×10^{-3}	7.3×10^{-3}
22	Ser	2.4×10^{-4}	5.2×10^{-2}	7.8×10^{-3}	2.7×10^{-2}
23	Lys	5.1×10^{-4}	2.6×10^{-2}	N.D.	2.5×10^{-2}
24	Pro	$>1.0\times10^{-3}$	1.4×10^{-2}	1.0×10^{-2}	1.7×10^{-2}

N.D.: not determined.

TABLE VI. Inhibition (IC_{50}) of Vertebrate Collagenase and Other Enzymes by Boc–Gly–X_3–Leu–Gly–NHOH

Compd. No.	X_3	Tadpole collagenase (M)	Bacterial collagenase (M)	Urease (M)	Thermolysin (M)
3	Pro	3.3×10^{-5}	3.0×10^{-2}	5.5×10^{-2}	3.4×10^{-2}
13	Hyp	3.8×10^{-4}	6.2×10^{-3}	4.4×10^{-4}	1.2×10^{-2}
14	Gly	3.4×10^{-4}	5.3×10^{-3}	1.8×10^{-4}	8.3×10^{-3}
15	Ala	5.0×10^{-5}	5.6×10^{-3}	6.3×10^{-4}	8.5×10^{-3}
16	D-Pro	5.8×10^{-4}	8.2×10^{-3}	6.5×10^{-3}	2.4×10^{-2}

subsites at the cleavage site of type I collagen require at least tetrapeptidyl derivative to give maximum inhibitory activity, introduction of D-form of amino acids to P_2–P_1 subsites was attempted to develop a proteinase-resistant collagenase inhibitor. To our surprise, introduction of D-Leu–D-Ala (compound **33** in Table IV), an enantiomeric dipeptide unit against L-Leu–L-Ala, to P_2–P_1 subsites showed collagenase inhibitory activity as high as that of its parent inhibitor carrying L-Leu–L-Ala (compound **32** in Table IV), although substitution with D-Leu–L-Ala[1] or L-Leu–D-Ala, a diastereomeric dipeptide unit (compound **28** in Table III), to the same subsites resulted in a marked decrease in the inhibitory activity. This again indicates that S_2–S_1 subsites of vertebrate collagenase constitute an extended bulky pocket to accommodate enantiomeric Leu–Ala dipeptide unit. Bz–Gly–Pro–D-Leu–D-Ala–NHOH thus prepared showed little reduction in collagenase inhibitory activity even after overnight incubation with pronase, trypsin or leukocyte elastase at pH 7.5 and 37 °C, while the inhibitory activity of its parent compound containing L-Leu–L-Ala decreased with incubation time

under the same conditions (data not shown).

Experimental

General experimental procedures used in this paper are the same as described in the previous paper.[1] The coupling reactions using DCC–HOBt and their post-treatments were performed as follows. DCC solution in CH_2Cl_2 was added at −15 °C to a dimethylformamide (DMF) solution containing both the components to be coupled and HOBt and the reaction mixture was stirred at 5 °C overnight. After removal of DC urea by filtration, the filtrate was evaporated *in vacuo*. The residue was dissolved in AcOEt and washed with 1 N HCl, H_2O, 10% Na_2CO_3 and finally with H_2O, then dried over $MgSO_4$ and evaporated *in vacuo*. The resulting residue was purified by chromatography on silica gel and/or by recrystallization from appropriate solvents.

All catalytic hydrogenations of *O*-benzyl ethers were performed in MeOH in the presence of 5% Pd–C at atmospheric pressure.

Melting points were determined on a Yanagimoto melting apparatus (Kyoto) without correction. Specific rotations were measured with a Jasco DIP-140 apparatus (Tokyo). The purity of all new compounds was monitored by analytical thin-layer chromatography (TLC) on Merck silica gel plates in the following solvent systems: Rf^1, $CHCl_3$–MeOH–AcOH (80:10:5, v/v); Rf^2, *n*-BuOH–AcOH–H_2O (4:1:1, v/v); Rf^3, $CHCl_3$–MeOH (14:1, v/v); Rf^4, $CHCl_3$–MeOH–AcOH (95:5:3, v/v) and Rf^5, *n*-BuOH–AcOH–H_2O (4:2:1, v/v).

FITC-labeled collagen (bovine type I collagen, K-21) was obtained from Cosmo Bio (Tokyo). Bacterial collagenase (collagenase-Sterile, type IA-S, *Clostridium histolyticum*), and furylacryloyl–Gly–Leu–NH_2 was purchased from Sigma Chemical Co. (St. Louis, MO). Urease (jack bean, 100 U/mg grade) was from Toyobo Co. (Osaka). Thermolysin (lyophilized, 7000 U/mg, *Bacillus thermoproteolyticus* ROKKO) from Wako Pure Chemical Industries (Osaka) and urea from Nakarai Chemicals (Kyoto).

Boc–Leu–Gly–NHOH (**1**), Boc–Pro–Leu–Gly–NHOH (**2**), Bod–Gln–Gly–NHOH (**5**) and Boc–Pro–Gln–Gly–NHOH (**6**) were prepared as described in the previous paper.[1]

Boc–Gly–Pro–Leu–Gly–NHOH (3) As a typical example of tetrapeptidyl hydroxamic acid synthesis, Boc–Pro–Leu–Gly–NHOBzl[1] (9.87 g, 20.1 mmol) was treated with 4.2 N HCl/AcOEt (50 ml) at room temperature for 1 h and evaporated *in vacuo*, and further dried in a vacuum desiccator with solid NaOH. The residue was coupled with Boc–Gly–OH (3.36 g, 19.2 mmol) by triethylamine (TEA, 4.22 ml, 30.2 mmol), DCC (5.16 g, 25.0 mmol) and HOBt (2.60 g, 19.2 mmol). The product was purified by chromatography on a silica gel with AcOEt to give Boc–Gly–Pro–Leu–Gly–NHOBzl (5.30 g, 50%) as colorless oil. $[\alpha]_D^{25}$ −68.6° (c=1.0, EtOH), Rf^1, 0.72; Rf^3, 0.37; single ninhydrin-positive spot.

Catalytic hydrogenation of Boc–Gly–Pro–Leu–Gly–NHOBzl (5.30 g, 9.68 mmol) by the conventional procedure gave compound **3** (3.71 g, 84%) as a white powder from MeOH–Et_2O. mp 90—104 °C, $[\alpha]_D^{25}$ −84.3° (c=1.0, EtOH), Rf^1, 0.34; Rf^2, 0.67; single ninhydrin-positive spot. *Anal.* Calcd for $C_{20}H_{35}N_5O_7$: C, 52.5; H, 7.71; N, 15.3. Found: C, 52.45; H, 7.53; N, 15.11. Compounds **7** and **13** were prepared in the same manner from Boc–Pro–Gln–Gly–NHOBzl[1] and Boc–Pro–Leu–Gly–NHOBzl[1] respectively.

7: A hygroscopic white power. $[\alpha]_D^{25}$ −67.1° (c=1.0, EtOH), Rf^1, 0.08; $Rf^2$0.43; single ninhydrin-positive spot. *Anal.* Calcd for $C_{19}H_{32}N_6O_8$: C, 48.29; H, 6.82; N, 17.78. Found: C, 48.55; H, 7.01; N, 17.5.

12: A white powder from MeOH–Et_2O. mp 94—99 °C, $[\alpha]_D^{25}$ −80.6° (c=1.0, EtOH), Rf^1, 0.42; Rf^2, 0.66; single ninhydrin-positive spot. *Anal.* Calcd for $C_{21}H_{37}N_5O_7 \cdot 1/2H_2O$: C, 52.48; H, 7.96; N, 14.57. Found: C, 52.63; H, 7.81; N, 14.55.

Boc–Pro–Gly–Pro–Leu–Gly–NHOH (4) Boc–Pro–Gly–Pro–Leu–Gly–NHOBzl was prepared from Boc–Gly–Pro–Leu–Gly–NHOBzl (3.0 g, 5.48 mmol) was Boc–Pro–OH (1.10 g, 5.11 mmol) as described for the preparation of Boc–Gly–Pro–Leu–Gly–NHOBzl. The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (15:1, v/v) to give Boc–Pro–Gly–Pro–Leu–Gly–NHOBzl (2.70 g, 82%) as colorless oil, $[\alpha]_D^{25}$ −106° (c=1.0, EtOH), Rf^1, 0.70; Rf^3, 0.29; single ninhydrin-positive spot.

Catalytic hydrogenation of Boc–Pro–Gly–Pro–Leu–Gly–NHOBzl (0.30 g, 0.46 mmol) by the conventional procedure gave compound **4** (0.19 g, 74%) as a white powder from MeOH–Et_2O. mp 115—120 °C, $[\alpha]_D^{25}$ −120.8° (c=0.5, EtOH), Rf^1, 0.31; Rf^2, 0.52; single ninhydrin-positive spot. *Anal.* Calcd for $C_{25}H_{42}N_6O_8 \cdot H_2O$: C, 52.43, H, 7.74, N, 14.67. Found: C, 52.23; H, 7.48; N, 14.67.

H–Gly–Pro–Leu–Gly–NHOH AcOH (8) As a typical example of tetrapeptide synthesis, compound **3** (100 mg, 0.22 mmol) was treated with 4.2 N HCl/AcOEt at room temperature for 1 h and concentrated *in vacuo*. The residue was passed through a PA-308 anion exchange column (AcO^- form) and purified by partition chromatography (Sephadex G-25, *n*-BuOH : AcOH : H_2O = 4 : 1 : 5). The product thus obtained was lyophilized to give compound **8** (50 mg, 55%) as a hygroscopic white powder. $[\alpha]_D^{25}$ −87.2° (c=0.5, EtOH), Rf^5, 0.24; single ninhydrin-positive spot. *Anal.* Calcd for $C_{17}H_{31}N_5O_7 \cdot 3/2H_2O$: C, 45.93; H, 7.70; N, 15.75. Found: C, 45.89; H, 7.62; N, 15.70. Compound **9** was prepared in the same manner from compound **12**.

9: A hygroscopic white powder. $[\alpha]_D^{25}$ −79.1° (c=1.0, EtOH), Rf^2, 0.19; Rf^5, 0.24; single ninhydrin-positive spot. *Anal.* Calcd for $C_{18}H_{33}N_5O_7 \cdot H_2O$: C, 48.09; H, 7.84; N, 15.58. Found: C, 48.01; H, 7.85; N, 15.28.

Ac–Gly–Pro–Leu–Gly–NHOH (10) The Boc- group of Boc–Gly–Pro–Leu–Gly–NHOBzl (0.55 g, 1.0 mmol) was removed by treatment with 4.2 N HCl/AcOEt. TEA (0.20 ml, 1.43 mmol), HOBt (0.14 g, 1.04 mmol) and AcOSu (0.19 g, 1.2 mmol) were added to 0 °C to a DMF solution of the HCl salt thus obtained and the reaction mixture was stirred for 1 h at room temperature and concentrated *in vacuo*. The residue was dissolved in AcOEt, and washed with 1 N HCl, H_2O, 10% Na_2CO_3 and finally with H_2O. The organic layer was dried over $MgSO_4$ and evaporated *in vacuo*. The residue was purified by chromatography on a silica gel with $CHCl_3$–MeOH (20:1, v/v), followed by recrystallization from AcOEt–*n*-Hexane to give Ac–Gly–Pro–Leu–Gly–NHOBzl (0.32 g, 65%) as a colorless crystal. mp 105—109 °C, $[\alpha]_D^{25}$ −76.1° (c=1.0, EtOH), Rf^1, 0.48; Rf^3, 0.13; single ninhydrin-positive spot. *Anal.* Calcd for $C_{24}H_{35}N_5O_6$: C, 58.88; H, 7.20; N, 14.30. Found: C, 58.82; H, 7.34; N, 14.11.

Catalytic hydrogenation of Ac–Gly–Pro–Leu–Gly–NHOBzl (0.28 g, 0.57 mmol) by the conventional procedure gave compound **10** (0.21 g, 92%) as a white powder from MeOH–Et_2O. mp 90—115 °C, $[\alpha]_D^{25}$ −91.3° (c=1.0, EtOH), Rf^1, 0.13; Rf^2, 0.43; single ninhydrin-positive spot. *Anal.* Calcd for $C_{17}H_{29}N_5O_6 \cdot 1/2H_2O$: C, 49.99; H, 7.40; N, 17.14. Found: C, 49.98; H, 7.34; N, 16.87. Compound **11** was prepared in the same manner from Boc–Gly–Pro–Leu–Gly–NHOBzl using Bz–Cl instead of AcOSu.

11: A white powder from MeOH–Et_2O. mp 110—123 °C, $[\alpha]_D^{25}$ −77.4° (c=1.0, EtOH), Rf^1, 0.23; Rf^2, 0.60; single ninhydrin-positive spot. *Anal.* Calcd for $C_{22}H_{31}N_5O_6$: C, 57.25; H, 6.77; N, 15.17. Found: C, 57.25; H, 6.79; N, 15.24.

Boc–Gly–Hyp–Leu–Gly–NHOH (13) Boc–Hyp–Leu–Gly–NHOBzl was prepared from Boc–Leu–Gly–NHOBzl[1] (3.89 g, 9.89 mmol) and Boc–Hyp–OH (1.99 g, 8.60 mmol) as described for the preparation of Boc–Pro–Leu–Gly–NHOBzl.[1] The product was purified by recrystallization from AcOEt to give Boc–Hyp–Leu–Gly–NHOBzl (2.45 g, 56%) as colorless crystals. mp 168—173 °C, $[\alpha]_D^{25}$ −50.8° (c=1.0, EtOH), Rf^3, 0.40; Rf^4, 0.19; single ninhydrin-positive spot. *Anal.* Calcd for $C_{25}H_{38}N_4O_7$: C, 59.27; H, 7.56; N, 11.05. Found: C, 59.16, H; 7.66; N, 10.93.

Boc–Gly–Hyp–Leu–Gly–NHOBzl was prepared from Boc–Hyp–Leu–Gly–NHOBzl (2.0 g, 3.95 mmol) and Boc–Gly–OSu (1.27 g, 4.35 mmol) in the same manner as described for the preparation of Ac–Gly–Pro–Leu–Gly–NHOBzl. The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (30:1, v/v) to give Boc–Gly–Hyp–Leu–Gly–NHOBzl (1.50 g, 67%) as colorless oil. $[\alpha]_D^{25}$ −55.5° (c=1.0, EtOH), Rf^3, 0.37; Rf^4, 0.13; single ninhydrin-positive spot.

Catalytic hydrogenation of Boc–Gly–Hyp–Leu–Gly–NHOBzl (0.75 g, 1.33 mmol) by the conventional procedure gave compound **13** (0.60 g, 95%) as a white powder from MeOH–Et_2O. mp 178—183 °C, $[\alpha]_D^{25}$ −73.8° (c=1.0, EtOH), Rf^1, 0.10; Rf^2, 0.51; single ninhydrin-positive spot. *Anal.* Calcd for $C_{20}H_{35}N_5O_8 \cdot H_2O$: C, 48.87; H, 7.58; N, 14.24. Found: C, 48.82; H, 7.63; N, 14.28. Compounds **14**, **15** and **16** were prepared in the same manner from Boc–Gly–Leu–Gly–NHOBzl, Boc–Ala–Leu–Gly–NHOBzl and Boc–D-Pro–Leu–Gly–NHOBzl, respectively.

14: A white powder, mp 120—129 °C, $[\alpha]_D^{25}$ −12.5° (c=1.0, EtOH), Rf^1, 0.18; Rf^2, 0.65; single ninhydrin-positive spot. *Anal.* Calcd for $C_{17}H_{31}N_5O_7 \cdot 1/2H_2O$: C, 47.87; H, 7.56; N, 16.42. Found: C, 47.72; H, 7.57; N, 16.45.

15: A white powder, mp 148—152 °C, $[\alpha]_D^{25}$ −42.2° (c=1.0, EtOH), Rf^1, 0.24; Rf^2, 0.67; single ninhydrin-positive spot. *Anal.* Calcd for $C_{18}H_{33}N_5O_7$: C, 50.10; H, 7.70; N, 16.23. Found: C, 50.20; H, 7.65; N, 16.11.

16: A white powder, mp 98—105 °C, $[\alpha]_D^{25}$ +18.4° (c=1.0, EtOH), Rf^1, 0.55; Rf^2, 0.70; single ninhydrin-positive spot. *Anal.* Calcd for $C_{20}H_{35}N_5O_7$: C, 52.50; H, 7.71; N, 15.30. Found: C, 52.55; H, 7.63; N, 15.28.

Boc–Gly–Pro–D-Leu–Gly–NHOH (17) As a typical example of tetrapeptide synthesis containing D-amino acid, Boc–D-Leu–Gly–NHOBzl

was prepared from Boc-D-Leu-OH·H_2O (2.53 g, 10.1 mmol) and Boc-Gly-NHOBzl (3.36 g, 12.0 mmol) as described for the preparation of Boc-Leu-Gly-NHOBzl.[1] The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (50:1, v/v) to give Boc-D-Leu-Gly-NHOBzl (3.56 g, 90%) as colorless oil. $[\alpha]_D^{25}$ +7.8° (c=1.0, EtOH), Rf^4, 0.56, single ninhydrin-positive spot.

Boc-Gly-Pro-D-Leu-Gly-NHOBzl was prepared from Boc-D-Leu-Gly-NHOBzl (2.26 g, 5.74 mmol) and Boc-Gly-Pro-OH (1.42 g, 5.22 mmol) as described for the preparation of Boc-Gly-Pro-Leu-Gly-NHOBzl. The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (50:1, v/v), followed by reprecipitation from AcOEt to give Boc-Gly-Pro-D-Leu-Gly-NHOBzl (1.1 g, 39%) as a hydroscopic white powder. $[\alpha]_D^{25}$ −6.2° (c=1.0, EtOH), Rf^3, 0.18; Rf^4, 0.25; single ninhydrin-positive spot. *Anal.* Calcd for $C_{27}H_{41}N_5O_7$: C, 59.21: H, 7.54; N, 12.78. Found: C, 59.05; H, 7.46; N, 12.84.

Catalytic hydrogenation of Boc-Gly-Pro-D-Leu-Gly-NHOBzl (0.56 g, 1.02 mmol) by the conventional procedure gave compound **17** (0.30 g, 64%) as a white powder from AcOEt–*n*-Hexane. mp 90—100 °C, $[\alpha]_D^{25}$ −20.3° (c=1.0, EtOH), Rf^1, 0.50; Rf^2, 0.73; single ninhydrin-positive spot. *Anal.* Calcd for $C_{20}H_{35}N_5O_7 \cdot 1/3H_2O$: C, 51.82; H, 7.75; N, 15.11. Found: C, 52.01; H, 7.64; N, 14.78. Compounds **18**—**24** were prepared in the same manner from the corresponding dipeptide derivatives and Boc-Gly-Pro-OH, respectively.

18: A white powder. mp 90—100 °C, $[\alpha]_D^{25}$ −64.9° (c=1.0, EtOH), Rf^1, 0.12; Rf^2, 0.49; single ninhydrin-positive spot. *Anal.* Calcd for $C_{19}H_{31}N_5O_9 \cdot 1/2H_2O$: C, 47.29; H, 6.65; N, 14.51. Found: C, 47.10, H, 6.62; N, 14.48.

19: A white powder. mp 178—180 °C, $[\alpha]_D^{25}$ −52.3° (c=1.0, DMF), Rf^1, 0.16; Rf^2, 0.44; single ninhydrin-positive spot. *Anal.* Calcd for $C_{16}H_{27}N_5O_7$: C, 47.87; H, 6.77; N, 17.44. Found: C, 47.79; H, 6.71; N, 17.54.

20: A white powder. mp 123—129 °C, $[\alpha]_D^{25}$ −78.3° (c=1.0, EtOH), Rf^1, 0.37; Rf^2, 0.64; single ninhydrin-positive spot. *Anal.* Calcd for $C_{20}H_{35}N_5O_7 \cdot 1/3H_2O$: C, 51.82; H, 7.75; N, 15.11. Found: C, 51.70; H, 7.57; N, 15.18.

21: A white powder. mp 166—171 °C, $[\alpha]_D^{25}$ −89.7° (c=1.0, MeOH), Rf^1, 0.44; Rf^2, 0.71; single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{33}N_5O_7$: C, 56.20; H, 6.76; N, 14.24. Found: C, 56.19; H, 6.71; N, 14.31.

22: A white powder. mp 92—105 °C, $[\alpha]_D^{25}$ −72.1° (c=1.0, EtOH), Rf^1, 0.12; Rf^2, 0.45; single ninhydrin-positive spot. *Anal.* Calcd for $C_{17}H_{29}N_5O_8 \cdot 1/3H_2O$: C, 46.68; H, 6.83; N, 16.01. Found: C, 46.61; H, 6.91; N, 15.78.

23: A hygroscopic white powder (purified by partition chromatography as described for compound **8**. $[\alpha]_D^{25}$ −56.1° (c=1.0, EtOH), Rf^2, 0.25; single ninhydrin-positive spot. *Anal.* Calcd for $C_{20}H_{36}N_6O_6 \cdot CH_3CO_2H \cdot 1/2H_2O$: C, 50.27; H, 7.86; N, 15.98. Found: C, 50.11; H, 7.86; N, 15.78.

24: A hygroscopic white powder. $[\alpha]_D^{25}$ −98.1° (c=1.0, EtOH), Rf^1, 0.31; Rf^2, 0.46; single ninhydrin-positive spot. *Anal.* Calcd for $C_{19}H_{31}N_5O_7$: C, 51.69; H, 7.07; N, 15.86. Found: C, 51.51; H, 7.26; N, 15.65.

Boc-Gly-Pro-Leu-β-Ala-NHON (25) Boc-β-Ala-NHOBzl was prepared from Boc-β-Ala-OH (9.46 g, 50 mmol) and HCl·NH_2OBzl (12 g, 75 mmol) as described for the preparation of Boc-Gly-NHOBzl.[1] The product was purified by recrystallization from AcOEt to give Boc-β-Ala-NHOBzl (12.5 g, 85%) as a colorless crystal. mp 102—103 °C, Rf^3, 0.57; Rf^4, 0.80; single ninhydrin-positive spot. *Anal.* Calcd for $C_{15}H_{22}N_2O_4$: C, 61.20; H, 7.53; N, 9.51. Found: C, 61.01; H, 7.55; N, 9.49.

Boc-Leu-β-Ala-NHOBzl was prepared from Boc-β-Ala-NHOBzl (4.42 g, 15.0 mmol) and Boc-Leu-OH (3.15 g, 13.6 mmol) as described for the preparation of Boc-D-Leu-Gly-NHOBzl. The product was purified by chromatography on a silica gel with AcOEt–*n*-Hexane (5:1, v/v) to give Boc-Leu-β-Ala-NHOBzl (5.49 g, 99%) as colorless oil. $[\alpha]_D^{25}$ −17.8° (c=1.0, EtOH), Rf^1, 0.80; Rf^3, 0.53; single ninhydrin-positive spot.

Boc-Gly-Pro-Leu-β-Ala-NHOBzl was prepared from Boc-Leu-β-Ala-NHOBzl (4.07 g, 10 mmol) and Boc-Gly-Pro-OH (2.46 g, 10 mmol) as described for the preparation of Boc-Gly-Pro-D-Leu-Gly-NHOBzl. The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (25:1, v/v) to give Boc-Gly-Pro-Leu-β-Ala-NHOBzl (3.98 g, 71%) as colorless oil. $[\alpha]_D^{25}$ −60.1° (c=1.0, EtOH), Rf^1, 0.73; Rf^3, 0.33; single ninhydrin-positive spot.

Catalytic hydrogenation of Boc-Gly-Pro-Leu-β-Ala-NHOBzl (0.75 g, 1.34 mmol) by the conventional procedure gave compound **25** (0.49 g, 78%) as a white powder from MeOH–Et_2O. mp 100–112 °C, $[\alpha]_D^{25}$ −70.2° (c=1.0, EtOH), Rf^1, 0.38; Rf^2, 0.64; single ninhydrin-positive spot. *Anal.* Calcd for $C_{21}H_{37}N_5O_7$: C, 53.48; H, 7.90; N, 14.85. Found: C, 53.78; H, 8.08; N, 14.56. Compounds **26**—**29** were prepared in the same manner from the corresponding dipeptide derivatives, respectively.

26: A white powder. mp 80—93 °C, $[\alpha]_D^{25}$ −56.9° (c=1.0, EtOH), Rf^1, 0.38; Rf^2, 0.64; single ninhydrin-positive spot. *Anal.* Calcd for $C_{22}H_{39}N_5O_7 \cdot 1/2H_2O$: C, 53.42; H, 8.15; N, 14.16. Found: C, 53.38; H, 8.31; N, 13.87.

27: A white powder. mp 110—118 °C, $[\alpha]_D^{25}$ −85.0° (c=1.0, EtOH), Rf^1, 0.39; Rf^2, 0.67; single ninhydrin-positive spot. *Anal.* Calcd for $C_{21}H_{37}N_5O_7 \cdot 1/2H_2O$: C, 52.48; H, 7.96; N, 14.57. Found: C, 52.77; H, 7.72; N, 14.71.

28: A white powder. mp 110—120 °C, $[\alpha]_D^{25}$ −89.3° (c=1.0, EtOH), Rf^1, 0.55; Rf^2, 0.70; single ninhydrin-positive spot. *Anal.* Calcd for $C_{21}H_{37}N_5O_7 \cdot 2/5H_2O$: C, 52.68; H, 7.95; N, 14.62. Found: C, 52.80; H, 7.83; N, 14.54.

29: A white powder. mp 116—123 °C, $[\alpha]_D^{25}$ −113.6° (c=0.5, EtOH), Rf^1, 0.53; Rf^2, 0.73; single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{41}N_5O_7 \cdot 1/2H_2O$: C, 54.31; H, 8.32; N, 13.76. Found: C, 54.31, H, 8.04; N, 13.76.

Bz-Gly-Pro-Leu-Ala-NHOH (32) As a typical example of Bz-tetrapeptide derivative synthesis, Bz-Gly-Pro-Leu-Ala-NHOBzl was prepared from Boc-Leu-Ala-NHOBzl[1] (4.89 g, 12.0 mmol) and Bz-Gly-Pro-OH (2.76 g, 10.0 mmol) as described for the preparation of Boc-Gly-Pro-Leu-Gly-NHOBzl. The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (50:1, v/v) to give Bz-Gly-Pro-Leu-Ala-NHOBzl (4.63 g, 82%) as colorless oil. $[\alpha]_D^{25}$ −73.2° (c=1.0, EtOH), Rf^1, 0.72; Rf^3, 0.22; single ninhydrin-positive spot.

Catalytic hydrogenation of Bz-Gly-Pro-Leu-Ala-NHOBzl (0.57 g, 1.0 mmol) by the conventional procedure gave compound **32** (0.36 g, 75%) as a white powder from MeOH–Et_2O. mp 202—204 °C, $[\alpha]_D^{25}$ −71.7° (c=1.0, DMF), Rf^1, 0.78; Rf^2, 0.75; single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{33}N_5O_6 \cdot 1/2H_2O$: C, 57.01; H, 7.07; N, 14.45. Found: C, 57.14; H, 6.99; N, 14.72. Compounds **33**—**35** were prepared in the same manner from the corresponding dipeptide derivatives and Bz-Gly-Pro-OH, respectively.

33: Colorless needles from MeOH. mp 172—174 °C, $[\alpha]_D^{25}$ +10.8 (c=1.0, DMF), Rf^1, 0.63; Rf^2, 0.70; single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{33}N_5O_6 \cdot 2/3H_2O$: C, 54.96; H, 7.21; N, 13.93. Found: C, 55.04; H, 7.07; N, 13.86.

34: A white powder. mp 130—135 °C, $[\alpha]_D^{25}$ −74.0° (c=0.5, EtOH), Rf^1, 0.35; Rf^2, 0.66; single ninhydrin-positive spot. *Anal.* Calcd for $C_{26}H_{39}N_5O_6$: C, 60.33; H, 7.59; N, 13.52. Found: C, 60.38; H, 7.42; N, 13.28.

35: A white powder (from MeOH–Et_2O). mp 187—190 °C, $[\alpha]_D^{25}$ +25.6° (c=0.5, EtOH), Rf^1, 0.69; Rf^2, 0.74; single ninhydrin-positive spot. *Anal.* Calcd for $C_{26}H_{39}N_5O_6$: C, 60.33; H, 7.59; N, 13.52. Found: C, 60.21; H, 7.65; N, 13.34.

Boc-Gly-Pro-Leu-Sar-NHOH (30) As a typical example of Sar-containing tetrapeptide synthesis, Boc-Sar-NHOBzl was prepared from Boc-Sar-OH (4.73 g, 25 mmol) and HCl·NH_2OBzl (6.0 g, 37.5 mmol) as described for the preparation of Boc-Gly-NHOBzl.[1] The product was purified by recrystallization from AcOEt–*n*-Hexane to give Boc-Sar-NHOBzl (6.62 g, 90%) as a colorless crystal. mp 102—103 °C, Rf^3, 0.85; Rf^4, 0.69; single ninhydrin-positive spot. *Anal.* Calcd for $C_{15}H_{22}N_2O_4$: C, 61.2; H, 7.53; N, 9.51. Found: C, 61.11; H, 7.55; N, 9.36.

Boc-Gly-Pro-Leu-Sar-NHOBzl was prepared from Boc-Sar-NHOBzl (0.60 g, 2.04 mmol) and Boc-Gly-Pro-Leu-OH (0.70 g, 1.8 mmol) as described for the preparation of Boc-Gly-Pro-Leu-Gly-NHOBzl. The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (20:1, v/v) to give Boc-Gly-Pro-Leu-Sar-NHOBzl (0.75 g, 77%) as a hygroscopic white powder. $[\alpha]_D^{25}$ −67.0° (c=1.0, EtOH), Rf^3, 0.44; Rf^4, 0.49; single ninhydrin-positive spot.

Catalytic hydrogenation of Boc-Gly-Pro-Leu-Sar-NHOBzl (0.36 g, 0.64 mmol) was performed by the conventional procedure. The product was purified by chromatography on a silica gel with $CHCl_3$–MeOH (30:1, v/v), followed by reprecipitation from MeOH–Et_2O to give compound **30** (75 mg, 25%) as a hygroscopic white powder. $[\alpha]_D^{25}$ −79.8° (c=1.0, EtOH), Rf^1, 0.35; Rf^2, 0.54; single ninhydrin-positive spot. *Anal.* Calcd for $C_{21}H_{37}N_5O_7 \cdot 1/2H_2O$: C, 52.48; H, 7.96; N, 14.57. Found: C, 52.55; H, 7.96; N, 14.30. Compounds **36** and **37** were prepared in the same manner from the corresponding Sar-containing dipeptide derivatives, respectively.

36: A pale yellow powder. mp 102—110 °C, $[\alpha]_D^{25}$ −94.5° (c=1.0,

EtOH), Rf^1, 0.44; Rf^2, 0.58; single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{33}N_5O_6 \cdot 1/2H_2O$: C, 57.01; H, 7.07; N, 14.45. Found: C, 57.20; H, 7.04; N, 14.49.

37: A colorless powder. mp 110—119 °C, $[\alpha]_D^{25}$ −36.9° (c=1.0, DMF), Rf^1, 0.49; Rf^2, 0.60; single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{33}N_5O_6 \cdot 1/2H_2O$: C, 57.01; H, 7.07; N, 14.45. Found: C, 57.23; H, 7.10; N, 14.31.

Enzyme Inhibition Assay Inhibitory activities of tetrapeptidyl hydroxamic acids against collagenase (tadpole and bacterial) were assayed using FITC-labeled collagen as substrate, as reported previously.[9] Inhibitory activity against urease was assayed by measuring pH changes with phenol red due to hydrolysis of urea, as described by K. Kobashi *et al.*[10] Inhibitory activity against thermolysin was assayed using furylacryloyl–Gly–Leu–NH_2 as substrate, as previously reported by Feder.[11]

Acknowledgement The authors thank Dr. N. Nishino, Associate Professor of Kyushu Institute of Technology, for his valuable suggestions through this work. The authors are also grateful to Dr. K. Nakahashi, Fuji Chemical Industries, Ltd., for his continuous support.

References and Notes

1) Part I: S. Odake, T. Okayama, M. Obata, T. Morikawa, S. Hattori and Y. Nagai, *Chem. Pharm. Bull.*, **38**, 1007 (1990).
2) H. Tschesche (ed.), "Proteinases in Inflammation and Tumor Invasion", Walter de Gruyter, Berlin, 1986.
3) L. M. Matrisian, *Trends in Genetics*, **6**, 121 (1990).
4) W. M. Moore and C. A. Spilburg, *Biochemistry*, **25**, 5189 (1986).
5) Y. Masui, T. Takemoto, S. Sakakibara, H. Hori and Y. Nagai, *Biochem. Med.*, **17**, 215 (1977).
6) *a*) R. D. Gray, H. H. Saneii and A. F. Spatola, *Biochem. Biophys. Res. Commun.*, **101**, 1251 (1981); *b*) D. K. Donald, M. M. Hann, J. Saunder and H. J. Wadsworh, U.S. Patent 4595700 (1986) [*Chem. Abstr.*, **105**, 209393 (1986)]; *c*) U. B. Goli and R. E. Galardy, *Biochemistry*, **25**, 7136 (1986); *d*) J. M. Delaisse, Y. Eeckhout, C. Sear, A. Galloway, K. McCullagh and G. Vaes, *Biochem. Biophys. Res. Commun.*, **133**, 483 (1985); *e*) J. P. Dickens, D. K. Donald, G. Kneen and W. R. Mckay, U.S. Patent 4599361 (1986) [*Chem. Abstr.*, **105**, 153550 (1986)]; *f*) B. K. Handa, W. H. Johnson and P. J. Machin, Eur. Patent 236872 (1987) [*Chem. Abstr.*, **108**, 167973 (1988)]; *g*) A. Shaw and D. J. Wolanin, Eur. Patent 231081 (1987) [*Chem. Abstr.*, **108**, 150980 (1988)]; *h*) K. A. Mookhtiar, C. K. Marlowe, P. A. Bartlett and H. Van Wart, *Biochemistry*, **26**, 1962 (1987); *i*) Z. P. Kortylewicz and R. E. Galardy, *J. Med. Chem.*, **33**, 263 (1990).
7) W. König and R. Geiger, *Chem. Ber.*, **103**, 2024 (1970).
8) H. Hori and Y. Nagai, *Biochim. Biophys. Acta*, **566**, 211 (1979).
9) Y. Nagai, H. Hori, S. Hattori, Y. Sunada, K. Terato and R. Hashida, *Ensho*, **4**, 123 (1984) (in Japanese).
10) K. Kobashi, K. Kumaki and J. Hase, *Biochim. Biophys. Acta*, **227**, 492 (1971).
11) J. Feder, *Biochem. Biophys. Res. Commun.*, **32**, 326 (1968).

Comparative Studies of the Colony-Promoting Activity of Porcine Kidney Extract with Several Interleukins and Colony-Stimulating Factors

Ikuo KASHIWAKURA,* Yukitoshi HAYASE, and Yoshinari TAKAGI

Hokkaido Institute of Pharmaceutical Sciences, 7–1, Katsuraoka-cho, Otaru, 047–02, Japan. *Received November 13, 1990*

Porcine kidney extracts (PKE) possess colony-promoting activity (CPA) which stimulates primitive hematopoietic cells in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF), but PKE itself does not stimulate colony formation on murine bone marrow cells.

We have compared the CPA of PKE with that of recombinant cytokines or CSFs such as interleukin-1 alpha (IL-1α), IL-3, IL-6, granulocyte colony-stimulating factor (G-CSF), GM-CSF and macrophage colony-stimulating factor (CSF-1). All of these factors were less potent than PKE.

Furthermore, the combinations of IL-1α or PKE with G-CSF, GM-CSF, IL-3 or IL-6 were examined in the presence of one of these factors such as CSF. It is found that PKE acts synergistically with G-CSF, GM-CSF, IL-3 and IL-6, showing enhancement ratios of 10, 2.5, 4.2 and 30, respectively. The combination of IL-1α resulted in poor colony formation in contrast with those of PKE, except for CSF-1.

These results suggest that the CPA of the factor(s) in PKE differ from the cytokines and CSFs tested in this study, and is significantly affected by various types of CSF.

Keywords colony-promoting activity; granulocyte/macrophage colony-forming unit; kidney extract; interleukine-1 alpha; colony-stimulating factor

Granulocyte/macrophage colony-stimulating factors (GM-CSF) stimulate the proliferation and differentiation of granulocyte/macrophage colony-forming units (CFU-GM) to form colonies in agar cultures.[1,2] On the other hand, there are some reports about factors named colony-promoting activity (CPA) which enhance colony formation in the presence of CSF but do not stimulate colony formation by themselves.[3-7]

Recently we found a CPA in the extracts of porcine kidney (PKE).[8] PKE may act on immature granulocyte/macrophage progenitors, which are younger than CSF-responsive CFU-GM, and enhance the induction into a cell cycle from a G_0 state.

Stanley *et al.* found a growth factor, hemopoietin 1 (H-1), which synergistically acted with CSF-1 to support the proliferation of primitive CFU progenitor cells, but did not stimulate colony formation.[9] Since H-1 also acts synergistically with interleukin-3 (IL-3) to induce a remarkable increase in colony number on bone marrow cells (BMC) from 5-fluorouracil (5-FU)-treated mice, it was inferred that H-1 is a growth factor that acts on multipotent stem cells which are more primitive than the IL-3-responsive cells.[10] Mochizuki *et al.* have demonstrated that H-1 is identical with IL-1α.[11]

PKE alone also did not support colony formation of murine BMC, but did enhance colony formation in the presence of GM-CSF,[8] showing a similarity to IL-1α.

In this paper, we compared the characteristics of CPA between PKE and several interleukins and CSFs, and the effects of PKE and IL-1α in combination with those growth factors are also discussed.

Experimental

Preparation of PKE PKE was prepared as previously described.[12] Briefly, porcine kidneys were homogenized with distilled water. The homogenates were centrifuged at 1×10^5 *g* and the clear supernatants thus obtained were lyophilized. The lyophilized material was dissolved in distilled water, and a saturated ammonium sulfate solution was added to the solution. The precipitate from a 50 to 65% saturation of ammonium sulfate was collected, dialyzed against distilled water and lyophilized. The lyophilized material was dissolved in distilled water and the insoluble material was removed by centrifugation. The clear supernatant obtained was dialyzed against phosphate-buffered saline (PBS), passed through a membrane filter (0.45 μm pore size, Fuji Photo Film Co., ltd.), and stored at −20 °C until use. The same preparation was used throughout the experiment. The protein content of this preparation was 15 mg per 1 ml. The results of repeated experiments showed good reproducibility on CPA of the preparations, and no detectable inactivation of CPA was observed during several months under the above storage condition.

Mice Male mice of ddY strain (Sankyo Labo Service Corporation, Sapporo) aged 6—10 weeks were used.

Preparation of Abdominal Wall Conditioned Medium (AWCM) AWCM was prepared as previously described.[12] Briefly, mouse abdominal wall was incubated with an F-10 medium (Gibco Laboratories, New York) containing 20% horse serum (HS) (Gibco) at 37 °C in 5% CO_2 for 4 d. The conditioned medium (CM) obtained was passed through a membrane filter (0.45 μm in pore size), and stored at −20 °C until use. Concentration of AWCM in cultures was determined as 20% (v/v) from dose–response curves of colony-stimulating activity on murine BMC. The CSF in AWCM stimulates granulocyte/macrophage colony formation, and is stable for several months under the above storage condition.

Growth Factors Recombinant human interleukin-1 alpha (IL-1α, specific activity $>1 \times 10^7$ U/mg) and recombinant human interleukin-6 (IL-6, specific activity $>2 \times 10^8$ U/mg) were purchased from Boehringer Mannheim GmbH (W. Germany). Murine interleukin-3 (IL-3, specific activity $>1 \times 10^7$ U/mg), recombinant murine granulocyte/macrophage colony-stimulating factor (GM-CSF, specific activity 5×10^7 U/mg) and recombinant human macrophage colony-stimulating factor (CSF-1, specific activity 1×10^8 U/mg) were purchased from Genzyme, Inc (Boston). Recombinant human granulocyte colony-stimulating factor (G-CSF, specific activity 2×10^8 U/mg) was purchased from Amersham International plc (UK). The optimal dose (plateau level) of each factor was determined from dose-response curves of colony formation in agar cultures by murine BMC. Doses of these factors per culture plate were as follows: (1) G-CSF, 250 units; (2) GM-CSF, 100 units; (3) CSF-1, 200 units; (4) IL-3, 200 units; (5) IL-6, 4000 units.

CPA Assay The assay for CPA was carried out as described by Tsurusawa *et al.*[7] Briefly, 5×10^4 BMC from a mouse femur were cultured in 35 mm plastic Petri dishes (SH-S3512SW, Terumo, Tokyo) in 1 ml of a nutrient mixture F-10 medium supplemented with 0.32% Bacto-agar (Difco Laboratories, Detroit, MI), 20% HS, CSFs, and test samples. As a source of CSF, AWCM, G-CSF, GM-CSF, CSF-1, IL-3 or IL-6 was used in this study. After 7 d of incubation at 37 °C in a humidified atmosphere of 5% CO_2 in air, colonies consisting of 50 or more cells were scored.

From a dose–response study, 50 μl per dish of PKE was considered to be optimal for promoting activity as discribed previously.[8] PKE alone

did not support colony formation, even at 100 μl.

CPA is expressed as the ratio of the colony number in the presence of the test sample to that in its absence.

Preincubation with a Polyclonal Antibody against IL-1α The polyclonal antibody against IL-1α (anti-IL-1α) was purchased from Genzyme, Inc. One mg of anti-IL-1α is capable of neutralizing 1000 units of the relevant form (alpha or beta) of natural or recombinant human IL-1. PKE (50 μl) or IL-1α (30 U) were preincubated with anti-IL-1α (50 μg) at 37 °C with gentle agitation. After 2 h, each incubation mixture was diluted with PBS containing 5% HS and was immediately assayed for CPA.

Results

CPA of Various Growth Factors Alone and in Combination We examined the effects of multiple growth factors on colony formation stimulated by AWCM as a source of GM-CSF. The results are shown in Figs. 1 and 2. In the presence of PKE, the number of colonies increased to 2.1 times greater than with AWCM alone. And in the presence of IL-1α (10 to 100 U/dish), the addition of 30 U resulted in a maximum increase of 1.5 times (Fig. 1). The number of colonies also increased to 1.4 times by the addition of IL-3 (200 U) to the cultures, but no remarkable increase was observed by the addition of G-CSF, GM-CSF, CSF-1 or IL-6 (Fig. 2).

We also examined the CPA of various combinations of the factors. As shown in Fig. 3, all combinations examined enhanced colony formation, but were less effective than PKE alone.

Influence of Anti-IL-1α on the CPA of PKE PKE and IL-1α treated with anti-IL-1α were assayed for CPA. As shown in Table I, the CPA of IL-1α was neutralized by the addition of anti-IL-1α, whereas that of PKE was not affected by the treatment of anti-IL-1α. In addition, anti-IL-1α ranging from 5—100 U did not affect the CPA of PKE (data not shown).

Comparison of the CPA between PKE and IL-1α in the Presence of Several Growth Factors as a Source of CSF PKE and/or IL-1α were assayed for CPA in the presence

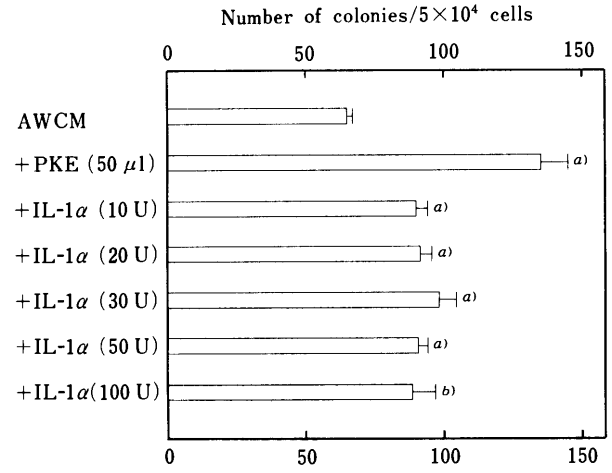


Fig. 1. The Effect of PKE and IL-1α on Colony Formation Supported by AWCM

Number of colonies stimulated with AWCM plus PKE or IL-1α. Dose of PKE was determined from dose–response curves of CPA in agar culture by murine BMC. Numbers represent mean ± S.E. of 6 dishes from two separate experiments. *a*) $p<0.001$, *b*) $p<0.05$ by *t* test.

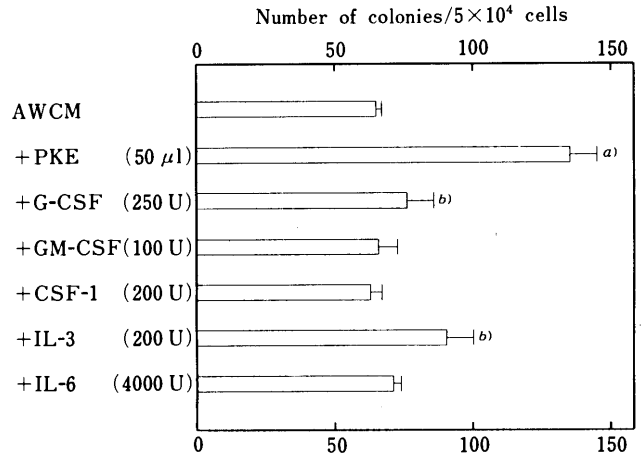


Fig. 2. The Effect of PKE and Various Cytokines on Colony Formation Supported by AWCM

Number of colonies stimulated with AWCM plus PKE or recombinant CSF. Dose of individual CSFs was determined from dose-response curves of colony formation in agar culture by murine BMC. Numbers represent mean ± S.E. of 6 dishes from two separate experiments. *a*) $p<0.001$, *b*) $p<0.05$ by *t* test.

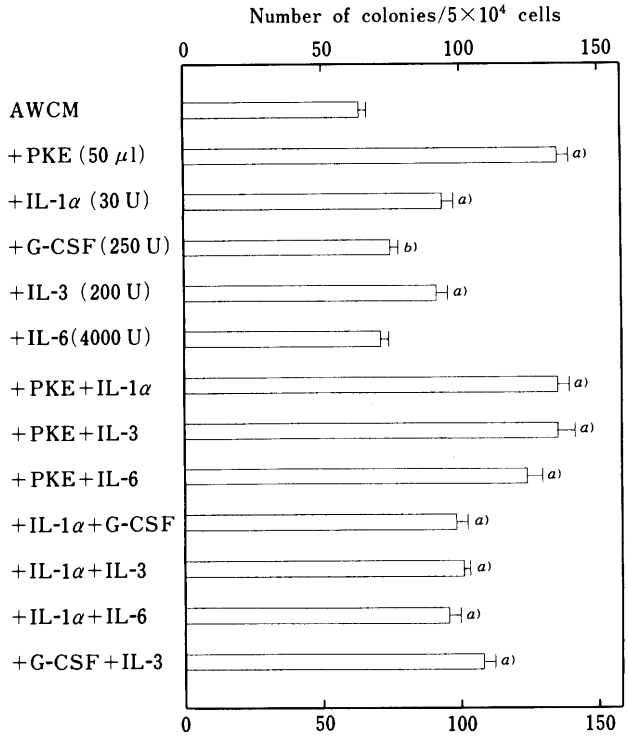


Fig. 3. The Effect of Various Combinations of PKE and Cytokines on Colony Formation Supported by AWCM

Number of colonies stimulated with AWCM plus PKE, recombinant CSF and multiple combinations of each factor. Numbers represent mean ± S.E. of 6 dishes from two separate experiments. *a*) $p<0.001$, *b*) $p<0.05$ by *t* test.

TABLE I. Neutralization of Colony-Promoting Activity IL-1α or PKE by Anti-IL-1α

Addition to culture	No. of colonies[a] /5 × 10⁴ cells	CPA
PBS	69.0 ± 3.4	1.00
PBS + anti-IL-1α[b]	68.6 ± 3.5	0.99
IL-1α[c]	100.0 ± 9.0	1.45[e]
IL-1α + anti-IL-1α	79.8 ± 5.1	0.97
PKE[d]	177.6 ± 9.6	2.57[f]
PKE + anti-IL-1α	177.4 ± 5.3	2.57[f]

a) Mean ± S.E. of 6 dishes from two separate experiments. *b*) anti-IL-1α: 50 μg. *c*) IL-1α: 30 U. *d*) PKE: 50 μl. *e*) $p<0.01$, *f*) $p<0.001$ by *t* test.

TABLE II. The Effect of IL-1α and PKE on Colony Formation Supported by Various CSFs

CSF	No. of colonies/5 × 10^4 cells[a]			
	PBS	PKE[b]	IL-1α[c]	PKE+IL-1α
G-CSF[d]	15.9±1.4 (1.00)	162.3±8.8[i] (10.2)	17.8±1.4 (1.12)	135.8±7.6[i] (8.54)
GM-CSF[e]	60.0±1.4 (1.00)	150.9±5.7[i] (2.52)	78.3±3.1[i] (1.31)	143.6±3.5[i] (2.39)
CSF-1[f]	23.4±1.9 (1.00)	26.0±2.6 (1.11)	46.8±5.5[j] (2.00)	54.6±8.0[j] (2.33)
IL-3[g]	26.3±1.7 (1.00)	111.7±3.4[i] (4.25)	50.0±2.6[i] (1.90)	104.3±6.7[i] (3.97)
IL-6[h]	3.2±0.65 (1.00)	96.7±6.7[i] (30.2)	8.0±0.82[j] (2.5)	98.5±2.3[i] (30.8)

a) Mean±S.E. of 6 or 9 dishes from two or three separate experiments. *b*) PKE: 50 μl. *c*) IL-1α: 30 U. *d*) G-CSF: 250 U. *e*) GM-CSF: 100 U. *f*) CSF-1: 200 U. *g*) IL-3: 200 U. *h*) IL-6: 4000 U. Each value in parentheses represents CPA. *i*) $p<0.001$ *vs.* PBS. *j*) $p<0.01$ *vs.* PBS.

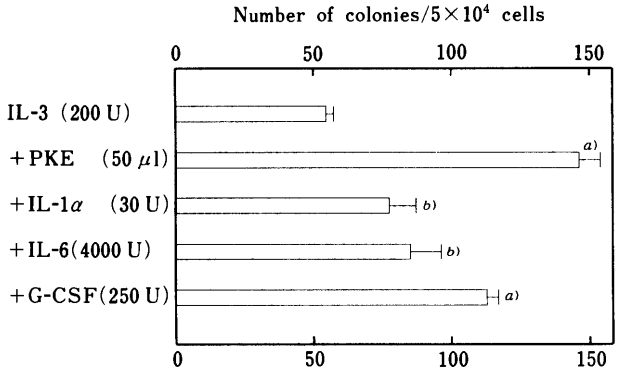


Fig. 4. The Effect of PKE, IL-1α, G-CSF, and IL-6 on Colony Formation Supported by IL-3

Number of colonies stimulated with IL-3 plus G-CSF, IL-6 IL-1α and PKE. Numbers represent mean±S.E. of 6 dishes from two separate experiments. *a*) $p<0.001$, *b*) $p<0.05$ by *t* test.

of G-CSF, GM-CSF, CSF-1, IL-3 or IL-6 as a source of CSF. The results are shown in Table II. By the addition of PKE in combination with G-CSF or IL-6, the number of colonies was 10 and 30 times when compared to G-CSF or IL-6 alone, respectively. When G-CSF or IL-6 was combined with IL-1α, the number of colonies was 1.1 and 2.5 times greater than with G-CSF or IL-6 alone. Furthermore, the combination of PKE plus GM-CSF or IL-3 resulted in much greater CPA than the combination of IL-1α plus GM-CSF or IL-3. On the other hand, the combination of IL-1α plus CSF-1 resulted in a 2.0 times increase in comparison to that of CSF-1 alone, and PKE plus CSF-1 resulted in no synergistic increase in colony number.

Since it has been reported that IL-3 acts synergistically with G-CSF and IL-6 to stimulate murine hemopoietic progenitors, and the synergistic effect of G-CSF or IL-6 is higher than that of IL-1α,[13] we tested the synergistic effects of PKE with IL-3. As shown in Fig. 4, in agreement with others, the combination of IL-3 plus G-CSF or IL-6 resulted in higher CPA than the combination of IL-3 plus IL-1α. However, the combination of PKE plus IL-3 showed much greater activity than the above combinations.

Discussion

In order to investigate the functional difference of of PKE from IL-1α, G-CSF, GM-CSF, CSF-1, IL-3, and IL-6, we examined the CPA of each factor alone and in combination in agar cultures of murine BMC in the presence of AWCM as a source of CSF. The comparable activity to PKE was not obtained in all cases (Figs. 1—3). PKE alone did not stimulate colony formation,[8] while G-CSF, GM-CSF, CSF-1, IL-3, and IL-6 were able to stimulate colony formation by themselves. The results also demonstrated that the CPA of PKE depended upon the type of CSF.

Since IL-1α is known to act synergistically with various growth factors, we compared the CPA of PKE and IL-1α in the presence of various growth factors as a source of CSF (Table II). The combination of PKE plus G-CSF, GM-CSF, IL-3 or IL-6 resulted in much higher CPA than the combination of IL-1α plus one of those factors. However, the combination of PKE plus CSF-1 failed to exhibit the promoting activity, whereas IL-1α plus CSF-1 resulted in an increase of macrophage colonies. It was indicated that the synergistic effect of IL-1α for G-CSF, GM-CSF, IL-3 and IL-6 was significantly less than that of PKE, except for CSF-1. On the other hand, the CPA of PKE was not blocked by anti-IL-1α, showing the probable difference in active site or chemical structure between PKE and IL-1α (Table I). The results concerning IL-1α were consistent with earlier studies by Stanley *et al.*[9]

CSF-1 is known to act on more mature progenitor cells than the IL-3-responsive celles.[14] Thus the target cells of G-CSF, GM-CSF, IL-3 and IL-6 may differ from those of CSF-1 at the differentiation stage. And it could be estimated that the target cell population of CSF-1 and PKE is quite different; possibly the target cell populations of PKE is more primitive than that of CSF-1. This is probably the combination of PKE plus CSF-1 results in no synergistic increase in colony number.

Ogawa *et al.* have reported that hemopoietic growth factors could be classified into three groups according to their functional properties[15]: (1) action on the early stage of stem cells, that is, lineage-nonspecific factors; (2) action on the late stage of stem cells, that is, lineage-specific factors; (3) factors affecting dormant stem cells.

IL-3 and GM-CSF support the proliferation of multipotential hemopoietic progenitors,[16] corresponding to Ogawa's lineage-nonspecific factors, whereas erythropoietin (Epo), CSF-1 and G-CSF act on progenitors commited to the erythrocyte, macrophage and granulocyte lineages, corresponding to the lineage-specific factors.[17-20] However, it has been shown that G-CSF acts on the commited progenitor and induces it to leave a G_0 state and enter a cell cycle to enhance cell proliferations.[21] IL-1α and IL-6 are known to support proliferation of dormant stem cells through a shortening of the G_0 period.[10,11,22]

Recently, Ikebuchi *et al.*[13] have shown that G-CSF and IL-6 act synergistically with IL-3 to stimulate the proliferation of murine hemopoietic progenitor cells, and these synergistic effects are greater than in combination with IL-1α. Then, we assayed the synergistic activity of PKE with IL-3. The combination of PKE plus IL-3 resulted in much greater activity than the other combinations (Fig. 4).

It is suggested that the target cells of PKE are mainly in a G_0 state and PKE enhances the induction into a cell cycle by our previous results in such a way that the proportion of deoxyribonucleic acid synthesizing CSF-responsive cells

is increased by preincubation with PKE, and the PKE-responsive cells are more primitive than CSF-responsive cells.[8,12] Although the functional properties of PKE partially overlap with those of IL-6 or G-CSF, it could be concluded that the active factor(s) in PKE differ from the others since the action spectrum of these factors are different from one another. On the other hand, CPA of PKE has an apparent molecular weight of 140000 (on high-performance liquid chromatography), and is heat labile and trypsin- and *N*-ethylamaleimide-sensitive but dithiothreitol- and periodate-resistant.[8,12]

These findings suggest that the factor(s) in PKE is different from the cytokines tested in this study.

Stanley *et al.* suggested that IL-1α was a synergistic factor (SF) of IL-3 and M-CSF in the CM of the human bladder carcinoma cell line, 5637 cells.[10] McNiece *et al.* suggested the presence of a factor in 5637 cell CM, which is distinct from G-CSF, GM-CSF and IL-6, which synergizes with IL-1 to produce the SF effect.[23] Furthermore, SF(s) was also found in human spleen and placental CM, and it acted synergistically with CSF-1 and IL-3.[24,25] Since PKE did not enhance the colony formation supported by CSF-1, the active factor(s) in PKE seems to differ from these SF.

Most recently, it has been demonstrated that IL-4 has a synergistic effect toward various growth factors.[26,27] IL-4 alone does not support colony formation, but supports the formation of erythroid, granulocyte, macrophage or mast cell colonies in combination with Epo, G-CSF, CSF-1 or IL-3, respectively. However, at least, the main factor in PKE differs from IL-4, since IL-4 does not enhance granulocyte/macrophage colony formation in the presence of GM-CSF or IL-3.

References

1) M. Paran and L. Sachs, *J. Cell. Physiol.*, **72**, 247 (1968).
2) G. Pigoli, A. Waheed, and R. K. Shadduck, *Blood*, **59**, 408 (1982).
3) K. Tsuneoka, Y. Takagi, K. Hirashima, and M. Shikita, *Exp. Hematol.*, **6**, 445 (1978).
4) S. Bol and N. Williams, *J. Cell. Physiol.*, **102**, 233 (1980).
5) Y. Kajigaya, K. Ikuta, Y. Koiso, T. Funabiki, and S. Matsuyama, *Acta Haematol. Jpn.*, **50**, 575 (1987).
6) H. Izumi, M. Tsurusawa, T. Miyanomae, K. Kumagai, and KJ. Mori, *Leuk. Res.*, **7**, 155 (1983).
7) M. Tsurusawa, T. Miyanomae, H. Izumi, and KJ. Mori, *Leuk. Res.*, **7**, 167 (1983).
8) I. Kashiwakura, Y. Hayase, and Y. Takagi, *Yagkugaku Zasshi*, **108**, 984 (1988).
9) S. H. Bartelmez and E. R. Stanley, *J. Cell. Physiol.*, **122**, 370 (1985).
10) E. R. Stanley, A. Bartocci, D. Patinkin, M. Rosendaal, and T. R. Bradley, *Cell*, **45**, 667 (1986).
11) D. Y. Mochizuki, J. R. Eisenman, P. J. Conlon, A. D. Larsen, and R. J. Tushinski, *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 5267 (1987).
12) I. Kashiwakura, Y. Hayase, and Y. Takagi, *Chem. Pharm. Bull.*, **39**, 425 (1991).
13) K. Ikebuchi, J. N. Ihle, Y. Hirai, G. G. Wong, S. C. Clark, and M. Ogawa, *Blood*, **72**, 2007 (1988).
14) K. Koike, E. R. Stanley, J. N. Ihle, and M. Ogawa, *Blood*, **67**, 859 (1986).
15) M. Ogawa, *Hematopoietic Growth Factors*, **3**, 453 (1989).
16) Y. Sonoda, Y-C. Yang, G. G. Wong, S. C. Clark, and M. Ogawa, *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 4360 (1988).
17) C. J. Gregory, *J. Cell. Physiol.*, **89**, 289 (1976).
18) P. L. Ouellette and F. C. Monette, *J. Cell. Physiol.*, **105**, 181 (1980).
19) Y. Ishizaka, K. Motoyoshi, K. Hatake, M. Saito, F. Takaku, and Y. Miura, *Exp. Hematol.*, **14**, 1 (1986).
20) D. Metcalf and N. A. Nicola, *J. Cell. Physiol.*, **116**, 198 (1983).
21) K. Ikebuchi, S. C. Clark, J. N. Ihle, L. M. Souza, and M. Ogawa, *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 3445 (1988).
22) K. Ikebuchi, G. G. Wong, S. C. Clark, J. N. Ihle, Y. Hirai, and M. Ogawa, *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 9035 (1987).
23) I. K. McNiece, A. B. Kriegler, and P. J. Quesenberry, *Blood*, **73**, 919 (1989).
24) A. B. Kriegler, T. R. Bradley, E. Januszewicz, G. S. Hodgson, and E. F. Elms, *Blood*, **60**, 503 (1982).
25) I. K. McNiece, T. R. Bradley, A. B. Kriegler, and G. S. Hodgson, *Exp. Hematol.*, **14**, 856 (1986).
26) K. Kishi, J. N. Ihle, D. L. Urdal, and M. Ogawa, *J. Cell. Physiol.*, **133**, 463 (1989).
27) Y. Sonoda, T. Okuda, S. Yokota, T. Maekawa, Y. Shizumi, H. Nishigaki, S. Misawa, H. Fujii, and T. Abe, *Blood*, **75**, 1615 (1990).

Properties of Androsterone-Sulfating Sulfotransferase in Female Rat Liver

Hiroshi HOMMA, Tsutomu SASAKI, and Michio MATSUI*

Kyoritsu College of Pharmacy, Shibakoen, Minato-ku, Tokyo 105, Japan. *Received November 19, 1990*

Some properties of androsterone (AD)-sulfating sulfotransferase (ST) present in female rat livers were characterized. Based on the substrate specificities of the enzyme preparation obtained by anion exchange chromatography and 3'-phosphoadenosine 5'-phosphate (PAP)-agarose affinity chromatography, AD-ST was supposed to be among isoenzymes of hydroxysteroid STs. The identity of the AD-ST with the isoenzymes of hydroxysteroid ST, however, remains unclear at present. The enzyme preparation revealed a wide range of native molecular weight with a major M_r of some 600000. The AD-ST did not appear to have a homogeneous isoelectric point, because the enzymatic activity was spread over a wide range of the pH gradient, centering around pH 6.6 on chromatofocusing. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the AD-ST showed a subunit with M_r of 30000, which was similar to the hydroxysteroid STs purified previously. Under denaturing conditions the subunit was demonstrated to be composed of three protein species containing distinct pI values (pI 6.1, 6.7 and 7.2). The AD-ST was thus supposed to be an oligomer with high molecular weight, in which the subunits of different pI values are assembled in various association numbers.

Keywords androsterone; hydroxysteroid; sulfotransferase; sulfation; subunit; isoelectric point; oligomer; rat liver

Introduction

Sulfation is an important conjugation reaction and is catalyzed by sulfotransferase (ST), which transfers sulfuric acid from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) not only to various xenobiotics but also to many endogenous compounds such as steroid hormones and neurotransmitters.[1-3] Evidence for the existence of ST isoenzymes, especially in rat liver has been provided by purification of several hydroxysteroid STs (EC 2.8.2.2),[2,4] phenol STs (EC 2.8.2.1),[5] bile salt STs (EC 2.8.2.14),[6-9] and estradiol ST.[10,11]

We have previously observed age-related alterations of the activity of androsterone (AD)-sulfating ST in rat livers.[12] The ST activity increased after birth in parallel in both male and female rats and reached the highest activity at about 20 d of age. In male rats, the activity was subsequently decreasing, while in female rats, after a temporal decline, the activity increased again after 40 d of age and remained at a high level in female adults. The activity in adult females is about 15-fold higher than in adult males. Such marked difference was not observed with other hydroxysteroid-sulfating STs.[13,14] We have been studying the molecular mechanism of these age-related changes of the AD-ST activity and in this communication we characterize some properties of the AD-ST obtained from female rat livers.

Materials and Methods

Materials [9,11-^{3}H]Androsterone (60 Ci/mmol) and [1-^{14}C]4-nitrophenol (56 mCi/mmol) were obtained from New England Nuclear Co., Boston, MA, U.S.A. and Amersham International, Buckinghamshire, England, respectively. Diethylaminoethyl (DEAE)-Toyopearl 650S (superfine) and DEAE-cellulose DE-52 were products of Tosoh Co., Tokyo, Japan and Whatman BioSystems, Ltd., Maidstone, Kent, England, respectively. 3'-Phosphoadenosine 5'-phosphate (PAP)-agarose (type 2), Mono P HR 5/5, standard proteins of ferritin, aldolase and bovine serum albumin (BSA) were acquired from Pharmacia, Uppsala, Sweden. A stainless steel column (i.d. 7.5 × 600 mm) packed with TSK gel G3000SW was a product of Tosoh Co. *N*-Ethylmaleimide (NEM), phenylmethanesulfonyl fluoride (PMSF), Tosyl-lysine chloromethyl ketone (TLCK), diethylpyrocarbonate, phenylglyoxal, androsterone, 17β-estradiol, estrone, taurolithocholic acid, deoxycorticosterone, hydrocortisone, myoglobin of horse heart and β-lactoglobulin A of bovine milk were purchased from Sigma Chemical Co., St. Louis, MO., U.S.A. Cholesterol, dehydroepiandrosterone, 4-nitrophenol, 2-naphthol and polyethylene glycol 20000 were obtained from Wako Chemical Ind., Ltd., Osaka, Japan. PAPS was prepared following the method of Singer.[15] All other reagents were of the highest grade available.

Enzyme Assay AD-ST activity was assayed by a slight modification of the method described previously.[12] Briefly, the reaction mixture (500 μl) contained 100 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 20 μM ethylenediaminetetra acetic acid (EDTA), 100 μM PAPS, a preparation of enzyme and 86 μM [^{3}H]androsterone (350 dpm/nmol). The mixture was incubated at 37 °C for 30 min. The reaction was terminated by placing the tubes in an ice-bath, followed by the addition of 4 ml of dichloromethane. After vigorous mixing, the aqueous and organic phases were separated by centrifugation and a portion of the upper aqueous phase was removed for the determination of radioactivity. For determination of the pH optimum, 500 mM Tris–acetate (pH 5.0—8.0) was included in the incubation mixtures instead of 100 mM Tris–HCl (pH 7.4).

4-Nitrophenol ST activity was determined colorimetrically as described previously[12] or radiometrically as follows: the assay mixture (500 μl) consisted of 100 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 20 μM EDTA, 100 μM PAPS, a preparation of enzyme and 63 μM [1-^{14}C]4-nitrophenol (380 dpm/nmol). The mixture was incubated at 37 °C for 30 min and the tube was placed on ice. One-tenth milliliter of 2 M phosphate buffer (pH 2.1) and 6 ml of a mixture of ether–chloroform (1 : 1 (v/v)) were quickly added. After mixing thoroughly, the aqueous and organic phases were separated by centrifugation and the radioactivity in the aqueous phase was counted. This radiometric assay is a more sensitive method than the colorimetric assay of our previous report,[12] in which the decrease of the absorbance of 4-nitrophenol was measured. Other assay methods were as described previously.[16]

Purification of AD-ST AD-ST was purified from liver cytosol of female Wistar rats essentially following the method previously reported.[16] Briefly liver cytosol was prepared from 8-week old female rats and subjected to both anion exchange chromatography and PAP-agarose affinity chromatography. The step of desalting with Sephadex G-100 column was omitted and DEAE-cellulose or DEAE-Toyopearl 650S was used for the anion exchange chromatography. By the anion exchange chromatography, all of the AD-ST activity in the cytosol was recovered in the eluate with NaCl gradient. As described previously,[16] the AD-ST activity was separated from the 4-nitrophenol- and cortisol-ST activities by DEAE-cellulose chromatography. The AD-ST fraction which contained the highest activity of AD-ST and low (or little) activities of 4-nitrophenol- and cortisol-STs was pooled. This fraction corresponded to "peak I" in the previous report[16] and contained 43% of the AD-ST activity applied on the column. Subsequently this fraction was applied onto a PAP-agarose affinity column and the AD-ST was eluted according to the method of Singer.[17] The AD-ST activity was specifically eluted with 4 mM adenosine diphosphate (ADP), and about 62% of the activity applied was recovered in this ADP-eluate. The final preparation had a specific activity of 7.0 nmol/min/mg protein and was purified about 30-fold relative to liver cytosol. In some experiments, AD-ST fraction obtained by PAP-agarose chromatography was again applied onto the same column PAP-agarose and this time the AD-ST activity was eluted with a linear

gradient of 0—10 mM ADP.

High Performance Liquid Chromatography (HPLC) Gel Filtration Chromatography The molecular weight of the native AD-ST was determined by HPLC gel filtration chromatography. A portion of the fraction of AD-ST purified by PAP-agarose chromatography was concentrated by Centricon-10 (Amicon) and injected onto columns of TSKgel G3000SW (two i.d. 7.5 × 600 mm stainless steel columns in tandem). The column was eluted at a flow rate of 0.4 ml/min with 50 mM Tris–HCl (pH 7.4), 3 mM 2-mercaptoethanol, 0.1 mM EDTA and 250 mM sucrose (buffer A) which also included 0.2 M NaCl. The liquid chromatograph consisted of a Toso HLC-803D liquid delivery pump equipped with a Toso UV-8 model II ultraviolet detector. Elution with buffer A alone resulted in a poor recovery of the AD-ST activity (about 3%), while the inclusion of NaCl in the elution buffer significantly improved the recovery (76%). The fractions of 0.4 ml were collected and assayed for AD-ST activity. The column was calibrated with standard proteins of ferritin (440 kilodaltons (kDa)), aldolase (158 kDa) and BSA (67 kDa). Void volume was determined by blue dextran (Pharmacia).

Chromatofocusing on a Mono P Column A portion of the AD-ST fraction obtained by PAP-agarose chromatography was passed through a NAP-10 column (Pharmacia) to exchange the solvent for a starting buffer of chromatofocusing (25 mM triethanolamine–iminodiacetic acid, pH 8.3), and was applied onto a Mono P HR 5/5 column. The column was eluted at a flow rate of 0.5 ml/min with elution buffer (pH 5.0), which was made of Polybuffer 96, Polybuffer 74 and iminodiacetic acid according to the instructions of the supplier. The liquid chromatograph was as described previously.[18] The AD-ST activity and pH value were determined in each fraction (0.5 ml) and approximately 72% of the activity applied was recovered in the eluate.

Inactivation of AD-ST by Chemical Modifying Reagents Affinity-purified AD-ST fraction was preincubated for 30 min at 25 °C in 50 mM Tris–HCl (pH 7.4) with NEM, PMSF or TLCK of 0, 0.5 or 2 mM. Subsequently, the residual enzyme activity was assayed as described above in 500 mM Tris–acetate (pH 6.0) instead of 100 mM Tris–HCl (pH 7.4). Before the treatment with NEM, the AD-ST fraction was dialyzed against a buffer (buffer A minus 2-mercaptoethanol) to remove 2-mercaptoethanol in the sample without any loss of the enzyme activity. PMSF was dissolved in isopropanol and introduced into tubes to a final concentration of 2% (v/v) isopropanol. About 70% of the initial activity was recovered after incubation with the vehicle alone (2% (v/v) isopropanol solution). For the treatment with diethylpyrocarbonate, the purified AD-ST fraction was preincubated in 50 mM Tris–HCl (pH 6.5) together with the reagent which was dissolved in ethanol. Incubation with the vehicle alone (final 4% (v/v) ethanol solution) resulted in the recovery of 84% of the initial activity. The purified AD-ST fraction was also treated in 125 mM sodium bicarbonate (pH 7.9) with phenylglyoxal of 0, 2.5 or 5.0 mM before the measurement of the residual enzyme activities. The percent inhibition was calculated relative to the enzyme activity with the vehicle alone.

Electrophoresis Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was done as described by Laemmli.[19] Two dimensional gel electrophoresis was performed following the method of O'Farrell.[20] Briefly, in the first dimension, 4% polyacrylamide gel isoelectrofocusing was done in the presence of 2% Nonidet P-40, 8.5 M urea and 2% Pharmalyte 3—10 (Pharmacia). After 12.5% polyacrylamide gel electrophoresis was carried out in the second dimension, protein spots were localized by Coomassie blue staining or silver staining. In some experiments, horse heart myoglobin (pI = 6.8, 7.2, subunit M_r = 16950) and bovine milk β-lactoglobulin A (pI = 5.1, subunit M_r = 18400) were mixed with the sample preparation and electrophoresed. These proteins were used as internal markers for the determination of pI values. Protein was assayed by the method of Lowry *et al.*[21] or the method of Bradford[22] using Bio-Rad reagents and BSA as standard.

Results

HPLC Gel Filtration Chromatography The AD-ST preparation which was obtained by the procedures described in Materials and Methods was electrophoresed on SDS–polyacrylamide gel (Fig. 1). The major protein band with subunit M_r of 30000 was observed in addition to several faint bands with higher M_r. In order to determine the molecular weight of the native form of the AD-ST, the affinity-purified preparation was applied onto HPLC gel filtration chromatography (Fig. 2). On TSK gel G3000 SW column chromatography, the activity was eluted just behind void volume and appeared as a broad peak, suggesting that the AD-ST had a wide range of molecular weight. The fractions containing AD-ST activity invariably displayed the protein band with a subunit M_r of 30000 on SDS–PAGE. The elution position of the highest activity corresponded to M_r of approximately 600000, based on calculations with standard proteins (Fig. 2).

Isoelectric Point of AD-ST The pI value of the affinity-purified AD-ST was measured by chromatography on a Mono P column, a pre-packed column for chromato-

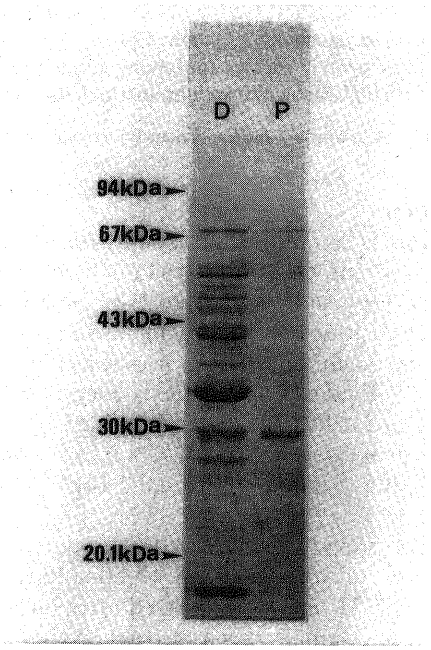


Fig. 1. SDS–PAGE

The pooled fraction of DEAE-cellulose chromatography (lane D) and the purified fraction by PAP-agarose chromatography (lane P) were electrophoresed together with marker proteins of known molecular weights (94 kDa; phosphorylase b, 67 kDa; BSA, 43 kDa; ovalbumin, 30 kDa; carbonic anhydrase, 20.1 kDa; soybean trypsin inhibitor). Proteins were detected by Coomassie blue staining.

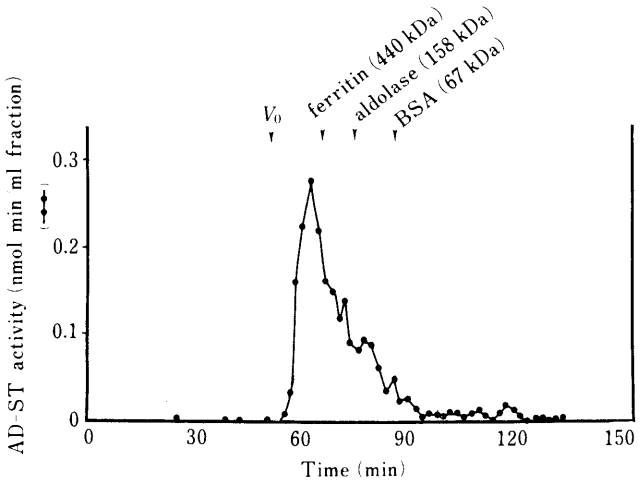


Fig. 2. HPLC Gel Filtration Chromatography of AD-ST

A portion of affinity-purified AD-ST (0.82 mg in 0.5 ml) was injected onto a TSKgel G3000SW column and the column was eluted as described in Materials and Methods. Void volume (V_0, determined with blue dextran) and positions of elutions of standard proteins are also indicated in this figure (ferritin with molecular weight of 440 kDa, aldolase, 158 kDa and BSA, 67 kDa).

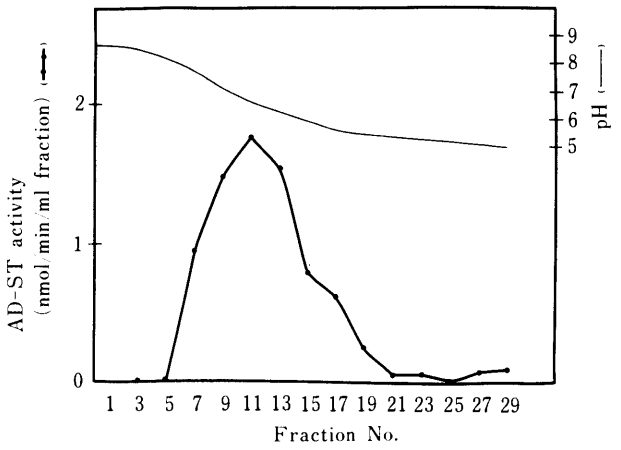


Fig. 3. Chromatofocusing of AD-ST on a Mono P Column

A portion of PAP-agarose-purified AD-ST which contained 0.78 mg protein in 3 ml starting buffer was analyzed by chromatofocusing on a mono P HR 5/5 column. AD-ST activity and pH value were determined all every other fractions. Details are described in Materials and Methods. About 72% of the AD-ST activity applied was recovered in the eluates.

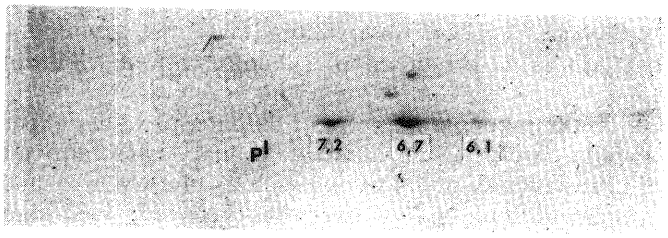


Fig. 4. Two Dimensional PAGE

Affinity-purified AD-ST was analyzed by two dimensional gel electrophoresis. Corresponding region including three spots of the proteins was presented with pI values of each spot.

focusing. The pH gradient from 8.0 to 5.0 was generated and the elution profile is shown in Fig. 3. The AD-ST activity was eluted over a wide range of the pH gradient with the highest activity appearing at pH 6.6. The fractions containing the AD-ST activity invariably showed the protein band with subunit M_r of 30000 on SDS–PAGE.

Two Dimensional Gel Electrophoresis Polyacrylamide gel isoelectrofocusing of the native from of the affinity-purified AD-ST was unsuccessful, probably because the native form was too large to get into the gel. On the contrary, the AD-ST was focussed in polyacrylamide gel in the presence of a nonionic detergent, Nonidet P-40 and urea, although its activity was lost. Figure 4 shows the result of two dimensional gel electrophoresis in which isoelectrofocusing was carried out in the first dimension in the presence of Nonidet P-40 and urea, followed by SDS–PAGE in the second dimension. AD-ST was focussed into three spots of proteins with different pI values. Calculated from the mobilities of the internal pI markers, the pI values of these spots were determined to be 6.1, 6.7 and 7.2 (Fig. 4). The relative amounts of the pI 6.7 and 7.2 spots were apparently comparable to each other, while the pI 6.1 spot was a minor component. A portion of the affinity-purified AD-ST was further purified by rechromatography on a PAP-agarose column with a linear gradient of 0—10 mM of ADP before the analysis by two dimensional gel electrophoresis. The activity appeared in a wide range of the ADP gradient, and the homogeneous fraction with the highest activity still revealed the two protein spots by silver staining on the electrophoretogram. The most acidic spot of the protein was undetectable, probably because of its low content (data not shown).

Other Properties of AD-ST Chemical modifying reagents specific for certain amino acids were examined for their effects on AD-ST activity. TLCK and diethylpyrocarbonate (both are specific for histidine) effectively inactivated the activity: with 0.5 and 2 mM TLCK, respectively, 42% and 100% of the activity was inhibited and with 0.5 mM diethylpyrocarbonate, 100% was inhibited. On the contrary, little inhibition was observed with NEM (specific for cysteine), PMSF (specific for serine or threonine) and phenylglyoxal (for arginine); with 2 mM NEM, 2 mM PMSF and 5 mM phenylglyoxal, respectively, 9.7%, 9.2% and 14% inhibitions were observed.

In order to investigate substrate preference of the purified AD-ST, various phenols and steroids were included in the reaction mixture and their inhibitory effects on the sulfation of radiolabeled AD were determined (Table I). Deoxycorticosterone, 17β-estradiol and dehydroepiandrosterone were extensive inhibitors, while estrone and taurolithocholic acid were less effective. Hydrocortisone was marginally inhibitory and 2-naphthol as well as cholesterol were totally ineffective.

In the pH range from 5.0 to 8.0, maximal activity was observed at pH 6.0.

Discussion

As indicated in Table I, the activity of the AD-sulfating ST obtained from female rat livers was effectively inhibited by dehydroepiandrosterone and 17β-estradiol, which were preferential substrates of hydroxysteroid STs.[23-26] By contrast, little inhibition was observed either with 2-naphthol, a preferred substrate for phenol STs[27] or with cholesterol, a substrate for cholesterol ST.[28] Taurolithocholic acid also had some inhibitory effect on the activity toward AD in accordance with the suggestion that an isoenzyme of bile acid ST is the same protein as an isoform of hydroxysteroid ST.[9] Thus AD-ST was supposed to be among isoforms of hydroxysteroid STs because of these substrate preferences.

An inactivation study with phenylglyoxal indicated that phenol ST of rat liver required arginine residue for its activity.[29,30] This is not the case with AD-ST, suggesting that the structure of the active site of the two STs is dissimilar. In contrast to the observation that little inhibition of AD-ST activity was observed with NEM, which is a specific modifying reagent for cysteine, the sulfhydryl group was reported to be essential for the phenol ST activity in male rat liver[30] and human platelet[31] as well as for the hydroxysteroid STs in female rat liver.[17,26,32] AD-ST may have a distinct structure of the active site from the hydroxysteroid STs so far examined. Studies with TLCK and diethylpyrocarbonate demonstrated that histidine residue may participate in the AD-ST activity. Histidine was also shown to be involved in the phenol ST activity, which was isolated from human intestinal bacterium.[33] How histidine residue is involved in the activity of AD-ST remains to be elucidated.

Several isoenzymes of hydroxysteroid STs have been

TABLE I. Inhibition of AD-ST Activity by Various Compounds

Compound	Concentration (μM)	% inhibition
Dehydroepiandrosterone	5	10
	20	30
Hydrocortisone	5	2
	20	5
Deoxycorticosterone	5	19
	20	41
17β-Estradiol	5	18
	20	40
Estrone	5	6
	20	16
Taurolithocholic acid	5	8
	20	20
2-Naphthol	5	4
	20	0
Cholesterol	5	0
	20	1

The activity of AD-ST at pH 6.0 (500 mM Tris–acetate) was assayed at 37 °C for 30 min in the presence of various compounds. The effects of these compounds on the sulfation of [^{3}H]-AD are presented as percent inhibition.

purified from female rat livers by the Jakoby group,[4,23,24] by Singer and his co-workers[17,25,26] and by Barnes *et al.*[9] and several properties of these enzymes were extensively studied. Among these hydroxysteroid ST isoenzymes, ST1,[23] ST2,[25] BAST1[9] and STI[17] were reported to be oligomers with large molecular weight and to be composed of subunits with M_r of nearly 30000: ST1 (native M_r is 180 kDa, subunit M_r is 28 kDa), ST2 (native M_r 290 kDa, subunit M_r 32 kDa), BAST1 (native $M_r > 200$ kDa, subunit M_r 30 kDa) and STI (native M_r 156 kDa, subunit M_r 30 kDa). Optimum pH for these ST isoenzymes was pH 6.0 for ST1, pH 5.5 for ST2, pH 6.0 for BAST1 and pH 6.0 for STI, respectively. The AD-ST studied in this communication had a pH optimum of 6.0 and had a native M_r of some 600000, which was composed of a subunit of M_r of 30000. These properties were quite similar to those of the hydroxysteroid ST isoenzymes described above, although the identity of the AD-ST with the hydroxysteroid ST isoenzymes is unclear at present.

The pI value of the native AD-ST was determined to be 6.6 by chromatofocusing. The AD-ST activity however, was eluted over a wide range of the pH gradient. In contrast, phenol ST of male rat liver which was recently purified by our group was eluted in a sharp and symmetrical peak by Mono P chromatofocusing (data not shown). Phenol ST III and IV, purified by Sekura and Jakoby[34] was also focussed in a narrow region on isoelectrofocusing. Since the elution profile of the AD-ST by chromatofocusing suggested that the AD-ST did not have a homogeneous isoelectric point, the native AD-ST was subsequently dissociated and denatured into its subunits and was then analyzed by isoelectrofocusing. The enzyme was composed of subunits with M_r of 30000 and with different pI values (pI 6.1, 6.7 and 7.2). In addition, the native AD-ST had a wide range of molecular weight as observed in Fig. 2. Based on these lines of evidence, it was supposed that the AD-ST was an oligomer in which the subunits with distinct pI values were associated with each other in different numbers and that the AD-ST, therefore, showed multiple native M_r as well as a wide range of isoelectric point. Ryan and Carrol[32] purified dehydroepiandrosterone ST from female rat liver and observed that the enzymatic activity was spread over a wide range of pH on isoelectrofocusing. They also reported that the enzyme did not chromatograph as a single molecular species on gel filtration. These properties were quite similar to those of the AD-ST described in this study. The earlier authors, however, did not describe the subunit of the enzyme and consequently its molecular weight and pI value are unknown. The pI values of the purified STs, ST1, ST2, BAST1 and STI were not examined under denaturing conditions.

It is possible that the three protein spots with different pI values are ascribable to the impurity of the final preparation. The affinity-purified AD-ST, however, still revealed at least two protein spots even after rechromatography on PAP-agarose. We have recently obtained two purified preparations of AD-ST from weanling female rats (20 days of age) as well as from adult rats (100 days of age). It was found that the pI values of the native forms of these two preparations were distinct. Furthermore, the relative amounts of the subunits were consistently different in these two preparations on two dimensional gel electrophoresis.[35] These observations favor the assumption that AD-ST is composed of subunits with different pI values.

It is still unknown whether the different pI values of the subunits results from different primary structures or posttranslational modifications such as glycosylation and phosphorylation. No evidence has been obtained that cytosolic STs are glycosylated. By immunoblot analysis, the three subunit molecules with distinct pI values were revealed to be present in fresh liver cytosol,[36] indicating that the three protein spots did not result from artifacts of the protein purification.

Recently Ogura *et al.*[37] purified a ST from female rat liver, which activated various carcinogenic hydroxymethyl-arenes and characterised it as one of the isoforms of hydroxysteroid STs. They also isolated many complementary deoxyribonucleic acid (cDNA) clones of the enzyme from rat liver cDNA library with polyclonal antisera raised against it and elucidated the nucleotide sequences of two cDNAs sharing a strong homology and encoding approximately 33 kDa proteins.[38,39] This indicated that there existed at least two subunit molecules which had the same molecular weight but had different primary structures and possibly had different pI values. Further investigations are necessary on the primary structures of the subunits with different pI values described in this communication.

References and Notes

1) G. J. Mulder and W. B. Jakoby, "Conjugation Reactions in Drug Metabolism," ed. by G. J. Mulder, Taylor & Francis, London, 1990, pp. 107—161.
2) S. S. Singer, "Biochemical Pharmacology and Toxicology," Vol. 1, ed. by D. Zakim and D. A. Vessey, John Wiley & Sons Inc., New York, 1985, pp. 95—159.
3) W. B. Jakoby, M. W. Duffel, E. S. Lyon, and S. Ramaswamy, "Progress in Drug Metabolism," Vol. 8, ed. by J. W. Bridges and L. F. Chasseaud, Tayloy & Francis, London, 1984, pp. 11—33.
4) E. S. Lyon, C. J. Marcus, J-L. Wang, and W. B. Jakoby, *Methods Enzymol.*, 77, 206 (1981).
5) R. D. Sekura, M. W. Duffel, and W. B. Jakoby, *Methods Enzymol.*, 77, 197 (1981).
6) L. J. Chen, *Methods Enzymol.*, 77, 213 (1981).

7) H. Takikawa, A. Stolz, and N. Kaplowitz, *FEBS Lett.*, **207**, 193 (1986).
8) R. H. Collins, L. Lack, K. M. Harman, and P. G. Killenberg, *Hepatology*, **6**, 579 (1986).
9) S. Barnes, E. S. Buchina, R. J. King, T. McBurnett, and K. B. Taylor, *J. Lipid Res.*, **30**, 529 (1989).
10) J. M. Green and S. S. Singer, *Can. J. Biochem. Cell Biol.*, **61**, 15 (1983).
11) Y. Sugiyama, A. Stolz, M. Sugimoto, J. Kuhlenkamp, T. Yamada, and N. Kaplowitz, *Biochem. J.*, **224**, 947 (1984).
12) M. Matsui and H. K. Watanabe, *Biochem. J.*, **204**, 441 (1982).
13) S. S. Singer, D. Giera, J. Johnson, and S. Sylvester, *Endocrinology*, **98**, 963 (1976).
14) S. S. Singer, *Biochim. Biophys. Acta*, **539**, 19 (1978).
15) S. S. Singer, *Anal. Biochem.*, **96**, 34 (1979).
16) M. Matsui and F. Nagai, *J. Pharmacobio-Dyn.*, **8**, 1048 (1985).
17) S. S. Singer, *Arch. Biophys. Biochem.*, **196**, 340 (1979).
18) H. Takanashi, H. Homma, and M. Matsui, *Chem. Pharm. Bull.*, **37**, 1583 (1989).
19) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
20) P. H. O'Farrell, *J. Biol. Chem.*, **250**, 4007 (1975).
21) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
22) M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
23) E. S. Lyon and W. B. Jakoby, *Arch, Biophys. Biochem.*, **202**, 474 (1980).
24) C. J. Marcus, R. D. Sekura, and W. B. Jakoby, *Anal. Biochem.*, **107**, 296 (1980).
25) S. S. Singer, J. Gebhart, and E. Hess, *Can. J. Biochem.*, **56**, 1028 (1978).
26) S. S. Singer and L. Bruns, *Can. J. Biochem.*, **58**, 660 (1980).
27) W. B. Jakoby, R. D. Sekura, E. S. Lyon, C. J. Marcus, and J-L. Wang, "Enzymatic Basis of Detoxication," Vol. 2, ed. by W. B. Jakoby, Academic Press, Inc., New York, 1980, pp. 199—228.
28) J. I. Rearick, P. W. Albro, and A. M. Jetten, *J. Biol. Chem.*, **262**, 13069 (1987).
29) R. T. Borchardt and C. S. Schasteen, *Biochem. Biophys. Res. Commun.*, **78**, 1067 (1977).
30) R. T. Borchardt, C. S. Schasteen, and S-E. Wu, *Biochim. Biophys. Acta*, **708**, 280 (1982).
31) J. A. Heroux and J. A. Roth, *Mol. Pharmacol.*, **34**, 194 (1988).
32) R. A. Ryan and J. Carrol, *Biochim. Biophys. Acta*, **429**, 391 (1976).
33) D-H. Kim, L. Konishi, and K. Kobashi, *Biochim. Biophys. Acta*, **872**, 33 (1986).
34) R. D. Sekura and W. B. Jakoby, *Arch. Biochem. Biophys.*, **211**, 352 (1981).
35) M. Takahashi, H. Homma, and M. Matsui, unpublished observations.
36) We have recently obtained a further purified and homogeneous preparation of AD-ST and have raised rabbit polyclonal antisera against it (I. Nakagome, H. Homma, and M. Matsui, unpublished observations).
37) K. Ogura, T. Sohtome, A. Sugiyama, H. Okuda, A. Hiratsuka, and T. Watabe, *Mol. Pharmacol.*, **37**, 848 (1990).
38) K. Ogura, J. Kajita, H. Narihata, T. Watabe, S. Ozawa, K. Nagata, Y. Yamazoe, and R. Kato, *Biochem. Biophys. Res. Commun.*, **165**, 168 (1989).
39) K. Ogura, J. Kajita, H. Narihata, T. Watabe, S. Ozawa, K. Nagata, Y. Yamazoe, and R. Kato, *Biochem. Biophys. Res. Commun.*, **166**, 1494 (1990).

Effects of a High α-Linolenate and High Linoleate Diet on Hemolysis and Lipid Peroxidation of Rat Erythrocytes

Keiko SAKAI and Harumi OKUYAMA*

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabedori, Mizuho-ku, Nagoya 467, Japan.
Received November 26, 1990

α-Linolenic acid (18:3*n*−3) is known to autoxidize several fold faster than linoleic acid (18:2*n*−6). Feeding a high α-linolenate or a high linoleate diet to rats resulted in significant changes in the *n*−3/*n*−6 ratios of 20 and 22 carbon highly unsaturated fatty acids in erythrocytes. However, the rates of hemolysis observed in N_2- or O_2-atmosphere were similar between the two dietary groups. No significant amounts of conjugated dienes were detected and no measurable changes in the fatty acid compositions were observed during the incubations, indicating that the hemolysis occurred without involving significant lipid peroxidation. When stimulated with a free radical initiator, [2,2′-azobis-(2-amidinopropane)dihydrochloride] (AAPH), hemolysis occurred more rapidly, conjugated dienes formed and unsaturated/saturated ratios of phospholipid fatty acids decreased. However, no statistically significant difference was observed in these parameters of the two dietary groups. These results indicate that hemolysis occurs without involving lipid peroxidation but is accelerated by free radicals through lipid peroxidation, and that the difference in autoxidizabilities of α-linolenate and linoleate is not reflected in the rates of hemolysis and autoxidation in rat erythrocytes.

Keywords dietary fatty acid; α-linolenate; linoleate; hemolysis; lipid peroxidation; erythrocyte; α-linolenic acid; linoleic acid

Introduction

Beneficial effects of feeding α-linolenic acid as compared with linoleic acid have been recognized in several animal models of chronic diseases.[1-4] However, unfavorable side effects have been suspected for long-term feedings of *n*−3 fatty acids since *n*−3 fatty acids are generally more easily autoxidizable than the corresponding *n*−6 fatty acids with the same carbon chains.[5-7] In fact, the rate of lipid peroxidation of membrane lipids is reported to be faster in the fish oil supplemented group than in the control diet group.[8-10] According to a free radical theory, free radicals generated in cells attack polyunsaturated fatty acids (PUFA) to form lipid peroxides, which in turn decompose to produce more free radicals. This kind of chain reaction is presumed to enhance the progress of aging, tumorigenesis and atherosclerosis.[11-14] Researchers who discuss human nutrition based on this free radical theory often advise people not to take a large amount of *n*−3 fatty acids. However, the results of our *in vivo* experiments were against this autoxidation-related lipid peroxide theory; a high α-linolenate diet, as compared with a high linoleate diet, suppressed aging[1,15] as well as tumorigenesis[16,17] in rats. Thus, the autoxidizability of fatty acids in diets does not appear to be directly correlated with the ability to produce and proliferate free radicals *in vivo*.

In this paper, we examined a possible relationship among the autoxidizability of dietary fatty acids, peroxidizability of membrane lipids and red cell stability. Use of a high α-linolenate (perilla seed oil) diet and a high linoleate (safflower seed oil) diet would allow us to examine mainly the effects of α-linolenate and linoleate because these two diets are roughly similar in their amounts of saturated fatty acids, mono unsaturated fatty acids and tocopherols.

Materials and Methods

Diets and Animals Male Sprague-Dawley rats (Shizuoka Laboratory Animals Co., Shizuoka, Japan) at 3 weeks of age were fed experimental diets for 17 or 37 weeks. The diet consisted of conventional laboratory chow (Nihon Clea Co., Tokyo, Japan) treated with hexane to remove endogenous lipids and then supplemented with vegetable oils (10 or 15%) and a vitamin mixture (2%) (Nihon Clea Co.).[18] Either safflower seed oil (rich in linoleate) or perilla seed oil (rich in α-linolenate) (Ohta Oil Co., Okazaki, Japan) was used. Each oil contained 0.1% vitamin E and the diet contained a total of 25 mg vitamin E/100 g. Diets with peroxide values below 50 meq/kg were served. These diets brought about no differences in growth rates and in appearance as compared with a conventional diet. The fatty acid compositions of the diets are given in Table I.

Preparation of Washed Erythrocytes and Hemolysis Assay Rats were anesthetized with nembutal and exsanguinated by abdominal aorta in the presence of 3.13% sodium citrate as an anticoagulant (1 : 5, v/v). Red cells were prepared by centrifugation and washed three times with phosphate-buffered saline.[19] Erythrocytes were suspended in phosphate-buffered saline (pH 7.4) containing 20 mM glucose at a packed cell volume of 2.5%. To keep the suspension sterile during incubations, penicillin (200 unit/ml, Meiji), streptomycin (1 mg/ml, Meiji) and fungizone (10 μl/ml, Gibco) were added. Either oxygen (95% O_2 and 5% CO_2) or nitrogen (N_2) were gassed through the incubation mixture for 2 min. Hemolysis was followed at 540 nm by measuring the hemoglobin content of the supernatant after its conversion into cyanomethemoglobin (Wako hemoglobin kit, Wako Co., Ltd.).

When the effect of 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), an azo compound, was examined, AAPH was added at a concentration of 74 mM to 20% erythrocytes suspended in phosphate-buffered saline (pH 7.4). The mixture was shaken gently during the incubation at 37 °C.

Analysis of Fatty Acid Composition and Measurement of Conjugated Dienes Lipids were extracted from erythrocytes with chloroform : methanol (2 : 1, v/v), and phospholipids were separated by thin-layer chromatography.

TABLE I. Fatty Acid Composition of the Diets[a]

Fatty acid	% of total fatty acids	
	High linoleate (safflower)-diet	High α-linolenate (perilla)-diet
14:0	0.1	0.1
16:0	7.9	8.1
18:0	1.8	2.3
18:1*n*−9	17.3	11.8
18:2*n*−6	17.1	74.2
18:3*n*−3	51.7	1.0
20:4*n*−6	0.1	0.0
20:5*n*−3	0.7	0.7
22:6*n*−3	0.3	0.3
n−3/*n*−6	3.0	0.02

a) The diet contained 10% high linoleate oil (safflower oil) or high α-linolenate oil (perilla oil). The fatty acid compositions (w/w %) were quantitated by gas chromatography. Fatty acids are expressed by the carbon chain: the number of double bond, and the first double bond numbered from the methyl terminus is designated as *n*−9, *n*−6 or *n*−3.

Fatty acids were analyzed as methyl esters by gas chromatography as described previously.[20] Conjugated dienes were estimated by measuring the absorbance at 233 nm ($\varepsilon = 23000\,M^{-1}cm^{-1}$) in cyclohexane solution.[21,22]

Results

Fatty acid compositions of erythrocyte phospholipids are shown in Fig. 1. Despite a significant difference in the proportions of linoleate and α-linolenate in the diets, the proportion of linoleate varied little and no significant amount of α-linolenate was found even in the high α-linolenate group. The major difference was observed in the 20 and 22 carbon highly unsaturated fatty acids (20 : 4*n*−6 *vs.* 20 : 5*n*−3 and 22 : 4*n*−6 *vs.* 22 : 5*n*−3).

Hemolysis of erythrocyte suspension was followed for up to 7 d. Under the conditions, the extent of hemolysis reached a plateau level (70%) within 24 h (Fig. 2). The rate of hemolysis tended to be slightly higher in the high linoleate group than in the high α-linolenate group but the difference was not statistically significant. When incubation atmosphere (O_2–CO_2) was replaced with nitrogen, the rate of hemolysis was affected a little. Quite similar results were obtained by using diets with 15% oils instead of 10% (data not shown).

Fatty acid compositions of the erythrocytes were determined before and after the incubations. Even after 7 d of incubation, no significant changes in the fatty acid compositions were observed (data not shown). These results indicate that the highly unsaturated fatty acids (HUFA) are quite resistant to autoxidation when present in the membranes in the presence of antibiotics, which was confirmed by measuring the production of conjugated dienes; very little conjugated diene was detected (below 10 nmol/mg fatty acids).

When a free radical generating agent, AAPH, was added to the incubation mixture at a concentration of as high as 74 mM, hemolysis occurred more rapidly[23] (data not shown). Changes in the fatty acid compositions of the erythrocytes during the 3 h incubation with AAPH were observed as shown in Fig. 3. The decrease in the proportions of 20 : 4*n*−6 and 22 : 6*n*−3 with a concomitant increase in the proportion of palmitate (16 : 0) was clearly seen in the high linoleate group and similar changes were also observed in the high α-linolenate group. In accordance with the decrease in highly unsaturated fatty acids, significant amounts of conjugated dienes were formed (Fig. 4). However, no statistically significant difference was observed between the two dietary groups.

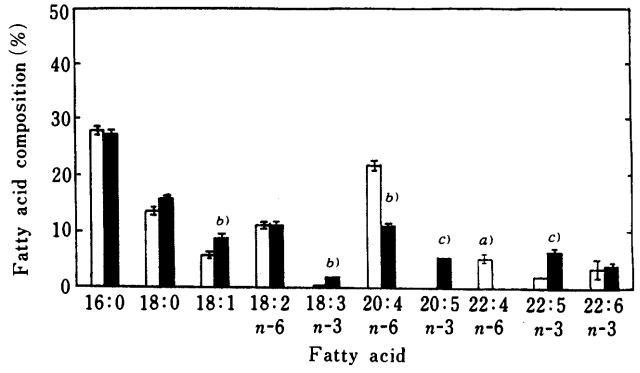


Fig. 1. Fatty Acid Compositions of Rat Erythrocyte Total Lipids

Sprague-Dawley rats were fed a diet supplemented with a high linoleate oil (safflower oil) or a high α-linolenate oil (perilla oil) for 17 weeks. Figures represent means ± S.E. for 3 rats. Statistical significance by Student's *t*-test is shown as *a*) $p<0.05$, *b*) $p<0.01$ or *c*) $p<0.001$ for the high linoleate *vs.* the high α-linolenate groups. ■, high-α-linolenate; □, high-linoleate.

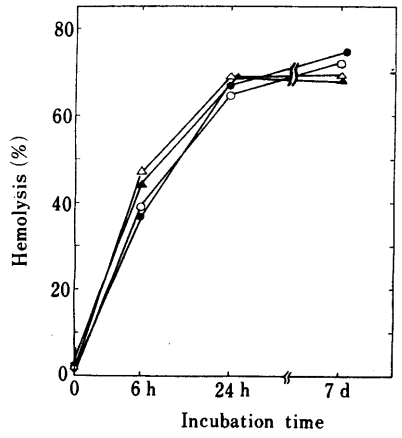


Fig. 2. Effect of Dietary *n*−3/*n*−6 Balance on Hemolysis of Rat Erythrocytes

Each point represents the mean for 3 rats, each assayed in duplicate. Rats fed either the high α-linolenate or high linoleate diet for 7 weeks were used.

	N_2	O_2
High α-linolenate:	●	○
High linoleate:	▲	△

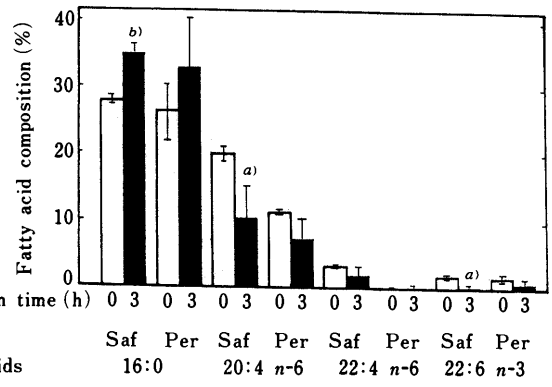


Fig. 3. Effect of AAPH on Fatty Acid Compositions of Total Lipids of Erythrocytes

Values are means ± S.E. for 3 or 4 rats. Statistical significance by Student's *t*-test is shown as *a*) $p<0.05$ or *b*) $p<0.01$.

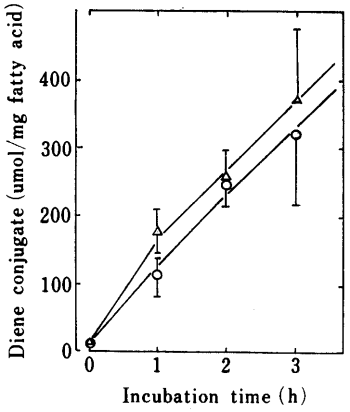


Fig. 4. AAPH-Stimulated Lipid Peroxidation Measured as Diene Conjugate Formation

Sprague-Dawley rats were fed test diets for 37 weeks. AAPH was added at a concentration of 74 mM to 20% erythrocytes suspended in phosphate buffered saline (pH 7.4). Each point represents the mean ± S.E. of determinations for 4 to 5 rats. △, high-linoleate; ○, high-α-linolenate.

Discussion

Cell damages by free radicals and lipid peroxides have been clearly shown *in vitro* in the absence of appropriate amounts of antioxidants.[24-27] However, vitamins E and C serve as endogenous antioxidants and the causal relationship between the lipid peroxide formation *in vivo* and pathogenesis of tumor, aging and atherosclerosis still remains to be clarified. The present results indicate that HUFA in the membranes are quite resistant to autoxidation in the presence of endogenous antioxidants; no significant diene formations nor losses of HUFA were observed even after 7 d of incubation under sterile conditions.

Although conjugated dienes are formed from the methylene bridges of fatty acids and are more specific indices of lipid peroxidation, their measurement is semiquantitative and less sensitive than TBA-RS.[28] Under similar conditions, Einsele *et al.*[19] observed volatile hydrocarbon formation possibly as a consequence of lipid peroxidation in human red blood cells, but the amount of hydrocarbons produced was very little; below 4 nmol/mg fatty acid/5 d (unpublished observations). Recently, Fraga *et al.*[29] examined the effects of dietary PUFA on the lability of red blood cells in humans. Although arachidonic acid content was correlated with lipid peroxidation in the stronger oxidative conditions (in the presence of hydroperoxide), variations in PUFA content in the human diet did not affect the rate of oxidative reactions in red blood cell membranes. While preparing this manuscript, Miyagawa reported that lipid peroxide concentrations measured by chemiluminescens were not significantly different in the liver, brain or blood of rats fed diets containing perilla oil, safflower oil or olive oil.[30]

We have previously shown that these high α-linolenate and high linoleate diets induce no measurable difference in erythrocyte deformabilities, whole blood viscosities or hematological indices in rats.[31] Therefore, the beneficial effects of increasing α-linolenate in diets, as compared with linoleate, which were observed in suppressing the aging process and tumorigenesis *in vivo*,[15-17,32] can not be ascribed to the difference in the peroxidizabilities of $n-3$ and $n-6$ fatty acids nor in the physicochemical properties of membranes so far measured. Needless to say, the balance of $n-3$ and $n-6$ eicosanoids, which are other kinds of peroxidation products of HUFA, is affected by these diets and is probably deeply involved in these diseases.[33] However, easily oxidizable compounds are generally good antioxidants. Therefore, we point out another possibility that easily autoxidizable fatty acids serve more effectively *in vivo* as free radical scavengers rather than free radical propagators, trapping generated free radicals. The resultant lipid peroxides would be converted to more stable hydroxy fatty acids in the presence of appropriate amounts of endogenous antioxidants (vitamins E, C and glutathione) and peroxidases. In their absence, HUFA may propagate free radicals which damage cellular components, leading to the enhancement of pathogenic disorders. This interpretation is consistent with an observation that a free radical initiator, AAPH, induced lipid peroxidation only after the consumption of endogenous vitamin E in erythrocytes[23,34] as reported in details by Frei *et al.*[35] The results by Hammer *et al.*[8] are also consistent with this interpretation; the rate of ascorbate-induced lipid peroxidation in rat liver microsomes was faster in the group with fish oil supplement than in the group with corn oil supplement with more vitamin E, but was even slower in the fish oil group in the presence of comparable amounts of vitamin E in the diets. Relatively good correlation observed *in vitro* between the degree of unsaturation of membrane lipids and peroxidizability of the membranes appear not to be applicable directly to the phenomena *in vivo*.

References

1) T. Shimokawa, A. Moriuchi, T. Hori, M. Saito, Y. Naito, H. Kabasawa, Y. Nagae, M. Matsubara, and H. Okuyama, *Life Sci.*, **43**, 2067 (1988).
2) A. Hashimoto, M. Katagiri, S. Torii, J. Dainaka, A. Ichikawa, and H. Okuyama, *Prostaglandins*, **36**, 3 (1988).
3) S. Watanabe, E. Suzuki, N. Kojima, Y. Suzuki, and H. Okuyama, *Chem. Pharm. Bull.*, **37**, 1572 (1989).
4) O. Adam, G. Wolfram, and N. Zollner, *J. Lipid Res.*, **27**, 421 (1986).
5) Y. Yamamoto, E. Niki, and Y. Kamiya, *Bull. Chem. Soc. Jpn.*, **55**, 1548 (1982).
6) J. P. Cosgrove, D. F. Church, and W. A. Pjryor, *Lipids*, **22**, 299 (1987).
7) S-Y. Cho, K. Miyashita, T. Miyazawa, K. Fujimoto, and T. Kaneda, *J. Am. Oli Chem. Soc.*, **64**, 876 (1987).
8) C. T. Hammer and E. D. Wills, *Biochem. J.*, **174**, 585 (1978).
9) J. Mounie, B. Faye, M. Magdalou, F. Goudonnet, R. Truchot, and G. Siest, *J. Nutr.*, **116**, 2034 (1986).
10) B. E. Leibovitz, M. L. Hu, and A. L. Tappel, *Lipids*, **25**, 125 (1990).
11) D. Harman, *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 7124 (1981).
12) D. Harman, *Age*, **7**, 111 (1984).
13) K. Yagi, *Chem. Phys. Lipids*, **45**, 337 (1987).
14) M. E. Begin, *Chem. Phys. Lipids*, **45**, 269 (1987).
15) N. Yamamoto, Y. Okaniwa, S. Mori, M. Nomura, and H. Okuyama, *J. Gerontol.*, **46**, B17 (1990).
16) T. Hori, A. Moriuchi, H. Okuyama, T. Sobajima, K. Tamiya-Koizumi, and K. Kojima, *Chem. Pharm. Bull.*, **35**, 3925 (1987).
17) M. Hirose, A. Masuda, N. Ito, K. Kamano, and H. Okuyama, *Carcinogenesis*, **11**, 731 (1990).
18) N. Yamamoto, M. Saitoh, A. Moriuchi, M. Nomura, and H. Okuyama, *J. Lipid Res.*, **28**, 144 (1987).
19) H. Einsele, M. R. Clements, and H. Remmer, *Arch. Toxicol.*, **60**, 163 (1987).
20) K. Sakai, K. Ueno, Y. Ogawa, and H. Okuyama, *Chem. Pharm. Bull.*, **34**, 2944 (1986).
21) J. A. Buege and S. D. Aust, "Method in Enzymology," Vol. 52, Academic Press, New York, 1978, p. 302.
22) M. J. Gibian and P. Vandenberg, *Anal. Biochem.*, **163**, 343 (1987).
23) M. Miki, H. Tamai, M. Mino, Y. Yamamoto, and E. Niki, *Arch. Biochem. Biophys.*, **258**, 373 (1987).
24) T. Nakayama, M. Kaneko, and M. Kodama, *Agric. Biol. Chem.*, **50**, 261 (1986).
25) V. M. Samokyszyn, D. M. Miller, D. W. Reif, and S. D. Aust, *J. Biol. Chem.*, **264**, 21 (1989).
26) A. Benedetti, A. F. Casini, M. Ferrali, and M. Comporti, *Biochem. J.*, **180**, 303 (1979).
27) S. K. Jain, *J. Biol. Chem.*, **259**, 3391 (1984).
28) M. L. Hu, E. N. Frankel, B. E. Leibovitz, and A. L. Tappel, *Lipids*, **119**, 1574 (1989).
29) C. G. Fraga, A. L. Tappel, B. E. Leibovitz, F. Kuypers, D. Chiu, J. M. Lacono, and D. S. Kelley, *Lipids*, **25**, 111 (1990).
30) T. Miyazawa, *Nippon Nogeikagaku Kaishi*, **64**, 831 (1990).
31) K. Sakai, H. Okuyama, K. Kon, N. Maeda, M. Sekiya, T. Shiga, and R. C. Reitz, *Lipids*, **25**, 793 (1990).
32) K. Kamano, H. Okuyama, R. Konishi, and H. Nagasawa, *Anticancer Res.*, **9**, 1903 (1990).
33) W. E. M. Lands, "Fish and Human Health," Academic Press, Inc., Orlando, 1986.
34) Y. Yamamoto, E. Niki, J. Eguchi, Y. Kamiya, and H. Shimasaki, *Biochim. Biophys. Acta*, **819**, 29 (1985).
35) B. Frei, R. Stocken, and B. N. Ames, *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 9748 (1988).

Lipid Peroxidation of the Erythrocyte Membrane Caused by Stimulated Polymorphonuclear Leukocytes in the Presence of Ferritin

Toshiaki MIURA* and Taketo OGISO

Department of Biochemistry in Hokkaido Institute of Pharmaceutical Sciences, 7-1 Katsuraokacho, Otaru, 047-02, Japan. *Received November 30, 1990*

Lipid peroxidation of erythrocyte membrane was caused by phorbol myristate acetate (PMA)-stimulated polymorphonuclear leukocytes (PMN) in the presence of ferritin. PMN themselves were not peroxidized. A lag period was observed before the start of the peroxidation reaction. In contrast, ferritin iron was continuously released by PMA-stimulated PMN, suggesting that accumulation of free iron in the reaction system was important for proceeding of the peroxidation reaction. Superoxide dismutase, catalase, hydroxyl radical scavengers and an iron chelator, diethylenetriaminepenta-acetic acid, inhibited the lipid peroxidation, indicating that the lipid peroxidation is initiated by a hydroxyl radical generated from the interaction of H_2O_2 with ferrous iron released from ferritin.

Keywords lipid peroxidation; erythrocyte membrane; polymorphonuclear leukocyte; ferritin

During inflammation, neutrophils and macrophages infiltrate tissues and produce reactive oxygen metabolites such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2).[1] These compounds not only attack microorganisms[2] but also cause tissue damage including deoxyribonucleic acid (DNA) degradation[3] and dysfunction of microtubules.[4] However, neither O_2^- nor H_2O_2 is considered to be of sufficient reactivity to directly initiate oxidation of cellular constituents.[5] Therefore, the detrimental effects of O_2^- and H_2O_2 have been thought to be due to more reactive radicals such as hydroxyl radicals ($HO^\cdot$). The conversion from O_2^- and H_2O_2 to $HO^\cdot$ requires trace metals.[6] The most widely accepted mechanism is the iron-catalyzed Haber–Weiss reaction.[7]

Iron in tissues except for heme-iron is predominately stored as ferritin, a large multisubunit protein in which iron is stored as ferric micelles.[8] It is known that iron release from ferritin requires reduction to the ferrous state[9] and O_2^- can readily release iron from ferritin.[10] Biemond *et al.* demonstrated that iron in ferritin was rapidly mobilized by stimulated neutrophils.[11] These findings suggest a possibility that the presence of ferritin enhances the tissue damages caused by infiltrated neutrophils. If highly reactive oxygen radicals are produced by infiltrated neutrophils, they may primarily attack the cell membrane. Xanthine oxidase which generates O_2^- and H_2O_2 during its conversion of xanthine to urate is capable of releasing iron from ferritin and promoting the lipid peroxidation of microsomal liposomes.[12] However, the lipid peroxidation caused by polymorphonuclear leukocytes (PMN) has still not been investigated. In the present study, we used the erythrocyte membrane as a model membrane to demonstrate that PMA-stimulated PMN caused lipid peroxidation of the plasma mambrane in the presence of ferritin.

Experimental

Materials Horse spleen ferritin, superoxide dismutase (SOD, from bovine erythrocytes), catalase (from bovine liver, thymol free) and phorbol myristate acetate (PMA) were obtained from Sigma Chemical Co., Ltd., St. Louis, Mo. U.S.A. Bathophenanthroline sulfonate was from Dojindo Laboratories Co. Other chemicals were obtained as analytical grade from commercial suppliers.

Preparation of PMN PMN were isolated from peritoneal cavities of Hertley guinea pig to which 5% casein (15—29 ml/animal) had been injected 12 h before the animals were killed. PMN were washed three times with physiological saline, treated hypoosmotically to lyse erythrocytes if necessary, and were suspended in Krebs-Ringer phosphate buffer pH 7.4 at a concentration of 10^8 PMN/ml.

Preparation of Ferritin and Erythrocyte Membrane To remove a trace iron, commercial ferritin was incubated at 4 °C in 10 mM phosphate buffer pH 7.4 containing 10 mM ethylenediaminetetra acetic acid (EDTA) and dialyzed against 10 mM phosphate buffer pH 7.4. The dialysis was continued for 1 d with three changes of buffer. Apoferritin was prepared as described previously.[13] Erythrocyte membrane of guinea pigs were prepared by the method described in a previous paper.[14]

Iron Release from Ferritin and Lipid Peroxidation Iron release from ferritin was continuously monitored in the reaction mixture contained 5×10^6 cells/ml, 10 ng of PMA, 1 mM bathophenanethroline sulfonate and ferritin in Krebs–Ringer phosphate buffer. Lipid peroxidation was determined according to Uchiyama *et al.*[15] The reaction was terminated by mixing 3 ml of samples with 1.0 ml of 4% phosphoric acid and 0.6% thiobarbituric acid (TBA). To prevent formation of TBA reactive products during the heating, 0.1 ml of 0.1% butylated hydroxytoluene in dimethylsulfoxide was added to the reaction mixture. After heating for 15 min at 100 °C, the solution was cooled and 3 ml of butanol was added. After centrifugation for 2 min at $1500 \times g$, absorbance by the butanol layer at 535—520 nm was measured with a Shimazu spectrophotometer UV-3000.

Results

PMN-Induced Lipid Peroxidation of Erythrocyte Membrane As summarized in Table I, PMN stimulated with PMA in the presence of ferritin could produce malondialdehyde (MDA) from the erythrocyte membrane. Neither PMA nor PMN alone induced the lipid peroxidation. In the absence of ferritin, no significant MDA formation was observed. Displacement of ferritin by apoferritin did not cause any lipid peroxidation, indicating involvement of ferritin iron in the lipid peroxidation. Omission of erythrocyte membranes from the reaction

TABLE I. Characteristics of Lipid Peroxidation of the Erythrocyte Membrane Caused by PMA-Stimulated PMN

Conditions	MDA formed (nmol/h)
Complete	11.5±1.6
−PMA	0.6±0.3
−PMN	0.3±0.2
−erythrocyte membrane	0.7±0.1
−ferritin	0.3±0.2

Complete reaction mixture contained 5×10^6 cells/ml, 10 ng of PMA, erythrocyte membrane (400 μg protein) and 100 μg ferritin in 3.0 ml of Krebs–Ringer phosphate buffer pH 7.4. The reaction mixture was incubated for 1 h at 37 °C. MDA was determined as described in Experimental. Values represent mean±S.D. of triplicate experiments.

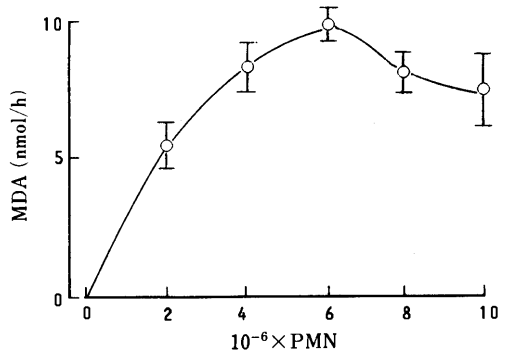


Fig. 1. Effect of PMN Number on the Lipid Peroxidation of Erythrocyte Membrane

Reaction mixture contained various numbers of PMN, 10 ng/ml of PMA, 33 μg/ml of ferritin and 400 μg protein of erythrocyte membrane in 3.0 ml of Krebs–Ringer phosphate buffer. Values represent the mean ± S.D. of triplicate experiments.

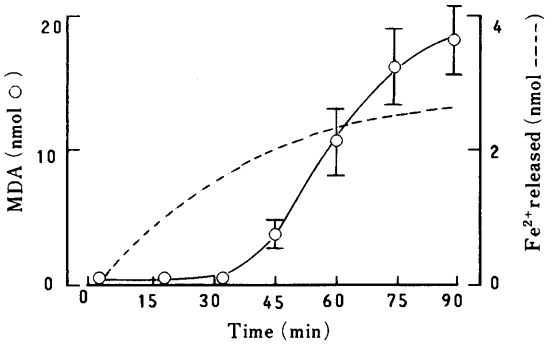


Fig. 2. Time Course of the Lipid Peroxidation and Iron Release from Ferritin

MDA formation was determined in the reaction mixture containing 5×10^6 cells/ml, 10 ng of PMA, 400 μg protein of erythrocyte membrane and 33 μg/ml of ferritin in Krebs–Ringer phosphate buffer. Each point represents the mean ± S.D. of triplicate experiments. Iron release from ferritin was measured as described in the Experimental section.

