Ab Initio Molecular Orbital Study of Reactivity of Active Alkyl Groups. II. Deprotonation of Some Acyclic Carbonyl Compounds with Methoxide Anion

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The present report describes studies on the reaction mechanisms of an α-proton abstraction from acetone (MMK), methyl ethyl ketone (MEK), methyl isopropyl ketone (MIPK) and methyl *tert*-butyl ketone (MTBK) using CH_3ONa in CH_3OD solution. The activation energies for the deuterium exchange reaction of active methyl group of the ketones MMK, MEK, MIPK and MTBK were 11.87, 12.14, 12.29 and 13.14 kcal·mol^{-1}, respectively. The deprotonation reaction mechanisms of the methyl alkyl ketones with methoxide anion were studied by *ab initio* molecular orbital method. Optimized geometries and energies of all complexes consisting of two molecules were calculated by 6-31+G basis set. The correlation between the activation energies and the differences in calculated energies of the transition state (E_{TS}) and the H-bonded encounter complex I (E_I) was fairly good. It is shown that the mechanisms of the deprotonation reactions can be explained with a simple model consisting of a methyl alkyl ketone and a methoxide anion.

Keywords acyclic carbonyl compound; methyl alkyl ketone; active alkyl group; deuterium exchange; deprotonation; methoxide anion; rate constant; activation energy; *ab initio* MO method

The electrophilic displacement reactions of active alkyl compounds (AH) with electrophilic reagents (E) using base catalysts (B^-) are shown by Eqs. 1 and 2. There are several types of base catalysts, almost all of which involve a proton transfer in at least one step. A prior ionization of the starting material is followed by a reaction with reagents to give the products:

$$AH + B^- \rightleftharpoons A^- + BH \quad (1)$$

$$A^- + E \rightarrow \text{products} \quad (2)$$

There are a number of reports on these reactions,[1] which fall into two groups depending on whether the rate determining step is Eq. 1 or 2. Examples of the former include the deuterium exchange and the halogenation reactions for many organic compounds containing an acidic hydrogen atom. Examples of the latter reactions include the condensation of acetone to diacetone alcohol and many similar condensations in organic chemistry, such as the Claisen and Michael reactions. Recently the detailed mechanisms of the electrophilic reactions have been studied and have been noteworthy in the synthesis of acyclic ketone derivatives, especially in regioselective and stereospecific synthesis.[2]

The first deprotonation step shown in Eq. 1 consists of the following three items, *i.e.*, (i) formation of an H-bonded encounter complex I (C-I), A–H···B^-, (ii) proton transfer *via* a complex TS (TS), [A··H··B]$^-$ to form a new complex II (C-II), A^-···H–B, and (iii) separation into an anion molecule A^- and a neutral HB, as shown in Chart 1.

In general, it depends on the bulkiness of the substituents in the ketones or in the bases, whether the configurations of the enolates produced in the deprotonation step of the ketones with the bases become (*Z*)- or (*E*)-form (Fig. 1). Models of a six or five-membered ring in the transition state have been proposed by Ireland and Moreland to predict and explain the compositions of (*Z*)- or (*E*)-form of the enolates.[3]

We reported previously a study on the mechanisms of deprotonation reactions of acetaldehyde and methane with three bases (NH_2^-, OH^-, F^-) by *ab initio* molecular orbital (MO) method using the 6-31+G basis set. The order of the deprotonation ability of these bases, *i.e.*, $NH_2^- > OH^- > F^-$ could be reasonably explained when the deformation energy was taken into account in calculating the energies of the complexes in each reaction pathway.[4]

In the present study, to further investigate the mechanism of the first step shown in Chart 1, five acyclic carbonyl compounds, CH_3COR (MHK: R=H, MMK: R=CH_3, MEK: R=C_2H_5, MIPK: R=$CH(CH_3)_2$, and MTBK: R=$C(CH_3)_3$) were used, and the rate constants of the deuterium exchange reactions of CH_3COR with CH_3OD were measured in the presence of CH_3ONa using gas chromatography/mass spectrometry (GC/MS). The activation energies for the reactions of four methyl alkyl ketones were determined from the Arrhenius plots. Furthermore, an *ab initio* MO calculation on the deprotonation step was carried out using a simple model consisting of two molecules in which the oxygen atom of CH_3O^- approached the hydrogen atom of the active alkyl group of CH_3COR along the axis of the C–H bond. Optimized geometries and energies of complexes in each case were calculated using the 6-31+G basis set. In this paper we studied the reactivities of a proton of the methyl and alkyl group in

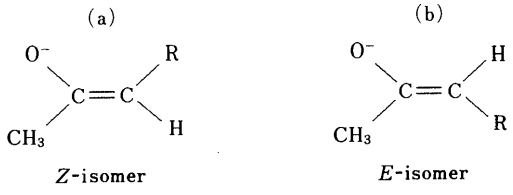

Fig. 1

$$AH + B^- \rightleftharpoons A\text{–}H\cdots B^- \rightleftharpoons [A\cdots H\cdots B]^- \rightleftharpoons A^-\cdots H\text{–}B \rightleftharpoons A^- + HB$$

reactants C-I TS C-II products

Chart 1

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CH_3COR by means of the chemical kinetics and the MO calculations of the chemical reaction energies.

Experimental

Instruments GC/MS of reaction products was obtained by the electron impact (EI) on a Shimadzu GC-MS 6020 instrument. A glass column (50 cm × 2.5 mm i.d.) packed with Porapak P was used. The temperature of the column oven was maintained at 120 °C. The carrier gas was helium with a linear velocity of 20 ml·min^{-1}. The temperature of the injection port and the transfer line was kept at 140 °C and that of the ion source at 250 °C. Ionization energy and trap current were 20 eV and 70 μA, respectively. The accelerating voltage was 3.5 kV. A thermo-regulator used for the reactions was a Model K4R (Lauda, West Germany).

Materials CH_3OD and methyl alkyl ketones of guaranteed reagent grade were purchased from Merck and Tokyo Kasei Kogyo Co., Ltd., respectively. Carbonyl compounds were purified by distillation before use. Concentrations of CH_3COR in CH_3OD were as follows: acetone (MMK); 0.0379 M, methyl ethyl ketone (MEK); 0.0337 M, methyl isopropyl ketone (MIPK); 0.0326 M, methyl *tert*-butyl ketone (MTBK); 0.0225 M. The CH_3OD solutions of CH_3ONa (0.025%, 0.05%) were prepared from 0.1% solution (0.0437 M).

Kinetic Measurement A reaction solution was prepared by adding a CH_3OD solution of CH_3ONa (0.1 ml) to a CH_3OD solution of CH_3COR (0.5 ml). The deuterium exchange reactions of the alkyl groups were carried out at desired temperature for the determination of rate constants and activation energies of the deprotonation reactions (MMK: 0.025%; 33—15 °C, 0.05%; 18—6 °C, 0.1%; 6—−6 °C, MEK: 0.025%; 39—21 °C, 0.05%; 24—15 °C, 0.1%; 12—0 °C, MIPK: 0.025%; 39—21 °C, 0.05%; 27—15 °C, 0.1%; 18—3 °C, MTBK: 0.025%; 42—24 °C, 0.05%; 30—18 °C, 0.1%; 24—6 °C). The residual amounts of active alkyl protons were measured at regular time and temperature intervals (3 min, 3 °C, respectively) by molecular and fragment ions in the mass spectra. The mass spectra were measured 3 times under the same reaction conditions as described above and the rate of the reaction was determined by the decrease in intensity of the ions. It was assumed that the bond cleavage of deuterated alkyl ketones was the same pattern as that of a starting material in mass spectra.

Computational Procedure The molecular orbital calculations were carried out with the GAUSSIAN80[5a] and GAUSSIAN82[5b] programs using the SCF method with 6-31+G[5c] basis set, which incorporates diffuse functions not only on the oxygen atoms of CH_3O^- and the conjugate acid CH_3OH, but also on the carbon atom in the enolates participating in the reaction. No diffuse function was contained in the basis set for the other first row atoms in the starting material. Geometry optimization was performed by the energy gradient method.[6] As a model of the deprotonation process, the oxygen atom of CH_3O^- approached the active hydrogen atom of methyl or alkyl groups along the axis of the C–H bond.

Results and Discussion

Deuterium Exchange Reaction of Active Alkyl Groups Using Methoxide Anion in Methanol-d_1 The rate determining step in the reaction shown in Eqs. 1 and 2 depends strongly on the species of electrophilic reagents. In this study, deuterium exchange reactions were carried out to determine the rate constants and the activation energies of the first step, *i.e.*, the deprotonation step for acyclic ketones. The rate constants of the reaction of active alkyl groups with CH_3O^- were determined by measuring the time-course of the decrease in intensity of the fragment ion peaks, *e.g.* $[CH_3CO]^+$, or the molecular ion peak M^+ in the mass spectra as shown in Fig. 2.

In this reaction, deuterium atoms were consecutively substituted for hydrogen atoms in active alkyl groups. The mass number of the fragment ion $[CH_3CO]^+$ increased in single increments from *m/z* 43 to *m/z* 46 with the lapse of reaction time. The relative intensity (RI) of the fragment ion peak *e.g.*, of *m/z* 43 was determined by the ratio of the intensity of ion peak *m/z* 43 to the sum of the intensities of four peaks, *m/z* 43, 44, 45, and 46. According to Beutelman, the isotope effect (k_H/k_D) for the enolate formation of 2-methyl-3-pentanone with lithium diisopropylamine in tetrahydrofuran is about 3.[7] Strictly speaking, correction of the isotope effect has to be made by analysis of the data, but in the present study this was precluded. To minimize the consequences of errors inherent in the method of analysis, the rates were evaluated from the variation of the relative intensities of the ion in the first half-life.

Time-Course of Decrease in Intensity of Fragment Ion Peaks in Mass Spectra of Methyl Alkyl Ketones The mass spectrum of MMK showed the molecular ion M^+ at *m/z* 58 together with the fragment ion $[CH_3CO]^+$ at *m/z* 43 as the base peak (Fig. 2a). The intensities of M^+ and the ion at *m/z* 43 decreased, the mass number of each ion increased one at a time with the progress of the deuterium exchange reactions, and the intensities of the ions having larger mass numbers increased successively. The rate of the decrease in

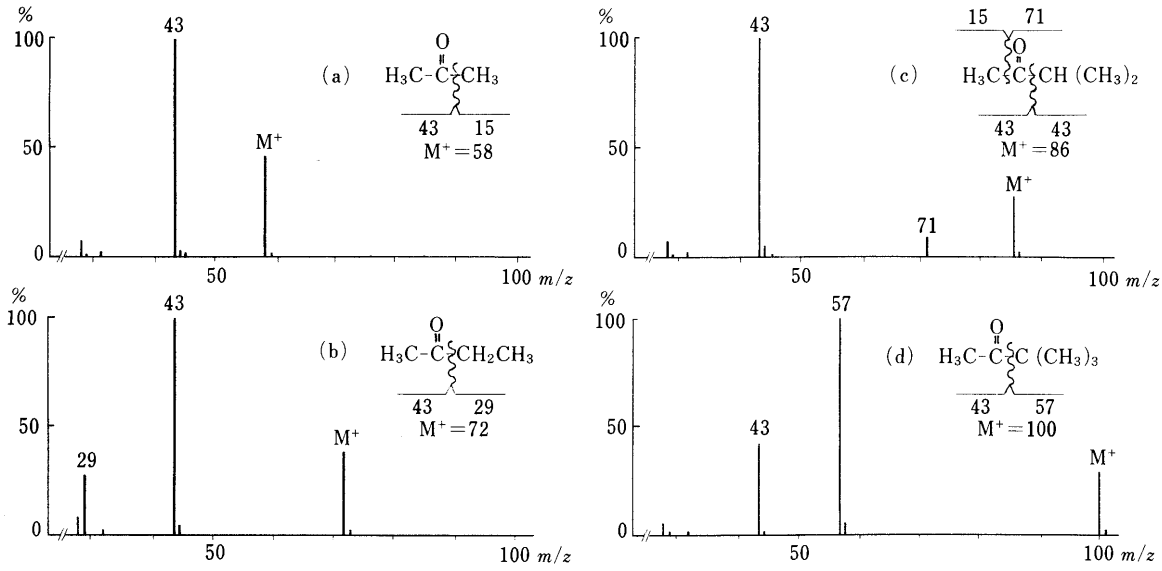

Fig. 2. Mass Spectra of Methyl Alkyl Ketones CH_3COR
(a), MMK; (b), MEK; (c), MIPK; (d), MTBK.

intensity of M^+ was twice that of the ion at m/z 43.

The mass spectrum of MEK gave the molecular ion M^+ at m/z 72 together with the fragment ions $[CH_3CH_2]^+$ at m/z 29 and $[CH_3CO]^+$ at m/z 43 as the base peak, respectively (Fig. 2b). With the progress of the deuterium exchange reactions, the intensities of M^+ and of the ions at m/z 43 and 29 decreased, the mass number of each ion increased one by one, and the intensities of the ions having larger mass numbers increased successively. The rate of the reduction in intensity of the ion at m/z 43 was about 3 times faster than that of the ion at m/z 29. The rate of the decrease in intensity of M^+ coincided with the sum of those of the individual ions at m/z 43 and 29. This indicates that a CH_3CO–CH_2CH_3 bond cleaves exclusively.

The mass spectrum of MIPK showed the molecular ion M^+ at m/z 86, accompanied by the duplicate ions $[CH(CH_3)_2]^+$ and $[CH_3CO]^+$ at m/z 43 as the base peak (Fig. 2c). In addition, cleavage of the CH_3–$COCH(CH_3)_2$ bond in the molecule resulted in the formation of a characteristic ion $[COCH(CH_3)_2]^+$ at m/z 71. The intensities of M^+ and the fragment ions at m/z 43 and 71 decreased and the mass number of each ion increased one by one with the progress of the deuterium exchange reactions, and the intensities of the ions having larger mass numbers increased successively. The rate of the decrease in intensity of the ion at m/z 71 was very slow compared with that of the ion at m/z 43.

The mass spectrum of MTBK gave the molecular ion M^+ at m/z 100 together with the ions $[CH_3CO]^+$ at m/z 43 and $[C(CH_3)_3]^+$ at m/z 57 as the base peak, respectively (Fig. 2d). As the deuterium exchange reactions proceeded, the intensities of M^+ and the fragment ion at m/z 43 decreased, the mass number of each ion increased one by one, respectively, and the ions having larger mass numbers increased successively. On the other hand, no change in time. The rate of intensity decrease of M^+ was in fair agreement with that of the ion at m/z 43. The above results in the mass spectrum indicate that only the CH_3CO–$C(CH_3)_3$ bond is cleaved.

The rate of the deuterium exchange reaction of active alkyl hydrogen atom using a base catalyst CH_3O^- can be represented by Eq. 3, where k is the rate constant and $[CH_3COR]$ stands for the concentration of CH_3COR in CH_3OD solution. This reaction is pseudo-first-order, since CH_3O^- is a catalyst and its concentration remains constant during a run (Eq. 4).

$$v = k[CH_3O^-][CH_3COR] \quad (3)$$

$$v = k'[CH_3COR] \quad (4)$$

$$k' = k[CH_3O^-]$$

The deuterium exchange reaction was treated as effectively irreversible during the first half-life. The values k were estimated by the rate of decrease in intensities of the molecular ion M^+ and the fragment ions. Logarithms of the relative intensities of the ions were plotted against time and a linear relationship between them was obtained with a good correlation coefficient ($r=0.99$) in all cases except in MIPK ($r=0.95$). The rate constants k of the reaction of CH_3COR with CH_3O^- at 25 °C are summarized in Table I.

The rate constants k_{Me}, k_R, and k_M shown in the table were obtained by calculation based on the decrease in intensities of the ions $[CH_3CO]^+$, $[R]^+$, and M^+ in the mass spectra, respectively. The sum of k_{Me} and k_R for both alkyl groups across carbonyl group is expected to be equal to k_M. Actually, it was confirmed that both $2\times k_{Me}$ and k_M for MMK have the same values within experimental errors. In the case of MEK, the sum of k_{Me} and k_R was in good agreement with k_M, and for MTBK, k_{Me} was in accord with k_M. The value of the rate constant k_{Me} for acetyl group in MIPK could not be determined directly from the intensity

TABLE I. Rate Constants (k) of Deuterium Exchange Reaction of CH_3COR with CH_3O^- at 25 °C ($M^{-1}\cdot s^{-1}$)

CH_3COR	R	k_{Me}	k_R	k_M	$k_{Me}/k_{Me(MMK)}$	
					This work	Warkentin[a]
MMK	CH_3	0.549	—	1.116	1.0	1.0
MEK	CH_2CH_3	0.295	0.082	0.381	0.54	0.5
MIPK	$CH(CH_3)_2$	0.222	0.042	0.302	0.40	0.34
MTBK	$C(CH_3)_3$	0.117	—	0.116	0.21	0.16

a) Ref. 8e.

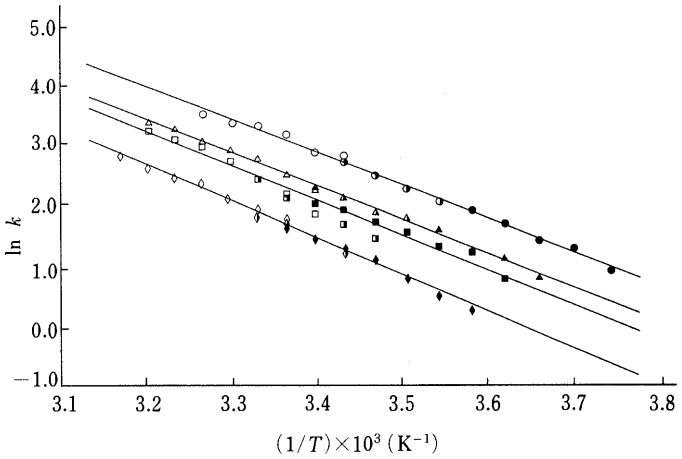

Fig. 3. Arrhenius Plots for Deuterium Exchange Reaction of Active Methyl Group of CH_3COR

○, MMK (correlation coefficient $r=0.997$); △, MEK ($r=0.998$); □, MIPK ($r=0.981$); □, MTBK ($r=0.999$). Open symbols: 0.025% CH_3ONa, half-closed symbols: 0.05% CH_3ONa, closed symbols: 0.1% CH_3ONa.

TABLE II. Calculated Energies for Reactants, Products and Complexes of Methyl Groups of CH_3COR with CH_3O^- in Deprotonation Reaction of CH_3COR with CH_3O^- (a.u.)

CH_3COR	R	E_R	E_I	E_{TS}	E_{II}	E_P
MHK	H	−267.20278	−267.23094	−267.22489	−267.24717	−267.22669
MMK	CH_3	−306.23487	−306.26353	−306.25366	−306.27374	−306.24960
MEK	CH_2CH_3	−345.25439	−345.28295	−345.27304	−345.29351	−345.26957
MIPK	$CH(CH_3)_2$	−384.27143	−384.30276	−384.29216	−384.31360	−384.29431
MTBK	$C(CH_3)_3$	−423.28712	−423.32100	−432.30771	−423.32896	−423.31015

of the ion at m/z 43, because this ion was comprised of two components $[CH_3CO]^+$ and $[CH(CH_3)_2]^+$ as described above. The rate constant k_{Me}, therefore, was calculated from the difference between k_M and k_R for the fragment ion at m/z 71. The value of the rate constant k_R obtained from the peak at m/z 71 involved some experimental errors, because of the weak intensity of the peak and a slight decrease in intensity of the peak with the lapse of time. The ratio $k_{Me}/k_{Me(MMK)}$ decreases in proportion with bulk of the alkyl groups, and these values are similar to those ascertained by Warkentin *et al.* using nuclear magnetic resonance (NMR) spectrometry.[8] The value of the rate constant per hydrogen atom $k_{Me}/3$ for methyl group is about twice that for methylene group $k_R/2$ in the case of MEK. Such comparison of the rate constants per hydrogen atom must be considered when the reactivities of the reaction sites of ketones are investigated. The reaction of a hydrogen atom of MMK proceeds about 4 times as fast as that of a methylene hydrogen atom of MEK.

The activation energies of the deuterium exchange reactions of four ketones, MMK, MEK, MIPK and MTBK were evaluated from the slopes of Arrhenius plots as shown in Fig. 3. The activation energies of each active methyl group in MMK, MEK, MIPK and MTBK were 11.87, 12.14, 12.29 and 13.14 kcal·mol^{-1}, respectively. In addition, the activation energies for a methylene proton in MEK and a methine proton in MIPK were 13.87 and 14.83 kcal·mol^{-1}, respectively.

Optimized Geometries and Energies of Complexes for Deprotonation Reaction of Active Alkyl Groups with Methoxide Anion The mechanisms for the deprotonation reactions of MHK, MMK, MEK, MIPK, and MTBK with CH_3O^- were studied by the *ab initio* MO method with 6-31+G basis set. First, the stable geometries of reactants in a ground state were calculated, and then a model consisting of two molecules, *i.e.*, a ketone CH_3COR and a base CH_3O^-, was used to calculate the geometries and the energies of complexes in the deprotonation process. In the simple model, the oxygen atom of CH_3O^- approached the hydrogen atom of CH_3COR along the axis of the alpha C–H bond in the ketones having the most stable conformation. The calculated energies of reactants (E_R), products (E_P), and complexes (E_I, E_{TS}, E_{II}) in the reaction of CH_3COR with CH_3O^- are presented in Table II.

The reaction of MHK with NH_2^- or OH^- showed single-well potential-energy curves as reported previously.[4] On the other hand, the reaction profiles for the deprotonation of CH_3COR with CH_3O^- differed from that of MHK with OH^- and indicated double-well potential-energy curves in all reaction systems as shown in Fig. 4.

First, as CH_3O^- approaches a hydrogen atom of the active alkyl group in CH_3COR, a stable C-I is formed. Further approach of CH_3O^- to the hydrogen atom lengthens the C–H bond and leads to the formation of a stable C-II *via* the transition state TS. Finally, enolate and methanol are produced. The differences in energies between each of the complexes are all shown in Table III. The difference between the reactivity of MHK with OH^- and with CH_3O^- may be attributable to the steric effects and the proton affinity of the bases (OH^-: 390.7, CH_3O^-: 379.2 kcal·mol^{-1}).[9] The interaction energies for the complex C-I consisting of MHK with OH^- and with CH_3O^- were −17.8 and −16.7 kcal·mol^{-1}, respectively,[4] whereas

Table III. Differences between Calculated Energies of Complexes of Methyl Groups of CH_3COR with CH_3O^- (kcal·mol^{-1})

CH_3COR	R	E_P-E_R	E_I-E_R	$E_{TS}-E_I$	$E_{II}-E_{TS}$	E_P-E_{II}
MHK	H	−15.00	−17.67	3.79	−13.98	12.85
MMK	CH_3	−9.24	−17.98	6.20	−12.60	15.14
MEK	CH_2CH_3	−9.52	−17.92	6.22	−12.84	15.02
MIPK	$CH(CH_3)_2$	−14.36	−19.66	6.65	−13.45	12.10
MTBK	$C(CH_3)_3$	−14.45	−21.26	8.34	−13.34	11.80

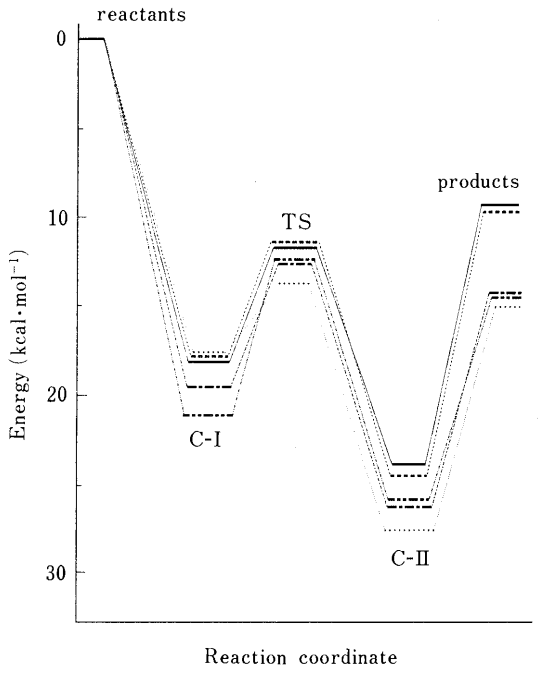

Fig. 4. Comparison of Reaction Profiles for the Deprotonation of Methyl Group of CH_3COR with CH_3O^- Using 6-31+G Basis Set
MHK, ·····; MMK, ——; MEK, – – –; MIPK, ——·——; MTBK, ——··——.

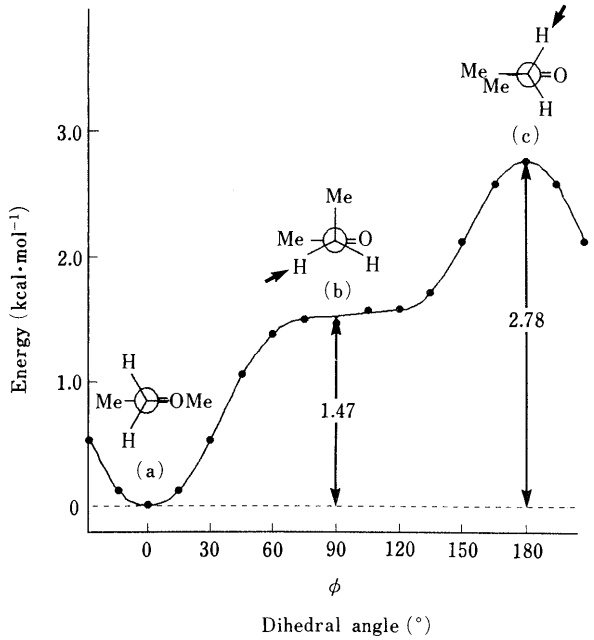

Fig. 5. Potential Energy for C_2–C_3 Bond Rotation from 0° (a) to 180° (c) of a Dihedral Angle

TABLE IV. Calculated Energies for Reactants, Products and Complexes of MEK or MIPK with CH_3O^- in Deprotonation Reaction of MEK or MIPK with CH_3O^- (a.u.)

Compound	Reaction site	E_R	E_I	E_{TS}	E_{II}	E_P
MEK	CH_3	−345.25439	−345.28295	−345.27304	−345.29351	−345.26957
	$CH_2CH_3(Z)$[a]	−345.25439	−345.28306	−345.26970	−345.29022	−345.26472
	$CH_2CH_3(E)$[b]	−345.25439	−345.28093	−345.26744	−345.28368	−345.26217
MIPK	CH_3	−384.27143	−384.30276	−384.29216	−384.31360	−384.29431
	$CH(CH_3)_2$	−384.27143	−384.30091	−384.28596	−384.30312	−384.28313

a) Product has a *Z*-form. b) Product has an *E*-form.

TABLE V. Differences between Calculated Energies of Complexes of MEK or MIPK with CH_3O^- (kcal·mol^{-1})

Compound	Reaction site	E_P-E_R	E_I-E_R	$E_{TS}-E_I$	$E_{II}-E_{TS}$	E_P-E_{II}
MEK	CH_3	−9.52	−17.92	6.22	−12.84	15.02
	$CH_2CH_3(Z)$[a]	−6.48	−17.99	8.39	−12.88	16.00
	$CH_2CH_3(E)$[b]	−4.88	−16.65	8.47	−10.19	13.50
MIPK	CH_3	−14.36	−19.66	6.65	−13.46	12.10
	$CH(CH_3)_2$	−7.34	−18.49	9.38	−10.77	12.54

a) Product has a *Z*-form. b) Product has an *E*-form.

there was a distinct difference between the reaction profile for the deprotonation of MHK with OH^- and with CH_3O^-. In the reaction of MHK with OH^-, no transition state was obtained,[4] while the reaction profile for the deprotonation of MHK with CH_3O^- had a double-well potential-energy surface ($E_{TS}-E_I=3.79$ kcal·mol^{-1}). From the above results, it may be assumed that the difference of reactivity between OH^- and CH_3O^- with MHK is mainly due to the steric effect in TS.

In general, the reactivities of the reactants increase with the gap between the energies of the reactants and those of the products under the dominance of the thermodynamic control. The differences E_P-E_R of calculated energies were in conflict with the experimental data for the deuterium exchange reactions shown in Table I. On the other hand, the differences $E_{TS}-E_I$ enlarged with the bulk of substituents "R" in CH_3COR and reasonably explained the reactivities of the methyl groups in CH_3COR. The values of $E_{TS}-E_I$ for the reactions of the ketones, MHK, MMK, MEK, MIPK and MTBK with CH_3O^- were 3.79, 6.20, 6.22, 6.65 and 8.34 kcal·mol^{-1} respectively, as shown in Table III. The differences $E_{TS}-E_I$ for the reactions of MMK or MTBK with CH_3O^- increased by a factor of about 1.6 or 2.2 compared with that for MHK, respectively.

Both MEK and MIPK have two different α-protons, that is, methyl and methylene protons in MEK, methyl and methine protons in MIPK. All of the carbon and oxygen atoms of the enolate produced by the deprotonation of methine proton in MIPK lie on the same plane; therefore, no regioisomers exist in the optimized geometries. On the other hand, in MEK, (*Z*)- and (*E*)-isomers shown in Fig. 1 are produced by the deprotonation of methylene proton. It is well known that the favored structure of MEK has the conformation shown in Fig. 5a.[10] A stable conformation of MEK (a) leads to an unstable conformation (c) with additional energy of about 2.8 kcal·mol^{-1} through a relatively unstable conformation (b) by the rotation of ethyl group as shown.

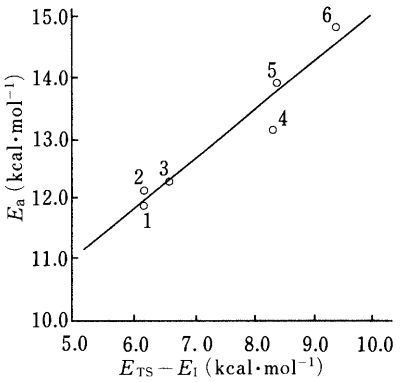

Fig. 6. Relation between Observed Activation Energies and Differences of Calculated Energies of Complexes ($E_{TS}-E_I$)

1, MMK; 2, MEK–methyl group; 3, MIPK–methyl group; 4, MTBK–methyl group; 5, MEK–ethyl group; 6, MIPK–isopropyl group.

A (*Z*)-enolate is produced by a deprotonation of methylene proton in the stable conformation. To obtain an (*E*)-enolate, it is necessary to rotate an ethyl group at least 90 degrees from stable conformation and then to deprotonate an appropriate proton on methylene carbon atom with CH_3O^-. The arrows in Fig. 5 indicate the deprotonated proton leading to the (*E*)-enolate. The calculated energies of complexes for two reaction sites in the unsymmetrical ketones and for the formation of (*Z*)- and (*E*)-isomer from MEK are shown in Table IV. The difference in energies between each of the complexes is summarized in Table V.

The differences E_P-E_R indicate that methyl group reacts thermodynamically more easily than ethyl or isopropyl group with CH_3O^-. The differences $E_{TS}-E_I$ for the deprotonation reaction of methyl group are smaller than those of another alkyl group, *i.e.*, a proton in methyl group reacts more easily than that in ethyl or isopropyl group with CH_3O^-, and this is consistent with the experimental data shown in Table I. In the present study the reaction of CH_3COR with CH_3O^- was carried out at *ca.* 0—30 °C in a protic solvent. Under these reaction conditions it is generally said that a thermodynamic enolate is formed.[11] Consequently, it can be considered that the (*Z*)-enolate is generated from the ketone in stable form shown in Fig. 5a in the deuterium exchange reaction. Therefore, circle number 5 in Fig. 6 is designated using the values of $E_{TS}-E_I$ calculated from the stable structure in the process of the formation of the (*Z*)-enolate. Figure 6 shows the plots of the observed activation energies of the deuterium exchange reactions of methyl alkyl ketones *versus* the $E_{TS}-E_I$ shown in Tables III and V. A linear relationship between the

TABLE VI. Optimized Geometries of Reactants, Products and Complexes between $CH_3COCH_2CH_3$ with CH_3O^-

Geometrical parameter	Optimized geometries r(Å), ∠(°)								
	Reactants	$-\underline{CH}_3$[a]				$-\underline{CH}_2CH_3$[a]			
		C-I	TS	C-II	Products	C-I	TS	C-II	Products
Bond length									
$r(C^1-O^2)$	1.219	1.229	1.243	1.282	1.281	1.229	1.245	1.290	1.289
$r(C^1-C^3)$	1.506	1.496	1.448	1.381	1.379	1.507	1.520	1.525	1.539
$r(C^3-H^4)$	1.080	1.082	1.083	1.077	1.076	1.082	1.082	1.086	1.083
$r(C^3-H^5)$	1.085	1.089	1.087	1.076	1.078	1.086	1.088	1.087	1.087
$r(C^3-H^6)$	1.085	1.096	1.350	2.198	—	1.084	1.082	1.085	1.087
$r(C^1-C^7)$	1.511	1.512	1.527	1.534	1.550	1.500	1.443	1.375	1.369
$r(C^7-C^8)$	1.526	1.525	1.526	1.531	1.528	1.523	1.522	1.508	1.509
$r(C^7-H^9)$	1.088	1.086	1.085	1.086	1.088	1.097	1.382	2.162	—
$r(C^7-H^{10})$	1.088	1.088	1.089	1.089	1.088	1.092	1.090	1.079	1.079
$r(C^8-H^{11})$	1.084	1.086	1.087	1.089	1.090	1.085	1.088	1.084	1.091
$r(C^8-H^{12})$	1.082	1.083	1.083	1.086	1.082	1.084	1.084	1.095	1.091
$r(C^8-H^{13})$	1.082	1.084	1.082	1.081	1.082	1.086	1.093	1.092	1.091
$r(H^{6(9)}-O^{14})$	—	1.858	1.277	0.968	0.950	1.855	1.244	0.968	0.950
$r(O^{14}-C^{15})$	1.399	1.398	1.408	1.430	1.436	1.399	1.407	1.432	1.436
$r(C^{15}-H^{16})$	1.107	1.103	1.096	1.082	1.076	1.103	1.097	1.085	1.076
$r(C^{15}-H^{17})$	1.107	1.103	1.093	1.085	1.082	1.103	1.092	1.081	1.082
$r(C^{15}-H^{18})$	1.107	1.104	1.093	1.081	1.082	1.103	1.093	1.082	1.082
Bond angle									
$\angle O^2C^1C^3$	121.1	123.4	125.1	126.7	127.3	120.8	118.0	115.4	115.8
$\angle C^1C^3H^4$	109.8	110.4	112.8	120.1	120.4	110.4	109.7	109.5	108.4
$\angle C^1C^3H^5$	110.6	109.2	113.3	121.5	121.6	110.0	109.4	108.9	111.7
$\angle C^1C^3H^6$	110.6	109.4	109.3	90.0	—	109.0	111.2	113.2	111.7
$\angle O^2C^1C^7$	121.8	121.2	118.7	115.5	116.0	123.9	125.3	126.5	126.3
$\angle C^1C^7H^8$	113.9	114.3	114.1	112.3	113.3	114.5	116.2	122.1	121.7
$\angle C^1C^7H^9$	107.9	106.8	107.9	110.7	108.9	106.6	106.1	90.0	—
$\angle C^1C^7H^{10}$	107.9	107.5	107.4	107.0	108.9	107.1	111.2	119.3	120.1
$\angle C^7C^8H^{11}$	110.4	110.5	110.8	111.6	111.1	109.7	110.5	109.3	110.9
$\angle C^7C^8H^{12}$	111.1	110.9	110.7	110.7	110.6	110.6	110.3	113.0	112.7
$\angle C^7C^8H^{13}$	111.1	111.4	110.9	109.3	110.6	112.2	113.5	112.8	112.7
$\angle C^1C^{3(7)}O^{14}$	—	109.4	109.3	90.0	—	106.6	106.1	90.0	—
$\angle H^{6(9)}O^{14}C^{15}$	—	169.2	114.6	111.0	114.4	177.6	116.0	110.9	114.4
$\angle O^{14}C^{15}H^{16}$	114.0	113.5	112.3	111.2	105.9	113.4	112.3	111.0	105.9
$\angle O^{14}C^{15}H^{17}$	114.0	113.4	112.0	111.1	111.4	113.6	111.5	107.8	111.4
$\angle O^{14}C^{15}H^{18}$	114.0	113.6	111.9	107.3	111.4	113.5	112.5	110.0	111.4
Dihedral angle									
$\angle O^2C^1C^3H^4$	0.0	−15.4	18.6	6.4	0.0	−3.9	−24.3	−54.1	0.0
$\angle O^2C^1C^3H^5$	120.6	104.2	146.8	176.6	180.0	116.8	94.3	62.7	119.8
$\angle O^2C^1C^3H^6$	−120.6	−138.4	−101.4	−90.0	—	−126.2	−146.0	−176.0	−119.8
$\angle C^3C^1O^2C^7$	180.0	180.0	180.0	180.0	180.0	180.0	180.0	180.0	180.0
$\angle O^2C^1C^7C^8$	0.0	1.2	10.3	48.3	0.0	9.8	−14.8	−6.8	0.0
$\angle O^2C^1C^7H^9$	123.2	125.6	133.8	172.3	122.3	134.6	105.8	90.0	—
$\angle O^2C^1C^7H^{10}$	−123.2	−122.2	−112.1	−71.0	−122.3	−112.9	−146.0	−176.0	180.0
$\angle C^1C^7C^8H^{11}$	180.0	179.6	181.0	186.5	180.0	178.8	175.5	15.6	180.0
$\angle C^1C^7C^8H^{12}$	59.7	59.6	60.7	66.3	59.0	60.1	56.6	−103.5	59.8
$\angle C^1C^7C^8H^{13}$	−59.7	−59.8	−58.2	−52.7	−59.0	−60.2	−63.4	135.7	−59.8
$\angle O^2C^1C^{3(7)}O^{14}$	—	−138.4	−101.4	−90.0	—	134.6	105.8	90.0	—
$\angle C^1C^{3(7)}O^{14}C^{15}$	—	100.4	83.6	52.5	—	−177.8	−191.7	−46.7	—
$\angle H^{6(9)}O^{14}C^{15}H^{16}$	—	0.1	−2.3	−42.6	180.0	−2.1	−15.6	−91.2	180.0
$\angle H^{6(9)}O^{14}C^{15}H^{17}$	60.0	120.1	117.7	78.3	61.3	−122.1	−135.1	149.8	61.3
$\angle H^{6(9)}O^{14}C^{15}H^{18}$	−60.0	−119.9	−122.0	−162.7	−61.3	117.9	104.6	29.6	−61.3

a) Underlines indicate the deprotonated protons.

differences $E_{TS}-E_I$ and the activation energies with the good correlation coefficient ($r=0.966$) is obtained. The good correlation indicates that the environment of each complex, *e.g.*, solvation, *etc.*, resembles closely the others in the deprotonation process of the reaction of acyclic carbonyl compounds with CH_3O^- and that the simple model consisting of ketone and CH_3O^-, can explain quite reasonably the reactivities of the deprotonation reactions dealt with in this paper.

The optimized geometries of reactants, products and complexes between MEK with CH_3O^- are presented in Table VI, and in Fig. 7 each of the complexes is represented by an ORTEP drawing.

The geometries of C-I and C-II are similar to those of reactants and products, respectively. A proton which migrates from MEK to CH_3O^- is situated in the neighborhood of the middle position between the oxygen atom of CH_3O^- and the carbon atom of alkyl group in TS. The distances between hydrogen and carbon or oxygen atom are 1.382 and 1.244 Å, respectively. A dihedral angle

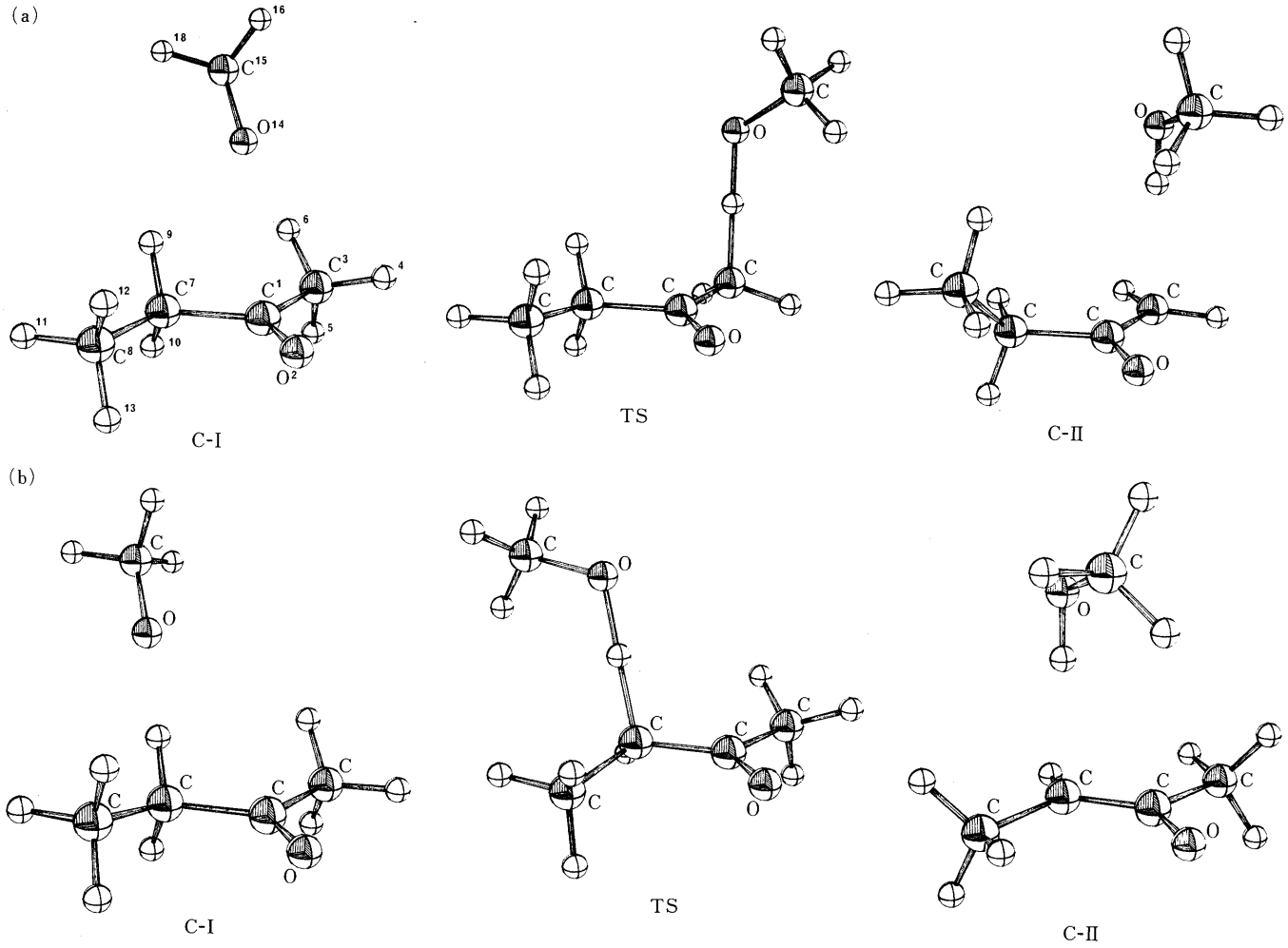

Fig. 7. ORTEP Drawing of Complexes of $CH_3COCH_2CH_3$ with CH_3O^-

(a), complex of methyl group of MEK with CH_3O^-; (b), complex of ethyl group of MEK with CH_3O^-; which gives (*Z*)-enolate.

between the carbonyl C=O bond and the alpha C–H bond is 105.8 degrees in TS. The calculated geometries in TS are similar to those described in previous papers.[12] Methyl group of CH_3O^- moves from a sterically stable position (Fig. 7, C-I) toward the oxygen atom of carbonyl group by rotating around the axis of C–H bond, and at the same time, CH_3O^- abstracts the proton of the active alkyl group as shown in Fig. 7, C-II. It can be said that the mechanism of the deprotonation reaction of CH_3COR with CH_3O^- in CH_3OH is illustrated reasonably by the simple model shown in Chart 1 and Fig. 7.

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Sequence-Dependent Interaction of Acidic Amino Acid with Guanine Base in Tryptophan-Containing Dipeptides: Spectroscopic Studies[1)]

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As a model study to investigate the sequence dependence of a peptide for the interaction with nucleic acid base, four kinds of tryptophan-containing dipeptides [Trp–Glu, Glu–Trp, Trp–Gly and Gly–Trp] were synthesized, and their abilities in forming the complexes with guanine base were examined by fluorescence and proton nuclear magnetic resonance (^{1}H-NMR) methods. The fluorescence titration of each dipeptide with 7-methylguanosine-5′-phosphate (m^7GMP) indicated that although the stacking interaction dominates the binding in each peptide, the carboxyl side chain of Glu plays an additional role in the binding with the base. The effect of Glu residue and its sequence dependence for the interaction was more clearly demonstrated by the ^{1}H-NMR titration. Since the association constants determined from the downfield shift of the guanine NH_2 resonance exhibited the same tendency as those from the upfield shift of the guanine N7-methyl protons [Trp–Glu > Trp–Gly > Glu–Trp > Gly–Trp], the close cooperation between the hydrogen bond and stacking interactions was suggested to be important for the tight binding of the peptides to the guanine base. Further, it was suggested that the peptide which contains an aromatic amino acid at the N-terminal side and an acidic one at the C-terminal side has an advantage in forming such a complex.

Keywords tryptophan-containing dipeptide; guanine base; molecular association; sequence dependence; stacking interaction; hydrogen bonding; fluorescence; ^{1}H-NMR

Introduction

The ability of a protein to specifically recognize a nucleotide or a nucleic acid sequence is essential for a variety of biological functions. The precise recognition is achieved by the specific noncovalent interaction between the constituent chemical groups of both molecules, in addition to their dimensional similarities as is observed between the deoxyribonucleic acid (DNA) groove and protein helix or sheet structure.[2)]

As observed in the tertiary structures of double-stranded DNA fragments or protein–nucleotide complexes, hydrogen bond pairings between the polar groups and aromatic stacking interactions function importantly as main forces for such a specific recognition; among several kinds of interaction modes, these have the ability to strictly fix the interacting species, as evidenced by the design of model receptors showing high selectivity for a particular molecule.[3)] Thus, it is of special importance in understanding the essence of molecular recognition mechanism between the protein and nucleic acid to investigate what kind of combination among many functional amino acids can selectively interact with a nucleic acid base by virtue of the coupling of these two interaction modes. In contrast with the many reports on the interactions between individual amino acids and nucleotide or nucleoside units,[4)] however, systematic studies on the peptides showing high selectivity for binding with a target nucleic acid base appear to be rather few.[5)]

As a series of studies to design peptides which exhibit a high affinity for the guanine base, we recently prepared cocrystals of the tryptophanylglutamic acid (Trp–Glu) and 7-methylguanosine-5′-phosphate (m^7GMP) molecules, and analyzed their structure by the X-ray diffraction method.[1,6)] The crystal structure showed the first example of a coupled contribution of triple hydrogen bonds between the guanine base and peptide backbone chain and of prominent stacking interaction between the guanine base and tryptophan indole ring, as is illustrated in Chart 1. Although the binding mode appears to be specific enough to select only the guanine base, the Glu side chain did not participate in the hydrogen bond with the base, contrary to the general acceptance that the carboxyl anion is specifically able to form a hydrogen bond pairing only with the guanine base.[7)] Thus, it is quite interesting to examine (1) whether such interactions observed in crystal are also observed in the solution state, (2) how the Glu interacts with the base, if this is not the case, and (3) the effect of the dipeptide sequence on the interaction with the base. This paper deals with the interactions of m^7G base with four kinds of tryptophan-containing dipeptides in solution state, studied by fluorescence and proton nuclear magnetic resonance (^{1}H-NMR) methods.

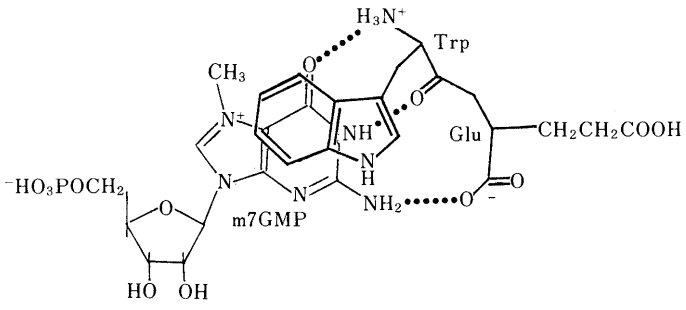

Chart 1

Experimental

Materials m^7GMP formate and 7-methylguanosine (m^7Guo) chloride were synthesized from GMP and guanosine, respectively, according to the method described previously.[8)] The dipeptides were also synthesized from respective amino acids by the usual liquid phase peptide condensation; Trp–Glu and Glu–Trp were obtained as the ammonium salts.

Fluorescence Measurements The fluorescence intensity of Trp indole ring in each peptide was measured on a Hitachi F-3000 spectrometer utilizing a Hg–Xe arc lamp, where a 10-nm slit and a 1-cm path length were employed. Sample solutions were prepared using 20 mM Tris–HCl buffer (pH = 7.6). For the quantitative measurements, the temperature of the sample solution was controlled by circulating temperature-controlled water through a brass cuvette holder. Fluorescence titrations were performed by adding 3.75—37.50 μl aliquots of 10 mM m^7GMP to a 3 ml solution of 5 M dipeptide, so as to vary the concentration ratio of

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[m^7GMP]/[dipeptide] from 2.5 to 25.0. All fluorescence measurements were corrected for the sample dilution in the course of the titration experiment. The intensities of emission spectra were measured at 353 nm, where the excitations were performed at 290 nm.

The equilibrium binding constant K_a between the two molecules was obtained from the Eadie–Hofstee equation[9]:

$$\Delta F = -\frac{1}{K_a[M]}\frac{\Delta F}{} + \Delta F_c$$

where ΔF is the difference in the fluorescence intensities of the peptide between the states in the absence and presence of m^7GMP at the concentration of [M], and ΔF_c is the difference between the fluorescence intensities for dipeptide alone and completely complexed with m^7GMP. The value of K_a was obtained from the slope calculated by the least-squares linear regression analysis.

^{1}H-NMR Measurements ^{1}H-NMR spectra were measured on a Varian XL-300 (300 MHz for ^{1}H, Fourier transform mode) spectrometer equipped with a temperature-control accessory (accuracy to 1 °C). Since the peptides are less soluble in aqueous solution, samples were dissolved in DMSO (dimethyl sulfoxide)-d_6, where m^7Guo was used instead of m^7GMP because of the solubility. The chemical shifts were measured with respect to an internal reference to TMS (tetramethylsilane). NMR titration was performed by adding 5—50 μl aliquots of 250 mM dipeptide to a 5 ml solution of 5 mM m^7Guo, so as to change the ratio of [dipeptide]/[m^7Guo] from 0.5 to 5.0 with an interval of 0.5.

Two kinds of association constants were estimated from the chemical shift changes of m^7Guo N2 amino and N7 methyl protons, respectively. The chemical shifts of guanine NH_2 protons shifted downfield in the presence of the dipeptide, while those of CH_3 protons, conversely, showed upfield shifts. The K_a values using the NH_2 and CH_3 protons were obtained from the Eadie–Hofstee and Benesi–Hildebrand[10] equations, respectively;

$$\Delta\delta = -\frac{1}{K_a[M]}\frac{\Delta\delta}{} + \Delta\delta_C \quad \text{for} \quad NH_2$$

$$\frac{1}{\Delta\delta} = \frac{1}{K_a\Delta\delta_C[M]}\frac{1}{} + \frac{1}{\Delta\delta_C} \quad \text{for} \quad CH_3$$

where $\Delta\delta = \delta_0 - \delta$ and $\Delta\delta_C = \delta_C - \delta$, and δ_0, δ and δ_C are the chemical shifts of protons (NH_2 or CH_3) of m^7Guo in the absence and the presence of dipeptide at the concentration of [M], and of m^7Guo completely complexed with dipeptide.

Results

Fluorescence of m^7GMP and Dipeptide Complexes The fluorescence emission spectra of tryptophan-containing dipeptide as a function of m^7GMP concentration were measured. Upon complex formation, a decrease in the dipeptide fluorescence intensity at 353 nm (λ_{max}) was observed. Such fluorescence quenching has been attributed to the π–π stacking interaction between the Trp indole ring and the m^7G base. The association constants of m^7GMP–dipeptide complexes were estimated from the relative fluorescence intensity changes. An Eadie–Hofstee plot for Trp–Glu–m^7GMP is shown in Fig. 1. The association constant (K_a) values for four dipeptides are given in Table I.

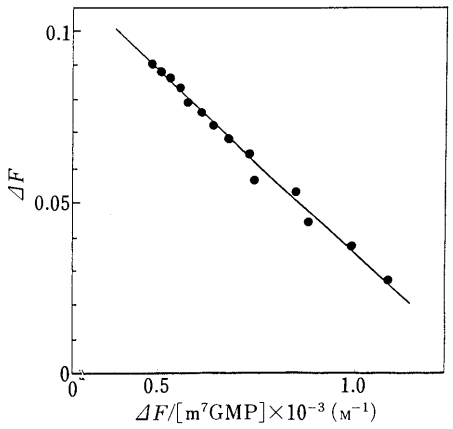

Fig. 1. Eadie–Hofstee Plot of the Trp Fluorescence Quenching as a Function of m^7GMP Concentration in Trp–Glu–m^7GMP Pair

^{1}H-NMR Measurements (1) Stoichiometry of m^7Guo–Dipeptide Complex The stoichiometry of the m^7Guo–dipeptide complex was examined by plotting the chemical shifts of m^7Guo N7–CH_3 protons as a function of molar fraction {[m^7Guo]/([m^7Guo]+[dipeptide])}, where the total concentration of [m^7Guo]+[dipeptide] was kept constant (Job plot[11]). Figure 2 shows the result for the Trp–Glu–m^7Guo pair. The maximum of the Job plot occurs at 0.5 mole fraction, implying the stoichiometry of 1 : 1. Other pairs also yielded Job plots suggesting equimolar

Table I. Association Constants between m^7GMP and Dipeptides Determined by Fluorescence Titration

Dipeptide	K_a (M^{-1})[a]
Trp–Glu	7.75×10^3
Trp–Gly	4.96×10^3
Glu–Trp	7.80×10^3
Gly–Trp	6.59×10^3

a) The estimated error is less than $\pm0.05\times10^3$.

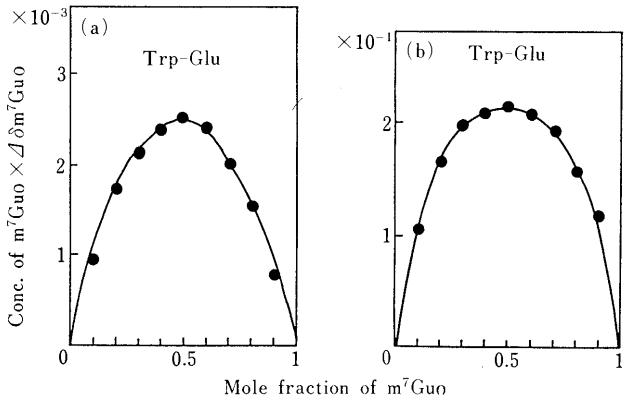

Fig. 2. Job Plot of Chemical Shifts of m^7Guo N7-Methyl (a) and N2-NH_2 (b) Protons at 30 °C

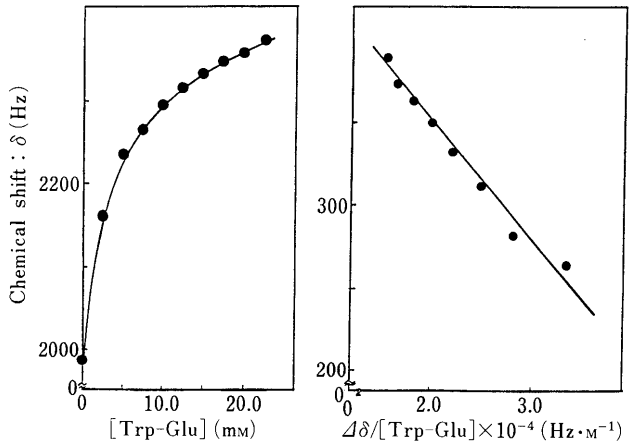

Fig. 3. (a) Downfield Change of m^7Guo N2-Amino Proton Chemical Shifts as a Function of Trp–Glu Concentration
(b) Eadie–Hofstee Plot of $\Delta\delta$ (m^7G-N2 Amino Protons) *vs.* Trp–Glu Concentration

TABLE II. Association Constants between m^{7}Guo and Dipeptides Determined by the Downfield Shifts of m^{7}Guo N2-Amino Proton Chemical Shifts

Dipeptide	K_a (M^{-1})[a]
Trp–Glu	2.77×10^2
Trp–Gly	1.28×10^2
Glu–Trp	1.07×10^2
Gly–Trp	0.82×10^2

a) The estimated error is less than $\pm 0.04 \times 10^2$.

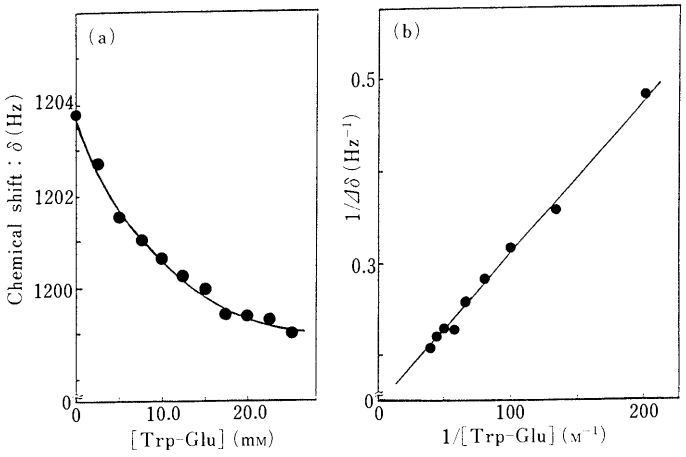

Fig. 4. (a) Upfield Change of m^{7}Guo N7-Methyl Proton Chemical Shifts as a Function of Trp–Glu Concentration
(b) Benesi–Hildebrand Plot of $\Delta\delta$ (m^{7}G N7-Methyl Protons) *vs.* Trp–Glu Concentration

TABLE III. Association Constants between m^{7}Guo and Dipeptides Determined by the Upfield Shifts of m^{7}Guo N7-Methyl Proton Resonances

Dipeptide	K_a (M^{-1})[a]
Trp–Glu	9.00×10
Trp–Gly	8.40×10
Glu–Trp	5.95×10
Gly–Trp	2.75×10

a) The estimated error is less than $\pm 0.05 \times 10$.

complex formations.

(2) Association Constants of the Complexes Two kinds of association constants were estimated for the complex pairs. One is based on the changes of m^{7}G amino protons and the other on those of N7-methyl protons. As shown in Fig. 3a, the chemical shifts of amino protons shifted downfield in proportion to the increase of dipeptide concentration. This phenomenon could be interpreted as a result of the participation of this amino proton in the hydrogen bond with the peptide acceptor atom. The treatment according to the Eadie–Hofstee equation gave good linearity within the experimental error (see Fig. 3b). The association constants for four dipeptides are given in Table II.

An alternative way to estimate the strength of interaction is to utilize the ring-current effect by the aromatic ring stacking. The chemical shifts of guanine N7-methyl protons shifted upfield in proportion to the increase of dipeptide concentration, as shown in Fig. 4a. This is clearly due to the ring stacking between the Trp indole ring and m^{7}G base. The treatment of the data by the Benesi–Hildebrand equation[12] also gave a good linearity (see Fig. 4b). The association constants obtained are listed in Table III.

Discussion

The stacking interaction of Trp indole ring with guanine base and its sequence dependence were investigated by fluorescence quenching. Since the quenching is mainly caused by the ring stacking interaction, the association constants in Table I reflect the order of stacking interaction. No significant preference was observed for these four dipeptides, and their results could be interpreted as indicating that the stacking degrees between the Trp indole ring and the m7G base are nearly the same in such a dilute solution, revealing no clear dependence for the amino acid sequence.

However, a noticeable characteristic was observed for the contribution of the Glu residue to the interaction, *i.e.*, the higher K_a value of Trp–Glu or Glu–Trp than that of Trp–Gly or Gly–Trp, respectively. This shows the contribution of the Glu side chain to the binding with m^{7}G base, probably by the hydrogen bond formation.

The sequence dependence of each amino acid and the participation of Glu residue for the binding with m7G base were, on the other hand, clearly evidenced by the ^{1}H-NMR measurements. The difference from the fluorescence result would be due to: (1) the physicochemical properties of solvents used being different from each other, *i.e.*, the stacking and hydrogen bonding interactions are predominant and stable in the aqueous and DMSO solutions, respectively, and (2) the concentrations of respective molecules being much (about 10 times) higher than those in the fluorescence experiment. Although it is reported[13] that the guanine base tends to self-associate in the solution into highly stable, regular structure, the molecular could be thought to behave as an unordered state in the concentration range used.[8] Furthermore, the Job plot showed that all of the interaction pairs have the stoichiometries of 1 : 1 molecular associations. Thus, the ^{1}H-NMR data were treated as the equimolar interaction state.

The ring-current effect due to the π–π aromatic stacking interaction between the Trp indole ring and the m^{7}G base shifts the chemical shifts of the protons directly attached to the ring toward the upfield side. Nevertheless, the guanine N2 amino protons were significantly moved to the downfield side with the increase of the dipeptide concentration. Thus, this is responsible for the participation of the m^{7}G amino group in the hydrogen bonds. Some acceptor atoms in the dipeptides are acceptable for the hydrogen bond formations. However, the carboxyl group of the Glu side chain may be the most probable candidate because the association constants in Table II show a high preference for this residue in the order Trp–Glu > Trp–Gly and Glu–Trp > Gly–Trp. In DMSO solution, the Glu side chain would exist as a carboxyl anion as judged from the NMR spectra and the usage of ammonium salt of dipeptide. The hydrogen bond pairing of carboxyl anion to the guanine N1 imino and N2 amino groups has been suggested by NMR[7] and X-ray crystallographic[14] studies. The N1 imino proton of guanine base was not detected as a result of the N7-methylation. This could be due to (1) the fast H–D exchange with H_2O which was slightly involved in DMSO solvent, or (2) the

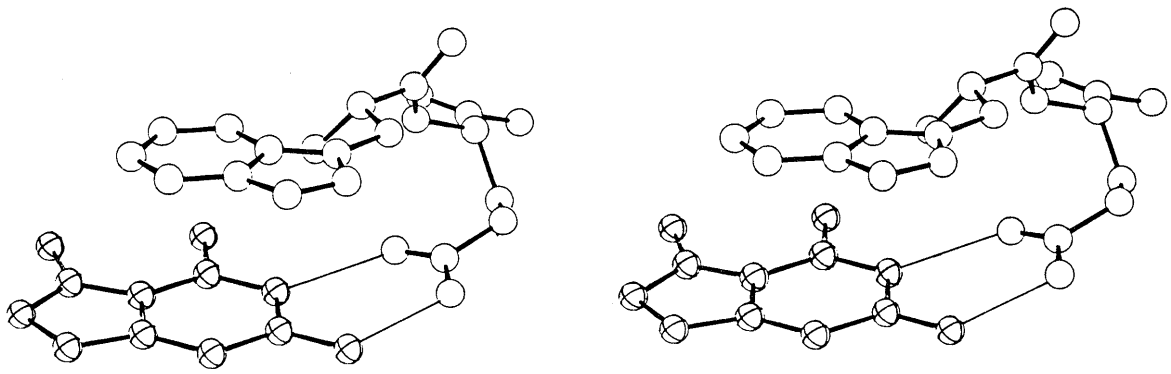

Fig. 5. Stereoscopic View of a Possible Binding Mode between m^7G Base and Trp–Glu in Solution
The thin lines represent hydrogen bonds.

enolate tautomerism of m^7G, as was pointed by Carberry *et al.*[15] In the case of (2), the formation of hydrogen-bond pairing *via* N1 imino group becomes impossible. Thus, a definite hydrogen bonding mode cannot be proposed at present. However, it is quite clear that the hydrogen bond formation with Glu carboxyl side chain participates importantly in the binding with the m^7G base, in addition to the participation of the other polar atoms such as the backbone oxygens in the hydrogen bonds, as was observed in the crystal (Chart 1); the latter interaction is important for the binding of Trp–Gly or Gly–Trp with m7GuO molecule.

In contrast, the values given in Table II also show the close relationship between the amino acid sequence and the association constant, as is obvious from the order Trp–Glu > Glu–Trp and Trp–Gly > Gly–Trp. This indicates that the m7G amino group is in a highly favorable state to form the complex when the Trp residue is located at the N-terminal side.

The sequence dependence in dipeptide and the importance of Glu residue for the binding with m^7G base were also suggested by the K_a values estimated from the upfield shift (ring-current effect) of the base N7-methyl protons by the stacking interaction with Trp indole ring (Table III). The order or Trp–Glu > Trp–Gly and Glu–Trp > Gly–Trp implies that the ring stacking interaction is strengthened by the hydrogen bonding of the Glu residue to the base. On the other hand, it is conceivable from the order of Trp–Glu > Glu–Trp and Trp–Gly > Gly–Trp that the location of the Trp residue at the N-terminal side is highly superior in its stacking interaction with the base.

As is shown in Chart 1, the crystal structure of m^7GMP–Trp–Glu complex showed no direct participation of the Glu carboxyl side chain in the binding with the base, which is a characteristic difference from the interaction mode observed in the solution. Such a difference results from the difference between the ionization states of Glu carboxyl side group in the crystal (neutral) and solution (anion).[1] However, it was rather easy to build up the interaction modes which were quite compatible with the NMR data from the conformational parameters shown in Chart 1. A tentative interaction mode in solution is shown in Fig. 5; the molecular geometry was energetically optimized by a molecular mechanics program MMFF.[16]

The present study clearly showed the necessity of the coupled cooperation of hydrogen bonding and stacking interactions for the tight binding of the guanine base by peptide, and further, the importance of the amino acid sequence was also demonstrated to achieve such cooperation effectively. These factors are important in the specific recognition of guanine nucleotide by G-protein or of messenger ribonucleic acid capped structure by the cap binding protein (eIF-4E).

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Spectroscopic Study on Interaction of Nucleic Acid Base with Tryptophan-Containing Tripeptides: Acetyl-Trp-X-Trp-$NHCH_3$ (X = Gly, Asn, Asp, Gln and Glu)[1)]

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As part of a series of peptides designed to have binding ability selective for each of the nucleic acid bases, five tripeptides consisting of *N*-acetyl-Trp-X-Trp-$NHCH_3$ (X = Gly, Asn, Asp, Gln and Glu) were synthesized, and their abilities to form complexes with four different nucleotides were examined by the fluorescence and phase distribution methods. The association constants obtained indicated that, depending on the sort of X residue, the peptides showed a variation in their interaction with guanosine monophosphate (GMP), while no noticeable selectivity was observed for other nucleotides adenosine monophosphate (AMP), uridine monophosphate (UMP) and cytidine monophosphate (CMP). The binding mode of *N*-acetyl-Trp-Asp-Trp-$NHCH_3$ for the guanine base was further investigated using the proton nuclear magnetic resonance (^{1}H-NMR) method. The mode was suggested to involve intimate cooperation of (1) the hydrogen bond formation between the carboxyl group of the Asp side chain and the guanine C2-amino group, and (2) the stacking interaction of the base with two terminal Trp residues of the peptide. Such interaction was strengthened by the protonation of the guanine base. A tentative binding mode is proposed based on these results.

Keywords tryptophan-containing tripeptide; nucleic acid base; interaction; hydrogen bonding; stacking interaction; fluorescence; phase distribution; ^{1}H-NMR

Introduction

Selective nucleotide or deoxyribonucleic acid (DNA) sequence recognition by an enzyme is an important fundamental biological process in living cells. In addition to geometrical complementarity between the tertiary structures of DNA and enzymes, one possible motif for such precise recognition could involve the coupling of hydrogen bonding to the polar atoms of the nucleic acid base with a stacking interaction perpendicular to its base plane. This kind of interaction pattern has been observed in guanine recognition in the crystal structures of ribonuclease (RNase) T1–guanosine-2′-monophosphate (2′GMP),[2)] elongation factor-Tu–guanosine diphosphate (GDP)[3)] and c-H-*ras* oncogene p21–GDP[4)] complexes, where the guanine base is tightly held by multiple hydrogen bonds with polar amimo acids, and is also sandwiched between two neighboring hydrophobic amino acid residues. Further, the importance of such a coupled interaction for molecular recognition has been evidenced by synthetic model receptors which show high selectivity for a particular molecule.[5)]

By contrast, the effort to design an oligopeptide exhibiting high selectivity for a specific nucleic acid base appears to be rather minimal, although detailed analysis of the binding mode at the atomic level provides important information for understanding the precise recognition mechanism between a nucleic acid and protein. As part of a series of base-recognizing peptide design, therefore, this paper deals with spectroscopic studies of the interaction of tripeptides consisting of *N*-acetyl-Trp-X-Trp-$NHCH_3$ (X = Gly, Asn, Asp, Gln and Glu) (abbreviated as WGW, WNW, WDW, WQW and WEW, respectively) with four different nucleic acid bases; these protected tripeptides were designed under the expectation that each nucleic acid base sandwiched by two terminal Trp residues would be differentiated by different hydrogen bond formations with the polar side chains of X residues.

Experimental

Syntheses of Protected Tripeptides All peptides were synthesized from respective L-amino acids by the usual liquid phase peptide condensation. The N- and C-terminals of the peptides were blocked with acetyl and methylamide groups, respectively. The peptides were purified by high performance liquid chromatography (HPLC) on an octadecyl silica (ODS) column (20 mm × 150 mm Capcell Pack C18, Shiseido, Japan) with a linear gradient of acetonitrile (20—60%, 60 min) in a 10 mM triethylammonium acetate buffer (pH 7.0) at a flow rate of 4 ml/min. The purities (>95%) were confirmed by peak assignments of proton nuclear magnetic resonance (^{1}H-NMR) spectra.

Fluorescence Titration Fluorescence spectra were measured on a JASCO FP-770F (Nihon-Bunko, Japan). For quantitative measurements, the temperature of the sample solution was controlled by circulating thermostatically regulated water through the brass cuvette holder (20.0 ± 0.3 °C). Sample solutions were prepared by using either a 20 mM Tris–HCl (pH = 7.5) or 50 mM KCl–HCl (pH = 2.0) buffer solution. The emission spectra excited at 290 nm were measured at respective λ_{max}, where a 10 nm slit and a 1 cm path length were employed. The absorbance of the peptide was adjusted below 0.1, thus avoiding any correction for self-quenching due to the inner-filter effect.

Fluorescence titrations were performed by adding 3.75—37.50 μl aliquots of 10 mM nucleotide to a 3 ml solution of 5 μM peptide, so as to vary the concentration ratio of [nucleotide]/[peptide] from 2.5 to 25.0. All fluorescence measurements were corrected for the sample dilution in the course of the titration experiment. The quenching was estimated as the difference (ΔF) between the fluorescence intensities of the peptide in the presence (F_a) and absence (F_0) of a nucleotide, where $\Delta F = F_0 - F_a$. The effect of the nucleotide itself for quenching was also taken into account. The association constant K_a was obtained from the Eadie–Hofstee equation[6)]:

$$\Delta F = -\frac{1}{K_a}\frac{\Delta F}{[\text{nucleotide}]} + \Delta F_c \qquad (1)$$

where the ΔF_c is the quenching of the peptide completely complexed with the nucleotide. The slope of the Eadie–Hofstee plot was determined by least-squares linear regression analysis.

Phase Distribution Measurement Equilibration of the peptide between a 50 mM Tris–HCl buffer solution (1 ml, pH = 7.5) and *n*-butanol (1 ml) was carried out in a stoppered tube at 20 ± 1 °C. The concentration of nucleotide completely dissolved in the buffer solution varied from 0 to 0.1 M. 1 mM of the peptide was added into the tube and was shaken constantly during 2 d, then the phase was allowed to separate for one day. After the dilution, the peptide concentration in both phases was determined from the peak height on the HPLC chart by elution using an ODS column (Capcell Pack) and 20% acetonitrile, or a linear gradient of 20—60% acetonitrile: flow rate = 0.4 ml/min, detection = 280 nm for adenosine monophosphate (AMP), 290 nm for GMP and cytidine monophosphate

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(CMP), and 285 nm for uridine monophosphate (UMP). The apparent association constant K_{app} was estimated by the equation[7]:

$$\frac{K}{K_0} = K_{app}[\text{nucleotide}]_w + 1 \quad (2)$$

where K/K_0 represents the ratio of the distribution coefficient of dipeptide in the presence and absence of a nucleotide, and $[\text{nucleotide}]_w$ represents the concentration of nucleotide dissolved in the aqueous phase. The measurement was repeated three to four times and then averaged.

Peptide–Guanine Base Interaction by ^{1}H-NMR Method ^{1}H-NMR spectra were measured on a Varian XL-300 spectrometer. Samples were dissolved in dimethylsulfoxide-d_6 (DMSO-d_6), and the chemical shifts were measured with respect to an internal reference of tetramethylsilane (TMS). Because of the solubility, guanosine (Guo) or 7-methylguanosine (m7Guo) ammonium salt was used instead of its nucleotide in NMR experiments at 20 ± 1 °C.

NMR titration was performed by adding 5—50 μl aliquots of 250 mM peptide to a 5 ml solution of 5 mM nucleoside in such a way that the ratio of [peptide]/[nucleoside] changed from 0.5 to 5.0 with an interval of 0.5. From the chemical shift changes of Guo or m7Guo protons by the coexistence of the peptide, the strength of the interaction was estimated.

Results and Discussion

Nucleotide–Peptide Interaction by Fluorescence Intensity The fluorescence emission spectra of the Trp indole ring in peptides were measured in a Tris–HCl buffer (pH = 7.5) as a function of nucleotide concentration. When coexisting with a nucleotide, a decrease in the fluorescence intensity of the peptide was observed. Since such a fluorescence quenching could be mainly attributed to the π–π stacking interaction between the Trp indole ring and the purine or pyrimidine ring of nucleic acid base,[8] the association constants evaluated by Eq. 1 reflect the strength of the interaction between aromatic rings. The fluorescence quenching titration and its Eadie–Hofstee plot for the WDW–GMP pair is exemplified in Fig. 1. The association constants for all pairs are summarized in Table I. In order to estimate which peptide exhibits a specific interaction with the nucleotide, WGW was treated as a standard peptide for the interaction, *i.e.*, if the association constant of WGW for a given nucleotide was larger than that of another peptide, it could be interpreted that the latter peptide exhibited no selectivity for its nucleotide concerning the hydrogen bonding interaction, because the interaction of the former peptide would have been due to mainly the stacking interaction between the Trp indole ring and the nucleic base. Thus, it appears from Table I that all peptides synthesized were not fittable for interaction with AMP, CMP and UMP, while a selectivity for the interaction with GMP was observable for the WDW, WQW and WEW.

Nucleotide–Peptide Interaction by Phase Distribution Measurement The phase distributions of WGW, WNW, WDW and WEW peptides between the aqueous buffer containing 5′-GMP and the organic *n*-butanol are exemplified in Fig. 2. According to Eq. 2, the apparent association constants are given in Table II, where the K_{app} values for the WEW–nucleotide pairs could not be accurately determined because of the overlapping of HPLC peaks between both molecules. The good linearity of the plot over the whole concentrantion range studied, and the intercept close to the unity, suggest the reliability of this method in estimating the interaction. A significant interaction was shown for the WDW–GMP pair. Contrary to the fluorescence data, the interactions for the WNW–AMP and –CMP pairs were also indicated to be significant. The association constants determined by this method are based on the contribution of all forces interacting between both molecules, while those by the fluorescence method are largely affected by the stacking interaction.

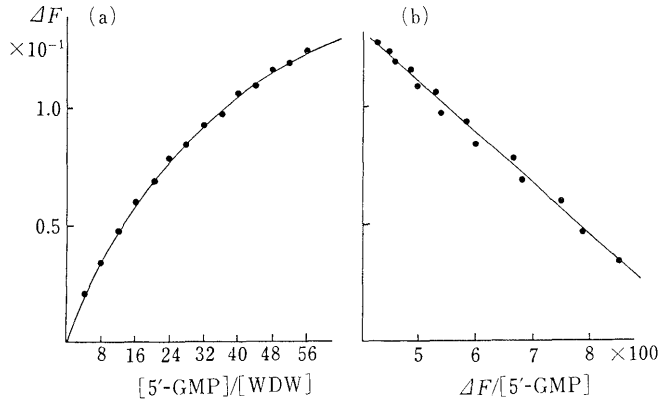

Fig. 1. Trp Fluorescence Quenching of WDW as a Function of 5′-GMP Concentration (a) and Its Eadie–Hofstee Plot (b)

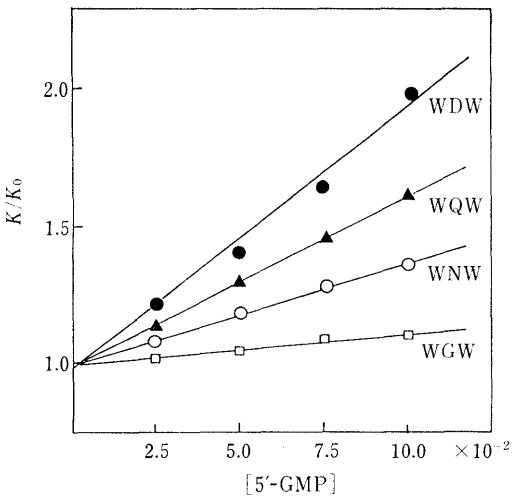

Fig. 2. Phase Distribution Plots of Four Kinds of Tripeptides as a Function of 5′-GMP Concentration

TABLE I. Association Constant ($K_a \times 10^3$, M^{-1}) between Nucleotide and Tripeptide, Determined by Fluorescence Titration[a]

	WGW	WNW	WDW	WQW	WEW
AMP	3.3 (3)	3.2 (2)	3.4 (3)	4.0 (1)	2.8 (2)
GMP	3.5 (1)	3.5 (1)	4.7 (3)	4.7 (2)	5.6 (3)
CMP	5.3 (5)	5.4 (3)	4.5 (3)	4.5 (4)	3.8 (3)
UMP	2.7 (4)	1.7 (2)	2.4 (3)	2.7 (4)	2.5 (3)

a) The standard errors are given in respective parentheses.

TABLE II. Apparent Association Constant (K_{app}, $l \cdot M^{-1}$) between Nucleotide and Tripeptide, Determined by Phase Distribution[a]

	WGW	WNW	WDW	WQW	WEW
AMP	4.0	8.2	3.6	2.4	—
GMP	1.2	3.1	12.5	6.8	—
CMP	0.7	6.5	5.5	—[b]	—
UMP	2.0	1.9	—[b]	1.6	—

a) The error was less than 10%. b) The value was negative.

Therefore, the relatively high K_{app} of the WNW–AMP and –CMP pairs may reflect the preferable hydrogen bond formation between both molecules. Judging from the data given in Tables I and II, it could be concluded that among the peptides synthesized, the interaction of the WDW–GMP pair is significant and it is mainly due to the coupling of the stacking and hydrogen bonding interactions.

Guanine Base–Peptide Interaction by the ^{1}H-NMR Method The fluorescence and phase distribution methods suggested that the peptides synthesized, as a whole, exhibited an interesting variation in their interaction with guanine base, according to the coupling degree of the hydrogen bond and stacking interactions. In order to examine the contribution of these interactions for the binding of guanine base to peptides more clearly, the changes of the base proton chemical shifts, which were caused by the coexistence of a peptide, were measured (Table III), where the guanosine, instead of GMP, was used because of its solubility. The guanine C2-NH_2 protons significantly shifted toward the downfield side by the existence of a peptide. As expected, the change was significant for the interaction with WDW. Since the change in shift of the base which coexisted with WGW was not very significant (*ca.* 0.3 Hz), such a large shift could have been primarily due to the direct hydrogen bond formation with the oxygen atom of the Asp carboxyl side chain. On the other hand, the chemical shift of the guanine H8 proton could also be monitored as an indicator of the interaction. Since it is suggested[9] that the H8 proton can participate in a hydrogen bond formation with a neighboring acceptor atom such as O5′, the chemical shift of this proton would indicate two different effects, *i.e.*, an upfield shift caused by a stacking interaction with the Trp aromatic ring and a downfield shift caused by the hydrogen bond formation. When the shift change of the H8 proton in the interaction with WGW is considered as a standard for such interaction effects, however, the significant stacking interaction can be observed only for the WDW. Thus, both the coupled hydrogen bonding and stacking interactions for the Guo–WDW pair was suggested. On the other hand, the downfield shifts of the Guo H8 proton by the WQW or WEW would suggest the participation of this proton in the hydrogen bond, and this is in conflict with the preferable stacking interaction suggested by the fluorescence or phase distribution method. This discrepancy may be due to the differences of the sample concentrations used and of the guanine nucleotide and nucleoside, although this is not definite.

Guanine Base–Peptide Interaction in Acidic Solution The systematic interaction studies in neutral states suggested that, among all pairs of four nucleic acid bases and five peptides, the WXW peptides showed a binding selectivity for the guanine base depending on the hydrogen bonding interaction with the X residue. On the other hand, it is known[10] that N-quarternalization of the guanine base significantly strengthens the π–π stacking interaction with aromatic amino acid. In order to examine the effect of the guanine base protonation on the interaction with these peptides, therefore, the fluorescence measurements were done in an acidic condition of pH = 2.0 (Table IV). For the ^{1}H-NMR measurements, m7Guo was used as a protonated model of the guanine base (Table V).[11] The stacking interactions of the guanine base with WDW or WEW peptides was significantly strengthened under the acidic condition, while the WQW was little affected by the base protonation (see Table IV). When the downfield shift of m7Guo C2-NH_2 protons and the upfield shift of the N7-CH_3 protons were compared with each other, the interaction of m7Guo with WDW was most significant. These results suggest that the coupling of the hydrogen bonding by the D carboxyl side group and the stacking interaction by the W indole ring is very important for the binding of the N-protonated guanine base; in other words, the strengthening of the stacking interaction leads to an increase in the hydrogen bonding interaction and *vice versa*. Such a coupled interaction is moderately observed for the WEW peptide.

In order to estimate a possible interaction between the guanine base and WDW in solution state, a model was built using an IRIS2400 graphics computer, and was energetically optimized by molecular mechanics calculation (program MMFF[12]). The result is given in Fig. 3. When the guanine base is sandwiched with two terminal Trp residues of WDW, the formation of a hydrogen bond is possible between the carboxyl oxygen atom of the Asp side chain and the guanine C2-NH_2 group in a neutral solution.[13] This binding mode would also be possible in an acidic solution, in which the carboxyl group of the Asp

TABLE III. Difference (in Hz[a]) between Guo C2-NH_2 and H8 Proton Chemical Shifts in Absence and Equimolar Presence of Tripeptide at 20 °C

	C2-NH_2	H-8
WGW	0.3	0.4
WNW	1.0	0.8
WDW	11.6	−0.8
WQW	1.5	2.9
WEW	2.2	3.4

a) The mean error was less than 0.2 Hz.

TABLE IV. Association Constants between GMP and Tripeptide in an Acidic Solution (pH = 2.0), Determined by Fluorescence Titration[a]

Tripeptide	Association constant ($K_a \times 10^3$, M^{-1})
WGW	4.2 (3)
WNW	4.1 (2)
WDW	7.9 (3)
WQW	5.2 (3)
WEW	11.2 (4)

a) The standard errors are given in respective parentheses.

TABLE V. Difference (in Hz[a]) between m^{7}Guo C2-NH_2 and N7-CH_3 Proton Chemical Shifts in Absence and Equimolar Presence of Tripeptide at 20 °C

	C2-NH_2	N7-CH_3
WGW	4.3	−0.3
WNW	53.1	−0.6
WDW	243.0	−2.8
WQW	65.3	−0.1
WEW	133.9	−1.9

a) The mean error was less than 0.2 Hz.

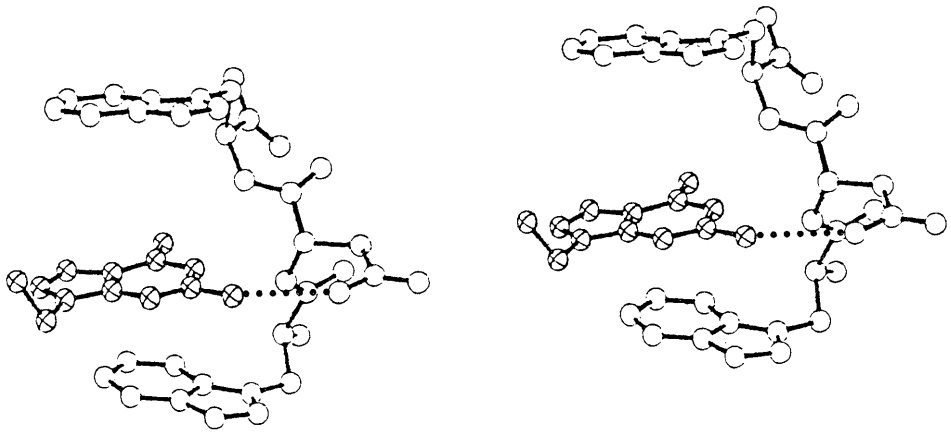

Fig. 3. A Stereoscopic View of Possible Binding Mode of WDW Tripeptide for Guanine Base in Solution State
The dotted line represents a hydrogen bond.

side chain takes a neutral form.

In order to form the hydrogen bond pairing between the carboxyl group of the Asp side chain and the guanine N1H and C2-NH_2 groups, which could be considered very important for revealing specific molecular recognition, it would be necessary to use a peptide longer than the present tripeptide, in which the Trp residue is separated by at least more than one residue from the Asp.

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11) For the ^{1}H-NMR experiments, the H8 proton of m7Guo could not be used as an indicator for the interaction, because of its cationic character caused by the N7-methylation,[10a] which consequently shifted it toward the downfield side. It is interesting to note that a similar downfield shift of the H8 proton of Guo is observed by N7 protonation in an acidic solution. Therefore, it appears reasonable to consider the m7Guo as a substituent for Guo under an acidic condition.
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Crystal Structure of Benzene : Picric Acid Complex

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The crystal structure of the benzene : picric acid complex has been elucidated by X-ray crystal analysis. Benzene and picric acid molecules are stacked alternately along the a-axis, making their molecular planes parallel to each other. Two crystallographically independent benzene : picric acid complexes exist in an asymmetric unit.

Keywords benzene; picric acid; π-complex; benzene picrate; crystal structure; X-ray analysis

Picric acid forms stable picrates with various organic molecules through π-bonding or ionic bonding, and such picrates have been very useful for identification and qualitative analysis. But there have been only a few studies on their crystal structures, apart from naphthalene,[1] anthracene,[2] and pyridine picrates.[3] As it seemed desirable to determine the crystal structures and the bonding mode of picrates of basic compounds, we have investigated the crystal structure of phenanthrene : picric acid complex,[4] and revised the crystal structure of pyridine picrate.[5]

Benzene is one of the most basic aromatic compounds and it is of interest to elucidate the crystal structure of the picrate. However, it is rather difficult to get crystals of benzene picrate suitable for X-ray analysis. This may be the reason why no previous study has been conducted on this crystal structure. After many attempts, crystals of benzene picrate suitable for X-ray analysis were obtained. We present here the crystal structure of benzene : picric acid complex.

Experimental

The picrate used for X-ray analysis was prepared by the saturation of picric acid in benzene. The melting point was determined to be 82—84 °C, measured according to the reported method.[6] As the crystals quickly lost benzene to yield a yellow powder on exposure to air, a crystal was coated with epoxy resin and used for X-ray analysis. The diffraction intensities were measured on a Rigaku AFC-5R diffractometer with graphite-monochromated Mo K_α ($\lambda = 0.71069$ Å) radiation at 23 °C. Crystal data and other information are summarized in Table I. The $\omega - 2\theta$ scan mode with a scan rate of 16°/min was employed with the ω scan range $(1.20 + 0.3 \tan\theta)°$. A total of 2220 reflections with $|F_O| > 3\sigma(|F_O|)$ were collected up to 2θ of 55°. The collected reflection intensities were corrected for Lorentz and polarization factors, but not for absorption. The structures were solved by direct methods using the program MITHRIL.[7] The non-hydrogen atoms were refined by the full-matrix least-squares method with anisotropic temperature factors. The positions of all hydrogen atoms were calculated. Two crystallographically independent

TABLE I. Crystallographic Details for Benzene Picrate

Formula	$(C_6H_6 \cdot C_6H_3N_3O_7)_2$
Formula weight	307.22
Crystal system	Monoclinic
Space group	$P2_1/a$
Size mm	$0.4 \times 0.4 \times 0.4$
Lattice parameters	
a (Å)	14.083 (7)
b (Å)	12.920 (5)
c (Å)	14.898 (5)
β (°)	91.30 (4)
Z value	4
V (Å^3)	2710 (2)
μ (Mo$K\alpha$)/cm^{-1}	1.19
D_x (g/cm^3)	1.506
Function minimized	$\Sigma w(\|F_O\| - \|F_C\|)^2$
w	$4F_O^2/\sigma^2(F_O^2)$[a]

a) This formula is equivalent to the usual weight ($w = 1/\sigma^2(F_O)$). Using the observed intensity I, the formula $1/\sigma^2(F_O)$ can be rewritten with $\sigma(I)$ ($\equiv \sigma(F_O^2)$) as follows: $\sigma(I) = 2F_O\sigma(F_O)$, where $I = F_O^2$, then $1/\sigma^2(F_O) = 4F_O^2/\sigma^2(F_O^2)$.

TABLE II. Fractional Coordinates ($\times 10^4$) and Equivalent Isotropic Thermal Parameters (B_{eq}/Å^2) of Benzene Picrate with Estimated Standard Deviations in Parentheses

Atom	x	y	z	B_{eq}
O 1A	5478 (3)	6064 (4)	9955 (3)	8.6 (3)
O 2A	5518 (4)	7357 (5)	11218 (3)	10.3 (4)
O 3A	5791 (5)	8970 (5)	10934 (4)	12.3 (4)
O 4A	5866 (5)	10257 (4)	7985 (4)	11.7 (4)
O 5A	5604 (4)	9259 (4)	6858 (3)	9.2 (3)
O 6A	5007 (4)	5644 (4)	7332 (4)	10.6 (4)
O 7A	5615 (4)	4946 (4)	8480 (4)	10.8 (4)
N 1A	5631 (4)	8055 (6)	10705 (5)	8.7 (4)
N 2A	5677 (4)	9405 (5)	7658 (4)	7.6 (3)
N 3A	5366 (4)	5688 (5)	8068 (4)	6.8 (3)
C 1A	5524 (4)	6857 (5)	9423 (4)	5.7 (3)
C 2A	5591 (4)	7894 (6)	9716 (4)	5.7 (3)
C 3A	5655 (5)	8746 (5)	9181 (5)	6.9 (4)
C 4A	5599 (4)	8535 (5)	8268 (4)	5.5 (3)
C 5A	5509 (4)	7544 (5)	7916 (4)	5.0 (3)
C 6A	5477 (4)	6709 (5)	8490 (4)	5.2 (3)
C 7A	3119 (5)	6561 (6)	9495 (7)	8.5 (5)
C 8A	3050 (5)	7041 (7)	8750 (5)	8.0 (5)
C 9A	2985 (5)	8059 (7)	8733 (6)	8.1 (5)
C10A	3006 (5)	8589 (5)	9489 (8)	9.8 (5)
C11A	3096 (6)	8100 (10)	10287 (6)	10.9 (6)
C12A	3159 (5)	7025 (8)	10302 (6)	9.8 (5)
O 1B	1927 (3)	5927 (3)	4995 (3)	6.0 (2)
O 2B	2030 (4)	7228 (4)	6290 (3)	8.0 (3)
O 3B	1746 (4)	8825 (4)	6055 (3)	8.0 (3)
O 4B	2111 (4)	10259 (3)	3141 (3)	8.3 (3)
O 5B	1920 (4)	9275 (3)	1975 (3)	8.9 (3)
O 6B	2420 (4)	5572 (3)	2455 (3)	8.7 (3)
O 7B	1425 (4)	5005 (3)	3412 (4)	9.4 (3)
N 1B	1891 (4)	7960 (4)	5783 (3)	5.5 (3)
N 2B	2004 (4)	9414 (4)	2782 (3)	6.7 (3)
N 3B	1916 (4)	5666 (4)	3112 (4)	6.2 (3)
C 1B	1928 (4)	6771 (4)	4475 (4)	4.7 (3)
C 2B	1922 (4)	7787 (4)	4826 (3)	4.1 (3)
C 3B	1943 (4)	8642 (4)	4272 (4)	4.2 (3)
C 4B	1972 (4)	8506 (4)	3372 (4)	4.1 (3)
C 5B	1983 (4)	7543 (4)	2985 (3)	4.5 (3)
C 6B	1944 (4)	6695 (4)	3540 (4)	4.6 (3)
C 7B	4397 (5)	6329 (6)	4301 (7)	8.8 (5)
C 8B	4467 (5)	7020 (9)	3656 (5)	10.0 (5)
C 9B	4481 (5)	8046 (6)	3894 (8)	11.6 (6)
C10B	4418 (7)	8282 (7)	4783 (9)	13.1 (7)
C11B	4348 (6)	7560 (10)	5390 (6)	11.6 (7)
C12B	4342 (6)	6591 (7)	5144 (7)	9.6 (6)

$B_{eq} = (4/3)\,\Sigma_i\Sigma_j\beta_{ij}\boldsymbol{a}_i\boldsymbol{a}_j$.

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benzene : picric acid complexes existing in an asymmetric unit were represented by the suffix letters A and B. At the final refinement, 1967 reflections, out of 2220 unique reflections with $|F_o|>4\sigma(|F_o|)$ were used. Final R was 0.074 ($R_w=0.091$). Atomic scattering factors were taken from International Tables for X-Ray Crystallography (1974).[8] The final positional and thermal parameters of benzene picrate are listed in Table II. No peaks larger than $0.5\,e\text{Å}^{-3}$ were found in the last difference electron density maps. All calculations were performed using the TEXSAN[9] crystallographic software package of Molecular Structure Corporation.

Discussion

A perspective drawing with the atomic-numbering system is given in Fig. 1. Dihedral angles of benzene picrate and the angles between the overlapping planes are listed in Table III. The bond distances and angles are presented in Tables IV and V, respectively. As shown in Table III, in complexes A and B, the picric acid molecules are differently located. A large conformational difference is found at the dihedral angles of the phenyl ring and the nitro group (*e.g.*, N3–O6–O7: 24.6° for A and 39.0° for B). The phenyl ring of picric acid and the benzene ring in complexes A and B are planar. The average deviation of all the atomic positions from their respective ring planes (mean square planes of the phenyl or benzene ring atoms) is only 0.006 Å (0.001—0.018 Å). The molecules of both picric acid and benzene lie approximately in (010) parallel to each other, stacking alternately. As shown in Fig. 2, complexes A and B form one-dimensional columns along the a-axis. The plane of the phenyl ring A makes angles of 8.3 and 2.2° with the overlapping planes of the benzene ring A and benzene ring A^i (i, $0.5+x$, $1.5-y$, z), and the plane of the phenyl ring B makes angles of 2.8 and 3.5° with the overlapping planes of the benzene B and B^{ii} (ii, $-0.5+x$, $1.5-y$, z). The average interplanar distances between the phenyl ring of picric acid and the neighboring benzene rings in the complexes are 3.51 (phenyl ring A–benzene ring A), 3.52 (phenyl ring A–benzene ring A^i), 3.49 (phenyl ring B–benzene ring B), and 3.54 Å (phenyl ring B–benzene ring B^{ii}). The overlap diagrams of the complexes A and B are slightly different from each other (Fig. 3). The overlapping of the phenyl ring and the neighboring benzene rings are calculated from the overlapping areas between the phenyl ring and the projection of the benzene ring on

Table III. Selected Dihedral Angles (°) of Benzene Picrate and the Angles between Overlapping Planes

	Plane 1	Plane 2	(°)
Dihedral angles	Phenyl ring A	N1–O2–O3 A	6.2
		N2–O4–O5 A	7.9
		N3–O6–O7 A	24.6
	Phenyl ring B	N1–O2–O3 B	11.7
		N2–O4–O5 B	8.2
		N3–O6–O7 B	39.0
Angles between the overlapping planes	Phenyl ring A	Benzene ring A	8.3
		Benzene ring A^i (i, $0.5+x$, $1.5-y$, z)	2.2
	Phenyl ring B	Benzene ring B	2.8
		Benzene ring B^{ii} (ii, $-0.5+x$, $1.5-y$, z)	3.5

Table IV. Bond Lengths (Å) of Benzene Picrate with Estimated Standard Deviations in Parentheses

Complex	A	B
O1–C1	1.299 (7)	1.338 (6)
O2–N1	1.195 (8)	1.223 (6)
O3–N1	1.249 (8)	1.208 (6)
O4–N2	1.231 (7)	1.223 (6)
O5–N2	1.208 (7)	1.219 (6)
O6–N3	1.198 (7)	1.227 (6)
O7–N3	1.187 (7)	1.193 (6)
N1–C2	1.487 (8)	1.444 (7)
N2–C4	1.451 (8)	1.467 (7)
N3–C6	1.467 (8)	1.474 (7)
C1–C2	1.412 (9)	1.414 (7)
C1–C6	1.404 (8)	1.396 (7)
C2–C3	1.363 (9)	1.380 (7)
C3–C4	1.388 (8)	1.354 (7)
C4–C5	1.388 (8)	1.372 (7)
C5–C6	1.378 (7)	1.375 (7)
C7–C8	1.27 (1)	1.32 (1)
C7–C12	1.34 (1)	1.30 (1)
C8–C9	1.32 (1)	1.37 (1)
C9–C10	1.32 (1)	1.36 (1)
C10–C11	1.35 (1)	1.30 (1)
C11–C12	1.39 (1)	1.31 (1)

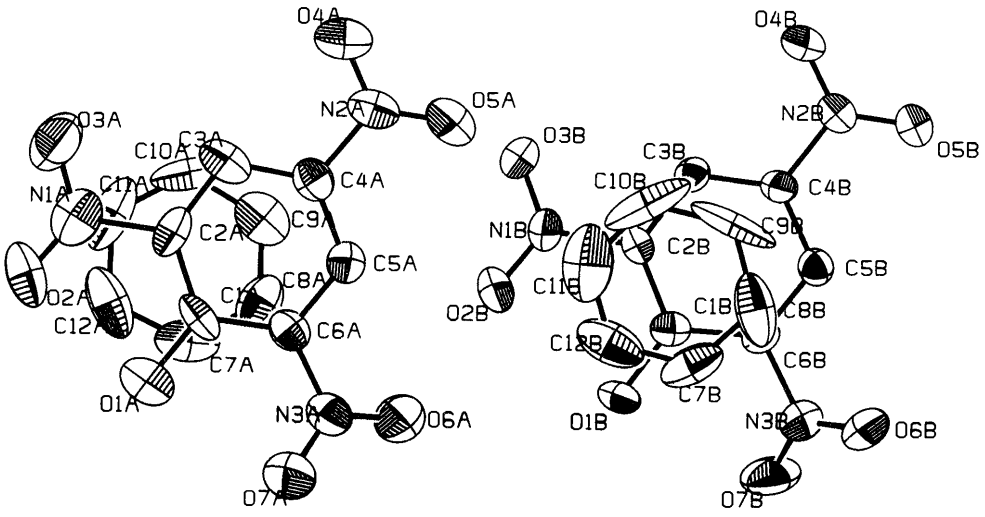

Fig. 1. Perspective Drawing of the Structure of Benzene Picrate with the Atomic-Numbering System Viewed along the *a*-Axis
Hydrogen atoms are excluded for clarity.

TABLE V. Bond Angles (°) of Benzene Picrate with Estimated Standard Deviations in Parentheses

Complex	A	B
O2–N1–O3	124.4 (7)	122.2 (5)
O2–N1–C2	121.7 (7)	118.9 (5)
O3–N1–C2	113.9 (7)	118.9 (5)
O4–N2–O5	122.9 (7)	124.8 (5)
O4–N2–C4	117.6 (6)	117.2 (5)
O5–N2–C4	119.4 (6)	118.0 (5)
O6–N3–O7	123.2 (7)	125.4 (6)
O6–N3–C6	118.3 (6)	115.1 (5)
O7–N3–C6	118.5 (6)	119.5 (6)
O1–C1–C2	124.4 (6)	122.8 (5)
O1–C1–C6	119.7 (7)	121.4 (5)
C2–C1–C6	115.9 (6)	115.8 (5)
N1–C2–C1	116.1 (6)	120.6 (5)
N1–C2–C3	117.7 (7)	117.9 (5)
C1–C2–C3	126.2 (6)	121.4 (5)
C2–C3–C4	114.3 (7)	119.3 (5)
N2–C4–C3	117.3 (6)	119.4 (5)
N2–C4–C5	119.0 (6)	118.2 (5)
C3–C4–C5	123.7 (6)	122.4 (5)
C4–C5–C6	119.5 (6)	118.0 (5)
N3–C6–C1	123.4 (6)	119.6 (5)
N3–C6–C5	116.2 (5)	117.3 (5)
C1–C6–C5	120.4 (6)	123.1 (5)
C8–C7–C12	124.3 (8)	122.2 (9)
C7–C8–C9	120.4 (8)	117.9 (9)
C8–C9–C10	120.1 (8)	117.8 (8)
C9–C10–C11	120.6 (8)	121.5 (9)
C10–C11–C12	119.1 (8)	119 (1)
C7–C12–C11	115.4 (8)	121.2 (1)

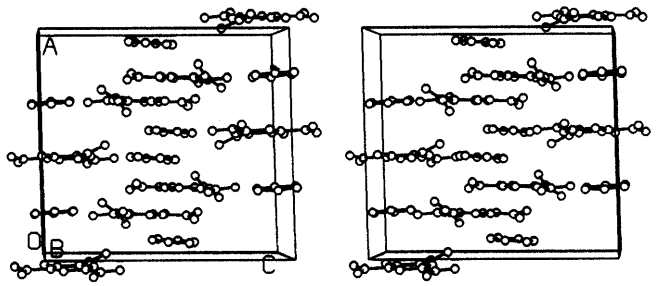

Fig. 2. Stereoscopic Drawings of the Molecular Packing of Benzene Picrate Viewed along *b*-Axis

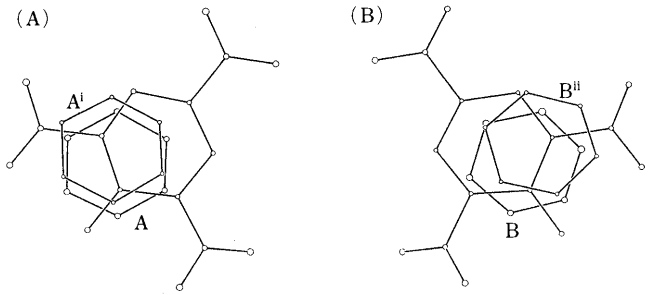

Fig. 3. Overlap of Picric Acid and Benzene Molecules in Sections Parallel to the Phenyl Ring

A, complex A; B, complex B. (i, $0.5+x$, $1.5-y$, z; ii, $-0.5+x$, $1.5-y$, z)

it, as shown in Fig. 3. They were 47% (both phenyl ring A–benzene ring A and phenyl ring A–benzene ring A^{i}), 57% (phenyl ring B–benzene ring B), and 48% (phenyl ring B–benzene ring B^{ii}). Consequently the π-electron clouds of the neighboring rings are thought to be interacting by overlapping each other. The crystal structure of benzene picrate is mainly stabilized by the Van der Waals contacts between the neighboring molecules. In the crystal of naphthalene picrate,[1] structural disorder is observed, but not in that of phenanthrene picrate.[4] In benzene picrate, there is no clear structural disorder, but as shown in Table IV, the bond lengths between C7 and C8, C8 and C9, and C9 and C10 in benzene A and C7 and C8, C7 and C12, C10 and C11, and C11 and C12 in benzene B are rather short as compared with the average value (1.39 Å). These facts are presumably a result of the superposition of two or more disordered benzene molecules, and this may be supported by the large temperature factors of the atoms of benzene molecules.

In previous studies[1,2,4] on the crystal structure of picrates of aromatic compounds, two types of hydrogen bonding have been seen. One of them is intermolecular hydrogen bonding, as found in naphthalene picrate,[1] where hydrogen bonds link pairs of picric acid molecules through phenolic hydroxyl groups and also nitro groups. The other is intramolecular hydrogen bonding between the hydroxyl group and nitro group, as found in the crystal of phenanthrene picrate.[4] But in the case of benzene picrate, no intermolecular or intramolecular hydrogen bonding is found.

In naphthalene picrate,[1] structural disorder is observed, but not in phenanthrene picrate.[4] In benzene picrate, as mentioned above, there is no clear structural disorder, but the possibility of disorder of benzene molecules remains.

As for the packing mode of the picrates of aromatic compounds, in all cases described above[1,2,4] including benzene picrate, picric acid and aromatic molecules are parallel to each other and stacked alternately. Distances between the planes of the two molecules are within the range of 3.4—3.54 Å.

Based on the results of X-ray analysis of naphthalene,[1] anthracene,[2] phenanthrene,[4] benzene picrates and pyridinium picrate,[5] it seems clear that the stable picrates are formed through ionic bonding in the case of aromatic base, and through π-bonding in the case of other aromatic compounds.

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Revised Interpretations of Electronic Substituent Constants Using Electron Number Analysis and Molecular Electrostatic Potential

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The substituent constants for sigma-inductive effect, called iota or σ_χ, were reproduced by calculation using the Takano–Hosoya–Iwata electron number (THIEN). The differences in electron numbers at a distance of R from a central atom were most effective in reproducing the sigma-inductive substituent constants, when R was set equal to 132.5 pm. On the other hand, averaged molecular electrostatic potentials reproduced well the substituent constants for electronic field effects, called σ_I or σ_F. These results indicate that the electronic substituent constants can be determined non-empirically by calculation based on quantum chemistry.

Keywords *ab initio* MO method; electron number; molecular electrostatic potential; sigma-inductive effect; field effect; substituent effect; substituent constant; regression analysis

Theoretical interpretation of substituent effects has been a long-standing problem in the field of physical organic chemistry. Recently, several important studies[1–5] on non-empirical estimations of some substituent constants have been carried out. For example, Marriot *et al.*[1–3] studied the substituent constant, especially the field effect and the σ-inductive effect using the *ab initio* molecular orbital (MO) method. They reached the following conclusions. (1) Field effect is explicable in terms of polarization in the π system of ethene when H–X (X is a substituent) lies close; σ_F was defined as $\sigma_F = -35.5\Delta q_H(\alpha)$, where q_H means the Mulliken's atomic populations at the hydrogen atoms and $\Delta q_H(\alpha)$ means the changes of the q_H values by the H–X molecule. (2) The σ-inductive effect is explicable in terms of the differences in formal charge between H–H and H–X; σ_χ was defined as $\sigma_\chi = 1 - q_H$.

Topsom[4] used molecular electrostatic potentials (*MEP*) at a certain point near the H–X or CH_3–X molecules in order to obtain the theoretical σ_F values. He introduced the following equations,

$$\sigma_{F(\text{theo.})} = 0.0635 MEP_{HX} \quad (1)$$

$$\sigma_{F(\text{theo.})} = 0.0357 MEP_{CH_3X} \quad (2)$$

Recently, Niwa[5] carried out the separation of inductive effects from mesomeric effects in aromatic systems by means of energy decomposition analysis.[6] He modeled the substituent effects using both H–X and C_6H_5–X perturbed with point charges, and obtained good correlations between the δES and the σ_I, where ES means the electrostatic component of the interaction energies.

Despite these studies,[1–5] we consider that some problems still remain unresolved. (1) The σ-inductive effect is defined using Mulliken's population analysis.[7] Nevertheless, it is well known that population analysis has poor accuracy; for example, the overlap population between a pair of atoms is artificially divided into halves for the paired atom, and the assignment is made with respect to the origin of the atomic orbital functions but not to the real spatial distribution. (2) In the case of electric field effects, all of the molecules or the point charges giving the perturbations are fixed at one point near the perturbed molecules whereas actually these molecules or charges move around the other molecules.

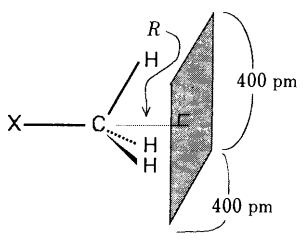

Fig. 1. A Plane on Which the *MEP* Calculations are Made

R is the distance from the methyl carbon to the center of the plane and is variable.

In this report, we have adopted Takano–Hosoya–Iwata's electron number analysis[8] (THIEN analysis) instead of Mulliken's population analysis[7] in order to explain the σ-inductive effects, since we considered that the THIEN analysis is superior to the population analysis in its accuracy. Furthermore, THIEN analysis does not involve the above-mentioned difficulties because this method gives the electron number in an arbitrary space.

In addition to this attempt, we have used the averaged *MEP*'s near the perturbed molecules. Thus more detailed information has been obtained with regard to the theoretical background of the Hammett type substituent constants.

Calculation

THIEN analysis was carried out by using the revised version of the IMSPAK[9] program package. The version used is equipped with subroutine programs for the THIEN analyses coded by Takano *et al.*,[10] and for Sakai–Huzinaga's model potential routine for the molecule containing a bromine atom coded by us. We calculated the electron number $N(R)$ and the difference electron number $\Delta N(R)$[8] in the sphere around the carbon atom in CH_3–X as defined by Takano *et al.*[8] The radius of the sphere was 25, 62.5, 100, 137.5, and 170 pm. *MEP* were calculated using the subroutine program, DENPOT.[11] The basis set used was Tatewaki–Huzinaga's MINI-1.[12] The use of the MIDI-2 basis set, instead of the MINI-1 did not improve the results.[13]

Geometries of the CH_3–X or the H–X molecules used for the MO calculations were the experimental ones[14] for X = H, CH_3, C_2H_5, CCH, CHO, COOH, $COCH_3$, CN, NO_2, NH_2, OH, OCH_3, F, SH, Cl, and Br or the optimized ones based on the modified neglect of diatomic overlap

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TABLE I. THIEN Analyses for Carbon Atom in CH_3–X

X	q_C	$N(25)$	$N(62.5)$	$N(100)$	$N(137.5)$	$N(170)$	$\Delta N(25)$	$\Delta N(62.5)$	$\Delta N(100)$	$\Delta N(137.5)$	$\Delta N(170)$
H	6.5758	1.0950	2.1763	2.9535	4.1060	5.4363	−0.0161	−0.0145	−0.0555	−0.0338	0.0550
CH_3	6.4137	1.0951	2.1734	2.9407	4.0770	5.4036	−0.0161	−0.0166	−0.0644	−0.0510	0.0386
C_2H_5	6.4195	1.0952	2.1754	2.9503	4.1007	5.4450	−0.0160	−0.0155	−0.0592	−0.0412	0.0483
CCH	6.4576	1.0945	2.1719	2.9383	4.0824	5.4306	−0.0167	−0.0198	−0.0745	−0.0702	0.0058
CHO	6.4576	1.0951	2.1764	2.9528	4.1034	5.4462	−0.0161	−0.0147	−0.0579	−0.0420	0.0389
COOH	6.4468	1.0954	2.1769	2.9547	4.1068	5.4498	−0.0158	−0.0137	−0.0530	−0.0297	0.0624
COMe	6.4582	1.0935	2.1774	2.9575	4.1131	5.4568	−0.0158	−0.0137	−0.0526	−0.0301	0.0587
CN	6.4635	1.0945	2.1723	2.9399	4.0859	5.4360	−0.0167	−0.0197	−0.0743	−0.0705	0.0028
NC	6.3353	1.0947	2.1667	2.9208	4.0520	5.4091	−0.0165	−0.0247	−0.0927	−0.1098	−0.0639
NO_2	6.3282	1.0958	2.1726	2.9406	4.0795	5.4195	−0.0153	−0.0176	−0.0673	−0.0637	−0.0026
NH_2	6.3476	1.0952	2.1714	2.9364	4.0768	5.4304	−0.0159	−0.0191	−0.0727	−0.0706	−0.0041
OH	6.2583	1.0953	2.1672	2.9220	4.0520	5.4154	−0.0158	−0.0230	−0.0872	−0.1017	−0.0590
OCH_3	6.2374	1.0954	2.1671	2.9218	4.0516	5.4185	−0.0157	−0.0231	−0.0874	−0.1029	−0.0624
F	6.1669	1.0952	2.1604	2.8970	4.0042	5.3652	−0.0158	−0.0284	−0.1078	−0.1439	−0.1270
SiH_3	6.6782	1.0961	2.1881	2.9908	4.1599	5.4745	−0.0151	−0.0030	−0.0173	0.0307	0.1357
SH	6.4949	1.0960	2.1759	2.9454	4.0682	5.3451	−0.0151	−0.0136	−0.0570	−0.0500	0.0128
Cl	6.4182	1.0960	2.1709	2.9265	4.0289	5.2882	−0.0150	−0.0182	−0.0741	−0.0853	−0.0433
Br	6.4777	1.0964	2.1734	2.9336	4.0365	5.2807	−0.0146	−0.0155	−0.0656	−0.0715	−0.0280

q_C: Mulliken's atomic populations at the carbon atom.

TABLE II. Correlation Coefficients Matrix between $N(R)$, $\Delta N(R)$, and Populations

X	q_C	q_H	$N(25)$	$N(62.5)$	$N(100)$	$N(137.5)$
q_C	1.0000					
q_H	−0.0994	1.0000				
$N(25)$	0.0944	−0.0266	1.0000			
$N(62.5)$	0.9059	0.0591	0.1197	1.0000		
$N(100)$	0.8723	0.1038	0.0090	0.9876	1.0000	
$N(137.5)$	0.7429	0.1971	−0.2249	0.8854	0.9471	1.0000
$N(170)$	0.1886	0.3636	−0.5653	0.3806	0.5189	0.7623
$\Delta N(25)$	0.1262	−0.0664	0.7887	0.2289	0.1084	−0.1638
$\Delta N(62.5)$	0.8899	0.0687	0.2330	0.9880	0.9561	0.8154
$\Delta N(100)$	0.8852	0.0936	0.1611	0.9953	0.9782	0.8659
$\Delta N(137.5)$	0.8754	0.1331	0.0472	0.9889	0.9916	0.9206
$\Delta N(170)$	0.8509	0.1633	−0.0733	0.9582	0.9787	0.9477

	$N(170)$	$\Delta N(25)$	$\Delta N(62.5)$	$\Delta N(100)$	$\Delta N(137.5)$	$\Delta N(170)$
$N(170)$	1.0000					
$\Delta N(25)$	−0.5875	1.0000				
$\Delta N(62.5)$	0.2656	0.3638	1.0000			
$\Delta N(100)$	0.3528	0.2880	0.9952	1.0000		
$\Delta N(137.5)$	0.4637	0.1568	0.9709	0.9893	1.0000	
$\Delta N(170)$	0.5507	0.0106	0.9225	0.9536	0.9868	1.0000

q_H: Mulliken's atomic populations at the hydrogen atom.

TABLE III. Correlation Coefficients between Electronic Substituent Parameters and THIEN Values

	ι[20]	σ_x[5]	σ_F[3]	$^1J_{CC}$[1]
q_C	−0.89699	−0.78558	−0.59268	−0.90305
$N(25)$	−0.15254	−0.01860	0.09496	0.13599
$N(62.5)$	−0.87146	−0.78236	−0.59508	−0.90141
$N(100)$	−0.83866	−0.76151	−0.60410	−0.90894
$N(137.5)$	−0.73637	−0.69433	−0.61290	−0.89440
$N(170)$	−0.23637	−0.29828	−0.43932	−0.56124
$\Delta N(25)$	−0.16422	−0.04333	0.12835	0.13980
$\Delta N(63.5)$	−0.88561	−0.79443	−0.58474	−0.86920
$\Delta N(100)$	−0.87973	−0.79686	−0.59877	−0.88690
$\Delta N(137.5)$	−0.87705	−0.81339	−0.63283	−0.91886
$\Delta N(170)$	−0.86971	−0.83158	−0.66414	−0.94238
ι	—	0.92128	0.78140	0.90305
σ_x	0.92128	—	0.80706	0.88295
σ_F	0.78140	0.80706	—	0.81802
$^1J_{CC}$	0.90305	0.88295	0.81802	—

(MNDO) MO method[15] for X=NC and SiH_3. MNDO MO calculations were carried out using the MNDOC[16] program which is a revised version of the QCPE program.[17]

Molecular orbital calculations in this work were performed on a NEAC S2000 plus SX-2N system at the Computation Center Osaka University, a FACOM M780/30 plus VP400E/VP200 system at the Data Processing Center, Kyoto University, and a HITAC M680H plus S820/60 system at the Computation Center at the Institute for Molecular Sciences. Statistical analyses were performed on personal computers, NEC PC9801/M/VX and EPSON PC286V using the program package for multivariate statistical analysis, MVA version 1.2[18] developed by three of us and co-workers.

Results and Discussion

σ-Inductive Effects Firstly, we calculated the electron number in the sphere, $N(R)$, and the difference electron number, $\Delta N(R)$, around the methyl carbon atom in CH_3–X (X=H, CH_3, C_2H_5, CCH, CHO, COOH, $COCH_3$, CN, NC, NO_2, NH_2, OH, OCH_3, F, SiH_3, SH, Cl, and Br), where R means the radius of the sphere. The results of THIEN analyses and population analyses are compared in Table I, and the correlation coefficients matrix is shown in Table II. Although significant correlations are observed between the populations and the $\Delta N(R)$ values except $\Delta N(25\,\text{pm})$,[19] $N(R)$ values are not always correlated adequately. The reason for the latter situation is considered to be that $N(137.5\,\text{pm})$ and $N(170\,\text{pm})$ consist of the electrons belonging not only to the methyl carbon atom but also to the hydrogen atoms and the substituents.

Although these linear relationships between the $\Delta N(R)$ values and the populations, are significant, it can not be concluded that the residuals consist of only the experimental errors or nonphysical errors. Since the THIEN analysis is apparently a better approximation than the population analysis, a better correlation is expected between the $\Delta N(R)$

TABLE IV. ΔMEP Values for the 34 Molecules

	CH_3–X					H–X				
	$R=200$ pm	$R=300$ pm	$R=400$ pm	$R=500$ pm	$R=600$ pm	$R=200$ pm	$R=300$ pm	$R=400$ pm	$R=500$ pm	$R=600$ pm
H	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CH_3	−0.0028	−0.0016	−0.0009	−0.0005	−0.0003	−0.0006	−0.0003	−0.0002	−0.0001	−0.0001
C_2H_5	−0.0031	−0.0017	−0.0010	−0.0006	−0.0004	−0.0009	−0.0004	−0.0003	−0.0002	−0.0001
CCH	0.0096	0.0060	0.0040	0.0028	0.0021	0.0120	0.0067	0.0039	0.0024	0.0016
CHO	0.0186	0.0125	0.0088	0.0065	0.0050	0.0269	0.0167	0.0109	0.0076	0.0055
COOH	0.0108	0.0073	0.0052	0.0039	0.0030	0.0146	0.0091	0.0060	0.0042	0.0031
COMe	0.0064	0.0045	0.0034	0.0026	0.0021	0.0099	0.0061	0.0041	0.0028	0.0021
CN	0.0056	0.0034	0.0022	0.0015	0.0010	0.0147	0.0082	0.0049	0.0031	0.0021
NC	0.0221	0.0146	0.0102	0.0075	0.0058	0.0346	0.0214	0.0139	0.0096	0.0070
NO_2	0.0016	0.0011	0.0008	0.0006	0.0005	0.0088	0.0054	0.0035	0.0024	0.0017
NH_2	0.0279	0.0182	0.0126	0.0092	0.0070	0.0460	0.0279	0.0180	0.0123	0.0089
OH	0.0075	0.0048	0.0032	0.0023	0.0017	0.0203	0.0123	0.0078	0.0053	0.0038
OCH_3	0.0075	0.0048	0.0032	0.0023	0.0017	0.0201	0.0121	0.0076	0.0051	0.0036
F	0.0175	0.0112	0.0076	0.0054	0.0040	0.0332	0.0199	0.0125	0.0084	0.0060
SiH_3	0.0016	0.0013	0.0010	0.0008	0.0007	−0.0014	−0.0018	−0.0010	−0.0006	−0.0003
SH	0.0143	0.0094	0.0065	0.0048	0.0036	0.0195	0.0114	0.0074	0.0051	0.0037
Cl	0.0204	0.0132	0.0090	0.0065	0.0049	0.0298	0.0175	0.0111	0.0075	0.0054

TABLE V. Correlation Coefficients Matrix between MEP Values

	CH_3–X						
	q_C	q_H	$R=200$	$R=300$	$R=400$	$R=500$	$R=600$
q_C	1.0000						
q_H	−0.0719	1.0000					
CH_3–X ($R=200$)	−0.3491	−0.8342	1.0000				
CH_3–X ($R=300$)	−0.3369	−0.8379	0.9994	1.0000			
CH_3–X ($R=400$)	−0.3259	−0.8422	0.9977	0.9994	1.0000		
CH_3–X ($R=500$)	−0.3160	−0.8437	0.9953	0.9980	0.9996	1.0000	
CH_3–X ($R=600$)	−0.3043	−0.8453	0.9919	0.9956	0.9982	0.9995	1.0000
H–X ($R=200$)	−0.5809	−0.6857	0.9556	0.9506	0.9449	0.9393	0.9317
H–X ($R=300$)	−0.5918	−0.6793	0.9523	0.9482	0.9434	0.9387	0.9319
H–X ($R=400$)	−0.5795	−0.6831	0.9550	0.9520	0.9483	0.9444	0.9385
H–X ($R=500$)	−0.5704	−0.6829	0.9558	0.9538	0.9508	0.9478	0.9427
H–X ($R=600$)	−0.5591	−0.6846	0.9575	0.9562	0.9540	0.9515	0.9472

	H–X				
	$R=200$	$R=300$	$R=400$	$R=500$	$R=600$
H–X ($R=200$)	1.0000				
H–X ($R=300$)	0.9990	1.0000			
H–X ($R=400$)	0.9976	0.9994	1.0000		
H–X ($R=500$)	0.9953	0.9979	0.9995	1.0000	
H–X ($R=600$)	0.9930	0.9960	0.9985	0.9996	1.0000

The unit of R is pm.

TABLE VI. Correlation Coefficients between MEP Values and σ_I

	σ_I		F	
	CH_3–X	H–X	CH_3–X	H–X
$R=200$ pm	0.93440	0.95462	0.92011	0.94822
$R=300$ pm	0.93232	0.95817	0.91822	0.95278
$R=400$ pm	0.93070	0.95687	0.91632	0.95483
$R=500$ pm	0.92676	0.95405	0.91283	0.95504
$R=600$ pm	0.92222	0.95088	0.90807	0.95355

values and the physical properties of the carbon atom representing σ-inductive effects. The correlation coefficients between the THIEN and the σ-inductive substituent constants (ι[20] and σ_χ) is shown in Table III together with those between the THIEN and the ^{13}C–^{13}C (ipso–ortho) indirect coupling constant ($^1J_{CC}$) in monosubstituted benzenes.

The results show that the σ_χ-$\Delta N(R)$ correlations are better than the ι-$\Delta N(R)$ ones. Hence σ_χ value should be a more useful substituent constant than ι as regards to the σ-inductive effects. Furthermore, $^1J_{CC}$ value most significantly correlate with ΔN(170 pm) whereas σ_χ needs a non-linear relationship to give the best statistics.[1] This fact shows that the $\Delta N(R)$ value may give the best fit to physical properties representing the σ-inductive effects. The results that the $\Delta N(R)$-$^1J_{CC}$ correlation at 170 pm is better than those at the smaller R values and that the π-resonance effect shows small contributions to the variations of the $^1J_{CC}$ are thought to be consistent with the well-known facts that the main contribution to the indirect coupling constants is the Fermi contact term and that the J value varies in proportion to bond order value as expressed in the equation presented by McConnell and Robertson.[21]

Field Effects MEP calculations were carried out at $21\times21\times5$ points near the CH_3–X and the H–X fragment as shown in Fig. 1. Table IV shows the averaged ΔMEP values at the 21×21 points in a plane, where $\Delta MEP = MEP_{R-X} - MEP_{R-H}$ (R = CH_3 or H). The correlation coefficients matrix between the average values of the ΔMEP's is shown in Table V. The correlations between the ΔMEP's near the CH_3–X and the H-X are not so good, as expected, because the interactions between the methyl group and the substituent X can not be neglected as mentioned by Topsom.[4] This result shows that the electric field effects for various systems can not be represented accurately by using the substituent constants.

Swain's F[22] and Taft's σ_I values which represent the electronic inductive effect show good correlations against the MEP values near the H–X (Table VI). Topsom[4] placed the positive charge at 4A from the hydrogen atom of H–X in order to calculate the theoretical σ_F values. Although the ΔMEP values at 4A from the H–X showed slightly better correlations, other values also showed similar correlations within the statistical confidential limits because the averaged values were used. Hence there is no need to place the positive charge at 4A from the hydrogen atom of

H–X when one uses averaged ΔMEP values to obtain the theoretical substituent constants representing the electric field effects.

Conclusions

(1) The electronic inductive effects can be expressed by using electron number differences as defined by Takano *et al.*[8]

(2) The ^{13}C–^{13}C (ipso–ortho) indirect coupling constant ($^1J_{CC}$) in monosubstituted benzenes highly correlates with the $\Delta N(R)$ values.

(3) The averaged ΔMEP values for the H–X systems can reproduce the substituent constants representing the electric field effects. One can estimate the electric field effects for various systems by using the averaged ΔMEP values.

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Asymmetric Reduction of 2-Aminobenzophenone Using Yeast, *Rhodosporidium toruloides*

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(±)-*N*-Isonicotinoyl-2-amino-5-chlorobenzhydrol (1) is a rice plant growth regulator which shortens the second leaf sheaths. One of the enantiomers, (*S*)-1, was obtained by microbiological asymmetric reduction of 2-amino-5-chlorobenzophenone using *Rhodosporidium toruloides* followed by isonicotinoylation. Several substituted benzhydrol derivatives were also prepared by use of the same biological method and converted to *N*-isonicotinoyl compounds. The growth-regulating activities of these compounds were evaluated.

Keywords 2-aminobenzophenone; 2-aminobenzhydrol; asymmetric reduction; microbiological reduction; yeast

(±)-*N*-Isonicotinoyl-2-amino-5-chlorobenzhydrol (**1**) exhibits a strong rice plant growth-regulating activity.[1,2] For the purpose of developing a still more active compound, both of the enantiomers, (*S*)-**1** and (*R*)-**1**, have been prepared by optical resolution of (±)-2-amino-5-chlorobenzhydrol **3** using *d*-tartaric acid, followed by isonicotinoylation. Their absolute configurations have also been determined by X-ray crystallographic analysis.[3] Comparison of the growth-regulating effects of these compounds showed that (*S*)-**1** is significantly more effective, particularly at low concentrations.[2]

Thus, we wished to prepare selectively a variety of derivatives of (*S*)-**1**, as well as (*S*)-**1** itself, in order to evaluate the structure–activity relationship. However, it was considered that application of the previously used resolution method for the resolution of the (±)-**1** derivatives would be tedious. Thus, a new and efficient method for the direct synthesis of (*S*)-**1** and its derivatives was developed by use of microbial asymmetric reduction.

The asymmetric reductions of 3- or 4-nitrobenzophenone with baker's yeast have been reported[4] to produce the corresponding (*S*)-alcohols and the aminobenzophenone derivatives. Formation of the aminoalcohols has not been observed in these reductions. Thus we carried out a screening experiment[5,6] on reduction of **2** using various microorganisms such as yeasts (*Candida* species,[7] *Hansenula* species,[7] *Rhodotorula* species[7]), bacterias (*Escherichia* species, *Salmonella* species, *Bacillus* species), and molds (*Aspergillus* species, *Penicillum* species).

Reduction took place only when *Rhodosporidium toruloides*, one of the yeasts, was used in the culture medium. Even in this case, reduction proceeded very slowly and the yield was low (11% yield, 3 d at 30 °C, Table I, run 1), but the product was found to be the benzhydrol (**3**) from its infrared (IR) and nuclear magnetic resonance (NMR) spectra. The absolute structure and the optical purity of this product were determined by high-pressure liquid chromatography (HPLC) using a Chiralpac OP (Daicel) column.

The assignment of these peaks was achieved by comparing them with those of authentic samples whose absolute configurations had been determined by X-ray crystallographic analysis (Fig. 1).[3] Namely, the peak with shorter retention time was found to correspond to that of the (*S*)-enantiomer and the peak with longer retention time to that of the (*R*)-enantiomer. Using this technique, the above reduction product was found to be the (*S*)-enantiomer and its optical purity was determined as 93%ee.

In order to establish the optimal conditions, the relation between yield and reaction period was followed.[8] As shown

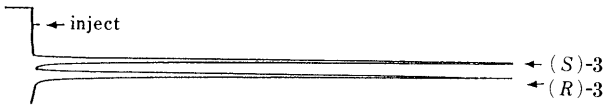


Fig. 1. HPLC Chromatogram of Each Enantiomer ((*S*)-**3** and (*R*)-**3**)

Column: Chiralpac OP (Daicel), mobile phase: 85% aqueous methanol, flow rate: 1 ml/min, detection: 254 nm.

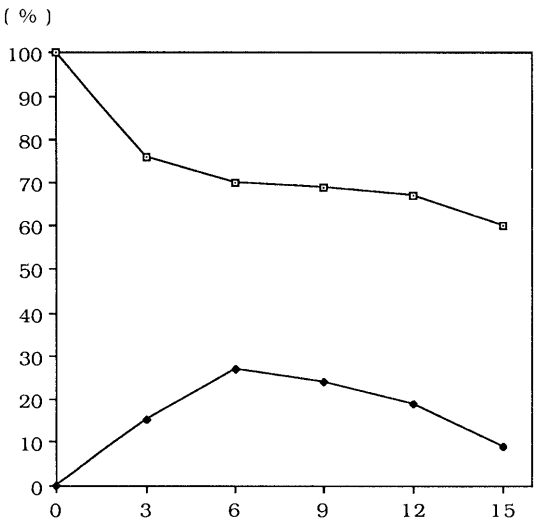
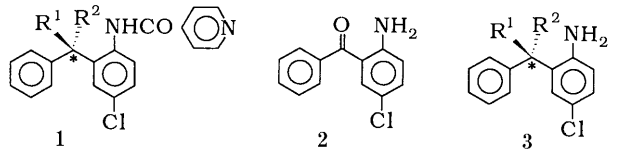


Fig. 2. Relation of Yield and Reaction Period

Substrate **2** (50 mg) in culture medium (100 ml) was incubated at 30 °C. □, **2** (residual percent); ◆, (*S*)-**3** (yield percent).



(±)-**1** : racemic mixture, inabenfide
(*S*)-**1** : R^1=H, R^2=OH (*S*-configuration)
(*R*)-**1** : R^1=OH, R^2=H (*R*-configuration)
(*S*)-**3** : R^1=H, R^2=OH
(*R*)-**3** : R^1=OH, R^2=H

Chart 1

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in Fig. 2, the yield of (*S*)-**3** increased with the decrease of **2** and became maximum after 6 to 7 d, but did not exceed 30%. Thus, our efforts were next focussed on increasing the yield of (*S*)-**3**.

In order to circumvent the low solubility of **2** in the culture medium, the effect of dispersing agents was studied. As dispersing agents, polysorbate derivatives (Tween 20, 40, 60, 80 and 85, Span 80 and 85) were selected. A mixture of **2** and a dispersing agent was added to a culture medium and the whole was shaken for 3 d at 30 °C. The results are listed in Table I. As shown in Table I, the yields of (*S*)-**3** increased when Tween 60, 80, and 85 were added (runs 4, 5, and 6). The significant feature of the effect of dispersing agents was that (S)-**3** was produced in extremely high optical yields (runs 2, 3, 4, and 5). The prolonged reaction (7 d) using Tween 80 gave the best result (60%; 99%ee).

This method was successfully applied to the preparation of optically active benzhydrol derivatives (**5**) from substituted 2-aminobenzophenone derivatives (**4**). The absolute configuration of **5b** was determined as *S* by X-ray analysis (Fig. 3).[9] The absolute configurations of the other compounds, **5a**, **c**, **d**, have not been determined yet, but are presumed to be *S* since they were prepared in the same way as (*S*)-**5b**. These compounds were finally converted to the isonicotinamide derivatives (**6**), by reaction with isonicotinoyl chloride.[10]

Yields and optical purity of compounds **5** are listed in Table II.

The activity of compounds **6a**—**d** against rice plant growth was compared with those of (±)-**1**, (*S*)-**1**, and (*R*)-**1**. A solution of one of compounds **6** at a suitable concentration (0.1, 1, or 10 ppm) was added to germination test petri dishes, and 1 week after the seeding date the second leaf sheaths of rice were measured. The growth-regulating activity of these compounds was evaluated in terms of the ratio of the length of the second leaf sheaths to that of the control.

Table I. Effect of Adding Dispersing Agents

Run	Dispersing agent	(*S*)-**3** (mg) (Yield (%))	Optical purity (% ee)
1[a]	None	5.7 (11.3)	93
2[a]	Tween 20	7.8 (15.5)	99
3[a]	Tween 40	9.1 (18.0)	99
4[a]	Tween 60	11.7 (23.2)	99
5[a]	Tween 80	11.8 (23.4)	99
6[a]	Tween 85	12.1 (24.0)	96
7[a]	Span 80	3.3 (6.5)	90
8[a]	Span 85	5.3 (10.5)	87
9[b]	Tween 80	18.1 (59.8)	99

a) The substrate **2** (50 mg) in culture medium (100 ml) containing dispersing agent (1 ml) was incubated at 30 °C for 3 d. *b*) The substrate **2** (30 mg) in culture medium (200 ml) containing dispersing agent (0.3 ml) was incubated at 30 °C for 7 d.

Table II. Benzhydrol Derivatives **5** Prepared from Substituted 2-Aminobenzophenones **4**[a]

	Yield (%)	Optical purity (% ee)[c]	Recovery of **4** (%)
5a	10	65	69
5b[b]	40	99	48
5c	20	99	43
5d	23	99	68

a) The substrate **4** (500 mg) in culture medium (1000 ml) was incubated at 30 °C for 3 d. *b*) The configuration of **5b** was found to be *S*-form by X-ray crystallographic analysis. *c*) Optical purity was determined by HPLC.

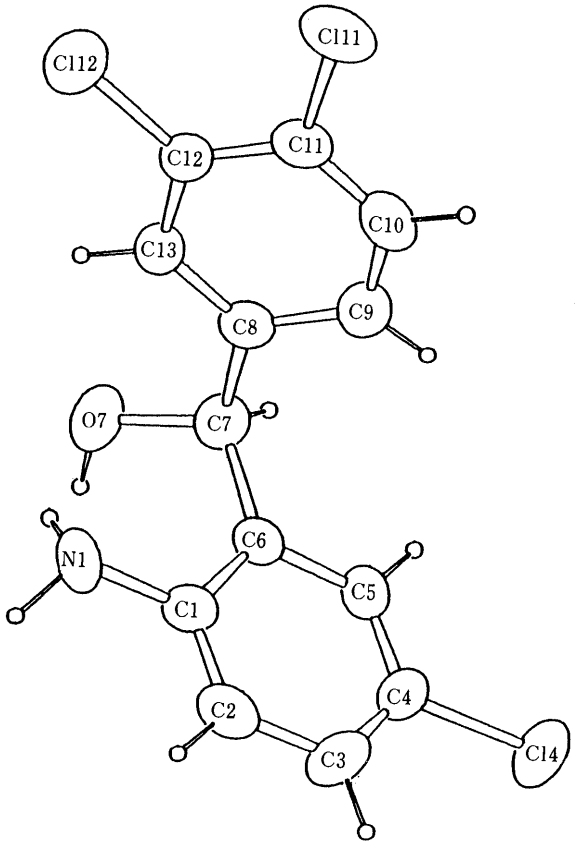
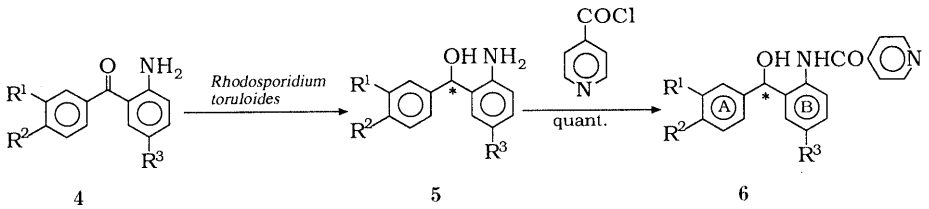


Fig. 3. ORTEP Drawing of (*S*)-**5b**



	R^1	R^2	R^3
a	H	F	Cl
b	Cl	Cl	Cl
c	H	Cl	F
d	Cl	H	Cl

Chart 2. Preparation of Substituted 2-Aminobenzhydrol and Its *N*-Isonicotinoyl Derivatives

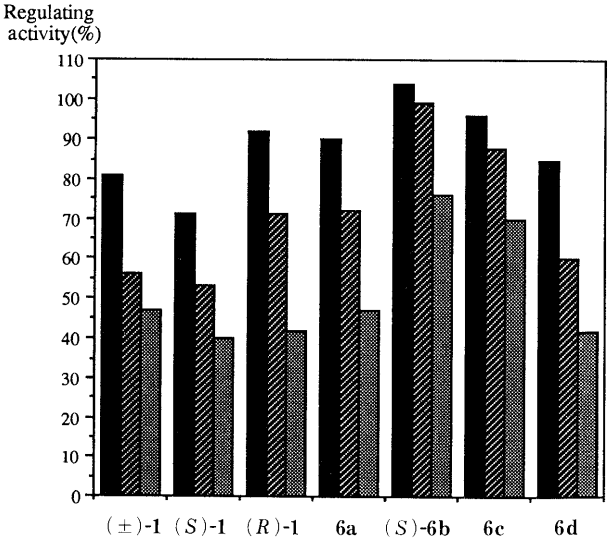


Fig. 4. Comparison of Regulating Activity of Compounds **1** and **6**
■, 0.1 ppm; ▨, 1 ppm; ▩, 10 ppm.

As reported already, the regulating activity of (±)-**1**, (*S*)-**1** and (*R*)-**1** is in the order of (*S*)-**1**>(±)-**1**>(*R*)-**1**.[2] In the cases of (*S*)-**6** having substituents on the aromatic A ring, the activities were equal to or lower than that of the (*R*)-enantiomer **1**, even though compounds **6** are in the optically active (*S*)-form which is expected to have a higher activity than the racemate or (*R*)-enantiomer.

Among the compounds so far examined, the (*S*)-isomer **1** gave the best result. Thus, a large-scale cultivation at high substrate concentration is now being examined for the large scale production of (*S*)-**1**.

Experimental

Melting points were measured with a Mettler FP61 melting point apparatus and are uncorrected. IR spectra (KBr) were measured on a Hitachi 270-30 IR spectrophotometer. NMR spectra were measured on a JEOL GX-400 instrument. Spectra were taken as 5—10% (w/v) solutions in $CDCl_3$ or CD_3OD with Me_4Si as an internal reference. HPLC was carried out on a Shimadzu SCL-6A (Daicel Chiralpac OP column (10 μm, 4.6 i.d. × 250 mm)). A Perkin-Elmer model 241 MC polarimeter was used to measure $[\alpha]_D$.

(*S*)-2-Amino-5-chlorobenzhydrol ((*S*)-3) Asymmetric reduction of **2** with *Rhodosporidium toruloides* was carried out according to the reported fermentation procedure.[5] A test tube (25 mm × 200 mm) containing 10 ml of culture medium comprising 5% glucose, 0.1% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.05% urea, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2 \cdot 2H_2O$, 0.1% yeast extract, a trace of mineral solution (0.1% $FeSO_4 \cdot 7H_2O$, 0.1% $MnCl_2 \cdot 4H_2O$, 0.1% $ZnSO_4 \cdot 7H_2O$; 0.2 ml per 100 ml of culture medium) and tap water (pH 7.0) was inoculated with *Rhodosporidium toruloides* and the yeast was cultured at 30 °C for 2 d with continuous shaking. Then 1 ml of the above seed culture was transferred to 200 ml of the same medium. After cultivation for 3d, a mixture of 30 mg of the substrate **2** and 0.3 ml of Tween 80 was added and cultivation was continued for a further 7 d under the same conditions. *n*-Hexane and ethyl acetate were added to the reaction mixture, and the whole was filtered with the aid of Celite. The organic layer of the filtrate was washed with saturated aqueous NaCl, then dried over $MgSO_4$. Removal of the solvent gave an oily product, which was subjected to preparative thin layer chromatography (silica gel, 20 cm × 20 cm: solvent, *n*-hexane–ethyl acetate (3:1)) and finally crystallized from ethyl acetate–*n*-hexane to provide (*S*)-**3** (18.1 mg, 60%) as colorless needles, mp 131—132 °C. *Anal.* Calcd for $C_{13}H_{12}ClNO$: C, 66.81; H, 5.18; Cl, 15.17; N, 5.99. Found: C, 66.98; H, 5.19; Cl, 15.43; N, 5.95. IR ν_{max} cm^{-1}: 3232 (OH), 1606 (NH_2). NMR (CD_3OD) δ: 5.66 (1H, s, CHPh), 6.56—7.29 (8H, m, Ar-H).

2-Amino-5-chloro-4′-fluorobenzhydrol (5a) The reaction was conducted in the same way as described above. Colorless needles (from ethyl acetate–*n*-hexane), mp 125—127 °C. *Anal.* Calcd for $C_{13}H_{11}ClFNO$: C, 62.04; H, 4.41; Cl, 14.09; N, 5.57. Found: C, 61.90; H, 4.36; Cl, 14.18; N, 5.48. IR ν_{max} cm^{-1}: 3180 (OH), 1606 (NH_2). NMR ($CDCl_3$) δ: 2.55 (1H, br s, OH), 3.94 (2H, br s, NH_2), 5.77 (1H, s, CHPh), 6.59—7.36 (7H, m, Ar-H).

2-Amino-5,3′,4′-trichlorobenzhydrol (5b) Colorless needles (from ethyl acetate–*n*-hexane), mp 156—158 °C. *Anal.* Calcd for $C_{13}H_{10}Cl_3NO$: C, 51.60; H, 3.33; Cl, 35.15. N, 4.63; Found: C, 51.50; H, 3.32; Cl, 35.25; N, 4.61. IR ν_{max} cm^{-1}: 3124 (OH), 1614 (NH_2). NMR ($CDCl_3$) δ: 2.62 (1H, br s, OH), 3.97 (2H, br s NH_2), 5.73 (1H, s, CHPh), 6.60—7.51 (6H, m, Ar-H).

2-Amino-4′-chloro-5-fluorobenzhydrol (5c) Colorless needles (from ethyl acetate–*n*-hexane), mp 110—112 °C. *Anal.* Calcd for $C_{13}H_{11}ClFNO$: C, 62.04; H, 4.41; Cl, 14.09; N, 5.57. Found: C, 62.05; H, 4.38; Cl, 14.08; N, 5.50. IR ν_{max} cm^{-1}: 3150 (OH), 1630 (NH_2). NMR ($CDCl_3$) δ: 2.62 (1H, br s, OH), 3.76 (2H, br s, NH_2), 5.79 (1H, s, CHPh), 6.61—7.36 (7H, m, Ar-H).

2-Amino-5,3′-dichlorobenzhydrol (5d) Colorless needles (from ethyl acetate–*n*-hexane), mp 137—138 °C. *Anal.* Calcd for $C_{13}H_{11}Cl_2NO$: C, 58.23; H, 4.13; Cl, 26.44; N, 5.22. Found: C, 58.21; H, 4.13; Cl, 26.31; N, 5.19. IR ν_{max} cm^{-1}: 3136 (OH), 1598 (NH_2). NMR ($CDCl_3$) δ: 2.62 (1H, br s, OH), 3.95 (2H, br s, NH_2), 5.76 (1H, s, CHPh), 6.59—7.41 (7H, m, Ar-H).

(*S*)-*N*-Isonicotinoyl-2-amino-5-chlorobenzhydrol ((*S*)-1) A mixture of (*S*)-**3** (50 mg) and the hydrochloride of isonicotinoyl chloride (46 mg) in ethyl acetate (2 ml) was stirred for 16 h at room remperature. After the addition of saturated aqueous $NaHCO_3$, the reaction mixture was stirred for several minutes. The organic layer was washed with saturated aqueous NaCl, then dried over $MgSO_4$. Removal of the solvent gave **1b** (70 mg, 99%) as colorless needles (from ethyl acetate–*n*-hexane), mp 163—165 °C. *Anal.* Calcd for $C_{19}H_{15}ClN_2O_2$: C, 67.36; H, 4.46; Cl, 10.46; N, 8.27. Found: C, 67.20; H, 4.45; Cl, 10.65; N, 8.23. $[\alpha]_D^{22}$ +16.4° (*c*=1, MeOH). IR ν_{max} cm^{-1}: 3060 (OH), 1686 (NHCO). NMR (CD_3OD) δ: 5.87 (1H, s, CHPh), 7.11—8.61 (12H, m, Ar-H).

***N*-Isonicotinoyl-2-amino-5-chloro-4′-fluorobenzhydrorol (6a)** The reaction was conducted in the same way as noted above. Colorless needles (from ethyl acetate–*n*-hexane), mp 176—177 °C. *Anal.* Calcd for $C_{19}H_{14}ClFN_2O_2$: C, 63.96; H, 3.96; Cl, 9.94; N, 7.85. Found: C, 63.94; H, 3.90; Cl, 9.98; N, 7.80. $[\alpha]_D^{22}$ +8.0° (*c*=1, MeOH). IR ν_{max} cm^{-1}: 3050 (OH), 1690 (NHCO). NMR (CD_3OD) δ: 5.95 (1H, s, CHPh), 7.00—8.62 (11H, m, Ar-H).

***N*-Isonicotinoyl-2-amino-5,3′,4′-trichlorobenzhydrol (6b)** Colorless needles (from ethyl acetate–*n*-hexane), mp 176—177 °C. *Anal.* Calcd for $C_{19}H_{13}Cl_3N_2O_2$: C, 55.98; H, 3.21; Cl, 26.09; N, 6.87. Found: C, 55.95; H, 3.24; Cl, 26.07; N, 6.89. $[\alpha]_D^{22}$ −25.9° (*c*=1, MeOH). IR ν_{max} cm^{-1}: 3080 (OH), 1683 (NHCO). NMR (CD_3OD) δ: 5.81 (1H, s, CHPh), 6.98—8.64 (10H, m, Ar-H).

***N*-Isonicotinoyl-2-amino-4′chloro-5-fluorobenzhydrol (6c)** Colorless needles (from ethyl acetate–*n*-hexane), mp 152—153 °C. *Anal.* Calcd for $C_{19}H_{14}ClFN_2O_2$: C, 63.96; H, 3.96; Cl, 9.94; N, 7.85. Found: C, 63.70; H, 4.01; Cl, 9.85; N, 7.78. $[\alpha]_D^{22}$ −18.3° (*c*=1, MeOH). IR ν_{max} cm^{-1}: 3100 (OH), 1686 (NHCO). NMR (CD_3OD) δ: 5.84 (1H, s, CHPh), 7.00—8.61 (11H, s, Ar-H).

***N*-Isonicotinoyl-2-amino-5,3′-dichlorobenzhydrol (6d)** Colorless needles (from ethyl acetate–*n*-hexane), mp 167—169 °C. *Anal.* Calcd for $C_{19}H_{14}Cl_2N_2O_2$: C, 61.14; H, 3.78; Cl, 19.00; N, 7.51. Found: C, 61.16; H, 3.78; Cl, 18.92; N, 7.34. $[\alpha]_D^{22}$ +1.6° (*c*=1, MeOH). IR ν_{max} cm^{-1}: 3070 (OH), 1689 (NHCO). NMR (CD_3OD) δ: 5.83 (1H, s, CHPh), 7.00—8.62 (11H, m, Ar-H).

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8) Five reactions were started at the same time and one was quenched every three days. The starting material and product were isolated.
9) *Acta Crystallogr.*, in preparation.
10) All reactions proceeded quantitatively.

Direct Transformation of *O*-Glycoside into Glycosyl Bromide with the Combination of Trimethylsilyl Bromide and Zinc Bromide

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A novel direct transformation of *O*-glycoside into glycosyl bromide by using trimethylsilyl bromide and zinc bromide has been developed. Treatment of various glycosides (3a—5a, 10, 12 and 14) with trimethylsilyl bromide and zinc bromide in dichloromethane afforded the glycosyl bromides (6, 11, 13 and 15) in 53, 85, 94, 47, 41 and 40% yields, respectively.

Keywords glycosyl bromide; trimethylsilyl bromide; zinc bromide; glucosamine; glucose; galactose; mannose; *O*-glycoside

Glycosyl bromides have been used as key intermediates in glycosidic bond formation.[1] They are generally prepared from the corresponding 1-*O*-acyl derivative by utilizing hydrogen bromide.[2] Recently, several methods have been reported for the direct conversion of *O*-glycosides into glycosyl halides.[3] In our preceding paper,[4] we reported that the combination of trimethylsilyl bromide (TMSBr) and zinc bromide ($ZnBr_2$) was a good catalyst for anomerization of the β-glycoside of D-glucosamine to the α-glycoside. In this glycoside anomerization reaction, glycosyl bromide was obtained as a by-product under certain reaction conditions. From these results, we suspected that the combination of TMSBr and $ZnBr_2$ could be employed as a halogenating agent for the preparation of glycosyl bromides from *O*-glycosides. In this paper we describe a facile method for converting *O*-glycosides to glycosyl bromides by using TMSBr and $ZnBr_2$.[5]

The starting materials (**3a—5a**, **3b—5b**, **10**, **12** and **14**) for this bromination reaction are shown in Chart 1. Glucosamine derivatives (**3a—5a**, **3b—5b**) were prepared from **2**[6] by Fischer's glycosidation followed by acetylation and separation using silica gel column chromatography. The other substrates (**10**, **12** and **14**)[3f] were prepared from commercially available methyl glycopyranosides by standard acetylation.

Direct transformation of *O*-glycosides (**3a—5a**, **3b—5b**) into glycosyl bromide (**6**) was investigated. The results are summarized in Table I. TMSBr has been used for the direct conversion of methyl glycosides into the corresponding glycosyl bromide.[3b] However, when the α-glycosides (**3a—5a**) were treated with 1.0 eq of TMSBr in dichloromethane at room temperature for 24 h, no reaction occurred. When the β-methyl glycoside (**3b**) was treated with 1.0 eq of TMSBr, no 1-bromo sugar (**6**) was obtained, whereas anomerization took place to give α-methyl glycoside (**3a**) in 6% yield along with 93% recovery of the starting material (Table I, run 1). In a similar reaction of β-isopropyl glycoside (**4b**), the anomerized product (**4a**) was obtained in 43% yield along with 51% recovery of the starting material (Table I, run 2). When β-benzyl glycoside (**5b**) was treated with TMSBr (1.0 eq), however, a 14% yield of an inseparable mixture of the bromide (**6**)[6] and the anomerized product (**5a**) in a ratio of 1 : 1 was obtained together with 86% recovery of the starting material (**5b**) (Table I, run 3). Thus, attempted transformation of *O*-glycosides into glycosyl bromide by use of TMSBr alone was found to be difficult, and so we turned our attention to the bromination with the combination of TMSBr and $ZnBr_2$.

When an α-methyl glycoside (**3a**) was treated with TMSBr (1.0 eq) and $ZnBr_2$ (1.0 eq) in dichloromethane at room temperature for 5 h, the bromide (**6**) was obtained in 35% yield along with 63% recovery of the starting material

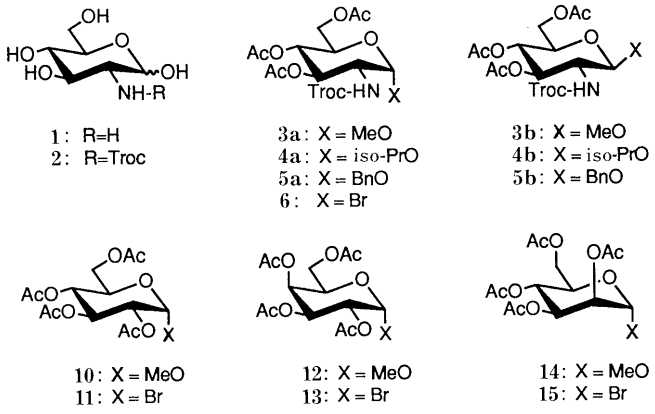

Chart 1

TABLE I. Transformation of *O*-Glycoside (**3a—5a**, **3b—5b**, **10**, **12** and **14**) into Glycosyl Bromides (**6**, **11**, **13** and **15**)

Run	Substrate	Reagent (eq)	Time[a] (h)	Products (% yield)[b]
1	**3b**	TMSBr(1.0)	24	**3a** (6), **3b** (93)[c]
2	**4b**	TMSBr(1.0)	24	**4a** (43), **4b** (51)[c]
3	**5b**	TMSBr(1.0)	24	**6** (7),[d] **5a** (7), **5b** (86)[c]
4	**3a**	TMSBr(1.0)–$ZnBr_2$(1.0)	5	**6** (35), **3a** (63)[c]
5	**3b**	TMSBr(1.0)–$ZnBr_2$(1.0)	5	**6** (43), **3a** (51)
6	**4a**	TMSBr(1.0)–$ZnBr_2$(1.0)	4	**6** (46), **4a** (53)[c]
7	**4b**	TMSBr(1.0)–$ZnBr_2$(1.0)	4	**6** (41), **4a** (45)
8	**5a**	TMSBr(1.0)–$ZnBr_2$(1.0)	5	**6** (37),[d] **5a** (50)[c]
9	**5b**	TMSBr(1.0)–$ZnBr_2$(1.0)	5	**6** (81),[d] **5a** (16)
10	**3a**	TMSBr(3.0)–$ZnBr_2$(1.0)	24	**6** (53), **3a** (43)[c]
11	**4a**	TMSBr(3.0)–$ZnBr_2$(1.0)	15	**6** (85)
12	**4b**	TMSBr(3.0)–$ZnBr_2$(1.0)	15	**6** (88)
13	**5a**	TMSBr(3.0)–$ZnBr_2$(1.0)	24	**6** (94)
14	**10**	TMSBr(3.0)–$ZnBr_2$(1.0)	24	**11** (47), **10** (9)[c]
15	**12**	TMSBr(3.0)–$ZnBr_2$(1.0)	24	**13** (41), **12** (4)[c]
16	**14**	TMSBr(3.0)–$ZnBr_2$(1.0)	24	**15** (40), **14** (9)[c]

a) All reactions were carried out in dichloromethane at room temperature. *b*) Isolated yield unless otherwise noted. *c*) The starting material was recovered. *d*) The bromide (**6**) and α-glycoside (**5a**) could not be separated by column chromatography, so the yields of **6** and **5a** were determined by ^{1}H-NMR.

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(Table I, run 4). In a similar reaction of the β-methyl glycoside (**3b**), the bromide (**6**) and the anomerized product (**3a**) were obtained in 43 and 51% yields, respectively (Table I, run 5). These results indicate that an active species generated from TMSBr and $ZnBr_2$ participates in the cleavage of the glycoside bond and subsequent bromination. When isopropyl and benzyl glycosides (**4a—b**, **5a—b**) were used as starting materials, similar results were obtained (Table I, runs 6—9). When the α-methyl glycoside was treated with 3.0 eq of TMSBr and $ZnBr_2$ (1.0 eq), the yield of the bromide (**6**) was improved to 53% (Table I, run 10). When the isopropyl glycosides (**4a—b**) were subjected to bromination with 3.0 eq of TMSBr and $ZnBr_2$ (1.0 eq), no starting material (**4a—b**) or anomerized product (**4a**) was found, and the bromide (**6**) was obtained in 85% yield from **4a** and 88% yield from **4b** (Table I, runs 11—12). Treatment of α-benzyl glycoside (**5a**) with TMSBr (3.0 eq) and $ZnBr_2$ (1.0 eq) afforded the bromide (**6**) in 94% yield.

We have succeeded in developing a novel direct transformation of glucosamine *O*-glycoside into glycosyl bromide with the combined use of TMSBr and $ZnBr_2$. To ascertain the limits of this methodology, we then investigated the bromination of some other hexopyranosides (**10**, **12** and **14**). The results are summarized in Table I. When the hexopyranosides (**10**, **12** and **14**) were treated with 3.0 eq of TMSBr in dichloromethane at room temperature for 24 h, no reaction occurred. When the reaction was carried out with the combined use of TMSBr (3.0 eq) and $ZnBr_2$ (1.0 eq) at room temperature for 24 h, the corresponding glycosyl bromides (**11**, **13** and **15**) were obtained in 47, 41 and 40% yield, respectively (Table I, runs 14—16). As the reaction time was lengthened to increase consumption of the starting material, the formation of several unidentified polar products increased.

We have postulated a mechanism for the formation of glycosyl bromide (**6**) catalyzed by TMSBr and $ZnBr_2$ as shown in Chart 2. The reaction of the other hexopyranosides (**10**, **12** and **14**) was also presumed to proceed *via* similar mechanisms. Thus, oxonium salts (**8b**), which were formed by the reaction of β-glycosides (**7b**) and an active species generated from TMSBr and $ZnBr_2$, collapsed to **9** with liberation of the trimethylsilyl ether. Next, the reaction seemed to proceed *via* two pathways. The bromide (**6**) was presumed to have been formed by bromination of the intermediate (**9**) with the resulting activated brominium anion. The anomerization of **7b** to the thermodynamically more stable α-glycosides (**7a**) would be accounted for by the conversion of **9** to intermediates (**8a**), followed by the liberation of TMSBr. The conversion of α-glycosides (**7a**) to the bromide (**6**) seemed to proceed *via* the reverse of the pathway stated above. When excess TMSBr was present in the reaction system, the pathway from **9** to **6** was considered to be predominant.

In summary, a novel transformation of *O*-glycoside into glycosyl bromide was achieved by the use of TMSBr and $ZnBr_2$. The reaction conditions are extremely mild, and this new method should be widely applicable in carbohydrate chemistry.

Experimental

Melting points were determined on a Yanagimoto melting point apparatus, and are uncorrected. Infrared (IR) spectra were taken on a Hitachi 270-30 IR spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained in deuteriochloroform on a JEOL GSX 500 spectrometer (500 MHz). Chemical shifts are reported in parts per million relative to tetramethylsilane (δ units) as an internal standard. Optical rotations were measured with a Horiba SEDA 200 polarimeter. Column chromatography was performed with Merck Silica gel 60 (70—230 mesh).

Methyl 3,4,6-Tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α- and -β-D-glucopyranoside (3a, 3b) A 5% HCl–dioxane mixture (25 ml) was added to a suspension of **2**[6] (4.6 g, 12.8 mmol) in MeOH (100 ml), and the mixture was heated under reflux for 2 h. The solution was concentrated under reduced pressure to give the methyl glycoside, which was treated with pyridine (30 ml) and Ac_2O (20 ml) for 1 h at room temperature. The solution was concentrated under reduced pressure and the residue was partitioned between AcOEt and 5% $NaHCO_3$. The separated organic layer was washed with water, 1 N HCl and water, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was chromatographed on silica gel (200 g) using chloroform–acetone (50 : 1) to give 4.8 g (76%) of **3a** and 1.5 g (24%) of **3b**, as colorless crystals, in that order of elution.

3a: mp 119—124 °C. $[\alpha]_D^{25}$ +64.7° (c=1.45, $CHCl_3$). *Anal.* Calcd for $C_{16}H_{22}Cl_3NO_{10}$: C, 38.85; H, 4.48; N, 2.83. Found: C, 38.90; H, 4.59; N, 2.97. IR (KBr): 3376, 1755, 1554, 1452, 1374, 1230, 1170 cm^{-1}. ^{1}H-NMR δ: 2.00, 2.03, 2.10 (each 3H, s, $OCOCH_3$), 3.43 (3H, s, OCH_3), 3.96 (1H, ddd, J=10.0, 4.0, 2.0 Hz, H-5), 4.06 (1H, dd, J=10.0, 3.0 Hz, H-2), 4.11 (1H, dd, J=12.0, 2.0 Hz, H-6), 4.26 (1H, dd, J=12.0, 4.0 Hz, H-6′), 4.63 (1H, d, J=11.9 Hz, $COOCH_2CCl_3$), 4.78 (1H, d, J=3.0 Hz, H-1), 4.81 (1H, d, J=11.9 Hz, $COOCH_2CCl_3$), 5.11 (1H, t, J=10.0 Hz,

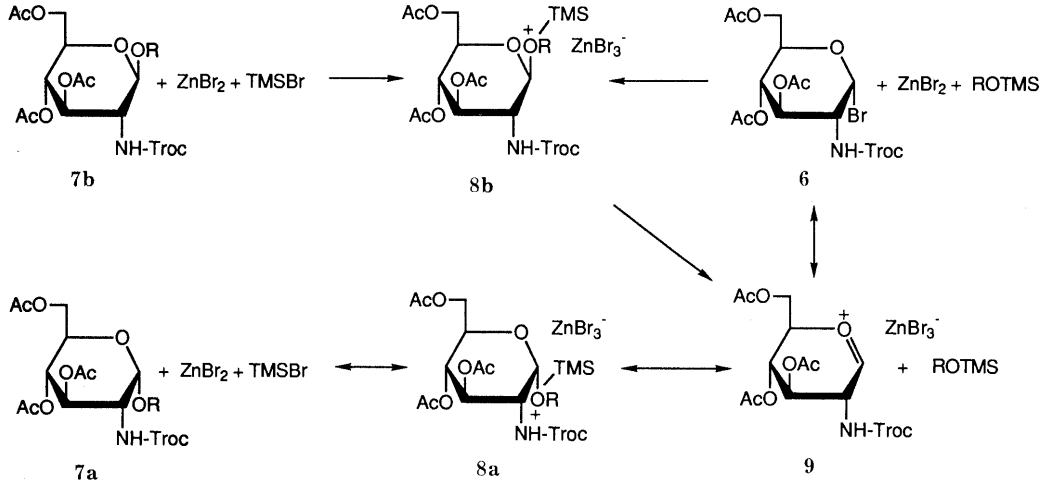

Chart 2

H-4), 5.26 (1H, t, J=10.0 Hz, H-3), 5.26 (1H, d, J=10.0 Hz, NH).

3b: mp 85—93 °C. $[\alpha]_D^{25}$ +15.8° (c=1.00, $CHCl_3$). *Anal.* Calcd for $C_{16}H_{22}Cl_3NO_{10}\cdot H_2O$: C, 37.48; H, 4.72; N, 2.73. Found: C, 37.36; H, 4.34; N, 2.85. IR (KBr): 3352, 2920, 1746, 1536, 1455, 1371, 1239, 1167 cm^{-1}. ^{1}H-NMR δ: 2.03 (6H, s, $OCOCH_3$), 2.09 (3H, s, $OCOCH_3$), 3.52 (3H, s, OCH_3), 3.63 (1H, q, J=9.0 Hz, H-2), 3.71 (1H, m, H-5), 4.15 (1H, dd, J=12.0, 2.5 Hz, H-6), 4.30 (1H, dd, J=12.0, 4.0 Hz, H-6'), 4.55 (1H, d, J=8.0 Hz, H-1), 4.65 (1H, d, J=11.9 Hz, $COOCH_2CCl_3$), 4.81 (1H, d, J=11.9 Hz, $COOCH_2CCl_3$), 5.08 (1H, t, J=10.0 Hz, H-4), 5.19 (1H, br, NH), 5.30 (1H, t, J=10.0 Hz, H-3).

Isopropyl 3,4,6-Tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α- and -β-D-glucopyranoside (4a, 4b) Compounds **4a** and **4b** were prepared from **2** as described for the preparation of **3a** and **3b**, in 61 and 7% yields.

4a: Colorless crystals. mp 122—125 °C. $[\alpha]_D^{25}$ +95.7° (c=0.70, $CHCl_3$). *Anal.* Calcd for $C_{18}H_{26}Cl_3NO_{10}$: C, 41.36; H, 5.01; N, 2.68. Found: C, 41.20; H, 5.26; N, 2.72. IR (KBr): 3300, 2980, 1755, 1545, 1377, 1240 cm^{-1}. ^{1}H-NMR δ: 1.18, 1.25 (each 3H, d, J=6.4 Hz, CH_3), 2.01, 2.04, 2.09 (each 3H, s, $OCOCH_3$), 3.91 (1H, m, CH), 4.05 (2H, m, H-2, H-5), 4.09 (1H, dd, J=12.0, 2.4 Hz, H-6), 4.25 (1H, dd, J=12.0, 4.0 Hz, H-6'), 4.68 (1H, d, J=11.9 Hz, $COOCH_2CCl_3$), 4.77 (1H, d, J=11.9 Hz, $COOCH_2CCl_3$), 4.98 (1H, d, J=4.0 Hz, H-1), 5.09 (1H, t, J=10.0 Hz, H-4), 5.19 (1H, d, J=10.0 Hz, NH), 5.24 (1H, t, J=10.0 Hz, H-3).

4b: Colorless crystals. mp 149—151 °C. $[\alpha]_D^{25}$ +3.8° (c=0.64, $CHCl_3$). *Anal.* Calcd for $C_{18}H_{26}Cl_3NO_{10}$: C, 41.36; H, 5.01; N, 2.68. Found: C, 41.44; H, 5.17; N, 2.79. IR (KBr): 3400, 2990, 1755, 1545, 1380, 1230 cm^{-1}. ^{1}H-NMR δ: 1.14, 1.24 (each 3H, d, J=6.4 Hz, CH_3), 2.02, 2.03, 2.08 (each 3H, s, $OCOCH_3$), 3.50 (1H, m, H-2), 3.70 (1H, m, H-5), 3.95 (1H, m, CH), 4.12 (1H, dd, J=12.0, 2.4 Hz, H-6), 4.26 (1H, dd, J=12.0, 5.0 Hz, H-6'), 4.68 (1H, d, J=11.9 Hz, $COOCH_2CCl_3$), 4.75 (1H, br, H-1), 4.76 (1H, d, J=11.9 Hz, $COOCH_2CCl_3$), 5.04 (1H, t, J=10.0 Hz, H-4), 5.13 (1H, br, NH), 5.37 (1H, t, J=10.0 Hz, H-3).

Benzyl 3,4,6-Tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α- and -β-D-glucopyranoside (5a, 5b) Compounds **5a** and **5b** were prepared from **2** as described for the preparation of **3a** and **3b**, in 34 and 16% yields.

5a: A colorless oil. $[\alpha]_D^{25}$ +82.4° (c=0.50, $CHCl_3$). ^{1}H-NMR δ: 2.00, 2.03, 2.11 (each 3H, s, $OCOCH_3$), 4.00 (1H, m, H-5), 4.04 (1H, m, H-2), 4.08 (1H, dd, J=12.0, 4.0 Hz, H-6), 4.25 (1H, dd, J=12.0, 4.0 Hz, H-6'), 4.55 (1H, d, J=11.0 Hz, CH_2Ph), 4.64 (1H, d, J=11.9 Hz, $COOCH_2CCl_3$), 4.73 (1H, d, J=11.0 Hz, CH_2Ph), 4.75 (1H, d, J=11.9 Hz, $COOCH_2CCl_3$), 4.99 (1H, d, J=4.0 Hz, H-1), 5.12 (1H, t, J=10.0 Hz, H-4), 5.28 (1H, t, J=10.0 Hz, H-3), 7.33—7.40 (5H, m, arom. H).

5b: Colorless crystals. 122—123 °C. $[\alpha]_D^{25}$ −22.7° (c=0.70, $CHCl_3$). *Anal.* Calcd for $C_{22}H_{26}Cl_3NO_{10}$: C, 46.36; H, 4.88; N, 2.58. Found: C, 46.29; H, 4.59; N, 2.45. IR (KBr): 3370, 2956, 1752, 1725, 1545, 1458, 1374, 1296 cm^{-1}. ^{1}H-NMR δ: 2.02 (6H, s, $OCOCH_3$), 2.11 (3H, s, $OCOCH_3$), 3.66 (1H, m, H-5), 3.73 (1H, m, H-2), 4.17 (1H, dd, J=12.0, 2.0 Hz, H-6), 4.30 (1H, dd, J=12.0, 5.0 Hz, H-6'), 4.62 (2H, d, J=11.0 Hz, $COOCH_2CCl_3$), 4.71 (2H, s, CH_2Ph), 4.92 (1H, d, J=12.0 Hz, H-1), 5.03 (1H, br, NH), 5.09 (1H, t, J=10.0 Hz, H-4), 5.23 (1H, d, J=10.0 Hz, H-3), 7.30—7.36 (5H, m, arom. H).

Typical Procedure for Reaction of *O*-Glycoside with TMSBr A solution of **5b** (71 mg, 0.11 mmol) and TMSBr (16 mg, 0.11 mmol) in dichloromethane (2 ml) was stirred for 24 h at room temperature. After concentration of the reaction mixture, the residue was chromatographed on silica gel (6 g) with chloroform to give an inseparable 1 : 1 mixture of **6** and **5a** (8 mg), and subsequently 61 mg (86%) of the starting material (**5b**) was recovered.

General Procedure for Bromination with Combined Use of Trimethylsilyl Bromide and Zinc Bromide $ZnBr_2$ (45 mg, 0.20 mmol) was added to a solution **3b** (100 mg, 0.20 mmol) and TMSBr (31 mg, 0.20 mmol) in dichloromethane (2 ml), and stirring was continued for 5 h at room temperature. The reaction mixture was diluted with AcOEt, washed with 5% $NaHCO_3$ and water, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was chromatographed on silica gel (20 g) with chloroform–acetone (50 : 1) to give 47 mg (43%) of **6** and 51 mg (51%) of **3a**, in order of elution.

6: A colorless foam. $[\alpha]_D^{25}$ +127.0° (c=1.11, $CHCl_3$). ^{1}H-NMR δ: 2.05, 2.07, 2.11 (each 3H, s, $OCOCH_3$), 4.14 (2H, m, H-2, H-6), 4.27 (1H, m, H-5), 4.33 (1H, dd, J=12.2, 3.9 Hz, H-6'), 4.67 (1H, d, J=12.2 Hz, $COOCH_2CCl_3$), 4.82 (1H, d, J=12.2 Hz, $COOCH_2CCl_3$), 5.25 (1H, t, J=9.8 Hz, H-3), 5.31 (1H, d, J=9.3 Hz, NH), 5.37 (1H, t, J=9.8 Hz, H-4), 6.53 (1H, d, J=3.4 Hz, H-1).

1-Bromo-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose (11) $ZnBr_2$ (124 mg, 0.55 mmol) was added to a solution of **10** (200 mg, 0.55 mmol) and TMSBr (252 mg, 1.65 mmol) in dichloromethane (10 ml), and stirring was continued for 24 h at room temperature. The reaction mixture was diluted with AcOEt and washed with 5% $NaHCO_3$ and water, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was chromatographed on silica gel (20 g) using hexane–AcOEt (5 : 1) to give 106 mg (47%) of **11** and 17 mg (9%) of **10** in order of elution.

11: A colorless oil. $[\alpha]_D^{25}$ +177.1° (c=1.45, $CHCl_3$), [lit.[3f] $[\alpha]_D^{25}$ +196°]. ^{1}H-NMR δ: 2.04, 2.05, 2.10, 2.10 (each 3H, s, $OCOCH_3$), 4.13 (1H, dd, J=12.5, 2.0 Hz, H-6), 4.30 (1H, ddd, J=9.8, 4.2, 2.0 Hz, H-5), 4.33 (1H, dd, J=12.5, 4.2 Hz, H-6'), 4.84 (1H, dd, J=9.8, 4.2 Hz, H-2), 5.16 (1H, t, J=9.8 Hz, H-4), 5.56 (1H, t, J=9.8 Hz, H-3), 6.61 (1H, d, J=4.2 Hz, H-1).

1-Bromo-2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranose (13) Compound **13** was prepared from **12** as described for preparation of **11**, in 41% yield.

13: A colorless oil. $[\alpha]_D^{25}$ +201.6° (c=1.03, $CHCl_3$). [lit.[3f] $[\alpha]_D^{25}$ +212°]. ^{1}H-NMR δ: 2.01, 2.06, 2.11, 2.15 (each 3H, s, $OCOCH_3$), 4.11 (1H, dd, J=11.2, 6.6 Hz, H-6), 4.19 (1H, dd, J=11.2, 6.6 Hz, H-6'), 4.49 (1H, t, J=6.6 Hz, H-5), 5.05 (1H, dd, J=10.5, 3.9 Hz, H-2), 5.41 (1H, dd, J=10.5, 3.2 Hz, H-3), 5.52 (1H, d, J=3.2 Hz, H-4), 6.70 (1H, d, J=3.9 Hz, H-1).

1-Bromo-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranose (15) Compound **15** was prepared from **14** as described for preparation of **11**, in 40% yield.

15: A colorless oil. $[\alpha]_D^{25}$ +124.6° (c=1.09, $CHCl_3$). [lit.[3f] $[\alpha]_D^{25}$ +122°]. ^{1}H-NMR δ: 2.01, 2.08, 2.11, 2.18 (each 3H, s, $OCOCH_3$), 4.14 (1H, dd, J=12.5, 2.0 Hz, H-6), 4.22 (1H, ddd, J=10.3, 4.9, 2.0 Hz, H-5), 4.33 (1H, dd, J=12.5, 4.9 Hz, H-6'), 5.37 (1H, t, J=10.3 Hz, H-4), 5.45 (1H, dd, J=3.4, 1.5 Hz, H-2), 5.72 (1H, dd, J=10.3, 3.4 Hz, H-3), 6.30 (1H, d, J=1.5 Hz, H-1).

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Preparation of Heptakis(6-*O*-(*p*-tosyl))-β-cyclodextrin and Heptakis(6-*O*-(*p*-tosyl))-2-*O*-(*p*-tosyl)-β-cyclodextrin and Their Conversion to Heptakis(3,6-anhydro)-β-cyclodextrin

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Heptakis(6-*O*-(*p*-tosyl))-β-cyclodextrin and heptakis(6-*O*-(*p*-tosyl))-2-*O*-(*p*-tosyl)-β-cyclodextrin were prepared by the reaction of β-cyclodextrin with *p*-tosyl chloride in pyridine. They were converted to heptakis(3,6-anhydro)-β-cyclodextrin, constituted from alternative ($^1C_4$) glucose units.

Keywords cyclodextrin; oligosaccharide; enzyme model; heptakis(6-*O*-(*p*-tosyl))-β-cyclodextrin; heptakis(6-*O*-(*p*-tosyl))-2-*O*-(*p*-tosyl)-β-cyclodextrin; alternative cyclodextrin; 3,6-anhydro-cyclodextrin; heptakis(3,6-anhydro)-β-cyclodextrin; hexakis(3,6-anhydro)-α-cyclodextrin

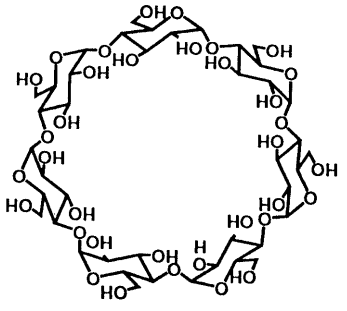
Cyclodextrins and chemically modified cyclodextrins have attracted much attention in basic and applied scientific research.[2] In most of the modification studies, the basic structure of cyclodextrins constituted from normal ($^4C_1$) glucose units, as shown in **1**, was not changed. However, for wide-ranging studies on molecular recognition in host–guest relationships, we need a variety of cyclodextrins having cavities different from those of normal cyclodextrins. Recently, novel types of cyclodextrins having half-chair glucose units (2,3-anhydroglucose units)[3b–f] or $^1C_4$ glucose units (3,6-anhydroglucose units)[3f,g,4] as partial constituents have been reported.

In this report, we describe alternative cyclodextrins (**2** and **3**) constituted from only $^1C_4$ conformers, which are restricted by intramolecular bridges.[5] In addition, we describe a convenient preparation of the starting material, heptakis(6-*O*-(*p*-tosyl))-β-cyclodextrin (**4**) and heptakis(6-*O*-(*p*-tosyl))-2-*O*-(*p*-tosyl)-β-cyclodextrin (**5**).

Results and Discussion

Preparation of Polysulfonylated β-Cyclodextrins (4 and 5) Heptakis(6-*O*-(*p*-tosyl))-β-cyclodextrin (**4**) has been prepared and used as a synthetic intermediate of an enzyme model.[6] We prepared sulfonylated cyclodextrins by the reaction of lyophilized β-cyclodextrin with *p*-tosyl chloride in pyridine with monitoring by thin-layer chromatography (TLC). The reaction was stopped by addition of H_2O when the main products were 6-*O*-heptatosylate **4** and octatosylate **5**. The products were separated and purified by reversed-phase column chromatography with aqueous CH_3CN gradient elution (Fig. 1). The high-performance liquid chromatographic (HPLC) pattern of the reaction mixture is shown in Fig. 2.

The structures were determined from the fast atom bombardment mass (FABMS) and nuclear magnetic resonance (NMR) spectra. The number of sulfonyl groups in **4** was determined from the FABMS and ^{1}H-NMR spectra. The ^{13}C-NMR spectrum (Fig. 3) showed only six signals due to glucose carbons, suggesting it was constituted from identical glucose units. This spectrum was very similar to that of hexakis(6-*O*-mesitylenesulfonyl)-α-cyclodextrin (**6**).[7] The large downfield shift of C6 and the small upfield shift of C5, as compared with those of β-cyclodextrin,



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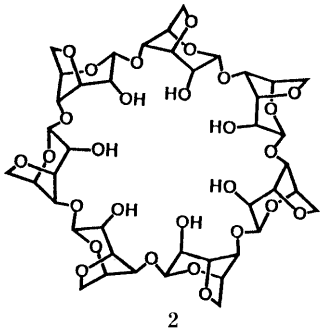

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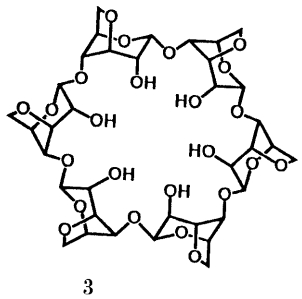
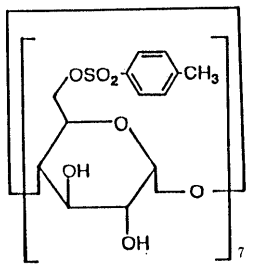


3

Chart 1



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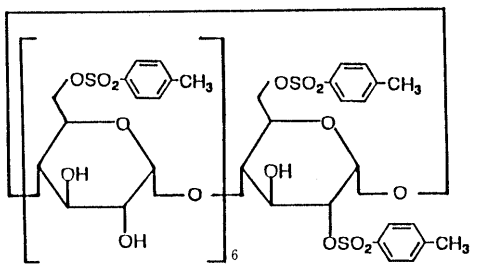

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Chart 2

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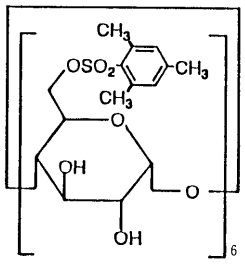


6

Chart 3

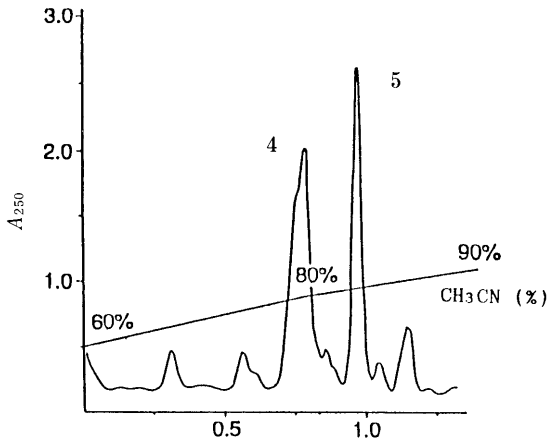


Fig. 1. Reversed-Phase Column Chromatography of the Mixture of Heptakis(6-*O*-(*p*-tosyl))-*β*-cyclodextrin (**4**) and Heptakis(6-*O*-(*p*-tosyl))-2-*O*-(*p*-tosyl)-*β*-cyclodextrin (**5**)

A gradient of CH_3CN was applied.

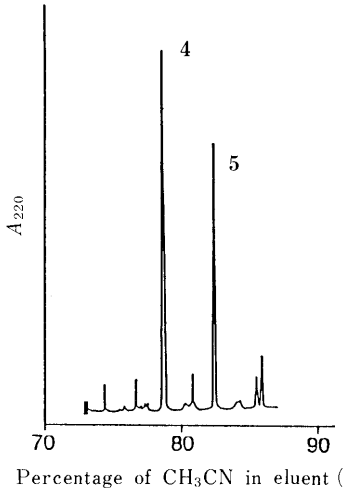


Fig. 2. Reversed-Phase HPLC of the Mixture of Heptakis(6-*O*-(*p*-tosyl))-*β*-cyclodextrin (**4**) and Heptakis(6-*O*-(*p*-tosyl))-2-*O*-(*p*-tosyl)-*β*-cyclodextrin (**5**)

A gradient of CH_3CN was applied.

indicated the sulfonylation of the 6-OH groups. Moreover, the ^{1}H-NMR spectrum of **4** showed a similar pattern to that of **6**.[7] These spectra demonstrated that **4** is heptakis(6-*O*-(*p*-tosyl))-*β*-cyclodextrin.

The number of sulfonylations in **5** was also determined from the FABMS spectrum. The ^{13}C-NMR spectrum of **5** showed the same six signals of substituted glucose units as

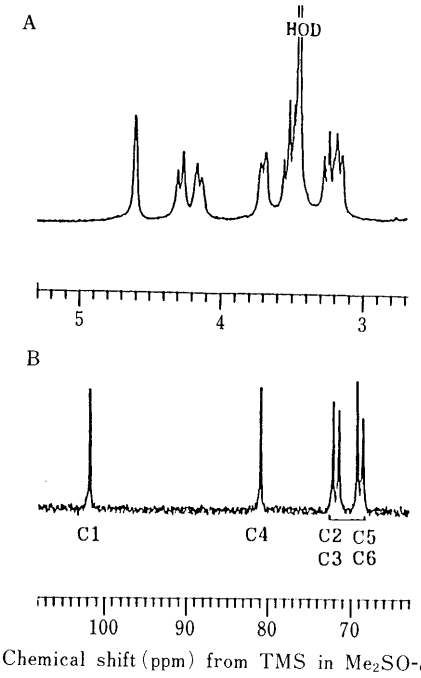
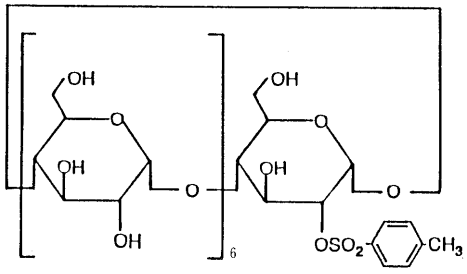


Fig. 3. ^{1}H-NMR (A) and ^{13}C-NMR (B) Spectra of the Cyclodextrin Part of Heptakis(6-*O*-(*p*-tosyl))-*β*-cyclodextrin (**4**) in Me_2SO-d_6



7

Chart 4

those of heptakis(6-*O*-(*p*-tosyl))-*β*-cyclodextrin (**4**) and the peaks of another substituted glucose unit (Fig. 4). Ueno and Breslow demonstrated that sulfonylation of 2-OH of *β*-cyclodextrin caused the downfield shift of C2 (*α* carbon) and the upfield shift of C1 (*β* carbon) signals.[3a] The shift pattern of compound **5** was similar to that of 2-*O*-(*p*-tosyl)-*β*-cyclodextrin (**7**), indicating that another sulfonylation was on 2-OH of the cyclodextrin. From these spectra, it appears that all of the 6-OH groups and one of the 2-OH groups of compound **5** were sulfonylated.

Sulfonylations with sulfonyl chloride in pyridine generally occur at primary hydroxyl groups of cyclodextrins. The preferential sulfonylation of 6-OH might occur in the present reaction, followed by the selective sulfonylation of 2-OH. This selectivity for 2-OH would be due to the lower reactivity of 3-OH as a consequence of the hydrogen bond formation between the 3-OH proton and the oxygen atom of 2-OH.[8] Also, the acidity of 2-OH owing to both the hydrogen bond between 2-OH and 3-OH and the proximity of 2-OH to the electron-withdrawing acetal functionality would favor this selective sulfonylation.[9]

Conversion of Polysulfonylated Cyclodextrins to Alternative Cyclodextrins The reaction of heptakis(6-*O*-(*p*-tosyl))-

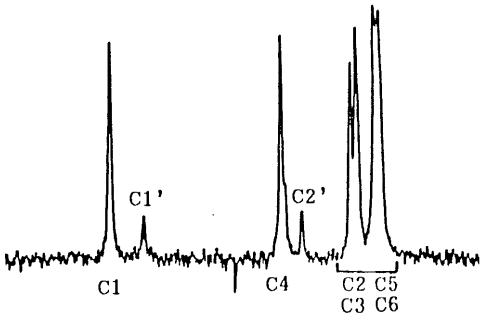


Fig. 4. ^{13}C-NMR Spectrum of the Cyclodextrin Part of Heptakis(6-*O*-(*p*-tosyl))-2-*O*-(*p*-tosyl)-*β*-cyclodextrin (**5**) in Me_2SO-d_6

The chemical shifts, in ppm from TMS, are 101.6 (C1), 97.7 (C1'), 80.9, (C4), 78.3 (C2'), and 72.2, 71.5, 69.2, 68.6 (C2, C3, C5, C6).

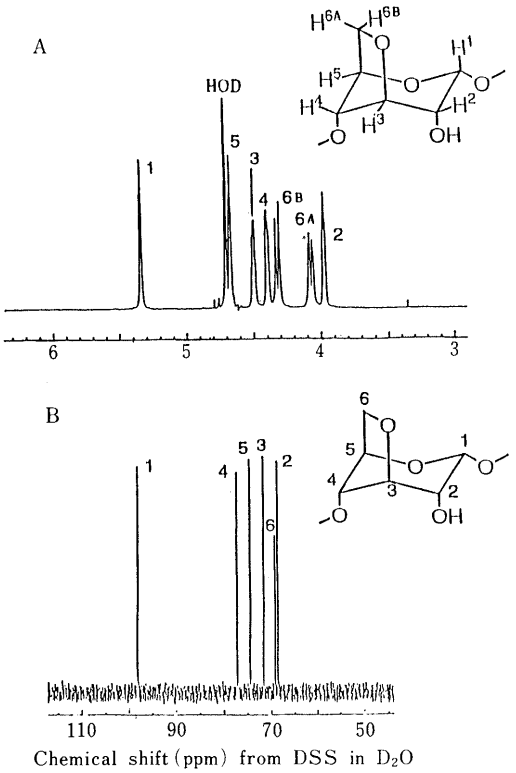


Fig. 5. ^{1}H-NMR (A) and ^{13}C-NMR (B) Spectra of Heptakis(3,6-anhydro)-*β*-cyclodextrin (**2**) in D_2O

β-cyclodextrin (**4**) with KOH in aqueous MeOH caused the disappearance of the starting material, and a new non-developing spot was observed on TLC. The reaction mixture was neutralized and desalted to give the alternative cyclodextrin **2** in high yield (95.7%).

This compound showed a single peak in reversed-phase HPLC (3% aqueous CH_3CN, 0.5 ml/min) and simple signal patterns in the ^{1}H- and ^{13}C-NMR spectra (Fig. 5). The signals of the ^{1}H-NMR spectrum were assigned by comparing their chemical shifts and coupling constants with reported values[4a] of methyl 3,6-anhydroglucoside. The ^{13}C-NMR signals were assigned on the basis of the C–H correlated spectroscopy (C–H COSY) two dimensional NMR spectrum (Fig. 6). The similarity of the ^{13}C- and ^{1}H-NMR spectra to those of methyl 3,6-anhydroglucoside and related sugars[3f,g,4] demonstrated that **2** is heptakis(3,6-anhydro)-*β*-cyclodextrin. The FABMS spectrum contained

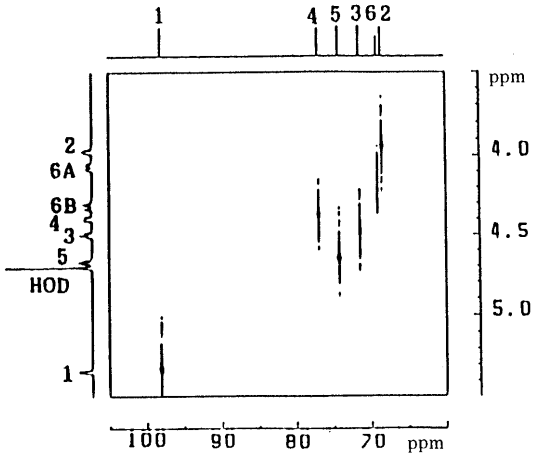


Fig. 6. C–H COSY Two Dimensional NMR Spectrum of Heptakis(3,6-anhydro)-*β*-cyclodextrin (**2**) in D_2O

the corresponding molecular ion, supporting the structure determination. The *α*-cyclodextrin analogue **3** was similarly prepared from hexakis(6-*O*-mesitylenesulfonyl)-*α*-cyclodextrin (**6**).

Heptakis(6-*O*-(*p*-tosyl))-2-*O*-(*p*-tosyl)-*β*-cyclodextrin (**5**) could be converted to heptakis(3,6-anhydro)-*β*-cyclodextrin (**2**) by the same procedure. The reactions of 2-*O*-sulfonyl cyclodextrins with alkali generally result in 2,3-anhydride formation on the glucose units of cyclodextrins.[3b-f] Therefore, either of the two types of reactions, 2,3-anhydride formation or 3,6-anhydride formation, could occur on the 2,6-bis(*O*-(*p*-tosyl))-glucose unit of **5**. The fact that the major product was heptakis(3,6-anhydro)-*β*-cyclodextrin (**2**) suggested that the rate of 3,6-anhydride formation would be far greater than that of 2,3-anhydride formation in the case of **5**. Also, it appears that 3,6-anhydride formation was followed by the hydrolysis of the 2-*O*-sulfonate.

Normal cyclodextrin cavities are "V" shaped, with the secondary hydroxyl sides more open than the primary hydroxyl sides. A consideration based on molecular model construction suggested that such a molecular shape was not retained in **2**, where the cavity is "V" shaped with the primary hydroxyl side more open than the opposite side. Also, all of the secondary hydroxyl groups (2-OH) are axial and directed to the inside of the cavity. Therefore, the secondary side surrounded by seven axial hydroxyl groups may be electron-dense and may bind metal ions. A preliminary study on the FABMS spectra of mixtures of **2** or **3** and alkali metal ions showed selective ion binding depending on the kind of alternative cyclodextrins used.

Experimental

The melting points (mp) were not corrected. Optical rotations were measured with a JASCO DIP-181 polarimeter. ^{1}H- and ^{13}C-NMR spectra were determined with JEOL FX-100, JNM GX-270, and JNM GSX-500 instruments. FABMS spectra were recorded with a JEOL JMS DX-303 spectrometer. TLC was run on precoated silica gel plates (Art 5554, Merck). Spot detection was carried out by exposure to UV light and/or staining with 0.1% 1,3-dihydroxynaphthalene in EtOH–H_2O–H_2SO_4 (200 : 157 : 43 (v/v)). The developing solvent for TLC was *n*-C_3H_7OH–AcOEt–H_2O (7 : 7 : 2 (v/v)). A prepacked column (Lobar column LiChroprep RP18, 25 × 310 mm, Merck) was used for reversed-phase column chromatography. HPLC was performed on a Hitachi L-6000 with a TSKgel ODS 80TM column (4.6 × 250 mm, 5*μ*m, Tosoh). An IE-Labo (Tosoh) was

used for desalting.

Preparation of Polysulfonylated β-Cyclodextrin A lyophilized β-cyclodextrin **1** (200 mg, 1.76×10^{-4} mol) was added to *p*-tosyl chloride (675 mg, 3.53×10^{-3} mol) in pyridine (8 ml) and the mixture was stirred for 5.25 h at room temperature, followed by addition of H_2O (10 ml). The reaction mixture was concentrated *in vacuo* and the residue was taken up in 60% aqueous CH_3CN (50 ml). It was neutralized, and applied to a reversed-phase column. After elution with 60% aqueous CH_3CN (300 ml), a gradient from 60% aqueous CH_3CN (300 ml) to 80% aqueous CH_3CN (300 ml) was applied to give **4** (94 mg, 24.0%) and **5** (64 mg, 15.3%).

4: ^{13}C-NMR(Me_2SO-d_6) and ^{1}H-NMR(Me_2SO-d_6) see Fig. 3. FABMS *m/z*: 2213($M+H^+$)

5: ^{13}C-NMR(Me_2SO-d_6) and ^{1}H-NMR(Me_2SO-d_6) see Fig. 4. FABMS *m/z*: 2368($M+H^+$)

Preparation of Heptakis(3,6-anhydro)-β-cyclodextrin (2) A solution of heptakis(6-*O*-(*p*-tosyl))-β-cyclodextrin (**4**) (400 mg, 1.81×10^{-4} mol) in 1 N KOH–75% aqueous MeOH (50 ml) was kept at 60°C for 2 h. After neutralization with dilute HCl, the reaction mixture was concentrated in *in vacuo* and the residue was taken up in H_2O (50 ml). Desalting and lyophilization gave heptakis(3,6-anhydro)-β-cyclodextrin (**2**) (175 mg, 95.7%).

Heptakis(6-*O*-(*p*-tosyl))-2-*O*-(*p*-tosyl)-β-cyclodextrin (**5**) (300 mg, 1.27×10^{-4} mol) gave **2** (117 mg, 91.9%) when treated according to the same procedure.

2: mp 224—225 °C. $[\alpha]_D^{22}$ −27° (*c*=0.91, H_2O). ^{13}C-NMR (D_2O) and ^{1}H-NMR (D_2O) see Fig. 5. FABMS *m/z*: 1009 ($M+H^+$). *Anal.* Calcd for $(C_6H_8O_4)_7 \cdot 4H_2O$: C, 46.66; H, 5.92. Found: C, 46.81; H, 5.76.

Preparation of Hexakis(3,6-anhydro)-α-cyclodextrin (3) A solution of hexakis(6-*O*-mesitylenesulfonyl)-α-cyclodextrin (**6**) (33 mg, 1.60×10^{-5} mol) in saturated aqueous $Ba(OH)_2$ (4 ml) was kept at 40 °C for 87 h. The reaction mixture was neutralized by adding CO_2, filtered, and applied to a short reversed-phase column (Sep-Pak, Waters). After eluting with H_2O (40 ml), 1% aqueous EtOH (10 ml), 3% aqueous EtOH (10 ml), and 5% aqueous EtOH (10 ml) were applied to give **3** (11 mg, 75.1%).

3: mp 225—228 °C. $[\alpha]_D^{22}$ −8° (*c*=0.18, H_2O). ^{13}C-NMR (D_2O) δ: 70.6, 71.2, 73.8, 76.7, 79.8, 100.4. FABMS *m/z*: 887($M+Na^+$), 903($M+K^+$). *Anal.* Calcd for $(C_6H_8O_4)_6 \cdot 6H_2O$: C, 44.44; H, 6.17. Found: C, 44.06; H, 5.90.

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New Coumarins from *Citrus* Plants

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Four new coumarins, named peroxytamarin (1), *cis*-casegravol (5), citrusarin-A (7), and citrusarin-B (8), were isolated from root of *Citrus* plants and their structures were elucidated by chemical and spectrometric methods. Citrusarin-B (8), a coumarin having both a dimethylpyran ring and a dihydrofuran ring in the molecule, was also synthesized.

Keywords coumarin; *Citrus*; Rutaceae; peroxytamarin; tamarin; casegravol; citrusarin; heteronuclear multiple bond connectivity

During our phytochemical studies of *Citrus* plants (Rutaceae), many kinds of new coumarins and acridone alkaloids have been isolated and characterized.[1] We report here the isolation of four new coumarins named peroxytamarin (**1**), *cis*-casegravol (**5**), and citrusarin-A (**7**), and -B (**8**), and their structural elucidation by chemical and/or spectral methods. Peroxytamarin (**1**) and *cis*-casegravol (**5**) were isolated from root of *Citrus sulcata* HORT. *ex* TAKAHASHI (Japanese name: sanbo-kan) together with many kinds of known coumarins and acridones. Citrusarin-A (**7**) and -B (**8**) are regioisomers, and both coumarins were also obtained from root of *C. Hassaku* HORT. *ex* Y. TANAKA (Japanese name: hassaku).

Results and Discussion

Structure of Peroxytamarin (1) Peroxytamarin (**1**) was isolated as a colorless oil, $[\alpha]_D$ $-5.3°$ ($CHCl_3$). The high-resolution mass spectrum (HR-MS) gave the molecular formula as $C_{15}H_{16}O_5$. The ultraviolet (UV) bands at λ_{max} 222, 252, 297 and 324 nm, infrared (IR) absorption at ν_{max} 1725 cm^{-1}, AB-type signals at δ_H 7.61 and 6.23 (each doublet, $J=9.4$ Hz), a methoxy signal at δ_H 3.91, and two 1H singlets at δ_H 7.25 and 6.79 in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum indicated the presence of 7-methoxy-6-substituted coumarin skeleton[2] in the molecule. Further, in the ^{1}H-NMR spectrum, ABX-type signals at δ_H 4.58 (1H, dd, $J=8.0$, 5.2 Hz), 2.92 (1H, dd, $J=14.1$, 5.2 Hz) and 2.87 (1H, dd, $J=14.1$, 8.0 Hz), two 1H singlets at δ_H 5.00 and 4.96 assignable to an *exo*-methylene protons, and a 3H singlet at δ_H 1.82 due to an allyl methyl group appeared. The 1H double-doublet at δ_H 4.58 in the ^{1}H-NMR spectrum and IR band at ν_{max} 3450 cm^{-1} together with the occurrence of typical mass fragments at *m/z* 260 and 258 corresponding to $[M^+ - \cdot O]$ and $[M^+ - H_2O]$, respectively, in the electron impact mass spectrum (EI-MS) suggested the presence of a hydroperoxy moiety in the molecule. These spectral data coupled with the appearance of a base fragment peak at *m/z* 189 arising from cleavage at the benzylic position indicated the structure of $[-CH_2-CH(OOH)-C(CH_3)=CH_2]$ for the side chain attached to C-6. For confirmation of the structure, the following two chemical reactions were carried out. 1) The hematoporphyrin-sensitized photo-oxygenation[3] of suberosin (**2**)[4] in oxygen gas gave two isomeric peroxygenated products. One of them was found to be identical with natural **1** by IR, UV, ^{1}H-NMR and mass spectrometric comparisons. The structure of the other reaction product was assigned as formula **3** on the basis of spectrometric analyses (see Experimental). 2) Treatment of peroxytamarin (**1**) with triphenylphosphine gave a colorless oil, $[\alpha]_D+8.9°$ ($CHCl_3$), which was found to be identical with tamarin (**4**), except for a difference of $[\alpha]_D$ value. Tamarin (**4**) was first isolated from *Ruta pinnata*,[5] and then from *Amyris balsamifera* by Burke and Parkins,[6] and *R*-stereochemistry was proposed for the specimen having $[\alpha]_D+27.3°$ ($CHCl_3$). From the results of the ^{1}H-NMR analysis using a chiral shift reagent, **4** derived from natural peroxytamarin (**1**) was to contain only about 5% excess of one of the enantiomers. According to the proposal by Bruke and Parkins,[6] the absolute stereochemistry of the negative $[\alpha]_D$ enantiomer of **1** was assigned as *R*, because **4** derived from **1** showed a positive $[\alpha]_D$ value. These results led us to conclude the structure of the major enantiomer of peroxytamarin to be as represented by formula **1**. The first isolation of a coumarin hydroperoxide from a plant was reported by Crombie *et al.*[7] in 1970. This is the fourth example of the isolation of a peroxygenated coumarin from a natural source.[8]

Structure of *cis*-Casegravol (5) *cis*-Casegravol (**5**) was obtained as a colorless oil, $[\alpha]_D$ $-12.1°$ ($CHCl_3$). The chemical ionization mass spectrum (CI-MS) using ammonia as a reactant gas showed the molecular ion $[M+NH_4]^+$ at *m/z* 294. The UV bands at λ_{max} 208, 255, 284 and 320 nm, IR band at ν_{max} 1720 cm^{-1}, and ^{1}H-NMR signals at δ_H 7.64 (1H, d, $J=9.4$ Hz, H-4), 6.26 (1H, d, $J=9.4$ Hz, H-3), and 3.94 (3H, OCH_3) suggested the 7-methoxycoumarin nucleus. The appearance of other AB-type doublets at δ_H 7.39 and 6.88 (each 1H, d, $J=8.7$ Hz) assignable to H-5 and H-6, respectively, indicated the presence of a side chain at C-8. The remaining ^{1}H-NMR signals showed the presence of a *Z*-disubstituted double bond [δ_H 6.30 and 5.96 (each 1H, d, $J=12.4$ Hz)], a methyl group attached to an oxygenated carbon [δ_H 1.25 (3H, s)], and a hydroxymethylene moiety [δ_H 3.57 and 3.36 (each 1H, d, $J=11.1$ Hz)]. These data together with the observation of mass fragments at *m/z* 259 and 245 corresponding to $[M^+ - \cdot OH]$ and $[M^+ - \cdot CH_2OH]$ ions, respectively, suggested the structure of the side chain as $[-CH=CH-C[(OH)CH_3]-CH_2OH]$ having *Z*-configuration. On the basis of these results, we proposed the structure of *cis*-casegravol to be as shown by formula **5**, except for the absolute

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stereochemistry. The racemic *E*-isomer named casegravol (**6**) was isolated from *Casearia graveolens* in 1980 and characterized.[9]

Structures of Citrusarin-A (7) and -B (8) Citrusarin-A (**7**) and -B (**8**) were isolated as pale yellow oils having $[\alpha]_D$ +5.2° and +3.0° ($CHCl_3$), respectively, and were found to have the same molecular formula $C_{19}H_{20}O_4$ by HR-MS analyses.

Citrusarin-A (**7**) showed UV bands at λ_{max} 210, 226, 284, and 340 nm and IR band at ν_{max} 1720 cm^{-1}. In the ^{1}H-NMR spectrum, two pairs of AB-type doublets at δ_H 7.94 and 6.07 (each 1H, d, J=9.5 Hz) and at δ_H 6.45 and 5.55 (each 1H, d, J=9.9 Hz), accompanied with signals of two methyl groups attached to oxygenated carbon at δ_H 1.46 (6H, s), were assignable to α- and β-protons on an α,β-unsaturated carbonyl system and two protons on the dimethylbenzopyran ring system, respectively. The lower chemical shift value of H-4 at δ_H 7.94 and the absence of other proton signals in the aromatic proton region in the ^{1}H-NMR spectrum, together with the results of the UV spectrum, suggested the presence of a 5,7-dioxygenated 6,8-disubstituted coumarin nucleus[2] having a dimethylpyran ring system in the molecule. The ramaining proton signals coupled with carbon signals in the ^{13}C-NMR spectrum were assigned to geminal methyls attached to a benzylic carbon [δ_H 1.52 and 1.26 (each 3H, s) and δ_C 43.99] and a secondary methyl [δ_H 1.39 (3H, d, J=6.6 Hz)] attached to an oxygenated methine carbon [δ_H 4.48 (1H, q, J=6.6 Hz) and δ_C 91.06]. The appearance of H–C long-range correlations in the ^{1}H detected heteronuclear multiple bond connectivity (HMBC) spectrum between the oxygenated methine carbon at δ_C 91.06 and two methyl protons at δ_H 1.26 and 1.52, which further correlated to an aromatic carbon at δ_C 114.25 and a quaternary carbon at δ_C 43.99 indicated the presence of 2,3,3-trimethyldihydrobenzofuran system in the molecule. Based on these spectral data together with the HMBC data shown by arrows in Fig. 1, the structure of citrusarin-A should be depicted by either formula **7** or **8**.

On the other hand, citrusarin-B (**8**) showed UV bands at λ_{max} 204, 230, 293 and 328 nm and the IR band at ν_{max} 1720 cm^{-1}. The ^{1}H-NMR signal pattern of citrusarin-B resembled to that of citrusarin-A (**7**), except for some differences of chemical shifts of the signals due to H-4 and H-4′ (Table I). The mass fragmentation pattern of citrusarin-B showed a close similarity to that of **7**. The higher chemical shift value of H-4 [δ_H 7.75 (1H, d, J=9.5 Hz)] corresponds to that of coumarin bearing no oxygenated substituent at C-5.[2] However, in the HMBC spectrum (Fig. 2), observation of three-bond H–C correlations of H-4 (δ_H 7.75) to two oxygenated aromatic carbons at δ_C 149.89 and 156.17 suggested the presence of an *O*-substituent at C-5, as in the molecule of **7**. These data together with the HMBC data shown by arrows in Fig. 2 implied that citrusarin-B should be represented either by structure **8** or **7**. Therefore, citrusarin-A and -B were found to be regioisomers with regard to the location of the dimethylpyran and dihydrofuran rings attached to the 5,7-oxygenated coumarin nucleus.

The location of the pyran ring (or the dihydrofuran ring) either at C-5,6 or C-7,8 could not be confirmed by H–C long-range correlations in the HMBC spectra (Figs. 1 and

Table I. ^{1}H- and ^{13}C-NMR Data for Citrusarin-A (**7**) and -B (**8**)

Carbon No.	Citrusarin-A (**7**)		Citrusarin-B (**8**)	
	δ_H	δ_C	δ_H	δ_C
2		161.23		161.38
3	6.07 (1H, d, 9.5)	109.84	6.11 (1H, d, 9.5)	110.54
4	7.94 (1H, d, 9.5)	139.25	7.75 (1H, d, 9.5)	138.60
4a		103.64		99.06
5		150.27		156.17
6		101.84		117.91
7		158.09		153.17
8		114.25		102.88
8a		151.12		149.89
2′		77.86		77.44
3′	5.55 (1H, d, 9.9)	127.81	5.56 (1H, d, 9.9)	126.98
4′	6.45 (1H, d, 9.9)	115.90	6.80 (1H, d, 9.9)	115.63
2″	4.48 (1H, d, 6.6)	91.06	4.49 (1H, d, 6.6)	91.14
3″		43.99		44.11
2′-CH_3	1.46 (6H, s)	28.06	1.46 (3H, s)	28.09
		27.96	1.47 (3H, s)	28.05
2″-CH_3	1.39 (3H, d, 6.6)	14.23	1.40 (3H, d, 6.6)	14.27
3″-CH_3	1.52 (3H, s)	25.54	1.42 (3H, s)	25.59
	1.26 (3H, s)	21.17	1.18 (3H, s)	21.07

Spectra were measured at 400 (^{1}H) and 100 (^{13}C) MHz in $CDCl_3$. Values are in δ (ppm). Figures in parentheses are coupling constants (J) in Hz. Assignments were confirmed by H–H and H–C correlation spectroscopy (COSY) and HMBC (J=8 Hz) spectrometric analyses.

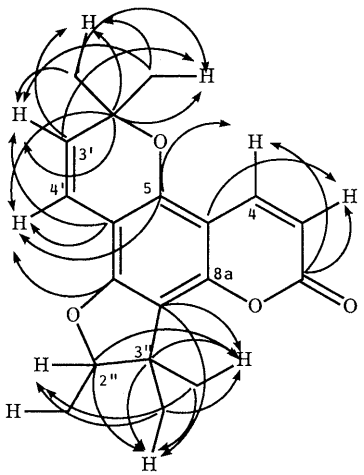


Fig. 1. CH Long-Range Correlations in the HMBC Spectrum (J=8 Hz) of Citrusarin-A (**7**)

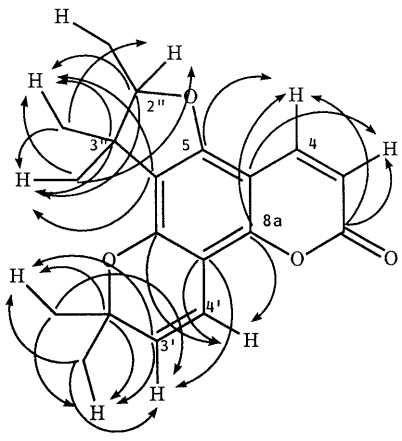
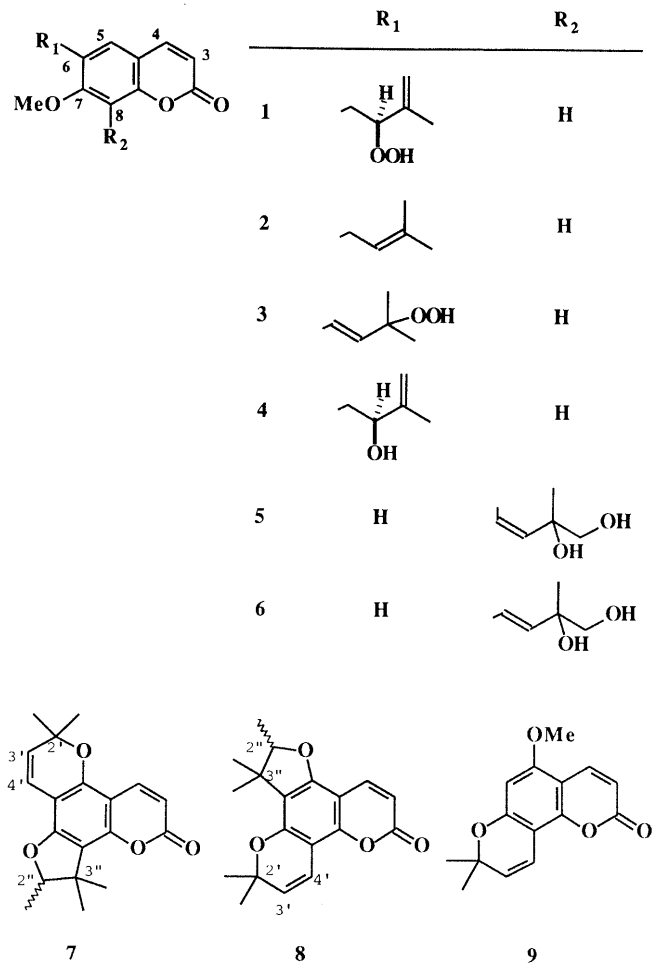
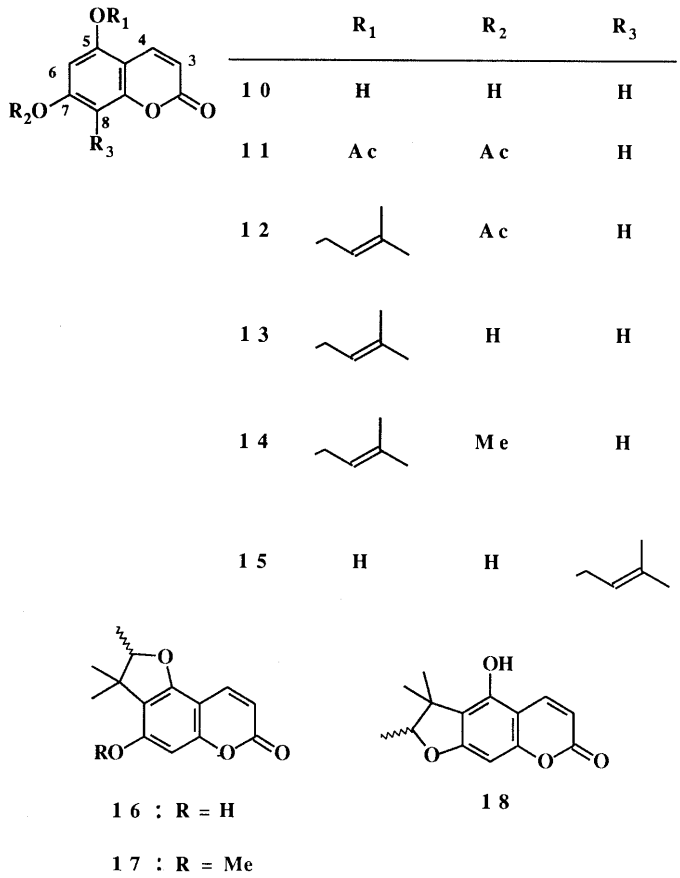


Fig. 2. CH Long-Range Correlations in the HMBC Spectrum (J=8 Hz) of Citrusarin-B (**8**)



7 8 9

Chart 1



16 : R = H

17 : R = Me

18

Chart 2

2) of citrusarin-A and -B, because the problem of the assignment of oxygenated aromatic carbon signals at δ_C 149.89 and 156.17 in the spectrum of citrusarin-B and at δ_C 150.27 and 151.12 in that of citrusarin-A, to either C-8a and C-5 or C-5 and C-8a, respectively, remained. The similarity of the chemical shift of the signal at δ_H 6.80 due to H-4′ on the pyran ring of citrusarin-B (**8**) to that of 5-methoxyseselin (**9**)[10] at δ_H 6.79 suggested the structure **8** for citrusarin-B.

To confirm this, we synthesized citrusarin-B (**8**) from 5,7-dihydroxycoumarin (**10**),[11] which was easily derived from phloroglucinol and ethyl propiolate. Treatment of the diacetate (**11**) obtained from **10** with 1-bromo-3-methylbut-2-ene in acetone in the presence of anhydrous potassium carbonate gave **12**,[12] which was hydrolyzed to afford the 5-prenylated coumarin (**13**).[12] The location of the prenyl ether at C-5 in **13** was confirmed by observation of nuclear Overhauser effect (NOE) between the *O*-methyl protons (δ_H 3.85) and two *meta*-coupled aromatic protons at δ_H 6.29 and 6.41 in the ^{1}H-NMR spectrum of the corresponding methyl ether (**14**).[12] A mixture of **13** and sodium acetate was heated at 190 °C for 20 min[13] gave three kinds of reaction products in 38 (A), 29 (B), and 27 (C) % yields. The spectroscopic data (UV, IR, and ^{1}H-NMR) of the major product (A) were in agreement with those of **15** reported by Murray and Jorge.[14] The ^{1}H-NMR spectra of the other products (B and C) showed the same signal pattern (see Experimental), which suggested the presence of a 2,3,3-trimethyldihydrofuran ring in the molecules, instead of a prenyl moiety and one of the *meta*-coupled aromatic protons on **13**. These data suggested the structures of B and C to be **16** and **18** (or *vice versa*), respectively. These products were considered to have been formed by Claisen rearrangement of the prenyl moiety on **13** followed by cyclization to give *ortho*-phenolic hydroxy groups. For confirmation of the structure, **14** was treated[13] with sodium acetate at 190 °C, as in the case of **13**, to give a cyclization product **17**, which was found to be identical with the *O*-methyl ether of B by spectrometric comparisons (UV, IR, MS, and ^{1}H-NMR). Therefore, we assigned the structures **16** for B and **18** for C.[15] Further treatment[16] of **16** with 3-chloro-3-methylbut-1-yne followed by cyclization at reflux temperature in diethylaniline afforded a colorless oil (**8**) in 37% yield, and this was found to be identical with natural citrusarin-B by spectrometric comparisons (UV, IR, MS, and ^{1}H-NMR). On the basis of the above results, the structures of citrusarin-A and -B were established as **7** and **8**, respectively, except for the absolute stereochemistry.

Experimental

Melting point was measured on a micromelting point hot-stage apparatus (Yanagimoto). ^{1}H- and ^{13}C-NMR spectra were recorded on GX-270 (JEOL) and GX-400 (JEOL) spectrometers, respectively, in $CDCl_3$, unless otherwise stated. Chemical shifts are shown in δ values (ppm) with tetramethylsilane (TMS) as an internal reference. HMBC spectra were measured at J=8 Hz on the GX-400. All mass spectra were measured under electron impact (EI) conditions, unless otherwise stated, using an M-80 (Hitachi) or a JMS-HX-110 (JEOL) spectrometer having a direct inlet system. UV spectra were recorded on a UVIDEC-610C double-beam

spectrophotometer (JASCO) in methanol, IR spectra on a IR-810 (JASCO) in $CHCl_3$, and optical rotations on a DIP-181 (JASCO) in $CHCl_3$. The preparative thin layer chromatographies (TLC) were done on Kieselgel 60 F_{254} (Merck).

Isolation and Separation of Peroxytamarin (1) and *cis*-Casegravol (5) The dried root (185 g) of *Citrus sulcata* HORT. *ex* TAKAHASHI (Japanese name: sanbo-kan) grown in the orchard of Okitsu Branch, Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries, Shimizu, Sizuoka, was extracted with acetone at room temperature. The acetone extract (9.5 g) was chromatographed over silica gel with benzene–hexane (1 : 1 and then 10 : 1), benzene, benzene–acetone (20 : 1, 10 : 1, 4 : 1, and then 2 : 1), acetone, and methanol successively to give nine fractions. Each fraction was subjected to preparative TLC using appropriate mixtures of benzene, hexane, acetone, $CHCl_3$, isopropyl ether, ethyl acetate, CH_2Cl_2, and methanol as developing solvents to obtain 21 kinds of coumarins and 4 kind of acridones, as stated below. From fraction 4: tamarin[5,6] 15.2 mg, hopeyhopin[17] 2.6 mg, demethylauraptenol[18] 5.2 mg, seselin[19] 319 mg, xanthyletin[20] 2.2 mg, and a new coumarin, peroxytamarin (**1**) 17.7 mg. From fractions 4 and 5: demethylsuberosin[21] 2.9 mg, osthenol[22] 35 mg, osthenon[23] 14.7 mg, junosmarin[24] 4.0 mg, 5-hydroxyseselin[25] 3.9 mg, scoparone[26] 4.1 mg, nordentatin[27] 3.1 mg, natsucitrine-I[28] 5.0 mg, citprssine-I[26,29] 3.4 mg, 5-hydroxynoracronycine[29] 8.2 mg, and citracridone-I[29] 13.1 mg. From fraction 5: umbelliferone[30] 3.8 mg, *cis*-osthenon[31] 5.6 mg, and bisosthenon[32] 3.1 mg. From fraction 6: 7-methoxy-8-formylcoumarin[33] 6.1 mg, kiyomal[34] 1.1 mg, casegravol[9] 4.8 mg, (+)-decurusidinol[35] 18.3 mg, and a new coumarin. *cis*-casegravol (**5**) 1.9 mg. Known compounds were fully characterized by UV, IR, ^{1}H-NMR, and MS.

Peroxytamarin (1) Colorless oil. $[\alpha]_D$ −5.3° (c=0.12, $CHCl_3$). UV λ_{max} nm: 222, 244 (sh), 252, 297, 324. IR ν_{max} cm^{-1}: 3450 (br), 1725, 1620, 1560. ^{1}H-NMR δ: 8.23 (1H, br, OOH), 7.61 (1H, d, J=9.4 Hz, H-4), 7.25 (1H, s, H-5), 6.79 (1H, s, H-8), 6.23 (1H, d, J=9.4 Hz, H-3), 5.00 and 4.96 (each 1H, s, *exo*-CH_2), 4.58 (1H, dd, J=5.2, 7.9 Hz), 3.91 (3H, s, OCH_3), 2.92 (1H, dd, J=14.1, 5.2 Hz), 2.87 (1H, dd, J=14.1, 8.0 Hz), 1.82 (3H, s). MS m/z (%): 276 (M^+, 3), 260 (8), 258 (21), 206 (33), 205 (12), 191 (15), 190 (99), 189 (100), 177 (11), 175 (10), 162 (19), 161 (23), 160 (14), 159 (47), 147 (23). HR-MS Calcd for $C_{15}H_{16}O_5$: 276.0996. Found: 276.0982.

Photo-oxygenation[3] of Suberosin (2) Oxygen gas was bubbled through a solution of suberosin (**2**)[4] (50 mg) in pyridine (5 ml) containing hematoporphyrin (5 mg), and the solution was irradiated with a high-pressure Hg lamp using a Pyrex glass filter for 2 h. Then, the solvent was evaporated off. The residue was subjected to silica gel preparative TLC to afford 19.2 and 20.1 mg of **1** and **3**, respectively. Compound **1** was found to be identical with natural peroxytamarin by spectrometric comparisons (IR, UV, ^{1}H-NMR, and MS). **3**: Light yellow oil. IR ν_{max} cm^{-1}: 3450 (br), 1725, 1620, 1560. UV λ_{max} nm: 206, 223, 256, 296, 306, 339. ^{1}H-NMR δ: 7.63 (1H, d, J=9.4 Hz), 7.53 (1H, s), 6.89 (1H, d, J=16.5 Hz), 6.78 (1H, s), 6.34 (1H, d, J=16.5 Hz), 6.27 (1H, d, J=9.4 Hz), 3.92 (3H, s), 1.48 (6H, s). MS m/z (%): 276 (M^+, 11), 260 (34), 245 (55), 243 (20), 205 (34), 204 (28), 203 (64), 190 (22), 189 (100), 175 (21), 149 (30). HR-MS Calcd for $C_{15}H_{16}O_5$: 276.0996. Found: 276.0988.

Treatment of Natural Peroxytamarin (1) with Triphenylphosphine A methanolic solution (1 ml) of **1** (2.3 mg) and Ph_3P (1 mg) was stirred for 1 h at room temperature. The solvent was evaporated off *in vacuo*. The residue was subjected to preparative TLC to afford a colorless oil (**4**) (1.7 mg), $[\alpha]_D$ +8.9° (c=0.083, $CHCl_3$), IR ν_{max} cm^{-1}: 3050, 1720, 1620, 1560. UV λ_{max} nm: 206, 222, 253, 297, 328. ^{1}H-NMR δ: 7.63 (1H, d, J=9.4 Hz, H-4), 7.29 (1H, s, H-5), 6.81 (1H, s, H-8), 6.26 (1H, d, J=9.4 Hz, H-3), 4.94 (1H, s, H-4′), 4.85 (1H, s, H-4′), 4.32 (1H, dd, J=4.0, 8.7 Hz, H-2′), 3.91 (3H, s, 7-OCH_3), 3.01 (1H, dd, J=4.0, 14.1 Hz, H-1′), 2.76 (1H, dd, J=8.7, 14.1 Hz, H-1′), 1.83 (3H, s, 3′-CH_3). This was found to be identical with tamarin (**4**) by comparisons of the IR, UV, and ^{1}H-NMR data with those in the literature.[5] Tamarin (**4**), $[\alpha]_D$ +8.9° ($CHCl_3$), derived from natural **1** was found to contain only 5% excess of one of the enantiomers by ^{1}H-NMR analysis using a chiral shift reagent, tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato]europium-(III). According to the literature,[6] the major enantiomer (**4**) having positive $[\alpha]_D$ can be assigned as having *R*-configuration in the chiral center on the side chain.

***cis*-Casegravol (5)** Colorless oil. $[\alpha]_D$ −12.1° (c=0.102, $CHCl_3$). UV λ_{max} nm: 208. 255, 284, 320. IR ν_{max} cm^{-1}: 3630, 3450 (br), 1720, 1600. ^{1}H-NMR δ: 7.63 (1H, d, J=9.4 Hz, H-4), 7.41 (1H, d, J=8.4 Hz, H-5), 6.86 (1H, d, J=8.4 Hz, H-6), 6.78 (1H, d, J=12.4 Hz, H-1′), 6.49 (1H, d, J=12.4 Hz, H-2′), 6.25 (1H, d, J=9.4 Hz, H-3), 3.90 (3H, s, 7-OCH_3), 2.25 (3H, s, 3′-CH_3). CI-MS m/z 294 [M+NH_4]$^+$. MS m/z (%): 260 (23), 259 (100), 257 (12), 245 (45), 231 (17), 219 (19), 213 (14), 203 (23).

Isolation of Citrusarin-A (7) and -B (8) The dried root (3200 g) of *Citrus Hassaku* HORT. *ex* Y. TANAKA (Japanese name: hassaku) was extracted with acetone at room temperature. The acetone extract (485 g) was subjected to silica gel column chromatography with benzene. The benzene eluate was further chromatographed on silica gel eluted successively with hexane and benzene–hexane (1 : 9 and then 1 : 4). The eluates was subjected repeatedly to preparative TLC with benzene–ethyl acetate (9 : 1), hexane–benzene (1 : 9), isopropyl ether, acetone–hexane (1 : 9), ethyl acetate–benzene (1 : 19), and/or acetone–hexane (1 : 19) to obtain citrusarin-A (**7**) (210 mg) and citrusarin-B (**8**) (5.1 mg) as well as other known compounds, which were characterized by spectral analysis (UV, IR, and ^{1}H-NMR).[36]

Citrusarin-A (7) Pale yellow oil. 0.00065% yield from the dry root. $[\alpha]_D$ +5.2° (c=0.172, $CHCl_3$). UV λ_{max} nm: 210, 226, 284, 340. IR ν_{max} cm^{-1}: 1720, 1640, 1625, 1610. HR-MS Calcd for $C_{19}H_{20}O_4$ 312.1359. Found: 312.1353. MS m/z (%): 312 (M^+, 58), 298 (35), 297 (100), 241 (11).

Citrusarin-B (8) Pale yellow oil. 0.000016% yield from the dry root. $[\alpha]_D$ +3.0° (c=0.148, $CHCl_3$). UV λ_{max} nm: 204, 230, 239 (sh), 247 (sh), 285 (fl), 293, 328. IR ν_{max} cm^{-1}: 1720, 1640, 1620. HR-MS Calcd for $C_{19}H_{20}O_4$: 312.1360. Found: 312.1358. MS m/z (%): 312 (M^+, 35), 298 (34), 297 (100), 295 (14), 241 (11), 149 (28), 141 (11).

5,7-Diacetoxycoumarin (11) A mixture of **10**[11] (7.4 g), acetyl anhydride (100 ml), and pyridine (7 ml) was refluxed for 2 h. The reaction mixture was poured into ice water, neutralized with $NaHCO_3$, and then extracted with ethyl acetate. The organic layer was washed with diluted aqueous $NaHCO_3$ and water, dried with anhydrous $MgSO_4$, and then concentrated to dryness to give **11** (6.3 g) as colorless prisms. **11**: mp 124—125 °C. UV λ_{max} nm: 206, 290, 313. IR ν_{max} cm^{-1}: 1780, 1740, 1630. ^{1}H-NMR δ_H: 7.73 (1H, d, J=9.7 Hz, H-4), 7.04 (1H, d, J=2.0 Hz, H-6 or -8), 6.98 (1H, d, J=2.0 Hz, H-8 or -6), 6.40 (1H, d, J=9.7 Hz, H-3), 2.40 (3H, s, OAc), 2.33 (3H, s, OAc). MS m/z (%): 262 (M^+, 7), 220 (22), 179 (12), 178 (100), 150 (48).

5-(2-Enyl-3-methylbut)oxy-7-acetoxycoumarin (12)[12] A mixture of **11** (30 mg) and 1-bromo-3-methylbut-2-ene (20.4 mg) in acetone (4.5 ml) was stirred in the presence of anhydrous K_2CO_3 (94 mg) for 14 h at room temperature. The reaction mixture was filtered and the filtrate was concentrated to dryness. The residue was dissolved in H_2O and extracted with ethyl acetate. The organic layer was washed with 5% K_2CO_3 and H_2O, and dried with anhydrous $MgSO_4$. The solvent was evaporated off. The residue was subjected to preparative TLC ($CHCl_3$: acetone = 40 : 1) to give **12** (9.0 mg) and **11** (5.0 mg) as colorless needles. **12**: mp 128—129 °C (lit.[12] mp 127—129 °C). UV λ_{max} nm: 210, 240, 304. IR ν_{max} cm^{-1}: 1770, 1738, 1618. ^{1}H-NMR δ_H: 8.05 (1H, d, J=9.8 Hz, H-4), 6.69 (1H, d, J=2.0 Hz, H-6 or -8), 6.51 (1H, d, J=2.0 Hz, H-8 or, -6), 6.28 (1H, d, J=9.8 Hz, H-3), 5.48 (1H, t, J=6.7 Hz, H-2′), 4.59 (2H, d, J=6.7 Hz, H-1′), 2.33 (3H, s, OAc), 1.82 (3H, s, CH_3), 1.75 (3H, s, CH_3). MS m/z (%): 288 (M^+, 2), 220 (57), 178 (83), 150 (21), 149 (21), 81 (12), 70 (16), 69 (100).

5-(2-Enyl-3-methylbut)oxy-7-hydroxycoumarin (13)[12] A methanolic solution (2 ml) of **12** (5.1 mg) and 1% aqueous $NaHCO_3$ (0.1 ml) was refluxed for 30 min, neutralized with diluted HCl, and then extracted with ethyl acetate. The organic layer was dried with anhydrous $MgSO_4$ and the solvent was evaporated off. The residue was subjected to preparative TLC (CH_2Cl_2: acetone = 20 : 1) to give **13** (4.9 mg) as colorless prisms. **13**: mp 134—136 °C. UV λ_{max} nm: 208, 249, 257, 330. IR ν_{max} cm^{-1}: 3300 (br), 1720, 1615. ^{1}H-NMR δ_H: 8.04 (1H, d, J=9.4 Hz, H-4), 6.63 (1H, d, J=2.0 Hz, H-6 or -8), 6.33 (1H, d, J=2.0 Hz, H-8 or -6), 6.11 (1H, d, J=9.4 Hz, H-3), 5.46 (1H, t, J=6.7 Hz, H-2′), 4.55 (2H, d, J=6.7 Hz, H-1′), 1.79 (3H, s, CH_3), 1.73 (3H, s, CH_3). MS m/z (%): 246 (M^+, 14), 179 (12), 178 (83), 150 (41), 70 (10), 69 (100).

***O*-Methylation of 13** A mixture of **13** (30 mg), anhydrous K_2CO_3 (18 mg) and methyl iodide (76.5 mg) in acetone (10 ml) was refluxed for 3 h. K_2CO_3 was filtered off and the filtrate was subjected to preparative TLC ($CHCl_3$: acetone = 20 : 1) to give colorless needles (**14**, 29.9 mg). **14**: mp 88—91 °C. UV λ_{max} nm: 208, 246, 255, 328. IR ν_{max} cm^{-1}: 1730, 1615. ^{1}H-NMR δ_H: 8.00 (1H, d, J=9.8 Hz, H-4), 6.41 (1H, d, J=2.0 Hz, H-6 or H-8), 6.29 (1H, d, J=2.0 Hz, H-8 or H-6), 6.14 (1H, d, J=9.8 Hz, H-3), 5.48 (1H, t, J=6.7 Hz, H-2′), 4.57 (2H, d, J=6.7 Hz, H-1′), 3.85 (3H, s, OCH_3), 1.82 (3H, s, CH_3), 1.76 (3H, s, CH_3). MS m/z (%): 260 (M^+, 19), 193 (22), 192 (99), 164 (60), 163 (14), 149 (17), 135 (15), 70 (11), 69 (100). Irradiation of the methoxy protons at δ_H 3.85 gave **4** and 12% NOE of the protons signals at δ_H 6.41 and 6.29, respectively.

Cyclization Reaction[13] of 13 A mixture of **13** (10 mg) and sodium

acetate (20 mg) was heated in an oil bath at 190 °C for 20 min. The residue was dissolved in a small amount of acetone and subjected to preparative TLC (iso-Pr_2O : acetone = 20 : 1) to give three kinds of pale yellow oils, A (*Rf*: 0.21) (**15**), B (*Rf*: 0.58) (**16**), and C (*Rf*: 0.46) (**18**) in 38, 29, and 27% yields, respectively. A (**15**):[14] UV λ_{max} nm: 208, 259, 331. IR ν_{max} cm^{-1}: 3400 (br), 1720, 1615. ^{1}H-NMR (acetone-d_6) δ_H: 8.03 (1H, d, J = 9.8 Hz, H-4), 6.66 (1H, s, OH), 6.48 (1H, s, H-6), 6.03 (1H, d, J = 9.8 Hz, H-3), 5.24 (1H, t, J = 7.0 Hz, H-2'), 3.40 (2H, d, J = 7.0 Hz, H-1'), 1.81 (3H, s, CH_3), 1.64 (3H, s, CH_3). MS *m/z* (%): 246 (M^+, 27), 191 (35), 97 (20), 83 (26), 73 (21), 71 (33), 70 (24), 69 (100). B (**16**): UV λ_{max} nm: 212, 258, 328. IR ν_{max} cm^{-1}: 3260 (br), 1710, 1630. ^{1}H-NMR δ_H: 7.82 (1H, d, J = 9.4 Hz, H-4), 6.57 (1H, s, H-8), 6.13 (1H, d, J = 9.4 Hz, H-3), 4.52 (1H, q, J = 6.4 Hz, H-2'), 1.78 (1H, br d, OH), 1.46 (3H, s, CH_3), 1.41 (3H, d, J = 6.4 Hz, CH_3), 1.21 (3H, s, CH_3). MS *m/z* (%): 246 (M^+, 41), 232 (21), 231 (100), 203 (19), 149 (46), 101 (12). C (**18**)[15]: UV λ_{max} nm: 210, 258, 331. IR ν_{max} cm^{-1}: 3400 (br), 1720, 1630. ^{1}H-NMR δ_H: 7.93 (1H, d, J = 9.8 Hz, H-4), 6.72 (1H, s, H-8), 6.39 (1H, s, OH), 6.16 (1H, d, J = 9.8 Hz, H-3), 4.45 (1H, q, J = 6.4 Hz, H-2'), 1.49 (3H, s, CH_3), 1.39 (3H, d, J = 6.4 Hz, CH_3), 1.24 (3H, s, CH_3). MS *m/z* (%): 246 (M^+, 41), 232 (17), 231 (100), 203 (16), 149 (31).

Cyclization Reaction[13] of 14 A mixture of **14** (20 mg) and sodium acetate (40 mg) was heated in an oil bath at 190 °C for 20 min. The reaction mixture was dissolved in acetone. The acetone solution was subjected to preparative TLC (hexane : acetone = 4 : 1) to give colorless prisms (**17**) (5.7 mg) together with the starting material (**14**) (5.3 mg). **17**: mp 107—110 °C. UV λ_{max} nm: 212, 228, 256 (sh), 322. IR ν_{max} cm^{-1}: 1730, 1630. ^{1}H-NMR δ_H: 7.77 (1H, d, J = 9.7 Hz, H-4), 6.35 (1H, s, H-8), 6.14 (1H, d, J = 9.7 Hz, H-3), 4.49 (1H, q, J = 6.4 Hz, H-2'), 3.86 (3H, s, OCH_3), 1.39 (3H, d, J = 6.4 Hz, CH_3), 1.40 (3H, s, CH_3), 1.16 (3H, s, CH_3). MS *m/z* (%): 260 (M^+, 54), 246 (20), 245 (100), 217 (17), 135 (16), 105 (73).

***O*-Methylation of 16** A mixture of **16** (10 mg) and methyl iodide (15 mg) in acetone (3 ml) was refluxed for 35 min in the presence of anhydrous K_2CO_3 (125 mg). The reaction mixture was filtered. The filtrate was subjected to preparative TLC ($CHCl_3$: hexane = 4 : 1) to give colorless prisms, mp 105—110 °C (10.9 mg). This product was found to be identical with **17** derived from **14** by UV, IR, ^{1}H-NMR, and MS comparisons.

Citrusarin-B (8) from 16 A mixture of **16** (40.7 mg), 3-chloro-3-methylbut-1-yne (169 mg), and anhydrous K_2CO_3 (338 mg) in acetone (8 ml) containing one drop of dimethylformamide was refluxed for 8 h. During the reaction, the butyne (169 mg) was added six times to the reaction mixture at intervals of 70 min. The reaction mixture was filtered, and the filtrate was concentrated to dryness. The residue was dissolved in diethylaniline (2 ml) and heated at 220 °C for 1 h in N_2 gas.[16] Diluted HCl was added to the mixture and the solution was extracted with diethyl ether. The extract was dried over anhydrous $MgSO_4$, and the solvent was evaporated off. The residue was subjected to preparative TLC (hexane : acetone = 4 : 1, and then 2 : 1) to give a pale yellow oil (19 mg), which was found to be identical with natural citrusarin-B (**8**) by spectrometric comparisons (UV, IR, ^{1}H-NMR, and MS).

References and Notes

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Stereoselective Synthesis of Both Half Segments for (−)-Sarcophytonin A and (−)-Sarcophytoxide[1)]

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A stereoselective synthesis of a dihydrofuran derivative (5) and an allylhalide (8), possible precursors of the cembranes (−)-sarcophytonin A and (−)-sarcophytoxide, is described. The optically active dihydrofuran, right half, segment (49) was synthesized starting from a naturally occurring monoterpene, (−)-carvone. It was converted into the phenylselenide (14) *via* three steps. Oxidation of 14 with *m*-chloroperbenzoic acid and then treatment with pyridine yielded the epoxy olefin (29). Cleavage of the epoxide with boron trifluoride etherate gave the methoxy alcohol (31). Bromoetherification with *N*-bromosuccinimide selectively yielded the *cis*-hexahydro-1-oxaindan derivative (36) having a new chirality at the C-6 position. Reductive debromination of 36 followed by oxidation and then enol silylation yielded the less substituted silyl enol ether (39). Ozonolysis of 39 followed by methylation and elimination of methanol with potassium *tert*-butoxide and then reduction with 9-borabicyclo[3.3.1]nonane (9-BBN) gave the dihydrofuran derivative (41). Protection as the silyl ether (42) and phenylselenenylation and then treatment with hydrogen peroxide yielded the unsaturated ester (45). Reduction followed by thiophenylation of the resulting hydroxyl group afforded the dihydrofuran derivative (49) as a right half segment. Left half segments, the chloride (53) and the bromide (54), were synthesized starting from geraniol *via* several steps.

Keywords sarcophine; sarcophytonin A; sarcophytoxide; carvone; bromoetherification; cembrane; synthesis; diterpene; geraniol; dihydrofuran

The cembranoid diterpenes, a widely distributed class of marine natural products, have been the focus of considerable synthetic effort.[2)] Syntheses of cembrenes[3)] and cembrenols[4)] have been reported, while the more complex cembranolides have recently been receiving attention.[5)] (+)-Sarcophine (**1**) is a cembranolide repellent agent protecting soft-coral against predators. It was isolated from *Sarcophytum glaucum* in the Red Sea and identified as **1** by single crystal X-ray analysis in 1974.[6)] Its absolute configuration, (2*S*,7*S*,8*S*), was established by interpretation of the circular dichroism (CD) spectra.[7)] Other closely related compounds, (−)-(2*R*,7*R*,8*R*)-sarcophytoxide (**2**), its (−)-(2*R*,7*S*,8*S*)-stereoisomer (**2**), isosarcophytoxide (**3**) and their enantiomers were also isolated from genus *Sarcophyton*[8)] or *Lobophytum*.[9)] (−)-Sarcophytonin A (**4**), an oil $[\alpha]_D$ −92°, has been isolated from *Sarcophyton glaucum* collected at the island of Ishigaki, Okinawa, by Kobayashi and co-workers.[10)] The structure was established by comparing its spectral data with those of (+)-(2*S*,7*S*,8*S*)-sarcophytoxide (**2**) but the absolute configuration had not been determined. Bowden *et al.*[8c,9)] and Frincke *et al.*[8e)] reported some chemical correlations of these cembranoids, and discussed their absolute stereochemistry. Bowden and co-workers[9)] determined the absolute configuration of (−)-sarcophytonin A (**4**) at the C-2 position to be *R*.

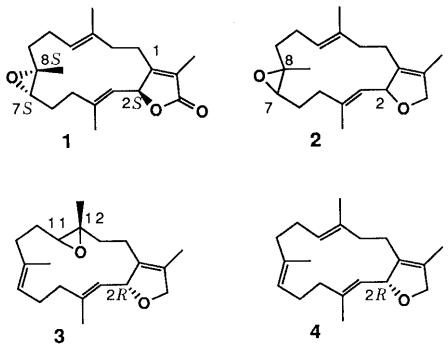

Chart 1

Results and Discussion

We planned the total synthesis of optically active sarcophine (**1**) and related compounds. Frincke and co-workers[8e)] found that air oxidation of (+)-sarcophytoxide (**2**) afforded (+)-sarcophine (**1**) in a good yield. Now, the target molecules of the total synthesis are sarcophytoxide (**2**), isosarcophytoxide (**3**), sarcophytonin A (**4**) and their enantiomers. We describe here some initial studies on the stereoselective synthesis of an intermediate with potential for elaboration to cembrane systems (**2**, **3** and **4**) having a dihydrofuran moiety as described above.

Our synthetic plan calls for the construction of two major segments, the dihydrofuran (**5**) and the bifunctional diene (**8**) or its epoxy derivatives (**9** and **10**). Sequential coupling of these segments *via* extrusion first of MY and then XZ completes the synthesis.[11)] We foresaw several advantages in this strategy. Four optical active isomers at the epoxide and the dihydrofuran moieties in the structure of sarcophytoxide (**2**) could be synthesized by the combination of the (+)- or (−)-epoxides and the (−)- or (+)-dihydrofuran segments. The syntheses of the dihydrofuran segment (**5**) and bifunctional diene (**8**) are the subject of this report.

Synthesis of the Right Half, Dihydrofuran, Segment Carvone, easily obtainable as a naturally occurring monoterpene, was selected as the starting material of the (+)- or (−)-dihydrofuran segment (**5**). The chirality of C-2 of **5** was induced from that of (−)-carvone by an intramolecular etherification of the cyclohexenyl alcohol (**7**), as shown in Chart 2.