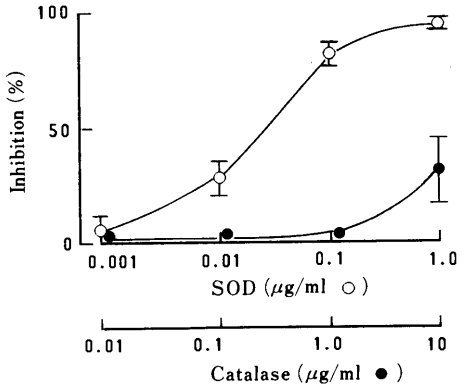


Fig. 3. Effect of SOD and Catalase on the Lipid Peroxidation

Various concentrations of SOD and catalase were added to the reaction mixture contained 5×10^6 cells/ml, 10 ng of PMA, 400 μg protein of erythrocyte membrane and 33 μg/ml of ferritin in Krebs–Ringer phosphate buffer. After incubation for 1 h at 37 °C, MDA was determined.

system did not cause MDA formation, indicating that PMN themselves were not peroxidized. Figure 1 shows the relationship between the number of PMN and lipid peroxidation. MDA formation was proportional to the number of PMN lower than 6×10^6 cells/ml, but fell off slightly at a higher concentration. Therefore, all data presented here obtained with the number of cell at 5×10^6 cells/ml.

TABLE II. Effect of Hydroxyl Radical Scavengers and DETAPAC on the Lipid Peroxidation

Additions	Conc. (mM)	MDA formed (nmol/h)	Inhibition (%)
None	—	10.7±2.5	—
Mannitol	10.0	6.1±2.3	43.0
	100.0	1.8±0.3	83.2
Benzoate	10.0	7.8±2.5	27.1
DETAPAC	1.0	0.6±0.2	94.4

Except for lack of SOD or catalase, conditions were the same as described in Fig. 3. Values represent mean ± S.D. of triplicate experiments.

Lipid Peroxidation and Iron Release from Ferritin As shown in Fig. 2, a lag period of about 30 min was observed before significant lipid peroxidation occurred. After the lag period, the peroxidation reaction rapidly proceeded with time. In contrast, ferritin iron was continuously released by PMA-stimulated PMN. To determine a possible role of O_2^- and H_2O_2 in the lipid peroxidation, experiments were performed in the presence of SOD or catalase. As shown in Fig. 3, as little as 0.1 μg/ml of SOD inhibited the lipid peroxidation by about 90%. This amount of heat-denatured SOD had no inhibitory effect on the lipid peroxidation, however, 1.0 μg/ml of the heat-denatured enzyme had some activity. Iron release from ferritin was completely inhibited by 10 μg/ml SOD (data not shown). Catalase had an inhibitory effect at a concentration of 10 μg/ml on the lipid peroxidation but not on iron release from ferritin. No amount of heat-denatured catalase did not significantly inhibited the lipid peroxidation. These results indicate that the lipid peroxidation of erythrocyte membrane is dependent upon O_2^- and H_2O_2.

Involvement of Hydroxyl Radical in the Lipid Peroxidation It is considered that O_2^- or H_2O_2 is insufficient to initiate the lipid peroxidation. Therefore, we investigated a possibility that HO· was involved in the lipid peroxidation. As shown in Table II, mannitol and benzoate which were hydroxyl radical scavengers inhibited the lipid peroxidation in concentration dependent fashion. An iron chelator, diethylenetriaminepentaacetic acid (DETAPAC), which was proposed to prevent the reduction of ferric iron by O_2^-,[16] completely inhibited the lipid peroxidation. These results suggest that the lipid peroxidation of the erythrocyte membrane is initiated by HO· which is generated *via* an O_2^- dependent Fenton reaction (Haber–Weiss reaction).

Discussion

Neutrophils themselves are damaged by their own oxidative products such as O_2^- or H_2O_2, leading to decreased functions in the cells.[17] Phagocyting neutrophils also inactivate their own granulocyte enzymes.[18] Asbeck *et al.* have demonstrated that an excess of iron inhibits the phagocytic function of PMN.[19] In the present study, however, the PMN plasma membrane was not peroxidized by oxygen radicals. This finding expects that some strong antioxidant systems are present in the cells. We determined SOD contents in PMN used here as 0.2 μg/10^7 cells. This amount of SOD almost agreed with that reported by Ishigame *et al.*[20] and was enough to inhibit the peroxidation reaction caused by PMA-stimulated PMN. Furthermore,

viability of PMN, assessed by measuring extracellularly leaked lactate dehydrogenase (LDH), was over 90% in the course of the lipid peroxidation reaction, indicating that PMN themselves are resistant to the toxicity of lipid peroxides.

Superoxide can easily penetrate biological membranes.[21] Weiss has demonstrated that O_2^- generated from PMA-stimulated neutrophils interacts with intracellular hemoglobin to cause damage to erythrocyte membranes.[22] The present study demonstrated that lipid peroxidation of the erythrocyte membrane was caused by PMA-stimulated PMN in the presence of ferritin with a lag period of about 30 min. On the other hand, ferritin iron was continuously released by PMA-stimulated PMN, probably leading to accumulation of free iron in the reaction system. The reduction of Fe^{3+} to Fe^{2+} in the ferritin core is essential[9] for iron release from ferritin. Presumably, the accumulation of the free iron plays a crucial role for the initiation of the lipid peroxidation. The hydroxyl radical is the most widely proposed initiator of lipid peroxidation. The present results suggests that O_2^- generated in PMN could release iron from ferritin with which H_2O_2 generated from O_2^- could react to produce $HO^\cdot$ *via* Haber–Weiss reaction.

Neutrophils stimulated with opsonized zymosan in the absence of iron causes the production of ethylene which may result from the interaction of $HO^\cdot$ with methional.[23,24] However, free radicals other than $HO^\cdot$ have been suggested in similar reactions.[25-27] By spin trapping, Britgan *et al.* demonstrated that human neutrophils stimulated with PMA could not produce $HO^\cdot$ unless iron is present.[28,29] We can not completely rule out a possibility that generation of another species like the ferryl or perferryl iron is involved in the lipid peroxidation. Deoxyribose is oxidized by $HO^\cdot$ to form TBA reactive products.[30] By using this method, we tried to detect $HO^\cdot$ in the reaction system of PMN. However, a significant degradation of deoxyribose was not observed. Probably, $HO^\cdot$ generated in the present experiment may be too low to be detected by deoxyribose degradation.

During inflammation, infiltrated PMN may be stimulated with various materials. Superoxide produced by stimulated PMN possibly releases iron from the ferritin to enhance the damage of tissue. Plasma membranes seem to be extremely susceptible to the attack of stimulated PMN.

References

1) B. M. Babior, R. S. Kipnes and J. T. Curnutte, *J. Clin. Invest.*, **52**, 741 (1973).
2) R. B. Johnston, B. Keele, H. P. Misra, J. E. Lehmeyer, L. S. Webb and K. V. Baehner, *J. Clin. Invest.*, **55**, 1357 (1975).
3) H. C. Birnboim, *Science*, **215**, 1247 (1982).
4) J. M. Oliver, D. F. Albetini and R. D. Berlin, *J. Cell. Biol.*, **71**, 921 (1976).
5) D. T. Sawyer and J. S. Valentine, *Acc. Chem. Res.*, **14**, 393 (1981).
6) B. Halliwell and J. M. C. Gutteridge, *Biochem. J.*, **219**, 1 (1984).
7) J. M. McCord and D. Day, Jr., *FEBS Lett.*, **86**, 139 (1978).
8) P. M. Harrison, *Semin. Hematol.*, **14**, 55 (1977).
9) T. Jones, R. Spencer and C. Walsh, *Biochemistry*, **17**, 4011 (1978).
10) B. J. Bolann and R. J. Ulvik, *Biochem. J.*, **243**, 55 (1987).
11) P. Biemond, H. G. Van Eijk, A. T. G. Swaak and J. F. Koster, *J. Clin. Invest.*, **73**, 1576 (1984).
12) C. E. Thomas, L. A. Morehouse and S. D. Aust, *J. Biol. Chem.*, **260**, 3275 (1985).
13) T. Miura and K. Sakurai, *Life Sci.*, **43**, 2145 (1988).
14) T. Miura and T. Ogiso, *Chem. Pharm. Bull.*, **30**, 3662 (1982).
15) M. Uchiyama and M. Mihara, *Anal. Biochem.*, **86**, 271 (1978).
16) G. R. Buettner, T. P. Doherty and L. K. Patterson, *FEBS Lett.*, **158**, 143 (1983).
17) R. L. Baehner, L. A. Boxer, J. M. Allen and J. Davis, *Blood*, **50**, 327 (1977).
18) A. A. Voetman, R. S. Weening, M. N. Hamers and L. J. Meerhof, *J. Clin. Invest.*, **67**, 1541 (1981).
19) B. S. Van Asbeck, J. J. M. Marx, A. Struyvenberg, J. H. Van Kats and J. Verhoef, *J. Immunol.*, **132**, 851 (1984).
20) K. Ishigame and Y. Nishi, *Clin. Chem.*, **31**, 1094 (1985).
21) R. E. Lynch and I. Fridovich, *J. Biol. Chem.*, **253**, 1838 (1978).
22) S. J. Weiss, *J. Biol. Chem.*, **255**, 9912 (1980).
23) A. I. Tauber and B. M. Babior, *J. Clin. Invest.*, **60**, 374 (1977).
24) S. J. Weiss, P. K. Rustagi and A. F. LeBulio, *J. Exp. Med.*, **147**, 316 (1978).
25) S. J. Klebanoff and H. Rosen, *J. Exp. Med.*, **148**, 490 (1978).
26) W. A. Pryor and R. H. Tang, *Biochem. Biophys. Res. Commun.*, **81**, 498 (1978).
27) A. I. Tauber, T. G. Gabif and B. M. Babior, *Blood*, **53**, 666 (1979).
28) B. E. Britigan, G. M. Rosen, Y. Chai and M. S. Cohen, *J. Biol. Chem.*, **261**, 4426 (1986).
29) B. E. Britigan, G. M. Rosen, B. Y. Thompson, Y. Chai and M. S. Cohen, *J. Biol. Chem.*, **261**, 17026 (1986).
30) J. M. C. Gutteridge, *FEBS Lett.*, **128**, 343 (1981).

Some Cytotoxicological Aspects of Ethyl and Fluoroethyl Alkanesulfonates in *Escherichia coli*: Role of Fluorine Substitution

Yuko OHTA, Kazuhiko TAKAHASHI, Kohfuku KOHDA, and Yutaka KAWAZOE*

Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabedori, Mizuoho-ku, Nagoya 467, Japan. *Received December 10, 1990*

Several fluoroethyl derivatives of alkanesulfonates and *N*-nitrosourea were tested for cytotoxicity and mutagenicity in *E. coli* K12 AB1157. Cytotoxicity was potentiated by fluorine substitution in the alkyl moiety of the ethylating agents. Mutagenicity was strongly suppressed by fluorine substitution in the alkanesulfonates, but not in the *N*-nitrosourea. The capacity to induce the SOS repair network was suppressed, as was mutagenicity, in alkanesulfonates, but not in *N*-nitrosourea. The potentiating effect of fluorine on the cytotoxicity of alkanesulfonates seems to be due to an as yet unknown killing mechanism. An appreciable suppressive effect on the mutagenicity and the SOS induction is worth notice for the biological role of fluorine substitution in alkylating agents.

Keywords fluorine effect; alkylating agent; fluoroethyl; cytotoxicity; mutagenicity; genotoxicity; alkanesulfonate

Fluorine-substitution sometimes produces improvements in the medicinal efficacy of lead compounds. It is thought that this modulation of the biological characteristics of some chemicals stems from the ability of fluorine to mimic hydrogen in terms of steric requirements for binding biological molecules, including enzymes and receptor sites, but to act differently from hydrogen in terms of electronic effect on certain biological reaction processes.[1,2] We previously reported[1] that the cytotoxicity of alkyl alkanesulfonates towards cultured leukemia L1210 cells increased dramatically by fluorine-substitution of the parent sulfonates, including anti-CML (chronic myeloid leukemia) busulfan. The cytotoxicity of a series of alkyl alkanesulfonates was subjected to multi-regression analysis to determine the relationship between structure and cytotoxicity. Correlation was satisfactorily described as a linear combination of the rate of hydrolysis (alkylating ability) and the capacity factor for partition. However, for some derivatives containing fluorine in the alkyl moiety of alkanesulfonates there was no correlation.[1] The cytotoxicity of 2-fluoroethyl and 2,2-difluoroethyl trifluoroethanesulfonate was significantly greater than expected from the regression equation. In connection with deoxyribonucleic acid (DNA)-death thereby concerned, the present study was undertaken to explore the biological role of fluorine substitution in the cytotoxicity and mutagenicity of several simple ethylating agents in *E. coli*. Three pairs of ethylating and 2-fluoroethylating agents were chosen as simple model compounds: ethyl methanesulfonate (EMS)[1] and 2-fluoroethyl methanesulfonate (EMS(0-1))[1]; ethyl trifluoroethanesulfonate (EES(3-0))[1] and 2-fluoroethyl trifluoroethanesulfonate (EES(3-1))[1]; and *N*-ethyl-*N*-nitrosourea (ENU)[3] and *N*-(2-fluoroethyl)-*N*-nitrosourea (F-ENU).[4] 2,2-Difluoroethyl and 2,2,2-trifluoroethyl esters[1] of trifluoroethanesulfonic acid (EES(3-2) and EES(3-3), respectively) were also examined.

Materials and Methods

The alkylating agents used were EMS, EMS(0-1), EES, EES(3-0), EES(3-1), EES(3-2), EES(3-3), ENU, and F-ENU, and all were prepared in our laboratory as previously reported.[1,2] The tester strain for the mutation assay, *E. coli* K12 AB1157,[5] was a gift from Dr. M. Sekiguchi of Kyushu University. The tester strain for the assay of gene expression was *E. coli* CSH26 (*ara*, *Δ(lac-pro)*, *thi*)[6] transformed with pSK1002 plasmid carrying the *umuDC'-lacZ'* fused gene,[7] which was also a gift from Dr. Sekiguchi. K-Medium was an M9 medium[6] (consisting of 42 mM Na_2HPO_4, 22 mM KH_2PO_4, 0.05% NaCl, 19 mM NH_4Cl, 0.1 mM $CaCl_2$, and 1 mM $MgSO_4$) supplemented with 1% casamino acids and 1% glucose. The semi-enriched medium (SEM) agar plate was prepared with Vogel–Bonner's medium E (consisting of 9.5 mM citric acid, 57 mM K_2HPO_4, 17 mM $NaNH_4HPO_4$, and 0.81 mM $MgSO_4$) supplemented with 0.4% glucose, 0.004% casamino acids, 1 μg/ml thiamine, 100 μg/ml of each amino acid (histidine, proline, threonine, and leucine), and 1.5% agar.

Assay for Mutagenicity The cells cultured overnight in an LB medium[6] (consisting of 1% Bacto tryptone, 0.5% Bacto yeast extract, and 1% NaCl) were collected by centrifugation and resuspended in an equal volume of 1/15 M phosphate buffer (pH 6.8). To 0.2 ml of the cell suspension, 0.75 ml of the phosphate buffer and 0.05 ml of dimethyl sulfoxide containing the test compound were added. This mixture was incubated at 37 °C for 30 min with shaking. To measure the number of surviving cells, the reaction mixture was appropriately diluted with the buffer and layered on an SEM plate with 2.5 ml of 0.7% molten top agar containing 100 μg/ml arginine. The plates were incubated at 37 °C for 2 d, and the number of colonies was counted. For measurement of revertant colonies, the cells in the residual reaction mixture were collected by centrifugation, resuspended in 1 ml of the buffer and then layered on an SEM plate with 2 ml of 0.7% molten top agar. The plates were incubated at 37 °C for 2 d and the number of Arg^+ revertant colonies formed was counted. The mutation frequency was calculated as $(M-M_0)/N$, where M and M_0 are the number of revertant colonies obtained from the test compound-containing reaction mixture and the control mixture, respectively, and N is the number of surviving colonies obtained from 1 ml of the test compound-containing reaction mixture.

Assay for Activation of Gene Expression by β-Galactosidase Activity The assay for β-galactosidase induction was carried out as previously described.[8] Briefly, an overnight culture of the tester strain grown in LB medium containing 30 μg/ml of ampicillin was inoculated into a 100-fold volume of *k*-medium containing 30 μg/ml of ampicillin. This was incubated at 37 °C for 2 h. To 1.9 ml of the cell culture, 0.1 ml of dimethyl sulfoxide containing the test compound was added. After incubation at 37 °C for 2 h with shaking, β-galactosidase activity was quantified using the method of Miller.[6]

Results

Cytotoxicity in *E. coli* K12 AB1157 Cells Cells were treated for a 30 min period with several ethylating and fluoroethylating agents. Cytotoxicity after 48 h incubation was expressed as IC_{10}, the concentration which produces 90% killing or 10% survival (Table I). IC_{10} values varied widely (over a 10^4-fold range) and a similar variation was noted in the rate of hydrolysis (alkylating ability).[1,3] In order to compare the cytotoxic efficacy of these agents, the term "consumed dose (mM)" was used in the present study to indicate the dose consumed during exposure of the cells for 90% killing, (initial concentration for 90% inhibition) − (final concentration after 30 min exposure). The consumed dose was calculated using $C_0(1-e^{-kt})$, where C_0

TABLE I. Cytotoxicity of Ethylating and Fluoroethylating Agents in *E. coli* K12 AB1157

Alkylating agent	Alkyl moiety	Hydrolysis rate[a] (1/h)	Capacity factor[b]	IC_{10} (mM)	Consumed dose[c] for 90% killing (mM)	F-Effect on[d] consumed dose (90% killing)
Methanesulfonates						
EMS	CH_3CH_2–	0.105	0.366	360.00	18.4	(1.00)
EMS (0-1)	FCH_2CH_2–	0.0034	nd	>300.00	>0.51	>0.03
Ethanesulfonate						
EES	CH_3CH_2–	0.109	0.574	245	13.0	
Trifluoroethanesulfonates						
EES (3-0)	CH_3CH_2–	5.78	0.811	2.40	2.27	(1.00)
EES (3-1)	FCH_2CH_2–	2.10	0.644	0.62	0.40	0.18
EES (3-2)	F_2CHCH_2–	2.77	0.789	0.25	0.19	0.08
EES (3-3)	F_3CCH_2–	nd	1.161	0.14	nd	
Nitrosoureas						
ENU	CH_3CH_2–	2.79	1.673	54.00	40.6	(1.00)
F-ENU	FCH_2CH_2–	10.4	1.373	9.5	9.45	0.23

a) Pseudo-first order rate constant (k) for *N*-nitrosoureas in 1/15 M phosphate buffer (pH 6.8) and for sulfonates in 1/4 M phosphate buffer (pH 7.4) (refs. 3 and 1, respectively). *b*) A measure for partition property estimated from the retention time by HPLC (refs. 1 and 3). *c*) Dose consumed for 90% killing, *i.e.*, mM-decrease in the concentration of the agent within a given cell-exposure period. *d*) Cytotoxic efficacy of fluoroethylation *vs.* ethylation: Ratio of the consumed dose of a fluoroethylating agent resulting in 90% growth inhibition versus that of the corresponding ethylating agent with the same leaving group. nd: Not determined.

is the initial concentration for 90% killing, k is the pseudo-first order rate constant for hydrolysis in a phosphate buffer at 37 °C as previously reported,[3] and t is 0.5 h of exposure, the period required under the present assay conditions for lethal toxicity and mutagenicity. Provided that cytotoxicity of the agents may be attributed to their alkylating ability, the efficacy of fluorine substitution to produce lethal damage in the cell is expressed as the ratio of the consumed dose (the dose consumed for bioalkylations and hydrolysis) of the fluoroethylating agent *versus* that of the corresponding ethylating agent with the same leaving group. The alkylation mechanism, including chemoselectivity, relates to the type of DNA lesion to be induced in the cell, and depends largely on the class of the leaving group. The effect of fluorine substitution is shown as "F-effect on the consumed dose for 90% killing" in Table I. It is apparent that fluorine substitution markedly increased the cytotoxicity of the sulfonates, as well as that of *N*-nitrosourea.

Table I also includes the capacity factor (k'),[1,3] which is a measure of partition property estimated from the retention time on high-performance liquid chromatography (HPLC). As we previously reported,[1] the cytotoxicity of alkyl alkanesulfonates in cultured leukemia L1210 cells correlated well with the pseudo-first order rate constant for hydrolysis (k) and the capacity factor k', and can be described as their linear combination, unless fluorine atom(s) are substituted in the alkyl moiety of the sulfonates[1]:

$$\log(1/IC_{50}) = 0.487 \log k + 0.392 \log k' + 0.271 \quad (1)$$
$$(r = 0.997, n = 7)$$

The equation indicates that the higher rate of hydrolysis and greater lipophilicity of the sulfonate enhances their cytotoxicity in this mammalian cell line. In the above study,[1] it was revealed that fluoroethyl sulfonates, EES(3-1) and EES(3-2), showed much lower IC_{50} values (0.04 mM in both cases) than the values calculated by the regression Eq. 1 (0.44 and 0.36 mM, respectively) obtained from non-fluorine alkylating agents. Based on the tentative assumption that the cytotoxicity of non-fluorine sulfonates toward *E. coli* in the present study could also be described by a linear combination of k and k', a correlation might be expressed for EMS, EES, and EES(3-0), as follows, although this is not a regression equation because there are only three data points:

$$\log(1/IC_{10}) = 1.099 \log k + 0.761 \log k' - 1.148 \quad (2)$$

It is worth noting that the observed IC'_{10}s of EES(3-1) and EES(3-2), 0.62 and 0.25 mM, respectively, are considerably lower than those calculated by the above Eq. 2 which gave 8.69 and 5.50 mM, respectively.

TABLE II. Mutagenicity of Ethylating and Fluoroethylating Agents in *E. coli* K12 AB1157

	MF at IC_{10}[a] ($\times 10^6$)	F-Effect[b] on MF/IC_{10}	F-Effect on[c] SOS induction
Methanesulfonates			
EMS	5.6	(1.00)	0.33
EMS (0-1)	0.0[d]	<0.014	0.06
Ethanesulfonates			
EES	2.0		0.29
Trifluoroethanesulfonates			
EES (3-0)	1.5	(1.00)	0.36
EES (3-1)	0.0[d]	<0.053	0.14
EES (3-2)	0.0[d]	<0.053	0.18
EES (3-3)	0.0[d]	<0.053	0.07
Nitrosoureas			
ENU	40.00	(1.00)	(1.00)
F-ENU	25.00	0.63	0.84

a) Mutation frequency at IC_{10}. *b*) Fluorine effect on mutagenicity: Ratio of (MF at IC_{10}) of a fluoroethylating agent *versus* that of the corresponding ethylating agent with the same leaving group. *c*) Ratio of the maximum level of activation of *umuDC* gene expression: Gene activating capacity at an equi-toxic dose to that of ENU. *d*) Background level of mutation frequency in *E. coli* K12 AB1157 was approximately 8×10^{-8}.

Mutagenicity in *E. coli* K12 AB1157 Cells Mutagenicity is given in Table II in terms of "mutation frequency (MF) at IC''_{10}, that is, the mutagenicity at an equi-toxic dose or the relative efficacy of mutagenicity to cytotoxicity. It is noteworthy that none of the fluoroethylating alkanesulfonates were demonstrated to be mutagenic, although all the corresponding ethylating agents were mutagenic to some degree. Fluoroethyl nitrosourea was as mutagenic as

its parent ENU. The F-effect on mutagenicity at an equitoxic dose is given as the ratio of mutation frequency at IC_{10} induced by a fluoroethylating agent *versus* that of the corresponding parent ethylating agent, as shown in Table II. It is probable that the fluorine substitution in the alkyl moiety of the sulfonates greatly enhanced cytotoxic efficacy compared to the mutagenic efficacy of the sulfonates. Alternatively, and less probable, it suppressed their mutagenic efficacy itself. In contrast, a fluoroethyl derivative of *N*-nitrosourea showed mutagenic efficacy as high as the parent ENU at an equi-toxic dose.

Capacity to Activate DNA Repair Genes It is known that cytotoxic and mutagenic DNA damage is effectively repaired by DNA repair enzyme systems. In *E. coli*, one of the inducible regulatory networks of the genes encoding DNA repair enzymes is the SOS network which includes at least 15 genes such as *recA*, *lexA*, *uvrA*, and *umuDC*.[9-11] Most of these provide for error-free rescue from various types of DNA damage that can lead to killing and mutation. However, *umuDC* is an error-prone repair gene leading to mutation. Since induction of the SOS network is known to be triggered by a variety of chemical injuries to DNA, an ability to induce the SOS repair network is often used as a marker for genotoxicity of chemicals, including alkylating agents.[9-12] The capacity to induce this SOS repair network was assayed by measurement of the β-galactosidase activity in *E. coli* CSH26 transformed with a plasmid carrying the *umuDC'-lacZ'* fused gene.[7] The values listed in Table II are the ratio of the maximum level of gene activation normalized with that induced by ENU, a typical ethylating agent (details to be published elsewhere). The maximum level of activation corresponds roughly to the gene activating capacity at an equi-toxic dose (IC_{50}). It is worth noting that fluorine substitution suppressed the induction of the SOS network, judging from remarkable suppressions of *umuDC* gene expression. We can provide no explanation for this suppression at present.

Discussion

In the present study, the cytotoxicological aspects of alkylation with the fluoroethyl group were investigated using fluoroethyl alkanesulfonates and *N*-nitrosourea. These fluoroethylating agents are known to react with the nucleophilic sites of the nucleic acid bases.[13,14] It was confirmed that some hydrolyzed products, *i.e.*, 2-fluoroethanol, 2,2,2-trifluoroethanol, and 2,2,2-trifluoroethanesulfonic acid, were proven to be non-cytotoxic by themselves. Since none of the fluoroethylating sulfonates exhibited mutagenicity, even at toxic doses, it appears that fluorine substitution strongly suppressed the mutagenicity of the parent ethylating sulfonates. Remarkable suppression of the SOS inducing ability supports this possibility. Alternatively, fluorine substitution may so greatly enhance the cytotoxic capacity of the sulfonates that mutagenicity could not emerge in the observable dose range. In other words, it seems that enhanced cytotoxicity induced by fluorine substitution on the alkyl moiety of sulfonates is not attributed to an increase in genotoxic alkylation leading to DNA death. Therefore, the present results, together with our previous findings[1] using the mammalian cell line L1210, suggest that an as yet unknown mechanism may be operating in the cytotoxicity of fluoroethyl sulfonates, distinct from their alkylating ability and partition property. Taking into account that fluorine substitution in ENU did not produce any appreciable effects on its cytotoxicity and mutagenesis, it is strongly suggested that fluoroethyl sulfonates, but not nitrosoureas, might produce a cytotoxic effect through an as yet unknown mechanism, which might be related to the inhibitory effect on enzymes or the induction of disorder in membrane functions of the cell.

Acknowledgements Thanks are due to Professor Mutsuo Sekiguchi of Kyushu University for the tester strains of bacteria. This work was partly supported by a Grant-in-Aid for Scientic Research from the Ministry of Education, Science and Culture of Japan.

References

1) Y. Ohta, K. Kohda, H. Kimoto, T. Okano, and Y. Kawazoe, *Chem. Pharm. Bull.*, **36**, 2410 (1988).
2) T. Kato, Y. Ohta, Y. Suzumura, K. Kohda, H. Kimoto, and Y. Kawazoe, *Jpn. J. Cancer Res.*, **79**, 1048 (1988).
3) K. Kohda, S. Ninomiya, K. Washizu, K. Shiraki, M. Ebie, and Y. Kawazoe, *Mutat. Res.*, **177**, 219 (1987).
4) W. Lijinski and A. W. Andrews, *Mutat. Res.*, **68**, 1 (1979).
5) P. Howard-Flanders, E. Simson, and L. Theriot, *Genetics*, **49**, 237 (1964).
6) J. H. Miller, "Experiments in Molecular Genetics," Cold Spring Harbor Laboratory, New York, 1972.
7) H. Shinagawa, T. Kato, T. Ise, K. Makino, and A. Nakata, *Gene*, **23**, 167 (1983).
8) K. Takahashi, M. Sekiguchi, and Y. Kawazoe, *Mutat. Res.*, **230**, 127 (1990).
9) G. C. Walker, *Microbiol. Rev.*, **48**, 60 (1984).
10) J. W. Little and D. W. Mount, *Cell*, **29**, 11 (1982).
11) E. M. Witkin, *Bacteriol. Res.*, **40**, 869 (1976).
12) Y. Oda, S. Nakamura, I. Oki, T. Kato, and H. Shinagawa, *Mutat. Res.*, **147**, 219 (1985).
13) D. T. Beranek, *Mutat. Res.*, **231**, 11 (1990).
14) W. P. Tong, M. C. Kirk, and D. B. Ludlum, *Biochem. Pharmacol.*, **32**, 2011 (1983).

Human Pre-interleukin 1α and β: Structural Features Revealed by Limited Proteolysis

Yoshiro KOBAYASHI,*[,1] Joost J. OPPENHEIM, and Kouji MATSUSHIMA

Laboratory of Molecular Immunoregulation, Biological Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD 21701-1013 U.S.A. *Received December 10, 1990*

Both pre-interleukin 1α and β (pre IL 1α and β) are proteolytically processed into extracellular mature forms of IL 1α and β. Since pre IL 1α is shown to be biologically active, there may be other reasons for the proteolytic processing of IL 1α and presumably, for IL 1β also. In order to examine the possibility that structural stabilization may be associated with the proteolytic processing of pre IL 1α and β, we investigated the structural features of pre IL 1α and β by the combination of limited proteolysis and immunoprecipitation with antibodies to the NH_2-terminal halves or COOH-terminal halves of pre IL 1α or β. Both trypsin and V8 protease digested the NH_2-terminal halves of pre IL 1α and β more easily than the COOH-terminal halves of pre IL 1α and β, yielding structurally stabilized "mature" forms of IL 1. Both trypsin and V8 protease yielded a fragment similar in size to mature IL 1α from pre IL 1α. In contrast, trypsin digested pre IL 1β into fragments smaller in size than mature IL 1β, while V8 protease yielded a fragment similar in size to mature IL 1β. Furthermore, mature IL 1β, once processed and released from cells, was resistant to trypsin. Since the COOH-terminal half of pre IL 1β is more susceptible to protease digestion than the extracellular mature form of IL 1β, which consists of the same COOH-half, the proteolytic processing of pre IL 1β appears to yield mature IL 1β with a more protease-resistant stable tertiary structure.

Keywords pre IL 1α; pre IL 1β; limited proteolysis; immunoprecipitation; proteolytic processing; structural stabilization

There are at least two types of IL 1, termed IL 1α and β. Both IL 1α and β are synthesized as precursor forms of 271 and 269 amino acid residues respectively.[2] The precursor forms are proteolytically cleaved between Phe^{118} and Leu^{119} in the case of IL 1α by calpain,[3] or between Asp^{116} and Ala^{117} in the case of IL 1β, yielding extracellular mature forms of 153 amino acid residues of the COOH-terminal halves of the precursors.[2]

The precursor form of IL 1β, whether produced by *in vitro* transcription–translation from a complementary deoxyribonucleic acid (cDNA) clone[4] or obtained from activated human monocytes,[5] is biologically inactive. In contrast, the recombinant IL 1α precursor, produced either by *in vitro* translation of messenger ribonucleic acid (mRNA) from activated human monocytes and hybrid selected by an IL 1α cDNA, or by *in vitro* transcription–translation from a cDNA clone, is presumed to be biologically active based on its receptor binding capability.[6] Since it is known that receptor binding is not always followed by cell activation, as exemplified by a mutant IL 1β,[7] and furthermore, that the specific activity of mature IL 1α is 10 times higher than that of the truncated form of pre IL 1α,[8] there may be other reasons why pre IL 1α needs to be proteolytically processed into mature IL 1α; one of them is that, as previously proposed, proteolytic processing may somehow be coupled with release,[3] and the other is that structural stabilization may be associated with proteolytic processing.

In this study, we investigated the structural features of pre IL 1α and β by limited proteolysis and immunoprecipitation with antibodies to the NH_2-terminal halves or COOH-terminal halves of pre IL 1α or β. The results lead us to propose that proteolytic processing of pre IL 1α and β may yield structurally stabilized mature forms of IL 1 and protect the resultant mature IL 1 against protease attack which may occur at the site of inflammation.

Materials and Methods

Materials L-[^{35}S]Methionine (Tran^{35}S-label, 1037 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). V8 protease was purchased from Baehringer Mannheim Biochemicals (Indianapolis, IN). TPCK trypsin was purchased from Sigma (St. Louis, MO). Recombinant human IL 1α and recombinant human IL 1β were generous gifts from Dainippon Pharmaceutical Company (Osaka, Japan) and Roche Inc. (Nutley, NJ) respectively. Rabbit anti-human IL 1α antibodies and anti-human IL 1β antibodies, which are also called antibodies to the COOH-terminal halves of IL 1α or β, were made by immunizing rabbits with each recombinant cytokines.[8] These two antibodies were found to be specific for IL 1α and β respectively as evidenced by Western blotting analysis,[8] neutralization assay[8] and immunoprecipitation (this study, data not shown).

Radiolabeling of Human Monocytes with L-[^{35}S]Methionine Normal peripheral blood mononuclear cells were isolated from the buffy coat of healthy human donors (National Institutes of Health Blood Bank, Bethesda, MD) by Ficoll-Hypaque density sedimentation. Cells enriched in monocytes were obtained by adherence to plastic, which yielded nearly 90% nonspecific esterase positive cells (monocytes). These cells were stimulated with lipopolysaccharide (LPS) (10 μg/ml; *Escherichia coli*, 055: B55, DIFCO, Detroit, MI) for 2 or 16 h as previously described.[8] LPS-activated monocytes were labeled by incubation for an additional 2 h at 37 °C with 0.1 mCi L-[^{35}S]methionine (Tran^{35}S-label, 1037 Ci/mmol, ICN Radiochemicals, Irvine, CA) in 1 ml of a methionine-free RPMI 1640 medium containing 1% dialyzed fetal calf serum (FCS). After incubation, monocytes were exposed to cold methionine for 2 h. After harvesting the culture supernatants, monocytes were extracted with 9 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) in Dulbecco's phosphate buffered saline (D-PBS) in the presence of protease inhibitors (ethylene glycol bis-β3-aminoethyl ether *N,N,N',N'*-tetraacetic acid (EGTA) 5 mM, phenylmethanesulfonyl fluoride 1 mM, leupeptin 50 μg/ml). Supernatants and extracts of methionine labeled cells were immunoprecipitated by specific antibodies and protein A-Sepharose (Pharmacia, Uppsala, Sweden) as previously described, followed by analysis on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE).[8] Labeled proteins were detected by fluorography using Kodak XAR film.

Preparation of Antibodies to the NH_2-Terminal Halves of Pre IL 1α or β To prepare antibodies to the NH_2-terminal halves of pre IL 1α or β in rabbits, two kinds of fusion protein expression vectors were constructed by using the pRIT2T vector (Pharmacia).

One is pRIT2T/5-N which was predicted to encode the chimeric protein consisting of protein A and the NH_2-terminal half portion of pre IL 1α (residue 1 to 112) with one additional isoleucine residue at the carboxy terminus.[8] This fusion protein was produced by N4830-1 cells harboring pRIT2T/5-N after shifting the temperature from 30 to 42 °C, and was subjected to purification by an anti-Ia-17β chain monoclonal immunoglobulin G (IgG) antibody coupled to Sepharose.

The other fusion protein expression vector is pRIT2T/ProAIL1B#6, which consisted of the construct between pRIT2T digested with *Sma* I

and *Bam* HI and pDC-8 (a generous gift of Dr. Don Carter, UpJohn Company, Kalamazoo, MI) digested with *Sac* I and *Hind* III. After inserting a *Sac* I/*Hind* III fragment of pDC-8 into pUC19, the plasmid was digested by *Hind* III, followed by a filling-in reaction, attachment of *Bam* HI linker and self ligation, digestion with *Eco* RI, another filling-in reaction and digestion with *Bam* HI. The resultant fragment (0.4 kb) has a blunt end as well as *Bam* HI end, which can be inserted into *Sma* I/*Bam* HI sites of pRIT2T. This was predicted to encode the chimeric protein consisting of protein A and the NH_2-terminal half portion of pre IL 1β (residue 1 to 133). This fusion protein was also produced by bacteria harboring pRIT2T/ProAIL1B#6 and was purified in the same way as the chimeric protein consisting of protein A and the NH_2-terminal half portion of pre IL 1α.

These two chimeric proteins were used to prepare antibodies against the NH_2-terminal halves of pre IL 1α and β respectively. Rabbits were immunized by 50 μg of each chimeric protein initially emulsified with complete adjuvant, and subsequently with incomplete adjuvant, for the 2nd, 3rd and 4th booster injections. Each antiserum was documented to effectively immunoprecipitate pre IL 1α or β before use.

Results

Characterization of Trypsin-Treated Labeled Cell Lysates by Immunoprecipitation with Anti-pre IL 1α or β Antibodies

Susceptibility of the protein substrate to protease digestion is known to be influenced by many factors, including the tertiary structure of the protein substrate. In general, the more relaxed the structure of the protein substrate, the more susceptible to digestion by protease. Therefore, limited proteolysis can be used as a probe to distinguish the relaxed part of the protein from the compact part of the protein.

Therefore, we performed limited proteolysis of L-[^{35}S]-methionine labeled cell lysates with trypsin followed by immunoprecipitation with antibodies to the NH_2-terminal halves or the COOH-terminal halves (mature halves) of pre IL 1α or β (Fig. 1) in order to investigate the structural features of pre IL 1α and β. The antigen specificity of the antibodies to the COOH-terminal halves of pre IL 1α or β has been confirmed as previously described.[8] One of the reasons we chose to use trypsin is that there are a similar number of trypsin cleavable sites between the NH_2-terminal portions and the COOH-terminal portions of pre IL 1α and β (Table I).

The treatment of labeled cell lysates with 2 μg/ml of trypsin, followed by immunoprecipitation with anti-pre IL 1α antibodies, yielded two fragments from pre IL 1α on SDS PAGE (Fig. 1, columns 4 and 14). As shown in Fig. 1 column 4, antibodies to the COOH-terminal half of pre IL 1α (anti-Cα antibodies) immunoprecipitated a 24 kilodaltons (kDa) fragment and an 18 kDa fragment as well as intact pre IL 1α (doublet; 35 and 33 kDa). On the other hand, antibodies to the NH_2-terminal half of pre IL 1α (anti-Nα antibodies) immunoprecipitated a 24 kDa fragment and a 16 kDa fragment as well as intact pre IL 1α (doublet; 35 and 33 kDa) as shown in Fig. 1 column 14. (We don't know the exact origin of the band with a molecular weight of 28 kDa, but it may be nonspecific, because even in column 11 (no trypsin) this band was seen.)

In contrast, the treatment of labeled cell lysates with 20 μg/ml of trypsin, followed by immunoprecipitation with anti-pre IL 1α antibodies, yielded only one fragment with a molecular weight of 18 kDa from pre IL 1α on SDS PAGE, which was immunoprecipitated by anti-Cα antibodies (Fig. 1 column 5), but not by anti-Nα antibodies (Fig. 1 column 15).

TABLE I. Number of Potential Trypsin or V8 Protease Cleavable Amino Acid Residues in Pre IL 1α and β

	Pre IL 1α		Pre IL 1β	
	NH_2-Terminal	COOH-Terminal	NH_2-Terminal	COOH-Terminal
Trypsin				
Lys	10	11	5	15
Arg	3	3	3	3
V8 protease				
Asp	6	10	12	7
Glu	7	8	9	11

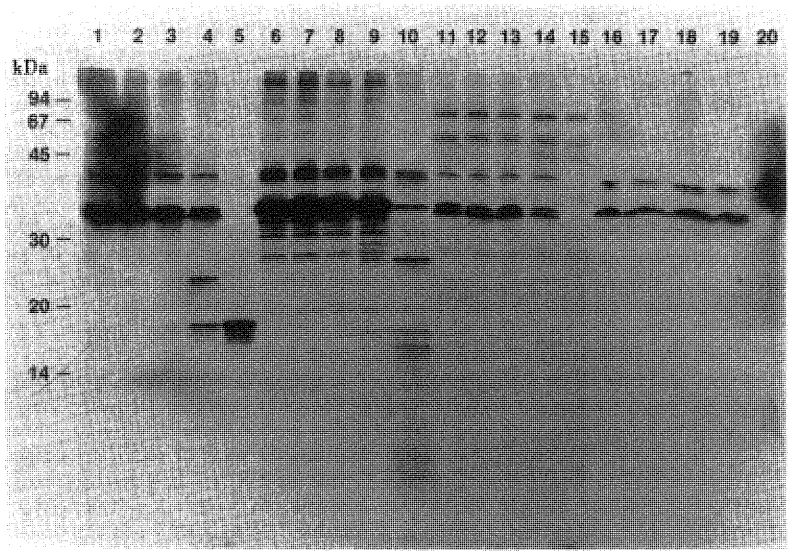


Fig. 1. Limited Proteolysis of Labeled Cell Lysates with Trypsin Followed by Immunoprecipitation with Anti-pre IL 1α or β Antibodies

L-[^{35}S]Methionine labeled cell lysates were treated with graded amounts of trypsin for 30 min at 37 °C, followed by the addition of soybean trypsin inhibitor and immunoprecipitation with anti-Cα, Cβ, Nα or Nβ antibodies, respectively. The immunoprecipitates were then analyzed on SDS PAGE, followed by autoradiography. Columns 1 to 5: anti-Cα, columns 6 to 10: anti-Cβ, columns 11 to 15: anti-Nα, columns 16 to 20: anti-Nβ. Columns 1, 6, 11, 16: no trypsin, columns 2, 7, 12, 17: 0.02 μg/ml of trypsin, columns 3, 8, 13, 18: 0.2 μg/ml of trypsin, columns 4, 9, 14, 19: 2 μg/ml of trypsin, columns 5, 10, 15, 20: 20 μg/ml of trypsin.

The treatment of labeled cell lysates with 20 μg/ml of trypsin, followed by immunoprecipitation with anti-pre IL 1β antibodies, yielded three fragments with molecular weights of 26, 17 and 14.5 kDa from pre IL 1β on SDS PAGE, all of which were immunoprecipitated only by antibodies to the COOH-terminal half of pre IL 1β (anti-Cβ antibodies) (Fig. 1 column 10), but not by antibodies to the NH_2-terminal half of pre IL 1β (anti-Nβ antibodies) (Fig. 1 column 20). It should be noted here that this anti-Nβ antibody could immunoprecipitate intact pre IL 1β (Fig. 1 columns 16 to 19) as well as the 31 and 29 kDa fragments of pre IL 1β (Fig. 3 column 18, see below).

Limited Proteolysis of Pre IL 1α and β with Trypsin Since the treatment of labeled cell lysates with 20 μg/ml of trypsin yielded fragment(s) that were immunoprecipitable only with anti-Cα or β antibodies (Fig. 1 columns 5, 10, 15 and 20), it is possible that the NH_2-terminal halves of pre IL 1α and β may be degraded into smaller fragments with trypsin. In order to test this possibility directly, we performed limited proteolysis of immunoprecipitated pre IL 1α and β with trypsin, followed by analysis on SDS PAGE.

The treatment of pre IL 1α with 0.02 μg/ml of trypsin yielded three fragments with molecular weights of 24, 18 and 16 kDa from pre IL 1α as shown in Fig. 2 column 2. On the other hand, 2 μg/ml of trypsin degraded pre IL 1α to yield an 18 kDa fragment and very small fragments, indicating that the NH_2-terminal half of pre IL 1α is degraded into smaller fragments and that the COOH-terminal half remains intact.

In contrast, treatment of pre IL 1β with trypsin totally degraded the COOH-terminal half 18 kDa fragment, as shown in Fig. 2 columns 7 and 8.

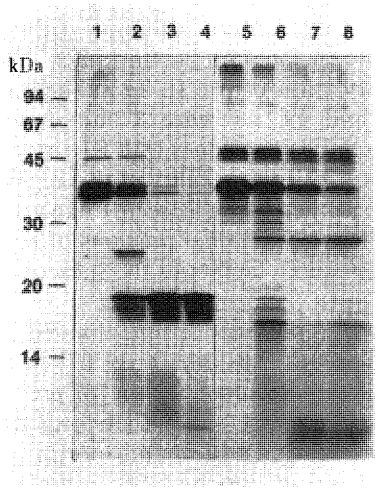


Fig. 2. Limited Proteolysis of Pre IL 1α and β with Trypsin

L-[^{35}S]Methionine labeled cell lysates were immunoprecipitated with anti-Cα or Cβ antibodies respectively. The same volumes of immunoprecipitates were then treated with graded amounts of trypsin for 30 min at 37°C, followed by boiling, analysis on SDS PAGE and autoradiography. Columns 1 to 4: pre IL 1α, columns 5 to 8: pre IL 1β. Columns 1, 5: no trypsin, column 2: 0.02 μg/ml of trypsin, columns 3, 6: 0.2 μg/ml of trypsin, columns 4, 7: 2 μg/ml of trypsin, column 8: 20 μg/ml of trypsin.

Effect of V8 Protease on Labeled Cell Lysates as Evaluated by Immunoprecipitation with Anti-pre IL 1α and β Antibodies In order to confirm the structural difference between the NH_2-terminal half portions and the COOH-terminal half portions of pre IL 1α and β, we performed limited proteolysis of L-[^{35}S]methionine labeled cell lysates with another protease, V8 protease, followed by immunoprecipitation with anti-Nα or Cα antibodies or with anti-Nβ or Cβ antibodies. We chose to use V8 protease for the same reason as trypsin (Table I).

The treatment of labeled cell lysates with 10 μg/ml of V8 protease, followed by immunoprecipitation with anti-Cα antibodies, yielded two fragments with molecular weights of 25 and 18 kDa from pre IL 1α on SDS PAGE (Fig. 3 column 4). As shown in Fig. 3 column 8, anti-Nα antibodies immunoprecipitated the 25 kDa fragment but not the 18 kDa fragment.

In contrast, treatment of labeled cell lysates with 100 μg/ml of V8 protease, followed by immunoprecipitation with anti-pre IL 1α antibodies, yielded only one fragment

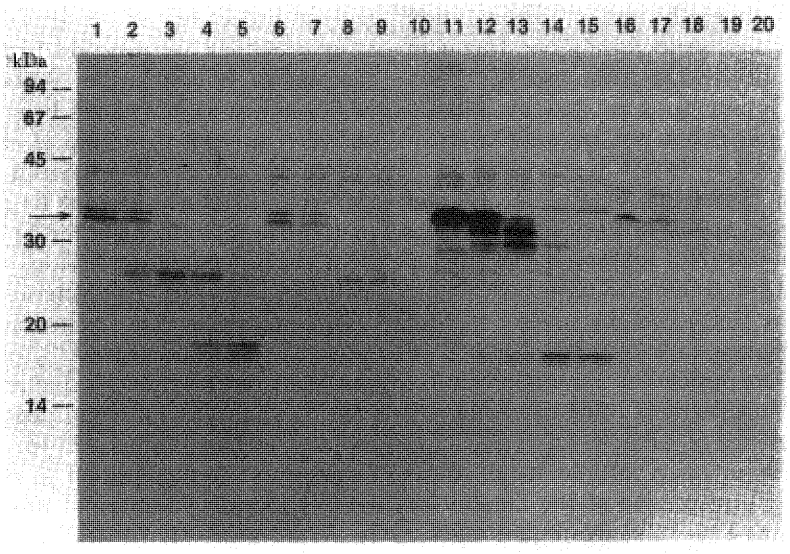


Fig. 3. Limited Proteolysis of Labeled Cell Lysates with V8 Protease Followed by Immunoprecipitation with Anti-pre IL 1α and β Antibodies

Essentially the same experiments as Fig. 1 were carried out by using graded amounts of V8 protease in 20 mM phosphate buffer (pH 7). Columns 1 to 5: anti-Cα, columns 6 to 10: anti-Nα, columns 1 to 15: anti-Cβ, columns 16 to 20: anti-Nβ. Columns 1, 6, 11, 16: no V8 protease, columns 2, 7, 12, 17: 0.1 μg/ml of V8 protease, columns 3, 8, 13, 18: 1 μg/ml of V8 protease, columns 4, 9, 14, 19: 10 μg/ml of V8 protease, columns 5, 10, 15, 20: 100 μg/ml of V8 protease.

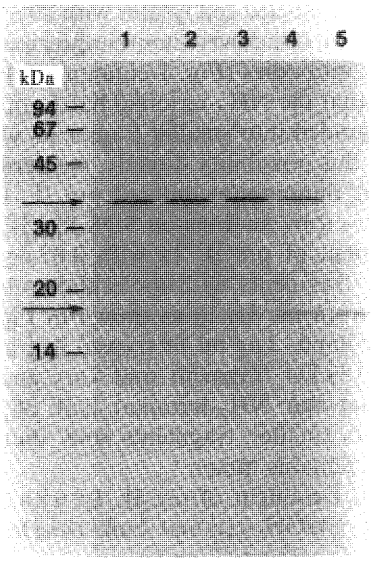


Fig. 4. Comparison of Trypsin Sensitivity of Pre IL 1β and Mature IL 1β

L-[^{35}S]Methionine labeled culture supernatants were prepared from fresh monocytes, cultured and labeled for 2 h in the presence of LPS, followed by chasing with cold methionine for an additional 2 h. After immunoprecipitation with anti-Cβ, the same volumes of immunoprecipitates were treated with graded amounts of trypsin for 30 min at 37 °C, followed by the addition of a soybean trypsin inhibitor, analysis on SDS PAGE and autoradiography. Column 1: no trypsin, column 2: 0.02 μg/ml of trypsin, column 3: 0.2 μg/ml of trypsin, column 4: 2 μg/ml of trypsin, column 5: 20 μg/ml of trypsin.

with a molecular weight of 18 kDa from pre IL 1α on SDS PAGE, which was immunoprecipitated by anti-Cα antibodies (Fig. 3 column 5), but not by anti-Nα antibodies (Fig. 3 column 10).

The treatment of labeled cell lysates with 1 μg/ml of V8 protease, followed by immunoprecipitation with anti-pre IL 1β antibodies, yielded two fragments with molecular weights of 31 and 29 kDa from pre IL 1β on SDS PAGE, which were immunoprecipitated by anti-Cβ antibodies (Fig. 3 column 13) as well as by anti-Nβ antibodies (Fig. 3 column 18).

In contrast, the treatment of labeled cell lysates with 100 μg/ml of V8 protease, followed by immunoprecipitation with anti-pre IL 1β antibodies, yielded only one fragment with a molecular weight of 18 kDa from pre IL 1β on SDS PAGE, which was immunoprecipitated by anti-Cβ antibodies (Fig. 3 column 15), but not by anti-Nβ antibodies (Fig. 3 column 20).

Comparison of Trypsin Sensitivity of Pre IL 1β and Mature IL 1β Since trypsin appeared to digest pre IL 1β into smaller fragments in size than mature IL 1β (Fig. 2 columns 7 and 8), pre IL 1β must contain trypsin cleavable site(s) in the pre region as well as in the mature region. Therefore, we compared the trypsin sensitivity of pre IL 1β and mature IL 1β, which were purified by immunoprecipitation from labeled culture supernatants of fresh monocytes cultured for 2 h in the presence of LPS.

The apparently constant intensity of the band corresponding to mature IL 1β, irrespective of the amount of trypsin used (Fig. 4 columns 1 to 5), suggests that mature IL 1β is not degraded into smaller molecules by trypsin. In contrast, increasing amounts of trypsin appeared to degrade pre IL 1β into smaller molecules which could hardly be detected (Fig. 4 column 5).

Discussion

In this paper, we demonstrate that the NH_2-terminal halves of pre IL 1α and β are more easily digested with either trypsin or V8 protease than the COOH-terminal halves. This is not due to the difference in the number of protease cleavable sites using these particular proteases in each half of pre IL 1α and β (Table I). Consequently, this suggests that the NH_2-terminal halves of pre IL 1α and β have a less tight configuration than the COOH-terminal halves.

Trypsin digestion of pre IL 1α has unique results. As shown in Fig. 1 column 4 and Fig. 2 column 2, pre IL 1α appears to have two similar trypsin susceptible sites. In contrast, proteolytic fragmentation in the other cases, namely digestion of pre IL 1α by V8 protease and that of pre IL 1β either by trypsin or by V8 protease, appeared to proceed from each amino terminus. Although we don't know as yet the explanation for this difference, it is possible that phosphorylation of pre IL 1α at the serine residue(s) (residues 87 and 89; reference 8 and unpublished data) close to the tetrabasic residues (KKRR; residues 82 to 85) might influence the susceptibility of this region (KKRR) to trypsin. In the other cases, it seems likely that the initial cleavage of the NH_2-terminal portions of pre IL 1α and β, even at the distal end, facilitates the subsequent proteolytic cleavage.

There is one report concerning limited proteolysis of recombinant pre IL 1β with trypsin[9] in which it was suggested that the precursor region (of pre IL 1β) may be structurally less compact and, therefore, more protease-sensitive than the COOH-terminal portion, and that the NH_2-terminal portion may become unstable once removed from the COOH-terminal sequence. Our data supports this hypothesis, extends this view to pre IL 1α, and defines the patterns of limited proteolysis in greater detail.

We also demonstrate that, while pre IL 1β was degraded by trypsin into smaller molecules than mature IL 1β, mature IL 1β, once correctly processed and released, apparently became resistant to trypsin digestion. Therefore, these findings support the possibility that the appropriate proteolytic processing of pre IL 1β may yield stable tertiary structures that are resistant to further protease attack at the site of inflammation. Although we didn't find any proteases for IL 1α which behave like trypsin for IL 1β, correctly processed mature IL 1α was found to be much more resistant to chymotrypsin than pre IL 1α (data not shown). Therefore, proteolytic processing may also yield mature IL 1α with stable tertiary structures.

In the inflammatory lesion, there are many proteases including elastase, collagenase, cathepsins and plasmin which might degrade the extracellular matrix as well as bioactive proteins such as cytokines. Since the substrate specificity of plasmin is similar to that of trypsin, it is possible that plasmin might degrade pre IL 1β into smaller molecules than mature IL 1β. In contrast, elastase and cathepsin G were reported to produce biologically active IL 1β containing additional amino acids relative to mature IL 1β from pre IL 1β.[10] Therefore, it is conceivable that the balance of each protease may determine the level of the bioactive IL 1(β) molecule.

In summary, proteolytic processing of pre IL 1β may be required for the expression of full biological activity[2] as

well as for yielding mature IL 1β with stable tertiary structures (this study). Although in the case of pre IL 1α the possibility cannot be excluded that proteolytic processing is associated with the enhancement of biological activities, there may be two other requirements for proteolytic processing; one is the coupling of processing with release through the interaction of phosphorylated pre IL 1α with acidic phospholipids in the inner side of the plasma membrane,[3,11] and the other may be the generation of a stable tertiary structure of IL 1α (suggested by this study).

Acknowledgment We would like to thank Ms. Karen Blodgett for her excellent technical assistance.

References and Notes

1) Present address: *Department of Biomolecular Science, Faculty of Science, Toho University, 2-2-1, Miyama, Funabashi, Chiba 274, Japan.*
2) Y. Kobayashi, K. Matsushima, and J. J. Oppenheim, "Interleukin-1, Inflammation and Disease," ed. by R. Bomford, and B. Henderson, Elsevier Science Publishers B. V., Amsterdam, 1989, p. 47.
3) Y. Kobayashi, K. Yamamoto, T. Saido, H. Kawasaki, J. J. Oppenheim, and K. Matsushima, *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 5548 (1990).
4) C. J. March, B. Mosley, A. Larsen, D. P. Cerretti, G. Braedt, V. Price, S. Gillis, C. S. Henney, S. R. Kronheim, K. Grabstein, P. J. Conlon, T. P. Hopp, and D. Cosman, *Nature* (London), **315**, 641 (1985).
5) K. Matsushima, M. Taguchi, E. J. Kovacs, H. A. Young, and J. J. Oppenheim, *J. Immunol.*, **136**, 2883 (1986).
6) B. Mosley, D. L. Urdal, K. S. Prickett, A. Larsen, D. Cosman, P. J. Conlon, S. Gillis, and S. K. Dower, *J. Biol. Chem.*, **262**, 2941 (1987).
7) L. Gehrke, S. A. Jobling, B. McDonald, L. J. Rosenwasser, and P. E. Auron, *J. Biol. Chem.*, **265**, 5922 (1990).
8) Y. Kobayashi, E. Appella, M. Yamada, T. D. Copeland, J. J. Oppenheim, and K. Matsushima, *J. Immunol.*, **140**, 2279 (1988).
9) D. J. Hazuda, R. L. Webb, P. Simon, and P. Young, *J. Biol. Chem.*, **263**, 1689 (1989).
10) D. J. Hazuda, J. Strickler, F. Kueppers, P. L. Simon, and P. R. Young, *J. Biol. Chem.*, **265**, 6318 (1990).
11) Y. Kobayashi, J. J. Oppenheim, and K. Matsushima, *J. Biochem.* (Tokyo), **107**, 666 (1990).

Cisplatin Suppository: Preparation, Release Characteristics and Clinical Evaluation

Yutaka NISHIOKA,*[,a] Syojiro KYOTANI,[a] Masahiko KUSUNOSE,[a] Hisashi MIMOTO,[a] Takashi HAMADA,[b] Masafusa ASAI,[b] and Yusuke SAGARA[b]

Department of Pharmacy, Kochi Madical School Hospital[a] and Department of Obstetrics and Gynecology, Kochi Medical School,[b] Kohasu Okoh-cho, Nankoku, Kochi 783, Japan. *Received September 20, 1990*

Cisplatin (CDDP) has attracted attention as a chemotherapeutic agent for the treatment of uterine endometrial carcinoma but causes serious side effects, including renal toxicity. CDDP suppositories containing NaCl at different concentrations were prepared to enhance the efficacy and to reduce the side effects of CDDP. The release characteristics, melting point and viscosity of the suppositories were first studied. The rate of CDDP release increased as the NaCl concentration increased: it was 12% 12 h after administration of suppositories containing no NaCl, but 32% with 0.2% NaCl. The melting point was raised by addition of NaCl: 35.5 °C without NaCl and 36.5 °C with 0.2% NaCl. Addition of 0.2% NaCl doubled the viscosity. Clinically, the suppository containing 0.06% NaCl was given to 3 patients with endometrial carcinoma twice a week for 3 weeks to examine serum CDDP levels and endometrial absorption. Patients with endometrial carcinoma showed different peak plasma platinum (Pt) levels which were as low as 0.12, 0.06 and 0.22 μg Pt/ml with similar patterns of change in the level. Radiographic analysis revealed many Pt particles in sections of necrosed endometria after 21d of the treatment. No side effects of CDDP were found in biochemical testing or subjective symptoms.

Keywords cisplatin; sodium chloride; suppository; endometrial carcinoma

The incidence of uterine endometrial carcinoma has been rising steadily and diverse methods are used for its treatment. In particular, cisplatin (CDDP) has been reported to be effective against endometrial carcinoma and has attracted attention.[1-3] Chemotherapy using CDDP is often combined with radiotherapy to enhance the effects of the latter and to improve the patient's prognosis.[4] However, CDDP caused serious renal toxicity in these studies. Therefore, we tried local chemotherapy with a CDDP suppository to enhance the effects of CDDP and to reduce its side effects. We measured the release and content of CDDP, the melting point and the viscosity of the suppository. In addition, a CDDP suppository was combined with radiotherapy in 3 patients with endometrial carcinoma to examine, serum CDDP concentration and endometrial absorption.

Experimental

Reagents CDDP powder was obtained by the following method. Commercially available CDDP for injection (Nippon Kayaku Co.) was dried using an evaporator. The powder so obtained was transferred to a beaker and was mixed thoroughly after adding water and then filtered. The same procedure was repeated five times to remove NaCl. Since CDDP is thought to be degraded by heat and light during the drying process in the evaporator, the powder was analyzed by high performance liquid chromatography (HPLC) for the presence of the degradation products. At the same time, the purity of the powder was measured to see if the desalting process was complete. The material which was completely desalted, as confirmed by HPLC, and which was devoid of degradation products was designated as the CDDP a powder.

HPLC was carried out using a Hitachi L-6000 apparatus equipped with a Shodex OH pak B804 (500 × 8 mm i.d., Showa Denko) and Hitachi L-4000 ultraviolet (UV) monitor (210 nm). Saline solution employed as a mobile phase at a flow rate of 1.0 ml/min. Witepsol H-15 and E-75 were purchased from Maruishi Seiyaku. All other reagents employed were commercial special-grade products.

Preparation of CDDP Suppositories CDDP powder and NaCl were dissolved in water and the solution was evaporated. The resultant solid was finely pulverized in an agate mortar, mixed with a base [a mixture of Witepsol H-15 and E-75 (6:4)] and melted at 50 °C. The melted mixture was cooled to 45 °C and then poured into a metal mold kept at 37 °C to form a rod suppository. The content of CDDP in the suppository was 20 mg/g. Since Witepsol H-15 was used as a substrate, it was thought that free OH residues in the substrate could react with CDDP and could produce highly toxic CDDP hydrate products such as mono- and di-aquocomplexes, and that the concentration of Cl^- had a great effect on the complex formation. Thus, NaCl was added to increase the stability of CDDP and to suppress the formation of complexes.

Melting Point and Viscosity The melting point was measured with a melting-point measuring device (MP-21, Yamato) by increasing temperature at the rate of 1 °C/min. A rotating viscosity meter (Model ED, Tokyo Keiki) was used for determination of viscosity.

A suppository was finely crushed and softened on a plate at 37 °C for 10 min. The viscosity was measured at 37 ± 0.1 °C, and expressed as an average value obtained at various shear rates.

CDDP Release Test A suppository release tester (TMS-103, Toyama Sangyo) was used. Normal saline (100 ml) as a release solution was poured into a release cell, which was immersed in a thermostatic tank maintained at 37 °C. A cell (nitrate cellulose membrane, pore size 3 μm) containing a suitable amount of the sample was immersed in this tank.

The contents of the cell were stirred (50 rpm) with a stirring rod, and aliquots of the solution were serially taken. The CDDP content in the release solution was measured with an atomic absorption spectrophotometer (Hitachi Z-9000).

Administration of CDDP Suppository and Blood Sampling Subjects were 3 patients with endometrial carcinoma: stage III (A: 41 kg, 76 years), stage I b-II (B: 72 kg, 74 years) and stage III (C: 65 kg, 68 years). A CDDP suppository was inserted into the uterus twice a week (9:00 a.m., Tuesday and Friday) for 3 weeks. Three milliliters of blood were collected from the brachial vein in a heparinized tube 0.5 and 1 d after the administration, and then every day. Plasma was separated and used for the test. Radiotherapy was a combined treatment in the 3 patients: intrauterine radiation of 3 Gy for 10 d and extracorporeal radiation of 2 Gy for 30 d in patient A; intrauterine radiation of 4 Gy for 10 d and extracorporeal radiation of 2 Gy for 25 d in patient B; intrauterine radiation of 4 Gy for 10 d and extracorporeal radiation of 2 Gy for 30 d in patient C. Plasma CDDP levels were determined using atomic absorption spectrophotometry.

Effects on liver and renal function, such as glutamate oxaloacetate transaminase (GOT) and creatinine, were studied with a biochemical analyzer (Vision analyzer).

Before the drug was administered, the doctor in charge fully explained to the patient the purpose, method, expected effects and dangers, and it was administered with the consent of the patient.

Plasma CDDP levels were determined 21 d after administration was started and biochemical examination was evaluated until 30 d afterwards.

CDDP in Endometrium Samples from the endometrium were endoscopically collected before and 21 d after the treatment was started, fixed in phosphate buffered formalin, dried and embedded. Sections of 90 nm in thickness were prepared with a Cu grid. Sections thus prepared

were U-Pb double stained. All sections were observed using a trace element X-ray analyzer (JEM-1200EX, LINK 860-500J, Nihon Denshi) after carbon evaporation.

Cytology The endometrium was cytologically examined before and after the combination therapy of CDDP suppository with radiotherapy to evaluate the therapeutic effects.

Results

Melting Point and Viscosity Melting points and viscosity of the suppositories are listed in Table I. Addition of NaCl raised the melting point: 35.5 °C when there was no NaCl and 36.5 °C with 0.2% NaCl. Addition of 0.2% NaCl doubled the viscosity.

CDDP Release from Suppository The CDDP release profiles from suppositories at various NaCl concentrations are shown in Fig. 1. The time required for 50% release was about 84 h with no NaCl and 30 h with 0.2% NaCl. The rate of release after 72 h was 42%, 88%, 99% and 100% at concentrations of NaCl corresponding to 0%, 0.06%, 0.1% and 0.2%, respectively.

For clinical application, the suppository containing 0.06% NaCl was selected on the ground that it is used twice

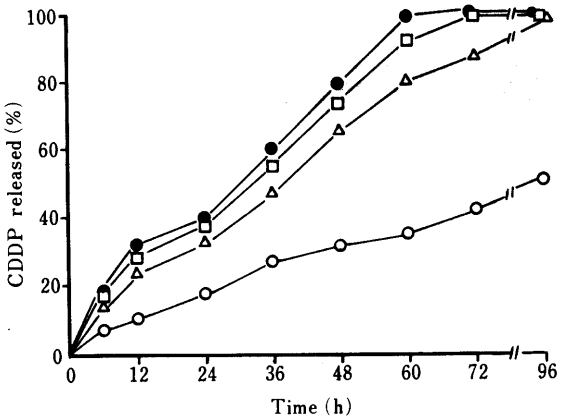


Fig. 1. Effect of NaCl on the CDDP Release Profiles from the CDDP Suppository

Concentration of NaCl: ○, 0%; △, 0.06%; □, 0.10%; ●, 0.20%.

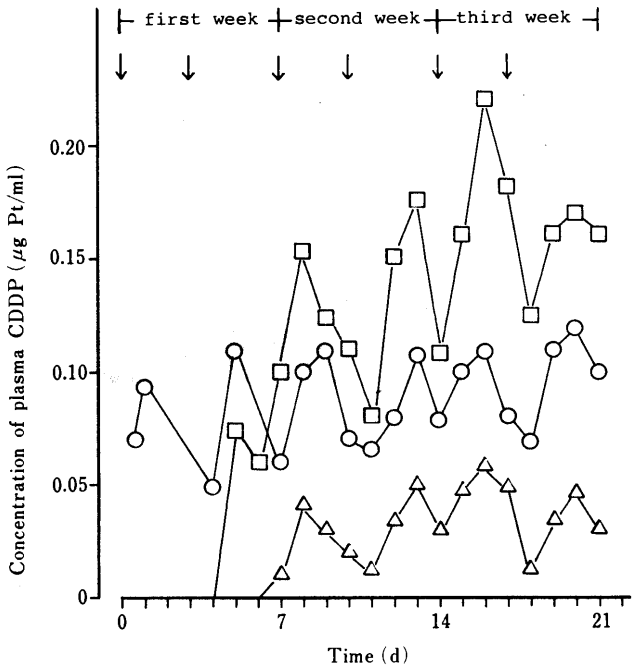


Fig. 2. Plasma CDDP Concentration after Administration of the CDDP Suppository

Patient: ○, (A); △, (B); □, (C). The arrow indicates the time at which the CDDP (20 mg) suppository was administered on Tuesday and Friday.

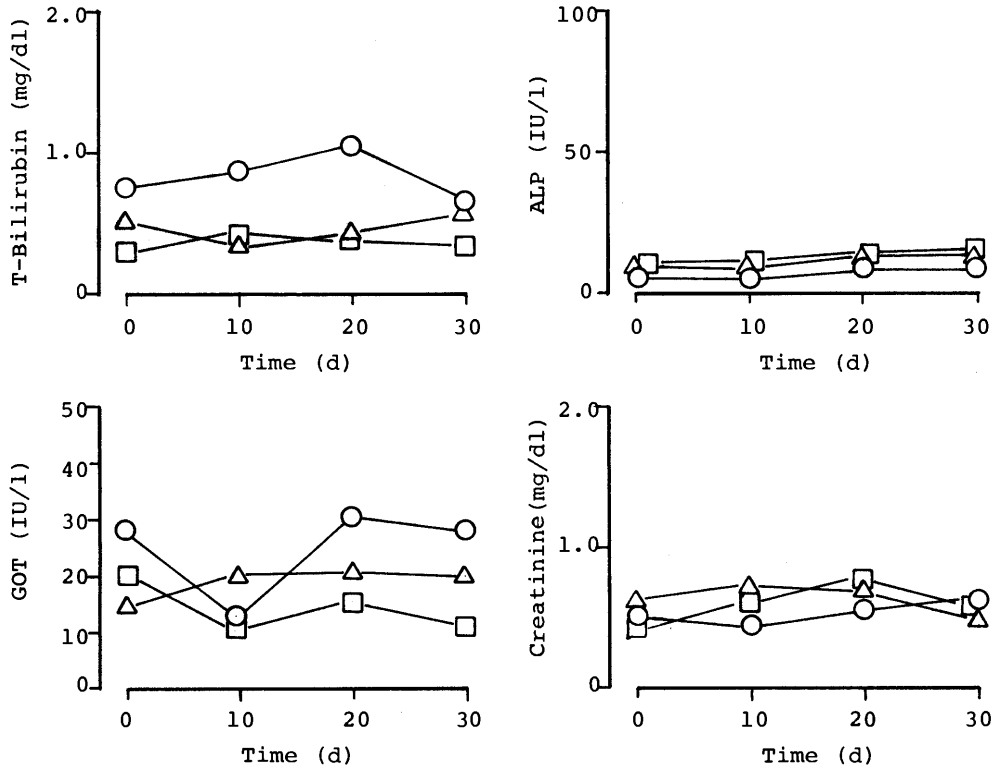


Fig. 3. Effect of the CDDP Suppository on the Liver and Renal Function

Patient: ○, (A); △, (B); □, (C).

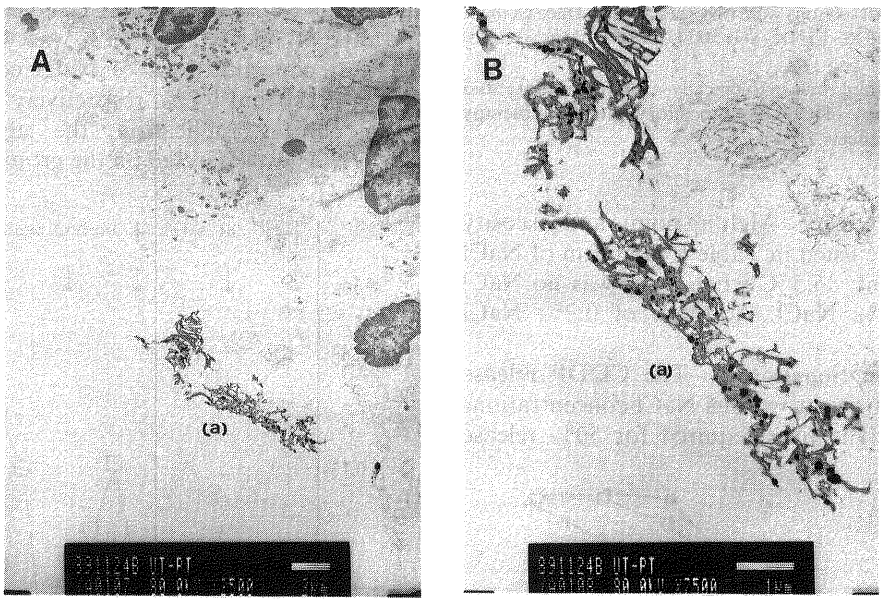


Fig. 4. Electron Micrograph
(A) was transmission electron microscopy, (B) was the enlarged image of area (a) in (A).

TABLE I. Effect of NaCl on Melting Point and Viscosity

Concentration of NaCl (%)	Melting point (°C)	Viscosity (η cP)
0	35.5±0.2	12.8±0.3
0.06	35.9±0.1	20.5±0.2
0.10	36.1±0.1	21.9±0.3
0.20	36.5±0.1	25.7±0.4

Mean±S.D. ($n=5$).

a week and the CDDP release was 88% after 72 h and 100% after 96 h.

Plasma CDDP Level and Biochemical Examination Figure 2 shows plasma CDDP levels after the suppository containing 0.06% NaCl was given. Plasma CDDP was detected in patient A on day 0.5; patient B, day 7; and patient C, day 5 after administration. The plasma levels differed among the patients and the peak levels were 0.12, 0.06 and 0.22 μg Pt/ml in patients A, B, and C, respectively.

The suppository did not influence the patient biochemical examination (Fig. 3).

X-Ray Analysis of CDDP in the Endometrium Figure 4 shows electron microscopic pictures of the endometrium on patient A. (A) Transmission electron microscopy showed that the membranous structure and organellae had been destroyed by tissue necrosis. A nucleus and some microsomes are shown. (B) An enlarged image of area (a) in (A), showing many black spots. The spots were analyzed by X-ray.

Four Pt peaks (Mα, Lα, L$\alpha+\beta$, Lβ) were detected. CDDP Pt was also identified in other spots (Fig. 5).

Cytological Examination Cytological examination showed therapeutic effects of the suppository: change from type 5 to type 2 in patient A, from type 3A to type 1 in patient B and from type 5 to type 2 in patient C.

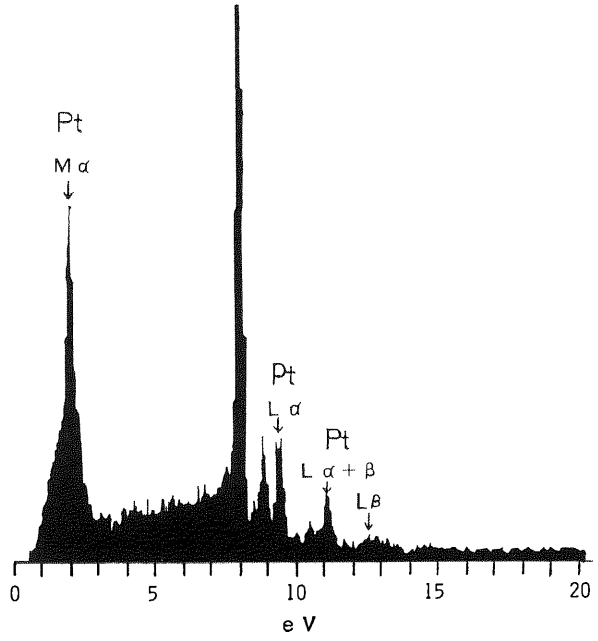


Fig. 5. X-Ray Diffraction Pattern

Discussion

In vitro, the release of CDDP from the suppository was markedly increased by the addition of NaCl, possibly because of the enhanced solubility of CDDP, reduced substitution activity of Cl^- ions in CDDP molecules, and inhibited hydrate formation. The melting point and viscosity were markedly affected by the NaCl content. Particularly, the viscosity was enhanced by the addition of NaCl, suggesting that added NaCl may prolong the intrauterine stay of the suppository.

In vivo, changes in the plasma CDDP level differed among the patients, possibly because of the difference in CDDP absorption in affected cells and in the distribution volume, which is dependent on the body weight. X-ray analysis showed a number of CDDP particles in necrotized endometrial cells collected 21 d after the administration was

started, and cytological examination revealed a therapeutic effect of the suppository. Therefore, it was suggested that the CDDP suppository enhanced the antitumor activity of CDDP. Low plasma CDDP concentrations (0.12, 0.06 and 0.22 μg Pt/ml) and no abnormality in patients biochemical examinations indicated that the suppository reduced the side effects of CDDP. We will study the absorption process in the future.

References

1) M. W. Pasmanter, M. Coleman and R. T. Silver, *Cancer Treat. Rep.*, **69**, 539 (1985).
2) G. Doppe, V. K. Malviya, E. Zebella, *Wiener Klinische Wochenchrift*, **96**, 747 (1984).
3) S. Kitayama, M. Iwahashi, M. Yamamoto and R. Nakano, *Jpn. J. Cancer Chemother.*, **16**, 2099 (1989).
4) H. Sato, K. Fujiwara, T. Azuma and K. Akiya, *Jpn. J. Cancer Chemother.*, **15**, 2443 (1988).

A Spray-Drying Method for Mass Production of Liposomes[1)]

Hiroshi KIKUCHI,* Hitoshi YAMAUCHI and Sadao HIROTA[2)]

Pharmaceutical Formulation Research Center, Research Institute, Daiichi Pharmaceutical Co., Ltd., 16–13, Kita-kasai 1-chome, Edogawa-ku, Tokyo 134, Japan. *Received October 22, 1990*

A spray-drying method for the mass production of liposomes was developed: lipids were dissolved in a volatile organic solvent such as chloroform in which, in some cases, a core material such as mannitol was additionally suspended, and the organic solution or suspension was then spray-dried. Addition of core material particles or the use of hydrogenated lecithins increased the recovery of the lipid mixture prepared by spray-drying. Since the obtained spray-dried product was very amorphous, it could be easily hydrated with an aqueous solution, and lipid vesicles (liposomes) were spontaneously formed by agitating. These spray-dried (SD) liposomes were characterized in comparison with the traditional liposomes known as Bangham's liposomes.

Incorporation of cholesterol into the SD-liposomal membrane was confirmed by differential scanning calorimetry and gel filtration chromatography. Incorporation of charged lipids was confirmed by the zeta potential of liposomes. The size distribution of the unextruded SD-liposomes was similar to that of Bangham's liposomes, and a more homogeneous size distribution could be obtained by the extrusion technique. When glucose and dextran were used as water-soluble model drugs, the encapsulation efficiency was 15—65% depending on the total lipid concentration. Increasing the practical surface area of the lipid mixture using a core material was considered to cause a noticeably high encapsulation efficiency of dextran into the SD-liposomes.

This spray-drying method was found to be useful and valuable for the mass production of liposomes.

Keywords spray-drying; liposome; core material; X-ray diffraction; crystallinity; differential scanning calorimetry; gel filtration chromatography; zeta potential; particle size distribution; encapsulation efficiency

While lipid vesicles called liposomes have been used widely as a model membrane system, they have also been utilized in various applications related to a drug delivery system.[3)] Though verious techniques have been proposed for preparing liposomes in a test tube or flask scale,[4)] most of them are not suitable for an industrial scale, and that is one reason why liposomal products have not become more popular. Some novel methods have recently been proposed for preparing liposomes, such as the freeze-drying method,[5)] the stable plurilamellar vesicles (SPLV) method,[6)] and the dry, free-flowing granular product (proliposomes) method.[7)] But there have not been many methods for the mass production of liposomes. Novel preparation methods are needed which are suitable for the industrial production of liposomes with ease and good reproducibility.

We previously used a spray dryer for the stabilization of liposomal products during storage[8)]; namely, a liposomal aqueous dispersion prepared in advance was spray-dried so that it could be stored stably. In this work, we used the same spray dryer to prepare a dried lipid mixture (spray-dried product), which was then easily hydrated with an aqueous solution containing a water-soluble model drug to form a liposomal dispersion. Here, an organic solution of the lipid mixture with or without a core material was spray-dried for later use in preparing liposomes. We call this novel method the spray-drying method. This paper describes this preparation procedure and compares the characteristics of the prepared liposomes with those by the lipid film method introduced by Bangham *et al.*[9)]

Experimental

Materials Egg phosphatidylcholine (EggPC) and hydrogenated EggPC (H-EggPC), whose iodine values were 66 g/100 g and 0 g/100 g, respectively, were obtained from Asahi Chemical Ind. Co., Ltd. (Tokyo, Japan). Hydrogenated soya phosphatidylcholine (H-SoyaPC) whose iodine value was less than 3 g/100 g was from Lucas Meyer (Hamburg, West Germany). L-α-Dipalmitoylphosphatidylcholine (DPPC) was purchased from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). Cholesterol (Chol), dicetylphosphate (DCP) and stearylamine (SA) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Mannitol and glucose were from Kishida Chemical Co., Ltd. (Osaka, Japan), and Dextran T-40 and Sepharose CL-4B were both from Pharmacia Fine Chemicals AB (Uppsala, Sweden). They were used as received without further purification. All other chemicals were commercial products of reagent grade.

Preparation of Liposomes by Spray-Drying Method The spray-dried products were prepared by a laboratory type spray dryer, a Pulvis Mini-Spray, model GA-31 (Yamato Scientific Co., Ltd., Tokyo, Japan). To begin with, the lipids in various molar ratios were dissolved in chloroform (bp 61—62 °C) or dichloromethane (bp 39—40 °C). The amount of organic solvent was varied from 50 to 500 ml (usually 100 ml) and the total lipid concentration was from 8.4 to 176 mM. As shown in Fig. 1, this organic solution of lipids fed by the quantitative peristaltic pump was sprayed into a drying chamber through a spray nozzle whose orifice size was 400 μm. The inlet flow rate of the solution was about 9 ml/min and the atomizing pressure was 1.0 kg/cm^2. The inlet amount of hot air was 0.4 m^3/min. Inlet temperature of the chamber was controlled at 55±15 °C, and the outlet temperature was 40±10 °C. Though the spray-dried product was essentially obtained from the reservoir attached to the cyclone, the portion which adhered to the wall of the cyclone was also used finally with that from the reservoir as the final spray-dried product, since there were practically no problems in preparing liposomes. This mixed spray-dried product was stored in the refrigerator until the next step in the procedure began.

When a core material was used, an appropriate amount of mannitol, which had previously been sieved through a 100 mesh (150 μm) screen, was suspended in the organic solvent in which the lipids had previously been dissolved. This organic suspension was stirred in a beaker, and other operating conditions were the same as described above.

Next, the pre-weighed spray-dried product was put into a test tube or a beaker. The former was used when the amount of liposomal dispersion was less than 10 ml, and the latter was used when the dispersion was more than 100 ml. The spray-dried product without a core material was hydrated with an isotonic 0.28 M glucose aqueous solution, while that with a core material was hydrated with distilled water alone, a 0.14 M glucose aqueous solution or a 1% (w/v) dextran T-40 aqueous solution. Glucose was used not only as a water-soluble model drug but also as an isotonizing agent; dextran was used only as a model drug. Here, since mannitol itself had osmotic pressure in an aqueous solution, the amount of spray-dried product used was weighed so that the final osmotic pressure could be isotonic after hydration with an aqueous solution. On the scale of a test tube (4—10 ml), it was shaken on a Vortex mixer for more than 5 min above the

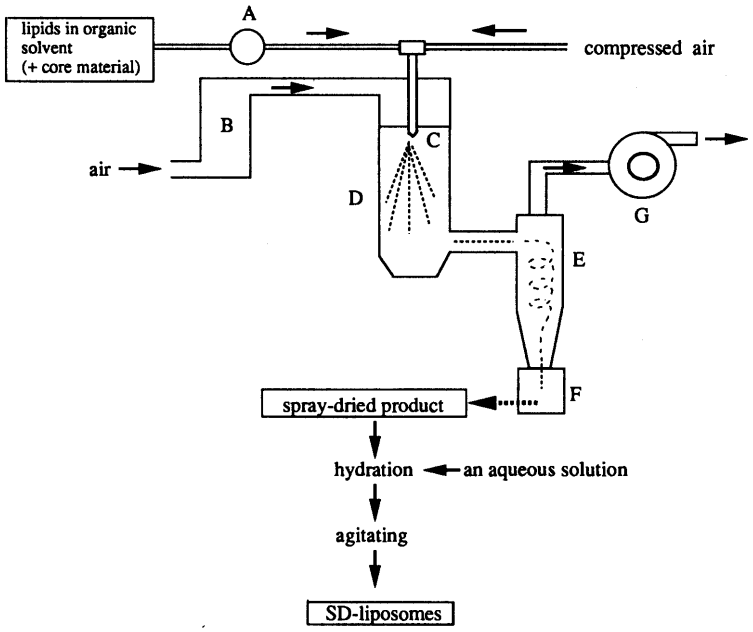


Fig. 1. Schematic Diagram of Spray-Drying Method

A, peristaltic pump; B, heater; C, spray nozzle; D, drying chamber; E, cyclone; F, reservoir; G, aspirator.

gel–liquid crystalline phase transition temperature (Tc) of the lipid materials. On the scale of a beaker (100—500 ml), the hydrated dispersion was homogenized by a T.K. Homo Mixer, type M (Tokushu Kika Kogyo Co., Ltd., Osaka, Japan) for more than 5 min above Tc. The total lipid concentration was from 3.2 to 46 mM.

In some cases, in order to obtain a more homogeneous size distribution of the liposomes, the liposomal dispersion was extruded once above Tc through a polycarbonate membrane filter with 0.2 μm pore size as described by Olson *et al.*[10]

In this report, the liposomes prepared by this spray-drying method are hereafter referred to as SD-liposomes.

Preparation of Liposomes by Bangham's Method As the control liposomes, multilamellar vesicles were prepared by the conventional method introduced by Bangham *et al.*[9] The lipids were dissolved in chloroform in a small round-bottomed flask. The chloroform was then evaporated off under vacuum, and after the lipid film was dried it was hydrated with a 0.28 M glucose aqueous solution. The flask was agitated on a Vortex mixer for more than 5 min until the lipid film had been freed from the bottom of the flask. The temperature was maintained above Tc of the lipid materials.

These liposomes are hereafter called Bangham's liposomes.

X-Ray Diffraction The crystallinities of the different types of lipid mixtures were determined. For physical mixture of lipids, after gentle mixing of the lipid powders in an agate mortar, an adequate amount of the mixture was transferred to a glass holder for measurement. For Bangham's lipid film, the following procedure was performed: after the chloroform solution of the lipid mixture was put on the glass holder, it was air-dried to dryness at room temperature and the residual solvent was removed in a desiccator under reduced pressure for 1 h. For the spray-dried product without a core material, the product itself was used. In each case approximately the same amount of lipid mixture was used.

A Geiger Flex 2012 diffractometer (Rigaku Denki Co., Ltd., Tokyo, Japan) was used at room temperature for measurement. The X-ray source was copper-K_α with a nickel filter. A goniometer was scanned at 2°/min between 40° and 4° (2θ).

Differential Scanning Calorimetry (DSC) DSC measurements were performed on a Du Pont 910 Differential Scanning Calorimeter (9900 Thermal Analysis System). Twenty μl of the dispersion of SD-liposomes or Bangham's liposomes was put into an aluminum pan, which was then hermetically sealed. The heating rate was 2 °C/min.

Gel Filtration Chromatography (GFC) One ml of the SD-liposomal dispersion was applied to a 2.2 i.d. × 42 cm column of Sepharose CL-4B and eluted with phosphate-buffered saline (PBS). The fraction size was 4.1 ml, and the concentrations of DPPC and Chol in fractions were determined by enzymatic assay using a Phospholipids B-test Wako and a Cholesterol B-test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively.

Particle Size Distribution and Zeta Potential of Liposomes The mean particle size, peak particle size and polydispersity index (PDI) of liposomes were determined by quasi-elastic laser light scattering (QELS) measurements using a Zatasizer II (Malvern Instruments Ltd., England). The light source was 5 mW He–Ne laser at a wavelength of 632.8 nm. Data analysis was performed using the principles of photon correlation spectroscopy.[11] The zeta potential of liposomes was determined by the same instrument. For this purpose the principles of laser doppler electrophoresis were used,[12] and the electrophoretic mobility was converted into the zeta potential by the Smoluchowski equation.[13]

Encapsulation Efficiency In order to estimate the encapsulation efficiencies of glucose and dextran T-40 into the SD-liposomes, the unencapsulated drugs were separated from the liposome-encapsulated drugs. For glucose, 0.5 ml of the liposomal dispersion was dialyzed in cellophane tubing (Union Carbide Co., Chicago, U.S.A.) against 1 l of saline for 5 h at room temperature, and the saline was changed 3 or 4 times during dialysis. For dextran, 1 ml of the liposomal dispersion was applied to a 2.2 i.d. × 42 cm column of Sepharose CL-4B and eluted with PBS. The amount of glucose or dextran encapsulated in the liposomes was determined as follows: glucose and dextran were extracted into a water phase according to the procedure by Bligh and Dyer[14] with slight modifications. Two ml of methanol, 1 ml of chloroform, another 1 ml of chloroform and then 1.2 ml of water were added in this order to 0.8 ml of the liposomal dispersion with shaking. The mixture was finally separated to an upper (water–methanol) phase and a lower (chloroform) phase by centrifugation at 3000 rpm for 5 min. Glucose or dextran extracted into an upper phase was then assayed by the phenol–sulfuric acid method proposed by Dubois *et al.*[15] It was confirmed in advance that mannitol had no influence on the color development.

Results and Discussion

Factors Affecting the Recovery of Lipid Mixture Prepared by Spray-Drying The recoveries of lipid mixture prepared by spray-drying are shown in Table I. In this experiment, chloroform or dichloromethane was used as a volatile organic solvent to solubilize lipids, and mannitol was used as a core material. In spray-drying without a core material, the recovery of lipid mixture was estimated by determination of the weight of product collected from the reservoir and cyclone. In spray-drying with a core material, it was estimated by determination of the amount of lecithin in the product using a Phospholipids B-test Wako.

When the organic solution of lipids without a core material was spray-dried, scant product was obtained from the reservoir and cyclone, because a large portion of the dried lipid mixture adhered to the wall of the drying chamber.

The reasons mannitol was used as a core material were not only to prevent the dried lipid mixture from adhesion to the wall of the chamber, but also to make the spray-dried product hydrate effectively by increasing the surface area of the lipid mixture. Actually, as shown in Table I, the recoveries of lipid mixture became higher when the concentration of core material was increased. Furthermore, the hydration of spray-dried product became much easier than expected (data not shown).

However, in the case of EggPC, satisfactory recovery was not possible because of the adhesion of lipid mixture to the wall of the chamber; this was true even if the core material particles were suspended in an organic solution. According to the X-ray diffraction pattern of anhydrous lecithin which was recrystallized from chloroform (data not shown), it was confirmed that EggPC, whose gel–liquid crystalline phase transition temperature was relatively low, was more amorphous than DPPC, H-EggPC and H-SoyaPC at room temperature. This result therefore suggests that the crystallinity of lipids has great influence on the production procedure of spray-drying.

Chloroform and dichloromethane which have different boiling points were used as organic solvents, but no difference in the effect was observed between the two solvents in this experiment (detailed data not shown).

TABLE I. Recoveries of Lipid Mixture Prepared by Spray-Drying

Lipid composition	Molar ratio	Total lipid concentration (mM)	Core material[a] (%)	Recovery[b] of lipid mixture
EggPC/Chol/DCP	5:5:0.5	8.4	0	Poor
		42	0	Poor
	7:3:1	36	30	Poor
EggPC/Chol/SA	7:3:1	63	10	Poor
DPPC alone	—	20	0	Good
		40	10	Excellent
		50	30	Excellent
DPPC/Chol/DCP	5:5:0.5	42	0	Poor
	7:3:1	126	0	Poor
		39	30	Excellent
		63	10	Good
DPPC/Chol/SA	5:5:1	44	0	Poor
		44	5	Poor
H-SoyaPC/Chol/DCP	5:5:1	36	15	Excellent
	7:3:1	145	0	Good
H-SoyaPC/Chol/SA	7:3:1	126	0	Poor
H-EggPC/Chol/DCP	5:5:1	42	0	Poor
		36	30	Excellent
		88	10	Excellent
		176	30	Excellent
	7:3:1	157	0	Good
H-EggPC/Chol/SA	5:5:1	36	15	Excellent
	7:3:1	36	15	Excellent
Ref. Chol alone	—	17	15	Excellent
DCP alone	—	3.3	15	Excellent
SA alone	—	6.9	15	Excellent

a) Mannitol at the indicated concentration was suspended in chloroform or dichloromethane whose amount was varied from 50 to 500 ml. *b*) Evaluated according to the recovery of lipid mixture from the reservoir and cyclone: excellent, recovery > 50%; good, 10% < recovery ≦ 50%; poor, recovery ≦ 10%.

Mannitol was used as a core material, but it was suggested that other sugars such as glucose, sucrose, dextran could also be used. Especially if disaccharides or polysaccharides were used, it was expected that the recovery would become higher because of increase of the usable amount of core material. Considering the final isotonicity of the liposomal dispersion, disaccharides or polysaccharides could be used in a larger quantity than mannitol or glucose.

This experiment was performed under restricted spray-drying conditions. Further examination is considered to be necessary for optimization of operating conditions and other factors.

Crystallinity of Lipid Mixture Prepared by Spray-Drying The X-ray diffraction patterns of the physical mixture of lipids, Bangham's lipid film and the spray-dried product composed of DPPC, Chol and DCP in a molar ratio of 7:3:1 are shown in Fig. 2. While the diffraction pattern of the physical mixture of lipids showed some sharp peaks attributable to each lipid material, some of these peaks disappeared in the patterns of Bangham's lipid film and the spray-dried product. It was confirmed that the spray-dried product was more amorphous than Bangham's

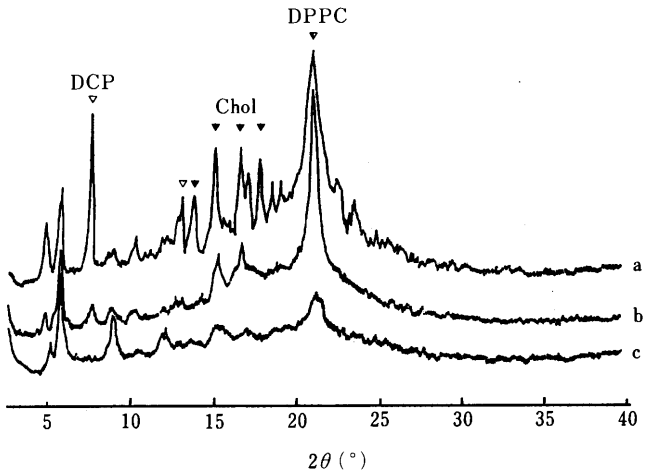


Fig. 2. X-Ray Diffraction Patterns of Different Types of Lipid Mixtures

a) Physical mixture of lipids; b) Bangham's lipid film; c) spray-dried product. The lipid mixture was composed of DPPC, Chol and DCP in a molar ratio of 7:3:1. The triangular symbols (▽, ▼ and ▽) indicate the peaks attributable to DPPC, Chol and DCP, respectively.

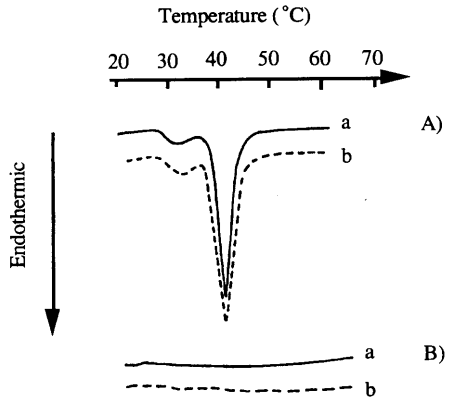


Fig. 3. DSC Curves of Bangham's Liposomes and SD-Liposomes Composed of DPPC Alone or DPPC/Chol/DCP Mixture

A) 42 mM DPPC alone; B) 42 mM DPPC/18 mM Chol/6 mM DCP mixture; a) Bangham's liposomes; b) SD-liposomes. Twenty μl of each sample was put into a pan.

lipid film. This phenomenon could explain why the spray-dried product could be very easily hydrated with water.

The fact that the spray-dried products could be stored for long periods prior to hydration was considered another advantage of this spray-drying method. Actually, even spray-dried products which had been stored in a refrigerator for more than 3 years could be hydrated with water very easily to form a liposomal dispersion.

Incorporation of Chol into the SD-Liposomal Membrane DSC is a well-developed technique applied to the study of the thermal behavior of lipids.[16] The thermal behaviors of SD-liposomes and Bangham's liposomes obtained from the differential scanning calorimeter are shown in Fig. 3. Both types of liposomes composed of 42 mM DPPC alone or a 42 mM DPPC/18 mM Chol/6 mM DCP mixture were prepared on a scale of 4 ml. For the SD-liposomes the spray-dried product without a core material was hydrated with a 0.28 M glucose aqueous solution. For Bangham's liposomes the lipid film was hydrated with the same solution. Both types of liposomes composed of DPPC alone exhibited two similar endothermic transitions, that is, pre-transition and main transition.[17] In the same way, these transitions of both types of liposomes composed of the DPPC/Chol/DCP mixture disappeared. From these results and a generalization that Chol at more than 20 mol percent makes the narrow peak of DPPC at Tc disappear,[18] it was indicated that Chol could be incorporated into the SD-liposomal membrane as well as Bangham's liposomes.

GFC is also very useful for characterization of the liposomes. Figure 4 shows the gel filtration profiles of SD-liposomes composed of 7 mM DPPC, 3 mM Chol and 1 mM DCP. After the spray-dried product with a core material was hydrated with 100 ml of distilled water and homogenized, the SD-liposomal dispersion was extruded through a polycarbonate membrane filter with 0.2 μm pore size. There was no residue remaining on the top of the column where the dispersion was applied, and the respective recoveries of DPPC and Chol from all of the collected fractions were almost 100%. Not only the above results in DSC but also the co-elution pattern of DPPC and Chol in the void volume of GFC as shown in Fig. 4 indicates that Chol could certainly be incorporated into the SD-liposomal membrane.

Incorporation of Charged Lipids into the SD-Liposomal Membrane Figure 5 shows an example of histograms of the electrophoretic mobilities of liposomes determined by laser doppler electrophoresis method. The SD-liposomes used here were the same as used for GFC. For Bangham's liposomes the lipid film of the same lipid composition was hydrated with a 0.28 M glucose aqueous solution. Both types of liposomal dispersion were extruded through a 0.2 μm polycarbonate membrane filter. There was no difference in the electrophoretic mobilities between Bangham's liposomes

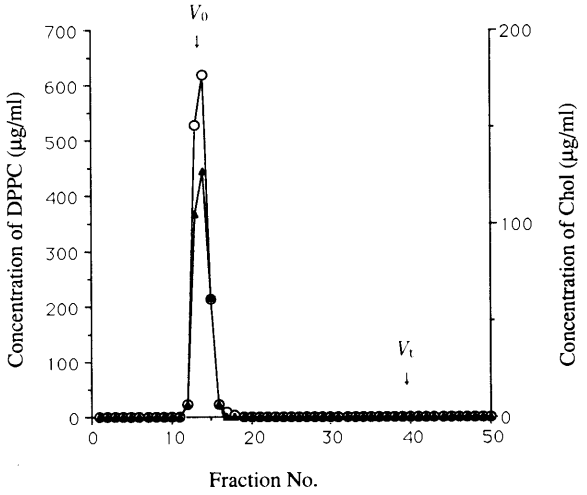


Fig. 4. Gel Filtration Profiles of SD-Liposomes

One ml of SD-liposomes composed of 7 mM DPPC, 3 mM Chol and 1 mM DCP was applied to a 2.2 cm × 42 cm column of Sepharose CL-4B, and the fraction sizes were 4.1 ml. ○, concentration of DPPC; ▲, concentration of Chol; V_0, void volume; V_t, total volume.

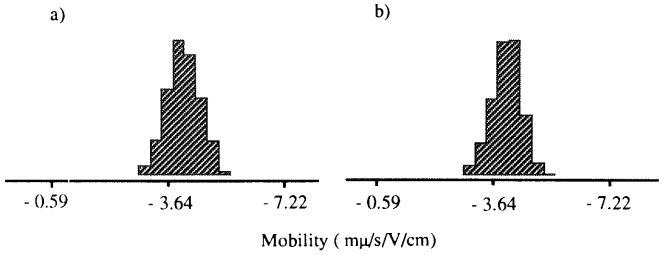


Fig. 5. Histograms of Electrophoretic Mobilities of Bangham's Liposomes and SD-Liposomes Determined by Laser Doppler Method

a) Bangham's liposomes; b) SD-liposomes; lipid composition was 7 mM DPPC, 3 mM Chol and 1 mM DCP. The zeta potentials of liposomes were a) −54.2 mV and b) −53.7 mV, respectively.

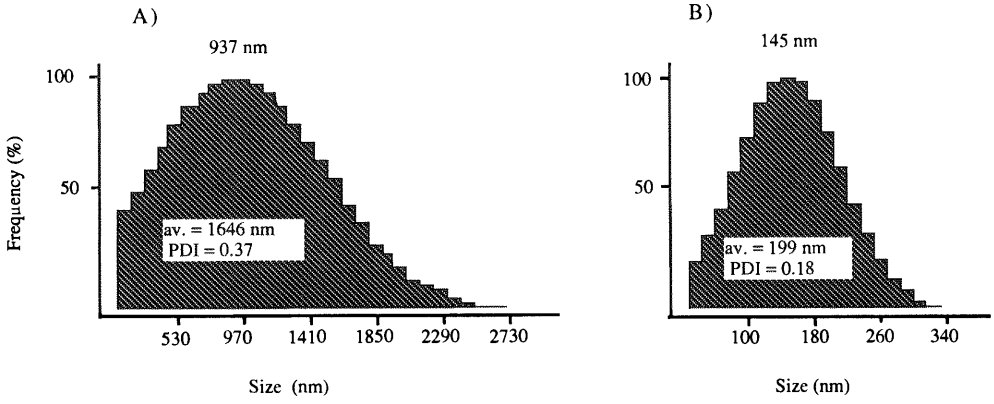


Fig. 6. Size Distributions of Unextruded and Extruded SD-Liposomes Determined by Quasi-Elastic Laser Light Scattering

SD-liposomes composed of 7 mM DPPC, 3 mM Chol and 1 mM DCP were extruded through a 0.2 μm polycarbonate membrane filter. A) Without extrusion; B) after extrusion through a 0.2 μm filter.

and the SD-liposomes. The zeta potential of the former was −54.2 mV and of the latter was −53.7 mV according to the Smoluchowski equation. Similarly, there were no significant differences between these two types of liposomes composed of various lipid compositions (data not shown). It was suggested from these results that the charged lipids could be incorporated into the SD-liposomal membrane as well as Bangham's liposomes.

Size Distribution of the SD-Liposomes Figure 6 shows an example of size distributions of the SD-liposomes determined by QELS, and the liposomes used here were the same as used for GFC and the electrophoretic mobility measurement. The mean particle size, peak particle size and PDI of the SD-liposomes, which were homogenized by a homo-mixer and were not extruded through a membrane filter, were 1646, 937 nm and 0.37, respectively. These data were very consistent with those of Bangham's liposomes. The size distribution of SD-liposomes after extrusion through a 0.2 μm polycarbonate membrane filter became more homogenous. The mean particle size, peak particle size and PDI were 199, 145 nm and 0.18, respectively. Though the data are not shown, similar results were observed in other lipid compositions or on other scales of more than 100 ml. These results indicate that the SD-liposomes are able to be easily extruded through a polycarbonate membrane filter to obtain a more homogeneous size distribution of liposomes.

Encapsulation Efficiencies of Water-Soluble Model Drugs into the SD-Liposomes The encapsulation efficiencies of water-soluble model drugs into the SD-liposomes are shown in Fig. 7. Glucose was used as a low molecular weight model drug and dextran T-40 as a high molecular weight model drug. The SD-liposomes used here were all from the spray-dried product with a core material, and were composed of various lipid compositions. The amounts of dispersions varied from 100 to 500 ml. As shown in this figure, the encapsulation efficiency was increased with increase of the amount of lipids irrespective of the lipid composition. The encapsulation efficiency of glucose was a little better than that of dextran. These results indicate that the lipid vesicles prepared by spray-drying method have an aqueous space within a continuous closed bilayer as well as the traditional liposomes. However, it is noted that the encapsulation efficiency of the high molecular weight drug was more than 50%, because this high efficiency has been rarely acquired by conventional preparation methods except the reverse-phase evaporation vesicles (REV) method introduced by Szoka and Papahadjopoulos.[19] The increase of the surface area of the lipid mixture by using a core material is thought perhaps to have influenced on the availability of lipids. The temporary hyperisotonization and the subsequent explosion inside of the spray-dried product owing to the penetration of water may have had some additional effect.

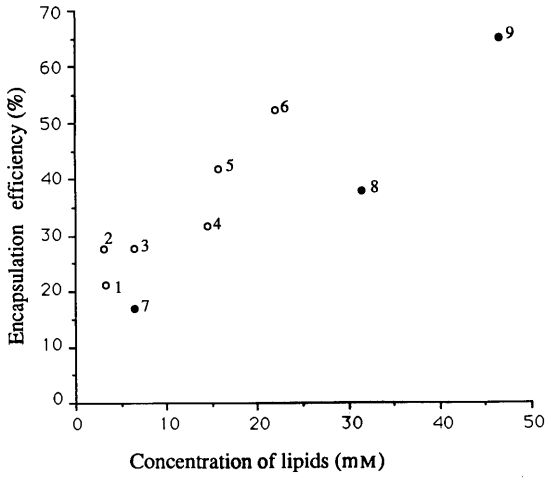


Fig. 7. Encapsulation Efficiencies of Water-Soluble Model Drugs into SD-Liposomes

○, glucose; ●, dextran T-40. The lipid compositions and the amounts of dispersions were as follows: 1, DPPC/Chol/DCP (7:3:1, molar ratio), 500 ml; 2, H-EggPC/Chol/DCP (5:5:1), 300 ml; 3, H-SoyaPC/Chol/DCP (5:5:1), 250 ml; 4, H-EggPC/Chol/DCP (5:5:1), 200 ml; 5, DPPC/Chol/DCP (7:3:1), 100 ml; 6, H-EggPC/Chol/DCP (5:5:1), 300 ml; 7, EggPC/Chol/DCP (7:3:1), 100 ml; 8, H-EggPC/Chol/SA (5:5:1), 150 ml; 9, H-EggPC/Chol/SA (7:3:1), 100 ml.

Advantages of Spray-Drying Method for Mass Production of Liposomes Compared with Bangham's Method Based on the above results, Table II summarizes the characteeristics of the spray-drying method without or with a core material for preparing liposomes in comparison with Bangham's method. Though volatile organic solvents must be used in each method, the spray-drying method has the advantage of ease of operation. It is very difficult by Bangham's method to prepare liposomes uniform in quality with good reproducibility, because the procedure of evaporating organic solvents greatly affects the final characteristics of liposomes. On the other hand, control of spray-drying is considered very easy if the optimum operating conditions are fixed.

The item of practical surface area of a lipid mixture is related to the hydration rate of lipids, the formation rate of liposomes and the encapsulation efficiencies of drugs. The practical surface area of a mixture prepared by the spray-drying method is much larger than that of a mixture (lipid film) prepared by Bangham's method. While the surface area of the spray-dried product, especially with a core material, is almost equal to the total surface area of core material particles, that of Bangham's lipid film is almost equal to the wall space of the round-bottomed flask.

The item of versatility in lipid composition is related to

Table II. Characteristics of Spray-Drying Method Compared with Bangham's Method

Evaluated items	Bangham's method	Spray-drying method	
		Without a core material	With a core material
Organic solvents	Used	Used	Used
Operation	Not easy	Easy	Easy
Lipid mixture			
Reproducibility	Not good	Good	Good
Practical surface area	Small	Middle	Large
Hydration rate	Not fast	Not fast	Fast
Versatility in composition	Large	Small	Large
Storage	Not easy	Easy	Easy
Encapsulation efficiency of a high molecular weight drug	Not high	Not done[a)]	High
Mass production	Difficult	Not easy	Easy

a) Not tested since recoveries of lipid mixture were very low.

the recovery of lipid mixture. As mentioned above, the recovery was very low in the case of the spray-dried product without a core material, since a large portion of the dried lipid mixture adhered to the wall of the drying chamber. This sole disadvantage of the spray-drying method could be greatly improved by using a core material, that is, the recovery of lipid mixture was very much increased. In the case of EggPC, whose gel–liquid crystalline phase transition temperature was relatively low, satisfactory recovery could not be obtained irrespective of existence of a core material. Further examinations for ways to improve this are necessary.

With respect to the storage of lipid mixtures before hydration, the spray-drying method has an advantage. It is, of course, not easy to store Bangham's lipid film which is on the wall of a round-bottomed flask for a long time, owing to the restriction of storage space. However, in many cases spray-dried products are obtained in the powdered state from the reservoir and cyclone, making the storage of products much easier.

Perhaps because of the increase of practical surface area of lipid mixture when a core material was used, the encapsulation efficiency of a high molecular weight drug into the SD-liposomes was very high as shown in Fig. 7. The temporary hyperisotonization and the subsequent explosion inside of the spray-dried product owing to the penetration of water are thought to have some additional effect on this. This characteristic has been rarely acquired by conventional preparation methods.

It is concluded that this novel method of preparing liposomes by spray-drying, especially with a core material, is very useful and valuable from the viewpoint of mass production. A core material such as mannitol, which is non-toxic and injectable, was shown to play an important role in this spray-drying method. This method does use chloroform, which is a volatile organic solvent, but if chemical companies, which supply lipid materials separately, could product and supply spray-dried products according to the orders of the pharmaceutical companies, this would be very convenient for the mass production of liposomes by pharmaceutical plants.

Acknowledgment The authors thank Yamato Scientific Co., Ltd. for providing them with facilities and technical assistance in using a spray dryer.

References and Notes

1) A part of this work was presented at the 106th Annual Meeting of the Pharmaceutical Society of Japan, Chiba, April 1986, and at the 7th International Symposium on Surfactants in Solution, Ottawa, October 1988.
2) Present address: *School of Pharmaceutical Sciences, University of Shizuoka, Yada, Shizuoka 422, Japan.*
3) G. Gregoriadis (ed.), "Liposome Technology," CRC Press, Inc., Florida, 1984; M. Furusawa, E. Kumazawa, K. Morishita, K. Murakami, S. Shibamura, S. Maeda, S. Hirota, H. Kikuchi, K. Yachi and M. Sawada, "Recent Progress of Life Science Technology in Japan," ed. by Y. Ikawa and A. Wada, Academic Press/Harcourt Brace Jovanovich Japan, Inc., Tokyo, 1989, pp. 225—234.
4) F. Szoka and D. Papahadjopoulos, *Ann. Rev. Biophys. Bioeng.*, **9**, 467 (1980).
5) T. Ohsawa, H. Miura and K. Harada, *Chem. Pharm. Bull.*, **32**, 2442 (1984).
6) S. M. Gruner, R. P. Lenk, A. S. Janoff and M. J. Ostro, *Biochemistry*, **24**, 2833 (1985).
7) N. I. Payne, P. Timmins, C. V. Ambrose, M. D. Ward and F. Ridgway, *J. Pharm. Sci.*, **75**, 325 (1986); N. I. Payne, I. Browning and C. A. Hynes, *ibid.*, **75**, 330 (1986).
8) H. Kikuchi, H. Yamauchi, K. Yachi and S. Hirota, the Symposium in the 107th Annual Meeting of the Pharmaceutical Society of Japan, Kyoto, April 1987; *idem*, *Chem. Pharm. Bull.*, in preparation.
9) A. D. Bangham, M. M. Standish and J. C. Watkins, *J. Mol. Biol.*, **13**, 238 (1965).
10) F. Olson, C. A. Hunt, F. C. Szoka, W. J. Vail and D. Papahadjopoulos, *Biochem. Biophys. Acta*, **557**, 9 (1979).
11) D. E. Koppel, *J. Chem. Phys.*, **57**, 4814 (1972); J. C. Brown and P. N. Pusey, *ibid.*, **62**, 1136 (1975).
12) J. C. Earnshaw and M. W. Steer (eds.), "The Application of Laser Light Scattering to the Study of Biological Motion," Plenum Press, New York, 1983.
13) R. J. Hunter, "Zeta Potential in Colloid Science," Academic Press, London, 1981.
14) E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.*, **37**, 911 (1969).
15) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
16) D. Chapman, R. M. Williams and B. D. Ladbrooke, *Chem. Phys. Lipids*, **1**, 445 (1967).
17) S. Mabrey-Graud, "Liposomes: From Physical Structure to Therapeutic Applications," ed. by C. G. Knight, Elsevier/North-Holland Biomedical Press, Amsterdam, 1981, pp. 105—138.
18) S. Mabrey, P. L. Mateo and J. M. Strutevant, *Biochemistry*, **17**, 2464 (1978).
19) F. Szoka and D. Papahadjopoulos, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 4194 (1978).

Preparation of Powdered Redispersible Vitamin E Acetate Emulsion by Spray-Drying Technique

Hirofumi TAKEUCHI,[a] Hideto SASAKI,[a] Toshiyuki NIWA,[a] Tomoaki HINO,[a] Yoshiaki KAWASHIMA,*[,a] Keizou UESUGI,[b] Masanori KAYANO,[c] and Yasuo MIYAKE[d]

Gifu Pharmaceutical University,[a] 5–6–1 Mitahora-higashi, Gifu 502, Japan, Research Laboratories, Eisai Co., Ltd.,[b] Kawashima, Hashima, Gifu 483, Japan, Eisai Co., Ltd.,[c] 4–6–1 Koishikawa, Bunkyo-ku, Tokyo 112, Japan, and Honjo Factory, Eisai Co., Ltd.,[d] 2–3–14 Minami, Honjo, Saitama 367, Japan. *Received November 19, 1990*

Vitamin E acetate (VEA) was transformed into powdered form by a spray-drying technique and the water dispersible or drug releasing property of the dried particle was evaluated. The powdered VEA was prepared by spray-drying emulsified VEA with colloidal silica (Aerosil 200) and a disintegrant such as low-substituted-hydroxypropylcellulose (L-HPC). VEA in the spray-dried particle was chemically stable in storage longer than three years. On being dispersed in water with gentle shaking, the spray-dried particle released a large number of VEA droplets into the water, which formed a stable emulsion without additional stirring processes. This VEA releasing was mainly due to water uptake and swelling of the disintegrant formulated into the particle. The drug releasing property of the spray-dried particle significantly varied with the type of additives formulated, *i.e.*, surfactant and disintegrant. The constituent ratio of the excipients (Aerosil 200 to disintegrant) influenced not only the VEA releasing but also the flowing property of the spray-dried particle. Based on these findings, an optimum formulation for the powdered VEA with both good drug releasing and flowing properties was established.

Keywords vitamin E acetate; spray-drying; dry emulsion; disintegrant; drug release; redispersion

Introduction

There are a lot of oily drugs such as various lipophilic vitamins or oily solutions of water-insoluble drugs formulated into oral dosage form. The oily ingredients can be filled into soft gelatin capsules. A tablet and a hard gelatin capsule containing oily drugs, which are converted to powder form with excipients, are also popular dosage forms. In each dosage form, a good drug dissolution in the gastrointestinal tract is essential to ensure sufficient bioavailability. Especialy, in developing the solid dosage forms of the oily materials, much attention should be paid to the drug releasing properties of the preparations.

The relationship between the dosage forms and the extent of bioavailability has been investigated.[1] Recently, it has been demonstrated that a hard gelatin capsule filled with an oily solution of *dl*-α-tocopherol is superior in bioavailability to the corresponding tablet form.[2] The relatively poor bioavailability is attributed to less adequate drug release properties. It has been pointed out that different commercial preparations of powdered oily drugs showed different water dispersibility, drug dissolution and bioavailability.[3] Although many patents[4–7] are claiming various formulations and preparation methods of the powdered oily drugs, there are few papers evaluating the effects of preparative formulations on water dispersibility, drug releasing property and bioavailability.

The aim of the present study is to design a powder dosage form of an oily drug having good water dispersibility and drug releasing property by using a spray-drying technique. *dl*-α-Tocopherol acetate (VEA) was used as a model of oily materials. VEA containing powders were prepared by spray-drying emulsified VEA with various excipients. The effects of the type and the amount of additives on the water dispersibility and drug releasing property of the spray-dried products were investigated to determine the optimum formulation.

Materials and Methods

Materials VEA was received from Eisai Co., Ltd. Colloidal silica (Aerosil 200), low-substituted hydroxypropylcellulose (L-HPC) and microcrystallinecellulose–sodiumcarboxymethylcellulose (Avicel RC-591NF) were obtained from Aerosil Japan Co., Ltd., Shin-etsu Chemical Co., Ltd. and Asahi Chemical Industry Co., Ltd., respectively. Other excipients were received from each supplier listed in the note of Tables I and II. An analytical reagent grade of polyoxyethylenesorbitanmonolaurate (Tween 20) was used without further purification. Polyoxyethylene–polyoxypropylene-blockcopolymer (Pluronic F-68), sugar ester (DK F-160, DK SS) and purified Soya lecithin (Epikuron 200), which were obtained from Asahidenka Co., Ltd., Daiichi Kogyo Seiyaku Co., Ltd. and Nikko Chemical Co., Ltd. respectively, were used as obtained.

Preparation of Spray-Dried VEA Particles Spray-dried VEA particles were prepared as follows. Aerosil 200 and/or additives (20 g) were suspended in an aqueous solution dissolving 1 g of surfactant (965 ml).

TABLE I. Effect of the Type of Additive on Water Dispersibility of Spray-Dried Particle with VEA

Additive	VEA content (%)	Dispersity value (%)	
		D (5)	*D* (60)
L-HPC	35.6	98.6	69.4
Avicel RC-591 NF	27.2	96.1	66.5
HPMC[a]	25.7	79.6	39.9
MCC[b]	33.4	11.1	10.3
Whey protein powder[c]	27.7	56.0	4.9
Lactose[d]	33.4	3.9	2.3
No additive	33.0	2.5	1.0

a) Hydroxypropylmethylcellulose, Shin-etsu Chemical Co. *b*) Microcrystallinecellulose, Asahi Chemical Industry Co. *c*) Taiyo Kagaku Co. *d*) Meggle Co.

TABLE II. Effect of the Type of Disintegrant on VEA Releasing Property of Spray-Dried Particle in Water

Disintegrant	VEA content (%)	*R* (60) (%)
L-HPC (LH-21)	35.6	69.4
L-HPC (LH-31)	31.2	64.6
PCS[a]	36.6	4.5
Croscarmellose-sodium[b]	31.1	6.2
CMC-Ca[c]	38.4	5.0
No disintegrant	33.0	1.0

a) Partly pregelatinized starch, Asahi Chemical Industry Co. *b*) Asahi Chemical Industry Co. *c*) Carboxymethylcellulose calcium, Gotoku Pharmaceutical Enterprise Inc.

VEA (15 g) was emulsified into the suspension by agitating with a homomixer (T.K. auto homomixer, Tokushukika Kogyo Co., Ltd.) at 6000 rpm for 10 min. The resultant VEA emulsion with excipients was fed into a spray-dryer (L-12 type, Okawara Kakoki Co., Ltd.) by a peristaltic pump at a rate of 50 ml/min. Spray-drying conditions were as follows: inlet air temperature, 220 °C; outlet air temperature, 125±5 °C; rotation speed of atomizer, 16500 rpm.

A corresponding VEA granule was prepared by the wet granulation method. VEA (3 g), L-HPC (0—4 g), Aerosil (0—4 g) and 2 ml of a 10% aqueous solution of Tween 20 were mixed with an appropriate amount of distilled water by using a mortar and a pestle. The resultant wet mass was extruded to cylindrical granules through a 12 mesh sieve. After drying at room temperature at reduced pressure for a day, the granules of 32 to 10 mesh fraction were used for the experiment.

VEA Content in the Spray-Dried Particle VEA in the spray-dried particle (360 mg) was extracted with methanol (9.5 ml) by shaking with a shaker (V-S type, Iwaki Co., Ltd.) at 240 strokes per minute for more than 1 h. Insoluble ingredients contained in the sample solution were removed by centrifuging at 3000 rpm for 10 min and filtrating through a membrane filter (0.3 μm). The filtrate (0.5 ml) diluted with methanol (9.5 ml) was subjected to determine the VEA content by high performance liquid chromatography (HPLC). The stationary and mobile phases were Nucleosil 5C18 packed in a column (150×4.6 mm i.d.) and methanol, respectively, operated at an ambient temperature and the rate of 1.0 ml/min. A sample solution (20 μl) was injected into the chromatograph (UVIDEC-100-V, Japan Spectroscopic Co., Ltd.) and the VEA was detected with the UV detector at 284 nm.

Evaluation of Water Dispersibility and VEA Releasing Property of Spray-Dried Particles As a dispersion test, spray-dried VEA (360 mg) was dispersed into distilled water (9.5 ml) in a test tube by hand-shaking (10 times) at room temperature. The test tube was allowed to stand for an appropriate period (5 min or 1 h), and an aliquot (0.5 ml) was sucked up through a syringe at the middle point of the dispersed phase. VEA droplets dispersed and those remaining in the particle in the sample solution were solubilized by adding an appropriate amount of methanol and successively shaking it with a shaker at 240 strokes per minute for more than 1 h. The amount of VEA in the methanol solution was determined by HPLC after removing the insoluble ingredients by the same method as described above. For determination of the amount of VEA released from the particle, the sample solution from which the dispersed particle was removed by centrifugation at 2000 rpm for 3 min before adding methanol was applied to the analysis.