Synthetic Route A (−)-Carvone was converted into the selenide (**14**) according to the procedure of Miyashita *et al.*[12)] The intermediate, selenoketone (**11**; mp 79—80 °C),[13)] was purified by repeated recrystallization to a constant specific rotation value ($[\alpha]_D$) of −120°. The pure selenide (**11**) was reduced with lithium aluminum hydride to the β-alcohol (**12**), and its hydroxyl group was protected with a tetrahydropyranyl group. The selenide (**14**) was treated with borane–tetrahydrofuran (THF) complex at −65 °C followed by excess hydrogen peroxide and 3 N potassium

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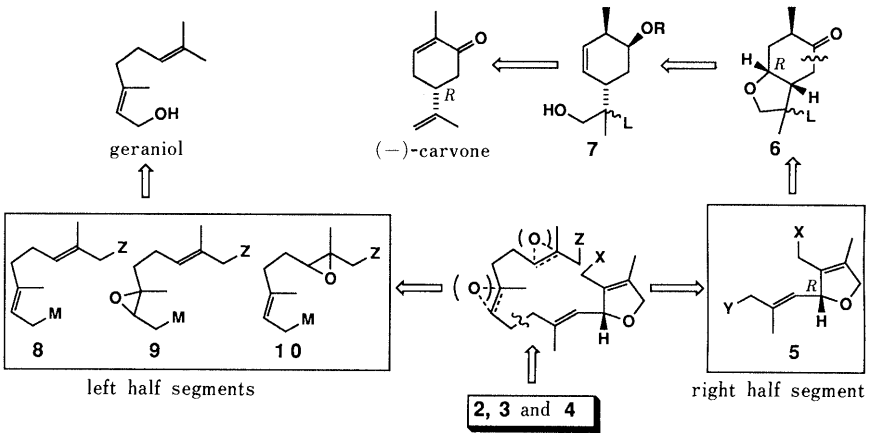


Chart 2

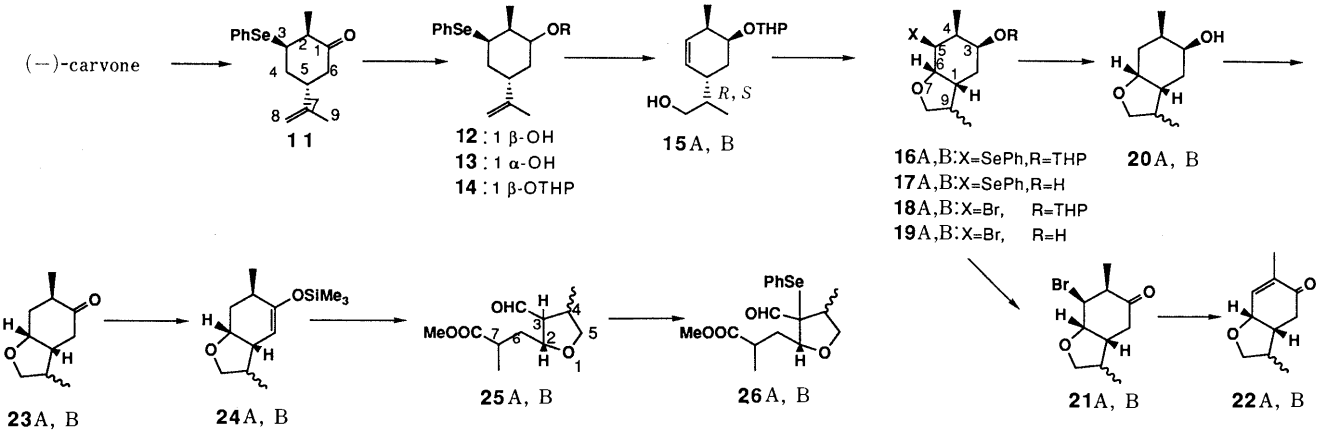


Chart 3

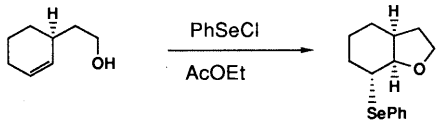


Chart 4

hydroxide to afford a mixture of the diastereoisomers of the primary alcohols (**15**) in 74% yield. These reactions, hydroboration and *in situ* elimination of the selenoxide, have good regioselectivities, but resulted in a poor stereoselectivity. The structure of **15** was confirmed by its proton nuclear magnetic resonance (^{1}H-NMR) spectrum exhibiting olefinic proton and C-8 methylene proton signals at δ 5.55 (2H, br s) and δ 3.38 (2H, m), respectively. We required regio- and stereo-selective etherification of the hydroxyolefin (**15**) in order to construct the dihydrofuran moiety of the right half segment. Clive and co-workers[14] reported that the selenoetherification of 3-(2-hydroxyethyl)-cyclohexene gave the *cis*-hexahydro-1-oxaindane skeleton (Chart 4). Treatment of **15** with phenylselenenyl chloride in ethyl acetate at −65 °C selectively afforded a mixture of the phenylselenides (**16**A and B), which was then treated with 10% sulfuric acid in acetone to yield the desired phenylselenides (**17**A and B), having *cis*-hexahydro-1-oxaindane structure, in 37 and 58% yields, respectively. The structures of the hydrooxaindanes were confirmed as follows. The relative stereochemistry of the phenylseleno

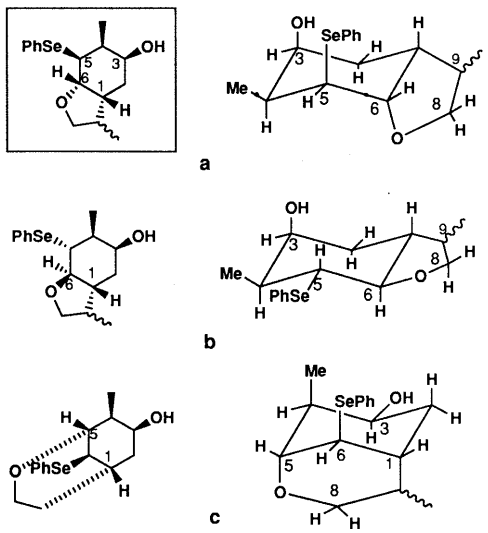


Fig. 1. The Possible Structures of **17**

group and C(6)–O bond should be *trans* from the reaction mechanism.[14] Therefore, three structures (**a**, **b** and **c**) as shown in Fig. 1 were possible for **17**.[15] The structure **c**, a six-membered cyclization product, was eliminated from the following transformations as described later, and the structures **a** and **b**, five-membered cyclization products, were supported by the experimental results of Clive *et al.*[14]

The configuration of the ring system of **17B** was confirmed to be *cis*(**a**) not *trans*(**b**) by the ^{1}H-NMR spectrum using a shift reagent, tris(dipivaloylmethanato)europium, as shown in Figs. 2 and 3; the signals due to C-5 (t, $J=4$ Hz) and C-6 (t, $J=4$ Hz) equatorial protons were observed. The other cyclic ether (**17A**) was considered to be an epimer of **17B** at C-9 from the spectral data, which were similar to those of **17B**. The removal of the phenylseleno groups of **17A** and B was effected with W-2 Raney nickel in acetone to afford **20A** and its C-9 epimer (**20B**) in good yields. Their ^{1}H-NMR spectra, exhibiting C-6 proton signals at δ4.02 (q, $J=4$ Hz) (**20A**) and δ4.01 (q, $J=3$ Hz) (**20B**), also supported the *cis*-bicyclo structure. Other etherification methods were examined. Bromoetherification[12,16] of

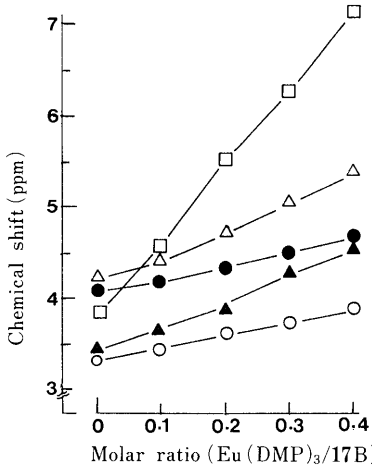


Fig. 2. The Chemical Shifts Values of the C-3, 5, 6 and 8 Proton Signals of **17B** Using Shift Reagent, Eu(DPM)$_3$

□, 3-H; ▲, 5-H; △, 6-H; ○, ●, 8-H.

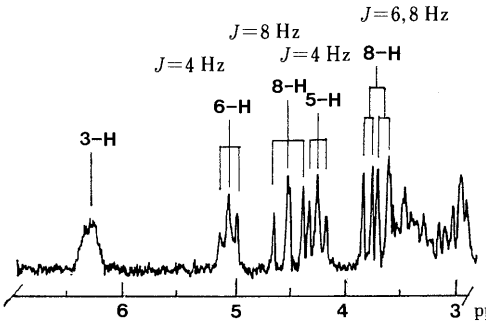


Fig. 3. The ^{1}H-NMR (at 60 MHz) Spectrum of **17B** in the Presence of 0.3 mol Equivalent of Eu(DMP)$_3$ in $CDCl_3$

15, effected with *N*-bromosuccinimide in acetonitrile at 0 °C, gave **18A** and B in 32 and 55% yields, respectively. The resulting bromides (**18A** and B) were then treated with pyridinium *p*-toluenesulfonate (PPTS) in ethanol to give quantitative yields of the bromoalcohols (**19A**, oil and **19B**, mp 112—113 °C). These structures were supported by the ^{1}H-NMR data, which were similar to those of the selenide (**17**). Jones oxidation of these alcohols followed by dehydrobromination with 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) at room temperature quantitatively yielded the α,β-unsaturated ketones (**22A** and B) within a few minutes. This experimental result, presumed to arise through antiperiplanar elimination, supported the proposed structures (**19**). The bromides (**19A** and B) were treated with tributyltin hydride in benzene to afford the *cis*-bicycloethers in 82—86% yields; these products were identical with **20A** and **20B** obtained by the selenoetherification method. Unfortunately, acid-catalyzed etherification,[17] a more direct method, of the alcohols (**15**) using concentrated hydrochloric acid in ethanol gave **20A** and **20B** but only in 12 and 32% yields, respectively. Consequently, the bromoetherification procedure was decided to be the best method for the synthesis of the hexahydrooxaindane skeleton.

After Jones oxidation of the alcohols (**20A** and B), the resulting ketones (**23A** and B) were regioselectively converted into the kinetically controlled less substituted silyl enol ethers (**24A** and B) by the use of lithium diisopropylamide and trimethylsilyl chloride at −78 °C. Ozonolysis followed by reductive work-up using methyl sulfide of both silyl enol ethers was performed in a mixture of methanol and methylene chloride in the presence of a pinch of sodium bicarbonate at −78 °C. The resulting crude carboxylic acids were methylated to give the formylated methyl carboxylates (**25A** and B) in 90 and 97% yields, respectively. These structures were confirmed from the ^{1}H-NMR spectra exhibiting the formyl proton signals at δ9.74 (d, $J=7$ Hz) for **25A** and δ9.63 (d, $J=4$ Hz) for **25B**. According to the enamine procedure of Williams and Nishitani,[18] the aldehyde (**25B**) was treated with piperidine at room temperature followed by phenylselenenyl chloride at −110 °C to afford a nearly 2:1 mixture of the epimeric selenides (**26Ba** and **26Bb**) in 66% combined yield. Unfortunately, oxidation of the major selenide (**26Ba**) with *m*-chloroperbenzoic acid (*m*CPBA) at −78 °C and *in situ* elimination of the selenoxide yielded the undesired α,β-unsaturated aldehyde (**27**) in 97%

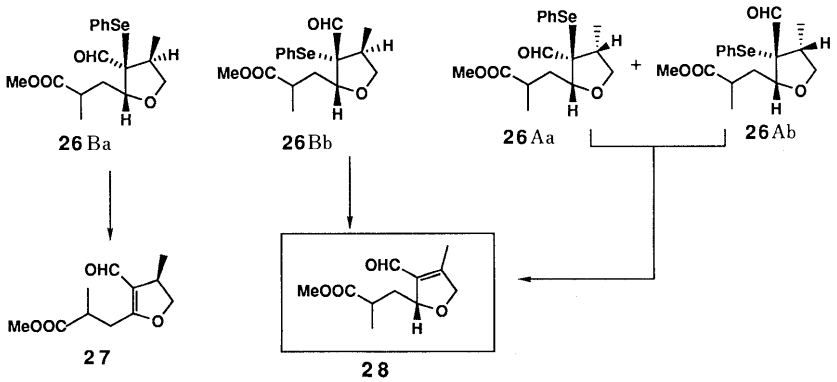


Chart 5

yield. The same treatment of the minor isomeric selenide (**26**Bb) gave a quantitative yield of the desired α,β-unsaturated aldehyde (**28**). These structures were confirmed by the following spectral data, **28**: IR: 1740, 1675 cm^{-1}; ^{1}H-NMR δ: 2.12 (3H, brs, 4-Me), 9.88 (1H, s, CHO), **27**: IR: 1740, 1653 cm^{-1}; ^{1}H-NMR δ: 1.15 and 1.16 (each 3H, d, J=7 Hz, 4,7-Me), 9.50 (1H, s, CHO). These experimental results, involving *syn*-elimination of selenoxide, suggested the stereochemistry of the above selenides (**26**Ba and **26**Bb) to be as shown in Chart 5. Phenylselenenylation of the other aldehyde (**25**A) *via* the enamine yielded a nearly 3:1 mixture of the epimeric selenides (**26**Aa and **26**Ab), exhibiting formyl proton signals at δ 9.28 (s) and 9.54 (d, J=1.5 Hz), in 39% combined yield. Treatment of the mixture with *m*CPBA gave the desired unsaturated aldehyde (**28**), but only in 28% yield. The yield of the conversion of the saturated aldehydes into the desired α,β-unsaturated aldehyde (**28**) was too low for this method to be useful as a the synthetic tool. Consequently, this approach had to be abandoned, and we sought a more effective approach.

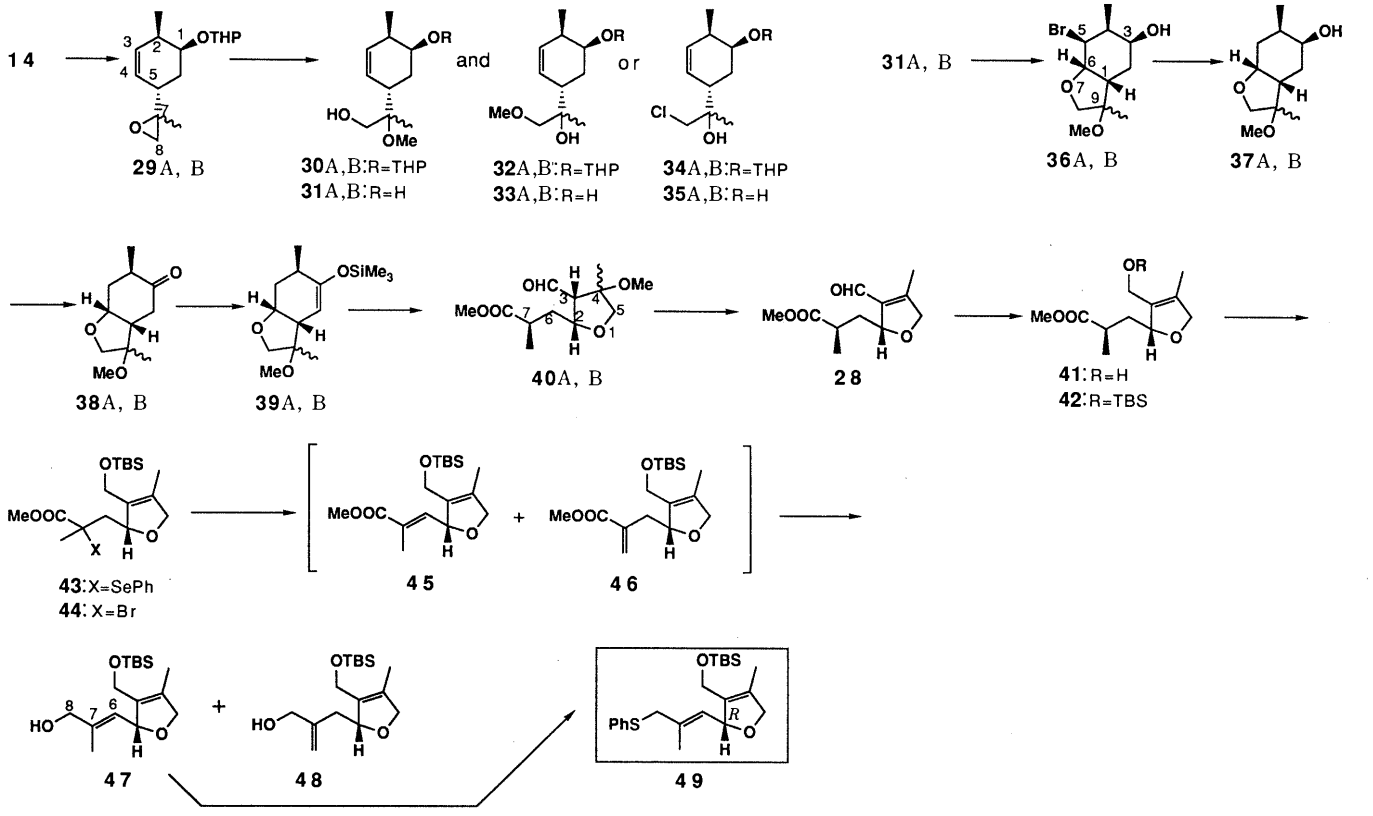
Synthetic Route B The main disadvantage of the above synthetic method is the formation of the undesired unsaturated aldehyde (**27**) because of the lack of stereoselectivity of the phenylselenenylation of the aldehydes (**25**A and B). In order to solve this problem, we placed a leaving group at the β-position (C-4) to the formyl group of the saturated aldehydes (**25**).

Treatment of the selenide (**14**) derived from (−)-carvone with 2.5 eq of *m*CPBA at −50 °C and then with pyridine in carbon tetrachloride at reflux temperature afforded the epoxy olefins (**29**) in 96% yield. The ^{1}H-NMR spectrum suggested that the epoxide was a mixture of diastereoisomers (**29**A and B). We needed a leaving group at the C-7 position of **15** in order to introduce an appropriate double bond selectively. The mixture of the epoxides (**29**) was treated with methanol in the presence of an acid under several conditions. These results are summarized in Table I. The opening of the epoxy group followed by removal of the tetrahydropyranyl protecting group was best effected with 3 eq of boron trifluoride etherate at −60—−30 °C for 20 h to give the expected primary alcohols (**31**A; oil and **31**B; mp 101—103 °C) along with the tertiary alcohol (**33**) in yields of 35, 31 and 14%, respectively (entry 4 in Table I). The former products (**31**A and B) appeared to be stereoisomeric by spectral criteria, but no effort was made to ascertain their stereochemistry. Regio- and stereoselective cyclization of **31** was performed by the bromoetherification method as described for the synthesis of **18**.

TABLE I. Opening of the Epoxy Group of **29** Using Acid

Entry	Acid[a]	(eq)	Time (h)	Temp. (°C)	Yield (%)				
					29	**30**+**32**	**31**	**33**	**34**+**35**
1	$TiCl_4$	(1.5)	4	−60—−40	0	0	0	0	87
2	BF_3–OEt_2	(1.0)	48	−60—−40	39	Trace	37	12	
3		(2.0)	48	−60—−30	16	37	28	11	
4		(3.0)	20	−60—−30	8	5	66	14	

a) The following acids were also examined for this reaction, but a complex mixture or the starting material was obtained. Complex mixture: Et_2AlCl, $AlCl_3$, $SnCl_4$, PPTS, $Mg(ClO_4)_2$, H_2SO_4. Starting material: $MgCl_2$, $ZnCl_2$.



A, B indicate the stereoisomer of the methyl group in the structures **29**—**40**

Chart 6

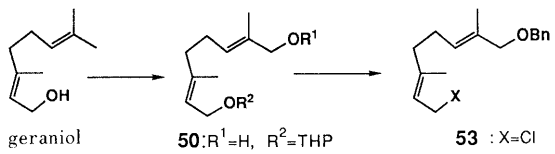
The primary alcohols (**31**A and B) were treated with *N*-bromosuccinimide in acetonitrile at −5 °C to afford the bromoethers (**36**A; oil and **36**B; mp 100—100.5 °C), having a *cis*-hexahydro-1-oxaindane skeleton, in fairly good yields. The structures of **36**A and B were confirmed from their ^{1}H-NMR spectra, analogous to those of **19**A and B, along with the reaction mechanism. Reductive debromination of the bromide (**36**A and B) with tributyltin hydride followed by Swern oxidation afforded the bicycloketones (**38**A and B) in good yields.

Trimethylsilylation of the kinetic enolate derived from the ketone (**38**) by the use of lithium diisopropylamide at −78 °C gave the less substituted silyl enol ethers (**39**A and B) quantitatively. The silyl enol ethers (**39**A and B) were treated with ozone in a mixture of methylene chloride and methanol in the presence of sodium bicarbonate at −78 °C, followed by methyl sulfide and then diazomethane to afford the methoxy aldehydes (**40**A and B) quantitatively. The configuration of the aldehydes at the C-7 position was expected to be R, but exact determination was not essential in this synthesis. The methoxy aldehyde (**40**A) and its epimer (**40**B) were treated with potassium *tert*-butoxide in THF at 0 °C to give a single α,β-unsaturated aldehyde in nearly 90% yield. The aldehyde was identical with the desired aldehyde (**28**) derived from the phenylselenide (**26**Bb). Reduction of the unsaturated aldehyde (**28**) to the allylic alcohol (**41**) could be effected with 9-borabicyclo-[3.3.1]nonane (9-BBN), but not with sodium borohydride and cerium chloride[19] or lithium tri-*tert*-butoxyaluminohydride.[20] Protection as the *tert*-butyldimethylsilyl ether (**42**) followed by phenylselenenylation afforded the phenylselenide (**43**) quantitatively. Unfortunately, oxidation of the selenide (**43**) with hydrogen peroxide and *in situ* elimination of the selenoxide gave rise to a nearly 2:3 mixture of regioisomers of the unsaturated ester, **45** and **46**, quantitatively. This ratio was calculated from the intensity of the olefinic proton signals appearing at δ 6.51 (br d, J=8 Hz) for **45**, and δ 5.67 and 6.20 (each d, J=1 Hz) for **46** in the ^{1}H-NMR spectrum. The value of the chemical shift (δ 6.51) of the internal olefinic proton of **45** suggested the *E* configuration of the double bond.[21] The other doublet signal around 6 ppm, expected for the *Z* isomer of **45**, was not observed at all in this mixture. Another method was examined to improve the yield of **45**. Bromination of the ester (**42**) was effected with lithium diisopropylamide and carbon tetrabromide at −78 °C, giving a quantitative yield of the bromoester (**44**). Dehydrobromination of the bromide (**44**) with lithium carbonate in hot *N*,*N*-dimethylformamide (DMF) gave a nearly 1.7:1 mixture of **45** and **46** in 85% yield. Isomerization of the double bond of the external olefin (**46**) to the internal olefin (**45**) by the use of several acids and bases was examined. When the 2:1 mixture of the olefins (**45** and **46**) was treated with NaH in THF at room temperature, a nearly 4:1 mixture of **45** and **46** was obtained in 54% yield. This experimental result can be explained in terms of the decomposition or the polymerization of **46**. Reduction of a nearly 2:1 mixture of the esters (**45** and **46**) with diisobutylaluminium hydride in toluene at −30 °C followed by separation gave a 51% yield of **47** along with a 27% yield of **48**. The allylic alcohol (**47**), exhibiting a doublet signal due to the vinyl proton at δ 5.32 in the ^{1}H-NMR spectrum, could be transformed into a halide, a tosylate, a sulfide or a selenide in the dihydrofuran (right half) segment (**5**). Douben and co-workers[5b] reported the synthesis of crassin acetate, in which they used the combination of the allylsulfide moiety and the tosylate for C–C bond formation. We selected the phenylsulfide (**49**) as a nucleophilic right half segment. The allylic alcohol (**47**) was treated with diphenyldisulfide and tributylphosphine to afford the (2*R*)-phenylsulfide (**49**) ($[\alpha]_D = -37.9°$) in 58% yield. The structure of the sulfide (**49**) thus obtained was supported by the spectral evidence. The ^{1}H-NMR spectral data of the sulfide (**49**) clorsely resembled those of sarcophytonin A (**4**) and sarcophytoxide (**3**), as shown in Table II.

TABLE II. Comparison of ^{1}H-NMR Data of **47** and **49** with Those of the Natural Products

Compound	C-3 H	C-2 H (C-6 H)[a]
47	5.32 (br d, J=9 Hz)	5.55 (m)
49	5.17 (br d, J=10 Hz)	5.47 (m)
(−)-Sarcophytonin-A[10]	5.06 (br d, J=10 Hz)	5.48 (d, J=10, 4.5 Hz)
(−)-Sarcophytoxide[8c]	5.28 (d, J=10 Hz)	5.55 (m)
(+)-Sarcophytoxide[8a]	5.23 (d, J=10 Hz)	5.54 (m)
(+)-Sarcophytoxide[8e]	5.12 (d, J=10 Hz)	5.36 (br d)

a) This assignment was made for **47** and **49**.



geraniol

50: R^1=H, R^2=THP
51: R^1=Bn, R^2=THP
52: R^1=Bn, R^2=H

53 : X=Cl
54 : X=Br

Chart 7

Synthesis of Left Half Segments (53 and 54) The hydroxyl group of **50**, prepared from geraniol by the modified method of Mori and co-workers,[22] was protected with a benzyl group to give **51**, which was then treated with PPTS in warm ethanol, giving **52**. The allylic alcohol (**52**) was converted into the allylchloride (**53**) in 71% yield by the use of *sym*-collidine, methanesulfonyl chloride and lithium chloride in DMF.[23] The alcohol (**52**) also converted into the allylbromide (**54**) in 87% yield.

Thus, the syntheses of the right half, dihydrofuran, segment and the left half segment of sarcophytonin A were achieved. Studies on the synthesis of sarcophytonin A, involving two C–C bond formations between the right and left half segments, are under way in our laboratory.

Experimental

All melting points were measured with a Yanaco hot-stage micro melting point apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on a JEOL FX-100 (100 MHz) or on a Hitachi R-24B (60 MHz) spectrometer. All NMR spectra were recorded in $CDCl_3$ and are reported in parts per million (ppm) relative to tetramethylsilane (TMS) (δ=0.0) or $CHCl_3$ (δ=7.26) unless otherwise noted. The following abbreviations are used for the signal patterns: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, and br=broad. Infrared (IR) spectra were run on a Hitachi 215 spectrophotometer. Ultraviolet (UV) spectra were obtained on a Hitachi 200-10 spectrophotometer using ethanol as a solvent. Mass spectra (MS) were recorded at 70 eV on a D-300 (low resolution) or on a Hitachi M-80 (high resolution) spectrometer using a direct inlet system.

High performance liquid chromatography (HPLC) was conducted on a Prep-pack cartridge silica column (57 mm i.d. × 300 mm) using a Waters LC 3000 system. Optical rotations were recorded on a JASCO DIP-360 digital polarimeter. Fuji-Davison Silica gel BW-127ZH (100—270 mesh) containing 2% fluorescence indicator F_{254} was used for column chromatography with a quartz column. Analytical thin-layer chromatography (TLC) was carried out using Merck Silica gel HF_{254}.

(2*S*,3*R*,5*S*)-2-Methyl-5-(1-methylethenyl)-3-(phenylseleno)-cyclohexanone (11) The method of Miyashita and co-workers was employed.[12] A solution of diphenyldiselenide (15.6 g, 49.9 mmol) in ethanol (190 ml) was treated with $NaBH_4$ (3.8 g, 99.9 mmol) at 0 °C. After the evolution of hydrogen gas was completed, acetic acid (9.85 ml, 176 mmol) was added, and the resulting colorless solution was stirred for 1 h. A solution of (−)-carvone (10.0 g, 66.6 mmol) in 39 ml of ethanol was added, and the resulting mixture was stirred for 3 h. The solvent was removed, then water and ether were added to the residue. The layers were separated, and the organic extracts were washed with 30% sodium carbonate solution and brine, and dried over $MgSO_4$. Removal of the solvent followed by recrystallization from hexane–ether afforded 13.0 g (64.0% yield) of the phenylselenide (**11**) as colorless needles, mp 79—80 °C.[12,13] $[\alpha]_D$ −119.9° (c=0.21, $CHCl_3$). *Anal.* Calcd for $C_{16}H_{20}OSe$: C, 62.54; H, 6.56. Found: C, 62.64; H, 6.64. IR (KBr) cm^{-1}: 3080, 1705, 1645, 1580. ^{1}H-NMR δ: 1.20 (3H, d, J=7 Hz, 2-Me), 1.65 (3H, br s, 8-Me), 1.8—3.2 (6H, m, 2,4,5,6-H), 3.95 (1H, q, J=4 Hz, 3-H), 4.70 (2H, br s, 9-H), 7.0—7.7 (5H, m, aromatic-H). MS m/z (rel. int. %): 308 (M^+, 42), 157 (22), 151 (M^+−PhSe, 100). High-resolution MS, Calcd for $C_{16}H_{20}OSe$ (M^+): 308.0678. Found: 308.0675.

(2*S*,3*R*,5*S*)-2-Methyl-5-(1-methylethenyl)-3-(phenylseleno)-cyclohexanols (12 and 13) The method of Miyashita and co-workers was employed.[12] A solution of the phenylselenoketone (**11**) (1.0 g, 3.28 mmol) in dry ether (15 ml) was treated with $LiAlH_4$ (125 mg, 3.28 mmol) at 0 °C with stirring for 5 min. The excess reagent was quenched by addition of wet ether, and the precipitate was filtered. The residue on the filter paper was washed several times with ether, and the combined organic layer was washed with brine, and then dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 10% EtOAc–hexane to give 797 mg (78.5%) of the β-alcohol (**12**) as an oil and 200 mg (19.7%) of the α-alcohol (**13**) as colorless needles, mp 97—98 °C (ether–hexane). Data for **12** (1*S*): $[\alpha]_D$ −107.3° (c=0.28, $CHCl_3$). IR (KBr) cm^{-1}: 3450, 3060, 1640, 740. ^{1}H-NMR δ: 1.20 (3H, d, J=6.5 Hz, 2-Me), 1.72 (3H, s, 7-Me), 1.80—2.20 (5H, m, 2,4,6-H), 2.84 (1H, br t, J=11 Hz, 5-H), 3.56 (1H, q, J=4 Hz, 3-H), 3.98 (1H, br, 1-H), 4.72 (2H, br s, 8-H), 7.00—7.70 (5H, m, aromatic-H). MS m/z (rel. int. %): 310 (M^+, 8), 292 (M^+-H_2O, 3), 157 ($PhSe^+$, 29), 153 (M^+−PhSe, 32), 135 (M^+−PhSe−H_2O, 100). High-resolution MS, Calcd for $C_{16}H_{22}OSe$ (M^+): 310.0834. Found: 310.0829. Data for **13** (1*R*): $[\alpha]_D$ −152.6° (c=0.21, $CHCl_3$). *Anal.* Calcd for $C_{16}H_{22}OSe$: C, 62.00; H, 7.35. Found: C, 61.90; H, 7.21. IR (film) cm^{-1}: 3450, 3080, 1655, 740. ^{1}H-NMR δ: 1.23 (3H, d, J=6 Hz, 2-Me), 1.67 (3H, br s, 7-Me), 2.84 (1H, tt, J=12, 4 Hz, 5-H), 3.2—3.8 (2H, m, 1,3-H), 3.48 (1H, br d, J=10 Hz, 1-H), 3.62 (1H, m, 3-H), 4.70 (2H, br s, 8-H), 7.00—7.70 (5H, m, aromatic-H). MS m/z (rel. int. %): 310 (M^+, 14), 157 ($PhSe^+$, 44), 153 (M^+−PhSe, 100), 135 (M^+−PhSe−H_2O, 78). High-resolution MS, Calcd for $C_{16}H_{22}OSe$ (M^+): 310.0834. Found: 310.0830.

(1*S*,2*S*,3*R*,5*S*)-2-Methyl-5-(1-methylethenyl)-3-(phenylseleno)-cyclohexanol Tetrahydropyranyl Ether (14) A solution of **12** (797 mg, 2.58 mmol), 3,4-dihydro-2*H*-pyran (0.47 ml, 5.16 mmol) and PPTS (130 mg, 0.516 mmol) in dry CH_2Cl_2 (12 ml) was stirred at room temperature for 7 h. Ether and 10% $NaHCO_3$ were added, and the organic layer was washed with water and brine, and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 5% EtOAc–hexane to give 1.02 g (quant.) of **14** as a colorless oil. $[\alpha]_D$ −71.9° (c=1.02, $CHCl_3$). IR (film) cm^{-1}: 3070, 1625, 1007. ^{1}H-NMR δ: 1.22, 1.36 (each 3/2H, d, J=7 Hz, 2-Me), 1.76 (3H, br s, 7-Me), 2.90 (1H, m, 5-H), 4.55 (3H, m, vinyl-H and -OTHP), 7.16—7.72 (5H, m, aromatic-H). MS m/z (rel. int. %): 394 (M^+, 13), 294 (7), 237 (M^+−SePh, 5), 85 (100). High-resolution MS, Calcd for $C_{21}H_{30}OSe$ (M^+): 394.1408. Found: 394.1406.

(1*S*,2*R*,5*S*)-5-(1-Hydroxypropan-2-yl)-2-methyl-3-cyclohexenol Tetrahydropyranyl Ether (15) Borane–THF complex (1.0 M THF solution) (18.9 ml, 18.9 mmol) was added dropwise over 20 min to a solution of **14** (2.5 g, 6.3 mmol) in 5 ml of THF at −65 °C. The mixture was stirred until the starting material disappeared on a TLC plate, and was warmed to 0 °C. Then 35% hydrogen peroxide (6.1 ml, 62.8 mmol) was added over 30 min, followed by 3 N NaOH (5.7 ml). The resulting reaction mixture was stirred at 65 °C for 15 h, then cooled to 0 °C, and saturated K_2CO_3 solution was added. The whole was extracted with ether. The extracts were washed with brine, and dried over $MgSO_4$. Removal of the solvent left a brown oil, which was purified by column chromatography with 20% EtOAc–hexane to give 1.20 g (74%) of **15** as a light yellow oil. IR (film) cm^{-1}: 3400, 3050, 1050. ^{1}H-NMR δ: 0.8—1.6 (6H, 2,7-Me), 2.20—2.40 (2H, m, 2,5-H), 3.38 (3H, m, 1-H, CH_2–OH), 4.63, 4.80 (each 1/2H, −OTHP), 5.55 (2H, br s, vinyl-H). MS m/z (rel. int. %): 152 (M^+−THPOH, 16), 121 (18), 95 (9), 85 (100).

Synthesis of (1*R*,3*S*,4*R*,6*R*,9*R*)- and (1*R*,3*S*,6*R*,9*S*)-3-Hydroxy-4,9-dimethyl-7-oxabicyclo[4.3.0]nonanes (20A and 20B). (a) Phenylselenoetherification Method Intramolecular Cyclization of the Alcohol **15**: The method of Clive and co-workers was employed.[14] A solution of **15** (1.25 g, 4.9 mmol) and phenylselenenyl chloride (938 mg, 4.9 mmol) in ethyl acetate (18 ml) was stirred at −65 °C under argon for 3 h. Removal of the solvent left 2.0 g of a dark yellow oil, which was purified by column chromatography with 20% EtOAc–hexane to give a mixture of the tetrahydropyranyl (THP)–ethers (**16**A and B) (1.22 g, 60%), **17**A (301 mg, 14.9%) and **17**B (268 mg, 13.3%). The mixture of (1*R*,3*S*,4*S*,5*S*,6*S*,9*R*)- and (1*R*,3*S*,4*S*,5*S*,6*S*,9*S*)-4,9-dimethyl-5-phenylseleno-3-(tetrahydropyran-2-yloxy)-7-oxabicyclo[4.3.0]nonanes (**16**A and B) was obtained as a colorless oil. IR (film) cm^{-1}: 3070, 1580, 1040, 1000. ^{1}H-NMR δ: 0.98—1.22 (6H, 4,9-Me), 4.65, 4.84 (each 1/2H, −OTHP), 7.10—7.70 (5H, m, aromatic-H). MS m/z (rel. int. %): 410 (M^+, 7), 157 ($PhSe^+$, 9), 151 (11), 85 (100). (1*R*,3*S*,4*S*,5*S*,6*S*,9*R*)-3-Hydroxy-4,9-dimethyl-5-phenylseleno-7-oxabicyclo[4.3.0]nonane (**17**A): colorless needles, mp 113—114 °C (hexane–ether). $[\alpha]_D$ −16.2° (c=0.16, $CHCl_3$). *Anal.* Calcd for $C_{16}H_{22}O_2Se$: C, 59.07; H, 6.82. Found: C, 59.07; H, 6.92. IR (KBr) cm^{-1}: 3450, 3070, 1550, 1020. ^{1}H-NMR δ: 0.96 (3H, d, J=7 Hz, 9-Me), 1.28 (3H, d, J=7 Hz, 4-Me), 2.1—2.8 (4H, m), 3.50 (1H, m, 5-H), 3.52 (1H, dd, J=8, 10 Hz, 8-H), 3.97 (1H, br s, 3-H), 4.02 (1H, t, J=8 Hz, 8-H), 4.32 (1H, t, J=3 Hz, 6-H), 7.18—7.66 (5H, m, aromatic H). MS m/z (rel. int. %): 326 (M^+, 32), 169 (M^+−PhSe, 14), 168 (M^+−PhSeH, 58), 157 (10), 156 (21), 151 (M^+−PheH−H_2O, 34), 124 (100). (1*S*,3*S*,4*S*,5*S*,6*S*,9*S*)-3-Hydroxy-4,9-dimethyl-5-phenylseleno-7-oxabicyclo[4.3.0]nonane (**17**B): a colorless oil. $[\alpha]_D$ −4.21° (c=1.32, $CHCl_3$). IR (film) cm^{-1}: 3420, 3070, 1580, 1010. ^{1}H-NMR δ: 1.05 (3H, d, J=6 Hz, 9-Me), 1.20 (3H, d, J=6 Hz, 4-Me), 3.32—3.70 (2H, m, 5,8-H), 3.88 (1H, m, 3-H), 4.04—4.40 (2H, m, 6,8-H), 4.18 (1H, d, J=8 Hz, 8-H), 4.28 (1H, t, J=4 Hz, 6-H), 7.10—7.70 (5H, m, aromatic-H). MS m/z (rel. int. %): 326 (M^+, 19), 168 (M^+−PhSe, 38), 157 (12), 156 (23), 124 (100). High-resolution MS, Calcd for $C_{16}H_{22}O_2Se$ (M^+): 326.0783. Found: 326.0777.

Removal of the THP Protecting Group of the Bicyclononanes (**16**A and B): A solution of the mixture of the THP ethers (**16**A and **16**B) (1.20 g, 2.9 mmol) described above in acetone (10 ml) and 10% sulfuric acid (2.5 ml) was stirred at 50 °C for 72 h. Water was added, and the whole was extracted with ether. The extracts were washed with 10% $NaHCO_3$ and brine, and then dried over $MgSO_4$. Removal of the solvent left 1.0 g of a yellow oil, which was subjected to column chromatography. Elution with 20% EtOAc–hexane afforded first the alcohol **17**A (288 mg, 30%), mp 113 °C, and then **17**B (657 mg, 69%). These spectral data were coincident with those of the authentic specimens described above.

Reductive Elimination of the Phenylseleno Group of **17**A: A solution of **17**A (85 mg, 0.26 mmol) in acetone (2 ml) was stirred with W2-Raney nickel (1.2 g) at room temperature for 7 h. The nickel was removed by filtration and washed several times with acetone. The filtrate was concentrated, and the residue was purified by column chromatography with 20% EtOAc–hexane to give 31 mg (70% yield) of the oxabicyclononane (**20**A) as a colorless oil. $[\alpha]_D$ −2.2° (c=0.86, $CHCl_3$). IR (film) cm^{-1}: 3450, 1030, 1000. ^{1}H-NMR δ: 0.96 (6H, d, J=7 Hz, 4,9-Me), 2.40—2.80 (1H, m), 3.46 (1H, dd, J=8, 10 Hz, 8-H), 3.92 (1H, m, $W_{1/2}$=6 Hz, 3-H), 4.00 (1H, t, J=8 Hz, 8-H), 4.02 (1H, q, J=4 Hz, 6-H). MS m/z (rel. int. %): 170 (M^+, 4), 169 (6), 152 (M^+-H_2O, 49), 137 (17), 111 (100). High-resolution MS, Calcd for $C_{10}H_{18}O_2$ (M^+): 170.1305. Found: 170.1298.

Reductive Elimination of the Phenylseleno Group of 17B: The selenide (**17**B) (80 mg, 0.25 mmol) was treated with W2-Raney nickel (1.2 g) in acetone (2 ml) at room temperature for 3 h. The crude product was purified by column chromatography with 20% EtOAc–hexane to give 37 mg (88%) of **20**B as a colorless oil. $[\alpha]_D$ −0.85° (c=0.22, $CHCl_3$). IR (film) cm^{-1}: 3450, 1030, 1000. ^{1}H-NMR δ: 0.95 (3H, d, J=7 Hz, 9-Me), 1.04 (3H, d, J=7 Hz, 4-Me), 3.34 (1H, dd, J=4, 9 Hz, 8-H), 3.80 (1H, m, $W_{1/2}$=7 Hz, 3-H), 4.01 (1H, q, J=3 Hz, 6-H), 4.31 (1H, dd, J=8, 9 Hz, 8-H). MS m/z (rel. int. %): 170 (M^+, 5), 169 (6), 152 (M^+-H_2O, 57),

137 (19), 111 (16), 98 (100). High-resolution MS, Calcd for $C_{10}H_{18}O_2$ (M^+): 170.1305. Found: 170.1300.

(b) Bromoetherification Method Intramolecular Cyclization of **15**: A solution of **15** (307 mg, 1.2 mmol) and *N*-bromosuccinimide (279 mg, 1.6 mmol) in dry acetonitrile (10 ml) was stirred at 0 °C for 5 min under argon. Water and ether were added, then the organic layer was washed with 10% Na_2CO_3 and brine and dried over $MgSO_4$. Removal of the solvent left an oil (500 mg), which was purified by column chromatography to give the bromides **18**A (130 mg), an oil, and **18**B (220 mg), an oil, in 32 and 55% yields, respectively. (1*R*,3*S*,4*S*,5*S*,6*S*,9*R*)-5-Bromo-4,9-dimethyl-3-(tetrahydropyran-2-yloxy)-7-oxabicyclo[4.3.0]nonane (**18**A): $[\alpha]_D$ −3.5° (c=1.2, $CHCl_3$). IR (film) cm^{-1}: 1015, 1000. ^{1}H-NMR δ: 1.00 (3H, d, J=7 Hz, 9-Me), 1.13, 1.25 (3H, d, J=7 Hz, 4-Me), 3.51 (1H, m), 3.52, 4.03 (each 1H, t, J=8 Hz, 8-H), 3.95 (2H, m), 4.32 (2H, m, 5,6-H), 4.66, 4.77 (each 1/2H, m, OTHP). MS m/z (rel. int. %): 233, 233 (M^+ −THP, 3), 169 (M^+ −THP−Br, 14), 85 (100). (1*R*,3*S*,4*S*,5*S*,6*S*,9*S*)-5-Bromo-4,9-dimethyl-3-(tetrahydropyran-2-yloxy)-7-oxabicyclo[4.3.0]-nonane (**18**B): $[\alpha]_D$ +3.4° (c=2.0, $CHCl_3$). IR (film) cm^{-1}: 1010, 1005. ^{1}H-NMR δ: 1.02 (3H, d, J=6 Hz, 9-Me), 1.12, 1.14 (each 3/2H, d, J=7 Hz, 4-Me), 2.30—2.64 (1H, m), 3.75—4.40 (5H, m), 4.54—4.80 (1H, m, OTHP). MS m/z (rel. int. %): 233, 231 (M^+ −THP, 3), 169 (M^+ −THP−Br, 47), 133 (24), 93 (27), 85 (100).

Removal of the THP Protecting Group of the Bromides (**18**A and **18**B): A solution of the THP ether (**18**A or **18**B) (200 mg) and a catalytic amount of PPTS in ethanol (5 ml) was heated at 45 °C for 6 h under argon. Water and EtOAc were added and the organic layer was washed with 10% $NaHCO_3$ and brine, and then dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 20% EtOAc–hexane to give the pure alcohol (**19**A or **19**B) in quantative yield. (1*R*,3*S*,4*S*,5*S*,6*S*,9*R*)-5-Bromo-3-hydroxy-4,9-dimethyl-7-oxabicyclo[4.3.0]nonane (**19**A): a slightly yellow oil: $[\alpha]_D$ −3.5° (c=3.7, $CHCl_3$). IR (film) cm^{-1}: 3450, 1025. ^{1}H-NMR δ: 1.00 (3H, d, J=6 Hz, 4 or 9-Me), 1.22 (3H, d, J=6 Hz, 9 or 4-Me), 3.49 (1H, dd, J=8, 10 Hz, 8-H), 3.92 (1H, m, 3-H), 4.03 (1H, t, J=8 Hz, 8-H), 4.20—4.43 (2H, m, 5,6-H). MS m/z (rel. int. %): 250, 248 (M^+, 9), 169 (M^+−Br, 9), 151 (11), 97 (100). High-resolution MS, Calcd for $C_{10}H_{17}BrO_2$ (M^+): 248.0410. Found: 248.0382. (1*R*,3*S*,4*S*,5*S*,6*S*,9*S*)-5-Bromo-3-hydroxy-4,9-dimethyl-7-oxabicyclo[4.3.0]nonane (**19**B), colorless plates, mp 112—113 °C: *Anal.* Calcd for $C_{10}H_{17}BrO_2$: C, 48.21; H, 6.88; Br, 32.07. Found: C, 48.04; H, 6.85; Br, 32.21. $[\alpha]_D$ −4.9° (c=0.85, $CHCl_3$). IR (KBr) cm^{-1}: 3500, 1090, 1060, 1030. ^{1}H-NMR δ: 1.05, 1.15 (each 3H, d, J=7 Hz, 4,9-Me), 3.40 (1H, dd, J=7, 9 Hz, 8-H), 3.70—4.50 (4H, m, 3,5,6,8-H). MS m/z (rel. int. %): 250, 248 (M^+, 4), 153 (M^+−Br, 8), 151 (12), 97 (100). High-resolution MS, Calcd for $C_{10}H_{17}BrO_2$ (M^+): 248.0411. Found: 248.0403.

Reductive Debromination of the Bromoalcohol (**19**A): A solution of **19**A (366 mg, 1.47 mmol) and tributyltin hydride (0.5 ml, 1.91 mmol) in dry benzene (6 ml) was stirred at 65 °C for 3 h. Removal of the solvent left an oil (857 mg), which was purified by column chromatography with 30% EtOAc–hexane, providing pure **20**A (204 mg, 82% yield). The physical and spectral data were coincident with those of an authentic specimen.

Reductive Debromination of the Bromoalcohol (**19**B): Treatment of **19**B (300 mg, 1.18 mmol) with tributyltin hydride (0.45 ml, 1.56 mmol) under the same conditions as described for **19**A afforded 176 mg of the alcohol (**20**B) in 86% yield. The alcohol was identical with an authentic specimen of **20**B.

(c) Acid Catalyzed Etherification of the Primary Alcohol (15) A solution of **15** (100 mg, 0.39 mmol) in 5 ml of ethanol containing 1 ml of 36% HCl was heated at reflux temperature for 48 h. After cooling, it was extracted with ether, and the extract was washed with 10% $NaHCO_3$ and brine, and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 20% EtOAc–hexane to give 8 mg (12% yield) of **20**A and 22 mg (32% yield) of **20**B.

(1*R*,3*S*,4*S*,6*R*,9*R*)-4,9-Dimethyl-7-oxabicyclo[4.3.0]-4-nonen-3-one (22A) Jones reagent (0.23 ml, 0.6 mmol) was added to a solution of **19**A (150 mg, 0.6 mmol) in a 2:1 mixture of acetone and ether (3 ml), and the reaction mixture was stirred at 0 °C for 5 min. Saturated $NaHCO_3$ and ether were added, and the organic layer was washed with brine and dried over $MgSO_4$. Removal of the solvent left the crude bromoketone (**21**A), which was treated with DBU (90 mg, 0.6 mmol) in benzene (3 ml) at 0 °C for 5 min. The reaction mixture was diluted with EtOAc, and washed with 5% HCl, water and brine, then dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 30% EtOAc–hexane to afford 99 mg (quant.) of **22**A as a colorless oil. $[\alpha]_D$ −5.9° (c=0.89, $CHCl_3$). IR (film) cm^{-1}: 1680, 1650, 1013. ^{1}H-NMR δ: 0.95 (3H, d, J=6 Hz, 9-Me), 1.30 (3H, br s, 4-Me), 3.55, 4.00 (each 1H, dd, J=7, 8 Hz, 8-H), 4.47 (1H, m, $W_{1/2}$=8 Hz, 6-H), 6.65 (1H, m, $W_{1/2}$=8 Hz, 5-H). MS m/z (rel. int. %): 166 (M^+, 100), 138 (81), 135 (67), 123 (99), 109 (69). High-resolution MS, Calcd for $C_{10}H_{14}O_2$ (M^+): 166.0993. Found: 166.0996.

(1*R*,3*S*,4*S*,6*R*,9*S*)-4,9-Dimethyl-7-oxabicyclo[4.3.0]-4-nonen-3-one (22B) The bromoalcohol **19**B (85 mg, 0.34 mmol) was treated with Jones reagent, and then DBU by the same method as described for the synthesis of **19**A, providing **22**B as a light yellow oil in quantative yield. $[\alpha]_D$ −4.6° (c=0.68, $CHCl_3$). IR (film) cm^{-1}: 1680, 1650, 1015. ^{1}H-NMR δ: 1.05 (3H, d, J=6 Hz, 9-Me), 1.30 (3H, br s, 4-Me), 3.40, 4.08 (each 1H, br t, J=8 Hz, 8-H), 4.70 (1H, m, $W_{1/2}$=11 Hz, 6-H), 6.50 (1H, m, $W_{1/2}$=6 Hz, 5-H). MS m/z (rel. int. %): 166 (M^+, 53), 138 (50), 123 (61), 109 (50), 69 (80), 41 (100).

(1*R*,4*R*,6*R*,9*R*)- and (1*R*,4*R*,6*R*,9*S*)-4,9-Dimethyl-7-oxabicyclo[4.3.0]-nonan-3-ones (23A and 23B) A solution of **20** (360 mg, 2.1 mmol) in acetone (7 ml) was treated with Jones reagent (1.2 ml) at 0 °C for 5 min. Saturated $NaHCO_3$ solution and ether were added, and the organic layer was washed with brine. Removal of the solvent left an oil, which was purified by column chromatography with 20% EtOAc–hexane to give the ketone (**23**). **23**A: An oil. Yield, starting from 51 mg of **20**A, 45 mg (89%). $[\alpha]_D$ −6.6° (c=3.0, $CHCl_3$). IR (film) cm^{-1}: 1710, 1035, 1020. ^{1}H-NMR δ: 0.95, 1.02 (each 3H, d, J=7 Hz, 4,9-Me), 1.65 (1H, ddd, J=3, 13, 15 Hz, 5α-H), 2.18—3.00 (6H, m), 3.65 (1H, dd, J=8, 10 Hz, 8-H), 4.10 (1H, q, J=3 Hz, 6-H), 4.12 (1H, t, J=8 Hz, 8-H). MS m/z (rel. int. %): 168 (M^+, 82), 153 (19), 127 (8), 125 (6), 111 (14), 109 (44), 97 (100). High-resolution MS, Calcd for $C_{10}H_{16}O_2$ (M^+): 168.1149. Found: 168.1155. **23**B: An oil. Yield, starting from 360 mg of **20**B, 310 mg (87%). $[\alpha]_D$ −1.9° (c=1.36, $CHCl_3$). IR (film) cm^{-1}: 1712, 1040. ^{1}H-NMR δ: 1.02, 1.07 (each 3H, d, J=7 Hz, 4,9-Me), 1.12—2.00 (2H, m), 2.10—2.70 (5H, m), 3.32 (1H, dd, J=8, 9 Hz, 8-H), 4.14 (1H, dd, J=7, 9 Hz, 8-H), 4.31 (1H, td, J=4, 7 Hz, 6-H). MS m/z (rel. int. %): 168 (M^+, 100), 153 (9), 127 (4), 125 (4). High-resolution MS, Calcd for $C_{10}H_{16}O_2$ (M^+): 168.1149. Found: 168.1158.

(1*S*,4*R*,6*R*,9*R*)- and (1*S*,4*R*,6*R*,9*S*)-4,9-Dimethyl-3-trimethylsilyloxy-7-oxabicyclo[4.3.0]-2-nonenes (24A and 24B) A THF solution of **23** (1.0 mmol) was added to a chilled (−78 °C) THF (3 ml) solution of lithium diisopropylamide (1.5 mmol), prepared from 0.2 ml (1.5 mmol) of diisopropylamine and 1.5 mmol of *n*-butyllithium hexane solution at 0 °C, and the resulting reaction mixture was stirred at −78 °C for 1 h. Chlorotrimethylsilane (0.2 ml, 1.5 mmol) was added to the reaction mixture, and the solution was stirred for 2 h at the same low temperature. Saturated $NaHCO_3$ and hexane were added, and the organic layer was washed with brine and dried over $MgSO_4$, Removal of the solvent left an oil, which was purified by column chromatography with 30% EtOAc–hexane to give pure silyl ether (**24**). **24**A: An oil. Yield, starting from 170 mg (1.5 mmol) of **23**A, 200 mg (83%). IR (film) cm^{-1}: 1660, 1255, 995, 845. ^{1}H-NMR (at 60 MHz) δ: 0.20 (9H, s, $SiMe_3$), 1.00, 1.08 (each 3H, d, J=7 Hz, 4,9-Me), 3.18 (1H, dd, J=7, 8 Hz, 8-H), 3.79 (1H, t, J=7 Hz, 8-H), 4.18 (1H, br, 6-H), 4.58 (1H, m, $W_{1/2}$=6 Hz, 2-H). MS m/z (rel. int. %): 240 (M^+, 38), 209 (13), 181 (48), 156 (18), 141 (19), 91 (69), 73 (100). High-resolution MS, Calcd for $C_{13}H_{24}O_2Si$ (M^+): 240.1520. Found: 240.1532. **24**B: An oil. Yield, starting from 310 mg (1.8 mmol) of **23**B, 411 mg (93%). $[\alpha]_D$ −1.0° (c=0.48, $CHCl_3$). IR (film) cm^{-1}: 1660, 1255, 1200, 840. ^{1}H-NMR (at 60 MHz) δ: 0.18 (9H, s, $SiMe_3$), 1.10, 1.12 (each 3H, d, J=6 Hz, 4,9-Me), 3.30 (1H, dd, J=5, 8 Hz, 8-H), 3.90 (1H, dd, J=6, 8 Hz, 8-H), 4.20 (1H, m, 6-H), 4.62 (1H, br d, J=3 Hz, 2-H). MS m/z (rel. int. %): 240 (M^+, 55), 209 (17), 181 (59), 156 (24), 141 (23), 83 (100). High-resolution MS, Calcd for $C_{13}H_{24}O_2Si$ (M^+): 240.1520. Found: 240.1532.

(2*R*,3*R*,4*R*)- and (2*R*,3*R*,4*S*)-3-Formyl-2-[2-(methoxycarbonyl)propyl]-4-methyl-tetrahydrofurans (25) Ozone was introduced into a solution of **24** (200 mg, 0.83 mmol) in wet CH_2Cl_2–methanol (4:1, 7 ml) containing a pinch of $NaHCO_3$ at −78 °C until the color of the solution changed to pale purple. The resulting mixture was stirred for 30 min at the same low temperature. After addition of methylsulfide (0.3 ml, 4.2 mmol), the resulting mixture was slowly warmed to room temperature and stirred for 1 h. The solvent was removed, and the residue was treated with an excess of ethereal diazomethane. Removal of the solvent left an oil, which was purified by column chromatography with 20% EtOAc–hexane to give the pure aldehyde (**25**). **25**A: An oil. Yield, starting from 200 mg (0.83 mmol) of **24**A, 160 mg (90%). IR (film) cm^{-1}: 1730, 1700, 1190. ^{1}H-NMR δ: 1.08, 1.16 (each 3H, d, J=7 Hz, 4,7-Me), 1.61 (1H, ddd, J=5, 7, 14 Hz, 6-H), 2.10 (1H, m, 6-H), 3.65 (3H, s, COOMe), 3.71 (1H, dd, J=8, 10 Hz, 5-H), 3.82—4.22 (2H, m, 2,5-H), 9.74 (1H, d, J=7 Hz, CHO). MS m/z

(rel. int. %): 213 (M^+-1, 1), 199 (M^+-Me, 8), 196 (M^+-H_2O, 4), 183 (M^+-OMe, 26), 156 (34), 127 (49), 113 (36), 88 (100). **25B**: An oil. Yield, starting from 175 mg (0.73 mmol) of **24B**, 152 mg (97%). IR (film) cm^{-1}: 1740, 1720, 1380, 1360, 1040. ^{1}H-NMR δ: 1.08, 1.17 (each 3H, d, $J=6$ Hz, 4,7-Me), 1.55 (1H, ddd, $J=4$, 6, 14 Hz, 7-H), 2.00 (1H, ddd, $J=7$, 10, 14 Hz, 6-H), 2.50 (2H, m), 3.30 (1H, dd, $J=7$, 8 Hz, 5-H), 3.65 (3H, s, COOMe), 4.04—4.30 (2H, m, 2,5-H), 9.63 (1H, d, $J=4$ Hz, CHO). MS *m/z* (rel. int. %): 213 (M^+-1, 0.5), 183 (M^+-OMe, 36), 156 (35), 127 (70), 113 (88), 88 (100).

Phenylselenenylation of 25B A solution of **25B** (127 mg, 0.59 mmol) and piperidine (55 mg, 0.65 mmol) in benzene (3 ml) was stirred with molecular sieves 3A at room temperature for 26 h. Removal of the molecular sieves followed by evaporation of the solvent gave a colorless oil. The resulting crude enamine was dissolved in anhydrous THF (3 ml), and was cooled to -110 °C. Then a solution of phenylselenenyl chloride (120 mg, 0.62 mmol) in THF (2 ml) was added, and the resulting mixture was stirred for 1 h. Water and ether were added, and the organic layer was separated, then washed with 10% $NaHCO_3$ and brine and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 20% EtOAc–hexane to give the phenylselenides **26Ba** (94 mg) and **26Bb** (50 mg) in 43 and 23% yields, respectively. (2*R*,3*R*,4*S*)-3-Formyl-2-[2-(methoxycarbonyl)propyl]-4-methyl-3-phenylseleno-tetrahydrofuran (**26Ba**): A pale yellow oil. $[\alpha]_D$ +14.3° ($c=0.47$, $CHCl_3$). IR (film) cm^{-1}: 1740, 1705. ^{1}H-NMR δ: 0.99, 1.14 (each 3H, d, $J=7$ Hz, 4,7-Me), 2.52 (1H, br q, $J=8$ Hz), 3.00 (1H, m), 3.30—3.76 (4H, COOMe, 5-H), 3.92—4.16 (2H, m), 7.20—7.70 (5H, m, aromatic-H), 9.49 (1H, s, CHO). MS *m/z* (rel. int. %): 339 (M^+-OMe, 5), 240 (91), 213 (36), 181 (28), 160 (100). High-resolution MS, Calcd for $C_{17}H_{22}O_4Se$ (M^+): 370.0682. Found: 370.0702. (2*R*,3*S*,4*S*)-3-Formyl-2-[2-(methoxycarbonyl)propyl]-4-methyl-3-phenylseleno-tetrahydrofuran (**26Bb**): A pale yellow oil. $[\alpha]_D$ +24.7° ($c=0.48$, $CHCl_3$). IR (film) cm^{-1}: 1730, 1700. ^{1}H-NMR δ: 1.04, 1.20 (each 3H, d, $J=7$ Hz, 4,7-Me), 2.37—2.85 (2H, m), 3.34 (1H, m), 3.68 (3H, s, COOMe), 4.06—4.52 (2H, m), 7.16—7.64 (5H, m, aromatic-H), 9.55 (1H, s, CHO). MS *m/z* (rel. int. %): 339 (M^+-OMe, 5), 240 (89), 213 (50), 181 (29), 160 (100), 131 (18). High-resolution MS, Calcd for $C_{17}H_{22}O_4Se$ (M^+): 370.0682. Found: 370.0694.

Phenylselenenylation of 25A The aldehyde (**25A**) (120 mg, 0.56 mmol) was treated with piperidine (53 mg, 0.62 mmol), and then with phenylselenenyl chloride (107 mg, 0.56 mmol) by the same method as described for the synthesis of **26B**, affording 80 mg (39% yield) of a mixture of phenylselenides (**26A**a and Ab) as a pale yellow oil. IR (film) cm^{-1}: 1740, 1673, 1065, 1000. ^{1}H-NMR (at 60 MHz) δ: 1.00, 1.22, 1.32 (6H, d, $J=7$ Hz, 4,7-Me), 3.3—4.4 (4H, m), 3.64 (3H, s, COOMe), 7.10—7.63 (5H, m, aromatic-H), 9.28 (0.7H, s, CHO), 9.54 (0.3H, d, $J=1.5$ Hz, CHO).

(4*S*)-3-Formyl-2-[2-(methoxycarbonyl)propyl]-4-methyl-4,5-dihydrofuran (27) The selenoaldehyde (**26Ba**) (50 mg, 0.14 mmol) was treated with 80% *m*CPBA (32 mg, 0.15 mmol) in CH_2Cl_2 (5 ml) at -78 °C for 15 min. Removal of the solvent gave a yellow oil, which was purified by column chromatography with 30% EtOAc–hexane to give 28 mg (97% yield) of the unsaturated aldehyde (**27**). IR (film) cm^{-1}: 1740, 1653, 1630. ^{1}H-NMR (at 60 MHz) δ: 1.15, 1.16 (each 3H, d, $J=7$ Hz, 4,7-Me), 2.73 (3H, m, 4,6-H), 3.60 (3H, s, COOMe), 4.00 (1H, dd, $J=4$, 9 Hz, 5-H), 4.45 (1H, t, $J=9$ Hz, 5-H), 9.50 (1H, s, CHO).

(2*R*)-3-Formyl-2-[2-(methoxycarbonyl)propyl]-4-methyl-2,5-dihydrofuran (28) The selenoaldehyde (**28Bb**) (45 mg, 0.12 mmol) was treated with 80% *m*CPBA (31 mg, 0.13 mmol) in CH_2Cl_2 (5 ml) at -78 °C for 30 min. Ether and 10% Na_2CO_3 were added, and the organic layer was washed with water and brine, then dried over $MgSO_4$. Removal of the solvent gave 50 mg of a yellow oil, which was purified by column chromatography with 30% EtOAc–hexane to give 26 mg (quant.) of **28** as a colorless oil. $[\alpha]_D$ −57.3° ($c=0.2$, $CHCl_3$). UV λ_{max} nm (ε): 256 (7000). IR (film) cm^{-1}: 1740, 1675. ^{1}H-NMR δ: 1.20 (3H, d, $J=7$ Hz, 7-Me), 1.92 (2H, m, 6-H), 2.12 (3H, br s, 4-Me), 2.66 (1H, quintet, $J=7$ Hz, 7-H), 3.62 (3H, s, COOMe), 4.63 (2H, m, $W_{1/2}=8$ Hz, 5-H), 5.06 (1H, m, $W_{1/2}=14$ Hz, 2-H), 9.88 (1H, s, CHO). MS *m/z* (rel. int. %): 212 (M^+, 1), 194 (7), 181 (10), 152 (8), 111 (100), 83 (48). High-resolution MS, Calcd for $C_{11}H_{16}O_4$ (M^+): 212.1047. Found: 212.1051.

Treatment of the Selenoaldehyde (26A) with *m*CPBA A mixture of selenoaldehydes (**26A**a and Ab) (49 mg, 0.13 mmol) was treated with *m*CPBA (32 mg, 0.14 mmol) in CH_2Cl_2 (5 ml) at -78 °C for 20 min. The resulting crude product was purified by column chromatography with 30% EtOAc–hexane to give 8 mg (28% yield) of **28**.

(1*S*,2*R*,5*S*)-7,8-Epoxy-2,7-dimethyl-1-(tetrahydropyranyl)oxy-3-cyclohexene (29) *m*CPBA (80%, 6.85 g, 31.8 mmol) was added to a solution of **14** (5.0 g, 12.7 mmol) in dry CH_2Cl_2 (5 ml) at -50 °C. The resulting mixture was stirred at -50 °C for 19 h and then warmed to room temperature. The reaction mixture was diluted with CH_2Cl_2, washed with 1 N NaOH, water and brine, and dried over $MgSO_4$. Removal of the solvent gave a crude oil, which was used for the following elimination reaction without purification. The crude product was stirred at 50 °C in dry CCl_4 (250 ml) with pyridine (2.5 ml, 25.4 mmol) for 1.5 h. Removal of the solvent left an oil, which was purified by column chromatography with 20% EtOAc–hexane to give 3.10 g (96% yield) of **29** as a colorless oil. $[\alpha]_D$ −100.7° ($c=1.13$, $CHCl_3$). IR (film) cm^{-1}: 3025, 1135, 1115, 1035, 1025. ^{1}H-NMR δ: 0.98, 1.10 (each 3/2H, d, $J=7$ Hz, 2-Me), 1.27 (3H, s, 7-Me), 2.46—2.72 (2H, m, 8-H), 4.52—4.76 (1H, −OTHP), 5.36—5.78 (2H, m, olefinic-H). MS *m/z* (rel. int. %): 252 (M^+, 0.1), 151 (2), 150 (4), 93 (21), 85 (100). High-resolution MS, Calcd for $C_{15}H_{24}O_3$ (M^+): 252.1724. Found: 252.1737.

(1*S*,2*R*,5*S*)-5-(1-Hydroxy-2-methoxypropan-2-yl)-2-methyl-3-cyclohexenols (31) Boron trifluoride etherate (1.48 ml, 12.0 mmol) was added dropwise to a solution of **29** (1.01 g, 4.0 mmol) in 26 ml of absolute methanol at -60 °C under an argon atmosphere. The reaction mixture was stirred at -60——30 °C for 20 h, and then sodium bicarbonate was added. The methanol was evaporated off, and water and chloroform were added to the resulting residue. The organic layer was washed with brine and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography followed by HPLC with ethyl acetate. The first fraction gave 111 mg (13.8% yield) of a diastereoisomeric mixture of (1*R*,2*R*,5*S*)-5-(2-hydroxy-1-methoxypropan-2-yl)-2-methyl-3-cyclohexanols (**33**) as a colorless oil. The second and the third fractions afforded 280 mg (34.9% yield) of **31A** as a colorless oil and 245 mg (30.6% yield) of **31B** as colorless plates, mp 101—103 °C (from EtOAc–hexane), respectively. **33**: IR (film) cm^{-1}: 3425, 3030, 1155, 1110, 1090, 1070, 1050. ^{1}H-NMR δ: 0.96—1.16 (6H, 2,7-Me), 3.15—3.64 (5H, m, 8-H, OMe), 3.98 (1H, br s, $W_{1/2}=11$ Hz, 1-H), 5.32—5.92 (2H, m, olefinic-H). MS *m/z* (rel. int. %): 200 (M^+, 0.3), 199 (0.2), 185 (M^+-Me, 5), 155 (3), 153 (3), 137 (21), 89 (100). High-resolution MS, Calcd for $C_{11}H_{20}O_3$ (M^+): 200.1412. Found: 200.1418. **31A**: $[\alpha]_D$ −83.8° ($c=1.14$, $CHCl_3$). IR (film) cm^{-1}: 3425, 3030, 1165, 1120, 1085, 1050. ^{1}H-NMR δ: 1.05 (3H, d, $J=7$ Hz, 2-Me), 1.14 (3H, s, 7-Me), 3.24 (3H, OMe), 3.52 (2H, ABq, $J=11$, 15 Hz, 8-H), 3.98 (1H, m, $W_{1/2}=10$ Hz, 1-H), 5.32—5.82 (2H, m, olefinic-H). MS *m/z* (rel. int. %): 185 (M^+-Me, 0.2), 169 (M^+-OMe, 6), 151 (11), 89 (100). **31B**: $[\alpha]_D$ −106.1° ($c=0.98$, $CHCl_3$). *Anal.* Calcd for $C_{11}H_{20}O_3$: C, 64.97; H, 10.07. Found: C, 64.91; H, 10.25. IR (KBr) cm^{-1}: 3350, 3020, 2820, 1165, 1120, 1085, 1065. ^{1}H-NMR δ: 1.06 (3H, d, $J=7$ Hz, 2-Me), 1.07 (3H, s, 7-Me), 3.25 (3H, s, OMe), 3.50 (2H, ABq, $J=11$, 15 Hz, 8-H), 3.97 (1H, br s, $W_{1/2}=10$ Hz, 1-H), 5.52—5.82 (2H, m, 3,4-H). MS *m/z* (rel. int. %): 169 (M^+-OMe, 7), 151 (11), 89 (100).

(1*R*,3*S*,4*S*,5*S*,6*S*)-5-Bromo-3-hydroxy-9-methoxy-4,9-dimethyl-7-oxabicyclo[4.3.0]nonanes (36A and B) A solution of **31A** (2.13 g, 10.65 mmol) in dry acetonitrile (106 ml) was treated with 2.47 g (13.84 mmol) of *N*-bromosuccinimide at -5 °C under an argon atmosphere. The resulting solution was stirred at the same temperature for 3 h, and then water and CH_2Cl_2 were added. The organic layer was washed with 10% $Na_2S_2O_3$ and half saturated brine, and then dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 50% EtOAc–hexane to give 2.82 g (94.9% yield) of **36A** as a colorless oil. **36B** (2.93 g, quant.) was also obtained from **31B** (2.03 g, 10.11 mmol) by the same method as described above. **36A**: $[\alpha]_D$ −14.1° ($c=1.06$, $CHCl_3$). IR (film) cm^{-1}: 3450, 1070, 1030. ^{1}H-NMR δ: 1.24 (3H, d, $J=7$ Hz, 4-Me), 1.26 (3H, s, 9-Me), 3.24 (3H, s, OMe), 3.73, 3.91 (each 1H, d, $J=10$ Hz, 8-H), 3.90 (1H, br s, $W_{1/2}=7$ Hz, 3-H), 4.34 (1H, dd, $J=3$, 4 Hz, 5-H), 4.68 (1H, t, $J=3$ Hz, 6-H). MS *m/z* (rel. int. %): 280, 278 (M^+, 0.4), 265, 263 (M^+-Me, 0.7), 199 (M^+-Br, 17), 198 (M^+-HBr, 51), 167 ($M^+-HBr-OMe$, 76), 151 ($M^+-Br-OMe-OH$, 100), 89 (86), 88 (97). High-resolution MS, Calcd for $C_{11}H_{19}BrO_3$ (M^+): 278.0517. Found: 278.0532. **36B**: Colorless needles, mp 100—100.5 °C (from ether–hexane). $[\alpha]_D$ +64.6° ($c=0.19$, $CHCl_3$). *Anal.* Calcd for $C_{11}H_{19}BrO_3$: C, 47.33; H, 6.86; Br, 28.62. Found: C, 47.40; H, 6.95; Br, 28.62. IR (KBr) cm^{-1}: 3480, 1070, 580. ^{1}H-NMR δ: 1.10 (3H, d, $J=7$ Hz, 4-Me), 1.24 (3H, s, 9-Me), 3.18 (3H, s, OMe), 3.43, 4.14 (each 1H, d, $J=10$ Hz, 8-H), 4.16 (1H, m, $W_{1/2}=8$ Hz, 3-H), 4.32 (1H, t, $J=3$ Hz, 6-H), 4.37 (1H, dd, $J=3$, 4 Hz, 5-H). MS *m/z* (rel. int. %): 280, 278 (M^+, 0.2), 265, 263 (M^+-Me, 0.8), 199 (M^+-Br, 14), 198 (M^+-HBr, 36), 167 ($M^+-HBr-OMe$, 74), 151 (82), 89 (100). High-resolution MS, Calcd for $C_{11}H_{19}BrO_3$ (M^+): 278.0517. Found: 278.0524.

(1*R*,3*S*,4*R*,6*S*)-3-Hydroxy-9-methoxy-4,9-dimethyl-7-oxabicyclo[4.3.0]-nonanes (37) A solution of **36**A (309 mg, 1.1 mmol) and tributyltin hydride (0.39 ml, 1.44 mmol) in dry benzene (11 ml) was refluxed for 3 h. Removal of the solvent left an oil, which was purified by column chromatography with 50% EtOAc–hexane to give 238 mg (quant.) of **37**A as a colorless oil. **37**B (775 mg, 98%) was obtained as a colorless oil from **36**B (1.10 g, 3.94 mmol) by the same method as described above. **37**A: $[\alpha]_D$ +0.86° (c=0.93, $CHCl_3$). IR (film) cm^{-1}: 3460, 1205, 1180, 1130, 1075, 1035. ^{1}H-NMR δ: 0.95 (3H, d, J=7 Hz, 4-Me), 1.21 (3H, s, 9-Me), 2.11—2.38 (1H, m, 1-H), 3.21 (3H, s, OMe), 3.67, 3.86 (each 1H, d, J=12 Hz, 8-H), 3.82 (1H, m, $W_{1/2}$=8 Hz, 3-H), 4.30 (1H, q, J=4 Hz, 6-H). MS m/z (rel. int. %): 200 (M^+, 1), 185 (M^+-Me, 3), 172 (M^+-H_2O, 2), 168 (M^+-MeOH, 75), 137 (44), 88 (100). High-resolution MS, Calcd for $C_{11}H_{20}O_3$ (M^+): 200.1411. Found: 200.1434. **37**B: $[\alpha]_D$ +34.1° (c=1.16, $CHCl_3$). IR (film) cm^{-1}: 3450, 1210, 1075, 1035. ^{1}H-NMR δ: 0.94 (3H, d, J=7 Hz, 4-Me), 1.21 (3H, d, J=1 Hz, 9-Me), 2.14 (1H, ddd, J=3, 7, 12 Hz, 1-H), 3.21 (3H, s, OMe), 3.61, 3.87 (each 1H, d, J=12 Hz, 8-H), 3.85 (1H, m, $W_{1/2}$=8 Hz, 3-H), 4.11 (1H, q, J=3 Hz, 6-H). MS m/z (rel. int. %): 185 (M^+-Me, 3), 169 (M^+-OMe, 10), 168 (M^+-MeOH, 63), 137 (40), 88 (100).

(1*R*,4*R*,6*S*)-9-Methoxy-4,9-dimethyl-7-oxabicyclo[4.3.0]nonan-3-ones (38) The method of Mancuso and Swern was employed.[23] Dimethylsulfoxide (0.17 ml, 2.3 mmol) was added to a solution of oxalyl chloride (0.12 ml, 1.3 mmol) in dry CH_2Cl_2 at −60 °C. The mixture was stirred for 3 min and then **37**A (201 mg, 1.0 mmol) was added. Stirring was continued for an additional 5 min. Triethylamine (0.7 ml, 5.0 mmol) was added and the resulting mixture was stirred at −60 to −30 °C for 1 h. Water and CH_2Cl_2 were added, and the organic layer was washed with brine and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 30% EtOAc–hexane to give 179 mg (90.2% yield) of **38**A as a colorless oil. **38**B (748 mg, 97.5%) was obtained as a colorless oil from 775 mg (3.87 mmol) of **37**B by the same method as described above. **38**A: $[\alpha]_D$ +12.0° (c=0.72, $CHCl_3$). IR (film) cm^{-1}: 1715, 1265, 1130, 1070, 1050. ^{1}H-NMR δ: 1.00 (3H, d, J=7 Hz, 4-Me), 1.24 (3H, s, 9-Me), 3.22 (3H, s, OMe), 3.81, 3.98 (each 1H, d, J=10 Hz, 8-H), 4.38 (1H, q, J=4 Hz, 6-H). MS m/z (rel. int. %): 198 (M^+, 5), 183 (M^+-Me, 1), 166 (M^+-MeOH, 8), 88 (100). High-resolution MS, Calcd for $C_{11}H_{18}O_3$ (M^+): 198.1255. Found: 198.1256. **38**B: $[\alpha]_D$ −44.5° (c=1.00, $CHCl_3$). IR (film) cm^{-1}: 1710, 1260, 1120, 1050. ^{1}H-NMR δ: 1.06 (3H, d, J=7 Hz, 4-Me), 1.27 (3H, s, 9-Me), 3.12 (3H, s, OMe), 3.33, 4.07 (each 1H, d, J=10 Hz, 8-H), 4.32 (1H, dt, J=5, 4 Hz, 6-H). MS m/z (rel. int. %): 198 (M^+, 8), 183 (M^+-Me, 2), 166 (M^+-MeOH, 9), 88 (100). High-resolution MS, Calcd for $C_{11}H_{18}O_3$ (M^+): 198.1255. Found: 198.1264.

(1*R*,4*R*,6*S*)-9-Methoxy-4,9-dimethyl-3-trimethylsilyloxy-7-oxabicyclo-[4.3.0]non-2-ene (39A) A THF (1 ml) solution of **38**A (179 mg, 0.9 mmol) was added to a solution of 1.17 mmol of lithium diisopropylamide, prepared from diisopropylamine (1.17 mmol) and 1.5 M *n*-butyl lithium–hexane solution in 18 ml of THF at 0 °C, at −78 °C under an argon atmosphere. The mixture was stirred for 30 min, and then chlorotrimethylsilane (0.18 ml, 1.36 mmol) was added to the resulting enolate. The mixture was stirred at the same low temperature (−78 °C) for 1 h, and then 10% $NaHCO_3$ was added. The whole was extracted with ether, and the extract was washed with cold brine, and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 20% EtOAc–hexane to give 233 mg (95% yield) of **39**A as a colorless oil. **39**B (150 mg, 97% yield) was obtained as a colorless oil from 113 mg (0.57 mmol) of **38**A by the same method as described above. **39**A: $[\alpha]_D$ −29.2° (c=1.09, $CHCl_3$). IR (film) cm^{-1}: 1665, 1265, 1140, 1070, 850. ^{1}H-NMR δ: 0.08 (9H, s, $SiMe_3$), 0.91 (3H, d, J=7 Hz, 4-Me), 1.12 (3H, s, 9-Me), 3.14 (3H, s, OMe), 3.42, 3.70 (each 1H, d, J=10 Hz, 8-H), 4.29 (1H, m, 6-H), 4.46 (1H, br d, J=3 Hz, 2-H). MS m/z (rel. int. %): 270 (M^+, 18), 255 (M^+-Me, 1), 238 (M^+-MeOH, 5), 182 (100), 73 (Me_3Si^+, 71). High-resolution MS, Calcd for $C_{14}H_{25}O_3Si$ (M^+): 270.1649. Found: 270.1636. **39**B: $[\alpha]_D$ −33.8° (c=0.31, $CHCl_3$). IR (film) cm^{-1}: 1660, 1255, 1130, 1065, 845. ^{1}H-NMR δ: 0.04 (9H, s, $SiMe_3$), 0.99 (3H, d, J=7 Hz, 4-Me), 1.21 (3H, s, 9-Me), 3.20 (3H, s, OMe), 3.79, 3.97 (each 1H, d, J=10 Hz, 8-H), 4.28—4.60 (2H, m, 2,6-H). MS m/z (rel. int. %): 270 (M^+, 15), 255 (M^+-Me, 21), 239 (6), 182 ($M^+-Me-SiMe_3$, 82), 73 ($SiMe_3$, 100). High-resolution MS, Calcd for $C_{14}H_{26}O_3Si$ (M^+): 270.1649. Found: 270.1636.

(2*S*,3*S*,4*R*)-3-Formyl-4-methoxy-2-[2-(methoxycarbonyl)propyl]-4-methyl-tetrahydrofuran (40) Ozone was introduced into a solution of **39**A (492 mg, 1.82 mmol) in a mixture of methanol and CH_2Cl_2 (3:1, 30 ml) containing a pinch of $NaHCO_3$ at −78 °C until the color of the solution changed to pale purple. The mixture was stirred for 30 min at −78 °C, and nitrogen gas was introduced into it. Methylsulfide (1.4 ml, 18 mmol) was added and the reaction mixture was stirred at −78 °C for 30 min and then at room temperature for 1 h. The solvent was removed, and the residue was dissolved in ether, then treated with an excess of ethereal diazomethane. After removal of the solvent, water and ethyl acetate were added to the residue. The organic layer was washed with brine, and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 30% EtOAc–hexane to give 482 mg (quant.) of **40**A as a colorless oil. The aldehyde (**40**B) (125 mg, 94.3% yield) was obtained as a colorless oil from 150 mg (0.55 mmol) of **39**B by the same method as described above. **40**A: $[\alpha]_D$ +38.1° (c=0.53, $CHCl_3$). IR (film) cm^{-1}: 1740 (br), 1205, 1100. ^{1}H-NMR δ: 1.18 (3H, d, J=7 Hz, 7-Me), 1.32 (3H, s, 4-Me), 3.22 (3H, s, OMe), 3.56, 3.71 (each 1H, d, J=10 Hz, 5-H), 3.64 (3H, s, COOMe), 4.00—4.44 (1H, m, 2-H), 9.61 (1H, d, J=6 Hz, CHO). MS m/z (rel. int. %): 243 (M^+-1, 5), 227 (3), 216 (4), 187 (28), 155 (21), 129 (49), 95 (53), 88 (78), 43 (100). **40**B: $[\alpha]_D$ +14.7° (c=0.35, $CHCl_3$). IR (film) cm^{-1}: 1740 (br), 1205, 1180, 1080. ^{1}H-NMR δ: 1.18 (3H, d, J=7 Hz, 7-Me), 1.31 (3H, s, 4-Me), 3.22 (3H, s, OMe), 3.66 (3H, s, COOMe), 3.85, 3.98 (each 1H, d, J=10 Hz, 5-H), 4.15—4.45 (1H, m, 2-H), 9.62 (1H, d, J=6 Hz, CHO). MS m/z (rel. int. %): 243 (M^+-1, 10), 227 (1), 187 (73), 155 (41), 129 (61), 125 (58), 95 (58), 88 (100).

(2*R*)-3-Formyl-2-[2-(methoxycarbonyl)propyl]-4-methyl-2,5-dihydrofuran (28) A solution of **40**A (482 mg, 1.97 mmol) and potassium *tert*-butoxide (44 mg, 0.4 mmol) in dry THF (40 ml) was stirred at 0 °C for 30 min. Saturated NH_4Cl and ether were added, and the organic layer was washed with brine and then dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 30% EtOAc–hexane to give 372 mg (89.6% yield) of **28** as a colorless oil. **40**B (41 mg) was also treated with potassium *tert*-butoxide by the same method as described above to give 32 mg (89.6% yield) of **28**. The dihydrofuran (**28**) thus obtained was identical with an authentic specimen of **28**.

(2*R*)-3-Hydroxymethyl-2-[2-(methoxycarbonyl)propyl]-4-methyl-2,5-dihydrofuran (41) A 0.5 M 9-BBN THF solution (14.4 ml, 7.2 mmol) was added to a solution of **28** (480 mg, 2.26 mmol) in THF (22 ml). The mixture was stirred at room temperature for 2 h, and then 2 N NaOH (3.5 ml) and 35% H_2O_2 (1.8 ml) were added. The mixture was stirred for 3 h, and extracted with EtOAc. The extract was washed with brine and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 50% EtOAc–hexane to give 461 mg (95.0% yield) of **41** as a colorless oil. $[\alpha]_D$ +2.0° (c=0.69, $CHCl_3$). IR (film) cm^{-1}: 3420, 1735, 1230, 1170, 1005. ^{1}H-NMR δ: 1.20 (3H, d, J=7 Hz, 7-Me), 1.68 (3H, s, 4-Me), 2.70 (1H, q, J=7 Hz, 7-H), 3.68 (3H, s, COOMe), 4.18 (2H, br s, CH_2OH), 4.48 (2H, m, $W_{1/2}$=10 Hz, 5-H), 4.94 (1H, m, $W_{1/2}$=17 Hz, 2-H). MS m/z (rel. int. %): 197 (M^+-OH, 2), 196 (M^+-H_2O, 1), 183 (M^+-OMe, 7), 168 (23), 125 (22), 97 (40), 33 (100).

(2*R*)-3-[(*tert*-Butyldimethylsilyl)oxymethyl]-2-[2-(methoxycarbonyl)-propyl]-4-methyl-2,5-dihydrofuran (42) A mixture of **41** (85 mg, 0.40 mmol), imidazole (87 mg, 1.27 mmol) and *tert*-butyldimethylsilyl chloride (96 mg, 0.64 mmol) in dry DMF (5 ml) was stirred at room temperature for 10 h. The reaction mixture was poured into ice-water, and extracted with ether. The extract was washed with brine and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 20% EtOAc–hexane to give 127 mg (97% yield) of **42** as a colorless oil. $[\alpha]_D$ +13.2° (c=1.11, $CHCl_3$). IR (film) cm^{-1}: 1735, 1250, 1060, 1005, 835. ^{1}H-NMR δ: 0.06 (6H, s, $SiMe_2$), 0.86 (9H, s, Si-*tert*-Bu), 1.18 (3H, d, J=7 Hz, 7-Me), 1.64 (3H, br s, 4-Me), 2.69 (1H, q, J=7 Hz, 7-H), 3.63 (3H, s, COOMe), 4.19 (2H, br d, J=5 Hz, 3-CH_2O), 4.45 (2H, br s, 5-H), 4.90 (1H, m, $W_{1/2}$=17 Hz, 2-H). MS m/z (rel. int. %): 328 (M^+, 0.2), 271 (M^+-tert-Bu, 55), 239 (M^+-tert-Bu−MeOH, 35), 227 (35), 95 (100). High-resolution MS, Calcd for $C_{17}H_{32}O_4Si$ (M^+): 328.2067. Found: 308.2067.

(2*R*)-3-[(*tert*-Butyldimethylsilyl)oxymethyl]-2-[2-(methoxycarbonyl)-2-phenylselenopropyl]-4-methyl-2,5-dihydrofuran (43) A THF (1 ml) solution of **42** (57 mg, 0.17 mmol) was added to a solution of 0.26 mmol of lithium diisopropylamide in anhydrous THF (4 ml), prepared from diisopropylamine (0.26 mmol) and 1.5 M *n*-butyllithium hexane solution (0.26 mmol), at −78 °C. The mixture was stirred at −78 °C for 30 min, and then a mixture of diphenyldiselenide (123 mg, 0.344 mmol) and hexamethylphosphoric triamide (0.06 ml, 0.344 mmol) in THF (1 ml) was added. The reaction mixture was stirred at the same low temperature for an additional 20 min. Saturated NH_4Cl and ether were added, and the

organic layer was washed with brine and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 20% EtOAc–hexane to give 84 mg (quant.) of **43** as a colorless oil. $[\alpha]_D$ −2.6° (c=1.02, $CHCl_3$). IR (film) cm^{-1}: 3075, 1730, 1250, 1115, 1065, 1010, 835. ^{1}H-NMR δ: 0.03 (6H, s, $SiMe_2$), 0.87 (9H, s, Si-*tert*-Bu), 1.61 (6H, br s, 4,7-Me), 3.41, 3.56 (each 3/2H, s, COOMe), 4.30 (2H, br s, 3-CH_2O), 4.41 (2H, br s, 5-H), 4.90 (1H, m, $W_{1/2}$=20 Hz, 2-H), 7.08—7.62 (5H, m, aromatic-H). MS m/z (rel. int. %): 427 (M^+ − *tert*-Bu, 2), 326 (M^+ − PhSe, 1), 269 (5), 227 (35), 95 (100).

(2*R*)-2-[2-Bromo-2-(methoxycarbonyl)propyl]-3-[(*tert*-butyldimethylsilyl)oxymethyl]-4-methyl-2,5-dihydrofuran (44) A solution of **43** (50 mg, 0.15 mmol) in THF (4 ml) was added to a solution of 0.22 mmol of lithium diisopropylamide at −78 °C and the mixture was stirred for 30 min. A THF solution of carbon tetrabromide (100 mg, 0.3 mmol) was then added, and the reaction mixture was stirred at −78 °C for 15 min. Saturated NH_4Cl and ether were added, and the organic layer was washed with brine and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 20% EtOAc–hexane to give 63 mg (quant.) of the bromide (**44**) as a colorless oil. $[\alpha]_D$ −14.5° (c=0.56, $CHCl_3$). IR (film) cm^{-1}: 1735, 1250, 1118, 1065, 1045, 840. ^{1}H-NMR δ: 0.11 (6H, s, $SiMe_2$), 0.94 (9H, s, Si-*tert*-Bu), 1.64 (3H, br s, 4-Me), 2.01 (3H, br s, 7-Me), 3.72 (3H, s, COOMe), 4.17 (2H, br s, 2-CH_2O), 4.35 (2H, br s, 5-H), 5.90 (1H, m, $W_{1/2}$=15 Hz, 2-H). $[\alpha]_D$ −14.5° (c=0.56, $CHCl_3$). IR (film) cm^{-1}: 1735, 1250, 1118, 1065, 1045, 840. ^{1}H-NMR δ: 0.11 (6H, s, $SiMe_2$), 0.94 (9H, s, Si-*tert*-Bu), 1.64 (3H, br s, 4-Me), 2.01 (3H, br s, 7-Me), 3.72 (3H, s, COOMe), 4.17 (2H, br s, 3-CH_2O), 4.35 (2H, br s, 5-H), 5.90 (1H, m, $W_{1/2}$=15 Hz, 2-H). MS m/z (rel. int. %): 352, 350 (M^+ − *tert*-Bu, 0.8), 328 (M^+ − Br, 0.1), 227 (20), 95 (100).

(2*R*)-3-[(*tert*-Butyldimethylsilyl)oxymethyl]-2-[2-(methoxycarbonyl)-4-methyl-(1*E*)-1- and 2-propenyl]-2,5-dihydrofurans (45 and 46) a) Synthesis from the Selenide (**43**): A 35% hydrogen peroxide solution (0.17 ml) and acetic acid (0.05 ml) were added to a solution of **43** (138 mg, 0.29 mmol) in THF (7 ml) at 0 °C. The resulting mixture was stirred at 0 °C for 30 min and then at room temperature for 1 h. Then 10% $NaHCO_3$ and ether were added, and the organic layer was washed with brine and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 20% EtOAc–hexane to give 92 mg (quant.) of a 2:3 mixture of **45** and **46** as a colorless oil. IR (film) cm^{-1}: 1730, 1640, 1260, 1210, 1145, 1050, 845. ^{1}H-NMR δ: 0.09 (6H, s, $SiMe_2$), 0.91, 0.92 (9H, s, Si-*tert*-Bu), 1.67 (21/5H, br s, 4-Me, and 7-Me of **45**), 3.73 (3H, s, COOMe), 4.24 (2H, br s, 3-CH_2O), 4.33—4.56 (2H, m, 5-H), 4.56—4.82 (3/5H, m, 2-H of **46**), 5.38—5.72 (2/5H, m, 2-H of **45**), 5.67, 6.20 (each 3/5H, d, J=1 Hz, =CH_2 of **46**), 6.51 (2/5H, br d, J=8 Hz, 6-H of **45**). MS m/z (rel. int. %): 326 (M^+, 0.8), 311 (M^+ − Me, 1), 269 (M^+ − *tert*-Bu, 32), 227 (51), 95 (100). High-resolution MS, Calcd for $C_{17}H_{30}O_4Si$ (M^+): 326.1912. Found: 326.1930.

b) Synthesis from the Bromide (**44**): A mixture of **44** (100 mg, 0.25 mmol), Li_2CO_3 (73 mg, 0.98 mmol) and LiBr (28 mg, 0.32 mmol) in dry DMF (6 ml) was heated at 120 °C for 15 h. Water and EtOAc were added, and the organic layer was washed with brine and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 20% EtOAc–hexane to give 69 mg (85% yield) of a 1.7:1 mixture of **45** and **46**.

(2*R*)-3-[(*tert*-Butyldimethylsilyl)oxymethyl]-2-[3-hydroxy-2-methyl-(1*E*)-1-propenyl]-4-methyl-2,5-dihydrofuran (47) A solution of a 2:1 mixture of **45** and **46** (99 mg, 0.30 mmol) in dry toluene (10 ml) was treated with 0.94 M diisobutylaluminum hydride hexane solution (1.28 ml, 1.2 mmol) at −30 °C for 10 min. The excess reagent was quenched with 5% HCl, and extracted with EtOAc. The extract was washed with cold 5% HCl, 10% $NaHCO_3$ and brine and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography. Elution with 20% EtOAc–hexane gave 24 mg (27% yield) of **48**, and further elution with 30% EtOAc–hexane gave 50 mg (56% yield) of **47**. **47**: $[\alpha]_D$ −0.41° (c=0.96, $CHCl_3$). IR (film) cm^{-1}: 3400, 1255, 1070, 1040, 835. ^{1}H-NMR δ: 0.03 (6H, s, $SiMe_2$), 0.86 (9H, s, Si-*tert*-Bu), 1.66 (3H, br s, 4-Me), 1.75 (3H, br d, J=1 Hz, 7-Me), 3.99 (2H, br s, 8-H), 3.96, 4.20 (each 1H, d, J=12 Hz, 3-CH_2OSi), 4.48 (2H, br s, 5-H), 5.32 (1H, br d, J=9 Hz, 6-H), 5.55 (1H, m, 2-H). MS m/z (rel. int. %): 298 (M^+, 3), 267 (17), 241 (M^+ − *tert*-Bu, 5), 75 (100). High-resolution MS, Calcd for $C_{16}H_{30}O_3Si$ (M^+): 298.1962. Found: 298.1937. **48**: (2*R*)-3-(*tert*-Butyldimethylsilyloxymethyl)-2-(2-hydroxymethyl-2-propenyl)-4-methyl-2,5-dihydrofuran, a colorless oil. $[\alpha]_D$ +13.8° (c=0.56, $CHCl_3$). IR (film) cm^{-1}: 3425, 1255, 1070, 1045, 835. ^{1}H-NMR δ: 0.05 (6H, s, $SiMe_2$), 0.87 (9H, s, Si-*tert*-Bu), 1.64 (3H, br s, 4-Me), 4.05 (2H, s, 8-H), 4.21 (2H, m, 3-CH_2OSi), 4.28—4.54 (3H, m, 2,5-H), 4.89, 5.02 (each 1H, d, J=1 Hz, olefinic-H). MS m/z (rel. int. %): 298 (M^+, 0.1), 283 (M^+ − Me, 0.1), 267 (0.5), 241 (M^+ − *tert*-Bu, 2), 227 (59), 95 (100). High-resolution MS, Calcd for $C_{16}H_{30}O_3Si$ (M^+): 298.1962. Found: 298.1958.

(2*R*)-3-[(*tert*-Butyldimethylsilyl)oxymethyl]-4-methyl-2-[3-phenylthio-2-methyl-(1*E*)-1-propenyl]-2,5-dihydrofuran (49) The method of Nakagawa and Hata was employed.[24] A mixture of **47** (41 mg, 0.14 mmol), diphenyldisulfide (90 mg, 0.41 mmol), tributylphosphine (0.10 ml, 0.41 mmol) and pyridine (0.06 ml) was stirred at room temperature for 1 h, then extracted with EtOAc. The extract was washed with 10% HCl, 1 N NaOH and brine, and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 20% EtOAc–hexane to give 30 mg (57.7% yield) of **49** as a colorless oil. $[\alpha]_D$ −37.9° (c=0.25, $CHCl_3$). IR (film) cm^{-1}: 3060, 1575, 1250, 1060, 1040, 1020, 835. ^{1}H-NMR δ: 0.01 (6H, s, $SiMe_2$), 0.85 (9H, s, Si-*tert*-Bu), 1.62 (3H, br s, 4-Me), 1.86 (3H, d, J=1 Hz, 7-Me), 3.41, 3.61 (each 1H, br d, J=12 Hz, 8-H), 3.62, 4.08 (each 1H, d, J=12 Hz, 3-CH_2OSi), 4.45 (2H, br s, 5-H), 5.17 (1H, dd, J=1, 10 Hz, 6-H), 5.47 (1H, m, $W_{1/2}$=14 Hz, 2-H), 7.04—7.34 (5H, m, aromatic-H). MS m/z (rel. int. %): 390 (M^+, 0.8), 367 (0.8), 333 (M^+ − *tert*-Bu, 5), 281 (M^+ − SPh, 100), 109 (PhS^+, 10), 95 (18). High-resolution MS, Calcd for $C_{16}H_{34}O_2SSi$ (M^+): 390.2404. Found: 390.2035.

1-Benzyloxy-2,6-dimethyl-8-(tetrahydropyran-2-yloxy)-(2*E*,6*E*)-2,6-octadiene (51) A mixture of **50** (3.55 g, 14 mmol), prepared from geraniol by the method of Mori and co-workers,[22] and 60% sodium hydride (670 mg, 16.8 mmol) in dry THF was stirred at room temperature for 1 h. Tetrabutylammonium iodide (515 mg, 1.4 mmol) and benzyl bromide (1.95 ml, 16.7 mmol) were added and the resulting mixture was stirred at room temperature for 4 h. Ice-water and ether were added, and the organic layer was separated, washed with brine and then dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 30% EtOAc–hexane to give **51** (3.61 g, 75% yield) as a colorless oil. IR (film) cm^{-1}: 1665, 1200, 1115, 1075, 1025. ^{1}H-NMR δ: 1.66 (6H, br s, 2,6-H), 2.00—2.32 (4H, m, 4,5-H), 3.88 (2H, s, 1-H), 4.10 (2H, m, 8-H), 4.42 (2H, s, OCH_2Ph), 4.59 (1H, m, OTHP), 5.35 (2H, br t, J=7 Hz, 3,7-H), 7.29 (5H, s, aromatic-H). MS m/z (rel. int. %): 259 (M^+ − OTHP, 0.8), 91 (100), 85 (65).

8-Benzyloxy-3,7-dimethyl-(2*E*,6*E*)-2,6-octadien-1-ol (52) A solution of **51** (3.61 g, 10.5 mmol) and PPTS (526 mg, 2.1 mmol) in ethanol (90 ml) was stirred at 55 °C for 2 h. Removal of the solvent followed by column chromatography of the residue with 30% EtOAc–hexane afforded **52** (2.10 g, 77% yield) as a colorless oil. IR (film) cm^{-1}: 3450, 1665, 1065, 1025. ^{1}H-NMR δ: 1.67 (6H, br s, 3,7-Me), 3.87 (2H, s, 8-H), 4.12 (2H, d, J=7 Hz, 1-H), 4.43 (2H, s, OCH_2Ph), 5.39 (2H, br t, J=7 Hz, 4,5-H), 7.30 (5H, s, aromatic-H). MS m/z (rel. int. %): 229 (M^+ − CH_2OH, 0.4), 91 (100).

8-Benzyloxy-3,7-dimethyl-(2*E*,6*E*)-2,6-octadienyl Chloride (53) A solution of lithium chloride (212 mg, 5.0 mmol) in dry DMF (10 ml) was added to a mixture of **52** (1.20 g, 4.6 mmol) and *sym*-collidine (0.73 ml, 5.5 mmol). Methanesulfonyl chloride (0.43 ml, 5.5 mmol) was added dropwise to the mixture at 0 °C, and the whole was stirred for 4 h, then poured into ice-water. The product was extracted with hexane and ether. The extracts were washed with cold 5% HCl, $NaHCO_3$ and brine and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 10% EtOAc–hexane to give 1.19 g (92.3% yield) of **53** as a colorless oil. IR (film) cm^{-1}: 1660, 1255, 1065, 1025. ^{1}H-NMR δ: 1.67, 1.73 (each 3H, d, J=1 Hz, 3,7-Me), 2.00—2.26 (4H, m, 4,5-H), 3.88 (2H, 2,8-H), 4.05 (2H, d, J=7 Hz, 1-H), 4.42 (2H, s, OCH_2Ph), 5.27—5.54 (2H, m, 2,6-H), 7.29 (5H, s, aromatic-H). MS m/z (rel. int. %): 243 (M^+ − Cl, 0.2), 242 (M^+ − HCl, 0.2), 91 (100).

8-Benzyloxy-3,7-dimethyl-(2*E*,6*E*)-2,6-octadienyl Bromide (54) A solution of lithium bromide (367 mg, 4.23 mmol) in dry DMF (6 ml) was added to a mixture of **52** (500 mg, 1.9 mmol) and *sym*-collidine (268 mg, 2.2 mmol). Methanesulfonyl chloride (0.1 ml, 2.3 mmol) was added dropwise to the mixture at 0 °C, and the whole was stirred for 2 h, then poured into ice-water. The product was extracted with ether. The extracts were washed with cold 5% HCl, 10% $NaHCO_3$ and brine and dried over $MgSO_4$. Removal of the solvent left an oil, which was purified by column chromatography with 10% EtOAC–hexane to give 540 mg (87% yield) of **54** as a colorless oil. IR (film) cm^{-1}: 1665, 1260, 1095, 1070, 1030. ^{1}H-NMR δ: 1.68, 1.73 (each 3H, d, J=1 Hz, 3,7-Me), 1.96—2.38 (4H, m, 4,5-H), 3.89 (2H, s, 8-H), 4.07 (2H, d, J=7 Hz, 1-H), 4.43 (2H, s, OCH_2Ph), 5.26—5.56 (2H, m, 2,6-H), 7.30 (5H, s, aromatic-H). MS m/z (rel. int. %): 243 (M^+ − Br, 0.2), 242 (M^+ − HBr, 0.1), 91 (100).

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New Carbazole Alkaloids from *Murraya euchrestifolia*

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In a further study of the constituents of stem bark of *Murraya euchrestifolia* HAYATA collected in Taiwan, five new monomeric carbazoles named murrayaline-B (1), -C (2) and -D (3), pyrayafoline-E (5) and euchrestine-E (7), one carbazolequinone named murrayaquinone-E (9), and two dimeric carbazoles named bismurrayafoline-C (11) and -D (12) were isolated along with known carbazoles. The structures of new alkaloids were elucidated by spectrometric and/or chemical methods. The new monomeric carbazoles were found to have 2-oxygenated 3-*C*-substituted carbazole nuclei and the new dimeric carbazoles to have symmetrical structures containing a 1-oxygenated 3-methylcarbazole skeleton.

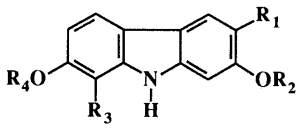
Keywords *Murraya*; *Murraya euchrestifolia*; Rutaceae; carbazole alkaloid; murrayaline; pyrayafoline; euchrestine; murrayaquinone; bismurrayafoline; dimer

Systematic studies of the chemical constituents of *Murraya* plants have been conducted by our group,[1,2] and we showed that *M. euchrestifolia* HAYATA collected in Taiwan contained many kinds of carbazole alkaloids as major components.[1] This paper describes the results of a further examination of the constituents of stem bark of *M. euchrestifolia*.[1b]

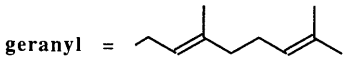
The acetone extract of the stem bark of the plant was subjected successively to silica gel column and preparative thin layer chromatographies (TLC) to give new carbazoles named murrayaline-B (**1**), -C (**2**) and -D (**3**), pyrayafoline-E (**5**), and euchrestine-E (**7**), one carbazolequinone, murrayaquinone-E (**9**), and two dimeric carbazoles, bismurrayafoline-C (**11**) and -D (**12**) along with some known carbazoles. All new monomeric carbazole alkaloids isolated from the stem bark on this occasion belong to the class of 2-oxygenated 3-*C*-substituted carbazoles, being different from the 1-oxygenated carbazole analogues isolated from the root bark of the same plant.[1c,f]

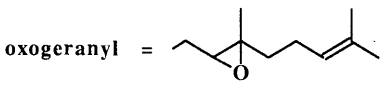
Results and Discussion

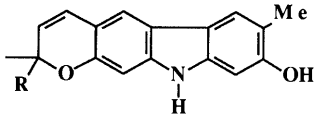
Structure of Murrayaline-B (1), -C (2), and -D (3) Murrayaline-B (**1**) was obtained as yellow prisms, mp 240—242 °C, from acetone and the molecular formula was determined as $C_{15}H_{13}NO_3$ by high-resolution mass spectrometry (HR-MS). The ultraviolet (UV) spectrum showed bands at λ_{max} 223, 259 (sh), 303, and 380 nm, which were similar to those of murrayaline-A[3] (**4**) [λ_{max} 226, 260 (sh), 304, and 383 nm] reported previously by us.[1d] The proton nuclear magnetic resonance (^{1}H-NMR) spectrum in acetone-d_6 suggested the presence of an aryl methyl [δ 2.34 (3H, s)], a methoxy [δ 4.02 (3H, s)], *ortho*-located aromatic protons [δ 6.89 and 8.16 (each 1H, d, J=8.7 Hz)], and *para*-located aromatic protons [δ 7.20 and 7.73 (each 1H, s)]. Among these aromatic protons, a lower-field singlet at δ 7.73 and a doublet at δ 8.16 were assignable to deshielded H-4 and H-5, respectively, on the carbazole nucleus.[4] A sharp 1H singlet at δ 10.58 was typical of a proton of an aldehyde group attached to C-8 on the carbazole nucleus.[1d,f] Two deuterium-exchangeable broad singlets were observed at δ 8.42 and 10.79. In nuclear Overhauser effect (NOE) experiments, irradiation of the methoxy (δ 4.02) and the aryl methyl signals (δ 2.34) showed 8 and 6% enhancements of the signals at δ 6.89 (H-6), one of the AB-type protons, and at δ 7.73 (H-4), respectively, indicating the location of the methoxy and the aryl methyl groups at C-7 and C-3, respectively. *O*-Methylation with methyl iodide in acetone in the presence of anhydrous potassium carbonate gave a pale yellow oil, which was found to be identical with murrayaline-A (**4**)[1d,4] by comparisons of infrared (IR) and ^{1}H-NMR spectra.



	R_1	R_2	R_3	R_4
1	Me	H	CHO	Me
2	CHO	H	CHO	Me
3	CHO	H	geranyl	Me
4	Me	Me	CHO	Me
7	Me	H	oxogeranyl	H
8	Me	H	geranyl	H

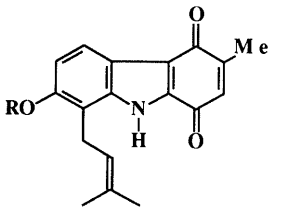






5 : R = $-CH_2CH_2CH=C(CH_3)_2$

6 : R = Me



9 : R = H

10 : R = Me

Chart 1

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Murrayaline-C (**2**) was isolated as a pale yellow powder. The HR-MS indicated the molecular formula as $C_{15}H_{11}NO_4$. The ^{1}H-NMR (in acetone-d_6) spectrum showed a similar signal pattern to that of murrayaline-B (**1**), except for the lack of an aryl methyl signal and the appearance of two additional lower-field sharp 1H singlets at δ 9.95 and 11.41 (Table I) along with the signal of an aldehyde proton (δ 10.80) at C-8. Among these lower-field 1H singlets, the signal at δ 9.95 was typical of a proton of an aldehyde group attached to C-3 on the carbazole nucleus.[1d] Another signal at δ 11.41 was assignable to a strongly hydrogen-bonded hydroxy proton located *ortho* to the aldehyde group. On the basis of these spectral data, the structure of murrayaline-C was proposed to be as shown by formula **2**.

Murrayaline-D (**3**) was isolated as a pale brown oil. The molecular formula, $C_{24}H_{27}NO_3$, was determined by HR-MS. The ^{1}H-NMR spectrum showed the signals due to a methoxy (δ 3.95), an aldehyde (δ 9.98) attached to C-3,[1d] *ortho*-located H-5 and H-6 [δ 7.88 and 6.95 (each 1H, d, J=8.8 Hz), respectively], and *para*-located H-1 and H-4 (δ 7.40 and 8.08, respectively). The remaining signals at δ 1.56 (3H, s), 1.60 (3H, s), 1.91 (3H, s), 2.10 (4H, m), 3.68 (2H, d, J=7.3 Hz), 5.08 (1H, m), and 5.36 (1H, t, J=7.3 Hz) in the ^{1}H-NMR spectrum were assignable to protons of a geranyl moiety [$-CH_2CH=C(CH_3)-CH_2CH_2CH=C(CH_3)_2$]. Observation of mass fragment ions at m/z 308 and 254 arising from loss of [$\cdot C_5H_9$] and [$\cdot C_9H_{15}$] from the molecular ion also suggested the presence of the geranyl side chain in the molecule. In NOE experiments, irradiation of the benzylic protons at δ 3.68 resulted in 5% area increase of the allyl methyl signal at δ 1.91, suggesting the *E*-configuration of the double bond on the side chain. Irradiation of the methoxy signal at δ 3.95 gave 11% enhancement of the doublet at δ 6.95 (H-6), indicating the location of the methoxy group at C-7. These results led us to conclude that murrayaline-D has the structure **3**.

Structure of Pyrayafoline-E (5) Pyrayafoline-E (**5**) was obtained as a pale brown oil, $[\alpha]_D$ 0° ($CHCl_3$) and was found to have the molecular formula $C_{23}H_{25}NO_2$ by HR-MS. The UV spectrum was similar to that of pyrayafoline-B (**6**).[1e] The signal pattern of the ^{1}H-NMR spectrum of this alkaloid (Table I) also resembled that of pyrayafoline-B (**6**), except for the appearance of signals at δ 5.10 (1H, t, J=7.3 Hz), 2.13 (2H, m), 1.70 (2H, m), 1.65 (3H, s), and 1.57 (3H, s) instead of the signal due to one of the methyls attached to the oxygenated carbon on the structure **6**. On the basis of these spectral data together with the observation of a characteristic mass fragment peak at m/z 264 [M^+-83] resulting from loss of [$-CH_2CH_2CH=C(CH_3)_2$] from the molecular ion, we proposed the structure **5** for pyrayafoline-E.

Structure of Euchrestine-E (7) Euchrestine-E (**7**) was obtained as a racemate. The molecular formula was determined as $C_{23}H_{27}NO_3$ by HR-MS. The ^{1}H-NMR spectrum showed the signals due to an aryl methyl (δ 2.38), deshielded H-4 and H-5 at δ 7.65 and 7.66, which appeared as a singlet and a doublet coupled to H-6 at δ 6.73 (d, J=8.4 Hz), respectively, as well as three D_2O exchangeable protons. Two dimensional H–H correlation spectroscopy

TABLE I. ^{1}H-NMR Data for Murrayalines, Pyrayafolines, Euchrestines, and Murrayaquinones

	1	**2**	**3**	**4**	**5**	**6**[1e]	**7**	**8**[1e]	**9**	**10**[1f]
H-1	7.20	6.95	7.40	7.31	6.78	6.74	6.81	6.81	—	—
2	8.42 (OH)	11.41 (OH)		4.04 (3H, OMe)	4.73 (br, OH)	4.94 (br, OH)	4.90 (OH)[a]	—	6.45 (d, 1.5)	6.42 (d, 1.5)
3	2.34 (3H, Me)	9.95 (CHO)	9.98 (CHO)	2.31 (3H, Me)	2.38 (3H, Me)	2.37 (3H, Me)	2.38 (3H, Me)	2.38 (3H, Me)	2.14 (3H, Me)	2.13 (3H, Me)
H-4	7.73	8.08	8.08	7.77	7.62	7.62	7.65	7.65	—	—
H-5	8.16 (d, 8.7)	8.10 (d, 8.8)	7.88 (d, 8.8)	8.20 (d, 8.4)	7.47	7.49	7.66 (d, 8.4)	7.63 (d, 8.4)	7.95 (d, 8.8)	7.98 (d, 9)
H-6	6.89 (d, 8.7)	6.85 (d, 8.8)	6.95 (d, 8.8)	6.93 (d, 8.4)	—	—	6.73 (d, 8.4)	6.70 (d, 8.4)	6.88 (d, 8.8)	7.02 (d, 9)
7	4.02 (3H, OMe)	4.03 (3H, OMe)	3.95 (3H, OMe)	3.91 (3H, OMe)	—	—	4.50 (OH)[a]	—		3.91 (3H, OMe)
8	10.58 (CHO)	10.60 (CHO)	—	10.60 (CHO)	6.75	6.71	3.99 (t, 4.4)	5.37 (t, 6.7)	5.32 (t, 6.8)	5.23 (t, 7)
			5.36 (t, 7.3)				5.07 (m)	5.07 (m)	3.56 (d, 6.8)	3.56 (d, 7)
			5.08 (m)				3.10 (dd, 4.4, 16.5)	3.59 (2H, d, 7.1)		
			3.68 (2H, d, 7.3)				2.84 (dd, 4.4, 16.5)			
			2.10 (4H, m)				2.17 (2H, m)	2.12 (4H, m)		
			1.91 (3H)				1.70 (2H, m)			
			1.60 (3H)				1.64 (3H)	1.88 (3H)	1.88 (3H)	1.85 (3H)
			1.56 (3H)				1.56 (3H)	1.65 (3H)	1.80 (3H)	1.74 (3H)
							1.40 (3H)	1.58 (3H)		
NH	10.79	10.52	8.54		7.72	7.70	7.63	7.73	9.00	9.07
Others	—	—	6.17	—	6.51 (d, 9.8)	6.48 (d, 9.6)				
					5.53 (d, 9.8)	5.58 (d, 9.6)				
					5.10 (t, 7.3)					
					2.13 (2H, m)					
					1.70 (2H, m)					
					1.65 (3H)	1.46 (6H)				
					1.57 (3H)					
					1.42 (3H)					

Values are δ ppm. Figures in parentheses are coupling constants in Hz. Each signal corresponds to 1H, and observed as a singlet, unless otherwise stated. Spectra of **1** and **4** were taken in acetone-d_6, and those of others in $CDCl_3$. *a*) Assignments may be reversed.

(H–H COSY) revealed the presence of three directly coupled protons [δ 2.84 (1H, dd, J=4.4, 16.5 Hz), 3.10 (1H, dd, J=4.4, 16.5 Hz), and 3.99 (1H, t, J=4.4 Hz)] due to a benzylic methylene and an adjacent methine bearing an oxirane ring. An additional methylene proton signal [δ 2.17 (2H, m)] coupled both with an olefinic proton [δ 5.07 (1H, m)] and other methylene protons [δ 1.70 (2H, m, overlapped with methyls)], along with signals of two allyl methyls (δ 1.64 and 1.56) and a methyl (δ 1.40) attached to an oxygenated carbon, were also observed. This ^{1}H-NMR spectral pattern resembled that of euchrestine-C (**8**)[1e] (Table I) except for signals due to some protons on the side chain. These spectral data and the occurrence of a characteristic mass fragment ion at m/z 226 produced by the cleavage of the benzylically activated bond in the molecular ion suggested the presence of a side chain having an oxirane ring, such as $-CH_2CH(O)C(CH_3)-CH_2CH_2-CH=C(CH_3)_2$.

From the results stated above, we assumed the structure of euchrestine-E to be shown by the formula **7**, except for the stereochemistry.

Structure of Murrayaquinone-E (9) Murrayaquinone-E (**9**) was isolated as a brown oil. The molecular formula, $C_{18}H_{17}NO_3$, was obtained from its HR-MS. The UV spectrum showed a close resemblance to that of murrayaquinone-B[1f] (**10**), thus suggesting a carbazole-1,4-quinone structure for the compound. The IR spectrum also showed bands at ν_{max} 1650 and 1620 cm^{-1} typical of carbazole-1,4-quinone, along with the bands at ν_{max} 3430 (NH) and 3400 (OH) cm^{-1}. The ^{1}H-NMR spectrum showed signals of an aryl methyl [δ 2.14 (3H, d, J=1.5 Hz)] long-range-coupled with an olefinic proton at δ 6.45 (1H, d, J=1.5 Hz), *ortho*-located protons [δ 7.95 and 6.88 (each 1H, d, J=8.8 Hz) assignable to H-5 and H-6, respectively], and protons corresponding to a prenyl side chain [δ 5.32 (1H, t, J=6.8 Hz), 3.56 (2H, d, J=6.8 Hz), 1.88 (3H, s), and 1.80 (3H, s)]. These ^{1}H-NMR signal patterns were similar to those of murrayaquinone-B (**10**)[1f] (Table I) except for the lack of a methoxy signal at δ 3.91 in the spectrum of **10**. On the basis of these spectral data, we proposed the structure **9** for murrayaquinone-E.

Structures of Bismurrayafoline-C (11) and -D (12) Bismurrayafoline-C (**11**) was isolated as a pale yellow oil. The HR-MS analysis established the molecular formula $C_{46}H_{52}N_2O_4$ for this alkaloid. The presence of a 1-oxygenated carbazole nucleus in the molecule was easily deduced from the UV spectrum [λ_{max} 226, 235, 260 (sh), 285 (fl), 311, 335 (sh) nm].[4] The ^{1}H-NMR spectrum suggested the presence of an aryl methyl [δ 2.48 (s)], *ortho*-located protons [δ 7.69 (H-5) and 6.73 (H-6) (each d, J=8.4 Hz)], and a deshielded H-4 [δ 7.84 (s)] on the carbazole nucleus[4] along with signals assignable to a geranyl moiety (Table II). These signal patterns were similar to those of murrayafoline-B (**14**)[1f] except for the lack of an H-2 signal and appearance of signals due to a geranyl moiety instead of signals due to a prenyl group in the spectrum of **14**. Observation of only half the number of proton signals expected from the molecular formula suggested that bismurrayafoline-C (**11**) has a completely symmetrical structure containing two monomeric carbazole, murrayafoline-B (**14**), units. Such a structure was supported by the following NOE experiments on **11**. Irradiation of the aryl methyl signal at δ 2.48 gave 8% enhancement of the H-4 (and H-4′) signal at δ 7.84. Irradiation of the benzylic signal of the side chain at δ 3.42 caused 10 and 5% area increases of the signals of the allyl methyl at δ 1.42 and N-H at δ 7.55, respectively. Further, in the NOE studies of the dimethyl ether (**13**) obtained by the treatment of **11** with diazomethane, irradiation of the methoxy signal at δ 3.90 caused a 14% enhancement of the doublet at δ 6.85 (H-6 and H-6′), suggesting the location of the oxygenated substituent at C-7 (and C-7′). Irradiation of another methoxy signal at δ 3.40 gave area increases of the N-H signal at δ 7.85 and a singlet at δ 2.52 due to an aryl methyl which was considered to be located on another carbazole nucleus. Irradiation of the aryl methyl signal at δ 2.52 caused 10 and 6% area increases of the lower-field singlet at δ 7.85 (H-4 and H-4′) and the methoxy signal at δ 3.40 on the other carbazole unit,

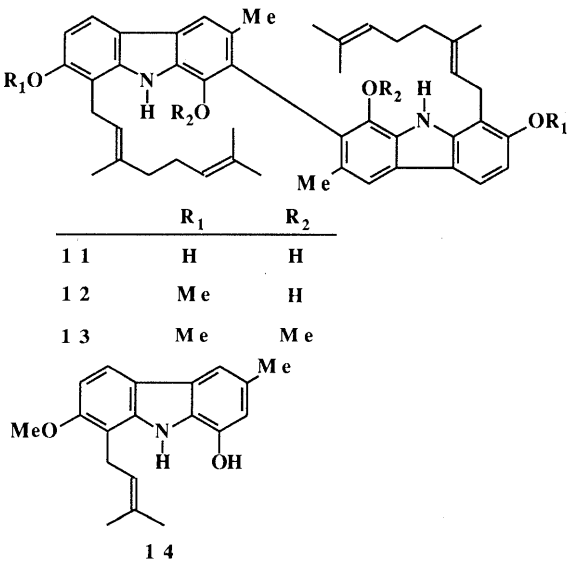

Chart 2

TABLE II. ^{1}H-NMR Data for Bismurrayafolines and Murrayafoline-B (**14**)

	11	**12**	**13**	**14**[1f]
1-OCH_3	—	—	3.40 (6H)	—
H-2	—	—	—	6.56 (1H)
3-CH_3	2.48 (6H)	2.48 (6H)	2.52 (6H)	2.42 (3H)
H-4	7.84	7.85	7.85	7.32 (1H)
H-5	7.69 (d, 8.4)	7.77 (d, 8.5)	7.80 (d, 8.8)	7.72 (1H, d, 8)
H-6	6.73 (d, 8.4)	6.85 (d, 8.4)	6.85 (d, 8.8)	6.82 (1H, d, 8)
7-OCH_3	—	3.89 (6H)	3.90 (6H)	3.90 (3H)
8	5.16 (t, 7.5)	5.08 (t, 6.8)	5.14 (t, 7.3)	5.30 (1H, t, 8)
	4.85 (t, 7.5)	4.77 (t, 6.8)	4.86 (4H, m)	
	3.42 (4H, m)	3.45 (2H, m)	3.52 (m)	3.60 (1H, d, 8)
		3.41 (2H, m)	3.41 (m)	
	1.75 (4H, m)	1.6—1.4 (8H, m)[a]	1.73 (m)	1.88 (3H)
	1.67 (4H, m)		1.59 (m)	1.72 (3H)
	1.60 (6H)	1.56 (12H)	1.58 (6H)	
	1.47 (6H)		1.44 (6H)	
	1.42 (6H)	1.42 (6H)	1.41 (6H)	
OH	5.19	5.17	—	4.99 (1H)
	5.05			
NH	7.84	7.85	7.85	7.94 (1H)

Values are δ ppm. Figures in parentheses are coupling constants in Hz. Each signal corresponds to 2H, and appears as a singlet, unless otherwise stated. *a*) Overlapped with methyl signals.

respectively. These findings together with the presence of a lower-field singlet due to H-4 (and H-4′), and the lack of a signal assignable to H-2 (and H-2′) in the ^{1}H-NMR spectra of both bismurrayafoline-C (**11**) and its dimethyl ether (**13**), revealed the location of the linkage of the two monomeric carbazole units at C-2 and C-2′. These spectral results led us to propose the structure **11** for bismurrayafoline-C.

Bismurrayafoline-D (**12**) was also isolated as colorless prisms, mp 198—200 °C, from acetone, and gave the molecular formula $C_{48}H_{56}N_2O_4$, and difference of CH_2 compared with bismurrayafoline-C (**11**), by HR-MS analysis. The ^{1}H-NMR spectrum of bismurrayafoline-D (**12**) showed a similar signal pattern to that of **11**, except for an additional singlet at δ 3.89 due to a methoxy group (Table II). The characteristic mass fragments due to a geranyl moiety at *m/z* 293 ($M^{2+} - \cdot C_5H_9$) and 239 ($M^{2+} - \cdot C_9H_{15}$) along with a double charged molecular ion at *m/z* 362 (M^{2+}) were observed. Treatment of this alkaloid with diazomethane afforded a methyl ether (**13**), which was found to be identical with the dimethyl ether (**13**) derived from **11**. In the NOE experiment on bismurrayafoline-D (**12**), irradiation of the methoxy signal at δ 3.89 resulted in 13% enhancement of the doublet at δ 6.85 due to H-6 (and H-6′) indicating the location of the methoxy group at C-7 (and C-7′). These results led us to assign structure **12** to bismurrayafoline-D.

Experimental

All melting points were measured on a micromelting point hot-stage apparatus (Yanagimoto). ^{1}H- and ^{13}C-NMR, differential NOE, and H–H COSY spectra were recorded on GX-270 (JEOL) or GX-400 (JEOL) spectrometers in $CDCl_3$, unless otherwise stated. Chemical shifts are shown in δ values (ppm) with tetramethylsilane (TMS) as an internal reference. Electron impact (EI-) and HR-MS were taken with M-80 (Hitachi) and JMS-HX-110 (JEOL) spectrometers having a direct inlet system, respectively. UV spectra were recorded on a UVIDEC-610C double-beam spectrophotometer (Jasco) in methanol, IR spectra on an IR-810 (Jasco) in $CHCl_3$, optical rotation on a DIP-181 (Jasco) in $CHCl_3$. The preparative TLC were done on Kieselgel 60 F_{254} (Merck).

Extraction and Separation The dried stem bark (925 g) of *Murraya euchrestifolia* HAYATA collected at Kuantaochi, Nantou Hsien, Taiwan in May, 1989 was extracted with acetone. The acetone extract was subjected to silica gel column chromatography eluted successively with benzene, benzene–iso-Pr_2O (1 : 1), benzene–acetone (9 : 1), and benzene–acetone (4 : 1). Each fraction was further subjected to preparative TLC to give eight new carbazoles as well as sixteen known carbazoles as stated below. From the benzene eluate: mahanimbine[5] (64.7 mg), murrayafoline-A[1f] (117.1 mg), and girinimbine[6] (4.7 mg). From the benzene–isopropyl ether eluate: pyrayafoline-E (**5**) (1.2 mg), murrayaquinone-E (**9**) (0.9 mg), murrayaline-D (**3**) (1.7 mg), euchrestine-E (**7**) (1.5 mg), euchrestine-A[1e] (9.1 mg), -B[1e] (3.9 mg), -C[1e] (50.9 mg) and -D[1e] (1.5 mg), pyrayafoline-B[1e] (21.6 mg), -C[1e] (6.0 mg) and -D[1e] (49.8 mg), bismurrayafoline-C (**11**) (6.8 mg) and -D (**12**) (1.5 mg), murrayanine[7] (61.6 mg), isomurrayafoline-B[1b] (66.1 mg), bismurrayafoline-B[1g] (14.9 mg), murrayaquinone-D[1f] (2.6 mg), and mahanine[5] (12.4 mg). From the benzene–acetone (9 : 1) eluate: murrayaline-B (**1**) (14.0 mg) and murrayaline-C (**2**) (1.5 mg). From the benzene–acetone (4 : 1) eluate: koenoline[8] (24.3 mg). Known components were fully characterized by UV, IR, ^{1}H-NMR, and MS analyses.

Murrayaline-B (1) Yellow prisms. mp 240—242 °C (acetone). UV λ_{max} nm: 223, 259 (sh), 303, 380. IR ν_{max} cm^{-1}: 3400, 3380, (br), 1660, 1635, 1600. EI-MS *m/z* (%): 255 (M^+, 100), 240 (26), 212 (19), 184 (28), 149 (13), 135 (45). HR-MS Calcd for $C_{15}H_{13}NO_3$: 255.0895. Found: 255.0935. NOE: Irradiation of 7-OCH_3 (δ 4.02)—8% enhancement of H-6 (δ 6.89). Irradiation of 3-CH_3 (δ 2.34)—6% enhancement of H-4 (δ 7.73).

Murrayaline-C (2) A pale yellow powder. UV λ_{max} nm: 239 (sh), 251 (sh), 267, 307 (sh), 329. IR ν_{max} cm^{-1}: 3420, 3400 (br), 1660, 1640, 1610. EI-MS *m/z* (%): 269 (M^+, 100), 254 (26), 226 (15), 198 (25), 149 (10), 135 (23). HR-MS Calcd for $C_{15}H_{11}NO_4$: 269.0688. Found: 269.0706. NOE: Irradiation of 7-OCH_3 (δ 4.03)—9.3% enhancement of H-6 (δ 6.85).

Murrayaline-D (3) A pale brown oil. UV λ_{max} nm: 243, 255 (sh), 287, 330, 357, 364, 368. IR ν_{max} cm^{-1}: 3450, 3400 (br), 1680, 1620, 1582. EI-MS *m/z* (%): 377 (M^+, 9), 308 (5), 254 (12), 149 (40), 135 (72). HR-MS Calcd for $C_{24}H_{27}NO_3$: 377.1989. Found: 377.1984. NOE: Irradiation of 7-OCH_3 (δ 3.95)—11% enhancement of H-6 (δ 6.95). Irradiation of CH_2 (δ 3.68)—5% enhancement of CH_3 (δ 1.91).

Pyrayafoline-E (5) A pale brown oil. $[\alpha]_D$ 0° (c=0.0007, $CHCl_3$). UV λ_{max} nm: 233 (sh), 252, 284, 330 (sh), 354. IR λ_{max} cm^{-1}: 3600, 3470, 3380 (br), 1625. EI-MS *m/z* (%): 347 (M^+, 14), 264 (43), 149 (46), 135 (95). HR-MS Calcd for $C_{23}H_{25}NO_2$: 347.1883. Found: 347.1883.

Euchrestine-E (7) A pale brown oil. $[\alpha]_D$ 0° (c=0.0007, $CHCl_3$). CD (c=0.0007, MeOH): No absorption was observed in the 210—500 nm range. UV λ_{max} nm: 212, 238, 264, 308 (sh), 316. IR ν_{max} cm^{-1}: 3600, 3460, 3400 (br), 1620, 1600. EI-MS *m/z* (%): 365 (M^+, 100), 264 (15), 226 (98), 225 (92), 196 (15). HR-MS Calcd for $C_{23}H_{27}NO_3$: 365.1989. Found: 365.1993.

Murrayaquinone-E (9) A brown oil. UV λ_{max} nm: 232, 262, 406. IR ν_{max} cm^{-1}: 3430, 3400, 1650, 1620. EI-MS *m/z* (%): 295 (M^+, 41), 278 (19), 240 (62). HR-MS Calcd for $C_{18}H_{17}NO_3$: 295.1207. Found: 295.1205.

Bismurrayafoline-C (11) A pale yellow oil. UV λ_{max} nm: 226, 235, 260 (sh), 285 (fl), 311, 335 (sh). IR ν_{max} cm^{-1}: 3600, 3480, 3450, 3425, 3380 (br), 1620. EI-MS *m/z* (%): 696 (M^+, 100), 264 (31), 226 (18). HR-MS Calcd for $C_{46}H_{52}N_2O_4$: 696.3924. Found: 696.3893. The results of NOE: Irradiation of the aryl methyl (δ 2.48)—8% enhancement of H-4 (and H-4′) (δ 7.84). Irradiation of the benzylic CH_2 (δ 3.42)—10 and 5% enhancements of the allyl methyl (δ 1.42) and N-H (δ 7.55), respectively.

Bismurrayafoline-D (12) Colorless prisms. mp 198—200 °C (acetone). UV λ_{max} nm: 224, 238, 260 (sh), 285 (sh), 310, 334 (sh). IR ν_{max} cm^{-1}: 3540, 3450, 1610. EI-MS *m/z* (%): 724 (M^+, 100), 601 (6), 479 (20), 362 (M^{2+}, 8), 293 (8), 239 (30). HR-MS Calcd for $C_{48}H_{56}N_2O_4$: 724.4237. Found: 724.4276. NOE: Irradiation OCH_3 (δ 3.89)—13% enhancement of H-6 (and H-6′) (δ 6.85).

Methylation of 11 and 12 Treatment of a methanolic solution (2 ml) of **11** (2.5 mg) or **12** (0.8 mg) with an excess amount of ether solution of diazomethane followed by preparative TLC with hexane–acetone (3 : 1) gave the dimethyl ether (**13**) (2 mg and 0.5 mg, respectively), as colorless oils. **13**: UV λ_{max} nm: 226, 241, 255 (sh), 286, 308, 335 (sh). IR ν_{max} cm^{-1}: 3450, 1610. NOE: Irradiation of 7-OCH_3 (δ 3.90)—14% enhancement of H-6 (and H-6′) (δ 6.85). Irradiation of 1-OCH_3 (δ 3.40)—2 and 4% enhancements of N-H (δ 7.85) and 3′-CH_3 (δ 2.52), respectively. Irradiation of 3-CH_3 (δ 2.52)—10 and 6% enhancements of H-4 (and H-4′) (δ 7.85) and 1′-OCH_3 (δ 3.40), respectively.

References and Notes

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Phytochemical Studies on Meliaceous Plants. VII.[1] The Structures of Two New Ionone Glucosides from *Melia toosendan* SIEB. *et* ZUCC. and a Novel Type of Selective Biooxidation by a Kind of Protease

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Two new ionone glucosides, melia-ionosides A and B, were isolated from leaves of *Melia toosendan* SIEB. *et* ZUCC. (Meliaceae) and their structures were elucidated based on combinations of chemical, spectral, and X-ray analytical studies. In addition, a novel type of selective bio-oxidation by a kind of protease (Molsin) was found during the course of the structural elucidation.

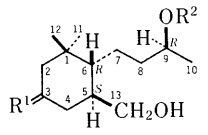
Keywords *Melia toosendan*; Meliaceae; leaf; ionone glucoside; melia-ionoside A; melia-ionoside B; bio-oxidation; Molsin; saturated ionone alcohol

In our continuing phytochemical studies on meliaceous plants, we have already characterized from the leaves of *Melia toosendan* SIEB. *et* ZUCC.[2] (Meliaceae) two pregnane steroids[3] and a pregnane glucoside, toosendanoside.[4] We have also briefly reported the structural elucidation of two new ionone glucosides, melia-ionosides A (**1**) and B (**2**), by means of two-dimensional nuclear magnetic resonance (2D-NMR) spectroscopy, X-ray analysis, and chemical reactions in the preceding communication.[1] This paper presents a full account of the isolation and structural elucidation of the glucosides **1** and **2**. A novel type of bio-oxidation found during the course of the structural study is also reported in detail.

Results and Discussion

The *n*-butanol fraction of the methanol extract from the plant material was subjected to repeated and precise separation by column and high-performance liquid chromatography (HPLC) to isolate **1**, a white powder, $[\alpha]_D^{20}$ $-35.1°$ and **2**, a white powder, $[\alpha]_D^{20}$ $-9.0°$ in yields (from the methanol extract) of 0.007 and 0.003%, respectively.

Both **1** and **2** showed hydroxy absorption bands (3350 and 1075 cm^{-1}) in the infrared (IR) spectra and possessed the same molecular formula $C_{19}H_{36}O_8$, based on elemental analyses and the presence of the common $(M-H)^-$ ion (the base peak) at *m/z* 391 in the negative ion fast atom bombardment mass (FAB-MS) spectra. As the next step of the structural elucidation, detailed studies of the proton and carbon-13 nuclear magnetic resonance (^1H- and ^{13}C-NMR) spectra of **1** and **2** were performed with the aid of the INEPT (insensitive nuclei enhanced by polarization transfer) method and 2D techniques such as ^1H–^1H and ^{13}C–^1H shift-correlated spectroscopy (COSY) and nuclear Overhauser effect spectroscopy (NOESY), and as shown in Tables I and II, all protons and carbons of both **1** and **2** were assigned.



1: R^1 = α-*O*-β-D-glucopyranosyl, β-H (*S*); R^2 = H
2: R^1 = α-OH, β-H (*S*); R^2 = β-D-glucopyranosyl
3: R^1 = α-OH, β-H (*S*); R^2 = H
4: R^1 = O; R^2 = H
5: R^1 = $NNHC_6H_3(NO_2)_2$; R^2 = H

Chart 1

Based on the established assignments, it was inferred that both **1** and **2** contain a β-D-glucopyranosyl ($^4C_1$ form) residue[5] and an ionone (**3**) as the common aglycone, that is, these ionone glucosides (**1** and **2**) are isomers as regards the glucosyl position on **3**.

On acidic hydrolysis, both glucosides afforded the common and genuine aglycone, the ionone (**3**), $C_{13}H_{26}O_3$, a colorless oil, $[\alpha]_D^{20}$ $-6.8°$ and D-glucose. The ^1H- and ^{13}C-NMR assignments established for **3** (Tables I and II) indicated that the relative structure of the ionone is as shown

TABLE I. ^1H-NMR (400 MHz) Data for **1**, **2**, and **3**[a]

	1[b]	**2**[b]	**3**[c]
2α-H	1.95, dd, 14.0, 2.8	1.88, dd, 13.7, 3.4	1.92, dd, 13.8, 3.1
2β-H	1.42, dd, 14.0, 3.4	1.47, dd, 13.7, 3.4	1.52, dd, 13.8, 3.4
3β-H	4.42, tt, 3.4, 2.8	4.43, quin, 3.4	4.47, tt, 3.4, 3.1
4α-H	2.67, ddd, 11.9, 2.8, 1.8	2.47, ddd, 13.7, 3.4, 2.4	2.52, ddd, 13.4, 3.1, 2.4
4β-H	1.6[d]	1.8[d]	1.9[d]
5α-H	2.27, m, $W_{h/2}$ = 19.0[e]	2.30, m, $W_{h/2}$ = 19.0[e]	2.38, m, $W_{h/2}$ = 19.0[e]
6β-H	1.10, ddd, 10.4, 5.2, 2.6	1.15, ddd, 10.4, 5.5, 1.8	1.25, ddd, 10.6, 5.2, 2.6
7-H_2	1.5,[d] 1.7[d]	1.6,[d] 1.8[d]	1.7,[d] 1.8[d]
8-H_2	1.6,[d] 1.98, m	1.7,[d] 2.12, m	1.7,[d] 2.06, m
9α-H	4.0[d]	4.1[d]	4.06, m
13-H_2	3.86, dd, 10.7, 6.7 4.14, dd, 10.7, 3.1	3.91, dd, 10.7, 6.1 4.09, dd, 10.7, 3.4	3.99, dd, 10.3, 6.3 4.18, dd, 10.3, 3.2
10-H_3	1.38, d, 6.4	1.40, d, 6.1	1.40, d, 6.1
11-H_3	1.22, s	1.32, s	1.39, s
12-H_3	0.94, s	1.02, s	1.05, s
1′-H	4.95, d, 7.9	4.98, d, 7.9	
2′-H	4.01, dd, 7.9, 8.9	4.02, dd, 7.9, 8.9	
3′-H	4.28, t, 8.9	4.26, t, 8.9	
4′-H	4.19, dd, 8.9, 9.5	4.19, dd, 8.9, 9.2	
5′-H	3.92, ddd, 9.5, 5.5, 2.1	3.97, ddd, 9.2, 5.5, 2.4	
6′-H_2	4.33, dd, 11.9, 5.5 4.52, dd, 11.9, 2.1	4.35, dd, 11.9, 5.5 4.54, dd, 11.9, 2.4	

a) Chemical shifts are in δ values relative to internal TMS, and are followed by multiplicities and coupling constants (Hz). Assignments for these compounds were made with the aid of ^1H–^1H COSY, NOESY, and ^{13}C–^1H COSY methods. *b*) In Py.-d_5 with a few drops of D_2O. *c*) In Py.-d_5. *d*) Both multiplicity and coupling constant were obscure, due to partial overlapping. *e*) Peak width at half height ($W_{h/2}$) is given in Hz.

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Table II. ^{13}C-NMR (100.5 MHz) Data for **1**—**4** (δ_C, ppm from TMS in Py.-d_5)[a]

	1[b]	2[b]	3	4
1-C	34.70 (s)	34.73 (s)	34.79 (s)	39.10 (s)
2-C	46.36 (t)	47.81 (t)	48.09 (t)	56.50 (t)
3-C	73.92 (d)	66.55 (d)	66.70 (d)	211.19 (s)
4-C	34.23 (t)	38.39 (t)	38.72 (t)	45.39 (t)
5-C	37.78 (d)	37.78 (d)	38.13 (d)	44.48 (d)
6-C	48.22 (d)	48.68 (d)	48.62 (d)	46.49 (d)
7-C	25.65 (t)	24.79 (t)	25.93 (t)	25.74 (t)
8-C	41.73 (t)	38.30 (t)	41.94 (t)	41.45 (t)
9-C	67.55 (d)	76.26 (d)	67.59 (d)	67.47 (d)
10-C	24.44 (q)	21.89 (q)	24.47 (q)	24.44 (q)
11-C	23.59 (q)	23.98 (q)	23.95 (q)	20.90 (q)
12-C	31.71 (q)	31.83 (q)	31.89 (q)	29.93 (q)
13-C	65.15 (t)	65.36 (t)	65.51 (t)	63.83 (t)

a) Assignments and multiplicities (in parentheses) were determined based on INEPT and ^{1}H–^{13}C COSY experiments. *b*) Data for the D-glucosyl moiety in **1** and **2** are as follows. **1**: 102.57 (d, 1′), 75.46 (d, 2′), 78.87 (d, 3′), 71.89 (d, 4′), 78.31 (d, 5′), 62.98 (t, 6′) and **2**: 103.52 (d, 1′), 75.20 (d, 2′), 78.35 (d, 3′), 71.68 (d, 4′), 78.14 (d, 5′), 62.75 (t, 6′).

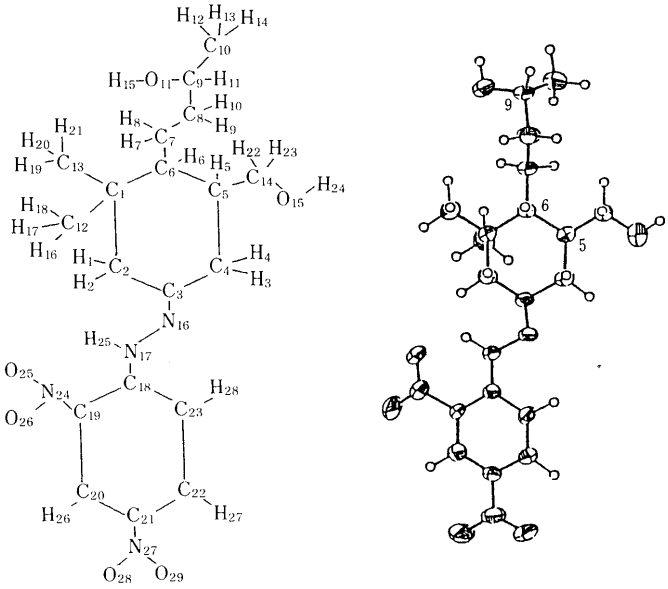

Fig. 1

in the formula **3** or its antipode, except for the configuration of the 9-OH group on the side chain. The identification of D-glucose was carried out by paper partition chromatography (PPC), high-performance thin layer chromatography (HPTLC), and gas liquid chromatography (GLC).

The whole relative structure of **3** was determined by a combination of the following chemical study and X-ray analysis. During the course of the chemical derivations of **1**, **2**, and **3**, a novel type of selective bio-oxidation was found. When incubated with Molsin (protease type XIII from *Aspergillus saitoi*),[6] the ionone (=triol) (**3**) gave the corresponding 3-keto derivative (**4**), $C_{13}H_{24}O_3$, a colorless oil, $[\alpha]_D^{20}$ +24.6° in 84% yield as a result of a novel selective bio-oxidation reaction, in which only the secondary axial 3-OH group among the primary and two secondary hydroxyls in **3** was oxidized. In a similar treatment with Molsin, both glucosides **1** and **2** also afforded the oxidized aglycone (**4**) (61 and 78% yield, respectively), presumably by usual hydrolysis followed by the selective oxidation.

As the next step, X-ray analysis was undertaken. The oily ketone (**4**) was, in the usual manner, transformed into the corresponding crystalline 2,4-dinitrophenylhydrazone (**5**), $C_{19}H_{28}N_4O_6$. Crystals of **5** were grown in ethyl acetate as fine red plates of mp 148—150 °C and subjected to a single crystal X-ray analysis. A crystal with dimensions of 0.40×0.15×0.05 mm was used for collection of X-ray diffraction data at 295 K on an automatic four circle diffractometer equipped with an X-ray tube (Cu $K\alpha$, λ=1.5418 Å) and a graphite monochromator. The cell parameters were determined by least-squares fitting with 20 reflections in the range of $28° < 2\theta < 40°$. The crystal data are as follows: M_r=408.3; monoclinic, space group $P2_1$, Z=2, unit cell dimensions; a=15.613(3), b=8.149(1), c=8.141(1) Å, β=91.32(2)°, V=1035.5(3) Å^3, D_m=1.29(1) by flotation on aqueous potassium citrate solution, D_x=1.309 Mg·m^{-3}; μ=8.31 cm^{-1} for Cu $K\alpha$ radiation; F(000)=436. The intensities of 1859 unique reflections were obtained within the 2θ range of 125°, in the ω-2θ scan mode with a scan speed of ω=6°/min. The structure was solved by the direct method, SAYTAN87.[7] All 28 hydrogen atoms were found in the difference Fourier map at the estimated positions. Refinement of the structure by the block-diagonal least-squares method converged R to 0.061 for 1504 reflections with $|F_O| = 3\sigma(F_O)$, applying unit weight for each F_{obs}, anisotropic temperature factors for non-hydrogen atoms, and isotropic ones for hydrogen atoms. The atomic scattering factors were taken from International Tables for X-Ray Crystallography.[8]

The right-hand diagram in Fig. 1 shows a perspective view (ORTEP[9]) of the molecular structure of **5**, including an ionone framework with (5*S*, 6*R*, 9*R*) or (5*R*, 6*S*, 9*S*) configuration, and the left hand one shows the numbering system of the molecule **5** used in the present X-ray study. The final atomic parameters are listed in Table III and the bond lengths and angles in Table IV.

Figure 2 shows the presence of three kinds of hydrogen bonds, *i.e.*, an intramolecular one between N17 and O25 (N17---O25=2.619(6)Å) and two intermolecular bonds between O11 and O28 (O11---O28=2.899(7) Å) and between O15 and O11 (O15---O11=2.805 (7) Å) in the crystal structure. The 2,4-dinitrophenylhydrazone part in molecule **5** appears to play an important role in the ready crystallization of **5**, which must be supported by the above-metioned intermolecular hydrogen bonds (see Fig. 2) and van der Walls contacts [see Fig. 3 (PLUTO[10])] observed in the crystal structure of **5**.

Based on this X-ray analysis results, the relative structure for the ketone (**4**) is defined as either the structure (**4**) or its optical antipode. However, the structure (**4**) had already been assigned to nigakialcohol, which gives a positive circular dichroism (CD) Cotton effect ($[\theta]$+310) at 294 nm (c=0.083, EtOH).[11] The CD behavior of the ketone (**4**), $[\theta]$ +276 and 294 nm (c=0.086, EtOH) was in agreement with that of nigakialcohol, and this finding indicates that the ketone has the absolute structure shown in the formula **4**. Furthermore, the accumulated lines of evidence demonstrated that the common aglycone is the ionone (**3**) with (3*S*, 5*S*, 6*R*, 9*R*) chiral centers.

As the final step of the structural elucidation, the location of the β-D-glucosyl residue on the aglycone (**3**) was confirmed in both glucosides, **1** and **2**. In the ^{13}C-NMR

TABLE III. Final Atomic Coordinates and Isotropic Temperature Factors of **5** with e.s.d.'s in Parentheses

Atom	*x*	*y*	*z*	B_{eq} or *B*
C1	1.1061 (3)	0.0844 (9)	−0.3193 (7)	3.20 (6)
C2	1.0371 (4)	−0.0161 (9)	−0.2299 (8)	3.53 (6)
C3	1.0185 (3)	0.0562 (9)	−0.0665 (6)	2.96 (6)
C4	1.0964 (4)	0.0735 (10)	0.0466 (7)	3.64 (7)
C5	1.1692 (4)	0.1676 (9)	−0.0367 (7)	3.35 (6)
C6	1.1882 (3)	0.0974 (9)	−0.2088 (6)	2.93 (6)
C7	1.2610 (3)	0.1920 (9)	−0.2958 (7)	3.38 (7)
C8	1.3390 (4)	0.0840 (11)	−0.3172 (8)	4.32 (7)
C9	1.4100 (3)	0.1586 (11)	−0.4195 (7)	4.21 (9)
C10	1.4422 (4)	0.3233 (11)	−0.3579 (9)	5.02 (9)
O11	1.3786 (3)	0.1693 (10)	−0.5851 (5)	6.73 (10)
C12	1.0688 (4)	0.2539 (10)	−0.3635 (8)	4.30 (7)
C13	1.1265 (4)	−0.0110 (12)	−0.4782 (8)	5.10 (9)
C14	1.2496 (4)	0.1622 (12)	0.0755 (7)	4.73 (10)
O15	1.2308 (3)	0.2392 (9)	0.2246 (6)	5.66 (7)
N16	0.9468 (3)	0.1048 (8)	−0.0089 (5)	3.32 (5)
N17	0.8772 (3)	0.0927 (8)	−0.1186 (5)	3.50 (6)
C18	0.7973 (3)	0.1156 (8)	−0.0619 (6)	2.93 (6)
C19	0.7235 (3)	0.1263 (9)	−0.1662 (6)	3.16 (7)
C20	0.6422 (4)	0.1484 (10)	−0.1086 (7)	3.96 (8)
C21	0.6327 (3)	0.1634 (10)	0.0561 (7)	3.69 (7)
C22	0.7022 (3)	0.1568 (8)	0.1667 (7)	3.08 (6)
C23	0.7825 (4)	0.1332 (9)	0.1080 (6)	3.18 (6)
N24	0.7302 (3)	0.1179 (9)	−0.3453 (6)	4.60 (7)
O25	0.8003 (3)	0.0907 (8)	−0.4065 (5)	5.13 (7)
O26	0.6653 (3)	0.1372 (12)	−0.4284 (5)	8.40 (13)
N27	0.5473 (3)	0.1892 (11)	0.1215 (7)	5.68 (10)
O28	0.5411 (3)	0.2293 (9)	0.2636 (6)	6.01 (8)
O29	0.4855 (3)	0.1603 (13)	0.0314 (6)	9.37 (16)
H1	0.981 (4)	−0.026 (10)	−0.303 (8)	3.4
H2	1.058 (4)	−0.131 (10)	−0.207 (8)	3.4
H3	1.119 (4)	−0.047 (10)	0.077 (8)	3.7
H4	1.077 (4)	0.137 (10)	0.156 (8)	3.7
H5	1.152 (4)	0.295 (10)	−0.053 (8)	3.4
H6	1.209 (4)	−0.028 (10)	−0.190 (8)	3.1
H7	1.278 (4)	0.298 (10)	−0.224 (8)	3.6
H8	1.240 (4)	0.233 (10)	−0.415 (8)	3.6
H9	1.320 (5)	−0.028 (11)	−0.378 (9)	4.5
H10	1.363 (5)	0.055 (11)	−0.198 (9)	4.4
H11	1.467 (5)	0.083 (12)	−0.412 (8)	4.8
H12	1.493 (5)	0.366 (11)	−0.438 (9)	4.9
H13	1.466 (5)	0.308 (11)	−0.235 (9)	4.9
H14	1.391 (5)	0.410 (11)	−0.354 (9)	4.9
H15	1.421 (5)	0.218 (13)	−0.655 (10)	7.0
H16	1.117 (5)	0.325 (11)	−0.427 (9)	4.6
H17	1.048 (5)	0.316 (11)	−0.253 (9)	4.5
H18	1.014 (5)	0.237 (11)	−0.441 (8)	4.6
H19	1.175 (5)	0.059 (11)	−0.550 (9)	5.0
H20	1.069 (5)	−0.015 (11)	−0.552 (9)	5.0
H21	1.148 (5)	−0.129 (11)	−0.454 (9)	5.0
H22	1.303 (5)	0.223 (11)	0.014 (9)	5.2
H23	1.267 (5)	0.036 (11)	0.097 (9)	5.1
H24	1.280 (5)	0.235 (12)	0.292 (9)	5.4
H25	0.888 (4)	0.053 (10)	−0.234 (8)	3.4
H26	0.588 (4)	0.154 (11)	−0.192 (8)	4.2
H27	0.690 (4)	0.166 (9)	0.297 (7)	3.1
H28	0.835 (4)	0.130 (10)	0.191 (7)	3.2

$B_{eq} = (4/3)\Sigma_i\Sigma_j B_{ij}\boldsymbol{a}_i\boldsymbol{a}_j$ for non-hydrogen atoms.

spectra (Table II), the C-3 carbon of **1** resonated downfield (by +7.22 ppm) from the corresponding signal for **3**. In contrast, the C-2 and C-4 carbons of **1** resonated upfield (by −1.73 and −4.49 ppm, respectively) from those of **3**. On the other hand, C-9 and both C-8 and C-10 of **2** were respectively shifted downfield (C-9; +8.67 ppm) and upfield (C-8 and C-10; −3.64 and −2.58 ppm, respectively)

TABLE IV. Bond Lengths (Å) and Angles (°) for non Hydrogen Atoms of **5** with e.s.d.'s in Parentheses

Bond lengths:

C1–C2	1.554 (11)	C1–C6	1.555 (11)	C1–C12	1.530 (12)
C1–C13	1.554 (13)	C2–C3	1.487 (11)	C3–C4	1.511 (11)
C3–N16	1.286 (10)	C4–C5	1.538 (11)	C5–C6	1.547 (11)
C5–C14	1.536 (13)	C6–C7	1.565 (11)	C7–C8	1.516 (12)
C8–C9	1.525 (12)	C9–C10	1.501 (13)	C9–O11	1.428 (12)
C14–O15	1.407 (12)	N16–N17	1.338 (10)	N17–C18	1.354 (10)
C18–C19	1.417 (10)	C18–C23	1.414 (10)	C19–C20	1.378 (11)
C19–N24	1.461 (11)	C20–C21	1.358 (12)	C21–C22	1.394 (11)
C21–N27	1.462 (12)	C22–C23	1.362 (10)	N24–O25	1.235 (11)
N24–O26	1.217 (13)	N27–O28	1.210 (12)	N27–O29	1.217 (14)

Bond angles:

C2–C1–C6	109.5 (6)	C2–C1–C12	108.7 (6)
C2–C1–C13	107.5 (7)	C6–C1–C12	112.5 (7)
C6–C1–C13	110.1 (7)	C12–C1–C13	108.3 (7)
C1–C2–C3	111.0 (6)	C2–C3–C4	114.0 (6)
C2–C3–N16	129.2 (7)	C4–C3–N16	116.8 (7)
C3–C4–C5	112.0 (7)	C4–C5–C6	111.5 (6)
C4–C5–C14	109.0 (7)	C6–C5–C14	110.7 (7)
C1–C6–C5	112.0 (6)	C1–C6–C7	111.6 (6)
C5–C6–C7	112.5 (6)	C6–C7–C8	111.0 (6)
C7–C8–C9	115.1 (7)	C8–C9–C10	114.9 (8)
C8–C9–O11	107.0 (7)	C10–C9–O11	111.5 (8)
C5–C14–O15	108.5 (8)	C3–N16–N17	115.1 (6)
N16–N17–C18	119.2 (6)	N17–C18–C19	123.1 (7)
N17–C18–C23	121.2 (7)	C19–C18–C23	115.8 (6)
C18–C19–C20	122.9 (7)	C18–C19–N24	121.4 (7)
C20–C19–N24	115.7 (7)	C19–C20–C21	118.4 (8)
C20–C21–C22	121.7 (8)	C20–C21–N27	119.7 (8)
C22–C21–N27	118.6 (7)	C21–C22–C23	119.6 (7)
C18–C23–C22	121.6 (7)	C19–N24–O25	119.7 (7)
C19–N24–O26	118.2 (8)	O25–N24–O26	122.1 (9)
C21–N27–O28	118.8 (8)	C21–N27–O29	118.1 (9)
O28–N27–O29	123.2 (9)		

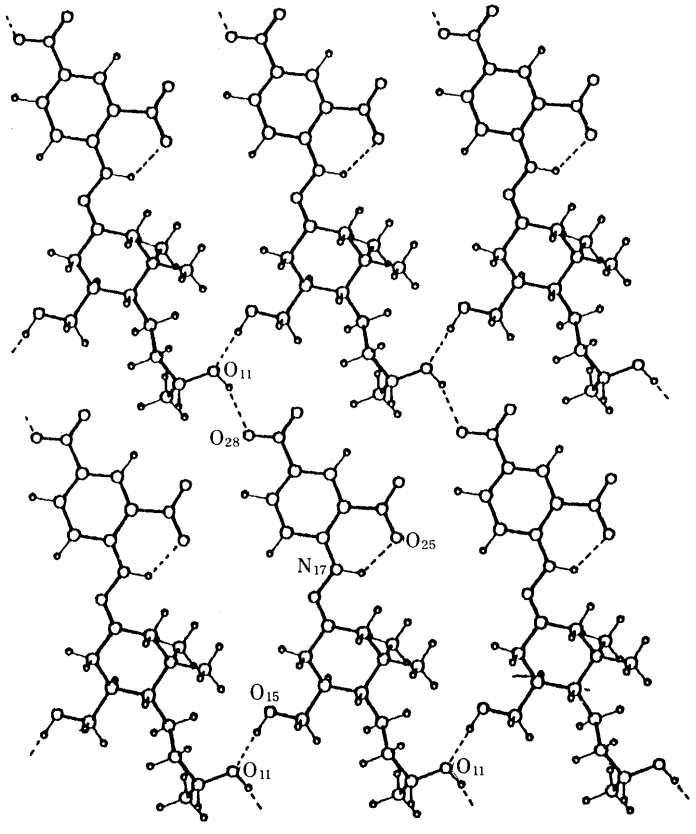

Fig. 2

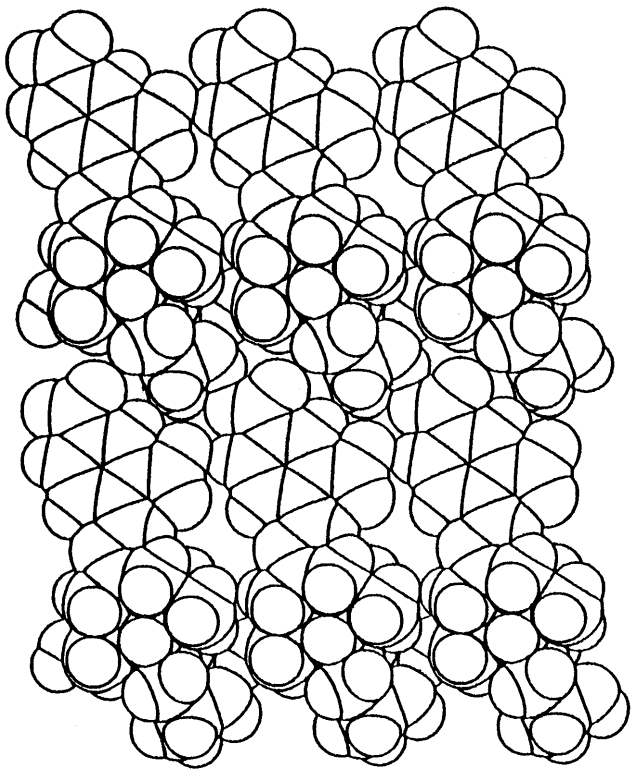

Fig. 3

compared with those for **3**. In addition, in the NOESY spectra of **1** and **2**, there were typical cross peaks between 3β-H and the glucosyl anomeric proton in **1** and between 9-H and the glucosyl anomeric proton in **2**. These NMR studies proved that the β-D-glucosyl moiety is linked at 3α-OH of the aglycone (**3**) in **1** and at 9-OH of the aglycone in **2**. Based on the accumulated evidence, the absolute structures of melia-ionosides A and B are defined as **1** and **2**, respectively.

This is the first time that **1** and **2** have been found in nature, and their common aglycone (**3**) is a new saturated ionone.

Experimental

All melting points were recorded on a Yanagimoto micro melting point apparatus and are uncorrected. The spectral data were obtained on the following instruments: optical rotation on a JASCO DIP-140, IR on a JASCO A-302, ^{1}H- and ^{13}C-NMR on a JEOL GX-400, EI- and accurate MS (ionization valtage; unless otherwise stated, 30 eV) and negative ion FAB-MS (matrix; triethanolamine) on a JEOL JNM-DX300, and CD on a JASCO J-500C. GLC was carried out on a Shimadzu GC-7AG under the following conditions: column, 1.5% SE-52 on Chromosorb WAW DMCS (2 m × 3 mm i.d.); detector, hydrogen flame ionization detecter; column temperature, 160 °C; carrier N_2 gas, 30 ml/min. As silica gel for column chromatography, Kieselgel 60 (Merck, 230—400 mesh) was used. Preparative HPLC was carried out on a Kusano instrument (Kusano Scientific Co., Tokyo) with a KPW-10 micro pump and a Shodex SE-31 differential refractometer under the following conditions: column, a reversed-phase Kusano octadecyl silica (ODS) (10 cm × 22 mm i.d.) and a Kusano Si-10 prepacked column (10 cm × 22 mm i.d.); flow rate, 3 ml/min. In the purification of the 2,4-dinitrophenylhydrazone (**5**), a Waters HPLC system [an M45J pump, an R-401 differential refractometer and a silica-phase μ-Porasil column (30 cm × 7.8 mm i.d.)] was used, with an eluant flow of 3 ml/min of *n*-hexane–AcOEt (1 : 3). The X-ray intensity data were measured on a Rigaku AFC-5 automatic diffractometer at room temperature. The Molsin (protease type XIII from *Aspergillus saitoi*) used in this work was a commercial product (Sigma Chem. Co., Lot. No. 104F-0124).

Plant Material and Extraction The same procedure as mentioned in refs. 3 and 4 was employed.

Isolation of Glucosides 1 and 2 The MeOH extract (287 g) obtained from the air-dried leaves of *M. toosendan* was suspended in H_2O and the suspension was extracted successively with petroleum ether (500 ml × 3), $CHCl_3$ (500 ml × 4), and *n*-BuOH (400 ml × 2). The residue (52.4 g) obtained from the *n*-BuOH layer was subjected to silica gel column chromatography [1 kg; eluted with $CHCl_3$–MeOH–H_2O (35 : 20 : 4)] and subsequent Sephadex LH-20 column chromatography (100 g; eluted with MeOH) to afford a fraction (2.87 g) containing **1**, **2**, and toosendanoside.[4] Successive HPLC separation of the fraction on ODS [eluent, MeOH–H_2O (1 : 1)] and Si-10 [eluent, $CHCl_3$–MeOH (6 : 1)] columns gave pure toosendanoside and a mixture (138 mg) containing **1** and **2**. This mixture was further purified by ODS HPLC separation [eluent, MeOH–H_2O (5 : 6)] to furnish pure glucosides **1** (21 mg) and **2** (9.5 mg), respectively.

Melia-ionoside A (**1**): A white powder (from a mixture of a large amount of C_6H_6 and a few drops of MeOH), $[\alpha]_D^{20}$ −35.1° (c=0.43, py.). IR (KBr) cm^{-1}: 3350, 1075. Negative ion FAB-MS m/z [%]: 391 [(M−H)$^-$, 100]. EI-MS m/z (%): 213 (M$^+$+H−162−H_2O, 5), 195 (M$^+$+H−162−2H_2O, 30), 177 (M$^+$+H−162−3H_2O, 100). ^{1}H- and ^{13}C-NMR: see Tables I and II, respectively. *Anal.* Calcd for $C_{19}H_{36}O_8$: C, 58.14; H, 9.25. Found: C, 58.13; H, 9.22.

Melia-ionoside B (**2**): A white powder (from the same solvent mixture as in the case of **1**), $[\alpha]_D^{20}$ −9.0° (c=0.15, py.). IR (KBr) cm^{-1}: 3350, 1075. Negative ion FAB-MS m/z [%]: 391 [(M−H)$^-$, 100]. EI-MS m/z (%): 355 (M$^+$−H−2H_2O, 4), 231 (M$^+$+H−162, 8), 213 (M$^+$+H−162−H_2O, 43), 195 (M$^+$+H−162−2H_2O, 69), 177 (M$^+$+H−162−3H_2O, 100). ^{1}H- and ^{13}C-NMR: see Tables I and II, respectively. *Anal.* Calcd for $C_{19}H_{36}O_8 \cdot 1/2H_2O$: C, 56.84; H, 9.29. Found: C, 57.05; H, 9.04.

Acidic Hydrolysis of 1 A solution of **1** (26 mg) in MeOH (6 ml) and 10% H_2SO_4 (3 ml) was refluxed for 4 h, poured into ice-water, and extracted with AcOEt (60 ml × 1 and 40 ml × 3) and *n*-BuOH (60 ml × 2), successively. Each of the AcOEt and *n*-BuOH extracts was washed with 5% aqueous $NaHCO_3$ and then H_2O, and dried over $MgSO_4$. The AcOEt and *n*-BuOH extracts were combined and evaporated to dryness. The residual product was purified by silica gel column chromatography [eluent, $CHCl_3$–MeOH (10 : 1) to give an aglycone (**3**) (12 mg, 78.4% yield), colorless oil, $[\alpha]_D^{20}$ −6.8° (c=0.10, py.). IR ($CHCl_3$) cm^{-1}: 3605, 3400. EI- and accurate MS (ionization voltage, at 20 eV) m/z (%): 231.195 (M$^+$+H, Calcd for $C_{13}H_{27}O_3$ 231.196, 3), 230.188 (M$^+$, Calcd for $C_{13}H_{26}O_3$ 230.188, 1), 212.178 (M$^+$−H_2O, Calcd for $C_{13}H_{24}O_2$ 212.178, 6), 194.168 (M$^+$−2H_2O, Calcd for $C_{13}H_{22}O$ 194.167, 39), 176 (M$^+$−3H_2O, 23), 122 (100). Negative ion FAB-MS m/z [%]: 229 [(M−H)$^-$, 30]. ^{1}H- and ^{13}C-NMR: see Tables I and II, respectively. The aqueous layer of the hydrolysate was neutralized with Amberlite IRA-45 (OH$^-$ form) and evaporated under reduced pressure. The residual product was subjected to PPC [iso-PrOH–*n*-BuOH–H_2O (7 : 1 : 2) as the developing solvents; developed twice; aniline hydrogen phthalate for detection], HPTLC [Merck Si 50000 type; *n*-BuOH–py.–H_2O (75 : 15 : 10) as the developing solvents], and GLC [after trimethylsilylation with *N,O*-bis(trimethylsilyl)-trifluoroacetamide–py.] to demonstrate the presence of glucose.

Acidic Hydrolysis of 2 A solution of **2** (60 mg) in 10% H_2SO_4 (5 ml) and MeOH (10 ml) was refluxed for 4h. In the same manner as in the case of acidic hydrolysis of **1**, the reaction mixture was worked up. The aglycone **3** (25.4 mg, 72.2% yield) and glucose were isolated and identified.

Enzymatic Hydrolysis of 1 A suspension of Molsin (protease type XIII from *Aspergillus saitoi*) (400 mg) in 0.2 M citric acid–0.2 M Na_2HPO_4 buffer (pH 4.0; 3 ml) was added to a solution of **1** (13 mg) in EtOH (0.4 ml). The reaction mixture was stirred at 37 °C for 63 h, then poured into H_2O, and extracted with AcOEt (60 ml × 1 and 40 ml × 3) and *n*-BuOH (50 ml × 2), successively. The AcOEt and *n*-BuOH extracts were each washed with H_2O, and dried over $MgSO_4$. The two extracts were combined and evaporated to dryness. The residue was purified by silica gel column chromatography [eluent, $CHCl_3$–MeOH (7 : 1)] to give the corresponding 3-keto derivative (**4**) (4.6 mg, 61% yield) of the aglycone (**3**), together with a small amount of **3** (1 mg, 13% yield). The physical and spectral properties of **4** are as follows: colorless oil, $[\alpha]_D^{20}$ +11.7° (c=0.185, py.) and $[\alpha]_D^{20}$ +24.6° (c=0.35, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3620, 3410 (OH), 1700 (C=O). EI- and accurate MS m/z (%): 228.172 (M$^+$, Calcd for $C_{13}H_{24}O_3$ 228.173, 1), 213.149 (M$^+$−CH_3, Calcd for $C_{12}H_{21}O_3$ 213.149, 14), 210.162 (M$^+$−H_2O, Calcd for $C_{13}H_{22}O_2$ 210.162, 25), 195.139 (M$^+$−H_2O−CH_3, Calcd for $C_{12}H_{19}O_2$ 195.139, 23), 192.151 (M$^+$−2H_2O, Calcd for $C_{13}H_{20}O$ 192.151, 6), 177.128 (M$^+$−2H_2O−CH_3, Calcd for $C_{12}H_{17}O$ 177.128, 35), 83 (100). Negative ion FAB-MS m/z [%]: 227 [(M−H)$^-$, 45]. ^{1}H-NMR (py.-d_5) δ : 0.80 (3H, s, 11-H$_3$), 1.04 (3H, s, 12-H$_3$), 1.38 (3H, d, J=6.1 Hz,

10-H_3), 1.6 (1H, m, 7-H), 1.7 (3H, m, 6β-H, 7-H, and 8-H), 1.93 (1H, m, $W_{1/2}$=18.0 Hz, 5α-H), 2.02 (1H, m, 8-H), 2.15 (1H, dd, J=13.1, 2.1 Hz, 2α-H), 2.36 (1H, d, J=13.1 Hz, 2β-H), 2.70 (1H, ddd, J=14.0, 5.2, 2.1 Hz, 4α-H), 2.76 (1H, dd, J=14.0, 11.9, 4β-H), 4.0 (1H, m, 9-H), 3.92 (1H, dd, J=10.7, 2.8 Hz), 4.01 (1H, dd, J=10.7, 4.9 Hz) (13-H_2). ^{13}C-NMR: see Table II. CD (c=0.086, EtOH) [θ] (nm): +276 (294) (positive maximum) and (c=0.050, $CHCl_3$) [θ] (nm): +336 (287) (positive maximum).

Enzymatic Hydrolysis of 2 A suspension of Molsin (700 mg) in 0.2 M citric acid–0.2 M Na_2HPO_4 buffer (pH 4.0, 5 ml) was added to a solution of **2** (21.0 mg) in EtOH (0.8 ml). The mixture was incubated with stirring at 37 °C for 120 h, then worked up and purified in the same manner as in the case of enzymic hydrolysis of **1**. The 3-keto derivative (**4**) (9.5 mg, 78% yield) was obtained as a sole product.

Bio-oxidation of 3 A suspension of Molsin (120 mg) in 0.2 M citric acid–0.2 M Na_2HPO_4 buffer (pH 4.0, 3 ml) was added to a solution of **3** (8.2 mg) in EtOH (0.3 ml). The mixture was allowed to react under stirring at 37 °C for 96 h, and worked up and purified in the same manner as described for the enzymic hydrolysis of **1** and **2**. The corresponding 3-keto derivative (**4**) (6.8 mg) was obtained in high yield (84%) as a sole reaction product.

Conversion of 4 to the Corresponding 2,4-Dinitrophenylhydrazone (5) A 0.5 ml aliquot of a 2,4-dinitrophenylhydrazine solution (0.07 mM), prepared by adding concentrated H_2SO_4 (0.2 ml), H_2O (0.3 ml), and 95% aqueous EtOH (1 ml) successively to 2,4-dinitrophenylhydrazine (0.04 g), was added dropwise to a solution of **4** (4.9 mg, 0.021 mM). The reaction mixture was allowed to stand for 2d at room temperature, poured into ice-H_2O, and extracted with AcOEt (30 ml × 4). The combined AcOEt layer was washed with aqueous $NaHCO_3$ and H_2O successively, dried over $MgSO_4$, and evaporated to dryness. The residue was subjected to purification by a Waters HPLC system to give the corresponding 2,4-dinitrophenylhydrazone (**5**) (3.0 mg) of **4**. Recrystallization from AcOEt afforded red plates of mp 148—150 °C. EI-MS m/z (%): 408 (M^+, 50), 390 ($M^+ - H_2O$, 39), 81 (100). ^{1}H-NMR (acetone-d_6) δ: 0.82 (3H, s, 11-H_3), 1.14 (3H, d, J=6.1, 10-H_3), 1.19 (3H, s, 12-H_3), 1.4 (1H, m, 6β-H), 1.5 (3H, m, 7-H_2 and 8-H), 1.6 (1H, m, 8-H), 1.74 (1H, m, 5α-H), 2.16 (1H, d, J=13.7 Hz, 2β-H), 2.38 (1H, dd, J=13.4, 12.8 Hz, 4β-H), 2.65 (1H, dd, J=13.7, 2.4 Hz, 2α-H), 2.71 (1H, ddd, J=13.4, 4.6, 2.4 Hz, 4α-H), 3.71 (1H, m, 9-H), 3.64 (1H, dd, J=11.2, 6.6 Hz), 3.77 (1H, dd, J=11.2, 3.4 Hz) (13-H_2), 8.04 (1H, d, J=9.5 Hz, aromatic 6-H), 8.38 (1H, dd, J=9.5, 2.7 Hz, aromatic 5-H), 9.00 (1H, d, J=2.7 Hz, aromatic 3-H), 11.22 (1H, s, –NH–).

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References and Notes

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Resin Glycosides. XI.[1] Operculins III, IV, IX, X, XVI, XVII and XVIII, New Ether-Soluble Resin Glycosides of Rhizoma Jalapae Braziliensis (Root of *Ipomoea operculata*)

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Ten ether-soluble resin glycosides (jalapins), operculins IX—XVIII, were isolated from Rhizoma Jalapae Braziliensis (roots of *Ipomoea operculata*). Operculins III and IV previously obtained, and operculins IX, X, XVI, XVII and XVIII were characterized on the bases of chemical and spectral data. All of them have a common glycosidic acid, operculinic acid B, with a macrocyclic ester structure and *n*-decanoyl and/or *n*-dodecanoyl residues as organic acid groups.

Keywords resin glycoside; Rhizoma Jalapae Braziliensis; *Ipomoea operculata*; Convolvulaceae; operculin III; operculin IV; operculin IX; operculin X; operculin XVI; operculin XVII; operculin XVIII

In the preceding paper,[2] we reported the isolation of eight ether-soluble resin glycosides (jalapins) named operculins I—VIII, and the structure elucidation of operculins I, II, V, VII and VIII, from Rhizoma Jalapae Braziliensis (roots of *Ipomoea operculata* (Gomes) MART). All of them were found to possess characteristic macrocyclic ester structures similar to those of the jalapins[1,3-6] so far isolated from Convolvulaceae plants, but they have *n*-dodecanoic acid and/or *n*-decanoic acid in place of the hitherto known isobutyric, 2-methylbutyric, tiglic and nilic acids.

Further separation of fractions 5, 6, and 13 obtained from the jalapin fraction[2] afforded ten new resin glycosides, operculins IX—XVIII. This paper deals with the structure elucidation of operculins III (**1**), IV (**2**) (previously isolated[2]), IX (**3**), X (**4**), XVI (**5**), XVII (**6**) and XVIII (**7**), which have a common glycosidic acid, operculinic acid B.[7]

Operculin III (**1**), colorless needles (MeOH–H_2O), mp 115—125 °C, $[\alpha]_D^{25}$ −25.7° (c=2.6, MeOH), $C_{10}H_{124}O_{26}$, showed an $(M-H)^-$ ion peak at m/z 1379 in the negative ion fast atom bombardment-mass spectrum (negative

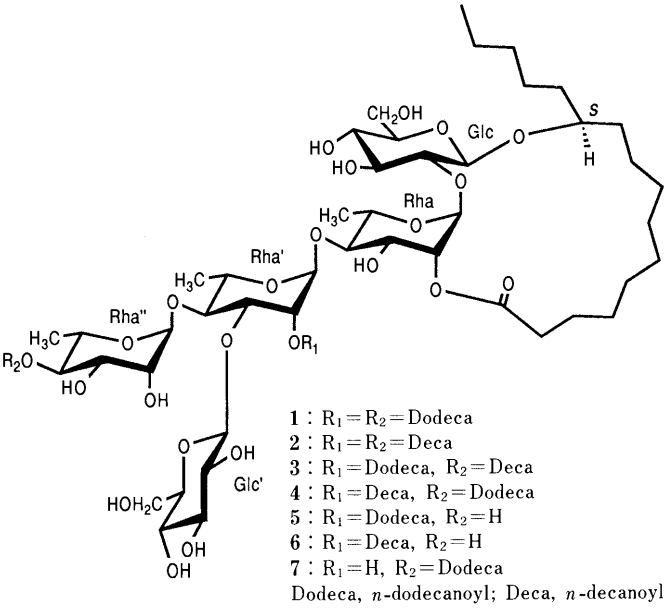

Fig. 1. Structures of **1**—**7**

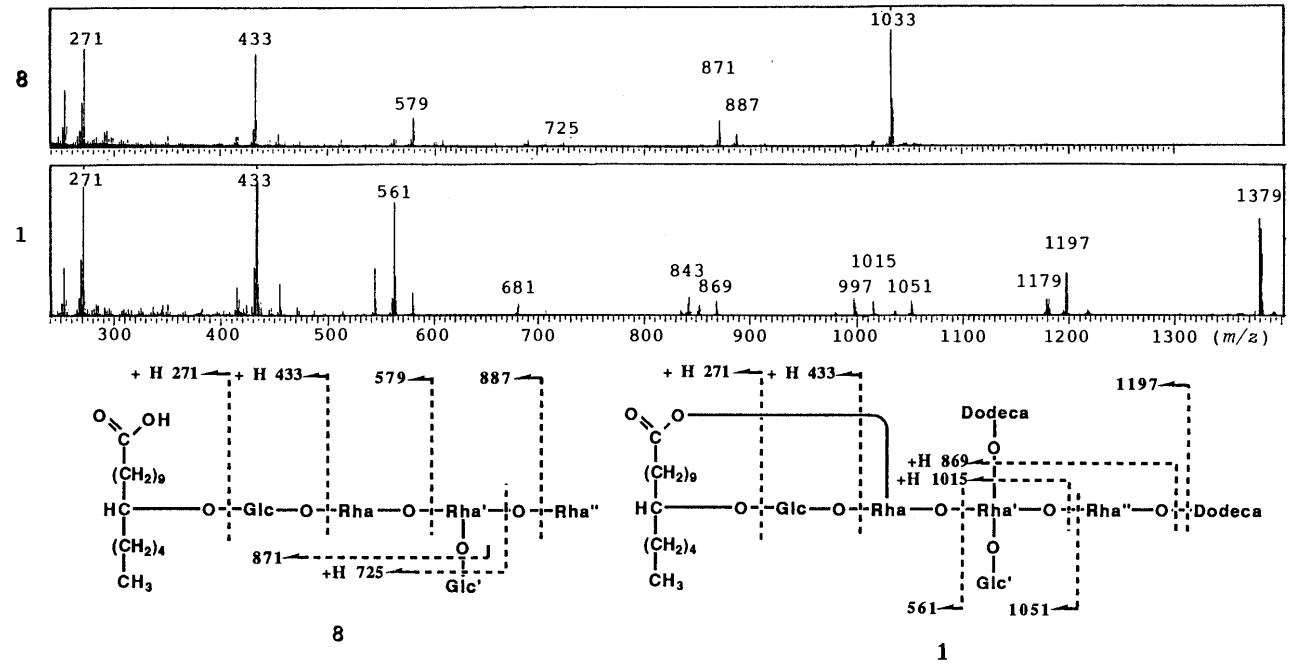

Fig. 2. Negative FAB-MS of **1** and **8**

Glc, glucopyranosyl; Rha, rhamnopyranosyl; Dodeca, *n*-dodecanoyl.

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TABLE I. ^{13}C-NMR Spectral Data for **1**, **2**, **3**, **4**, **5**, **6** and **7** (in Pyridine-d_5, 100 MHz)

		1	2	3	4	5	6	7
Glc	-1	104.4	104.4	104.5	104.5	104.5	104.5	104.4
Glc	-2	82.0	81.9	82.0	82.0	81.9	81.9	81.8
Glc	-3	76.6	76.5	76.6	76.6	76.6	76.6	76.6
Glc	-4	71.8	71.8	71.9	71.9	71.9	71.9	71.8
Glc	-5	77.9	77.9	78.0	77.9	78.0	78.0	77.9
Glc	-6	62.8	62.8	62.9	62.9	62.9	62.9	62.9
Glc′	-1	105.4	105.4	105.5	105.4	105.5	105.5	105.3
Glc′	-2	75.2	75.1	75.2	75.2	75.2	75.2	75.1
Glc′	-3	78.5	78.4	78.5	78.5	78.5	78.5	78.4
Glc′	-4	71.5	71.4	71.5	71.5	71.5	71.5	71.8
Glc′	-5	78.1	78.1	78.2	78.1	78.2	78.2	78.2
Glc′	-6	62.9	62.9	63.0	63.0	63.0	63.0	62.9
Rha	-1	98.6	98.6	98.6	98.6	98.6	98.6	98.5
Rha	-2	73.5	73.5	73.6	73.5	73.5	73.5	73.6
Rha	-3	69.4	69.4	69.5	69.4	69.4	69.4	69.7
Rha	-4	81.3	81.3	81.3	81.3	81.4	81.4	81.9
Rha	-5	69.0	69.0	69.0	69.0	69.1	69.1	69.2
Rha	-6	19.1	19.0	19.1	19.1	19.1	19.1	19.1
Rha′	-1	99.9	99.9	100.0	99.9	100.0	100.0	103.6
Rha′	-2	73.1	73.1	73.1	73.1	73.2	73.2	71.8
Rha′	-3	80.2	80.1	80.2	80.2	80.3	80.2	82.4
Rha′	-4	78.6	78.6	78.6	78.6	78.3	78.3	79.0
Rha′	-5	68.5	68.5	68.5	68.5	68.7	68.7	68.7
Rha′	-6	18.9	18.8	18.9	18.9	18.9	18.9	19.0
Rha″	-1	103.2	103.1	103.2	103.2	103.5	103.5	102.9
Rha″	-2	72.4	72.3	72.5	72.4	72.5	72.5	72.6
Rha″	-3	70.3	70.3	70.4	70.3	72.9	72.9	70.3
Rha″	-4	75.5	75.4	75.5	75.5	74.0	74.0	75.5
Rha″	-5	68.2	68.1	68.2	68.2	70.8	70.8	68.0
Rha″	-6	18.0	18.0	18.1	18.0	18.5	18.5	18.0
Ag	-11	82.8	82.8	82.8	82.8	82.8	82.8	82.8
Ag	-16	14.3	14.2	14.3	14.3	14.3	14.3	14.3
C=O		173.6	173.6	173.7	173.6	173.7	173.7	173.5
		173.5	173.4	173.5	173.5	173.4	173.4	173.3
		173.3	173.3	173.4	173.4			

δ in ppm from tetramethylsilane (TMS). Glc, glucopyranosyl; Rha, rhamnopyranosyl; Ag, aglycone ((*S*)-jalapinolic acid). All assignments are based on the HETCOR spectral data.

FAB-MS) (Fig. 2). It afforded, on alkaline hydrolysis, *n*-dodecanoic acid and a glycosidic acid which was identical with operculinic acid B (**8**), that is, (11*S*)-11-hydroxyhexadecanoic acid ((*S*)-jalapinolic acid) 11-*O*-*β*-D-glucopyranosyl-(1→3)-*O*-[α-L-rhamnopyranosyl-(1→4)]-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside.[7]

Compound **1** exhibited signals due to three carboxyl (δ 173.4, 173.5, 173.7) and five anomeric (δ 98.6, 100.0, 103.2, 104.3, 105.4) carbons in the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum, indicating that **1** consists of 1 mol of **8** and 2 mol of *n*-dodecanoic acid (Table I).

The ^{1}H-NMR signals of **1** were assigned with the aid of ^{1}H–^{1}H shift-correlated two dimensional (2D)-NMR (COSY) and nuclear Overhauser effect 2D-NMR (NOESY) spectra. When compared with the signals of **8**, 2-H of the first rhamnose (Rha), 2-H of the second rhamnose (Rha′) and 4-H of the third rhamnose (Rha″) were shifted downfield by 1.35, 1.14, 1.58 ppm, respectively, and instead of the signal at δ 2.50 (2H, t) in **8**, two signals at δ 2.30, 2.40 (each 1H), due to the nonequivalent 2-H_2 of the jalapinolic acid group were observed (Fig. 3). These observations indicated that **1** has, like all the ether-soluble resin glycosides (jalapins) so far isolated,[1-6] a macrocyclic ester structure formed by

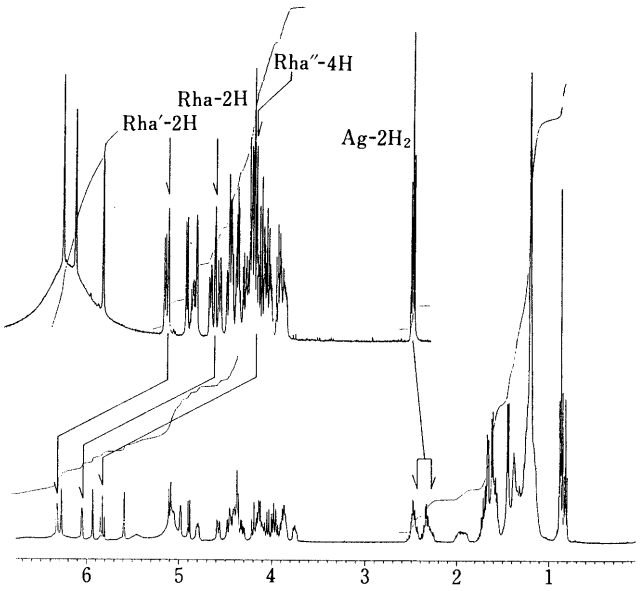

Fig. 3. ^{1}H-NMR Spectra of **1** and **8** (Upper) (in Pyridine-d_5, 400 MHz)

linkage between the carboxyl group of the jalapinolic acid and one of the hydroxyl groups in the sugar moiety of **8**, and that the three ester groups are located at 2-OH of Rha, 2-OH of Rha′ and 4-OH of Rha″.

In the negative FAB-MS of **1** and **8** (Fig. 2), along with the fragment peaks observed at *m/z* 271 [272 (jalapinolic acid)−H]$^-$ and 433 [272+162 (hexose unit)−H]$^-$, **1** showed a strong fragment peak at *m/z* 561 [272+162+128 (146 (6-deoxyhexose unit)−18(H_2O)−H]$^-$ in place of that at *m/z* 579 [272+162+146−H]$^-$ observed in the spectrum of **8**. The difference of 18 mass units suggested that the ester linkage of jalapinolic acid is placed in Rha.[4]

To confirm the above suggestion, **1** was converted to the peracetate (**9**). Its electron impact mass spectrum (EI-MS) revealed fragment ion peaks at *m/z* 331, 413, 723 and 1071, which are respectively ascribable to the fragments a, c, d and g as shown in Fig. 4. Therefore, the two *n*-dodecanoic acid groups should be attached to 2-OH of Rha′ and 4-OH of Rha″ and hence the ester linkage of jalapinolic acid groups is located at 2-OH of Rha.

Taking the *J* values of the anomeric and methine proton signals due to the sugar moiety into account, the conformations of the rhamnopyranose units of **1** are concluded to be $^1C_4$ and those of glucopyranose units are $^4C_1$ (Table II).

Consequently, the structure of operculin III (**1**) was defined as (*S*)-jalapinolic acid 11-*O*-*β*-D-glucopyranosyl-(1→3)-*O*-[4-*O*-*n*-dodecanoyl-α-L-rhamnopyranosyl-(1→4)]-*O*-(2-*O*-*n*-dodecanoyl)-α-L-rhamnopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside, intramol. 1, 2″-ester (Fig. 1).

Operculin IV (**2**), a white powder (MeOH–H_2O), mp 117—120 °C, $[\alpha]_D^{22}$ −22.2° (*c*=5.4, MeOH), $C_{66}H_{116}O_{26}$, furnished, on alkaline hydrolysis, *n*-decanoic acid and **8**. Compound **2** showed the (M−H)$^-$ ion peak at *m/z* 1323 together with the same fragment peaks as those of **1** at *m/z* 561, 433, 271 in the negative FAB-MS and three carboxyl carbon signals at δ 173.4, 173.5, 173.6 in the ^{13}C-NMR spectrum (Table I). Further, the ^{1}H-NMR spectrum of **2** was quite similar to that of **1** and, in particular, the chemical

TABLE II. ^{1}H-NMR Spectral Data for **1**—**8** (in Pyridine-d_5, 400 MHz)

		1	**2**	**3**	**4**
Glc	-1	4.89, d (7.3)	4.84, d (7.2)	4.88, d (7.6)	4.87, d (7.6)
Glc	-2	3.89, dd (7.3, 8.6)	3.84, dd (7.2, 8.0)	3.88, dd (7.6, 9.0)	3.86, dd (7.6, 9.0)
Glc	-3	4.16, dd (8.6, 8.6)	4.09, dd (8.0, 8.0)	4.14, dd (9.0, 9.0)	4.14, dd (9.0, 9.0)
Glc	-4	4.12, dd (8.6, 8.6)	4.05[a]	4.10, dd (9.0, 9.0)	4.10, dd (8.0, 8.0)
Glc	-5	3.86, ddd (2.5, 6.4, 8.6)	3.80, ddd (3.0, 5.0, 9.0)	3.84, ddd (3.5, 5.5, 9.0)	3.84, ddd (3.0, 5.2, 8.0)
Glc	-6	4.32, dd (6.4, 11.9)	4.26, dd (5.0, 11.0)	4.30, dd (5.5, 11.5)	4.29, dd (5.2, 12.0)
		4.47, dd (2.5, 11.9)	4.41, dd (3.0, 11.0)	4.45, dd (3.5, 11.5)	4.43, dd (3.0, 12.0)
Glc′	-1	5.10, d (7.6)	5.03, d (7.8)	5.09, d (7.5)	5.07, d (7.4)
Glc′	-2	3.98, dd (7.6, 8.8)	3.92, dd (7.8, 9.0)	3.97, dd (7.5, 8.5)	3.95, dd (7.4, 9.0)
Glc′	-3	4.05, dd (8.8, 8.8)	4.00, dd (9.0, 9.0)	4.04, dd (8.5, 8.5)	4.02, dd (9.0, 9.0)
Glc′	-4	3.96, dd (8.8, 8.8)	3.89, dd (9.0, 9.0)	3.94, dd (8.5, 8.5)	3.92, dd (9.0, 9.0)
Glc′	-5	3.75, ddd (3.0, 6.0, 8.8)	3.71, ddd (2.8. 6.0, 9.0)	3.75, ddd (2.5, 6.0, 8.5)	3.75, ddd (3.0, 6.0, 9.0)
Glc′	-6	4.10, dd (6.0, 11.5)	4.03[a]	4.09, dd (6.0, 12.0)	4.06, dd (6.0, 11.5)
		4.40[a]	4.36[a]	4.38, dd (2.5, 12.0)	4.36, dd (3.0, 11.5)
Rha	-1	5.59, d (1.2)	5.55, d (1.0)	5.59, d (1.5)	5.57, d (1.5)
Rha	-2	6.05, dd (1.2, 3.0)	5.98, dd (1.0, 3.0)	6.02, dd, (1.5, 3.0)	6.01, dd (1.5, 3.2)
Rha	-3	5.07[a]	5.00, dd (3.0, 9.5)	5.05, dd (3.0, 9.5)	5.03, dd (3.2, 9.2)
Rha	-4	4.19, dd (9.4, 9.4)	4.13, dd (9.5, 9.5)	4.18, dd (9.5, 9.5)	4.16, dd (9.2, 9.2)
Rha	-5	4.42[a]	4.40[a]	4.43[a]	4.42[a]
Rha	-6	1.62, d (6.1)	1.59, d (6.0)	1.62, d (6.1)	1.60, d (6.1)
Rha′	-1	5.93, d (1.5)	5.85, d (1.0)	5.91, d (1.5)	5.88, d (1.6)
Rha′	-2	6.32, dd (1.5, 3.1)	6.27, dd (1.0, 3.5)	6.31, dd (1.5, 3.5)	6.30, dd (1.6, 3.5)
Rha′	-3	4.80, dd (3.1, 8.6)	4.74, dd (3.5, 9.0)	4.79, dd (3.5, 9.5)	4.77, dd (3.5, 9.0)
Rha′	-4	4.38[a]	4.33[a]	4.37, dd (9.5, 9.5)	4.36, dd (9.0, 9.0)
Rha′	-5	4.37[a]	4.34[a]	4.37[a]	4.35[a]
Rha′	-6	1.67, d (5.5)	1.64, d (5.5)	1.67, d (5.5)	1.65, d (5.5)
Rha″	-1	6.27, d (1.5)	6.19, d (1.5)	6.25, d (1.5)	6.23, d (1.5)
Rha″	-2	4.98, dd (1.5, 3.4)	4.91, dd (1.5, 3.0)	4.95, dd (1.5, 3.0)	4.94, dd (1.5, 3.5)
Rha″	-3	4.58, dd (3.4, 9.2)	4.51, dd (3.0, 9.0)	4.55, dd (3.0, 9.5)	4.54, dd (3.5, 9.5)
Rha″	-4	5.82, dd (9.2, 9.2)	5.75, dd (9.0, 9.0)	5.80, dd (9.5, 9.5)	5.78, dd (9.5, 9.5)
Rha″	-5	4.39[a]	4.35[a]	4.40[a]	4.38[a]
Rha″	-6	1.44, d (6.4)	1.41, d (6.5)	1.45, d (6.4)	1.43, d (6.4)
Ag	-2	2.29, ddd (4.0, 8.0, 14.0)	2.28, ddd (4.0, 7.5, 14.5)	2.30, ddd (4.0, 8.0, 15.0)	2.28, ddd (4.0, 8.0, 14.5)
Ag	-2	2.44[a]	2.43[a]	2.44[a]	2.43[a]
Ag	-11	3.83, m	3.84, m	3.86[a]	3.85[a]
Ag	-16	0.82, t (7.0)	0.82, t (7.0)	0.83, t (7.0)	0.82, t (7.0)
Org	-2	2.34, ddd (4.5, 7.0, 7.0)	2.32, ddd (3.5, 7.0, 7.0)	2.35[a]	2.34[a]
		2.48, ddd (3.0, 7.0, 7.0)	2.46, ddd (2.0, 7.0, 7.0)	2.47[a]	2.47[a]
CH_3		0.87, t (7.0)	0.85, t (7.0)	0.85, t (7.0)	0.85, t (7.0)
		0.87, t (7.0)	0.85, t (7.0)	0.88, t (7.0)	0.87, t (7.0)

		5	**6**	**7**	**8**
Glc	-1	4.89, d (7.5)	4.89, d (7.2)	4.88, d (7.5)	4.97, d (7.3)
Glc	-2	3.88, dd (7.5, 9.0)	3.88, dd (7.2, 9.0)	3.88, dd (7.5, 9.0)	4.21, dd (7.3, 9.0)
Glc	-3	4.15, dd (9.0, 9.0)	4.15, dd (9.0, 9.0)	4.15, dd (9.0, 9.0)	4.25[a]
Glc	-4	4.11, dd (9.0, 9.0)	4.11, dd (9.0, 9.0)	4.10, dd (9.0, 9.0)	4.12, dd (9.0, 9.0)
Glc	-5	3.85[a]	3.84[a]	3.83, ddd (3.0, 5.0, 9.0)	3.89, ddd (2.5, 5.5, 9.0)
Glc	-6	4.30, dd (4.0, 11.5)	4.30, dd (4.0, 11.5)	4.30, dd (5.0, 11.0)	4.33, dd (5.5, 11.5)
		4.46, dd (2.8, 11.5)	4.45, dd (2.5, 11.5)	4.44, dd (3.0, 11.0)	4.49, dd (2.5, 11.5)
Glc′	-1	5.09, d (7.5)	5.09, d (7.5)	5.12, d (7.5)	5.20, d (7.6)
Glc′	-2	3.98, dd (7.5, 9.0)	3.97, dd (7.5, 9.0)	3.93, dd (7.5, 9.0)	3.97, dd (7.6, 9.0)
Glc′	-3	4.05, dd (9.0, 9.0)	4.04, dd (9.0, 9.0)	4.07, dd (9.0, 9.0)	4.14, dd (9.0, 9.0)
Glc′	-4	3.94, dd (9.0, 9.0)	3.93, dd (9.0, 9.0)	3.97, dd (9.0, 9.0)	4.09, dd (9.0, 9.0)
Glc′	-5	3.75, ddd (3.0, 6.0, 9.0)	3.75, ddd (2.5, 6.0, 9.0)	3.76, ddd (2.0, 5.5, 9.0)	3.95[a]
Glc′	-6	4.09, dd (5.0, 11.5)	4.09[a]	4.07, dd (5.5, 11.0)	4.28, dd (5.5, 11.5)
		4.40[a]	4.40[a]	4.39, dd (2.0, 11.0)	4.51, dd (2.0, 11.5)
Rha	-1	5.59, d (1.2)	5.58, d (1.5)	5.59, d (1.5)	6.31, d (1.5)
Rha	-2	6.02, dd (1.2, 3.2)	6.02, dd (1.5, 3.0)	5.98, dd (1.5, 3.2)	4.66, dd (1.5, 3.0)
Rha	-3	5.04, dd (3.2, 9.0)	5.03, dd (3.0, 9.0)	5.04, dd (3.2, 9.5)	4.60, dd (3.0, 9.5)
Rha	-4	4.16, dd (9.0, 9.0)	4.16, dd (9.0, 9.0)	4.15, dd (9.5, 9.5)	4.25, dd (9.5, 9.5)
Rha	-5	4.42[a]	4.42[a]	4.46[a]	4.90, dq (9.5, 6.1)
Rha	-6	1.60, d (6.0)	1.59, d (6.1)	1.65, d (6.1)	1.70, d (6.1)
Rha′	-1	5.87, d (1.5)	5.87, d (1.5)	5.96, d (1.8)	5.88, d (1.5)
Rha′	-2	6.31, dd (1.5, 3.0)	6.31, dd (1.5, 3.0)	5.12[a]	5.16, dd (1.5, 2.5)
Rha′	-3	4.78, dd (3.0, 9.0)	4.78, dd (3.0, 9.0)	4.69, dd (3.0, 9.0)	4.71, dd (2.5, 8.5)
Rha′	-4	4.38[a]	4.37[a]	4.47, dd (9.0, 9.0)	4.48, dd (8.5, 8.5)
Rha′	-5	4.33[a]	4.33[a]	4.35[a]	4.40[a]

TABLE II. (continued)

	5	6	7	8
Rha′ -6	1.65, d (6.0)	1.64, d (6.0)	1.63, d (6.0)	1.61, d (6.1)
Rha″ -1	6.25, d (1.2)	6.24, d (1.5)	6.19, d (1.5)	6.18, d (1.2)
Rha″ -2	4.97, dd (1.2, 3.0)	4.96, dd (1.5, 3.0)	4.91, dd (1.5, 3.0)	4.86, dd (1.2, 3.5)
Rha″ -3	4.51, dd (3.0, 9.0)	4.50, dd (3.0, 9.0)	4.56, dd (3.0, 9.5)	4.41, dd (3.5, 9.0)
Rha″ -4	4.29, dd (9.0, 9.0)	4.29, dd (9.0, 9.0)	5.76, dd (9.5, 9.5)	4.20, dd (9.0, 9.0)
Rha″ -5	4.38[a]	4.37[a]	4.35[a]	4.32[a]
Rha″ -6	1.67, d (6.1)	1.66, d (6.1)	1.37, d (6.4)	1.57, d (6.1)
Ag -2	2.48, ddd (4.0, 8.0, 15.0)	2.48, ddd (4.0, 8.5, 15.0)	2.41, ddd (4.0, 8.0, 15.0)	2.50, t (7.3)
Ag -2	2.30, ddd (4.0, 8.0, 15.0)	2.29, ddd (4.0, 8.1, 15.0)	2.26, ddd (4.0, 8.0, 15.0)	
Ag -11	3.88[a]	3.88[a]	3.88[a]	4.05[a]
Ag -16	0.83, t (7.0)	0.83, t (7.0)	0.83, t (7.0)	0.91, t (6.7)
Org -2	2.34, ddd (2.0, 7.0, 7.0)	2.34, ddd (2.0, 7.0, 7.0)	2.47, ddd (2.0, 7.0, 7.0)	
CH_3	0.87, t (7.0)	0.85, t (7.0)	0.87, t (7.0)	

δ in ppm from TMS (coupling constants (J) in Hz are given in parentheses). Glc, glucopyranosyl; Rha, rhamnopyranosyl; Ag, aglycone ((*S*)-jalapinolic acid); Org, *n*-decanoyl or *n*-dodecanoyl. *a*) Signals are overlapping. All assignments are based on the ^1H–^1H COSY and NOESY spectral data.

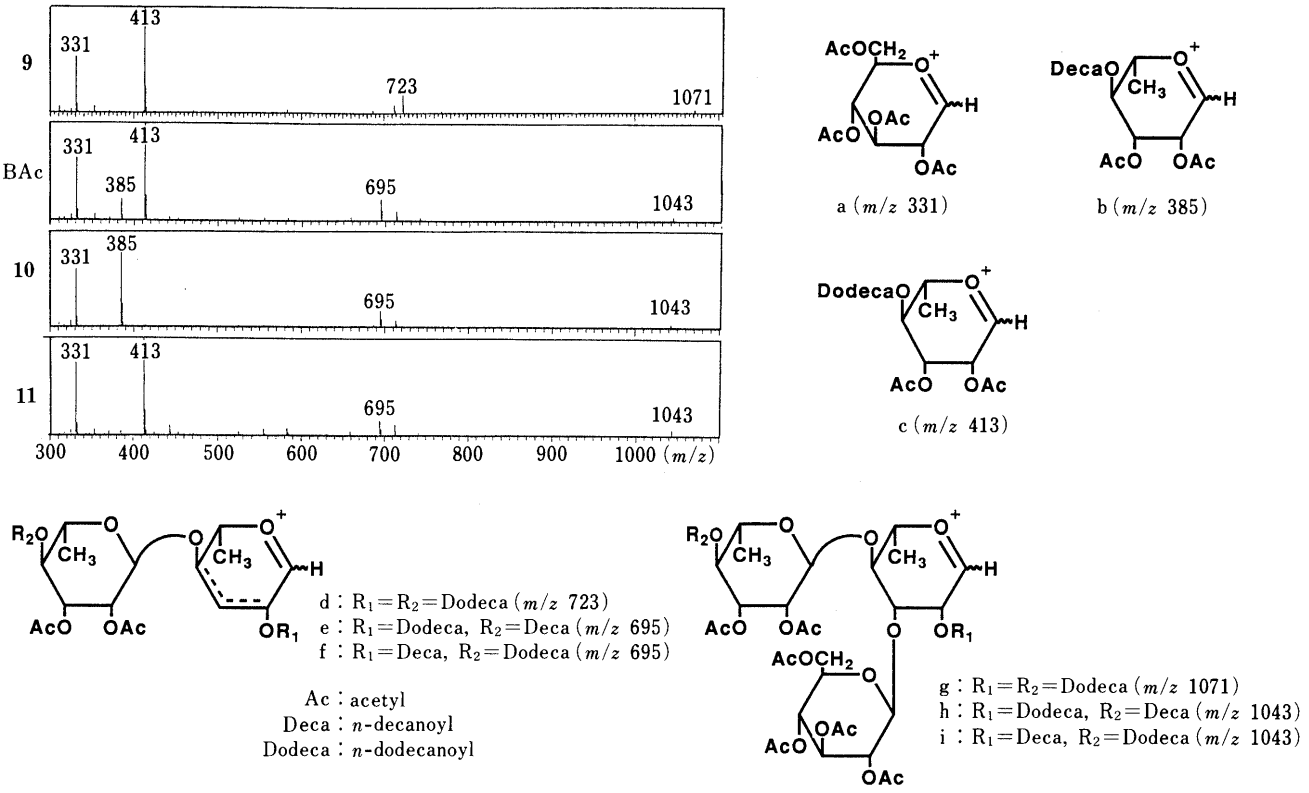

Fig. 4. EI-MS of **9**, BAc, **10** and **11**

shifts of the signals, which are subject to acylation shift, due to 2-H of Rha, 2-H of Rha′ and 4-H of Rha″ were identical to those of **1** (Table II).

Accordingly, **2** was characterized as (*S*)-jalapinolic acid 11-*O*-β-D-glucopyranosyl-(1→3)-*O*-[4-*O*-*n*-decanoyl-α-L-rhamnopyranosyl-(1→4)]-*O*-(2-*O*-*n*-decanoyl)-α-L-rhamnopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside, intramol. 1, 2″-ester (Fig. 1).

Fraction B[8] previously obtained,[2] gave, on alkaline hydrolysis, *n*-dodecanoic acid and *n*-decanoic acid in the ratio of about 1 : 1 [gas chromatography (GC)] along with **8**. The negative FAB-MS showed the $(M-H)^-$ ion peak at m/z 1351, which is 28 mass units (C_2H_4) less than that of **1**, together with the same fragment peaks as those of **1** at m/z 561, 433, 271, and the ^1H-NMR spectrum was quite similar, including the chemical shifts of the signals due to 2-H of Rha (δ 6.01), 2-H of Rha′ (δ 6.30) and 4-H of Rha″ (δ 5.58), to that of **1**. These data suggested that the structure of fraction B is analogous to that of **1**, with one of the *n*-dodecanoic acid residues in **1** having been replaced by an *n*-decanoic acid residue.

The EI-MS of the peracetate of fraction B (BAc) showed fragment peaks at m/z 331, 385, 413, 695, 1043 assignable to the fragments a, b, c, e and/or f, as well as h and/or i shown in Fig. 4. Coexistence of the fragments b and c indicated that fraction B is a mixture composed of two compounds in which the organic acid groups are mutually interchanged between Rha′ and Rha″. Therefore, we attempted further separation under several conditions.

Finally, preparative recycling high performance liquid

chromatography (HPLC) on an octadecyl silica (ODS) column using MeOH as an eluent afforded operculin IX (**3**), a white powder, mp 115—125 °C, $[\alpha]_D^{23}$ −26.2° (c = 0.5, MeOH), $C_{68}H_{120}O_{26}$, and operculin X (**4**), a white powder, mp 116—127 °C, $[\alpha]_D^{23}$ −26.6° (c = 2.3, MeOH), $C_{68}H_{120}O_{26}$.

Although the ^{1}H- and ^{13}C-NMR spectra (Tables I and II) and negative FAB-MS of **3** and **4** were almost identical with those of fraction B, the EI-MS of the peracetate of **3** (**10**) showed the peak at m/z 385 but no peak at m/z 413 observed in BAc. In contrast, that of the peracetate of **4** (**11**) exhibited the peak at m/z 413 but no peak at m/z 385 (Fig. 4).

Consequently, the structures of **3** and **4** were respectively concluded to be (*S*)-jalapinolic acid 11-*O*-β-D-glucopyranosyl-(1→3)-*O*-[4-*O*-*n*-decanoyl-α-L-rhamnopyranosyl-(1→4)]-*O*-(2-*O*-*n*-dodecanoyl)-α-L-rhamnopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside, intramol. 1, 2″-ester and its constitutional isomer in which the acyl groups of **3** are interchanged, as shown in Fig. 1.

Operculin XVI (**5**), a white powder, mp 129—134 °C, $[\alpha]_D^{25}$ −38.9° (c = 1.0, MeOH), $C_{58}H_{102}O_{25}$, furnished, on alkaline hydrolysis, *n*-dodecanoic acid and **8**. Compound **5** exhibited the $(M-H)^-$ ion peak at m/z 1197 together with the same fragment peaks as those of **1** at m/z 561, 433, 271 in the negative FAB-MS and two carboxyl carbon signals at δ 173.1, 173.6 in the ^{13}C-NMR spectrum (Table I).

The ^{1}H-NMR spectrum of **5** exhibited, in comparison with that of **1**, an upfield shift (1.49 ppm) of 4-H of Rha″ and loss of the signals attributable to one *n*-dodecanoic acid residue, while the other proton signals due to the sugar moieties remained almost unshifted (Table II).

Consequently, the structure of operculin XVI (**5**) was defined as (*S*)-jalapinolic acid 11-*O*-β-D-glucopyranosyl-(1→3)-*O*-[α-L-rhamnopyranosyl-(1→4)]-*O*-(2-*O*-*n*-dodecanoyl)-α-L-rhamnopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside, intramol. 1, 2″-ester (Fig. 1).

Operculin XVII (**6**), a white powder, mp 131—135 °C, $[\alpha]_D^{25}$ −39.1° (c = 1.0, MeOH), $C_{56}H_{98}O_{25}$, afforded, on alkaline hydrolysis, *n*-decanoic acid and **8**. The negative FAB-MS of **6** showed the $(M-H)^-$ ion peak at m/z 1169, which is 28 mass units less than that of **5**, together with the same fragment peaks as those of **5** at m/z 561, 433, 271, and further, the ^{1}H-NMR spectrum of **6** was quite similar to that of **5** except for the signals due to the organic acid moiety (Table II).

Accordingly, the structure of **6** was concluded to be a homolog of **5**, in which the *n*-dodecanoyl group of **5** is replaced by an *n*-decanoyl group (Fig. 1).

Operculin XVIII (**7**), a white powder, mp 130—133 °C, $[\alpha]_D^{25}$ −40.8° (c = 1.0, MeOH), $C_{58}H_{102}O_{25}$, was hydrolyzed with alkali to afford *n*-dodecanoic acid and **8**. The negative FAB-MS of **7** was almost superimposable on that of **5**; the same $(M-H)^-$ and fragment ion peaks were seen at m/z 1181, 561, 433, 271. Therefore, **7** is considered to be a positional isomer of **5**.

The ^{1}H-NMR spectrum of **7**, compared with that of **5**, showed a considerable downfield shift (1.47 ppm) of 4-H of Rha″ along with an upfield shift (1.19 ppm) of 2-H of Rha′, but the other proton signals due to the sugar moiety remained almost unshifted (Table II).

Consequently, **7** was characterized as (*S*)-jalapinolic acid 11-*O*-β-D-glucopyranosyl-(1→3)-*O*-[4-*O*-*n*-dodecanoyl-α-L-rhamnopyranosyl-(1→4)]-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranoside, intramol. 1, 2″-ester (Fig. 1).

Experimental

All instruments and materials used were as cited in the preceding report[2] unless otherwise specified. The separation procedure of compounds **1**, **2** and fraction B[8] was also described in the preceding paper.[2]

1: IR (KBr) cm^{-1}: 3400 (OH), 1725 (C=O). Negative FAB-MS m/z: see Fig. 2. *Anal.* Calcd for $C_{70}H_{124}O_{26}$: C, 60.85; H, 9.05. Found: C, 60.61; H, 9.12.

2: IR (KBr) cm^{-1}: 3400 (OH), 1725 (C=O). Negative FAB-MS m/z (%): 1323 (88) $[M-H]^-$, 1169 (21) [1323−154 (decanoic acid unit)]$^-$, 1023 (6) [1169−146 (6-deoxyhexose unit)]$^-$, 561 (35) [1023−146−154−162 (hexose unit)]$^-$, 433 (100) [561−128 (6-deoxyhexose unit−H_2O)]$^-$, 271 (62) [433−162]$^-$. *Anal.* Calcd for $C_{66}H_{116}O_{26}$: C, 59.80; H, 8.82. Found: C, 59.98; H, 8.92.

Fraction B: A white powder, mp 108—115 °C, $[\alpha]_D^{25}$ −25.9° (c = 2.7, MeOH). IR (KBr) cm^{-1}: 3400 (OH), 1725 (C=O). Negative FAB-MS m/z (%): 1351 (56) $[M-H]^-$, 1197 (14) [1351−154]$^-$, 1169 (6) [1351−182 (dodecanoic acid unit)]$^-$, 561 (46) [869−146−162]$^-$, 433 (100) [561−128]$^-$, 271 (50) [433−162]$^-$. *Anal.* Calcd for $C_{68}H_{120}O_{26}$: C, 60.34; H, 8.94. Found: C, 60.45; H, 9.01.

Isolation of Operculins IX–XVIII Fraction 5[2] was chromatographed over silica gel (Merck art. 9385, 3.7 cm i.d. × 30 cm, $CHCl_3$: MeOH : H_2O = 10 : 2 : 0.1→8 : 2 : 0.2→7 : 3 : 0.5→MeOH) to give five fractions, fr. 32 (354 mg), fr. 33 (206 mg), fr. 34 (199 mg), fr. 35 (289 mg), fr. 36 (493 mg). Fraction 33, 34, 35 were each subjected to repetitive preparative HPLC on an Inertsil ODS-2 column (GL Sciences, 2.0 cm i.d. × 25 cm, MeOH) to afford fr. 37 (22 mg), operculin XIV (27 mg), operculin XIII (42 mg) and fr. 38 (64 mg) from fr. 33, fr. 39 (72 mg), operculin XV (31 mg) and fr. 40 (81 mg) from fr. 34, and fr. 41 (16 mg), **6** (18 mg), **5** (30 mg), **7** (17 mg) and fr. 42 (28 mg) from fr. 35. Reversed-phase chromatography over Fuji-gel ODS G3 (Fuji Gel Co., 3.6 cm i.d. × 22 cm, 96% MeOH) of fr. 6[2] furnished fr. 43 (10 mg), fr. 44 (224 mg), fr. 45 (385 mg), fr. 46 (865 mg). Preparative HPLC on the Inertsil ODS-2 column (97% MeOH) of fr. 45 yielded fr. 47 (80 mg) and fr. 48 (241 mg). Fraction 48 was subjected repeatedly to preparative HPLC on a Kusano CIG Si gel column (2.2 cm i.d. × 10 cm, $CHCl_3$: MeOH = 20 : 1) to give operculin XI (100 mg) and operculin XII (73 mg). Preparative HPLC on a Hibar RP-8 column (Merck, 2.5 cm i.d. × 25 cm, 92% MeOH) of fr. 13[2] gave operculin XII (28 mg) and fr. 49 (98 mg).

Fraction B (100 mg) was subjected to recycling HPLC (TSK-gel ODS 120T, Tosoh, 2.15 cm i.d. × 30 cm, MeOH, 6 cycles) to afford **3** (13 mg) and **4** (70 mg).

3: IR (KBr) cm^{-1}: 3400 (OH), 1725 (C=O). Negative FAB-MS m/z (%): 1351 (70) $[M-H]^-$, 1169 (20) [1351−182]$^-$, 1051 (6) [1351−146−154]$^-$, 1015 (6) [1169−154]$^-$, 869 (8) [1351−154−146−182]$^-$, 561 (74) [853−2×146]$^-$, 433 (100) [561−128]$^-$, 271 (91) [433−162]$^-$. *Anal.* Calcd for $C_{68}H_{120}O_{26}$: C, 60.34; H, 8.94. Found: C, 60.36: H, 8.95.

4: IR (KBr) cm^{-1}: 3400 (OH), 1725 (C=O). Negative FAB-MS m/z (%): 1351 (66) $[M-H]^-$, 1197 (21) [1351−154]$^-$, 1169 (6) [1351−182]$^-$, 869 (5) [1197−146−182]$^-$, 561 (55) [869−146−162]$^-$, 433 (100) [561−128]$^-$, 271 (58) [433−162]$^-$. *Anal.* Calcd for $C_{68}H_{120}O_{26}$: C, 60.34; H, 8.94. Found: C, 60.34: H, 8.94.

5: IR (KBr) cm^{-1}: 3400 (OH), 1725 (C=O). Negative FAB-MS m/z (%): 1197 (58) $[M-H]^-$, 1051 (5) [1197−146]$^-$, 1015 (25) [1197−182]$^-$, 869 (5) [1015−146]$^-$, 853 (6) [1015−162]$^-$, 561 (24) [853−2×146]$^-$, 433 (100) [561−128]$^-$, 271 (28) [433−162]$^-$. *Anal.* Calcd for $C_{58}H_{102}O_{25}$: C, 58.08; H, 8.57. Found: C, 58.22; H, 8.67.

6: IR (KBr) cm^{-1}: 3400 (OH), 1725 (C=O). Negative FAB-MS m/z (%): 1169 (49) $[M-H]^-$, 1015 (24) [1169−154]$^-$, 853 (7) [1015−162]$^-$, 561 (23) [853−2×146]$^-$, 433 (100) [561−128]$^-$, 271 (28) [433−162]$^-$. *Anal.* Calcd for $C_{56}H_{98}O_{25}$: C, 57.42; H, 8.43. Found: C, 57.38; H, 8.38.

7: IR (KBr) cm^{-1}: 3400 (OH), 1725 (C=O). Negative FAB-MS m/z (%): 1197 (42) $[M-H]^-$, 1015 (8) [1197−182]$^-$, 869 (10) [1015−146]$^-$, 853 (5) [1015−162]$^-$, 561 (10) [869−146−162]$^-$, 433 (100) [561−128]$^-$, 271 (37) [433−162]$^-$. *Anal.* Calcd for $C_{58}H_{102}O_{25}$: C, 58.08; H, 8.57. Found: C, 57.96; H, 8.72.

Operculin XI: A white powder, mp 105—116 °C, $[\alpha]_D^{22}$ −62.7° (c = 0.9,

MeOH).

Operculin XII: A white powder, mp 109—117 °C, $[\alpha]_D^{22}$ −33.0° (c=0.8, MeOH).

Operculin XIII: A white powder, mp 137—140 °C, $[\alpha]_D^{21}$ −28.1° (c=1.0, MeOH).

Operculin XIV: A white powder, mp 133—135 °C, $[\alpha]_D^{21}$ −31.1° (c=1.0, MeOH).

Operculin XV: A white powder, mp 134—140 °C, $[\alpha]_D^{21}$ −32.9° (c=1.0, MeOH).

Alkaline Hydrolysis of 1, 2, 5, 6, 7 and Fraction B Suspensions of **1** (10 mg), **2** (11 mg), **5** (7 mg), **6** (4 mg), **7** (7 mg) and fraction B (11 mg) in 3% KOH (2 ml) were each heated at 95 °C for 1 h. The reaction mixture was adjusted to pH 4 with 1 N HCl, then diluted with H_2O (10 ml), and extracted with ether (3 × 5 ml). The ether layer was washed with H_2O, dried over $MgSO_4$ and then treated with diazomethane in ether. After removal of the solvent the residue was subjected to GC (column, Unisole 3000, 3.2 mm i.d. × 2 m glass column; column temperature, 160 °C; carrier gas, N_2 (1.25 kg/cm^2), t_R (min): 8.35 (methyl *n*-dodecanoate) for **1**, 4.18 (methyl *n*-decanoate) for **2**, 8.36 (methyl *n*-dodecanoate) for **5**, 4.18 (methyl *n*-decanoate) for **6**, 8.38 (methyl *n*-dodecanoate) for **7** and 4.18 (methyl *n*-decanoate), 8.37 (methyl *n*-dodecanoate) for fraction B.

The aqueous layer was desalted by chromatography over MCI-gel CHP 20P to give a white powder (glycosidic acid) (6 mg from **1** (mp 165—169 °C), 7 mg from **2** (mp 166—170 °C), 4 mg from **5** (mp 168—173 °C), 3 mg from **6** (mp 167—175 °C), 4 mg from **7** (168—174 °C), 7 mg from fraction B (167—170 °C). The ^{1}H-NMR spectra of glycosidic acids (in pyridine-d_5, 400 MHz) were each superimposable on that of an authentic sample of operculinic acid B (**8**).[7]

Acetylation of 1, 3, 4 and Fraction B A solution of **1** (17 mg), **3** (8 mg), **4** (10 mg) or fraction B (18 mg) in Ac_2O–pyridine (1 : 1, 2 ml) was left to stand at room temperature overnight. The solvent was removed under an N_2 stream to give a white powder, **9** (22 mg from **1**), **10** (10 mg from **3**), **11** (13 mg from **4**), or BAc (23 mg from fraction B).

9: mp 58—61 °C, $[\alpha]_D^{15}$ −17.2° (c=1.6, MeOH). IR (KBr) cm^{-1}: 1750 (C=O). EI-MS m/z: see Fig. 4.

10: mp 55—58 °C, $[\alpha]_D^{28}$ −17.0° (c=0.7, MeOH). IR (KBr) cm^{-1}: 1750 (C=O). EI-MS m/z: see Fig. 4.

11: mp 59—63 °C, $[\alpha]_D^{23}$ −17.7° (c=0.9, MeOH). IR (KBr) cm^{-1}: 1750 (C=O). EI-MS m/z: see Fig. 4. BAc: mp 59—61 °C, $[\alpha]_D^{15}$ −17.2° (c=1.0, MeOH). IR (KBr) cm^{-1}: 1750 (C=O). EI-MS m/z: see Fig. 4.

BAc: mp 59—61 °C, $[\alpha]_D^{15}$ −17.2° (c=1.0, MeOH). IR (KBr) cm^{-1}: 1750 (C=O), no OH band, EI-MS m/z: see Fig. 4.

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8) Fraction B is identical to compound B which was previously reported.[2]

Synthesis of (+)- and (−)-*cis*-α-Irones

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A stereocontrolled total synthesis of natural (+)-*cis*-α-irone (1) is described. The key intermediate (±)-hemiacetal 15 was prepared from the diene 10 in six steps. The crucial optical resolution of (±)-15 was achieved by initial stereoselective conversion into the corresponding *l*-menthyl acetals 16 and 17 followed by separation and hydrolysis. One of the optically pure enantiomers of 15 was transformed to (−)-18 and then to (+)-1 in seven steps. The synthetic (+)-1 was found to be identical with the natural (+)-*cis*-α-irone. The isomeric (−)-*cis*-α-irone was also synthesized from the other enantiomer of 15, *via* (+)-18.

Keywords *cis*-α-irone; stereocontrolled synthesis; optically active alcohol; optical resolution; diastereomeric acetal; cyclic hemiacetal; phenylselenyl cyanide; tri-*n*-butylphosphine; absolute configuration

Five isomeric irones, (+)-*cis*-α- (**1**), (−)-*trans*-α- (**2**), (+)-β- (**3**), (+)-*cis*-γ- (**4**) and (+)-*trans*-γ-irone (**5**), have been isolated as main constituents of natural iris oil.[1] They have violet-like fragrance and are used as components of perfumes. Many syntheses of these irones have already been reported.[2] However, in most of the previous works, acid-catalyzed cyclization of polyenes has been used for the construction of cyclohexene derivatives which constitute the framework of the irones and thus a mixture of regioisomers with respect to the double bond in the cyclohexene ring is usually produced. In fact, only (±)-*cis*-α- (**1**)[2y] and (±)-*trans*-γ-irone (**5**)[2s,x,v] have been synthesized stereoselectively. As for the optically active isomers, the stereoselective synthesis of (+)-*trans*-γ-irone (**5**) is the only example thus far reported.[2v] We now report the first stereocontrolled total synthesis of (+)-*cis*-α-irone (**1**) and unnatural (−)-*cis*-α-irone (**1**).

In an effort to synthesize the taxane skeleton (*e.g.* taxusin **6**), we have reported the stereoselective synthesis of the cyclohexene derivative **8** starting from the diene **7**.[3] Here, it should be noticed that the substitution pattern of the cyclohexene ring in **8** is closely related to that of **1** and in particular, the configurations of all of these substitutions are the same as those in **1**. Therefore, it is expected that a congener of **8** could be useful as an intermediate for the synthesis of **1**. Moreover, we have observed that **8** gives the corresponding ethyl acetal **9** as a sole product on ethanol/*dl*-10-camphorsulfonic acid (CSA) treatment,[4] which suggests that when appropriate optically active alcohols are used, a mixture of two optically active diastereomeric acetals should be produced stereoselectively. Thus, if it is possible to separate these isomers by simple chromatogrphy, optically active **8** or its congeners will be produced after the subsequent hydrolysis.

Taking this possibility into account, (±)-**15**, a congener

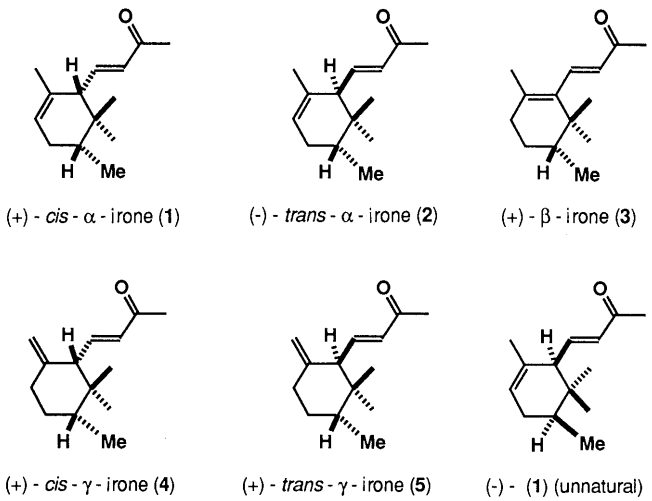

Fig. 1

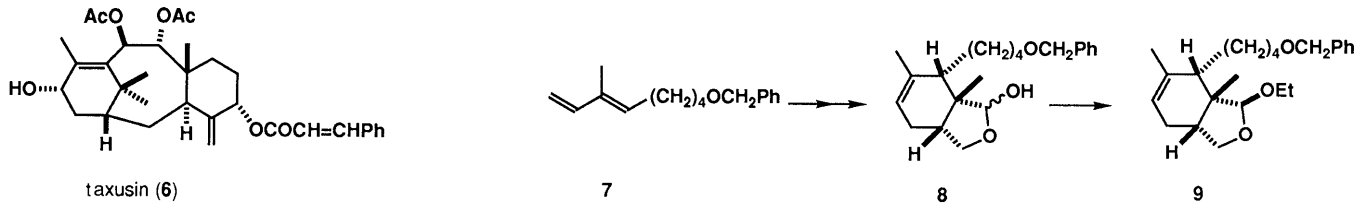

Fig. 2

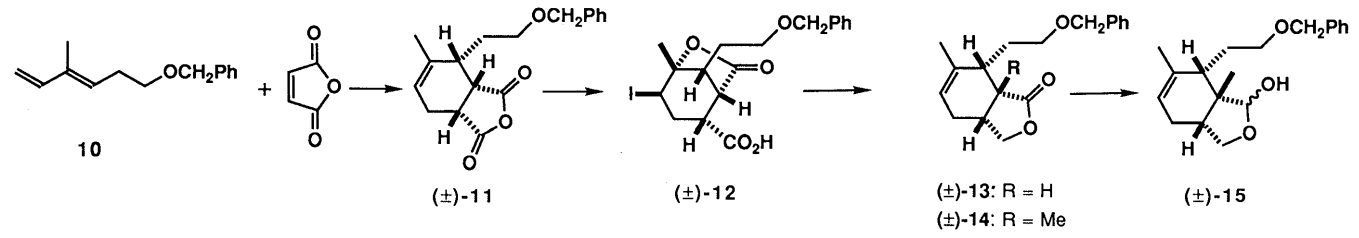

Fig. 3

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of (±)-**8** with the (2-benzyloxy)ethyl group at the C-7α position was prepared and subjected to an optical resolution according to the suggestion described above. The resolution procedure worked extremely well an optically pure (+)- and (−)-**15**, which are required for the synthesis of optically active irones, were obtained with high efficiency.

Maleic anhydride was heated with the diene **10**[5] in benzene to give the anhydride **11** in 97% yield, which was converted into the γ-lactone **13** *via* the iodo lactone **12** in 42% yield through 4 steps (hydrolysis, iodo-lactonization, BH_3 reduction and Zn reduction) in the same way as described in the preceding paper.[3] Methylation of **13** with lithium diisopropylamide (LDA)/MeI afforded **14** exclusively in 82% yield, and this product was reduced with diisobutylaluminum hydride (DIBAH) in toluene at −78 °C, yielding the cyclic hemiacetal (±)-**15** as a stereoisomeric mixture at the C-1 position.

Next, (±)-**15** was converted into the corresponding acetals using optically active alcohols such as borneol, isomenthol and menthol. In all cases, a mixture of two diastereoisomers was obtained stereoselectively out of the four possible isomers, as expected. Among the optically active alcohols tested, *d*- and *l*-menthols were found to give the best results for both preparation of the acetal and separation (SiO_2 chromatography) of the resultant diastereoisomers. Thus, (±)-**15** was treated with *l*-menthol in the presence of CSA in benzene with azeotropic removal of water to give an 1 : 1 mixture of two diastereoisomers, and SiO_2 column chromatography (hexane–AcOEt) afforded **16** as the less polar cyclic hemiacetal and **17** as the more polar one. The less polar hemiacetal **16** was hydrolyzed by boiling in dioxane–water with a small amount of *p*-toluenesulfonic acid (TsOH) and the resultant hemiacetal was treated with hydrazine and NaOH in diethylene glycol (110—210 °C). The alcohol (+)−**18**, $[\alpha]_D^{20}$ +4.3°, was obtained in 90% yield from **16**. In the same way, the polar hemiacetal **17** was also converted into the alcohol (−)-**18**, $[\alpha]_D^{20}$ −4.1°, in 92% yield. The absolute configurations of the chiral **18** thus obtained could not be determined at this stage and so both (+)- and (−)-**18** were used for further synthesis.

The alcohol (−)-**18** was mesylated ($MsCl/Et_3N$, 0 °C) and then reduced with $LiEt_3BH$ (tetrahydrofuran (THF), reflux) to give (−)-**20** in 85% yield. The benzyl group was removed reductively with Li in liquid NH_3 giving the alcohol (−)-**21** in 94% yield. The aldehyde (−)-**22** obtained by pyridinium dichromate (PDC) oxidation of (−)-**21** was treated with phenylselenyl cyanide and tri-*n*-butylphosphine in THF and then oxidized with 15% hydrogen peroxide. The resulting *E*/*Z* mixture (*E*:*Z*=5:2) of the α,β-unsaturated nitriles (−)-**23** was converted into the α,β-unsaturated ketones by MeLi treatment (THF, 0 °C) followed by acid hydrolysis according to the procedure reported by Yoshikoshi and co-workers.[2v] Silical gel chromatography (hexane–AcOEt) of the product afforded the *Z*-α,β-unsaturated ketone, $[\alpha]_D^{22}$ +50.7°, and the *E*-isomer, $[\alpha]_D^{22}$ +111.4°, in 16% and 64% yields, respectively, from (−)-**21**. The spectral data (infrared (IR), proton nuclear magnetic resonance (^{1}H-NMR), gas chromatography-mass spectrometry (GC-MS) including $[\alpha]_D$ of the major product were identical with those of natural (+)-*cis*-α-irone (**1**). The absolute structure of the minor product can thus be shown as (+)-**24**. In the same way, the alcohol (+)-**18** was converted into unnatural (−)-*cis*-α-irone (**1**), $[\alpha]_D^{21}$ −119.2°.

Since the absolute configuration of (+)-**1** is the same as that of the A-ring of taxane-type diterpenes, the chiral alcohol (−)-**18** is expected to be a potent key intermediate for the synthesis of these diterpenes and other related terpenes (*e.g.* pallescensin C (**25**)[6]). Irones are used as a component of perfumes as described before. Among several intermediates prepared herein, the aldehydes (−)- and (+)-**22** and unnatural (−)-*cis*-α-irone (**1**) were also found to have a characteristic aroma.

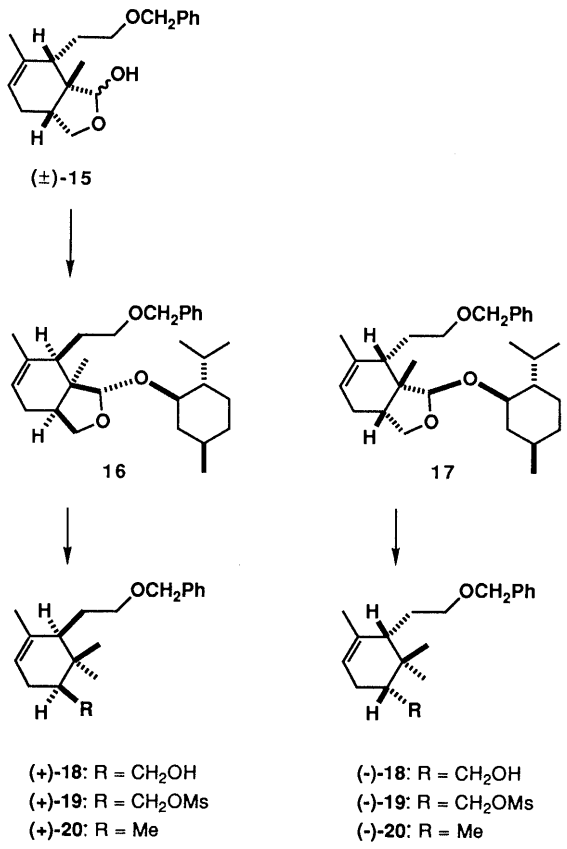

Fig. 4

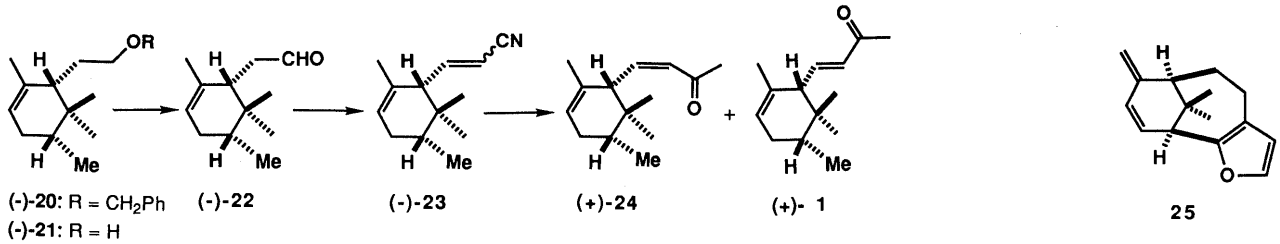

Fig. 5

Experimental

The 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 with a JASCO A-3 spectrometer. Mass spectra (MS) were obtained with a Hitachi RMU-6M mass spectrometer and high resolution MS (HRMS) were recorded on a Hitachi M-80 GC-MS spectrometer. $[\alpha]_D$ was measured on a Perkin-Elmer model 241 MC polarimeter. All reactions were carried out under N_2 or Ar.

(1*R,2*S**,3*R**)-3-(2-Benzyloxy)ethyl-4-methyl-1,2,3,6-tetrahydrophthalic Anhydride (11)** A mixture of 6-benzyloxy-3-methyl-1,3-hexadiene (**10**)[5] (4.14 g, 20.5 mmol), maleic anhydride (2.05 g, 21 mmol) and hydroquinone (10 mg) in benzene (70 ml) was refluxed for 19 h with stirring. After removal of the solvent, the residue was chromatographed on SiO_2 (hexane–AcOEt (5 : 1)) to give (±)-**11** (5.98 g, 97.2% yield) as a colorless oil. IR (CCl_4): 1860, 1770 cm^{-1}. ^{1}H-NMR δ: 1.64—2.91 (5H, m), 1.75 (3H, s), 3.20—3.42 (2H, m), 3.64 (2H, dd, J=4.9, 6.1 Hz), 4.51 (2H, s), 5.62—5.88 (1H, m), 7.32 (5H, s). MS *m/z*: 300 (M^+). HRMS Calcd for $C_{18}H_{20}O_4$ (M^+) *m/z*: 300.136. Found *m/z*: 300.133.

(3a*R,7*S**,7a*S**)-7-(2-Benzyloxy)ethyl-6-methyl-3a,4,7,7a-tetrahydrophthalide (13) *via* the Iodo Lactone 12** A mixture of the anhydride (±)-**11** (5.98 g, 19.9 mmol) and a saturated aqueous $NaHCO_3$ solution (200 ml) was stirred at room temperature for 2 h. An aqueous solution of iodine (5.4 g, 21.2 mmol) and KI (21 g) was added dropwise to the above mixture at room temperature with vigorous stirring and the reaction mixture was stirred for 17 h. Then the mixture was acidified with diluted HCl and extracted with AcOEt. The extract was washed with a 5% aqueous $Na_2S_2O_3$ solution and brine, dried ($MgSO_4$), and evaporated to afford the iodo lactone (±)-**12** (9.12 g) as a crystalline compound which could be used for the next reaction. Recrystallization from Et_2O–pentane gave (±)-**12** (5.90 g, 66.7% yield) as colorless prisms, mp 114—115 °C. IR (Nujol): 1780, 1705 cm^{-1}. ^{1}H-NMR δ: 1.10—2.10 (2H, m), 1.56 (3H, s), 2.30—2.70 (2H, m), 2.80—3.24 (3H, m) 3.56 (2H, s), 4.35—4.60 (1H, m), 4.53 (2H, s), 7.34 (5H, m).

A solution of the iodo lactone (±)-**12** (5.46 g, 12.3 mmol) in THF (20 ml) was added dropwise to a stirred solution of $BH_3 \cdot Me_2S$ complex (1.97 ml, 19.7 mmol) in THF (50 ml) and then $B(OMe)_3$ (2.2 ml, 20 mmol) was added at room temperature. The reaction mixture was stirred at room temperature for 7 h and extracted with $CHCl_3$ after quenching with water at 0 °C. The extract was washed with a saturated aqueous $NaHCO_3$ solution, dried ($MgSO_4$), and concentrated to dryness, affording the crude alcohol (4.58 g) as a gum. A mixture of this alcohol (4.58 g) and Zn dust (12 g) in AcOH (70 ml) was heated at 90 °C for 1 h with stirring. After filtration of the cooled mixture using Celite, the filtrate was concentrated under reduced pressure, diluted with CH_2Cl_2, washed with a saturated aqueous $NaHCO_3$ solution and brine, and dried ($MgSO_4$). Removal of the solvent gave an oil, which was chromatographed on SiO_2(hexane–AcOEt, (5 : 1)) to yield the lactone (±)-**13** (2.22 g, 63% yield) as a colorless oil. IR (neat): 1770 cm^{-1}. ^{1}H-NMR δ: 1.74 (3H, d, J=2.3 Hz), 1.60—3.00 (7H, m), 3.62 (2H, dd, J=5, 6.7 Hz), 4.51 (2H, s), 5.40—5.74 (1H, m), 7.32 (5H, m). MS *m/z*: 286 (M^+). HRMS Calcd for $C_{18}H_{22}O_3$ (M^+) *m/z*: 286.157. Found *m/z*: 286.153.

(3a*R,7*S**,7a*S**)-7-(2-Benzyloxy)ethyl-6,7a-dimethyl-3a,4,7,7a-tetrahydrophthalide (14)** A solution of (±)-**13** (2.22 g, 7.76 mmol) in THF (10 ml) was added dropwise to a stirred THF solution of LDA prepared from diisopropylamine (1.62 ml, 11.6 mmol) and *n*-BuLi (9.31 mmol) in THF (40 ml) at −65 °C. After 30 min, methyl iodide (1 ml, 16 mmol) was added and the mixture was stirred at the same temperature for 4 h. The reaction was quenched with a saturated aqueous NH_4Cl solution. The extract of the reaction mixture with AcOEt was washed with diluted HCl and brine, dried ($MgSO_4$), and concentrated. The resulting crude product was chromatographed on SiO_2 (hexane–AcOEt, (5 : 1)) to afford the lactone (±)-**14** (1.91 g, 82% yield) as a colorless oil. IR (neat): 1770 cm^{-1}. ^{1}H-NMR δ: 1.24 (3H, s), 1.50—2.64 (6H, m), 1.79 (3H, d, J=1.6 Hz), 3.46 (2H, t, J=6.4 Hz), 3.60—4.66 (2H, m), 4.49 (2H, s), 5.34—5.64 (1H, m), 7.31 (5H, s). MS *m/z*: 300 (M^+). HRMS Calcd for $C_{19}H_{24}O_3$ (M^+) *m/z*: 300.172. Found *m/z*: 300.172.

Diastereomeric Mixture of (3a*R,7*S**,7a*S**)-7-(2-Benzyloxy)-ethyl-6,7a-dimethyl-1-hydroxy-3a,4,7,7a-tetrahydro-(1,3*H*)-isobenzofuran (15)** DIBAH (1 M solution of toluene, 7.5 ml) was added dropwise to a stirred solution of (±)-**14** (1.51 g, 5 mmol) in THF (30 ml) at −65 °C and the mixture was stirred for 1 h. The reaction mixture was poured into 1 M tartaric acid aqueous solution and extracted with Et_2O. The extract was washed with a saturated aqueous $NaHCO_3$ solution and brine, and dried ($MgSO_4$). Removal of the solvent followed by SiO_2 column chromatography (hexane–AcOEt (5 : 1)) gave a mixture of the cyclic hemiacetal (±)-**15** (1.17 g, 77.4% yield) as a colorless oil. IR (neat): 3400 cm^{-1}. ^{1}H-NMR δ: 1.09 and 1.13 (3 : 2, 3H, each s), 1.75 (3H, br s), 3.26—4.38 (5H, m), 4.50 and 4.55 (3 : 2, 2H, each s), 4.93—5.27 (1H, m), 5.27—5.55 (1H, m), 7.32 (5H, s).

(1*S*,3a*R*,7*S*,7a*S*)-7-(2-Benzyloxy)ethyl-6,7a-dimethyl-1-*l*-menthoxy-3a,4,7,7a-tetrahydro-(1,3*H*)-isobenzofuran (16) and (1*R*,3a*S*,7*R*,7a*R*)-7-(2-Benzyloxy)ethyl-6,7a-dimethyl-1-*l*-menthoxy-3a,4,7,7a-tetrahydro-(1,3*H*)-isobenzofuran (17) A mixture of the cyclic hemiacetal (±)-**15** (830 mg, 2.75 mmol), *l*-menthol (860 mg, 5.5 mmol), and CSA (10 mg) in benzene (40 ml) was refluxed for 30 min with azeotropic removal of water and then concentrated under reduced pressure. Column chromatography on SiO_2 (benzene) of the residue gave the less polar acetal **16** (540 mg, 44.6% yield), the more polar acetal **17** (250 mg, 20.7% yield) and a mixture of **16** and **17** (250 mg, 20.7 % yield) as a colorless gum. A mixture of hexane and AcOEt (9 : 1) can also be used as the eluent.

The Less Polar Acetal **16**: $[\alpha]_D^{23}$ −166.1° (*c*=1.22, CH_2Cl_2). IR (neat): 2930, 2880, 1455, 1370, 1090 cm^{-1}. ^{1}H-NMR (400 MHz) δ: 0.78 (3H, d, J=6.8 Hz), 0.88 (3H, d, J=7.1 Hz), 0.89 (3H, d, J=6.3 Hz), 1.01 and 1.75 (3H each, s), 3.38—3.52 (4H, m), 4.09 (1H, t, J=8.3 Hz), 4.51 (2H, s), 4.95 (1H, s), 5.40 (1H, br s), 7.35 (5H, m). MS *m/z*: 302 (M^+ −138), 285 (M^+ −155). HRMS Calcd for $C_{19}H_{26}O_3$ ($M^+ - C_{10}H_{18}$) *m/z*: 302.188. Found *m/z*: 302.185.

The More Polar Acetal **17**: $[\alpha]_D^{23}$ +85.6° (*c*=2.30, CH_2Cl_2). IR (neat): 2960, 2940, 2880, 1455, 1370, 1090 cm^{-1}. ^{1}H-NMR (400 MHz) δ: 0.72 and 0.88 (3H each, d, J=7.1 Hz), 0.89 (3H, d, J=6.3 Hz), 1.05, and 1.75 (3H each, s), 3.20—3.26 (1H, m), 3.36—3.53 (3H, m), 4.14 (1H, t, J=8.3 Hz), 4.50 (2H, s), 4.84 (1H, s), 5.41 (1H, br s), 7.34 (5H, m). MS *m/z*: 302 (M^+ −138), 285 (M^+ −155). HRMS Calcd for $C_{19}H_{26}O_3$ ($M^+ - C_{10}H_{18}$) *m/z*: 302.188. Found *m/z*: 302.184.

Conversion of the Hemiacetals 16 and 17 into the Optically Active Alcohol (+)- and (−)-18 (1) Preparation of (4*R*,6*S*)-6-(2-Benzyloxy)ethyl-4-hydroxymethyl-1,5,5-trimethylcyclohexene ((+)-**18**): A mixture of **16** (540 mg, 1.2 mmol), dioxane (20 ml), water (10 ml) and TsOH (10 mg) was refluxed for 1 h. After addition of $NaHCO_3$ (10 mg), the solvents were removed under reduced pressure. The ethereal extract of the residue was dried ($MgSO_4$) and concentrated. Column chromatography on SiO_2 (hexane–AcOEt (1 : 1)) of the resulting oil provided the cyclic hemiacetal **15** (340 mg, 91.6% yield) as a colorless oil.

A mixture of **15** (340 mg, 1.1 mmol,) NaOH (360 mg), and 80% NH_2NH_2 (0.6 ml) in diethylene glycol (5 ml) wa heated at 100 °C (bath temperature) for 35 min. Then the temperature was raised gradually to 210 °C for 1 h with removal of water and maintained at 210 °C for 2 h. After cooling to room temperature, the reaction mixture was diluted with water and extracted with Et_2O. The extract was washed with brine, dried ($MgSO_4$) and concentrated. The crude oil obtained was chromatographed on SiO_2 (hexane–AcOEt (5 : 1)), affording (+)-**18** (280 mg, 90.3% yield) as a colorless oil. $[\alpha]_D^{20}$ +4.3° (*c*=1.60, EtOH). IR (CCl_4): 3630 cm^{-1}. ^{1}H-NMR δ: 0.77 and 0.99 (3H each, s), 1.67 (3H, br s), 3.1—3.7 (3H, m), 3.86 (1H, dd, J=4.1, 10.4 Hz), 4.52 (2H, s), 5.20—5.50 (1H, m), 7.32 (5H, s). MS *m/z*: 170 (M^+ −18). HRMS Calcd for $C_{19}H_{26}O$ ($M^+ - H_2O$) *m/z*: 270.198. Found *m/z*: 270.196.

(2) Preparation of (4*S*,6*R*)-6-(2-Benzyloxy)ethyl-4-hydroxymethyl-1,5,5-trimethylcyclohexene ((−)-**18**): According to the above procedure, **17** (847 mg, 1.93 mmol) was treated with TsOH (16 mg), dioxane (32 ml) and water (16 ml) and subjected to SiO_2 column chromatography, affording the cyclic hemiacetal **15** (545 mg, 93.7% yield) accompanied with the starting **17** (27 mg, 3.2% recovery).

The cyclic hemiacetal **15** (545 mg, 1.81 mmol) was reduced by treatment with 80% NH_2NH_2 (1.2 ml), NaOH (1.75 g) and diethylene glycol (9 ml) and SiO_2 column chromatography of the crude product gave (−)-**18** (477 mg, 91.8% yield) as a colorless oil, whose spectral data were coincident with those of (+)-**18** except for $[\alpha]_D^{20}$ −4.1° (*c*=1.80, EtOH).

Methanesulfonylation of 18 (1) Preparation of (4*S*,6*R*)-6-(2-Benzyloxy) ethyl-4-methanesulfonyloxymethyl-1,5,5-trimethylcyclohexene ((−)-**19**. Methanesulfonyl chloride (0.45 ml) was added dropwise to a stirred solution of (−)-**18** (374 mg, 1.30 mmol) and Et_3N (1 ml) in CH_2Cl_2 (7 ml) at 0 °C. After being stirred for 30 min, the reaction mixture was diluted with CH_2Cl_2, washed with 1 N HCl and brine, and dried ($MgSO_4$). Removal of the solvent followed by SiO_2 chromatography (hexane–AcOEt (5 : 1)) yielded (−)-**19** (468 mg, 91.7% yield) as a colorless oil. $[\alpha]_D^{21}$ −7.7° (*c*=4.96, CH_2Cl_2). IR (CCl_4): 2950, 1365, 1175 cm^{-1}. ^{1}H-NMR δ: 0.80, 1.01 and 2.98 (3H each, s), 1.68 (3H, br s), 3.2—3.75 (2H, m), 4.03 (1H, dd, J=8.4, 9.4 Hz), *ca.* 4.42 (1H, dd, J=3.5, 9.4 Hz), 4.51 (2H, s), 5.15—5.45 (1H, m), 7.32 (5H, s). MS *m/z*: 275 (M^+ −91), 257 (M^+ −109).

(2) Preparation of (4*R*,6*S*)-6-(2-Benzyloxy)ethyl-4-methanesulfonyloxymethyl-1,5,5-trimethylcyclohexene ((+)-**19**): Mesylation of (+)-**18** (461 mg, 1.62 mmol) with methanesulfonyl chloride (0.51 ml) and Et_3N (1.1 ml) in CH_2Cl_2 (8 ml) gave (+)-**19** (561 mg, 94.4% yield), $[\alpha]_D^{20}$ +8.6° (*c*=4.17, CH_2Cl_2).

Reduction of 19 (1) Preparation of (4*R*,6*R*)-6-(2-Benzyloxy)ethyl-1,4,5,5-tetramethylcyclohexene ((−)-**20**): A solution of $LiEt_3BH$ (Super-Hydride) in THF (1 M solution, 2.50 ml) was added to a stirred solution of (−)-**19** (424 mg, 1.16 mmol) in THF (1.2 ml) at room temperature. The mixture was stirred for 1 h at 50 °C and cooled on an ice bath. An excess of MeOH was added carefully to the mixture and the solvent was evaporated off under reduced pressure. The resulting gum was subjected to SiO_2 column chromatography (hexane–AcOEt (19 : 1), affording (−)-**20** (291 mg, 92.4% yield) as a colorless oil. $[\alpha]_D^{22}$ −7.3° (*c*=4.89, CH_2Cl_2). IR (neat): 2960, 2900, 1460, 1370, 1110 cm^{-1}. ^{1}H-NMR δ: 0.65 and 0.91 (3H, each, s), 0.85 (3H, d, *J*=6 Hz), 1.67 (3H, br s), 3.2—3.75 (2H, m), 4.52 (2H, s), 5.2—5.5 (1H, m), 7.32 (5H, s). MS *m/z*: 181 (M^+−91), 164 (M^+ −108). HRMS Calcd for $C_{12}H_{21}O$ ($M^+-C_7H_7$) *m/z*: 181.159. Found *m/z*: 181.157.

(2) Preparation of (4*S*,6*S*)-6-(2-Benzyloxy)ethyl-1,4,5,5-tetramethylcyclohexene ((+)-**20**): Reduction of (+)-**19** (493 mg, 1.36 mmol) with Super-Hydride (1 M, 2.84 ml) in THF (1.3 ml) gave (+)-**20** (346 mg, 94.4% yield) after SiO_2 chromatography, $[\alpha]_D^{20}$ +7.6° (*c*=2.80, CH_2Cl_2).

Reductive Debenzylation of 20 (1) Preparation of (4*R*,6*R*)-6-(2-Hydroxy)ethyl-1,4,5,5-tetramethylcyclohexene ((−)-**21**): Sodium metal (90 mg) was added to a stirred solution of (−)-**20** (278 mg, 1.02 mmol) in THF (1 ml) and liquid NH_3 (5 ml), and the whole mixture was stirred for 1 h. The reaction was quenched by addition of an excess of solid NH_4Cl and NH_3 was allowed to evaporate at room temperature. The residue was extracted with Et_2O. The extract was washed with brine, dried ($MgSO_4$) and concentrated. Column chromatography on SiO_2 (hexane–AcOEt (5 : 1)) of the residue gave (−)-**21** (174 mg, 93.5% yield) as a colorless oil. $[\alpha]_D^{21}$ −18.0 (*c*=3.91, CH_2Cl_2). IR (CCl_4): 3620 cm^{-1}. ^{1}H-NMR δ: 0.66 and 0.92 (3H each, s), 0.86 (3H, d, *J*=5.8 Hz), 1.70 (3H, br s), 3.2—4.0 (2H, m), 5.2—5.5 (1H, m). MS *m/z*: 182 (M^+), 167 (M^+ −15). HRMS Calcd for $C_{12}H_{22}O$ (M^+) *m/z*: 182.167. Found *m/z*: 182.169.

(2) Preparation of (4*S*,6*S*)-6-(2-Hydroxy)ethyl-1,4,5,5-tetramethylcyclohexene ((+)-**21**): From (+)-**20** (314 mg, 1.15 mmol), Na (120 mg), THF (1.5 ml) and liquid NH_3 (7 ml), (+)-**21** (203 mg, 96.7% yield) was obtained. $[\alpha]_D^{21}$ +18.4° (*c*=2.84, CH_2Cl_2).

Oxidation of 21 (1) Preparation of (4*R*,6*R*)-(1,4,5,5-Tetramethyl-1-cyclohexen-6-yl)acetaldehyde ((−)-**22**): A solution of (−)-**21** (168 mg, 0.92 mmol) in CH_2Cl_2 (5 ml) was treated with PDC (890 mg) for 20 h at room temperature. The reaction mixture was diluted with Et_2O, and filtered through Florisil, then the filtrate was concentrated. The resulting oil was subjected to SiO_2 column chromatography (hexane–AcOEt (9 : 1)), affording the aldehyde (−)-**22** (133 mg, 80% yield) as a colorless oil. $[\alpha]_D^{21}$ −21.5° (*c*=1.36, CH_2Cl_2). IR (CCl_4); 2700, 1725 cm^{-1}. ^{1}H-NMR δ: 0.63 and 0.92 (3H each, s), *ca.* 0.89 (3H, d, *J*=*ca.* 6 Hz), 1.55 (3H, br s), 2.49 (2H, d, *J*=1.5 Hz), 5.2—5.5 (1H, m), 9.89 (1H, dd, *J*=1.5, 2.1 Hz). MS *m/z*: 180 (M^+), 165 (M^+ −15). HRMS Calcd for $C_{12}H_{20}O$ (M^+) *m/z*: 180.151. Found *m/z*: 180.154.

(2) Preparation of (4*S*,6*S*)-(1,4,5,5-Tetramethyl-1-cyclohexen-6-yl)acetaldehyde ((+)-**22**): Using PDC (790 mg) and CH_2Cl_2 (6 ml), (+)-**21** (188 mg, 1.03 mmol) was converted into (+)-**22** (137 mg, 74% yield). $[\alpha]_D^{23}$ +23.5° (*c*=2.49, CH_2Cl_2).

Conversion of the Aldehydes 22 into the *E*/*Z* Mixture of α,β-Unsaturated Nitriles 23 *via* Seleno Cyanides (1) Preparation of a Mixture of *E*- and *Z*-Isomers of (4*R*,6*S*)-(1,4,5,5,-Tetramethyl-1-cyclohexen-6-yl)acrylonitrile ((−)-**23**): A solution of tri-*n*-butylphosphine (178 mg, 1.76 eq) in THF (2 ml) was added to a stirred solution of (−)-**22** (90 mg, 0.5 mmol) and phenylselenyl cyanide (160 mg, 1.75 eq) in THF (3 ml) at room temperature. After being stirred for 3 h, the reaction mixture was concentrated under reduced pressure and the residue was purified by SiO_2 column chromatography (hexane–AcOEt (49 : 1)) to give a mixture (5 : 2) of diastereoisomers of the seleno cyanide (126 mg, 72.6% yield) as a pale yellow oil. IR (CCl_4): 2220 cm^{-1}. ^{1}H-NMR δ: 0.56 and 0.87 (3H × 2/7 each, s), 0.65 and 0.93 (3H × 5/7 each, s), 0.85 (3H, d, *J*=6 Hz), 1.68 (3H, br s), 3.5—4.0 (1H, m), 5.25—5.45 (1H, m), 7.1—7.55 (3H, m), 7.55—7.85 (2H, m).

Aqueous hydrogen peroxide (15%, 0.80 ml, *ca.* 10 eq) was added to a stirred solution of a mixture of the seleno cyanide (124 mg, 0.36 mmol) obtained above and pyridine (0.05 ml) in CH_2Cl_2 (2 ml) at room temperature. The reaction mixture was stirred for 2 h and then diluted with Et_2O. The solution was washed with brine, dried ($MgSO_4$) and concentrated. Column chromatography on SiO_2 (hexane–AcOEt (49 : 1)) of the resulting oil afforded a mixture of *E*- and *Z*-isomers (*ca.* 5 : 2 ratio) of (−)-**23** (48 mg, 71.1% yield) as a colorless oil. IR (CCl_4): 2220, 1625 cm^{-1}. ^{1}H-NMR δ: 0.70 (3H, s), *ca.* 0.88 (3H, d, *J*=5.8 Hz), 0.87 (3H × 5/7, s), 0.94 (3H × 2/7, s), 1.54 (3H, d, *J*=1 Hz), 5.45 (5/7H, dd, *J*=0.5, 16.3 Hz), 5.50 (2/7H, d, *J*=11 Hz), 5.3—5.6 (1H, m), 6.54 (5/7H, dd, *J*=10.8, 16.3 Hz), 6.34 (2/7H, q, *J*=11 Hz). MS *m/z*: 189 (M^+).

(2) Preparation of a Mixture of *E*- and *Z*-Isomers of (4*S*,6*R*)-(1,4,5,5-Tetramethyl-1-cyclohexen-6-yl)acrylonitrile ((+)-**23**): From (+)-**22** (85 mg, 0.47 mmol)/THF (3 ml), phenylselenyl cyanide (152 mg, 1.75 eq) and tri-*n*-butylphosphine (137 mg, 1.75 eq)/THF (2 ml), a mixture (5 : 2) of diastereoisomers of the seleno cyanide (123 mg, 75.7% yield) was obtained.

The porduct (118 mg, 0.34 mmol) was converted into (+)-**23** (42 mg, 65% yield) as a mixture (5 : 2) of stereoisomers.

Preparation of Optically Active *cis*-α-Irone (1) and Its *Z*-Isomer (24) from 23 (1) (+)-*cis*-α-Irone (**1**) and the *Z*-Isomer ((+)-**24**): An ethereal solution of MeLi (1.5 M, 0.45 ml) was added to a stirred solution of (−)-**23** (47 mg, 0.25 mmol) obtained above in Et_2O (2.5 ml) at −78 °C. The reaction mixture was gradually warmed to −30 °C during 2 h and then maintained at −5 °C for 30 min. The reaction was quenched with a saturated aqueous NH_4Cl solution at 0 °C. After being stirred for 10 min, the mixture was diluted with Et_2O, dried ($MgSO_4$) and concentrated. The residue was chromatographed on SiO_2 (hexane–AcOEt (40 : 1)) to give successively (+)-**24** (8 mg, 15.6% yield) as a colorless oil and (+)-**1** (33 mg, 64.4% yield) as a colorless oil.

The Less Polar (+)-**24**: $[\alpha]_D^{22}$ +50.7° (*c*=0.80, CH_2Cl_2). IR (CCl_4): 1670, 1615, 1170 cm^{-1}. ^{1}H-NMR δ: 0.69, 0.84 and 2.23 (3H each, s), 0.85 (3H, d, *J*=5.6 Hz), 1.54 (3H, d, *J*=1.5 Hz), 3.8—4.2 (1H, m), 5.3—5.6 (1H, m), 5.90 (1H, t, *J*=11.6 Hz), 6.34 (1H, d, *J*=11.6 Hz). MS *m/z*: 206 (M^+). HRMS Calcd for $C_{14}H_{22}O$ (M^+) *m/z*: 206.167. Found *m/z*: 206.166.

The More Polar (+)-*cis*-α-Irone (**1**): $[\alpha]_D^{22}$ +111.4° (*c*=1.21, CH_2Cl_2). IR (CCl_4): 1670, 1617 cm^{-1}. ^{1}H-NMR δ: 0.71, 0.86 and 2.27 (3H each, s) 0.88 (3H, d, *J*=5.7 Hz), 1.53 (3H, d, *J*=1.3 Hz), 5.4—5.65 (1H, m), 6.12 (1H, d, *J*=15.9 Hz), 6.63 (1H, dd, *J*=10.3, 15.9 Hz). MS *m/z*: 206 (M^+). HRMS Calcd for $C_{14}H_{22}O$ (M^+) *m/z*: 206.167. Found *m/z*: 206.167. These spectral data and *Rf* values of thin-layer chromatography (TLC) analysis were identical with those of natural (+)-**1** including $[\alpha]_D$ value, lit.[1a] $[\alpha]_D^{22}$ +109° (CH_2Cl_2).

(2) (−)-*cis*-α-Irone (**1**) and the *Z*-Isomer ((−)-**24**): Treatment of the mixture of (+)-**23** (39 mg, 0.21 mmol) in Et_2O (2.5 ml) with a solution of MeLi in Et_2O (1.5 M, 0.38 ml) followed by SiO_2 chromatography in the same manner as in the case of (−)-**23** afforded (−)-**24** (10 mg, 23.6% yield) and (−)-**1** (28 mg, 66% yield). The less polar (−)-**24**. $[\alpha]_D^{21}$ −37.7° (*c*=0.83, CH_2Cl_2). The more polar (−)-**1**. IR, ^{1}H-NMR and mass spectra and TLC behavior of the synthetic **1** were identical with those of natural (+)-*cis*-α-irone (**1**) except for $[\alpha]_D^{21}$ −119.2° (*c*=0.25, CH_2Cl_2).

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Synthesis of Methyl Esters of AF-Toxin IIa and IIc, Toxins to Japanese White Pear Produced by *Alternaria alternata* Strawberry Pathotype

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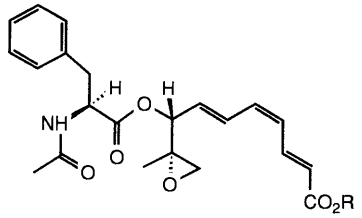
Methyl esters of AF-toxin IIa and IIc, toxic compounds to Japanese white pear produced by *Alternaria alternata* strawberry pathotype, were synthesized as the optically active forms starting from vitamin C as a chiral material.

Keywords AF-toxin; host-specific toxin; plant pathology; isoleucine; Mitsunobu reaction; α-hydroxy-2-methylpentanoic acid; Wadsworth–Emmons reaction; esterification

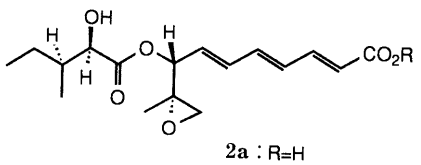
The fungi *Alternaria alternata* strawberry pathotype produce several kinds of compounds named AF-toxin, which are toxic not only to the host plant but also to Japanese white pear. Some of them were isolated in pure forms and the structures were elucidated by Nakatsuka *et al.*[1] AF-Toxins are closely related structurally to AK-toxins[2] in the C-11 trienoic acid moiety. Because of their biological activity and their novel structures, there have been several reports[3-5] concerning the synthesis of these toxins and their esters. We also reported the synthesis of the methyl ester (**1**) of AK-toxin II and pointed out that the stereochemistries of two chiral centers in the C-11 trienoic acid moiety played an important role in the toxicity–structure relationship.[6] As a continuation of our synthetic work on the toxins, we report here the synthesis of the methyl esters (**3** and **2**) of AF-toxin IIa and IIc, which differ from each other in the double bond geometry of the trienoic acid moiety. When we initially examined the synthesis of AF-toxins, the exact stereochemistry of the α-hydroxy-β-methylpentanoic acid moiety in the toxins had not been firmly elucidated.[7] Therefore, preparation of the diastereoisomeric acids (*threo* and *erythro*-forms) in optically active forms was required to complete the synthesis of these toxin methyl esters.

Treatment of isoleucine (**4**) with sodium nitrite in acetic acid gave the α-acetoxy-β-methylpentanoic acid (**5**) as a result of retention of the configuration of an α-amino group.[8] Esterification of the acid (**5**) with benzyl alcohol in benzene in the presence of *p*-toluenesulfonic acid with removal of water afforded a mixture consisting of the α-acetoxy-ester (**6**) and α-hydroxy-ester (**7**), the former of which was easily converted to the latter by hydrolysis with lithium carbonate in methanol. After protection of the hydroxyl group with a *tert*-butyldiphenylsilyl (TBDPS) group, hydrogenation of the resulting ester (**8**) on palladium carbon furnished the acid (**9**) in good yield. Esterification of the acid (**9**) with the oxide-ester (**10**)[6] with dicyclohexylcarbodiimide (DCC) in the presence of 4-pyrrolidinopyridine[9] gave the ester (**11**) in 86% yield without racemization at the α-carbon of the acid (**9**). De-silylation of the ester (**11**) with tetrabutylammonium fluoride (TBAF) gave the hydroxy-ester (**12**) which has the entire carbon framework corresponding to AF-toxin IIc methyl ester. Although the proton nuclear magnetic resonance (^{1}H-NMR, 400 MHz) spectrum of the synthetic compound (**12**) exhibited a close similarity to that of AF-toxin IIc methyl ester reported in the literature,[1] the chemical shifts of the primary (0.88, 3H, t) and secondary methyl (0.99, 3H, d) groups in the synthesized ester (**12**) showed remarkable differences from the methyl ester obtained from natural sources (0.97, 3H, t and 0.87, 3H, d). Thus, we aimed at the synthesis of the diastereoisomeric α-hydroxy-acid (**13**). Treatment of the α-hydroxy-ester (**7**) with ethyl diazodicarboxylate and triphenylphosphine in formic acid[10] gave the formyl ester (**14**). Mild hydrolysis of **14** with lithium carbonate in methanol gave the hydroxy-ester (**15**). Its ^{1}H-NMR spectrum and $[\alpha]_D$ value are different from those of the hydroxy-ester (**7**) mentioned above. The same reaction sequence on the hydroxy-ester (**15**) as for the ester (**7**) gave the acid (**16**) in 68% overall yield. The same reaction sequence (condensation of **10** and **16** followed by the de-silylation reaction) furnished AF-toxin IIc methyl ester (**2b**) in 75% yield. Accomplishment of the synthesis was confirmed by the identity of the ^{1}H-NMR spectral data of the synthetic compound with reported values.[1]

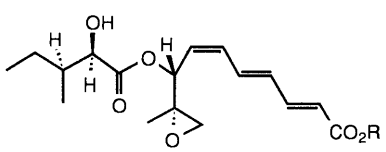
Next, we turned our attention to the synthesis of AF-toxin IIa methyl ester (**3b**), which has a 6,7-*cis* double bond in the trienoic acid moiety and shows strong toxicity to Japanese white pear. An attempt to form the *cis* olefin by Wittig reaction of the aldehyde (**17**)[6] with methyl 4-triphenylphosphonium crotonate and lithium methoxide[6] was unsuccessful, resulting in formation of the *trans–trans* ester (**18**), identical with an authentic sample,[6] in low yield. Then, we planned to form the *cis* double bond by a partial



1a : R=H
1b : R=Me



2a : R=H
2b : R=Me



3a : R=H
3b : R=Me

Chart 1

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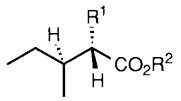
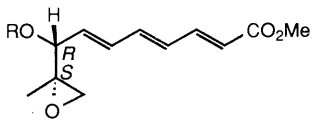
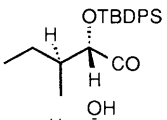


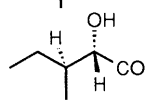
4 : R^1= NH_2; R^2= H
5 : R^1= OAc; R^2= H
6 : R^1= OAc; R^2= CH_2Ph
7 : R^1= OH; R^2= CH_2Ph
8 : R^1= OTBDPS; R^2= CH_2Ph
9 : R^1= OTBDPS; R^2= H



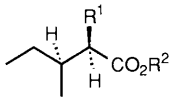
10 : R= H



11 : R=

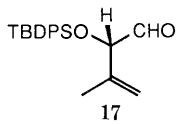


12 : R=



13 : R^1= OTBDPS; R^2=CH_2Ph
14 : R^1= OCHO; R^2=CH_2Ph
15 : R^1= OH; R^2= CH_2Ph
16 : R^1= OTBDPS; R^2= H

Chart 2



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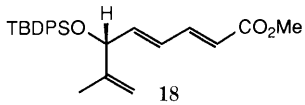

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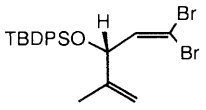

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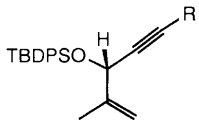
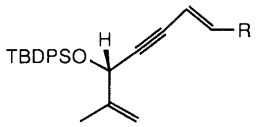
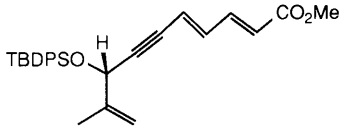


20 : R= H
21 : R= CHO



22 : R= CO_2Me
23 : R= CH_2OH
24 : R= CHO



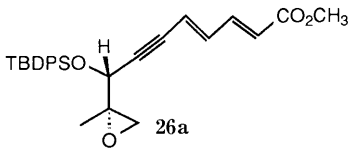
25

Chart 3

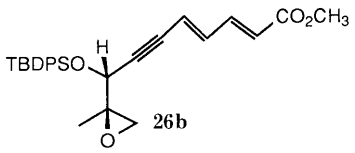
reduction of a triple bond.

Treatment of the aldehyde (**17**)[6] with carbon tetrabromide and triphenylphosphine[11] gave the dibromide (**19**) in 73% yield. The bromide was smoothly transformed to the acetylene (**20**) by treatment with *n*-butyllithium followed by water in 65% yield. Lithiation of the acetylene (**20**) with *n*-butyllithium and treatment of the resulting lithio compound with dimethylformamide (DMF) in tetrahydrofuran (THF) at −78 °C gave the aldehyde (**21**) in 86% yield. Direct treatment of the reaction mixture of the acetylene formation reaction with DMF did not give a good result. The aldehyde (**21**) was subjected to a Wadsworth–Emmons reaction with trimethyl phosphonoacetate, affording the ester (**22**) in 87% yield. The structure of this product was confirmed by its ^{1}H-NMR spectrum, which showed signals at δ 5.97 (1H, J=16 Hz) and 6.63 (1H, dd, J=16 and 1.8 Hz) assigned to two olefinic protons on a newly formed *trans*-double bond. Reduction of **22** with diisobutylaluminum hydride (DIBAL-H) followed by oxidation (MnO_2) gave the aldehyde (**24**) in good yield. Wadsworth–Emmons reaction on the aldehyde (**24**) gave the ester (**25**) having a *trans* diene. Epoxidation of **25** with *m*-chloroperbenzoic acid (*m*CPBA) gave a mixture (revealed by its ^{1}H-NMR spectrum (400 MHz)) of the oxides (**26a** and **26b**), but both oxides showed the same *Rf* values on thin layer chromatography with several solvent systems. An attempt to isolate each oxide in pure form was unsuccessful.

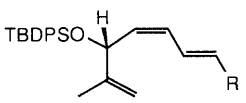
Hydrogenation of **22** with Lindlar catalyst gave the *cis–trans* diene-ester (**27**) in 87% yield. Its ^{1}H-NMR spectrum exhibited two doublet signals at δ 5.91 (t, J=11.7 Hz) and 5.71 (dd, J=11.7 and 8.4 Hz), confirming the *cis* geometry of the newly formed double bond. The same reaction sequence on **27** (DIBAL-H reduction, manganese dioxide oxidation, and Wadsworth–Emmons reaction) gave the *cis–trans–trans*-trienoic acid ester (**29**). The structure of **29** was also confirmed by its ^{1}H-NMR spectrum (7.04 and δ 5.80 (1H each, J=15.4 Hz)). Oxidation of **29** with *m*CPBA gave a mixture of two diastereoisomers (**30a**) and (**30b**) in a 1 : 1 ratio. In this case, both isomers were isolated in pure forms by preparative thin layer chromatography and flash chromatography. Stereostructures of both oxides were proposed on the basis of their ^{1}H-NMR spectra. Thus, one of the isomers showed signals at δ 2.53 and 2.64 (1H each, J=4.6 Hz) and the other showed signals at δ 2.55 and 2.63 (1H each, J=4.9 Hz), respectively, as a pair of AB-quartets assigned to the methylene protons of the oxide moiety. It is possible to



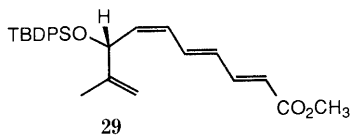
26a



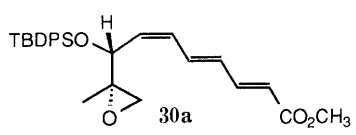
26b



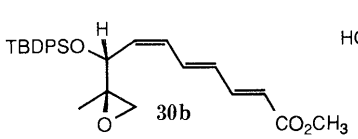
27 : R= CO_2Me
28 : R= CHO



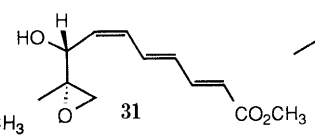
29



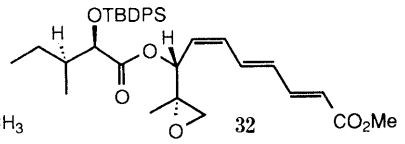
30a



30b



31



32

Chart 4

discriminate the structures of these compounds based on the fact[6] that an oxide exhibiting larger chemical shift difference of the AB-signal has (*R*) and (*S*) configuration at the carbons bearing the silyloxy and the oxide oxygen, respectively. Thus, the former (**30a**) has (*R*) and (*S*) configurations and the other (**30b**) has (*R*) and (*R*) configurations at these carbons. The discrimination was ultimately confirmed by successful synthesis of AF-toxin IIa methyl ester. Treatment of **30a** with TBAF gave the (8*R*)-(9*S*)-hydroxy-*cis*–*trans*–*trans* ester (**31a**) in 45% yield. The acylation of **31** with the α-silyloxy-acid (**16**) in the same manner as mentioned above gave the ester (**32**) in 40% yield. Deprotection of the TBDPS group of **32** with TBAF in methylene chloride afforded AF-toxin IIa methyl ester (**3b**). Success in the synthesis of AF-toxin II methyl ester was confirmed by comparison of the ^{1}H-NMR spectral data with those[1] of AF-toxin II methyl ester obtained from natural sources. The synthetic AF-toxin IIa and IIc are toxic to Japanese white pear, as evaluated on leaves of the plant.

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Shimadzu IR-408 spectrometer in chloroform. ^{1}H-NMR spectra were recorded on JEOL PMX-60, JEOL FX 90Q, and JNM-GX 400 NMR spectrometers with tetramethylsilane as an internal standard and chemical shifts are given in δ (ppm). Optical rotations were measured with a JASCO DIP-181 digital polarimeter and high-resolution mass (HR-MS) spectra were taken with a JEOL JMS-DX303 instrument. Column chromatography was performed with Kieselgel 60G (70—230 mesh) and flash column chromatography was performed with Kieselgel 60G (Art 7731). Homogeneities of the compounds cited in this report were confirmed by examination of the ^{1}H-NMR spectra and by thin layer chromatography.

2(*S*)-Acetoxy-3(*S*)-methylpentanoic Acid (5) $NaNO_2$ (4.8 g, 69.6 mmol) was added in portions to a stirred solution of L-isoleucine (**4**) (7.84 g, 59.8 mmol) in AcOH (72 ml) over 4.5 h at 30—35 °C, and the whole was allowed to stand overnight at room temperature. The solvent was evaporated off *in vacuo* to give a residue, which was shaken vigorously with a mixture of ether (120 ml), water (10 ml) and concentrated HCl (6 ml). After washing of the ethereal layer with water, the ethereal layer was extracted with 10% aqueous Na_2CO_3. The aqueous extracts were combined, and acidified with concentrated HCl, and extracted with ether. The ethereal layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated to dryness to give the acid (**5**) (8.9 g, 85%) as a yellow oil. IR ($CHCl_3$): 1720, 1740 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 0.85 (3H, t, J=7.2 Hz), 1.01 (3H, d, J=7.2 Hz), 1.30—1.62 (3H, m), 2.15 (3H, s), 4.95 (1H, d J=4.8 Hz), 10.3 (1H, s), $[\alpha]_D^{25}=+14.6°$ (c=1.00, EtOH). MS m/z: 174 (M^+).

Benzyl 2(*S*)-Acetoxy-3(*S*)-methylpentanoate (6) and Benzyl 2(*S*)-Hydroxy-3(*S*)-methylpentanoate (7) A mixture of the acid (**5**) (5.0 g, 28.7 mmol), *p*-toluenesulfonic acid (0.5 g) and benzyl alcohol (10.0 g, 92.6 mmol) in dry benzene (60 ml) was refluxed overnight with azeotropic removal of water under argon. The solvent was evaporated off *in vacuo* and the residue was dissolved in ether (100 ml). The ethereal solution was washed with 3% aqueous Na_2CO_3, and water, dried with $MgSO_4$ and concentrated to dryness to give a residue, which was chromatographed on silica gel in hexane–chloroform (1 : 1). Elution with the same solvent gave the benzyl acetoxypentanoate (**6**) (2.25 g, 32%) and the hydroxypentanoate (**7**) (2.40 g, 35.3%) in that order as colorless oils.

Benzyl 2(*S*)-Acetoxy-3(*S*)-methylpentanoate (**6**): IR ($CHCl_3$): 1740 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 0.88 (3H, t, J=7.2 Hz), 0.96 (3H, d, J=7.2 Hz), 1.15—1.90 (3H, m), 2.10 (3H, s), 4.93 (1H, d, J=4.8 Hz), 5.17 (2H, s), 7.33 (5H, br s). $[\alpha]_D^{27}=-27.6°$ (c=1.25, EtOH). HR-MS m/z: Calcd for $C_{15}H_{20}O_4$ (M^+): 264.1362. Found: 264.1341.

Benzyl 2(*S*)-Hydroxy-3(*S*)-methylpentanoate (**7**): IR ($CHCl_3$): 1725, 3540 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 0.86 (3H, t, J=7.2 Hz), 0.98 (3H, d, J=7.2 Hz), 1.15—1.90 (3H, m), 2.72 (1H, d, J=6.2 Hz), 4.12 (1H, dd, J=6.2, 4.8 Hz), 5.20 (2H, s), 7.35 (5H, s). $[\alpha]_D^{27}=-11.8°$ (c=1.00, EtOH). HR-MS m/z: Calcd for $C_{13}H_{18}O_3$ (M^+): 222.1256. Found: 222.1252.

Hydrolysis of the Acetate (6) A mixture of the acetoxycarboxylate **6** (270 mg, 1.0 mmol) and lithium carbonate (45 mg, 1.2 mmol) in methanol (10 ml) was stirred at room temperature for 15 h. The reaction mixture was concentrated *in vacuo* to afford a residue, which was extracted with ether. The ethereal solution was washed with 3% aqueous HCl, 3% aqueous Na_2CO_3 and water, and dried with $MgSO_4$. Removal of the solvent gave **7** (172 mg, 76%).

Benzyl 2(*S*)-(*tert*-Butyldiphenylsiloxy)-3(*S*)-methyl Pentanoate (8) A mixture of 7 (2.9 g, 13 mmol), *tert*-butylchlorodiphenylsilane (4.7 g, 17 mmol) and imidazole (1.2 g, 17 mmol) in dry DMF (20 ml) was stirred at room temperature for 12 h, diluted with 3% aqueous NH_4Cl, and extracted with ether. The ethereal extract was washed with 3% aqueous NH_4Cl, 3% aqueous Na_2CO_3 and water, dried with $MgSO_4$ and concentrated. The residue was chromatographed on silica gel in hexane–acetone (100 : 2) to give the TBDPS-ester (**8**) (4.5 g, 75%) as a colorless oil. IR ($CHCl_3$): 1740 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 0.82 (3H, t, J=6.8 Hz), 0.85 (3H, d, J=6.8 Hz), 1.08 (9H, s), 1.03—1.92 (3H, m), 4.16 (1H, d, J=4.6 Hz), 4.77 (2H, s), 7.20—7.81 (15H, m). $[\alpha]_D^{26}=-44.8°$ (c=1.35, EtOH). MS m/z: 460 (M^+).

2(*S*)-(*tert*-Butyldiphenylsiloxy)-3(*S*)-methylpentanoic Acid (9) A suspension of the TBDPS-ester (**8**) (1.0 g, 2.2 mmol) and 10% palladium carbon (2.0 g) in ethanol (50 ml) was stirred under H_2 for 2 h. The reaction mixture was filtered and the filtrate was concentrated *in vacuo* to afford the acid (**9**) (470 mg, 75%) as a colorless oil. IR ($CHCl_3$): 3520—2510, 1770, 1720 cm^{-1}. ^{1}H-NMR (60 MHz in $CDCl_3$): 0.78 (3H, t, J=6.8 Hz), 0.85 (3H, d, J=6.8 Hz), 1.11 (9H, s), 1.01—1.85 (3H, m), 4.19 (1H, d, J=4.6 Hz), 7.21—7.85 (10H, m). HR-MS m/z: Calcd for $C_{22}H_{30}O_3Si$ (M^+): 370.1965. Found: 370.1977.

Methyl 9(*S*),10-Epoxy-8(*R*)-(2′(*S*)-*tert*-butyldiphenylsiloxy-3′(*S*)-methylpentanoyloxy)-9-methyl-deca-(*E*,*E*,*E*)-trienoate (11) A solution of DCC (332 mg, 1.5 mmol) and the acid (**9**) (596 mg, 1.5 mmol) in methylene chloride (4 ml) was stirred at room temperature under argon for 1.5 h, then the oxide-ester (**10**)[5b] (110 mg, 0.49 mmol) and 4-pyrrolidinopyridine (40 mg) were added and the resulting mixture was stirred at the same temperature for 12 h, diluted with ether (30 ml) and filtered. The filtrate was washed with 3% aqueous Na_2CO_3, 3% aqueous NH_4Cl and brine, dried with $MgSO_4$ and concentrated. The residue was chromatographed on silica gel in hexane–ethyl acetate (100 : 4) to give the ester (**11**) (269 mg, 95%) as a pale yellow oil. IR ($CHCl_3$): 1750, 1720 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 0.79 (3H, t, J=7.2 Hz), 0.93 (3H, d, J=7.2 Hz), 1.09 (9H, s), 1.14 (3H, s), 1.14—1.96 (3H, m), 2.47 (1H, d, J=4.8 Hz), 2.61 (1H, d, J=4.8 Hz), 3.74 (3H, s), 4.23 (1H, d, J=4.2 Hz), 4.99 (1H, d, J=7.3 Hz), 5.58 (1H, dd, J=14.3, 7.3 Hz), 5.81 (1H, d, J=15.4 Hz), 6.05—6.49 (3H, m), 7.14—7.72 (11H, m). $[\alpha]_D^{17}=-67.7°$ (c=0.98, EtOH). HR-MS m/z: Calcd for $C_{34}H_{44}O_6Si$ (M^+): 576.2908. Found: 576.2912.

The Diastereoisomer (12) of AF-Toxin IIc Methyl Ester (2a) TBAF (0.21 ml, 0.2 mmol, 1 M in THF) was added dropwise to a solution of the ester (**11**) (60 mg, 0.1 mmol) in dry THF (2 ml) at −10 °C under argon and the resulting mixture was stirred at the same temperature for 10 min and at room temperature for 1 h, and then diluted with ether (30 ml). The ethereal solution was washed with brine, dried with $MgSO_4$ and concentrated. The residue was chromatographed on silica gel in chloroform to afford the hydroxy-ester (**12**) (24 mg, 68%) as a pale yellow oil. IR ($CHCl_3$): 3540, 1750, 1720 cm^{-1}. ^{1}H-NMR (400 MHz in $CDCl_3$): 0.89 (3H, t, J=7.2 Hz), 0.99 (3H, d, J=7.2 Hz), 1.17—1.36 (2H, m), 1.37 (3H, s), 1.87 (1H, m), 2.64 (1H, d, J=4.8 Hz), 2.79 (1H, d, J=4.8 Hz), 3.76 (3H, s), 4.13 (1H, d, J=4.3 Hz), 5.32 (1H, d, J=7.5 Hz), 5.80 (1H, dd, J=14.5, 7.5 Hz), 5.93 (1H, d, J=15.8 Hz), 6.36 (1H, dd, J=15.1, 11.5 Hz), 6.43 (1H, dd, J=14.5, 10.8 Hz), 6.52 (1H, dd, J=15.1, 10.8 Hz), 7.29 (1H, dd, J=15.8, 11.5 Hz), $[\alpha]_D^{16}=+3.0°$ (c=0.67, EtOH). HR-MS m/z: Calcd for $C_{18}H_{26}O_6$ (M^+): 338.1730. Found: 338.1787.

Benzyl 2(*R*)-Formyloxy-3(*S*)-methylpentanoate (14) Diethylazodicarboxylate (226 mg, 1.3 mmol) in dry ether (2 ml) was added dropwise to a solution of the (*S*)-benzyl ester (**7**) (144 mg, 0.65 mmol), triphenylphosphine (340 mg, 1.3 mmol) and formic acid (60 mg, 1.3 mmol) in dry ether (4 ml) and the resulting mixture was stirred at room temperature for 24 h, diluted with ether (35 ml), and filtered. The filtrate was washed with 3% aqueous $NaHCO_3$, 3% aqueous NH_4Cl and water, dried with $MgSO_4$ and concentrated. The residue was chromatographed on silica gel in hexane–ethyl acetate (100 : 4) to give the formyl-ester (**14**) (166 mg, 72%) as a colorless oil. IR ($CHCl_3$): 1720 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 0.90 (3H, t, J=7.2 Hz), 0.92 (3H, d, J=7.2 Hz), 1.18—1.51 (2H, m), 1.69—2.13 (1H, m), 5.19 (2H, s), 5.24 (1H, d, J=4.3 Hz), 7.34 (5H, s),

8.16 (1H, s). $[\alpha]_D^{22} = +23.3°$ ($c = 1.20$, EtOH). MS m/z: 256 (M^+).

Benzyl 2(*R*)-Hydroxy-3(*S*)-methylpentanoate (15) A mixture of the formyl ester (**14**) (650 mg, 2.6 mmol) and lithium carbonate (260 mg, 3.1 mmol) in methanol–water (14 : 1) was stirred at room temperature for 2 h and concentrated *in vacuo* to give a residue, which was extracted with ether. The ethereal solution was washed with 3% aqueous Na_2CO_3, 3% aqueous NH_4Cl and water, and dried with $MgSO_4$. Removal of the solvent afforded the hydroxy-ester (**15**) (554 mg, 96%) as a colorless oil. IR ($CHCl_3$): 3500, 1715 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 0.78 (3H, d, $J = 7.2$ Hz), 0.92 (3H, t, $J = 7.2$ Hz), 1.00—1.98 (3H, m), 2.67 (1H, d, $J = 5.8$ Hz), 4.22 (1H, dd, $J = 3.2$, 5.8 Hz), 5.21 (2H, s), 7.32 (5H, s). $[\alpha]_D^{23} = +9.0°$ ($c = 1.05$, EtOH). HR-MS m/z: Calcd for $C_{13}H_{18}O_3$ (M^+): 222.1256. Found: 222.1244.

2(*R*)-(*tert*-Butyldiphenylsiloxy)-2(*S*)-methylpentanoic Acid (16) By use of the same procedure as described for the preparation of the acid (**9**) from **7**, the hydroxy-ester (**15**) (256 mg, 1.15 mmol) gave the acid (**16**) (374 mg, 87% overall yield) as a colorless oil. IR ($CHCl_3$): 3520—2510, 1760, 1720 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 0.76 (3H, t, $J = 7.2$ Hz), 0.91 (3H, d, $J = 7.2$ Hz), 1.11 (9H, s), 1.10—1.83 (3H, m), 4.19 (1H, d, $J = 4.1$ Hz), 7.24—7.73 (10H, m). $[\alpha]_D^{22} = +18.5°$ ($c = 1.01$, EtOH). HR-MS m/z: Calcd for $C_{22}H_{30}O_3Si$ (M^+): 370.1965. Found 370.1952.

AF-Toxin IIc Methyl Ester (2a) By use of the procedure as described for the preparation of **12**, coupling of the epoxy-ester (**10**) (123 mg, 0.33 mmol) and **16** (150 mg, 0.40 mmol) gave AF-toxin IIc methyl ester (**2a**) (72 mg, 65% overall yield) as a colorless oil. IR ($CHCl_3$): 1620, 1720, 1720 cm^{-1}. ^{1}H-NMR (400 MHz in $CDCl_3$): 0.86 (3H, d, $J = 6.9$ Hz), 0.98 (3H, t, $J = 7.4$ Hz), 1.36 (3H, s), 1.36—1.40 (2H, m), 1.83 (1H, m), 2.61 (1H, d, $J = 5.8$ Hz), 2.63 (1H, d, $J = 4.8$ Hz), 2.78 (1H, d, $J = 4.8$ Hz), 3.75 (3H, s), 4.23 (1H, dd, $J = 2.9$, 5.8 Hz), 5.35 (1H, d, $J = 7.3$ Hz), 5.80 (1H, dd, $J = 7.3$, 14.8 Hz), 5.94 (1H, d, $J = 15.3$ Hz), 6.38 (1H, dd, $J = 11.1$, 14.5 Hz), 6.43 (1H, dd, $J = 10.8$, 14.8 Hz), 6.53 (1H, d, $J = 14.5$, 10.8 Hz), 7.29 (1H, dd, $J = 11.1$, 15.3 Hz). HR-MS m/z: Calcd for $C_{18}H_{26}O_6$ (M^+): 338.1730. Found: 338.1787.

1,1-Dibromo-3(*R*)-*tert*-butyldiphenylsiloxy-4-methylpenta-1,4-diene (19) A solution of triphenylphosphine (1.48 g, 4 mmol) in anhydrous methylene chloride (3 ml) was added dropwise to a solution of carbon tetrabromide (633 mg, 2.0 mmol) in anhydrous methylene chloride (6 ml) under argon at 0 °C. The mixture was stirred at the same temperature for 5 min, then zinc powder (262 mg, 4 mmol) and the aldehyde (**17**)[6] (388 mg, 1 mmol) were added alternately and the resulting mixture was stirred at room temperature for 3 h and diluted with ether (50 ml). The ethereal solution was washed with water, dried with $MgSO_4$ and concentrated to give a residue, which was chromatographed on silica gel in chloroform. Elution with the same solvent afforded the dibromide (**19**) (360 mg, 73%). IR ($CHCl_3$): 1625 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 1.09 (9H, s), 1.72 (3H, s), 4.67 (1H, d, $J = 9.0$ Hz), 4.95 (1H, br s), 5.18 (1H, br s), 6.40 (1H, d, $J = 9.0$ Hz), 7.26—7.71 (10H, m). $[\alpha]_D^{21} = -80.6°$ ($c = 1.01$, EtOH). MS m/z: 494 (M^+).

3(*R*)-*tert*-Butyldiphenylsiloxy-4-methylpent-4-en-1-yne (20) *n*-Butyllithium (1 ml, 1.7 M in hexane) was added to a solution of the dibromide (**19**) (350 mg, 0.7 mmol) in dry THF (3 ml) at −78 °C under argon and the resulting mixture was stirred at the same temperature for 1 h, and at 25 °C for 1 h. After addition of water (2 ml), the reaction mixture was stirred at room temperature for 30 min and diluted with ether (50 ml). The ethereal solution was washed with water, dried with $MgSO_4$, and concentrated *in vacuo* to leave a residue, which was chromatographed on silica gel in chloroform. Elution with the same solvent gave the acetylene (**20**) (154 mg, 65%) as a colorless oil. IR ($CHCl_3$): 3290 cm^{-1}, ^{1}H-NMR (90 MHz in $CDCl_3$): 1.09 (9H, s), 1.85 (3H, br s), 2.35 (1H, d, $J = 2.2$ Hz), 4.37 (1H, m), 4.82 (1H, br s), 4.94 (1H, br s), 7.24—7.82 (10H, m). $[\alpha]_D^{24} = -47.3°$ ($c = 1.00$, EtOH). MS m/z: 334 (M^+).

4(*R*)-*tert*-Butyldiphenylsiloxy-5-methyl-hex-5-en-1-ynal (21) *n*-Butyllithium (1 ml, 2.3 mmol) was added dropwise to a solution of the acetylene (**20**) (480 mg, 1.48 mmol) in dry THF (5 ml) at −78 °C under argon, and the mixture was stirred at the same temperature for 2 h. Then DMF (0.1 ml) was added and the resulting mixture was stirred at −78 °C for 4 h and diluted with ether (30 ml). The ethereal solution was washed with water, dried with anhydrous $MgSO_4$ and concentrated *in vacuo* to give a residue, which was chromatographed on silica gel in hexane–ether (100 : 5). Elution with the same solvent afforded the aldehyde (**21**) (450 mg, 86%) as a colorless oil. IR ($CHCl_3$): 2200, 1665 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 1.09 (9H, s), 1.84 (3H, br s), 4.88 (1H, br s), 4.91 (1H, br s), 5.04 (1H, br s), 7.31—7.78 (10H, m), 9.30 (1H, d, $J = 0.7$ Hz). MS m/z: 326 (M^+).

Wadsworth–Emmons Reaction of the Aldehyde (21) A solution of trimethylphosphonoacetate (98 mg, 0.54 mmol) in dry benzene (2 ml) was added dropwise to a suspension of sodium hydride (13 mg, 0.45 mmol, 60% in oil) in benzene (5 ml) at room temperature and the resulting mixture was stirred at room temperature under argon for 30 min. Then a solution of the aldehyde (**21**) (130 mg, 0.36 mmol) in dry benzene (2 ml) was added and the whole was stirred at room temperature for 30 min, and diluted with ether (30 ml). The ethereal solution was washed with 3% aqueous $NaHCO_3$, 3% aqueous NH_4Cl and water, dried with $MgSO_4$ and concentrated to give a residue, which was chromatographed on silica gel in hexane–ether (100 : 2). Elution with the same solvent afforded the diene-acetylene (**22**) (130 mg, 87%) as a colorless oil. IR ($CHCl_3$): 1710 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 1.08 (9H, s), 1.84 (3H, br s), 3.73 (3H, s), 4.87 (2H, br s), 5.02 (1H, m), 5.97 (1H, d, $J = 15.8$ Hz), 6.63 (1H, dd, $J = 15.8$, 1.8 Hz), 7.24—7.79 (10H, m). $[\alpha]_D^{24} = -95.0°$ ($c = 1.30$, EtOH). HR-MS m/z: Calcd for $C_{26}H_{30}O_3Si$ (M^+): 418.1965. Found: 418.1942.

6(*R*)-*tert*-Butyldiphenylsiloxy-7-methyl-octa-2,7-dien-4-yn-1-ol (23) DIBAL-H (0.2 ml, 0.36 mmol, 25% (w/w) in *n*-hexane) was added to a solution of the ester (**22**) (100 mg, 0.24 mmol) in anhydrous methylene chloride (2 ml) at −78 °C under argon. The resulting mixture was stirred at the same temperature for 1 h. After addition of brine and chloroform (50 ml) to the mixture, the whole was filtered. The chloroform solution was washed with brine, dried with $MgSO_4$ and concentrated to give a residue, which was chromatographed on silica gel in benzene. The benzene eluate afforded the alcohol (**23**) (84 mg, 90%). IR ($CHCl_3$): 3600, 2255 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 1.08 (9H, s), 1.84 (3H, s), 4.14 (2H, dd, $J = 4.9$, 1.5 Hz), 4.83 (2H, br s), 4.98 (1H, br s), 5.60 (1H, dd, $J = 15.9$ Hz, 1.5 Hz), 6.04 (1H, dt, $J = 15.9$, 4.9 Hz), 7.27—7.28 (10H, m). $[\alpha]_D^{23} = -123°$ ($c = 1.01$, EtOH). MS m/z: 390 (M^+).

Oxidation of the Allyl Alcohol (23) A mixture of the allyl alcohol (**23**) (260 mg, 0.67 mmol) and active MnO_2 (1.5 g, 17 mmol) in methylene chloride (10 ml) was stirred at room temperature for 30 min. The reaction mixture was filtered and the filtrate was concentrated to afford the aldehyde (**24**) (217 mg, 87%) as a pale yellow oil. IR ($CHCl_3$): 2220, 1680 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 1.09 (9H, s), 1.85 (3H, s), 4.91 (2H, br s), 5.05 (1H, br s), 6.15 (1H, dd, $J = 15.8$, 7.1 Hz), 6.46 (1H, d, $J = 15.8$ Hz), 7.24—7.80 (10H, m), 9.48 (1H, d, $J = 7.1$ Hz). MS m/z: 388 (M^+).

Methyl 8(*R*)-*tert*-Butyldiphenylsiloxy-9-methyl-deca-2(*E*),4(*E*),9-trien-6-ynoate (25) By use of the procedure as described for the preparation of the ester (**22**), the aldehyde (**24**) (200 mg, 0.52 mmol) gave the ester (**25**) (192 mg, 84%) as a colorless oil. IR ($CHCl_3$): 2400, 1710 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 1.08 (9H, s), 1.85 (3H, br s), 3.75 (3H, s), 4.86 (2H, br s), 5.03 (1H, br s), 5.78 (1H, d, $J = 15.4$ Hz), 5.92 (1H, d, $J = 15.2$ Hz), 6.33 (1H, dd, $J = 15.4$, 11.1 Hz), 7.20 (1H, dd, $J = 15.2$, 11.1 Hz), 7.26—7.81 (10H, m). $[\alpha]_D^{26} = -198.0°$ ($c = 1.02$, EtOH). HR-MS m/z: Calcd for $C_{28}H_{32}O_3Si$ (M^+): 444.2122. Found: 444.2128.

Methyl 6(*R*)-*tert*-Butyldiphenylsiloxy-7-methyl-oct-2(*E*),4(*E*),7-trienoate (27) A mixture of quinoline (30 mg, 0.24 mmol), Lindlar catalyst (30 mg) and the methyl ester (**22**) (100 mg, 0.24 mmol) in ethyl acetate (5 ml) was stirred at 0 °C under H_2 for 2 d and filtered. The filtrate was concentrated to give a residue, which was submitted to flash chromatography in hexane–ether (9 : 1). Elution with the same solvent afforded the triene ester (**27**) (87 mg, 87%) as a colorless oil. IR ($CHCl_3$): 1705, 1640 cm^{-1}. ^{1}H-NMR (400 MHz in $CDCl_3$): 1.07 (9H, s), 1.65 (3H, s), 3.70 (3H, s), 4.83 (1H, br s), 4.95 (1H, d, $J = 8.4$ Hz), 5.09 (1H, br s), 5.69 (1H, d, $J = 15.4$ Hz), 5.71 (1H, dd, $J = 11.7$, 8.4 Hz), 5.91 (1H, dd, $J = 11.7$, 11.7 Hz), 7.04 (1H, dd, $J = 15.4$, 11.7 Hz), 7.29—7.66 (10H, m). $[\alpha]_D^{17} = +30.1°$ ($c = 1.01$, EtOH). HR-MS m/z: Calcd for $C_{26}H_{32}O_3Si$ (M^+): 420.2122. Found 420.2132.

6(*R*)-*tert*-Butyldiphenylsiloxy-7-methyl-oct-2(*E*),4(*E*),7-trienal (28) By use of the procedure described for the preparation of **24** from **22**, the *cis–trans*-diene ester (**27**) (100 mg, 0.24 mmol) gave the aldehyde (**28**) (79 mg, 85% overall yield) as a colorless oil. IR ($CHCl_3$): 1675, 1630 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 1.07 (9H, s), 1.68 (3H, s), 4.89 (1H, br s), 4.91 (1H, d, $J = 10.8$ Hz), 5.12 (1H, br s), 5.71—6.16 (2H, m), 6.74 (1H, dd, $J = 10.8$, 15.1 Hz), 7.24—7.71 (11H, m), 9.22 (1H, d, $J = 7.9$ Hz). MS m/z: 390 (M^+).

Wadsworth–Emmons Reaction of the Aldehyde (28) By use of the method described for the preparation of **22**, the aldehyde (**28**) (300 mg, 0.77 mmol) gave the *cis–trans–trans* ester (**29**) (295 mg, 86%) as a colorless oil. IR ($CHCl_3$): 1700, 1620 cm^{-1}. ^{1}H-NMR (400 MHz in $CDCl_3$): 1.06 (9H, s), 1.65 (3H, s), 3.73 (3H, s), 4.82 (1H, br s), 4.88 (1H, d, $J = 8.8$ Hz), 5.07 (1H, br s), 5.59 (1H, dd, $J = 8.8$, 11.0 Hz), 5.80 (1H, d, $J = 15.4$ Hz), 5.90 (1H, dd, $J = 11.0$, 11.0 Hz), 6.10 (1H, dd, $J = 15.7$, 10.6 Hz), 6.17 (1H, dd, $J = 15.7$, 11.0 Hz), 7.04 (1H, dd, $J = 15.4$, 10.6 Hz). $[\alpha]_D^{17} = +5.5°$

($c=0.80$, EtOH). HR-MS m/z: Calcd for $C_{28}H_{34}O_3Si$ (M^+): 446.2278. Found: 446.2262.

Epoxidation of the Ester (29) A mixture of the ester (**29**) (320 mg, 0.72 mmol) and *m*CPBA (186 mg, 1.1 mmol) in methylene chloride (8 ml) was allowed to stand overnight at room temperature in the dark, and then 10% aqueous $NaHSO_3$ and chloroform was added. The chloroform solution was washed with 3% aqueous Na_2CO_3, 3% aqueous NH_4Cl and water, and dried with $MgSO_4$. Removal of the solvent gave a residue, which was submitted to flash chromatography in ether–hexane (1 : 9). Elution with the same solvent afforded the epoxide (**30a**) (200 mg, 60%) as the faster-running portion and the epoxide (**30b**) (130 mg, 39%) as the slower-running one.

Methyl 8(*R*)-*tert*-Butyldiphenylsiloxy-9(*S*),10-epoxy-9-methyl-deca-2(*E*),4(*E*),6(*Z*)-trienoate (**30a**): IR ($CHCl_3$): 1705, 1620 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 1.07 (9H, s), 1.39 (3H, s), 2.53 (1H, d, $J=4.6$ Hz), 2.64 (1H, d, $J=4.6$ Hz), 3.75 (3H, s), 4.26 (1H, d, $J=8.8$ Hz), 5.60—6.09 (5H, m), 6.97 (1H, dd, $J=10.2$, 15.2 Hz), 7.25—7.73 (10H, m). $[\alpha]_D^{15}=+16.1°$ ($c=1.60$, EtOH). HR-MS m/z: Calcd for $C_{28}H_{34}O_4Si$ (M^+): 462.2227. Found: 462.2202.

Methyl 8(*R*)-*tert*-Butyldiphenylsiloxy-9(*R*),10-epoxy-9-methyl-deca-2(*E*),4(*E*),6(*Z*)-trienoate (**30b**): IR ($CHCl_3$): 1705, 1620 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 1.10 (9H, s), 1.36 (3H, s), 2.55 (1H, d, $J=4.9$ Hz), 2.63 (1H, d, $J=4.9$ Hz), 3.73 (3H, s), 3.90 (1H, d, $J=5.5$ Hz), 5.60—6.35 (5H, m), 7.10—7.74 (11H, m). $[\alpha]_D^{15}=-28.9°$ ($c=1.26$, EtOH). HR-MS m/z: Calcd for $C_{28}H_{34}O_4Si$ (M^+): 462.2227. Found: 462.2206.

Methyl 9(*S*),10-Epoxy-8(*R*)-Hydroxy-9-methyl-deca-2(*E*),4(*E*),6(*Z*)-trienoate (31) TBAF (3.12 ml, 3 mmol, 1 M in THF) was added dropwise to a solution of **30a** (200 mg, 0.45 mmol) in dry THF (5 ml) at -10 °C, and the resulting mixture was stirred at room temperature for 1 h then diluted with ether (50 ml). The ethereal solution was washed with brine, dried with $MgSO_4$ and concentrated to give a residue, which was chromatographed on silica gel in hexane–chloroform (1 : 4). Elution with the same solvent gave the alcohol (**31**) (117 mg, 68%) as a colorless oil. IR ($CHCl_3$): 3630, 1700, 1620 cm^{-1}. ^{1}H-NMR (90 MHz in $CDCl_3$): 1.33 (3H, s), 2.30 (1H, br s), 2.62 (1H, d, $J=4.6$ Hz), 2.96 (1H, d, $J=4.6$ Hz), 3.75 (3H, s), 4.64 (1H, d, $J=8.9$ Hz), 5.40—6.93 (6H, m). MS m/z: 224 (M^+).

Acylation of 31 with the Acid (16) By use of the method described for the preparation of **11**, the *cis–trans–trans* hydroxy-ester (**31**) (25 mg, 0.11 mmol) gave the ester (**32**) (36 mg, 40%) as an oil. IR ($CHCl_3$): 1745, 1705, 1620 cm^{-1}. ^{1}H-NMR (400 MHz in $CDCl_3$): 0.81 (3H, t, $J=7.3$ Hz), 0.94 (3H, d, $J=7.0$Hz), 1.08 (9H, s), 1.22 (3H, s), 1.45—1.75 (3H, m), 2.50 (1H, d, $J=4.8$ Hz), 2.63 (1H, d, $J=4.8$ Hz), 3.77 (3H, s), 4.22 (1H, d, $J=3.3$ Hz), 5.26 (1H, dd, $J=9.3$, 10.6 Hz), 5.47 (1H, d, $J=9.3$ Hz), 5.93 (1H, d, $J=15.4$ Hz), 6.17 (1H, dd, $J=10.6$, 11.3 Hz), 6.35 (1H, dd, $J=14.6$, 11.4 Hz), 6.79 (1H, dd, $J=14.6$, 11.3 Hz), 7.28—7.65 (11H, m). HR-MS m/z: Calcd for $C_{34}H_{44}O_6Si$ (M^+): 576.2908. Found: 576.2898.

AF-Toxin IIa Methyl Ester (3b) By use of the procedure described for the preparation of **12**, the ester (**32**) (24 mg, 0.041 mmol) gave AF-toxin IIa methyl ester (**3b**) (9 mg, 64%) as an oil. IR ($CHCl_3$): 3550, 1735 cm^{-1}. ^{1}H-NMR (400 MHz in $CDCl_3$): 0.87 (3H, d, $J=7.0$ Hz), 0.97 (3H, t, $J=7.3$ Hz), 1.36—1.40 (2H, m), 1.38 (3H, s), 1.83 (1H, m), 2.59 (1H, d, $J=5.7$ Hz), 2.62 (1H, d, $J=4.8$ Hz), 2.78 (1H, d, $J=4.8$ Hz), 3.76 (3H, s), 4.19 (1H, dd, $J=2.7$, 5.7 Hz), 5.52 (1H, dd, $J=9.2$, 10.6 Hz), 5.81 (1H, d, $J=9.2$ Hz), 5.95 (1H, d, $J=15.4$ Hz), 6.33 (1H, dd, $J=10.6$, 11.4 Hz), 6.41 (1H, dd, $J=11.4$, 15.0 Hz), 6.93 (1H, dd, $J=11.4$, 15.0 Hz), 7.37 (1H, dd, $J=11.4$, 15.4 Hz), HR-MS m/z: Calcd for $C_{18}H_{26}O_6$ (M^+): 338.1730. Found: 338.1761.

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Novel Synthesis of Three Types of C-Terminal Components of Renin Inhibitors from Unnatural (2*S*,3*S*)-Tartaric Acid[1)]

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The addition reaction of cyclohexylmethylmagnesium bromide with the imine prepared from unnatural (2*S*,3*S*)-tartaric acid was found to proceed in a highly stereoselective manner in the presence of cerium(III) chloride. A chelation-controlled mechanism could explain the stereochemical outcome of the addition reaction. The addition product could be elaborated into three types of C-terminal components of renin inhibitors by employing oxidative cleavage of the 1,2-diol moiety, epoxide formation with inversion of configuration, and epoxide opening with a nucleophile.

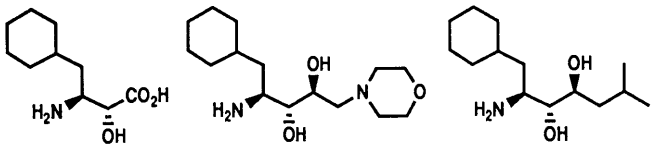
Keywords renin inhibitor; C-terminal component; 1,2-amino alcohol; 1,2,3-amino diol; Grignard reagent; cerium(III) chloride; chelation control; oxidative cleavege; epoxide formation; epoxide opening

Renin is a highly specific aspartic acid protease which cleaves a decapeptide fragment from angiotensinogen to generate angiotensin I.[2)] While no biological activity is observed for angiotensin I, the octapeptide angiotensin II produced from angiotensin I by angiotensin-converting enzyme shows a potent vasoconstricting activity and stimulates the release of aldosterone. Thus, renin inhibitors are currently the object of intensive research aimed at development of novel antihypertensive drugs.[2)]

Some of the renin inhibitors recently developed as promising antihypertensive agents with oral efficacy involve either (2*R*,3*S*)-3-amino-2-hydroxy-4-cyclohexylbutyric acid [(2*R*,3*S*)-cyclohexylnorstatine] (**1**),[3)] (2*S*,3*R*,4*S*)-4-amino-5-cyclohexyl-1-morpholino-2,3-pentanediol (**2**),[4)] or (2*S*,3*R*,4*S*)-2-amino-1-cyclohexyl-6-methyl-3,4-heptanediol (**3**)[5)] as their C-terminal component. These unusual amino alcohols (**1**, **2**, and **3**) were originally synthesized from (*S*)-phenylalanine[3,5)] or D-glucose,[4)] and ingeniously incorporated into renin inhibitors as isosteres of the Leu–Val scissile site in angiotensinogen. Recently, several novel synthetic routes to **1** from (*S*)-phenylalanine[6)] or (*R*)-mandelic acid[7)] and to **2** and **3** from optically active epoxy alcohols[8,9)] have also been reported.

With the aim of preparing **1**, **2**, and **3** from readily available unnatural (2*S*,3*S*)-tartaric acid (**4**), the novel synthetic route depicted in Chart 1 was designed. Thus, the amino alcohols (**1**, **2**, and **3**) could be derived from the amine (**6**) by manipulating its terminal protected diol moiety. Addition of cyclohexylmethylmagnesium bromide with the imine (**5**) would afford **6** if the addition reaction proceeds with the desired high stereoselectivity. Preparation of **5** might be readily achieved from **4** according to the reported procedures.[7,10,11)] After many unsuccessful attempts,[12)] we found that the addition reaction of cyclohexylmethylmagnesium bromide with **5** took place in a highly stereoselective manner in the presence of cerium(III) chloride, yielding **6** as a sole product. The addition product (**6**) could be elaborated to **1**, **2**, and **3** by sequential chemical manipulations.

This report details highly stereoselective syntheses of **1**, **2**, and **3** from **4** accomplished by featuring the novel addition reaction of a Grignard reagent with an imine in the presence of cerium(III) chloride.[13)]

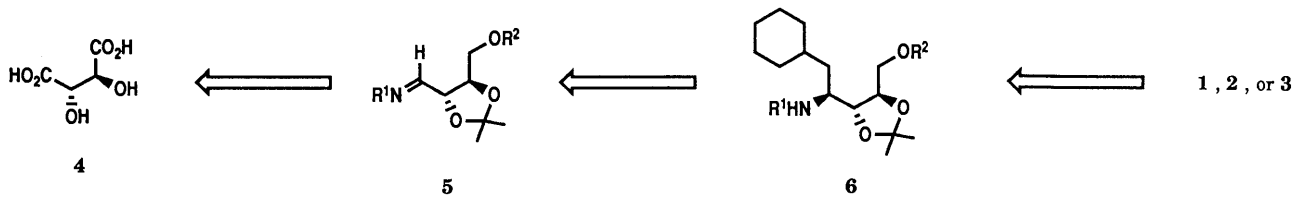


1 **2** **3**

Fig. 1

Results and Discussion

Synthesis of (2*R*,3*S*)-Cyclohexylnorstatine (1) 4-*O*-Benzyl-2,3-isopropylidene-D-threose (**7**) was prepared from **4** according to the reported procedure.[10)] Condensation of **7** with benzylamine ($BnNH_2$) in the presence of anhydrous magnesium sulfate readily afforded the imine (**8**) in a quantitative yield.[7,11)] Initially, the imine (**8**) was allowed to react with cyclohexylmethylmagnesium bromide in various solvents. However, contrary to our expectation, no addition product could be produced and complete recovery of **8** was always observed. After these unsuccessful experiments, it was found, as shown in Chart 2, that when cyclohexylmagnesium bromide was first treated with cerium(III) chloride in a mixture of ether and tetrahydrofuran and the resulting cerium(III) complex was reacted with **8**, the addition reaction could proceed smoothly in a highly stereoselective manner, giving rise to the amine



4 **5** **6** **1**, **2**, or **3**

Chart 1

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(**9**), $[\alpha]_D^{20}$ +12.3° (chloroform), as a sole product in 75% yield (*vide infra*).[13]

Since stereoselective addition reaction of an organocopper(I) reagent with an imine had been reported,[15] the reaction of **8** with cyclohexylmethylcopper(I) was next examined. Interestingly, treatment of **8** with cyclohexylmethylcopper(I) in the presence of boron trifluoride-etherate in a mixture of ether and tetrahydrofuran according to the reported procedure[15] was found to give the undesired diastereomer (**13**), $[\alpha]_D^{20}$ +26.7° (chloroform), as a sole product in 52% yield (*vide infra*). The organocopper(I) reagent could be produced *in situ* from cyclohexylmethylmagnesium bromide and copper(I) iodide at −78 °C prior to the addition reaction.

Comparison of the 400 MHz proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **9** with that of **13** established that each addition product (**9** or **13**) was not contaminated with the other diastereomer (**13** or **9**). Taking into account of the accuracy of 400 MHz ^{1}H-NMR spectra, the stereoselectivities for the two sorts of addition reactions could be calculated as more than 98%. The stereochemistries of diastereometric **9** and **13** could be firmly established at the stages of the oxazolidin-2-one derivatives (**10** and **14**) (*vide infra*). As shown in Fig. 2, highly stereoselective formation of **9** may be explained by a chelation-controlled mechanism (A) in which M represents magnesium(II), cerium(III) or a complex of both cations. On the other hand, the dipolar model (B) or the Felkin–Anh model (C) may account for the highly stereoselective addition of cyclohexylmethylcopper(I) with **8**, as claimed for a similar addition reaction.[15]

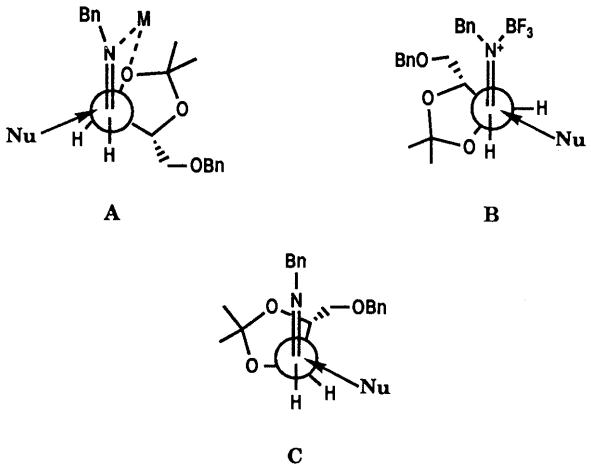
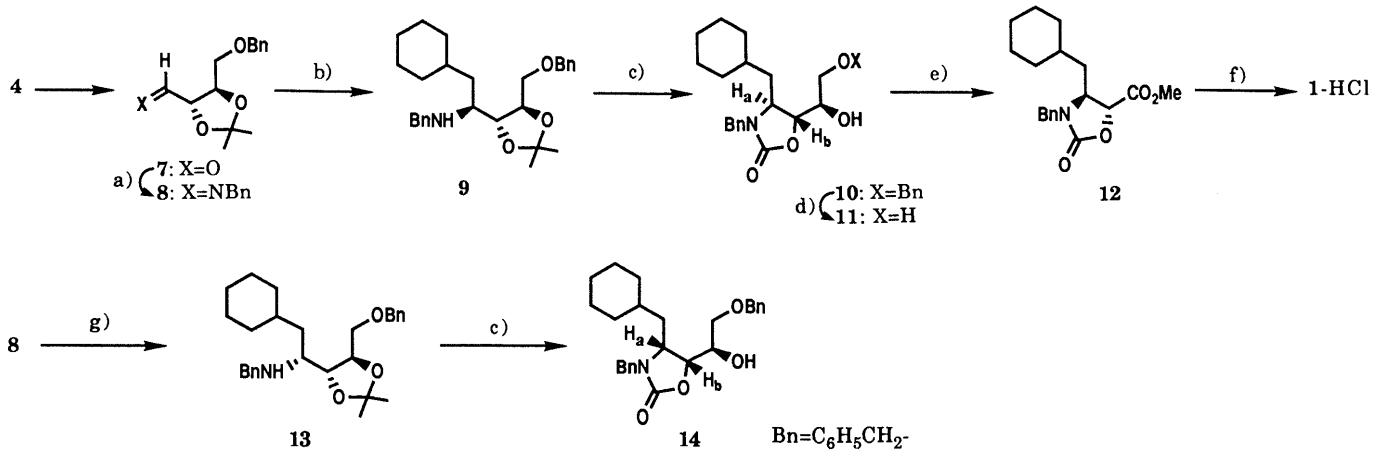


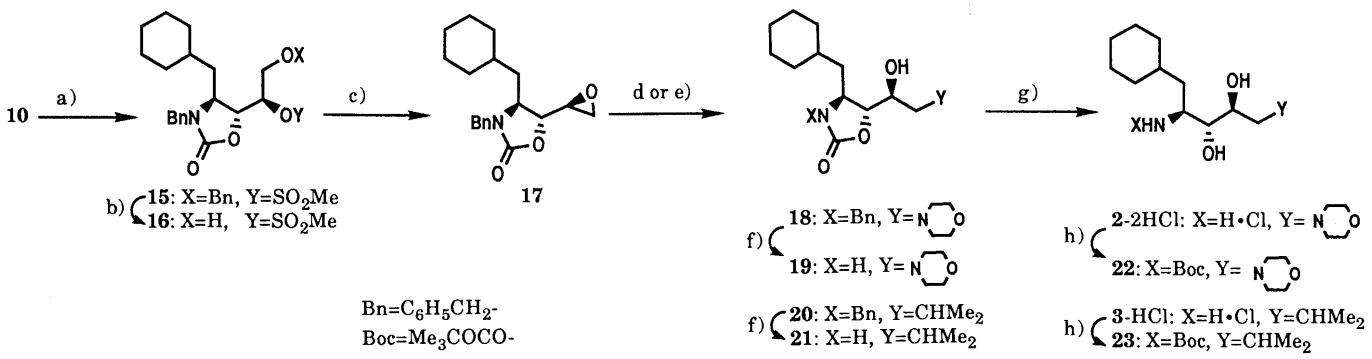
Fig. 2

With **9** in hand, elaboration **9** to **1** was next attempted. Thus, methoxycarbonylation of **9** followed by deacetalization and oxazolidin-2-one formation produced **10**, $[\alpha]_D^{20}$ −29.7° (chloroform), in 90% overall yield. The same sequential treatment of **13** gave **14**, $[\alpha]_D^{20}$ −31.6° (chloroform), in 94% overall yield. The coupling constants between H_a and H_b protons in **10** and **14** were found to be 5.1 and 8.5 Hz, respectively. Based on these spectral features as well as successful synthesis of **1** from **10**, the absolute stereochemistries of **10** and **14**, namely those of **9** and **13**, could be rigorously assigned as depicted. Hydrogenation of **10** quantitatively gave the diol (**11**), which, on oxidative cleavage and subsequent esterification, produced the methyl ester (**12**) in 73% combined yield. This was readily derived to the hydrochloride of **1** (**1**·HCl) in 76% overall yield by sequential alkaline hydrolysis of the oxazolidin-2-one moiety, salt formation, hydrogenolysis of the N-benzyl group, removal of inorganic salt with an ion exchange resin, and salt formation. The hydrochloride of **1** (**1**·HCl) thus produced exhibited mp 191—192 °C (dec.) and $[\alpha]_D^{20}$ −13.6° (1 M HCl) [lit.,[6] mp 190 °C (dec.) and $[\alpha]_D^{20}$ −12.4° (1 M HCl)][16] and the same ^{1}H-NMR spectrum as that of an authentic sample.[3c,6]

Synthesis of (2*S*,3*R*,4*S*)-4-Amino-5-cyclohexyl-1-morpholino-2,3-pentanediol (2) and (2*S*,3*R*,4*S*)-2-Amino-1-cyclohexyl-6-methyl-3,4-heptanediol (3) With completion of the synthesis of **1**·HCl from **9**, highly stereo- and regioselective elaboration of **9** to **2** and **3** was next attempted by employing epoxide formation with inversion of configuration followed



a) $BnNH_2$ (1.0 eq)–anhyd. $MgSO_4$ in PhMe, 0 °C, 1.5 h, 100% b) i) $C_6H_{11}CH_2MgBr$ (5.0 eq)–$CeCl_3$ (5.0 eq) in Et_2O–THF, −30 °C, 5 h ii) **8** in Et_2O–THF, −30 °C, 2 h, then, r.t., 12 h, 75% from **8** c) i) $ClCO_2Me$–anhyd. K_2CO_3 in THF, 0 °C, 7 h ii) 80% aq AcOH, 80 °C, 5 h iii) 10% KOH in MeOH, r.t., 3.5 h, 90% (**9**) from **10** or 94% (**14**) from **13** d) H_2 (1 atm)–20% $Pd(OH)_2$/C in MeOH, r.t., overnight, 100% e) i) $RuCl_3 \cdot 3H_2O$–$NaIO_4$ in CCl_4–MeCN–H_2O, r.t., 2 h ii) $TMSCHN_2$ in PhH–MeOH, r.t., 1 h, 73% from **11** f) i) NaOH in MeOH–H_2O, 90 °C, overnight ii) aq HCl iii) H_2 (1 atm)–20% $Pd(OH)_2$/C in MeOH, r.t., overnight iv) Dowex AG 50W-X2, H^+-form v) aq HCl, 76% from **12** g) i) $C_6H_{11}CH_2MgBr$ (2.7 eq)–CuI (2.7 eq) in Et_2O–THF, −78 °C, 10 min ii) **8** in Et_2O–THF, −78 °C, 1 h, then, r.t., 15 h, 52% from **8**



a) $MeSO_2Cl$ (1.3 eq) in Py, 0 °C, 3 h, 96% b) H_2 (1 atm)–20% $Pd(OH)_2/C$ in MeOH, r.t., 2 d, 98% c) NaOMe (8.3 eq) in THF, 0 °C, 0.5 h, 94% d) morpholine (2.1 eq) in MeOH, r.t., 1 d, 94% e) $Me_2CHMgCl$ (3.0 eq)–CuI (0.16 eq) in Et_2O, 1.5 h, 62% f) Na in liq. NH_3, −78 °C, 2 h, 78% (**19**) from **18** or 51% (**21**) from **20** g) 37% HCl, reflux, 3 d, 100% (**2**·2HCl or **3**·HCl) from **19** or **21** h) *tert*-Boc_2O (2.0 eq)–Et_3N (3.0 eq) in $CHCl_3$, r.t., 1 or 2 h, 75% (**22**) from **19** or 84% (**23**) from **21**

Chart 3

by epoxide opening with a nucleophile.

Thus, as shown in Chart 3, mesylation of **10** followed by hydrogenolysis of the mesylate (**15**) produced the alcohol (**16**) in 94% combined yield. Treatment of **16** with sodium methoxide afforded the epoxide (**17**), $[\alpha]_D^{20}$ −7.6° (chloroform), as a sole product. That the epoxide formation took place in a highly stereoselective manner with inversion of configuration could be definitely established by the successful synthesis of **2** and **3** from **17**. Reaction of **17** with morpholine effected highly regioselective epoxide opening, giving rise to the alcohol (**18**), $[\alpha]_D^{20}$ −55.2° (chloroform), in 94% yield. Similar highly regioselective epoxide opening could also be achieved by the reaction of **17** with isopropylmagnesium chloride in the presence of copper(I) iodide, affording the alcohol (**20**), $[\alpha]_D^{20}$ −35.0° (chloroform), in 62% yield. Reductive removal of the N-benzyl groups involved in **18** and **20** produced the unprotected oxazolizin-2-one derivatives (**19** and **21**), **19**: mp 173—174 °C, $[\alpha]_D^{20}$ −79.8° (chloroform) and **20**: mp 162—162.5 °C, $[\alpha]_D^{20}$ −81.2° (chloroform), in 78% and 51% yields, respectively. Subsequent acidic hydrolysis of **19** and **21** afforded quantitative yields of **2** and **3** as their hydrochlorides (**2**·2HCl and **3**·HCl). The hydrochlorides of the 1,2,3-amino diols (**2**·2HCl and **3**·HCl) were characterized as their N-*tert*-butoxycarbonyl derivatives (**22** and **23**) prepared by treatment with di-*tert*-butyl dicarbonate and triethylamine. The carbamates (**22** and **23**) showed mp 143.5—144.5 °C, $[\alpha]_D^{20}$ −15.7° (chloroform) [lit.,[8] mp 143—145 °C, $[\alpha]_D^{20}$ −15.8° (chloroform)] and mp 134—135 °C, $[\alpha]_D^{20}$ −67.4° (chloroform) [lit.,[5c] mp 130—131 °C, $[\alpha]_D^{20}$ −64.91° (chloroform)], respectively. Spectral data of **22** and **23** were found to be identical with those reported.[5e,8] Successful syntheses of **2**·2HCl and **3**·HCl from **17** definitely established the steric course of epoxide formation and the regiochemistry of epoxide opening.

As mentioned above, we have succeeded in developing a highly stereoselective addition reaction of cyclohexylmethylmagnesium bromide with **8** in the presence of cerium(III) chloride. The addition product (**9**) could be elaborated to three types of C-terminal components of renin inhibitors (**1**, **2**, and **3**). The novel addition reaction developed here may further find a wide application in the syntheses of various optically active amines.

Experimental

All melting points were determined with a Yamato MP-21 melting point apparatus and a Yamato micro melting point apparatus and are uncorrected. Measurements of optical rotations were performed with a Horiba SEPA-200 automatic digital polarimeter. ^1H- and ^{13}C-NMR spectra were measured with Hitachi R-90H (90 MHz) and Brucker AM-400 (400 MHz) spectrometers. All signals were expressed in ppm using tetramethylsilane (0.00 ppm) (in $CDCl_3$) or HDO (4.70 ppm) (in D_2O) as an internal standard (δ-value). The following abbreviations are used: singlet (s), doublet (d), triplet (t), quartet (q) and broad (br). Infrared (IR) spectra measurements were carried out with a JASCO A-202 diffraction grating infrared spectrometer. Mass spectra (MS) were taken with Hitachi RMU-6MG (regular mass spectra) and Hitachi M-80A [high resolution and secondary ionization mass spectra (HRMS and SIMS)] mass spectrometers. Unless otherwise noted, all reactions were performed using anhydrous solvents. Tetrahydrofuran and ether freshly distilled from sodium benzophenone ketyl were mainly used. Wako Gel C-200 and C-300 were used as adsorbents for column chromatography. The following abbreviations are used for solvents and reagents: acetic acid (AcOH), acetonitrile (CH_3CN), benzene (PhH), carbon tetrachloride (CCl_4), cerium(III) chloride ($CeCl_3$), chloroform ($CHCl_3$), copper(I) iodide (CuI), ether (Et_2O), ethyl acetate (EtOAc), hexane (C_6H_{14}), methanol (MeOH), palladium(II) hydroxide ($Pd(OH)_2$), pyridine (Py), ruthenium(III) chloride ($RuCl_3$), tetrahydrofuran (THF), toluene (PhMe), triethylamine (Et_3N).

4-*O*-Benzyl-2,3-*O*-isopropylidine-L-threose (7) According to the reported procedure,[10] this compound could be prepared from **4** in 5 steps. The aldehyde (**7**) obtained as a slightly yellow oil showed bp. 180 °C (1 mmHg) (bath temp.) [(lit.,[10] bp 140—150 °C (0.6 mmHg) (bath temp.)]. ^1H-NMR ($CDCl_3$) δ: 1.40, 1.47 (6H, two s, $CH_3 \times 2$), 3.50—3.77 (2H, m, CH_2O), 4.07—4.39 (2H, m, CHCH), 4.59 (2H, s, CH_2Ph), 7.31 (5H, s, C_6H_5), 9.72 (1H, br s, CHO). This ^1H-NMR spectrum was identical with that reported.[6]

(4*R*,5*R*)-4-Benzyliminomethyl-5-benzyloxymethyl-2,2-dimethyl-1,3-dioxolane (8) Benzylamine (0.225 ml, 2.06 mmol) and anhydrous $MgSO_4$ (602 mg, 5.00 mmol) were added to a solution of **7** (505 mg, 2.02 mmol) in PhMe (10 ml) cooled in an ice bath, and the mixture was stirred at the same temperature for 1.5 h.[7,11] After being warmed to room temperature, the mixture was filtered. The filtrate was concentrated *in vacuo* to give crude **8** as a pale yellow oil (701 mg, quantitative yield). ^1H-NMR δ: 1.44 (6H, two s, $CH_3 \times 2$), 3.49—3.73 (2H, m, CH_2O), 4.12—4.45 (2H, m, CHCH), 4.45—4.81 (4H, m, $CH_2Ph \times 2$), 7.10—7.37 (10H, m, $C_6H_5 \times 2$), 7.79 (1H, br d, $J = 5$ Hz, CH=N). Since **8** was found to be fairly unstable, it was directly subjected to the next addition reaction.

(4*R*,5*R*)-4-[(*S*)-(1-*N*-Benzylamino-2-cyclohexyl)ethyl]-5-benzyloxymethyl-2,2-dimethyl-1,3-dioxolane (9) Anhydrous $CeCl_3$ prepared by heating $CeCl_3 \cdot 7H_2O$ (4.00 g, 10.7 mmol) at 130—140 °C for 5 h *in vacuo*, was suspended in THF (20 ml) at room temperature. The suspension was sonicated for 1 h, then stirred for 2 h at room temperature. A solution of cyclohexylmethylmagnesium bromide in Et_2O (1.0 M solution, 10.1 ml, 10.1 mmol) was added to the suspension of anhydrous $CeCl_3$ in THF prepared above with stirring at −30 °C, and stirring was continued at the same temperature for 0.5 h. A solution of crude **8** (701 mg, 2.02 mmol) in THF (10 ml) was added to the reaction mixture with stirring at −30 °C.

Stirring was continued for 2 h at the same temperature, then the reaction mixture was gradually warmed to room temperature, stirred for an additional 12 h, then poured into saturated $NaHCO_3$. The mixture was extracted with EtOAc. The ethyl acetate extracts were combined, washed successively with water and saturated NaCl, dried over anhydrous $MgSO_4$, then filtered. Concentration of the filtrate *in vacuo* gave an oily residue, which was purified by column chromatography (PhMe : EtOAc = 100 : 1 → 50 : 1) to give pure **9** as a colorless oil (660 mg, 75% from **8**), $[\alpha]_D^{20}$ +12.3° (c = 1.22, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.77—1.73 (13H, m, $C_6H_{11}CH_2$), 1.40, 1.42 (6H, two s, $CH_3 \times 2$), 2.66—2.74 (1H, m, $PhCH_2NHCH$), 3.56 (1H, dd, J = 10.3, 5.5 Hz, one of CH_2O), 3.57 (1H, dd, J = 10.3, 5.5 Hz, one of CH_2O), 3.75 (1H, d, J = 13.1 Hz, one of $PhCH_2N$), 3.85 (1H, d, J = 13.1 Hz, one of $PhCH_2N$), 3.88 (1H, dd, J = 7.9, 4.0 Hz, $CHCHCH_2O$), 4.24 (1H, ddd, J = 7.9, 5.5, 4.4 Hz, $CHCHCH_2O$), 4.56 (2H, s, OCH_2Ph), 7.19—7.35 (10H, m, $C_6H_5 \times 2$). IR (neat): 2930, 2870 cm^{-1}. MS m/z: 438 ($[M+1]^+$), 422, 340, 216. HRMS: Calcd for $C_{28}H_{40}NO_3$ ($[M+1]^+$): 438.3005. Found: 438.2987.

(4*R*,5*R*)-4-[(*R*)-(1-*N*-Benzylamino-2-cyclohexyl)ethyl]-5-benzyloxymethyl-2,2-dimethyl-1,3-dioxolane (13) A solution of cyclohexylmethylmagnesium bromide in Et_2O (1.0 M solution, 1.25 ml, 1.25 mmol) was added to a suspension of CuI (248 mg, 1.30 mmol) in THF (2 ml) with stirring at −78 °C. Stirring was continued at the same temperature for 10 min, then boron trifluoride-etherate (0.155 ml, 1.26 mmol) was added and the reaction mixture was further stirred for 5 min. A solution of crude **8** (82 mg, 0.233 mmol) in THF (1 ml) was added to the reaction mixture at −78 °C. The mixture was stirred at the same temperature for 1 h, gradually warmed up to room temperature, and further stirred for 15 min. After being poured into saturated $NaHCO_3$, the mixture was extracted with EtOAc. The organic extracts were combined, washed successively with H_2O and saturated NaCl, then dried over anhydrous $MgSO_4$. Filtration followed by concentration *in vacuo* gave an oily residue, which was purified by column chromatography (PhMe : EtOAc = 100 : 1) to give pure **13** as a pale yellow oil (52% from **8**), $[\alpha]_D^{20}$ +26.7° (c = 1.05, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.70—1.72 (13H, m, $C_6H_{11}CH_2$), 1.40, 1.41 (6H, two s, $CH_3 \times 2$), 2.81 (1H, dt, J = 7.4, 4.6 Hz, $PhCH_2NHCH$), 3.57 (1H, dd, J = 10.3, 5.8 Hz, one of CH_2O), 3.67 (1H, dd, J = 10.3, 3.6 Hz, one of CH_2O), 3.77 (1H, d, J = 13.1 Hz, one of $PhCH_2N$), 3.83 (1H, d, J = 13.1 Hz, one of $PhCH_2N$), 3.90 (1H, dd, J = 7.8, 4.6 Hz, $CHCHCH_2O$), 4.15 (1H, ddd, J = 7.9, 5.8, 3.6 Hz, $CHCHCH_2O$), 4.58 (1H, d, J = 12.2 Hz, one of OCH_2Ph), 7.18—7.40 (10H, m, $C_6H_5 \times 2$). IR (neat): 2940, 2850 cm^{-1}. MS m/z: 437 (M^+), 422, 340, 216.

(4*S*,5*R*)-3-Benzyl-5-[(*R*)-(2-benzyloxy-1-hydroxy)ethyl]-4-cyclohexylmethyloxazolidin-2-one (10) a) (4*R*,5*R*)-4-[(*S*)-(1-(*N*-Benzyl-*N*-methoxycarbonyl)amino-2-cyclohexyl)ethyl]-5-benzyloxymethyl-2,2-dimethyl-1,3-dioxolan: Anhydrous K_2CO_3 (435 mg, 3.15 mmol) and methyl chloroformate (0.24 ml, 3.11 mmol) was added to a solution of **9** (262 mg, 0.598 mmol) in THF (6.5 ml) with stirring in an ice bath, and the mixture was stirred at the same temperature for 7 h. After being diluted with EtOAc, the ethyl acetate solution was washed successively with dilute HCl, H_2O, and saturated NaCl, dried over anhydrous $MgSO_4$, then filtered. Concentration of the filtrate *in vacuo* gave an oily residue, which was purified by column chromatography (PhMe : EtOAc = 100 : 1) to give the carbamate as a colorless oil (288 mg, 97%), $[\alpha]_D^{20}$ −24.9° (c = 1.05, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.40—2.10 (13H, m, $C_6H_{11}CH_2$), 1.38 (6H, s, $CH_3 \times 2$), 3.69 (3H, s, CO_2CH_3), 4.61 (4H, s, $PhCH_2 \times 2$), 3.46—4.50 (5H, m, $NCHCHCHCH_2O$), 7.27 (5H, s, one of C_6H_5), 7.33 (5H, s, one of C_6H_5). IR (neat): 2950, 2875, 1690 cm^{-1}. MS m/z: 495 (M^+), 480, 437, 274.

b) (2*R*,3*R*,4*S*)-4-(*N*-Benzyl-*N*-methoxycarbonyl)amino-1-benzyloxy-5-cyclohexylpentane-2,3-diol: The carbamate (242 mg, 0.487 mmol) obtained in a) was dissolved in 80% AcOH (12 ml) and the acidic solution was stirred at 80 °C for 5 h. Concentration *in vacuo* gave the crude diol, which was immediately subjected to the next step. ^{1}H-NMR ($CDCl_3$) δ: 0.45—1.90 (13H, m, $C_6H_{11}CH_2$), 3.73 (3H, s, CO_2CH_3), 7.27 (5H, s, one of C_6H_5), 7.32 (5H, s, one of C_6H_5), Other signals could not be assigned.

c) **10**: The crude diol obtained in b) was dissolved in a 10% solution of KOH in MeOH (3 ml), and the solution was stirred at room temperature for 3.5 h. The reaction mixture was neutralized with 1 M HCl and extracted with EtOAc. The ethyl acetate extracts were combined, washed successively with H_2O and saturated NaCl, dried over anhydrous $MgSO_4$, then filtered. Concentration of the filtrate *in vacuo* gave a residue, which was purified by column chromatography (PhMe : EtOAc = 10 : 1) to give **10** as a colorless oil [189 mg, 92% (2 steps)], $[\alpha]_D^{20}$ −29.7° (c = 1.25, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.67—1.70 (13H, m, $C_6H_{11}CH_2$), 2.33 (1H, br s, OH), 3.53 (1H, dd, J = 9.6, 7.3 Hz, one of CH_2O), 3.58 (1H, dd, J = 9.6, 5.1 Hz, one of CH_2O), 3.64—3.75 (2H, m, $NCHCHCHCH_2O$), 4.09 (1H, d, J = 15.3 Hz, one of $PhCH_2N$), 4.20 (1H, dd, J = 5.0, 2.8 Hz, $NCHCHCH-CH_2O$), 4.52 (2H, s, OCH_2Ph), 4.79 (1H, d, J = 15.3 Hz, one of $PhCH_2N$), 7.24—7.38 (10H, m, $C_6H_5 \times 2$). IR (neat): 3300, 2940, 2875, 1735 cm^{-1}. MS m/z: 423 (M^+), 332, 302, 284. HRMS: Calcd for $C_{26}H_{33}NO_4$ (M^+): 423.2407. Found: 423.2431.

(4*R*,5*R*)-3-Benzyl-5-[(*R*)-(2-benzyloxy-1-hydroxy)ethyl]-4-cyclohexylmethyloxazolidin-2-one (14) a) (4*R*,5*R*)-4-[(*R*)-(1-(*N*-Benzyl-*N*-methoxycarbonyl)amino-2-cyclohexyl)ethyl]-5-benzyloxymethyl-2,2-dimethyl-1,3-dioxolane: Anhydrous K_2CO_3 (35.8 mg, 0.259 mmol) and methyl chloroformate (0.02 ml, 0.0514 mmol) were added to a solution of **13** (22.5 mg, 0.259 mmol) in THF (1.1 ml) with stirring in an ice bath, and the mixture was stirred at the same temperature for 9.5 h. Treatments of the reaction mixture in the same manner as described for the preparation of **10** gave the crude carbamate after concentration of the ethyl acetate extracts. This was immediately subjected to the next acidic hydrolysis. ^{1}H-NMR ($CDCl_3$) δ: 0.45—2.00 (13H, m, $C_6H_{11}CH_2$), 1.28, 1.37 (6H, two s, $CH_3 \times 2$), 3.70 (3H, s, CO_2CH_3), 7.26 (5H, s, one of C_6H_5), 7.33 (5H, one of C_6H_5). Other signals could not be assigned.

b) (2*R*,3*R*,4*R*)-4-(*N*-Benzyl-*N*-methoxycarbonyl)amino-1-benzyloxy-5-cyclohexylpentane-2,3-diol: The crude carbamate obtained in a) was treated with 80% AcOH (1 ml) in the same manner as described for the preparation of **10**, giving the crude diol after concentration of the reaction mixture *in vacuo*. This was immediately subjected to the next step. ^{1}H-NMR ($CDCl_3$) δ: 0.60—1.90 (13H, m, $C_6H_{11}CH_2$), 3.80 (3H, s, CO_2CH_3), 7.27 (5H, s, one of C_6H_5), 7.31 (5H, one of C_6H_5). Other signals could not be assigned.

c) **14**: The crude diol obtained in b) was dissolved in a 10% solution of KOH in MeOH (1 ml) and the solution was stirred at room temperature for 3.5 h. Treatments of the reaction mixture in the same manner as described for the preparation of **10** gave pure **14** as a colorless oil [20.4 mg, 94% (3 steps)] after purification by column chromatography (PhMe : EtOAc = 20 : 1 → 10 : 1), $[\alpha]_D^{20}$ −31.6° (c = 0.823, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.65—1.84 (13H, m, $C_6H_{11}CH_2$), 2.22 (1H, br d, J = 5.5 Hz, OH), 3.56 (1H, dd, J = 9.2, 5.7 Hz, one of CH_2O), 3.65 (1H, dd, J = 9.2, 7.5 Hz, one of CH_2O), 3.77 (1H, ddd, J = 9.6, 8.5, 4.1 Hz, $PhCH_2NCH$), 3.99 (1H, br q, J = 5.9 Hz, $CHCH_2O$), 4.13 (1H, d, J = 15.5 Hz, one of $PhCH_2N$), 4.43 (1H, dd, J = 8.5, 1.2 Hz, $CHCHCH_2O$), 4.55 (1H, d, J = 11.8 Hz, one of $PhCH_2O$), 4.56 (1H, d, J = 11.8 Hz, one of $PhCH_2O$), 4.78 (1H, d, J = 15.5 Hz, one of $PhCH_2N$), 7.24—7.38 (10H, m, $C_6H_5 \times 2$). IR (neat): 3400, 2920, 2850, 1720 cm^{-1}. MS m/z: 423 (M^+), 332, 326, 314. HRMS: Calcd for $C_{26}H_{33}NO_4$(M^+): 423.2407. Found: 423.2420.

(4*S*,5*R*)-3-Benzyl-4-cyclohexyl-5-[(*R*)-(1,2-dihydroxy)ethyl]oxazolidin-2-one (11) A mixture of **10** (158 mg, 0.372 mmol) and 20% $Pd(OH)_2$ on carbon (catalytic amount) in MeOH (3 ml) was stirred under a hydrogen atmosphere (1 atm) overnight at room temperature. The catalyst was filtered off, and the filtrate was concentrated *in vacuo* to give **11** as a colorless solid (125 mg, quantitative yield). Recrystallization from EtOAc–C_6H_{14} gave an analytical sample of **11** as colorless crystals, mp 101—102 °C and $[\alpha]_D^{20}$ −43.1° (c = 0.594, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.70—1.80 (13H, m, $C_6H_{11}CH_2$), 2.20 (1H, t, J = 5.9 Hz, CHOH), 2.65 (1H, d, J = 6.6 Hz, CHOH), 3.55 (1H, m, CHOH), 3.64 (1H, m, $PhCH_2NCH$), 3.72 (2H, dd, J = 5.8, 5.5 Hz, CH_2OH), 4.10 (1H, d, J = 15.3 Hz, one of $PhCH_2N$), 4.20 (1H, d, J = 5.1, 3.3 Hz, $CHCHCH_2O$), 4.79 (1H, d, J = 15.3 Hz, one of $PhCH_2N$), 7.20—7.40 (5H, m, C_6H_5). IR (KBr): 3440, 3250, 2930, 2885, 1720, 1440, 1042, 702 cm^{-1}. MS m/z: 333 (M^+), 236, 176, 91. *Anal.* Calcd for $C_{19}H_{24}NO_4$: C, 68.44; H, 8.16; N, 4.20. Found: C, 68.22; H, 8.25; N, 4.19.

Methyl (4*S*,5*R*)-3-Benzyl-4-cyclohexylmethyloxazolidin-2-one-5-carboxylate (12) $RuCl_3 \cdot 3H_2O$ (catalytic amount) and $NaIO_4$ (512 mg, 2.40 mmol) was added to a solution of **11** (162 mg, 0.485 mmol) in a mixture of CCl_4, MeCN, and H_2O (2 : 2 : 3) (6.3 ml). After stirring at room temperature for 2 h, the reaction mixture was extracted with $CHCl_3$. The chloroform extracts were combined, dried over anhydrous $MgSO_4$, then filtered. Concentration of the filtrate *in vacuo* gave crude (4*S*,5*R*)-3-benzyl-4-cyclohexylmethyloxazolidin-2-one-5-carboxylic acid, which was dissolved in a mixture of PhH and MeOH (4 : 1) (3 ml). An excess amount of trimethylsilyldiazomethane (10% hexane solution) was added to the solution of the crude carboxylic acid. After stirring at room temperature for 1 h, the mixture was concentrated *in vacuo*. The concentration residue was purified by column chromatography (C_6H_{14} : EtOAc = 9 : 1 → 4 : 1) to give **12** as a colorless oil [117 mg, 73% (2 steps)], $[\alpha]_D^{20}$ −21.5° (c = 1.92, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.70—1.70 (13H, m, $C_6H_{11}CH_2$), 3.58 (1H, dt, J = 9.8, 3.8 Hz, $PhCH_2NCH$), 3.78 (3H, s, CO_2CH_3), 4.04 (1H, d, J = 15.3 Hz, one of $PhCH_2N$). 4.55 (1H, d, J = 3.8 Hz, $CHCO_2Me$), 4.83 (1H, d, J = 15.3 Hz, one of $PhCH_2N$), 7.20—7.40 (5H, m, C_6H_5). IR (neat): 2950, 2870, 1775, 1760, 1240, 702 cm^{-1}. MS m/z: 331 (M^+),

234, 205, 91.

(2*R*,3*S*)-3-Amino-4-cyclohexyl-2-hydroxybutyric Acid Hydrochloride [(2*R*,3*S*)-Cyclohexylnorstatine Hydrochloride] (1·HCl) Two molar NaOH solution (4.0 ml) was added to a solution of **12** (127 mg, 0.384 mmol) in MeOH (0.6 ml), and the mixture was stirred overnight at 90 °C. After being acidified (pH = 1) with 1.0 M HCl, the mixture was concentrated *in vacuo*. Addition of MeOH to the residue followed by filtration and concentration *in vacuo* gave crude (2*R*,3*S*)-3-benzylamino-4-cyclohexyl-2-hydroxybutyric acid hydrochloride, which was immediately dissolved in MeOH (6 ml). Twenty percent $Pd(OH)_2$ on carbon (catalytic amount) was added to the methanolic solution prepared above, and the whole mixture was stirred under a hydrogen atmosphere (1 atm) overnight at room temperature. Filtration followed by concentration *in vacuo* gave a residue, to which was added 6.0 M HCl (4 ml). After stirring at 100 °C for 2 h, concentration *in vacuo* gave crude (2*R*,3*S*)-3-amino-4-cyclohexyl-2-hydroxybutyric acid hydrochloride. This was dissolved in a small amount of H_2O, and the aqueous solution was poured onto a column of ion exchange resin (AG50W-X2). After being washed with H_2O, the column was eluted with 25% NH_4OH. The alkaline eluate was concentrated *in vacuo* to give a residue, which was dissolved in 1.0 M HCl. Concentration of the acidic solution to a small volume *in vacuo* gave **1**·HCl as colorless crystals [69.0 mg, 76% (5 steps)]. Recrystallization from H_2O gave an analytical sample of **1**·HCl, mp 191—192 °C (dec.) and $[\alpha]_D^{20}$ −13.6° (*c* = 0.633, 1 M HCl) [lit.,[6] mp 190 °C (dec.) and $[\alpha]_D^{20}$ −12.4° (*c* = 0.482, 1 M HCl)].[12] ^{1}H-NMR (D_2O) δ: 0.8—1.80 (13H, m, $C_6H_{11}CH_2$), 3.67 (1H, dt, *J* = 7.3, 3.5 Hz, CHN), 4.34 (1H, d, *J* = 3.5 Hz, CHO). This ^{1}H-NMR spectrum was identical with that reported.[3c] IR (KBr): 3900—2600, 3350, 2940, 1725, 1485, 1400, 1075 cm^{-1}. MS (SIMS) *m/z*: 202 ([M − Cl])$^+$), 126.

(4*S*,5*R*)-3-Benzyl-4-cyclohexylmethyl-5-[(*R*)-(2-benzyloxy-1-methanesulfonyloxy)ethyl]oxazolidin-2-one (15) Methanesulfonyl chloride (70 μl, 0.90 mmol) was added to a solution of **10** (306 mg, 0.723 mmol) in pyridine (2 ml) cooled in an ice bath, and the mixture was stirred in an ice bath for 3 h. After being diluted with EtOAc, the ethyl acetate solution was washed successively with H_2O and saturated NaCl, and dried over anhydrous $MgSO_4$. Filtration and concentration *in vacuo* gave a residue, which was purified by column chromatography (C_6H_{14} : EtOAc = 4 : 1→3 : 2) to give pure **15** as a colorless oil (347 mg, 96%). ^{1}H-NMR ($CDCl_3$) δ: 0.70—1.70 (13H, m, $C_6H_{11}CH_2$), 2.87 (3H, s, SO_2CH_3), 3.65 (2H, m, NCH and one of CH_2OBn), 3.70 (1H, dd, *J* = 10.6, 4.9 Hz, one of CH_2OBn), 4.12 (1H, d, *J* = 15.1 Hz, one of $PhCH_2N$), 4.38 (1H, dd, *J* = each 4.0 Hz, $CHCH_2O$), 4.47 (1H, d, *J* = 11.7 Hz, one of OCH_2Ph), 4.52 (1H, d, *J* = 11.7 Hz, one of OCH_2Ph), 4.68 (1H, m, $CHOSO_2$), 4.71 (1H, d, *J* = 15.1 Hz, one of $PhCH_2N$), 7.24—7.38 (10H, m, $C_6H_5 \times 2$). IR (neat): 3050, 2950, 2875, 1760, 1180, 922, 702 cm^{-1}. MS *m/z*: 501 (M^+), 410 ([M − $C_6H_5CH_2$])$^+$), 404.

(4*S*,5*R*)-3-Benzyl-4-cyclohexylmethyl-5-[(*R*)-(2-hydroxy-1-methanesulfonyloxy)ethyl]oxazolidin-2-one (16) Twenty percent $Pd(OH)_2$ on carbon (80 mg, catalytic amount) was added to a solution of **15** (645 mg, 1.29 mmol) in MeOH (5 ml), and the mixture was stirred under a hydrogen atmosphere (1 atm) at room temperature for 2 d. Filtration and concentration *in vacuo* gave crude **16** as a colorless oil (517 mg, 98%). This was immediately subjected to the next reaction. ^{1}H-NMR ($CDCl_3$) δ: 0.70—1.70 (13H, m, $C_6H_{11}CH_2$), 2.34 (1H, t, *J* = 6.0 Hz, OH), 2.87 (3H, s, SO_2CH_3), 3.66 (1H, m, CHN), 3.84 (2H, m, CH_2OH), 4.09 (1H, d, *J* = 14.9 Hz, one of $PhCH_2N$), 4.40 (dd, *J* = 4.5, 3.4 Hz, CHOCO), 4.60 (1H, ddd, *J* = 6.3, 4.7, 3.4 Hz, $CHOSO_2$), 4.75 (1H, d, *J* = 15.0 Hz, one of $PhCH_2N$), 7.30—7.40 (5H, m, C_6H_5). IR (neat): 3700—3200, 2945, 2855, 1740, 1442, 1175, 915, 702 cm^{-1}. MS *m/z*: 411 (M^+), 314 ([M − $C_6H_{11}CH_2$])$^+$), 218, 91.

(4*S*,5*R*)-3-Benzyl-4-cyclohexylmethyl-5-[(*S*)-oxiran-2-yl]oxazolidin-2-one (17) Sodium methoxide (560 mg, 10.4 mmol) was added to a solution of **16** (515 mg, 1.25 mmol) in THF (10 ml) with stirring in an ice bath. Stirring was continued in the ice bath for 30 min, then the reaction mixture was diluted with EtOAc, washed succesively with H_2O and saturated NaCl, and then dried over $MgSO_4$. Filtration and concentration *in vacuo* gave a residue, which was purified by column chromatography (C_6H_{14} : EtOAc = 19 : 1→3 : 1) to give **17** as a colorless oil (370 mg, 94%), $[\alpha]_D^{20}$ −7.6° (*c* = 0.706, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.70—1.70 (13H, m, $C_6H_{11}CH_2$), 2.68 (1H, dd, *J* = 4.7, 2.5 Hz, one of CH_2O), 2.84 (1H, dd, *J* = 4.7, 4.0 Hz, one of CH_2O), 2.94 (1H, ddd, *J* = 6.1, 4.0, 2.5 Hz, $CHOCH_2$), 3.50 (1H, ddd, *J* = 9.8, 4.2, 3.9 Hz, CHN), 3.84 (1H, dd, *J* = 6.1, 4.2 Hz, CHOCO), 4.05 (1H, d, *J* = 15.2 Hz, one of $PhCH_2N$), 4.83 (1H, d, *J* = 15.2 Hz, one of $PhCH_2N$), 7.25—7.38 (5H, m, C_6H_5). IR (neat): 2950, 2855, 1760, 1422, 1240, 702 cm^{-1}. MS *m/z*: 315 (M^+), 218 ([M − $C_6H_{11}CH_2$])$^+$), 91.

(4*S*,5*R*)-3-Benzyl-4-cyclohexylmethyl-5-[(*S*)-(1-hydroxy-2-morpholin-4-yl)ethyl]oxazolidin-2-one (18) A mixture of **17** (70.5 mg, 0.224 mmol) and morpholine (40 μl, 0.459 mmol) in MeOH (1.5 ml) was stirred at room temperature for 1 d. After being diluted with EtOAc, the ethyl acetate solution was washed successively with H_2O and saturated NaCl, and dried over anhydrous $MgSO_4$. Filtration and concentration *in vacuo* gave a residue, which was purified by column chromatography (C_6H_{14} : EtOAc = 1 : 7) to give **18** as a colorless caramel (84.4 mg, 94%), $[\alpha]_D^{20}$ −55.2° (*c* = 0.782, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.70—1.70 (13H, m, $C_6H_{11}CH_2$), 2.34 (1H, dd, *J* = 12.5, 10.0 Hz, one of $CH_2NCH_2CH_2O$), 2.39 (2H, m, NCH_2CH_2O), 2.55 (1H, dd, *J* = 12.5, 3.5 Hz, one of $CH_2NCH_2CH_2O$), 2.58 (2H, m, NCH_2CH_2O), 3.53 (1H, ddd, *J* = 10.0, 7.2, 3.5 Hz, CHOH), 3.59 (1H, m, CHN), 3.68 (4H, m, CH_2OCH_2), 3.96 (1H, dd, *J* = 7.2, 3.6 Hz, CHOCO), 4.05 (1H, d, *J* = 15.1 Hz, one of $PhCH_2N$), 4.81 (1H, d, *J* = 15.1 Hz, one of $PhCH_2N$), 7.26—7.38 (5H, m, C_6H_5). IR (neat): 3700—3200, 2948, 2855, 1755, 1442, 1120, 702 cm^{-1}. MS *m/z*: 401 ([M − 1]$^+$), 340, 258, 100 ([CH_2 = $N(CH_2CH_2)_2O$]$^+$).

(4*S*,5*R*)-3-Benzyl-4-cyclohexylmethyl-5-[(*S*)-(1-hydroxy-3-methyl)butyl]oxazolidin-2-one (20) A solution of isopropylmagnesium chloride in Et_2O (1.0 M solution, 0.70 ml, 0.70 mmol) was added to a suspension of CuI (7 mg, 0.037 mmol) in Et_2O (2 ml) with stirring at 0 °C. Stirring was continued at the same temperature for 10 min, then a solution of **17** (73.4 mg, 0.233 mmol) in Et_2O (1 ml) was added to the reaction mixture at 0 °C. The whole was stirred at the same temperature for 1.5 h, then diluted with EtOAc. The ethyl acetate solution was washed successively with saturated $NaHCO_3$ and saturated NaCl, and dried over anhydrous $MgSO_4$. Filtration and concentration *in vacuo* gave a residue, which was purified by column chromatography (C_6H_{14} : Et_2O = 2 : 1) to give **20** as a colorless caramel (51.8 mg, 62%), $[\alpha]_D^{20}$ −35.0° (*c* = 0.982, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.84 (3H, d, *J* = 6.6 Hz, CH_3), 0.91 (3H, d, *J* = 6.7 Hz, CH_3), 0.70—1.70 (16H, m, $C_6H_{11}CH_2$ and CH_2CHMe_2), 3.50 (1H, m, CHN), 3.67 (1H, m, CHOH), 4.03 (1H, t, *J* = 4.2 Hz, CHOCO), 4.07 (1H, d, *J* = 15.2 Hz, one of $PhCH_2N$), 4.80 (1H, d, *J* = 15.2 Hz, one of $PhCh_2N$), 7.24—7.38 (5H, m, C_6H_5). IR ($CHCl_3$): 2940, 2850, 1740, 1442, 1092 cm^{-1}. MS *m/z*: 359 (M^+), 262, 176, 91.

(4*S*,5*R*)-4-Cyclohexylmethyl-5-[(*S*)-(1-hydroxy-2-morpholin-4-yl)ethyl]-oxazolidin-2-one (19) A solution of **18** (84.4 mg, 0.210 mmol) in Et_2O (3 ml) was added to liquid ammonia (5 ml) containing sodium metal (*ca.* 50 mg, 2.2 mmol) at −78 °C. Stirring was continued at the same temperature for 2 h, then the reaction was quenched by adding NH_4Cl and liquid ammonia was evaporated off. Water was added to the evaporation residue and the aqueous mixture was extracted with EtOAc. The ethyl acetate extracts were combined, washed with saturated NaCl, then dried over anhydrous $MgSO_4$. Filtration and concentration *in vacuo* gave a residue, which was purified by column chromatography (C_6H_{14} : EtOAc = 1 : 4→EtOAc) to give **19** as colorless crystals (50.8 mg, 78%). An analytical sample of **19** was prepared by recrystallization from EtOAc, mp 173—174 °C and $[\alpha]_D^{20}$ −79.8° (*c* = 0.694, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.80—1.80 (13H, m, $C_6H_{11}CH_2$), 2.39 (1H, dd, *J* = 12.6, 9.9 Hz, one of $CH_2NCH_2CH_2O$), 2.46 (1H, m, NCH_2CH_2O), 2.66 (1H, dd, *J* = 12.6, 3.6 Hz, one of $CH_2NCH_2CH_2O$), 2.64 (2H, m, NCH_2CH_2O), 3.71 (4H, m, CH_2OCH_2), 3.79 (1H, ddd, *J* = 9.9, 7.1, 3.6 Hz, CHO), 3.92 (1H, m, CHN), 3.97 (1H, dd, *J* = 7.1, 4.7 Hz, CHOCO), 5.54 (1H, br s, NH). IR ($CHCl_3$): 2940, 2855, 1740, 1118, 1090 cm^{-1}. MS *m/z*: 313 ([M + 1]$^+$), 130,100. *Anal.* Calcd for $C_{16}H_{28}N_2O_4 \cdot 0.2H_2O$: C, 60.81; H, 9.06; N, 8.87. Found: C, 60.89; H, 9.09; N, 8.72.

(4*S*,5*R*)-4-Cyclohexyl-5-[(*S*)-(1-hydroxy-3-methyl)butyl]oxazolidin-2-one (21) Treatment of **20** (118 mg, 0.328 mmol) in the same manner as described for **18** gave **21** as colorless crystals (44.9 mg, 51%) after purification by column chromatography (C_6H_{14} : EtOAc = 4 : 1→3 : 2). An analytical sample of **21** was obtained by recrystallization from EtOAc, mp 162—162.5 °C and $[\alpha]_D^{20}$ −81.2° (*c* = 0.739, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.93 (3H, d, *J* = 6.6 Hz, CH_3), 0.97 (3H, d, *J* = 6.7 Hz, CH_3), 0.80—1.90 (16H, m, $C_6H_{11}CH_2$ and CH_2CHMe_2), 2.40 (1H, br s, OH), 3.92 (2H, m, CHN and CHOH), 4.02 (1H, dd, *J* = 5.4, 3.7 Hz, CHOCO), 5.45 (1H, br s, NH). IR ($CHCl_3$): 2940, 2855, 1758, 1092 cm^{-1}. MS *m/z*: 270 ([M + 1]$^+$), 251 ([M − H_2O]$^+$), 212, 122, 86. *Anal.* Calcd for $C_{15}H_{27}NO_3 \cdot 0.1H_2O$: C, 66.44; H, 10.11; N, 5.16. Found: C, 66.51; H, 10.10; N, 5.12.

(2*S*,3*R*,4*S*)-4-Amino-5-cyclohexyl-1-morpholin-4-yl-2,3-pentanediol Dihydrochloride (2·2HCl) A mixture of **19** (37.2 mg, 0.119 mmol) and concentrated HCl (3 ml) was heated at reflux for 3 d. Concentration *in vacuo* gave **2**·2HCl as a colorless caramel (48.4 mg, quantitative yield). ^{1}H-NMR (D_2O) δ: 0.70—1.70 (13H, m, $C_6H_{11}CH_2$), 3.10 (2H, m, NCH_2CH_2O), 3.18 (1H, dd, *J* = 13.5, 10.5 Hz, one of $CH_2NCH_2CH_2O$),

3.22 (1H, dd, J=13.5, 2.8 Hz, one of $CH_2NCH_2CH_2O$), 3.46 (3H, m, $CHNH_3^+$ and NCH_2CH_2O), 3.56 (1H, dd, J=7.0, 2.6 Hz, $CH(OH)$-$CH(OH)CH_2$), 3.75 (2H, m, CH_2OCH_2), 3.98 (2H, m, CH_2OCH_2), 4.06 (1H, ddd, J=10.5, 7.0, 2.8 Hz, $CH(OH)CH_2$). ^{13}C-NMR (D_2O) δ: 28.27, 28.31, 28.65, 35.17, 35.35, 35.63, 39.80, 51.93, 53.80, 56.24, 62.13, 66.28 (C×2), 67.98, 72.99. SIMS m/z: 287 ($[M+1-2HCl]^+$), 200, 100. This compound (**2**·2HCl) was further characterized as **22**.

(2*S*,3*R*,4*S*)-2-Amino-1-cyclohexyl-6-methyl-3,4-heptanediol Hydrochloride (3·HCl) A mixture of **21** (42.8 mg, 0.159 mmol) and concentrated HCl (4 ml) was heated at reflux overnight. Concentration of the mixture *in vacuo* gave **3**·HCl as a colorless caramel (47.7 mg, quantitative yield). ^{1}H-NMR (D_2O) δ: 0.85 (3H, d, J=6.6 Hz, CH_3), 0.89 (3H, d, J=6.7 Hz, CH_3), 0.80—1.80 (16H, m, $C_6H_{11}CH_2$ and CH_2CHMe_2), 3.56 (2H, m, CHO and CHN), 3.82 (1H, m, CHO). SIMS m/z: 244 ($[M+1-HCl]^+$), 126, 55. This compound was further characterized as **23**.

(2*S*,3*R*,4*S*)-4-*N*-*tert*-Butoxycarbonylamino-5-cyclohexyl-1-morpholin-4-yl-2,3-pentanediol (22) Di-*tert*-butyl dicarbonate (48.0 mg, 0.220 mmol) and Et_3N (30 μl, 0.22 mmol) were added to a solution of **2**·2HCl (15.1 mg, 0.042 mmol) in CH_2Cl_2 (0.3 ml), and the mixture was stirred at room temperature for 1 h. After being diluted with EtOAc and H_2O, the ethyl acetate layer was separated, washed with saturated NaCl, then dried over anhydrous $MgSO_4$. Filtration and concentration *in vacuo* gave a residue, which was purified by column chromatography (EtOAc) to give **22** as colorless crystals (12.2 mg, 75%). Recrystallization from EtOAc–C_6H_{14} gave an analytical sample of **22**, mp 143.5—144.5 °C and $[\alpha]_D^{20}$ −15.7° (c=0.82, $CHCl_3$) [lit.,[8] mp 143.5—145 °C and $[\alpha]_D^{20}$ −15.8° (c=1.07, $CHCl_3$)]. ^{1}H-NMR ($CDCl_3$) δ: 0.80—1.82 (13H, m, $C_6H_{11}CH_2$), 1.46 (9H, s, $C(CH_3)_3$), 2.52 (2H, m, CH_2N $(CH_2CH_2)_2O$), 2.60—2.85 (4H, m, $N(CH_2CH_2)_2O$), 3.43 (2H, m, $CH(OH)CH(OH)$), 3.71 (4H, m, $N(CH_2CH_2)_2O$), 3.97 (1H, ddd, J=14.2, 9.7, 4.7 Hz, $CHNHCO$), 4.66 (2H, two br d, J=each 9.5 Hz, NH and OH). IR (KBr): 3000—3600, 2940, 2860, 1678 cm^{-1}. MS m/z: 387 (M^+), 313, 100. These ^{1}H-NMR and IR spectra of **22** were identical with those reported.[8] HRMS: Calcd for $C_{20}H_{39}N_2O_5$: 387.2859. Found: 387.2867.

(2*S*,3*R*,4*S*)-2-*tert*-Butoxycarbonylamino-1-cyclohexyl-6-methyl-3,4-pentanediol (23) A mixture of **3**·HCl (47.0 mg, 0.159 mmol), di-*tert*-butyl dicarbonate (70.0 mg, 0.321 mmol), and Et_3N (67 μl, 0.48 mmol) in $CHCl_3$ (1 ml) was stirred at room temperature for 2 h. The mixture was worked up in the same manner as described for the preparation of **22** to give **23** as colorless crystals (45.9 mg, 84%) after purification by column chromatography (C_6H_{14} : EtOAc = 3 : 1→1 : 1). Recrystallization from cyclohexane gave an analytical sample of **23**, mp 134—135 °C and $[\alpha]_D^{20}$ −67.4° (c=1.17, $CHCl_3$) [lit.,[5c] mp 130—131 °C and $[\alpha]_D^{20}$ −64.91° (c=2.20, $CHCl_3$)]. ^{1}H-NMR ($CDCl_3$) δ: 0.80—1.50 (15H, m, $C_6H_{11}CH_2$ and CH_2CHMe_2), 0.89 (3H, d, J=6.6 Hz, CH_3), 0.95 (3H, d, J=6.7 Hz, CH_3), 1.45 (9H, s, $C(CH_3)_3$), 1.94 (1H, m, $CHMe_2$), 3.20 (1H, m, $CH(OH)CH(OH)CH_2$), 3.33 (1H, m, $CH(OH)CH(OH)CH_2$), 4.04 (1H, m, CHN), 4.16 (1H, d, J=4.3 Hz, OH), 4.54 (1H, d, J=9.0 Hz, NH). IR ($CHCl_3$): 3450, 2940, 1680, 1502, 1160 cm^{-1}. These ^{1}H-NMR and IR spectra of **23** were identical with those reported.[5c] MS m/z: 344 ($[M+1]^+$), 288, 226, 57. *Anal.* Calcd for $C_{19}H_{37}NO_4$: C, 66.44; H, 10.86; N, 4.08. Found: C, 66.32; H, 10.77; N, 3.87.

References and Notes

1) Part of this work has been the subject of two preliminary communications. *a*) T. Matsumoto, Y. Kobayashi, Y. Takemoto, Y. Ito, T. Kamijo. H. Harada, and S. Terashima, *Tetrahedron Lett.*, **31**, 4175 (1990); *b*) Y. Kobayashi, K. Nakatani, Y. Ito, and S. Terashima, *Chemistry Lett.*, **1990**, 1709.

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12) At the outset of this work, the addition reaction of cyclohexylmethylmagnesium bromide with 4-*O*-benzyl-2,3-isopropylidene-D-threose (**7**), the precursor of **5**, was extensively studied in various solvents and in the presence of Lewis acids such as cerium(III) chloride, copper(I) iodide–boron trifluoride and zinc(II) chloride. While mixtures of two diastereomeric alcohols could be obtained in 31—70% yields, their formation ratios were found to be unrewarding (45 : 55—77 : 23). T. Matsumoto, Y. Ito, and S. Terashima, unpublished results.

13) While many successful results have so far been reported for the addition reactions of organocerium(III) reagents with various carbonyl compounds,[14] the addition reaction of organocerium(III) reagent with an imine has never been reported.

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16) Although the melting point and optical rotation of **1**·HCl had been reported as mp 172—175 °C and $[\alpha]_D^{23}$ −11.2° (c=2.0, H_2O) by Kiso *et al.*,[3c] an authentic sample of **1**·HCl prepared at Sagami was found to show mp 190 °C (dec.), $[\alpha]_D^{20}$ −12.4° (c=0.482, 1 M HCl), and $[\alpha]_D^{20}$ −12.2° (c=2.05, H_2O).[6] Accordingly, the values in parenthesis reported in ref. 6 were used as the melting point and optical rotation of authentic **1**·HCl.

Studies on the Synthesis of Condensed Pyridazine Derivatives. IV.[1] Synthesis and Anxiolytic Activity of 2-Aryl-5,6-dihydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-ones and Related Compound[2]

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A series of 2-aryl-5,6-dihydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-ones and related compounds were synthesized and evaluated for their ability to displace ^{3}H-diazepam from rat brain membranes *in vitro*, and to prevent bicuculline induced convulsions in mice *in vivo*.

Compounds with a 4′-methoxyphenyl (36) or 4′-chlorophenyl group (37, 39—42) as 2-aryl substituents showed prominent activities in both the *in vitro* and *in vivo* tests. Among them, 2-(4′-chlorophenyl)-5,6-dihydro- (37) and 2-(4′-chlorophenyl)-5,6-dihydro-10-fluoro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-one 7-oxides (41) showed activity twice as potent as diazepam in an anticonflict test (Vogel type, rats) while exhibiting less muscle relaxation (rotarod test, mice) and augmentation of γ-aminobutyric acid-induced chloride current (I_{Cl}) in isolated frog sensory neurones than diazepam. Compound 37 (Y-23684) was selected from this series as a candidate for further development. The structure–activity relationships are discussed.

Keywords benzodiazepine receptor; binding assay; antibicuculline test; anticonflict test; anxiolytic activity; sulfoxide; γ-aminobutyric acid; (1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-one; structure–activity relationship

Since the discovery of chlordiazepoxide and diazepam (DZ), the 1,4-benzodiazepines (BZs) have been the most widely used anxiolytics. In addition to their anxiolytic activity, the BZs possess undesirable effects, *e.g.*, muscle relaxation, sedation, and a hypnotic effect. Recently, research of anxiolytics has focused on discovering potent anxioselective agents[3] which do not exhibit the undesirable effects. Anxioselective activity may be achieved by partial agonists[4] at the BZ receptor (BZR) complex. Most of the ligands which are characterized as partial agonists to BZR are approximately planar shaped,[5] constructing a rigidly condensed ring system; β-carboline,[5c] pyrazolo[4,3-*c*]-quinoline,[6] imidazo[1,5-*a*][1,4]benzodiazepine,[7] triazolo-[4,3-*b*]pyridazine,[8] and imidazo[1,2-*a*]pyrimidine.[9]

We have previously reported that (4a*R**,6*R**)-2-(4-chlorophenyl)-4a,5-dihydro-2*H*-(1)benzothiopyrano[4,3-*c*]pyridazin-3(4*H*)-one 6-oxide (**1**), which has a six–six–six tricyclic condensed ring system, possesses an anxioselective property and behaves as a BZR partial agonist.[10] In the course of the research program on anxioselective compounds, we synthesized 2-aryl-5,6-dihydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-ones and related compounds, which have a six–seven–six tricyclic ring system designated as the general formula **2**. We describe here the detailed

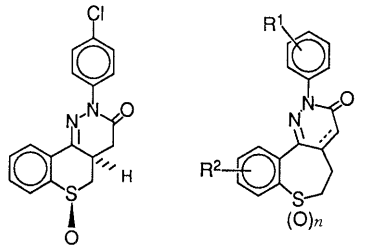

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Chart 1

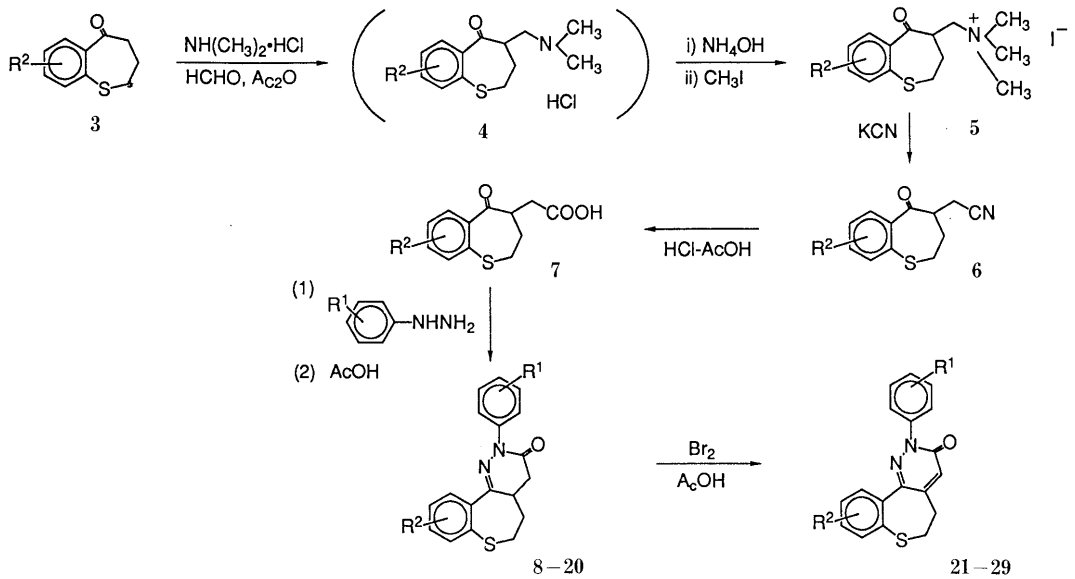

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Chart 2

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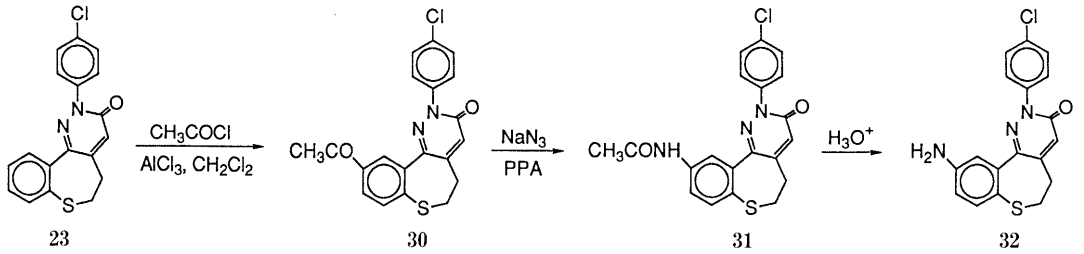


23 30 31 32

Chart 3

synthesis, biological activity, and structure–activity relationships of the (1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-ones (**2**).

Chemistry The 2-aryl-4,4a,5,6-tetrahydro- (**8**—**20**) and 2-aryl-5,6-dihydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-ones (**21**—**29**) were prepared by the synthetic route shown in Chart 2.

The key intermediates **7**, 5-oxo-2,3,4,5-tetrahydro-1-benzothiepin-4-acetic acids, were prepared according to the method in our previous paper.[1] The ketones **3** were converted to the Mannich products **4**. Reaction of **4** with iodomethane gave the quaternary salts **5**, which were converted to the γ-ketonitrile derivatives **6** in a reaction with an aqueous methanolic solution of potassium cyanide. The intermediates **7** were obtained by hydrolysis of **6** (Table IV).

The desired 2-aryl-4,4a,5,6-tetrahydro-(1)benzothiepino-[5,4-*c*]pyridazin-3(2*H*)-ones (**8**—**20**) were furnished by treatment of **7** with an ethanolic solution of phenylhydrazine derivatives followed by cyclization in acetic acid under reflux.

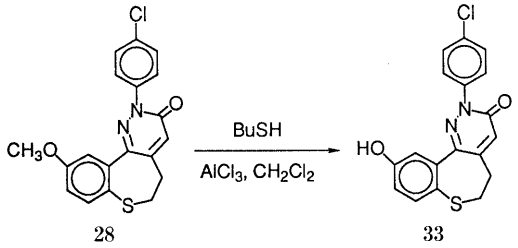
The 2-aryl-5,6-dihydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-ones (**21**—**29**) were prepared by oxidation of the corresponding 4,4a,5,6-tetrahydro compounds with bromine in acetic acid.

The 10-acetyl (**30**), 10-acetylamino (**31**), and 10-amino (**32**) compounds were prepared by the synthetic sequence shown in Chart 3. The Friedel–Crafts reaction of **23** with acetyl chloride in dichloromethane afforded **30**, which underwent the Schmidt rearrangement reaction with sodium azide in polyphosphoric acid (PPA) to give **31**. Compound **32** was obtained from **31** in good yield by hydrolysis with hydrochloric acid under reflux. The 10-hydroxy compound (**33**) was prepared by treating the 10-methoxy compound (**28**) with butylmercaptan and $AlCl_3$ in dichloromethane according to the method of Node *et al.*[11] as shown in Chart 4.

The sulfoxide (7-oxide) derivatives (**35**—**43**) were prepared by oxidation of the corresponding sulfides (**21**—**28**, **30**) with hydrogen peroxide in formic acid (Chart 5).

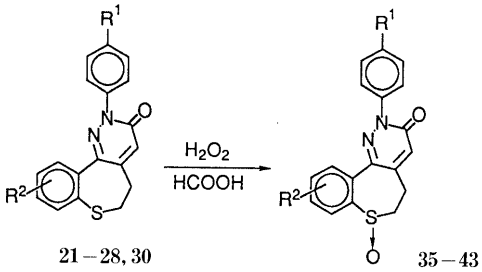
The 3-thioxo compound (**34**) was obtained by treatment of **23** with Lawesson's reagent in benzene (Chart 6). Physicochemical properties of compounds **2** are summarized in Tables I and II.

The structural assignment of compound **37** was also confirmed by X-ray analysis as shown in Fig. 1.



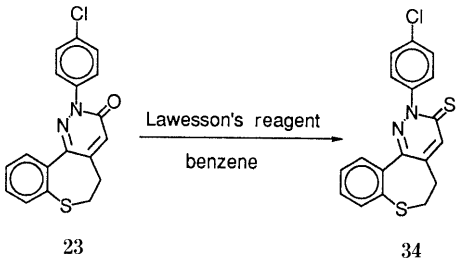
28 33

Chart 4



21–28, 30 35–43

Chart 5



23 34

Chart 6

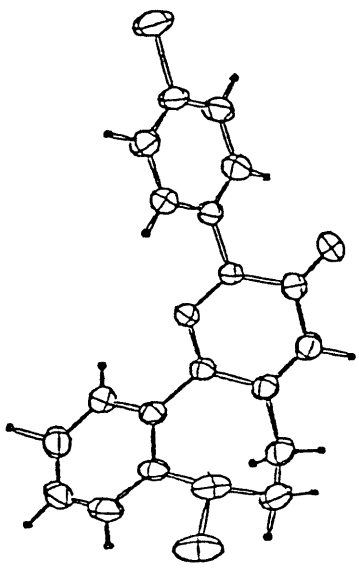
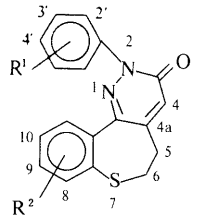


Fig. 1. X-Ray Crystal Structure of **37** by ORTEP Drawing

Results and Discussion

Pharmacology The (1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-ones were primarily examined for their ability to displace ^{3}H-diazepam from rat brain membranes *in vitro* and to prevent bicuculline-induced convulsions (anti-BCL)

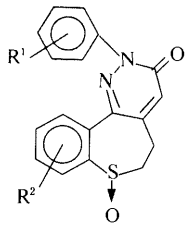
TABLE I. Physicochemical and Biological Data for 2-Aryl-4,4a,5,6-tetrahydro- and 2-Aryl-5,6-dihydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-ones



Compd. No.	R^1	R^2	4–4a[b] Bond	Yield (%)	mp (°C)[c] Recryst. solv.	Formula	Analysis (%) Calcd (Found) C	H	N	[^{3}H]Diazepam binding assay K_i (nM)	Antibicu.[d] ED_{50} mg/kg, *p.o.*
8	4′-OCH_3	H	1	53	143—145 EtOH	$C_{19}H_{18}N_2O_2S$	67.43 (67.48)	5.36 5.38	8.28 8.24)	46	>100
9	H	H	1	67	138–140 EtOH	$C_{18}H_{16}N_2OS$	70.10 (70.27)	5.23 5.25	9.08 9.06)	670	>100
10	4′-Cl	H	1	85	140—142 EtOH	$C_{18}H_{15}ClN_2OS$	63.06 (63.10)	4.41 4.41	8.17 8.04)	70	36.2
11	3′-Cl	H	1	43	122—123 MeOH	$C_{18}H_{15}ClN_2OS$	63.06 (63.09)	4.41 4.35	8.17 8.09)	>1000	N.T.
12	2′-Cl	H	1	18	148—150 EtOH	$C_{18}H_{15}ClN_2OS$	63.06 (62.85)	4.41 4.40	8.17 8.11)	>1000	N.T.
13	4′-CH_3	H	1	53	143—145 EtOH	$C_{19}H_{18}N_2OS$	70.78 (71.07)	5.63 5.63	8.69 8.52)	160	>100
14	4′-Br	H	1	50	135—137 MeOH	$C_{18}H_{15}BrN_2OS$	55.82 (56.06)	3.90 3.80	7.23 7.24)	110	49.9
15	4′-F	H	1	47	146—147 EtOH	$C_{18}H_{15}FN_2OS$	66.24 (65.70)	4.63 4.60	8.58 8.55)	610	>100
16	4′-Cl	10-Cl	1	66	141—143 MeOH	$C_{18}H_{14}Cl_2N_2OS$	57.30 (57.54)	3.74 3.79	7.43 7.48)	200	>100
17	4′-Cl	10-CH_3	1	40	151—153 EtOH	$C_{19}H_{17}ClN_2OS$	63.95 (64.17)	4.80 4.78	7.85 7.80)	180	>100
18	4′-Cl	10-OCH_3	1	64	137—138 EtOH–$CHCl_3$	$C_{19}H_{17}ClN_2O_2S$	61.20 (61.23)	4.60 4.61	7.51 7.42)	38	38.4
19	4′-Cl	10-F	1	47	141—142 EtOH	$C_{18}H_{14}ClFN_2OS$	59.92 (59.93)	3.91 4.02	7.76 7.73)	90	12.5
20	4′-OCH_3	10-Cl	1	34	159—161 EtOH	$C_{19}H_{17}ClN_2OS$	61.20 (61.18)	4.60 4.52	7.51 7.43)	56	>100
21	4′-OCH_3	H	2	80	134—135 EtOH	$C_{19}H_{16}N_2O_2S$	67.84 (67.89)	4.79 4.97	8.33 8.25)	3	4.8
22	4′-Br	H	2	44	208—209 MeOH	$C_{18}H_{13}BrN_2OS$	56.12 (56.27)	3.40 3.58	7.27 7.20)	16	29.5
23	4′-Cl	H	2	88	185—186 EtOH	$C_{18}H_{13}ClN_2OS$	63.43 (63.48)	3.84 3.79	8.22 8.18)	11	5.5
24	4′-Cl	10-Cl	2	80	215—217 EtOH–$CHCl_3$	$C_{18}H_{12}Cl_2N_2OS$	57.61 (57.72)	3.22 3.21	7.47 7.53)	11	22.0
25	4′-Cl	9-Cl	2	88	191—193 EtOH–$CHCl_3$	$C_{18}H_{12}Cl_2N_2OS$	57.61 (57.52)	3.22 3.47	7.47 7.40)	63	7.9
26	4′-Cl	8-Cl	2	50	166—168 EtOH	$C_{18}H_{12}Cl_2N_2OS$	57.61 (57.72)	3.22 3.24	7.47 7.40)	29	29.4
27	4′-Cl	10-F	2	80	162—163 IPA	$C_{18}H_{12}ClFN_2OS$	60.25 (60.38)	3.37 3.35	7.81 7.80)	14	2.2
28	4′-Cl	10-OCH_3	2	87	205—206 MeOH	$C_{19}H_{15}ClN_2O_2S$	61.55 (61.79)	4.08 4.15	7.55 7.55)	2.3	8.1
29	4′-Cl	10-CH_3	2	67	182—184 EtOH	$C_{19}H_{15}ClN_2OS \cdot 1/2H_2O$	62.72 (62.54)	4.44 4.05	7.70 7.58)	13	19.7
30	4′-Cl	10-Ac	2	47	178—179 EtOH–$CHCl_3$	$C_{20}H_{15}ClN_2O_2S \cdot 1/3H_2O$	61.77 (61.79)	4.06 3.92	7.20 7.15)	22	11.4
31	4′-Cl	10-NHAc	2	71	258—260 EtOH–$CHCl_3$	$C_{20}H_{16}ClN_3O_2S$	60.37 (60.08)	4.05 3.96	10.56 10.50)	750	>100
32	4′-Cl	10-NH_2	2	67	242—244 EtOH–$CHCl_3$	$C_{18}H_{14}ClN_3OS$	60.76 (60.63)	3.97 3.86	11.81 11.81)	30	>100
33	4′-Cl	10-OH	2	65	233—235 MeOH	$C_{18}H_{13}ClN_2O_2S$	60.59 (60.31)	3.67 3.69	7.85 7.64)	17	>100
34[e]	4′-Cl	H	2	64	258—259 $CHCl_3$–EtOH	$C_{18}H_{13}ClN_2S_2$	60.58 (60.64)	3.67 3.63	7.85 7.42)	490	>100

a) Ac, acetyl; AcNH, acetylamino. *b*) 1, single bond; 2, double bond. *c*) IPA, isopropyl alcohol. *d*) Antibicu., antibicuculline. N.T., no test. *e*) 3-Thioxo compound.

TABLE II. Physicochemical and Biological Data for 2-Aryl-5,6-dihydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-one 7-Oxides



Compd. No.	R^1	$R^{2a)}$	Yield (%)	mp (°C) Recryst. solv.	Formula	Analysis (%) Calcd (Found) C	H	N	[^{3}H]Diazepam binding assay K_i (nM)	Antibicu.[b] ED_{50} mg/kg, *p.o.*
35	4′-Br	H	73	227 MeOH–$CHCl_3$	$C_{18}H_{13}BrN_2O_2S$	53.88 (53.81)	3.27 3.31	6.98 7.00)	34	5.9
36	4′-OCH_3	H	86	200—201 MeOH	$C_{19}H_{16}N_2O_3S$	64.76 (64.77)	4.58 4.75	7.95 7.82)	18	0.7
37	4′-Cl	H	74	233—234 EtOH	$C_{18}H_{13}ClN_2O_2S$	60.59 (60.79)	3.67 3.64	7.85 7.86)	41	1.2
38	4′-Cl	10-Cl	40	239—241 MeOH	$C_{18}H_{12}Cl_2N_2O_2S$	55.26 (55.47)	3.10 3.30	7.16 7.02)	11	3.2
39	4′-Cl	9-Cl	78	208—209 EtOH	$C_{18}H_{12}Cl_2N_2O_2S$	55.26 (55.17)	3.10 3.35	7.16 7.06)	97	0.8
40	4′-Cl	8-Cl	78	217—219 MeOH	$C_{18}H_{12}Cl_2N_2O_2S$	55.26 (55.01)	3.10 3.08	7.16 7.09)	11	0.7
41	4′-Cl	10-F	77	233—235 MeOH	$C_{18}H_{12}ClFN_2O_2S$	57.68 (57.69)	3.23 3.26	7.47 7.44)	36	0.7
42	4′-Cl	10-OCH_3	48	209—210 MeOH	$C_{19}H_{15}ClN_2O_3S$	59.00 (58.80)	3.91 4.14	7.24 7.12)	3.9	1.0
43	4′-Cl	10-Ac	53	231—232 EtOH–$CHCl_3$	$C_{20}H_{15}ClN_2O_3S \cdot 1/3H_2O$	59.33 (59.31)	3.90 3.73	6.92 6.83)	40	7.9
Diazepam									5.8	0.4

a) Ac, acetyl. *b*) Antibicu., antibicuculline.

TABLE III. Comparative Biological Data for Representative Compounds

Compd. No.	[^{3}H]Diazepam binding K_i (nM)	Antibicuculline ED_{50} mg/kg, *p.o.*	Anticonflict (Water-lick test) MED mg/kg, *p.o.*	Rota rod ED_{50} mg/kg, *p.o.*	Ratio (Rotarod/anticonflict)	Relative I_{cl}
36	18	0.7	25	157	6	1.54
37	41	1.2	5	158	32	1.64
39	97	0.8	5	35	7	1.61
40	11	0.7	25	>250	>10	1.75
41	36	0.7	5	107	21	1.75
42	3.9	1.0	10	N.T.[a]		1.91
Diazepam	5.8	0.4	10	1.6	0.2	2.40

a) N.T., no test.

in mice *in vivo* (Tables I and II).

Compounds that exhibited activity in both of the primary screens were further studied by the following assays: anticonflict test (water-lick test),[12] rotarod test,[13] and electrophysiological test on the γ-aminobutyric acid (GABA)-induced chloride current (concentration-clamp technique).[14] The results are summarized in Table III.

The tests described above were selected in order to evaluate anxioselectivity. The anticonvulsant properties of the compounds were evaluated by using the $GABA_A$ receptor antagonist bicuculline. The anticonflict test was adapted from Vogel *et al.*,[12] and is considered to be a reliable method for identifying potential anxiolytic activity. The concentration-clamp technique appears to be useful for evaluating the efficacy of the compounds on the responses mediated by the GABA receptor complex.[14b] The details of these tests are given in the experimental section.

Structure–Activity Relationships The K_i (nM) values and anti-BCL activity (ED_{50} values) were used as preliminary selection criteria and a probe for further synthesis. Among the 2-aryl-4,4a,5,6-tetrahydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-ones (**8**—**15**), compounds which had such substituents for R^1 as a 4′-chloro, 4′-bromo, 4′-methoxy, or 4′-methyl group on the phenyl ring at the 2-position revealed an appreciable affinity for BZR. The unsubstituted compound (**9**) and 4′-fluoro compound (**15**) had a reduced affinity for BZR. In contrast, the 3′-chloro compound (**11**) and 2′-chloro compound (**12**) did not exhibit BZR affinity ($K_i > 10^3$ nM). The 4′-methoxy compound (**8**) showed the highest affinity to BZR among **8**—**15**.

In the *in vivo* test, 4′-chloro (**10**) and 4′-bromo (**14**) compounds showed anti-BCL activities. However, com-

pounds with other substituents for R^1 were inactive in the anti-BCL test, showing no apparent correlation to the affinity to BZR.

Compounds (**16**—**20**) were examined regarding how the substituent R^2 on the benzothiepine ring affected pharmacological activity. The 10-methoxy compound (**18**) seemed to have the best K_i value among them, though a remarkable improvement over the activity of the unsubstituted counterpart (**10**) was not observed. In the anti-BCL test, the 10-fluoro compound (**19**) showed more enhanced activity than compound **10**.

On the basis of the above mentioned structure–activity studies on the 4–4a single bond (s-type) series (**8**—**20**), the 2-aryl-5,6-dihydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-ones (**21**—**33**) having a 4–4a double bond (d-type) were then tested to determine whether the modification of the same series of compounds could improve the affinity to BZR and anti-BCL activity. The d-type compounds (**21**—**24**, **27**—**29**) exhibited a higher affinity to BZR than the corresponding s-type compounds. Among the chloro-substituted compounds at the 8, 9 or 10-position as R^2, the 10-chloro compound (**24**) showed the highest affinity for BZR. The 10-acetyl (**30**), 10-amino (**32**), and 10-hydroxy (**33**) compounds also revealed a high affinity for BZR, whereas the 10-acetylamino compound (**31**) showed low affinity. Among these d-type compounds, the K_i values of compound **21** with a 4′-methoxy group as R^1, and **28** with a 10-methoxy group as R^2, were superior to that of DZ.

Moreover, the d-type compounds revealed a marked improvement in *in vivo* anti-BCL activity. Compounds with a 4′-chloro group as R^1 (**23**—**30**) exhibited remarkable anti-BCL activity. Among them, the unsubstituted compound (**23**), or compounds with a 9-chloro (**25**), 10-fluoro (**27**) or 10-methoxy (**28**) group as R^2 had an oral ED_{50} of less than 10 mg/kg in the anti-BCL test. The 10-amino (**32**) and 10-hydroxy (**33**) compounds, however, did not show *in vivo* activity despite a high affinity for BZR. These results might be attributed to the low penetration of both compounds (**32**, **33**) through the gastrointestinal membrane and/or the blood-brain barrier. The 4′-bromo compound (**22**) as R^1 exhibited moderate activity in the anti-BCL test compared with the 4′-chloro compounds. The 4′-methoxy compound (**21**) showed prominent anti-BCL activity with an oral ED_{50} of 4.8 mg/kg. The thioxo compound (**34**) at the 3-position showed reduced affinity to BZR and exhibited no activity *in vivo*. These results suggest that the carbonyl oxygen at the 3-position in the (1)benzothiepino-[5,4-*c*]pyridazine skeleton is an essential moiety to interact with the BZR complex, for instance, a hydrogen bond acceptor.[5c]

On the basis of our previous data,[10] compounds with a sulfoxide group at 7-position (**35**—**43**) were synthesized in order to improve the *in vivo* activity of the d-type compounds having potent anti-BCL activity. All sulfoxides synthesized showed 3 to 30 times stronger activity in the anti-BCL test and tended to have less BZR affinity than the corresponding d-type compounds. Compounds with a 4′-chloro group as R^1 in combination with no substituent (**37**) or a 9-chloro (**39**), 8-chloro (**40**), 10-fluoro (**41**), or 10-methoxy (**42**) substituent as R^2 showed prominent anti-BCL activity with an oral ED_{50} of 0.7 to 1.2 mg/kg. The 4′-methoxy compound (**36**) as R^1 also revealed remarkable anti-BCL activity (ED_{50} = 0.7 mg/kg). Also, the solubility of the sulfoxide **37** in water at 25 °C was 1×10^{-3} w/v%, being 10 times higher than that of the corresponding sulfide **23** (1×10^{-4} w/v%). Therefore, it is suggested that the enhanced *in vivo* activity of these sulfoxides reflects their improved oral bioavailability.

Table III gives comparative biological data for the compounds (**36**, **37**, **39**—**42**) chosen for further studies in comparison with those for DZ. Compound **37**, **39** and **41** among those selected showed a remarkable improvement in the ratio of minimum effective dose (MED) values in a model of antianxiety (anticonflict test) to ED_{50} values of muscle relaxant effects (rotarod test) when compared to DZ, as shown in Table III. This data suggests that the above listed compounds have more potent anxiolytic effects and weaker muscle relaxant properties.

Recent electrophysiological studies[14] by use of a concentration-clamp technique in isolated frog sensory neurones showed that (1) all full agonists to BZR increased the peak amplitude of chloride current (I_{cl}) elicited by GABA, and (2) partial agonists showed a dose-dependent augmentation of the GABA response, although at about half the amplitude of full agonists. In the present study, the relative I_{cl} value for DZ was 2.40, but the representative compounds showed reduced efficacy with relative I_{cl} values of 1.54 to 1.91. This data suggests that these compounds have partially agonistic properties.

Compounds **37** and **41** were judged to have especially anxioselective properties because these compounds had higher efficacy in the anticonflict test, more reduced activities in the muscle relaxation test, and a lower I_{cl} value than DZ.

Based on the favorable characteristics described above, as well as on detailed biological[15] and toxicological[16] evaluations, 2-(4′-chlorophenyl)-5,6-dihydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-one 7-oxide (**37**, Y-23684) was selected as a candidate for further development. Y-23684 is currently undergoing additional studies to further evaluate its potential as an anxioselective anxiolytic agent, namely as a BZR partial agonist.

Experimental

Melting points were determined on a Buchi 530 melting point apparatus and are uncorrected. Infrared (IR) spectra were taken on a JASCO IR-810 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL-FX100 spectrometer unless otherwise noted and chemical shifts were given in ppm with tetramethylsilane as an internal standard. Mass spectra (MS) were measured with a JEOL JMS-01SG-2 spectrometer.

2,3,4,5-Tetrahydro-1-benzothiepin-5-ones (3) 7-Chloro (**3c**),[17] 7-methyl (**3d**),[18] 7-methoxy (**3e**),[19] 7-fluoro (**3f**)[17] and unsubstituted (**3g**)[20] compounds were prepared according to the published methods. 9-Chloro (**3a**) and 8-chloro (**3b**) compounds were synthesized by treating 2-chlorothiophenol and 3-chlorothiophenol, respectively, with γ-butyrolactone in ethanolic sodium ethoxide, followed by cyclization with PPA according to the method of Traynelis and Love.[20]

9-Chloro-2,3,4,5-tetrahydro-1-benzothiepin-5-one (**3a**): Colorless prisms (from hexane), mp 71—72 °C. *Anal.* Calcd for $C_{10}H_9ClOS$: C, 56.47; H, 4.26. Found: C, 56.62; H, 4.35. IR ν_{max}^{KBr} cm^{-1}: 1680 (C=O). MS *m/z*: 212 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.20—2.47 (2H, m), 2.96—3.10 (4H, m), 7.15 (1H, t, *J*=8 Hz, ArH), 7.45 (1H, dd, *J*=2, 8 Hz, ArH), 7.71 (1H, dd, *J*=2, 8 Hz, ArH).

8-Chloro-2,3,4,5-tetrahydro-1-benzothiepin-5-one (**3b**): Oil. *Anal.* Calcd for $C_{10}H_9ClOS$: C, 56.47; H, 4.26. Found: C, 56.75; H, 4.15. IR $\nu_{max}^{liq.film}$ cm^{-1}: 1680 (C=O). MS *m/z*: 212 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.13—2.42 (2H, m), 2.93—3.12 (4H, m), 7.21 (1H, dd, *J*=2, 8 Hz, ArH), 7.47 (1H, d, *J*=2 Hz, ArH), 7.78 (1H, d, *J*=8 Hz, ArH).

5-Oxo-2,3,4,5-tetrahydro-1-benzothiepin-4-acetic Acids (7) A typical example is given to represent the general procedure.

9-Chloro-2,3,4,5-tetrahydro-4-trimethylammoniomethyl-1-benzothiepin-5-one Iodide (**5a**): A solution of 11.3 g (0.139 mol) of dimethylamine hydrochloride in 11.3 g (0.139 mol) of 37% HCHO was stirred at room temperature for 0.5 h. Acetic anhydride (50 ml) was added dropwise at 70—90 °C and the mixture was then kept at 70—75 °C for 1 h. To the solution was added **3a** (20 g, 0.094 mol), and the mixture was stirred at 70—75 °C for 3 h. After removal of the solvent under reduced pressure, acetone (50 ml) and isopropylether (50 ml) were added to the residue and the mixture was allowed to stand at room temperature for 3 h. The crystals formed were collected by filtration, dissolved in chilled water, neutralized with 28% NH_4OH (12 ml), and extracted by $CHCl_3$. The extract was washed with water, dried over $MgSO_4$, and evaporated below 40 °C. The residue was dissolved in acetone (150 ml), and iodomethane (17 g, 0.12 ml) was added to the resulting solution below 5 °C in an ice bath. After the mixture was allowed to stand at room temperature for 3 h, the crystals formed were collected by filtration and washed with acetone to give **5a** (31 g, 81%). Recrystallization from EtOH gave colorless needles, mp 213—214 °C (dec.). *Anal.* Calcd for $C_{14}H_{19}ClIOS$: C, 40.84; H, 4.65; N, 3.40. Found: C, 40.93; H, 4.71; N, 3.39. IR ν_{max}^{KBr} cm^{-1}: 1680 (C=O). ^{1}H-NMR (DMSO-d_6) δ: 1.80—2.73 (3H, m), 2.96 (9H, s, $N(CH_3)_3$), 3.36—3.51 (2H, m), 3.81—4.24 (2H, m), 7.30 (1H, t, J=8 Hz, ArH), 7.56 (1H, dd, J=2, 8 Hz, ArH), 7.67 (1H, dd, J=2, 8 Hz, ArH).

9-Chloro-5-oxo-2,3,4,5-tetrahydro-1-benzothiepin-4-acetonitrile (**6a**): To a solution of **5a** (20.6 g, 0.05 mol) in methanol (300 ml) was added a solution of KCN (3.9 g, 0.06 mol) in water (50 ml) dropwise at room temperature. The solution was stirred at room temperature for 1 h and poured into ice-water. The resulting mixture was extracted with AcOEt. The extract was washed with water, dried over $MgSO_4$, and concentrated *in vacuo*. After the addition of isoproryl alcohol (IPA) to the residue, the crystals formed were collected by filtration and recrystallized from EtOH to afford **6a** (10.5 g, 83%) as colorless needles, mp 115—116 °C. *Anal.* Calcd for $C_{12}H_{10}ClNOS$: C, 57.27; H, 4.01; N, 5.56. Found: C, 57.27; H, 4.06; N, 5.55. IR ν_{max}^{KBr} cm^{-1}: 1680 (C=O), 2240 (CN). MS m/z: 251 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 1.96—2.24 (1H, m), 2.33—2.95 (4H, m), 3.11—3.44 (1H, m), 3.70—4.02 (1H, m), 7.18 (1H, t, J=8 Hz, ArH), 7.49 (1H, dd, J=2, 8 Hz, ArH), 7.75 (1H, dd, J=2, 8 Hz, ArH).

9-Chloro-5-oxo-2,3,4,5-tetrahydro-1-benzothiepin-4-acetic Acid (**7a**): To a solution of conc. HCl (10 ml) and acetic acid (10 ml) was added **6a** (5 g, 0.02 mol). The solution was refluxed for 3 h, and poured into ice-water. The precipitate was collected by filtration, washed with water, and recrystallized from IPA to give **7a** (4.3 g, 80%) as colorless needles, mp 195—197 °C. IR ν_{max}^{KBr} cm^{-1}: 1680 (C=O), 1700 (COOH). MS m/z: 270 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 1.87—3.32 (6H, m), 3.73—4.06 (1H, m), 7.17 (1H, t, J=8 Hz, ArH), 7.45 (1H, dd, J=2, 8 Hz, ArH), 7.63 (1H, dd, J=2, 8 Hz, ArH).

Other compounds (**7b—g**) in Table IV were similarly prepared from the corresponding **3b—g**.

2-Aryl-4,4a,5,6-tetrahydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-ones (8—20) A typical example is given to represent the general procedure.

Method A: 2-(4′-Methoxyphenyl)-4,4a,5,6-tetrahydro-(1)benzothiepino-[5,4-*c*]pyridazin-3(2*H*)-one (**8**): A mixture of **7g** (4 g, 0.017 mol), 4-methoxyphenylhydrazine hydrochloride (2.2 g, 0.02 mol), and sodium acetate (1.8 g, 0.022 mol) in EtOH (100 ml) was refluxed overnight. After evaporation of the solvent, the residue was dissolved in acetic acid (50 ml). The mixture was refluxed for 2 h, poured into ice-water, and extracted with AcOEt. The extract was washed successively with aq. saturated $NaHCO_3$, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was chromatographed on a silica gel column using $CHCl_3$ as an eluent to give **8** (3.0 g, 53%), which was recrystallized from EtOH to give colorless needles, mp 143—145 °C. IR ν_{max}^{KBr} cm^{-1}: 1680 (CON). MS m/z: 338 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.00—3.09 (6H, m), 3.57—3.84 (1H, m), 3.82 (3H, s, OCH_3), 6.93 (2H, d, J=9 Hz, ArH), 7.17—7.39 (3H, m, ArH), 7.72—7.81 (1H, m, ArH).

Other compounds (**9—20**) in Table I were prepared in a similar manner.

2-Aryl-5,6-dihydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-ones (21—29) Typical examples are given to represent the general procedure.

Method B: 2-(4′-Chlorophenyl)-5,6-dihydro-(1)benzothiepino[5,4-*c*]-pyridazin-3(2*H*)-one (**23**): To a solution of **10** (16.4 g, 0.048 mol) in acetic acid (500 ml), bromine (9.2 g, 0.057 mol) was added dropwise at 40—50 °C. The reaction mixture was stirred at 40—45 °C for 1 h and poured into ice-water. The precipitate was collected by filtration, washed with water, and recrystallized from EtOH to give **23** (14.4 g, 88%) as colorless needles. mp 185—186 °C. IR ν_{max}^{KBr} cm^{-1}: 1680 (C=O). MS m/z: 340 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.73 (2H, t, J=7 Hz, $SCH_2\underline{CH_2}$), 3.26 (2H, t, J=7 Hz, $S\underline{CH_2}CH_2$), 6.89 (1H, s, C=C$\underline{H}$CO), 7.43 (2H, d, J=9 Hz, ArH), 7.38—7.64 (4H, m, ArH), 7.70 (2H, d, J=9 Hz, ArH).

Other compounds (**21**, **22**, **24**, **27—29**) in Table I were prepared in a similar manner.

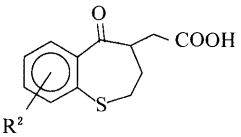
Method C: 9-Chloro-2-(4′-chlorophenyl)-5,6-dihydro-(1)benzothiepino-[5,4-*c*]pyridazin-3(2*H*)-one (**25**): A mixture of **7b** (7 g, 0.026 mol), 4-chlorophenylhydrazine hydrochloride (6.1 g, 0.034 mol), and sodium acetate (2.9 g, 0.034 mol) in EtOH (100 ml) was refluxed overnight. Work-up in a manner similar to that described in method A gave the crude intermediate 9-chloro-2-(4′-chlorophenyl)-4,4a,5,6-tetrahydro-(1)-benzothiepino[5,4-*c*]pyridazin-3(2*H*)-one (**7g**) as a pale yellow oil. To a solution of this crude intermediate in acetic acid (100 ml), bromine (3.3 g, 0.021 mol) was added dropwise at 40—45 °C. Work-up as in method B gave **25** (6.1 g, 63%) as a colorless powder (from EtOH–$CHCl_3$), mp 191—193 °C. IR ν_{max}^{KBr} cm^{-1}: 1680 (C=O). MS m/z: 375 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.73 (2H, t, J=7 Hz, $SCH_2\underline{CH_2}$), 3.27 (2H, J=7 Hz, $S\underline{CH_2}CH_2$), 6.70 (1H, s, C=C$\underline{H}$CO), 7.42 (2H, d, J=9 Hz, ArH), 7.42—7.68 (3H, m, ArH), 7.67 (2H, d, J=9 Hz, ArH).

Another compound (**26**) in Table I was prepared in a similar manner.

10-Acetyl-2-(4′-chlorophenyl)-5,6-dihydro-(1)benzothiepino[5,4-*c*]-pyridazin-3(2*H*)-one (30) To an ice-cooled solution of $AlCl_3$ (26 g, 0.19 mol) in CH_2Cl_2 (200 ml) was added acetylchloride (10 g, 0.13 mol) and the mixture was stirred at 0—10 °C for 0.5 h. After addition of **23** (20 g, 0.059 mol), the mixture was refluxed for 7 h and then poured onto crushed ice. The resulting mixture was extracted with CH_2Cl_2. The extract was washed with water, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was chromatographed on a silica gel column using $CHCl_3$ as an eluent to give **30** (10.5 g, 47%), which was recrystallized from MeOH to afford a colorless powder, mp 178—179 °C. IR ν_{max}^{KBr} cm^{-1}: 1670 (C=O), 1685 (C=O). MS m/z: 382 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.63 (3H, s, $COCH_3$), 2.75 (2H, t, J=7 Hz, $SCH_2\underline{CH_2}$), 3.31 (2H, t, J=7 Hz, $S\underline{CH_2}CH_2$), 6.92 (1H, s, C=CHCO), 7.43 (2H, d, J=9 Hz, ArH), 7.68 (2H, d, J=9 Hz, ArH), 7.71 (1H, d, J=8 Hz, ArH), 7.97 (1H, dd, J=2, 8 Hz, ArH), 8.12 (1H, d, J=2 Hz, ArH).

10-Acetylamino-2-(4′-chlorophenyl)-5,6-dihydro-(1)benzothiepino[5,4-*c*]-pyridazin-3(2*H*)-one (31) To a suspension of **30** (5.4 g, 0.014 mol) in PPA (55 g) was added NaN_3 (1.4 g, 0.021 mol) by portions, and the mixture was stirred at room temperature for 2 h. The mixture was poured into ice-water and extracted with $CHCl_3$. The extract was washed successively with aq. saturated $NaHCO_3$, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was recrystallized from EtOH–$CHCl_3$ to give **31** (4.0 g, 71%) as a colorless powder, mp 258—260 °C. IR ν_{max}^{KBr} cm^{-1}: 1670 (C=O), 3300 (CONH). MS m/z: 397 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.12 (3H, s,

TABLE IV. Physicochemical Data for 5-Oxo-2,3,4,5-tetrahydro-1-benzothiepin-4-acetic Acids



Compd. No.	R^2	Yield[a] (%)	mp (°C) Recryst. solv.[b]	Formula	Analysis (%) Calcd (Found) C	H
7a	9-Cl	57	195—197 IPA	$C_{12}H_{11}ClO_3S$	53.23 (53.02)	4.10 3.89)
7b	8-Cl	54	144—147 IPA	$C_{12}H_{11}ClO_3S$	53.23 (53.48)	4.10 4.20)
7c	7-Cl	53	205—207 EtOH	$C_{12}H_{11}ClO_3S$	53.23 (53.26)	4.10 4.20)
7d	7-CH_3	46	203—205 IPA	$C_{13}H_{14}O_3S$	62.38 (62.57)	5.64 5.75)
7e	7-OCH_3	42	204—206 IPA	$C_{13}H_{14}O_4S$	58.63 (58.78)	5.30 5.37)
7f[c]	7-F	69	193—195 IPA			
7g[c]	H	67	171—173 EtOH			

a) Yield from **3**. *b*) IPA, isopropyl alcohol. *c*) Lit. (reference 1).

$COCH_3$), 2.70 (2H, t, $J=7$ Hz, SCH_2CH_2), 3.20 (2H, t, $J=7$ Hz, SCH_2CH_2), 6.87 (1H, s, C=CHCO), 7.38 (2H, d, $J=9$ Hz, ArH), 7.64 (2H, d, $J=9$ Hz, ArH), 7.54—7.75 (2H, m, ArH), 7.93 (1H, s, ArH), 7.25—7.75 (1H, br, CONH).

10-Amino-2-(4′-chlorophenyl)-5,6-dihydro-(1)benzothiepino[5,4-*c*]-pyridazin-3(2*H*)-one (32) A mixture of **31** (8.9 g, 0.022 mol) and conc. HCl (90 ml) in EtOH (150 ml) was refluxed for 1 h. The resulting solution was poured into ice-water, neutralized with K_2CO_3, and extracted with $CHCl_3$. The extract was washed with water, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was recrystallized from EtOH–$CHCl_3$ to give **32** (5.2 g, 67%) as a colorless powder, mp 242—244 °C. IR ν_{max}^{KBr} cm^{-1}: 1670 (C=O), 3330 (NH_2), 3460 (NH_2). MS *m/z*: 355 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.70 (2H, t, $J=7$ Hz, SCH_2CH_2), 3.15 (2H, t, $J=7$ Hz, SCH_2CH_2), 4.04 (2H, br s, NH_2), 6.71 (1H, dd, $J=3$, 8 Hz, ArH), 6.87 (1H, s, C=CHCO), 6.91 (1H, d, $J=3$ Hz, ArH), 7.38 (1H, d, $J=8$ Hz, ArH), 7.42 (2H, d, $J=9$ Hz, ArH), 7.69 (2H, d, $J=9$ Hz, ArH).

2-(4′-Chlorophenyl)-5,6-dihydro-10-hydroxy-(1)benzothiepino[5,4-*c*]-pyridazin-3(2*H*)-one (33) To a suspension of $AlCl_3$ (5 g, 0.038 mol) in CH_2Cl_2 (30 ml), butyl mercaptan (5 ml) was added at 0—5 °C and stirred for on additional 0.5 h. After the addition of **28** (5 g, 0.013 mol), the mixture was stirred at room temperature for 0.5 h and then poured into ice-water. The precipitate was collected by filtration, washed with water, and recrystallized from MeOH to give **33** (3.1 g, 65%) as colorless needles, mp 233—235 °C. IR ν_{max}^{KBr} cm^{-1}: 1660 (C=O), 3200 (OH). MS *m/z*: 356 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.71 (2H, t, $J=7$ Hz, SCH_2CH_2), 3.19 (2H, t, $J=7$ Hz, SCH_2CH_2), 3.65 (1H, br s, OH), 6.88 (1H, dd, $J=3$, 8 Hz, ArH), 6.89 (1H, s, C=CHCO), 7.08 (1H, d, $J=3$ Hz, ArH), 7.42 (2H, d, $J=9$ Hz, ArH), 7.49 (1H, d, $J=8$ Hz, ArH), 7.68 (2H, d, $J=9$ Hz, ArH).

2-Aryl-5,6-dihydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-one 7-Oxides (35—43) A typical example is given to represent the general procedure.

2-(4′-Chlorophenyl)-5,6-dihydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-one 7-Oxide (**37**): To a stirred solution of **23** (6.5 g, 0.019 mol) in formic acid (65 ml) was added dropwise 30% H_2O_2 (2.2 g, 0.019 mol) below 5 °C. The mixture was stirred at 5—10 °C for 1 h and poured into ice-water. The precipitate was collected by filtration and washed with water. Recrystallization from EtOH gave **37** (5.0 g, 74%) as colorless needles, mp 233—234 °C. IR ν_{max}^{KBr} cm^{-1}: 1680 (C=O). MS *m/z*: 356 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.70—3.25 (3H, m), 3.83—4.11 (1H, m), 6.97 (1H, s, C=CHCO), 7.44 (2H, d, $J=9$ Hz, ArH), 7.60—7.82 (5H, m, ArH), 7.90—7.97 (1H, m, ArH).

Other compounds (**35**, **36**, **38**—**43**) in Table II were prepared in a similar manner.

2-(4′-Chlorophenyl)-5,6-dihydro-(1)benzothiepino[5,4-*c*]pyridazin-3(2*H*)-thione (34) A mixture of **23** (9 g, 0.026 mol) and the Lawesson's reagent (5.7 g, 0.014 mol) in benzene (100 ml) was refluxed for 7 h. After the mixture was allowed to stand at room temperature for 3 h, the precipitated solid was collected by filtration and recrystallized from EtOH–$CHCl_3$ to give **34** (6.0 g, 64%) as a yellow powder, mp 258—259 °C. MS *m/z*: 356 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.71 (2H, t, $J=7$ Hz, SCH_2CH_2), 3.28 (2H, t, $J=7$ Hz, SCH_2CH_2), 7.36—7.68 (8H, m, ArH), 7.76 (1H, s, C=CHCS).

Benzodiazepine Receptor Binding Assay Preparation of a synaptosome fraction and ^{3}H-diazepam (^{3}H-DZ) binding studies were carried out according to the method of Mohler and Okada.[21] Crude synaptosomal membranes were suspended in a 50 mM Tris–HCl buffer (pH 7.4) containing 120 mM NaCl and 5 mM KCl. The reaction was started by the addition of a 900 μl aliquot of crude synaptosomal membranes to 100 μl solution containing ^{3}H-DZ (final concentration was 2 nM) and a known concentration of test compounds. After the mixture was incubated for 20 min at 0 °C, the binding was stopped by adding 3 ml of ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 120 mM NaCl and 5 mM KCl. The samples were then filtered under vacuum through Whatman GF/B filters and immediately washed 4 times with 3 ml of ice-cold buffer. The radioactivity on the filters was measured by a liquid scintillation counter. Binding in the presence of 1 μM unlabelled DZ was defined as nonspecific binding. Specific binding was defined as the difference between the total binding and the nonspecific binding. The experiments were carried out in triplicate. The K_i values were determined by the relationship $K_i = IC_{50}/(1+c/K_d)$, where IC_{50} was the concentration of the test compounds which caused a 50% reduction of the specific binding *vs.* the control, c was the concentration of ^{3}H-DZ (2 nM), and K_d was the dissociation constant determined by Scatchard's plot.

Anticonvulsant Test (Antibicuculline Test) The experiment was practiced by a modification of the method of Lippa and Regan.[22] Groups of 7—14 ddY male mice were challenged with bicuculline (0.6 mg/kg i.v.) 1 h after theoral administration of the test compounds. The ED_{50} values were calculated by the probit method as the dose which prevented tonic extension in half of the animals.

Anticonflict Test (Water-Lick Test) The experiment was carried out by a modification of the method of Vogel *et al.*[13] Groups of 10—14 Wister rats were deprived of water for 72 h before the tests began. The rats were placed in a plexiglass conflict test box (light compartment: 38 × 38 × 20 cm, dark compartment: 10 × 10 × 20 cm). A water bottle with a stainless steel spout was fitted to the middle of the outside so that the spout extended 3 cm into the box at 10 cm above the grid floor. A drinkometer circuit (Ohara Inc., Nihon Koden) was connected to the spout and the number of licks was counted. The rats were placed in the apparatus where an electric shock (0.2—0.3 mA, 0.3 s) was given once every 20th lick. After the rats were received the first electric shock, the number of shocks were recorded during the subsequent 3-min test period. The test compounds were administered orally 1 h before the test. The MED was defined as the lowest dose to produce a statistically significant difference in the punished responses from 0.5% methylcellusolve-treated (One-way Anova test; $p<0.05$).

Rotarod Test The experiment was carried out according to the method described by Dunham and Miya.[13] Groups of 10 ddY male mice were used. The mice were gently placed on the rod (2.8 cm in diameter rotating at 11 rpm.) 1 h after oral administration of the test compounds. The ED_{50} value was calculated by the probit method as the dose which caused half of the animals to drop from the rotarod within 1 min.

GABA-Induced Response in Isolated Sensory Neurones of Frogs Sensory neurons of frogs were isolated, and electrophysical experiments were carried out according to the method of Akaike *et al.*[14] Neurones were voltage-clamped at a holding membrane potential of −50 mV with a single-electrode voltage-clamp system. Test compounds were applied by using a concentration-clamp technique. When the peak Cl^- current (I_{Cl}) elicited by 3×10^{-6} M GABA alone was presented as 1, the augmentative I_{Cl} of the test compounds in the presence of 3×10^{-6} M GABA was measured. The results were presented as relative I_{Cl} values.

X-Ray Crystallographic Analysis A crystal of **37** for X-ray crystallographic analysis was obtained from a methanol solution as colorless prisms. The crystal data is as follows: $C_{18}H_{13}ClN_2O_2S$, monoclinic, $P2_1/c$, $a=14.677$ (2), $b=6.946$ (1), $c=16.157$ (2) Å, $\beta=104.56$ (1), $V=1594.2$ (4) Å^3, $Z=4$, D(Calcd) = 1.49, μ(Cu$K\alpha$) = 34.60, F(000) = 736. Intensities were collected on an Enraf-Nonius CAD4 diffractometer with graphite-monochromated Cu$K\alpha$ radiation ($\lambda=1.5418$ Å). 2328 unique reflections with $I>2.3\ \sigma(I)$ were used for the refinement. The structure was solved by the direct method. Atomic parameters were refined by a block-diagonal method and the final R value was 0.044.

Supplementary Material Available Tables of final atomic positional parameters, atomic thermal parameters, and bond distances and angles of compound **37** are available. Ordering information is given on the current masthead page.

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Synthesis and Biological Activity of 11-[4-(Cinnamyl)-1-piperazinyl]-6,11-dihydrodibenz[*b*,*e*]oxepin Derivatives, Potential Agents for the Treatment of Cerebrovascular Disorders[1)]

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A series of 11-[4-(cinnamyl)-1-piperazinyl]-6,11-dihydrodibenz[*b*,*e*]oxepins and related compounds were synthesized and evaluated for their protective activities against complete ischemia, normobaric hypoxia, lipidperoxidation and convulsion. Structure–activity relationship studies of this series led to the finding of (*E*)-1-(3-fluoro-6,11-dihydrodibenz[*b*,*e*]oxepin-11-yl)-4-(3-phenyl-2-propenyl)piperazine dimaleate (50), AJ-3941 with the most appropriate property for combined pharmacological activities.

Compound 50 also shows an inhibitory effect against cerebral edema as well when orally given to rats.

Keywords cerebrovascular disorder; anti-ischemic effect; antihypoxic effect; anti-lipidperoxidation; anticonvulsion; anti-cerebral edema; 11-[4-(cinnamyl)-1-piperazinyl]-6,11-dihydrodibenz[*b*,*e*]oxepin; structure–activity relationship

As far as the brain is concerned, the most common disorders in middle-age and late life are cerebrovascular disorders such as cerebral infarction and hemorrhage.[2)] These cerebrovascular disorders are mainly caused by the cessation of blood flow or hypoperfusion. A decrease in oxygen supply to the brain results in a failure of energy metabolism which triggers a cascade of many concomitant events such as a massive increase in the release of excitatory transmitters and an influx of calcium into the cells, leading to the development of ischemic convulsion.[3,4)] These events cause a vicious circle in energy deficiency, and ultimately lead to irreversible cerebral dysfunction which results in the extensive death of neurons.[5)] Oxygen-derived free radicals, morever, are abundantly produced when oxygen supply is restored in a tissue whose antioxidative capacity has been reduced by ischemia/hypoxia.[6)] Lipidperoxidation due to the free radical reaction has been considered to be one of the basic deteriorative mechanisms the cerebrovascular disorders.[6,7)]

In view of the pathogenesis of cerebral dysfunctions, it seems to us that one attractive approach to the development of useful therapeutic agents for cerebrovascular disorders is to design compounds having concurrent protective activities against ischemia/hypoxia, convulsion, and lipidperoxidation.

With synthetic and pharmacological interests in dibenzoheterotricyclic compounds, we have studied such compounds for several years and recently reported the synthesis of *trans*-2-(6,6a,7,8,9,10,10a,11-octahydro-11-oxodibenzo[*b*,*e*]thiepin-3-yl)propionic acid (**1**) and its oxepin analogue **2** with an antiinflammatory activity,[8)] and of 11-[4-[4-(4-fluorophenyl)-1-piperazinyl]butyryl]amino-6,6a,7,8,9,10,-10a,11-octahydrodibenzo[*b*,*e*]thiepin (**3**) and its dibenzo analogue **4** with a calcium antagonistic activity.[9,10)] As a continuation of our research project, we planned to search for agents useful for the treatment of cerebrovascular disorders.

We had previously synthesized various 11-amino-6,11-dihydrodibenzo[*b*,*e*]thiepin derivatives **5** in order to know the fundamental structure emerging protective activities against ischemia/hypoxia, convulsion and lipidperoxidation.[11)] Among the compounds prepared, the 4-cinnamyl-1-piperazinyl derivative **38** was found to possess such activities (Table I). Compound **38** is similar in structure to cinnarizine (**6**)[12)] and flunarizine (**7**),[13)] which are both being used in practice for the treatment of cerebrovascular disorders. Protective effects of **38** against ischemia/hypoxia compared favorably with those of cinnarizine and flunarizine as shown in Table I. This finding prompted us to study further structure–activity relationships (SARs) of the related dibenzotricycles having a side chain(s) on the ring system.

Chemistry 6,11-Dihydrodibenzo[*b*,*e*]thiepin- and -oxepin-11-ones (**11**—**15** and **16**—**30**) were prepared by modified procedures of the reported methods[14)] (Chart 2). Condensation of phthalides **8** with thiophenols and phenols gave the carboxylic acids **9** and **10**, respectively, which were used in the next step without further purification. 2-(Phenylthiomethyl)benzoic acids **9** were treated with polyphospholic acid (PPA) to give ketones **11**—**15** in moderate yields (method A), whereas 2-(phenoxymethyl)-benzoic acids **10**, however, always afforded a complex mixture, probably owing to their lability under the acidic conditions employed. However, on treatment of **10** with trifluoroacetic anhydride in the presence of a catalytic amount of boron trifluoride at room temperature, the cyclization proceeded smoothly to give ketones **16**—**30** in

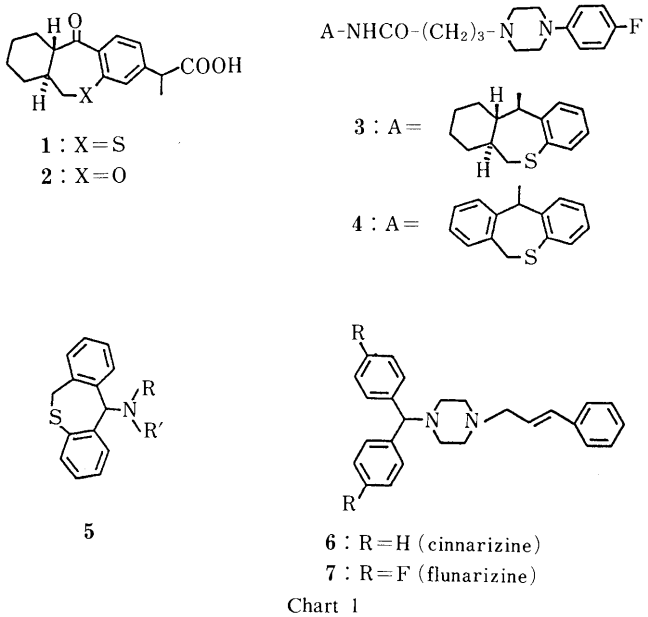

Chart 1

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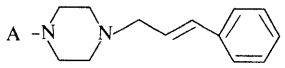
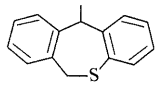
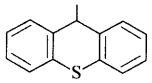
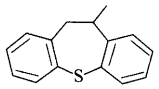
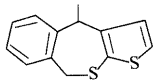
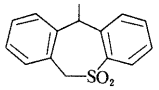
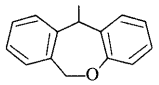
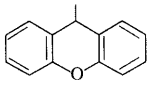
TABLE I. Physicochemical and Pharmacological Data for the Tricyclic Derivatives **38**—**44**



Compd. No.	A	Method[a]	Yield[b] (%)	mp (°C)	Recrystn. solvent	Formula	Analysis (%) Calcd (Found) C	H	N	S	Complete ischemia MED[c] (mg/kg)	Normobaric hypoxia score[d]	Anti-lipid-peroxidation IC_{50}[e] (μM)	Anti-MES[f]
38		E	63	203—206	EtOH–Et_2O	$C_{27}H_{28}N_2S \cdot C_2H_2O_4$[h]	69.30 (69.09	6.02 6.22	5.57 5.38	6.38 6.60)	10	+	8.2	0/3
39		E	51	118—120	Et_2O	$C_{26}H_{26}N_2S$	78.35 (78.56	6.58 6.71	7.03 6.96	8.04 8.31)	10	−	12.4	0/3
40		E	13	101—103	EtOH–Et_2O	$C_{27}H_{28}N_2S \cdot C_2H_2O_4$[h]	69.30 (69.27	6.02 6.11	5.57 5.32	6.38 6.15)	30	−	9.8	0/3
41		E	59	150—152	Et_2O	$C_{25}H_{26}N_2S_2$	71.73 (71.70	6.34 6.37	6.69 6.46	15.32 15.18)	30	−	8.6	1/3
42		E	18	217—220	EtOH	$C_{27}H_{28}N_2O_2S \cdot C_2H_2O_4$[g]	65.15 (64.94	5.66 5.75	5.24 5.22	6.00 6.06)	>100	+	19.5	0/3
43		E	67	142—145	EtOH–Et_2O	$C_{27}H_{28}N_2O \cdot 2C_4H_4O_4$[h]	66.87 (67.03	5.77 5.85	4.46 4.36)		100	+	5.4	2/3
44		E	71	119—120	MeOH	$C_{26}H_{26}N_2O$	81.64 (81.39	6.85 6.87	7.32 7.30)		100	−	9.2	1/3
Cinnarizine (**6**)											100	−	6.2	0/3
Flunarizine (**7**)											30	−	3.3	3/3

a) See Experimental. *b*) Yields are not optimal and are of the last step in the reaction sequence. *c*) Minimal effective dose, *p.o.* *d*) Symbols represent the increased percent change from control of survival time against normobaric hypoxia at 100 mg/kg, *p.o.*: −, <0%; +, 0—30%; ++, >30%. *e*) IC_{50} values for the malondialdehyde formation in rat brain mitochondria. *f*) Numerals indicate number of animals which showed anticonvulsant activity to number of tested. Anticonvulsant activity was determined at 2 h after oral administration of 100 mg/kg. *g*) Oxalate. *h*) Maleate.

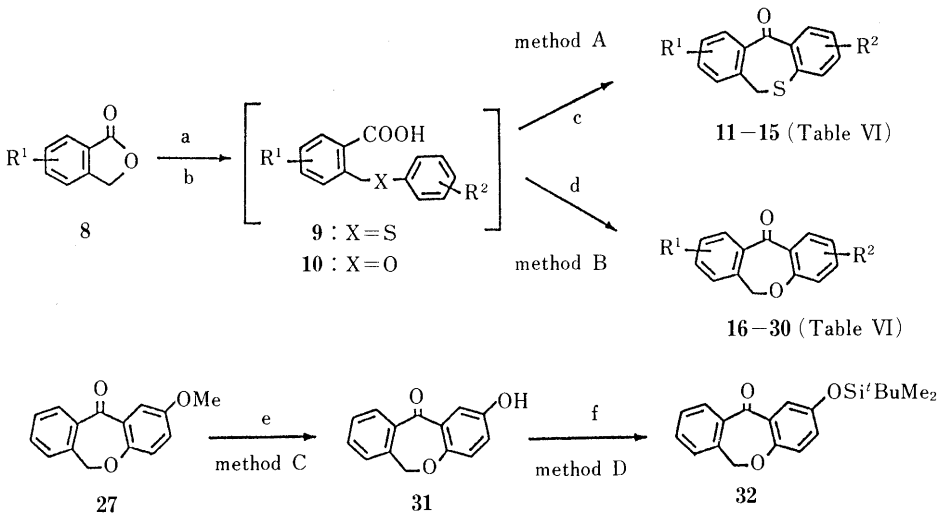
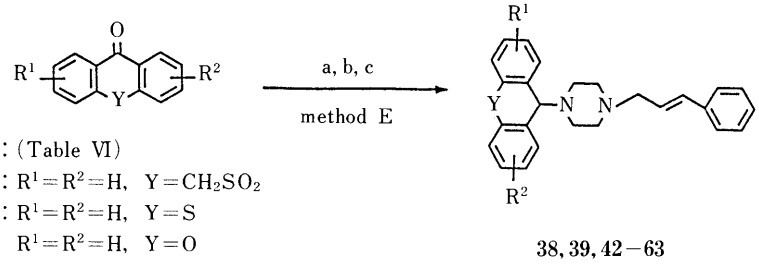


Chart 2

excellent yields (method B). 2-(Phenylthiomethyl)benzoic acids **9** failed to cyclize under the same conditions, resulting in a recovery of the starting materials probably because of less electron-donating character of sulfur atoms than that of oxygen atoms.

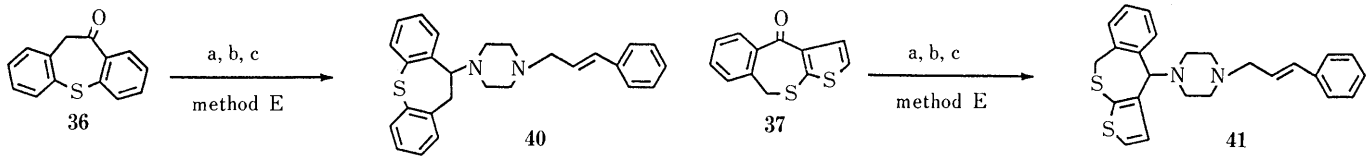
Demethylation (method C) of the 2-methoxy group of **27**



11—30 : (Table VI)
33 : $R^1=R^2=H$, $Y=CH_2SO_2$
34 : $R^1=R^2=H$, Y=S
35 $R^1=R^2=H$, Y=O

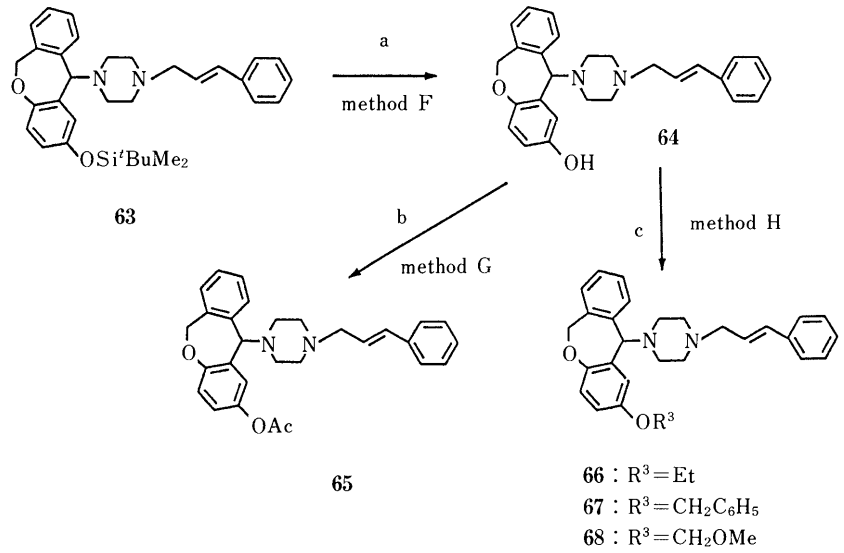
38, 39, 42—63

(Tables I and II)



a : $NaBH_4$/MeOH b : $SOCl_2$ c : (*E*)-cinnamylpiperazine-CH_2Cl_2

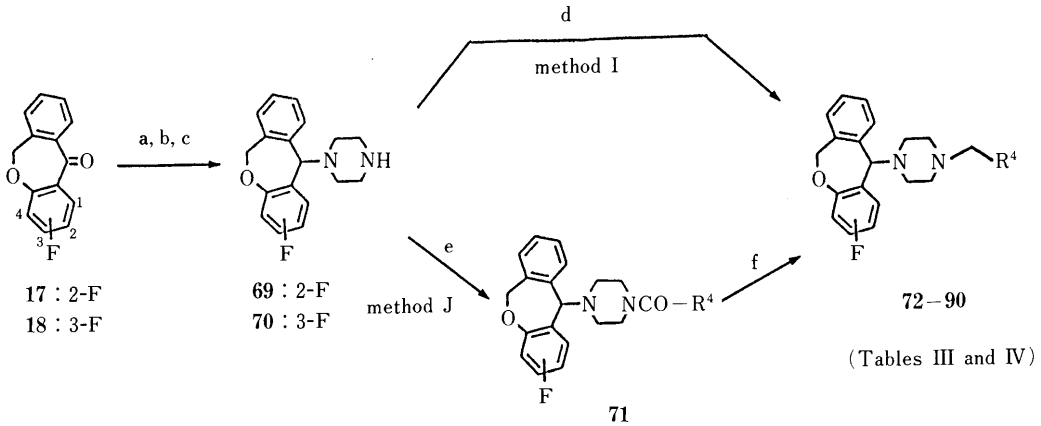
Chart 3



66 : $R^3=Et$
67 : $R^3=CH_2C_6H_5$
68 : $R^3=CH_2OMe$

a : *n*-$Bu_4N^+F^-$-THF b : Ac_2O-Py c : NaH-THF, then R^3Cl THF, tetrahydrofuran

Chart 4

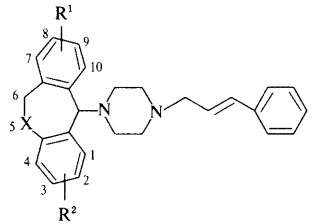


(Tables III and IV)

a : $NaBH_4$-MeOH b : $SOCl_2$ c : anhydrous piperazine-CH_2Cl_2
d : R^4CH_2Cl-toluene e : R^4COOH, WSC-CH_2Cl_2 f : Vitride-THF

Chart 5

TABLE II. Physicochemical and Pharmacological Data for the Dibenzo[*b*,*e*]thiepin and -oxepin Derivatives **45**—**68**



Compd. No.	X	R^1	R^2	Method[a]	Yield[b] (%)	mp (°C)	Recrystn. solvent	Formula	Analysis (%) Calcd (Found) C	H	N	F	S	Complete ischemia MED[c] (mg/kg)	Normobaric hypoxia score[d]	Anti-lipid-peroxidation IC_{50}[e] (μM)	Anti-MES[f]
45	S	H	2-F	E	47	132—135	EtOH–Et_2O	$C_{27}H_{27}FN_2S \cdot C_2H_2O_4$[g] $\cdot 0.5H_2O$	65.78 (65.94)	5.71 5.62	5.29 5.32	3.57 3.59	6.16 6.09)	30	+	4.8	0/3
46	S	H	3-F	E	39	195—198	EtOH–Et_2O	$C_{27}H_{27}FN_2S \cdot C_2H_2O_4$[g]	66.90 (66.75	5.61 5.56	5.38 5.41	3.65 3.54	6.16 6.04)	30	++	11.1	0/3
47	S	H	4-F	E	32	205—207	EtOH	$C_{27}H_{27}FN_2S \cdot C_2H_2O_4$[g]	66.90 (66.63	5.61 5.61	5.38 5.34	3.65 3.75	6.16 6.27)	100	+	14.9	0/3
48	S	H	2-OMe	E	36	128—129	EtOH–Et_2O	$C_{28}H_{30}N_2OS \cdot C_4H_4O_4$[h] $\cdot 0.5H_2O$	67.70 (67.70	6.21 6.24	4.93 4.68	— —	5.65 5.32)	3	—	>150	0/3
49	O	H	2-F	E	55	142—144	EtOH–Et_2O	$C_{27}H_{27}FN_2O \cdot 2C_4H_4O_4$[h]	65.01 (65.32	5.46 5.57	4.33 4.52	2.94 3.05)		30	+	9.1	3/3
50	O	H	3-F	E	62	137—138	EtOH	$C_{27}H_{27}FN_2O \cdot 2C_4H_4O_4$[h]	65.01 (64.99	5.46 5.47	4.33 4.28	2.94 2.85)		10	++	7.8	3/3
51	O	H	4-F	E	49	139—141	Et_2O	$C_{27}H_{27}FN_2O$	78.23 (77.98	6.57 6.61	6.76 6.71	4.58 4.56)		100	—	17.8	0/3
52	O	8-F	2-F	E	39	134—136	EtOH–Et_2O	$C_{27}H_{26}F_2N_2O \cdot 2C_4H_4O_4$[h]	63.25 (62.96	5.16 5.20	4.21 4.27	5.72 5.65)		30	+	>150	3/3
53	O	9-F	2-F	E	59	157—160	EtOH–Et_2O	$C_{27}H_{26}F_2N_2O \cdot 2C_4H_4O_4$[h]	63.25 (63.02	5.16 5.28	4.21 4.22	5.72 5.71)		30	+	>150	0/3
54	O	8-F	3-F	E	40	127—132	EtOH–Et_2O	$C_{27}H_{26}F_2N_2O \cdot 2C_4H_4O_4$[h]	63.25 (62.96	5.16 5.42	4.21 4.08	5.72 5.58)		30	+	>150	1/3
55	O	9-F	3-F	E	39	144—146	EtOH	$C_{27}H_{26}F_2N_2O \cdot 2C_4H_4O_4$[h]	63.25 (62.95	5.16 5.27	4.21 3.94	5.72 5.54)		10	+	150	0/3
56	O	H	2,3-F	E	60	107—108	EtOH–Et_2O	$C_{27}H_{26}F_2N_2O \cdot 2C_4H_4O_4$[h] $\cdot 0.5H_2O$	62.40 (62.40	5.24 4.97	4.16 4.05	5.64 5.53)		30	—	>150	2/3
57	O	H	3-Cl	E	59	138—139	EtOH–Et_2O	$C_{27}H_{27}ClN_2O \cdot 2C_4H_4O_4$[h]	63.39 (63.22	5.32 5.35	4.22 4.02)			30	—	37.1	0/3
58	O	H	3-Me	E	48	195—200	EtOH–Et_2O	$C_{28}H_{30}N_2O \cdot 2C_2H_2O_4$[g] $\cdot 0.5H_2O$	64.26 (64.10	5.89 5.88	4.93 4.67)			>100	—	>150	0/3
59	O	H	2-OMe	E	51	142—145	EtOH–Et_2O	$C_{28}H_{30}N_2O_2 \cdot 2C_4H_4O_4$[h]	65.64 (65.50	5.82 5.82	4.25 4.27)			3	++	>150	1/3
60	O	H	3-OMe	E	62	162—164	EtOH–Et_2O	$C_{28}H_{30}N_2O_2 \cdot 2C_4H_4O_4$[h] $\cdot H_2O$	63.90 (63.77	5.96 5.59	4.14 4.44)			30	—	>150	0/3
61	O	H	4-OMe	E	60	138—140	EtOH–Et_2O	$C_{28}H_{30}N_2O_2 \cdot 2C_4H_4O_4$[h]	65.64 (65.46	5.82 5.78	4.25 4.31)			30	—	>150	0/3
62	O	H	2,3-OMe	E	33	150—151	EtOH–Et_2O	$C_{29}H_{32}N_2O_3 \cdot 2C_4H_4O_4$[i] $\cdot 2.5H_2O$	60.56 (60.41	6.18 5.97	3.82 3.96)			30	—	150	0/3
63	O	H	2-OSiTBD[j]	E	87	138–139	EtOH–Et_2O	$C_{33}H_{42}N_2O_2Si \cdot 2C_4H_4O_4$[h]	64.89 (65.14	6.64 6.68	3.69 3.74)			100	—	>150	0/3
64	O	H	2-OH	F	69	162—166	EtOH–Et_2O	$C_{27}H_{28}N_2O_2 \cdot C_4H_4O_4$[h] $\cdot 0.5H_2O$	69.26 (69.25	6.19 5.86	5.21 5.15)			30	+	12.3	0/3
65	O	H	2-OAc	G	78	150—152	Et_2O	$C_{29}H_{30}N_2O_3$	76.63 (76.37	6.65 6.67	6.15 6.05)			>100	+	>150	0/3
66	O	H	2-OEt	H	56	177—179	EtOH–Et_2O	$C_{29}H_{32}N_2O_2 \cdot 1.5C_4H_4O_4$[i]	68.39 (68.10	6.23 6.31	4.56 4.44)			30	—	NT[m]	0/3
67	O	H	2-OBz[k]	H	62	176—178	EtOH–Et_2O	$C_{34}H_{34}N_2O_2 \cdot 2C_4H_4O_4$[i] $\cdot 0.25H_2O$	68.23 (68.03	5.79 5.75	3.79 3.75)			>100	+	NT[m]	0/3
68	O	H	2-OMOM[l]	H	55	159—160	EtOH–Et_2O	$C_{29}H_{32}N_2O_3 \cdot 1.5C_4H_4O_4$[i]	66.65 (66.33	6.07 6.11	4.44 4.32)			100	+	NT[m]	0/3

a—*h*) See footnotes in Table I. *i*) Fumarate. *j*) *tert*-Butyldimethylsilyloxy group. *k*) Benzyloxy group. *l*) Methoxymethyloxy group. *m*) NT, not tested.

with pyridinium hydrochloride furnished the 2-hydroxy compound **31** without cleavage of the oxygen bridge portion; however an attempt at conversion of the 3-methoxy analogue **28** to the corresponding 3-hydroxy compound was unsuccessful under the same conditions, affording a complex mixture. *tert*-Butyldimethylsilylation of **31** by the usual manner[15] yielded **32** (method D).

Introduction of an (*E*)-cinnamylpiperazinyl group into the tricyclic system was accomplished by method E (Chart 3). Thus, reduction of ketones **11**—**30** and **33**—**37** with sodium borohydride afforded the corresponding alcohols, which on subsequent treatment with thionyl chloride were converted to corresponding chloro derivatives; finally, the subsequent substitution reaction of the chloro derivatives with (*E*)-cinnamylpiperazine gave the 4-cinnamylpiperazin-1-yl derivatives **38**—**63** (Tables I and II).

Desilylation of the *tert*-butyldimethylsilyloxy group of **63** with tetrabutylammonium fluoride (method F) gave the 2-hydroxy derivative **64**, which was subsequently treated with acetic anhydride in pyridine (method G) to afford acetate **65**, or treated with an appropriate alkyl halide (method H) to give the corresponding ethers **66**—**68** (Chart 4) (Table II).

A 4-substituted piperazinyl group, other than (*E*)-cinnamylpiperazine, was introduced into 2- and 3-fluorodibenz[*b*,*e*]oxepins **17** and **18** *via* the 11-piperazinyl derivatives **69** and **70** (Chart 5). Treatment of **70** with alkyl halides (method I) gave the N-alkyl derivatives **72** and **74**. The reaction of **69** and **70** with an appropriate carboxylic acid (R^4COOH) using a condensing agent, 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (WSC), followed by reduction of **71** with sodium bis(2-methoxyethoxy)aluminium hydride (Vitride) (method J) produced the corresponding N-alkyl derivatives **73** and **76**—**90** (Tables III and IV).

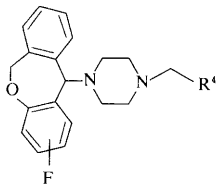
The structures of all compounds thus prepared were confirmed by their proton nuclear magnetic resonance (^{1}H-NMR) spectra and elemental analyses.

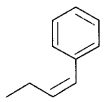
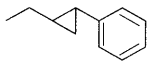
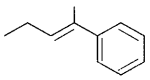
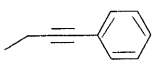
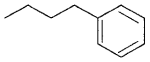
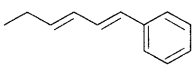
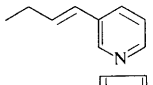
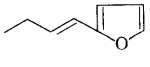
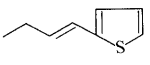
Results and Discussion

Protective activities against ischemia and hypoxia were assessed by the survival time of mice with on oral administration of the test compound under complete ischemic and normobaric hypoxic conditions. Anti-lipidperoxidative activity was determined using a rat brain mitochondrial fraction. The anticonvulsive effect was measured by maximal electroshock-induced seizure (MES) in mice. The detailed procedures are described in Experimental. The results are summarized in Tables I—IV; data for cinnarizine (**6**) and flunarizine (**7**) are included for comparison in Table I.

Modification of the tricyclic system of **38** was first

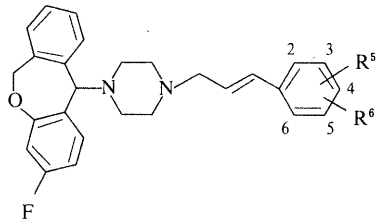
TABLE III. Physicochemical and Pharmacological Data for the 11-(Piperazinyl)dibenz[*b*,*e*]oxepin Derivatives **72**—**80**



Compd. No.	F	R^4	Method[a]	Yield[b] (%)	mp (°C)	Recrystn. solvent	Formula	Analysis (%) Calcd (Found) C	H	N	F	Complete ischemia MED[c] (mg/kg)	Normobaric hypoxia score[d]	Anti-lipidperoxidation IC_{50}[e] (μM)	Anti-MES[f]
72	3-F		I	48	121—124	EtOH –Et_2O	$C_{27}H_{27}FN_2O \cdot 2C_4H_4O_4$[h]	65.01 (64.84	5.46 5.65	4.33 4.09	2.94 2.86)	10	++	33.7	2/3
73	3-F		J	37	118—122	EtOH –Et_2O	$C_{28}H_{29}FN_2O \cdot 1.5C_2H_2O_4$[g]	66.06 (66.30	5.72 5.62	4.97 5.13	3.37 3.43)	30	–	>150	0/3
74	3-F		I	56	130—135	EtOH	$C_{28}H_{29}FN_2O \cdot 2C_4H_4O_4$[h] $\cdot 0.25H_2O$	65.00 (65.01	5.68 5.73	4.21 3.91	2.86 2.82)	30	–	93.3	0/3
75	—[i]		I	18	132—137	EtOH –Et_2O	$C_{27}H_{26}N_2O \cdot 2C_4H_4O_4$[h] $\cdot 0.5H_2O$	66.13 (66.36	5.55 5.48	4.41 4.53)		100	+	>150	0/3
76	3-F		J	66	192—193	EtOH	$C_{27}H_{29}FN_2O \cdot C_2H_2O_4$[g]	68.76 (68.70	6.17 6.07	5.53 5.48	3.75 3.81)	30	+	>150	1/3
77	2-F		J	30	142—144	EtOH –Et_2O	$C_{29}H_{29}FN_2O \cdot 2C_4H_4O_4$[h] $\cdot 0.5H_2O$	65.19 (65.19	5.62 5.60	4.11 4.11	2.79 2.64)	100	+	>150	0/3
78	2-F		J	32	109—112	EtOH	$C_{26}H_{26}FN_3O \cdot 2C_2H_2O_4$[g] $\cdot 0.25H_2O$	56.25 (56.28	5.51 5.23	6.56 6.44	2.97 2.95)	30	–	>150	0/3
79	2-F		J	62	106—108	EtOH –Et_2O	$C_{25}H_{25}FN_2O_2 \cdot 2C_4H_4O_4$[h] $\cdot 0.5H_2O$	61.39 (61.67	5.31 5.41	4.34 4.28	2.94 2.86)	>100	–	84.6	0/3
80	2-F		J	55	116—120	EtOH –Et_2O	$C_{25}H_{25}FN_2OS \cdot 2C_2H_2O_4$[g] $\cdot 0.5H_2O$	57.14 (57.40	4.96 4.85	4.60 4.53	3.12 3.03)	30	+	>150	0/3

a—*h*) See footnotes in Table I. *i*) Fluorine atom is unsubstituted.

TABLE IV. Physicochemical and Pharmacological Data for the 11-[4-(Cinnamyl)-1-piperazinyl]dibenz[*b*,*e*]oxepin Derivatives **81**—**90**



Compd. No.	R^5	R^6	Method[a]	Yield[b] (%)	mp (°C)	Recrystn. solvent	Formula	Analysis (%) Calcd (Found) C	H	N	F	Complete ischemia MED[c] (mg/kg)	Normobaric hypoxia score[d]	Anti-lipidperoxidation IC_{50}[e] (μM)	Anti-MES[f]
81	H	4-F	J	66	145—146	EtOH	$C_{27}H_{26}F_2N_2O$	74.98 (74.88)	6.06 6.35	6.48 6.41	8.78 8.85)	30	+	112.3	0/3
82	H	4-Br	J	55	130—133	EtOH–Et_2O	$C_{27}H_{26}BrFN_2O \cdot 2C_4H_4O_4$[h]	57.94 (58.14	4.72 4.88	3.86 3.80	2.62 2.64)	30	+	>150	0/3
83	H	4-Me	J	39	138—141	EtOH	$C_{28}H_{29}FN_2O \cdot 2C_4H_4O_4$[h]	65.45 (65.15	5.64 5.93	4.24 4.27	2.88 3.16)	30	+	>150	0/3
84	H	4-SMe	J	51	144—145	Et_2O	$C_{28}H_{29}FN_2OS$	73.01 (72.73	6.35 6.42	6.08 6.05	4.12 4.15)	100	−	>150	0/3
85	H	4-CF_3	J	34	146—148	EtOH–Et_2O	$C_{28}H_{26}F_4N_2O \cdot 2C_4H_4O_4$[h]	60.50 (60.74	4.80 4.50	3.92 3.89	10.63 10.39)	30	+	>150	0/3
86	H	4-OMe	J	44	153—154	EtOH	$C_{28}H_{29}FN_2O_2$	75.65 (75.46	6.58 6.85	6.30 6.10	4.28 4.36)	30	+	>150	0/3
87	H	3-OMe	J	51	136—140	EtOH–Et_2O	$C_{28}H_{29}FN_2O_2 \cdot 2C_4H_4O_4$[h]	63.90 (64.07	5.51 5.72	4.14 4.09	2.81 2.82)	100	+	>150	0/3
88	H	2-OMe	J	48	118—120	EtOH–Et_2O	$C_{28}H_{29}FN_2O_2 \cdot 2C_4H_4O_4$[h] $\cdot 0.75H_2O$	62.65 (62.94	5.62 5.77	4.06 3.77	2.75 2.73)	>100	+	>150	0/3
89	3-OMe	4-OMe	J	53	113—115	EtOH–Et_2O	$C_{29}H_{31}FN_2O_3 \cdot 2C_4H_4O_4$[h]	62.88 (62.99	5.56 5.28	3.96 3.94	2.69 2.66)	100	+	>150	0/3
90	2-OMe	5-OMe	J	46	155—156	EtOH	$C_{29}H_{31}FN_2O_3$	73.40 (73.52	6.58 6.83	5.90 5.87	4.00 3.92)	30	−	>150	0/3

a—*h*) See footnotes in Table I.

examined (Table I). Contraction of the central seven-membered ring into a six-membered congener as **38**→**39** and alteration of the site of the ring juncture of the left-hand phenyl ring as **38**→**40** caused a complete loss of the protective effects against normobaric hypoxia and MES. Replacement of the right-hand phenyl ring of **38** by a thiophene ring, giving **41**, resulted in a decrease in protective effects against complete ischemia and normobaric hypoxia. Oxidation of the sulfur atom of **38** gave the sulfone derivative **42**, of which activities against complete ischemia and lipidperoxidation became lower than those of the parent compound **38**. Then we prepared the oxygen-bridged tricyclic analogue (**43**) of **38**. Compound **43** compares favorably with **38**, on the whole, in the screening results, although it requires further improving the anti-ischemic activity. Contraction of the central ring of **43**, giving **44**, resulted in a loss of the protective effect against normobaric hypoxia. Compounds **38** and **43**, therefore, were selected as lead tricycles to be subjected to further modifications of substituents on the phenyl ring of 6,11-dihydrodibenzo-[*b*,*e*]thiepin and -oxepin nuclei.

Comparison of cinnarizine (**6**) with flunarizine (**7**) indicates that the presence of fluorine atoms in the benzhydryl moiety causes an increase in activities, as shown in Table I. Therefore, the fluoro-substituted 6,11-dihydrodibenzo[*b*,*e*]thiepin and -oxepin derivatives **45**—**47** and **49**—**56** were prepared. Among them, compound **46** with a fluorine atom at the 3-position has more potent protective activity against normobaric hypoxia than the unsubstituted congener **38**, but is less active against complete ischemia (Table II).

Introduction of the fluorine atom into the 2- and 3-positions of 6,11-dihydrodibenz[*b*,*e*]oxepin **43** (giving **49** and **50**, respectively) enhanced activities against complete ischemia, normobaric hypoxia and MES. Noteworthy is compound **50** (3-F) which is equipotent to **38** in anti-ischemic and anti-lipidperoxidative activities and superior to **38** in activities against normobaric hypoxia and MES. Interestingly, the presence of an additional fluorine atom on the opposite (**52**—**55**) and the same (**56**) phenyl ring of 6,11-dihydrodibenz[*b*,*e*]oxepin is deleterious to activities, thus leading to a striking loss of anti-lipidperoxidative activity and to a considerable decrease in protective effects against normobaric hypoxia, MES and/or complete ischemia.

Substituents such as 3-chloro (**57**), 3-methyl (**58**), and 3- and 4-methoxy (**66** and **61**) groups were of no advantage to an enhancement of activities over the fluoro group. It is of interest that the introduction of a 2-methoxy group (**48** and **59**) considerably increased the protective effect against complete ischemia, of which potency was approximately three times that of **50** (3-F). The 2-hydroxy derivative **64** shows potent anti-lipidperoxidative activity. However, the activities against MES of **59** and **64** are lower than that of **50**. Esterification of the hydroxy group of **64**, giving the acetoxy derivative **65**, markedly reduced activities. Alkylation of the hydroxy group (as in **66**—**68**) caused no increase in activities. Consequently, the optimal substituent was the fluorine atom at the 3-position of the 6,11-dihydrodibenz[*b*,*e*]oxepin nucleus. The allyl portion between

the terminal phenyl group and the piperazinyl moiety of **50** was modified (Table III); the modification includes geometric isomerization of the double bond (*trans* **50** into *cis* **72**), addition of a methylene group into the double bond (**73**), substitution at the β-styryl carbon by a methyl group (**74**), unsaturation of the double bond to a triple bond (**75**), saturation of the double bond (**76**), enlongation of the conjugation (**77**), and conversion of the phenyl ring into a hetero aromatic ring (**78**—**80**). All of these modifications, however, resulted in a decrease of the protective activities against lipidperoxidation and MES.

Finally we examined SARs of substituents on the phenyl ring of the (*E*)-cinnamylpiperazinyl moiety of **50** (Table IV). Introduction of a substituent on the phenyl ring caused a slight decrease in protective effects against complete ischemia and normobaric hypoxia, and diminished anti-lipidperoxidative and anti-MES activities. From the present SAR studies, it followed that compound **50** was optimal. Its protective effects against complete ischemia and normobaric hypoxia were superior to those of flunarizine and its other activities were almost equipotent to flunarizine. Compound **50** also significantly inhibited cerebral edema when orally given to rats in a dose of 30 mg/kg at 30 min before ligation, whereas flunarizine showed no significant inhibition at the same dose (Table V).

TABLE V. Effect of Compound **50** and Flunarizine on Brain Edema Induced by Bilateral Ligation of Common Carotid Arteries in Rats[a]

	Dose (mg/kg, *p.o.*)	No. of rats	Specific gravity of cerebral cortex (mean ± S.E.)
Sham control	—	10	1.0467 ± 0.0002[b]
Vehicle control	—	10	1.0431 ± 0.0006
Compound **50**	30	10	1.0453 ± 0.0004[b]
Sham control	—	10	1.0466 ± 0.0003[b]
Vehicle control	—	10	1.0417 ± 0.0008
Flunarizine (**7**)	30	10	1.0435 ± 0.0006

a) See Experimental. *b*) Significantly different from the value of the respective vehicle control ($p < 0.01$).

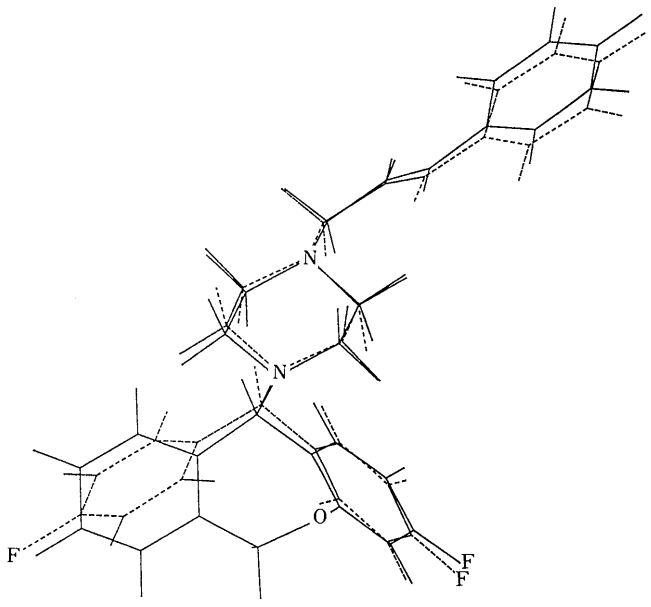


Fig. 1. Superimposition of Compound **50** (Bold Lines) and Flunarizine (Dotted Lines) by the Program COMPAR

The right-hand phenyl rings of the 3-fluoro-6,11-dihydrodibenz[*b*, *e*]oxepin nucleus and the 4,4′-difluorobenzhydryl moiety, and the (*E*)-cinnamylpiperazinyl groups were subjected to a least-squares fit.

In order to get insight into the cause of the difference in activities between compound **50** and flunarizine, semi-empirical molecular orbital calculations (using the AM1 method[16]) were performed with the MOL-GRAPH[17] molecular modeling system for their minimum-energy structures. The results are given in Fig. 1, where a common sequence of atoms comprising the cinnamylpiperazinyl and phenyl rings was superimposed by the program COMPAR.[18] Overall spatial structures of the two molecules are quite similar except that the phenyl rings of flunarizine are flexible in conformation, but those of **50** are restricted by a linkage between the two phenyl rings. Hence the difference in the protective activities between compound **50** and flunarizine may be concluded to result from an apparent difference in mobility of the phenyl rings.

In conclusion, compound **50** is overall the most potent against complete ischema, normobaric hypoxia, lipidperoxidation and MES, and also inhibits cerebral edema caused by ischemia. Compound **50** (AJ-3941), (*E*)-1-(3-fluoro-6,11-dihydrodibenz[*b*,*e*]oxepin-11-yl)-4-(3-phenyl-2-propenyl)piperazine dimaleate, therefore, is worthy of further evaluation as a potential agent for the treatment of cerebrovascular disorders. A correlation of the stereochemistry at the 11-position of AJ-3941 with biological activities will be discussed in a separate paper.

Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus, and are uncorrected. ^{1}H-NMR spectra were obtained on a Varian XL-300 spectrometer with tetrametylsilane as an internal standard. Mass (MS) spectra were taken on a JEOL D-300 mass spectrometer. Column chromatography was carried out on Merck Silica gel 60. The organic extract was dried over anhydrous magnesium sulfate unless otherwise noted.

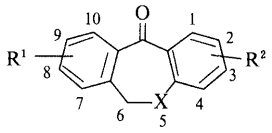
The following known compounds were prepared according to the cited literature: 6,11-dihydrodibenzo[*b*,*e*]thiepin-11-one 5,5-dioxide (**33**)[20]; 10,11-dihydrodibenzo[*b*,*f*]thiepin-10-one (**36**)[22]; 4,9-dihydrobenzo-[*e*]thieno[2,3-*b*]thiepin-4-one (**37**)[23]; 5- and 6-fluorophthalides (**9** and **10**)[24]; (*Z*)-1-chloro-3-phenyl-2-propene[25]; 2-phenylcyclopropylacetic acid[26]; 1-chloro-3-phenyl-2-butene[27]; 1-chloro-3-phenyl-2-propyne[28]; (*E*,*E*)-5-phenyl-1,3-pentadienoic acid[29]; (*E*)-3-(3-pyridinyl)-2-propenoic acid[30]; (*E*)-3-(2-thienyl)- and 3-(2-furyl)-2-propenoic acids[31]; (*E*)-3-(4-methylthiophenyl)-2-propenoic acid[32] and (E)-3-(4-bromophenyl)-2-propenoic acid.[33] (*E*)-3-(4-Fluoro-, 4-methoxy- and 4-trifluoromethylphenyl)-2-propenoic acids, 3-(4-methyl-, 2-methoxy-, 3-methoxy-, 4-methoxy-, 2,5-dimethoxy- and 3,4-dimethoxyphenyl)-2-propenoic acids, phthalide, thioxanthone (**34**), xanthone (**35**) and 3-phenylpropionic acid were purchased from Aldrich Chemical Co., Ltd.

Method A. 2-Fluoro-6,11-dihydrodibenzo[*b*,*e*]thiepin-11-one (12) A mixture of phthalide (**8**; 23 g, 171 mmol), 3-fluorothiophenol (22 g, 172 mmol) and anhydrous potassium carbonate (25 g, 180 mmol) in dimethylformamide (DMF) was heated at 100 °C for 2 h, cooled to room temperature and poured into water. After the solution was acidified with hydrochloric acid, the resulting precipitate was collected and dried to give 2-[(3-fluorophenyl)thiomethyl]benzoic acid (**9**; 39 g, 87%), MS *m/z*: 244 (M^+). A stirred mixture of the crude product and PPA (200 g) was heated at 100 °C for 3 h, poured into ice water, and then extracted with toluene. The extract was successively washed with 20% sodium hydroxide, water and brine. The solvent was evaporated and the resulting solid was recrystallized from ethanol to give **12** (32 g, 81%) (Table VI). ^{1}H-NMR ($CDCl_3$) δ: 4.06 (2H, s), 7.08—7.53 (5H, m), 7.65 (1H, dd, J=8, 2 Hz), 7.95 (1H, J=11, 4 Hz).

Compounds **11** and **13**—**15** were prepared according to the same method (Table VI).

Method B. 3-Fluoro-6,11-dihydrodibenz[*b*,*e*]oxepin-11-one (18) A mixture of phthalide (**8**, 20 g, 149 mmol) and sodium 3-fluorophenoxide (20 g,

TABLE VI. Physicochemical Data for 6,11-Dihydrodibenzo[*b*,*e*]thiepin- and -oxepin-11-ones **11**—**32**



Compd. No.	X	R^1	R^2	Method[a]	Yield[b] (%)	mp (°C)	Recrystn. solvent	Formula	Analysis (%) Calcd (Found) C	H	S	X[c]
11	S	H	H	A	33	87—89[d]	EtOH	$C_{14}H_{10}OS$	74.31	4.45	14.17	
									(74.26	4.44	14.22)	
12	S	H	2-F	A	41	102—103[e]	EtOH	$C_{14}H_9FOS$	68.83	3.71	13.13	7.78
									(68.90	3.80	13.01	7.66)
13	S	H	3-F	A	32	100—101	EtOH	$C_{14}H_9FOS$	68.83	3.71	13.13	7.78
									(68.79	3.54	12.99	7.69)
14	S	H	4-F	A	38	104—105	EtOH	$C_{14}H_9FOS$	68.83	3.71	13.13	7.78
									(68.72	3.69	13.11	7.51)
15	S	H	2-OMe	A	19	92—94[f]	EtOH	$C_{15}H_{12}O_2S$	70.29	4.72	12.51	
									(70.53	4.58	12.29)	
16	O	H	H	B	79	66—68[g]	EtOH	$C_{14}H_{10}O_2$	79.98	4.79		
									(80.13	4.65)		
17	O	H	2-F	B	89	131—133	EtOH	$C_{14}H_9FO_2$	73.68	3.97	—	8.32
									(73.64	3.77	—	8.18)
18	O	H	3-F	B	82	80—81	EtOH	$C_{14}H_9FO_2$	73.68	3.97	—	8.32
									(73.39	3.78	—	8.09)
19	O	H	4-F	B	81	119—123	AcOEt	$C_{14}H_9FO_2$	73.68	3.97	—	8.32
									(73.51	4.16	—	8.17)
20	O	8-F	2-F	B	85	129—130	AcOEt	$C_{14}H_8F_2O_2$	68.30	3.28	—	15.43
									(68.44	3.01	—	15.18)
21	O	9-F	2-F	B	80	130—132	AcOEt	$C_{14}H_8F_2O_2$	68.30	3.28	—	15.43
									(68.14	3.22	—	15.31)
22	O	8-F	3-F	B	91	153—155	AcOEt	$C_{14}H_8F_2O_2$	68.30	3.28	—	15.43
									(68.31	3.35	—	15.33)
23	O	9-F	3-F	B	80	133—141	AcOEt	$C_{14}H_8F_2O_2$	68.30	3.28	—	15.43
									(68.28	3.35	—	15.27)
24	O	H	2,3-F	B	71	140—142	EtOH	$C_{14}H_8F_2O_2$	68.30	3.28	—	15.43
									(68.17	3.33	—	15.44)
25	O	H	3-Cl	B	79	106—107	EtOH	$C_{14}H_9ClO_2$	68.72	3.71	—	14.49[h]
									(68.66	3.53	—	14.28[h])
26	O	H	3-Me	B	89	68—71[i]	Et_2O	$C_{15}H_{12}O_2$	80.34	5.39		
									(80.30	5.43)		
27	O	H	2-OMe	B	83	93—94[j]	EtOH	$C_{15}H_{12}O_3$	74.99	5.03		
									(75.16	4.98)		
28	O	H	3-OMe	B	75	104—105	EtOH	$C_{15}H_{12}O_3$	74.99	5.03		
									(74.88	4.87)		
29	O	H	4-OMe	B	81	72—73	MeOH	$C_{15}H_{12}O_3$	74.99	5.03		
									(74.84	4.77)		
30	O	H	2,3-OMe	B	90	119—121	AcOEt	$C_{16}H_{14}O_4$	71.10	5.22		
									(70.99	5.30)		
31	O	H	2-OH	C	68	168—169	EtOH	$C_{14}H_{10}O_3$	74.33	4.46		
									(74.42	4.49)		
32	O	H	2-OSiTBD[k]	D	91	Oil		$C_{20}H_{24}O_3Si$	70.55	7.10		
									(70.32	7.09)		

a, *b*) See footnotes in Table I. *c*) X represents fluorine unless otherwise noted. *d*) Lit.[14] mp 86—87 °C. *e*) Lit.[14] mp 101—105 °C. *f*) Lit.[19] mp 94—96 °C. *g*) Lit.[20] mp 69—70 °C. *h*) For chlorine. *i*) Lit.[21] mp 71—72 °C. *j*) Lit.[21] mp 93—94 °C. *k*) *tert*-Butyldimethylsilyloxy group.

149 mmol) was heated at 200 °C for 1 h, and cooled to room temperature. The mixture was dissolved in water and acidified with hydrochloric acid. The resulting precipitate was collected and dried to give 2-[(3-fluorophenyl)oxymethyl]benzoic acid (**10**, 27 g, 74%), MS *m/z*: 246 (M^+). Boron tifluoride etherate (3 g, 21 mmol) was added to a solution of the crude product and trifluoroacetic anhydride (25 g, 119 mmol) in dichloromethane (300 ml). The mixture was stirred at room temperature for 1 h and then extracted with toluene. The extract was successively washed with 20% sodium hydroxide, water and brine, and concentrated to dryness. The resulting solid was recrystallized from ethanol to give **18** (23 g, 92%) (Table VI). ^{1}H-NMR ($CDCl_3$) δ: 5.21 (2H, s), 6.74—6.91 (2H, m), 7.37 (1H, dd, J=8, 2 Hz), 7.44—7.63 (2H, m), 7.91 (1H, dd, J=8, 3 Hz), 8.28 (1H, dd, J=10, 8 Hz).

Compounds **16**, **17** and **19**—**30** were prepared according to the same method (Table VI).

Method C. 2-Hydroxy-6,11-dihydrodibenz[*b*,*e*]oxepin-11-one (31) A mixture of **27** (11 g, 46 mmol) and pyridine hydrochloride (25 g, 216 mmol) was heated at 180 °C for 2 h and cooled to room temperature. After the addition of water, the resulting aqueous mixture was extracted with chloroform. The extract was successively washed with water and brine. The solvent was evaporated and the resulting solid was crystallized from ethanol to give **31** (7.4 g, 71%) (Table VI). ^{1}H-NMR ($CDCl_3$) δ: 5.18 (2H, s), 6.12 (1H, br s), 6.90 (1H, d, J=8 Hz), 7.12 (1H, dd, J=9, 3 Hz), 7.17 (1H, dd, J=8, 2 Hz), 7.44—7.63 (2H, m), 7.89 (1H, d, J=3 Hz), 7.96 (1H, dd, J=8, 2 Hz).

Method D. 2-(*tert*-Butyldimethylsilyloxy)-6,11-dihydrodibenz[*b*,*e*]oxepin-11-one (32) A solution of *tert*-butyldimethylsilyl chloride (3.3 g, 22 mmol) in DMF (10 ml) was added to a solution of **31** (5.0 g, 22 mmol)

and imidazole (3.0 g, 44 mmol) in dry DMF (100 ml) at room temperature. The mixture was stirred for 1 h and then water was added. The resulting aqueous mixture was extracted with toluene. The extract was successively washed with water and brine. After the solvent was evaporated, the resulting oil was chromatographed on silica gel with 30% hexane in toluene as an eluant to give **32** (6.8 g, 90%) as a colorless oil (Table VI). ^{1}H-NMR ($CDCl_3$) δ: 0.21 (6H, s), 1.00 (9H, s), 5.18 (2H, s), 6.94 (1H, dd, J=12, 1 Hz), 7.12 (1H, dd, J=12, 4 Hz), 7.36 (1H, dd, J=8, 2 Hz), 7.43—7.61 (2H, m), 7.67 (1H, dd, J=3, 1 Hz), 7.94 (1H, dd, J=8, 2 Hz).

Method E. (*E*)-1-(3-Fluoro-6,11-dihydrodibenz[*b*,*e*]oxepin-11-yl)-4-(3-phenyl-2-propenyl)piperazine Dimaleate (50) Sodium borohydride (1 g, 26 mmol) was added portionwise to a solution of **18** (5.0 g, 22 mmol) in methanol (50 ml) at 0 °C. The reaction mixture was stirred for 20 min, and water was added. The aqueous mixture was extracted with toluene. The extract was successively washed with water and brine. The solvent was evaporated to give 3-fluoro-6,11-dihydrodibenz[*b*,*e*]oxepin-11-ol (4.9 g, 97%), MS *m*/*z*: 230 (M^+). A mixture of the crude product and thionyl chloride (5 g, 42 mmol) was stirred for 10 min at room temperature. After evaporation of the excess thionyl chloride, the resulting residue was dissolved in dichloromethane, and (*E*)-4-(3-phenyl-2-propenyl)piperazine (12 g, 59 mmol) was added. The mixture was stirred for 1 h at room temperature, and then 5% sodium hydroxide was added. The solution was extracted with chloroform. The extract was successively washed with water and brine, and dried over anhydrous potassium carbonate. After removal of the solvent, the residue was chromatographed on silica gel with chloroform as an eluant to give an oil, which was crystallized from ethanol to give a free base of **50** (5.8 g, 62%), mp 135—136 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.2—2.7 (8H, m), 3.15 (2H, d, J=7 Hz), 3.89 (1H, s), 3.89 (1H, s), 4.70 (1H, d, J=11 Hz), 6.18—6.34 (1H, m), 6.44—6.56 (3H, m), 6.81 (1H, d, J=12 Hz), 7.05 (1H, m), 7.15—7.38 (9H, m). *Anal.* Calcd for $C_{27}H_{27}FN_2O$: C, 78.23; H, 6.57; N, 6.76; F, 4.58. Found: C, 78.03; H, 6.59; N, 6.73; F, 4.51. Maleic acid was added to a solution of the free base in ethanol. The resulting precipitate was collected by filtration to give the maleate **50** (Table II).

Compounds **38**—**49** and **51**—**63** were prepared according to the same method (Tables I and II).

Method F. (*E*)-1-(2-Hydroxy-6,11-dihydrodibenz[*b*,*e*]oxepin-11-yl)-4-(3-phenyl-2-propenyl)piperazine Maleate (64) Tetrabutylammonium fluoride (3.0 g, 12 mmol) was added to a solution of **63** (5.2 g, 9.9 mmol) in tetrahydrofuran (THF, 100 ml) at room temperature. The mixture was stirred for 30 min, and then taken up in water. The aqueous mixture was extracted with chloroform. The extract was successively washed with water and brine. The solvent was evaporated and the resulting oil was chromatographed on silica gel with 1% methanol in chloroform as an eluant to afford the free base of **64** (2.8 g, 69%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 2.2—2.8 (8H, m), 3.15 (2H, d, J=7 Hz), 3.73 (1H, s), 4.67 (1H, d, J=12 Hz), 6.14—6.29 (1H, m), 6.47—6.73 (4H, m), 7.10—7.32 (10H, m). *Anal.* Calcd for $C_{27}H_{28}N_2O_2$: C, 78.61; H, 6.84; N, 6.79. Found: C, 78.75; H, 6.66; N, 6.73. This oil was dissolved in ethanol, and maleic acid was added. The resulting precipitate was collected by filtration to give the maleate **64** (Table II).

Method G. (*E*)-1-(2-Acetoxy-6,11-dihydrodibenz[*b*,*e*]oxepin-11-yl)-4-(3-phenyl-2-propenyl)piperazine (65) Acetic anhydride (0.5 g, 4.9 mmol) was added to a solution of **64** (1.0 g, 2.4 mmol) in dry pyridine (10 ml) at room temperature. After stirring for 3 h, the mixture was concentrated and the resulting oil was chromatographed on silica gel with chloroform as an eluant to give an oil, of which crystallization from ethyl acetate–hexane gave **65** (0.86 g, 78%) (Table II). ^{1}H-NMR ($CDCl_3$) δ: 2.22—2.72 (8H, m), 2.27 (3H, s), 3.12 (2H, d, J=7 Hz), 3.82 (1H, s), 4.68 (1H, d, J=12 Hz), 6.16—6.32 (1H, m), 6.48 (1H, d, J=14 Hz), 6.76—6.93 (4H, m), 7.17—7.39 (9H, m).

Method H. (*E*)-1-(2-Ethoxy-6,11-dihydrodibenz[*b*,*e*]oxepin-11-yl)-4-(3-phenyl-2-propenyl)piperazine Fumarate (66) Sodium hydride (1.0 g, 2.5 mmol, 60% dispersion in mineral oil) was added potionwise to a solution of **64** (1.0 g, 2.4 mmol) in dry DMF (10 ml) at 0 °C. After stirring for 5 min, iodoethane (0.4 g, 2.6 mmol) was added. The mixture was stirred for 1 h, and then taken up in water. The aqueous mixture was extracted with ether. The extract was successively washed with water and brine. The ether was evaporated to leave an oil, which was chromatographed on silica gel with chloroform as an eluant to give the free base of **66** (0.6 g, 56%). ^{1}H-NMR ($CDCl_3$) δ: 1.36 (3H, t, J=7 Hz), 2.20—2.70 (8H, m), 3.10 (2H, d, J=7 Hz), 3.82 (1H, s), 3.93 (2H, q, J=7 Hz), 4.67 (1H, d, J=12 Hz), 6.16—6.32 (1H, m), 6.47 (1H, d, J=9 Hz), 6.65—6.77 (4H, m), 7.15—7.38 (9H, m). *Anal.* Calcd for $C_{29}H_{32}N_2O_2$: C, 79.06; H, 7.32; N, 6.36. Found: C, 79.00; H, 7.30; N, 6.33. This oil was dissolved in ethanol, and fumaric acid was added. The resulting precipitate was collected by filtration to give the fumarate **66** as colorless crystals (Table II).

Compounds **67** and **68** were prepared according to the same method (Table II).

1-(2-Fluoro- and 3-fluoro-6,11-dihydrodibenz[*b*,*e*]oxepin-11-yl)piperazine (69 and 70) Sodium borohydride (1 g, 26 mmol) was added portionwise to a solution of **17** (5.0 g, 22 mmol) in methanol (50 ml) at 5 °C. The mixture was stirred for 20 min, and water was added. The aqueous mixture was extracted with toluene. The extract was successively washed with water and brine. The solvent was evaporated to give 2-fluoro-6,11-dihydrodibenz[*b*,*e*]oxepin-11-ol (4.9 g), MS *m*/*z*: 230 (M^+). A mixture of the crude product and thionyl chloride (5 g, 42 mmol) was stirred for 10 min at room temperature. After evaporation of the excess thionyl chloride, the residue was dissolved in dichloromethane, and anhydrous piperazine (5.7 g, 66 mmol) was added. The mixture was stirred for 2 h at room temperature, and then concentrated to give an oil, which was chromatographed on silica gel with 10% methanol in chloroform, giving 1-(2-fluoro-6,11-dihydrodibenz[*b*,*e*]oxepin-11-yl)piperazine (**69**) (4.5 g, 68%) as an oil, MS *m*/*z*: 298 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.22—2.50 (5H, m), 2.75—2.87 (4H, m), 3.81 (1H, s), 4.71 (1H, d, J=12 Hz), 6.69—6.92 (4H, m), 7.13—7.32 (4H, m). *Anal.* Calcd for $C_{18}H_{19}FN_2O$: C, 72.46; H, 6.42; F, 6.37; N, 9.39. Found: C, 72.60; H, 6.45; F, 6.26; N, 9.22.

Compound **18** was treated in the same manner as described above. 1-(3-Fluoro-6,11-dihydrodibenz[*b*,*e*]oxepin-11-yl)piperazine (**70**) was obtained as an oily product in 76% yield. MS *m*/*z*: 298 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 2.18 (1H, s), 2.19—2.51 (4H, m), 2.78—2.86 (4H, m), 3.89 (1H, s), 4.71 (1H, d, J=13 Hz), 6.46—6.58 (2H, m), 6.85 (1H, d, J=12 Hz), 7.00—7.10 (1H, m), 7.18—7.32 (4H, m). *Anal.* Calcd for $C_{18}H_{19}FN_2O$: C, 72.46; H, 6.42; F, 6.37; N, 9.39. Found: C, 72.51; H, 6.49; F, 6.20; N, 9.36.

Method I. (*E*)-1-(3-Fluoro-6,11-dihydrodibenz[*b*,*e*]oxepin-11-yl)-4-(3-phenyl-2-butenyl)piperazine Maleate (74) 1-Chloro-3-phenyl-2-butene (1.2 g, 7.2 mmol) was added to a solution of **70** (2.1 g, 7.0 mmol) in toluene (100 ml). The mixture was heated to reflux for 8 h, cooled to room temperature, and then taken up in chloroform. The organic phase was successively washed with 10% sodium hydroxide, water and brine. The solvent was evaporated to leave an oil, which was chromatographed on silica gel with chloroform as an eluant to give the free base of **74** (1.7 g, 56%), MS *m*/*z*: 428 (M^+). This free base was dissolved in ethanol, and maleic acid was added. The resulting precipitate was collected by filtration to give the maleate **74** (Table III).

Compounds **72** and **75** were prepared according to the same method (Table III).

Method J. (*E*)-1-(3-Fluoro-6,11-dihydrodibenz[*b*,*e*]oxepin-11-yl)-4-[3-(4-fluorophenyl)-2-propenyl]piperazine (81) WSC (2.1 g, 11 mmol) was added to a solution of **70** (3.1 g, 10 mmol) and (*E*)-3-(4-fluorophenyl)-2-propenoic acid (1.7 g, 10 mmol) in dichloromethane (100 ml) at room temperature. After stirring for 1 h, followed by the addition of water, the aqueous mixture was extracted with chloroform. The extract was successively washed with water and brine to give crude (*E*)-1-(3-fluoro-6,11-dihydrodibenz[*b*,*e*]oxepin-11-yl)-4-[3-(4-fluorophenyl)-1-oxo-2-propenyl]piperazine **71** (3.0 g, 66%), MS *m*/*z*: 436 (M^+). A mixture of the crude product **71** and sodium bis(2-methoxyethoxy)aluminium hydride (Vitride, available from Nacalai Tesque, Inc.; 3 g, 70% solution in toluene) was stirred for 2 h, then poured into ice-water and extracted with chloroform. The extract was successively washed with water and brine. The solvent was evaporated and the residue was chromatographed on silica gel with chloroform as an eluant to give an oil, which was crystallized from ethanol to give **81** (2.9 g, 66%) (Table IV). ^{1}H-NMR ($CDCl_3$) δ: 2.20—2.65 (8H, m), 3.12 (2H, d, J=7 Hz), 3.89 (1H, s), 4.70 (1H, d, J=11 Hz), 6.08—6.22 (1H, m), 6.41—6.57 (3H, m), 6.81 (1H, d, J=12 Hz), 6.92—7.10 (3H, m), 7.17—7.35 (6H, m).

Compounds **73**, **76**—**80** and **82**—**90** were prepared according to the same method (Tables III and IV).

Conformational Analysis Initial atomic coordinates for calculation of the molecular orbital of the (*E*)-cinnamyl group in compound **50** and flunarizine, and of the 4,4′-difluorobenzhydryl moiety in flunarizine were determined by the X-ray crystallographic data for cinnarizine.[34] The 3-fluoro-6,11-dihydrodibenz[*b*,*e*]oxepin nuclei of compound **50** was refered to the data from X-ray crystallography of 10,11-dihydrodibenzo[*a*,*d*]cycloheptene,[35] of which the C-11 methylene group was replaced by on oxygen atom. AM1 calculations[16] with geometry optimization in MOPAC[36] were carried out. Molecular modelings were performed using the commercially available program MOL-GRAPH.[17] The program COMPAR[18] was used for the superimposition.

Biological Assays Complete Ischemia[37]: A group of five mice was

decapitated at 2 h after the administration of the test compounds. The persistent time of gasping movements of the isolated head was determined and expressed as a minimal effective dose (MED) which was significantly different from the control ($p < 0.05$).

Normobaric Hypoxia[37]: At 2 h after the oral administration of test compounds (100 mg/kg), a group of five mice was introduced into a 2.5-liter plastic chamber through which their was a current of a mixture of N_2 and O_2 (96 : 4). The survival time, *i.e.* the time between the start of the introduction of the mice into the hypoxic gas chamber and the end of respiration, was measured.

Lipidperoxidation[38]: A mixture of mitochondrial fraction with test compounds was incubated for 30 min with ascorbic acid (50 μM) and ferrous sulfate (20 μM) at 30 °C. Malondealdehyde (MDA) formed in a sample mitochondrial fraction was determined by the thiobarbituric acid reaction.

Convulsion[39]: Maximal electroshock seizures were induced by the method of Swinyard.[40] A group of three mice was subjected to a 60 Hz current of 25 mA for 0.2 s delivered through corneal electrodes after the oral administration of test compounds (100 mg/kg). Complete abolition of the hindlimb tonic extensor components of seizures was taken as evidence of anticonvulsant activity.

Brain Edema[37,41]: The test compounds (30 mg/kg) were orally given to a group of eight to ten rats. After 30 min the bilateral common carotid arteries of the rats were doubly ligated under ether anesthesia. The rats were killed by decapitation at 5 h after the ligation. Specific gravity of the cerebral cortex was determined according to the gradient column method of Marmarou *et al.*

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Chemical Modification of an Antitumor Alkaloid Camptothecin: Synthesis and Antitumor Activity of 7-*C*-Substituted Camptothecins

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A radical substitution reaction of 20(*S*)-camptothecin (1) with methanol furnished 7-hydroxymethylcamptothecin (2). Reaction of 1 with primary alcohols higher than methanol gave 7-alkylcamptothecins (4), of which alkyl groups were one carbon less than the alcohols used and also 7-hydroxyalkylcamptothecins (5). For the preparation of 7-alkylcamptothecin (4), aldehydes were used as a radical source and several alkylated derivatives were synthesized. 7-Acyloxymethyl derivatives (6), 7-carbaldehyde (7), iminomethyl derivatives (10), acid (11), esters (12) and amides (13) were synthesized starting from 2. 7-Ethyl- (4b) and 7-propylcamptothecin (4c), acyloxymethyl compounds 6a, 6c and ethyl ester (12b) exhibited higher antitumor activity than 1 against L1210 in mice.

Keywords 20(*S*)-camptothecin; homolytic alkylation; 7-alkylcamptothecin; 7-hydroxymethylcamptothecin; 7-ethylcamptothecin; 5-*O*-substituted camptothecin; antitumor activity; leukemia L1210

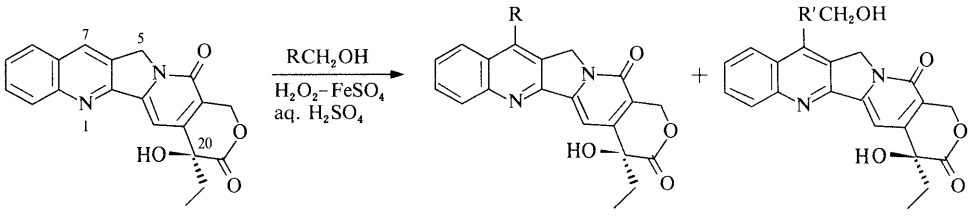
20(*S*)-Camptothecin (**1**) is an alkaloid which was first isolated from *Camptotheca acuminata* (Nyssaceae) by Wall and co-workers in 1966.[1] It has attracted much attention because of its significant antitumor activity in various murine tumor systems. In clinical trials, however, the high toxicity of this alkaloid has restricted its therapeutic usefulness.[2]

We have been conducting a study on chemical modification of **1** to obtain derivatives with less toxicity.[3] We wish to report here the synthesis of 7-*C*-substituted camptothecins and their antitumor activities against leukemia L1210 in mice.

Chemistry As pointed out by Minisci and Porta, homolytic alkylation of heteroaromatic bases can be performed in aqueous acidic media: alkyl radicals add at the electron-deficient positions of the protonated bases.[4] Treatment of **1** with hydrogen peroxide and ferrous sulfate in an aqueous methanol–sulfuric acid solution furnished 7-hydroxymethylcamptothecin (**2**) in 82% yield. The formation of **2** was also observed by heating **1** with hydroxylamine-*O*-sulfonic acid (HSA)[5] as a suspension in aqueous methanol or with ammonium persulfate in a mixture of methanol–sulfuric acid.[4] But in the latter cases the yields were low. Application of homolytic reaction using glycolic acid and ammonium persulfate in the presence of silver ion[6] resulted in the formation of 5-hydroxycamptothecin (**3a**, 10% yield) in addition to **2** (19% yield) and trace amounts of camptothecin 1-oxide.

We also examined the radical substitution reaction of **1** with primary alcohols such as ethanol, propanol, butanol and isobutanol. The predominant product of the reaction was 7-alkylcamptothecins (**4**), of which the alkyl groups were one carbon less than the alcohols used having lost the carbinol moiety. 7-Hydroxyalkylcamptothecins (**5**) were also formed by this reaction. Displacement reaction of the hydrogen atom of the alcohols was observed at each position except for the α- and tertiary carbon. For example, using ethanol as a radical source, two products were isolated

TABLE I. Results of the Homolytic Reactions of **1** with Primary Alcohols Yielding 7-Alkyl- (**4**) and 7-Hydroxyalkylcamptothecins (**5**)



1: 20(*S*)-camptothecin **4** **5**

Alcohol	4	R	Yield (%)	5	R'CH$_2$OH	Yield (%)
CH_3CH_2OH	**a**	$-CH_3$	40	**a**	$-CH_2CH_2OH$	32
$CH_3CH_2CH_2OH$	**b**	$-C_2H_5$	40	**b**	$-CH(CH_3)CH_2OH$	22
				c	$-CH_2CH_2CH_2OH$	12
$CH_3CH_2CH_2CH_2OH$	**c**	$-C_3H_7$	35	**d**	$-CH(C_2H_5)CH_2OH$	20
				e	$-CH(CH_3)CH_2CH_2OH$	15
				f	$-CH_2CH_2CH_2CH_2OH$	5
$(CH_3)_2CHCH_2OH$	**d**	$-CH(CH_3)_2$	48	**g**	$-CH_2CH(CH_3)CH_2OH$	12
$(CH_3)_2CHCH_2CH_2OH$	**e**	$-CH_2CH(CH_3)_2$	8	**h**	$-CH_2CH(CH_3)CH_2CH_2OH$	15
$CH_3(CH_2)_3CH_2OH$	**f**	$-C_4H_9$	9		Not isolated	
$C_6H_5CH_2CH_2OH$	**g**	$-CH_2C_6H_5$	33		Not isolated	
$C_6H_5CH_2CH_2CH_2OH$	**h**	$-CH_2CH_2C_6H_5$	14		Not isolated	
Cyclic-$C_6H_{11}CH_2OH$	**i**	–cyclic-C_6H_{11}	23		Not isolated	

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TABLE II. Preparation of 7-Alkylcamptothecins (**4**) by the Reaction of **1** with Aldehydes

Aldehyde	Product	R	Yield (%)
C_2H_5CHO	**4b**	$-C_2H_5$	77
C_3H_7CHO	**4c**	$-C_3H_7$	60
C_4H_9CHO	**4f**	$-C_4H_9$	48
$C_6H_5CH_2CHO$	**4g**	$-CH_2C_6H_5$	51
n-$C_7H_{15}CHO$	**4j**	$-C_7H_{15}$	38

through silica gel column chromatography. The compound with a higher *Rf* value on thin layer chromatography (TLC) was 7-methylcamptothecin (**4a**), and the other was 7-(2-hydroxyethyl)camptothecin (**5a**); they were obtained in 40% and 32% yields, respectively.[7] In the case of propanol, three products were obtained in a similar manner; the compound with the highest *Rf* value was 7-ethyl derivative (**4b**) and the others were 7-(1-hydroxy-2-propyl)- and 7-(3-hydroxy-1-propyl)camptothecins (**5b** and **5c**).

In the case of pentanol or higher alcohols, 7-alkyl derivatives were isolated in lower yields due to poor solubility of the alcohols in the reaction medium. The 7-hydroxyalkyl derivatives could not be isolated from the complex mixtures.

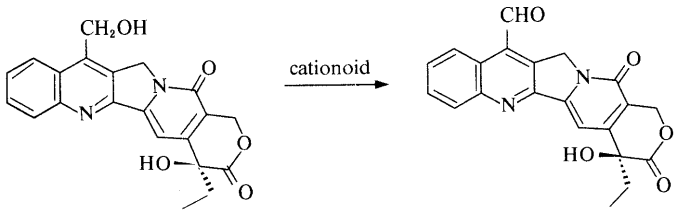
Attempted synthesis of 7-*tert*-butyl derivative using 2,2-dimethylpropanol furnished only 7-methylcamptothecin (**4a**) in a low yield. The reaction with secondary alcohol such as isopropanol gave small amounts of **4a** and 7-(2-hydroxy-1-propyl)camptothecin (**5i**) in 11% yield.

7-Methylcamptothecin (**4a**) was produced in 79% yield by the reaction with *tert*-butylhydroperoxide as the radical source in the presence of ferrous ion.[4] For the preparation of 7-ethylcamptothecin (**4b**), we examined the use of propionaldehyde, diethylketone and propionic acid as the radical source.[8] The best result was obtained with the aldehyde (77% yield), on the other hand the reactions with the ketone and the acid afforded **4b** in 22% and 13% yields, respectively, together with a complex mixture. Further examples of the alkylation of **1** using aldehydes are presented in Table II.

As the reaction mechanism, for example with propanol, it was speculated that the alcohol yields a propoxy radical and three possible carbon radicals (α, β and γ position) by the action of the hydroxyl radical formed under the conditions. The propoxy radical undergoes self-decomposition to the more reactive ethyl radical. In the reaction, 7-ethyl (**4b**) and hydroxyalkyl compounds (**5b** from the β-radical, **5c** from the γ-radical) were isolated, but not the product of the α-radical, probably due to its reduced reactivity (stabilized by the α-oxygen).[4] With branched alcohols such as 2-methylpropanol and 3-methylbutanol, the tertiary radicals did not attack **1** possibly due to steric reasons. The aldehydes afford the corresponding ketyl radicals by the action of the hydroxyl radical, which decompose to alkyl radicals and carbon monoxide, and then the alkylation reactions occur predominantly to give 7-alkyl compounds (**4**).

We synthesized various 7-*C*-substituted derivatives starting from 7-hydroxymethylcamptothecin (**2**). Acylation of **2** with acid anhydride gave the acyloxymethyl compound (**6**) in good yield, whereas acid chlorides such as benzoyl and phenylacetyl chlorides afforded 7-formylcamptothecin (**7**) in addition to the corresponding acyloxymethyl compounds (**6**). The treatment with *p*-toluenesulfonyl chloride in hot pyridine and with the chlorinating agents showed in Table III effected the conversion of **2** to **7**. Compound **2** was also converted into **7** by heating in aqueous sulfuric or acetic acid.