Water dispersibility and VEA releasing property were evaluated by dispersity value ($D(t)$) and percent released ($R(t)$) at time t determined by the following equations

$$D(t)=\frac{\text{VEA(d)}}{\text{VEA(c)}}\times 100\ (\%) \quad (1)$$

$$R(t)=\frac{\text{VEA(r)}}{\text{VEA(c)}}\times 100\ (\%) \quad (2)$$

where, VEA(d) is the total amount of VEA dispersed in the test solution at time t, VEA(r) is the amount of VEA released in the test solution at time t and VEA(c) is the VEA content of the spray-dried particle tested.

Results and Discussion

VEA Releasing Property of the Spray-Dried Particle with Colloidal Silica (Aerosil 200) VEA emulsified in water by using Tween 20 as an emulsifying agent was spray-dried with Arerosil 200 in order to be converted to a powder form as described in the experimental section. Figure 1 is a microscopicphotograph of the resultant particle, showing that the particle was spherical and 10 to 50 μm in diameter.

The initial dispersity value of the spray-dried particle redispersed in water reached 100%. However, the value dropped quickly to a few percent in a few minutes. This result suggested that the spray-dried particle settled down without releasing VEA droplets. It was observed microscopically that the particle kept a spherical shape without being swelled or disintegrated when dispersed in water. Thus, it was confirmed that the spray-dried VEA with Aerosil did not release a large amount of VEA by such a relatively gentle dispersing method as hand-shaking.

It was found in the previous paper[8] that Tween 20 adsorbed on Aerosil 200 surface in a monolayer in the aqueous solution and that Aerosil particles adsorbed with Tween 20 were firmly agglomerated with hydrohobic interaction of the lipophilic segment of surfactant molecules during spray-drying. The rigidity and lipophilic properties of agglomerated particles might cause the poor release of VEA when they are redispersed in aqueous medium. The VEA releasing property of the spray-dried particle was improved by increasing the amount of surfactant formulated up to ten times of that in the formulation described in the experimental section. This is caused by multilayer adsorption of the surfactant on the Aerosil particle, which leads to loose agglomeration of the particles. The excess amount of surfactant makes the resultant agglomerates hydrophilic. However, so much surfactant incorporation should be avoided because of a dosing risk such as irritation to the gastrointestinal tract. Moreover, an increase in the amount of surfactant in the spray-drying formulation led to low recovery and poor flowing property of the resultant particles.

Improvement in VEA Releasing Property by Additives To improve the VEA releasing property, various kinds of additives such as a disintegrant and a stabilizer were incorporated into the spray-drying formulation as an alternative method. The additives were incorporated according to the formulation described in Chart 1. The VEA content and dispersity values ($D(5)$ and $D(60)$) of the particle are summarized in Table I, where $D(5)$ and $D(60)$ are the water dispersity values at 5 and 60 min after standing, respectively. It was found that dispersibility of the spray-dried particle was considerably improved by introducing the additives. The improvement in dispersity values of the spray-dried particle with Avicel RC-591NF or HPMC was attributed to the improvement in the stability of the suspension of the spray-dried particle, since a considerable amount of intact spray-dried particle was observed in the withdrawn sample to determine the amount of VEA dispersed. Actually, $R(60)$ values of these particles were less than 22%. The

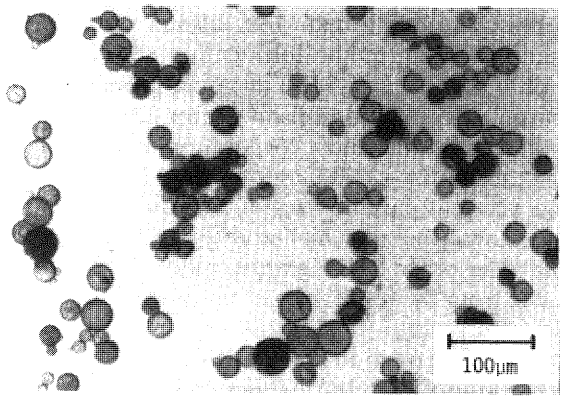


Fig. 1. Spray-Dried Particles with VEA

vitamin E acetate	15 g
Aerosil 200	5 g
Tween 20	1 g
additive (disintegrant)	15 g
water	964 g

Chart 1. Formulation of VEA Emulsion for Spray-Drying

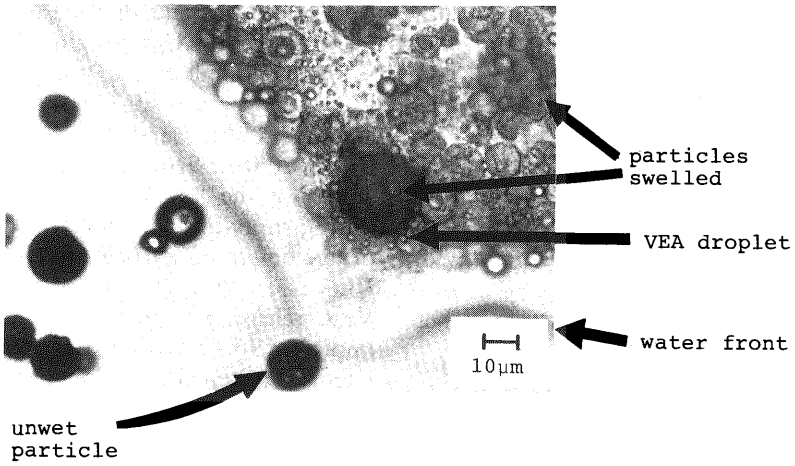


Fig. 2. Release of VEA Droplets from Spray-Dried Particles with L-HPC

improvement in suspension stability is attributed to the increase in the viscosity of the dispersing medium. In the case of Avicel RC-591NF, the gel structure formed with sodiumcarboxymethylcellulose dissolved from the particle was assumed to be responsible for the increase in the suspension stability. As the particle settled down gradually, the dispersity decreased with increasing standing time.

On the other hand, in the case of L-HPC formulation, the spray-dried particle was not observed in the withdrawn sample especially at 60-min standing. The VEA releasing property value ($R(60)$) of this particle is 69.4%, and the $R(60)$ value is equal to the $D(60)$ value (Tables I and II). This result means that the particle with L-HPC effectively released VEA droplets, which formed a stable emulsion. Microscopic analysis showed that the particle with L-HPC considerably swelled and released many VEA droplets when a small amount of water was added to them on a slide glass (Fig. 2). This phenomenon was similar to that observed for a spray-dried tolbutamide with a disintegrant reported in our previous paper.[9]

Dispersibilities of Spray-Dried VEA Particle with Disintegrant Based on the finding that L-HPC formulated into the spray-dried particle improved the VEA releasing, the effect of the type of disintegrant on the VEA releasing property was investigated. The disintegrants were incorporated according to the formulation shown in Chart 1. The VEA content and VEA releasing property ($R(60)$) of the particles with various disintegrants are shown in Table II. It was found that the incorporation of L-HPC gave the most excellent drug releasing property. LH-21 and LH-31 having different particle sizes of 37.8 and 26.9 μm, respectively, showed the same high ability of facilitating the drug release. The other disintegrants could not promote VEA releasing from the resultant particle as much as the L-HPC particle did.

The weight fraction of L-HPC in the formulation influenced the VEA releasing and flowing properties of the spray-dried particle. The respective dispersity values of $D(5)$ and $D(60)$ ($=R(60)$) after 5 and 60 min standing are plotted as a function of the weight fraction of L-HPC in the formulation (Fig. 3). The amount of VEA released from the particle increased with an increase in the weight fraction of L-HPC up to 87.5%. At a low weight fraction of L-HPC, particularly below 25%, VEA was not released from the

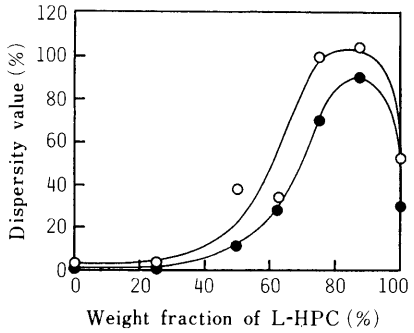


Fig. 3. Effect of the Weight Fraction of L-HPC in the Formulation (L-HPC + Aerosil 200) on VEA Releasing Property of Spray-Dried Particle in Water

○, $D(5)$; ●, $D(60)$.

particle. If Aerosil was not formulated, the particle released relatively large VEA droplets, which coalesced and floated up to the surface of the solution. The change in drug releasing property is photographically shown in Fig. 4. The formulation of Aerosil was found to affect the flowing property of the resultant particle. The flowability parameter in Kawakita's equation[10] applied to a tapping test of the particles was improved with an increase in the amount of Aerosil, *i.e.*, a decrease in the amount of L-HPC in the formulation. The values were 0.4, 0.3 and 0.26 for the particles having different L-HPC contents, 87.5, 75 and 62.5%, respectively. Considering both the VEA releasing property and the flowing property of the particles, the optimum constituent ratio of L-HPC to Aerosil was concluded to be 5—7.

The type of surfactant formulated in the particle also influenced the drug releasing property as shown in Table III. Tween 20 or Pluronic F-68 offered the resultant spray-dried particle a good drug releasing property. When lecithin was formulated in the preparation, the VEA releasing property was extremely depressed probably because of its low hydrophilic–lipophilic-balance (HLB) value. However, a sucrose ester (DK F-160 and DS SS) in a solid state did not increase the drug releasing property so much in spite of its high HLB value. These results suggested that not only the affinity of the surfactant adsorbed on the particles for water but also the dissolution property of the surfactant should be taken into account to elucidate the VEA

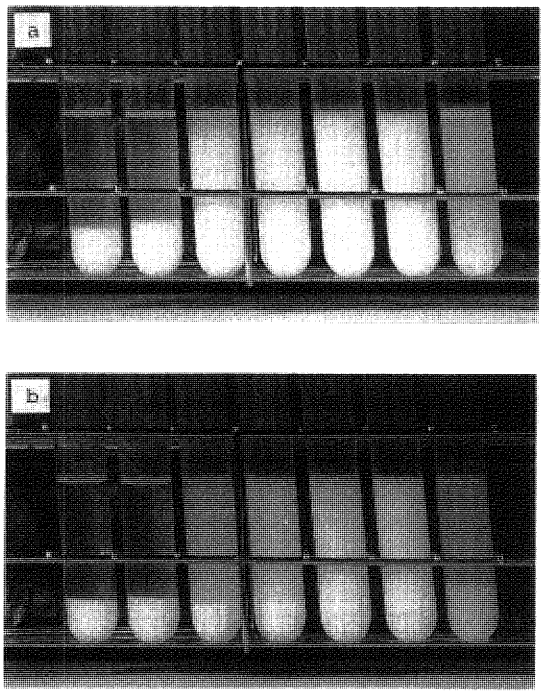


Fig. 4. VEA Emulsions Released from Spray-Dried Particles with 0, 25, 50, 62.5, 75, 87.5 and 100% (from Left to Right) Weight Fraction of L-HPC in the Formulation (L-HPC + Aerosil 200) at 5 min (a) and 1 h (b) after Hand-Shaking

TABLE III. Effect of the Type of Surfactant on VEA Releasing Property of Spray-Dried Particle in Water

Surfactant	VEA content (%)	R (60) (%)
Tween 20[a]	35.6	69.4
Tween 80[b]	39.3	25.6
Pluronic F-68	40.8	75.7
HCO 60[c]	40.2	41.4
DK F-160	35.3	9.7
DK SS	27.2	7.2
Lecithin	42.5	1.6

a) Kishida Chemical Co. *b*) Polyoxyethylene sorbitan monooleate, Kishida Chemical Co. *c*) Polyoxyethylene hydrogenated castor oil, Nikko Chemicals Co.

dispersing property of the spray-dried particle, because the emulsion droplets of VEA released from the particles could be stabilized only with the dissolved surfactant in an aqueous medium.

Comparison of the Spray-Dried Particle with Wet Massed Granule The water dispersibility of the spray-dried particle was compared with that of the wet massed granule with the same formulation. The $D(60)$ value for the wet massed granule was lower than that for the spray-dried particle, although the $D(5)$ value of the wet massed granule was as high as that of the spray-dried particle. When the weight fraction of L-HPC varied, the $D(60)$ value still remained low as shown in Fig. 5. VEA in the wet massed granule remained entrapped in the solid mass although the granule was disintegrated by the disintegrating function of L-HPC formulated. Such a difference in the VEA releasing property might be attributed to the difference in the dispersibility of VEA in the particle. VEA in the spray-dried particle could be finely divided and well dispersed, and was responsible for the good VEA releasing property.

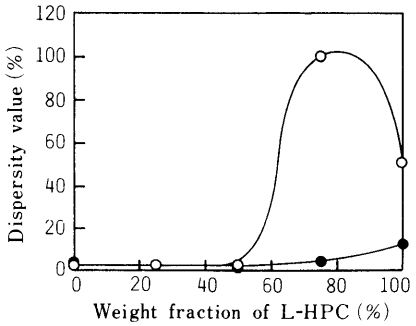


Fig. 5. Effect of the Weight Fraction of L-HPC in the Formulation (L-HPC + Aerosil 200) on VEA Releasing Property of Wet Massed Granule in Water

○, D (5); ●, D (60).

Chemical Stability of VEA in the Spray-Dried Particle From a practical point of view, the chemical stability of VEA in the spray-dried particles was investigated. The VEA in the present preparation was chemically stable for a long time. The spray-dried particles (Aerosil : L-HPC = 1 : 3) were stored in a closed styrol bottle at room temperature in the dark for more than three years. The powder appeared unchanged after storage and revealed approximately the same drug content as that of a freshly prepared one, only 1.5% of VEA being decomposed. This long term stability is acceptable for practical use. Further, the dispersibility of the spray-dried particle in water was reserved as a freshly prepared one.

Conclusion

It was confirmed that spray-dried VEA with surfactant (Pluronic F-68 or Tween 20), colloidal silica (Aerosil 200) and disintegrant (L-HPC) had a good VEA releasing property which was highly dependent on the type of constituents and their formulating ratios. Incorporation of the disintegrant into the particle made it possible to reduce the amount of surfactant which is enough to cause the drug releasing. The present powdered oily system can provide a novel solid dosage form of not only an oily drug but also an oily solution of a poorly water soluble drug with improved bioavailability.

References

1) F. S. Hom and J. J. Miskel, *J. Pharm. Sci.*, **59**, 827 (1970).
2) T. Tokumura, Y. Machida, Y. Tsushima, M. Kayano, and T. Nagai, *Chem. Pharm. Bull.*, **35**, 4592 (1987).
3) K. Minakuchi, F. Shono, K. Teraoka, K. Miyata, and M. Takasugi, *Yakuzaigaku*, **47**, 93 (1987).
4) Hoffmann La Roche Co., U.S. Patent 3962384 (1976) [*Chem. Abstr.*, **85**, 51771*q* (1976)].
5) Nisshin Seifun Co., Nippon Kokai Tokkyo Koho, 56-3326 (1981) [*Chem. Abstr.*, **81**, 111481*z* (1974)].
6) Sankyo Shokuhin Co., Nippon Kokai Tokkyo Koho, 57-181009 (1982) [*Chem. Abstr.*, **98**, 40609*k* (1983)].
7) BASF Co., U.S. Patent 4395422 (1983) [*Chem. Abstr.*, **98**, 40596*d* (1983)].
8) Y. Kawashima, H. Takeuchi, H. Sasaki, T. Handa, Y. Miyake, M. Kayano, and K. Uesugi, *Funtaikougaku Kaishi*, **25**, 574 (1988).
9) H. Takeuchi, T. Handa, and Y. Kawashima, *J. Pharm. Pharmacol.*, **39**, 769 (1987).
10) K. Kawakita and K. H. Lüdde, *Powder Technol.*, **4**, 61 (1970).

New Methods for Preparing Cyclodextrin Inclusion Compounds. IV. Enhancement of Combining Molar Ratio by Using a Ground Mixture in Heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin and Benzoic Acid System

Yoshinobu NAKAI,* Keiji YAMAMOTO, Toshio OGUCHI, Etsuo YONEMOCHI, and Takehisa HANAWA

Faculty of Pharmaceutical Sciences, Chiba University, 1–33 Yayoicho, Chiba 260, Japan. *Received November 27, 1990*

The inclusion compound of heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin (DMβCD) with benzoic acid was prepared by heating the ground mixture in a sealed container. The formation of the inclusion compound was investigated quantitatively as a functions of heating temperature, heating time, and grinding time. Differential scanning calorimetry (DSC) measurements were made of the ground mixture at different grinding times. From the DSC measurements, it was found that the temperature of crystallization of an amorphous ground mixture increased with an increase in grinding time. When the ground mixture was heated in a sealed container, the combining molar ratio of benzoic acid to DMβCD increased as the heating temperature increased. The experimental results indicated that the inclusion compound was obtained more effectively by heating the ground mixture rather than by heating the physical mixture.

Keywords heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin; benzoic acid; ground mixture; inclusion compound; sealed heating; combining molar ratio; DSC; crystallization

Introduction

In the previous paper, we have reported that the inclusion compound of heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin (DMβCD) with benzoic acid was obtained by heating a mixture of each component in a glass ampule[1] (sealed heating method). The combining molar ratio of benzoic acid to DMβCD increased with an increase of heating temperature, mixing molar ratio, and heating time. The 1 : 1 inclusion compound, however, was not prepared by this method, while the stoichiometry of the inclusion compound obtained by the coprecipitation method was 1 : 1.[1]

Nakai *et al.* demonstrated that the co-grinding method is a useful method for obtaining an amorphous molecular dispersion system.[2-6] Grinding has also caused an inclusion formation when cyclodextrins were chosen as grinding materials.[7-9]

We already tried the use of a ground mixture briefly for the sealed heat method in the previous paper.[10] In this paper, to increase the combining molar ratio, we prepared the ground mixture of DMβCD and benzoic acid as a heating sample for the sealed heat method, and investigated the properties of the ground mixture during the heating process using differential scanning calorimetry (DSC), Fourier transform infrared spectrometry (FTIR), and X-ray diffractometry.

Experimental

Materials DMβCD was purchased from Toshin Chemical Co. and used without further purification. Benzoic acid was of a reagent grade.

Preparation of Ground Mixture The ground mixture of DMβCD and benzoic acid was prepared by the addition of equimolar benzoic acid to DMβCD, followed by grinding for a definite time (5 s—15 min). The vibrational mill used was a Heiko Seisakusho model TI-200, of which cells were made of tungsten carbide.

Heating of Mixture in a Sealed Container The ground mixture (300 mg) was sealed in a 2 ml glass ampule, then heated at a definite temperature (60—90 °C) for period of 0.5—0.6 h using an oil bath. After washing the samples with diethyl ether to remove the excess free benzoic acid,[11] the amounts of benzoic acid included in DMβCD were determined spectrophotometrically as reported previously.[1]

Thermal Analysis The thermal behavior of ground samples was measured by DSC using a Du Pont TA9900 thermal analysis system under N_2 stream. The sample weight was about 2.0 mg. The sample pan for liquid samples was used. The heating rate was 10 °C/min.

X-Ray Diffraction Powder X-ray diffraction patterns were measured using a Rigakudenki 2027 diffractometer. Conditions: target Cu, filter Ni, voltage 30 kV, current 5 mA, receiving slit 0.15 mm, count range 2000 cps, scanning speed 4°/min.

Infrared (IR) Absorption Spectroscopy This was carried out with a Nicolet Fourier transform infrared spectrophotometer using Nujol method.

Results and Discussion

Figure 1 shows the X-ray diffraction patterns of DMβCD and benzoic acid equimolar mixtures in various states. The diffraction pattern of the physical mixture showed the diffraction peaks of DMβCD crystals at $2\theta = 10.3$, 12.3, 17.0, and 19.0°, and those of benzoic acid at $2\theta = 8.5$ and 21.5°. The crystalline diffraction pattern changed to an

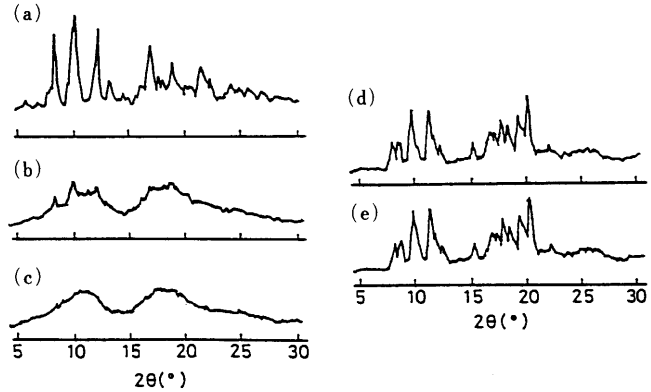


Fig. 1. X-Ray Diffraction Patterns of DMβCD-Benzoic Acid Equimolar Mixtures

(a) Physical mixture of DMβCD-benzoic acid. (b) Ground for 1 min. (c) Ground for 5 min. (d) After heating of 5 min ground mixture in ampule at 90 °C for 6 h. (e) Inclusion compound.

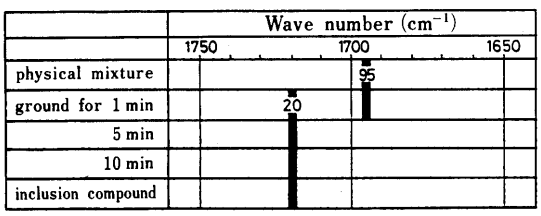


Fig. 2. Schematic Changes of IR Spectra of DMβCD-Benzoic Acid Equimolar Mixtures

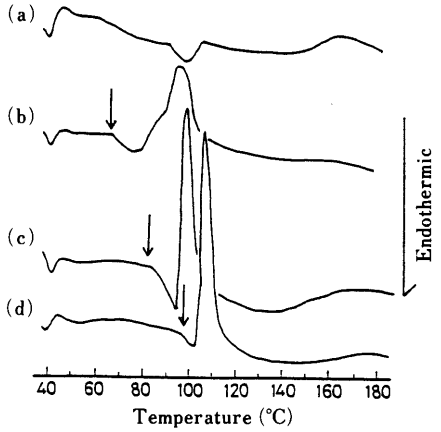


Fig. 3. DSC Curves of Ground Mixtures of DMβCD-Benzoic Acid

Molar ratio = 1 : 1. (a) Physical mixture. (b) Ground for 15 s. (c) Ground for 1 min. (d) Ground for 5 min.

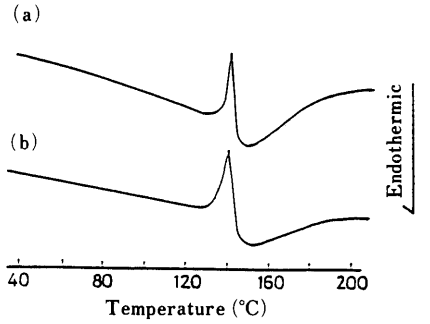


Fig. 4. DSC Curves of Ground DMβCD

(a) Ground for 1 min. (b) Ground for 5 min.

amorphous one with grinding time, and the five minutes of grinding made a halo X-ray diffraction pattern.

When the ground sample was heated at 90 °C for 6 h, reflection peaks were observed on the diffractogram. From comparison with the diffraction pattern of the inclusion compound prepared by the coprecipitation method, the crystallized sample was determined as a crystalline inclusion compound.

Figure 2 shows the changes of IR spectra of the mixtures of DMβCD and benzoic acid as a function of grinding time. In the case of the physical mixture, the carbonyl stretching vibrational band of benzoic acid was observed at 1695 cm^{-1}. This peak shifted to a higher frequency and the 10 min ground sample showed the same pattern with the inclusion compound having a 1720 cm^{-1} peak. This indicated the destruction of the hydrogen bond between dimeric benzoic acids in crystal,[11] as well as the inclusion formation of benzoic acid to the DMβCD cavity in the course of grinding. Figure 3 shows the changes in DSC curves of equimolar mixtures of DMβCD and benzoic acid with the progress of grinding. The physical mixture showed an endothermic peak at 93 °C, which was due to the inclusion formation.[12] The 15 s ground mixture showed a successive change from an endothermic peak at 66 °C to an exothermic peak at 95 °C. Similar thermal characteristics were also obtained for the 1 and 5 min ground samples. It was ascertained from the comparison of X-ray diffraction patterns that the successive changes in DSC curves of the ground mixture were due to

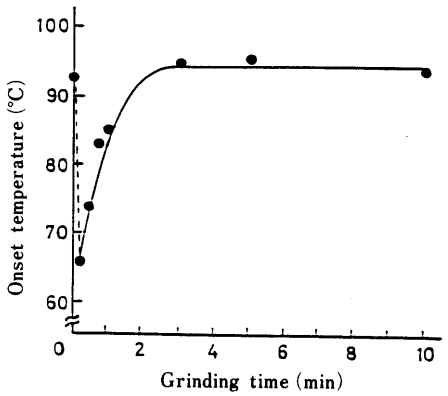


Fig. 5. Variation of DSC Onset Temperature of Inclusion Formation with Grinding Time

crystallization of the inclusion compound. The temperature of crystallization increased with an increase of grinding time; that is, the 5 min ground mixture crystallized at 10 °C higher than the 1 min ground sample. Figure 4 shows the DSC curves of DMβCD ground alone for 1 and 5 min. Both of the curves showed the exothermic peaks due to the crystallization of DMβCD at the same temperature, 139 °C, indicating that crystallization of ground DMβCD was not affected by the grinding time, in contrast with the ground mixture of DMβCD and benzoic acid. The change in the endothermic temperatures of the ground mixture, which are shown by arrows in Fig. 3, are illustrated in Fig. 5 as a function of grinding time. The endothermic temperature suddenly fell to 66 °C after 15 s grinding, then increased with an increase of grinding time up to 3 min, and after 3 min the temperature became constant at 95 °C. An equimolar mixture of amorphous DMβCD and benzoic acid showed the same DSC pattern as the 15 s ground mixture.[2] Since DMβCD was readily changed to the amorphous state by 15 s, these results indicated that the coexistence of amorphous DMβCD and crystalline benzoic acid caused the sudden decrease of the endothermic peak due to sublimation and inclusion. The results of the X-ray diffraction and IR absorption measurements suggest that the amorphous benzoic acid was obtained after a few minutes of grinding. The higher shift of the endothermic temperature with increasing grinding time up to 2 min should be explained by the increase in the amorphous portion of benzoic acid in DMβCD. In the case of grinding in a short period such as 15 s, the monomolecular dispersion of benzoic acid was not sufficiently completed, that is, benzoic acid existed in both the crystalline state and dispersed state interacting with DMβCD. The sublimation of benzoic acid from the crystals could rapidly take place at a low temperature in the ground mixture, because a sink condition of sublimed benzoic acid would be obtained by the inclusion reaction on the DMβCD surface. In sustained grinding, a complete dispersion—an amorphous inclusion compound— could be obtained, where the benzoic acid molecules interacted with DMβCD molecules and the sublimation of benzoic acid was inhibited. This complete dispersion could produce a small enthalpy change due to a phase transition at a high and constant temperature. From these results, it can be said that the sublimed benzoic acid molecules of the crystal react with DMβCD forming the inclusion compound, and this reaction accelerates the

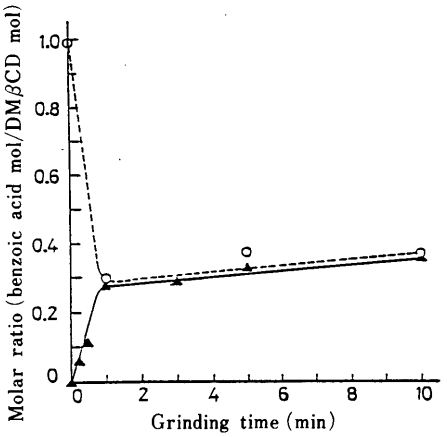


Fig. 6. Variation of Inclusion Molar Ratio with Grinding Time

▲, DMβCD-benzoic acid physical mixture (molar ratio = 1 : 1); ○, DMβCD-benzoic acid inclusion compound prepared by coprecipitation.

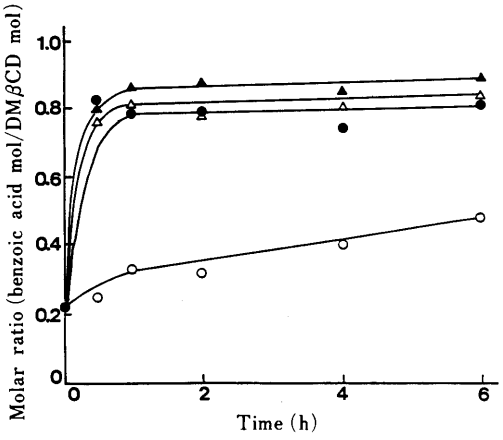


Fig. 7. Inclusion Behavior of Benzoic Acid to DMβCD in the 1 min Ground Mixture by Heating in Ampule at Various Temperatures

○, 60 °C; ●, 70 °C; △, 80 °C; ▲, 90 °C.

crystallization of the inclusion compound.

In order to determine the amounts of benzoic acid included in DMβCD during the grinding, a change in the combining molar ratio was investigated as a function of grinding time.

The solid line in Fig. 6 shows the changes of combining molar ratio in the physical mixture as a function of grinding time. The combining molar ratio increased at the initial grinding time. However, the ratio became almost constant after 1 min grinding. This pattern is similar to the changes of the DSC onset temperature with grinding time shown in Fig. 5. The broken line in Fig. 6 shows changes in the combining molar ratio of a crystalline inclusion compound prepared by the coprecipitation method as a function of grinding time. The combining molar ratio decreased rapidly to 0.30 after 1 min grinding. After this, values were in good accordance with the values of the ground mixture.

As for the ground mixture, while the crystal components readily turned into an amorphous state at the initial grinding time, and benzoic acid molecules were included in the DMβCD cavity by grinding, the inclusion was not as complete as that obtained in the crystalline inclusion complex. Therefore, it seems likely that the included benzoic acid was easily washed out by diethyl ether from the ground mixture.

As for the coprecipitated complex, the included benzoic acid was not washed out by diethyl ether from the crystalline complex. When grinding of the complex proceeded, however, the coprecipitate changed from crystalline to amorphous, and the combining molar ratio decreased by the grinding. This result was explained by presuming the existence of an incomplete inclusion of benzoic acid in the ground coprecipitated complex as well as in the ground mixture. The IR spectra in Fig. 2 suggested the inclusion formation by grinding, and it seems unlikely that the grinding of the inclusion compound causes a release of benzoic acid molecules from DMβCD cavities. Therefore, this consideration seems reliable.

Figure 7 shows the change in the molar ratio of a 1 min ground mixture as a function of heating time. A glass ampule containing the ground mixture was heated at 60, 70, 80, and 90 °C respectively. With the heating at 60°C, the formation of a crystalline inclusion compound was not

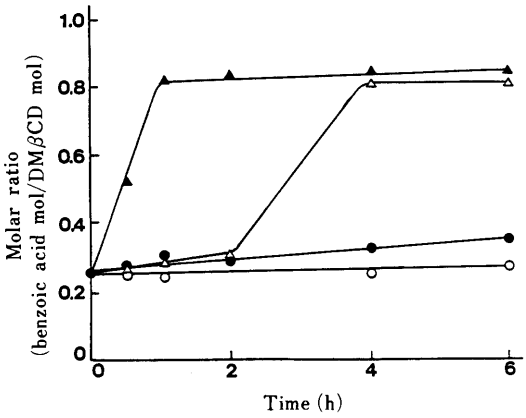


Fig. 8. Inclusion Behavior of Benzoic Acid to DMβCD in the 5 min Ground Mixture by Heating in Ampule at Various Temperatures

○, 60 °C; ●, 70 °C; △, 80 °C; ▲, 90 °C.

observed even after 6 h of heating, and the combining molar ratio was as low as 0.48. With heating at temperatures higher than 70 °C, however, formation of crystalline inclusion compound took place within 30 min of heating, and high combining molar ratios of 0.81—0.89 were obtained.

Figure 8 shows the change in the molar ratio of a 5 min ground mixture at 60, 70, 80, and 90 °C respectively. With heating at 60 and 70 °C, crystallization of the inclusion compound was not observed on an X-ray diffractogram after of 6 h heating, and the combining molar ratio did not increase during heating time. When the ground mixture was heated at 80 °C, the formation of a crystalline inclusion compound was observed after heating for 2 h, and showed a sudden increase in combining molar ratios, from 0.24 to 0.83. Furthermore, with heating at 90 °C, the formation of a crystalline inclusion compound occurred within 30 min. The results indicate that the grinding time affected both the crystallization temperature and the combining molar ratio.

Figure 9 shows a comparison of the combining molar ratios of the physical mixture, 1 min ground mixture and 5 min ground mixture when they were heated for 6 h at 60, 70, 80, 90 °C. With heating at 80 and 90 °C, the ground mixtures showed high combining molar ratios of 0.82 and 0.87, which were about 1.4 times higher than that of physical mixture. With heating at 60 and 70 °C, the com-

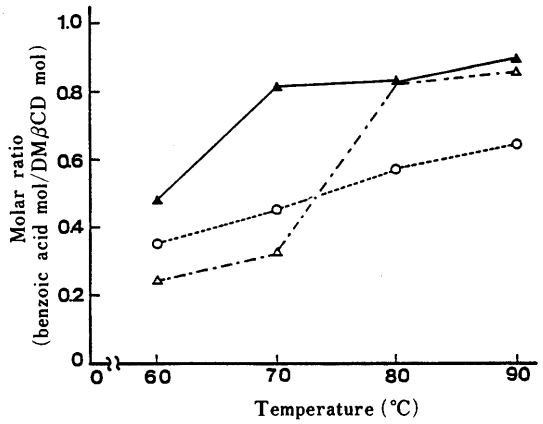


Fig. 9. Comparison of Combining Molar Ratio of Benzoic Acid to DMβCD in Various Grinding Time Samples

○, DMβCD-benzoic acid physical mixture (molar ratio = 1 : 1); ▲, ground for 1 min; △, ground for 5 min.

bining molar ratio of the 1 min ground mixture was about twice that of the 5 min ground mixture. The difference in the combining molar ratio with grinding time may be explained on the basis of the thermal behavior shown in Fig. 3, that is, the crystallization temperature of the 5 min ground mixture was about 10 °C higher than that of 1 min ground mixture. Thus, temperatures of 60 and 70 °C were not sufficient to crystallize the 5 min ground mixture.

In conclusion, the combining molar ratio of benzoic acid to DMβCD was increased by heating the ground mixture in a glass ampule, and grinding time affected the properties of the ground mixtures, such as the temperature of the formation of the crystalline inclusion compound and the combining molar ratio.

References

1) Y. Nakai, K. Yamamoto, T. Oguchi, E. Yonemochi, and T. Hanawa, *Chem. Pharm. Bull.*, **38**, 1345 (1990).
2) Y. Nakai, K. Yamamoto, E. Fukuoka, and S. Nakajima, *Chem. Pharm. Bull.*, **25**, 3340 (1977).
3) Y. Nakai, E. Fukuoka S. Nakajima, and Y. Iida, *Chem. Pharm. Bull.*, **26**, 2983 (1978).
4) Y. Nakai, S. Nakajima, K. Yamamoto, K. Terada, and T. Konno, *Chem. Pharm. Bull.*, **26**, 3419 (1978).
5) Y. Nakai, S. Nakajima, K. Yamamoto, K. Terada, and T. Konno, *Chem. Pharm. Bull.*, **28**, 652 (1980).
6) K. Kawano and Y. Nakai, *Yakugaku Zasshi*, **103**, 1060 (1983).
7) Y. Nakai, S. Nakajima, K. Yamamoto, K. Terada, and T. Konno, *Chem. Pharm. Bull.*, **28**, 1552 (1980).
8) Y. Nakai, K. Yamamoto, K. Terada, and K. Akimoto, *Chem. Pharm. Bull.*, **32**, 685 (1984).
9) G. A. El-Gendy, K. Terada, K. Yamamoto, and Y. Nakai, *Int. J. Pharm.*, **31**, 25 (1986).
10) Y. Nakai, K. Yamamoto, K. Terada, and D. Watanabe, *Chem. Pharm. Bull.*, **35**, 4609 (1987).
11) Y. Nakai, K. Yamamoto, K. Terada, T. Oguchi, H. Saito, and D. Watanabe, *Chem. Pharm. Bull.*, **37**, 1055 (1989).
12) Y. Nakai, K. Yamamoto, T. Oguchi, E. Yonemochi, and T. Hanawa, The 108th Annual Meeting of Pharmaceutical Society of Japan, Hiroshima, April 1988.

Interaction of Recombinant Human Interferon-γ with Liposomes

Hiroshi ISHIHARA,[a] Toshifumi HARA,[a] Yukihiko ARAMAKI,[a] Seishi TSUCHIYA,*[,a] and Kaoru HOSOI[b]

Tokyo College of Pharmacy,[a] 1432–1 Horinouchi, Hachioji, Tokyo 192–03, Japan and Meiji Seika Kaisya Ltd.,[b] 760 Morooka-cho, Yokohama, Kanagawa 222, Japan. *Received December 17, 1990*

The interaction of recombinant human interferon-γ (IFN) with egg phosphatidylcholine liposomes was studied. IFN which binds to liposomes was dependent on the liposomal charge and pH, and a preferential binding was observed in negatively charged liposomes at pH 7.4—10. Electron-microscopic observation showed that the increased liposomal turbidity induced by IFN was due to liposomal aggregation, and the increased turbidity could be decreased by the addition of NaCl. Thus, ionic binding may participate in this interaction. But, when the incubation time was longer, the liposomal aggregation was not decreased by the addition of NaCl, and the leakage of the entrapped marker, calcein, was observed. Electron-microscopic analysis showed that this leakage resulted from the morphological change of liposomes. From these findings, ionic binding may participate in the interaction between IFN and liposomes and then develop a morphological change in negatively charged liposomes under the neutral pH condition.

Keywords interferon-γ; liposome; interaction; binding; aggregation; leakage

Liposomes are currently being explored as a device for drug delivery in order to increase the effect of chemotherapeutic agents.[1] Interferon-γ (IFN) is a peptide which has antiviral,[2] antiproliferative[3] and immunoregulatory[4] effects, and is used in the treatment of viral hepatitis[5,6] and certain kinds of cancer.[7,8]

Previously, we reported the encapsulation of IFN into asialofetuin-labeled liposomes (AF-liposomes)[9] and their receptor-mediated uptake by isolated rat hepatocytes.[9-12] In the preparation process of IFN-encapsulated AF-liposomes, a high apparent entrapment efficiency was obtained, which suggests that IFN interacts with the liposomal membrane. On the other hand, the interaction of peptides such as insulin,[13] neurotoxins[14] and immunomodulators,[15] with the lipid bilayer examined and induced some increase in the membrane permeability of liposomes and the liposomal fusion.

Thus, in this report, the features of the interaction between IFN and liposomes were investigated through IFN binding to liposomes, the changes in liposomal aggregation, and the leakage of calcein under the conditions of different pH and/or ionic strength. Ionic binding may participate in the interaction between IFN and liposomes, and then develop a morphological change in negatively charged liposomes.

Experimental

Materials Recombinant human IFN (Meiji Seika Kaisya Ltd., Tokyo, Japan) with antiviral activity ranging from 1×10^7 to 2×10^7 units/mg was dissolved in 50 mM phosphate buffer (pH 5.5) containing 2 mM dithiothreitol and stored at 4 °C. Before the experiments, the IFN solution was dialyzed against physiological saline at 4 °C for 4 h to exclude the effect of the phosphate buffer on the following experiments. Egg yolk phosphatidylcholine (PC, average molecular weight; 774) was kindly provided by Nippon Fat and Oil Co., Ltd. (Tokyo). Cholesterol (Chol) and calcein were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Egg yolk phosphatidic acid (PA, average molecular weight; 726) and stearylamine (SA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

Preparation of Liposomes Saline-loaded liposomes were prepared by the vortexing method. Liposome suspension was extruded through a polycarbonate membrane (pore size 0.4 μm, Nuclepore Inc., Pleasanton, CA, U.S.A.) and washed four times by centrifugation at 12000 × *g* for 10 min.

Liposomes containing 90 mM calcein (pH 7.4, 97% self-quenching) were prepared by the reverse-phase evaporation technique,[16] and extruded through a polycarbonate membrane (pore size; 0.2 μm). Unencapsulated calcein was removed by gel filtration on a Sepharose 4B column (Pharmacia LKB Biotechnology, Uppsala, Sweden).

The concentration of phospholipids in the liposome suspension was measured as inorganic phosphorus by the method of Chen *et al.*[17] The composition of liposomes was indicated with the molar ratio of the component.

Binding Assay Liposomes composed of various membrane components (5—10 μmol lipid) were suspended in 1 ml of buffered saline solution having different pH (3—12), and incubated with 50 μg of IFN at 4 °C for 30 min. The buffer solutions containing 0.9% NaCl were as follows: pH 3—5: 10 mM citric acid–Na_2HPO_4, pH 7.4: 10 mM Tris–HCl, pH 9—10: 10 mM glycine–NaOH, pH 11—12: 10 mM Na_2HPO_4–NaOH. The incubated mixture was centrifuged at 22000 × *g* for 10 min, and the IFN concentration in the supernatant was determined by the method of Lowry *et al.*[18] The amount of IFN bound to the liposomes was expressed as percent relative to used IFN.

Fluorescence Leakage Assay Liposomes (200 nmol lipid) encapsulating self-quenched calcein were incubated in 2 ml of buffered-saline solution (pH 7.4—11) with IFN (25—100 μg). The fluorescence was measured at 37 °C (Ex. 495 nm, Em. 520 nm) with a fluorescence spectrophotometer Model 650-40 (Hitachi Co., Ltd., Tokyo, Japan), and the fluorescence intensity was expressed as percent of the fluorescence obtained by the addition of Triton X-100 (final concentration; 0.1%). The leakage rate was defined as the maximum differential value in the change of the relative fluorescence.

Turbidity Development Liposomes were incubated in 2 ml of buffered-saline solution (pH 7.4—11) at 37 °C with a varying amount (50—200 μg) of IFN. After rapid mixing, the turbidity was measured as absorbance at 400 nm with a spectrophotometer model U-3200 (Hitachi Co., Ltd.) for 5 or 25 min. The initial absorbance of liposome suspension was adjusted to 0.3 (2.4 μmol lipid/2 ml) and the changes in turbidity were indicated as Δ absorbance.

Electron Microscopic Observation Liposomes (molar ratio; PC : PA : Chol = 4 : 3 : 6, 500 nmol of lipid), which were incubated with or without IFN (50 μg) in 10 mM Tris–HCl buffered saline (pH 7.4, 1 ml) at 37 °C for 1, 5, or 30 min, were placed on a 300-mesh copper grid. After 1 min, excess liposome suspension was removed with the edge of a piece of filter paper. The liposomes on the grid were negatively stained with 3% phosphotungstic acid, pH 7.4, for 1 min. These samples were observed with an electron microscope model H-600 (Hitachi Co., Ltd.).

Results

Binding of IFN to Liposomes Figure 1 shows the binding of IFN to liposomes of various lipid compositions at pH 7.4. IFN hardly bound to neutral (molar ratio; PC : Chol = 7 : 6) and positively charged (PC : SA : Chol = 6 : 1 : 6) liposomes, but bound to negatively charged (PC : PA : Chol) liposomes. The more PA that was contained in the negatively charged liposomes, the more binding was observed. Figure

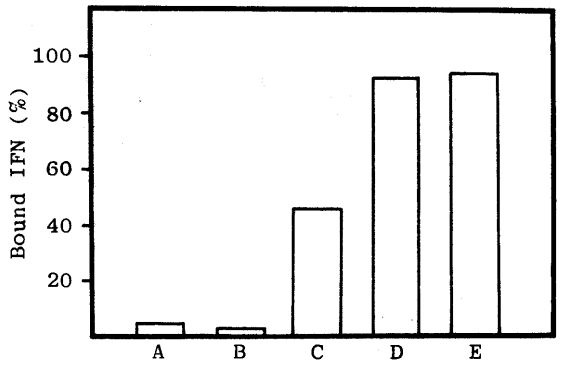


Fig. 1. Effect of Lipid Composition on the Binding of IFN to Liposomes

IFN was added to each liposomal suspension (10 g IFN/mol lipid) in 10 mM Tris–HCl buffered saline (1 ml, pH 7.4). The experiment was carried out at 4 °C. Each bar indicates the average of two experiments. A, PC:Chol=7:6; B, PC:SA:Chol=6:1:6; C, PC:PA:Chol=6:1:6; D, PC:PA:Chol=5:2:6; E, PC:PA:Chol=4:3:6.

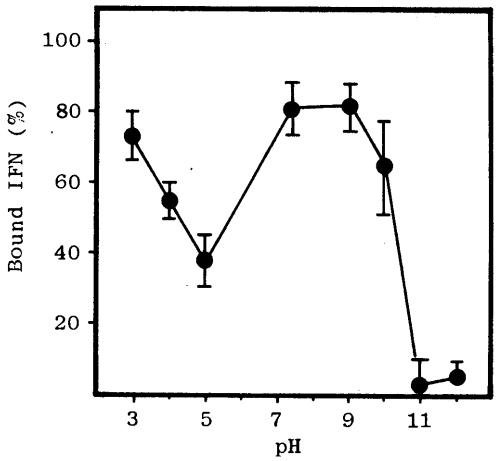


Fig. 2. pH Dependency of the Binding of IFN to Negatively Charged Liposomes

Each point represents the mean ± S.D. ($n=3$). Liposomes (PC:PA:Chol=6:1:6) were incubated with IFN (5 g IFN/mol lipid) in various pH of buffered saline solution (1 ml). The experiment was carried out at 4°C.

2 shows the effect of pH on the IFN binding to negatively charged (PC:PA:Chol=6:1:6) liposomes. IFN showed a higher rate of binding at the pH region of 7.4—10, but hardly bound to the liposomes at and above pH 11.

Leakage of Calcein from Liposomes Figure 3 is a typical result of the calcein leakage from negatively charged (PC:PA:Chol=4:3:6) and neutral (PC:Chol=7:6) liposomes induced by the addition of IFN in 10 mM Tris–HCl buffered saline (pH 7.4). In the case of the negatively charged liposomes, the intensity of fluorescence began to increase after a lag time of several minutes, and it reached about 60% relative to the total fluorescence intensity after an adequate incubation time. On the other hand, calcein was not leaked from the neutral liposomes by the addition of IFN.

At pH 7.4, the leakage rate of calcein from negatively charged liposomes increased with increasing PA content and IFN concentration (Fig. 4). Table I indicates that the leakage rate of calcein from negatively charged liposomes (PC:PA:Chol=4:3:6) was affected by pH.

Turbidity Development and Its Decrease By the addition of IFN to the liposomal suspension at pH 7.4 (Fig. 5), an increase in turbidity was observed in negatively charged

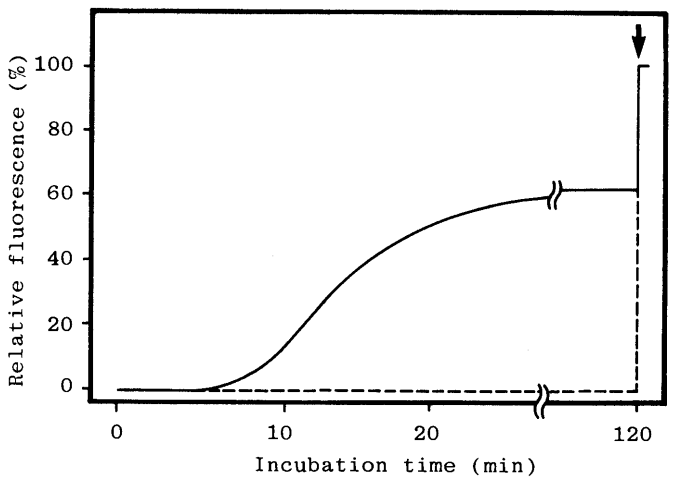


Fig. 3. Typical Leakage of Calcein from Liposomes Induced by IFN

Negatively charged (PC:PA:Chol=4:3:6, —) and neutral (PC:Chol=7:6, ---) liposomes encapsulating 90 mM calcein were incubated with IFN (100 μg IFN; 500 g/mol lipid) at 37 °C under neutral conditions (pH 7.4). The arrow indicates the addition of Triton X-100 to disrupt liposomes.

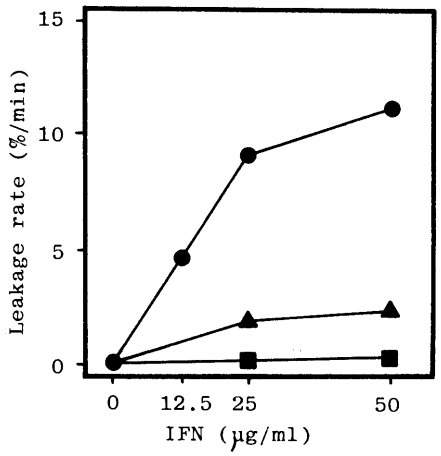


Fig. 4. Effect of Lipid Composition on the Leakage Rate of Calcein from Liposomes by IFN

Liposomes encapsulating 90 mM calcein were incubated with IFN (125—500 g IFN/mol lipid) at 37 °C under the neutral conditions (pH 7.4). Each point indicates the average of two experiments. ●, PC:PA:Chol=4:3:6; ▲, PC:PA;Chol=6:1:6; ■, PC:Chol=7:6.

TABLE I. Effect of pH on the IFN-Induced Leakage of Calcein from Liposomes

pH	Leakage rate (%/min)
7.4	11.1
9.0	7.0
11.0	4.0

Lipid composition; PC:PA:Chol=4:3:6. One hundred micrograms of IFN was added to liposome suspension (670 g IFN/mol lipid).

liposomes (PC:PA:Chol=4:3:6), but not in neutral liposomes (data not shown). The intensity of turbidity in the suspension of the negatively charged liposomes was dependent on IFN concentration.

Table II shows the effects of pH and NaCl concentration on the turbidity development of negatively charged liposomes (PC:PA:Chol=4:3:6). At pH 7.4, the turbidity development until 5 min after the IFN addition was inversely proportional to the NaCl concentration, and when the NaCl

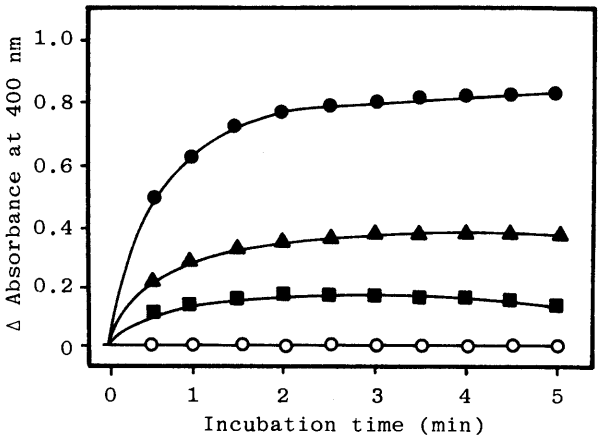


Fig. 5. Effect of IFN Concentration on Turbidity Development in Liposome Suspension

Liposomes and IFN (20.8—83.3 g IFN/mol lipid) were incubated at 37 °C under neutral conditions (pH 7.4). Lipid composition; PC : PA : Chol = 4 : 3 : 6. ●, 100 μg IFN/ml; ▲, 50 μg IFN/ml; ■, 25 μg IFN/ml; ○, without IFN.

Table II. Effect of pH and NaCl Concentration of IFN-Induced Turbidity Development of Liposome Suspension

pH	NaCl conc. (%)	Δ Absorbance
7.4	0.45	1.18
7.4	0.90	0.39
7.4	1.80	0.09
9.0	0.90	0.14
11.0	0.90	0.02

Lipid composition; PC : PA : Chol = 4 : 3 : 6. One hundred micrograms of IFN was added to liposome suspension (41.7 g IFN/mol lipid).

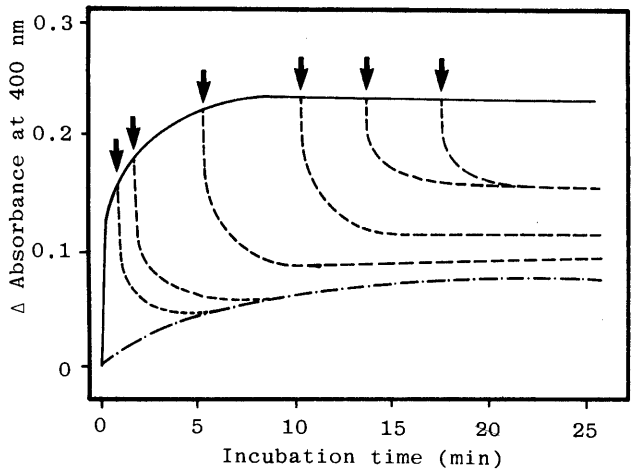


Fig. 6. Effect of NaCl Addition on the Turbidity Development Induced by IFN

Each arrow indicates the timing of NaCl addition after IFN addition (83.3 g IFN/mol lipid, pH 7.4). Lipid composition; PC : PA : Chol = 4 : 3 : 6. ——; IFN (100 μg) was added. —·—; NaCl (final 2.7%) was added. – – –; NaCl (final 2.7%) was added after the addition of IFN (100 μg).

concentration remained constant (0.9%), a lower turbidity development was observed in a higher pH region.

The results in Fig. 6 show the change in the IFN-induced turbidity by the addition of NaCl. When NaCl (final concentration; 2.7%) was added to negatively charged liposomes (PC : PA : Chol = 4 : 3 : 6), a slight increase in turbidity was observed (control experiment). When NaCl was added to the liposomal suspension at 1 or 2 min after

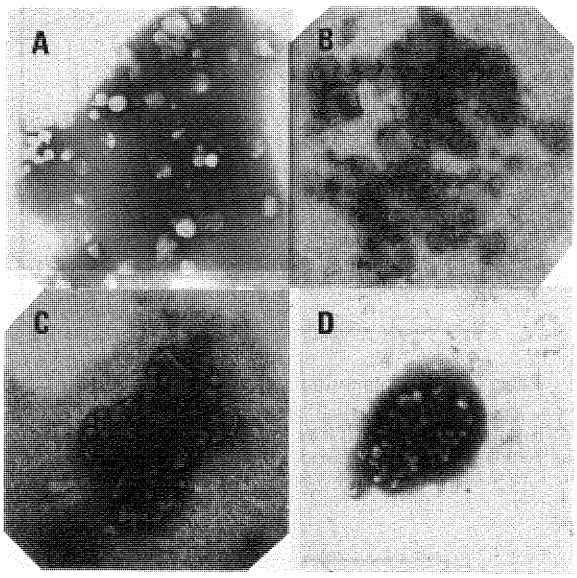


Fig. 7. Nagative-Stain Electron Micrographs of Liposomes

Liposomes (PC : PA : Chol = 4 : 3 : 6, 500 nmol lipids) were incubated with IFN (50 μg/ml) under the neutral conditions (pH 7.4) and were stained with 3% phosphotungstic acid at indicated time. A, before the addition of IFN; B, 1 min after the addition of IFN; C, 5 min after the addition of IFN; D, 30 min after the addition of IFN; magnification, × 10000.

the IFN addition, the increased turbidity induced by IFN decreased to the control level. However, the addition of NaCl at 5—17 min after the IFN addition could not decrease the turbidity to the control level.

Electron Microscopy The effects of IFN on the change in the size and morphology of liposomes were examined with electron microscopic observation. One minute after the addition of IFN liposomal aggregation appeared, and with a lapse of time the aggregation advanced and each liposome became smaller in size (Fig. 7).

Discussion

The binding of IFN to liposomes was dependent on the liposomal charge and pH, and a preferential binding was observed in negatively charged liposomes at pH 7.4—10 (Figs. 1 and 2). The IFN-induced turbidity development in the suspension of negatively charged liposomes at pH 7.4 was dependent on the IFN concentration (Fig. 5), and was inversely proportional to the NaCl concentration (Table II). Because the pI value of IFN has been reported to be 8.6—10.4,[9,19] this binding may be caused by the ionic interaction between positively ionized IFN and negatively charged PA, one of the components of liposomal phospholipids. This consideration may be supported by the fact that human IFN is quite basic in character, having 27 basic amino acids, lysine and arginine, and especially in that some of them are localized on the amino acid sequences of 89—92 (Lys–Lys–Lys–Arg) and 131—134 (Lys–Arg–Lys–Arg).[20] From the results of Fig. 6, the presence of two kinds of interactions between negatively charged liposomes and IFN are suggested: one is an ionic interaction which aggregates negatively charged liposomes and decreases by the addition of NaCl, and the other is a non-ionic interaction which may accompany a leakage of entrapped calcein and does not decrease by the addition of NaCl.

The lag time observed in the leakage of calcein (pH 7.4)

from negatively charged liposomes (Fig. 3) was in accordance with the time at which the complete reversion of IFN-induced turbidity development could no longer be observed by the addition of NaCl (Fig. 6). Thus, the leakage of calcein may be a subordinate phase following the transition from ionic to non-ionic interaction. If the calcein leakage results from the latter step, a change in membrane fluidity or liposomal fusion should occur. But no proof for either of these phenomena could be obtained using the fluorescence polarization or the resonance energy transfer technique (data not shown). Electron microscopic analysis showed that the liposomal aggregation grew tight and the liposomal size gradually became smaller with incubation time after adding IFN. Thus, the calcein leakage may have resulted from this morphological change of liposomes induced by IFN.

Under alkaline pH conditions, the calcein leakage (Table I) and turbidity development (Table II) in negatively charged liposomes were lower than those under neutral pH conditions. However, at pH 9.0, IFN showed a high binding to negatively charged liposomes (Fig. 2). Since the ionization of IFN hardly occurs at pH 9.0, IFN may bind to negatively charged liposomes through a non-ionic rather than an ionic interaction. Walter *et al.* reported that the polylysine-induced fusion of liposomes containing an acidic phospholipid, phosphatidylserine, occurred at an acidic pH, in which condition the negative charge of phosphatidylserine is considered to be neutralized.[21] Oku *et al.* reported that the tumor necrosis factor increased the permeability of negatively charged liposomes by hydrophobic interaction under the acidic condition, but the tumor necrosis factor did not affect the permeability of neutral liposomes.[22] Polylysine is a basic peptide and the tumor necrosis factor is an acidic peptide. From these reports, the neutralization of a negative charge on the liposomal membrane may be important to the perturbation of the liposomal membrane. However, in the case of IFN, the ionic interaction with a negatively charged phospholipid under the neutral pH condition is the trigger for aggregation and calcein leakage. Thus, the cationic portions of an IFN molecule may bind to the negatively charged phospholipid and then induce a change in the integrity of liposomes. At pH 9.0, IFN does not ionize, and the observed binding (Fig. 2) may be caused by non-ionic interaction through a hydrophobic portion developed in an IFN molecule. To aggregate liposomes, an IFN molecule needs more than two binding sites for cross-linking of liposomes. The decrease in liposomal aggregation at pH 9—11 (Table II), indicates a reduction of multivalent binding between IFN and liposomal membrane. This consideration is based on the finding that the net charge and the conformation of peptides, such as polyglutamate, in aqueous solution are affected by the pH and ionic strength of the solution.[23] The magnitude of the cationic and hydrophobic portions in IFN molecules, which interact with the liposomal surface, may change according to the characteristics of the solution in which IFN is dissolved. IFN may show an affinity to liposomes through different types of interactions at different pH and ionic strength.

The above findings suggest that IFN bound to the outer surface of the liposome bilayer should be removed to keep negatively charged liposomes stable.

References

1) M. J. Ostro (ed.), "Liposomes: from Biophysics to Therapeutics," Marcel Dekker, New York and Basel, 1987.
2) I. M. Kerr and R. E. Brown, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 256 (1978).
8) Y. Ozaki, M. P. Edelstein, and D. S. Duck, *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 1242 (1988).
4) R. E. Garmer, U. Kuruganti, C. W. Czarniecki, H. H. Chiu, and J. E. Domer, *Infect. Immun.*, **57**, 1800 (1989).
5) G. L. Davis and J. H. Hoohnagle, *Hepatology*, **6**, 1038 (1986).
6) A. M. D. Bisceglie, V. K. Rustgi, C. Kassianides, M. L. Melman, Y. Dark, J. G. Waggoner, and J. H. Hoofnagle, *Hepatology*, **11**, 266 (1990).
7) E. H. Kaplan, S. T. Rosen, D. B. Narris, H. H. Roenigk, Jr., S. R. Saks, and P. A. Bunn, Jr., *J. Natl. Cancer Inst.*, **82**, 208 (1990).
8) K. Sriskandan, P. Garner, J. Watkinson, K. W. Pettingale, D. Brinkley, F. M. B. Calman, and D. E. H. Tee, *Cancer Chemother. Pharmacol.*, **18**, 63 (1986).
9) H. Ishihara, T. Hara, Y. Aramaki, S. Tsuchiya, and K. Hosoi, *Pharm. Res.*, **7**, 542 (1990).
10) T. Hara, H. Ishihara, Y. Aramaki, and S. Tsuchiya, *Int. J. Pharmaceut.*, **42**, 69 (1988).
11) T. Hara, H. Ishihara, Y. Aramaki, and S. Tsuchiya, *Int. J. Pharmaceut.*, **67**, 123 (1991).
12) H. Ishihara, Y. Hayashi, T. Hara, Y. Aramaki, S. Tsuchiya, and K. Koike, *Biochem. Biophys. Res. Commun.*, **174**, 839 (1991).
13) J. H. Wiessner, H. Mar, D. G. Baskin, and K. J. Hwang, *J. Pharm. Sci.*, **75**, 259 (1986).
14) C. Montecucco, G. Schiavo, and B. R. Dasgupta, *Biochem. J.*, **259**, 47 (1989).
15) T. Yoshimura and S. Sone, *J. Biol. Chem.*, **262**, 4597 (1987).
16) F. Szoka and D. Papahadjopoulos, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 4194 (1978).
17) P. S. Chen, Jr., T. Y. Toribara, and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).
18) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
19) J. A. Kant and T. L. Steck, *J. Biol. Chem.*, **248**, 8457 (1973).
20) P. W. Gray, D. W. Leung, D. Pennica, E. Yelverton, R. Najarian, C. C. Simonsen, R. Derynck, P. J. Sherwood, D. M. Wallace, S. L. Berger, A. D. Levinson, and D. V. Goeddel, *Nature* (London), **295**, 503 (1982).
21) A. Walter, C. J. Steer, and R. Blumenthal, *Biochim. Biophys. Acta*, **861**, 319 (1986).
22) N. Oku, R. Araki, H. Araki, S. Shibamoto, F. Ito, T. Nishihara, and M. Tsujimoto, *J. Biochem.* (Tokyo), **102**, 1303 (1987).
23) M. Hatano, M. Yoneyama, and Y. Sato, *Biopolymers*, **12**, 895 (1973).

Properties of Novel Aldose Reductase Inhibitors, M16209 and M16287, in Comparison with Known Inhibitors, ONO-2235 and Sorbinil

Katsuaki KATO,*[,a] Kazuo NAKAYAMA,[a] Masahiro MIZOTA,[a] Ichitomo MIWA[b] and Jun OKUDA[b]

Department of Pharmacology, Fuji Central Research Laboratory, Mochida Pharmaceutical Co., Ltd.,[a] 1-1-1 Kamiya, Kita-ku, Tokyo 115, Japan and Department of Clinical Biochemistry, Faculty of Pharmacy, Meijo University,[b] Tempaku-ku, Nagoya 468, Japan. *Received September 17, 1990*

Properties and efficacies of novel aldose reductase (AR) inhibitors, M16209 (1-(3-bromobenzo[*b*]furan-2-ylsulfonyl)hydantoin) and M16287 (1-(3-chlorobenzo[*b*]furan-2-ylsulfonyl)hydantoin), were examined *in vitro* and *in vivo*, compared with known AR inhibitors, ONO-2235 and sorbinil. These four compounds inhibited partially purified aldose reductases from various origins, and the potencies of M16209 and M16287 were on the whole similar to ONO-2235, and were greater than that of sorbinil. The IC_{50} values of the four AR inhibitors did not substantially depend on the substrate used. Kinetic studies of inhibition of partially purified bovine lens (BLAR) revealed that M16209, M16287 and sorbinil were uncompetitive with glyceraldehyde and noncompetitive with nicotineamide adenine dinucleotide phosphate (NADPH), whereas ONO-2235 was noncompetitive with both glyceraldehyde and NADPH. Aldose reductase became less sensitive to the four inhibitors as enzyme purification progressed, although the susceptibility to inhibition was partially reversed by incubation with dithiothreitol. In addition, the four compounds slightly affected those enzymes of carbohydrate and glutathione metabolism which were tested. M16209 and M16287 prevented sorbitol accumulation in isolated rat tissues as potently as ONO-2235 and sorbinil. M16209 and M16287 were effective in the prevention of galactosemic cataracts and amelioration of diabetic neuropathy with almost the same potency, while ONO-2235 was effective only in neuropathy, and sorbinil was effective in galactosemic cataracts and diabetic neuropathy with a different potency. These results indicate that M16209 and M16287 are potent aldose reductase inhibitors, which could be applicable to treatment for diabetic complications.

Keywords aldose reductase inhibitor; M16209; M16287; ONO-2235; sorbinil; sorbitol; diabetic complication; cataract; motor nerve conduction velocity

Introduction

Aldose reductase (AR, E.C. 1.1.1.21) catalyzes the reduction of D-glucose to its corresponding sugar alcohol, sorbitol, and it belongs to a larger family of aldehyde reductases that are monomeric, nicotineamide adenine dinucleotide phosphate (NADPH)-dependent oxidoreductases. Aldose reductases distribute widely in mammalian tissues, including lens, Schwann cells in nerve, endothelial cells in aorta, Müller cells in retina, and certain cells in the medulla and cortex of the kidney.[1-3] It is believed that excessive conversion of glucose to sorbitol in these tissues containing AR leads to the pathogenesis of diabetic complications, such as cataracts, neuropathy and retinopathy. Recent studies showed that AR inhibitors reduced tissue sorbitol in diabetic animals and tissue galactitol in galactosemic rats and exhibited beneficial effects in animal models of diabetic neuropathy and cataracts.[4-6] Furthermore, several AR inhibitors have been found to be of some therapeutic value in clinical trials,[7-9] although they have not yet been launched in most of countries, including Japan and United States.

Among a variety of structurally different compounds possessing AR inhibiting properties, 1-[(substituted phenyl)-sulfonyl]hydantoins were found to be one of the most potent inhibitors of the enzyme, according to Okuda and his co-workers, who have searched a series of hydantoin derivatives.[10,11] We have conducted structural alterations of aryl moieties of 1-(arylsulfonyl)hydantoins and found 1-[(substituted benzofuranyl)sulfonyl]hydantoins were also effective inhibitors both *in vitro* and *in vivo*.

In the present study, we describe the inhibitory activities of M16209 (1-(3-bromobenzo[*b*]furan-2-ylsulfonyl)hydantoin) and M16287 (1-(3-chlorobenzo[*b*]furan-2-ylsulfonyl)-hydantoin), chemically novel hydantoin derivatives (see Fig. 1 for chemical structures of these compounds), against AR from various sources in comparison with ONO-2235, a carboxylic acid-type AR inhibitor, and sorbinil, a spirohydantoin-type AR inhibitor. We also describe the effects of M16209 and M16287 on sorbitol accumulation in isolated rat tissues and on the activities of enzymes of carbohydrate and glutathione metabolism. Furthermore, we report the preventive and/or ameliorative effects of these compounds on experimentally-induced galactosemic cataracts and diabetic neuropathy.

Materials and Methods

Materials Reduced nicotinamide adenine dinucleotide (NADH) disodium salt (grade III), nicotineamide adenine dinucleotide (NAD) sodium salt (grade V), phosphoenolpyruvate monosodium salt, adenosine triphosphate (ATP) disodium salt (grade I), D-fructose 6-phosphate disodium salt, D-(−)-3-phosphoglyceric acid disodium salt, pyruvic acid sodium salt (type II), D-sorbitol, alcohol dehydrogenase (from equine liver) glucose-6-phosphate dehydrogenase (from baker's yeast, type IX), glyceraldehyde-3-phosphate dehydrogenase (from rabbit muscle), hexokinase (from bovine heart, type X), lactate dehydrogenase (from rabbit muscle, type II), phosphofructokinase (from rabbit muscle, type III), pyruvate kinase (from rabbit muscle, type II), 6-phosphogluconate dehydrogenase (from sheep liver), sorbitol dehydrogenase (from sheep liver), aldolase (from rabbit muscle, type IV), α-glycerophosphate dehydrogenase-triose phosphate isomerase (from rabbit muscle, type III), 3-phosphoglycerate phosphokinase (type I, from rabbit muscle), glutathione peroxidase (from bovine erythrocytes), glutathione reductase (type IV, from baker's yeast), streptozotocin, *tert*-butyl hydroperoxide and glutathione oxidized form (grade III) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). D-Glucose 6-phosphate disodium salt, NADPH, adenosine diphosphate (ADP) disodium salt and 6-phosphogluconate trisodium salt (Oriental Yeast Co., Ltd., Osaka, Japan), DL-glyceraldehyde, galactose and glutathione reduced form (Wako Pure Chemicals Industries, Ltd., Osaka, Japan), phenylisocyanate (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan), Sephadex G-75 and DEAE-Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden), Matrex gel red A (Amicon Corp., Danvers, MA, U.S.A.), and all other chemicals used were of the highest purity available. AR inhibitors, M16209, M16287, ONO-2235 and sorbinil were synthesized in our laboratory, and dissolved in dimethyl sulfoxide to give desired concentrations. Bovine eyes and kidneys were obtained from

animals slaughtered freshly at the local slaughterhouse, and lenses were removed and kept frozen at −40 °C until needed. Rat lenses for the preparation of AR were obtained from male Sprague-Dawley strain rats 6 weeks of age, and canine lenses were from mongrel dogs weighing 10—15 kg immediately after killing by bleeding *via* carotid artery under anesthesia with sodium pentobarbital. Fresh human placentas were obtained after parturition and kept frozen until needed. Lenses, sciatic nerves and erythrocytes for the measurement of sorbitol accumulation *in vitro* were obtained from male Wistar strain rats 7 to 8 weeks of age under anesthesia with sodium pentobarbital. Male Sprague-Dawley strain rats 5 to 6 weeks of age and male Wistar strain rats 7 weeks of age were used for the study of galactose-induced cataracts and streptozotocin-induced diabetic neuropathy, respectively. Rats were obtained from Japan SLC (Hamamatsu, Japan).

Determination of Aldose Reductase Activities and the Effects of Inhibitors The aldose reductase activity was assayed at 30 °C according to the method of Inagaki *et al.*,[12] with a reaction mixture (1.0 ml) containing 0.4 M ammonium sulfate, 0.1 M sodium phosphate buffer (pH 6.2), 10 mM DL-glyceraldehyde and 0.16 mM NADPH. As assay substrate, 100 mM glucose, 50 mM galactose or 15 mM glucuronate were used instead of 10 mM glyceraldehyde when the activity dependence on the assay substrate was examined. The concentrations of DL-glyceraldehyde or NADPH were varied when the inhibition pattern for AR was examined. The reaction was initiated by the addition of an enzyme, and the activity was measured by recording the decrease in absorbance at 340 nm with a Shimadzu UV-2100s spectrophotometer (Shimadzu Corp., Kyoto, Japan). Non-specific reduction of NADPH in the absence of the substrate was subtracted from the total activity. One unit of the enzyme was defined as the amount of enzyme which catalyzed the oxidation of 1 μmol of NADPH per minute. The effect of an inhibitor on the enzyme activity was determined by the addition of 10 μl of the inhibitor solution in dimethyl sulfoxide (DMSO) at the desired concentration. Inhibition rate (%) was calculated regarding the enzyme activity with DMSO alone as 100%. The concentration of inhibitors required to produce 50% inhibition (IC_{50}) was determined by plotting the %-remaining activity against the log concentration of the inhibitor.

Preparation of Crude Aldose Reductases Aldose reductases were partially purified from various tissues according to the method of Inagaki *et al.*[12] Tissues were homogenized by a Polytron homogenizer (Kinematika, Luzern, Switzerland) in 5 vol of 5 mM sodium phosphate buffer (pH 7.4) containing 1 mM 2-mercaptoethanol at 4 °C, and the homogenates were fractionated by 40—75% ammonium sulfate. The precipitates were dissolved and dialyzed three times against 10 vol of the same buffer and the dialysate was centrifuged to remove insoluble materials.

Purification of Aldose Reductases Aldose reductases from the bovine lens was purified by the method of Inagaki *et al.*[12] The partially purified enzyme prepared by 40—75% ammonium sulfate fractionation as described above was applied to a DEAE-Sephacel column and eluted with a 5—150 mM linear phosphate buffer (pH 7.4) gradient. The fractions with the enzyme activity were concentrated by Amicon YM 5 pressure dialysis. The concentrated sample was dialyzed against a 2-mercaptoethanol-free 5 mM sodium phosphate buffer (pH 7.4) and applied to a Matrex gel red A column. After washing, the column was developed with 0.33 mM NADPH in the same buffer, and the fractions containing the enzyme activity were collected. The pooled eluate was then applied to a Sephadex G-75 gel column pre-equilibrated with a 5 mM sodium phosphate buffer (pH 7.4) containing 1 mM 2-mercaptoethanol and eluted with the same buffer. The purified enzyme was stored at −40 °C until enzyme assay.

Effects of Dithiothreitol on the Activity of Purified Aldose Reductase and on Its Susceptibility to Inhibitors Purified aldose reductase from bovine lenses was incubated on an ice bath with 5 mM dithiothreitol (DTT) for as long as 40 min. Aldose reductase activities in the presence of 0.2% DMSO or AR inhibitors were measured before, immediately after and 40 min after incubation with DTT.

Measurement of Activities of Carbohydrate and Glutathione Metabolism Enzymes Sorbitol dehydrogenase activity was measured spectrophotometrically from the change of concentration of NADH at 340 nm according to the method of Wolff,[13] and glutathione reductase and glutathione peroxidase were measured spectrophotometrically from the change of concentration of NADPH at 340 nm according to the method of Horn[14] and to the method of Awashi *et al.*,[15] respectively. Other enzyme activities were determined spectrophotometrically by measuring the rate of change of NADH or NADPH at 340 nm at 37 °C. Glycine buffer (0.1 M, pH 9.6) was used for the alcohol dehydrogenase assay and triethanolamine buffer (0.05 M, pH 7.4) was used for the other enzyme assays. The assay mixture for each enzyme was as follows: glucose-6-phosphate dehydrogenase, 1.3 mM glucose 6-phosphate, 0.4 mM NADP, 6 mM ethylenediaminetetraacetic acid (EDTA), 8 mM $MgCl_2$; 6-phosphogluconate dehydrogenase, 1.1 mM 6-phosphogluconate, 0.4 mM NADP, 6 mM EDTA, 8 mM $MgCl_2$; hexokinase, 3.7 mM glucose, 1.7 mM ATP, 0.4 mM NADP, 0.013 mg/ml glucose-6-phosphate dehydrogenase, 8 mM $MgCl_2$; phosphofructokinase, 1.7 mM fructose-6-phosphate, 0.83 mM ATP, 0.15 mM NADH, 0.013 mg/ml α-glycerophosphate dehydrogenase-triosephosphate isomerase, 0.4 mg/ml aldolase, 6 mM EDTA, 8 mM $MgCl_2$; glyceraldehyde-3-phosphate dehydrogenase, 10 mM 3-phosphoglycerate, 1.1 mM ATP, 0.15 mM NADH, 0.033 mg/ml 3-phosphoglycerate phosphokinase, 6 mM EDTA, 8 mM $MgCl_2$; pyruvate kinase, 1.33 mM phosphoenolpyruvate, 2 mM ADP, 0.15 mM NADH, 0.017 mg/ml lactate dehydrogenase, 6 mM EDTA, 8 mM $MgCl_2$, 75 mM KCl; lactate dehydrogenase, 1.4 mM pyruvic acid, 0.15 mM NADH; alcohol dehydrogenase, 3.3% ethanol, 5 mM NAD; AR inhibitors were added at 1×10^{-5} M in final concentration, and the remaining activity was calculated and expressed as a percentage of the control value measured without the addition of an AR inhibitor.

Measurement of Sorbitol Accumulation *in Vitro* Lenses and sciatic nerves were incubated at 37 °C in 5 ml of buffer A (see below) containing 50 mM glucose in the presence of AR inhibitors at desired concentrations according to the method of Miwa *et al.*[11] Buffer A (pH 7.4) contained 110 mM NaCl, 3.8 mM KCl, 0.54 mM $MgSO_4$, 0.9 mM $KHCO_3$, 0.27 mM NaH_2PO_4, 0.23 mM KH_2PO_4, 1.25 mM $CaCl_2$ and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Incubation was conducted for 4 h for lenses and 8 h for sciatic nerves, after which the tissues were washed and stored at −40 °C until sorbitol measurement. Each experiment was run in triplicate. The sorbitol content in these tissues was measured by high performance liquid chromatography (HPLC), measuring UV-absorbing carbamate derivatives of sorbitol at 240 nm as described by Miwa *et al.*[16]

Erythrocytes from heparinized blood were centrifuged at $600 \times g$ and washed three times with cold saline. One ml of packed cells was incubated for 3 h with agitation in 3 ml of medium equilibrated with 95% O_2 and 5% CO_2 according to the method of Terashima *et al.*[17] The medium was a Krebs–Ringer bicarbonate buffer (pH 7.4) containing 25 mM glucose. The sorbitol content in erythrocytes was determined by the method of Malone *et al.*,[18] which follows the reduction of NAD by sorbitol dehydrogenase with an excitation wavelength at 360 nm and an emission wavelength at 460 nm using a Shimadzu model FDU-3 fluorophotometer (Shimadzu Corp., Kyoto, Japan). The number of each experiment ranged from three to five.

Protein Determination The protein concentration was quantified by the method of Lowry *et al.*[19]

Induction and Examination of Cataracts in Rats Rats were fed a 30% galactose diet, prepared by mixing galactose with commercial powder chow, for 6 d. The rats were orally administered at around 10 a.m. with M16209 (10 and 30 mg/kg/d), M16287 (10 and 30 mg/kg/d), ONO-2235 (50 mg/kg/d) and sorbinil (3 mg/kg/d) suspended in a 5 g/dl gum arabic solution from the first day of galactose feeding. Galactose-fed untreated rats were administered the vehicle alone. Non-galactosemic rats fed with ordinary chow were used as the normal control. Morphological investigation was performed using a slit-lamp (Kowa SC-6, Nagoya) on the 6th day of galactose feeding. Progression of the cataracts was evaluated according to the method of Sippel,[20] and the appearance of apparent opacification in the peripheral region of a lens was regarded as the onset of cataract formation.

Induction of Diabetic Neuropathy in Rats Fourteen days after the injection of streptozotocin (30 mg/kg, i.v.), rats with serum glucose levels of around 500 mg/dl and with motor nerve conduction velocities (MNCV) of between 29 and 36 m/s were divided into groups so that serum glucose levels and MNCV would not be significantly different among them. The rats were orally administered with M16209 (10 and 30 mg/kg/d), M16287 (10 and 30 mg/kg/d), ONO-2235 (30 mg/kg/d) and sorbinil (30 mg/kg/d) suspended in a 5 g/dl gum arabic solution for 14 d. Untreated control rats were administered the vehicle alone and non-diabetic rats were used as the normal control.

Measurement of MNCV Sciatic MNCV was measured one day before the start of treatment and one day after the final administration. The measurement of MNCV was performed under sodium pentobarbital anesthesia in an air-conditioned room (22—24 °C) according to the method of Robertson and Sima[21] with some modifications. Briefly, the sciatic nerve was stimulated with two needle electrodes. Supramaximal 0.1 ms stimuli were delivered at 1 Hz. The action potentials were recorded at the peroneal muscle on the ankle with an electromyograph with a

storage oscilloscope (7S12, NEC San-ei, Tokyo). The MNCV (m/s) was calculated by dividing the distance (35 mm) between the two stimulation points by the difference in latency.

Results

Inhibition of Partially Purified Aldose Reductases The specific activities of partially purified enzymes from rat lens (RLAR), bovine lens (BLAR), bovine kidney (BKAR), canine lens (CLAR) and human placenta (HPAR) were 0.027, 0.0028, 0.024, 0.0023, and 0.0066 units/mg, respectively. AR inhibitory activities of M16209, M16287, ONO-2235 and sorbinil were dependent on the enzyme studied, and the order of susceptibility to inhibition was RLAR > BLAR > CLAR > BKAR > HPAR (Table I). The potency of M16209 and M16287 in AR inhibiting activity was much higher than sorbinil for all enzymes examined. RLAR and BLAR were more sensitive to ONO-2235 than M16209 and M16287, whereas BKAR and HPAR were more sensitive to M16209 and M16287 than ONO-2235.

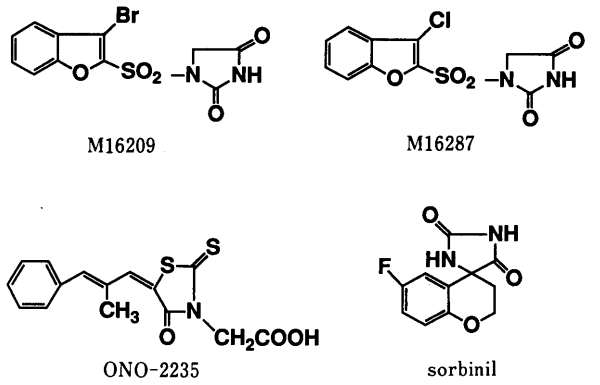


Fig. 1. Chemical Structures of M16209, M16287, ONO-2235 and Sorbinil

TABLE I. Susceptibility to Inhibition of Aldose Reductases from Various Sources

Enzyme	IC_{50} (μM)			
	M16209	M16287	ONO-2235	Sorbinil
RLAR	0.12	0.08	0.06	0.60
BLAR	0.24	0.25	0.07	1.3
BKAR	4.5	4.1	11.0	20<
CLAR	1.2	0.42	0.53	9.1
HPAR	9.3	7.1	14.2	20<

Aldose reductases partially purified by 40—75% ammonium sulfate fractionation from rat lens (RLAR), bovine lens (BLAR), bovine kidney (BKAR), canine lens (CLAR) and human placenta (HPAR) were used with DL-glyceraldehyde as a substrate. Each enzyme amount added in the reaction mixture ranged from 0.058 to 0.21 units/ml, while the specific activity was ranged from 0.0023 to 0.027 units/mg protein.

TABLE II. Dependence of Inhibition of Bovine Lens Aldose Reductase on Assay Substrate

Substrate	IC_{50} (μM)			
	M16209	M16287	ONO-2235	Sorbinil
Glyceraldehyde	0.24	0.25	0.07	1.3
Glucose	0.37	0.19	0.11	1.0
Galactose	0.18	0.11	0.04	0.78
Glucuronate	0.35	0.18	0.09	1.9

An enzyme preparation obtained by 40—75% ammonium sulfate fractionation was used.

Dependence of Inhibition on Assay Substrate Whether or not the potencies of AR inhibitors were affected by assay substrate was examined. For each of the AR inhibitors, only a slight difference in IC_{50} values against partially purified BLAR was observed when the assay substrate was varied (Table II).

Kinetic Study of Inhibition Kinetic study of the inhibition was conducted with partially purified BLAR using glyceraldehyde and NADPH as a substrate. As shown in Fig. 2, M16209 and M16287 exhibited apparent parallel lines characteristic of uncompetitive inhibition in Lineweaver–Burk plots with respect to glyceraldehyde, but a reciprocal plot in the presence of high drug concentration became nonlinear with respect to glyceraldehyde. Sorbinil also showed a similar kinetic pattern of inhibition, whereas ONO-2235 was noncompetitive to glyceraldehyde. At high concentrations of ONO-2235 and sorbinil, reciprocal plots became nonlinear with respect to glyceraldehyde. As summarized in Table III, all of the inhibitors were noncompetitive to NADPH.

Inhibition of Purified Aldose Reductases BLAR was pu-

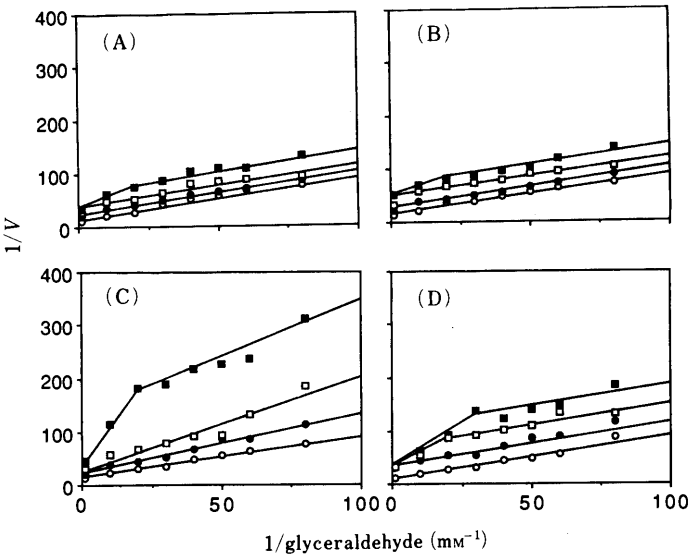


Fig. 2. Effects of (A) M16209, (B) M16287, (C) ONO-2235 and (D) Sorbinil on Lineweaver–Burk Plots of Bovine Lens Aldose Reductase Activity with DL-Glyceraldehyde as a Substrate

An enzyme preparation obtained by 40—75% ammonium sulfate fractionation was used. The ordinate represents the reciprocal of initial velocity expressed as the change in absorbance at 340 nm per minute. The abscissa represents the reciprocal of DL-glyceraldehyde concentration ranging from 0.0125×10^{-3} to 1×10^{-3} M. (A) ○, 0.0 μM; ●, 0.15 μM; □, 0.30 μM; ■, 0.45 μM. (B) ○, 0.0 μM; ●, 0.15 μM; □, 0.30 μM; ■, 0.45 μM. (C) ○, 0.0 μM; ●, 0.05 μM; □, 0.10 μM; ■, 0.20 μM. (D) ○, 0.0 μM; ●, 1.0 μM; □, 2.0 μM; ■, 3.0 μM.

TABLE III. Kinetic Pattern of Inhibition against Bovine Lens Aldose Reductase

Substrate	Type of inhibition			
	M16209	M16287	ONO-2235	Sorbinil
Glyceraldehyde	UC[a)]	UC[a)]	NC[a)]	UC[a)]
NADPH	NC	NC	NC	NC

An enzyme preparation obtained by 40—75% ammonium sulfate fractionation was used. UC, uncompetitive; NC, noncompetitive. *a)* The reciprocal plot became nonlinear at higher inhibitor concentrations.

TABLE IV. Effects of Purification on Susceptibility of Bovine Lens Aldose Reductase to Inhibition

Purification step	IC_{50} (μM)			
	M16209	M16287	ONO-2235	Sorbinil
40—75% $(NH_4)_2SO_4$	0.24	0.25	0.07	1.3
DEAE-Sephacel	1.5	0.73	1.3	20<
Sephadex G-75	5.9	3.2	12.6	20<

Specific activities of preparations after ammonium sulfate fractionation, DEAE-Sephacel and Sephadex G-75 were 0.0035, 0.167 and 3.65 units/mg protein, respectively. Enzyme amount of each purification step added in the reaction mixture ranged from 0.060 to 0.14 units/ml.

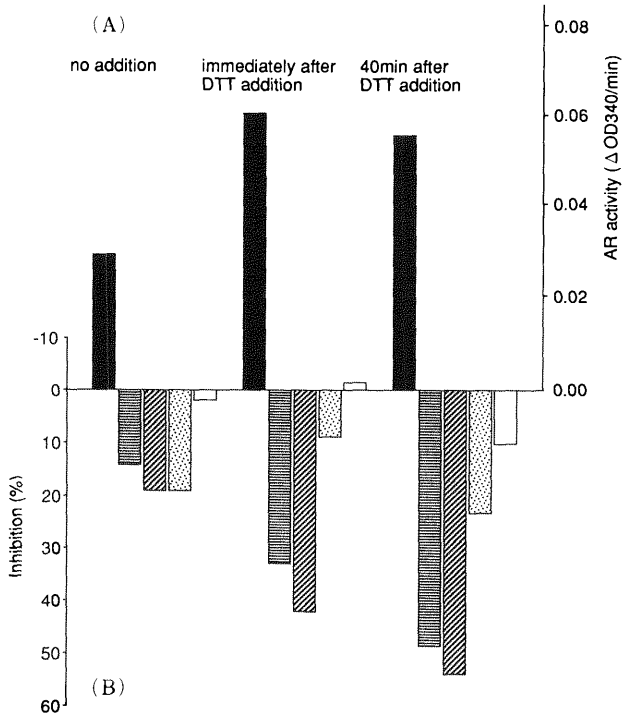


Fig. 3. Effects of Dithiothreitol on (A) the Activity of Bovine Lens Aldose Reductase and (B) Susceptibility of Purified Bovine Lens Aldose Reductase to AR Inhibitors

■, AR activity; ▤, M16209 (1 μM); ▨, M16287 (1 μM); ▩, ONO-2235 (0.5 μM); □, sorbinil (3 μM).

rified 3295-fold with the specific activity of 3.65 units/mg protein. Table IV shows that IC_{50} values of AR inhibitors became larger as BLAR purification progressed. The orders of potency of AR inhibitors in purification steps were ONO-2235 > M16209 = M16287 > sorbinil in ammonium sulfate fractionation, M16287 > ONO-2235 = M16209 > sorbinil in DEAE-Sephacel chromatography and M16287 > M16209 > ONO-2235 > sorbinil in Sephadex G-75 chromatography, respectively.

Effects of Dithiothreitol on the Activity of Purified Aldose Reductase and Its Susceptibility to Inhibition Purified BLAR showed a 2-fold increase in activity immediately after incubation with DTT, and a further increase in activity was not observed up to 40 min after incubation with DTT. The sensitivities of BLAR to AR inhibitors were also increased by incubation with DTT and were increased rather gradually up to 40 min after incubation with DTT; however, the sensitivities of BLAR to ONO-2235 increased slightly compared with other AR inhibitors.

TABLE V. Effects of Aldose Reductase Inhibitors on Activities of Enzymes of Carbohydrate and Glutathione Metabolism

Enzyme	% of control			
	M16209	M16287	ONO-2235	Sorbinil
SDH (sheep liver)	101	104	90	109
G6PDH (baker's yeast)	100	99	97	87
6PGDH (sheep liver)	102	99	100	94
HK (bovine heart)	94	91	97	97
PFK (rabbit muscle)	100	104	100	92
G3PDH (rabbit muscle)	73	92	107	87
PK (rabbit muscle)	88	85	80	82
LDH (rabbit muscle)	85	96	94	86
ALDH (equine liver)	101	94	98	108
GR (baker's yeast)	103	107	106	110
GPOX (bovine erythrocytes)	100	99	94	98

Inhibitors were added at 1×10^{-5} M in final concentration. SDH, sorbitol dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; HK, hexokinase; PFK, phosphofructokinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PK, pyruvate kinase; LDH, lactate dehydrogenase; ALDH, alcohol dehydrogenase; GR, glutathione reductase; GPOX, glutathione peroxidase.

TABLE VI. Inhibition of Sorbitol Accumulation in Isolated Rat Tissues

Tissue	IC_{50} (μM)			
	M16209	M16287	ONO-2235	Sorbinil
Lens	0.85	1.2	0.84	1.9
Sciatic nerve	1.8	3.1	2.0	5.7
Erythrocyte	2.8	3.1	5.6	2.2

Effects of AR Inhibitors on Enzymes of Carbohydrate and Glutathione Metabolism The effects of AR inhibitors on the enzyme activities of carbohydrate metabolism pathways such as the polyol pathway, glycolytic pathway and pentose phosphate pathway were examined using commercially available enzymes at a drug concentration of 1×10^{-5} M. As summarized in Table VI, M16209, M16287, ONO-2235 and sorbinil showed no or marginal effect on sorbitol dehydrogenase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, hexokinase, phosphofructokinase and alcohol dehydrogenase, glutathione reductase and glutathione peroxidase. However, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase were weakly affected by these inhibitors.

Sorbitol Accumulation in Isolated Tissues Table V shows the inhibitory effects of AR inhibitors on sorbitol accumulation in sciatic nerves, lenses and erythrocytes from rats. The potencies of AR inhibitors varied depending on the tissue used, while the differences in IC_{50} values among AR inhibitors in a given tissue and the differences in IC_{50} values of a given AR inhibitor among tissues were relatively small. As for lenses, the IC_{50} values were larger than those obtained with a partially purified enzyme from the lens, and the differences in IC_{50} values among AR inhibitors were smaller than those observed in enzyme inhibition.

Effects of AR Inhibitors on Galactose-Induced Cataracts in Rats All the lenses of galactose-fed untreated rats developed cataract formation on the 6th day of galactose feeding. As shown in Table VII, cataract formation of galactosemic rats was prevented almost completely by M16209 at 30 mg/kg/d, and completely by M16287 at

TABLE VII. Effects of Aldose Reductase Inhibitors on Galactose-Induced Cataracts in Rats

Group		Incidence of cataracts (%)
Galactose-fed		
Untreated		100 (40)[a]
+M16209	10 mg/kg	70 (10)
	30 mg/kg	10 (10)
+M16287	10 mg/kg	20 (10)
	30 mg/kg	0 (10)
+ONO-2235	50 mg/kg	90 (10)
+Sorbinil	3 mg/kg	0 (10)

Results are expressed as the percentage of incidence of cataract formation on the 6th day of galactose-feeding. *a*) Numbers of eyes observed are shown in parentheses.

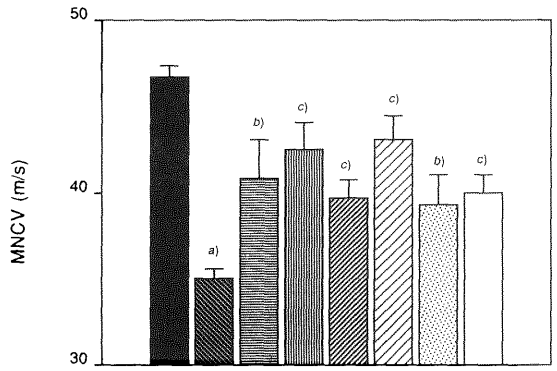


Fig. 4. Effects of M16209, M16287, ONO-2235 and Sorbinil on Motor Nerve Conduction Velocities in Streptozotocin-Induced Diabetic Rats

Each column and bar represents the mean±S.E. of 20 rats (normal control, untreated control) or 5 rats (M16209, 10; M16209, 30; M16287, 10; M16287, 30; ONO-2235, 30; sorbinil, 30 mg/kg). *a*) Significantly different from normal control ($p<0.01$). *b*) Significantly different from untreated control ($p<0.05$). *c*) Significantly different from untreated control ($p<0.01$). ■, normal control; ▨, untreated control; ▤, M16209 10 mg/kg; ▦, M16209 30 mg/kg; ▨, M16287 10 mg/kg; ▨, M16287 30 mg/kg; ▨, ONO-2235 30 mg/kg; □, sorbinil 30 mg/kg.

30 mg/kg/d and sorbinil at 3 mg/kg/d, respectively. ONO-2235, administered at as much as 50 mg/kg/d, however, showed almost no preventive effects on cataract formation in this model. The order of potency of AR inhibitors in the prevention of cataract formation was sorbinil > M16287 > M16209 ≫ ONO-2235.

Effects of AR Inhibitors on MNCV in Streptozotocin-Induced Diabetic Rats Diabetic, untreated control rats showed a significant delay in MNCV two weeks after treatment with streptozotocin (STZ), compared with the normal control. The delay in MNCV of STZ diabetic rats was ameliorated by every AR inhibitor tested. M16209 and M16287 were slightly more potent than ONO-2235 and sorbinil. The effects of ONO-2235 at 30 mg/kg/d and sorbinil at 30 mg/kg/d were almost comparable to those of M16209 at 10 mg/kg/d and M16287 at 10 mg/kg/d.

Discussion

M16209 and M16287 inhibited partially purified ARs from various sources with IC_{50} values ranging from 0.12×10^{-6} to 9.3×10^{-6} M and 0.08×10^{-6} to 7.1×10^{-6} M, respectively. On the other hand, ONO-2235 and sorbinil inhibited them with IC_{50} values ranging from 0.06×10^{-6} to 14.2×10^{-6} M and 0.60×10^{-6} to more than 20×10^{-6} M, respectively (Table I). Such differences in the susceptibility of various ARs to inhibition have been reported previously,[22-24] and it was suggested that the evaluation of AR inhibitors for clinical use may require the use of human AR from the appropriate target tissue.[24] We found that the order of susceptibility of various ARs to inhibition was RLAR > BLAR > HPAR, which is in good agreement with previous findings.[25]

Although the physiological substrate of AR has been regarded as glucose, AR shows rather broad substrate specificity.[26,27] A recent study indicated that AR became more susceptible to some AR inhibitors, including sorbinil, when glucose, as distinct from 4-nitrobenzaldehyde, was used as a substrate.[28] We found in the present study, however, that the difference in substrate did not markedly affect AR inhibition when glyceraldehyde, glucose, galactose and glucuronate were used as substrates.

The inhibitory potencies of M16209 and M16287 against purified BLAR were 25-fold and 13-fold less than those against the crude enzyme obtained by ammonium sulfate fractionation. This decrease in inhibitory activity against the final enzyme preparation was more marked for ONO-2235, which became 180-fold less effective. An apparent decrease in susceptibility to inhibition has previously been reported to occur with increased enzyme purification.[29,30] In order to investigate the reason for such differences in susceptibility to inhibition, we examined the effect of dithiothreitol, a reducing agent, on the activity of purified BLAR and on the susceptibilities of enzymes to AR inhibitors. The results indicate that sulfhydryl residues in AR are involved either in the enzyme catalytic activity or in the enzyme-inhibitor interaction. It was also suggested that purification and storage of the enzyme in 2-mercaptoethanol-containing buffers might have altered the catalytic activity of the enzyme as well as the sensitivity of AR to inhibition. This observation is similar to the result of Bhatnagar *et al.*,[31] who studied the interaction between human placental or kidney AR and sorbinil.

In kinetic studies with BLAR, M16209 and M16287 displayed uncompetitive inhibition to glyceraldehyde and noncompetitive inhibition to NADPH. As the concentration of the inhibitor increased, however, reciprocal plots became nonlinear with respect to glyceraldehyde (Table III, Fig. 2). Sorbinil exhibited the same type of inhibition as M16209 and M16287, while ONO-2235 displayed noncompetitive inhibition with respect to both glyceraldehyde and NADPH. Terashima *et al.*[17] reported that ONO-2235 inhibited RLAR uncompetitively with respect to glyceraldehyde. On the other hand, sorbinil has been shown to be uncompetitive with respect to glyceraldehyde using BLAR,[6] being consistent with our results. The reason for the discrepancy in inhibition type for ONO-2235 is unclear, but the difference in enzyme source may be concerned. A recent study showed that the kinetic inhibition pattern for human kidney AR with respect to glyceraldehyde and NADPH varied among a series of AR inhibitors and that this variation was attributed to a difference in the drug binding site on the enzyme.[32] Our results, therefore, suggest that the binding site of M16209 and M16287 may be the same as that of sorbinil.

Since sciatic nerves and lenses are the target tissues of diabetic complications,[33,34] and since the sorbitol content in erythrocytes has been shown to be a good indicator of *in vivo* polyol pathway activity in diabetic patients,[18,35] we

examined whether M16209 and M16287 really suppressed the reduction of glucose to sorbitol in these tissues. M16209 and M16287 effectively suppressed the polyol accumulation in the tissues, and no marked differences in inhibitory potency were observed among M16209, M16287, ONO-2235 and sorbinil. Their IC_{50} values, however, were about 10-fold higher in the suppression of polyol accumulation *in vitro* than those in direct enzyme inhibition studies, with the exception of sorbinil, which showed relatively similar values in both studies. The reason for such differences in IC_{50} values between tissue study and enzyme study may be explained by the existence of connective tissue and/or plasma membrane in the tissue study. It was also suggested that sorbinil may reach the enzyme in the tissue more easily than the other inhibitors.

In addition, we examined whether AR inhibitors affected the enzymes of carbohydrate and glutathione metabolism including NAD(P)H-requiring oxidoreductases. M16209 and M16287, as well as ONO-2235 and sorbinil, hardly affected the enzymes of carbohydrate metabolism and glutathione metabolism, which may suggest that M16209 and M16287 are more specific for aldose reductase than for the other oxidoreductases.

Further, we demonstrate in this report the efficacies of M16209 and M16287 in an experimental model of diabetic complications, in comparison with those of ONO-2235 and sorbinil, respectively. In the cataract model induced by a galactose diet, M16209 and M16287 showed preventive effects and their efficacies were approximately ten times less than that of sorbinil. ONO-2235 failed to show any suppressive effects on cataract formation in this model, even at higher dose. On the other hand, the delay in MNCV of streptozotocin-induced diabetic rats was ameliorated by the four AR inhibitors, among which M16209 and M16287 were slightly more potent than the others. From these observations *in vivo*, M16209 and M16287 were demonstrated to be almost equipotently effective in both models: cataracts and neuropathy, unlike ONO-2235 and sorbinil.

This study indicates that M16209 and M16287 are potent inhibitors of aldose reductases from both animals and human tissues. They also effectively suppress sorbitol accumulation in isolated rat tissues. Moreover, they are effective in animal models of diabetic complications. As M16209 and M16287, given orally to mice at 1 g/kg, do not show any noticeable symptoms in a preliminary study of acute toxicity, it is concluded that the two compounds could be offered for further investigation as new drugs for diabetic complications.

Acknowledgements The authors wish to thank Miss Tomoko Hatsuya and Miss Akemi Kurita for excellent assistance.

References

1) M. A. Ludvigson and R. L. Sorenson, *Diabetes*, **29**, 438 (1980).
2) M. A. Ludvigson and R. L. Sorenson, *Diabetes*, **29**, 450 (1980).
3) Y. Akagi, Y. Yajima, P. F. Kador, T. Kuwabara and J. H. Kinoshita, *Diabetes*, **33**, 562 (1984).
4) D. Dvornik, N. Simard-Duquesne, M. Krami, K. Sestanj, K. H. Gabby, J. H. Kinoshita, S. D. Varma and L. O. Merola, *Science*, **182**, 1146 (1973).
5) S. D. Varma, A. Mizuno and J. H. Kinoshita, *Science*, **195**, 205 (1977).
6) M. J. Peterson, R. Sarges, C. E. Aldinger and D. P. MacDonald, *Metabolism*, **28**, 456 (1979).
7) N. E. Pitts, K. Gundersen, D. J. Mehta, F. Vreeland, G. L. Shaw, M. L. Peterson and J. Collier, *Drugs*, **32** (Suppl. 2), 30 (1986).
8) N. Hotta, H. Kakuta, M. Kimura, H. Fukasawa, N. Koh, H. Terashima, M. Iida and N. Sakamoto, *Diabetes*, **32** (Suppl. 1), 98A (1983).
9) J. Jaspan, R. Maselli, K. Herold and C. Bartkus, *Lancet*, II, 758 (1983).
10) K. Inagaki, I. Miwa, T. Yashiro and J. Okuda, *Chem. Pharm. Bull.*, **30**, 3244 (1982).
11) I. Miwa, M. Hirano, K. Inagaki, C. Belbeloc'h and J. Okuda, *Biochem. Pharmacol.*, **36**, 2789 (1987).
12) K. Inagaki, I. Miwa and J. Okuda, *Arch. Biochem. Biophys.*, **216**, 337 (1982).
13) J. B. Wolff, "Methods in Enzymology," Vol. 1, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York, 1955, pp. 348—350.
14) H.-D. Horn, "Methods of Enzymatic Analysis," ed. by H. U. Bergmeyer, Academic Press, New York, 1975, pp. 876—877.
15) Y. C. Awasthi, E. Beutler and S. K. Srivastava, *J. Biol. Chem.*, **250**, 5144 (1951).
16) I. Miwa, M. Kanbara, H. Wakazono and J. Okuda, *Anal. Biochem.*, **173**, 39 (1988).
17) H. Terashima, K. Hama, R. Yamamoto, M. Tsuboshima, R. Kikkawa, I. Hatanaka and Y. Shigeta, *J. Pharmacol. Exp. Ther.*, **229**, 226 (1983).
18) J. I. Malone, G. Knox, S. Benford and T. A. Tedesco, *Diabetes*, **29**, 861 (1980).
19) O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
20) T. O. Sippel, *Invest. Ophthalmol.*, **5**, 568 (1966).
21) D. M. Robertson and A. A. F. Sima, *Diabetes*, **29**, 60 (1980).
22) P. Müller, O. Hockwin and C. Ohrloffi, *Ophthalmic Res.*, **17**, 115 (1985).
23) P. F. Kador, J. H. Kinoshita, W. H. Tung and L. T. Chylack, *Invest. Ophthalmol. Vis. Sci.*, **19**, 980 (1980).
24) P. F. Kador, L. O. Merola and J. H. Kinoshita, *Docum. Ophtal. Proc. Series*, **18**, 117 (1979).
25) D. Dvornik, "Aldose Reductase Inhibition. An Approach to the Prevention of Diabetic Complications," ed. by D. Porte, McGraw-Hill, New York, 1987, pp. 221—323.
26) S. Hayman, M. F. Lou, L. O. Merola and J. H. Kinoshita, *Biochim. Biophys. Acta*, **128**, 474 (1966).
27) P. K. Pottinger, *Biochem. J.*, **104**, 663 (1967).
28) R. Poulsom, *Biochem. Pharmacol.*, **36**, 1577 (1987).
29) P. F. Kador, J. H. Kinoshita and N. E. Sharpless, *J. Med. Chem.*, **28**, 841 (1985).
30) P. F. Kador, T. Shiono and J. H. Kinoshita, *Invest. Ophthalmol. Vis. Sci.*, **24** (Suppl. 1), 267 (1983).
31) A. Bhatnagar, S. Liu, B. Das and S. K. Srivastava, *Mol. Pharmacol.*, **36**, 825 (1989).
32) A. Bhatnagar, S. Liu, B. Das, N. H. Ansari and S. K. Srivastava, *Biochem. Pharmacol.*, **39**, 1115 (1990).
33) K. H. Gabby and E. S. Cathcart, *Diabetes*, **23**, 460 (1974).
34) R. S. Clements, J. P. Weaver and A. I. Winegrad, *Biochem. Biophys. Res. Commun.*, **37**, 347 (1969).
35) J. I. Malone, H. Leavengood, M. J. Peterson, M. M. O'brien, M. G. Page and C. E. Aldinger, *Diabetes*, **33**, 45 (1984).

Inhibitory Effect of 2-(*E*-2-Alkenoylamino)ethyl Alkyl Sulfides on Gastric Ulceration in Rats. II. Structure and Activity Relationships of 2-(*E*-*n* or *Z*-*n*-Decenoylamino)ethyl Alkyl Sulfides

Isao KOHDA,*[,a] Masakazu IWAI,[a] Masahiro WATANABE,[a] Yoshio ARAKAWA,[a] Chikara FUKAYA,[a] Kazumasa YOKOYAMA,[a] Yasuhiro KOHAMA,[b] and Tsutomu MIMURA[b]

Central Research Laboratories, The Green Cross Corporation,[a] Shodai Ohtani 2-1180-1 Hirakata, Osaka 573, Japan and Faculty of Pharmaceutical Sciences, Osaka University,[b] Yamadaoka 1-6, Suita, Osaka 565, Japan. *Received December 17, 1990*

The analogues of 2-(*E*-*n* or *Z*-*n*-decenoylamino)ethyl carbamoylmethyl sulfide, including the modifications of sulfide portion, double bond in decenoyl chain and alkyl sulfide moiety, were synthesized and their inhibitory effects on stress-induced ulceration in rats were compared.

Replacing the sulfura atom by methylene group or oxygen atom reduced the effect of potency. Saturation of the double bond in the decenoyl chain tended to reduce the anti-ulcerogenic activity in rats. There was no relationship between the position of double bond in decenoyl chain and the pharmacological activity. On the other hand, compounds with *E*-configuration showed stronger anti-ulcer activity than the corresponding *Z*-type of compounds. Among 9 kinds of S substituted alkyl groups for carbamoylmethyl, 2-(*E*-2-decenoylamino)ethyl 2-cyclohexylethyl sulfide showed the most potent anti-ulcerogenic activity in rats and also showed the lowest acute toxicity in mice.

Keywords 2-(*E*-2-alkenoylamino)ethyl alkyl sulfide; 2-(*E*-2-decenoylamino)ethyl cyclohexylethyl sulfide; anti-ulcerogenic activity; acute toxicity

We have reported that human immunoglobulin G (IgG) showed anti-ulcerogenic[1] and anti-inflammatory[2] activities after reductive cleavage of interchain disulfide bonds, though the native IgG showed no such activities. It was suggested that chemical modification of S–S bonds in the hinge region was essential to the appearance of these pharmacological activities. We have also reported the anti-ulcerogenic activities of various saturated[3] and unsaturated[4] fatty acids and investigated the relationship between alkyl or alkenyl chain lengths and pharmacological activities. In an effort to develop a new anti-ulcerogenic drug based on this evidence, we previously investigated the pharmacological activities of a series of 2-(*E*-2-alkenoylamino)ethyl alkyl sulfides which were synthesized from various *E*-2-unsaturated fatty acids and cystamine.[5] In this series, 2-(*E*-2-decenoylamino)ethyl carbamoylmethyl sulfide (I-1-a) was the most potent inhibitor of gastric ulceration in rats.

In the present article, a structure–activity study of I-1-a has been made in an attempt to test the effects of modifications of the sulfide portion, double bond in decenoyl chain and alkyl sulfide moiety on the appearance of the anti-ulcerogenic activity.

Experimental

Synthesis of 2-(*E*-2-Decenoylamino)ethyl Carbamoylmethyl Sulfide (I-1-a) Neat $SOCl_2$ was added dropwise to a *E*-2-decenoic acid at 0 °C. This solution was stirred for 4 h under reflux, and then the excess $SOCl_2$ was removed by evaporation. The residual oil was purified by distillation, affording *E*-2-decenoyl chloride as a pale yellow oil. One normal NaOH was added to a stirred suspension of cystamine dihydrochloride in water at 0 °C to pH 8.0. The solution was added with *E*-2-decenoyl chloride in CH_2Cl_2, and stirred for 1 h at room temperature. After the reaction, ethyl acetate (EtOAc) and 0.5 N HCl were added to the solution and suspended. The organic layer was removed and washed with water, 5% $NaHCO_3$, water, and brine. The EtOAc solution was dried over $MgSO_4$ and evaporated. The residual solid was crystallized from EtOAc, affording the *N*,*N*′-bis(*E*-2-decenoyl)cystamine. Bu_3P was added to a stirred suspension of this compound in 90% MeOH/water at 0 °C, and stirred for 1 h at room temperature. Iodoacetamide and 1 N NaOH were added to the solution and stirred for 1.5 h at room temperature. After the reaction, an off-white solid was precipitated by the addition of water. The suspension was filtered, and the solid was washed with water, affording I-1-a as a white crystal, mp 151.0—151.5 °C. IR ν_{max}^{KBr} cm^{-1}: 3370, 3300, 3170, 2920, 2850, 1650, 1620, 1540, 970. ^{1}H-NMR (DMSO-d_6 + $CDCl_3$) δ: 0.90 (3H, br t, J = 6 Hz), 2.15 (2H, br s), 2.65 (2H, t, J = 6 Hz), 3.10 (2H, s), 3.40 (2H, br dt, J = 6.6 Hz), 5.80 (1H, d, J = 16 Hz), 6.65 (1H, dt, J = 16, 7 Hz), 6.90 (br t, 1H), 7.30 (br s, 1H), 7.90 (br s, 1H). HRMS *m*/*z*: 287.1745 [M + H] (287.1792 Calcd for $C_{14}H_{26}N_2O_2S$ + H).

Synthesis of 5-(*E*-2-Decenoylamino)pentanamide (I-2-a) Neat $SOCl_2$ was added dropwise to 5-bromopentanoic acid at 0 °C. This solution was stirred for 4 h under reflux, and then the excess $SOCl_2$ was removed by evaporation. The residual oil was purified by distillation, affording 5-bromopentanoyl chloride as an oil. IR ν_{max}^{film} cm^{-1}: 1800. 5-Bromopentanoyl chloride was added to a stirred solution of 28% NH_4OH at 0 °C, and stirred for 45 min at the same temperature. Chloroform was added and suspended. The organic layer was removed, and the aqueous layer was extracted with chloroform. The chloroform layer was combined, washed with brine, dried over $MgSO_4$ and then evaporated, affording 5-bromopentanamide as a colorless crystal. IR ν_{max}^{KBr} cm^{-1}: 1650, 1630. Dibenzylamine and anhydrous K_2CO_3 were added to a stirred solution of 5-bromopentanamide in dry dimethylformamide (DMF), and stirred for 87 h at room temperature. EtOAc and water was added to the solution and suspended. The organic layer was removed, and the aqueous layer was extracted with EtOAc. The EtOAc layer was combined, washed with water and brine, dried over $MgSO_4$, and then evaporated. The residue was chromatographed on silica gel (9 : 1 EtOAc–MeOH), affording 5-dibenzylaminopentanamide. A solution of 5-dibenzylaminopentanamide in MeOH was treated with 10% Pd/C and stirred under H_2 atmosphere for 43 h. The suspension was filtered through celite and evaporated, affording 5-aminopentanamide. 2-Decenoyl chloride was added dropwise to a stirred solution of 5-aminopentanamide in 0.6 N NaOH at 0 °C, and stirred for 30 min at room temperature. The suspension was filtered, and the solid was washed with water and ether. The solid was dried *in vacuo*, affording I-2-a as a needle-shaped white crystal, mp 184.0—185.5 °C. IR ν_{max}^{KBr} cm^{-1}: 3360, 3400, 3180, 1645, 1630. ^{1}H-NMR (DMSO-d_6) δ: 0.86 (3H, m), 1.10—1.60 (14H, m), 2.00—2.20 (4H, m), 3.00—3.13 (2H, m), 5.86 (1H, d, J = 15.3 Hz), 6.58 (1H, dt, J = 15.3, 6.8 Hz), 6.63—6.80 and 7.18—7.30 (2H, br s), 7.80—7.90 (1H, br t). HRMS *m*/*z*: 269.2231 [M + H] (269.2227 Calcd for $C_{15}H_{28}N_2O_2$ + H).

Synthesis of 2-(*E*-2-Decenoylamino)ethyl Carbamoylmethyl Ether (I-3-a) 1.58 N *n*-BuLi/Hex was added dropwise to a stirred solution of dibenzylamine in dry hexamethylphosphoramide (HMPA) and dry tetrahydrofuran (THF) at −70 °C. The scarlet solution was then stirred for 45 min at the same temperature. Ethylenebromohydrin in dry THF was added dropwise to the solution. After the addition, the solution was gradually warmed to room temperature, dry EtOAc was added, and the solution was stirred for 30 min. The solution was diluted with EtOAc, washed with water and brine, dried over $MgSO_4$, and then evaporated. The residual oil was chromatographed on silica gel (1 : 3 EtOAc–hexane), affording *O*-acetyl-*N*,*N*-dibenzylethanolamine as a colorless oil. IR ν_{max}^{film}

cm^{-1}: 1740. Anhydrous K_2CO_3 was added to a stirred solution of *O*-acetyl-*N*,*N*-dibenzylethanolamine in MeOH and stirred for 30 min at room temperature. EtOAc and water added to the solution and suspended. The organic layer was removed, washed with water and brine, dried over $MgSO_4$, and then evaporated. The residual oil was chromatographed on silica gel (1:2 EtOAc–hexane), affording *N*,*N*-dibenzylethanolamine. IR ν_{max}^{film} cm^{-1}: 3400. A suspension of 35% KH in oil was washed with hexane and dry THF was added to it. *N*,*N*-Dibenzylethanolamine in dry THF was added dropwise at 0 °C to the suspension. After the addition, iodoacetamide in dry THF was added dropwise to the solution, and stirred overnight. EtOAc, water and 1 N NaOH were added and suspended. The organic layer was removed, washed with water and brine, dried over $MgSO_4$, and then evaporated. The residue was chromatographed on silica gel (9:1 EtOAc–MeOH), affording *N*,*N*-dibenzyl-*O*-carbamoylmethylethanolamine as a crystal. IR ν_{max}^{KBr} cm^{-1}: 3450, 3200, 1680. A solution of *N*,*N*-dibenzyl-*O*-carbamoylmethylethanolamine in MeOH was treated with 10% Pd/C and stirred under H_2 atmosphere for 19 h. The suspension was filtered through celite and evaporated, affording *O*-carbamoylmethylethanolamine. *E*-2-Decenoyl chloride was added dropwise to a stirred solution of *O*-carbamoylmethylethanolamine in 1 N NaOH and water at 0 °C, and stirred for 30 min at room temperature. EtOAc was added to the solution and suspended. The organic layer was removed, and the aqueous layer was extracted with EtOAc. The EtOAc layer was combined, washed with water and brine, dried over $MgSO_4$, and then evaporated. The residual solid was chromatographed on octadecyl silica (ODS) [high performance liquid chromatography (HPLC)], affording I-3-a as a crystal powder, mp 129.0—130.0 °C. IR ν_{max}^{KBr} cm^{-1}: 3410, 3310, 3200, 1630, 1225. ^{1}H-NMR (DMSO-d_6) δ: 0.86 (3H, m), 1.10—1.50 (10H, m), 2.05—2.20 (2H, m), 3.25—3.38 (2H, m), 3.46 (2H, t, J=5.2 Hz), 3.80 (2H, s), 5.90 (1H, d, J=15.4 Hz), 6.62 (1H, dt, J=15.4, 6.8 Hz), 7.10—7.45 (2H, br s), 8.01 (1H, br t). HRMS: *m/z*: 271.1975 [M+H] (271.2020 Calcd for $C_{14}H_{26}N_2O_3$+H).

Synthesis of 2-Decanoylaminoethyl Carbamoylmethyl Sulfide (I-1-b) The procedures used for I-1-a were repeated with decanoic acid, affording I-1-b as a white crystal, mp 141.0—142.0 °C. IR ν_{max}^{KBr} cm^{-1}: 3370, 3320, 3170, 1650, 1540. ^{1}H-NMR ($CF_3COOH+CDCl_3$) δ: 0.89 (3H, br t, J=6 Hz), 1.10—2.00 (14H, m), 2.30—3.20 (4H, m), 3.48 (2H, s), 3.60—3.90 (2H, m), 7.80 (1H, br t), 8.10 (2H, br s). HRMS *m/z*: 289.1920 [M+H] (289.1948 Calcd for $C_{14}H_{28}N_2O_2S$+H).

Synthesis of 2-(*E*-3-Decenoylamino)ethyl Carbamoylmethyl Sulfide (I-1-c) The procedures used for I-1-a were repeated with *E*-3-decenoic acid, affording I-1-c as a crystal powder, mp 126.5—127.0 °C. IR ν_{max}^{KBr} cm^{-1}: 3380, 3320, 3180, 1640, 1540, 960. ^{1}H-NMR (DMSO-d_6) δ: 1.90—2.03 (2H, m), 2.56—2.66 (2H, m), 2.79 (2H, d, J=4.4 Hz), 3.08 (2H, s), 3.16—3.27 (2H, m), 5.35—5.60 (2H, m). HRMS *m/z*: 287.1794 [M+H] (287.1792 Calcd for $C_{14}H_{26}N_2O_2S$+H).

Synthesis of 2-(*Z*-3-Decenoylamino)ethyl Carbamoylmethyl Sulfide (I-1-d) The procedures used for I-1-a were repeated with *Z*-3-decenoic acid, affording I-1-d as a crystal powder, mp 116.0—117.0 °C. IR ν_{max}^{KBr} cm^{-1}: 3380, 3320, 3180, 1640, 1540. ^{1}H-NMR (DMSO-d_6) δ: 1.95—2.07 (2H, m), 2.56—2.65 (2H, m), 2.87 (2H, d, J=5.8 Hz), 3.08 (2H, s), 3.17—3.28 (2H, m), 5.36—5.56 (2H, m). HRMS *m/z*: 287.1812 [M+H] (287.1792 Calcd for $C_{14}H_{26}N_2O_2S$+H).

Synthesis of 2-(*E*-4-Decenoylamino)ethyl Carbamoylmethyl Sulfide (I-1-e) The procedures used for I-1-a were repeated with *E*-4-decenoic acid, affording I-1-e as a crystal powder, mp 140.0—142.0 °C. IR ν_{max}^{KBr} cm^{-1}: 3370, 3300, 1645, 1635, 960. ^{1}H-NMR (DMSO-d_6) δ: 187—2.00 (2H, m), 2.00—2.24 (4H, m), 2.56—2.65 (2H, m), 3.08 (2H, s), 3.17—3.28 (2H, m), 5.28—5.50 (2H, m). HRMS *m/z*: 287.1807 [M+H] (287.1792 Calcd for $C_{14}H_{26}N_2O_2S$+H).