TABLE III. Synthesis of Aldehyde (**7**) by the Reaction of **2** with Cationoid Reagents



2 **7**

Cationoid	Solvent	Temp (°C)	Time (h)	Yield (%)
H_2SO_4	H_2O	Reflux	30.5	30
CH_3COOH	Neat	Reflux	5.5	68
$BF_3 \cdot OEt_2$	TCE[a]–dioxane	Reflux	14.5	26
$POCl_3$	$CHCl_3$–dioxane	Reflux	2.0	34
$SOCl_2$	$CHCl_3$–dioxane	Reflux	14.5	57
TsCl[b]	Pyridine	80—90	4.5	69
Ph_3P–CCl_4	DMF[c]	80—90	10.0	56

a) Tetrachloroethane. *b*) *p*-Toluenesulfonyl chloride. *c*) *N*,*N*-Dimethylformamide.

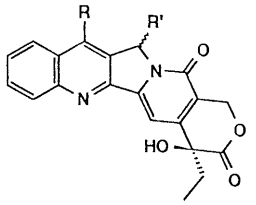
Heating of **2** in methanol or ethanol in the presence of 15% (v/v) sulfuric acid furnished ethers (**8a**, 38.6%, **8b**, 20.7%) and acetals (**9a**, 17%, **9b**, 16%), the latter being hydrolyzed into **7**. By heating in butanol containing 15% (v/v) sulfuric acid, **2** was converted into 7-butoxymethyl derivative (**8c**) in 42% yield. When **2** was treated with 0.2% (v/v) sulfuric acid, 7-dibutoxymethyl derivative (**9c**) was obtained in 39% yield as the major product.

Aldehyde **7** was converted into the iminomethyl derivative (**10**) by reaction with carbonyl reagents in a mixture of ethanol–pyridine.

Chromic acid oxidation of **2** furnished carboxylic acid (**11**) in good yield. Esterification of **11** in methanol in the presence of sulfuric acid resulted in the formation of the ester in poor yield in spite of heating for a long period of time, whereas, treatment of the potassium salt of the acid with alkylating agents gave esters (**12**) in good yields.

Carboxamides, **13**, were synthesized from **11** with amines by using ethyl chloroformate and dicyclohexylcarbodiimide (DCC) as condensing agents. Aminolysis of methyl ester (**12a**) with diethylamine resulted in the formation of the acid (**11**).

Several 5-*O*-substituted camptothecins were prepared. Compound **3a** was conveniently obtained by oxidation of **1** with iodine in the presence of potassium carbonate in *N*,*N*-dimethylformamide (DMF). The nuclear magnetic resonance (NMR) spectrum suggested that **3a** was a mixture (1 : 1) of the diastereomers with respect to the configuration at the C-5. By heating in methanol and in butanol with boron trifluoride etherate, the hemiaminal, **3a**, was converted into the 5-methoxyl or 5-butoxyl derivatives (**3b** and **3c**), respectively. 5-Acyloxyl derivatives **3d** and **3e** were



3, 6, 8—13

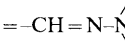
3a:	R=–H	R′=–OH
3b:	R=–H	R′=–OCH_3
3c:	R=–H	R′=–OC_4H_9
3d:	R=–H	R′=–$OCOCH_3$
3e:	R=–H	R′=–$OCOC_6H_5$
6a:	R=–CH_2OCOCH_3	R′=–H
6b:	R=–$CH_2OCOC_2H_5$	R′=–H
6c:	R=–$CH_2OCOC_3H_7$	R′=–H
6d:	R=–$CH_2OCOC_4H_9$	R′=–H
6e:	R=–$CH_2OCOCH_2C_6H_5$	R′=–H
6f:	R=–$CH_2OCOC_6H_5$	R′=–H
8a:	R=–CH_2OCH_3	R′=–H
8b:	R=–$CH_2OC_2H_5$	R′=–H
8c:	R=–$CH_2OC_4H_9$	R′=–H
9a:	R=–$CH(OCH_3)_2$	R′=–H
9b:	R=–$CH(OC_2H_5)_2$	R′=–H
9c:	R=–$CH(OC_4H_9)_2$	R′=–H
10a:	R=–CH=N–OH	R′=–H
10b:	R=–CH=N–NH_2	R′=–H
10c:	R=–CH=N–$NHCH_3$	R′=–H
10d:	R=–CH=N–NHC_6H_5	R′=–H
10e:	R=–CH=N– 	R′=–H
10f:	R=–CH=N–$NHCONH_2$	R′=–H
11:	R=–COOH	R′=–H
12a:	R=–$COOCH_3$	R′=–H
12b:	R=–$COOC_2H_5$	R′=–H
12c:	R=–$COOCH_2C_6H_5$	R′=–H
13a:	R=–$CONH_2$	R′=–H
13b:	R=–$CONHC_2H_5$	R′=–H
13c:	R=–$CONHCH_2C_6H_5$	R′=–H
13d:	R=–$CON(CH_3)_2$	R′=–H
13e:	R=–CONH-cyclic C_6H_{11}	R′=–H

Chart 1

prepared by the ordinary method. The diastereomers of **3d** were separated and characterized (Experimental).

Results and Discussion

The antitumor activities of the derivatives synthesized herein are presented in Table IV, compared with camptothecin (**1**). 7-Alkyl derivatives **4b** and **4c**, 7-acyloxymethylcamptothecins (**6a** and **6c**) and ethyl ester (**12b**) exhibited higher antitumor activity than **1**. In the 7-alkylcamptothecin series, 7-ethylcamptothecin (**4b**) showed 453 T/C% with 9 cured mice in 10 tested mice. The highest activity observed was in the derivative with the ethyl substituent and the compounds having lower or higher alkyl substituents than these which resulted in a decrease or loss of activity. The derivatives possessing α-branched alkyl groups (such as **4e**, **4f** and **5e**) had no activity.

Water-soluble derivatives, **10e** (as HCl salt) or **11** (as Na salt) showed slight activity. Neither the carboxamides, **13**, nor 5-*O*-substituted camptothecins, **3**, had any activity.

In general, more hydrophilic substituents caused a decrease of activity while the derivatives with more lipophilic substituents showed higher activity.

Significant antitumor activity was already reported for the 7-ethyl derivative, **4b**, in other experimental systems.[9] Further modification, for example introduction of substituents into the A-ring of **4b**, will be published elsewhere in the near future.[10]

TABLE IV. Antitumor Activity of 7-*C*- and 5-*O*-Substituted Camptothecin Derivatives (L1210 in Mice)

Compd. No.	T/C%[a] Total dose (mg/kg)		
	10	25	40
1	180	313 (4/10)[b]	120
2	NT[c]	163	92
3a	92	99	102
3b	102	NT	98
3c	NT	89	98
3d	NT	101	98
3e	NT	102	99
4a	NT	160	61[e]
4b	182	453 (9/10)	81[e]
4c	182	326 (4/10)	82[e]
4d	NT	76	74[e]
4f	110	116	76[e]
4h	120	176	120[e]
5b	NT	77	76[e]
5e	NT	77	77[e]
6a	136	390 (4/10)	78
6b	NT	202 (2/10)	76
6c	136	390 (4/10)	77
6d	126	235	159
6e	NT	168	99
6f	NT	160	224 (1/10)
7	136	302 (5/10)	285
8a	320 (1/6)[d]	NT	80[e]
8b	120[d]	NT	80[e]
8c	120[d]	NT	160[e]
9a	148	129	75
9b	127	199 (1/10)	86
10a	NT	212 (1/10)	242
10b	128	182	134
10e[f]	145	185	113
11[g]	121	126	128
12a	NT	173	161 (2/10)
12b	162	340 (4/10)	76
12c	NT	115	162
12d	NT	127	131
13a	100	102	106
13b	NT	100	102
13c	NT	100	102
13d	NT	99	110

a) T/C% = (the mean survival time of treated mice)/(the mean survival time of untreated control mice). *b*) Figures in parentheses indicate (number of cured mice/number of tested mice). *c*) NT: not tested. *d*) 12.5 mg/kg. *e*) 50 mg/kg. *f*) Administerd as HCl salt in saline. *g*) Administered as Na salt in saline.

Experimental

All the melting points (with decomposition) are uncorrected. Infrared (IR) spectra were determined on a JASCO DIR-40 spectrometer. ^{1}H-NMR spectra were measured on a JEOL JNM-FX 100 (99.6 MHz) with tetramethylsilane (TMS) as the internal standard. Mass spectra (MS) and Ultraviolet (UV) spectra were recorded on a JEOL JMS-DX 300 mass spectrometer and on a Shimadzu DIP-140 spectrophotometer, respectively. Optical rotation values were obtained with a JASCO DIP-140 spectrometer.

7-Hydroxymethylcamptothecin (2) To a suspension of **1** (3.00 g, 8.6 mmol) in a mixture of MeOH (90 ml) and H_2O (75 ml), 75% H_2SO_4 (75 ml) was added dropwise, and then $FeSO_4 \cdot 7H_2O$ (2.40 g, 8.6 mmol) was added. To the ice-cold mixture, 30% H_2O_2 (15 ml, 6.6 mmol) was added dropwise for 2 h with stirring. The mixture was stirred at room temperature for 14 h and then diluted with H_2O, the precipitate was

collected on a celite pad by suction. The pad was eluted with hot DMF and the eluent was evaporated to dryness, and the residue was recrystallized to give **2** (2.70 g, 82% yield) as pale yellow prisms. mp 274—276 °C (pyridine). IR (KBr) ν: 1770, 1665, 1605 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 5.27 (2H, d, J=4 Hz, 7-C$\underline{H}_2$OH), 5.40 (2H, s, C_5-$\underline{H}_2$), 5.80 (2H, s, C_{17}-$\underline{H}_2$), 5.80 (1H, t, J=4 Hz, 7-CH$_2$O$\underline{H}$), 6.50 (1H, s, 20-O$\underline{H}$), 7.33 (1H, s, C_{14}-$\underline{H}$), 7.60—8.21 (4H, m, arom). MS m/z: 378.1283 (M^+). Calcd for $C_{21}H_{18}N_2O_5$: 378.1209. *Anal.* Calcd for $C_{21}H_{18}N_2O_5$: C, 66.66; H, 4.79; N, 7.40. Found: C, 66.02; H, 4.61; N, 7.57.

Preparation of 2 with HSA To a suspension of **1** (100 mg, 0.287 mmol) in MeOH (200 ml), HSA (100 mg, 0.881 mmol) was added. The mixture was heated for 36 h while adding HSA (100 mg, 0.881 mmol) every 3 h during heating. The insoluble material was collected by suction and dissolved in a mixture of DMF–pyridine (1 : 1, 50 ml). To the solution, acetic anhydride (300 mg, 2.94 mmol) was added. The mixture was warmed at 70—80 °C for 18 h and then evaporated to dryness. The residue was dissolved in $CHCl_3$ (200 ml) and washed with an aqueous NaCl solution. The organic layer was separated and dried over $MgSO_4$, filtered and evaporated. The residue was purified through silica gel column chromatography with $CHCl_3$. The combined fractions containing 7-acetoxymethyl-20-*O*-acetylcamptothecin were evaporated to dryness. The residue was dissolved in MeOH (20 ml) containing 1 N NaOH (10 ml) and the mixture was heated at 70—80 °C for 18 h. The mixture was condensed and then the residue was acidified with 1 N HCl (10 ml). The precipitate was collected and washed with acetone (10 ml) to give **2** (32 mg, 28.7% yield).

Preparation of 2 with $(NH_4)_2S_2O_8$ To a suspension of **1** (100 mg, 0.287 mmol) in MeOH (25 ml), 75% H_2SO_4 (10 ml) was added in an ice-bath. To the mixture an aqueous solution of $(NH_4)_2S_2O_8$ (15 g, 65.7 mmol, in 100 ml H_2O) was added dropwise over 2 h while heating at 100—110 °C. The mixture was diluted with ice-H_2O (100 ml) and the precipitate was extracted with a 1 : 1 mixture of $CHCl_3$–dioxane (500 ml) and then $CHCl_3$ (100 ml × 3). The combined extracts were dried over $MgSO_4$, filtered, and evaporated to dryness. The residue was dissolved in MeOH (200 ml) at 50—60 °C. The insoluble material was filtered out by suction and the filtrate was evaporated to dryness. The residue was crystallized from DMF–dioxane to give **2** (40 mg, 36.9% yield).

Preparation of 2 Using Glycolic Acid To a solution of **1** (50 mg, 0.143 mmol) in 75% H_2SO_4 (3 ml) containing glycolic acid (500 mg, 6.57 mmol) and silver nitrate (250 mg, 1.13 mmol), an aqueous solution of $(NH_4)_2S_2O_8$ (3 g, 13.1 mmol, in 15 ml H_2O) was added dropwise over 2 h while heating at 100—110 °C. The mixture was diluted with ice-H_2O (100 ml) and the precipitate was extracted with $CHCl_3$ (100 ml × 3). The combined extracts were dried over $MgSO_4$, filtered, and evaporated to dryness. The residue was purified by preparative TLC with 5% MeOH–$CHCl_3$ to give **2** (10.3 mg, 19.0% yield), **3a** (5 mg, 10% yield) and camptothecin 1-oxide which was identified with a synthetic sample. The *N*-oxide was obtained by the following method: A solution of **1** (3.0 g) in acetic acid (800 ml) containing 30% H_2O_2 (50 ml) was heated at 70—80 °C for 3.5 h. The mixture was condensed at 45—55 °C to about one-third, and the residue was poured into ice-H_2O (3 l). The precipitate was collected by suction. The solid was purified by recrystallization. Camptothecin 1-oxide: Yield 90.6%. Yellow-orange needles, mp 254 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) ν: 1745, 1645, 1590 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.88 (3H, t, J=7 Hz), 1.87 (2H, q, J=7 Hz), 5.28 (2H, s), 5.42 (2H, s), 6.51 (1H, s), 7.78—7.97 (2H, m), 8.09 (1H, s), 8.17—8.23 (2H, m), 8.63 (1H, dd, J=2, 8 Hz). MS m/z: 364 (M^+).

5-Hydroxycamptothecin (3a) To a solution of **1** (1.17 g, 3.7 mmol) and K_2CO_3 (556 mg, 4.0 mmol) in DMF (300 ml), I_2 (930 mg, 4.0 mmol) was added. After stirring at room temperature for 4 h, the mixture was passed through a celite pad by suction. The filtrate was evaporated to dryness, and the residue was recrystallized to give **3a** (1.03 g, 84% yield) as colorless prisms. mp 232—234 °C (acetone). IR (KBr) ν: 1743, 1655, 1597 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 5.41 (2H, br s), 6.90 (0.5H, s), 6.98 (0.5H, s), 7.21 (0.5H, s), 7.24 (0.5H, s), 7.65—8.21 (5H, m), 8.66 (1H, s, C_7-H). MS m/z: 364.1041 (M^+). Calcd for $C_{20}H_{16}N_2O_5$: 364.1053.

5-Methoxycamptothecin (3b) A solution of **3a** (224 mg, 0.62 mmol) in MeOH (40 ml) containing $BF_3 \cdot OEt_2$ (515 mg, 3.4 mmol) was heated under reflux for 6 h. The mixture was condensed, and the residue was purified through silica gel column chromatography with $CHCl_3$ to give **3b** (153 mg, 79% yield) as pale yellow needles. mp 255 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) ν: 1750, 1660, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.02 (3H, t, J=8 Hz), 1.92 (2H, q, J=8 Hz), 3.52 (1.5H, s, 5-OMe), 3.68 (1.5H, s, 5-OMe), 5.32 (1H, d, J=19 Hz), 5.72 (1H, d, J=19 Hz), 6.72 (0.5H, s, C_5-H), 6.89 (0.5H, s, C_5-H), 7.60 (1H, br), 7.50—8.40 (4H, m), 8.41 (1H, s). MS m/z: 387.1214 (M^+). Calcd for $C_{21}H_{18}N_2O_5$: 378.1209.

5-Butoxycamptothecin (3c) A solution of **3a** (224 mg, 0.62 mmol) in butanol (40 ml) containing $BF_3 \cdot OEt_2$ (515 mg, 6.4 mmol) was refluxed for 6 h. The mixture was worked up as described above to afford **3c** (170 mg, 66% yield) as pale yellow needles. mp 245 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) ν: 1765, 1670, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.02 (3H, t, J=8 Hz), 0.86—1.80 (7H, br m), 4.00 (4H, m), 5.22 (1H, d, J=18 Hz), 5.69 (1H, d, J=18 Hz), 6.77 (0.5H, s), 6.87 (0.5H, s), 7.33—8.40 (4H, m), 7.61 (1H, s), 8.41 (1H, s). MS m/z: 420 (M^+).

5-Acetoxycamptothecin (3d) To a solution of **3a** (300 mg, 0.82 mmol) in pyridine (20 ml), acetic anhydride (105 mg, 1.0 mmol) was added dropwise. The mixture was stirred at room temperature for 3 h and then evaporated to dryness. The residue was purified with preparative high performance liquid chromatography (HPLC) (Richrosorb SI-60 column, 0.5% MeOH–$CHCl_3$). **3d-1**: Yield 41%. Colorless needles, mp 202—205 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) ν: 1760, 1670, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.06 (3H, t, J=7 Hz), 1.90 (2H, q, J=7 Hz), 2.195 (3H, s), 5.22 (1H, d, J=16 Hz), 5.64 (1H, d, J=16 Hz), 7.62 (1H, s), 7.91 (1H, s), 7.68—8.26 (4H, m), 8.45 (1H, s). MS m/z: 406.1176 (M^+). Calcd for $C_{22}H_{18}N_2O_6$: 406.1158. $[\alpha]_D^{25}$ +117.3° (c=0.0052, pyridine). **3d-2**: Yield 36%. Pale yellow needles, mp 258—261 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) ν: 1760, 1670, 1625, 1595 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.90 (2H, q, J=7 Hz), 2.192 (3H, s), 5.22 (1H, d, J=17 Hz), 5.67 (1H, d, J=17 Hz), 7.62 (1H, s), 7.96 (1H, s), 7.70—8.30 (4H, m), 8.46 (1H, s). MS m/z: 406.1134 (M^+). Calcd for $C_{22}H_{18}N_2O_6$: 406.1158. $[\alpha]_D^{25}$ −123° (c=0.0033, pyridine).

5-Benzoyloxycamptothecin (3e) Yield 68%. Pale yellow needles, mp 260 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) ν: 1760, 1740, 1670, 1610 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.02 (3H, t, J=7 Hz), 2.00 (2H, q, J=7 Hz), 5.23 (1H, d, J=18 Hz), 5.56 (1H, d, J=18 Hz), 7.33—8.40 (9H, m), 7.21 (0.5H, s), 7.32 (0.5H, s), 8.72 (1H, s). MS m/z: 468.1296 (M^+). Calcd for $C_{27}H_{20}N_2O_6$: 468.1314.

Reaction of 1 with Alcohols or Aldehydes, General Procedure To a suspension of **1** (1.00 g, 2.9 mmol) containing $FeSO_4 \cdot 7H_2O$ (300 mg, 1.1 mmol) and an alcohol or aldehyde (2 ml) in H_2O (20 ml), conc. H_2SO_4 (11 ml) was added dropwise. To the mixture 30% H_2O_2 (720 mg, 6.4 mmol) was added dropwise with stirring in an ice-salt bath. The mixture was stirred at room temperature for 3 h and then diluted with H_2O. The precipitate was extracted with $CHCl_3$ (100 ml × 3). The combined extracts were evaporated, and the residue was passed through silica gel column chromatography with 2% MeOH–$CHCl_3$.

4a: Pale yellow needles, mp 280—281 °C (pyridine–MeOH). IR (KBr) ν: 1755, 1650, 1600 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.91 (3H, t, J=8 Hz), 1.88 (2H, q, J=8 Hz), 2.79 (3H, s), 5.26 (2H, s), 5.41 (2H, s), 6.43 (1H, s), 7.34 (1H, s), 7.57—8.32 (4H, m). MS m/z: 362 (M^+). *Anal.* Calcd for $C_{21}H_{18}N_2O_4$: C, 69.60; H, 5.00; N, 7.73. Found: C, 69.42; H, 4.92; N, 7.76.

Preparation of 4a Using *tert*-Butylhydroperoxide To a suspension of **1** (500 mg, 1.43 mmol) in H_2O (15 ml) containing $FeSO_4 \cdot 7H_2O$ (150 mg, 0.55 mmol) and AcOH (1.5 ml), conc. H_2SO_4 (8 ml) was added dropwise, and then *tert*-butylhydroperoxide (900 mg, 10.0 mmol) was added dropwise with stirring in an ice-salt bath. The mixture was stirred at room temperature for 1 h and then diluted with H_2O. The precipitate was extracted with $CHCl_3$ (1.5 l). The extract was evaporated, and the residue was washed with acetone and then recrystallized to give **4a** (409 mg, 79.0% yield).

4b: Pale yellow needles, mp 258—261 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) ν: 1750, 1650, 1595 cm^{-1}. ^{1}H-NMR (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), 5.21 (2H, s), 5.24 (1H, d, J=16 Hz), 5.57 (1H, d, J=16 Hz), 7.49 (1H, s), 7.44—8.21 (4H, m). MS m/z: 376.1399 (M^+). Calcd for $C_{22}H_{20}N_2O_4$: 376.1422. *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.

Preparation of 4b Using Diethylketone To a suspension of **1** (1.00 g, 2.9 mmol) in H_2O (20 ml) containing $FeSO_4 \cdot 7H_2O$ (300 mg, 1.1 mmol), AcOH (15 ml) and diethyl ketone (1.29 g, 15 mmol), conc. H_2SO_4 (11 ml) was added dropwise. To the mixture 30% H_2O_2 (720 mg, 6.4 mmol) was added dropwise with stirring in an ice-bath. The mixture was stirred at room temperature for 48 h and then diluted with H_2O. The precipitate was extracted with $CHCl_3$ (100 ml × 3). The combined extracts were evaporated, and the residue was passed through silica gel column chromatography with $CHCl_3$ to give **4b** (24 mg, 22% yield).

Preparation of 4b Using Propionic Acid To a suspension of **1** (1.00 g,

2.9 mmol) in H_2O (20 ml) containing $FeSO_4 \cdot 7H_2O$ (300 mg, 1.1 mmol) and propionic acid (1.10 g, 15 mmol), conc. H_2SO_4 (11 ml) was added dropwise. To the mixture 30% H_2O_2 (720 mg, 6.4 mmol) was added dropwise with stirring in an ice-bath. The mixture was stirred at room temperature for 48 h and then diluted with H_2O. The precipitate was extracted with $CHCl_3$ (100 ml × 3). The combined extracts were evaporated, and the residue was passed through silica gel column chromatography with $CHCl_3$ to give **4b** (17 mg, 13% yield).

4c: Pale yellow needles, mp 260—261 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) *v*: 1745, 1650, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, t, J=7 Hz), 1.08 (3H, t, J=8 Hz), 1.25—2.05 (4H, m), 3.26 (2H, t, J=8 Hz), 5.24 (2H, s), 5.35 (1H, d, J=17 Hz), 5.69 (1H, d, J=17 Hz), 7.67 (1H, s), 7.55—8.29 (4H, m). MS *m/z*: 390 (M^+). *Anal.* Calcd for $C_{23}H_{24}N_2O_4$: C, 70.75; H, 5.68; N, 7.18. Found: C, 70.90; H, 5.65; N, 7.12.

4d: Pale yellow needles, mp 258—259 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) *v*: 1750, 1645, 1595 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=8 Hz), 1.54 (6H, d, J=7 Hz), 1.90 (2H, q, J=8 Hz), 3.9—4.1 (1H, m), 5.37 (2H, s), 5.29 (1H, d, J=17 Hz), 5.75 (1H, d, J=17 Hz), 7.63 (1H, s), 7.45—8.63 (4H, m). MS *m/z*: 390 (M^+).

4e: Pale yellow needles, mp 198—200 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) *v*: 1740, 1650, 1595 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.07 (3H, t, J=7 Hz), 1.07 (6H, d, J=7 Hz), 1.93 (2H, q, J=7 Hz), 2.12—2.41 (1H, m), 3.09 (2H, d, J=7 Hz), 5.29 (1H, d, J=16 Hz), 5.37 (2H, s), 5.54 (1H, J=16 Hz), 7.68 (1H, s), 7.55—8.29 (4H, m). MS *m/z*: 404 (M^+).

4f: Pale yellow needles, mp 206—207 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) *v*: 1745, 1680, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.80—2.04 (12H, m), 3.14 (2H, t, J=7 Hz), 5.23 (2H, s), 5.26 (1H, d, J=17 Hz), 5.73 (1H, d, J=17 Hz), 7.62 (1H, s), 7.28—7.88 (2H, m), 7.96—8.26 (2H, m). MS *m/z*: 404 (M^+). *Anal.* Calcd for $C_{24}H_{24}N_2O_4$: C, 71.27; H, 5.98; N, 6.93. Found: C, 71.11; H, 5.95; N, 7.01.

4g: Pale yellow needles, mp 263—265 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) *v*: 3360, 1735, 1650, 1590 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, t, J=8 Hz), 1.89 (2H, q, J=8 Hz), 4.58 (2H, s), 5.14 (2H, s), 5.26 (1H, d, J=16 Hz), 5.73 (1H, d, J=16 Hz), 7.00—7.34 (5H, m), 7.68 (1H, s), 7.55—8.23 (4H, m). MS *m/z*: 438 (M^+).

4h: Pale yellow needles, mp 260—262 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) *v*: 1745, 1655, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.02 (3H, t, J=8 Hz), 4.19 (2H, q, J=8 Hz), 3.47 (2H, t, J=7 Hz), 3.80 (2H, t, J=7 Hz), 4.78 (2H, s), 5.24 (1H, d, J=17 Hz), 5.70 (1H, d, J=17 Hz), 6.98—7.40 (5H, m), 7.61 (1H, s), 7.51—8.38 (4H, m). MS *m/z*: 452 (M^+).

4i: Pale yellow needles, mp 260—262 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) *v*: 1745, 1655, 1595 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=8 Hz), 1.20—2.18 (12H, m), 3.70—3.72 (1H, m), 5.39 (2H, s), 5.30 (1H, d, J=17 Hz), 5.72 (1H, d, J=17 Hz), 7.67 (1H, s), 7.50—7.85 (2H, m), 8.16—8.27 (2H, m). MS *m/z*: 430 (M^+).

5a: Colorless needles, mp 264—266 °C (EtOH). ^{1}H-NMR (DMSO-d_6) δ: 1.00 (3H, t, J=7 Hz), 1.92 (2H, q, J=7 Hz), 3.42 (2H, t, J=6 Hz), 3.99 (2H, m), 4.72 (1H, t, J=4 Hz), 5.35 (2H, s), 5.25 (1H, d, J=17 Hz), 5.65 (1H, d, J=17 Hz), 6.01 (1H, s), 7.58 (1H, s), 7.5—7.9 (2H, m), 8.1—8.20 (2H, m). MS *m/z*: 392.1352 (M^+). Calcd for $C_{22}H_{20}N_2O_5$: 392.1369.

5b: Pale yellow needles, mp 257—259 °C (EtOH). ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.48 (3H, d, J=6 Hz, 7-CH(CH_3)CH_2OH), 3.8—4.0 (3H, m), 4.90 (1H, t, J=5 Hz), 5.43 (4H, s), 6.50 (1H, s), 7.34 (1H, s), 7.6—7.9 (2H, m), 8.16 (1H, dd, J=1, 8 Hz), 8.37 (1H, d, J=7 Hz). MS *m/z*: 406.1530 (M^+). Calcd for $C_{23}H_{22}N_2O_5$: 406.1529.

5c: Pale yellow needles, mp 210—212 °C (EtOH–*n*-C_6H_{14}). ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.80—1.95 (4H, m), 3.28—3.32 (2H, m), 3.51 (2H, t, J=6 Hz), 4.68 (1H, br), 5.37 (2H, s), 5.43 (2H, s), 6.49 (1H, s), 7.33 (1H, s), 7.5—7.9 (2H, m), 8.1—8.3 (2H, m). MS *m/z*: 406.1548 (M^+). Calcd for $C_{23}H_{22}N_2O_5$: 406.1529.

5d: Colorless needles, mp 228—229 °C (EtOH). ^{1}H-NMR (DMSO-d_6) δ: 0.88 (6H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 1.97—2.25 (2H, m), 3.90 (2H, br), 4.33 (1H, br), 4.83 (1H, br), 5.39 (1H, s), 5.44 (2H, s), 6.52 (1H, s), 7.35 (1H, s), 7.61—7.94 (2H, m), 8.18 (1H, d, J=7 Hz), 8.41 (1H, d, J=8 Hz). MS *m/z*: 420 (M^+).

5e: Colorless needles, mp 214—216 °C (EtOH). ^{1}H-NMR (DMSO-d_6) δ: 0.90 (3H, t, J=7 Hz), 1.51 (3H, d, J=7 Hz), 1.89 (2H, q, J=7 Hz), 1.92—2.20 (2H, m), 3.30—3.50 (2H, m), 3.80 (1H, br), 4.59 (1H, t, J=5 Hz), 5.39 (2H, s), 5.44 (2H, s), 6.53 (1H, s), 7.35 (1H, s), 7.63—7.94 (2H, m), 8.19 (1H, dd, J=1, 8 Hz), 8.42 (1H, d, J=8 Hz). MS *m/z*: 420.1679 (M^+). Calcd for $C_{24}H_{24}N_2O_5$: 420.1862.

5f: Colorless needles, mp 197—199 °C (EtOH–*n*-C_6H_{14}). ^{1}H-NMR (DMSO-d_6) δ: 0.90 (3H, t, J=7 Hz), 1.60—1.70 (4H, m), 1.88 (2H, q, J=7 Hz), 3.20 (2H, br), 3.47 (2H, q, J=6 Hz), 4.42 (1H, t, J=5 Hz), 5.26 (2H, s), 5.43 (2H, s), 6.51 (1H, s), 7.32 (1H, s), 7.63—7.91 (2H, m), 8.10—8.30 (2H, m). MS *m/z*: 420.1673 (M^+). Calcd for $C_{24}H_{24}N_2O_5$: 420.1682.

5g: Pale yellow needles, mp 211—213 °C (EtOH). ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, d, J=7 Hz), 1.04 (3H, t, J=7 Hz), 1.90 (2H, q, J=7 Hz), 2.83—3.04 (1H, m), 3.42—3.80 (4H, m), 5.20—5.83 (4H, m), 7.66 (1H, s), 7.57—7.88 (2H, m), 8.12—8.27 (2H, m). MS *m/z*: 420 (M^+).

5h: Colorless needles, mp 222—224 °C (EtOH). ^{1}H-NMR ($CDCl_3$) δ: 1.01 (3H, d, J=7 Hz), 1.02 (3H, t, J=7 Hz), 1.60—1.99 (4H, m), 2.25 (1H, br), 2.94—3.39 (2H, m), 3.7—4.0 (2H, m), 5.24 (2H, s), 5.28 (1H, d, J=17 Hz), 5.68 (1H, d, J=17 Hz), 7.5—7.8 (3H, m), 8.03—8.17 (2H, m). MS *m/z*: 434 (M^+).

5i: Pale yellow needles, mp 237—239 °C (EtOH–$CHCl_3$). ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.28 (3H, d, J=6 Hz), 1.88 (2H, q, J=7 Hz), 3.04—3.47 (2H, m), 4.00—4.10 (1H, m), 4.85 (1H, d), 5.34 (2H, s), 5.43 (2H, s), 6.51 (1H, s), 7.34 (1H, s), 7.63—7.93 (2H, s), 8.12—8.34 (2H, m). MS *m/z*: 406 (M^+).

7-Acyloxymethylcamptothecin (6), General Procedure To a cooled solution of **2** in pyridine, an acylating agent (1.2 eq) was added dropwise. The mixture was stirred at room temperature for a few hours and then evaporated to dryness. The residue was purified through silica gel column chromatography with $CHCl_3$.

6a: Yield 78%. Pale yellow needles, mp 277—279 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) *v*: 1770, 1650, 1595 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, t, J=7 Hz), 1.89 (2H, q, J=7 Hz), 2.18 (3H, s), 5.44 (2H, s), 5.26 (1H, d, J=17 Hz), 5.72 (1H, d, J=17 Hz), 5.71 (2H, s), 7.70 (1H, s), 7.58—8.28 (4H, m). MS *m/z*: 420.1369 (M^+). Calcd for $C_{23}H_{20}N_2O_6$: 420.1314. *Anal.* Calcd for $C_{23}H_{20}N_2O_6$: C, 65.71; H, 4.79; N, 6.66. Found: C, 65.36; H, 4.73; N, 6.43.

6b: Yield 86%. Pale yellow needles, mp 279—280 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) *v*: 1740, 1655, 1595 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.05 (3H, t, J=7 Hz), 1.18 (3H, t, J=7 Hz), 1.91 (2H, q, J=7 Hz), 2.47 (2H, q, J=7 Hz), 5.28 (1H, d, J=17 Hz), 5.70 (1H, d, J=17 Hz), 5.44 (4H, s), 5.71 (2H, s), 7.63 (1H, s), 7.70—8.30 (4H, m). MS *m/z*: 434 (M^+). *Anal.* Calcd for $C_{24}H_{22}N_2O_6$: C, 66.35; H, 5.10; N, 6.45. Found: C, 66.37; H, 5.07; N, 6.33.

6c: Yield 71%. Pale yellow needles, mp 252—254 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) *v*: 1750, 1740, 1665, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.94 (3H, t, J=7 Hz), 1.05 (3H, t, J=7 Hz), 1.69 (2H, sextet, J=7 Hz), 1.91 (2H, q, J=7 Hz), 2.42 (2H, t, J=7 Hz), 5.28 (1H, d, J=16 Hz), 5.72 (1H, d, J=16 Hz), 5.44 (2H, s), 5.69 (2H, s), 7.63 (1H, s), 7.58—8.25 (4H, m). MS *m/z*: 448 (M^+). *Anal.* Calcd for $C_{25}H_{24}N_2O_6$: C, 66.95; H, 5.39; N, 6.25. Found: C, 66.80; H, 5.30; N, 6.28.

6d: Yield 66%. Pale yellow needles, mp 240—242 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) *v*: 1740, 1650, 1595 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.94 (6H, d, J=7 Hz), 1.04 (3H, t, J=7 Hz), 1.91 (2H, q, J=7 Hz), 2.31 (2H, d, J=7 Hz), 3.71 (1H, m), 5.30 (1H, d, J=16 Hz), 5.69 (1H, d, J=16 Hz), 5.42 (2H, s), 5.72 (2H, s), 7.65 (1H, s), 7.57—8.27 (4H, m). MS *m/z*: 462 (M^+).

6e: Yield 49%. Pale yellow needles, mp 252—253 °C ($CHCl_3$–*n*-C_6H_{14}). IR (KBr) *v*: 1760, 1665, 1600 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.89 (2H, q, J=7 Hz), 5.40 (2H, s), 5.42 (2H, s), 5.45 (2H, s), 5.79 (2H, s), 7.25 (5H, s), 7.35 (1H, s), 7.60—8.30 (8H, m). MS *m/z*: 496 (M^+).

6f: Yield 58%. Pale yellow needles, mp 298 °C (MeOH). IR (KBr) *v*: 1770, 1730, 1675, 1610 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.09 (3H, t, J=8 Hz), 1.93 (2H, q, J=8 Hz), 5.55 (4H, br s), 5.92 (2H, s), 7.47 (1H, s), 7.54—8.30 (9H, m). MS *m/z*: 482 (M^+). *Anal.* Calcd for $C_{27}H_{22}N_2O_6$: C, 69.70; H, 4.60; N, 5.81. Found: C, 69.35; H, 5.81; N, 5.62.

Reaction of 2 with Phenylacetyl Chloride A mixture of **2** (100 mg, 0.264 mmol) and phenylacetyl chloride (200 mg, 1.29 mmol) in DMF–pyridine (1 : 1, 100 ml) was stirred at 90—100 °C for 6 h. The mixture was evaporated to dryness and the residue was purified through silica gel column chromatography with $CHCl_3$ to give **6e** (74 mg, 56.6% yield) and **7** (19 mg, 19.1% yield).

7-Formylcamptothecin (7), Typical Procedure A suspension of **2** (200 mg, 0.53 mmol) in AcOH (100 ml) was heated under reflux for 5.5 h. The mixture was evaporated to dryness and the residue was purified through silica gel column chromatography with 5% MeOH–$CHCl_3$ to give **6a** (19 mg, 9% yield) and **7** (135 mg, 68% yield) as orange needles, mp 256—260 °C (aq. AcOH). IR (KBr) *v*: 1750, 1690, 1655, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.18 (H, t, J=8 Hz), 1.93 (2H, q, J=8 Hz), 5.31 (1H, d, J=16 Hz), 5.80 (1H, d, J=16 Hz), 5.63 (2H, s), 7.68 (1H, s), 7.85—7.95 (2H, m), 8.35—8.41 (1H, m), 8.77—8.83 (1H, m), 11.20 (1H, s). MS *m/z*: 376 (M^+).

7-Methoxy- and 7-Dimethoxymethylcamptothecin (8a and 9a) A solu-

tion of **2** (100 mg, 0.26 mmol) in a 1 : 1 mixture of MeOH–dioxane (40 ml) containing conc. H_2SO_4 (3 ml) was heated under reflux for 35 h. The mixture was condensed, the residue was poured into H_2O, and the precipitate was extracted with $CHCl_3$ (100 ml × 3). The combined extracts were dried over $MgSO_4$, filtered and evaporated to dryness. The residue was purified through silica gel column chromatography with 1% MeOH–$CHCl_3$.

8a: Yield 40 mg, 39%. Pale yellow needles, mp 252—257 °C (MeOH–$CHCl_3$). IR (KBr) ν: 1755, 1655, 1600 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.94 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 3.31 (3H, s), 5.20 (2H, s), 5.36 (2H, s), 5.46 (2H, s), 6.51 (1H, s), 7.39 (1H, s), 7.60—8.30 (4H, m). MS m/z: 392 (M^+).

9a: Yield 19 mg, 17%. Pale yellow needles, mp 222—224 °C ($CHCl_3$–n-C_6H_{14}). IR (KBr) ν: 1750, 1655, 1595 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.05 (3H, t, J=7 Hz), 1.90 (2H, q, J=7 Hz), 3.40 (3H, s), 3.41 (3H, s), 5.29 (1H, d, J=16 Hz), 5.77 (1H, d, J=16 Hz), 5.49 (2H, s), 6.25 (1H, s), 7.76 (1H, s), 7.67—8.34 (4H, m). MS m/z: 422 (M^+). *Anal.* Calcd for $C_{23}H_{22}N_2O_6$: C, 65.39; H, 5.25; N, 6.63. Found: C, 65.47; H, 5.19; N, 6.59.

7-Ethoxy- and 7-Diethoxymethylcamptothecin (8b and 9b) A solution of **2** (200 mg, 0.5 mmol) in a 1 : 1 mixture of EtOH and dioxane (40 ml) containing conc. H_2SO_4 (6 ml) was refluxed for 7 h. The reaction mixture was worked up as described above to give **8b**: Yield 27 mg, 21%. Pale yellow needles, mp 139—142 °C (EtOH–$CHCl_3$). IR (KBr) ν: 1740, 1655, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.38 (3H, t, J=7 Hz), 1.83 (2H, q, J=7 Hz), 3.81 (2H, q, J=7 Hz), 5.18 (2H, s), 5.27 (1H, d, J=17 Hz), 5.76 (1H, d, J=17 Hz), 5.43 (2H, s), 7.65 (1H, s), 7.65—8.28 (4H, m). MS m/z: 406 (M^+). *Anal.* Calcd for $C_{23}H_{22}N_2O_5$: C, 67.96; H, 5.46; N, 6.89. Found: C, 67.69; H, 5.35; N, 6.94. **9b**: Yield 24 mg, 19%. Pale yellow needles, mp 223—224 °C ($CHCl_3$–n-C_6H_{14}). IR (KBr) ν: 1740, 1655, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.17 (3H, t, J=7 Hz), 1.26 (3H, t, J=7 Hz), 1.28 (3H, t, J=7 Hz), 1.90 (2H, q, J=7 Hz), 3.6—3.8 (4H, m), 5.29 (1H, d, J=16 Hz), 5.76 (1H, d, J=16 Hz), 5.50 (2H, s), 6.36 (1H, s), 7.66 (1H, s), 7.64—7.87 (2H, m), 8.17—8.39 (2H, m). MS m/z: 450 (M^+). *Anal.* Calcd for $C_{25}H_{26}N_2O_6$: C, 66.65; H, 5.82; N, 6.22. Found: C, 66.28; H, 5.95; N, 6.09.

7-Butoxymethylcamptothecin (8c) A suspension of **2** (100 mg, 0.26 mmol) in butanol (30 ml) containing conc. H_2SO_4 (5 ml) was heated under reflux for 2.5 h. The mixture was worked up as described above to give **4c**: Yield 48 mg, 42%. Pale yellow needles, mp 142—144 °C (EtOH–n-C_6H_{14}). IR (KBr) ν: 1745, 1660, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.97 (3H, t, J=7 Hz), 1.03 (3H, t, J=6 Hz), 1.10—1.80 (4H, m), 1.90 (2H, q, J=7 Hz), 3.70 (2H, t, J=6 Hz), 5.17 (2H, s), 5.28 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 5.42 (2H, s), 7.66 (1H, s), 7.50—8.30 (4H, m). MS m/z: 434 (M^+). *Anal.* Calcd for $C_{25}H_{26}N_2O_5$: C, 69.11; H, 6.03; N, 6.45. Found: C, 69.24; H, 5.98; N, 6.48.

7-Dibutoxymethylcamptothecin (9c) A solution of **2** (100 mg, 0.26 mmol) in butanol (50 ml) containing conc. H_2SO_4 (8 mg) was heated under reflux for 0.5 h. The mixture was worked up as described above to give **9c** (52 mg, 39% yield) as pale yellow needles, mp 107—111 °C ($CHCl_3$–n-C_6H_{14}). IR (KBr) ν: 1750, 1660, 1610 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7 Hz), 1.11 (3H, t, J=7 Hz), 1.12 (3H, t, J=7 Hz), 1.14—1.79 (8H, m), 1.90 (2H, q, J=7 Hz), 3.47—3.67 (4H, m), 5.29 (1H, d, J=16 Hz), 5.77 (1H, d, J=16 Hz), 5.50 (2H, s), 6.36 (1H, s), 7.68 (1H, s), 7.50—7.80 (2H, m), 8.20—8.40 (2H, m). MS m/z: 506 (M^+).

Hydrolysis of 8b A solution of **8b** (250 mg, 0.555 mmol) in 20% HCl (40 ml) was stirred at room temperature for 18 h. The mixture was diluted with ice-H_2O and the precipitate was extracted with $CHCl_3$ (200 ml × 3). The combined extracts were evaporated and the residue was recrystallized from aqueous AcOH to give **7** (187 mg, 89.6% yield).

7-Iminomethylcamptothecins (10), General Procedure A mixture of **5** (350 mg, 0.93 mmol) and a carbonyl reagent (2.8 mmol) in a mixture of EtOH (70 ml) and pyridine (10 ml) was heated under reflux for 0.5 h. The mixture was condensed, and the residue was recrystallized.

10a: Yield 87%. Yellow prisms, mp 255—257 °C (EtOH). IR (KBr) ν: 1740, 1655, 1590 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.9 (3H, t, J=8 Hz), 1.92 (2H, q, J=8 Hz), 5.34 (2H, s), 5.43 (2H, s), 7.63 (1H, s), 7.75—8.34 (4H, m), 9.26 (1H, s), 12.54 (1H, s). MS m/z: 391 (M^+).

10b: Yield 71%. Yellow prisms, mp 262—265 °C (MeOH). IR (KBr) ν: 1755, 1655, 1590 cm^{-1}. MS m/z: 390 (M^+).

10c: Yield 74%. Yellow needles, mp 203—205 °C (EtOH). IR (KBr) ν: 1740, 1650, 1600 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.95 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 3.13 (3H, d, J=4 Hz), 5.35 (2H, s), 5.20 (1H, d, J=14 Hz), 5.60 (1H, d, J=14 Hz), 6.25 (1H, br s), 7.43 (1H, s), 7.50—8.80 (4H, m), 10.15 (1H, s). MS m/z: 404 (M^+).

10d: Yield 71%. Yellow needles, mp 205—208 °C (EtOH). IR (KBr) ν: 1735, 1655, 1600 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.97 (3H, t, J=7 Hz), 1.90 (2H, q, J=7 Hz), 5.24 (1H, d, J=16 Hz), 5.64 (1H, d, J=16 Hz), 5.48 (2H, s), 6.90 (1H, br s), 7.00—8.90 (9H, m), 7.48 (1H, s), 11.24 (1H, br s). MS m/z: 466 (M^+).

10e: Yield 83%. Colorless needles, mp 250 °C (EtOH). IR (KBr) ν: 1743, 1655, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.05 (3H, t, J=7 Hz), 1.89 (2H, q, J=7 Hz), 2.44 (3H, s), 2.72 (4H, t, J=8 Hz), 3.53 (4H, t, J=8 Hz), 5.41 (2H, s), 5.31 (1H, d, J=16 Hz), 5.73 (1H, d, J=16 Hz), 7.62 (1H, s), 7.40—8.30 (5H, m). MS m/z: 473 (M^+).

10f: Yield 91%. Pale yellow needles, mp 283 °C (EtOH). IR (KBr) ν: 1740, 1690, 1655, 1585 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.90 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 5.43 (2H, s), 5.35 (2H, s), 6.65 (2H, br), 7.35 (1H, s), 7.80—8.30 (6H, m), 8.86 (1H, s), 10.85 (1H, s).

Camptothecin 7-Carboxylic Acid (11) To a solution of **2** (1.00 g, 2.6 mmol) in conc. H_2SO_4 (10 ml), sodium dichromate (1.00 g, 3.82 mmol) was added with stirring in an ice-salt bath. The mixture was stirred at room temperature for 2 h and then poured into H_2O; the precipitate was collected by suction, and the solid was purified by recrystallization to give **11** (815 mg, 79% yield) as yellow needles, mp 280 °C (pyridine). IR (KBr) ν: 1760, 1650, 1595 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.91 (3H, t, J=7 Hz), 1.90 (2H, q, J=7 Hz), 5.31 (2H, s), 5.41 (2H, s), 6.50 (1H, br s), 7.30 (1H, s), 7.65—8.84 (4H, m). MS m/z: 392.0995 (M^+). Calcd for $C_{21}H_{16}N_2O_6$: 392.1002.

Esters (12), General Procedure To a solution of **11** (200 mg, 0.51 mmol) in DMF (30 ml), K_2CO_3 (200 mg, 1.1 mmol) and an alkylating reagent (5 eq, methyl p-toluenesulfonate, ethyl bromide, benzyl chloride) were added. The mixture was stirred at room temperature for 2 h and then passed through a celite pad by suction. The filtrate was evaporated to dryness, and the residue was purified through silica gel column chromatography with $CHCl_3$.

12a: Yield 87%. Yellow needles, mp 245—246 °C ($CHCl_3$–n-C_6H_{14}). ^{1}H-NMR ($CDCl_3$) δ: 1.05 (3H, t, J=8 Hz), 1.91 (2H, q, J=8 Hz), 4.17 (3H, s), 5.49 (2H, s), 5.32 (1H, d, J=17 Hz), 5.74 (1H, d, J=17 Hz), 7.66 (1H, s), 7.74—7.88 (2H, m), 8.27—8.89 (2H, m). MS m/z: 406 (M^+). *Anal.* Calcd for $C_{22}H_{18}N_2O_6$: C, 65.02; H, 4.46; N, 6.89. Found: C, 64.95; H, 4.51; N, 6.84.

12b: Yield 89%. Yellow needles, mp 239—241 °C ($CHCl_3$–n-C_6H_{14}). ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.56 (3H, t, J=7 Hz), 1.90 (2H, q, J=7 Hz), 4.63 (2H, q, J=7 Hz), 5.47 (2H, s), 5.32 (1H, d, J=17 Hz), 5.72 (1H, d, J=17 Hz), 7.61 (1H, s), 7.70—7.86 (2H, m), 8.27—8.85 (2H, m). MS m/z: 420 (M^+). *Anal.* Calcd for $C_{23}H_{20}N_2O_6$: C, 65.71; H, 4.79; N, 6.66. Found: C, 65.84; H, 4.80; N, 6.63.

12c: Yield 79%. Yellow needles, mp 208—210 °C ($CHCl_3$–n-C_6H_{14}). ^{1}H-NMR ($CDCl_3$) δ: 1.04 (6H, t, J=7 Hz), 1.45—1.98 (6H, m), 4.57 (2H, q, J=7 Hz), 5.46 (2H, s), 5.31 (1H, d, J=17 Hz), 5.73 (1H, d, J=17 Hz), 7.64 (1H, s), 7.69—7.76 (2H, m), 8.24—8.84 (2H, m). *Anal.* Calcd for $C_{25}H_{24}N_2O_6$: C, 66.95; H, 5.49; N, 6.24. Found: C, 66.90; H, 5.49; N, 6.24.

12d: Yield 91%. Yellow needles, mp 237—240 °C ($CHCl_3$–n-C_6H_{14}). ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 5.40 (2H, s), 5.30 (1H, d, J=17 Hz), 5.72 (1H, d, J=17 Hz), 5.59 (2H, s), 7.39—7.83 (7H, m), 7.61 (1H, s), 8.21—8.81 (2H, m). *Anal.* Calcd for $C_{28}H_{22}N_2O_6$: C, 69.70; H, 4.60; N, 5.81. Found: C, 69.40; H, 4.51; N, 5.56.

Preparation of 12a with Sulfuric Acid in MeOH A solution of **11** (100 mg, 0.255 mmol) in MeOH (40 ml) containing conc. H_2SO_4 (0.5 ml) was heated under reflux for 36 h. The mixture was diluted with ice-water (100 ml) and the solution was extracted with $CHCl_3$ (100 ml × 2). The combined extracts were dried over $MgSO_4$, filtered and evaporated *in vacuo*. The residue was purified through silica gel column chromatography with $CHCl_3$ to give **12a** (23 mg, 22.2% yield).

Amides (13), General Procedure To a solution of **11** (500 mg, 1.3 mmol) in CH_2Cl_2 (100 ml) containing Et_3N (0.8 ml), $ClCO_2Et$ (0.8 ml, 8.8 mmol) was added dropwise with stirring in an ice-salt bath. The mixture was stirred for 0.5 h and then ammonia or amine (5 eq) was added. The mixture was stirred at room temperature for 3 h and evaporated to dryness. The residue was passed through silica gel column chromatography with 2% MeOH–$CHCl_3$.

13a: Yield 90%. Yellow needles, mp 293—295 °C (pyridine–MeOH). IR (KBr) ν: 1740, 1675, 1660, 1590 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.9 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 5.32 (2H, s), 5.48 (2H, s), 6.52 (1H, s), 7.37 (1H, s), 7.74—8.34 (6H, m). MS m/z: 391 (M^+).

13b: Yield 66%. Yellow needles, mp 263—266 °C (aq. MeOH). IR (KBr) ν: 1760, 1640, 1580 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.25 (3H, t, J=7 Hz), 1.87 (2H, q, J=7 Hz), 3.30 (2H, m), 5.28 (2H, s),

5.43 (2H, s), 6.52 (1H, s), 7.37 (1H, s), 7.83—8.26 (4H, m), 8.96 (1H, m). MS *m/z*: 419 (M^+).

13c: Yield 78%. Yellow needles, mp 267—269 °C (aq. MeOH). IR (KBr) *v*: 1740, 1655, 1590 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.88 (2H, t, J=7 Hz), 4.62 (2H, d, J=6 Hz), 5.28 (2H, s), 5.43 (2H, s), 6.52 (1H, s), 7.30—7.50 (6H, m), 7.75—8.28 (4H, m), 9.42 (1H, m). MS *m/z*: 481 (M^+).

13d: Yield 90%. Yellow needles, mp 283—285 °C (aq. MeOH). ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 2.80 (3H, s), 3.21 (3H, s), 5.01 (1H, d, J=17 Hz), 5.40 (1H, d, J=17 Hz), 5.43 (2H, s), 6.53 (1H, s), 7.37 (1H, s), 7.75—8.28 (4H, m). MS *m/z*: 419 (M^+).

13e: To a suspension of **11** (100 mg, 0.225 mmol) in dioxane (50 ml), DCC (100 mg, 0.485 mmol) was added. The mixture was stirred at room temperature for 30 min, to the mixture cyclohexylamine (2 ml) was added dropwise. The mixture was stirred at room temperature for 6 h and worked-up as described above to give **13e** (29% yield) as yellow needles, mp 252—254 °C (aq. EtOH). IR (KBr) *v*: 1755, 1660, 1595 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.3—1.9 (12H, m), 3.90 (1H, br), 5.24 (2H, s), 5.42 (2H, s), 6.49 (1H, s), 7.37 (1H, s), 7.73—8.24 (4H, m), 8.78 (1H, d, J=8.5 Hz). MS *m/z*: 473 (M^+).

Antitumor Activity L1210 leukemia cells (10^5) were implanted intraperitoneally (i.p.) in 7 week old BDF_1 female mice on day 0, and 10 mice were used at each dose. Samples were suspended in saline and the suspension was administered i.p. days 1 to 5. The control mice were injected with saline and cured mice were calculated on day 40.

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Triazole Antifungals. IV.[1] Synthesis and Antifungal Activities of 3-Acylamino-2-aryl-2-butanol Derivatives

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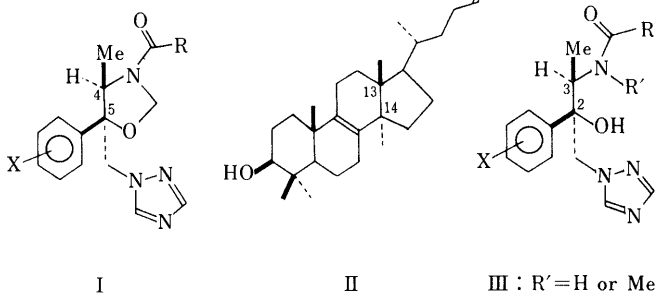
New triazole compounds were designed and synthesized as potential inhibitors of the fungal cytochrome P-450 14α-demethylase. In testing for antifungal activity against a mouse systemic *Candida albicans* infection, (2*R*,3*R*)-3-acylamino-2-aryl-2-butanol derivatives III exhibited remarkably high efficacy after oral or parenteral administration. The structure–activity relationships of these amidoalcohols were evaluated.

Keywords antifungal activity; 1,2,4-triazole; 3-acylamino-2-aryl-1-(triazol-1-yl)-2-butanol; cytochrome P-450 inhibitor; structure–activity relationship; oral activity

In a previous paper,[2] we reported the synthesis and antifungal activities of triazole compounds I, which have a methyl-substituted oxazolidine ring. Remarkably potent *in vivo* activity was observed in this series of derivatives. The antifungal activities of these oxazolidines were presumed to arise from the potential ability of I to inhibit the fungal cytochrome P-450 14α-demethylase. This enzyme catalyzes 14-demethylation of lanosterol (II) in the biosynthesis of ergosterol,[3] which is an important constituent of the fungal membrane. A structural similarity between the oxazolidine I and lanosterol was hypothesized to be an essential factor accounting for the antifungal potency of I. The oxazolidine I was favorably overlaid to fit to the lanosterol skeleton in a manner such that the 5β-aromatic ring and the oxazolidine ring of I are located on the B and D rings of the steroid, and that the 4β-methyl group and the methylene carbon atom of the 5α-triazolylmethyl group of I respectively occupy the positions of the 13β-methyl and 14α-methyl groups of lanosterol. The N-acyl group of I was regarded as corresponding to the 17-side chain of lanosterol.

The 4*R*,5*R* absolute configuration, as well as the location of the methyl substituent at the C(4) position of I, was identified as a key structural element of antifungal potency. Changing the stereochemistry at the C(4) and/or C(5) position or 4-demethylation resulted in a profound decrease in activity, confirming the validity of the hypothesis described above.

There is, however, some divergence in overlaying the oxazolidine I onto lanosterol. As can be seen by inspection of the Dreiding model, the four carbon atoms, Me–C(13)–C(14)–Me, of lanosterol adopt a nearly *anti* coplaner conformation, whereas X-ray crystallographic analysis of the oxazolidine I [X=2,4-F_2, R=4-$(CF_3)C_6H_4$] revealed that the dihedral angle between the 4-methyl group and the 5-methylene group of I was *ca.* 137°.[4] Meanwhile, X-ray crystallographic analysis of the azidoalcohol (2*R*,3*R*)-**2b**, one of the synthetic intermediates for I, established that the four carbon centers, Me–C(3)–C(2)–CH_2, of **2b** are approximately *anti* coplanar.[5] This conformation of **2b** can fit more favorably around these centers to match lanosterol than the oxazolidine I, and **2b** was found to exhibit fairly good *in vivo* antifungal activity, superior to that of ketoconazole. From these facts, we were interested in the antifungal activity of the acylaminoalcohol derivatives represented by the general formula III, which corresponds to the ring-opened analog of the oxazolidine I. It was anticipated that a better fitting of molecule III than oxazolidine I to the space, which should be occupied by lanosterol in the binding site of the cytochrome P-450 14α-demethylase, could be obtained by rotating the C(2)–C(3) bond appropriately, and that triazole III might form a stable complex with the enzyme, resulting in good inhibitory activity.



I II III : R′=H or Me

Chart 1

A variety of acylaminoalcohol derivatives (**7**—**49**), as shown in Chart 2, was synthesized, as racemates and/or in optically active forms, by acylation of aminoalcohol intermediates **3** and **4**. Racemic aminoalcohols **3a**—**c** were provided as described previously from the epoxides **1a**—**c** *via* the azidoalcohols **2a**—**c**. Preparation of the optically active aminoalcohol **3b** has already been reported.[2] Stereoselective synthesis of enantiomeric epoxides, (2*R*,3*S*)- and (2*S*,3*R*)-**1a**, and azidoalcohols, (2*R*,3*R*)- and (2*S*,3*S*)-**2a**, key intermediates for the preparation of optically active aminoalcohols, **3a** and **4a**, has been disclosed.[1] *N*-Methylaminoalcohols **4a** and **4c** were obtained by lithium aluminum hydride reduction of the oxazolidine derivatives **5a** and **5c**, which were formed by treatment of **3a** and **3c**, respectively, with paraformaldehyde.[2] These *N*-methylaminoalcohols were alternatively prepared by a ring-opening reaction of the epoxides **1a** and **1c** with methylamine on heating in methanol, although the 2,4-difluorophenyl analog **4a** was accompanied by a substantial amount of the indoline derivative **6**, which was shown to be formed by cyclization of **4a**.

For structure–activity relationship studies, some addi-

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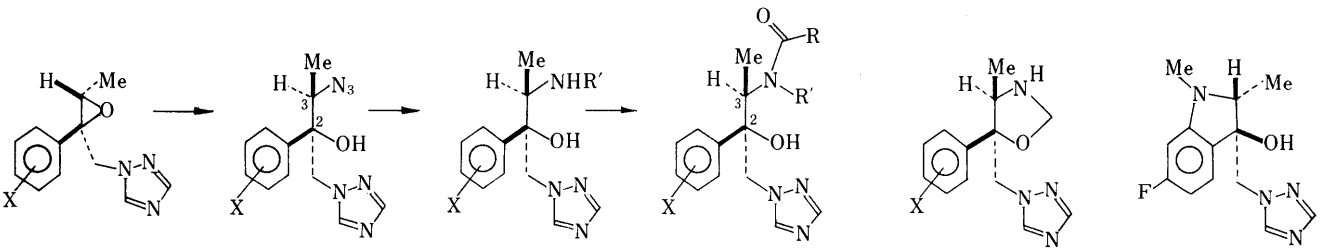


1a, b, c **2a, b, c** **3a, b, c** : R′=H; **4a, c** : R′=Me **7–44** : R′=H; **45–49** : R′=Me **5a, c** **6**

a : X=2, 4-F_2
b : X=2, 4-Cl_2
c : X=4-Cl

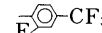
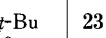
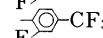
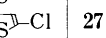
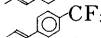
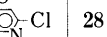
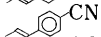
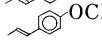
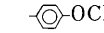
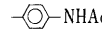
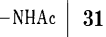
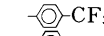
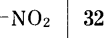
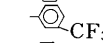
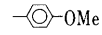
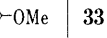
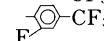
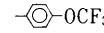
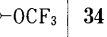
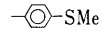
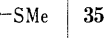
	X	R′	R		X	R′	R		X	R′	R
7	2, 4-Cl_2	H	H	22	2, 4-Cl_2	H		37	2, 4-F_2	H	
8	2, 4-Cl_2	H	*tert*-Bu	23	2, 4-Cl_2	H		38	4-Cl	H	
9	2, 4-F_2	H		24	2, 4-F_2	H		39	2, 4-F_2	H	
10	2, 4-F_2	H		25	2, 4-F_2	H		40	2, 4-F_2	H	
11	2, 4-F_2	H		26	2, 4-F_2	H		41	2, 4-F_2	H	
12	2, 4-F_2	H		27	2, 4-F_2	H		42	2, 4-F_2	H	
13	2, 4-F_2	H		28	2, 4-F_2	H		43	2, 4-F_2	H	
14	2, 4-F_2	H	Ph	29	2, 4-F_2	H		44	2, 4-F_2	H	
15	2, 4-F_2	H		30	2, 4-F_2	H		45	2, 4-F_2	Me	
16	2, 4-F_2	H		31	2, 4-F_2	H		46	2, 4-F_2	Me	
17	2, 4-Cl_2	H		32	2, 4-Cl_2	H		47	2, 4-F_2	Me	
18	4-Cl	H		33	2, 4-F_2	H		48	4-Cl	Me	
19	4-Cl	H		34	2, 4-Cl_2	H		49	2, 4-F_2	Me	
20	2, 4-Cl_2	H		35	4-Cl	H					
21	2, 4-Cl_2	H		36	2, 4-F_2	H					

Chart 2

50 **51** : X=OMs; **52** : X=N_3; **53** : X=NH_2 **54**

57 **58**

55 : R=H
56 : R=CO-

Chart 3

tional analogs (racemates), as shown in Chart 3, were synthesized and tested for antifungal activity. The (2*R**, 3*S**) amidoalcohol **54** was prepared from diol **50**[2] in the usual manner *via* **51**, **52** and **53**. The demethyl derivative **56** was obtained from the aminoalcohol **55**,[2] and the sulfonamide **57** was obtained from **3b**. The phthalimide analog **58** was formed by heating the aminoalcohol **3a** with 4-chlorophthalic anhydride in toluene in the presence of triethylamine.

The *in vitro* antifungal activities of these acylaminoalcohols and their analogs were evaluated on Sabouraud dextrose agar media. Most of the compounds were shown to have slight or some degree of activity against *Trichophyton rubrum*. As observed with the oxazolidine derivatives I, however, almost all of the synthesized compounds described above were inactive against most of the other yeast and fungi (*Candida albicans*, *Cryptococcus neoformans*, *Mucor mucedo*, *Aspergillus fumigatus*, *Microsporum gypseum* and *Trychophyton mentagrophytes*) at a concentration of 50 μg/ml. It is known that there is no good correlation between *in vitro* and *in vivo* activities of azole antifungals, as was also seen in the oxazolidine series I. Therefore, these triazoles were subjected to animal model studies of fungal infection. The results of *in vivo* studies in mice in systemic *Candida albicans* infection are summarized

TABLE I. Comparative Antifungal Efficacy of Acylaminoalcohol Derivatives (**7**—**49**,**54** and **56**) and Related Compounds (**57** and **58**) against Systemic Infection of *Candida albicans*[a]

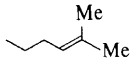
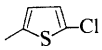
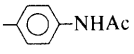
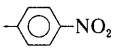
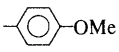
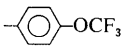
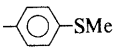
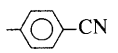
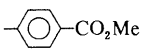
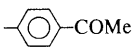
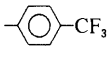
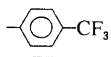
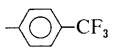
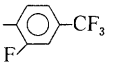
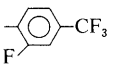
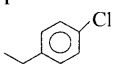
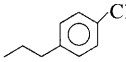
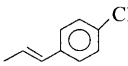
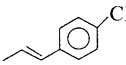
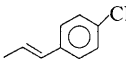
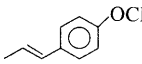
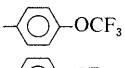
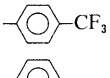
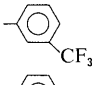
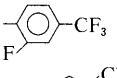
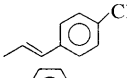
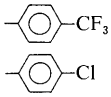
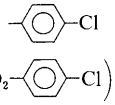
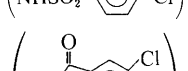
Compd.[b]	X	R′	R	Stereochemistry	Dose (mg/kg)	Route	% survival rate on day			
							3	9	14	21
7	2, 4-Cl_2	H	H	2*R**, 3*R**	20	*p.o.*	100	30	10	0
						i.p.	100	30	0	
8	2, 4-Cl_2	H	*tert*-Bu	2*R**, 3*R**	20	*p.o.*	20	0		
						i.p.	40	20	0	
9	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	10	10	10	10
						i.p.	0			
10	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	30	10	0	
						i.p.	10	0		
11	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	80	60	60	30
						i.p.	100	70	60	60
12	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	100	60	10	0
						i.p.	100	80	50	50
13	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	100	70	40	30
						i.p.	100	40	10	0
14	2, 4-F_2	H	Ph	2*R**, 3*R**	20	*p.o.*	60	10	10	10
						i.p.	20	0		
15	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	40	0		
						i.p.	90	40	30	0
16	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	100	100	20	0
						i.p.	80	70	40	20
17	2, 4-Cl_2	H		2*R**, 3*R**	20	*p.o.*	100	90	70	60
						i.p.	100	100	100	100
18	4-Cl	H		2*R**, 3*R**	20	*p.o.*	40	0		
						i.p.	20	0		
19	4-Cl	H		2*R**, 3*R**	20	*p.o.*	100	90	90	90
						i.p.	100	100	100	100
20	2, 4-Cl_2	H		2*R**, 3*R**	20	*p.o.*	80	80	40	30
						i.p.	80	80	40	20
21	2, 4-Cl_2	H		2*R**, 3*R**	20	*p.o.*	100	100	100	100
						i.p.	100	100	100	100
22	2, 4-Cl_2	H		2*R**, 3*R**	20	*p.o.*	30	10	10	0
						i.p.	0			
23	2, 4-Cl_2	H		2*R**, 3*R**	20	*p.o.*	0			
						i.p.	0			
24	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	100	80	40	30
						i.p.	100	100	100	70
25	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	20	10	0	
						i.p.	30	20	20	0
26	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	100	100	90	60
						i.p.	100	100	80	50
27	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	70	70	60	40
						i.p.	80	50	20	10
28	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	100	100	100	70
						i.p.	100	100	100	60
29	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	100	100	90	70
						i.p.	100	100	90	70
30	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	10	0		
						i.p.	0			
31	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	100	60	40	30
						i.p.	100	100	90	90
32	2, 4-Cl_2	H		2*R**, 3*R**	20	*p.o.*	100	100	70	30
						i.p.	100	100	100	80
33	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	100	100	100	90
						i.p.	100	100	100	90
(−)-**33**	2, 4-F_2	H		2*R*, 3*R*	20	*p.o.*	100	100	100	100
						i.p.	100	100	100	100
(+)-**33**	2, 4-F_2	H		2*S*, 3*S*	20	*p.o.*	50	10	10	10
						i.p.	30	0		
34	2, 4-Cl_2	H		2*R**, 3*R**	20	*p.o.*	100	100	100	100
						i.p.	100	100	100	100
(−)-**34**	2, 4-Cl_2	H		2*R*, 3*R*	2.5	*p.o.*	100	100	100	100
						i.p.	100	100	100	100

TABLE I. (continued)

Compd.[b]	X	R′	R	Stereochemistry	Dose (mg/kg)	Route	% survival rate on day			
							3	9	14	21
					1.25	*p.o.*	60	60	60	50
						i.p	90	90	90	80
					0.63	*p.o.*	30	30	30	30
						i.p	80	80	80	80
35	4-Cl	H		2*R**, 3*R**	20	*p.o.*	100	100	100	90
						i.p.	100	100	100	100
36	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	100	50	10	10
						i.p.	100	20	0	
37	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	100	100	100	100
						i.p.	100	100	100	100
38	4-Cl	H		2*R**, 3*R**	20	*p.o.*	100	100	100	100
						i.p.	100	100	100	100
39	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	60	30	0	
						i.p.	10	0		
40	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	100	80	80	40
						i.p.	100	90	30	0
41	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	100	100	60	60
						i.p.	100	100	100	100
42	2, 4-F_2	H		2*R**, 3*R**	20	*p.o.*	100	100	90	60
						i.p.	90	90	90	70
(−)-**43**	2, 4-F_2	H		2*R*, 3*R*	20	*p.o.*	100	100	50	40
						i.p.	100	100	60	50
(−)-**44**	2, 4-F_2	H		2*R*, 3*R*	20	*p.o.*	100	100	100	80
						i.p.	100	100	100	100
(−)-**45**	2, 4-F_2	Me		2*R*, 3*R*	20	*p.o.*	100	100	70	40
						i.p.	100	100	70	40
46	2, 4-F_2	Me		2*R**, 3*R**	20	*p.o.*	100	100	100	100
						i.p.	100	100	100	90
47	2, 4-F_2	Me		2*R**, 3*R**	20	*p.o.*	100	70	40	20
						i.p.	100	80	50	40
48	4-Cl	Me		2*R**, 3*R**	20	*p.o.*	100	100	100	100
						i.p.	100	100	100	100
49	2, 4-F_2	Me		2*R**, 3*R**	20	*p.o.*	100	100	80	60
						i.p.	100	100	100	80
54	4-Cl	H		2*R**, 3*S**	20	*p.o.*	20	10	0	
						i.p.	0			
56	2, 4-F_2	H		2*R** (3-demethyl)	20	*p.o.*	100	50	10	0
						i.p.	100	20	0	
57	2, 4-Cl_2			2*R**, 3*R**	20	*p.o.*	10	0		
						i.p.	50	0		
58	2, 4-F_2			2*R**, 3*R**	20	*p.o.*	100	40	20	0
						i.p.	100	50	10	0
Ketoconazole					20	*p.o.*	100	50	30	10
						i.p.	100	50	20	0
Fluconazole					20	*p.o.*	100	100	70	60
					6.25	*p.o.*	100	90	70	60
					3.13	*p.o.*	100	90	60	30
Control (no drug)							0			

a) *In vivo* activity was determined in mice (each group consisted of ten male mice, 5 weeks old, of the ddY strain) that were infected systemically using an intravenous challenge of 6 to 9×10^6 cells of *Candida albicans* 427. The triazole was administered orally (*p.o.*) or intraperitoneally (i.p.) at 1, 4, and 24 h post-infection. *b*) Compounds are racemic unless otherwise indicated. The amides **8**—**10** are oxalic acid salts, (−)-**33**, (+)-**33** and (−)-**44** are HCl salts, and (−)-**45** and **58** are HNO_3 salts.

in Table I. The compounds were administered orally (*p.o.*) or intraperitoneally (i.p.) at 1, 4, and 24 h post-infection. All control mice (no drug) died within 2 d after infection. The antifungal efficacy of the compounds was compared with that of ketoconazole and fluconazole, both of which are currently used for the treatment of fungal diseases, including deep-seated mycoses.

The structure–activity relationships were evaluated initially by varying the N-acyl groups in a series of compounds with 2*R**,3*R** stereochemistry. No significant difference

was observed between the *p.o.* and i.p. activities of the compounds tested. Compound **7**, which has an N-formylamino function, was somewhat active, though it was not very potent, and the analogs **8** and **9**, which have a bulky alkyl group, showed significantly decreased activity. The latter compound, **9**, was of initial interest because of the likeness in shape of its acyl group to the 17-side chain of lanosterol. Amides **10**, **14** and **15**, with an unsubstituted (hetero)aromatic ring, showed no significant activity, although the unsubstituted thenoyl counterpart **11** showed good activity. Amides **12** and **13**, with a chlorine-substituted heteroaromatic ring, also showed high activity. These heteroaromatic amide analogs, however, were excluded from any further consideration of modification, because of their relatively less potent activity compared with the corresponding benzamide congener. The benzamide derivatives, **16** and **20**, which have an acetamido or methylthio group at the 4-position of the benzoyl group, expressed some potency, whereas the 4-methoxybenzamide **18** exhibited very poor activity, presumably as a result of rapid metabolism of both the methoxy group and the relatively electron-rich benzene ring of the acyl moiety. The trifluoromethoxy derivative **19** showed excellent activity, as did the nitro (**17**) and the cyano (**21**) compound. These compounds possess an electron-withdrawing group at the 4-position of the benzoyl group, whose benzene ring is thereby expected to be resistant to metabolism. On the other hand, the compounds with either an ester (**22**) or acetyl (**23**) function on the benzoyl group showed no significant activity, which is presumably due to the metabolic instability of these substituents. Monohalogenation at the 4-position of the benzoyl group enhanced antifungal potency, as can be seen from **24**, **31** and **32**, whereas the 3-fluorobenzoyl derivative **25** showed only poor activity. The difluoro series **26**—**29** exhibited increased potency. All 4-(trifluoromethyl)benzoyl derivatives, **33**, **34** and **35**, and the 2-fluoro-4-(trifluoromethyl)benzoyl derivatives, **37** and **38**, showed the highest activity, because after being administered *p.o.* or i.p., almost all mice survived as long as 3 weeks after infection. This activity was more potent than that of fluconazole. In contrast, the activity of the 2-(trifluoromethyl)benzoyl analog **36** was less potent. The phenylacetyl derivative **39**, a homolog of **31**, had no significant activity. The phenethyl analog **40** showed good activity, and its dehydro analog **41**, a cinnamamide derivative, enhanced *in vivo* potency. Other cinnamamides, **42**, (−)-**43** and (−)-**44**, retained the potent activity of the parent benzamide derivatives.

The *N*-methylbenzamides, (−)-**45**, **46**—**48**, and the *N*-methylcinnamamide **49** also expressed remarkably high activity. The (2*R**,3*S**)-benzamidoalcohol **54**, a diastereoisomer of **35**, was almost devoid of activity, and the demethyl analog **56** showed significantly less potency than the methyl equivalent **31**. Comparison of the activity of the enantiomeric pair of the benzamidoalcohols, (−)-**33** and (+)-**33**, revealed that antifungal activity resides in (−)-**33**, which has a 2*R*,3*R* absolute configuration, as was expected from the previous results of the oxazolidines I and also from the aforementioned discussion of the structural similarity between III and lanosterol. Interestingly, the sulfonamide **57**, an analog of the benzamide **32**, did not show any significant *in vivo* activity. The phthalimide derivative **58** was less potent than the amide analog **31**.

TABLE II. The ED_{50} Values of Selected Compounds in Mice Infected Systemically with *Candida albicans*

Compound	Route	ED_{50} (mg/kg) on day	
		14	15
32	*p.o.*	7.07	
	i.p.	3.84	
33	*p.o.*		0.86
	i.p.		<0.63
(−)-**33**	*p.o.*		<0.31
	i.p.		<0.31
(−)-**34**	*p.o.*	1.06	
	i.p.	<0.63	
35	*p.o.*		1.19
	i.p.		1.71
37	*p.o.*		<0.63
	i.p.		<0.63
38	*p.o.*		0.88
	i.p.		0.90
46	*p.o.*		0.66
	i.p.		<0.63
48	*p.o.*		1.31
	i.p.		1.97
Ketoconazole	*p.o.*		31.4
	i.p.		39.9
Fluconazole	*p.o.*		3.00
	i.p.		3.04

The ED_{50} values were determined by the probit method in mice (each group consisted of ten male mice, 5 weeks old, of the ddY strain) that were infected systemically using an intravenous challenge of 6 to 9×10^6 cells of *Candida albicans* 427. Compounds were tested at appropriate doses selected from the series 10, 5, 2.5, 1.25, 0.63, 0.31 mg/kg by *p.o.* or i.p. administration at 1, 4, and 24 h post-infection. All compounds are free bases except for **32** (oxalic acid salt) and (−)-**33** (HCl salt).

The structure–activity relationship patterns of the amidoalcohol variants discussed here are parallel with those of the oxazolidine derivatives I. The ED_{50} values (the dose of compound required to cause 50% of the treated animals to survive) for the selected amidoalcohols in a murine model of systemic candidiasis were evaluated, and the results are given in Table II. Antifungal potency of these amidoalcohols and the oxazolidines I was compared by the ED_{50} values. The ED_{50}'s (on day 14) of the typical oxazolidine (I: X = 2,4-Cl_2, R = 4-chlorobenzoyl; racemate, oxalic acid salt) were determined as 8.52 mg/kg, *p.o.* and 10.0 mg/kg, i.p., and those of the amidoalcohol equivalent **32** (racemate, oxalic acid salt) were 7.07 mg/kg, *p.o.* and 3.84 mg/kg, i.p.; the amidoalcohol showed slightly more potent activity than the oxazolidine counterpart. Among the racemic triazoles tested, the 2-fluoro-4-(trifluoromethyl)benzoyl derivative **37** was the most potent against *Candida albicans* infection; the ED_{50} (on day 15) was <0.63 mg/kg for both *p.o.* and i.p. administration, which was at least 5 times more potent than fluconazole, whose ED_{50}'s (on day 15) were 3.00 mg/kg, *p.o.* and 3.07 mg/kg, i.p.

In a preliminary toxicity study, all five mice (ddY strain, male, 5 weeks old) tested survived after oral dosing of 1000 mg/kg of **33** (racemate) once daily for 4 d.

Further evaluation of these amidoalcohols is in progress.

Experimental

Melting points are uncorrected. Infrared spectra (IR) were recorded on a JASCO A-2 spectrometer and proton nuclear magnetic resonance spectra (^{1}H-NMR) on a Varian EM-360L spectrometer in $CDCl_3$ using

TABLE III. 3-Acylamino-2-aryl-1-(1*H*-1,2,4-triazol-1-yl)-2-butanols (**7**—**49**, and **54**) and Related Compounds (**56**—**58**)

Compd.	Yield (%)	mp (°C) (Solvent[a]) [Oxalic acid salt: mp (°C)]	IR ν_{max} cm^{-1} (State) Optical rotation	^{1}H-NMR ($CDCl_3$) δ[b]	Formula	Analysis % or MS *m/z* Calcd (Found)				
						C	H	N	Halogen	
7	57	233—237 (A–H)	3200, 1679, 1656, 1138 (KBr)	0.95 (3H, d, 7), 4.51 (1H, d, 14), 5.45 (1H, d, 14), 5.45 (1H, q, 7), 7.0—7.7 (3H, m), 7.75 (1H, s), 8.25 (2H, s)[c]	$C_{13}H_{14}Cl_2N_4O_2$	47.43 (47.61	4.29 4.36	17.02 16.78)		
8	94	Oil [156—160]	3650, 1650, 1506 ($CHCl_3$)	0.86 (3H, d, 7), 1.27 (9H, s), 4.27 (1H, d, 14), 5.33 (1H, br d), 5.37 (1H, br), 5.62 (1H, d, 14), 6.24 (1H, br d), 7.08 (1H, dd, 9, 2), 7.32 (1H, d, 2), 7.52 (1H, d, 9), 7.80 (1H, s), 7.84 (1H, s)	$C_{17}H_{22}Cl_2N_4O_2$	387, 385 ($M^+ + 1$), 369, 327, 302, 257, 256, 197, 128, 85, 70 (100%)				
9	65	Oil [143—145]	3450, 1660, 1500 ($CHCl_3$)	0.92 (3H, d, 7), 1.66 (6H, s), 2.34 (4H, d, 3), 4.37 (1H, d, 14), 4.68 (1H, q, 7), 4.96 (1H, d, 14), 6.5—7.7 (3H, m), 7.76 (1H, s), 7.97 (1H, s)[c]	$C_{19}H_{24}F_2N_4O_2$	379, 378 (M^+), 296, 224, 186, 154, 141, 127, 82, 70, 44 (100%)				
10	87	Oil [174—176]	3430, 1658, 1595, 1515 ($CHCl_3$)	1.00 (3H, d, 7), 4.50 (1H, d, 14), 4.9 (1H, m), 5.06 (1H, d, 14), 5.25 (1H, br), 6.5—7.6 (6H, m), 7.80 (1H, s), 7.87 (1H, s)	$C_{17}H_{16}F_2N_4O_3$	363 ($M^+ + 1$), 343, 281, 224 (100%), 141, 138, 127, 95, 82, 70				
11	76	166—167 (A–H)	3430, 1645, 1530, 1500 ($CHCl_3$)	1.03 (3H, d, 7), 4.52 (1H, d, 14), 4.95 (1H, m), 5.09 (1H, d, 14), 5.4 (1H, br), 6.6—7.8 (6H, m), 7.79 (1H, s), 7.90 (1H, s)	$C_{17}H_{16}F_2N_4O_2S$	53.96 (54.21	4.26 4.33	14.81 14.89	F 10.04 F 10.29)	
12	40	191—193 (E–H)	3400, 3270, 1620, 1550 (KBr)	0.99 (3H, d, 7), 4.43 (1H, d, 14), 4.96 (1H, m), 5.02 (1H, d, 14), 5.36 (1H, br s), 6.3—7.1 (3H, m), 6.91 (1H, d, 4), 7.1—7.6 (1H, m), 7.36 (1H, d, 4), 7.81 (2H, s)	$C_{17}H_{15}ClF_2N_4O_2S$	49.46 (49.64	3.66 3.81	13.57 13.31	Cl 8.59 Cl 8.55)	
13	77	188—190 (A–H)	3430, 1662, 1583, 1500 ($CHCl_3$)	1.01 (3H, d, 7), 4.46 (1H, d, 14), 5.0 (1H, m), 5.05 (1H, d, 14), 5.3 (1H, br), 6.6—7.0 (2H, m), 7.2—7.6 (1H, m), 7.45 (1H, d, 9), 7.80 (2H, s), 8.18 (1H, dd, 9, 2.5), 8.92 (1H, d, 2.5)[c]	$C_{18}H_{16}ClF_2N_5O_2$	53.01 (52.86	3.95 3.86	17.17 17.30	Cl 8.69 Cl 8.32)	
14	76	133—134 (A–H)	3380, 1658, 1616, 1518 (KBr)	1.02 (3H, d, 7), 4.48 (1H, d, 14), 4.97 (1H, br s), 5.05 (1H, m), 5.09 (1H, d, 14), 6.6—7.1 (3H, m), 7.3—7.7 (4H, m), 7.7—8.1 (2H, m), 7.81 (1H, s), 7.86 (1H, s)	$C_{19}H_{18}F_2N_4O_2$	61.28 (61.19	4.87 4.77	15.05 15.17	F 10.20 F 10.08)	
15	58	187—189 (A–H)	3460, 1657, 1500 ($CHCl_3$)	1.08 (3H, d, 7), 4.54 (1H, d, 14), 5.0 (1H, m), 5.11 (1H, d, 14), 5.2 (1H, br), 6.6—7.1 (3H, m), 7.2—8.1 (8H, m), 8.40 (1H, s)	$C_{23}H_{20}F_2N_4O_2$	65.40 (65.66	4.77 4.69	13.26 12.93	F 8.99 F 8.71)	
16	51	254—255.5 (A–H)	3405, 3325, 1694, 1643, 1529 (KBr)	1.05 (3H, d, 7), 4.47 (1H, d, 14), 4.97 (1H, m), 5.03 (1H, d, 14), 6.6—7.1 (2H, m), 7.1—7.5 (1H, m), 7.5—8.2 (4H, m), 7.73 (1H, s), 7.81 (1H, s)[c]	$C_{21}H_{21}F_2N_5O_3$	58.74 (58.72	4.93 5.00	16.31 16.28	F 8.85 F 9.05)	
17	66	257—258.5 (M–A)	3375, 3300, 1650, 1556 (KBr)	1.14 (3H, d, 7), 4.89 (1H, d, 14), 4.98 (1H, m), 5.86 (1H, d, 14), 5.89 (1H, br), 7.15 (1H, dd, 9, 2), 7.47 (1H, d, 2), 7.81 (1H, d, 9), 7.91 (1H, s), 8.37 (1H, s)[d]	$C_{19}H_{17}Cl_2N_5O_4$	50.68 (50.45	3.81 3.78	15.55 15.31)		
18	71	200—202 (A–B)	3450, 1645, 1604, 1493 ($CHCl_3$)	1.03 (3H, d, 7), 3.85 (3H, s), 4.47 (1H, d, 14), 4.70 (1H, m), 4.72 (1H, d, 14), 5.33 (1H, br), 6.74 (1H, br d, 9), 6.94 (2H, d, 8), 7.27 (4H, s), 7.65 (1H, s), 7.80 (1H, s), 7.84 (2H, d, 8)	$C_{20}H_{21}ClN_4O_3$	59.93 (59.59	5.28 5.21	13.98 13.83)		
19	60	172—177 (A–H)	3360, 1638, 1543, 1502 (KBr)	1.03 (3H, d, 7), 4.45 (1H, d, 14), 4.65 (1H, m), 4.74 (1H, d, 14), 5.30 (1H, s), 6.82 (1H, br d, 9), 7.30 (4H, s), 7.34 (2H, d, 9), 7.66 (1H, s), 7.82 (1H, s), 7.93 (2H, d, 9)	$C_{20}H_{18}ClF_3N_4O_3$	52.81 (52.55	3.99 4.16	12.32 12.20	F 12.53 F 12.54)	
20	41	167—168 (A–H)	3300, 1627, 1545 (KBr)	0.98 (3H, d, 7), 2.51 (3H, s), 4.42 (1H, d, 15), 5.47 (2H, m), 5.67 (1H, d), 6.83 (1H, br d), 7.11 (1H, dd, 9, 2), 7.32 (2H, d, 8), 7.39 (1H, d, 2), 7.58 (1H, d, 9), 7.80 (1H, s), 7.82 (2H, d, 8), 7.86 (1H, s)	$C_{20}H_{20}Cl_2N_4O_2S$	53.22 (53.04	4.47 4.58	12.41 12.39	Cl 15.71 Cl 15.14)	
21	74	246—248 (A)	3520, 3250, 2220, 1660, 1554 (KBr)	1.11 (3H, d, 7), 4.83 (1H, d, 15), 5.00 (1H, br), 5.84 (1H, d, 15), 7.15 (1H, dd, 9, 2), 7.44 (1H, d, 2), 7.80 (1H, d, 9), 7.85 (2H, d, 8), 8.25 (2H, d, 8), 8.36 (2H, s)[d]	$C_{20}H_{17}Cl_2N_5O_2$	55.83 (55.95	3.98 3.87	16.28 16.02)		
22	82	122—130 (dec.) (M)	3560, 3330, 1711, 1634, 1560 (KBr)	1.01 (3H, d, 7), 3.92 (3H, s), 4.61 (1H, d, 15), 5.42 (1H, br), 5.51 (1H, d, 15), 6.11 (1H, s), 7.23 (1H, dd, 9, 2), 7.51 (1H, d, 2), 7.63 (1H, d, 9), 7.8—8.3 (4H, m), 8.15 (2H, s)[e]	$C_{21}H_{20}Cl_2N_4O_4$	54.44 (54.63	4.35 4.33	12.09 11.88)		
23	57	169—172 (A–H)	3430, 3110, 1677, 1650, 1525 (KBr)	0.98 (3H, d, 7), 2.63 (3H, s), 4.45 (1H, d, 15), 5.49 (2H, br), 5.67 (1H, d, 15), 6.90 (1H, br d), 7.12 (1H, dd, 9, 2), 7.38 (1H, d, 2), 7.58 (1H, d, 9), 7.82 (1H, s), 7.95 (1H, s), 8.06 (2H, s)	$C_{21}H_{20}Cl_2N_4O_3$	56.39 (56.00	4.51 4.48	12.53 12.24	Cl 15.85 Cl 15.39)	
24	89	205 (A)	3280, 1634, 1530 (KBr)	1.04 (3H, d, 7), 4.47 (1H, d, 15), 4.96 (1H, m), 5.08 (1H, d, 15), 6.5—7.1 (2H, m), 7.1—7.9 (1H, m), 7.19 (2H, t, 9), 7.78 (1H, s), 7.97 (2H, dd, 9, 6), 8.01 (1H, s)[f]	$C_{19}H_{17}F_3N_4O_2$	58.46 (58.62	4.39 4.71	14.35 14.25	F 14.60 F 14.52)	
25	44	164—165 (A–E)	3400, 3280, 1638, 1553 (KBr)	1.02 (3H, d, 7), 4.47 (1H, d, 15), 4.96 (1H, m), 5.09 (1H, d, 15), 5.38 (1H, br), 6.5—7.0 (3H, m), 7.2—7.9 (5H, m), 7.82 (2H, s)	$C_{19}H_{17}F_3N_4O_2$	58.46 (58.38	4.39 4.41	14.35 14.17	F 14.60 F 14.85)	
26	37	165—166 (A)	3370, 3230, 1668, 1535 (KBr)	1.02 (3H, d, 7), 4.51 (1H, d, 14), 4.99 (1H, br), 5.06 (1H, d, 14), 5.32 (1H, br d), 6.5—7.7 (5H, m), 7.81 (2H, s), 7.9—8.4 (1H, m)	$C_{19}H_{16}F_4N_4O_2$	55.88 (55.94	3.95 3.81	13.72 13.73	F 18.61 F 18.59)	
27	39	150—151 (E—H)	3420, 1662, 1528 (KBr)	1.02 (3H, d, 7), 4.52 (1H, d, 14), 4.99 (1H, br), 5.07 (1H, d, 14), 5.32 (1H, br d), 6.6—7.1 (3H, m), 7.1—7.6 (3H, m), 7.6—8.1 (1H, m), 7.82 (2H, s)	$C_{19}H_{16}F_4N_4O_2$	55.88 (56.11	3.95 4.07	13.72 13.82	F 18.61 F 18.61)	
28	38	168—169 (E–H)	3400, 3300, 1637, 1540 (KBr)	1.01 (3H, d, 7), 4.45 (1H, d, 14), 5.00 (1H, br), 5.07 (1H, d, 14), 5.39 (1H, br d), 6.5—7.1 (3H, m), 7.1—8.0 (4H, m), 7.83 (2H, s)	$C_{19}H_{16}F_4N_4O_2$	55.88 (56.12	3.95 4.18	13.72 13.51	F 18.61 F 18.37)	
29	39	178 (E–H)	3420, 1640, 1620, 1595 (KBr)	1.02 (3H, d, 7), 4.43 (1H, d, 14), 4.98 (1H, br), 5.06 (1H, d, 14), 5.39 (1H, br s), 6.4—7.1 (3H, m), 7.1—7.6 (4H, m), 7.81 (2H, s)	$C_{19}H_{16}F_4N_4O_2$	55.88 (55.40	3.95 4.42	13.72 13.46	F 18.61 F 18.87)	
30	47	111—116 (A–B–H)	3425, 1680, 1500 ($CHCl_3$)	0.98 (3H, d, 7), 4.53 (1H, d, 14), 5.09 (1H, d, 14), 5.1 (2H, m), 6.6—7.8 (4H, m), 7.82 (1H, s), 7.85 (1H, s)	$C_{19}H_{13}F_7N_4O_2$	49.36 (49.02	2.83 3.23	12.12 10.02)		

31	76	221—222 (A–H)	3310, 1635, 1536 (KBr)	1.02 (3H, d, 7), 4.50 (1H, d, 15), 4.95 (1H, m), 5.06 (1H, d, 15), 6.5—7.1 (2H, m), 7.1—7.9 (1H, m), 7.50 (2H, d, 8), 7.77 (1H, s), 7.90 (2H, d, 8), 8.00 (1H, s)[f]	$C_{19}H_{17}ClF_2N_4O_2$	56.10 (56.30)	4.21 (4.23)	13.77 (13.68)	Cl	8.71 (8.67)
32	74	217—219 (E–H)	3430, 1655, 1585, 1505 ($CHCl_3$)	0.95 (3H, d, 7), 4.40 (1H, d, 15), 5.46 (2H, m), 5.63 (1H, d, 15), 6.88 (1H, br d), 7.11 (1H, dd, 9, 2), 7.35 (1H, d, 2), 7.45 (2H, d, 8), 7.54 (1H, d, 9), 7.78 (1H, s), 7.83 (2H, d, 8), 7.84 (1H, s)	$C_{19}H_{17}Cl_3N_4O_2$	51.89 (51.64	3.90 3.95	12.74 12.48)		
33	77	201—205 (A–H)	3375, 1666, 1540 (KBr)	1.08 (3H, d, 7), 4.50 (1H, d, 15), 4.98 (1H, m), 5.11 (1H, d, 15), 6.6—7.2 (2H, m), 7.2—7.9 (1H, m), 7.77 (1H, s), 7.80 (2H, d, 8), 8.10 (1H, s), 8.11 (2H, d, 8)[f]	$C_{20}H_{17}F_5N_4O_2$	54.55 (54.75	3.89 4.07	12.72 12.85)		
(2*R*,3*R*)-(−)-**33**	81	Oil [140—148][h]	$[\alpha]_D$ −105° (c=0.96, $CHCl_3$)	Identical with that of **33**	$C_{20}H_{17}F_5N_4O_2$	441 (M^++1), 421, 358, 224, 216, 173 (100%), 145, 131, 127, 82, 70				
(2*S*,3*S*)-(+)-**33**	82	Oil [140—148][h]	$[\alpha]_D$ +106° (c=0.98, $CHCl_3$)	Identical with that of **33**	$C_{20}H_{17}F_5N_4O_2$					
34	67	186 (A–H)	3360, 1665, 1530 (KBr)	0.99 (3H, d, 7), 4.40 (1H, d, 15), 5.49 (2H, m), 5.68 (1H, d, 15), 6.84 (1H, br d), 7.10 (1H, dd, 9, 2), 7.37 (1H, d, 2), 7.56 (1H, d, 9), 7.79 (2H, d, 9), 7.83 (1H, s), 7.87 (1H, s), 8.04 (2H, d, 9)	$C_{20}H_{17}Cl_2F_3N_4O_2$	50.76 (51.14	3.62 3.59	11.84 11.82)		
(2*R*,3*R*)-(−)-**34**	75	88—91 (A–H)	$[\alpha]_D$ −115° (c=1.01, $CHCl_3$)	Identical with that of **34**	$C_{20}H_{17}Cl_2F_3N_4O_2$	475, 473 (M^++1), 455, 453, 392, 390, 258, 256, 216, 173 (100%), 145, 70				
35	62	204—207 (A–H)	3450, 1660, 1525, 1500 ($CHCl_3$)	1.04 (3H, d, 7), 4.48 (1H, d, 14), 4.76 (1H, d, 14), 4.4—5.0 (2H, m), 7.1 (1H, br d), 7.28 (4H, s), 7.69 (2H, d, 9), 7.69 (1H, s), 7.78 (1H, s), 7.99 (2H, d, 9)	$C_{20}H_{18}ClF_3N_4O_2$	54.74 (54.41	4.13 4.51	12.77 12.76	F	12.99 12.95)
36	69	184—186 (A)	3370, 3220, 1671, 1500 (KBr)	1.06 (3H, d, 7), 4.55 (1H, d, 15), 4.96 (1H, m), 5.08 (1H, d, 15), 6.5—7.1 (2H, m), 7.2—7.9 (1H, m), 7.40 (1H, s), 7.64 (2H, br s), 7.76 (1H, s), 8.00 (1H, s)[f]	$C_{20}H_{17}F_5N_4O_2$	54.55 (54.23	3.89 4.07	12.72 12.30)		
37	48	176—177 (M)	3360, 3250, 1668, 1546 (KBr)	1.02 (3H, d, 7), 4.52 (1H, d, 15), 5.06 (1H, d, 15), 5.36 (1H, br s), 6.5—7.1 (2H, m), 7.1—7.8 (4H, m), 7.81 (2H, br s), 8.1—8.5 (1H, m)	$C_{20}H_{16}F_6N_4O_2$	52.41 (52.31	3.52 3.82	12.22 12.54	F	24.87 24.96)
38	68	223—225 (A–H)	3310, 1668, 1534 (KBr)	0.93 (3H, d, 7), 4.57 (1H, d, 14), 4.63 (1H, m), 4.83 (1H, d, 14), 5.67 (1H, s), 7.38 (4H, s), 7.5—8.1 (3H, m), 7.73 (1H, s), 8.10 (1H, s), 8.46 (1H, br d, 9)[g]	$C_{20}H_{17}ClF_4N_4O_2$	52.58 (52.82	3.75 3.96	12.26 12.38	F	16.63 16.61)
39	77	185—186 (A–H)	3420, 3280, 1644 (KBr)	0.92 (3H, d, 7), 3.42 (1H, br s), 3.68 (2H, s), 4.48 (1H, d, 14), 4.70 (1H, m), 4.90 (1H, d, 14), 5.95 (1H, br s), 6.5—7.6 (3H, m), 7.63 (1H, s), 8.26 (1H, s)[e]	$C_{20}H_{19}ClF_2N_4O_2$	57.08 (57.08	4.55 4.61	13.31 13.06	F	9.03 8.75)
40	66	133—134 (A–H)	3270, 1646, 1546 (KBr)	0.79 (3H, d, 7), 2.3—2.8 (2H, m), 2.8—3.2 (2H, m), 3.91 (1H, d, 15), 4.64 (1H, br), 4.76 (1H, d, 15), 5.17 (1H, br d), 6.05 (1H, br d), 6.5—7.0 (2H, m), 7.0—7.6 (1H, m), 7.21 (4H, s), 7.78 (1H, s), 7.81 (1H, s)	$C_{21}H_{21}ClF_2N_4O_2$	58.00 (58.09	4.87 4.81	12.88 12.74)		
41	26	204—206 (A–H)	3450, 1665, 1622, 1500 ($CHCl_3$)	1.00 (3H, d, 7), 4.49 (1H, d, 14), 4.9 (1H, m), 5.05 (1H, d, 14), 5.45 (1H, br s), 6.53 (1H, d, 16), 6.5—7.0 (3H, m), 7.1—7.6 (5H, m), 7.66 (1H, d, 16), 7.80 (1H, s), 7.84 (1H, s)	$C_{21}H_{19}ClF_2N_4O_2$	58.27 (58.68	4.42 4.45	12.94 12.75	F	8.78 8.59)
42	93	166—167 (A–H)	3300, 1657, 1617 (KBr)	1.00 (3H, d, 7), 4.51 (1H, d, 14), 5.0 (1H, m), 5.08 (1H, d, 14), 5.39 (1H, br s), 6.64 (1H, d, 16), 6.4—7.1 (3H, m), 7.2—7.4 (1H, m), 7.65 (4H, s), 7.77 (1H, d, 16), 7.84 (2H, s)	$C_{22}H_{19}F_5N_4O_2$	56.65 (56.95	4.11 4.47	12.01 12.02	F	20.37 20.18)
(2*R*,3*R*)-(−)-**43**	71	209—210 (A–H)	$[\alpha]_D$ −83° (c=1.04, $CHCl_3$)	0.98 (3H, d, 7), 4.46 (1H, d, 14), 4.9 (1H, m), 5.03 (1H, d, 14), 5.4 (1H, br), 6.59 (1H, d, 16), 6.5—7.5 (3H, m), 7.2—7.6 (1H, m), 7.65 (4H, s), 7.72 (1H, d, 16), 7.80 (1H, s), 7.85 (1H, s)	$C_{22}H_{19}F_2N_5O_2$	62.40 (62.33	4.52 4.55	16.54 16.40	F	8.97 8.87)
(2*R*,3*R*)-(−)-**44**	72	Oil [60 (dec.)][h]	$[\alpha]_D$ −56° (c=0.78, $CHCl_3$)	0.98 (3H, d, 7), 4.48 (1H, d, 14), 4.9 (1H, m), 5.03 (1H, d, 14), 6.53 (1H, d, 16), 6.5—7.7 (8H, m), 7.70 (1H, d, 16), 7.77 (1H, s), 7.83 (1H, s)	$C_{22}H_{19}F_5N_4O_3$	483 (M^++1), 400, 258, 224, 215 (100%), 161, 82, 70				
(2*R*,3*R*)-(−)-**45**	69	Oil [103—106][i]	$[\alpha]_D$ −106° (c=1.39, $CHCl_3$)	1.14 (3H, d, 7), 3.18 (3H, s), 4.42 (1H, d, 14), 5.30 (1H, br), 5.32 (1H, d, 14), 6.5—7.1 (2H, m), 7.1—7.8 (1H, m), 7.30 (2H, d, 10), 7.47 (2H, s)	$C_{21}H_{19}F_5N_4O_3 \cdot HNO_3$	47.29 (47.44	3.78 4.06	13.13 12.87	F	17.81 17.70)
46	73	144—146 (E)	3110, 1638, 1610 (KBr)	1.17 (3H, d, 7), 3.16 (3H, s), 4.43 (1H, d, 14), 5.30 (1H, br), 5.35 (1H, d, 14), 6.4—7.1 (2H, m), 7.2—8.2 (5H, m), 7.60 (1H, s), 7.69 (1H, s)	$C_{21}H_{19}F_5N_4O_2$	55.51 (55.64	4.21 4.49	12.33 12.12	F	20.90 20.90)
47	52	155—157.5 (E–H)	3430, 3170, 1646, 1501 (KBr)	1.16 (3H, d, 7), 3.16 (3H, s), 4.43 (1H, d, 14), 5.22 (1H, br), 5.33 (1H, d, 14), 5.46 (1H, br), 6.5—7.1 (2H, m), 7.2—8.2 (5H, m), 7.72 (1H, s), 7.78 (1H, s)	$C_{21}H_{19}F_5N_4O_2$	55.51 (55.54	4.21 4.38	12.33 12.24	F	20.90 20.75)
48	54	174—175 (A–H)	3420, 1680, 1626 ($CHCl_3$)	1.12 (3H, d, 7), 3.11 (3H, s), 4.48 (1H, d, 14), 4.91 (1H, d, 14), 5.20 (2H, br), 7.1—7.9 (3H, m), 7.29 (4H, s), 7.69 (1H, s), 7.78 (1H, s)	$C_{21}H_{19}ClF_4N_4O_2$	53.57 (53.31	4.07 4.22	11.90 11.21	F	16.14 16.01)
49	61	169—171 (E–H)	3430, 3100, 1648, 1494 (KBr)	1.08 (3H, d, 7), 3.34 (3H, s), 4.32 (1H, d, 14), 5.13 (1H, br), 5.24 (1H, d, 14), 5.37 (1H, br), 6.6—6.9 (2H, m), 7.17 (1H, d, 15), 7.2—8.1 (5H, m), 7.43 (1H, s), 7.47 (1H, s), 7.78 (1H, d, 15)	$C_{22}H_{21}ClF_2N_4O_2$	59.13 (59.47	4.74 4.98	12.54 12.40	F	8.50 8.28)
54	60	175—177 (B–H)	3430, 1659, 1621, 1599 ($CHCl_3$)	1.41 (3H, d, 6.5), 4.60 (2H, s), 4.5—5.0 (1H, m), 6.41 (1H, br d, 9), 7.29 (4H, s), 7.65 (4H, s), 7.82 (1H, s), 7.87 (1H, s)	$C_{20}H_{18}ClF_3N_4O_2$	54.74 (54.77	4.13 4.18	12.77 12.64	F	12.99 12.83)
56	87	170—172 (E–C)	3400, 1655 ($CHCl_3$)	3.8—4.0 (2H, m), 4.62 (2H, s), 5.85 (1H, br), 6.6—7.0 (3H, m), 7.3—7.7 (1H, m), 7.35 (2H, d, 9), 7.61 (2H, d, 9), 7.83 (1H, s), 8.09 (1H, s)	$C_{18}H_{15}ClF_2N_4O_2$	55.04 (55.24	3.85 3.65	14.26 14.11)		
57	40	104—108 (E)	3390, 1588 ($CHCl_3$)	0.64 (3H, d, 6.5), 4.56 (1H, dq, 9, 6.5), 4.78 (1H, d, 15), 5.5 (1H, br), 5.54 (1H, d, 15), 5.60 (1H, d, 9), 7.05 (1H, dd, 8.5, 2), 7.31 (1H, d, 2), 7.47 (1H, d, 8.5), 7.51 (2H, d, 9), 7.80 (1H, s), 7.85 (1H, s), 7.92 (2H, d, 9)	$C_{18}H_{17}Cl_3N_4O_3S$	45.44 (45.47	3.60 3.72	11.78 11.76)		
58	98	Foam [143—150][i]	3380, 1770, 1705, 1615 ($CHCl_3$)	1.35 (3H, d, 7), 4.40 (1H, d, 15), 4.90 (1H, d, 15), 5.16 (1H, q, 7), 5.3 (1H, br), 6.6—7.1 (2H, m), 7.54 (1H, s), 7.4—8.1 (4H, m), 8.13 (1H, s)	$C_{20}H_{15}ClF_2N_4O_3$	435, 433 (M^++1), 352, 350, 334, 332, 279, 277, 224 (100%), 210, 208, 141				

a) Recrystallization solvent: A, AcOEt; H, hexane; B, benzene; E, ether; M, MeOH; C, $CHCl_3$. *b*) Chemical shifts are given with proton numbers, absorption patterns, and coupling constants in Hz in parentheses. *c*) CD_3OD was used as a solvent. *d*) Pyridine-d_5 was used as a solvent. *e*) DMF-d_7 was used as a solvent. *f*) A mixture of $CDCl_3$ and CD_3OD (10 : 1) was used as a solvent. *g*) DMSO-d_6 was used as a solvent. *h*) HCl salt. *i*) HNO_3 salt.

Me_4Si as an internal standard. Mass spectra (MS) were obtaind on a JEOL JMS D300 spectrometer. Optical rotations were determined on a Perkin-Elmer 141 spectrometer at 25 °C. Thin-layer chromatography (TLC) was performed on TLC plates, Silica gel 60 F_{254} precoated, layer thickness 0.25 mm (E. Merck), and spots were made visible by ultraviolet (UV) irradiation, by spraying with vanadic acid–sulfuric acid followed by heating, or by iodine treatment. Chromatography columns were prepared with silica gel (60—110 mesh, Kanto Chemical Co., Inc.) and preparative TLC was carried out on plates of Silica gel 60 F_{254}, layer thickness 2 mm (E. Merck). The amount of silica gel used and the developing solvents are shown in parentheses. The abbreviations used are as follows: s, singlet; d doublet; dd, doublet of doublets; dq, doublet of quartets; t, triplet; q, quartet; qd, quartet of doublets; m, multiplet; br, broad.

(2*R*,3*R*)-3-Amino-2-(2,4-difluorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol [(2*R*,3*R*)-3a] A solution of (2*R*,3*R*)-**2a**[1] (5.00 g) in EtOH (100 ml) was shaken with 10% palladium–carbon (1.30 g) under an H_2 atmosphere for 1 h. The catalyst was filtered off using Celite, and the filtrate was concentrated under reduced pressure to give (2*R*,3*R*)-**3a** (4.56 g, 100%) as a solid, which was recrystallized from benzene to afford a pure specimen, mp 154—155 °C: $[\alpha]_D$ −73° (c=1.06, $CHCl_3$). *Anal.* Calcd for $C_{12}H_{14}F_2N_4O$: C, 53.73; H, 5.26; N, 20.89. Found: C, 53.59; H, 5.35, N, 20.71. IR ν_{max} (KBr) cm^{-1}: 3210, 1620, 1600. The NMR spectrum was identical with that of the corresponding racemate.[2]

(2*R,3*R**)-2-(2,4-Difluorophenyl)-3-(*N*-methylamino)-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol (4a) and the (2*R*,3*R*) Enantiomer [(2*R*,3*R*)-4a]** i) A solution of (4*R**,5*R**)-5-(2,4-difluorophenyl)-4-methyl-5-[(1*H*-1,2,4-triazol-1-yl)methyl]oxazolidine[2] (**5a**, 941 mg, 3.36 mmol) in tetrahydrofuran (THF, 25 ml) was added dropwise to a suspension of lithium aluminum hydride (127 mg, 3.36 mmol) in THF (8 ml), with ice-cooling and stirring. After stirring at the same temperature for 30 min, a 1 N aqueous solution of NaOH (0.5 ml) was added and the whole was stirred for an additional 1 h. At the end of this time, the mixture was filtered using Celite, and the filtrate was concentrated under reduced pressure to afford **4a** (868 mg, 92%) as a solid, which was used without further purification for the next reaction. An analytical sample of **4a**, mp 141—145 °C, was obtained by recrystallization from AcOEt–hexane. *Anal.* Calcd for $C_{13}H_{16}F_2N_4O$: C, 55.31; H, 5.71; F, 13.46; N, 19.85. Found: C, 55.39; H, 5.78; F, 13.63; N, 20.01. IR ν_{max} (KBr) cm^{-1}: 3210, 1610, 1580, 1499. NMR ($CDCl_3$) δ: 0.90 (3H, dd, J=7, 2 Hz), 2.48 (3H, s), 2.96 (1H, qd, J=7, 2 Hz), 4.80 (2H, s), 6.55—7.1 (2H, m), 7.1—7.7 (1H, m), 7.77 (1H, s), 7.96 (1H, s).

In a similar way, (2*R*,3*R*)-**4a** was obtained as a crude cake in 95% yield from (4*R*,5*R*)-**5a**, which was prepared as described previously[2] by heating equimolar amounts of (2*R*,3*R*)-**3a** and paraformaldehyde in benzene. An analytical sample of (2*R*,3*R*)-**4a**, mp 168—170 °C, $[\alpha]_D$ −97.4° (c=0.96, $CHCl_3$), was obtained by recrystallization from AcOEt–MeOH. The NMR spectrum was identical with that of the racemate **4a** described above.

ii) A 40% methylamine solution in MeOH (0.7 ml) was added to a solution of **1a**[2] (racemate, 180 mg) in MeOH (1 ml), and the whole in a sealed tube was heated at 70 °C for 21 h. The solvent was evaporated off under reduced pressure to give a viscous oil (202 mg), which was shown by the NMR spectrum to be a mixture of **4a**, **6** and **1a** in approximately a 3 : 1 : 1 ratio.

This reaction in a scale using 1.00 g of **1a** (racemate) at a higher temperature (100 °C) during a longer time (46 h) afforded an almost pure product **6** as a solid, which was purified by recrystallization from AcOEt–benzene to give a pure specimen (943 mg, 84%), mp 170—173 °C. *Anal.* Calcd for $C_{13}H_{15}FN_4O$: C, 59.53; H, 5.76; F, 7.24; N, 21.36. Found: C, 59.62; H, 5.89; F, 6.95; N, 21.24. IR ν_{max} (KBr) cm^{-1}: 3138, 1621, 1602, 1514, 1492. NMR (DMF-d_7) δ: 1.35 (3H, d, J=6.5 Hz), 2.73 (3H, s), 3.32 (1H, q, J=6.5 Hz), 4.28 (1H, d, J=15 Hz), 4.42 (1H, d, J=15 Hz), 5.85 (1H, br), 6.1—6.5 (3H, m), 7.84 (1H, s), 8.34 (1H, s).

(2*R,3*R**)-2-(4-Chlorophenyl)-3-(*N*-methylamino)-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol (4c)** i) Following a procedure similar to that described for **4a**, **4c** was prepared as a cake in 90% yield by treatment of **5c**[2] (racemate) with lithium aluminum hydride in THF. MS m/z: 281 (M^++1), 223, 222, 198, 139, 125, 111, 70, 58 (100%). NMR ($CDCl_3$) δ: 0.96 (3H, d, J=6.5 Hz), 2.38 (3H, s), 2.55 (1H, q, J=6.5 Hz), 3.41 (1H, s), 4.46 (1H, d, J=15 Hz), 4.80 (1H, d, J=15 Hz), 7.29 (4H, s), 7.83 (1H, s), 8.05 (1H, s).

ii) A 40% methylamine solution in MeOH (12 ml) was added to a solution of **1c**[2] (racemate, 3.00 g) in MeOH (15 ml), and the whole in a sealed tube was heated at 70 °C for 24 h. Removal of the solvent by evaporation *in vacuo* afforded **4c** (3.45 g) as a solid, which was used without further purification for the next reaction. The NMR spectrum was identical with that of the sample obtained above.

(2*R,3*R**)-2-(2,4-Difluorophenyl)-3-formamido-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol (7)** A solution of *N*-formylimidazole in CH_2Cl_2, prepared *in situ* from formic acid (42 mg, 0.91 mmol) and *N*,*N*′-carbonyldiimidazole (147 mg, 0.91 mmol) in CH_2Cl_2 (1.5 ml), was added to a solution of **3b**[2] (racemate, 120 mg, 0.40 mmol) in CH_2Cl_2 (0.5 ml). The mixture was stirred at room temperature for 10 h, after which it was diluted with AcOEt, washed successively with water and brine, and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure gave a solid, which was recrystallized from AcOEt–hexane to give **7** (75 mg, 57%) as a colorless powder, mp 233—237 °C. Spectroscopic data and elementary analysis data are given in Table III.

(2*R,3*R**)-2-(2,4-Dichlorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)-3-[(trimethylacetyl)amino]-2-butanol (8)** Trimethylacetyl chloride (100 mg, 0.83 mmol) was added to a solution of **3b** (150 mg, 0.50 mmol) in pyridine (2.5 ml), with stirring at 0 °C. After being stirred at the same temperature for 30 min, the mixture was concentrated under reduced pressure and the residue was partitioned between AcOEt and a diluted aqueous solution of $NaHCO_3$. The organic layer was washed with brine, dried and concentrated *in vacuo* to afford an oily residue, which was chromatographed on silica gel (2 g, benzene–AcOEt, 4 : 1—1 : 1, v/v) to give **8** (180 mg, 94%) as an oil. Spectroscopic data is given in Table III.

The amide **8** (140 mg) formed its oxalic acid salt (120 mg), mp 156—160 °C, on being mixed with 1 eq of oxalic acid in ether.

(2*R,3*R**)-2-(2,4-Difluorophenyl)-3-[(5-methyl-4-hexenoyl)amino]-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol (9)** A solution of 5-methyl-4-hexenoic acid (192 mg, 1.50 mmol) and *N*,*N*′-carbonyldiimidazole (243 mg, 0.15 mmol) in THF (5 ml) was warmed at 40 °C for 30 min. To this solution was added **3a**[2] (racemate, 200 mg, 0.75 mmol), and the mixture was stirred at 60—65 °C for 2.5 h. After cooling, the mixture was partitioned between AcOEt and a 5% aqueous solution of $NaHCO_3$. The organic layer was collected, washed with brine and dried over $MgSO_4$. The solvent was evaporated off under reduced pressure to give an oily residue, which was chromatographed on silica gel (10 g, benzene–AcOEt, 1 : 2—1 : 4, v/v) to afford **9** (193 mg, 65%) as an oil. Spectroscopic data of **9** is given in Table III.

The amide **9** formed its oxalic acid salt, mp 143—145 °C, on being mixed with 1 eq of oxalic acid in AcOEt–hexane.

(2*R,3*R**)-3-Acylamino-2-aryl-1-(1*H*-1,2,4-triazol-1-yl)-2-butanols (10—42), the (2*R*,3*R*) Enantiomers [(−)-33, (−)-34, (−)-43 and (−)-44] and the (2*S*,3*S*) Enantiomer [(+)-33]** As a typical example, the preparation of (−)-**33** is described. 4-(Trifluoromethyl)benzoyl chloride (218 mg, 1.04 mmol) was added to a solution of (2*R*,3*R*)-**3a** (200 mg, 0.75 mmol) in pyridine (2 ml), with stirring and ice-cooling. After 1 h, MeOH (0.3 ml) was added to the mixture, which was then stirred for 10 min. The solvent was removed under reduced pressure, and a diluted aqueous solution of $NaHCO_3$ was added to the residue. Extraction with AcOEt and evaporation of the solvent *in vacuo* gave an oily residue, which was chromatographed on silica gel (10 g, benzene–AcOEt, 1 : 1, v/v) to afford (−)-**33** (266 mg, 81%) as an oil, $[\alpha]_D$ −105° (c=0.96, $CHCl_3$). Spectroscopic data is given in Table III.

The amide (−)-**33** formed its HCl salt, mp 140—148 °C, on being mixed with a 4 N HCl dioxane solution (1 eq) in AcOEt–benzene.

In a similar way, other amidoalcohols were prepared by acylation of the aminoalcohols **3a**—**c** with the corresponding acyl chloride. Crystalline compounds were purified by recrystallization of the crude products from an appropriate solvent. The amide **32** was converted to its oxalic acid salt, mp 222—226 °C, on being mixed with 1 eq of oxalic acid in AcOEt–hexane. Oily products were purified by column chromatography, then converted to crystalline salts by mixing them with oxalic acid or HCl in an organic solvent. The yields, physical and spectroscopic data and elementary analysis data (or MS data) of these amidoalcohols are given in Table III.

(2*R*,3*R*)-2-(2,4-Difluorophenyl)-3-[*N*-methyl-*N*-[4-(trifluoromethoxy)-benzoyl]amino]-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol [(−)-45] and the (2*R,3*R**)-*N*-Methylamidoalcohols (46—49)** As a typical example, the preparation of **46** is described. 4-(Trifluoromethyl)benzoyl chloride (375 mg, 1.80 mmol) was added to a solution of **4a** (428 mg, 1.50 mmol) and triethylamine (404 mg, 4.00 mmol) in CH_2Cl_2 (8 ml). The mixture was stirred at room temperature for 30 min. At the end of this time, MeOH (0.5 ml) was added to this mixture and stirring was continued for 15 min, after which the solvent was removed by evaporation under reduced pressure and a diluted aqueous solution of $NaHCO_3$ was added to the residue. Extraction with AcOEt and evaporation of the solvent gave a

crystalline mass, which was recrystallized from ether to afford **46** (504 mg, 73%), mp 144—146 °C. Spectroscopic data and elementary analysis data are given in Table III.

In a similar way, other *N*-methylamidoalcohols were prepared by acylation of *N*-methylaminoalcohol **4a** or **4c** with the corresponding acyl chloride. The amide (−)-**45** was obtained from (−)-**4a** as an oil, which formed its HNO_3 salt, mp 103—106 °C, on being mixed with 1 eq of concentrated HNO_3 in ether. Amides **47**—**49** were crystalline and purified by recrystallization from an appropriate solvent. The yields, physical and spectroscopic data and elementary analysis data of these *N*-methylamidoalcohols are given in Table III.

(2*R,3*S**)-2-(4-Chlorophenyl)-3-methanesulfonyloxy-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol (51)** Methanesulfonyl chloride (140 mg, 1.31 mmol) was added to a solution of **50**[2] (racemate, 157 mg, 0.59 mmol) in pyridine (2 ml) with stirring at 0 °C. After 2 h, the mixture was partitioned between ether and water. The organic layer was washed with brine and dried. Evaporation of the solvent under reduced pressure gave **51** (193 mg, 95%) as an oil, which was used without further purification for the next reaction. NMR ($CDCl_3$) δ: 1.27 (3H, d, J=6 Hz), 2.97 (3H, s), 4.6 (1H, br), 4.66 (2H, s), 4.85 (1H, q, J=6 Hz), 7.1—7.4 (4H, m), 7.78 (1H, s), 8.03 (1H, s).

(2*R,3*S**)-3-Azido-2-(4-chlorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol (52)** A mixture of **51** (193 mg, 0.56 mmol), sodium azide (188 mg, 2.89 mmol), ammonium chloride (30 mg, 0.56 mmol) and *N*,*N*-dimethylformamide (DMF, 3 ml) was stirred at 120 °C for 2 h. After cooling, the mixture was partitioned between benzene and water. The organic layer was washed with brine and concentrated *in vacuo* to give an oily residue, which was purified by column chromatography (5 g, AcOEt–hexane, 2 : 1, v/v) to give **52** (150 mg, 92%) as an oil. MS *m/z*: 295, 293 (M^+ + 1), 224, 222 (100%), 141, 139, 127, 125. IR ν_{max} ($CHCl_3$) cm^{-1}: 3410, 2110. NMR ($CDCl_3$) δ: 1.11 (3H, d, J=6.5 Hz), 3.55 (1H, q, J=6.5 Hz), 4.58 (2H, s), 5.14 (1H, br s), 7.2—7.6 (4H, m), 7.82 (1H, s), 8.01 (1H, s).

(2*R,3*S**)-2-(4-Chlorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)-3-[[4-(trifluoromethyl)benzoyl]amino]-2-butanol (54)** A solution of **52** (122 mg, 0.42 mmol) in EtOH (3 ml) was shaken with 10% palladium–carbon (35 mg) under an H_2 atmosphere for 1 h. The catalyst was filtered off using Celite, and the filtrate was concentrated under reduced pressure to give the aminoalcohol **53** (111 mg, 100%) as an oil, which was then dissolved in pyridine (1.5 ml) and treated with 4-(trifluoromethyl)benzoyl chloride (97 mg, 0.47 mmol) at 0 °C for 30 min. Work-up in the usual manner, followed by purification by column chromatography (5 g, AcOEt–hexane, 2 : 1, v/v), and then by recrystallization from benzene–hexane gave **54** (109 mg, 60% yield from **52**), mp 175—177 °C. Spectroscopic data and elementary analysis data are given in Table III.

(±)-1-[(4-Chlorobenzoyl)amino]-2-(2,4-difluorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)-2-propanol (56) To a solution of **55**[2] (racemate, 81 mg, 0.32 mmol) in pyridine (1 ml) was added 4-chlorobenzoyl chloride (72 mg, 0.41 mmol), with stirring at 0 °C. After 10 min, the mixture was partitioned between AcOEt and water. The organic layer was washed with brine and concentrated *in vacuo* to give a crystalline residue, which was recrystallized from $CHCl_3$–ether to afford **56** (109 mg, 87%), mp 170—172 °C. Spectroscopic data and elementary analysis data are given in Table III.

(2*R,3*R**)-3-[(4-Chlorobenzenesulfonyl)amino]-2-(2,4-dichlorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol (57)** A solution of **3b** (racemate, 50 mg, 0.166 mmol) and 4-chlorobenzenesulfonyl chloride (53 mg, 0.25 mmol) in pyridine (1 ml) was stirred at 0 °C. After quenching the reaction with MeOH (0.1 ml), the mixture was partitioned between AcOEt and water. The organic layer was dried and concentrated *in vacuo* to give an oily residue, which was purified by column chromatography (1 g, AcOEt–benzene, 1 : 3, v/v) to yield **57** (32 mg, 40%) as a solid. Recrystallization from ether gave an analytical sample, mp 104—108 °C. Spectroscopic data and elementary analysis data are given in Table III.

(2*R,3*R**)-3-(4-Chlorophthalimido)-2-(2,4-difluorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol (58)** A solution of **3a** (150 mg, 0.56 mmol), 4-chlorophthalic anhydride (124 mg, 0.68 mmol) and triethylamine (5 mg, 0.05 mmol) in toluene (4 ml) was heated under reflux for 4 h with azeotropic removal of water. The mixture was then diluted with AcOEt and washed with a diluted aqueous solution of $NaHCO_3$. The organic layer was dried and the solvent was distilled off under reduced pressure. The residue was purified by preparative TLC (AcOEt) to give **58** (238 mg, 98%) as a colorless foam. Spectroscopic data of **58** is given in Table III.

The imide **58** formed its HNO_3 salt, mp 143—150 °C, on being mixed with 1 eq of concentrated HNO_3 in ether.

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Synthesis of Optically Active Lipopeptide Analogs from the Outer Membrane of *Escherichia coli*

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The synthesis of optically active lipopeptide derivatives has been accomplished by the use of chiral glycerol derivatives. Lipopeptide derivatives with (*R*)-glycerol moieties showed higher mitogenic activities than those with the (*S*)-configuration. *N*-2,2,2-Trichloroethoxycarbonyl lipopeptide derivatives increased mitogenic activity.

Keywords peptide synthesis; lipoprotein; mitogenic activity; chiral glycerol derivative; *S*-[2,3-bis(palmitoyloxy)propyl]-*N*-trichloroethoxycarbonyl pentapeptide

Lipoprotein[1] from the outer membrane of *Escherichia coli* and other Enterobacteriaceae is a potent polyclonal activator for *B* lymphocytes. It is composed of 58 amino acids with one amidelinked- and two ester linked-fatty acids attached to *S*-(2,3-dihydroxypropyl)cysteine at the N-terminus, which contains a mixture of different fatty acids, palmitic acid being the main component (Chart 1).

To determine the molecular structure responsible for the biological activities of lipoprotein, a series of oligopeptide analogs of its *N*-terminal part containing only palmitoyl residues were synthesized.[2,3] *S*-[2,3-Bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-seryl-(*S*)-asparaginyl-(*S*)-alanine was an active mitogen and polyclonal B lymphocyte activator *in vitro* and *in vivo*.[4-6] It also supplements *Salmonella vaccines*.[7]

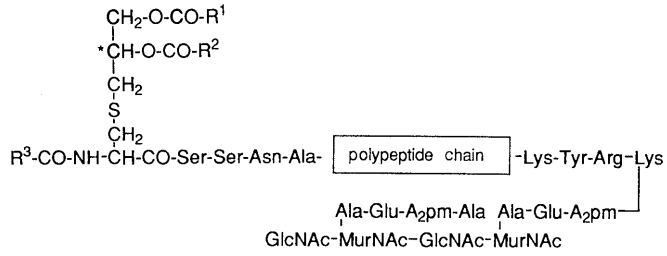

Chart 1. Lipoprotein

In the preceding paper,[8] we have reported a new synthesis of *S*-[2,3-bis(palmitoyloxy)-(2*R* and 2*S*)-propyl]-*N*-palmitoyl-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-seryl-(*S*)-asparaginyl-(*S*)-alanine (**1** and **3**) and their *N*-2,2,2-trichloroethoxycarbonyl (Troc) derivatives (**2** and **4**) by using *N*-(2,2,2-trichloroethoxycarbonyl)cysteinyl intermediates, which prevent a racemization of their cysteinyl parts in the condensation steps. The biological assay results of these compounds indicated that the natural [(2*R*)-propyl] type **1** has a higher activity than the unnatural [(2*S*)-propyl] type **3**, and that their Troc derivatives increase mitogenic activities.[9] Comparison of the activities among compounds **1**, **2**, **3** and **4** showed that the most active was compound **2**. Therefore, we focused our attention on compound **2**. In order to discover more highly active derivatives and structure–activity relationships, we synthesized from the lipopenta- to the lipomonopeptide trichloroethoxycarbonyl derivatives **2**, **8**, **7**, **6** and **5**. Likewise to find out the effect of a trichloroethoxycarbonyl group on mitogenic activity, we synthesized derivatives **9** and **10**. We wish to report

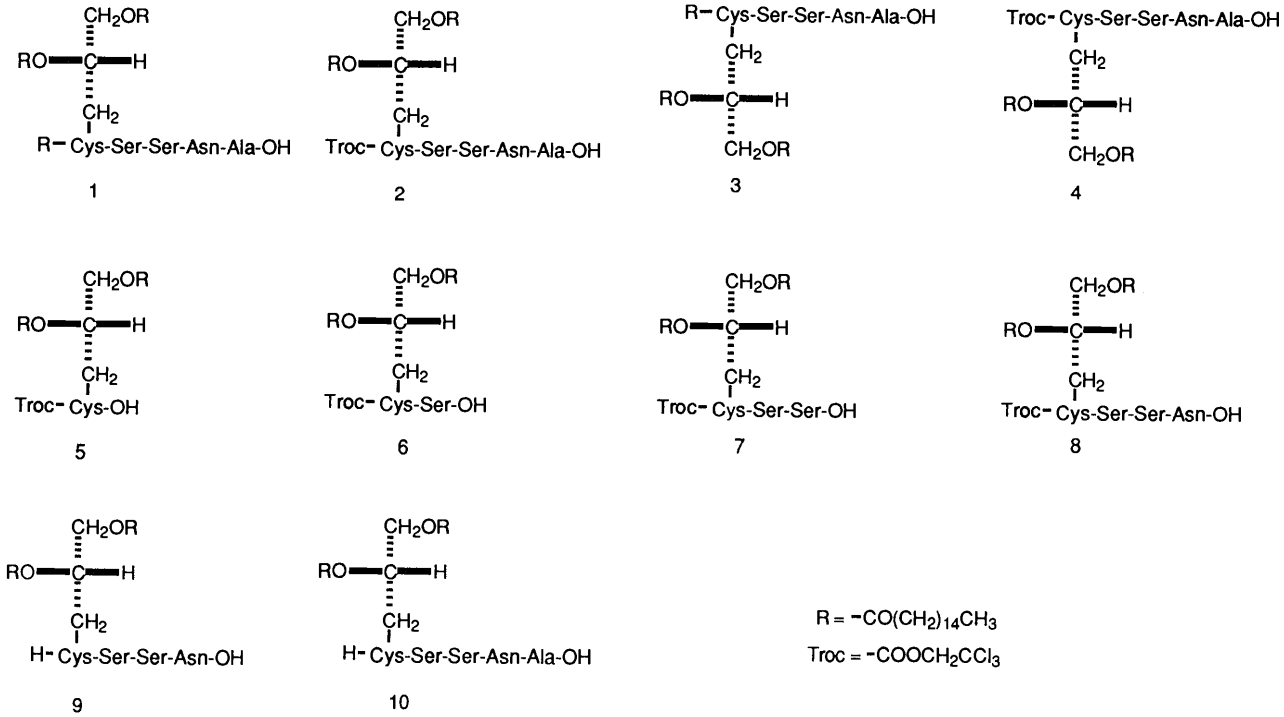

Chart 2

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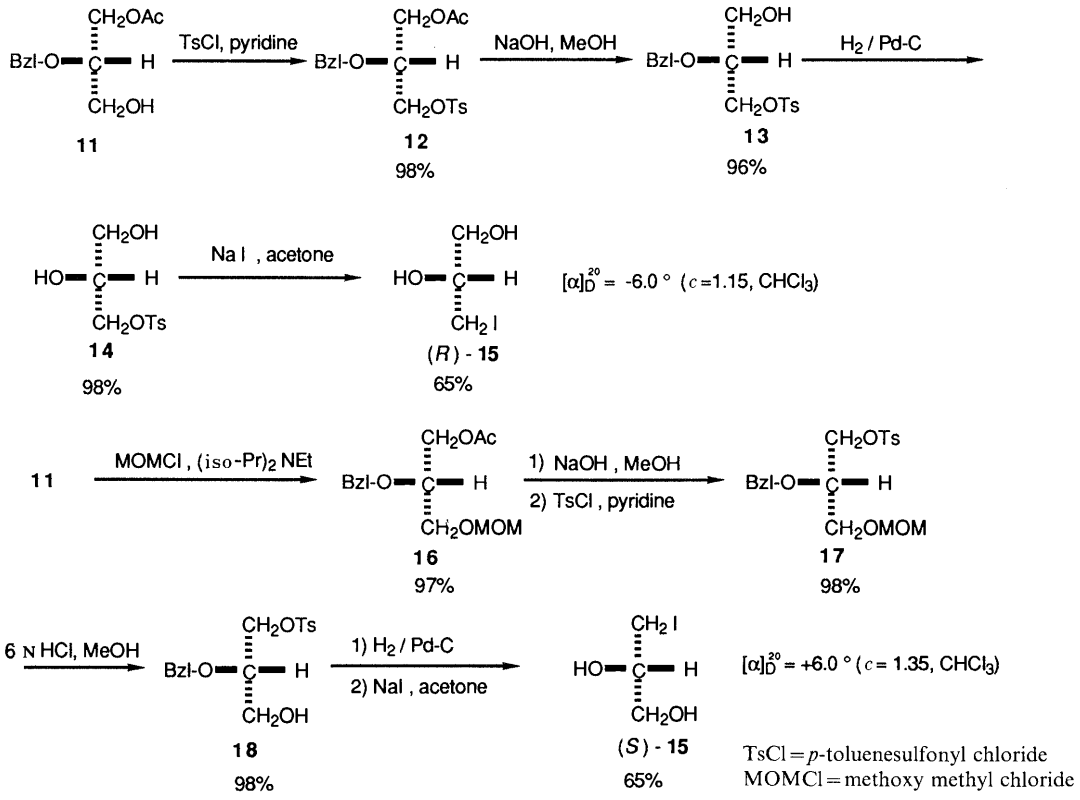


Chart 3

here the synthetic details and mitogenic activity of these derivatives (**1**—**10**) (Chart 2).

K. H. Wiesmuller *et al.*[2] have reported the synthesis of lipopeptides from racemic 3-bromo-1,2-propanediol. Therefore, we have synthesized lipopeptides from optically active glycerol derivatives. Natural lipopeptide **1** can be obtained from (*R*)-1-iodoglycerol (*R*)-**15**. On the other hand, unnatural lipopeptide **3** can be obtained from (*S*)-1-iodoglycerol (*S*)-**15**.

Compounds (*R*)-**15** and (*S*)-**15** were synthesized according to the reaction sequence shown in Chart 3. K. Achiwa *et al.*[10] reported that a chiral glycerol derivative (*S*)-1-*O*-acetyl-2-*O*-benzyl glycerol **11** is prepared by lipase-catalyzed asymmetric transesterification. Thus compound **11** was prepared according to Achiwa's method. Treatment of **11** with *p*-toluenesulfonyl chloride in pyridine followed by hydrolysis with sodium hydroxide in ethanol gave **13** in 94% yield from **11**. Hydrogenolysis of **13** over 5% Pd–C in ethanol gave **14** in 98% yield. Treatment of **14** with NaI in a pressure bottle afforded (*R*)-**15** in 65% yield. (*S*)-**15** was synthesized in a following step. Treatment of **11** with methoxymethyl chloride in CH_2Cl_2 gave **16** in 97% yield. Hydrolysis of **16** with sodium hydroxide in ethanol followed by treatment with *p*-toluenesulfonyl chloride in CH_2Cl_2 afforded **17** in 98% yield. After demethoxymethylation of **17** with 6 N HCl in MeOH, the resulting **18** was hydrogenated over 5% Pd–C in ethanol, and treated with NaI in a pressure bottle to afford (*S*)-**15** in 65% yield. These compounds (*R*)-**15** and (*S*)-**15** showed $[\alpha]_D^{22}$ $-6.0°$ ($c=1.35$, $CHCl_3$) and $[\alpha]_D^{22}$ $+6.0°$ ($c=1.23$, $CHCl_3$), respectively.

Compounds **1**, **2**, **3**, **4** and **5** were synthesized according to the reaction sequence shown in Chart 4. The starting material, **19**, was prepared according to the method reported by K. H. Wiesmuller *et al.*[2] N-protection of **19** with 2,2,2-trichloroethoxychloroformate (3 eq) in pyridine, followed by reduction with dithioerythritol (4 eq) in $CHCl_3$ in the presence of triethylamine (3 eq) afforded **21**, which was used without further purification. In coupling **21** with glycerol moieties, the natural lipopeptide **1** could be obtained by (*R*)-**15** and the unnatural **3** by (*S*)-**15**. Compounds **1**, **2** and **5** were synthesized from (*R*)-**15** *via* the following steps. Reaction of **21** with (*R*)-**15** in dimethylformamide (DMF) in the presence of *N*,*N*-diisopropylethylamine (4 eq) gave **22** (55% from **20**). Esterification of **22** with palmitoyl chloride (2 eq) and *N*,*N*-diisopropylethylamine (4 eq) in CH_2Cl_2 in the presence of a catalytic amount of 4-dimethylaminopyridine (DMAP), followed by deprotection of the *tert*-butyl group of **23** with trifluoroacetic acid, afforded **5** in 69% yield from (*R*)-**15**. Compound **5** was employed for coupling with tetrapeptide H–Ser(Bu^t)–Ser(Bu^t)–Asn–Ala–OBu^t **30**, which was prepared by stepwise chain elongation using the DCC–HOBt method[11] as shown in Chart 4. **24** was condensed with Z (carbobenzoxy)–Asn–OH by the DCC–HOBt method in DMF in the presence of *N*-methylmorpholine to give **25**[11] in 68% yield. The Z group of **25** was removed by hydrogenation, and the free base **26** was coupled to Z–Ser(Bu^t)–OH to afford **27** in 76% yield. In the same way, **27** was hydrogenated to give **28**, which was coupled to Z–Ser(Bu^t)–OH to afford **29** in 47% yield. The Z group of **29** was removed by hydrogenation to afford **30**[2], which is the same partially protected tetrapeptide as reported by K. H. Wiesmuller *et al.*[2] Compound **31** was obtained by coupling **5** with **30**[2] in DMF by the DCC–HOBt method in 50% yield. Deprotection of all

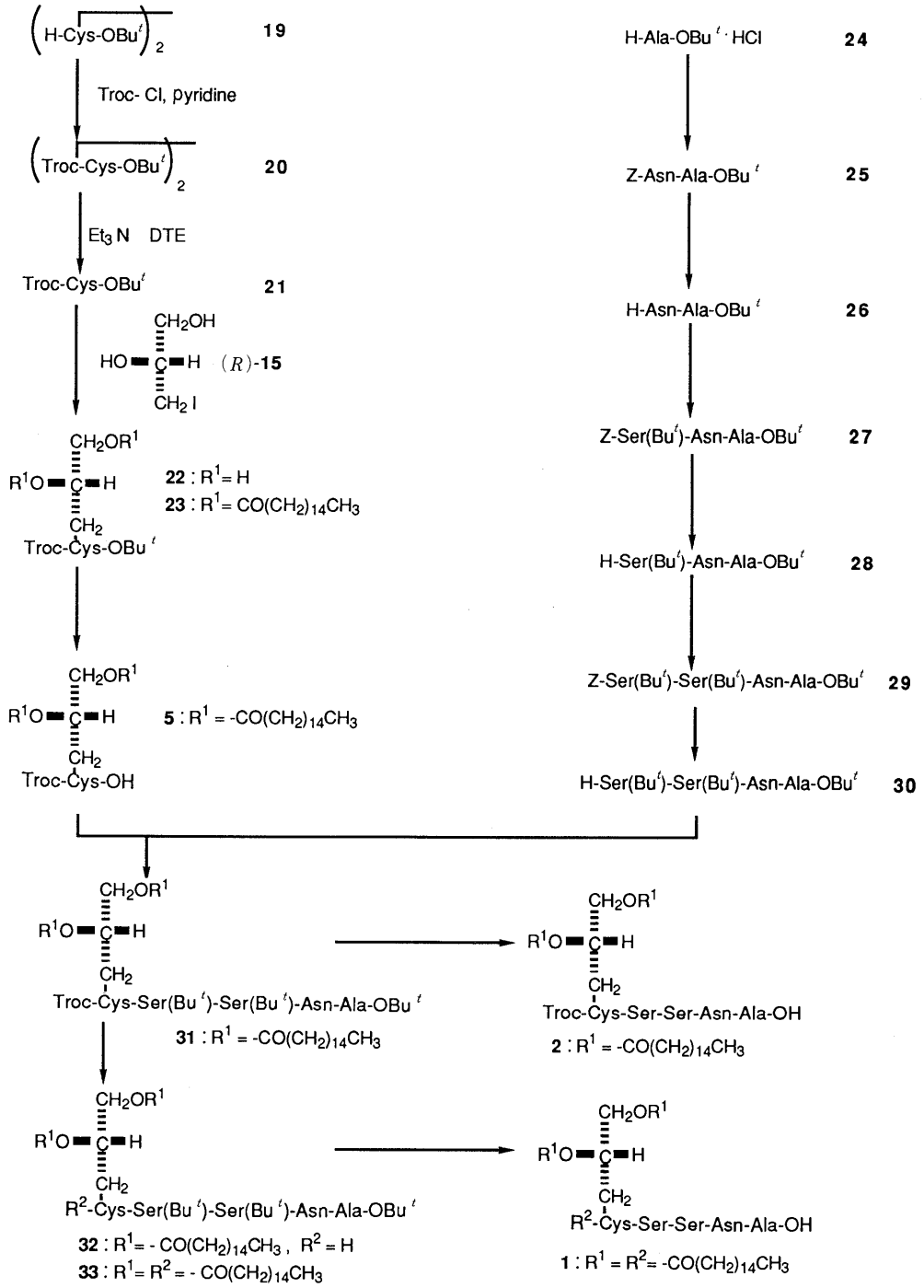


Chart 4

tert-butyl groups of **31** was carried out by treatment with trifluoroacetic acid to give **2** in 45% yield. In the same route, the unnatural Troc derivative **4** was synthesized by using (*S*)-**15** in place of (*R*)-**15**. The trichloroethoxycarbonyl group of **31** was removed by treatment with zinc in acetic acid to give **32**, which was then acylated in the presence of a catalytic amount of DMAP with palmitoyl chloride and *N*,*N*-diisopropylethylamine in CH_2Cl_2 to afford **33**. The final deprotection of all *tert*-butyl groups of **33** was carried out by treatment with trifluoroacetic acid to give **1** (53% yield from **31**). In the same way, unnatural compound **3** was synthesized by using (*S*)-**15** in place of (*R*)-**15**.

Compounds **6**, **7** and **8** were synthesized according to the reaction sequence shown in Chart 5. Compound **5** was condensed with the peptides **34**, **35** and **36** by the DCC–HOBt method in DMF to give **37** (57%), **38** (67%) and **39** (52%), respectively. The final deprotection of all *tert*-butyl groups of **37**, **38** and **39** was carried out by treatment with trifluoroacetic acid to give **6** (50%), **7** (53%) and **8** (67%), respectively.

Compounds **9** and **10** were synthesized according to the reaction sequence shown in Chart 6. The trichloroethoxycarbonyl groups of **36** and **31** were removed by treatment

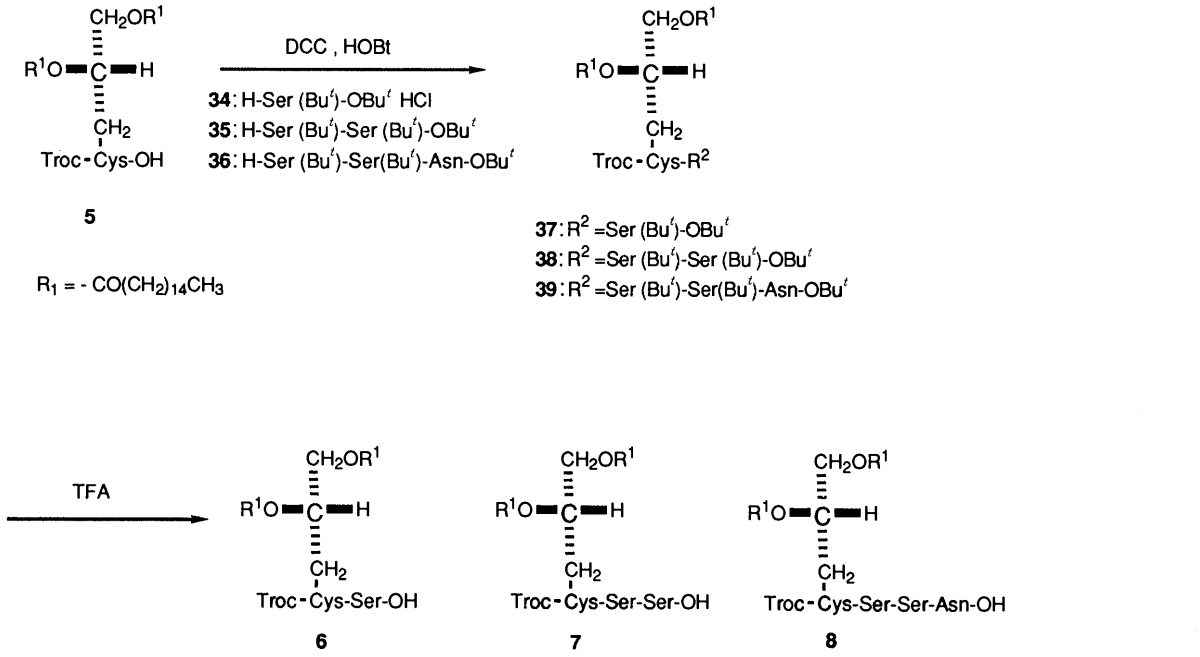
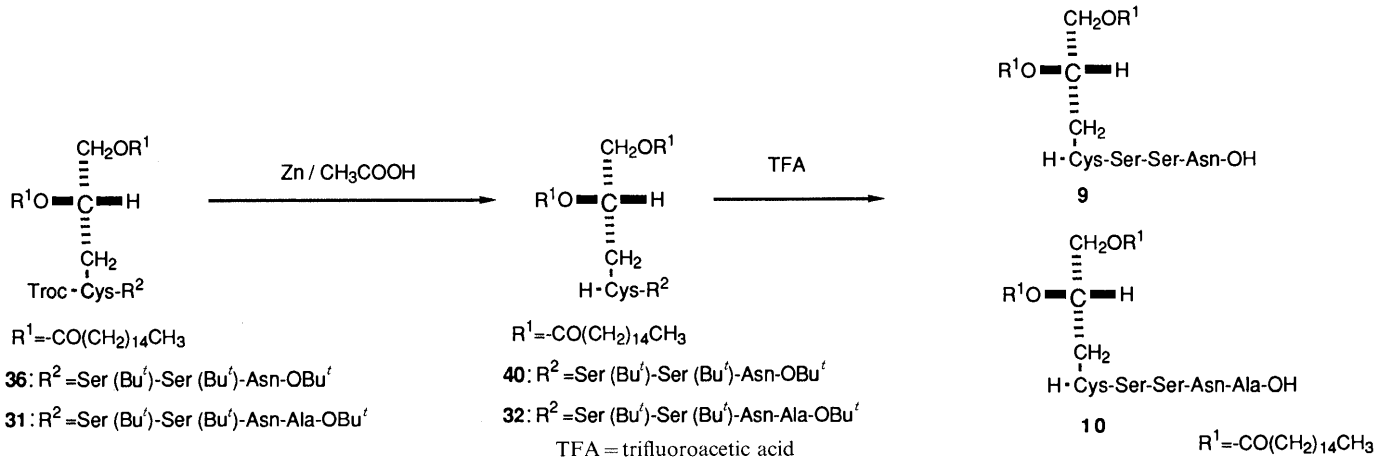


Chart 5



TFA = trifluoroacetic acid

Chart 6

with zinc in acetic acid to give **40** (84%) and **32** (85%), respectively. The final deprotection of all *tert*-butyl groups of **40** and **32** was carried out by treatment with trifluoroacetic acid to give **9** (58%) and **10** (64%).

The structures of **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9** and **10** were supported by elemental analysis and confirmed by analysis of the infrared (IR), proton nuclear magnetic resonance (^{1}H-NMR) and fast atom bombardment mass spectrum (FAB-MS) spectra. The mitogenic activities of all compounds (**1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9** and **10**) were measured. Of compounds **1**, **2**, **3** and **4**, **1** and **4** maintained the same degree of activity. The activity of **2** was greatly enhanced, while the activity of compound **3** was weak. These results indicated that the natural [(2*R*)-propyl] type **1** has higher activity than the unnatural [(2*S*)-propyl] type **3**, and that the Troc derivative increases mitogenic activity. Comparison of compounds **2**, **8**, **7**, **6** and **5** indicated that after shortening the peptide chain from the lipopenta- to the lipomonopeptide, mitogenic activity is still maintained in a high concentration. However, in a low concentration, the activity of **2** and **8** was still maintained, but **6**, **7** and **5** exhibited weak activity. In compounds **2**, **9** and **10**, the mitogenic activities of **2**, **9** and **10** were maintained in a high concentration; however, in a low concentration, only the activity of **2** and **10** was still maintained.

Experimental

Melting points were determined on a micro melting point BY-1 (Yazawa) and are uncorrected. Optical rotations were measured on a JASCO DIP-140 digital polarimeter. IR spectra were taken on JASCO IR-810 IR spectrophotometers and are given in cm^{-1}. ^{1}H-NMR spectra were recorded on a JEOL JNM-FX90Q (90 MHz) spectrophotometer in $CDCl_3$. Chemical shifts are given in δ (ppm) downfield from tetramethylsilane, and the abbreviations of signal patterns are as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. Thin layer chromatography (TLC) was performed on silica gel (Kiesel $60F_{254}$ on aluminium sheets, Merck). All compounds were located by spraying the TLC plate with 10% phosphomolybdic acid in ethanol and heating it on a hot plate. Preparative TLC was performed on a preparative layer chromatography plate (Kieselgel $60F_{254}$ 2 and 0.5 mm, Merck). Column chromatography was performed on silica gel (Kieselgel 60, 70—230 mesh, Merck).

(*R*)-1-*O*-Acetyl-2-*O*-benzyl-3-tosylglycerol (12) *p*-Toluenesulfonyl chloride (19 g, 0.10 mol) was added to a stirred solution of (*S*)-1-*O*-acetyl-2-*O*-benzylglycerol[10] (13 g, 0.06 mol) in pyridine (60 ml) at 0 °C and the mixture was stirred for 15 h at room temperature. The reaction mixture was poured onto ice-H_2O (100 ml) and extracted with CH_2Cl_2 (100 ml). The organic layer was washed with 1 N HCl (150 ml × 1) and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel with isopropylether (IPE)–$CHCl_3$ (1 : 10) as an eluent to give **12** (22 g 98%) as a colorless oil. **12**: $[\alpha]_D^{22}$ +13.5° (*c* = 1.22, $CHCl_3$). IR (neat): 1742 (ester), 1365, 1178 (SO_2) cm^{-1}. ^{1}H-NMR: 1.99 (3H, s), 2.44 (3H, s), 3.64—3.96 (1H, m), 4.12, (4H, d, *J* = 4.9 Hz), 4.57 (2H, s), 7.30 (5H, s), 7.31 (2H, d, *J* = 8.0 Hz).

(*R*)-1-*O*-Tosyl-2-*O*-benzylglycerol (13) 25% ammonium hydroxide (10 ml) was added to a stirred solution of **12** (22 g, 0.06 mol) in methanol at room temperature and the mixture was stirred for 15 h at the same temperature. The reaction mixture was concentrated *in vacuo* and extracted with CH_2Cl_2 (100 ml). The organic layer was washed with water (50 ml × 3) and brine, and concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel with $CHCl_3$–EtOH (20 : 1) as an eluent to give **13** (18 g, 96%) as a colorless oil. **13**: $[\alpha]_D^{22}$ +29.5° (*c* = 1.01, $CHCl_3$). IR (neat): 1360, 1380 (SO_2) cm^{-1}. ^{1}H-NMR: 2.41 (3H, s), 3.49—3.73 (4H, m), 4.12 (2H, d, *J* = 4.6 Hz), 7.27 (5H, s), 7.30 (2H, d, *J* = 8.3 Hz), 7.76 (2H, d, *J* = 8.3 Hz).

(*R*)-1-*O*-Tosylglycerol (14) **13** (18.4 g, 0.06 mol) was hydrogenated over 5% Pd–C as a catalyst in ethanol (100 ml). After removal of Pd–C, the filtrate was concentrated *in vacuo* to give **14** (13.7 g, 98%) as a colorless crystal. The product was used without further purification. **14**: mp 54—56 °C, $[\alpha]_D^{22}$ −8.36° (*c* = 1.02, MeOH). IR (KBr): 3400 (OH), 1365, 1180 (SO_2) cm^{-1}. ^{1}H-NMR: 2.30 (3H, s), 2.91 (2H, br s), 3.55—3.97 (5H, m), 7.28 (2H, d, *J* = 8.0 Hz), 7.53 (2H, d, *J* = 8.0 Hz).

(*R*)-1-Iodoglycerol (*R*-15) A mixture of **14** (1.9 g, 9.4 mmol) and NaI (3.63 g, 24 mmol) in acetone (5 ml) was stirred for 9 h at 90 °C in a pressure bottle. The reaction mixture was filtrated and the filtrate was concentrated *in vacuo*. Ether (150 ml) was added to the residue and the mixture was stirred for a while and extracted with ether. The organic layer was filtered off and 0.1 N sodium thiosulfate was added to the filtrate to make it colorless. After washing with brine (100 ml) and drying over $MgSO_4$, the solvent was concentrated *in vacuo*. The residue was washed with *n*-hexane (100 ml × 3) and collected by suction to give (*R*)-**15** (1.1 g, 65%) as yellow needles. (*R*)-**15**: mp 33—35 °C, $[\alpha]_D^{22}$ −6.0° (*c* = 1.35, $CHCl_3$). IR (KBr): 3330 (OH) cm^{-1}. ^{1}H-NMR: 2.60—3.20 (2H, br s), 3.23 (2H, d, *J* = 6.0 Hz), 3.40—3.90 (3H, br s).

(*S*)-1-*O*-Acetyl-2-*O*-benzyl-3-*O*-methoxymethylglycerol (16) Methoxymethyl chloride (6.5 g, 81 mmol) in CH_2Cl_2 (20 ml) was added to a stirred mixture of **11** (15 g, 67 mmol) and *N*,*N*-diisopropylethylamine (13 g, 0.10 mol) in CH_2Cl_2 (80 ml) at 0 °C. After being stirred for 15 h at room temperature, the reaction mixture was washed with water (50 ml × 3) and brine (50 ml), dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel with *n*-hexane–AcOEt (15 : 1) as an eluent to give **16** (33 g, 97%) as a colorless oil.

(*S*)-1-*O*-Tosyl-2-*O*-benzyl-3-*O*-methoxymethylglycerol (17) A solution of NaOH (2.7 g, 67 mmol) in EtOH (50 ml) was added to **16** (18 g, 67 mmol) in EtOH (80 ml) at 0 °C. After being stirred for 1 h at the same temperature, the mixture was neutralized with 6 N HCl and removed off EtOH *in vacuo*. The residue was extracted with CH_2Cl_2 (100 ml) and the organic layer was washed with brine (50 ml), dried over $MgSO_4$ and concentrated *in vacuo* to give (*R*)-1-*O*-methoxymethyl-2-*O*-benzylglycerol (15 g, 100%). *p*-Toluenesulfonyl chloride (20 g, 0.10 mol) in CH_2Cl_2 (30 ml) was added to a stirred solution of (*R*)-1-*O*-methoxymethyl-2-*O*-benzylglycerol (15 g, 0.67 mol) and triethylamine (10 g, 0.10 mol) in CH_2Cl_2 (80 ml) at 0 °C. After being stirred for 15 h, the reaction mixture was washed with water (50 ml × 3) and brine (50 ml × 1), dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel with *n*-hexane–AcOEt (5 : 1) as an eluent to give **17** (25 g, 98%) as a colorless oil.

(*S*)-1-*O*-Tosyl-2-*O*-benzylglycerol (18) 6 N HCl (15 ml) in MeOH (30 ml) was added to **17** (25 g, 65 mmol) in MeOH (80 ml). After being stirred for 8 h at 60 °C, the reaction mixture was neutralized with 2 N NaOH aq., and MeOH was removed *in vacuo*. The residue was extracted with CH_2Cl_2 (100 ml × 3) and the organic layer was washed with brine (100 ml × 1), dried over $MgSO_4$ and concentrated *in vacuo* to give **18** (21 g, 98%) as a colorless oil.

(*S*)-1-Iodoglycerol (*S*-15) **18** was hydrogenated over Pd–C as a catalyst in EtOH (50 ml). The mixture was treated by the same procedure described in the preparation of compound **14** to give (*S*)-1-*O*-tosylglycerol (6.5 g, 99%) as a colorless oil. NaI (3.6 g, 24 mmol) was added to (*S*)-1-*O*-tosylglycerol (1.9 g, 8.1 mmol) in acetone (5 ml). The mixture was treated by the same procedure described in the preparation of the compound **15** to give (*S*)-**15** (1.1 g, 65%) as yellow needles. (*S*)-**15**: mp 32—34 °C, $[\alpha]_D^{22}$ +6.0° (*c* = 1.23, $CHCl_3$). IR (KBr): 3340 (OH) cm^{-1}. ^{1}H-NMR: 2.60—3.20 (2H, br s), 3.23 (2H, d, *J* = 6.0 Hz), 3.40—3.90 (3H, br s).

***N*,*N*-Di-2,2,2-trichloroethoxycarbonylcystine Di-*tert*-butyl Ester (20)** *N*-2,2,2-Trichloroethoxycarbonyl chloride (1.0 g, 4.8 mmol) in CH_2Cl_2 (5 ml) was added dropwise under stirring to the solution of **19** (0.53 g, 1.5 mmol) and pyridine (0.50 g, 6.3 mmol) in CH_2Cl_2 (30 ml). After being stirred for 3 h at room temperature, CH_2Cl_2 (50 ml) was added and washed with 5% citric acid, 5% $NaHCO_3$ aq. and water (50 ml × 3 each). The CH_2Cl_2 layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel with *n*-hexane–AcOET (10 : 1) as an eluent to give **20** (0.47 g, 60%) as a yellow oil. **20**: IR (neat): 3330 (NH), 1728 (ester) cm^{-1}. ^{1}H-NMR: 1.60 (18H, s), 3.26 (4H, d, *J* = 5.6 Hz), 3.80—4.3 (2H, m), 4.85 (4H, s), 5.8 (2H, br s).

***N*-2,2,2-Trichloroethoxycarbonylcysteine *tert*-Butyl Ester (21)** **20** (0.85 g, 1.6 mmol) in $CHCl_3$ (50 ml) was reduced with dithioerythritol (1.0 g, 6.5 mmol) in the presence of triethylamine (0.48 g, 0.48 mmol). After being stirred for 2 h under argon, the solution was washed with 5% citric acid and brine (30 ml × 3 each). After drying over $MgSO_4$, the solvent was removed *in vacuo* to give **21** as a yellow oil. **21** was used without further purification because **21** was easily oxidized in air. **21**: ^{1}H-NMR: 1.60 (9H, s), 3.12 (2H, dd, *J* = 9.0 Hz, *J* = 4.2 Hz), 4.40—4.87 (1H, m), 4.70 (2H, s), 5.83 (1H, br s).

***S*-{2,3-Dihydroxy-(2*R*)-propyl}-*N*-2,2,2-trichloroethoxycarbonylcysteine *tert*-Butyl Ester (*R*-22)** (*R*)-**15** (0.43 g, 2.1 mmol) was added to **21** (0.68 g, 1.9 mmol) in DMF (5 ml) in the presence of *N*,*N*-diisopropylethylamine (1.0 g, 7.7 mmol). After being stirred for 15 h at room temperature, CH_2Cl_2 (50 ml) was added to the reaction mixture and the mixture was washed with 1 N HCl (40 ml × 2) and brine (50 ml × 3). After drying over $MgSO_4$, the solvent was concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel with $CHCl_3$–MeOH (15 : 1) as an eluent to give (*R*)-**22** (0.49 g, 59%) as a yellow oil. (*R*)-**22**: FAB-MS *m*/*z*: 426 $(M+H)^+$. IR (neat): 3330 (OH, NH), 1739 (ester) cm^{-1}. ^{1}H-NMR: 1.47 (9H, s), 2.80—3.17 (6H, m), 3.60 (2H, br s), 4.27—4.57 (2H, m), 4.75 (2H, s), 6.00 (1H, d, *J* = 7.0 Hz).

***S*-{2,3-Bis(palmitoyloxy)-(2*R*)-propyl}-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteine *tert*-Butyl Ester (*R*-23)** Palmitoyl chloride (0.83 g, 3.0 mmol) in CH_2Cl_2 (5 ml) was added to a stirred solution of (*R*)-**22** (0.64 g, 15 mmol), 4-dimethylaminopyridine (46 mg, 0.38 mmol) and *N*,*N*-diisopropylethylamine (0.78 g, 6.0 mmol) in CH_2Cl_2 (30 ml) at 0 °C. After being stirred for 5 h at room temperature, CH_2Cl_2 (30 ml) was added to the reaction mixture. The CH_2Cl_2 solution was washed with 5% citric acid, 4% $NaHCO_3$ aq. (50 ml × 3 each) and brine (50 ml × 1), dried over $MgSO_4$ and concentrated *in vacuo*, the residue was precipitated as a solid by cooling at −20 °C from MeOH–$CHCl_3$ (3 : 1) to give (*R*)-**23** (1.0 g, 73%) as a white powder. (*R*)-**23**: mp 43 °C, $[\alpha]_D^{22}$ +2.1° (*c* = 1.04, $CHCl_3$). FAB-MS *m*/*z*: 902 $(M+H)^+$. IR (KBr): 3298 (NH), 1739 (ester) cm^{-1}. ^{1}H-NMR: 0.89 (6H, t, *J* = 5.8 Hz), 1.23 (28H, s), 1.49 (9H, s), 2.10—3.17 (6H, m), 4.10—4.33 (2H, m), 4.71 (2H, s), 5.93 (1H, br s).

***S*-{2,3-Bis(palmitoyloxy)-(2*R*)-propyl}-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteine (5)** CF_3COOH (2 ml) was added to (*R*)-**23** (0.41 g, 0.46 mmol) at room temperature. After being stirred for 1 h, the mixture was evaporated *in vacuo* and CH_2Cl_2 (50 ml) was added to the residue. After washing with water (30 ml × 3), the solution was dried over $MgSO_4$ and evaporated to dryness. The residue was precipitated to a solid by cooling at −20 °C from MeOH–$CHCl_3$ (3 : 1) to give **5** as a colorless powder. **5**: mp 31—33 °C, $[\alpha]_D^{22}$ +13.7° (*c* = 1.21, $CHCl_3$). FAB-MS *m*/*z*: 868 $(M+Na)^+$. IR (KBr): 3334 (OH, NH), 1740 (ester) cm^{-1}. ^{1}H-NMR: 0.93 (6H, t, *J* = 5.8 Hz), 1.33 (28H, s), 2.20—3.27 (6H, m), 3.76—4.33 (2H, m), 4.80 (2H, s), 6.03 (1H, d, *J* = 7.0 Hz). *Anal.* Calcd for $C_{41}H_{74}Cl_3NO_8S \cdot H_2O$: C, 56.90; H, 8.85; N, 1.62. Found: C, 57.42; H, 8.79; N, 1.67.

L-Alanine *tert*-Butyl Ester Hydrochloride (24) Z–Ala–OBu^t (3.8 g, 13 mmol) was hydrogenated over 5% Pd–C as a catalyst in EtOH (80 ml) for 3 h at room temperature. After removal of the catalyst, HCl (0.47 g, 13 mmol) in EtOH was added to the filtrate. The filtrate was evaporated *in vacuo* to give **24** (2.1 g, 87%) as a white powder.

***N*-Carbobenzoxy-L-asparaginyl-L-alanine *tert*-Butyl Ester (25)** Z–Asn–OH (1.1 g, 4.0 mmol), dicyclohexylcarbodiimide (0.91 g, 4.4 mmol) and 1-hydroxybenzotriazole (0.61 g, 4.0 mmol) were added under stirring to solution of **24** (0.72 g, 4.0 mmol) and *N*-methylmorphorine (0.41 g, 4.0 mmol) in dimethylformamide (3 ml). After being stirred for 15 h,

N,*N*-dicyclohexylurea (DCUrea) was filtered off and the filtrate was concentrated *in vacuo*. The residue was dissolved in AcOEt (10 ml) and DCUrea was filtered off again. After evaporation of the solvent, the residue was dissolved in CH_2Cl_2 (100 ml) and washed with 5% citric acid, 4% $NaHCO_3$ aq. (50 ml × 3, each) and brine (50 ml × 1). After drying over $MgSO_4$, the solvent was evaporated *in vacuo*. The residue was dissolved in a small amount of $CHCl_3$ and precipitated by the addition of *n*-hexane and dried *in vacuo* to give **25** (1.1 g, 68%) as a white powder. **25**: mp 144—147 °C, $[\alpha]_D^{22}$ +10.5° (*c* = 1.0, $CHCl_3$). *Anal.* Calcd for $C_{19}H_{27}N_3O_6$: C, 57.84; H, 7.16; N, 10.64. Found: C, 57.45; H, 7.07; N, 10.62.

L-Asparaginyl-L-alanine *tert*-Butyl Ester (26) **25** (0.20 g, 0.5 mmol) was hydrogenated over 5% Pd–C as a catalyst in EtOH (30 ml) for 3 h at room temperature. After removal of Pd–C, the filtrate was concentrated *in vacuo* to give **26** (0.13 g, 97%) as a white powder.

***N*-Carbobenzoxy-*O-tert*-butyl-L-seryl-L-asparaginyl-L-alanine *tert*-Butyl Ester (27)** HOBt (0.12 g, 0.77 mmol) and DCC (0.16 g, 0.77 mmol) were added to a solution of Z–Ser(Bu^t)–OH (0.23 g, 0.77 mmol) and **26** (0.20 g, 0.77 mmol) in DMF (3 ml). After being stirred for 15 h at room temperature, DCUrea was filtered off and the filtrate was concentrated *in vacuo*. The residue was dissolved in AcOEt (10 ml) and DCUrea was filtered off again. The filtrate was concentrated *in vacuo* and CH_2Cl_2 was added to the residue. The CH_2Cl_2 layer was washed with 4% $NaHCO_3$ aq. (50 ml × 3) and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was dissolved in a small amount of $CHCl_3$ and precipitated to a solid by the addition of *n*-hexane. The colorless product was filtrated, washed with *n*-hexane and dried *in vacuo* to give **27** (0.31 g, 76%) as a white powder. **27**: mp 139—142 °C, $[\alpha]_D^{22}$ +14.8° (*c* = 0.4, $CHCl_3$). *Anal.* Calcd for $C_{26}H_{40}N_4O_8 \cdot 1/2H_2O$: C, 57.21; H, 7.58; N, 10.27. Found: C, 57.56; H, 7.37; N, 10.14.

***O-tert*-Butyl-L-seryl-L-asparaginyl-L-alanine *tert*-Butyl Ester (28)** **27** (0.80 g, 1.5×10^{-3} mol) was hydrogenated over 5% Pd–C as a catalyst in EtOH (50 ml) for 3 h at room temperature. The mixture was treated by the same procedure described in the preparation of compound **26** to give **28** (0.13 g, 97%) as a white powder.

***N*-Carbobenzoxy-*O-tert*-butyl-L-seryl-*O-tert*-butyl-L-seryl-L-asparaginyl-L-alanine *tert*-Butyl Ester (29)** HOBt (0.50 g, 3.3×10^{-3} mol) and DCC (0.74 g, 3.6×10^{-3} mol) was added to a solution of Z–Ser(Bu^t)–OH (0.97 g, 3.3×10^{-3} mol) and **28** (1.3 g, 3.3×10^{-3} mol) in DMF (5 ml). After being stirred for 15 h at room temperature, the mixture was treated by the same procedure described in the preparation of compound **27** to give **29** (1.1 g, 47%) as a white powder. **29**: mp 127—131 °C, $[\alpha]_D^{22}$ +19.8° (*c* = 1.02, $CHCl_3$). FAB-MS *m*/*z*: 680 $(M+H)^+$. *Anal.* Calcd for $C_{33}H_{53}N_5O_{10}$: C, 58.30; H, 7.86; N, 10.30. Found: C, 58.45; H, 7.75; N, 10.32.

***O-tert*-Butyl-L-seryl-*O-tert*-butyl-L-seryl-L-asparaginyl-L-alanine *tert*-Butyl Ester (30)** **29** (0.78 g, 1.2×10^{-3} mol) was hydrogenated over 5% Pd–C as a catalyst in EtOH (50 ml) for 3 h at room temperature. The mixture was treated by the same procedure described in the compound **26** to give **30** (0.55 g, 88%) as a white powder.

***S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteinyl-*O-tert*-butyl-(*S*)-seryl-*O-tert*-butyl-(*S*)-seryl-(*S*)-asparaginyl-(*S*)-alanine *tert*-Butyl Ester (*R*-31)** HOBt (60 mg, 3.8×10^{-4} mol) and DCC (90 mg, 4.1×10^{-4} mol) were added to a solution of **5** (0.32 g, 3.8×10^{-3} mol) and **30** (0.21 g, 3.8×10^{-3} mol) in DMF (3 ml). After being stirred for 15 h, the mixture was treated by the same procedure described in the preparation of compound **27**. The residue was precipitated to a solid by cooling at −20 °C from $CHCl_3$–MeOH (1 : 3) to give (*R*)-**31** (0.25 g, 50%) as a white powder. (*R*)-**31**: mp 132—134 °C, $[\alpha]_D^{22}$ +3.8° (*c* = 1.0, $CHCl_3$). FAB-MS *m*/*z*: 1374 $(M+H)^+$. *Anal.* Calcd for $C_{66}H_{119}Cl_3N_6O_{15}S$: C, 57.65; H, 8.72; N, 6.11. Found: C, 57.63; H, 8.63; N, 5.92.

***S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-(*R*)-cysteinyl-*O-tert*-butyl-(*S*)-seryl-*O-tert*-butyl-(*S*)-seryl-(*S*)-asparaginyl-(*S*)-alanine *tert*-Butyl Ester (*R*-32)** Zinc powder (0.75 g) was added to a stirred solution of (*R*)-**31** (0.15 g, 1.1×10^{-4} mol) in CH_3COOH (2 ml). After being stirred for 15 h at room temperature, CH_2Cl_2 (50 ml) was added to the filtrate and zinc powder was filtered off. The filtrate was washed with sat. $NaHCO_3$ aq. (50 ml × 3) and brine. The CH_2Cl_2 layer was dried over $MgSO_4$ and concentrated *in vacuo* to give (*R*)-**32** (0.13 g, 96%), which was used without further purification. (*R*)-**32**: mp 118—121 °C, $[\alpha]_D^{22}$ −3.6° (*c* = 1.2, $CHCl_3$). FAB-MS *m*/*z*: 1200 $(M+H)^+$.

***S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-*N*-palmitoyl-(*R*)-cysteinyl-*O-tert*-butyl-L-seryl-*O-tert*-butyl-L-seryl-L-asparaginyl-L-alanine *tert*-Butyl Ester (*R*-33)** Palmitoyl chloride (14 mg, 5.0×10^{-5} mol) in CH_2Cl_2 (2 ml) was added to a stirred solution of (*R*)-**32** (60 mg, 5.0×10^{-5} mol), 4-dimethylaminopyridine (2.0 mg, 1.3×10^{-5} mol) and *N*,*N*-diisopropylethylamine (26 mg, 2.0×10^{-4} mol) in CH_2Cl_2 (10 ml) at 0 °C. After being stirred for 5 h at room temperature, the mixture was treated by the same procedure for the preparation of (*R*)-**23** to give (*R*)-**33** (54 mg, 76%) as a white powder. (*R*)-**33**: mp 187—189 °C, $[\alpha]_D^{22}$ −1.9° (*c* = 0.86, $CHCl_3$). FAB-MS *m*/*z*: 1438 $(M+H^+)$. *Anal.* Calcd for $C_{79}H_{148}SN_6O_{14}$: C, 65.98; H, 10.37; N, 5.84. Found: C, 65.60; H, 10.40; N, 5.44.

***S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-*N*-palmitoyl-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-seryl-(*S*)-asparaginyl-(*S*)-alanine (1)** CF_3COOH (2 ml) was added to **33** (70 mg, 4.9×10^{-3} mol). After being stirred for 1 h at room temperature, the mixture was concentrated *in vacuo* and the residue was precipitated to a solid by cooling at −20 °C from MeOH–$CHCl_3$ (3 : 1) to give **1** (33 mg, 53%) as a white powder. **1**: mp 211—213 °C, $[\alpha]_D^{22}$ +56.5° (*c* = 1.02, $CHCl_3$). FAB-MS *m*/*z*: 1270 $(M+H)^+$. IR (KBr): 3324 (OH, NH), 1732 (ester), 1627, 1537 (amide) cm^{-1}. *Anal.* Calcd for $C_{67}H_{124}N_6O_{14} \cdot 2H_2O$: C, 61.57; H, 9.87; N, 6.43. Found: C, 61.83; H, 9.60; N, 6.05.

***S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-seryl-(*S*)-asparaginyl-(*S*)-alanine (2)** CF_3COOH (2 ml) was added to (*R*)-**31** (90 mg, 6.6×10^{-5} mol). After being stirred for 1 h at room temperature, the mixture was concentrated *in vacuo* and the residue was precipitated to a solid by cooling at −20 °C from MeOH–$CHCl_3$ (3 : 1) to give **2** (32 mg, 45%) as a white powder. **2**: mp 205—207 °C, $[\alpha]_D^{22}$ +9.20° (*c* = 1.00, $CHCl_3$). FAB-MS *m*/*z*: 1205 $(M+H)^+$. IR (KBr): 3300 (OH, NH), 1736 (ester), 1662, 1537 (amide) cm^{-1}. *Anal.* Calcd for $C_{54}H_{95}Cl_3N_6O_{15}$: C, 53.75; H, 7.93; N, 6.96. Found: C, 53.56; H, 8.17; N, 6.54.

***S*-{2,3-Dihydroxy-(2*S*)-propyl}-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteine *tert*-Butyl Ester (*S*-22)** (*S*)-**15** (0.98 g, 4.8×10^{-3} mol) was added to **21** (1.6 g, 4.4×10^{-3} mol) in DMF (5 ml) in the presence of *N*,*N*-diisopropylethylamine (2.3 g, 1.8×10^{-2} mol). After being stirred for 15 h at room temperature, CH_2Cl_2 (50 ml) was added to the reaction mixture and the mixture was washed with 1 N HCl (40 ml × 2) and brine (50 ml × 3). After drying over $MgSO_4$, the solvent was concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel with $CHCl_3$–MeOH (15 : 1) as an eluent to give (*S*)-**22** (1.2 g, 62%) as an yellow oil. (*S*)-**22**: FAB-MS *m*/*z*: 426 $(M+H)^+$. IR (neat): 3330 (OH, NH), 1739 (ester) cm^{-1}.

***S*-{2,3-Bis(palmitoyloxy)-(2*S*)-propyl}-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteine *tert*-Butyl Ester (*S*-23)** Palmitoyl chloride (0.46 g, 1.7×10^{-3} mol) in CH_2Cl_2 (5 ml) was added to a stirred solution of (*S*)-**22** (0.34 g, 8.0×10^{-4} mmol), 4-dimethylaminopyridine (25 mg, 2.0×10^{-4} mmol) and *N*,*N*-diisopropylethylamine (0.41 g, 3.2×10^{-3} mol) in CH_2Cl_2 (30 ml) at 0 °C. After being stirred for 5 h at room temperature, CH_2Cl_2 (30 ml) was added to a reaction mixture. The CH_2Cl_2 solution was washed with 5% citric acid, 4% $NaHCO_3$ aq. (50 ml × 3 each) and brine (50 ml × 1), dried over $MgSO_4$ and concentrated *in vacuo*. The residue was precipitated to a solid by cooling at −20 °C from MeOH–$CHCl_3$ (3 : 1) to give (*S*)-**23** (0.48 g, 70%) as a white powder. (*S*)-**23**: mp 45—46 °C, $[\alpha]_D^{22}$ −2.1° (*c* = 1.04, $CHCl_3$). FAB-MS *m*/*z*: 902 $(M+H)^+$. IR (KBr): 3366 (NH), 1732 (ester) cm^{-1}.

***S*-{2,3-Bis(palmitoyloxy)-(2*S*)-propyl}-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteine (*S*-5)** CF_3COOH (2 ml) was added to (*S*)-**23** (0.15 g, 1.7×10^{-4} mol). After being stirred for 1 h at room temperature, the mixture was concentrated *in vacuo* and added to CH_2Cl_2 (50 ml). After washing with water (30 ml × 3), the solution was dried over $MgSO_4$ and evaporated to dryness. The residue was precipitated to a solid by cooling at −20 °C from MeOH–$CHCl_3$ (3 : 1) to give (*S*)-**5** (0.11 g, 80%) as a colorless powder. (*S*)-**5**: mp 31—33 °C, $[\alpha]_D^{22}$ +9.7° (*c* = 1.42, $CHCl_3$). FAB-MS *m*/*z*: 868 $(M+Na)^+$. IR (KBr): 3298 (OH, NH), 1739 (ester) cm^{-1}.

***S*-[2,3-Bis(palmitoyloxy)-(2*S*)-propyl]-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteinyl-*O-tert*-butyl-(*S*)-seryl-*O-tert*-butyl-(*S*)-seryl-(*S*)-asparaginyl-(*S*)-alanine *tert*-Butyl Ester (*S*-31)** HOBt (27 mg, 1.7×10^{-4} mol) and DCC (40 mg, 2.0×10^{-4} mol) were added to the solution of **5** (0.14 g, 1.7×10^{-4} mol) and **30** (95 mg, 1.7×10^{-4} mol) in DMF (3 ml). After being stirred for 15 h, the mixture was treated by the same procedure described in the preparation of compound **27**. The residue was precipitated to a solid by cooling at −20 °C from $CHCl_3$–MeOH (1 : 3) to give (*S*)-**31** (0.11 g, 46%) as white powder. (*S*)-**31**: mp 146—148 °C, $[\alpha]_D^{22}$ −6.6° (*c* = 0.80, $CHCl_3$). FAB-MS *m*/*z*: 1374 $(M+H)^+$. IR (KBr): 3288 (NH), 1739 (ester), 1639, 1541 (amide) cm^{-1}.

***S*-[2,3-Bis(palmitoyloxy)-(2*S*)-propyl]-(*R*)-cysteinyl-*O-tert*-butyl-(*S*)-seryl-*O-tert*-butyl-(*S*)-seryl-(*S*)-asparaginyl-(*S*)-alanine *tert*-Butyl Ester (*S*-32)** Zinc powder (0.75 g) was added to a stirred solution of (*S*)-**31** (0.15 g, 1.1×10^{-4} mol) in CH_3COOH (2 ml). After being stirred for 15 h,

CH_2Cl_2 (50 ml) was added to the filtrate and zinc powder was filtered off. The filtrate was washed with sat. $NaHCO_3$ aq. (50 ml × 3) and brine. The CH_2Cl_2 layer was dried over $MgSO_4$ and concentrated *in vacuo* to give (*S*)-**32** (0.13 g, 96%), which was used without further purification. (*S*)-**32**: mp 122—124 °C, $[\alpha]_D^{22}$ +3.4° (*c* = 1.2, $CHCl_3$). FAB-MS *m/z*: 1200 $(M+H)^+$. IR (KBr): 3296 (NH_2), 1740 (ester), 1642, 1537 (amide) cm^{-1}.

***S*-[2,3-Bis(palmitoyloxy)-(2*S*)-propyl]-*N*-palmitoyl-(*R*)-cysteinyl-*O*-*tert*-butyl-(*S*)-seryl-*O*-*tert*-butyl-(*S*)-seryl-L-asparaginyl-(*S*)-alanine *tert*-Butyl Ester (*S*-33)** Palmitoyl chloride (28 mg, 1.0×10^{-4} mol) in CH_2Cl_2 (2 ml) was added to a stirred solution of (*S*)-**32** (0.12 g, 1.0×10^{-4} mol), 4-dimethylaminopyridine (3.0 mg, 2.5×10^{-5} mol) and *N*,*N*-diisopropylethylamine (26 mg, 4.1×10^{-4} mol) in CH_2Cl_2 (10 ml) at 0 °C. After being stirred for 5 h at room temperature, the mixture was treated by the same preparation procedure as (*R*)-**23** to give (*S*)-**33** (0.10 g, 75%) as a white powder. (*S*)-**33**: mp 194 °C, $[\alpha]_D^{22}$ +4.3° (*c* = 1.02, $CHCl_3$). FAB-MS *m/z*: 1438 $(M+H)^+$. IR (KBr): 3295 (NH), 1728 (ester), 1628, 1538 (amide) cm^{-1}.

***S*-[2,3-Bis(palmitoyloxy)-(2*S*)-propyl]-*N*-palmitoyl-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-seryl-(*S*)-asparaginyl-(*S*)-alanine (3)** CF_3COOH (2 ml) was added to (*S*)-**33** (84 mg, 5.9×10^{-5} mol). After being stirred for 1 h at room temperature, the mixture was evaporated *in vacuo* and the residue was precipitated to a solid by cooling at −20 °C from MeOH–$CHCl_3$ (3 : 1) to give **3** (40 mg, 53%) as a white powder. **3**: mp 210—212 °C, $[\alpha]_D^{22}$ −28.3° (*c* = 0.86, $CHCl_3$). FAB-MS *m/z*: 1270 $(M+H)^+$. IR (KBr): 3296 (OH, NH), 1736 (ester), 1639, 1538 (amide) cm^{-1}. *Anal.* Calcd for $C_{67}H_{124}N_6O_{14} \cdot 3H_2O$: C, 60.79; H, 9.90; N, 6.35. Found: C, 61.00; H, 9.88; N, 6.14.

***S*-[2,3-Bis(palmitoyloxy)-(2*S*)-propyl]-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-seryl-(*S*)-asparaginyl-(*S*)-alanine (4)** CF_3COOH (2 ml) was added to (*S*)-**31** (75 mg, 5.5×10^{-5} mol). After being stirred for 1 h, the mixture was evaporated *in vacuo* and the residue was precipitated to a solid by cooling at −20 °C from MeOH–$CHCl_3$ (3 : 1) to give **4** (27 mg, 45%) as a white powder. **4**: mp 204—207 °C, $[\alpha]_D^{22}$ +16.6° (*c* = 1.00, $CHCl_3$). FAB-MS *m/z*: 1205 $(M+H)^+$. IR (KBr): 3302 (OH, NH), 1737 (ester), 1629, 1538 (amide) cm^{-1}. *Anal.* Calcd for $C_{54}H_{95}Cl_3N_6O_{15}$: C, 53.75; H, 7.93; N, 6.96. Found: C, 53.56; H, 8.34; N, 6.47.

***S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteinyl-*O*-*tert*-butyl-(*S*)-serine *tert*-Butyl Ester (37)** **5** (0.13 g, 1.6×10^{-4} mol), DCC (36 mg, 1.8×10^{-4} mol), and HOBt (24 mg, 1.6×10^{-4} mol) were added under stirring to a solution of **34** (40 mg, 1.6×10^{-4} mol) and *N*-methylmorphorine (16 mg, 1.6×10^{-4} mol) in DMF (3 ml). After being stirred for 15 h at room temperature, the mixture was treated by the same procedure described in the preparation of compound **26**, and chromatographed on silica gel with *n*-hexane–AcOEt (7 : 1) as an eluent to give **37** (95 mg, 57%) as a colorless oil. **37**: $[\alpha]_D^{22}$ +9.7° (*c* = 1.9, $CHCl_3$). FAB-MS *m/z*: 1046 $(M+H)^+$: IR (neat): 3300 (NH), 1738 (ester), 1658, 1528 (amide) cm^{-1}.

***S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteinyl-*O*-*tert*-butyl-(*S*)-seryl-*O*-*tert*-butyl-(*S*)-serine *tert*-Butyl Ester (38)** HOBt (80 mg, 5.2×10^{-4} mol) and DCC (36 mg, 1.8×10^{-4} mol) was added to the solution of **5** (0.20 g, 2.4×10^{-4} mol) and **35** (85 mg, 2.4×10^{-4} mol) in DMF (2 ml). After being stirred for 15 h at room temperature, the mixture was treated by the same procedure described in the preparation of compound **27**, and subjected to column chromatography on silica gel with *n*-hexane–AcOEt (7 : 1) as an eluent to give **38** (0.19 g, 67%) as a colorless oil. **38**: $[\alpha]_D^{22}$ +15.3° (*c* = 2.1, $CHCl_3$). FAB-MS *m/z*: 1189 $(M+H)^+$. IR (neat): 3280 (NH), 1737 (ester), 1638, 1524 (amide) cm^{-1}.

***S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteinyl-*O*-*tert*-butyl-(*S*)-seryl-*O*-*tert*-butyl-(*S*)-seryl-(*S*)-asparagine *tert*-Butyl Ester (39)** HOBt (25 mg, 1.6×10^{-4} mol) and DCC (40 mg, 2.0×10^{-4} mol) were added to a solution of **5** (0.15 g, 1.6×10^{-4} mol) and **36** (78 mg, 1.6×10^{-4} mol) in DMF (2 ml). After being stirred for 15 h at room temperature, the mixture was treated by the same procedure described in the preparation of the compound **27** to give **39** as a white powder. **39**: mp 129—131 °C, $[\alpha]_D^{22}$ +12.7° (*c* = 1.25, $CHCl_3$). FAB-MS *m/z*: 1305 $(M+H)^+$. IR (KBr): 3288 (NH), 1742 (ester), 1641, 1539 (amide) cm^{-1}.

***S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteinyl-(*S*)-serine (6)** CF_3COOH (2 ml) was added to **34** (0.13 g, 1.2×10^{-4} mol). After being stirred for 1 h at room temperature, the mixture was treated by the same procedure described in the preparation of compound **1** to give **6** (0.10 g, 86%) as a white powder. **6**: mp 50—52 °C, $[\alpha]_D^{22}$ +6.4° (*c* = 0.52, $CHCl_3$). FAB-MS *m/z*: 933 $(M+H)^+$. IR (neat): 3324 (OH, NH), 1741 (ester), 1666, 1537 (CONH). *Anal.* Calcd for $C_{44}H_{79}Cl_3N_2O_{10}S \cdot H_2O$: C, 55.46; H, 8.57; N, 2.94. Found: C, 56.05; H, 8.46; N, 2.94.

***S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-serine (7)** CF_3COOH (2 ml) was added to **35** (0.19 g, 1.6×10^{-4} mol). After being stirred for 1 h at room temperature, the mixture was treated by the same procedure described in the preparation of the compound **1** to give **7** (0.10 g, 86%) as a white powder. **7**: mp 102—105 °C; $[\alpha]_D^{22}$ +3.99° (*c* = 1.04, $CHCl_3$). FAB-MS *m/z*: 1020 $(M+H)^+$. IR (neat): 3314 (OH, NH), 1741 (ester), 1633, 1537 (CONH) cm^{-1}. *Anal.* Calcd for $C_{47}H_{84}Cl_3N_3O_{12}S \cdot H_2O$: C, 54.30; H, 8.34; N, 4.04. Found: C, 54.65; H, 8.20; N, 3.94.

***S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-*N*-2,2,2-trichloroethoxycarbonyl-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-serine-(*S*)-asparagine (8)** CF_3COOH (2 ml) was added to **36** (60 mg, 5.0×10^{-5} mol). After being stirred for 1 h at room temperature, the mixture was treated by the same procedure described in the preparation of compound **1** to give **8** (0.10 g, 86%) as a white powder. **8**: mp 183—185 °C, $[\alpha]_D^{22}$ −9.46° (*c* = 1.13, $CHCl_3$: MeOH = 1 : 1). FAB-MS *m/z*: 1135 $(M+H)^+$. IR (neat): 3302 (OH, NH), 1731 (ester), 1632, 1537 (CONH) cm^{-1}. *Anal.* Calcd for $C_{51}H_{90}Cl_3N_5O_{14}S$: C, 53.94; H, 7.99; N, 6.16. Found: C, 53.90; H, 8.00; N, 5.74.

***S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-(*R*)-cysteinyl-*O*-*tert*-butyl-(*S*)-seryl-*O*-*tert*-butyl-(*S*)-seryl-(*S*)-asparagine *tert*-Butyl Ester (40)** Zinc powder (0.65 g) was added to a stirred solution of **36** (0.13 g, 1.0×10^{-4} mol) in CH_3COOH (2 ml). After being stirred for 15 h at room temperature, CH_2Cl_2 (50 ml) was added to the mixture and zinc powder was filtered off. The filtrate was washed with sat. $NaHCO_3$ aq. (50 ml × 3) and brine. The CH_2Cl_2 layer was dried over $MgSO_4$ and concentrated *in vacuo* to give **37** (94 mg, 84%) as a white powder, which was used without further purification. **37**: mp 91—93 °C, $[\alpha]_D^{22}$ +3.3° (*c* = 1.84, $CHCl_3$). FAB-MS *m/z*: 1129 $(M+H)^+$. IR (KBr): 3292 (OH, NH), 1741 (ester), 1641, 1542 (amide) cm^{-1}.

***S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-seryl-(*S*)-asparagine (9)** CF_3COOH (2 ml) was added to **40**. After being stirred for 1 h at room temperature, the mixture was treated by the same procedure described in the preparation of compound **1** to give **9** (45 mg, 58%) as a white powder. **9**: mp 142—145 °C, $[\alpha]_D^{22}$ +6.4° (*c* = 0.38, $CHCl_3$). FAB-MS *m/z*: 961 $(M+H)^+$. IR (neat): 3322 (OH, NH), 1738 (ester), 1663, 1541 (amide) cm^{-1}.

***S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-seryl-(*S*)-asparaginyl-alanine (10)** CF_3COOH (2 ml) was added to **32** (75 mg, 6.3×10^{-5} mol). After being stirred for 1 h at room temperature, the mixture was treated by the same procedure described in the preparation of compound **1** to give **10** (45 mg, 58%) as a white powder. **10**: mp 198—200 °C, $[\alpha]_D^{22}$ +31.6° (*c* = 0.22, $CHCl_3$). FAB-MS *m/z*: 1032 $(M+H)^+$. IR (neat): 3296 (OH, NH), 1738 (ester), 1666, 1537 (amide) cm^{-1}.

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Central Depressant Effects of N^3-Substituted 6-Azauridines in Mice

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Central depressant effects in mice of N^3-substituted 6-azauridines (6-AzUd) (1) were examined by intracerebroventricular (i.c.v.) injection. Eleven derivatives including alkyl-, benzyl-, xylyl- and phenylethyl-substitution onto the N^3-position of 1 were synthesized and their pharmacological effects were evaluated using hypnotic activity, locomotor activity, motor incoordination and pentobarbital-induced sleep prolongation as indices. Six of 12 compounds showed the hypnotic activity. At a dose of 2 μmol/mouse, the mean sleeping time induced by 1, N^3-benzyl-6-AzUd (7), N^3-*o*-xylyl-6-AzUd (8), N^3-*m*-xylyl-6-AzUd (9), N^3-*p*-xylyl-6-AzUd (10) and N^3-α-phenylethyl-6-AzUd (11) was 14, 11, 45, 12, 9 and 16 min, respectively. These derivatives and N^3-β-phenylethyl-6-AzUd (12) (1.5 μmol/mouse) significantly prolonged pentobarbital-induced (40 mg/kg, i.p.) sleeping time, whereas none of the N^3-alkylated derivatives (methyl-, ethyl-, *n*-propyl-, *n*-butyl- and allyl-substitution) exerted the hypnotic activity or pentobarbital-induced sleep prolongation. Nucleoside 1 and its xylyl-derivatives (1.5 μmol/mouse) significantly decreased locomotor activity of mice, their effects paralleled the hypnotic activity. These compounds (1.5 μmol/mouse) also produced motor incoordination and potentiated the effect of diazepam-induced motor incoordination. These results indicate that 1 and its benzyl-related derivatives, but not alkyl-derivatives have a depressant effect on the central nervous system.

Keywords 6-azauridine; alkyl-derivative; benzyl-derivative; hypnotic activity; locomotor activity; motor incoordination; sleep prolongation; N^3-substituted nucleoside; xylyl-derivative

Uridine has been identified as one of the sleep-promoting substances isolated from the brainstem of sleep-deprived rats.[1] In connection with this finding, our previous studies have shown that N^3-substituted derivatives of uridine and thymidine exert central depressant effects involving hypnotic activity.[2-4] These studies suggested the importance of a sugar moiety as well as the substitution of benzyl-related groups on the N^3-position of uracil or thymine to exhibit the hypnotic activity. 6-Azauridine (6-AzUd) (**1**), a pyrimidine nucleoside, was reported to have antitumor activity and cytotoxicity.[5,6] Welch *et al.*[7] reported that 6-azauracil, a pyrimidine base, showed hypnotic activity at a relatively higher dose of 2.0 to 3.0 g/kg i.p. In connection with our recent study on the central depressant effects of N^3-substituted nucleosides, the present paper describes pharmacological effects of 6-AzUd derivatives in mice.

Experimental

Animals Male ddN mice weighing 22 to 28 g were used throughout the experiments. Mice were kept in an air-conditioned room (24±2 °C) with controlled lighting (8:00 to 20:00 light period). They were given food and water *ad libitum*.

Chemicals Sodium pentobarbital and halogenated alkyls were purchased from Tokyo Kasei Kogyo Co., Ltd.; **1** from Aldrich Chemical Co.; diazepam from Yamanouchi Pharmaceuticals, Ltd.

Syntheses of N^3-Substituted 1 N^3-Substituted 6-AzUds were prepared by the methods described previously.[3,8,9] Briefly, **1** dissolved in dimethyl sulfoxide (DMSO) and acetone was reacted with halogenated alkyls in the presence of a base (NaOH or Na_2CO_3). The product was purified by column chromatography with a solvent system of chloroform–ethyl acetate–methanol (5:5:1).

Analytical data of the derivatives prepared were as follows:

N^3-Methyl-6-AzUd (**2**): Yield 52%, mp 127—128 °C. ^{1}H-NMR (DMSO-d_6) δ: 2.50 (3H, s, N–CH_3), 3.26—4.37 (2H, m, 5′-CH_2), 4.52—5.37 (3H, m, 2′-, 3′- and 4′-H), 5.98 (1H, d, J=2 Hz, 1′-H), 7.72 (1H, s, 5-H). MS *m/z*: 260 (M^++1)

N^3-Ethyl-6-AzUd (**3**): Yield 63%, oil. ^{1}H-NMR ($CDCl_3$) δ: 1.23 (3H, t, CH_3), 3.47—4.80 (7H, s and m, 2′-, 3′- and 4′-H, 5′-CH_2, N–CH_2), 6.20 (1H, d, J=2 Hz, 1′-H), 7.62 (1H, s, 5-H). MS *m/z*: 274 (M^++1).

N^3-*n*-Propyl-6-AzUd (**4**): Yield 64%, oil. ^{1}H-NMR ($CDCl_3$) δ: 0.95 (3H, t, CH_3), 1.26—2.04 (2H, m, –CH_2–), 3.20—4.73 (7H, s and m, 2′-, 3′-, and 4′-H, 5′-CH_2, N–CH_2), 6.20 (1H, d, J=2 Hz, 1′-H), 7.62 (1H, s, 5-H). MS *m/z*: 288 (M^++1).

N^3-*n*-Butyl-6-AzUd (**5**): Yield 84%, oil. ^{1}H-NMR ($CDCl_3$) δ: 1.05 (3H, t, CH_3), 1.13—1.83 (4H, m, –CH_2–CH_2–), 3.63—4.67 (7H, s and m, 2′-, 3′- and 4′-H, 5′-CH_2, N–CH_2), 6.18 (1H, d, J=2 Hz, 1′-H), 7.53 (1H, s, 5-H). MS *m/z*: 302 (M^++1).

N^3-Allyl-6-AzUd (**6**): Yield 23%, oil. ^{1}H-NMR ($CDCl_3$) δ: 3.48—4.67 (7H, s and m, 2′-, 3′- and 4′-H, 5′-CH_2, N–CH_2), 5.07—5.52 (2H, d, J=4 Hz, CH_2=), 5.57—5.88 (1H, m, =CH–), 6.07—6.25 (1H, d, J=2 Hz, 1′-H), 7.57 (1H, s, 5-H). MS *m/z*: 286 (M^++1).

N^3-Benzyl-6-AzUd (**7**): Yield 73%, mp 125—127 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.97—5.00 (5H, 2′-, 3′- and 4′-H, 5′-CH_2), 5.53 (2H, s, N–CH_2), 6.78 (1H, d, J=2 Hz, 1′-H), 7.93—8.40 (6H, m, 5-H and aryl-H). MS *m/z*: 335 (M^+).

N^3-*o*-Xylyl-6-AzUd (**8**): Yield 90%, mp 120—122 °C. ^{1}H-NMR (DMSO-d_6) δ: 2.40 (3H, s, CH_3), 3.70—5.45 (7H, s and m, 2′-, 3′- and 4′-H, 5′-CH_2, N–CH_2), 6. 03 (1H, d, J=2 Hz, 1′-H), 7.02—7.48 (4H, m, aryl-H), 7.85 (1H, s, 5-H). MS *m/z*: 349 (M^+).

N^3-*m*-Xylyl-6-AzUd (**9**): Yield 98%, mp 49—51 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.30 (3H, s, CH_3), 3.15—4.53 (5H, m, 2′-, 3′- and 4′-H, 5′-CH_2), 4.97 (2H, s, N–CH_2), 6.12 (1H, d, J=2 Hz, 1′-H), 6.97—7.35 (4H, m, aryl-H), 7.50 (1H, s, 5-H). MS *m/z*: 349 (M^+).

N^3-*p*-Xylyl-6-AzUd (**10**): Yield 93%, oil. ^{1}H-NMR ($CDCl_3$) δ: 2.28 (3H, s, CH_3), 3.24—4.53 (5H, m, 2′-, 3′- and 4′-H, 5′-CH_2), 5.64 (2H, s, N–CH_2), 6.11 (1H, d, J=2 Hz, 1′-H), 7.04—7.38 (4H, q, aryl-H), 7.47 (1H, s, 5-H). MS *m/z*: 349 (M^+).

N^3-α-Phenylethyl-6-AzUd (**11**): Yield 37%, mp 55—57 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.78 (3H, d, J=5 Hz, CH_3), 3.21—4.47 (7H, s and m, 2′-, 3′- and 4′-H, 5′-CH_2, N–CH_2), 6.10 (1H, d, J=2 Hz, 1′-H), 7.19—7.59 (6H, m, 5-H and aryl-H). MS *m/z*: 349 (M^+).

N^3-β-Phenylethyl-6-AzUd (**12**): Yield 22%, mp 137—139 °C. ^{1}H-NMR (DMSO-d_6) δ: 1.23—1.95 (2H, m, aryl-CH_2), 3.85—5.50 (7H, s and m, 2′-, 3′- and 4′-H, 5′-CH_2, N–CH_2), 6.08 (1H, d, J=2 Hz, 1′-H), 7.45 (5H, s, aryl-H), 7.77 (1H, s, 5-H). MS *m/z*: 349 (M^+).

Drug Administration N^3-Substituted 6-AzUds were suspended in saline containing 3% Tween 80 and injected intracerebroventricularly (i.c.v.).[10] Sodium pentobarbital (40 mg/kg) dissolved in saline and diazepam (5 mg/kg) suspended in the same vehicle as the test compounds were administered intraperitoneally (i.p.).

Pharmacological Experiments Sleeping time was measured as the period between loss and recovery of the righting reflex. Prolongation effects of N^3-substituted 6-AzUds on pentobarbital-induced sleep were assessed by the injection of sodium pentobarbital (40 mg/kg, i.p.) 15 min after the administration of test compounds. Locomotor activity of mice was recorded on a Digiscan activity monitor coupled with a Digiscan computer (Omnitec Electronics Inc.) as described previously.[11] Motor incoordination was assessed by the bar test described previously.[12] In addition, the interaction of **1** and its xylyl derivatives with diazepam (5 mg/kg, i.p.) was also carried out in this index. Statistical significance of difference was calculated by one way of variance, individual data were compared by

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the F-test. The data for motor incoordination were analyzed by the χ^2-test.

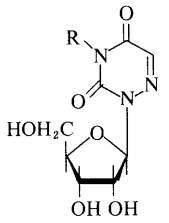
Results and Discussion

Effects of **1** and its derivatives on pentobarbital-induced sleep are described in Table I. As shown, none of the N^3-alkylated derivatives examined (1.5 μmol/mouse, i.c.v.) significantly prolonged pentobarbital-induced sleeping time, while nucleoside **1** and its benzyl-related derivatives (benzyl-, xylyl- and phenylethyl-derivatives) significantly prolonged the sleeping time at the same dose. The prolongation effect was in the following order in potency: **11** (% of control, 248), **8** (225), **12** (219), **7** (188), **9** (181), **1** (169) and **10** (165). These results showed the same tendency as the previous finding in N^3-substituted derivatives of uridine,[3] the only difference being that **1** itself showed a significant prolongation on pentobarbital-induced sleep while uridine exerted none. The result indicates that **1** has some depressant effect on the central nervous system. Table I also summarizes the hypnotic activity of **1** and its derivatives. Six of the 12 compounds examined exhibited the hypnotic activity; among them, that of **8** was the highest. Nucleoside **1** and other derivatives, **7**, **9**, **10** and **11** showed almost the same potency. At a dose of 2.0 μmol/mouse, the mean sleeping time was 9 to 16 min. This is the first case nucleoside itself being reported a hypnotic. Welch *et al.*[7] reported that 6-azauracil showed hypnotic activity in mice and rats, while **1** did not produce any hypnosis in mice. The activity of **8** is comparable to that of N^3-benzyluridine.[2]

Table II summarizes the effects of **1** and its xylyl-derivatives on locomotor activity of mice. At a dose of 1.5 μmol/mouse, **1**, **8**, **9** and **10** reduced activity by 40, 3, 19 and 34%, respectively. The result demonstrates that **1** could decrease the locomotor activity of mice, and that xylyl-derivatives have a stronger effect than the parent nucleoside, as has been reported for thymidine.[4]

Motor incoordination induced by **1** and its benzyl and xylxy derivatives is shown in Fig. 1. Nucleoside **1** induced motor incoordination for 40 min after administration (1.5 μmol/mouse). The effect of **9** and **10** was almost the same as that of **1**. Compound **8**, which exhibited the highest activity in hypnosis and pentobarbital-induced sleep

TABLE I. Central Depressant Effects of 6-AzUd and Its Derivatives



R		Sleeping time (min)			Pentobarbital-induced sleep prolongation[a]
		1.5[b]	2.0[b]	3.0[b]	1.5[b]
H	(**1**)	6±2	14±5	—	169±21[c]
CH_3	(**2**)	None	None	None	83± 4
C_2H_5	(**3**)	None	None	None	111± 5
n-C_3H_7	(**4**)	None	None	None	96±12
n-C_4H_9	(**5**)	None	None	None	108± 8
$CH_2CH=CH_2$	(**6**)	None	None	None	120± 9
$CH_2C_6H_5$	(**7**)	9±3	11±2	14± 3	188±21[d]
o-$CH_2C_6H_4CH_3$	(**8**)	25±3	45±9	144±12	225±27[d]
m-$CH_2C_6H_4CH_3$	(**9**)	None	12±1	17± 3	181± 8[d]
p-$CH_2C_6H_4CH_3$	(**10**)	2±1	9±1	12± 2	165± 8[d]
$CH(CH_3)C_6H_5$	(**11**)	None	16±4	23± 8	248±31[d]
$CH_2CH_2C_6H_5$	(**12**)	None	None	None	219±23[d]

a) % of control. The mean sleeping time of control mice was 30±2 min. *b*) Dose (μmol/mouse, i.c.v.). Data are expressed as the mean±S.E. from 6 to 10 mice. *c*) and *d*) indicate significant difference from the control value with $p<0.05$ and $p<0.01$, respectively.

TABLE II. Effects of 6-AzUd and Its Xylyl Derivatives on Locomotor Activity

Compounds	Dose (μmol/mouse)	Total distance (inches)	% of control
Control	3%Tween 80–saline	1634±245	100
1	1.5	651±260[a]	40
8	1.5	44± 23[b]	3
9	1.5	306±144[b]	19
10	1.5	556±300[a]	34

Data are expressed as the mean±S.E. of 7 to 9 mice. Compounds tested were injected i.c.v. *a*) and *b*) indicate significant difference from the control value with $p<0.05$ and $p<0.01$, respectively.

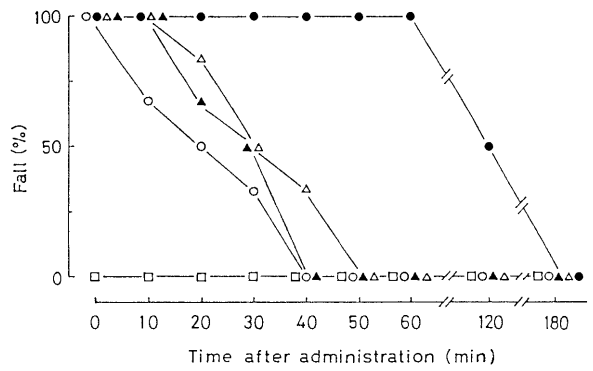


Fig. 1. Motor Incoordination Induced by 6-AzUd and Its Xylyl-Derivatives

Each point indicates % of mice ($N=6$) that fell from the bar within 30 s. Each compound tested was injected at a dose of 1.5 μmol/mouse i.c.v. □—□, control (3% Tween 80–saline); ○—○, 6-AzUd; ●—●, N^3-o-xylyl-6-AzUd; △—△, N^3-m-xylyl-6-AzUd; ▲—▲, N^3-p-xylyl-6-AzUd.

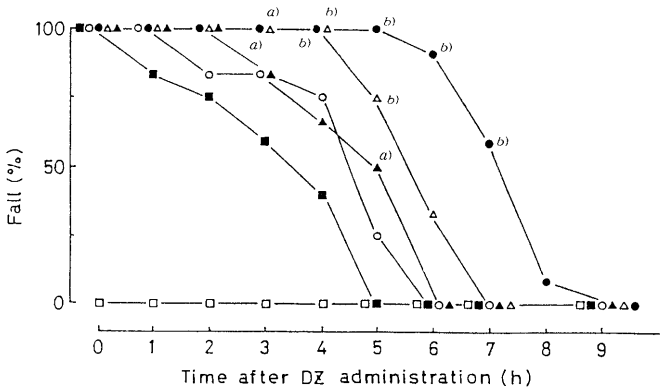


Fig. 2. Effects of 6-AzUd and Its Xylyl-Derivatives on Diazepam-Induced Motor Incoordination

Each point indicates % of mice ($N=12$) that fell from the bar within 30 s. Each compound tested was injected at a dose of 1.5 μmol/mouse i.c.v. Diazepam (5 mg/kg, i.p.) was administered 15 min after the injection of the test compounds. □—□, control (3% Tween 80-saline); ■—■, diazepam; ○—○, 6-AzUd + diazepam; ●—●, N^3-o-xylyl-6-AzUd + diazepam; △—△, N^3-m-xylyl-6-AzUd + diazepam; ▲—▲, N^3-p-xylyl-6-AzUd + diazepam. *a*) Significantly different from diazepam group ($p<0.05$). *b*) Significantly different from diazepam group ($p<0.01$).

prolongation, also showed the strongest effect in that it took 180 min to recover from motor incoordination. The effects of these compounds were additive to that of diazepam as shown in Fig. 2. The potentiating effect of the compounds tested on diazepam-induced motor incoordination was in the following order: **8**, **9**, **10** and **1**.

We have shown that N^3-benzyluridine inhibited the binding of a benzodiazepine agonist, flunitrazepam to the benzodiazepine receptor.[13] The potentiation by N^3-substituted **1** on the effect of diazepam suggests that these derivatives may exert their effects through the benzodiazepine receptor.

In conclusion, the present study strongly supports our previous findings[2-4] that N^3-substituted nucleosides possess hypnotic and sedative activities, and that the introduction of benzyl-related groups onto the N^3-position is an important factor in exhibiting the central depressant effects of nucleoside derivatives.

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Four New Phenolic Glycosides from *Polygala tenuifolia*

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Four new phenolic glycosides, tenuifolisides A (1), B (2), C (3), and D (4) together with a known phenolic glycoside, β-D-(3-*O*-sinapoyl)-fructofuranosyl-α-D-(6-*O*-sinapoyl)-glucopyranoside (5) were isolated from the roots of *Polygala tenuifolia*. The structures of these new compounds were characterized as β-D-[3-*O*-(3,4,5-trimethoxycinnamoyl)]-fructofuranosyl-α-D-[6-*O*-(*p*-hydroxybenzoyl)]-glucopyranoside (1), β-D-(3-*O*-sinapoyl)-fructofuranosyl-α-D-[6-*O*-(*p*-hydroxybenzoyl)]-glucopyranoside (2), β-D-[3-*O*-(3,4,5-trimethoxycinnamoyl)]-fructofuranosyl-α-D-(6-*O*-sinapoyl)-glucopyranoside (3), and 1,5-anhydro-[6-*O*-(3,4,5-trimethoxycinnamoyl)]-D-glucitol (4), respectively, on the basis of chemical and spectral evidence including two dimensional nuclear magnetic resonance (2D-NMR) studies.

Keywords *Polygala tenuifolia*; Polygalaceae; phenolic glycoside; tenuifoliside A; tenuifoliside B; tenuifoliside C; tenuifoliside D

The roots of *Polygala tenuifolia* WILLD. (Polygalaceae) are used as an expectorant, tonic and sedative agent under the names Onji in Japan and Yuan zhi in China. Various xanthones[1-4] and saponins[5,6] have been isolated from this material by Fujita *et al.* and Shoji *et al.* This paper describes the isolation and structure determination of four new phenolic glycosides, tenuifolisides A (**1**), B (**2**), C (**3**), and D (**4**) together with a known glycoside, β-D-(3-*O*-sinapoyl)-fructofuranosyl-α-D-(6-*O*-sinapoyl)-glucopyranoside (**5**) from this material.

Tenuifoliside A (**1**) was obtained as a yellow amorphous powder and its fast atom bombardment mass spectrum (FAB-MS) showed a significant peak due to $[M+Na]^+$ at *m/z* 705. The constitution of this peak was determined to be $C_{31}H_{38}O_{17}Na$ from the high-resolution FAB-MS. The infrared (IR) spectrum of **1** showed the presence of hydroxyl group (3412 cm^{-1}), conjugated ester (1704 cm^{-1}), double bond (1632 cm^{-1}) and aromatic ring (1608, 1588 cm^{-1}). The ultraviolet (UV) spectrum of **1** in MeOH exhibited typical absorptions of cinnamoyl derivatives at λ_{max} 202, 235, 256, 309 nm. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** in CD_3OD (Table I) showed the signals belonging to a 3,4,5-trimethoxycinnamoyl group [three methoxyl signals at δ 3.79 (3H, s), and 3.86 (6H, s), two *trans* olefinic proton signals at δ 6.54 and 7.72 (each 1H, d, J=15.9 Hz), and two aromatic proton signals at δ 6.95 (2H, s)] and a *p*-hydroxybenzoyl group [four aromatic proton signals at δ 6.76 and 7.87 (each 2H, d, J=8.9 Hz)]. The ^{1}H-NMR spectrum also showed the doublet signal [δ 5.50 (J=3.7 Hz)] which was due to the anomeric proton in the α-glucopyranose moiety of sucrose.[7]

Alkaline hydrolysis of **1** with 3% KOH–MeOH gave methyl 3,4,5-trimethoxycinnamate and methyl *p*-hydroxybenzoate and sucrose (**6**). From these observations, **1** was

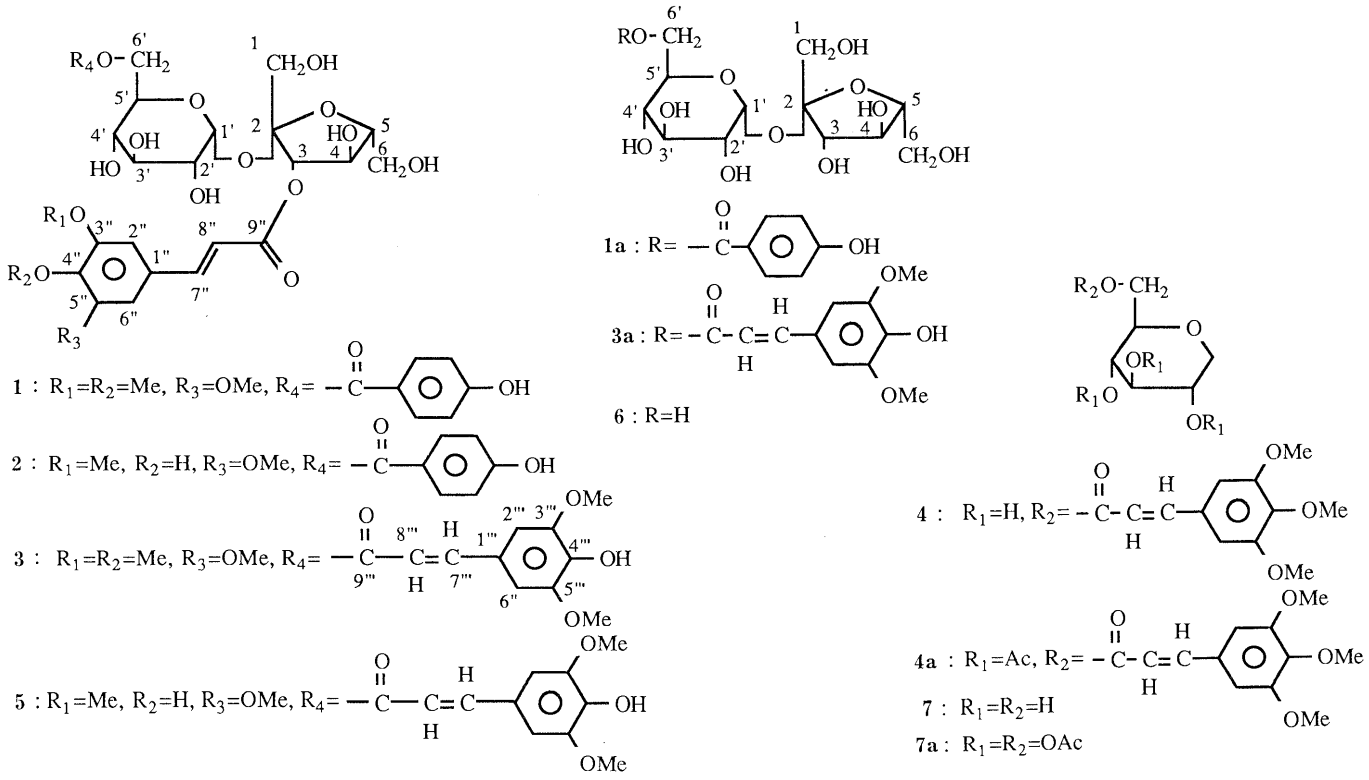

Chart 1

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TABLE I. ^{1}H-NMR Spectral Data for Compounds **1**—**5**, **1a**, **3a**, and **4a** in CD_3OD (500 MHz)

Proton	1	1a[a]	2	3	3a[a]	5	5[a]	4	4a[a]
H-1a	3.61, d (12.2)	3.40, d (12.6)	3.61, d (12.2)	3.59, d (12.1)	3.41, s	3.61, d (12.2)	3.38, s		
H-1b	3.66, d (12.2)	3.42, d (12.6)	3.66, d (12.2)	3.63, d (12.1)	3.41, s	3.65, d (12.2)	3.38, s		
H-3	5.47, d (7.9)	3.90, d (8.2)	5.47, d (8.0)	5.51, d (8.0)	3.91, d (8.2)	5.51, d (8.1)	5.41, d (8.0)		
H-4	4.37, t (7.9)	3.79, t (8.2)	4.38, t (8.0)	4.50, t (8.0)	3.48, t (8.2)	4.51, t (8.1)	4.21, t (8.0)		
H-5	3.93, ddd (7.9, 6.6, 3.0)	3.58, ddd (8.2, 6.6, 2.9)	3.93, ddd (8.0, 6.6, 3.0)	3.98, ddd (8.0, 6.9, 3.3)	3.60—3.65, m	3.96, ddd (8.1, 6.9, 3.3)	3.84, ddd (8.0, 7.0, 3.6)		
H-6a	3.73, dd (12.1, 3.0)	3.47, dd (11.5, 2.9)	3.71, dd (12.2, 3.0)	3.83, dd (12.1, 3.3)	3.56, m	3.86, dd (12.1, 3.3)	3.64, dd (11.9, 3.6)		
H-6b	3.81, dd (12.1, 6.6)	3.53, dd (11.5, 6.6)	3.80, dd (12.2, 6.6)	3.90, dd (12.1, 6.9)	3.60—3.65, m	3.91, dd (12.1, 6.9)	3.81, dd (11.9, 7.0)		
H-1′	5.50, d (3.7)	5.22, d (3.7)	5.53, d (3.7)	5.51, d (3.8)	5.23, d (3.8)	5.53, d (3.8)	5.30, d (3.7)	3.20, t (10.7) 3.90, dd (10.7, 5.4)	3.42, dd (11.2, 10.6) 4.11, dd (11.2, 5.7)
H-2′	3.48, dd (9.7, 3.7)	3.26, dd (9.6, 3.7)	3.48, dd (9.7, 3.7)	3.48, dd (9.7, 3.8)	3.27, dd (9.7, 3.8)	3.50, dd (9.7, 3.8)	3.31, dd (9.6, 3.7)	3.50, ddd (10.7, 9.0, 5.4)	4.98, ddd (10.6, 9.4, 5.7)
H-3′	3.69, dd (9.7, 8.9)	3.55, t (9.6)	3.70, dd (9.7, 8.9)	3.67, dd (9.7, 8.7)	3.54, dd (9.7, 8.8)	3.69, dd (9.7, 8.9)	3.49, brt (9.6)	3.32, m	5.23, t (9.4)
H-4′	3.46, dd (10.0, 8.9)	3.24, dd (9.9, 9.6)	3.46, dd (10.0, 8.9)	3.31, dd (9.9, 8.7)	3.14, dd (10.0, 8.8)	3.38, dd (9.7, 8.9)	3.18, brt (9.5)	3.32, m	5.07, dd (9.9, 9.4)
H-5′	4.23, ddd (10.0, 4.9, 2.1)	4.04, ddd (9.9, 4.9, 1.9)	4.24, ddd (10.0, 6.5, 1.8)	4.28, ddd (9.9, 7.2, 1.6)	4.01, ddd (10.0, 6.5, 1.8)	4.29, ddd (9.7, 7.2, 1.5)	4.12, ddd (9.5, 7.3, 1.6)	3.43, ddd (9.5, 6.0, 2.1)	3.79, ddd (9.9, 3.9, 3.3)
H-6′a	4.42, dd (12.0, 4.9)	4.25, dd (11.9, 4.9)	4.14, dd (11.7, 6.5)	4.21, dd (11.7, 7.2)	4.14, dd (11.7, 6.5)	4.23, dd (11.6, 7.2)	4.18, dd (11.6, 7.3)	4.28, dd (11.9, 6.0)	4.29, dd (12.4, 3.9)
H-6′b	4.64, dd (12.0, 2.1)	4.36, dd (11.9, 1.9)	4.31, dd (11.7, 1.8)	4.68, dd (11.7, 1.6)	4.31, dd (11.7, 1.8)	4.68, dd (11.6, 1.5)	4.64, dd (11.6, 1.6)	4.51, dd (11.9, 2.1)	4.31, dd (12.4, 3.3)
H-2″,6″	6.95, 2H, s		6.92, 2H, s	6.94, 2H, s		6.90, 2H, s[c]	7.00, 2H, s[c]		
H-7″	7.72, d (15.9)		7.70, d (15.8)	7.69, d (15.9)		7.67, d (15.8)[d]	7.55, d (15.9)[d]		
H-8″	6.54, d (15.9)		6.45, d (15.8)	6.54, d (15.9)		6.44, d (15.8)[e]	6.48, d (15.9)[e]		
3″,5″-OMe	3.86, 6H, s		3.87, 6H, s	3.87, 6H, s		3.86, 6H, s[f]	3.78, 6H, s[f]		
4″-OMe	3.79, 3H, s		—	3.79, 3H, s		—	—		
H-2‴,6‴	7.87, d (8.9)	7.79, d (8.8)	7.91, d (8.8)	6.89, 2H, s	6.99, 2H, s	6.88, 2H, s[c]	7.01, 2H, s[c]	6.91, 2H, s	6.93, 2H, s
H-3‴,5‴	6.76, d (8.9)	6.85, d (8.8)	6.82, d (8.8)	—	—	—	—	—	—
H-7‴	—	—	—	7.59, d (15.8)	7.53, d (15.8)	7.59, d (15.8)[d]	7.61, d (15.9)[d]	7.63, d (15.9)	7.64, d (16.0)
H-8‴	—	—	—	6.56, d (15.8)	6.44, d (15.8)	6.42, d (15.8)[e]	6.54, d (15.9)[e]	6.49, d (15.9)	6.48, d (16.0)
3‴,5‴-OMe	—	—	—	3.85, 6H, s	3.78, 6H, s	3.84, 6H, s[f]	3.81, 6H, s[f]	3.86, 6H, s	3.88, 6H, s
4‴-OMe	—	—	—	—	—	—	—	3.79, 3H, s	3.81, 3H, s
OAc									2.00, 2.02, 2.04, s

J values in Hz. Abbreviations: br = broad, d = doublet, m = multiplet, s = singlet, t = triplet. *a*) Compounds **1a**, **3a** and **5** were measured in DMSO-d_6 and **4a** was done in CD_3OD–$CDCl_3$ (9 : 1). *b*) All assignments were confirmed by the ^{1}H–^{1}H COSY spectrum. *c*—*f*) Assignments may be interchangeable in each column.

assumed to be the compound which 3,4,5-trimethoxycinnamoyl and *p*-hydroxybenzoyl groups attached to two hydroxyl groups of the sucrose moiety.

The substitutive positions of 3,4,5-trimethoxycinnamoyl and *p*-hydroxybenzoyl groups in the sucrose moiety of **1** were revealed by the ^{1}H- and carbon-13 (^{13}C)-NMR (Table II) spectral analysis and partial hydrolysis as follows. Assignments of proton and carbon signals of **1** were achieved by the ^{1}H–^{1}H homonuclear and ^{13}C–^{1}H heteronuclear shift correlation spectra (^{1}H–^{1}H COSY and ^{13}C–^{1}H COSY). The arrangement of ester linkage was determined by the correlation spectroscopy *via* long range coupling (COLOC) spectrum of **1** (Fig. 1), that is, the cross peaks between H-3 (δ 5.47) of fructose moiety and the carbonyl carbon (δ 167.88) of 3,4,5-trimethoxycinnamoyl group, and H-6′ (δ 4.64) of glucose moiety and the carbonyl carbon (δ 168.61) of *p*-hydroxybenzoyl group were exhibited. This indicates that 3,4,5-trimethoxycinnamoyl and *p*-hydroxybenzoyl groups attach to the C-3 and C-6′ hydroxyl groups of sucrose (**6**), respectively. Treatment of **1** with 5% K_2CO_3–70% MeOH gave methyl 3,4,5-trimethoxycinnamate and a partial hydrolysis product, $C_{30}H_{36}O_{17}$ (**1a**). The H-3 signal in the ^{1}H-NMR spectrum of **1a** appeared upfield compared with that of **1**, indicating that 3,4,5-trimethoxycinnamoyl group attaches to the C-3 hydroxyl group of **1a**.

Consequently, the structure of tenuifoliside A was determined as β-D-[3-*O*-(3,4,5-trimethoxycinnamoyl)]-fructofuranosyl-α-D-[6-*O*-(*p*-hydroxybenzoyl)]-glucopyranoside (**1**).

Tenuifoliside B (**2**) was obtained as a yellow amorphous powder and its high-resolution FAB-MS gave an $[M+Na]^+$ ion peak at *m/z* 691.1882 ($C_{30}H_{36}O_{17}Na$). The ^{1}H-NMR spectrum of **2** (Table I) showed the signals belonging to a sinapoyl group [the methoxyl signals at δ 3.87 (6H, s), two *trans* olefinic proton signals at δ 6.45 and 7.70 (each 1H, d, J = 15.8 Hz), and two aromatic proton signals at δ 6.92 (2H, s)] and a *p*-hydroxybenzoyl group (four aromatic proton signals at δ 6.82 and 7.91 (each 2H, d, J = 8.8 Hz)] in addition to the signals due to the sucrose moiety. By comparisons of the ^{1}H- and ^{13}C-NMR spectra of **2** with those of **1**, it was assumed that the 3,4,5-trimethoxycinnamoyl and sinapoyl groups attached to the C-3 and C-6′ hydroxyl groups of sucrose. Treatment of **2** with 5% K_2CO_3–70% MeOH gave methyl sinapate and 6′-(*p*-hydroxybenzoyl)-sucrose (**1a**), indicating that a sinapoyl group attached to the C-3 hydroxyl group of **1a**.

On the basis of the above observations, tenuifoliside B was determined as β-(3-*O*-sinapoyl)-fructofuranosyl-α-D-[6-*O*-(*p*-hydroxybenzoyl)]-glucopyranoside (**2**).

Tenuifoliside C (**3**) was obtained as a yellow amorphous powder and its high-resolution FAB-MS gave an $[M+Na]^+$ ion peak at *m/z* 791.2403 ($C_{35}H_{44}O_{19}Na$). The ^{1}H-NMR spectrum of **3** showed the signals due to a sinapoyl group [two methoxyl signals at δ 3.85 (6H, s), two aromatic proton signals at δ 6.89 (2H, s), and two *trans* olefinic

TABLE II. ^{13}C-NMR Spectral Data for Compounds **1**—**5**, **1a**, **3a**, and **4a** in CD_3OD (50 MHz)

Carbon	1	1a[a]	2	3	3a[a]	5	4	4a[a,b]
1	65.70	62.05	65.64	65.81	62.20	65.73		
2	104.97	103.89	104.90	104.93	103.68	104.87		
3	79.88	76.95	79.63	79.58	76.76	79.37		
4	74.21	74.36	74.08	74.31	74.29	74.20		
5	84.25	82.49	84.13	84.44	82.44	84.30		
6	63.46	62.37	63.40	63.87	62.45	63.76		
1′	93.21	91.57	93.12	92.79	91.19	92.68	71.03	67.59
2′	73.20	71.43	73.14	73.19	71.28	73.10	71.34	70.46
3′	74.99	72.60	74.91	75.19	72.57	75.08	79.86	75.27
4′	71.68	69.95	71.60	72.04	70.07	71.93	71.87	70.11
5′	72.64	63.58	72.51	72.61	69.92	72.49	80.02	77.58
6′	64.98		65.10	65.68	63.70	65.55	65.30	63.50
1″	131.54		126.56	131.54		125.86		
2″	107.05		107.09	107.07		107.02[c]		
3″	154.84		149.43	154.85		149.58[d]		
4″	141.46		139.70	141.47		140.57[e]		
5″	154.84		149.43	154.85		149.58[d]		
6″	107.05		107.09	107.07		107.02[c]		
7″	147.33		148.00	147.32[c]		147.12[f]		
8″	117.83		115.43	117.87		114.87[g]		
9″	167.88		168.23[c]	167.88		168.41[h]		
3″,5″-OMe	56.88		56.93	56.88		56.84[i]		
4″-OMe	61.24			61.22				
1‴	120.42	119.42	122.09	126.66	122.60	125.86	131.57	131.47
2‴	133.08	131.35	133.02	107.07	106.27	107.20[c]	106.89	106.90
3‴	117.15	115.42	116.24	149.50	148.18	149.62[d]	154.85	154.79
4‴	166.43	162.85	163.59	139.69	140.90	140.85[e]	141.45	141.44
5‴	117.15	115.42	116.24	149.50	148.18	149.62[d]	154.85	154.79
6‴	133.08	131.35	133.02	107.07	106.27	107.20[c]	106.89	106.90
7‴	168.61	165.65	168.20[c]	147.26[c]	145.49	147.43[f]	146.53	146.86
8‴				115.92	113.39	115.33[g]	118.08	117.68
9‴				169.13	166.77	169.21[h]	168.65	168.11
3‴,5‴-OMe				56.95	55.97	56.88[i]	56.80	56.79
4‴-OMe							61.22	61.22

a) Compounds **1a** and **3a** were measured in DMSO-d_6 and **4a** was done in CD_3OD–$CDCl_3$ (9:1). *b*) Other signals: δ 19.76, 20.55, 20.66, 171.36, 171.47, 171.82 ($3\times COCH_3$). *c*—*i*) Assignments may be interchangeable in each column. All assignments were confirmed by the ^{13}C–^{1}H COSY spectrum.

proton signals at δ 6.46 and 7.59 (each 1H, d, $J=15.8$ Hz)] and a 3,4,5-trimethoxycinnamoyl group [three methoxyl signals at δ 3.79 (3H, s) and 3.87 (6H, s), two aromatic proton signals at δ 6.94 (2H, s), and two *trans* olefinic proton signals at δ 6.54 and 7.69 (each 1H, d, $J=15.9$ Hz)]. The ^{1}H- and ^{13}C-NMR spectra of **3** closely resembled those of **1** except for acid moieties. By comparisons of the ^{1}H- and ^{13}C-NMR spectra of **3** with those of **1**, **3** was assumed to be the compound which 3,4,5-trimethoxycinnamoyl and sinapoyl groups attached to the C-3 and C-6′ hydroxyl groups of the sucrose moiety.

Treatment of **3** with 5% K_2CO_3–70% MeOH gave methyl 3,4,5-trimethoxycinnamate and a partial hydrolysis product, $C_{23}H_{32}O_{15}$ (**3a**). Furthermore, hydrolysis of **3a** with 3% KOH–MeOH gave methyl sinapate and sucrose (**6**), indicating that **3a** was the sinapoyl ester of sucrose. From these hydrolytic reactions, **3** was confirmed to be the 3,4,5-trimethoxycinnamoyl and sinapoyl ester of sucrose. The arrangement of ester linkages was confirmed by the COLOC spectrum of **3** (Fig. 2), that is, the cross peaks between H-3 (δ 5.51) of the fructose moiety and a carbonyl carbon at δ 167.88 of the 3,4,5-trimethoxycinnamoyl group, and H-6′ (δ 4.68) of glucose moiety and the carbonyl carbon at δ 169.13 of the sinapoyl group were exhibited. This indicated that the 3,4,5-trimethoxycinnamoyl group in **3** attached to the C-3 hydroxyl group of sucrose and that the sinapoyl group attached to the C-6′ hydroxyl group. On the basis of the above observations, tenuifoliside C was determined to be β-D-[3-*O*-(3,4,5-trimethoxycinnamoyl)]-fructofuranosyl-α-D-(6-*O*-sinapoyl)-glucopyranoside (**3**).

Compound **5**, $C_{34}H_{42}O_{19}$, was obtained as a yellow amorphous powder. The ^{1}H-NMR spectrum of **5** (in CD_3OD) showed the signals belonging to two sinapoyl groups (four methoxyl signals at δ 3.84 and 3.86 (each 6H, s), four aromatic proton signals at δ 6.88 and 6.90 (each 2H, s), also four *trans* olefinic proton signals at δ 6.42, 6.44, 7.59, 7.67 (each 1H, d, $J=15.8$ Hz)]. The ^{1}H- and ^{13}C-NMR spectra of **5** closely resembled those of **3** except for the acid moieties. By comparisons of the ^{1}H- and ^{13}C-NMR spectra of **5** with those of **3**, it was assumed that a 3,4,5-trimethoxycinnamoyl group in **3** was replaced by a sinapoyl group in **5**. Treatment of **5** with 5% K_2CO_3–70% MeOH gave methyl sinapate and **3a**. The H-3 signal in the ^{1}H-NMR spectrum of **3a** (in dimethylsulfoxide (DMSO)-d_6) appeared of 1.5 ppm upfield compared with that of **5** (in DMSO-d_6), indicating that one sinapoyl group in **5** attached to the C-3 hydroxyl group of **3a**.

On the basis of the above observations, **5** was identified as β-D-(3-*O*-sinapoyl)-fructofuranosyl-α-D-(6-*O*-sinapoyl)-glucopyranoside, which has been already isolated from *Raphanus sativus*.[8]

Tenuifoliside D (**4**) was obtained as a white amorphous

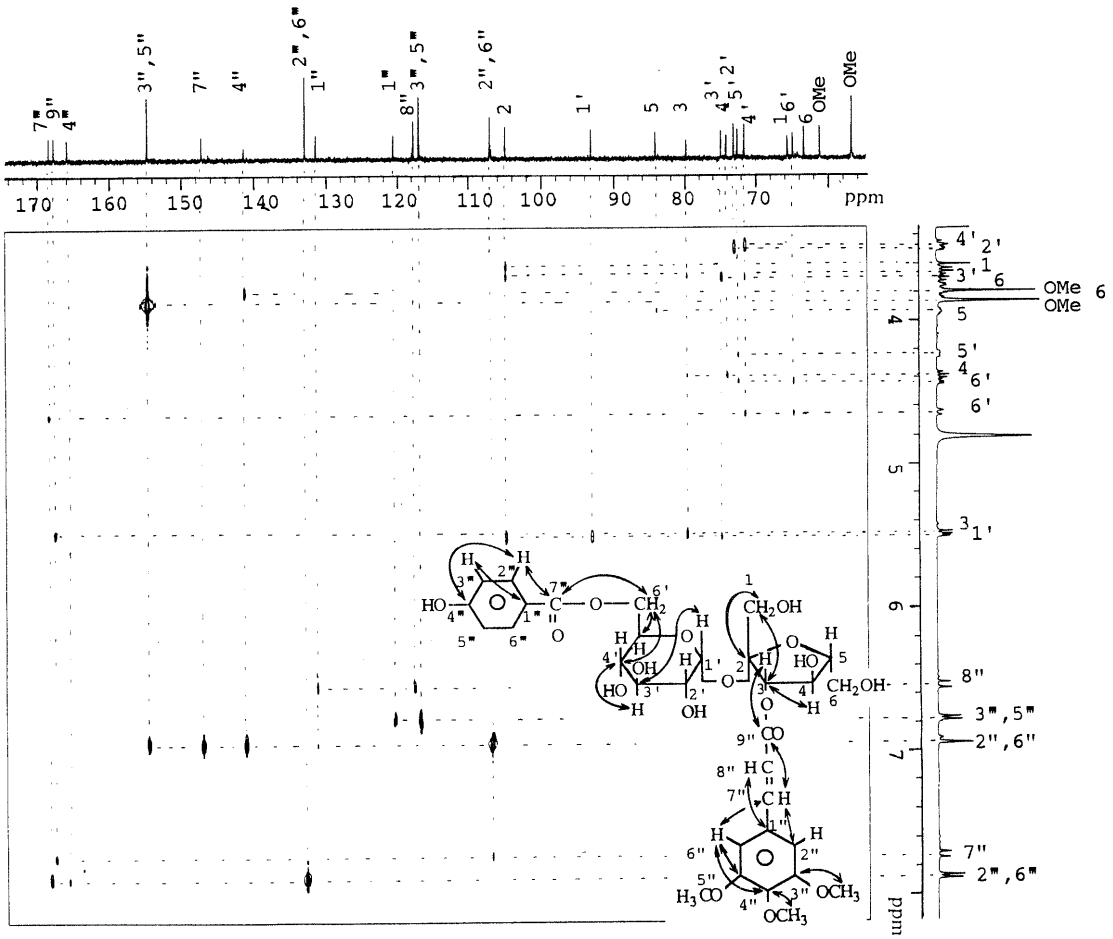

Fig. 1. COLOC Spectrum of **1** in CD_3OD (J_{CH} = 5.5 Hz)

powder and its high-resolution FAB-MS gave an $[M+Na]^+$ ion peak at *m/z* 407.1309 ($C_{18}H_{24}O_9Na$). The ^{1}H-NMR spectrum of **4** showed the signals attributed to a 1,5-anhydroglucitol moiety (δ 3.20 (1H, t, J = 10.7 Hz), 3.90 (1H, dd, J = 10.7, 5.4 Hz) (C-1 methylene), 3.50 (1H, ddd, J = 10.7, 9.0, 5.4 Hz, H-2), 3.32 (2H, m, H-3 and H-4), 3.43 (1H, ddd, J = 9.5, 6.0, 2.1 Hz, H-5), 4.28 (1H, dd, J = 11.9, 6.0), 4.51 (1H, dd, J = 11.9, 2.1 Hz) (C-6 methylene)] in addition to the signals due to a 3,4,5-trimethoxycinnamoyl group. Hydrolysis of **4** with 5% K_2CO_3–70% MeOH gave methyl 3,4,5-trimethoxycinnamate and compound **7**. Acetylation of **7** gave **7a**, which was identified as 1,5-anhydro-D-glucitol tetraacetate.[9] These facts indicated that **4** is the 3,4,5-trimethoxycinnamoyl ester of 1,5-anhydro-D-glucitol (**7**).

The substitutive position of an ester linkage was revealed by ^{1}H-NMR spectral analysis of **4** and its triacetate (**4a**). The C-2, 3 and 4 methine proton signals in the ^{1}H-NMR spectrum of **4a** showed 1.48—1.93 ppm downfield shifts compared with those of **4**. On the other hand, the C-6 methylene proton signals of **4** didn't show downfield shift by acetylation. This indicated that the 3,4,5-trimethoxycinnamoyl group attached to the C-6 hydroxyl group of **7**. On the basis of the above observations, tenuifoliside D was determined to be 1,5-anhydro-[6-*O*-(3,4,5-trimethoxycinnamoyl)]-D-glucitol (**4**).

Tenuifoliside D is the first phenylpropanoid ester of 1,5-anhydro-D-glucitol from plants of genus *Polygala*. Up to present, a few esters of the phenylpropanoid acids with sucrose have been found from *P. amarella*[7] and *P. chamaebuxus*[10] in the family Polygalaceae.

Experimental

All the melting points were taken on a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-360 polarimeter. UV spectra were taken with a Hitachi U-3200 spectrophotometer and IR spectra with a Hitachi 270-30 IR spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were taken on Bruker AM-500, JEOL JNM EX-400 and JEOL JNM FX-200 spectrometer with tetramethylsilane (TMS) as an internal standard. ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY, COLOC spectra were obtained with the usual pulse sequence and data processing was performed with the standard Bruker and JEOL softwares. Electron impact (EI) and high-resolution EI-MS were obtained with a JEOL, JMS-DX-300 mass spectrometer, and FAB-MS and high-resolution FAB-MS with a Kratos Concept 1H mass spectrometer. For silica gel column chromatography, Kieselgel 60 (Merck) was used. Kieselgel 60 F_{254} (Merck precoated plate) was used for preparative thin layer chromatography (prep. TLC) and spots were detected under UV (254 nm).

Extraction and Separation of the Constituents of Roots of *Polygala tenuifolia* The dried roots (4.8 kg) of *P. tenuifolia* were pulverized and extracted three times with boiling MeOH (20 l, 3 h). The concentrated methanolic extract (1084.8 g) was dissolved in H_2O and extracted with ether and BuOH successively. The BuOH solution was concentrated to give a dark mass (638.8 g). A part (408 g) of this mass was chromatographed on Sepabeads SP-207 (Mitsubishi Chemical Industries Limited) (2.6 l), developing with H_2O (7 l), 20% MeOH (6.5 l), 40% MeOH (6.5 l) and then MeOH (6.5 l). The MeOH elute was concentrated to give a residue (182.71 g), which was chromatographed on SiO_2 (1.15 kg) with a mixture of $CHCl_3$–MeOH. The details of this chromatography are given in Table III.

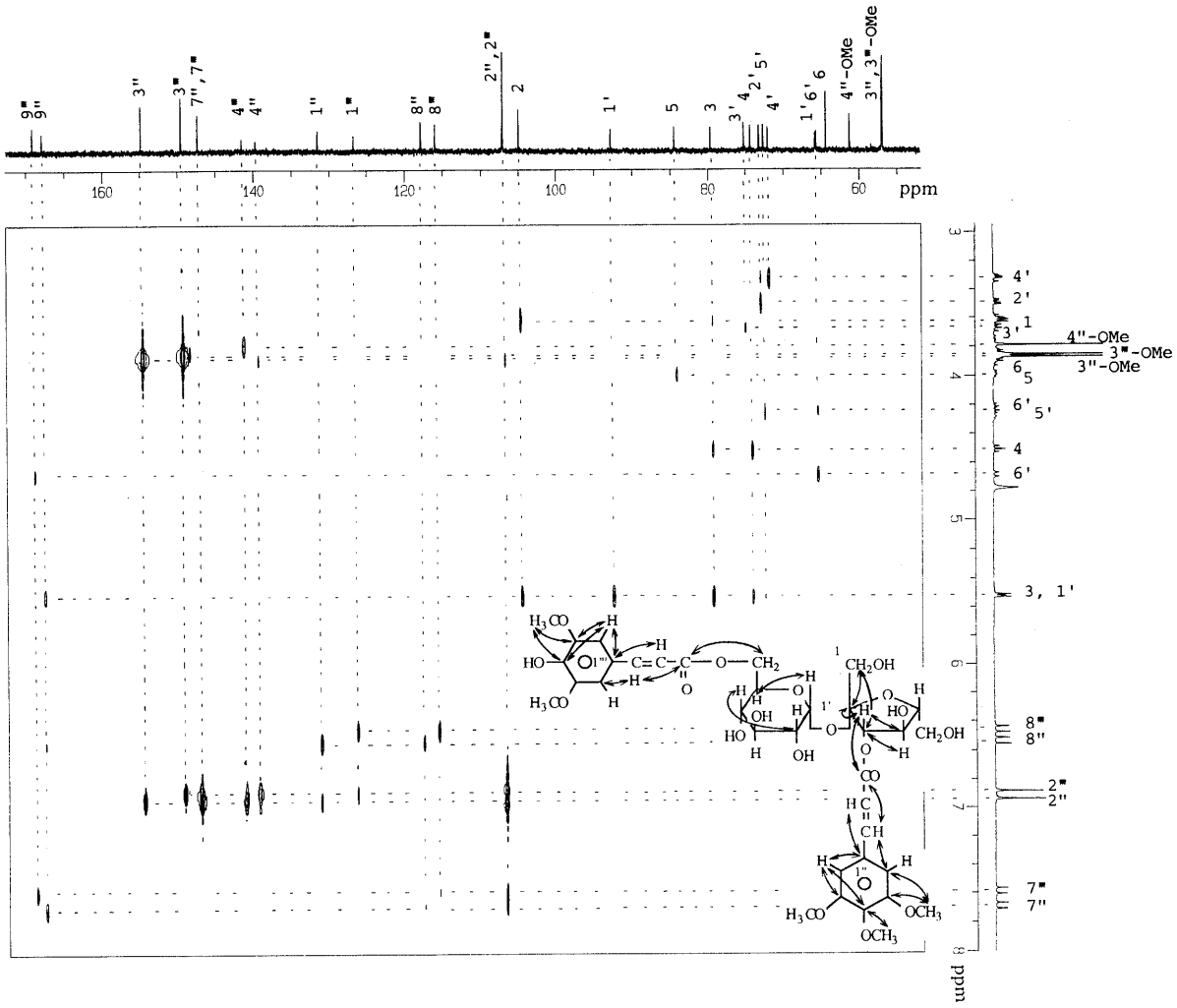

Fig. 2. COLOC Spectrum of **3** in CD_3OD (J_{CH} = 5.5 Hz)

TABLE III. Silica Gel Column Chromatography of the MeOH Eluate from Sepabeads SP-207 Column Chromatography of the BuOH Extract

Fraction No.	Solvent	Volume (l)	Yield (g)
1	$CHCl_3$–MeOH (95 : 5)	3	0.20
2	$CHCl_3$–MeOH (90 : 10)	0.5	0.58
3	$CHCl_3$–MeOH (85 : 15)	2.5	5.01
4	$CHCl_3$–MeOH (80 : 20)	1	7.61
5	$CHCl_3$–MeOH (80 : 20)	2	22.13
6	$CHCl_3$–MeOH (70 : 30)	0.5	16.19
7	$CHCl_3$–MeOH (50 : 50)	2.5	55.91
8	$CHCl_3$–MeOH (30 : 70)	1	44.07
	MeOH	1	
	MeOH–H_2O (90 : 10)	2.5	

Fraction 3 (5.01 g) was chromatographed on SiO_2 with $CHCl_3$–MeOH (14 : 1) and $CHCl_3$–MeOH (17 : 3). The fraction eluted with $CHCl_3$–MeOH (14 : 1) was concentrated to give a residue (964 mg), which was purified by prep. TLC [i) $CHCl_3$–MeOH (9 : 1), *Rf* 0.45; ii) EtOAc–MeOH–H_2O (16 : 3 : 1), *Rf* 0.73] to give **4** (209 mg, yield 0.0097%). The fraction eluted with $CHCl_3$–MeOH (17 : 3) was concentrated to give a residue (706 mg), which was purified by prep. TLC [i) $CHCl_3$–MeOH (9 : 1), *Rf* 0.2; ii) EtOAc–MeOH–H_2O (16 : 3 : 1), *Rf* 0.73] to give **3** (158 mg). Fraction 4 (7.61 g) was chromatographed on SiO_2 with $CHCl_3$–MeOH (17 : 3) and $CHCl_3$–MeOH (4 : 1). The fraction eluted with $CHCl_3$–MeOH (17 : 3) was concentrated to give a residue (482 mg), which was purified by prep. TLC [$CHCl_3$–MeOH (9 : 1), *Rf* 0.2] to give **3** (240 mg, total 398 mg, yield 0.019%). The fraction eluted with $CHCl_3$–MeOH (4 : 1) was concentrated to give a residue (6.50 g), which was rechromatographed on SiO_2 with EtOAc–MeOH–H_2O (33 : 6 : 1) to give **1** (2.785 g, yield 0.13%). Fraction 5 (22.13 g) was repeatedly chromatographed on SiO_2 with $CHCl_3$–MeOH (17 : 3) to give **5** (5.088 g, yield 0.24%). Fraction 6 (16.19 g) was repeatedly chromatographed on SiO_2 with $CHCl_3$–MeOH (4 : 1) to give crude **2** (721 mg). This was purified by prep. TLC [$CHCl_3$–MeOH (3 : 1), *Rf* 0.51] to give **2** (473 mg, yield 0.022%).

Tenuifoliside A (**1**): A yellow amorphous powder, $[\alpha]_D^{24}$ −20.5° (*c* = 1.52, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3412 (OH), 1704 (C=O), 1632 (C=C), 1608, 1588 (aromatic ring). UV λ_{max}^{MeOH} nm (log ε): 202 (4.51), 235 (4.31), 256 (4.21), 309 (4.22). Positive ion FAB-MS *m/z*: 705 $[M+Na]^+$. High-resolution FAB-MS, Calcd for $C_{31}H_{38}O_{17}Na$ ($[M+Na]^+$): 705.2007. Found: 705.2048.

Tenuifoliside B(**2**): A yellow amorphous powder, $[\alpha]_D^{24}$ −20.8° (*c* = 1.01, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1702 (C=O), 1632 (C=C), 1602 (aromatic ring). UV λ_{max}^{MeOH} nm (log ε): 201 (4.44), 246 (4.30), 331 (4.19). Positive ion FAB-MS *m/z*: 691 $[M+Na]^+$. High-resolution FAB-MS, Calcd for $C_{30}H_{36}O_{17}Na$ ($[M+Na]^+$): 691.1851. Found: 691.1882.

Tenuifoliside C (**3**): A yellow amorphous powder, $[\alpha]_D^{24}$ −53.6° (*c* = 1.51, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3416 (OH), 1708 (C=O), 1632 (C=C), 1606, 1586 (aromatic ring). UV λ_{max}^{MeOH} nm (log ε): 201 (4.55), 235 (4.51), 319 (4.50). Positive ion FAB-MS *m/z*: 791 $[M+Na]^+$. High-resolution FAB-MS, Calcd for $C_{35}H_{44}O_{19}Na$ ($[M+Na]^+$): 791.2375. Found: 791.2403.

Tenuifoliside D (**4**): A white amorphous powder, $[\alpha]_D^{24}$ +24.6° (*c* = 1.66, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3412 (OH), 1708 (C=O), 1636 (C=C), 1584 (aromatic ring). UC λ_{max}^{MeOH} nm (log ε): 202 (4.24), 232 (4.21), 307 (4.20). Positive ion FAB-MS *m/z*: 407 $[M+Na]^+$, 384 $[M]^+$. High-resolution FAB-MS, Calcd for $C_{18}H_{24}O_9Na$ ($[M+Na]^+$): 407.1318. Found: 407.1309.

β-D-(3-*O*-Sinapoyl)-fructofuranosyl-α-D-(6-*O*-sinapoyl)-glucopyranoside (5) A yellow amorphous powder, $[\alpha]_D^{24}$ −28.9° (*c* = 1.11, MeOH). IR

ν_{max}^{KBr} cm^{-1}: 3396 (OH), 1704 (C=O), 1632 (C=C), 1606 (aromatic ring). UV λ_{max}^{MeOH} nm (log ε): 201 (4.60), 225 (4.57), 237 (4.56), 330.8 (4.58). Positive ion FAB-MS *m/z*: 777 $[M+Na]^+$. High-resolution FAB-MS, Calcd for $C_{34}H_{42}O_{19}Na$ ($[M+Na]^+$): 777.2218. Found: 777.2179.

Hydrolysis of 1 with 5% K_2CO_3–70% MeOH A solution of **1** (107 mg) in 5% K_2CO_3–70% MeOH (2 ml) at 25 °C for 40 min, then neutralized with 1 N HCl and concentrated to give a residue. The residue was purified by prep. TLC [$CHCl_3$–MeOH (7:3)] to give **1a** (43 mg) and colorless prisms (from EtOH) (43 mg), mp 94—95 °C. IR ν_{max}^{KBr} cm^{-1}: 1698 (C=O), 1632 (C=C), 1584 (aromatic ring). EI-MS *m/z* (%): 252 (M^+, 100), 237 (59), 222 (16), 221 (14). High-resolution MS, Calcd for $C_{13}H_{16}O_5$ (M^+): 252.0998. Found: 252.1000. ^{1}H-NMR (in $CDCl_3$) δ: 3.81 (3H, s), 3.88 (3H, s), 3.98 (6H, s), 6.35 (1H, d, J=15.9 Hz), 6.75 (2H, s), 7.61 (1H, d, J=15.9 Hz). This was identical (mp, IR, ^{1}H-NMR, MS) with methyl 3,4,5-trimethoxycinnamate, which was derived from methylation of sinapic acid (Tokyo Kasei Industries Limited) with CH_2N_2.

1a: A pale brown amorphous powder, $[\alpha]_D^{24}$ +24.1° (*c*=0.746, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 1698 (C=O), 1610 (aromatic ring). UV λ_{max}^{MeOH} nm (log ε): 202 (4.10), 208 (sh, 4.00), 258 (4.03). Positive ion FAB-MS *m/z*: 485 $[M+Na]^+$. High-resolution FAB-MS, Calcd for $C_{19}H_{26}O_{13}Na$ ($[M+Na]^+$): 485.1271. Found: 485.1276.

Hydrolysis of 1 A solution of **1** (29 mg) in 3% KOH–MeOH (2 ml) was stirred at room temp. for 1.5 h, then neutralized with 1 N HCl and concentrated to give a residue. This residue was purified by prep. TLC [$CHCl_3$–MeOH–H_2O (14:6:1)] to give methyl 3,4,5-trimethoxycinnamate (5.5 mg), methyl *p*-hydroxybenzoate (5 mg) and sucrose (**6**). Methyl *p*-hydroxybenzoate: colorless prisms (from MeOH–H_2O) (5 mg), mp 123—124 °C. EI-MS *m/z* (%): 152 (M^+, 35), 121 (100), 93 (24). ^{1}H-NMR (in $CDCl_3$) δ: 3.89 (3H, s), 5.90 (1H, br s, OH), 6.86 (2H, d, J=9.0 Hz), 7.95 (2H, d, J=9.0 Hz). IR ν_{max}^{KBr} cm^{-1}: 3304 (OH), 1680 (C=O), 1608, 1588 (aromatic ring). This was identified as methyl *p*-hydroxybenzoate with an authentic sample (Tokyo Kasei Industries Limited).

Sucrose (**6**): Syrup, $[\alpha]_D^{23}$ +30.8° (*c*=0.721, MeOH). ^{13}C-NMR (in C_5D_5N) δ: 61.90 (CH_2), 62.98 (CH_2), 63.83 (CH_2), 71.23 (CH), 72.81 (CH), 74.29 (CH), 75.33 (CH), 79.16 (CH), 83.48 (CH), 93.36 (CH), 105.34 (C). This was identified by comparison of the ^{13}C-NMR data with those of an authentic sample.[11]

Hydrolysis of 2 A solution of **2** (60 mg) in 5% K_2CO_3–70% MeOH (2 ml) was stirred at 25 °C for 50 min, then neutralized with 1 N HCl and concentrated to give a residue. This residue was purified by prep. TLC [$CHCl_3$–MeOH (3:1)] to give unchanged **2** (12 mg), a pale brown amorphous powder (**1a**) (21 mg) and a white powder (from ether–MeOH) (5 mg), mp 88—89 °C. IR ν_{max}^{KBr} cm^{-1}: 3532 (OH), 1698 (C=O), 1606 (aromatic ring). EI-MS *m/z* (%): 238 (M^+, 100), 207 (41), 175 (31), 163 (26), 119 (22). ^{1}H-NMR (in $CDCl_3$) δ: 3.80 (3H, s), 3.91 (6H,s), 5.84 (1H, br s, OH), 6.30 (1H, d, J=15.9 Hz), 6.76 (2H, s), 7.60 (1H, d, J=15.9 Hz). This compound was identical (mp, IR, ^{1}H-NMR, MS) with methyl sinapate, which was derived from methylation of sinapic acid with CH_2N_2.

A Pale Brown Powder (**1a**): $[\alpha]_D^{24}$ +21.7° (*c*=0.347, MeOH). High-resolution FAB-MS, Calcd for $C_{19}H_{26}O_{13}Na$ ($[M+Na]^+$): 485.1271. Found: 485.1276. This compound was identified as **1a** with an authentic sample by direct comparison ($[\alpha]_D$, IR, FAB-MS, ^{1}H-NMR).

Hydrolysis of 3 A solution of **3** (70 mg) in 5% K_2CO_3–70% MeOH (2 ml) was stirred at 20 °C for 30 min, then neutralized with 1 N HCl and concentrated to give a residue. This was purified by prep. TLC [$CHCl_3$–MeOH (3:1)] to give **3a** (47 mg) and colorless prisms (from EtOH) (14 mg), mp 93—94 °C, which was identified as methyl 3,4,5-trimethoxycinnamate with an authentic sample by direct comparison (mp, MS, ^{1}H-NMR).

3a: A pale yellow amorphous powder, $[\alpha]_D^{24}$ +27.4° (*c*=0.365, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3408 (OH), 1700 (C=O), 1632 (C=C), 1608 (aromatic ring). UV λ_{max}^{MeOH} nm (log ε): 200 (4.25), 225 (sh, 4.12), 240 (4.19), 329 (4.26). Positive ion FAB-MS *m/z*: 571 $[M+Na]^+$. High resolution FAB-MS, Calcd for $C_{23}H_{32}O_{15}Na$ ($[M+Na]^+$): 571.1639. Found: 571.1639. Negative ion FAB-MS *m/z*: 547 $[M-H]^-$, 223 $[(CH_3)(OH)C_6H_2CH=CH-COO]^-$.

Hydrolysis of 3a A solution of **3a** (21 mg) in 3% KOH–MeOH (2 ml) was stirred at room temperature for 1.5 h, then neutralized with 1 N HCl and concentrated to give a residue. This was purified by prep. TLC [$CHCl_3$–MeOH–H_2O (14:6:1)] to give methyl sinapate (4.5 mg) and sucrose (**6**), syrup, $[\alpha]_D^{24}$ +29.2° (*c*=0.75, MeOH), which was identified by comparison of ^{13}C-NMR data (in C_5D_5N) with those of an authentic sample.[11]

Acetylation of 4 A solution of **4** (35 mg) in a mixture of Ac_2O (0.25 ml) and pyridine (0.4 ml) was allowed to stand at room temperature overnight, then diluted with ether. The ethereal solution was washed with 1 N HCl, 5% $NaHCO_3$, then H_2O and concentrated to give a triacetate (**4a**) as colorless prisms (from ether–hexane), mp 69—70.5 °C, $[\alpha]_D^{24}$ +32.8° (*c*=1.34, MeOH). IR ν_{max}^{KBr} cm^{-1}: 1756, 1722 (C=O), 1584 (aromatic ring). Positive ion FAB-MS *m/z*: 517 $(M)^+$.

Hydrolysis of 4 A solution of **4** (60 mg) in 5% K_2CO_3–70% MeOH (2 ml) was stirred at room temperature for 20 min, then neutralized with 1 N HCl and concentrated. The residue was purified by prep. TLC (1-PrOH–EtOAc–H_2O (7:2:1)] to give methyl 3,4,5-trimethoxycinnamate (27 mg) and syrup (**7**), which was acetylated with Ac_2O (0.4 ml) and pyridine (0.8 ml) at room temperature overnight to give colorless prisms (from ether–hexane) (28 mg), mp 72—73 °C, $[\alpha]_D^{24}$ +37.1° [*c*=0.75, EtOH–H_2O (1:1)]. IR ν_{max}^{KBr} cm^{-1}: 1756, 1722 (C=O), 1584 (aromatic ring). ^{1}H-NMR δ in $CDCl_3$: 2.02 (3H, s), 2.03 (6H, s), 2.09 (3H, s) (4×OAc), 3.34 (1H, dd, J=11.2, 10.6 Hz, H-1α), 3.68 (1H, ddd, J=10.0, 4.9, 2.3 Hz, H-5), 4.15 (1H, dd, J=11.2, 5.7 Hz, H-1β), 4.13 (1H, dd, J=12.4, 2.3 Hz), 4.35 (1H, dd, J=12.4, 4.9 Hz) (H-6), 5.01 (1H, ddd, J=10.6, 9.5, 5.7 Hz, H-2β), 5.02 (1H, dd, J=10.0, 9.4 Hz, H-4β), 5.20 (1H, t, J=9.4 Hz, H-3α). Positive ion FAB-MS *m/z*: 333 $[M+H]^+$. High-resolution FAB-MS, Calcd for $C_{14}H_{21}O_9$ ($[M+H]^+$): 333.1186. Found: 333.1173. This compound was identified as 1,5-anhydro-D-glucitol tetraacetate (**7a**) by the comparison of physical data with those reported in the literature.[9]

Hydrolysis of 5 A solution of **5** (40 mg) in 5% K_2CO_3–70% MeOH (2 ml) was stirred at room temperature for 1 h, then neutralized with 1 N HCl and concentrated. The residue was purified by prep. TLC ($CHCl_3$–MeOH (7:3)] to give methyl sinapate (6 mg) and a yellow amorphous powder (17 mg), $[\alpha]_D^{24}$ +29.0° (*c*=0.545, MeOH), which was identified as **3a** by direct comparison ($[\alpha]_D$, IR, ^{1}H-NMR, MS) with an authentic sample.

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Two New Sesquineolignans from the Bark of *Illicium dunnianum*

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Two new sesquineolignans, named dunnianol (1) and isodunnianol (2), respectively, were isolated from the bark of *Illicium dunnianum* together with the known biphenylneolignan, magnolol (3). Compounds 1 and 2 have a similar triphenyl-type neolignan structure to that of macranthol (4), which we previously isolated from the pericarps of *I. macranthum*. Their structures were elucidated by means of the detailed analysis of their ^{1}H- and ^{13}C-nuclear magnetic resonance spectra of 1, 2, and their acetylation and methylation products, including two-dimensional NMR spectra, comparing with those of magnolol, isomagnolol and macranthol.

Keywords *Illicium dunnianum*; Illiciaceae; sesquineolignan; dunnianol; isodunnianol; magnolol; honokiol; isomagnolol; macranthol; triphenyl-neolignan

The constituents of the families of the Magnoliales, especially the genus of Magnoliaceae[1] and Lauraceae,[2] are characterized by the presence of a series of unusual biphenylneolignans. While Illiciaceae once belonged to Magnoliaceae, no biphenyls have been isolated so far. As a part of a research program dealing with screening toxic sesquiterpenes from *Illicium* plants,[3] we previously isolated a triphenylneolignan, macranthol (**4**),[4] which has a non-symmetrical structure, from the bark of *Illicium macranthum*. The presence of a symmetrical structure of compound "magnolol (**3**)", and non-symmetrical structure of compound "honokiol (**5**)" in biphenyls prompted us to reinvestigate the other *Illicium* plants to isolate the symmetrical triphenylneolignan.

We previously reported the isolation of three pseudoanisatin-like sesquiterpenes, 6-deoxypseudoanisatin, dunnianin and 6-deoxydunnianin, from the bark of *Illicium dunnianum* TUTCHER (Illiciaceae),[5] which is one of the toxic *Illicium* plants in China. Reinvestigation of the MeOH extract of the bark of *I. dunnianum* resulted in the isolation of two new triphenyl-neolignans (**1**) and (**2**) together with a well-known biphenyl compound, magnolol (**3**).

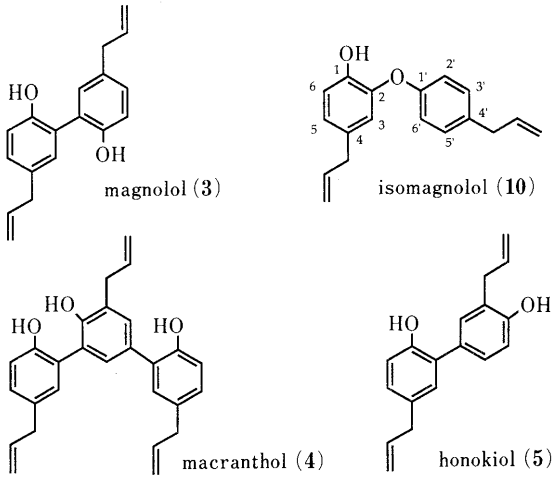

Fig. 1

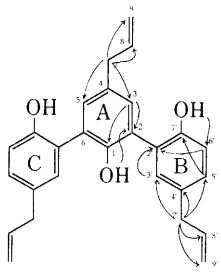

Fig. 2. Structure of Dunnianol (**1**) and the ^{1}H–^{13}C Long-Range Correlations

The EtOAc soluble part of the MeOH extract (180 g) of the bark of *I. dunnianum* (1.19 kg) was subjected to counter-current distribution using the solvent system of EtOAc–H_2O to give fractions of 1—68 as we reported before.[5] Of these fractions, 6-deoxypseudoanisatin, dunnianin and 6-deoxydunnianin were isolated from fractions of 41—55. Separation and purification of fractions 56—63 by repeated silica gel chromatography afforded crystalline compound (**1**) (998.5 mg) and oily compound (**2**) (118.8 mg), which we named dunnianol and isodunnianol, respectively, along with magnolol (**3**) (28.0 mg).

Dunnianol (**1**) was obtained as colorless needles, mp 132—133 °C, and its molecular formula, $C_{27}H_{26}O_3$, the same as that of **4**, was determined by mass spectrum (MS) (*m/z*: 398 [M^+]) and elemental analysis, and responded positively to the iron(III) chloride test. The feature of the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** was very similar to that of macranthol (**4**) indicating a type of triphenylneolignan for **1**. But, the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **1** showed only fifteen signal peaks in spite of the molecular formula indicated 27 carbons, suggesting it to be a symmetrical structure for **1**.

Dunnianol (**1**) afforded a methyl-derivative (**6**) by treatment with CH_3I and K_2CO_3 in acetone, and an acetyl-derivative (**7**) with a mixture of 4,4′-dimethylaminopyridine (DMAP), Ac_2O and pyridine, respectively. The molecular ion peak of these compounds in MS suggested that these are trimethoxy (*m/z*: 440) and triacetoxy (*m/z*: 524) derivatives, respectively. These results indicated **1** has three phenolic hydroxyl groups. The symmetrical structure of **1** was also supported by the methoxy- and acetoxy-methyl signals in the ^{1}H-NMR spectra of **6** and **7**, respectively; *i.e.* the signals of three methoxy groups appeared as a 3H-singlet at δ_H 3.19 and a 6H-singlet at δ_H 3.78 in the ^{1}H-NMR spectrum of **6**, and the same property was shown in the case of acetoxy signals (δ_H 1.66 (3H, s) and 2.08 (6H, s)) in the ^{1}H-NMR spectrum of **7**.

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TABLE I. ^{1}H-NMR Data for Dunnianol (**1**) and Isodunnianol (**2**) (400 MHz, in $CDCl_3$; *J* (Hz) in Parentheses)

Protons	Dunnianol (**1**)	Protons	Isodunnianol (**2**)
3,5	7.17 s	3	6.72 d (1.8)
7	3.46 d (6.6)	5	6.90 d (1.8)
8	5.99	7	3.29 d (6.7)
	ddt (18.7, 11.7, 6.6)	8	5.90 ddt (16.1, 9.5, 6.7)
9a	5.13 dd (18.7, 1.8)	9a	5.03 dd (9.5, 1.6)
9b	5.14 dd (11.7, 1.8)	9b	5.04 dd (16.1, 1.5)
3′,3″	7.14 d (2.2)	2′,6′	6.99 d (8.4)
5′,5″	7.15 dd (8.4, 2.2)	3′,5′	7.19 d (8.4)
6′,6″	6.97 d (8.4)	7′	3.39 d (5.9)
7′,7″	3.39 d (6.6)	8′	5.98 ddt (16.1, 9.6, 5.9)
8′,8″	5.98	9′a,9″a	5.09 dd (9.6, 1.5)
	ddt (17.2, 10.0, 6.6)	9′b,9″b	5.10 dd (16.1, 1.5)
9′a,9″a	5.07 dd (10.0, 1.9)	3″	7.12 d (2.2)
9′b,9″b	5.10 dd (17.2, 1.9)	5″	7.13 dd (8.8, 2.2)
		6″	7.01 d (8.8)
		7″	3.39 d (5.9)
		8″	5.95 ddt (16.1, 9.6, 5.9)

Assignments were aided by ^{1}H–^{1}H 2D COSY and HMQC spectra.

TABLE II. ^{13}C-NMR Data for Dunnianol (**1**) and Isodunnianol (**2**) (100 MHz, in $CDCl_3$),[a] and Related Compounds, Magnolol (**3**)[b] and Isomagnolol (**10**)[c]

Carbons	**1**	**3**	**2**	**10**
1	147.7		141.7	143.9
2	125.4		144.2	145.9
3	131.3		117.8	119.2
4	134.0		132.7	132.6
5			126.5	124.8
6			125.5	116.2
7	39.4		39.5	
8	137.2		137.3	
9	116.2		115.6	
1′	151.4	151.0	154.6	155.4
2′	124.4	124.5	118.5	117.9
3′	131.6	131.4	130.1	129.9
4′	133.2	133.3	135.9	135.3
5′	130.0	129.8	130.1	129.9
6′	117.2	116.8	118.5	117.9
7′	39.4		39.5	
8′	137.5		137.8	
9′	115.8		116.0	
1″			151.8	
2″			125.0	
3″			131.0	
4″			133.3	
5″			129.7	
6″			117.5	
7″			39.5	
8″			137.1	
9″			116.0	

a) Assignments were made with the aid of HMQC and HMBC spectra. *b*, *c*) Data were taken from references 6 and 2*c*, respectively.

The detailed analysis of ^{1}H- and ^{13}C-NMR spectra of **1** was undertaken with the aid of ^{1}H–^{1}H 2-dimensional correlation spectroscopy (2D COSY), heteronuclear multiple bond correlation (HMBC), and heteronuclear multiple quantum coherence (HMQC) spectroscopies. As a result of these assignments, it was elucidated that the carbon signals for B- (and C-) ring were almost identical with those of magnolol (**3**),[6] and positions 3 and 5 in the A-ring have protons. Thus, considering the symmetrical structure, two

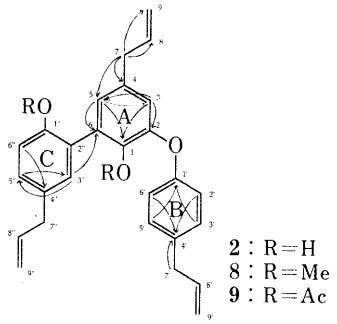

Fig. 3. Structure of Isodunnianol (**2**) and the ^{1}H–^{13}C Long-Range Correlations

phenolic arylpropanoids (B- and C-rings) are linked to positions 2 and 6 of the A-ring. Therefore, the structure of dunnianol (**1**) was determined as shown in Fig. 2.

Isodunnianol (**2**), a colorless oil, was also positive to the iron(III) chloride test. The molecular formula of **2**, $C_{27}H_{26}O_3$, was the same as those of **1** and **4**. The ^{1}H-NMR spectrum of **2** also resembled those of macranthol (**4**) and dunnianol (**1**), suggesting it to be a triphenyl-sesquineolignan. In spite of the same molecular formula as those of **1** and **4**, compound **2** afforded dimethoxy- (**8**) and diacetoxy-derivatives (**9**) suggesting the presence of only two phenolic hydroxyl groups. Thus, another oxygen should be included in an ether-linkage.

According to the unambiguous assignments of the ^{1}H- and ^{13}C-NMR spectra of **2** with the aid of ^{1}H–^{1}H 2D COSY, HMBC, and HMQC spectra, it was clarified that the carbon signals for aromatic C-ring were almost identical with those of magnolol (**3**),[6] and the carbon signals for A and B-rings resembled those of isomagnolol (**10**), which is the isomer of magnolol (**3**), and was isolated from the roots of *Sassafras randaiense* (Lauraceae).[2c] The presence of a *p*-substituted benzene ring (B-ring) was also supported by AA′BB′ signals at δ_H 6.99 (2H, d, J=8.4 Hz) and 7.19 (2H, d, J=8.4 Hz), and the proton signals for a catechol moiety (A-ring) appeared as AB type signals at δ 6.72 and 6.90 (each 1H, d, J=1.8 Hz), respectively. On the other hand, the carbon signal of isomagnolol at δ_C 116.2 corresponding to the position C-6 in A-ring showed a distinct downfield shift to δ_C 125.5 in the ^{13}C-NMR spectrum of **2**, indicating the C-ring links to position 6 in the A-ring.

To confirm a linkage pattern of three arylpropanoids in the structure of **2**, nuclear Overhauser effect (NOE) experiments were performed on the methylation product (**8**). Two methoxyl signals were shown in the ^{1}H-NMR spectrum of **8** at δ_H 3.59 and 3.77. When the signal at δ_H 3.77 was irradiated, a doublet signal at δ_H 6.89 (1H, J= 8.0 Hz, H-6″) in the C-ring was enhanced. An irradiation of the signal at δ_H 3.59 produced NOEs at δ_H 6.93 (2H, d, J=8.4 Hz, H-2′, 6′) in the B-ring and at δ_H 7.08 (1H, d, J=2.6 Hz, H-3″) in the C-ring. Therefore, these results confirmed the structure proposed for **2**.

Biphenyls are of interest because of the known pharmacological activities.[7] Although macranthol (**4**) was found to be biologically non-active by our research, the above compounds obtained here are now under investigation.

Experimental

The melting point was determined on a Yanagimoto micro melting point

apparatus and is uncorrected. ^{1}H- and ^{13}C-NMR spectra were taken with JEOL GX-400 spectrometers. NOE and 2D COSY experiments were performed on this apparatus. Chemical shifts are expressed in δ(ppm) values with tetramethylsilane as an internal standard. Electron impact mass spectrum (EI-MS) was recorded on a JEOL JMS-DX-303 spectrometer. Infrared (IR) spectra were recorded on a JASCO IR-180 or Shimadzu IR-408. Ultraviolet (UV) absorption maxima were measured on a Hitachi 323 spectrometer.

Extraction and Isolation The bark (1.19 kg) of *I. dunnianum* was extracted with methanol (6 l), and successive separation was undertaken as previously reported.[5] The EtOAc-soluble part was fractionated by counter-current distribution using the solvent system of EtOAc–H_2O to give fractions of 1—68. 6-Deoxypseudoanisatin, dunnianin and 6-deoxydunnianin were obtained from the residue from fractions of 21—55. Repeated silica gel column chromatography [*n*-hexane–EtOAc (7 : 3)] of fractions of 56—63 afforded magnolol (**3**) (28.0 mg), a crude compound of **1**, and an oily compound **2**, isodunnianol (118.8 mg). The crude compound of **1** was recrystallized from *n*-hexane–EtOAc to give colorless needles, dunnianol (**1**) (998.5 mg).

Although **3** was not obtained as crystals, this compound was suggested as magnolol because its MS showed a molecular ion at *m/z* 266 and the appearance of ^{1}H-NMR spectrum of **3** was identical with that of the figure reported in the reference.[1c]

Dunnianol (1) Colorless needles from *n*-hexane–EtOAc, mp 132—133 °C, EI-MS *m/z*: 398 (M^+). IR ν_{max} (KBr) cm^{-1}: 3260, 3080, 2970, 2900, 1638, 1495, 1415, 970, 910, 610. UV λ_{max} (EtOH) nm (ε): 223 (49800), 246 (29000), 296 (6300), 340 (5700). *Anal.* Calcd for $C_{27}H_{26}O_3$: C, 81.38; H, 6.58. Found: C, 81.29; H, 6.70.

Methylation of Dunnianol (1) **1** (20 mg) was dissolved in a mixture of K_2CO_3 (0.5 g), CH_3I (0.6 ml) and dry acetone (20 ml), and the mixture was refluxed for 3 h. After the solution was cooled down, a grain of K_2SO_3 was filtered off. The filtrate was evaporated to dryness under reduced pressure, and the residual oil was chromatographed on silica gel [*n*-hexane–EtOAc (1 : 9)] to give a methylated compound (**6**) as an oil (16 mg). EI-MS *m/z*: 440(M^+), 426 (M^+-14). ^{1}H-NMR δ($CDCl_3$): 3.19 (3H, s, –OMe (C-1)), 3.35 (4H, d, J=7.0 H, H-7′,7″), 3.40 (2H, d, J=7.0 Hz, H-7), 3.78 (6H, s, –OMe (C-1′, 1″)), 5.02—5.17 (6H, m), 5.92—6.08 (3H, m), 6.90 (2H, d, J=8.1 Hz, H-6′, 6″), 7.07 (2H, s, H-3,5), 7.13 (2H, dd, J=8.1, 2.6 Hz, H-5′, 5″), 7.14 (2H, d, J=2.6 Hz, H-3′, 3″).

Acetylation of Dunnianol (1) **1** (10 mg) was dissolved in a mixture of dry pyridine (0.5 ml) and Ac_2O (0.5 ml) with a grain of DMAP (2 mg), and the solution was left overnight at room temperature, then evaporated to dryness under reduced pressure. This residue was chromatographed on silica gel [*n*-hexane–EtOAc (1 : 9)] to give an oily acetylated compound (**7**) (9 mg). EI-MS *m/z*: 524 (M^+), 482, 440, 398. ^{1}H-NMR δ ($CDCl_3$): 1.66 (3H, s, –OAc), 2.08 (6H, s, –OAc), 3.39 (4H, d, J=7.0 Hz, H-7′, 7″), 3.41 (2H, d, J=8.8 Hz, H-7), 5.06—5.14 (6H, m), 5.91—6.03 (3H, m), 7.08 (2H, d, J=8.1 Hz, H-6′, 6″), 7.12 (2H, s, H-3, 5), 7.14 (2H, d, J=2.2 Hz, H-3′, 3″), 7.20 (2H, dd, J=8.1, 2.2 Hz, H-5′, 5″).

Isodunnianol (2) Colorless oil, $C_{27}H_{26}O_3$, EI-MS *m/z*: 398 (M^+), 264 (M^+ – *p*-allylphenoxy group). IR ν_{max} (Nujol) cm^{-1}: 3350, 3080, 2900, 1637, 1605, 1590, 1425, 997, 910.

Methylation of Isodunnianol (2) **2** (25 mg) was methylated with the same manner as **1** to give a methylated compound (**8**) as an oil (22 mg). EI-MS *m/z*: 426 (M^+). ^{1}H-NMR δ($CDCl_3$): 3.32 (2H, d, J=6.6 Hz, H-7), 3.35 (4H, d, J=6.2 Hz, H-7′, 7″), 3.59 (3H, s, –OMe (C-1″)), 3.77 (3H, s, –OMe (C-1)), 5.02—5.08 (6H, m), 5.89—6.03 (3H, m), 6.80 (1H, d, J=1.8 Hz, H-3), 6.87 (1H, d, J=1.8 Hz, H-4), 6.89 (1H, d, J=8.0 Hz, H-6″), 6.93 (2H, d, J=8.4 Hz, H-2′,6′), 7.08 (1H, d, J=2.6 Hz, H-3″), 7.12 (2H, d, J=8.4 Hz, H-3′,5′), 7.13 (1H, dd, J=8.0, 2.6 Hz, H-5″). ^{13}C-NMR δ ($CDCl_3$): 148.5 (C-1), 148.6 (C-2), 120.5 (C-3), 133.6 (C-4), 126.8 (C-5), 131.8 (C-6), 155.3 (C-1′), 117.3 (C-2′, 6′), 129.6 (C-3′,5′), 135.4 (C-4′), 156.3 (C-1″), 127.5 (C-2″), 131.5 (C-3″), 134.0 (C-4″), 128.7 (C-5″), 111.0 (C-6″), 55.8 (–OMe (C-1″)), 60.8 (–OMe (C-1)), 39.3, 39.5, 39,6, 115.5, 115.6, 116.0, 137.1, 137.7, 137.9 (signals of allyl groups).

Acetylation of Isodunnianol (2) **2** (15 mg) was acetylated with the same manner as **1** to give an acetylated compound (**9**) as an oil (11 mg). EI-MS *m/z*: 482 (M^+), 440, 398. ^{1}H-NMR δ ($CDCl_3$): 1.97 (3H, s, –OAc), 2.08 (3H, s, –OAc), 3.33 (2H, d, J=7.0 Hz), 3.36 (2H, d, J=6.6 Hz), 3.40 (2H, d, J=6.6 Hz), 5.04—5.12 (6H, m), 5.86—6.02 (3H, m) (allyl protons), 6.82 (1H, d, J=2.2 Hz, H-3), 6.87 (1H, d, J=2.2 Hz, H-5), 6.94 (2H, d, J=8.8 Hz, H-2′, 6′), 7.08 (1H, d, J=8.1 Hz, H-6″), 7.13 (2H, d, J=8.8 Hz, H-3′, 5′), 7.15 (1H, d, J=2.2 Hz, H-3″), 7.21 (1H, dd, J=8.1, 2.2 Hz, H-5″).

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Saponins from *Amaranthus hypochondriacus*

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Four triterpenoid saponins were isolated from *Amaranthus hypochondriacus* which are grain crops in the Nepal, Mexico and South America. Their structures were elucidated based on spectral evidence to be: (1) 3-*O*-α-L-rhamnopyranosyl(1→3)-β-D-glucuronopyranosyl-2β,3β-dihydroxyolean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester. (2) 3-*O*-α-L-rhamnopyranosyl(1→3)-β-D-glucuronopyranosyl-2β,3β-dihydroxyolean-12-en-23-al-28-oic acid 28-*O*-β-D-glucopyranosyl ester; (3) 3-*O*-α-L-rhamnopyranosyl(1→3)-β-D-glucuronopyranosyl-2β,3β-dihydroxy-30-norolean-12,20(29)-dien-28-oic acid 28-*O*-β-D-glucopyranosyl ester. (4) 3-*O*-α-L-rhamnopyranosyl(1→3)-β-D-glucuronopyranosyl-2β,3β-dihydroxy-30-norolean-12,20(29)-dien-23-al-28-oic acid 28-*O*-β-D-glucopyranosyl ester.

Keywords *Amaranthus hypochondriacus*; Amaranthaceae; amaranthus-saponin; oleanane triterpenoid; food source

Several amaranth species have been cultivated in the Old and New World since ancient times as grain crops, pot herbs, ornamentals and dye plants.[1] The potential of both grain and vegetable amaranth as a food resource has been reviewed extensively by Teutonico and Knorr.[2] Amaranth was a major grain in the pre-conquest Aztec empire.[3] Ancient Mexicans formed idols out of a dough made seeds of the crop they called "huahtli", which has been identified as grain amaranth.[4] Recently, because of its excellent nutritional quality, there has been renewed interest in amaranth as a vegetable food source. The crude protein content of grain amaranth ranges from 12.5 to 17.6% dry matter, this is higher than in most common grains except soybeans.[5]

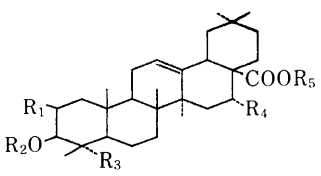
This paper deals with the isolation and structural elucidation of saponins from grain of *Amaranthus hypochondriacus* (Amaranthaceae) which was cultivated in Japan.

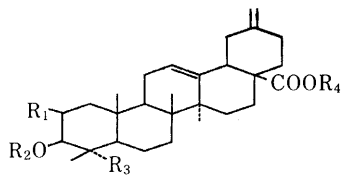
The grains were extracted with 80% methanol, and the extract was subjected to column chromatography on highly porous polymer by successively eluting with 30% methanol, 60% methanol, methanol and chloroform. The methanol eluate was suggested to be a complex mixture of a number of saponins, based on a thin layer chromatographic examination. The eluate was separated by chromatography on silica gel followed by repeated column chromatography on a Lichroprep RP-18, and final purification was carried out by high performance liquid chromatography (HPLC) on reversed-phase material (RP-18) affording four new saponins, amaranthus-saponins I—IV (**1**—**4**).

Amaranthus-saponin I (**1**) showed an $[M-H]^-$ ion at *m/z* 955 on the negative fast-atom bombardment mass spectrum (FAB-MS), indicating its molecular weight to be 956. Compound **1** afforded a sapogenin (**5**), D-glucuronic acid, D-glucose and L-rhamnose on acid hydrolysis. The ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectrum of **1** revealed the presence of two oxygenated carbons (δ 69.6 and 90.5), a pair of olefinic carbons (δ 123.3 and 144.0), one ester carbon (δ 176.4) and three anomeric carbons (δ 95.7, 102.4 and 104.6). It was found that the ^{13}C-NMR signals of **5** were composed of those due to the carbons of the A ring of asterogenic acid (**6**), isolated from *Aster tataricus*,[6] and those due to the carbons of B, C, D and E rings of oleanolic acid,[7] leading to the identification of **5** as 2β-hydroxyoleanolic acid. The ^{13}C-NMR spectrum of **1** indicated that **1** is a 3,28-*O*-bisglycosylated 2β-hydroxyoleanolic acid.

On treatment with an anhydrous LiI–2,6-lutidine–methanol reagent,[8] compound **1** yielded an anomeric mixture of methyl glucoside by comparison of its ^{13}C-NMR data with those of an authentic sample and a prosapogenin (**7**).

Compound **7** was converted into a methyl ester with CH_2N_2 followed by treatment with $NaBH_4$ in MeOH. Reduced **7** was subjected to permethylation followed by methanolysis. The resulting methylated alditol mixture was





	R_1	R_2	R_3	R_4	R_5
1	OH	-glcUA^{3}rha	CH_3	H	-glc
2	OH	-glcUA^{3}rha	CHO	H	-glc
5	OH	H	CH_3	H	H
6	OH	H	CH_3	OH	CH_3
7	OH	-glcUA^{3}rha	CH_3	H	H
8	H	-glcUA^{2}glc	CHO	H	-xyl^{2}rha^{4}xyl^{3}xyl
9	OH	-glcUA^{3}rha	CHO	H	H

	R_1	R_2	R_3	R_4
3	OH	-glcUA^{3}rha	CH_3	-glc
4	OH	-glcUA^{3}rha	CHO	-glc
10	H	-ara	CH_3	-glc^{6}glc^{4}rha

glcUA : β-D-glucuronopyranosyl
rha : α-L-rhamnopyranosyl
glc : β-D-glucopyranosyl
ara : α-L-arabinopyranosyl
xyl : β-D-xylopyranosyl

Chart 1

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acetylated with Ac_2O–C_5H_5N. The sugar sequence analysis by gas chromatography-mass spectrometory (GC-MS) showed a 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-glucitol and 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-rhamnitol.[9]

The structure of **1** was concluded to be 3-*O*-α-L-rhamnopyranosyl(1→3)-β-D-glucuronopyranosyl-2β,3β-dihydroxyolean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester.

Amaranthus-saponin II (**2**) showed an $[M-H]^-$ ion at *m/z* 969, indicating its molecular weight to be 970. The ^{13}C-NMR and ^{1}H-NMR spectra of **2** indicated that **2** has an aldehyde group (^{13}C-NMR: δ 206.2 and ^{1}H-NMR: δ 9.60 (1H, s)) and six methyl groups. Their ^{13}C chemical shifts were like those of **1**, except for the appearance of an aldehyde group and loss of one methyl gruop. In the ^{13}C-NMR spectrum of **2**. C-2, C-3, C-5, C-10 and C-24 signals were shifted upfield by 1.7, 5.9, 6.4, 0.7 and 7.2 ppm, respectively, and C-4 and C-6 were shifted downfield by 15.6 and 1.6 ppm, respectively, in comparison with those of **1**. The above mentioned difference resembled those between chikusetsusaponin V[10] and thladioside H1[11] (**8**). These results suggested that an aldehyde group of **2** is located at C-23. Compound **2** yielded and anomeric mixture of methyl glucoside and a prosapogenin (**9**) by selective cleavage of the ester–glycoside linkage (*vide supra*). The sugar linkage of C-3 of **9** exhibited a good coincidence with those of **7** in the ^{13}C-NMR spectrum and these date were supported by the result of sugar sequence analysis of **9** by GC–MS.

The structure of **2** was concluded to be 3-*O*-α-L-rhamnopyranosyl(1→3)-β-D-glucuronopyranosyl-2β,3β-dihydroxyolean-12-en-23-al-28-oic acid 28-*O*-β-D-glucopyranosyl ester.

The negative FAB-MS of amaranthus-saponin III (**3**) exhibited an $[M-H]^-$ ion at *m/z* 939, indicating its molecular weight to be 940. On acid hydrolysis, **3** afforded D-glucose, L-rhamnose and D-glucuronic acid, while a genuine aglycone of this saponin was not obtained owing to the acid-catalyzed modification.[12] The ^{13}C-NMR spectrum suggested that **3** is a noroleanane type-triterpenoid glycoside. Carbon signals observed at δ 143.4, 122.9, 148.5 and 107.3 and proton signals at δ 5.39 (1H, t) and 4.74 (2H, s) showed the presence of one trisubstituted double bond and one *exo*-methylene group in **3**.

It was found that the ^{13}C-NMR spectrum of **3** was composed of signals due to the carbons of the A, B and C rings of **1**, and those due to the carbons of D and E rings of ciwujianoside C1[13] (**10**). Furthermore, in the ^{13}C-NMR spectrum of **3**, the signals due to sugar moieties were found to be almost superimposable on those of **1**. These observations led to the formulation of **3** as 3-*O*-α-L-rhamnopyranosyl(1→3)-β-D-glucuronopyranosyl-2β,3β-dihydroxy-30-norolean-12,20(29)-dien-28-oic acid 28-*O*-β-D-glucopyranosyl ester.

The negative FAB-MS of amaranthus-saponin IV (**4**) showed an $[M-H]^-$ ion at *m/z* 953, indicating its molecular weight to be 954. The ^{13}C-NMR and ^{1}H-NMR spectrum of **4** indicated that **4** has one aldehyde group (^{13}C-NMR: δ 206.2 and ^{1}H-NMR: δ 9.63 (1H, s)) and one *exo*-methylene group (^{13}C-NMR: δ 148.3, δ 107.3 and ^{1}H-NMR: δ 4.73 (2H, s)). It was found that carbon signals of **4** were composed of signals from the carbons of the A, B and C rings of **2** and those from the carbons of the D and E rings of **3**. Furthermore, the carbon signals caused by the sugar moieties of **4** were found to be almost superimposable on those of **1**, leading to the structure of **4** as 3-*O*-α-L-rhamnopyranosyl(1→3)-β-D-glucuronopyranosyl-2β,3β-dihydroxy-30-norolean-12,20(29)-dien-23-al-28-oic aicd 28-*O*-β-D-glucopyranosyl ester.

Recently, Namba and his colleagues[14] have reported the inhibitory effect of oleanolic acid saponins obtained from *Anemone flaccida* on the reverse transcriptase from a ribonucleic acid (RNA) tumor virsus. Compounds **1**, **2**, **3** and **4** (10^{-4} M) did not have inhibitory activity of reverse transcriptase of the avian myeloblastosis virus (AMV) in the presence of poly (rA) and oligo $(dT)_{12-18}$ activated calf thymus deoxyribonucleic acid (DNA) and 70S RNA template primer.

TABLE I. ^{13}C-NMR Chemical Shifts of Compounds **1**, **2**, **3**, **4** and Related Compounds in C_5D_5N

Carbon No.	Oleanolic acid	1	2	3	4	5	6	8	10
Aglycone moiety									
1	38.9	45.1	45.0	44.2	44.9	45.0	45.0	38.0	38.9
2	28.2	69.6	67.9	69.8	68.0	71.5	71.4	25.1	26.5
3	78.0	90.5	84.6	89.6	84.5	78.4	78.3	82.0	88.7
4	39.4	38.8	54.4	39.0	53.3	38.8	38.8	54.9	39.5[a]
5	55.8	56.0	49.6	56.0	48.3	56.0	56.0	48.6[a]	55.9
6	18.8	18.5	20.1	18.2	20.1	18.7	18.6	20.6	18.5
7	33.3	33.1	32.4	33.1	32.4	33.4	33.5	32.5	33.0
8	39.8	40.1	40.2	40.0	40.2	40.0	40.0	40.1	39.9[a]
9	48.1	48.4	48.4	48.3	47.9	48.6	47.6	47.8[a]	48.0
10	37.4	37.1	36.4	37.0	36.3	37.4	37.4	36.2	36.9
11	23.8	23.4	23.3	23.5	23.4	23.8	23.9	23.6	23.6[b]
12	122.5	123.3	122.8	122.9	122.4	123.0	122.8	122.5	123.3
13	144.8	144.0	144.1	143.4	143.3	144.9	144.4	144.0	143.2
14	42.0	42.3	42.3	42.2	42.2	42.4	42.1	42.2	41.6
15	28.3	28.1	28.1	28.1	28.0	28.3	35.9	28.4	28.2
16	23.8	24.0	24.0	23.8	23.9	24.0	74.4	23.6	23.5[b]
17	46.7	47.0	46.9	47.3	47.2	46.7	49.1	47.2	47.3[c]
18	42.0	41.7	41.7	47.6	47.5	42.1	41.3	41.9	47.5[c]
19	46.7	46.2	46.1	41.6	41.6	46.5	47.0	46.7	42.0
20	31.0	30.7	30.7	148.5	148.3	31.0	30.8	30.7	148.3
21	34.3	34.0	33.9	30.1	30.0	34.3	35.9	34.1	30.4
22	33.3	32.6	32.5	37.6	37.6	33.3	32.5	32.5	37.8
23	28.7	29.9	206.2	29.4	206.2	30.3	30.2	210.0	28.2
24	16.5	18.5	11.3	18.5	11.3	18.2	18.1	10.0	16.9
25	15.5	16.8	17.1	16.4	17.0	16.7	16.7	15.7	15.6
26	17.5	17.7	17.6	17.5	17.6	17.5	17.3	17.3	17.4
27	26.2	26.1	26.1	26.1	26.0	26.3	27.2	25.8	26.0
28	180.2	176.4	176.4	175.7	175.7	180.2	177.7	176.4	175.7
29	33.3	33.1	33.1	107.3	107.3	33.3	33.1	33.1	107.4
30	23.8	23.6	23.6			23.8	24.6	23.6	
OMe							51.7		
Sugar moiety									
3-*O*-Sugar glcUA-1		104.6	103.7	106.7	103.8				
		75.6	74.9	75.5	74.2				
		81.7	81.5	82.1	81.5				
		71.8	71.6	71.6	71.6				
		76.1	76.1	77.6	76.2				
		176.1	176.4	175.7	175.6				
rha-1		102.4	102.4	102.9	102.4				
		72.5	72.5	71.1	72.5				
		72.8	72.7	72.8	72.8				
		74.2	74.2	74.1	74.2				
		69.6	69.6	69.8	69.6				
		18.6	18.6	18.6	18.6				
28-*O*-Sugar glc-1		95.7	95.7	95.8	95.8				
		74.1	74.1	74.0	74.0				
		79.2	79.2	79.3	79.2				
		71.1	71.1	72.5	71.1				
		78.8	78.8	78.8	78.8				
		62.2	62.2	62.2	62.3				

a—*c*) Assignments may be interchanged in each column.

Experimental

All melting points were measured on a Yanaginoto micro melting points apparatus and are uncorrected. ^{1}H-NMR 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. GC-MS was taken on a JEOL JMS-SX 102 spectrometer: column, Neutra Bond-1 25 m × 0.25 mm i.d. 0.4 μm, ionizing volt 70 eV, accelerating volt 1.5 kV, carrier gas He 1 ml/min, injection temp. 200 °C, column temp. 100—200 °C, separator temp. 250 °C; for column chromatography, Kieselgel 60H (Art. 7736, Merck), LiChroprep RP-18 (25—40 μm, Merck) and Diaion HP-20 (Mitsubishi Chem. Ind. Co., Ltd., Tokyo, Japan) were used. All solvent systems for chromatography were homogeneous.

Extraction and Separation Air-dried grains of *Amaranthus hypochondriacus* (2 kg), cultivated in the Experimental Station of Medicinal Plants, Hiroshima University School of Medicine, were extracted with hot 80% aqueous MeOH. The 80% MeOH extract was concentrated and the residue (71.5 g) was chromatographed on a column of Diaion HP-20 by elution with 30% aqueous MeOH, 60% squeous MeOH, MeOH, and finally, with $CHCl_3$.

A saponin mixture (8.2 g) eluted with MeOH was separated by chromatography on a silica gel column with $CHCl_3$–MeOH–H_2O (30 : 8 : 1, 30 : 12 : 2, 30 : 17 : 3, 30 : 20 : 5, 30 : 24 : 6 and 30 : 36 : 15 successively) to give 48 fractions (Fr. 1—48), in order of elution. Fr. 37 was applied to combination of reverse phase column, Lichroprep RP-18 (solvent: 65% MeOH) and preparative HPLC (TSK-gel ODS-120-T(7.6 × 250 mm i.d.), UV_{210} flow rate: 2.5 ml/min, solvent: 70% MeOH (0.05% trifluoroacetic acid) to afford four saponins, called amaranthus-saponins I—IV. Each saponin and prosapogenin was treated with diazomethane for elementary analysis.

I (**1**): A white powder, $[\alpha]_D$ +23.3° (c=0.43, pyridine). Negative FAB-MS $[M-H]^-$ m/z 955. ^{1}H-NMR (C_5D_5N) δ: 0.85, 0.89, 1.12, 1.22, 1.29, 1.30, 1.49 (each 3H, s), 1.71 (1H, d, CH_3 of rhamnose), 4.78 (1H, d, J=7.8 Hz, anomeric H of glucuronic acid), 6.24 (1H, s, anomeric H of rhamnose), 6.28 (1H, d, J=7.9 Hz, anomeric H of glucose), 5.43 (1H, br s, H-12). ^{13}C-NMR (C_5D_5N) data of **1** are given in Table I. Amaranthus-saponin I methyl ester: *Anal.* Calcd for $C_{49}H_{78}O_{19}\cdot 2H_2O$: C, 58.43; H, 8.21. Found: C, 58.37; H, 8.11.

II (**2**): A white powder, $[\alpha]_D$ +9.2° (c=0.87, MeOH). Negative FAB-MS $[M-H]^-$ m/z 969. ^{1}H-NMR (C_5D_5N) δ: 0.85, 0.89, 1.08, 1.19, 1.51, 1.56 (each 3H, s), 1.66 (3H, d, CH_3 of rhamnose), 4.74 (1H, d, J=7.9 Hz, anomeric H of glucuronic acid), 6.18 (1H, s, anomeric H of rhamnose), 6.27 (1H, d, J=7.9 Hz, anomeric H of glucose), 5.40 (1H, br s, H-12), 9.60 (1H, s, CHO). ^{13}C-NMR data are given in Table I. Amaranthus-saponin II methyl ester: *Anal.* Calcd for $C_{49}H_{76}O_{20}\cdot 3H_2O$: C, 56.63; H, 7.95. Found: C, 56.59; H, 8.01.

III (**3**): A white powder, $[\alpha]_D$ +22.0° (c=0.41, pyridine). Negative FAB-MS $[M-H]^-$ m/z 939. ^{1}H-NMR (C_5D_5N) δ: 1.09, 1.24, 1.27, 1.34, 1.44 (each 3H, s), 1.71 (3H, d, CH_3 of rhamnose), 4.97 (1H, d, J=8.0 Hz, anomeric H of glucuronic acid), 6.26 (1H, d, J=8.1 Hz, anomeric H of glucose), 6.31 (1H, s, anomeric H of rhamnose), 4.74 (2H, br s, $=CH_2$), 5.39 (1H, t, H-12). ^{13}C-NMR data are given in Table I. Amaranthus-saponin III methyl ester: *Anal.* Calcd for $C_{48}H_{74}O_{19}\cdot 2H_2O$: C, 58.17; H, 7.93. Found: C, 58.09; H, 7.90.

IV (**4**): A white powder, $[\alpha]_D$ +71.9° (c=0.32, MeOH). Negative FAB-MS $[M-H]^-$ m/z 953. ^{1}H-NMR (C_5D_5N) δ: 1.06, 1.16, 1.50, 1.56 (each 3H, s), 4.79 (1H, d, J=7.9 Hz, anomeric H of glucuronic acid), 6.22 (1H, s, anomeric H of rhamnose), 6.24 (1H, d, J=8.1 Hz, anomeric H of glucose), 4.73 (2H, br s, $=CH_2$), 5.40 (1H, br s, H-12), 9.63 (1H, s, CHO). ^{13}C-NMR data are given in Table I. Amaranthus-saponin IV methyl ester: *Anal.* Calcd for $C_{48}H_{72}O_{20}\cdot 2H_2O$: C, 57.36; H, 7.62. Found: C, 57.41; H, 7.58.

Selective Cleavage of the Ester Glycosyl Linkages of 1 and 2 A solution of **1** (42.0 mg), anhydrous LiI (50.0 mg) and 2,6-lutidine (1.2 ml) in anhydrous MeOH was refluxed for 40 h at 140 °C under N_2 stream. After cooling, the reaction mixture was diluted with 50% MeOH (6 ml), neutralized with Amberlite MB-3 resin and evaporated to dryness. The residue was chromatographed on a column of Diaion HP-20 (30% MeOH and MeOH) to afford **7** (12 mg) and methyl (α and β)-D-glucopyranosides, identified by the ^{13}C-NMR spectrum data. By the same method, **2** (38 mg) gave **9** (10 mg) and an anomeric mixture of methyl glucopyranoside.

Compound **7**: A white powder, $[\alpha]_D$ +5.6° (c=0.36, pyridine). ^{1}H-NMR (C_5D_5N) δ: 0.93, 0.98, 1.02, 1.27, 1.32, 1.34, 1.43 (each 3H, s), 1.71 (3H, d, CH_3 of rhamnose), 4.95 (1H, d, J=7.7 Hz, anomeric H of glucuronic acid), 6.35 (1H, s, anomeric H of rhamnose), 5.45 (1H, br s, 12H). Compound **7** methyl ester: *Anal.* Calcd for $C_{44}H_{70}O_{14}\cdot H_2O$: C, 62.83; H, 8.63. Found: C, 62.89; H, 8.57.

Compound **9**: A white powder, $[\alpha]_D$ +22.9° (c=0.35, pyridine). ^{1}H-NMR (C_5D_5N) δ: 0.96, 1.00, 1.01, 1.31, 1.47, 1.67 (each 3H, s), 1.72 (3H, d, CH_3 of rhamnose), 4.91 (1H, d, J=7.7 Hz, anomeric H of glucuronic acid), 6.27 (1H, s, anomeric H of rhamnose), 5.46 (1H, br s, 12-H), 9.69 (1H, s, CHO). Compound **9** methyl ester: *Anal.* Calcd for $C_{44}H_{68}O_{15}\cdot 3H_2O$: C, 59.31; H, 8.37. Found: C, 59.20; H, 8.29.

Sequence Analysis by GC-MS Compound **1** was treated with excess ethereal diazomethane and the reaction mixture was allowed to stand overnight. The product was obtained after removal of the solvent followed by treatment with $NaBH_4$ (25 mg) in methanol at room temperature for 2 h. The reaction mixture was then neutralized with AcOH and subjected to column chromatography, affording the crude product which was purified by silica gel column chromatography to furnish the reduced product. To a solution of reduced product (5 mg) in dimethylformamide (DMSO, 300 μl) was added a saturated solution of NaH in DMSO (300 μl). The mixture was sonicated at room temperature for 1 h. To this mixture was added CH_3I (500 μl) under cooling and the mixture was further sonicated at room temperature for 30 min. The reaction mixture was diluted with H_2O and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O, dried, and concentrated to dryness. The residue was treated with 90% HCOOH (2 ml) at 100 °C for 1 h. The reaction mixture was evaporated to remove HCOOH. The esidue was treated with 10%-H_2SO_4 (2 ml) at 100 °C for 4 h. To the reaction mixture was added $BaCO_3$ and the resulting precipitate was filtered and washed with H_2O. The filtrate and washing were combined and concentrated, and 50% MeOH (2 ml) was added. To this solution was added $NaBH_4$ (25 mg). After standing at room temperature for 2 h, the mixture was acidified by passage through a column of Dowex 50W-X8 (H^+ form) and concentrated to dryness. Boric acid in the residue was removed by repeated (four times) co-distillation with MeOH. The resulting methylated alditol mixture was acetylated with Ac_2O–C_5H_5N (1 : 1, 1 ml) at room temperature overnight. The reagent was removed by co-distillation with n-hexane. The methylated alditol acetate mixture thus obtained was subjected to GC-MS.

Acid Hydrolysis of 1, 2, 3 and 4 A solution of **1** (10 mg) in 10% H_2SO_4–EtOH (1 : 1, 3.5 ml) was refluxed for 4 h. The reaction mixture was diluted with H_2O and then extracted with Et_2O. The Et_2O layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The H_2O layer was neutralized with Amberlite MB-3 ion exchange resin and evaporated to dryness. The resulting monosaccharides were trimethylsilylated with TMS and identified by gas-liquid chromatography (GLC) comparison with authentic samples.

Compound **1** afforded a aglycone (**5**), D-glucose, L-rhamnose and D-glucuronic acid. Compound **2** gave a aglycone, D-glucose, L-rhamnose and D-glucuronic acid. Compounds **3** and **4** afforded D-glucose, L-rhamnose and D-glucuronic acid, while a genuine aglycone of these saponins was not obtained owing to the acid-catalyzed modification.

Compound **5**: A white powder, $[\alpha]_D$ +28.6° (c=0.14, pyridine). ^{1}H-NMR (C_5D_5N) δ: 0.96, 1.02, 1.09, 1.28, 1.32, 1.38, 1.53 (each 3H, s), 3.33 (1H, m, 18-H), 3.45 (1H, d, J=4.0 Hz, 3-H), 4.40 (1H, m, 2-H), 5.53 (1H, br s, 12-H). ^{13}C-NMR data of **5** is listed in Table I. Compound **5** methyl ester: *Anal.* Calcd for $C_{31}H_{50}O_4$: C, 76.50; H, 10.36. Found: C, 76.63; H, 10.29.

AMV Reverse Transcriptase Assay The methods described by Nishio *et al.*[15] were modified to assay the reverse transcriptase activity of AMV.

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Synergistic Action of Phenolic Signal Compounds and Carbohydrates in the Induction of Virulence Gene Expression of *Agrobacterium tumefaciens*

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Virulence (*vir*) gene expression of *Agrobacterium tumefaciens* is activated by plant phenolic compounds such as α-hydroxyacetosyringone (HOAS), acetosyringone (AS), methyl syringate, coniferyl alcohol and sinapyl alcohol. Inositol was found to be a potentiating factor of *vir*-inducing activity, which enhanced the *vir*-inducing activity of AS and HOAS in a synergistic manner, in particular at a low concentration of AS and HOAS. Of the other sugars tested D-glucose, L-rhamnose, D-xylose and D-galacturonic acid, the main components of plant cell wall polysaccharides, remarkedly potentiated the *vir*-inducing activity of AS, indicating the cooperative action of the signal compounds and sugars in *Agrobacterium* infection to plants.

Keywords *Agrobacterium*; *vir* gene; induction; acetosyringone; α-hydroxyacetosyringone; synergism; signal compound; inositol; glucose

Agrobacterium tumefaciens is a soil pathogen harboring a tumor inducing (Ti) plasmid which contains the transferred deoxyribonucleic acid (T-DNA) region to be transferred and integrated into plant genomic DNA to form crown galls.[1] This process of gene transfer is extensively used as vectors to transfer foreign genes into plants.[2,3] Ti plasmid has several loci (*virA*, *-B*, *-C*, *-D*, *-E* and *-G*) in the virulence (*vir*) region and their expression is a prerequisite for T-DNA transfer and integration.[4,5] Although the overall process of T-DNA transformation is very complex and not completely clarified,[6,7] expression of *vir* genes is recognized as an early event in T-DNA transformation.[8]

The *vir* genes (*B*, *C*, *D*, *E* and *G*) were expressed at a high level during co-cultivation of plant cells with *Agrobacterium* and the factors that induced *vir* genes expression were shown to be plant cell metabolites.[8] Following this observation two phenolic compounds, acetosyringone (AS, 3′,5′-dimethoxy-4′-hydroxy-acetophenone) and α-hydroxyacetosyringone (HOAS, 3′,5′-dimethoxy-4′-hydroxy-α-hydroxy-acetophenone), were identified as *vir* gene inducing compounds from tobacco hairy root cultures.[9] Recently, methyl syringate was isolated and identified as a *vir*-inducing compound of the vine tree, and coniferyl and sinapyl alcohols as those of pea seedlings.[10,11] Several phenolic compounds having similar functional groups to AS were reported to induce *vir* gene expression and the structural requirement for *vir*-inducing compounds were also discussed.[9,12,13] As a part of the studies to identify the intrinsic *vir*-inducing compounds of plants other than tobacco, we investigated belladonna hairy root cultures for their *vir*-inducing compounds. During preliminary fractionation experiments of the culture medium of belladonna hairy roots, *vir*-inducing activity was found in rather non-polar fractions, whereas polar fractions which have no *vir*-inducing activity significantly enhanced the activity of non-polar fractions in a synergistic manner. It was of interest to clarify this synergism at the chemical level and we investigated the potentiating compounds from the culture medium of hairy roots. This paper reports the identification of inositol as a potentiator for *vir*-inducing activity of AS and HOAS, and also describes the potentiating activity of other carbohydrates.

Results

In our studies on *vir*-inducing factors of belladonna hairy root cultures HOAS and AS were identified as active compounds.[14] In contrast to tobacco hairy root cultures however HOAS was the major *vir*-inducing factor in belladonna hairy roots.[14] During the course of fractionation and isolation of *vir*-inducing compounds it was found that the activity of a fraction containing AS and HOAS was less than that of the original activity of culture medium. A rather polar fraction having no *vir*-inducing activity enhanced significantly the activity of *vir*-inducing factors. A concentrated solution of Murashige–Skoog's (MS) medium which was used as a reference for the concentrated medium of belladonna hairy root cultures also showed the potentiating activity. Following this finding the stock solutions of MS medium which are routinely used for plant tissue cultures were tested for their potentiating activity for AS and HOAS. The potentiating activity was found in the organic stock solution of MS medium and we tested each constituent for its potentiating activity. Inositol was finally identified as the compound responsible for enhancing the *vir*-inducing activity of signal compound (Table I).

To clarify the optimum concentration of inositol in enhancing the *vir*-inducing activity of AS and HOAS *vir*-inducing activity was measured at various concentrations of inositol and the results appear in Fig. 1. The concentration of inositol to elicit the maximum activity of AS and HOAS

TABLE I. Detection of Inositol as a Potentiator for *vir*-Inducing Activity of AS

Treatment	β-Galactosidase activity (units)
AS 25 μM	254 (32)
AS 25 μM + MS soln. 1—4[a] 0.15 ml	244 (50)
AS 25 μM + MS soln. 1—4 0.3 ml	220 (42)
AS 25 μM + MS soln. 1—4 0.5 ml	230 (45)
AS 25 μM + MS soln. 5[b] 0.1 ml	500 (117)
AS 25 μM + inositol 5 mM	490 (85)
AS 0 μM + inositol 10 mM	0

The *vir* gene expression assays were carried out as described in Experimental. MS stock solution was sterilized by filtering. The numbers in parentheses were average deviation. *a*) Murashige–Skoog plant tissue culture medium (inorganic stock solution ×10) containing Murashige-Skoog plant salt mixture. *b*) Murashige–Skoog plant tissue culture medium (organic stock solution ×200) containing inositol, nicotinic acid, pyridoxine, thiamine and glycine; and only inositol has shown the potentiating effect.

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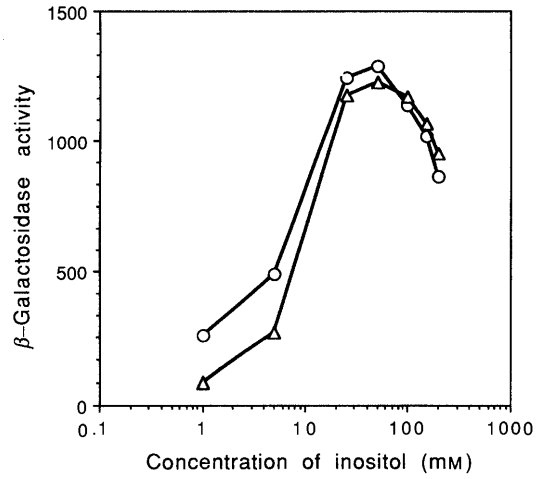

Fig. 1. Enhancement of *vir*-Inducing Activity of AS/HOAS by Inositol in Different Concentration

—○—, AS (25 μM); —△—, HOAS (25 μM).

TABLE II. Effects of Inositol on *vir*-Inducing Activity of Phenolics

Test compound	Inositol	Concentration of test compound				
		1 μM	5 μM	25 μM	50 μM	100 μM
AS	0	2 (2)	30 (20)	254 (32)	471 (84)	788 (189)
AS	50 mM	21 (0)	559 (80)	1290 (245)	1327 (205)	1377 (125)
HOAS	0	0	4 (1)	64 (30)	170 (41)	420 (123)
HOAS	50 mM	21 (25)	433 (128)	1105 (167)	1250 (193)	1279 (102)
Syringic acid	0	ND	0	12 (4)	16 (7)	24 (15)
Syringic acid	50 mM	ND	0	30 (11)	131 (58)	301 (117)
2,4-Dihydroxy-acetophenone	0	ND	0	0	0	0
2,4-Dihydroxy-acetophenone	50 mM	ND	0	0	0	0

The values were units of β-galactosidase activity. Assays were conducted as described in Experimental. The numbers in parentheses were average deviation. No β-galactosidase activity was detected in the control in which only 50 mM of inositol was co-incubated with bacteria. ND: not determined.

was 50 mM when the dose dependent experiments were carried out at a fixed concentration of 25 μM for AS and HOAS. The *vir*-inducing compounds induce *vir* gene expression in a lower concentration range (μM), whereas the potentiation of *vir*-inducing activity by inositol requires a higher concentration range (mM).

Some phenolic compounds other than AS and HOAS were reported to have *vir*-inducing activity.[9,12,13] We chose AS as a high *vir*-inducing compound, HOAS as a medium *vir*-inducing compound, syringic acid as a low *vir*-inducing compound, and 2,4-dihydroxyacetophenone as the compounds having no *vir*-inducing activity and tested the potentiating effect of inositol on these phenolics (Table II). 2,4-Dihydroxyacetophenone was chosen because it is an acetophenone derivative as AS and HOAS. The *vir*-inducing activity of AS, HOAS and syringic acid was enhanced 10—100 folds in the presence of inositol. The potentiation of *vir*-inducing activity was very high at a lower concentration of AS and HOAS. Though AS induced *vir* gene expression several times higher than HOAS, the difference of *vir*-inducing activity disappeared in the presence of 50 mM inositol, indicating that inositol acted to elicit the maximum activity of AS and HOAS. Syringic acid has a low *vir*-inducing activity without inositol, however its activity was enhanced to a higher level by the co-operation of inositol. Inositol gave no effect to 2,4-dihydroxyacetophenone which has no activity of *vir* gene expression. The results clearly indicate that inositol is a potentiating factor for enhancing the *vir*-inducing activity of signal compounds in a synergistic manner.

TABLE III. Effects of Carbohydrates on *vir*-Inducing Activity of AS

Carbohydrate	β-Galactosidase activity (units)	
	25 mM	50 mM
None	254 (32)	254 (32)
D-Xylose	1533 (177)	1249 (137)
L-Rhamnose	1597 (134)	1449 (141)
L-Fucose	226 (23)	246 (36)
D-Fucose	1343 (52)	1277 (13)
D-Glucose	1357 (157)	1274 (157)
L-Arabinose	912 (226)	841 (224)
D-Mannose	798 (192)	750 (177)
D-Fructose	264 (32)	249 (16)
L-Sorbose	110 (15)	121 (17)
Sucrose	288 (26)	266 (48)
Maltose	1201 (186)	1077 (163)
Lactose	841 (1)	703 (17)
Trehalose	222 (5)	222 (36)
Cellobiose	772 (91)	810 (1)
Raffinose	279 (35)	287 (17)
Dulcitol	586 (200)	754 (220)
D-Mannitol	224 (25)	186 (20)
D-Sorbitol	244 (17)	200 (21)
Inositol	1245 (260)	1290 (245)
D-Galacturonic acid	1445 (34)	1205 (44)
D-Gluconic acid	1477 (17)	902 (144)

Carbohydrates were added to induction media with 25 μM of AS and cocultivated with bacteria. Induction assay was carried out as described in Experimental. The numbers in parentheses were average deviation.

Next we examined the potentiating effect of various kinds of carbohydrates, since inositol is a cyclic polyol but is usually regarded as a member of the carbohydrates that are widely distributed among plants (Table III). Several carbohydrates exhibited potentiating activity against the *vir*-induction by AS, though they did not induce *vir* gene expression by themselves.

In monosaccharides, aldoses such as D-glucose, L-arabinose, L-rhamnose, D-fucose and D-mannose enhanced the *vir*-inducing activity of AS, while ketoses such as D-fructose and L-sorbose showed no activity. D-Galacturonic acid and D-gluconic acid also showed the potentiating effect of *vir*-inducing activity of AS at a level comparable to inositol. The potentiation of *vir*-inducing activity was not limited to monosaccharides, as reducing disaccharides having 1,4-glycosidic linkage, maltose, lactose and cellobiose, also had the potentiating effect. The effect on the disaccharides was not caused by glucose formed by hydrolysis, since glucose was not detected in the medium of *Agrobacterium* which had been incubated with disaccharides (data not shown). Of the polyol tested, D-mannitol and D-sorbitol were not active, and inositol, cyclic polyol, and dulcitol, linear polyol, enhanced *vir*-inducing activity. However, the potentiating effect of dulcitol was much less compared to that of inositol.

It is of interest to clarify the mechanism of action of the potentiating activity. As mentioned in the experimental section the *vir*-inducing activity was monitored by measuring β-galactosidase activity of *Agrobacterium* which was cultured in the presence of AS or HOAS with inositol.

TABLE IV. Effects of Inositol on *vir*-Inducing Activity of AS and HOAS

Treatment	β-Galactosidase activity (units)
Inositol without AS/HOAS	0
AS without inositol	254 (32)
AS with inositol (i)	1290 (245)
AS with inositol (ii)	250 (43)
AS with inositol (iii)	264 (35)
HOAS without inositol	64 (30)
HOAS with inositol (i)	1105 (167)
HOAS with inositol (ii)	70 (24)
HOAS with inositol (iii)	68 (35)

The *vir* gene expression assays were conducted in 25 μM of AS/HOAS and 50 mM of inositol as described. The numbers in parentheses were average deviation. (i): inositol was added to the mixture of AS/HOAS and bacteria at the time when incubation started. (ii): inositol was added to the β-galactosidase reaction mixture after incubation with AS/HOAS was completed. (iii): AS, HOAS and inositol were separately incubated with bacteria during the incubation period, and then mixed with each other before β-galactosidase assay.

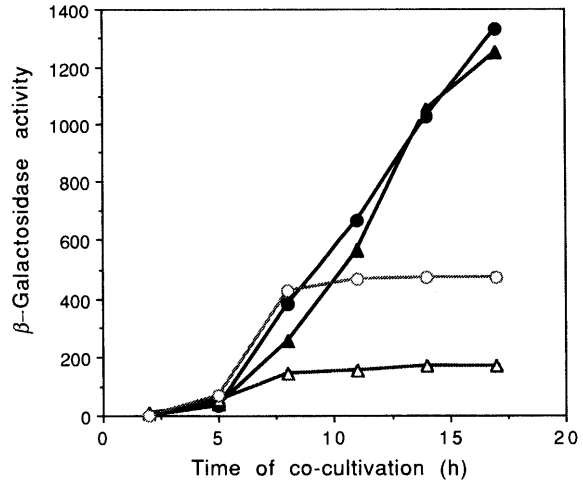

Fig. 2. Time Course of *vir* Gene Induction by AS/HOAS and Inositol

Each induction was stopped at the indicated time and β-galactosidase activity was measured. —▲—, HOAS (50 μM) + inositol (50 mM); —●—, AS (50 μM) + inositol (50 mM); —△—, HOAS (50 μM); —○—, AS (50 μm).

The enhancement of β-galactosidase activity might be caused by the direct effect of inositol to β-galactosidase in the incubation mixture of an enzyme assay. The other possibility was the synergistic action between *vir*-inducing compounds and *Agrobacterium*. AS/HOAS and inositol were incubated with *Agrobacterium* in two different experimental conditions, (i) and (ii) (Table IV). In experiment (i) AS/OHAS and inositol were added to the incubation medium of *Agrobacterium* to test the synergistic effect of inositol on the induction of β-galactosidase activity by the *vir*-inducing factors. Experiments (ii) were designed to test the direct enhancing action of inositol to β-galactosidase. Experiments (iii) were control incubations in which AS/HOAS and inositol were incubated separately. The co-cultivation of inositol with AS/HOAS and *Agrobacterium* was found to be essential for enhancing the *vir*-inducing activity which was measured as β-galactosidase activity. The β-galactosidase reaction itself was not enhanced by the addition of inositol and AS/HOAS.

In order to clarify time dependency in the synergistic effect of the potentiators and activators during induction of *vir* gene expression, the time-course of the *vir*-inducing activity of AS and HOAS was tested in the presence and absence of inositol (Fig. 2). Since β-galactosidase activity

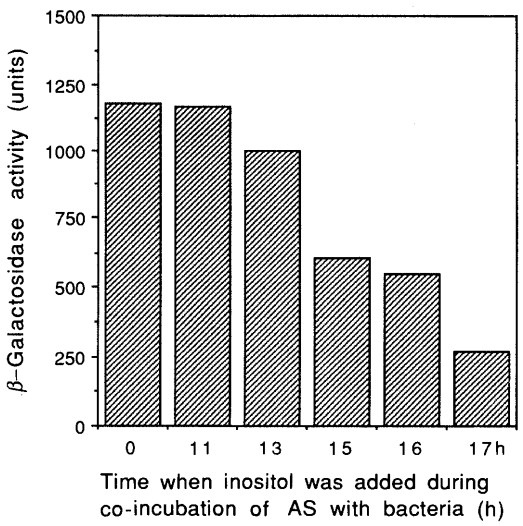

Fig. 3. Effect on *vir*-Inducing Activity of AS by Inositol at Various Incubation Times

Six tubes were incubated with AS (25 μM) for 17 h to induce *vir* gene. Inositol (50 mM) was added to each tube at the indicated time and induction was continued. 273 units were shown in the control in which no inositol was added.

was calculated as units per bacterial concentration (OD at 600 nm), indicated β-galactosidase activities were shown as units per bacterial concentration. The level of expression per bacterial concentration became maximum after 8 h of incubation without inositol, whereas the level of expression increased until the end of incubation (17 h) when inositol was present in the incubation mixture. This result demonstrates that inositol acted to remove the suppression that appeared after 8 h of incubation.

In the next experiments inositol was added at various times to the cultures of *Agrobacterium* which were incubated with AS from 0 h (Fig. 3). In the case of the addition at 11 h, β-galactosidase activity was the same as when it was added at the start of incubation. When inositol was added at 13, 15 and 16 h, the potentiating effects were respectively 85%, 51% and 47% of the maximum value. This result means that the suppression against *vir* gene activation by AS was partially removed by the addition of inositol at later periods of incubation.

Discussion

Some factors of plants induce the expression of *vir* genes which is a prerequisite for the transfer and integration of T-DNA into plant cells. This fact indicates that chemical signals from plant cells play very important roles in the early step of plant transformation, including the recognition of plant cells which are susceptible to the transformation by *Agrobacterium*. In this context inositol was identified as a potentiator of *vir*-inducing activity. Inositol can remarkably enhance the activity of *vir*-inducing compounds, though inositol itself can not induce the expression of *vir* genes. Although several *vir*-inducing compounds have been reported,[9,12,13] the synergistic effect between phenolics and sugars may indicate that plant factors related to the induction of *vir* gene expression were not limited to the *vir*-inducing compounds and that carbohydrates might also play a positive function in the process.

Our results demonstrated that many carbohydrates can outstandingly enhance the activity of *vir*-inducing com-

pounds. During the course of studies on *vir* gene inducing factors in monocotyledonous plants which are normally not susceptible to *Agrobacterium* infection, Machida *et al.* found that D-glucose, D-galactose and L-arabinose markedly enhanced the induction level of *vir* gene expression by AS.[15] A lower concentration (1 mM) of sugar also showed potentiation of *vir*-inducing activity at a high level[15] (our data not shown). It is important to note that most of the active sugars are widely present in plants and they would be responsible for plant transformation.

Wounded plant cells are known to be susceptible to *Agrobacterium* infection and the wounding of plant cells stimulates the production of *vir*-inducing compounds.[9,10] The results so far obtained by us and by Machida *et al.*[15] indicate that D-glucose, D-xylose, L-rhamnose, D-galactose, L-arabinose, D-galacturonic acid and cellobiose have the potentiating activity. It is worth noting here that they are the major constituents of plant cell wall[16] and that inositol phosphates, phytates, are also known to be contained as storage compounds in seeds and rapidly hydrolyzed at the onset of germination.[17] It is highly probable that these sugars are released from the cell wall by the action of induced enzymes when plant tissues are wounded. The *vir*-inducing signal compound is supplied by other metabolism and can synergistically activate the bacterium present close to the wounded cells.

As shown in Tables II and III, the potentiators, carbohydrates, showed a remarkable effect in enhancing the *vir*-inducing activity (10—100 fold), in particular, at a lower concentration of the *vir*-inducing compounds and to lower active compounds such as syringic acid. Although the *vir*-inducing compounds are supplied from wounded tissue, they are not always present at a high concentration enough to induce *vir* gene expression.[9,10] In some plants signal compounds of lower *vir*-inducing activity only may be available. The presence of carbohydrates as the potentiators provides the possibility that *vir* genes are effectively expressed by their synergistic effect to induce plant transformation even in unfavorable circumstances if the carbohydrates are not present.

The results shown in Figs. 2 and 3 are suggestive of the mechanism of the synergistic effect of sugars. When inositol was co-cultivated with AS and *Agrobacterium* from the beginning of incubation, the potentiating effect on *vir*-inducing activity lasted until the end of incubation (17 h) (Fig. 2). No practical difference was observed between AS and HOAS on the potentiating effect of inositol (Fig. 2 and Table III), indicating inositol elicits the maximum activity of AS and HOAS. When inositol was added to the incubation mixture later than 11 h, the final activities (at 17 h) of β-galactosidase were less than the maximum value observed in an experiment in which inositol was present from the beginning (Fig. 3). The potentiation of *vir*-inducing activity by inositol is interpreted as the result of the release of repression that was observed in the experiments without inositol. It has been pointed out that the *vir*-inducing compounds act to a receptor or sensor protein of Ti plasmid such as *virA* to trigger a secondary messenger system.[18] On the other hand carbohydrates also act to virA protein, since the periplasmic region of the virA protein is proven to be important in the potentiation by glucose.[19] These results suggest that sugars can release the repression of the signal transduction process starting from virA protein which is bound with the signal compound. Another series of experiments would be necessary to clarify the exact mechanism of this desaturation phenomenon caused by carbohydrates.

Experimental

D-Xylose, L-rhamnose, L-fucose, D-fucose, L-arabinose, D-mannose, D-glucose, D-fructose, L-sorbose, sucrose, maltose, lactose, raffinose, dulcitol, D-mannitol, inositol, D-galacturonic acid, D-gluconic acid and 2,4-dihydroxyacetophenone were purchased from Wako Pure Chemical Industries, Japan. Trehalose, cellobiose and syringic acid were purchased from Tokyo Kasei Kogyo Co., Ltd. AS was purchased from Aldrich Chem. Co. D-Sorbitol was purchased from Nakarai Kagaku Yakuhin Co., Ltd. HOAS was synthesized from AS as described in a previous paper.[10]

The phenolic compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted in induction buffer[10] to a final concentration of 0.4% DMSO. The carbohydrates were directly dissolved in the induction buffer. The solutions were sterilized by filtering.

A *Tn3–lacZ* transposon system in which the production of β-galactosidase (the *lacZ* gene product) is controlled by *virB* locus fused with *lacZ* is used in the detection and measurement of *vir*-inducing activity. We used *Agrobacterium* strain A348 pSM243 cd (contains *virB*: *lacZ* fusion plasmid) to monitor β-galactosidase activity as *vir*-inducing activity. Cultures of bacteria and *vir*-inducing assay were carried out as described in the other report.[10] Each value represents the average of the results of two to five independent experiments.

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Phospholipid-Binding Properties of Calphobindin-II(Annexin VI), an Anticoagulant Protein from Human Placenta

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Calphobindin-II (CPB-II, annexin VI), is a calcium dependent phospholipid binding protein that can be classified as a member of the annexin family.

The phospholipid-binding properties of CPB-II were investigated by measuring the binding constants of [^{125}I]-CPB-II using phospholipid vesicles consisting of 80% phosphatidylcholine and 20% phosphatidylserine. A dissociation constant (K_d) of CPB-II with the phospholipid vesicles was determined to be 0.2 to 0.3 nM in the presence of Ca^{2+} ranging from 0.3 to 30 mM. The number of CPB-II capable of binding to the phospholipid vesicles at 0.3 mM Ca^{2+} decreased to about 1/2 in the presence of Ca^{2+} of more than 1 mM. Prothrombin and factor X were effective in competing with the binding of CPB-II to the phospholipid vesicles, although their affinities were lower by two or three orders of magnitude than that of unlabeled CPB-II at 30 mM Ca^{2+}. Competitive effects of CPB-II, calphobindin-I (CPB-I, annexin V) and calphobindin-III (CPB-III, annexin III) on binding of [^{125}I]-CPB-II to phospholipid vesicles, were similarly observed.

Keywords calphobindin; annexin; phospholipid; blood coagulation; dissociation constant; calcium phospholipid binding protein; placental protein

Introduction

Phospholipid surfaces are recognized to play crucial roles in catalyzing procoagulant reactions of blood coagulation.[1,2] Stimulation of platelets with several substances has been reported to result in an increase of the capacity to expose acidic phospholipids on the extracellular surface of their cell membranes, correlating with an increase in the rate of thrombin generation by the prothrombinase complex.[3] Treatments of endothelial cells with endotoxin, interleukin-I or tumor necrosis factor have also been demonstrated to result in an increase in the amount of a tissue factor acting as an initiator for blood coagulation at the site of cell damage in association with membrane phospholipids.[4-8]

Surveys for plasma of patients suffering from autoimmune disorders led to the detection of an autoantibody to phospholipids, termed lupus anticoagulant, that acts as a spontaneously acquired inhibitor for blood coagulation.[9] Reacting with phospholipids,[10] lupus anticoagulant inhibits phospholipid-dependent coagulation reaction *in vitro*.[11] This finding suggests that the inhibitory mechanism of lupus anticoagulant is closely related to the differential affinities between the anticoagulant and blood coagulation factors for phospholipids. Similarly, calphobindins (CPBs) from human placenta have been shown to inhibit blood coagulation by their phospholipid-binding capacities in a Ca^{2+} dependent manner.[12-14]

In this paper, we report that CPB-II binds to phospholipid with a high affinity, and that this binding property is sufficient to account for its anticoagulant activity.

Materials and Methods

Materials 1-α-Phosphatidylcholine dipalmitoyl (PC), bovine brain 1-α-phosphatidyl-L-serine (PS), human prothrombin, and human factor X were obtained from Sigma Chemical Co., MO. (U.S.A.). [^{125}I]-Sodium iodide was from Du Pont de Nemours & Co., Inc., DE. (U.S.A.). Rabbit lung tissue factor (Lyoplastin) was a product of Mochida Pharmaceutical Co., Ltd., Tokyo. Human plasma was purchased from Ortho Diagnostic Systems Inc., NJ. (U.S.A.). Enzymobeads were obtained from Bio-Rad Lab., CA. (U.S.A.).

Protein Purification CPB-I, CPB-II, and CPB-III were prepared from term placetae as described previously.[12-14]

Bovine prothrombin and factor X were purified according to the methods of Hashimoto *et al.*[15]

[^{125}I]-Labeling of CPB-II For preparation of [^{125}I]-labeled CPB-II, 400 μl of CPB-II solution (2 mg/ml) was mixed with 100 μl of enzymobeads (1 vial/500 μl of water), 200 μl of 400 mM phosphate buffer (pH 7.2), 3 μl of 1 mM potassium iodide, 30 μl of [^{125}I]-sodium iodide (74 MBq/100 μl) and 50 μl of 2% β-D-glucose, and incubated for 20 min at 25 °C. The reaction mixture was centrifuged at 3000 × *g* for 1 min to precipitate enzymobeads. The supernatant was dialyzed against 5 mM phosphate buffer (pH 7.4) and stored at 4 °C.

Preparation of Phospholipid Vesicles PC (12.5 mg) and PS (3.125 mg) were dissolved in $CHCl_3$ and evaporated to dryness by blowing nitrogen gas. The lipid mixture was suspended in 5 ml of 50 mM Tris–HCl buffer (pH 7.4) containing 100 mM NaCl and sonicated three times in a sonicator (Microson, Heat Systems Ultrasonics Inc.) at 23 kHz for 30 s. The resultant phospholipid vesicles were centrifuged at 105000 × *g* for 10 min, and the precipitates were resuspended in 5 ml of the same buffer and again sonicated as above. Phospholipid vesicles thus obtained were stored in the buffer at 4 °C.

Assay for Coagulant Activity Coagulant activity was assayed by measuring modified prothrombin time (mPT) using Coagtec TE-600 (Erma Inc., Tokyo) as described previously.[12,13] In order to evaluate mPT in the presence of [^{125}I]-CPB-II, 100 μl of tissue factor (a stock solution diluted with 20 mM $CaCl_2$ to a final concentration of 100 μg/ml) was added to an equal volume of samples containing 2, 4, 6, 8, and 10 μg/ml of labeled or unlabeled CPB-II in Tris–HCl buffer (pH 7.4), respectively, and mixed well. For the control, 100 μl of the buffer was added. After incubation at 37 °C for 3 min, 200 μl of human plasma (2 fold-diluted with saline) was added to the mixture and incubated to measure the initiation time of clot formation. Tissue factor solutions (100 μl of 0.5 mg/ml) containing 5 to 70 mM $CaCl_2$ were used to examine the effects of Ca^{2+} concentrations on mPT in the presence of CPB-II (10 μg/ml).

Recalcification time was determined for measurement of the initiation time for clot formation in a reaction mixture (300 μl) consisting of 100 μl each of a sample containing CPB-II (10 μg/ml), human plasma and 10 to 70 mM $CaCl_2$ in a siliconized test tube (1 × 10 cm).

Binding Assay Binding assay was performed in a reaction mixture (a total volume of 2 ml) consisting of 400 μl of sample, 100 μl of [^{125}I]-CPB-II (1 nM), 500 μl of phospholipid vesicles (4 μg of phospholipid) and 1 ml of $CaCl_2$ (0.6 to 60 mM) in 50 mM Tris–HCl buffer (pH 7.4) containing 100 mM NaCl, 0.02% (w/v) NaN_3 and 0.1% (w/v) bovine serum albumin at 25 °C.

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Unlabeled CPB-II was diluted with the same buffer to the following concentrations (0, 25, 50, 75, 100, 150, 200, 300, 500, 1000 ng/ml corresponding to a final concentration of 0.05 to 2.75 nM) and added to the mixture in order to determine the dissociation constant of CPB-II. Effects of blood coagulation factors or CPBs on binding of CPB-II to phospholipid vesicles were investigated using the samples containing blood coagulation factor X (final concentrations of 2.7 to 400 nM), prothrombin (final concentrations of 3.2 nM to 3.2 μM), unlabeled CPB-II (final concentrations of 0.08 to 81 nM or 0.05 to 55 nM), and/or CPB-I and CPB-III (final concentrations of 0.12 to 60 nM). After incubation for 30 min, the mixtures were centrifuged at 105000 $\times g$ for 10 min to precipitate the phospholipid vesicles. The radioactivities of the supernatants and precipitates were determined with an Aloka ARG-251 autowell gamma system.

Determination of protein was performed by the method of Lowry *et al.*[16] using bovine serum albumin as the standard.

Results

Properties of [^{125}I]-CPB-II Thirty ng of [^{125}I]-CPB-II and 1 μg of CPB-II were mixed and then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of Laemmli.[17] After staining with Coomassie brilliant blue R-250, a single protein band was detected in the gel, which then was dried and sliced into 2 mm wide strips. The radioactivity of each strip was determined. Location of the radioactivity on the zone corresponding to the protein band in the gel indicated the comigration of native and iodinated CPB-IIs (data not shown).

Anticoagulant activity of labeled CPB-II was measured by the mPT method and compared with that obtained for unlabeled CPB-II. Similar dose-response curves were obtained with both samples under standard assay conditions, indicating that labeled CPB-II can be used for inhibition studies on blood coagulation (Fig. 1).

Effect of Ca^{2+} on Anticoagulant Activity of CPB-II Effect of Ca^{2+} concentrations on the anticoagulant activity of CPB-II was examined by measuring mPT and recalcification time as described in Materials and Methods. Curves of clotting time *versus* Ca^{2+} concentrations in Fig. 2 reveal that optimum Ca^{2+} concentrations minimizing clotting time are 20 and 25 mM in mPT and recalcification time assays, respectively. The ability of CPB-II to prolong the clotting times as determined by the two methods increased remarkably with Ca^{2+} concentrations lower than the optima, while the protein's clotting time-prolongation effect gradually decreased at Ca^{2+} concentrations above the optima.

Phospholipid Binding Capacity Binding of CPB-II to phospholipid vesicles has been demonstrated qualitatively

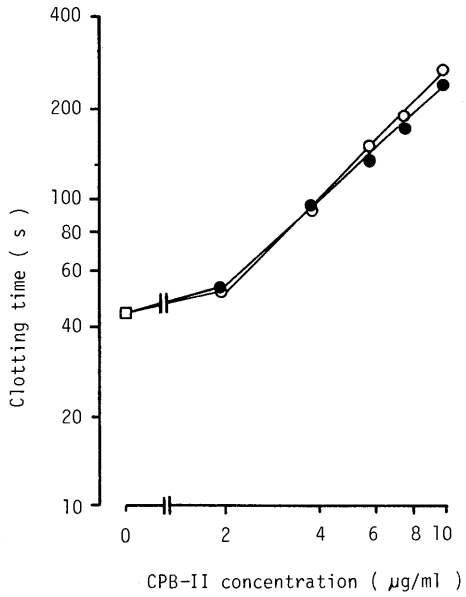

Fig. 1. Anticoagulant Activities of [^{125}I]-CPB-II and Unlabeled CPB-II on the mPT

A sample was mixed with tissue factor (0.1 mg/ml with 20 mM Ca^{2+}). After 3 min, coagulation was started by the addition of human plasma. [^{125}I]-CPB-II (●), unlabeled CPB-II (○).

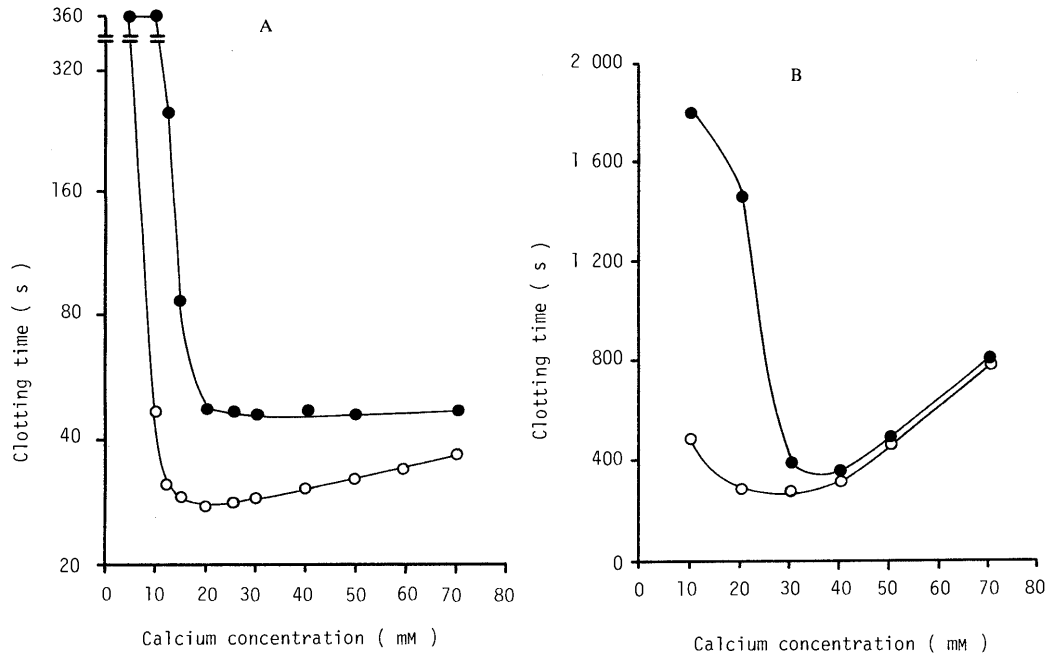

Fig. 2. The Effect of Ca^{2+} Concentrations on the mPT and the Recalcification Time with and without CPB-II

The mPT and the recalcification time were performed as described in Materials and Methods. A) The mPT, CPB-II (10 μg/ml) (●), none (○); B) the recalcification time, CPB-II (10 μg/ml) (●), none (○).

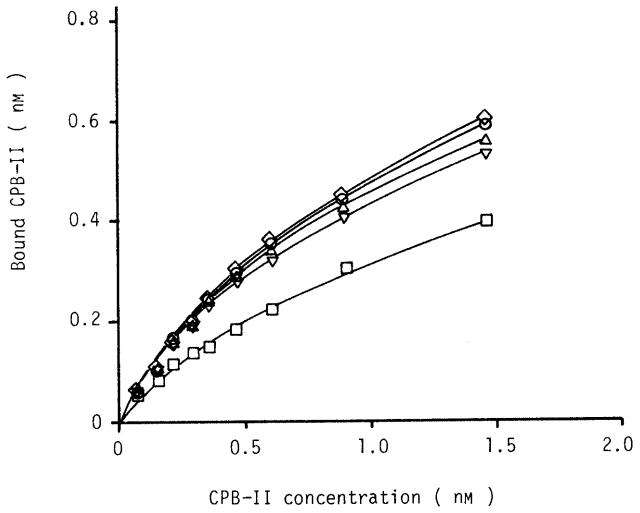

Fig. 3. The Influence of Ca^{2+} Concentrations on the Binding of CPB-II to Phospholipid Vesicles

[^{125}I]-CPB-II (0.05 nM), unlabeled CPB-II, and phospholipid vesicles (1 μg of phospholipid) were mixed with Ca^{2+} concentrations of 0.3 mM (□), 1 mM (▽), 3 mM (△), 10 mM (○), and 30 mM (◇). Dissociation constant and stoichiometry are given in Table I.

TABLE I. Determination of Dissociation Constant and Stoichiometry for Binding of CPB-II to Phospholipid Vesicles ($n=3$)

Ca^{2+} concentration (mM)	K_d (nM)	Stoichiometry (CPB-II nM/μg phospholipid)
0.3	0.33±0.13	0.39±0.10
1	0.25±0.07	0.82±0.16
3	0.24±0.03	0.85±0.32
10	0.21±0.04	0.92±0.30
30	0.24±0.06	0.85±0.24

Mean±S.D.

as reported previously.[13] In this work, further assay using [^{125}I]-CPB-II allowed quantitative analysis of the interaction of CPB-II with phospholipid vesicles. As revealed from the curves in Fig. 2A, B, changes in the anticoagulant activity of CPB-II at various Ca^{2+} concentrations might indicate the Ca^{2+} dependent binding of the protein to phospholipid vesicles. Figure 3 shows the curves plotting stoichiometry (the amounts [nM] of CPB-II capable of binding to phospholipid vesicles) against different concentrations (nM) of CPB-II in the presence of Ca^{2+} ranging from 0.3 to 30 mM. A dissociation constant (K_d) was approximated to be 0.2 to 0.3 nM from the curve plotting bound CPB-II (nM) against concentrations of CPB-II (nM) in the figure. Table I summarizes the K_d values (nM) and specific binding (nM of CPB-II/μg of phospholipid) of CPB-II at Ca^{2+} concentrations of 0.3 to 30 mM. The K_d values and the amounts of CPB-II capable of binding to phospholipid were shown to be relatively constant despite changes in Ca^{2+} concentrations of 1 to 30 mM. The binding capacity of CPB-II, however, decreased to about 1/2 at 0.3 mM Ca^{2+}.

Effects of Blood Coagulant Factors Since the phospholipid binding properties of CPB-II in the calcium concentration range of 1 to 30 mM were consistent, the shifting of optimum calcium concentration with CPB-II on the measurement of coagulation in Fig. 2A, B may be due to the difference in phospholipid binding properties between

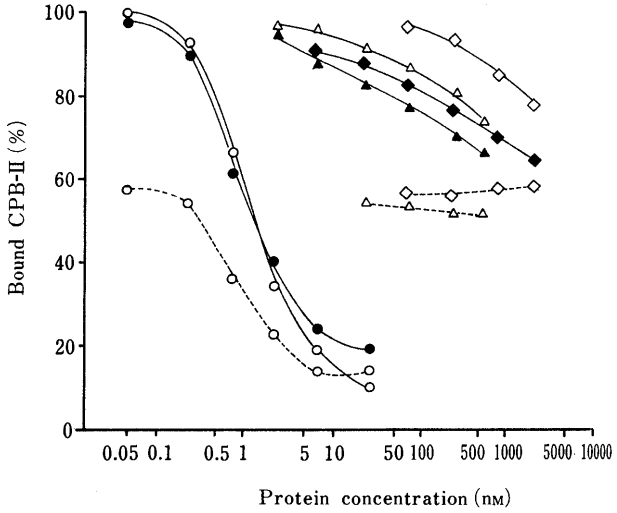

Fig. 4. Competition of Unlabeled CPB-II, Factor X, and Prothrombin with [^{125}I]-CPB-II for Binding to Phospholipid Vesicles

Phospholipid vesicles (1 μg of phospholipid), [^{125}I]-CPB-II (0.05 nM), and Ca^{2+} were mixed with unlabeled CPB-II (○, ●), factor X (△, ▲), or prothrombin (□, ■). Ca^{2+} concentration of 0.3 mM (○···○, △···△, □···□), 3 mM (○—○, △—△, □—□), 30 mM (●—●, ▲—▲, ■—■). The percentages of [^{125}I]-CPB-II bound were calculated by dividing by control in the absence of competitors at 3 mM Ca^{2+}.

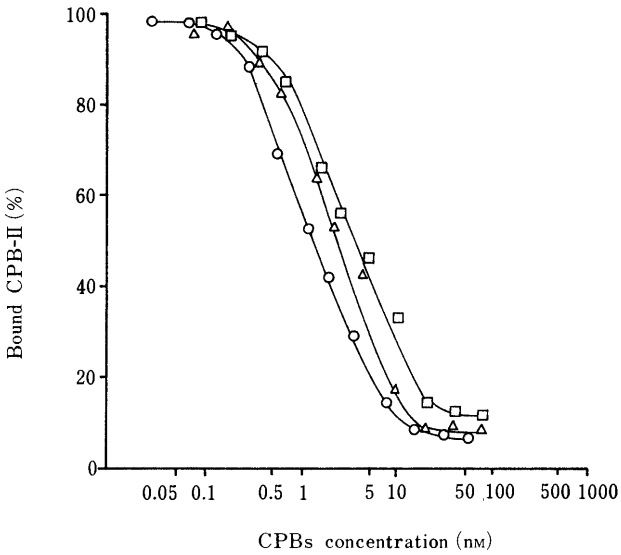

Fig. 5. Competition of CPBs-I, II, and III with [^{125}I]-CPB-II for Binding to Phospholipid Vesicles

Phospholipid vesicles (1 μg of phospholipid), [^{125}I]-CPB-II (0.05 nM), and Ca^{2+} (3 mM) were mixed with unlabeled CPB-II (○), CPB-I (△), or CPB-III (□). The percentages of [^{125}I]-CPB-II bound were calculated by dividing by control in the absence of competitor.

human blood coagulation factors and CPB-II. In order to verify the effect on coagulation, competition assays were carried out by adding unlabeled CPB-II, human prothrombin or human factor X to the reaction mixture containing [^{125}I]-CPB-II, phospholipid vesicles and different concentrations of Ca^{2+} (Fig. 4). Unlabeled CPB-II clearly competed the binding of labeled CPB-II to phospholipid vesicles in a dose-dependent manner at Ca^{2+} concentrations of 3 and 30 mM, while its competition capacity decreased markedly at 0.3 mM Ca^{2+}. Similar competitive effects were found with higher concentrations of human blood coagulant factor X and prothrombin at 3 and 30 mM Ca^{2+}, and these decreased significantly at 0.3 mM

Ca^{2+}. About 300 nM and 1 μM of factor X and prothrombin are required for displacement of approximately 30% of labeled CPB-II at 30 mM Ca^{2+}, respectively, and their competition rates at 3 mM Ca^{2+} are several times lower than those obtained at 30 mM Ca^{2+}. No appreciable competition was observed with these proteins at 0.3 mM Ca^{2+}. Similar results were obtained in competition assays using bovine blood coagulant factor X and prothrombin (data not shown).

Effects of Other Calphobindins Competition assays were made using CPB-I, unlabeled CPB-II and CPB-III in the reaction system at 3 mM Ca^{2+} as above. Figure 5 shows that similar dose-dependent curves to that of CPB-II were obtained with CPB-I and CPB-III, respectively, indicating the same order of binding capacities to phospholipid vesicles for these proteins as that of unlabeled CPB-II.

Discussion

The dissociation constant (K_d) of bovine vascular anticoagulant (VAC) for its binding to phospholipid vesicles has been estimated to be 6 nM by Reutelingsperger *et al.*[18] Recently, Tait *et al.*[19] and Andree *et al.*[20] reported independently that K_d values for binding to phospholipid vesicles of human VAC-α and placental anticoagulant protein-I (PAP-I), both of which can be classified as CPB-I or annexin V, are less than 0.2 and 0.1 nM, respectively. Differences in the K_d values are attributed to species-specific phospholipid-binding capacities of bovine and human proteins by Andree *et al.*,[20] but on the other hand, high homology in primary structures of these proteins has been reported.[21]

K_d values were estimated to be 0.2 to 0.3 nM for human CPB-II in phospholipid binding which were comparable with that obtained for human annexin V (CPB-I) in our preliminary work (unpublished data). This was further supported by the findings in this work showing that CPB-I, CPB-II and CPB-III could serve as competitive inhibitors with similar potencies as assayed by their respective effects on the binding of [^{125}I]-CPB-II to phospholipid vesicles.

The K_d value of CPB-II was not influenced to any appreciable extent when determined in the presence of different concentrations of Ca^{2+} (0.3 to 30 mM). On the contrary, the amount of CPB-II capable of binding to phospholipid vesicles decreased distinctly at 0.3 mM Ca^{2+} as compared with that in the presence of Ca^{2+} in excess of 1 mM. The structure of CPB-II contains eight internally repeated sequences.[22] Only one of these eight Ca^{2+}-binding sites is available in the absence of phospholipid vesicles, while the addition of phospholipid vesicles to the reaction mixture enabled CPB-II to bind to 8 mol Ca^{2+}.[22] Hence, the presence of a suitable amount of phospholipids and Ca^{2+} is likely to be essential for the conformational changes of CPB-II to convert the fully active Ca^{2+}-binding form.

Bindings and affinities to phospholipid vesicles have been investigated for vitamin K-dependent blood coagulation factors such as prothrombin,[23,24] factor IX,[23,25] factor X,[23,24] protein C[26] and other factors. A comparison of dissociation constants (K_d values) of these factors and calphobindins revealed that the K_d value of CPB-II was on a similar order of magnitude to those of CPB-I and CPB-III but lower than those of coagulation factors except for factors Va[24,26-28] and Xa, either of which was able to bind to thrombin-stimulated platelets with comparable K_d value.[29,30] Human prothrombin and factor X were shown to act as competitive inhibitor for CPB-II in a binding assay but their affinities to phospholipid vesicles were estimated to be about 1/1000 that of CPB-II. Obviously, the anticoagulant activity of CPB-II varied depending upon Ca^{2+} concentrations as is evident from comparison of the curves plotting the clotting times *versus* Ca^{2+} concentrations in Fig. 2A, B. Effect of CPB-II to prolong the clotting time is notable at Ca^{2+} concentrations below the optima while no marked influence was observed with increasing Ca^{2+} concentrations above this. These results might be due not only to the differences in the affinities of CPB-II and other blood coagulation factors toward phospholipid vesicles but also to their differential responses to Ca^{2+} concentrations. Chap *et al.*[31] and Tait *et al.*[19] have reported that some Ca^{2+}-binding proteins of the annexin family exhibit anticoagulant activity, and their phospholipid-binding potencies vary significantly. Data of Tait and his colleagues[19] demonstrated that annexin II was much less effective as an anticoagulant than annexins III, IV and V, consistent with its lower affinity for phospholipid. Similar phospholipid-dependent inhibitory activities have been noted with lupus anticoagulants.[9] These findings with annexins support strongly that the anticoagulant activity of CPB-II, a Ca^{2+}-binding protein, is correlated with its affinity in binding to phospholipid vesicles.

It has been generally accepted that exposure of acidic phospholipids to the cell surface is the initial event that triggers the procoagulant mechanism. Phospholipids stimulate protein C activation through the thrombin-thrombomodulin complex[32] and the conversion of a latent plasminogen activator inhibitor-I to the active form.[33] Ross *et al.*[34] recently, presented evidence demonstrating the identity of annexin-III(CPB-III) with inositol 1,2-cyclic phosphate 2-phosphohydrolase, a key enzyme of the phosphatidylinositol signaling pathway and the stimulation of the enzyme activity in the presence of phospholipids. Although biochemical information on CPB-II is accumulating in our laboratory, the participation of this protein in physiological events in human placenta and other tissues remains to be unraveled. Further physiological and pathological investigations will be needed to clarify the functions of CPB-II in cells and tissues.

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Purification and Characterization of an Actin-, Calmodulin- and Tropomyosin-Binding Protein from Chicken Gizzard Smooth Muscle

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An actin-binding protein (p33) has been purified from chicken gizzard smooth muscle. The homogenous protein has a molecular weight near 33000 as determined by both sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and size exclusion chromatography. Its binding ability to F-actin remained after heating at 95 °C for 4 min. Immunoblot analyses indicated that p33 was not a degradation product from higher molecular components. The binding of p33 to F-actin was saturable in a molar ratio of about one p33 to 2—3 actin molecules with an apparent binding constant of $6.6 \times 10^7\ M^{-1}$. p33 also bound to calmodulin and tropomyosin. The bindings of p33 to F-actin and tropomyosin were regulated by calmodulin in a Ca^{2+}-dependent fashion. In addition to actin, caldesmon and tropomyosin, p33 was contained in the native thin filaments prepared from smooth muscle. Other actin-binding proteins, including α-actinin, caldesmon and filamin, had little effect on p33 binding to actin filaments. These results demonstrate that p33 may function in actin-based cellular processes which are mediated by Ca^{2+} and calmodulin.

Keywords actin; calmodulin; tropomyosin; binding protein; smooth muscle; native thin filament; binding site

Actin-containing microfilaments are found in nearly all eukaryotic cells and are responsible for a number of cellular activities including cell motility, cell shape change, cytokinesis, secretion and adhesion to substratum.[1–3] The organization of actin filaments is modulated by a variety of actin-binding proteins that have been classified into several categories based on their biochemical properties. In a variety of smooth muscle, it is generally considered that Ca^{2+} can initiate and modulate the contractile response derived from actin and myosin filaments, and calmodulin acts as a transducer of the intracellular Ca^{2+} signal. There are dual regulatory mechanisms of smooth muscle contraction. One is the phosphorylation of myosin light chain catalyzed by myosin light chain kinase,[4–6] and the other is actin-mediated regulation by caldesmon.[7,8] Ca^{2+} and calmodulin can lead to the activation of actomyosin adenosine triphosphatase (ATPase) activity *via* the activation of myosin light chain kinase activity and the inhibition of caldesmon–actin interaction. Myosin light chain kinase and caldesmon also bind to actin[9,10] and tropomyosin,[11–13] respectively, and their bindings are regulated by Ca^{2+} and calmodulin. Taken together, these observations suggest that calmodulin-dependent actin-binding proteins would participate in the contractile systems. In addition, it is not clear whether or not a troponin-T-like protein is present in smooth muscle.

During the isolation of caldesmon from chicken gizzard smooth muscle, we observed a low-molecular-weight protein which could bind to F-actin, calmodulin and tropomyosin. In this paper, we describe the purification procedure and functional characterization of this protein.

Materials and Methods

Preparation of Proteins Actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt,[14] and was further gel filtered through Sephadex G-150.[15] The following proteins were isolated from chicken gizzard smooth muscle using published methods: caldesmon,[16] tropomyosin,[17] α-actinin[17] and filamin.[17] Calmodulin was purified from porcine brain according to the method of Isobe *et al.*[18] Native thin filaments were prepared from chicken gizzard muscle as described by Marston and Lehman.[19]

Purification of an Actin-Binding Protein (p33) Unless otherwise noted, all purification procedures were carried out at 4 °C. Fresh chicken gizzard muscles were homogenized in 6 volumes of 50 mM imidazole–HCl buffer (pH 6.9) containing 2 mM $MgCl_2$, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM ethylene glycol bis(2-aminoethyl ether)tetraacetic acid (EGTA) and 0.3 M KCl by Polytron®, heated at 95 °C for 4 min, and then chilled rapidly on ice. The suspension was centrifuged at $50000 \times g$ for 30 min and the precipitate was discarded. Solid ammonium sulfate was added to give 30% saturation and the resulting precipitate collected by centrifugation was dialyzed overnight against 20 mM Tris–HCl buffer (pH 7.5) containing 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, 1 mM EGTA and 50 mM KCl (buffer A). The dialysate was clarified by centrifugation and applied to a carboxymethyl (CM)-cellulose column (2×14 cm) equilibrated with buffer A. The column was washed with the same buffer and the bound protein was eluted with a linear concentration gradient of KCl, ranging from 0 to 350 mM of buffer A. The p33 fractions were combined and concentrated using Aquacide II. The concentrated solution was subjected to gel filtration on an ultrogel AcA-44 column (3×85 cm), which was equilibrated and eluted with buffer A containing 350 mM KCl. p33 fractions were collected and stored on ice.

Preparation of Antiserum Antisera against p33 were raised in rabbits by subcutaneous injection of about 300 μg of protein in complete Freund's adjuvant. Rabbits were boostered bimonthly with 150 μg of protein in incomplete Freund's adjuvant. The rabbits were bled 2 weeks after the last injection.

Electrophoresis and Immunoblotting Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli,[20] using 0.8 mm-thick slabs stained with Coomassie blue R-250. Immunoblots were performed by a modification of the method of Towbin *et al.*[21] Proteins were electrophoretically transfered to a nitrocellulose membrane (Schleicher & Schuell). After incubating the filter in 5% bovine serum albumin and 1% calf serum in 10 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl (Tris-buffered saline) for 4 h at 25 °C to block nonspecific binding sites, it was incubated for 2 h at 37 °C with diluted antisera (1 : 3000) to p33 in 5% bovine serum albumin in Tris-buffered saline. The filters were then treated with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (IgG) for 2 h at 37 °C. The immunoreactive bands were visualized by using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as a substrate for alkaline phosphatase.[16]

Other Methods The interaction of p33 with F-actin was measured by the cosedimentation assay.[16,22] Calmodulin and tropomyosin were coupled to cyanogen bromide-activated Sepharose 4B following the procedures outlined by Pharmacia. About 5 mg calmodulin and 2.5 mg tropomyosin were linked to 1 ml of Sepharose. Protein concentration was determined by a Bio-Rad coomassie blue protein assay using bovine serum albumin as a standard, or spectrophotomeric measurements using $E_{cm}^{1\%}$ at 290 nm of 6.5 for actin,[23] $E_{cm}^{1\%}$ at 278 nm of 2.9 for tropomyosin[24] and $E_{cm}^{1\%}$ at 276 of 1.8 for calmodulin.[25]

Results

Purification of p33 from Chicken Gizzard Smooth Muscle When crude extract from chicken gizzard was mixed

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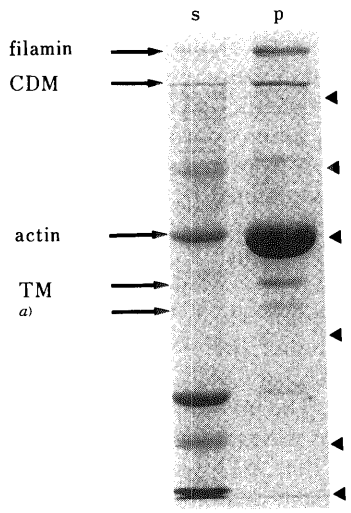

Fig. 1. Sedimentation Analysis of Actin-Binding Proteins in Chicken Gizzard Smooth Muscle

Chicken gizzards were extracted in buffer A and centrifuged at 100000 × *g* for 60 min at 4 °C. Obtained supernatant was incubated with F-actin for 60 min at 25 °C in 5 mM 1,4-piperazinediethanesulphonic acid (PIPES)–NaOH (pH 7.0), 1 mM $MgCl_2$, 1 mM ATP, 100 mM NaCl in a final volume of 200 μl. The supernatants (s) and pellets (p) were separated and analyzed by SDS–PAGE. The arrowheads indicate the positions of molecular weight markers: phosphorylase b (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000) and lysozyme (14400), from top to bottom. CDM, TM, and *a*) indicate caldesmon, tropomyosin and 33000 protein, respectively.

with skeletal muscle F-actin, incubated for 60 min at 25 °C and then centrifuged at 100000 × *g* for 30 min at 25 °C, a number of components including filamin, caldesmon and tropomyosin cosedimented with F-actin. In addition to these proteins, a 33000 kilodaltons (kDa) polypeptide (p33) estimated by SDS–PAGE also bound to F-actin (Fig. 1). Since p33 was soluble after heating at 95 °C for 4 min and retained its actin binding activity, we utilized this characteristic for its purification.

Figure 2A shows the elution profile of CM-cellulose chromatography. Fractions 39 through 43 were collected and used for further purification. The sample was applied to an ultrogel AcA-44 column equilibrated with buffer A containing 350 mM KCl. The elution profile and the polypeptide composition are shown in Fig. 2B. Fractions 81 through 86 were collected and used as the purified p33. Analysis by microplate procedure showed that the yield was about 35% (12 mg of purified p33 from 200 g of chicken gizzard).

The preparation gave a single band with a molecular weight of 33000 on SDS–PAGE (Fig. 3) and we called it p33. The molecular weight of the native protein was estimated to be 30000 by gel filtration (Protein pak 125 HPLC and Sephadex G-100 columns) indicating p33 exists as a monomer. Immunoblot analysis showed that the polyclonal antibodies against p33 reacted specifically with p33 and did not crossreact with other polypeptides in chicken gizzard smooth muscle.

Interaction of p33 with Actin, Tropomyosin and Calmodulin

The binding of p33 to F-actin is examined by sedimentation assay and the results are plotted in the form of a Scatchard plot (Fig. 4). Control experiments showed that p33 did not sediment by itself and also had no influence on the amount of actin sedimented after this centrifugation. The amount of p33 in actin pellet progressively increased by increasing

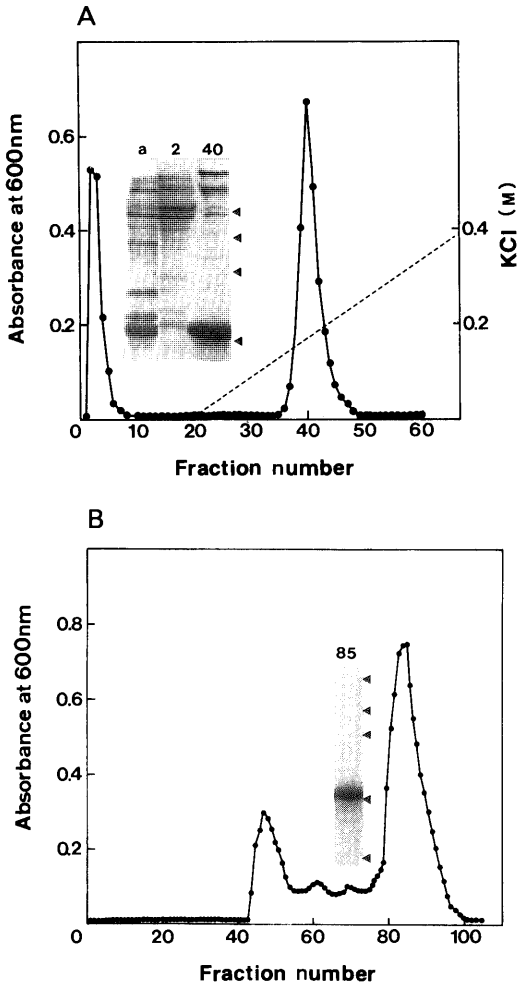

Fig. 2. Chromatographic Purification Steps of p33

A) The 0—30% ammonium sulfate fraction was applied to a 2 × 10 cm CM-cellulose column equilibrated with buffer A. After washing with the buffer, the column was eluted with 140 ml gradient from 0 to 350 mM KCl of buffer A at a flow rate of 10 ml/h. Fractions of 3 ml each were collected and analyzed for protein using the Bio-Rad dye binding assay. The dotted line represents the KCl concentration. B) The concentrated CM-cellulose fractions were applied at a flow rate of 12 ml/h to a 3 × 85 cm ultrogel AcA-44 column equilibrated with buffer A containing 350 mM KCl. Fractions (5 ml) were collected and analyzed for protein. The inset shows the profile for SDS–PAGE for applied sample (a) and fractions 2 and 40 (A) and fraction 85 (B). The small arrowheads on the right indicate the positions of the molecular weight markers described in the legend to Fig. 1.

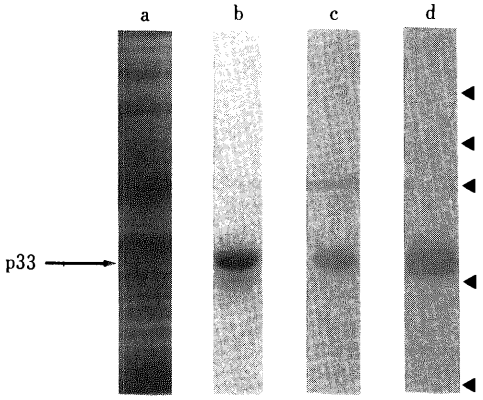

Fig. 3. SDS–PAGE and Immunoblot

Chicken gizzard homogenate (a, c) and purified p33 (b, d) were separated in 12.5% SDS–PAGE. (a, b) and (c, d) indicate coomassie blue-stained gels and immunoblots with anti-p33 polyclonal antibodies, respectively.

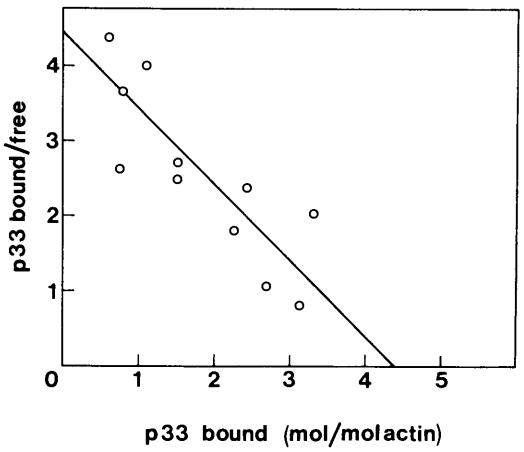

Fig. 4. Scatchard Plot of the Binding of p33 to F-Actin

F-Actin (7 μM) was incubated with various concentrations of p33 as indicated for 30 min at 25 °C. The details of the cosedimentation assay are described in the legend to Fig. 1. The protein bands were stained with coomassie blue and scanned in a densitometer (Shimadzu CS9000) at 570 nm.

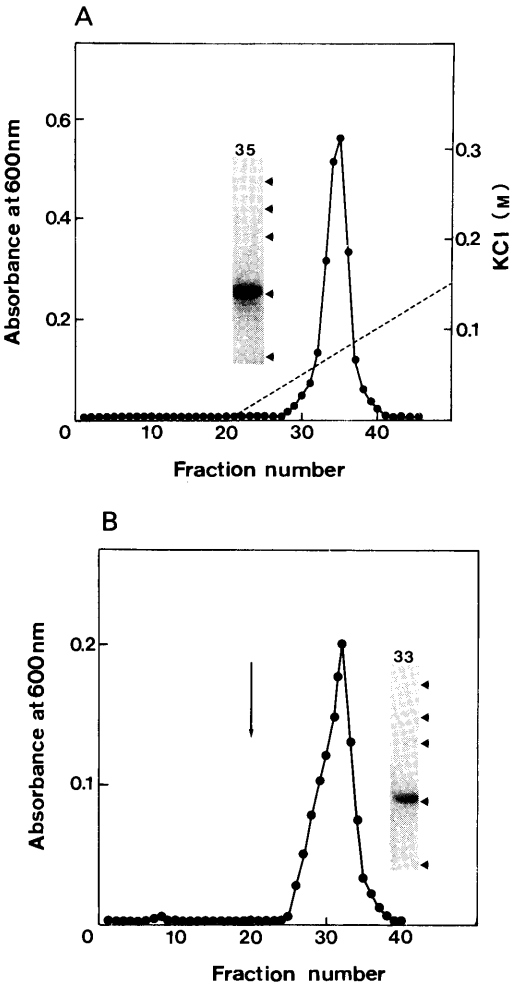

Fig. 5. Tropomyosin- and Calmodulin-Sepharose 4B Affinity Chromatographies of p33

A) The 0.6×9 cm column was equilibrated with 20 mM Tris-HCl (pH 7.0) containing 0.1 mM DTT (buffer C), and purified p33 (1 mg) was applied. The column was washed with the above buffer and the bound protein was eluted with a linear gradient of 0—150 mM KCl (---). B) Purified p33 (0.5 mg) was applied to a calmodulin-Sepharose column (0.6×11 cm) equilibrated with buffer B. The column was washed with buffer B and at the point indicated by arrow, the bound protein was eluted with 1 mM EGTA of buffer B without 0.5 mM $CaCl_2$. Both fractions (1 ml) were collected and analyzed for protein. The insets show the SDS-polyacrylamide gel profiles for fractions 35 (A) and 33 (B).

the concentration of p33. Saturation was achieved at an approximate molar ratio of one p33 to 2—3 actin monomers and an apparent association constant was $6—7 \times 10^7\ M^{-1}$.

We investigated whether p33 could bind to tropomyosin and calmodulin by affinity chromatography. When purified p33 was applied to a tropomyosin-Sepharose column, most of the protein was retained by the column (Fig. 5A). The binding appeared to be relatively weak because bound p33 was eluted at 60—80 mM KCl under our experimental conditions. Purified p33 was also applied to a calmodulin-Sepharose 4B column which had been equilibrated with 10 mM Tris-HCl buffer (pH 7.0) containing 0.1 mM DTT, 50 mM KCl and 0.5 mM $CaCl_2$ (buffer B) and washed with buffer B (Fig. 5B). The bound protein was eluted with 1 mM EGTA of buffer B without 0.5 mM $CaCl_2$. Electrophoretic analysis showed that p33 was recovered in the bound fractions. These results demonstrated that p33 was an actin-, calmodulin- and tropomyosin-binding protein.

Effects of Ca^{2+} and Calmodulin on p33–Actin and p33–Tropomyosin Interactions Calmodulin is well known to be a Ca^{2+}-dependent regulatory protein. F-actin sedimentation experiments were also done to examine the effect of calmodulin on the binding of p33 to F-actin (Fig. 6). After centrifugation of the three proteins at p33 : actin : calmodulin = 1 : 6 : 5 (molar ratio), calmodulin released about 50% of p33 from actin filaments in the presence of Ca^{2+} (pair d), while this release was not observed in the presence of 0.2 mM EGTA (pair c). Similar results were obtained when tropomyosin was present in the assay mixture (data not shown).

We also examined the effect of Ca^{2+}/calmodulin on the interaction between p33 and tropomyosin by affinity chromatography (Fig. 7). The binding of p33 to tropomyosin was not affected by 0.2 mM EGTA or 0.5 mM $CaCl_2$, and calmodulin did not bind to the column irrespective of Ca^{2+} concentration. In the presence of EGTA, p33 adsorbed on the column was not released by calmodulin and most of the protein was eluted by 200 mM KCl. On

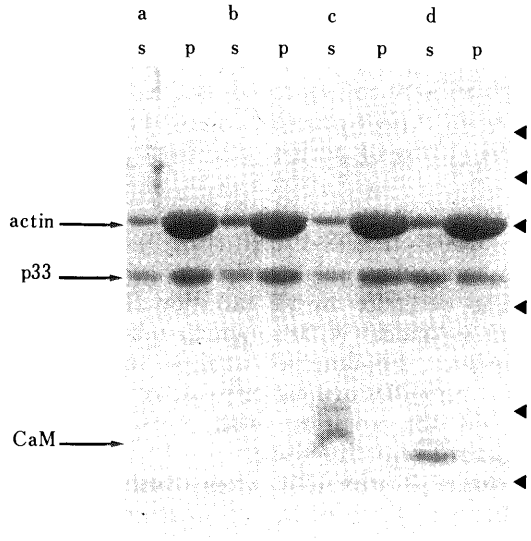

Fig. 6. Sedimentation Analysis of the Interaction between p33 and F-Actin in the Presence or Absence of Ca^{2+} and Calmodulin

The concentrations of p33, F-actin and calmodulin were 3, 6 and 15 μM, respectively. Pair a, 0.2 mM EGTA; pair b, 0.5 mM $CaCl_2$; pair c, 0.2 mM EGTA + calmodulin; pair d, 0.5 mM $CaCl_2$ + calmodulin.

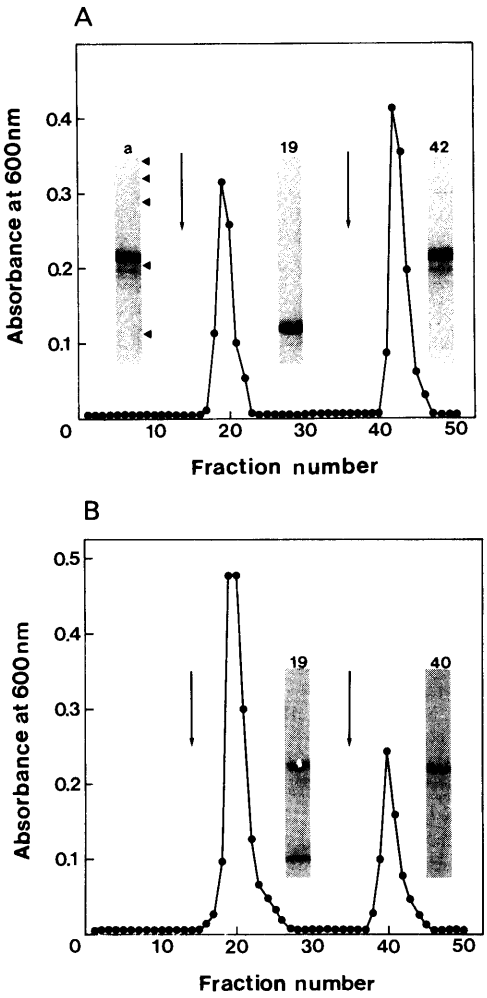

Fig. 7. Effect of Ca^{2+} and Calmodulin on the Binding of p33 to Tropomyosin–Sepharose 4B

p33 (1 mg) was applied to the tropomyosin–Sepharose column as described in the legend to Fig. 5. The column was washed with buffer C containing 0.2 mM EGTA (A) or 0.5 mM $CaCl_2$ (B). At the first point, calmodulin (1 mg) which was dissolved in buffer C containing 0.2 mM EGTA (A) or 0.5 mM $CaCl_2$ (B) was applied to the column. Then bound protein was eluted with the buffer containing 250 mM NaCl at the second point. Fractions of 1 ml each were collected and analyzed for protein. The inset shows the SDS–polyacrylamaide gel profiles for the applied sample (a) and fractions 19 and 42 (A) and fractions 19 and 40 (B).

the other hand, the addition of calmodulin in the presence of Ca^{2+} released most of the p33 bound to the column.

p33 is a Component of Native Thin Filaments Since p33 can bind to F-actin, calmodulin and tropomyosin, and its bindings to actin and/or tropomyosin are regulated by Ca^{2+} and calmodulin, this protein is most likely to be associated with the contractile processes of smooth muscle. We prepared myofibrils and native thin filaments from chicken gizzard, and their constituents were analyzed by SDS–PAGE (Fig. 8). In addition to actin, tropomyosin, caldesmon and myosin (only in myofibrils), a 33000 polypeptide was found in the myofibrils and the native thin filament fractions (lanes 1 and 2), and the polyclonal antibodies raised against p33 cross-reacted with the polypeptide (lanes 3 and 4).

Effects of Actin–Binding Proteins on the Interaction between p33 and F-Actin In order to determine whether p33 binding site(s) on actin directly involved the binding regions of other actin-binding proteins in smooth muscle, we examined the effects of α-actinin, caldesmon and filamin

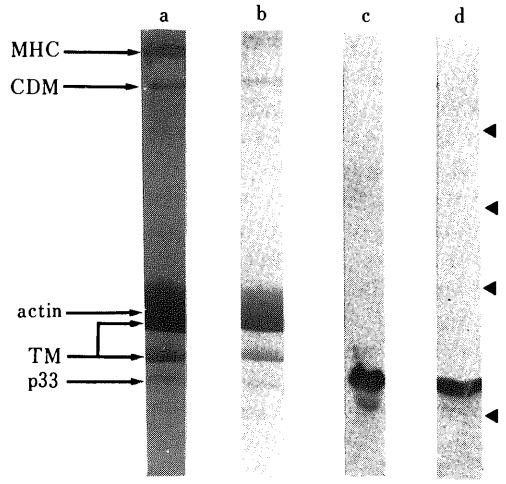

Fig. 8. SDS–PAGE and Immunoblot of Myofibrils and Native Thin Filaments

Chicken gizzard myofibrils (a, c) and native thin filaments (b, d) were separated in 12.5% SDS–PAGE. (a, b) and (c, d) indicate coomassie blue-stained gels and immunoblots with anti-p33 polyclonal antibodies, respectively. MHC, myosin heavy chain.

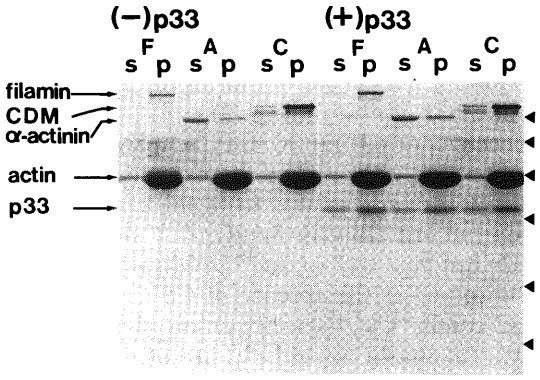

Fig. 9. Sedimentation Analysis of the Interaction between p33 and F-Actin in the Presence of α-Actinin, Caldesmon and Filamin

The experimental conditions were the same as for Fig. 6. The concentrations of α-actinin, caldesmon and filamin were 0.06, 0.08 and 0.05 mg/ml, respectively.

on p33 binding to F-actin by sedimentation analyses (Fig. 9). The binding of p33 to F-actin was significantly changed in the presence of these proteins, and behaved *vice versa.*

Discussion

In the present experiments, we describe the purification and characterization of an actin-, calmodulin- and tropomyosin-binding protein (p33) with a molecular weight of 33000 from chicken gizzard. The properties of p33 reported here are apparently very similar to those of caldesmon (M.W. 120000 on SDS–PAGE), an abundant F-actin-, calmodulin- and tropomyosin-binding protein whose F-actin and tropomyosin binding abilities can be modulated by Ca^{2+} and calmodulin.[7,8,12,16,22,26,27] Moreover, this protein exhibit a similar apparent molecular weight on SDS–PAGE as an actin-, calmodulin- and tropomyosin-binding fragment (M.W. 35000—38000) digested from caldesmon.[22,26,27] Immunoblot analysis showed that the antibodies raised against p33 are specific for p33 and do not recognize other proteins in chicken gizzard muscle (Fig. 3). The apparent association constants of caldesmon, filamin, villin, vinculin and synapsin I to F-actin have been calculated to be 10^5—10^6 M^{-1},[28] 1.3×10^7 M^{-1},[29] 7×10^5

M^{-1},[30] $2.5 \times 10^7\ M^{-1}$ [31] and $1.2—1.9 \times 10^6\ M^{-1}$,[32] respectively. The maximum bindings reach 1 mol of protein to 6—28 mol (caldesmon), 12 mol (filamin), at least 10 mol (villin), 100 mol (vinculin) and 2—3 mol (synapsin I) of actin monomers as determined by sedimentation analyses. The estimated K_a value ($6.6 \times 10^7\ M^{-1}$) for the binding of p33 to F-actin is adequate as an actin-binding protein and the maximum amount of p33 to bind F-actin is relatively high compared with those of other actin-binding proteins.

This protein is likely to be basic as judged from its elution profile from a CM-cellulose column (Fig. 2A), while actin, calmodulin and tropomyosin are acidic proteins.[24,34] However, the bindings of p33 to these proteins seem to be caused not merely by ionic interactions. This is supported by the following observations. The binding of p33 to calmodulin was dependent on Ca^{2+} concentrations (Fig. 5B), and p33 bindings to actin and tropomyosin were regulated by calmodulin in a Ca^{2+}-dependent fashion (Figs. 6 and 7). Although tropomyosin has been reported to block the binding of actin-binding proteins such as α-actinin,[34] filamin,[35] gelsolin,[36] villin[30] and heat shock protein[37] to F-actin and their functions, the interaction between p33 and F-actin was also observed in the presence of tropomyosin. In addition, α-actinin, caldesmon and filamin from chicken gizzard smooth muscle did not affect the binding of p33 to F-actin and p33 *vice versa* (Fig. 9).

It has been reported that two Ca^{2+}-dependent membrane-binding proteins, calcimedins, with molecular weights of 67000 and 35000, are isolated from chicken gizzard muscle and belong to the annexin family.[38,39] Some proteins of this family can bind to F-actin.[40,41] However, the bindings are not observed in the absence of Ca^{2+}, and calmodulin does not confer Ca^{2+}-sensitivity on these interactions. Recently, Takahashi *et al.*[42,43] isolated an actin- and calmodulin-binding protein from chicken gizzard and called this protein calponin. Immunologically, calponin reacted with the antibodies against troponin T from skeletal muscle. However, we could not detect any polypeptides in skeletal muscle crude extract to crossreact with antibodies to p33 (data not shown). The elution of p33 from calmodulin-Sepharose was only dependent on Ca^{2+} concentrations, while calponin release occurred at over 100 mM NaCl in addition to EGTA. At present, we have no definite evidence that p33 is distinct from calponin. More recently, Lehman[44] has shown that an actin-binding protein with a molecular weight of 35000 is not associated with chicken gizzard thin filaments. As shown in Fig. 8, p33 is included in the myofibrils and native thin filaments. A similar polypeptide has also been observed in the native thin filaments reported by Ngai *et al.*[45] It may be due to differences in experimental conditions, especially in the concentrations of Triton X-100 and ATP. Further experiments are necessary to examine the distribution and localization of p33. The present results demonstrate that p33 may play a role in microfilament- and calmodulin-mediated cellular processes in smooth muscle.

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Inhibition of Transcription by Mammalian Ribonucleic Acid Polymerase II: Effects of Diethylstilbestrol and Its Analogues

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We examined the effects of diethylstilbestrol (1), *Z,Z*-dienestrol (2), *E,E*-dienestrol (3), indenestrol A (4), indenestrol B (5) and estradiol (6) on the activity of *in vitro* accurate transcription from adenovirus 2 major late promoter by mammalian ribonucleic acid (RNA) polymerase II using partially purified transcription factors from HeLa cell nuclear extract as well as on RNA polymerase II activity assayed for random incorporation of ribonucleotides. 1, 2, and 3 inhibited both activities, whereas 4 and 5 were inhibitory only in the transcription activity at concentrations higher than 10 μg/ml. However 6 had no effects on both activities.

Keywords transcription; RNA polymerase II; diethylstilbestrol; indenestrol A; indenestrol B; estradiol; dienestrol

trans-Diethylstilbestrol (**1**) is a potent synthetic estrogen capable of eliciting biological responses similar to those induced by endogenous 17β-estradiol.[1] In recent years, studies[2] in humans and mice have suggested that *in utero* exposure to **1** induces cancerous lesions of the reproductive tract. The mechanism of these effects of **1** is unknown, in spite of many studies covering metabolic activation,[3] deoxyribonucleic acid (DNA) binding,[4] radical formation[5] or DNA breaks.[6] We have reported that **1** and its derivatives inhibit microtubule assembly *in vitro*.[7] Also, ^{2}H-labeled **1** has been analyzed by means of deuterium nuclear magnetic resonance (NMR), which demonstrated its intercalation into multibilayers of egg phosphatidylcholine.[8] Moreover, we have elucidated that **1** and its derivatives caused aneuploidy induction and disturbance of microtubule distribution in Chinese hamster V79 cells.[9] These findings suggest that within cells, **1** does not act directly on DNA.

Accurate transcription initiation by mammalian ribonucleic acid (RNA) polymerase II *in vitro* is recognized as a complex reaction involving multiple factors, and is a major regulatory step in many biological phenomena.[10-12] Several laboratories, including ours, have identified proteins which are required for correct transcription initiation. Some of these factors have been partially purified and characterized,[13-19] and can be separated functionally into two or more groups.

We are interested in the direct effect of diethylstilbestrol (**1**) on the transcription factors and mammalian RNA polymerase II in eukaryotic transcription. Therefore, in the present study, we examined the effect of **1**, *Z,Z*-dienestrol (**2**) and indenestrol A (**4**),[20] the latter two being the products of *in vivo* and *in vitro* oxidative metabolism[3] of **1**, *E,E*-dienestrol (**3**) and indenestrol B (**5**), which are chemical analogs of **3** and **4**, respectively, and estradiol (**6**), which is the most potent naturally occurring estrogen in mammals for *in vitro* transcription by mammalian RNA polymerase II using the partially purified transcription factors and mammalian RNA polymerase II.

Experimental

Materials Diethylstilbestrol (**1**), *E,E*-dienestrol (**3**), and estradiol (**6**) were purchased from Sigma Chemical Co. Syntheses of *Z,Z*-dienestrol (**2**) and indenestrols A and B (**4** and **5**) were performed as described elsewhere.[20] Chart 1 shows the chemical structures of the six drugs used in this study. Hind III was purchased from Takara Shuzo Co., Ltd. [α-^{32}P]uridine triphosphate (UTP) was from Du Pont-New England Nuclear. Other materials were of reagent grade.

Plasmids and Template The template used for transcription was a plasmid, pAd2Δ, which contained the adenovirus type 2 major late (ML) promoter sequence. The length of the transcript resulting from run-off assay was 186 nucleotides when this template was digested with Hind III. The template used for RNA polymerase activity was denatured salmon sperm DNA.

Preparation of Transcription Factors HeLa cell nuclear extract (7.5 g) was prepared as described,[21] and transcription factors, FA, FB, FC, FD, FE, and FF were resolved by chromatographic procedures as reported previously.[19]

***In Vitro* Transcription Activity** *In vitro* transcription assay was carried out in a 27.5-μl reaction volume containing 11 mM Tris–HCl (pH 7.9), 6 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 55 mM NaCl, 10% glycerol, 500 μM each of citidine triphosphate (CTP), guanosine triphosphate (GTP) and adenosine triphosphate (ATP), 25 μM [α-^{32}P]UTP (approximately 2 μCi), 0.5 μg of pAd2Δ and transcription factors as indicated in Figures. The addition of drug/acetone was usually 1 μl. The reaction was started by the addition of the nucleoside triphosphate mixture. After incubation at 29 °C for 60 min, the reaction was stopped by adding 100 μl of 20 mM Tris–HCl pH 7.9, 1 mM EDTA, 0.3 M NaCl, 7 M urea, and 0.5% sodium dodecyl sulfate (SDS). Transcripts were purified and analyzed by electrophoresis on 6% polyacrylamide–7 M urea gel. The gels were then exposed to Fuji X-ray film at −80 °C with an intensifying acreen. The inhibition activities were determined by densitometric analysis of the exposed X-ray film using a Bio-rad model 620 video densitometer.

Preparation of RNA Polymerase II RNA polymerase II was partially purified from calf thymus as described by Hodo and Blatti[21] except that the final step of purification on a column of agarose A-1.5 m capped with Sephadex A-25 was omitted. The purity of RNA polymerase II was estimated to be 94% among RNA polymerases by α-amanitin inhibition assay.

Assay of RNA Polymerase Activity The assays were done in a final volume of 27.5-μl containing 10 mM Tris–HCl (pH 7.9), 100 mM NaCl, 1.8 mM $MnCl_2$, 180 μM each of GTP. CTP and ATP, 9 μM [α-^{32}P] UTP (approximately 2 μCi), and 8 μg of DNA. The reaction was initiated by the addition of 5 μl of enzyme and incubated at 37 °C for 30 min; 30 μl aliquots were withdrawn from each tube after the addition of 2.5 μl of 50 mM UTP, and spotted onto DEAE-cellulose paper filters. The filters were washed six times for 5 min each in 5% Na_2HPO_4, then in distilled water and in 95% EtOH, and finally dried under an infrared lamp. Radioactivity was determined by placing each disc in 10 ml of aqueous counting scintillant (ACS II: Amersham Corporation).

Other Procedures SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli,[22] and gels were stained with Ag-stain obtained from Daiichi. Protein concentration was measured by the dye-binding method[23] or absorbance at 280 nm using bovine serum albumin as a standard.

Results and Discussion

First, we examined the effect of diethylstilbestrol (**1**) on *in vitro* accurate transcription from adenovirus 2 major late promoter by mammalian RNA polymerase II using partially

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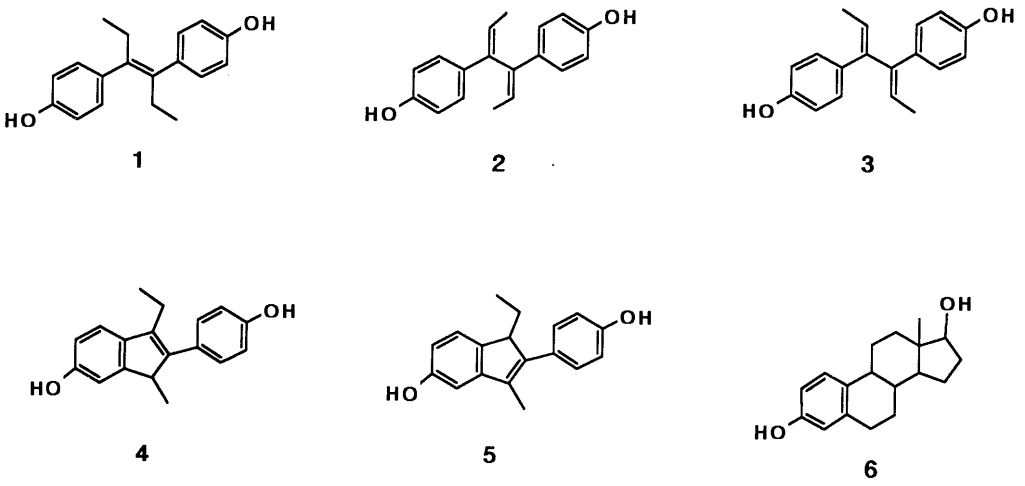

Chart 1

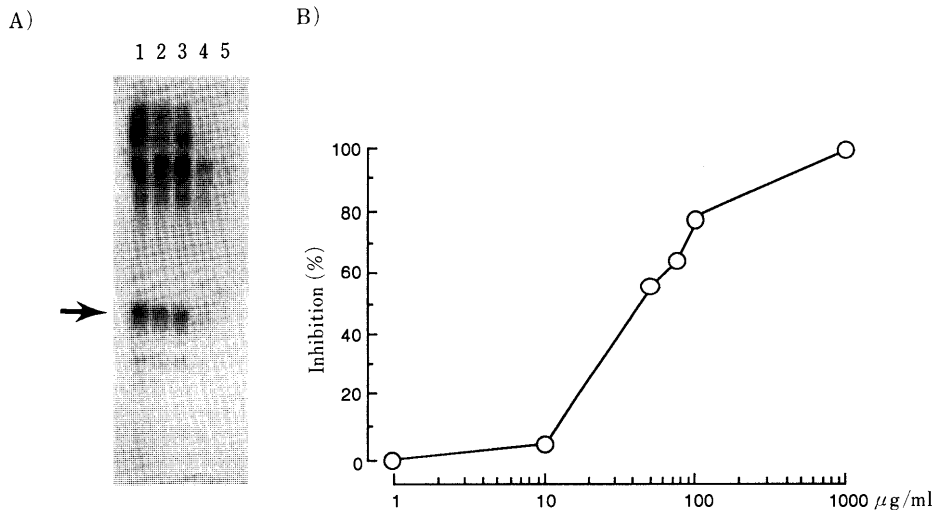

Fig. 1. Effect of Diethylstilbestrol (**1**) on Transcription by RNA Polymerase II

In vitro transcription of pAd2*Δ* was assayed as described in Experimental by using FA (3.0 μl), FB (1.5 μl), FC (3.0 μl), FD (3.0 μl), FE (4.0 μl), FF (1.5 μl) and purified RNA polymerase II (1.0 μl). Arrow indicates correct transcript. A) An autoradiogram from the results of the assay: lane 1, control; lane 2, 1 μg/ml; lane 3, 10 μg/ml; lane 4, 100 μg/ml; lane 5, 1000 μg/ml. B) Densitometric analysis of autoradiogram. Doses were 1, 10, 50, 75, 100, 1000 μg/ml. Each value (%) represents the average of densitometric analyses of autoradiogram from three experiments.

purified transcription factors from HeLa cell nuclear extract as a standard system. Figure 1 shows the dose–response autoradiogram and densitometric analysis of the autoradiogram based on the assay in the presence of **1**. At concentrations lower than 10 μg/ml, **1** did not affect the transcription activity. However, **1** inhibited the activity at more than 50 μg/ml. This result is interesting, since the concentration of **1** was the same as that for the inhibition of *in vitro* microtubule polymerization.[7a] This experiment was performed on the assumption that the inhibition of transcription by **1** may be due to interaction with transcription factors and/or RNA polymerase II. Next, we examined the effect of **1** on RNA polymerase II activity assayed by the random incorporation of ribonucleotides, in which denatured salmon sperm DNA was used as a template in the presence of $MnCl_2$ as a divalent cation. As shown in Table I, **1** had inhibitory activity on mammalian RNA polymerase II itself. The activity of **1** on RNA polymerase II itself was weaker than that on the transcription at 100 μg/ml.

Consequently, we examined the effects of *Z*,*Z*-dienestrol,

TABLE I. Effects of Compounds (**1**—**6**) on RNA Polymerase II Activity

Concentration (μg/ml)	Compounds	Inhibition (%)
1	**1**	16
	2	0
	3	0
	4	0
	5	0
	6	0
10	**1**	36
	2	3
	3	12
	4	0
	5	0
	6	0
100	**1**	55
	2	24
	3	62
	4	0
	5	0
	6	0

Each value represents the average of three experiments.

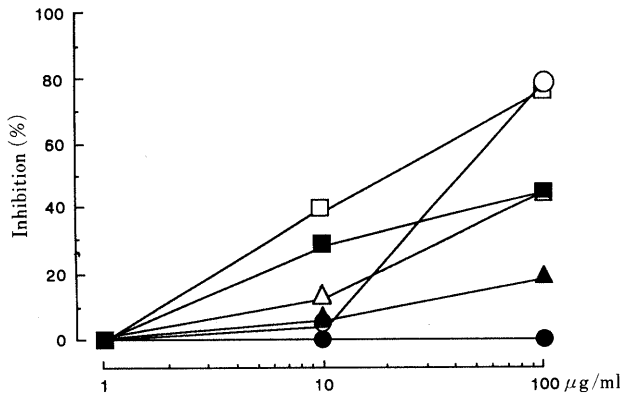

Fig. 2. Effects of Drugs on Transcription by RNA Polymerase II

Each value (%) represents the average of densitometric analyses of autoradiogram from three experiments. ○, diethylstilbestrol (**1**); ■, *Z,Z*-dienestrol (**2**); □, *E,E*-dienestrol (**3**); ▲, indenestrol A (**4**); △, indenestrol B (**5**); ●, estradiol (**6**).

indenestrol A, *E,E*-dienestrol, and indenestrol B on *in vitro* transcription activity. As shown in Fig. 2, *E,E*-dienestrol (**3**) and indenestrol B (**5**) gave higher inhibitory activity than *Z,Z*-dienestrol (**2**) and indenestrol A (**4**), respectively. Next, we examined the effects of the drugs on RNA polymerase II itself, as seen in Table I. These two kinds of experiments indicated that **2** and **3** inhibited not only the transcription activity but also RNA polymerase II activity as is the case with microtubule polymerization. Moreover, the result show that indenestrols (**4** and **5**) had an inhibitory effect only on the transcription factors, since inhibitory activities of the indenestrols on RNA polymerase II itself were not observed.

We also tested the effect of estradiol (**6**), the most potent naturally occurring estrogen in mammals. However, in spite of the reported inducibility of mitotic non-disjunction by 200 μM (54.4 μg/ml) estradiol in HeLa cells,[24] the results of turbidity measurement and electron microscopy have indicated that it has no ability to interact with microtubules or microtubule protein *in vitro*.[7a] On the other hand, we previously discovered that estradiol had toxic activity on Chinese hamster V79 cells, and inhibited microtubule polymerization in cultured V79 cells.[25] Therefore, we examined the effects of estradiol (**6**) on transcription by mammalian RNA polymerase II and on RNA polymerase II itself. As seen in Fig. 2 and Table I, **6** did not affect the activities of transcription as well as RNA polymerase II itself. These results suggest that the target of the inhibitory activity of estradiol was neither general transcription factors nor RNA polymerase II itself.

As part of our series of studies aimed at achieving a better understanding of the mechanism of action of tumorigenic stilbene estrogens, the direct effects of diethylstibestrol (**1**) and its analogues and estradiol (**6**) on the transcription activity of mammalian RNA polymerase II were clarified in the present investigation. Present clear-cut results would not be obtained if a nuclear extract was used as an assay system.

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Constituents of the Seed of *Malva verticillata*. VII.[1] Structural Features and Reticuloendothelial System-Potentiating Activity of MVS-I, the Major Neutral Polysaccharide

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The structural features of MVS-I, the major neutral polysaccharide isolated from the seeds of *Malva verticillata* L., were elucidated by controlled Smith degradation, methylation analysis, partial acid hydrolysis and enzymic degradation studies. It has a backbone chain composed of β-1,3-linked D-glucose and D-galactose residues having branches composed of α-1,5-linked L-arabinosyl β-1,4-linked D-galactose and of β-1,4-linked D-galactosyl β-1,3-linked D-glucose residues at position 6 of a part of D-galactose units as side chains. MVS-I showed remarkable reticuloendothelial system-potentiating activity in a carbon clearance test.

Keywords *Malva verticillata*; seed; MVS-I; polysaccharide structure; immunological activity; reticuloendothelial system; Smith degradation; partial hydrolysis; enzymic degradation; methylation analysis

MVS-I is the major neutral polysaccharide obtained from the seed of *Malva verticillata* L. (Malvaceae).[2] The seed of this plant is an Oriental crude drug used as a diuretic, laxative and galactopoietic. As the other glycans of this material, the isolation and structural features of two neutral polysaccharides (MVS-IIA and MVS-IIG),[3] three acidic polysaccharides (MVS-IIIA, MVS-IVA and MVS-VI)[1,4,5] and the major peptidoglycan (MVS-V)[6] were reported in previous papers. These substances were examined for anti-complementary and hypoglycemic activities.[7] Among them, MVS-I showed especially remarkable activities. The polysaccharide is composed of L-arabinose: D-galactose: D-glucose in the molar ratio of 3:6:7.[2] The present paper describes controlled Smith degradation, partial acid hydrolysis, enzymic degradation and methylation analysis of the products, and presents its structural features and reticuloendothelial system (RES)-potentiating activity.

Materials and Methods

Isolation of Polysaccharide This was performed as described in a previous report.[2]

Periodate Oxidation The polysaccharide (100 mg) was oxidized with 0.05 M sodium metaperiodate (50 ml) at 4 °C in the dark. The periodate consumption was measured by a spectrophotometric method,[8] and the oxidation was completed after 5 d. The reaction mixture was successively treated with ethylene glycol (0.5 ml) at 4 °C for 1 h and sodium borohydride (0.5 g) at 4 °C for 16 h, then adjusted to pH 5.0 by addition of acetic acid. The solution was concentrated and applied to a column (5 × 85 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 31 to 34 were combined, concentrated and lyophilized. Yield, 86 mg.

Controlled Smith Degradation The product (300 mg) obtained by periodate oxidation was dissolved in 0.5 N sulfuric acid (25 ml). After standing at 26 °C for 16 h, the solution was neutralized with barium carbonate. The filtrate was concentrated and applied to a column (5 × 85 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 31 to 34 were combined, concentrated and lyophilized. Yield, 89 mg.

Methylation The sample (5 mg) was dissolved in dimethyl sulfoxide (0.5 ml), then finely powdered sodium hydroxide (20 mg) and methyl iodide (0.1 ml) were added to the solution. The whole was stirred at room temperature for 1 h. All procedures were carried out under nitrogen. Chloroform and water (2 ml each) were then added to the reaction mixture, and the whole was extracted three times with chloroform (2 ml each). The combined extract was washed three times with water (6 ml each), then dried over sodium sulfate. The filtrate was concentrated to dryness, and the residue was dissolved in a chloroform–methanol mixture (2:1), then applied to a column (1 × 17 cm) of Sephadex LH-20. The column was eluted with the same solvent, and fractions of 1 ml were collected. The eluates obtained from tubes 3 to 6 were combined and concentrated. The infrared (IR) spectra of final residues showed no hydroxyl group absorption. Yield, 3 mg.

Analysis of Methylated Products The products were hydrolyzed with diluted sulfuric acid in acetic acid, then reduced and acetylated as previously described.[9] The partially methylated alditol acetates obtained were analyzed by gas chromatography-mass spectrometry (GC-MS) using a fused silica capillary column (0.32 mm i.d. × 30 m) of SP-2330 (Supelco Co.) with a programmed temperature increase of 4 °C per min from 160 to 220 °C at a helium flow of 1 ml per min. GC-MS was performed with a JEOL JMS-GX303 mass spectrometer. The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in GC and the main fragments in MS are listed in Table I.

Partial Acid Hydrolysis and Isolation of Products The controlled Smith degradation product (192 mg) was dissolved in 0.5 M trifluoroacetic acid and heated at 80 °C for 8 h. After evaporation for the removal of acid, the residue was dissolved in water and applied to a column (5 × 80 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method.[10] Fraction 1 was obtained from tubes 51 to 56, fr. 2 from tubes 57 to 59, fr. 3 from tubes 60 to 62, and fr. 4 from tubes 63 to 70. The yields were 24.9, 9.7, 38.0 and 110.0 mg, respectively.

Analysis of Products Thin-layer chromatography (TLC) was carried out on Merck precoated Kieselgel 60 plates using *n*-butanol–acetic acid–water (2:1:1, v/v) as a developing solvent. High-performance liquid chromatography (HPLC) of *p*-aminobenzoyl derivatives purified using a column (6 × 22 mm) of PBA-60 (Amicon Co.)[11] was performed with a column (4.6 × 250 mm) of TSK-gel Amide-80 (Tosoh Co.) and detected by absorption at 304 nm. The HPLC system used consisted of a Hitachi L-6200 intelligent pump, a JASCO Uvidec-100 spectrophotometer and a Hitachi D-2000 chromatointegrator. Elution was done at a flow rate of 1 ml per min at room temperature using a linear gradient of acetonitrile–

TABLE I. Relative Retention Times on GC and Main Fragments in MS of Partially Methylated Alditol Acetates

	Relative retention time[a]	Main fragments (*m/z*)
1,4-Ac_2-2,3,5-Me_3-L-arabinitol	0.69	43, 45, 71, 87, 101, 117, 129, 161
1,4,5-Ac_3-2,3-Me_2-L-arabinitol	1.13	43, 87, 101, 117, 129, 189
1,5-Ac_2-2,3,4,6-Me_4-D-glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,3,5-Ac_3-2,4,6-Me_3-D-glucitol	1.39	43, 45, 87, 101, 117, 129, 161
1,3,5-Ac_3-2,4,6-Me_3-D-galactitol	1.34	43, 45, 87, 101, 117, 129, 161
1,4,5-Ac_3-2,3,6-Me_3-D-galactitol	1.44	43, 45, 87, 99, 101, 113, 117, 233
1,3,5,6-Ac_4-2,4-Me_2-D-galactitol	2.01	43, 87, 117, 129, 189

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Abbreviations: Ac = acetyl; Me = methyl (*e.g.*, 1,4-Ac_2-2,3,5-Me_3- = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-).

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water (75:25 to 50:50) for 20 min after sample injection. Retention times of glucose, laminaribiose, laminaritriose and laminaritetraose were 3.3, 4.4, 6.0 and 8.1 min.

Enzymic Degradation and Isolation of Products The polysaccharide (200 mg) was dissolved in 0.05 M acetate buffer (pH 6.0, 10 ml) and β-glucosidase preparation from almonds (10 mg of Emulsin, 900 units per mg, Tokyo Kasei Co.) was added. The solution was incubated with a few drops of toluene at 37 °C for 24 h, then applied to a column (5 × 15 mm) of Dowex 50W-X8 (H^+). The eluate with water was applied to a column (5 × 80 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method. Fraction a-1 was obtained from tubes 33 to 38 and fr. b-1 from tubes 62 to 66. Their yields were 165.0 and 10.9 mg. Fraction a-1 was treated with the same enzyme preparation once again, and the resulting solution was applied to the two columns successively as described above. Fraction a-2 was obtained from tubes 31 to 35 and fr. b-2 from tubes 61 to 65. The yields were 135.6 and 19.0 mg, respectively.

Analysis of Degradation Products Molecular masses were estimated by gel chromatography. The sample (3 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0), and applied to a column (2.6 × 94 cm) of Toyopearl HW-55F, pre-equilibrated and developed with the same buffer. Fractions of 5 ml were collected and analyzed by the phenol–sulfuric acid method. Standard pullulans (Shōwa Denkō Co.) having known molecular masses were run on the column to obtain a calibration curve.

Component sugars were analyzed by GC after conversion of the hydrolyzate into alditol acetates as described previously.[12]

Phagocytic Activity This was measured as described in a previous report.[12] The sample and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved and suspended in physiological saline and dosed i.p. (20 or 50 mg/kg body weight) once a day. The phagocytic index, K, was calculated by means of the following equation:

$$K=(\ln OD_1-\ln OD_2)/(t_2-t_1)$$

where OD_1 and OD_2 are the optical densities at times t_1 and t_2, respectively. Results were expressed as the arithmetic mean ± S.D. of five male mice (ICR-SPF). The comparison of results was performed by means of Student's t-test.

Results

The hot water extract obtained from the seed of *Malva verticillata* was applied to a column chromatography of diethylaminoethyl (DEAE)-Sephadex A-25. The eluate with water was subjected to affinity chromatography on concanavalin A (Con A)-Sepharose. The passed-through fraction was dialyzed and purified by gel chromatography with Sephadex G-25, then the major neutral polysaccharide, MVS-I, was obtained after lyophilization.[2] The polysaccharide was homogeneous on electrophoresis and gel chromatography, and it was composed of L-arabinose, D-galactose and D-glucose in the molar ratio of 3:6:7.[2] Its molecular mass was estimated to be 7.7×10^4. The minimal repeating unit of MVS-I is composed of terminal α-L-arabinofuranose, α-1,5-linked L-arabinofuranose, β-1,3-linked D-galactopyranose, β-1,4-linked D-galactopyranose, β-3,6-branched D-galactopyranose, terminal β-D-glucopyranose and β-1,3-linked D-glucopyranose residues in the ratio of 3:3:2:6:4:1:13.[2]

The controlled Smith degradation[13] of MVS-I by periodate oxidation and reduction followed by mild hydrolysis yielded a polymer composed of D-galactose and D-glucose in a molar ratio of 6:13. Its molecular mass was 2.4×10^4. The controlled Smith degradation product was methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.[14] The methylated product was hydrolyzed, then converted into the partially methylated alditol acetates. Analysis by GC-MS revealed derivatives of 2,3,4,6-tetra-*O*-methyl D-glucose, 2,4,6-tri-*O*-methyl D-glucose, 2,4,6-tri-*O*-methyl D-galactose and 2,4-di-*O*-methyl D-galactose in a molar ratio of 1:12:5:1.

The result of methylation analysis revealed that it was mainly composed of β-1,3-linked D-galactose and D-glucose units and that a part of the D-galactose residues in the backbone carries side chains having terminal D-glucose at position 6.

On the other hand, the controlled Smith degradation product was partially hydrolyzed with dilute trifluoroacetic acid under the condition providing the maximum yield of total oligosaccharides. After removal of the acid, the products were isolated by chromatography with Sephadex G-25. In addition to galactose and glucose in an approximate ratio of 2:3 (fr. 4), three oligosaccharide fractions (frs. 1 to 3) were obtained. Laminaritetraose, laminaritriose and laminaribiose were identified in these fractions by HPLC of *p*-aminobenzoyl derivatives and by TLC. No galactose-containing oligosaccharide was obtained under this condition. This result suggested the presence of linear oligosaccharide chains composed of β-1,3-linked D-glucose units in MVS-I.

MVS-I was treated with β-glucosidase preparation from almonds followed by chromatography with Sephadex G-25. A high molecular mass fraction (fr. a-1) was treated once more with the same enzyme preparation. The final high molecular mass fraction (fr. a-2) is tentatively designated as EDP. The yields of the monosaccharide fraction (frs. b-1 and b-2) of the primary and secondary enzymic degradations were 5.4% and 11.5%, respectively. The yields of fr. a-1 and EDP were 82.5% and 82.2%, respectively. Fraction b-1 contained glucose, galactose and arabinose in an approximate ratio of 5:1:1, and fr. b-2 had glucose, galactose and arabinose in an approximate ratio of 12:1:1.

EDP was homogeneous on gel chromatography, and its molecular mass was estimated to be 2.4×10^4. It was composed of L-arabinose, D-galactose and D-glucose in the molar ratio of 6:9:4. The methylation analysis of EDP was performed in the similar manner to those described above, and GC-MS revealed derivatives of 2,3,5-tri-*O*-methyl L-arabinose, 2,3-di-*O*-methyl L-arabinose, 2,3,6-tri-*O*-methyl D-galactose, 2,4,6-tri-*O*-methyl D-galactose, 2,4-di-*O*-methyl D-galactose and 2,4,6-tri-*O*-methyl D-glucose in a molar ratio of 3:3:3:3:3:4. These results suggested that EDP was appeared from MVS-I by the loss of side chains composed of both β-1,4-linked D-galactose and β-1,3-linked D-glucose. Glycosidase activities of the enzyme preparation on these types of glycosidic linkage were ascertained using lactitol and laminaribitol as substrates. Weak α-L-arabinofuranosidase activity of the enzyme preparation used was also confirmed using a larch wood arabinogalactan (Sigma Co.) as the substrate. It caused about a 6% liberation of terminal arabinose residues in the polysaccharide. In the side chains, β-1,4-linked D-galactosyl units must be located on the outer side of β-1,3-linked D-glucosyl units because of the loss of the former group by the controlled Smith degradation.

Mild treatment of MVS-I with 0.05 M trifluoroacetic acid at 100 °C for 3 h caused partial liberation of some component sugars. The liberation rates of arabinose, galactose and glucose were 90.6%, 16.4% and 1.8%, respectively. This result showed the presence of arabinosyl arabinose units in the terminals of side chains in MVS-I.

Based on the accumulated evidence described above, it

side chain A ↓ 6 side chain B ↓ 6

$[\rightarrow 3\ \beta\text{-D-Glc}p\ 1\rightarrow]_a[\rightarrow 3\ \beta\text{-D-Gal}p\ 1\rightarrow]_b[\rightarrow 3\ \beta\text{-D-Glc}p\ 1\rightarrow]_c[\rightarrow 3\ \beta\text{-D-Gal}p\ 1\rightarrow]_d[\rightarrow 3\ \beta\text{-D-Glc}p\ 1\rightarrow]_e[\rightarrow 3\ \beta\text{-D-Gal}p\ 1\rightarrow]_f$

average feature of side chain A: α-L-Ara$f$ 1→5 α-L-Ara$f$ 1→4 β-D-Gal$p$ 1→

average feature of side chain B: β-D-Glc$p$ 1→[4 β-D-Gal$p$ 1→]$_3$[3β-D-Glc$p$ 1→]$_9$

Chart 1. Possible Structural Units of MVS-I

$(a+c+e):b:d:f=4:3:2:1$.

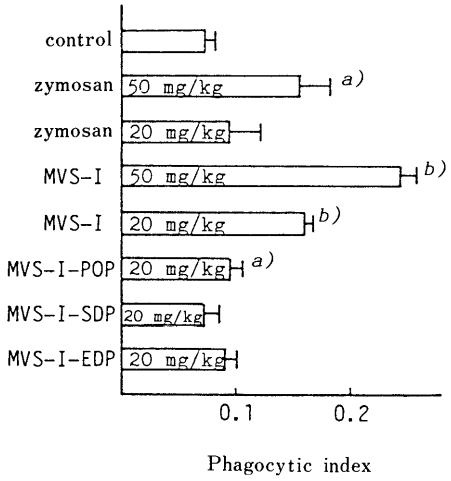

Fig. 1. Effects of MVS-I and Its Degradation Products on Phagocytosis

Significantly different from the control, *a*) $p<0.01$, *b*) $p<0.001$.

can be concluded that MVS-I has the structural features shown in Chart 1.

The effects of MVS-I, and its periodate oxidation and reduction product (POP), the controlled Smith degradation product (SDP) and EDP on the RES were demonstrated by a modification[12] of the *in vivo* carbon clearance test[15] using zymosan as a positive control. As shown in Fig. 1, MVS-I showed remarkable activity. POP showed significant activity, however, neither SDP nor EDP showed RES activity.

Discussion

We have obtained three neutral polysaccharides, MVS-I,[2] MVS-IIA and MVS-IIG,[3] and three acidic polysaccharides, MVS-IIIA,[4] MVS-IVA[5] and MVS-VI,[1] from the seed of *Malva verticillata*. As one of their immunological activities, especially remarkable anti-complementary activity was found in MVS-I, MVS-IIA and MVS-VI.[1,7] In addition, both MVS-IIIA and MVS-IVA also showed considerable anti-complementary activity.[7] Further, these two acidic polysaccharides showed remarkable RES-potentiating activity in the carbon clearance test, while this activity of MVS-VI was relatively low.[1] The phagocytic index was remarkably increased, suggesting the marked activation of RES by i.p. injection of MVS-I.

MVS-IIIA, -IVA and -VI possess acidic arabino-3,6-galactan structure as their major parts. We have already isolated and characterized saposhnikovan A[12] from the root and rhizome of *Saposhnikovia divaricata*, ukonans A[16] and B[17] from the rhizome of *Curcuma longa*, glycyrrhizan UA[18] from the root of *Glycyrrhiza uralensis*, and glycyrrhizan GA[19] from the stolon of *G. glabra* var. *glandulifera* as the other RES active polysaccharides belonging to this structural group.

MVS-I possesses both α-1,5-linked L-arabinofuranosyl and β-3,6-branched D-galactosyl units. However, unlike typical arabino-3,6-galactan, the major part of this polysaccharide is occupied by β-1,3-linked D-glucose residues with dispersed β-1,3-linked D-galactose units. In addition, it has considerable β-1,4-linked D-galactose residues and no hexuronic acid. Thus MVS-I is a novel RES active neutral polysaccharide having a new structural type. Two kinds of side chains were found in it. They are composed of β-1,4-linked D-galactosyl β-1,3-linked D-glucosyl units and α-1,5-linked L-arabinosyl β-1,4-linked D-galactosyl units. The removal of one of each caused the disappearance of the RES-potentiating activity.

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Purification and Characterization of a Nuclease from *Lentinus edodes*

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An endonuclease with 3′-nucleotidase activity (nuclease Le1) was purified from fruit bodies of *Lentinus edodes* in a single band on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The apparent molecular weight of nuclease Le1 was about 27000. The nuclease was inactivated in the presence of ethylenediaminetetraacetic acid (EDTA) and reactivated by the addition of Zn^{2+}. Hydrolysis of poly U by the nuclease showed many intermediate size oligomers prior to the formation of 5′-uridine monophosphate (UMP). Therefore, it was concluded that nuclease Le1 was a Zn^{2+}-endonuclease similar to P1-nuclease from *Penicillium citrinum*. The nuclease was very sensitive to ionic strength, but pH-profiles of the hydrolysis of four 3′-nucleotides were very similar to those of P1 nuclease from *P. citrinum*.

Keywords nuclease; 3′-nucleotidase; *Lentinus edodes*; purification; amino acid composition; zinc-reactivation; molecular weight

5′-Guanylic acid is known as the major component of the tasty substance in *Lentinus edodes*. It is evident that the production of this compound is correlated to the nucleic acid metabolism in *L. edodes*, in which several ribonucleic acid (RNA) degrading enzymes are reportedly present.[1-4] In a previous paper, we isolated one of the enzymes, ribonuclease (RNase) Le2, from the fruit bodies of *L. edodes* and characterized it as a base non-specific and adenylic acid preferential RNase.[5] In this paper, we report the purification of another RNA degrading enzyme from *L. edodes* and characterize it as a Zn^{2+}-endonuclease with 3′-nucleotidase activity.

Materials and Methods

Substrate Yeast RNA is a product of Kojin Co. (Tokyo). 3′-Nucleotides, 5′-nucleotides and salmon sperm deoxyribonucleic acid (DNA) were the products of Wako Pure Chemicals (Osaka). Homopolynucleotides were the products of Yamasa (Chiba).

Reagents Molecular weight marker proteins were obtained from Oriental Yeast Co. (Tokyo). The reagents for polyacrylamide gel electrophoresis (PAGE) were purchased from Wako Pure Chemicals. Phospho-cellulose and diethylaminoethyl (DEAE)-cellulose were obtained from Serva (West Germany). Sephadex G-50, and SP-Sephadex C-50 were obtained from Pharmacia (Uppsala). DEAE-toyopearl 650M, Butyl-toyopearl, QAE-toyopearl 550C and TSK-gel G2000SW for high performance liquid chromatography (HPLC) were the products of Toso (Tokyo).

Enzyme Source Fresh fruit bodies of *L. edodes* were supplied by Mori Mushroom Research Institute (Kiryu). Alkaline phosphatase from *Escherichia coli* was purchased from Wako Pure Chemicals.

Enzyme Assay (a) RNase assay was carried out as described in the previous paper[5] with RNA as substrate at pH 4.75 and 37 °C. The enzyme unit was defined as the absorbancy increase per 5 min under the experimental conditions described above.

(b) Hydrolyses of DNA, homopolynucleotides, and 3′- or 5′-nucleotides were followed by the method of Fujimoto *et al.*[6] Twenty mM of buffer (0.4 ml) containing 1 mg nucleic acid or nucleotide was treated with 10 μl of enzyme solution at 37 °C for 10 min. The reaction was terminated by the addition of an equal volume of 0.2 M Tris–HCl buffer (pH 9.1). To the reaction mixture was added 0.25 unit of alkaline phosphatase and the release of Pi was measured as described by Chen *et al.*[7]

Protein Concentration Protein concentration was measured colorimetrically according to Smith *et al.*[8] using bovine serum albumin as standard.

Sodium dodecylsulfate (SDS)-PAGE SDS-PAGE was conducted in 12.5% polyacrylamide gel according to the method of Laemmli.[9] The gel was stained with a silver staining kit (Daiichi Kagaku, Tokyo). Molecular weight of nuclease was determined by SDS-PAGE using molecular weight marker proteins.

Isoelectric Focusing Electrofocusing on 5% polyacrylamide gel was performed according to the procedure of Dale and Latner[10] using ampholine covering pH 3.5—10.0 after overnight electrophoresis at 200 V: voltage was then raised to 400 V for 2 h. The pI value of nuclease was estimated by comparing the mobility of nuclease with that of pI markers (pI 4.1—10.6).

Amino Acid Analysis Amino acid analysis was done by the method of Bidlingmeyer *et al.*[11] with a Pico Tag™ amino acid system (Waters). Phenylthiocarbamoyl amino acids were separated by reversed phase HPLC with a Pico-Tag™ column. The separation conditions were previously described.[5] Tryptophan content was determined by the method of Pajot.[12]

Edman Degradation Nuclease Le1 was subjected to automated Edman degradation using an Applied Biosystem 477A protein sequencer with a 120A PTH-analyzer according to Hewick *et al.*[13]

Hydrolysis of RNA with a Purified Nuclease RNA (0.5 mg) was dissolved in 50 mM acetate buffer (pH 4.75) and incubated with 5 μg of enzyme at 37 °C. Aliquots were withdrawn at appropriate intervals and chromatographed as described.[5]

Time Course of Hydrolysis of Poly U Checked by Gel-Filtration To 0.2 ml Poly U solution containing 0.5 mg poly U, 5 μl of enzyme (*ca.* 0.5 u) was added. The reaction mixture was incubated at 37 °C. Fifty μl aliquots were withdrawn at an appropriate time and injected to the column of TSK-gel G2000SW equilibrated with 50 mM ammonium acetate buffer (pH 6.0). The column was eluted with the same buffer at the flow rate of 0.5 ml/min. The eluate was monitored by the absorbancy at 260 nm.

Zn^{2+} Content Zn^{2+} content in nuclease Le1 was determined with a Shimadzu atomic absorption spectrometer A 660 at 213.8 nm using 3 nmol of nuclease.

Results

Purification of RNA Hydrolyzing Enzyme We previously found two RNA hydrolyzing enzymes in the fresh fruit bodies of *L. edodes*.[5] One of them was identified as the base non-specific and adenylic acid preferential RNase.[5] In this paper, we purified an another enzyme. During the course of purification described below, 0.1 mM $ZnCl_2$ was added to the buffers used.

Step 1: The crude extract of fresh fruit bodies of *L. edodes* (1.5 kg) was extracted with 3.75 l of 10 mM sodium acetate buffer (pH 6.0) as described.[5]

Step 2: To 6 l of crude extract, 600 g of DEAE-cellulose (wet weight) pre-equilibrated with 10 mM sodium acetate buffer (pH 6.0) was added and the suspension was stirred gently at 0 °C for 30 min. The DEAE-cellulose-adsorbed protein was collected by suction and washed with 3 l of the buffer, then suspended in the same buffer. The column packed with the DEAE-cellulose was eluted with a linear

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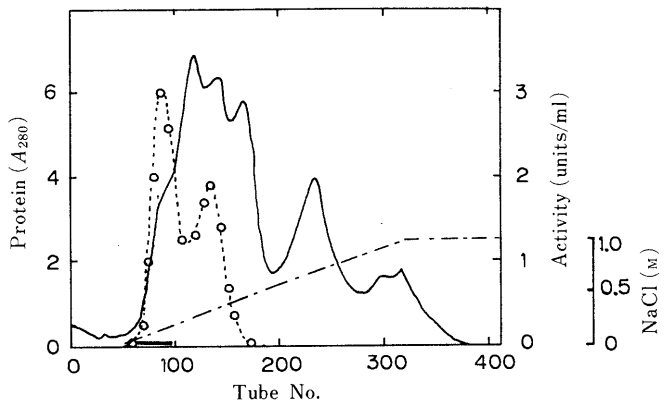

Fig. 1. DEAE-Cellulose Column Chromatography of Crude Extract

The crude extract of step 1 was adsorbed on a column (6 × 40 cm) of DEAE-cellulose equilibrated with 10 mM sodium acetate buffer (pH 6.0). After washing with 3 l of the same buffer, the column was eluted with a linear gradient of 0—1 M NaCl in 4 l of the same buffer. Fifteen ml fractions were collected, and those indicated by a heavy bar were pooled. ——, A_{280}; ○, RNase activity; —·—, NaCl concentration.

gradient of 0—1 M NaCl in 4 l of the same buffer. Fifteen ml fractions were collected (Fig. 1). The enzyme activity was separated into two fractions, the first (tube Nos. 70—99, RNase Le1 fraction) and the second (tube Nos. 100—180, RNase Le2 fraction).

Step 3: The RNase Le1 fraction was concentrated *in vacuo*, then dialyzed against deionized water. The pH of the dialyzate was adjusted to 6.0, and the precipitate formed was eliminated by centrifugation at 10000 rpm for 15 min. The supernatant was fractionated by gel-filtration on Sephadex G-50 (4 × 210 cm, Pharmacia) equilibrated with 10 mM sodium acetate buffer (pH 6.0). Ten ml fractions were collected. The active RNase fractions (tube Nos. 55—80) were pooled.

Step 4: SP-Sephadex C-50 Column Chromatography: The active RNase fraction of step 3 was dialyzed against deionized water. The pH of the dialyzate was adjusted to 5.0 and put on a column of SP Sephadex C-50 (3 × 30 cm, Pharmacia) equilibrated with 10 mM sodium acetate buffer (pH 5.0). Ten ml fractions were collected. The enzymatic activity was found in the unadsorbed fraction (tube Nos. 22—33).

Step 5: DEAE-Toyopearl 650M Column Chromatography: The active RNase fraction of step 4 was dialyzed against deionized water. The pH of dialyzate was adjusted to 6.0 and put on a column of DEAE-toyopearl 650M (1 × 25 cm, Toso) equilibrated with 10 mM sodium acetate buffer (pH 6.0). The column was eluted with a linear gradient of 200 ml of 10 mM sodium acetate buffer (pH 6.0) and 200 ml of sodium acetate buffer (pH 6.0) containing 0.3 M NaCl. Three ml fractions were collected. Active RNase fractions were pooled (tube Nos. 54—60).

Step 6: Butyl-Toyopearl Column Chromatography: The active RNase fraction of step 5 was dialyzed against 0.1 M phosphate buffer (pH 6.5) and solid ammonium sulfate was added to the dialyzate to make 1.2 M. The enzyme solution was applied on a column of Butyl-toyopearl (1 × 20 cm) equilibrated with the same buffer described above but containing 1.2 M ammonium sulfate (200 ml). The column was eluted with a linear gradient of 200 ml of the equilibrating buffer and the same buffer without ammonium sulfate. Three ml fractions were collected. The active RNase fraction (tube Nos. 89—100) was collected.

Step 7: QAE-Toyopearl 550C Column Chromatography: The active RNase fraction of step 6 was dialyzed against deionized water, and the pH of the solution was adjusted to 6.0. The enzyme solution was applied on a column of QAE-toyopearl (1 × 20 cm). The column was eluted with the linear gradient of between 200 ml of the same buffer and the same buffer containing 0.25 M NaCl. Three ml fractions were collected. The active RNase fractions (tube Nos. 121—126) were pooled.

Step 8: HPLC on TSK-Gel G2000SW: The active RNase

Table I. Purification of Nuclease Le1 from the Caps of Fruit Bodies of *L. Edodes* (1.5 kg)

Step	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Yield (%)
1. Crude extract	5940	34900	0.17	100
2. DEAE-cellulose	1420	2700	0.52	23.9
3. Sephadex G-50	1250	781	1.60	21.0
4. SP-Sephadex	760	281	2.70	12.8
5. DEAE-toyopearl	428	6.40	66.8	7.2
6. Butyl-toyopearl	214	2.69	79.6	3.6
7. QAE-toyopearl	187	2.18	85.7	3.1
8. TSK-gel G2000SW	113	0.88	128	1.9

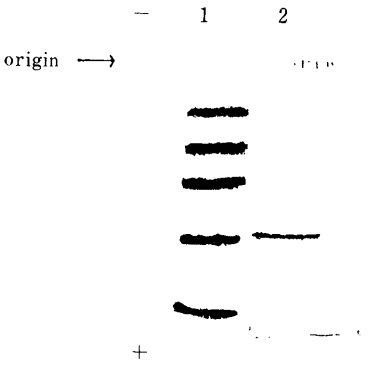

Fig. 2. SDS–PAGE of Nuclease Le1

SDS–PAGE of the purified RNase fraction Le1 (nuclease Le1) stained with silver staining kit. Lane 1, standard proteins of molecular weight 74000, 49600, 37200, 24800, 12400; lane 2, RNase fraction Le1.

Table II. Effect of Divalent Cations and EDTA on the Enzymatic Activity of Nuclease Le1

Addition	Enzymatic activity (%)
None	100 (27)
Zn^{2+}	100 (89)
Mg^{2+}	100 (31)
Mn^{2+}	100 (28)
Ca^{2+}	100 (27)
Fe^{2+}	98 (27)
Sr^{2+}	98 (25)
Ba^{2+}	98 (24)
Cd^{2+}	85 (18)
Ni^{2+}	75 (17)
Hg^{2+}	37 (12)
Cu^{2+}	39 (8)
EDTA	27 (27)

The figures in parentheses indicate the effect of divalent cation on the enzyme pre-treated with EDTA (27% activity).

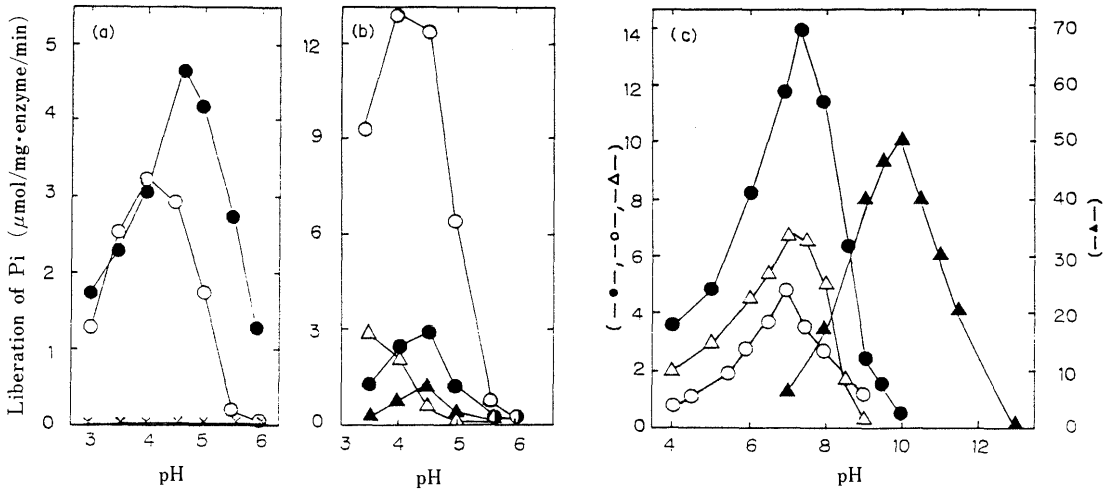

Fig. 3. Effect of pH on the Rate of Hydrolysis of RNA, DNA, Homopolynucleotides and Four 3′-Nucleotides by Nuclease Le1

Substrate concentration: 2.5 mg/ml. Enzyme concentration; 0.002—0.13 u. Buffers used were: 0.1 M citrate buffer for pH 3—6.5, 0.1 M Mes buffer for pH 6—7, 0.1 M Tris–HCl buffer for pH 7—9, 0.1 M glycine–NaOH buffer for pH 9—11.5 and KCl–NaOH for pH 12—13. (a) ●, RNA; ○, heat denatured DNA; ×, double stranded DNA. (b) ●, poly A; ○, poly U; ▲, poly G; △, poly C. (c) ●, 3′-AMP; ○, 3′-UMP; ▲, 3′-GMP; △, 3′-CMP.

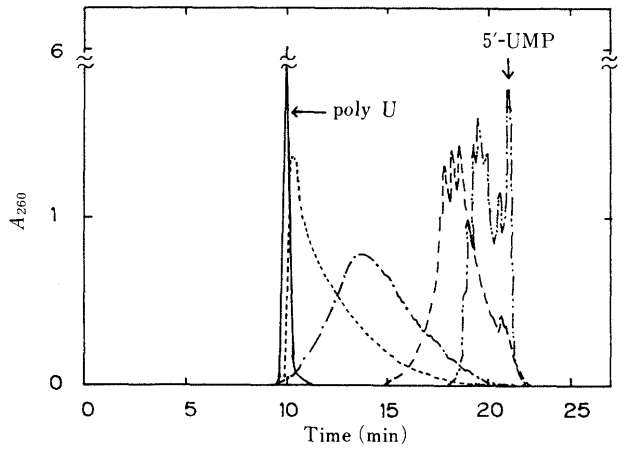

Fig. 4. Time Course of Degradation of Poly U by Nuclease Le1

The digestion products of poly U by nuclease Le1 were separated by gel filtration on TSK-gel G2000SW. ——, 0 time; – – –, 1 min; —·—, 2 min; — —, 5 min; —··—, 30 min. Arrow at retention time 21.6 min indicates the elution position of 5′-UMP. Other experimental conditions were as described in Materials and Methods.

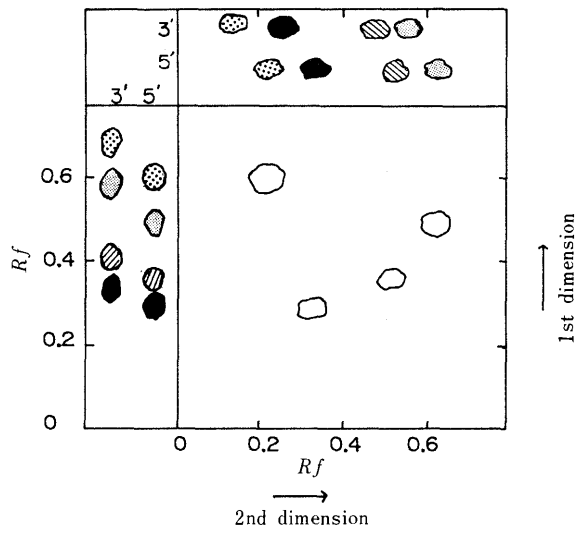

Fig. 5. Hydrolysis Products of RNA by Nuclease Le1

The hydrolysis products of 24 h were applied to two-dimensional paper chromatography. Solvents used for the first and second dimensions were isobutyric acid; 0.5 N NH_4OH (10 : 6, v/v) and 2-propanol : water : saturated ammonium sulfate in water : 0.1 M sodium citrate–phosphate buffer (pH 6.0) (2 : 80 : 20, v/v), respectively. Dark spot, GMP; heavy dotted spot, AMP; hatched spot, UMP and light dotted spot, CMP.

fraction of step 7 was concentrated to 0.2 ml and gel-filtrated with TSK-gel G2000SW HPLC (0.78 × 30 cm) equilibrated with 50 mM ammonium acetate buffer (pH 6.0), and eluted with the same buffer. Five-tenth ml fractions were collected. The active RNase fractions (tube Nos. 17—20) were pooled.

The RNase fraction (RNase Le1) was purified 753 fold, at a yield of *ca.* 2%. Purification procedures are summarized in Table I. The RNase Le1 thus purified gave a single band on SDS-PAGE (Fig. 2).

Effect of Divalent Cations and EDTA At 1 mM concentration, among the various divalent cations and EDTA tested with RNA as substrate, Cu^{2+}, Hg^{2+} inhibited markedly the RNase activity and inhibited moderately the Ni^{2+} and Cd^{2+}. However, the RNase activity was inhibited by EDTA. The EDTA inactivated enzyme was markedly reactivated with Zn^{2+} (Table II). This evidence seems to suggest that the RNase Le1 fraction is not a simple RNase, but a Zn^{2+}-enzyme like P1 nuclease from *Penicillium citrinum*,[14] and S1 nuclease from *Saccharomyces cerevisiae*.[14]

Substrate Specificity In order to characterize the RNase Le1, the enzymatic activities towards DNA, homopolynucleotides, 3′-nucleotides and 5′-nucleotides were measured at various pHs. RNase Le1 fraction hydrolyzed heat-denatured DNA as well as RNA, but not double stranded DNA, though the optimum pH for DNA cleavage was somewhat different from that of RNA (Fig. 3). This evidence revealed Le1 to be a nuclease specific for single stranded RNA and DNA. The enzyme was active towards 3′-nucleotides but not 5′-nucleotides (data not shown). The rate of hydrolysis of RNA is about 1.45 fold that of single stranded DNA. Since this enzyme works both for DNA and RNA, we renamed it nuclease Le1.

As shown in Fig. 4, when poly U was hydrolyzed by nuclease Le1, various sizes of polynucleotides were observed

before the formation of 5′-uridine monophosphate (UMP). It was therefore, evident that the nuclease was an endonuclease with 3′-nucleotidase activity.

Nuclease Le1 hydrolyzed RNA and accumulated four 5′-nucleotides (Fig. 5). Thus it was a 5′-former nuclease.

Other Properties of Nuclease Le1 Molecular weight of nuclease Le1 estimated from SDS-PAGE was about 27000 (Fig. 2). The pI of nuclease Le1 obtained from disc gel electrophoresis[10] was 4.6 (data not shown). As shown in Fig. 6, the nuclease Le1 activity was markedly inhibited by the increase of ionic strength as compared to that of RNase Le2 coexisting in *L. edodes*.[5] When we incubated the nuclease at 37 °C for 1 h, nuclease Le1 was stable between pH 5—6, and became unstable below pH 4 and above 7.0 (Fig. 7). Zn^{2+} Content of nuclease Le1 contains 1.5—2.0 atoms of Zn^{2+} per mol enzyme. From this evidence and the results described above, it was evident that nuclease Le1 was a Zn^{2+} endonuclease.

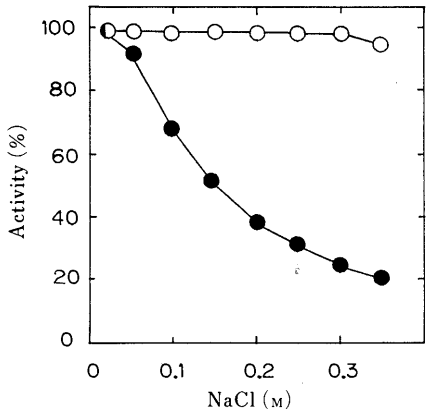

Fig. 6. Effect of Salt Concentration on Nuclease Le1 Activity

Details of the experimental conditions are the same as the assay method (a) with RNA as substrate. ●, nuclease Le1; ○, RNase Le2.

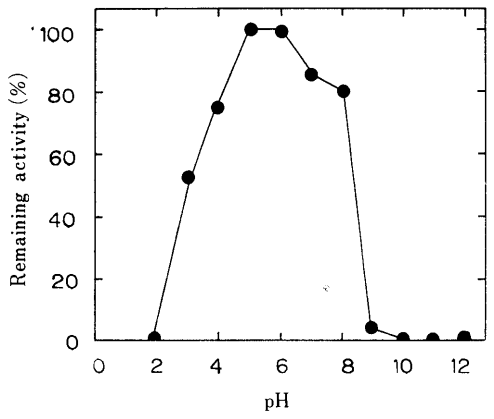

Fig. 7. pH Stability of Nuclease Le1

Nuclease Le1 (0.6 u) was incubated at various pH and 37 °C for 1 h, then 1/10 volume of 2 M acetate buffer (pH 4.75) was added to the reaction mixtures. The enzymatic activities of each incubation mixture were measured with the assay method (a) at pH 4.75.

Amino Acid Composition of Nuclease Le1 The amino acid composition of nuclease Le1 is shown in Table III and that of a typical nuclease, P1 nuclease is shown for comparison. Half cystine content was the same as that of P1 nuclease. The results also show the similarity between these two enzymes.

Amino-Terminal Sequence of Nuclease Le1 The amino terminal amino acid sequence of nuclease Le1 was determined by Edman degradation (Fig. 8). The amino terminal sequence is very similar to that of *Penicillium* enzyme[5] indicating the homogeneity of nuclease. The sequence homology between two enzymes of the first 19 residues so far determined is 63%.

Discussion

Accumulation of 5′-nucleotides during the processing of edible mushrooms was observed by Mouri *et al.*,[1] and they suggested the participation of some RNA cleaving enzymes in the production of these nucleotides. Kadowaki *et al.*[2] also described the presence of nuclease which forms 5′-nucleotides in dry shii-take (*L. edodes*) fruit body. Endo *et al.*[3] partially purified three 3′-nucleotidase fractions from shii-take, and reported their properties. In this paper, we purified only one nuclease with 3′-nucleotidase activity in the homogeneous state as judged by SDS-PAGE. The enzymatic properties of this purified nuclease, such as pH optimum and pH-activity profile of 3′-nucleotidase towards

Table III. Amino Acid Composition of Nuclease Le1

Amino acid	Experimental value[a]	Nuclease from *Penicillium* sp.[b]
Trp	3.8 (4)	9
Lys	5.9 (6)	7
His	5.7 (6)	11
Arg	9.0 (9)	6
Asp	21.0 (21)	32
Thr	15.5 (16)	17
Ser	22.5 (23)	29
Glu	24.6 (25)	22
Pro	18.3 (18)	8
Gly	18.3 (18)	21
Ala	30.5 (31)	34
1/2Cys	4.0 (4)	4
Val	19.4 (19)	4
Met	4.2 (4)	3
Ile	15.9 (16)	18
Leu	20.4 (20)	18
Tyr	8.6 (9)	14
Phe	12.9 (13)	4
Total	263	270

a) Calculated assuming Arg residue as 9.0. The amino acid contents of Ser, Thr, and Met are the values extrapolated to zero time from the data of 24 and 48 h hydrolysis. Parenthesis indicates the nearest integer. *b*) Amino acid composition of the nuclease from *Penicillium* sp. was obtained from the literature.[18]

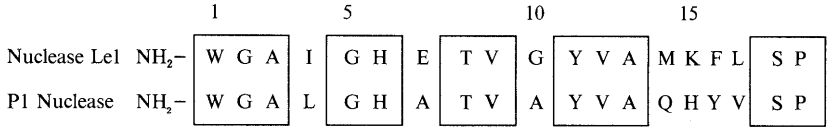

Fig. 8. *N*-Terminal Amino Acid Sequence of Nuclease Le1

The sequence of nuclease from *P. citrinum* is also included. Common sequences between these two enzymes are blocked.

four 3′-nucleotides, were essentially the same as those of the three 3′-nucleotidase fractions reported by Endo *et al.*[3] The production of three species of nuclease in dry shii-take is probably due to some processing of 3′-nucleotidase during the drying sequence. As shown above, nuclease Le1 is a Zn^{2+} enzyme, so we added 0.1 mM $ZnCl_2$ to the buffers used for purification to prevent the loss of the enzyme.

As described above, we showed that nuclease Le1 contains about 2 atoms of Zn^{2+}. Therefore, it belonged to the same category as P1 nuclease and S1 nuclease found in fungi.[14,15] As shown in Fig. 3, nuclease Le1 liberated inorganic phosphate from 3′-nucleotides, but not from 5′-nucleotides. The optima for three 3′-nucleotides, 3′-UMP, 3′-cytidine monophosphate (CMP) and 3′-adenosine monophosphate (AMP) was 7.0—7.5 and that of 3′-guanosine monophosphate (GMP) was about 10.0. The results shown in this figure are very similar to those observed for P1 nuclease.[16] Thus, nuclease Le1 seems to be a very similar enzyme as P1 nuclease.

The primary structures of this kind of nuclease were determined by Iwamatsu *et al.*[17] only for *Saccharomyces cerevisiae* nuclease (S1 nuclease) and by Tabata *et al.*[18] and Maekawa *et al.*[19] for *P. citrinum* and related species. The N-terminal sequence of nuclease Le1 is very similar to that of the *Penicillium* enzyme, and the sequence homology is 63%. But even in unidentical amino acid residues, we observed several substitution in these two nucleases between amino acid of similar nature, such as Leu to Val, Phe to Tyr and Ile to Leu. Since other fungi such as *Flammulina velutipes* contain similar nucleases,[20] elucidation of the structure of nuclease Le1 might provide useful information on their mechanism of action and also on the chemotaxonomy of fungi.

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Enzymatic Sulfation of Polyphenols Related to Tannins by Arylsulfotransferase

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This report discusses a novel type of arylsulfotransferase (AST) which was derived from human intestinal bacterium sulfated polyphenolic compounds when *p*-nitrophenyl sulfate (PNS) was taken as a donor substrate. (+)-Catechin, (±)-catechin, (−)-epicatechin and (−)-epicatechin gallate were better substrates than tyramine. (−)-Epigallocatechin and (−)-epigallocatechin gallate were slightly worse substrates than tyramine. Although gallic acid was a bad substrate, alkyl gallate esters were better substrates than tyramine. The degree of acceptor specificity increased in proportion to the length of the alkyl group up to the carbon number of five. Pedunculagin, geraniin and corilagin were less effective than tyramine. Rosmarinic acid and penta-*O*-galloyl-β-D-glucose were similarly well sulfated. Two products, 4′-monosulfate and 4′,5-disulfate of (+)-catechin, were detected at a two-fold molar excess of PNS over (+)-catechin. When (+)-catechin-4′-monosulfate as an acceptor was enzymatically sulfated with PNS as a donor, only the 4′,5-disulfate was produced. Thus, arylsulfotransferase was useful for the convenient preparation of sulfate esters of polyphenols at their specific hydroxyl groups.

Keywords arylsulfotransferase; (+)-catechin; polyphenol; sulfate conjugation; intestinal bacteria

Introduction

Sulfate conjugation is widely known as one of the major metabolic pathways for the detoxification of endogenous and exogenous phenolic compounds. Arylsulfotransferase (EC 2.8.2.1) was first discovered in guinea pig liver,[1] and thereafter was found in the brain, kidney, intestinal epithelial cells and so on.[2-4] The enzyme catalyzes the transfer of sulfate from 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to aliphatic and phenolic alcohol groups, and forms sulfate conjugates. A similar type of enzyme was also found in the plant kingdom.[5]

Tannins are polyphenolic compounds of various chemical structures and are divided into two main classes, hydrolyzable tannins and condensed tannins. Several studies have been reported on their complexation with proteins or metal ions, and on their biological activities which involved the inhibitory effect of (−)-epigallocatechin gallate on protein kinase C[6] and carcinogenesis.[7]

We discovered a novel type of arylsulfotransferase from the human intestinal bacterium, *Eubacterium* A-44.[8] This enzyme catalyzes the transfer of sulfate groups from phenol sulfate esters, but not from PAPS, to other phenols with strict specificity. The bacterial enzyme is several thousand-fold more active than the enzymes from mammalian and plant origins, and is expected to be useful for the preparation of sulfated phenols.

We have already reported that this enzyme effectively sulfates polyphenols, such as chalcones, xanthones and flavones.[9] Therefore, human intestinal bacteria may play an important role in the sulfation of flavones and tannins ingested as components of vegetable food. The present paper describes the enzymatic sulfation of monomeric polyphenols such as gallic acid and catechin derivatives, and also of some condensed tannins, by the bacterial arylsulfotransferase, and the position of sulfated hydroxyl groups of (+)-catechin used as a typical example.

Materials and Methods

Chemicals *p*-Nitrophenyl sulfate (PNS) was purchased from Sigma Chemicals, Co. (U.S.A). Tyramine was purchased from Nacalai Tesque (Japan) and diethylaminoethyl (DEAE)-cellulose was from Brown Co. (U.S.A). General anaerobic medium (GAM) broth was from Nissui Seiyaku Co., Ltd. (Japan). Methyl, ethyl, *n*-propyl, *n*-butyl and isoamyl gallate were supplied from Fuji Chemical (Japan), and lauryl and stearyl gallate were purchased from Tokyo Kasei (Japan). Rosmarinic acid (from *Perilla frutescens* BRITTON var. *crispa* DECNE.),[10] pedunculagin (from *Casuarina stricta* AIT.),[11] pentagalloylglucose (=1,2,3,4,6-penta-*O*-galloyl-β-D-glucose, from gallotannin mixture in Chinese gall),[12] geraniin (from *Geranium thunbergii* SIEB. *et* ZUCC.)[13] and corilagin (from *G. thunbergii*)[13] were prepared according to cited references.

Gallic acid, ellagic acid, chlorogenic acid, (+)-catechin and (±)-catechin were purchased from Sigma Chemical, Co. (U.S.A.). (−)-Epicatechin was purchased from Aldrich Chemical Co., Inc. (U.S.A.), (−)-epicatechin gallate, (−)-epigallocatechin and (−)-epigallocatechin gallate were from Kurita Ind., Ltd. (Japan). All other chemicals were of analytical reagent grade.

Partial Purification of Arylsulfotransferase *Eubacterium* A-44, isolated from human feces, was cultured under the same procedure as described in our previous report,[8] except that 1 mM PNS was added to the culture medium as an inducer in place of phenolphthalein disulfate.[14] The crude extract of the enzyme was prepared according to the method described in our previous report[15] and did not exhibit any arylsulfatase activity when PNS was used as a substrate. The extract was applied to a column of DEAE-cellulose and partially purified under the same procedure.[9] The resulting enzyme solution (12.6 units/ml, 29.8 mg protein/ml) was used for the sulfation of polyphenols. The assay method and the definition of the enzyme activity were described in our previous reports.[8,15]

Enzymatic Sulfation of Polyphenols Tannins and related polyphenols were dissolved in dimethylsulfoxide (DMSO). A reaction mixture contained 0.17 ml of 1 mM acceptor, 0.034 ml of 200 mM PNS, 0.048 ml of 25 mM L-ascorbic acid, 0.048 ml of 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) and 0.7 ml of 0.1 M Tris–HCl buffer (pH 8.0). The reaction was started by the addition of 0.1 ml of arylsulfotransferase preparation (12.6 units/ml) to the reaction mixture, which was incubated for 15 min at 37 °C. The final concentration of EDTA (48 μM) had little effect on the enzyme activity. The initial velocity of the sulfation reaction was measured by monitoring absorbance at 405 nm and the acceptor activity was obtained by comparison with the enzymatic activity using tyramine as a control acceptor.

Enzymatic Preparation of Sulfated (+)-Catechin A reaction mixture contained 17 ml of 20 mM (+)-catechin, 3.4 ml of 200 mM PNS, 4.8 ml of 25 mM L-ascorbic acid, 4.8 ml of 1 mM EDTA-2Na, 70 ml of 0.1 M Tris–HCl buffer (pH 8.0) and 10 ml of arylsulfotransferase preparation (12.6 units/ml). The mixture was incubated for 24 h at 37 °C. The reaction mixture was lyophilized and the resulting residue was subjected to preparative paper partition chromatography (PPC) through the same procedure in our previous report,[9] except that *n*-BuOH–EtOH–H_2O (5:1:4, v/v/v) was used as a solvent system in place of *n*-BuOH–AcOH–H_2O (4:1:2, v/v/v).

Structural Determination of Catechin Sulfates Nuclear magnetic resonance (NMR) spectra were measured on a JEOL JNM-GX 400 spec-

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trometer in acetone-d_6, dimethylsulfoxide-d_6, or methanol-d_4 solutions using tetramethylsilane as an internal standard. Fast atom bombardment mass spectrum (FAB-MS) was obtained with a JEOL JMS-SX 102 spectrometer.

Analysis of the Reaction Products by Two-Dimensional Cellulose Thin-Layer Chromatography (TLC) Electrophoresis The reaction products were spotted on a cellulose TLC plate (Merck, 10 × 20 cm) and developed with *n*-BuOH–EtOH–H_2O (5 : 1 : 4, v/v/v). The plate was air-dried and subjected to high voltage electrophoresis for 60 min in an HCOOH–AcOH H_2O buffer (33 : 147 : 1820, v/v/v), pH 1.9 at 1.5 kV by electrophoretic combined equipment (Bio-Rad Power Supply Model 3300, Pharmacia Flat Bed Apparatus FBF-3000, mgw Lauda RMT, RM6). After electrophoresis, the plate was dried, spots were detected under a ultraviolet (UV) lamp (365 nm), and then their mobilities were determined.

Results

Bacterial arylsulfotransferase was used for the sulfation of several polyphenols. Some of them tended to be oxidized spontaneously by atmospheric oxygen during the sulfation reaction. Therefore, our spectrophotometric analysis needed to be carried out in the presence of an antioxidant, such as ascorbic acid, which prevented their oxidation most effectively. Because iron chelated ascorbic acid is known to be an oxidant, 48 μM EDTA was added to the reaction mixture in order to eliminate the influence of such undesired oxidation and coloration. Inhibition of the enzyme activity by EDTA at the final concentration was negligible.

Polyphenols in the present investigation, except for gallic acid, ellagic acid and chlorogenic acid, were rapidly sulfated with PNS as a donor substrate, (Table I). (+)-Catechin as well as (±)-catechin, (−)-epicatechin and (−)-epicatechin gallate were better substrates than tyramine, which is used as an acceptor substrate in a standard assay. (−)-Epigallocatechin and (−)-epigallocatechin gallate were slightly worse substrates than tyramine. Alkyl gallate esters, such as methyl-, ethyl-, *n*-propyl-, *n*-butyl-, isoamyl- and lauryl gallate, were better substrates than tyramine. The degree of the acceptor specificity increased in proportion to the length of alkyl group up to the carbon number of five. Lauryl gallate was also sulfated as well, but stearyl gallate was less sulfated among the alkyl gallate esters examined. Pedunculagin, geraniin and corilagin were less effective than tyramine. Rosmarinic acid and penta-*O*-galloyl-β-D-glucose were similarly well sulfated. Numerous condensed tannins are composed of catechin and analogs in various combinations, though there are many exceptions in certain plant species. We had already reported the chemical structures of enzymic sulfated quercetin as a typical flavonoid example.[9] Therefore, in the present study we investigated the position of sulfated hydroxyl groups of catechin as a component of tannins.

A two-fold molar excess of PNS over catechin was incubated in the reaction mixture for 24 h at 37 °C for the preparation of sulfated (+)-catechin. The reaction products were concentrated *in vacuo* until the concentration was appropriate for PPC. Two new spots were detected as products with low mobility in *n*-BuOH–EtOH–H_2O compared with catechin, PNS and *p*-nitrophenol (PNP) (lane 5, in Fig. 1). They were designated in terms of product I (of higher *Rf* value) and product II (of lower *Rf* value). PNP derived from a donor substrate, PNS, was clearly observed along with a small amount of remaining PNS, while no PNP was detected in the absence of the enzyme (lane 4, in Fig. 1). These results suggest that products I and II were

TABLE I. Sulfation of Tannins

Acceptors	Activity (%)
Tyramine	100
Gallic acid[a]	3.4
Ellagic acid[a]	6.6
Chlorogenic acid[a]	2.9
(+)-Catechin[a]	437
(±)-Catechin[a]	241
(−)-Epicatechin[a]	231
(−)-Epicatechin gallate[b]	194
(−)-Epigallocatechin[b]	78.8
(−)-Epigallocatechin gallate[b]	84.0
Methyl gallate	281
Ethyl gallate	420
n-Propyl gallate	438
n-Butyl gallate	467
Isoamyl gallate	487
Lauryl gallate	273
Stearyl gallate	32.4
Pedunculagin	31.3
Corilagin	35.2
Rosmarinic acid	159
Penta-*O*-galloyl β-D-glucose	182
Geraniin	24.2

A reaction mixture contained 0.17 ml of 1 mM acceptor (*a*) 20 mM, *b*) 2 mM) under the standard assay conditions as described in Materials and Methods. Activity for tyramine as an acceptor at the corresponding concentration was taken as 100.

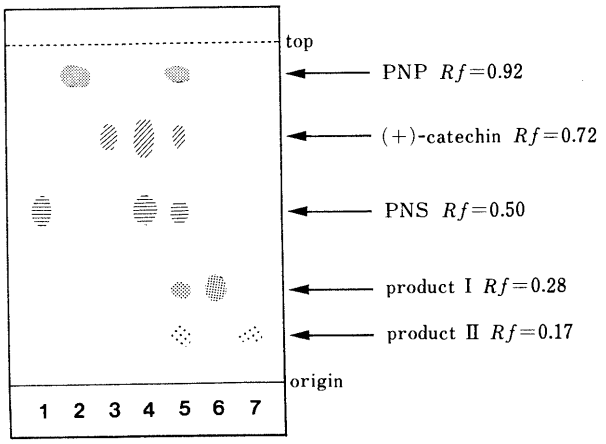

Fig. 1. Paper Partition Chromatogram of Reaction Products

Preparation and analysis of the reaction products were described in Materials and Methods.

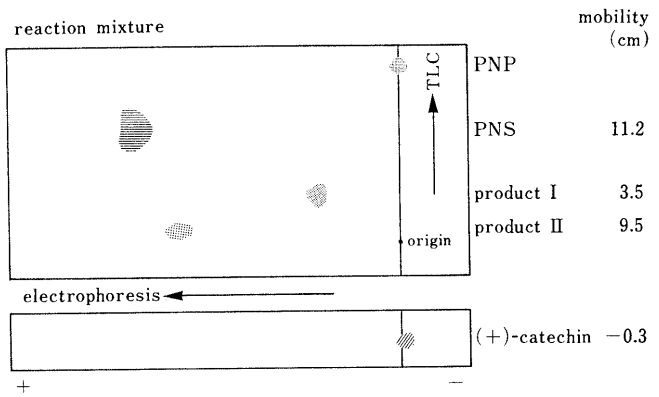

Fig. 2. TLC and Electrophoresis of Reaction Mixture

Solvent system; TLC, *n*-BuOH–EtOH–H_2O (5 : 1 : 4, v/v/v); high voltage thin-layer electrophoresis, 1000 V, 60 min, pH 1.9, HCOOH–AcOH–H_2O (33 : 147 : 1820, v/v/v).

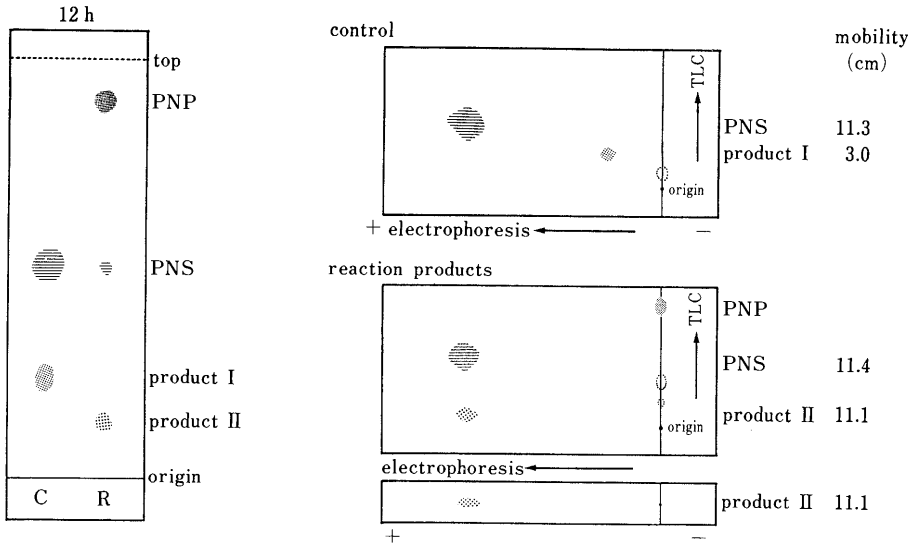

Fig. 3. Sulfation of Product I

C, control, product I+PNS; R, reaction product, product I+PNS+AST. TLC and electrophoresis were carried out under the same procedure described in Fig. 2.

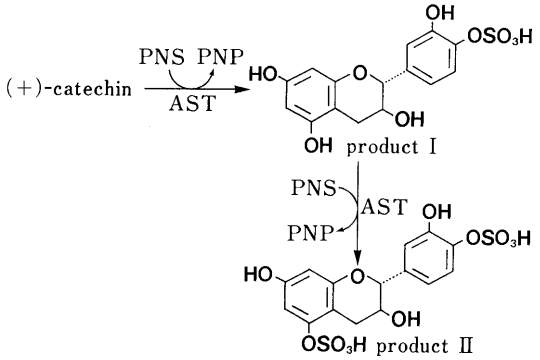

Chart 1. Successive Sulfation of (+)-Catechin by Arylsulfotransferase

sulfated catechin. A mixture of products I and II was prepared in a large scale, and subjected to preparative PPC in the same solvent system. Each band was cut out and extracted with water. The extract was concentrated to dryness. The products were analyzed by PPC with the same development and it was confirmed that they were well separated (lanes 6 and 7, in Fig. 1).

It is known that sulfation causes a change in the polarity of the compound. Thus, the determination of the number of sulfates by electrophoretic experiment would be important to structural elucidation. Two-dimensional development combining cellulose TLC and electrophoresis provided a clearer separation (Fig. 2). Product II was more mobile in the electrophoretograms than product I, although (+)-catechin and PNP were not mobile. The mobilities of products I and II were 3.5 and 9.5 cm to anode, respectively, under the present conditions. Based on these results, it appeared that product II contained more sulfate groups than product I. Therefore, it is assumed that product I is an intermediate between (+)-catechin and product II. Product II was produced from product I when the isolated product I was incubated again for 12 h under the same reaction conditions (Fig. 3). These results suggested that product I was first formed as an intermediate and then product II was formed when PNS was in excess in molar over catechin (Chart 1).

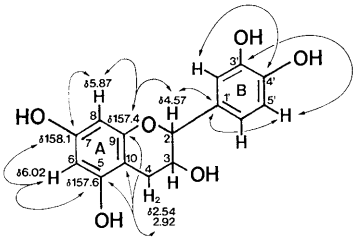

Fig. 4. Significant Long-Range Correlations Observed in the ^{1}H–^{13}C Long-Range COSY Spectrum of (+)-Catechin (**1**) in Acetone-d_6

Prior to analyzing the structure of products I and II, we carried out a re-examination of the proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra of (+)-catechin by means of two-dimensional (2-D) NMR techniques and found that the previous assignments[16,17] of ^{13}C-signals for C-5, C-7 and C-9 must be revised.

In the ^{1}H–^{13}C long-range correlation spectroscopy (COSY) spectrum of (+)-catechin, the quaternary carbon at δ 157.4 (C-9) showed long-range correlations with 4-H_2 (δ 2.45 and 2.92) and 2-H (δ 4.57) and also with the aromatic proton at δ 5.87 (8-H), while the quaternary carbon at δ 157.6 (C-5) exhibited long-range correlations with 4-H_2 (δ 2.54 and 2.92) and with the aromatic proton at δ 6.02 (6-H) (see Fig. 4). It followed that the carbon signals at δ 157.4 and δ 157.6 are unambiguously assigned to C-9 and C-5, respectively, and also the proton signals at δ 5.87 and δ 6.02 to 8-H and 6-H, respectively. On the other hand, the quaternary carbon at δ 158.1 correlated with both 8-H and 6-H, and were thus assigned reasonably to C-7. Some other significant long-range correlations observed are also shown by arrows in Fig. 4. Complete assignments of ^{1}H- and ^{13}C-signals are given in Table II.

Product I exhibited a quasi-molecular ion peak at m/z 369 $[M-H]^-$, corresponding to the molecular formula $C_{15}H_{14}O_9S$, in negative ion fast atom bombardment mass spectrum (FAB-MS). The ^{1}H- and ^{13}C-NMR spectra, analyzed with the aid of ^{1}H–^{1}H COSY and ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) techniques, showed marked downfield shifts of ^{1}H- and ^{13}C-

TABLE II. ^{1}H- and ^{13}C-NMR Data for (+)-Catechin (**1**), Product I (**2**) and Product II (**3**) (Coupling Constants in Parentheses)

Position	1				2		3	
	δH[a)]	δC[a,c)]	δH[b)]	δC[b,c)]	δH[a)]	δC[a,d)]	δH[a)]	δC[a,d)]
2	4.57 d (7.5)	83.6 d	4.57 d (7)	83.1 d	4.61 d (7.6)	83.2 d	4.65 d (7.5)	83.5 d
3	3.98 ddd (8, 7.5, 5.5)	69.6 d	4.01 ddd (8, 7, 5)	68.8 d	3.97 ddd (8, 7.6, 5.5)	69.7 d	3.98 ddd (8, 7.5, 5.5)	69.5 d
4	2.50 dd (16, 8) 2.85 dd (16, 5.5)	29.2 t	2.54 dd (16.6, 8) 2.92 dd (16.6, 5)	29.2 t	2.50 dd (16, 8) 2.84 dd (16, 5.5)	29.3 t	2.71 dd (16.5, 8) 3.09 dd (16.5, 5.5)	29.8 t
5	—	158.3 s	—	157.6 s	—	158.3 s	—	153.5 s
6	5.93 d (2.5)	97.1 d	6.02 d (2.5)	96.6 d	5.92 d (2.2)	97.2 d	6.62 d (2)	104.1 d
7	—	158.6 s	—	158.1 s	—	158.6 s	—	158.3 s
8	5.86 d (2.5)	96.3 d	5.87 d (2.5)	95.9 d	5.86 d (2.2)	96.3 d	6.17 d (2)	101.5 d
9	—	157.7 s	—	157.4 s	—	157.5 s	—	157.2 s
10	—	101.6 s	—	101.1 s	—	101.6 s	—	107.5 s
1′	—	133.0 s	—	132.6 s	—	139.6 s	—	139.4 s
2′	6.84 d (2)	116.0 d	6.90 d (2)	115.7 d	6.95 d (2)	117.9 d	6.96 d (2)	117.9 d
3′	—	147.0 s	—	146.07 s	—	151.1 s	—	151.1 s
4′	—	147.0 s	—	146.13 s	—	141.8 s	—	141.8 s
5′	6.76 d (8)	116.9 d	6.80 d (8)	116.2 d	7.27 d (8)	124.6 d	7.29 d (8)	124.6 d
6′	6.72 dd (8, 2)	120.8 d	6.76 dd (8, 2)	120.5 d	6.85 dd (8,2)	120.5 d	6.87 dd (8, 2)	120.6 d

δ Values in ppm and coupling constants in Hz. Multiplicities of carbon signals were determined by means of the distortionless enchancement by polarization transfer method and were indicated as s, d, t and q. *a, b*) Measured in MeOH-d_4 and acetone-d_6, respectively. *c*) Signal assignments are based on the analyses of the ^{1}H–^{13}C and ^{1}H–^{13}C long-range COSY spectra. *d*) Signal assignments are based on the analyses of the HMQC and HMBC spectra.

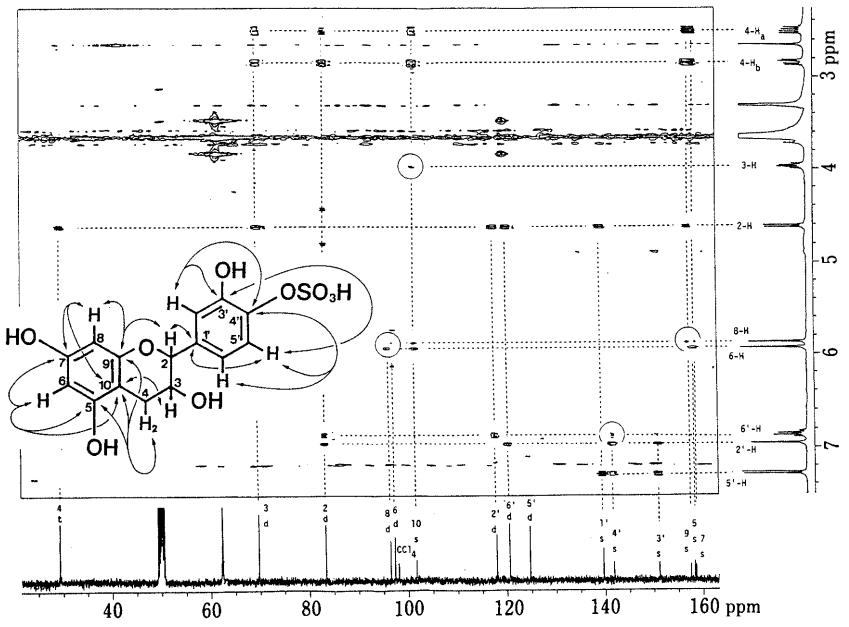

Fig. 5. HMBC Spectrum of Product I (**2**) in Methanol-d_4

8 mg, 40 h run, J_{CH}=6 Hz.

signals assignable to the 5′-methine group in the ring-B, suggesting that the sulfation took place at the 3′- or 4′-hydroxyl group.

Further, in the ^{1}H-detected heteronuclear multiple bond connectivity (HMBC) spectrum (Fig. 5), the quaternary carbon at 139.6 (C-1′) showed long-range correlation with 2-H (δ 4.61, d, J=7.6 Hz) and 6′-H (δ 6.85, dd, J=8.0, 2.0 Hz), while the quaternary carbon at δ 141.8 (C-4′) showed long-range correlation with 2′-H (δ 6.95, d, J=2.0 Hz), 5′-H (δ7.27, d, J=8.0 Hz) and 6′-H (δ 6.85). Therefore, the former carbon was assigned to C-1′ and the latter to C-4′, unambiguously. On the other hand, the quaternary carbon at 151.1 (C-3′) showed long-range correlations with 2′-H (δ 6.95) and 5′-H (δ 7.27) and was

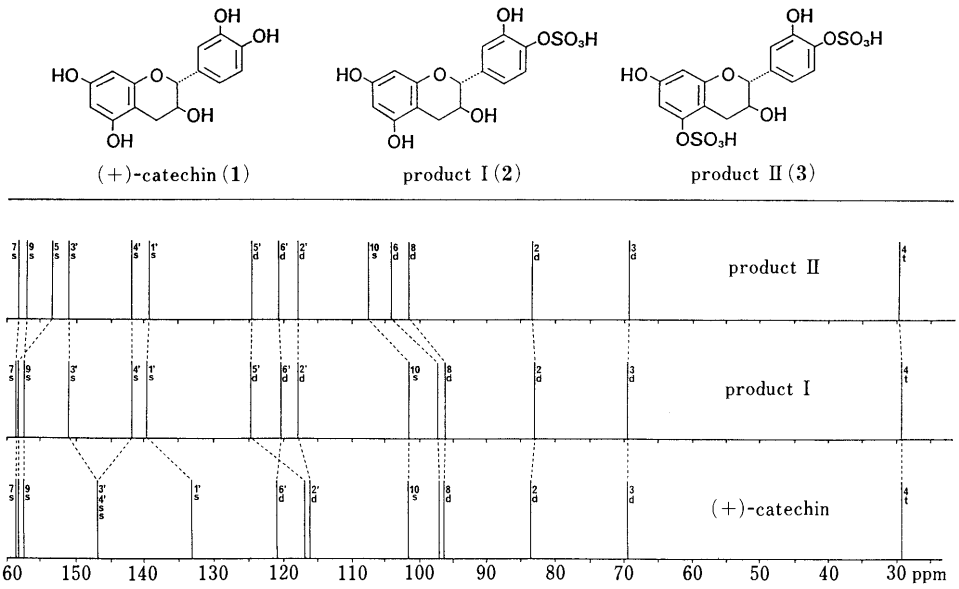

Fig. 6. Comparison of the ^{13}C-NMR Signals with (+)-Catechin (**1**), Product I (**2**) and Product II (**3**)

assigned to C-3′ unambiguously. In a similar manner, all quaternary carbons in ring-A were assigned as shown in Fig. 5.

Now, comparison of the ^{13}C-NMR date of product I with those of (+)-catechin disclosed an upfield shift of the signal due to C-4′ by 5.21 ppm, and downfield shifts of the signals due to C-3′, C-1′ and C-5′ by about 4—7 ppm[5] (Table II). This finding led us to conclude that the structure of product I is represented by formula **2** (Fig. 6).

Product II had the molecular formula $C_{15}H_{14}O_{12}S_2$ as proven by the negative ion FAB-MS, which revealed a quasi-molecular ion peak at *m/z* 449. The ^{1}H-NMR spectrum of product II showed a pattern similar to that of product I, but it was characterized by the downfield shifts of the ^{1}H-signals assignable to 6-H and 8-H in ring-A (Table II). The ^{13}C-NMR spectrum could also be analyzed completely by the use of HMBC and the results are compiled in Table II. Comparison of the ^{13}C-NMR data of product II with that of product I indicated that the signal due to C-5 shifted upfield by 4.81 ppm, while the signals due to C-6, C-8 and C-10 shifted downfield (6.83, 5.19 and 5.96, respectively). Thus, the structure of product II was determined to be **3** (Fig. 6).

Discussion

Tannins, as well as flavonoids, are widely distributed in the plant kingdom and are ingested daily as components of vegetable food. Human intestinal flora play an important role in the metabolism of such vegetable components, for examle, by β-glycosidase for glycosides,[18,19] and by C-ring cleavage to a flavonoid.[20] Through studies on the metabolism of oriental medicine components by human intestinal bacteria, we discovered a novel type of arylsulfotransferase, which catalyzes a specific trans-sulfation reaction between phenolic hydroxyl groups. We have already reported the sulfation of flavons, chalcones and xanthones by an enzyme from human intestinal bacterium[14] and established the chemical structure of sulfated quercetin.[9] The present study demonstrated that tannins and alkyl gallates were also effectively sulfated by the enzyme using PNS as a donor substrate, and the chemical structures of sulfated (+)-catechins, 4′-mono- and 5,4′-disulfated (+)-catechins were elucidated. Therefore, ingested polyphenols may be enzymatically sulfated in the intestine, and converted to be more soluble in water and less absorbable. Such sulfate conjugation in the intestine may represent one of the detoxication mechanisms of exogeneous polyphenols, though the sulfate donors have not yet been proven in the human intestinal tract. The position and number of sulfate groups incorporated to tannins have not yet been established, but will be clarified in the near future.

As alkyl gallate esters were better substrates than gallic acid (Table I), it seems that the presence of a carboxylic anion has an adverse effect on enzymatic sulfation. This result is similar to the fact that tyrosine methyl ester was sulfated more than tyrosine.[14] Since pedunculagin, corilagin and geraniin were less sulfated than tyramine, the presence of biphenyl also has an unfavorable influence on the sulfation.

Spectrometric analysis using an excess of PNS as a donor substrate showed that only one hydroxy group among many hydroxyl groups of pedunculagin, corilagin, geraniin, rosmarinic acid and penta-*O*-galloyl-β-D-glucose was sulfated (data not shown).

These findings suggest that some acidic amino acids and hydrophobic moieties of arylsulfotransferase might be present in the neighborhood of the active site of the enzyme, but we suggested previously that tyrosine and histidine residues are involved in the active site of the enzyme.[14] From the data on the chemical structure of sulfated catechins, a certain phenolic hydroxyl group(s) of polyphenols is specifically and rapidly sulfated. Therefore, the enzymatic trans-sulfation may be a useful method for the preparation of tannins sulfated at specific positions.

Acknowledgment This study was supported in part by a Grant-in-Aid (No. 01480491) from the Ministry of Education, Science and Culture of Japan. We wish to thank Mr. K. Tanaka of the National Research Institute of Police Science for the FAB-MS measurements, Miss M. Kamitani and Mr. K. Dohi for their technical assistance and Miss S. Takayanagi for her secretarial assistance.

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Antibacterial Activity of a New Tetracyclic Quinolone, No. 5290, against Norfloxacin- and Ciprofloxacin-Resistant Strains of *Staphylococcus aureus*

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The antibacterial activity of a new tetracyclic quinolone, No. 5290, against 25 strains of *Staphylococcus aureus* clinically isolated in Japan in 1988—1989 was determined. The minimum inhibitory concentrations (MICs) of No. 5290 against both quinolone-susceptible (MIC: norfloxacin $\leq$ 6.25 μg/ml, ciprofloxacin $\leq$ 1.56 μg/ml) and 4 out of 5 norfloxacin- and ciprofloxacin-moderately resistant strains (MIC: 25 μg/ml $\leq$ norfloxacin $\leq$ 50 μg/ml, 3.13 μg/ml $\leq$ ciprofloxacin $\leq$ 12.5 μg/ml) were 0.05 μg/ml. Similar findings were obtained on the quinolone-resistant mutants derived by norfloxacin- or KB-5246-selection from quinolone-susceptible clinical isolates of *S. aureus*. The uptake of No. 5290 into a quinolone-susceptible strain of *S. aureus* was 2.47 μg/mg dry cell and the uptake in norfloxacin- and ciprofloxacin-moderately resistant strains was comparable to that in the quinolone-susceptible strain. The uptake of No. 5290 in both the quinolone-susceptible strain, and norfloxacin- and ciprofloxacin-moderately resistant, and No. 5290-susceptible strains was only slightly influenced by the treatment of bacteria with carbonyl cyanide *m*-chlorophenylhydrazone. These findings indicate that: (i) No. 5290 has potent antibacterial activity against quinolone-susceptible strains of *S. aureus*, and the potent activity might be due to a high uptake caused by an ineffective efflux of No. 5290. (ii) No. 5290 also has potent antibacterial activity against norfloxacin- and ciprofloxacin-moderately resistant strains, the reason for which could not be explained by the efflux.

Keywords tetracyclic quinolone; antibacterial agent; uptake; efflux; *Staphylococcus aureus*; quinolone resistance; hydrophobicity

Introduction

We have reported that the tetracyclic quinolone-derivative, KB-5246, demonstrates a broad antibacterial spectrum, and potent antibacterial activity.[1] The potent antibacterial activity of KB-5246 against the quinolone-susceptible strains of *Staphylococcus aureus* could be explained by its high uptake drug due to ineffective efflux.[2] As KB-5246 had a higher hydrophobicity than norfloxacin or ciprofloxacin,[3] the good permeability of the quinolones in *S. aureus* might be correlated with their hydrophobicities.

Then we compared the antibacterial activity and the uptake in *S. aureus* of a new tetracyclic quinolone derivative (Fig. 1), No. 5290 which is more hydrophobic than KB-5246, with those of KB-5246.

Materials and Methods

Drugs No. 5290 (9,1-methyliminomethano-7-fluoro-8-(4-methyl-1-piperazinyl)-5-oxo-5*H*-thiazolo[3,2-*a*]quinoline-4-carboxylic acid) and norfloxacin were synthesized in our laboratory (Fig. 1).

Test Strains Clinical isolates of *S. aureus* used in this study were the same as the strains used previously.[2] They were donated by Dr. Deguchi and were isolated in 1988—1989 in Japan.

Determination of Hydrophobicity The hydrophobicity was estimated from the retention time on high-pressure liquid chromatography (HPLC) as reported previously.[3]

Determination of the Minimum Inhibitory Concentrations (MICs) The MICs were determined by the two-fold agar dilution method. The media used for preculture and the MIC determination were sensitivity test broth (Nissui Seiyaku Co., Ltd.) and sensitivity test agar (Nissui Seiyaku Co., Ltd.). An overnight broth culture (about 10^8 CFU/ml) was diluted to 10^{-2} and about 10^4 CFU was inoculated onto a drug-containing agar surface. The MIC was scored after 18—20 h of incubation at 37 °C. The MIC was defined as the lowest drug concentration which prevented visible growth of microorganisms.

Uptake of No. 5290 in *S. aureus* The uptake of No. 5290 was measured as reported previously.[2] Bacterial cells were grown to OD_{660} = 0.6. No. 5290 was added to the bacterial suspension to a concentration of 10 μg/ml, and the suspensions were incubated for 10 min at 37 °C. After the suspension was washed with 1 ml of saline by centrifugation, the obtained precipitate was suspended in 5% acetic acid. The suspension was immersed in boiling water for 5 min and centrifuged. The amount of No. 5290 in the resulting supernatant was defined as the uptake. Carbonyl cyanide *m*-chlorophenylhydrazone (cccp) was added 5 min before the addition of No. 5290. The concentration in the supernatant was determined by HPLC using an Inertsil ODS-2 column (Gasukuro Kogyo Co., Ltd.), a mobile phase composed of 85 : 15.5% acetic acid : methanol, a flow rate of 1.5 ml/min, and detection wavelength of 270 nm at column temperature of 40 °C.

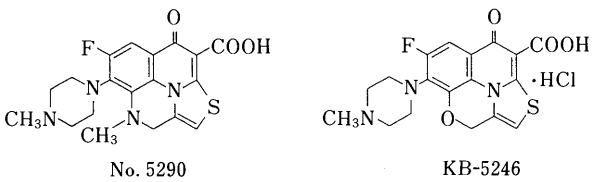

Fig. 1. Chemical Structures of 2 Tetracyclic Quinolones

Results

Susceptibilities of Clinical Isolates of *S. aureus* to No. 5290 Figure 2 shows the susceptibilities of quinolone-susceptible *S. aureus* strains clinically isolated in 1988—1989 to No. 5290. The peak-concentration of No. 5290-susceptible strains on the distribution of susceptibilities was 0.05 μg/ml, which indicates the potent antibacterial

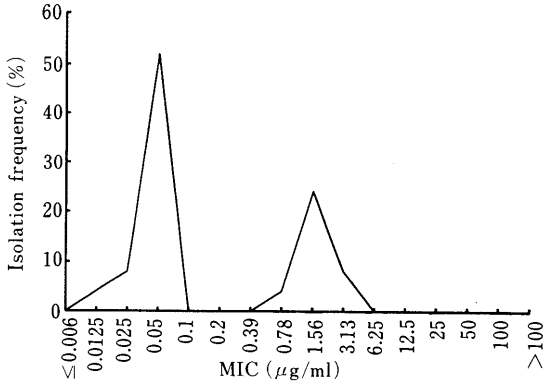

Fig. 2. Susceptibilities of *S. aureus* to No. 5290

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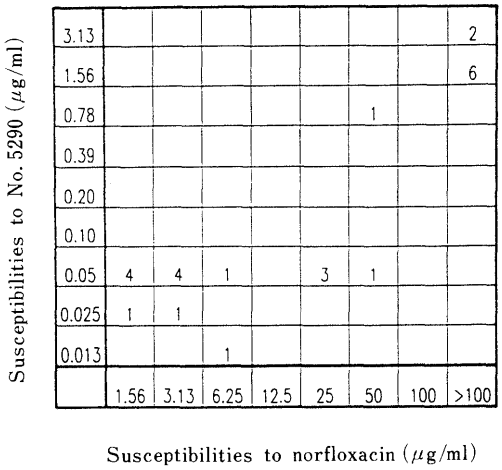

Fig. 3. Relationship between No. 5290 and Norfloxacin
Numerals in columns indicate the numbers of strains.

TABLE I. Mutation Frequency and Susceptibility to No. 5290 of Quinolone Resistant Mutants of *S. aureus*

Parental strains	Mutant-selection by drug (selection-conc. (μg/ml))	Mutation frequency ($\log_{10}$)	MIC range (μg/ml)
			No. 5290
INK-1			0.05
	Norfloxacin (6.25)	−6.6	0.05
	Norfloxacin (12.5)	−7.3	0.10
	KB-5246 (0.39)	−7.1	0.05—0.10
INK-3			0.05
	Norfloxacin (12.5)	−7.4	0.05—0.10
	KB-5246 (0.39)	−7.2	0.05
INK-5			0.05
	Norfloxacin (6.25)	−7.3	0.05—0.10
	KB-5246 (0.20)	−6.2	0.05—0.10
INK-6			0.05
	Norfloxacin (12.5)	−8.1	0.05
	KB-5246 (0.20)	−8.1	0.05
INK-8			0.05
	Norfloxacin (12.5)	−6.4	0.05—0.10
	KB-5246 (0.39)	−6.4	0.05
INK-21			0.05
	Norfloxacin (6.25)	−7.4	0.05
	KB-5246 (0.39)	−7.7	0.05
INK-22			0.05
	Norfloxacin (6.25)	−7.2	0.05
	KB-5246 (0.20)	−6.1	0.05

activity of No. 5290 against quinolone-susceptible strains. The distribution of susceptibility to No. 5290 demonstrated only two peaks. The peak of No. 5290-susceptible strains (peak concentration 0.05 μg/ml) had no shoulder.

Figure 3 shows the susceptibility distribution of the clinical isolates to No. 5290 *vs.* norfloxacin. The MIC of No. 5290 against 4 strains among 5 norfloxacin-moderately resistant strains (MIC; 25 μg/ml ≤ norfloxacin ≤ 50 μg/ml) was 0.05 μg/ml, while the MIC of the other strain was 0.78 μg/ml. The susceptibility of the 4 strains to No. 5290 was similar to that of typical norfloxacin-susceptible strains. Although several norfloxacin-moderately resistant strains of *S. aureus* were classified into typical No. 5290-susceptible strains, 8 strains resistant to 100 μg/ml of norfloxacin were all resistant to 0.78 μg/ml of No. 5290, and they were classified into No. 5290-resistant strains.

Susceptibilities of Quinolone-Resistant Mutants of *S. aureus* to No. 5290 Susceptibilities of spontaneous quinolone-resistant mutants to No. 5290 were determined (Table I). Spontaneous resistant mutants were prepared as described previously.[2] We previously reported that most of the spontaneous resistant mutants are susceptible to 0.20 μg/ml of KB-5246, and resistant to 12.5 μg/ml of norfloxacin and 1.56 μg/ml of ciprofloxacin.[2] However, the MIC of KB-5246 against most of the mutants was 2 to 4-fold higher than the MIC against the parent strains. To our surprise, the MIC of No. 5290 against these mutants was not altered or at most 2-fold higher than that against the parent strains. The MIC of No. 5290 against YK-134 and 136, which were resistant mutants from INK-1 (clinical isolates) selected by norfloxacin, was comparable to the MIC against INK-1 (0.05 μg/ml).[2] These findings were in agreement with the finding that norfloxacin-moderately resistant clinical isolates were classified into No. 5290-susceptible strains (Fig. 2).

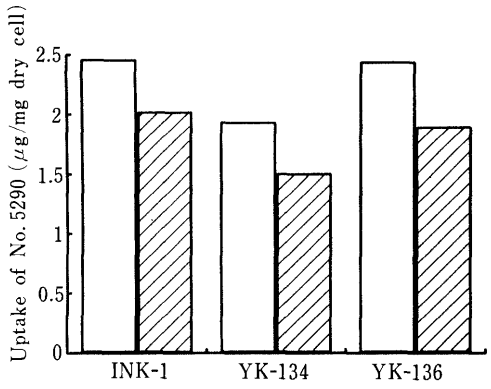

Fig. 4. Uptake of No. 5290 in *S. aureus*
Open column: control, hatched column: +200 μM cccp.

HPLC Retention Time of No. 5290 The hydrophobicity of No. 5290 was estimated from the retention time obtained by HPLC. The retention time of No. 5290 was 5.89 min.

Uptake of No. 5290 in *S. aureus* The uptake of No. 5290 in *S. aureus* was measured (Fig. 4). The uptake of No. 5290 in the quinolone-susceptible strain, INK-1 (MIC: norfloxacin, 3.13 μg/ml; No. 5290, 0.10 μg/ml), was 2.47 μg/mg dry cell. The energy-dependent efflux of the quinolones has been reported to contribute to the mechanism of resistance of the quinolones on *S. aureus*, and the energy for the efflux was supplied from the respiratory chain.[4] Then we examined the effect of an uncoupler on the uptake of No. 5290. We used cccp as an uncoupler. The uptake of No. 5290 was only slightly influenced by cccp. This suggested that an ineffective efflux of No. 5290 in *S. aureus* might be one of the reasons for the potent antibacterial activity against quinolone susceptible strains of *S. aureus*. The uptake of No. 5290 in two norfloxacin-moderately resistant mutants (YK-134 and 136, MIC: norfloxacin, 25 μg/ml; No. 5290, 0.10—0.20 μg/ml) was comparable to that in the parent INK-1 strain. The uptake of No. 5290 in these mutants was only slightly influenced by cccp.

Discussion

The following conclusions were drawn from the findings obtained on No. 5290 and KB-5246[2] using the same

methods and strains: (i) No. 5290 is more hydrophobic than KB-5246, which is more hydrophobic than ciprofloxacin and norfloxacin. (ii) The antibacterial activity of No. 5290 against the quinolone-susceptible *S. aureus* was comparable to that of KB-5246. (iii) Although the MICs of KB-5246 for 2 of the 5 norfloxacin- and the ciprofloxacin-moderately resistant strains of *S. aureus* was 4-fold higher than the peak-MIC against KB-5246-susceptible strains, the MIC of No. 5290 did not shift from the peak-MIC against No. 5290-susceptible strains. (iv) The uptake of No. 5290 in the quinolone-susceptible strain (INK-1) was about 40-fold higher than that of norfloxacin, and about 2-fold lower than that of KB-5246. The uptake of No. 5290, KB-5246, and norfloxacin in norfloxacin and ciprofloxacin-moderately resistant, KB-5246-slightly resistant, and No. 5290-susceptible mutant strains was not altered from that in the parent strain. (vi) Different in the case of norfloxacin, No. 5290 showed an ineffective efflux in both the quinolone-susceptible and norfloxacin- and ciprofloxacin-moderately resistant strains (Fig. 4).

These results suggest that the more potent antibacterial activity of No. 5290 and KB-5246 against *S. aureus* than norfloxacin may be due to a high uptake caused by an ineffective efflux. The hydrophobicity of No. 5290 and KB-5246 might contribute to their ineffective efflux.

The reason why the antibacterial activity of No. 5290 and KB-5246 against the quinolone-susceptible strain was not correlated with the uptake may be explained by a factor other than permeability, *i.e.* the different inhibitory activity on deoxyribonucleic acid (DNA) gyrase of *S. aureus*.

Against norfloxacin- and ciprofloxacin-moderately resistant strains, No. 5290 demonstrated more potent antibacterial activity than KB-5246, the reason for which could also be explained by the factor other than permeability, *i.e.* the different inhibitory activity on DNA gyrase.

Previous studies have demonstrated that one of the mechanisms of quinolone-resistance in *S. aureus* is an energy-dependent efflux.[4,5] Our results suggest that another factor should contribute to the resistant mechanisms of *S. aureus*. Several mechanisms may be responsible for the resistance. The effect of No. 5290 and KB-5246 on DNA gyrase of *S. aureus* is under investigation.

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Synthesis of Rat Parathymosin α Fragment 1—28 and Examination of Its Inhibitory Activity towards the Restoring Activity of Thymosin α_1 on the Impaired T-Lymphocytes of Uremic Patients[1)]

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A fragment corresponding to *N*-terminal octaeicosapeptide of rat parathymosin α was synthesized by assembling 5 peptide fragments, followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole (molar ratio 1:1) in trifluoroacetic acid in the presence of dimethylselenium. Incubation of impaired T-lymphocytes isolated from uremic patients with the synthetic parathymosin α fragment 1—28 showed no immunological restoring effect, but when it was administered together with thymosin α_1, it appeared to suppress the restoring effect of the thymosin α_1 on the impaired T-lymphocytes of uremic patients.

Keywords parathymosin α fragment 1—28 synthesis; trifluoromethanesulfonic acid deprotection; impaired T-lymphocyte blastogenic response; restoring effect; restoring effect suppression

Cellular immunity is known to be impaired in uremic patients. This impairment has been implicated in a high susceptibility to infections and an increased incidence of malignancy. The thymus plays an essential role in the development and maintenance of cellular immune competence.

Thymosin α_1, a component of the thymus-derived polypeptide mixture thymosin fraction 5, has been shown to exhibit various activity *in vitro* immunological assays.[2,3)] *In vivo*, thymosin α_1 seems to play an important regulatory role in the late stages of T-lymphocyte differentiation.[2,4)]

In previous papers,[5-8)] we reported the syntheses of deacetylthymosin α_1, thymosin α_1 and its fragments, and showed that synthetic thymosin α_1 and some of its C-terminal fragments could have a restoring effect on impaired cell-mediated immunological functions.

	1				5					10
parathymosin α	Ac–	Ser–	Lys–	Ser–	Glu–	Val–	Glu–	Ala–	Ala–	Ala–	Glu–
prothymosin α	Ac–	Ser–	Asp–	Ala–	Ala–	Val–	Asp–	Thr–	Ser–	Ser–	Glu–
	11				15					20	
parathymosin α	Leu–	Ser–	Ala–	Lys–	Asp–	Leu–	Lys–	Glu–	Lys–	Lys–	
prothymosin α	Ile–	Thr–	Thr–	Lys–	Asp–	Leu–	Lys–	Glu–	Lys–	Lys–	
	21				25						
parathymosin α	Asp–	Lys–	Val–	Glu–	Glu–	Lys–	Ala–	Gly–			
prothymosin α	Glu–	Val–	Val–	Glu–	Glu–	Ala–	Glu–	Asn–			

Fig. 1. Comparison of the N-Terminal Amino Acid Sequences of Prothymosin α and Parathymosin α

The positions of identity are underlined.

In 1985, Haritos *et al.*[9)] reported that a peptide parathymosin α, containing about 105 amino acid residues, had been isolated from the rat thymus, and the sequence of the first 30 residues at the N-terminus had been determined. In this region, a 43% structural identity is shown between thymosin α_1 and parathymosin α.

Preliminary assay results suggest that parathymosin α may act to modulate the immunoenhancing activity exhibited by prothymosin α.

In our preceding paper,[6)] we reported evidence of impaired immune function in patients with chronic uremia. We also reported[5-8)] that the synthetic thymosin α_1 and some of its fragments show a restoring effect on impaired cell-mediated immunological functions.