Synthesis of 2-(*Z*-4-Decenoylamino)ethyl Carbamoylmethyl Sulfide (I-1-f) The procedures used for I-1-a were repeated with *Z*-4-decenoic acid, affording I-1-f as crystal powder, mp 121.5—124.0 °C. IR ν_{max}^{KBr} cm^{-1}: 3370, 3310, 1625, 1615. ^{1}H-NMR (DMSO-d_6) δ: 1.90—2.27 (6H, m), 2.55—2.64 (2H, m,), 3.07 (2H, s), 3.16—3.27 (2H, m), 5.21—5.41 (2H, m). HRMS *m/z*: 287.1828 [M+H] (287.1792 Calcd for $C_{14}H_{26}N_2O_2S$+H).

Synthesis of 2-(*E*-5-Decenoylamino)ethyl Carbamoylmethyl Sulfide (I-1-g) The procedures used for I-1-a were repeated with *E*-5-decenoic acid, affording I-1-g as a crystal powder, mp 130.5—131.5 °C. IR ν_{max}^{KBr} cm^{-1}: 3380, 3320, 3180, 1640, 1540, 960. ^{1}H-NMR (DMSO-d_6) δ: 0.83—0.90 (3H, m), 1.16—1.36 (4H, m), 1.45—1.60 (2H, m), 1.88—2.09 (6H, m), 2.56—2.65 (2H, s), 3.08 (2H, s), 3.17—3.33 (2H, m), 5.24—5.50 (2H, m). HRMS: *m/z*: 287.1819 [M+H] (287.1792 Calcd for $C_{14}H_{26}N_2O_2S$+H).

Synthesis of 2-(*Z*-5-Decenoylamino)ethyl Carbamoylmethyl Sulfide (I-1-h) The procedures used for I-1-a were repeated with *Z*-5-decenoic acid, affording I-1-h as a crystal powder, mp 115.0—115.5 °C. IR ν_{max}^{KBr} cm^{-1}: 3380, 3320, 3180, 1640, 1540. ^{1}H-NMR (DMSO-d_6) δ: 0.83—0.90 (3H, m), 1.17—1.37 (4H, m), 1.44—1.59 (2H, m), 1.92—2.10 (6H, m), 2.55—2.65 (2H, s), 3.08 (2H, s), 3.17—3.33 (2H, m), 5.24—5.42 (2H, m). HRMS *m/z*: 287.1809 [M+H] (287.1792 Calcd for $C_{14}H_{26}N_2O_2S$+H).

Synthesis of 2-(*E*-6-Decenoylamino)ethyl Carbamoylmethyl Sulfide (I-1-i) The procedures used for I-1-a were repeated with *E*-6-deceonoic acid, affording I-1-i as a crystal powder, mp 135.5—137.0 °C. IR ν_{max}^{KBr} cm^{-1}: 3370, 3310, 3180, 1645, 1635, 1540, 965. ^{1}H-NMR (DMSO-d_6) δ: 0.85 (3H, t, J=7.4 Hz), 1.23—1.55 (6H, m), 1.80—2.09 (6H, m), 2.57—2.64 (2H, m), 3.08 (2H, s), 3.17—3.27 (2H, m), 5.25—5.47 (2H, m). HRMS *m/z*: 287.1812 [M+H] (287.1792 Calcd for $C_{14}H_{26}N_2O_2S$+H).

Synthesis of 2-(*Z*-6-Decenoylamino)ethyl Carbamoylmethyl Sulfide (I-1-j) The procedures used for I-1-a were repeated with *Z*-6-decenoic acid, affording I-1-j as a crystal powder, mp 115.5—117.0 °C. IR ν_{max}^{KBr} cm^{-1}: 3370, 3310, 3180, 1645, 1635, 1545. ^{1}H-NMR (DMSO-d_6) δ: 0.86 (3H, t, J=7.4 Hz), 1.19—1.57 (6H, m), 1.92—2.10 (6H, m), 2.57—2.64 (2H, s), 3.08 (2H, s), 3.17—3.27 (2H, m), 5.25—5.41 (2H, m). HRMS *m/z*: 287.1791 [M+H] (287.1792 Calcd for $C_{14}H_{26}N_2O_2S$+H).

Synthesis of 2-(*E*-2-Decenoylamino)ethyl 4-Methyl-1-piperazinylcarbonylmethyl Sulfide (II-1-a) The procedures used for I-1-a were repeated with chloroacetic acid, affording 2-(*E*-2-decenoylamino)ethyl carboxymethyl sulfide. *N*,*N*′-Dicyclohexylcarabodiimide and *N*-methylpiperazine in dry chloroform was added dropwise to a stirred solution of 2-(*E*-2-decenoylamino)ethyl carboxymethyl sulfide in dry chloroform at 0 °C, and stirred for 3.75 h at room temperature. The suspension was filtered, and the filtrate was evaporated. The residue was chromatographed on silica gel (93:7 chloroform–MeOH). The obtained crystal was crystallized from EtOAc/hexane (1:4), affording II-1-a as a needle-shaped white crystal, mp 77—78 °C. IR ν_{max}^{KBr} cm^{-1}: 3280, 2920, 2770, 1665, 1620, 1550. ^{1}N-NMR ($CDCl_3$), δ: 0.88 (3H, br t), 1.15—1.55 (10H, m), 2.10—2.22 (2H, m), 2.32 (3H, s), 2.37—2.52 (4H, m), 2.75—2.85 (2H, m), 3.45—3.70 (6H, m), 5.83 (1H, dt, J=15.3, 1.5 Hz), 6.80 (1H, br t), 6.84 (1H, dt, J=15.3, 7.0 Hz). HRMS *m/z*: 370.2478 [M+H] (370.2526 Calcd for $C_{19}H_{35}N_3O_2S$+H).

Synthesis of 2-(*E*-2-Decenoylamino)ethyl 2-Cyclohexylethyl Sulfide (III-1-a) The procedures used for I-1-a were repeated with 2-bromoethylcyclohexane, affording III-1-a as yellow paste. IR ν_{max}^{KBr} cm^{-1}: 3250, 2920, 1660, 1625, 1540. ^{1}H-NMR ($CDCl_3$) δ: 0.78—1.05 (5H, m), 1.06—1.55 (16H, m), 1.55—1.80 (5H, m), 2.10—2.25 (2H, m), 2.48—2.62 (2H, m), 2.69 (2H, t, J=15.3, 1.4 Hz), 5.92 (1H, br t), 6.84 (1H, dt, J=15.3, 7.0 Hz). HRMS *m/z*: 340.2684 [M+H] (340.2672 Calcd for $C_{20}H_{37}NOS$+H).

Synthesis of 2-(*E*-2-Decenoylamino)ethyl Cyclohexylmethyl Sulfide (IV-1-a) The procedures used for I-1-a were repeated with iodomethylcyclohexane, affording IV-1-a as yellow paste. IR ν_{max}^{KBr} cm^{-1}: 3300, 1660, 1620, 1540. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, br t), 0.90—1.58 (16H, m), 1.58—1.91 (5H, m), 2.10—2.26 (2H, m), 2.42 (2H, d, J=6.8 Hz), 2.67 (2H, t, J=6.5 Hz), 3.50 (2H, q, J=6.2 Hz), 5.78 (1H, dt, J=15.3, 1.5 Hz), 5.97 (1H, br t), 6.84 (1H, dt, J=15.3, 7.0 Hz). HRMS *m/z*: 326.2494 [M+H] (326.2515 Calcd for $C_{19}H_{35}NOS$+H).

Synthesis of 2-(*E*-2-Decenoylamino)ethyl 2-Phenylethyl Sulfide (V-1-a) The procedures used for I-1-a were repeated with 2-bromoethylbenzene, affording V-1-a as a needle-shaped white crystal, mp 53.0—53.3 °C. IR ν_{max}^{KBr} cm^{-1}: 3280, 2910, 1660, 1620, 1540. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, br t), 1.15—1.55 (10H, m), 2.10—2.28 (2H, m), 2.69 (2H, t, J=6.4 Hz), 2.73—2.98 (4H, m), 3.49 (2H, q, J=6.2 Hz), 5.75 (1H, dt, J=15.3, 1.4 Hz), 5.89 (1H, br t), 6.83 (1H, dt, J=15.3, 7.0 Hz), 7.16—7.36 (5H, m). HRMS *m/z*: 334.2165 [M+H] (334.2203 Calcd for $C_{20}H_{31}NOS$+H).

Synthesis of 2-(*E*-2-Decenoylamino)ethyl Phenylmethyl Sulfide (VI-1-a) The procedures used for I-1-a were repeated with benzyl bromide, affording VI-1-a as a needle-shaped white crystal, mp 52.0—52.5 °C. IR ν_{max}^{KBr} cm^{-1}: 3300, 2920, 1665, 1625, 1545, 700. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, br t), 1.11—1.53 (10H, m), 2.00—2.21 (2H, m), 2.58 (2H, t, J=6.4 Hz), 3.44 (2H, q, J=6, 2 Hz), 3.71 (2H, s), 5.74 (1H, dt, J=15.2, 1.4 Hz), 6.05 (1H, br t), 6.81 (1H, dt, J=15.3, 7.0 Hz), 7.20—7.33 (5H, m). HRMS *m/z*: 320.2027 [M+H] (320.2047 Calcd for $C_{19}H_{29}NOS$+H).

Synthesis of 2-(*E*-2-Decenoylamino)ethyl *n*-Butyl Sulfide (VII-1-a) The procedures used for I-1-a were repeated with *n*-butyl bromide, affording VII-1-a as a scaly-shaped white crystal, mp 48.0—48.5 °C. IR ν_{max}^{KBr} cm^{-1}: 3220, 3070, 2920, 2850, 1660, 1620, 1550, 1465, 975. ^{1}H-NMR ($CDCl_3$) δ; 0.70—1.10 (6H, m), 1.10—1.90 (14H, m), 2.40—2.90 (4H, m). HRMS *m/z*: 286.2189 [M+H] (286.2203 Calcd for $C_{16}H_{31}NOS$+H).

Synthesis of 2-(*E*-2-Decenoylamino)ethyl Methyl Sulfide (VIII-1-a) The procedures used for I-1-a were repeated with methyl iodide, affording VIII-1-a as a needle-shaped white crystal, mp 51.0—51.5 °C. IR ν_{max}^{KBr} cm^{-1}:

3300, 3090, 2930, 2860, 1665, 1630, 1545, 980. ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, m), 1.08—1.80 (10H, br s), 2.10 (3H, s), 1.90—2.50 (2H, m), 2.65 (2H, t, J=6 Hz), 3.50 (2H, dt, J=6, 6 Hz), 5.82 (1H, dt, J=15, 1.3 Hz), 5.90—6.50 (1H, br t), 6.88 (1H, dt, J=15, 6.6 Hz). HRMS: m/z: 244.1694 [M+H] (244.1734 Calcd for $C_{13}H_{25}NOS$+H).

Synthesis of 2-(*E*-2-Decenoylamino)ethyl (1-Methyl-2-piperidyl)methyl Sulfide (IX-1-a) The procedures used for I-1-a were repeated with 1-methyl-2-chloromethylpiperidine, affording IX-1-a as yellow paste. IR ν_{max}^{KBr} cm^{-1}: 3300, 3090, 2920, 2870, 2800, 1665, 1630, 1540, 975, 755. ^{1}H-NMR ($CDCl_3$) δ: 1.10—2.00 (16H, m), 2.00—2.50 (6H, m), 2.50—3.10 (6H, m). HRMS m/z: 341.2642 [M+H] (341.2625 Calcd for $C_{19}H_{36}N_2OS$+H).

The abbreviated name and structural formula of each synthesized compound is shown in Fig. 1.

Stress-Induced Gastric Ulcer Male Wistar rats weighing 230—250 g and fasted for 24 h were used as experimental animals. The rats were subjected to stress following the method of Takagi and Okabe,[6] in which animals were immobilized in a stress cage and immersed vertically in a water bath at 22±1 °C to the level of the xiphoid process. Each sample was suspended with 10% HCO-60® (polyoxyethylene harden castor oil; Nikko Chemicals) in saline and administered perorally, immediately or 1.5 h before the stress treatment. The animals were sacrificed 7 h later and their stomachs were removed, inflated with 10 ml of saline and immersed in 1% formalin solution for 5 min. The stomach was incised along the greater curvature and examined for gastric lesions. Total length (mm) of all lesions in the glandular portion of the stomach was used as an ulcer index.

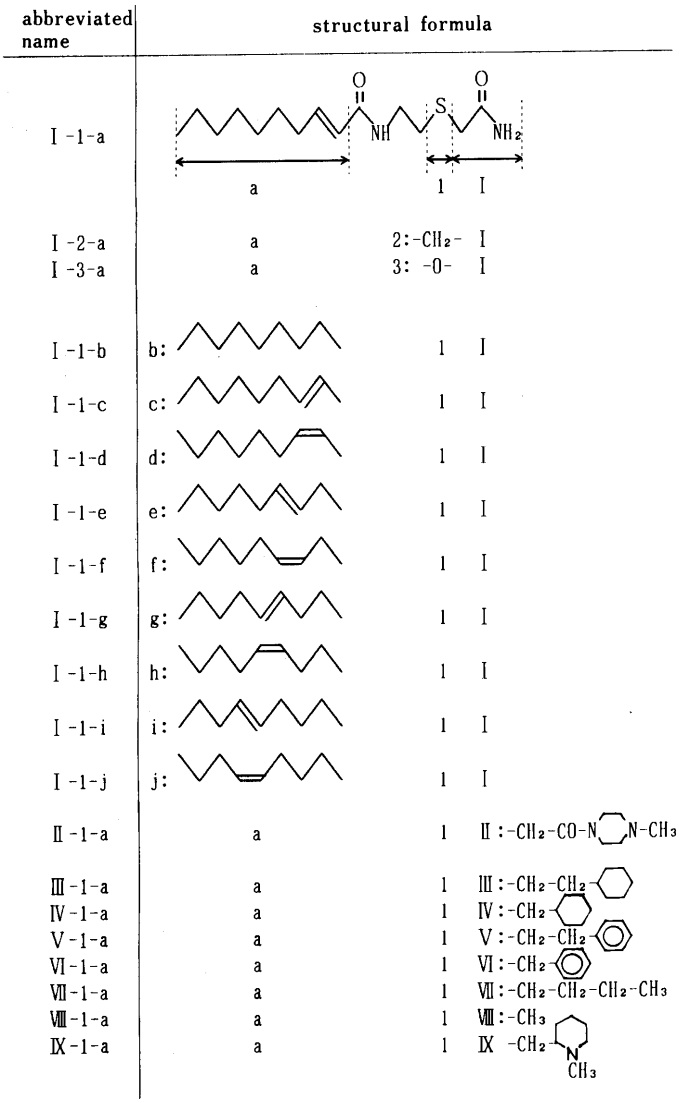


Fig. 1. The Abbreviated Name and Structural Formula of Each Synthesized Compound

Acute Toxicity Test in Mice Acute toxicity was studied in male ddy mice weighing 18—21 g. Five mice in each group were used for the experiment. Each sample, suspended in 10% HCO-60 in saline, was administered at doses of 500, 2000, 8000 mg/kg perorally at a volume of 40 ml/kg. After the administration of a single dose of each test sample, the behavior of the animals was observed for 6 h, then they were caged and fed as usual for 7 days. Number of dead animals and the change of body weight in each mouse were observed every day until the end of the experiment.

Statistics Results were expressed as the mean±S.E. Student's t test was applied to evaluate the significance of differences between the mean of the control group and the means of sample-adiministered groups.

Results

The inhibitory effects of I-2-a and I-3-a replacing sulfur atom by methylene group and oxygen atom given at doses of 2 and 5 mg/kg, *p.o.*, on stress-induced ulceration in rats were compared with that of I-1-a. I-2-a and I-3-a showed significant anti-ulcerogenic activity, but their potencies were less than that of I-1-a (Table I).

The effect of modification of unsaturation in decenoyl chain on stress-induced ulceration was tested. At doses of 5 and 2 mg/kg perorally immediately before the stress treatment, I-1-b with saturated fatty acid showed somewhat less pharmacological activity than I-1-a with unsaturated fatty acid (Table II).

The effect of position and configuration of unsaturation in various 2-(*E-n* or *Z-n*-decenoylamino)ethyl carbamoylmethyl sulfides, given at a dose of 5 mg/kg, *p.o.* is shown in Fig. 2. I-1-c, -e and -g retained inhibitory potency comparable to I-1-a and I-1-d, -f and -j almost completely lost it. There was no relationship between the position of

TABLE I. Effect of I-1-a, I-2-a and I-3-a on Stress-Induced Ulceration in Rats

Treatment	Dose (mg/kg)	Ulcer index	Inhibition (%)
Control[a]	—	25.7±4.2	—
I-1-a	5	8.2±1.3[c]	68.1
	2	13.0±3.1[b]	49.4
I-2-a	5	16.2±2.0	37.0
	2	17.2±3.0	33.1
Control[a]		24.1±3.0	—
I-1-a	5	9.6±2.8[c]	60.1
	2	10.4±2.1[c]	56.4
I-3-a	5	12.7±2.3[c]	47.3
	2	19.1±3.7	20.8

All values represent the mean±S.E. of 8 rats. *a*) HCO-60, 10% in saline. Each sample was perorally administered immediately before the restraint and water-immersion stress loading. Significantly different from the control group: *b*) $p<0.05$, *c*) $p<0.01$.

TABLE II. Effect of I-1-a and I-1-b on Stress-Induced Ulceration in Rats

Treatment	Dose (mg/kg)	Ulcer index	Inhibition (%)
Control[a]	—	17.2±2.2	—
I-1-a	5	4.3±0.7[d]	75.0
	2	7.9±0.8[c]	54.1
I-1-b	5	9.1±0.8[c]	47.1
	2	11.4±1.7[b]	33.1

All values represent the mean±S.E. of 8 rats. *a*) HCO-60, 10% in saline. Each sample was perorally administered immediately before the restraint and water-immersion stress loading. Significantly different from the control group: *b*) $p<0.05$, *c*) $p<0.01$, *d*) $p<0.001$.

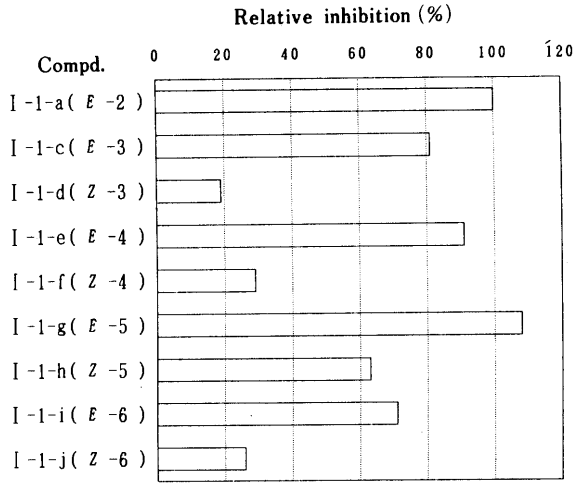


Fig. 2. Effect of Position and Configuration of Unsaturation in Various 2-(*E*-*n* or *Z*-*n*-Decenoylamino)ethyl Carbamoylmethyl Sulfides on Stress-Induced Ulceration in Rats

Each column represents relative percent inhibition of ulcer index in various 2-(*E*-*n* or *Z*-*n*-decenoylamino)ethyl carbamoylmethyl sulfides against that in the control group. The inhibitory activity of I-1-a was considered to 100%. Each sample was administered perorally (5 mg/kg) immediately before the restraint and water-immersion stress loading.

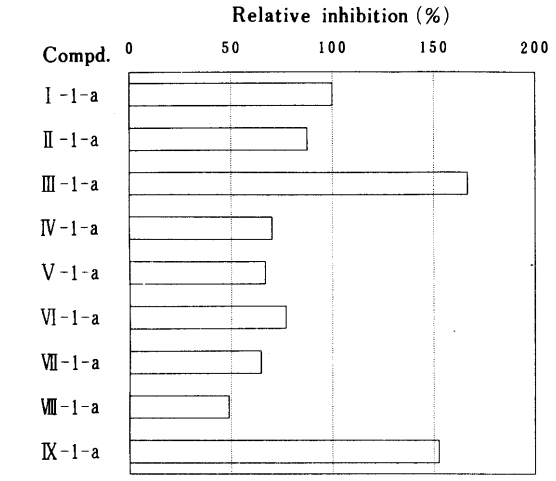


Fig. 3. Effect of Alkyl Group in Various 2-(*E*-2-Decenoylamino)ethyl Alkyl Sulfides on Stress-Induced Ulceration in Rats

Each column represents relative percent inhibition of ulcer index in various 2-(*E*-2-decenoylamino)ethyl alkyl sulfides against that in the control group. The inhibitory activity of I-1-a was considered to 100%. Each sample was administered perorally (equimolar dose of 5 mg/kg of I-1-a) 1.5 h before the restraint and water-immersion stress loading.

unsaturation and the pharmacological activity. On the other hand, compounds with *E*-configuration showed stronger pharmacological activity than the corresponding *Z*-type compounds.

The effect of S-alkyl group in various 2-(*E*-2-decenoylamino)ethyl alkyl sulfides, given at an equimolar dose of 5 mg/kg of I-1-a perorally 1.5 h before the stress treatment, was examined. As shown in Fig. 3, III-1-a with cyclohexylethyl group and IX-1-a wth (1-methyl-2-piperidyl)-methyl group showed highly improved anti-ulcerogenic activity over I-1-a. The ulcer indices of control, I-1-a and III-1-a were 22.0 ± 3.6, 12.8 ± 1.6 ($p < 0.05$) and 6.7 ± 1.7 ($p < 0.01$), respectively. The other analogues with different S-alkyl groups were less active than I-1-a.

TABLE III. Effect of Alkyl Group in Various 2-(*E*-2-Decenoylamino)ethyl Alkyl Sulfides on Acute Toxicity in Mice

Treatment	Dose (mg/kg)	Mortality	Toxic symptom
I-1-a	8000	0/5	Not observed
	2000	0/5	Not observed
	500	0/5	Not observed
II-1-a	2000	0/5	Mild
	500	0/5	Mild
III-1-a	2000	0/5	Not observed
	500	0/5	Not observed
IV-1-a	2000	0/5	Not observed
	500	0/5	Not observed
V-1-a	2000	0/5	Serious
	500	0/5	Not observed
VI-1-a	2000	0/5	Not observed
	500	0/5	Not observed
VII-1-a	200	0/5	Not observed
	500	0/5	Not observed
VIII-1-a	2000	0/5	Serious
IX-1-a	2000	5/5	Severe
	500	5/5	Severe

Each sample, suspended in 10% HCO-60 in saline, was administered perorally at a volume of 40 ml/kg.

The acute toxicities of I-1-a—IX-1-a were tested in mice. No death or toxic symptoms were observed in mice given I-1-a, III-1-a, IV-1-a or VI-1-a at any examined doses (*p.o.*) during the experimental periods (Table III). IX-1-a, however, showed serious toxicity and mortality was 5/5.

Discussion

We have reported that several sulfur-containing amino acids and amines, especially cystamine, showed strong gastric secretion inhibitory activity.[5] Further, we found that in a series of 2-(*E*-2-alkenoylamino)ethyl alkyl sulfides which were synthesized from cystamine and various *E*-2-unsaturated fatty acids, I-1-a showed the most potent anti-ulcerogenic activity.[5] Recently, it has been noted that sulfur-containing compounds show multi-pharmacological activities such as radio-preventive,[7] cytoprotective,[8] and anti-rheumatoid[9] effects. To elucidate the necessity of sulfide in I-1-a, compounds replacing the sulfur atom by methylene group or oxygen atom were synthesized and their anti-ulcerogenic activities were tested. I-2-a and I-3-a showed reduced pharmacological activity, but did not lose it. It seems that sulfide linkage is not essential but is more favorable than methylene linkage or ether linkage for showing the pharmacological activity, probably due to subtle differences of steric interactions among the atoms around the sulfur.

From the relationship studies between alkyl or alkenyl chain lengths and anti-ulcerogenic activity of various saturated and unsaturated fatty acids, we found *E*-2-decenoic acid to be the most effective.[4] Further, we discovered I-1-a to be the strongest anti-ulcerogenic compound in a series of hybrids which were synthesized from various *E*-2-unsaturated fatty acids and cystamine.[5] These results led us also to prepare analogues with modifications of unsaturation in the decenoyl chain. Saturation of the decenoyl chain resulted in some degree of potency loss. It seems likely that unsaturation relates to intestinal absorption rather than to action mechanism of

the compounds, because the saturated compound retains significant potency. There was no relationship between the position of unsaturation and the potency, while the configuration of unsaturation was important to the appearance of anti-ulcerogenic activity. Generally, most naturally occurring unsaturated fatty acids are *Z*-configuration. It is reported that many *Z*-configurated fatty acids in natural products change to *E*-type in processing to foods,[10] and that the *E*-type fatty acids are absorbed from the digestive tract and oxidized *in vivo* as well as *Z*-type fatty acids.[11] In the case of fatty acid alone, *Z*- and *E*-configurated fatty acids are absorbed to a similar extent. On the other hand, fatty acid moiety in the derivatives of I-1-a probably related to the absorpiton rate of the compounds, as indicated by the result that *E*-configuration was essential to the appearance of the anti-ulcerogenic activity.

I-1-a with carbamoylmethyl as S-alkyl group is insoluble in water and many organic solvents, indicating its low bioavailability *in vivo*. Attempts have been made to increase the potency of I-1-a by replacing the carbamoylmethyl group with appropriate S-alkyl groups. Two compounds replacing S-alkyl group, III-1-a and IX-1-a were selected for more being potent inhibitors of ulceration than I-1-a.

Since no toxic symptom was observed at any tested dose in mice, III-1-a may be practical and useful as an anti-ulceration drug, but this requires further studies on its action mechanism, metabolism and so on.

Acknowledgements We are grateful to Mr. Satoru Inoue and Mr. Tadashi Okano for their technical assistance.

References

1) T. Mimura, T. Terada, M. Iwai, I. Kohda, S. Take, K. Maeda, and S. Aonuma, *J. Pharmacobio-Dyn.*, **6**, 397 (1983).
2) T. Mimura, K. Tsujikawa, H. Nakajima, M. Okabe, Y. Kohama, M. Iwai, and K. Yokoyama, *J. Pharmacobio-Dyn.*, **9**, 46 (1986).
3) T. Mimura, H. Tsujibo, N. Muto, S. Otsuka, and S. Aonuma, *Chem. Pham. Bull.*, **28**, 1077 (1980).
4) T. Mimura, I. Kohda, K. Maeda, M. Iwai, Y. Sasaki, S. Aonuma, and T. Momose, *J. Pharmacobio-Dyn.*, **6**, 527 (1983).
5) M. Iwai, I. Kohda, C. Fukaya, Y. Naito, K. Yokoyama, H. Nakajima, K. Tsujikawa, M. Okabe, and T. Mimura, *Chem. Pharm. Bull.*, **35**, 4616 (1987).
6) K. Takagi and S. Okabe, *Jpn. J. Pharmacol.*, **18**, 9 (1968).
7) M. V. Vasin, B. I. Davydov, and V. V. Antipov, *Radiobiologiya*, **11**, 517 (1971).
8) P. Van Caneghem and M. L. Beaumariage, *Strahlentherapoe*, **145**, 663 (1973); S. Szabo, J. S. Treier, and P. W. Frankel, *Science*, **214**, 200 (1981).
9) J. T. Lamount, A. S. Ventola, E. A. Manll, and S. Szabo, *Gastroenterology*, **84**, 306 (1983); S. Szabo, *ibid.*, **87**, 228 (1984); H. Fujimura, Y. Hiramatsu, Y. Yanagihara, A. Koda, H. Nagai, K. Uda, T. Ito, and H. Yamauchi, *Nippon Yakurigaku Zasshi*, **76**, 117 (1980).
10) C. R. Scholfield, V. L. Davison, and H. J. Dutten, *J. Am. Oil Chem. Soc.*, **44**, 648 (1967).
11) F. Bickerstaffe and E. F. Annison, *Biochem. J.*, **118**, 433 (1970).

Constituents of the Roots of *Boerhaavia diffusa* L. III.[1] Identification of Ca^{2+} Channel Antagonistic Compound from the Methanol Extract

Nzunzu LAMI,[a] Shigetoshi KADOTA,[a] Tohru KIKUCHI,*[,a] and Yasunori MOMOSE[b]

Research Institute for Wakan-Yaku (Oriental Medicines)[a] and Faculty of Medicine,[b] Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan. *Received January 25, 1991*

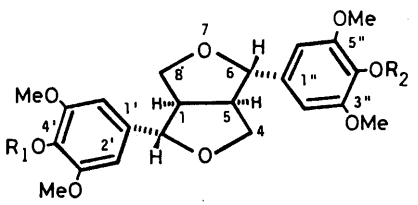
Two known lignans, liriodendrin and syringaresinol mono-β-D-glucoside, have been isolated from the methanol extract of the roots of *Boerhaavia diffusa* L. (Nyctaginaceae), and the former compound was found to exhibit a significant calcium (Ca^{2+}) channel antagonistic effect in frog heart single cells using the whole-cell voltage clamp method. Reexamination of the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra of these compounds was also carried out by the use of two-dimensional NMR techniques including a ^{1}H-detected heteronuclear multiple bond connectivity (HMBC) experiment, and it was found that the previous signal assignments for C-1′ and C-4′ have to be revised.

Keywords calcium channel antagonistic action; *Boerhaavia diffusa*; Nyctaginaceae; liriodendrin; syringaresinol mono-β-D-glucoside; HMBC

In previous papers,[1,2] we reported the isolation and structure determination of 3-*O*-(6′-palmitoyl-β-D-glucopyranosyl)sitosterol and three new rotenoids named boeravinones A, B, and C from the ether extract of the roots of *Boerhaavia diffusa* L. (Nyctaginaceae), which is used as a traditional medicine in Nepal, Sri Lanka, India, and East Africa. In a further study, we have examined the constituents of the methanol extract, which showed a calcium (Ca^{2+}) channel antagonistic activity[3] in frog heart single cells. This paper deals with the isolation and identification of two known lignans, liriodendrin (**1**) and syringaresinol mono-β-D-glucoside (**2**), and the Ca^{2+} channel antagonistic action of compound **1**.

The methanol extract of *B. diffusa*, obtained as described in a previous paper,[2] was separated roughly into the chloroform-soluble, butanol-soluble, ethanol-soluble, and ethanol-insoluble fractions (Chart 2). Among these fractions, three (not the ethanol-insoluble one) showed significant Ca^{2+} channel antagonistic effect in an electrophysiological bioassay.

The ethanol-soluble fraction was further separated by a combination of Iatrobeads column chromatography and preparative thin layer chromatography (TLC) to give compound **1** (**1**) along with glycerol and a complex mixture of sugars. On the other hand, the butanol-soluble fraction was extracted with 1.5% hydrochloric acid solution at room temperature and the insoluble material, which contained a mixture of phenolic compounds, was separated by filtration. Then the aqueous layer was treated as shown in Chart 2, giving an additional crop of compound **1** together with sucrose and a mixture of sugars. The chloroform-soluble fraction was also separated in a similar manner (Chart 2) to give compound **2** (**2**) together with mixtures of fatty acids and sterol glucosides.



1: $R_1 = R_2 = \beta$-D-glc
2: $R_1 = \beta$-D-glc, $R_2 = H$

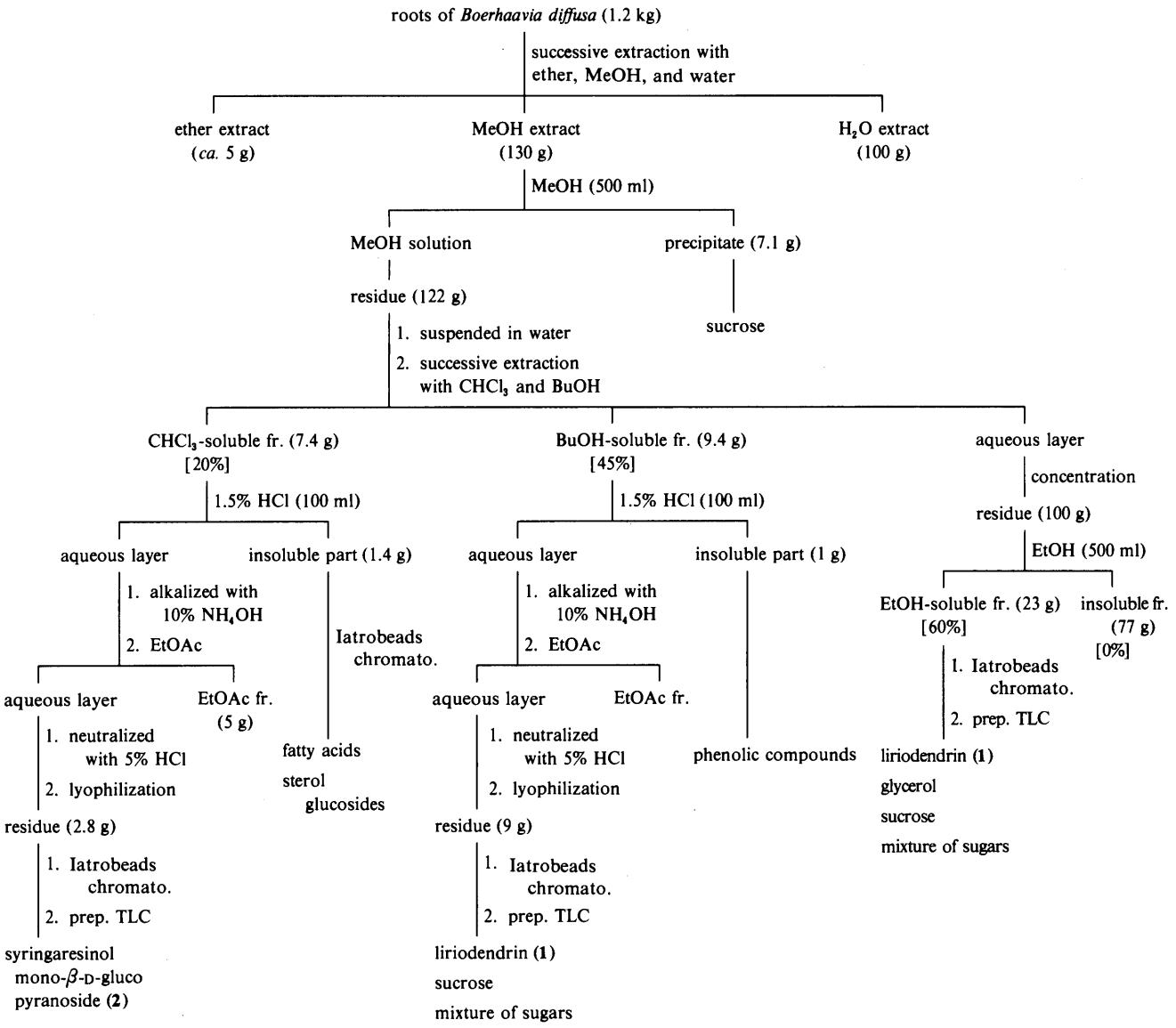
Chart 1

Compound **1** (**1**) was obtained as colorless needles, mp 256—259 °C, and showed $[\alpha]_D^{28}$ −13.5° (pyridine). It showed characteristic absorption bands at 272 and 281_{sh} nm (log ε: 3.35 and 3.27, respectively) in the ultraviolet (UV) spectrum, and a strong hydroxyl absorption at 3450 cm^{-1} and aromatic absorptions at 1597, 1510, and 1465 cm^{-1} in the infrared (IR) spectrum. The positive ion fast atom bombardment mass spectrum (FAB-MS) of **1** revealed the quasi-molecular ion peak at *m/z* 743 $[M+H]^+$, while the negative ion FAB-MS showed the $[M-H]^-$ peak at *m/z* 741. These MS data coupled with the combustion analysis data indicated the molecular formula of **1** to be $C_{34}H_{46}O_{18}$.

The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** gave a rather simple pattern, showing singlet signals due to methoxy methyl(s) and aromatic proton(s) at δ_H 3.76 and 6.65 ppm, respectively, along with signals in the 3.03—4.95 ppm region, which could be interpreted as being due to the protons of glucose and propane units based on the results of proton-proton shift correlation spectroscopy (^{1}H–^{1}H COSY) and ^{1}H–^{13}C COSY (Table I).

These spectral data suggested that compound **1** may be a lignan glucoside having a highly symmetric structure. Then, we measured the ^{1}H-detected heteronuclear multiple bond connectivity (HMBC)[4] spectrum to establish the connectivities of these benzene, glucose, and propane units. As can be seen in Fig. 1, the quaternary carbon at δ_C 133.7 (C-4′) showed long-range correlations with the protons at δ_H 6.65 (2′,6′-H) and 4.88 (G1-H), indicating the connection of the glucose moiety with the benzene ring at the C-4′ position. On the other hand, the quaternary carbon at δ_C 137.1 (C-1′) showed long-range correlations with the protons at δ_H 6.65 (2′,6′-H), 4.67 (2-H), and 3.08 (1-H), and the tertiary carbons at δ_C 104.6 (C-2′,6′) were correlated only with the proton at δ_H 4.67 (2-H). In turn, the carbon at δ_C 85.1 (C-2) showed correlations with 2′,6′-H (δ_H 6.65) and 8-H_2 (or 4-H_2) (δ_H 3.83 and 4.21). Further, the carbon at δ_C 152.6 (C-3′,5′) showed correlations with the methoxyl protons (δ_H 3.76) and with 2′,6′-H (δ_H 6.65). These data disclosed the connectivities of the propane unit and methoxy group(s) with the benzene ring at the C-1′ and C-3′,5′ positions.

Eventually, compound **1** was identified as liriodendrin (**1**),[5] a known symmetric lignan, by direct spectral com-



[ ]: numbers in brackets indicate the inhibition % of Ca^{2+} current at a concentration of 1 mg/ml

Chart 2

parisons with an authentic sample. It should be noted that the previous ^{13}C-signal assignments[5] for C-1′ (and 1″) and C-4′ (and 4″) were revised (Table I).

Compound **2** (**2**), is a minor constituent obtained as an amorphous solid, and showed $[\alpha]_D^{28}$ $-10°$ (MeOH). It gave UV and IR spectra similar to those of **1**. The positive ion FAB-MS of **2** showed the quasi-molecular ion peak at *m/z* 603 $[M+Na]^+$, corresponding to the molecular formula $C_{28}H_{36}O_{13}$, which differed by one glucose unit from that of **1**.

The ^1H- and ^{13}C-NMR spectra of **2**, analyzed with the aid of ^1H–^1H COSY, ^1H–^{13}C COSY, and HMBC, indicated the presence of one glucose, two benzene rings, two propane units and four methoxyl groups in the molecule (Table I), and the overall spectral patterns resembled those of **1**, except for the lack of signals corresponding to one glucose unit.

On the basis of the above spectral evidence, compound **2** was concluded to be syringaresinol mono-β-D-glucoside (**2**).[5c] The physico-chemical and spectral data were in good agreement with those published.[5c] Further, the previous assignments of ^{13}C-NMR signals for C-1′ and C-4′ were revised on the basis of the HMBC experimental result.[6]

Next, the Ca^{2+} channel antagonistic activity of liriodendrin (**1**) was examined. In frog atrium, a regenerative action potential can be obtained even after the transient inward sodium current has been inhibited completely by tetrodotoxin (TTX, 3×10^{-6} M). The peak height and the duration of the slow response are both modulated strongly by a transmembran calcium current which we designate I_{Ca}. Fig. 2 illustrates a control 'slow response', as well as a response obtained 15 min after bath application of liriodendrin (10^{-5} M). Liriodendrin produces a significant decrease in both the height and the duration of the slow response, but causes no measurable change in either the resting membrane potential (−90 mV) or the rate of negative repolarization of −70 mV. These findings suggest

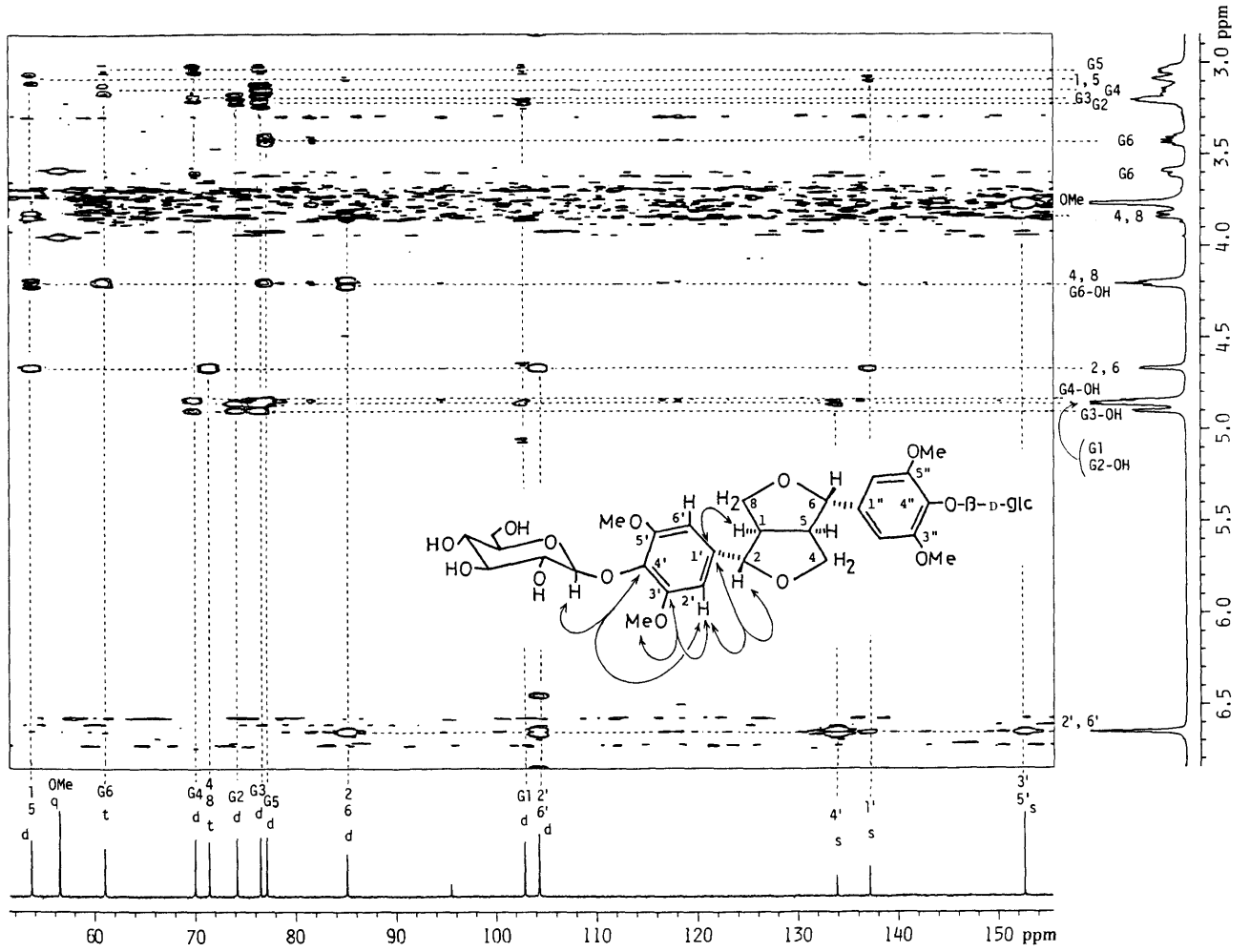


Fig. 1. HMBC Spectrum of Liriodendrin (**1**) in DMSO-d_6 (Sample 60 mg, J_{CH} = 8.3 Hz, 25 °C, 15 h Run)

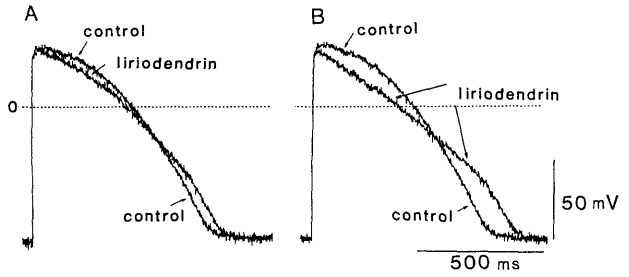


Fig. 2. Effects of Liriodendrin (**1**) on Slow Action Potentials in Bullfrog Atrial Cells

Slow action potentials were obtained by applying tetrodotoxin (3×10^{-6} M) to block the sodium channel current. The cell was stimulated at a frequency of 0.2 Hz. A: liriodendrin 10^{-5} M. B: liriodendrin 10^{-4} M.

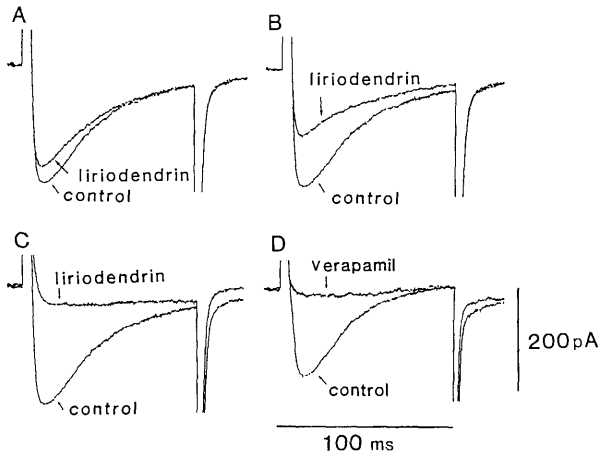


Fig. 3. Effects of Liriodendrin and Verapamil on Ca^{2+} Channel Currents in Bullfrog Atrial Single Cells

Na^+ channel current was blocked by tetrodotoxin at 3×10^{-6} M. Ca^{2+} current traces before and after 15 min application of liriodendrin at 10^{-6} M (A), 10^{-5} M (B), or 10^{-4} M (C) or verapamil at 10^{-6} M are superimposed. Holding potential was −90 mV. D is a different cell from A, B, and C.

that liriodendrin (**1**) reduces I_{Ca} quite selectively, but no conclusive information could be obtained at this stage.

To test further the mechanism of liriodendrin action(s) the whole-cell voltage clamp method[7] was employed. Fig. 3 shows the effect of liriodendrin (**1**) on the calcium current, I_{Ca}, which generates the slow response, in comparison with that of verapamil. In this experiment, the isolated atrial cell was voltage-clamped at its normal resting membrane potential (−90 mV), and a series of graded depolarizing or hyperpolarizing clamp steps, each 100 ms in duration, were applied. Liriodendrin (**1**) at 10^{-5} M markedly decreased the amplitude of I_{Ca} (approx. 40% of the control). However, it has no significant effect on the activation threshold or the apparent reversal potential for I_{Ca} (Fig. 3). These actions of liriodendrin (**1**) on I_{Ca} were dose-dependent and

TABLE I. 400 MHz ^{1}H- and 100 MHz ^{13}C-NMR Data for **1** and **2** in DMSO-d_6 (Coupling Constants in Parenthesis)

Position	1		2	
	δ_H	δ_C	δ_H	δ_C
1	3.08 m	53.6 d	3.08 m	53.6 d
2	4.67 d (4.0)	85.1 d	4.66 d (4.5)	85.0 d
4	4.21 dd (9.0, 6.0)	71.4 t	4.19[b] dd (6.0, 2.0)	71.1[c] t
	3.83 dd (9.0, 3.5)		3.79 m	
5			3.06 m	53.5 d
6	(same as those at the		4.61 d (4.5)	85.3 d
8	1-, 2-, and 4-positions)		4.17[b] dd (6.5, 2.0)	71.2[c] t
			3.79 m	
1′	—	137.1 s	—	137.1 s
2′,6′	6.65 s	104.6 d	6.65 s	104.1 d
3′,5′	—	152.6 s	—	152.6 s
4′	—	133.7 s	—	133.6 s
1″			—	131.3 s
2″,6″	(same as those at the		6.59 s	103.6 d
3″,5″	1′—6′-positions)		—	148.8 s
4″			—	134.9 s
G1	4.88 m	102.7 d	4.88 m	102.7 d
G2	3.20 m	74.1 d	3.20 m	74.1 d
G3	3.19 m	76.5 d	3.20 m	76.4 d
G4	3.14 m	69.9 d	3.16 m	69.8 d
G5	3.03 m	77.2 d	3.03 m	77.1 d
G6	3.41 dt (12.0, 5.5)	60.9 t	3.42 dd (12.0, 1.3)	60.8 t
	3.60 ddd (12.0, 5.5, 2.0)		3.57 dd (12.0, 5.0)	
3′,5′-OMe	3.76 s	56.4 q	3.76 s	56.4 q
3″,5″-OMe	3.76 s	56.4 q	3.75 s	55.9 q
G2-OH	4.94[a] d (5.0)			
G3-OH	4.95[a] d (5.0)			
G4-OH	4.89 m			
G6-OH	4.26 t (5.5)			

δ values in ppm and coupling constants in Hz. Multiplicities of carbon signals were determined by means of the DEPT method and are indicated as s, d, t, and q. *a*, *b*, and *c*) Assignments may be interchanged.

reversible.

It is now well known that Ca^{2+} channel antagonists act on cardiac muscles to cause a relaxation and a reduction of heart rate. Thus, some of them are used effectively for the treatment of cardiovascular diseases such as hypertension, ischemia, and arrhythmia. In recent years, there have been many reports concerning the screening of traditional herbal medicines for Ca^{2+} antagonistic activity.[8] Ichikawa *et al.*[8c] and Chen *et al.*[8e] reported that some lignans such as trachelogenin, pinoresinol, pinoresinol dimethyl ether, and fargesones A and B have a Ca^{2+} antagonistic activity, as determined by measuring the mechanical contraction of the taenia coli of the guinea pig. Only tetrandrine,[3,9] a bisbenzylisoquinoline alkaloid from a plant of *Stephania* species, and tanshinone,[3,10] a diterpene from a plant of *Salvia* species, have so far been reported as naturally occurring Ca^{2+} channel antagonists which have been studied by electrophysiological techniques. Apparently our present result provides the first example of a water-soluble lignan glucoside having a Ca^{2+} antagonistic action, as found by the electrophysiological method. It must be emphasized that liriodendrin (**1**) is a member of the fused tetrahydrofuran-type lignans, which are a very common class of lignans in the plant kingdom. Further studies on the activities of related lignans and lignan glucosides should be interesting.

Experimental

Melting points were determined with Kofler-type apparatus and are uncorrected. Optical rotations were measured in MeOH solutions on a JASCO DIP-140 digital polarimeter at 28 °C. UV spectra were taken with a Shimadzu 202 UV spectrometer in MeOH solutions and IR spectra with a JASCO IRA-2 or a Nicolet DX FT-IR spectrometer in KBr. ^{1}H-NMR and ^{13}C-NMR spectra were taken on a JEOL JNM-GX 400 spectrometer with tetramethylsilane as an internal standard. Chemical shifts are recorded in δ values and coupling constants in hertz (Hz). Multiplicities of ^{13}C-NMR signals were determined by means of the distortionless enhancement by polarization transfer (DEPT) method. ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY, and HMBC spectra were obtained with the JEOL standard pulse sequences and data processing was performed with JEOL standard software. FAB-MS was obtained with a JEOL SX-102 spectrometer (matrix: glycerol or *m*-nitrobenzyl alcohol). Column chromatography was done with Iatrobeads. Preparative TLC was carried out on Merck Kieselgel GF_{254} plates and the plates were examined under UV light. Extraction of substances from silica gel was done with MeOH–$CHCl_3$ (3 : 7) and solutions were concentrated *in vacuo*. TLC analyses were done on Merck Kieselgel GF_{254} plates and spots were detected by the use of 1% $Ce(SO_4)_2$–aqueous H_2SO_4 (10%) reagent. For drying organic solutions, anhydrous $MgSO_4$ was used.

Isolation of Liriodendrin (1) and Syringaresinol Mono-β-D-glucoside (2) The MeOH extract (130 g) obtained from the air dried roots of *B. diffusa* (1.2 kg), which was described in a previous paper,[2] was employed in this experiment. The extract was dissolved again in MeOH (about 500 ml) and the insoluble crystalline precipitate (7.1 g) was separated by filtration. This precipitate was identified as sucrose by direct ^{1}H-NMR comparison with an authentic sample. The MeOH filtrate was evaporated to dryness and the residue (122 g) was suspended in water (650 ml) and fractionated by successive extractions with $CHCl_3$ (500 ml × 3) and BuOH (500 ml × 4) to give the $CHCl_3$ fraction (7.5 g), BuOH fraction (9.4 g), and water fraction (*ca.* 100 g).

The water fraction was concentrated *in vacuo*. The residue (*ca.* 100 g) was dissolved in EtOH (500 ml) and the insoluble material (77 g) was separated by filtration. The EtOH solution was concentrated to dryness *in vacuo*, giving the EtOH-soluble fraction (23 g). This was subjected to column chromatography on Iatrobeads (250 g) and eluted successively with MeOH–$CHCl_3$ (1 : 2, 1 : 1, and 2 : 1; 1900, 760, and 650 ml, respectively), MeOH (700 ml), and MeOH–H_2O (8 : 1 and 8 : 3, 900 ml each). A fraction (1.2 g) eluted with MeOH–$CHCl_3$ (1 : 2) was triturated with MeOH to give liriodendrin (**1**) (24 mg). The mother liquor was further purified by preparative TLC with EtOAc–MeOH–H_2O (6 : 2 : 1) to give an additional crop of liriodendrin (**1**) (11 mg) and glycerol (500 mg). On the other hand, fractions eluted with MeOH–$CHCl_3$ (2 : 1 and 1 : 1) contained sucrose and a mixture of sugars, respectively.

The BuOH fraction (8.9 g) was extracted with 1.5% aqueous HCl (100 ml) at room temperature and the insoluble material (1 g), which contained a mixture of phenolic compounds,[11] was separated by filtration. The acidic aqueous solution was slightly alkalized by the addition of 10% NH_4OH and washed with EtOAc (200 ml × 3). The alkaline aqueous solution was then neutralized by the careful addition of 5% HCl and evaporated by lyophilization. The residue (9 g) was subjected to column chromatography on Iatrobeads (140 g) and eluted successively with EtOAc–MeOH–H_2O (6 : 2 : 1, 2500 ml), MeOH (600 ml), and MeOH–H_2O (1 : 1, 500 ml). A fraction (*ca.* 4 g) eluted with EtOAc–MeOH–H_2O (6 : 2 : 1) was again chromatographed on Iatrobeads (200 g) with EtOAc–MeOH–H_2O (6 : 2 : 0.5). Fractions which showed a dark spot on TLC, examined under UV light, were combined (*ca.* 290 mg) and further purified by preparative TLC with EtOAc–MeOH–H_2O (6 : 2 : 0.5) to give liriodendrin (**1**) (26 mg). On the other hand, fractions eluted with MeOH and MeOH–H_2O (1 : 1) afforded sucrose (1.8 g) and a mixture of sugars (*ca.* 4 g), respectively.

The $CHCl_3$ fraction (7.4 g) was also extracted with 1.5% HCl (100 ml) and the insoluble material (1.4 g), which contained a complex mixture of fatty acids and sterol glucosides, was removed by filtration. The acidic aqueous solution was slightly alkalized with 10% NH_4OH, then washed with EtOAc, and the aqueous layer was neutralized by the addition of 5% HCl. Lyophilization of this aqueous solution gave a residue (2.8 g), which was chromatographed on an Iatrobeads column (60 g). A fraction (150 mg) eluted with MeOH–EtOAc (1 : 5) was further purified by preparative TLC with EtOAc–MeOH–HCO_2H (20 : 10 : 1) to give syringaresinol mono-β-D-glucoside (**2**) (20 mg) as an amorphous solid.

Liriodendrin (**1**): White needles (from MeOH), mp 256—259 °C, $[\alpha]_D^{28}$ −13.5° (c=0.59, pyridine). IR ν_{max} cm^{-1}: 3450 (OH), 1597, 1510, 1465 (phenyl). UV λ_{max} nm (log ε): 272 (3.35) and 281$_{sh}$ (3.27). ^{1}H- and ^{13}C-NMR: Table I. Positive ion FAB-MS *m/z*: 743 $[M+H]^+$; negative

ion FAB-MS *m/z*: 741 $[M-H]^-$. *Anal.* Calcd for $C_{34}H_{46}O_{18}$: C, 54.98; H, 6.24. Found C, 54.66; H, 6.00.

Syringaresinol Mono-β-D-glucoside (2): Amorphous solid, $[\alpha]_D^{28}$ −10° (c=1.4, MeOH). IR ν_{max} cm^{-1}: 3350 (OH), 1590, 1510, 1460 (phenyl). UV λ_{max} nm (log ε): 270 (3.02). ^{1}H- and ^{13}C-NMR: Table I. Positive ion FAB-MS *m/z*: 603 $[M+Na]^+$.

Assay of Ca^{2+} Current Inhibitory Activity Cell Dispersion: Adult bullfrogs (*Rana catesbeiana*) were pithed and their hearts were removed and transferred to a dissecting dish containing standard Ringer's solution. The right atrium was separated and several large pieces of tissue were cut off and placed in a second dish containing calcium-free Ringer's solution. Single atrial cells were obtained by using an enzymatic dispersion procedure (0.15% collagenase, 0.1% bovine pancreatic trypsin, and 0.1% bovine albumin) as described by Hume and Giles.[12] The cell suspension was taken into a chamber (1 ml) on the stage of an inverted bipolar phase-contrast microscope (Olympus, IMt-2, Tokyo, Japan). After the single cells had settled at the bottom of the chamber, the frog Ringer's solution was perfused with 95% O_2 and 5% CO_2 mixed gas. Ringer's solution had the following composition (mM): NaCl, 90.6; $NaHCO_3$, 20; KCl, 2.5; $MgCl_2$, 5.0; $CaCl_2$, 2.5; and glucose, 10 (pH 7.4, adjusted with NaOH). The temperature of the perfusate in the experimental chamber was maintained at 20—22 °C, and perfusion was started at a rate of 4 ml/min.

Electrophysiology: The membrane potentials were measured by using a conventional current clamp method. A glass microelectrode (Radnoti Glass Technology, CA, U.S.A.) was constructed with a two-stage vertical microelectrode puller (Narishige, PP-83, Tokyo, Japan). The electrode resistance was 3—5 megohm when filled with 150 mM KCl. A single cell was impaled by a glass microelectrode by suction, after positioning by using a hydraulic micromanipulator (Narishige, MO-102, Tokyo, Japan). The potential difference between the intracellular microelectrode and a bath reference electrode was measured using a microelectrode amplifier (Nihon Kohden, MEZ-8201, Tokyo, Japan). An action potential was elicited by an intracellular stimulus (15—20 nA, 1 ms, 0.2 Hz). The current pulse was controlled by an electronic stimulator (Nihon Kohden, SEN-7203, Tokyo, Japan).

The whole-cell voltage clamp technique[7] was employed to measure the transmembran Ca^{2+} current through Ca^{2+} channels in frog atrial single cells. A patch amplifier (Yale MK V, CT, USA) was used to record the whole cell membrane currents. Data were displayed on a storage oscilloscope (Cos 5020-ST, Kikusui, Tokyo, Japan) and simultaneously recorded on a PCM data recording system (RP-880, NF Electronic Instruments, Tokyo, Japan) at a bandwidth of DC to 10 kHz.

Drugs and Solutions: The drugs used were as follows: collagenase (Sigma), trypsin (Sigma), albumin (Sigma), and tetrodotoxin (Sankyo). Liriodendrin and drugs were applied by changing the perfusion solution to one which contained the substance at a desired concentration.

Acknowledgements We are grateful to Dr. N. Matsukura of Jujo-seishi Co., Ltd., for a gift of authentic liriodendrin. Thanks are also due to Mr. K. Tanaka of the National Research Institute of Police Science for FAB-MS measurements. This work was supported in part by a Grant-in-Aid for Overseas Scientific Survey (No. 58041031) and a Grant-in-Aid for Scientific Research (No. 01571145) from the Ministry of Education, Science and Culture of Japan. One of the authors (N.L.) is grateful to the Japanese Government for a scholarship.

References and Notes

1) Part II: N. Lami, S. Kadota, Y. Tezuka, and T. Kikuchi, *Chem. Pharm. Bull.*, **38**, 1558 (1990).
2) S. Kadota, N. Lami, Y. Tezuka, and T. Kikuchi, *Chem. Pharm. Bull.*, **37**, 3214 (1989).
3) W. G. Nayler, "Calcium Antagonists," Academic Press, London, 1988.
4) *a*) A. Bax and M. F. Summers, *J. Am. Chem. Soc.*, **108**, 2093 (1986); *b*) A. Bax, A. Aszalos, Z. Dinya, and K. Sudo, *ibid.*, **108**, 8056 (1986).
5) *a*) D. J. Shivanand, J. J. Hoffman, J. R. Cole, M. S. Tempesta, and R. B. Bates, *J. Org. Chem.*, **45**, 1327 (1980); *b*) T. Deyama, *Chem. Pharm. Bull.*, **31**, 2993 (1983); *c*) H. Kobayashi, H. Karasawa, T. Miyase, and S. Fukushima, *ibid.*, **33**, 1452 (1985).
6) Revised assignments are consistent with the empirical glycosylation shifts in phenolic compounds, which are reported to be 0.2—2.0 ppm upfield at the *ipso* and *ortho* carbons and 3.0—5.0 ppm downfield at the *para* carbon. See P. K. Agrawal (ed.), "Carbon-13 NMR of Flavonoids," Elsevier Science Publishers B. V., Amsterdam, 1989, p. 293.
7) O. P. Hamill, A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth, *Pflugers. Arch.*, **391**, 85 (1981).
8) *a*) T. Kozawa, K. Sakai, M. Uchida, T. Okuyama, and S. Shibata, *J. Pharm. Pharmacol.*, **33**, 317 (1981); *b*) J. Yamahara, S. Miki, H. Murakami, T. Sawada, and H. Fujimura, *Yakugaku Zasshi*, **105**, 449 (1985); *c*) K. Ichikawa, T. Kinoshita, S. Nishibe, and U. Sankawa, *Chem. Pharm. Bull.*, **34**, 3514 (1986); *d*) J. Yamahara, S. Miki, H. Matsuda, and H. Fujimura, *Yakugaku Zasshi*, **106**, 888 (1986); *e*) C. C. Chen. Y. L. Huang, H. T. Chen, Y. P. Chen, and H. Y. Hsu, *Planta Medica*, 438 (1988); *f*) K. Ichikawa, T. Kinoshita, and U. Sankawa, *Chem. Pharm. Bull.*, **37**, 345 (1989).
9) D. Fang and M. Jiang, *J. Hypertens.*, **4** (suppl. 6), S150 (1986).
10) L. Patmore and R. L. Whiting, *British J. Pharmacol.*, **75**, 149 (1982).
11) Separation and structure elucidation of the phenolic compounds will be reported in a forthcoming paper.
12) J. Hume and W. Giles, *J. Gen. Physiol.*, **78**, 19 (1981).

Imidazo[1,2-*a*]pyridines. I. Synthesis and Inotropic Activity of New 5-Imidazo[1,2-*a*]pyridinyl-2(1*H*)-pyridinone Derivatives[1)]

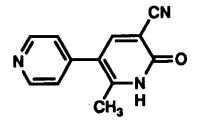
Motosuke YAMANAKA,* Kazutoshi MIYAKE, Shinji SUDA, Hideto OHHARA and Toshiaki OGAWA

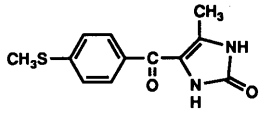
Eisai Tsukuba Research Laboratories, 5-1-3, Tokoudai, Tsukuba, Ibaraki 300-26, Japan. *Received December 21, 1990*

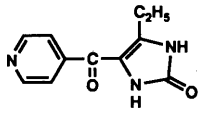
A series of 1,2-dihydro-5-imidazo[1,2-*a*]pyridinyl-2(1*H*)-pyridonones was synthesized and evaluated for positive inotropic activity. 1,2-Dihydro-5-imidazo[1,2-*a*]pyridin-6-yl-6-methyl-2-oxo-3-pyridinecarbonitrile (11a) hydrochloride monohydrate (E-1020) was found to be a potent and selective inhibitor of phosphodiesterase III and a long-acting, potent, orally active positive inotropic agent. Additional imidazo[1,2-*a*]pyridin-2-yl (3a), -3-yl (16), -7-yl (20) and -8-yl (24a) compounds were also prepared. Altering the pyridine substitution from the 2-position to the 6-position produced a 2-fold increase in the i.v. cardiotonic potency (ED_{50}) from 52 to 23 μg/kg, while substitution at the 3-, 7- or 8-position reduced potency. In the 2-positional isomers, introduction of halogen groups enhanced the activity and 3-chloro-1,2-dihydro-5-(6-fluoroimidazo[1,2-*a*]pyridin-2-yl)-6-methyl-2(1*H*)-pyridinone (3u) was the most potent (i.v. ED_{50} 11 μg/kg) in this series. E-1020 is presently under development for the treatment of congestive heart failure.

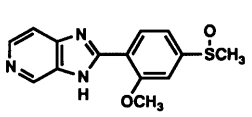
Keywords cardiotonic agent; positive inotropic activity; imidazo[1,2-*a*]pyridine; 5-imidazo[1,2-*a*]pyridinyl-2(1*H*)-pyridinone; structure–activity relationship; phosphodiesterase III inhibitor

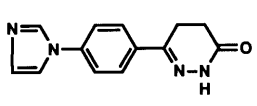
Congestive heart failure (CHF) is a major cause of death in patients with coronary artery disease. For 200 years, digitalis glycosides have been used for the treatment of CHF.[2,3)] Their use, however, is limited by their narrow therapeutic index and their propensity to cause life-threatening arrhythmias. Oral ineffectiveness and chronotropic liability prevent the use of sympathomimetic amines, dobutamine and dopamine, in chronic therapy of CHF. Therapy with vasodilators has been found to be effective in reducing the workload of the heart. There is now clinical and experimental evidence which demonstrates the advantages of combining positive inotropic stimulation with vasodilating activity to achieve maximum improvement in cardiac performance.[4,5)] Since inotropic agents increase myocardial oxygen consumption, whereas vasodilators enhance fiber shortening without changing or actually decreasing oxygen demand, these two activities may have additive effects on cardiac output.

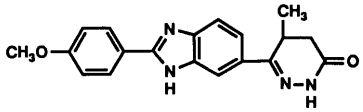

milrinone


enoximone


piroximone


isomazole


imazodan


pimobemdan

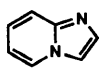

I

Chart 1

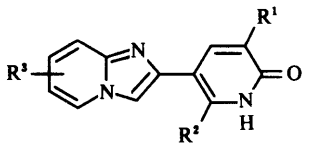
Recently several orally effective cardiotonic agents, milrinone,[6)] enoximone,[7)] piroximone,[8)] isomazole,[9)] imazodan[10)] and pimobendan,[11)] have been described as possessing these activities and some of them are at present being subjected to clinical evaluation for the treatment of CHF (Chart 1). Mechanistically, these drugs appear to drive their inotropic and vasodilator effects, at least in part, from selective inhibition of cyclic adenosine monophosphate (AMP) specific phosphodiesterase (PDE III) activity resulting in an increase in cellular cyclic AMP level.[12)] PDE I catalyzes the hydrolysis of cyclic AMP and cyclic guanosine monophosphate (GMP), but it has not been reported that selective PDE I inhibitors exert inotropic activity. Although there are a few PDE II inhibitors having cardiotonic activity, it has not been established that such activity is due to their inhibitory effects on cardiac PDE II. The nonselective "first generation" phosphodiesterase inhibitor like theophylline produces inotropic activity with a number of side effects, including tachycardia, tremor and increased rate of respiration.[13)]

The considerable therapeutic need prompted us to search for a potent, safe and orally effective agent which has these dual activities and is efficacious for a long period. Stimulated by some similarity in the structures of the agents described above, we focused our interest on imidazo[1,2-*a*]pyridine (I, Chart 1). This was thought to be a fused structure of the imidazole and the phenyl ring in imazodan holding one nitrogen in common, and the nitrogen at 1-position of I was thought to be the one derived from the pyridine in milrinone and piroximone. I also maintains a fused imidazole structure in isomazole and pimobendan. Connection of I at certain positions with the right residues of the agents in Chart 1 was expected to elicit positive inotropic activity. We report here the synthesis and inotropic activities of a novel class of 5-imidazo[1,2-*a*]pyridin-6-yl-2(1*H*)-pyridinones and their regioisomers.

Chemistry

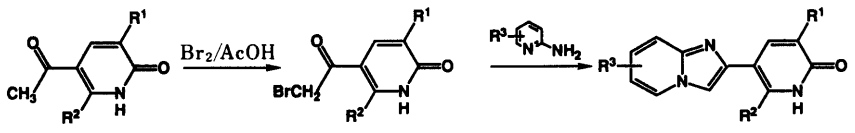
The compounds used in this study were prepared by different routes, depending on the site of 2-pyridinone ring substitution. The synthesis of 2-positional isomers,

TABLE I. 5-Imidazo[1,2-*a*]pyridinones (**3a—u**)



Compd.	R^1	R^2	R^3	mp (°C) (Solvent[a])	Yield[b] (%)	Formula	Analysis (%) Calcd (Found)		
							C	H	N
3a	CN	CH_3	H	>300 (A)	78	$C_{14}H_{10}N_4O$	67.18 (67.19)	4.04 4.00	22.39 22.57)
3b	CN	C_2H_5	H	275 (dec.) (B)	73	$C_{15}H_{12}N_4O$	68.16 (68.08)	4.59 4.49	21.20 21.19)
3c	CN	CH_3	6-CH_3	>290 (B)	71	$C_{15}H_{12}N_4C$ ·HCl	59.90 (59.74)	4.37 4.58	18.68 18.28)
3d	CN	C_2H_5	6-CH_3	278—280 (C)	65	$C_{16}H_{14}N_4O$ ·1/2H_2O	66.88 (67.28)	5.27 5.02	19.50 19.60)
3e	CN	CH_3	6-F	>300 (B)	42	$C_{14}H_9FN_4O$ ·HCl	62.68 (62.48)	3.39 3.41	20.89 20.67)
3f	CN	CH_3	8-F	>300 (B)	50	$C_{14}H_9FN_4O$ ·2/3H_2O	59.99 (59.76)	3.72 3.52	20.00 20.03)
3g	CN	CH_3	6-CN	>300 (D)	36	$C_{15}H_9N_5O$ ·2/3H_2O	62.71 (62.91)	3.52 3.52	24.38 24.47)
3h	CN	C_2H_5	6-CN	>300 (D)	59	$C_{16}H_{11}N_5O$	66.42 (66.54)	3.84 3.97	24.21 24.43)
3i	CN	CH_3	6-OCH_3	>300 (B)	42	$C_{15}H_{12}N_4O_2$ ·1.6H_2O	58.28 (58.68)	4.96 4.91	18.13 17.79)
3j	CN	CH_3	6-CF_3	>300 (B)	46	$C_{15}H_9F_3N_4O$	56.60 (56.56)	2.86 3.08	17.61 17.88)
3k	H	CH_3	H	>300 (dec.) (B′)	67	$C_{13}H_{11}N_3O$ ·HCl·1/3H_2O	58.31 (58.36)	4.79 4.66	15.70 15.86)
3l	H	CH_3	6-CH_3	>290 (E)	55	$C_{14}H_{13}N_3O$ ·HCl·2/3H_2O	58.42 (58.66)	5.36 5.12	14.60 14.87)
3m	H	CH_3	6-F	>300 (F)	24	$C_{13}H_{10}FN_3O$ ·HBr	45.63 (45.62)	3.84 3.86	12.28 12.23)
3n	Br	CH_3	H	>290 (B′)	66	$C_{13}H_{10}BrN_3O$ ·HCl	45.82 (45.57)	3.26 3.26	12.33 12.24)
3o	Br	CH_3	6-CH_3	>290 (dec.) (B)	67	$C_{14}H_{12}BrN_3O$ ·HCl	47.39 (46.99)	3.70 3.69	11.85 11.81)
3p	Br	CH_3	6-Cl	>280 (dec.) (B′)	64	$C_{13}H_9BrClN_3O$ ·HCl	41.61 (41.61)	2.69 2.80	11.20 11.02)
3q	Br	CH_3	6-F	289—290 (D)	35	$C_{13}H_9BrFN_3O$	48.45 (48.24)	2.82 2.80	13.04 12.75)
3r	Br	CH_3	6-CN	>300 (D)	41	$C_{14}H_9BrN_4O$ ·2/5H_2O	49.98 (50.31)	2.94 2.88	16.66 16.30)
3s	Br	CH_3	6-CF_3	>300 (F)	56	$C_{14}H_9BrF_3N_3O$	45.17 (45.36)	2.44 2.52	11.29 11.01)
3t	Cl	CH_3	H	296—297 (E)	51	$C_{13}H_{10}ClN_3O$	60.11 (60.03)	3.89 3.95	16.18 16.03)
3u	Cl	CH_3	6-F	305—307 (E)	26	$C_{13}H_9ClFN_3O$	56.21 (56.30)	3.27 3.38	15.13 15.13)

a) Recrystallization solvents: A, only filtration; B, DMF; B′, DMF–HCl–EtOH; C, methyl ethyl ketone–MeOH; D, DMF–H_2O; E, MeOH; F, DMF–MeCN. *b*) Not optimized.



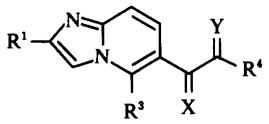
1a—e **2a—e** **3a—u**

a : R^1=CN, R^2=CH_3 **b** : R^1=CN, R^2=C_2H_5 **c** : R^1=H, R^2=CH_3 **d** : R^1=Br, R^2=CH_3 **e** : R^1=Cl, R^2=CH_3

Chart 2

5-imidazo[1,2-*a*]pyridin-2-yl-2(1*H*)-pyridinones **3a—u**[14] (Table I), was accomplished by condensation of requisite 5-bromoacetyl-1-pyridinones **2a—e**, which were obtained by bromination[15] of 5-acetyl-2-pyridinones **1a—e**, with 2-aminopyridines (Chart 2). 5-Acetyl-3-cyano-6-methyl-2-pyridinone **1a** was prepared according to the procedure in

TABLE II. 6-Propyl and Butylimidazo[1,2-*a*]pyridines



Compd.	R^1	R^3	R^4	X	Y	mp (or bp)[a] (°C)	Yield[b] (%)	^{1}H-NMR ($CDCl_3$) δ (ppm)
8a	H	H	CH_3	H_2	CH_2	(118—122[c])	71	1.70 (3H, s, CH_3), 3.28 (2H, s, CH_2), 4.80 (1H, d, J=1 Hz, =CH), 4.90 (1H, d, J=1 Hz, =CH), 7.02 (1H, dd, J=2, 9 Hz, 7-H), 7.52 (1H, d, J=1 Hz, 3-H), 7.56 (1H, d, J=9 Hz, 8-H), 7.58 (1H, d, J=1 Hz, 2-H), 7.92 (1H, br s, 5-H)
8b	CH_3	H	CH_3	H_2	CH_2	Oil	82	1.70 (3H, s, CH_3), 2.44 (3H, s, CH_3), 3.25 (2H, s, CH_2), 4.78 (1H, s, =CH), 4.87 (1H, s, =CH), 6.97 (1H, dd, J=2, 9 Hz, 7-H), 7.28 (1H, s, 3-H), 7.44 (1H, d, J=9 Hz, 8-H), 7.84 (1H, br s, 5-H)
8c	H	CH_3	CH_3	H_2	CH_2	Oil	66	1.74 (3H, s, CH_3), 2.50 (3H, s, CH_3), 3.34 (2H, s, CH_2), 4.56 (1H, s, =CH), 4.82 (1H, s, =CH), 7.02 (1H, d, J=10 Hz, 7-H), 7.46 (1H, d, J=1 Hz, 3-H), 7.50 (1H, d, J=10 Hz, 8-H) 7.66 (1H, J=1 Hz, 2-H)
8d	CH_3OCH_2	H	CH_3	H_2	CH_2	Oil	80	1.70 (3H, s, CH_3), 3.26 (2H, s, CH_2), 3.48 (3H, s, OCH_3), 4.61 (2H, s, OCH_2), 4.76 (1H, s, =CH), 4.86 (1H, s, =CH), 6.99 (1H, dd, J=2, 9 Hz, 7-H), 7.46 (1H, d, J=9 Hz, 8-H), 7.48 (1H, s, 3-H), 7.86 (1H, br s, 5-H)
8e	ph	H	CH_3	H_2	CH_2	125—126	17	1.70 (3H, s, CH_3), 3.27 (2H, s, CH_2), 4.78 (1H, s, =CH), 4.87 (1H, s, =CH), 7.01 (1H, dd, J=2, 9 Hz, 7-H), 7.20—7.52 and 7.78—7.98 (5H, m, C_6H_5), 7.53 (1H, d, J=9 Hz, 8-H), 7.77 (1H, s, 3-H), 7.96 (1H, d, br s, 5-H)
8f	H	H	C_2H_5	H_2	CH_2	(120—124[d])	32	1.04 (3H, t, J=7 Hz, CH_3), 2.00 (2H, q, J=7 Hz, CH_2), 3.29 (2H, s, CH_2), 4.76 (1H, d, J=1 Hz, =CH), 4.88 (1H, d, J=1 Hz, =CH), 6.98 (1H, dd, J=2, 9 Hz, 7-H), 7.50 (1H, s, 3-H), 7.52 (1H, d, J=9 Hz, 8-H), 7.56 (1H, s, 2-H), 7.90 (1H, br s, 5-H)
8g	CH_3	H	C_2H_5	H_2	CH_2	Oil	39	1.02 (3H, t, J=7 Hz, CH_3), 1.99 (2H, q, J=7 Hz, CH_2), 2.38 (3H, s, CH_3), 3.25 (2H, s, CH_2), 4.71 (1H, s, =CH), 4.86 (1H, s, =CH), 6.92 (1H, dd, J=2, 9 Hz, 7-H), 7.24 (1H, s, 3-H), 7.39 (1H, d, J=9 Hz, 8-H), 7.80 (1H, br s, 5-H)
9a	H	H	CH_3	H_2	O	(155—159[e])	71	2.24 (3H, s, CH_3), 3.70 (2H, s, CH_2), 6.95 (1H, dd, J=2, 9 Hz), 7.56 (1H, s, 3-H), 7.60 (1H, d, J=9 Hz, 8-H), 7.64 (1H, s, 2-H), 8.03 (1H, br s, 5-H)
9b	CH_3	H	CH_3	H_2	O	60—61	74	2.22 (3H, s, CH_3), 2.44 (3H, s, CH_3), 3.66 (2H, s, CH_2), 6.94 (1H, dd, J=2, 9 Hz, 7-H), 7.30 (1H, s, 3-H), 7.47 (1H, d, J=9 Hz, 8-H), 7.93 (1H, br s, 5-H)
9c	H	CH_3	CH_3	H_2	O	73—75	64	2.12 (3H, s, CH_3), 2.44 (3H, s, CH_3), 3.71 (2H, s, CH_2), 6.92 (1H, d, J=9 Hz, 7-H), 7.42 (1H, d, J=1 Hz, 3-H), 7.46 (1H, d, J=9 Hz, 8-H), 7.61 (1H, d, J=1 Hz, 2-H)
9d	CH_3OCH_2	H	CH_3	H_2	O	80—81.5	38	2.24 (3H, s, CH_3), 3.48 (3H, s, OCH_3), 3.68 (2H, s, CH_2), 4.60 (2H, s, OCH_2), 6.94 (1H, dd, J=2, 9 Hz, 7-H), 7.48 (1H, d, J=9 Hz, 8-H), 7.50 (1H, s, 3-H), 7.90 (1H, br s, 5-H)
9e	ph	H	CH_3	H_2	O	144—147	74	2.24 (3H, s, CH_3), 3.68 (2H, s, CH_2), 6.96 (1H, dd, J=2, 9 Hz, 7-H), 7.24—7.55 and 7.82—8.10 (6H, m, C_6H_5 and 5-H), 7.58 (1H, d, J=9 Hz, 8-H), 7.80 (1H, s, 3-H)
9f	H	H	C_2H_5	H_2	O	Oil	40	1.06 (3H, t, J=7 Hz, CH_3), 2.52 (2H, q, J=7 Hz, CH_2), 3.64 (2H, s, CH_2), 6.94 (1H, dd, J=2, 9 Hz, 7-H), 7.50 (1H, s, 3-H), 7.53 (1H, d, J=9 Hz, 8-H), 7.56 (1H, s, 2-H), 7.98 (1H, br s, 5-H)
9g	CH_3	H	C_2H_5	H_2	O	Oil	60	0.94 (3H, t, J=7 Hz, CH_3), 2.30 (3H, s, CH_3), 2.40 (2H, q, J=7 Hz, CH_2), 3.48 (2H, s, CH_2), 6.76 (1H, dd, J=2, 9 Hz, 7-H), 7.12 (1H, s, 3-H), 7.30 (1H, d, J=9 Hz, 8-H), 7.76 (1H, br s, 5-H)
10a	H	H	CH_3	$CHN(CH_3)_2$	O	177—178	75	2.04 (3H, s, CH_3), 2.80 (6H, s, $N(CH_3)_2$), 7.03 (1H, dd, J=2, 9 Hz, 7-H), 7.55 (1H, s, 3-H), 7.57 (1H, d, J=9 Hz, 8-H), 7.63 (2H, s, 2-H and =CH), 7.94 (1H, br s, 5-H)
10b	CH_3	H	CH_3	$CHN(CH_3)_2$	O	210—219	68	2.02 (3H, s, CH_3), 2.78 (6H, s, $N(CH_3)_2$), 6.98 (1H, dd, J=2, 9 Hz, 7-H), 7.32 (1H, s, 3-H), 7.48 (1H, d, J=9 Hz, 8-H), 7.66 (1H, s, =CH), 7.87 (1H, J=2 Hz, 5-H)
10c	H	CH_3	CH_3	$CHN(CH_3)_2$	O	122—123	83	1.95 (3H, s, CH_3), 2.48 (3H, s, CH_3), 2.74 (6H, s, $N(CH_3)_2$), 7.04 (1H, d, J=10 Hz, 7-H), 7.47 (1H, s, 3-H), 7.52 (1H, d, J=10 Hz, 8-H), 7.68 (2H, s, 2-H and =CH)
10d	CH_3OCH_2	H	CH_3	$CHN(CH_3)_2$	O	163—165	62	2.03 (3H, s, CH_3), 2.78 (6H, s, $N(CH_3)_2$), 3.48 (3H, s, OCH_3), 4.60 (2H, s, OCH_2), 6.98 (1H, dd, J=2, 9 Hz, 7-H), 7.46 (1H, d, J=10 Hz, 8-H), 7.50 (1H, s, 3-H), 7.60 (1H, s, =CH), 7.86 (1H, br s, 5-H)

TABLE II. (continued)

Compd.	R^1	R^3	R^4	X	Y	mp (or bp)[a] (°C)	Yield[b] (%)	^{1}H-NMR ($CDCl_3$) δ (ppm)
10e	ph	H	CH_3	$CHN(CH_3)_2$	O	>253 (dec.)	60	2.05 (3H, s, CH_3), 2.76 (6H, s, $N(CH_3)_2$), 6.97 (1H, dd, J=2, 9 Hz, 7-H), 7.20—7.55 and 7.78—8.01 (6H, m, 8-H and C_6H_5), 7.76 (1H, s, 3-H), 8.18 (1H, s, =CH), 8.28 (1H, brs, 5-H)
10f	H	H	C_2H_5	$CHN(CH_3)_2$	O	114—115	69	1.01 (3H, t, J=7 Hz, CH_3), 2.28 (2H, q, J=7 Hz, CH_2), 2.77 (6H, s, $N(CH_3)_2$), 7.00 (1H, dd, J=2, 9 Hz, 7-H), 7.52 (1H, d, J=1 Hz, 3-H), 7.62 (1H, d, J=1 Hz, 2-H), 7.64 (1H, s, =CH), 9.92 (1H, brs, 5-H)
10g	CH_3	H	C_2H_5	$CHN(CH_3)_2$	O	130—131	52	0.92 (3H, t, J=7 Hz, CH_3), 2.21 (2H, q, J=7 Hz, CH_2), 2.36 (3H, s, CH_3), 2.70 (6H, s, $N(CH_3)_2$), 6.90 (1H, dd, J=2, 9 Hz, 7-H), 7.24 (1H, s, 3-H), 7.36 (1H, d, J=9 Hz, 8-H), 7.56 (1H, s, =CH), 7.80 (1H, brs, 5-H)

a) Purified by column chromatography on silica gel excluding **8a**, **f** and **9a**. *b*) Not optimized. *c*) Boiling point under 0.5 mmHg. *d*) Boiling point under 0.3 mmHg. *e*) Boiling point under 0.4 mmHg.

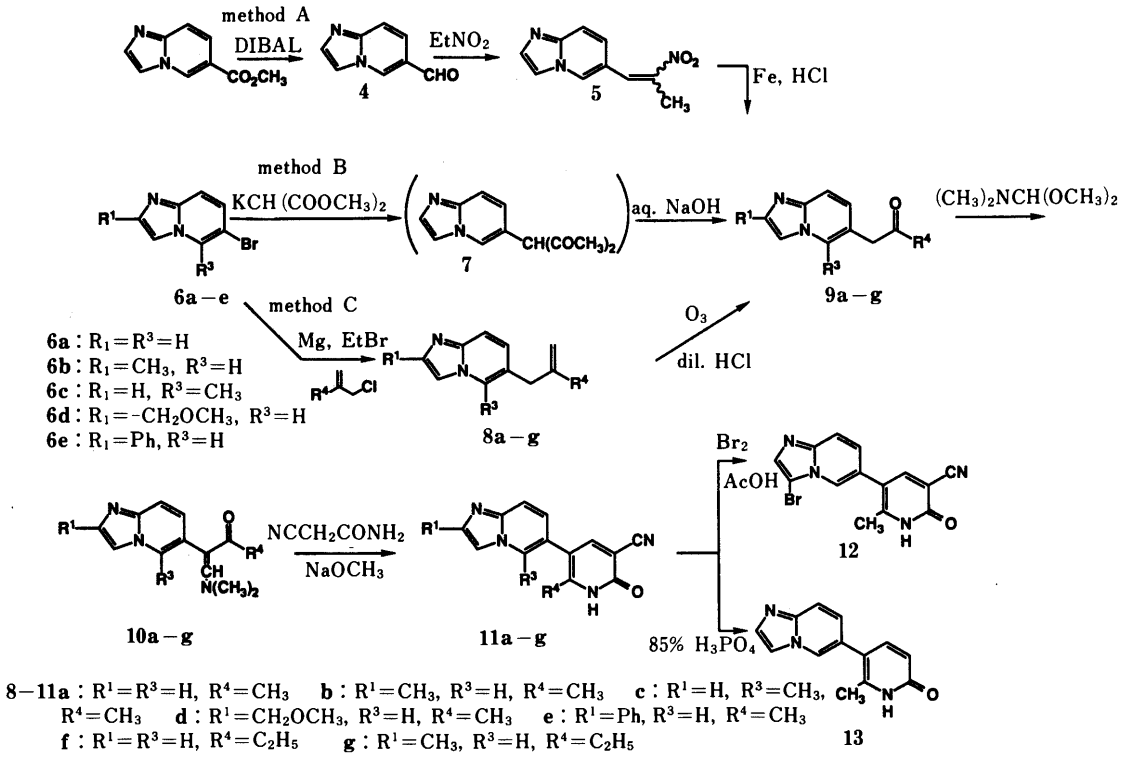


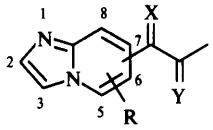
Chart 3

the literature.[16] 6-Ethyl derivative **1b** was also prepared in the same manner, but the purification of **1b** was not successful. Therefore crude **1b** was brominated without further purification to **2b**, and then purified. 5-Acetyl-2-pyridinone **1c** was obtained by treatment of **1a** with 50% H_2SO_4 followed by heating in Dowtherm A instead of using another procedure reported by Kato *et al.*[17] 5-Acetyl-3-bromo and 3-chloro-2-pyridinone **1d—e** were obtained by treatment of **1c** with 48% HBr and conc. HCl in the presence of hydrogen peroxide in 50 and 9.6% yield, respectively.

Formation of 2-pyridinone ring was carried out at the last step in the synthesis of other positional isomers; namely, 3-, 6-, 7- and 8-positional isomers (Tables IV and V) were prepared *via* the key intermediates, imidazo[1,2-*a*]pyridinyl-2-propanones.

For the synthesis of 6-positional isomers, three methods were examined (Chart 3). The first involved condensation of imidazo[1,2-*a*]pyridine-6-carboxaldehyde **4** with nitroethane and successive reduction of 6-(2-nitro-1-propenyl)-imidazo[1,2-*a*]pyridine **5** with iron powder in hydrochloric acid and EtOH (method A). The second was the reaction of 6-bromoimidazo[1,2-*a*]pyridine **6a**[18] with potassium acetoacetonate in the presence of cuprous iodide followed by treatment with aq NaOH (method B). These methods afforded imidazo[1,2-*a*]pyridin-6-yl-2-propanone **9a** in 6.1 and 37.6% yield, respectively, but were not efficient for the preparation of other 6-yl derivatives. The third method was therefore explored. After conversion of **6a** by the Grignard cross coupling reaction with 3-chloro-2-methylpropene to 6-isobutenylimidazo[1,2-*a*]pyridine **8a**, ozonolysis of **8a** under acidic conditions provided **9a** in 50% yield from **6a** (method C). Other 6-yl derivatives **9b—g** (Table II) were readily prepared according to method C. Conversion of the ketones **9a—g** to the pyridinones **11a—g** was accomplished

TABLE III. 3-, 7- and 8-Propylimidazo[1,2-*a*]pyridines



Compd.	R	Position of 	X	Y	mp (°C)[a]	Yield (%)[b]	^{1}H-NMR ($CDCl_3$) δ (ppm)
15	H	3	H_2	CH_2	64—66	41	1.72 (3H, s, CH_3), 3.56 (2H, s, CH_2), 4.64 (1H, s, =CH), 4.86 (1H, s, =CH), 6.70 (1H, ddd, J=1, 7, 8 Hz, 6-H), 7.05 (1H, ddd, J=1, 7, 9 Hz, 7-H), 7.38 (1H, s, 2-H), 7.52 (1H, dt, J=1, 2, 9 Hz, 8-H), 7.82 (1H, dt, J=1, 1, 7 Hz, 5-H)
20	H	7	H_2	CH_2	Oil	12	1.70 (3H, s, CH_3), 3.32 (2H, s, CH_2), 4.78 (1H, s, =CH), 4.86 (1H, s, =CH), 6.64 (1H, dd, J=2, 8 Hz, 6-H), 7.34 (1H, br s, 8-H), 7.48 and 7.54 (each, 1H, s, 2- and 3-H), 8.01 (1H, d, J=8 Hz, 5-H)
29	6-CH_3	8	H_2	CH_2	Oil	39	1.78 (3H, s, CH_3), 2.28 (3H, s, CH_3), 3.70 (2H, s, CH_2), 4.90 (1H, s, =CH), 4.80 (1H, s, =CH), 6.82 (1H, s, 7-H), 7.46 and 7.54 (each, 1H, d, J=1 Hz, 2- and 3-H), 7.98 (1H, s, 5-H)
16	H	3	H_2	O	83—85	69	2.18 (3H, s, CH_3), 3.94 (2H, s, CH_2), 6.74 (1H, ddd, J=1, 8, 8 Hz, 6-H), 7.12 (1H, ddd, J=1, 8, 8 Hz, 7-H), 7.50 (1H, s, 2-H), 7.56 (1H, dd, J=1, 8 Hz, 8-H), 7.84 (1H, dd, J=1, 8 Hz, 5-H)
21	H	7	H_2	O	Oil	22,[e] 75[f]	2.22 (3H, s, CH_3), 3.73 (2H, s, CH_2), 6.62 (1H, dd, J=2, 9 Hz, 6-H), 7.36 (1H, t like s, 8-H), 7.48 and 7.52 (each, 1H, d, J=1 Hz, 2- and 3-H), 8.01 (1H, dd, J=2, 9 Hz, 5-H)
30a	H	8	H_2	O	68—69	47	2.28 (3H, s, CH_3), 4.08 (2H, s, CH_2), 6.70 (1H, 1H, dd, J=8, 8 Hz, 6-H), 6.96 (1H, d, J=8 Hz, 8-H), 7.52 and 7.54 (each, 1H, s, 2- and 3-H), 8.01 (1H, d, J=8 Hz)
30b	6-CH_3	8	H_2	O	78—80	80	2.24 (3H, s, 2 × CH_3), 4.08 (2H, s, CH_2), 6.86 (1H, br s, 7-H), 7.49 and 7.54 (each, 1H, s, 2- and 3-H), 7.86 (1H, br s, 5-H)
17	H	3	$CHN(CH_3)_2$	O	142—143	60	1.82 (3H, s, CH_3), 2.2—3.0 (6H, br s, $N(CH_3)_2$), 6.86 (1H, ddd, J=1, 7, 9 Hz, 6-H), 7.22 (1H, ddd, 2, 7, 9 Hz, 7-H), 7.52 (1H, s, =CH), 7.66 (1H, dd, J=1, 9 Hz, 8-H), 7.84 (1H, dd, J=1, 9 Hz, 5-H), 8.01 (1H, s, 2-H)
22	H	7	$CHN(CH_3)_2$	O	142—144	74	2.04 (3H, s, CH_3), 2.78 (6H, s, $N(CH_3)_2$), 6.66 (1H, dd, J=2, 8 Hz, 6-H), 7.32 (1H, br s, 8-H), 7.54—7.60 (2H, m, 2, 3-H), 7.52 (1H, s, =CH), 8.04 (1H, dd, J=2, 8 Hz, 5-H)
31a	H	8	$CHN(CH_3)_2$	O	164—166	73	2.01 (3H, s, CH_3), 2.70 (6H, s, $N(CH_3)_2$), 6.88 (1H, dd, J=8 Hz, 7.01 (1H, dd, J=2, 8 Hz, 7-H), 7.60 and 7.62 (each, 1H, d, J=1 Hz, 2- and 3-H), 7.78 (1H, s, =CH), 8.10 (1H, dd, J=2, 8 Hz, 5-H)
31b	6-CH_3	8	$CHN(CH_3)_2$	O	212—214	69	2.02 (3H, s, CH_3), 2.36 (3H, s, CH_3), 2.68 (6H, s, $N(CH_3)_2$), 6.84 (1H, d, J=2 Hz, 7-H), 7.50 and 7.58 (each, 1H, d, J=1 Hz, 2- and 3-H), 7.76 (1H, s, =CH), 7.88 (1H, br s, 5-H)

a) Purified by column chromatography on silica gel. *b*) Not optimized. *c*) Reduction of the nitropropenyl derivative. *d*) Ozonolisis of the methylpropenyl derivative.

by minor modification of the general procedure of Lesher and Philino.[19] Treatment of the ketones **9a—g** with *N*,*N*-dimethylformamide dimethylacetal in dimethylformamide (DMF) or toluene provided the enamino ketones **10a—g** (Table II). The pyridinones **11a—g** were obtained by condensation of **10a—g** with cyanoacetamide in the presence of sodium methoxide in DMF or EtOH. Treatment of **11a** with bromine in AcOH gave the brominated product **12** (the position of bromine is discussed later), and with 85% H_3PO_4 provided the decyanated product **13**.

The key intermediates **16**, **21** and **30a—b** (Table III) of 3-, 7- and 8-yl regioisomers were prepared by method A or C, depending on ease of preparation of starting materials. In the case of 7-yl isomer **21**, both methods were used. The Grignard cross coupling reaction of **14**,[20] **19**[21] and **28** provided isobutenyl derivatives **15**, **20** and **29** which were converted to **16**, **21** and **30b** by ozonolysis under acidic conditions. Treatment of 7- and 8-imidazo[1,2-*a*]pyridinecarboxaldehydes **24** and **26** with nitroethane followed by reduction with iron powder also provided imidazo[1,2-*a*]pyridinyl-2-propanones **21** and **30a**. These were converted to pyridinones, **18**, **23** and **32a—b** in the same manner as mentioned above (Chart 4).

The position of bromine of **12** was presumed to be the 3-position of imidazo[1,2-*a*]pyridine (IM) in view of **14**, and confirmed by comparison of Nuclear Overhauser effects (NOE's) and coupling constants of **11a** and **12** (free base) in the proton nuclear magnetic resonance (^{1}H-NMR) (400 MHz) spectra in deuteriodimethyl sulfoxide (DMSO-d_6). Irradiation of 5-H resonance at 8.59 ppm in the spectrum of **11a** gave NOE enhancement of 11.2% and 14.7%, respectively, in two resonances at 8.16 (singlet) and 7.92 ppm (doublet of doublets), assigned to 4-H of pyridinone (PN) and 3-H of IM. On the other hand, irradiation of 5-H at 8.35 ppm in the spectrum of **12** caused only a weak enhancement of 4-H of PN at 8.19 ppm (due to near chemical shift) and no increase of intensity of the signal at 7.77 ppm. In addition, the 3-H at 7.92 ppm of **11a** had J_{2H-3H}=1.1 and J_{3H-8H}=0.8 Hz, whereas the

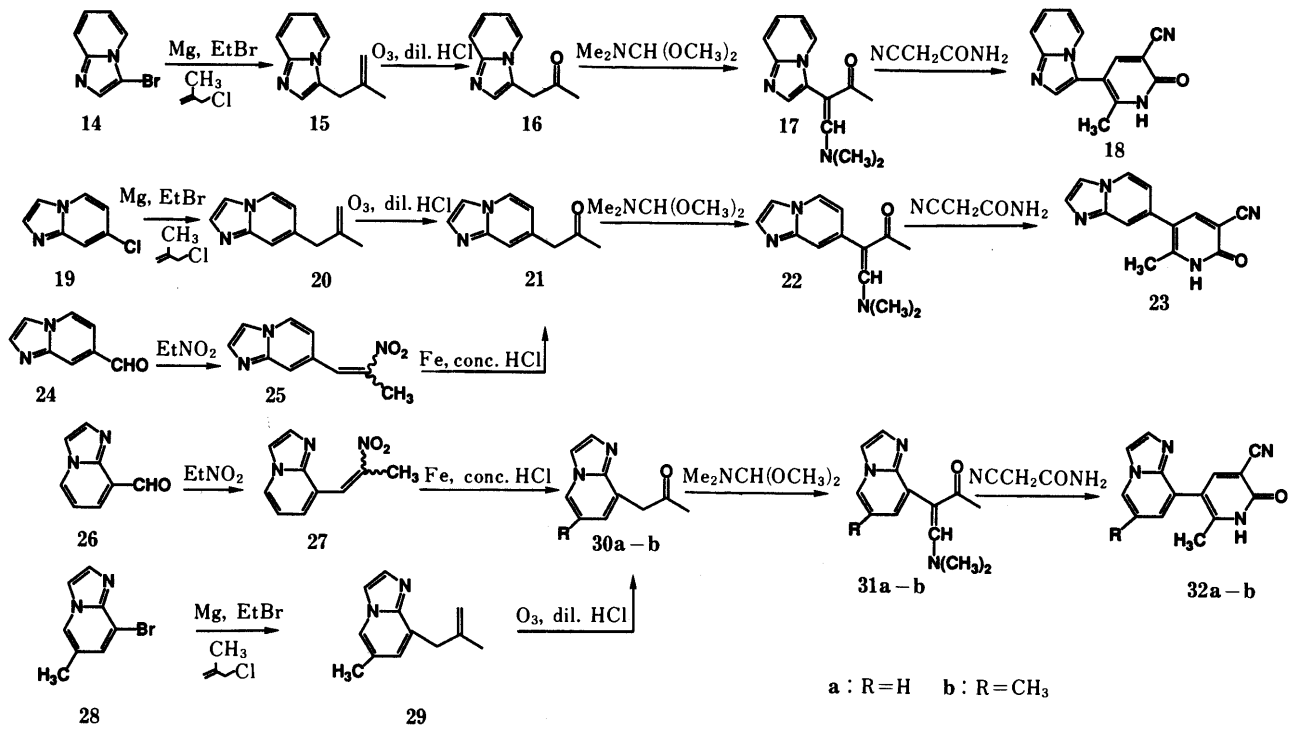
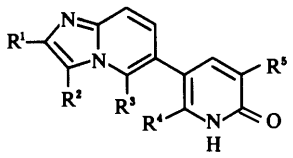


Chart 4

TABLE IV. 5-Imidazo[1,2-*a*]pyridin-6-yl-2(1*H*)-pyridinones (**11a—g, 12, 13**)



Compd.	R^1	R^2	R^3	R^4	R^5	mp (°C) (Solvent[a])	Yield[b] (%)	Formula	Analysis (%) Calcd (Found)		
									C	H	N
11a	H	H	H	CH_3	CN	>300 (B)	51	$C_{14}H_{10}N_4O \cdot HCl \cdot H_2O$	55.16 (55.26	4.30 4.40	18.39 18.44)
11b	CH_3	H	H	CH_3	CN	>260 (dec.) (E)	45	$C_{15}H_{12}N_4O \cdot HCl \cdot 1/6H_2O$	59.30 (59.28	4.42 4.58	18.44 18.56)
11c	H	H	CH_3	CH_3	CN	>300 (E)	15	$C_{15}H_{12}N_4O \cdot 1/6H_2O$	67.39 (67.49	4.65 4.70	20.96 20.90)
11d	CH_3OCH_2	H	H	CH_3	CN	>270 (dec.) (E)	35	$C_{16}H_{14}N_4O_2 \cdot H_2O$	61.52 (61.68	5.17 5.23	17.94 17.68)
11e	C_6H_5	H	H	CH_3	CN	>300 (B)	43	$C_{20}H_{14}N_4O \cdot 1/2H_2O$	71.62 (71.63	4.51 4.51	16.70 16.58)
11f	H	H	H	C_2H_5	CN	250—252 (E)	37	$C_{15}H_{12}N_4O \cdot HCl \cdot 3/5H_2O$	57.82 (57.93	4.60 4.58	17.99 17.63)
11g	CH_3	H	H	C_2H_5	CN	276—278 (E)	35	$C_{16}H_{14}N_4O$	69.05 (69.28	5.07 5.35	20.13 20.26)
12	H	Br	H	CH_3	CN	>300 (E)	55	$C_{14}H_{19}BrN_4O \cdot HBr \cdot 2/3H_2O$	39.83 (39.59	2.70 2.95	13.27 12.88)
13	H	H	H	CH_3	H	290—292 (G)	50	$C_{13}H_{11}N_3O$	69.31 (69.57	4.93 5.11	18.66 18.67)

a) Recrystallization solvent: see the footnote of Table I. G, EtOH–ether. *b*) Not optimized.

resonance at 7.77 ppm of **12** was a singlet and showed no correlation with 8-H. These results supported that the position of bromine was the 3-position of IM.

Biological Results and Discussion

The pyridinones in Tables I, IV and V were evaluated for inotropic activity intravenously in an acutely instrumented anesthetized dog model and orally in a chronically instrumented conscious dog model. Brief description of the method is included in the experimental section. Heart rate, myocardial contractility (derived by measuring dP/dt max of left ventricular pressure), and

TABLE V. 5-Imidazo[1,2-*a*]pyridin-3-,7- and 8-yl-2(1*H*)-pyridinones (**18**, **23**, **32a** and **32b**)

Compd.	mp (°C) (Solvent[a])	Yield[b] (%)	Formula	Analysis (%) Calcd (Found) C	H	N
18	>300 (B)	54	$C_{14}H_{10}N_4O \cdot HCl \cdot 4/5H_2O$	55.83 (55.86	4.23 4.36	18.60 18.51)
23	>290 (E)	33	$C_{14}H_{10}N_4O \cdot 1/6H_2O$	66.39 (66.67	4.12 4.42	22.13 21.83)
32a	276—278 (E)	23	$C_{14}H_{10}N_4O \cdot 5/4H_2O$	61.63 (61.84	4.55 4.57	20.54 20.63)
32b	>300 (E)	35	$C_{15}H_{12}N_4O \cdot HCl \cdot 0.03H_2O$	59.78 (59.48	4.38 4.55	18.60 19.00)

a, *b*) See footnote in Table I.

TABLE VI. Cardiovascular Profile of 5-Imidazo[1,2-*a*]pyridinyl-2(1*H*)-pyridinones in Anesthetized Dogs after i.v. Administration

Compd.	*n*[a]	Dose (mg/kg)	% change LV$dP/dt_{max}$[b]	HR[c]	MAP[d]	ED_{50}[e] (μg/kg)
3a	5	0.100	75	18	−17	52±10
3b	2	0.300	15	4	−5	>300
3c	2	0.100	22	11	−15	>300
3d	2	0.300	19	3	−12	>300
3e	3	0.100	97	27	−27	31±14
3f	3	0.100	60	36	−15	58±11
3g	2	0.100	86	9	−12	32
3h	2	0.300	26	−1	−10	>300
3i	2	0.300	47	6	−8	>300
3j	2	0.300	16	4	−3	>300
3k	2	0.300	84	26	−9	123
3l	2	0.300	12	9	−6	>300
3m	2	0.300	43	18	−18	195
3n	3	0.100	82	30	−27	33±6
3o	2	0.100	31	19	−8	177
3p	2	0.300	57	23	−30	193
3q	2	0.300	62	22	−40	27
3r	2	0.300	25	8	−10	>300
3s	2	0.300	17	11	−7	>300
3t	2	0.100	47	21	−32	91
3u	2	0.030	86	32	−31	11
11a	6	0.100	99	28	−9	23±2
11b	2	0.100	131	40	−21	18
11c	2	0.300	30	10	−10	>300
11d	2	0.300	57	14	−7	218
11e	2	1.000	30	9	−8	>1000
11f	2	0.100	89	17	−9	52
11g	2	0.100	24	13	−21	197
12	2	0.300	76	12	−13	172
13	2	0.100	52	12	−21	87
18	2	1.000	43	8	1	>1000
23	2	1.000	25	11	−11	>1000
32a	2	0.300	5	8	7	>300
32b	2	1.000	6	3	1	>1000
Milrinone	6	0.100	98	33	−18	25±6

a) Number of experiments. *b*) Maximum rate of rise in left ventricular pressure. *c*) Heart rate. *d*) Mean arterial pressure. *e*) Values are doses that produced 50% increase in LV$dP/dt_{max}$ and are expressed as the mean ± S.E.M. When two determinations were made, the values shown is the arithmetic mean.

systolic and diastolic blood pressure were recorded. Dose response curves were determined with at least three doses of each compound.

Cardiovascular data in anesthetized dogs after intravenous administration are summarized in Table VI.

TABLE VII. Guinea Pig. Right Ventricular Papillary Muscle Contractility

Compound	*n*[a]	% change from control 1×10^{-6}	1×10^{-5}	1×10^{-4}[b]
3a	5	37±5.3	64± 5.6	84± 7.4
11a	8	35±6.1	83±11.1	120±18.3
18	3		5± 2.9	91± 2.5
23	4		12± 4.8	67±15
Milrinone	9	33±7.5	83± 8.8	129±12

a) The number of experiments. *b*) Concentration of compounds (M).

TABLE VIII. Effect of Cardiotonic Agents on Myocardial Contractility in Conscious Dogs Following Oral Administration

Compd.	*n*[a]	mg/kg	% increase[b] LV$dP/dt_{max}$	HR	Duration[c] (h)
11a	3	0.3	27±3[d]	6±4	3
	4	1.0	37±4[d]	6±3	6
	3	3.0	69±9[d]	14±7	8
Milrinone	3	0.3	30±7[d]	21±4[d]	2
	3	1.0	59±17[d]	57±17[d]	4

a) Number of experiments. *b*) Values are maximum response from control average ± S.E.M. *c*) Values are maximum numbers of hours after administration that the inotropic response was significant at $p<0.05$ compared to control. *d*) Significant difference from control, $p<0.05$.

TABLE IX. IC_{50} Values of Guinea Pig. Phosphodiesterase

Compound	IC_{50} (M) PDE I	PDE II	PDE III
11a	1.8×10^{-4}	1.0×10^{-4}	6.3×10^{-7}
Milrinone	1.5×10^{-4}	1.1×10^{-4}	7.6×10^{-7}

Among a series of 2-positional isomers, **3a** produced substantial inotropic responses ($ED_{50}=52\,\mu g/kg$) when administered intravenously to anesthetized dogs. Introduction of a fluorine or cyano group into the 6-position of imidazo[1,2-*a*]pyridine enhanced this activity (ED_{50} of **3e** and **3g** were 31 and 32 μg/kg, respectively), while that of methoxy, methyl and trifluoromethyl groups reduced it ($ED_{50}>300\,\mu g/kg$). Replacement of the cyano group in the pyridinone rings of **3a**, **3c**, **3e** and **3g** with bromine led to an increase in activity for **3n** and **3o** ($ED_{50}=33$ and 177 μg/kg) and retention of activity for **3q** ($ED_{50}=27$ μg/kg), but diminished potency for **3r** ($ED_{50}>300\,\mu g/kg$). The most striking effect of fluorine substitution and replacement of the cyano group was shown in **3u** ($ED_{50}=11\,\mu g/kg$), which had a fluorine in the 6-position of the imidazo[1,2-*a*]pyridine, and a chlorine in the 3-position of pyridinone. Replacement of methyl with ethyl or cyano group with hydrogen in the pyridinone ring resulted in decreased potency. The inotropic response of those compounds which had halogen groups in the pyridinone ring was of shorter duration than that of compounds having a cyano group (data not shown).

Among a series of 6-positional isomers, **11a** produced dose related increases in myocardial contractility ($ED_{50}=23\,\mu g/kg$), and was 2 times more potent than **3a**. Methyl substitution in the 2-position of imidazo[1,2-*a*]pyridine

TABLE X. ^{1}H-NMR (90 MHz) Spectra of **3b—u**, **11a—g**, **18**, **23** and **32a—b**

Compound	δ (ppm) in DMSO-d_6
3b	1.22 (3H, t, J=7 Hz, CH_3), 2.99 (2H, q, J=7 Hz, CH_2), 6.93 (1H, ddd, J=1, 7, 7 Hz, 6-H), 7.27 (1H, ddd, J=1, 7, 8 Hz, 7-H), 7.57 (1H, dd, J=1, 8 Hz, 8-H), 8.18 (1H, s, 3-H), 8.47 (1H, s, 4-H of PN), 8.55 (1H, dd, J=1, 7 Hz, 5-H), 12.84 (1H, brs, NH)
3c	2.52 (3H, s, CH_3), 7.77 (1H, dd, J=2, 9 Hz, 7-H), 7.86 (1H, d, J=9 Hz, 8-H), 8.35 (1H, s, 3-H), 8.44 (1H, s, 4-H of PN), 8.62 (1H, brs, 5-H), 12.96 (1H, brs, NH)
3d	1.21 (3H, t, J=7 Hz, CH_3), 2.28 (3H, s, CH_3), 3.00 (2H, q, J=7 Hz, CH_2), 7.13 (1H, dd, J=1, 9 Hz, 7H), 7.49 (1H, d, J=9 Hz, 8-H), 8.08 (1H, s, 3-H), 8.34 (1H, d, J=1 Hz, 5-H), 8.45 (1H, s, 4-H of PN)
3e	(400 MHz) 2.56 (3H, s, CH_3), 7.85 (1H, ddd, J=2.4, 9.1, 9.9 Hz, 7-H), 7.94 (1H, dd, J=4.8, 9.9 Hz, 8-H), 8.39 (1H, s, 3-H), 8.47 (1H, s, 4-H of PN), 9.10 (1H, t like s, 5-H), 13.05 (1H, brs, NH)
3f	2.61 (3H, s, CH_3), 6.88 (1H, ddd, J=5, 7, 8 Hz, 6-H), 7.16 (1H, ddd, J=2, 8, 11 Hz, 7-H), 8.29 (1H, d, J=3 Hz, 3-H), 8.39 (1H, dd, J=2, 7 Hz, 5-H), 8.54 (1H, s, 4-H or PN), 12.82 (1H, brs, NH)
3g	2.61 (3H, s, CH_3), 7.52 (1H, dd, J=2, 9 Hz, 7-H), 7.75 (1H, d, J=9 Hz, 8-H), 8.28 (1H, s, 3-H), 8.55 (1H, s, 4-H of PN), 9.35, 1H, d, J=2 Hz, 5-H), 12.70 (1H, brs, NH)
3h	1.21 (3H, t, J=7 Hz, CH_3), 2.97 (2H, q, J=7 Hz, CH_2), 7.51 (1H, dd, J=2, 9 Hz, 7-H), 7.74 (1H, d, J=9 Hz, 8-H), 8.28 (1H, s, 3-H), 8.49 (1H, s, 4-H of PN), 9.35 (1H, d, J=2 Hz, 5-H), 12.72 (1H, brs, NH)
3i	2.52 (3H, s, CH_3), 3.26 (3H, s, OCH_3), 7.02 (1H, dd, J=2, 10 Hz, 7-H), 7.50 (1H, d, J=10 Hz, 8-H), 8.07 (1H, s, 3-H), 8.22 (1H, d, J=2 Hz, 5-H), 8.50 (1H, s, 4-H of PN), 12.78 (1H, brs, NH)
3j	2.62 (3H, s, CH_3), 7.52 (1H, dd, J=2, 10 Hz, 7-H), 7.78 (1H, d, J=10 Hz, 8-H), 8.28 (1H, s, 3-H), 8.55 (1H, s, 4-H of PN), 12.81 (1H, brs, NH)
3k	2.45 (3H, s, CH_3), 6.36 (1H, d, J=9 Hz, 3-H of PN), 7.30—7.50 (1H, m, 6-H), 7.74 (1H, d, J=9 Hz, 4-H of PN), 7.84—7.92 (2H, m, 7- and 8-H), 8.36 (1H, s, 3-H), 8.83 (1H, dt, J=1, 7 Hz, 5-H), 12.92 (1H, brs, NH)
3l	2.44 (3H, s, CH_3), 6.34 (1H, d, J=10 Hz, 3-H of PN), 7.66 (1H, dd, J=2, 9 Hz, 7-H), 7.70 (1H, d, J=10 Hz, 4-H of PN), 7.80 (1H, d, J=9 Hz, 8-H), 8.23 (1H, s, 3-H), 8.62 (1H, brs, 5-H), 12.64 (1H, brs, NH)
3m	2.42 (3H, s, CH_3), 6.39 (1H, d, J=10 Hz, 3-H of PN), 7.70 (1H, d, J=10 Hz, 4-H of PN), 7.84—8.00 (1H, m, 7-H), 7.90—8.08 (1H, m, 8-H), 8.36 (1H, s, 3-H), 9.04—9.20 (1H, m, 5-H), 12.90 (1H, brs, NH)
3n	2.46 (3H, s, CH_3), 7.36—7.54 (1H, m, 6-H), 7.78—8.02 (2H, m, 7 and 8-H), 8.28 (1H, s, 4-H of PN), 8.44 (1H, s, 3-H), 8.86 (1H, dt, J=1, 1, 7 Hz, 5-H), 12.60 (1H, brs, NH)
3o	2.44 (6H, s, 2 × CH_3), 7.74 (1H, dd, J=1, 9 Hz, 7-H), 7.88 (1H, d, J=9 Hz, 8-H), 8.22 (1H, s, 4-H of PN), 8.34 (1H, s, 3-H), 8.66 (1H, brs, 5-H), 12.60 (1H, brs, NH)
3p	2.44 (3H, s, CH_3), 7.70 (1H, dd, J=2, 9 Hz, 7-H), 7.86 (1H, d, J=9 Hz, 8-H), 8.27 (1H, s, 4-H of PN), 9.03 (1H, t like s, 5-H), 12.50 (1H, brs, NH)
3q	2.48 (3H, s, CH_3), 7.18—7.48 (1H, m, 7-H), 7.63 (1H, dd, J=5, 9 Hz, 8-H), 8.13 (1H, s, 3-H), 8.38 (1H, s, 4-H of PN), 8.63—8.78 (1H, m, 5-H), 12.56 (1H, brs, NH)
3r	2.50 (3H, s, CH_3), 7.50 (1H, dd, J=2, 9 Hz, 7-H), 7.73 (1H, d, J=9 Hz, 8-H), 8.22 (1H, s, 3-H), 8.40 (1H, s, 4-H of PN), 9.30 (1H, d, J=2 Hz, 5-H), 12.40 (1H, brs, NH)
3s	2.50 (3H, s, CH_3), 7.50 (1H, dd, J=2, 10 Hz, 7-H), 7.77 (1H, d, J=10 Hz, 8-H), 8.24 (1H, s, 4-H of PN), 8.40 (1H, s, 3-H), 9.19 (1H, brs, 5-H), 12.37 (1H, brs, NH)
3t	2.52 (3H, s, CH_3), 6.91 (1H, ddd, J=1, 7, 7 Hz, 6-H), 7.26 (1H, ddd, J=1, 7, 9 Hz, 7-H), 7.56 (1H, d, J=9 Hz, 8-H), 8.13 (1H, s, 3-H), 8.23 (1H, s, 4-H of PN), 8.51 (1H, dt, J=1, 1, 7 Hz, 5-H), 12.28 (1H, brs, NH)
3u	2.46 (3H, s, CH_3), 7.34 (1H, ddd, J=2, 8, 10 Hz, 7-H), 7.62 (1H, dd, J=6, 10 Hz, 8-H), 8.13 (1H, s, 3-H), 8.20 (1H, s, 4-H of PN), 8.70 (1H, m, 5-H), 12.21 (1H, brs, NH)
11a	(HCl salt) 2.34 (3H, s, CH_3), 7.94 (1H, d, J=9 Hz, 7-H), 8.06 (1H, d, J=9 Hz, 8-H), 8.20 (1H, s, 4-H of PN), 8.25 and 8.37 (each, 1H, d, J=2 Hz, 2- and 3-H), 9.02 (1H, brs, 5-H), 12.94 (1H, brs, NH)
11b	2.30 (3H, s, CH_3), 2.50 (3H, s, CH_3), 7.88 (1H, dd, J=2, 9 Hz, 7-H), 7.93 (1H, d, J=9 Hz, 8-H), 8.05 (1H, s, 3H), 8.20 (1H, s, 4-H of PN), 8.90 (1H, brs, 5-H), 12.90 (1H, brs, NH)
11c	2.10 (3H, s, CH_3), 2.42 (3H, s, CH_3), 7.08 (1H, d, J=10 Hz, 7-H), 7.50 (1H, d, J=10 Hz, 8-H), 7.66 and 7.88 (each, 1H, d, J=1 Hz, 2- and 3-H), 8.02 (1H, s, 4-H of PN), 12.68 (1H, brs, NH)
11d	2.28 (3H, s, CH_3), 3.32 (3H, s, OCH_3), 4.49 (2H, s, OCH_2), 7.18 (1H, dd, J=2, 9 Hz, 7-H), 7.52 (1H, d, J=9 Hz, 8-H), 7.82 (1H, s, 3-H), 8.14 (1H, s, 4-H of PN), 8.56 (1H, brs, 5-H), 12.70 (1H, brs, NH)
11e	2.32 (3H, s, CH_3), 7.23 (1H, dd, J=2, 10 Hz, 7-H), 7.20—7.58 and 7.86—8.08 (5H, m, C_6H_5), 7.62 (1H, d, J=10 Hz, 8-H), 8.17 (1H, s, 4H of PN), 8.37 (1H, s, 3-H), 8.55 (1H, brs, 5-H), 12.80 (1H, brs, NH)
11f	1.11 (3H, t, J=8 Hz, CH_3), 2.53 (2H, q, J=8 Hz, CH_2), 7.91 (1H, bd, J=9 Hz, 7-H), 8.04 (1H, d, J=9.2 Hz, 8-H), 8.18 (1H, s, 4-H of PN), 8.24 and 8.36 (each, 1H, d, J=2 Hz, 2- and 3-H), 8.96 (1H, brs, 5-H), 12.90 (1H, brs, NH)
11g	1.09 (3H, t, J=7 Hz, CH_3), 2.33 (3H, s, CH_3), 2.52 (2H, q, J=7 Hz, CH_2), 7.08 (1H, dd, J=2, 9 Hz, 7-H), 7.45 (1H, d, J=9 Hz), 7.65 (1H, s, 3-H), 8.10 (1H, s, 4-H of PN), 8.44 (1H, brs, 5-H), 12.68 (1H, brs, NH)
18	2.20 (3H, s, CH_3), 7.42—7.60 (1H, m, 6-H), 7.88—8.12 (2H, m, 7- and 8-H), 8.24 (1H, s, 4-H of PN), 8.28 (1H, s, 2-H), 8.66 (1H, d, J=7 Hz, 5-H), 12.80 (1H, brs, NH)
23	2.32 (3H, s, CH_3), 6.92 (1H, dd, J=2, 8 Hz, 6-H), 7.56 and 7.96 (each, 1H, s, 2- and 3-H), 7.62 (1H, d, J=2 Hz, 8-H), 8.16 (1H, s, 4-H of PN), 8.56 (1H, d, J=8 Hz, 5-H), 12.64 (1H, brs, NH)
32a	2.20 (3H, s, CH_3), 6.92 (1H, t, J=7, 8 Hz, 6-H), 7.16 (1H, dd, J=2, 8 Hz, 7-H), 7.52 and 7.92 (each, 1H, d, J=1 Hz, 2- and 3-H), 8.14 (1H, s, 4-H of PN), 8.54 (1H, dd, J=2, 7 Hz, 5-H), 12.80 (1H, brs, NH)
32b	2.16 (3H, s, CH_3), 2.43 (3H, s, CH_3), 7.74 (1H, brs, 8-H), 8.13 and 8.33 (each, 1H, br d, 2- and 3-H), 8.15 (1H, s, 4-H of PN), 8.75 (1H, brs, 5-H), 12.74 (1H, brs, NH)

PN: Pyridinone ring.