These results prompted us to synthesize the N-terminal peptide fragment corresponding to amino acids 1—28 of parathymosin α. This paper deals with the synthesis of parathymosin α fragment 1—28, with an examination of the immunological effect of this peptide and our synthetic thymosin α_1[7)] on the impaired blastogenic response of T-lymphocytes of uremic patients. From a synthetic viewpoint, compared with our previous syntheses of deacetylthymosin α_1,[8)] thymosin α_1[7)] and its fragments,[5-7)] the thioanisole-mediated trifluoromethanesulfonic acid (TFMSA) deprotection procedure[10,11)] was applied in the final step of the present synthesis instead of catalytic hydrogenation or hydrogen fluoride.

According to the scheme shown in Fig. 2, the oc-

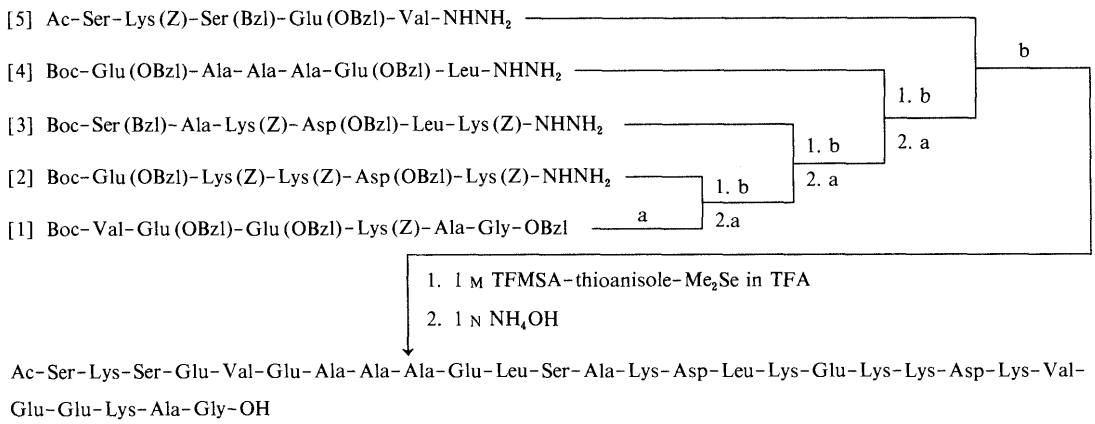

Fig. 2. Synthetic Route to Parathymosin α Fragment 1—28

a, TFA–anisole; b, azide.

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taeicosapeptide corresponding to sequence 1—28 of parathymosin α was synthesized. Amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA[10] were employed, *i.e.*, Lys(Z), Ser(Bzl), Asp(OBzl), Glu(OBzl) and Gly–OBzl. These protecting groups survive mostly intact under careful TFA treatment for removal of Boc or Z(OMe) group, employed as a temporary α-amino protecting group. Five protected intermediate peptide fragments, Ac–(1—5)–NHNH–Troc [XI], Boc–(6—11)–NHNH–Troc [XV], Boc–(12—17)–NHNH–Troc [X], Boc–(18—22)–NHNH–Troc [VIII] and Boc–(23—28)–OBzl [1], were chosen to construct the full sequence. In order to prepare the peptide hydrazides containing Asp(OBzl) or Glu(OBzl), these four fragments were synthesized with Troc–$NHNH_2$.[11] This Troc group is known to be cleaved by Zn[12] in AcOH without affecting other functional groups.

Throughout the syntheses of these intermediates and fragments, the purity of every intermediate was checked by thin-layer chromatography (TLC), elemental analysis and amino acid analysis. The analytical results were within ±0.44% of theoretical values in all cases.

The five fragments, [5], [4], [3], [2] and [1], were prepared in a stepwise manner starting from a protected C-terminal amino acid by the Su active ester procedure,[13] except for introduction of Ac–Ser, for which the azide procedure[14] was employed. The resulting protected peptide fragments, [XI], [XV], [X] and [VIII], were treated with Zn[11,12] in AcOH to remove the Troc group, and the zinc acetate was removed by treatment with EDTA to give the required hydrazides in analytical pure forms. The hydrazine test on the thin-layer chromatograms and the elemental analysis data were consistent with the homogeneity of the desired products. The five fragments were assembled successively from the C-terminal fragment to the N-terminal fragment using the azide procedure[14] according to the routes illustrated in Fig. 2.

The amount of acyl component in each fragment condensation was increased from 1.5 to 4 eq as the chain elongation progressed in order to secure complete condensation. The solubility of the protected intermediates in DMF decreased remarkably with chain elongation. Consequently, a mixture of DMF–DMSO had to be employed for the subsequent condensation reactions. Some of the intermediates were purified by repeated precipitation from DMF with MeOH, and others were purified by gel-filtration on Sephadex LH-20 using DMF as an eluent.

Throughout the synthesis, Gly was taken as the diagnostic amino acid in acid hydrolysis. By comparing of the recovery of Gly with that of newly incorporated amino acids, satisfactory incorporation of each fragment in each condensation was confirmed.

Starting with the C-terminal hexapeptide ester corresponding to positions 23 to 28 of parathymosin α [1], four fragments, Boc–(18—22)–$NHNH_2$ [2], Boc–(12—17)–$NHNH_2$ [3], Boc–(6—11)–$NHNH_2$ [4] and Ac–(1—5)–$NHNH_2$ [5], were successively condensed by the azide procedure[14] to give the protected octaeicosapeptide ester corresponding to the parathymosin α fragment 1—28.

In the final step of the synthesis, the protected octaeicosapeptide ester was treated with 1 M TFMSA–thioanisole in TFA in the presence of Me_2Se. Me_2Se was employed to facilitate acidic cleavage of the protecting groups.[15] The deprotected peptide was precipitated with dry ether, converted to the corresponding acetate with Amberlite IRA-400 (acetate form) and then treated with 1 N NH_4OH to reverse a possible N → O shift at the Ser residues.[16] The product was purified by gel-filtration on Sephadex G-25, followed by partition column chromatography on Sephadex G-25 according to Yamashiro.[17] Analysis of the main fractions by TLC using Partridge's solvent system[18] revealed the presence of one major chlorine-tolidine-positive spot and one minor spot. The crude octaeicosapeptide was further purified by preparative TLC developed with Partridge's solvent system. The purified peptide so obtained was found to be a single component from the results of TLC using two different solvent systems. The purified product was then subjected to Sephadex G-25 column chromatography as described above. The amino acid ratios in the acid hydrolysate of XXIV agreed well with theoretical values. The peptide also gave a single spot on paper electrophoresis and exhibited a single peak on HPLC. The molecular weight of the synthetic peptide was ascertained by FAB-MS spectrometry.

The immunological effect of the synthetic parathymosin α fragment 1—28 and thymosin α_1[7] was examined by the JIMRO (Japan Immunoresearch Laboratories Co., Ltd.) fluorometric blast-formation test according to Itoh and Kawai.[19] Responses of T-lymphocytes to mitogenic stimulation were lower in uremic patients than those of normal persons. The *in vitro* effect of the synthetic peptides on the impaired PHA response of T-lymphocytes from uremic patients is shown in Table I.

Comparison of the stimulation index (SI) values of the blastogenic transformation of T-lymphocytes into lymphoblasts with mitotic activity upon PHA stimulation shows that, in the case of the uremic patients investigated, the synthetic thymosin α_1 exhibited a restoring effect at a dose of 1 μg/ml, but the synthetic parathymosin α fragment 1—28 had no restoring effect on the impaired mitotic activity induced by PHA stimulation under the same conditions. Apparently, the synthetic parathymosin α fragment 1—28 is considerably far less active than thymosin α_1 in our *in*

TABLE I. Effect of the Synthetic Parathymosin α Fragment 1—28 and Thymosin α_1 on the Impaired PHA-Stimulation of T-Lymphocytes of Uremic Patients

Peptide	Dose (μg/ml)	SI[a,b]
—[c]	—	284.3±51.4
—[d]	—	106.5±50.7[g]
Thymosin α_1[d,e]	1.0	231.6±48.4[h]
Parathymosin α fragment 1—28[d,e]	1.0	111.2±49.3
Thymosin α_1 + parathymosin α fragment 1—28[d,e]	1.0+1.0	126.3±51.6
Thymosin α_1 + H–Gly–Gly–His–OH[d,e,f]	1.0+1.0	227.9±52.1[h]

a) Each value represents the mean±S.D. of triplicate measurements. *b*) SI (Stimulation index) was calculated according to the following formula: $SI = (I_2 - I_0/I_1 - I_0) \times 100$, where I_2 = mean fluorescence intensity of PHA-activated lymphocytes, I_1 = fluorescence intensity of PHA-nonactivated lymphocytes and I_0 = fluorescence intensity of ethidium bromide. *c*) Normal venous lymphocytes. *d*) Uremic patiens' lymphocytes. *e*) Incubation was carried out at 37°C in a humidified atmosphere of 5% CO_2 in air at 37°C for 12 h in the presence of each synthetic peptide or both. *f*) Control: purchased from the Peptide Institute Inc., Osaka. *g*) $p<0.05$, when compared to the normal persons using Student's *t* test. *h*) $p<0.01$, when compared to the uremic patients using Student's *t* test.

vitro assay test. Interestingly, when the synthetic parathymosin α fragment 1—28 was administered together with thymosin α_1, it appeared to neutralize the immunoenhancing effect of the latter at a dose of 1 μg/ml.

These results suggest that parathymosin α fragment 1—28 may act to modulate the immunoenhancing activity exhibited by thymosin α_1. In the case of normal subjects, *in vitro* addition of these peptides had no effect on the mitotic activity induced by PHA stimulation under the same conditions (data not shown).

Experimental

General experimental procedures used in this paper are essentially the same as described in the previous papers.[20,21] Azides were prepared according to Honzl and Rudinger[14] with isoamyl nitrite. Melting points are uncorrected. Rotations were measured with an Atago Polax machine (cell length: 10 cm). The amino acid compositions of the hydrolysates were determined with a Hitachi type 835-50 amino acid analyzer. HPLC was conducted with a Shimadzu LC-3A apparatus coupled to a YMC AM-312 column (6.0 × 150 mm). FAB-MS spectra was obtained on a Auto spec Q instrument (UQ Analytical Co., England) mass spectrometer equipped with an OPUS data processor. Solutions were concentrated in a rotary evaporator under reduced pressure at a temperature of 30—45 °C. Z(OMe) or Boc groups of the protected peptides were removed by TFA–anisole treatment. The resulting amino components were chromatographed on silica gel plates (Kieselgel G, Merck) and *Rf* values refer to the following solvent system: Rf^1 $CHCl_3$–MeOH–H_2O (8 : 3 : 1). The final product corresponding to the N-terminal octaeicosapeptide fragment of parathymosin α was chromatographed on cellulose plates (Merck). Rf^2 value refers to the Partridge system,[18] and Rf^3 value refers to BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24).[22] Patient selection: two uremic patients who were suffering from recurrent infections diseases were selected. Examination of the cellular immunocompentence of these patients revealed a significant decrease in blast-formation by PHA. ^{3}H-Thymidine incorporation values of these patients were 12160 and 11641 cpm respectively (normal values: 41694—42319 cpm). Venous blood was obtained from these uremic patients for the fluorometric blast-formation test. Venous blood samples from three healthy donors were used as a control. The fluorescence excitation spectrum was measured with an Oyo-Bunko ulog-flouspec 11 A fluorometer. Kits for the fluorometric blast-formation test were purchased from Japan Immunoresearch Laboratories Co., Ltd., Japan. After each coupling reaction, each product was purified by one of two procedures. Procedure A: For purification of protected peptides soluble in EtOAc, the extract was washed with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O, then dried over $MgSO_4$ and concentrated. The residue was reprecipitated or recrystallized from appropriate solvents. Procedure B: For purification of protected peptides almost insoluble in EtOAc, the reaction mixture was poured into ice-chilled 5% citric acid with stirring. The powder thereby formed was washed with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The dried product was recrystallized or reprecipitated from appropriate solvents. Preparations of protected intermediates were repeated several times in order to obtain sufficient quantities for the next step.

Boc–Ala–Gly–OBzl [I] H–Gly–OBzl·Tos (3.4 g) was dissolved in DMF (20 ml) containing NMM (1.2 ml). To this solution, Boc–Ala–OSu (3.2 g) was added, and the mixture was stirred at room temperature for 6 h. The reaction mixture was purified by procedure A, followed by reprecipitation from EtOAc with petroleum ether: Yield 3.1 g (86%), mp 80—85 °C, $[\alpha]_D^{21}$ −21.3° (c = 1.0, DMF), Rf^1 0.52, single ninhydrin-positive spot. *Anal.* Calcd for $C_{17}H_{24}N_2O_5 \cdot H_2O$: C, 57.62; H, 7.40; N, 7.90. Found: C, 57.59; H. 7.58; N, 8.02.

Boc–Lys(Z)–Ala–Gly–OBzl [II] I (3 g) was treated with TFA–anisole (20 ml–4 ml) in an ice-bath for 40 min, and TFA was removed by evaporation. The residue was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 2 h, and then dissolved in DMF (15 ml) containing NMM (1 ml). To this solution, Boc–Lys(Z)–OSu (4.4 g) was added, and the mixture was stirred at room temperature for 6 h. The product was pufified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane: Yield 4.1 g (76%), mp 79—84 °C, $[\alpha]_D^{21}$ −9.7° (c = 1.0, DMF), Rf^1 0.54, single ninhydrin-positive spot. *Anal.* Calcd for $C_{31}H_{42}N_4O_8 \cdot 2H_2O$: C, 58.66; H, 7.31; N, 8.83. Found: C, 58.47; H, 7.56; N, 8.95.

Boc–Glu(OBzl)–Lys(Z)–Ala–Gly–OBzl [III] This compound was prepared essentially in the same manner as described for the preparation of II by using II (3.2 g) and Boc–Glu(OBzl)–OSu (2.4 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane: Yield 3 g (71%), mp 92—98 °C, $[\alpha]_D^{21}$ −11.6° (c = 1.0, DMF), Rf^1 0.64, single ninhydrin-positive spot. *Anal.* Calcd for $C_{43}H_{55}N_5O_{11} \cdot H_2O$: C, 61.78; H, 6.87; N, 8.38. Found: C, 61.81; H, 7.13; N, 8.06.

Boc–Glu(OBzl)–Glu(OBzl)–Lys(Z)–Ala–Gly–OBzl [IV] This compound was prepared essentially in the same manner as described for the preparation of II using III (2.8 g) and Boc–Glu(OBzl)–OSu (1.6 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with ether: Yield 2.6 g (74%), mp 114—123 °C, $[\alpha]_D^{21}$ −8.2° (c = 1.0, DMF), Rf^1 0.67, single ninhydrin-positive spot. *Anal.* Calcd for $C_{55}H_{68}N_6O_{14}$: C, 63.69; H, 6.61; N, 8.10. Found: C, 63.40; H, 6.86; N, 7.82.

Boc–Val–Glu(OBzl)–Glu(OBzl)–Lys(Z)–Ala–Gly–OBzl [I] This compound was prepared essentially in the same manner as described for the preparation of II by using IV (2.1 g) and Boc–Val–OSu (692 mg). The product was purified by procedure B, followed by reprecipitation from MeOH with ether: Yield 2 g (83%), mp 123—129 °C, $[\alpha]_D^{21}$ −13.4° (c = 1.0, DMF), Rf^1 0.69, single ninhydrin-positive spot. *Anal.* Calcd for $C_{60}H_{77}N_7O_{15} \cdot 2H_2O$: C, 61.47; H, 6.97; N, 8.36. Found: C, 61.28; H, 7.16; N, 8.54.

Boc–Asp(OBzl)–Lys(Z)–NHNH–Troc [V] This compound was prepared essentially in the same manner as described for the preparation of II by using Boc–Lys(Z)–NHNH–Troc (2.3 g) and Boc–Asp(OBzl)–OSu (1.9 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with petroleum ether: Yield 2.1 g (70%), mp 74—82 °C, $[\alpha]_D^{21}$ −7.4° (c = 1.0, DMF), Rf^1 0.53, single ninhydrin-positive spot. *Anal.* Calcd for $C_{33}H_{42}Cl_3N_5O_{10}$: C, 49.38; H, 5.66; N, 9.36. Found: C, 49.03; H, 5.38; N, 9.71.

Boc–Lys(Z)–Asp(OBzl)–Lys(Z)–NHNH–Troc [VI] This compound was prepared from V (2 g) and Boc–Lys(Z)–OSu (1.4 g) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with *n*-hexane: Yield 2.1 g (75%), mp 91—97 °C, $[\alpha]_D^{21}$ −10.1° (c = 1.0, DMF), Rf^1 0.59, single ninhydrin-positive spot. *Anal.* Calcd for $C_{47}H_{60}Cl_3N_7O_{13} \cdot H_2O$: C, 53.49; H, 5.92; N, 9.29. Found: C, 53.48; H, 6.17; N, 9.46.

Boc–Lys(Z)–Lys(Z)–Asp(OBzl)–Lys(Z)–NHNH–Troc [VII] This compound was prepared from VI (2 g) and Boc–Lys(Z)–OSu (1 g) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from MeOH with ether: Yield 1.8 g (72%), mp 110—116 °C, $[\alpha]_D^{21}$ −12.6° (c = 1.0, DMF), Rf^1 0.64, single ninhydrin-positive spot. *Anal.* Calcd for $C_{61}H_{78}Cl_3N_9O_{16} \cdot 2H_2O$: C, 54.85; H, 6.19; N, 9.44. Found: C, 54.64; H, 6.42; N, 9.58.

Boc–Glu(OBzl)–Lys(Z)–Lys(Z)–Asp(OBzl)–Lys(Z)–NHNH–Troc [VIII] This compound was prepared from VII (1.7 g) and Boc–Glu(OBzl)–OSu (597 mg) essentially as described for the preparation of II. The product was purified by procedure B, followed by recrystallization from hot EtOAc: Yield 1.4 g (70%), mp 121—128 °C, $[\alpha]_D^{21}$ −7.5° (c = 1.0, DMF), Rf^1 0.69, single ninhydrin-positive spot. *Anal.* Calcd for $C_{73}H_{91}Cl_3N_{10}O_{19} \cdot H_2O$: C, 57.05; H, 6.10; N, 9.11. Found: C, 56.87; H, 6.38; N, 9.46.

Boc–Glu(OBzl)–Lys(Z)–Lys(Z)–Asp(OBzl)–Lys(Z)–NHNH$_2$ [2] VIII (1.3 g) in a mixture of AcOH (6 ml) and DMF (6 ml) was treated with Zn dust (545 mg) at 4 °C for 12 h. The solution was filtered, and the residue was treated with 3% EDTA and then with $NaHCO_3$ to adjust the pH to neutral. The resulting gelatinous mass was washed with H_2O and reprecipitated from DMF with H_2O: Yield 1 g (83%), mp 146—153 °C, $[\alpha]_D^{21}$ −13.2° (c = 1.0, DMF), Rf^1 0.57, single hydrazin-test-positive spot. *Anal.* Calcd for $C_{70}H_{90}N_{10}O_{17} \cdot 3H_2O$: C, 60.16; H, 6.92; N, 10.02. Found: C, 59.86; H, 7.19; N, 10.27.

Boc–Ala–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–NHNH–Troc [IX] This compound was prepared essentially in the same manner as described for the preparation of II by using Z(OMe)–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–NHNH–Troc[8] (1.5 g) and Boc–Ala–OSu (394 mg). The product was purified by procedure A, followed by reprecipitation from EtOAc with ether; Yield 1.2 g (80%), mp 102—110 °C, $[\alpha]_D^{21}$ −13.7° (c = 1.0, DMF), Rf^1 0.63, single ninhydrin-positive spot. *Anal.* Calcd for $C_{56}H_{76}Cl_3N_9O_{15} \cdot H_2O$: C, 55.61; H, 6.50; N, 10.42. Found: C, 55.38; H, 6.89; N, 10.27.

Boc–Ser(Bzl)–Ala–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–NHNH–Troc [X] This compound was prepared from IX (1.2 g) and Boc–Ser(Bzl)–OSu (432 mg) essentially as described for the preparation of II. The product was purified by procedure B, followed by reprecipitation from MeOH with ether: Yield 1.1 g (79%), mp 118—125 °C, $[\alpha]_D^{21}$ −12.7° (c = 1.0, DMF), Rf^1 0.66, single ninhydrin-positive spot. *Anal.* Calcd for

$C_{66}H_{87}Cl_3N_{10}O_{17}\cdot 2H_2O$: C, 55.25; H, 6.39; N. 9.76. Found: C, 55.02, H, 6.64; N, 9.48.

Boc–Ser(Bzl)–Ala–Lys(Z)–Asp(OBzl)–Leu–Lys(Z)–NHNH$_2$ [3] This compound was prepared from X (1 g) and Zn dust (467 mg) essentially as described for the preparation of [2]. The product was recrystallized from MeOH: Yield 801 mg (90%), mp 129—137 °C, $[\alpha]_D^{21}$ −16.5° (c=1.0, DMF), Rf^1 0.49, single hydrazin-test-positive spot. *Anal.* Calcd for $C_{63}H_{86}N_{10}O_{15}\cdot 3H_2O$: C, 59.23; H, 7.26; N, 10.96. Found: C, 59.01; H, 7.49; N, 10.58.

Boc–Glu(OBzl)–Leu–NHNH–Troc [XI] This compound was prepared from Boc–Leu–NHNH–Troc (2.2 g) and Boc–Glu(OBzl)–OSu (2.4 g) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with petroleum ether: Yield 2.6 g (79%), mp 90—94 °C, $[\alpha]_D^{21}$ −28.9 °C (c=1.0, DMF), Rf^1 0.55, single ninhydrin-positive spot. *Anal.* Calcd for $C_{26}H_{37}Cl_3N_4O_8\cdot H_2O$: C, 47.46; H, 5.98; N, 8.52. Found: C, 47.59; H, 6.26; N, 8.21.

Boc–Ala–Glu(OBzl)–Leu–NHNH–Troc [XII] This compound was prepared assentially in the same manner as described for the preparation of II by using XI (2.2 g) and Boc–Ala–OSu (1.1 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with n-hexane: Yield 2 g (83%), mp 86—95 °C, $[\alpha]_D^{21}$ −17.3° (c=1.0, DMF), Rf^1 0.62, single ninhydrin-positive spot. *Anal.* Calcd for $C_{29}H_{42}Cl_3N_5O_9$: C, 48.99; H, 5.95; N, 9.85. Found: C, 48.72; H, 6.28; N, 9.90.

Boc–Ala–Ala–Glu(OBzl)–Leu–NHNH–Troc [XIII] This compound was prepared essentially in the same manner as described for the preparation of II by using XII (1.8 g) and Boc–Ala–OSu (787 mg). The product was purified by procedure A, followed by reprecipitation from EtOAc with n-hexane: Yield 1.6 g (80%), mp 96—102 °C, $[\alpha]_D^{21}$ −14.2° (c=1.0, DMF), Rf^1 0.59, single ninhydrin-positive spot. *Anal.* Calcd for $C_{32}H_{47}Cl_3N_6O_{10}\cdot H_2O$: C, 48.04; H, 6.17; N, 10.50. Found; C, 47.93; H, 6.43; N, 10.24.

Boc–Ala–Ala–Ala–Glu(OBzl)–Leu–NHNH–Troc [XIV] This compound was prepared from XIII (1.3 g) and Boc–Ala–OSu (525 mg) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with ether: Yield 1.2 g (86%), mp 108—116 °C, $[\alpha]_D^{21}$ −19.3° (c=1.0, DMF), Rf^1 0.63, single ninhydrin-positive spot. *Anal.* Calcd for $C_{35}H_{52}Cl_3N_7O_{11}\cdot H_2O$: C, 48.25; H, 6.25; N, 11.25. Found: C, 48.23; H, 6.49; N, 11.02.

Boc–Glu(OBzl)–Ala–Ala–Ala–Glu(OBzl)–Leu–NHNH–Troc [XV] This compound was prepared from XIV (1.1 g) and Boc–Glu(OBzl)–OSu (597 mg) essentially as described for the preparation of II. The product was purified by procedure B, followed by reprecipitation from DMF with H_2O: Yield 1.2 g (86%), mp 126—133 °C, $[\alpha]_D^{21}$ −12.8° (c=1.0, DMF), Rf^1 0.68, single ninhydrin-positive spot. *Anal.* Calcd for $C_{47}H_{65}Cl_3N_8O_{14}\cdot 2H_2O$: C, 52.35; H, 6.45; N, 10.39. Found: C, 52.18; H, 6.77; N, 10.53.

Boc–Glu(OBzl)–Ala–Ala–Ala–Glu(OBzl)–Leu–NHNH$_2$ [4] This compound was prepared from XV (1.1 g) and Zn dust (654 mg) essentially as described for the preparation of [2]. The product was reprecipitated from DMF with ether: Yield 864 mg (89%), mp 149—157 °C, $[\alpha]_D^{21}$ −19.7° (c=1.0, DMF), Rf^1 0.54, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{44}H_{64}N_8O_{12}\cdot 3H_2O$: C,55.61; H, 7.42; N, 11.78. Found: C, 55.61; H, 7.86; N, 11.39.

Boc–Glu(OBzl)–Val–NHNH–Troc [XVI] This compound was prepared from Boc–Val–NHNH–Troc (2 g) and Boc–Glu(OBzl)–OSu (2.4 g) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with n-hexane: Yield 2.6 g (81%), mp 72—76 °C, $[\alpha]_D^{21}$ −10.2° (c=1.0, DMF), Rf^1 0.65, single ninhydrin-positive spot. *Anal.* Calcd for $C_{25}H_{35}Cl_3N_4O_8\cdot H_2O$: C, 46.63; H, 5.79; N, 8.70. Found: C, 46.60; H, 5.96; N, 8.82.

Boc–Ser(Bzl)–Glu(OBzl)–Val–NHNH–Troc [XVII] This compound was prepared essentially in the same manner as described for the preparation of II by using XVI (2.2 g) and Boc–Ser(Bzl)–OSu (1.4 g). The product was purified by procedure A, followed by reprecipitation from EtOAc with n-hexane: Yield 2.1 g (75%), mp 90—98 °C, $[\alpha]_D^{21}$ −8.3° (c=1.0, DMF), Rf^1 0.72, single ninhydrin-positive spot. *Anal.* Calcd for $C_{35}H_{46}Cl_3N_5O_{10}\cdot H_2O$: C, 51.20; H, 5.89; N, 8.53. Found: C, 51.33; H, 6.12; N, 8.87.

Boc–Lys(Z)–Ser(Bzl)–Glu(OBzl)–Val–NHNH–Troc [XVIII] This compound was prepared from XVII (2 g) and Boc–Lys(Z)–OSu (1.3 g) essentially as described for the preparation of II. The product was purified by procedure A, followed by reprecipitation from EtOAc with n-hexane: Yield 2 g (74%), mp 94—99 °C, $[\alpha]_D^{21}$ −14.1° (c=1.0, DMF), Rf^1 0.64, (single ninhydrin-positive spot. *Anal.* Calcd for $C_{49}H_{64}Cl_3N_7O_{13}\cdot 2H_2O$: C, 53.43; H, 6.22; N, 8.90. Found: C, 53.09; H, 6.51; N, 8.66.

Ac–Ser–Lys(Z)–Ser(Bzl)–Glu(OBzl)–Val–NHNH–Troc [XIX] XVIII (1.4 g) was treated with TFA–anisole (14 ml–2.8 ml) as usual and the resulting powder was dissolved in DMF (10 ml) together with NMM (0.28 ml). A solution of Ac–Ser–NHNH$_2$ (214 mg) in DMF (3 ml) was chilled in dry ice-80% EtOH bath to −60 °C. To this solution, 4 N HCl in dioxane (0.52 ml) was added, followed by isoamyl nitrite (0.3 ml). The mixture was stirred for 20 min until the hydrazine-test was negative. The mixture was neutralized with NMM (0.28 ml) at −60 °C. To a cold solution of the tetrapeptide, a cold solution of Ac–Ser–N_3 was added and stirred at −10 °C for 36 h. The mixture was poured into cold 5% citric acid. To the suspension thereby formed, 50% NH_4OAc was added dropwise with stirring to form a precipitate. The precipitate was collected and washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The dried product was reprecipitated from MeOH with ether: 1.1 g (73%), mp 118—127 °C, $[\alpha]_D^{21}$ −23.6° (c=1.0, DMF), Rf^1 0.66, single chlorine–tolidine-positive spot. *Anal.* Calcd for $C_{49}H_{63}Cl_3N_8O_{14}\cdot 3H_2O$: C, 51.25; H, 6.06; N, 9.76. Found: C, 51.43; H, 6.41; N, 9.72.

Ac–Ser–Lys(Z)–Ser(Bzl)–Glu(OBzl)–Val–NHNH$_2$ [5] This compound was prepared from XIX (1 g) and Zn dust (568 mg) essentially as described for the preparation of [2]. The product was recrystallized from hot MeOH: Yield 721 mg (89%), mp 140—147 °C, $[\alpha]_D^{21}$ −71.6° (c=1.0, DMF), Rf^1 0.52, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{46}H_{61}N_8O_{12}\cdot H_2O$: C, 59.03; H, 6.78; N, 11.97. Found: C, 58.79; H, 7.10; N, 11.65.

Synthesis of Protected Parathymosin α Fragment 1—28 Successive azide condensations of five fragments were carried out according to Fig. 2. Prior to condensation, the Boc group was removed from the respective amino component (1 ml per 0.1 g of the peptide) in the presence of anisole (10 eq) in an ice-bath for 40 min. The TFA-treated sample was precipitated with dry ether, dried over KOH pellets *in vacuo* for 2 h, and dissolved in DMF or DMF–DMSO (1 : 1) containing NMM (1 : 1 eq). The corresponding azide (the amount was increased from 1.5 to 4 eq as chain elongation progressed) in DMF or DMF–DMSO (1 : 1) and NMM (1 : 1 eq) was added to the above ice-chilled solution and the mixture was stirred at −10 °C until the solution became negative to the ninhydrin-test. The mixture was neutralized by adding a few drops of AcOH and poured into ice-chilled 5% citric acid with stirring. The precipitate thereby formed was successively washed with 5% citric acid, H_2O and MeOH. The dried product was

TABLE II. Characterization of the Protected Parathymosin α Fragment 1—28 and Its Intermediates

	Puri. proc. (Yield %)	mp (°C)	Rf^1	$[\alpha]_D^{21}$ (c=1.0, DMSO)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
Boc–(18—28)–OBzl	B (71)	140—148	0.62	−17.6	$C_{125}H_{155}N_{15}O_{30}\cdot 6H_2O$	61.14	6.86	8.56
						(60.87	6.97	8.42)
Boc–(12—28)–OBzl	B (63)	152—161	0.69	−12.4	$C_{183}H_{229}N_{23}O_{43}\cdot 9H_2O$	61.10	6.91	7.18
						(61.22	7.18	8.59)
Boc–(6—28)–OBzl	A (55)	164—173	0.59	−15.7	$C_{222}H_{281}N_{29}O_{53}\cdot 11H_2O$	60.57	6.94	9.23
						(60.48	7.24	9.50)
Boc–(1—28)–OBzl	A (65)	159—167	0.67	−26.4	$C_{263}H_{330}N_{35}O_{63}\cdot 13H_2O$	60.47	6.87	9.39
						(60.16	7.13	9.42)

A, precipitation from DMF or DMSO with MeOH; B, gel-filtration on Sephadex LH-20.

purified by one of the following procedures. A: Precipitation from DMF with MeOH. B: Gel-filtration on Sephadex LH-20 using DMF containing 3% H_2O as an eluent. In procedure B, eluates (4 ml each fractions) were examined by measuring the ultraviolet (UV) absorption at 260 nm and fractions corresponding to the front main peak were combined. The solvent was removed by evaporation and the residue was treated with ether to afford a powder. The purification procedure, yield, physical constants and analytical data of protected parathymosin α fragment 1—28 and its intermediates are listed in Tables II and III.

Ac–Ser–Lys–Ser–Glu–Val–Glu–Ala–Ala–Ala–Glu–Leu–Ser–Ala–Lys–Asp–Leu–Lys–Glu–Lys–Lys–Asp–Lys–Val–Glu–Glu–Lys–Ala–Gly–OH (Corresponding to Parathymosin α Fragment 1—28) [XXIV] The protected octaeicosapeptide ester (100 mg) was treated with 1 M TFMSA–thioanisole in TFA (4 ml) in the presence of Me_2Se (100 μl) in an ice-bath for 110 min, then dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets for 2 h and dissolved in 1 N AcOH (5 ml). The solution, after being stirred with Amberlite IRA-400 (acetate form, approximately 1 g) for 30 min, was filtered. The pH of the filtrate was adjusted to pH 8.0 with 1 N NH_4OH, and after 30 min to pH 6.0 with 1 N AcOH, and the solution was lyophilized. The residue was dissolved in 2% AcOH (2 ml), applied to a column of Sephadex G-25 (3.0 × 96 cm), and eluted with the same solvent. Fractions of 4 ml each were collected per 20 min, and the absorption at 230 nm was determined. Fractions corresponding to the front main peak (tube Nos. 68—79) were combined and the solvent was removed by lyophilization. The Sephadex-purified sample was dissolved in a small amount of the upper phase of BuOH–AcOH–H_2O (4 : 1 : 5). The solution was applied to a column of Sephadex G-25 (3.0 × 94 cm) previously equilibrated with the lower phase of the above solvent system. The column was developed with the same upper phase. Individual fractions (4 ml each) were collected and the absorbancy at 230 nm was determined. The fractions corresponding to the main peak (tube Nos. 60—68) were combined. The solvent was removed by evaporation. Analysis by TLC revealed the presence of two chlorine-tolidine-positive spots with Rf^2 0.05 (main) and 0.31 (minor). The crude peptide was subscribed to preparative TLC (cellulose phase, 20 × 40 cm) using the solvent system of BuOH–AcOH–H_2O (4 : 1 : 5, upper phase). The zone corresponding to Rf^2 0.05 was separated and extracted with 2% AcOH. The extract was concentrated to a small volume, applied to a Sephadex G-25 column (3.0 × 96 cm), and eluted with 2% AcOH. The main fractions containing a single component were combined and the solvent was removed by lyophilization: Yield 8.3 mg (14%), $[\alpha]_D^{21}$ −79.5° (c = 0.3, 2% AcOH), Rf^2 0.05, Rf^3 0.08, single chlorine–tolidine-positive spot. The synthetic peptide exhibited a single spot on paper electrophoresis: Toyo Roshi No. 51 (2 × 40 cm), acetate buffer at pH 2.86, mobility 2.1 cm from the origin toward the cathode after running at 1.5 mA, 600 V for

TABLE III. Amino Acid Ratios in 6 *N* HCl Hydrolysates of the Protected Parathymosin α Fragment 1—28 and Its Intermediates[a)]

	Protected peptides				Residue
	18—28	12—28	6—28	1—28	
Gly	1.00	1.00	1.00	1.00	1
Ala	1.04	2.04	5.06	5.07	5
Leu		0.97	1.96	2.02	2
Val	0.95	0.95	0.91	1.91	2
Ser		0.94	0.93	2.93	3
Asp	0.93	1.90	1.99	1.97	2
Glu	2.99	3.03	4.92	5.89	6
Lys	4.06	5.91	5.92	7.04	7

a) The results are expressed as ratios to the value for Gly, which was taken as the diagnostic amino acid in acid hydrolysates.

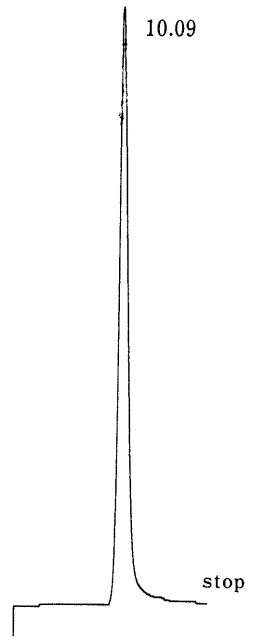

Fig. 3. HPLC of the Synthetic Parathymosin α Fragment 1—28

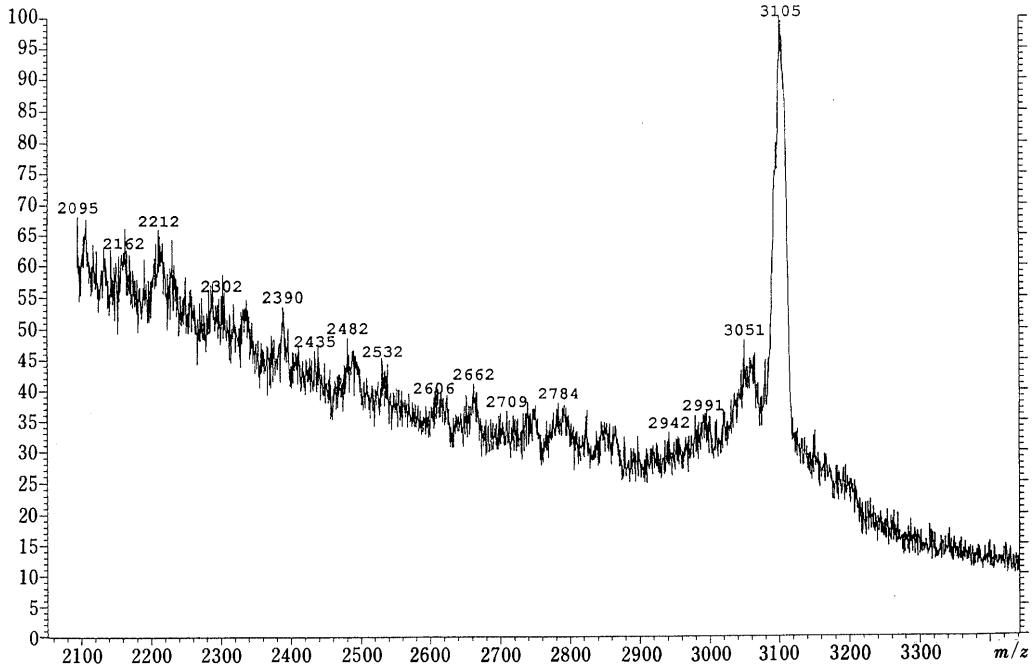

Fig. 4. FAB-MS of the Synthetic Parathymosin α Fragment 1—28

75 min. Amino acid ratios in 6 N HCl hydrolysate: Gly 1.00, Ala 5.07, Leu 1.95, Val 1.98, Ser 2.90, Asp 2.03, Glu 5.90, Lys 7.04 (recovery of Gly 83%). The synthetic peptide exhibited a single peak on HPLC using an analytical YMC AM-312 column (6.0 × 150 mm) at a retention time of 10.09 min, when eluted with a gradient of acetonitrile 32 to 45% in 0.1% TFA at a flow rate of 1 ml per min (Fig. 3). FAB-MS *m/z*: 3105 $(M+H)^+$.

Fluorometric Blast-Formation Test A 3-ml aliquot of venous blood from uremic patients was drawn into a syringe containing 25U/ml of heparin, and the mixed with 3 ml of PBS. Lymphocytes were isolated in a Hypaque-Ficoll gradient.[23] Isolated lymphocytes were adjusted to 1.0×10^6/ml with PBS. The lymphocytes were cultured in 0.5 ml of RPMI 1640 (Gibco) with FCS (Dainippon Pharmaceutical Co.) in microplates. Cultures of each combination were incubated at 37 °C in the presence of the peptide in a humidified atmosphere of 5% CO_2 in air for 12 h and PHA (0.125%, 0.5 ml) was added to each well. Incubation was continued under the same conditions for 60 h. T-lymphocytes in each well were transfered to a test tube and centrifuged for 10 min at 240 *g*, and an aliquot of 0.125% SDS was added to the residue and stirred for 20 min at room temperature; lymphocytes were completely destroyed and solubilized by this procedure. An ethidium bromide solution was added to the above solution and the mixture was stirred for 15 min at room temperature. The fluorescence excitation spectrum was measured according to the method of Itoh and Kawai.[19]

References and Notes

1) Abbreviations used: TFA, trifluoroacetic acid; Z, benzyloxycarbonyl; OBzl, benzyl ester; Z(OMe), *p*-methoxybenzyloxycarbonyl; Ac, acetyl; Boc, *tert*-butoxycarbonyl; Troc, β,β,β-trichloroethoxycarbonyl; Bzl, benzyl; NMM, *N*-methylmorpholine; OSu, *N*-hydroxysuccinimide ester; Su, *N*-hydroxysuccinimide; DMF, dimethylformamide, DMSO, dimethylsulfoxide; AcOH, acetic acid; MeOH, methanol; EtOAc, ethyl acetate; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; PHA, phytohemagglutinin; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PRMI, Rosewell Park Memorial Institute; FCS, fetal calf serum; FAB-MS, fast atom bombardment mass spectrometry; Tos, *p*-toluenesulfonic acid; EtOH, ethanol; NH_4OAc, ammonium acetate.

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Freeze-Drying of Liposomes Prepared by Sonication and Extrusion Techniques

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Factors such as lipid composition and liposome size, which affect the stability of liposomes during the freeze-drying process, were investigated. The addition of 9 mol% of charged lipids to egg-phosphatidylcholine (eggPC) had little effect on liposome stability. The addition of cholesterol decreased the retention percentage (*R*%) of entrapped calcein and increased the size of liposomes after freeze-drying. Destabilization occurred in the gel state of eggPC/cholesterol binary system. In liposome systems prepared using the extrusion technique, the *R*% decreased through freeze-drying as their size increased. Similar damage to liposomes was observed only by hyperosmotic shock: Considerable leakage occurred for large liposomes when the medium was changed from hyperosmotic to isotonic. Consequently, liposome leakage by freeze-drying likely occurs at the rehydration process.

Keywords sonicated liposome; extrusion method; retention; liposome stability; freeze-drying; charged lipid; cholesterol; liposome size; hyperosmotic treatment

Introduction

Freezing[1,2] and freeze-drying[3,4] of liposomes have been recently challenged with stabilizers such as saccharides. The stabilization mechanism of saccharides has been investigated by several workers,[2,5-7] while there is less research related to the effect of the characteristics of the liposomes, such as lipid composition or size, on their stability. Concerning composition, Harrigan *et al.*[8] investigated the effect of phosphatidylglycerol on the stability of liposomes composed of egg-phosphatidylcholine (eggPC) during dehydration, and they pointed out the importance of the type of lipid head group in such a system. Charged lipids are often added to liposomes in order to stabilize them against aggregation and fusion, and to control the membrane permeability.[9] Crowe *et al.*[10] reported that the elimination of the charged lipids decreased the stability of liposomes during freeze-drying. But the reason for destabilization was not clear. Cholesterol is often added to liposomes to improve their stability in blood.[9] But cholesterol's effect on liposome stability during freeze-drying is not known. As to the size of liposomes, some workers reported that stability decreased as liposome size increased during freeze-thawing,[11] dehydration[8] and freeze-drying,[10] with a sufficient amount of saccharides. But the reason for such instability is also still obscure.

We investigated three factors affecting the stability of liposomes during freeze-drying with a sufficient amount of saccharides: The type of charged lipids, the amount of cholesterol, and the size of the liposomes.

Experimental

Materials Egg yolk-L-α-phosphatidylcholine (eggPC) was supplied from Asahi Kasei Co., Ltd., and was used without further purification. Bovine brain L-α-phosphatidyl-L-serine (PS), dicetyl phosphate (DCP) and stearylamine (SA) were purchased from Sigma Chemical Co., Ltd. *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE) was obtained from Molecular Probes Co., Ltd. *N*-(Lissamine-rhodamine-B-sulfonyl)dioleoyl-L-α-phosphatidylethanolamine (Rh-PE) was supplied from Avanti Co., Ltd. Cholesterol, trehalose and sucrose were obtained from Wako Pure Chemical Co., Ltd. Saccharides were used after drying *in vacuo* at room temperature on P_2O_5 for 3 d. Calcein was obtained from Dojin (Kumamoto, Japan). Water was purified by deionization and distillation.

Preparation of Liposomes Aliquots of a lipid solution in chloroform–methanol were placed in a round-bottom 20-ml flask. After an evaporation of the solvent, the residual film was dried under vacuum over-night. The lipid film was hydrated with a 10 mM Tris–HCl buffer solution containing 70 mM calcein and 50 mM saccharide (pH = 7.2). The dispersion was vortexed to obtain multilamellar vesicle dispersion (MLV). The MLV dispersion was sonicated in ice water with nitrogen, bubbling for 30 min with a probe-type sonicator (Tomy UD-200). Vesicles by the extrusion technique (VET) were prepared as described by Mayer *et al.*[12] The MLV dispersions were frozen in liquid nitrogen and thawed in a 40 °C bath before extrusion. This process was repeated five times. The frozen and thawed MLV systems were successively extruded through polycarbonate filters with 600, 400, 200, 100 and 50 nm pore diameters. The extrusion was repeated five times for 600, 400 and 200 nm filters and ten times for 100 and 50 nm filters, respectively. The designation "VETs" with a subscription indicating the pore size of the filter as described by Mayer's *et al.*[12] is also used in this paper. The negative stain electron micrograph of each system showed that most of the sonicated liposomes in the VET_{50} system were unilamellar liposomes, and that VET_{100} and other larger liposomes include a considerable number of oligolamellar and multilamellar liposomes. Untrapped calcein was removed by gel filtration (Sephadex G50, 10 mM Tris–HCl buffer or 10 mM Tris–HCl buffer with 365 mM sucrose as an eluent). The lipid concentration was determined by phosphorus analysis.[13]

Freeze-Drying Normally, 100 μl-aliquots of liposome dispersion were freeze-dried in glass vials (diameter: 15 mm) in a chamber freeze-dryer (RL-10NA by Kyowa-Shinku Co., Ltd). The sample was frozen to a terminal temperature of −45 °C at a cooling rate of about 5 °C/min, and dried at a pressure of 0.01 Torr for 18 h. The temperature was held at −45 °C. After that, the shelf temperature was set at 25 °C, and drying was continued at about 0.003 Torr for 6 h. The samples were rehydrated to their original volume with distilled water.

Calculation of Percent Retention of Calcein (*R*%) The percentage of calcein retained after freeze-drying (*R*%) was determined by measuring the fluorescence intensity at 520 nm with excitation at 490 nm using a Jasko FB-550 fluorometer. *R*% is calculated as described by Crowe *et al.*[10]

Liposome Size Liposome size was measured by the quasi-elastic laser light scattering method (QLS). The QLS apparatus was a model LPA-3100 with a He–Ne ion laser ($\lambda = 628.3$ nm) manufactured by Otsuka Electronics Co., Ltd. The weight average diameter ($\bar{D}_w$) and distribution of particle size were determined by histogram methods.[14] As for the viscosity and refractive index, the values for each saccharide solution were used.

Fusion Fusion of liposomes by freeze-drying was estimated by probe dilution assay of the resonance energy transfer technique as described by Struck.[15] The efficiency of energy transfer (E) before and after freeze-drying was obtained from the fluorescence emission intensity of NBD-PE at 530 nm (excitation 460 nm) by Eq. 1,

$$E = 1 - F/F_0 \quad (1)$$

F_0 and F are fluorescence intensities in the presence and absence of Triton X-100, respectively.

Osmotic Shocks The initially-prepared vesicles contained 10 mM Tris–HCl, 70 mM calcein and 50 mM sucrose. Osmotic shrinkage was provided by placing the initially-prepared liposome dispersion in an

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appropriated hyperosmolar sucrose solution. The osmotic gradient was 1.0 M. The percentage of leakage of calcein was measured fluorometrically both before and after the hyperosmotic dispersion was diluted by a Tris–HCl buffer solution to an isotonic condition.

Long-Term Stability After keeping each freeze-dried liposome system used in the present study at both −25 °C and 4 °C for one month, a further decrease in R% or destruction of the liposomes was not observed.

Results and Discussion

Effect of the Concentrations of Trehalose and PC The relation between the retention percentage (R%) of sonicated liposomes composed of pure eggPC after freeze-drying and the trehalose concentration outside the sonicated liposomes, [trehalose], is investigated at various phospholipid concentrations, [eggPC]. Fig. 1a shows the relation at [eggPC] of 4 mM. Maximum R% is about 90%, which is obtained at the molar ratio of trehalose to phospholipid above 4. Fig. 1b summarizes the relation between [trehalose] and [eggPC] at certain constant values of R%. At [eggPC] over about 2 mM, there is a linear relationship between [trehalose] and [eggPC] for each R%. All the extrapolated lines pass through the zero point, indicating that R% depends only on the molar ratio of trehalose to phospholipid, not on the bulk concentration of trehalose. [Trehalose] required for the same R% increases with a decrease in [eggPC] in the low concentration range (<2 mM). The reason for the deviations is not obvious at the present. All experiments described hereafter are performed in a linear region. The [trehalose] is kept isotonic with an inside solution of liposomes, because the osmotic swelling of liposomes is known to introduce a structural change in the liposome membranes.[16]

Lipid Composition Charge: The R% value of sonicated liposomes containing 9 mol% of acidic lipids (PS, DCP) or cationic lipid (SA) are shown in Table I. No significant effect is observed by the addition of SA. The R% of anionic lipids slightly decreased. From these results the addition of 9 mol% of charged lipids had no notable influence on the stability of liposomes during freeze-drying with a sufficient amount of saccharides. By contrast, Crowe *et al.*[10] reported that the absence of anionic lipids (PS) decreased the stability of liposomes during freeze-drying. The reason for the discrepancy between our results and theirs is not clear at the present.

Cholesterol: The values of R% of sonicated liposomes and VET50 systems composed of various ratios of egg-PC to cholesterol in the presence of a sufficient amount of trehalose after freeze-drying are shown in Fig. 2. The decrease of R% is observed in both systems with an increase in cholesterol content, especially above 30 mol%. The mean size of the liposomes containing cholesterol increases after freeze-drying, as shown in Fig. 3, suggesting the fusion of liposomes. In order to get direct information on the fusion of liposomes, the value of energy transfer efficiency (E) in each system is measured. E values in liposomes without cholesterol are the same, 0.43, before and after freeze-drying, while those for the system containing 35 mol% cholesterol decrease from 0.39 to 0.27, indicating fusion in this system.

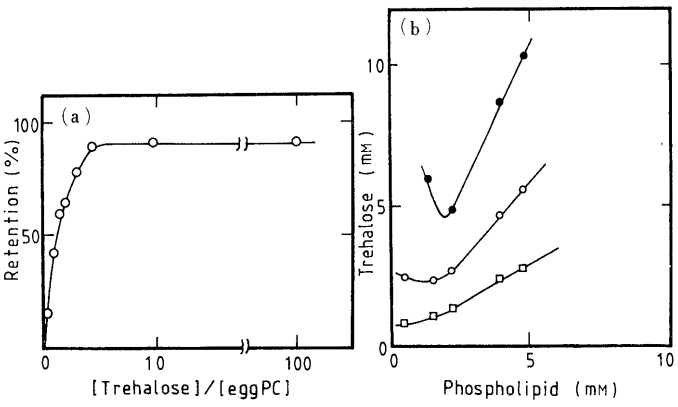

Fig. 1a. Relation between R% and [Trehalose]/[eggPC] in Sonicated Liposome System

Fig. 1b. Relation between Concentrations of Trehalose and EggPC at x% Retention

□, x=30%; ○, x=50%; ●, x=70%.

TABLE I. Effect of the Charged Lipids on R%

Composition		R%
EggPC		88.7±0.4
EggPC : SA	(10 : 1)	88.7±1.0
EggPC : DCP	(10 : 1)	83.4±0.5
EggPC : PS	(10 : 1)	84.2±1.0

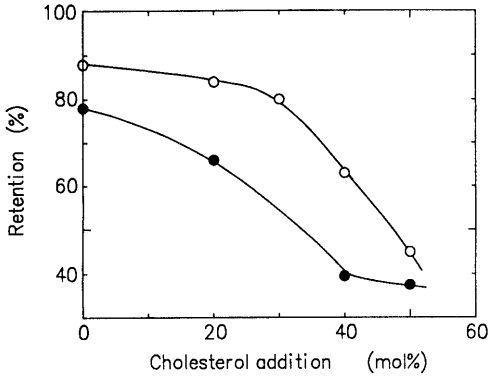

Fig. 2. Relation between R% and Cholesterol Addition (mol%)

○, in sonicated liposome system; ●, in VET_{50} system.

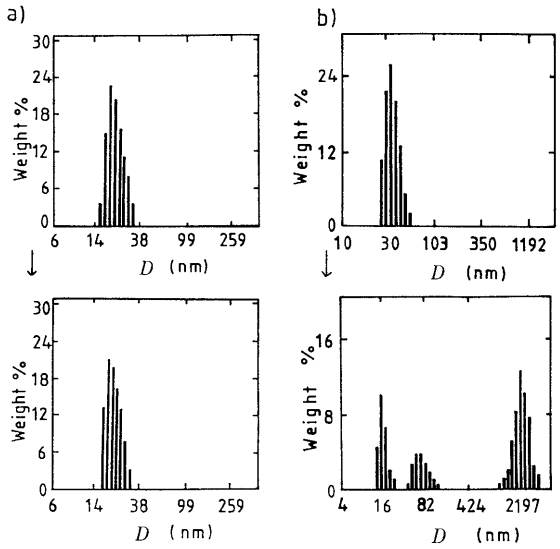

Fig. 3. Size Distribution of Sonicated Liposomes before (above) and after (below) the Freeze-Drying

a) Without cholesterol; *b*) containing 40 mol% cholesterol.

These results show that cholesterol destabilizes the liposome during freeze-drying.

Harrigan *et al.*[8] investigated the $R\%$ of entrapped carboxyfluorescein of liposomes with 100 nm diameter containing various amount of cholesterol with trehalose as a cryoprotectant, by dehydration at room temperature, and found no effect from cholesterol on $R\%$ up to 40 mol%. This is completely different from the present results. The discrepancy may arise from the difference between the dehydration and freeze-drying processes. In the latter process, lipids should pass through the gel state. By contrast, in the former process the lipids must remain only in a liquid-crystalline state, because the phase transition temperature (T_c) of lipids in a dehydrated state is known to be depressed by the addition of saccharides.[6] Therefore, the damage of liposomes observed in the present system most probably occurs in their gel state.

Damage is probably induced by the phase separation in the gel state. The fusion of liposomes and the leakage of entrapped calcein often occur in the phase separation state.[17,18] A phase separation of the present binary system in the gel state has not been proven. But, such phase separation in the gel state is reasonably expected, since a clear phase separation has been reported[19] in the liquid-crystalline state of the present binary system. As a matter of fact, in the binary dipalmitoylphosphatidylcholine–cholesterol system, phase separation has been reported both in gel and liquid-crystalline states.[19]

Liposome Size The values of $R\%$ and $\bar{D}_w$ before and after the freeze-drying with a sufficient amount of sucrose for sonicated liposome and VETs composed of pure egg PC systems are summarized in Table II. Sonicated liposome and VET_{50} systems show similar behavior regarding $R\%$; that is, the $R\%$ decreases as the liposome size increases. No increase in $\bar{D}_w$ by freeze-drying was found in any systems, indicating that the fusion of liposomes is not the reason for a decrease in $R\%$.

It has already been pointed out that liposome size is an important factor in its stability during freeze-thawing,[11] dehydration[8,20] and freeze-drying.[10] Hauser *et al.*[20] showed that the spray drying of an SUV system was possible with high marker retention. Crowe *et al.*[10] reported that liposomes with diameters of 50—100 nm were most stable during freeze-drying, and that both SUV and the larger liposomes were unstable even with sufficient amounts of trehalose. Madden *et al.*[8] observed that 70—100 nm diameter liposomes showed the highest $R\%$ following the dehydration (70%), and that $R\%$ decreased as the liposome size increased. Talsma *et al.*[11] reported that 140 nm diameter liposomes showed the highest retention during the freeze-thawing. Our results show good agreement with these reported results, except for that by Crowe *et al.*[10] which showed a low stability of the SUV system. They proposed that SUV was unstable because of its high curvature, which induced the fusion of liposomes. In our system, such fusion during freeze-drying is completely prevented by the addition of saccharides such as trehalose and sucrose. The reason for the discrepancy between the two results is mainly the difference in experimental conditions. Liposomes in their system were in an osmotic swelling state. The SUV system was liable to fuse because of its high curvature. In the swelling state, fusion may occur more easily. Another difference is the condition of the freeze-drying process. In our experiments, the sublimation of ice is completed at $-45\,^{\circ}C$, followed by drying at 25 °C. On the other hand, in their system, the temperature in the sublimation process was not clearly described. Talsma *et al.*[21] reported that storage at $-25\,^{\circ}C$ after the initial freezing in liquid nitrogen, even in the presence of 5% trehalose, caused significant leakage in the freeze-thawing cycle, while storage at $-50\,^{\circ}C$ did not. They proposed that the instability was caused by mechanical damage liposomes suffered as a result of ice crystal growth and/or recrystallization proceeding in the system during storage at $-25\,^{\circ}C$. The low stability of the SUV system results from the temperature rise during the sublimation stage in their system. Ice crystals may indeed have grown in their system at that time, causing mechanical damage to the liposomes. Such damage may have triggered the fusion observed in their swelled SUV system during freeze-drying. This data well demonstrates the importance of the freeze-drying condition on the stability of liposomes.

Our results and literatures[8,10,11,20] indicated that the threshold size over which the liposome stability decreases during freezing or dehydration is around 70 nm. The leakage observed in larger liposomes systems during freeze-drying possibly occurs when a considerable amount of water permeates from inside to outside the liposomes or *vice versa* during freeze-drying and rehydration. A similar flow of water can be induced by osmotic shock, setting liposome dispersion in a hyperosmotic solution and then taking them back to an isotonic condition. We investigated the percent leakage of each liposome system by such osmotic shock. Little leakage in the hyperosmotic condition was found in any system (<1%). On the other hand, they showed significant leakage after they were returned to an isotonic condition, depending on liposome size, as shown in Fig. 4. Liposomes larger than 200 nm in diameter are well known

TABLE II. Retention% and $\bar{D}_w$ before and after the Freeze-Drying with Sucrose of SUV and VETs Systems

System	Trapped volume (l/mol egg-PC)	$R\%$	$\bar{D}_w$ (nm)	
			Before	After
SUV	0.18	88	26± 8	29± 7
VET_{50}	0.96	87	67±16	62±20
VET_{100}	1.42	75	111±24	103±23
VET_{200}	1.77	62	172±26	170±29
VET_{400}	2.14	55	266±107	259±122

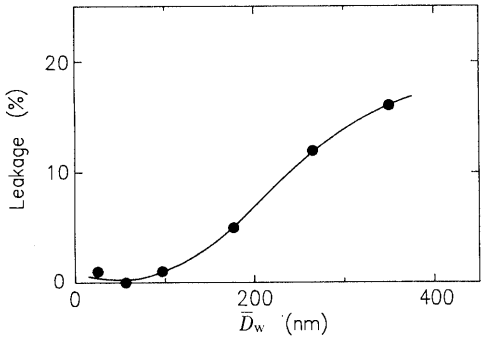

Fig. 4. Relation between Percent Leakage by Hyperosmotic Shock and $\bar{D}_w$

to suffer from lysis under hypoosmotic conditions.[22] The present result shows that hyperosmotic treatment also decreases an ability to retain entrapped compounds in larger liposome systems. The leakage observed during freeze-drying occurred mainly during the rehydration process, in which a considerable amount of water permeated from outside to inside the liposomes. Such leakage cannot be prevented by the addition of saccharides. Kano and Fendler reported that osmotic shrinkage changed the microviscosity of the polar interior and surface of liposomes and also altered hydrocarbon fluidity.[23] With the increase of liposome size, the number of multilamellar membrane increases. Large liposomes probably cannot maintain the membrane permeability barrier when the microenvironment of the liposome changes in such a way. It may be a phenomenological aspect of the lack of structural rigidity[10] of the multilamellar membrane.

The inhomogeneities in the distribution of saccharides in the multilamellar system may be another reason for the instability of larger liposomes. Crowe *et al.*[24] found such inhomogeneities in the freeze-dried MLV system composed of dipalmitoylphosphatidylcholine prepared in a trehalose solution by their thermal study. Because of the inhomogeneous distribution, the local amount of saccharides per lipid molecule in some parts of the multilamellar membrane became less than the minimum amount necessary for protection, which thus induced a decrease in $R\%$.

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Enhancement Effect of Lauric Acid on the Rectal Absorption of Propranolol from Suppository in Rats

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In a previous paper, we have demonstrated that medium chain fatty acids significantly enhance the *in vitro* rectal absorption of propranolol (PL) and that the enhancement may be partly due to the formation of a complex with a fatty acid at a 1 : 1 molar ratio. To confirm *in vivo* the enhancement effect of lauric acid on PL absorption, PL suppositories with lauric acid at various molar ratios were administered to rat rectum. PL absorption from Witepsol and macrogol suppositories with lauric acid at a 1 : 1 molar ratio was much larger than that after PL alone and the 1 : 2 or 1 : 3 molar ratio ones. The bioavailability (BA) after administration of the 1 : 1 molar ratio suppository (PL, 4 mg/kg) was 1.6- and 2.1-fold for the Witepsol and macrogol formulations respectively, compared with that after PL alone. A similar result was obtained with the PL solid dispersion suppository with lauric acid at a 1 : 1 molar ratio, showing a 1.7-fold higher BA compared with PL alone. The release of PL from the macrogol suppository was significantly faster at a 1 : 1 molar ratio than that of other preparations, but not so in the solid dispersion suppository. There was not good agreement between the release rates of PL from the suppositories and the plasma levels after dosing. These results supported the concept that a portion of PL, by forming a 1 : 1 complex with lauric acid, would penetrate across the rectal mucosa more easily than PL alone.

Keywords propranolol; rectal absorption; lauric acid; absorption enhancement; complex formation; rat; propranolol release; suppository

Propranolol (PL) is a non-selective β-adrenergic blocking agent used in the treatment of hypertention and cardiac arrhythmia. However, the bioavailability of oral dosage forms of PL is low and varies highly between individuals.[1-3] The low bioavailability and the variation have been attributed to extensive drug metabolism in the liver, first-pass effects.[4] Rectal administration may be useful for drugs with low bioavailability due to first-pass metabolism.[5] In fact, the hepatic first-pass effect of PL can be avoided by the rectal administration.[6]

In a previous paper, we demonstrated that medium chain fatty acids significantly enhance the *in vitro* rectal absorption of PL and that the enhancement may be partly related to the formation of a complex with a fatty acid at a 1 : 1 molar ratio.[7]

Based on the results of our previous studies,[7,8] lauric acid was selected for evaluation of the *in vivo* absorption enhancing effect. The present study had two objectives: 1) to clarify whether the rectal absorption of PL is enhanced in the presence of lauric acid; 2) to demonstrate *in vivo* the complex mechanism for the enhancement of PL rectal absorption.

Materials and Methods

Materials PL hydrochloride was a generous gift of Ono Pharmaceutical Industry Co. Verapamil hydrochloride, an internal standard for high performance liquid chromatography (HPLC), was purchased from Sigma Chemical Co. Witepsol H-15, macrogol 6000, 4000 and 1500 (JP grade) and fatty acids (99%) were obtained from Mitsuba Boeki Co. and Nakalai Tesque Co., respectively. All other chemicals were of analytical or special grade. Free PL was prepared from the hydrochloride salt by the method previously described[9] and used throughout this study. Male Wistar rats weighing 200—250 g were used throughout this experiment. The animals, maintained on a MF diet (Oriental Yeast Co.) for 3—4 d prior to the experiment, were divided at random into 3—4 groups, each consisting of 4—6 rats. On the day before the experiment, the jugular vein was cannulated with silicon tubing.[10,11]

Intravenous (i.v.) Administration PL hydrochloride dissolved in saline was administered intravenously through the tubing at a 4 mg/kg (free PL equivalent) dose. After administration, blood samples were withdrawn from the cannulated jugular vein periodically into a heparinized syringe. The plasma was separated immediately by centrifugation and stored frozen until assay.

Preparation of Solid Dispersion of PL A 40% solid dispersion of PL in macrogol 6000 was prepared using a melt method. Macrogol 6000 was melted in a beaker heated at 65—70 °C. PL was added to the melted macrogol with constant stirring until a clear liquid was observed. This liquid was poured into a glass motar cooled in ice. The mass was triturated and sieved through a 60-mesh sieve.

Preparation of Suppository The suppositories were prepared by the fusion method at a temperature as low as possible, using Witepsol H-15 or macrogol (4000 : 1500 = 3 : 1, w/w) as the bases. In some cases, the PL-macrogol solid dispersion was added to the melt of macrogol with or without lauric acid and mixed immediately before the solidification. One cm length of the suppository (diameter 4 mm) contained 1 or 2 mg PL with or without lauric acid (molar ratio to PL, 1—3 : 1). Details of the composition are listed in Table I.

***In Vitro* Release Test** The release test of PL from suppositories was carried out by the method of Muranishi *et al.*, using a cylinder filter paper and 0.067 M phosphate buffer, pH 7.3, 50 ml.[12]

***In Vivo* Rectal Absorption Experiment** The animals were fasted for 24 h before and during the experiment of rectal administration. Each rat received a PL suppository rectally at a 4 or 8 mg/kg dose. The suppository was inserted to a depth of 2 cm from the anus, and the anus was closed with an adhesive, Aronalpha (Konishi Co.). Blood samples were withdrawn from the cannulated jugular vein periodically. The plasma was stored frozen until assay.

TABLE I. Composition of PL Suppositories

No.	PL (mg)	Lauric acid (mg)	Macrogol 1500 (g)	Macrogol 4000 (g)	Witepsol H-15 (g)	Molar ratio of PL : adjuvant
1	Powder 10	—	—	—	1.16	—
2	Powder 10	7.7	—	—	1.15	1 : 1
3	Powder 20	—	—	—	1.15	—
4	Powder 20	15.5	—	—	1.13	1 : 1
5	Powder 10	—	0.38	1.14	—	—
6	Powder 10	7.7	0.38	1.13	—	1 : 1
7	Powder 10	15.5	0.38	1.13	—	1 : 2
8	Powder 10	23.2	0.37	1.13	—	1 : 3
9	PL-S[a] 10	—	0.37	1.13	—	—
10	PL-S[a] 10	7.7	0.37	1.12	—	1 : 1
11	PL-S[a] 10	15.5	0.37	1.11	—	1 : 2
12	PL-S[a] 10	23.2	0.37	1.10	—	1 : 3

a) A 25 mg of the 40% solid dispersion of PL in macrogol 6000 (10 mg as PL) was used.

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Determination of PL PL was determined by the method of Katayama *et al.*[13] described in a previous paper.[9]

Analysis of Data Kinetic parameters were calculated by using the least-squares fit program, MULTI.[14] The *in vitro* release rate (nmol/min$^{1/2}$) was calculated from the slope of the percent release *vs.* time curve. The plasma concentration data after i.v. administration were fitted to the equation[15]:

$$C_t = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t}$$

where C_t is the drug concentration at time, t and A, α, B and β are the biexponential equation constants. The half-life ($t_{1/2}$) of the terminal phase was calculated as $t_{1/2,\beta} = 0.693/\beta$. The area under the plasma concentration–time curve (AUC) after i.v. administration was calculated by the following equation:

$$AUC = A/\alpha + B/\beta$$

The area under the first moment curve ($AUMC$) and the mean residence time (MRT) were calculated by means of the following equations:

$$AUMC = A/\alpha^2 + B/\beta^2$$

$$MRT = AUMC/AUC$$

The AUC after rectal administration was determined by the trapezoidal method to the last observed data point. The residual area beyond the last sampling time was estimated as C'/k_z, where C' is the last observed concentration and k_z is the slope of the terminal log-linear phase. The absolute bioavailability was calculated using the AUC values. The plasma PL concentrations after a single rectal dosing were fitted to the 1-compartment model including a first-order absorption process and the parameters were determined by the simulation.

The means of all data are presented with their standard deviation (mean ± S.D.). Student's t-test was utilized to determine a significant difference between the groups, $p < 0.05$ being taken as the minimum level of significance.

Results

Plasma Concentration of PL after Single i.v. Administration The plasma concentrations after a single i.v. administration of PL hydrochloride (4 mg/kg) are shown in Fig. 1. Since the plasma decay curve after dosing showed biexponential kinetics, as well as other reports,[6,9,16] it was analyzed by the 2-compartment open model. Pharmacokinetic parameters calculated by using the data are listed in Table II. The $t_{1/2,\beta}$ was 0.75 ± 0.08 h, indicating the rapid elimination of the drug in rats.

Plasma Concentration of PL after Rectal Administration of Suppositories The plasma PL concentration profiles and the kinetic parameters after dosing of Witepsol H-15 suppositories, as a lipid soluble carrier, are shown in Fig. 2 and Table III, respectively. The addition of lauric acid at a 1 : 1 molar ratio significantly increased the maximum plasma concentration (C_{max}), AUC and bioavailability compared with those without fatty acid ($p < 0.05$). A high dose (8 mg/kg) of PL especially enhanced the values to 2—3.5-fold. The increased availability at a high dose may be due to the relatively decreased first-pass effect. However, MRT was not altered in the presence or absence of lauric acid at a 1 : 1 molar ratio.

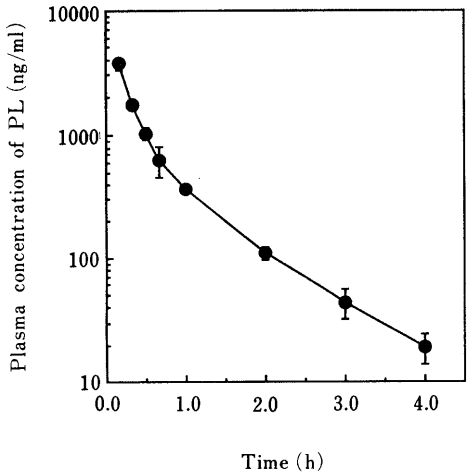

Fig. 1. Plasma Concentration of PL after a Single i.v. Administration of PL Hydrochloride

Each point represents the mean ± S.D. ($n = 4$). The i.v. dose was 4 mg/kg.

TABLE II. Pharmacokinetic Parameters of PL after i.v. Administration

Parameter	Estimate	Parameter	Estimate
A (ng/ml)	7404.3 ± 1676.0	V_{dss} (l/kg)	0.94 ± 0.16
α (/h)	5.19 ± 0.99	K_{10} (/h)	3.70 ± 0.67
B (ng/ml)	714.5 ± 77.7	K_{12} (/h)	1.11 ± 0.32
β (/h)	0.94 ± 0.11	K_{21} (/h)	1.31 ± 0.13
$t_{1/2,\beta}$ (h)	0.75 ± 0.08	CL_{tot} (l/h/kg)	1.84 ± 0.09
AUC (h·ng/ml)	1672.0 ± 73.2	$AUMC$ (h^2·ng/ml)	1141.3 ± 96.5
V_1 (l/kg)	0.52 ± 0.14	MRT (h)	0.68 ± 0.07
V_2 (l/kg)	0.42 ± 0.08		

Each value represents the mean ± S.D. ($n = 4$). The dose was 4 mg/kg.

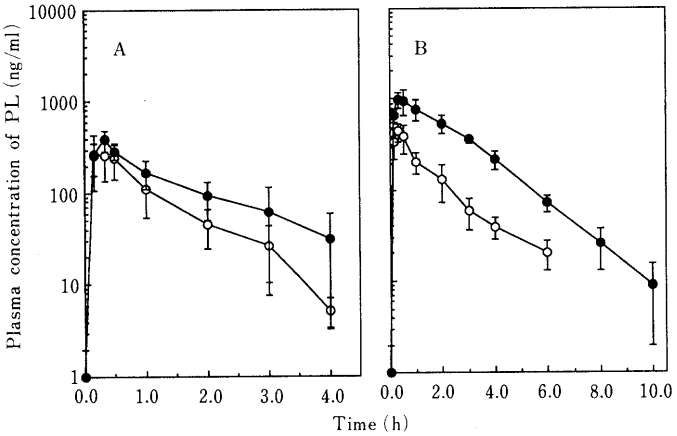

Fig. 2. Plasma Concentration of PL after a Single Rectal Administration of Witepsol H-15 Suppositories with or without Lauric Acid (LA) at 1 : 1 Molar Ratio

Each point represents the mean ± S.D. ($n = 6$—8). The dose was 4 mg/kg (A) and 8 mg/kg (B). —○—, PL only; —●—, PL : LA = 1 : 1.

TABLE III. Pharmacokinetic Parameters Following Rectal Administration of PL

No.	C_{max} (ng/ml)	AUC (ng·h/ml)	Bioavailability (%)	MRT (h)
1	191.1 ± 27.0	382.1 ± 129.4	22.9 ± 9.5	1.27 ± 0.13
2	393.8 ± 100.1[b]	623.6 ± 163.2[b]	37.3 ± 9.8[b]	1.49 ± 0.43
3	453.8 ± 100.0	757.5 + 123.5	22.7 + 4.3	2.25 ± 0.52
4	984.2 ± 177.7[a]	2641.3 ± 440.0[a]	79.0 ± 13.2[a]	2.34 ± 0.33
5	298.9 ± 55.9	288.7 ± 71.7	17.3 ± 4.3	0.97 ± 0.11
6	400.6 ± 90.5[b]	612.2 ± 113.6[a]	36.6 ± 6.8[a]	0.97 ± 0.58
7	189.1 ± 28.9[a]	331.8 ± 119.1	19.8 ± 7.1	2.37 ± 0.95[b]
8	130.5 ± 8.6[a]	302.7 ± 68.9	18.1 ± 5.3	1.95 ± 0.32[b]
9	283.9 ± 25.9	602.7 ± 125.9	36.0 ± 7.5	2.06 ± 0.50
10	528.1 ± 110.9[b]	934.0 ± 170.5[b]	55.9 ± 12.2[b]	2.26 ± 0.50
11	204.7 ± 29.3	377.2 ± 122.1	22.6 ± 7.3	1.44 ± 0.25
12	150.3 ± 41.0[a]	361.5 ± 106.7[b]	21.6 ± 6.4[b]	1.89 ± 0.20

Each value represents the mean ± S.D. ($n = 3$—8). *a*) $p < 0.01$ and *b*) $p < 0.05$, respectively, compared with the suppository without lauric acid.

To confirm whether the enhancement effect of lauric acid occurs in a water soluble carrier, macrogol suppositories with PL and lauric acid were prepared and applied to rats. In addition, to obtain the sustained release preparation of PL, the solid dispersion of PL in macrogol 6000 was prepared for the rectal absorption experiment. The plasma concentration profiles and parameters are shown in Fig. 3 and Table III, respectively. The plasma levels after administration of the 1 : 1 molar ratio suppositories were much higher than after the PL alone suppositories over all the postdose periods. The plasma levels after dosing the suppositories with a 1 : 2 or 1 : 3 molar ratio to lauric acid were low at the first time point measured compared with those after the 1 : 1 molar ratio and PL alone, but slightly higher levels were observed thereafter. The bioavailability after administration of the 1 : 1 molar ratio suppository was 2.1-fold compared with that after PL alone. These results indicate that PL at a 1 : 1 molar ratio to lauric acid is absorbed more rapidly than PL alone through the rectal mucosa, while PL at a higher lauric acid molar ratio is absorbed relatively slowly. The comparatively slower elimination of PL after dosing the suppositories with lauric acid, compared with that after i.v. administration, indicated that the PL suppositories would be useful for a prolonged effect of the drug.

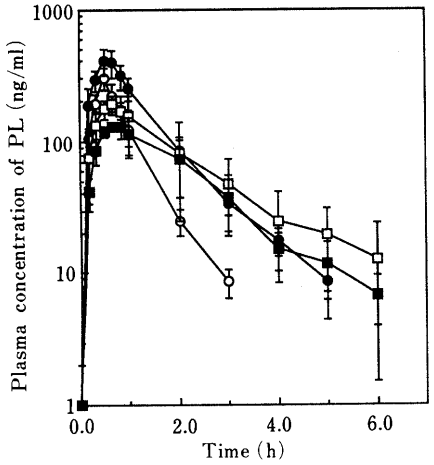

Fig. 3. Plasma Concentration of PL after a Single Rectal Administration of Macrogol Suppositories with or without Lauric Acid (LA) at Various Molar Ratios

Each point represents the mean ± S.D. ($n=4$). The dose was 4 mg/kg. —○—, PL only; —●—, PL : LA = 1 : 1; —□—, PL : LA = 1 : 2; —■—, PL : LA = 1 : 3.

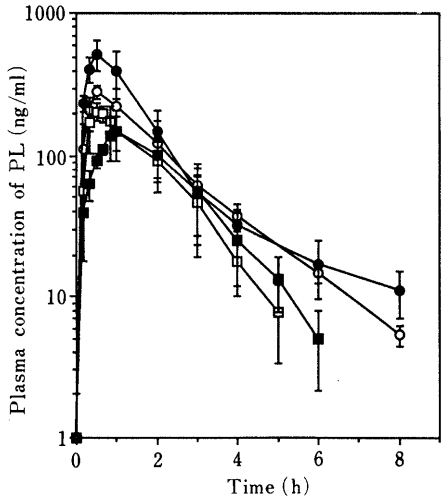

Fig. 4. Plasma Concentration of PL after a Single Rectal Administration of PL-Solid Dispersion (PL-S) Suppositories with or without Lauric Acid (LA) at Various Molar Ratios

Each point represents the mean ± S.D. ($n=3$—4). The dose was 4 mg/kg. —○—, PL-S only; —●—, PL-S : LA = 1 : 1; —□—, PL-S : LA = 1 : 2; —■—, PL-S : LA = 1 : 3.

The plasma concentrations after administration of the solid dispersion suppositories are depicted in Fig. 4. The kinetic parameters are shown in Table III. The plasma levels of PL after administration of the solid dispersion suppository were sustained much longer than those after dosing the macrogol suppository, having a higher *MRT* value (No. 9, 2.06 h) compared with No. 5 (0.97 h). The PL solid dispersion with lauric acid (molar ratio 1 : 1) also gave a higher plasma concentration of PL compared with the preparation without lauric acid, resulting in a higher C_{max} (1.9-fold) and bioavailability (1.7-fold). At increased molar ratios of lauric acid (*e.g.*, 1 : 2 and 1 : 3) the low but sustained plasma levels of PL were also obtained, as well as the macrogol suppositories (Fig. 3). These phenomena may be explained by the sustained absorption of PL in the presence of excess fatty acid. The high C_{max} and bioavailabilities after administration of the 1 : 1 molar ratio suppositories of both Witepsol and macrogol demonstrated that a considerable portion of PL, after forming a complex with lauric acid, would rapidly be absorbed through the rectal membrane, as suggested previously.[7]

Release of PL from Suppositories The release profiles of PL from macrogol suppositories are shown in Fig. 5A. The

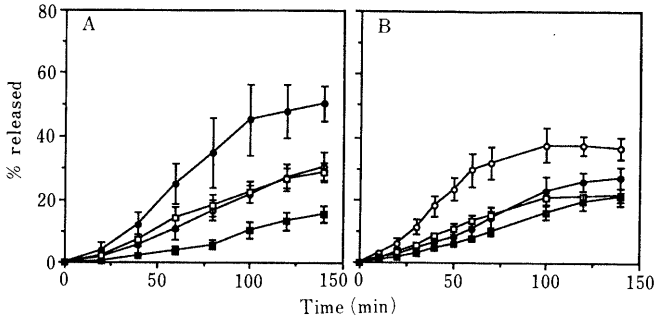

Fig. 5. Release Profiles of PL from Macrogol and PL-Solid Dispersion (PL-S) Suppositories with or without Lauric Acid (LA) at Various Molar Ratios

A, PL macrogol suppositories; B, PL-S suppositories. Each point represents the mean ± S.D. ($n=3$—6). A) —○—, PL only; —●—, PL : LA = 1 : 1; —□—, PL : LA = 1 : 2; —■—, PL : LA = 1 : 3. B) —○—, PL-S only; —●—, PL-S : LA = 1 : 1; —□—, PL-S : LA = 1 : 2; —■—, PL-S : LA = 1 : 3.

TABLE IV. Parameters of PL Release Test

No.	Release rate (nmol/min$^{1/2}$)	Lag time (min)
5	186.1 ± 28.8	29.5 ± 6.6
6	396.2 ± 107.0[a)]	28.0 ± 3.0
7	162.6 ± 31.7	23.0 ± 10.7
8	131.6 ± 31.6	48.7 ± 10.7
9	292.0 ± 93.8	14.5 ± 6.2
10	197.6 ± 24.0	30.2 ± 10.2
11	128.8 ± 11.9[a)]	14.6 ± 1.4
12	129.2 ± 23.5[a)]	27.7 ± 3.8

Each value represents the mean ± S.D. ($n=3$—6). *a)* $p<0.05$ compared with the suppository with or without lauric acid.

released amount of PL was significantly larger at a 1 : 1 molar ratio than that of PL alone, the 1 : 2 and 1 : 3 molar ratios to lauric acid. The higher molar ratio (1 : 3) of lauric acid extremely decreased the release rate compared with that at the 1 : 1 molar ratio, probably due to the retention of drug in excess fatty acid. The release rate and lag time calculated are shown in Table IV. The release profiles of PL from the solid dispersion suppositories are depicted in Fig. 5B. The release of PL from the PL dispersion suppository was much larger than that of PL dispersion-lauric acid suppositories. The release of PL from the dispersion-lauric acid suppositories was relatively smaller than that of corresponding macrogol suppositories except for the 1 : 3 preparation, as shown in Table IV. The release data of macrogol suppositories approximately agreed with the *in vivo* results (C_{max} and bioavailability) after dosing the preparations, but the data of PL dispersion suppositories did not.

Discussion

There has been renewed interest in the rectal administration of drugs as a possible way to partially bypass the liver in humans and rats.[6,17,18] de Boer *et al.* showed that the mean oral and rectal bioavailabilities of PL in rats are 3.1 and 101.1%, respectively.[6] This indicates that PL is a drug highly metabolized by the first-pass elimination. Thus, considerable work has been devoted to the improvement of PL bioavailability by rectal administration.[6,19,20] Our results showed that the mean rectal bioavailability of PL without lauric acid was increased by a factor of 6—7 compared with the mean oral systemic one (3.1%[6]). The addition of lauric acid at a 1 : 1 molar ratio to PL further increased the bioavailability by a factor of 12 (Table III). The enhanced bioavailability after the rectal administration would therefore be due to a substantial avoidance of the hepatic first-pass effect.

Kajii *et al.* showed that sodium caprylate can increase the permeability of rat small intestinal brush border membrane (BBM) through the perturbation of the membrane, promoting the absorption of water-soluble and poorly absorbed drugs.[21] However, their promotion mechanism may considerably be different from our mechanism, because they suggest that caprylate may at least act on the plasma membrane proteins of BBM. The complex with lauric acid, which was demonstrated in our previous paper,[8] appears to mainly penetrate a lipoidal pathway, transcellular pathway, within the rectum, because the complex has lipophilic characteristics[7,8] as well as PL.

Following oral administration of PL hemisuccinate, plasma PL levels are eight times higher than after an equivalent dose of PL hydrochloride.[22] This is due to the rapid absorption of the ester and the bypassing of glucuronide formation during absorption.[22] The complex formed, at least partially, in this study would also be rapidly absorbed through the rectal membrane. This is suggested from the result shown in Fig. 3.

The results obtained from the *in vivo* and *in vitro*[7] rectal absorption experiments indicate that the enhancement of PL absorption by lauric acid and medium chain fatty acids would partly but fairly be due to the complex formation mechanism. The complex may readily partition into the bulk lipid phase of rectal mucosa and many PL in the tissue may be prevented from binding to tissue components such as proteins and glycoproteins by the formation of a complex with fatty acids. Walter and Kurz suggested that drugs, including PL, are bound to proteins and glucosaminoglycans.[23] Although our results indicated that a 1 : 1 complex with lauric acid was absorbed more easily through the rectal membrane than PL alone (Figs. 2 and 3), the enhancement (1.6—3.5-fold) by lauric acid in rectal absorption was much smaller than that (15-fold)[8] in percutaneous absorption. This may be mainly explained by the partial dissociation of the complex in the rectal lumen, owing to the presence of aqueous fluid. In a previous paper, we have shown that the apparent partition coefficient of PL (0.2 mM) at the 1 : 1 molar ratio to lauric acid is higher than that of PL alone and that the value at a diluted concentration (0.02 mM) is extremely decreased.[7] This indicates that the complex dissociates in the rectal lumen when the concentration is diluted.

Since fatty acids including caprylate can lower the phase transition temperature of artificial phospholipid membrane[24] and disorder the synaptosomal plasma membrane,[25] these factors may be partly related to the enhancing effect of fatty acids on the rectal absorption of PL. However, the complex formation is likely to be an important mechanism for the enhancement.

The slow release of PL from the macrogol suppositories at increased lauric acid molar ratios (*e.g.*, 1 : 2 and 1 : 3) may be due to the ability of excess fatty acid to retain the drug in the base. This is suggested by the higher apparent partition coefficient of PL at the 1 : 3 molar ratio compared with that at the 1 : 1 molar ratio.[7]

There has been a great interest in the pharmaceutical field in using solid dispersions to improve the dissolution rate and the bioavailability of poorly water-soluble drugs.[26-28] The faster dissolution rate from the PL dispersion (No. 9, Table IV), when compared with that of macrogol suppository (No. 5), agreed well with the results of other studies.[26-28] The slow dissolution rate of the PL dispersion-fatty acid suppositories, observed in this study, may be ascribed to the perturbation of dispersion, such as the reaggregation of drug particles in the carriers and the formation of drug-rich surface layers by the fatty acid.[29-31] Since there was not good agreement between the release rates of PL from suppositories and the plasma drug concentrations after dosing, the absorption process of PL does not seem to be dissolution rate limited. This further strengthens our concept that the complex formation would be, at least partly, a mechanism for the enhancement effect of fatty acids.

In conclusion, PL at a 1 : 1 molar ratio to lauric acid was absorbed most rapidly through the rectal membrane and gave the highest bioavailability. Therefore, the present results evoked the possibility that a considerable portion of PL, by forming a 1 : 1 complex with lauric acid, would penetrate across the mucosal membrane, probably through a lipoidal pathway. The results obtained from the *in vivo* experiment agreed well those of the *in vitro* study.[7]

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Stability of Human Insulin in Solutions Containing Sodium Bisulfite[1)]

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The stabilities of human insulin (HI) in aqueous solutions were investigated in the pH range of 4.0—7.0 in the presence ($1.0—3.0 \times 10^{-3}$ M) and absence of sodium bisulfite (SBS) both in the dark and under scattered light (1000 lux) using high performance liquid chromatography. Increasing concentrations of SBS tended to degrade HI. In the presence of SBS, with an increase in the pH value, the stability of HI decreased in the pH range of 4.0—7.0. There was a partial difference in the stability of HI in the presence of SBS in the dark and that under scattered light.

HI was stabilized by glucose in the presence of SBS in the dark, and the stability of HI was revealed to depend on the concentration of free SBS. The reason for this phenomenon was postulated to be the formation of bisulfite–glucose adduct.

Keywords sodium bisulfite; human insulin; degradation; stabilization; bisulfite–glucose adduct

Sodium bisulfite (SBS) used as a stabilizer in injectable preparations is known to degrade various drugs including thiamine,[2,3)] fursultiamine,[3)] gabexate mesilate,[4)] nafamostat mesilate,[5)] urokinase[6)] and bovine insulin.[7,8)] Considering the pharmaceutical stability of insulin injection in an intravenous admixture (mixed infusion) containing SBS, however, there are no reports on detailed kinetic studies for the degradation reaction on bovine and porcine insulin with SBS. These insulins and human insulin have two disulfide inter-chain bonds linking the A and B chains and an intra-chain bond linking residues 6 and 11 on the A chain.[9)]

Recently, a human insulin (HI) having fewer adverse reactions (allergy *etc.*) than other insulins was developed. HI is becoming a major insulin preparation, and is often added to various infusion solutions. In view of the compatibility of the mixed infusion, the stability of HI injection in aqueous solution containing SBS was investigated by high-performance liquid chromatography (HPLC) in the pH range of 4.0—7.0 in the presence and absence of SBS both in the dark and under scattered light (1000 lux).

In addition, many infusion solutions contain glucose, which has an aldehyde group and forms bisulfite (BS)–glucose adduct[3,10)] in the presence of SBS. Therefore, the stabilization of HI in the presence of SBS by glucose was attempted. In order to clear the stabilization mechanism, the relationship between the stability of HI in the mixed solution of SBS and glucose and the free SBS concentration was studied. The free SBS concentrations were measured stoichiometrically by the complementary tristimulus colorimetry (CTS method)[3,11-13)] using pyridoxal phosphate (PLP).

Experimental

Materials HI injection (Humalin R U-40®, lot No. 3DF58B) was supplied by the Shionogi Co., Ltd. HI, SBS, glucose, PLP and the other agents were commercial products of special grade. Buffer solutions, *i.e.*, 0.05 M acetate buffer (pH 4.0 and 5.0), 0.05 M phosphate buffer (pH 6.0 and 7.0) were adjusted to an ionic strength (μ) 0.5 with sodium chloride.

Samples and Storage Conditions The stability of HI injection in aqueous solution (approximately 0.08 unit/ml, *ca.* 0.5 μM) was studied in the pH range of 4.0—7.0 in the presence ($1.0—3.0 \times 10^{-3}$ M) and absence of SBS in the dark and under scattered light (indoor 1000 lux) at 25 °C. Test solutions were stored in glass bottles of 500 ml in inner volume with each glass stopper.

To evaluate the influence of glucose on the stability of HI in the presence of SBS, glucose was added to the solution of HI injection (approximately 0.08 unit/ml) buffered at pH 6.0 and 7.0 in the presence of SBS (2.0×10^{-3} M).

Determination of HI HI was measured by HPLC. Equipment: a Hitachi 655-11 high-performance liquid chromatograph, a Hitachi 655A UV detector, and a Shimadzu C-R3A chromatopack were used. Measurement conditions: column, Zorbax TMS (4.6 mm i.d. × 250 mm); mobile phase, 0.1 M ammonium phosphate buffer (pH 2.0)–acetonitrile (74 : 26); flow rate, 1.0 ml/min; column temperature, 40 °C; and detection wavelength, 210 nm. As an internal standard, ethyl *p*-hydroxybenzoate was employed, and a 10 μl aliquot was injected into the HPLC.

Measurement of the Equilibrium Constant for the BS–Glucose Adduct 1) Measurement of Absorption Spectra: PLP and glucose have an aldehyde group, respectively, so form each 1 : 1 adduct of BS[3,10,11)] in the presence of SBS. The BS addition reaction with PLP and glucose is a competition reaction. PLP and BS–PLP adduct have an absorption maximum at 386 and 328 nm, respectively. Glucose, however, has only slight absorption in this ultraviolet (UV) region.

To evaluate the equilibrium constants of the BS–PLP adduct, various amounts of SBS ($0—3.6 \times 10^{-3}$ M) were added to the PLP solutions (9.0×10^{-4} M) buffered at pH 6.0 and 7.0 at 25 °C in 0.1 M phosphate buffer ($\mu = 0.5$). The UV spectra of these solutions were measured using a Hitachi model 200-20 spectrophotometer with an isothermal cell holder (25 °C) in an air-conditioned room at 25 ± 1 °C. Subsequently, various amounts of glucose (5—20%) were added to the mixed solutions of PLP (9.0×10^{-4} M) and SBS (1.0 or 2.0×10^{-4} M) buffered at pH 6.0 and 7.0. These UV spectra were measured similarly to calculate the equilibrium constants of the BS–glucose adduct based on the same constants of the BS–PLP adduct.

2) CTS Method: The three ranges for the CTS method were selected (u range 323—332 nm, v range 345—354 nm, w range 381—390 nm) from the obtained UV spectra. From the sum of 10 absorbance readings in each range, the color point and molar fraction were calculated by the CTS method[12,13)] based on previous papers.[3,11)] The main complementary tristimulas parameters and the equilibrium constants obtained in the present study were listed in Table I.

Results and Discussion

Determination of HI by HPLC Method for the determination of HI by HPLC have been reported by Tokunaga *et al.*[14)] This method was not suitable for the

TABLE I. The Equilibrium Constants of the BS–PLP and BS–Glucose Adduct at pH 6.0 and 7.0[a)]

pH	E_{PLP}[b)]	E_{BS-PLP}[b)]	K_{BS-PLP}[c)]	$K_{BS-glucose}$[d)]
6.0	1.035×10^5	1.105×10^5	25310 ± 288	2.01 ± 0.367[e)]
7.0	9.910×10^4	1.006×10^5	7579 ± 113	1.17 ± 0.193[f)]

a) At 25 °C; 9.0×10^{-4} M PLP in 0.1 M phosphate buffer ($\mu = 0.5$). *b*) *E* represents each overall absorptivity. *c*) 0—3.6×10^{-3} M SBS was used. *d*) 5—20% glucose was used. *e*) 1.0×10^{-4} M SBS was used. *f*) 2.0×10^{-4} M SBS was used.

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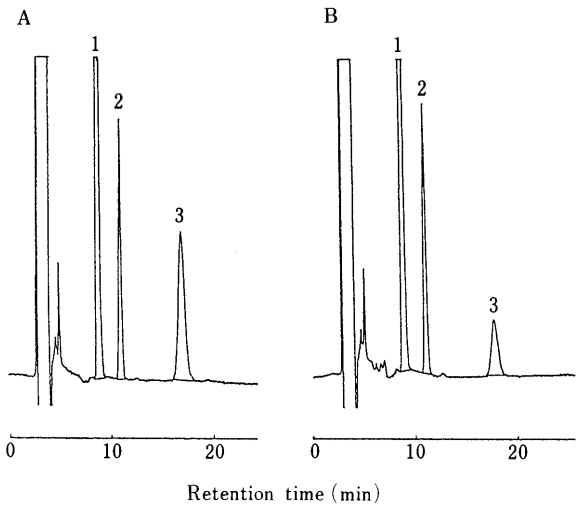

Fig. 1. HPLC Chromatograms of HI

(A) HI injection. (B) HI (approximately 0.08 unit/ml) in the presence of SBS (2×10^{-3} M) in 0.05 M phosphate buffer (pH 6.0) in the dark at 25 °C and $\mu = 0.5$ (6 h incubation). Peak 1, *m*-cresol; peak 2, internal standard (ethyl *p*-hydroxybenzoate); peak 3, HI.

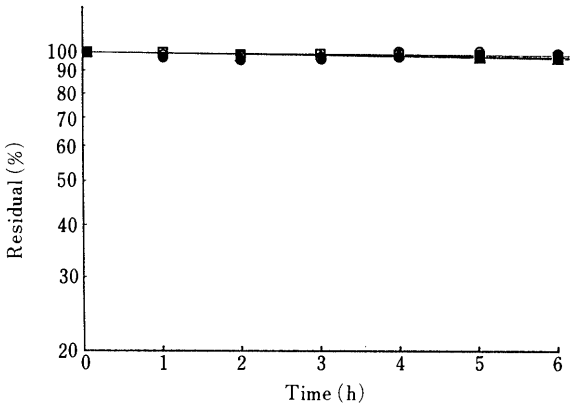

Fig. 2. Semilogarithmic Plots for the Degradation of HI in the Absence of SBS in the pH Range of 4.0—7.0 in the Dark at 25 °C and $\mu = 0.5$

Initial concentration of HI, approximately 0.08 unit/ml; ●, in 0.05 M acetate buffer (pH 4.0); ○, in 0.05 M acetate buffer (pH 5.0); □, in 0.05 M phosphate buffer (pH 6.0); △, in 0.05 M phosphate buffer (pH 7.0).

separation of HI regarding the HI solution containing SBS. The reason for this seemed to be attributed to the fact that the inter-chain disulfide bonds of insulin are cleaved by SBS.[7,8,15] Therefore, new conditions for the determination of HI were studied. The conditions described in Experimental allowed determination of HI in the presence of SBS (Fig. 1). Peak 1 corresponded to *m*-cresol which was contained in HI injection as a preservative. The degradation products of HI were eluted jointly in the first peak. A calibration curve was produced using 0.04—0.16 unit/ml solutions of HI based on the peak area obtained by the HPLC method. The calibration curve showed a linear relationship in the concentration range ($y = 583x - 0.9118$) $r = 0.9957$.

Stability of HI in the Presence and Absence of SBS in the Dark or under Scattered Light No significant degradation was observed in the time courses of the residual % of HI in the absence of SBS in the pH range of 4.0—7.0 and in the presence of SBS (2.0×10^{-3} M) at pH 4.0. An adsorption of insulin on the surface of the container was well known.[16-18] The residual ratios of HI in the pH range

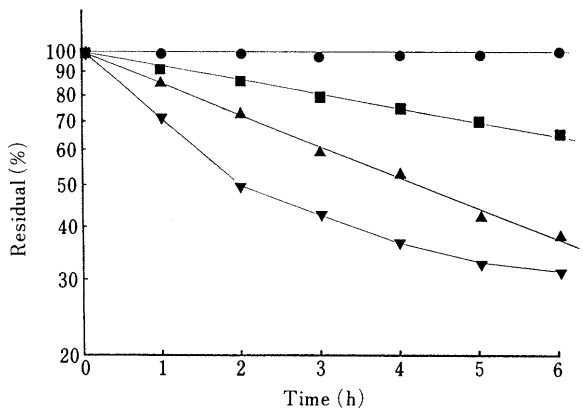

Fig. 3. Semilogarithmic Plots for the Degradation of HI in the Presence of SBS in Phosphate Buffer (pH 7.0) in the Dark at 25 °C and $\mu = 0.5$

Initial concentration of HI, approximately 0.08 unit/ml; ●, [SBS] = 0 M; ■, $[SBS]_{total} = 1 \times 10^{-3}$ M; ▲, $[SBS]_{total} = 2 \times 10^{-3}$ M; ▼, $[SBS]_{total} = 3 \times 10^{-3}$ M.

of 4.0—7.0 after 0 h storage were 87.6±1.94% in this experiment, and this extent was considered to be rather small.[18] Based on these results, HI concentration after 0 h storage was regarded as 100% residual in view of the kinetic analysis for the degradation of HI by SBS. No significant degradation was observed in the time courses of the residual % of HI in the absence of SBS in the pH range of 4.0—7.0 in the dark (Fig. 2). Therefore, the adsorption of HI was considered to be momentarily equilibrated.[18]

In contrast, significant degradation of HI was observed in the presence of SBS in the pH range of 5.0—7.0. The semilogarithmic plots for the time course of the degradation of HI in the presence of SBS (0—3.0×10^{-3} M) at pH 7.0 in the dark are shown in Fig. 3. The semilogarithmic plots for the degradation of HI in the presence of SBS (0—2.0×10^{-3} M) gave straight lines in the course of 6 h (Fig. 3). Nevertheless, the same plots for the degradation of HI in the coexistence of 3.0×10^{-3} M SBS did not give any straight lines in the course of 6 h (Fig. 3).

Sulfitolysis *i.e.* the scission of disulfides by BS or sulfite has frequently been used to cleave disulfide bonds in proteins. Referring to the scission of disulfide bond in a number of proteins by sulfite alone, the inter-chain disulfide bond generally reacts with SBS more easily than with the intra-chain bond.[8] It was reported that the two inter-chain bonds of insulin react with sulfite causing the insulin to split into its component A and B chains, and the 6—11 intra-chain bond on the A chain does not react with sulfite alone.[7,8]

It is well known that the scission of disulfides by sulfitolysis forms *S*-sulfonated derivative ($R\text{-}S\cdot S\text{-}R + SO_3^{2-} \rightleftarrows R\text{-}S^- + R\text{-}S\cdot SO_3^-$).[19,20] The reaction is reversible and will proceed under conditions where the concentration of $R\text{-}S^-$ is small.[8] Therefore, the degradation of disulfides by SBS was shown to obey a reversible biomolecular reaction.[21] However, it was also reported that the initial reaction of some disulfides by SBS below pH 9 was apparently a first-order reaction.[21] From these considerations, the initial degradation of HI by SBS (0—2.0×10^{-3} M) was practically regarded as an apparent first-order reaction.

On the other hand, the time courses of the residual % of HI in the presence of SBS under scattered light (Fig. 4)

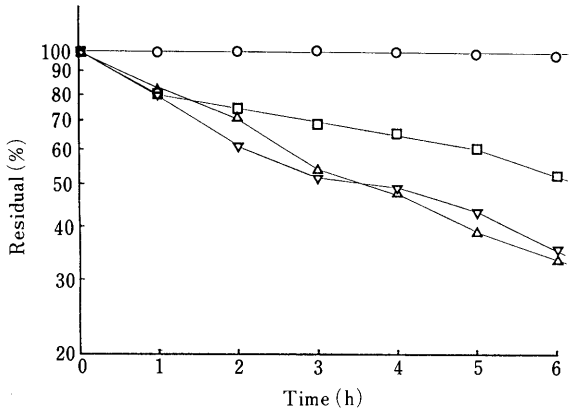

Fig. 4. Semilogarithmic Plots for the Degradation of HI in the Presence of SBS in 0.05 M Phosphate Buffer (pH 7.0) under Scattered Light at 25 °C and $\mu=0.5$

Initial concentration of HI, approximately 0.08 unit/ml; ○, $[SBS]=0$ M; □, $[SBS]_{total}=1\times10^{-3}$ M; △, $[SBS]_{total}=2\times10^{-3}$ M; ▽, $[SBS]_{total}=3\times10^{-3}$ M.

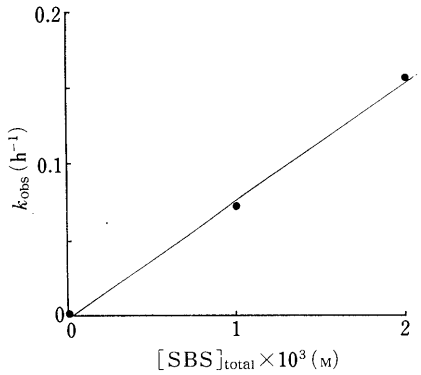

Fig. 5. Relationship between the Total SBS Concentration and Apparent First-Order Degradation Rate Constant for the Degradation of HI in the Presence of SBS in the Dark in 0.05 M Phosphate Buffer (pH 7.0, $\mu=0.5$) at 25 °C

Initial concentration; HI, approximately 0.08 unit/ml.

were complicated, especially at 3.0×10^{-3} M of SBS concentration under the influence of light. The existence of a free radical chain mechanism for the aerobic oxidation of BS to sulfate had been well documented.[22] It has also been reported that the oxidative radicals formed during autooxidation of BS were regulated by light.[6,23,24] For these reasons, the difference in the stabilities of HI in the presence of SBS under scattered light and in the dark seemed attributable to the light-induced chain oxidation of BS.

Effects of SBS Concentrations The apparent first-order degradation rate constants (k_{obs}) of HI at an initial concentration of approximately 0.08 unit/ml at various SBS concentrations was evaluated at 25 °C in the pH range of 5.0—7.0 in the dark. Typical plots for SBS concentration *vs.* apparent first-order degradation rate constants of HI at pH 7.0 yielded a straight line as shown in Fig. 5. The second-order rate constant in the degradation of HI by SBS (k_{SBS}) obtained from the slope of the line was 79.0 M^{-1} h^{-1}. The intercept of this line on the ordinate, which corresponded to the rate constant of HI in the absence of SBS (k_0) was regarded as 0, therefore the rate constant of HI in the presence of SBS (k_{obs}) can be represented as in Eq. 1 in this kinetic study.

$$k_{obs}=k_{SBS}\cdot[SBS]_{total} \quad (1)$$

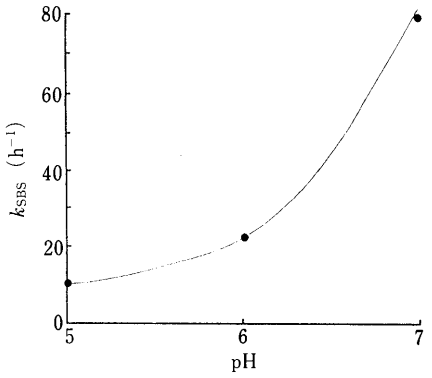

Fig. 6. Second-Order Rate Constant for the Degradation of HI by SBS in the Dark at 25 °C and $\mu=0.5$ as a Function of pH

Initial concentration of HI, 0.08 unit/ml.

pH-Profiles At various pHs, the degradation of HI at approximately 0.08 unit/ml in 0—2.0×10^{-3} M SBS concentration was studied at 25 °C in the dark. The second-order rate constants in the degradation of HI by SBS (k_{SBS}) at various pHs obtained from the slope of the lines by the same treatment as for pH 7.0 (Fig. 5). The pH-profiles of the second-order rate constants in the degradation of HI by SBS (k_{SBS}) are shown in Fig. 6. Figure 6 shows that the lower the pH value was, the lower the rate constants of HI degradation by SBS became in the pH range of 5.0—7.0 in the dark.

The dissociation constants of SBS were reported as $K_1=1.72\times10^{-2}$ (*ca.* $pK_1=1.8$) and $K_2=6.24\times10^{-8}$ (*ca.* $pK_2=7.2$),[25] therefore the equilibrium between BS and sulfite was considered to be formed in the pH range of 5.0—7.0.

Though the three dimensional structure of HI and the charge distribution in the vicinity of the disulfide bonds of HI were considered to be changed with the change of pH, it was assumed for practical kinetic analysis that these factors change only slightly in a relatively small pH range of 5.0—7.0.

From these considerations, the stability of HI in the presence of SBS was regarded to depend on the equilibrium between BS (HSO_3^-) and sulfite (SO_3^{2-}). Therefore k_{SBS} must be divided to $k_{HSO_3^-}$ and $k_{SO_3^{2-}}$, and Eq. 2 can be obtained from the kinetic equation and the dissociation equilibrium SBS.

$$k_{SBS}=\frac{k_{HSO_3^-}\cdot K_1\cdot[H^+]+k_{SO_3^{2-}}\cdot K_1\cdot K_2}{[H^+]^2+K_1\cdot[H^+]+K_1\cdot K_2} \quad (2)$$

where k_{HSO^3-} is the apparent second-order rate constant for the degradation of HI by BS, $k_{SO_3^{2-}}$ is the apparent second-order rate constant for the degradation of HI by sulfite. These rate constants were estimated by the least-squares method (Simplex method[26]), and $k_{HSO_3^-}=9.50$ M^{-1} h^{-1} and $k_{SO_3^{2-}}=196.6$ M^{-1} h^{-1} were obtained. These results show that the effect of sulfite on the degradation of HI was 20 times greater than that of BS. The degradation of HI in the presence of SBS in the pH range of 5.0—7.0 can be represented as in Eq. 3.

$$k_{obs}=[SBS]\cdot\frac{k_{HSO_3^-}\cdot K_1\cdot[H^+]+k_{SO_3^{2-}}\cdot K_1\cdot K_2}{[H^+]^2+K_1\cdot[H^+]+K_1\cdot K_2} \quad (3)$$

Employing Eq. 3, the residual % of HI stored for a

constant time (t) at a condition was estimated by Eq. 4. Stability estimation is useful for evaluating the compatibility of an intravenous admixture (mixed infusion) of HI containing SBS as an additive.

$$\text{residual } \% = 10^{-(k_{obs}t/2.303)} \times 100 \quad (4)$$

Effects of Glucose on the Degradation of HI We studied the stabilization effects of glucose on HI in the presence of SBS in the pH range of 6.0—7.0, in which HI was relatively unstable. The semilogarithmic plots for the degradation of HI (initial concentration approximately 0.08 unit/ml) in the coexistence of glucose (0—20%) and SBS (2.0×10^{-3} M) at pH 7.0 in the dark gave straight lines (Fig. 7). The residual of HI after storage for 2 h in the coexistence of SBS (2.0×10^{-3} M) and glucose (20% (w/v), *ca.* 1.1 M) at pH 7.0 and 6.0 were 90.9 and 98.2%, respectively. From these findings, the mixed infusion of HI containing SBS with glucose (about 20%) was considered to be rather stable even in the pH range 6.0—7.0.

We subsequently studied the stabilization mechanism of glucose for HI in the presence of SBS in the pH range of 6.0—7.0. The degradation rate constants for HI incubated at pH 7.0 and 25°C in the coexistence of SBS (2.0×10^{-3} M) and glucose (0—20%) in the dark were plotted against the concentration of glucose (Fig. 8). As shown in Fig. 8, HI was stabilized by glucose concentration dependently. However, a linearity was not observed in these plots.

We previously revealed that stability for thiamine, fursultiamine[3] and urokinase[6] in the coexistence of SBS and glucose depended on the concentration of free SBS. The degradation rates of HI were assumed to depend mainly on the concentrations of free SBS.

The equilibrium of the BS–glucose addition reaction is represented as Eq. 5, and the equilibrium constant of the BS–glucose adduct ($K_{BS-glucose}$) is represented as Eq. 6. The equilibrium of the chemical species are represented by Eqs. 7 and 8. The concentration of the BS–glucose adduct was much smaller than the concentration of free glucose in this experimental condition, therefore the total glucose concentration can be represented approximately as in Eq. 8′. From Eqs. 6—8′, the concentration of free SBS can be expressed as Eq. 9.

$$[SBS]_{free} + [glucose]_{free} \underset{}{\overset{K_{BS-glucose}}{\rightleftarrows}} [BS\text{–glucose}] \quad (5)$$

$$K_{BS-glucose} = \frac{[BS\text{–glucose}]}{[SBS]_{free} \cdot [glucose]_{free}} \quad (6)$$

$$[SBS]_{total} = [SBS]_{free} + [BS\text{–glucose}] \quad (7)$$

$$[glucose]_{total} = [glucose]_{free} + [BS\text{–glucose}] \quad (8)$$

$$[glucose]_{total} \fallingdotseq [glucose]_{free} \quad (8')$$

$$[SBS]_{free} = \frac{[SBS]_{total}}{1 + K_{BS-glucose} \cdot [glucose]_{total}} \quad (9)$$

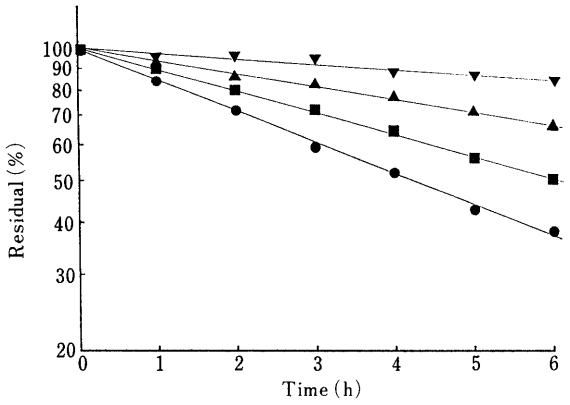

Fig. 7. Semilogarithmic Plots for the Degradation of HI in the Coexistence of Glucose and SBS in Phosphate Buffer (pH 7.0) in the Dark at 25 °C and $\mu = 0.5$

Initial concentration of HI, approximately 0.08 unit/ml; $[SBS]_{total} = 2 \times 10^{-3}$ M; ●, glucose = 0%; ■, glucose = 5%; ▲, glucose = 10%; ▼, glucose = 20%.

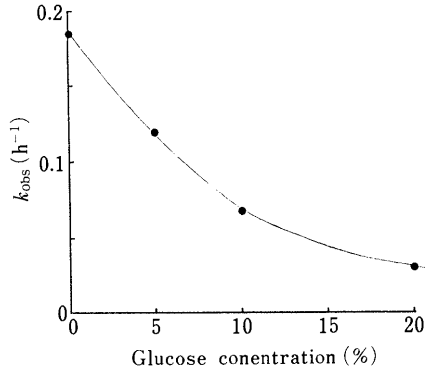

Fig. 8. Relationship between the Glucose Concentration and the Apparent First-Order Degradation Rate Constant of HI in the Presence of SBS in the Dark in 0.05 M Phosphate Buffer of pH 7.0 with $\mu = 0.5$ at 25 °C

Initial concentration; HI, approximately 0.08 unit/ml; SBS, 2×10^{-3} M.

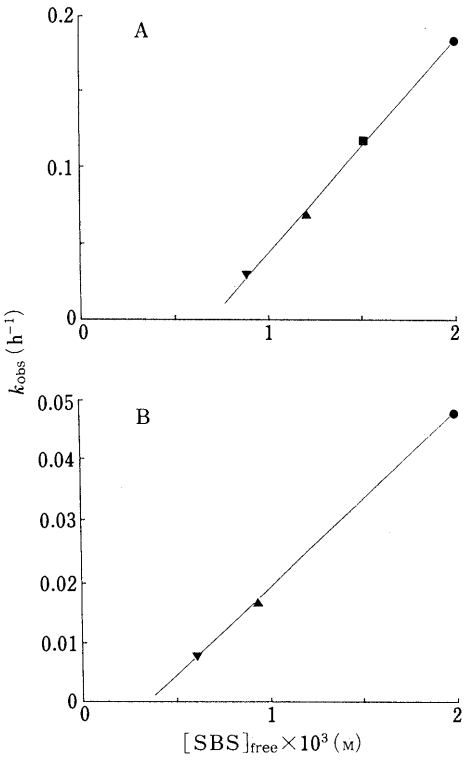

Fig. 9. Relationship between the Free SBS Concentration and Apparent First-Order Degradation Rate Constant of HI in the Dark in the Coexistence of Glucose and SBS in 0.05 M Phosphate Buffer (pH 7.0 and 6.0, $\mu = 0.5$) at 25 °C

(A) pH 7.0, (B) pH 6.0. Initial concentration; HI, approximately 0.08 unit/ml; $[SBS]_{total} = 2 \times 10^{-3}$ M; ●, glucose = 0%; ■, glucose = 5%; ▲, glucose = 10%; ▼, glucose = 20%.

where $K_{BS-glucose}$ means the equilibrium constant of the BS–glucose adduct (BS–glucose), and [ ] expresses the molar concentration of each chemical species. The measurement of the equilibrium constants for the BS–glucose adduct at pH 6.0 and 7.0 was described in Experimental.

The relationship between the degradation rate constants of HI at pH 7.0 in the coexistence of SBS (2.0×10^{-3} M) and glucose (0—20%) in the dark and the concentrations of free SBS is shown in Fig. 9A, and the plots gave a straight line. Similarly, the plots between the degradation rate constants of HI at pH 6.0 and the concentrations of free SBS gave a straight line (Fig. 9B). From these results, the stability of HI in the solution containing SBS and glucose was inferred to depend largely on the concentration of free SBS.

Another stabilization mechanism for HI was also suggested from the fact that each regression line in Fig. 9 did not pass through each origin. Brange *et al.* reported that the physical stability of insulin was slightly increased by the addition of glucose.[27] Nevertheless, this stabilization mechanism was not discussed in the report. A study by Lougheed *et al.* showed that the alcohols such as glycerol (30—50%) and isopropanol (10—50%) are moderately effective stabilizers for insulin.[28] The mechanism for this phenomenon had been postulated to be the reduction in the polarity of the environment of the insulin molecule.[28] Moreover, glucose also slightly reduces the polarity of the aqueous solution.[29] For these reasons, it was suggested that the reduction of the polarity by the addition of glucose also contributed to the stabilization of HI in the presence of SBS.

Considering the practical stability estimation, the concentration of free SBS in the coexistence of glucose could be estimated by Eq. 9 as previously described. The degradation rate constant of HI could be estimated practically from the concentration of free SBS and regression lines at pH 7.0 and pH 6.0 shown in Fig. 9A and 9B, respectively. Therefore, the residual % of HI in a mixed infusion stored for a chosen constant time (t) at a constant glucose concentration in the coexistence of SBS (about 2.0×10^{-3} M) at pH 6.0 or 7.0 can be estimated by Eq. 4.

From these kinetic studies, it may be possible to evaluate the compatibility of a mixed infusion of HI containing SBS in the absence or presence of glucose, and to find the optimal conditions.

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References and Notes

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Physicochemical Properties of Nitrofurantoin Anhydrate and Monohydrate and Their Dissolution

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The anhydrate and monohydrate of nitrofurantoin were characterized by X-ray diffraction analysis, infrared (IR) spectroscopy, thermal analysis and scanning electron microscopy. The X-ray diffraction patterns and the IR spectra of the anhydrate and monohydrate were significantly different, respectively. The differential thermal analysis (DTA) curve of the anhydrate showed endothermic and exothermic peaks at 272 and 276 °C, respectively, with a loss of weight due to decomposition on the thermogravimetry curve. The DTA curve of the monohydrate showed an endothermic peak at 120—128 °C with a loss of weight due to the dehydration of 1 mol/mol of crystal water, and endothermic and exothermic peaks at 273 and 276 °C, respectively, accompanying decomposition. The initial dissolution rate of anhydrate and monohydrate in JP XI, 2nd fluid (pH 6.8) at 37 °C were measured to estimate for solubility by using a rotating disk method. The solubilities of the anhydrate and monohydrate were estimated to be 47.8 and 27.4 mg/100 ml, respectively.

Keywords nitrofurantoin; anhydrate; monohydrate; X-ray powder diffractometry; solubility; dissolution rate; thermal behavior; pseudopolymorphism

Introduction

Nitrofurantoin[1] is widely used as an urinary tract antibacterial drug, however, it has bioavailability problems. Formulation factors of the drug preparation, mainly particle size,[2] affect the dissolution rate, bioavailability in humans and incidence of side-effects.[3] The USP XXII monograph for nitrofurantoin tablets requires not less than 25% of the labeled amount of drug to be dissolved for 60 min in a pH 7.2 phosphate buffer. Gouda *et al.*[4] and Ebian *et al.*[5,6] reported that the dissolution rate and bioavailability of nitrofurantoin commercial tablets in humans decreased after 1—8 weeks of storage at different relative humidities and higher temperatures. They concluded that the dissolution rate decreased by the physicochemical change involving an agglomeration of the particles. Marshall and York[7] reported three modifications of nitrofurantoin (two anhydrate and a monohydrate) which were recrystallized from formic acid or aqueous solutions of formic acid, and discussed the relation between the particle shape and the properties of recrystallization solvents. Previously, we reported briefly the characterization of anhydrate and monohydrate forms of the drug.[8] In this paper, we investigated the physicochemical properties of the anhydrate and monohydrate nitrofurantoin, and their dissolution behaviors were investigated by using a dispersed amount method and a rotating disk method.

Experimental

Materials A bulk nitrofurantoin powder (lot 11085) was obtained from Fukujyu Pharmaceutical Co., Ltd., Japan. The anhydrate was obtained by the recrystallization method using any of the hot saturated solutions of organic solvents described below: methanol, ethanol, isopropanol, *n*-butanol, isobutanol, *n*-amylalcohol, ethyl acetate, chloroform, carbon tetrachloride, methylene chloride, acetone, methyl ethyl ketone, benzene, *o*-xylene, cyclohexanone, dimethyl formamide, trichloroethane, *n*-hexane, *n*-heptane, dioxane, acetonitrile, propylene glycol, ethyl ether and *n*-hexyl alcohol. The saturated solutions were allowed to stand at room temperature, and then the separated crystals were filtered and dried *in vacuo* for 2 h at room temperature. The monohydrate was also obtained by the recrystallization method; the hot saturated aqueous solution of the drug was allowed to stand at room temperature and then the separated crystals were filtrated and dried *in vacuo* for 2 h at room temperature.

Micromeritic Characterization The true density of the crystals was determined by using an air comparison pycnometer (model 930; Beckman-Toshiba Co.). The specific surface area (S_w) was measured with a gas adsorption instrument (Flow sorb, model 2300, Shimadzu Co.) using the Brunauer–Emmett–Teller (BET) gas adsorption method. The micromeritic parameters are summarized in Table I.

X-Ray Powder Diffraction Analysis X-Ray powder diffraction profiles were taken at room temperature with an X-ray diffractometer (XD-3A, Shimadzu Co.). The operating conditions were as follows: target, Cu; filter, Ni; voltage 20 kV, current, 5 mA; receiving slit, 0.1 mm; time constant, 1 s; counting range, 60 kcpm; scanning speed 1° 2θ/min.

Infrared (IR) Spectroscopy IR spectra were taken by the Nujol mull method on an IR spectrophotometer (type 270-30, Hitachi Co.).

Elemental Analyses Elemental analyses of the samples were performed for atoms C, H and N with a CHN corder (model MT-2, Yanaco Co.).

Nuclear Magnetic Resonance (NMR) Spectroscopy $(CD_3)_2CO$ was used as a solvent with *ca.* 3% concentration of the sample solution. The proton NMR spectra were recorded at 200 MHz on a NMR instrument (model XL-200; Varian).

Thermal Analysis Differential thermal analysis (DTA) and thermogravimetry (TG) were performed with type DTG-30 and DTA-TG instruments (Shimadzu Co.). Differential scanning calorimetry (DSC) was carried out with a type 3100 instrument (Mac Science Co.). The operating conditions in an open-pan system were as follows: sample weight, 5 mg; heating rate, 10 °C/min; N_2 gas flow rate, 50 ml/min.

Scanning Electron Microscopy Scanning electron photomicrographs of samples were taken with a scanning electron microscope (model JSM-T20, Jeol Co.) at a magnification of × 1500.

Dissolution Study (1) The Dispersed Amount Method: The dissolution profiles of the anhydrate and monohydrate were investigated in JP XI, 2nd fluid (pH 6.8). An excess amount (500 mg) of sample was introduced into 300 ml of dissolution medium in a 1000-ml round-bottomed flask with a plastic cover. The flask was fixed on the sample holder in a thermostatically regulated water bath maintained at 37±0.5 °C, and stirred by a paddle at 200 rpm. Aliquots (5 ml) of the solution were withdrawn at appropriate time intervals with a syringe through a 0.8-μm membrane filter and suitably diluted with the dissolution medium. The concentration of the drug was measured spectrophotomerically (UV 160A, Shimadzu Co.) at 370 nm. The resultant loss in volume was compensated by adding the dissolution medium maintained at the same temperature.

TABLE I. Micromeritic Parameters of Anhydrate and Monohydrate

Sample	Density ± S.D.[a] (g/cm^3)	S_w[b] (m^2/g)	D[c] (μm)
Anhydrate	1.60±0.01	1.43	2.64
Monohydrate	1.53±0.01	1.11	3.54

a) Standard deviation (n=4). *b*) Specific surface area. *c*) Surface area diameter.

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(2) Rotating Disk Method: The pellets for the rotating disk method were prepared by the following method: A 300-mg sample of powder was compressed in a cylindrical die of 1.5-cm diameter at 140 kg/cm^2 using an accurate compression/tension testing machine (Autograph model IS-5000, Shimadzu Co.) and then the compressed pellet was kept for 5 min in the die at that compression pressure. The ejected pellet was fixed to a dissolution holder with bee wax so that only one side of the pellet surface was exposed. The pellet fixed to the holder was set up on the dissolution instrument. The initial dissolution profiles of the anhydrate and monohydrate were investigated in JP XI, 2nd fluid (pH 6.8) at 37±0.5 °C. The sample holder was rotated at 630±5 rpm in 100 ml of dissolution medium in a 1000-ml round bottomed flask with a plastic cover. The solution was introduced into a quartz flow-through cell with a peristaltic pump and the concentration was determined spectrophotometrically at 370 nm.

Results and Discussion

X-Ray Diffraction Analysis of Anhydrate and Monohydrate Figure 1 shows the X-ray diffraction profiles of the anhydrate and monohydrate. The diffraction angles and the characteristic diffraction intensities are also summarized in Table II. The anhydrate showed main diffraction peaks at 14.4 and 28.8°, while the monohydrate at 10.1, 12.3, 13.9, 21.5 and 27.2°, indicating that both modifications are significantly different in diffraction patterns. The results of the X-ray diffraction pattern suggested that the monohydrate had a different crystalline structure from that of the anhydrate.

The results of the elemental analysis of the anhydrate and monohydrate are summarized in Table III. The observed values were in good agreement with the calculated values for any of the anhydrate and monohydrate. Based on the results that the proton NMR spectra of both modifications were identical to the data shown in a reference,[1] it was confirmed that no impurities were contained in the anhydrate and monohydrate crystals.

IR Spectra of Anhydrate and Monohydrate Figure 2 shows the IR spectra of the anhydrate and monohydrate. The IR spectrum of the drug was explained by Cadwallader and Jun[1] as follows: The absorption band at 3280 cm^{-1} was attributable to the NH group, the band at 1786—1740 cm^{-1} was attributable to the C=O group in the hydantoin ring, and the band at 1515—1342 cm^{-1} was attributable to the α nitrofuran ring. The IR spectrum of the anhydrate showed the following absorption band: The band at 3325 cm^{-1} was attributable to the NH group, the bands at 1828, 1803 and 1750 cm^{-1} were attributable to the C=O group in the hydantoin ring, and the bands at 1580 and 1540 cm^{-1} were attributable to the α-nitrofuran

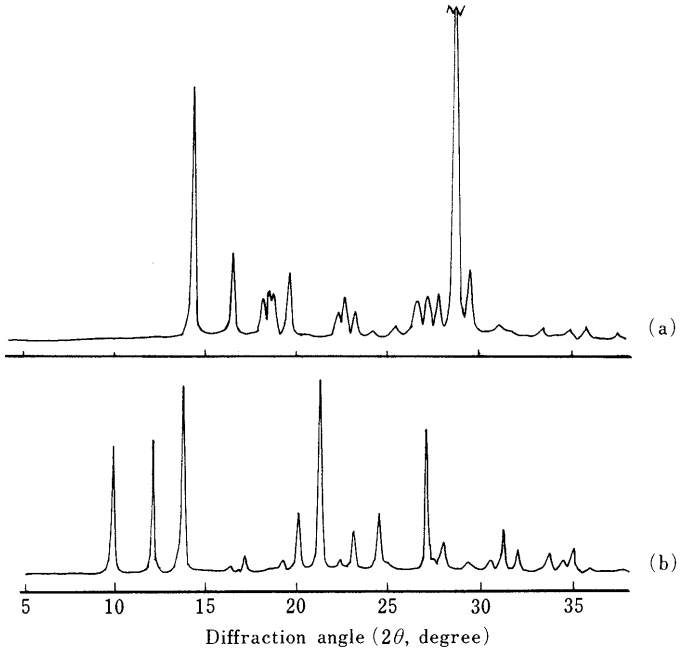

Fig. 1. X-Ray Powder Diffraction Profiles of Anhydrate and Monohydrate
(a) Anhydrate, (b) monohydrate.

TABLE II. X-Ray Powder Diffraction Angles and Relative Diffraction Intensities of Anhydrate and Monohydrate

Anhydrate			Monohydrate		
2θ (°)	d (Å)	I/I_0	2θ (°)	d (Å)	I/I_0
14.4	6.15	0.45	10.1	8.76	0.66
16.5	5.37	0.15	12.3	7.21	0.68
18.2	4.87	0.07	13.9	6.37	1.00
18.5	4.80	0.09	17.3	5.14	0.09
18.8	4.72	0.09	19.6	4.56	0.07
19.6	4.52	0.12	20.3	4.38	0.29
22.3	3.99	0.05	21.5	4.15	0.97
22.7	3.92	0.08	22.6	3.93	0.06
23.2	3.83	0.05	23.3	3.82	0.20
25.5	3.49	0.03	24.6	3.62	0.29
26.6	3.35	0.07	27.2	3.28	0.71
27.2	3.28	0.08	28.0	3.19	0.13
27.8	3.21	0.08	29.6	3.02	0.07
28.8	3.10	1.00	30.7	2.91	0.08
29.6	3.02	0.13	31.3	2.86	0.22
31.1	2.88	0.03	32.1	2.79	0.10
33.5	2.68	0.02	33.8	2.65	0.09
35.0	2.56	0.02	34.5	2.60	0.06
35.5	2.51	0.03	35.1	2.56	0.10

TABLE III. Elemental Analysis of Anhydrate and Monohydrate

Sample	Formula	Calcd (%)			Found (%)		
		C	H	N	C	H	N
Anhydrate	$C_8H_6N_4O_5$ (M.W.=238.16)	40.34	2.54	23.53	40.14	2.41	23.44
Monohydrate	$C_8H_6N_4O_5 \cdot H_2O$ (M.W.=256.16)	37.48	3.12	21.86	37.63	2.94	21.65

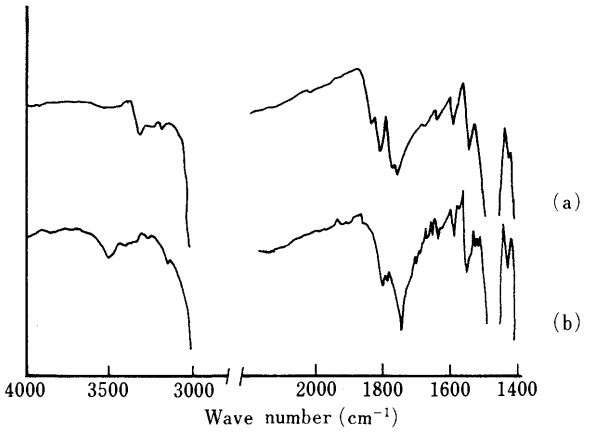

Fig. 2. IR Spectra of Anhydrate and Monohydrate
(a) Anhydrate, (b) monohydrate.

ring. The monohydrate exhibited an absorption peak at 3600—3450 cm^{-1} attributable to the hydroxyl group. The band at 3170 cm^{-1} was attributable to the NH group, the bands at 1798 and 1743 cm^{-1} were attributable to the C=O group in the hydantoin ring, and the bands at 1580 and 1540 cm^{-1} were attributable to the α-nitrofuran ring. The absorption pattern at 1800—1600 cm^{-1} of anhydrate, which was attributable to the C=O group in the hydantoin ring, seemed to be different from that of the monohydrate because the bands within this wavelength range were affected by an intermolecular action between the NH group of the hydantoin ring and a water molecule in a crystal lattice.

Thermal Behaviors of Anhydrate and Monohydrate Figure 3 shows the DTA and TG curves of the anhydrate and monohydrate. The DTA curve of the anhydrate showed an endothermic peak at 272 °C and the subsequent exothermic peak at 276 °C with a loss of weight on the TG curve. The endo- and exothermic peaks were due to decomposition after melting. The DTA curve of the monohydrate showed an endothermic peak at 120—128 °C with a 7.05% loss of weight on the TG curve, a second endothermic peak at 273 °C, and then the subsequent exothermic peak at 276 °C with a loss of weight on the TG curve. These facts suggested that the first endothermic peak was due to dehydration of 1 mol/mol of crystal water (Calcd 7.03%), and the second endo- and exothermic peaks were due to decomposition after melting. The heat of dehydration of the monohydrate was measured to be 38.0±0.9 kJ/mol ($n=3$) from the DSC curve. The X-ray diffraction pattern obtained for the monohydrate maintained at 150 °C for 10 min was identical to that of the anhydrate. This result suggests that the monohydrate transformed to the anhydrate by dehydration.

Morphological Characterization Figure 4 shows the scanning electron photomicrographs of the anhydrate and monohydrate. The monohydrate formed long needle-like crystals with a smooth surface, while the anhydrate existed in particles of 20—30 μm in diameter with a jagged surface.

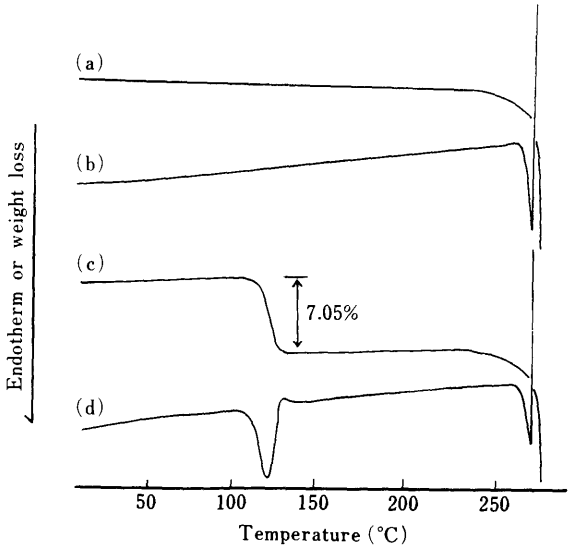

Fig. 3. DTA and TG Thermograms of Anhydrate and Monohydrate

(a) TG of anhydrate, (b) DTA of anhydrate, (c) TG of monohydrate, (d) DTA of monohydrate.

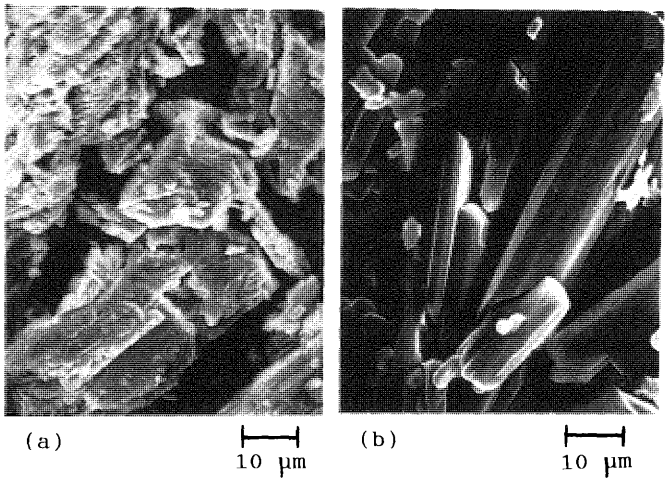

Fig. 4. Scanning Electron Photomicrographs of Nitrofurantoin Anhydrate and Monohydrate (×1500)

(a) Anhydrate, (b) monohydrate.

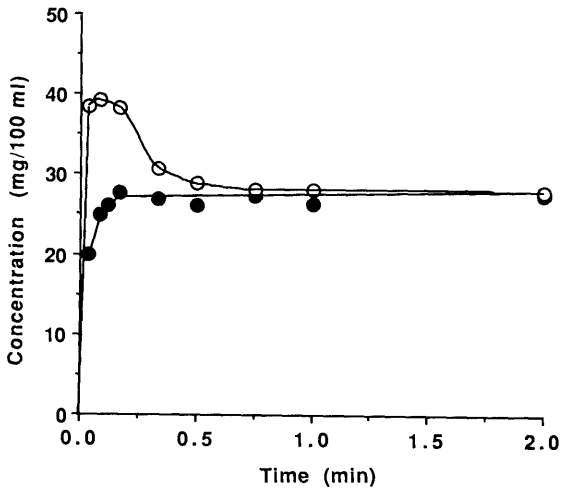

Fig. 5. Dissolution Profiles of Anhydrate and Monohydrate at pH 6.8 at 37 °C

○, anhydrate; ●, monohydrate.

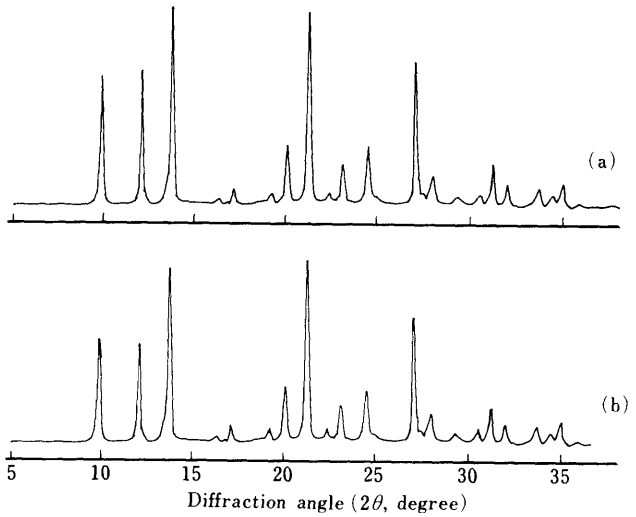

Fig. 6. Change of X-Ray Powder Diffraction Profiles of Anhydrate after Dissolution Test

(a) Intact monohydrate, (b) the precipitate.

TABLE IV. Solubility of Anhydrate and Monohydrate

Sample	$J^{a)}$ (mg/ml·min) ±S.D.[d)]	$C_{s1}{}^{b)}$ (mg/100 ml) ±S.D.[d)]	$C_{s2}{}^{c)}$ (mg/100 ml) ±S.D.[d)]
Anhydrate	$2.19\times10^{-3}\pm0.15\times10^{-3}$	47.8±3.3	39.3
Monohydrate	$1.26\times10^{-3}\pm0.01\times10^{-3}$	—	27.4±0.5

a) Initial dissolution rate. *b*) Solubility obtained by initial dissolution rate constant (Fig. 6). *c*) Solubility obtained by amount dispersion method. *d*) Standard deviation ($n=3$).

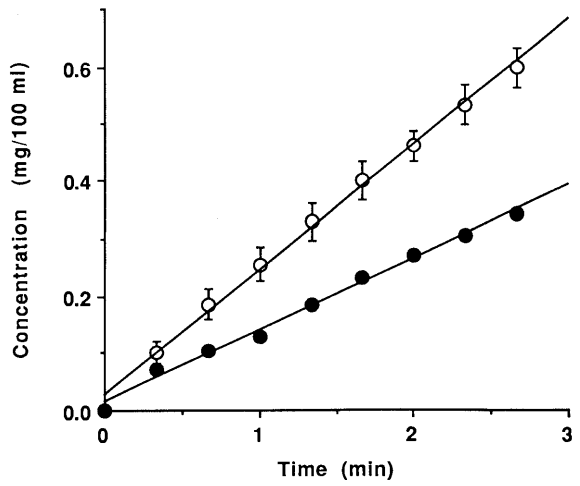

Fig. 7. Initial Dissolution Profiles of Anhydrate and Monohydrate at pH 6.8 at 37 °C by Using Rotating Disk Method (630 rpm)

○, anhydrate; ●, monohydrate.

The polycrystals were formed from many single crystals of less than 4—10 μm in diameter.

Dissolution Behaviors in JP XI, 2nd Fluid at 37 °C of the Anhydrate and Monohydrate Figure 5 shows the dissolution profiles of the anhydrate and monohydrate by the dispersed amount method. The anhydrate showed characteristic convex dissolution curves with a maximal solubility, whereas the monohydrate showed only a normal dissolution curve. The dissolution curve of the anhydrate obtained by the dispersed amount method were similar to those of theophylline involving a crystallization process together with a phase change from the anhydrate to monohydrate.[9)] After the dissolution experiment for the anhydrate, the X-ray powder diffraction patterns of crystallized precipitate coincided with those of the intact monohydrate as shown in Fig. 6. This result suggests that the dissolution behavior of the anhydrate involved a crystallization process together with a phase change from anhydrate to monohydrate. The solubilities of the monohydrate and the maximum concentration (apparent solubility) of the anhydrate are summarized in Table IV in which the apparent solubility was not the true value because the transformation rate of the anhydrate to the monohydrate was very high.

Figure 7 shows the initial dissolution curves of the anhydrate and monohydrate by the rotating disk method. The dissolution process of the anhydrate and monohydrate followed the Noyes-Whitney-Nernst equation. Kanke and Sekiguchi[10)] derived Eq. 1 from Noyes-Whitney-Nernst equation under the sink condition, since the apparent dissolution rate constant of the meta-stable form can be assumed to be equal to that of the stable form.

$$C_{sa}=C_{sh}(J_a/J_h) \quad (1)$$

where C_{sa} is the solubility of anhydrate, C_{sh} is the solubility monohydrate, J_a is the dissolution rate of anhydrate and J_h is the dissolution rate of monohydrate.

The solubility of anhydrate obtained from the initial dissolution rate was larger than that obtained from the dispersed amount method. This suggested that the maximum concentration in dissolution profiles of anhydrate (Fig. 5) did not reach an equilibrium value since the recrystallization rate from anhydrate to monohydrate was faster than the dissolution rate.

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Increase in the Plasma Protein Binding of Weakly Basic Drugs in Carbon Tetrachloride-Intoxicated Rats

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Plasma protein binding of weakly basic drugs such as propranolol and quinidine was determined in rats with carbon tetrachloride (CCl_4)-induced hepatic disease. Free fractions of propranolol and quinidine in the plasma of rats at 24 h after CCl_4-intoxication were decreased by 41 and 30%, respectively, compared to those of control rats. An addition of Tris (butoxyethyl) phosphate (TBEP), a specific displacer for basic drugs from α_1-acid glycoprotein (AGP), to the plasma increased the free fractions of the basic drugs, resulting in no difference in the extent of the plasma free fraction of each drug between control and CCl_4-intoxicated rats.

Plasma concentration of AGP in CCl_4-intoxicated rats was elevated 2.7-fold of that in control rats at 24 h after the CCl_4 intoxication and reached a peak of 4.8-fold elevation at 48 h. A regression analysis revealed a high degree of positive correlation between ratios of bound to free fraction of propranolol and plasma concentrations of AGP. These results suggest that the plasma protein binding of the basic drugs was increased mainly due to the rise in the plasma AGP concentration in CCl_4-intoxicated rats.

Keywords plasma protein binding; α_1-acid glycoprotein; hepatic disease; carbon tetrachloride; weakly basic drug

Introduction

Protein binding in the blood is an improtant determinant of modified drug pharmacokinetics in the disease state. In patients with hepatic diseases, the binding of many acidic drugs to plasma proteins is frequently decreased.[1] The decreased plasma protein binding of drug has been related to either the reduction of concentrations of plasma proteins such as albumin and lipoproteins or the binding competition between acidic drugs and endogenous substances such as bilirubin and free fatty acids on binding sites of those plasma proteins.[2] In experimental model animals with D-galactosamine-induced hepatic disease, a reduction of albumin concentration and the resultant decrease in the binding of phenytoin to the plasma protein have been observed.[3]

On the other hand, the plasma protein binding of basic drugs in patients with hepatic diseases is not conclusive despite many clinical reports.[4] Basic drugs associate with α_1-acid glycoprotein (AGP) as well as with albumin and lipoproteins.[5] The plasma level of AGP has been reported to fluctuate even under ill conditions of the liver[4,6] in comparision with concentrations of albumin and lipoproteins which are mostly in the direction of reduction in patients with hepatic diseases.[2a,b] Therefore, AGP is considered to play a more important role than albumin and lipoproteins in the variability of the plasma binding of basic drugs in such patients.[4,6] However, little information on the change of the plasma protein binding of basic drugs in relation to the change of the concentrations of plasma proteins under the hepatic disease state is available.

This study was undertaken to examine the effect of experimental liver disease caused by carbon tetrachloride (CCl_4)-intoxication on the plasma protein binding of commonly used basic drugs such as propranolol and quinidine, and on the plasma concentration of AGP in rats. The plasma binding of salicylic acid, a model acidic drug, was also examined.

Materials and Methods

Chemicals *dl*-[^{3}H]Propranolol (27 Ci/mmol) and [^{14}C]salicylic aicd (56 mCi/mmol) were purchased from Dupon/NEN Research Products. Unlabeled *dl*-propranolol-HCl, quinidine sulfate and sodium salicylic acid were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). All other chemicals used were of reagent grade.

Animals Male Wister rats weighing 250—300 g on a normal laboratory diet were used. CCl_4 was dissolved in olive oil (1 : 4, v/v). The mixture was intraperitoneally administered to rats at a dose of 0.5 ml per 100 g body weight. Control rats received an injection of olive oil alone. Blood was collected by a heparinized-syringe from a venae cava inferior of rats under ethyl ether anesthesia. The blood samples were centrifuged at 10000 rpm to obtain plasma.

Binding Experiments The extent of the binding of drup to plasma proteins was determined through an ultrafiltration procedure. 0.9 ml of plasma were mixed with 0.1 ml of 1.0 M phosphate buffer, pH 7.4 containing a drug. The final concentration of drug in the plasma sample was 1 μg/ml. In the case of the competitive binding study with Tris (butoxyethyl) phosphate (TBEP), 5 μl of an ethanol solution of TBEP was added to each milliliter of plasma sample to give a final TBEP concentration of 200 μg/ml. After being incubated for 10 min at room temperature (20—23 °C), an aliquote (50 μl) was removed from each plasma compartment by filtration through Molcut II LGC (Millipore-Japan) of molecular sieve 10000. The amount of drug in the filtrate was assessed by measuring either radioactivity for propranolol and salicylic acid or intensity of fluorescence for quinidine. The ratio of the free fraction of drug in plasma was calculated as follows:

$$F = C_f / C_p$$

Where C_f = a drug concentration in filtrate
C_p = a drug concentration in plasma

Analytical Method AGP was purified from rat serum collected at 48 h after a turpentine injection according to the procedure described by Urban *et al.*[7] The amount of AGP in the plasma was measured by a single radial immunodiffusion analysis with purified AGP as a standard.[8] The protein assay measured the amount of AGP within the range of 500 ng to 5 μg in each test hole. Lipoproteins were isolated from plasma by adding potassium bromide and centrifuging at 105000 *g* for 48 h.[9] The amount of protein was measured by the method of Lowry *et al.*[10] The albumin concentration was determined with an Albumin B-Test kit (Wako Pure Chemicals Co., Ltd.). Glutamic pyruvic transaminase (GPT) concentration was determined with a Shino Test kit (Shino Test Co., Ltd., Tokyo, Japan).

Data Analysis Statistical analysis was performed with a Student's *t* test. Values are presented as the means ± S.E.

Results

***In Vitro* Plasma Protein Binding of Propranolol, Quinidine, and Salicylic Acid** The plasma protein binding of two basic drugs (propranolol and quinidine) and one acidic drug (salicylic acid) was determined in control and CCl_4-intoxicated rats 24 h after the intraperitoneal injection of

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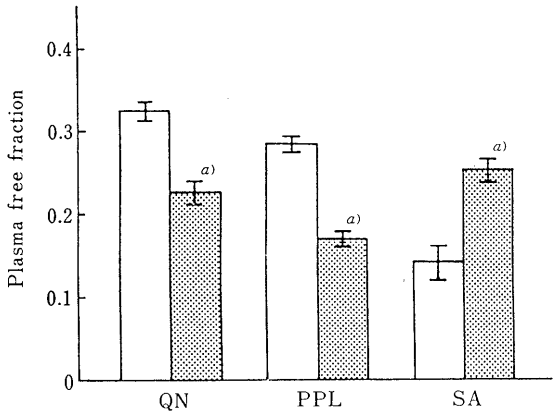

Fig. 1. Binding of Various Drugs to the Plasma Proteins from the Control and CCl_4-Intoxicated Rats

Animals were sacrificed 24 h after the injection. Plasma samples containing each drug at a concentration of 1 μg/ml were ultrafiltrated. Plasma free fractions of the drug were expressed as ratios of the amount of drug in the filtrate to the total amount of drug. Values are the means $\pm$ S.E. of six to nine animals. Significantly different from control; *a)* $p<0.01$. QN, quinidine; PPL, propranolol; SA, salicylic acid. ▭, control; ▩, CCl_4.

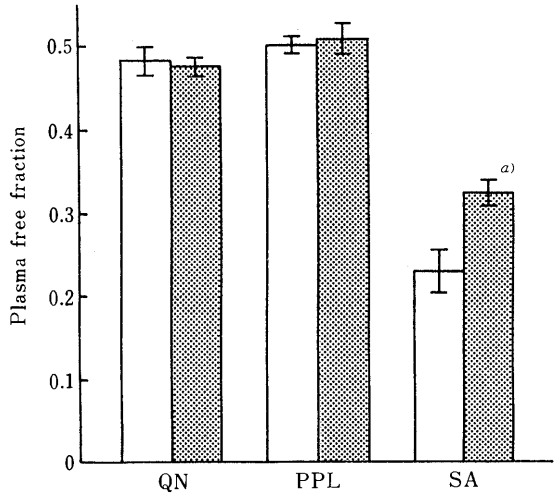

Fig. 2. Inhibitory Effect of TBEP on the Binding of Various Drugs to the Plasma Proteins from the Control and CCl_4-Intoxicated Rats

The same plasma samples as those used in Fig. 1 were subjected to the experiments. Plasma samples containing each drug and TBEP (200 μg/ml) were ultrafiltrated. Other experimental conditions were the same as those in Fig. 1. Values are the means $\pm$ S.E. of six to nine animals. Significantly different from control; *a)* $p<0.05$. QN, quinidine; PPL, propranolol; SA, salicylic acid. ▭, control; ▩, CCl_4.

olive oil and CCl_4–olive oil mixture, respectively. Plasma samples containing each drug at a concentration of 1 μg/ml were ultrafiltrated and the amounts of drug in the filtrates were measured (Fig. 1). The plasma free fraction of salicylic acid in the CCl_4-intoxicated group was increased by 71% compared to that in the control group. In contrast, plasma free fractions of propranolol and quinidine in the intoxicated group were decreased by 41 and 30% compared to those in the control group, respectively. The binding experiment was also performed in the presence of TBEP (200 μg/ml) which is known to displace several basic drugs from AGP.[11] The results are shown in Fig. 2. By an addition of TBEP to the plasma samples, plasma free fractions of propranolol and quinidine in the CCl_4-intoxicated group increased more than those in the control group. As a consequence, the difference in the plasma protein binding of the two basic drugs between the control and CCl_4-intoxicated groups was completely cancelled. On the other hand, the difference of the plasma protein binding of salicylic acid between the two groups remained in the same degree even after the TBEP treatment. These results suggest that the increased plasma protein binding of the basic drugs in CCl_4-intoxicated rats was mainly due to the increase in the plasma AGP level.

TABLE I. The Plasma Concentrations of Total Proteins, Albumin, Lipoproteins and AGP in Control and CCl_4-Intoxicated Rats

Constituent	Concentration (mg/ml plasma)	
	Control	CCl_4
Total proteins	73.2 $\pm$ 1.34	70.2 $\pm$ 1.13
Albumin	41.7 $\pm$ 0.07	40.6 $\pm$ 0.07
Lipoproteins	1.21 $\pm$ 0.073	0.62 $\pm$ 0.042[a)]
AGP	0.168 $\pm$ 0.012	0.460 $\pm$ 0.025[a)]

Animals were sacrificed 24 h after the injection. Values are the means $\pm$ S.E. of six to nine animals. Significantly different from control; *a)* $p<0.001$.

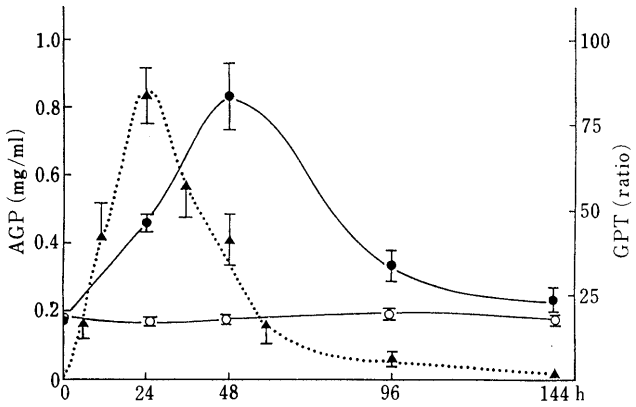

Fig. 3. Changes in the Plasma Concentrations of AGP and GPT in the Rat after the CCl_4-Intoxication

Animals were sacrificed at definite times after the injection. Values are the means $\pm$ S.E. of six to eight animals. The AGP level: ○, control group; ●, CCl_4 group. The GPT levels (▲) were expressed as ratios of the plasma concentrations in the CCl_4-intoxicated rats to those in normal rats.

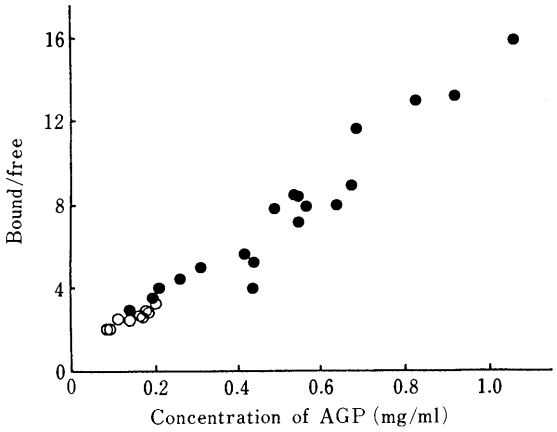

Fig. 4. Relationship between the Ratios of Bound to Free Fraction of Propranolol and the Concentration of AGP in the Plasma from Rats

Animals were sacrificed at various intervals after the injection. ○, control group; ●, CCl_4 group. Regression line $y=13.95x+0.415$; $r=0.978$; $p<0.001$.

Plasma Concentrations of Total Protein, Albumin, Lipoproteins and AGP Table I shows the concentrations of total protein, albumin, lipoproteins and AGP in the plasma from control and CCl_4-intoxicated rats 24 h after an injection.

The concentrations of total protein and albumin were not significantly altered by the CCl_4-intoxication. The concentration of lipoproteins in the intoxicated group was lowered by half compared to the control value. On the other hand, there was a marked elevation in AGP concentration in the intoxicated group. The mean values of AGP concentration were 168 μg/ml in the control group and 460 μg/ml in the intoxicated group, representing, a 2.7 fold increase in the level of the glycoprotein in the latter group. Following the CCl_4-intoxication, the AGP concentration reached its peak of 4.8 fold higher than the control value at 48 h and then declined gradually toward the control level over the following 72 h (Fig. 3). The level of GPT reached a maximum after 24 h and then fell down.

To assess the effect of the change in AGP concentration on the plasma protein binding of the basic drugs, the binding of propranolol to the plasma protein was determined simultaneously with the measurement of the plasma AGP concentration in all the experimental animals. The ratios of bound to free fraction of propranolol were plotted *versus* the AGP concentration (Fig. 4). These plots showed a good linear line with a correlation coefficient of $r=0.978$.

Discussion

In this study we examined the plasma protein binding of two commonly used basic drugs such as propranolol and quinidine, and one acidic drug, salicylic acid in experimental hepatic disease rats caused by CCl_4-intoxication. The plasma protein binding of salicylic acid was decreased in CCl_4-intoxicated rats, which is consistent with the results obtained in patients with hepatic diseases.[1] The decrease in plasma protein binding of the acidic drug seems to be due to the appearance of endogenous binding inhibitors.[2] In contrast to the acidic drug, the plasma protein binding of propranolol and quinidine was markedly increased in CCl_4-intoxicated rats. The competitive binding study with TBEP was performed since the ligand can selectively displace basic drugs from binding sites on AGP.[11] The difference in the plasma protein binding of the basic drugs between control and intoxicated rats was completely abolished by an addition of TBEP into the plasma. This result suggested that the increased plasma protein binding of the basic drugs was largely due to an elevation in the plasma AGP level in CCl_4-intoxicated rats. In fact, the rise in the plasma AGP concentration following the CCl_4 intoxication was demonstrated by using an immunological method (Table I). Goldberg *et al.* reported previously an acute-phase response in hepatic disease rats which were caused by oral intubation of CCl_4.[12] However, the plasma protein binding of the drug was not referred to in their paper. We found a high degree of correlation between the ratios of bound to free fraction of propranolol and the AGP concentration (Fig. 4). This finding further confirmed AGP as a major determinant of the plasma protein binding of basic drugs in rats with CCl_4-induced hepatic disease. It has been recognized that the increase in AGP level occurs in pathological states associated with inflammatory disease.[13] It is known that CCl_4-intoxication causes acute necrosis of parenchymal cells in the liver and the lesion is followed by infiltration of reticuloendothelial cells.[14] Hence, the rise in plasma AGP level in CCl_4-intoxicated rats seems to be a reflection of changes in the immunoregulatory system. Hepatic diseases in humans are likely caused by many factors which are different from CCl_4-intoxication. The present study at least showed the possibility that liver cells produce AGP despite severe damage.

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Effects of Diazepam Administration on Melatonin Synthesis in the Rat Pineal Gland *in Vivo*

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The effect of diazepam (DZP) on melatonin synthesis in rat pineal gland was investigated *in vivo*. Subcutaneous injection of DZP (3 mg/kg) 1 h before the start of darkness significantly suppressed nocturnal elevations of pineal *N*-acetylserotonin (NAS) and melatonin contents in rats, and caused a 2-h delay in reaching the maximum melatonin level in the dark phase. DZP treatment also markedly suppressed the dark-induced increase of pineal *N*-acetyltransferase activity, which catalyzes the rate-limiting step in melatonin synthesis, but had no effect on hydroxyindole-*O*-methyltransferase activity, which catalyzes the final step of melatonin formation. Pineal norepinephrine and dopamine contents, in contrast, were not altered by DZP injection. The distribution rate of DZP to the brain reached the highest level 30 min after a single injection, while that to the pineal gland was observed 5 h later (*i.e.*, 4 h after the start of darkness). It is clear that the inhibitory effect of DZP on melatonin synthesis in rat pineal gland appears concomitantly with the increase in the distribution volume of DZP into this gland. These results suggest that the inhibitory effect of DZP on melatonin synthesis results from the drug's direct action on the rat pineal gland.

Keywords diazepam; pineal gland; melatonin; *N*-acetylserotonin; *N*-acetyltransferase; hydroxyindole-*O*-methyltransferase

Introduction

Several psychiatric disorders such as stress,[1] insomnia,[2] depression,[3] and schizophrenia[4] are thought to be related to changes in pineal gland function. Many investigations with animals and humans have been undertaken to elucidate the relationship between pineal melatonin and psychotropic drugs.[5–7] These reports suggest that further studies on the relationship between the pineal gland and psychiatric disease would be worthwhile. We earlier reported that diazepam (DZP) injection induced a dose-dependent decrease of melatonin content in rat pineal gland during the dark phase.[8] However, the biochemical mechanism of melatonin synthesis inhibition induced by DZP administration *in vivo* is not known.

The purpose of this study was to elucidate whether or not the inhibitory effect of DZP on melatonin synthesis results from direct action of the substance on the rat pineal gland. We present data on changes in the content of pineal indoleamines and the activities of pineal *N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT), which are involved in melatonin synthesis, as well as data on the distribution of DZP to rat pineal gland and brain after a single DZP injection.

Experimental

Materials DZP was purchased as an injectable solution (Cercine®) from Takeda Chemical Co., Ltd. (Japan). Acetyl-[1-^{14}C]coenzyme A (2.01 GBq/mmol) and *S*-adenosyl-L-[methyl-^{14}C]methionine (2.13 GBq/mmol) were purchased from New England Nuclear (Boston, U.S.A.). All other reagents were of the highest quality commercially available.

Animals Male Wistar rats, weighing 250—300 g, were used in the experiments. Animals were housed at constant temperature (24±1 °C) and humidity (55±5%) under a 12-h light-dark cycle (lights on from 7:00 p.m. to 7:00 a.m.) for 2 weeks before the experiment. Food (MF, Oriental Yeast) and water were provided *ad libitum*.

Effect of DZP Administration on Pineal Melatonin Synthesis DZP (3 mg/kg) was administered subcutaneously to rats ($n=30$) at 6:00 a.m. (1 h before the onset of the dark phase). Control animals ($n=30$) received 0.9% saline solution with an equal volume of DZP solution at 6:00 a.m. Five rats in the DZP-treated group and five in the control group were killed by decapitation at 3, 4, 5, 6, 7 and 8 h after DZP injection. The pineals were rapidly dissected, frozen on dry ice and stored at −80 °C until assayed for indoleamine content and the activities of NAT and HIOMT. During the dark phase, decapitation and pineal dissection were performed with the aid of a dim red light.

Effect of DZP Administration on Pineal Catecholamine DZP (3 mg/kg) was injected subcutaneously into rats at 6:00 a.m. ($n=5$). As a control ($n=5$), 0.9% saline solution was administered at 6:00 a.m. All the animals were killed by decapitation at 4 h after injection (*i.e.*, 3 h after the onset of darkness). Pineals were rapidly removed, frozen on dry ice and stored at −80 °C prior to high performance liquid chromatography (HPLC) analysis.

Distribution of DZP to Rat Pineal Gland and Brain after a Single DZP Injection DZP (3 mg/kg) was administered subcutaneously to rats ($n=32$) at 6:00 a.m. Four animals were killed at each of the following times after the injection: 5, 15, 30 min, 1, 3, 5, 7 and 9 h. The pineals and brains were removed, frozen on dry ice, and stored at −80 °C until required for assays.

Concurrent Determination of Indoleamine Content and Enzymatic Activity in the Same Rat Pineal Gland Indoleamines were extracted from rat pineal gland and measured by HPLC as previously reported.[9] The assay of NAT and HIOMT activities in the same pineal gland was modified from the radioimmunoassay methods of Champney *et al.*[10] and Deguchi and Axelrod.[11] Just before the assay, each pineal gland was homogenized in 50 μl of 50 mM phosphate buffer (pH 6.8). For the NAT assay, 35 μl of homogenate was incubated at 37 °C for 20 min in the presence of a mixture containing 60 nM tryptamine, 2 μl of 0.76 nM acetyl-[1-^{14}C]coenzyme A (1.4 kBq/sample) and 50 mM phosphate buffer (pH 6.8). The reaction was stopped by the addition of 100 μl of 0.5 M borate buffer (pH 10). The reaction product, *N*-acetyl[^{14}C]tryptamine, was extracted with 2 ml of toluene–isoamyl alcohol mixture (7:3). A 1.6 ml aliquot of the organic extract was allowed to evaporate to dryness in a glass scintillation vial. Radioactivity was quantified by liquid scintillation spectroscopy after adding 8 ml of scintillant. Results were expressed as nanomoles of *N*-acetyltryptamine formed per pineal per hour. For the HIOMT assay, 10 μl of pineal homogenate was thawed and 30 μl of 0.05 M phosphate buffer (pH 11.3) was added to raise the pH of the homogenate to 7.9. The mixture was incubated at 37 °C for 60 min in the presence of 20 μl of 50 nM *N*-acetylserotonin (NAS), 1 μl of *S*-adenosyl-L-[methyl-^{14}C]methionine (0.74 kBq/sample), and 40 μl of 0.05 M phosphate buffer (pH 7.9). The subsequent procedure was identical to that in the NAT assay. Results were expressed as nanomoles of melatonin formed per pineal per hour.

Determination of Catecholamines in Rat Pineal Gland Extraction and determination of catecholamines in rat pineal gland were carried out by the HPLC-ED (electrochemical detection) method previously developed.[12] Briefly, individual pineals were sonicated at 0—4 °C for 10 s using a Sonicator model W-10 (Heat Systems Ultrasonics, Inc.) in 0.1 M perchloric acid and centrifuged at 1500*g* at 0 °C for 10 min. Catecholamines in the supernatant were extracted by adsorption on alumina. Catecholamines thus extracted were determined using a HPLC-ED system.

Determination of DZP in Rat Pineal Gland and Brain Pineal was homogenized in 100 μl of 0.2 M borate buffer (pH 9.0) containing

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prazepam (150 ng/ml) as an internal standard. Brain was homogenized in 2 ml (prazepam, 30 ng) of the same buffer. These samples were extracted by vortexing for 1 min with a mixed solvent of dichloromethane–pentane–hexane (8 : 1 : 1, v/v) using 400 μl for pineal or 8 ml for brain. After centrifugation for 10 min at 10000*g*, the organic layer was taken and evaporated to dryness at room temperature under nitrogen. The residue thus obtained was dissolved in 100 μl of methanol, and the solution was filtered through a Column Guard® (Millipore). Fifty microliters of the filtrate was injected into the HPLC system. Separation was done on a Shim-pack CLC-ODS column (15 cm × 6 mm i.d.) with an ODS guard column (5 cm × 4.6 mm i.d.), using a mobile phase of 0.02 M phosphate buffer (pH 6)–methanol–acetonitrile (30 : 55 : 15, v/v). The flow rate was set at 1.4 ml/min. Monitoring was done at 230 nm with a spectrophotometric detector (Shimadzu SPD-6AV).

Results

Effect of DZP Administration on Pineal Melatonin, 5-Hydroxytryptamine (5-HT), and NAS Contents Time courses of the changes in pineal indoleamine levels of control and DZP-administered (3 mg/kg) rats are illustrated in Fig. 1. Significant reduction in pineal melatonin levels caused by DZP was shown at 4 and 5 h after the start of darkness (69%, $p<0.01$ and 15%, $p<0.01$, respectively).

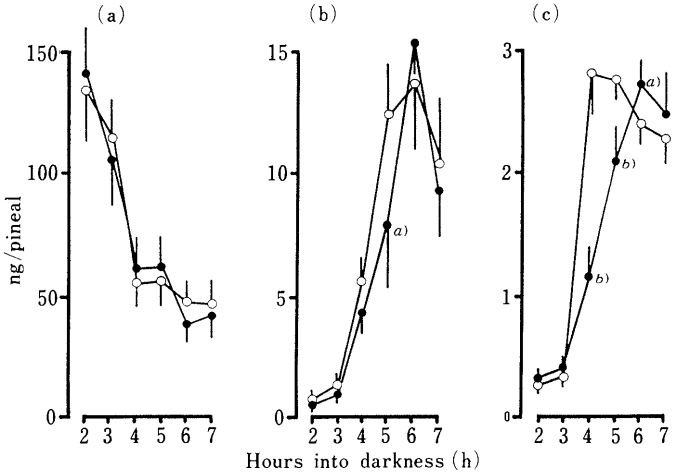

Fig. 1. Time Course of the Effects of DZP Administration (3 mg/kg) on Rat Pineal 5-HT (a), NAS (b) and Melatonin (c) Levels during the Dark Phase

Diazepam was injected subcutaneously 1 h before the onset of darkness. Each value is the mean ± S.E. of 5 rats. ○—○, control; ●—●, DZP. *a*) $p<0.05$. *b*) $p<0.01$.

However, at 6 h (*i.e.*, 7 h after DZP treatment), pineal melatonin content in DZP-treated rats was equal to the maximum levels seen in control rats. Pineal NAS content in DZP injected rats began to decrease from 4 h after the start of darkness and showed a significant decrease (38%, $p<0.05$) at 5 h, but at 6 h there was no significant difference from the control. On the other hand, DZP treatment did not alter the content of pineal 5-HT, which is the precursor of NAS and melatonin.

Effect of DZP Administration on Pineal NAT and HIOMT Activities Figure 2 shows the time courses of pineal NAT and HIOMT activities for control and DZP-treated rats. NAT activity was significantly reduced in DZP-treated rats compared with saline-injected control rats at 3 h (50%, $p<0.01$) and 4 h (32%, $p<0.02$) after the onset of darkness; however, no significant difference in this activity was observed at 5 or 6 h. Pineal HIOMT activity showed no significant variation in DZP-injected and control rats.

Effect of DZP Administration on Pineal Norepinephrine (NE) and Dopamine (DA) Contents In pineal glands of DZP-treated rats 2.69 ± 0.65 ng/gland of NE and 0.62 ± 0.15 ng/gland of DA were found at 3 h after the onset of darkness (*i.e.*, 4 h after DZP treatment) (Table I). No significant differences in pineal NE and DA contents were found between the control and DZP-treated rats. These values are in good agreement with those reported by Fujiwara *et al.*[13] and by Brownstein and Axelrod.[14]

Distribution of DZP to Rat Pineal Gland and Brain after a Single DZP Injection Figure 3 shows the time courses of the distribution of DZP to rat pineal gland and brain after a single DZP treatment. DZP in brain was detected at 5 min after the injection and the maximum DZP

Table I. Pineal Catecholamine Levels 4 h after DZP Administration

Drug	NE	DA
	ng/pineal	
Control	2.78 ± 0.6	0.69 ± 0.2
DZP (3 mg/kg, s.c.)	2.69 ± 0.6	0.62 ± 0.2

Each value represents the mean ± S.E. of 4 rats.

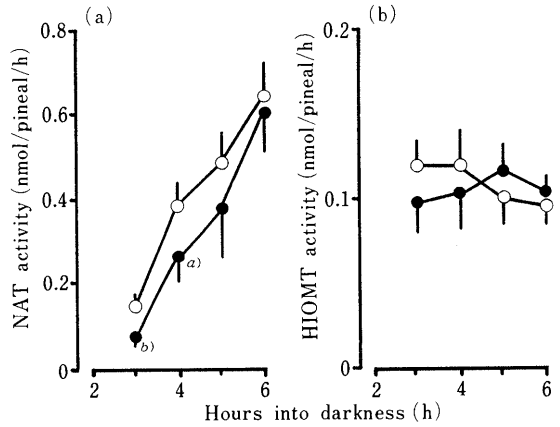

Fig. 2. Time Course of the Effects of DZP Administration (3 mg/kg) on Rat Pineal NAT (a) and HIOMT (b) Activities during the Dark Phase

DZP was injected subcutaneously 1 h before the onset of darkness. Each value is the mean ± S.E. of 5 rats. ○—○, control; ●—●, DZP. *a*) $p<0.02$. *b*) $p<0.01$.

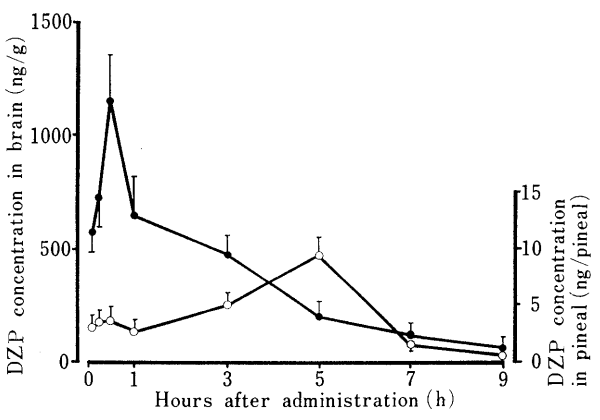

Fig. 3. Time Courses of DZP Concentration in Pineal and Brain after a Single s.c. Injection of DZP (3 mg/kg) in Male Wistar Rats

DZP was injected subcutaneously 1 h before the onset of darkness. Each value is the mean ± S.E. of 4 rats. ○—○, pineal; ●—●, brain.

concentration in brain appeared at 30 min following DZP treatment. In pineal gland, DZP was detected at 5 min after treatment, and began to increase from 1 h after treatment, reaching the maximum (9.1 ± 1.5 ng/pineal) at 5 h (*i.e.*, 4 h after the onset of darkness).

Discussion

We reported earlier that DZP injection induced a significant decrease in pineal melatonin content in rats without altering the contents in 5-HT and NAS measured at 4 h after the start of darkness.[8] In this paper we described the time course of the effects of DZP administration on the rat pineal melatonin production. A single DZP injection did not affect 5-HT content or HIOMT activity in the pineal gland in the dark phase, while it suppressed the initial dark-induced elevations in pineal NAT activity, and in NAS and melatonin content (Figs. 1 and 2). As a result, a single DZP administration to rats produced a 2 h phase delay in the melatonin rhythm in the dark phase of the light-dark cycle. These results indicate that the transitory inhibition of nocturnal elevation in the contents of pineal NAS and melatonin is a direct result of suppression of NAT activity. Because the pharmacological or physiological significance of the phase-shifting effect of DZP on melatonin synthesis in rat pineal gland is not known, the possibility of melatonin involvement in benzodiazepines exerting their effects as anti-anxiety or hypnotic drugs should be examined further.

In mammals, a major pacemaker for the generation of many circadian rhythms appears to be localized in the suprachiasmatic nucleus (SCN) of the hypothalamus.[15] Van Reeth *et al.*[16] demonstrated that the phase-shifting effects of the benzodiazepine triazolam on the circadian rhythm of locomotor activity in free-running hamsters may be due to a direct effect of this drug within the SCN. The SCN of the hypothalamus has been suggested to initiate a stimulus which enhances pineal NAT cyclicity in the rat.[17] If the site of action for the inhibitory effects of DZP on pineal melatonin synthesis is the SCN, one would expect marked inhibition of the release of NE from the adrenergic nerve endings stimulating the pineal gland. Further, the variation in the release of NE from the sympathetic terminals stimulating the gland would be reflected in the NE turnover in the rat pineal gland.[14] Thus, we tried to determine the contents of pineal NE and DA, the precursor of NE, at 3 h after the start of darkness (*i.e.*, 4 h after DZP injection) in order to estimate the NE release from nerve terminals. However, no significant difference was found in pineal NE or DA content between the control and DZP treatment groups (Table I). Thus, we speculated that the inhibitory effects of DZP on pineal melatonin synthesis are not due to a direct effect of DZP on the SCN.

We also investigated the time course of DZP concentration in rat pineal gland after a single DZP administration in order to explore the possibility that DZP may act directly on this gland. Little work has been done on DZP distribution to rat pineal. The DZP distribution to rat whole brain reached maximum at 30 min after DZP injection (Fig. 3); this result is similar to those of Friedman *et al.*[18] and Rahman *et al.*[19] However, DZP concentration in rat pineal gland began to increase from 1 h after the drug administration and reached maximum at 5 h after injection (*i.e.*, 4 h after the onset of darkness). The time course of DZP concentration in rat pineal gland was in good accordance with that of the inhibitory effects of DZP on pineal melatonin synthesis, as illustrated in Figs. 1 and 2. The results may indicate that the inhibitory effect of DZP on melatonin synthesis results from direct action of DZP on the rat pineal gland.

In conclusion, a single DZP treatment temporarily suppressed the dark-induced initial elevation of pineal NAT activity, and caused a 2-h delay in attainment of maximum melatonin level in the dark phase.

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Effects of Adrenergic Blockers or Bicuculline on Diazepam Induced Changes in Rat Pineal Melatonin Synthesis *in Vivo* and *in Vitro*

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The effect of propranolol (PPL), phenoxybenzamine (PBZ) or bicuculline (BCL) on the diazepam (DZP)-induced changes of pineal melatonin synthesis in male rats was examined *in vivo* and *in vitro*. Administration of PBZ did not affect the inhibitory action of DZP on pineal melatonin synthesis *in vivo*. A single injection of PPL inhibited the pineal melatonin synthesis similarly to the administration of DZP alone, but the two drugs together did not exhibit additive or synergistic effects on the melatonin synthesis. Significant decreases in the *N*-acetyltransferase (NAT) activity and the *N*-acetylserotonin (NAS) and melatonin contents were observed in the BCL-injected group, being greater than those in the DZP-treated group. Unexpectedly, however, the combination treatment of DZP and BCL caused an increase in the NAT activity and melatonin content compared with the BCL-alone group. Incubation with DZP at higher concentrations resulted in an increase of pineal NAT activity *in vitro*, but this increase was inhibited by preincubation with PPL, PBZ or BCL. DZP treatment thus appeared to have different effects on pineal NAT activity *in vivo* and *in vitro*. These results suggest that both a GABAergic mechanism and peripheral benzodiazepine (BZP) receptors in rat pineal gland may be involved in the modulation of melatonin synthesis by DZP.

Keywords diazepam; rat pineal gland; melatonin synthesis; bicuculline

Introduction

Benzodiazepines (BZP) are widely used in the treatment of anxiety, stress-related conditions, insomnia, and other mental disorders.[1] The drugs potentiate the action of the neurotransmitter γ-aminobutyric acid (GABA) through BZP receptors.[2] Melatonin is a major secretory product of the pineal gland and displays BZP-like pharmacological properties.[3] Several investigations have been made to elucidate the effect of benzodiazepine on pineal functions,[4] but it is not clear whether the pineal gland mediates or modulates the pharmacological effects of BZP. We have reported that diazepam (DZP) administration to rats induced a phase delay in the pineal melatonin rhythm during the dark phase, suppressing the activity of *N*-acetyltransferase (NAT) without causing a reduction in the activity of hydroxyindole-*O*-methyltransferase (HIOMT) *in vivo*.[5] The purpose of this investigation was to clarify the mechanism of DZP action on the rat pineal gland by examining the effect of propranolol (PPL), phenoxybenzamine (PBZ) or the GABA antagonist bicuculline (BCL) on the DZP-induced changes of rat pineal melatonin synthesis *in vivo* and *in vitro*.

Experimental

Materials The following drugs were used: DZP (Cercine inj.® and crystalline diazepam; Takeda Chemical Co. Ltd., Osaka), *dl*-propranolol hydrochloride (PPL, Wako Pure Chemical Industries, Ltd., Osaka), phenoxybenzamine hydrochloride (PBZ, Nakarai Chemicals, Kyoto), (+)-bicuculline (BCL, Sigma Chemical Co., St. Louis, MO). For *in vivo* studies with DZP, Cercine injectable solution was used, while crystalline DZP was suspended in 0.9% saline just prior to use in the *in vitro* experiments. For *in vivo* and *in vitro* studies, PPL and PBZ were dissolved in 0.9% saline, BCL was dissolved in 0.1 N HCl and diluted with saline prior to use. Acetyl-[1-^{14}C]coenzyme A (2.01 GBq/mmol) and *S*-adenosyl-L-[methyl-^{14}C]methionine (2.13 GBq/mmol) were purchased from New England Nuclear (Boston, U.S.A.). All other reagents were of the highest quality commercially available.

Animals Male Wistar rats, weighing 250—300 g, were used in the experiments. Animals were housed at constant temperature (24±1 °C) and humidity (55±5%) under a 12 h light-dark cycle (lights on from 7:00 p.m. to 7:00 a.m.) for 2 weeks before the experiment. Food (MF, Oriental Yeast) and water were provided *ad libitum*.

Effects of PPL, PBZ or BCL on DZP-Induced Changes of Rat Pineal Melatonin Synthesis *in Vivo* DZP (3 mg/kg) was administered subcutaneously to rats ($n=5$) at 6:00 a.m. (1 h before the onset of the dark phase). Control animals ($n=5$) received an injection of 0.9% saline solution equal in volume to the DZP solution at 6:00 a.m. Ten rats were given an intraperitoneal injection of PPL (3 mg/kg) at 5:30 a.m., and five of the PPL-treated group also received a subcutaneous DZP injection (3 mg/kg) at 30 min after the PPL injection. In the same manner as described for PPL treatment, PBZ (3 mg/kg) and BCL (3 mg/kg) were administered to other groups of rats. These drug-treated or control rats were killed by decapitation at 5 h after DZP or saline injection (*i.e.*, 4 h after the start of darkness). The pineals were rapidly removed, frozen on dry ice and stored at −80 °C until required for assays. Experimental manipulation and decapitation during the dark phase of the light-dark cycle were performed with the aid of a dim red light.

Effects of PPL, PBZ or BCL on DZP-Induced Changes of Pineal NAT and HIOMT Activities *in Vitro* Non-treated rats were killed by decapitation at 4 h after the start of darkness and the pineals were rapidly dissected. Each gland was preincubated individually in 300 μl of Tyrode's solution at 37 °C for 5 min prior to addition of a drug. Experiment I: The preincubated gland was individually incubated once again in the presence of DZP suspension (final concentration: 10^{-3}—10^{-10} M) at 37 °C for 30 min. As a control ($n=10$), the gland was incubated in the absence of DZP at 37 °C for 30 min. Experiment II: After preincubation, the gland was individually incubated again at 37 °C for 10 min in PPL solution (10^{-3}—10^{-11} M), PBZ solution (10^{-3}—10^{-7} M) or BCL solution (10^{-3}—10^{-7} M). Then, these glands preincubated with PPL, PBZ or BCL were incubated individually with DZP suspension (10^{-3} M) at 37 °C for 30 min. After incubation with those drugs in experiments I or II, the pineal glands were rinsed twice with 1 ml of fresh cold Tyrode's solution, immediately frozen on dry ice and stored at −80 °C until assayed for NAT or HIOMT activity.

Determination of Indoleamine and Measurement of NAT and HIOMT Activities in Rat Pineal Gland To examine the indoleamine contents and the activities of NAT and HIOMT in rat pineal gland, extraction and measurement of indoleamines were carried out according to the method previously reported.[6] The activities of NAT and HIOMT in individual rat pineal glands were measured according to the modified radiochemical procedure described previously.[5]

Results

Effects of PPL, PBZ or BCL on DZP-Induced Changes of Rat Pineal Melatonin Synthesis *in Vivo* Figures 1, 2 and 3 show the effects of PPL, PBZ or BCL injection on the DZP-induced changes of rat pineal melatonin synthesis.

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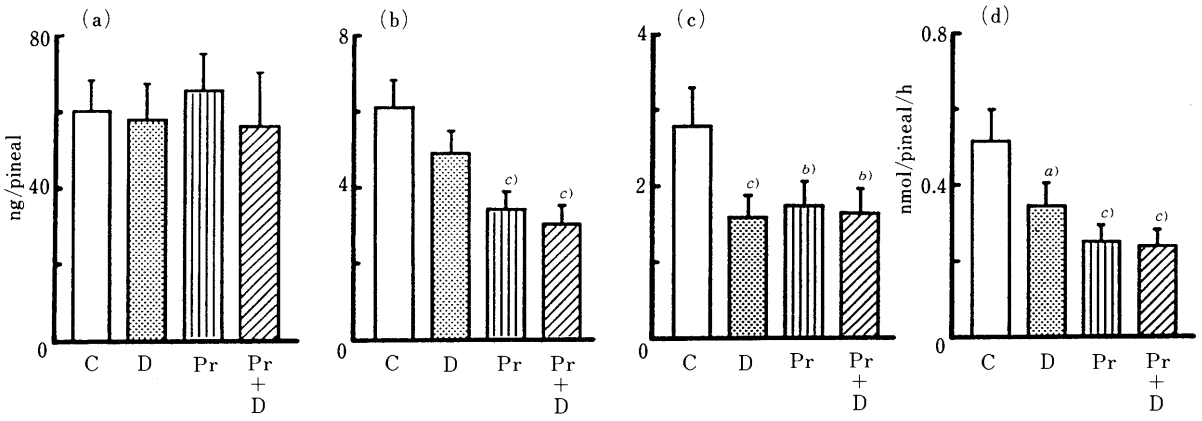

Fig. 1. Effect of DZP Administration with PPL on Melatonin Synthesis in Rat Pineal Gland

(a) 5-HT; (b) NAS; (c) melatonin; (d) NAT. Each value is the mean±S.E. of 5 rats. C, control; D, DZP; Pr, PPL; Pr+D, PPL+DZP. *a*) $p<0.05$. *b*) $p<0.02$. *c*) $p<0.01$.

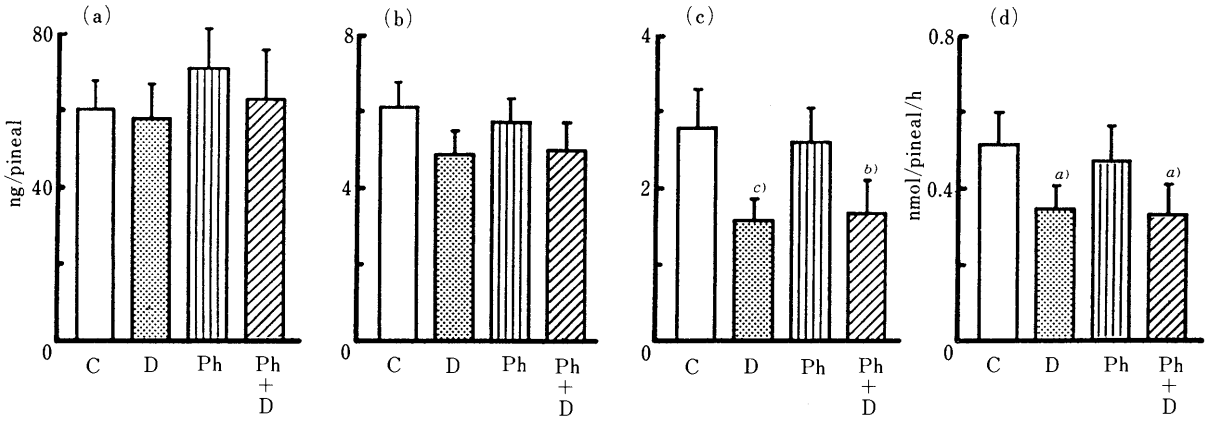

Fig. 2. Effect of DZP Administration with PBZ on Melatonin Synthesis in Rat Pineal Gland

(a) 5-HT; (b) NAS; (c) melatonin; (d) NAT. Each value is the mean±S.E. of 5 rats. C, control; D, DZP; Ph, PBZ; Ph+D, PBZ+DZP. *a*) $p<0.05$. *b*) $p<0.02$. *c*) $p<0.01$.

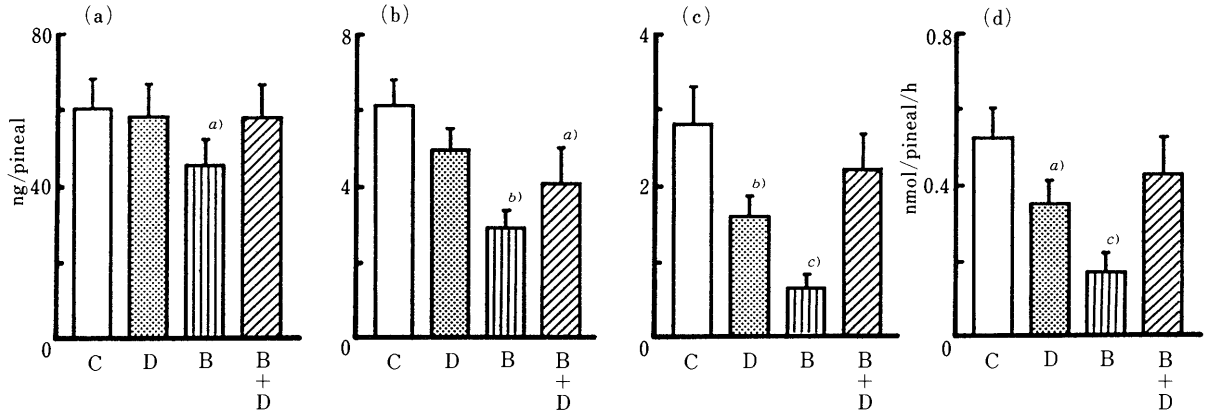

Fig. 3. Effect of DZP Administration with BCL on Melatonin Synthesis in Rat Pineal Gland

(a) 5-HT; (b) NAS; (c) melatonin; (d) NAT. Each value is the mean±S.E. of 5 rats. C, control; D, DZP; B, BCL; B+D, BCL+DZP. *a*) $p<0.05$. *b*) $p<0.01$. *c*) $p<0.001$.

A single injection of DZP (3 mg/kg, s.c.) resulted in remarkable inhibition of rat pineal NAT activity (33%, $p<0.05$) and reduction of melatonin content (43%, $p<0.01$) at 5 h after DZP treatment. PPL (3 mg/kg, i.p.) also significantly reduced the NAT activity (52%, $p<0.01$) and decreased the contents of NAS (44%, $p<0.01$) and melatonin (38%, $p<0.02$) with no change in 5-hydroxytryptamine (5-HT) content compared with the control group (Fig. 1). However, the administration of DZP (3 mg/kg s.c.) at 30 min after PPL (3 mg/kg, i.p.) injection resulted in no significant difference in rat pineal melatonin synthesis compared with the single PPL or single DZP injection group (Fig. 1). PBZ (3 mg/kg, i.p.) administration had no effect on pineal melatonin synthesis (Fig. 2), and the inhibitory effects of DZP on rat pineal melatonin synthesis were still clearly observed in PBZ-pretreated rats.

TABLE I. Effect of DZP on Rat Pineal NAT and HIOMT Activities *in Vitro*

	NAT	HIOMT
	pmol/pineal/h	
Control (10)	372±44	75± 9
DZP 10^{-3} M (4)	655±96[a]	82±10
10^{-4} M (4)	481±88[a]	80±10
10^{-5} M (4)	400±56	79±10
10^{-6} M (4)	377±42	76± 7
10^{-7} M (4)	373±46	79±10
10^{-8} M (4)	369±53	78± 9
10^{-9} M (4)	364±54	73± 8
10^{-10} M (4)	371±40	77±10

Intact rats were killed by decapitation at 4 h after the start of darkness and the pineals were rapidly dissected for the experiments. Each value represents the mean±S.E. of the number of glands indicated in parentheses. *a)* $p<0.01$.

TABLE II. Effect of PPL, PBZ or BCL on DZP-Induced NAT Activity in Incubated Rat Pineal Gland

M	M	NAT activity (pmol/pineal/h)
DZP 10^{-3}		655± 96
DZP 10^{-3}	+PPL 10^{-3}	371± 58[a]
	+PPL 10^{-5}	370± 66[a]
	+PPL 10^{-7}	364± 65[a]
	+PPL 10^{-9}	533±107
	+PPL 10^{-11}	667±135
DZP 10^{-3}	+PBZ 10^{-3}	370± 67[a]
	+PBZ 10^{-5}	475±106
	+PBZ 10^{-7}	661±128
DZP 10^{-3}	+BCL 10^{-3}	377± 75[a]
	+BCL 10^{-5}	364± 60[a]
	+BCL 10^{-7}	484± 97[b]

Each value represents the mean±S.E. of 4 rats. *a)* $p<0.01$. *b)* $p<0.05$.

Figure 3 shows the effects of BCL treatment on pineal melatonin synthesis. BCL loading at 3 mg/kg greatly reduced NAT activity (70%, $p<0.01$) and NAS (53%, $p<0.01$) and melatonin (78%, $p<0.001$) contents, as well as 5-HT content (25%, $p<0.05$). However, the administration of DZP at 30 min after BCL injection resulted in only slight inhibition of the melatonin synthesis in rat pineal gland in comparison with the controls.

Effects of PPL, PBZ or BCL on DZP-Induced Changes of Pineal NAT and HIOMT Activities *in Vitro* When pineal gland was incubated with DZP (10^{-3}—10^{-10} M) for 30 min *in vitro*, DZP at lower concentrations (below 10^{-4} M) showed no effect on NAT activity. The NAT activity was significantly increased ($p<0.01$) with higher concentrations (10^{-3} or 10^{-4} M) of DZP, while the pineal HIOMT activity was not affected by DZP (Table I). Preincubation with PPL (10^{-7} to 10^{-3} M), PBZ (10^{-3} M) or BCL (10^{-7} to 10^{-3} M) inhibited the increase in pineal NAT activity caused by the incubation with DZP (10^{-3} M) (Table II).

Discussion

The rat pineal gland is innervated by postganglionic sympathetic nerve fibers originating bilaterally in the superior cervical ganglia.[7] It is generally accepted that the activity of NAT, the rate-limiting enzyme in melatonin synthesis, is controlled transsynaptically by the release of norepinephrine (NE), which interacts with both α- and β-adrenergic receptors on rat pinealocytes.[8] Psychotropic drugs, including antidepressant[9] and anticonvulsant[4d] drugs, alter the sensitivity of adrenergic receptors in rat pineal gland, and thus influence melatonin synthesis in the gland.

We reported earlier that the administration of some clinically used psychotropic drugs brought about significant changes in rat pineal melatonin levels during the dark phase.[4a] The time at which pineal melatonin content reached maximum was delayed 2 h by the administration of DZP, and the time course of the inhibitory effect of DZP on the pineal melatonin synthesis was in good accordance with that of the distribution of DZP into the gland.[5] From these results, we concluded that the inhibition of pineal melatonin synthesis by DZP administration might be a result of direct action of DZP on the gland. Morton[4d] suggested that the reduction in pineal NAT activity caused by anticonvulsants including DZP could be due, at least in part, to the blockade of β-adrenergic receptors on pinealocytes. On the other hand, a study by Zatz and Brownstein[4b] showed that the sedative drug did not reduce the NAT activity through a direct effect upon the pineal gland. Whether DZP acts on the rat pineal gland directly or indirectly remains unclear.

In order to elucidate the mechanism of DZP action on the rat pineal gland, the effects of DZP administration in combination with adrenergic blockers on the pineal melatonin synthesis were investigated in male rats. The results of β-adrenergic blocker PPL injection were similar to those reported by Deguchi and Axelrod[10] and Parfitt *et al.*,[11] who showed that administration of PPL inhibits the normal night-time rise in melatonin synthesis by blocking the β-adrenergic receptors on pinealocytes. Namely, PPL itself attenuated the NAT activity and decreased the contents of NAS and melatonin to a degree equivalent to or exceeding that caused by single DZP injection (Fig. 1). As a result, the apparent inhibitory effect of DZP on pineal melatonin synthesis could not be observed after pretreatment with PPL. On the other hand, the administration of an α-adrenergic blocking agent PBZ did not affect the pineal melatonin biosynthesis at the dose used in this experiment. Consequently, the administration of DZP at 30 min after PBZ injection still resulted in the suppression of melatonin synthesis (Fig. 2). These observations suggest that the inhibition of pineal melatonin synthesis may be caused by the direct effects of DZP on the functions of pineal β-receptors. In addition to the foregoing investigations using adrenergic blockers, the effect of the GABA antagonist BCL was also examined *in vivo*. Our finding that pineal melatonin synthesis was suppressed by the acute administration of the GABAergic agent DZP suggested that GABAergic mechanisms may be involved in the modulation of melatonin synthesis in rat pineal gland. In fact, the GABA system has been associated with habenula complex, which has been shown to innervate this gland.[12] Mata *et al.*[13] concluded that GABA does not stimulate or modulate the adrenergic regulation of NAT. Ebadi *et al.*[14] and Balemann *et al.*,[15] however, proposed that GABA may be a modulator of melatonin synthesis. In this study, although the actions of DZP and BCL on GABA neurotransmission were generally contrary, the GABA

antagonist BCL itself greatly depressed melatonin synthesis in pineal gland (Fig. 3). These results may reflect the fact[16] that BCL does not act as a simple GABA antagonist, and occasionally can antagonize and potentiate GABA. The attenuation of pineal melatonin synthesis caused by pretreatment with BCL was restored by the administration of DZP (Fig. 3). The above finding that DZP has an opposite effect on melatonin synthesis under conditions of altered GABA neurotransmission suggests that the mechanism of the inhibitory effect of DZP on pineal melatonin synthesis is complicated. Furthermore, we found that the effects of DZP on the level of pineal 5-HT were quite different from those of BCL, although a single injection of DZP or BCL inhibited melatonin synthesis in rat pineal gland (Fig. 3).

Based on these results, we speculate that DZP acts through another binding site, and also acts on GABA and/or β-adrenergic receptors. The possible site of action may be peripheral BZP receptors in rat pineal gland. There is evidence that the gland contains peripheral BZP receptors which are not GABA-modulated and are associated with catecholamine nerve terminals.[17] The functional role of peripheral BZP receptors in the rat pineal gland remains to be clarified. A study by Lowenstein *et al.*[4e] has shown that this gland takes up and retains BZP. They pointed out a link between the pharmacological action of melatonin and BZP receptor function in rats.[4g] We have also demonstrated that the inhibitory effect of DZP on pineal melatonin synthesis was in good accordance with the time course of DZP distribution to the pineal gland *in vivo*.[5] These observations suggest that peripheral BZP receptors in rat pineal gland are involved in the modulation of melatonin synthesis, and that DZP might affect the pineal melatonin synthesis by acting on these receptors.

To confirm the results obtained in the *in vivo* study and to examine whether the pharmacological active site of DZP is located inside the rat pineal gland, intact rat pineals were incubated with DZP suspension *in vitro*. Interestingly, DZP treatment had opposite effects *in vivo* and *in vitro* upon pineal NAT activity. Incubation with DZP at higher concentrations (10^{-3} to 10^{-4} M) induced an increase of NAT activity (Table I), and this result was similar to that of a study reported by Matthew *et al.*[4c] This increase of NAT activity caused by incubation with DZP (10^{-3} M) was prevented by preincubation with PPL, PBZ or BCL (Table II). These *in vitro* results suggest that DZP acts on the rat pineal gland directly through certain receptors involved in melatonin synthesis. This difference between the results *in vivo* and *in vitro* may be due to a difference in the physiological state of the gland.

In conclusion, although the present results *in vivo* and *in vitro* did not define the site of the inhibitory action of DZP on melatonin synthesis in intact rat pineal gland, both GABA and peripheral BZP receptors may be involved in the modulation of this synthesis. We further found that DZP has an opposite action on the pineal melatonin synthesis under conditions of altered GABA neurotransmission.

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Aryloxyacetic Acid Diuretics with Uricosuric Activity. II. Substituted [(4-Oxo-4*H*-1-benzopyran-7-yl)oxy]acetic Acids and the Related Compounds

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Di- and tri-substituted [(4-oxo-4*H*-1-benzopyran-7-yl)oxy]acetic acids, and 4-oxo-3-phenyl-4*H*-furo[2,3-*h*]-[1]benzopyran-8-carboxylic acid were synthesized and tested for natriuretic and uricosuric activities. Among the compounds tested, 3,5-disubstituted [(4-oxo-4*H*-1-benzopyran-7-yl)oxy]acetic acids (6c—f, h, n and x) showed potent natriuretic and uricosuric activities, whereas 4-oxo-3-phenyl-4*H*-furo[2,3-*h*][1]benzopyran-8-carboxylic acid (6dd) possessed only potent natriuretic activity. The structure–activity relationships are also discussed.

Keywords [(5-chloro-4-oxo-4*H*-1-benzopyran-7-yl)oxy]acetic acid; natriuretic activity; uricosuric activity; structure–activity relationship; 4-oxo-3-phenyl-4*H*-furo[2,3-*h*][1]benzopyran-8-carboxylic acid

In the previous study[1] for diuretics with uricosuric activity, we discovered that [(5-chloro-4-oxo-3-phenyl-4*H*-1-benzopyran-7-yl)oxy]acetic acid (**6a**) showed potent natriuretic and uricosuric activities as well as moderate antihypertensive activity. In order to obtain diuretics with uricosuric activity, the investigation has been extended to the synthesis of di-(2,5- and 3,5-) and tri-(3,5,6-) substituted [(4-oxo-4*H*-1-benzopyran-7-yl)oxy]acetic acids and 4-oxo-3-phenyl-4*H*-furo[2,3-*h*][1]benzopyran-8-carboxylic acid, and to the evaluation of their compounds for natriuretic and uricosuric activities. As a result of this research, we found that some of the 3,5-disubstituted [(4-oxo-4*H*-1-benzopyran-7-yl)oxy]acetic acids which were new aryloxyacetic acids exhibited potent natriuretic and uricosuric activities.

Herein we report the synthesis, the biological evaluation, and structure–activity relationships for these new aryloxyacetic acids.

Chemistry Most of the [(4-oxo-4*H*-1-benzopyran-7-yl)oxy]acetic acids (**6**) were synthesized according to the route shown in Chart 1.

The 7-methoxy-4-oxo-4*H*-1-benzopyrans (**3**) were prepared by methods B, C, D and E described in the literature[2] from 1-acyl-2-hydroxy-4-methoxybenzenes (**2**), which were obtained from the substituted 3,5-dimethoxybenzenes (**1**) and substituted acetyl chlorides under typical Friedel–Crafts reaction (method A). Demethylation of **3** with anhydrous aluminum chloride in benzene afforded the hydroxy derivatives **4** in high yields (method F). Treatment of **4** with ethyl bromoacetate gave the esters **5** (method G), which were hydrolyzed to yield **6** (method H).

The 5-trifluoromethyl compound **6t** was prepared in a

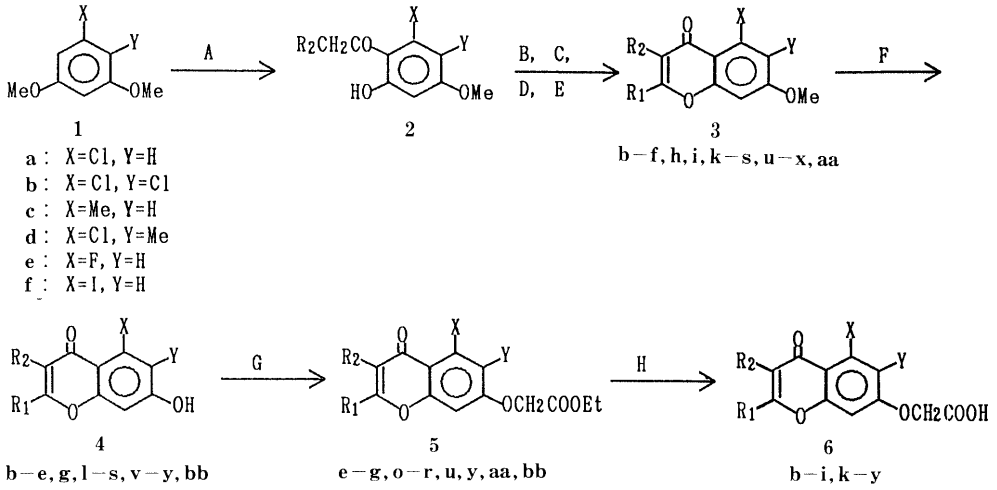

Chart 1

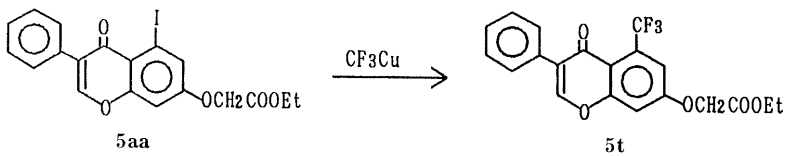

Chart 2

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good yield from ethyl [(4-oxo-3-phenyl-5-trifluoromethyl-4*H*-1-benzopyran-7-yl)oxy]acetate (**5t**), which was synthesized from the 5-iodobenzopyran derivative **5aa** by reaction with trifluoromethyl iodide in hexamethylphosphoramide (HMPA) in the presence of copper powder in a sealed tube (Chart 2).[3]

The compounds **6i** and **6j** having a methoxy and a hydroxy group at position 2 on the 3-phenyl group, respectively, were synthesized by the route shown in Chart 3. Demethylation of 7-methoxy-3-(2-methoxyphenyl)benzopyran (**3i**) with excess anhydrous aluminum chloride yielded the 7-hydroxy-3-(2-methoxyphenyl)benzopyran derivative containing a small amount of the 7-hydroxy-3-(2-hydroxyphenyl)benzopyran derivative. Reaction of the mixture with ethyl bromoacetate followed by methylation with methyl iodide gave ethyl [(5-chloro-3-(2-methoxyphenyl)-4-oxo-4*H*-1-benzopyran-7-yl)oxy]acetate (**5i**), which was converted to the 3-(2-hydroxyphenyl)benzopyran derivative **5j** by demethylation with boron tribromide at −78 °C. Reaction of **5j** with boron tribromide in dichloromethane under reflux gave **6j**,[4] while hydrolysis of **5i** afforded **6i** (Chart 1).

The compound **6h** and **6k** having a *p*-methoxy group on the 3-phenyl group were prepared by the route shown in Chart 4 and Chart 1, respectively. Alkylation of **4g** and **4bb** with an equimolar amount of ethyl bromoacetate in the presence of potassium carbonate gave the esters **5g** and **5bb** in high yields, respectively. The substitution at 7-OH, but not at 4-OH, on the 3-phenyl groups was proved from the fact that C-3 and C-5 proton signals (at δ 6.96 and 6.92) on the 3-phenyl group of **7** having a 4-acetoxy group were shifted to a lower field than those (at δ 6.60 and 6.56) of **5bb** on the proton nuclear magnetic resonance (^{1}H-NMR) spectra. The compounds **5g** and **5bb** were converted to **5h** and **5k**, respectively, according to the route shown in Chart 4.

The compound **6y**, which has a phenyl group at position 2, was prepared *via* **3y** (Chart 5). Friedel–Crafts reaction of **1a** with acetyl chloride according to the method A gave the acetophenone derivatives, which consisted of a mixture of 2-chloro-6-hydroxy-4-methoxyacetophenone and 4-chloro-2-hydroxy-6-methoxyacetophenone (1 : 1). Reaction of the mixture with benzaldehyde in potassium hydroxide solution yielded the α,β-unsaturated ketones **8** and **9**, which were easily separated by silica gel column chromatography. The compounds **8** and **9** were heated in the presence of iodine in dimethyl sulfoxide (DMSO) to give **3y** and the 2-phenyl-5-methoxybenzopyran derivative **10**, respectively (method I).[5] Compound **3y** was converted to **6y** by the route shown in Chart 1.

The chemical structure of **3y** was confirmed on the basis of the following findings. On the ^{1}H-NMR spectrum, the signal attributable to the 5-OH group of **11** which was prepared from **10** according to method F was shifted to a lower field (at δ 12.74) than that (at δ 11.02) of the 7-OH group of **4y** obtained by demethylation of **3y**. The comparison of **11** with **4y** on a TLC showed that the *Rf* value of **11** was much higher than that of **4y** (see

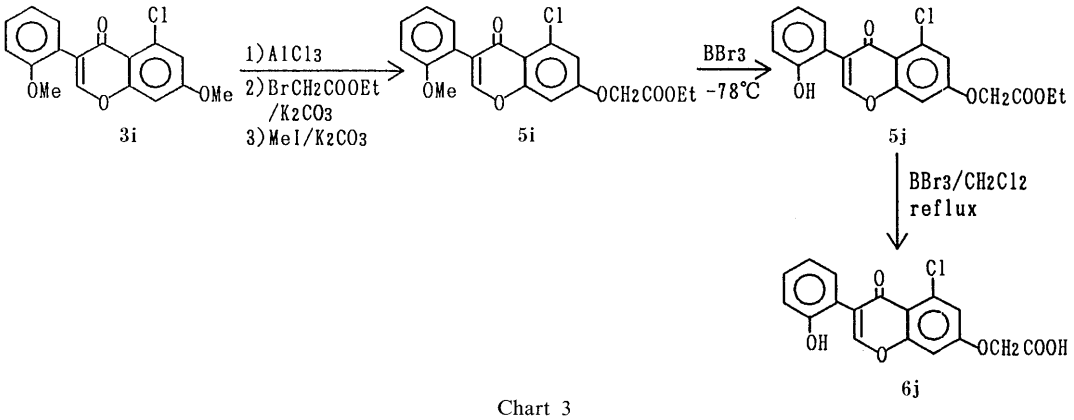


Chart 3

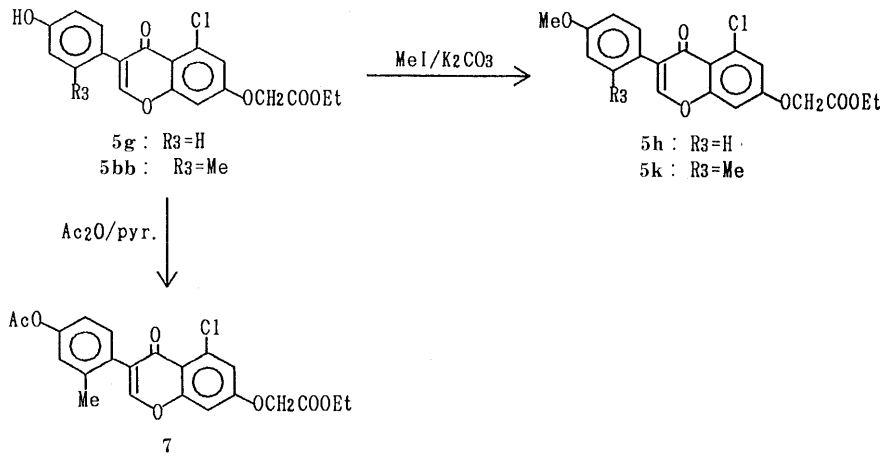


Chart 4

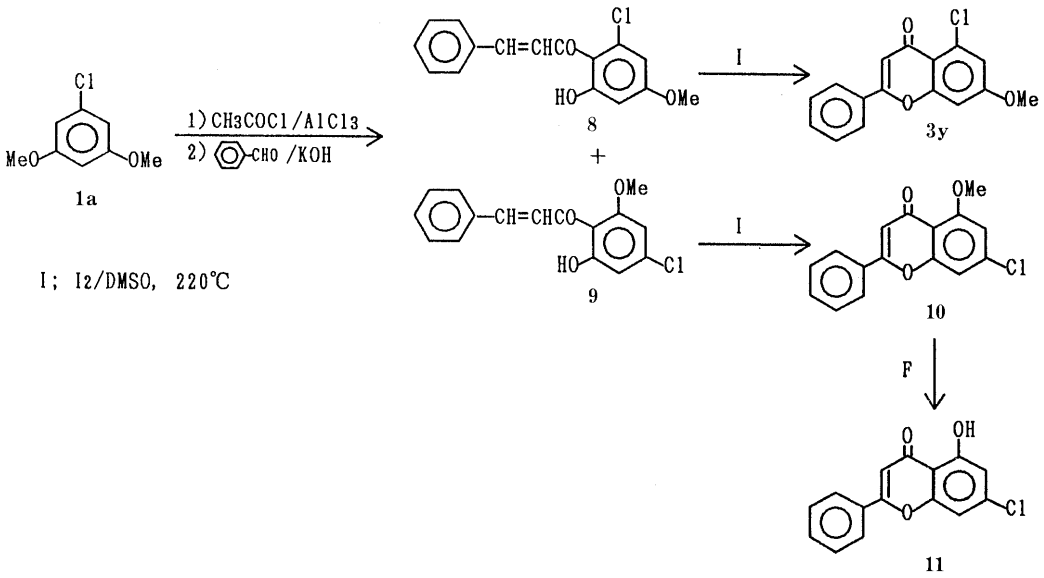


Chart 5

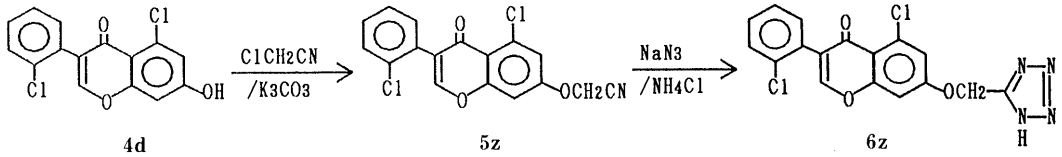


Chart 6

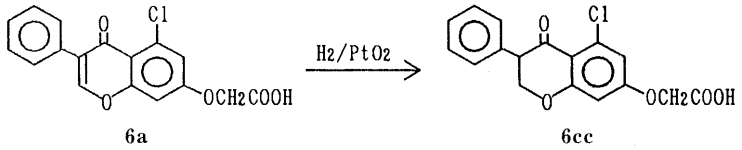


Chart 7

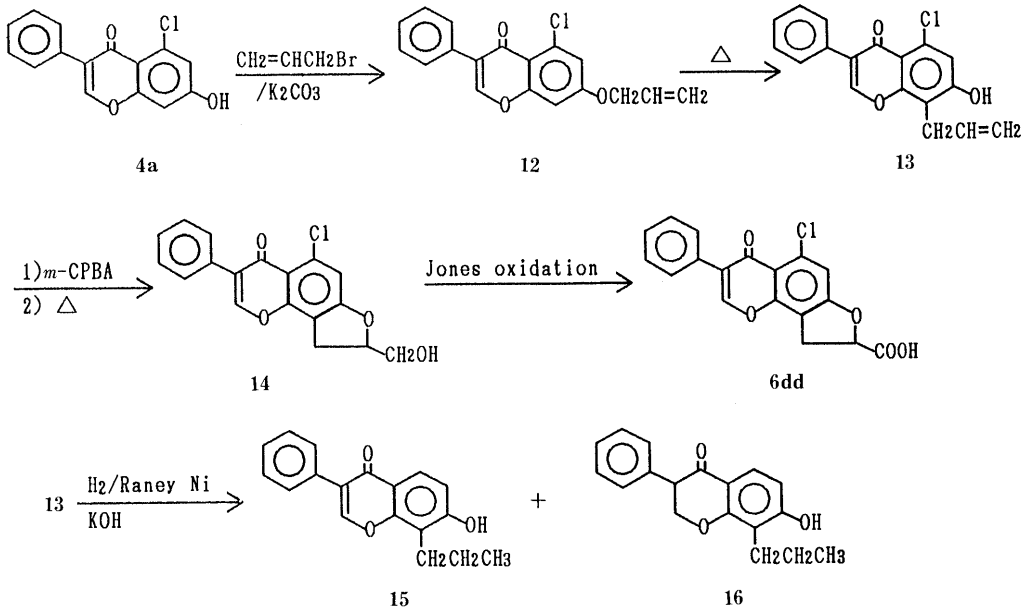


Chart 8

Experimental). The data show that compound **11** forms a hydrogen bond between the 5-hydroxy and the 4-carbonyl groups. On the other hand, the compound **4y** has a free phenolic hydroxy group at position 7.

The tetrazole derivative **6z** was prepared according to the route shown in Chart 6. Reaction of the 7-hydroxy

derivative **4d** with chloroacetonitrile gave the cyano compound **5z**, which was converted to **6z** by reaction with sodium azide in the presence of ammonium chloride.[6]

The 2,3-dihydro compound **6cc** was prepared from **6a** by catalytic hydrogenation in the presence of platinum oxide (Chart 7).

The furo[2,3-*h*][1]benzopyran derivative **6dd** was prepared according to the route shown in Chart 8. Allylation of **4a** and subsequent Claisen rearrangement gave the 8-allyl derivative **13**. Epoxidation of **13** with *m*-chloroperbenzoic acid followed by heating at 120—140 °C gave the alcohol **14**. Jones oxidation of **14** yielded **6dd** in a low yields.[7] The structure of **6dd** was confirmed by the fact that **15** and **16**, which were obtained by hydrogenation of **13** in the presence of Raney nickel,[8] showed *ortho* coupling between C-5 and C-6 protons on their ^{1}H-NMR spectra, respectively (see experimental section).

Structure–Activity Relationships. Saluresis-Diuresis The excretion of urine, Na^+, K^+, and Cl^- was measured in the experiments conducted in rats, but for brevity, only the Na^+ excretion is reported here. The excretion of Cl^- and urine generally paralleled that of the Na^+; thus, either of these could have been used for relative potency comparisons. The results are summarized in Table I and the data are shown as ratios to the controls. The excretion of Na^+ in the control groups was 0.33 meq/kg·B.W. Tienilic acid, indacrinone and furosemide were used as reference compounds.

Table I. Natriuretic Activities of Substituted [(4-Oxo-4*H*-1-benzopyran-7-yl)oxy]acetic Acids and the Related Compounds

Compd. No.	Natriuretic (Rat)[a]	Compd. No.	Natriuretic (Rat)[a]	Compd. No.	Natriuretic (Rat)[a]
6a	3.37	**6l**	4.12	**6w**	1.15[c]
6b	1.78	**6m**	2.56	**6x**	3.29
6c	3.12	**6n**	3.85	**6y**	1.17[c]
6d	3.50	**6o**	1.48[c]	**6z**	0.80[c]
6e	3.43	**6p**	2.45	**6cc**	3.51
6f	4.15	**6q**	2.76	**6dd**	5.60
6g	0.80[c]	**6r**	1.98	**6ee**	1.19[c]
6h	3.35	**6s**	1.68	TA[b,d]	1.80
6i	0.97[c]	**6t**	2.71	MK-196[e]	2.26
6j	1.20[c]	**6u**	1.27[c]	Fr[f]	4.60
6k	2.98	**6v**	0.85[c]		

a) Ratio to control (treated/control value) is shown. Animal: SLC-Wistar, 100 mg/kg, *p.o.*/5 h. *b*) Dose: 300 mg/kg *p.o.* *c*) The difference from the control is not statistically significant. *d*) TA is "tienilic acid". *e*) MK-196 is "indacrinone". *f*) Fr is "furosemide".

Table II. Uricosuric Activities of Substituted [(4-Oxo-4*H*-1-benzopyran-7-yl)oxy]acetic Acids and the Related Compounds

Compd. No.	Uricosuric[a]	Compd. No.	Uricosuric[a]	Compd. No.	Uricosuric[a]
6a	++	**6l**	+[c]	**6x**	+
6b	+[b]	**6m**	+	**6cc**	+[b]
6c	+	**6n**	+	**6dd**	−[c]
6d	+	**6q**	++	TA	+
6e	+	**6r**	++[b]	MK-196	+
6f	+	**6s**	+[b]		
6h	+	**6t**	+		

a) Excretion of uric acid (%, FEua, 100 mg/kg, *p.o.* in rats): $-\leqq 0$, $0 < + \leqq 50$, $50 < ++ \leqq 150$, $150 < +++$. *b*) 200 mg/kg, *p.o.* in rats. *c*) 30 mg/kg, *p.o.* in rats.

In considering the structural requirement for natriuretic activity within this series, it is convenient to divide these compounds into four groups, the first group: substituted [(4-oxo-4*H*-1-benzopyran-7-yl)oxy]acetic acids (**6a**—**y**), the second group: 5-[(5-chloro-3-(2-chlorophenyl)-4-oxo-4*H*-1-benzopyran-7-yl)oxymethyl]tetrazole (**6z**), the third group: [(5-chloro-2,3-dihydro-4-oxo-3-phenyl-4*H*-1-benzopyran-7-yl)oxy]acetic acid (**6cc**), and the fourth group: 5-chloro-8,9-dihydro-4-oxo-3-phenyl-4*H*-furo[2,3-*h*][1]benzopyran-8-carboxylic acid (**6dd**).

Among the first group, the relative effectiveness of the substituents at position 5 in producing natriuretic activity was $Cl > Me \geqq CF_3 \gg F \geqq H$ (**6a** > **6q** ≧ **6t** ≫ **6u** ≧ **6ee**). The 5,6-disubstituted compounds **6v** and **6w** lacked natriuretic activity. From these results, it was found that the 5-Cl compounds showed the most diuretic activity in this series. This is surprising, since in the earlier studies the relative activity contributed by the substituents between the carbonyl group and the oxyacetic acid side chain are generally as follows; vicinal di Cl group ≫ mono Cl group at the *ortho* position of carbonyl group.[9]

Variants at position 3 of the 4-oxo-4*H*-1-benzopyran ring system were evaluated in a sub-group of the compound in which the Cl group was held constant at position 5. Among the compounds having the substituted phenyl group at position 3, the 2-F compound **6f** had a most potent effect on the natriuretic response and its activity was comparable with that of furosemide. Compounds **6c**, **d**, **e** and **h** demonstrated almost equivalent activity to **6a**. On the other hand, introducing *p*-Cl (**6b**), *p*-OH (**6g**) or *o*-OMe (**6i**) group into the 3-phenyl ring markedly decreased the activity. Introduction of the *p*-OMe group into the 3-phenyl ring of **6e** demonstrated only slightly less activity than that of **6e** (**6e** > **6k**). These results suggest that, in general, introducing an *ortho* substituent into the 3-phenyl group shows more potent activity than that of a *para* substituent except for compounds (**6h**, **6i**) having a methoxy group (**6d** > **6b**, **6f** > **6c**, **6j** ≧ **6g**).

Among the 3-alkyl compounds, the 3-isopropyl compound **6l** showed comparable activity with furosemide. The cyclopentyl compound **6m** also had more potent activity than that of MK-196. Among the 3-aralkyl compounds, the 3-benzyl compound **6n** retained more potent activity than that of **6a**, but the 3-phenethyl compound **6p** was less active and the introduction of an *o*-Cl group into the 3-benzyl group considerably decreased natriuretic effects (**6n** > **6a** > **6p** > **6o**).

In order to study the effect of a substituent on position 2 for natriuretic activity, the relative potency of **6x** and **6y** was compared with that of **6a**. Introducing a 2-Me group into **6a** retained almost equipotent activity to that of **6a**. However, the 2-phenyl compound **6y** having no substituent on position 3 exhibited significantly weak potency.

From these results, it is likely that substituents such as alkyl, aralkyl, phenyl and substituted phenyl groups at position 3 of the 4-oxo-4*H*-1-benzopyran ring nucleus are necessary for potent activity.

The tetrazole analogue **6z** in the second group completely lost the activity.

Reduction of 2,3-double bond of **6a** to afford the compound **6cc** in the third group retained equipotent activity to that of **6a**.

In regard to the fourth group, ring annelation of the oxyacetic acid chain to position 8 of the 4-oxo-4*H*-1-benzopyran ring demonstrated a large increase in potency (**6dd**). This result paralleled previous findings on tienilic acid and indeno[5,4-*b*]furan-2-carboxylic acids.[7,10]

The data presented above indicate that in the 4-oxo-4*H*-1-benzopyran series.

(1) Substituents at position 5 in these compounds are important for the natriuretic activity.

(2) Substituents such as bulky alkyl, aralkyl, phenyl and substituted phenyl group at position 3 are required for the natriuretic activity.

(3) The ring annelation of the oxyacetic acid chain to position 8 of the 4-oxo-4*H*-1-benzopyran ring nucleus enhances the natriuretic activity.

Uricosuric Activity The compounds which displayed potent diuretic activity were tested for uricosuric activities. As shown in Table II, compounds **6a** and **6q** showed more potent uricosuric activity than MK-196 and TA. The other compounds had equipotent activity to MK-196 and TA except for **6b**, **6l**, **6s**, **6cc**. From these results it was difficult to relate the uricosuric activity with the structures in this series. As observed in this series, the uricosuric activities did not run parallel with the natriuretic activities.

In this series, [(5-chloro-3-(2-methylphenyl)-4-oxo-4*H*-1-benzopyran-7-yl)oxy]acetic acid (**6e**) showed a good balance of diuretic, uricosuric and the other biological activities. We therefore selected **6e** for further investigation, and evaluation of the compound is in progress.

Experimental

Melting points were determined on a Yanagimoto melting point apparatus and are uncorrected. The ^{1}H-NMR spectra were taken on a Hitachi R40 and JEOL JNM-FX90Q spectrometers with tetramethylsilane (TMS) as an internal standard. Signal multiplicities are represented by s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), m (multiplet). Chemical shifts are expressed in δ values and the coupling constants in Hz. For column chromatography, silica gel (Kieselgel 60, 70—230 mesh, Merck) was used. Infrared (IR) spectra were recorded in KBr tablet with Hitachi 285 and Hitachi 270-30 spectrophotometers.

Starting Materials and Standard Drugs Starting materials **1b—f** were prepared according to a similar manner to the reported method.[11] (4-Methoxy-2-methylphenyl)acetyl chloride, which was the starting material of **6k**, was prepared according to the reported method.[12] [(5-Chloro-4-oxo-3-phenyl-4*H*-1-benzopyran-7-yl)oxy]acetic acid (**6a**) and [(4-oxo-3-phenyl-4*H*-1-benzopyran-7-yl)oxy]acetic acid (**6ee**) were prepared according to the methods previously reported.[1]

2-Substituted and 2,3-Disubstituted 1-Acyl-6-hydroxy-4-methoxybenzenes (2) (Method A) Compounds **2** were prepared in a similar manner to that previously reported[1] and used for the next synthetic stage after removing the starting materials by silica gel column chromatography.

5-Chloro-3-(4-chlorophenyl)-7-methoxy-4-oxo-4*H*-1-benzopyran (3b) (Method B) Compound **3b** was prepared from the corresponding compound **2** according to a manner similar to that of the previous paper.[1]

Compounds **3c**, **d**, **q**, **v**, **w** and **aa** were prepared in a similar manner and the results are shown in Table III.

5-Chloro-7-methoxy-3-(2-methylphenyl)-4-oxo-4*H*-1-benzopyran (3e) (Method C) The crude compound **2** (R_2 = 2-methylphenyl, X = Cl, Y = H, 303 g, 1.04 mol) was dissolved in a mixture of 1,4-dioxane (1 l) and ethyl formate (1.3 l, 17.6 mol), and then 60% NaH (113 g, 2.82 mol) was added in small portions to the solution under ice-cooling with stirring. The mixture was heated at 40 °C for 3 h. The reaction mixture was poured into ice-cooled 20% HCl and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O, dried (Na_2SO_4) and concentrated to dryness *in vacuo*. The residue was heated at 160 °C under reduced pressure for 1 h, and recrystallized from C_6H_6 to give **3e** (250 g, 79.6%).

Compounds **3f**, **h**, **i**, **k**, **n**, **o**, **p**, **r**, **s** and **u** were prepared in a similar manner and the results are shown in Table III.

5-Chloro-3-isopropyl-7-methoxy-4-oxo-4*H*-1-benzopyran (3l) (Method D)[2a] The crude compound **2** ($R_2 = CH(CH_3)_2$, X = Cl, Y = H, 45.3 g, 0.187 mol) was dissolved in ethyl formate (230 ml), and Na wire (12.4 g, 0.54 mol) was added to the solution, and then the reaction mixture was stirred at room temperature for 3 h. After the Na wire was completely dissolved, the reaction mixture was heated at 60 °C with stirring for 2 h. The reaction mixture was poured into ice-cooled 20% HCl (1 l), and the organic layer was separated. The aqueous layer was extracted with $CHCl_3$. The organic layer and the $CHCl_3$ layer were combined, washed successively with H_2O and brine, dried (Na_2SO_4), and then concentrated *in vacuo* to dryness. The resulting residue was heated at 160 °C for 1 h under reduced pressure. Recrystallization of the residue from C_6H_6 gave **3l** (17.6 g, 38.4%).

Compound **3m** was prepared in a similar manner and the results are shown in Table III.

5-Chloro-7-methoxy-2-methyl-4-oxo-3-phenyl-4*H*-1-benzopyran (3x) (Method E) Compound **3x** was prepared from the corresponding compound **2** and ethyl orthoacetate according to the reported method.[2a]

The results are shown in Table III.

5-Chloro-7-methoxy-4-oxo-2-phenyl-4*H*-1-benzopyran (3y) (Method I)[5] A mixture of **8** (7.25 g, 25.1 mmol) and I_2 (0.25 g, 2 mmol) in Me_2SO (120 ml) was heated at 220 °C with stirring for 40 min. After cooling to room temperature, the reaction mixture was poured into H_2O (800 ml). The resulting precipitates were collected by filtration and purified by silica gel (200 g) column chromatography to give **3y** (3.70 g, 51.4%).

The results are shown in Table III.

5-Chloro-7-hydroxy-3-(2-methylphenyl)-4-oxo-4*H*-1-benzopyran (4e) (Method F) Compound **4e** was prepared from **3e** according to a manner similar to that of the previous paper.[1]

Compounds **4b—d**, **l—s** and **v—y** were prepared in a similar manner and the results are shown in Table IV.

5-Chloro-7-hydroxy-3-(4-hydroxy-2-methylphenyl)-4-oxo-4*H*-1-benzopyran (4bb) Compound **4bb** was prepared from **3k** and anhydrous $AlCl_3$ (6 eq) according to a manner similar to method F.

Compound **4g** was prepared in a similar manner and the results are shown in Table IV.

Ethyl [(5-Chloro-3-(2-methylphenyl)-4-oxo-4*H*-1-benzopyran-7-yl)-oxy]acetate (5e) (Method G) Compound **5e** was prepared from **4e** according to a manner similar to that of the previous paper.[1]

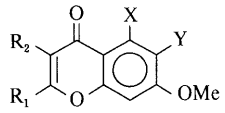
Compounds **5f**, **o—r**, **u**, **y** and **aa** were prepared in a similar manner and the results are shown in Table V.

Ethyl [(5-Trifluoromethyl-4-oxo-3-phenyl-4*H*-1-benzopyran-7-yl)oxy]-acetate (5t) A copper powder (9.5 g) and hexamethylphosphoric triamide (30 ml) were placed in a stainless steel-made reaction tube, and trifluoromethyl iodide (6 ml, 50 mmol) was added to the mixture with cooling by dry ice-acetone. The mixture was heated at 125 °C with stirring for 2 h. After cooling and then adding **5aa** (3.5 g, 7.7 mmol), the solution was heated at 45 °C with stirring for 12 h under an atmosphere of nitrogen in a closed system. The reaction mixture was poured into C_6H_6–AcOEt (1 : 1, v/v, 500 ml), and ice-cold H_2O (500 ml) was added, followed by stirring for 30 min. The resulting precipitates were removed by filtration through celite, and the filtrate was washed with H_2O, and then dried (Na_2SO_4). The solvent was removed *in vacuo* to give a residue. The resulting residue was purified by silica gel (300 g) column chromatography, and then recrystallized from EtOH to yield **5t** (2.65 g, 88%).

The results are shown in Table V.

Ethyl [(5-Chloro-3-(2-methoxyphenyl)-4-oxo-4*H*-1-benzopyran-7-yl)-oxy]acetate (5i) A mixture of **3i** (20 g, 63 mmol) and anhydrous $AlCl_3$ (42 g, 0.32 mol) in C_6H_6 (200 ml) was refluxed with stirring for 3 h. After cooling, the solvent was removed by decantation, and ice-cold H_2O (1 l) was added to the resulting residue. The reaction mixture was then agitated vigorously. The resulting crystals were collected by filtration, washed with hot H_2O, and then dried to give a mixture of 5-chloro-7-hydroxy-3-(2-methoxyphenyl)-4-oxo-4*H*-1-benzopyran and 5-chloro-7-hydroxy-3-(2-hydroxyphenyl)-4-oxo-4*H*-1-benzopyran (18.8 g, the mixture comprised 67 per cent 5-chloro-7-hydroxy-3-(2-methoxyphenyl)-4-oxo-4*H*-1-benzopyran and 33 per cent 5-chloro-7-hydroxy-3-(2-hydroxyphenyl)-4-oxo-4*H*-1-benzopyran). Ethyl bromoacetate (10 g, 60 mmol) and K_2CO_3 (8.3 g, 60 mmol) were added to the mixture (17 g) in Me_2CO (500 ml), and then the mixture was refluxed with stirring for 4 h. After cooling, the solvent was removed *in vacuo*, and ice-cooled 10% HCl (excess) was added to the resulting residue. The reaction mixture was then extracted with $CHCl_3$. The $CHCl_3$ layer was dried (Na_2SO_4), concentrated *in vacuo*, and then purified by silica gel (500 g) column chromatography with C_6H_6–AcOEt (1 : 1, v/v) as an eluent to give a mixture (14 g) of **5i** and **5j**. K_2CO_3 (2.9 g,

TABLE III. Di- and Tri-Substituted 7-Methoxy-4-oxo-4*H*-1-benzopyrans (**3**)



Compd. No.	R_1	R_2	X	Y	mp (°C) (Solv.)	Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	IR (KBr) cm^{-1}	Solv.[d]	^{1}H-NMR Chemical shift (δ)[e]
3b	H	*p*-ClC_6H_4	Cl	H	264—267 (C_6H_6)	11.8[b]	$C_{16}H_{10}Cl_2O_3$	59.83 (59.47	3.14 3.44)	1648	C	7.82 (1H, s), 7.27 (4H, s), 6.97 (1H, d, J=3), 6.78 (1H, d, J=3), 3.81 (3H, s)
3c	H	*p*-FC_6H_4	Cl	H	216—217 (C_6H_6)	22.6[b]	$C_{16}H_{10}ClFO_3$	63.07 (62.92	3.31 3.50)	1640	C	7.86 (1H, s), 7.50—6.90 (5H, m), 6.80 (1H, d, J=3), 3.91 (3H, s)
3d	H	*o*-ClC_6H_4	Cl	H	159—162[a]	33.9[b]	$C_{16}H_{10}Cl_2O_3$	59.83 (59.86	3.14 3.21)	1640	C	7.70 (1H, s), 7.50—7.00 (4H, m), 6.94 (1H, d, J=3), 6.73 (1H, d, J=3), 3.86 (3H, s)
3e	H	*o*-MeC_6H_4	Cl	H	151—153 (EtOH)	51.0[b]	$C_{17}H_{13}ClO_3$	67.89 (67.94	4.36 4.66)	1640	C	7.69 (1H, s), 7.40—7.10 (4H, m), 6.98 (1H, d, J=3), 6.79 (1H, d, J=3), 3.88 (3H, s), 2.55 (3H, s)
3f	H	*o*-FC_6H_4	Cl	H	150—151 (EtOH)	37.7[b]	$C_{16}H_{10}ClFO_3$	63.07 (63.03	3.31 3.36)	1664	C	7.78 (1H, s), 7.60—7.00 (4H, m), 6.91 (1H, d, J=3), 6.72 (1H, d, J=3), 3.85 (3H, s)
3h	H	*p*-$MeOC_6H_4$	Cl	H	161—162 (EtOH)	36.2[b]	$C_{17}H_{13}ClO_4$	64.47 (64.18	4.14 4.16)	1652	C	8.20 (1H, s), 7.40 (2H, d, J=9), 7.05 (2H, s), 6.92 (2H, d, J=9), 3.87 (3H, s), 3.77 (3H, s)
3i	H	*o*-$MeOC_6H_4$	Cl	H	168—169 (EtOH)	31.1[b]	$C_{17}H_{13}ClO_4$	64.46 (64.43	4.14 4.17)	1650	D	8.08 (1H, s), 7.45—6.80 (4H, m), 7.03 (2H, s), 3.87 (3H, s), 3.70 (3H, s)
3k	H	*o*-Me, *p*-MeO $-C_6H_3$	Cl	H	141—142 (EtOH)	26.3[b]	$C_{18}H_{15}ClO_4$	65.36 (65.25	4.57 4.61)	1640	C	7.60 (1H, s), 7.00 (1H, d, J=8), 6.92 (1H, br s), 6.71 (2H, s), 6.68 (1H, d like, J=8), 3.86 (3H, s), 3.77 (3H, s), 2.20 (3H, s)
3l	H	$(Me)_2CH$	Cl	H	177—179 (C_6H_6)	37.0[b]	$C_{13}H_{13}ClO_3$	61.79 (62.03	5.19 5.23)	1640	C	7.54 (1H, s), 6.94 (1H, d, J=3), 6.71 (1H, d, J=3), 3.87 (3H, s), 3.30—2.80 (1H, m), 1.20 (6H, d, J=7)
3m	H	cyclo-C_5H_9	Cl	H	114—115 (C_6H_6)	51.0[b]	$C_{15}H_{13}ClO_3$	64.63 (64.70	5.42 5.36)	1610	C	6.93 (1H, d, J=3), 6.71 (1H, d, J=3), 3.86 (3H, s), 3.30—2.80 (1H, m), 2.3—1.2 (8H, m)
3n	H	$C_6H_5CH_2$	Cl	H	161—163 (EtOH)	31.5[b]	$C_{17}H_{13}ClO_3$	67.89 (67.94	4.36 4.46)	1608	C	7.30 (1H, s), 7.20 (5H, s), 6.83 (1H, d, J=2), 6.59 (1H, d, J=2), 3.78 (3H, s), 3.70 (2H, s)
3o	H	*o*-$ClC_6H_4CH_2$	Cl	H	146—148 (EtOH)	29.6[b]	$C_{17}H_{12}Cl_2O_3$	60.91 (60.73	3.61 3.76)	1650	C	7.60—7.10 (5H, m), 6.95 (1H, d, J=3), 6.70 (1H, d, J=3), 3.86 (5H, s)
3p	H	$C_6H_5(CH_2)_2$	Cl	H	132—133 (EtOH)	16.0[b]	$C_{18}H_{15}ClO_3$	68.68 (68.88	4.80 4.90)	1652	C	7.37 (1H, s), 7.20 (5H, s), 6.90 (1H, d, J=3), 6.65 (1H, d, J=3), 3.85 (3H, s), 3.10—2.50 (4H, m)
3q	H	C_6H_5	Me	H	120—122 (EtOH)	19.0[b]	$C_{17}H_{14}O_3$	76.68 (76.69	5.30 5.45)	1620	C	7.70 (1H, s), 7.60—7.20 (5H, m), 6.60 (2H, br s), 3.78 (3H, s), 2.80 (3H, s)
3r	H	*o*-MeC_6H_4	Me	H	135—136 (EtOH)	14.4[b]	$C_{18}H_{16}O_3$	77.12 (77.17	5.75 5.89)	1655	C	7.70 (1H, s), 7.50—7.00 (4H, m), 6.72 (2H, s), 3.90 (3H, s), 2.84 (3H, s), 2.23 (3H, s)
3s	H	*o*-ClC_6H_4	Me	H	154—156 (C_6H_6–*n*-C_6H_{14})	30.0[b]	$C_{17}H_{13}ClO_3$	67.89 (68.17	4.36 4.54)	1645	C	7.78 (1H, s), 7.60—7.20 (4H, s), 6.73 (2H, s), 3.89 (3H, s), 2.83 (3H, s)
3u	H	C_6H_5	F	H	112—114 (EtOH)	40.8[b]	$C_{16}H_{11}FO_3$	71.72 (71.35	4.10 4.25)	1642	C	7.70 (1H, s), 7.60—7.10 (5H, m), 6.54 (1H, m), 6.48 (1H, dd, J=3, 12), 3.78 (3H, s)
3v	H	C_6H_5	Cl	Cl	216—218 (C_6H_6)	48.1[b]	$C_{16}H_{10}Cl_2O_3$	59.83 (59.45	3.14 3.16)	1645	D	7.83 (1H, s), 7.50—7.20 (5H, m), 6.86 (1H, s), 3.97 (3H, s)
3w	H	C_6H_5	Cl	Me	168—169 (C_6H_6–*n*-C_6H_{14})	38.3[b]	$C_{17}H_{13}ClO_3$	67.89 (68.25	4.36 4.45)	1640	C	7.83 (1H, s), 7.70—7.30 (5H, m), 6.76 (1H, s), 3.94 (3H, s), 2.34 (3H, s)
3x	Me	C_6H_5	Cl	H	167—168 (EtOH)	38.3[b]	$C_{17}H_{13}ClO_3$	67.89 (67.77	4.36 4.43)	1650	C	7.50—7.10 (5H, m), 6.85 (1H, d, J=2), 6.68 (1H, d, J=2), 3.84 (3H, s), 2.21 (3H, s)
3y	C_6H_5	H	Cl	H	152—154 (EtOH)	50.0[c]	$C_{16}H_{11}ClO_3$	67.02 (66.66	3.87 3.63)	1641	C	7.85—7.65 (2H, m), 7.50—7.30 (3H, m), 6.85 (1H, d, J=2), 6.78 (1H, d, J=2), 6.60 (1H, s), 3.86 (3H, s)
3aa	H	C_6H_5	I	H	171—172 (MeOH)	10.8[b]	$C_{16}H_{11}IO_3$	50.82 (50.87	2.93 3.00)	1640	C	7.75 (1H, s), 7.53 (1H, d, J=3), 7.50—7.00 (5H, m), 6.73 (1H, d, J=3), 3.77 (3H, s)

a) Recrystallized from $Cl(CH_2)_2Cl$–EtOH. *b*) Yield from the corresponding compound **1**. *c*) Yield from **8**. *d*) C is $CDCl_3$, D is DMSO-d_6. *e*) Coupling constants (J) are given in Hz.

21 mmol) and MeI (6.0 g, 42.6 mmol) were added to the mixture (8.0 g) of **5i** and **5j** in Me_2CO (200 ml), and then the mixture was refluxed with stirring for 3 h. After cooling, an insoluble material was removed by filtration and the filtrate was concentrated to dryness *in vacuo*. The resulting residue was recrystallized from EtOH to give **5i** (7.0 g, 55.2% from **3i**).

The results are shown in Table V.

Ethyl [(5-Chloro-3-(2-hydroxyphenyl)-4-oxo-4*H*-1-benzopyran-7-yl)-oxy]acetate (5j) (from 5i) A solution of **5i** (2.5 g, 6.4 mmol) in dry CH_2Cl_2 (50 ml) was cooled at −78 °C by dry ice-acetone, and BBr_3 (0.9 ml, 9.7 mmol) was added dropwise to the solution at the same temperature. The reaction solution was stirred at 0 °C for 1 h, poured into ice-cooled 10% HCl (100 ml), and then extracted with $CHCl_3$. The $CHCl_3$ layer was dried (Na_2SO_4) and concentrated *in vacuo* to dryness. The resulting residue was recrystallized from EtOH to give **5j** (2.2 g, 91%).

The results are shown in Table V.

Ethyl [(5-Chloro-3-(4-methoxy-2-methylphenyl)-4-oxo-4*H*-1-benzo-pyran-7-yl)oxy]acetate (5k) A mixture of **5bb** (3.1 g, 8 mmol), K_2CO_3 (9.14 g, 66 mmol) and MeI (19.0 g, 134 mmol) in Me_2CO (80 ml) was refluxed with stirring for 12 h. The reaction mixture was poured into ice-cold H_2O (300 ml) and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O, dried (Na_2SO_4), and concentrated *in vacuo* to give **5k**, (3.57 g) as an oily residue. Compound **5k** was used for the next synthetic stage without further purification.

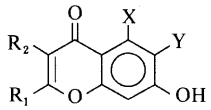
Compound **5h** was prepared in a similar manner and the results are shown in Table V.

[(5-Chloro-3-(2-chlorophenyl)-4-oxo-4*H*-1-benzopyran-7-yl)oxy]ace-tonitrile (5z) A mixture of **4d** (1.4 g, 4.6 mmol), K_2CO_3 (0.7 g, 5.1 mmol), KI (0.15 g, 0.9 mmol) and $ClCH_2CN$ (0.38 g, 5.0 mmol) in Me_2CO (50 ml) was refluxed for 8 h. H_2O (200 ml) was added to the reaction mixture, and the resulting precipitates were collected by filtration and then recrystallized from EtOH to give **5z** (1.4 g, 87.9%).

The results are shown in Table V.

Ethyl [(5-Chloro-3-(4-hydroxy-2-methylphenyl)-4-oxo-4*H*-1-benzo-pyran-7-yl)oxy]acetate (5bb) Compound **5bb** was prepared from **4bb** and

TABLE IV. Di- and Tri-Substituted 7-Hydroxy-4-oxo-4*H*-1-benzopyrans (**4**)



Compd. No.	R_1	R_2	X	Y	mp (°C) (Solv.)	Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	IR (KBr) cm^{-1}	Solv.[d]	^{1}H-NMR Chemical shift (δ)[e]
4b	H	*p*-ClC_6H_4	Cl	H	>290 (EtOH)	87.0[a]	$C_{15}H_8Cl_2O_3$	58.66 (58.64	2.63 2.81)	1630	D	8.33 (1H, s), 7.70—7.40 (4H, m), 6.95 (1H, d, J=2), 6.86 (1H, d, J=2)
4c	H	*p*-FC_6H_4	Cl	H	>290 (EtOH)	85.2[a]	$C_{15}H_8ClFO_3$	61.98 (61.81	2.77 3.00)	1635	D	8.31 (1H, s), 7.56 (2H, dd, J=6, 9), 7.24 (2H, dd, J=9, 9), 6.94 (1H, d, J=2), 6.85 (1H, d, J=2)
4d	H	*o*-ClC_6H_4	Cl	H	288—290 (EtOH–C_6H_6)	68.0[a]	$C_{15}H_8Cl_2O_3$	58.66 (58.89	2.63 2.70)	1640	D	8.28 (1H, s), 7.70—7.30 (4H, m), 6.99 (1H, d, J=3), 6.90 (1H, d, J=3)
4e	H	*o*-MeC_6H_4	Cl	H	>290 (MeOH)	96.0[a]	$C_{16}H_{11}ClO_3$	67.02 (67.11	3.87 3.98)	1634	D	8.13 (1H, s), 7.40—7.00 (4H, m), 6.95 (1H, d, J=2), 6.87 (1H, d, J=2), 2.15 (3H, s)
4g	H	*p*-HOC_6H_4	Cl	H	>300 (DMF–EtOH)	98.0[b]	$C_{15}H_9ClO_4$	62.41 (62.66	3.14 3.35)	1635	D	10.96 (1H, br s), 9.44 (1H, br s), 8.09 (1H, s), 7.28 (2H, d, J=8), 6.90—6.70 (4H, m)
4l	H	$(Me)_2CH$	Cl	H	256—260 (dec.) (EtOH–C_6H_6)	75.2[a]	$C_{12}H_{11}ClO_3$	60.19 (60.39	4.65 4.68)	1640	D	7.92 (1H, s), 6.89 (1H, d, J=2), 6.77 (1H, d, J=2), 3.20—2.80 (1H, m), 1.14 (6H, d, J=7)
4m	H	cyclo-C_5H_9	Cl	H	282—284 (C_6H_6)	99.0[a]	$C_{14}H_{13}ClO_3$	61.43 (61.73	5.16 4.91)	1640	D	7.95 (1H, s), 6.93 (1H, d, J=3), 6.81 (1H, d, J=3), 3.20—2.70 (1H, m), 2.2—1.2 (8H, m)
4n	H	$C_6H_5CH_2$	Cl	H	297—300 (dec.) (EtOH)	98.0[a]	$C_{16}H_{11}ClO_3$	67.02 (66.68	3.87 3.86)	1630	D	10.97 (1H, s), 8.02 (1H, s), 7.20 (5H, m), 6.82 (1H, d, J=3), 6.72 (1H, d, J=3), 3.60 (2H, s)
4o	H	*o*-$ClC_6H_4CH_2$	Cl	H	295—296 (EtOH)	99.0[a]	$C_{16}H_{10}Cl_2O_3$	59.83 (59.86	3.14 3.18)	1647	D	7.95 (1H, s), 7.27 (4H, m), 6.93 (1H, d, J=2), 6.82 (1H, d, J=2), 3.74 (2H, s)
4p	H	$C_6H_5(CH_2)_2$	Cl	H	240—241 (dec.) (EtOH)	99.8[a]	$C_{17}H_{13}ClO_3$	67.89 (67.75	4.36 4.42)	1640	D	7.83 (1H, s), 7.16 (5H, s), 6.83 (1H, d, J=3), 6.69 (1H, d, J=3), 2.90—2.50 (4H, m)
4q	H	C_6H_5	Me	H	252—253 (EtOH)	88.0[a]	$C_{16}H_{12}O_3$	76.48 (76.40	4.79 4.92)	1625	D	8.10 (1H, s), 7.60—7.10 (5H, m), 6.64 (2H, s), 2.70 (3H, s)
4r	H	*o*-MeC_6H_4	Me	H	247—248 (C_6H_6)	75.2[a]	$C_{17}H_{14}O_3$	76.67 (76.18	5.31 5.40)	1630	C	8.30 (1H, s), 7.50—7.20 (4H, m), 7.08 (2H, s), 2.84 (3H, s), 2.22 (3H, s)
4s	H	*o*-ClC_6H_4	Me	H	228—230 (MeOH)	46.0[a]	$C_{16}H_{11}ClO_3$	67.03 (66.71	3.87 3.99)	1640	D	10.75 (1H, br s), 8.11 (1H, s), 7.65—7.25 (4H, m), 6.72 (2H, s), 2.70 (3H, s)
4v	H	C_6H_5	Cl	Cl	>290 (DMF–EtOH)	89.2[a]	$C_{15}H_8Cl_2O_3$	58.60 (59.01	2.63 2.81)	1625	D	8.18 (1H, s), 7.50—7.20 (5H, s), 6.93 (1H, s)
4w	H	C_6H_5	Cl	Me	>300 (DMF–MeOH)	83.5[a]	$C_{16}H_{11}ClO_3$	67.03 (66.85	3.87 3.97)	1630	D	11.20 (1H, s), 8.25 (1H, s), 7.65—7.30 (5H, m), 6.89 (1H, s), 2.25 (3H, s)
4x	Me	C_6H_5	Cl	H	>300 (EtOH)	99.0[a]	$C_{16}H_{11}ClO_3$	67.02 (66.79	3.87 3.90)	1620	D	7.40—7.10 (5H, m), 6.83 (1H, d, J=3), 6.74 (1H, d, J=3), 2.16 (3H, s)
4y	C_6H_5	H	Cl	H	>300 (DMF–MeOH)	99.0[a]	$C_{15}H_9ClO_3$	66.07 (65.93	3.33 3.38)	1629	D	11.02 (1H, br s), 8.00—7.85 (2H, m), 7.60—7.40 (3H, m), 7.01 (1H, d, J=1), 6.83 (1H, d, J=1), 6.75 (1H, s)
4bb	H	*o*-Me, *p*-HO $-C_6H_3$	Cl	H	290—292 (dec.) (EtOH–C_6H_6)	98.6[c]	$C_{16}H_{11}ClO_4$	63.48 (63.70	3.66 3.92)	1600	D	9.12 (1H, br s), 7.88 (1H, s), 6.79 (2H, br s), 6.78 (1H, d, J=11), 6.55 (1H, s), 6.50 (1H, dd, J=2, 11), 3.27 (1H, br s), 2.00 (3H, s)

a) Yield from the corresponding methoxy derivative **3**. *b*) Yield from **3h**. *c*) Yield from **3k**. *d*) D is DMSO-d_6, C is $CDCl_3$. *e*) Coupling constants (J) are given in Hz.

ethyl bromoacetate (1 eq) according to a manner similar to method G.