(**11b**) retained the activity of **11a**, whereas substitution in other positions or with other substituents did not offer any advantage over **11a** and only resulted in decreased potency.

Regioisomers at the positions 3, 7 and 8 of the imidazo[1,2-*a*]pyridine moiety, **18**, **23** and **32a**, were less potent than **3a** and **11a**. The weak activities of these isomers were also shown in guinea pig papillary muscles (Table VII). The drastic decreases in the activity of 7-yl isomer

23 in comparison with that of 6-yl isomer **11a** shows the orientation of nitrogen which may function as a hydrogen-bond-acceptor appears to be a critical determinant of inotropic potency.

The selected compounds, **3e** and **11a** (**3n** and **3u** were not chosen due to their short duration) were further examined for oral activity in conscious dogs. Table VIII shows comparative data obtained from **11a** and milrinone. The inotropic response of **11a** at a dose of 1 mg/kg (maximum response: a 37% increase) lasted in excess of 6 h without a significant increase in heart rate. The same dose of milrinone produced a 60% increase in contractility, but the effect was of relatively short duration and the effect on heart rate was greater than that of **11a**. According to electrophysiological studies, the positive chronotropic effect of **11a** in isolated guinea pig sinus nodes was lower than that of milrinone. The reason was the magnitude of the increase in the slope of slow diastolic depolarization, and the shortening of the action potential duration caused by **11a** was less than that caused by milrinone.[22] The mechanism of inotropic action of milrinone and related compounds appears to involve, at least at part, the selective inhibition of the low-K_m, cyclic AMP specific phosphodiesterase (PDE III) that is present in myocardial cells as mentioned above. **11a** was consequently investigated for its ability to inhibit cardiac PDE III, and demonstrated a potent inhibitory effect. The inhibitory effects for PDE I and PDE II were significantly less (Table IX).

In conclusion, a series of 5-imidazo[1,2-*a*]pyridinyl-2(1*H*)-pyridinone possessing potent cardiotonic properties has been discovered. These new agents also retain potent inhibitory activity of cardiac PDE III. On the basis of extensive pharmacological and toxicological evaluations, **11a** hydrochloride monohydrate (E-1020)[23] was selected for development for the management of congestive heart failure.

Experimental

Melting points were determined on a Yamato Model MP 12 capillary melting point apparatus and are uncorrected. ^{1}H-NMR spectra (90 MHz and 400 MHz) were obtained on a JEOL FX-90Q or a JEOL JNM-GX400 spectrometer. Chemical shifts are expressed in values (ppm) with tetramethylsilane as an internal standard. Elemental analyses were within ±0.4% of the calculated values, except where noted otherwise. The reported yields for the procedures obtained were not optimized.

5-Acetyl-6-methyl-2(1*H*)-pyridinone (1c) A mixture of **1a**[16] (12.8 g, 72.6 mmol) in 50% H_2SO_4 (100 ml) was refluxed for 7.5 h. After cooling, the reaction mixture was adjusted to pH 2 with 20% NaOH solution. The precipitates were collected by filtration, washed with water and dried to give 6.9 g (49%) of 5-acetyl-1,2-dihydro-6-methyl-2-oxo-3-pyridinecarboxylic acid, mp 236—238 °C. *Anal.* Calcd for $C_9H_9NO_4$: C, 55.38; H, 4.66; N, 7.17. Found: C, 55.41; H, 4.62; N, 7.19. A mixture of the acid (30.5 g) in Dowtherm A (100 ml) was refluxed for 3 h. After cooling, the precipitates were collected by filtration and recrystallized from EtOH to afford 18.4 g (78%) of **1c**, mp 196—198 °C. ^{1}H-NMR ($CDCl_3$): 2.47 (2H, s, $COCH_3$), 2.70 (3H, s, CH_3), 6.48 (1H, d, J=11 Hz, 3-H), 7.90 (1H, d, J=11 Hz, 4-H), 12.32 (1H, br s, NH).

5-Acetyl-3-bromo-6-methyl-2(1*H*)-pyridinone (1d) To a stirred mixture of **1c** (5.4 g, 35.7 mmol) in 4.5 ml of 48% HBr, 7 ml (72 mmol) of 35% H_2O_2 was added dropwise at 40—60 °C, then the mixture was stirred at 60 °C for 1 h. After cooling, the solid materials were collected by filtration and were recrystallized from MeOH to afford 4.12 g (50%) of **1d**, mp 216—217 °C. *Anal.* Calcd for $C_8H_8BrNO_2$: C, 41.76; H, 3.51; N, 6.09. Found: C, 41.93; H, 3.46; N, 6.08. ^{1}H-NMR ($CDCl_3$): 2.48 (3H, s, $COCH_3$), 2.70 (3H, s, CH_3), 8.24 (1H, s, 4-H), 12.90 (1H, br s, NH).

5-Acetyl-3-chloro-6-methyl-2(1*H*)-pyridinone (1e) To a stirred mixture of **1c** (13.5 g, 89.3 mmol) in 35 ml of conc. HCl, 17.4 ml (179 mmol) of 35% H_2O_2 was added dropwise at 40—60 °C, and the mixture was stirred at 60 °C for 1 h. After cooling, the reaction mixture was neutralized with K_2CO_3 solution and extracted with $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated. The residue was twice chromatographed on silica gel with AcOEt–hexane (7 : 3), and recrystallized from EtOH to give 1.6 g (9.6%) of **1e**, mp 182—183 °C. *Anal.* Calcd for $C_8H_8ClNO_2$: C, 51.76; H, 4.35; N, 7.55. Found: C, 51.60; H, 4.24; N, 7.56. ^{1}H-NMR ($CDCl_3$): 2.46 (3H, s, $COCH_3$), 2.70 (3H, s, CH_3), 7.98 (1H, s, 4-H), 13.0 (1H, br s, NH).

5-Bromoacetyl-1,2-dihydro-6-methyl-2-oxo-3-pyridinecarbonitrile (2a) To a suspension of **1a** (19.3 g, 109.7 mmol) in 30% HBr–AcOH (3 ml) and AcOH (160 ml) was added dropwise Br_2 (5.79 ml, 109.7 mmol), and the mixture was heated at 50—60 °C with stirring until the red color of bromine disappeared (for about 1 h). The precipitates were collected by filtration, washed with ether and recrystallized from DMF–MeOH to afford 20.3 g (73%) of **2a**, 210—212 °C. ^{1}H-NMR (DMSO-d_6): 2.54 (3H, s, CH_3), 4.72 (2H, s, CH_2), 8.68 (1H, s, 4-H), 12.44 (1H, br s, NH). **2c—e** were prepared similarly. **2c**: Yield 54%, mp 204—206 °C. ^{1}H-NMR ($CDCl_3$): 2.74 (3H, s, CH_3), 4.23 (2H, s, CH_2), 6.48 (1H, d, J=10 Hz, 3-H), 7.88 (1H, d, J=10 Hz, 4-H), 12.40 (1H, br s, NH). **2d**: Yield 70% (purified by chromatography with AcOEt–hexane = 8 : 2), mp 192—194 °C. ^{1}H-NMR ($CDCl_3$): 2.72 (3H, s, CH_3), 4.22 (2H, s, CH_2), 8.22 (1H, s, 4-H), 12.42 (1H, br s, NH). **2e**: Yield 10% (purified by chromatography with AcOEt : hexane = 8 : 2), mp 179—181 °C. ^{1}H-NMR ($CDCl_3$): 2.76 (3H, s, CH_3), 4.22 (2H, s, CH_2), 8.04 (1H, s, 4-H), 12.22 (1H, br s, NH).

5-Bromoacetyl-6-ethyl-1,2-dihydro-2-oxo-3-pyridinecarbonitrile (2b) **1b** was obtained as a mixture with 1,2-dihydro-6-methyl-2-oxo-5-(*n*-propanoyl)-3-pyridinecarbonitrile (the ratio estimated by ^{1}H-NMR was 2 : 3) in accordance with the method for **1a** but replacing acetylacetone with 2,4-hexanedione. The mixture (7.0 g) was brominated similarly and recrystallized three times from AcOEt–hexane to give 0.9 g of **2b** (23%), mp 183—185 °C. ^{1}H-NMR (DMSO-d_6): 1.16 (3H, t, J=7 Hz, CH_3), 2.86 (2H, q, J=7 Hz, CH_2), 4.76 (2H, s, CH_2), 8.72 (1H, s, 4-H), 12.23 (1H, br s, NH).

1,2-Dihydro-5-imidazo[1,2-*a*]pyridin-2-yl-6-methyl-2-oxo-3-pyridinecarbonitrile (3a) (General Procedure) 2-Aminopyridine (2.4 g, 25.5 mol) was added portionwise to a boiling clean solution of **2a** (2 g, 8.4 mmol) in CH_3CN (250 ml). After refluxing for 1.5 h, the precipitates were collected by filtration while hot and washed with CH_3CN, acetone and EtOH to yield 1.54 g (77.8%) of pure **3a** (Table I). ^{1}H-NMR (CF_3COOD): 2.4 (3H, s, CH_3), 7.56 (1H, ddd, J=2, 5, 9 Hz, 6-H of imidazo[1,2-*a*]pyridine (IM)), 7.9—8.16 (1H, m, 7-H of IM), 8.0 (1H, s, 8-H of IM), 8.06 (1H, s, 3-H of IM), 8.27 (1H, s, 4-H of pyridinone (PN)), 8.64 (1H, d, J= 9 Hz, 5-H of IM). Compounds **3b—u** were prepared similarly and the results are listed in Table I. HCl salts of some compounds were prepared by treatment of hot solutions of free bases in DMF with HCl–EtOH.

Methyl 6-Imidazo[1,2-*a*]pyridinecarboxylate After a mixture of bromoacetaldehyde diethyl acetal (11.38 g, 57.5 mmol), H_2O (40 ml) and conc. HCl (1.15 ml, 11.3 mmol) was stirred vigorously at room temperature for 2.5 h, it was heated in an 80 °C oil bath for 40 min to give a clear solution. The cold solution was treated with portions of $NaHCO_3$ (6.28 g, 74.8 mmol) and methyl 2-amino-5-pyridinecarboxylate[24] (7 g, 46 mmol). The mixture was stirred overnight at room temperature. The precipitates were collected by filtration, washed with a small volume of water and dried over P_2O_5 to give 7.5 g (99%) of methyl 6-imidazo[1,2-*a*]pyridinecarboxylate, mp 145—146 °C, which was used in the next reaction without futher purification. ^{1}H-NMR ($CDCl_3$): 3.94 (3H, s, CH_3), 7.62—7.74 (4H, m, 2-, 3-, 7- and 8-H), 8.92 (1H, t like s, 5-H). Methyl 7-imidazo-[1,2-*a*]pyridinecarboxylate was similarly prepared in 89% yield from methyl 2-amino-4-pyridinecarboxylate,[25] mp 143—144 °C. ^{1}H-NMR ($CDCl_3$): 3.97 (3H, s, CH_3), 7.40 (1H, dd, J=2, 7 Hz, 6-H), 7.70 (1H, d, J=1 Hz, 2-H), 7.80 (1H, d, J=1 Hz, 3-H), 8.19 (1H, dd, J=1, 7 Hz, 5-H), 8.37 (1H, br s, 8-H).

Methyl 8-Imidazo[1,2-*a*]pyridinecarboxylate A solution of 2-amino-3-pyridinecarbonitrile[26] (9.5 g, 79.7 mmol) and bromoacetaldehyde diethylacetal (50 g, 253.7 mmol) in *n*-butanol (100 ml) was refluxed overnight. The precipitates were collected by filtration and dissolved in H_2O (200 ml). The solution was adjusted to pH 8 with sat. $NaHCO_3$ solution, extracted with $CHCl_3$, washed with brine and dried over $MgSO_4$. Removal of solvent *in vacuo* gave 8.2 g (72%) of 8-imidazo[1,2-*a*]pyridinecarbonitrile, mp 167—169 °C, which was used in the next reaction without further purification. ^{1}H-NMR ($CDCl_3$): 6.90 (1H, dd, J=7, 7 Hz, 6-H), 7.79 (1H, d, J=1 Hz, 2-H), 8.36 (1H, dd, J=1, 7 Hz, 5-H). A solution of 8-imidazo[1,2-*a*]pyridinecarbonitrile (9 g, 62.9 mmol) in MeOH and conc. H_2SO_4 (25 g) was refluxed for 2 d. After solvent was removed *in*

vacuo, ice was added to the residue. Then the solution was adjusted to pH 8 with 20% NaOH and sat. $NaHCO_3$ solution, and extracted with $CHCl_3$. The organic layer was washed with brine and dried over $MgSO_4$. After removal of solvent, the residue was chromatographed on silica gel with AcOEt–MeOH (95:5) to give 3.9 g (38%) of methyl 8-imidazo[1,2-*a*]pyridinecarboxylate, mp 70—72 °C. ^{1}H-NMR ($CDCl_3$): 4.00 (3H, s, CH_3), 6.82 (1H, dd, J=8, 8 Hz, 6-H), 7.66 (1H, d, J=1 Hz, 3-H), 7.72 (1H, d, J=1 Hz, 2-H), 7.94 (1H, dd, J=2, 8 Hz, 5-H), 8.30 (1H, dd, J=2, 8 Hz, 7-H).

The following compounds were prepared according to the method of Hand and Paudler[27] like methyl 6-imidazo[1,2-*a*]pyridinecarboxylate.

6-Bromoimidazo[1,2-*a*]pyridine (**6a**): Yield 71%, bp 123—125 °C (1.5 mmHg), mp 76—78 °C (lit.[18] mp 53—55 °C). ^{1}H-NMR ($CDCl_3$): 7.21 (1H, dd, J=2, 10 Hz, 7-H), 7.48 (1H, d, J=10 Hz, 8-H), 7.58 (1H, d, J=1 Hz, 2-H), 7.64 (1H, d, J=1 Hz, 3-H), 8.30 (1H, dd, J=1, 2 Hz, 5-H).

6-Bromo-5-methylimidazo[1,2-*a*]pyridine (**6c**) (from 2-Amino-5-bromo-6-methylpyridine)[28]: Yield 76%, mp 122—124 °C (recrystallized from cyclohexane). ^{1}H-NMR ($CDCl_3$): 2.74 (3H, s, CH_3), 7.26 (1H, d, J=9 Hz, 7-H), 7.42 (1H, d, J=9 Hz, 8-H), 7.46 (1H, s, 3-H), 7.64 (1H, s, 2-H).

8-Bromo-6-methylimidazo[1,2-*a*]pyridine (**28**) (from 2-Amino-3-bromo-5-methylpyridine)[29]: Yield 71%, mp 70.5—71.5 °C (recrystallized from cyclohexane). ^{1}H-NMR ($CDCl_3$): 2.28 (3H, s, CH_3), 7.28 (1H, d, J=2 Hz, 7-H), 7.56 (1H, d, J=1 Hz, 3-H), 7.62 (1H, d, J=1 Hz, 2-H), 7.88 (1H, d, J=2 Hz, 5-H).

6-Bromo-2-methylimidazo[1,2-*a*]pyridine (**6b**) and 6-Bromo-2-phenylimidazo[1,2-*a*]pyridine (**6e**) were obtained by the procedure of Godovikova and Gol'dfab.[30]

6-Bromo-2-methoxymethylimidazo[1,2-*a*]pyridine (6d) A solution of 2-amino-5-bromopyridine (19.9 g, 114 mmol) and ethyl bromopyruvate (25.8 g, 132.3 mmol) in dimethoxyethane was stirred at room temperature for 2 h. The precipitates were collected by filtration and refluxed in EtOH (700 ml) for 3 h. After removing solvent, the residue was dissolved in H_2O. The solution was adjusted to pH 8 with sat. $NaHCO_3$ solution and extracted with $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$ and evaporated to afford 22.2 g (72%) of ethyl 6-bromoimidazo[1,2-*a*]pyridine-2-carboxylate, mp 126—128 °C, which was used without further purification. ^{1}H-NMR ($CDCl_3$): 1.44 (3H, t, J=7 Hz, CH_3), 4.44 (2H, q, J=7 Hz, CH_2), 7.28 (1H, s, dd, J=2, 10 Hz, 7-H), 7.58 (1H, d, J=10 Hz, 8-H), 8.12 (1H, s, 3-H), 8.28 (1H, d, J=2 Hz, 5-H). To a stirred solution of ethyl 6-bromoimidazo[1,2-*a*]pyridinyl-2-carboxylate (12 g, 44.6 mmol) in CH_2Cl_2 (150 ml). 1 M solution of diisobutylaluminum hydride (DIBAL) in CH_2Cl_2 (100 ml) was added dropwise at −5—0 °C under N_2. The mixture was stirred for 3 h at 0—10 °C, then 3.5 ml of MeOH was added at −40 °C and 4.5 ml of H_2O was added at 0 °C with stirring. After dissolving solids by adding 6 N HCl, the solution was alkalized with 20% NaOH solution and extracted three times with $CHCl_3$ (300 ml). The combined organic extracts were washed with brine and dried and evaporated to solid. Chromatography on silica gel, eluting with $CHCl_3$–MeOH (98:2), gave 6.8 g (66.3%) of 6-bromo-2-hydroxymethylimidazo[1,2-*a*]pyridine, mp 137 °C. ^{1}H-NMR ($CDCl_3$): 3.2—4.2 (1H, br s, OH), 4.82 (2H, s, CH_2), 7.18 (1H, dd, J=2, 9 Hz, 7-H), 7.48 (1H, d, J=9 Hz, 8-H), 7.50 (1H, s, 3-H), 8.20 (1H, m, 5-H). To a solution of 6-bromo-2-hydroxymethylimidazo[1,2-*a*]pyridine (2.85 g, 12.3 mmol) in CH_2Cl_2 (200 ml) was added thionyl chloride (1.6 g, 13.4 mmol) at 5 °C, and the mixture was stirred at room temperature for 2 h. After ice and sat. aq. $NaHCO_3$ solution (50 ml) were added, the organic layer was separated, washed with brine and evaporated to give crude 6-bromo-2-chloromethylimidazo[1,2-*a*]pyridine (3.08 g) which was used without further purification. The mixture of 6-bromo-2-chloromethylimidazo-[1,2-*a*]pyridine (3.0 g) and 1.3 g (24 mmol) of NaOMe in MeOH (50 ml) was refluxed for 3 h. After solvent was evaporated, the residue was dissolved in $CHCl_3$. The solution was washed with brine and dried over $MgSO_4$. Solvent was removed *in vacuo*, and the residue was chromatographed on silica gel with $CHCl_3$–MeOH (98:2) to give 2.8 g (95.4%) of **6d**, mp 104—106 °C. ^{1}H-NMR ($CDCl_3$): 3.46 (3H, s, OCH_3), 4.73 (2H, s, CH_2), 7.20 (1H, dd, J=2, 9 Hz, 7-H), 7.46 (1H, d, J=9 Hz, 8-H), 7.56 (1H, s, 3-H), 8.20 (1H, br s, 5-H).

1-Imidazo[1,2-*a*]pyridin-6-yl-2-propanone (9a) (General Procedure) Method A To a solution of methyl 6-imidazo[1,2-*a*]pyridinecarboxylate (49 g, 298.5 mmol) in CH_2Cl_2 (500 ml), 1.0 M solution of DIBAL in CH_2Cl_2 (400 ml, 1.34 mmol) was added dropwise at −60 °C under N_2 over a 3 h period. The excess DIBAL was decomposed with MeOH and then water. The solids were removed by filtration and washed with MeOH. After removal of solvent *in vacuo*, the crude product was chromatographed on silica gel with $CHCl_3$–MeOH (98:2) to afford 7.5 g (17.2%) of pure 6-imidazo[1,2-*a*]pyridinecarboxaldehyde **4**, mp 146—148 °C. ^{1}H-NMR ($CDCl_3$): 7.64—7.80 (4H, m, 2-, 3-, 7- and 8-H), 8.70 (1H, m, 5-H), 9.96 (1H, s, CHO). 7- and 8-imidazo[1,2-*a*]pyridinecarboxaldehydes, **24** and **26**, were similarly prepared. **24**: Yield 23%, mp 143—144 °C. ^{1}H-NMR ($CDCl_3$): 7.34 (1H, dd, J=2, 7 Hz, 6-H), 7.76 (1H, s, 2-H), 7.86 (1H, d, J=1H, 3-H), 8.13 (1H, dd, J=1, 2 Hz, 8-H), 8.22 (1H, d, J=7 Hz, 5-H), 10.00 (1H, s, CHO). **26**: Yield 57%, mp 103—104 °C. ^{1}H-NMR ($CDCl_3$): 6.90 (1H, dd, J=7, 7 Hz, 6-H), 7.66 (1H, d, J=1 Hz, 3-H), 7.72 (1H, d, J=1 Hz, 2-H), 7.76 (1H, dd, J=2, 7 Hz, 7-H), 8.30 (1H, dd, J=2, 7 Hz, 5-H). A mixture of **4** (6.9 g, 47.2 mmol), nitroethane (10.6 g, 141.2 mmol), *n*-butylamine (30 drops) in EtOH (40 ml) was refluxed for 14 h, then some of ethylamine was added, and the mixture was refluxed for an additional 18 h. The solids were removed by filtration while hot EtOH (50 ml) and Et_2O (150 ml) were added and the solids were removed by filtration again. After removal of solvent *in vacuo*, the residue was purified twice by recrystallization from EtOH to give 1.14 g (11.9%) of 6-(2-nitro-1-propenyl)imidazo[1,2-*a*]pyridine **5**, mp 190—192 °C (dec.). ^{1}H-NMR ($CDCl_3$): 2.52 (3H, d, J=1 Hz, CH_3), 7.26 (1H, dd, J=2, 9 Hz, 7-H), 7.66 (1H, d, J=1 Hz, 3-H), 7.70 (1H, d, J=9 Hz, 8-H), 7.73 (1H, d, J=1 Hz, 2-H), 8.04 (1H, d, J=1 Hz, =CH), 8.30 (1H, d, J=2 Hz, 5-H). 7- and 8-(2-Nitro-1-propenyl)imidazo[1,2-*a*]pyridine, **25** and **27**, were prepared similarly. **25**: Yield 48%, mp 135—137 °C. ^{1}H-NMR ($CDCl_3$): 2.53 (3H, d, J=1 Hz, CH_3), 6.86 (1H, dd, J=2, 7 Hz, 6-H), 7.68 (1H, dd, J=1, 2 Hz, 8-H), 7.76 (2H, br s, 2- and 3-H), 8.05 (1H, br s, =CH), 8.20 (1H, dd, J=1, 7 Hz, 5-H). **27**: Yield 25% mp 158—160 °C. ^{1}H-NMR ($CDCl_3$): 2.46 (3H, d, J=1 Hz, CH_3), 6.85 (1H, dd, J=8, 8 Hz, 6-H), 7.22 (1H, d, J=8 Hz, 7-H), 7.63 (2H, s, 2- and 3-H), 8.16 (1H, d, J=8 Hz, 5-H), 8.48 (1H, d, br s, =CH). A vigorously stirred mixture of 1.14 g (5.6 mmol) of **5**, Fe powder (2.35 g), $FeCl_2 \cdot (H_2O)_x$ (0.1 g) in EtOH (25 ml)–H_2O (25 ml) was heated at 80 °C and treated dropwise with 2.5 ml of conc. HCl. Upon refluxing for an additional 1 h, the hot reaction mixture was filtered. After removal of solvent *in vacuo*, the residue was made basic with $NaHCO_3$ solution and extracted with $CHCl_3$. The $CHCl_3$ extract was washed with brine, dried over $MgSO_4$, and evaporated to give an oil, which was purified by chromatography on silica gel with $CHCl_3$–MeOH (99:1) to afford 0.5 g (51.2%) of **9a** (bp is shown at method C). ^{1}H-NMR ($CDCl_3$): 2.24 (3H, s, CH_3), 3.70 (2H, s, CH_2), 6.95 (1H, dd, J=2, 9 Hz, 7-H), 7.56 (1H, br s, 3-H), 7.60 (1H, d, J=9 Hz, 8-H), 7.64 (1H, s, 2-H), 8.03 (1H, m, 5-H). **21** and **30a** were prepared similarly and the results are listed in Table III.

Method B A mixture of **6a** (1.97 g, 10 mmol), potassium acetoacetonate (6.91 g, 50 mmol), dried potassium iodide (1.66 g, 10 mmol), and cuprous iodide (0.1 g, 0.5 mmol) in DMF was stirred at 100 °C for 15 h under N_2. To the cooled reaction mixture 20% solution of NaOH (30 ml) was added and the mixture was stirred at room temperature for 3 h. Then, the mixture was adjusted to pH 1 with conc. HCl, and washed with $CHCl_3$ (3 × 100 ml). The aqueous layer was made basic with excess $NaHCO_3$, saturated with NaCl and extracted with $CHCl_3$. The $CHCl_3$ extract was washed with brine, dried over $MgSO_4$, and evaporated to give a dark brown oil, which was purified by silica gel chromatography ($CHCl_3$: MeOH = 99 : 1) to afford 655 mg (37.6%) of **9a**.

Method C A solution of ethylbromide (8.25 g, 76 mmol) in THF (14 ml) was added dropwise to magnesium turning (24.5 g, 1 mol) under N_2, and to the resulting mixture a solution of **6a** (49.25 g, 0.25 mol) and ethylbromide (74.25 g, 0.68 mol) in tetrahydrofuran (THF) (300 ml) was added dropwise over a 40 min-period maintaining the temperature at 50 to 60 °C. After completion of the addition, the reaction mixture was refluxed for 1 h. To the stirred reaction mixture, a solution of 3-chloro-2-methylpropene (97.5 g, 1.08 mol) in THF (200 ml) was added dropwise at 0 to 10 °C, and the mixture was then refluxed for 2 h. After cooling, a solution of ammonium chloride (50 g) in water (500 ml) was added dropwise to the mixture, and then toluene (250 ml), hexane (200 ml) and water (200 ml) were added. The organic layer was separated, washed twice with brine, and dried over $MgSO_4$. After removal of solvent *in vacuo*, the product was purified by distillation under reduced pressure to give 30.5 g (70.9%) of 6-isobutenylimidazo[1,2-*a*]pyridine **8a**, boiling at 118—122 °C/0.5 mmHg. ^{1}H-NMR ($CDCl_3$): 1.70 (3H, s, CH_3), 3.28 (2H, s, CH_2), 4.80 (1H, d, J=1 Hz, H of $=CH_2$), 4.90 (1H, d, J=1 Hz, H of $=CH_2$), 7.02 (1H, dd, J=2, 9 Hz, 7-H), 7.52 (1H, d, J=1 Hz, 3-H), 7.56 (1H, d, J=9 Hz, 8-H), 7.72 (1H, d, J=1 Hz, 2-H), 7.92 (1H, br s, 5-H). **8b—e**, **15**, **20** and **29** were prepared similarly and the results are listed in Tables II and III. Ozone produced by an ozone generator (Nihon Ozone 0-10-3) was introduced to a solution of **8a** (20 g, 116.1 mmol) in conc. HCl (12.3 g), water (45 ml) and MeOH (45 ml) at −5 to 0 °C. The endpoint of the reaction was confirmed by thin layer chromatography (TLC). After

completion of reaction, a solution of sodium sulfite (30.6 g) in water (160 ml) was added dropwise under cooling at a rate that did not exceed 20 °C. Then, $NaHCO_3$ (22 g) and an appropriate amount of NaCl were added as solid and the mixture was extracted with $CHCl_3$. The organic layer was washed twice with brine and dried over $MgSO_4$. After removal of solvent *in vacuo*, the product was purified by distillation under reduced pressure to provide 14.2 g (70.5%) of **9a** boiling at 155—159 °C/0.4 mmHg. **9b—e**, **16**, **21** and **30b** were prepared similarly. *Via* method C but replacing 3-chloro-2-methylpropene with 3-chloro-2-ethylpropene, 1-imidazo-[1,2-*a*]pyridin-6-yl-2-butanones, **9f** and **9g**, were obtained. These results are listed in Tables II and III.

4-Dimethylamino-3-(6-imidazo[1,2-*a*]pyridinyl)-3-buten-2-one (10a) (General Procedure) A mixture of **9a** (33.17 g, 0.19 mol) and *N*,*N*-dimethylformamide dimethylacetal (45.4 g, 0.38 mol) in DMF (200 ml) was stirred at 80 °C for 1 h. The solution was concentrated under reduced pressure and the residue was purified by silica gel chromatography with $CHCl_3$–MeOH (97 : 3) to afford 32.46 g (74.5%) of **10a**, mp 176—178 °C. *Anal.* Calcd for $C_{13}H_{15}N_3O$: C, 68.10; H, 6.59; N, 18.33. Found: C, 67.91; H, 6.67; N, 18.34. ^{1}H-NMR ($CDCl_3$): 2.04 (3H, s, CH_3), 2.80 (6H, s, $N(CH_3)_2$), 7.03 (1H, dd, J=2, 9 Hz, 7-H), 7.55 (1H, s, 3-H), 7.57 (1H, d, J=9 Hz, 8-H), 7.63 (2H, s, 2-H and =CH), 7.90 (1H, br s, 5-H). **10b—g**, **17**, **22** and **31a, b** were prepared similarly and the results are listed in Tables II and III.

1,2-Dihydro-5-imidazo[1,2-*a*]pyridin-6-yl-6-methyl-2-oxo-3-pyridinecarbonitrile (11a) Hydrochloride Monohydrate (General Procedure) To a solution of **10a** (23.5 g, 0.102 mol) in DMF (230 ml) was added 2-cyanoacetamide (9.48 g, 0.113 mol) and $NaOCH_3$ (12.2 g, 0.226 mol) and the mixture was heated at 80—90 °C for 12 h. DMF was evaporated under reduced pressure, and the residue was dissolved in water and washed with $CHCl_3$. After the pH of the aqueous layer was adjusted to 6.5 with AcOH (5 ml), the precipitated crystals were collected by filtration and washed with water. The crystals were dissolved in 2.5% NaOH solution (200 ml) and treated with chacol. PH of the solution was adjusted to 6.5 with AcOH (7 ml) and the precipitates were collected by filtration and washed with water, CH_3CN and ether. This was recrystallized from DMF to give **11a** (13 g, 50.9%), mp >300 °C. *Anal.* Calcd for $C_{14}H_{10}N_4O$: C, 67.18; H, 4.04; N, 22.39. Found: C, 67.17; H, 4.02; N, 22.56. ^{1}H-NMR (400 MHz, DMSO-d_6): 2.29 (3H, s, CH_3), 7.23 (1H, dd, J=1.8, 9.5 Hz, 7-H of IM), 7.60 (1H, ddd, J=0.8, 1.1, 9.5 Hz, 8-H of IM), 7.61 (1H, d, J=1.1 Hz, 2-H of IM), 7.92 (1H, dd, J=0.8, 1.1 Hz, 3-H of IM), 8.16 (1H, s, 4-H of PN), 8.58 (1H, dd, J=1.1, 1.8 Hz, 5-H of IM), 12.76 (1H, br s, NH). To a hot solution of **11a** (12.1 g) in DMF (180 ml) was added HCl–EtOH to give hydrochloride (13.5 g) of **11a**, mp >300 °C. *Anal.* Calcd for $C_{14}H_{10}N_4O \cdot HCl \cdot H_2O$: C, 55.16; H, 4.30; N, 18.39. Found: C, 55.26; H, 4.40; N, 18.44. Compounds **11b—g**, **18**, **23** and **32a—b** were prepared similarly and the results are listed in Tables IV and V.

5-(3-Bromoimidazo[1,2-*a*]pyridin-6-yl)-1,2-dihydro-6-methyl-2-oxo-3-pyridinecarbonitrile Hydrobromic Acid (12) To a solution of **11a** (0.3 g, 1.2 mmol) in AcOH (10 ml) was added bromine (0.2 g, 1.25 mmol) in AcOH (1 ml) and the mixture was warmed at 30 °C for 30 min. The precipitates were collected by filtration, washed with ether and recrystallized twice from MeOH to give 0.3 g (55%) of **12**, mp >300 °C. ^{1}H-NMR (DMSO-d_6): 2.30 (3H, s, CH_3), 7.81 (1H, dd, J=2, 9 Hz, 7-H of IM), 7.99 (1H, d, J=9 Hz, 8-H of IM), 8.24 (1H, s, 4-H of PN), 8.32 (1H, s, 2-H of IM), 8.68 (1H, d, J=2 Hz, 5-H of IM), 12.98 (1H, br s, NH). A suspension of **12** (0.15 g) in water (30 ml) was adjusted to pH 8 with 28% NH_4OH with stirring. The precipitates were collected by filtration, washed with water and MeOH, and recrystallized from MeOH to give 70 mg of free base of **12**, mp 274—276 °C (dec.). ^{1}H-NMR (400 MHz, DMSO-d_6): 2.28 (3H, s, CH_3), 7.35 (1H, dd, J=1.8, 9.5 Hz, 7-H of IM), 7.69 (1H, dd, J=0.75, 9.5 Hz, 8-H of IM), 7.77 (1H, s, 2-H of IM), 8.19 (1H, s, 4-H of PN), 8.35 (1H, dd, J=0.7, 1.8 Hz, 5-H of IM), 12.76 (1H, br s, NH).

5-Imidazo[1,2-*a*]pyridin-6-yl-6-methyl-2(1*H*)-pyridinone (13) A solution of **11a** (1 g, 4 mmol) in 85% (v/v) phosphoric acid (10 ml) was refluxed for 18 h. After cooling, water (50 ml) was added and the solution was adjusted to pH 8 with 28% NH_4OH. The precipitates were extracted with $CHCl_3$ and the extract was washed with brine and dried over $MgSO_4$. After removal of the solvent under reduced pressure, the residue was recrystallized from EtOH–ether to give 0.4 g (50%) of **13**, mp 290—292 °C. ^{1}H-NMR ($CDCl_3$): 2.38 (3H, s, CH_3), 6.52 (1H, d, J=9 Hz, 4-H of PN), 7.06 (1H, dd, J=2, 10 Hz, 7-H of IM), 7.40 (1H, d, J=9 Hz, 3-H of PN), 7.52—7.70 (3H, m, 2-, 3- and 8-H of IM), 8.00 (1H, d, J=2 Hz, 5-H of IM), 12.62 (1H, br s, NH).

Pharmacological Methods and Materials 1. Anesthetized Dog Studies: Using mongrel dogs of either sex (10—15 kg) under artificial respiration and anesthetization with halothane-nitrous oxide, the cardiotonic effect of compounds was evaluated. Aortic pressure was recorded with a catheter inserted into the aorta and connected to a pressure transducer. The left ventricular pressure was recorded with a micro tip pressure transducer (Millar PC-360) inserted into the left ventricle. The heart rate was monitored by means of a tachograph triggered by the left ventricular pressure pulse. As an index of cardiac contractility, LV dP/dt_{max}, was recorded. Depending on solubility of the agent, compounds were dissolved in saline, diluted hydrochronic acid or polyethylene glycol, and were administered intravenously.

2. Conscious Dog Studies: Male beagle dogs (10—13 kg) were chronically instrumented to monitor left ventricular pressure and heart rate. Under halothane-nitrous oxide anesthesia, a precalibrated Konigsberg P6.5 pressure transducer was implanted into the left ventricle through a stab wound at the apex. After recovery from surgery, a period of about 1 week was allowed to train the dogs to lie quietly. This conditioning was necessary to obtain stable, reproducible results from day to day. As an index of cardiac contractility, LV dP/dt_{max} was recorded. Drugs were administered orally in gelatin capsules.

3. Isolated Heart Muscle Preparations: Male guinea pigs of Hartley strain, weighing 300—500 g, were stunned with a blow on the head and exsanguinated. The heart was excised, and the right atrium and thin papillary muscles (diameter: 0.5—1 mm) from the right ventricle were rapidly isolated. The tissues were mounted in organ baths of 6-ml capacity which were filled with a modified Krebs solution of the following composition (mmol/l): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.3; KH_2PO_4, 1.2; $NaHCO_3$, 25.0 and glucose, 11.0. The solution was maintained at 37 °C and equilibrated with a mixture of 95% O_2 and 5% CO_2 to make the pH 7.4. Two platinum electrodes were attached close to the base of the papillary muscle to stimulate the muscle with rectangular pulses of 3 ms duration and voltage 20% above the threshold. The basic stimulation frequency was 1 Hz. Contractile force was recorded isometrically by means of a force transducer (TB-611T; Nihon-Koden, Tokyo, Japan) connected to a pen recorder. The resting tension applied to papillary muscles and right atria was adjusted to produce the maximum developed tension. Spontaneous beating rate of the atria was counted with a heart rate tachometer (AT-601G; Nihon-Koden) which was triggered by tension signals. An equilibration time of at least 60 min preceded the commencement of each experiment. Drugs were directly applied to the bathing solution.

4. Measurement of Phosphodiesterase Activity: Fractions of phosphodiesterase (PDE) were prepared using elution chromatography according to a method similar to that reported by Thompson *et al.*[31] Hearts from guinea pigs were homogenized and sonicated at 4 °C in 5 volumes of 10 mmol/Tris–HCl buffer (pH 7.5) containing 2 mmol/l $MgCl_2$ and 1 mmol/l dithiothreitol. The homogenate was centrifuged at 9300 × *g* for 20 min and the supernatant was again centrifuged at 30000 × *g* for 20 min. The supernatant fraction thus obtained was applied to a column (DEAE-Toyopearl 650S; Toso, Tokyo, Japan). Three fractions of PDE activity (fractions I, II and III) were eluted with an acetate gradient. Each fraction was concentrated by ultrafiltration with PM-10 membrane (Amicon, Danvers, MA, U.S.A.), diluted with 65% ethyleneglycol and stored at −20 °C. PDE activity was determined basically according to the method reported by Thompson *et al.* Briefly, an appropriate dilution of each of the three fractions of the enzyme was incubated at 30 °C in 0.2 ml of medium containing 40 mmol/Tris–HCl, 10 mmol/$MgCl_2$, 3.75 mmol/l 2-mercaptoethanol, 25 μg bovine serum albumin, 1 μmol/l [^{3}H] cyclic AMP and a test compound. After incubation for 5 min, the reaction was terminated by boiling the medium and then cooling in an ice bath. The reaction mixture was incubated for an additional 10 min with 0.05 ml of 1 mg/ml snake venom. This reaction was terminated by the addition of 0.5 ml of a slurry consisting of 1 part resin AG-X2 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and 3 parts water. The tube containing the mixture was allowed to stand at 4 °C for at least 10 min and centrifuged at 6800 × *g* for 90 s. An aliquot (0.45 ml) of the supernatant was transferred to a vial containing ACS scintillator (Amersham, Buckinghamshire, England) and the radioactivity was determined by a liquid scintillation counter (LSC753; Aloka, Tokyo, Japan).

References and Notes

1) This work was presented in part at the 194th National Meeting of the American Chemical Society, New Orleans, Louisiana, Aug 1987, Abstracts of Papers, MEDI 58.
2) D. T. Mason, R. Zelis, G. Lee, J. Hughes, J. Spann and E. A. Amsterdam, *Am. J. Cardiol.*, **27**, 546 (1971).

3) D. T. Mason, E. A. Amsterdam and G. Lee, "Congestive Heart Failure," Dun-Donnelly, New York, 1976, p. 321.
4) R. R. Miller, A. R. Palomo, B. S. Brandon, C. J. Hartley and M. A. Quinones, *Am. Heart J.*, **102**, 500 (1981).
5) S. H. Taylor, B. Silke and G. I. C. Nelson, *Eur. Heart J.*, **3**, 19 (1982).
6) A. A. Alousi, J. M. Canter, M. J. Monternaro, D. J. Fort and R. A. Ferrari, *J. Cardiovasc. Pharmacol.*, **5**, 792 (1983).
7) R. A. Schnettler, R. C. Dage and J. M. Grisar, *J. Med. Chem.*, **25**, 1477 (1982).
8) R. C. Dage, L. E. Robel, C. P. Hseih and J. K. Woodward, *J. Cardiovasc. Pharmacol.*, **6**, 35 (1984).
9) D. W. Robertson, E. E. Beedle, J. H. Krushinski, G. D. Pollock, H. Wilson, V. L. Wyss and J. S. Hayes, *J. Med. Chem.*, **28**, 717 (1985).
10) J. A. Bristol, I. Sircar, W. H. Moos, D. B. Evans and R. E. Weishaar, *J. Med. Chem.*, **27**, 1099 (1984); I. Sircar, B. L. Duell, G. Bobowski, J. A. Bristol and D. B. Evans, *ibid.*, **28**, 1405 (1985).
11) J. C. A. Van Meel, *Arzneim.-Forsch.*, **35**, 284 (1985).
12) A. A. Alousi, G. P. Stankus, J. C. Stuart and L. H. Walton, *J. Cardiovasc. Pharmacol.*, **5**, 804 (1983); T. Kariga, J. L. Wille and R. C. Dage, *ibid.*, **6**, 50 (1984); R. E. Weishaar, M. H. Cain and J. A. Bristol, *J. Med. Chem.*, **28**, 537 (1985).
13) R. E. Weishaar, M. H. Cain and J. A. Bristol, *J. Med. Chem.*, **28**, 537 (1985); R. E. Weishaar, S. D. Burrows, D. C. Kobylarz, M. M. Quade and D. B. Evans, *Biochem. Pharmacol.*, **35**, 787 (1986).
14) Some of the 3-pyridinecarbonitriles were disclosed in Japan, patent, J 86-10557, by Jurszky *et al.* after we finished synthesizing them, but no *in vivo* data were shown there.
15) The similar procedure was described by G. Y. Lesher and B. Singh in U.S. Patent 4469699 (1984) [*Chem. Abstr.*, **101**, 211159*a* (1984)].
16) S. R. Baker, L. Crombie, R. V. Done and D. A. Slack, *J. Chem. Soc., Perkin Trans. 1*, **1979**, 677.
17) T. Kato, M. Sato and A. Wagi, *J. Heterocycl. Chem.*, **18**, 603 (1981).
18) L. Almirante, A. Mugnaini, L. P. Fritz and E. Provincial, *Boll. Chim. Farm.*, **105**, 32 (1966) [*Chem. Abstr.*, **65**, 700*b* (1966)].
19) G. Y. Lesher and R. E. Philino, U.S. Patent 4313951 (1982) [*Chem. Abstr.*, **97**, 216005*f* (1982)].
20) W. W. Paulder and H. L. Belwitt, *J. Org. Chem.*, **30**, 4081 (1978).
21) J. P. Panolini and R. K. Robins, *J. Heterocycl. Chem.*, **2**, 53 (1965). They reported mp 49—50 °C as 1/2 hydrate, but we obtained **19** of mp 75—77 °C, recrystallized from AcOEt–isopropyl ether–hexane after silica gel chromatography. ^{1}H-NMR ($CDCl_3$): 6.74 (1H, dd, J=2, 8 Hz, 6-H), 7.54 and 7.61 (each, 1H, s, 2- and 3-H), 7.62 (1H, d, J=2 Hz, 8-H), 8.04 (1H, d, J=8 Hz, 5-H).
22) Details will be described elsewhere.
23) T. Ogawa, H. Ohhara, H. Tsunoda, J. Kuroki and T. Shoji, *Arzneim-Forsch./Drug Res.*, **39**, 33 (1989); H. Ohhara, T. Ogawa, M. Takeda, H. Katoh, Y. Daiku and T. Igarashi, *ibid.*, **39**, 38 (1989); M. Ohoka, M. Honda, S. Morioka Y. Yamori and K. Moriyama, *Cardiovasc. Drug Ther.*, **3** (Suppl. 2), 618 (1989); S. Ishikawa, M. Honda, M. Ohoka, S. Morioka and K. Moriyama, *ibid.*, **3** (Suppl. 2), 595 (1989); H. Tanio, T. Kumada and Y. Himura, *ibid.*, **3** (Suppl. 2), 634 (1989); I. Ikuma, H. Ochi, T. Shimada, H. Toda, S. Morioka and K. Moriyama, *ibid.*, **3** (Suppl. 2), 593 (1989); H. Kuzuo, R. Murakami, T. Ohta, S. Morioka and K. Moriyama, *ibid.*, **3** (Suppl. 2), 604 (1989); H. Satoh and M. Endo, *Jpn. J. Pharmacol.*, **52**, 215 (1990); H. Kodaka, M. Sugimachi, M. Toyama, K. Sunakawa and M. Nakamura, *Jpn. Circ. J.*, **54** (Suppl.), Abstr. 0044 (1990).
24) G. Ferrari, *Boll. Chim. Farm.*, **96**, 542 (1957) [*Chem. Abstr.*, **52**, 7313*g* (1958)].
25) G. Ferrari and E. Marcon, *Farmaco* (Pavia) *Ed. sci.*, **13**, 485 (1958) [*Chem. Abstr.*, **53**, 7162*b* (1959); L. W. Deady, O. L. Korytsky and J. E. Rowe, *Aust. J. Chem.*, **35**, 2025 (1982).
26) E. C. Taylor and A. J. Crovetti, *J. Org. Chem.*, **19**, 1633 (1954).
27) Imidazo[1,2-*a*]pyridines as starting materials were prepared by minor modification of the procedure of E. S. Hand and W. W. Paulder, *J. Org. Chem.*, **43**, 2900 (1978).
28) R. Adams and A. W. Schreker, *J. Am. Chem. Soc.*, **71**, 1186 (1949). We used AcOH instead of 20% H_2SO_4 as solvent.
29) W. J. Link, R. F. Borne and F. L. Setliff, *J. Heterocycl. Chem.*, **4**, 641 (1967).
30) S. N. Godovikova and Gol'dfab, *Izv. Akad. Nauk SSSR, Ser. Khim.*, **8**, 1434 (1965) [*Chem. Abstr.*, **63**, 16334 (1965)].
31) W. J. Thompson, W. L. Terasaki, P. M. Epstein and S. J. Strada, *J. Adv. Cyclic Nucleotide Res.*, **10** 69 (1979).

Sophoraflavanones H, I and J, Flavonostilbenes from *Sophora moorcroftiana*[1)]

Yoshiaki SHIRATAKI,*[,a] Mami NOGUCHI,[a] Ichiro YOKOE,[a] Tsuyoshi TOMIMORI,[b] and Manki KOMATSU[a]

Faculty of Pharmaceutical Sciences, Josai University,[a] 1–1 Keyakidai, Sakado, Saitama 350–02, Japan and School of Pharmacy, Hokuriku University,[b] 3 Ho, Kanagawa-machi, Kanazawa 920–11, Japan. *Received December 20, 1990*

Three new prenylflavanones, sophoraflavanones H, I and J, with resveratrol residues, were isolated from the roots of *Sophora moorcroftiana* (Leguminosae).

The structures were determined by spectroscopic methods. Therefore, these were new types of natural products which, when combined with flavanones and stilbenes, were named flavonostilbenes.

Keywords *Sophora moorcroftiana*; Leguminosae; flavonostilbene; prenylflavanone; stilbene; resveratrol; sophoraflavanone H; sophoraflavanone I; sophoraflavanone J

The seeds of *Sophora moorcroftiana* BENTH. *ex.* BAKER (Leguminosae) are used in China as a crude drug known as "shā shēng huái" (砂生槐). In a previous paper, we reported the structural determination of two new flavonoids from the ether soluble fraction of the methanol extracts of the roots of this plant.[2)] Further investigations of the ether extract have now led to the isolation of three new flavanones, sophoraflavanones H, I and J (**1**—**3**), together with the known compounds, licoisoflavone A,[3)] euchrestaflavanone A,[4)] and phaseollidin.[5)] In this paper, we report the isolation and characterization of these three new compounds.

Sophoraflavanone H (**1**) was obtained as a yellow amorphous powder, mp 158—162 °C (dec.), $[\alpha]_D$ −114°. Elemental analysis and the fast atom bombardment mass spectrum (FAB-MS) indicated a mass of 582, agreeing with the formula $C_{34}H_{30}O_9$. It showed a positive Mg–HCl test. The infrared (IR) spectrum of **1** indicated the presence of hydroxyl (3400 cm^{-1}), carbonyl (1640 cm^{-1}) and aromatic (1620, 1520 cm^{-1}) rings. The ultraviolet (UV) spectrum absorptions at 289 (log ε 4.36) and $336_{(sh)}$ (log ε 3.70) nm, which shifted in the presence of $AlCl_3$ to 311 and 388 nm and NaOAc to 297 and 329 nm, indicated that the skeleton of **1** was a 5,7-dihydroxyflavanone.

The presence of ABX-type signals in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum (Table I) at δ 2.75 (1H, dd, J=16.9, 2.6 Hz), δ 3.12 (1H, dd, J=16.9, 13.6 Hz) and δ 5.71 (1H, dd, J=13.6, 2.6 Hz), attributable to the heterocyclic ring (C-ring) protons and the signals at δ 75.6 (d) and 42.8 (t) in ^{13}C-NMR spectrum, also supported that **1** was a flavanone derivative.

The FAB-MS spectrum of **1** showed major ion peaks at m/z 564, 362, 221 and 165. The ion peak at m/z 564 ($M^+ - H_2O$) showed the presence of a 2′-hydroxyl group in the flavanone nucleus.[2)] The ion peaks at m/z 362 and 221 were derived from a retro-Diels–Alder fragmentation of the C-ring. In view of the ^{1}H-NMR spectral and UV spectral data, the ion peak at m/z 221 must have been derived from the A-ring. This ion loses C_4H_8 to yield the ion peak at m/z 165; therefore, the A-ring contains a 3,3-dimethylallyl group and two hydroxyl groups.

The ^{1}H-NMR spectrum of **1** also shows the presence of a 3,3-dimethylallyl group [δ 1.48, 1.53 (each 3H, each s, $CH_3 \times 2$), 3.17 (2H, br d, J=7.0 Hz), 5.16 (1H, br t, J=7.0 Hz)] on an aromatic ring. In the aromatic region (δ 6.00—7.30) of the spectrum, three singlet signals appeared. The signals of δ 6.02 (1H, s), 6.52 (1H, s) and 7.24 (1H, s) were assigned to H-6 or H-8, H-3′ and H-6′, respectively.[6)] The signals of seven protons appeared as an A_2B_2 type [δ 6.86 (2H, d, J=8.4 Hz) and δ 7.23 (2H, d, J=8.4 Hz)] and an ABB′ type [δ 6.22 (2H, d, J=2.2 Hz) and δ 6.29 (1H, t, J=2.2 Hz)]. These were assigned to the protons of 1,4-disubstituted benzene and 1,3,5-trisubstituted benzene. The appearance of an AB type protons at δ 4.39 (1H, d, J=7.3 Hz) and δ 5.46 (1H, d, J=7.3 Hz) suggests the presence of a 2,3-diaryl-2,3-dihydrobenzofuran nucleus in **1** which compares with miyabenols A, B, C,[7)] α-viniferin,[8)] ε-viniferin,[7,8)] scirpusins A and B.[9)] Furthermore, the ^{1}H-NMR spectrum of **1** reveals six hydroxyls [δ 8.4—8.9 (5H, br) and 12.19 (1H, s, chelated with C-4 carbonyl); both of which are exchangeable in D_2O]. On the basis of the above data, it was suggested that **1** is a prenylflavanone having a 2,3-diaryl-2,3-dihydrofuran.

The position of the 3,3-dimethylallyl group on the A-ring was shown to be at C-8 according to the long-range selective proton decoupling (LSPD) method in the ^{13}C-NMR spectrum as follows. In the proton non-decoupled ^{13}C-NMR spectrum, a signal at δ 96.4 was observed as a double doublet (J=160.8, 6.6 Hz) which changed to a

TABLE I. ^{1}H-NMR Spectral Data for Sophoraflavanone H (**1**), Sophoraflavanone I (**2**) and Sophoraflavanone J (**3**) (270 MHz, δ from TMS in Acetone-d_6; J (Hz) in Parenthesis)

Protons	1	2	3
H-2	5.71 (dd, 13.6, 2.6)	5.70 (dd, 13.6, 2.6)	5.68 (dd, 13.6, 2.6)
H-3_{eq}	2.75 (dd, 16.9, 2.6)	2.76 (dd, 17.2, 2.6)	2.76 (dd, 17.2, 2.6)
H-3_{ax}	3.12 (dd, 16.9, 13.6)	3.08 (dd, 17.2, 13.6)	3.10 (dd, 17.2, 13.6)
H-6	6.02 (s)	6.01 (s)	—
H-3′	6.52 (s)	6.52 (s)	6.51 (s)
H-6′	7.24 (s)	7.25 (s)	7.23 (s)
H-1″	3.17 (br d, 7.0)	2.45—2.58 (m)	3.32[a)] (br d, 7.0)
H-2″	5.16 (br t, 7.0)	2.45—2.58 (m)	5.10 (br t, 7.0)
H-3″	—	1.96—2.07 (m)	—
H-4″	1.53 (s)	4.97 (br t, 7.0)	1.75 (s)
H-5″	1.48 (s)	—	1.64 (s)
H-6″	—	1.57 (s)	3.23[a)] (br d, 7.0)
H-7″	—	1.49 (s)	5.18 (br t, 7.0)
H-8″	—	—	—
H-9″	—	4.47 (br s)	1.54 (s)
H-10″	—	1.50 (s)	1.46 (s)
H-2‴,6‴	7.23 (d, 8.4)	7.23 (d, 8.8)	7.23 (d, 8.4)
H-3‴,5‴	6.86 (d, 8.4)	6.86 (d, 8.8)	6.85 (d, 8.4)
H-7‴	5.46 (d, 7.3)	5.44 (d, 7.7)	5.45 (d, 7.3)
H-8‴	4.39 (d, 7.3)	4.42 (d, 7.7)	4.37 (d, 7.3)
H-10‴,14‴	6.22 (d, 2.2)	6.22 (d, 2.2)	6.20 (d, 2.2)
H-12‴	6.29 (t, 2.2)	6.28 (t, 2.2)	6.27 (t, 2.2)
5-OH	12.19 (s)	12.22 (s)	12.52 (s)
OH × 5	8.4—8.9 (br)	8.2—9.0 (br)	8.1—8.5 (br)

Assignments were made with the aid of the ^{1}H–^{13}C 2D COSY and COLOC spectra. *a*) Assignments may be interchangeable in each column.

doublet ($J=160.8$ Hz) when the chelated hydroxyl proton at the C-5 position (δ 12.19) was selectively irradiated.[4b] So, the signal of δ 96.4 could be assigned to the C-6 carbon and the 3,3-dimethylallyl group must be located at C-8.

Since, two important cross peaks were observed in the ^1H–^{13}C shift correlated two dimensional (2D) NMR (COSY) spectrum of **1**, the carbon signals of δ 97.7 and 124.2 were assigned to C-3′ and C-6′ (Table II). Accordingly, the substituted pattern of the B-ring was concluded to be 1′,2′,4′ and 5′, and the 2,3-diaryl-2,3-dihydrofuran moiety was located at C-4′ and C-5′. The orientation of 2,3-diaryl-2,3-dihydrofuran to the B-ring was clearly determined by ^1H–^{13}C long-range COSY (COLOC) spectral analysis. That is, the 2D NMR spectrum of **1** led to precise assignments of the carbon signals [δ 57.7 (d, C-8‴), 94.4 (d, C-7‴), 107.3 (d, C-10‴ and C-14‴) and 128.4 (d, C-2‴ and C-6‴)]. The cross peaks between δ 57.7 (C-8‴)/δ 6.22 (H-10‴, H-14‴), δ 94.4 (C-7‴)/δ 7.23 (H-2‴, H-6‴), δ 107.3 (C-10‴, C-14‴)/δ 4.39 (H-8‴) and δ 6.29 (H-12‴), and δ 128.4 (C-2‴, C-6‴)/δ 5.46 (H-7‴) and 6.86 (H-3‴, H-5‴) were observed in the COLOC spectrum (Table II). From these results, it was clear that the 4-hydroxyphenyl group was located at C-7‴ and the 3,5-dihydroxyphenyl group was located at C-8‴.

The stereochemistry of **1** has been assigned from the results of circular dichroism (CD) spectrum and 2D nuclear Overhauser effect correlation spectroscopy (NOESY) experiments.

The relative configuration of **1** with the B-ring equatorial to the C-ring was assigned by the high value of $J_{2ax,3ax}=13.6$ Hz. The CD spectrum of **1** exhibited n-π^* and π-π^* Cotten effects at 333 and 300 nm, respectively, of opposite signs, indicating that **1** is a (2*S*) absolute configuration.[10]

The relative stereochemistry of C-7‴ and C-8‴ in **1** has been assigned from the results of NOESY experiments. NOE interactions were observed between H-7‴/H-10‴ (14‴), H-8‴/H-2‴ (6‴), H-3_{ax}/H-6′ and H-6′/H-10‴ (14‴), so the relative configuration of the two aryl groups were *trans* (Fig. 1). Furthermore, in the CD spectrum, **1** showed a negative maximum at 238 nm likes (−)-ε-viniferin,[11] so the absolute configuration was concluded as (7‴*R*, 8‴*R*).

Consequently, sophoraflavanone H was established to be structure **1**.

Sophoraflavanone I (**2**) was obtained as a yellow amorphous powder, mp 139—141° (dec.), $[\alpha]_D$ −100°. Elemental analysis and the FAB-MS indicated a mass of 650, agreeing with the formula $C_{39}H_{38}O_9$. It showed a positive Mg–HCl test. The UV and IR spectra were very similar to those of **1**, and the FAB-MS showed major fragment ions at *m/z*

Table II. NMR Spectral Data for Sophoraflavanone H (**1**) (270 MHz, δ from TMS in Acetone-d_6)

Assignment	^{13}C-NMR δ_C	Correlated ^{1}H δ_H	Long-range correlated ^{1}H ($J=10$ Hz)
C-2	75.6 (d)	5.71	3.12
C-3	42.8 (t)	2.75	—
		3.12	—
C-4	198.1 (s)	—	2.75, 3.12
C-5	163.0 (s)	—	6.02, 12.19
C-6	96.4 (s)	6.02	12.19
C-7	164.8 (s)	—	3.17
C-8	108.4 (s)	—	3.17, 6.02
C-9	161.6 (s)	—	3.17
C-10	103.2 (s)	—	6.02, 12.19
C-1′	122.7 (s)	—	4.39, 6.52
C-2′	156.0 (s)	—	6.52, 7.24
C-3′	97.7 (d)	6.52	—
C-4′	158.3 (s)	—	7.24
C-5′	119.2 (s)	—	5.71, 6.52
C-6′	124.2 (d)	7.24	5.71
C-1″	22.3 (t)	3.17	—
C-2″	123.4 (d)	5.16	1.48, 1.53, 3.17
C-3″	131.5 (s)	—	3.17
C-4″	25.8 (q)	1.53	1.48
C-5″	17.7 (q)	1.48	1.53
C-1‴	132.9 (s)	—	4.39, 6.86
C-2‴,6‴	128.4 (d)	7.23	5.46, 6.86
C-3‴,5‴	116.2 (d)	6.86	7.23
C-4‴	161.6 (s)	—	7.23
C-7‴	94.4 (d)	5.46	7.23
C-8‴	57.7 (d)	4.39	6.22
C-9‴	145.9 (s)	—	4.39, 5.46
C-10‴,14‴	107.3 (d)	6.22	4.39, 6.29
C-11‴,13‴	159.7 (s)	—	6.22, 6.29
C-12‴	102.3 (d)	6.29	6.22
C_5–OH		12.19	

Table III. NMR Spectral Data for Sophoraflavanone I (**2**) (270 MHz, δ from TMS in Acetone-d_6)

Assignment	^{13}C-NMR δ_C	Correlated ^{1}H δ_H	Long-range correlated ^{1}H ($J=10$ Hz)
C-2	75.7 (d)	5.70	3.08
C-3	43.0 (t)	2.76	—
		3.08	—
C-4	198.3 (s)	—	2.76
C-5	163.0 (s)	—	6.01, 12.22
C-6	96.3 (s)	6.01	12.22
C-7	165.2 (s)	—	2.45—2.58
C-8	108.0 (s)	—	2.45—2.58, 6.01
C-9	162.0 (s)	—	2.45—2.58
C-10	103.1 (s)	—	6.01, 12.22
C-1′	122.7 (s)	—	4.42, 6.52
C-2′	155.9 (s)	—	6.52, 7.25
C-3′	97.7 (d)	6.52	—
C-4′	158.3 (s)	—	7.25
C-5′	119.2 (s)	—	5.70, 6.52
C-6′	124.2 (d)	7.25	5.70
C-1″	27.9 (t)	2.45—2.58	—
C-2″	47.8 (d)	2.45—2.58	1.50, 2.45—2.58, 4.47
C-3″	31.9 (t)	1.96—2.07	2.45—2.58
C-4″	124.5 (d)	4.97	1.49, 1.57
C-5″	131.5 (s)	—	1.49, 1.57
C-6″	25.9 (q)	1.57	1.49
C-7″	17.9 (q)	1.49	1.57
C-8″	148.9 (s)	—	1.50, 1.96—2.07, 2.45—2.58
C-9″	111.2 (t)	4.47	1.50
C-10″	18.9 (q)	1.50	4.47
C-1‴	132.9 (s)	—	4.42, 6.86
C-2‴,6‴	128.4 (d)	7.23	5.44, 6.86
C-3‴,5‴	116.1 (d)	6.86	7.23
C-4‴	161.6 (s)	—	7.23
C-7‴	94.4 (d)	5.44	7.23
C-8‴	57.7 (d)	4.42	6.22
C-9‴	145.8 (s)	—	4.42, 5.44
C-10‴,14‴	107.3 (d)	6.22	4.42, 6.28
C-11‴,13‴	159.7 (s)	—	6.22, 6.28
C-12‴	102.3 (d)	6.28	6.22
C_5–OH		12.22	

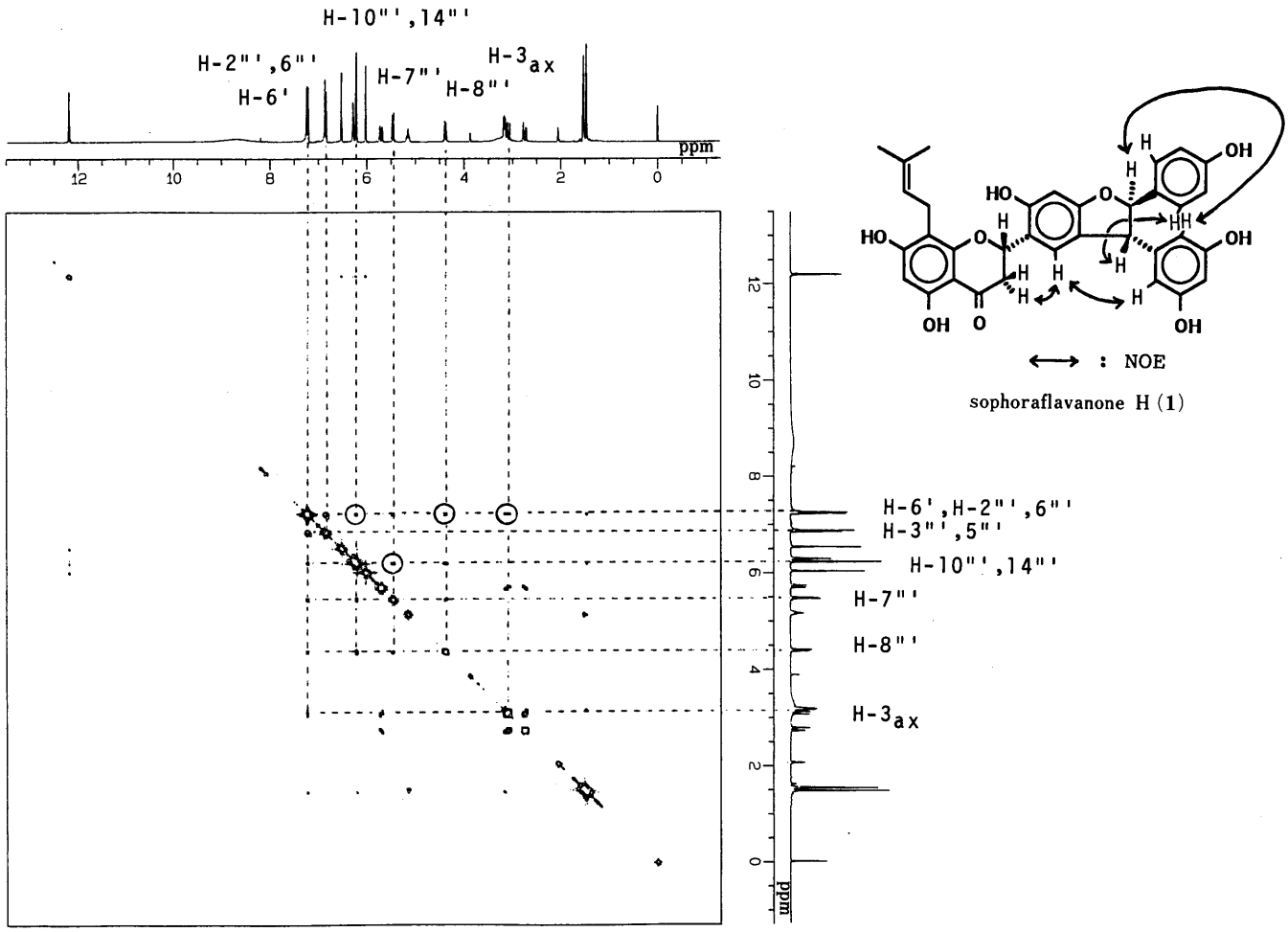


Fig. 1. Stereostructure of **1** Revealed by the 2D NOESY Spectrum

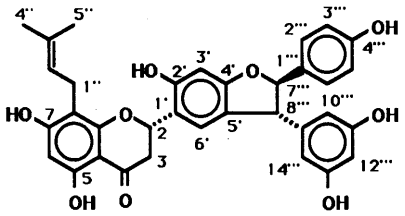
632 ($M^+ - H_2O$), 362 (B-ring), 289 (A-ring) and 165 (A-ring$-C_9H_{16}$). Therefore, the A-ring contains one C_{10} unit and two hydroxyl groups.

The ^{1}H-NMR spectrum of **2** was also similar to that of **1**, excluding the signals of δ 1.49, 1.50, 1.57 (each 3H, each s), δ 1.96—2.07 (2H, m), δ 2.45—2.58 (3H, m), δ 4.47 (2H, br s), δ 4.97 (1H, br t) which were assigned to a lavandulyl group. The position of the lavandulyl group on the A-ring was shown to be at C-8 by a ^{1}H–^{13}C COLOC spectrum and a chemical shift value, δ 12.22 assignable to a hydroxyl proton at C-5.[12] That is to say, in the ^{1}H–^{13}C COLOC spectrum, a cross peak between δ 12.22 assignable to a hydroxyl proton at C-5 and δ 96.3 assigned to C-6 was observed, and hence the lavandulyl group was located at C-8. The orientation of 2,3-diaryl-2,3-dihydrofuran was clearly determined by ^{1}H–^{13}C COLOC spectral analysis. That is, the 2D NMR spectrum of **2** led to precise assignments of the carbon signals [δ 57.7 (d, C-8‴), 94.4 (d, C-7‴), 107.3 (d, C-10‴ and C-14‴) and 128.4 (d, C-2‴ and C-6‴)]. The cross peaks between δ 57.7 (C-8‴)/δ 6.22 (H-10‴, H-14‴), δ 94.4 (C-7‴)/δ 7.23 (H-2‴, H-6‴), δ 107.3 (C-10‴, C-14‴)/δ 4.42 (H-8‴) and δ 6.28 (H-12‴), and δ 128.4 (C-2‴, C-6‴)/δ 5.44 (H-7‴) and 6.86 (H-3‴, H-5‴) were observed in the COLOC spectrum (Table III). From these results, it was clear that the 4-hydroxyphenyl group was located at C-7‴ and the 3,5-dihydroxyphenyl group was located at the C-8‴. Furthermore, the ^{1}H–^{13}C COSY and COLOC spectral experiments established complete signal assignments (Table III). Regarding the stereochemistry of **2**, the CD and NOESY spectra were very similar to those of **1**, so **2** also has a (2*S*, 7‴*R*, 8‴*R*) configuration.

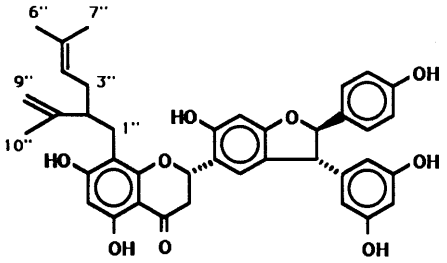
Consequently, the structure of sophoraflavanone I was concluded to be structure **2**.

Sophoraflavanone J (**3**) was obtained as a yellow amorphous solid, $[\alpha]_D$ −75°. Elemental analysis and the FAB-MS indicated a mass of 650, agreeing with the formula $C_{39}H_{38}O_9$. It showed a positive Mg–HCl test. The UV and IR spectra were very similar to those of **1** and the FAB-MS showed major fragment ions at *m/z* 632 ($M^+ - H_2O$), 362 (B-ring), 289 (A-ring) and 179 (A-ring$-C_4H_7 \times 2$). Therefore, the A-ring contains two 3,3-dimethylallyl groups and two hydroxyl groups. The ^{1}H-NMR spectrum of **3** was also similar to that of **1**, excluding the signals of δ 1.64, 1.75 (each 3H, each s, $CH_3 \times 2$), 3.32 (2H, br d, J=7.0 Hz), and 5.10 (1H, br t, J=7.0 Hz) which were assigned to another 3,3-dimethylallyl group, but it cannot be seen whether the signals near δ 6.0 are assignable to H-6 or H-8 in the flavanone nucleus (Table I). From these results, two 3,3-dimethylallyl groups were located at both C-6 and C-8. The other partial structure of **3** was the same as **1** and **2** according to ^{1}H-NMR spectral analysis.

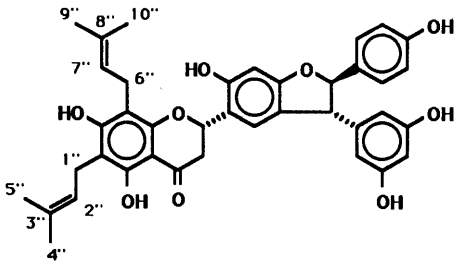
About the stereochemistry of **3**, the CD spectrum was very similar that of **1**, so **3** also has a (2*S*, 7‴*R*, 8‴*R*) configuration.



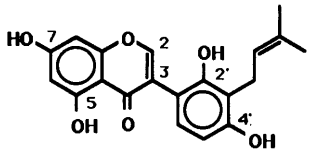
sophoraflavanone H (1)



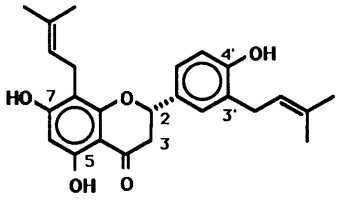
sophoraflavanone I (2)



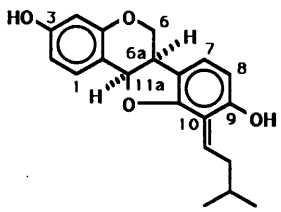
sophoraflavanone J (3)



licoisoflavone A



euchrestaflavanone A



phaseollidin

Consequently, the structure of sophoraflavanone J was concluded to be structure **3**.

These were new natural products which were combined with flavanones and stilbenes, although flavonolignans[13] (*e.g.* silybin, silydianin and silychristin) which were combined with flavanones and phenylpropans were also isolated from the seeds of *Silybum marianum*. Therefore, we proposed here that these new natural products be named flavonostilbenes.

Sophoraflavanones H, I and J (**1**—**3**) should be biosynthesized by oxidative coupling of 5,7,2′,4′-tetrahydroxy 8-(3,3-dimethylallyl)flavanone, 5,7,2′,4′-tetrahydroxy 8-lavandulylflavanone, 5,7,2′,4′-tetrahydroxy 6,8-di(3,3-dimethylallyl)-flavanone and resveratrol as postulated previously in biogenesis of the scirpusins A, B[9] and oligostilbenes.[11]

Furthermore, licoisoflavone A, euchrestaflavanone A, and phaseollidin were also isolated. These are new components of this plant.

Experimental

All melting points were determined on a Yanagimoto MP-S3 micro melting point apparatus and are uncorrected. IR and UV spectra were taken on Nihon Bunko IR-810 and UVIDEC-430 machines, respectively. ^{1}H- and ^{13}C-NMR spectra were obtained on a JEOL JNM GX-270 FT NMR spectrometer at 270 and 67.9 MHz, respectively, and chemical shifts are given in δ (ppm) with tetramethylsilane (TMS) as an internal standard (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad). The multiplicities (s, d, t, q) of carbon signals were determined by means of gated decoupling with NOE. MS were taken on a JEOL JMS DX-300 mass spectrometer with a direct inlet system. The $[\alpha]_D$ was recorded at 589 nm on a JASCO J-20A spectrometer. Column chromatography was performed on silica gel (Merck, Kieselgel 60 Art. 7734 and Fuji Davison silica gel BW-820 MH). Thin layer chromatography (TLC) was conducted on a Wakogel B-5FM, Analtech Silica gel GF and high performance TLC (HPTLC-Fertigplatten RP-18 WF 254S Art. 13124, Merck). The solvent systems were benzene : ethyl acetate = 1 : 1, MeOH : H_2O = 3 : 1, respectively. The spots were detected by spraying with diluted H_2SO_4 followed by heating.

Extraction and Separation The dried roots of *Sophora moorcrofitiana*, which were collected in Jomsom, Nepal in September, 1986 (1.1 kg) were extracted four times with boiling MeOH. The MeOH extract (228 g) was shaken with Et_2O and H_2O. The Et_2O extract was concentrated (121 g) and chromatographed on silica gel using benzene–ethyl acetate (10 : 0—1 : 1) as solvents (each fraction was checked by TLC) to give *l*-maackiain (1.3 g), licoisoflavone B (783 mg), sophoraflavanone B (401 mg), medicagol (60 mg), a mixture of licoisoflavone A, phaseollidin and euchrestaflavanone A, sophoraisoflavone A (1.6 g), sophoraflavanone G (599 mg), a mixture of sophoraflavanones H, I and J, and calycosin (307 mg) in that order. The mixture of licoisoflavone A, phaseollidin and euchrestaflavanone A were subjected to low-pressure liquid chromatography (LC) on octadecyl chemically bonded silica gel (ODS) (CPO-HS-221-20, C.I.G. Column system, Kusano Sci. Man. Co., Ltd., Japan) using MeOH : H_2O = 3 : 1 to yield licoisoflavone A (383 mg), phaseollidin (26 mg) and euchrestaflavanone A (38 mg). The mixture of sophoraflavanones H, I and J were also subjected to LC on ODS using MeOH : H_2O = 2 : 1 to yield sophoraflavanones H (374 mg), I (51 mg), and J (6 mg).

Sophoraflavanone H (1) Yellow amorphous powder (benzene), mp 158—162 °C (dec.), $[\alpha]_D^{22}$ −114° (c = 1.0, MeOH), Mg–HCl test (+). *Anal.* Calcd for $C_{34}H_{30}O_9 \cdot H_2O$: C, 67.98; H, 5.37. Found: C, 67.93; H, 5.50. Positive FAB-MS m/z: 583 $(M+H)^+$, 564, 362, 221, 165. UV λ_{max}^{MeOH} nm (log ε): 289 (4.36), $336_{(sh)}$ (3.70). UV $\lambda_{max}^{MeOH+AlCl_3}$ nm (log ε): 311 (4.40), 388 (3.60). UV $\lambda_{max}^{MeOH+NaOAc}$ nm (log ε): 297 (4.23), 329 (4.44). CD ($c = 2 \times 10^{-3}$, MeOH) $\Delta\varepsilon^{24}$: −30.42 (238) (negative maximum), −2.65 (262) (positive maximum), −4.85 (275) (negative maximum), −3.09 (285) (positive maximum), −14.58 (300) (negative maximum), +3.08 (333) (positive maximum). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1640 (C=O), 1620, 1520 (arom. C=C). ^{1}H- and ^{13}C-NMR; see Tables I and II.

Sophoraflavanone I (2) Yellow amorphous powder (benzene), mp 139—141 °C (dec.), $[\alpha]_D^{22}$ −100° (c = 1.0, MeOH), Mg–HCl test (+). *Anal.* Calcd for $C_{39}H_{38}O_9 \cdot 1/2H_2O$: C, 69.26; H, 5.88. Found: C, 69.20; H, 5.99. Positive FAB-MS m/z: 651 $(M+H)^+$, 632, 362, 289, 165. UV λ_{max}^{MeOH} nm (log ε): 289 (4.30), $343_{(sh)}$ (3.56). UV $\lambda_{max}^{MeOH+AlCl_3}$ nm (log ε): 313 (4.31), 391 (3.55). UV $\lambda_{max}^{MeOH+NaOAc}$ nm (log ε): 301 (4.17), 333 (4.36). CD ($c = 2 \times 10^{-3}$, MeOH) $\Delta\varepsilon^{24}$: −25.26 (238) (negative maximum), 0 (259) (positive maximum), −2.46 (275) (negative maximum), −1.97 (285) (positive maximum), −11.82 (300) (negative maximum), +2.46 (335) (positive maximum). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1640 (C=O), 1605, 1500 (arom. C=C). ^{1}H- and ^{13}C-NMR; see Tables I and III.

Sophoraflavanone J (3) Yellow amorphous solid (MeOH), $[\alpha]_D^{22}$ −75° (c = 1.0, MeOH), Mg–HCl test (+). *Anal.* Calcd for $C_{39}H_{38}O_9 \cdot 3/2H_2O$: C, 69.11; H, 6.10. Found: C, 68.91; H, 6.11. Positive FAB-MS m/z: 651 $(M+H)^+$, 632, 362, 289, 179. UV λ_{max}^{MeOH} nm (log ε): 291 (4.32), $342_{(sh)}$ (3.55). UV $\lambda_{max}^{MeOH+AlCl_3}$ nm (log ε): 318 (4.36), 402 (3.35). UV $\lambda_{max}^{MeOH+NaOAc}$ nm (log ε): 288 (4.06), 329 (4.39). CD ($c = 4 \times 10^{-3}$, MeOH) $\Delta\varepsilon^{24}$: −21.32 (236) (negative maximum), −1.85 (262) (positive maximum), −2.14 (270) (negative maximum), −0.53 (285) (positive maximum), −8.71 (300) (negative maximum), +0.66 (345) (positive maximum). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1640 (C=O), 1605, 1500 (arom. C=C). ^{1}H-NMR; see Table I.

Licoisoflavone A Pale yellow needles (benzene–MeOH), mp 110—113 °C. ^{1}H-NMR (DMSO-d_6): 1.63, 1.72 (each 3H, each s, Me × 2), 3.28 (2H, br d, J=6.8 Hz, Ar–C$\underline{H}_2$–CH=), 5.22 (1H, br t, J=6.8 Hz, –CH_2–C$\underline{H}$=C<), 6.25 (1H, d, J=2.2 Hz, H-6), 6.34 (1H, d, J=8.3 Hz, H-5′), 6.40 (1H, d, J=2.2 Hz, H-8), 6.76 (1H, d, J=8.3 Hz, H-6′), 8.11 (1H, s, H-2), 8.26, 9.31, 10.86 (each 1H, each br s, OH; exchangeable in D_2O), 12.87 (1H, s, C_5-OH; exchangeable in D_2O). ^{13}C-NMR (DMSO-d_6): 17.7 (q, Me), 22.4 (t, Ar–C$\underline{H}_2$–CH=), 25.5 (q, Me), 98.9 (d, C-6), 104.7 (s, C-10), 106.7 (d, C-5′), 109.5 (s, C-3′), 115.4 (s, C-1′), 121.1 (d, C-3), 123.5 (d, –CH_2–C$\underline{H}$=C<), 128.6 (d, C-6′), 129.5 (s, –CH=C<), 154.0 (s, C-2′), 155.6 (d, C-2), 156.3 (s, C-4′), 157.7 (s, C-9), 161.8 (s, C-5), 164.1 (s, C-7), 181.0 (s, C-4). The spectral data for this compound was identical with that reported for licoisoflavone A.

Euchrestaflavanone A Colorless needles (benzene–$CHCl_3$), mp 145—147 °C. ^{1}H-NMR (acetone-d_6): 1.61 (6H, s, Me × 2), 1.72 (6H, s, Me × 2), 2.75 (1H, dd, J=16.9, 2.9 Hz, H_{eq}-3), 3.12 (1H, dd, J=16.9, 12.5 Hz, H_{ax}-3), 3.22 (2H, br d, J=7.3 Hz, Ar–C$\underline{H}_2$–CH=), 3.36 (2H, br d, J=7.3 Hz, Ar–C$\underline{H}_2$–CH=), 5.21 (1H, br t, J=7.3 Hz, –CH_2–C$\underline{H}$=C<), 5.37 (1H, br t, J=7.3 Hz, –CH_2–C$\underline{H}$=C<), 5.42 (1H, dd, J=12.5, 2.9 Hz, H-2), 6.02 (1H, s, H-6), 6.89 (1H, d, J=8.1 Hz, H-5′), 7.22 (1H, dd, J=8.1, 2.2 Hz, H-6′), 7.31 (1H, d, J=2.2 Hz, H-2′), 8.98 (2H, br s, OH × 2; exchangeable in D_2O), 12.15 (1H, s, C_5-OH; exchangeable in D_2O). ^{13}C-NMR (acetone d_6): 17.9 (q, Me × 2), 22.3 (t, Ar–C$\underline{H}_2$–CH=), 25.9 (q, Me × 2), 29.1 (t, Ar–C$\underline{H}_2$–CH=), 43.5 (t, C-3), 79.9 (d, C-2), 96.4 (d, C-6), 103.3 (s, C-10), 108.3 (s, C-8), 115.7 (d, C-5′), 123.5 (d, –CH_2–C$\underline{H}$=C<), 123.7 (d, –CH_2–C$\underline{H}$=C<), 126.0 (d, C-2′), 128.8 (d, C-6′), 128.9 (s, C-3′), 131.1 (s, –CH=C<), 131.2 (s, –CH=C<), 132.7 (s, C-1′), 156.0 (s, C-4′), 161.1 (s, C-9), 163.0 (s, C-5), 165.0 (s, C-7), 197.6 (s, C-4). This was identified by direct comparison (mixed melting point, $[\alpha]_D$, TLC, IR, MS, ^{1}H-NMR and ^{13}C-NMR) with an authentic sample isolated from *E. japonica*.

Phaseollidin Yellow powder (benzene–$CHCl_3$), mp 67—69 °C. $[\alpha]_D^{22}$ −220° (c=1.0, MeOH). ^{1}H-NMR (acetone-d_6): 1.61, 1.73 (each 3H, each s, Me × 2), 3.26 (2H, br d, J=7.0 Hz, Ar–C$\underline{H}_2$–CH=), 3.54 (1H, m, H-6a), 3.57 (1H, dd, J=10.8, 10.5 Hz, H-6_{ax}), 4.24 (1H, m, H-6_{eq}), 5.27 (1H, br t, J=7.0 Hz, –CH_2–C$\underline{H}$=C<), 5.46 (1H, d, J=6.6 Hz, H-11a), 6.36 (1H, d, J=2.6 Hz, H-4), 6.39 (1H, d, J=7.7 Hz, H-8), 6.56 (1H, dd, J=8.4, 2.6 Hz, H-2), 6.95 (1H, d, J=7.7 Hz, H-7), 7.34 (1H, d, J=8.4 Hz, H-1), 8.4 (2H, br s, OH × 2; exchangeable in D_2O). ^{13}C-NMR (acetone-d_6): 17.9 (q, Me), 23.5 (t, Ar–C$\underline{H}_2$–CH=), 25.9 (q, Me), 40.9 (d, C-6a), 67.2 (t, C-6), 78.9 (d, C-11a), 103.9 (d, C-4), 108.1 (d, C-8), 110.4 (d, C-2), 112.0 (s, C-10), 113.2 (s, C-1a), 119.0 (s, C-7a), 122.7 (d, C-7), 123.6 (d, –CH_2–C$\underline{H}$=C<), 131.3 (s, –CH=C<), 133.1 (d, C-1), 156.8 (s, C-9), 157.7 (s, C-4a), 159.6 (s, C-3), 159.7 (s, C-10a). The spectral data for this compound was identical with that reported for phaseollidin.

Acknowledgments We are grateful to Misses M. Seto and Y. Nagasawa for their helpful assistance throughout the course of this work. Thanks are also due to Mr. S. Yamaguchi of Josai University for the NMR spectral measurements. Y. S. is grateful to President T. Matsu-ura, Josai University, for a grant to support a part of this work.

References and Notes

1) Part XXIV in the series "Studies on the Constituents of *Sophora* Species." For Part XXIII, See Y. Shirataki, T. Tsuzuku, I. Yokoe, R. T. Hirano, and M. Komatsu, *Chem. Pharm. Bull.*, **38**, 1712 (1990). This paper also forms Part 2 in the series "Constituents of the Roots of *Sophora moorcroftiana*"; for Part 1, See Y. Shirataki *et al.*[2]
2) Y. Shirataki, I. Yokoe, M. Noguchi, T. Tomimori, and M. Komatsu, *Chem. Pharm. Bull.*, **36**, 2220 (1988).
3) T. Kinoshita, T. Saitoh, and S. Shibata, *Chem. Pharm. Bull.*, **26**, 141 (1978).
4) *a*) Y. Shirataki, M. Komatsu, I. Yokoe, and A. Manaka, *Chem. Pharm. Bull.*, **29**, 3033 (1981); *b*) Y. Shirataki, I. Yokoe, M. Endo, and M. Komatsu, *ibid.*, **33**, 444 (1985).
5) D. R. Perrin and C. P. Whittle, *Tetrahedron Lett.*, **1972**, 1673.
6) Y. Shirataki, A. Manaka, I. Yokoe, and M. Komatsu, *Phytochemistry*, **21**, 2959 (1982).
7) K. Suzuki, T. Shimizu, J. Kawabata, and J. Mizutani, *Agric. Biol. Chem.*, **51**, 1003 (1987).
8) P. Langcake and R. J. Pryce, *Phytochemistry*, **16**, 1193, 1452 (1977); *Idem*, *Experientia*, **33**, 151 (1977); S. Kitanaka, T. Ikezawa, K. Yasukawa, S. Yamanouchi, M. Takido, H. K. Sung, and I. H. Kim, *Chem. Pharm. Bull.*, **38**, 432 (1990).
9) K. Nakajima, H. Taguchi, T. Endou, and I. Yoshioka, *Chem. Pharm. Bull.*, **26**, 3050 (1978).
10) W. Gaffield, *Tetrahedron*, **26**, 4093 (1970).
11) H. Kurihara, J. Kawabata, S. Ichikawa, and J. Mizutani, *Agric. Biol. Chem.*, **54**, 1097 (1990); H. Kurihara, J. Kawabata, S. Ichikawa, M. Mishima, and J. Mizutani, *Phytochemistry*, **30**, 649 (1991).
12) M. Iinuma, T. Tanaka, M. Mizuno, Y. Shirataki, I. Yokoe, M. Komatsu, and F. A. Lang, *Phytochemistry*, **29**, 2667 (1990).
13) H. Wagner, L. Hörhammer, and R. Münster, *Naturwissenschften*, **52**, 305 (1965); A. Pelter and R. Hänsel, *Chem. Ber.*, **108**, 790, (1975); R. Hänsel, J. Schulz, and A. Pelter, *ibid.*, **108**, 1482 (1975); H. Wagner, O. Seligmann, M. Seitz, D. Abraham, and J. Sonnenbichler, *Z. Naturforsch.*, **31B**, 876 (1976); H. Wagner, V. M. Chari, M. Seitz, and I. Riess-Maurer, *Tetrahedron Lett.*, **1978**, 381.

Heats of Dissolution of Thiamine Disulfide–Fatty Acids Complexes in Ethanol

Shoko YOKOYAMA,*[,a] Fumio UEDA,[b] Akiko KANEKO,[a] and Tadao FUJIE[a]

Kyoritsu College of Pharmacy,[a] 1–5–30, Shibakoen, Minato-ku, Tokyo 105, Japan and Research Laboratory, Kawai Seiyaku Co., Ltd.,[b] 6–3–5, Nakano-ku, Tokyo 164, Japan. *Received January 9, 1991*

The heats of dissolution (ΔH_d) of thiamine disulfide (TDS)–fatty acids (FA) complexes, $(FA)_6$ (TDS), were measured at 310.15 K in ethanol using a calorimetric technique, where the FA are tetradecanoic acid (C14), pentadecanoic acid (C15), hexadecanoic acid (C16), heptadecanoic acid (C17) and octadecanoic acid (C18). The values of ΔH_d were 432.8, 475.0, 493.9, 541.0 and 558.5 kJ mol^{-1} for $(C14)_6$ (TDS), $(C15)_6$ (TDS), $(C16)_6$ (TDS), $(C16)_6$ (TDS), $(C17)_6$-(TDS) and $(C18)_6$ (TDS), respectively. The values of ΔH_d of $(FA)_6$(TDS) increased by increasing the carbon numbers (n) of the constituent fatty acids. However, the plots of ΔH_d of $(FA)_6$ (TDS) against n showed a zig-zag pattern which indicates an upward convex at an odd-numbered position, while the plots[1] of ΔH_d of FA against n indicate a single line pattern.

The differences between ΔH_d of $(FA)_6$ (TDS) and ΔH_d of (6FA + TDS) were 23—37 kJ mol^{-1} for even-numbered FA and 33—41 kJ mol^{-1} for odd-numbered FA, indicating a stronger binding force for $(FA)_6$ (TDS) formed from odd-numbered FA than those formed from even-numbered FA. Furthermore, the estimated values of the binding force between FA and TDS are very small, leading to a conclusion that $(FA)_6$ (TDS) is a clathrate (or an inclusion compound) formed by van der Waals forces and hydrophobic interactions between FA and TDS.

Keywords dissolution heat; thiamine disulfide; fatty acid; complex; ethanol; calorimetric technique; binding force; van der Waals force; hydrophbic interaction; clathrate

It has been reported that fatty acids (FA) form crystalline complexes with thiamine disulfide (TDS) whose molar ratio is 6:1.[2] In addition, it is suggested from studies on the release of TDS from TDS–FA complexes that the binding force between FA and TDS is not very strong[3] and that TDS–FA complexes have the property of an inclusion compound.[4] However, further details have not yet been obtained.

Heats of dissolution (ΔH_d) is the difference in thermal quantities between the solid and solution states, and ΔH_d is accompanied by various changes in the states: one of which is the breaking down of the crystal structure. When a solute is dissolved in a solvent, it is necessary to break the binding forces, hydrogen bond and/or van der Waals forces *etc.*, between the solute molecules. A little information on the molecular structure can be obtained by the measurement of ΔH_d. From these points of view, we tried to measure the ΔH_d of TDS–FA complexes, $(FA)_6$ (TDS). For the measurement of ΔH_d, it is necessary to choose a proper solvent in which FA and TDS are easily dissolved. Furthermore, solvents in which FA exist as monomeric species are good the estimation of the binding forces between FA and TDS.

ΔH_d of FA in ethanol has been measured,[1] and it has been shown that the plots of ΔH_d of FA against the carbon numbers (n) in the FA indicate a linear pattern.[1] On the other hand, the plots of the release rate constant of TDS from $(FA)_6$ (TDS) against n showed a zig-zag pattern which indicates a downward convex at the odd-numbered positions, though the release rate constant decreased rather regularly with an increase of the alkyl chain length for only even-numbered or odd-numbered FA.[4] The delayed release rate for $(FA)_6$ (TDS) composed of odd-numbered FA may be due to a stronger interaction between odd-numbered FA and TDS as compared with even-numbered FA and TDS. It is, therefore, expected that the plots of ΔH_d of $(FA)_6$-(TDS) against n will show a zig-zag pattern which indicates an upward convex at the odd-numbered positions. It is very interesting to know the ΔH_d of $(FA)_6$ (TDS).

From these points of view, ΔH_d of $(FA)_6$ (TDS) in ethanol were measured. ΔH_d of TDS in ethanol was also measured. Furthermore, the binding force concerned in the formation of $(FA)_6$ (TDS) was estimated by using the values of ΔH_d of FA[1] and TDS, and the structure of $(FA)_6$ (TDS) was inferred.

Experimental

Materials Tetradecanoic acid (C14), pentadecanoic acid (C15), hexadecanoic acid (C16), heptadecanoic acid (C17) and octadecanoic acid (C18) were the same as those used previously.[1] $(FA)_6$ (TDS) was prepared as previously described.[2–4] Ethanol was the same as that used previously.[1] Cyclohexane of guaranteed reagent grade was obtained from Wako Pure Chemical Industries, Ltd. TDS in the $(FA)_6$ (TDS) was found[2] to be anhydrous according to elementary analysis, although TDS used for the preparation of $(FA)_6$ (TDS) was hydrate. Anhydrate TDS used for the measurement of ΔH_d was obtained from the following treatment: About 1.5 g of $(FA)_6$ (TDS) was added to 100 ml of cyclohexane at about 40 °C in order to dissolve only FA. TDS is insoluble in cyclohexane under these conditions. A residue was collected by filtration, washed in cyclohexane, and dried. This residue did not melt at the melting points of FA and $(FA)_6$-(TDS), and did not change into a brown color at 130 °C, and it decomposed with bubbling at about 175 °C. (A hydrate TDS changes into brown color at 130 °C and decomposes with bubbling at 160 °C). Furthermore, this residue was confirmed to be anhydrate TDS from the elementary analysis.

Measurement of ΔH_d The apparatus for the measurement of ΔH_d was a twin differential conduction-type microcalorimeter as described in the previous paper.[1] About 0.2 g of $(FA)_6$ (TDS) sealed into glass ampules was immersed into 100 ml of ethanol (final concentration is 1.00×10^{-3} mol dm^{-3} for each $(FA)_6$ (TDS)). For TDS, ΔH_d was measured at various concentrations. About 0.01—0.2 g of TDS sealed into glass ampules were immersed into 100 ml of ethanol (final concentration is 2.00×10^{-4}—4.00×10^{-3} mol dm^{-3}). The sealed ampules were kept in a calorimeter until thermal equilibrium was established. All measurements were carried out at 310.15 K. The reproducibility was estimated by measuring ΔH_d for each sample at least five times. The uncertainty was determined by twice the standard deviation of the mean of five experiments.

Results

Values of ΔH_d of TDS The final solution concentrations of TDS were 2.00×10^{-4}, 1.00×10^{-3} and 4.00×10^{-3} mol dm^{-3}. TDS dissolved endothermally in ethanol. The values of ΔH_d of TDS at three concentrations are shown in Table I.

TABLE I. Heats of Dissolution, ΔH_d, of TDS in Ethanol at 310.15 K with Varying Final Concentrations

C (mol dm^{-3})	ΔH_d (kJ mol^{-1})
2.00×10^{-4}	47.1 ± 1.2
1.00×10^{-3}	46.5 ± 0.5
4.00×10^{-3}	46.7 ± 0.5

TABLE II. Heats of Dissolution, ΔH_d, of $(FA)_6$(TDS) in Ethanol at 310.15 K, the Values of ΔH_d of (6FA + TDS), and the Differences, $\Delta\Delta H_d$, between $(FA)_6$(TDS) and (6FA + TDS)

FA	ΔH_d (kJ mol^{-1})		$\Delta\Delta H_d$ (kJ mol^{-1})
	$(FA)_6$(TDS)[a]	6FA[1] + TDS	
C14	432.8 ± 1.5	410.3	22.5
C15	475.0 ± 2.6	442.3	32.7
C16	493.9 ± 1.9	462.7	31.2
C17	541.0 ± 2.4	500.3	40.7
C18	558.5 ± 2.5	521.1	37.4

a) Final concentration is 1.00×10^{-3} mol dm^{-3}.

ΔH_d was nearly constant within this concentration range. This is a phenomenon similar to that observed for the ΔH_d of C18 in ethanol.[1]

Values of ΔH_d of $(FA)_6$(TDS) As described in the previous paper,[1] no concentration dependence of ΔH_d of C18 has been found within the concentration range of 1×10^{-3}—2×10^{-2} mol dm^{-3}. Furthermore, no concentration dependence of ΔH_d of TDS was found within the concentration range of 2×10^{-4}—4×10^{-3} mol dm^{-3}, as described in the previous section. ΔH_d of $(FA)_6$ (TDS) was, therefore, measured at a constant concentration (final solution concentration of $(FA)_6$ (TDS) was 1×10^{-3} mol dm^{-3}), and the results are summarized in Table II. ΔH_d increased with an increase in carbon numbers (n) of the constituent FA.

Discussion

Effect of Alkyl Chain Length of FA on ΔH_d of $(FA)_6$-(TDS) The values of ΔH_d of $(FA)_6$ (TDS) were plotted against n, and shown in Fig. 1. In the results, the plots of ΔH_d *vs.* n displayed a zig-zag pattern which indicates an upward convex at odd-numbered positions. This phenomenon suggests a stronger binding force for $(FA)_6$ (TDS) formed from odd-numbered FA, $(C_{2a-1})_6$ (TDS), than from even-numbered FA, $(C_{2a})_6$ (TDS), because the ΔH_d of FA[1] increases linearly by increasing n.

The zig-zag line can be separated by two individual lines among $(FA)_6$ (TDS), with the even-numbered FA and the odd-numbered FA drawn by two dotted lines as in Fig. 1. The upper line represents $(C_{2a-1})_6$ (TDS) and the lower one $(C_{2a})_6$ (TDS), where the dotted line for $(C_{2a-1})_6$ (TDS) was drawn according to the slope for $(C_{2a})_6$ (TDS) since two runs were conducted for $(C_{2a-1})_6$ (TDS). The difference between the two dotted lines is obtained as approximately 13 kJ mol^{-1}. The $\{(CH_2)_2\}_6$-increment of ΔH_d is obtained as 61.50 ± 0.25 kJ (mol $-\{(CH_2)_2\}_6-)^{-1}$ from the slope of the dotted line for $(C_{2a})_6$ (TDS). One mole of $(FA)_6$ (TDS) is composed of six moles of FA. Therefore, the (CH_2)-increment of FA was obtained as 5.125 ± 0.03 kJ (mol $-CH_2-)^{-1}$. The increment in ΔH_d of FA as a function of the number of methylene groups ($-CH_2-$) in the alkyl chain has been obtained[1] as 4.40 ± 0.10 kJ (mol $-CH_2-)^{-1}$. The (CH_2)-increment of ΔH_d obtained from the values of ΔH_d of $(FA)_6$ (TDS) is slightly larger in comparison with that obtained from the value[1] of FA.

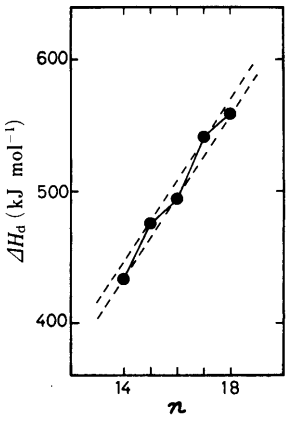


Fig. 1. Heats of Dissolution, ΔH_d, of $(FA)_6$ (TDS) in Ethanol
Temperature: 310.15 K.

Binding Force between FA and TDS The following bases and postulations were used to estimate the binding force between FA and TDS: (1) $(FA)_6$ (TDS) is not formed from the dissolved FA and TDS in an ethanol solution;[2] (2) for the comparison of ΔH_d among $(C14)_6$ (TDS)—$(C18)_6$-(TDS), the heat of breaking the interaction between the ethanol molecules can be eliminated from consideration, because this process is common in the dissolution of each $(FA)_6$ (TDS).

The values of summation of ΔH_d of six moles of FA[1] and one mole of TDS were calculated, and summarized in Table II, where 46.5 kJ mol^{-1} at 1.00×10^{-3} mol dm^{-3} was used as the value of ΔH_d of TDS. The values of ΔH_d of $(FA)_6$ (TDS) are larger than those of (6FA + TDS). The differences ($\Delta\Delta H_d$) were shown in the righthand column of Table II. The value of $\Delta\Delta H_d$ for $(C_{2a-1})_6$ (TDS) was slightly larger than that for $(C_{2a})_6$ (TDS) whose alkyl chain of FA is one carbon number longer, though the values of $\Delta\Delta H_d$ increased rather regularly with an increase of the alkyl chain length for only even-numbered or odd-numbered FA. It is suggested that the binding force between FA and TDS for $(C_{2a-1})_6$ (TDS) is stronger than that $(C_{2a})_6$ (TDS).

Regarding the release of TDS from $(FA)_6$ (TDS), the following has been found:[4] the plots of the release rate constant of TDS from $(FA)_6$ (TDS) against the carbon numbers of the constituent FA show a zig-zag pattern which indicates a downward convex at odd-numbered positions; and the plots of the positive values of activation enthalpy $\Delta H^{\ddagger}$ for the release of TDS from $(FA)_6$ (TDS) against the carbon numbers of the constituent FA show a zig-zag line which indicates an upward convex at odd-numbered positions. These phenomena can be explained by the values of $\Delta\Delta H_d$ for $(C_{2a})_6$ (TDS) and $(C_{2a-1})_6$ (TDS).

Regarding the mechanism of the fomation of $(FA)_6$-(TDS), which is obtained as a crystal in a 1,2-dichloroethane solution, it has been suggested[5] that FA forms a hydrogen-bonded cyclic hexamer similar to a reversed micelle in 1,2-dichloroethane, and also that TDS is solubilized in

the FA association in the first step. The distance of hydrogen bond (O–H···O) is shortened (about 0.15 Å) by crystallization.[6] It has been considered[5] that the shortening of the (O–H···O) distance leads to a new or strengthened interaction between FA and TDS and contributes to forming the crystaline $(FA)_6$ (TDS). On the other hand, solid FA forms cyclic dimers by hydrogen bonds.[7] In ethanol, *n*-mers of FA completely dissociate to monomers.[1] If it is assumed that the heat of breaking the hydrogen bonds between three cyclic dimers and one cyclic hexamer is the same, it follows that $\Delta\Delta H_d$ denotes the enthalpy of interaction between FA and TDS. Unfortunately, the values of $\Delta\Delta H_d$ are not exactly equal to the binding forces between FA and TDS because the enthalpies of the lattice fusion of FA and TDS in the $(FA)_6$ (TDS) may be not equal to those of FA alone and TDS alone. However, it may be possible to discuss approximately the magnitude of the binding force between FA and TDS. TDS has functional groups of $-NH_2$, –OH and –CHO, and FA has a functional group of –COOH. The enthalpies of hydrogen bond formation have been reported as $-26.7\,kJ\,mol^{-1}$ per hydrogen bond for the dimer of acetic acid[8] and $-28\,kJ\,mol^{-1}$ per hydrogen bond for the dimer of C18.[9] Furthermore, the enthalpies of the dimerization of schiff bases have been repoted[10] as about -35——$-40\,kJ\,mol^{-1}$. A comparison between these hydrogen bond values and the values of $\Delta\Delta H_d$ indicates that $(FA)_6$ (TDS) is formed by a weak binding force between FA and TDS roughly similar to single hydrogen bonding. However, it is unnatural that $(FA)_6$ (TDS) is formed by only one hydrogen bond between six molecules of FA and one molecule of TDS. It is, therefore, suggested that $(FA)_6$-(TDS) is formed by van der Waals forces and hydrophobic interactions between FA and TDS. This consideration supports the inference[4] that $(FA)_6$ (TDS) may be an inclusion compound.

Conclusion

The plots of ΔH_d of $(FA)_6$ (TDS) against *n* showed a zig-zag pattern which indicates an upward convex at odd-numbered positions, though ΔH_d increased by increasing *n*. The difference, $\Delta\Delta H_d$, between ΔH_d of $(FA)_6$-(TDS) and ΔH_d of (6FA + TDS) for $(C_{2a-1})_6$ (TDS) was slightly larger than that for $(C_{2a})_6$ (TDS), indicating that $(C_{2a-1})_6$ (TDS) is formed by stronger binding forces than $(C_{2a})_6$ (TDS). The value of $\Delta\Delta H_d$ was small as 23—$41\,kJ\,mol^{-1}$. It is suggested that $(FA)_6$ (TDS) may be a clathrate or an inclusion compound formed by weak binding forces, van der Waals forces and hydrophobic interactions, between FA and TDS.

References

1) S. Yokoyama and T. Fujie, *Chem. Pharm. Bull.*, **38**, 2347 (1990).
2) F. Ueda, T. Higashi, Y. Ayukawa, A. Takada, T. Fujie, and A. Kaneko, *Bitamin*, **61**, 57 (1987).
3) F. Ueda, T. Higashi, Y. Ayukawa, T. Fujie, and S. Yokoyama, *Chem. Pharm. Bull.*, **37**, 2545 (1989).
4) S. Yokoyama, F. Ueda, and T. Fujie, *Chem. Pharm. Bull.*, **38**, 1819 (1990).
5) S. Yokoyama and T. Fujie, *Chem. Pharm. Bull.*, **38**, 2249 (1990).
6) M. Davies and B. Kybett, *Nature* (London), **200**, 776 (1963).
7) E. von Sydow, *Ark. Kemi.*, **9**, 231 (1956).
8) G. Allen, J. G. Watkinson, and K. H. Webb, *Spectrochim. Acta*, **22**, 807 (1966).
9) D. S. Sarkadi and J. H. De Boer, *Recl. Trav. Chim. Pays-Bas*, **76**, 628 (1957).
10) V. Buss and L. Simon, *J. Chem. Soc., Chem. Commun.*, **1986**, 1032.

Pyrrolizidine Alkaloids from *Parsonsia laevigata* in Okinawa Island (Studies on *Parsonsia*. V)

Fumiko ABE,[a] Tatsuo YAMAUCHI,*,[a] Shiryo YAGA[b] and Kazuo MINATO[c]

Faculty of Pharmaceutical Sciences, Fukuoka University,[a] 8–19–1 Nanakuma, Jonan-ku, Fukuoka 814–01, Japan and Department of Forestry, Faculty of Agriculture,[b] Museum, Faculty of Agriculture,[c] University of the Ryukyus, Nishihara-cho, Okinawa 903–01, Japan. *Received December 10, 1990*

Nine pyrrolizidine alkaloids including three new compounds, were isolated from *Parsonsia laevigata* collected in Okinawa Island. No courtship pheromone with a dihydropyrrolizine framework was detectable in hair-pencil of *Idea leuconoe*, whose worms feed on the leaves of *Parsonsia laevigata*.

Keywords *Parsonsia laevigata*; Apocynaceae; pyrrolizidine alkaloid; cyclic pyrrolizidine alkaloid; 14-deoxyparsonsianine; 14-deoxyparsonsianidine; 12-seco-14-deoxyparsonsianine-13-methylester; *Idea leuconoe*

During studies on the constituents of *Parsonsia laevigata* ALSTON, we have described the structure determinations of several new pyrrolizidine alkaloids, parsonine,[1] parsonsianine,[2] parsonsianidine and its 17-methyl derivative,[3] as well as lignans[4] from the leaves of *Parsonsia laevigata* ALSTON collected in Kikai Island and Iriomote Island. For a comparative study of the alkaloid pattern of this plant in the Ryukyu Islands, the leaves collected in Okinawa Island were examined. This paper deals with the isolation of nine alkaloids, including three new alkaloids.

From the fresh leaves, after homogenization with MeOH, nine alkaloids were isolated (compounds **1**—**9**). Compounds **1**, **2** and **3** were identified as parsonsianine, parsonsianidine, and 17-methylparsonsianidine, respectively by direct comparisons with authentic samples obtained from the leaves collected in Iriomote Island. In the same way, compounds **4**, **5** and **6** were confirmed to be spiraline,[3,5] parsonsine,[5] and heterophylline,[5] respectively, based on comparisons of their physical constants, and assignments from the nuclear magnetic resonance (NMR) spectra and electron impact (EI)-mass spectra (MS).

Compound **7** was obtained as fine prisms, mp 159—164 °C (dec.). Based on the high resolution (HR)-MS (*m/z*: 425.205), the molecular formula was considered to be $C_{21}H_{31}NO_8$. In the ^{1}H-NMR spectrum, the presence of one each of ethyl, methyl and isopropyl groups as well as a retronecine moiety was suggested, as in **1**. A singlet peak observed at δ4.40, due to H-14 of **1**, however, was no longer detected in **7**, but signals due to methylene protons were observed at δ2.66 and δ2.84 (J=16 Hz) as an AB quartet pattern. In the ^{13}C-NMR spectrum, signals corresponding to C-13 and C-14 in **1** showed upfield shifts in **7** by −3.0 and −30.9 ppm, respectively. The fragment peak a (Chart 1) in the EI-MS of **7** was observed at *m/z* 296.149 ($C_{15}H_{22}NO_5$), while that of **1** at *m/z*: 312.148 ($C_{15}H_{22}NO_6$).[2,3] Thus, **7** was determined to be 14-deoxyparsonsianine.

Based on the HR-MS, **8** (mp 185—192 °C (dec.)) was considered to be $C_{22}H_{33}NO_8$, 14 mass unit (mu) more than **7**. From a comparison of the ^{1}H-NMR spectra of **8** and **7**, one of the two methyl groups of the isopropyl residue in **7** seemed to have been transformed into an ethyl group by addition of an extra methyl group. Fragment peak a was observed at *m/z* 310.165 ($C_{16}H_{24}NO_5$), 14 mu more than that of **7**, showing **8** to be a 21-methyl derivative of **7**, 14-deoxyparsonsianidine.[6]

The EI-MS of **9** afforded an M^+ peak at *m/z* 457.230 ($C_{22}H_{35}NO_9$), and three carbonyl carbon signals were

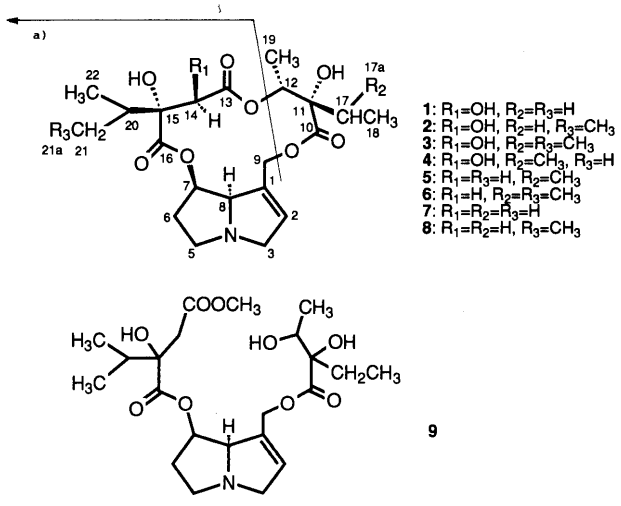


Chart 1

TABLE I. ^{1}H Chemical Shifts of **1**, **7**, **8** and **9**, δ (ppm) from Tetramethylsilane (TMS) in $CDCl_3$ (J (Hz) in Parentheses)

H	1[a]	7	8	9[a]
2	5.85 (br s)	5.89 (br s)	5.89 (br s)	5.84 (br s)
3	3.54 (dt, 14, 3)	3.40 (br d, 14)	3.38 (br d, 15)	3.51 (br d, 15)
	3.97 (dd, 14, 1)	3.99 (dd, 15, 2)	3.99 (dd, 15, 2)	4.24 (br d, 15)
5	2.82 (q, 9)	2.60 (q, 9)	2.60 (m)	2.82 (m)
	3.39 (br t, 9)	3.38 (t, 9)	3.39 (t, 9)	3.68 (m)
6	2.20—2.30	2.10 (m)	2.06 (m)	2.25—2.30
		2.15 (m)	2.15 (m)	
7	5.53 (br s)	5.54 (br s)	5.56 (br s)	5.47 (br s)
8	4.43 (br s)	4.36 (br s)	4.35 (br s)	4.91 (br s)
9	4.44 (dd, 14, 2)	4.37 (dd, 13, 2)	4.38 (dd, 13, 1)	4.61 (br d, 13)
	5.32 (d, 14)	5.20 (d, 13)	5.20 (d, 13)	4.93 (d, 13)
12	4.95 (q, 6)	5.08 (q, 6)	5.08 (q, 6)	3.96 (q, 6)
14	4.40 (s)	2.66 (d, 16)	2.65 (d, 16)	2.72 (d, 15)
		2.84 (d, 16)	2.87 (d, 16)	2.84 (d, 15)
17	1.60 (m)	1.59 (m)	1.60 (m)	1.59 (m)
	1.81 (m)	1.76 (m)	1.76 (m)	1.65 (m)
18	0.92 (t, 7)	0.92 (t, 7)	0.92 (t, 7)	0.86 (t, 7)
19	1.29 (d, 6)	1.26 (d, 6)	1.26 (d, 6)	1.19 (d, 6)
20	2.28 (sep, 7)	1.80 (sep, 7)	1.50 (m)	1.87 (sep, 7)
21	0.88 (d, 7)	0.85 (d, 7)	1.20 (m)	0.89 (d, 6)
21a			0.90 (t, 7)	
22	1.12 (d, 7)	0.97 (d, 7)	0.96 (d, 7)	0.96 (d, 6)
				3.67 (s, $-COOCH_3$)

a) Dissolved in $CDCl_3$–CD_3OD.

TABLE II. ^{13}C Chemical Shifts of **1**, **7**, **8** and **9**, δ (ppm) from TMS in $CDCl_3$

C	1[a]	7	8	9[a]
1	132.2	132.4	132.4	132.7
2	127.3	129.7	129.7	126.1
3	62.5	63.6	63.6	61.5
5	52.2	52.9	52.9	53.5
6	34.8	35.0	35.1	33.9
7	78.2	77.5	77.5	74.9
8	76.2	76.7	76.6	75.2
9	61.8	61.9	61.9	60.8
10	172.9	173.2	173.2	175.3
11	80.7	80.8	80.8	81.7
12	78.5	76.0	76.0	71.5
13	170.2	167.2	167.2	171.9
14	73.6	42.7	42.7	41.3
15	81.6	76.7	76.6	77.7
16	174.1	176.8	177.0	174.1
17	27.6	27.7	27.7	27.8
18	7.5	7.9	7.9	8.0
19	12.4	13.0	13.0	16.8
20	34.1	35.7	42.8	35.6
21	16.2	16.2	24.1	16.3
21a			12.7	
22	17.9	16.9	12.7	17.0
			$-OCH_3$	51.9

a) Dissolved in $CDCl_3$–CD_3OD.

observed besides the signals due to a retronecine moiety. Based on the degree of unsaturation, a macrocyclic structure as in **7** and **8** is unlikely in **9**. Since a 3H singlet signal was observed at δ 3.67 (^{13}C-NMR: δ 51.9 ppm), the presence of a carbomethoxyl residue was suggested, along with one each of ethyl, methyl, and isopropyl residues and one methylene group. A comparison of the NMR signals with those of **7** revealed that H-12 and C-12 showed upfield shifts of -1.12 and -4.5 ppm in **9**, respectively, indicating the splitting of the ester linkage in the macrocyclic ring. The structure of **9** was therefore assigned as 12-seco-14-deoxyparsonsianine-13-methylester. The possibility that the splitting of the ester linkage in **9** occurred during the MeOH extraction can not be excluded.

Worms of *Idea leuconoe* ERICHSON (Danaidae) feed on the leaves of this plant. In contrast to the case of other *Danaus* butterflies,[7] no courtship pheromone having a dihydropyrrolizine framework, such as danaidone, danaidal or hydroxydanaidal, was not detected from the hair-pencil of the male butterfly. Other constituents of the hair-pencil extract remain to be identified.

Experimental

Melting points were taken on a hot stage and are uncorrected. ^1H- and ^{13}C-NMR spectra were recorded on a JEOL GX-400 spectrometer in $CDCl_3$ unless otherwise mentioned. Chemical shifts are given in δ values referred to internal tetramethylsilane (TMS), and the following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, sep = septet, m = multiplet, br s = broad singlet, br d = broad doublet, dd = doublet of doublets. Electron impact (EI)-MS were recorded on a JEOL D-300-FD spectrometer. Optical rotations were measured on a JASCO DIP 360 polarimeter. Spots on thin layer chromatography (TLC) plates were detected by spraying the plates first with chloranil in benzene (saturated), and then with 5% *p*-dimethylaminobenzaldehyde in diluted H_2SO_4.

Extraction and Isolation of Alkaloids Fresh leaves of *Parsonsia leavigata* Alston (2.6 kg) collected at Okinawa Island in January 1989, were homogenized and then percolated with MeOH. The MeOH percolate was concentrated *in vacuo* and filtered. The filtrate was extracted with benzene and then $CHCl_3$. The benzene and $CHCl_3$ extract were combined and subjected to silica gel column chromatography with $CHCl_3$–MeOH–H_2O (7:1:1—7:2:1). Each fraction was further chromatographed on a silica gel column with AcOEt–MeOH–H_2O (7:1:6—5:1:4) to give **1** (55 mg), **2** (17 mg), **3** (6 mg), **4** (17 mg), **5** (8 mg), **6** (5 mg), **7** (70 mg), **8** (12 mg) and **9** (31 mg). Compounds **1** (parsonsianine), **2** (parsonsianidine) and **3** (17-methylparsonsianidine) were identified by direct comparison of NMR spectra and TLC behavior with those of authentic samples.[2,3] **4** (Spiraline): Solid, $[\alpha]_D^{30}$ +66° (c=0.60, MeOH). EI-MS m/z: 455.2150 (Calcd for $C_{22}H_{33}NO_9$: 455.2155). ^1H-NMR[8] ($CDCl_3$–CD_3OD) δ: 0.89, 1.11 (3H, each, d, J=6 Hz, H-21,22), 0.99, 1.04 (3H each, d, J=6 Hz, H-17a, 18), 1.28 (3H, d, J=6 Hz, H-19), 1.93 (1H, sep, J=6 Hz, H-17), 2.20—2.30 (3H, m, H-6, 20), 2.82 (1H, m, H-5a), 3.37 (1H, t, J=9 Hz, H-5b), 3.57, 3.96 (1H each, br d, J=15 Hz, H-3), 4.40 (1H, s, H-14), 4.44 (1H, br s, H-8), 4.49, 5.30 (1H, each, br d, J=13 Hz, H-9), 5.24 (1H, q, J=6 Hz, H-12), 5.49 (1H, br s, H-7), 5.88 (1H, br s, H-2). **5** (Parsonsine): mp 158—159 °C, $[\alpha]_D^{29}$ +21.0° (c=1.25, MeOH). EI-MS m/z: 439.2208 (Calcd for $C_{22}H_{33}NO_8$: 439.2206). ^1H-NMR δ: 0.85, 0.97 (3H each, d, J=6 Hz, H-21, 22), 0.97, 1.04 (3H each, d, J=6 Hz, H-17a, 18), 1.26 (3H, d, J=6 Hz, H-19), 1.80, 1.90 (1H each, sep, J=6 Hz, H-17, 20), 2.11, 2.20 (1H each, m, H-6), 2.62 (1H, m, H-5a), 2.64, 2.84 (1H each, d, J=16 Hz, H-14), 3.39, 4.02 (1H each, d, J=15 Hz, H-3), 4.40, 5.20 (1H each, d, J=13 Hz, H-9), 4.42 (1H br s, H-8), 5.33 (1H, q, J=6 Hz, H-12), 5.54 (1H, br s, H-7), 5.91 (1H, br s, H-2). **6** (Heterophylline): mp 186—188 °C, $[\alpha]_D^{29}$ +19.8° (c=0.20, MeOH). EI-MS m/z: 453.2365 (Calcd for $C_{23}H_{35}NO_8$: 453.2363). ^1H-NMR[8] δ: 0.90 (3H, t, J=6 Hz, H-21a), 0.971, 0.975, 1.04 (3H each, d, J=6 Hz, H-17a, 18, 22), 1.20 (2H, m, H-21), 1.26 (3H, d, J=6 Hz, H-19), 1.48 (1H, m, H-20), 1.90 (1H, sep, J=6 Hz, H-17), 2.08 (1H, dd, J=14, 6 Hz, H-6a), 2.26 (1H, m, H-6b), 2.63, 2.87 (1H each, d, J=16 Hz, H-14), 2.60, 3.54 (1H each, m, H-5), 3.42, 4.11 (1H each, br d, J=14 H, H-3), 4.43, 5.20 (1H, each, br d, J=13 Hz, H-9), 4.53 (1H, br s, H-8), 5.33 (1H, q, J=6 Hz, H-12), 5.61 (1H, br s, H-7), 5.91 (1H, br s, H-3).

14-Deoxyparsonsianine (7) Fine prisms from MeOH, mp 159—164 °C (dec.), $[\alpha]_D^{30}$ +33.5° (c=0.80, MeOH). EI-MS m/z: 425.2052 (Calcd for $C_{21}H_{31}NO_8$: 425.2050), 296.1490 (Calcd for $C_{15}H_{22}NO_5$: 296.1497).

14-Deoxyparsonsianidine (8) Fine needles from MeOH, mp 185—192 °C (dec.), $[\alpha]_D^{29}$ +25.3° (c=0.50, MeOH). EI-MS m/z: 439.2205 (Calcd for $C_{22}H_{33}NO_8$: 439.2206), 310.1654 ($C_{16}H_{24}NO_5$: 310.1654).

12-Seco-14-deoxypasonsianine-13-methylester (9) A solid, $[\alpha]_D^{31}$ +31.6° (c=0.70, MeOH). EI-MS m/z: 457.2304 (Calcd for $C_{22}H_{35}NO_9$: 457.2311)

Examination of the Hair-pencil Extract Hair-pencils were collected from 3 groups of 5 male butterflies of *Idea leuconoe* Erichson captured in Okinawa Island in Sept. 1989, June 1990 and Sept. 1990, and homogenized with cold CH_2Cl_2. Each CH_2Cl_2 solution was concentrated *in vacuo*. The residue was developed on TLC in parallel with authentic samples of danaidone and 7-hydroxydanaidal (solvents: benzene–acetone 4:1; $CHCl_3$–MeOH–H_2O 7:2:1; EtOAc–MeOH–H_2O 9:1:0.1) and no spots having similar staining to danaidone and hydroxydanaidal were observed in the three lots with chloranil/*p*-dimethylaminobenzaldehyde reagent.

Acknowledgements We thank Ms. Y. Iwase, J. Honda and Mr. H. Hanazono of Fukuoka University for NMR and MS measurements. This work was supported in part by the grant from the Central Research Institute of Fukuoka University.

References and Notes

1) F. Abe and T. Yamauchi, *Chem. Pharm. Bull.*, **35**, 4661 (1987).
2) F. Abe, T. Nagao, H. Okabe, T. Yamauchi, N. Marubayashi and I. Ueda, *Chem. Pharm. Bull.*, **38**, 2127 (1990).
3) F. Abe, T. Nagao, H. Okabe and T. Yamauchi, *Phytochemistry*, **30**, 1737 (1991).
4) F. Abe and T. Yamauchi, *Phytochemistry*, **28**, 1737 (1989).
5) J. A. Edgar, N. J. Eggers and G. B. Russell, *Tetrahedron Lett.*, **21**, 2657 (1980).
6) The configuration at C-20 in **8**, as well as in **2**, remains to be determined.
7) H. Komae, A. Nishi, T. Tanaka, N. Hayashi, C. Wesons and T. Kuwahara, *Biochemical Systematics and Ecology*, **10**, 181 (1982).
8) ^1H-NMR data were not given in reference 5.

Synthesis of Cobalt(II) and Nickel(II) Complexes of Ceclor (Cefaclor) and Preliminary Experiments on Their Antibacterial Character

Zahid Hussain CHOHAN

Department of Chemistry, Islamia University, Bahawalpur, Pakistan. *Received September 10, 1990*

Cobalt(II) and nickel(II) complexes of the antibacterial drug Ceclor have been synthesized and characterized on the basis of their elemental analysis, molar conductance, magnetic moment and electronic and infrared spectral data. These complex have been, then subjected to screening for their antibacterial properties against bacterial species such as *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli*. In comparison to uncomplexed Ceclor, the metal complexes have been shown to be more antibacterial.

Keywords metal ion; antibacterial drug; pharmacological assay; antimicrobial character

Introduction

The relationship between the metal ions and the bioability of drugs occasioned interest over since there arose indications[1-4] that metal chelates of ligands which have anticancer activity are more carcinostatic than the free ligand itself. The apparent role of metal ions in the treatment of such ailments is definite, but just how is still a matter of conjecture.[5,6]

In order to gain information and to understand the possible mode of action, a study programme commenced in this laboratory concerns the formation of metal complexes by a number of chelating agents which are known to have a biological function or are likely to possess antibacterial activity. And, in continuation of the same, in this paper synthesis and coordinating properties of cobalt(II) and nickel(II) complexes of the antibacterial drug Ceclor are reported, and the relative changes that occur in the drug action of Ceclor upon complexation are explored.

Ceclor (**1**) is a broad spectrum antibacterial agent which is commonly used against gram-positive and gram-negative microorganisms.[7] This antibacterial drug (ligand) contains common donor atoms such as nitrogen, oxygen and sulphur, which easily coordinate with cobalt(II) and nickel(II) ions to form complexes. The two Ceclor molecules are expected to act as a bidentate ligand occupying four coordination sites, while the remaining two are filled by water molecules thus giving octahedral complexes. The evidence concerning the structure and coordinating properties of these prepared complexes was based on their spectroscopic and magnetic moment properties.

The synthesized metal–Ceclor complexes when compared with pure Ceclor for their antibacterial activity, were found to be more active against bacterial species including *Streptococcus pyogenes* (a), *Streptococcus pneumoniae* (b), *Staphylococcus aureus* (c) and *Escherichia coli* (d).

Experimental

Materials and Methods All chemicals used for synthesis and structure determination were of a reagent grade and used as such. Ceclor was obtained from Eli Lilly and Company Limited, England and used without further purification.

Elemental analysis of C, H and N was carried out on a Coleman automatic analyzer. The magnetic measurements were done on solid complexes using the Gouy method. Electronic spectra were studied on a Beckman spectrophotometer model DU using glass cells of 1 cm thickness. Infrared spectra were recorded on a Perkin-elmer 521 spectrophotometer.

Determination of Metal Contents Metal contents of the complexes were determined by treating the complexes first with boiling concentrated nitric acid and then by evaporating them to dryness repeatedly until the organic matter was completely destroyed. The residue was extracted with water and filtered. The filtrate was further used for estimation of cobalt and nickel content using the reported method.[8]

Synthesis of Metal Complexes The usual method consisted of mixing an aqueous solution (30 ml) of metal chloride salt (1 mmol) with an aqueous solution (20 ml) of ligand (2 mmol). The mixture was refluxed near the boiling point for 2 h. The resulting solution was cooled, filtered and later reduced to a small volume (15 ml). The concentrated solution was left overnight, which resulted in the formation of crystals. The product thus formed was separated and recrystallized from water.

Antibacterial Studies Antibacterial activity of the complexes was carried out in the Department of Pathology, Qaid-e-Azam Medical College, Bahawalpur. Antibacterial activity of the prepared complexes against bacterial species, obtained from different patients carrying the bacteria *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli* was determined using the paper disc diffusion method.

Preparation of Discs: A metal complex (30 μg) in dimethylformamide (DMF) (0.01 ml) was applied on a paper disc prepared from blotting paper (3 mm size) with the help of a micropipett. The discs were left in an incubator for 48 h at 40 °C and then applied on bacteria grown agar plates.

Preparation of Agar Plates: For this purpose, minimal agar was used for the growth of specific bacterial species. For *Streptococcus* and *Staphylococcus* species, a blood agar base with low pH was used. The blood agar base (40 g) was first suspended in cold distilled water (1 l) and heated to boiling. It was then sterilized at 120 °C for 15 min and later allowed to cool at 50 °C. Then 5% sterile defibrinated cow or sheep blood was added to it and the mixture was poured into previously washed and sterilized petri dishes which were stored at 4 °C for inoculation. For the preparation of agar plates for *E. coli*, Mac Conkey agar (50 g), obtained from Merck Chemical Company, was suspended in freshly distilled water (1 l). It was allowed to soak for 15 min and then boiled with constant shaking in a water bath until the agar was completely dissolved. The mixture was autoclaved for 15 min at 120 °C and then poured into previously washed and sterilized petri dishes and stored at 4 °C for inoculation.

Procedure of Inoculation: Inoculation was done with the help of a platinium wire loop which was first made red hot on a flame, allowed to cool in air and then used for the application of previously described bacterial strains. The preculture was first prepared in 2 ml of a nutrient broth by selecting a suitable bacterial colony, and later on it was transfered to a nutrient broth which was incubated for 2 h at 37 °C. Then 500 μl of the culture was spread on the specific agar plates, which was incubated for 24 h at 37 °C.

Application of Disc: A sterilized forcep was used for the application of a paper disc on the already inoculated agar plates. When the disc was applied, it was then incubated at 37 °C for 24 h.

Results and Discussion

The analytical data and properties of the complexes are reported in Tables I and II. The complexes are soluble in water, DMF, sparingly soluble in ethanol and insoluble in

TABLE I. Magnetic Moment and Elemental Analysis Data of the Complexes

Complexes	mp (°C)	Elemental analysis % Calcd (Found) C	H	N	M. moment μ_{eff} (B.M)
$[Co(C_{15}H_{14}ClN_3O_4S)_2(OH_2)_2]Cl_2$(A)	180—183	39.92 (39.91)	3.55 (3.57)	9.31 (9.31)	4.45
$[Ni(C_{15}H_{14}ClN_3O_4S)_2(OH_2)_2]Cl_2$(B)	195—197	40.00 (40.08)	3.55 (3.56)	9.33 (9.36)	3.28

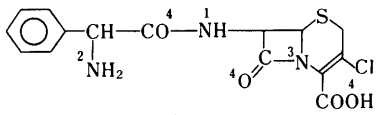
TABLE II. Infrared and Spectral Data of the Complexes

Complexes	IR (cm^{-1})	λ_{max} (cm^{-1})
A	3430, 3210, 3020, 2950, 1620, 1380, 1115, 990, 735, 690	22100, 15180, 9430
B	3400, 3230, 3030, 1630, 1460, 1110, 1030, 880, 725, 670	32500, 15200, 9550

non-polar solvents. Molar conductance of aqueous solutions of cobalt ($255\,\Omega^{-1}$/mol) and nickel ($230\,\Omega^{-1}$/mol) complexes have been shown to conduct electricity, which in turn suggests their ionic nature.[9] Furthermore, conductometric titration of the complexes with silver nitrate shows the presence of two anionic chloride ions probably lying outside the coordination sphere.

Elemental analysis data has shown these complexes to have 1:2 (M:L) stoichiometry. The room temperature magnetic moment values for both cobalt and nickel complexes lie within the range for their observed octahedral geometries.[10] The magnetic moment (μ_{eff}) for the cobalt complex (4.45 B.M) is expected to contain an odd number of electrons. Similarly, the μ_{eff} value for the nickel complex (3.28 B.M) also lies in the range consistent to a spin-free octahedral environment.[11]

Although Ceclor contains a number of potential donor atoms (nitrogen, oxygen and sulfur), the model of the ligand shows that it can only act as bidentate and preferentially bond with metal atoms through its N^1, N^2 or N^3, O^4 atoms. A comparative scrutiny of the infrared spectral data of the complexes with those of the uncomplexed ligand indicates that a strong stretching vibration of carbonyl (–C=O) at 1620 cm^{-1} in the ligand is not effected upon complexation. Also, a vibration corresponding to the (M–S) bond in the range 330—350 cm^{-1} was not detectable in the spectra of complexes. These observations, therefore, indicate that the metal atom is not coordinated with the oxygen or sulfur atoms. However, the complexes showed two characteristic bands (not observable in the free ligand) in the region 720—670 cm^{-1} attributed to (M–N) modes respectively[12] which hint at the coordination of the ligand to metal atoms through N^1 and N^2 groups.



1

The solution spectra of the complexes in visible and near infrared regions exhibited bands due to d–d transitions. Cobalt(II) complexes exhibited three distinct bands at 22100, 15180 and 9430 cm^{-1}. The first band is assigned to a metal→ligand charge transfer band because of its high intensity corresponding to transition $^4T_{1g}\rightarrow{}^4T_{1g}(P)$. The band at 15180 cm^{-1} is assigned to the transition $^4T_{1g}(F)\rightarrow{}^4A_{2g}(\nu)$ and at 9430 cm^{-1} due to transition $^4T_{1g}(F)\rightarrow{}^4T_{2g}(F)$. Nickel(II) complexes also exhibited three typical bands at 23500, 15200 and 9555 cm^{-1} corresponding to the transitions $^3A_{2g}\rightarrow{}^3T_{2g}(F)(\nu_3)$, $^3A_{2g}\rightarrow{}^3T_{1g}(F)(\nu_2)$ and $^3A_{2g}\rightarrow{}^3T_{2g}(F)(\nu_1)$, respectively, in an octahedral field.[13]

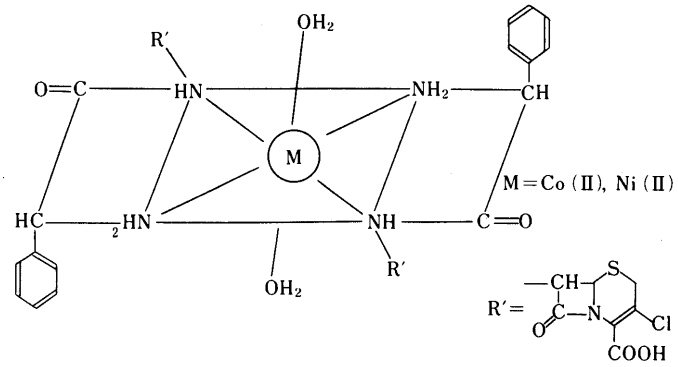


Fig. 1. Proposed Structure for $[M(L)_2(OH_2)_2]^{2+}$ Ions

TABLE III. Antibacterial Activity Data of the Complexes

Ceclor[a] and complexes	Microbial species a	b	c	d
Ceclor	+	++	++	+
A	++	+++	+++	++
B	+++	+++	+++	+++

a = *Streptococcus pyogenes*, b = *Streptococcus pneumoniae*, c = *Staphylococcus aureus*, d = *Escherichia coli*. Inhibition zone measured in diameter +, 6—10 mm; ++, 10—16 mm; +++, 16—22 mm. *a*) Uncomplexed antibacterial drug.

The two water molecules around the metal atom are assumed to have a trans arrangement due to their bulky molecules of the ligand at larger distances from each other, which gives a more stable geometry to the complex.

Antibacterial Properties of the Complexes The metal complexes were tested for their antibacterial activity against bacterial species (a), (b), (c) and (d) in comparison with pure, uncomplexed Ceclor. The complexes were tested at a concentration of 30 μg/0.01 ml in DMF using the paper disc diffusion method. The zone of inhibition (in diameter) was measured. The results of these studies (Table III) show that metal ions significantly increase the antibacterial activity of Ceclor upon coordination against all bacterial species. It is, however, not clear why the metal ions enhance the potency of such ligands, but it is definite[14-16] that metal atoms do play this important role. Further work to investigate this role of metal ions by determining the lethal dose of the complex in biological systems and their pharmacological screening is in progress.

Acknowledgement The author is highly indebted to the Department of Pathology, Qaid-e-Azam Medical College, Bahawalpur, for its help in

undertaking the antibacterial studies.

References

1) A. Albert, *Aust. J. Sci.*, **30**, 1 (1967).
2) K. B. Swedo and J. H. Enemark, *J. Chem. Ed.*, **56**, 70 (1970).
3) D. R. Williams, *Inorg. Chim. Acta Rev.*, **8**, 123 (1972).
4) D. R. Williams, *Chem. Rev.*, **72**, 203 (1972).
5) J. Schubert, *Sci. Am.*, **214**, 40 (1966).
6) S. Kirschner, Y. K. Wei, D. Francis and J. H. Berjman, *J. Med. Chem.*, **9**, 369 (1966).
7) A. G. Gilman, L. S. Goodman and A. Gilman, "The Pharmacological Basis of Therapeutics," 6th Ed., McMillan Publishing Co., 1980.
8) A. I. Vogel, "A Textbook of Quantitative Inorganic Analysis," 3rd Ed., Longmans, London, 1961.
9) W. J. Geary, *Coord. Chem. Rev.*, **7**, 81 (1971).
10) V. B. Rama, D. D. Singh, P. Singh and M. Toetia, *Transt. Met. Chem.*, **6**, 36 (1981).
11) A. B. P. Lever, *Inorg. Chem.*, **4**, 763 (1965).
12) K. Nakamoto, "Infrared Spectra of Inorganic and Coordination Compounds," John Willy, New York, 1977.
13) C. J. Balhausen, "Introduction to Ligand Field," McGraw Hill, New York, 1962.
14) Z. H. Chohan and A. Khaliq, *Pak. J. Med. Res.*, **28**, 92 (1989).
15) Z. H. Chohan and M. U. H. Ansari, *Pak. J. Pharmacol.*, **6**, 21 (1989).
16) Z. H. Chohan, *Pak. J. Pharmacol.*, in press.

Fluoromethylated and Hydroxymethylated Derivatives of *N*-Methyl-D-aspartate Receptor Antagonist 1-[1-(2-Thienyl)cyclohexyl]piperidine

Syuji TSUKIYAMA,[a] Atsushi HASHIMOTO,[b] Souta KATAYAMA,[c] Toru NISHIKAWA,[b] Akihiro TOBE,[c] and Minoru MAEDA*,[a]

Faculty of Pharmaceutical Sciences, Kyushu University,[a] Maidashi 3–1–1, Higashi-ku, Fukuoka 812, Japan, Division of Mental Disorder Research, National Institute of Neuroscience,[b] NCNP, 4–1–1, Ogawa-Higashi, Kodaira, Tokyo 187, Japan, and Research Center, Mitsubishi Kasei Corporation,[c] Kamoshida, Midori-ku, Yokohama 227, Japan. *Received December 13, 1990*

Derivatives with fluoromethyl and hydroxymethyl groups on the cyclohexyl ring of 1-[1-(2-thienyl)cyclohexyl]piperidine (TCP), a noncompetitive antagonist of *N*-methyl-D-aspartate (NMDA) receptor, were tested in a radioligand binding assay to evaluate their ability to inhibit [^{3}H]TCP binding by rat brain homogenates. The potencies of these compounds as antagonists of NMDA and L-glutamate responses were also compared using a rat cortical slice preparation. One of the analogs, *cis*-2-hydroxymethyl-*r*-1-(*N*-piperidyl)-1-(2-thienyl)cyclohexane (5) was found to show a high affinity (IC_{50} = 16 nM) for the phencyclidine (PCP) binding sites, very close to that of TCP, and to be 38-fold more potent in binding than its *trans* isomer. Fluoromethyl and hydroxymethyl substitutions at C_4 position of the cyclohexyl ring of TCP clearly reduced the affinity by at least one order of magnitude relative to TCP.

Keywords *cis*-2-hydroxymethyl-*r*-1-(*N*-piperidyl)-1-(2-thienyl)cyclohexane; *trans*-2-hydroxymethyl-*r*-1-(*N*-piperidyl)-1-(2-thienyl)cyclohexane; *N*-methyl-D-aspartate (NMDA) receptor; NMDA receptor antagonist; *in vitro* binding; 1-[1-(2-thienyl)-cyclohexyl]piperidine (TCP); phencyclidine binding site

1-[1-(2-Thienyl)cyclohexyl]piperidine (TCP), a derivative of the dissociative anaesthetic phencyclidine (PCP), has become a preferred ligand for characterizing the PCP receptor and PCP-induced effects, since it binds with a higher affinity and a greater specificity than PCP or other PCP-like drugs.[1,2] Recent biochemical studies have shown that part of the recognition sites of ^{3}H-labeled TCP are located within ion channels coupled to the *N*-methyl-D aspartate (NMDA) subtype of L-glutamate receptor. Autoradiographic studies using [^{3}H]TCP or [^{3}H]L-glutamate suggested a loss of the NMDA receptor ion channel complex in Alzheimer-type dementia or Huntington's disease brains.[3,4]

Numerous PCP and TCP analogs have been synthesized and evaluated for receptor affinity.[5–7] There is a recent suggestion that TCP analog labeled with positron-emitting isotopes might be a suitable *in vivo* marker for neurodegenerative disease using positron emission tomography (PET) scanning.[3] Of the commonly used positron-emitting isotopes, the relatively long half-life of ^{18}F ($T_{1/2}$ = 110 min) has made this nuclide particularly attractive.[8] The radiochemical synthesis of ^{18}F-labeled 1-[1-(2-thienyl)cyclohexyl]-4-fluoropiperidine has recently been reported by Kiesewetter *et al.*[9] It has been shown that TCP and PCP analogs with methyl substituents on the cyclohexyl ring possess a higher affinity than PCP,[7,10] although modifications of the cyclohexyl portion of the molecule have been universally detrimental to the binding affinity at the PCP binding sites.[11] We are especially interested in the investigation of the effect of the molecule on the affinity and selectivity for the PCP receptors, and the biological consequences of such alterations. Another aim of this research is to develop a ^{18}F-labeled radiopharmaceutical for possible use as PET scanning agents. This paper describes the synthesis of *cis* and *trans*-2-hydroxymethyl-*r*-1-(*N*-piperidyl)-1-(2-thienyl)cyclohexane. Moreover, these compounds together with three other analogs of TCP having a hydroxymethyl or fluoromethyl group at the C-4 position of the cyclohexyl ring, the synthesis of which has been previously reported,[12] were evaluated for their affinity to the PCP receptor and for their ability to antagonize excitatory amino acid induced depolarization in rat cortical slice preparation.

All TCP derivatives described in this paper were prepared by using the general method of Geneste *et al.*[13] which would be a possible route for access to the preparation of ^{18}F-labeled analogs. Thienylmagnesium bromide was condensed with the cyclohexanone (1) to give the tertiary

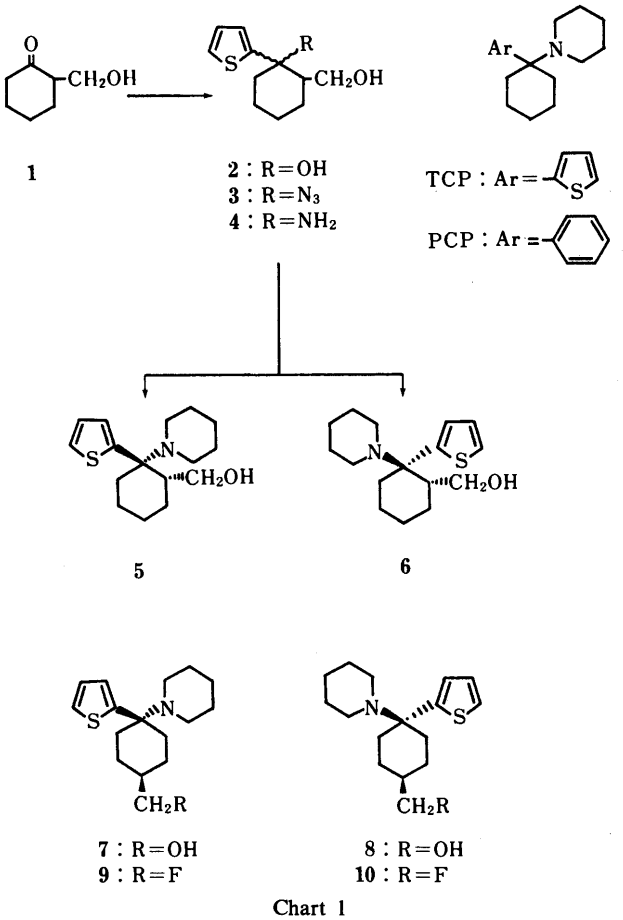


Chart 1

alcohol (**2**). Treatment with sodium azide and trichloroacetic acid followed by reduction of the resulting azide (**3**) gave the amine (**4**). Heating of the amine with 1,5-dibromopentane gave the *cis* and *trans* isomer (**5** and **6**) of C-2 hydroxymethylated compounds. The preparation of 4-hydroxymethylated and 4-fluoromethylated analogs (**7**—**10**) has been previously described.[12] The racemic *cis* and *trans* isomers (**5** and **6**) have distinctive proton nuclear magnetic resonance (^{1}H-NMR) profiles and can be differentiated easily from one another. The signals due to the C-2 oxymethylene protons in the *cis* (**5**) were observed at δ 3.62 (dd, J=10.6, 4.0 Hz) and 4.35 ppm (t, J=10.5 Hz), whereas those in the *trans* (**6**) appeared at δ 3.57 (dd, J=10.2, 5.9 Hz) and 3.82 ppm (t, J=10 Hz). This difference is considered to be a result of differential shielding by the thienyl ring in the two isomers, consistent with the results obtained with other analogs previously reported.[14] In addition, one of the oxymethylene protons at δ 3.82 ppm in the *trans* (**6**) was found to show cross peaks with the aromatic protons at δ 7.18 and 7.28 ppm in the two-dimentional nuclear Overhauser enhancement and exchange spectroscopy (NOESY) spectra, which is consistent with the close proximity of the groups. On the other hand, such close peaks were not observed in *cis* isomer (**5**). Furthermore, X-ray crystallography of the *cis* isomer (**5**) confirmed the hydroxymethyl at C-2 as axial with the thienyl ring occupying the axial position.[15] Efforts to convert the C-2 hydroxymethyl moiety to the C-2 fluoromethylated derivatives by direct displacement with diethylaminosulfur trifluoride (DAST) or by substitution of sulfonate esters with fluoride ion were uniformly unsuccessful. Thus, a different approach must be devised to prepare the C-2 fluoromethylated analogs.

It has been shown that drugs acting at PCP receptors such as the dissociative anesthetics antagonize NMDA-induced polarization in a noncompetitive manner.[6,16] The antagonistic potencies to depolarization induced by the excitatory amino acid of the compounds were determined *in vitro* using a rat cortical slice preparation. The results are summarized in Table I. All the compounds tested produced a blockade of depolarizing responses to NMDA in a concentration-dependent manner with differences in activity between the *cis* and *trans* isomeric pairs. On the other hand, they were ineffective against the block of depolarizing responses to L-glutamate except the C-2 hydroxymethylated analog (**5**) with a 43% inhibition at 10 μM. The *cis* (**5**) caused 59% and 97% decreases of the NMDA responses at 1 μM and 10 μM respectively which proved to be the most potent compound in the present work. The C-4 fluoromethylated analog (**9**) was less potent, a 72% decrease of the NMDA response being observed in the presence at a concentration of 10 μM. The corresponding *cis* isomer (**10**) was not determined due to the difficulty in solubility.

The receptor binding affinity of the compounds assessed on the basis of competitive inhibition of [^{3}H]TCP binding to rat brain tissues is shown in Table II. For comparison we followed the same experimental protocol to test PCP and TCP in our system. The displacement curves from which the data of Table II were obtained are all monophasic, indicating the displacement of [^{3}H]TCP from an apparent single class of binding sites. It is noteworthy that compound

TABLE I. Blockade of L-Glutamate and *N*-Methyl-D-aspartate-Induced Depolarization by TCP Analogs on Rat Cortical Slices

Compd.	Concn. (μM)	n	% inhibition	
			L-Glutamate	*N*-Methyl-D-aspartate
5	0.1	2	1.9± 5.5	9.6± 3.7
	1	4	27.7± 4.1[a]	59.6± 5.3[a]
	10	2	43.4± 6.7	97.2± 2.8
6	1	2	−1.3± 1.3	1.7± 1.7
	10	2	6.7± 3.4	20.7±20.7
7	1	5	−9.5±10.7	−11.7± 5.3
	10	5	−2.7±12.0	55.5± 5.4[a]
8	1	3	−4.0± 5.9	2.5± 6.8
	10	5	7.9± 4.1	16.8± 4.4
9	1	4	−7.0±10.6	6.7± 3.0
	10	4	11.4±14.3	72.6± 2.3[a]

Values are means with S.E.M. of the indicated number (n) of experiments. *a*) $p<0.01$.

TABLE II. Evaluation of Binding of TCP Analogs to PCP Receptors by Displacement of [^{3}H]TCP Bound to Rat Brain Homogenates

Compd.	IC_{50} (nM)[a]
5	16± 2
6	620± 53
7	580± 48
8	3300±470
9	170± 16
PCP[b]	52± 7
TCP[c]	7.94±0.03

a) Values are means with S.E.M. of the data obtained from three separate experiments for each compound. Nonspecific binding is defined as [^{3}H]TCP binding in the presence of 100 μM PCP. *b*) Phencyclidine, 1-(1-phenylcyclohexyl)piperidine. *c*) 1-[1-(2-Thienyl)cyclohexyl]piperidine.

5 with thienyl and hydroxymethyl occupying the axial position showed the best binding characteristics with a IC_{50} of 16 nM for the PCP binding sites, very close to that of TCP and slightly more potent than PCP. All other compounds tested were found to be less potent in binding than PCP itself. A separate binding study showed that inhibition of [^{3}H]glutamate, [^{3}H]CGS-19755 ([piperidinyl-^{3}H]-4-(3-phosphonoprop-1-yl)piperazine-2-carboxylic acid), [^{3}H]kainate and [^{3}H]AMPA ([5-methyl-^{3}H]amino-3-hydroxy-5-methylisoxazole-4-propionic acid) binding by compound **5** in rats were ineffective in all cases ($IC_{50}>100$ μM).[17] There is also a considerable difference in affinity between the *cis* and *trans* pairs, as observed in the electrophysiological study. The stereoselectivity in receptor binding indicates a high degree of spatial arrangement recognized by the PCP receptor binding sites, and suggests that the binding conformation of TCP analogs adopts the axial thiophene position, which is in accord with known structure–activity relationships in the PCP series.[4,14]

The ultimate goal of fluorinated compounds obtained is to conduct *in vivo* imaging for mapping of the NMDA receptor complex. It is the primary properties of receptor binding affinity and lipophilicity that are pertinent in predicting the utility of new compounds for receptor imaging with PET. Generally, compounds with a dissociation constant of the 10 nM or lower range are required for *in vivo* receptor based imaging.[8] Thus, the fluori-

nated compound **9**, lacking sufficient receptor affinity, may not be a suitable ligand for *in vivo* PCP receptor imaging by PET. The PCP receptor model developed by Beart *et al.*[18] provides that a cleft in the upwards direction, capable of accomodating lipophilic groups in the region of the cyclohexane ring of PCP, is one of the three secondary binding sites for the interaction of PCP ligands. Both NMDA antagonism determination and radioligand studies suggest that substitution of the fluoromethyl moiety for the hydroxymethyl group in compound **5**, leading to the more lipophilic analog, should confer at least similar affinity of the ligand for the PCP binding sites. We are presently trying to prepare this fluoro analog following a different approach as well as other derivatives of TCP with increased binding affinities.

Experimental

All melting points are uncorrected. ^{1}H-NMR spectra were recorded on a JNM GX-270 or JEOL FX-100 spectrometer with tetramethylsilane as internal reference. Infrared (IR) spectra were recorded on a JASCO IR Report 100 spectrometer and mass spectra (MS) were obtained with a JEOL JMS D-300 mass spectrometer. Column chromatography was performed on Kieselgel 60 (70—230 mesh, Merck). Thin-layer chromatography (TLC) on Silica gel 60F 254 (Merck) was used to monitor the reactions and to ascertain the purity of reaction products. During workup, organic extracts were routinely dried over anhydrous Na_2SO_4 and evaporated with a rotary evaporator under reduced pressure. [^{3}H]TCP (47.8 Ci/mmol) was purchased from New England Nuclear Research Products. Phencyclidine (PCP) hydrochloride and 1-[1-(2-thienyl)cyclohexyl]piperidine (TCP) hydrochloride were gifts from Sumitomo Pharmaceutical Company (Osaka, Japan). The C-4 fluoromethylated and hydroxymethylated derivatives (**7**—**10**) of TCP were prepared as previously reported.[12] All other reagents were obtained from commercial sources.

2-Hydroxymethyl-1-(2-thienyl)cyclohexanol (2) A solution of 2-thienylmagnesium bromide was prepared by the dropwise addition of 2-bromothiophene (10.2 g) to a vigorously stirred mixture of magnesium turnings (1.52 g) in dry ether (30 ml). A solution of 2-hydroxymethylcyclohexanone (**1**) (4.0 g) [prepared from ethyl 2-oxocyclohexanecarboxylate by ketalization with ethylene glycol, reduction with $LiAlH_4$, and then acidic hydrolysis with tartaric acid in a 51% overall yield] in dry ether (20 ml) was added dropwise. After stirring for 24 h, aqueous NH_4Cl was added to the mixture and it was extracted with ether. The etheral layer was dried and evaporated to dryness. Column chromatography on silica gel of the residue ($CHCl_3$: acetone = 95 : 5, v/v) afforded the tertiary alcohol (**2**) (2.94 g, 44%) as an oil. IR (neat): 3360, 2930, 1430 cm^{-1}. MS *m/z*: 212 (M^+).

***cis*- and *trans*-2-Hydroxymethyl-*r*-1-(*N*-piperidyl)-1-(2-thienyl)cyclohexane (5 and 6)** A solution of the alcohol **2** (1.72 g) in $CHCl_3$ (15 ml) was added to a stirred suspension of NaN_3 (1.80 g) and trichloroacetic acid (4.53 g) in $CHCl_3$ (15 ml) at 0 °C. The mixture was stirred for 5 h at the same temperature. After the addition of water and neutralization with concentrated aqueous NH_3, the mixture was extracted with $CHCl_3$. The organic layer was dried and evaporated to the residue containing the two isomeric azides (**3**). The crude azides were dissolved in ether (20 ml), which was then added dropwise to a solution of $LiAlH_4$ (736 mg) in ether (20 ml). After refluxing for 12 h, the reaction was quenched by the addition of aqueous NH_4Cl. The mixture was extracted with ether and washed with 10% HCl. The acidic fraction was made neutral with aqueous NH_3 and extracted with $CHCl_3$. The combined organic phase was dried and evaporated to dryness. The residue of the amine (**4**) (1.74 g) was dissolved in dry acetone (8 ml) and to it was added 1,5-dibromopentane (3.79 g). The mixture was heated at 65 °C for 48 h. Anhydrous K_2CO_3 (2.23 g) was added and the mixture was heated at 65 °C for a further 48 h. After filtration, ether was added to the filtrate and it was extracted with 10% HCl. The acidic fraction was made neutral with concentrated aqueous NH_3 and extracted with $CHCl_3$. The combined organic phase was dried and evaporated. Column chromatography of the residue on silica gel (EtOAc) yielded in the earlier fractions the *trans* (**6**) (329 mg, 8.5%) as a solid, mp 65 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.92 (m, 1H), 1.28—1.52 (m, 9H), 1.66—1.71 (m, 2H), 1.81—1.96 (m, 3H), 2.08—2.12 (m, 1H), 2.28 (br s, 2H), 2.42—2.52 (m, 1H), 2.71 (br s, 1H), 3.57 (dd, J = 10.2, 5.9 Hz, 1H), 3.82 (t, J = 10 Hz, 1H), 7.03 (dd, J = 4.9, 3.6 Hz, 1H), 7.18 (d, J = 3.0 Hz, 1H), 7.28 (dd, J = 5.0, 1.1 Hz, 1H). IR ($CHCl_3$): 3320 2940, 1450 cm^{-1}. MS *m/z*: 279 (M^+). *Anal.* Calcd for $C_{16}H_{25}NOS$: C, 68.77; H, 9.02; N, 5.01. Found: C, 68.91; H, 9.01; N, 4.96. Later column fractions gave the *cis* (**5**) (96 mg, 2.5%) as colorless prisms after recrystallization from ether, mp 123—125 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.13—1.38 (m, 5H), 1.44—1.65 (m, 6H), 1.70—1.85 (m, 3H), 1.98—2.09 (m, 2H), 2.24 (d, 2H), 2.88—2.92 (m, 2H), 3.62 (dd, J = 10.6, 4.0 Hz, 1H), 4.35 (t, J = 10.5 Hz, 1H), 6.90 (dd, J = 3.5, 1.1 Hz, 1H), 7.03 (dd, J = 5.0, 3.5 Hz, 1H), 7.23 (dd, J = 4.9, 1.2 Hz, 1H). IR ($CHCl_3$): 3320, 2940, 1450 cm^{-1}. MS *m/z*: 279 (M^+). *Anal.* Calcd for $C_{16}H_{25}NOS$: C, 68.77; H, 9.02; N, 5.01. Found: C, 68.81; H, 8.99; N, 4.82.

Electrophysiological Study A cortical slice of rat brain was prepared in a manner similar to that described by Harrison and Simmonds.[16] Wistar male rats (approximately 200 g) were killed by decapitation and their brains were rapidly removed. A coronal section (600 μm thick) through the level of the corpus callosum was cut in continuously gassed (95% O_2 and 5% CO_2) artificial cerebrospinal fluid of the following composition (mM): NaCl, 124; $MgSO_4$, 1.3; KCl, 2; KH_2PO_4, 1.25; $NaHCO_3$, 25; $CaCl_2$, 2.4; D-glucose, 11, at room temperature. A cortical wedge (1 mm wide) was cut from these slices and mounted in a two-compartment bath, with the ventral margin of the cortical tissue laying almost entirely within one compartment, and the white matter entirely within the other. The chamber containing the cortical tissue was continuously perfused with a Mg^{2+} free medium at a rate of 2 ml/min. The dc potential between the two-compartment was monitored *via* Ag/AgCl electrodes and a high-input impedance amplifier was continuously displayed on a chart recorder through low-pass filter (< 0.5 Hz). Excitatory amino acid agonists were applied for periods of 30 s. Control responses were obtained to test the application of L-glutamate (2 mM) and NMDA (30 μM). In a 30 min application of the test compounds, population depolarizations by L-glutamate and NMDA were recorded.

Radioligand Binding Assay [^{3}H]TCP binding to PCP receptors was performed by the method of Yoshida *et al.* and Ogita *et al.*[19] with slight modification. The forbrains from Wistar male rats (200—250 g) were homogenized in 30 vols. of 50 mM Tris–HCl buffer (pH 7.7 at 25 °C) with a Brinkman Polytron (settings 6, 30 s). The homogenate was centrifuged at 40000 × *g* for 20 min and resuspended in 30 vols. of 5 mM Tris–HCl buffer. The suspension was stored at −80 °C until use. For binding experiments, thawed membranes were centrifuged (40000 × *g*, 20 min). The pellet was resuspended in 120 vols. of 5 mM Tris–HCl buffer containing 0.04% Triton X-100 and stirred at 4 °C for 10 min. The suspension was centrifuged two times (40000 × *g*, 20 min) and finally resuspended in 25 vols. of 5 mM Tris–HCl buffer. The incubation medium consisted of 0.5 ml of 5 mM Tris–HCl buffer containing 5 nM [^{3}H]TCP (47.8 Ci/mmol), 100 μg membrane protein, various amounts of test compounds, 30 μM L-glutamate and 100 μM glycine. Test substances were dissolved in dimethyl sulfoxide (DMSO) and dilutions were made with the buffer so that there was not more than 0.5% of DMSO. Nonspecific binding was determined in the presence of 100 μM PCP. Following a 90 min equilibration at 25 °C, the binding reaction was terminated by vacuum filtration on Whatman GF/C filters presoaked in 0.25% of polyethylenimine. The filters were rinsed twice with 5 ml of ice-cooled 5 mM Tris–HCl buffer and extracted in an Aquasol-2 (NEN) scintillation cocktail. The bound radiolabeled ligand was measured by liquid scintillation spectrometry. IC_{50} values were determined by log-probit analysis.

Acknowledgements The authors are grateful to Mr. Y. Tanaka, Miss Y. Soeda and Mr. R. Isobe for ^{1}H-NMR and MS measurements and to the staff of the Central Analysis Room of Kyushu University for elemental analyses.

References and Notes

1) J. Vignon, R. Chicheportiche, M. Chicheportiche, J. M. Kamenka, P. Geneste, and M. Lazdunski, *Brain Res.*, **280**, 194 (1983).
2) P. C. Contreras, R. Quirion, and T. L. Q'Donohue, *Neurosci. Lett.*, **67**, 101 (1986).
3) W. F. Maragos, D. C. M. Chu, A. B. Young, C. J. D'Amate, and J. B. Penny, Jr, *Neurosci. Lett.*, **74**, 371 (1987).
4) A. P. Kozikowski and Y.-P. Pang, *Mol. Pharmacol.*, **37**, 352 (1990); A. B. Young, J. T. Greenamyre, Z. Hollingsworth R. Albin, C. D'Amato, I. Shoulson, and J. B. Penney, *Science*, **241**, 981 (1988).
5) J. P. Vincent, B. Kartalovoky, P. Geneste, J. M. Kamenka, and M. Lazdunski, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4678 (1979).
6) E. H. F. Wong, J. A. Kemp, T. Priestley, A. R. Knight, G. N.

Woodruff, and L. L. Iversen, *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 7104 (1986).
7) I. Chaudieu, J. Vignon, M. Chicheportiche, A. EL Harfi, J. M. Kamenka, and R. Chicheportiche, *Eur. J. Med. Chem.*, **22**, 359 (1987); Y. Itzhak, *Eur. J. Pharmacol.*, **136**, 231 (1987).
8) J. J. Frost and H. N. Wagner Jr. (ed.), "Quantitative Imaging," Raven Press, New York, 1990.
9) D. O. Kisewetter, C. Rice, M. Mattson, and R. D. Finn, *J. Labelled Compds. Radiopharm.*, **27**, 277 (1989).
10) J.-P. Vincent, J.-N. Bidard. M. Lazdunski, G. Romey, Y. Tourneur, and J. Vignon, *Fed. Proc.*, **42**, 2570 (1983).
11) A. Thurkauf, P. Hillery, M. V. Mattson, A. E. Jacobson, and K. C. Rice, *J. Med. Chem.*, **31**, 1625 (1988); R. L. McQuinn, E. J. Cone, H. E. Shannon, and T.-P. Su, *ibid.*, **24**, 1429 (1981).
12) M. Maeda, S. Tsukiyama, T. Fukumura, K. Orita, and M. Kojima, *Appl. Radiat. Isot.*, **42**, 563 (1991).
13) P. Geneste, P. Herrmann, J. M. Kamenka, and A. Pons, *Bull. Soc. Chim. Fr.*, **7**, 1619 (1975).
14) P. Geneste, J. M. Kamenka, M. S. N. Ung, P. Herrmann, R. R. Goudal, and G. Trouiller, *Eur. J. Med. Chem.*, **14**, 301 (1979).
15) M. L. Pennec, Y. T. Osano, S. Tsukiyama, M. Maeda, and T. Matsuzaki, *Anal. Sci.*, **6**, 785 (1990).
16) N. L. Harrison and M. A. Simmonds, *Br. J. Pharmacol.*, **84**, 381 (1985).
17) Compound (**5**) was found to possess only a low potency ($IC_{50} = 7\ \mu M$) in displacing the σ-opioid ligand [+]-[^{3}H]SKF10047 from the binding sites in rat brainstem.
18) D. T. Manallack, M. G. Wong, M. Costa, P. R. Andrews, and P. M. Beart, *Mol. Pharmacol.*, **34**, 863 (1988).
19) Y. Yoshida, T. Nishikawa, Y. Tanii, and K. Takahashi, *Brain Res.*, **499**, 179 (1989); K. Ogita and Y. Yoneda, *Biochem. Biophys. Res. Commun.*, **153**, 510 (1988).

Metal-Containing Components in Medicinal Plants. II.[1] Convenient Identification of Some *Datura* Species by Peptide Mapping of Their Ferredoxins

Yoshiki MINO

Osaka University of Pharmaceutical Sciences, 2-10-65, Kawai, Matsubara, Osaka 580, Japan. *Received November 15, 1990*

A new method for plant identification is proposed in which each ferredoxin (Fd) is isolated from small amounts of fresh leaves of five species of genus *Datura* and the tryptic digest of each *S*-carboxymethlated Fd is subsequently compared by peptide mapping using reversed-phase high-performance liquid chromatography (HPLC). This method makes it possible to analyze five samples in 4 or 5 d. The results obtained for the five plants indicate the HPLC patterns which reflect amino acid sequences of Fd to possibly be correlated to their morphological similarity.

Keywords *D. metel* (*D. alba*); *D. innoxia*; *D. stramonium* var. *stramonium*; *D. stramonium* var. *tatula*; *D. arborea*; *Datura* species; ferredoxin; peptide mapping; identification; high-performance liquid chromatography

In pharmacognosy it is important to identify closely related medicinal plants scientifically. The most reliable method is to analyze whole genes from each plant; however, even at the present time, this is too difficult. It may be possible to identify such plants by the amino acid sequences of their common proteins that reflect the nucleotide base sequences of the corresponding parts of the genes. In the course of our studies to elucidate metal-containing components of medicinal plants, we found that iron in the anionic soluble fraction from *Datura* species is present mainly as ferredoxin (Fd), one of the iron–sulfur proteins.[1,2] Amino acid analysis for each Fd indicated slight differences in amino acid composition among them, suggesting the possibility that the amino acid composition or sequence of the protein provides valuable information for identification of *Datura* species.

In this study, to establish a rapid identification method for *Datura* plants by differences in amino acid sequences of *Datura* Fds, we minimized the scale of starting leaves and purified Fds rapidly by a mini-column, fast protein liquid chromatography (FPLC), and high performance gel chromatography. Peptide mapping by reversed-phase high performance liquid chromatography (RP-HPLC) was used to detect differences in amino acid sequences. By this method, it is possible to analyze five *Datura* plant samples in 4 or 5 d. The results may provide valuable information about the taxonomy of *Datura* species.

Experimental

Apparatus Isolation and analyses for Fds and its related substances were carried out on a Waters HPLC system (model 510 pumps, M680 gradient controller, and M441 absorbance detector) equipped with a stainless steel column, μ-Bondasphere C18-100 Å (3.9 mm i.d. × 15 cm) or Protein-pack 125 (7.8 mm i.d. × 30 cm) (Waters Assoc.). The purification of Fds was performed on a Waters W650 protein system using Mono Q column (HR5/5, Pharmacia).

Materials *Datura metel* L. (*D. alba* NEES) (chousen asagao in Japanese), *D. innoxia* MILL. (kechousen asagao), *D. stramonium* var. *stramonium* (shirobana youshu chousen asagao), and *D. stramonium* var. *tatula* (youshu chousen asagao) were cultivated in an herb garden at this university. *D. arborea* L. (kodachi chousen asagao) were cultivated in a greenhouse of the university. *D. metel* (*D. alba*) has white corollas, glabrous stem and leaves, and very short spines or tubercle on the capsules. *D. innoxia* has white corollas, leaves and stem covered with soft hairs, and capsules covered with long, slender spines. *D. stramonium* var. *stramonium* has white corollas, ovoid and spiny capsules, glabrous green stem, and glabrous, ovata and sinuately dentate leaves. *D. stramonium* var. *tatula* has lavender corollas and purple stem. The other features are the same as those of *D. stramonium* var. *tatula*. *D. arborea*, the tree *Datura*, has white large corollas, woody stem, and long and slender capsules.

Recommended Procedures Isolation of Fds from Each Type of *Datura* Leaves: Homogenize the frozen fresh *Datura* leaves (10 g) in an ice bath with 20 ml of 0.02 M Tris–HCl buffer, pH 7.5, and filter the homogenate through two layers of cloth mesh. After centrifugation at 16000 × *g*, apply the supernatant onto a mini-column (7 mm i.d. × 6 cm, Muromachi Kagaku) containing 2.5 ml of diethylaminoethyl (DEAE) cellulose (DE52, Whatman), equilibrated with 10 ml of 1 M Tris–HCl, pH 7.5, followed by 10 ml of 0.02 M Tris–HCl buffer. Wash the column with the buffer containing 0.2 M NaCl (10 ml) and then elute the desired fraction with the same buffer containing 0.4 M NaCl (10 ml). Dilute the eluent (10 ml) to 25 ml with the NaCl free buffer, and apply the solution onto another mini-column containing 1 ml of the pre-equilibrated DEAE cellulose, followed by elution of 1 ml of the buffer containing 1 M NaCl. The desired portion is recovered in the 1 ml. Inject the desalted solution by gel filtration (NAP-10 column, Pharmacia) into the Mono Q column [gradient of 0—0.75 M NaCl in 0.02 M Tris–HCl], and collect the Fd fraction shown as the sharp peak at *ca.* 38 min (retention time, t_R) in the case of the ultraviolet (UV) (420 nm) detection (Fig. 1). After concentrating this fraction to *ca.* 100 μl by ultrafiltration (Centricon 10, Amicon), inject *ca.* 100 μl of the solution into the Protein-pack 125 column (mobile phase: the buffer containing 0.2 M NaCl). Pool the reddish fraction at 15 min (t_R) (see Fig. 2), and concentrate the solution to about 50 μl by Centricon 10. The resulting solution is almost pure Fd.

Preparation for Apo-Fd and S-Carboxymethylated (Cm-) Fd: Transfer the almost pure Fd solution (50 μl) to a microtube (Eppendorf tube, 1.5 ml). Add 1.8 M trichloroacetic acid (TCA) (25 μl) to the solution, collect the precipitate by convenient centrifugation, and wash it with 20 μl of 0.6 M TCA by centrifugation. Carboxymethylation of the obtained apo-Fd is performed according to a slightly modified method of Crestfield *et al.*[3] Dissolve the precipitate in 100 μl of 0.4 M Tris–HCl buffer, pH 8.6, containing 6 M guanidine HCl and 0.2% ethylenediaminetetraacetic acid (EDTA), and add 4 μl of β-mercaptoethanol. Allow to stand for 3 h in anerobic condition, and add 10.7 mg of iodoacetic acid (IAA) in 40 μl of 1 M NaOH. Again allow the solution to stand for 15 min after the addition of 25 μl of 1 M NaOH to adjust the pH to neutral. Change the buffer containing IAA using NAP-10 to 0.02 M Tris–HCl, pH 7.5. All steps after the addition of IAA should be conducted without exposure to light to prevent sub-reaction. Next, isolate pure Cm-Fd by RP-HPLC (μ-Bondasphere C18, trifluoroacetic acid (TFA)/CH_3CN/water solvent system [A = 0.1% TFA; B = 0.1% TFA, 100% CH_3CN] with a gradient program [23% B—48% B in 24 min; flow rate 1 ml/min). The main peak (500 μl) at 16 min (t_R) is collected in a microtube; change the solvent to 0.1 M NH_4HCO_3 (pH 8.5) for trypsin digestion.

Trypsin Digestion and Comparison of HPLC Patterns: Add L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (40 μg) to the buffer solution containing Cm-Fd and allow it to react at 37 °C for 3 h. Add 10% TFA solution (35 μl) to 700 μl of digestion solution, and inject into the reversed-phase column (μ-Bondasphere C18, the same solvent system as used in the isolation of Cm-Fd with the gradient program [0% B—40% B in 50 min]; flow rate 1 ml/min). The obtained chromatograms are then compared with each other.

Results and Discussion

To establish a routine method to determine the differences

in the amino acid sequence of Fd, attempts were made to scale down the amount of starting material (leaves) to only 10 g and simplify the procedure. In previous experiments,[1] since about 1 kg of *Datura* leaves was used as starting material, comparatively large amounts of resin such as DEAE cellulose and Sephadex G-75 and preparative-size columns were required. In this study, a mini-column, FPLC and HPLC were primarily used for rapid analysis. First, a mini-column containing 2.5 ml of DEAE cellulose was used for the adsorption of Fd. The adsorbed Fd was eluted with the 0.02 M Tris–buffer, pH 7.5, containing 0.4 M NaCl, whereas no Fd was eluted with the buffer containing 0.2 M NaCl. Consequently, the Fd fraction adsorbed from *Datura* leaves was eluted from the mini-column with the buffer containing 0.4 M NaCl after washing the column with the buffer containing 0.2 M NaCl to remove extraneous substances. A crude Fd fraction (1.5 ml) was obtained by concentrating the resulting solution (10 ml) using another mini-column containing 1 ml of DEAE cellulose and desalting by gel filtration (NAP-10).

Figure 1 shows the FPLC chromatograms of the crude Fd fractions eluted with 0.4 M NaCl buffer. Even at this step, Fd appeared as a sharp peak at about 40 min (t_R). The pool of the peak was further purified by high performance gel chromatography (Protein-pack 125, Waters) (Fig. 2). The peak at 17 min (t_R) is pure Fd. After treatment of TCA followed by carboxymethylation, Cm-Fd was finally purified by RP-HPLC as seen in Fig. 3. Note that four Cm-Fds from *metel* (*alba*), *innoxia*, *stramonium* var. *stramonium*, and *stramonium* var. *tatula* have their main peaks at the same retention time (15.0 min), whereas the main peak of *D. arborea* Cm-Fd appeared at a different retention time (15.6 min). Indeed, the mixture of *stramonium* var. *tatula* and *arborea* Cm-Fds exhibited two peaks (the second arising from *arborea* Cm-Fd). This suggests that the primary structure of *arborea* Fd is somewhat different from those of other *Datura* Fds.

Figure 4 shows HPLC chromatograms of peptide

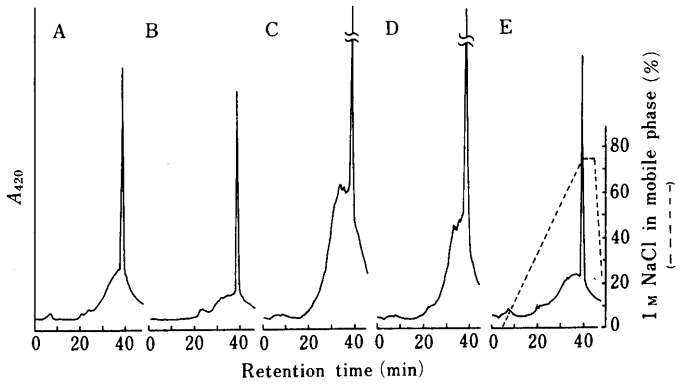


Fig. 1. FPLC of the Crude Fd Obtained by Elution from Mini-Column (DE52) with 0.4 M NaCl

A, *Datura metel* (*D. alba*); B, *D. innoxia*; C, *D. stramonium* var. *stramonium*; D, *D. stramonium* var. *tatula*; E, *D. arborea*. Column, Mono Q (HR5/5); flow rate, 0.5 ml/min; gradient elution of 0—0.75 M NaCl in 0.02 M Tris–HCl (pH 7.5).

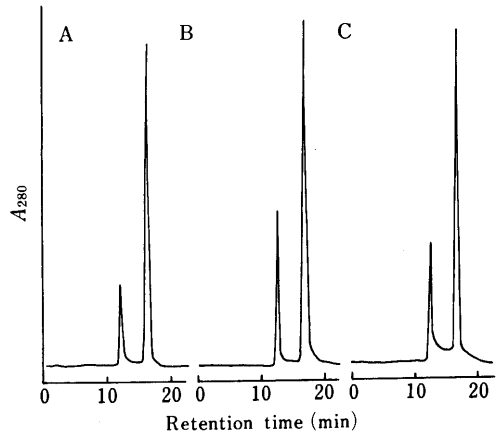


Fig. 2. High-Performance Gel Chromatography of the Fd Fractions after the FPLC (Mono Q)

A, *D. innoxia*; B, *D. stramonium* var. *stramonium*; C, *D. arborea*. Column, Protein-pack 125 (7.8 mm i.d. × 30 cm); flow rate, 0.5 ml/min; mobile phase, 0.02 M Tris–HCl (pH 7.5) containing 0.2 M NaCl.

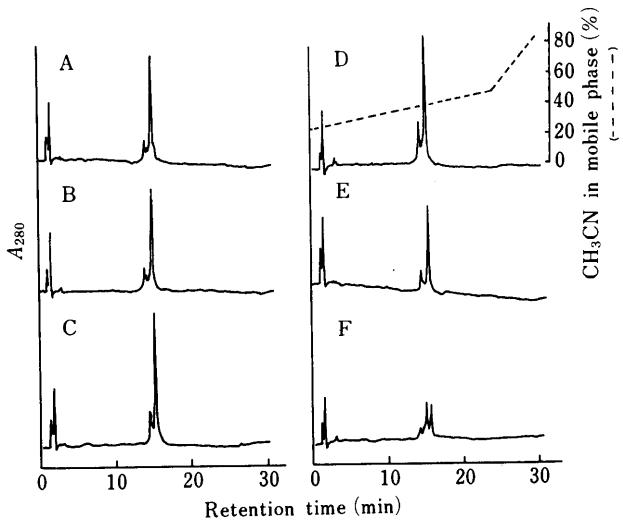


Fig. 3. HPLC of Each *Datura* Cm-Fd

A, *Datura metel* (*D. alba*); B, *D. innoxia*; C, *D. stramonium* var. *stramonium*; D, *D. stramonium* var. *tatula*; E, *D. arborea*; F, *tatula* + *arborea*. Column, μ-Bondasphere C18 (3.9 mm i.d. × 15 cm); flow rate, 1 ml/min, mobile phase, 0.1% TFA in H20/0.1% TFA in CH_3CN.

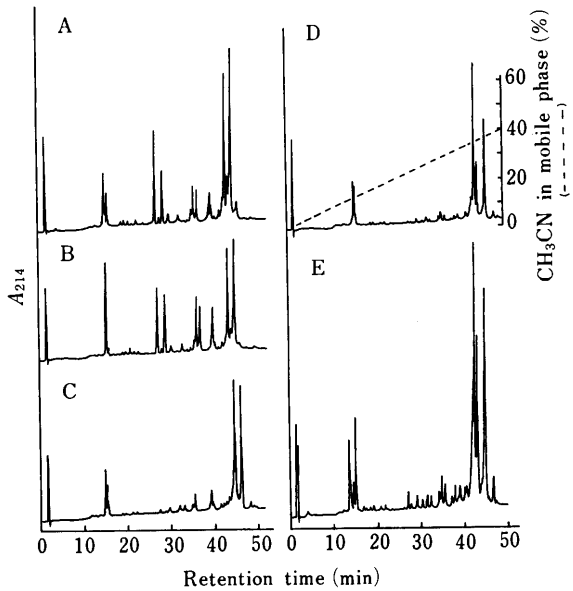


Fig. 4. HPLC of the Tryptic Digest of Each *Datura* Cm-Fd

A, *Datura metel* (*D. alba*); B, *D. innoxia*; C, *D. arborea*; D, *D. stramonium* var. *stramonium*; E, *D. stramonium* var. *tatula*. Gradient program was different from that in Fig. 3, although other chromatographic conditions were the same.

mixtures of tryptic digestion from each *Datura* Fd. These are thought to be more sensitive to differences in the amino acid sequence of Fd than those for the whole Cm-Fd. *D. metel* (*D. alba*) and *innoxia* gave very close chromatograms in which only the ratios of peak heights slightly differed. Thus, the chromatograms were virtually identical. *D. stramonium* var. *stramonium* and var. *tatula* gave identical chromatograms. Their peak patterns at around 45 min (t_R) differed from those for *D. metel* (*D. alba*) and *innoxia*. One clear distinction is the two peaks at 27 min (t_R) observed only in the former group (Fig. 4). In our previous report,[1] amino acid analyses on four *Datura* Fds indicated *metel* (*alba*) and *innoxia* to possess two arginine residues in each molecule, whereas *stramonium* var. *stramonium* and *stramonium* var. *tatula*, only one. Since trypsin hydrolyzes the peptide bond at the carboxyl side of arginine and lysine residue, the arginine-containing long peptide may possibly be degraded by this enzyme to form the two additional shorter peptides which appear as the peaks at 27 min (t_R). However, the corresponding long peptide must remain unchanged in *stramonium* and *tatula* Fds. The chromatogram of the peptide mixture from *arborea* Fd, having main peaks at 44.8 and 46.0 min (t_R), is apparently distinguished from those of any groups (42.4 and 44.0 min for the *alba* group; 43.2, 44.0, and 45.6 min for the *stramonium* group). Of interest is the fact that morphologically analogous plants, *D. metel* (*D. alba*) and *D. innoxia*, or *D. stramonium* var. *stramonium* and *D. stramonium* var. *tatula*, exhibit the same HPLC patterns, although three groups belonging to different sections in genus *Datura* give distinct patterns. *D. metel* (*D. alba*) and *D. innoxia*, *D. stramonium* var. *stramonium* and *D. stramonium* var. *tatula*, and *D. arborea* belong to the *Dutra* section, *Stramonium* section, and *Brugmansia* section, respectively. The latter is thought to differ considerably from other sections of the genus *Datura*, the so-called herbaceous *Datura*. In practice, the species forming the section *Brugmansis* were first placed in a distinct genus by Person in 1805.[4] This seems consistent with the finding that *D. arborea* Fd exhibits a chromatogram distinct from others, even in HPLC of Cm-Fd.

Some confusion still remains in naming of the two varieties of *D. stramonium*. *D. stramonium* with white flowers and *D. tatula* with purple flowers, both having spiny capsules, were proposed by Linnaeus in 1753 and 1762, respectively, as two distinct species. Other botanists, however, were inclined to regard them rather as varieties. The breeding experiments of several botanists have proved that white-flowered *D. stramonium* L. and the purple-flowered *D. tatula* L. differ only by a single pair of genes and that they belong to a single species.[4] In addition, the breeding experiments of Tabata *et al.* on morphological characters and alkaloid content support the proposal by Blakeslee and others that *D. stramonium* L. and *D. tatula* L. should be considered two varieties of a single species.[5] So, *D. stramonium* var. *stramonium* and *D. stramonium* var. *tatula* are used here, as many present botanists do. In this connection, it is of interest that these two *Datura* plants gave reasonably the same chromatograms in this study. More detailed study on the taxonomy of *Datura* species should be advanced by careful determination of the primary structures of each Fds.

In conclusion, this method, in which each Fd is isolated from a small amount of plant leaves and the HPLC patterns of the tryptic digest of Cm-Fds are subsequently compared to determine whether amino acid sequences are identical, should be found useful for identifying closely related plants scientifically.

Acknowledgments The author is grateful to Dr. Toshiro Matsuoka, Kyoto Pharmaceutical University, for supplying *Datura metel* (*D. alba*), *D. stramonium* var. *stramonium*, and *D. stramonium* var. *tatula*, and to Prof. Yukio Noro, Faculty of Pharmacy, Meijo University, for his valuable comments on naming Datura species. Thanks are also due to Emeritus Prof. Nagayo Ota for his helpful discussion.

References

1) Inorganic Chemical Approaches to Pharmacognosy. XI. Part X: Y. Mino, *Syoyakugaku Zasshi*, **45**, 145 (1991).
2) D. I. Arnon and B. B. Buchanan, *Methods Enzymol.*, **23**, 413 (1971).
3) A. M. Crestfield, S. More, and W. H. Stein, *J. Biol. Chem.*, **238**, 622 (1963).
4) S. Satina and A. G. Avery, "Blakeslee: Genus Datura," ed. by A. G. Avery, S. Satina, and J. Rietsema, Ronald Press Company, New York, 1959.
5) M. Tabata, N. Hiraoka, and M. Konoshima, *Syoyakugaku Zasshi*, **24**, 65 (1970).

Chikusetsusaponin. VI. A New Saponin from the Rhizome of *Panax pseudo-ginseng* var. *angustatus* HARA

Hiroshi KOHDA,*[,a] Seiji TANAKA,[a] Yasutoshi YAMAOKA,[a] Hiromasa IZUMI,[b] Mariko NUNO,[c] Susumu ISODA,[d] Katsumi GOTOH,[e] Takashi WATANABE,[f] Shigeki KATSUKI[g] and Motoyoshi SATAKE[h]

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine,[a] Kasumi, Minami-ku, Hiroshima 734, Japan, Medicinal Plant Garden of Tokyo College of Pharmacy,[b] Horinouchi, Hachioji, Tokyo 192–03, Japan, Medicinal Plant Garden, Faculty of Pharmaceutical Sciences, Teikyo University,[c] Suarashi, Sagamiko-machi, Kanagawa 199–01, Japan, Medicinal Plant Garden of School of Pharmaceutical Sciences, Showa University,[d] 1–5–8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan, Garden of Medicinal Plants, Kyoto Pharmaceutical University,[e] Hinohayashi-cho 39, Fushimi-ku, Kyoto 601–13, Japan, Medicinal Plant Garden, School of Pharmaceutical Sciences, Kitasato University,[f] Kitasato 2–15–1, Sagamihara, Kanagawa 228, Japan, National Institute of Hygienic Sciences, Tanegashima Medicinal Plant Research Station,[g] Nakatane-cho Noma, Kumage-gun, Kagoshima 891–36, Japan, and National Institute of Hygienic Sciences, Tsukuba Medicinal Plant Research Station,[h] Yatabe-cho, Tsukuba, Ibaraki 305, Japan. *Received November 26, 1990*

A novel saponin named chikusetsusaponin VI was isolated from the rhizome of *Panax pseudo-ginseng* var. *angustatus* HARA and its structure was determined as 20(*S*)-protopanaxadiol 3-*O*-β-D-glucopyranosyl(1→2)-[β-D-xylopyranosyl-(1→6)]-β-D-glucopyranosido-20-*O*-β-D-glucopyranosyl(1→6)-β-D-glucopyranoside.

Keywords *Panax pseudo-ginseng* var. *angustatus*; Araliaceae; chikusetsusaponin VI; hosoba-ninjin; ginsenoside; saponin

In our series of studies on the evaluation[1] of wild materials collected as crude drugs, we have now investigated *Panax pseudo-ginseng* var. *angustatus* HARA (Araliaceae) so-called "hosoba-ninjin."[2] Isolation and structure determination of a number of saponins from "chikusetsu-ninjin," a rhizome of *P. pseudo-ginseng* subsp. *japonicus* HARA (synonym, *P. japonicus* C. A. MEYER) have been reported.[3] This time, a new saponin named chikusetsusaponin VI (**1**) was isolated from the dried rhizome of *P. pseudo-ginseng* var. *angustatus* along with five known saponins. This paper deals with the structure elucidation of **1**.

The MeOH extract of the dried rhizome of *P. pseudo-ginseng* var. *angustatus*, collected at the foot of Mt. Fuji, was treated as described in the experimental section to afford notoginsenoside R_2[4] (140 mg), ginsenoside Rf[5] (15 mg), chikusetsusaponin III (120 mg), IVa (20 mg), IV (200 mg), V (300 mg) and VI (20 mg).

Chikusetsusaponin VI (**1**) was obtained as a powder, $[\alpha]_D^{19}$ −10.3° (c=1.07, pyridine), and its molecular formula was determined as $C_{59}H_{100}O_{27}$ on the basis of the elemental analysis and the negative fast-atom bombardment mass spectrum (negative FAB-MS); $[M-H]^-$ at m/z 1239. Furthermore, negative fragment ions at m/z 1107 [M−H−pentose]$^-$, m/z 1077 [M−H−hexose]$^-$, m/z 915 [M−H−hexose−hexose]$^-$ and m/z 783 [M−H−hexose−hexose−pentose]$^-$ were observed.

The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **1** showed two olefinic carbon signals [δ 125.92 (d, C-24) and δ 130.99 (s, C-25)] and five anomeric carbon signals [δ 98.07, 104.93, 105.30, 105.86, 105.95]. The signals due to the aglycone moiety were in good agreement with those of 20(*S*)-protopanaxadiol (20(*S*)-ppd),[6] which was one of the main aglycones of ginsenosides. Moreover, **1** demonstrated the glycosylation shifts around C-3 and C-20 as observed for 3,20-*O*-bis-desmoside of 20(*S*)-ppd[7] (see Table I).

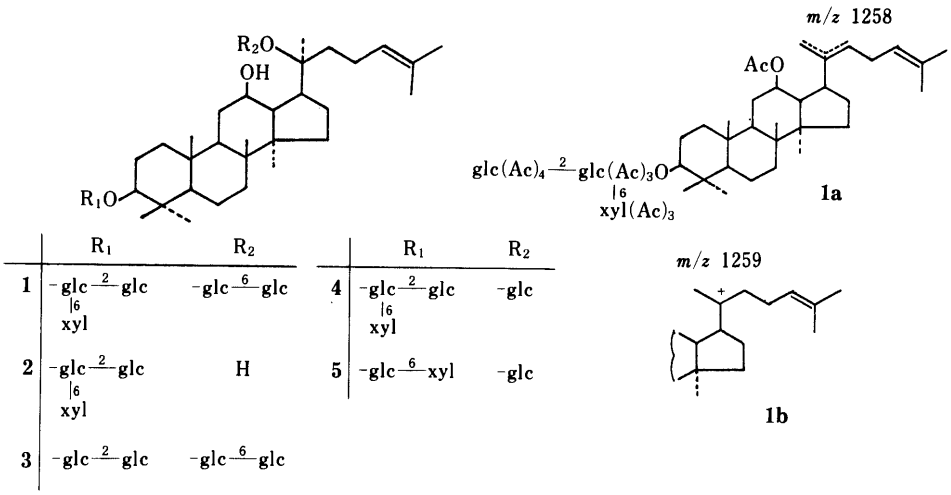
Comparison of the ^{13}C-NMR spectrum of **1** with those of chikusetsusaponin III (**2**) and ginsenoside Rb_1 (**3**) revealed that the signals due to the sugar moiety of **1** consisted of those due to the 3-*O*-β-D-glucopyranosyl (1→2)-[β-D-xylopyranosyl (1→6)]-β-D-glucopyranosyl moiety of **2** and those assigned to the 20-*O*-β-gentiobiose moiety of **3**. The mass spectra of acetate of **1** exhibited a pair of ions, m/z 1258 and m/z 1259 due to the prosapogenin moiety (1_a+1_b)[8] and ions at m/z 259 and m/z 331, characteristic of terminal pentose and terminal hexose.

On acid hydrolysis, **1** afforded as a modified aglycone, panaxadiol, and glucose and xylose as sugar components. On mild acid hydrolysis with aqueous acetic acid, **1** afforded gentiobiose along with a pair of prosapogenins,[9] which

TABLE I. ^{13}C-NMR Data of **1** and Related Compounds in C_5D_5N

Carbon	Compounds				Sugar moieties		
	2	**1**	**3**		**2**	**1**	**3**
1	39.1	39.4	39.1	C-3 Glc 1	104.9	105.0	105.0
2	27.9	26.7	26.6	Glc 2	83.0	83.1	82.9
3	88.9	89.1	89.3	Glc 3	77.9	78.1	77.2
4	39.6	39.7	39.6	Glc 4	71.0	71.3	71.5
5	56.3	56.5	56.3	Glc 5	76.4	76.4	78.0
6	18.5	18.4	18.6	Glc 6	69.9	70.2	62.6
7	35.1	35.1	35.1	Glc 1	105.7	105.9	105.6
8	39.9	40.1	39.9	Glc 2	76.8	77.0	76.7
9	50.3	50.2	50.1	Glc 3	77.9	78.2	78.8
10	36.8	37.0	36.8	Glc 4	71.5	71.1	71.5
11	32.0	30.8	30.8	Glc 5	77.9	77.9	78.0
12	71.0	70.2	70.1	Glc 6	62.6	62.8	62.6
13	48.3	49.5	49.3	Xyl 1	105.7	105.9	
14	51.6	51.5	51.3	Xyl 2	74.6	74.9	
15	31.3	30.8	30.8	Xyl 3	77.9	78.1	
16	27.0	26.7	26.6	Xyl 4	71.0	71.7	
17	54.6	51.7	51.6	Xyl 5	67.0	67.0	
18	16.3[a)]	16.2	16.2	C-20 sugars			
19	15.7[a)]	16.2	16.2	Glc 1		98.1	97.9
20	72.9	83.5	83.5	Glc 2		75.2	74.9
21	27.0	22.3	22.6	Glc 3		79.1	78.0
22	35.9	36.2	36.1	Glc 4		71.5	71.5
23	22.9	23.1	23.1	Glc 5		77.0	76.7
24	126.3	125.9	125.8	Glc 6		70.0	71.5
25	130.7	130.9	131.0	Glc 1		105.2	105.0
26	25.8	25.7	25.8	Glc 2		74.7	74.9
27	17.7	17.9	17.9	Glc 3		78.2	78.0
28	28.0	28.1	28.0	Glc 4		71.7	71.5
29	16.5[a)]	16.5	16.5	Glc 5		77.9	78.0
30	17.0[a)]	17.5	17.3	Glc 6		62.8	62.6

a) Assignments may be interchangeable within the same column.



	R_1	R_2
1	-glc(2→1)glc, 6→1 xyl	-glc(6→1)glc
2	-glc(2→1)glc, 6→1 xyl	H
3	-glc(2→1)glc	-glc(6→1)glc
4	-glc(2→1)glc, 6→1 xyl	-glc
5	-glc(6→1)xyl	-glc

Chart 1

were identical with a 20-epimeric pair of **2** prepared from **2** itself under the same condition. This suggested **1** to be 20-*O*-gentiobioside of **2**.

On enzymatic hydrolysis with takadiastase, **1** yielded two prosapogenins (**4** and **5**). One (**5**) was identified as 20-*O*-*β*-D-glucopyranosyl-20(*S*)-protopanaxadiol 3-*O*-*β*-D-xylopyranosyl (1→6)-*β*-D-glucopyranoside by comparison of thin layer chromatography (TLC) behavior and ^{13}C-NMR spectrum and the other (**4**) was characterized as 20-*O*-*β*-D-glucopyranosyl-20(*S*)-protopanaxadiol 3-*O*-*β*-D-glucopyranosyl (1→2)-[*β*-D-xylopyranosyl(1→6)]-*β*-D-glucopyranoside based on the detailed analysis of ^{13}C-NMR spectrum (Table I).

Accordingly, **1** was deduced to be 20-*O*-*β*-D-glucopyranosyl (1→6)-*β*-D-glucopyranosyl-20(*S*)-protopanaxadiol 3-*O*-*β*-D-glucopyranosyl (1→2)-[*β*-D-xylopyranosyl (1→6)]-*β*-D-glucopyranoside.

Our present result provided the first example of saponin having five monosaccharide units from the taxon within the *P. pseudo-ginseng* species. From a geographical point of view, the existence of chikesetsusaponin VI of *P. pseudo-ginseng* subsp. *japonicus* and other *Panax* spp. is in progress.

Experimental

General Methods Optical rotation was measured with a Union PM-101 automatic polarimeter. ^{1}H- and ^{13}C-NMR spectra were measured on a JEOL GX-400 spectrometer in a C_5D_5N solution using tetramethylsilane (TMS) as an internal standard. For gas liquid chromatography (GLC), a Shimadzu GC-6A apparatus was used with a glass column of 1.5% SE-30 on Chromosorb W, 4 mm × 2 m; detector, FID; injection temperature 200 °C; column temperature 180 °C; carrier gas, N_2, 1.0 kg/cm^2. EI-MS was taken on a JEOL JMS-SX 102 spectrometer by the direct inlet method. FAB-MS was performed on a JEOL JMS D-300 instrument.

For column chromatography, Kieselgel 60H (Art. 7736, Merck), LiChroprep RP-18 (25—40 *μ*m) and Diaion HP-20 (Mitsubishi Chem. Ind. Co., Ltd., Tokyo, Japan) were used. All solvent systems for chromatography were homogeneous.

Extraction and Separation of Saponins The air-dried rhizome of *Panax pseudo-ginseng* var. *angustatus* (14 g), collected in Fujiyoshida, Yamanashi-prefecture, Japan, were extracted with hot MeOH and then with hot 80% aqueous MeOH to give an MeOH extract. The extracts were combined and evaporated to dryness. The residue MeOH extract (5.1 g) was chromatographed on a column of Diaion HP-20 by elution with 40% aqueous MeOH, MeOH and finally with $CHCl_3$.

A saponin mixture (2.15 g) eluted with MeOH was separated by chromatography on silica gel with $CHCl_3$–MeOH–H_2O (30:10:1, 15:7:1, 15:10:2 successively) to give six fractions, fr.-1 to fr.-6. Fraction-1 was crystallized from MeOH–EtOAc to give notoginsenoside-R_2 (yield 1%). The other fractions were further chromatographed individually on a reverse phase column, Lichroprep RP-18 (60% or 65% MeOH), to give ginsenoside Rf (15 mg) from fr.-2, chikusetsusaponin III (**2**, 120 mg) from fr.-3, chikusetsusaponin IVa (20 mg) from fr.-4, chikusetsusaponin IV (200 mg) from fr.-5, chikusetsusaponin V (350 mg) and VI (**1**, 30 mg) from fr.-6. Identification of the known saponins was carried out by comparison of TLC on a precoated plate (Merck) of SiO_2 ($CHCl_3$–MeOH–H_2O (15:6:1)) and/or ODS (60% MeOH), and ^{13}C-NMR, ^{1}H-NMR spectroscopy and mass spectroscopy of the acetates or TMSi ethers. Chikusetsusaponin VI (**1**) white powder $[\alpha]_D^{19}$ −10.3° (*c*=1.07, pyridine), negative FAB-MS, *m/z* 1239 ([M−H]$^-$), 1107 ([M−H]$^-$−pentose), 1077 ([M−H]$^-$−hexose), 915 ([M−H]$^-$−hexose−hexose) and 783 ([M−H]$^-$−hexose−hexose−pentose). ^{1}H-NMR (C_5D_5N) *δ*: 5.50 (1H, br s, 24-H), 4.84 (1H, d, *J*=7.7 Hz, anomeric H), 4.91 (1H, d, *J*=7.3 Hz, anomeric H), 5.07 (1H, d, *J*=7.7 Hz, anomeric H), 5.11 (1H, d, *J*=7.9 Hz, anomeric H), 5.30 (1H, d, *J*=7.5 Hz, anomeric H). ^{13}C-NMR data are given in Table I. *Anal.* Calcd for $C_{59}H_{100}O_{27}\cdot 3H_2O$: C, 54.70; H, 8.25. Found: C, 54.78; H, 8.41.

Acid Hydrolysis of 1 Hydrolysis of **1** (3 mg) was carried out with 10% HCl in H_2O–dioxane (1:1) at 80 °C for 4 h. The reaction mixture was concentrated to dryness by blowing N_2 gas flow at room temperature. TLC: on Silica gel $60F_{254}$ with solvent $CHCl_3$–MeOH–H_2O. The residue was trimethylsilylated with *N*-trimethylsilylimidazole (TMSi) (0.5 ml) in a sealed micro-tube at 80 °C for 2 h, followed by dilution with H_2O and then extraction with *n*-C_6H_{14}. The *n*-C_6H_{14} layer was worked up in the usual manner and concentrated to dryness under a N_2 gas flow at room temperature. A solution of the residue in *n*-C_6H_{14} was subjected to GLC analysis. TMSi-glucose, TMSi-arabinose and TMSi-xylose were identified by comparison of the retention times with those of authentic samples.

Partial Hydrolysis of 1 A solution of **1** (60 mg) in 50% AcOH was heated at 70 °C for 4 h. The reaction mixture was diluted with H_2O and extracted with 1-BuOH (saturated with H_2O). The BuOH layers were concentrated to dryness (51 mg) and the residue was purified by column chromatography on silica gel with $CHCl_3$–EtOAc–MeOH–H_2O, giving prosapogenin as a white powder (15 mg), which was identified by comparison of the ^{13}C-NMR spectrum and TLC behavior with those of an authentic sample obtained from chikusetsusaponin III by the same treatment. The aqueous layer was deionized on Amberlite NM-3 and concentrated to dryness *in vacuo* to give gentiobiose which was identified by comparison of the ^{13}C-NMR spectrum of an authentic sample.

Enzymatic Hydrolysis of 1 A solution of **1** (60 mg) and takadiastase A (60 mg, Tanabe Pharm. Co., Ltd., Osaka, Japan) in 10 ml of McIlvaine buffer (pH 4) was incubated at 38 °C for 2 d. The reaction mixture was diluted with water and then extracted with 1-BuOH. The 1-BuOH extract was washed with H_2O and evaporated to dryness, and the residue was subjected to chromatography on Lichroprep Rp-18 to give two prosapogenins, **4** (37 mg) and **5** (10 mg). **4** and **5** was identified as 20-*O*-*β*-D-glucopyranosyl-chikusetsusaponin III and 20-*O*-*β*-D-glucopyranosyl-chikusetsusaponin Ia on comparison of the ^{13}C- and ^{1}H-NMR spectral

data, respectively. The aqueous layer was passed through the column of Amberlite MB-3 and evaporated to dryness. The carbohydrate components of the hydrolysate were identified as glucose and xylose by TLC and gas chromatography.

Acknowledgements We are grateful to Dr. O. Tanaka, Dr. R. Kasai and Dr. K. Ohtani of Hiroshima University for their valuable advice. Thanks are also due to Professor Nishioka and Dr. M. Shoyama of Kyushu University for measurement of elementary analysis.

References and Notes

1) K. Gotoh, H. Izumi, M. Nuno, S. Isoda, H. Kohda, and M. Satake, *Shoyakugaku Zasshi*, **42**, 51 (1988); H. Yamaguchi, H. Matsuura, R. Kasai, O. Tanaka, M. Satake, H. Kohda, H. Izumi, M. Nuno, S. Katsuki, S. Isoda, J. Shoji, and K. Gotoh, *Chem. Pharm. Bull.*, **36**, 4177 (1988).
2) H. Hara, *Shokubutsu Kenkyu Zasshi*, **45**, 197 (1970).
3) N. Kondo and J. Shoji, *Yakugaku Zasshi*, **88**, 325 (1968); N. Kondo, K. Aoki, H. Ogawa, R. Kasai, and J. Shoji, *Chem. Pharm. Bull.*, **18**, 1558 (1970); T. D. Lin, N. Kondo, and J. Shoji, *ibid.*, **24**, 253 (1976).
4) J. Zhou, M. Z. Wu, S. Taniyasu, H. Besso, O. Tanaka, Y. Saruwatari, and T. Fuwa, *Chem. Pharm. Bull.*, **29**, 2844 (1981).
5) S. Sanada, N. Kondo, J. Shoji, O. Tanaka, and S. Shibata, *Chem. Pharm. Bull.*, **22**, 2407 (1974).
6) J. Asakawa, R. Kasai, K. Yamasaki, and O. Tanaka, *Tetrahedron*, **33**, 1935 (1977).
7) R. Kasai, M. Suzuo, J. Asakawa, and O. Tanaka, *Tetrahedron Lett.*, **1977**, 175.
8) T. Komori, O. Tanaka, and Y. Nagai, *Organic Mass Spectrometry*, **9**, 744 (1974); R. Kasai, K. Matsuura, O. Tanaka, S. Sanada, and J. Shoji, *Chem. Pharm. Bull.*, **25**, 3277 (1977).
9) S. Shibata, T. Ando, and O. Tanaka, *Chem. Pharm. Bull.*, **14**, 1157 (1966).

Physagulin C, a New Withanolide from *Physalis angulata* L.[1)]

Kazushi SHINGU,[a] Nobuhiro MARUBAYASHI,[b] Ikuhiko UEDA,[b] Shoji YAHARA[a] and Toshihiro NOHARA*,[a]

Faculty of Pharmaceutical Sciences, Kumamoto University,[a] Oe-honmachi 5–1, Kumamoto 862, Japan and Research Laboratories, Yoshitomi Pharmaceutical Industries Ltd.,[b] 955 Koiwai, Yoshitomi-cho, Chikujo-gun, Fukuoka 871, Japan. *Received December 20, 1990*

A new withanolide, physagulin C was isolated from the methanolic extract of the fresh leaves and stems of *Physalis angulata* L. (Solanaceae) and the structure was determined to be (20*S*,22*R*)-15α-acetoxy-5β(6),16β(17)-diepoxy-4β,14β-dihydroxy-1-oxo-witha-2,24-trienolide by spectroscopic means and X-ray analysis.

Keywords *Physalis angulata*; Solanaceae; withanolide; X-ray analysis; physagulin C

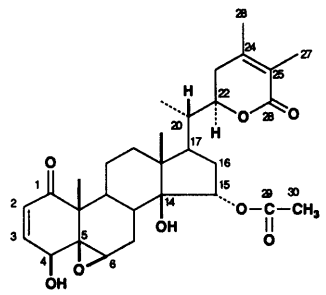
Through searching for constituents possessing antitumor activities from solanaceous plants, we previously found some withanolide derivatives from *Tubocapsicum anomalum* MAKINO,[2)] *Datura metel* L.[3)] and *Datura tatura* L.[4)] As an extended study of searching for withanolides among solanaceous plants, we have now isolated a new withanolide from *Physalis angulata* L.

Physagulin C (**1**), $C_{30}H_{38}O_9$, colorless needles (from 1-BuOH saturated with water), mp 242.5—243.0 °C, exhibited characteristic absorption bands at 1750 cm^{-1} (acetyl), 1714 cm^{-1} (α,β-unsaturated δ-lactone) and 1670 cm^{-1} (α,β-unsaturated ketone) in the infrared (IR) spectrum. The positive fast atom bombardment mass spectrum (FAB-MS) displayed peaks at *m/z* 543 $[M+H]^+$, 525 $[M-H_2O+H]^+$ and 507 $[M-2H_2O+H]^+$. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum (pyridine-d_5) for **1** (Table I) showed signals due to two tertiary methyl groups [δ 1.26 (3H, s, H_3-18) and δ 1.78 (3H, s, H_3-19)], a secondary methyl group [δ 0.99 (3H, d, J=7.0 Hz, H_3-21)] and two vinyl methyl groups [δ 1.68 (3H, s, H_3-27) and 1.87 (3H, s, H_3-28)] besides signals due to two olefinic proton signals [δ 6.40 (1H, d, J=9.8 Hz, H-2) and δ 7.26 (1H, dd, J=9.8 and 6.2 Hz, H-3)] and three oxymethine signals [δ 3.96 (1H, d, J=6.2 Hz, H-4), 3.26 (1H, br s, H-6) and 4.43 (1H, m, H-22)], which suggested the presence of a 1-one-2-ene-4β-hydroxy-5β(6)-epoxy system on rings A and B in the ergostane skeleton and of a normal side chain moiety such as a typical withanolide, physapubenolide (**2**).[5)] Furthermore, additional signals at δ 2.09 (3H, br s), δ 5.21 (1H, s) and δ 3.72 (1H, s) could be assigned to an acetoxymethyl, a proton geminal to the acetoxy group and a proton adjacent to the epoxy group,

TABLE I. ^{1}H-NMR Assignments for Physagulin C (**1**)[a)] and Physapubenolide (**2**)[b)]

	1	2
H-2	6.40 (d, J=9.8 Hz)	6.18 (d, J=10.0 Hz)
H-3	7.26 (dd, J=9.8, 6.2 Hz)	6.95 (dd, J=10.0, 5.5 Hz)
H-4	3.96 (d, J=6.2 Hz)	3.79 (d, J=5.5 Hz)
H-6	3.26 (br s)	3.36 (m, $W_{1/2h}$=4 Hz)
H-15	5.21 (s)	4.97 (d, J=4.4 Hz)
H-16	3.72 (s)	
H_3-18	1.26 (s)	1.11 (s)
H_3-19	1.78 (s)	1.40 (s)
H_3-21	0.99 (d, J=7.0 Hz)	1.02 (d, J=6.8 Hz)
H-22	4.43 (m)	4.32 (dt)
H_3-27	1.68 (s)	1.87 (s)
H_3-28	1.87 (s)	1.94 (s)
H_3-30	2.09 (s)	1.99 (s)

a) In pyridine-d_5. b) In $CDCl_3$.



physapubenolide (**2**)

TABLE II. ^{13}C-NMR Data for Physagulin C (**1**)[a)] and Physapubenolide (**2**)[b)]

	1	2		1	2
C-1	202.5	202.6	C-16	58.7	33.8
C-2	131.1	131.5	C-17	75.9	52.3
C-3	145.6	143.5	C-18	15.0	15.6
C-4	69.6	69.6	C-19	16.4	17.2
C-5	63.1	63.4	C-20	34.0	37.5
C-6	60.4	62.7	C-21	13.8	17.2
C-7	25.7	26.1	C-22	77.9	78.5
C-8	39.4	40.3	C-23	32.7	31.2
C-9	35.1	36.1	C-24	In solvent	149.5
C-10	47.8	47.9	C-25	121.3	121.9
C-11	19.7	21.8	C-26	165.6	166.8
C-12	30.9	41.4	C-27	12.2	12.4
C-13	46.3	46.1	C-28	20.6	20.5
C-14	80.9	84.1	C-29	169.3	169.8
C-15	76.8	80.8	C-30	19.8	21.5

a) In pyridine-d_5. b) In $CDCl_3$.

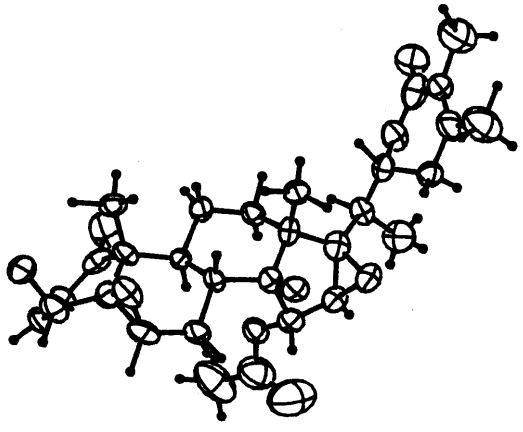
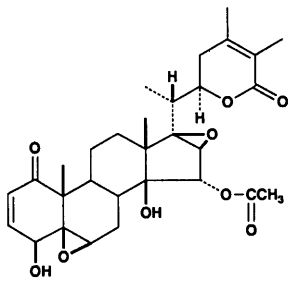


Fig. 1. ORTEP Drawing of Physagulin C (**1**)

respectively. On the other hand, the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum (pyridine-d_5) for **1** (Table II) also indicated an occurrence of the 1-one-2-ene-4β-hydroxy-5β(6)-epoxy moiety at δ 202.5 (s, C-1), 131.1 (d, C-2), 145.6 (d, C-3), 69.6 (d, C-4), 63.1 (s, C-5) and 60.4 (d, C-6) and a side chain residue at C-17 including the α,β-unsaturated δ-lactone function at δ 34.0 (d, C-20), 13.8 (q, C-21), 77.9 (d, C-22), 32.7 (t, C-23), 12.2 (q, C-27) and 20.6 (q, C-28). The remaining signals disclosed the presence of a carbon (δ 80.9, s) bearing a tertiary hydroxy group, a carbon (δ 76.8, d) germinal to an acetoxyl group and two carbons (δ 58.7, d and 75.9, s) bearing an epoxy ring. A signal due to C-24 was hidden by a solvent at around δ 149 ppm.



physagulin C (**1**)

TABLE III. Atomic Positional Parameters ($\times 10^4$) and Equivalent Isotropic Thermal Parameters of Physagulin C (**1**)

Atom	X	Y	Z	B
O1	1838 (8)	2167 (4)	4315 (12)	6.82 (33)
O4	1683 (7)	3067 (3)	9063 (13)	4.89 (26)
O5	3358 (6)	2406 (3)	10198 (12)	4.74 (26)
O14	5586 (6)	1241 (3)	9042 (10)	3.53 (21)
O15	3424 (6)	702 (3)	7034 (11)	3.98 (22)
O16	6133 (6)	522 (3)	6844 (13)	4.50 (24)
O22	7180 (6)	779 (3)	1132 (12)	4.44 (24)
O26	8044 (8)	1024 (3)	−1257 (12)	6.09 (31)
O29	3163 (10)	9 (5)	8258 (21)	11.61 (56)
C1	1821 (11)	2204 (4)	6031 (16)	4.09 (36)
C2	866 (8)	2195 (4)	6911 (17)	3.75 (34)
C3	718 (9)	2370 (5)	8453 (17)	4.23 (38)
C4	1594 (10)	2570 (5)	9469 (16)	4.45 (38)
C5	2535 (10)	2302 (4)	9007 (18)	4.15 (35)
C6	2894 (10)	1947 (5)	10312 (17)	4.66 (40)
C7	3521 (9)	1532 (4)	9766 (15)	3.68 (34)
C8	4132 (8)	1644 (4)	8058 (14)	2.95 (31)
C9	3423 (8)	1802 (4)	6599 (15)	2.79 (30)
C10	2802 (8)	2258 (4)	7003 (14)	3.17 (31)
C11	4040 (9)	1870 (4)	4856 (16)	3.52 (33)
C12	4599 (9)	1416 (4)	4332 (15)	3.36 (33)
C13	5383 (9)	1279 (4)	5818 (18)	3.71 (34)
C14	4842 (8)	1223 (4)	7602 (15)	3.18 (30)
C15	4427 (9)	708 (4)	7682 (16)	3.57 (33)
C16	5069 (9)	437 (4)	6458 (18)	3.58 (34)
C17	5687 (9)	761 (4)	5336 (18)	4.21 (37)
C18	6241 (9)	1630 (4)	5920 (19)	3.85 (35)
C19	3316 (10)	2714 (4)	6481 (16)	4.02 (36)
C20	6052 (9)	616 (4)	3416 (16)	3.55 (33)
C21	6084 (11)	84 (5)	3138 (21)	5.67 (45)
C22	7046 (9)	853 (4)	3001 (16)	3.29 (31)
C23	7954 (9)	655 (4)	3882 (17)	3.80 (34)
C24	8888 (9)	881 (5)	3157 (20)	5.37 (43)
C25	8908 (9)	1058 (4)	1621 (18)	3.88 (35)
C26	8038 (11)	963 (4)	353 (22)	5.74 (45)
C27	9774 (13)	1295 (7)	687 (26)	8.43 (64)
C28	9774 (12)	912 (7)	4396 (26)	8.19 (63)
C29	2815 (11)	358 (6)	7580 (26)	7.75 (60)
C30	1765 (11)	433 (7)	7105 (25)	9.96 (71)

In order to determine the obscure structure of **1**, especially with respect to the location and configurations of the epoxy, acetoxy and hydroxy groups in the molecule, we conducted a single crystal X-ray diffraction study on **1**. The ORTEP drawing and their atomic parameters were shown in Fig. 1 and Table III, respectively.

As a result, the location of all the functional groups of the epoxy, acetoxy and hydroxy groups in **1** were determined as above, and the asymmetric centers at C-14, C-15, C-16, C-17, C-20, and C-22 were also concluded as *S*, *S*, *S*, *R*, *R*, and *R* configurations, respectively, as shown in the formula: (20*R*,22*R*)-15α-acetoxy-5β(6),16β(17)-diepoxy-4β,14β-dihydroxy-1-oxo-witha-2,24-trienolide. The carbon signals were attributable as listed in Table II.

Physagulin C (**1**) has been characterized as a withanolide possessing the highly oxygenated structure on rings A, B and D.

Experimental

Melting point was determined on a Yanagimoto micromelting point apparatus and was uncorrected. Optical rotation was on a JASCO DIP-360 automatic digital polarimeter and circular dichroism (CD) spectrum on a JASCO J-50A spectropolarimeter. The IR spectrum was recorded with a Hitachi IR spectrometer, model 270-30. The ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL JNM-GX 400 NMR spectrometer and chemical shifts are given on a δ (ppm) scale with tetramethylsilane (TMS) as an internal standard. The FAB-MS was measured with JEOL DX-303 HF spectrometer and taken in a glycerol matrix containing NaI. Thin layer chromatography was performed on precoated Kiselgel 60 F_{254} (Merck) and detection was achieved by spraying 10% H_2SO_4 followed by heating. Column chromatography was carried out on Kieselgel (70—230 mesh and 230—400 mesh, Merck) and Sephadex LH-20 (Pharmacia Fine Chem.).

Extraction and Separation The fresh leaves and stems (2.0 kg) of *Physalis angulata* L. (Solanaceae) harvested in the botanical garden at Fukuoka University in September 1988, were extracted with MeOH and the extract was partitioned between 1-BuOH and water. The 1-BuOH layer (38 g) was subjected repeatedly to column chromatography over silica gel using $CHCl_3$–MeOH–H_2O=1:0:1→8:2:0.1→0:1:0 and Sephadex LH-20 using MeOH to give physagulin C (**1**, 270 mg).

Physagulin C (1) Colorless needles, mp 242.5—243.0 °C, from 1-BuOH saturated with water. $[\alpha]_D^{31}$ +105.8° (c=0.52, MeOH). Positive FAB-MS m/z: 543 $[M+H]^+$, 525 $[M-H_2O+H]^+$, 507 $[M-2H_2O+H]^+$. IR ν_{max}^{KBr} cm^{-1}: 3456, 2984, 2928, 1750, 1714, 1670, 1464, 1434, 1320, 1308, 1298, 1288. CD (c=0.052, MeOH) [θ] (nm): +17800 (340) (positive peak), +75400 (247) (positive maximum peak). Crystal Data: $C_{30}H_{38}O_9$, M=542.63, orthorhombic, space group, $P2_12_12_1$, Z=4; cell constants, a=13.372 (2), b=28.089 (4), c=7.500 (1) Å, V=2817.1 (7) Å^3, D_x=1.279 gcm^{-3}, $F(000)$=1160, $\mu(CuK_\alpha)$=0.736 mm^{-1}, R=0.087, for unique 1424 reflections. Intensity data were collected on a Enraf-Nonius CAD4F-11 difractometer with graphite-monochromated CuK_α radiation (λ=1.5418 Å) by the ω–2θ scan technique, and were corrected for background by Lorentz and polarization effects, but absorptions were ignored. The crystal structures were solved by direct method with the MULTAN 11/82 program. All atomic parameters, anisotropic temperature factors for non-hydrogen atoms and isotropic ones for hydrogen atoms were refined by a block-diagonal matrix least-squares method.

Acknowledgement We would like to express our thanks to Prof. H. Okabe, Faculty of Pharmaceutical Sciences, Fukuoka University, for a supply of fresh leaves and stems of *physalis angulata* L.

References and Notes

1) This work is Part XXI in a series of studies on the constituents of the solanaceous plants.
2) *a*) K. Yoshida, K. Shingu, S. Yahara, T. Nohara, N. Marubayashi, I. Ueda, K. Miyahara and T. Kawasaki, *Tetrahedron Lett.*, **29**, 673 (1988); *b*) K. Shingu, N. Marubayashi, I. Ueda, S. Yahara and T. Nohara, *Chem. Pharm. Bull.*, **38**, 1107 (1990).

3) *a*) K. Shingu, T. Kajimoto, Y. Furusawa and T. Nohara, *Chem. Pharm. Bull.*, **35**, 4359 (1987); *b*) K. Shingu, Y. Furusawa and T. Nohara, *ibid.*, **37**, 2132 (1989); *c*) K. Shingu, Y. Furusawa, N. Marubayashi, I. Ueda, S. Yahara and T. Nohara, *ibid.*, **38**, 2866 (1990).
4) K. Shingu, S. Yahara and T. Nohara, *Chem. Pharm. Bull.*, **38**, 3485 (1990).
5) E. Glotter, M. Sahai, I. Kirson and H. E. Gottlieb, *J. Chem. Soc., Perkin Trans. 1*, **1985**, 2241 (1985).

Products from Nileprost, 5-Cyano-16-methylprostacyclin, and Its β-Cyclodextrin Complex under Heating

Michiaki YONEDA* and Yasushi OHKAWA

Tokyo Research Laboratories, Kowa Co., Ltd., 2-17-43 Noguchi-cho, Higashimurayama-shi, Tokyo 189, Japan. *Received October 19, 1990*

Products from nileprost (NP) and its β-cyclodextrin (CD) complex under heating was investigated. Stability of NP under thermal conditions was improved by complexation. The product from the complex is an ester between the carboxyl group of NP and the hydroxyl group of CD, and the NP moiety is fixed at the interior of the cavity of the CD moiety. Main products from NP are also esters between two molecules.

Keywords nileprost; prostacyclin analogue; inclusion complex; β-cyclodextrin; heating

It is well known that prostacyclin (PGI_2) has remarkable biological activity, but its vinyl ether moiety is easily hydrolyzed in aqueous solution.[1] This chemical instability has limited the utilization of PGI_2 for clinical treatment. Nileprost (NP, Fig. 1) with an electron withdrawing cyano group at the 5-position is one of the analogues synthesized in order to improve the stability of PGI_2.[2] In the previous paper, we noted that the introduction of the cyano group is very effective for the stabilization of the vinyl ether moiety and that the degradation mode of NP is different from that of PGI_2.[3] Furthermore, we investigated the stability of NP under thermal conditions and found that NP gradually changes to different compounds, even under normal storage conditions (room temperature). We also investigated the stability of a complex between NP and β-cyclodextrin (CD). Recently, many studies of complexes of various drugs with CDs have been done for improvment of the stability and solubility and for many other aims.[4]

This paper describes the structure determination of products from NP and its inclusion complex with CD under heating.

Experimental

Instruments Nuclear magnetic resonance (NMR) spectra were measured with a JEOL JNM-GSX 270 spectrometer. Tetramethylsilane (TMS) was used as an internal standard. The chemical shifts are given in ppm from TMS. ^1H–^1H correlation spectroscopy (COSY) and ^{13}C–^1H-COSY were used for the assignment. Fast atom bombardment mass spectra (FABMS) were measured with a JEOL JMS-D300 mass spectrometer equipped with a FAB gun (Xe, 6 kV) at an accelerating voltage of 1.5 kV. *m*-Nitrobenzyl alcohol (NBA) and glycerol were used as matrices, and a mixture of NBA, polyethylene glycol (PEG) 1000, PEG 400 and 5% NaCl (16:4:2:1) as both the matrix and the standard for positive high-resolution measurements. Infrared (IR) absorption spectra were measured by the KBr disc method with a JASCO FT/IR-3 spectrophotometer. High performance liquid chromatography (HPLC) was performed with a Hitachi 655A-12 equipped with a Hitachi 655A-21 ultraviolet (UV) absorption detector.

Materials and Reagents NP and its CD complex[5] were supplied by Schering AG (Berlin, Germany). A 0.02 M phosphate buffer solution (pH 3.5) was prepared by mixing 0.02 M KH_2PO_4 and 0.02 M H_3PO_4. Deionized water was used in all experiments. Other reagents used were of an analytical reagent grade.

Isolation of Products Product **1** was isolated by preparative HPLC. The conditions are as follows: column, μBondapak-C_{18} (8 i.d. × 300 mm, Waters Assoc.); oven, 30 °C; mobile phase, water–MeCN (3:1); flow rate, 3.0 ml/min; and detector, UV 240 nm. Products **2**—**4** were separated by preparative thin-layer chromatography (TLC) with $CHCl_3$–MeOH–conc.-NH_4OH (12:6:1) as the developing solvent. *Rf*-Values of NP, **2** and a mixture of **3** and **4** are about 0.2, 0.3 and 0.5, respectively. The mixture of **3** and **4** was separated to **3** (upper band) and **4** (lower band) by preparative silica gel TLC with AcOEt–AcOH (20:1) as the developing solvent.

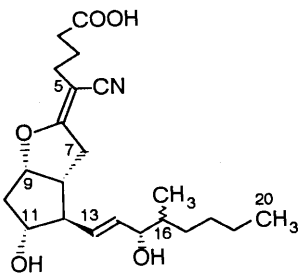


Fig. 1

Results and Discussion

HPLC of Products Figure 2 shows liquid chromatograms of NP and its CD complex before and after heating. Three peaks from NP and one peak from the complex appeared after heating. The stability of NP under thermal conditions was fairly improved by the complexation, although NP gradually changed to different compounds even at room temperature. The structures of the numbered peaks in Fig. 2 were determined.

Structure Determination of the Products Negative FAB-MS of product **1** gave the $[M-H]^-$ ion at *m/z* 1506 corresponding to $[NP+CD-H_2O-H]^-$. The integral value of the ^1H-nuclear magnetic resonance (^1H-NMR) spectrum of **1** also indicated that **1** consists of NP moiety and CD moiety in 1:1 ratio. IR spectra of **1** gave absorption

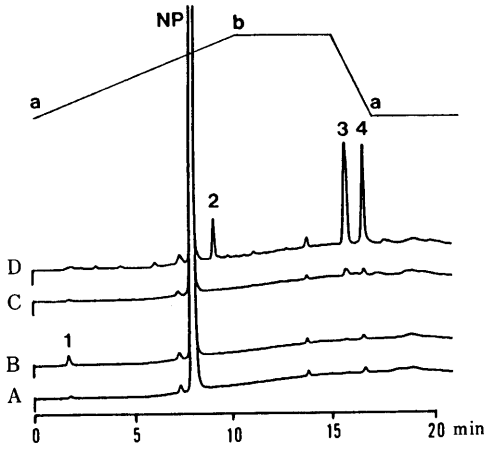


Fig. 2. Liquid Chromatograms of NP and Its CD Complex

A, the complex in a refrigerator; B, the complex at 60 °C for a week; C, NP in a refrigerator; D, NP at 60 °C for a week. HPLC conditions: column, TSK gel ODS-80TM 4.6 mm × 150 mm (TOSOH); detector, UV 240 nm; oven, 40 °C; mobile phase, (a) 0.02 M phosphate buffer (pH 3.5)–MeCN (3:2), (b) 0.02 M phosphate buffer (pH 3.5)–MeCN (3:7); flow rate, 1 ml/min.

bands at 2203 and 1732 cm^{-1}, indicating the existence of a cyano group and an ester carbonyl group, respectively, whereas the carboxyl band of NP is at 1711 cm^{-1}. Analytical data of **1** was as follows: C, 49.84; H, 6.86; N, 0.94 (Calcd for $C_{64}H_{101}NO_{39}\cdot 2H_2O$: C, 49.77; H, 6.85; N, 0.91). These results indicate the formation of ester-linking between NP and CD. For the structure of the ester, there are two possibilities: (a) NP moiety is at the interior of the cavity of CD or (b) at outside of the cavity. If the structure is the former, NMR signals of protons at the interior of the cavity should shift from the chemical shifts of corresponding protons of NP alone and CD alone and exhibit no concentration-dependent change. By contrast, if the latter is true, the concentration-dependent change is expected.[4a] Figure 3 shows the chemical shifts of ^{1}H-NMR spectra of product **1** measured in the range of 1.4×10^{-2}—3.6×10^{-4} mol, and those of a 1 : 1 mixture of NP and CD measured in a similar concentration range.

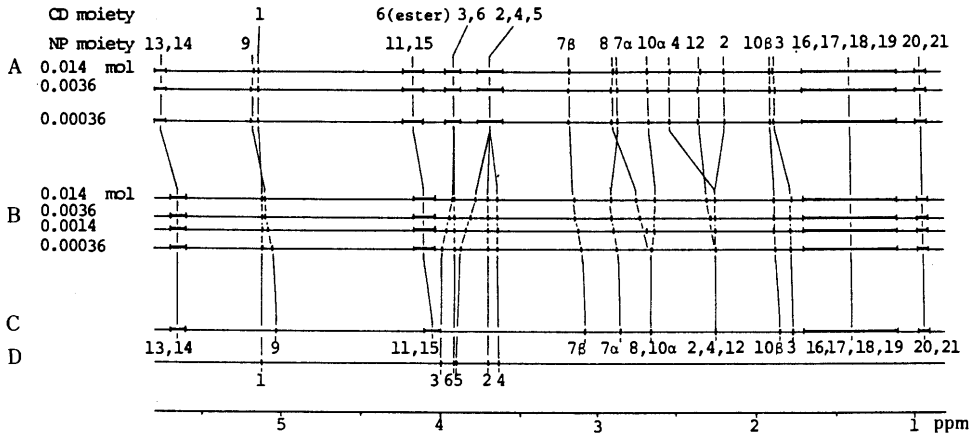


Fig. 3. Proton Chemical Shifts of (A) Product **1**, (B) a 1 : 1 Mixture of NP and CD, (C) NP and (D) CD
▬, broad overlapping peak. ^{1}H-NMR spectra were measured with D_2O using tetramethylsilane as an external standard.

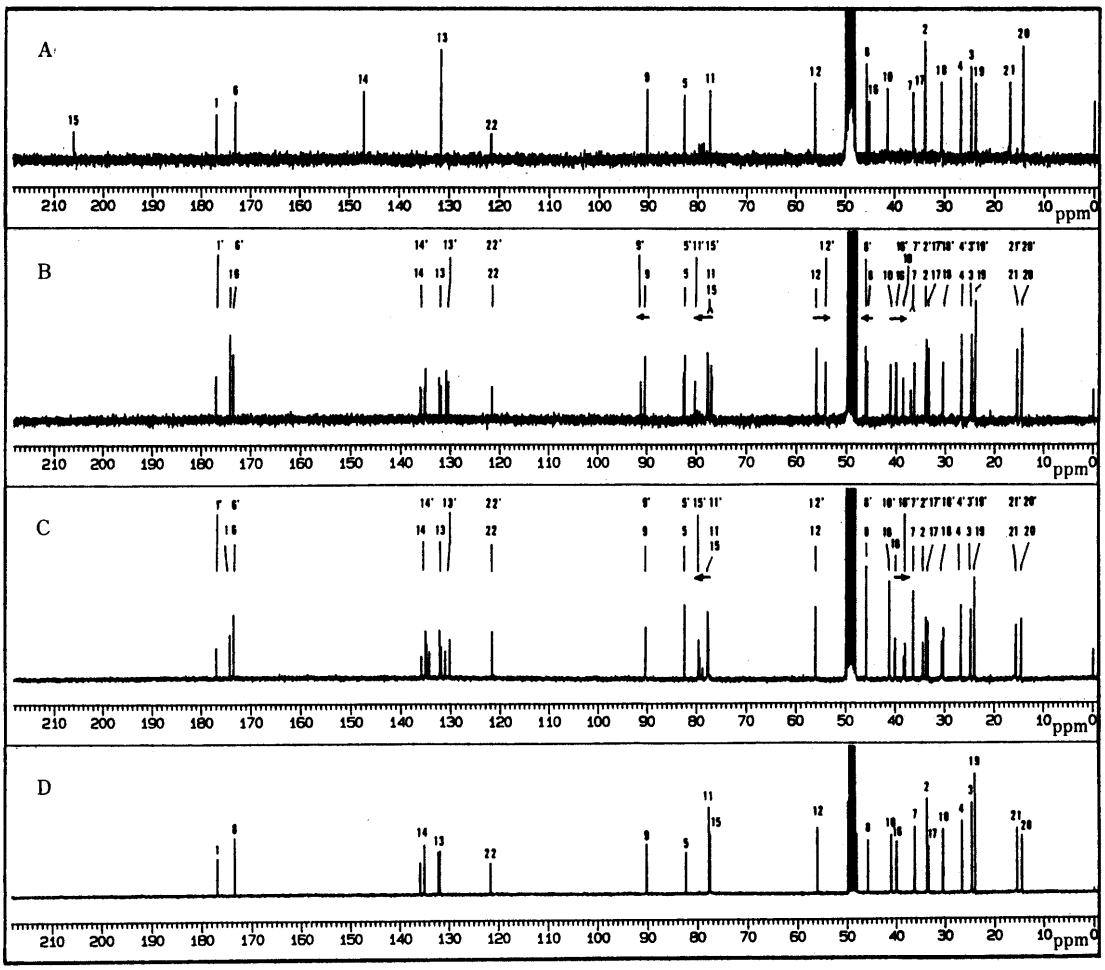


Fig. 4. ^{13}C-NMR Spectra of Products **2**—**4** and NP in CD_3OD
A, product **2**; B, product **3**; C, product **4**; D, NP.

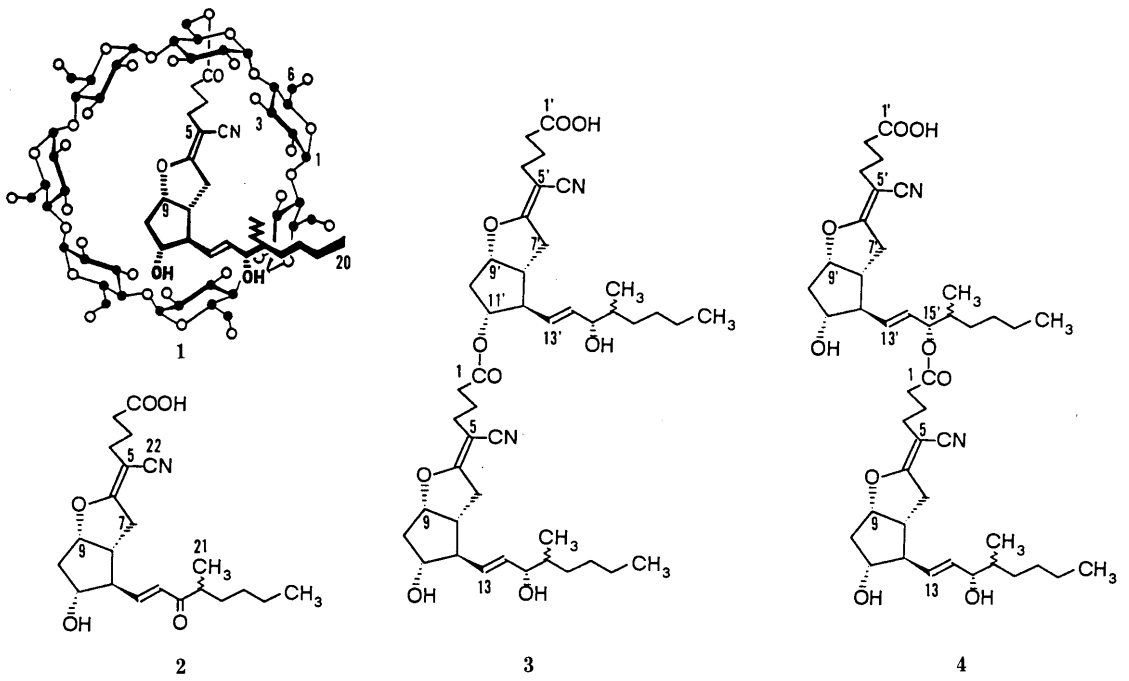


Fig. 5. Structure of the Products

In the spectra of the mixture of NP and CD, while the concentration increased, protons of NP at 7α-, 7β-, 8-, 9- and 12-positions shifted to a lower field, and those of the glucose units of CD at 3- and 5-positions shifted to a higher field (Fig. 3B), indicating a concentration-dependent interaction between NP and the cavity of CD. On the other hand, the spectra of **1** showed no concentration-dependence and, even under the lowest concentration of **1**, proton chemical shifts of the NP moiety of **1** at 4-, 8-, 9-, 13- and 14-positions were lower field and that of the CD moiety at 5-position higher field than those of corresponding protons in the highest concentration of the mixture of NP and CD (Fig. 3A), indicating that the interaction between NP and CD moieties of **1** is large and constant. To explain the above results, the NP moiety of **1** must be fixed at the interior of the cavity of CD moiety. The integral value of the signal of **1** at *ca.* 4.2 ppm corresponds to four protons. Two of those were considered to be assigned to the methylene of a glucose unit ester-linked to the NP moiety. Hydrolysis of **1** gave NP. From these results, product **1** was determined to be the structure shown in Fig. 5.

In FABMS of product **2**, the molecular ion region in the negative mode exhibited an $[M-H]^-$ ion at *m/z* 388 and that in the positive mode an $[M+Na]^+$ ion at *m/z* 412, which was confirmed as $[C_{22}H_{31}NO_5Na]^+$ ($[NP-H_2+Na]^+$) by *m/z* 412.2099. IR spectrum of **2** gave absorption bands at 2203, 1711 and *ca.* 1680 cm^{-1},[6] indicating the existence of a keto-carbonyl group. The ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectrum is shown in Fig. 4A. The signal of C-15 shifted from 77.8 ppm in NP to 206.1 ppm, indicating a conversion from the hydroxyl group of NP at C-15 to a carbonyl group. Thus, product **2** was determined to be a 15-keto compound.

In FABMS studies of product **3**, the molecular ion region in the negative mode exhibited an $[M-H]^-$ ion at *m/z* 763 and that in the positive mode an $[M+Na]^+$ ion at *m/z* 787, which was confirmed as $[C_{44}H_{64}N_2O_9Na]^+$ ($[2NP-H_2O+Na]^+$) by *m/z* 787.4523. Similarly, product **4** gave an $[M-H]^-$ ion and an $[M+Na]^+$ ion at the same mass numbers to those of **3**, respectively, and the formula of the $[M+Na]^+$ ion was also confirmed to be the same as that of **3** by *m/z* 787.4535. IR spectrum of **3** gave absorption bands at 2203, 1732 and 1711 cm^{-1}, indicating the existance of an ester carbonyl group. Product **4** also gave bands similar to those of **3**. These results indicate the ester formation between the carboxyl group and hydroxyl group (11- or 15-positions) of another molecule. ^{13}C-NMR spectra of **3** and **4** are shown in Figs. 4B and 4C, respectively. The chemical shifts of signals of carbons numbered without a prime (Fig. 5) correspond closely to those of NP excepting a carbonyl group. It is known that esterification of secondary aliphatic alcohol causes a 4 ppm downfield shift for α-carbon, a 3 ppm upfield shift for β-carbon and a 2 ppm downfield shift for γ-carbon.[7] Similar shifts, which are noted by the arrows in the figures, were observed for **3** and **4**. The spectrum of **3** shows a change around the 11-position and of **4** a change around the 15-position. Thus, **3** and **4** were determined to be dimers esterified at the 11- and 15-positions, respectively.

Determined structures of the products are shown in Fig. 5. The product from the complex is an ester between NP and CD and has a structure where the NP moiety is fixed at the interior of the cavity of CD. This result is the first to show that such a compound is produced from the CD complex. The main products from NP are esters between the carboxyl group of NP and the hydroxyl group of another molecule.

Acknowledgement We wish to thank Schering AG for supplying NP and its CD complex.

References and Notes

1) Y. Chiang, M. J. Cho, B. A. Euser and A. J. Kresge, *J. Am. Chem. Soc.*, **108**, 4192 (1986).

2) W. Skuballa, B. Radüchel and H. Vorbrüggen, *Tetrahedron Lett.*, **29**, 4285 (1988).
3) M. Yoneda and Y. Ohkawa, *Chem. Pharm. Bull.*, **38**, 2507 (1990).
4) For example: *a*) K. Uekama, *Yakugaku Zasshi*, **101**, 857 (1981); *b*) A. Yoshida, M. Yamamoto, T. Irie, F. Hirayama and K. Uekama, *Chem. Pharm. Bull.*, **37**, 1059 (1989); *c*) K. Nakanishi, M. Masada, T. Nadai and K. Miyajima, *ibid.*, **37**, 211 (1989); *d*) R. D. Voyksner, F. P. Williams, C. S. Smith, D. L. Koble and H. H. Seltzman, *Biomed. Environ. Mass Spectrom.*, **18**, 1071 (1989).
5) The ratio of NP and CD is 2:5 and the ratio was confirmed by titration with 0.1 N NaOH.
6) The wave numbers of carbonyl groups of products **2**—**4** are slightly inaccurate because of broad overlapping bands.
7) F. W. Wehrli and T. Wirthlin, "Interpretation of Carbon-13 NMR Spectra," Heydon and Son Ltd., London, 1978.

Hydroxylation of Phenylalanine by Myeloperoxidase–Hydrogen Peroxide System

Sadaki FUJIMOTO,* Susumu ISHIMITSU, Satoru HIRAYAMA, Naoko KAWAKAMI, and Akira OHARA

Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607, Japan. *Received October 19, 1990*

When phenylalanine was incubated with myeloperoxidase (MPO) and hydrogen peroxide (H_2O_2), *p*-tyrosine, *m*-tyrosine and *o*-tyrosine were identified as hydroxylated products. With inactivated MPO, no significant amount of tyrosines was formed. Tyrosine formation was dependent on reaction time and the MPO concentration. The addition of Cl^- at lower concentration to the MPO–H_2O_2 system resulted in increase of tyrosine formation, though Cl^- at higher concentration reduced the formation. The tyrosine formation by both MPO–H_2O_2 and MPO–H_2O_2–Cl^- systems was significantly prevented by hydroxyl radical scavengers such as mannitol, benzoate and formate. Superoxide dismutase was also an effective inhibitor of tyrosine formation from phenylalanine by MPO–H_2O_2 system.

Keywords myeloperoxidase; oxygen radical; phenylalanine; hydroxylation; *o*-tyrosine; *m*-tyrosine

Both superoxide radical (O_2^-)[1] and hydrogen peroxide (H_2O_2)[2] are generated by neutrophils upon exposure to appropriate stimuli, and the formation of hydroxyl radical (OH·) in the medium containing stimulated neutrophils might therefore be anticipated. There are reports dealing with detection of OH· in the medium containing stimulated neutrophils, utilizing a spin-trapping technique of OH·,[3-5] oxidation of methional to ethene by OH·,[6] and hydroxylation of aromatic compounds by OH·.[7,8] We have recently demonstrated that the hydroxylation of phenylalanine occurs in the medium containing stimulated neutrophils and the hydroxylation might be caused by OH· generated in the medium.[9] The peroxidase of neutrophil, myeloperoxidase (MPO) (EC 1.11.1.7), is capable of catalyzing the oxidation of chloride ion to yield HOCl,[10] which is a powerful oxidant and thought to be an important species for the microbicidal function of neutrophils.[11,12] It is interesting in connection with the hydroxylation of phenylalanine by stimulated neutrophils observed in our previous paper[9] to know if the hydroxylation of phenylalanine is catalyzed by MPO in the presence of H_2O_2. The hydroxylation of aromatic compound by MPO in the presence of H_2O_2 has not yet been observed. In this paper, we examined the action of MPO to phenylalanine in the presence of H_2O_2 and observed that the MPO–H_2O_2 system caused the hydroxylation of phenylalanine.