Compound **5g** was prepared in a similar manner and the results are shown in Table V.

[(5-Chloro-3-(2-methylphenyl)-4-oxo-4*H*-1-benzopyran-7-yl)oxy]acetic Acid (6e) (Method H) Compound **6e** was prepared from the corresponding compound **5e** according to a similar manner as the previous method.[1]

Compounds **6b—d**, **f—i** and **k—y** were prepared in a similar manner and the results are shown in Table VI.

[(5-Chloro-3-(2-hydroxyphenyl)-4-oxo-4*H*-1-benzopyran-7-yl)oxy]-acetic Acid (6j) BBr_3 (3 ml, 32.3 ml) was added dropwise to a solution of **5j** (1.7 g, 4.5 mmol) in dry CH_2Cl_2 (100 ml) with cooling by dry ice-acetone at −78 °C. After the addition, the reaction mixture was stirred at room temperature for 2 h, and then the mixture was refluxed for 3 h. After cooling, the solvent was evaporated *in vacuo*, and 10% HCl (100 ml) was added to the resulting residue with stirring. The resulting precipitates were collected by filtration, washed with H_2O, dried, and recrystallized from EtOH to give **6j** (1.0 g, 64%).

The results are shown in Table VI.

5-[(5-Chloro-3-(2-chlorophenyl)-4-oxo-4*H*-1-benzopyran-7-yl)oxymethyl]tetrazole (6z) NaN_3 (0.26 g, 4.0 mmol) and NH_4Cl (0.21 g, 3.93 mmol) were added to a solution of **5z** (1.2 g, 3.5 mmol) in Me_2NCHO (60 ml). The mixture was heated at 110 °C for 12 h. The reaction mixture was poured into ice-cold H_2O (100 ml) and acidified with concentrated HCl. The precipitated crystals were collected by filtration, washed with H_2O and then dried to give **6z** (1.1 g, 80.8%).

The results are shown in Table VI.

[(5-Chloro-2,3-dihydro-4-oxo-3-phenyl-4*H*-1-benzopyran-7-yl)oxy]-acetic Acid (6cc) Compound **6a** (1.0 g, 3.0 mmol) in MeOH (200 ml) was catalytically hydrogenated with PtO_2 under atmospheric pressure with an absorption of 67 ml of H_2 gas. After removal of the catalyst by filtration, the solvent was evaporated to dryness *in vacuo*. The resulting residue was recrystallized from $CHCl_3$ to give **6cc** (0.4 g, 40%).

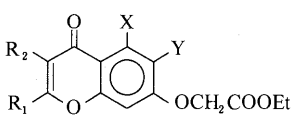
The results are shown in Table VI.

5-Chloro-8,9-dihydro-4-oxo-3-phenyl-4*H*-furo[2,3-*h*][1]benzopyran-8-carboxylic Acid (6dd) Jones reagent (175 ml)[7] was added dropwise to a stirred and cooled solution of **14** (30.5 g, 92.8 mmol) in Me_2CO (1.2 l) at such a rate that the internal temperature did not exceed 20±5 °C. The resulting mixture was stirred at 25 °C for 3 h. Then the reaction mixture was poured into ice-cold H_2O (2 l) and the solution was cocentrated *in vacuo* to one-third. The resulting precipitates were collected by filtration, washed with H_2O, and recrystallized from EtOH to give **6dd** (8.45 g, 26.6%).

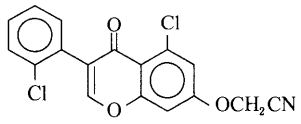
The results are shown in Table VI.

Ethyl [(3-(4-Acetoxy-2-methylphenyl)-5-chloro-4-oxo-4*H*-1-benzopyran-7-yl)oxy]acetate (7) A solution of **5bb** (0.194 g, 0.5 mmol) and AcCl (59 mg, 0.75 mmol) in dry pyridine (2.5 ml) was stirred at room temperature for 18 h. The reaction mixture was poured into ice-cooled 20% HCl solution and extracted with Et_2O. The extract was washed with H_2O, dried (Na_2SO_4) and concentrated *in vacuo* to yield an oily residue. The crude product was purified by silica gel (10 g) column chromatography with $CHCl_3$ as an eluent to give **7** (192 mg, 89.1%) as an oily product. ^{1}H-NMR

TABLE V. Di- and Tri-Substituted Ethyl [(4-Oxo-4*H*-1-benzopyran-7-yl)oxy]acetates (**5**) and the Related Compound (**5z**)



A

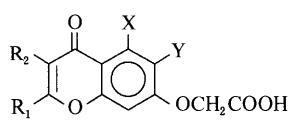


B

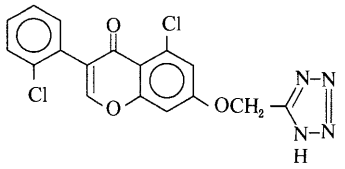
Compd. No.	Structure	R_1	R_2	X	Y	mp (°C) (Solv.)	Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	IR (KBr) cm^{-1}	Solv.[i]	^{1}H-NMR Chemical shift (δ)[j]
5e	A	H	*o*-MeC_6H_4	Cl	H	122—123 (EtOH)	89.0[a]	$C_{20}H_{17}ClO_5$	64.43 (64.76	4.60 4.76)	1756, 1650	C	7.70 (1H, s), 7.40—7.10 (4H, m), 7.03 (1H, d, J=3), 6.78 (1H, d, J=3), 4.70 (2H, s), 4.30 (2H, q, J=7), 2.24 (3H, s), 1.32 (3H, t, J=7)
5f	A	H	*o*-FC_6H_4	Cl	H	117—118 (EtOH)	78.0[b]	$C_{19}H_{14}ClFO_5$	60.57 (60.63	3.75 3.66)	1760, 1640	C	7.77 (1H, s), 7.50—6.90 (4H, m), 6.95 (1H, d, J=3), 6.70 (1H, d, J=3), 4.65 (2H, s), 4.26 (2H, q, J=6), 1.30 (3H, t, J=6)
5g	A	H	*p*-HOC_6H_4	Cl	H	186—187 ($ClCH_2CH_2Cl$)	76.0[a]	$C_{19}H_{15}ClO_6$	60.57 (60.82	4.01 4.09)	1745, 1640	D	9.44 (1H, s), 8.18 (1H, s), 7.28 (2H, d, J=9), 7.08 (2H, s), 6.75 (2H, d, J=9), 4.96 (2H, s), 4.16 (2H, q, J=6), 1.23 (3H, t, J=6)
5h	A	H	*p*-$MeOC_6H_4$	Cl	H	121—122 (EtOH–$CHCl_3$)	88.0[c]	$C_{20}H_{17}ClO_6$	61.78 (61.63	4.41 4.43)	1745, 1640	D	8.20 (1H, s), 7.40 (2H, d, J=9), 7.07 (2H, s), 6.92 (2H, d, J=9), 4.95 (2H, s), 4.17 (2H, q, J=6), 3.77 (3H, s), 1.23 (3H, t, J=6)
5i	A	H	*o*-$MeOC_6H_4$	Cl	H	135—136 (EtOH)	34.0[d]	$C_{20}H_{17}ClO_6$	61.78 (61.65	4.41 4.47)	1740, 1645	D	8.10 (1H, s), 7.45—6.70 (4H, m), 7.10 (2H, s), 4.96 (2H, s), 4.16 (2H, q, J=7), 3.70 (3H, s), 1.23 (3H, t, J=7)
5j	A	H	*o*-HOC_6H_4	Cl	H	142—143 (EtOH)	91.0[e]	$C_{19}H_{15}ClO_6$	60.89 (60.51	4.03 4.13)	1740, 1650	D	9.22 (1H, s), 8.10 (1H, s), 7.10 (2H, s), 7.25—7.00 (2H, m), 6.90—6.70 (2H, m), 4.97 (2H, s), 4.16 (2H, q, J=7), 1.22 (3H, t, J=7)
5k	A	H	*o*-Me, *p*-MeO –C_6H_3	Cl	H	Oil	98.0[f]					C	7.61 (1H, s), 6.97 (1H, d, J=9), 6.95 (1H, br s), 6.73 (2H, s), 6.66 (1H, d like, J=9), 4.66 (2H, s), 4.24 (2H, q, J=6), 3.77 (3H, s), 2.20 (3H, s), 1.30 (3H, t, J=6)
5o	A	H	*o*-$ClC_6H_4CH_2$	Cl	H	123—125 (EtOH)	88.0[a]	$C_{20}H_{16}Cl_2O_5$	58.98 (58.55	3.96 3.98)	1758, 1647	C	7.60—7.20 (5H, m), 6.98 (1H, d, J=3), 6.66 (1H, d, J=3), 4.67 (2H, s), 4.28 (2H, q, J=7), 3.87 (2H, s), 1.30 (3H, t, J=7)
5p	A	H	$C_6H_5(CH_2)_2$	Cl	H	114—115 (EtOH)	85.5[a]	$C_{21}H_{19}ClO_5$	65.20 (65.24	4.95 4.93)	1752, 1650	C	7.33 (1H, s), 7.16 (5H, s), 6.93 (1H, d, J=3), 6.62 (1H, d, J=3), 4.64 (2H, s), 4.25 (2H, q, J=8), 3.0—2.5 (4H, m), 1.30 (3H, t, J=8)
5q	A	H	C_6H_5	Me	H	127—129 (EtOH)	78.5[a]	$C_{20}H_{18}O_5$	71.00 (70.87	5.36 5.35)	1756, 1644	C	7.80 (1H, s), 7.60—7.20 (5H, m), 6.76 (1H, d, J=2), 6.65 (1H, d, J=2), 4.68 (2H, s), 4.28 (2H, q, J=7), 2.85 (3H, s), 1.30 (3H, t, J=7)
5r	A	H	*o*-MeC_6H_4	Me	H	99—100 (EtOH)	97.4[a]	$C_{21}H_{20}O_5$	71.58 (71.83	5.72 5.76)	1756, 1642	C	7.68 (1H, s), 7.30—7.10 (4H, m), 6.78 (1H, d, J=3), 6.67 (1H, d, J=3), 4.69 (2H, s), 4.30 (2H, q, J=7), 2.83 (3H, s), 2.24 (3H, s), 1.32 (3H, t, J=7)
5t	A	H	C_6H_5	CF_3	H	139—140 (EtOH)	88.0[g]	$C_{20}H_{15}F_3O_5$	61.23 (61.34	3.95 4.02)	1748, 1662	C	7.79 (1H, s), 7.70—7.00 (6H, m), 6.90 (1H, d, J=3), 4.70 (2H, s), 4.25 (2H, q, J=7), 1.30 (3H, t, J=7)
5u	A	H	C_6H_5	F	H	109—110 (EtOH)	89.0[b]	$C_{19}H_{15}FO_5$	66.66 (66.45	4.42 4.34)	1751, 1635	C	7.74 (1H, s), 7.60—7.20 (5H, m), 6.58 (1H, m), 6.58 (1H, dd, J=3, 9), 4.63 (2H, s), 4.24 (2H, q, J=7), 1.28 (3H, t, J=7)
5y	A	C_6H_5	H	Cl	H	173—174 (EtOH)	78.0[a]	$C_{19}H_{15}ClO_5$	63.60 (63.47	4.21 4.26)	1737, 1638	D	7.85—7.70 (2H, m), 7.55—7.35 (3H, m), 6.90 (1H, d, J=3), 6.77 (1H, d, J=3), 6.56 (1H, s), 4.65 (2H, s), 4.24 (2H, q, J=8), 1.26 (3H, t, J=8)
5z	B					174—175 (EtOH)	85.5[h]	$C_{17}H_9Cl_2NO_3$	58.98 (59.08	2.62 2.75	4.05 4.05)	1645	C	7.73 (1H, s), 7.50—7.15 (4H, m), 6.97 (1H, d, J=3), 6.85 (1H, d, J=3), 4.81 (2H, s)
5aa	A	H	C_6H_5	I	H	155—156 (EtOH)	81.8[b]	$C_{19}H_{15}IO_5$	50.69 (50.80	3.36 3.38)	1740, 1645	C	7.80 (1H, s), 7.60 (1H, d, J=3), 7.60—7.10 (5H, m), 6.78 (1H, d, J=3), 4.65 (2H, s), 4.25 (2H, q, J=7), 1.30 (3H, t, J=7)
5bb	A	H	*o*-Me, *p*-HO –C_6H_3	Cl	H	188—190 (AcOEt)	63.0[a]	$C_{20}H_{17}ClO_6$	61.78 (61.73	4.41 4.41)	1750, 1640	D	8.02 (1H, s), 7.08 (2H, s), 6.89 (1H, d, J=8), 6.60 (1H, br s), 6.56 (1H, d like, J=8), 4.96 (2H, s), 4.15 (2H, q, J=6), 2.05 (3H, s), 1.22 (3H, t, J=6)

a) Yield from the corresponding hydroxy derivative **4**. *b*) Yield from the corresponding methoxy derivative **3**. *c*) Yield from **5g**. *d*) Yield from **3i**. *e*) Yield from **5i**. *f*) Yield from **5bb**. *g*) Yield from **5aa**. *h*) Yield from **4d**. *i*) C is $CDCl_3$, D is DMSO-d_6. *j*) Coupling constants (J) are given in Hz.

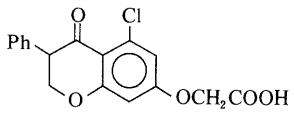
TABLE VI. Di- and Tri-Substituted [(4-Oxo-4*H*-1-benzopyran-7-yl)oxy]acetic Acids (**6b—y**) and the Related Compounds (**6z**, **6cc**, **6dd**)



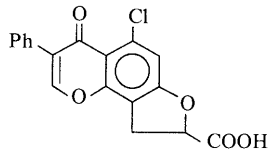
A



B



C



D

Compd. No.	Structure	R_1	R_2	X	Y	mp (°C) (Solv.)	Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	IR (KBr) cm^{-1}	Solv.[f]	^{1}H-NMR Chemical shift (δ)[g]
6b	A	H	*p*-ClC_6H_4	Cl	H	254—257 (MeCN)	77.0[a]	$C_{17}H_{10}Cl_2O_5$	55.91 (55.52	2.76 2.90)	1740, 1645	D	8.40 (1H, s), 7.70—7.40 (4H, m), 7.16 (2H, s), 4.93 (2H, s)
6c	A	H	*p*-FC_6H_4	Cl	H	265—267 (MeCN)	81.3[a]	$C_{17}H_{10}ClFO_5$	58.55 (58.54	2.89 3.04)	1742, 1646	D	8.39 (1H, s), 7.58 (2H, dd, *J*=6, 9), 7.25 (2H, dd, *J*=9, 9), 7.17 (2H, s), 4.92 (2H, s)
6d	A	H	*o*-ClC_6H_4	Cl	H	193—195 (C_6H_6)	73.0[a]	$C_{17}H_{10}C_{12}O_5$	55.91 (56.09	2.76 2.97)	1754, 1730, 1640	D	8.32 (1H, s), 7.70—7.30 (4H, m), 7.20 (2H, s), 4.94 (2H, s)
6e	A	H	*o*-MeC_6H_4	Cl	H	191—192 (MeCN)	86.0[b]	$C_{18}H_{13}ClO_5$	62.71 (62.89	3.80 3.97)	1722, 1644	D	8.20 (1H, s), 7.30—7.10 (6H, m), 4.94 (2H, s), 2.16 (3H, s)
6f	A	H	*o*-FC_6H_4	Cl	H	171—173 (MeCN)	86.0[b]	$C_{17}H_{10}ClFO_5$	58.55 (58.68	2.89 3.17)	1754, 1604	D	8.30 (1H, s), 7.50—7.10 (4H, m), 7.13 (2H, s), 4.90 (2H, s)
6g	A	H	*p*-HOC_6H_4	Cl	H	>300 (DMF-H_2O)	80.0[b]	$C_{17}H_{11}ClO_6$	58.89 (58.64	3.19 3.40)	1734, 1605	D	8.14 (1H, s), 7.28 (2H, d, *J*=9), 7.06 (2H, d, *J*=9), 7.04 (2H, s), 4.86 (2H, s)
6h	A	H	*p*-$MeOC_6H_4$	Cl	H	240—244 (DMF-H_2O)	84.0[b]	$C_{18}H_{13}ClO_6$	59.93 (59.93	3.63 3.82)	1740, 1644	D	8.21 (1H, s), 7.40 (2H, d, *J*=9), 7.06 (2H, s), 6.92 (2H, d, *J*=9), 4.86 (2H, s), 3.75 (3H, s)
6i	A	H	*o*-$MeOC_6H_4$	Cl	H	184—185 (MeCN)	78.0[b]	$C_{18}H_{13}ClO_5$	59.93 (60.26	3.63 3.67)	1730, 1640	D	8.10 (1H, s), 7.50—6.80 (4H, m), 7.07 (2H, s), 4.87 (2H, s), 3.72 (3H, s)
6j	A	H	*o*-HOC_6H_4	Cl	H	188—189 (EtOH)	64.0[b]	$C_{17}H_{11}ClO_6$	58.89 (58.82	3.19 3.41)	1740, 1718, 1614	D	9.22 (1H, s), 8.10 (1H, s), 7.30—6.95 (2H, m), 7.07 (2H, s), 6.90—6.65 (2H, m), 4.87 (2H, s)
6k	A	H	*o*-Me, *p*-MeO -C_6H_3	Cl	H	221—223 (MeCN)	86.0[b]	$C_{19}H_{16}ClO_6$	60.89 (60.67	4.03 4.03)	1770, 1635	D	8.05 (1H, s), 7.07 (2H, s), 7.02 (1H, d, *J*=10), 6.76 (1H, br s), 6.73 (1H, d like, *J*=10), 4.87 (2H, s), 3.75 (3H, s), 2.12 (3H, s)
6l	A	H	$(Me)_2CH$	Cl	H	172—174 (EtOH-C_6H_6)	89.0[a]	$C_{14}H_{13}ClO_5$	56.67 (56.63	4.42 4.44)	1730, 1640	D	7.93 (1H, s), 7.02 (2H, s), 4.86 (2H, s), 3.20—2.60 (1H, m), 1.14 (6H, d, *J*=7)
6m	A	H	cyclo-C_5H_9	Cl	H	197—199 (MeCN)	77.0[a]	$C_{16}H_{15}ClO_5$	59.54 (59.67	4.68 4.84)	1720, 1650	D	8.04 (1H, s), 7.08 (2H, s), 4.89 (2H, s), 3.20—2.70 (1H, m), 2.20—1.20 (8H, m)
6n	A	H	$C_6H_5CH_2$	Cl	H	156—159 (MeCN)	68.4[a]	$C_{18}H_{14}ClO_4$	62.53 (62.28	4.08 4.07)	1770, 1742, 1640	D	8.06 (1H, s), 7.20 (5H, s), 7.01 (2H, s), 4.84 (2H, s), 3.62 (2H, s)
6o	A	H	*o*-$ClC_6H_4CH_2$	Cl	H	170—172 (EtOH-H_2O)	89.0[b]	$C_{18}H_{12}Cl_2O_5$	57.01 (56.86	3.19 3.33)	1767, 1638	D	8.05 (1H, s), 7.60—7.20 (4H, m), 7.12 (2H, s), 4.91 (2H, s), 3.75 (2H, s)
6p	A	H	$C_6H_5(CH_2)_2$	Cl	H	157—158 (MeCN)	96.3[b]	$C_{19}H_{15}ClO_5$	63.60 (63.69	4.21 4.27)	1746, 1647	D	7.90 (1H, s), 7.16 (5H, s), 7.00 (2H, br s), 4.85 (2H, s), 2.80—2.50 (4H, m)
6q	A	H	C_6H_5	Me	H	208—210 (MeCN)	76.2[b]	$C_{18}H_{14}O_5$	69.67 (69.76	4.55 4.64)	1740, 1630	D	8.20 (1H, s), 7.60—7.30 (5H, m), 6.93 (1H, d, *J*=2), 6.85 (1H, d, *J*=2), 4.80 (2H, s), 2.72 (3H, s)
6r	A	H	*o*-MeC_6H_4	Me	H	195—196 (AcOEt-C_6H_6)	87.0[b]	$C_{19}H_{16}O_5$	70.36 (70.38	4.97 5.00)	1750, 1728, 1630	C	7.74 (1H, s), 7.40—7.10 (4H, m), 6.79 (2H, s), 4.70 (2H, s), 2.79 (3H, s), 2.20 (3H, s)
6s	A	H	*o*-ClC_6H_4	Me	H	198—200 (70% EtOH)	81.8[a]	$C_{18}H_{13}ClO_5$	62.79 (62.71	3.94 3.80)	1740, 1720, 1630	D	8.22 (1H, s), 7.65—7.30 (4H, m), 6.96 (1H, s), 6.90 (1H, s), 4.85 (2H, s), 2.72 (3H, s)
6t	A	H	C_6H_5	CF_3	H	259—261 (MeCN)	75.0[a]	$C_{18}H_{11}F_3O_5$	59.35 (59.40	3.04 3.15)	1734, 1652	D	8.35 (1H, s), 7.60—7.10 (7H, m), 4.95 (2H, s)
6u	A	H	C_6H_5	F	H	169—170 (MeCN)	31.0[b]	$C_{17}H_{11}FO_5$	64.97 (64.72	3.52 3.66)	1742, 1630	D	8.25 (1H, s), 7.60—7.20 (5H, m), 6.95 (1H, m), 6.87 (1H, dd, *J*=2, 14), 4.86 (2H, s)
6v	A	H	C_6H_5	Cl	Cl	289—290 (dec.) (DMF-MeCN)	70.9[a]	$C_{17}H_{10}Cl_2O_5$	55.91 (55.72	2.76 2.97)	1740, 1640	D	8.33 (1H, s), 7.60—7.20 (6H, m), 5.00 (2H, s)
6w	A	H	C_6H_5	Cl	Me	>300 (DMF-MeOH)	67.9[a]	$C_{18}H_{13}ClO_4$	62.71 (62.56	3.80 4.00)	1740, 1645	D	8.31 (1H, s), 7.60—7.20 (5H, m), 7.11 (1H, s), 4.93 (2H, s), 2.33 (3H, s)
6x	A	Me	C_6H_5	Cl	H	184—186 (EtOH)	61.0[a]	$C_{18}H_{13}ClO_5$	62.71 (62.87	3.80 3.95)	1782, 1598	D	7.50—7.10 (5H, m), 7.03 (2H, s), 4.87 (2H, s), 2.17 (3H, s)
6y	A	C_6H_5	H	Cl	H	265—268 (DMF-MeCN)	70.2[b]	$C_{17}H_{11}ClO_5$	61.73 (61.63	3.35 3.51)	1758, 1635	D	8.08—7.83 (2H, m), 7.68—7.38 (3H, m), 7.24 (1H, d, *J*=3), 7.01 (1H, d, *J*=3), 6.80 (1H, s), 4.86 (2H, s)
6z	B					192—195 (MeCN)	81.4[c]	$C_{17}H_{10}Cl_2N_4O_3$	52.46 (52.63	2.59 2.82	14.40 13.98)	1640	D	8.25 (1H, s), 7.56—7.16 (6H, m), 5.64 (2H, s)
6cc	C					171—173 ($CHCl_3$)	40.0[d]	$C_{17}H_{13}ClO_5$	61.36 (61.35	3.94 4.07)	1715, 1675	D	7.30 (5H, br s), 6.75 (1H, d, *J*=3), 6.58 (1H, d, *J*=3), 5.00—4.60 (4H, m), 4.11 (1H, dd, *J*=5, 8)
6dd	D					268—271 (EtOH)	26.6[e]	$C_{18}H_{11}ClO_5$	63.08 (63.09	3.24 3.38)	1725, 1620	D	8.32 (1H, s), 7.65—7.30 (5H, m), 7.15 (1H, s), 5.55 (1H, dd, *J*=7, 10), 3.76 (1H, dd, *J*=10, 16), 3.41 (1H, dd, *J*=7, 16)

a) Yield from the corresponding hydroxy derivative **4**. *b*) Yield from the corresponding ester derivative **5**. *c*) Yield from **5z**. *d*) Yield from **6a**. *e*) Yield from **14**. *f*) D is DMSO-d_6, C is $CDCl_3$. *g*) Coupling constants (*J*) are given in Hz.

(DMSO-d_6): 8.14 (1H, s), 7.12 (1H, d, $J=9$), 7.12 (2H, s), 6.96 (1H, br s), 6.92 (1H, d like, $J=9$), 4.97 (2H, s), 4.06 (2H, q, $J=6$), 2.26 (3H, s), 2.14 (3H, s), 1.22 (3H, t, $J=6$). MS m/z: 430 (M^+).

3-(2-Chloro-6-hydroxy-4-methoxyphenyl)-1-phenyl-1-propen-3-one (8) and 3-(4-Chloro-2-hydroxy-6-methoxyphenyl)-1-phenyl-1-propen-3-one (9) A mixture of 2-chloro-6-hydroxy-4-methoxyacetophenone and 4-chloro-2-hydroxy-6-methoxyacetophenone was prepared from **1a** (60 g, 0.35 mol) and acetyl chloride (27.3 g, 0.35 mol) according to the method A. The mixture was recrystallized from hexane to give crystals (53.3 g, the ratio of 2-chloro-6-hydroxy-4-methoxyacetophenone and 4-chloro-2-hydroxy-6-methoxyacetophenone was about 1 : 1 in ^{1}H-NMR spectrum).

The acetophenone derivatives (13 g, 64.8 mmol) were dissolved in a solution of benzaldehyde (6.9 g, 64.8 mmol) in EtOH (10 ml) with heating at 80 °C. KOH solution (KOH (26 g, 0.46 mol)/H_2O (8 ml)) was added to the solution at room temperature, and then the mixture was stirred vigorously at 50—60 °C for 12 h. The reaction mixture was poured into ice-cold H_2O (200 ml) and extracted with Et_2O. The extract was dried (Na_2SO_4), concentrated *in vacuo* and purified by silica gel (200 g) column chromatography with C_6H_6–hexane (2 : 1, v/v) as an eluent to afford **8** (2.0 g, 10.7%) and **9** (4.4 g, 23.5%). Compound **8**: mp 134—135 °C (EtOH). *Anal.* Calcd for $C_{16}H_{13}ClO_3$: C, 66.55; H, 4.54. Found: C, 66.31; H, 4.51. ^{1}H-NMR ($CDCl_3$): 7.74 (2H, s), 7.70—7.20 (5H, m), 6.60 (1H, d, $J=2$), 6.35 (1H, d, $J=2$), 3.92 (3H, s). IR (KBr) cm^{-1}: 1635, 1580, 1410, 1215. Compound **9**: mp 118—119 °C (EtOH). *Anal.* Calcd for $C_{16}H_{13}ClO_3$: C, 66.55; H, 4.54. Found: C, 66.60; H, 4.56. ^{1}H-NMR ($CDCl_3$): 7.67 (2H, s), 7.64—7.45 (2H, m), 7.45—7.25 (3H, m), 6.51 (1H, d, $J=3$), 6.35 (1H, d, $J=3$), 3.80 (3H, s). IR (KBr) cm^{-1}: 1630, 1560, 1340, 1215, 1145, 980.

7-Chloro-5-methoxy-4-oxo-2-phenyl-4*H*-1-benzopyran (10) Compound **10** was prepared in 88.6% yield from **9** (1.44 g, 5 mmol) according to the method I. mp 195—197 °C (EtOH). *Anal.* Calcd for $C_{16}H_{11}ClO_3$: C, 67.02; H, 3.87. Found: C, 66.69; H, 3.74. ^{1}H-NMR ($CDCl_3$): 7.92—7.67 (2H, m), 7.57—7.32 (3H, m), 7.06 (1H, s), 6.72 (1H, s), 6.60 (1H, s), 3.95 (3H, s). IR (KBr) cm^{-1}; 3080, 1665, 1605, 1125.

7-Chloro-5-hydroxy-4-oxo-2-phenyl-4*H*-1-benzopyran (11) Compound **11** was prepared in 58.7% yield from **10** (286 mg, 1 mmol) according to the method F. mp 171—172 °C (EtOH). *Anal.* Calcd for $C_{15}H_9ClO_3$: C, 66.07; H, 3.33. Found: C, 65.91; H, 3.32. ^{1}H-NMR (DMSO-d_6): 12.74 (1H, s), 8.06—7.94 (2H, m), 7.64—7.44 (3H, m), 7.26 (1H, d, $J=2$), 7.04 (1H, s), 6.80 (1H, d, $J=2$). IR (KBr) cm^{-1}; 3077, 1660, 1618, 1454, 1265, 1010.

Rf value of **4y** on a thin layer chromatography (TLC) (E. Merck, Art5714 Kieselgel 60 F_{254}) using $CHCl_3$ as developing solvent was 0.03. On the other hand, that of compound **11** was 0.49.

7-Allyloxy-5-chloro-4-oxo-3-phenyl-4*H*-1-benzopyran (12) A stirred mixture of **4a** (5.03 g, 18.4 mmol), K_2CO_3 (2.73 g, 19.7 mmol) and allyl bromide (2.46 g, 20.3 mmol) in dimethylformamide (DMF) (40 ml) was heated at 50—70 °C for 2 h, and then poured into H_2O (200 ml) to give **12** (5.67 g, 98.3%) as white powder. mp 107—108 °C (C_6H_6–hexane). *Anal.* Calcd for $C_{18}H_{13}ClO_3$: C, 69.13; H, 4.19. Found: C, 69.22, H, 4.33. ^{1}H-NMR ($CDCl_3$): 7.83 (1H, s), 7.70—7.30 (5H, m), 7.02 (1H, d, $J=2$), 6.80 (1H, d, $J=2$), 6.32—5.80 (1H, m), 5.60—5.25 (2H, m), 4.63 (2H, d, $J=5$). IR (KBr) cm^{-1}: 3080, 3060, 1650, 1625, 1600, 1555.

8-Allyl-5-chloro-7-hydroxy-4-oxo-3-phenyl-4*H*-1-benzopyran (13) A solution of **12** (5.47 g, 17.5 mmol) in diethyl aniline (50 ml) was refluxed for 4 h. The reaction mixture was poured into cold, 20% HCl (excess) and the precipitated crystals were collected by filtration to give **13** (3.83 g, 70.0%). mp 223—226 °C (C_6H_6). *Anal.* Calcd for $C_{18}H_{13}ClO_3$: C, 69.13; H, 4.19. Found: C, 68.97, H, 4.35. ^{1}H-NMR ($CDCl_3$): 7.91 (1H, s), 7.80 (1H, br s), 7.70—7.30 (5H, m), 7.22 (1H, s), 6.30—5.70 (1H, m), 5.30—4.95 (2H, m), 3.56 (2H, d, $J=6$). IR (KBr) cm^{-1}: 3400—2800, 1635, 1595, 1580.

5-Chloro-8,9-dihyrdro-8-hydroxymethyl-4-oxo-3-phenyl-4*H*-furo[2,3-*h*]-[1]benzopyran (14) A mixture of **13** (0.31 g, 1.0 mmol) and 70% *m*-chloroperbenzoic acid (0.28 g, 1.1 mmol) in CH_2Cl_2 (20 ml) was stirred at room temperature for 42 h. The reaction mixture was extracted with $CHCl_3$, and the $CHCl_3$ layer was washed with saturated $NaHCO_3$ (30 ml × 3), dried (Na_2SO_4), and then concentrated *in vacuo* to give pale yellow powder (0.33 g). The resulting product was heated at 120—140 °C for 15 min to yield **14** (0.29 g, 89%) as brownish crystals. mp 156—158 °C (C_6H_6–AcOEt). *Anal.* Calcd for $C_{18}H_{13}ClO_4$: C, 65.76; H, 3.99. Found: C, 65.60; H, 4.15. ^{1}H-NMR ($CDCl_3$): 7.81 (1H, s), 7.65—7.30 (5H, m), 6.90 (1H, s), 5.35—5.00 (1H, m), 4.10—3.65 (2H, m), 3.60—3.00 (2H, m), 1.84 (1H, br s). IR (KBr) cm^{-1}: 3440, 3050, 1635, 1600, 1580.

7-Hydroxy-4-oxo-3-phenyl-8-propyl-4*H*-1-benzopyran (15) and 2,3-Dihydro-7-hydroxy-4-oxo-3-phenyl-8-propyl-4*H*-1-benzopyran (16) A solution of **13** (0.31 g, 1.0 mmol) and KOH (0.11 g, 2 mmol) in MeOH (12 ml) was catalytically hydrogenated with Raney nickel (R-100, Nikko's Chemicals) (0.5 g) under atmospheric pressure. The catalyst was filtered off and the filtrate was concentrated *in vacuo*. H_2O (30 ml) was added to the resulting residue, and the mixture was acidified with concentrated HCl to yield brownish powder. The crude product was collected by filtration and separated by preparative TLC using MeOH–$CHCl_3$ (2 : 98, v/v) as developing solvent to afford **15** (60 mg, 21.6%) and **16** (60 mg, 21.4%). Compound **15**: mp 227—229 °C (C_6H_6). *Anal.* Calcd for $C_{18}H_{16}O_3$: C, 77.12; H, 5.75. Found: C, 77.42; H, 5.81. ^{1}H-NMR ($CDCl_3$): 8.06 (1H, d, $J=9$), 8.04 (1H, s), 7.70—7.20 (5H, m), 6.91 (1H, d, $J=9$), 6.39 (1H, s), 2.86 (2H, t, $J=7$), 1.85—1.45 (2H, m), 1.02 (3H, t, $J=7$). IR (KBr) cm^{-1}: 3400—2500, 1625, 1590, 1565, 1500. Compound **16**: mp 157—158 °C (C_6H_6). *Anal.* Calcd for $C_{18}H_{18}O_3$: C, 76.57; H, 6.43. Found: C, 76.34; H, 6.37. ^{1}H-NMR ($CDCl_3$): 7.76 (1H, d, $J=9$), 7.50—7.20 (5H, m), 6.49 (1H, d, $J=9$), 5.28 (1H, s), 4.66 (2H, d, $J=7$), 3.90 (1H, t, $J=7$), 2.64 (2H, t, $J=8$), 1.80—1.20 (2H, m), 0.97 (3H, t, $J=7$). IR (KBr) cm^{-1}: 3500—3000, 2970, 2940, 2880, 1660, 1600, 1585, 1500.

Biological Activities Saluretic and uricosuric activities were evaluated by the method described in the previous paper.[1] The results are shown in Tables I and II.

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Pharmacologic Analysis of 7-*O*-Ethyl-fangchinoline-Induced Vasodilation Properties in Isolated Perfused Common Carotid Arteries of Wistar Kyoto Rats and Spontaneously Hypertensive Rats

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Using the cannula insertion method, we investigated vascular effects of 7-*O*-ethyl-fangchinoline (TJN-220) derived from tetrandrine in isolated and perfused common carotid arteries of Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). A single dose of TJN-220 caused a vasodilation in a dose-related manner in arteries preconstricted by phenylephrine. The vasodilation was not inhibited by propranolol, a potent beta-adrenoceptor antagonist. A potent alpha-antagonist bunazosin inhibited the vasoconstriction to norepinephrine while TJN-220 did not modify the norepinephrine-induced constriction, indicating TJN-220 had no alpha-blocking activity. A potent calcium entry blocker, diltiazem, markedly attenuated the KCl-induced vasoconstriction, and TJN-220 slightly but significantly attenuated the KCl-induced one in large doses. The vasodilation of TJN-220 was not abolished after removing the endothelium by an intraluminal administration of saponin, although the ACh-induced dilation was completely abolished by it. A comparison of vascular responses in WKY and SHR revealed no significant differences. From these results, it is concluded that 1) a new tetrandrine derivative, TJN-220 has relatively long- lasting vasorelaxant properties, 2) the dilatory effects might not be related to adrenergic, muscarinic or endothelium-dependent mechanisms, and 3) the effects might partially be due to calcium entry antagonistic properties.

Keywords 7-*O*-ethyl-fangchinoline; tetrandrine; spontaneously hypertensive rat; cannula insertion method; calcium antagonist

Tetrandrine is one of alkaloids isolated from Chinese herb Radix *Stephaniae Tetrandrae*. Tetrandrine has been used clinically in the management of hypertension, especially hypertensive crisis and coronary heart diseases in China.[1,2] Experimental studies of tetrandrine on smooth muscle, cardiac papillary muscle, left atrial tissue and GH_3 anterior pituitary cells were reported.[3-6] These suggested that these effects of tetrandrine might be related to an inhibition of Ca^{2+} entry. Recently, several tetrandrine derivatives were studied to obtain an even more effective compound.[7] 7-*O*-Ethyl-fangchinoline (TJN-220) is a compound which is substituted at the 7-position to the ethyl group from the methyl group of tetrandrine.[7] Kawashima *et al.*[7] demonstrated that TJN-220 had more hypotensive potency than tetrandrine in unanesthetized and freely moving stroke-prone spontaneously hypertensive rats (SHRSP) however, they did not make a pharmacologic analysis. Thus, in the present study we tried pharmacologically to analyze vascular responses to TJN-220 in isolated common carotid arteries of Wister Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) using the cannula insertion method.[8,9]

Methods

Male WKY and SHR rats (weighing 150—250 g: aged 7—13 weeks) were used in the present study. After treatment with sodium heparin (200 units/kg, i.v.), rats were killed by rapid exsanguination, and common carotid arteries (0.8 to 1.3 cm in length and 0.8 to 1.2 mm in outer diameter) were carefully isolated. A stainless steel cannula with small holes at 2 mm distance from the distal sealed end (23 or 25 gauge; 0.5 to 0.6 mm in outer diameter and 3 cm in length) was inserted into each vessel segment to avoid injury of the intraluminal surface of the isolated vessel. Segments were set up in the bath as described by Tsuji and Chiba.[9] The perfusion solution contained (mmol/l): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, and glucose 10; and bubbled with 95% O_2 and 5% CO_2 to maintain the pH of the solution at 7.2—7.4. The bath and perfusion circuit were warmed at 37 °C with a thermopump (Model FE2, Haake, Karlsruhe, FRG). Speed of the perfusion pump was determined at a resting perfusion pressure of 40—60 mmHg. The vasodilation and vasoconstriction were presented as a decrease and an increase in perfusion pressure, respectively, which was continuously measured with an electronic manometer. TJN-220 used in the present study was obtained from the Research Institute for Biology and Chemistry, Tsumura Co., Ltd., and the procedure for its preparation has been published elsewhere.[10] Other drugs used were isoproterenol hydrochloride (Nikken, Tokyo), propranolol hydrochloride (Sumitomo, Tokyo), acetylcholine chloride (Daiichi, Tokyo), atropine sulfate, potassium chloride, diltiazem hydrochloride (Tanabe, Tokyo), norepinephrine hydrochloride (Sankyo, Tokyo), phenylephrine hydrochloride (Kowa, Tokyo), and bunazosin hydrochloride (Eisai, Tokyo). Drug solution was injected intraluminally into the isolated vessel from the rubber tubing close to the cannula in a volume of 0.01—0.03 ml for a period of 4 s.

Results are expressed as mean ± S.E. mean. Statistical significance between two data sets was tested by either Student's paired or unpaired *t* test. A probability level of $p < 0.05$ was considered statistically significant.

Results

Vasodilator Responses to TJN-220 and Isoproterenol in Isolated Common Carotid Arteries of WKY and SHR The vascular effect of TJN-220 in non-treated WKY and SHR common carotid arteries was tested but no vascular responses were observed. Thus, a preconstricted condition was induced by treatment with phenylephrine (1 μg/ml). The increase in perfusion pressure was approximately 30 mmHg, and each experiment was performed in a stable state. When

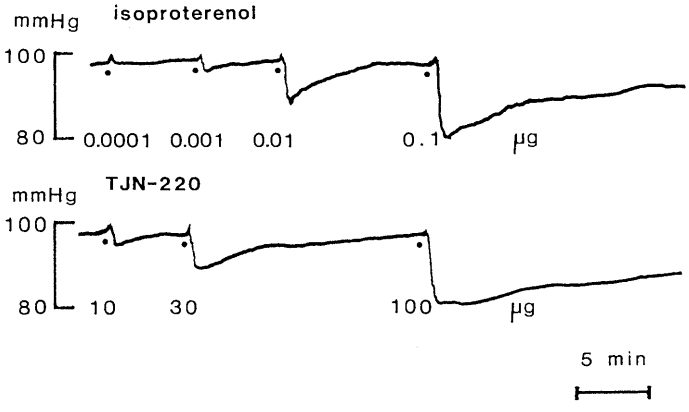

Fig. 1. Vascular Responses of Isolated and Perfused Common Carotid Arteries to Isoproterenol and TJN-220

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TABLE I. Vasodilation Effects of Isoproterenol, TJN-220 and Papaverine on Isolated Common Carotid Arteries of WKY and SHR

Drug	Dose (μg)	Maximum decrease in P.P.[a] (%)	Time to maximum response (min)	50% recovery time (min)	n
WKY					
Isoproterenol	0.01	36.7±4.1	0.5±0.03	1.2±0.1	10
	0.03	50.8±3.5	0.5±0.05	1.8±0.2	10
	0.1	67.8±4.3	1.0±0.1	3.9±0.5	8—10
TJN-220	30	25.6±2.9	1.4±0.2	3.8±0.6	7—9
	100	51.8±5.0	2.2±0.3	8.8±1.0	8—10
Papaverine	100	100	4.6±0.6	—	6
SHR					
Isoproterenol	0.01	39.6±2.1	0.5±0.02	1.0±0.1	10
	0.03	53.8±2.4	0.5±0.02	1.4±0.2	10
	0.1	68.4±3.3	0.8±0.05	3.5±0.4	8—10
TJN-220	30	19.5±2.5	1.0±0.2	4.5±0.7	8
	100	37.2±4.6	1.8±0.4	7.8±1.2	8—10
Papaverine	100	100	4.7±0.5	—	6

a) P.P., perfusion pressure.

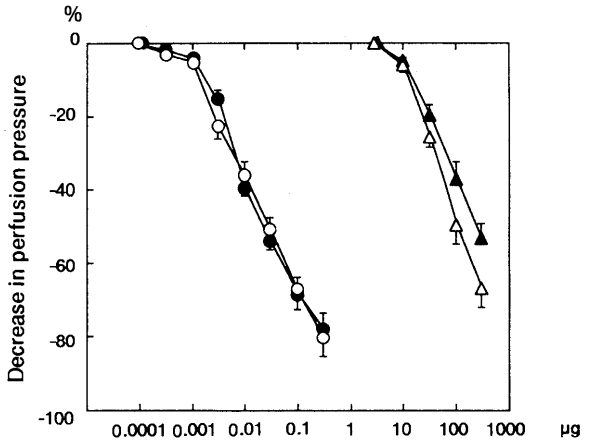

Fig. 2. Vasodilation to Isoproterenol and TJN-220 in Isolated Common Carotid Arteries of WKY and SHR Preconstricted by Phenylephrine

○, isoproterenol-induced vasodilation in WKY; ●, in SHR; △, TJN-220-induced vasodilation in WKY; ▲, in SHR. Each point and vertical bar represent the mean±S.E. (n=8—10).

a single dose of TJN-220 or a beta-adrenoceptor agonist, isoproterenol was intraluminally administered in vessels of both WKY and SHR, vasodilation was readily observed as a decrease in perfusion pressure (Fig. 1). Slight fluctuation just after drug administration was an artifact caused by injection of the drug solution. In each preparation, the maximum decrease in perfusion pressure by 100 μg papaverine was defined as a 100% decrease. In both WKY and SHR, the threshold doses for inducing a vasodilation to TJN-220 and isoproterenol were 10 μg and 0.0003 μg, respectively. These responses were induced in a dose-related manner (Fig. 2). The maximum decrease induced by TJN-220 at a dose of 300 μg was 67±5.5% in of WKY and 53±3.9% in SHR. The maximum decrease induced by 0.3 μg isoproterenol was 80% in WKY and 78% in SHR, showing no difference between the two preparations. The response to TJN-220 was markedly longer than that to isoproterenol (Table I). The vasodilation of 100 μg TJN-220 lasted 30—40 min, with 50% recovery time lasting 7—9 min in both preparations.

Effects of Propranolol on Vasodilations Induced by TJN-220 and Isoproterenol After administration of 1 μg propranorol, the vasodilation of isoproterenol was markedly inhibited in both WKY and SHR in the preconstricted state. In contrast, the vasodilation of TJN-220 was not clearly modified by propranolol, although it was attenuated slightly by propranolol in WKY. When a relatively large dose of TJN-220 (100—300 μg) was used,

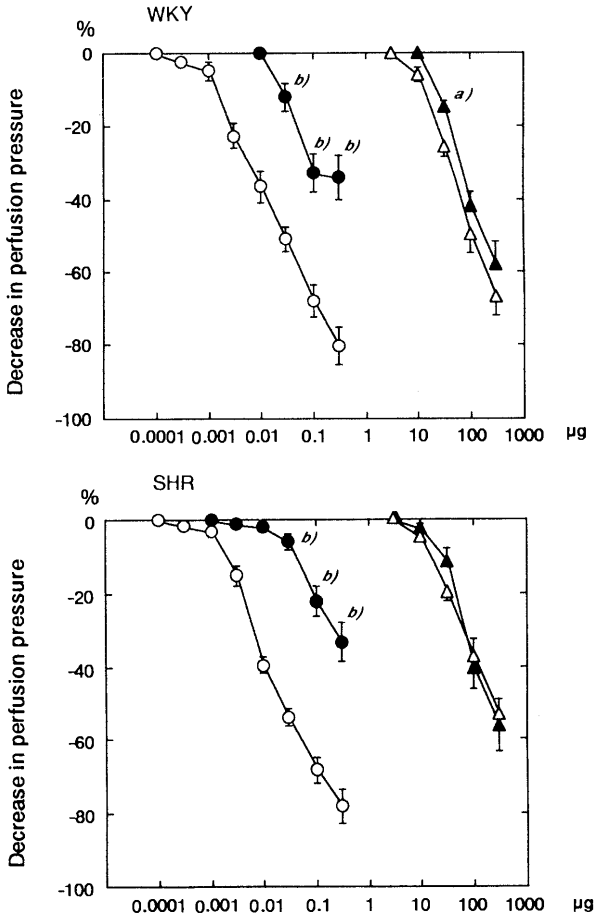

Fig. 3. Effects of 1 μg of Propranolol on Vasodilations Induced by Isoproterenol and TJN-220 in WKY and SHR Arteries

○, control of isoproterenol; ●, after administration of propranolol; △, control of TJN-220; ▲, after administration of propranolol. Each point and vertical bar represent the mean±S.E. (n=6—8). a) $p<0.05$, b) $p<0.005$, compared with controls.

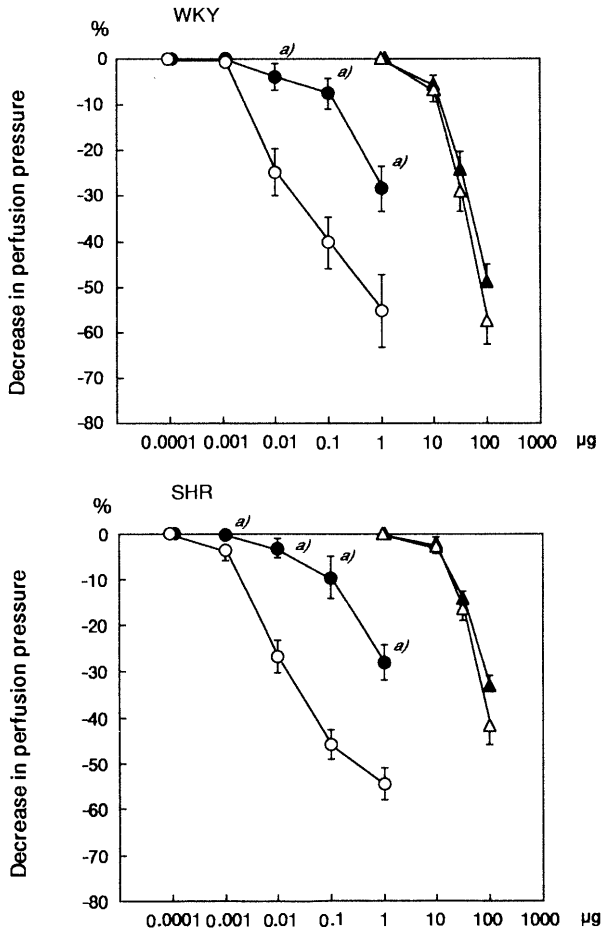

Fig. 4. Effects of 10 μg of Atropine on Acetylcholine- and TJN-220-Induced Vasodilations in WKY and SHR Arteries

○, control of acetylcholine; ●, after administration of atropine; △, control of TJN-220; ▲, after administration of atropine. Each point and vertical bar represent the mean ± S.E. (n=6—8). *a*) $p<0.05$, *b*) $p<0.005$, compared with the controls.

propranolol was injected just before each TJN-220 administration. Summarized data are shown in Fig. 3.

Effects of Atropine on Acetylcholine- and TJN-220-Induced Vasodilations Acetylcholine usually caused a vasodilation in a dose-related manner in vessels with the presence of the endothelium preconstricted by phenylephrine. After an administration of 10 μg atropine, the vasodilation induced by acetylcholine was significantly inhibited. However, the vasodilation induced by TJN-220 was not significantly influenced by atropine treatment (Fig. 4).

Effects of Endothelium Removal on Acetylcholine- and TJN-220-Induced Vasodilations In a preconstricted state, intraluminal saponin treatment (1—3 mg) readily removed the endothelium, because acetylcholine-induced dilation disappeared thereafter. Since saponin caused a long-lasting increase in perfusion pressure (approximately 20 mmHg), acetylcholine or TJN-220 was administered under a stable condition of perfusion pressure higher than control. In both WKY and SHR vessels, TJN-220-induced dilations were not modified after saponin treatment. Summarized data are shown in Fig. 5.

Effects of TJN-220 and Diltiazem on KCl-Induced Vasoconstrictions When KCl (1, 3 mg) was injected intraluminally, a vasoconstrictor response was clearly produced in

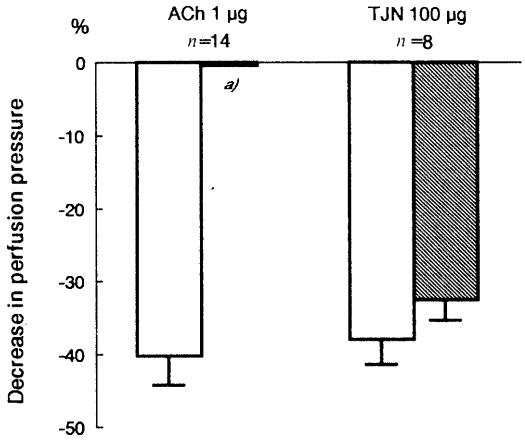

Fig. 5. Effects of Endothelium Removal on Vasodilation to Acetylcholine and TJN-220

ACh, acetylcholine; TJN, TJN-220. Open column, control; hatched column, after endothelium removal. Vertical bars represent the mean ± S.E. *a*) Significant different from control ($p<0.005$).

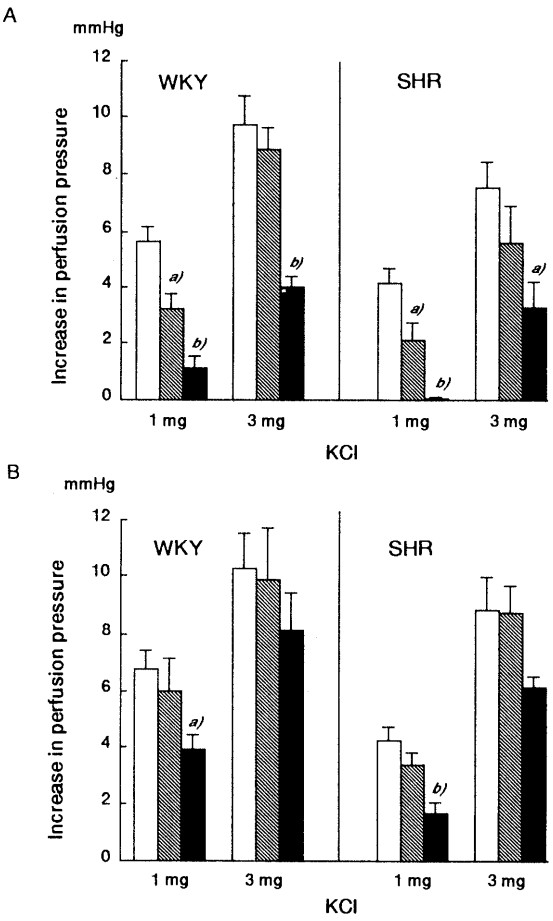

Fig. 6. Effects of Diltiazem and TJN-220 on KCl-Induced Vasoconstrictions

Open column, control; hatched column, after administration of 1 μg diltiazem (A) or 10 μg TJN-220 (B); closed column, after administration of 10 μg diltiazem (A) or 100 μg TJN-220 (B); vertical bar represent the mean ± S.E. (n=8). *a*) $p<0.05$, *b*) $p<0.005$, compared with controls.

a dose-related manner in both WKY and SHR common carotid arteries. The vasoconstriction to KCl was inhibited by pretreatment with diltiazem, a Ca^{2+} entry antagonist, or with TJN-220. In both WKY and SHR arteries, the vasoconstrictor responses to 1 mg KCl were decreased to

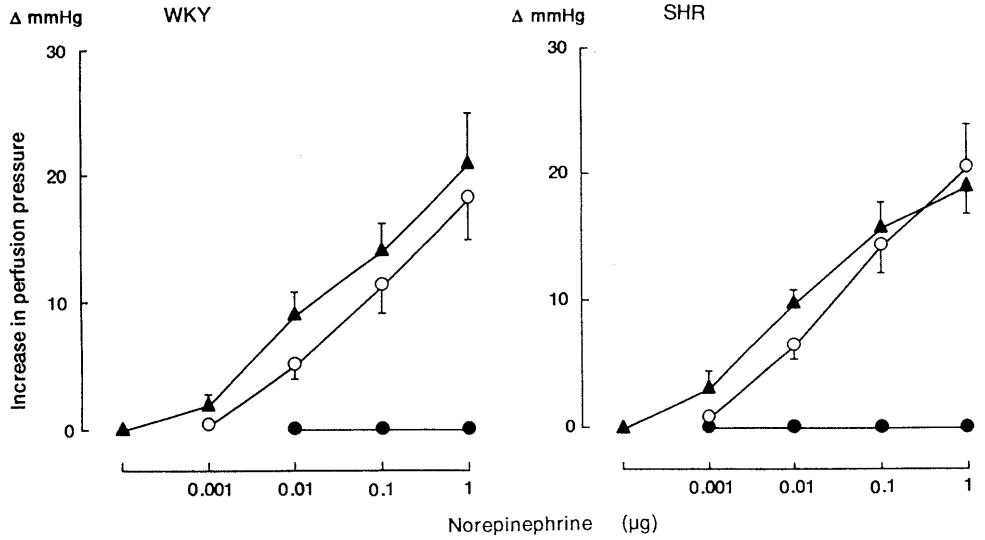

Fig. 7. Effects of Bunazosin (0.1—10 μg) and TJN-220 on Norepinephrine-Induced Vasoconstrictions in WKY and SHR Arteries

○, control; ●, after administration of bunazosin; ▲, after administration of TJN-220. Each point and vertical bar represent the mean ± S.E. (n=9—13). *a*) $p<0.05$, compared with control.

approximately 50% of control by 1 μg diltiazem, and were markedly inhibited by 10 μg diltiazem ($p<0.005$) (Fig. 6A). However, the effects of TJN-220 for the KCl-induced vasoconstriction were much less than those of diltiazem. After the 100 μg TJN-220 was administered, the vasoconstrictions induced by 1 mg and 3 mg KCl were reduced to approximately 40—60% and 70—80% of the controls, respectively (Fig. 6B).

Effects of TJN-220 and Bunazosin on Norepinephrine-Induced Vasoconstrictions When norepinephrine was administered into common carotid arteries, an increase in perfusion pressure was usually observed in a dose-related manner. After bunazosin (0.1—10 μg), an alpha-adrenoceptor antagonist, was injected, the vasoconstriction induced by norepinephrine was completely inhibited in both WKY and SHR carotid arteries. In both strains, TJN-220 did not suppress norepinephrine-induced constrictions, indicating that TJN-220 has no alpha-adrenoceptor blocking activity. In most preparations, TJN-220 slightly enhanced norepinephrine-induced vasoconstrictions but not significantly. Summarized data are shown in Fig. 7.

Discussion

When the cannula insertion method was used in isolated and perfused rat common carotid arteries, preconstriction was required to investigate the vascular effects of vasodilatory agents.[11] A potent beta-adrenoceptor agonist, isoproterenol induced a vasodilation in a dose-related manner. In contrast, TJN-220 caused less potent vasodilator responses, although its action was long-lasting.

Tetrandrine and several of its derivatives have a hypotensive effect of gradual onset and long duration of action after oral administration in SHRSP.[7] Among the derivatives, 7-*O*-methyl, 7-*O*-ethyl (TJN-220) and 7-*O*-isopropyl derivatives showed the most marked and almost equivalent hypotensive activity.[7]

In conscious normotensive Wistar rats, SHR, renal hypertensive and deoxycorticosterone (DOCA)-salt rats, i.v. administration of tetrandrine caused an abrupt and long-lasting decrease in mean arterial pressure, which proved dose-dependent.[12] After the injection of tetrandrine, the decrease of mean arterial pressure in conscious normotensive rats was slightly greater than that in SHR. In our experimental data of TJN-220, we were unable to detect a clear difference between WKY and SHR vessels.

The vasodilation to isoproterenol was readily inhibited by propranolol, while the vasodilation to TJN-220 was not, indicating that TJN-220 has no beta-adrenoceptor agonistic activity in vessels. In the isolated rabbit atrium, propranolol exhibited competitive antagonism with isoproterenol while tetrandrine did not, indicating that tetrandrine has no beta-adrenoceptor blocking activity in cardiac tissue.[13]

Acetylcholine produces vasodilator responses in preconstricted vessels existing in the intact endothelium. In this study, it induced a vasodilation in a dose-related manner in both WKY and SHR vessels. This vasodilation was markedly inhibited by atropine. The vasodilation of TJN-220, however was not modified by atropine in either WKY or SHR, indicating that TJN-220 has no muscarinic properties. Acetylcholine dilation was readily abolished by removing the endothelium,[14] while that of TJN-220 was not modified by such removal, indicating that TJN-220 induced no release of endothelium derived relaxing factor (EDRF).

Diltiazem significantly inhibited vasoconstriction induced by KCL in both WKY and SHR. TJN-220 also showed inhibitory effect on the KCl-induced constrictions, but a large dose was required and its blocking effects were not strong. Thus, the vasodilating effects of TJN-220 might, in part, be due to blocking of Ca^{2+} entry to vascular smooth muscle.

Norepinephrine produced a vasoconstriction in both WKY and SHR common carotid arteries in a dose-related manner. The pressor responses to exogenous norepinephrine were greater in SHR than in WKY perfused mesenteric arterial beds.[15] However, in the present study, the increase in perfusion pressure in SHR was slightly but not significantly greater than that in WKY. In both WKY and SHR isolated arteries, vasoconstrictions of norepinephrine were readily abolished by a potent alpha-adrenoceptor

antagonist, bunazosin, while TJN-220 did not influence the norepinephrine-induced vasoconstriction even in large doses. The vasodilation of TJN-220 was thus not explained by blocking action of alpha-adrenoceptors in isolated perfused common carotid arteries.

It was indicated that TJN-220 caused a long-lasting vasodilation in isolated WKY and SHR common carotid arteries, in part by a partial Ca^{2+} entry blocking action in addition to unknown vasodilatory mechanisms.

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Release Kinetics of Nicotinamide from Fatty Acid–Nicotinamide Equimolar Complexes. I.[1] Release Characteristics of Fatty Acid Complexes

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The rates of release of nicotinamide (NAA) from fatty acid (FA)–NAA complexes, FA–NAA, were determined in a JP XI dissolution test apparatus in 500 ml of JP XI disintegration test medium No. 1 at 37°C. The release rate constant (k) and the activation Gibbs energy ($\Delta G^{\ddagger}$) for the release of NAA from FA–NAA were estimated. The results obtained for FA–NAA were compared with previous results obtained for the thiamine disulfide (TDS) complex, $(FA)_6$-(TDS).

The plots of log k against the carbon number of the constituent FA (n) presented a zig-zag line which indicates a downward convex at an odd-numbered position. The plots of $\Delta G^{\ddagger}$ against n showed a zig-zag line with an upward convex at an odd-numbered position, though the positive value of $\Delta G^{\ddagger}$ increased rather regularly with an increase of n for either even-numbered or odd-numbered FA.

The phenomena that the plots of log k *vs.* n and $\Delta G^{\ddagger}$ *vs.* n show zig-zag lines due to the difference between even- and odd-numbered FA were the same as observed previously for the release of TDS from $(FA)_6$ (TDS).

Keywords nicotinamide; complex; fatty acid; odd-even effect; release; release rate constant; activation Gibbs energy

Higher saturated fatty acids (FA) form crystalline complexes with water-soluble drugs in 1,2-dichloroethane[2]: for example, thiamine disulfide (TDS) complex $(FA)_6$-(TDS)[2a] whose molar ratio of FA to TDS is 6:1, and nicotinamide (NAA) complex FA–NAA[2b] whose molar ratio of FA to NAA is 1:1. The release rate of TDS from $(FA)_6$ (TDS) has already been determined.[3] In the release study of TDS from $(FA)_6$ (TDS), the plot of the release rate constant (k) of TDS against the carbon number of the constituent FA (n) showed a zig-zag line which was represented by an alternative convex at an odd-numbered position, and k decreased rather regularly with an increase of n for either even-numbered or odd-numbered FA. This is considered due to the greater stability of the TDS complex formed with odd-numbered FA compared with the complex formed with even-numbered FA. This is also reflected in the melting points[2a] of $(FA)_6$ (TDS). It is very interesting to see whether the zig-zag patterns as observed for $(FA)_6$-(TDS) will be seen with other drug complexes formed with FA.

By contrast, the results of the heat of dissolution of $(FA)_6$-(TDS)[4] and the association of FA in 1,2-dichloroethane[5] suggested that $(FA)_6$ (TDS) has an inclusion compound property. Determining a common feature among FA–drug complexes by measuring the release kinetics gives an important clue to the application of FA–drug complexes in pharmaceutical and other fields. In the previous paper,[6] the times required for 50% and 80% release of NAA from FA–NAA were shown, and it was suggested that FA–NAA may be applicable to the preparation of a sustained-release drug formulation. This paper presents the release rate constant and the activation Gibbs energy for the release of NAA from FA–NAA. Furthermore, the results obtained for FA–NAA are compared with the results[3] obtained for $(FA)_6$ (TDS), and the effects of the difference in FA will be discussed.

Experimental

Materials NAA, tetradecanoic acid (C14), pentadecanoic acid (C15), hexadecanoic acid (C16), heptadecanoic acid (C17) and octadecanoic acid (C18) were the same as those used for the previous studies.[2b,6] FA–NAA was prepared as follows: FA and NAA were dissolved in warm 1,2-dichloroethane, and the solution was set aside to crystallize.[2b] The purity of each FA–NAA was examined by measuring the melting point of FA–NAA.[6] After it had been confirmed that no extra free FA and/or NAA was present, crystals of FA–NAA were passed through 48 and 60 mesh sieves, and the particles of 48—60 mesh[6] were taken for the release test.

Measurement of the Release of NAA from FA–NAA The release of NAA from FA–NAA was determined in a JP XI dissolution test apparatus (paddle method) in 500 ml of JP XI disintegration test medium No. 1 (pH 1.2) as described in the previous paper.[6] About 30 mg of FA–NAA was used in the test. Experiments were carried out at 37 °C.

Quantitative Analysis of NAA The concentration of released NAA was determined spectrophotometrically as previously described.[6]

Results

Rate Constants for the Release of NAA from FA–NAA FA–NAA, FA and NAA are in equilibrium in an aqueous solution (pH 1.2) as follows:

$$\text{FA–NAA solid} \rightleftharpoons \text{FA solid} + \text{NAA}$$

where released NAA is dissolved in the test medium. FA and FA–NAA are almost insoluble in an aqueous acidic medium, and the solubilities of FA–NAA and FA are negligible under the experimental conditions. The percentages of released NAA were calculated with respect to the theoretical total concentration of NAA which is contained in the 1:1 complex, FA–NAA. The equilibrium percent of released NAA was 88—95% under the experimental conditions. The details were shown in the previous paper.[6]

In the equilibrium equation, it is assumed that the concentration change of FA is negligible because of its insolubility. Furthermore, it is assumed that the formation of FA–NAA by the reversed reaction is negligible because the concentration of FA–NAA formed by the reversed reaction is sufficiently lower than that of released NAA. According to the assumptions, the reaction of pseudo first order can be applied in this system. The rate constant for the release of NAA was defined as follows:

$$\ln(x_e - x) = \ln x_e - kt \quad (1)$$

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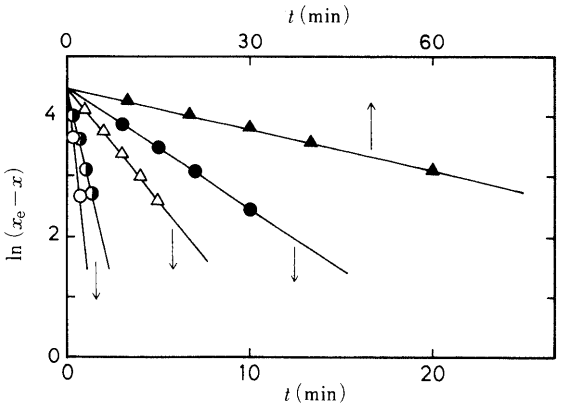


Fig. 1. Effect of FA on the Release of NAA from FA–NAA

Carbon numbers in FA: ○, 14; ◑, 16; ●, 18; △, 15; ▲, 17. Particle size: 48—60 mesh. Temperature: 37 °C.

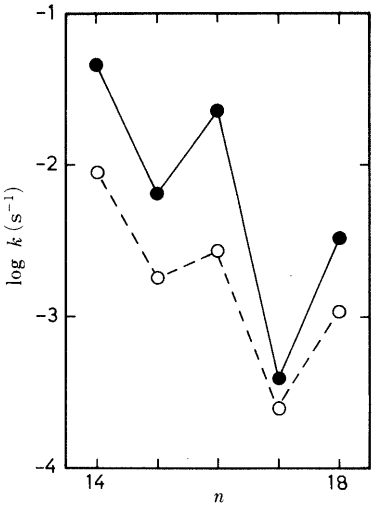


Fig. 2. Effect of FA on the Release Rate Constants (k) of NAA from FA–NAA and TDS[3] from $(FA)_6$(TDS)

Complex: ●, FA–NAA; ○, $(FA)_6$(TDS). Temperature: 37 °C.

where k is the rate constant of release, x is the percentage of NAA released from FA–NAA during time t, and x_e is the equilibrium percent of released NAA. Plots of $\ln(x_e - x)$ *vs.* t are presented in Fig. 1. As can be seen in Fig. 1, good linear relationships were obtained in all cases. It was confirmed that the release of NAA can be represented by the first order reaction. The values of release rate constant k were obtained from the slopes shown in Fig. 1. The values of log k obtained for C14–NAA—C18–NAA at 37 °C are plotted against n by closed circles in Fig. 2. The results[3] of the corresponding fatty acid complexes with TDS were also drawn for comparison. Plots of log k against n showed a zig-zag line with a downward convex at an odd-numberd position, which was quite similar to that of the TDS complexes.

Discussion

Activation Gibbs Energy for the Release of NAA from FA–NAA The activation Gibbs energy, $\Delta G^{\ddagger}$, for the release of NAA from FA–NAA can be represented in terms of the release rate constant, k, as follows:

$$\Delta G^{\ddagger} = -RT \ln k + RT \ln(k_B T/h) \quad (2)$$

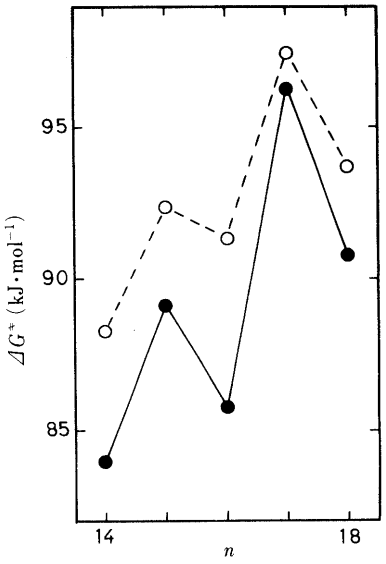


Fig. 3. Effect of FA on the Activation Gibbs Energies ($\Delta G^{\ddagger}$) for the Release of NAA from FA–NAA and TDS[3] from $(FA)_6$(TDS)

Complex: ●, FA–NAA; ○, $(FA)_6$(TDS). Temperature: 37 °C.

where R, k_B and h are the gas constant, Boltzmann constant and Planck constant, respectively. The values of $\Delta G^{\ddagger}$ were estimated from the values of k shown in Fig. 2, and the values of $\Delta G^{\ddagger}$ were plotted against n by closed circles in Fig. 3. The plots of the positive values of $\Delta G^{\ddagger}$ against n showed a zig-zag line with an upward convex at an odd-numbered position, indicating that the release of NAA from FA–NAA formed with odd-numbered FA is more disadvantageous than that from FA–NAA formed with even-numbered FA.

Comparison of k and $\Delta G^{\ddagger}$ between FA–NAA and $(FA)_6$-(TDS) In order to make a comparison between the release behaviors of NAA from FA–NAA and TDS from $(FA)_6$-(TDS), the previously obtained values of k[3] for $(FA)_6$(TDS) were shown by open circles in Fig. 2 together with the values of k for FA–NAA, where the measurement of k for $(FA)_6$-(TDS) was made under the same conditions as applied in this paper. As can be seen in Fig. 2, the absolute values of log k for FA–NAA and $(FA)_6$(TDS) formed with odd-numbered FA are larger than those formed with even-numbered FA whose alkyl chain length is one carbon number longer. The plots of log k *vs.* n show a similar zig-zag pattern in both cases of FA–NAA and $(FA)_6$ (TDS). In addition, it is found that the release rate of NAA from FA–NAA is faster than that of TDS from $(FA)_6$ (TDS). This phenomenon suggests that the binding force between FA and NAA is weaker than that between $(FA)_6$ and TDS. This leads to a further inference that FA–NAA does not consist of one molecule of FA and one molecule of NAA by a strong binding force but consists of n molecules of FA and n molecules of NAA by weakly physical binding modes.

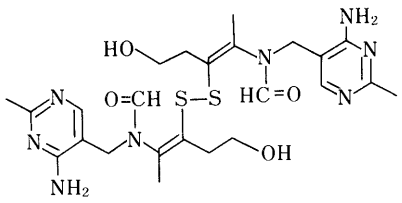
On the other hand, the previously obtained values[3] of $\Delta G^{\ddagger}$ at 37 °C for the release of TDS from $(FA)_6$ (TDS) were shown by open circles in Fig. 3 together with the values of $\Delta G^{\ddagger}$ for FA–NAA. As can be seen in Fig. 3, the positive values of $\Delta G^{\ddagger}$ for FA–NAA and $(FA)_6$ (TDS) formed with odd-numbered FA are larger than those formed with even-numbered FA whose alkyl chain length is one carbon number longer. The plots of $\Delta G^{\ddagger}$ *vs.* n show a similar zig-zag

pattern in the cases of both FA–NAA and $(FA)_6$ (TDS). In addition, it is found that the positive value of $\Delta G^{\neq}$ for FA–NAA is smaller than that for $(FA)_6$(TDS), indicating that the release of NAA from FA–NAA is easier than that of TDS from $(FA)_6$ (TDS).

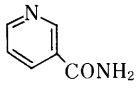
As described above, similar patterns with regard to the release of a drug from an FA–drug complex were found in the cases of both FA–NAA and $(FA)_6$ (TDS). FA–NAA may have a similar structure to $(FA)_6$ (TDS), though the molar ratio of FA to NAA is 1 : 1 for FA–NAA whereas the molar ratio of FA to TDS is 6 : 1 for (FA_6) (TDS).

Considering the weak interaction between FA and TDS estimated from the measurement[4] of the heat of dissolution of $(FA)_6$ (TDS), it was suggested that $(FA)_6$ (TDS) might be an inclusion compound. On the other hand, it was found in the infrared (IR) spectra[2] that the absorption band near 1700 cm^{-1} which is the characteristic of the carbonyl stretching vibration was shifted to higher frequency fields by the formation of either $(FA)_6$ (TDS) or FA–NAA. The similar patterns of IR spectra also imply that FA–NAA has a similar structure to $(FA)_6$ (TDS). Regarding higher and lower frequency shifts for the carbonyl stretching band, Nakai *et al.* reported[7] that the inclusion of *p*-acetoxydiphenyl into the β-cyclodextrin cavity caused a higher frequency shift whereas the formation of hydrogen bonding between *p*-acetoxydiphenyl and β-cyclodextrin caused a lower frequency shift. Taking account of the report[7] with regard to the IR spectra of an inclusion compound and a hydrogen-bonded compound, the IR spectra[2] of FA–NAA and $(FA)_6$ (TDS), the mechanism for the formation of FA–drug complexes in 1,2-dichloroethane,[5] and the release characteristics of $(FA)_6$-(TDS)[3] and FA–NAA, FA–NAA may be an inclusion compound, as may $(FA)_6$ (TDS).[3b,4] Recently, the existence of a new crystalline complex $(C18)_6$ $(NAA)_5$, whose molar ratio of C18 to NAA is 6 : 5, was confirmed.[8] Therefore, the structure formula of 1 : 1 complex FA–NAA may possibly be $(FA)_6$ $(NAA)_6$, in which six molecules of NAA are included in the $(FA)_6$ host structure. This suggestion leads to further inferences that FA–drug complexes formed in 1,2-dichloroethane have a basic structure composed of six molecules of FA, $(FA)_6$,[5] and that the differences in physiochemical properties shown in Figs. 2 and 3 are caused by the difference in the host structure composed of six molecules of odd-numbered FA or six molecules of even-numbered FA, although X-ray crystal structure analysis is necessary to discuss the structure of FA–NAA.

The diameter of the host cavity of $(FA)_6$ was estimated[5] to be 5.1—6.7 Å on the assumption that the host structure consists of six molecules of FA. This suggests a size similar



TDS



NAA

Chart 1

to the host cavity[9] of α- or β-cyclodextrin, although cyclodextrins in general includes a lipophilic compound whereas $(FA)_6$ includes a water-soluble compound. Regarding the inclusion of aromatic guest molecules in cyclodextrin, axial-, equatorial- and lid-type inclusions are known.[10] Taking account of the reports[10] and the structure formulas of TDS and NAA shown in Chart 1, the inclusion mode for NAA may differ from that for TDS: one molecule of TDS may be included axially in $(FA)_6$ whereas six molecules of NAA may be included equatorially in $(FA)_6$.

As described above, the release behavior of NAA from FA–NAA (whose molecular formula may possibly be $(FA)_6$-$(NAA)_6$) was similar to that[3] of TDS from $(FA)_6$ (TDS). In conclusion, it was suggested that the release characteristics of drugs from FA–drug complexes may depend on the basic structure composed of six molecules of FA.

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The Nephritogenic Glycopeptide from Rat Glomerular Basement Membrane. X.[1] Synthesis of an *N*-Triglycosyl Dipeptide and Characteristics of Its *cis–trans* Isomers

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Isomers of *O*-α-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→6)-*N*-[L-aspart-1-oyl-(L-proline)-4-oyl]-α-D-glucopyranosylamine have been prepared, as models for a derivative possibly present in the glomerular basement membrane of rats, by condensation of the corresponding dipeptide derivative (5) with triglycosylamine (4) in the presence of *O,O*-diethylcyanophosphonate, followed by deprotection of the trisaccharide–dipeptide derivative. During the deprotection process, *cis*- and *trans*-isomers containing proline were separated by silica gel column chromatography and also reversed-phase high performance liquid chromatography.

Keywords nephritogenoside; glycopyranosylamine; synthesis; *cis–trans* isomer; glomerulonephritis; triglycosyl dipeptide; HPLC; condensation

Shibata *et al.*[2] isolated and purified from the glomerular basement membrane of rats a compound named nephritogenoside that was active for the induction of glomerulonephritis in homologous animals.[3] Nephritogenoside is composed of three D-glucose units, α-D-Glc$_p$-(1→6)-β-D-Glc$_p$-(1→6)-D-Glc$_p$, and 21 amino acids [^{1}Asn–Pro–Leu–Phe–Gly–Ile–Ala–Gly–Glu–Asp–Gly–Pro–Thr–Gly–Pro–Ser–Gly–Ile–Val–Gly–^{21}Gln], and the reducing α-D-glucose unit is linked N-glucosidically to the N-terminal asparagine unit.[4] The synthesis of model glycoproteins and glycopeptides is important because these compounds may have significant biological properties. In our previous paper,[1] we reported the syntheses of the α and β anomers of an N-triglycosyl dipeptide by condensation of the corresponding D-glucosyl dipeptide derivatives with isomaltopyranosyl bromide.

The present synthesis of the glycopeptide differs from those previously reported[1] in that the trisaccharide α-azide was used to improve the α-azide reduction. We examined several conditions for the glycosylazide reduction in our previous paper.[1] In each case, the β-D-anomer was predominantly obtained. Condensation of 2,3-di-*O*-benzyl-α-D-glucopyranosyl azide (**1**) with 2,3,4,2′,3′,4′,6′-hepta-*O*-acetyl-α-D-isomaltosyl bromide (**2**) in the presence of mercuric cyanide in nitromethane afforded the *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-(1→6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→6)-2,3-di-*O*-benzyl-α-D-glucopyranosyl azide (**3**) in 42.4% yield. No other α-D-glycoside was observed in this reaction and the structure of **3** was unambiguously ascertained by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy. The signals due to H-1, H-1′, and H-1″ were observed at δ 5.23 (J=4.0), 4.58 (J=8.1), 5.10 (J=3.7 Hz), and signals due to C-1, C-1′, and C-1″ at δ 89.0, 102.2, 97.0 ppm, respectively. Use of Lindlar's catalyst in 1:1 tetrahydrofuran (THF)–methanol in the presence of a large amount of triethylamine gave the trisaccharide-α,β-D-glucopyranosylamine (**4**) in the ratio of 5:1 as determined from the ^{1}H-NMR spectrum. Compound **4** (mixture of α and β anomers) was coupled with the dipeptide, *N*-(*tert*-butoxycarbonyl)-L-aspartyl-L-proline methyl ester (**5**), in the presence of *O,O*-diethylcyanophosphonate (DEPC) to give *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-(1→6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→6)-2,3-di-*O*-benzyl-*N*-[*N*-(*tert*-butoxycarbonyl)-L-aspart-1-oyl)-(methyl L-prolinate)-4-oyl]-α,β-D-glucopyranosylamine in 64.1% yield. The isomers **6α** and **6β** have the same *Rf* value and could not be separated by chromatography. Removal of the *tert*-butoxycarbonyl and methyl ester groups of **6** with 85% formic acid gave **7α** (60.4%) and **7β** (12.1%) in the ratio of 5:1. Enhancement of stereoselectivity in favor of the α-product was thus achieved compared with our previous paper result[1] (α to β ratio 1:3). The acetyl group of **7α** was removed by sodium methoxide treatment to give the **8α**-*trans* and **8α**-*cis* compounds, respectively. These products were separated by silica gel column chromatography. In our previous paper, we reported this compound (**8α**) as a mixture of *cis–trans* isomers because double peptide peaks were seen in the ^{13}C-NMR spectra; this was anticipated because of the *cis–trans* isomerism present in this compound. In addition, ^{13}C-NMR evidence of *cis–trans* isomerism of the amide bond involving the nitrogen atom of the proline residue was reported by Thomas and Williams,[5] and Dorman and Bovery.[6] The assignments of the *cis*- and *trans*-forms were based on analogy with the N-acetyl group of proline, which preferentially takes the *trans*-form in all usual solvents. It is generally the case that the asparaginyl β-proton of the *trans* conformer is

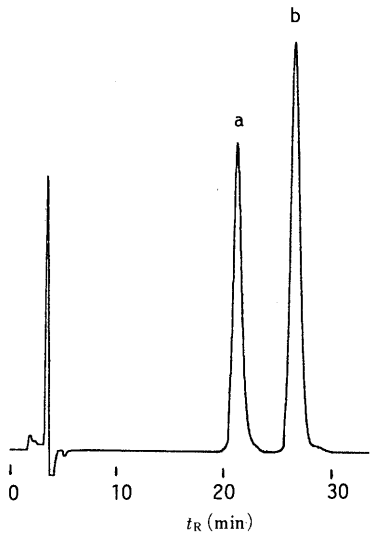

Fig. 1. HPLC Elution Profile of **8α**-*cis* and **8α**-*trans*

Peaks: a, **8α**-*cis*; b, **8α**-*trans*. Column, LiChrosphere; solvent, acetonitrile–THF–water (20:5:75); flow rate, 0.6 ml/min; detection, 210 nm.

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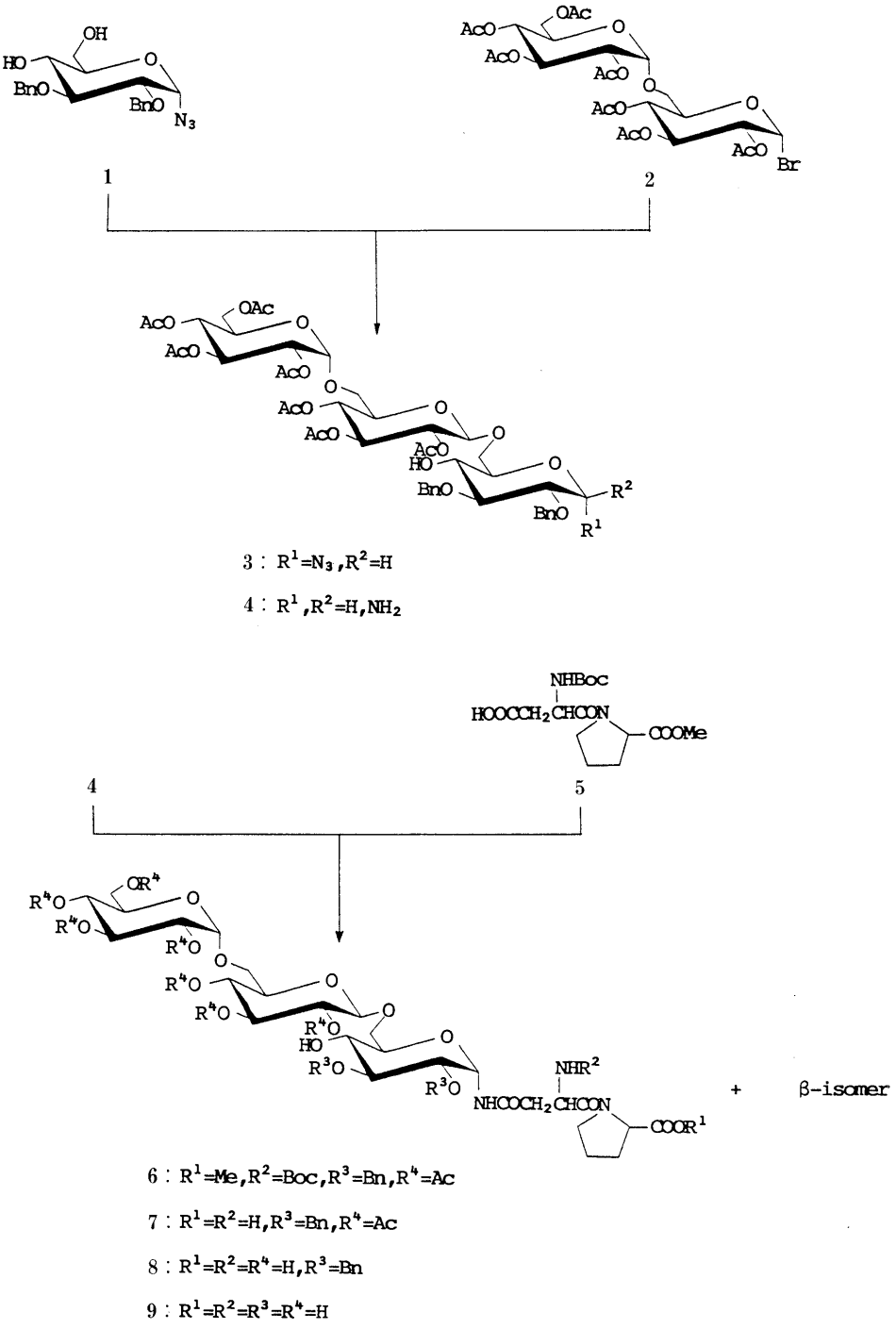

Chart 1

deshielded relative to that of the *cis* conformer. A downfield shift of the signal due to the *trans*-isomer (3.24 ppm for compound **8α**-*trans*, 3.06 ppm for compound **9α**-*trans*) as compared with the *cis*-isomer (2.81 ppm for compound **8α**-*cis*, 2.89 ppm for compound **9α**-*cis*) was observed. This suggests that the chemical shifts of these protons are influenced by the anisotropic effect of the prolyl carboxyl group.

The *cis* and *trans* isomers (**8α**-*cis*, **8α**-*trans*) were also separated by high performance liquid chromatography (HPLC) using a column of LiChrospher RP-18 (5 μm) with acetonitrile–THF–water (20 : 5 : 75) as the eluent, monitored by measuring the absorbance at 210 nm with a ultraviolet (UV) detector, as shown in Fig. 1. The *tran*-isomer is retained to a great extent on the stationary phase. The proportions of each isomer in **8α** were roughly calculated from the peak areas to be 45 : 55 for **8α**-*cis*, **8α**-*trans*, respectively. In the case of the debenzylated *N*-triglucosyl-dipeptide compound (**9α**), two peaks corresponding to **9α**-*cis* : **9α**-*trans* (45 : 55) were clearly obtained on a column of LiChrospher RP-18 with methanol–water (5 : 95) as the eluent. The *trans*/*cis* ratio in the -OBn trisaccharide dipeptide is qualitatively the same as that in the -OH compound. Hydrogenation of **8α**-*cis* and/or -*trans* afforded the corresponding *cis* and *trans* isomers of the target compound, *O*-α-D-glucopyranosyl-(1→6)-*O*-β-D-

glucopyranosyl-(1→6)-*N*-[L-aspart-1-oyl-(L-proline)-4-oyl]-α-D-glucopyranosylamine (**9α-*cis*, 9α-*trans***), in 89.9 and 93.3% yields, respectively. These are the desired key compounds for the completion of nephritogenoside synthesis.

Experimental

Optical rotations were measured with a JASCO DIP-4 digital polarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded with a GSX-400 instrument at room temperature; tetramethylsilane was the internal standard for solutions in $CDCl_3$ and CD_3OD, and sodium 4,4-dimethyl-4-silapentane-1-sulfonate for solutions in D_2O. Thin-layer chromatography (TLC) was conducted on precoated silica gel plates (Merck GF-254), and the compounds were detected by quenching of UV fluorescence and by spraying with 10% H_2SO_4 or a 5% methanolic ninhydrin solution. Column chromatography was carried out on silica gel (Merck Kieselgel 60). HPLC was performed on a Shimadzu LC-4A apparatus equipped with a Shimadzu SPD-6AV spectrometer and a LiChrospher RP-18 (5 μm) column (4.0 mm i.d. × 250 mm) [mobile phase: A) acetonitrile–THF–water, 20 : 5 : 75, B) methanol–water, 5 : 95]: flow rate 0.6 ml/min, detection: 210 nm.

2,3-Di-*O*-benzyl-α-D-glucopyranosyl Azide (1) 2,3-Di-*O*-benzyl-4,6-*O*-isopropylidene-α-D-glucopyranosyl azide[1] (8 g, 19 mmol) was treated with 80% acetic acid (60 ml) at 40 °C for 3 h. The solution was poured into ice-water, and extracted with chloroform. The extract was washed with water, and concentrated, and the residue was chromatographed on silica gel with 50 : 1 (v/v) chloroform–methanol. The fractions having *Rf* 0.70 (8 : 1, chloroform–methanol) were pooled and concentrated (6.8 g, 93.6%); $[\alpha]_D^{22}$ +74.4° (*c* = 2.5, chloroform). ^{1}H-NMR (CD_3OD) δ: 7.36—7.21 (10H, m, aromatic proton), 5.43 (1H, d, *J* = 4.0 Hz, H-1). *Anal.* Calcd for $C_{20}H_{23}N_3O_5 \cdot H_2O$: C, 59.54; H, 6.25; N, 10.42. Found: C, 59.93; H, 6.12; N, 10.13.

***O*-(2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl)-(1→6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→6)-2,3-di-*O*-benzyl-α-D-glucopyranosyl Azide (3)** 2,3,4,2′,3′,4′,6′-Hepta-*O*-acetyl-α-D-isomaltosyl bromide (**2**) (11 g, 15.7 mmol), mercuric cyanide (17.4 g), and molecular sieves 4 Å (14 g) were added to a solution of **1** (5.8 g, 15.7 mmol) in nitromethane (100 ml), and the suspension was stirred for 6 h at 55 °C under Ar gas. The solids were filtered off, and the filtrate was poured into ice water, and extracted with chloroform. The extract was washed with water, and concentrated, and the residual product was chromatographed on silica gel with 10 : 1 (v/v) benzene–acetone. The fractions containing the trisaccharide azide having *Rf* 0.40 (4 : 1, benzene–acetone) were pooled and concentrated (3.6 g, 42.4%). $[\alpha]_D^{23}$ +69.0° (*c* = 2.7, chloroform). ^{1}H-NMR ($CDCl_3$) δ: 7.37—7.27 (10H, m, aromatic proton), 5.23 (1H, d, *J* = 4.0 Hz, H-1), 5.10 (1H, d, *J* = 3.7 Hz, H-1″), 4.58 (1H, d, *J* = 8.1 Hz, H-1′), 2.03—2.00 (21H, each s, 7 × OAc). ^{13}C-NMR ($CDCl_3$) δ: 89.0 (C-1), 102.2 (C-1′), 97.0 (C-1″). *Anal.* Calcd for $C_{46}H_{57}N_3O_{22}$: C, 55.03; H, 5.72; N, 4.19. Found: C, 54.46; H, 5.69; N, 3.79.

***O*-(2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl)-(1→6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→6)-2,3-di-*O*-benzyl-*N*-[*N*-(*tert*-butoxycarbonyl)-L-aspart-1-oyl-(methyl L-prolinate)-4-oyl]-α,β-D-glucopyranosylamine (6α,6β)** A solution of **3** (1.16 g, 1.25 mmol) in 1 : 1, THF–methanol (36 ml) was hydrogenolyzed under atmospheric pressure in the presence of Lindlar's catalyst (1.18 g) and triethylamine (3.6 ml) for 24 h at room temperature. The catalyst was filtered off and the filtrate was evaporated to dryness to give α,β mixtures of the trisaccharide glycosylamine, *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-(1→6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl-(1→6)-2,3-di-*O*-benzyl-α,β-D-glucopyranosylamine (**4**) [^{1}H-NMR ($CDCl_3$) δ: 4.93 (d, *J* = 4.95 Hz, H-1α), 4.16 (d, *J* = 8.61 Hz, H-1β)]. The dipeptide, *N*-(*tert*-butoxycarbonyl)-L-aspartyl-L-proline methylester (**5**)[1] (0.41 g, 1.25 mmol), DEPC (0.25 ml), and triethylamine (0.19 ml) were added to a solution of **4** in *N*,*N*-dimethylformamide (15 ml). The mixture was stirred at 0 °C for 1 h, then left at room temperature for 24 h, diluted with ethyl acetate, washed with water, 10% citric acid solution, and water, dried, and concentrated to give a syrup, which was chromatographed on silica gel with 4 : 1 (v/v) benzene–acetone. The first eluate was evaporated to dryness to give **6α,6β** (0.83 g, 64.1%). The NMR assignments were in good agreement with those published previously.[1]

***O*-(2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl)-(1→6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→6)-*N*-[L-aspart-1-oyl-(L-proline)-4-oyl]-2,3-di-*O*-benzyl-α-D-glucopyranosylamine (7α) and Its β-Isomer (7β)** A solution of **6α,6β** (686.6 mg, 0.53 mmol) in 85% formic acid (12 ml) was stirred for 5 h at room temperature, and then extracted with chloroform. The organic layer was evaporated to dryness and the residue was chromatographed on silica gel with 50 : 1 (v/v) chloroform–methanol. The first eluate was evaporated to dryness to give **7α** (378.5 mg, 60.4%), *Rf* 0.66 (12 : 1, chloroform–methanol). The latter eluate gave **7β** (75.7 mg, 12.1%), *Rf* 0.49. These physicochemical properties were in agreement with previous data.[1]

O*-α-D-Glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→6)-*N*-[L-aspart-1-oyl-(L-proline)-4-oyl]-2,3-di-*O*-benzyl-α-D-glucopyranosylamine (8α)** This compound was obtained by the procedure described in a previous paper[1] by treating **7α** (48.9 mg) with sodium methoxide. The syrup was chromatographed on silica gel with chloroform–methanol–water, 65 : 35 : 10 (lower layer). The first eluate was evaporated to dryness to give **8α-*trans (20.1 mg, 54.6%), $[\alpha]_D^{20}$ +32.3° (*c* = 0.34, methanol), TLC (chloroform–methanol–water, 65 : 35 : 10, lower layer) *Rf* 0.42. ^{1}H-NMR (CD_3OD) δ: 7.39—7.25 (10H, m, aromatic proton), 5.65 (1H, d, *J* = 5.5 Hz, H-1), 4.89 (1H, s, H-1″), 4.66 (1H, d, *J* = 7.9 Hz, H-1′), 4.26 (1H, dd, *J* = 3.5, 9.0 Hz, asp-α), 3.97 (1H, dd, *J* = 3.4, 9.8 Hz, pro-α), 3.24 (1H, dd, *J* = 3.5, 17.3 Hz, asp-β-H_b), 2.67 (1H, dd, *J* = 9.0, 17.3 Hz, asp-β-H_a), 2.31 (1H, m, pro-β-H_a), 2.07—1.88 (3H, m, pro-β-H_b, pro-γ). The later eluate gave **8α-*cis*** (16.5 mg, 44.7%), $[\alpha]_D^{20}$ +53.1° (*c* = 0.28, methanol), TLC *Rf* 0.36. ^{1}H-NMR (CD_3OD) δ: 7.39—7.25 (10H, m, aromatic proton), 5.66 (1H, d, *J* = 5.3 Hz, H-1), 4.88 (1H, s, H-1″), 4.40 (1H, d, *J* = 7.7 Hz, H-1′), 4.29 (1H, dd, *J* = 4.2, 7.7 Hz, asp-α), 3.92 (1H, dd, *J* = 5.1, 10.6 Hz, pro-α), 2.94 (1H, dd, *J* = 7.7, 15.8 Hz, asp-β-H_a), 2.81 (1H, dd, *J* = 4.2, 15.8 Hz, asp-β-H_b), 2.35 (1H, m, pro-β-H_a), 2.36—1.88 (3H, m, pro-β-H_b, pro-γ). HPLC (mobile phase A): retention time, **8α-*cis*** 21.4 min, *trans* 26.7 min. *Anal.* Calcd for $C_{41}H_{57}N_3O_{19} \cdot 1/2H_2O$: C, 54.42; H, 6.46; N, 4.64. Found: for **10α-*trans***: C, 54.35; H, 6.17; N, 4.45. Found: for **10α-*cis***: C, 54.59; H, 6.64; N, 4.39.

O*-α-D-Glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→6)-*N*-[L-aspart-1-oyl-(L-proline)-4-oyl]-α-D-glucopyranosylamine (9α-*trans* and 9α-*cis*)** A suspension consisting of a solution of **8α-*trans (24.4 mg, 28 μmol) in 1 : 1, ethanol–water (4 ml) and 10% Pd–C (40 mg) was stirred for 24 h under H_2, and then filtered. The filtrate was concentrated to dryness. The residue was chromatographed on Sephadex G-10. The water eluate was lyophilized to give a white powder, **9α-*trans*** (18.2 mg, 93.3%). In the same way compound **9α-*cis*** (17.5 mg, 89.9%) was obtained from **8α-*cis*** (24.4 mg).

9α-*trans*: $[\alpha]_D^{18}$ +96.7° (*c* = 0.3, water), TLC (1 : 1 : 1 : 1, butanol–acetic acid–ethyl acetate–water) *Rf* 0.19. ^{1}H-NMR (D_2O) δ: 5.58 (1H, d, *J* = 5.7 Hz, H-1), 4.97 (1H, d, *J* = 3.7 Hz, H-1″), 4.53 (1H, d, *J* = 7.9 Hz, H-1′), 4.38 (1H, dd, *J* = 4.6, 5.5 Hz, asp-α), 3.97 (1H, dd, *J* = 4.6, 11.2 Hz, pro-α), 3.06 (1H, dd, *J* = 4.6, 17.0 Hz, asp-β-H_b), 2.99 (1H, dd, *J* = 5.5, 17.0 Hz, asp-β-H_a), 2.36 (1H, m, pro-β-H_a), 2.09 (1H, m, pro-β-H_b), 2.06—1.92 (2H, m, pro-γ). *Anal.* Calcd for $C_{27}H_{45}N_3O_{19} \cdot 2.5H_2O$: C, 42.63; H, 6.62; N, 5.52. Found: C, 42.42; H, 6.25; N, 5.18.

9α-*cis*: $[\alpha]_D^{18}$ +100.1° (*c* = 0.3, water), TLC *Rf* 0.19. ^{1}H-NMR (D_2O): δ: 5.59 (1H, d, *J* = 5.5 Hz, H-1), 4.97 (1H, d, *J* = 3.7 Hz, H-1″), 4.52 (1H, d, *J* = 7.9 Hz, H-1′), 4.40 (1H, dd, *J* = 4.6, 7.2 Hz, asp-α), 3.97 (1H, dd, *J* = 4.4, 11.1 Hz, pro-α), 2.97 (1H, dd, *J* = 7.2, 15.6 Hz, asp-β-H_a), 2.89 (1H, dd, *J* = 4.6, 15.6 Hz, asp-β-H_b), 2.36 (1H, m, pro-β-H_a), 2.09 (1H, m, pro-β-H_b), 2.09—1.87 (2H, m, pro-γ). *Anal.* Calcd for $C_{27}H_{45}N_3O_{19}$: C, 45.31; H, 6.34; N, 5.87. Found: C, 45.12; H, 6.62; N, 5.58. HPLC (mobile phase B): retention time **9α-*cis*** 15.7 min, *trans* 20.7 min.

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Conversion of D-Glucose into the β-Hydroxy-δ-lactone Moiety of Mevinic Acids and Congeners *via* D-Idose as a Key Chiral Intermediate

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(4*R*,6*S*)-4-Hydroxy-6-hydroxymethyl-3,4,5,6-tetrahydro-2*H*-pyran-2-one (1) and (4*R*,6*S*)-4-hydroxy-6-hydroxymethyl-2-methoxytetrahydropyran (2), chirons of the β-hydroxy-δ-lactone moiety of mevinic acids and congeners, were derived from 1,2,3,6-tetra-*O*-acetyl-α-D-idose (5), which is easily available from penta-*O*-acetyl-β-D-glucose (3).

Keywords idose; δ-lactone; mevinic acid; tetrahydropyran-2-one; tributyltin hydride; reductive deoxygenation; reductive rearrangement

Mevinic acids [*i.e.*, mevastatin (A)[1] and lovastatin (B)[2]] are potent inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme involved in one of the early steps of cholesterol biosynthesis.[3] Lovastatin (B) and a 3-hydroxymevastatin derivative (pravastatin sodium) are members of a new class of hypocholesterolemic agents and are in clinical use. Numerous studies[4] on the structure–activity relationships of HMG-CoA reductase inhibitors have shown that the chiral δ-lactone moiety in mevinic acids is essential for strong hypocholesterolemic activity and various types of (4*R*,6*S*)-6-arylethenyl-4-hydroxy-3,4,5,6-tetrahydro-2*H*-pyran-2-one (C) retain the activity. Thus, synthesis of (4*R*,6*S*)-4-hydroxy-6-hydroxymethyl-3,4,5,6-tetrahydro-2*H*-pyran-2-one (**1**) and (4*R*,6*S*)-4-hydroxy-6-hydroxymethyl-2-methoxytetrahydropyran (**2**), chirons of the δ-lactone moiety of mevinic acids and their congeners is of potential importance.

Among the published routes to the δ-lactone moiety, the conversion of a readily available carbohydrate such as D-glucose has been most studied. These conversions involve two deoxygenation reactions at C-2 and C-4 and an inversion of configuration at C-3 of D-glucose by various procedures.[5–9]

A special feature of our synthetic approaches to **1** and **2** is employment of 1,2,3,6-tetra-*O*-acetyl-α-D-idose (**5**) as a key chiral intermediate which is easily obtained from penta-*O*-acetyl-β-D-glucose (**3**).[10] The intermediate (**5**) appropriately sets up the 3(*R*)-configuration corresponding to the 4(*R*)-configuration of **1** and **2** and possesses a free C-4-hydroxy group, which is convenient for reductive deoxygenation.[11] For the deoxygenation at C-2, two methods are applicable: one is reductive rearrangement of 2-*O*-acetyl-1-halogenosugars[12] and the other is reductive elimination of 1-*O*-acetyl-2-halogenosugars (see Chart 2). Although the overall yields of the present approaches are not always superior to previously reported routes,[6] employment of **5** as a chiral pool made it possible to prepare **1** and **2** *via* comparatively few steps on a laboratory scale.[13]

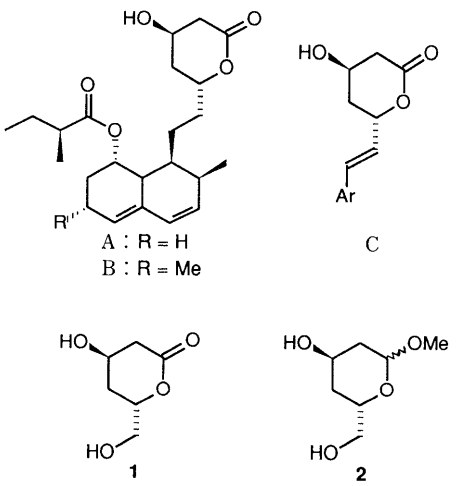

Chart 1

According to the modified Paulsen procedure,[10] the glucose (**3**) was allowed to react with antimony pentachloride to give the acetoxonium salt, which was subsequently treated with sodium acetate to give a mixture of D-idose derivatives (**4** and **5**) as rearranged products. Fortunately, the desired 4-hydroxy derivative (**5**) was obtained as a sole product upon treatment of the mixture of **4** and **5** with aqueous pyridine as a result of acetyl migration[14] (46.7% from **3**). After conversion of **5** to the 4-*O*-phenoxythiocarbonyl derivative (**6**), the reaction with tributyltin hydride resulted in smooth deoxygenation at C-4 of **5** to give the 4-deoxy derivative (**7**) (82% from **5**).

The 1-bromo derivative (**8**), which was quantitatively prepared by bromination of **7** using hydrogen bromide, was subjected to radical rearrangement leading to 1,3,6-tri-*O*-acetyl-2,4-dideoxy-β-D-idopyranose [(2*S*,4*R*,6*S*)-2,4-diacetoxy-6-acetoxymethyltetrahydropyran] (**9a**). Giese *et al.*[12] have shown that the reduction of 2-*O*-acylated or benzoylated glycosyl halides with low concentrations of tributyltin hydride provides 2-deoxysugars, and an important step in this radical chain reaction is the *cis*-selective migration of an ester group. Although the reductive rearrangement of 2-*O*-acylglucosyl bromides to 2-deoxy-α-D-glucoses proceeds in high yield, acylmannosyl bromide gives 2-deoxy-β-D-mannose with *cis*-selectivity in slightly lower yield, because the C–O bond at the anomeric center of the resulting 2-deoxysugar is situated in the less stable equatorial position.[12] In accordance with the previous observation, the *cis*-rearranged product (**9a**), which has β-configuration at the anomeric position was formed in moderate yield (60%) together with the debromination product (**14**) (40%) (reaction conditions: see the Experimental section). The structure of **9a** with the equatorial 2-acetoxy group in its preferred conformation was confirmed by nuclear magnetic resonance (NMR) spectral comparison with **9a**, which was previously prepared by an alternative route.[15] When the bromide (**11**) derived from **6** was treated with tributyltin hydride, a complex mixture of products was formed and **9a** was isolated in only a poor

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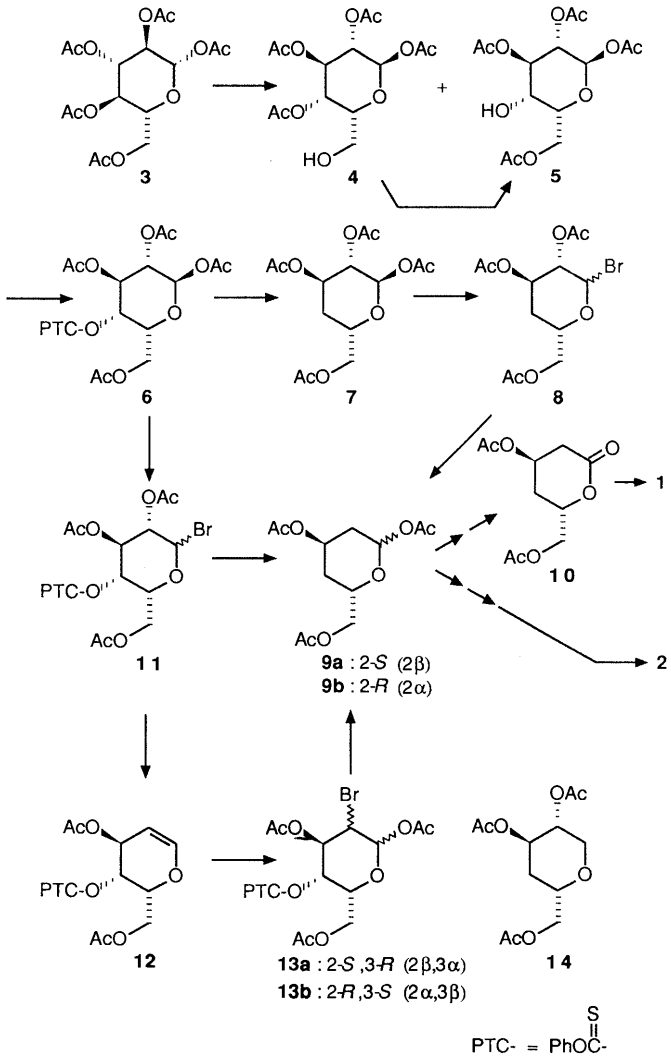

Chart 2

yield from the reaction mixture.

According to the procedure previously reported,[16] **9a** was hydrolyzed in aqueous dioxane and subsequently oxidized with bromine to give the lactone (**10**). Deprotection of **10** to **1** was achieved by using diluted hydrochloric acid. The deprotection, however, was always accompanied with the formation of the (3*R*,5*S*)-3,5,6-trihydroxyhexanoic acid and (6*S*)-6-hydroxymethyl-5,6-dihydro-2*H*-pyran-2-one through hydrolysis of the lactone portion and elimination of the 4-acetoxy group in **10**, respectively. The acetal (**2**) was obtained in 74% yield from **9a** in the previously reported manner.[16] Thus, in the present trial, **2** is preferable to **1** as a synthetic intermediate for the HMG-CoA reductase inhibitors. The spectral data of **1**[17] and **2**[16] were identical with those of authentic samples.

When **11** was treated with zinc in acetic acid,[18] the gulal derivative (**12**) was obtained in 44% yield. *trans*-Addition of acetyl hypobromite (CH_3COOBr, prepared from *N*-bromosuccinimide and acetic acid) to **12** resulted in the formation of a stereoisomeric mixture of 2-acetoxy-3-bromopyran derivative (**13a** and **13b**) in 60% yield. Upon treatment of **13a** and **13b** with tributyltin hydride, **9a** and its anomer (**9b**) were obtained in 32% and 48% yields after chromatography as a result of the concurrent occurrence of debromination and deoxygenation. The product (**9a**) was identical with a sample prepared by the foregoing method. The structure of the anomer (**9b**) was confirmed by comparison of its NMR spectrum with that of **9b** described in the literature.[16]

The overall yield of the mixture of **9a** and **9b** (9.7% from **3**) obtained by this route, however, is inferior to that by the foregoing route (22.5% **9a** from **3**).

Experimental

Melting points were determined on a Yanagimoto melting-point apparatus and are uncorrected. ^{1}H-NMR spectra were determined with a Hitachi–Perkin-Elmer R-20B 60 MHz instrument and a JEOL JNM-GX270 NMR spectrometer, using tetramethylsilane in $CDCl_3$ or sodium 2,2-dimethyl-2-silapentane-5-sulfonate in $(CD_3)_2SO$ as an internal standard. Chemical shifts are reported in ppm (δ) and J values in Hz. Mass spectra were taken on a JEOL JMS-D300 machine operating at 70 eV. Elemetal analyses were carried out at the Microanalytical Laboratory of our University. Column chromatography was carried out on silica gel (Wako gel C-300).

1,2,3,6-Tetra-*O*-acetyl-α-D-idopyranose (5) A solution of antimony pentachloride (15 ml, 110 mmol) in dry dichloromethane (10 ml) was added dropwise to a stirred solution of penta-*O*-acetyl-β-D-glucopyranose (**3**) (31.2 g, 80 mmol) in dry dichloromethane (100 ml) at 0 °C under an argon atmosphere. The acetoxonium salt appeared within 10—15 min. After 1 h, the resulting salt was filtered through a glass filter, washed with dry dichloromethane (100 ml), and dried *in vacuo*. The salt was added to a aqueous solution of sodium acetate (36 g of sodium acetate was dissolved in 100 ml of water) with stirring at 0 °C for 15 min. The reaction solution was extracted with chloroform. The organic layer was washed with water and saturated aqueous sodium chloride, and dried over anhydrous magnesium sulfate. Evaporation of the solvent under reduced pressure gave a syrup, which was a mixture of 1,2,3,4-tetra-*O*-acetyl-α-D-idopyranose (**4**) and 1,2,3,6-tetra-*O*-acetyl-α-D-idopyranose (**5**). The mixture was dissolved in 10% aqueous pyridine (100 ml) and left to stand at room temperature for 24 h. The solution was extracted with chloroform. The extract was washed with water, dil. HCl and water, and dried over anhydrous magnesium sulfate. Evaporation of the solvent under reduced pressure gave a syrup, which was triturated with dry ether to give 1,2,3,6-tetra-*O*-acetyl-α-D-idopyranose (**5**) as a white powder (13.0 g, 46.7%). This crude product was used in the next step without further purification. Recrystallization from ether gave a pure product (**5**), which was identified by comparison of its NMR spectra with those of an authentic sample.[10]

1,2,3,6-Tetra-*O*-acetyl-4-*O*-phenoxythiocarbonyl-α-D-idopyranose (6) Phenoxythiocarbonyl chloride (PTC-Cl) (2.32 ml, 13 mmol) was added dropwise to a mixture of **5** (3.48 g, 10 mmol) and 4-dimethylaminopyridine (DMAP) (2.44 g, 20 mmol) in dry acetonitrile (50 ml) at 0 °C under an argon atmosphere. The solution was stirred overnight at room temperature. The solvent was evaporated off under reduced pressure and the residue was purified by column chromatography on silica gel with benzene–ethyl acetate (10:1) to give 1,2,3,6-tetra-*O*-acetyl-4-*O*-phenoxythiocarbonyl-α-D-idopyranose (**6**) (4.80 g, 98%) as a colorless syrup. *Anal.* Calcd for $C_{21}H_{24}O_{11}S$: C, 52.06; H, 4.99. Found: C, 52.28; H, 5.12. ^{1}H-NMR ($CDCl_3$) δ: 7.39—7.01 (5H, m, O-Ph), 6.06 (1H, d, J=1.0 Hz, H-1), 5.37 (1H, m, H-2), 5.28 (1H, t, J=2.4 Hz, H-3), 4.85 (1H, m, H-4), 4.55 (1H, dt, J=2.0, 6.4 Hz, H-5), 4.26 (2H, m, H-6 and H-6'), 2.09, 2.08, 2.07, 2.02 (each 3H, each s, each OAc).

1,2,3,6-Tetra-*O*-acetyl-4-deoxy-α-D-idopyranose (7) A solution of the idopyranose (**6**) (4.84 g, 10 mmol), α,α'-azobis(isobutyronitrile) (AIBN) (200 mg, 1.2 mmol), and tributyltin hydride (2 ml, 7.2 mmol) in distilled toluene (50 ml) was degassed with argon for 20 min, and then heated at 80 °C for 2 h. Tributyltin hydride (1 ml, 3.6 mmol) was further added twice at every third hour. Then the reaction solution was stirred for 1 h at the same temperature. The solution was diluted with acetonitrile, and washed three times with the same volume of hexane. The acetonitrile layer was evaporated under reduced pressure and the residue was triturated with dry ether. The resulting precipitate was collected by filtration and recrystallized from dry ether to give 2.72 g (82%) of 1,2,3,6-tetra-*O*-acetyl-4-deoxy-α-D-idopyranose (**7**), mp 92 °C. *Anal.* Calcd for $C_{14}H_{20}O_9$: C, 50.60; H, 6.07. Found: C, 50.56; H, 6.11. ^{1}H-NMR ($CDCl_3$) δ: 6.03 (1H, d, J=1.0 Hz, H-1), 5.02 (1H, dd, J=1.0, 2.9 Hz, H-3), 4.81 (1H, t, J=1.0 Hz, H-2), 4.32 (1H, m, H-5), 4.14 (2H, dd, J=1.0, 5.9 Hz, H-6 and H-6'), 2.13 (3H, s, OAc), 2.12 (6H, s, OAc), 2.10 (3H, s, OAc), 2.00 (1H,

ddd, J=2.9, 11.7, 14.6 Hz, H-4), 1.76 (1H, d, J=14.6 Hz, H-4').

(2*S*,4*R*,6*S*)-2,4-Diacetoxy-6-acetoxymethyltetrahydropyran (1,3,6-Tri-*O*-acetyl-2,4-dideoxy-β-D-idopyranose) (9a) and (3*S*,4*R*,6*S*)-3,4-Diacetoxy-6-acetoxymethyltetrahydropyran (14) a): A 30% acetic acid solution of HBr (3 ml) was added to a solution of **7** (996 mg, 3 mmol) in acetic acid (2 ml) with stirring at room temperature. After 20 min, the straw-colored solution was taken up in dichloromethane (50 ml) and the mixture was washed twice with ice water, cold 1 M sodium bicarbonate, and water, then dried over anhydrous magnesium sulfate, filtered, and evaporated under reduced pressure to give 2,3,6-tri-*O*-acetyl-4-deoxy-D-idopyranosyl bromide (**8**) as a light yellow syrup. To a solution of the above-obtained product (**8**) and AIBN (50 mg, 0.3 mmol) in distilled toluene (200 ml), 0.2 ml of tributyltin hydride was added dropwise every 5 min twelve times at 80 °C under an argon atmosphere. The mixture was stirred for 2 h and the solvent was evaporated off under reduced pressure. The residue was dissolved in acetonitrile and the solution was washed three times with the same volume of hexane. The acetonitrile layer was evaporated under reduced pressure and the residue was chromatographed on silica gel with benzene–ethyl acetate (5 : 1) to give two products, **9a** and **14**, in 60%, 40% yields, respectively. (2*S*,4*R*,6*S*)-2,4-Diacetoxy-6-acetoxymethyltetrahydropyran (**9a**); syrup, *Anal.* Calcd for $C_{12}H_{18}O_7$: C, 52.55; H, 6.62. Found: C, 52.92; H, 6.87. ^{1}H-NMR ($CDCl_3$) δ: 6.00 (1H, dd, J=9.8, 2.5 Hz, H-2), 5.32 (1H, t, J=3.4 Hz, H-4), 4.18—4.11 (3H, m, H-6 and C<u>H</u>$_2$–OAc), 2.11, 2.09, 2.08 (each 3H, each s, each OAc), 2.02—1.50 (4H, m, H-3, H-3', H-5, H-5'). (3*S*,4*R*,6*S*)-3,4-Diacetoxy-6-acetoxymethyltetrahydropyran (**14**); syrup, *Anal.* Calcd for $C_{12}H_{18}O_7$: C, 52.55; H, 6.62. Found: C, 52.64; H, 6.77. ^{1}H-NMR ($CDCl_3$) δ: 5.05 (1H, d, J=2.9 Hz, H-4), 4.67 (1H, d, J=2.0 Hz, H-3), 4.20—3.80 (5H, m, H-2, H-2', H-6, C<u>H</u>$_2$–OAc), 2.13 (9H, s, 3×OAc), 1.92 (1H, ddd, J=2.9, 11.7, 14.1 Hz, H-5), 1.73 (1H, d, J=14.1 Hz, H-5').

b): A 30% acetic acid solution of HBr (3 ml) was added to a solution of **6** (1.46 g, 3 mmol) in acetic acid (2 ml) with stirring at room temperature. After 20 min, the solution was taken up in dichloromethane (50 ml) and the mixture was washed twice with ice water, dried over anhydrous magnesium sulfate, and evaporated under reduced pressure to give 2,3,6-tri-*O*-acetyl-4-*O*-phenoxythiocarbonyl-D-idopyranosyl bromide (**11**) as a syrup. To a solution of the above product (**11**) and AIBN (100 mg, 0.6 mmol) in distilled toluene (200 ml), 0.3 ml of tributyltin hydride was added dropwise every 5 min twelve times. The mixture was stirred for 2 h and the solvent was evaporated off under reduced pressure. The residue was dissolved in acetonitrile and the solution was washed three times with the same volume of hexane. The acetonitrile layer was evaporated under reduced pressure and the residue was chromatographed on silica gel with benzene–ethyl acetate (5 : 1) to give a mixture of (2*S*,4*R*,6*S*)-2,4-diacetoxy-6-acetoxymethyltetrahydropyran (**9a**) (9.3%) and (3*S*,4*R*,6*S*)-3,4-diacetoxy-6-acetoxymethyltetrahydropyran (**14**) (60%). The two products could not be separated by further column chromatography and the yields were determined from the integrals of the NMR signals.

(4*R*,6*S*)-4-Hydroxy-6-hydroxymethyl-3,4,5,6-tetrahydro-2*H*-pyran-2-one (1) According to the previously reported method,[16] **9a** was hydrolyzed and oxidized to give (4*R*,6*S*)-4-acetoxy-6-acetoxymethyl-3,4,5,6-tetrahydro-2*H*-pyran-2-one (**10**) (122 mg, 82%) as a syrup. This product was identified by comparison of its NMR spectra with those of an authentic sample.[16] A solution of **10** (280 mg, 1.2 mmol) and 2N HCl (5 ml) was stirred at room temperature for 24 h and then adjusted to pH 3 with a saturated aqueous solution of sodium bicarbonate. The solution was further stirred at room temperature for 24 h and evaporated under reduced pressure. The residue was chromatographed on silica gel with chloroform–methanol (10 : 1) to give three products. (4*R*,6*S*)-4-Hydroxy-6-hydroxymethyl-3,4,5,6-tetrahydro-2*H*-pyran-2-one (**1**) (40 mg, 23%); syrup. This product was identified by comparison of its NMR spectra with those of an authentic sample.[17] (3*R*,5*S*)-3,5,6-Trihydroxyhexanoic acid (38 mg, 22%); ^{1}H-NMR [$(CD_3)_2SO$] δ: 4.18 (1H, m, H-3), 3.70 (1H, m, H-5), 3.42 (2H, d, J=5.4 Hz, H-6 and H-6'), 2.52 (2H, dd, J=4.4, 12.7 Hz, H-2 and H-2'), 2.80—2.50 (2H, m, H-4 and H-4'). (6*S*)-6-Hydroxymethyl-5,6-dihydro-2*H*-pyran-2-one (8 mg, 5%); ^{1}H-NMR ($CDCl_3$) δ: 6.89 (1H, m, H-4), 5.95 (1H, ddd, J=1.0, 2.9, 9.8 Hz, H-3), 4.49 (1H, m, H-6), 3.80 (1H, dd, J=3.4, 12.5 Hz, one H of –C<u>H</u>$_2$–OH), 3.68 (1H, dd, J=4.9, 12.5 Hz, one H of –C<u>H</u>$_2$–OH), 2.52 (1H, m, H-5), 2.27 (1H, m, H-5')

(2*S*,3*R*,4*R*,5*S*,6*R*)-2,4-Diacetoxy-6-acetoxymethyl-3-bromo-5-phenoxythiocarbonyloxytetrahydropyran (1,3,6-Tri-*O*-acetyl-2-bromo-2-deoxy-4-*O*-phenoxythiocarbonyl-β-D-gulopyranose) (13a) and (2*R*,3*S*,4*R*,5*S*,6*R*)-2,4-Diacetoxy-6-acetoxymethyl-3-bromo-5-phenoxythiocarbonyloxytetrahydropyran (1,3,6-Tri-*O*-acetyl-2-bromo-2-deoxy-4-*O*-phenoxythiocarbonyl-β-D-idopyranose) (13b) A solution of $CuSO_4 \cdot 5H_2O$ (747 mg) in water (4 ml) was added to a mixture of sodium acetate (13.1 g) and zinc powder (7.2 g) in water (10 ml) and acetic acid (13 ml) under ice-salt cooling. A solution of **11** (10.2 g, 20 mmol) in acetic acid (10 ml) was dropped into the above solution for about 1 h under stirring with a mechanical stirrer at −10—−20 °C. After the addition, the mixture was further stirred for 3h, then filtered through Celite, and washed with 50% aqueous acetic acid. The combined filtrate was extracted with chloroform, and the organic layer was washed with cooled water, saturated aqueous solution of sodium bicarbonate, and water. The solution was dried over anhydrous magnesium sulfate and evaporated under reduced pressure to give a syrupy product. Purification by column chromatography on silica gel with benzene–ethyl acetate (10 : 1) gave 3,6-di-*O*-acetyl-2-deoxy-4-*O*-phenoxythiocarbonyl-D-xylohex-1-enitol (**12**) (3.19 g, 43.6%); ^{1}H-NMR ($CDCl_3$) δ: 7.45—7.12 (5H, m, O-Ph), 6.69 (1H, d, J=5.9 Hz, H-1), 5.58 (1H, m, H-4), 5.21 (1H, dd, J=2.4, 5.9 Hz, H-3), 5.13 (1H, dt, J=1.4, 5.9 Hz, H-2), 4.50—4.32 (3H, m, H-5, H-6, H-6'), 2.12 (6H, s, 2×OAc).

N-Bromosuccinimide (1.41 g, 7.92 mmol) was added to a solution of the gulal derivative (**12**) (2.67 g, 7.2 mmol) in acetic acid (15 ml). The mixture was stirred for 5 h at room temperature and extracted with chloroform. The extract was washed with water, a saturated aqueous solution of sodium bicarbonate, and water, and dried over anhydrous magnesium sulfate. The solvent was evaporated off under reduced pressure and the residue was chromatographed on silica gel with benzene–ethyl acetate (10 : 1) to give two main products (**13a** and **13b**). (2*S*,3*R*,4*R*,5*S*,6*R*)-2,4-Diacetoxy-6-acetoxymethyl-3-bromo-5-phenoxythiocarbonyloxytetrahydropyran (**13a**) was obtained in 24% yield as a syrup; ^{1}H-NMR ($CDCl_3$) δ: 7.47—7.12 (5H, m, O-Ph), 6.07 (1H, d, J=9.3 Hz, H-2), 5.70 (1H, dd, J=3.5, 3.9 Hz, H-4), 5.49 (1H, dd, J=1.5, 3.5 Hz, H-5), 4.49 (1H, dt, J=1.5, 5.8 Hz, H-6), 4.37—4.29 (2H, m, H-3 and one H of –C<u>H</u>$_2$–OAc), 4.18 (1H, dd, J=6.8, 11.8 Hz, one H of –C<u>H</u>$_2$–OAc), 2.23, 2.20, 2.09 (each 3H, each s, each OAc). (2*R*,3*S*,4*R*,5*S*,6*R*)-2,4-Diacetoxy-6-acetoxymethyl-3-bromo-5-phenoxythiocarbonyloxytetrahydropyran (**13b**) was obtained in 36% yield as a syrup; ^{1}H-NMR ($CDCl_3$) δ: 7.46—7.12 (5H, m, O-Ph), 6.33 (1H, d, J=2.4 Hz, H-2), 5.64 (1H, m, H-4), 4.90 (1H, dd, J=1.0, 2.5 Hz, H-5), 4.45—4.30 (3H, m, H-6 and –C<u>H</u>$_2$–OAc), 4.08 (1H, m, H-3), 2.15, 2.14. 2.11 (each 3H, each s, each OAc).

(2*S*,4*R*,6*S*)-2,4-Diacetoxy-6-acetoxymethyltetrahydropyran (1,3,6-Tri-*O*-acetyl-2,4-dideoxy-β-D-idopyranose) (9a) and (2*R*,4*R*,6*S*)-2,4-Diacetoxy-6-acetoxymethyltetrahydropyran (1,3,6-Tri-*O*-acetyl-2,4-dideoxy-α-D-idopyranose) (9b) A solution of the mixture of **13a** and **13b** (2.19 g, 4.3 mmol), AIBN (100 ml, 0.6 mmol), and tributyltin hydride (2.5 ml, 9 mmol) in distilled toluene (50 ml) was degassed with argon for 20 min, and then heated at 80 °C for 2 h. Tributyltin hydride (1 ml, 3.6 mmol) was further added and the solution was stirred for 1 h at the same temperature. The solution was diluted with acetonitrile, and washed with hexane. The acetonitrile layer was evaporated under reduced pressure and the residue was chromatographed on silica gel with benzene–ethyl acetate (5 : 1) to give a mixture of **9a** and its anomer (**9b**) (997 mg, 80%). (2*R*,4*R*,6*S*)-2,4-Diacetoxy-6-acetoxymethyltetrahydropyran (**9b**) was isolated as a syrup, *Anal.* Calcd for $C_{12}H_{18}O_7$: C, 52.55; H, 6.62. Found: C, 52.62; H, 6.88. ^{1}H-NMR ($CDCl_3$) δ: 6.18 (1H, d, J=3.4 Hz, H-2), 5.15 (1H, m, H-4), 4.38 (1H, m, H-6), 4.11 (2H, m, –C<u>H</u>$_2$–OAc), 2.15—1.70 (4H, m, H-3, H-3', H-5, H-5'), 2.09 (9H, s, 3×OAc).

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Efficient Preparation of Optically Active (*S*)-(−)-3-Methyl-γ-butyrolactone by Catalytic Asymmetric Hydrogenation Using Chiral *N*-Substituted Pyrrolidinebisphosphine Rhodium Complexes[1)]

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(*S*)-(−)-2-Methyl succinamic acid, which is a good precursor of (*S*)-(−)-3-methyl-γ-butyrolactone, can be prepared by homogeneous asymmetric hydrogenation of 2-methylene succinamic acid catalyzed by (2*S*,4*S*)-*N*-substituted-4-(diphenylphosphino)-2-[(diphenylphosphino)-methyl]pyrrolidine–rhodium complexes. Various *N*-substituted pyrrolidine-bisphosphines were synthesized to find the optimum ligand for this purpose and to compare the effects of the *N*-substituents.

Keywords *N*-substituted pyrrolidinebisphosphines ligand; asymmetric hydrogenation; rhodium-bisphosphine complex; (*S*)-(−)-3-methyl-γ-butyrolactone; (*S*)-(−)-2-methylsuccinamic acid

(*S*)-(−)-3-Methyl-γ-butyrolactone (**1**) is an important chiral building block for the synthesis of vitamin E, vitamin K, vitamin D_2 and dilichol.[2−5)] Though many attempts[6−9)] at asymmetric synthesis of optically active **1** have been made, highly optically active **1** has not been obtained. In order to establish a practical preparation of optically active **1**, we designed a synthetic pathway containing catalytic asymmetric hydrogenation as the key step (Chart 1).

Our study of the catalytic asymmetric hydrogenation step began with syntheses of various *N*-substituted pyrrolidinebisphosphine ligands (*N*-substituted PPMs). (2*S*,4*S*)-*N*-(*tert*-Butoxycarbonyl)-4-(diphenylphosphino)-2-[(diphenylphosphino)methyl]pyrrolidine, named BPPM, has already been found to be effective as a ligand for rhodium-catalyzed asymmetric hydrogenation of ketopantolactone, dehydroamino acid, itaconic acid, *etc.*[10−14)] When we achieved asymmetric hydrogenation of itaconic acid, *N*-substituted PPMs were also synthesized except *N*-carbamoyl PPMs.[15)] Recently, we found that MCCPM, one of the *N*-carbamoyl CPMs, was an especially efficient ligand in asymmetric hydrogenation of aminoketones rather than BCPM, which is one of the *N*-alkoxycarbonyl CPMs.[16)] So we prepared various PPM analogues, including *N*-carbamoyl PPMs (Chart 2).

BPPM was treated with trifluoroacetic acid (TFA) to give PPM in 94% yield and this was converted into various *N*-substituted PPMs. The abbreviations of the products, yields from PPM, melting points, and $[\alpha]_D$ values are listed in Table I.

As shown in Chart 1, the hydrogenation substrate, 2-methylenesuccinamic acid (**3**), was obtained by hydrolysis of itaconic anhydride (**2**) with 28% ammonium hydroxide. The asymmetric hydrogenation of **3** was carried out in the presence of 1—0.1 mol% of a cationic rhodium complex ($[Rh(COD)\cdot Ligand]^+ClO_4^-$) and an equimolar amount of triethylamine at room temperature for 20 h in methanol ([subst.] = 0.33 M) under an initial hydrogen pressure of 1—30 atm. These results are summarized in Table II and show that *N*-carbamoyl PPMs gave moderately better optical yields. Moreover, even at lower hydrogen pressure, *N*-carbamoyl PPM–rhodium complexes were very effective, resulting in nearly 80%ee. The steric or electronic effects of the *N*-substituent are considered to play an important role in forming the chirality of the complex and so these results suggest that the *N*-carbamoyl PPM–rhodium complex forms a favorable chiral array of the phenyl rings on the phosphines. On the other hand, the hydrogenation did not proceed at all when the BCPM which has a dicyclohexylphosphino group was used.

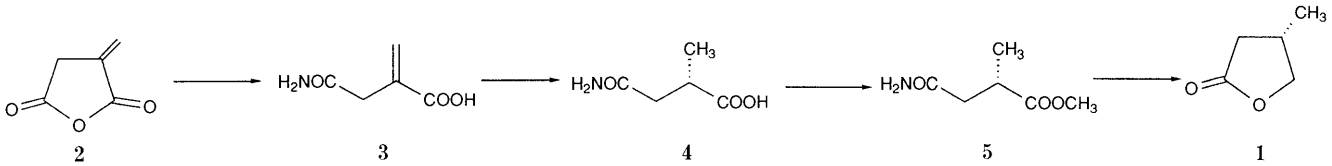
Recrystallization of the hydrogenation product (nearly 80%ee, entry 9 or 13) from methanol gave optically pure (*S*)-(−)-2-methylsuccinamic acid (**4**) in 62% yield from **3**. Then, **4** was esterified with dry hydrogen chloride in methanol to give (*S*)-(−)-2-methylsuccinamic acid methyl ester (**5**) in 90% yield. Reduction of **5** with $NaBH_4$–methanol in tetrahydrofuran (THF) followed by treatment with concentrated hydrochloric acid gave (*S*)-(−)-3-methyl-γ-butyrolactone (**1**) in 50% yield. The overall yield from **3** was 26.8%.

Higher enantioselectivity and catalytic activity may be required in the asymmetric hydrogenation step, but by using *N*-carbamoyl PPMs under atmospheric hydrogenation pressure, the optical yield of the hydrogenation product could be increased to 75—77%ee.

Thus optically active **4** could be obtained by a single recrystallization of the hydrogenation proudct, and we could complete the practical synthesis of (*S*)-(−)-3-methyl-γ-butyrolactone.

Experimental

All melting points were determined with a micro-melting point apparatus (Yanagimoto) and are uncorrected. Optical rotations were measured on a JASCO DIP-140 digital polarimeter. Infrared (IR) spectra were measured on a JASCO A-202 IR spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL JNM-



2 3 4 5 1

Chart 1

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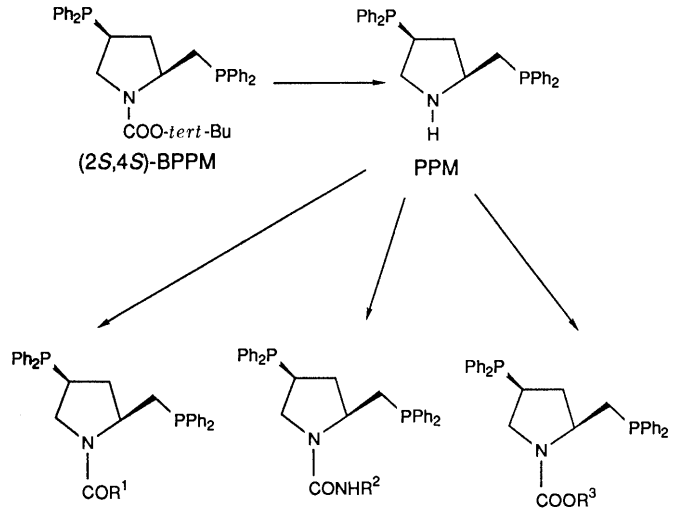


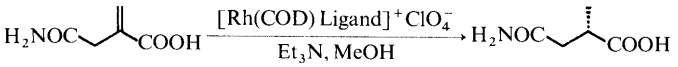
Chart 2. Synthesis of *N*-Substituted PPMs

TABLE I. *N*-Substituted PPMs

R in Chart 2		Abbreviation	Yield %	mp/°C[a]	$[\alpha]_D^{22}$/°[b]
R^1	H	FPPM	65.0	123	−36.4
	CH_3	APPM	70.0	—	−13.8
	Ph	BZPPM	91.2	162	−78.3
	$C(CH_3)_3$	PVPPM	75.0	151	−4.3
R^2	CH_3	MCPPM	94.9	—	−10.5
	Ph	PCPPM	76.2	182	−16.5
	$C(CH_3)_3$	BCPPM	73.1	122	−20.2
R^3	CH_3	MPPM	91.0	—	−38.2
	Ph	PPPM	98.6	—	−44.6

a) —, amorphous. *b*) $[\alpha]_D$, $c=0.5$ in benzene.

TABLE II. Asymmetric Hydrogenation of 2-Methylenesuccinamic Acid[a]



3 4

Entry	Ligand	[Subst.]/[Rh]	Convn. %[b]	Atm/h	ee%[c]
1	FPPM	100	100	30/20	64
2		100	13	5/20	43
3	APPM	100	100	30/20	61
4	BZPPM	100	100	30/20	63
5	PVPPM	100	100	30/20	48
6	MCPPM	1000	82	30/20	59
7		100	100	30/20	65
8		100	100	5/20	70
9		100	100	1/40	75
10	PCPPM	1000	80	30/20	62
11		100	100	30/20	66
12		100	100	5/20	71
13		100	100	1/40	77
14	BCPPM	100	100	30/20	64
15	MPPM	100	100	30/20	66
16	PPPM	100	100	30/20	59
17	BPPM	1000	22	30/20	53
18		100	100	30/20	60
19		100	5	5/20	—
20	BCPM	1000	Trace	30/20	—

a) All hydrogenations were carried out with [subst.] = 0.33 M in methanol at room temperature. *b*) Determined by ^{1}H-NMR analysis. *c*) %ee was calculated on the basis of maximum optical rotation of pure (*S*)-2-methylsuccinamic acid, $[\alpha]_D$ −17.7° ($c=2.0$, methanol), and all configurations are *S*-form.

FX90Q(90 MHz)FT NMR spectrometer using tetramethylsilane (TMS) as an internal standard and ^{31}P-NMR spectra were taken on a JEOL JNM-GX500 spectrometer (^{31}P, 202.35 MHz); the abbreviations of signal patterns are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Thin layer chromatography (TLC) was performed on silica gel (Kiesel $60F_{254}$ on aluminum sheet. Merck). Column chromatography was carried out on silica gel (Kiesel gel 60,70—230 mesh, Merck).

(2*S*,4*S*)-4-(Diphenylphosphino)-2-[(diphenylphosphino)methyl]pyrrolidine; PPM Trifluoroacetic acid (50 ml) was added under nitrogen to 11.04 g (20.0 mmol) of BPPM. The mixture was stirred at 0 °C for 1 h and then evaporated under reduced pressure. The residue was dissolved in 100 ml of dichloromethane and then washed with 100 ml of water, 100 ml of 2 N sodium hydroxide and 100 ml of saturated aqueous NaCl. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure to give 8.51 g of PPM (94.0%), mp 79—81 °C, $[\alpha]_D^{22}$ −15.1° ($c=1.0$, benzene). IR ν_{max}^{KBr} cm^{-1}: 2900 (NH). ^{1}H-NMR δ ($CDCl_3$): 1.13—3.27 (9H, m, $NHCH_2CHCH_2CHCH_2$-), 7.32 (20H, s, Ar-H).

(2*S*,4*S*)-4-(Diphenylphosphino)-2-[(diphenylphosphino)methyl]-*N*-formylpyrrolidine; FPPM PPM (1.81 g, 4.0 mmol) was added to 0.44 ml (4.8 mmol) of ice-cooled acetic anhydride and 0.2 ml (4.8 mmol) of formic acid. The mixture was stirred under nitrogen at 0—10 °C for 3 h, then evaporated under reduced pressure. The residue was dissolved in 20 ml of ethyl acetate and neutralized with 2 N sodium hydroxide and then washed with 10 ml of saturated aqueous NaCl. The organic layer was evaporated under reduced pressure. The residue was purified on a column (40 g) of silica gel (benzene : ethyl acetate = 9 : 1) to give 1.15 g of FPPM(65.0%), mp 122—123 °C, $[\alpha]_D^{22}$ −36.4° ($c=0.5$, benzene). IR ν_{max}^{KBr} cm^{-1}: 1620 (C=O). ^{1}H-NMR δ ($CDCl_3$): 1.80—2.55 (4H, m, CCH_2C, CH_2P), 2.60—3.45 and 3.50—4.60 (4H, m, $CHCH_2NCH$), 7.20—7.85 (20H, m, Ar-H), 8.15 (1H, d, CHO). *Anal.* Calcd for $C_{30}H_{29}NOP_2$: C, 74.83; H, 6.07; N, 2.91. Found: C, 74.97; H, 6.22; N, 2.88.

The preparation procedure for *N*-substituted PPMs was as described that for APPM. The reagents were used as follows; 0.57 ml of benzyl chloride for BZPPM, 0.59 ml pivaloyl chloride for PVPPM, 0.28 ml of methyl isocyanate for MCPPM, 0.54 ml of *tert*-butyl isocyanate for BCPPM, 0.37 ml of methyl chloroformate for MPPM, 0.61 ml of phenyl chloroformate for PPPM.

(2*S*,4*S*)-*N*-Acetyl-4-(diphenylphosphino)-2-[(diphenylphosphino)-methyl]pyrrolidine; APPM Acetyl chloride (0.35 ml, 4.8 mmol) was added under nitrogen to a mixture of 1.81 g (4.0 mmol) of PPM, 40 ml of dichloromethane and 0.65 ml (4.8 mmol) of triethylamine. The reaction was carried out at 0—5 °C for 2 h, then the reaction mixture was washed with water and dried over $MgSO_4$. The organic layer was evaporated under reduced pressure. The residue was purified on a column (20 g) of silica gel (benzene: ethyl acetate = 2 : 1).

Yield 70.1%, amorphous, $[\alpha]_D^{22}$ −13.8° ($c=0.5$, benzene). IR ν_{max}^{KBr} cm^{-1}: 1650 (C=O). ^{1}H-NMR δ ($CDCl_3$): 1.85 (3H, s, CH_3), 1.80—2.55 (4H, m, CCH_2C, CH_2P), 2.60—3.40 and 3.55—4.40 (4H, m, $CHCH_2$-NCH), 7.15—7.80 (20H, m, Ar-H). *Anal.* Calcd for $C_{31}H_{31}NOP_2$: C, 75.14: H, 6.31; N, 2.83. Found: C, 75.05; H, 6.16; N, 2.67.

(2*S*,4*S*)-*N*-Benzoyl-4-(diphenylphosphino)-2-[(diphenylphosphino)-methyl]pyrrolidine; BZPPM Yield 91.2%, mp 162 °C, $[\alpha]_D^{22}$ −78.3° ($c=0.5$, benzene). IR ν_{max}^{KBr} cm^{-1}: 1650 (C=O). ^{1}H-NMR δ ($CDCl_3$): 1.80—2.55 (4H, m, CCH_2C, CH_2P), 2.60—3.45 and 3.50—4.60 (4H, m, $CHCH_2NCH$), 7.20—7.85 (25H, m, Ar-H). *Anal.* Calcd for $C_{36}H_{33}NOP_2$: C, 77.54; H, 5.97; N, 2.51. Found: C, 77.62; H, 6.02; N, 2.59.

(2*S*,4*S*)-4-(Diphenylphosphino)-2-[(diphenylphosphino)methyl]-*N*-pivaloylpyrrolidine; PVPPM Yield 75.0%, mp 148—151 °C, $[\alpha]_D^{22}$ −4.3° ($c=0.5$, benzene). IR ν_{max}^{KBr} cm^{-1}: 1650 (C=O). ^{1}H-NMR δ ($CDCl_3$): 1.17 (9H, s, $C(CH_3)_3$), 1.80—2.50 (4H, m, CCH_2C, CH_2P), 2.60—3.40 and 3.55—4.40 (4H, m, $CHCH_2NCH$), 7.20—7.80 (20H, m, Ar-H). *Anal.* Calcd for $C_{34}H_{37}NOP_2$: C, 75.96; H, 6.94; N, 2.61. Found: C, 76.02; H, 7.02; N, 2.57.

(2*S*,4*S*)-4-(Diphenylphosphino)-2-[(diphenylphosphino)methyl]-*N*-methylcarbamoylpyrrolidine; MCPPM Yield 94.9%, amorphous, $[\alpha]_D^{22}$ −10.5° ($c=0.5$, benzene). IR ν_{max}^{KBr} cm^{-1}: 3320 (NH), 1680 (C=O). ^{1}H-NMR δ ($CDCl_3$): 1.70—2.40 (4H, m, CCH_2C, CH_2P), 2.50—3.47 and 3.50—3.89 (4H, m, $CHCH_2NCH$), 2.74 (3H, d, CH_3), 2.78 (1H, s, NH), 7.20— 7.82 (20H, m, Ar-H). *Anal.* Calcd for $C_{31}H_{32}N_2OP_2$: C, 72.93; H, 6.32; N, 5.49. Found: C, 73.03; H, 6.35; N, 5.28.

(2*S*,4*S*)-4-(Diphenylphosphino)-2-[(diphenylphosphino)methyl]-*N*-phenylcarbamoylpyrrolidine; PCPPM Yield 76.2%, mp 181—182 °C, $[\alpha]_D^{22}$ −16.5° ($c=0.5$, benzene). IR ν_{max}^{KBr} cm^{-1}: 3250 (NH), 1700 (C=O). ^{1}H-NMR δ ($CDCl_3$): 1.70—2.40 (4H, m, CCH_2C, CH_2P), 2.50—3.47 and

3.50—3.89 (4H, m, $CHCH_2NCH$), 5.80 (1H, s, NH), 7.20—7.82 (20H, m, Ar-H). *Anal.* Calcd for $C_{36}H_{34}N_2OP_2$: C, 75.51; H, 5.98; N, 4.89. Found: C, 75.25; H, 5.80; N, 4.72.

(2*S*,4*S*)-*N*-*tert*-Butylcarbamoyl-4-(diphenylphosphino)-2-[(diphenylphosphino)methyl]pyrrolidine; BCPPM Yield 73.1%, mp 120—122 °C, $[\alpha]_D^{22}$ −20.2° (*c*=0.5, benzene). IR ν_{max}^{KBr} cm^{-1}: 3450 (NH), 1700 (C=O). ^{1}H-NMR δ ($CDCl_3$): 1.22 (9H, s, $C(CH_3)_3$), 1.70—2.40 (4H, CCH_2C, CH_2P), 2.60—3.40 and 3.55—3.89 (4H, m, $CHCH_2NCH$), 3.40 (1H, s, NH), 7.20—7.70 (20H, m, Ar-H). *Anal.* Calcd for $C_{34}H_{38}N_2OP_2$: C, 73.90; H, 6.93; N, 5.07. Found: C, 74.04; H, 6.93; N, 5.04.

(2*S*,4*S*)-4-(Diphenylphosphino)-2-[(diphenylphosphino)methyl]-*N*-methoxycarbonylpyrrolidine; MPPM Yield 91.0%, amorphous, $[\alpha]_D^{22}$ −38.2° (*c*=0.5, benzene). IR ν_{max}^{KBr} cm^{-1}: 1700 (C=O). ^{1}H-NMR δ ($CDCl_3$): 1.80—2.50 (4H, m, CCH_2C, CH_2P), 2.60—3.40 and 3.55—4.40 (4H, m, $CHCH_2NCH$), 3.62 (3H, s, CH_3), 7.20—7.70 (20H, m, Ar-H). *Anal.* Calcd for $C_{31}H_{31}NO_2P_2$: C, 72.79; H, 6.11; N, 2.74. Found: C, 72.17; H, 6.17; N, 2.66.

(2*S*,4*S*)-4-(Diphenylphosphino)-2-[(diphenylphosphino)methyl]-*N*-phenoxycarbonylpyrrolidine; PPPM Yield 98.6%, amorphous, $[\alpha]_D^{22}$ −44.6° (*c*=0.5, benzene). IR ν_{max}^{KBr} cm^{-1}: 1710 (C=O). ^{1}H-NMR δ ($CDCl_3$): 1.80—2.50 (4H, m, CCH_2C, CH_2P), 2.60—3.40 and 3.55—4.40 (4H, m, $CHCH_2NCH$), 7.10—7.80 (25H, m, Ar-H). *Anal.* Calcd for $C_{36}H_{33}NO_2P_2$: C, 75.38; H, 5.80; N, 2.44. Found: C, 75.14; H, 5.94; N, 2.40.

Cationic rhodium complexes of these ligands were prepared in the same manner, as follows.

MCPPM(cyclo-octa-1,5-diene)rhodium Perchlorate: [Rh(COD)-MCPPM]$^+$·ClO_4^- Under a nitrogen atmosphere, acetylacetonato(cyclo-octa-1,5-diene)rhodium (500 mg, 1.6 mmol) was dissolved in THF (6 ml), and 70% perchloric acid (*d* 1.66, 230 mg) in THF (2 ml) was added. The mixture was stirred for 5 min, then MCPPM (816 mg, 1.6 mmol) was added at room temperature and stirring was continued for 5 min. The color of the solution turned deep red, and degassed dry ether (30 ml) was added all at once to give orange-red crystals of MCPPM–rhodium cationic complex in quantitative yield. $[\alpha]_D^{20}$ +43.8° (*c*=0.5, MeOH). ^{31}P-NMR δ: 16.4 ppm (dd, J_{Rh-P1}=140.2 Hz, J_{P1-P2}=38.1 Hz), 43.7 ppm (dd, J_{Rh-P2}=145.0 Hz, J_{P1-P2}=38.1 Hz).

BCPPM(cyclo-octa-1,5-diene)rhodium Perchlorate; [Rh(COD)-BCPPM]$^+$·ClO_4^- $[\alpha]_D^{22}$ +37.3° (*c*=0.5, MeOH). ^{31}P-NMR δ: 14.9 ppm (dd, J_{Rh-P1}=139.2 Hz, J_{P1-P2}=37.2 Hz), 43.5 ppm (dd, J_{Rh-P2}=145.0 Hz, J_{P1-P2}=37.2 Hz).

PCPPM(cyclo-octa-1,5-diene)rhodium Perchlorate; [Rh(COD)-PCPPM]$^+$·ClO_4^- $[\alpha]_D^{20}$ +17.7° (*c*=0.5, MeOH). ^{31}P-NMR δ:16.4 ppm (dd, J_{Rh-P1}=140.2 Hz, J_{P1-P2}=37.2 Hz), 43.9 ppm (dm, J_{Rh-P2}=145.0 Hz).

FPPM(cyclo-octa-1,5-diene)rhodium Perchlorate; [Rh(COD)FPPM]$^+$·ClO_4^- $[\alpha]_D^{20}$ +48.0° (*c*=0.5, MeOH). ^{31}P-NMR δ: A; 15.4 ppm (dd, J_{Rh-P1}=141.2 Hz, J_{P1-P2}=36.2 Hz), 45.4 ppm (dd, J_{Rh-P2}=145.0 Hz, J_{P1-P2}=36.2 Hz). B; 16.2 ppm (dd, J_{Rh-P1}=141.2 Hz, J_{P1-P2}=37.2 Hz), 45.2 ppm (dd, J_{Rh-P2}=145.0 Hz, J_{P1-P2}=37.2 Hz).

APPM(cyclo-octa-1,5-diene)rhodium Perchlorate; [Rh(COD)APPM]$^+$·ClO_4^- $[\alpha]_D^{20}$ +26.5° (*c*=0.5, MeOH). ^{31}P-NMR δ: A; 16.1 ppm (dd, J_{Rh-P1}=141.1 Hz, J_{P1-P2}=38.1 Hz), 45.4 ppm (dd, J_{Rh-P2}= 145.0 Hz, J_{P1-P2}=37.2 Hz). B; 16.7 ppm (dd, J_{Rh-P1}=142.0 Hz, J_{P1-P2}= 38.1 Hz), 46.2 ppm (dd, J_{Rh-P2}=145.0 Hz, J_{P1-P2}=38.1 Hz.

BZPPM(cyclo-octa-1,5-diene)rhodium Perchlorate; [Rh(COD)-BZPPM]$^+$·ClO_4^- $[\alpha]_D^{20}$ +6.5° (*c*=0.5, MeOH). ^{31}P-NMR δ: 14.1 ppm (dd, J_{Rh-P1}=140.2 Hz, J_{P1-P2}=36.2 Hz), 45.2 ppm (dd, J_{Rh-P2}=145.9 Hz, J_{P1-P2}=36.2 Hz).

PVPPM(cyclo-octa-1,5-diene)rhodium Perchlorate; [Rh(COD)-PVPPM]$^+$·ClO_4^- $[\alpha]_D^{20}$ +26.0° (*c*=0.5, MeOH). ^{31}P-NMR δ: 13.7 ppm (dm, J_{Rh-P1}=142.1 Hz), 44.9 ppm (dm, J_{Rh-P2}=145.0 Hz).

MPPM(cyclo-octa-1,5-diene)rhodium Perchlorate; [Rh(COD)-MPPM]$^+$·ClO_4^- $[\alpha]_D^{20}$ +27.6° (*c*=0.5, MeOH). ^{31}P-NMR δ: A; 16.0 ppm (dd, J_{Rh-P1}=140.7 Hz, J_{P1-P2}=37.2 Hz), 43.7 ppm (dd, J_{Rh-P2}=144.0 Hz, J_{P1-P2}=37.2 Hz). B; 16.2 ppm (dd, J_{Rh-P1}=141.1 Hz, J_{P1-P2}=38.2 Hz), 46.2 ppm (dd, J_{Rh-P2}=145.0 Hz, J_{P1-P2}=38.2 Hz).

PPPM(cyclo-octa-1,5-dienerhodium Perchlorate; [Rh(COD) PPPM]$^+$·ClO_4^- $[\alpha]_D^{20}$ +7.7° (*c*=0.5, MeOH). ^{31}P-NMR δ: A; 15.2 ppm (dd, J_{Rh-P1}=140.2 Hz, J_{P1-P2}=37.2 Hz), 43.6 ppm (dd, J_{Rh-P2}=145.0 Hz, J_{P1-P2}=37.2 Hz). B; 15.3 ppm (dd, J_{Rh-P1}=140.2 Hz, J_{P1-P2}=37.2 Hz), 44.6 ppm (dd, J_{Rh-P2}=145.0 Hz, J_{P1-P2}=37.2 Hz).

2-Methylenesuccinamic Acid (3) Itaconic anhydride (**2**, 112.1 g, 1.0 mol) was added to an ice-cooled 28% NH_4OH solution (163 g, 1.3 mol). The reaction was carried out at room temperature for 18 h. The reaction mixture was treated with 3 N HCl to give crystals. Recrystallization from ethanol (200 ml) gave 62.0 g of colorless crystals. Yield 48.0%, mp 150—152 °C. IR ν_{max}^{KBr} cm^{-1}: 3360 (OH), 3200 (NH), 1710 (COOH), 1670 (CH_2=C), 1640 ($CONH_2$). ^{1}H-NMR δ (dimethylsulfoxide (DMSO)-d_6): 3.08 (2H, s, CH_2CON), 5.65 and 6.09 (each 1H, each d, =CH_2), 6.83 and 7.31 (each 1H, each br s, NH_2).

(*S*)-(−)-2-Methylsuccinamic Acid (4) A typical asymmetric hydrogenation was carried out as follows. 2-Methylenesuccinamic acid (**3**, 2.58 g, 20 mmol), methanol (60 ml), triethylamine (2.78 ml, 20 mmol) and[Rh-(COD)·MCPPM]$^+$$ClO_4^-$ (102.1 mg, 0.2 mmol) were placed in a autoclave (300 ml). The air was immediately evacuated and replaced with nitrogen to 5 atm. This procedure was repeated 3—4 times and then the autoclave was pressurized with hydrogen to 5 atm. After 20 h, the completion of the hydrogenation was analyzed by ^{1}H-NMR. The reaction solution was treated with Amberlite 120B and then evaporated to give a brown oil. This was taken up in benzene and the solution was evaporated. Further extraction with benzene and evaporation give white crystals of **4**. Recrystallization once from methanol gave optically pure crystals (1.62 g, 12.4 mmol). Yield 62%, mp 72 °C, $[\alpha]_D^{22}$ −17.7° (*c*=2.0, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3440 (OH), 3220 (NH), 1705 (COOH), 1645 ($CONH_2$). ^{1}H-NMR δ (DMSO): 1.06 (3H, d, CH_3), 1.95—2.40 (2H, m, CH_2CO), 2.42—2.81 (1H, m, CH), 6.79 and 7.31 (each 1H, each br s, NH_2).

(*S*)-(−)-2-Methylsuccinamic Acid Methyl Ester (5) Compound **4** (2.48 g, 18.9 mmol) was dissolved in 45 ml of ice-cooled methanol and then hydrogen chloride gas was bubbled into the solution. The bubbling was continued with stirring at room temperature for 1 h, then the reaction mixture was evaporated to give a colorless oil. The oil was dissolved in 50 ml of chloroform and washed with saturated aqueous NaCl. The organic layer was dried over $MgSO_4$ and evaporated to obtain **5** as a colorless oil (2.30 g, 15.9 mmol). Yield 84%, $[\alpha]_D^{22}$ −4.47° (*c*=1.0, MeOH). IR ν_{max}^{NaCl} cm^{-1}: 3440 (OH), 3210 (NH), 1735 ($COOCH_3$), 1670 ($CONH_2$). ^{1}H-NMR δ ($CDCl_3$): 1.23 (3H, d, CH_3C), 2.15—2.76 (2H, m, CH_2), 2.65—3.10 (1H, m, $CHCH_3$), 3.70 (3H, s, $COOCH_3$), 5.99 (2H, br s, NH_2).

(*S*)-(−)-3-Methyl-γ-buthyrolactone (1) Compound **5** (1.45 g, 10 mmol), THF (40 ml) and sodium borohydride ($NaBH_4$, 0.76 g, 20 mmol) were mixed and stirred. Methanol (8 ml) was added dropwise to the solution at room temperature and the mixture was stirred for 2 h. Then 10 ml of water was added to quench the reaction. After being stirred for 1 h, the reaction mixture was evaporated to remove the organic solvent and then refluxed with 35%HCl (10 ml) for 1 h. The residue was extracted with benzene, dried over $MgSO_4$ and then evaporated. Distillation of the residue gave **1** as a colorless oil (0.50 g, 5.0 mmol). Yield 50%, bp 95—97 °C (15 mmHg), $[\alpha]_D^{22}$ −25.7° (*c*=1.0, MeOH), IR ν_{max}^{NaCl} cm^{-1}: 1770 (C=O). ^{1}H-NMR δ ($CDCl_3$): 1.16 (3H, d, CH_3), 2.11 (1H, d, H_E), 2.43—2.85 (2H, m, H_C, H_D), 3.87 (1H, d, H_B), 4.42 (1H, d, H_A).

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3′-*epi*-19-Norafroside and 12β-Hydroxycoroglaucigenin from *Asclepias curassavica*

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3′-*epi*-19-Norafroside, a 19-norcardenolide glycoside with dual linkages between a cardenolide and 4,6-dideoxy-galactos-2-ulose, and 12β-hydroxycoroglaucigenin were newly isolated from the stems of *Asclepias curassavica*, along with known glycosides and free cardenolides. The structures were determined by spectral and chemical methods.

Keywords cardenolide; 3′-*epi*-19-norafroside; 19-norcardenolide; doubly linked cardenolide glycoside; 4,6-dideoxy-D-galactopyranos-2-uloside; 12β-hydroxycoroglaucigenin; *Asclepias*

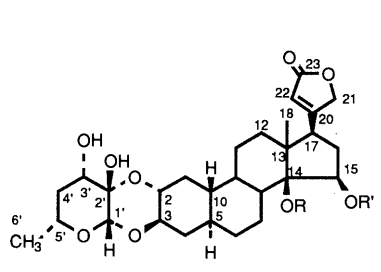
Asclepias is a well known genus containing doubly linked cardenolide glycosides[1] as well as *Calotropis*,[2] *Pergularia*,[3] and *Gomphocarpus*.[4] Larvae of *Danaus* butterflies feed on the leaves, the cardenolides being stored in the adult body as a defense substance.[5] Following studies on similar doubly linked glycosides from *Anodendron affine* DRUCE,[6] the isolation of the minor glycosides from *Asclepias curassavica* L. was attempted. This paper deals with the isolation and the structure determination of 3′-*epi*-19-norafroside and 12β-hydroxycoroglaucigenin[5] from the stems.

3′-*epi*-19-Norafroside (**1**) and 12β-hydroxycoroglaucigenin (**2**) were obtained along with known glycosides, calotropin (**3**), asclepin (**4**), calactin (**5**), calotoxin (**6**),[1] and free cardenolides, uzarigenin (**7**), and coroglaucigenin (**8**),[5] by usual procedures, including reversed-phase and regular-phase column chromatographies (Chart 1).

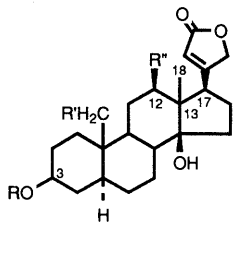
The high-resolution mass spectrum (HRMS) of **1** suggested a molecular formula of $C_{28}H_{40}O_9$. A shape singlet signal at δ 5.01 and a 3H doublet signal at δ 1.32 were assigned to an anomeric proton and 6′-methyl protons, respectively, in the sugar moiety, but only one angular methyl group (δ 0.99) was observed along with H-21a, b and H-22 in the usual multiplicity in the proton magnetic resonance (^{1}H-NMR) spectrum (Table I). Based on a comparison of the signals due to the sugar moiety of **1** with those of **3** in the ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectra as well as in the ^{1}H-NMR spectra, **1** was concluded to be a 4,6-dideoxy-D-galactopyranos-2-uloside[7] (Table I). The aglycone was therefore considered to be composed of 22 carbons. The 3H singlet at δ 0.99 showed cross peaks to C-13 (^2J) and C-17 (^3J) in the long-range coupling (COLOC) spectrum, and was assigned to 18-methyl protons. H-17 also showed ^3J coupling to C-12, C-14 and C-21.

Signals ascribable to H-2 and H-3 showed similar coupling patterns to those of **3**, suggesting their axial orientations in a 5α-cardenolide, as in other cardenolides in this plant. The sequence from H-3 to H-5 *via* H-4α, β was confirmed by the cross peak of each signal in the ^{1}H–^{1}H chemical shift correlation spectroscopy (COSY) spectrum. H-2 was located next to the methylene group, which further showed cross peaks to a methine proton assignable to H-10, indicating that **1** is a 19-norcardenolide. In the nuclear Overhauser effect (NOE) difference nuclear magnetic resonance (NMR) measurement, the irradiation of H-10 resulted in a response at the peaks due to H-2β, H-4β, H-6β, and H-8. The orientation of H-10 was therefore assigned to be β.

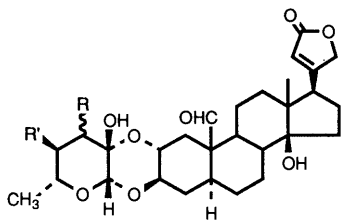
The presence of an additional secondary hydroxyl group was expected from the molecular formula and a doublet signal at δ 72.9 in the ^{13}C-NMR spectrum. The corresponding carbinyl methine proton was observed at δ 4.67 as a triplet signal (J = 7 Hz), suggesting the location of the hydroxyl group at ring D, possibly at C-15, as in 3′-epiafroside.[7a] In order to confirm the location and the orientation, **1** was reacted with $SOCl_2$. The two products (**1a** and **1b**) afforded the same $[M+H]^+$ peaks at m/z 567.226, and the carbinyl proton was shifted downfield from δ 4.67 to δ 5.35 (**1a**) or to δ 5.16 (**1b**), indicating the formation of a cyclosulfite ring between the 14β- and 15β-hydroxyl groups. The configurations of the cyclosulfite group were assigned based on the larger downfield shifts of H-15α in **1a** and of H-22 (δ 6.40 (**1b**), δ 6.25 (**1a**)) in **1b**, as



1 : R=R′=H
1a : R,R′= –S(=O)–
1b : R,R′= –S(=O)–



2 : R=H, R′=R″=OH
2a : R=Ac, R′=R″=OAc
7 : R=R′=R″=H
8 : R=R″=H, R′=OH



3 : R=α-OH, R′=H
4 : R=α-OAc, R′=H
5 : R=β-OH, R′=H
6 : R=R′=β-OH

Chart 1

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TABLE I. ^1H and ^{13}C Chemical Shifts of **1**—**3** and **8**, δ (ppm) from Tetramethylsilane (TMS) in Pyridine-d_5 (J (Hz) in Parentheses)

No.	**1**		**3**		**2**		**8**	
	C[a]	H	C	H	C[a]	H	C	H
1	33.9	α 1.07 (q, 12)	36.5	α 1.16 (t, 12)	32.1	α 0.95 (td, 14, 3)	33.1[b]	α 0.94 (td, 14, 3)
		β 1.99 (td, 3, 12)		β 2.48 (dd, 12, 4)		β 2.74 (td, 3, 14)		β 2.69 (td, 3, 14)
2	72.2	4.33 (td, 12, 4)	69.3	4.32 (td, 12, 4)	32.9		33.1[b]	
3	71.9	4.25 (td, 12, 4)	72.3	4.46 (td, 12, 4)	70.6	3.99 (m)	70.7	4.00 (m)
4	37.2	α 1.80 (td, 4, 12)	32.5	1.74 (td, 4, 12)	39.6	1.95 (bd, 12)	39.7[c]	
		β 1.25 (q, 12)		1.59 (q, 12)		1.82 (q, 12)		
5	40.9	1.02 (m)	42.5[b]		45.3		45.2	
6	33.7	α 1.64 (m)	27.9		28.8		28.9	
		β 0.95 (m)						
7	26.2	α 1.60 (m)	27.9		28.1		28.2	
		β 2.20 (qd, 1, 12)						
8	47.1	1.33 (td, 9, 2)	43.4[b]		42.1		42.3	
9	43.2		48.7		47.6		51.5	
10	46.5	0.71 (qd, 12, 3)	52.8		39.7		39.8	
11	26.4		22.2		32.6		23.5	
12	38.5		39.2		75.0	α 3.66 (dd, 11, 5)	40.5[c]	
13	48.8		49.7		56.8		50.3	
14	81.2		84.0		85.4		84.8	
15	72.9	α 4.67 (t, 9)	33.9		33.5		32.6[b]	
16	37.7		27.1		27.8		27.3	
17	49.3	2.62 (t, 8)	51.2	2.74 (dd, 9, 5)	46.6	3.76 (dd, 8, 7)	50.7	2.80 (dd, 9, 5)
18	16.3	0.99 (s)	15.2	0.90 (s)	10.3	1.28 (s)	16.4	1.07 (s)
19			207.8	10.00 (s)	59.1	4.09 (d, 11)	59.2	4.07 (d, 11)
						4.19 (d, 11)		4.16 (d, 11)
20	175.1		175.4		176.7		176.0	
21	73.7	4.99 (dd, 18, 1)	73.6	4.99 (dd, 18, 1)	74.0	5.11 (dd, 18, 1)	73.8	5.02 (dd, 18, 1)
		5.29 (dd, 18, 1)		5.24 (dd, 18, 1)		5.26 (dd, 18, 1)		5.30 (dd, 18, 1)
22	118.0	6.09 (br s)	117.8	6.10 (br s)	117.3	6.24 (br s)	117.6	6.11 (br s)
23	174.3		174.3		174.7		174.5	
1′	97.2	5.01 (s)	97.2	5.01 (s)				
2′	92.5		92.7					
3′	73.9	4.10 (dd, 12, 5)	73.8	4.12 (dd, 12, 5)				
4′	39.8	1.98 (td, 5, 12)	39.9	2.02 (td, 5, 12)				
		2.09 (q, 12)		2.12 (q, 12)				
5′	68.4	3.73 (m)	68.5	3.76 (m)				
6′	21.4	1.32 (d, 6)	21.5	1.37 (d, 6)				

a) Signal assignments were done based on the 2D-NMR (^{13}C–^1H COSY and/or COLOC) spectra. *b*, *c*) Signal assignments marked *b*) or *c*) may be reversed.

syn to H-15α in **1a** and *anti* in **1b**, respectively.[8] Compound **1** was thus determined to be 3′-*epi*-19-norafroside.

Compound **2** showed the presence of one primary and two secondary hydroxyl groups along with 14β-OH, based on the ^1H- and ^{13}C-NMR spectra and the molecular formula $C_{23}H_{34}O_6$ based on HRMS. The location of the H-17 signal at lower field (δ 3.76) and its upfield shift in **2**-triacetate (**2a**) suggested one of the secondary hydroxyls to be 12β-OH, as in digoxigenin (=12β-hydroxydigitoxigenin). The ^{13}C-NMR signals due to rings C and D in **2** were in good agreement with those of digoxigenin,[9] while the signals from rings A and B in **2** were in agreement with those of **8**. Compound **2** was thus characterized as 12β-hydroxycoroglaucigenin.

3′-Epiafroside was obtained from *Asclepias fruticosa* L. by Cheung *et al.*[7] and two doubly linked glycosides of 19-norcardenolides, 19-norgomphoside and 16-acetoxy-19-norgomphoside, were also reported from *Asclepias vestita*.[10] 3′-*epi*-19-Norafroside is the third 19-norcardenolide to have been isolated from a natural source. 3′-*epi*-19-Norafroside, but no corresponding 19-nor-derivatives of **3**—**5**, was obtained from this plant material. The possibility that 19-norcardenolides are artifacts formed through the autooxidation of 10-formylcardenolides was suggested.[10]

Experimental

The melting points were taken on a hot stage apparatus and are uncorrected. ^1H- and ^{13}C-NMR spectra were recorded on a JEOL GX-400 spectrometer in pyridine-d_5. Chemical shifts are given in δ values referred to internal tetramethylsilane (TMS), and the following abbreviations are used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br s=broad singlet, dd=doublet of doublets, td=triplet of doublets, qd=quartet of doublets. HRMS was recorded on a JEOL D-300-FD spectrometer. Optical rotations were measured on a JASCO DIP 360 polarimeter. The following solvent systems were used for silica gel column chromatographies and thin layer chromatography (TLC): benzene–acetone, $CHCl_3$–MeOH–H_2O (bottom layer), EtOAc–MeOH–H_2O (top layer). Spots on TLC plates were detected by spraying Kedde's reagent (1:1 mixture of 5% 3,5-dinitrobenzoic acid in MeOH and 2 N NaOH) or 10% H_2SO_4.

Extraction and Isolation of Cardenolide Glycosides *Asclepias curassavica* L. was cultivated in the medicinal plant garden of Fukuoka University and harvested in the fall of 1987. The air-dried stems (6.5 kg) were powdered and percolated with MeOH. The MeOH percolate was concentrated to 2 l and diluted with 2 l of H_2O. The deposit was removed by filtration and the filtrate was extracted with benzene, $CHCl_3$, and BuOH. Each extract was then subjected to column chromatographies on polystyrene gel (Mitsubishi CHP-20, eluted with H_2O–MeOH) and silica gel. The following cardenolides and glycosides were isolated as crystals and identified by ^1H- and ^{13}C-NMR and HRMS; calotropin (**3**, 509 mg), asclepin (**4**, 668 mg), calactin (**5**, 34 mg), calotoxin (**6**, 42 mg), uzarigenin (**7**, 188 mg), and coroglaucigenin (**8**, 375 mg), along with **1** and **2**.

3′-*epi*-19-Norafroside (1) Prisms from MeOH (75 mg), mp 230—233 °C, $[\alpha]_D^{26}$ +36.7° (c=1.07, $CHCl_3$). HRMS (FAB) m/z: 521.2747

(Calcd for $C_{28}H_{40}O_9+H$: 521.2750). Cross peaks in the COLOC spectrum: H-1/C-5, H-4α/C-10, H-6/C-8, -10, H-7/C-5, -9, H-8/C-12, -13, H-9/C-12, -14, H-11/C-8, -13, H-16/C-14, H-17/C-12, -14, -21, H-18/C-13, -17, H-21/C-20, H-22/C-21, -23, H-1′/C-2′, H-4′/C-2′, H-6′/C-4′. Compound **1** (20 mg) was dissolved in pyridine (1 ml) and $SOCl_2$ (0.1 ml) was added at 0 °C with stirring. The mixture was allowed to stand for 1.5 h and diluted with H_2O. The whole was extracted with $CHCl_3$ and the extract was purified on a silica gel column to afford cyclosulfites of **1** (**1a** and **1b**). **1a**: a solid, 9 mg, $[\alpha]_D^{29}$ +82.5° (c=0.4, MeOH). HRMS (FAB) m/z: 567.2263 (Calcd for $C_{28}H_{38}O_{10}S+H$: 567.2264. ^{1}H-NMR δ: 1.13 (3H, s, H-18), 1.32 (3H, d, J=6 Hz, H-6′), 3.73 (1H, m, H-5′), 4.12 (1H, dd, J=12, 5 Hz, H-3′), 4.25 (1H, td, J=12, 4 Hz, H-3), 4.34 (1H, td, J=12, 4 Hz, H-2), 4.84, 5.01 (1H each, dd, J=17, 2 Hz, H-21), 5.01 (1H, s, H-1′), 5.35 (1H, dd, J=7, 2 Hz, H-15), 6.25 (1H, br s, H-22). ^{13}C-NMR δ: 18.3 (C-18), 36.2 (C-16), 44.0 (C-8), 50.2 (C-13), 86.4 (C-15), 105.1 (C-14). **1b**: a solid, 7 mg, $[\alpha]_D^{29}$ +101.8° (c=0.3, MeOH). HRMS (FAB) m/z: 567.2258 (Calcd for $C_{28}H_{38}O_{10}S+H$: 567.2264). ^{1}H-NMR δ: 1.07 (3H, s, H-18), 1.31 (3H, d, J=6 Hz, H-6′), 3.73 (1H, m, H-5′), 4.13 (1H, dd, J=12, 5 Hz, H-3′), 4.28 (1H, td, J=12, 4 Hz, H-3), 4.36 (1H, td, J=12, 4 Hz, H-2), 4.91, 5.02 (1H each, dd, J=18, 1 Hz, H-21a, b), 5.03 (1H, s, H-1′), 5.16 (1H, dd, J=9, 6 Hz, H-15), 6.40 (1H, br s, H-22). ^{13}C-NMR δ: 18.7 (C-18), 35.6 (C-16), 41.0 (C-8), 48.1 (C-13), 85.7 (C-15), 108.7 (C-14).

12β-Hydroxycoroglaucigenin (2) Needles from MeOH (132 mg), mp 228—235 °C, $[\alpha]_D^{26}$ +136.4° (c=0.45, $CHCl_3$–MeOH (1:1)). HRMS (FAB) m/z: 429.2250 (Calcd for $C_{23}H_{34}O_6+Na$: 429.2253). The acetylation of **2** (10 mg) was carried out in the usual manner with pyridine and Ac_2O to afford **2**-triacetate (**2a**) as a solid, $[\alpha]_D^{29}$ +33.7° (c=0.75, MeOH). HRMS (FAB) m/z: 533.2748 (Calcd for $C_{29}H_{40}O_9+H$: 533.2751). ^{1}H-NMR δ: 1.21 (3H, s, H-18), 1.96, 2.04, 2.16 (3H each, s, –OAc), 3.14 (1H, dd, J=9, 6 Hz, H-17), 4.35, 4.48 (1H each, d, J=12 Hz, H-19), 4.85 (1H, m, H-3), 4.88 (1H, dd, J=12, 4 Hz, H-12), 5.03, 5.23 (1H each, dd, J=18, 1 Hz, H-21a, b), 5.74 (1H, s, –OH), 6.17 (1H, br s, H-22).

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Re-examination of the Synthesis of 7,8-Dimethoxy-2-methyl-3-(4′,5′-methylenedioxy-2′-vinylphenyl)isocarbostyril[1)]

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Oxidation of the enamine (6) to the isocarbostyril (7) was re-examined. Simply stirring a dimethylformamide (DMF) solution of 6 provided 7 with good reproducibility, in the presence of KCN under an oxygen atmosphere. The structure of the chloroform adduct (8) was determined by an X-ray analysis.

Keywords chelerythrine; protoberberine; Hofmann degradation; X-ray analysis; biomimetic synthesis; oxy functionality

Fully aromatized (quaternary) benzo[*c*]phenanthridine alkaloids (A) have attracted much attention because of their potent biological activities[2)] against leukemia L1210 and P-388 in mice. Considerable effects have, therefore, been directed towards the development of general and convenient methods for syntheses of these alkaloids.[3)] One of the authors (H.I.) has reported a versatile method for synthesis of fully aromatized benzo[*c*]phenanthridine alkaloids (A) from the appropriately substituted chalcones (B) *via* 2-aryl-1-tetralone derivatives (C),[4)] and another author (M.H.) has reported an efficient method for synthesis of A from protoberberine alkaloids (D) through a biomimetic route[5)] (see Chart 1).

Recently, 2,3,7,8-tetraoxygenated dihydrobenzo[*c*]-phenanthridine alkaloids (**1**) with a substituent group at C_6 have been isolated from *Xanthoxylum*,[6)] *Corydalis*,[7)] and *Hypecoum*[8)] plants and their structures elucidated (Chart 2). In this connection, we needed a relatively large amount of chelerythrine (**2**) in order to synthesize ailanthoidine (**1a**).[6b)] We planned to prepare **2** using the biomimetic method[5a)]; *e.g.* the transformation of berberine chloride (**3**) to chelerythrine (**2**) *via* the enamine (**6**) and the isocarbostyril (**7**) reported by Onda *et al.*,[9)] because **3** is commercially available and few synthetic steps are required. The details of these results are the subject of this paper.

Berberine chloride (**3**) was reduced with lithium aluminum hydride (LAH)[10)] in dry tetrahydrofuran (THF) at room temperature to afford the dihydro derivative (**4**), which was immediately methylated with dimethyl sulfate in refluxing benzene, providing the methomethylsulfate (**5**) in 73% yield from **3**. Hofmann degradation of **5** in 25% methanolic potassium hydroxide yielded the labile enamine (**6**), which was subsequently oxidized overnight with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in chloroform ($CHCl_3$) at room temperature according to the literature.[9)] However, in our hands we obtained the desired isocarbostyril (**7**) in a variable and low yield (5—25%) in contrast to Onda's report (44% yield). Attempts were made to improve the yield of **7** under various reaction conditions; DDQ oxidation in various solvents (benzene, carbon tetrachloride, methanol, and dioxane) and DDQ oxidation in $CHCl_3$ followed by potassium ferricyanide in the presence of 25% aqueous potassium hydroxide in refluxing methanol,[4d)] but they were not fruitful. Interestingly, on oxidizing **6** with DDQ in $CHCl_3$, a new compound, mp 161—163 °C, was always

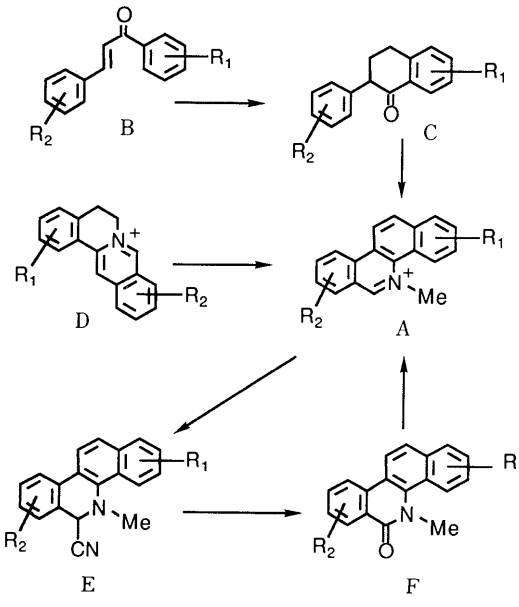

Chart 1

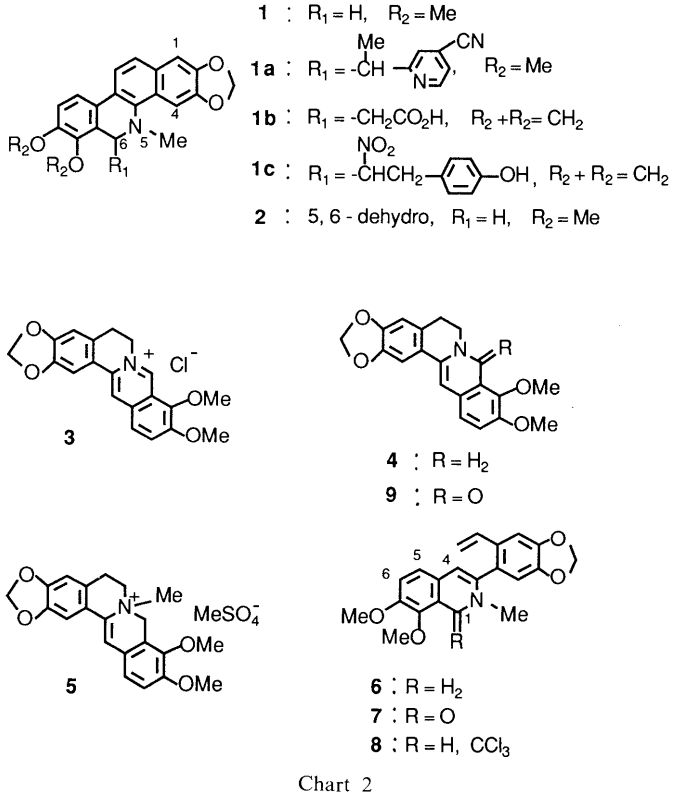

Chart 2

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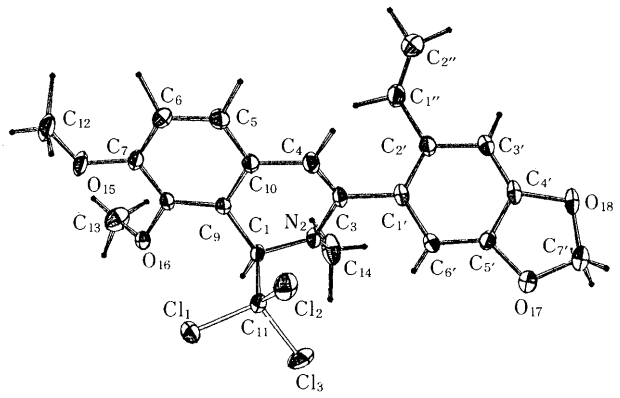

Fig. 1. Molecular Structure of the Chloroform Adduct (**8**)

TABLE I. Atomic Parameters for Non-hydrogen Atoms in the Chloroform Adduct (**8**)

	X	*Y* (×10000)	*Z*	*B*
Cl1	9722 (1)	8799 (2)	3407 (1)	4.7
Cl2	10029 (1)	8798 (2)	1783 (1)	4.7
Cl3	11278 (1)	8971 (3)	3054 (1)	6.0
O15	7888 (2)	3857 (7)	3282 (2)	4.0
O16	9334 (2)	4956 (5)	3851 (2)	2.9
O17′	12989 (2)	7818 (7)	384 (2)	4.6
O18′	13251 (2)	5331 (7)	−358 (2)	4.5
N2	11044 (2)	5315 (7)	2336 (2)	2.6
C1	10437 (3)	5891 (8)	2789 (3)	2.4
C1′	11517 (3)	4811 (9)	1040 (3)	2.8
C1″	11227 (4)	1569 (9)	626 (4)	4.0
C2′	11668 (3)	3253 (8)	591 (3)	2.9
C2″	11127 (4)	300 (11)	89 (5)	5.1
C3	10864 (3)	4838 (8)	1528 (3)	2.8
C3′	12269 (3)	3333 (9)	100 (3)	3.3
C4	10129 (3)	4547 (9)	1203 (3)	3.2
C4′	12658 (3)	4922 (9)	84 (3)	3.0
C5	8800 (3)	3705 (9)	1423 (3)	3.4
C5′	12507 (3)	6411 (8)	530 (3)	2.9
C6	8247 (3)	3482 (9)	1932 (4)	3.5
C6′	11950 (3)	6395 (8)	1021 (3)	2.9
C7	8414 (3)	3939 (9)	2749 (4)	2.9
C7′	13497 (3)	7104 (8)	−124 (3)	5.7
C8	9143 (3)	4579 (8)	3040 (3)	2.3
C9	9678 (4)	4962 (13)	2523 (5)	2.3
C10	9518 (3)	4453 (8)	1703 (3)	2.9
C11	10363 (3)	7943 (7)	2751 (3)	3.2
C12	7108 (3)	3532 (9)	2965 (3)	5.1
C13	9313 (3)	3417 (9)	4385 (3)	4.7
C14	11741 (4)	4593 (12)	2791 (4)	4.9

Estimated standard deviations are given in parentheses.

obtained, although in a very low yield, along with **7**. In its infrared (IR) spectrum no characteristic absorption band is observed and in its ^{1}H-nuclear magnetic resonance (NMR) spectrum a singlet signal assignable to C_1-H at δ 5.64 is seen. These findings indicate that the structure of the new compound can be represented by formula **8** in view of its elemental analysis ($C_{22}H_{20}Cl_3NO_4$). This assumption was supported by an X-ray analysis of **8** as shown in Fig. 1 and Table I. On the basis of these results, we speculated that **6** in $CHCl_3$ would produce **8** through oxidation (dehydrogenation) to the quaternary base followed by the addition of chloroform to the iminium double bond, since **6** was very labile and seemed susceptible to air-oxidation.

TABLE II. Results of Oxidation of Crude **6** to **7**

Run	Reaction conditions			Yield (%)[a]	
	Additive	O_2	Crown[b] ether	**7**	**9**
1	KCN	+	−	43.7	~3
2	KCN	+	+	47.7	1.5
3	KCN	−	−	17.7	1.6
4	—	+	−	Many spots on TLC	
5	$NaHCO_3$	+	−	Many spots on TLC	

a) Yields from methomethylsulfate (**5**) are given. *b*) Dicyclohexyl-18-crown-6 (*ca.* 10 mg) was used.

This speculation was confirmed by the production of **8** in 58% yield on stirring a solution of **6** in $CHCl_3$.[11,12] Subsequently, attempts were made to convert **8** to **7** by treatment with alkali or oxidizing reagents such as $K_3Fe(CN)_6$, MnO_2, pyridinium chlorochromate, pyridinium dichromate, and Ag_2O, but these reagents did not give satisfactory results.

In a previous paper,[13] we reported the preparation of the oxybase (F) by air-oxidation of the carbanion of the Ψ-cyanide (E) derived from the fully aromatized quaternary base (A). Since the Hofmann degradation product (**6**) was expected to produce the quaternary base by air-oxidation as mentioned above, we anticipated that the desired isocarbostyril (**7**) could be prepared in one-pot by sequential conversions involving quaternization by oxidation, addition of cyanide anion and reoxidation of the corresponding Ψ-cyanide. In practice, transformation of **6** to **7** was successfully carried out in the presence of potassium cyanide (KCN) in dimethylformamide (DMF). The results are given in Table II.

In conclusion, oxidation of **6** in the presence of KCN under an oxygen atmosphere (crown ether seems not to be required) provided the desired isocarbostyril (**7**) reproducibly, although in a moderate yield, along with a small amount of oxyberberine (**9**)[14] which was assumed to have arisen from dihydroberberine contaminating the crude Hofmann degradation product (**6**) (see runs 1 and 2 in Table II).

Experimental

Melting points were measured on a micro melting point hot-stage apparatus (Yanagimoto) and are uncorrected. IR spectra were recorded in Nujol on a Hitachi 215 spectrometer, and ^{1}H-NMR spectra in deuteriochloroform on JEOL FX-270 and/or JEOL GSX-400 spectrometers. The NMR data are reported in parts per million downfield from tetramethylsilane as an internal standard (δ 0.0) and coupling constants in hertz. Column chromatography was carried out on silica gel (Merck, Silica gel 60, No. 7734) or Florisil (Nacalai Tesque Inc., 100—200 mesh). Berberine chloride was purchased from Nacalai Tesque Inc. In general, the extract was dried over anhydrous potassium carbonate, then filtered, and the filtrate was evaporated to dryness under reduced pressure.

Dihydroberberine Methomethylsulfate (5) Berberine hydrochloride (8.63 g, 23.2 mmol) was added portionwise to a stirred suspension of LAH (4.09 g, 107.8 mmol)[10] in dry THF (340 ml) over a period of 15 min. The mixture was stirred for 1 h at room temperature under an argon atmosphere. The excess reagent was decomposed with ethyl acetate and wet ether, and the organic layer was decanted. The residue in benzene was chromatographed on Florisil (30 g). The benzene effluent (*ca.* 230 ml) was heated under reflux and dimethyl sulfate (21.99 g, 174.3 mmol) was added to the refluxing benzene solution. After refluxing for 2.5 h, the reaction mixture was cooled with ice-water and the resulting yellow precipitates were collected, then recrystallized from ethyl alcohol to provide the methomethylsulfate (**5**) (8.08 g, 73%), yellow prisms, mp 263—271 °C (dec.)

(lit.[15] mp 208—211 °C). *Anal.* Calcd for $C_{22}H_{25}NO_8S \cdot 3/4H_2O$: C, 55.38; H, 5.60; N, 2.94. Found: C, 55.37; H, 5.67; N, 2.65. ^{1}H-NMR (270 MHz in MeOH-d_4) δ: 3.12 (3H, s, NCH_3), 3.93 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.79 (1H, d, J=14.8 Hz, $NC\underline{H}_AH_B$), 5.10 (1H, d, J=14.8 Hz, $NCH_A\underline{H}_B$), 6.02 (1H, d, J=1.0 Hz, $-OC\underline{H}_AH_BO-$), 6.03 (1H, d, J=1.0 Hz, $-OCH_A\underline{H}_BO-$), 6.80 (1H, s, olefinic proton), 7.16 (1H, d, J=8.6 Hz, aromatic H), 7.27 (1H, d, J=8.6 Hz, aromatic H), 7.43 (1H, s, aromatic H), 7.48 (1H, s, aromatic H).

Hofmann Degradation of the Methomethylsulfate (5) The methomethylsulfate (**5**) (0.504 g, 1.06 mmol) was added at once to refluxing 25% methanolic potassium hydroxide (10 ml) and the mixture was heated under reflux for 20 min. The reaction mixture was poured into ice-water and extracted with ether. The crude Hofmann degradation product (**6**) (*ca.* 330 mg) was immediately used for the next step without purification because of its instability. ^{1}H-NMR (270 MHz in $CDCl_3$) δ: 2.50 (3H, s, NCH_3), 3.83 (3H, s, OCH_3), 3.84 (3H, s, OCH_3), 4.47 (2H, s, C_1-H_2), 5.12 (1H, dd, J=11.0, 0.9 Hz, $C_{2''}$-H_A), 5.18 (1H, s, C_4-H), 5.57 (1H, dd, J=17.5, 0.9 Hz, $C_{2''}$-H_B), 5.97 (2H, s, $-OCH_2O-$),6.58 (1H, d, J=8.4 Hz, aromatic H), 6.70 (1H, d, J=8.4 Hz, aromatic H), 6.71 (1H, s, aromatic H), 6.85 (1H, dd, J=17.5, 11.0 Hz, $C_{1''}$-H), 7.08 (1H, s, aromatic H).

Oxidation of the Hofmann Degradation Product (6) with DDQ in $CHCl_3$ A solution of **6** (*ca.* 330 mg) and DDQ (0.472 g, 2.08 mmol) in $CHCl_3$ (10 ml) was stirred overnight at room temperature. After filtration, the $CHCl_3$ solution was washed with 5% aqueous NaOH solution and water. The residue was chromatographed on silica gel (10 g). Elution with hexane–ethyl acetate (2:1) gave **8** (34.8 mg, 7.0%), mp 161—163 °C, (colorless prisms from ether–hexane). *Anal.* Calcd for $C_{22}H_{20}Cl_3NO_4$: C, 56.37; H, 4.30; N, 2.99. Found: C, 56.38; H, 4.25; N, 2.90. ^{1}H-NMR (400 MHz in $CDCl_3$) δ: 2.94 (3H, s, NCH_3), 3.90 (3H, s, OCH_3), 3.93 (3H, s, OCH_3), 5.09 (1H, dd, J=11.0, 0.9 Hz, $C_{2''}$-H_A), 5.45 (1H, s, C_1-H), 5.55 (1H, dd, J=17.4, 0.9 Hz, $C_{2''}$-H_B), 5.64 (1H, s, C_4-H), 5.98 (1H, d, J=1.5 Hz, $-OC\underline{H}_AH_BO-$), 6.01 (1H, d, J=1.5 Hz, $-OCH_A\underline{H}_BO-$), 6.87 (1H, d, J=8.2 Hz, C_5-H), 7.00 (1H, d, J=8.2 Hz, C_6-H), 7.07 (1H, dd, J=17.4, 11.0 Hz, $C_{1''}$-H), 7.10 (2H, s, $C_{3'}$-H and $C_{6'}$-H). Successive elution with the same solvent gave the isocarbostyril (**7**) (19.3 mg, 5.0%), mp 167—169 °C, (colorless plates from CH_2Cl_2–ether). (lit.,[9] mp 179—180 °C). *Anal.* Calcd for $C_{21}H_{19}NO_5$: C, 69.03; H, 5.24; N, 3.83. Found C, 68.98; H, 5.34; N, 3.82. ^{1}H-NMR (270 MHz in $CDCl_3$) δ: 3.23 (3H, s, NCH_3), 3.95 (3H, s, OCH_3), 4.03 (3H, s, OCH_3), 5.12 (1H, dd, J=10.9, 0.7 Hz, $C_{2''}$-H_A), 5.60 (1H, dd, J=17.5, 0.7 Hz, $C_{2''}$-H_B), 6.04 (2H, s, $-OCH_2O-$), 6.28 (1H, s, C_4-H), 6.43 (1H, dd, J=17.5, 10.9 Hz, $C_{1''}$-H), 6.72 (1H, s, $C_{3'}$-H), 7.13 (1H, s, $C_{6'}$-H), 7.20 (1H, d, J=8.6 Hz, C_6-H), 7.33 (1H, d, J=8.6 Hz, C_5-H). IR (Nujol): ν_{max} 1645 (C=O).

The Chloroform Adduct (8) A solution of **6** prepared by Hofmann elimination of the methomethylsulfate (0.67 g, 1.40 mmol) in $CHCl_3$ (20 ml) was stirred overnight at room temperature. The reaction solution was evaporated to dryness under reduced pressure. The residue was chromatographed on Florisil (15 g). Elution with benzene provided **8** (0.38 g, 58%), mp 161—163 °C (colorless prisms from ether–hexane).

General Procedure for Oxidation of the Hofmann Product (6) KCN (88 mg, 1.36 mmol) for runs 1, 2, and 3 or $NaHCO_3$ (108 mg, 0.779 mmol) for run 5 was added to a DMF solution (5 ml) of the crude enamine (**6**) prepared from methomethylsulfate (**5**) (0.251 g, 0.53 mmol). The reaction mixture was stirred for 21 h at 50—60 °C under the reaction conditions indicated in Table I. The mixture was diluted with water and extracted with ether. The residue was chromatographed on silica gel (10 g). Elution with hexane–ethyl acetate (2:1) provided the isocarbostyril (**7**), mp 168 °C. Successive elution with hexane : ethyl acetate (1:1) provided oxyberberine (**9**), mp 206—208 °C, (colorless prisms from methanol). (lit.,[12] mp 201—203 °C).

X-Ray Structure Determination of 8 Crystal Data: $C_{22}H_{20}Cl_3NO_4$, M=468.36, monoclinic, space group P_21/n, a=17.540(13), b=7.365(1), c=16.806(12) Å, β=97.21(6)°, U=2154.1 Å^3, Z=4, and D_c=1445 g/cm^3. Single crystals of **8** were prepared by slow crystallization from ether–hexane. A crystal with dimensions of 0.10×0.15×0.20 mm was used for data collection. The intensity data were collected on a Rigaku AFC-5FU diffractometer with monochromated Cu K_α radiation (λ=1.54178 Å) using the $\omega<30°<\omega$–2θ scan method at a 2θ scan speed of 4°/min. The structure was solved by the direct method using the MULTAN (UNICS-III system)[16] program and was refined by the full-matrix least-sequares method. The final R value was 0.075 for 3122 unique reflections [$F_O>3\sigma(F_O)$].

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The Oxidized Products of 8-Methoxypsoralen (8-MOP) with H_2O_2–NaOCl

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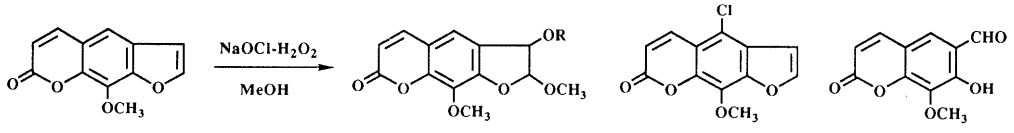
In order to obtain adducts of 8-methoxypsoralen (8-MOP, 1) and singlet oxygen ($^1O_2$), the oxidation of 1 with chemically generated singlet oxygen in H_2O_2–NaOCl was undertaken. Bioassay-directed fractionation of the crude oxidized products has led to the isolation and characterization of a novel derivative of 1, 2,3-dihydro-2,9-dimethoxy-3-hydroxy-7*H*-furo[3,2-*g*][1]benzopyran-7-one (2) as a substance inhibiting chemotactic activity of polymorphonuclear neutrophils toward anaphylatoxin $C5_a$ des Arg. The structure of 2 was determined from the spectroscopic data and by correlation with its acetate (2a). Furthermore, the oxidation of 1 in H_2O_2–NaOCl afforded 5-chloro-9-methoxy-7*H*-furo[3,2-*g*][1]benzopyran-7-one (3) and 6-formyl-7-hydroxy-8-methoxy-2*H*-1-benzopyran-2-one (4) along with 1, but they exhibited no such activity.

Keywords furocoumarin; 8-methoxypsoralen; singlet oxygen ($^1O_2$); photoadduct; chemotactic activity of polymorphonuclear neutrophil; anaphylatoxin $C5_a$ des Arg; 2,3-dihydro-2,9-dimethoxy-3-hydroxy-7*H*-furo[3,2-*g*][1]benzopyran-7-one

Linear furocoumarins, commonly named psoralens, are naturally occurring compounds which exhibit various biological activities associated with their sensitivity to light, and they have proved useful as drugs for the treatment of skin disease.[1] Their recent use in PUVA (psoralen + UVA-light) therapy for the treatment of psoriasis has generated much interest in their biological properties.[2] Previously, Mizuno *et al.* reported that the photoadduct of 8-methoxypsoralen (8-MOP, **1**) inhibited psoriasis leukotactic factor (PLF) activity,[3] a finding which was presumably related to the biological activity of **1**. Although the photoadduct of **1** with singlet oxygen may be involved in the biological activity,[4] the photoreaction of **1** with singlet oxygen has been little investigated. So far, Wasserman's and Logani's groups have isolated only 6-formyl-7-hydroxy-8-methoxy-2*H*-1-benzopyran-2-one (**4**) as the photoadduct of **1** with singlet oxygen.[5] They have not characterized other photoadducts because of low yields and have not examined the biological activity of **4**. Thus, we investigated the reaction of **1** and singlet oxygen chemically generated by H_2O_2–NaOCl,[6] and isolated a novel 8-MOP derivative (**2**) inhibiting chemotactic activity of polymorphonuclear neutrophilis (PMN) toward anaphylatoxin $C5_a$, by means of bioassay-directed fractionation of oxidized products of 8-MOP with H_2O_2–NaOCl, along with known derivatives (**3** and **4**). In addition, 6-formyl-7-hydroxy-8-methoxy-2*H*-1-benzopyran-2-one (**4**) was also prepared by the ozonolysis of **1** to examine its pharmacological activity. In this paper, we describe the structural elucidation of the 8-MOP (**1**) oxidized product, which inhibits chemotactic activity of PMN toward anaphylatoxin $C5_a$.

First of all, we examined the reactions of **1** with chemically generated singlet oxygen under various conditions such as decomposition of triphenylphosphite ozonide[7] and 9,10-diphenylanthracene peroxide,[8] and H_2O_2–NaOCl treatment.[6] 8-MOP (**1**) did not react and was recovered quantitatively under the former two conditions, but in the last case, **1** was consumed completely. The oxidation of **1** in methanol with 30% aqueous H_2O_2 and 5% aqueous NaOCl furnished a mixture of several 8-MOP oxidized products. Since extraction of the reaction mixture with organic solvents gave secondarily modified compounds on thin layer chromatographic (TLC) analysis, the resulting mixture was extracted using commercially available prepacked Extrelute 20 (Merck Co., Ltd.) (see Fig. 1). Namely, the reaction mixture was absorbed on Extrelute 20, then the column was eluted with EtOAc and MeOH successively. The EtOAc eluate with activity to inhibit PLF activity was further separated by preparative thin layer chromatography to afford six fractions. An active fraction 3 was purified by normal-phase high performance liquid chromatography (HPLC) to furnish **2** as a substance inhibiting chemotactic activity of PMN toward anaphylatoxin $C5_a$ des Arg.[9] On the other hand, fractions 1 and 2 were purified by silica gel column chromatography to yield **3** and **4**, respectively.

Compound **2** was shown to have the molecular formula $C_{13}H_{12}O_6$ from its electron impact mass spectrum (EI-MS) and high resolution mass spectra. The infrared (IR) spectrum of **2** showed a hydroxyl absorption band (3690, 3590 cm^{-1}), while the proton nuclear magnetic resonance (^{1}H-NMR) spectrum indicated the retention of the α-pyrone and aromatic moieties and the disappearance of the furan unit in **1**. In addition, one methoxyl group signal (δ3.61, 3H, s) and two oxymethine signals (δ5.04, 1H, s; δ5.54, 1H, s) were newly observed in the ^{1}H-NMR



8-MOP (1) **2 : R=H** **2a : R=Ac** **3** **4**

Chart 1

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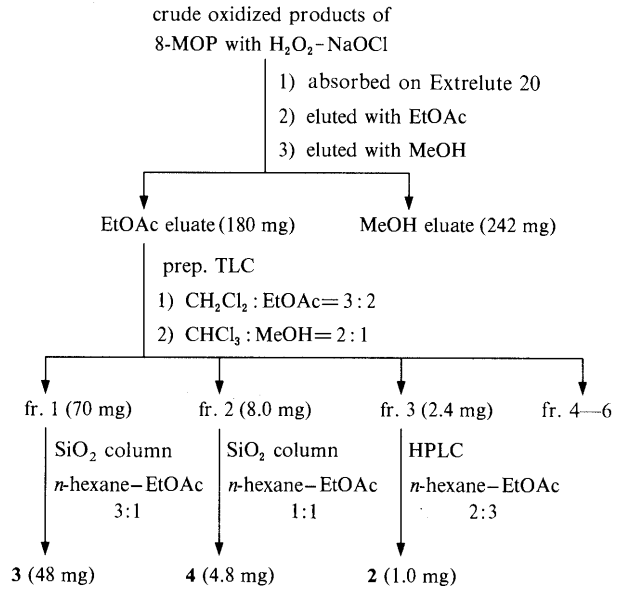

Fig. 1. Fractionation and Isolation of 8-MOP Oxidized Products

TABLE I. ^{13}C-NMR Data for **1**, **2**, and **2a** [δ_c, 100 MHz, $(CD_3)_2CO$]

	1	2	2a
2	148.1	116.3	112.7
3	107.8	76.5	77.5
3a	127.1	128.3	123.8
4	115.3	120.0	121.4
4a	117.6	115.3	115.7
5	145.5	145.2	145.1
6	114.5	113.6	113.9
7	160.5	160.5	160.2
8	148.5	149.2	149.8
9	133.4	132.7	132.9
9a	144.1	154.1	154.7
2-OMe		56.9	56.9
9-OMe	61.5	60.1	61.1
OAC			170.6 20.7

spectrum of **2**. Detailed comparison of the ^{13}C-NMR data for **2** with those for **1** has shown that two methine carbons (δ_c 116.3, 76.5) bearing oxygen are newly formed, which is indicative of the presence of a ketal moiety. Thus, the oxidized active product (**2**) was presumed to have added hydroxyl and methoxyl groups to the furan moiety of **1**.

In order to verify the positions of methoxyl and hydroxyl groups in **2**, it was acetylated with Ac_2O and pyridine. The ^{1}H-NMR spectrum of its acetate (**2a**) showed the signal due to 3-H (δ 5.93, 1H, s) shifted downfield in comparison with that of **2** (δ 5.04, 1H, s). However, the 2-H signals of **2a** and **2** appeared at similar chemical shifts. Consequently, the newly formed hydroxyl group was determined to be at C-3 and the structure of **2** was elucidated as 2,3-dihydro-2,9-dimethoxy-3-hydroxy-7*H*-furo[3,2-*g*][1]benzopyran-7-one, which is also substantiated by its ^{13}C-NMR spectrum (Table I). The relative stereochemistry of 2-OCH_3 and 3-OH was proved to be *trans* because of the absence of coupling between 2-H and 3-H.

The major product (**3**) in the reaction was indicated to contain chlorine in the molecule from its EI-MS, and it has the molecular formula $C_{12}H_7ClO_4$ from the high resolution mass spectrum. The ^{1}H-NMR spectrum of **3** is nearly the same as that of **1** except for the signal due to 5-H in **1**. Compound **3** was, therefore, identified as 5-chloro-9-methoxy-7*H*-furo[3,2-*g*][1]benzopyran-7-one.[10] Compound **4** was obtained as colorless needles having the molecular formula $C_{11}H_8O_5$. The IR spectrum of **4** showed a hydroxyl absorption band (3540 cm^{-1}) and two carbonyl absorption ones due to a lactone group and a formyl group (1740, 1660 cm^{-1}). The ^{1}H-NMR spectrum of **4** exhibited signals due to the formyl (δ 9.93, 1H, s) and the phenolic hydroxyl group (δ 11.30, 1H, br s) as well as ones due to α-pyrone and aromatic moieties, but it lacked signals ascribable to the furan unit. Thus, compound **4** was assigned as 6-formyl-7-hydroxy-8-methoxy-2*H*-1-benzopyran-2-one. On the other hand, the oxidative cleavage reaction of the double bond of the furan unit in **1** was examined under various conditions in order to obtain sufficient samples for bioassay. In consequence, we found that the ozonolysis of **1** in CH_2Cl_2 afforded **4** in 62% yield as a major product.

TABLE II. Effect of Chemotactic Activity of PMN toward Anaphylatoxin $C5_a$ des Arg

Compound	Migrated PMN
2	52±5
3	98±5
4	99±4

In order to examine inhibition of chemotactic activity of PMN toward anaphylatoxin $C5_a$ des Arg, the PMN migration test was performed by Boyden's method as modified by Snyderman and Pike.[11] The number of migrated PMN was expressed as a percentage of the absolute control and the test was run in triplicate. The PMN migration test result of each compound (**2**—**4**) is given in Table II.

In conclusion, we found that 2,3-dihydro-2,9-dimethoxy-3-hydroxy-7*H*-furo[3,2-*g*][1]benzopyran-7-one (**2**) inhibits PMN activity, by means of bioassay-directed separation of the products of 8-MOP in H_2O_2–NaClO. The compound was presumably formed *via* either an oxetane derivative[12] or 2-chloro-2,3-dihydro-3-hydroxy-9-methoxy-7*H*-furo-[3,2-*g*][1]benzopyran-7-one, but the pathway of formation of **2** was ambiguous. It should be noted that this is a first example of isolation of an 8-MOP derivative inhibiting chemotactic activity of PMN toward anaphylatoxin $C5_a$ des Arg.

Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. The EI-MS and high-resolution mass spectra were measured with a JEOL JMX DX-300 mass spectrometer, and the IR spectra with a JASCO IRA-2 spectrometer. The ultraviolet (UV) spectra were recorded with a Shimadzu UV 2100 spectrometer, and the ^{1}H-NMR and ^{13}C-NMR spectra with JEOL FX-100 and JEOL GSX-400 spectrometers using tetramethylsilane as an internal standard. The following abbreviations are used: s, singlet; d, doublet. TLC was performed on Merck precoated Kieselgel 60F_{254} plates. Column chromatography was carried out on Silica gel BW-200 (Fuji Davison Chemicals Co., Ltd.). For HPLC, a JASCO twincle-type instrument with a UV detector (JASCO UVIDEC 100-III) was used. Preparative TLC was run on Kieselgel 60PF_{254} plates preparated by ourselves. Extrelute 20 were purchased from Merck Co., Ltd.

Oxidation of 8-MOP with H_2O_2–NaOCl A 5% aqueous solution of NaOCl (35 ml) was added dropwise over 30 min to a water-cooled

solution of 8-MOP (600 mg) in MeOH (60 ml) and 30% aqueous H_2O_2 (4.5 ml), then the whole mixture was stirred at room temperature for 30 min. After removal of MeOH from the whole mixture under reduced pressure, the residual solution was absorbed on an Extrelute 20 column. The column was eluted with EtOAc (300 ml) and removal of the solvent from the eluate under reduced pressure gave crude 8-MOP oxidized products (180 mg).

Purification of the 8-MOP Oxidized Products The crude product (180 mg) was subjected to preparative TLC (CH_2Cl_2 : EtOAc = 3 : 2–$CHCl_3$: MeOH = 2 : 1) to give six fractions. Fraction 1 (70 mg) was purified by column chromatography (SiO_2 5 g, *n*-hexane : EtOAc = 3 : 1) to furnish **3** (48 mg). Fraction 2 (8.0 mg) was chromatographed on silica gel (3 g) with *n*-hexane : EtOAc = 1 : 1 to yield **4** (4.8 mg). Fraction 3 was subjected to normal-phase HPLC (Develosil 100-5, *n*-hexane : EtOAc = 2 : 3) to furnish **2** (1.0 mg).

2: An amorphous powder. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3690, 3590, 2925, 2850, 1730, 1720, 1620, 1580. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 3.61 (3H, s, 2-OMe), 4.10 (3H, s, 9-OMe), 5.04 (1H, s, 3-H), 5.54 (1H, s, 2-H), 6.25 (1H, d, J = 9.5 Hz, 6-H), 7.21 (1H, s, 4-H), 7.62 (1H, d, J = 9.5 Hz, 5-H). UV λ_{max}^{MeOH} nm (ε): 260 (6200), 321 (16000). MS m/z (%): 264 (M^+, 63), 232 ($M^+ - CH_3OH$, 16). High resolution MS m/z: Calcd for $C_{13}H_{12}O_6$: 260.063; Found: 260.064.

3: Colorless needles, mp 154—155 °C (EtOH). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2940, 2845, 1730, 1620, 1585. ^{1}H-NMR ($CDCl_3$, 100 MHz) δ: 4.28 (3H, s, 9-OMe), 6.46 (1H, d, J = 10.0 Hz, 6-H), 6.93 (1H, d, J = 2.2 Hz, 3-H), 7.72 (1H, d, J = 2.2 Hz, 3-H), 8.17 (1H, d, J = 10.0 Hz, 5-H). UV λ_{max}^{MeOH} nm (ε): 253 (13000), 266 (12000), 305 (8000). MS m/z (%): 252 (M^+, 34), 250 (M^+, 100), 237 ($M^+ - CH_3$, 16), 235 ($M^+ - CH_3$, 46). High resolution MS m/z: Calcd for $C_{12}H_7{}^{37}ClO_4$: 252.637. Found: 252.637. Calcd for $C_{12}H_7{}^{35}ClO_4$: 250.640. Found: 250.638.

4: Colorless needles, mp 185—187 °C (EtOH). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3500, 2945, 2850, 1740, 1625, 1585. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 4.07 (3H, s, 8-OMe), 6.34 (1H, d, J = 9.5 Hz, 3-H), 7.49 (1H, s, 5-H), 7.66 (1H, d, J = 9.5 Hz, 4-H), 9.93 (1H, s, 6-CHO), 11.3 (1H, s, 7-OH). UV λ_{max}^{MeOH} nm (ε): 264 (20000), 313 (10000). MS m/z (%): 220 (M^+, 100). High-resolution MS m/z: Calcd for $C_{11}H_8O_5$: 220.183. Found: 220.182.

Acetylation of 2 Ac_2O (0.5 ml) was added dropwise to an ice-cooled solution of **2** (5.0 mg) in pyridine (1.0 ml) and the whole mixture was allowed to stand at room temperature for 10 h. The reaction mixture was poured into ice-water, then the whole was extracted with EtOAc. The EtOAc extract was washed with 5% aqueous HCl, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl, then dried over $MgSO_4$. Removal of the solvent from the EtOAc extract under reduced pressure gave a product which was purified by column chromatography (SiO_2 5 g, *n*-hexane : EtOAc = 5 : 1) to furnish **2a** (5.8 mg, quant.).

2a: A white powder, mp 108—109 °C. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2940, 2840, 1740, 1730, 1620, 1580. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 2.10 (3H, s, OAc), 3.61 (3H, s, 3-OMe), 4.11 (3H, s, 9-OMe), 5.61 (1H, s, 2-H), 5.93 (1H, s, 3-H), 6.27 (1H, d, J = 9.6 Hz, 6-H), 7.30 (1H, s, 4-H), 7.61 (1H, d, J = 9.6 Hz, 5-H). UV λ_{max}^{MeOH} nm (ε): 262 (6800), 315 (14000). MS m/z (%): 306 (M^+, 61), 219 (100). High resolution MS m/z: Calcd for $C_{15}H_{14}O_7$: 306.074. Found: 306.074.

Preparation of 4 by Ozone Oxidation of 1 A solution of **1** (300 mg) in CH_2Cl_2 (50 ml) was bubbled through with ozonized oxygen at −78 °C for 2 h. The cooled solution was then bubbled through with nitrogen to remove excess ozone. After warming gradually to room temperature, the reaction mixture was treated with $(CH_3)_2S$ (1.0 ml) at room temperature for 10 min. The reaction mixture was poured into ice-water, then the whole was extracted with EtOAc. The EtOAc extract was washed with saturated aqueous NaCl and dried over $MgSO_4$. Removal of the solvent from the EtOAc extract under reduced pressure gave a product which was purified by column chromatography (SiO_2 10 g, *n*-hexane : EtOAc = 1 : 1) to furnish **4** (189 mg, 62%). The product was identical with **4** obtained in the previous H_2O_2–NaOCl treatment of **1** based upon a comparison of the physical data.

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The Hammett Acidity Function H_0 of Trifluoromethanesulfonic Acid–Trifluoroacetic Acid and Related Acid Systems. A Versatile Nonaqueous Acid System

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The acidity function (H_0) of mixtures of trifluoromethanesulfonic acid (TFSA) and trifluoroacetic acid (TFA) was measured. Nonaqueous acid systems based on TFSA, which is distillable, stable, non-oxidative and only weakly nucleophilic, can cover a wide range of acidity comparable to that of aqueous sulfuric acid, and should be useful for mechanistic and synthetic studies in organic chemistry. An H_0 value lower than-16 could be obtained by adding a small amount of SbF_5 to TFSA.

Keywords Hammett acidity function; trifluoromethanesulfonic acid; trifluoroacetic acid; antimony pentafluoride; nonaqueous acid system

Trifluoromethanesulfonic acid (TFSA) is a very strong acid, about a thousand times stronger than sulfuric acid. It is distillable, stable, non-oxidative and only weakly nucleophilic. Because of these advantages, this acid is very useful in various areas of chemistry.[1] Acidity is defined in terms of the Hammett acidity function H_0, as

$$H_0 = pK_{BH}^{+} - \log[BH^+]/[B] \quad (1)$$

in Eq. 1.[2] The acidity of strong acids, in particular of aqueous sulfuric acid, has been established by the use of a sophisticated selection of indicators[3a,b,4] (designated B in Eq. 1) as well as by advanced spectroscopic technology. Jorgenson and Hartter[3a] established a unified set of primary aniline derivatives as indicators for the determination of the acidity of acids weaker than 100% H_2SO_4. This indicator method was extended in due course to the determination of much stronger acids, mixture of sulfuric acid and Lewis acids, such as H_2SO_4–SO_3, H_2SO_4–HSO_3F, H_2SO_4–HSO_3Cl and H_2SO_4–$HB(HSO_4)_4$.[5] These acids are so strong that weaker bases such as aromatic nitro compounds are employed as indicators. Other super acid systems such as HSO_3F–SbF_5,[6-8] CF_3SO_3H–$B(SO_3CF_3)_3$,[9] $C_4F_9SO_3H$–SbF_5[10] and HF–SbF_5[8] were also studied similarly. However, nonaqueous acid system which can continuously cover the acidity range of aqueous sulfuric acid has been limited because only several acid systems (H_2SO_4–AcOH, H_2SO_4–trifluoroacetic acid (TFA), HF–TFA) have been reported.[11a,b] An acid system covering this region is important, because organic compounds bearing a nitro, nitrile, carbonyl, or hydroxy group behave as weak bases ($pK_{BH}^{+} = -12$ to -2),[12] and their protonation occurs in the acidity range of aqueous sulfuric acid (-2— -10). Because we have been interested in the formations of cation intermediates and their reactions in TFSA,[13] we needed weakly nucleophilic nonaqueous acid systems based on TFSA which would cover a wide range of acidity. Thus, we determined the acidity of mixtures of TFSA and TFA. We also obtained the acidity function of TFSA–SbF_5 covering the range of acidity to an H_0 value of below -16. This nonaqueous acid system is so strong and yet so mild that it should find extensive application in mechanistic and synthetic studies in organic chemistry.

Results

The acidity functions H_0 of TFSA, TFA and TFSA–TFA mixtures were determined by a modification of the procedure previously described using a set of nitroanilines and nitrobenzenes as indicators, with ultraviolet (UV) detection.[3,5,6] The H_0 values of TFSA and TFA were found to be -13.7 and -2.7, respectively. These values, though the former was diverging from the previously estimated value[14a,b] to a certain extent, are stable and reproducible.[15]

TABLE I. H_0 Values for the TFSA–TFA System[a]

% of TFSA[b]	$-H_0$	% of TFSA[b]	$-H_0$
0.0	2.7 (A, B)	43.5	11.5 (H)
0.9	7.7 (C, D)	53.4	11.8 (H)
1.1	7.8 (D)	62.4	12.2 (H)
1.6	8.0 (D)	72.8	12.5 (H)
3.1	8.4 (D)	84.9	12.9 (H)
3.4	8.4 (D)	91.1	13.0 (H)
5.7	8.9 (D)	92.9	13.2 (I)
8.0	9.4 (E, F)	94.6	13.3 (I)
11.2	9.7 (F)	95.5	13.3 (I)
11.4	9.7 (E, F)	96.3	13.4 (I)
22.1	10.6 (F)	98.2	13.5 (I)
26.9	10.9 (F)	99.1	13.6 (I)
32.4	11.2 (F)	100.0	13.7 (I)
43.0	11.3 (G)		

a) (A)—(G) represent the indicators used (shown in Table III). *b*) % means weight percentage.

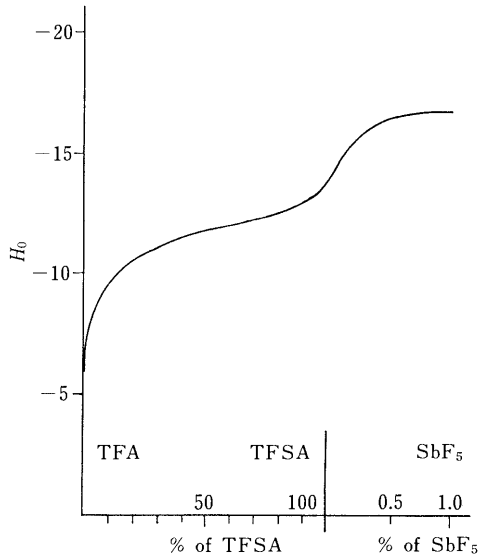

Fig. 1. H_0 Profile of TFA–TFSA–SbF_5 Acid Systems

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By mixing of TFSA and TFA we can obtain a nonaqueous acid system with acidity values between $H_0 = -2.7$ and -13.7 (Table I). A graphic representation is also shown in Fig. 1 Although Olah *et al.* reported that spectroscopic determination of the acidity of TFSA–SbF_5 was impossible because of strong absorption of the solvent in the UV region,[16] we found it possible to apply the UV method up to 1% (w/w) SbF_5 in TFSA, giving an H_0 value of -16.8 (Table II). We found that the addition of a small amount of SbF_5 to TFSA could extend the range of acidity rapidly. These mixed acids lack the shortcomings which are often encountered in the use of sulfuric acid, such as sulfonation reaction, oxidation, and nucleophilic attack of sulfate anion at electron-deficient centers. Another practical shortcoming of sulfuric acid is the difficulty of purification owing to its high boiling point and high viscosity. The high freezing point (6 °C) of sulfuric acids is also significantly disadvantageous for nuclear magnetic resonance (NMR) studies of unstable cationic intermediates at low temperature.[17] The present acid system does represent a better acid catalyst.[18]

Experimental

Materials TFSA was purchased from 3M Co., and was purified by rectification with a Widmer column (30 cm) under reduced pressure, followed by repeated distillation under reduced pressure (bp 102 °C (90 mmHg)). TFA was purchased from Wako Pure Chemical Co., and was purified by distillation with a small amount of potassium permanganate (0.1 g/kg) at atmospheric pressure, followed by repeated distillation (bp 73 °C (760 mmHg)). Antimony pentafluoride was available from Aldrich Co., and was used after distillation under reduced pressure (bp 81.5—82 °C (90 mmHg)). 2,6-Dichloro-4-nitroaniline (mp 75—75.5 °C), 6-bromo-2,4-dinitroaniline (mp 153.5—154 °C), 2,4,6-trinitroaniline (194.5—195 °C), 4-nitrotoluene (mp 51.7—52 °C), 2,4-dinitrotoluene (mp 71.5—72 °C) and 1,3,5-trinitrobenzene (mp 126.5—127 °C) were commercial products, and were purified by repeated recrystallization from ethanol. 4-Nitrotoluene (mp 51.7—52 °C) was also purchased and was purified by recrystallization from *n*-hexane. 4-Nitrofluorobenzene (bp 100 °C (24 mmHg)) and 2,4-dinitrofluorobenzene (bp 102—103 °C (0.45 mmHg)) were also available and were purified by distillation under reduced pressure. 2-Chloro-6-nitroanilline (mp 75—75.5 °C) and 3-bromo-2,4,6-trinitroaniline (mp 215—216 °C) were prepared as previously described[3a] and purified from *n*-hexane–benzene. 3-Methyl-2,4,6-trinitroaniline (mp 140—141 °C) was also prepared[3a] and purified by recrystallization from ethanol.

Preparation of Solutions (A) TFSA–TFA: Several milligrams of the indicator (required for sufficient UV absorption (approximate ionization ratio $I = 0.8$) in the subsequent spectrophotometric measurements) was weighed into a 10-ml volumetric flask and was diluted to the mark (10 ml) with TFA (when the mixed acid was less acidic than 90% (w/w) TFSA–10% TFA), or with TFSA (when the mixed acid was more acidic than 90% TFSA–10% TFA). A mixture of TFSA and TFA of known weights was prepared in a 25-ml volumetric flask. A 1-ml aliquot of the stock indicator (which was weighed) was withdrawn into a 10-ml volumetric flask. This indicator solution was then diluted to the mark with the prepared acid mixture, which was analytically weighed. At this stage the composition of the mixed acid was calculated in weight percentage. After mixing, the resultant solution was transferred into a 1-cm cell, which was fitted with a Teflon cap. Stock acid mixture was used as a reference sample.

(B) TFSA–SbF_5: A solution of the indicator in TFSA was prepared in a similar manner in a 10-ml volumetric flask. Weighed antimony pentafluoride was placed in 25-ml volumetric flask and made up with TFSA to 25 ml. A 1-ml aliquot of the stock indicator and a 3-ml aliquot of the stock acid sample were mixed in a 1-cm cell, which was sealed with a Teflon cap. The change of volume on mixing can be ignored, and the composition of the mixed acid was calculated in weight percentage. Neat TFSA was used as a reference sample.

Spectrophotometric Measurements All measurements were carried out with matched quartz glass 1-cm cells fitted with Teflon stoppers. Spectra were recorded at room temperature (25 °C) with a Shimadzu model UV-200S recording spectrophotometer. The UV absorptions were measured at the specified wavelength (Table III), at λ_{max} of the neutral base in the case of anilines indicators, and at the λ_{max} of the protonated base in the case of nitrobenzene indicators. The ionization ratios, I, were obtained from the expression $(\varepsilon_{BH^+} - \varepsilon)/(\varepsilon - \varepsilon_B)$, where ε_B and ε_{BH^+} are the extinction coefficients of the unprotonated and fully protonated forms of the base. The extinction coefficient of the indicator in any given solution, ε was measured directly from the absorption spectra. The UV spectral data (extinction coefficients and λ_{max}) of the indicators are also shown in Table III, together with the reported pK_{BH^+} values of the indicators.[2,3,5]

TABLE II. H_0 Values for the TFSA–SbF_5 System[a]

% of SbF_5[b]	$-H_0$
0.0	13.7 (I, J)
0.02	13.8 (I)
0.04	13.8 (I, J)
0.07	14.1 (I)
0.15	15.4 (K)
0.22	15.8 (K)
0.29	16.1 (K)
0.37	16.1 (K)
0.40	16.3 (K)
0.51	16.4 (K)
0.72	16.7 (K)
1.02	16.8 (K)

a) The superscripts represent the indicators used (shown in Table III). *b*) % means weight percentage.

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TABLE III. Ultraviolet Spectral Data of Indicators Used

	Indicator, nitroaniline	λ_{max} of B (nm)[a]	ε_B[e]	ε_{BH^+}[f]	pK_{BH^+}[g]
A	2-Chloro-6-nitroaniline	410[b]	4500	30	−2.46
B	2,6-Dichloro-4-nitroaniline	367[b]	12500	160	−3.24
C	6-Bromo-2,4-dinitroaniline	345[c]	12500	370	−6.71
D	3-Methyl-2,4,6-trinitroaniline	370[d]	6200	270	−8.37
E	3-Bromo-2,4,6-trinitroaniline[f]	370[c]	—	—	−9.62
F	2,4,6-Trinitroaniline	405[c]	8400	160	−10.10

	Indicator, nitrobenzene	λ_{max} of BH^{+h} (nm)	ε_B[k]	ε_{BH^+}[l]	pK_{BH^+}[m]
G	4-Nitrotoluene	378[i]	190	20800	−11.35
H	4-Nitrofluorobenzene	362[j]	160	16800	−12.44
I	2,4-Dinitrotoluene	336[j]	740	12300	−13.76
J	2,4-Dinitrofluorobenzene	332[j]	270	12000	−14.52
K	1,3,5-Trinitrobenzene	305[j]	530	8400	−16.04

a) Measured at an acid concentration of about two H_0 units below the pK. *b*) Measured in TFA–H_2O acid. *c*) Measured in TFA. *d*) A shoulder was used. *e*) Values at the λ_{max}. *f*) Values at the wavelength of λ_{max} of the free base, measured in TFSA. *g*) Values determined in ref. 3*a* and 5. *h*) Measured at an acid concentration of about two H_0 units above the pK. *i*) Measured in TFSA. *j*) Measured in TFSA–SbF_5. *k*) Values at the wavelength of the λ_{max} of the protonated base, measured in TFA. *l*) Values at the λ_{max}. *m*) Values determined in ref. 5.

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The Reactions of (−)-(*R*)-2-Aminomethylpyrrolidine(1,1-cyclobutanedicarboxylato)platinum(II), Carboplatin and Cisplatin with Guanosine: Kinetic Comparison of the Reactivities

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The reaction of a new antitumor platinum complex, (−)-(*R*)-2-aminomethylpyrrolidine(1,1-cyclobutanedicarboxylato)platinum(II) (1), with guanosine (Guo) in an aqueous solution was kinetically compared with the reactions of carboplatin (2) and cisplatin (3) with Guo using high performance liquid chromatography (HPLC) analyses and proton nuclear magnetic resonance (^{1}H-NMR).

Reactions of 1 and 2 with Guo proceeded according to second order kinetics; the 1,1-cyclobutanedicarboxylate (CBDCA) group of 1 and 2 was directly substituted with two N-7 of Guo.

The reaction of 3 followed a two-step course through the intermediate, *cis*-Pt(NH_3)$_2$Cl(Guo).

The reaction rates of the three platinum complexes with Guo were related to 3 ≫ 1 > 2. This result indicated that the CBDCA ligand (chelate ligand, bidentate ligand) coordinated to platinum more strongly than the Cl ligand (monodentate ligand), and the trans effect of the 2-aminomethylpyrrolidine ligand was stronger than that of the ammine ligand.

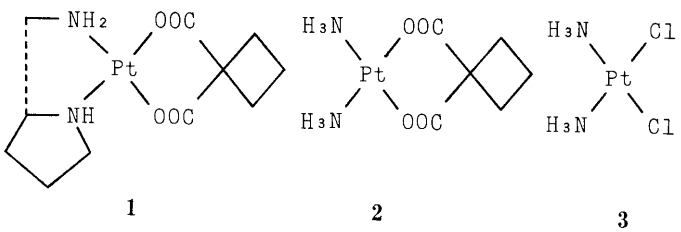
Keywords kinetics; antitumor platinum complex; guanosine; 2-aminomethylpyrrolidine Pt(II) complex; 1,1-cyclobutanedicarboxylato Pt(II) complex; bis(N_7-guanosine) Pt(II) complex; carboplatin; cisplatin

The antitumor platinum(II) complexes are represents by the general formula PtL_2X_2, where X_2 represents either two monodentate ligands or one bidentate anionic ligand, usually called leaving groups, and L_2 represents two monodentate ligands or one bidentate ammine ligand, usually called carrier ligands.

The activity of these complexes is believed to be due to bifunctional crosslinking with deoxyribonucleic acid (DNA). A number of fundamental studies have been carried out on the interaction of these complexes with DNA, especially with guanine bases, and on the structure elucidation of the reaction products.[1]

In the preceding paper,[2] we reported that (−)-(*R*)-2-aminomethylpyrrolidine(1,1-cyclobutanedicarboxylato)-platinum(II) (**1**) reacts with 2 eq of guanosine (Guo) at room temperature to form [(−)-(*R*)-2-aminoethylpyrrolidine]-bis(N_7-guanosine)platinum(II).

In order to explore the characteristic features of this reaction, we carried out reactions using other two antitumor platnium(II) complexes, *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II) (carboplatin, **2**) and *cis*-diamminedichloroplatinum(II) (cisplatin, **3**), with Guo under comparable conditions, and kinetically compared the reactivities of **1**, **2** and **3**. Thus, **1** and **2** were allowed to react with 2 eq Guo in an aqueous solution separately at 40 °C and 60 °C, **3** was similarly treated in a 0.9% sodium chloride injection at the same temperatures, and the respective reactions were followed by means of proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy and high performance liquid chromatography (HPLC).



1 2 3

Chart 1

Experimental

Materials Guo was obtained from Kanto Chemical Co., Inc.. (−)-(*R*)-2-Aminomethylpyrrolidine(1,1-cyclobutanedicarboxylato)-platinum(II) (**1**), *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II) (carboplatin, **2**) and *cis*-diamminedichloroplatinum(II) (cisplatin, **3**) were prepared according to the respective published methods.[3-5]

Reaction of 1, 2 or 3 with Guo in Aqueous Solution A solution of 20 mg (67 μmol) of Guo in 20 ml of water was treated with **1** (15 mg, 33 μmol) or **2** (12 mg, 33 μmol), and the mixture was allowed to stand separately at 40 °C or 60 °C in a water bath. The reaction of **3** was carried out in a similar way using 10 mg (33 μmol) of **3** and 20 mg of Guo in 20 ml of a 0.9% sodium chloride injection. Each of the reaction mixtures was analyzed at intervals by HPLC and ^{1}H-NMR spectroscopy. The ^{1}H-NMR spectra were measured with samples prepared by adding 5 ml of D_2O to 1 ml aliquots.

Measurements HPLC analyses were performed on a Shimadzu LC-6A liquid chromatograph with detection at 235 nm (in the case of **3**, 210 nm), on a Tosoh TSKgel G-Oligo-PW column (7.8 × 300 mm), using 0.1 M Na_2SO_4 aqueous solution as an eluent. The flow rate was 1 ml/min.

^{1}H-NMR spectra were recorded on a JEOL JNM-GX400 spectrometer operating in the Fourier transform mode with sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) as an internal standard, and the chemical shifts are given in ppm units.

Results and Discussion

The ligand substitution in square planar platinum(II) complexes has been shown to follow the S*N*2 mechanism.[6]

The rate laws are consistent with both solvent-assisted and second-order substitution pathways, and the reactions

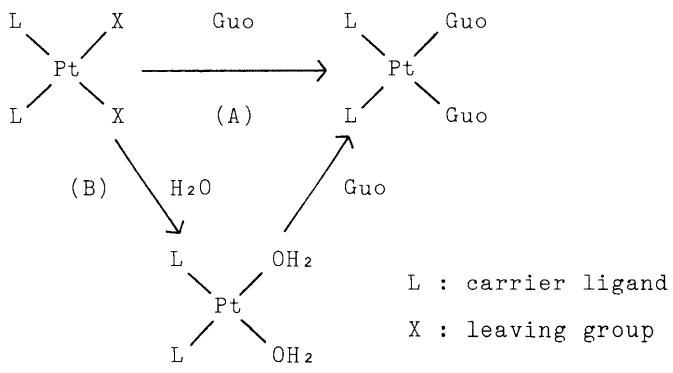


Chart 2

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under consideration may be conveniently schematized as Chart 2.

Pathway (A) is a direct nucleophilic substitution of X_2 in platinum(II) complexes with two Guo. Pathway (B) proceeds through the solvent-assisted substitution pathway, in which the first reaction is a nucleophilic substitution of X_2 with H_2O, and the second reaction is that of the aqua complexes with Guo.

Since the leaving group, 1,1-cyclobutanedicarboxylate (CBDCA), of **1** and **2** is not two monodentate (anion) ligands but a bidentate ligand chelating to the platinum, it appears likely to be stable enough not to be substituted with water. In fact, we checked the aqueous solution of **1** or **2** stored at 40 °C for 7 d by ^{1}H-NMR spectroscopy and HPLC, and confirmed that the liberation of CBDCA was not noticed at all, suggesting that the solvent (H_2O)-assiated pathway can be neglected.

Consequently, the reactions of **1** or **2** with Guo are reasonably presumed to follow pathway (A), and the equation to be considered is:

$$PtL_2X_2 \xrightarrow[k_1]{Guo} PtL_2X(Guo) \xrightarrow[k_2]{Guo} PtL_2(Guo)_2$$

Further, the checking of the reaction mixture of **1** or **2** with Guo by ^{1}H-NMR spectroscopy and HPLC demonstrated that the only detectable product was always that derived from 1 eq **1** or **2** and 2 eq Guo, and the formation of the product containing one Guo could not be observed at all (Figs. 1 and 2).

Accordingly, the binding of the second Guo may be a very rapid reaction ($k_1 \ll k_2$), and the reaction of **1** or **2** with Guo can be considered to proceed according to second order kinetics.

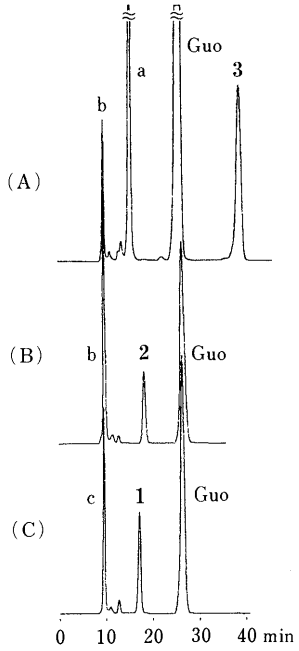

Fig. 1. HPLC Chromatogram of the Reaction Mixture of **1** (C), **2** (B) and **3** (A) with Guo

A: the residual ratio of **3** was 71.2% at 60 °C for 2 h. B: the residual ratio of **2** was 67.9% at 60 °C for 25 h. C: the residual ratio of **1** was 76.7% at 60 °C for 6 h. a: *cis*-[Pt(NH_3)$_2$Cl(Guo)]. b: *cis*-[Pt(NH_3)$_2$(Guo)$_2$], c: [(2-aminomethylpyrrolidine)Pt(Guo)$_2$]

$$-d[\text{Pt complex}]/dt = k[\text{Pt complex}][2(\text{Guo})]$$
$$= k_{obs}[\text{Pt complex}]^2$$

Residual ratio (α) is defined:

$$\alpha = [\text{Pt complex}]/[\text{Pt complex}]_0$$
$$1/\alpha = k_{obs}[\text{Pt complex}]_0 t + 1$$

Table I shows the residual ratios of **1** and **2** remaining in the reaction mixtures kept separately at 40 °C and 60 °C by HPLC at intervals. The residual ratios of **1** and **2** are in good proportion to those of Guo in both cases.

Plots of time *vs.* reciprocal of the residual ratios of **1** and **2** are found to be linear for all reactions as illustrated in Fig. 3. Thus, it can be concluded that the reactions of **1**

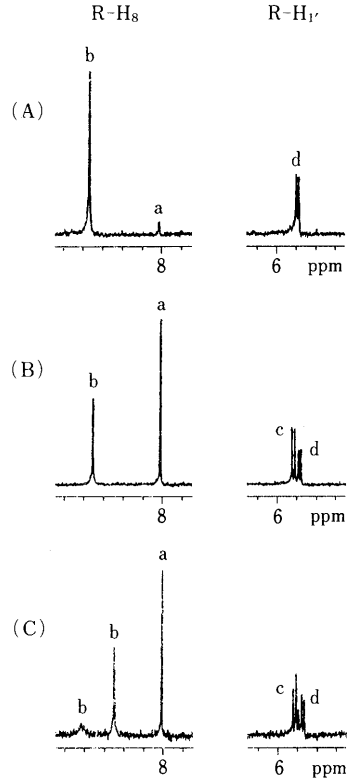

Fig. 2. Parts of ^{1}H-NMR Spectra of the Reaction Mixture of **1** (C), **2** (B) and **3** (A) with Guo at 60 °C for 24 h, Showing from Left to Right Guanine H_8 and the Sugar $H_{1'}$

a and c: free Guo, b and d: bound Guo to Pt through the N-7 atom.

TABLE I. Residual Ratios of the Reaction Mixtures of **1**, **2** and **3** with Guo at 40 °C and 60 °C

Time (h)	**1**		**2**		**3**	
	40 °C	60 °C	40 °C	60 °C	40 °C	60 °C
2	97.4% (98.0%)	90.1% (91.2%)	99.9% (100.9%)	97.2% (97.4%)	94.0% (96.7%)	71.2% (81.9%)
4	96.4% (97.5%)	82.9% (84.2%)	98.6% (98.9%)	93.8% (94.2%)	89.4% (95.0%)	52.4% (69.8%)
6	91.4% (92.9%)	76.7% (78.3%)	97.4% (98.1%)	90.9% (91.4%)	84.9%[a] (92.1%)	39.9% (59.9%)
24	79.3% (80.2%)	40.4%[b] (41.9%)	92.8% (—)	67.9%[c] (68.7%)	52.2% (70.0%)	10.5%[b] (26.0%)
k_{obs}[d]	4.56	34.6	2.16	11.8	—	—

Residual ratios of Guo corresponding to each of the Pt complexes respectively are in parentheses. *a*) 5 h, *b*) 26 h, *c*) 25 h, *d*) Second-order rate constant ($M^{-1}h^{-1}$).

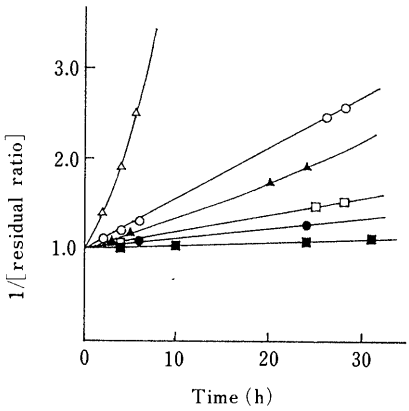

Fig. 3. Time *vs.* 1/[Residual Ratio] Profile of the Reaction Mixtures of **1**, **2** and **3** with Guo at 40 °C and 60 °C

—●—, **1**; —■—, **2**; —▲—, **3**; at 40 °C. —○—, **1**; —□—, **2**; —△—, **3**; at 60 °C.

and **2** with Guo proceed according to second order kinetics as anticipated.

Contrary to the above cases, it has been known that one or two chloride molecules of the leaving groups of **3** are replacable with H_2O to give the corresponding aqua-complexes.[7] Therefore, both pathways (A) and (B) in Chart 2 should be considered in the reaction of **3** with Guo. Nevertheless, taking into account the high chloride ion concentration in a 0.9% sodium chloride injection, it appears likely that pathway (B) is negligible[8] and the reaction proceeds through pathway (A) in this case, too.

The HPLC chromatogram of the reaction mixture of **3** with Guo (2 eq) in a 0.9% sodium chloride injection at 60 °C for 2 h exhibited a peak due to the intermediate,[9] *cis*-$Pt(NH_3)_2Cl(Guo)$, in addition to that of *cis*-$Pt(NH_3)_2(Guo)_2$ (Fig. 1). This observation evidently indicates that the reaction of **3** with Guo follows a two-step course ($k_1 > k_2$).[10] The shelf-life of *cis*-$Pt(NH_3)_2Cl(Guo)$ is comparatively long, and a prolonged reaction time or the use of excess Guo is required to complete the reaction.

Table I also shows the residual ratios of **3** remaining in the reaction mixtures kept separately at 40 °C and 60 °C by HPLC at intervals. The residual ratios of **3** are not in proportion to those of Guo in this case. As illustrated in Fig. 3, plots of time *vs.* reciprocal of the residual ratios of **3** are not linear in accord with the above observation. Thus, it can be concluded that the reaction of **3** with Guo in a 0.9% sodium chloride injection does not proceed according to second order kinetics, but is a stepwise reaction, different from the reaction of **1** and **2** with Guo in aqueous solution.

As seen from Fig. 3, the reaction rates of the three platinum complexes with Guo were found to follow a decreasing order: $\mathbf{3} \gg \mathbf{1} > \mathbf{2}$.

The large difference in the reactivities between **3** and **2**, having the same carrier ligands (NH_3), should be considered to be due to the different nature of the leaving groups, and it may be ascribed not only to the difference in binding-energies between Pt–Cl and Pt–OCO–, but also to the stabilizing effect owing to the chelation of the leaving group (bidentate ligand, CBDCA) to Pt in **2**.

On the other hand, comparison of the reactivities of **1** and **2** must be done by taking into account the nature of their carrier ligands, since the leaving group is the same (CBDCA). The difference in the reaction rates between **1** and **2** can be ascribed to the trans effect[6,11] of the carrier ligand. It may be assumed that the trans effect of the 2-aminomethylpyrrolidine ligand is stronger than that of ammine ligand (NH_3).

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A New Series of Antiallergic Agents. I. Synthesis and Activity of 11-(2-Aminoethyl)thio-6,11-dihydrodibenz[*b*,*e*]oxepin Derivatives

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A new series of 11-substituted 6,11-dihydrodibenz[*b*,*e*]oxepin derivatives was synthesized and evaluated for antiallergic activity. Convenient methods for the preparation of sulfides from alcohols were developed. Structure–activity relationships are described. Compound 7, 11-[2-(dimethylamino)ethyl]thio-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylic acid hydrochloride, was the most potent in the rat passive cutaneous anaphylaxis test (ED_{50} = 0.92 mg/kg *p.o.*). It had a potent inhibitory effect on anaphylactic bronchoconstriction in guinea pigs (ED_{50} = 0.029 mg/kg *p.o.*) and H_1 receptor antagonistic effect (K_i = 14 nM) with few central nervous system side effects. Additionally, an antagonistic effect against prostagrandin D_2-induced contraction of isolated guinea pig trachea (pA_2 = 5.73) was an attractive mechanism of action of the new antiallergic agent. Compound 7 was selected for further evaluation as KW-4994.

Keywords antiallergic agent; antiasthmatic agent; H_1-antihistaminic activity; receptor antagonist; anti-passive cutaneous anaphylaxis activity; structure–activity relationship; prostagrandin D_2; 6,11-dihydrodibenz[*b*,*e*]oxepin

Effective and orally active antiallergic agents with fewer side effects have been attractive targets for drug research in recent years.[1] Our research has been focused on syntheses of some new series of benzoxepin derivatives and their pharmacological properties.[2] From our efforts, we had found that compound **1**,[3] one of the 11-substituted-dibenz[*b*,*e*]oxepin derivatives, showed highly potent antiallergic activity. However, **1** was accompanied by undesirable central nervous system (CNS) side effects (*e.g.* inhibition of locomotor activity in mice). We thus attempted to introduce a hydrophilic substituent instead of a methyl group at the 2-position of **1** in the hope that the decrease in lipophilicity of the molecule might lead to a reduction of CNS side effects. We finally found **7**, 11-[2-(dimethylamino)ethyl]thio-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylic acid hydrochloride, which had potent antiallergic activity and was devoid of undesirable CNS side effects.