Materials and Methods

Phenylalanine, catalase (from bovine liver, 44000 units/mg) and superoxide dismutase (SOD) (from bovine erythrocytes, 3570 units/mg) were obtained from Sigma Chemical Co., sodium benzoate, mannitol and sodium formate were from Wako Pure Chemicals, and MPO (from human leukocytes) was from Green Cross Co. The MPO preparation was highly purified by chromatographic technique and electrophoretically homogeneous. The enzyme unit was determined by the guaiacol test.[13] Reaction mixtures containing phenylalanine (1 μmol), H_2O_2 (0.5 μmol) and MPO in the presence and absence of NaCl and radical scavengers, in 0.5 ml of 50 mM phosphate buffer (pH 6.0), were incubated at 35 °C. After incubation for indicated times, 50 μl of catalase (100 μg/ml) was added to stop the reaction. One hundred μl of the mixture was directly injected into high-performance liquid chromatography (HPLC). The *o*-, *m*- and *p*-tyrosines derived from phenylalanine were measured by HPLC under the conditions described previously.[14]

Results and Discussion

Formation of Tyrosine Isomers from Phenylalanine by MPO–H_2O_2 System Figure 1 shows the chromatograms of reaction mixtures obtained by reaction of phenylalanine with H_2O_2 in the presence (B) and absence (A) of MPO. A significant amount of tyrosine isomers was detected in the reaction mixture containing both MPO and H_2O_2 (Fig. 1B). The tyrosine-forming activity of the MPO–H_2O_2

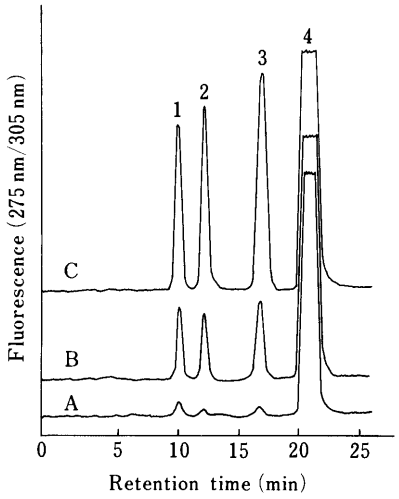


Fig. 1. Chromatogram of the Reaction Mixture

A, The reaction mixture contained phenylalanine and H_2O_2 in 0.5 ml of 50 mM phosphate buffer (pH 6.0). After 10 min of incubation, the reaction mixture was applied to HPLC as described in Materials and Methods. B, Same as A, except for addition of MPO (24 units). C, Same as B, except for addition of NaCl (5 mM). Peaks 1, 2, 3, and 4 correspond to *p*-tyrosine, *m*-tyrosine, *o*-tyrosine, and phenylalanine, respectively.

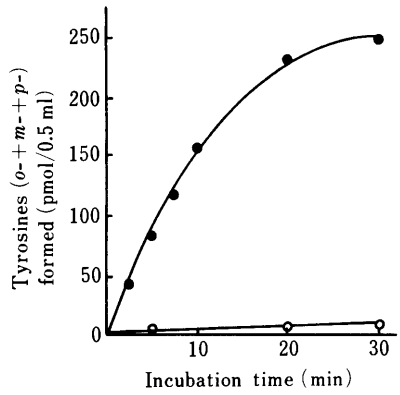


Fig. 2. Time Courses of Tyrosine Formation from Phenylalanine by the MPO–H_2O_2 System

Phenylalanine was incubated with H_2O_2 and MPO (24 units) (●) or heat treated MPO (○) under the conditions described in Materials and Methods. Samples were incubated for the indicated times, and then assayed as described. Heat treated MPO was prepared by incubation of MPO (24 units) in boiling water bath for 5 min.

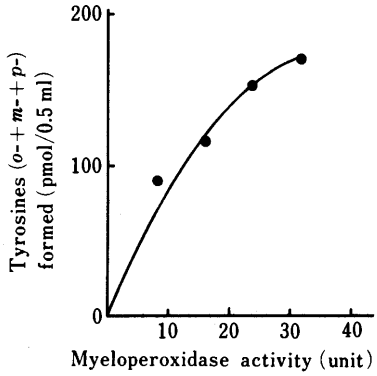


Fig. 3. Effect of MPO on Tyrosine Formation from Phenylalanine in the Presence of H_2O_2

Phenylalanine was incubated with H_2O_2 and the indicated amount of MPO under the conditions described in Materials and Methods. Samples were incubated for 10 min, and then assayed as described.

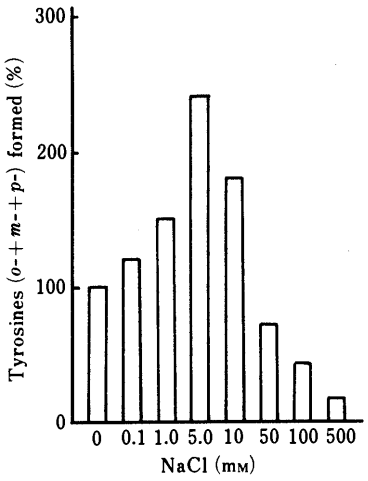


Fig. 4. Effect of Chloride Ions on Tyrosine Formation from Phenylalanine by the MPO–H_2O_2 System

Phenylalanine was incubated with MPO (24 units) and H_2O_2 in the presence of indicated amount of NaCl under the conditions described in Materials and Methods. Samples were incubated for 10 min, and then assayed as described.

system was lost with the deletion of H_2O_2 (data not shown) and greatly reduced by the omission of MPO (Fig. 1A). Although a trace amount of tyrosines was detected in the reaction with H_2O_2 alone (Fig. 1A), a part of the tyrosines detected was due to the contaminant of *p*-, *m*- and *o*-tyrosines in the phenylalanine preparation used in this experiment. The incubation of phenylalanine (1 μmol) with MPO (24 units) and H_2O_2 (0.5 μmol) in 0.5 ml of 50 mM phosphate buffer (pH 6.0) for 10 min caused the formation of 51.77 ± 5.06 pmol of *p*-tyrosine, 53.39 ± 7.52 pmol of *m*-tyrosine and 60.89 ± 0.39 pmol of *o*-tyrosine. In the following experiments, amounts of hydroxylated phenylalanine were expressed as a sum of *o*-, *m*- and *p*-tyrosines. Figure 2 shows the time courses of tyrosine formation by the reaction of phenylalanine with MPO or with heat-treated MPO in the presence of H_2O_2. With active MPO, significant amounts of tyrosines were formed and the formation was dependent on reaction time. The tyrosine-forming activity of the MPO–H_2O_2 system was greatly reduced on addition of heat inactivated MPO instead of active MPO. In addition, tyrosine formation by the MPO–H_2O_2 system increased with increasing MPO at the concentration employed (Fig. 3).

Effectors Modifying Tyrosine Formation from Phenylalanine by MPO–H_2O_2 System MPO is able to oxidize Cl^- to form HOCl, a powerful oxidant. We examined the effect of Cl^- on the formation of tyrosines from phenylalanine by the MPO–H_2O_2 system. The addition of NaCl to the reaction system containing H_2O_2 and MPO as described in Fig. 1B resulted in significant increase of tyrosine formation (Fig. 1C): Five mM of Cl^- increased tyrosine formation by the MPO–H_2O_2 system about 2.4 fold. Incubation of phenylalanine (1 μmol) with H_2O_2 (0.5 μmol), MPO (24 units) and NaCl (2.5 μmol) in 0.5 ml of 50 mM phosphate buffer (pH 6.0) at 35 °C for 10 min caused the formation of 114.2 ± 19.5 pmol of *p*-tyrosine, 132.6 ± 25.4 pmol of *m*-tyrosine, and 150.0 ± 10.8 pmol of *o*-tyrosine. The acceleration depended on NaCl added at lower concentration, below 5 mM, but at higher concentration, above 50 mM, significant inhibition was observed (Fig. 4). It has been noted that several α-amino acids including phenylalanine and tyrosine are oxidized by HOCl to the corresponding nitriles and aldehydes.[15] HOCl formation should be increased with increasing NaCl added to the MPO–H_2O_2 system. Therefore, the above inhibitory effect of NaCl at higher concentration may be due to further oxidation of tyrosines formed and/or to oxidation of phenylalanine as a substrate by HOCl produced largely in the reaction mixture. In fact, at higher concentration of NaCl, there was greater disappearance of phenylalanine from the reaction mixture than with the addition of 5 mM NaCl (data not shown). When taurine (10 mM), a scavenger of HOCl, was added to the MPO–H_2O_2–Cl^- system, the accelerating effect of Cl^- disappeared: Tyrosine formation by the MPO–H_2O_2–Cl^- system was 243% that by the MPO–H_2O_2 system. The addition of 10 mM taurine to the MPO–H_2O_2–Cl^- system

TABLE I. Effect of Radical Scavengers on Tyrosine Formation from Phenylalanine by the MPO–H_2O_2 System in the Presence and Absence of Chloride Ions

Scavengers added	Concentration	Tyrosines (*o*-+*m*-+*p*-) formed
A) MPO–H_2O_2 system		% of control
None (control)		100
Mannitol	1.0 mM	95
	10 mM	34
Sodium formate	1.0 mM	67
	10 mM	10
Sodium benzoate	1.0 mM	94
	10 mM	19
SOD	0.02 μg/0.5 ml	73
	0.2 μg/0.5 ml	24
	1.0 μg/0.5 ml	15
Heat treated SOD	1.0 μg/0.5 ml	105
B) MPO–H_2O_2–Cl^- system		
None (control)		100
Mannitol	10 mM	40
Sodium formate	10 mM	11
Sodium benzoate	10 mM	15
SOD	40 μg/0.5 ml	89

A) Phenylalanine was incubated with MPO (24 units), H_2O_2 and the specified amount of various substances under the conditions described in Materials and Methods. Samples were incubated for 10 min, and then assayed as described. Heat treated SOD, prepared by incubation in boiling water bath for 5 min. B) Same as A), except for addition of NaCl (5 mM).

resulted in reduction to 101%, though 10 mM taurine did not significantly affect tyrosine formation by the MPO–H_2O_2 system. These findings suggest that HOCl produced in the reaction mixture containing H_2O_2, MPO and Cl^- may participate in the hydroxylation of phenylalanine.

To obtain information on the hydroxylation mechanism of phenylalanine by the MPO–H_2O_2(–Cl^-) system, the effect of some radical scavengers on the hydroxylation was examined (Table I). Hydroxyl radical scavenger such as mannitol, sodium formate and sodium benzoate, at 10 mM, effectively prevented tyrosine formation by the MPO–H_2O_2 system. Inhibitory effect of the above hydroxyl radical scavengers was also observed in the MPO–H_2O_2–Cl^- system. MPO activity was not affected by 10 mM of the above radical scavengers (data not shown). Interestingly, tyrosine formation by the MPO–H_2O_2 system was strongly inhibited by SOD at low concentration, and this inhibition was abolished by heat treatment (Table I). In the MPO–H_2O_2–Cl^- system, SOD was found to be a much less effective inhibitor than in the MPO–H_2O_2 system. This may be due to the inactivation of SOD by HOCl produced in the reaction medium containing H_2O_2, MPO and Cl^-.

We have demonstrated with chemical systems and HPLC that OH· can mediate the formation of *o*-, *m*-, and *p*-tyrosines from phenylalanine, but that H_2O_2, O_2^-, and singlet oxygen cannot.[16] As shown in the present experiments, the incubation of phenylalanine in the medium containing H_2O_2 and MPO resulted in formation of hydroxylated phenylalanine, including *o*-, *m*- and *p*-tyrosines. The inhibitory effect of hydroxyl radical scavengers, such as mannitol, benzoate, and formate, on hydroxylation of phenylalanine by the MPO–H_2O_2 system suggest that OH· production in the medium containing MPO and H_2O_2 may occur. In addition, the strong inhibitory effect of SOD on the hydroxylation may suggest the implication of O_2^- in OH· generation in the medium containing H_2O_2 and MPO. The accelerating effect of NaCl at lower concentration (Fig. 4) may suggest that a larger OH· generation occurs in the MPO–H_2O_2–Cl^- system than in the MPO–H_2O_2 system. However, a possibility that HOCl produced in the reaction system directly causes the hydroxylation of phenylalanine to form tyrosines cannot be excluded, though hydroxylation of an aromatic compound by HOCl has not been observed to our knowledge.

The mechanism of O_2^- and OH· production by the MPO–H_2O_2(–Cl^-) system is obscure at the present time. Mason presented the relationships between oxygen, H_2O_2, electron donors, and iron protoporphyrin enzymes such as various peroxidases as follows[17]:

$$Fe_p^{3+} + H_2O_2 \longrightarrow Fe_p^{3+}H_2O_2 \text{ (complex I)}$$

$$\text{complex I} + e^- \longrightarrow Fe_p^{2+}O \text{ (complex II)}$$

in the presence of an excess of H_2O_2

$$\text{complex II} + H_2O_2 \longrightarrow Fe_p^{2+}O_2 \text{ (complex III)}$$

$$\text{complex III} - O_2 \longrightarrow Fe_p^{2+}$$

$$Fe_p^{2+} - e^- \longrightarrow Fe_p^{3+}$$

where Fe_p and e^- refer to iron protoporphyrin and electron, respectively. On the basis of the above relationships, we presumed the mechanism of O_2^- and OH· production by the MPO–H_2O_2(–Cl^-) system to be as follows: MPO is also an iron protoporphyrin enzyme. In the present experiments with MPO, phenylalanine or Cl^- may act as an electron donor to the complex I as presented above. If so, there may be a possibility that O_2 is reduced by e^- from Fe_p^{2+} to form O_2^- in the MPO–H_2O_2(–Cl^-) system. Then, O_2^- may interact with H_2O_2 to form OH· (Harber–Weiss reaction).[18]

$$O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + OH\cdot$$

In addition, OH· may also be formed by the reaction of O_2^- with HOCl produced in the MPO–H_2O_2–Cl^- system.

$$HOCl + O_2^- \longrightarrow OH\cdot + O_2 + Cl^-$$ [19]

Although further experiments are necessary to clarify the mechanism of O_2^- formation, the findings obtained in the present experiments may suggest the existence of an OH·-generating system by which MPO participates in the medium containing stimulated neutrophils.

References

1) B. M. Babior, R. S. Kipnes, and J. T. Curnutte, *J. Clin. Invest.*, **52**, 741 (1973).
2) G. Y. Lyer, D. M. F. Islam, and J. H. Quanstel, *Nature* (London), **192**, 535 (1961).
3) M. R. Green, H. A. O. Hill, M. J. Okolow-Zubkowska, and A. W. Segal, *FEBS Lett.*, **100**, 23 (1979).
4) B. E. Britigan, G. M. Rosen, Y. Chai, and M. S. Cohen, *J. Biol. Chem.*, **261**, 4426 (1986).
5) B. E. Britigan, G. M. Rosen, B. Y. Thompson, Y. Chai, and M. S. Cohen, *J. Biol. Chem.*, **261**, 17026 (1986).
6) A. I. Tauber and B. M. Babior, *J. Clin. Invest.*, **60**, 374 (1977).
7) M. S. Alexander, R. M. Husney, and A. L. Sagone, *Biochem. Pharmacol.*, **35**, 3649 (1986).
8) H. Kaur, I. Fagerheim, M. Grootreld, A. Puppo, and B. Halliwell, *Anal. Biochem.*, **172**, 369 (1988).
9) S. Fujimoto, S. Ishimitsu, H. Kanazawa, T. Mizutani, A. Ohara, and T. Hayakawa, *Agric. Biol. Chem.*, **51**, 2851 (1987).
10) J. E. Harrison and J. Schults, *J. Biol. Chem.*, **251**, 1371 (1976).
11) J. M. Albrich, C. A. McCarthy, and J. K. Hurst, *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 210 (1981).
12) A. R. J. Bakkenist, J. E. G. DeBoer, H. Plat, and R. Wever, *Biochim. Biophys. Acta*, **613**, 337 (1980).
13) B. Chance and A. C. Maehly, "Methods in Enzymology," Vol. 2, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York, 1955, p. 770.
14) S. Ishimitsu, S. Fujimoto, and A. Ohara, *J. Chromatogr.*, **378**, 222 (1986).
15) W. E. Pereira, Y. Hoyana, R. E. Summons, V. A. Bacon, and A. M. Duffield, *Biochim. Biophys. Acta*, **313**, 170 (1973).
16) S. Ishimitsu, S. Fujimoto, and A. Ohara, *Chem. Pharm. Bull.*, **32**, 752 (1984); *idem, ibid.*, **32**, 3645 (1984); *idem, ibid.*, **33**, 1552 (1985).
17) H. S. Mason, *Adv. Enzymol.*, **19**, 79 (1957).
18) F. Haber and J. Weiss, *Proc. R. Soc. Edin.*, **147**, 332 (1934).
19) C. A. Long and B. H. Bielski, *J. Phys. Chem.*, **84**, 555 (1980).

Immunohistochemical Demonstration of Calcium-Binding Protein Regucalcin in the Tissues of Rats: The Protein Localizes in Liver and Brain

Masayoshi YAMAGUCHI,*,[a] Mitsutaka ISOGAI,[b] Shinji KATO,[b] and Seiichi MORI[a]

Department of Environmental Biochemistry, School of Pharmaceutical Sciences, University of Shizuoka,[a] 395 Yada, Shizuoka 422, Japan and Tokai Research Laboratories, Daiichi Pure Chemicals Co., Ltd.,[b] 2117 Muramatsu, Tokai-mura, Ibaraki 319-11, Japan. *Received December 3, 1990.*

A calcium-binding protein, regucalcin, was isolated from rat liver cytosol. Rabbit-anti-regucalcin antiserum, which was raised against regucalcin conjugated by glutaraldehyde to bovine serum albumin, was applied to glutaraldehyde-fixed whole mounts and subsequently visualized using the peroxidase–antiperoxidase methods. Rat hepatic regucalcin immunoreactivity was most pronounced in the liver and brain of rats, while it was not seen in the duodenum, testicle, spleen, lung and smooth muscle (bladder), and appeared only slightly in the kidney and heart. Control experiments using non-immune sera or adsorbed anti-rat liver regucalcin antiserum showed no staining. The present finding suggests that regucalcin localizes in the liver and brain of rats.

Keywords regucalcin; calcium-binding protein; immunoreactivity; liver; brain; rat

It is well known that Ca^{2+} plays an important role in the regulation of many cell functions.[1] The role of Ca^{2+} in liver metabolism has been demonstrated in recent investigations.[2,3] Recently, it has been found that a novel calcium-binding protein (regucalcin) is distributed in the hepatic cytosol of rats.[4] The molecular weight of regucalcin was estimated to be 28800, and the Ca^{2+} binding constant was found to be 4.19×10^5 M^{-1} by equilibrium dialysis.[5] This protein has a reversible effect on the activation and inhibition of various enzymes by Ca^{2+} in liver cells.[6-9] Regucalcin may play a cell physiological role in the regulation of liver cell function related to Ca^{2+}.

On the other hand, it has not been reported whether regucalcin is distributed in other tissues besides the liver. The present investigation, therefore, was undertaken to clarify the immunohistochemical distribution of regucalcin in the tissues of rats. It was found that the immunoreactivity of regucalcin is most pronounced in the liver and brain.

Materials and Methods

Animals Male Wistar rats weighing 100—120 g were used. They were purchased from Japan SLC Inc. (Hamamatsu, Japan) and fed commercial laboratory chow (solid) containing 57.5% carbohydrate, 1.1% Ca, and 1.1% P, and distilled water, freely.

Isolation of Regucalcin Rats were killed by bleeding. The livers were perfused with a Tris–HCl buffer (pH 7.4, containing 100 mM Tris, 120 mM NaCl, 4 mM KCl, cooled to 4 °C). The livers were removed, cut into small pieces, suspended 1 : 4 in Tris–HCl buffer (pH 7.4) and homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at 5500*g* in a refrigerated centrifuge for 10 min and the supernatant was spun at 105000*g* for 60 min. Regucalcin in the 105000*g* supernatant (cytosol fraction) was purified to electrophoretic homogeneity by gel filtration on Sephadex G-75 and G-50 followed by ion exchange chromatography on diethylaminoethyl (DEAE)-cellulose, including assays for $^{45}Ca^{2+}$ binding activity, as reported previously.[4]

Antiserum Rabbits (from Japan SLC Inc., 2.5—3.0 kg), used to raise anti-(rat liver regucalcin) antibodies, were subcutaneously injected with 1.0 mg per animal of antigen emulsified with Freund's complete adjuvant, and then injected four more times at 2-week intervals with Freund's incomplete adjuvant. Animals were killed by bleeding 10 d after the last injection to obtain antiserum.

Immunohistochemical Procedure Rats were killed by bleeding, and the liver, kidney, heart, brain, duodenum, testicle, spleen, lung, and smooth muscle (bladder) were immediately obtained. The entire immunohistochemical procedure was carried out at room temperature.[10] All tissues were directly immersed in a fixation solution containing 4% formaldehyde and 0.25% reagent-grade glutaraldehyde in 125 mM of a phosphate buffer, pH 7.4, and then frozen in Freon 12 cooled with liquid N_2. Ultrathin (5 μm thick) cryosections, cut in an Ultracut microtome (Bright), were dried by air cooling. The whole mounts were dehydrated in 100% acetone for 10 min each. After extensive washing with 10 mM phosphate buffered saline (PBS; pH 7.4), endogenous peroxidase was inactivated with 5 mM periodic acid for 10 min. The preparations were then washed in PBS. The tissues were preincubated in an antibody diluting solution (normal rabbit immunoglobulin (IgG) from Cappel Co., Ltd.; 19.4 mg protein/ml) for 20 min. After a 5 min wash in ice-cold PBS, rabbit-anti-rat regucalcin antiserum diluted 1 : 200 was applied for 20 min. Following a further wash with ice-cold PBS, the preparations were incubated in goat-anti-rabbit IgG (from Cappel Co., Ltd.; 18.6 mg protein/ml) labeled with horseradish peroxidase for 20 min. After washes in ice-cold PBS, the tissues were preincubated for 10 min in 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.), 20 mg/100 ml, made up in 50 mM Tris, pH 7.6, followed by reaction for 10 min in a similar solution now containing 0.01% hydrogen peroxide. The tissues were then washed in ice-cold PBS for 5 min (× 3) and incubated in veronal-acetate buffered 1% methyl green solution, pH 4.0 for 10 min. After washing with water, the preparations were dehydrated, cleared, and mounted in coverglass. Specificity was tested by replacing the primary antiserum with either nonimmune rabbit serum at the same dilution as the anti-regucalcin antiserum or adsorbed anti-regucalcin antiserum.

Results

Immunostaining with anti-regucalcin antibodies was studied in whole mounts of the liver, brain, kidney, heart, duodenum, testicle, spleen, lung, and smooth muscle (bladder) from rats. In all four types of preparations studied, the application of antiregucalcin antibodies resulted in strong immunostaining of the liver and brain (Figs. 1 and 2). No immunostaining was seen in parallel experiments using adsorbed anti-regucalcin antibodies or non-immune serum. In the brain, immunostaining was prominent in the cortical cerebrum and hippocampus. Meanwhile, immunostaining was weak in the kidney and heart (Figs. 3 and 4), and it was not seen in the duodenum, testicle, spleen, lung, or smooth muscle (bladder) (data not shown).

Discussion

The most important part of the present result is the observation that a highly specific anti-regucalcin antibody selectively reacted with a subpopulation of the liver and brain in rats. Immunohistochemically, regucalcin existed in the liver and brain of rats. This adds definitive support to previous findings that regucalcin, which is isolated from rat liver cytosol, plays a cell physiological role in the regulation of liver cell function related to Ca^{2+}.[6-9]

Thus far, it has not been reported that regucalcin exists

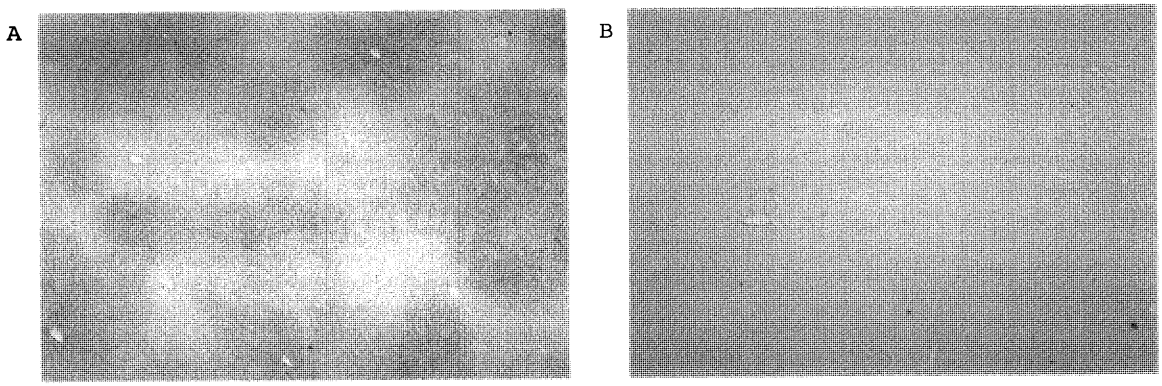


Fig. 1. Immunostaining of the Rat Liver Treated with Anti-regucalcin Antibodies

The immunostaining was strong in the application of anti-regucalcin antibodies (A) compared with parallel experiments using adsorbed anti-regucalcin antibodies (B). × 20.

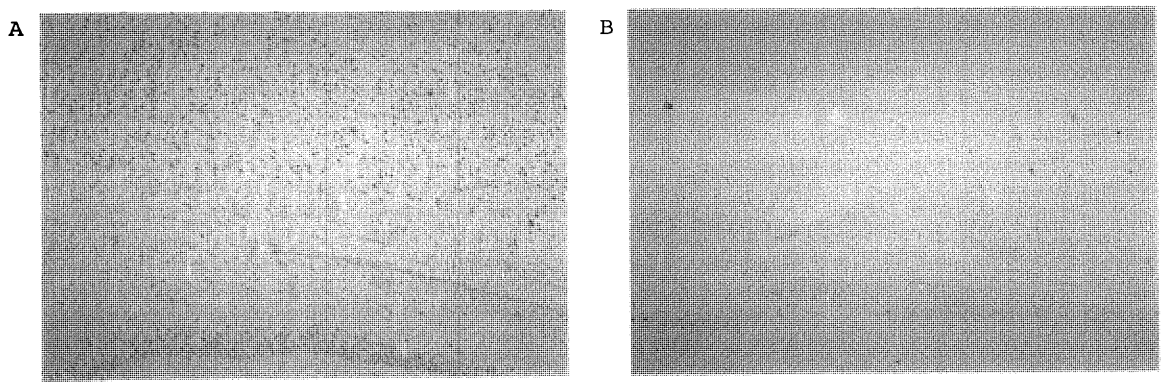


Fig. 2. Immunostaining of the Rat Brain Treated with Anti-regucalcin Antibodies

The immunostaining was strong in the application of anti-regucalcin antibodies (A) compared with parallel experiments using adsorbed anti-regucalcin antibodies (B). × 20.

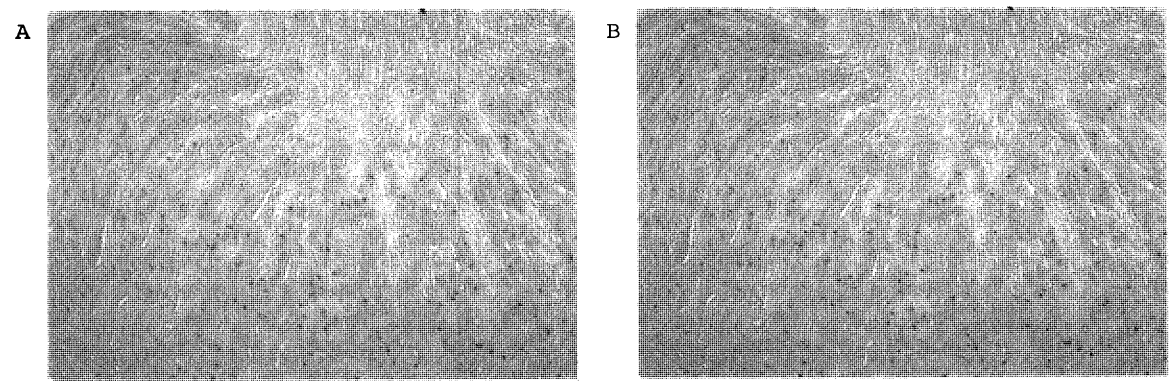


Fig. 3. Immunostaining of the Rat Kidney Treated with Anti-regucalcin Antibodies

The immunostaining was weak in the application of anti-regucalcin antibodies (A) compared with parallel experiments using adsorbed anti-regucalcin antibodies (B). × 20.

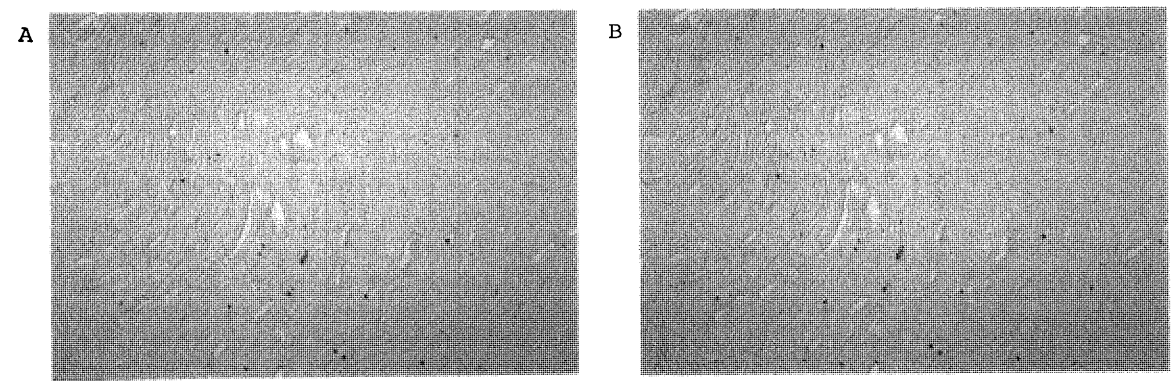


Fig. 4. Immunostaining of the Rat Heart Treated with Anti-regucalcin Antibodies

The immunostaining was weak in the application of anti-regucalcin antibodies (A) compared with parallel experiments using adsorbed anti-regucalcin antibodies (B). × 20.

in the brain. The present immunohistochemical demonstration of regucalcin in the rat brain suggests that regucalcin may play a role in brain function. Other calcium-binding proteins (calmodulin, S-100 protein, caligulin and calregulin) also exist in the brain.[11-13] More recently, it has been reported that calreticulin, which differs from regucalcin, is a candiate for a calsequestrin-like function in Ca^{2+}-storage compartments (calciosomes) of the liver and brain in rats.[14] Whether regucalcin has a regulatory role in the effect of Ca^{2+} on brain function, as well as in the liver, remains to be elucidated.

Acknowledgements This work was supported in part by a Grant-in-Aid for Scientific Research (C) (No. 02671006) from the Ministry of Education, Japan.

References

1) J. Rasmussen, *Science*, **170**, 404 (1970).
2) J. R. Williamson, R. H. Cooper, and J. B. Hoek, *Biochim. Biophys. Acta*, **639**, 243 (1981).
3) P. H. Reinhart, W. M. Taylor, and F. L. Bygrave, *Biochem. J.*, **223**, 1 (1984).
4) M. Yamaguchi and T. Yamamoto, *Chem. Pharm. Bull.*, **26**, 1915 (1978).
5) M. Yamaguchi and K. Sugii, *Chem. Pharm. Bull.*, **29**, 567 (1981).
6) M. Yamaguchi and H. Yoshida, *Chem. Pharm. Bull.*, **33**, 4489 (1985).
7) M. Yamaguchi and S. Mori, *Chem. Pharm. Bull.*, **36**, 321 (1988).
8) M. Yamaguchi and S. Mori, *Biochem. Med. Metab. Biol.*, **43**, 140 (1990).
9) M. Yamaguchi and S. Mori, *Mol. Cell. Biochem.*, **99**, 25 (1990).
10) K. R. Jessen, J. M. Hills, and M. J. Saftrey, *J. Neurosci.*, **6**, 1628 (1986).
11) W. Y. Cheung, *Science*, **202**, 19 (1980).
12) D. M. Waisman, J. Muranyi, and M. Ahmed, *FEBS Lett.*, **164**, 80 (1983).
13) D. M. Waisman, B. P. Salimath, and M. J. Anderson, *J. Biol. Chem.*, **260**, 1652 (1985).
14) S. Treves, M. De Mattel, M. Lanfredi, A. Villa, N. M. Green, D. H. MacLennan, J. Meldolesi, and T. Pozzan, *Biochem. J.*, **271**, 473 (1990).

Antibacterial Activity of Two Chalcones, Xanthoangelol and 4-Hydroxyderricin, Isolated from the Root of *Angelica keiskei* KOIDZUMI[1)]

Yoshihiko INAMORI, Kimiye BABA, Hiroshi TSUJIBO, Masahiko TANIGUCHI, Kouji NAKATA and Mitsugi KOZAWA*

Osaka University of Pharmaceutical Sciences, Kawai, Matsubara-shi, Osaka 580, Japan. *Received December 6, 1990*

Two chalcones, xanthoangelol (I) and 4-hydroxyderricin (II), isolated from the root of *Angelica keiskei* KOIDZUMI (Umbelliferae) showed antibacterial activity against gram-positive pathogenic bacteria. The activity of I on *Micrococcus luteus* IFO-12708 (minimum inhibitory concentration (MIC), 0.76 μg/ml) was the same potency as that of gentamicin, which is used as a standard. Although the activity of both chalcones on plant-pathogenic bacteria was lower than that of streptomycin sulfate, used as a positive control, they also exhibited growth-inhibitory effects. The antibacterial activity of I isolated from *Angelica keiskei* KOIDZUMI is being reported here for the first time. The growth-inhibitory effect of II on plant-pathogenic bacteria is also reported for the first time in this paper.

Keywords *Angelica keiskei*; Umbelliferae; chalcone; xanthoangelol; 4-hydroxyderricin; antibacterial activity; plant-pathogenic bacterium; gram-positive pathogenic bacterium; *Micrococcus luteus*

Angelica keiskei KOIDZUMI (Japanese name "ashitaba," Umbelliferae) is a sort of perennial herb growing mainly in Izu islands, and the Izu and Miura peninsulas near the Kanto District, a central area of Japan. In ancient times, this plant was called "kenso" in Japan, and was used in the treatment of small pox and food poisoning. Presently in Japan, this plant is used as a diuretic, analeptic and a lactogen. In addition, the fresh leaves of this plant and its dry powder are used for food. Kozawa *et al.* have already made a study on the components of this plant, isolating two chalcones,[2)] xanthoangelol (I, Chart 1) and 4-hydroxyderricin (II, Chart 1), together with seven coumarins,[3,4)] bergapten, xanthotoxin, angelicin, psoralen, columbianadin, archangelicin and 8(*S*),9(*S*)-angeloyloxyl-8,9-dihydroroseolol. Among these compounds, 4-hydroxyderricin (II) isolated from the root bark of *Lonchocarpus neuroscapha* BENTH. was already reported to inhibit the growth of gram-positive bacteria.[5)] However, no work has been done on the antibacterial activity of xanthoangelol (I).

In the present study, attention has been focused on the antibacterial activity of two chalcones, xanthoangelol (I) and 4-hydroxyderricin (II) against organisms including plant-pathogenic bacteria to obtain knowledge on the biological activity of the components.

Materials and Methods

Chemicals Two chalcones, xanthoangelol (I) and 4-hydroxyderricin (II),[2)] isolated from the root of *Angelica keiskei* KOIDZUMI (Umbelliferae) were used for antibacterial activity tests. Standards: Gentamicin (Sigma Chemical Co., Ltd., for pathogenic bacteria); streptomycin sulfate (Sigma Chemical Co., Ltd., for plant-pathogenic bacteria).

Microorganisms The pathogenic bacteria and plant-pathogenic bacteria listed in Tables I and II were used for the antibacterial activity tests.

Antimicrobial Activity Test This was carried out by the agar dilution test.[6)] The test bacterium (inoculum size: 10^6 cell/ml) was applied to heart infusion agar (Eiken Chemical Co., Ltd.) containing various concentrations of I, II, gentamicin and streptomycin sulfate. The plates were incubated at 37 °C for 18 h. However, in the case of the plant-pathogenic bacteria, the plates were incubated at 27 °C for 2 d. The minimum inhibitory concentration (MIC) was defined as the lowest drug concentration resulting in complete inhibition of visible growth.

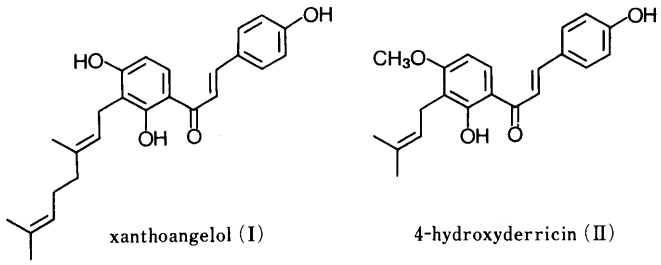


Chart 1

Results

Antibacterial Activities of Two Chalcones, Xanthoangelol (I) and 4-Hydroxyderricin (II) The antibacterial activities of two chalcones, I and II, were examined by the agar dilution test. The results are listed in Table I. Although the antibacterial activities of I and II were lower than that of gentamicin used as a standard, both chalcones showed antibacterial activities against gram-positive bacteria. In particular, the antibacterial activity of I on *Micrococcus luteus* IFO-12708 was strong and its MIC on this bacterium was 0.76 μg/ml. On the other hand, both compounds did not exhibit growth-inhibitory activity on all the gram-

TABLE I. Antibacterial Activity of Two Chalcones, Xanthoangelol (I) and 4-Hydroxyderricin (II)

Bacterium	Antibacterial activity (MIC[a]: μg/ml)		
	I	II	Gentamicin
Bacillus subtilis PCI-219	1.56	1.56	0.09
Bacillus subtilis ATCC-6633	1.56	1.56	0.09
Bacillus cereus FDA-5	1.56	3.12	0.045
Staphylococcus aureus 209-P	6.25	6.25	0.19
Staphylococcus aureus IFO-3060	3.12	3.12	0.19
Staphylococcus epidermidis IFO-3762	1.56	6.25	0.09
Micrococcus luteus IFO-12708	0.76	1.56	0.76
Escherichia coli IFO-12734	>100.00	>100.00	1.56
Escherichia coli IFO-12713	>100.00	>100.00	1.56
Escherichia coli IFO-3301	>100.00	>100.00	1.56
Proteus vulgaris IFO-3988	>100.00	>100.00	1.56
Proteus mirabilis ATCC-21100	>100.00	>100.00	1.56
Enterobacter cloacae IFO-12937	>100.00	>100.00	0.78
Salmonella typhimurium IFO-12529	>100.00	>100.00	0.19
Klebsiella pneumoniae IFO-3317	>100.00	>100.00	0.19
Citrobacter freundii IFO-12681	>100.00	>100.00	0.39
Serratia marcescens IFO-12648	>100.00	>100.00	0.78

Culture conditions, 37 °C, 18 h; medium, heart infusion agar; assay method, agar dilution test. *a*) Minimal inhibitory concentration.

TABLE II. Antibacterial Activity of Two Chalcones, Xanthoangelol (I) and 4-Hydroxyderricin (II), on Plant-Pathogenic Bacteria

Bacterium	Antibacterial activity (MIC[a]: μg/ml)		
	I	II	Streptomycin sulfate
Agrobacterium tumefaciens IFO-3058	1.00	0.75	0.40
Pseudomonas syringae pv. phaseolicola IFO-12656	1.00	0.75	0.10
Pseudomonas syringae pv. tabaci IFO-3508	1.00	0.75	0.10
Pseudomonas stutzeri IFO-12510	1.00	0.75	0.20
Corynebacterium michiganense IFO-12471	>100.00	>100.00	0.05

Culture conditions, 27 °C, 2 d; medium, heart infusion agar; assay method, agar dilution test. *a*) Minimal inhibitory concentration.

negative bacteria examined.

Antibacterial Activities of Two Chalcones, Xanthoangelol (I) and 4-Hydroxyderricin (II) on Plant-Pathogenic Bacteria The antibacterial activities of two chalcones, I and II, on plant-pathogenic bacteria were investigated by the agar dilution test. The results are listed in Table II. Although the antibacterial activities of I and II were lower than that of streptomycin sulfate, used as a positive control, both compounds showed antibacterial activities against all of the plant-pathogenic bacteria tested except for *Corynebacterium michiganense* IFO-12471.

Discussion

It was found that two chalcones, xanthoangelol (I) and 4-hydroxyderricin (II) isolated from the root of *Angelica keiskei* KOIDZUMI (Umbelliferae), showed antibacterial activities against gram-positive pathogenic bacteria (Table I). It should be emphasized that although the antibacterial activities of the two compounds were lower than that of gentamicin, used as a standard, the growth-inhibitory activity of I on *Micrococcus luteus* IFO-12708 (MIC: 0.76 μg/ml) was as strong as that of the standard. On the other hand, the two chalcones did not inhibit the growth of gram-negative bacteria. Regarding the antibacterial activity of the chalcone derivatives, it has already been reported by Goncalves De Lima *et al.*[5] that 4-hydroxyderricin (II) isolated from the root bark of *Lonchocarpus neuroscapha* BENTH. exhibited marked *in vitro* inhibitory activity against gram-positive bacteria. Our present results are in accordance with their report.[5] However, the antibacterial activity of I is reported for the first time in this paper. Although the antibacterial activity of two chalcones on plant-pathogenic bacteria was lower than that of streptomycin sulfate, used as a positive control, they did show anti-bacterial activities against these bacteria (Table II). In the case of plant-pathogenic bacteria, unlike pathogenic bacteria, the two compounds inhibited the growth of gram-negative bacteria. However, the reason is not clear at present. Further studies on the antibacterial activities of I and II against many plant-pathogenic bacteria seem to be desirable. The growth-inhibitory activities of the two chalcones, I and II, on plant-pathogenic bacteria is also being reported here for the first time.

Studies of the mechanism of antibacterial actions of the two chalcones and their acute toxicities in mice are in progress.

Acknowledgement The authors wish to express their deep gratitude to T. Hasegawa, Ph. D., Director of the Institute for Fermentation, Osaka, for his valuable advice during this project.

References and Notes

1) Part VI in the series "Chemical Components of *Angelica keiskei* KOIDZUMI;" For Part V see K. Baba, T. Kido, Y. Yoneda, M. Taniguchi and M. Kozawa, *Shoyakugaku Zasshi*, **44**, 235 (1990).
2) M. Kozawa, N. Morita, K. Baba and K. Hata, *Yakugaku Zasshi*, **98**, 636 (1978).
3) M. Kozawa, N. Morita, K. Baba and K. Hata, *Yakugaku Zasshi*, **98**, 210 (1978).
4) K. Hata and M. Kozawa, *Yakugaku Zasshi*, **81**, 1647 (1961).
5) O. Goncalves De Lima, J. F. De Mello, J. S. De Barros Coelho, F. D. De Andrade Lyra, M. Machado De Albuquerque, G. B. Marini-Bettolo, G. Delle Monache and F. Delle Monache, II, *Farmaco. Ed. Sci.*, **30**, 326 (1974).
6) Y. Inamori, H. Amino, M. Tsuboi, S. Yamaguchi and H. Tsujibo, *Chem. Pharm. Bull.*, **38**, 2296 (1990).

Inhibition of Experimental Pulmonary Metastasis of Lewis Lung Carcinoma by Orally Administered β-Glucan in Mice

Iwao SUZUKI,[a] Teruaki SAKURAI,[a] Koichi HASHIMOTO,[a] Shozo OIKAWA,[b] Akiyoshi MASUDA,[b] Masumi OHSAWA[b] and Toshiro YADOMAE*,[a]

Tokyo College of Pharmacy,[a] Horinouchi 1432-1, Hachioji, Tokyo 192-03, Japan and Nippon Beet Sugar Mfg., Co., Ltd.,[b] Chuo-ku, Tokyo 104, Japan. *Received December 7, 1990*

The inhibitory effect on experimental pulmonary metastasis of Lewis lung carcinoma (3LL) of SSG, a (1→3)-β-D-glucan obtained from the fungus *Sclerotinia sclerotiorum* IFO 9395, administered orally was examined in mice. Oral administration of SSG for 10 consecutive days just after the intravenous implantation of tumor cells significantly inhibited the experimental pulmonary metastasis of 3LL at a dose of 2000 μg. However, SSG administered orally involving other timings was less effective. In comparison with oral administration, SSG was effective when administered intraperitoneally for 10 consecutive days at a dose of 200 μg.

These results suggest that SSG given by both parenteral and nonparenteral routes is effective in the inhibition of experimental pulmonary metastasis of tumors.

Keywords (1→3)-β-D-glucan; SSG; *Sclerotinia sclerotiorum* IFO 9395; oral administration; pulmonary metastasis; Lewis lung carcinoma

Introduction

Various biological response modifiers (BRMs) have been studied for the therapy of cancer and microbial infections in humans and animals. Recently, considerable attention has been focused on the effects of orally administered BRMs.[1-5] The oral administration has an advantage of being easy to perform with essentially no accompanying pain and no serious complication.

SSG is a (1→3)-β-D-glucan produced from the liquid-cultured broth of a fungus, *Sclerotinia sclerotiorum* IFO 9395, belonging to Ascomycotina.[6] This glucan is a potent BRM and can exhibit antitumor and immunomodulating activities when administered by a parenteral or non-parenteral route.[7-11] The property of effectiveness as a BRM by oral administration is different from that of other β-1,3-glucans such as lentinan, shizophyllan and grifolan.[12,13]

Clinically, cancer therapy by BRM alone cannot cure a patient completely. Therefore, the use of BRMs after chemotherapy, radiotherapy or surgery to defend repullulation and metastasis of tumor cells is rational.[14,15] In view of these points, investigation of the effects of BRMs on the metastasis of tumor cells is important. Particularly, the application of BRMs which can be orally administered to patients is great value.

In this study, we examined the effect of SSG administered orally on the experimental pulmonary metastasis of Lewis lung carcinoma (3LL) in mice, and the effect was compared to that of the parenteral route.

Materials and Methods

Animals Specific-pathogen-free male mice of BDF_1 (C57BL/6 × DBA/2) and C57BL/6 mice were purchased from Japan SLC, Inc. (Shizuoka) and used at 6—8 weeks of age. The animals were bred under specific pathogen-free conditions.

Preparation of SSG A preparation method of SSG was previously described.[9] SSG contained less than 1% of protein and >98% carbohydrate. Lipopolysaccharide contamination of this preparation was less than 0.00014% (1.4 pg/μg of test sample).

Tumor 3LL was maintained by serial biweekly subcutaneous passage in C57BL/6 mice. A local tumor grown subcutaneously was removed aseptically and minced in 10 ml of Hank's balanced salt solution (HBSS; Nissui Seiyaku Co., Ltd., Tokyo) containing heparin (5 U/ml). Single tumor cell suspensions were prepared by passing the matter through a 150-mesh wire sieve. The isolated tumor cells were washed three times with HBSS, resuspended in fresh HBSS, and counted in a hemocytometer.

Administration of Sample SSG was administered according to the method described previously.[9] For oral or intraperitoneal administration, SSG was dissolved in distilled water or physiological saline, respectively.

Assay of Experimental Pulmonary Metastasis Experimental metastasis assay followed the method described previously.[16] Two hundred microliters of the suspension containing $1—3 \times 10^5$ 3LL cells was injected into the tail vein of BDF_1 mice (semi-syngeneic to 3LL) or C57BL/6 mice (syngeneic to 3LL). Two to three weeks after the implantation of tumor cells, the mice were anesthesized by intraperitoneal injection of 150 mg/kg of sodium pentbarbital (Nembutal®; Dainabot Co., Ltd., IL.) and autopsied. Pulmonary metastases were estimated by counting the numbers of metastatic nodules on the pulmonary surface after washing the lungs in physiological saline.

Statistics All results are expressed as mean ± standard deviation (S.D.). The significance of difference between means was determined by the Student's *t*-test or Wilcoxon's *u*-test. A value of $p<0.05$ was considered significant.

Results

Inhibitory Effect of Various Doses of SSG Administered Orally on the Experimental Pulmonary Metastasis of 3LL

In the previous study, we showed that oral administration of SSG significantly inhibited the growth of a solid form of IMC carcinoma at a dose of 2000 μg for 10 consecutive days,[9] and that the optimum dose of parenterally (intraperitoneally) administered SSG to exhibit antitumor activity in the solid form of the tumor-mice system (IMC carcinoma) was 250 μg every other day five times.[7] On the basis of these findings, we examined the ability of SSG administered orally to inhibit the experimental pulmonary metastasis of 3LL, and compared the effect to the intraperitoneal administration of SSG.

In the first experiment, we implanted 3×10^5 3LL cells into BDF_1 mice from the tail vein on day 0 and administered SSG orally or intraperitoneally. Fourteen days after the implantation, the effect of SSG on the inhibition of pulmonary metastasis was determined (Table I). In the oral administration system, SSG was administered for 10 consecutive days (days 1—10), and a significant inhibitory effect was observed in the mice administered SSG at a dose of 2000 μg (exp. 1). The effect seemed to be dose-dependent

TABLE I. Inhibitory Effect of Various Doses of SSG on Experimental Pulmonary Metastasis of 3LL[a]

Dose (μg)	Administration route[b]	No. of mice	Number of surface nodules of pulmonary metastasis (mean ± S.D.)[c]	Inhibition ratio (%)
Experiment 1				
500	*p.o.*	7	140.9 ± 44.9	−12.2
1000	*p.o.*	7	111.6 ± 44.2	11.1
2000	*p.o.*	6	80.7 ± 19.0[d]	35.7
4000	*p.o.*	7	128.4 ± 24.6	−2.2
Control	—	7	125.6 ± 41.1	0
Experiment 2				
50	i.p.	7	117.0 ± 43.7	1.6
100	i.p.	7	88.6 ± 26.9	25.5
200	i.p.	7	73.3 ± 34.1[d]	38.4
400	i.p.	7	85.1 ± 32.4	28.4
Control	—	7	118.9 ± 40.0	0

a) 3LL cells (3×10^5) were implanted *via* tail vein of BDF_1 mice on day 0. SSG was administered for 10 consecutive days (day 1—10). These mice were sacrificed on day 14 and the numbers of nodules of 3LL on the lung surface were counted. *b*) Sample was administered orally (*p.o.*) or intraperitoneally (i.p.). *c*) The significance was evaluated by means of Student's *t*-test against the control group. *d*) $p < 0.05$.

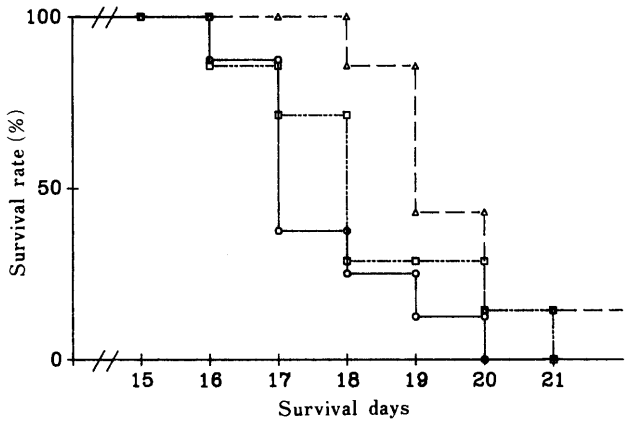


Fig. 1. Inhibitory Effect of SSG on the Survival Days of 3LL-Bearing Mice

Inhibitory effect of SSG on the survival days of BDF_1 mice (7 mice/group) with experimental pulmonary metastasis of 3LL. 3LL cells (3×10^5) were implanted *via* tail vein of BDF_1 mice on day 0. SSG was administered orally (2000 μg) or intraperitoneally (200 μg) for 10 consecutive days (days 1—10) and the mice were observed daily until day 21. ○—○, control; □·····□, SSG administered orally; △---△, SSG administered intraperitoneally.

in the range of 1000 to 2000 μg, but 4000 μg of SSG was less effective than 2000 μg. On the other hand, the administration of SSG for 10 consecutive days (200 μg, days 1—10) was effective in the intraperitoneal administration system (exp. 2), though the 5 administrations of SSG (days 1, 3, 5, 7 and 9) were not effective (data not shown). The inhibition seemed to be dose-dependent in the range of 50 to 200 μg, and the highest inhibitory effect was observed at 200 μg, but 400 μg of SSG was not effective. In the next experiment, BDF_1 mice were implanted with the same number of 3LL cells (3×10^5) on day 0 and were administered with SSG orally or intraperitoneally for 10 consecutive days (days 1—10), and their survival days were determined. The doses of SSG used for this experiment were within the effective doses determined by Table I. As shown in Fig. 1, all mice survived until day 15 and died off by pulmonary metastasis of 3LL after day 16. However, the survival days

TABLE II. Inhibitory Effect of SSG Administered Orally by Various Timings on Experimental Pulmonary Metastasis of 3LL[a]

Dose (μg)	Treatment days	Schedule times	No. of mice	Number of surface nodules of pulmonary metastasis (mean ± S.D.)[b]	Inhibition ratio (%)
Experiment 1 (C57BL/6 mice)					
2000	−4— 0	× 5	7	35.1 ± 9.3	−2.0
2000	1— 5	× 5	9	26.8 ± 6.8	22.1
2000	1— 10	× 10	9	20.7 ± 6.9[c]	39.8
Control	—	—	9	34.4 ± 14.6	0
Experiment 2 (BDF_1 mice)					
2000	−10— −1	× 10	5	49.8 ± 7.9	−3.3
2000	− 5— −1	× 5	5	40.4 ± 5.7	16.2
2000	− 5— 4	× 10	5	38.4 ± 5.3	19.9
2000	1— 5	× 5	3	48.0 ± 1.4	0.4
2000	1— 10	× 10	5	25.4 ± 8.2[c]	47.3
Control	—	—	5	48.2 ± 19.1	0

a) 3LL cells (1×10^5) were implanted *via* tail vein of C57BL/6 or BDF_1 mice on day 0. SSG was administered orally by various timings. These mice were sacrificed on day 21 and the numbers of nodules of 3LL on the lung surface were counted. *b*) The significance was evaluated by means of Student's *t*-test against the control group. *c*) $p < 0.05$.

of one group of mice given SSG intraperitoneally were increased significantly as compared with the control group ($p < 0.01$; Wilcoxon's *u*-test). In the case of oral administration, SSG slightly increased the survival days of mice. Numerous numbers of nodules of 3LL, 130—140, were observed on the lungs of all the dead mice.

Inhibitory Effect of SSG Administered Orally by Various Timings In this experiment, mice were implanted 1×10^5 3LL cells on day 0, and 2000 μg of SSG was administered orally on various time schedules (Table II). In an experiment (exp. 1), 3LL cells were implanted into C57BL/6 mice which are syngeneic to 3LL. In one group of mice which were given SSG for 10 consecutive days (days 1—10), significant inhibition of experimental pulmonary metastasis of 3LL was observed. However, the number of nodules of pulmonary metastasis in other groups of mice was almost similar to that of the control mice.

A similar result was obtained in the other experiment (exp. 2) using BDF_1 mice.

Discussion

In this study, we showed that SSG, a (1→3)-β-D-glucan obtained from *S. sclerotiorum* IFO 9395, administered by either an oral or intraperitoneal route, inhibited the experimental pulmonary metastasis of 3LL in mice. There have been few reports of inhibition of tumor metastasis by orally administered BRMs.[17,18] Particularly, there are few reports on the inhibitory effect of glucans administered orally.[12] In view of this, the efficacy of orally administered SSG to inhibit the experimental metastasis is very interesting.

In a previous report, we examined the antitumor effects of oral administration of SSG for 5 or 10 consecutive days. SSG significantly inhibited the growth of the solid form of tumor cells of Meth A fibrosarcoma or IMC carcinoma.[9] The optimal dose of SSG in the experiments was 2000 μg, and a significant inhibitory effect was observed only when SSG was administered orally for 5 or 10 consecutive days just after the implantation of tumor cells.[9] Similar to those results, the optimum dose of SSG was also 2000 μg for 10 consecutive days on the pulmonary metastasis of 3LL in

this study (Tables I, II).

As shown in Tables I and II, orally administered SSG significantly inhibited the experimental pulmonary metastasis of 3LL cells. However, the inhibitory effect was not observed on survival days of tumor-bearing mice, though SSG administered intraperitoneally was significantly effective (Fig. 1). Therefore, the inhibitory effect of SSG by an oral route was lower than that by an intraperitoneal route. This finding was also similar to the results obtained from an antitumor assay using the solid form of tumor cells.[7,9]

The mechanism of inhibition of pulmonary metastasis of 3LL by oral administration of SSG is not clear. It has been suggested that activated macrophages are important in inhibiting the metastasis of experimental tumors.[19] Fidler *et al.* reported that oral administration of a lipophilic analog of muramyl dipeptide, MTP-PE, systematically augmented the tumoricidal activity of macrophages including alveolar macrophages and peritoneal macrophages, and inhibited the experimental pulmonary metastasis of B16BL-6 cells.[4] In a previous paper, we described that oral administration of SSG enhanced the activity of peritoneal macrophages in mice.[11] Therefore, we assume that alveolar macrophages may also be activated by the oral administration of SSG.

On the other hand, it was reported that natural killer (NK) cells in the lungs are also important in the inhibition of lung metastasis.[20] In a case of oral administration of streptococcal preparation, OK-432, into mice, augmentation of NK activity in mesenteric lymph nodes and the suppression of metastasis of tumor cells to mesenteric lymph nodes was observed.[18] Oral administration of SSG augmented the NK activity in the systemic site (spleen) in mice,[9] therefore, it may be possible that SSG administered orally augments NK activity in lungs.

The process of cancer metastasis is complex and involves the detachment of cells from the primary tumor, penetration of the tumor cells through the host's connective tissues and capillary basement membranes, dissemination *via* blood circulation, arrest and extravasation into the target organ, and growth of the metastasizing cells into new tumor colonies.[16] In this study, we implanted 3LL cells *via* the tail vein and administered SSG orally for 10 consecutive days just after the implantation of tumor cells. It is considered that effector cells, including macrophages and NK cells, activated by the administration of SSG affect the processes of the establishment of 3LL cells in the lungs and/or on the growth of the metastasizing cells in new tumor colonies.

The micrometastasis of cancer cells which escape as a result of surgery, irradiation and chemotherapy occurs more often in lungs than in the other organs.[21] The combination therapy of BRMs and other cancer therapies is very useful in the eradication of cancer micrometastasis in lung tissue. Therefore, the investigation of BRMs which can inhibit the pulmonary micrometastasis of tumor cells by oral administration is of great value.

Acknowledgements This work was supported by a Grant-in-Aid from the Uehara Memorial Foundation. We express our thanks to Dr. N. Ohno for critical assessment of this study, and to Mr. T. Hayashi for technical assistance.

References

1) T. Fujii, H. Maeda, F. Suzuki and N. Ishihara, *J. Antibiot.*, **31**, 1079 (1978).
2) A. H. Bartal, D. Mordohovich, C. Lichtig, T. Mekori and E. Robinson, *Int. J. Immunopharmaco.*, **5**, 329 (1983).
3) H. U. Schorlemmer, K. Bosslet and H. H. Sedlacek, *Cancer Res.*, **43**, 4148 (1983).
4) I. J. Fidler, W. E. Fogler, A. F. Brownbill and G. Schumann, *J. Immunol.*, **138**, 4509 (1987).
5) G. Perdigón, M. E. N. de Macias, S. Alvarez, G. Oliver and A. P. de Ruiz Holgado, *Immunology*, **63**, 17 (1988).
6) N. Ohno, I. Suzuki and T. Yadomae, *Chem. Pharm. Bull.*, **34**, 1362 (1986).
7) I. Suzuki, K. Hashimoto and T. Yadomae, *J. Pharmacobio-Dyn.*, **11**, 527 (1988).
8) I. Suzuki, K. Hashimoto and T. Yadomae, *Int. J. Immunopharmaco.*, **10**, Suppl. 1, 91 (1988).
9) I. Suzuki, K. Hashimoto, N. Ohno, H. Tanaka and T. Yadomae, *Int. J. Immunopharmaco.*, **11**, 761 (1989).
10) K. Hashimoto, I. Suzuki, M. Ohsawa, S. Oikawa and T. Yadomae, *J. Pharmacobio-Dyn.*, **13**, 512 (1990).
11) I. Suzuki, H. Tanaka, A. Kinoshita, S. Oikawa, M. Osawa and T. Yadomae, *Int. J. Immunopharmaco.*, **12**, 675 (1990).
12) G. Chihara, *Rinsyo Meneki*, **17**, 200 (1985).
13) N. Ohno, Y. Adachi, I. Suzuki, S. Oikawa, K. Sato, M. Osawa and T. Yadomae, *J. Pharmacobio-Dyn.*, **9**, 861 (1986).
14) K. Ishihara, *Biotherapy*, **1**, 19 (1987).
15) T. Ooyama, H. Sakamoto, H. Yamada and Y. Esaki, *Biotherapy*, **2**, 601 (1988).
16) R. M. Abdallah, J. R. Starkey and G. G. Meadows, *J. Natl. Cancer Inst.*, **78**, 759 (1987).
17) F. Abe, M. Schnider, P. L. Black and J. E. Talmadge, *Cancer Immunol. Immunother.*, **29**, 231 (1989).
18) Y. Nakajima, M. Akimoto, M. Kimura, H. Iwasaki, S. Matano, H. Hirakawa and S. Mori, *Gan To Kagaku Ryoho*, **14**, 1229 (1987).
19) I. J. Fidler, *Cancer Res.*, **34**, 1074 (1974).
20) J. E. Talmadge, M. Schneider, M. Collins, H. Phillips, R. B. Herberman and R. H. Wiltrout, *J. Immunol.*, **135**, 1477 (1985).
21) G. Poste and I. J. Fidler, *Nature* (London), **283**, 139 (1980).

Percutaneous Absorption of Bromhexine in Rats

Taro OGISO,* Masahiro IWAKI and Sachiko TSUJI

Faculty of Pharmaceutical Sciences, Kinki University, 3-4-1 Kowakae, Higashi-Osaka 577, Japan. *Received December 14, 1990*

The percutaneous absorption of bromhexine (BH), an expectorant drug, through rat skin was examined *in vitro* and *in vivo*. BH in free base form penetrated better than the hydrochloride through the skin. When the *in vitro* penetration of BH was compared using Plastibase, macrogol and sucrose ester of fatty acid F-160 (DK-ester) formulations, the DK-ester formulation showed the best penetration of BH of the three. The addition of Azone (3%) or lauric acid (BH: lauric acid molar ratio, 1:1) considerably increased BH penetration to a relatively large penetration rate. The plasma levels of BH after *in vivo* application of the DK-ester formulation with Azone or lauric acid (0.6 g/3.8 cm^2) were also higher than those after the formulation without an enhancer, and a constant plasma level (20—50 ng/ml) was obtained during the application for 48 h. However, the bioavailability was low, 2.5 and 2.7% respectively. When the amount of BH remaining in DK-ester ointment and the skin after an 18-h application was measured, the BH content in the ointment was 88.6±8.0% for the formulation without Azone and 93.7±6.9% for that with Azone. The low penetration and low bioavailability observed will thus be due to the high drug retention of the base.

Keywords bromhexine; percutaneous absorption; ointment; rat; absorption enhancer; high drug retention; low bioavailability

Bromhexine (BH) is an expectorant drug, promoting bronchial secretion and having mucolytic properties. Although BH is widely used in human and veterinary medicine, the bioavailability is found to be considerably low; 2.4% in rat,[1] 6% in dog[2] and 26% in man.[3] However, successful therapy in man with BH can be achieved using very low doses (5—15 mg).[4] The low dosage, rapid biotransformation, and large distribution volume resulting from the lipophilic character of the drug[4-6] are the reasons for the very low plasma levels (nanogram range).

To avoid presystemic metabolism and to enhance the bioavailability of BH, we examined the potential of percutaneous absorption of the drug in rats. Additionally, the reason for the low plasma levels after percutaneous administration was analyzed by measuring BH levels in the skin and ointment after application.

Materials and Methods

Materials Reagent: BH hydrochloride and laurocapram (Azone) were generous gifts of Tokyo-Tanabe Pharmaceutical Co. and Nelson Research and Development Co., respectively. Santonin, an internal standard for gas chromatography (GLC), and imipramine hydrochloride, an internal standard for high performance liquid chromatography (HPLC), were obtained from Sigma Chemical Co. and Japan-Ciba Geigy Co., respectively. Plastibase (Taisho Pharmaceutical Co.), macrogol 400 and 4000 (Wako Pure Chemical Ind.) and sucrose ester of fatty acid (DK-ester) F-160 (Daiichi Industry Co.) were used as the ointment base. All other chemicals used were of reagent grade. A BH free base was prepared by the following procedure: To BH hydrochloride dissolved in water, 2N NaOH was added and BH was extracted twice with ether. The extract, after dehydration with Na_2SO_4 and filtration, was evaporated under a vacuum. The drug obtained was recrystallized from ethanol.

Animals: Male Wistar rats, weighing 200—260 g, were used throughout this study. The animals had free access to an MF diet (Oriental Yeast Co.) and water during the experiment.

Preparation of Ointment Details of the ointment composition are listed in Table I. For Rp. 1, BH was directly dissolved in Plastibase with or without Azone. For Rp. 2, BH dissolved in carbitol was added to a mixture of macrogol 400 and 4000 with or without Azone. For Rp. 3, DK-ester was mixed to hydroxyethyl cellulose, swollen with water, and the mixture was heated in boiling water for 10 min. After cooling, BH dissolved in carbitol with or without Azone (or lauric acid) was mixed with the above base.

***In Vitro* Percutaneous Penetration Experiment** The hair of the abdominal area of male Wistar rats was carefully removed with an electric razor 24 h prior to application of the formulation. Pieces (2×2 cm area) of full-thickness skin were freshly excised from the rats and the dermal side of the skin was soaked in a buffer solution (0.9% NaCl–10 mM phosphate buffer, pH 7.4) for 12 h at 5 °C to equilibrate the skin.[7] 0.15 g of formulation was uniformly spread over the stratum corneum surface of the skin, which was then mounted in a Franz diffusion cell (reservoir volume 13.0 ml, a 1.0 cm i.d. O-ring flange), and occluded with a sheet of aluminum foil. A gentamycin solution (10 mg/ml, Boehringer Mannheim) was added to the receptor fluid in the ratio of 1:100. The cells were incubated for 12 h at 37 °C.

***In Vivo* Percutaneous Absorption Experiment** The hair of the abdominal area of male Wistar rats was removed and the rat jugular vein was cannulated with silicone tubing (Phicon tube, SH No. 00, Fuji Systems) under pentobarbital anesthesia (50 mg/kg) 24 h prior to application of the formulation.[8,9] 0.6 g of a given formulation was uniformly spread over the shaved skin (3.8 cm^2, designated by attaching a silicone rubber sheet with a cut-out area, using α-cyanoacrylate) and immediately occluded with a sheet of aluminum foil and adhesive tape. The ointment remained in contact with the skin for 48 h. Blood samples (0.2 ml) were collected periodically after dosing through the tubing. The plasma was separated immediately by centrifugation and strored frozen until assay.

Determination of BH BH in the sample was determined by the method reported previously.[1]

Determination of BH in Ointment and Skin The remaining ointment after application for 18 h was collected. The skin was wiped off with gauze soaked in saline. Pieces (0.5 g) of skin were homogenized in 2 ml of saline using a micro homogenizer (Physcotron NS-10, Nichion). To 50 mg of ointment or skin homogenate, 0.75 ml of 6N NaOH and 0.1 ml of imipramine hydrochloride (4 mg/ml) solution was added, then the contents were extracted with 1.0 ml of ether. The extractions with 0.2N HCl and ether at alkaline pH were mutually repeated twice. The final ether layer

Table I. Composition of BH Ointments

Rp. 1	BH	2.0 g
	(Azone	3.0 g)
	Plastibase	98.0 g (95.0 g)
Rp. 2	Macrogol 4000	40.0 g
	Macrogol 400	30.0 g
	Carbitol	20.0 g
	BH	2.0 g
	(Azone	3.0 g)
	Purified water	ad 100.0 g
Rp. 3	DK-ester F-160	3.0 g
	Hydroxyethyl cellulose	7.0 g
	Carbitol	30.0 g
	BH	2.0 g
	(Azone[a]	3.0 g)
	Lauric acid[a]	1.1 g)
	Purified water	ad 100.0 g

a) Azone or lauric acid was added to the ointment.

was evaporated. The residue dissolved in 100 μl of a mobile phase (acetonitrile–methanol–0.01 N phosphate buffer, pH 7.0, 40:40:20, v/v) was filtrated through a Chromatodisc filter (0.45 μm, Biofield Co.). Three μl of the filtrate were injected into a column (4.6 mm × 25 cm) packed with LiChrospher 100 RP-8 (5 μm, Kanto Chemical, Tokyo), using a Shimadzu LC-6A liquid chromatograph equipped with a SPD-6AV UV-VIS detector. The flow rate was 1.0 ml/min and detection was at 254 nm.

Analysis of Data The area under the plasma concentration–time curve (AUC) after topical administration of BH was calculated by the trapezoidal method, and the AUC beyond the last observed plasma concentration (C_T) was extrapolated according to C_T/k_e, where k_e is the elimination rate constant.

The absolute bioavailability was calculated by the following equation:

$$\text{bioavailability (\%)} = \frac{AUC_{\text{p.c.}}\, dose_{\text{i.v.}}}{AUC_{\text{i.v.}}\, dose_{\text{p.c.}}} \times 100$$

The $AUC_{\text{i.v.}}$ was adopted from the previous paper.[1)]

Plasma concentration–time curves obtained after administration and the *in vitro* penetration data were analyzed by the iterative nonlinear least-squares regression procedure, MULTI.[10)] The means of all data are presented with their standard deviation (S.D.). Statistical analysis was performed using the non-paired Student's t-test, and a p-value of 0.05 or less was considered to be significant.

Results and Discussion

***In Vitro* Percutaneous Penetration of BH Hydrochloride and BH Base** The penetration of BH hydrochloride and the free base through rat skin was compared using a macrogol formulation with or without Azone (Rp. 2). The results are shown in Fig. 1. The hydrochloride from the formulation without Azone barely penetrated, while the free base penetrated appreciably. This result agreed with that in the case of propranolol absorption.[11)] The addition of Azone to the formulations considerably enhanced the permeation. In view of the results, the free base of BH was used for subsequent experiments. However, the free base may have a low thermodynamic activity, as suggested by its high melting point (dec. 237.5—238 °C) and low solubility.[12)] Since percutaneous absorption of drugs is affected by their physicochemical properties,[13)] BH may be not absorbed as much as expected.

***In Vitro* Percutaneous Penetration of BH after Application of Various Formulations** The penetration of BH through rat skin was compared using Plastibase, macrogol and DK-ester formulations. The results are shown in Fig. 2. The penetration of BH from Plastibase was extremely low, even if Azone was added in the base (Fig. 2). DK-ester formulation showed the best penetration of BH among the three formulations. When an absorption enhancer, either Azone or lauric acid, was added to the formulation, the penetration of BH was significantly increased. The penetration profiles always consisted of a lag time followed by a linear rise. The lag time tended to decrease slightly in the formulation with Azone or lauric acid. The apparent penetration rate (slope of the linear portion of the profile) was enhanced 1.5—2.5 fold in the presence of the enhancers, except in the Plastibase formulation.

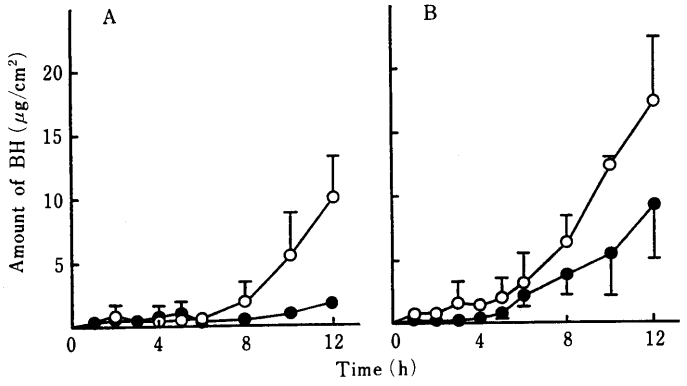


Fig. 1. Penetration Profiles of BH through Rat Skin after Application of Macrogol Ointment

A, BH·HCl; B, BH base. Each point represents the mean ± S.D. (n=3). Applied dose was 0.15 g/0.785 cm². ●, without Azone; ○, with Azone.

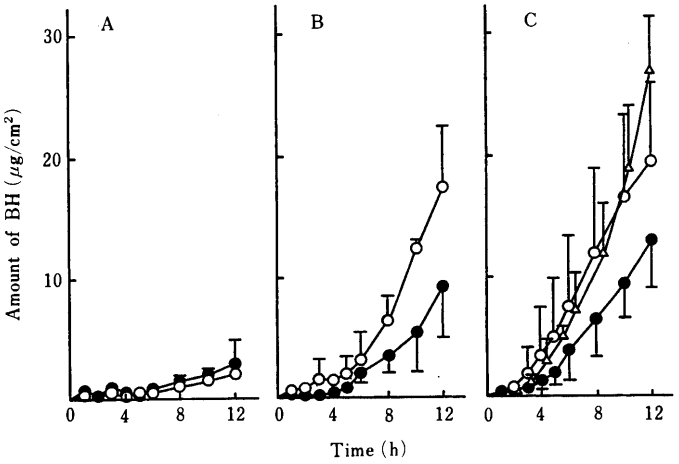


Fig. 2. Penetration Profiles of BH through Rat Skin after Application of Various Ointments

A, Plastibase; B, macrogol; C, DK-ester. Each point represents the mean ± S.D. (n=3—5). Applied dose was 0.15 g/0.785 cm². ●, without enhancer; ○, with Azone; △, with lauric acid.

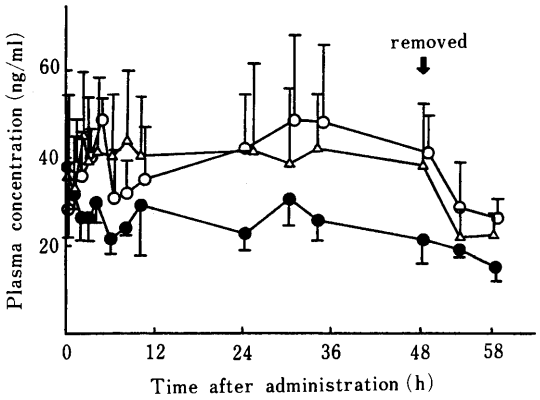


Fig. 3. Plasma Concentrations of BH after Percutaneous Administration of DK-ester Ointment

Each point represents the mean ± S.D. (n=3). Applied dose was 0.6 g/3.8 cm². The ointment was removed after 48 h. ●, without enhancer; ○, with Azone; △, with lauric acid.

TABLE II. Model-Independent Pharmacokinetic Parameters of BH Following Percutaneous and Oral Administrations

Parameter	DK-ester formulation (BH 12 mg/rat)			Oral (10 mg/kg)
	Without enhancer	+Azone	+LA[a)]	
$AUC_{0-\infty}$ (ng·h/ml)	1729±242	2920±680[b)]	2674±665[b)]	535±57
Bioavailability (%)	1.5±0.2	2.5±0.7	2.7±0.6[b)]	2.5±0.3

Each value represents the mean ± S.D. (n=3). *a)* Lauric acid. *b)* $p<0.05$ compared with no enhancer.

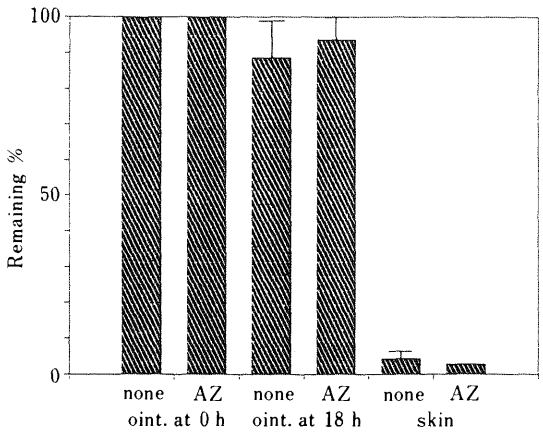


Fig. 4. Amount of BH Remaining in DK-ester Ointment and Rat Abdominal Skin after Application of the Ointment

Each bar represents the mean ± S.D. ($n = 3$). Application time of ointment was 18 h.

***In Vivo* Percutaneous Absorption of BH** DK-ester formulations, which showed good penetration of BH in the *in vitro* experiment, were used for the *in vivo* study. The plasma concentrations of BH after a 48-h application are depicted in Fig. 3. The plasma levels of BH after application of the formulation with Azone or lauric acid were much higher than those after the formulation without each enhancer. The model-independent pharmacokinetic parameters calculated are shown in Table II together with the oral data obtained previously.[1] The bioavailability was only 2.5 and 2.7% for the formulations with Azone or lauric acid, indicating low absorption. It is shown that the low drug level of BH in circulating blood (ng/ml) is sufficient for pharmacological effectiveness.[4] Therefore, the low plasma levels after percutaneous absorption would also be sufficient for pharmacological effect.

Amount of BH Remaining in Ointment and Skin after Application To confirm the low bioavailability and the cause, we determined the amount of BH remaining in ointment and the skin after an 18-h application. The results are shown in Fig. 4. The amount of BH remaining in the ointment was 88.6±8.0% for the formulation without Azone and 93.7±6.9% for the formulation with Azone. As a result, about 11 and 6% of the drug was released from the bases in 18 h, respectively. The low values were probably due to the high drug retention of the ointment base used. On the other hand, the amount in the skin was low, 4.3 and 2.8%, respectively. These results indicate that BH may be slowly released from the DK-ester formulation, due to the high lipophilicity of BH (octanol/water distribution constant $10^{6.2}$ [14]). Therefore, these characteristics of BH may also be used for the sustained release of the drug.

In conclusion, therefore, the present results lead us to postulate that BH was absorbed through rat skin, but the amounts absorbed during 48 h was considerably low. The percutaneous DK-ester formulations gave constant plasma levels after application to rat abdomen. The release of BH from DK-ester formulations was very slow and BH was largely retarded in the base.

References

1) M. Iwaki, T. Ogiso and Y. Ito, *J. Pharmacobio-Dyn.*, **13**, 475 (1990).
2) L. M. R. Vandecasteele-Thienpont, F. M. Belpaire, R. A. Braeckman and A. P. De Leenheer, *Arzneim.-Forsch.*, **30**, 1643 (1980).
3) E. Bechgaard and A. Nielsen, *Biopharm. Drug Dispos.*, **3**, 337 (1982).
4) F. Arch, *Arzneim.-Forsch.*, **13**, 480 (1963).
5) E. Schraven, F. W. Koss, J. Keck and G. Beisenherz, *Eur. J. Pharmacol.*, **1**, 445 (1967).
6) Z. Kopitar, R. Jauch, R. Hankwitz and H. Pelzer, *Eur. J. Pharmacol.*, **21**, 6 (1973).
7) B. W. Barry and S. L. Bennett, *J. Pharm. Pharmacol.*, **39**, 535 (1987).
8) R. A. Upton, *J. Pharm. Sci.*, **64**, 112 (1975).
9) S. K. Baker and S. Niazi, *J. Pharm. Sci.*, **72**, 1027 (1983).
10) K. Yamaoka, Y. Tanigawara, T. Nakagawa and T. Uno, *J. Pharmacobio-Dyn.*, **4**, 879 (1981).
11) T. Ogiso, Y. Ito, M. Iwaki and A. Shintani, *J. Pharmacobio-Dyn.*, **11**, 349 (1988).
12) M. Windholz (ed.), "Merck Index," 10th, Merck & Co., Inc., Rahway, N. J., U.S.A., 1983, p. 192.
13) B. Idson, *J. Pharm. Sci.*, **64**, 901 (1975).
14) J. Schmid and F. W. Koss, *J. Chromatogr.*, **277**, 71 (1982).

Pharmacokinetics of [6]-Gingerol after Intravenous Administration in Rats

Guohua DING,[a] Kohji NAORA,[a] Masakazu HAYASHIBARA,[a] Yoshihiro KATAGIRI,[a] Yoshihiro KANO[b] and Kikuo IWAMOTO*,[a]

Department of Pharmacy, Shimane Medical University Hospital,[a] 89–1 Enya-cho, Izumo, Shimane 693, Japan and Hokkaido Institute of Pharmaceutical Sciences,[b] 7–1 Katuraoka-cho, Otaru 047–02, Japan. *Received December 20, 1990*

A high-performance liquid chromatographic method to determine [6]-gingerol, a pungent constituent of ginger, in rat plasma was developed and a pharmacokinetic study was performed in rats. Quantitative analysis with high reproducibility was achieved for [6]-gingerol over the concentration range of 0.2—40 μg/ml. After bolus intravenous administration at a dose of 3 mg/kg, the plasma concentration–time curve was described by a two-compartment open model. [6]-Gingerol was rapidly cleared from plasma with a terminal half-life of 7.23 min and a total body clearance of 16.8 ml/min/kg. Serum protein binding of [6]-gingerol was 92.4%.

Keywords [6]-gingerol; pharmacokinetics; determination; intravenous administration; serum protein binding; rat; HPLC

The rhizome of ginger (*Zingiber officinale* ROSCOE) has been used not only as a seasoning spice but also as a useful crude drug in Chinese medicine, named "Shokyo." It is considered to be used as antiemetic, antitussive, stomach tonic, carminative, stimulant and diuretic.[1,2] [6]-Gingerol has been known to be an important pungent component of ginger.[3,4] It has been found that [6]-gingerol possesses various pharmacological effects, for example, inhibition of spontaneous motor activity, antipyretic and analgesic effects,[5] and cardiotonic effect.[6] However, little has been known about the pharmacokinetics of [6]-gingerol. The purpose of the present study is to develop a method for determination of [6]-gingerol in rat plasma with high-performance liquid chromatography (HPLC) and to clarify the pharmacokinetics of [6]-gingerol after bolus intravenous (i.v.) administration in rats.

Materials and Methods

Materials [6]-Gingerol was obtained from the rhizome of ginger. The details for the methods of isolation and purification have been given in a previous report.[7] All other reagents were commercial products of analytical grade.

Animals Male Wistar rats (Japan SLC Inc., Hamamatsu, Japan), weighing 350—419 g, were used. Under light anesthesia with ether each rat was cannulated with silicone tubing in the right jugular vein and fasted overnight (about 24 h) before the experiments.

Drug Administration [6]-Gingerol was uniformly dispersed in normal saline containing 5% Tween 80. The rats were given a bolus i.v. dose of 3 mg/kg of [6]-gingerol *via* the cannula.

HPLC Assay Extraction: To 150 μl of plasma sample, 500 μl of CH_3CN was added, and vortexed for 20 s. The mixture was centrifuged at 1.5×10^4 rpm for 5 min at room temperature and the supernatant (550 μl) obtained was evaporated to dryness at 40 °C under a gentle stream of N_2 gas. The residue was reconstituted in 75 μl of C_2H_5OH–H_2O (4:6) and an aliquot (20 μl) was injected into the chromatograph.

Chromatography: A Shimadzu Model LC-9A pump (Shimadzu, Kyoto, Japan), equipped with a Shimadzu Model SPD-6A variable-wavelength spectrophotometric detector, was used. Samples were chromatographed with an analytical column of Wakosil 5C18 (4.6 mm i.d. × 150 mm) at 40 °C. The mobile phase was CH_3CN–H_2O (45:55) with a flow-rate of 1.0 ml/min. The eluate was monitored by the spectrophotometric detector at 280 nm with a sensitivity of 0.005 a.u.f.s.

Calibration Graph: To blank plasma were added known amounts of [6]-gingerol in the final concentration range of 0.2—40 μg/ml. These plasma samples were treated according to the above determination procedure. The calibration curve was constructed by using the peak area of [6]-gingerol.

Reproducibility: Blood samples were obtained from the rats at appropriate times after the administration of [6]-gingerol. Aliquots (150 μl each) of the plasma samples were repeatedly analyzed according to the above procedure.

Accuracy: To the plasma samples which were obtained from the rat administered [6]-gingerol were added known amounts of [6]-gingerol and then this compound in the plasma was determined. The recovery of [6]-gingerol was calculated by comparing the experimental value with the corresponding theoretical value.

Pharmacokinetic Study [6]-Gingerol was administered to the rats and blood samples (about 330 μl) were withdrawn through the cannula into the heparinized tubes at 2, 4, 7, 10, 15, 20, 25, 30, 45 and 60 min after the administration. After centrifugation, the plasma was immediately separated and kept frozen until the analysis. Plasma concentration–time data after i.v. administration of [6]-gingerol was analyzed by a non-linear least squares regression program, MULTI.[8]

Serum Protein Binding Study About 2.5 ml of blood was collected from the rats at about 2 to 4 min after the administration of [6]-gingerol. The serum was immediately obtained by centrifugation with a serum separator (Fibrichin, Takazono Sangyo Co., Ltd., Osaka, Japan) and was utilized for the ultrafiltration method by using a micropartition system MPS-3 (Amicon Corp., Danvers, MA, U.S.A.). Since the preliminary experiments indicated that 11.5% of the [6]-gingerol was adsorbed by this system, we corrected the drug concentration in the filtrate by using this value. The extent of binding of [6]-gingerol to serum protein was estimated by the drug concentrations in the serum and the filtrate.

Results

Figure 1 shows representative chromatograms for a plasma blank and plasma sample obtained from the rat which was given [6]-gingerol. The peak for [6]-gingerol was well separated from the peaks which seemed to be derived from endogenous materials in the rat plasma. The retention time for [6]-gingerol was about 8.8 min.

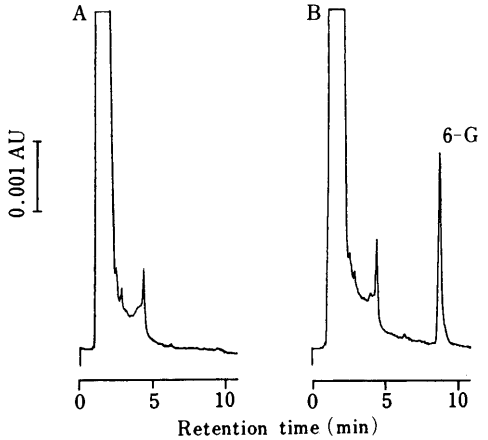


Fig. 1. Chromatograms of (A) a Plasma Blank and (B) a Plasma Sample Obtained 4 min after Bolus Intravenous Administration of [6]-Gingerol (3 mg/kg) to a Rat

TABLE I. Precision and Accuracy on the Determination of [6]-Gingerol (6-G) in Rat Plasma

Reproducibility[a]			Recovery[b]		
6-G level (μg/ml)		C.V. (%)	6-G level (μg/ml)		Mean (%)
Mean	S.D.		Added	Found	
35.8	0.7	2.0	None	3.47	—
2.65	0.04	1.7	1.00	4.50	103.2
0.310	0.015	4.8	10.0	13.2	97.3

a) Based on 10 determinations. b) Based on 5 determinations.

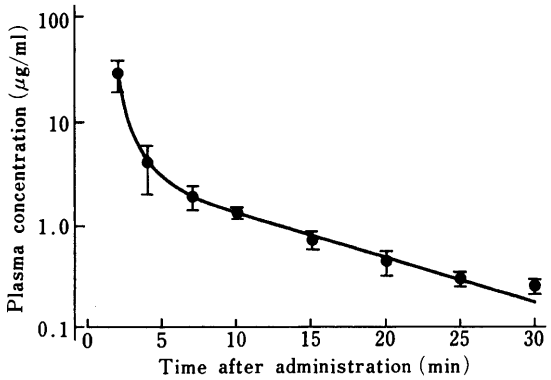


Fig. 2. Plasma Concentration–Time Profile of [6]-Gingerol after Bolus Intravenous Administration (3 mg/kg) to Rats

Each point and vertical bar represent the mean and S.D. of 6 rats.

TABLE II. Pharmacokinetic Parameters for [6]-Gingerol after Bolus Intravenous Administration (3 mg/kg) to Rats

Parameter[a]		Mean ± S.D.[b]
A	(μg/ml)	283.1 ± 197.8
B	(μg/ml)	3.44 ± 0.93
α	(min^{-1})	1.30 ± 0.33
β	($10^{-2} min^{-1}$)	9.69 ± 1.05
$t_{1/2\beta}$	(min)	7.23 ± 0.83
AUC	(μg·min/ml)	238.1 ± 114.0
CL	(ml/min/kg)	16.8 ± 10.9
V_c[c]	(ml/kg)	20.3 ± 18.7
V_p[d]	(ml/kg)	36.8 ± 41.5

a) Estimated by program MULTI [Weight(i) = 1/Ci]. b) Based on the data of 6 rats. c) Distribution volume of central compartment. d) Distribution volume of peripheral compartment.

Calibration line for [6]-gingerol was linear over the concentration range of 0.2—40 μg/ml. The regression equation was $y = 3484x - 642$, $r = 1.000$, where y is the peak-area of the drug, x is plasma concentration (μg/ml) of the drug and r is the correlation coefficient. The values of coefficient of variation (C.V.) for the peak-area at [6]-gingerol concentrations tested were within 8.1%. Similar results were also obtained when serum was used in place of plasma.

Table I shows the precision and accuracy data on the determination of [6]-gingerol. The precision of the determination of [6]-gingerol in plasma was examined by performing ten replicate analyses at three different concentrations of the drug in plasma samples. The C.V. values ranged from 1.7 to 4.8%. The recovery data was obtained when drug was added at two different concentrations to the rat plasma sample. The average recovery at both concentrations

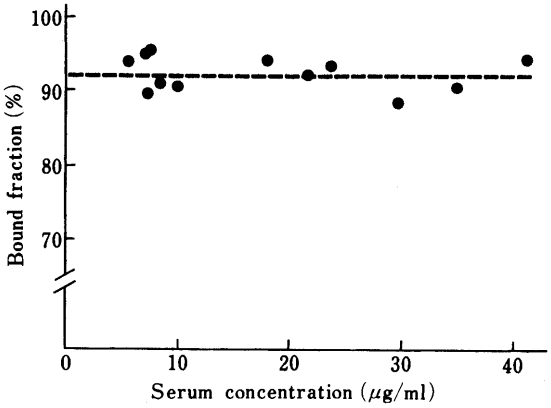


Fig. 3. Fraction of [6]-Gingerol Bound to Rat Serum Protein after Bolus Intravenous Administration (3 mg/kg)

Broken line indicates the mean value (= 92.4%) of bound fraction.

was nearly 100%.

After the bolus i.v. administration of 3 mg/kg of [6]-gingerol, plasma level of the drug declined with time in a biexponential pattern as shown in Fig. 2. A two-compartment open model was found to describe the data most adequately. The corresponding pharmacokinetic parameters which were estimated by the analysis of the data obtained from the individual rat are given in Table II. The elimination half-life at the terminal phase was 7.23 min and the total body clearance was 16.8 ml/min/kg.

The results of serum protein binding experiments are shown in Fig. 3. In the serum concentration range of 5.24—40.9 μg/ml, the bound fraction of [6]-gingerol was almost constant and its mean value was 92.4%.

Discussion

In order to elucidate the clinical effects of Chinese medical preparations, it is important to investigate their pharmacokinetics. Ginger is prescribed in about 50% of Chinese medical prescriptions.[7] Thus, we selected [6]-gingerol, an effective component of ginger, as a model compound and investigated the pharmacokinetics of this compound in rats. In the first place, a sensitive and selective method for the determination of [6]-gingerol in biological fluids was required to investigate the pharmacokinetics of this compound. The determination methods of [6]-gingerol in the "Zingiberis Rhizoma" by using gas chromatography-mass spectrometry (GC-MS),[3] HPLC[7] and supercritical fluid chromatography (SFC)[9] have been reported. However, no paper concerning the method in the biological fluids such as blood, plasma or serum has been published. We have developed a simple and precise method for determination of [6]-gingerol in the rat plasma by HPLC. The pretreatment procedure in the present determination method consisted of only two processes, the deproteinization and concentration, without any solvent extraction. Therefore, nothing was employed as the internal standard. The data listed in Table I indicated that the present method had satisfactory precision and accuracy in spite of the absence of an internal standard.

Using the present determination method we have performed a pharmacokinetic study on [6]-gingerol after i.v. administration. Limitation of sampling time-points as well as a large variation of the plasma concentrations in the

initial phase might yield relatively large standard deviation for several pharmacokinetic parameters. It was not possible to monitor plasma levels of [6]-gingerol beyond 30 min since its concentrations were quite close to the limit of sensitivity. However, the plasma concentration of [6]-gingerol at 30 min was less than about 0.1% of that attained immediately after i.v. administration. Therefore, it is thought that the plasma concentration–time profile has reached an elimination phase at about 30 min. From the present kinetic analysis, it was found that [6]-gingerol was cleared very rapidly from plasma with a short terminal half-life in rats.

It has been reported that the pharmacological effect was maintained until 180 min after i.v. administration at a dose of 3.5 mg/kg.[5] Thus for [6]-gingerol, there may be a small discrepancy or time-shift between the duration of pharmacological effect and the plasma level due to rapid clearance. The following possible explanations may be proposed for this: [6]-gingerol may be irreversibly sequestered in the tissues where it acts; [6]-gingerol may be effective at a much lower plasma concentration than those detected by the present assay; some active metabolites may be produced. It is also interesting that [6]-gingerol was cleared quickly from plasma in spite of the relatively high extent of binding to serum protein. From these aspects, it is possible that [6]-gingerol has a specific route or mechanism of distribution and elimination. Further detailed work is necessary to clarify the above discrepancy and specific disposition mechanism of [6]-gingerol and is now in progress.

References

1) L. M. Perry, "Medicinal Plants of East and Southeast Asia," The MIT Press, Boston, Mass., 1980, p. 443.
2) K. Akamatsu, "Wakanyaku," Ishiyaku Shuppan, Tokyo, 1980, p. 551.
3) Y. Masada, T. Inoue, K. Hashimoto, M. Fujioka and K. Shiraki, *Yakugaku Zasshi*, **93**, 318 (1973).
4) D. W. Connell and M. D. Sutherland, *Aust. J. Chem.*, **22**, 1033 (1969).
5) M. Suekawa, A. Ishige, K. Yuasa, K. Sudo, M. Aburada and E. Hosoya, *J. Pharmacobio-Dyn.*, **7**, 836 (1984).
6) N. Shoji, A. Iwasa, T. Takemoto, Y. Ishida and Y. Ohizumi, *J. Pharm. Sci.*, **71**, 1174 (1982).
7) Y. Kano, K. Saito, T. Sakurai, S. Kanemaki, M. Tanabe and M. Yasuda, *Shoyakugaku Zasshi*, **40**, 333 (1986).
8) K. Yamaoka, Y. Tanigawara, T. Nakagawa and T. Uno, *J. Pharmacobio-Dyn.*, **4**, 879 (1981).
9) K. Suto, K. Sagara and T. Mizutani, Abstract of Papers, 36th Annual Meeting of the Japanese Society of Pharmacognosy, Kumamoto, October 1989, p. 144.

Mitogenic and Colony-Stimulating Factor-Inducing Activities of Polysaccharide Fractions from the Fruit Bodies of *Dictyophora indusiata* FISCH.

Chihiro HARA,*[,a] Yoshio KUMAZAWA,[b] Kazunari INAGAKI,[b] Masahiro KANEKO,[b] Tadashi KIHO,[c] and Shigeo UKAI[c]

Shotoku Gakuen Women's Junior College,[a] 1–38, Nakauzura, Gifu 500, Japan, School of Pharmaceutical Sciences, Kitasato University,[b] 5–9–1, Shirokane, Minato-ku, Tokyo 108, Japan, and Gifu Pharmaceutical University,[c] 6–1, Mitahora-higashi 5-chome, Gifu 502, Japan. *Received October 16, 1990*

Biological effects (mitogenic and colony-stimulating factor (CSF)-inducing activities) of five homogeneous polysaccharides and a conjugated polysaccharide fraction isolated from the fruit bodies of *Dictyophora indusiata* FISCH. were investigated. Fucomannogalactan (T-3-Ad) and conjugated polysaccharide fraction (T-2-A) exhibited significant mitogenic and CSF-inducing activities. Among two β-(1→6)-branched (1→3)-β-D-glucans (T-4-N and T-5-N), only T-4-N showed both mitogenic and CSF-inducing effects. Partially *O*-acetylated (1→3)-α-D-mannans (T-2-HN and T-3-M′) did not show these effects.

Keywords polysaccharide; *Dictyophora indusiata*; mitogenic activity; colony-stimulating factor-inducing activity; acetylated (1→3)-α-D-mannan; branched (1→3)-β-D-glucan; fucomannogalactan

Immune responses are usually augmented or modulated by various types of agents including bacteria, bacterial products, fungal products, plant products, synthetic chemicals, and so on. Many investigators are working to develop immunopotentiators to combat cancer, to reverse iatrogenic immunosuppression, and to treat immunodeficiency and chronic infection. In previous studies,[1–10] we reported on the structural characterization and biological activities (antitumor[1] and anti-inflammatory[2,3]) of polysaccharides obtained from several fungi. Six homogeneous polysaccharides were isolated from the fruit bodies of *Dictyophora indusiata* FISCH., two partially *O*-acetylated (1→3)-α-D-mannans (T-2-HN[4,5] from 70% aqueous ethanol extract and T-3-M′[6] from hot water extract), three β-(1→6)-branched (1→3)-β-D-glucans (T-3-G[7] from hot water extract, T-4-N[8] from 2% sodium carbonate extract, and T-5-N[2,9] from 1 M sodium hydroxide extract), and a fucomannogalactan (T-3-Ad[10] from hot water extract). In addition to these, a conjugated polysaccharide fraction[11] (T-2-A) was obtained from 70% ethanol extract. In this paper, we report the mitogenic and colony-stimulating factor (CSF)-inducing activities of five polysaccharides, except for T-3-G, and a conjugated polysaccharide fraction from the fungus.

Materials and Methods

Material The dried fruit bodies of *D. indusiata* are commercially available in Hong Kong.

Mice Female 8- to 10-week-old C3H/He, C57BL/6 and ICR mice (Japan Shizuoka Laboratory Animal Center, Hamamatsu, Shizuoka, Japan) were used. The mice were housed under specific-pathogen-free conditions.

Preparation of Cells Spleen cells were prepared from female 10-week-old C3H/He mice as described previously.[12] Bone marrow cells were removed from the femurs of 8- to 10-week-old C57BL/6 mice, suspended in RPMI 1640 medium supplemented with 20% fetal bovine serum, 50 μM 2-mercaptoethanol, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 100 units of penicillin per ml and 100 μg of streptomycin per ml, and incubated for 1 h to remove adherent cells. Resultant nonadherent cells were suspended in the supplemented RPMI 1640 medium and used as bone marrow cells for assay of CSF.[13]

Determination of Mitogenic Activity Mitogenic activity was assessed by determining [^{3}H]-thymidine uptake into C3H/He spleen cells which were incubated in quadruplicate at 37 °C for 48 h with or without test samples as described previously.[14] Lipopolysaccharide (LPS) of *Escherichia coli* 055: B5 prepared by the method of Westphal (Difco Laboratories, Detroit, Mich., U.S.A.) was used as a standard. A value of more than 1.5 of stimulation index (S.I.) was taken as positive.

Determination of CSF-Inducing Activity CSF-inducing activity was determined as described previously.[13] Briefly, 100-μg test samples were injected intraperitoneally (i.p.) into 8-week-old ICR mice 3 h before collection of blood. Sera were separated from three mice per group, pooled in equal volumes and diluted at 1 : 8 or 1 : 16 with serum-free RPMI 1640 medium. Samples (0.2 ml) of the diluted sera (12.5 or 25 μl of serum per dish) were mixed with 0.2 ml of bone marrow cells (5×10^4), 0.2 ml of fetal bovine serum, and 0.4 ml of 2.2% methylcellulose. The mixture was plated in a total volume of 1 ml in 35-mm-diameter petri dishes and incubated in triplicate at 37 °C for 7 d in a humidified atmosphere of 5% CO_2–95% air. Murine granulocyte/monocyte CSF (GM-CSF; Genzyme Co., Boston, Mass., U.S.A.) was used as a standard. A cell aggregate composed of more than 50 granulocytes and/or macrophages was counted as one colony.

Results

Mitogenic Activity Mitogenic responses of polysaccharide fractions were determined by the enumeration of tritiated thymidine incorporated into cultured murine spleen cells by pulse labelling. The results are shown in Table I. The uptake of tritiated thymidine in the cells could be increased by the addition of T-2-A, T-3-Ad and T-4-N at doses of 10 μg per well, and T-2-A at a dose of 1 μg per well. In particular, the mitogenic potency of T-3-Ad at a dose of 10 μg per well was much stronger than those of T-2-A and T-4-N, although the activity was little or none at doses of 1 and 0.1 μg per well. T-2-HN, T-3-M′ and T-5-N were not effective at any dose.

CSF-Inducing Activity Table II shows the CSF-inducing

TABLE I. Mitogenic Activity of Polysaccharide Fractions Obtained from the Fruit Bodies of *Dictyophora indusiata* FISCH.[a]

Sample	[^{3}H]-Thymidine uptake[b] mean cpm ± S.D. (S.I.) Dose (μg/well)					
	0.1		1		10	
T-2-A	7291 ± 698	(1.1)	10806 ± 679[d]	(1.7)	17777 ± 1169[d]	(2.8)
T-2-HN	6856 ± 428	(1.1)	6724 ± 410	(1.0)	7530 ± 843	(1.2)
T-3-Ad	6009 ± 1048	(0.9)	9148 ± 1786	(1.4)	65287 ± 9298[d]	(10.2)
T-3-M′	5947 ± 767	(0.9)	6178 ± 2331	(1.0)	8567 ± 1388	(1.3)
T-4-N	7260 ± 677	(1.3)	6427 ± 3202	(1.0)	12499 ± 2790[c]	(1.9)
T-5-N	6971 ± 629	(1.1)	5868 ± 426	(0.9)	7127 ± 890	(1.1)
Lentinan	5211 ± 943	(0.8)	5472 ± 1293	(0.9)	7430 ± 809	(1.2)
LPS	25199 ± 8825[d]	(3.9)	37621 ± 1753[d]	(5.9)	N.t.[e]	

a) C3H/He spleen cells were stimulated for 48 h with the indicated dose of polysaccharide fractions. *b*) [^{3}H]-Thymidine uptake was measured by a pulse label for the last 4 h of incubation in quadruplicate cultures. Value of the control without test samples was 6426 ± 361. S.I. = stimulation index (cpm in the test sample/cpm in the control). Statistical analyses were carried out by the Student's *t*-test. *c*) $p < 0.01$. *d*) $p < 0.001$. *e*) N.t. = not tested.

TABLE II. CSF-Inducing Activity of Polysaccharide Fractions Obtained from the Fruit Bodies of *Dictyophora indusiata* FISCH.[a]

Sample	No. of colonies/5×10^4 bone marrow cells per dish[b] mean ± S.D.	
	12.5 μl/dish	25 μl/dish
Control	0	0
T-2-A	69 ± 6	147 ± 8
T-2-HM	0	6 ± 4
T-3-Ad	49 ± 2	139 ± 16
T-3-M′	0	12 ± 5
T-4-N	41 ± 3	118 ± 5
T-5-N	0	6 ± 3
Lentinan	0	4 ± 2
GM-CSF (33 units)	90 ± 2	124 ± 10

a) Three ICR mice per group were injected i.p. with 100-μg polysaccharide fractions in saline. Sera were separated from the blood 3 h after administration and pooled in equal volumes. *b*) The CSF-inducing activity in 12.5 or 25 μl of serum per dish was measured in triplicate.

activities of sera obtained from mice injected i.p. with test samples (100 μg each) 3 h earlier, as determined in triplicate cultures containing 5×10^4 C57B/6 bone marrow cells. Among the polysaccharide fractions tested, T-2-A, T-3-Ad and T-4-N exhibited significant CSF-inducing activities in a dose-dependent manner. The potency of CSF induction by T-2-A was somewhat stronger than those of T-3-Ad and T-4-N in both 12.5 and 25 μl serum per dish, in contrast, T-2-HN, T-3-M′ and T-5-N did not show the activity.

Discussion

The fruit bodies of *D. indusiata* have been used as a nutritious and invigorating food in China. In this study, it was found that two homogeneous polysaccharides (T-3-Ad and T-4-N) and a conjugated polysaccharide fraction (T-2-A), which were isolated from the fungus, exhibited both mitogenic and CSF-inducing activities in a dose-dependent manner. The three polysaccharide fractions were shown to have mitogenicity[15] for murine lymphocytes (B cells) and to induce CSF in the blood of mice, one of the mediators which stimulates a proliferation and differentiation of macrophages and/or lymphocytes.[16] The mitogenicity of T-3-Ad observed by the addition of 10 μg per well, in contrast to those of T-2-A and T-4-N, is more effective and the potency may be comparable to that of bacterial LPS, though it was not tested at a dose of 10 μg per well. Also, colony-stimulating activities induced by T-2-A, T-3-Ad and T-4-N are similar to each other and their activities cannot be distinguished from that of murine GM-CSF (33 units) in 25 μl of serum per dish.

Among the active polysaccharide fractions, T-3-Ad is a heterogalactan (molecular weight $\bar{M}w$: 1.4×10^4) which is composed of L-fucose, D-mannose and D-galactose in the molar ratio of 1.0 : 1.9 : 4.2,[10] while T-4-N is a branched (1→3)-β-D-glucan ($\bar{M}w$: *ca.* 5.5×10^6) with single, β-(1→6)-linked D-glucosyl side chains.[8] Both polymers are free from proteins and lipids. It is very interesting that T-5-N ($\bar{M}w$: 3.3×10^5)[2] and lentinan (isolated from the fruit bodies of *Lentinus edodes*[17]), which have almost the same chemical structure as that of T-4-N, did not show the mitogenic or CSF-inducing activity. From the results, the structure of branched (1→3)-β-D-glucans for developing mitogenicity or CSF induction seems to be related to the molecular weight, the degree of branching, localization of the side chains and so on.[18] Also, T-2-A is a conjugated polysaccharide fraction which is mainly composed of mannose and galactose (1.0 : 1.4) and probably carries lipid-like substances in the molecules.[11] However, it is not clear whether its mitogenic and CSF-inducing activities are due to the sugar portion or another portion in the molecules of T-2-A. Inactive polysaccharides (T-2-HN and T-3-M′), on the other hand, are partially *O*-acetylated (1→3)-α-D-mannans, which differ from each other in molecular weight and *O*-acetyl content.[4,6]

Thus, it has become apparent that the fruit bodies of *D. indusiata* contain some biologically active polysaccharide components which may prove useful for the enhancement of immune responses. Further studies are therefore needed to clarify the relationship between the structures and the biological activities of these polysaccharide fractions.

Acknowledgments The authors are grateful to Dr. H. Danbara of the Kitasato Institute for helpful discussion and to Dr. G. Chihara of National Cancer Center Research Institute for the donation of lentinan.

References and Notes

1) S. Ukai, T. Kiho, C. Hara, M. Morita, A. Goto, N. Imaizumi, and Y. Hasegawa, *Chem. Pharm. Bull.*, **31**, 741 (1983).
2) C. Hara, T. Kiho, Y. Tanaka, and S. Ukai, *Carbohydr. Res.*, **110**, 77 (1982).
3) S. Ukai, T. Kiho, C. Hara, I. Kuruma, and Y. Tanaka, *J. Pharmacobio-Dyn.*, **6**, 983 (1983).
4) S. Ukai, C. Hara, T. Kiho, and H. Hirose, *Chem. Pharm. Bull.*, **28**, 2647 (1980).
5) C. Hara, T. Kiho, and S. Ukai, *Carbohydr. Res.*, **111**, 143 (1982).
6) C. Hara, Y. Yokomori, T. Kiho, K. Nagai, and S. Ukai, *Carbohydr. Res.*, **173**, 332 (1988).
7) C. Hara, T. Kiho, and S. Ukai, *Carbohydr. Res.*, **145**, 237 (1986).
8) C. Hara, T. Kiho, and S. Ukai, *Carbohydr. Res.*, **117**, 201 (1983).
9) S. Ukai, C. Hara, and T. Kiho, *Chem. Pharm. Bull.*, **30**, 2147 (1982).
10) C. Hara, T. Kiho, and S. Ukai, Abstract of the 104th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, March 1984, p. 629.
11) Details on the structural characterization of T-2-A will be reported in the near future.
12) Y. Kumazawa, M. Matsuura, Y. Nakatsuru-Watanabe, M. Fukumoto, C. Nishimura, J. Y. Homma, M. Inage, S. Kusumoto, and T. Shiba, *Eur. J. Immunol.*, **14**, 109 (1984).
13) Y. Kumazawa, M. Matsuura, J. Y. Homma, T. Furuya, H. Takimoto, K. Inagaki, T. Nagumo, M. Kiso, and A. Hasegawa, *Infect. Immun.*, **57**, 1845 (1989).
14) Y. Kumazawa, M. Matsuura, T. Maruyama, J. Y. Homma, M. Kiso, and A. Hasegawa, *Eur. J. Immunol.*, **16**, 1099 (1986).
15) J. Anderson, F. Melchers, C. Galanos, and O. Lüderitz, *J. Exptl. Med.*, **137**, 943 (1973).
16) D. H. Pluznic and L. Sachs, *Exp. Cell Res.*, **43**, 553 (1966).
17) G. Chihara, J. Hamuro, Y. Y. Maeda, Y. Arai, and F. Fukuoka, *Cancer Res.*, **30**, 2776 (1970).
18) We earlier discussed in references 8) and 9) the structural differences of the branched (1→3)-β-D-glucans (T-3-G, T-4-N and T-5-N) from the fungus.

CONFORMATIONAL STUDIES OF CYCLO(L-Phe-L-Pro-Gly-L-Pro)$_2$ BY ^{13}C NUCLEAR MAGNETIC RESONANCE

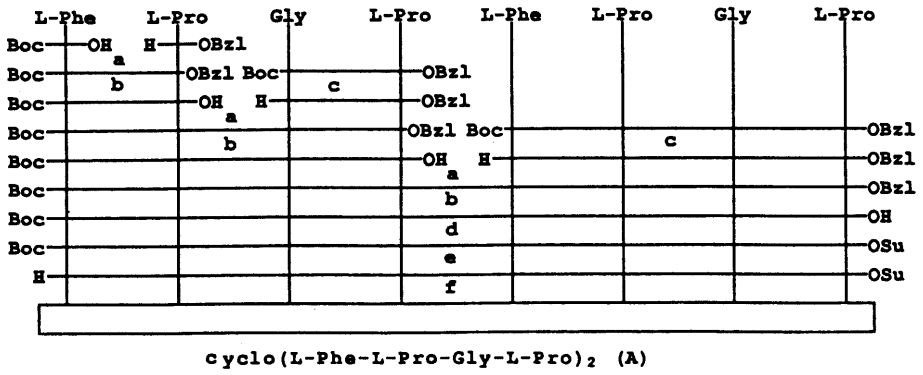
Takashi ISHIZU,* Ayumi FUJII, and Shunsaku NOGUCHI
Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, 985 Higashimura-cho, Fukuyama, Hiroshima, 729-02, Japan

The ^{13}C-NMR spectrum (Fig. 2,1) of cyclooctapeptide cyclo(L-Phe-L-Pro-Gly-L-Pro)$_2$ (A) in $CDCl_3$ suggested that its conformation involved the coexistence of two kinds of C_2-symmetric conformation with trans-trans-trans-trans and cis-trans-trans-trans forms. Adding 0.5 equivalent of CsSCN or one equivalent of DL-Phe-OMe·HCl to the solution of cyclopeptide (A) in $CDCl_3$ yielded ^{13}C-NMR spectra (Fig. 2,2 and Table I) which suggested a single C_2-symmetric conformation with trans-trans-trans-trans form, resulting from the formation of complexes with CsSCN or DL-Phe-OMe·HCl. The ^{13}C-NMR spectrum of complexes of A with DL-Phe-OMe·HCl displayed separete resonances[2] for C_γ, C_o,[1] C_m,[1] C_α, and C_β of D-Phe-OMe·HCl and L-Phe-OMe·HCl (Table I).

KEYWORDS cyclooctapeptide; ^{13}C-NMR; C_2-symmetric conformation; CsSCN; DL-Phe-OMe·HCl

Studies on complexes between " host and guest " molecules, such as the binding of an enzyme, a receptor, or an ionophore to its substrate, are widespread in chemistry and biology. However, it is often difficult to probe the detailed interaction and conformational change which occur in both components upon binding. To study this, we synthesized cyclooctapeptide cyclo(L-Phe-L-Pro-Gly-L-Pro)$_2$ (A) as a host model. The Pro residue allows a cis-trans isomerization of the peptide bond. This feature increases the number of available conformations of the peptide, which has been considered to be favorable for complex formation. In this communication we report its conformation and conformational changes, which took place in the formation of complexes with CsSCN or DL-Phe-OMe·HCl, monitored by the carbon-13 nuclear magnetic resonance (^{13}C-NMR).

Cyclooctapeptide cyclo(L-Phe-L-Pro-Gly-L-Pro)$_2$[3] (A) was synthesized by the liquid phase method the shown in Fig 1. The conformation of cyclopeptide (A) was determined by ^{13}C-NMR spectroscopy. Figure 2,1 shows the ^{13}C-NMR spectrum of A in $CDCl_3$ at 25°C. In the region of carbonyl group signals, four intense and three minor signals appeared at 171.23, 170.70, 169.82, 168.30 ppm, and at 173.59, 171.91, 169.16 ppm, respectively. Two signals appeared for each carbon atom of the L-Phe and L-Pro residues except for the L-Phe C_m atoms. On the other hand, only one signal for the Gly C_α atoms was observed at 42.59 ppm.



a) MA[1]
b) H_2/Pd-C
c) HCl/dioxane
d) HOSu/DCCI
e) TFA
f) pyridine

Fig. 1. Synthetic Route of Cyclo(L-Phe-L-Pro-Gly-L-Pro)$_2$ (A)

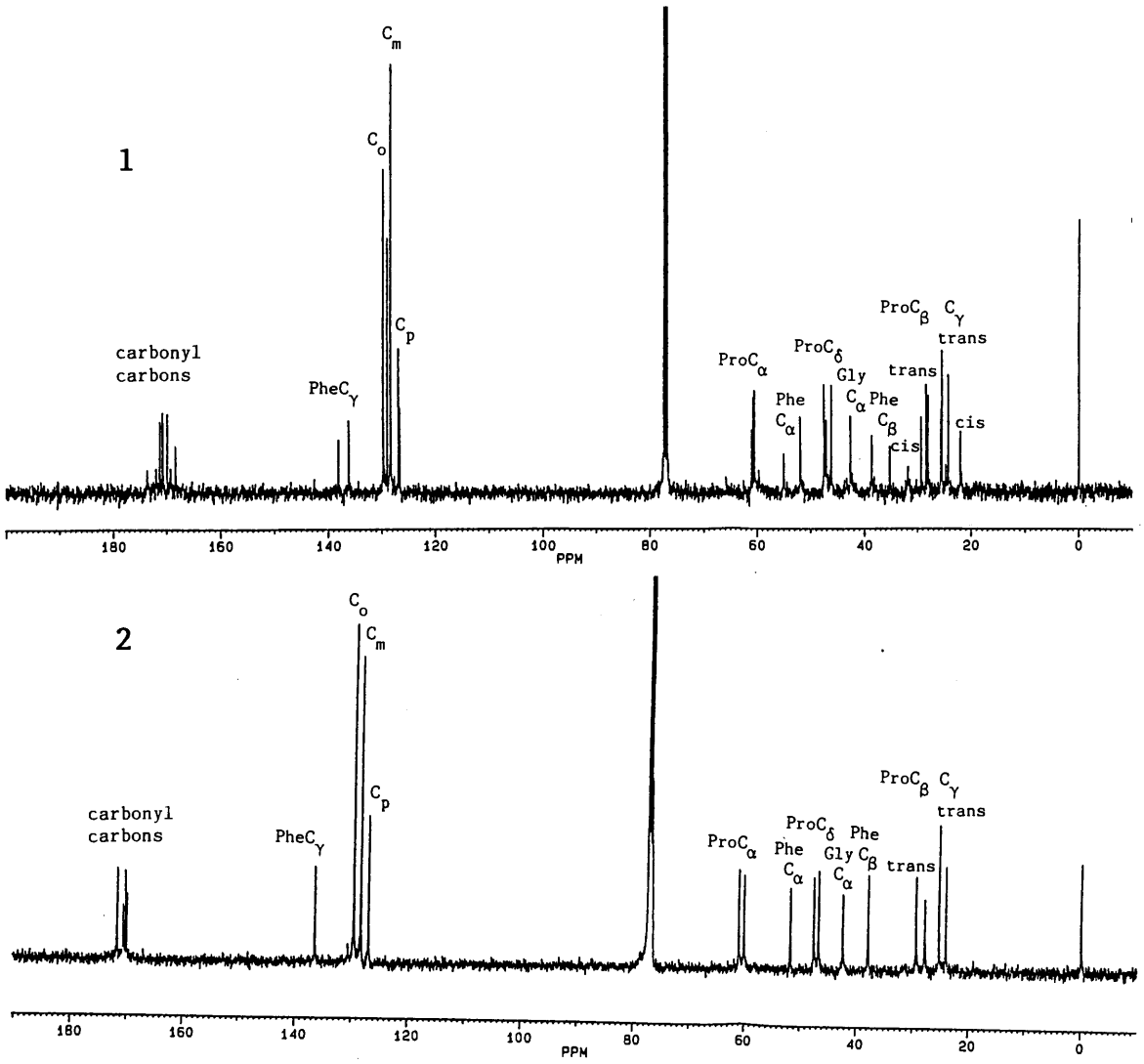


Fig. 2. ^{13}C-NMR (400MHz) Spectra of Cyclo(L-Phe-L-Pro-Gly-L-Pro)$_2$ (A)(1) and A + CsSCN (Molar Ratio = 2:1)(2) in $CDCl_3$.

The chemical shifts of the L-Pro C_β atoms at 29.28, 28.39, 28.00 ppm and C_γ atoms at 25.61, 25.43, 24.25 ppm indicated three trans Xxx-L-Pro peptide bonds (Xxx= L-Phe or Gly), and those of L-Pro C_β and C_γ atoms at 31.76 and 21.97 ppm indicated one cis bond, according to Wüthrich.[4] This suggested that the L-Phe-L-Pro peptide bonds coexist in trans and cis forms, while the Gly-L-Pro peptide bonds were all trans forms. This led to the conclusion that the conformation of cyclopeptide (A) coexists in two kinds of C_2-symmetric conformation with trans-trans-trans-trans and cis-trans-trans-trans forms in $CDCl_3$ (on the NMR time scale). The signals appearing at 51.92 and 38.62 ppm were assigned to the L-Phe C_α and C_β atoms of the trans-trans-trans-trans form, respectively, and the signals appearing at 55.00 and 35.21 ppm were assigned to those of cis-trans-trans-trans form, respectively, judging from the chemical shifts of the complexes of A with CsSCN or DL-Phe-OMe·HCl (Table I).

The ^{13}C-NMR spectrum of cyclopeptide (A) with CsSCN in $CDCl_3$ at 25°C is shown in Fig.2,2. Adding CsSCN (0.5 equivalent to A), decreased the total number of signals of the spectrum to almost half. Single signals appeared for each carbon atom of A. The L-Phe-L-Pro and Gly-L-Pro peptide bonds were assigned to all trans forms, because the chemical shifts of L-Pro C_β and C_γ atoms occurred at 29.40, 27.83 ppm and at 25.33, 24.10 ppm, respectively.[4] It is therefore concluded that only one C_2-symmetric conformation with trans-trans-trans-trans form resulted from the complex formation with CsSCN. No change in the ^{13}C-NMR spectrum (Fig. 2,2) occurred after the addition of more than 0.5 equivalent of CsSCN to A. This implied the formation of the 1:2 complex (Cs^+:A) in $CDCl_3$.

Adding one equivalent of DL-Phe-OMe·HCl to the solution ofcyclopeptide (A) in $CDCl_3$ at 25°C induced a conformational change in the ^{13}C-NMR spectrum (Table I) similar to that in the addition of CsSCN. The ^{13}C-NMR spectrum indicated that A took only one C_2-symmetric conformation, resulting from the formation of complexes with DL-Phe-OMe·HCl. Since the chemical shifts of Pro C_β and C_γ atoms occurred at 28.26, 26.59 ppm and at 25.10, 24.47 ppm, respectively, all L-Phe-L-Pro and Gly-L-Pro peptide bonds were trans forms.[4] No change in the ^{13}C-NMR spectrum (Table I) was observed after the addition of more than one equivalent of DL-Phe-OMe·HCl to A. This implied the formation of 1:1 complexes (DL-Phe-OMe·HCl:A) in $CDCl_3$.

Furthermore, the ^{13}C-NMR spectrum of the complexes of A with DL-Phe-OMe·HCl displayed seperate resonances for C_γ, C_o, C_m, C_α, and C_β of D-Phe-OMe·HCl and L-Phe-OMe·HCl (Table I).[2] This spectrum resulted from the formation of diasteromeric pairs of complexes. It was found from this result that cyclopeptide (A) distinguished a difference between D-Phe-OMe·HCl and L-Phe-OMe·HCl.