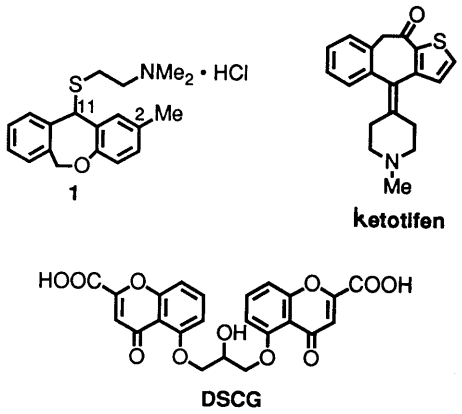

Fig. 1

Known antiallergic agents might be classified into two groups according to their chemical structures.[4] The basic agents such as ketotifen elicit their antiallergic effects by mainly antagonizing against histamine (H_1) and the acidic agents such as disodium cromoglycate (DSCG) by mainly inhibiting release of chemical mediators. From the viewpoint of the classification described above, **7** represents a new class of antiallergic agent having both acidic and basic moieties in one molecule. To our knowledge, only a few agents have been reported as such type of compound.[5] We expected such a new class of compounds to exhibit their

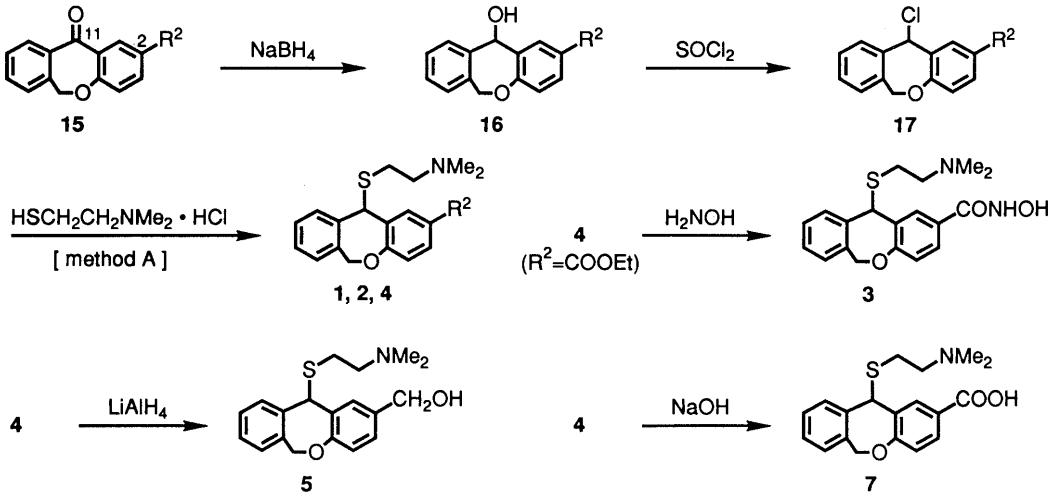

Chart 1

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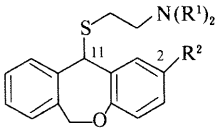
TABLE I. Substituted 6,11-Dihydrodibenz[*b*,*e*]oxepin Derivatives



Compd.[a] No.	R^1	R^2	mp (°C)	Recrystn.[b] solvent	H_1 % inhibn. 0.1 μM	M_1 % inhibn. 1 μM	PCA % inhibn. 10	PCA (mg/kg *p.o.*) 1
1	Me	Me	166—169	ET	93	24	97	66
2	Me	$CONH_2$	135—140 (dec.)	IP	91	79	59	20
3	Me	CONHOH	176—177	IP	91	24	51	61
4	Me	COOEt	169—171	IP	89	65	78	70
5	Me	CH_2OH	132—142	AC	93	23	92	41
6	H	COOH	250—253	IP	44	2	26	27
7	Me	COOH	233 (dec.)	IP	59	7	87	70
8	Et	COOH	128—130	IP	56	1	40	47
9	iso-Pr	COOH	250—252	IP	40	−2	66	58
10	Me	CH_2COOH	174—179	IP	84	−1	60	51
11	Me	CH(Me)COOH	123—124	EE	71	0	65	45
12	Me	$C(Me)_2COOH$	129—130	MT	74	16	66	69
13	Me	CH_2CH_2COOH	213—215	IE	61	1	78	58
14	Me	$CH_2CH_2CH_2COOH$	101—103	IE	75	3	57	21
Ketotifen					99	9	75	48

a) All compounds were obtained as HCl salts, except **2** (free base), **5** (fumaric acid salt), and **12** (free base). *b*) ET, ethanol; EE, diethyl ether; AC, acetone; MT, methanol; IE, diisopropyl ether; IP, isopropanol.

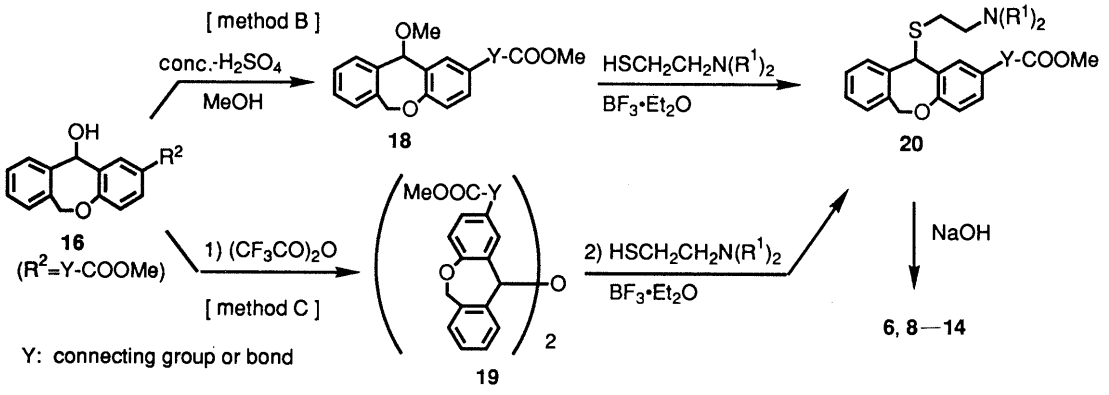


Chart 2

acitivity through new mechanisms of action.

In this paper, we describe the synthesis and structure–activity relationships of 11-substituted 6,11-dihydrodibenz[*b*,*e*]oxepin derivatives. Ketotifen, whose structure is also tricyclic, was used as a reference compound in our series of experiments.

Chemistry The 11-substituted dihydrodibenz[*b*,*e*]oxepin derivatives listed in Table I were prepared from appropriate aminoethanethiols and 11-alcohols **16** which were prepared by the $NaBH_4$ reduction of the corresponding ketones **15**[6,7] (Chart 1). In the initial stage of this study, we prepared the compounds having a varied substituent at the 2-position (**1**—**5**, and **7**). The chlorides **17** prepared by the reaction of **16** and $SOCl_2$ were extremely moisture-sensitive and immediately treated with 2-(dimethylamino)-ethanethiol in dimethylformamide (DMF) [method A]. The carboxylic acid **7** was prepared by the saponification of the ester **4**. The hydroxamic acid **3** was prepared by the treatment of **4** with hydroxylamine. The alcohol **5** was prepared by the reduction of **4** with lithium aluminum hydride ($LiAlH_4$).

Since **7**, which has a carboxyl group at C-2, was found to be the most favorable of the early cmpounds (**1**—**5**, **7**), a series of compounds having a carboxyl group as the terminal substituent of the dibenz[*b*,*e*]oxepin ring system (**6** and **8**—**14**) was prepared. These compounds were prepared *via* a methyl ether **18** [method B], or a dimeric ether **19** [method C] instead of an unstable chloride **17** (Chart 2). The sulfide formation in methods B and C can be explained in terms of a push-pull mechanism. A similar synthetic method for sulfides using zinc iodide has been reported.[8] In method C, the sequential treatments were performed in one-flask operation under very mild conditions: trifluoroacetic anhydride acted as a dehydrating agent to give **19**, which was converted to **20** in the presence of $BF_3 \cdot Et_2O$.

Results and Discussion

The compounds were tested for their inhibitory effects on the specific binding of [^{3}H]pyrilamine to guinea pig cerebellum histamine-1 (H_1) receptors,[9] the specific [^{3}H]-quinuclidinyl benzilate binding to rat striatum muscarinic acetylcholine (M_1) receptors,[10] and 48 h homologous

passive cutaneous anaphylaxis (PCA) in rats. These biological methods are described in the experimental section. The results are summarized and represented by percent inhibition in Table I. The H_1 receptor antagonistic effect was one of the mechanisms of antiallergic action,[11] whereas the M_1 receptor antagonistic was one of the indices of side effects such as suppression of salivary secretion and mydriasis.[12]

In the initial stage of this study, we focused on diminishing the undesirable CNS side effects of **1**. Our research began with replacement of the methyl group at the 2-position of **1** by a hydrophilic substituent (**2**—**5** and **7**). The results showed that inhibitory effects on both the H_1 receptor binding and rat PCA were retained, irrespective of a substituent at C-2, whereas affinity for the M_1 receptor was sensitive to the nature of the substituent. The carboxylic acid (**7**), which showed negligible M_1 receptor binding activity, turned out to be suitable for our purpose. Compound **7** was almost equipotent to **1** on the PCA test. Furthermore, it did not show any significant CNS effects up to a dose of 300 mg/kg *p.o.* These results encouraged us to pursue a series of compounds possessing a carboxyl group as the terminal of the substituent of the dibenzoxepin ring system (**6** and **8**—**14**). The effect of a substitution pattern of an amino group in the side chain was examined. From the data of several examples (**6**—**9**) in the 2-carboxyl series, dimethylamino group (**7**) was the most potent in the PCA test. We next examined the influence of the introduction of the connecting group between a carboxyl group and the dibenz[*b*,*e*]oxepin skeleton (C-2), fixing 2-[(2-dimethylamino)ethyl]thio side chain of **7**. Compounds **10**—**14** having a connecting group, as well as **7**, turned out to show significant inhibitory effects on both the PCA and H_1 receptor binding tests, and negligible M_1 receptor affinity. Although compounds **10**—**14** were somewhat more potent than **7** in the H_1 receptor binding assay, none of these compounds exhibited a significant increase in the PCA inhibitory activity.

Overall, **7** was the most potent in the rat PCA test with an ED_{50} value of 0.92 mg/kg *p.o.* and about 5 times more potent than ketotifen (ED_{50} = 5.1 mg/kg *p.o.*). Compound **7** exhibited a H_1 receptor antagonistic effect with a K_i value of 14 nM, whereas ketotifen had a K_i value of 0.31 nM. Observed H_1 receptor antagonistic activity alone could not explain the PCA inhibitory effect of **7**. Therefore, the mechanism of action of **7** might be different from that of ketotifen. Detailed analysis of the mechanism of action of **7** is now under investigation. From these results described above, compound **7** was selected for further pharmacological studies.

In order to elucidate efficacy on bronchial asthma, we examined the effect of **7** on immunoglobulin G_1 (IgG_1)-mediated bronchoconstriction in guinea pigs using the modified method reported by Konzett and Rössler.[13] Compound **7** inhibited the bronchoconstriction dose-dependently (0.003—0.3 mg/kg) with an ED_{50} value of 0.029 mg/kg *p.o.* Similarly ketotifen was also effective with an ED_{50} value of 0.009 mg/kg *p.o.*

Prostagrandin D_2 (PGD_2) has been identified as a potent bronchoconstrictor.[14] We therefore examined and found that **7** had an antagonistic effect against PGD_2-induced contraction of isolated guinea pig trachea (pA_2 = 5.73).[15] We deduced that the potent inhibitory effect of **7** on anaphylactic bronchoconstriction was based partly on the PGD_2 antagonistic effect.

In conclusion, we synthesized a new series of 11-(2-aminoethyl)thio-6,11-dihydrodibenz[*b*,*e*]oxepin derivatives and evaluated their antiallergic activity. Convenient methods for the preparation of sulfides were developed. Among the compounds synthesized, **7** had the most potent antiallergic activity with few CNS side effects. From the structural point of view, **7** represents the new class of antiallergic agent with both amino and carboxyl moieties in one molecule. It exhibited antihistaminic activity like a basic agent and a weak mediator release inhibitory effect[16] like an acidic agent. Additionally, it had an antagonistic effect against PGD_2, which was an attractive mechanism of action of the new antiallergic agents. It has a great safety margin (*e.g.* LD_{50} 771 mg/kg *p.o.*, mice) and a beneficial duration of action (6—9 h in rat PCA test). Compound **7** was seledted for further pharmacological evaluation as KW-4994. The detailed pharmacology and mechamisms of action of KW-4994 will be published elsewhere.

Experimental Section

Synthetic Procedures Melting points were determined with a Büchi-510 melting point apparatus and are uncorrected. Infrared spectra (IR) were recorded on a Shimadzu IR-400 spectrometer. Proton nuclear magnetic resonance spectra (^{1}H-NMR) were recorded on a JEOL PMX-60 (60 MHz), a Hitachi R-90H (90 MHz), or a JEOL GX-270 (270 MHz) spectrometer with Me_4Si as internal standard. Mass spectra (MS) were recorded on a JEOL D300 mass spectrometer. Elemental analyses were performed by the analytical department of our laboratories. For column chromatography, silica gel: Kieselgel 60 (Merck, 70—230 or 230—400 mesh) and highly porous synthetic resin: Diaion HP-10 (Mitsubishi Chem. Ind. Co., Ltd.) were used.

Methyl 11-Hydroxy-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylate (16c: R_2 = COOMe) To a suspension of methyl 11-oxo-6,11-dihydrodibenz-[*b*,*e*]oxepin-2-carboxylate[7] (200 g, 0.75 mol) in MeOH (1l), $NaBH_4$ (25 g, 0.66 mol) was added portionwise. After the addition was completed, the mixture was stirred at room temperature for 1 h. The mixture was treated with acetic acid (40 ml, 0.70 mol) at room temperature for 30 min. The resultant precipitate was filtered, washed successively with MeOH and H_2O, and dried. The crude product (136 g) was recrystallized from toluene to give 118 g (59%) of **16c**, mp 126—127 °C (lit.[6c] 85—87 °C). IR (KBr): 2950, 1710, 1240, 1015 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.86 (s, 3H), 5.01 and 6.05 (AB, J_{AB} = 12.4 Hz, 2H), 5.71 (s, 1H), 6.87 (d, J = 8.6 Hz, 1H), 7.2—7.5 (m, 4H), 7.84 (dd, J = 2.1, 8.6 Hz, 1H), 8.08 (d, J = 2.1 Hz, 1H). *Anal.* Calcd for $C_{16}H_{14}O_4$: C, 71.10; H, 5.22. Found: C, 71.00; H, 5.41.

Compounds **16a** (R^2 = Me, mp 90—91 °C), **16b** (R^2 = $CONH_2$, mp 248 °C (dec.)), **16d** (R^2 = COOEt, oil), **16e** (R^2 = CH_2COOMe, mp 85—87 °C), **16f** (R^2 = CH(Me)COOMe, oil), **16g** (R^2 = $C(Me)_2COOMe$, oil), **16h** (R^2 = CH_2CH_2COOMe, oil), **16i** (R^2 = $CH_2CH_2CH_2COOMe$, oil) were prepared by a similar method as described above from the corresponding ketones.[6,7]

Method A. Ethyl 11-[2-(Dimethylamino)ethyl]thio-6,11-dihydrodibenz-[*b*,*e*]oxepin-2-carboxylate Hydrochloride (4) **16d** (R^2 = COOEt) (11.4 g, 40.1 mmol) was dissolved in dry CH_2Cl_2 (300 ml), to which was added $SOCl_2$ (4.0 ml, 55.1 mmol) dropwise at 0 °C and the mixture was stirred at room temperature for 1 h. The reaction mixture was concentrated to give crude **17** (R^2 = COOEt), which was used without purification in the next reaction. A mixture of **17** (R^2 = COOEt)(12.7 g, 39.9 mmol), 2-(dimethylamino)ethanethiol hydrochloride (90%, 9.5 g, 60.4 mmol), and DMF (200 ml) was stirred at 70 °C under nitrogen atmosphere for 5 h. The solvent was evaporated under reduced pressure and the residue was dissolved in H_2O (500 ml). The solution was acidified to pH 1.0 with 4 N HCl, washed twice with ether and subsequently adjusted to pH 10.0 with 10 N NaOH. The reaction mixture was extracted with ether. The extract was washed with brine, dried, and concentrated. The residue was chromatoraphed on silica gel (AcOEt–triethylamine, 10 : 1) to give 10.9 g (74%) of the free base of **4** as an oil. IR (neat): 2970, 2770, 1710,

1460, 1250, 1115 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.37 (t, J=7.0 Hz, 3H), 2.15 (s, 6H), 2.3—2.9 (m, 4H), 4.32 (q, J=7.0 Hz, 2H), 4.85 and 6.45 (AB, J_{AB}=13.0 Hz, 2H), 5.04 (s, 1H), 6.7— 8.0 (m, 7H). MS m/z: 371 (M^+). This free base (8.0 g, 24.0 mmol) was dissolved in isopropanol (200 ml). To the solution was added 7 N HCl in isopropanol (10 ml, 70 mmol) and the mixture was stirred at room temperature for 1 h. The resultant precipitate was collected and recrystallized from isopropanol to give 7.5 g (84%) of **4** crystals. IR (KBr): 2930, 2670, 1720, 1610, 1240, 1130 cm^{-1}. *Anal.* Calcd for $C_{21}H_{25}NO_3S \cdot HCl$: C, 61.83; H, 6.42; N, 3.43. Found: C, 61.59; H, 6.70; N, 3.70.

Compounds **1** (R^2=Me) and **2** (R^2=$CONH_2$) were prepared by a similar method as described above from **16a** (R^2=Me) and **16b** (R^2=$CONH_2$), respectively. **1**: *Anal.* Calcd for $C_{19}H_{23}NOS \cdot HCl$: C, 65.22; H, 6.91; N, 4.00. Found: C, 65.20; H, 6.84; N, 3.84. **2**: *Anal.* Calcd for $C_{19}H_{22}N_2O_2S$: C, 66.64; H, 6.48; N, 8.18. Found: C, 66.93; H, 6.25; N, 8.00.

Method B. Methyl 11-(2-Aminoethyl)thio-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylate (20a: R^1=H; Y=bond). A mixture of **16c** (R^2=COOMe)(190 g, 0.70 mol), conc-H_2SO_4 (15 ml), and MeOH (500 ml) was refluxed for 1 h. After being cooled, H_2O (500 ml) was added and the mixture was stirred at room temperature for 1 h. The resultant precipitate was collected, washed with H_2O, dried, and recrystallized from MeOH to give 180 g (91%) of **18** (Y= bond) as crystals, mp 112—113 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.27 (s, 3H), 3.82 (s, 3H), 4.84 and 6.08 (AB, J_{AB}= 12.1 Hz, 2H), 4.99 (s, 1H), 6.73—8.16 (m, 7H). To a mixture of **18** (Y= bond)(3.7 g, 13.0 mmol), 2-aminoethanethiol (1.2 g, 15.6 mmol) and dry CH_2Cl_2 (70 ml) was added $BF_3 \cdot Et_2O$ (3.9 ml, 31.7 mmol) and the mixture was stirred at room temperature for 3 h. The reaction mixture was washed successively with 1 N NaOH and brine, dried, and concentrated. The residue was chromatographed on silica gel (AcOEt–triethylamine, 20:1) to give 4.1 g (96%) of **20a** as an oil. IR (neat): 3370, 1710, 1240, 1115 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.30 (s, 2H), 2.23—2.97 (m, 4H), 3.79 (s, 3H), 4.79 and 6.32 (AB, J_{AB}=13 Hz, 2H), 4.93 (s, 1H), 6.72 (d, J= 8.5 Hz, 1H), 6.94—7.33 (m, 4H), 7.65 (dd, J=2.2, 8.5 Hz, 1H), 7.83 (d, J=2.2 Hz, 1H). *Anal.* Calcd for $C_{18}H_{19}NO_3S$: C, 65.63; H, 5.81; N, 4.25. Found: C, 65.51; H, 5.62; N, 4.43.

Compounds **20c** (R^1=iso-Pr; Y=bond, oil) and **20e** (R^1=Me; Y= –CH(Me)–, oil) were prepared by a similar method as described above from the corresponding alcohols **16c** and **16f**, respectively. **20c**: ^{1}H-NMR ($CDCl_3$) δ: 0.90 (d, J=6.5 Hz, 12H), 2.46 (br s, 4H), 2.50—3.16 (m, 2H), 3.80 (s, 3H), 4.85 and 6.48 (AB, J_{AB}=12.5 Hz, 2H), 4.99 (s, 1H), 6.7—8.0 (m, 7H). High resolution MS m/z: Calcd for $C_{24}H_{31}NO_3S$ 413.2025. Found 413.2036 (M^+). **20e**: ^{1}H-NMR ($CDCl_3$) δ: 1.44 (d, 3H), 2.12 (s, 6H), 2.25—2.73 (m, 4H), 3.58 (s, 3H), 3.41—3.80 (m, 1H), 4.77 and 6.26 (AB, J_{AB}=13.0 Hz, 2H), 4.92 (s, 1H), 6.66—7.31 (m, 7H). High resolution MS m/z: Calcd for $C_{22}H_{27}NO_3S$ 385.1712. Found 385.1722 (M^+).

Method C. Methyl 11-[2-(Dimethylamino)ethyl]thio-6,11-dihydrodibenz-[*b*,*e*]oxepin-2-acetate (20d: R^1=Me; Y=–CH_2–) To a solution of **16e** (R^2=CH_2COOMe) (40 g, 0.14 mol) in dry CH_2Cl_2 (300 ml) was added trifluoroacetic anhydride (22 ml, 0.16 mol) dropwise and the mixture was stirred at room temperature for 2 h. The mixture was treated with 2-(dimethylamino)ethanethiol hydrochloride (25 g, 0.18 mol) and $BF_3 \cdot Et_2O$ (3 ml, 0.02 mol) at room temperature for 3 h. The reaction mixture was washed successively with 1 N NaOH and brine, dried, and concentrated. The residue was chromatographed on silica gel (AcOEt–triethylamine, 40:1) to give 32 g (61%) of **20d** as an oil. IR (neat): 1730 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.16 (s, 6H), 2.30—2.76 (m, 4H), 3.50 (s, 2H), 3.63 (s, 3H), 4.77 and 6.25 (AB, J_{AB}=13 Hz, 2H), 4.92 (s, 1H), 6.75 (d, J=8.0 Hz, 1H), 6.90—7.45 (m, 6H). *Anal.* Calcd for $C_{21}H_{25}NO_3S$: C, 67.90; H, 6.78; N, 3.77. Found: C. 67.85; H, 6.88; N, 3.59.

Compounds **20b** (R^1=Et, Y=bond, oil), **20f** (R^1=Me, Y=–$C(Me)_2$–, oil), **20g** (R^1=Me, Y=–CH_2CH_2–, oil) and **22h** (R^1=Me, Y=–CH_2CH_2–CH_2–, oil) were prepared by a similar method as described above from the corresponding materials. **20b**: ^{1}H-NMR ($CDCl_3$) δ: 0.84—1.14 (m, 6H), 2.28—2.77 (m, 8H), 3.80 (s, 3H), 4.84 and 6.44 (AB, J_{AB}=13.0 Hz, 2H), 5.03 (s, 1H), 6.7—8.0 (m, 7H). MS m/z: 385 (M^+). **20f**: ^{1}H-NMR ($CDCl_3$) δ: 1.55 (s, 6H), 2.16 (s, 6H), 2.1—2.7 (m, 4H), 3.63 (s, 3H), 4.83 and 6.31 (AB, J_{AB}=13.0 Hz, 2H), 6.7—7.4 (m, 7H). High resolution MS m/z: Calcd for $C_{23}H_{29}NO_3S$ 399.1868. Found 399.1875 (M^+). **20g**: ^{1}H-NMR ($CDCl_3$) δ: 2.14 (s, 6H), 2.14—2.97 (m, 8H), 3.61 (s, 3H), 4.79 and 6.24 (AB, J_{AB}=13.0 Hz, 2H), 4.92 (s, 1H), 6.63—7.31 (m, 7H). High resolution MS m/z: Calcd for $C_{22}H_{27}NO_3S$ 385.1712. Found 385.1733 (M^+). **20h**: ^{1}H-NMR ($CDCl_3$) δ: 2.15 (s, 6H), 1.75—2.78 (m, 10H), 3.62 (s, 3H), 4.81 and 6.25 (AB, J_{AB}=12.5 Hz, 2H), 4.93 (s, 1H), 6.66—7.36 (m, 7H). MS m/z: 399 (M^+).

11-[2-(Dimethylamino)ethyl]thio-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylic Acid Hydrochloride (7) A mixture of the free base of **4** (57.3 g, 0.16 mol), 4 N NaOH (120 ml), and EtOH (800 ml) was refluxed for 2 h.The reaction mixute was concentrated and diluted with H_2O. The solution was acidified to pH 5.7 with 4 N HCl. After stirring for 1 h at room temperature, the resultant precipitate was filtered, washed with H_2O and dried to give 51.5 g (94%) of the crude free base of **7**. ^{1}H-NMR (DMSO-d_6) δ: 2.13 (s, 6H), 2.3—2.8 (m, 4H), 5.05 and 6.26 (AB, J_{AB}= 12.5 Hz, 2H), 5.43 (s, 1H), 6.87 (d, J=8.5 Hz, 1H), 7.36—7.46 (m, 4H), 7.71 (dd, J=2,2, 8.5 Hz, 1H), 7.97 (d, J=2.2 Hz, 1H). This was converted to the hydrochloride in a similar manner to that used for the preparation of **4** to give 53.0 g of **7** (91%), as crystals. IR (KBr): 3400, 2700, 1700, 1620, 1240, 1200 cm^{-1}. *Anal.* Calcd for $C_{19}H_{21}NO_3S \cdot HCl \cdot 0.5H_2O$: C, 58.68; H, 5.96; N, 3.60. Found: C, 58.45; H, 5.73; N, 3.52.

Compounds **6** and **8**—**14** were saponificated by a similar method as described above from the corresponding materials. **6**: *Anal.* Calcd for $C_{17}H_{17}NO_3S \cdot HCl$: C, 58.03; H, 5.16; N, 3.98. Found: C, 57.99; H, 5.29; N, 3.98. **8**: *Anal.* Calcd for $C_{21}H_{25}NO_3S \cdot HCl$: C, 61.83; H, 6.42; N, 3.43. Found: C, 61.54; H, 6.48; N, 3.66. **9**: *Anal.* Calcd for $C_{23}H_{29}NO_3S \cdot HCl$: C, 63.36; H, 6.94; N, 3.21. Found: C, 63.49; H, 7.28; N, 3.55. **10**: *Anal.* Calcd for $C_{20}H_{23}NO_3S \cdot HCl$: C, 60.98; H, 6.14; N, 3.56. Found: C, 61.32; H, 6.25; N, 3.73. **11**: *Anal.* Calcd for $C_{21}H_{25}NO_3S \cdot HCl \cdot 0.5H_2O$: C, 60.49; H, 6.53; N, 3.36. Found: C, 60.74; H, 6.51; N, 3.40. **12**: *Anal.* Calcd for $C_{22}H_{27}NO_3S \cdot H_2O$: C, 65.48; H, 7.24; N, 3.47. Found:C, 65.86; H, 7.61; N, 3.09. **13**: *Anal.* Calcd for $C_{21}H_{25}NO_3S \cdot HCl$: C, 61.83; H, 6.42; N, 3.43. Found: C, 62.02; H, 6.72; N, 3.37. **14**: *Anal.* Calcd for $C_{22}H_{27}NO_3S \cdot HCl \cdot 0.5H_2O$: C, 61.31; H, 6.78; N, 3.25. Found: C, 61.67; H, 6.94; N, 3.17.

11-[2-(Dimethylamino)ethyl]thio-6,11-dihydrodibenz[*b*,*e*]oxepin-2-hydroxamic Acid Hydrochloride (3) To a solution of hydroxylamine hydrochloride (1.67 g, 25.8 mmol) in H_2O (20 ml) was added a solution of NaOH (2.4 g, 60.2 mmol) in H_2O (10 ml) under nitrogen atmosphere. A solution of the free base of **4** (6.4 g, 17.2 mmol) in dioxane (10 ml) was added dropwise and the mixture was stirred at room temperature for 17 h. The reaction mixture was concentrated under reduced pressure and then diluted with H_2O. The solution was washed with ether and subsequently acidified to pH 5.5 with 4 N HCl. Insoluble material was removed by filtration and the filtrate was concentrated. The precipitated material was filtered, washed with H_2O and dried to give 2.02 g (33%) of the crude free base of **3**. IR (KBr): 3200, 1640, 1260, 1010 cm^{-1}. MS m/z: 358 (M^+). This crude product was converted to the hydrochloride and purified in a similar manner to that used for the preparation of **4** to give 1.5 g of **3** (67%). IR (KBr): 1640, 1260, 1010 cm^{-1}. *Anal.* Calcd for $C_{19}H_{22}N_2O_3S \cdot HCl$: C, 57.79; H, 5.87; N, 7.09. Found: C, 57.96; H, 5.71; N, 7.43.

11-[2-(Dimethylamino)ethyl]thio-2-hydroxymethyl-6,11-dihydrodibenz-[*b*,*e*]oxepin Fumarate (5) To a solution of the free base of **4** (10.9 g, 29.4 mmol) in tetrahydrofuran (THF 300 ml) was added $LiAlH_4$ (0.8 g, 21.1 mmol) portionwise at 0 °C. After the addition was completed, the mixture was stirred at room temperature for 3.5 h. The excess reagent was quenched with H_2O and the resultant inorganic salts were removed by filtration. The filtrate was evaporated to give 9.6 g of the crude free base of **5** as an oil. IR (neat): 3400, 1500, 1460, 1230 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.10 (s, 6H), 2.38—2.84 (m, 2H), 3.52—3.93 (m, 2H), 4.49 (s, 2H), 4.81 and 6.24 (AB, J_{AB}=13.5 Hz, 2H), 4.96 (s, 1H), 6.61—7.47 (m, 7H). MS m/z: 329 (M^+). The oil (1.5 g, *ca.* 4.5 mmol) and fumaric acid (0.6 g, 5.17 mmol) was dissolved in hot acetone (500 ml, 50 °C). After being cooled to room temperature, the resultant precipitate was filtered, washed with acetone, and dried to give 1.28 g (64%) of **5**. *Anal.* Calcd for $C_{19}H_{23}NO_2S \cdot C_4H_4O_4$: C, 62.00; H, 6.11; N, 3.14. Found: C, 62.02; H, 6.09; N, 2.88.

Biological Evaluation Procedures. Histamine-1 (H_1) Receptor Binding Assay H_1 binding assay was performed according to the previously reported method[10] with minor modification. The cerebellum of male Hartley guinea pig was homogenized in 40 volumes (w/v) of ice cold 50 mM sodium–potassium phosphate buffer, pH 7.5, (abbreviated as buffer) by a polytron homogenizer (Kinematica). The homogenate was centrifuged at 35500 × *g* for 10 min at 4 °C and the precipitate was homogenized again in the same volumes of buffer by the polytron homogenizer and centrifuged at 35000 × *g* for 10 min. The resulting precipitate was resuspended in 100 volumes of buffer by a teflon homogenizer. Tissue homogenates (10 mg wet weight), 3.8 nM of [^{3}H]pyrilamine and 0.1 μM of drugs in the total volume of 1.1 ml were added to a polypropylene tube

and incubated for 30 min at 25 °C. Ice cold buffer (4 ml) was added to a reaction tube and the reaction was stopped by rapid vacuum filtration (cell harvester Brandel M-24-R) through a Whatman GF/C glass fiber filter. The filter was washed 3 times with 5 ml of ice cold buffer. The filter was transferred to a scintillation vial, to which 0.5 ml of MeOH and 8 ml of scintisol EX-H (Wako Pure Chemicals) were added to determine radioactivity by a liquid scintillation counter (Packard 4530). Nonspecific binding was determined in the presence of 1 μM astemizole.

Muscarinic Acetylcholine (M_1) Receptor Binding Assay The binding assay was carried out as in the previously described method[11] with minor modification. The striatum of the rat was homogenized in 10 volumes of distilled water with a Potter-El-vehjem homogenizer. This homogenate preparation was diluted to 200 volumes of the wet tissue with 50 mM sodium–potassium phosphate buffer, pH 7.4. The homogenate (5 mg wet weight), 1.26 nM of [^{3}H]quinuclidinyl benzilate, and 1 μM of drugs in the total volume of 1.1 ml were incubated at 37 °C for 60 min. Nonspecific binding was determined by the addition of 1 μM unlabeled dexetimide. The assay was terminated by rapid filration under reduced pressure over a Whatman GF/B filter. The filters were washed three times with 5 ml of ice-cold 50 mM sodium–potassium phosphate buffer, pH 7.4 and the radioactivity was counted by a liquid scintillation counter.

Effects on 48 h Homologous Passive Cutaneous Anaphylaxis (PCA) in Rats Rat reaginic antibody (IgE) raised to ovalbumin (OA) was prepared by the method of Stotland and Share.[17] Briefly, Wistar strain male rats were immunized by giving a subcutaneous injection of 1 ml of a suspension containing 1 mg OA, 20 mg aluminum hydroxide gel and 10^{10} killed Bordetella pertussis organisms and then bled 14 d after this sensitization. The antiserum was separated and kept at −80 °C. Groups of 3 Wistar male rats were used and 0.05 ml of anti-OA rat serum, diluted 1 : 8 with 0.9% saline, was injected intradermally at two points on the dorsum. After 48 h, the PCA reaction was induced by intravenous administration of an aqueous solution containing 2 mg of OA and 5 mg of Evans blue. Test compounds were administered orally 1 h before injection of the antigen. After 30 min, the animals were anesthetized with ethyl ether and the dorsal skin was removed to determine the extravasated dye at each reaction site. The amount of dye was extracted by the method of Katayama[18] and was quantified by spectrometry. The percent of inhibition of the PCA reaction was then calculated.

Effects on Anaphylactic Bronchoconstriction in Passively Sensitized Guinea Pigs The experiment was performed according to the method of Konzett and Rössler.[14] Groups of 8 to 12 Hartley strain male guinea pigs were sensitized passively by giving intraperitoneally 1 ml of IgG-like guinea pig antiserum against OA. After 24 h, the animals were anesthetized with urethane (1.2 g/kg i.p.) and tracheotomised and ventilated by means of a respiratory pump (75 strokes/min, stroke volume 6 ml). After eliminating the spontaneous respiration by injection of gallamine triethiodide (10 mg/kg i.v.), initial airway resistance was kept constant at 8 cm H_2O pressure by means of a water valve. The airway was connected to a bronchospasm transducer (type 7020, Ugo Basile, Milan, Italy). The animals were challenged with OA (1 mg/kg i.v.) and anaphylactic bronchoconstriction was recorded as the % of maximal overflow obtained by clamping off the trachea. Test drugs were administered orally 1 h before the OA challenge. The preventive effect of the test drugs was expressed as present inhibition as compared to the increase of overflow volume determined at a 5 min later antigen challenge in control animals. ED_{50} value, *i.e.*, the dose required for 50% inhibition of the anaphylactic bronchoconstriction, was calculated from the relation between the logarithmic dose and the percent of inhibition by the method of least squares.

Effect on PGD_2-Induced Contraction of Isolated Guinea Pig Tracheal Preparations The zig-zag tracheal strip preparations from guinea pigs were made according to the method of Emmerson and Mackay.[19] The preparations were suspended in organ baths containing 10 ml of Krebs–Henseleit's solution with indomethacin (1×10^{-6} M) in order to antagonize the compensatory release of dilator PG product during tracheal contraction. The bath fluid was kept at 37 °C and gassed with 5% O_2 in CO_2. The contractile responses to PGD_2 were measured using an isotonic transducer (TD-112S, Nihon Kohden). PGD_2 was cumulatively added to the bath to obtain a concentration–response curve. Each test drug was added 10 min before the addition of PGD_2. The pA_2 value of the test drug was calculated by the method of Takayanagi.[20]

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A New Series of Antiallergic Agents. II.[1] Synthesis and Activity of New 6,11-Dihydrodibenz[*b*,*e*]-oxepin-carboxylic Acid Derivatives

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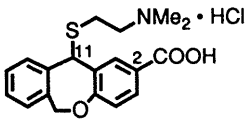
New methods for the preparation of multi-functionallized-6,11-dihydrodibenz[*b*,*e*]oxepins were developed. The structural requirements of KW-4994 (1), a promising orally active antiallergic agent, were defined. A carboxyl group at C-2 was critical for enhanced antiallergic activity of 1. The introduction of bromine atom at C-9 of 1 could elongate the duration of the action of the parent. Antiplatelet activity, a new pharmacological property of this series of compounds, was observed in one of the derivatives of 1.

Keywords antiallergic agent; antiasthmatic agent; H_1-antihistaminic activity; receptor antagonist; anti-passive cutaneous anaphylaxis activity; structure–activity relationship; antiplatelet activity; 6,11-dihydrodibenz[*b*,*e*]oxepin

Effective and orally active antiallergic agents with fewer side effects have been attractive targets for drug research in recent years.[2] In the preceding paper,[1] we reported the synthesis and activity of a new series of 11-substituted-dibenz[*b*,*e*]oxepin derivatives, including KW-4994 (**1**), one of our promising candidates for new antiallergic agents. From the structural point of view,[3] **1** represents a new class of antiallergic agent with both amino and carboxyl moieties in one molecule.



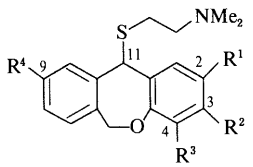
KW-4994 (1)

Fig. 1

In this paper we extended the scope of this novel series of antiallergic agents. The influence of the position of the carboxyl group was examined. Moreover, an additional substituent was introduced in the 4- or 9-position of **1** in order to obtain the compounds with more potent and/or longer duration of action.

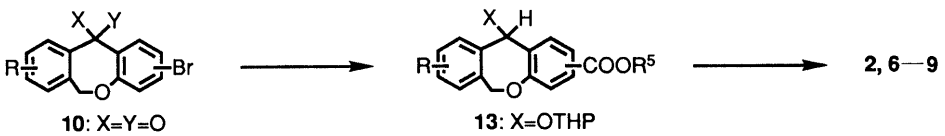
Chemistry Compounds listed in Table I were prepared from appropriate 11-alcohols and 2-(dimethylamino)-ethanethiol by the methods described in the preceding paper.[1] The 11-alcohols possessing a carboxyl group in the varied position of the oxepin ring system were appropriately prepared by the method as outlined in Chart 1. The ketones **10** were reduced with $NaBH_4$ and the resulting alcohols **11** were treated with dihydropyran/

TABLE I. Substituted 6,11-Dihydrodibenz[*b*,*e*]oxepin Derivatives



Compd.[a] No.	R^1	R^2	R^3	R^4	mp (°C)	Recrystn.[b] solvent	H_1 % inhibn. 0.1 μM	M_1 % inhibn. 1 μM	PCA % inhibn. (mg/kg *p.o.*) 100	10
1 (KW-4994)	COOH	H	H	H			59	7	95	87
2	COOH	H	Me	H	243—244 (dec.)	IP	20	2	99	47
3	COOH	H	H	Br	251—253 (dec.)	IP	83	2	70	62
4	COOH	H	H	SMe	230—232 (dec.)	IP	56	−1	N.T.	62
5	COOH	H	H	iso-Pr	215—217	IP	28	0	N.T.	63
6	H	COOH	H	H	95—110 (dec.)	TL	10	−1	16	N.T.
7	H	H	COOH	H	135 (dec.)	IP	0	−2	24	N.T.
8	Me	H	COOH	H	90 (dec.)	IP	−12	−4	28	N.T.
9	H	H	H	COOH	250—251 (dec.)	IP	24	5	38	N.T.

a) All compounds were obtained as HCl salts. *b*) IP, isopropanol; TL, toluene. N.T., not tested.



10: X=Y=O
11: X=OH, Y=H
12: X=OTHP, Y=H

13: X=OTHP
14: X=OH

Chart 1

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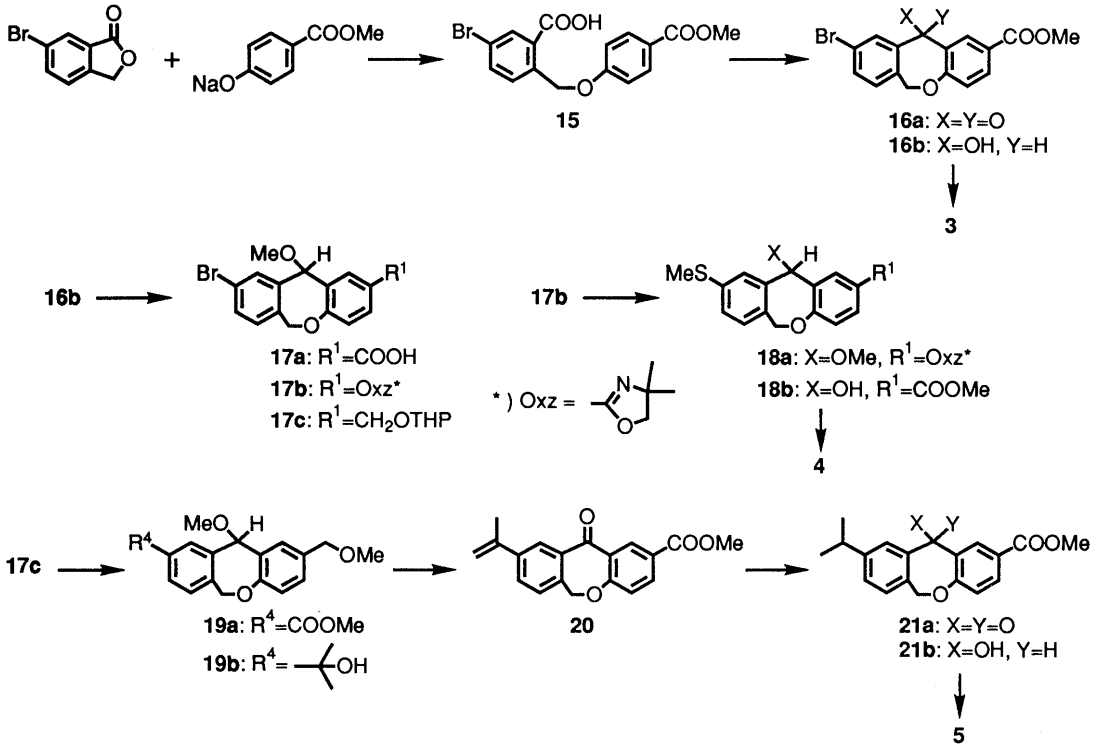

Chart 2

pyridinium *p*-toluenesulfonate (PPTS) to afford the tetrahydropyranyl (THP) ethers **12**. The bromides **12** were treated with 1) *n*-BuLi, 2) $(EtO)_2CO$ [method A] or 1) *n*-BuLi, 2) CO_2, 3) Me_2SO_4 [method B] to provide the esters **13**: Acid treatment of **13** afforded **14**, from which **2** and **6**—**9** were prepared.

The synthesis of **3**—**5** which were the analogues of **1** possessing an additional substituent at C-9 was outlined in Chart 2. For the preparation of the starting ketone **16a**, our newly developed procedure[4] based on the intramolecular Friedel–Crafts acylation of **15** turned out to be quite superior to the previously reported methods.[5,6] Merck and Daiich groups reported the synthetic methods for the preparation of 11-oxo-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylic acid, independently. The former method which involved the cyanation of bromide could not be applied for the synthesis of 9-bromo-2-carboxylic acid derivatives. The latter was unsatisfactory in yield. The dicarboxylic acid monoester **15** was treated with equimolecular of trifluoroacetic anhydride and subsequently with a catalytic amount of $BF_3 \cdot Et_2O$ to afford **16a** under very mild conditions.

$NaBH_4$ reduction of **16a** provided the alcohol **16b**, from which **3** was prepared. In addition, **17b** was prepared by the conversion of the hydroxyl and methoxycarbonyl groups of **16b** to methoxy and 4,4-dimethyl-oxazolin-2-yl groups, respectively. The resulting **17b** was treated with *n*-BuLi and then Me_2S to afford the sulfide **18a**. Simultaneous acidic cleavage of the oxazoline and ether groups of **18a** and the subsequent ester exchange reaction provided **18b**, which was converted to **4**. The alcohol **21b**, the precursor of **5**, was obtained by the reduction of the ketone **21a** prepared *via* 9-methoxycarbonyl derivative **19a**. Compound **17a** was reduced with $LiAlH_4$ and the resulting alcohol was protected to **17c**, which was converted to **19a** in a similar manner described in Chart 1. Compound **19b** prepared by the treatment of **19a** with MeMgBr was oxidized and dehydrated to **20**, which was hydrogenated to the final ketone **21a**.

Results and Discussion

The compounds synthesized were tested for their inhibitory effects on the specific binding of [^{3}H]pyrilamine to guinea pig cerebellum histamine-H_1 receptors (H_1),[7] the specific [^{3}H]quinuclidinyl benzilate binding to rat striatum muscarinic acetylcholine receptors (M_1),[8] and 48 h homologous passive cutaneous anaphylaxis (PCA) in rats. The results are summarized and represented by percent inhibition in Table I. Any of the compound synthesized showed negligible M_1 receptor binding affinity which was one of the indices of side effects such as suppression of salivary secretion and mydriasis.[9]

We examined the influence of the position of the carboxyl group on the activity. In the PCA test, 2-carboxylic acid (**1**) was the most potent compound and 9-carboxylic acid (**9**) was less potent than **1**. Compounds with a carboxyl group at C-3 or C-4 (**6**—**8**) were devoid of an inhibitory effect in this test. This tendency was also observed in the H_1 receptor binding assay. Based on the observed difference in potency between 2-COOH and 9-COOH, we presumed that the oxygen in the oxepin ring might play a crucial role for their antiallergic activity.

We next examined the influence of the introduction of an additional substituent at C-4 or C-9 of **1**. Such kind of modification had succeeded in improvement of the antiallergic activities of 5-oxo-5*H*-[1]benzopyrano[2,3-*b*]-pyridines[10] and pyrido[2,1-*b*]quinazolinecarboxylic acids.[11] Contrary to our expectation, compounds **2**—**5** failed to enhance the antiallergic activity of **1**. Comparative oral PCA duration studies for compounds **1**—**5** were performed in rats. All compounds were administered orally

at 1 mg/kg. Each inhibition of the PCA response was measured at various times following drug administration. Compound **3** exhibited significant inhibitory effect at 12 h after the dosing and its duration of action was longer than that of **1** (6—9 h). Therefore, the introduction of a bromine atom at C-9 as an additional substituent to **1** resulted in elongation of the action. The long duration of action is beneficial if an agent is to be useful in the clinical prophylaxis of allergic diseases, and the details of the effect of the additional substituent on the improved pharmacokinetics of **3** is now under investigation.

Of all the synthesized compounds in this study including **1**, only **8** was found to possess a potent antiplatelet activity in our screening experiments. It significantly inhibited a collagen-induced aggregation of rabbit platelet rich plasma at 3 μg/ml, whereas it showed negligible antiallergic and antihistaminic activities as described above.

In conclusion, we developed new procedures for the preparation of a new series of multi-functionallized-6,11-dihydrodibenz[*b*,*e*]oxepins and defined the structural requirements of KW-4994 (**1**). A carboxyl group at C-2 was critical for the enhanced antiallergic activity of **1**. Some of the compounds synthesized showed potent antiallergic activities, of which compound **3** exhibited a longer duration of action than **1**. Additionally, compound **8** is a new lead as an antiplatelet agent. Further modifications of these compounds are now in progress in our research laboratories.

Experimental

Synthetic Procedures Melting points were determined with a Büchi-510 melting point apparatus and are uncorrected. Infrared spectra (IR) were recorded on a Shimadzu IR-400 spectrometer. Proton nuclear magnetic resonance spectra (^{1}H-NMR) were recorded on a JEOL PMX-60 (60 MHz), a Hitachi R-90H (90 MHz), or a JEOL GX-270 (270 MHz) spectrometer with Me_4Si as internal standard. Mass spectra (MS) were recorded on a JEOL D300 mass spectrometer. Elemental analyses were performed by the analytical department of our laboratories.

Methyl 9-Bromo-11-oxo-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylate (16a) To a solution of methyl 4-hydroxybenzoate (11.9 g, 78.2 mmol) was added 28% MeONa in MeOH (5 ml, 78.2 mmol) and the solution was stirred. After being evaporated to dryness, 6-bromophtalide (20 g, 93.9 mmol) was added. The mixture was heated at 120 °C for 8 h. Acetic acid (5 ml, 78.2 mmol) and MeOH (30 ml) were added and stirring was continued at 50 °C for 1 h. The reaction mixture was diluted with water and adjusted to pH 5.6 with 2 N HCl. The resultant precipitate was collected and recrystallized from isopropanol to give 12.1 g (42%) of **15** as crystals, mp 184—187 °C. ^{1}H-NMR (DMSO-d_6) δ: 3.88 (s, 3H), 5.49 (s, 2H), 6.8—8.2 (m, 7H). IR (KBr): 3400, 1700 cm^{-1}. To a suspension of **15** (12.0 g, 32.9 mmol) in CH_2Cl_2 (300 ml) was added trifluoroacetic anhydride (4.7 ml, 32.9 mmol) under Ar atmosphere and the mixture was stirred at room temperature for 1 h. $BF_3 \cdot Et_2O$ (1 ml, 8.1 mmol) was added and the stirring was continued for a further 2 h under the same conditions. The reaction mixture was poured into ice-water. The organic layer was separated, washed successively with aqueous $NaHCO_3$ and brine, dried and concentrated. The residue was recrystallized from AcOEt to give 8.86 g (78%) of **16a** as crystals, mp 199—200 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.93 (s, 3H), 5.19 (s, 2H), 7.09 (d, J=8.6 Hz, 1H), 7.27 (d, J=8.0 Hz, 1H), 7.70 (dd, J=2.0, 8.0 Hz, 1H), 8.01 (d, J=2.0 Hz, 1H), 8.14 (dd, J=2.2, 8.6 Hz, 1H), 8.90 (d, J=2.2 Hz, 1H). *Anal.* Calcd for $C_{16}H_{11}BrO_4$: C, 55.36; H, 3.19. Found: C, 55.55; H, 3.19.

Methyl 9-Bromo-11-hydroxy-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylate (16b) To a solution of **16a** (11.90 g, 34 mmol) in MeOH (150 ml) was added $NaBH_4$ (0.79 g, 21 mmol) and the solution was stirred at room temperature for 3 h. After concentration, water was added and the mixture was extracted with AcOEt. The extract was washed with brine, dried, and concentrated to give **16b** (11.9 g, 100%), as a solid, mp 132—134 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.81 (s, 4H), 4.96 and 5.76 (AB, J_{AB}=14.1 Hz, 2H), 5.70 (s, 1H), 6.80 (d, J=8.5 Hz, 1H), 7.02—7.46 (m, 3H), 7.79 (dd, J=2.2, 8.5 Hz, 1H), 8.02 (d, J=2.2 Hz, 1H).

4-Bromo-11-tetrahydropyranyloxy-6,11-dihydrodibenz[*b*,*e*]oxepin (12a) The ketone **10a** (4-Br, R=H, mp 129—132 °C) was prepared from phtalide and 2-bromophenol by the same method as described in the preparation of **16a** (75%). The ketone **10a** was reduced to **11a** (4-Br, R=H), mp 94—96 °C) by the same method as described in the preparation of **16b**. The alcohol **11a** (17 g, 0.059 mmol) was treated with 2,3-dihydropyran (49.3 g, 0.59 mol) and pyridinium *p*-toluenesulfonate (PPTS) (1.47 g, 5.9 mol) in CH_2Cl_2 at room temperature for 2.5 h. The reaction mixture was washed with aqueous $NaHCO_3$, dried, and concentrated. The residue was chromatographed on silica gel (hexane–AcOEt, 5:1) to give 21.3 g (97%) of **12a** as an oil. ^{1}H-NMR ($CDCl_3$) δ:1.59 (br s, 6H), 3.24—4.13 (m, 2H), 4.53 and 4.73 (each br s, total 1H), 5.00 and 5.94 (AB, J_{AB}=12.0 Hz, 1H), 5.04 and 5.95 (AB, J_{AB}=12.5 Hz, 1H), 5.55 and 5.63 (each br s, total 1H), 6.66 (t, J=7.5 Hz, 1H), 7.03—7.54 (m, 6H). MS *m/z*: 376 and 374 (M^+).

Compounds **12b** (3-Br, R=H, oil), **12c** (4-Br, R=2-Me, mp 114—116 °C), **12d** (9-Br, R=H, oil), and **12e** (2-Br, R=4-Me, oil) were prepared by the same method as described above.

Ethyl 11-Hydroxy-6,11-dihydrodibenz[*b*,*e*]oxepin-4-carboxylate (14a) [Method A] To a solution of **12a** (41.5 g, 0.11 mol) in tetrahydrofuran (THF 330 ml) was added dropwise a 1.5 M solution of *n*-BuLi in hexane (73.3 ml, 0.11 mol) at −78 °C under Ar atmosphere. After being stirred for 10 min, the solution was added dropwise to a solution of diethyl carbonate (133 ml, 1.1 mol) in THF (220 ml) at −78 °C. The mixture was gradually warmed to room temperature with stirring. After an addition of H_2O (500 ml), the mixture was extracted with AcOEt. The extract was washed with H_2O, dried, and concentrated. The residue containing crude **13a** (4-COOEt, R=H) and *p*-TsOH·H_2O (2.1 g, 11 mmol) was dissolved in the mixture of 1,4-dioxane (220 ml) and H_2O (80 ml) and the mixture was stirred at 60 °C for 2.5 h. The mixture was extracted with AcOEt and the extract was washed successively with aqueous $NaHCO_3$ and brine. The organic layer was dried and concentrated. The residue was chromatographed on silica gel (toluene–AcOEt, 5:1) to give **14a** (4-COOEt, R=H, 10.0 g, 32%) as crystals, mp 147—148.5 °C (toluene). IR (KBr): 3350, 1660, 1595, 1440, 1220 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.36 (t, J=7.0 Hz, 3H), 3.53 (d, J=6.0 Hz, 1H), 4.32 (q, J=7.0 Hz, 2H), 5.21 and 5.58 (AB, J_{AB}=14.5 Hz, 2H), 5.73 (d, J=6.0 Hz, 1H), 6.78—7.75 (m, 5H). *Anal.* Calcd for $C_{17}H_{16}O_4$: C, 71.82; H, 5.67. Found: C, 71.78; H, 5.88.

Compounds **14c** (4-COOEt, R=H, oil) and **14e** (2-COOEt, R=4-Me, mp 159.5—160 °C) were prepared by the same method as described above from **12c** and **12e**, respectively.

Methyl 11-Hydroxy-6,11-dihydrodibenz[*b*,*e*]oxepin-9-carboxylate (14d) [Method B] To a solution of **12d** (20.8 g, 55 mmol) in THF (180 ml) was added dropwise a 1.5 M solution of *n*-BuLi in hexane (36.8 ml, 55 mmol) at −78 °C under Ar atmosphere. After being stirred for 10 min, the mixture was poured onto dry ice (50 g) and stirred at room temperature for 2 h. Then dimethyl sulphate (5.8 ml, 60.5 mmol) was added and the mixture was refluxed for 1 h. After addition of 1 N NaOH (20 ml) and H_2O (300 ml), the mixture was extracted with AcOEt. The extract was washed with brine, dried, and concentrated. The residue obtained and *p*-TsOH·H_2O (2.6 g, 13.7 mmol) were dissolved in a mixture of 1,4-dioxane (300 ml) and H_2O (150 ml) and the solution was stirred at 60 °C for 4 h. The mixture was extracted with AcOEt and the extract was washed successively with aqueous $NaHCO_3$ and brine. The organic layer was dried and concentrated. The residue was chromatographed on silica gel (toluene–AcOEt, 10:1 and then 2:1) to give **14d** (6.0 g, 49%) as a viscous oil. IR (neat): 3380, 1720, 1605, 1485, 1435, 1280, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.57 (s, 3H), 4.95 and 5.73 (AB, J_{AB}=13.0 Hz, 2H), 5.63 (s, 1H), 6.64—8.04 (m, 7H). High resolution MS *m/z*: Calcd for $C_{16}H_{14}O_4$ 270.0892. Found: 270.0888 (M^+).

Compound **14b** (3-COOMe, R=H, mp 101—103 °C) was prepared by the same method as described above from **12b**.

9-Bromo-11-methoxy-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylic Acid (17a) A mixture of **16b** (11.5 g, 33 mmol) and *p*-TsOH·H_2O (0.3 g, 1.6 mmol) in MeOH (165 ml) was refluxed for 1 h. NaOH (1 N, 50 ml) was added and the mixture was refluxed for 2 h. After concentration, the residue was dissolved in H_2O and acidified with 4 N HCl. The resultant precipitate was collected by filtration, washed with H_2O, and dried to give **17a** (11.3 g, 98%) as a solid, mp 249—250 °C. ^{1}H-NMR (DMSO-d_6) δ: 3.32 (s, 3H), 5.06 and 5.95 (AB, J_{AB}=12.6 Hz, 2H), 5.30 (s, 1H), 6.83—8.25 (m, 6H). MS *m/z*: 348 and 350 (M^+). *Anal.* Calcd for $C_{16}H_{13}BrO_4$: C, 55.04; H, 3.75. Found: C, 55.00; H, 3.85.

9-Bromo-2-(4,4-dimethyl-2-oxazolin-2-yl)-11-methoxy-6,11-dihydrodibenz[*b*,*e*]oxepin (17b) To a solution of **17a** (10.0 g, 28.6 mmol) and

pyridine (4.7 ml, 57.2 mmol) in CH_2Cl_2 (100 ml) was added a solution of $SOCl_2$ (2.7 ml, 37.2 mmol) in CH_2Cl_2 (20 ml) at 0 °C and the mixture was stirred at the same temperature for 30 min and then at room temperature for 2 h. After concentration, the residue was dissolved in CH_2Cl_2 (20 ml) and the solution was added to a solution of 2-amino-2-methyl-1-propanol (17.8 g, 200 mmol) in CH_2Cl_2 (150 ml) at 0 °C. The mixture was stirred at the same temperature for 1 h and then at room temperature overnight. The mixture was diluted with CH_2Cl_2. The organic solution was washed successively with aqueous $NaHCO_3$ and brine, dried, and concentrated to give a crude *N*-(1,1-dimethyl-2-hydroxy)ethyl-9-bromo-11-methoxy-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxamide (9.93 g) as an oil. This crude amide and pyridine (5 ml) were dissolved in CH_2Cl_2 (120 ml) and a solution of $SOCl_2$ (1.7 ml, 23.6 mmol) in CH_2Cl_2 (10 ml) was added at 0 °C. The mixture was refluxed for 6 h. Upon cooling, the mixture was washed with water, dried, and concentrated. The residue was chromatographed on silica gel (hexane–AcOEt, 2 : 1) to give **17b** (6.25 g, 54% from **17a**) as an amorphous solid. IR ($CHCl_3$): 2950, 1640, 1480, 1070 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.32 (s, 6H), 3.26 (s, 3H), 3.99 (s, 2H), 4.78 and 5.91 (AB, J_{AB}=11.7 Hz, 2H), 4.91 (s, 1H), 6.76 (d, J=8.5 Hz, 1H), 6.97—7.84 (m, 5H). *Anal.* Calcd for $C_{20}H_{20}BrNO_3$: C, 59.71; H, 5.01; N, 3.48. Found: C, 59.60; H, 5.12; N, 3.30.

9-Methylthio-2-(4,4-dimethyl-2-oxazolin-2-yl)-11-methoxy-6,11-dihydrodibenz[*b*,*e*]oxepin (18a) To a solution of **17b** (5.70 g, 14 mmol) in THF (56 ml) was added *n*-BuLi (1.5 M in hexane, 9.3 ml, 14 mmol) at −78 °C. After being stirred at the same temperature for 5 min, methyldisulfide (1.35 ml, 15 mmol) was added. The mixture was gradually warmed to room temperature over 4 h with stirring. After the addition of water, the mixture was extracted with AcOEt. The extract was washed with brine, dried, and concentrated. The residue was chromatographed on silica gel (hexane–AcOEt, 2 : 1) to give **18a** (2.29 g, 44%) as a viscous oil. IR (neat): 2960, 1735, 1645, 1490, 1240 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.31 (s, 6H), 2.38 (s, 3H), 3.24 (s, 3H), 3.98 (s, 2H), 4.75 and 5.96 (AB, J_{AB}=12.1 Hz, 2H), 4.91 (s, 1H), 6.75 (d, J=8.5 Hz, 1H), 7.04—7.27 (m, 3H), 7.66 (dd, J=2.2, 8.5 Hz, 1H), 7.84 (d, J=2.2 Hz, 1H). *Anal.* Calcd for $C_{21}H_{23}NO_3S$: C, 68.27; H, 6.27; N, 3.79. Found: C, 68.00; H, 6.51; N, 3.55.

Methyl 11-Hydroxy-9-methylmercapto-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylate (18b) A mixture of **18a** (2.0 g, 54 mmol), conc. HCl (0.7 ml), H_2O (6 ml), and 1,4-dioxane (18 ml) was heated at 90 °C for 2 h. Upon cooling, the mixture was extracted with AcOEt. The extract was washed successively with aqueous $NaHCO_3$ and brine, dried and concentrated. The residue was dissolved in MeOH (20 ml) and 28% MeONa in MeOH (0.2 ml) was added. After being refluxed for 3 h, the mixture was diluted with H_2O and extracted with AcOEt. The extract was washed with brine, dried, and concentrated. The residue was chromatographed (flash chromatography) on silica gel (hexane–AcOEt, 5 : 1 and then 2 : 1) to give **18b** (0.60 g, 35%) as an amorphous solid. IR (neat): 3400, 1710, 1605, 1250 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.43 (s, 3H), 3.83 (s, 3H), 4.96 and 5.87 (AB, J_{AB}=12.3 Hz, 2H), 5.64 (s, 1H), 6.79 (d, J=8.5 Hz, 1H), 7.10—7.30 (m, 3H), 7.77 (dd, J=2.2, 8.5 Hz, 1H), 8.01 (d, J=2.2 Hz, 1H). *Anal.* Calcd for $C_{17}H_{16}O_4S$: C, 64.54; H, 5.10. Found: C, 64.20; H, 5.33.

Methyl 11-Methoxy-2-methoxymethyl-6,11-dihydrodibenz[*b*,*e*]oxepin-9-carboxylate (19a) To a solution of **17a** (11.16 g, 30.7 mmol) in ether (120 ml) was added $LiAlH_4$ (1.17 g, 30.7 mmol) portionwise at 0 °C. After being stirred for 1 h, water (3 ml) was added dropwise at 0 °C. The resultant inorganic salts were filtered off and the filtrate was concentrated. A mixture of the residue obtained, dihydropyran (3.2 ml, 35 mmol), and PPTS (0.73 g, 2.9 mmol) in CH_2Cl_2 (150 ml) was stirred at room temperature for 3 h. The mixture was washed successively with aqueous $NaHCO_3$ and water, dried, and concentrated. The residue was chromatographed on silica gel (hexane–AcOEt, 4 : 1) to give **17c** (7.23 g, 59%) as a viscous oil. ^{1}H-NMR ($CDCl_3$) δ: 3.21 (s, 3H), 4.43 (s, 2H), 4.76 and 5.80 (AB, J_{AB}=12.5 Hz, 2H), 4.90 (s, 1H), 6.70—7.41 (m, 6H). To a solution of **17c** (4.0 g, 9.5 mmol) in THF (47.5 ml) was added dropwise a solution of 1.5 M *n*-BuLi in hexane (6.7 ml, 10.0 mmol) at −78 °C. After being stirred for 5 min at the same temperature, the mixture was poured onto dry ice (50 g) and warmed gradually to room temperature with stirring. Dimethyl sulfate (0.98 ml, 10.5 mmol) was added and the mixture was refluxed for 2.5 h. Upon cooling, the mixture was diluted with AcOEt. The organic solution was washed successively with aqueous $NaHCO_3$ and brine, dried, and concentrated. The residue and catalytic amount of *p*-TsOH·H_2O was dissolved in MeOH and the mixture was refluxed for 2 h. After concentration, the residue was poured into aqueous $NaHCO_3$ and extracted with AcOEt. The extract was washed with brine, dried, and concentrated. The residue was chromatographed on silica gel (hexane–AcOEt, 5 : 1) to give **19a** (1.92 g, 61%) as crystals, mp 93.5—95 °C. IR ($CHCl_3$): 1725, 1500, 1445, 1290 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.31 (s, 6H), 3.88 (s, 3H), 4.35 (s, 2H), 4.88 and 5.97 (AB, J_{AB}=12.3 Hz, 2H), 5.07 (s, 1H), 6.73—7.38 (m, 4H), 7.87—8.07 (m, 2H). *Anal.* Calcd for $C_{19}H_{20}O_5$: C, 69.50; H, 6.14. Found: C, 69.29; H, 6.40.

Methyl 11-Oxo-9-isopropenyl-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylate (20) To a solution of **19a** (1.57 g, 4.8 mmol) in THF (20 ml) was added dropwise MeMgBr (1 M in THF, 24.5 ml, 24.5 mmol) at 0 °C. The mixture was stirred at the same temperature for 2 h and then saturated NH_4Cl was added. The mixture was diluted with AcOEt. The organic solution was washed with brine, dried, and concentrated to give crude **19b** (1.54 g, 98%) as a viscous oil. ^{1}H-NMR ($CDCl_3$) δ: 1.48 (s, 6H), 3.27 (s, 6H), 4.31 (s, 2H), 4.77 and 5.93 (AB, J_{AB}=12.2 Hz, 2H), 4.94 (s, 1H), 6.67—7.43 (m, 6H). To a solution of the crude **19b** (1.52 g, 4.6 mmol) in absolute MeOH (20 ml) was added portionwise dried ceric ammonium nitrate (CAN) (5.07 g, 9.3 mmol) at room temperature and the mixture was stirred for 2 h. After the addition of H_2O (50 ml), the mixture was extracted with AcOEt. The extract was washed with brine, dried, and concentrated. The residue was chromatographed (flash chromatography) on silica gel (hexane–AcOEt, 2 : 1) to give 9-(1-hydroxy-1-methyl)ethyl-11-methoxy-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carbaldehyde (0.81 g, 56%) as an amorphous solid. IR ($CHCl_3$): 3500, 2960, 1680, 1600, 1565, 1490 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.51 (s, 6H), 3.30 (s, 3H), 4.88 and 6.05 (AB, J_{AB}=12.1 Hz, 2H), 5.02 (s, 1H), 6.86 (d, J=8.5 Hz, 1H), 7.12—7.82 (m, 5H), 9.76 (s, 1H). *Anal.* Calcd for $C_{19}H_{20}O_4$: C, 73.06; H, 6.45. Found: C, 72.98; H, 6.61. To a solution of the aldehyde (0.80 g, 2.6 mmol) in acetone (10 ml) was added Jones reagent at 0 °C. After the addition of isopropanol to destroy the excess Jones reagent, the resultant insoluble solid was filtered off and the filtrate was concentrated. The residue was dissolved in AcOEt and the organic solution was washed with H_2O, dried, and concentrated. The obtained crude crystals were triturated with diisopropylether to afford 9-(1-hydroxy-1-methyl)ethyl-11-oxo-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylic acid (0.64 g, 79%), mp 186.5—188 °C. *Anal.* Calcd for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16. Found: C, 69.10; H, 5.00. The mixture of the carboxylic acid (0.14 g, 0.45 mmol) and 2 drops of conc. H_2SO_4 in MeOH (4 ml) was refluxed for 5 h. Toluene (10 ml) was added and the mixture was heated to evaporate MeOH. After evaporation of MeOH was completed, the mixture was refluxed for 1 h. Upon cooling, the mixture was diluted with AcOEt. The organic solution was washed with brine, dried, and concentrated to give **20** (0.14 g, 100%) as a viscous oil. IR (neat): 1720, 1640, 1250 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.15 (s, 3H), 3.88 (s, 3H), 5.14 (br s, 1H), 5.17 (br s, 1H), 5.42 (br s, 1H), 7.03 (d, J=8.5 Hz, 1H), 6.95—7.33 (m, 2H), 7.61 (dd, J=2.2, 8.5 Hz, 1H), 7.90 (d, J=2.2 Hz, 1H), 8.06 (dd, J=2.4, 8.9 Hz, 1H), 8.78 (d, J=2.4 Hz, 1H). High resolution MS *m*/*z*: Calcd for $C_{19}H_{16}O_4$ 308.1049. Found: 308.1051 (M^+).

Methyl 11-Hydroxy-9-isopropyl-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylate (21b) A suspension of **20** (0.14 g, 0.45 mmol) and 10% Pd–C (14 mg) in EtOH (3 ml) was stirred under H_2 atmosphere for 2 h. The catalyst was filtered off and the filtrate was concentrated to give **21a** (0.14 g, 100%) as a viscous oil. IR ($CHCl_3$): 2960, 1710, 1610, 1255 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.25 (d, J=7.0 Hz, 6H), 2.93 (q, J=7.0 Hz, 1H), 3.89 (s, 3H), 5.18 (s, 2H), 7.04 (d, J=8.5 Hz, 1H), 7.27—7.43 (m, 2H), 7.73 (d, J=1.8 Hz, 1H), 8.09 (dd, J=2.2, 8.5 Hz, 1H), 8.92 (d, J=2.2 Hz, 1H). To a solution of **21a** (0.14 g, 0.45 mmol) in MeOH (3 ml) was added portionwise $NaBH_4$ (10 mg, 0.27 mmol) at room temperature. The mixture was stirred for 5 h and then diluted with AcOEt. The organic solution was washed with H_2O, dried, and concentrated to give **21b** (0.13 g, 93%) as an amorphous solid. IR ($CHCl_3$): 3400, 2950, 1705, 1615, 1240 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.22 (d, J=7.0 Hz, 6H), 2.85 (q, J=7.0 Hz, 1H), 3.83 (s, 3H), 4.96 and 5.99 (AB, J_{AB}=12.3 Hz, 2H), 5.66 (s, 1H), 6.83 (d, J=8.5 Hz, 1H), 7.18 (br s, 3H), 7.80 (dd, J=2.2, 8.5 Hz, 1H), 8.04 (d, J=2.2 Hz, 1H). *Anal.* Calcd for $C_{19}H_{20}O_4$: C, 73.06; H, 6.45. Found: C, 73.12; H, 6.66.

11-[2-(Dimethylamino)ethyl]thio-6,11-dihydrodibenz[*b*,*e*]oxepin-3-carboxylate Hydrochloride (6) 14b (3-COOMe, R=H, 6.8 g, 25 mmol) was dissolved in dry CH_2Cl_2 (100 ml), to which was added $SOCl_2$ (3.6 ml, 50 mmol) dropwise at 0 °C and the mixture was stirred at room temperature for 1 h. The reaction mixture was evaporated to dryness. A mixture of the resulting residue, 2-(dimethylamino)ethanethiol hydrochloride (90%, 7.1 g, 45 mmol), and dimethylformamide (DMF 100 ml) was stirred at 120 °C under nitrogen atmosphere for 3 h. The solvent was evaporated under reduced pressure and the residue was dissolved in H_2O (100 ml). The solution was acidified to pH 1.0 with 4 N HCl, washed twice with ether and subsequently adjusted to pH 12.0 with 10 N NaOH. The reaction mixture was extracted with ether. The extract was washed with

brine, dried, and concentrated. The residue was chromatographed on silica gel (AcOEt–triethylamine, 10:1) to give 7.7 g (86%) of methyl 11-[2-(dimethylamino)ethyl]thio-6,11-dihydrodibenz[*b*,*e*]oxepin-3-carboxylate as an oil. IR (neat): 2940, 1720, 1435, 1415, 1030 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 2.11 (s, 6H), 2.3—2.8 (m, 4H), 3.76 (s, 3H), 4.78 and 6.21 (AB, J_{AB} = 12.5 Hz, 2H), 4.94 (s, 1H), 6.9—7.6 (m, 7H). MS *m/z*: 357 (M^+). A mixture of this ester obtained (2.0 g, 5.6 mol), 1 N NaOH (10 ml), and EtOH (80 ml) was refluxed for 2 h. The reaction mixture was concentrated and diluted with H_2O. The solution was acidified to pH 5.7 with 4 N HCl. After stirring for 1 h at room temperature, the resultant precipitate was filtered, washed with water and dried to give 1.7 g (89%) of the crude free base of **6**. This crude free base (1.5 g, 4.4 mmol) was dissolved in isopropanol (15 ml). To the solution was added 8.2 N HCl in isopropanol (0.8 ml, 6.6 mmol) and the mixture was stirred at room temperature for 1 h. The resultant precipitate was collected and recrystallized from toluene to give 1.3 g (78%) of **6** as crystals. *Anal.* Calcd for $C_{19}H_{21}NO_3S \cdot HCl \cdot 0.75H_2O$: C, 58.01; H, 6.02; N, 3.56. Found: C, 58.18; H, 6.40; N, 3.71.

Compounds **2**—**5** and **7**—**9** were prepared by the same method as described above from **14e**, **16b**, **18b**, **21b**, **14a**, **14c**, and **14d**, respectively. **2**: *Anal.* Calcd for $C_{20}H_{23}NO_3S \cdot HCl$: C, 60.98; H, 6.14; N, 3.56. Found: C, 60.79; H, 6.28; N, 3.35. **3**: *Anal.* Calcd for $C_{19}H_{20}BrNO_3S \cdot HCl$: C, 49.74; H, 4.61; N, 3.05. Found: C, 49.61; H, 4.51; N, 3.20. **4**: *Anal.* Calcd for $C_{20}H_{23}NO_3S_2 \cdot HCl$: C, 56.39; H, 5.68; N, 3.29. Found: C, 56.20; H, 5.77; N, 3.29. **5**: *Anal.* Calcd for $C_{22}H_{27}NO_3S \cdot HCl \cdot 0.5H_2O$: C, 61.31; H, 6.78; N, 3.25. Found: C, 61.35; H, 6.66; N, 3.20. **7**: *Anal.* Calcd for $C_{19}H_{21}NO_3S \cdot HCl \cdot 0.25H_2O$: C, 59.37; H, 5.90; N, 3.64. Found: C, 59.53; H, 6.19; N, 3.33. **8**: *Anal.* Calcd for $C_{20}H_{23}NO_3S \cdot HCl$: C, 60.98; H, 6.14; N, 3.56. Found: C, 61.00; H, 6.49; N, 3.26. **9**: *Anal.* Calcd for $C_{19}H_{21}NO_3S \cdot HCl \cdot 0.25H_2O$: C, 59.37; H, 5.90; N, 3.64. Found: C, 59.48; H, 5.82; N, 3.36.

Biological Evaluation Procedures. Histamine-1 (H_1) Receptor Binding Assay H_1 binding assay was performed according to the previously reported method[7] with minor modification. The cerebellum of male Hartley guinea pig was homogenized in 40 volumes (w/v) of ice cold 50 mM sodium–potassium phosphate buffer, pH 7.5, (abbreviated as buffer) by a polytron homogenizer (Kinematica). The homogenate was centrifuged at 35500 × *g* for 10 min at 4 °C and the precipitate was homogenized again in the same volumes of buffer by the polytron homogenizer and centrifuged at 35000 × *g* for 10 min. The resulting precipitate was resuspended in 100 volumes of buffer by a teflon homogenizer. Tissue homogenates (10 mg wet weight), 3.8 nM of [^3H]-pyrilamine and various concentrations of drugs in a total volume of 1.1 ml were added to a polypropylene tube and incubated for 30 min at 25 °C. Ice cold buffer (4 ml) was added to a reaction tube and reaction was stopped by rapid vacuum filtration (cell harvester Brandel M-24-R) through a Whatman GF/C glass fiber filter. The filter was washed 3 times with 5 ml of ice cold buffer. The filter was transferred to a scintillation vial, to which 0.5 ml of MeOH and 8 ml of scintisol EX-H (Wako Pure Chemicals) were added to determine radioactivity by a liquid scintillation counter (Packard 4530). Nonspecific binding was determined in the presence of 1 μM astemizole.

Muscarinic Acetylcholine (M_1) Receptor Binding Assay The binding assay was carried out as in the previously described method[8] with minor modification. The striatum of the rat was homogenized in 10 volumes of distilled water with a Potter-El-vehjem homogenizer. This homogenate preparation was diluted to 200 volumes of the wet tissue weight with 50 mM sodium-potassium phosphate buffer, pH 7.4. The homogenate (5 mg wet weight), 1.26 nM of [^3H]quinuclidinyl benzilate, and various concentrations of drugs in a total volume of 1.1 ml were incubated at 37 °C for 60 min. Nonspecific binding was determined by the addition of 1 μM unlabeled dexetimide. The assay was terminated by rapid filtration under reduced pressure over a Whatman GF/B filter. The filters were washed three times with 5 ml of ice-cold 50 mM sodium–potassium phosphate buffer, pH 7.4 and the radioactivity was counted by a liquid scintillation counter.

Effects on 48 h Homologous Passive Cutaneous Anaphylaxis (PCA) in Rats Rats reaginic antibody (IgE) raised to ovalbumin (OA) was prepared by the method of Stotland and Share.[12] Briefly, Wistar strain male rats were immunized by a subcutaneous injection of 1 ml of a suspension containing 1 mg OA, 20 mg aluminum hydroxide gel and 10^{10} killed Bordetella pertussis organisms and then bled 14 d after this sensitization. The antiserum was separated and kept at −80 °C. Groups of 3 Wistar male rats were used and 0.05 ml of anti-OA rat serum, diluted 1:8 with 0.9% saline, was injected intradermally at two points on the dorsum. After 48 h, the PCA reaction was induced by intravenous administration of an aqueous solution containing 2 mg of OA and 5 mg of Evans blue. Test compounds were administered orally 1 h before injection of the antigen. After 30 min, the animals were anesthetized with ethyl ether and the dorsal skin was removed to determine the extravasated dye at each reaction site. The amount of dye was extracted by the method of Katayama[13] and was quantified by spectrometry. The percent inhibition of the PCA reaction was then calculated.

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A Synthetic Method Suitable for the Rapid Preparation of ^{13}N-Labeled Dermorphin Analogue, H–Tyr–D-Met(O)–Phe–Gly–NH_2 (SD-62)[1)]

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A synthetic method suitable for the preparation of ^{13}N-labeled dermorphin analogue, H–Tyr–D-Met(O)–Phe–Gly–NH_2 (SD-62), was established; *i.e.*, SD-62 was synthesized by a 5 min treatment of the active ester precursor with ammonia. When the ^{13}N-labeled SD-62, prepared by this method with [^{13}N]ammonia, was administered into mice, the time profile of the radioactivity accumulation in the brain paralleled well that of the analgesic activity.

Keywords positron emission tomography; dermorphin analogue; peptide synthesis; ^{13}N ammonia; active ester ammonolysis; brain uptake; analgesic activity

Recently, the positron emission tomography (PET) technique has been applied to study of the *in vivo* behavior of endogenous opioid peptides.[2)] Nagren *et al.*[3)] reported ^{11}C-labeled methionine-enkephalin and its analogues as the labeled peptides suitable for the PET technique. However, the labeling method was limited to methionine-containing peptides, and the labeled peptide showed rapid degradation *in vivo*. We now report a synthetic method suitable for the preparation of a ^{13}N-labeled dermorphin analogue, H–Tyr–D-Met(O)–Phe–Gly–NH_2 (SD-62), which can be easily applied to the PET technique. SD-62 has much higher opioid activity *in vitro* and *in vivo* than morphine or Met-enkephalin, as well as high stability in enzymatic digestion.[4)]

Our synthetic scheme for the preparation of ^{13}N-labeled SD-62 is illustrated in Fig. 1. We employ [^{13}N]ammonia as a labeling reagent for the ammonolysis of the precursor peptide, since [^{13}N]ammonia can be prepared by a fully automated system.[5)] As the precursor peptide for ammonolysis, an active ester form is employed. In this labeling method, no condensation reagent is necessary, which makes the following purification step easy, and no racemization occurs during ammonolysis since SD-62 possesses a Gly at its C-terminus.

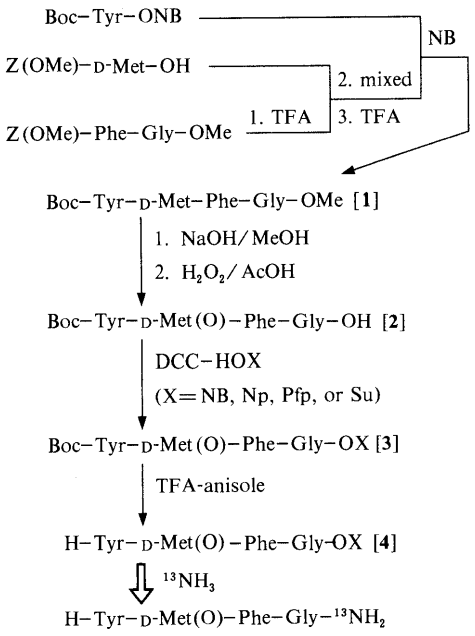

Fig. 1. Synthetic Scheme for the Preparation of ^{13}N-Labeled SD-62

The precursor of SD-62 [**4**] was prepared according to the scheme shown in Fig. 1. The published synthetic route to SD-62 starting from Gly–NH_2[6)] is not applicable to our labeling method because of the limited amount of $^{13}NH_3$ available. Starting with H–Phe–Gly–OMe prepared by the usual TFA treatment of a known dipeptide ester, Z(OMe)–Phe–Gly–OMe,[7)] N^{α}-protected D-Met and Tyr were condensed successively by the mixed anhydride[8)] and the NB active ester[9)] methods to give Boc–Tyr–D-Met–Phe–Gly–OMe [**1**]. The tetrapeptide methyl ester [**1**] was converted to tetrapeptide [**2**] by ordinary hydrolysis with NaOH in methanol followed by oxidation with H_2O_2 in AcOH. As the active ester of the precursor peptide [**4**], the NB, Np,[10)] Pfp,[11)] and Su[12)] esters were selected and the yield of ammonolysis of the peptide active ester was examined as described later. Each precursor peptide [**4**] was prepared by the condensation of tetrapeptide [**2**] and the corresponding alcohol using the dicyclohexylcarbodiimide (DCC) method followed by usual TFA treatment.

Next, ammonolysis of the precursor peptide was examined using nonradioactive NH_3. The Np active ester precursor [**4**] was treated with 28% aq. MH_3 and the product was purified by gel-filtration followed by FPLC (Fast Protein Liquid Chromatography, Pharmacia). The purified SD-62 had the same elution time on analytical high performance liquid chromatography (HPLC) as an authentic sample prepared by the known method[6)] and produced amino acids in the ratios predicted by theory.

In practical radiosynthesis with [^{13}N]ammonia, however, the labeled peptide should be prepared within a short time due to the short half-life of ^{13}N (9.96 min). Thus, the yield of ammonolysis of each precursor active ester peptide [**4**] in 5 min was examined using nonradioactive NH_3. The

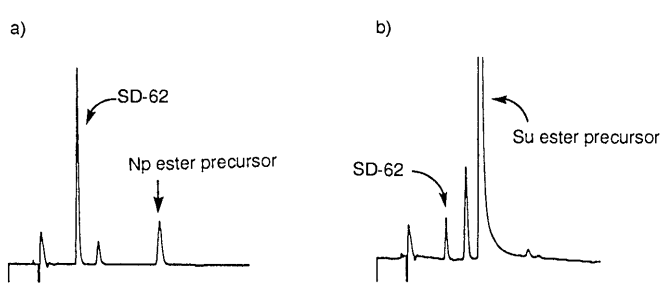

Fig. 2. Ammonolysis of the Active Ester Precursor [**4**] with aqueous NH_3

a, ammonolysis of the Np ester precursor; b, ammonolysis of the Su ester precursor.

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TABLE I. Yield of Ammonolysis of Precursor Peptide [4]

Precursor [4]	Yield (%)
Np ester	25
NB ester	29
Pfp ester	22
Su ester	5

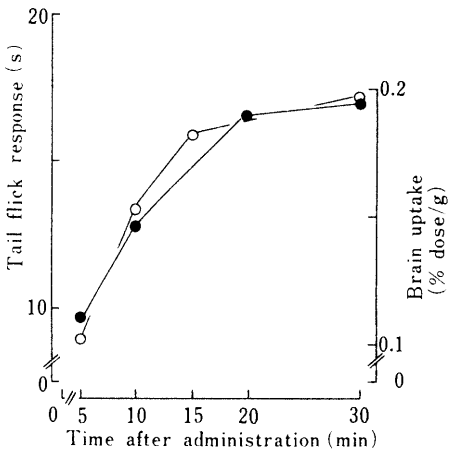

Fig. 3. Correlation of Brain Uptake and Analgesic Activity

●, brain uptake of ^{13}N-SD-62 (1.5 mg/kg, i.v.); ○, analgesic activity assayed by the tail flick test (0.8 mg/kg, i.v.).

conversion yield of each reaction mixture was estimated from the peak area of the resulting SD-62 on HPLC (Fig. 2). As shown in Table I, nearly the same conversion yields (22—29%) were obtained for all active ester peptides except for the Su ester precursor which gave a very low yield (5%). Each yield obtained with Np, NB, or Pfp ester precursor is moderate and suggests that the method can be applicable for radiosynthesis using [^{13}N]ammonia since ^{13}N nuclide has high specific radioactivity (0.053 pmol/mCi). In addition, the desired product can easily be purified from the reaction mixture using HPLC within 15 min.

From the experimental data described above; we concluded that our synthetic method is suitable for the preparation of [^{13}N]-labeled SD-62. When the [^{13}N]-labeled SD-62, prepared by our method, was administered into mice, the time profile of the radioactivity uptake into the brain closely paralleled that of analgesic activity (Fig. 3). The details of these biological results will be published elsewhere.

Experimental

Thin layer chromatography (TLC) was performed on silica gel (Kiesel-gel 60 F_{254}, Merck). *Rf* values refer to the following v/v solvent system: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1, lower phase), Rf_2 $CHCl_3$–MeOH (10:0.5), Rf_3 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2). Analytical HPLC was conducted with a Hitachi 655A. Amino acid analysis was conducted with a Hitachi L-8500.

Z(OMe)-D-Met-Phe-Gly-OMe Z(OMe)-Phe-Gly-OMe[7] (5.0 g, 12.5 mmol) was treated with TFA–anisole (8.8 ml–2.5 ml) in an ice-bath for 60 min, then TFA was removed by evaporation. The residue was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (15 ml) containing Et_3N (1.8 ml, 12.5 mmol). A mixed anhydride [prepared from 4.7 g (15.0 mmol) of Z(OMe)-D-Met-OH] in DMF (10 ml) was added to the above ice-chilled solution and the mixture was stirred for 1.5 h. The solvent was removed by evaporation and the product was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O and recrystallized from DMF with ether; yield 4.67 g (70%), mp 165—167 °C, $[\alpha]_D^{23}$ −20.2° (*c*=0.5, MeOH), Rf_2 0.52. *Anal.* Calcd. for $C_{26}H_{33}N_3O_7S$: C, 58.74; H, 6.26; N, 7.91. Found: C, 58.19; H, 6.32; N, 7.78.

TABLE II. Yield and Physical Constants of the Active Ester [3]

X	Yield (%)	mp (°C)	$[\alpha]_D^{21}$ (°)	Rf_1
Np	59	203—205	−15.0 (*c*=0.6, DMF)	0.51
NB	35	134—136	−36.0 (*c*=0.5, DMF)	0.44
Pfp	32	106 (dec.)	−28.0 (*c*=0.5, DMF)	0.67
Su	40	111—114	−28.0 (*c*=0.5, DMF)	0.40

Boc-Tyr-D-Met-Phe-Gly-OMe [1] The above protected tripeptide ester (4 g, 7.5 mmol) was treated with TFA–anisole–EDT (8.5 ml–2.3 ml–0.63 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMF (15 ml) containing Et_3N (1.1 ml, 7.5 mmol). Boc-Tyr-ONB [prepared by DCC (2.23 g, 10.8 mmol) coupling of Boc-Tyr-OH (2.5 g, 9 mmol) and HONB (1.77 g, 9.9 mmol) in tetrahydrofuran (THF) (10 ml)] and Et_3N (1.1 ml, 7.5 mmol) were added to the above solution and the mixture was stirred at 25 °C overnight. After evaporation of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, then dried over Na_2SO_4 and concentrated. The residue was recrystallized from MeOH with ether; yield 3.83 g (81%), mp. 173—175 °C, $[\alpha]_D^{22}$ +1.2° (*c*=1.0, MeOH), Rf_1 0.74. *Anal.* Calcd. for $C_{31}H_{42}N_4O_8S$: C, 59.03; H, 6.71; N, 8.88. Found: C, 59.20; H, 6.90; N, 8.68.

Boc-Tyr-D-Met(O)-Phe-Gly-OH [2] To an ice-chilled solution of the above tetrapeptide ester [1] (3.5 g, 5.6 mmol) in MeOH (30 ml) was added 1 N NaOH (6.7 ml), and the mixture was stirred for 2 h at an ice-bath temperature. The pH of the mixture was adjusted to 7 with citric acid, then the methanol was removed by evaporation. The resulting aqueous solution was acidified with 1 N HCl and extracted with AcOEt. The organic extract was washed with H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was reprecipitated from MeOH with ether to give Boc-Tyr-D-Met-Phe-Gly-OH; yield 2.60 g (76%), Rf_1 0.61. 35% H_2O_2 (0.51 ml, 4.5 mmol) was added to an ice-chilled solution of the above tetrapeptide (2.5 g, 4.1 mmol) in AcOH (10 ml) and the mixture was stirred at 25 °C for 1 h. The solvent of the mixture was removed by evaporation and the residue was recrystallized from MeOH with ether; yield 2.31 g (90%), mp. 134—136 °C, $[\alpha]_D^{22}$ +2.1° (*c*=1.0, MeOH), Rf_1 0.24. Amino acid ratios in 6 N HCl hydrolysate; Gly 1.00, Met 0.92, Tyr 1.02, Phe 0.99 (recovery of Gly 89%). *Anal.* Calcd. for $C_{30}H_{40}N_4O_9S \cdot 2H_2O$: C, 53.88; H, 6.63; N, 8.38. Found: C, 53.61; H, 6.64; N, 8.40.

Boc-Tyr-D-Met(O)-Phe-Gly-OX (X=Np, NB, Pfp or Su) [3] Boc-Tyr-D-Met(O)-Phe-Gly-OH was dissolved in THF together with a coresponding alcohol (1.1 eq) and DCC (1.1 eq) and the mixture was stirred for 12 h at 25 °C. The solution was filtered and the solvent of the filtrate was removed by evaporation. The residue was reprecipitated from THF with AcOEt; yield and physical constants of each active ester peptide are listed in Table II.

H-Tyr-D-Met(O)-Phe-Gly-NH$_2$ (SD-62) Boc-Tyr-D-Met(O)-Phe-Gly-ONp (100 mg, 0.13 mmol) was treated with TFA–anisole (2 ml–72 μl) for 1 h at an ice-bath temperature. The TFA of the mixture was removed by evaporation and the residue was triturated with ether. The resulting powder was dissolved in DMF (2 ml) and 28% NH_4OH (47 μl, 0.66 mmol) was added to the solution. After stirring for 1 h, the mixture was concentrated and the solution was applied to a column of Sephadex G-15 (3.2×45 cm), which was eluted with 1 N AcOH. The ultraviolet (UV) absorption at 280 nm was determined in each fraction (6 ml). The fractions corresponding to the front main peak were combined and the solvent was removed by lyophilization. The resulting powder was dissolved in 0.1% TFA (3 ml) and applied to a column (1.6×50 cm) packed with YMC-gel ODS-AQ 120A S-50, which was eluted with a linear gradient of CH_3CN (0—60%, 400 min) in 0.1% TFA at a flow rate of 3.0 ml/min. The eluate corresponding to the main peak was collected and the solvent was removed by lyophilization. The purified peptide exhibited a single peak (retention time 5.62 min, peak area 24467/nmol, determined by UV absorption measurement at 280 nm) on a Cosmosil 5C18ST (4.6×150 mm) column eluted with 14% CH_3CN in 0.1 M NaCl–HCl (pH 2.0) at a flow rate of 0.7 ml/min: yield 30 mg (43%), $[\alpha]_D^{24}$ +25.0° (*c*=0.5, 0.5 N AcOH), Rf_3 0.53. Amino acid ratios in 6 N HCl hydrolysate; Gly1.00, Met 0.81, Tyr 1.00, Phe 1.02 (recovery of Gly 92%).

Ammonolysis of Precursor Peptide [4] Each precursor active ester [4] prepared as stated above (5 mg) was dissolved in DMF–H_2O (0.1 ml–

0.5 ml), and 28% aqueous NH_3 (4 μl, 3 eq) was added to the solution. After 5 min, 10 μl of each mixture was applied to a Cosmosil 5C18ST (4.6 × 150 mm) column, which was eluted with 14% CH_3CN in 0.1 M NaCl–HCl (pH 2.0) at the flow rate of 0.7 ml/min. The amount of each resulting SD-62 (elution time 5.62 min) was estimated from the peak area. The typical elution profiles are shown in Fig. 2 and each conversion yield is lited in Table I.

References and Notes

1) The following abbreviations are used: Boc = *tert*-butyl oxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, NB = 5-norbornene-2,3-dicarboximidyl, Np = *p*-nitrophenyl, Pfp = pentafluorophenyl, Su = succinimidyl, TFA = trifluoroacetic acid, DMF = dimethylformamide, EDT = ethanedithiol.
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Ligustrinoside, a New Bisiridoid Glucoside from *Strychnos ligustrina*

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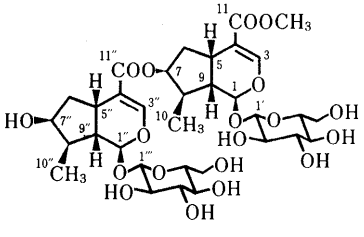
A new bisiridoid glucoside, ligustrinoside (1), along with three known iridoids, loganin, loganetin and loganic acid was isolated from the wood of *Strychnos ligustrina* BL. (Loganiaceae), collected in Indonesia. The structure of ligustrinoside (1) has been determined as an ester dimmer of loganin and loganic acid between C-7 and C-11.

Keywords *Strychnos ligustrina*; Loganiaceae; ligustrinoside; iridoid glucoside; loganin; loganetin; loganic acid

Strychnos ligustrina BL. is a widely distributed plant in tropical regions. The wood of this plant is called "kaju ular" and is used for the treatment of malaria in the Indonesian folkloric remedy "jamu". Earlier work on the constituents of this plant described the isolation of seven strychnos alkaloids.[1] This paper describes the characterization of a new bisiridoid glucoside, ligustrinoside (**1**), which was isolated from the wood of *S. ligustrina* collected on the Island of Java.

Ligustrinoside (**1**), a white powder, $[\alpha]_D$ −64.1° (MeOH), showed pseudomolecular ions at *m/z* 771 $[M+Na]^+$, 749 $[M+H]^+$ in positive ion fast-atom bombardment mass spectrometry (FAB-MS). From the FAB-MS and elementary analysis, the molecular formula was concluded to be $C_{33}H_{48}O_{19}$. The infrared (IR) spectrum of **1** showed the presence of hydroxyl (ν_{max} 3400 cm^{-1}) and α,β-unsaturated ester carbonyl groups (ν_{max} 1700, 1640, 1290 cm^{-1}).[2] Its ultraviolet (UV) spectrum exhibited absorption maximum at 235 nm due to a carbonyl enol ether chromophore typical of the iridoid.[2] Acetylation of **1** afforded a nona-acetate (**1a**), which showed molecular ions at *m/z* 1149 $[M+Na]^+$, 1127 $[M+H]^+$ in the FAB-MS and the presence of signals due to nine acetyls (δ 1.98—2.10 ppm) in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum. The ^{1}H-NMR spectrum of **1** showed characteristic signals for dimeric iridoid glycoside; a pair of signals due to the olefinic protons at δ 7.60, 7.67 (each 1H, d, J=1 Hz), methyl protons at δ 0.93, 1.23 (each 3H, d, J=7 Hz) and anomeric protons at δ 5.32, 5.39 (each 1H, d, J=8 Hz) and others as shown in Table I. Its carbon-13 (^{13}C)-NMR spectrum also showed a pair of signals due to the α,β-unsaturated ester groups at δ 167.0, 167.4, 151.1, 151.4, 112.5, 113.6, methyls at δ 13.3, 13.7 and anomeric carbons at δ 100.7, 100.8 suggesting that **1** was an ester dimmer of iridoid glycoside. Alkaline hydrolysis of **1** afforded loganic acid (**4**) which on subsequent methylation with diazomethane yielded loganin (**2**). These results suggested that the structure of **1** is an ester dimmer of loganin and loganic acid. The location of linkage was determined by comparing the ^{1}H- and ^{13}C-NMR chemical shifts of **1** with those of loganin (**2**). Marked downfield shifts for H-7 ($\Delta\delta$=1.0 ppm) and a C-7 ($\Delta\delta$=3.2 ppm) in **1** suggested that the structure of **1** was esterified between the hydroxyl group at C-7 of loganin and carboxylic acid group of loganic acid. On the basis of the above results, the structure of ligustrinoside was determined to be **1**.

In addition to **1**, the known iridoids loganin (**2**),[2]



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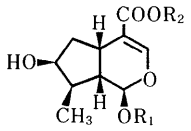

2 : R_1=glc, R_2=CH_3
3 : R_1=H, R_2=CH_3
4 : R_1=glc, R_2=H

Chart 1

TABLE I. ^{1}H- and ^{13}C-NMR Spectral Data for Compounds **1** and **2**

Position	**1**		**2**	
	H	C	H	C
1	5.58 d (4)	96.7	5.69 d (4)	97.5
3	7.60 d (1)	151.4	7.70 s	151.3
4	—	112.5	—	113.3
5	3.24 br q (8)	31.6	3.53 br q (8)	31.8
6	1.81 m	39.8	1.74 m	41.7
	2.40 m		2.65 ddd (14, 8, 1)	
7	5.27 m	76.6	4.27 t (6)	73.4
8	2.14 m	39.8	2.04 m	42.9
9	2.22 m	46.6	2.45 m	46.0
10	0.93 d (7)	13.3	1.21 d (7)	13.7
11	—	167.4		167.7
1′	5.32 d (8)	100.7	5.40 d (8)	100.8
2′	4.02 m	74.7	4.05 m	74.8
3′	4.23 m	73.5	4.27 m	78.5
4′	4.23 m	71.7	4.27 m	71.5
5′	3.98 m	78.7	4.05 m	78.8
6′	4.30 dd (12, 4)	62.8	4.39 br dd (12, 4)	62.7
	4.47 dd (12, 2)		4.57 br d (12)	
1″	5.62 d (4)	97.8		
3″	7.67 d (1)	151.1		
4″	—	113.6		
5″	3.48 br q (8)	31.8		
6″	1.72 m	43.1		
	2.60 ddd (14, 8, 1)			
7″	4.23 m	73.5		
8″	2.05 m	41.7		
9″	2.43 m	46.0		
10″	1.23 d (7)	13.7		
11″	—	167.0		
1‴	5.39 d (8)	100.8		
2‴	4.02 m	74.7		
3‴	4.23 m	73.5		
4‴	4.23 m	71.7		
5‴	4.23 m	78.4		
6‴	4.30 dd (12, 4)	62.7		
	4.47 br q (12)			
$COOCH_3$	3.56 s	51.0	3.58 s	50.9

The spectra were measured in C_5D_5N. Coupling constants (J in Hz) are given in parentheses. The signal assignments were based on spectral analysis of ^{1}H-^{1}H shift correlation spectra. Abbreviations: s=singlet, d=doublet, dd=doublet of doublets, t=triplet, br d=broad doublet, br q=broad quartet, m=multiplet.

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loganetin (**3**)[3] and loganic acid (**4**)[4] were isolated and identified by direct comparison with authentic samples. This is the first report on the isolation of compounds (**2**—**4**) from this plant.

Experimental

Melting points were taken on a Yanagimoto micro-melting point apparatus and are uncorrected. UV and IR spectra were recorded on a Hitachi 340 and a Hitachi 260-30 spectrophotometers, respectively. Optical rotations were measured on a JASCO DIP-4 digital polarimeter. The ^{1}H- and ^{13}C-NMR spectra were recorded with a JEOL JNM GX-400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrometer. Chemical shifts are given on the δ-scale (ppm downfield from tetramethylsilane as an internal standard) and coupling constants (J) in Hz. FAB-MS was run on a JEOL JMS DX-303 mass spectrometer. Medium-pressure liquid chromatography (MPLC) was carried out on silica gel (CQ-3, Fuji gel, 24 mm i.d. × 360 mm, detector: 254 nm).

Extraction and Isolation Dried crushed wood (4.4 kg) of *S. ligustrina*, collected on the Island of Java in 1989, was extracted for 3 h with *n*-hexane (3 l × 3) and MeOH (3 l × 3). The MeOH extract was concentrated under reduced pressure to give a residue (150 g), to which an equal volume of water was added. The aqueous solution was extracted with $CHCl_3$ (3 l), EtOAc (3 l) and *n*-BuOH (3 l), successively. The *n*-BuOH soluble extract (74 g) was chromatographed on Diaion HP-20 (Mitsubishi Kasei) with H_2O and an increasing content with MeOH as eluents. The fraction eluted with MeOH–H_2O (1 : 1) was evaporated to give a residue, which was fractionated sequentially by silica gel column chromatography ($CHCl_3$–MeOH–H_2O = 10 : 5 : 1 and 6 : 4 : 1) and MPLC ($CHCl_3$–MeOH–H_2O = 30 : 10 : 1, 10 : 5 : 1 and 6 : 4 : 1) to give ligustrinoside (**1**, 130 mg) along with known compounds, loganin (**2**, 160 mg), loganetin (**3**, 15 mg), loganic acid (**4**, 76 mg).

Ligustrinoside (**1**): White powder. $[\alpha]_D$ −64.1° (c = 2.1, MeOH). FAB-MS (m/z): 771 $[M+Na]^+$, 749 $[M+H]^+$. *Anal.* Found: C, 49.85; H, 6.46. Calcd for $C_{33}H_{48}O_{19}\cdot 5/2H_2O$: C, 49.93; H, 6.73. IR ν_{max} (KBr) cm^{-1}: 3400, 2900, 1700, 1640, 1290. UV λ_{max} (MeOH) nm (log ε): 235 (3.51). ^{1}H- and ^{13}C-NMR see Table I.

Acetylation of Ligustrinoside A mixture of ligustrinoside (**1**, 10 mg), acetic anhydride (1 ml), and pyridine (1 ml) was kept at room temperature for 30 min and treated as usual. Ligustrinoside nona-acetate (**1a**, 14 mg) was obtained as a white powder. FAB-MS m/z: 1149 $[M+Na]^+$, 1127 $[M+H]^+$. IR ν_{max} (KBr) cm^{-1}: 3400, 2950, 1710, 1650, 1080. ^{1}H-NMR (δ in C_5D_5N): 0.95 (3H, d, J = 6 Hz), 1.03 (3H, d, J = 6 Hz), 1.98, 1.99, 2.02, 2.03, 2.03, 2.05, 2.06, 2.07, 2.10 (each 3H, s), 2.25 (2H, m), 2.36 (2H, m), 3.68 (3H, s), 4.43 (2H, dt, J = 12, 2 Hz), 4.65 (2H, dt, J = 12, 4 Hz), 5.29 (2H, t, J = 4 Hz), 7.56 (1H, d, J = 2 Hz), 7.57 (1H, d, J = 2 Hz).

Alkaline Hydrolysis of Ligustrinoside A solution of ligustrinoside (**1**, 20 mg) in 1 N KOH (1 ml) was reactioned at 50 °C for 3.5 h. After cooling, the reaction mixture was neutralized with ion-exchange resin (Amberlite MB-3), evaporated to give a residue (**1c**, 8.6 mg). This compound was identified with loganic acid (from alkalysis of loganin), by direct comparison with authentic sample [thin layer chromatography (TLC), $[\alpha]_D$, IR, ^{1}H-NMR and MS spectra].

Methylation of Loganic Acid A solution of loganic acid (19.7 mg) in MeOH (0.5 ml) was treated with diazomethane for 1.5 h. The reaction mixture was evaporated and recrystallized with EtOAc–MeOH to give colorless needles (**2**, 18.4 mg). This compound was identified with loganin by direct comparison with authentic sample [TLC, $[\alpha]_D$, IR, ^{1}H-NMR and MS spectra].

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Fluorometric Determination of an Inverse Agonist to Benzodiazepine Receptors S-135 in Monkey Plasma by High Performance Liquid Chromatography

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A high performance liquid chromatographic method for the determination of an agent of benzodiazepine receptor inverse agonist (S-135) was developed. S-135 in monkey plasma, after its administration, was assayed using the method. S-135 is ionized in alkaline *N,N*-dimethylformamide (DMF), and fluoresces strongly at 490 nm on excitation at 290 nm. Extracted S-135 from a plasma sample was separated with an Asahipak GS-310H polyvinyl alcohol gel column and detected fluorometrically, with the eluate being mixed with a triethylamine–DMF mixture. S-135 in plasma is assayable with a coefficient of variation being less than 3.5% ($n=11$). The lower limit of the determination is 2 ng/ml. This method was applied as a highly sensitive and simple method to pharmacokinetic studies of S-135 after oral administration to cynomolgus monkeys.

Keywords benzodiazepine receptor inverse agonist; HPLC; ionization; polyvinyl alcohol gel; cynomolgus monkey; plasma

S-135, 2-(5-methylthien-3-yl)-2,5-dihydro-3*H*-pyrazolo-[4,3-*c*]quinolin-3-one, binds to benzodiazepine receptors with high affinity, and its pharmacological action is opposite to that of conventional benzodiazepine drugs. Pharmacological studies indicated that S-135 can be a useful drug for activating depressed brain function.[1,2] An analytical method was necessary to investigate the pharmacokinetics of S-135 at the animal level. The present paper describes a high performance liquid chromatographic (HPLC) method to fluorometrically determine S-135 in monkey plasma. S-135 fluoresces strongly in alkaline *N,N*-dimethylformamide (DMF). An HPLC system comprises separation and postcolumn ionization for the detection.

Experimental

Materials and Reagents All the chemicals were of a reagent or special grade.

Saturated Ammonium Sulfate Solution: Add 80 g of ammonium sulfate (reagent grade) to 100 ml of water and shake with a Direct mix, model TS-50 (Thermal Kagaku Sangyo Co., Ltd.) for 30 min. After allowing it to stand, decant the solution.

0.25 M Triethylamine–DMF Solution: Pipet 35 ml of triethylamine (reagent grade, Wako Pure Chemical Ind., Ltd.) into a 1000-ml volumetric fiask and dilute to the mark with DMF.

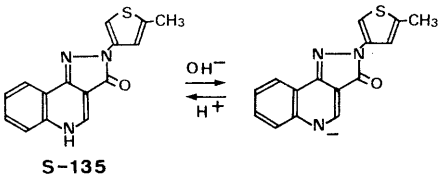
0.25 M Acetic Acid–DMF Solution: Dissolve 15 g of acetic acid (super special grade, Nacalai Tesque, Inc.) with DMF to make 1000 ml.

pH 8 Phosphate Buffer Solution: Dilute 4.5 ml of triethylamine with 1000 ml of water and then adjust pH to 8 by adding phosphoric acid (85%, reagent for biochemistry, Wako Pure Chemical Ind., Ltd.).

Standard Solution of S-135: Accurately weigh about 1.5 mg of S-135 into a 10-ml volumetric flask, then dissolve it in, and dilute to the mark with, acetonitrile–water (1 : 1). Prepare several solutions of varying concentration by diluting the solution with the mobile phase solvent, acetonitrile–pH 8 phosphate buffer solution (1 : 1).

HPLC Apparatus Liquid Chromatograph: A Shimadzu LC-6A pump, equipped with a Shimadzu RF-535 fluoresence detector and a Rheodyne model 7125 injection valve with a 500 μl sample loop, were used.

Analytical Column: Polyvinyl alcohol gel column, Asahipak GS-310H (25 cm × 7.6 mm i.d.) was used.



S-135
nonfluorescent fluorescent

Chart 1

Solvent Delivery Pump: An Eldex 30-A-S microquantitative pump linked to a high pressure damper was used.

Mixing Coil: A stainless-steel coil (1 m × 0.5 mm i.d.) was connected before the detector.

Data Integration System: A Shimadzu Chromatopac C-R4A TD data processor was used (Atten 2).

Chromatographic Conditions Flow rates of the mobile phase and the postcolumn reagent were 1 and 1.26 ml/min, respectively. The wavelength of the fluorescence detector was set at 490 nm with excitation at 290 nm with a sensitivity of 1 V full scale.

Collection of Plasma Sample Three each of male and female cynomolgus monkeys weighing 2.45 to 4.25 kg were used. After overnight fasting, a suspended solution of S-135 with 5% acacia solution was orally administered through a catheter. After administration at a dose of 25 mg/kg, blood (about 1.2 ml) was collected into a heparin-treated centrifuge tube at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 24 h. After centrifugation, separated plasma was used as a sample.

Assay Procedure Pipet 0.5 ml of plasma into a 15-ml centrifuge tube made of polypropylene (12.5 × 1.4 cm), add exactly 1 ml of saturated ammonium sulfate solution and mix well. Add 5 ml of ethyl acetate and shake with a shaker (an Iwaki KM shaker, type V-S) for 30 min. After centrifugation with a centrifuge (Kubota model KR-600P) at 3000 rpm at 4 °C for 5 min, transfer exactly 4.5 ml of the supernatant fluid into a 15-ml polypropylene centrifuge tube and evaporate off the solvent under a nitrogen stream at 30 °C. Add exactly 0.2 ml of acetonitrile saturated with *n*-hexane and 0.2 ml of *n*-hexane saturated with acetonitrile, and vortex the mixture with a Touch mixer (model MT-51, Yamato Kagaku Co., Ltd.) for 2 min. After centrifugation at 3000 rpm at 4 °C for 5 min, discard the *n*-hexane layer with an aspirator. Inject 10 μl of the acetonitrile layer and each standard solution into the column. Measure the peak areas of the sample and standard solutions. Make a standard curve and calculate the concentrations of S-135 in the plasma sample according to the following equation:

$$\text{plasma concentration of S-135 (ng/ml)} = \frac{\text{concentration (ng/ml)} \times 0.2 \times 5 \times 2 \times 100}{4.5 \times \text{recovery percent}}$$

The recovery value (%) is 92.5.

Results and Discussion

HPLC Conditions 1. Fluorescence Property S-135 is a weak acid with a pK_a of 9.44. It is fluorescent in organic solvents containing a base. The addition of a base enhanced the fluorescence intensity of S-135.[3] The emission maximum is at 492 nm in DMF upon excitation at 308 nm. The fluorescence intensity decreased with increasing water concentration. Therefore, the column eluate was mixed with DMF containing triethylamine to detect S-135 fluorometrically with high sensitivity.

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2. Column A polyvinyl alcohol gel column GS-310H separates materials in the mode of size exclusion chromatography. The merit is higher resolution of smaller molecules, more than 300.[4] Also, the application data[5] suggests that the column partly functions in a hydrophobic interaction like a reversed-phase. In the S-135 determination, a single sharp peak was obtained with the retention on the column, and with clear separation of the biological components in plasma.

3. Reagent Delivery Conditions The mobile phase was a pH 8 phosphate buffer and acetonitrile mixture (1 : 1) which effectively separates S-135 from plasma components. To make the mobile phase after elution from the column alkaline, the required amount of triethylamine–DMF solution sent post-column was examined. It is necessary that the triethylamine concentration in the mobile phase be less than 50 mM and the reagent solution more than 0.1 M to change the pH of the mobile phase to alkaline.

4. Flow Rate Two mixing coils, each 1 m in length and with internal diameters of 0.5 and 0.8 mm, were linked to the line of the post column. When 0.25 M of a triethylamine–DMF solution was flowed at 0.6 to 1.5 ml per min, the peak area increased with the rate to reach maximum values at 1.26 ml. Both coils were available at a flow rate greater than 1.26 ml/min.

Clean-Up of Plasma Sample A sample spiked with S-135 was extracted with ethyl acetate. Ammonium sulfate was added to plasma for deproteinization. The extract was dried under a nitrogen stream. The residue was dissolved in acetonitrile, and to eliminate lipophilic components, the solution was shaken with *n*-hexane. The acetonitrile layer was subjected to HPLC. The HPLC chromatogram for the treated plasma is shown in Fig. 1. No interfering peaks were found from monkey plasma (cynomolgus and rhesus) at the retention time of S-135 (Fig. 1B).

Recovery Test Cynomolgus monkey plasma spiked with S-135 at various concentrations (5—1000 ng/0.5 ml) was assayed. A linear relationship was obtained between the recovered and the added amounts. From the slope of the straight line, the recovery was calculated to be 92.5%. This value, measured in interday analyses, was reproducible.

Plasma Level of S-135 in Cynomolgus Monkeys S-135 was administered orally to cynomolgus monkeys. Figure 2 shows a plasma level *vs.* time profile after the administration. Each point shows the mean concentration observed using six monkeys.

Identification of an HPLC Peak in Plasma with S-135 When the eluate was mixed with an acidic solvent, such as DMF containing acetic acid, and sent to the fluorescence detector, the peak which was considered to be S-135 disappeared. This coincided with the fluorescence property of S-135. Figure 3 shows two chromatograms observed for the rhesus monkey plasma collected 1 h after administration of S-135 at the dose of 5 mg/kg (B), as well as its plasma treated by the acid (C). There are two other fluorescent peaks at the retention times: at 7.1 and 10.1 min in the chromatogram of Fig. 3B. These peaks could not been in plasma before the administration of S-135 (Fig. 3A), and disappeared following the addition of the acidic postcolumn reagent, a compound similar to S-135, possibly its metabolites.

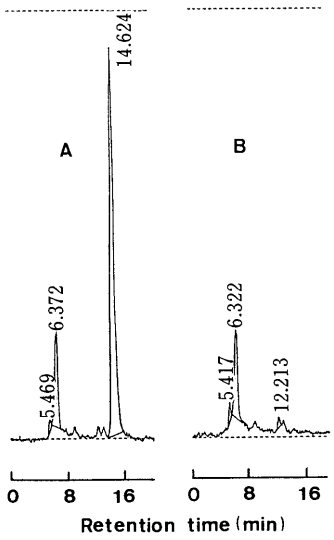

Fig. 1. Chromatograms of Cynomolgus Monkey Plasma Spiked with S-135 (50 ng/0.5 ml) (A) and the Control Plasma (B)

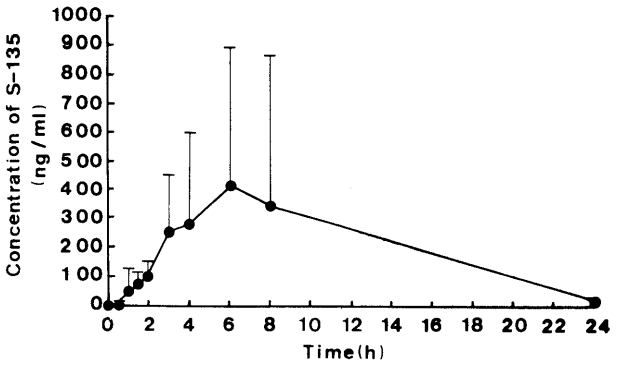

Fig. 2. Plasma Levels of S-135 after Oral Administration to Cynomolgus Monkeys ($n=6$, 25 mg/kg)

Each point represents the mean ± S.D.

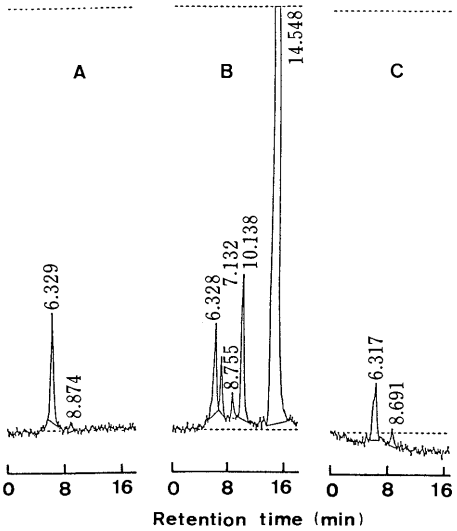

Fig. 3. Chromatograms of Plasma Sample Taken after Oral Administration to Rhesus Monkey (5 mg/kg)

A: Plasma collected before administration (reagent is 0.25 M Et_3N in DMF).
B: Plasma collected at 1 h after administration (reagent is 0.25 M Et_3N in DMF).
C: Plasma collected at 1 h after administration (reagent is 0.25 M AcOH in DMF).

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Optical Separation of Racemic 5-(*p*-Hydroxyphenyl)-5-phenylhydantoin by Reversed Phase High-Performance Liquid Chromatography Using Eluents Containing β-Cyclodextrin

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Both *S*-(−)- and *R*-(+)-enantiomers of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), a main oxidative metabolite of the achiral antiepileptic drug phenytoin, could be determined simply, sensitively and accurately using reversed phase high-performance liquid chromatography by using a methanol–monopotassium phosphate eluent containing β-cyclodextrin. Using this assay procedure, it was determined that an *S*-(−)-enantiomer was formed predominantly by the oxidation of phenytoin in isolated rat hepatocytes.

Keywords chiral separation; 5-(*p*-hydroxyphenyl)-5-phenylhydantoin; reversed phase high-performance liquid chromatography; β-cyclodextrin; metabolism; phenytoin; isolated rat hepatocyte

5-(*p*-Hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), a main oxidative metabolite of a prochiral antiepileptic agent, phenytoin (PHT), possesses an optically active center at the 5-position of a hydantoin nuclei (Fig. 1). The absolute configurations of the enantiomers of *p*-HPPH were assigned by Poupaert *et al.* in 1975 as *S*-(−)- and *R*-(+)-structures, respectively.[1] Ever since Murray *et al.* reported the metabolic inhibition of PHT by coadministered isoniazid (INH, antituberculotic) in tuberculous patients in 1962,[2] the clinically important drug interactions between PHT and INH,[3] as well as various co-administered drugs, have become of interest to many researchers[4] including the present authors.[3f,g] In their *in vivo* or *in vitro* studies, the formation amounts of *p*-HPPH are used as a reliable inhibition index of PHT metabolism.[3] However, there is no report of an investigation on the effects of the inhibitors regarding the stereochemistry of *p*-HPPH formation.

The optical resolution of *S*-(−)- and *R*-(+)-enantiomer of *p*-HPPH using high-performance liquid chromatography (HPLC) was performed by McClanahan *et al.* employing a β-cyclodextrin (β-CyD)-bonded chiral column.[5] Fritz *et al.* accomplished chiral ligand exchange chromatography by using an L-prolinamide derivative and nickel ion containing mobile phase.[6] We could also determine the enantiomers separately by direct HPLC on a cellulose tris-(4-methylbenzoate) column using a water–ethanol mobile phase.[7] Obstacles to the application of these methods are the high expense of the chiral columns, and/or the difficulty in obtaining the reagent. On the other hand, β-CyD, which is commercially available, has been applied as the mobile phase component in reversed phase HPLC for the resolution of some racemic compounds.[8] However, there is no report, to the best of our knowledge, on the separation of enantiomers of *p*-HPPH using the β-CyD inclusion method. Therefore, the authors examined this HPLC method prior to the stereochemical investigation of *p*-HPPH formation from an achiral starting compound, PHT, in the presence of enzymatic inhibitors.

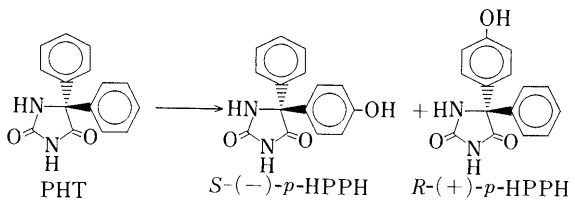

Fig. 1. Stereoselective Formation of *p*-HPPH from PHT

Experimental

Materials *p*-HPPH racemate was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), PHT and β-CyD were from Tokyo Chemical (Tokyo, Japan), and PRM (primidone) was of Dainihon Co. (Tokyo, Japan), respectively. MeOH and ethanol used for eluents were of HPLC grade from E. Merck (Darmstadt, F.R.G.). Other chemicals were of reagent grade. Extrelut column was obtained from E. Merck. The authentic samples of *R*-(+)- and *S*-(−)-*p*-HPPH were yielded by HPLC of racemic *p*-HPPH using a Chiralcel OJ column packed with cellulose tris-(4-methylbenzoate) (250 mm × 4.6 mm i.d., 10 μm particle size, Daicel Chem. Ind., Tokyo, Japan) and a water–ethanol (30 : 70) mobile phase, as described in our preceding paper[7]: $[\alpha]_{589}^{26}$ ($c = 2.85$, MeOH): +20.0° and −20.0° for *R*- and *S*-*p*-HPPH isomers.

Apparatus and HPLC Conditions A Shimadzu LC-6AD HPLC (Kyoto, Japan) was equipped with a ultraviolet (UV)-detector (Shimadzu SPD-6AV) or an electrochemical detector (ECD) (ECD-100, Eicom Co., Japan) and an HPLC column packed with ODS TSKgel 80TM (150 mm × 4 mm i.d., Toso Co., Japan). The column temperature was ambient (about 26 °C). The mobile phase was a mixture of 11.2 mM β-CyD in 1/30 M KH_2PO_4–MeOH (80 : 20), and the flow rate was 0.8 ml/min. The eluates were monitored at 228 nm. Optical rotations of both *p*-HPPH enantiomers were measured under 589 nm at 26 °C with a Nihon Bunko DIP-370 digital polarimeter (JASCO, Tokyo, Japan).

Preparation of Standard Isolated Rat Hepatocyte Suspensions of *p*-HPPH Enantiomers The isolated hepatocytes were prepared from male Wistar rats (200—220 g) according to the method of Moldéus *et al.*[9] For the examination of recovery and accuracy as well as reproducibility, the isolated hepatocyte suspensions were adjusted to 3×10^6 cells/ml followed by ultrasonification to decompose the cells. Then 0.37, 3.73, 9.32, 27.96 μM of *p*-HPPH racemate was added. The standard hepatocyte suspensions were obtained from these mixtures according to the purification procedure for the metabolic reaction mixtures, as described below. The recoveries were calculated by comparison of the HPLC peak areas of each *p*-HPPH enantiomer with those for respective isomers in the control ethanol solutions spiked with different amounts of *p*-HPPH racemate.

Incubation of PHT in Isolated Rat Hepatocytes After adding 79.3 μM of PHT to the hepatocyte suspension (3×10^6 cells/ml), incubation was performed at 37 °C under a 95%O_2–5%CO_2 atmosphere for 20 min, and the reaction mixture was frozen immediately to keep it at −40 °C until analysis. The mixture was treated under ultrasonification just before analysis, and centrifuged (1300*g*). The supernatants were poured into an Extrelut-1 column, and after 10 min the column was eluated with 2.5 ml of *tert*-butylmethyl ether. After drying the eluate, the residue was dissolved in 100 μl of MeOH, 20 μl aliquots of which were injected to HPLC.

Results and Discussion

Both enantiomers of *p*-HPPH could be resolved smoothly

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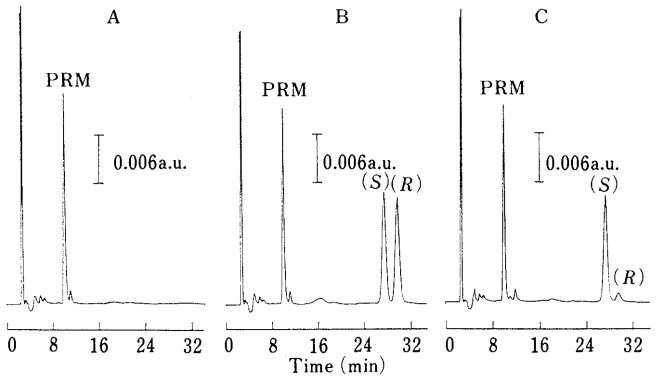

Fig. 2. Optical Resolution of *p*-HPPH Enantiomers in Isolated Rat Hepatocytes

(A) Standard hepatocyte suspension without adding *p*-HPPH. (B) Standard hepatocyte suspension spiked with 37.3 μM *p*-HPPH racemate. (C) Sample prepared after 20 min incubation in isolated rat hepatocytes using 79.3 μM PHT. Conditions: 150 mm × 4 mm i.d. ODS TSKgel 80 TM column; eluent, 11.2 mM β-CyD in 1/30 M KH_2PO_4 (80 : 20); flow rate, 0.8 ml/min.

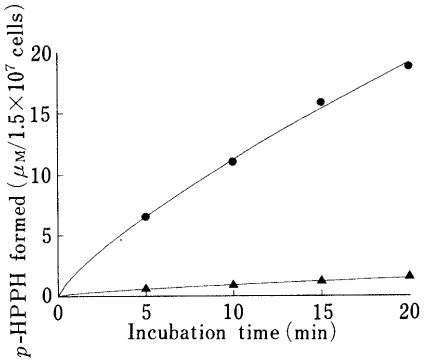

Fig. 3. Time Course of Formation of *S*-(−)-*p*-HPPH (●) and *R*-(+)-*p*-HPPH (▲) from PHT under Incubation in Isolated Rat Hepatocytes

Each point is the mean value of three experiments. Incubation conditions: see Fig. 2C.

and effectively by reversed phase HPLC using a 1/30 M KH_2PO_4–MeOH (80 : 20) eluent containing β-CyD (11.2 mM). The maximal resolution factor ($Rs = 7.5$) was obtained with concentrations in the region of 7.4 mM β-CyD and above, and the retention values became smaller as the β-CyD concentrations increased. However, when the concentration of β-CyD was above 12 mM, the eluent became highly viscous. From these facts, we determined to use an eluent containing 11.2 mM of β-CyD. By contrast, the more the MeOH concentration in the eluent increased the more the enantiomer separation became difficult. However, long retention times did result from lower MeOH concentrations. Considering both sufficient separation and the appropriate retention time, a 20% MeOH solution was used as the best eluent.

Figures 2A and 2B show the chromatograms obtained by injection of the sample aliquots from the isolated hepatocyte suspensions with or without spiking of racemic *p*-HPPH. Each peak separated was assigned as an *S*-(−)- or *R*-(+)-enantiomer by comparing the retention times to the authentic samples. The standard deviations of within-sample reproducibility were excellent, showing 4.4% and under 2% for the hepatocyte suspensions spiked with 0.37 μM of *p*-HPPH racemate and the higher concentrations, respectively ($n = 6$). The average recovery was 75—80% (S.D. < 3%), and the detection limits with a 20 μl injection were 39 and 36 pg for *S*- and *R*-enantiomers, respectively.

Applicability of the simple assay procedures to the metabolic experiments were proven using the isolated rat hepatocyte system. After adding 79.3 μM of PHT, the hepatocyte suspension was incubated for 20 min at 37 °C and the samples were collected at a fixed regular time interval followed by purification as described above. A typical chromatogram obtained from a 20 min incubation mixture was shown in Fig. 2C, which demonstrated that the oxidation was stereoselective and the main metabolite of PHT was *S*-(−)-*p*-HPPH. The formation ratio of *S*-enantiomer was about 92.5 ± 0.7% (mean ± S.D., $S/R = 12.3$), which remained unchanged over the incubation period (Fig. 3). The results are consistent with observations that the urine excretions of *p*-HPPH were stereospecific after administration of PHT to rats as well as humans.[1,6,7,10]

Under the HPLC conditions employed here, the relatively longer assay time is necessary, since the retention time of PHT was longer than 90 min after injection. However, using an ECD (ECD-100: +0.88 V *versus* an Ag/AgCl reference electrode) instead of a UV detector, the times needed for measurements could be shortened, since PHT could not be perceived by ECD, in that we observed no influence on the accuracy of the measurements. Thus, both enantiomers of *p*-HPPH can be determined simply, sensitively and accurately by reversed phase high-performance liquid chromatography using the β-CyD containing eluent. By using this simple analytical method, we are now beginning to explore the stereochemical features of PHT metabolism *in vitro* system, and in animals as well as in humans.

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Use of the *o*-Phenylenediamine Fluorescence System in the Enzymatic Assay of Serum Uric Acid

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A manual enzymatic method is described for sensitive fluorometric determination of uric acid in human serum. This method is based on an enzymatic reaction with uricase to form hydrogen peroxide from uric acid and the following oxidation of *o*-phenylenediamine with peroxidase and hydrogen peroxide for the production of a fluorescence compound. The specificity and the selectivity in the method are due to the uricase reaction and the fluorometry, respectively. The formed fluorescence in the reaction mixture is measured at 410 nm (an excitation) and 550 nm (an emission). This enzymatic method can determine uric acid at 30—1000 μM, with a between-assay relative standard deviation of 4.35% or less. A good correlation is obtained between the present method and the colorimetric kit method.

Keywords serum uric acid; *o*-phenylenediamine; fluorometry; uricase; peroxidase; hydrogen peroxide

Studies on the metabolism of purine have recently proceeded in the field of clinical medicine. One important study is the determination of uric acid in biological samples. Basically, the methods for determining uric acid can be divided into two groups; either those that utilize the reducing activity of uric acid or the enzymatic oxidation of uric acid with uricase.

Among the former methods, colorimetry[1] with phosphotungstic acid was most commonly employed. However, these colorimetric methods lacked specificity and required deproteinization because they were affected by reducing substances besides uric acid in biological samples. Recently, specificity has been increased by various combinations of uricase.[2] Other methods, using 1,10-phenanthroline,[3] 2,4,6-tripyridyl-*s*-triazine,[4] and 2-(5-nitro-2-pyridylazo)-5-(*N*-propyl-*N*-sulfopropylamino)phenol[5] required relatively large volumes (more than 50 μl) of sample.[6]

On the other hand, the latter methods using uricase can be classified into three types; the ultraviolet absorption method,[7] the uricase-catalase method,[8] and the uricase-peroxidase method.[9] The first method is not sensitive, therefore, the second or third colorimetric methods are practically available. However, these methods also require large volumes of sample. Therefore, more sensitive methods using fluorometry,[6,10] high performance liquid chromatography,[11] and chemiluminescence[12] were investigated. But in these methods, some faults were involved. For example, the time-consuming deproteinization process is adopted in the fluorometric methods and special equipment is required in the other methods. Therefore, these methods are not suitable for routine mass screening.

o-Phenylenediamine (*o*-PD), a typical hydrogen donor, is converted to 2,3-diaminophenazine, a highly fluorescence compound, by oxidation with peroxidase and hydrogen peroxide.[13] We have studied the application of fluorometry with *o*-PD to determine the uric acid concentration in serum by the deproteinization process using an ultrafilter membrane.

Experimental

Materials Chemicals, Reagents, and Apparatus: Uric acid, *o*-PD dihydrochloride, uricase from yeast (Candida utilis) (urate oxidase; urate; oxygen oxidoreductase, EC 1.7.3.3), ascorbate oxidase from cucumber (ascorbase, L-ascorbate; oxygen oxidoreductase, EC 1.10.3.3), peroxidase from horseradish (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7), sodium sulfite (Wako, Osaka, Japan), human serum albumin, cerulоplasmin (Sigma, St. Louis, Mo., U.S.A.), bilirubin, tripalmitin, and ascorbic acid (Wako) were used. A hemoglobin stock solution was prepared as previously described.[14] All other chemicals were of reagent grade.

The uricase solution (150 mU/ml) was prepared by dissolving uricase, authorized by Wako, in water. The ascorbate oxidase solution (50 U/ml) was prepared by dissolving authorized ascorbate oxidase powder in water. These solutions were stored by freezing at −20 °C and fused on the day of use. The uric acid stock solution was prepared as previously described.[5] Standard solutions were prepared by diluting the uric acid stock solution with water to give concentrations of up to 1000 μM. The *o*-PD solution (50 mM) was prepared by dissolving 90.5 mg of *o*-PD dihydrochloride in 10 ml of water.

Fluorescence spectra and fluorescence intensity were measured with a fluorescence spectrophotometer (model 650-40, Hitachi, Tokyo, Japan), setting at 10 mm slit-width in the excitation and emission monochrometers.

Methods Deproteinization: Filter 100 μl of sample or uric acid standard solution through a ultrafilter membrane (molecut II GC, Millipore, Bedford, Mass., U.S.A.).

Enzymatic Reaction: To the mixture of 3 ml of 0.1 M phosphate buffer (pH 8.0), 10 μl of 150 mU/ml uricase solution, and 10 μl of 50 U/ml ascorbate oxidase solution, add 20 μl of the deproteinized serum or uric acid standard solution, and mix well. After the addition of 100 μl of 100 mU/ml peroxidase solution and 100 μl of 50 mM *o*-PD solution, incubate at 37 °C for 15 min. Cool the reaction mixture in water, add 0.5 ml of 50 mM sodium sulfite solution, and mix well. Measure the fluorescence of the reaction mixture at 410 nm (an excitation) and 550 nm (an emission) within 60 min, using a blank solution in which deproteinized serum has been replaced with water.

For comparison, we also determined the uric acid concentration in serum or artificial samples by a colorimetric method[15] with uricase, peroxidase, and 4-aminoantipyrine, using an available kit (uric acid C-test Wako, Wako).

Results and Discussion

Reaction Conditions In the presence of uric acid, uricase, and peroxidase, *o*-PD is oxidized and then exhibits fluorescence in the reaction mixture (Fig. 1). The fluorescence-producing reagent, *o*-PD, was used as an indicator of hydrogen peroxide produced by the oxidation of uric acid with uricase. This fluorescence substance was a dimer of *o*-PD produced by oxidation in the presence of hydrogen peroxide with peroxidase.[16] An appropriate pH of 0.1 M phosphate buffer (pH 6.0—8.6) was 8.0, because of its high fluorescence production. The combination of 150 mU/ml uricase, 50 U/ml ascorbate oxidase, and 100 mU/ml peroxidase activities gave almost maximum fluorescence intensity when they were tested at the range of 0—1000 mU/ml, 0—500 U/ml, and 0—1000 mU/ml, respectively. The optimal concentrations of both *o*-PD and sodium sulfite were 50 mM when they were tested between

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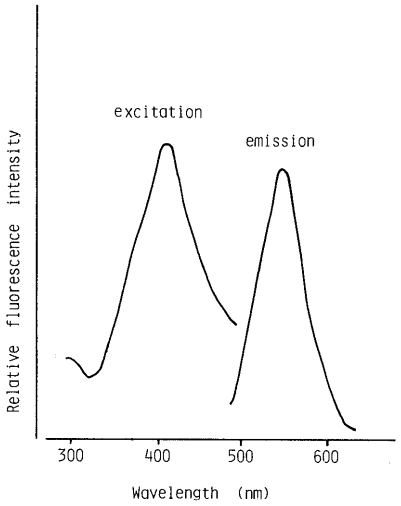

Fig. 1. Excitation and Emission Spectra of the Fluorescent Product in the Reaction Mixture

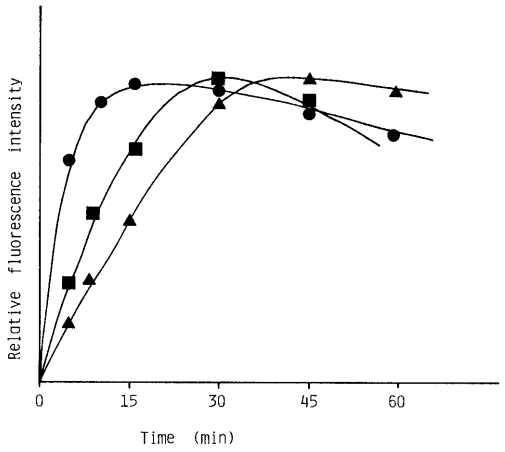

Fig. 2. Time-Course of the *o*-Phenylenediamine Oxidation Reaction at Different Temperatures

Uric acid concentration in sample: 1000 μM. Reaction temperatures: 25 (▲), 37 (●), and 45 (■) °C.

0 and 300 mM and 0 and 1000 mM.

Figure 2 shows the time-course for the *o*-PD oxidation reaction. The reaction proceeded more quickly at 37 °C, and reached its maximum within a period of 15 min. Therefore, the oxidation reaction was completed within 15 min at 37 °C. However, the fluorescence produced decreased gradually without a stabilizer. But the adoption of sodium sulfite after the reaction, produced fluorescence that was stable and gave a constant value for at least 60 min.

Effect of Interfering Substances The fluorometric methods[6,10] reported formerly involved the deproteinization process with heating at 95 °C in their procedures because the protains in sample plasma or serum may act as acceptors for hydrogen peroxide in the presence of peroxidase,[17] or as the effect of catalase.[18] In our method, we adopted the deproteinization process by ultrafiltration for simplicity and time-saving. Table I shows the recovery of uric acid in the serum sample containing each interfering substance, albumin, bilirubin, lipids, hemoglobin, ceruloplasmin, glucose, or absorbic acid.

Utility of Ascorbate Oxidase Table II shows the effect of ascorbic aid at concentrations of 0, 41.8, 83.7, 167.5, 335.0, and 500.0 μM in the absence or presence of 50 U/ml ascorbate oxidase. At the ascorbic acid concentration of 335.0 μM, the recovery of uric acid was 61.2% against the additional uric acid (485 μM). Generally, the concentration of ascorbic acid in the serum of a healthy subject is between 10 and 100 μM,[19] although values up to 250 μM are clinically important. Therefore, in our method, the effect of ascorbic acid (less than 500 μM) was eliminated by oxidation with ascorbate oxidase.

Accuracy The calibration graph was linear up to 1000 μM. The lower limit of detection was 30 μM, at a sample-to-blank relative fluorescence intensity ratio of 2.0. The typical regression equation of the curve was represented by $y = 1.057x + 31.386$ ($n = 11$, $r = 0.998$); the standard error of the slope was 0.064 ($n = 10$).

The normal concentration range of uric acid in healthy human serum was 178—416 μM for males and 118—356 μM for females.[20] These levels may vary in several diseases with abnormal purine metabolism. For example, the concentra-

Table I. Effect of Some Interfering Substances on Serum Uric Acid Determination

Substance	(concentration)	Uric acid[a] Recovery range (%)
Albumin	(22.5—90 g/l)	95.9—105.2
Bilirubin	(0.156—0.625 g/l)	94.6—101.7
Tripalmitin	(0.9—7.5 g/l)	99.3—104.9
Hemoglobin	(1.25—2.50 g/l)	99.1—105.9
Ceruloplasmin	(0.125—1.000 g/l)	95.2—102.8
Glucose	(1.8—30 mM)	96.5—103.0
Ascorbic acid	(41.8—500 μM)	94.3—105.5

a) Uric acid concentrations of samples were 194, 485, and 776 μM.

Table II. Effect of Ascorbic Acid on Serum Uric Acid Determination with or without Ascorbate Oxidase

Ascorbic acid (μM)	Ascorbate oxidase (U/ml)	Uric acid[a] Found (μM)	Recovery (%)
None	None	486	100.2
41.8	None	463	95.4
83.7	None	436	89.8
167.5	None	408	84.1
355.0	None	297	61.2
500.0	50	487	100.5

a) The concentration of uric acid in each sample was 485 μM.

Table III. Precision Data

Sample	*n*	Uric acid (μM) Mean	S.D.[a]	RSD[b] (%)
	Within-assay			
Serum I	5	194	3.4	1.75
Serum II	5	485	15.6	3.21
Serum III	5	776	26.0	3.35
	Between-assay			
Serum I	10	194	8.4	4.32
Serum II	10	485	14.5	2.98
Serum III	10	776	19.6	2.52

a) Standard deviation. *b*) Relative standard deviation.

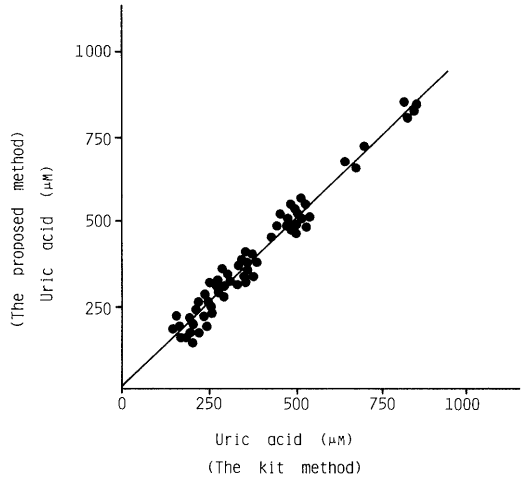

Fig. 3. Correlation between the Results Obtained by the Kit Method and the Proposed Method

tion is higher in the case of gout, Lesch–Nyhan syndrome, and chronic kidney disorders, and lower in the Fanconi syndrome. Therefore, in the proposed method, a determinable range of up to 1000 μM was investigated.

Precision Table III gives the results in five repeated within-assays of samples containing 194, 485, and 776 μM of uric acid and the results in ten repeated between-assays of frozen samples for daily analysis of the same uric acid concentrations for 10 d. The relative standard deviation (RSD) in the former assays was 1.75—3.35% and the latter RSD was 2.52—4.32%.

Comparison with the Other Method Figure 3 show the uric acid concentrations in sera from healthy subjects and artificial sera by the proposed method and the kit method. The correlation between these two methods was good ($n=61$, $r=0.994$); the regression equation of the graph was represented by $y=1.001x+0.923$.

Therefore, the method presented is expected to be useful in clinical practice.

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Comparison of Deacylation Rates of Chymotryptic Catalysis within an Enantiomeric Pair of *p*-Nitrophenyl Esters

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***p*-Nitrophenyl esters carrying a chiral acyl group were synthesized. These compounds were shown to meet the requirements of chymotrypsin for both the specific binding and the acylation. Therefore, the behavior of *p*-nitrophenyl esters with chymotrypsin is considered to be virtually identical to that of *p*-amidinophenyl esters with trypsin which were proposed as "inverse substrates" for the enzyme. These esters, derived from each pair of enantiomers, have been successfully used for the analysis of enantiomeric preference of chymotrypsin at the deacylation stage. The chiral requirement of the enzyme active site for the catalytic efficiency was discussed.**

Keywords chymotrypsin; deacylation rate; *p*-nitrophenyl ester; enantiomeric pair; inverse substrate; active site; catalytic efficiency

In our previous work,[1] it was shown that *p*-amidinophenyl esters are specifically hydrolyzed by trypsin. In these compounds the site-specific group for the enzyme, an amidinium group, is not included in the acyl moiety but in the leaving group. Thus a new term, "inverse substrate," was proposed for these esters. The esters provided a novel method for the specific introduction of an acyl group of a wide variety of acyl groups into a trypsin active site.[2] The design of esters has been extended to those carrying an asymmetric carbon in their acyl part and the catalytic efficiency of acyl trypsin derived from each pair of enantiomers has been compared with a view to analyzing the relationship between the chiral structure of the active site and the catalytic efficiency.[3]

It is known that a variety of *p*-nitrophenyl esters are specifically hydrolyzed by chymotrypsin. Thus, it could be considered that the interaction of *p*-nitrophenyl esters with chymotrypsin is essentially the same as that of *p*-amidinophenyl esters with trypsin. In this case the site-specific group for the enzyme is *p*-nitrophenyl group and it is included in the leaving part. The possibility of *p*-nitrophenyl esters acting as "inverse substrates" for chymotrypsin has been pointed out in our previous report.[1]

In this investigation *p*-nitrophenyl esters, those carrying an asymmetric carbon in their acyl part (**Ia—f**), were synthesized and the catalytic efficiency of acyl chymotrypsins deriving from each pair of enantiomers has been compared.

Results and Discussion

Kinetic Parameters for Chymotrypsin-Catalyzed Hydrolysis of Ia—f Chymotrypsin-catalyzed hydrolysis of **Ia—f** at pH 8.0 and 25 °C was analyzed spectrometrically by monitoring the liberation of *p*-nitrophenol at 405 nm. All the esters tested were hydrolyzed by chymotrypsin. After the rapid mixing of enzyme and *p*-nitrophenyl ester, fast acylation occurs and slow deacylation follows. In Fig. 1 the time course of the chymotrypsin-catalyzed reaction of **Ia**(*S*) was shown as an example. As a result, an acyl enzyme intermediate accumulates in the course of the steady-state hydrolysis. The acylation process was analyzed by a stopped-flow spectrophotometer with either the condition [E]≫[S] or [E]≪[S]. The reaction follows Michaelis–Menten kinetics, and the dissociation constants of enzyme-substrate complex, K_s, and the acylation rate constants, k_2, were determined from the double reciprocal plots of the acylation rates *vs.* either substrate concentrations (when [E]≪[S]) or enzyme concentrations (when [E]≫[S]). The deacylation rate constants, k_3, were determined from the steady-state catalytic rates under the condition [E]≪[S]. The rate constants for spontaneous hydrolysis of the

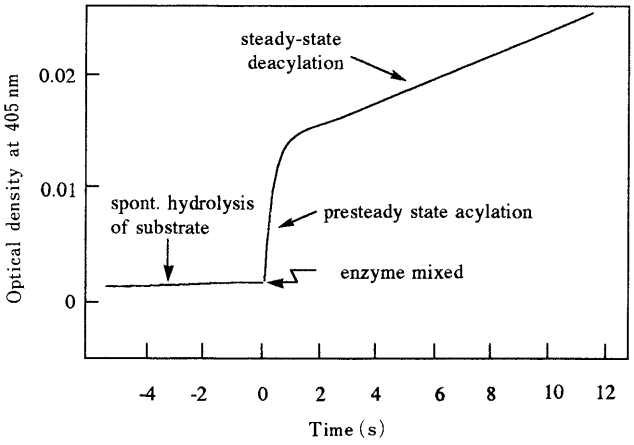

Fig. 1. Time Course of Chymotrypsin-Catalyzed Hydrolysis of **Ia**(*S*) under Steady-State Condition

The reaction was carried out in 0.05 M Tris–HCl buffer containing 0.02 M $CaCl_2$, pH 8.0 at 25 °C, $[S]_0$: 13.0 μM, $[E]_0$: 1.06 μM.

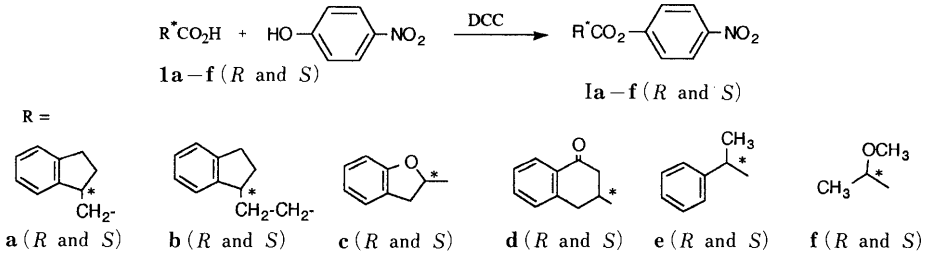

Chart 1

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TABLE I. Kinetic Parameters for Chymotrypsin-Catalyzed Reactions of Ia—f at pH 8.0[a]

Substrate	K_s (μM)	k_2 (s^{-1})	k_3 (s^{-1})	k_{sp} (s^{-1})	$k_3(R)/k_3(S)$[b]
Ia (*R*)	4.7	1.1	1.9×10^{-3}	5.3×10^{-5}	0.032 (0.14)
Ia (*S*)	12	1.8	5.9×10^{-2}	6.1×10^{-5}	
Ib (*R*)	6.6	1.7	5.2×10^{-3}	9.8×10^{-5}	0.012 (0.21)
Ib (*S*)	2.6	0.86	4.4×10^{-1}	1.0×10^{-4}	
Ic (*R*)[c]	—	—	12	3.8×10^{-3}	100 (56)
Ic (*S*)[c]	—	—	0.12	3.8×10^{-3}	
Id (*R*)	43	6.3	8.4×10^{-2}	7.1×10^{-5}	2.2 (1.2)
Id (*S*)	14	2.6	3.8×10^{-2}	7.2×10^{-5}	
Ie (*R*)	98	0.72	2.0×10^{-4}	1.3×10^{-5}	0.08 (0.32)
Ie (*S*)	120	0.42	2.4×10^{-3}	1.9×10^{-5}	
If (*R*)	71	2.7	1.0×10^{-1}	3.1×10^{-4}	14 (3.9)
If (*S*)	96	7.5	7.3×10^{-3}	2.5×10^{-4}	

a) The values are means of four runs. The standard errors are within 19% of respective means. *b*) The values for bovine trypsin determined by *p*-amidinophenyl esters in our previous report were listed in the brackets. *c*) Much faster acylation rate than deacylation rate was observed under steady-state condition. However, determination of K_s and k_2 was not feasible due to rapid acylation and facile spontaneous hydrolysis.

substrates, k_{sp}, are also determined. The values within a pair of enantiomers are almost identical. All these parameters are listed in Table I.

Comparison of Deacylation Rate Constants within a Pair of Enantiomers All the compounds **Ia—f** have strong binding affinity to chymotrypsin. The dissociation constants of the enzyme-substrate complex, K_s, are determined to be less 120 μM. The acylation rate constants are larger than the deacylation rate constants for all cases. We are especially interested in the deacylation rate constants. The inherent character of the enzyme as a protease will be exhibited at the deacylation stage,[2] and k_3 values in each enantiomeric pair are expected to exhibit information of the chiral requirement of the chymotrypsin active site.

The difference in k_3 values within pairs of enantiomers was analyzed. The ratios of the enantiomeric preference (R/S ratio) were determined as shown in Table I. In compounds **Ia**, **Ib** and **Ie**, enantiomers of *S*-series have larger k_3 values than their antipodes, but in **Ic**, **Id** and **If** the enantiomeric preference is opposite. The enantiomeric preference of trypsin, which was determined by *p*-amidinophenyl esters,[3] were listed in Table I for comparison. It is interesting that the enantiomeric preference of chymotrypsin is the same as that of trypsin, though the former is exhibited more pronouncedly than the latter in their extent.

It has been proposed that inverse substrates carrying a chiral acyl group are feasible for sensitive differentiation of tryptic enzymes of similar specificity.[4] It has been shown that trypsin and thrombin exhibit opposite enantiomeric preference in the deacylation of an indanylacetyl group (**1a**). In the present investigation, however, chymotrypsin and trypsin exhibited identical enantiomeric preference for all acyl groups tested including **1a**. It is well recognized that the active site in chymotrypsin is very similar to that in trypsin. Only Asp-189 and Gln-192 residues of chymotrypsin are replaced to Ser-189 and Met-192 for trypsin.[5] The resemblance of a three-dimensional structure of both active sites was revealed from X-ray analysis.[6,7] Present observation further revealed that the spatial arrangement of both active sites is similar in the distinguishing chiral structure of substrates.

Experimental

Materials α-Chymotrypsin was obtained from Worthington (lot CDI). Enzyme concentration was determined from titrated normality with *N-trans*-cinnamoylimidazole.[8] Optically pure carboxylic acids, **1a—f**, were prepared following the reported procedure.[3]

Synthesis of Substrates **Ia**(*R*): Dicyclohexylcarbodiimide (248 mg, 1.2 mmol) was added to a solution of **1a**(*R*)-acid (176 mg, 1.0 mmol) and *p*-nitrophenol (139 mg, 1.0 mmol) in ethyl acetate 5 ml. The reaction mixture was kept at 0 °C for 1 h and at room temperature for 12 h. Excess dicyclohexylcarbodiimide was converted to dicyclohexylurea by the addition of aqueous acetic acid. Precipitated urea was removed and the solvent was evaporated *in vacuo*. Recrystallization from ethanol gave 176 mg of colorless fine needles (59% yield), mp 80—82 °C, $[\alpha]_D^{20}$ −9.6 (c=1.6, $CHCl_3$). *Anal.* Calcd for $C_{17}H_{15}NO_4$: C, 68.68; H, 5.09; N, 4.71. Found: C, 68.63; H, 4.98; N, 4.65.

All other esters were prepared in the same manner as above.

Ia(*S*): Recrystallization from ethanol gave colorless fine needles (57% yield), mp 80—82 °C, $[\alpha]_D^{20}$ 9.4 (c=0.9, $CHCl_3$). *Anal.* Calcd for $C_{17}H_{15}NO_4$: C, 68.68; H, 5.09; N, 4.71. Found: C, 68.58; H, 5.04; N, 4.59.

Ib(*R*): Recrystallization from ethanol gave colorless leaflets (58% yield), mp 52—54 °C, $[\alpha]_D^{20}$ −16.7 (c=1.2, $CHCl_3$). *Anal.* Calcd for $C_{18}H_{17}NO_4$: C, 69.44; H, 5.50; N, 4.50. Found: C, 69.49; H, 5.48; N, 4.53.

Ib(*S*): Recrystallization from ethanol gave colorless leaflets (45% yield), mp 52—54 °C, $[\alpha]_D^{20}$ 15.8 (c=1.1, $CHCl_3$). *Anal.* Calcd for $C_{18}H_{17}NO_4$: C, 69.44; H, 5.50; N, 4.50. Found: C, 69.17; H, 5.51; N, 4.90.

Ic(*R*): Recrystallization from ethanol gave colorless needles (46% yield), mp 82—83 °C, $[\alpha]_D^{20}$ −22.7 (c=1.2, $CHCl_3$). *Anal.* Calcd for $C_{15}H_{11}NO_5$: C, 63.24; H, 3.84; N, 4.91. Found: C, 63.32; H, 3.84; N, 4.85.

Ic(*S*): Recrystallization from ethanol gave colorless needles (48% yield), mp 82—83 °C, $[\alpha]_D^{20}$ 25.3 (c=0.9, $CHCl_3$). *Anal.* Calcd for $C_{15}H_{11}NO_5$: C, 63.24; H, 3.84; N, 4.91. Found: C, 63.24; H, 3.84; N, 5.09.

Id(*R*): Recrystallization from ethanol gave colorless needles (36% yield), mp 154—155 °C, $[\alpha]_D^{20}$ 1.8 (c=1.0, $CHCl_3$). *Anal.* Calcd for $C_{17}H_{13}NO_5$: C, 65.59; H, 4.29; N, 4.50. Found: C, 65.38; H, 4.14; N, 4.38.

Id(*S*): Recrystallization from ethanol gave colorless needles (60% yield), mp 154—155 °C, $[\alpha]_D^{20}$ −1.5 (c=1.0, $CHCl_3$). *Anal.* Calcd for $C_{17}H_{13}NO_5$: C, 65.59; H, 4.29; N, 4.50. Found: C, 65.60; H, 4.12; N 4.69.

Ie(*R*): Recrystallization from *n*-hexane gave colorless needles (78% yield), mp 77—78 °C, $[\alpha]_D^{20}$ −127.2 (c=1.0, MeOH). *Anal.* Calcd for $C_{15}H_{13}NO_4$: C, 66.41; H, 4.83; N, 5.14. Found: C, 66.37; H, 4.82; N, 5.14.

Ie(*S*): Recrystallization from *n*-hexane gave colorless needles (85% yield), mp 76—77 °C, $[\alpha]_D^{20}$ −129.9 (c=1.0, MeOH). *Anal.* Calcd for $C_{15}H_{13}NO_4$: C, 66.41; H, 4.83; N, 5.14. Found: C, 66.40; H, 4.82; N, 5.04.

If(*R*): Recrystallization from ether–pet. ether gave colorless needles (67% yield), mp 68—69 °C, $[\alpha]_D^{20}$ 67.1 (c=1.3, $CHCl_3$). *Anal.* Calcd for $C_{10}H_{11}NO_5$: C, 53.33; H, 4.92; N, 6.22. Found: C, 53.38; H, 4.91; N, 6.22.

If(*S*): Recrystallization from ether–pet. ether gave colorless needles (64% yield), mp 68—69 °C, $[\alpha]_D^{20}$ −66.1 (c=1.7, $CHCl_3$). *Anal.* Calcd for $C_{10}H_{11}NO_5$: C, 53.33; H, 4.92; N, 6.22. Found: C, 53.31; H, 4.92; N, 6.23.

Determination of Kinetic Parameters Chymotrypsin-catalyzed hydrolysis of **Ia—f** was determined by monitoring the liberation of *p*-nitrophenol at 405 nm using a Union Giken stopped-flow spectrophotometer RA-401 and a Hitachi spectrophotometer 200-10 equipped with a thermostatically controlled water jacket. The reaction was carried out at pH 8.0 in 0.05 M Tris–HCl buffer containing 0.02 M $CaCl_2$ and 1.6% dimethylformamide at 25 °C. Determination of kinetic parameters was carried out following the method used for the analysis of trypsin-catalyzed hydrolysis of *p*-amidinophenyl esters.[3]

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Measurement of the Distribution Parameter in Solubilized Systems. III.[1] Measurement of the Distribution Parameter of Drugs Which Show Blue or Red Shifts in the Surfactant Solution and Its Utilization for Discrimination

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There are two types of shift of absorption spectrum at surfactant concentrations above the critical micelle concentration, one is the system shifting to a longer wavelength side (red shift; alkylparaben–sodium dodecyl sulfate (SDS) solution system, alkyl gallate–cetyl trimethylammonium bromide (CTAB) solution system, *etc.*) and the other is the system shifting to a shorter wavelength side (blue shift; sorbic acid–SDS solution system, benzoic acid–CTAB solution system, *etc.*). The distribution parameter values can be obtained through measuring the shift-length of the maximum absorption wavelength by the first derivative absorption spectrum (FDAS-SL method), regardless of their shift direction.

It was confirmed that in a case where discrimination by the absorption spectrum of drugs is difficult (for example, alkylparabens), measurement of the distribution parameter values is a good alternative. In the case of alkylparabens, discrimination was easily attained in the order of SDS (anionic) > CTAB (cationic) > octaethylene glycol monododecyl ether > polyoxyethylene (20) sorbitan monolaurate (nonionic) solution. Room temperature was the best condition for the measurement.

Keywords first derivative absorption spectrum; wavelength shift; blue shift; distribution parameter; partition coefficient; critical micelle concentration; discrimination; sodium dodecyl sulfate; alkylparaben; cetyl trimethylammonium bromide

Introduction

The absorption maximum of a drug in surfactant solution often shifts at surfactant concentrations above the critical micelle concentration (cmc). In previous reports,[1,2] we measured the shift-length (SL, mm, or nm (0.1 nm corresponded to 1 mm SL on the recorded chart)) of the maximum absorption wavelength by utilizing the first derivative absorption spectrum (FDAS), and obtained the distribution parameters and the partition coefficients. In this method, the FDAS is used to measure the SL of the absorption maximum wavelength, and so the method is called the FDAS-SL method (this method is different from the FDAS method[3-5] utilizing the change (=shift) of the wavelength of the drug).

The blue shift[6-9] or the red shift may occur according to a combination of the surfactants and the drugs. We previously reported the systems of the red shift.[1,2] In this report, some systems of the blue shift and the distribution parameters are stated. The degree of wavelength shift of a drug depends on the concentration of the surfactant, but not on the concentration of the drug. Therefore, the titration method in which the concentration of the surfactant was kept constant was used to improve the method shown in the previous report.

It is feasible to discriminate between the drugs by spectrophotometry, only when the differences of absorption spectra are evident. However, where the maximum absorption wavelengths of many drugs are concentrated in a narrow ultraviolet region, in many cases, discrimination by the absorption spectrum is difficult. For example, as the maximum absorption wavelengths of alkylparabens (methyl *p*-hydroxybenzoate (methylparaben), ethyl *p*-hydroxybenzoate (ethylparaben), propyl *p*-hydroxybenzoate (propylparaben) and butyl *p*-hydroxybenzoate (butylparaben)) are at 253—254 nm, it is not possible to discriminate between them by the absorption spectra only. However, it has been found that the discrimination of these four alkylparabens is clearly feasible by measuring the distribution parameter values (or the partition coefficient values).

Experimental

Materials Benzoic acid (minimum 99.5%), sorbic acid (minimum 98%), *o*-hydroxydiphenyl and dehydroxyacetic acid sodium salt were obtained from Wako Pure Chemical Ind., Ltd., cetyl trimethylammonium bromide (CTAB, minimum 99%), sodium dodecyl sulfate (SDS, minimum 99%), methylparaben, ethylparaben, propylparaben and butylparaben (minimum 99%) were obtained from Nakarai Chemicals, Ltd., and octaethylene glycol dodecyl ether (OED) was obtained from Nikko Chemicals Company, Tokyo, Japan. These chemicals were used as received.

Measurement of Absorption Spectra A Hitachi 557 dual-wavelength double-beam spectrophotometer (557) equipped with a Haake F2C thermostat (F2C) was used. Measurement conditions were as follows: scan speed 120 nm/min, slit width 2 nm, scale of absorption spectra from 0 to 1.

Measurement of FDAS The 557 spectrophotometer equipped with the F2C thermostat was used.

(i) Measurement of the Distribution Parameter by the Preparation of Various Concentration Solutions: Surfactant solutions of some concentration with or without a drug were placed in test tubes and kept at a constant temperature by a Tokyo Rikakikai T-80 thermostat. The surfactant solution was used as a reference. Cells holding the surfactant solutions with or without a drug were placed in the cell-holder of a 557 spectrophotometer, and the FDAS of different absorption spectra were taken after 4 min. (Alkylparaben–SDS, alkylparaben–CTAB, alkylparaben–Tween 20 and alkyl gallates–CTAB solution systems were measured by this method.)

(ii) Measurement of the Distribution Parameter by the Titration Method: Titration was performed by adding portions of the stock solution of α mM drug–β mM surfactant to 2000 μl of α mM drug solution, or of a β mM stock solution of surfactant to 2000 μl of aqua (control). In each concentration, the FDAS of different absorption spectra were taken after 4 min of each addition. In these methods (i) and (ii), measurement conditions were as follows: scale expansion 20 times, scan speed 6 or 12 nm/min, derivative width ($\Delta\lambda$) 5, slit width 2 nm, scale of absorption spectra from +0.5 to −0.5 or from +0.3 to −0.3. α or β were arbitrary numbers.

Calculation of the Distribution Parameter Values and the Partition Coefficient Values The point where the spectrum crosses the base line in the FDAS reflects the maximum absorption wavelength of the drug. Thus, the points in the surfactant solution and in the aqueous solution give the SL. We obtained the maximum SL (degree of shift = 1) by extrapolation of a plot of reciprocal of the SL on the ordinate against the reciprocal of the micelle concentration of surfactant on the abscissa (=the

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double-reciprocal plot).[10] The shift degree of each concentration was calculated in comparison with the maximum SL. The distribution parameter (K_{dp}) values and the partition coefficient (K_{pc}) values were obtained from the K_{dp} equation[1,2] and the K_{pc} equation,[1,2] respectively.

Measurement of the cmc of SDS Solution and CTAB Solution Following the method described in the previous paper,[1] the cmc measurement was carried out on a CD-35MII electric conductivity meter at 30 °C.

Results and Discussion

1) Measurement of the Distribution Parameter of the Drugs Which Show Blue Shift or Red Shift The blue shift or the red shift occurs at surfactant concentrations above the cmc. The circumstances of the blue shift or the red shift are the same except that the direction of the shift is reversed. The drugs incorporated into the micellar phase show the absorption maximum shift owing to the microenvironmental change (polar→non-polar). Moreover, the polarization by donor groups or suction groups of electron can determine the direction of the shifts. However, the distribution parameter values can be obtained regardless of the direction of the shift.

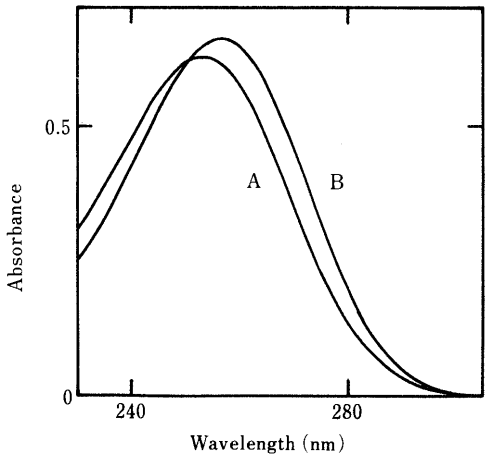

Fig. 1. Effect of CTAB on the Absorption Spectrum of 28.18 μM Sorbic Acid

A, in 1.28 mM CTAB solution; B, in H_2O.

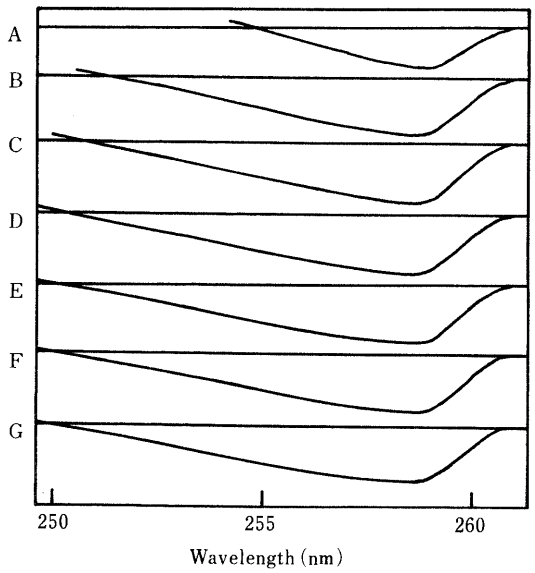

Fig. 2. FDAS of 37.81 μM Sorbic Acid

A, in H_2O; B, in 1.56 mM CTAB solution; C, 1.99 mM CTAB; D, 2.39 mM CTAB; E, 2.75 mM CTAB; F, 3.09 mM CTAB; G, 3.41 mM CTAB.

Methylparaben, ethylparaben, propylparaben and butylparaben in SDS, CTAB and polyoxyethylene (20) sorbitan monolaurate (Tween 20®) solutions, and 3,4,5-trihydroxybenzoic acid ethyl ester (ethyl gallate) and 3,4,5-trihydroxybenzoic acid propyl ester (propyl gallate) in CTAB solution which have been shown in the previous papers[1,2] were among the red shift systems. On the other hand, dehydroacetic acid sodium salt and sorbic acid in SDS solution and benzoic acid and sorbic acid in CTAB solution were among the blue shift systems (Fig. 1).

Figure 2 shows the FDAS of sorbic acid in the solution of surfactant concentration above the cmc. As the wavelength regions of about 258.5—261.5 nm in these FDAS are the calculating regions of first derivative function by 557 spectrophotometer, the curves do not show real FDAS in these regions. Namely, the contact of the curve and the base line around 261 nm means a false crossing-point

Substituting the slope and the intercept of the double-reciprocal plots and the cmc of surfactant (the cmc values of SDS, CTAB and OED at 30 °C are 8.1, 1.0 and 0.14 mM,[4] respectively) into the K_{dp} equation, we obtained the distribution parameter values, as shown in Table I. Moreover, Table II shows the partition coefficient values of the drug–CTAB systems. The micellar volume of the core and the Stern layer is assumed to be 0.999 ml/(g of CTAB).[11] From these results, the distribution parameter and the partition coefficient can be obtained from the red shift or the blue shift length in the solution of surfactant concentrations above the cmc.

2) Discrimination of Drugs Since methylparaben, ethylparaben, propylparaben and butylparaben in aqueous solution have the approximate wavelength of absorption maximum at 253—254 nm, it is impossible to discriminate

Table I. Distribution Parameter Values of Drugs at 30 °C

[A] in SDS solution	
Benzoic acid	0.13
Dehydroacetic acid sodium salt	0.44[a]
o-Hydroxydiphenyl	3.1
Sorbic acid	1.2[a]
[B] in CTAB solution	
Benzoic acid	2.3[a]
Dehydroacetic acid sodium salt	1.1
o-Hydroxydiphenyl	4.7
Sorbic acid	3.2[a]
[C] in OED solution	
Methylparaben	—
Ethylparaben	0.22
Propylparaben	0.26
Butylparaben	1.6
Benzoic acid	2.1
Dehydroacetic acid sodium salt	0.29
o-Hydroxydiphenyl	0.79
Sorbic acid	2.7

a) Blue shift system.

Table II. Partition Coefficient Values of Drugs in CTAB Solution

Benzoic acid	6300
Dehydroacetic acid sodium salt	3000
o-Hydroxydiphenyl	13000
Sorbic acid	8800

between them from the absorption spectra alone. We measured the distribution parameter values (or the partition coefficient values) of these drugs.[1] In SDS solution, the order of magnitude of the distribution parameter values was butylparaben > propylparaben > ethylparaben > methylparaben at any temperatures. When the alkyl chain length is longer (*i.e.*, the hydrophobic moiety is greater), the drug can be incorporated more easily into the micellar phase, and the degree of SL becomes larger. Therefore, the distribution parameter values become larger, too. These results in CTAB solution,[2] in Tween 20 solution[2] and in OED solution (Table I) show the same tendency as in SDS solution. And in the case of the alkyl gallate–CTAB system, the order of magnitude of the distribution parameter value was propyl gallate > ethyl gallate, and this result was similar to those in the alkylparaben–CTAB system.[2]

In four kinds of surfactant, the order of magnitude of the distribution parameter values is SDS > CTAB > OED > Tween 20.[1] Therefore, the order of simplicity of discrimination is anionic surfactant > cationic surfactant > nonionic surfactant. The difference of the distribution parameter values became clearly larger at a lower temperature. Consequently, in this experiment, the best results of discrimination of four alkylparabens were obtained from the distribution parameter values at room temperature in the anionic surfactant solution (SDS solution). Discrimination by the distribution parameter values can be evidently attained, but no difference of the absorption spectra is observed. However, this method is effective only when the drug shows a shift in the solution of surfactant concentration above the cmc.

In this experiment, it was confirmed that the measurement of the distribution parameter by the FDAS-SL method widened by the following advantages. 1) The distribution parameter and the partition coefficient of the drugs can be obtained regardless of the direction of the shift in the surfactant solution. This loosened the restrictions of drugs and surfactants for the measurement of the distribution parameter (or the partition coefficient). 2) The titration method has been adapted. This makes the preparation of various concentration solutions in the conventional methods dispensable. 3) When it is impossible to discriminate drugs from the absorption spectra alone, often the distribution parameter values can be utilized for the discrimination.

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Effect of CO_2 Laser Irradiation on the Surface Hardness of Self-Setting Hydroxyapatite Cement

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A novel approach to the use of self-setting hydroxyapatite cement in a dental cement system using CO_2 laser irradiation is proposed to increase the hardness of the cement. After setting, the cement was transformed to apatite which has a high affinity for hard tissues. The Vicker's hardness of the cement matrix was significantly increased by CO_2 laser irradiation.

Keywords laser irradiation; artificial hard tissue; Vicker's hardness; hydroxyapatite; self-setting cement; biomaterial

Introduction

Hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$, $M_r = 1004.62$), is an important mineral component of hard tissues such as teeth and bone. Therefore, when used in implanted artificial hard tissue, HAP has a high affinity for natural hard tissue *in situ*[1] and can be molded to fill spaces created by accidents or surgical operations on bones or teeth. It can also be used as a bonding material between bones or between bone and a prosthesis.[2] Brown and Chow[3] investigated the phase diagram of calcium phosphate, and proposed that a patented self-setting apatite cement with the same elementary chemical composition as bone and teeth be used as a new dental and bioconvertible material. But in practical applications, the apatite cement does not have adequate mechanical strength after setting. This is the most serious problem with this cement system. The surgical laser has proved to be a valuable treatment modality in many special surgical applications,[4] and Stern and Sognnaes[5] showed that when dental enamel surfaces of extracted human teeth were exposed to the ruby laser, the tendency toward subsurface demineralization was reduced. Also, Stern *et al.*[6] and Yamamoto *et al.*[7] reported that human enamel exposed to a CO_2 laser or an yttrium aluminum garnet laser is highly acid-resistant. Wong *et al.*[8] studied the dissolution kinetics of synthetic HAP exposed to a CO_2 laser in acidic media, using the rotating disk method. In this study, we tested the effect of CO_2 laser irradiation on apatite cement formation. The formation was accelerated by the irradiation, and it also hardened the cement. The physicochemical properties of the laser-irradiated cement were investigated.

Experimental

Materials Tetracalcium phosphate (TTCP, $Ca_4(PO_4)_2O$) and dicalcium phosphate dihydrate (DCPD, $CaHPO_4 \cdot 2H_2O$) powders were obtained from kyoritsu Ceramic Co., Japan and Nakalai Tesque Co., Japan. HAP used as seed crystal was synthesized by the precipitation method[8] at 105 °C. All other reagents were of analytical grade.

HAP Cement The patented apatite cement was prepared according to the procedure described by Brown and Chow,[3] except for the addition of HAP seed crystals.[9] The apatite cement powder system was a mixture of TTCP (1.83 g), DCPD (0.86 g) and HAP [1.79 g (40%) or 0.13 g (3%)] seed crystals. Five hundred milligrams of the cement powder was mixed and kneaded with 0.25 ml of 20 mM H_3PO_4. The paste was poured into a 13 mm diameter mold, and stored at 37 °C and 100% relative humidity (RH) for 24 h. The sample for X-ray powder diffractometry and Fourier transform infrared (FT-IR) spectroscopy was ground in an agate mortar after the cement fixed.

Laser Treatment The apparatus used for the laser treatment was modified from that reported by Wong *et al.*[8] It consisted of a CO_2 laser generater (model 60Z, Nippon Infrared Ind. Co.), a 10-ml platinum crucible and a moist nitrogen gas-supplying apparatus. The fixed cement or powder was irradiated 1—5 times by a CO_2 laser at 50 W for 2 s. The laser beam was 14 mm in diameter.

Measurement of Vicker's Hardness The hardness of the cement was measured by a Vicker's hardness tester (model M, Shimadzu Co.). A 1.0 kg load was applied to the cement for 10 s, and then the diagonal length of the indentation was measured. Vicker's hardness was calculated from the loaded weight and the area of dent on the surface, and was an average of 4 measurements.

X-Ray Powder Diffraction Analysis X-Ray powder diffraction profiles were taken at room temperature with an X-ray diffractometer (XD-3A, Shimadzu Co.). The operating conditions were as follows: target, Cu; filter, Ni; voltage 35 kV; current, 15 mA; receiving slit, 0.1 mm; time constant, 1 s; counting range, 1 kcps; scanning speed 1° 2θ/min.

Infrared (IR) Spectroscopy IR spectra (KBr disk method) of the powder sample of apatite cement were measured using an FT-IR spectrophotometer (type 1600, Perkin Elmer Co.).

Scanning Electron Microscopy (SEM) SEM photographs of samples were taken with a scanning electron microscope (model JSM-T20, Jeol Co.) at a magnification of × 10000.

Results and Discussion

Effect of Laser Irradiation on the Physicochemical Properties of HAP Cement Figure 1 shows the effect of CO_2 laser irradiation on the X-ray diffraction profiles of apatite cement. The fixed apatite cement samples before and after irradiation exhibited patterns which were characteristic of HAP. After irradiation the diffraction peaks of the cement

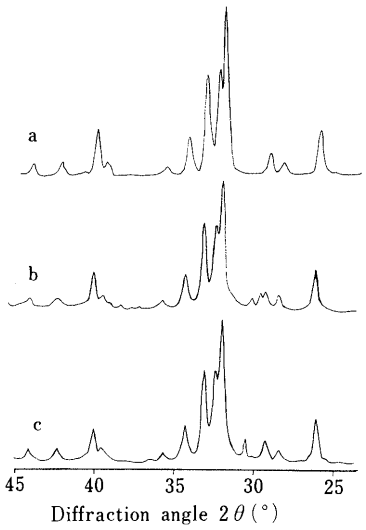

Fig. 1. Effect of Laser Irradiation on X-Ray Powder Diffraction Profiles of Hydroxyapatite Cement

a, synthetic hydroxyapatite; b, apatite cement containing 40% seed crystal; c, laser-irradiated cement containing 40% seed crystal.

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were somewhat sharper than those before irradiation. However, the diffraction peaks of the cements were broader than those of the synthetic HAP, indicating that they had an apatite with a low-crystallinity. Munemiya *et al.*[8] reported that the low-crystallinity apatite, which showed a broader X-ray diffraction profile, had a higher affinity for hard tissue than the high-crystallinity apatite. Therefore, we believe that after laser irradiation the hydroxyapatite-containing fixed cement has a high affinity for hard tissue because of its low crystallinity.

Figure 2 shows the effect of CO_2 laser irradiation on the FT-IR spectra of apatite cement. The apatite cements before and after irradiation had typical HAP IR spectra. The bands at 1410 and 1460 cm^{-1} in the HAP IR spectrum were due to carbonate ion impurities.[11] Legeros *et al.*[12] reported that the amount of carbonate ion included in the carbonate-containing apatite was lower after heat treatment at 400—600 °C. The absorption intensity of the hydroxyapatite cement in the wavenumber range from 1400 to 1500 cm^{-1} was lower after laser treatment. This indicates that the carbonate content in the cement was lower because the carbonate ion was transformed to CO_2 by the laser energy. Nelson *et al.*[13] reported that HAP crystals absorbed CO_2 laser light energy very well because the wavenumber of the CO_2 laser light was 943 cm^{-1} (10.6 μm) and adjusted to the IR absorption band of the phosphate group at 930—1100 cm^{-1}.

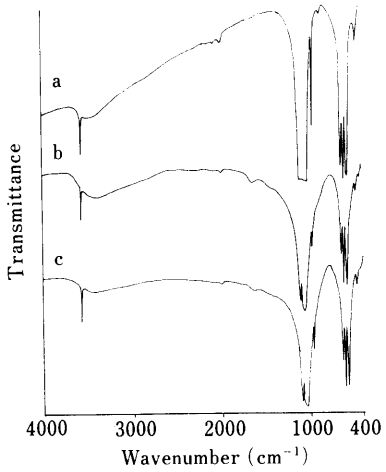

Fig. 2. Effect of Laser Irradiation on FT-IR Spectra of the Hydroxyapatite Cement

a, synthetic hydroxyapatite; b, apatite cement containing 40% seed crystal; c, laser-irradiated cement containing 40% seed crystal.

Figure 3 shows the SEM photographs of laser-irradiated apatite cement. The intact apatite cement aggregated, forming a surface by the particles less than 0.5 μm in diameter, but after irradiation these particles bonded together and formed secondary particles. This indicates that laser energy increased recrystallization of the surface of the apatite with lower crystallinity.

Effect of Laser Irradiation on the Surface Hardness of Apatite Cement The surface of tooth enamel which was irradiated at a low energy density (10—60 J/cm^2), had less subsurface acid demineralization than non-irradiated controls,[14] but the teeth decomposed after irradiation by > 100 J/cm^2.[15] Therefore, an irradiation density of 64.9 J/cm^2 (50 W, 2 s) was used in this investigation to prevent damage to surrounding hard tissue. Figure 4 shows the relationship between Vicker's hardness and laser

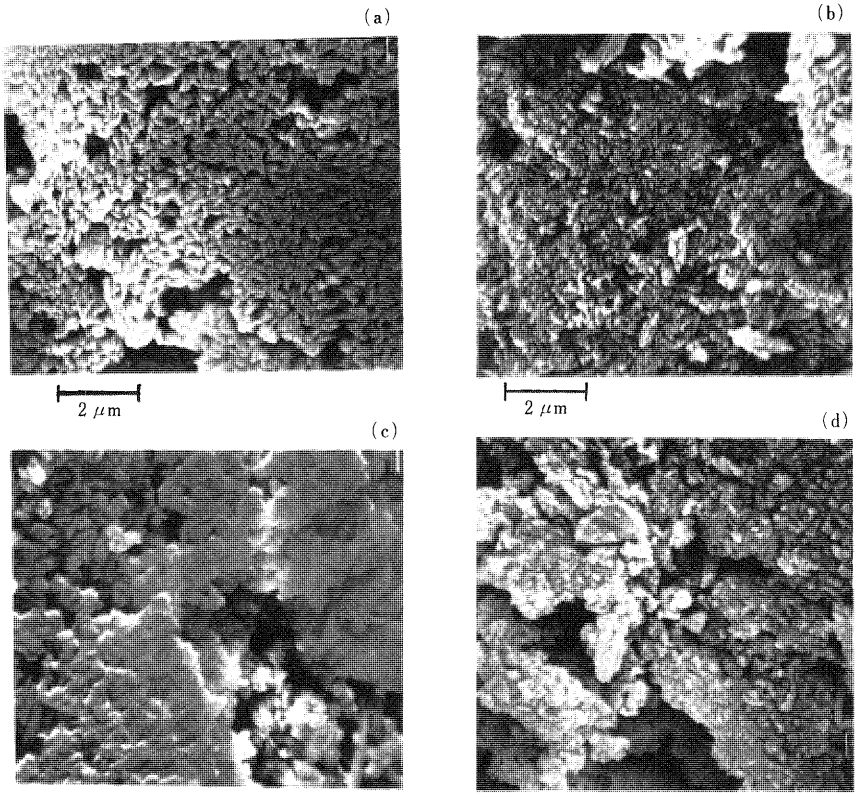

Fig. 3. SEM Photographs of Surface Morphology of Laser-Irradiated Hydroxyapatite Cement (× 10000)

a, intact apatite cement containing 40% seed crystal; b, laser-irradiated cement (50 W, 2 s × 5) containing 40% seed crystal; c, intact apatite cement containing 3% seed crystal; d, laser-irradiated cement (50 W, 2 s × 5) containing 3% seed crystal.

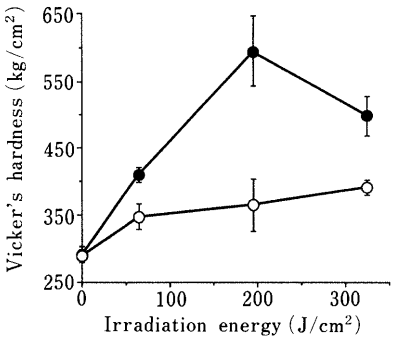

Fig. 4. Effect of Laser Irradiation Energy Density on the Surface Mechanical Strength of Hydroxyapatite Cement

○, apatite cement containing 40% seed crystal; ●, apatite cement containing 3% seed crystal.

irradiation energy. The surface mechanical strengths of laser-irradiated apatite cement containing 3% or 40% seed crystals noticeably increased with increased laser energy, and the laser-irradiated apatite cement containing 3% seed crystals was harder than that containing 40% seed crystals. This shows that the cement system containing a lower percentage of seed crystals was hardened to a greater extent by CO_2 laser irradiation. Munemiya *et al.*[8] reported that low-crystallinity HAP apatite had transformed into high-crystallinity HAP after heat treatment at 250—1250 °C. Therefore, the high crystallinity HAP was stable during laser irradiation and there was no crystal transformation required for the particles to bond. The HAP seed crystal used in this cement system had a high crystallinity, so there was no enhancement of the cement hardness by the laser irradiation. Therefore, the cement containing 3% of the seed crystals was harder than that containing 40%. We believe that the part of the lower crystallinity apatite which contained carbonate ion impurities (Fig. 2), which was transformed from TTCP and DCPD, was recrystallized to a higher crystallinity by the laser energy.

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The Relationship between Van der Waals Volume of 4-Substituent of Phenol and Sulfation in the Rat

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The influence of 4-substituent of phenol on the sulfate conjugation was studied in the rat. 4-Substituents used were 4-ethyl, 4-*tert*-butyl and 4-phenyl. The *in vivo* and *in vitro* sulfations decreased in the order of phenol, 4-ethylphenol, 4-*tert*-butylphenol and 4-phenylphenol. The activity of sulfotransferase was correlated with the van der Waals volume of 4-substituent. The modification of thiol and arginyl residues of cytosol protein showed a similar degree of inactivation of sulfotransferase to all substituents used. Inactivation by the modification of histidyl residue of the protein increased with the increase of the van der Waals volume of 4-substituent. This result suggests that histidyl residue recognized van der Waals volume of 4-substituent.

Keywords sulfotransferase; 4-substituent, phenol; van der Waals volume; protein modification

The formation of sulfate esters is well known as a route of metabolism for xenobiotics with a phenolic group. Substrate specificity of purified sulfotransferase in the rat liver has been studied.[1,2] Duffel and Jakoby[3] reported the effect of the substitution of phenol on sulfation from the Hammett theory. However, little is known of the effect of a substituent without an electronic factor or a wealky ionic substituent of phenol. This report used 4-ethyl, 4-*tert*-butyl and 4-phenyl substituents of phenol, and investigated to clarify the influence of these substituents on sulfation.

Experimental

Animals Male Wistar rats weighing 240—250 g were used.

Materials The following labelled compounds were used: phenol (^{14}C-u) was purchased from New England Nuclear. The 4-ethyl, 4-*tert*-butyl and 4-phenyl derivatives of phenol (^{14}C-u) were synthesized by Daiichi Pure Chemicals Co. Phenol and its derivatives were purchased from Kanto Chemicals Co. Sulfatase (type VI), *p*-chloromercuribenzoic acid (PCMB), 2,3-cyclohexanedione (CD) and diethylpyrocarbonate (DEP) were purchased from Sigma.

***In Vivo* Sulfate Formation** Labelled compounds (specific activity 50 μCi/mmol) were injected intravenously into rats at a dose of 120 μmol/kg. Urine and bile were collected during 24 h. Aliquots of excretes were subjected to thin layer chromatography (TLC).

Cytosol Preparation The rat liver was homogenized in 3 volumes of 0.25 M sucrose, and then centrifuged at 100000*g* for 1 h at 4 °C. The cytosolic fraction was used as an enzyme source.

***In Vitro* Sulfate Formation** The sulfate formation of labelled compounds (0.75 mM) was carried out by using the cytosol of the rat liver according to the method of Lyman and Poland.[4] After the reaction was stopped by the addition of a half volume of methanol, proteins were removed by centrifugation. Supernatant was subjected to TLC.

Identification of Sulfate TLC of the *in vitro* samples *versus* each of the substrates showed the occurrence of a spot with a *Rf* value lower than any of the substrates. When the *in vitro* samples were incubated with sulfatase (500 units) at 37 °C for 1 h, the aforementioned spots did not appear and only spots with *Rf* values corresponding to the substrates remained. Additionally, the spot with a low *Rf* value did not appear in the *in vitro* system lacking sodium sulfate. The spot of sulfate in the *in vivo* sample was confirmed by performing cochromatography with the sulfate which was obtained from the *in vitro* system.

Modification of Cytosol Protein The cytosol protein modification with PCMB was carried out in the manner of Suzuki *et al.*[5]: the mixture containing PCMB (5 μmol), cytosol (0.33 mg protein) and 0.1 ml of 0.01 M phosphate buffer (pH 7.0) was incubated at 20 °C for 15 min. The modification with CD was carried out in the manner of Patthy and Smith[6]: the mixture containing CD (1 μmol), cytosol (0.16 mg protein) and 0.1 ml of 0.2 M borate buffer (pH 7.5) was incubated at 20 °C for 1 h. The modification with DEP was carried out in the manner of Blumberg *et al.*[7]: the mixture containing DEP (0.8 μmol), cytosol (0.02 mg protein) and 0.1 ml of 0.1 M Tris–HCl buffer (pH 6.5) was incubated at 20 °C for 30 min.

Determination of Van der Waals Volume The van der Waals volume of 4-substituent was calculated according to Moriguchi *et al.*[8]

TLC and Radioactivity Assay Plates (Merck Silica gel F_{254}) were developed in *n*-butanol, 15% aq. ammonia (5:1). The radioactivity areas corresponding to sulfate were assayed in the manner previously reported.[9]

Results and Discussion

The sulfation of phenol and its derivatives were examined by the *in vitro* and *in vivo* experiments (Table I). The *in vitro* formation rate of sulfate decreased in the other of phenol, 4-ethylphenol, 4-*tert*-butylphenol and 4-phenylphenol. This result indicates that the increase of a relative mass of 4-substituent decreased the formation of sulfate. The cytosol fraction used in the present study contains sulfotransferases and the enzyme system to form adenosin-5′-phosphosulphate (PAPS). Thus, the difference in the

TABLE I. Influence of 4-Substituent of Phenol on *in Vivo* and *in Vitro* Sulfation

Substituents	Sulfation	
	in Vitro (nmol/min·mg protein)	*in Vivo* Sulfate in urine (μmol)
4-H	209 ±32	21.5±1.8
4-Ethyl	162 ±30	18.6±1.2
4-*tert*-Butyl	65.5±11.8	1.5±0.2
4-Phenyl	21.1± 2.7	0.2±0.0

Data are the mean±S.E. of five animals or five experiments.

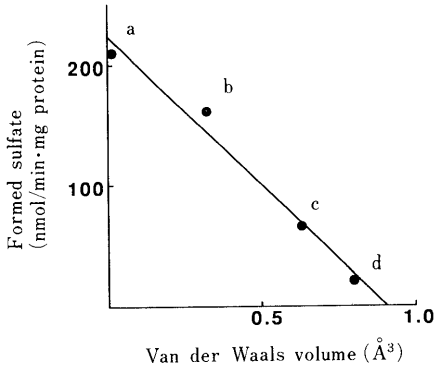

Fig. 1. Relationship between *in Vitro* Sulfation and Van der Waals Volume of 4-Substituent

a, 4-H; b, 4-ethyl; c, 4-*tert*-butyl; d, 4-phenyl.

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enzymatic activity among substrates reflects the degree of influence of 4-substituents, since PAPS might be formed equally. The *in vitro* data were used to examine the influence of 4-substitution on sulfation. Duffel and Jakoby[3] discussed the effect of electron-withdrawing 4-substituents of phenol on sulfation. However, another explanation would be possible for the effect of 4-substituents without an electronic factor and with a weak electronic factor on sulfation. Thus, the *in vitro* data in Table I was correlated with van der Waals volume of 4-substituent (Fig. 1). Equation 1 was calculated from the data in Fig. 1.

$$A = -246.4V_w + 222.9 \quad (r = 0.9792,\ s = 464.0) \qquad (1)$$

where A is the formation rate of sulfate, V_w is van der Waals volume of 4-substituent, r is the correlation coefficient and s is the standard deviation. The value of r in Eq. 1 indicates that sulfotransferase activity decreased by the increase of the van der Waals volume of 4-substituent. Sulfate amounts in the urine following the intravenous injection of labelled compounds decreased in the same order of 4-substituent as the *in vitro* sulfation. However, the correlation between the *in vivo* sulfation and V_w ($r = 0.9066$) was low compared with the *in vitro* result. No significant amount of sulfate was found in the biliary excretes. The evaluation of the relationship between the *in vivo* sulfation and 4-substituent seems to be not simple because 4-substituent may influence the duration of blood concentration, and the sulfate conjugation may compete with glucuronide conjugation. Despite these difficulties of the evaluation, the similarity in the profile of sulfation between *in vivo* and *in vitro* was observed; therefore, this result suggests that the enzyme response for 4-substituent of phenol reflected the *in vivo* sulfation.

To clarify the recognition site of 4-substituents in the enzyme, protein was modified by use of the following reagents: PCMB, modifier of the thiol group of protein, CD; modifier of arginyl residue, DEP; modifier of histidyl residue. As shown in Table II, the modifications with PCMB and CD decreased sulfation, showing no significant difference in the decrease ratio among substrates. This result suggests that amino acid with a thiol group and arginyl residue are located near the binding site of the hydroxy group of phenol and play a role in the interaction of the phenolic group with a sulfate donor. The inactivation of sulfotransferase by modification with DEP increased with the relative molecular mass of 4-substituent. Thus, the inactivation of the enzyme was correlated with van der Waals volumes of 4-substituent (Fig. 2). Using data in Fig. 2, the following equation was derived.

$$I = 53.91V_w + 43.39 \quad (r = 0.9667,\ s = 36.01) \qquad (2)$$

where I is the inhibition ratio by the modification. From the high correlation in Eq. 2, the histidyl residue of sulfotransferase is estimated to play a role in the recognition of the van der Waals volume of 4-substituent. The presence of sulfotransferase isozymes is well known.[1,2] The present work did not use purified sulfotransferase. Therefore, it is necessary to take into account that the present data reflected isozymes which catalyzed mainly substrate used, and these isozymes recognized van der Waals volume of 4-substituent with histidyl residues.

TABLE II. Inhibition of Sulfation of Phenol and Its Derivatives by Modification of Cytosol Protein

Substituents	Inactivation (%)		
	PCMB	CD	DEP
4-H	64.6	78.2	45.2
4-Ethyl	59.8	72.1	63.5
4-*tert*-Butyl	60.3	69.5	72.7
4-Phenyl	62.5	73.3	90.1

PCMB, *p*-chloromercuribenzoic acid; CD, 1,2-cyclohexanedione; DEP, diethylpyrocarbonate. Data are the mean of five experiments.

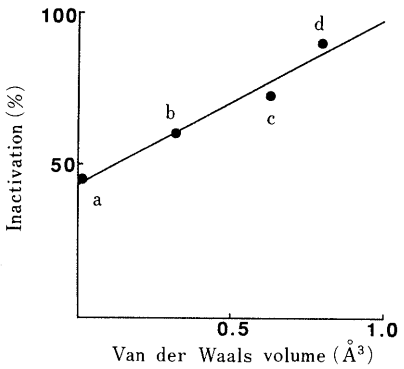

Fig. 2. Relationship between Inhibition of Sulfation by Modification of Histidyl Residue and Van der Waals Volume of 4-Substituent of Phenol

Each point represents the mean of five experiments. Key to 4-substituent corresponds to Fig. 1.

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EFFICIENT ENZYMATIC PREPARATION OF (+)- AND (–)-COREY LACTONE DERIVATIVES

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Two pairs of Corey lactone derivatives, (+)-**1**, (–)-**2** and (+)-**3**, (–)-**4** have been efficiently prepared in high optical purity by enzymatic esterification and enzymatic hydrolysis catalyzed by lipases from *Pseudomonas sp.*

KEYWORDS Corey lactone; lipase PS; lipase AK; enzymatic transesterification; enzymatic hydrolysis

Prostaglandins and prostacyclins are important biologically active compounds and a number of synthetic methods of preparing them are reported.[1,2] The (–)-Corey lactone derivative is one of the most important key intermediates in the synthesis of prostaglandins, prostacyclins and their derivatives. (–)-Corey lactone derivatives are mode by numerous methods such as the optical resolution method, the asymmetric Diels-Alder method, and so on, but there are some problems including high cost of reagents for optical resolution and/or, long reaction steps.[1] Recently enzymatic resolution of prochiral substrates is a well established procedure for the preparation of enantiomerically pure products.[3] We report here a more convenient preparation of (+)- and (–)-Corey lactones based on a lipase-catalyzed transesterification of (±)-**1** and enzymatic hydrolysis of (±)-**3** (Chart).[4]

First, we studied the enzymatic transesterification of (±)-Corey lactone diol **1**.[5] After several experiments using lipases in organic solvent, (+)-Corey lactone diol **1** and (–)-Corey lactone acetate **2** were obtained at ≥99% ee[6] in good yield respectively, using lipase AK (Amano, *Pseudomonas sp.*) and vinyl acetate as the acylating reagents.[7]

Next, we tested the enzymatic hydrolysis of (±)-Corey lactone acetate **3**.[8] Treatment of (±)-**3** with lipase PS (Amano, *Pseudomonas sp.*) in 0.1 M phosphate buffer pH 7.6 at 30 °C for 48 h gave (+)-acetate **3** and (–)-alcohol **4** both with ≥99% ee[9] in good yield.[10]

In summary, efficient and expeditious routes for the preparation of both enantiomers of Corey lactone derivatives, (±)-**1**, (±)-**3** under exceptionally mild and easy conditions are reported.[11] The use of these optically pure Corey lactone derivatives in the synthesis of didemnenone C, D and marine prostanoids is currently under investigation.[12]

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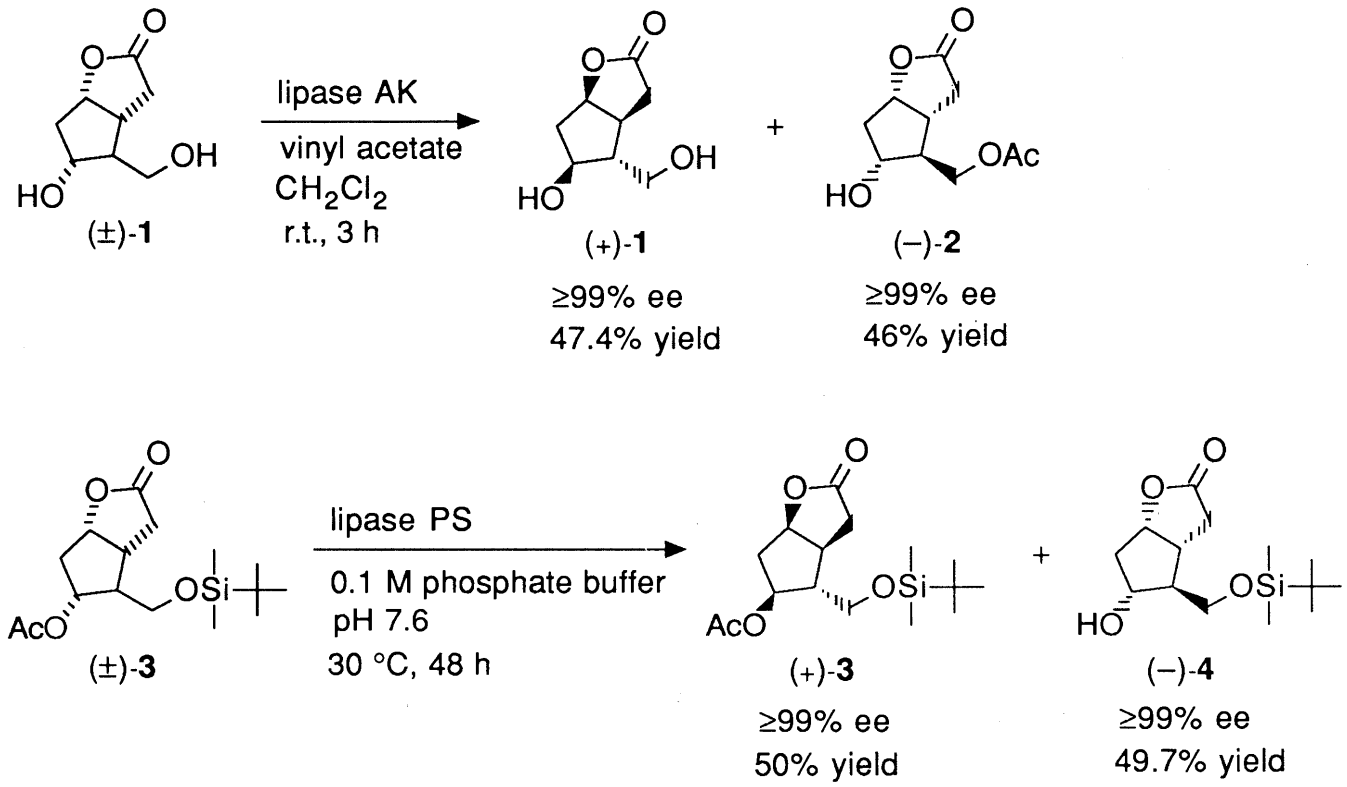

Chart

ACKNOWLEDGEMENT We thank Amano Pharmaceutical Co. LTD. for supplying us with the lipase AK and PS.

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5) The requisite precursor, (±)-diol **1** was prepared by the methods in the literature: a) P. A. Grieco, *J. Org. Chem.*, **37**, 2362 (1972); b) E. J. Corey and J. Mann, *J. Am. Chem. Soc.*, **95**, 6832 (1973); c) I. Tömösközi, L. Gruber, G. Kovács, I. Székely and V. Simonidesz, *Tetrahedron Lett.*, **1976**, 4639.

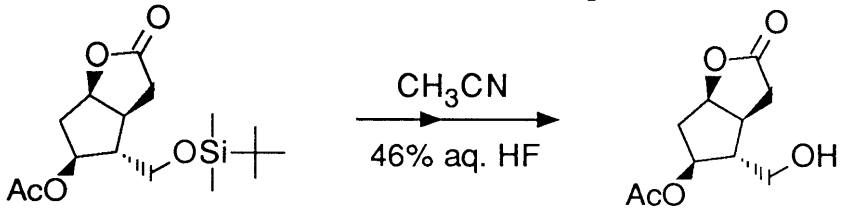
6) The enantio excess of (+)-**1** was determined by the comparison of the NMR spectrum (500 MHz) of the (+)-mono-Mosher ester of its primary alcohol

with the corresponding (+)-mono-Mosher ester of (±)-**1**. And the enantio excess of (–)-**2** was also determined by the comparison of the NMR spectrum (500 MHz) of its (+)-Mosher ester with the (+)-Mosher ester of (±)-**2**, which was prepared from (±)-**1**.

7) 109 mg of (±)-**1**, vinyl acetate (10 eq., 0.59 ml) and 100 mg lipase AK in CH_2Cl_2 were stirred at r.t. for 3 h. After filtration of the enzyme, the products were separated by silica gel column chromatography.

8) (±)-Acetate **3** was prepared by selective silylation of (±)-**1** followed by acetylation.

9) (+)-**3** was converted to (+)-**5** by acid treatment prior to derivatization.



(+)-**3** (+)-**5**

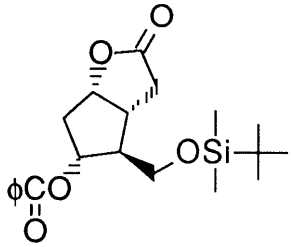
The enantio excess of (+)-**3** and (–)-**4** was determined by the comparison of the NMR spectrum (500 MHz) of their (+)-Mosher esters of (+)-**5** with (–)-**4** and the (+)-Mosher esters of (±)-**5** with (±)-**4**, which were prepared from (±)-**3**.

10) (±)-**3**, 2.3 g, and lipase PS, 1.15 g, were suspended in 230 ml phosphate the buffer and the mixture was stirred at 30 °C for 48 h. After filtration of the enzyme, the products were extracted with AcOEt. (+)-**3** and (–)-**4** were obtained by silica gel column chromatography.

11) All compounds have been fully characterized by ^1H NMR and MS spectrum. Data for optical rotations are as follows: $[\alpha]_D$ +31.6° (*c* 0.688; MeOH) (+)-**1** [lit.,[5c] $[\alpha]_D$ –43.4° (*c* 1.46, MeOH) for (–)-**1**] and –16.4° (*c* 1.158, $CHCl_3$) (–)-**2** [lit.,[13] $[\alpha]_D$ –17.9° (*c* 0.518, $CHCl_3$)]; $[\alpha]_D$ +48.0° (*c* 1.016, $CHCl_3$) (+)-**3** [lit.,[13] $[\alpha]_D$ –47.4° (*c* 1.294, $CHCl_3$) for (–)-**3**] and –14.2° (*c* 1.008, $CHCl_3$) (–)-**4** [lit.,[13] $[\alpha]_D$ –15.3° (*c* 1.004, $CHCl_3$)].

12) For earlier application of optically active Corey lactone in natural products synthesis, see; T. Sugahara, T. Ohike, M. Soejima, and S. Takano, *J. Chem. Soc., Perkin Trans. 1*, **1990**, 1824.

13) (–)-**2**, (–)-**3** and (–)-**4** were synthesized respectively from authentic (–)-**6**, which was supplied by NISSAN Chemical Industry Ltd.



(–)-**6**

(Received August 5, 1991)

SCHUBERTOSIDES A - D, NEW (22*S*)-HYDROXYCHOLESTANE GLYCOSIDES FROM *ALLIUM SCHUBERTII*

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Four new (22*S*)-hydroxycholestane glycosides, schubertosides A - D, have been isolated from the bulbs of *Allium schubertii*. Their structures were determined by spectroscopic analysis and hydrolysis. The advanced Mosher's method was applied to determine the C-22 absolute configuration.

KEYWORDS *Allium schubertii*; Liliaceae; (22*S*)-hydroxycholestane glycoside; schubertoside

Some *Allium* plants (Liliaceae) have long been used in medicine throughout the world.[1] The existence of steroidal saponins in the genus *Allium* was well documented.[2] Previously, we have isolated five new spirostanol saponins from the bulbs of *Allium giganteum* and *A. aflatunense*.[3] Here, we report the structures of new cholestane glycosides isolated from the bulbs of *A. schubertii*.

The *n*-BuOH-soluble phase of the MeOH extract of the plant material (5.5 kg) was chromatographed on silica gel, Diaion HP-20 and Cosmosil (ODS) to give **1** (576 mg), **2** (272 mg), **3** (54 mg) and **4** (115 mg) as white powders.

Compound **1** (schubertoside A),[4] $C_{39}H_{64}O_{12}$ (SI-MS, *m/z* 725 $[M + H]^+$), $[\alpha]_D$ -16.0° (MeOH), was hydrolyzed with 1*N* HCl to give D-galactose, L-rhamnose and an aglycon (**1a**), $C_{27}H_{44}O_3$ (EI-MS, *m/z* 417 $[M + H]^+$), $[\alpha]_D$ +36.0° ($CHCl_3$). The spectral data indicated the **1a** is a cholestane derivative with an α,β-unsaturated carbonyl group and two secondary hydroxyl groups. The presence of 3-oxo-4-ene group was shown by agreement of the ^{13}C signals due to the A and B rings between **1a** and (22*S*)-26-methyl-24-methylenecholest-4-en-3-one,[5] which was also supported by the fragment ion peaks at *m/z* 148 and 124 in the EI-MS.[5,6] Also, double resonance experiments in the ^1H-NMR spectrum and comparison of the ^{13}C-NMR data of **1a** with that of cholesterol proved the two hydroxyl groups to be located at the C-16 and C-22 positions. The configuration of the C-16 hydroxyl group was determined to be β as the H-18 methyl proton resonated at δ 1.06; that of cholesterol appeared at δ 0.70.[7] The advanced Mosher's method allowed us to assign the absolute configuration of the C-22 hydroxyl position as *S* (Fig. 1).[8] The ^1H- and ^{13}C-NMR spectra of **1** assigned the structure of the sugar moiety including the configurations at the anomeric centers as α-L-rhamnopyranosyl-(1→3)-β-D-galactopyranose, which was concluded to be linked at the C-16 hydroxyl group by the observation of the glycosylation-induced shift at the C-16 carbon signal of **1** as compared with that of **1a**. Thus, the structure of **1** was formulated as (22*S*)-16β,22-dihydroxycholest-4-en-3-one 16-*O*-α-L-rhamnopyranosyl-(1→3)-β-D-galactopyranoside.

Compound **2** (schubertoside B),[9] $C_{39}H_{66}O_{12}$ (SI-MS, *m/z* 749 $[M + Na]^+$), $[\alpha]_D$ -44.0° (MeOH), gave D-galactose, L-rhamnose and an aglycon (**2a**), $C_{27}H_{46}O_3$ (EI-MS, *m/z* 400 $[M - H_2O]^+$), $[\alpha]_D$ -62.0° ($CHCl_3$). The ^{13}C-NMR signals of **2** were in good agreement with those of **1** except for the signals arising from the A and B rings. The presence of a 3β-hydroxy-5-ene group was indicated by comparing of the ^{13}C assignments of **2** with those of cholesterol. So, the structure of **2** was determined to be (22*S*)-cholest-5-ene-3β,16β,22-triol 16-*O*-α-L-rhamnopyranosyl-(1→3)-β-D-galactopyranoside.

Compound **3** (schubertoside C),[10] $C_{45}H_{76}O_{17}$ (SI-MS, *m/z* 911 $[M + Na]^+$), $[\alpha]_D$ -58.0° (MeOH), was hydrolyzed

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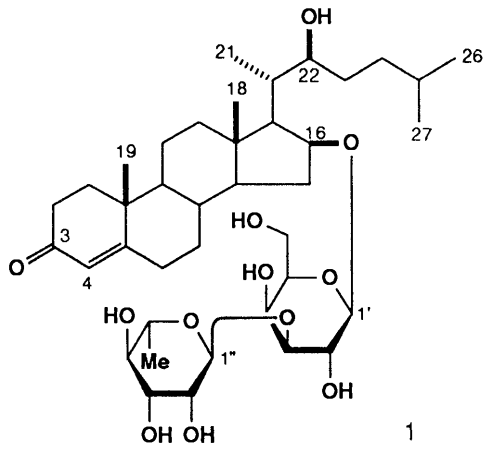

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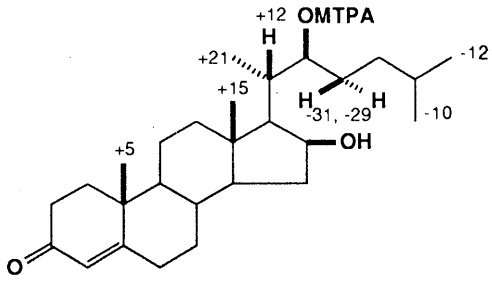
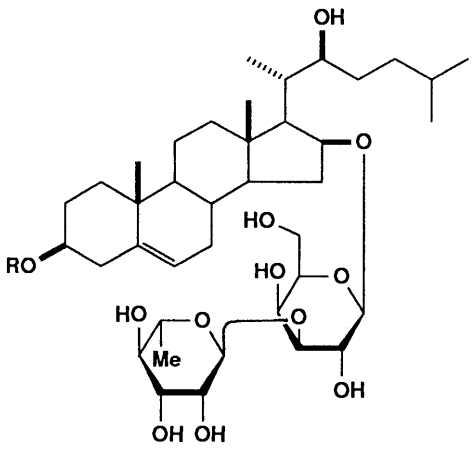


Fig. 1. Chemical Shift Differences between **1a** (*S*)-MTPA Ester and **1a** (*R*)-MTPA Ester
$\Delta\delta$ (Hz) = $\delta_{(S)\text{-MTPA}} - \delta_{(R)\text{-MTPA}}$

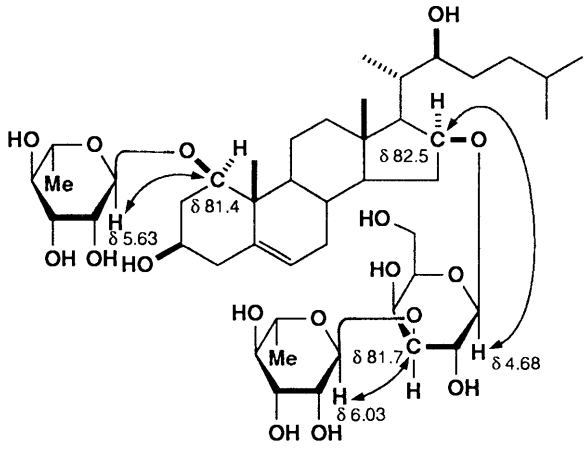
with β-glucosidase to provide D-glucose and **2**. In the ^{13}C-NMR spectrum of **3**, the signals of the C-3 and C-16 were shifted to lower fields (ca 7 ppm) by *O*-glycosylation compared with those of **2a**. The structure was characterized as (22*S*)-cholest-5-ene-3β,16β,22-triol 3-*O*-β-D-glucopyranosido,16-*O*-α-L-rhamnopyranosyl-(1→3)-β-D-galactopyranoside.

Compound **4** (schubertoside D),[11] $C_{45}H_{76}O_{17}$ (SI-MS, *m/z* 890 $[M + 2H]^+$), $[\alpha]_D$ -52.0° (MeOH), was hydrolyzed with 1*N* HCl to give D-galactose, L-rhamnose and an aglycon (**4a**), $C_{27}H_{46}O_4$ (EI-MS, *m/z* 416 $[M - H_2O]^+$), $[\alpha]_D$ -60.0° ($CHCl_3$). The spectral data of **4a** were essentially analogous with those of **2a** and suggestive of a cholestane derivative of the same type. In the ^{13}C-NMR spectrum of **4a**, downfield shifts of the signals due to the C-2, C-6, C-10 and C-11, accompanied by upfield shifts of the signals due to the C-3, C-5, C-9 and C-19 compared with those of **2a** indicated the presence of a 1β-hydroxyl group in addition to the 3β, 16β and (22*S*)-hydroxyl groups. The ^{13}C-NMR spectrum of **4** revealed the existence of two terminal α-L-rhamnopyranosyl units and a 3-substituted β-D-galactopyranosyl unit. The anomeric proton signals at δ 6.03 (br s, rhamnose), 5.63 (br s, rhamnose) and 4.68 (d, J = 7.7 Hz, galactose) showed long-range correlations with the ^{13}C signals at δ 81.7 (galactose C-3), 81.4 (aglycon C-1) and 82.5 (aglycon C-16), respectively, in the HMBC spectrum.[12] Accordingly, the structure of **4** was elucidated as (22*S*)-cholest-5-ene-1β,3β,16β,22-tetraol 1-*O*-α-L-rhamnopyranosido, 16-*O*-α-L-rhamnopyranosyl-(1→3)-β-D-galactopyranoside.

While a few (22*R*)-hydroxycholestane derivatives were reported,[13] to the best of our knowledge, schubertosides are the first (22*S*)-hydroxycholestane derivatives from natural origin. Cholestane bisdesmosides as schubertosides C and D are very rare in nature.[14]



R
2 H
3 Glc



4 ⌒: ^{1}H-^{13}C Long-Range Correlation

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4) IR (KBr) ν: 3460, 1660 cm^{-1}. UV (MeOH) λ: 242 nm (ε 13800). ^{1}H-NMR (C_5D_5N, 400 MHz) δ: 6.04 (1H, br s, H-1"), 4.68 (1H, d, J = 7.7 Hz, H-1'), 1.18 (3H, d, J = 7.0 Hz, H-21), 1.02 (3H, s, H-19), 0.96 (3H, s, H-18), 0.95 and 0.93 (each 3H, d, J = 6.4 Hz, H-26 and H-27). ^{13}C-NMR (C_5D_5N, 100.6 MHz) δ: (aglycon C-1 - C-27) 35.8, 34.4, 198.4, 124.1, 170.7, 32.9, 32.2, 35.2, 53.9, 38.7, 21.0, 39.8, 42.5, 54.4, 36.8, 82.4, 57.9, 12.7, 17.1, 35.9, 13.5, 72.1, 33.4, 36.9, 28.7, 23.1, 23.0; (galactose C-1' - C-6') 107.4, 73.4, 81.6, 70.0, 76.7, 62.0; (rhamnose C-1" - C-6") 104.1, 72.4, 72.7, 74.2, 69.7, 18.6.

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9) IR (KBr) ν: 3450 cm^{-1}. ^{1}H-NMR (C_5D_5N) δ: 6.04 (1H, br s, H-1"), 4.68 (1H, d, J = 7.7 Hz, H-1'), 1.19 (3H, d, J = 7.0 Hz, H-21), 1.07 (3H, s, H-19), 0.98 (3H, s, H-18), 0.95 and 0.93 (each 3H, d, J = 6.3 Hz, H-26 and H-27). ^{13}C-NMR (C_5D_5N) δ: (aglycon C-1 - C-27) 37.1, 32.1, 71.3, 43.5, 142.0, 121.3, 32.7, 31.9, 50.6, 36.9, 21.2, 40.1, 42.5, 55.3, 37.8, 82.5, 58.0, 12.8, 19.7, 36.0, 13.5, 72.1, 33.4, 36.8, 28.8, 23.1, 23.1; (galactose C-1' - C-6') 107.4, 73.5, 81.7, 70.0, 76.7, 62.0; (rhamnose C-1" - C-6") 104.1, 72.4, 72.7, 74.2, 69.8, 18.6.

10) IR (KBr) ν: 3400 cm^{-1}. ^{1}H-NMR (C_5D_5N) δ: 6.02 (1H, br s, H-1'''), 5.03 (1H, d, J = 7.7 Hz, H-1'), 4.67 (1H, d, J = 7.7 Hz, H-1"), 1.17 (3H, d, J = 7.0 Hz, H-21), 0.96 - 0.91 (12H, H-18, H-19, H-26 and H-27). ^{13}C-NMR (C_5D_5N) δ: (aglycon C-1 - C-27) 37.1, 30.3, 78.2, 39.4, 140.9, 121.9, 32.1, 31.8, 50.5, 37.0, 21.1, 40.0, 42.5, 55.3, 37.5, 82.5, 57.9, 12.8, 19.5, 35.9, 13.4, 72.1, 33.4, 36.8, 28.8, 23.1, 23.1; (glucose C-1' - C-6') 102.6, 75.4, 78.5, 71.8, 78.6, 62.9; (galactose C-1" - C-6") 107.4, 73.5, 81.7, 70.0, 76.7, 62.1; (rhamnose C-1"' - C-6"') 104.1, 72.4, 72.7, 74.2, 69.8, 18.6.

11) IR (KBr) ν: 3450 cm^{-1}. ^{1}H-NMR (C_5D_5N) δ: 6.03 (1H, br s, H-1'''), 5.63 (1H, br s, H-1'), 4.68 (1H, d, J = 7.7 Hz, H-1'), 1.23 (3H, s, H-19), 1.13 (3H, d, J = 7.0 Hz, H-21), 1.01 (3H, s, H-18), 0.94 (3H, d, J = 6.2 Hz, H-26 or H-27), 0.91 (3H, d, J = 6.3 Hz, H-26 or H-27). ^{13}C-NMR (C_5D_5N) δ: (aglycon C-1 - C-27) 81.4, 36.0, 68.1, 43.8, 139.2, 125.1, 31.6, 33.5, 50.9, 42.9, 24.8, 40.6, 42.3, 55.4, 37.3, 82.5, 58.1, 12.9, 14.6, 36.0, 13.8, 72.1, 33.4, 36.9, 28.8, 23.1, 23.1; (rhamnose C-1' - C-6') 97.8, 72.9, 73.0, 73.7, 70.7, 18.7; (galactose C-1" - C-6") 107.4, 73.5, 81.7, 70.0, 76.7, 62.1; (rhamnose C-1"' - C-6"') 104.1, 72.4, 72.7, 74.2, 69.8, 18.6.

12) Assignments of the ^{13}C signals of the C-1 (δ 81.4), C-16 (δ 82.5), C-3' (δ 81.7) with the similar chemical shifts were carried out by the ^{1}H-^{13}C COSY spectrum.

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BUDDLEJASAPONINS I - IV, FOUR NEW OLEANANE-TRITERPENE SAPONINS FROM THE AERIAL PARTS OF *BUDDLEJA JAPONICA* HEMSL.

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Four new oleanane-type triterpene saponins, named buddlejasaponins I (**1**), II (**2**), III (**3**) and IV (**4**) were isolated from the aerial parts of *Buddleja japonica* HEMSL., together with a known saponin saikosaponin a (**5**). Their structures were elucidated on the basis of the chemical and spectroscopic studies.

KEYWORDS *Buddleja japonica*; Buddlejaceae; buddlejasaponin; saikosaponin a; oleanane-triterpene saponin

Buddleja species are used in traditional medicine in many parts of the world[1] and phytochemical investigation has shown the presence of flavonoids,[2] iridoids,[3] sesquiterpenoids,[4] phenylethanoids[5] and lignans.[6] Houghton *et al.* reported the anti-hepatotoxic activity of the extract and constituents (acteoside, echinacoside, linarin) of *B. officinalis* L., *B. americana* L. and *B. globosa* HOPE.[7] We reported the isolation of acylated iridoid glycosides from the whole plants of *B. japonica* HEMSL.[8] Now we report the isolation of five saponins from the aerial parts of *B. japonica* HEMSL.

The MeOH extract of the dried aerial parts of *B. japonica* HEMSL. was partitioned between ether and water and then the water soluble portion was chromatographed on MITSUBISHI Diaion HP-20 and eluted successively with 50% MeOH and 100% MeOH. From 100% MeOH eluate, four new saponins, named buddlejasaponins I - IV, and a known saponin, saikosaponin a, were isolated by reversed-phase HPLC [ODS; CH_3CN-H_2O (30 : 70 → 38 : 62)]. Saikosaponin a (**5**) was identified by comparison of the spectral data with reported data.[9]

Buddlejasaponin I (**1**), amorphous powder, $[\alpha]_D^{25}$ +34.4° (*c*=0.91, MeOH), $C_{54}H_{88}O_{22}\cdot 5/2\ H_2O$ (Calcd. C, 57.18; H, 8.26. Found C, 57.30; H, 8.54), FAB-MS *m/z*: 1111 $[M + Na]^+$. ^{1}H-NMR [10]: δ 0.90 (3H, s, H_3-30), 0.94 (3H, s, H_3-29), 0.97 (3H, s, H_3-25), 1.05 (3H, s, H_3-24), 1.09 (3H, s, H_3-27), 1.37 (3H, s, H_3-26), 3.33 (1H, br d, *J*=7 Hz, H-28), 3.68 (1H, d, *J*=10 Hz, H-23), 4.14 (1H, dd, *J*=12, 5 Hz, H-3), 4.36 (1H, d, *J*=10 Hz, H-23), 4.39 (1H, d, *J*=7 Hz, H-28), 4.49 (overlapped, H-16), 4.94 (1H, d, *J*=8 Hz, H-1 of fuc), 5.21 (1H, d, *J*=8 Hz, H-1 of glc at C-3 of fuc), 5.56 (1H, d, *J*=8 Hz, H-1 of glc at C-2 of fuc), 5.65 (1H, dd, *J*=10, 3 Hz, H-12), 5.77 (1H, br s, H-1 of rham), 5.97 (1H, br d, *J*=10 Hz, H-11). Methanolysis gave saikogenin A (**6**),[11] while acid hydrolysis liberated fucose, glucose and rhamnose in the ratio 1:2:1, as the sugar moiety. Detailed proton spin decoupling experiments, difference NOE experiments on irradiation at each anomeric proton signal and an ^{1}H-^{13}C COSY spectrum informed us of the structure of buddlejasaponin I (**1**). In the ^{13}C-NMR spectrum, the glycosylation shifts supported the sugar linkage as shown.

Buddlejasaponin II (**2**), amorphous powder, $[\alpha]_D^{25}$ +28.2° (*c*=0.97, pyridine), $C_{53}H_{86}O_{22}\cdot 3\ H_2O$ (Calcd. C, 56.37; H, 8.21. Found C, 56.32; H, 8.33), FAB-MS *m/z*: 1097 $[M + Na]^+$. ^{1}H-NMR: δ 0.90 (3H, s, H_3-30), 0.94 (3H, s, H_3-29), 0.98 (3H, s, H_3-25), 1.06 (3H, s, H_3-24), 1.10 (3H, s, H_3-27), 1.38 (3H, s, H_3-26), 3.33 (1H, d, *J*=8 Hz, H-28), 3.71 (1H, d, *J*=11 Hz, H-23), 4.14 (1H, dd, *J*=12, 5 Hz, H-3), 4.36 (1H, d, *J*=11 Hz, H-23), 4.36 (overlapped, H-28), 4.49 (overlapped, H-16), 4.91 (1H, d, *J*=8 Hz, H-1 of fuc), 5.10 (1H, d, *J*=8 Hz, H-1 of xyl), 5.24 (1H, d, *J*=8 Hz, H-1 of glc at C-3 of fuc), 5.56 (1H, d, *J*=8 Hz, H-1 of glc at C-2 of fuc), 5.64 (1H, dd, *J*=10, 3 Hz, H-12), 5.98 (1H, br d, *J*=10 Hz, H-11). **2** gave NMR data similar to those of **1**. Acid hydrolysis liberated fucose, glucose and xylose in the ratio 1:2:1, as the sugar moiety. The difference NOE experiments on irradiation at the xylopyranosyl anomeric proton signal at δ 5.10 (1H, d, *J*=8 Hz) showed xylose to be present at the C-4 of glucose, which was attached to the C-3 of fucose.

Buddlejasaponin III (**3**), amorphous powder, $[\alpha]_D^{25}$ +38.1° (*c*=0.97, MeOH), $C_{47}H_{76}O_{17}\cdot 5/2\ H_2O$ (Calcd. C, 58.92; H, 8.52. Found C, 58.82 ; H, 8.76), FAB-MS *m/z*: 935 $[M + Na]^+$. ^{1}H-NMR: δ 0.90 (3H, s, H_3-30), 0.92 (3H, s, H_3-29), 0.94 (3H, s, H_3-25), 1.01 (3H, s, H_3-24), 1.10 (3H, s, H_3-27), 1.40 (3H, s, H_3-26), 3.33 (1H, d, *J*=7 Hz, H-28), 3.69 (1H, d, *J*=11 Hz, H-23), 4.26 (1H, dd, *J*=12, 5 Hz, H-3), 4.35 (1H, d, *J*=11 Hz, H-23), 4.38 (1H, d, *J*=7 Hz, H-28), 4.49 (overlapped, H-16), 4.95 (1H, d, *J*=8 Hz, H-1 of fuc), 5.10 (1H, d, *J*=8 Hz,

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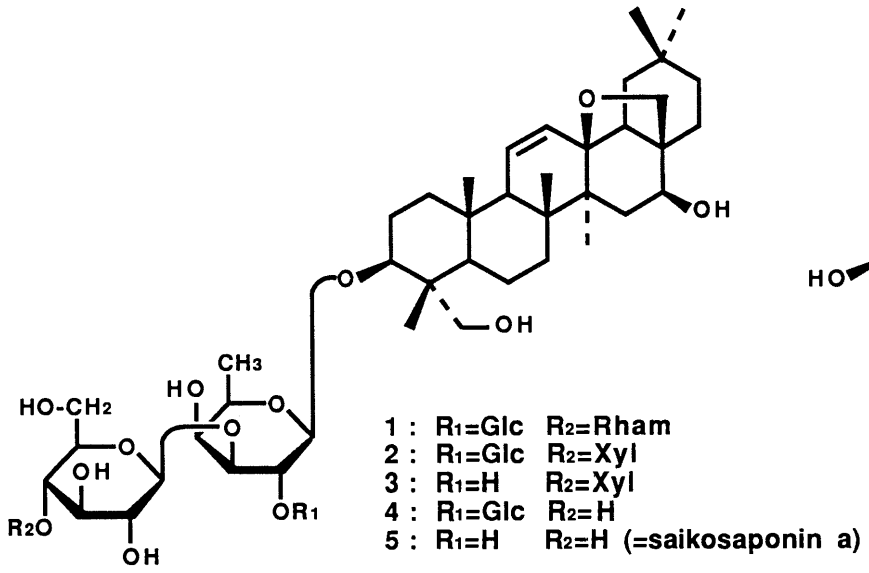
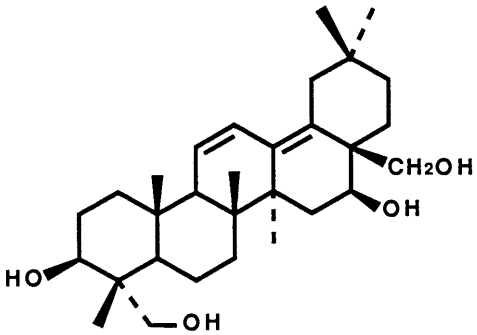


1 : R_1=Glc R_2=Rham
2 : R_1=Glc R_2=Xyl
3 : R_1=H R_2=Xyl
4 : R_1=Glc R_2=H
5 : R_1=H R_2=H (=saikosaponin a)



6 : saikogenin A

Chart 1

Table I. ^{13}C-NMR Chemical Shifts[12] and Yields

Comp.	1	2	3	4	5
Yield	0.47 %	0.23 %	0.08 %	0.03 %	0.03 %
Aglycone moiety					
C-3	82.6	82.6	81.8	82.7	81.7
C-11	132.2	132.2	132.2	132.2	132.2
C-12	131.2	131.2	131.2	131.2	131.2
C-16	64.1	64.1	64.1	64.1	64.1
C-23	64.7	64.6	64.2	64.7	64.1
C-28	73.0	73.0	73.1	73.1	73.0
Fucose moiety					
C-1	104.1	104.1	106.0	104.1	106.0
C-2	77.2	77.1	71.8	77.2	71.7
C-3	84.8	85.0	85.6	84.9	85.3
C-4	72.0	72.1	72.2	72.0	71.9
C-5	70.5	70.5	71.1	70.5	71.1
C-6	17.2	17.2	17.3	17.2	17.2
Glucose moiety (at C-3 of fuc)					
C-1	105.0	105.0	106.4	105.2	106.7
C-2	75.5	75.1	75.5	75.4	75.9
C-3	76.5	76.3	76.3	78.4	78.8
C-4	78.5	80.8	80.9	71.7	72.2
C-5	77.3	76.6	76.8	78.5	78.5
C-6	61.4	61.7	61.8	62.6	62.8
Glucose moiety (at C-2 of fuc)					
C-1	104.0	104.1		104.1	
C-2	76.3	76.3		76.3	
C-3	78.9	78.8		78.8	
C-4	72.2	72.2		72.3	
C-5	77.5	77.6		77.5	
C-6	63.2	63.2		63.2	
Xylose moiety					
C-1		105.5	105.6		
C-2		75.0	75.0		
C-3		78.4	78.4		
C-4		70.8	70.8		
C-5		67.4	67.4		
Rhamnose moiety					
C-1	102.8				
C-2	72.6				
C-3	72.8				
C-4	74.0				
C-5	70.4				
C-6	18.5				

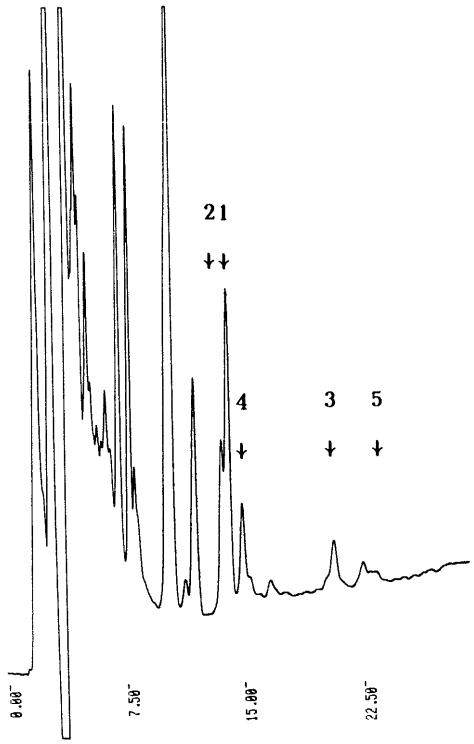


Fig 1. HPLC of 100% MeOH Eluate of Diaion HP-20 Column from MeOH Extract of *Buddleja japonica* Hemsl.

Conditions
Column YMC R-ODS-5 AQ 4.6 mm x 25 cm
Eluent CH_3CN-H_2O (35:65) → (45:55) 60 min linear grad.
Flow rate 1.0 ml/min
Detector 205 nm

fucose, glucose and xylose in the ratio 1:1:1. The NOE experiments and the ^{13}C-NMR data led us to conclude the linear sugar linkage structure.

Buddlejasaponin IV (**4**), amorphous powder, $[\alpha]_D^{25}$ +54.2° (*c*=0.99, MeOH), $C_{48}H_{78}O_{18}\cdot 7/2\ H_2O$ (Calcd. C, 57.30; H, 8.52. Found C, 57.48; H, 8.39), FAB-MS *m/z*: 965 $[M + Na]^+$. ^{1}H-NMR: δ 0.90 (3H, s, H_3-30), 0.94 (3H, s, H_3-29), 0.97 (3H, s, H_3-25), 1.06 (3H, s, H_3-24), 1.10 (3H, s, H_3-27), 1.38 (3H, s, H_3-26), 3.33 (1H, d, *J*=7 Hz, H-28), 3.70 (1H, d, *J*=11 Hz, H-23), 4.13 (1H, dd, *J*=12.5, 4 Hz, H-3), 4.36 (1H, d, *J*=11 Hz, H-23), 4.37 (1H, d, *J*=7 Hz, H-28), 4.49 (1H, dd, *J*=10, 6 Hz, H-16), 4.91 (1H, d, *J*=8 Hz, H-1 of fuc), 5.27 (1H, d, *J*=8 Hz, H-1 of glc at C-3 of fuc), 5.56 (1H, d, *J*=8 Hz, H-1 of glc at C-2 of fuc), 5.64 (1H, dd, *J*=10, 2.5 Hz, H-12), 5.97 (1H, br d, *J*=10 Hz, H-11). Acid hydrolysis liberated fucose and glucose in the ratio 1:2. The NOE experiments and the glycosylation shifts showed two glucoses to be present at the C-2 and C-3 of fucose.

New saponins **1-4** were composed of saikosaponin a and one or two more sugars. Until now, saikosaponins were isolated only from *Bupleurum* species (Umbelliferae), but these new saponins could be obtained easily and in high yield from *B. japonica* Hemsl. It is of common knowledge that saikosaponins are unstable to yield diene-type artifacts during extraction from *Bupleurum* species under neutral or acidic condition,[9] but this transformation did not occur during the extraction and isolation from this plant. Therefore, this plant is expected to serve as a medicinal plant.

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SYNTHESIS OF ATROPISOMERIC BIPHENYLBISPHOSPHINE, 6,6'-BIS(DICYCLOHEXYLPHOSPHINO)-3,3'-DIMETHOXY-2,2',4,4'-TETRAMETHYL-1,1'-BIPHENYL AND ITS USE IN RHODIUM(I)-CATALYZED ASYMMETRIC HYDROGENATION[1)]

Masanao MURATA, Naoko YAMAMOTO, Kiyoshi YOSHIKAWA, Toshiaki MORIMOTO, and Kazuo ACHIWA*
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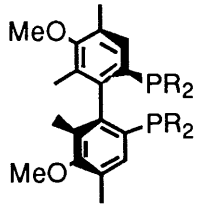
An optically pure atropisomeric biphenylbisphosphine, Cy-BIMOP, has been prepared, and its rhodium(I) complex showed higher catalytic activity and better enantioselectivity with the reverse configuration than BIMOP, *p*-MeO-BIMOP, and BINAP in the asymmetric hydrogentions of (*Z*)-α-acetamidocinnamic acid, itaconic acid, and its derivative.

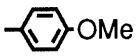
KEYWORDS atropisomeric biphenylbisphosphine; rhodium(I) complex; asymmetric hydrogenation; optical resolution; enantioselectivity; acetamidocinnamic acid; itaconic acid; piperonylidenesuccinic acid

In much progress in the development of asymmetric hydrogenations with the complexes of transition metal and bisphosphine ligands, atropisomeric biarylbisphosphines such as BINAP,[2)] BIPHEMP,[3)] and BICHEP[4)] have been recently utilized for highly efficient ruthenium(II)- and rhodium(I)-catalyzed asymmetric hydrogenations of several functionalized ketones and olefins.

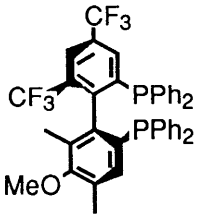
In previous communications,[5,6)] we reported the preparation of optically pure BIMOP (**1**), *p*-MeO-BIMOP (**2**), FUPMOP (**3**), and BIFUP (**4**), atropisomeric biphenylbisphosphines bearing electron-donating groups or/and electron-withdrawing groups on the biphenyl frameworks and the efficient asymmetric hydrogenations of β-keto ester and α,β-unsaturated carboxylic acid with their ruthenium(II) complex catalysts. We revealed that the steric effect and the electronic effect of the biphenyl framework have roles in the enantioselectivity and the catalytic activity, respectively, of the ruthenium(II)-catalyzed asymmetric hydrogenation of methyl acetoacetate, where BIMOP (**1**) and FUPMOP (**3**) bearing electron-donating groups at least on one phenyl group showed high catalytic activities, though BIFUP (**4**) bearing only electron-withdrawing groups exhibited much lower catalytic activity. These results prompted us to prepare a new atropisomeric biphenylbisphosphine bearing electron-donating groups on both the biphenyl framework and the phosphino groups.



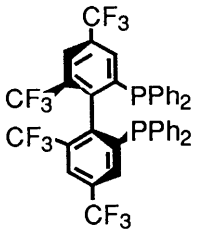
R=  : (*R*)-BIMOP (**1**)

R=  : (*R*)-*p*-MeO-BIMOP (**2**)

R=  : (*R*)-Cy-BIMOP (**5**)



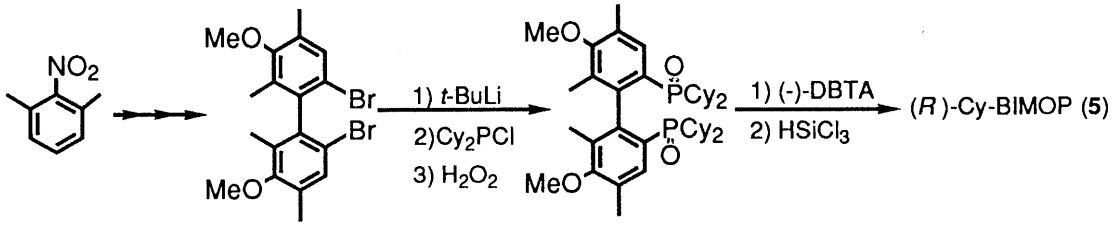
(*S*)-FUPMOP (**3**)



(*S*)-BIFUP (**4**)

This communication describes the synthesis of optically pure atropisomeric biphenylbisphosphine, 6,6'-bis(dicyclohexylphosphino)-3,3'-dimethoxy-2,2',4,4'-tetramethyl-1,1'-biphenyl (abbreviated to Cy-BIMOP) (**5**) and its use in rhodium(I)-catalyzed asymmetric hydrogenations of (*Z*)-α-acetamidocinnamic acid, itaconic acid, and its derivative.

Optically pure Cy-BIMOP (**5**) was prepared by a similar procedure reported previously as shown in Chart 1. 6,6'-Dibromo-3,3'-dimethoxy-2,2',4,4'-tetramethylbiphenyl (**6**)[5)] prepared via 5 steps from 2,6-dimethylnitrobenzene was dilithiated with *tert*-butyllithium in tetrahydrofuran (THF) at -70 °C and allowed to react with chlorodicyclohexylphosphine affording the corresponding bisphosphine, which was directly converted to racemic Cy-BIMOPO (**7**) in 68% overall yield from **6** by oxidation with hydrogen



6 **(*RS*)-7**

Chart 1

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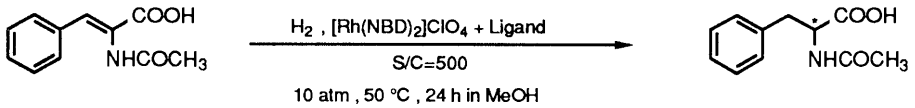
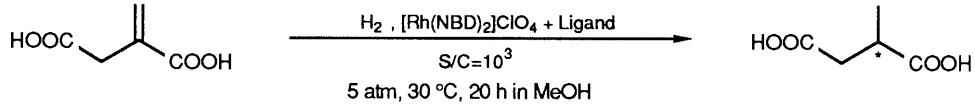
Table I. Asymmetric Hydrogenation of (*Z*)-α-Acetamidocinnamic Acid



Ligand	Convn.[b](%)	Optical Yield[c](%)	Confign.[d]
(*R*)-BINAP	42	15	*R*
(*R*)-BIMOP (**1**)	42	30	*R*
(*R*)-*p*-MeO-BIMOP (**2**)	100	44	*R*
(*R*)-Cy-BIMOP (**5**)	100	79	*S*

a) All hydrogenations were carried out in 0.1 M solution of the substrate. b) Determined by ^{1}H-NMR analysis.
c) Calculated on the basis of the reported value $[\alpha]_D^{20}$ +40.1° (c 1.00, MeOH) for the pure *S*-enantiomer.
d) Determined by the sign of optical rotation.

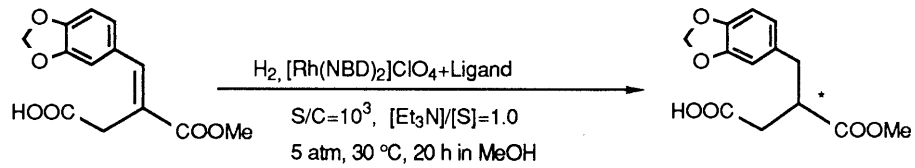
Table II. Asymmetric Hydrogenation of Itaconic Acid



Ligand	Convn.[b](%)	Optical Yield[c] (%)	Confign.[d]
(*R*)-BINAP	100	~0	---
(*R*)-BIMOP (**1**)	100	51	*S*
(*R*)-*p*-MeO-BIMOP (**2**)	100	57	*S*
(*R*)-Cy-BIMOP (**5**)	100	80	*R*

a) All hydrogenations were carried out in 0.5 M solution of the substrate. b) Determined by ^{1}H-NMR analysis.
c) Calculated on the basis of the reported value $[\alpha]_D^{20}$ +16.88° (c 2.16, EtOH) for the pure *R*-enantiomer.
d) Determined by the sign of optical rotation.

Table III. Asymmetric Hydrogenation of α–Piperonylidenesuccinic Acid Monomethyl Ester



Ligand	Convn.[b] (%)	Optical Yield[c] (%)	Confign.[d]
(*R*)-BINAP	24	~0	----
(*R*)-BIMOP (**1**)	72	56	*S*
(*R*)-*p*-MeO-BIMOP (**2**)	70	70	*S*
(*R*)-Cy-BIMOP (**5**)	100	73	*R*

a) All hydrogenations were carried out in 0.5 M solution of the substrate. b) Determined by ^{1}H-NMR analysis.
c) Calculated on the basis of the maximum optical rotation $[\alpha]_D^{20}$ +29.2° (c 2.00, MeOH) determined by HPLC analysis of the corresponding morpholino derivative on Chiralcel OC (Daicel).
d) Determined by the sign of optical rotation.

peroxide. Optical resolution of racemic **7** was achieved by using (2*R*, 3*R*)-(-)-2,3-*O*-dibenzoyltartaric acid ((-)-DBTA) as a resolving agent. Repeated recrystallization of the complex from methanol gave a diastereomerically pure complex of (*R*)-Cy-BIMOPO ((*R*)-**7**) and (-)-DBTA in 40% yield. Free (*R*)-**7** was obtained by treating the complex with aq. sodium hydroxide in dichloromethane. The phosphinyl groups were reduced by heating at 140 °C for 4 h with a large excess of trichlorosilane in chlorobenzene to afford optically pure (*R*)-Cy-BIMOP (**5**)[7] in 64% yield. The absolute configuration was determined by comparison of its CD spectrum with that of (*R*)-BIPHEMP.[8]

First, the ruthenium (II)-catalyzed asymmetric hydrogenation of methyl acetoacetate was carried out for evaluation of the capability of (*R*)-Cy-BIMOP (**5**) and for comparison with **1**, **2**, **3**, and **4**. Unexpectedly, the hydrogenation catalyzed by (*R*)-Cy-BIMOP (**5**)-ruthenium (II) complex did not proceed smoothly even under a hydrogen pressure of 90 atm at 30-40 °C for 40 h. As reported previously,[6] the (*S*)-BIFUP (**4**)-ruthenium(II) complex also showed very low catalytic activity. These results may suggest that the complex formation of the bisphosphine-ruthenium-monohydride by oxidative addition of hydrogen to the intermediate considered as a rate-determining step retards since, in Cy-BIMOP (**5**) bearing only electron-donating groups, the bisphosphine-ruthenium-monohydride complex is unstable and in BIFUP (**4**) bearing only electron-withdrawing groups the oxidative addition of hydrogen becomes slower.

We have already reported that bisphosphine ligands bearing electron-donating groups have important roles in effective asymmetric hydrogeantions of ketones and olefins using their rhodium(I) complex.[9] Therefore, we next carried out the asymmetric hydrogenation of functionalized olefins such as (*Z*)-α-acetamidocinnamic acid and itaconic acids with the rhodium(I) complex of (*R*)-Cy-BIMOP (**5**). Asymmetric hydrogenation of (*Z*)-α-acetamidocinnamic acid was carried out using the cationic rhodium(I) complex of BIMOPs (**1**, **2**, **5**) (molar ratio: substrate/catalyst (S/C)=500) in methanol under a hydrogen pressure of 10 atm at 50 °C for 24 h. The results summarized in Table I show that the ligands bearing more electron-donating groups have higher catalytic activity and better enantioselectivity. Itaconic acid and α-piperonylidenesuccinic acid half-ester were also hydrogenated using their cationic complex (S/C=1000) in methanol under a hydrogen pressure of 5 atm at 30 °C for 20 h. Tables II and III show that (*R*)-Cy-BIMOP (**5**) was the most effective ligand for exhibiting higher enantioselectivity and catalytic activity though all the reactions proceeded completely in itaconic acid under these conditions. Thus, in the the ligands having a biphenyl framework, it has been revealed that the electron-donating groups give higher catalytic activity and better enantioselectivity to the rhodium(I) complex catalyst. In addition, the direction of the enantioselection of (*R*)-Cy-BIMOP (**5**) bearing dicyclohexylphosphino groups was reverse to that of the other ones, **1**, **2**, and (*R*)-BINAP, bearing diphenylphosphino groups. Previously, it was reported that an isolated (*R*)-BINAP-rhodium(I) complex gave the (*S*)-products in high optical yields from (*Z*)-α-(acylamino)acrylic acids but the diene coordinated starting complex or a dinuclear complex showed much lower selectivity.[10] Although the reason for the different enantioselectivity between (*R*)-Cy-BIMOP (**5**) and the others is not clearly understood so far, it is likely that in the ligands of biarylbis(diphenylphosphine) type, a dinuclear rhodium complex formed *in situ* has higher catalytic activity with different enantioselectivity than a mononuclear rhodium complex, or an intermediary complex bearing the chelation of the olefin group and the α-carboxylic group to the rhodium shows higher activity than the complex bearing the β-carbonyl or carboxylic chelation, resulting in different selectivity.

Further investigations along this line are in progress to clarify the mechanism of the enantioselectivity in the hydrogenation of functionalized olefins.

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