Table I. ^{13}C-NMR Spectral Data (ppm, in $CDCl_3$) of Cyclo(L-Phe-L-Pro-Gly-L-Pro)$_2$ (A), A+CsSCN, and A+DL-Phe-OMe·HCl

Carbon[1]	A	A + CsSCN[a]	A + DL-Phe-OMe·HCl[b]	
			A	DL-Phe-OMe·HCl
C=O	173.59(s)[c]	171.65	171.36	
	171.91(s)	170.58	171.11	
	171.23(1)[c]	170.23	169.78	
	170.70(1)	169.93	169.61	
	169.82(1)			
	169.16(s)			
	168.30(1)			
$PheC_\gamma$	138.05	136.44	136.36	135.43(D)
	136.11			134.83(L)
$PheC_o$	129.70	129.53	129.68	130.02(D)
	128.95			129.97(L)
$PheC_m$	128.30	128.28	128.11	128.54(D)
				128.40(L)
$PheC_p$	126.88	126.87	126.72	127.07(DL)
	126.61			
$ProC_\alpha$	61.00	61.06	60.42	
	60.68	60.07	59.95	
	60.42			
$PheC_\alpha$	55.00	51.75	52.30	55.09(D)
	51.92			54.84(L)
$ProC_\delta$	47.51	47.57	47.71	52.77(DL,Me[e])
	47.23	46.70	46.43	
	47.06			
	46.14			
$GlyC_\alpha$	42.59	42.37	41.97	
$PheC_\beta$	38.62	37.85	38.84	36.76(D)
	35.21			36.56(L)
$ProC_\beta$	31.76(c)[d]	29.40(t)	28.26(t)	
	29.28(t)[d]	27.83(t)	26.59(t)	
	28.39(t)			
	28.00(t)			
$ProC_\gamma$	25.61(t)	25.33(t)	25.10(t)	
	25.43(t)	24.10(t)	24.47(t)	
	24.25(t)			
	21.97(c)			

a) Molar ratio = 2:1. b) Molar ratio = 1:1.
c) s,l are small, large signals, respectively.
d) c,t are cis, trans, respectively.
e) Me is a methyl carbon atom of DL-Phe-OMe·HCl.

ACKNOWLEDGEMENT The authors thank Dr. Hiroyuki Iwamoto (Department of Engineering Sciences, Faculty of Foodtechnology, Fukuyama University) for the measurement of ^{13}C-NMR (Bruker AM-400, 400 MHz).

REFERENCES AND NOTES

1) The following abbreviations are used: MA=mixed anhydride method, Boc=t-butoxycarbonyl, HOSu=N-hydroxysuccinimide, DCCI=dicyclohexylcarbodiimide, TFA=trifluoroacetic acid. C_o, C_m, and C_p mean carbon atoms of ortho, meta, and para positions of phenyl group of Phe, respectively.
2) C.M.Deber, and E.R.Blout, J.Am.Chem.Soc., 96, 7566 (1974).
3) Yield 33.8% (on the basis of Boc(L-Phe-L-Pro-Gly-L-Pro)$_2$OSu), mp 220°C, Anal. Calcd for $C_{42}H_{52}O_8N_8$ $2H_2O$: C, 60.56; H, 6,78; N, 13.45. Found: C, 60.23; H, 6.57; N, 13.17. FAB-MS m/z: 797 ($M+H^+$).
4) K.Wüthrich, (1976) NMR in Biological Research: Peptide and Protein, North-Holland Publ. Co., Amsterdam.

(Received March 8, 1991)

EFFECT OF MOLECULAR WEIGHT IN AMPHIPATHIC POLYETHYLENEGLYCOL ON PROLONGING THE CIRCULATION TIME OF LARGE UNILAMELLAR LIPOSOMES

Kazuo MARUYAMA,*[,a] Tsutomu YUDA,[b] Aki OKAMOTO,[a] Chiyoji ISHIKURA,[b] Shuji KOJIMA[c] and Motoharu IWATSURU[a]

Faculty of Pharmaceutical Sciences, Teikyo University,[a] Sagamiko, Kanagawa 199-01, Bohsei Pharmacy,[b] Isehara, Kanagawa 259-11, Research Institute for Biosciences, Science University of Tokyo,[c] Noda, Chiba 278, Japan

The effect in mice of the molecular weight of polyethyleneglycol on prolonging the circulation time of large unilamellar liposomes (LUVs) was examined using four different distearoyl N-(monomethoxy polyethyleneglycol succinyl) phosphatidylethanolamines (DSPE-PEGs). The molecular weights tested were 1000, 2000, 5000 and 12000. Incorporation of 6 mol% of DSPE-PEG in LUV composed of distearoylphosphatidylcholine (DSPC) / cholesterol (CH) (1:1 in molar ratio) increased the blood circulation half-life significantly more than those without DSPE-PEG derivatives. DSPE-PEGs with molecular weights of 1000 and 2000 prolonged the circulation time of liposomes more than other DSPE-PEGs with higher molecular weights, such as 5000 and 12000. Their effects are also higher than ganglioside GM1, a well described glycolipid with this effect. DSPC/CH LUV-incorporated DSPE-PEG with a molecular weight of 2000 displayed a high concentration in the blood, approximately 40% of the dose, 6 h after the injection.

KEYWORDS liposome; polyethyleneglycol; drug delivery system; reticuloendotherial system

Liposomal drug delivery systems have been widely researched as a sustained release system, as well as a targeted drug delivery system.[1] LUVs have the advantage of entrapping a large volume of the drug solution. However, there are some problems in this use of LUVs. The rapid removal of liposomes from blood circulation by cells of the reticuloendotherial system (RES) is a serious problem for the sustained release of the drugs in the bloodstream and the targeting to non-RES tissues.[2] A prolonged residence of the drug-entrapped liposomes in the circulation is important for their sustained release.[3] Thus it is important to develop LUVs which avoid the uptake by the RES and extend the circulation half-lives in vivo. Allen et al.[4,5] and Gabizon et al.[6] showed that the use of ganglioside GM1 as a lipid component of the liposomes allowed them to avoid or delay the uptake by RES. Recently, Klibanov et al.[7] showed that the conjugation of amphipathic PEG of 5000 molecular weight with liposomes significantly increased the blood circulation half-life of the liposomes more than those without PEG.

However, so far there is little evidence concerning the effect of PEGs with different molecular weights on prolonging the circulation time of liposomes. In this paper, we described the effects of four different PEGs, 1000, 2000, 5000 and 12000 in molecular weight, on the blood residence time of LUV composed of DSPC/CH (1:1 in molar ratio).

METHOD

High purity DSPC (COATSOME MC-8080) was kindly donated by Nippon Oil & Fats, Tokyo. Four kinds of monomethoxy polyethyleneglycol succinimidyl succinates (PEG-OSu), 1000, 2000, 5000

and 12000 in molecular weight, were also purchased from Nippon Oil & Fats. Hereafter, these compounds are abbreviated as PEG1000, 2000, 5000 and 12000, respectively. Distearoyl phosphatidylethanolamine (DSPE) was obtained from Sigma. Triethylamine and CH were purchased from Wako Pure Chemical. Ganglioside GM1 (GM1) was from Funakoshi.

DSPE derivatives of PEG (DSPE-PEG) were synthesized as described by Klibanov et al.[7] Large unilamellar liposomes (LUV, 180-200 nm in diameter) composed of DSPC/CH and 6 mol% of DSPE-PEG were prepared according to Szoka et al.[8] and extruded through two stacked 0.2 μm Nucleopore polycarbonate filters at 60°C in a thermostat-heated extrusion device (EXTRUDER, Lipex, Vancouver). Liposomes were sized on a System 4700 sub-micro particle analyzer (Malvern). ^{125}I-tyraminylinulin, synthesized according to Sommerman et al.,[9] was added during liposome preparation to make approximately 5 X 10^5 cpm/mouse of entrapped label.

Male ddY mice (3 per group) were injected in the tail vein (0.5 mg lipids/mouse). Data were expressed as percent of the injected dose accumulated per organ. The radioactivity accumulated in the lungs, heart and kidneys did not exceed 3% of the injected dose.

RESULTS AND DISCUSSION

Six mol% of DSPE-PEGs added to the LUVs in this study were enough to prolong circulation in preliminary experiments (data not shown).

As shown in Fig.1, liposomes composed of DSPC and CH were rapidly taken up by the RES (liver + spleen). By 3 h after injection, these liposomes had been cleared from the blood (16% of dose) and were located in the RES with 42% of the injected dose. On the other hand, LUVs conjugated with PEG showed that a large amount of the liposomes had stayed in the blood circulation by 6 h after the injection. Approximately 40% of the injected dose of LUV with PEG2000 remained in the blood and only 11% was in the RES 6 h after injection. LUV with PEG5000 also showed a high concentration (29% of dose) in the blood and little in the RES. Thus PEG2000 in DSPC/CH LUV prolonged the stay of liposomes more than PEG5000 did.

Table I shows the in vivo blood/RES ratio and the percentage of total remaining PEG-liposomes 3 h after injection. All of the liposomes, PEG1000, 2000, 5000 and 12000, displayed higher blood/RES ratios than the liposomes without PEG. Among these PEG-liposomes, the PEG2000 produced the highest blood/RES ratio (=4.8). PEG12000 produced the lowest ratio (=1.8). As shown in data of percent remaining in Table I, the relatively high stability of liposomes in vivo was in LUVs conjugated with low molecular

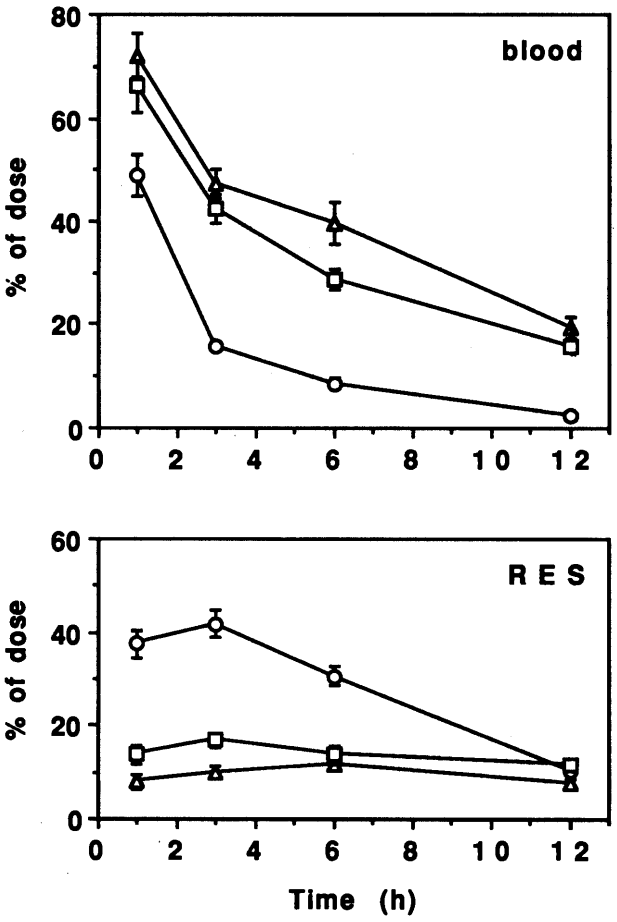


Fig. 1. Blood Clearance and RES Uptake of Liposomes after iv Injection

Data are expressed as mean (n=3). Bar indicates SD.
○ , liposomes composed of DSPC/CH;
△ , liposomes composed of DSPC/CH/PEG2000;
□ , liposomes composed of DSPC/CH/PEG5000.

weight PEG. ^{125}I-tyraminylinulin was used as a liposome marker. Since this compound can be rapidly eliminated to urine after leaking from fractured liposomes in bloodstream, it is a good marker for estimating the liposome stability in vivo.

Table I. The Effect of Molecular Weight of Polyethyleneglycol on Tissue Biodistribution of DSPC/CH LUVs in Mice

Liposomal composition	Blood	Liver (% of dose)	Spleen	Blood /RES	% remaining in vivo
(DSPC/CH)	15.7(0.4)	30.8(0.6)	11.0(1.2)	0.4	61.0(1.7)
PEG 1000	49.0(1.5)	9.2(1.2)	1.8(0.1)	4.4	65.1(2.8)
PEG 2000	47.1(0.7)	8.6(0.8)	1.2(0.2)	4.8	62.2(1.5)
PEG 5000	32.4(2.4)	12.1(0.6)	3.7(0.1)	2.1	55.9(1.9)
PEG12000	34.7(2.2)	16.3(2.4)	2.9(0.3)	1.8	54.1(1.8)
GM1	42.2(1.9)	16.6(1.7)	12.7(0.3)	1.4	76.1(2.7)

LUV (180-200nm in size) composed of DSPC/CH (1:1 in molar ratio) contained 6 mol% PEG or GM1 were injected and their biodistribution were estimated at 3h after iv injection. Results are expressed as means(SD) (n=3).

PEG can provide liposomes to prolong circulation time and to avoid uptake by the liver and spleen. As the present results show, these potential activities depended on the PEG's molecular weight. The order of their activities in DSPC/CH LUV was 1000=2000>5000=12000 in molecular weight. The mechanism of PEG's activities obtained here is not yet clear. It was suggested that PEGs provide a steric barrier which prevents close contact with cells of RES and/or the alleged opsonin molecules[10] in serum. However, further experiments are required to explain the mechanism. Our present data indicate that amphipathic PEGs even with low molecular weights such as 1000 or 2000 can significantly enhance the DSPC/CH liposomes concentration in the bloodstream in a given time. The use of selected and optimized PEGs will play an important role in liposomal drug delivery systems.

ACKNOWLEDGMENT We thank Mr. Suginaka of Nippon Oil & Fat Co., Ltd. for the supply of COATSOME MC-8080, and Mr. Yamano of Inui Trading Co., Ltd. for help with the EXTRUDER.

REFERENCES

1) "Liposomes as drug carriers. Recent trend and progress." ed. by G. Gregoriadis, John Wiley and Sons, Chichester, 1988.
2) K.J. Hwang, "Liposomes" ed. by M.J. Ostro, Marcel Dekker, New York, 1987, p.109.
3) T.M. Allen, T. Mehra, and C. Hansen, Eur. J. Pharmacol., 183, 197 (1990).
4) T.M. Allen and A. Chonn, FEBS Lett., 223, 42 (1987).
5) T.M. Allen, C. Hansen, and J. Rutledge, Biochim. Biophys. Acta, 981, 27 (1989).
6) A. Gabizon and D. Papahadjopoulos, Proc. Natl. Acad. Sci. USA, 85, 6949 (1988).
7) A.L. Klibanov, K. Maruyama, V.P. Torchilin, and L. Huang, FEBS Lett., 268, 235 (1990).
8) F. Szoka and D. Papahadjopoulos, Proc. Natl. Acad. Sci. USA, 75, 4194 (1978).
9) E.F. Sommerman, P.H. Pritchard, and P.R. Cullis, Biochem. Biophys. Res. Commun., 122, 319 (1984).
10) S.M. Moghimi and H.M. Patel, Biochim. Biophys. Acta, 984, 379 (1989).

(Received March 25, 1991)

THE FIRST ISOLATION OF LIGNAN TRI- AND TETRA-GLYCOSIDES[1)]

Junei KINJO, Katsura FUKUI, Hiroyuki HIGUCHI and Toshihiro NOHARA*

Faculty of Pharmaceutical Sciences, Kumamoto University, Oe-honmachi 5-1, Kumamoto 862, Japan

Three new syringaresinol glycosides have been isolated from Albizziae Cortex (the dried stem bark of *Albizzia julibrissin* DURAZZ.). Two of them were the first examples of lignan tri- and tetra-glycosides. Besides, syringaresinol diglucoside which is responsible for the pharmacological effect as tonics was also obtained.

KEYWORDS Albizziae Cortex; *Albizzia julibrissin*; Leguminosae; lignan; (-)-syringaresinol; lignan glycoside; lignan tri-glycoside; lignan tetra-glycoside; syringaresinol diglucoside

The dried stem bark of silktree *Albizzia julibrissin* DURAZZ.(Leguminosae) are used as tonics, to ease the mind and calm the nerves in China. During our studies on the constituents of leguminous plants, we have obtained three new syringaresinol glycosides(**3**~**5**) along with two known glycosides, (-)-syringaresinol-4-*O*-β-D-glucopyranoside(**1**) and 4,4'-bis-*O*-β-D-glucopyranoside(**2**)[2)] from this plant. This paper describes the structures of compounds **3**~**5**.

Compound **3**, a pale yellow amorphous powder, $[\alpha]_D$ -2.6°(MeOH) showed an $[M-H]^-$ ion peak at *m/z* 711 in the negative FAB-MS. An enzymatic hydrolysis of **3** gave compound **3a**, which was identified as (-)-syringaresinol by various tests. In the ^{13}C-NMR spectrum(Table I), the signals of **3** were in accord with those of **1** except for a downfield shift at C-2 (+2.9 ppm) and an upfield shift at C-1 (-2.2 ppm) in the β-D-glucosyl residue. In addition, since signals appeared due to the terminal β-D-apiosyl group, the structure of **3** was determined to be (-)-syringaresinol-4-*O*-β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside. This is a new lignan glycoside, although the pinoresinol derivative which has the same sugar unit was recently reported.[3)]

Compound **4**, a pale yellow amorphous powder, $[\alpha]_D$ -23.6°(MeOH) showed an $[M-H]^-$ ion peak at *m/z* 873 which has one more hexosyl moiety than **3** in the negative FAB-MS. The aglycone **4a** derived from **4** by enzymatic hydrolysis was identical to **3a**. In the ^{13}C-NMR spectrum of **4**, signals due to the lower unit(C-1~9, glc-1~6 and api-1~5) were in agreement with those of **3**, whereas signals due to the upper unit(C-1'~9' and glc'-1~6) were consistent with those of **2**. Therefore **4** was established as (-)-syringaresinol-4-*O*-β-D-apiofuranosyl-(1→2)-β-D-glucopyranosyl-4'-*O*-β-D-glucopyranoside. This is the first isolation of lignan tri-glycoside in nature.

Compound **5**, a pale yellow amorphous powder, $[\alpha]_D$ -30.7°(MeOH), gave **3a** in an enzymatic hydrolysis. On the other hand, the ^{13}C-NMR spectral data for **5** showed only 19 signals which were consistent with those of the lower unit (C-1~9, C-3,5-OMe, glc-1~6 and api-1~5) for **4**. Since **5** showed an $[M-H]^-$ ion peak at *m/z* 1005 in the negative FAB-MS, suggesting that it has one more pentosyl moiety than **4**, the structure of **5** was concluded to be (-)-syringaresinol-

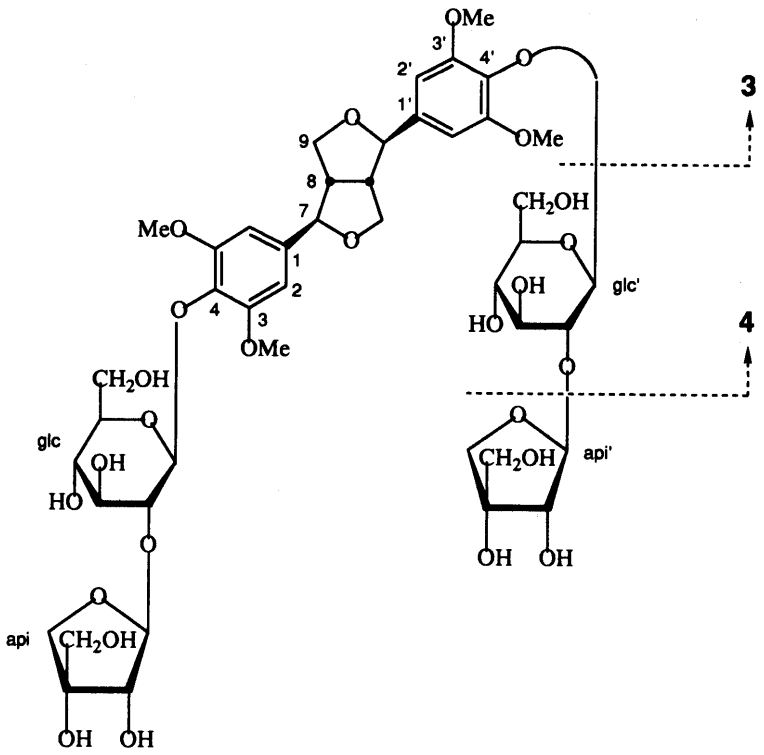
4,4'-bis-*O*-β-D-apiofuranosyl(1→2)-β-D-glucopyranoside. This is the first example of the isolation of lignan tetra-glycoside in nature.

Table I. ^{13}C-NMR Data of Compounds **1**~**5**(δ:ppm, in DMSO-d_6)

	1	**2**	**3**	**4**	**5**
C-1	133.6	133.8	133.3	133.3[a]	133.5
2	104.0	104.3	103.9	104.0[b]	104.1
3	152.5	152.7	152.7	152.7[c]	152.9
4	137.1	137.2	137.2	137.0[d]	137.2
5	152.5	152.7	152.7	152.7[c]	152.9
6	104.0	104.3	103.9	104.0[b]	104.1
7	85.2[a]	85.1	85.2[a]	85.0	85.1
8	53.6[b]	53.6	53.6[b]	53.5	53.6
9	71.2[c]	71.4	71.1[c]	71.3	71.4
1'	131.2	133.8	131.3	133.6[a]	133.5
2'	103.5	104.3	103.5	104.1[b]	104.1
3'	147.8	152.7	147.8	152.5[c]	152.9
4'	134.7	137.2	134.7	137.1[d]	137.2
5'	147.8	152.7	147.8	152.5[c]	152.9
6'	103.5	104.3	103.5	104.1[b]	104.1
7'	85.0[a]	85.1	84.9[a]	85.0	85.1
8'	53.5[b]	53.6	53.6[b]	53.5	53.6
9'	71.1[c]	71.4	71.1[c]	71.3	71.4
OMe	56.3(×2)	56.5(×4)	56.3(×2)	56.3(×4)	56.4(×4)
	55.9(×2)		55.9(×2)		
glc-1	102.7	102.7	100.5	100.5	100.6
2	74.0	74.2	76.9	77.0	77.1
3	76.4	76.5	76.1	76.1	76.2
4	69.8	69.9	69.9	69.9	70.1
5	77.1	77.2	76.7	76.9	76.9
6	60.8	61.0	60.9	60.9	61.0
api-1			108.4	108.4	108.5
2			76.7	76.7	76.8
3			79.2	79.2	79.4
4			73.9	73.9	73.9
5			64.5	64.5	64.7
glc'-1		102.7		102.6	100.6
2		74.2		74.0	77.1
3		76.5		76.4	76.2
4		69.9		69.8	70.1
5		77.2		77.0	76.9
6		61.0		60.8	61.0
api'-1					108.5
2					76.8
3					79.4
4					73.9
5					64.7

a)-d) In each vertical column may be changeable.

Meanwhile, (-)-syringaresinol diglucoside(**2**) from the Araliaceous plant was effective for stressed animals; so it reduces the normal physiological responses of the mammalian body to stress.[4] Very recently, (+)-syringaresinol diglucoside was also reported to have the same pharmacological effect.[5] Since Albizziae Cortex is used as a tonic in China, these glycosides(**2**, **4** and **5**) may be the active complexes in this folk medicine.



5

ACKNOWLEDGEMENTS The authors are grateful to Prof. Tatsuo Yamauchi and Dr. Fumiko Abe, Faculty of Pharmaceutical Sciences, Fukuoka University, for their valuable suggestions.

REFERENCES AND NOTES

1) Part 23 in the series of the studies of the leguminous plants.
2) L. A. Elyakova, A. K. Dzizenko and G. B. Elyakova, *Dakl. Akad. Naauk SSSR*, **165**, 562 (1965); S. D. Jorad, J. J. Hoffmann, J. R. Cole, M. S. Tempesta and R. B. Bates, *J. Org. Chem.*,**45**, 1327(1980); T. Deyama, T. Ikawa and S. Nishibe, *Chem. Pharm. Bull.*, **33**, 3651(1985); T. Miyase, A. Ueno, N. Takizawa, H. Kobayashi and H. Oguchi, *Chem. Pharm. Bull.*,**35**, 3713(1987).
3) F. Abe and T. Yamauchi, *Phytochemistry*, **28**, 1737(1989).
4) W. D. MacRae and G. H. N. Towers, *Phytochemistry*, **23**, 1207(1984) and see references cited therein.
5) S. Nishibe, H. Kinoshita, H. Takeda and G. Okano, *Chem. Pharm. Bull.*, **38**, 1763(1990).

(Received April 15, 1991)

STRUCTURES OF TWO NEW STEROIDAL GLYCOSIDES, SOLADULCOSIDES A AND B FROM *SOLANUM DULCAMARA*[1)]

Tomoyuki YAMASHITA,[a] Tomoko MATSUMOTO,[a] Shoji YAHARA,[a] Naotoshi YOSHIDA[b] and Toshihiro NOHARA*,[a]

Faculty of Pharmaceutical Sciences, Kumamoto University,[a] Oe-honmachi 5-1, Kumamoto 862, Japan and Faculty of Pharmaceutical Sciences, Hokkaido University,[b] Sapporo 060, Japan

The structures of two new steroidal glycosides named soladulcosides A and B, isolated from the aerial parts of *Solanum dulcamara* including new sapogenols, were elucidated as (22*R*,25*R*)-3β,15α,23α-trihydroxy-5α-spirostan-26-one 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside and (22*R*,25*R*)-3β,23α-dihydroxy-5α-spirostan-26-one 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside, respectively.

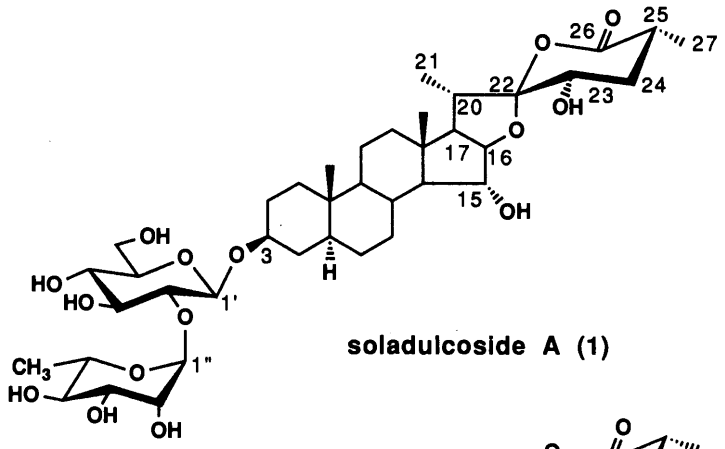
KEYWORDS *Solanum dulcamara*; Solanaceae; steroidal glycoside; soladulcoside

A European crude drug, *Solanum dulcamara* L. has been used to treat cancers, tumors, and warts since the old time of Galen.[2)] The constituents steroidal alkaloid glycosides[2)] and steroidal glycosides[3)] have been known. Now we have obtained two novel spirostanol steroidal glycosides, named soladulcosides A and B, which have a lactone ring in the sapogenol moiety, from the aerial parts of the above plant.

Soladulcoside A (**1**), a white powder, $[\alpha]_D$ -73.8°, showed a peak due to $[M+Na]^+$ at *m/z* 793 ($C_{39}H_{62}O_{15}Na$) in the positive FAB-MS and the IR spectrum showed an absorption band at 1764 cm^{-1} suggesting the presence of a carbonyl group. Acid hydrolysis of **1** provided glucose and rhamnose as sugar components. The ^{1}H-NMR spectrum displayed signals due to two tertiary methyl [δ 0.91(3H, s) and 0.99 (3H, s)] and three secondary methyl[1.16(3H, d, *J*=6.2 Hz), 1.29(3H, d, *J*=6.6 Hz) and 1.77(3H, d, *J*=5.7 Hz)] groups and two anomeric protons [δ 5.07(1H, d, *J*=7.3 Hz) and 6.37(1H, s)], indicating that **1** was a steroid diglycoside. Moreover, the ^{13}C-NMR spectrum showed an α-rhamnopyranosyl-(1→2)-β-glucopyranosyl moiety (Table I), an ester carbonyl signal at δ 180.6, four oxygenated methine signals at δ 76.8, 77.7, 79.0 and 92.0, and a spiroketal carbon signal at δ 109.9, which was closely analogous to tigogenin glycoside.[4)] In the ^{1}H-^{1}H COSY spectrum, there were correlations in a sequence of H_3-21[δ 1.29], H-20[δ 2.78(dq, *J*=6.6, 6.6 Hz)], H-17[δ 2.15(dd, *J*=6.6, 8.4 Hz)], H-16[δ 4.99(1H, dd, *J*=3.9, 8.4 Hz)] and H-15[δ 4.38(dd, *J*=3.9, 10.0 Hz)] and in a sequence of H_3-27[δ 1.16], H-25[δ 2.99(m)], H_2-24[δ 1.94(m) and 2.98(m)] and H-23[δ 4.69(br d, *J*=8.8 Hz)]. The evidence indicated two hydroxyl groups attached to C-15 and C-23 on tigogenin. All carbon signals in **1** could be assigned as listed in Table I by the ^{13}C-NMR and ^{1}H-^{13}C COSY, and signals at C-1~13 showed chemical shifts similar to those of tigogenin 3-O-glycoside.[4)] The location of the lactone ring was determined in the ^{1}H-^{13}C long-range COSY spectrum. Cross peaks were observed between H_3-27 and C-26(δ 180.6), and H-25 and C-26, indicating that the lactone ring was in F-ring. NOE's in **1** were observed between H_3-18(δ 0.99) and H-15; H-23 and H-25, and between H_3-27 and H_2-24, showing that the configurations of the two hydroxyl groups at C-15 and C-23 and methyl group at C-25 were α. Moreover, the CD spectrum ($[\Theta]_{222}$ -6.63×10^2) revealed that

the C-22 configuration was *R*.[5] So the structure of soladulcoside A(**1**) was (22*R*,25*R*)-3β,15α,23α-trihydroxy-5α-spirostan-26-one 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside.

Soladulcoside B (**2**), a white powder, $[\alpha]_D$ -79.2°, showed a peak due to $[M+Na]^+$ at *m/z* 923($C_{45}H_{72}O_{18}Na$) in the positive FAB-MS. The IR spectrum showed an absorption band at 1762 cm^{-1}. Acid hydrolysis of **2** yielded rhamnose and glucose. The ^{1}H-NMR spectrum exhibited three anomeric porton signals at δ 4.93(1H, d, *J*=6.2 Hz), 5.85(1H, s) and 6.37(1H, s) together with signals due to two tertiary methyl groups (each 3H, s, δ0.86 and 0.92), four secondary methyl group [each 3H, d, δ 1.20(*J*=7.3 Hz), 1.29(*J*=7.0 Hz), 1.62(*J*=5.9 Hz) and 1.75(*J*=5.9 Hz)]. In the ^{13}C-NMR spectrum, signals due to the C-1~13 and C-19~27 showed chemical shifts similar to those of **1**, but signals due to C-14, C-15, C-16 and C-17 were similar to the chemical shifts of tigogenin, suggesting that the aglycone in **2** should be a 15-dehydroxy derivative of **1**. Signals due to the sugar part in the ^{13}C-NMR spectrum of **2** were superimposable on those of solamargine (solasodine 3-*O*-β-chacotrioside).[6] Consequently, the structure of soladulcoside B (**2**) was determined to be (22*R*,25*R*)-3β,23α-dihydroxy-5α-spirostan-26-one 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside.[7]



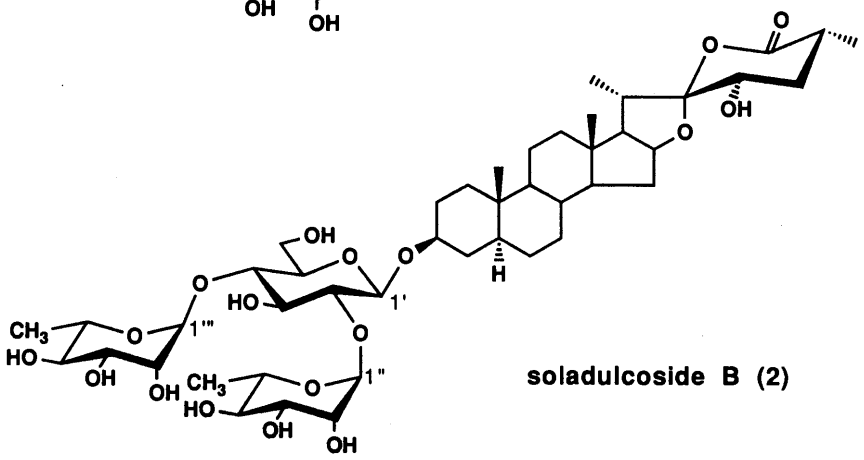


TABLE I. ^{13}C-NMR Data for **1**, **2** and Tigogenin (in pyridine d_5)

	1	**2**	Tigogenin		**1**	**2**
C-1	37.4	37.2	37.5	Glc 1'	99.8	99.8
2	30.0	29.9	32.5	2'	79.6	78.7
3	76.8	76.9	70.6	3'	78.1	77.9
4	34.4	34.4	39.3	4'	71.9	78.0
5	44.6	44.6	45.2	5'	78.3	77.9
6	29.2	28.9	29.1	6'	62.8	61.4
7	32.8	32.4	32.5	Rha 1"	102.2	102.1
8	35.9	35.0	35.4	2"	72.5	72.4[a]
9	54.6	54.3	54.5	3"	72.8	72.6[b]
10	36.1	35.8	35.8	4"	74.1	73.8[c]
11	21.3	21.2	21.1	5"	69.5	69.4[d]
12	40.9	40.1	40.3	6"	18.7	18.4[e]
13	41.4	41.4	40.8	Rha 1'"		102.9
14	60.7	56.3	57.6	2'"		72.5[a]
15	79.0	32.4	32.1	3'"		72.7[b]
16	92.0	82.1	81.1	4'"		74.0[c]
17	60.3	62.9	63.1	5'"		70.4[d]
18	18.1	16.7	16.7	6'"		18.6[e]
19	12.5	12.5	12.5			
20	37.3	37.2	42.0			
21	15.6	15.6	15.0			
22	109.9	110.0	109.2			
23	77.7	77.2	31.9			
24	31.1	31.1	29.3			
25	34.1	34.1	30.6			
26	180.6	180.7	66.9			
27	16.3	16.3	17.3			

Assignments a~e may be interchangeable.

REFERENCES AND NOTES

1) Part 22 in a series of the studies on the solanaceous plants.
2) S.M. Kupchan, S.J. Barboutis, J.R. Knox and C.A. Lau-Can, *Scieces*, **150**, 1827 (1965).
3) W. Guenter and K. Ulrich, *Arch. Pharm*, **316**, 678 (1983).
4) Y. Ding, Y. Chen, D. Wang and C. Yang, *Phytochemistry*, **28**, 2787 (1989).
5) J.P. Jennings, W. Klyne and P.M. Scopes, *Proc. Chem. Soc*, **1964**, 412; *idem*, *J. Chem. Soc*, **1965**, 7211.
6) S.B. Mahato, N.P. Sahu, A. N. Ganguly, R. Kasai and O. Tanaka, *Phytochemistry*, **19**, 2017 (1980).
7) The CD spectrum showed a peak of $[\Theta]_{221} = -1.34 \times 10^3$.

(Received April 15, 1991)

SYNTHESIS AND ANTITUMOR ACTIVITY OF 7-(*N*-GLYCOSYLAMINO)-INDOLO[3,2-*b*]QUINOLINES

Yasuo TAKEUCHI, Ming-rong CHANG, Kuniko HASHIGAKI, and Masatoshi YAMATO*

Faculty of Pharmaceutical Sciencies, Okayama University, Tsushima-naka 1-1-1, Okayama 700, Japan

Novel indolo[3,2-*b*]quinolines (**1d-g**), introduced at the 7-position with an *N*-glycosylamino group, were prepared and their antitumor activities against leukemia P388 in mice were examined. The *N*-Galactopyranosylamino derivative (**1e**) was a much more potent anti-leukemia compound (optimal dose = 25 mg/kg, T/C > 333%, cure 5/6) than lead compound **1a**.

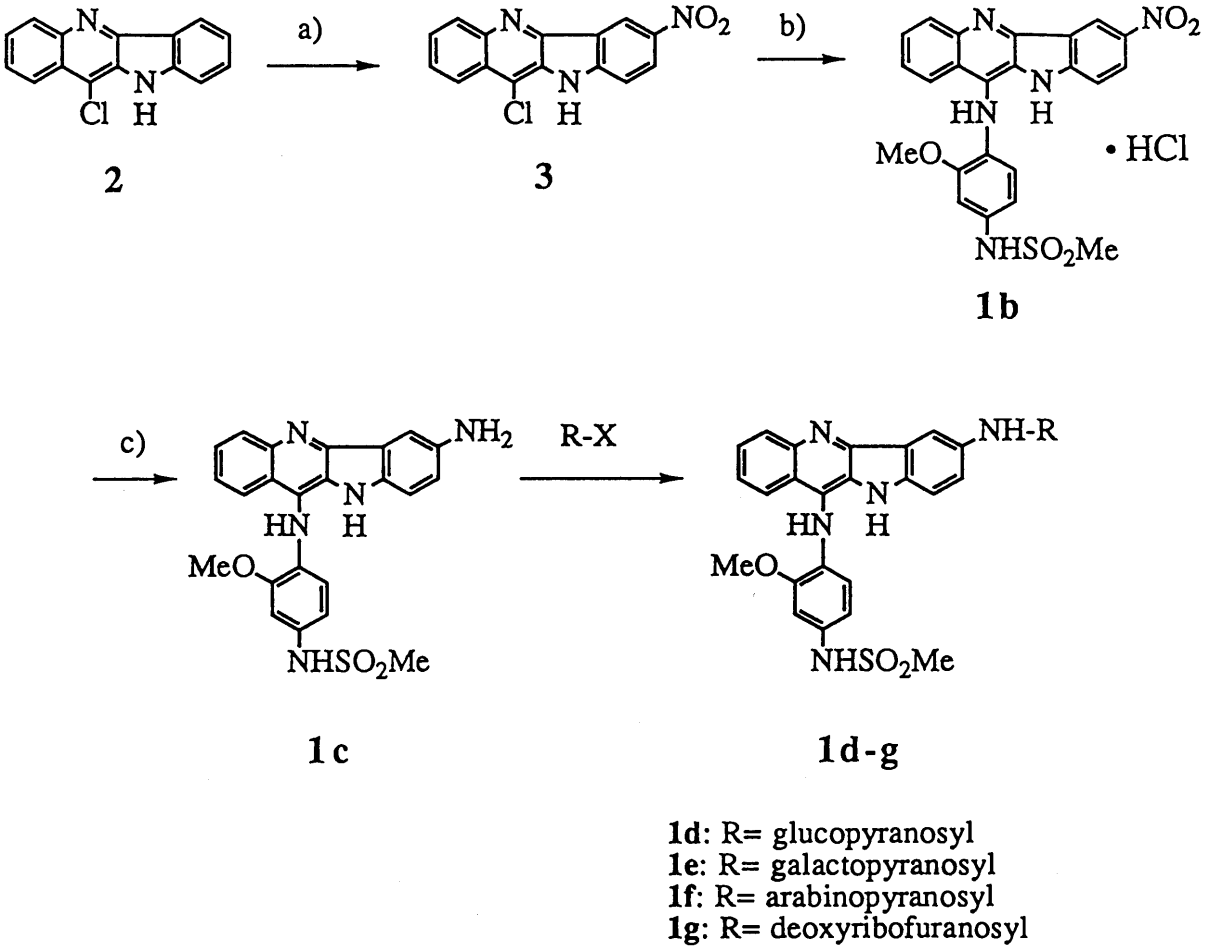
KEYWORDS indolo[3,2-*b*]quinoline; glycosylation; antitumor activity; intercalation; P388 leukemia

We have designed and synthesized novel fused tri- and tetra-cyclic quinolines with various side chains, aiming to develop a new-intercalative antitumor-active compound.[1,2] Among the compounds previously prepared, indoloquinoline derivative (**1a**)containing an *N*-{2-methoxy-4-[(methylsulfonyl)amino]phenyl}-amino group as a side chain shows the most potent activity against leukemia P388 in mice. In earlier studies, of the structure-activity relationship of this series, the slight structural modification of the chromophore moiety lead to dramatic changes in the compound's intercalative ability and in the antitumor activity. Searching for more effective indoloquinoline derivatives, we have synthesized a new type of **1a** containing a glycosylamino group on the chromophore. Glycosylation of a drug generally increases its bioavailability, its penetrability into target cells, and its biological activity.[3] This paper describes the synthesis and antitumor activity of 7-(*N*-glycosylamino)indolo[3,2-*b*]quinoline derivatives (**1d-g**).

The aglycon moiety, 7-aminoindolo[3,2-*b*]quinoline derivative (**1c**), was synthesized from an 11-chloro derivative[4] (**2**) through three steps as shown in Chart 1. Nitration of **2** with nitric acid in acetic acid afforded regioselectively 11-chloro-7-nitroindolo[3,2-*b*]quinoline (**3**) in 85% yield. Refluxing **3** with *N*-(4-amino-2-methoxyphenyl)methanesulfamide hydrochloride[5] in 2-ethoxyethanol gave **1b** in 65% yield. Compound **1b**, on hydrogenation over 10% Pd/C, gave **1c** in 78% yield.

As a glycosyl moiety, we selected the glucopyranosyl, galactopyranosyl, arabinopyranosyl, and deoxyribofuranosyl groups. The typical procedure of the glycosylation of **1c** is as follows: A mixture of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide[6] (560 mg, 1.5 mmol), dry DMF (30 ml), and dry pyridine (2 ml) was stirred at room temperature for 12 h under an argon atomosphere, to which **1c** (360 mg, 0.75 mmol) was added. After stirring at the same temperature for 1 day, the reaction mixture was worked up

in the usual way. The crude *O*-acetyl product was hydrolyzed with a saturated NH_3 in MeOH-H_2O (5:1) at room temperature for 3 days. A standard work-up and subsequent purification by recrystallization from MeOH gave **1e** in a 30% yield as a green crystal. After compounds **1d-f** were again converted to the corresponding *O*-acetyl derivatives, their structures were identified based on their spectral data. Thus, the relative configuration of the 1" and 2" carbons of the glycosylamino group in the *O*-acetyl derivatives of **1d** or **1e**[7] was assigned to the *trans* based on their large coupling constant, $J_{1",2"} \approx 8$ Hz, in their ^{1}H-NMR spectra. However, the relative configuration of the *O*-acetyl derivatives of **1f** and **1g** could not be assigned on the basis of their coupling constants.

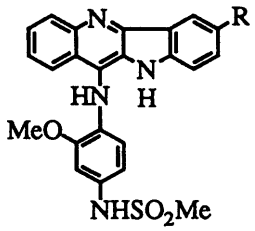


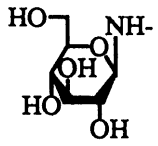
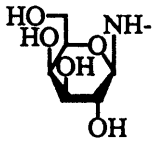
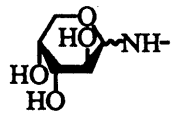
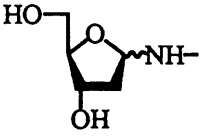
1d: R= glucopyranosyl
1e: R= galactopyranosyl
1f: R= arabinopyranosyl
1g: R= deoxyribofuranosyl

a) HNO_3 (d=1.42), r.t., 12 h; b) *N*-(4-Amino-2-methoxyphenyl)methanesulfonamide hydrochloride, $EtOCH_2CH_2OH$, reflux, 8 h; c) H_2, Pd-C, AcOH, r.t.

These 7-(*N*-glycosylamino)indolo[3,2-*b*]quinolines (**1d-g**) and their related compounds (**1a-c**) were evaluated against leukemia P388 in mice (Table I). Apparently, the introduction of a glycosyl group into the chromophore of lead compound **1a** greatly increased its antitumor potency. In this series, 7-(*N*-galactopyranosylamino)indolo[3,2-*b*]quinoline (**1e**) was the most potent antitumor compound [optimal dose = 25 mg, T/C > 333%, cure 5/6 (at day 30)] against P388.

TABLE I. Antitumor Activity of Indolo[3,2-*b*]quinolines



Compd. No.	R	Dose (mg/kg)[a]	T/C (%)[b]	Cure[c]
1a	H	12.5	203	2/6
		6.25	300	3/6
		3.13	177	
1b	NO_2	50	70	
		25	131	
		12.5	164	1/6
1c	NH_2	50	242	1/6
		25	200	
		12.5	171	
1d		50	90	
		25	145	
		12.5	213	2/6
1e		50	>333	5/6
		25	>333	5/6
		12.5	268	1/6
1f		50	119	
		25	>332	4/6
		12.5	290	
1g		50	185	
		25	140	
		12.5	114	

a) The dose listed was given i.p. once a day on days 1 and 5. b) T/C>120%, active. c) The cure rates were observed at day 30.

REFERENCES AND NOTES

1) M. Yamato, Y. Takeuchi, K. Hashigaki, Y. Ikeda, M-r. Chang, K. Takeuchi, M. Matsushima, T. Tsuruo, T. Tashiro, T. Tsukagoshi, and Y. Yamashita, *J. Med. Chem.*, 32, 1295 (1989).
2) M. Yamato, Y. Takeuchi, M-r. Chang, K. Hashigaki, T. Tsuruo, T. Tashiro, and S. Tsukagoshi, *Chem. Pharm. Bull.*, 38 3048 (1990).
3) T. Honda, M. Kato, M. Inoue, T. Shimamoto, K. Shima, T. Nakanishi, T. Yoshida, and T. Noguchi, *J. Med. Chem.*, 31, 1295 (1988).
4) K. Gorlitzer and J. Weber, *Arch. Pharm.*, 314, 852 (1981).
5) B. F. Cain, G. J. Atwell, and W. A. Denny, *J. Med. Chem.*, 18, 1111 (1975).
6) H. Paulsen and C. Kolar, *Chem. Ber.*, 112, 3190 (1979).
7) *O*-Tetraacetate of **1e**; mp 193—195°C (decomp.). IR (Nujol): 3600, 3380, 1760, 1740 cm^{-1}. ^1H-NMR (60 MHz, $CDCl_3$: DMSO-d_6:D_2O = 10:1:1) δ: 1.96, 2.01, 2.08, 2.14 (each 3H, each s, $COCH_3$), 2.89 (3H, s, SO_2CH_3), 4.00 (3H, s, OCH_3), 3.81—4.36 (3H, m, 5"H and CH_2), 5.12—5.49 (3H, m, 2"H, 3"H, and 4"H), 5.60 (1H, d, J = 8 Hz, 1"H), 6.30 (1H, d, J = 8 Hz, 5'H), 6.74 (1H, dd, J = 2 and 8 Hz, 6'H), 6.88—7.75 (5H, m), 7.93—8.55 (3H, m).

(Received April 18, 1991)

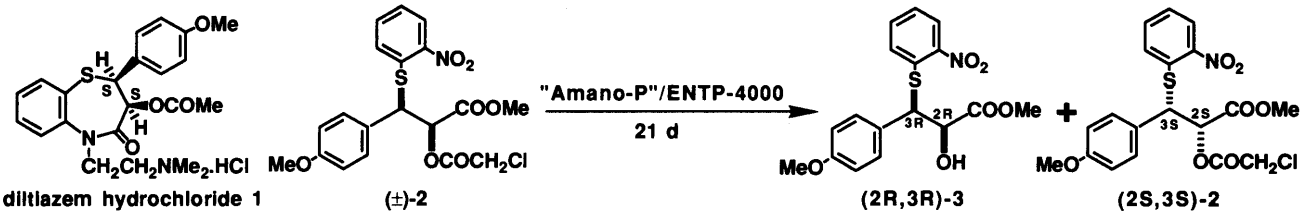
A LIPID-LIPASE AGGREGATE AS A NEW TYPE OF IMMOBILIZED ENZYME

Hiroyuki AKITA,*,[a] Isao UMEZAWA,[b] Hiroko MATSUKURA[b] and Takeshi OISHI*,[b]
School of Pharmaceutical Sciences, Toho University,[a] 2-2-1, Miyama, Funabashi, Chiba 274, Japan
Riken Institute (The Institute of Physical and Chemical Research),[b] 2-1, Hirosawa, Wako-shi, Saitama 351-01, Japan

Lipid-lipase aggregate was prepared by mixing an aqueous solution of lipase with a benzene solution of synthetic phospholipids. The precipitated solid aggregate catalyzed effectively an asymmetric hydrolysis of water-insoluble (±)-α-acyloxy ester 2 in water-saturated isopropyl ether producing (2S,3S)-2, a key intermediate for the medicinally active diltiazem hydrochloride 1.

KEYWORDS asymmetric hydrolysis; water-insoluble substrate; α-acyloxy ester; lipid-aggregated lipase; diltiazem hydrochloride

It is highly advantageous when an enzymatic reaction can be carried out in organic solvents. Immobilizing an enzyme is apparently a method of choice for protecting them from denaturation by organic solvents. In fact, (2S,3S)-2, the important chiral intermediate for medicinally active diltiazem hydrochloride 1, was obtained with high chemical (48%) and optical (98% ee) yields when (±)-α-acyloxy ester 2 was exposed to hydrolysis using lipases immobilized with photo-crosslinkable resin prepolymer (ENTP-4000) in a water-saturated mixture of isooctane and benzene (10:3).[1] However, there is a serious drawback in this hydrolysis that the reaction proceeds extremely slowly and requires 21 days to complete. We now report a new type of the immobilized enzyme, a lipid-lipase aggregate, which catalyze the same asymmetric hydrolysis of (±)-2 much more efficiently than lipases immobilized with ENTP-4000 in water-saturated isopropyl ether.

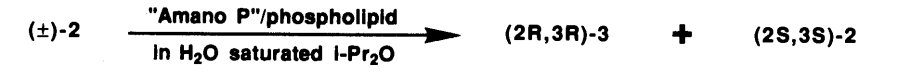


Several ways to minimize the denaturation of enzymes in organic solvent have been reported. Among them, a method using the reversed micellar system was appeared to be promising.[2,3] Recently, a lipid-coated lipase was reported to show a high activity for the synthesis of di- and tri-glycerides in organic solvents.[4] One advantage of the use of the reversed micellar system is that it is possible to design the structure of amphiphiles freely. Since our interest is in the asymmetric hydrolysis of esters, it is highly advantageous to avoid amphiphiles having ester linkages in their molecules. Thus, several phospholipid analogues (4-7)[5] having dihexadecyl ether chains instead of the usual fatty acid ester linkages were synthesized and used to prepare aggregates with lipases.

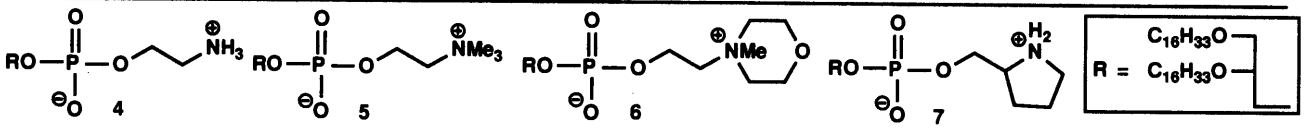
A typical procedure for the preparation of the lipid-lipase aggregate is as follows. A mixture of lipase "Amano P" (100 mg) from Pseudomonas sp. in water (5 ml) and dialkyl phospholipid analogues (50 mg) in benzene (40 ml)[6] was sonicated for 30 min at 0°C. The resulting precipitate was centrifuged at

3000 X g and the solvent was decanted. The residual precipitate was dried under reduced pressure to provide the dry aggregate. The amount of the dry aggregate depended on the dialkyl phospholipid analogues used. It ranged from 20 to 80 mg. The lipid-lipase aggregate thus obtained was insoluble in most organic solvents and aqueous media contrary to the lipid-coated lipase prepared by Okahata et al. which are freely soluble in organic solvents. Taking into account their insoluble properties in organic solvents, the present aggregate should be placed under the category of a new type of the immobilized enzyme. Their precise nature is now being examined.

The asymmetric hydrolysis of (±)-2 was investigated using the synthetic lipid-lipase aggregates in water-saturated isopropyl ether. The progress of the reaction was monitored by HPLC analysis using chiral column (CHIRALCEL OD). Selected data of the asymmetric hydrolysis are shown in Table .



Entry	"Aggregate" (mg)	Substrate (mg)	Time (h)	Product (yield; %)(optical purity; % ee)	
1	"Amano P"/ 4 (5)	(10)	(72)	(2R,3R)-3 (44.9)(>99)	(2S,3S)-2 (45.9)(99)
2	"Amano P"/ 5 (5)	(10)	(72)	(2R,3R)-3 (44.7)(>99)	(2S,3S)-2 (52.5)(87)
3	"Amano P"/ 6 (5)	(10)	(72)	(2R,3R)-3 (47.0)(>99)	(2S,3S)-2 (51.4)(93)
4	"Amano P"/ 6 (50)	(100)	(72)	(2R,3R)-3 (47.0)(97)	(2S,3S)-2 (50.0)(94)
5	"Amano P"/ 7 (5)	(10)	(48)	(2R,3R)-3 (50.0)(98)	(2S,3S)-2 (49.7)(>99)



The most exciting feature in the present experiments is that the reaction proceeded rapidly when these newly prepared immobilized lipases were used, compared to the previous cases where lipases immobilized with ENTP-4000 were used. For example, in entry 5, the hydrolysis was completed within 2 days and (2R,3R)-3 was produced with high chemical (50%) and optical (98% ee) yields. The desired (2S,3S)-2 remained unaffected by these conditions. The chemical (49.7%) and optical (>99% ee) yields of the remaining (2S,3S)-2 were also high.

The lipase "Amano P" immobilized by the aggregate formation with phospholipid analog were high active even in water-saturated diisopropyl ether. However, the relation between the structure of the amphiphile and a catalytic activity of the aggregate has not been fully elucidated. Further exploration of amphiphiles having a different type of a hydrophilic portion as well as a long-chain ether linkage would lead to a still more effective aggregate. Investigation along this line and an application of the present immobilization method to a wide variety of enzymes are now in progress.

ACKNOWLEDGEMENT The authors are grateful to TANABE SEIYAKU CO., LTD for FAB-MS measurement of the synthetic amphiphiles.

REFERENCES AND NOTES

1) H. Akita, Y. Enoki, H. Yamada, and T. Oishi, Chem. Pharm. Bull., 37, 2876 (1989).
2) P. Luthi and P. L. Luisi, J. Am. Chem. Soc., 106, 7285 (1984).
3) S. Morita, H. Narita, T. Matoba, and M. Kito, J. Am. Oil Chem., 61, 1571 (1984).
4) Y. Okahata, Y. Fujimoto and K. Ijiro, Tetrahedron Lett., 29, 5133 (1988); Y. Okahata and K. Ijiro, Chem. Commun., 1988, 1392.
5) Satisfactory analytical data were obtained for all new compounds. The details will be described elsewhere.
6) Since phospholipid analogues were insoluble in water, benzene was used as a solvent.

(Received April 18, 1991)

A NEW COMPLEX COMPOSED OF NICOTINAMIDE AND OCTADECANOIC ACID

Shoko YOKOYAMA,*[,a] Fumio UEDA,[b] and Tadao FUJIE [a]

Kyoritsu College of Pharmacy,[a] 1-5-30, Shibakoen, Minato-ku, Tokyo 105, Japan and Research Laboratory, Kawai Seiyaku Co., Ltd.,[b] 6-3-5, Nakano, Nakano-ku, Tokyo 164, Japan

A new crystalline complex composed of nicotinamide (NAA) and octadecanoic acid (C18) was isolated. The composition was determined by elementary and quantitative analysis. It was found that the stoichiometry of the complex was $(C18)_6(NAA)_5$.

KEYWORDS nicotinamide; octadecanoic acid; complex; elementary analysis; melting point; fatty acid

It has already been reported [1] that nicotinamide (NAA) forms a crystalline complex with octadecanoic acid (C18) whose molar ratio is 1:1. When the release of NAA from the 1:1 complex C18-NAA was examined in a JP XI dissolution test apparatus in JP XI disintegration test medium No. 1 (pH 1.2) at temperature intervals of 5 °C from 37 °C, it was found by a mere chance that NAA did not released more than 16% at temperatures below 22 °C: as an example, the released NAA at 22 °C was gradually reached 16% at 150 minutes, thereafter NAA did not released more than 16% even when the measurement continued for 5 hours. This indicated that there is another stable compound. So we isolated the new complex, and its physicochemical properties were investigated.

First, we tried to isolate a new complex by releasing NAA from C18-NAA. The accurate molar ratio of C18 to NAA in the C18-NAA used in the measurement was 1.00 : 1. NAA was released from C18-NAA in a JP XI dissolution test apparatus in JP XI disintegration test medium No. 1 at 22 °C, the particulars for the procedure was the same as that used [2] for the release of thiamine disulfide (TDS) from the TDS-fatty acid (FA) complex, $(FA)_6(TDS)$. The concentration of released NAA was determined spectrophotometrically at a wavelength of 261 nm. After 16% of the NAA had been released and it had been confirmed that not more than 16% of the NAA was released, the solid residue was collected by filtration, washed with distilled water, and sufficiently dried. This residue melted within a narrow temperature range of 87～89 °C, and no substances which melt at 69～71 °C (melting point of C18), 83～85 °C (melting point of C18-NAA) or 128～129 °C (melting point of NAA) were found. This indicated the existence of a single new compound. Furthermore, this indicated that the new compound is more thermostable than C18-NAA.

The molar ratio of C18 to NAA in the new compound was found to be 1.20 : 1 by the previously described [1] quantitative analysis of C18 and NAA in the compound. This molar ratio is a relative one based on the mole number of NAA. The 1.20 : 1 ratio can be converted to 6.00 : 5. Furthermore, it was confirmed that the stoichiometry of the new compound was expressed as $(C18)_6(NAA)_5$ by elementary analysis as shown in Table I.

On the other hand, the 6:5 complex $(C18)_6(NAA)_5$ could be obtained purely as a crystal by dissolving 3.25 g of C18 and 1.00 g of NAA in 200 ml of 1,2-dichloroethane. The solution was set aside to crystallize at not 35 °C [1] but at 20～25 °C. The concentrations of C18 and NAA were 5.71×10^{-2} mol dm^{-3} and 4.09×10^{-2} mol dm^{-3}, respectively. The molar ratio of C18 to NAA in the preparation conditions was 1.40 : 1.

Table I. Elementary Analysis of $(C18)_6(NAA)_5$ Obtained by the Release of NAA from C18-NAA

Formula	Analysis (%)	C	H	N
$(C_{18}H_{36}O_2)_6(C_6H_6N_2O)_5$	Calc.	71.52	10.70	6.05
	Found	71.47	10.81	6.01

The extra C18 does not participate in the formation of the $(C18)_6(NAA)_5$. It exists as a monomeric or a dimeric species, as it is in equilibrium in 1,2-dichloroethane as a monomer $\rightleftharpoons$ dimer $\rightleftharpoons$ hexamer.[3] The results of the elementary analysis thus obtained $(C18)_6(NAA)_5$ are shown in Table II.

Table II. Elementary Analysis of $(C18)_6(NAA)_5$ Obtained from the 1,2-Dichloroethane Solution of C18 and NAA

Formula	Analysis (%)	C	H	N
$(C_{18}H_{36}O_2)_6(C_6H_6N_2O)_5$	Calc.	71.52	10.70	6.05
	Found	71.63	10.65	6.04

A question may arise: why was the thermostable complex $(C18)_6(NAA)_5$ not obtained in the first place? This is considered to be related to the temperature for the formation of the complexes: C18-NAA may be formed at a higher temperature and $(C18)_6(NAA)_5$ may be formed at a lower temperature. The formation of the pure complex is easier at a higher temperature than that at a lower temperature to avoid coprecipitating the extra C18, and C18-NAA obtained in the first place.

Apparently the change in composition from C18-NAA to $(C18)_6(NAA)_5$ in aqueous acidic medium at 22°C is not accompanied by the quantitative change of C18 because of the insolubility of C18 in aqueous acidic medium. Possibly the transition of the tilting angle of the crystalline complex, as in the transition of FA between B polymorph and C polymorph may occur.

As described above, the existence of the new complex $(C18)_6(NAA)_5$ was confirmed. Taking into account the formation of $(C18)_6(NAA)_5$ by passing through 16% ($\fallingdotseq$ 1/6) release of NAA from C18-NAA, it is reasonable to regard the 1:1 complex C18-NAA as $(C18)_6(NAA)_6$. Furthermore, it appears that the FA-drug complexes formed in 1,2-dichloroethane have a basic structure which consists of six molecules [3] of FA, although X-ray crystal structure analysis is necessary to determine the structure of the FA-drug complex.

REFERENCES

1) F. Ueda, T. Higashi, Y. Ayukawa, A. Takada, T. Fujie, A. Kaneko, and S. Yokoyama, Bitamin, 62, 669 (1988).
2) S. Yokoyama, F. Ueda, and T. Fujie, Chem. Pharm. Bull., 38, 1819 (1990).
3) S. Yokoyama and T. Fujie, Chem. Pharm. Bull., 38, 2249 (1990).

(Received April 22, 1991)

ESTIMATING TISSUE PERMEABILITY AND OTHER BIOELECTRICAL PARAMETERS USING MEMBRANE VOLTAGE AND SHORT-CIRCUIT CURRENT

Sveinbjörn GIZURARSON,*[1] Shin-ichi TAMURA and Takeshi KURATA
National Institute of Health, Department of Pathology, 2-10-35 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

Evaluating the acute toxic effects of drugs or toxins is based mainly on studies which require the use of light microscopy. Recently, the effects of such substances on biological membranes, such as the nasal membrane, has been studied using the traditional Ussing chambers, which make it possible to study the transepithelial flux of drugs across membranes and to measure some bioelectrical parameters. A model is described, with which the changes in the membrane permeability, for sodium, potassium and chlorine, can be calculated directly, based on values obtained from the Ussing chamber system. Also, an experiment is described for evaluating the toxic effects of the cholera toxin B subunit, by measuring these changes in isolated rabbit nasal mucosa.

KEYWORDS bioelectrical parameter; conductance; model; nasal membrane; permeability; Ussing chamber

INTRODUCTION

The effects of various drugs or toxins on biological tissues, such as the nasal membrane, are of great importance when new drug and vaccine formulations are developed. Recently, the use of Ussing chambers have been used as a tool to measure the toxicity and the transepithelial flux of drugs[2] and toxins[3] across biological membranes, and to evaluate the ability of absorption enhancers to increase this transepithelial flux[2]. At the same time, bioelectrical parameters, such as the transepithelial potential difference (PD), short-circuit current (I_{sc}) and conductance (G_t), can be measured, using this system. The traditional Ussing chambers, as described by Ussing and Zerahn[4], have been widely used to characterize and understand the movements of ions through biological membranes. Now this system may be used to evaluate the immediate or acute toxic effects on tissue membranes, observing changes in PD, I_{sc} and G_t. Furthermore, the trans-epithelial flux of non-ionic molecules and peptides may be analyzed by using radioactive labeled molecules[2] or other sensitive methods[3]. The purpose of this communication is to describe mathematical models, which can be used to evaluate the ion permeability and conductance across tissue membranes, without using isotopes. The measurement of ion permeability before and after adding drugs can be a helpful tool to evaluate their effect on the membrane and in some cases understand their mode of action.

THEORY

Precise calculations of permeability and conductance for ions depend on measuring the movement of respective ions across cell membranes, using radioactive isotopes. Recently, Gordon and Macknight discussed and analyzed various applications of membrane potential equations to calculate bioelectrical parameters[5,6]. When the traditional Ussing chamber system is used, the voltage, as well as the short-circuit current, is measured automatically, then the sodium permeability (P_{Na}) can be calculated directly, using a rearrangement of the Goldman-Hodgkin-Katz flux equation (I)[5] where I_{sc} is the measured short-circuit current, $\phi = FV/RT$ and Na_o and Na_i designate the mucosal and cell sodium concentrations, respectively. V is the absolute membrane potential difference, R is the gas constant, T the absolute temperature in the system and F is the Faraday constant. Sodium transport accounts for almost 90% of the short-circuit current. Knowing P_{Na}, the chlorine permeability (P_{Cl}) can be calculated, by using the following equation (II) where y is

$$P_{Na}=\frac{I_{sc}(e^{\phi}-1)}{F\phi(Na_o e^{\phi}-Na_i)} \quad (I)$$

$$P_{Cl}=\frac{P_{Na}Na_o-P_{Na}Na_i e^{\phi}+I_{sc}\frac{RT}{F^2V}(1-e^{\phi})}{\frac{y}{1+y}(Cl_o e^{\phi}-Cl_i)} \quad (II)$$

$$P_K=\frac{P_{Na}(Na_o-Na_i e^{\phi})+P_{Cl}(Cl_i-Cl_o e^{\phi})}{K_i e^{\phi}-K_o} \quad \text{(III)}$$

$$g=\frac{z^2F^2P}{RT(e^{\phi}-1)^2}(c_o e^{\phi}(e^{\phi}-1-\phi)+c_i(\phi e^{\phi}+1-e^{\phi})) \quad \text{(IV)}$$

the ratio of cation currents ($y=I_{Na}/I_K$), which is 1 in the case of the Na–K–2Cl cotransporter Finally, the potassium permeability (P_K) can be calculated from equation (III). Knowing the permeability values for Na^+, K^+ and Cl^-, the conductance for each ion may be calculated by a derivative of the Goldman–Hodgkin–Katz flux equation (VI)[5], where z is the ionic charge and c_o and c_i are the mucosal and cell ion concentrations, respectively.

EXPERIMENTAL

To test the model, nasal tissue membranes were isolated from New Zealand White rabbits, as previously described[3]. After mounting the tissue in the chambers, the reservoirs were filled with bicarbonate Ringer solution, containing 150, 5 and 127 mM sodium, potassium and chlorine, respectively. The system was maintained at 37°C and equipped with oxygen, throughout the study. Prior to each experiment the electrochemical properties of the tissue membrane was checked. The I_{sc} was continuously measured, except for brief intervals during which the PD was fixed at −10 mV using a current/voltage clamp. Here the G_t was measured and compensated for the fluid between the PD bridge tips and tissue surface. The PD, I_{sc} and G_t were −11 mV, 220 μA/cm^2 and 20 mS/cm^2, respectively. Knowing these values, P_{Na}, P_K, P_{Cl}, g_{Na}, g_K and g_{Cl}, can be calculated using equations (I), (II), (III) and (IV). The results are shown in Table I. After measuring the basic bioelectrical parameters, the tissue may be exposed to a drug or toxin, which may change the permeability and the conductance of the tissue membrane. These changes can be used to evaluate the effect of the respective drug or toxin on the membrane. Table I show the acute effects of 1.8 nmol cholera toxin B subunit on rabbit nasal mucosa, measured 1 h after exposure. As seen in the table, the CTB increases the permeability and conductance of the membrane. This effect may be one of the mechanism for the extreme diarrhoea, coused by cholera.

Table I. Calculated Absolute Bioelectrical Parameters for Normal and Cholera Toxin B Subunit (CTB) Treated Rabbit Nasal Mucosa in Ussing Chamber System.

Bioelectrical parameters	Normal values	CTB treated values
Permeability (× 10^{-5}; cm/s)		
Sodium	2.38	9.9
Potassium	2.58	10.0
Chlorine	8.31	33.3
Conductance (× 10^{-4}; S/cm^2)		
Sodium	3.72	15.5
Potassium	3.50	13.6
Chlorine	13.54	54.3

ACKNOWLEDGEMENTS This work was supported by grant from the Japanese Health Science Foundation and from the National Research Concil of Iceland.

REFERENCES

1) Present address: S. Gizurarson, The Icelandic Drug Delivery Group, LYF hf, Gardaflöt 16–18, 210 Gardabær, Iceland.
2) M.A. Wheatley, J. Dent, E.B. Wheeldon, and P.L. Smith, *J. Contr. Rel.*, **8,** 167 (1988).
3) S. Gizurarson, S. Tamura, T. Kurata, K. Hashiguchi, and H. Ogawa, *Vaccine* (in press).
4) H.H. Ussing, and K. Zerahn, *Acta Physiol. Scand.*, **23,** 110 (1951).
5) L.G.M. Gordon, and A.D.C. Macknight, *J. Membrane Biol.*, **120,** 155 (1991).
6) L.G.M. Gordon, and A.D.C. Macknight, *J. Membrane Biol.*, **120,** 141 (1991).

(Received May 2, 1991)

ANTIVIRAL ACTIVITY OF POLYOXOMOLYBDOEUROPATE PM-104 AGAINST HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

Yoshio INOUYE,*,[a] Yoshiki TOKUTAKE,[a] Tetsuya YOSHIDA,[a] Akihiro YAMAMOTO,[b] Toshihiro YAMASE[c] and Shoshiro NAKAMURA[a]

Hiroshima University School of Medicine,[a] 1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan, Terumo Corporation R and D Center,[b] 1500 Inokuchi, Nakai-machi, Ashigarakami-gun, Kanagawa 259-01, Japan and Research Laboratory of Resources Utilization, Tokyo Institute of Technology,[c] 4259 Nagatsuta, Midori-ku, Yokohama 227, Japan

A polyoxomolybdoeuropate PM-104 $(NH_4)_{12}H_2[Eu_4(MoO_4)(H_2O)_{16}(Mo_7O_{24})_4]\cdot 13H_2O$ was found to be a potent inhibitor of the growth of human immunodeficiency virus type 1 (HIV-1), a causative agent of acquired immunodeficiency syndrome (AIDS). On the basis of TI_{50} [median cytotoxic concentration (CC_{50})/median effective concentration (EC_{50})], the *in vitro* anti-HIV-1 activity of PM-104 is favorably comparable to that of a heteropolyoxotungstate PM-19 $K_7[PTi_2W_{10}O_{40}]\cdot 6H_2O$, which is one of the most potent HIV-1 inhibitors among the polyoxometalates so far tested. The heteropolyoxomolybdate with a potent anti-HIV-1 activity is introduced for the first time in this communication.

KEYWORDS heteropolyoxomolybdate; heteropolyoxotungstate; human immunodeficiency virus type 1 (HIV-1); acquired immunodeficiency syndrome (AIDS); antiviral agent

Polyoxometalates are condensed oligomeric aggregate anions of transition-metal oxide, usually with their d^0 electronic configurations. The principal units that make up most polyoxometalates are MO_6 octahedra, with metal atoms (M) being surrounded by six oxygen atoms. Due to the energetic and structural factors, M is usually Mo or W, less frequently V, Nb or Ta, or mixtures of the elements. The MO_6 units in polyoxometalates can be linked together by a single oxygen atom (corner-sharing) or two oxygen atoms (edge-sharing). The polyoxometalates are further subcategorized into iso- and hetero-polyoxometalates. The former consist only of metal and oxygen atoms. The latter contain one or more "heteroatoms" in the molecule in addition to the metal and oxygen atoms. The polyoxometalates consisting of molybdenum and oxygen atoms are called polyoxomolybdates and those containing tungsten atoms instead of molybdenum atoms are called polyoxotungstates.

Some heteropolyoxotungstates, *i.e.*, $[SiW_{12}O_{40}]^{4-}$, $[BW_{12}O_{40}]^{5-}$, $[P_2W_{18}O_{62}]^{6-}$, $[As_2W_{18}O_{62}]^{6-}$ and $[Sb_9W_{21}O_{86}]^{19-}$, are potent inhibitors of cellular, bacterial and viral DNA and RNA polymerases and in non-toxic doses have antiviral effects both *in vitro* and *in vivo*.[1-11] HPA 23 $(NH_4)_{17}Na[NaSb_9W_{21}O_{86}]$ inhibited the transformation of mouse embryonic fibroblasts by murine sarcoma virus and the reverse transcriptase of murine leukemia virus,[2] and reduced the development of the disease caused by Friend leukemia or Moloney murine sarcoma virus.[3] HPA 23 was used in clinical trials for AIDS patients in both France and the U.S.A. without any clinical benefit. Later, it was proved that HPA 23 has little effect on the growth of HIV-1 *in vitro*.[12,13] Recently, we observed marked inhibition of the growth of Herpes simplex virus (HSV) and HIV-1 by some Keggin polyoxotungstates such as PM-19 $K_7[PTi_2W_{10}O_{40}]\cdot 6H_2O$ and PM-48 $K_{13}[Eu(SiW_{11}O_{39})_2]\cdot 30H_2O$.[13,14,15] Unlike polyoxotungstates, polyoxomolybdates seemed to be devoid of antiviral activity,[13,16] though there was antitumor activity in certain polyoxomolybdates, *e.g.*, PM-8 $(NH_3Pr^i)_6[Mo_7O_{24}]\cdot 3H_2O$.[17]

In our screening of various kinds of polyoxomolybdates for anti-HIV-1 substances, only PM-104 showed a potent activity; this is the first example of polyoxomolybdate with anti-HIV-1 potential.

PM-104 was prepared as reported previously.[18] The assay for the growth of HIV-1 was based on the bioassay system established by Harada *et al.* using HTLV-I-carrying MT-4 cells.[19] Briefly, MT-4 cells were infected for 1 h with HTLV-III_B, a standard strain of HIV-1, at the multiplicity of infection (moi) of 0.01. The cells were rinsed and resuspended in a fresh medium at 2.5×10^4 cells/ml. The 200-μl portions of the cell suspension were transferred to the wells of a flat-bottomed 96-well microtiter tray. After six-day culture in the absence or presence of various concentrations of test compounds, the cell viability was monitored by the absorbance at 595 nm attributed to the formazane dye formed by the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) by viable cells. Virus-specific antigens were assayed by an indirect immuno-fluorescence (IF) technique using serum from a patient with hemophilia and FITC-conjugated affinity-purified goat anti-human IgG.

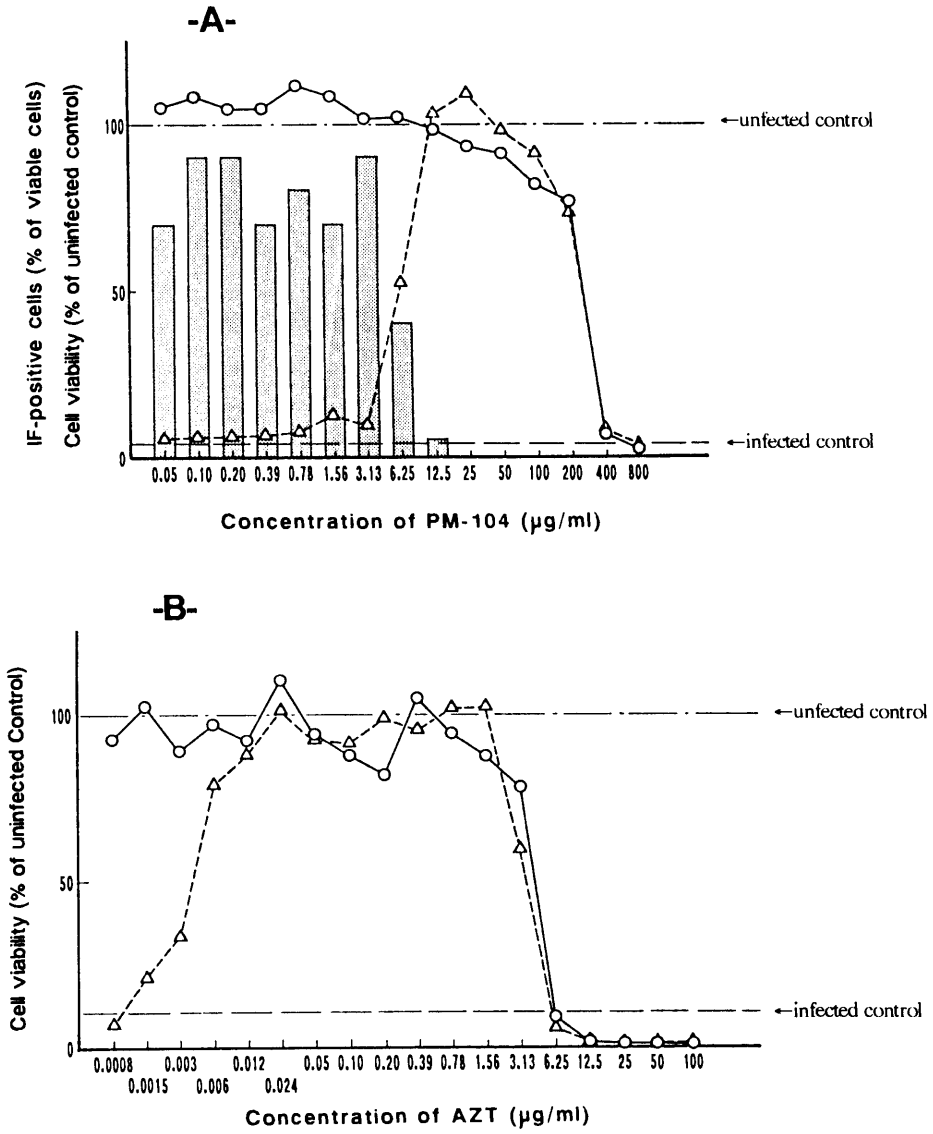


Fig. 1. Effects of PM-104 and AZT on the Growth of HIV-1 in MT-4 Cells

MT-4 cells were infected with HIV-1 (moi = 0.01) for 1 h. The drug was added to the culture and the cell number was adjusted at 5×10^3 cells/well in a total volume of 200μl. The cell viability and the percentage of IF-positive cells were measured 6 days after the viral infection.
O———O : Uninfected and drug-treated culture, Δ———Δ : infected and drug-treated culture, ▭ : IF-positive cells.

Fig. 1A shows the effect of PM-104 in reversing the cytopathic effect of HIV-1 on MT-4 cells, partially at 6.25 μg/ml and completely at concentrations higher than 12.5 μg/ml. There was not significant toxicity up to a concentration of 200 μg/ml. Since 3'-azido-3'-deoxythymidine (AZT) for treatment of AIDS is officially approved in most nations in the world, it was used as a reference compound. The results shown in Fig. 1B were obtained under the assay conditions described above. On the basis of TI_{50} values, AZT is by far superior to PM-104. As in the case of PM-19,[13] blockade of the life cycle of HIV-1 by PM-104 may exist at one of the early steps such as binding to cell surface receptor and uncoating (data not shown); these steps are completed during 1-h viral infection. In contrast, AZT works as a chain terminater in a DNA synthesis that starts several h after viral infection.[20] Therefore, comparing the results for both compounds should be done with care; AZT blocks even primary infection that is already insusceptible to PM-104 treatment under the present experimental conditions.

Besides severe side effects, patients treated with AZT for a long period exceeding 6 months often develop resistance to the drug.[21] Under these circumstances, the search for non-nucleoside antivirals are becoming more important than ever. Polyoxometalates can afford a variety of compounds, with biological activities remaining to be evaluated. The details of the screening of polyoxomolybdates for anti-HIV-1 substances, the mechanism by which PM-104 inhibits the growth of HIV-1 *in vitro* and the structure-activity correlation will be published elsewhere.

REFERENCES

1) D. Haapala, C. Jasmin, F. Sinoussi, J.-C. Chermann and M. Raynaud, *Biomedicine*, 19, 7 (1973).
2) C. Jasmin, N. Raybaud, J.-C. Chermann, D. Haapala, F. Sinoussi, C. Boy Loustau, C. Bonissol, P. Kona and M. Raynaud, *Biomedicine*, 18, 319 (1973).
3) C. Jasmin, J.-C. Chermann, G. Herve, A. Teze, P. Souchay, C. Boy-Loustau, N. Raybaud, F. Sinoussi and M. Raynaud, *J. Nat. Cancer Inst.*, 53, 469 (1974).
4) B. Schonfeld, G. Steinheider and O. Glemser, *Z. Naturforsch.*, 30b, 959 (1975).
5) J.-C. Chermann, F.C. Sinousi and C. Jasmin, *Biochem. Biophys. Res. Commun.*, 65, 1229 (1975).
6) J. Fischer, L. Ricard and R. Weiss, *J. Am. Chem. Soc.*, 98, 305 (1976).
7) D.V. Ablashi, D.R. Twardzik, J.M. Easton, G.R. Armstrong, J. Luetzeler, C. Jasmin and J.-C. Chermann, *Europ. J. Cancer*, 13, 713 (1977).
8) N. Larnicol, Y. Augery, C. Le Bousse-Kerdiles, V. Degiorgis, J.-C. Chermann, A. Teze and C. Jasmin, *J. Gen. Virol.*, 55, 17 (1981).
9) M. Herve, F. Barre-Sinoussi, J.-C. Chermann, G. Herve and C.Jasmin, *Biochem. Biophys. Res. Commun.*, 116, 222 (1983).
10) D. Dormont, B. Spire, F. Barre-Sinoussi, L. Montagnier and J.-C. Chermann, *Annales de Institute Pasteur/Virologie*, 136E, 75 (1985).
11) K. Ono, H. Nakane, F. Barre-Sinoussi and J.-C. Chermann, Eur. *J. Biochem.*, 176, 305 (1988).
12) J. Balzarini, H. Mitsuya, E. De Clercq and S. Broder, *Int. J. Cancer*, 37, 451 (1986).
13) Y. Take, Y. Tokutake, Y. Inouye, T. Yoshida, A. Yamamoto, T. Yamase and S. Nakamura. *Antiviral Res.*, 15, 113 (1991).
14) T. Yamse, K. Tomita, Y. Seto and H. Fujita. Proc. the 4th Int. Conf. Polymers in Medicine, Technomic Pub. Company Inc., Lancaster, Penn., (1991), in press.
15) Y. Inouye, Y. Take, Y. Tokutake, T. Yoshida, A. Yamamoto, T. Yamase and S. Nakamura. *Chem. Pharm. Bull.*, 38, 285 (1990).
16) C.L. Hill., M.S. Weeks and R.F. Schinazi. *J. Med. Chem.*, 33, 2767 (1990).
17) a) T. Yamase, H. Fujita and K. Fukushima, *Inorg. Chim. Acta*, 151, 15 (1988); b) K. Tomita, T. Yamase and K. Shishido, *Inorg. Chim. Acta*, 157, 167 (1989); c) T. Yamase and K. Tomita, Inorg. Chim. Acta, 169, 147 (1990).
18) a) H. Naruke and T. Yamase. *Denki Kagaku*, 58, 507 (1990); b) H. Naruke, T. Ozeki and T. Yamase. *Acta. Crystallogr.*, C47, 487 (1991).
19) S. Harada, Y. Koyanagi and N. Yamamoto, *Virology*, 14, 272 (1985).
20) R. Pauwels, K. Andries, J. Desmyter, D. Schols, M.J. Kukla, H.J. Breslin, A.Raeymaeckers, J.V. Gelder, R. Woestenborghs, J. Heykants, K. Schellekens, M.A.C. Janssen, E. De Clercq and A.J. Janssen, *Nature*, 343, 470 (1990).
21) B.A. Lader, G. Darby and D. Richman. *Science*, 243, 1731 (1989).

(Received May 7, 1991)

AN ENANTIOSELECTIVE SYNTHESIS OF (-)-NEONEPETALACTONE

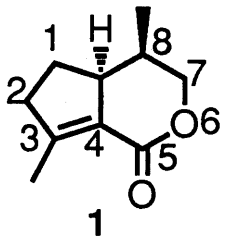
Toshio HONDA,*,[a] Hirohide ISHIGE,[a] Masayoshi TSUBUKI,[a] Koichi NAITO,[b] and Yukio SUZUKI[b]
Institute of Medicinal Chemistry, Hoshi University, Ebara 2-4-41, Shinagawa, Tokyo 142, Japan and Research and Development of Horiuchi Itaro Co., Ltd., Kumegawa-cho 5-29-7, Higashimurayama, Tokyo 189, Japan

(-)-Neonepetalactone (**1**) was synthesized using a rhodium acetate-catalyzad intramolecular cyclization of the γ,δ-unsaturated diazoketone (**7**), easily derived from (-)-carvone, as a key step.

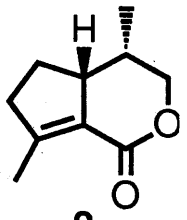
KEYWORDS (-)-neonepetalactone; rhodium acetate; γ,δ-unsaturated diazoketone; (-)-carvone; enantioselective synthesis

Neonepetalactone was isolated[1] from *Actinidia polygama* as a substance attractive to cats. Although the antipodal form (**1**) was first proposed for neonepetalactone, its absolute structure was unambiguously determined to be **2** by total synthesis.[2]

Recently we have developed[3] a novel carbon-carbon bond forming reaction by a rhodium acetate catalyzed cyclization of the γ,δ-unsaturated diazoketone (**3**) to give the cyclohexenone derivative (**4**) stereoselectively. As an extention of this work, we have applied this procedure to the synthesis of (-)-neonepetalactone (**1**).

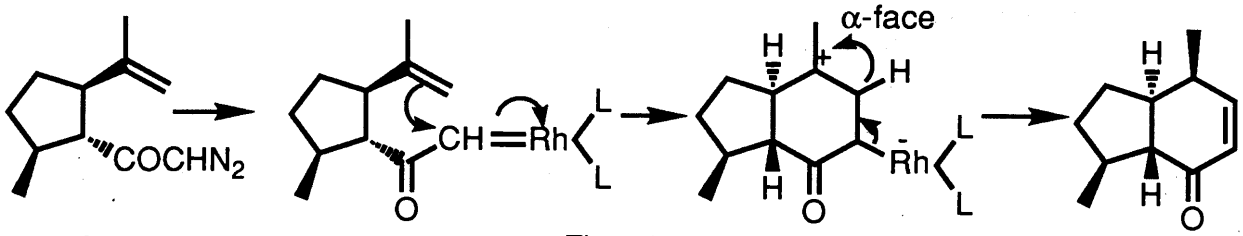


1



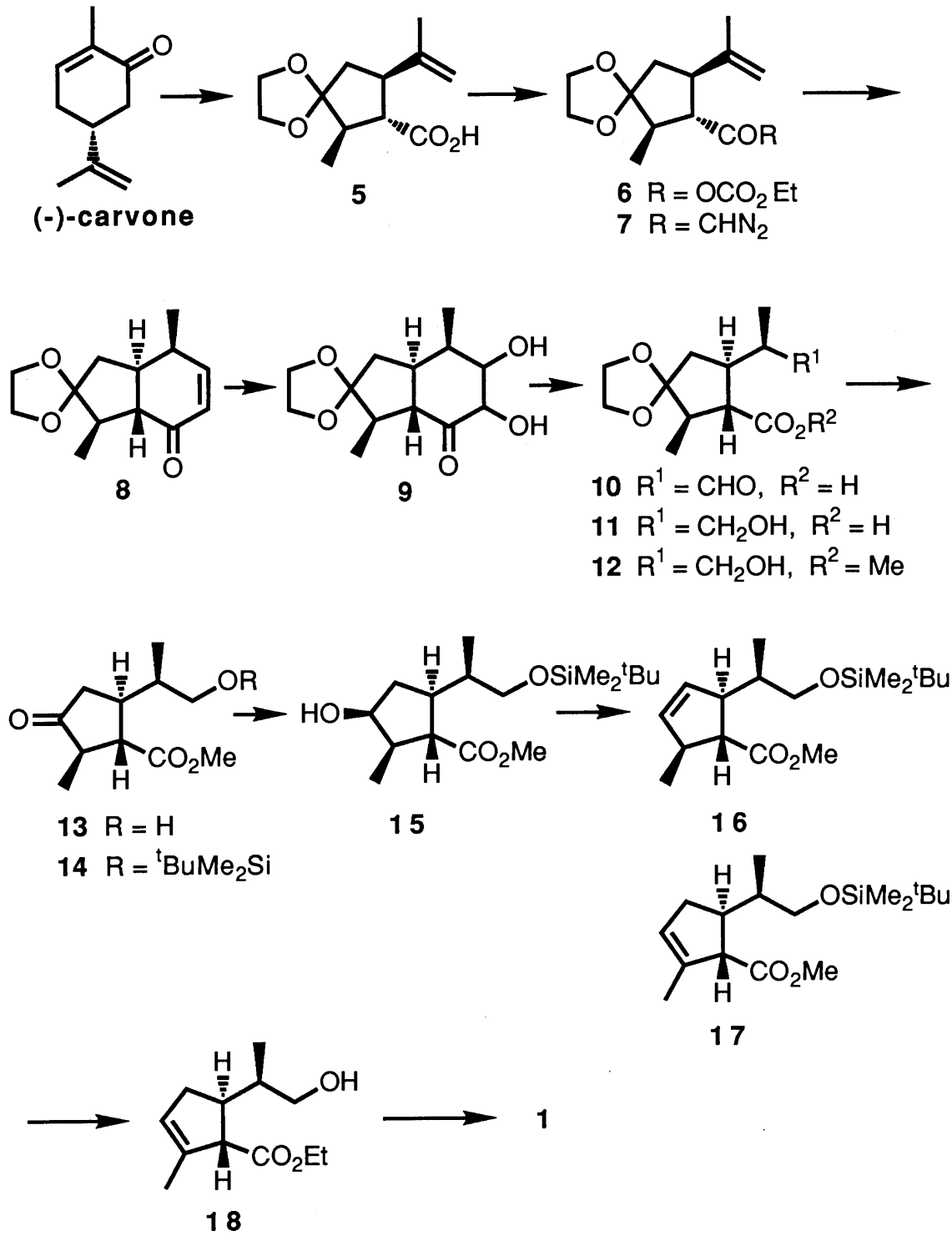
2

The starting carboxylic acid (**5**),[4] easily prepared from (-)-carvone, was converted into the diazoketone (**7**) *via* the mixed anhydride (**6**) by successive treatments of **5** with ethyl chloroformate and triethylamine, and with an excess of ethereal diazomethane. Cyclization of **7** in dichloromethane at room temperature for 1 h using rhodium acetate as a catalyst afforded the desired cyclohexenone derivative (**8**),[5] in 62.8% yield from **5**.



3 Fig. 1 **4**

The principal advantages offered in Fig.1 are not only the formation of a new carbon-carbon bond providing a cyclohexenone ring system but also the ready stereochemical control at the 8 position of neo-nepetalactone during the reductive elimination of the rhodium complex.



Treating of **8** with osmium tetroxide in tetrahydrofuran in the presence of pyridine gave the diol (**9**) which, on exposure with saturated aqueous sodium periodate solution in methylene chloride, furnished the acid (**10**) in 88% yield. Reduction of the formyl group of **10** with sodium borohydride, followed by esterification of the resulting acid (**11**) with an excess of ethereal diazomethane afforded the ester (**12**) in 85.8% yield in two steps. In order to complete the synthesis, introduction of the double bond for **12**

was achieved as follows. Deketalization of **12** by acid treatment gave the ketone (**13**) whose hydroxyl group was then protected as the silyl ether by the usual manner to give the silyl ether (**14**). Reduction of **14** with sodium borohydride yielded the alcohol (**15**) as a single stereoisomer, in 82.3% yield. Its stereochemistry was tentatively assigned to be **15** based on its NMR spectrum. Regioselective dehydration of **15** was first attempted under the various reaction conditions adopting the known procedures for secondary alcohols, such as base treatment of the corresponding tosylate or mesylate, and direct dehydration with phosphoryl chloride-pyridine or thionyl chloride-pyridine, however, none of the reactions gave satisfactory results in terms of the regioselectivity and the conversion yield.

Finally, treating of **15** with methyltriphenoxyphosphonium iodide in hexamethylphosphoramide[6] provided the olefins (**16**) and (**17**), which without separation was subjected to the isomerization of the double bond with rhodium chloride in ethanol in a sealed tube at 100°C to give the olefin (**18**),[7] along with the removal of the silyl group, in 78% yield from **15**. Interestingly, this migration afforded the tri-substituted olefin (**18**) without epimerization at the allylic position, and none of the tetra-substituted olefins were produced under such reaction conditions. Further isomerization of **18** with DBU in refluxing toluene brought about a lactone formation simultaneously to furnish (-)-neonepetalactone, $[\alpha]_D$ -166.6° (c=0.02, $CHCl_3$) {for natural configuration[2] $[\alpha]_D$ +166.8° (c=0.31, $CHCl_3$)}, in 85% yield, as a sole isolated product. Its spectroscopic data were identical with those reported.[2]

Since (+)-carvone is also readily available starting material, the present result constitutes the formal synthesis of natural neonepetalactone.

We are currently exploring this synthesis strategy to synthesize of other types of naturally occurring compounds.

ACKNOWLEDGMENT The present research is supported by Grants-in-Aid for Scientific Research (Grant No. 02670964) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1) T. Sakan, F. Murai, S. Isoe, S. B. Hyeon, and Y. Hayashi, *Nippon Kagaku Zasshi*, **90**, 507(1969).
2) T. Sakai, K. Nakajima, K. Yoshihara, T. Sakan, and S. Isoe, *Tetrahedron*, **36**, 3115(1980).
3) T. Honda, H. Ishige, M. Tsubuki, K. Naito, and Y. Suzuki, *J. Chem. Soc., Perkin Trans.1*, 954 (1991).
4) T. Kametani, Y. Suzuki, C. Ban, and T. Honda, *Heterocycles*, **26**, 1491(1987).
5) IRν(max): 1685 cm^{-1}. ^{1}H-NMR($CDCl_3$)δ: 1.63(3H, d, *J*=7.3 Hz, Me), 1.14(3H, d, *J*=4.9 Hz, Me), 1.75(1H, dd, *J*=12.8 and 12.8 Hz, 7-H), 1.91(1H, dd, *J*=6.7 and 12.8 Hz, 7-H), 5.89(1H, d, *J*=10.4 Hz, 3-H), 6.83(1H, dd, *J*=4.9 and 10.4 Hz, 4-H). MS: 222(M^+). $[\alpha]_D$ +266.6° (c=0.2, $CHCl_3$).
6) R. O. Hutchins, M. G. Hutchins, and C. A. Milewski, *J. Org. Chem.*, **37**, 4190(1972).
7) IRν(max): 1730 cm^{-1}. ^{1}H-NMR($CDCl_3$)δ: 0.87(3H, d, *J*=6.7 Hz, Me), 1.28(3H, t, *J*=7.3 Hz, Me), 1.69(3H, s, Me), 3.20(1H, d, *J*=7.9 Hz, 1-H), 3.45 and 3.54(each 1H, each dd, *J*=6.1 and 11.0 Hz, CH_2OH), 4.19(2H, q, *J*=7.3 Hz, CO_2CH_2), 5.45(1H, m, 4-H). MS: 212(M^+). $[\alpha]_D$ -32.0° (c=0.1, $CHCl_3$).

(Received May 9, 1991)

FIVE NEW NAPELLINE-TYPE DITERPENE ALKALOIDS FROM *ACONITUM LIANGSHANIUM*

Hiromitsu TAKAYAMA,[a] Feng-E WU,[b] Hisano EDA,[a] Kyoko ODA,[a] Norio AIMI,[a] and Shin-ichiro SAKAI*,[a]

Faculty of Pharmaceutical Sciences, Chiba University,[a] 1-33, Yayoi-cho, Chiba 260, Japan and Chengdu Institute of Biology, The Chinese Academy of Sciences,[b] Sichuan, Chengdu 610015, People Republic of China

The structures of five new C20-diterpene alkaloids having the napelline skeleton, *i.e.*, 12-*epi*-lucidusculine (**2**), 12-*epi*-19-dehydronapelline (**8**), 12-*epi*-19-dehydrolucidusculine (**9**), liangshanine (**3**), and liangshanone (**11**), found from the roots of *Aconitum liangshanium*, were determined by spectroscopic analysis and chemical reactions.

KEYWORDS *Aconitum liangshanium*; diterpene alkaloid; structure elucidation; absolute configuration; 12-*epi*-lucidusculine; 12-*epi*-19-dehydronapelline; 12-*epi*-19-dehydrolucidusculine; liangshanine; liangshanone

Our investigation on the alkaloidal constituents of *Aconitum liangshanium* W.Z. Wang, a plant native to Liang-mountain, above an altitude 4500 meters, in China, has led to the isolation and assignments of five new C20-diterpene alkaloids, *i.e.*, 12-*epi*-lucidusculine (**2**) (0.32% of the crude base), 12-*epi*-19-dehydronapelline (**8**) (0.37%), 12-*epi*-19-dehydrolucidusculine (**9**) (0.28%), liangshanine (**3**) (3.63%), and liangshanone (**11**) (0.27%), along with six known alkaloids, 12-*epi*-napelline (**1**) (6.42%), songorine (**10**) (0.21%), aconitine (1.96%), aconine (2.98%), neoline (0.10%), and senbusine A (7.73%). Senbusine A and 12-*epi*-napelline are the main bases of the roots of this plant.

The new alkaloid (**2**) was obtained as colorless prisms, mp 160-164°C, $[\alpha]_D^{21.5}$ -100° (*c*=0.91, $CHCl_3$), and its formula, $C_{24}H_{35}NO_4$, was confirmed by the high resolution mass spectrum (found; 401.2566). The mass spectral fission pattern {*m/z* 401 (M^+, 81%), 358 (M^+-$COCH_3$, 100%)}, and the IR absorption at 1730cm^{-1} showed an acetoxy group. In the ^{1}H-NMR spectrum, in addition to some characteristic signals in C20-diterpene alkaloids having the napelline-type skeleton {N-CH_2CH_3 (δ 0.98, 3H, t, *J*=7.2 Hz), C18-H_3 (δ 0.69, 3H, s), C20-H (δ 3.24, s), C1-H (δ 3.84, dd, *J*=6.0, 7.8 Hz), C17-H_2 (δ 5.15, s and 5.08, d, *J*=2.2 Hz)}, there were a singlet at δ 2.05 (3H, s, $COCH_3$) and a doublet at δ 5.50 (*J*=2.2 Hz, C15-H) . On hydrolysis with 5% KOH-aq. MeOH, **2** gave 12-*epi*-napelline (**1**)[1] {$[\alpha]_D^{11.5}$ -28.7° ($CHCl_3$)}. The signal at C15-H in **1** was upfield 1.3 ppm higher than the corresponding signal of **2**. Therefore, the structure of the new alkaloid (**2**) was concluded to be 12-*epi*-lucidusculine (12-*epi*-15-*O*-acetylnapelline).

The second new alkaloid (**8**) was an amorphous powder, $[\alpha]_D^{23}$ +56.8° (*c*=1.02, $CHCl_3$). The high resolution mass spectrum of **8** showed a molecular ion at *m/z* 357.2314, corresponding to the formula $C_{22}H_{31}NO_3$. This is 2 a.m.u. lower than the corresponding peak in napelline or 12-*epi*-napelline (**1**). The ^{1}H-NMR spectrum of **8** indicated the presence of an N-CH_2CH_3 (δ 1.01, 3H, t, *J*=7.3 Hz), a tertiary methyl (δ 0.82, 3H, s), an exomethylene (δ 5.40, s and 5.21, d, *J*=2.0 Hz) and two hydroxyls (δ 4.28, C15-H, and 4.16, C12-H) in the napelline skeleton. The new alkaloid was characterized by the appearance of the signals at δ 4.01 (d, *J*=5.3 Hz, C1-H) and at δ 3.69 (s, C19-H) in the ^{1}H-NMR spectrum and by the loss of molecule of acrolein (M^+-C_3H_4O, 100%) in the mass spectrum.[2] This indicated the presence of a C1-O-C19 ether linkage. The β-orientation of the hydroxy group at C12 was deduced by the analysis of the coupling patterns of C13-H (δ 2.82, dd, *J*=4.6, 8.8 Hz) and C12-H (δ 4.16, dd, *J*=8.8, 6.1 Hz).[1] The structure of **8** was proved by the chemical conversion from 12-*epi*-napelline (**1**). Compound (**1**) was treated with *N*-bromosuccinimide[3] in dry benzene at 90°C for 3 h to give the 19-dehydro derivative in 56% yield together with 28% of the starting material **1**. Semisynthetic **8** was identical with the natural product in all respects (co-tlc, IR, MS, ^{1}H-NMR, and $[\alpha]_D$).

The new alkaloid (**9**), $[\alpha]_D^{23.5}$ -9.6° (*c*=1.2, $CHCl_3$), was an amorphous powder. The high resolution

mass spectrum of **9** presents the molecular ion at *m/z* 399.2412, corresponding to the formula $C_{24}H_{33}NO_4$. The intensive peak at 1730 cm^{-1} in the IR spectrum and the appearance of a signal at δ 2.15 (3H, s) showed an acetoxy group in **9**. The loss of acrolein (M^+-C_3H_4O, 78%) in the mass spectral fragmentation and the characteristic signals at δ 4.02 (d, *J*=5.2 Hz, C1-H) and δ 3.69 (s, C19-H) in the ^{1}H-NMR spectrum indicated the presence of C1-O-C19 ether bond in the napelline-type compound {N-CH_2CH_3 (δ 1.00, 3H, t, *J*=7.2 Hz), C18-H_3 (δ 0.81, 3H, s), C20-H (δ 2.74, s), C13-H (δ 2.86, dd, *J*=8.5, 4.7 Hz), C12-H (δ 4.18, t-like, *J*=7 Hz), C15-H (δ 5.56, t, *J*=2.2 Hz), C17-H_2 (δ 5.19, 2H, br-s)}. On hydrolysis with 5% KOH-aq. MeOH, **9** gave 12-*epi*-19-dehydronapelline (**8**), which was identical with the natural product (co-tlc, MS, ^{1}H-NMR, and $[\alpha]_D$). In the ^{1}H-NMR spectra, a signal observed at δ 5.56 (C15-H) in **9** was shifted upfield at δ 4.28 in **8**. From these chemical and spectroscopic data, **9** was determined to be 12-*epi*-19-dehydrolucidusculine.

The new alkaloid (**3**), named liangshanine, was an amorphous powder, $[\alpha]_D^{10.5}$ -16.4° (*c*=1.5, $CHCl_3$). The high resolution mass spectrum presents the molecular ion *m/z* 373.2615 ($C_{23}H_{35}NO_3$), which is 14 a.m.u. higher than the corresponding peak in the spectrum of napelline. The ^{1}H-NMR spectrum showed the typical napelline-type signals due to N-CH_2CH_3 (δ 1.04, 3H, t, *J*=6.8Hz), C18-H_3 (δ 0.73, 3H, s), C17-H_2 (δ 5.31, d, *J*=0.8 Hz and 5.09, t like), C12-H (δ 4.16, dd, *J*=4.4, 9.1 Hz), and C15-H (δ 4.18, br-s). Most characteristic of this new compound was the presence of a methoxy group, that was indicated by the ^{1}H-(δ 3.31, 3H, s) and ^{13}C-NMR (δ 55.6) spectra. The placement of the methoxy function at the C1 position was deduced by the mass spectral fission pattern {M^+ (42%), M^+-OMe (100%)}[4] and by the comparison of the chemical shifts of the C1 and C2 positions in **3** with those of 12-*epi*-napelline (**1**) (Table I). The signals of C1-H and OCH_3 were overlapped in the ^{1}H-NMR spectrum of **3**, however, the orientation of the C1 methoxy group was α-equatorial as indicated by the coupling pattern of C1-H (δ 3.01, dd, *J*=9.8, 3.8 Hz) in the ^{1}H-NMR spectrum of the diacetyl derivative of **3**.

To determine the structure of liangshanine indicated by the spectroscopic analysis to be 1-*O*-methyl-12-*epi*-napelline, we synthesized **3** from **1**. The preliminary experiments on the selective acylation of **1**

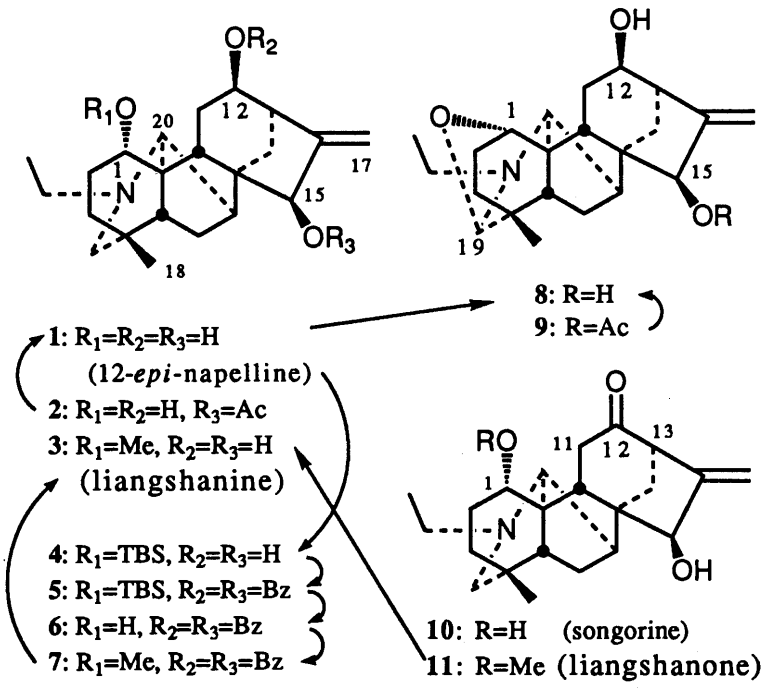


Table I. ^{13}C-NMR Data and Assignments

C	(1)	(3)	(11)
1	69.6	80.7	80.4
2	31.2	25.8	25.8
3	36.3	38.1	37.9
4	34.1	34.5	34.3
5	48.4	50.7	50.4
6	23.6	23.3	22.9
7	44.0	44.6	44.1
8	50.8	51.4	49.8
9	37.6	38.2	35.9
10	52.8	51.4	51.3
11	29.5	28.8	38.0
12	67.0	67.4	210.7
13	44.0	44.4	54.1
14	32.6	32.6	31.7
15	76.8	77.0	77.3
16	154.8	155.4	151.3
17	111.6	111.1	111.4
18	26.2	26.0	26.0
19	57.9	57.0	56.9
20	66.1	66.3	66.4
N-CH_2	51.7	51.2	51.1
CH_3	12.7	13.5	13.6
OCH_3	—	55.6	55.5

Assignments were made by using 2D-NMR technique (δ in $CDCl_3$).

indicated that the C1 hydroxy group was the most reactive of the three hydroxyls. So **1** was treated with 1.5 equiv. of *tert*-butyldimethylsilyl (TBS) chloride in CH_2Cl_2 in the presence of triethylamine at 0°C for 2 h to provide the C_1-OTBS derivative **4** in 57% yield. The remaining hydroxy groups at C12 and C15 in **4** were benzoylated in 70% yield with benzoyl chloride/dimethylaminopyridine in pyridine. Then the silyl protective group in **5** was removed in 78% yield by treating with HF-pyridine in THF to yield the alcohol (**6**). The free hydroxy group at C1 in **6** was methylated in 27% yield with CH_2N_2 in the presence of 0.5 eq of BF_3-Et_2O[5] at room temperature for 6.5 h (44% of the starting material was recovered). Finally, benzoyl esters in **7** were hydrolyzed with 5% KOH in aq. MeOH to furnish 1-*O*-methyl-12-*epi*-napelline. Semisynthetic **3** was identical with natural liangshanine (**3**) in all respects (co-tlc, MS, ^{1}H-NMR, $[\alpha]_D$).

The fifth new alkaloid (**11**) obtained as an amorphous powder, $[\alpha]_D^{22.5}$ -101° (*c*=1.1, $CHCl_3$), was named liangshanone. The high resolution mass spectrum of **11** showed the molecular ion *m/z* 371.2458 ($C_{23}H_{33}NO_3$), which is 2 a.m.u. lower than the corresponding peak in liangshanine (**3**). The ^{1}H-NMR spectrum of **11** showed the characteristic napelline-type signals due to N-CH_2CH_3 (δ 1.06, 3H, t, *J*=6.9 Hz), C18-H_3 (δ 0.75, 3H, s), C17-H_2 (δ 5.29, dd, *J*=1.2, 2.6 Hz, and 5.20, s), and C15-H (δ 4.34, br-s). The presence of the C1-α-OMe group as in **3** was indicated by the appearance of signals at δ 3.28 (3H, s) and δ 3.30 (1H, dd, *J*=10.5, 6.6 Hz) in the ^{1}H-NMR spectrum and by the fragmentation pattern {M^+ (84%), M^+-OMe (100%)} in the mass spectrum. Furthermore, the intensive peak at 1710 cm^{-1} in the IR spectrum and the characteristic signal at δ 210.7 in the ^{13}C-NMR spectrum indicated the presence of a carbonyl function. The position of the carbonyl group in **11** was deduced by comparing the ^{13}C-NMR spectra of **3** and **11** (Table I). Thus, the signals at the C11 and C13, α positions to C12 (δ 210.7, C=O), in **11** were downfield 9.2 and 9.4 ppm, respectively, lower than the corresponding signals of liangshanine (**3**). By reduction with $LiAlH_4$ in THF at room temperature, **11** gave two epimeric alcohols, 1-*O*-methyl-12-*epi*-napelline (**3**) and 1-*O*-methyl-napelline, in 16% and 45% yield, respectively. The former isomer (**3**) was identical with natural liangshanine (**3**). From these spectroscopic data and the chemical reaction, the structure of **11** was assigned to be 1-*O*-methylsongorine.

Songorine (**10**), the absolute configuration was known,[6] and liangshanone (**11**) exhibited very similar CD curves of the carbonyl chromophores.[7] As mentioned above, all of the new alkaloids were chemically related to each other including the specific rotation. So their absolute configuration was established to be the (-)-kaurene type as with other aconite diterpene alkaloids.

Liangshanine (**3**) and liangshanone (**11**) are the first examples of C20-diterpene alkaloids bearing the methoxy group in the molecule.

ACKNOWLEDGEMENT We are grateful to Professors S. H. Hong and A. N. Lao, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, for a generous supply of natural 12-*epi*-napelline and its ^{1}H-NMR and IR spectra.

REFERENCES AND NOTES

1) Z. G. Chen, A. N. Lao, H. C. Wang, and S. H. Hong, *Heterocycles*, **26**, 1455 (1987).

2) a) S. W. Pelletier and S. W. Page, "*Diterpene Alkaloids*" in "*The Alkaloids*", (Specialist Periodical Reports) ed. by J. E. Saxton, The Chem. Soc., London, 1973, Vol. 3, p. 239: b) K. Wada, H. Bando, and T. Amiya, *Heterocycles*, **23**, 2473 (1985).

3) T. Amiya, *Bull. Chem. Soc. Jpn.*, **33**, 644 (1960).

4) O. E. Edwards, "*Diterpene Alkaloids*" in "*The Alkaloids*", (Specialist Periodical Reports) ed. by J. E. Saxton, The Chem. Soc., London, 1971, Vol. 1, p.369.

5) E. Müller and W. Rundel, *Angew. Chem.*, **70**, 105 (1958).

6) T. Okamoto, M. Natsume, Y. Iitaka, A. Yoshino, and T. Amiya, *Chem. Pharm. Bull.*, **13**, 1270 (1965)

7) CD spectra songorine (**10**); (c=0.011, methanol) $[\theta]_\lambda$ (nm): 0 (330), -14100 (297) (negative max.), 0 (259), +13400 (positive max.) (225), +1800 (220). liangshanone (**11**); (c=0.008, methanol) $[\theta]_\lambda$ (nm): 0 (330), -12800 (297) (negative max.), 0 (255), +14500 (224) (positive max.), +1800 (220).

(Received May 10, 1991)

EFFICIENT PREPARATION OF 32-OXYGENATED LANOSTEROL DERIVATIVES

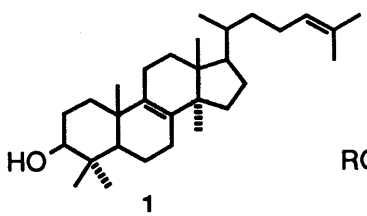
Yukiko TAKANO and Masuo MORISAKI*
Kyoritsu College of Pharmacy, Minatoku, Tokyo 105, Japan

An efficient synthesis of 32-hydroxy- and 32-oxo-lanost-8-en-3β-ol (**2** and **4**) from the 8-ene-15-ketone **7** was described.

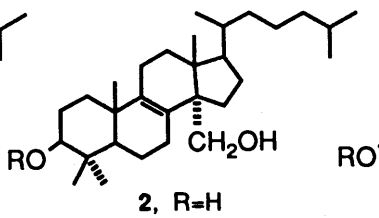
KEYWORDS 32-hydroxylanost-8-en-3β-ol; 32-oxo-lanost-8-en-3β-ol; benzyloxymethylation; 14-demethylase; antimycotic agent; hypocholesterolemic agent

One of the key steps in the biosynthesis of cholesterol (in mammals) and ergosterol (in fungi) is the 14-demethylation of lanosterol (**1**). So 14-demethylase is a potential target for the development of hypocholesterolemic and/or antimycotic agents. This reaction is considered to proceed through the intermediacy of 32-hydroxy- and 32-oxo-lanosterol. To elucidate the precise mechanism of this carbon-carbon bond cleavage reaction, adequate samples of these 32-oxygenated lanosterol derivatives are required. These 32-oxygenated compounds should also be useful for preparing sterol-based inhibitors of 14-demethylase.[1] However, no satisfactory method for their chemical preparation has hitherto been reported.[2] Here we describe an efficient synthesis of Δ^8-24,25-dihydro analogs **2** and **4**, based on the recently reported method of 14α-alkylation of the 15-oxo-8-ene system, which in turn was straightforwardly prepared from the 7-ene derivative.[3]

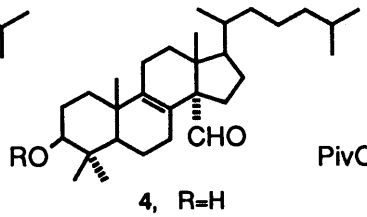
To introduce the 14α-hydroxymethyl group, we first envisaged the hydroxymethylation of an appropriate 8,14-dien-15-ol silyl ether derived from the 8-en-15-one **6** or **7**.[3] When the 15-ketone **6** was treated with LDA/trimethylsilyl chloride, only the 8,15-dienol TMS ether **8** was obtained in a good yield. Similarly treatment of **6** with 2,6-lutidine/triethylsilyl trifluoromethanesulfonate afforded quantitatively the 8,15-dienol TES ether **9** . The 8,15-diene structures but not the expected 8,14-diene counterpart were evident from ^{1}H-NMR, δ 4.5 ppm(16-H) and ^{13}C-NMR, δ 157.3 and 101.9 ppm (C-15 and -16 of **9**, respectively). Reaction of the enol silyl ethers **8** and **9** with methyl lithium followed by methyl iodide or



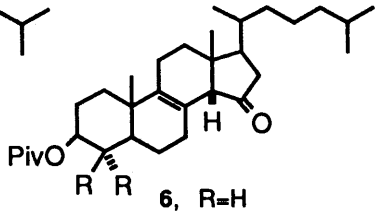
1



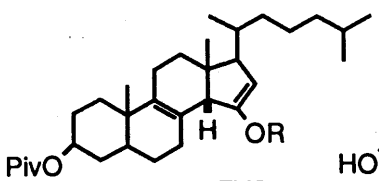
2, R=H
3, R=Ac



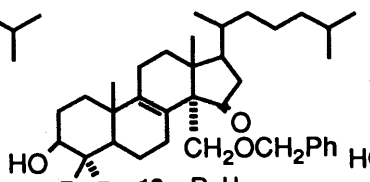
4, R=H
5, R=Ac



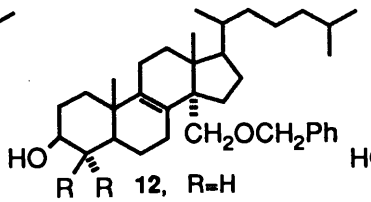
6, R=H
7, R=Me



8, R=TMS
9, R=TES



10, R=H
11, R=Me



12, R=H
13, R=Me

14

formaldehyde, gave the 16-methyl or 16-hydroxymethyl compounds, respectively. These results are in marked contrast to those of Aranda *et al.*[4] We then turned our attention to benzyloxymethylation of the 15-ketone. When the 15-ketone **6** was treated with NaH/dioxane or LDA/THF followed by benzyloxymethyl chloride,[5] a complex mixture was produced. However, when the latter alkylation was done on the enolate anion formed by t-BuOK/t-BuOH, we obtained, after saponification, the 14α-benzyloxymethyl derivative **10**, ^{1}H-NMR, δ 0.77 (13-Me), 0.93 (10-Me), 3.38 (d, *J*=9 Hz, 32-H), 3.68 (d, *J*=9 Hz, 32-H), 4.38 (d, *J*=12 Hz, benzylic) and 4.45(d, *J*=12 Hz, benzylic), in 60% yield, together with a trace (less than 5%) of the tentatively assigned 14β-isomer. Although the C-14 stereochemistry of **10** was not secured at this stage, it was definitely determined as 14α, based upon the results of analogous reactions of 4,4-dimethyl analog **7**, leading to the known 14α-hydroxymethyl lanostane derivative **2** (*vide infra*). The reaction of **10** with 80% hydrazine hydrate/diethylene glycol followed by the addition of KOH afforded the deoxygenated compound **12**, in 50% yieled: ^{1}H-NMR, δ 0.70 (13-Me), 0.95 (10-Me), 3.31 (d, *J*=8 Hz, 32-H), 3.41 (d, *J*=8 Hz, 32-H), 4.39 (d, *J*=12 Hz, benzylic), 4.53 (d, *J*=12 Hz, benzylic). Catalytic hydrogenation of **12** with 5% Pd-C in ethanol gave 14α-hydroxymethyl-5β-cholest-8-en-3β-ol (**14**), mp 195-197 °C in 75% yield: MS, *m/z* 398 (M-H_2O), 385 (M-H_2O-side chain, base peak), ^{1}H-NMR, δ 0.71 (13-Me), 1.00 (10-Me), 3.20 (d, *J*=11 Hz, 32-H), 3.63 (d, *J*=11 Hz, 32-H).

By analogous procedures as described above, 32-oxygenated lanosterol derivatives, **2** and **4** were then prepared. Thus the 4,4-dimethyl-15-ketone **7** [3] was treated with benzyloxymethyl chloride/t-BuOK/t-BuOH, and the crude alkylation product **11** was then deoxygenated by Huang-Minlon reduction. The resulting 14α-benzyloxymethyl compound **13** (49% yield from **7**), was subjected to hydrogenolysis with H_2/5% Pd-C/EtOH to afford 32-hydroxy-24,25-dihydrolanosterol (**2**, 72%), mp 172-174 °C (ref [2c] mp 173-174 °C). To synthesize 32-oxo derivative **4**, compound **13** was subjected to acetylation and then debenzylation as described above, to yield the 32-hydroxy-3-acetate **3** (72%), mp 130-131 °C, and subsequent oxidation of **3** with pyridinium chlorochromate in dichloromethane provided the aldehyde **5**, mp 147-150 °C in 79% yield. Finally treatment of **5** with 1% KOH-MeOH-benzene gave 32-oxo-24,25-dihydrolanosterol (**4**), mp 178-180 °C (ref [6] mp 177-179 °C).

The present work established an efficient route to synthesize 32-hydroxy- and 32-oxo-lanost-8-en-3β-ol.

REFERENCES AND NOTES

1) a) A. B. Cooper, J. J. Wright, A. K. Ganguly, J. Desai, D. Loebenberg, R. Parmegiani, D. S. Feingold and I. D. Sud, *J. Chem. Soc., Chem. Commun.*, **1989**, 89; b) L. L. Frye and C. H. Robinson, *J. Org. Chem.*, **55**, 1579 (1990); c) S. F. Tuck, C. H. Robinson and J. V. Silverton, *J. Org. Chem.*, **56**, 1260 (1991).
2) a) S. Eguchi, K. Ebihara and M. Morisaki, *Chem. Pharm. Bull.*, **36**, 4638 (1988); b) Y. Sonoda, Y. Tanoue, M. Yamaguchi and Y. Sato, *Chem. Pharm. Bull.*, **35**, 394 (1987); c) E. Parish and G. J. Schroepfer Jr., *J. Lipid Res.*, **22**, 859 (1981); d) P. L. Batten, T. J. Bentley, R. B. Boar, R. W. Draper, J. F. McGhie and D. H. R.Barton, *J. Chem. Soc. Perkin Trans 1*. **1972**, 739.
3) S. Araki, S. Eguchi and M. Morisaki, *Chem. Pharm. Bull.*, **38**, 1796 (1990).
4) G. Aranda, M. Fetizon and N. Tayeb *Tetrahedron*, **41**, 5661 (1985). They have claimed that the reaction of methyl 3α,12α-diacetoxy-15-oxo-5β,14β-chol-8-en-24-oate with trimethylsilyl iodide/pyridine gave the 15-trimethylsilyloxy-8,14-diene, which was then converted to the 14α-methyl derivative by the action of methyl lithium. The presence of the 12α-acetoxyl group in their substrate might bias the course of enolate formation.
5) C. L. Graham and F. J. McQuillin, *J. Chem. Soc.* **1963**, 4634; T. H. Chan and A. E. Schwerdtfeger, *J. Org. Chem.*, **56**, 3294 (1991)
6) J. M. Trzaskos, M. F. Favata, R. T. Fischer and S. H. Stam, *J. Biol. Chem.*, **262**, 12261 (1987).

(Received May 13, 1991)