Mechanism of the Photochemical Reaction of 10-(9*H*-Xanthenylidene)-9(10*H*)-anthracenone[1)]

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A novel mechanism of the photochemical reaction of 10-(9*H*-xanthenylidene)-9(10*H*)-anthracenone (XA) was proposed on the basis of absorption spectrum changes observed upon photoirradiation, and the temperature effects upon the quantum yields of the radical formation and cyclization reaction.

The cyclization reaction of XA has a negative apparent activation energy value (−8.5 kcal/mol), while the radical formation from XA has a positive activation energy value (7.9 kcal/mol). The negative value of the apparent activation energy of the cyclization reaction was explained as a rasult of the competing effect of radical scavenging by oxygen associated with the conformation change which occurs with larger activation energy but smaller steric restriction compared to the cyclization reaction.

Keywords activation energy; conformational change; photocyclization; radical; reaction mechanism; UV-spectrum

Introduction

Thermochromic as well as photochromic substances have attracted significant interest from chemists in recent years. Especially, thermochromic ethylenes have been determined to posess the unique reversible conformational change due to twisting around the central bridge. As reported in previous papers,[2)] the anion radicals of those compounds are considerably twisted about the central bridge. Thus, the reversible conformational changes of those compounds can be explained in terms of a decrease in the double-bond character of the central bridge caused by electronic excitation.

Some of those compounds are photosensitive. For example, 10-(9*H*-xanthenylidene)-9(10*H*)-anthracenone (XA) undergoes both the radical formation and the cyclization reaction when it is irradiated by the light of its main absorption band.[3)]

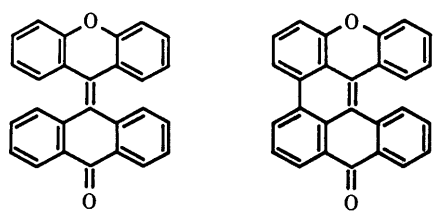
The main purpose of this paper is to elucidate the mechanism of the photochemical reaction of XA associated with the conformational change and, particularly, to give a reasonable explanation for the negative value of the apparent activation energy observed in the photochemical cyclization process.

Experimental

The synthesis and purification of XA were done according to the literature models.[4)] Sample solutions of XA were prepared by solving them in spectrograde tetrahydrofuran and by adjusting the concentration to 5×10^{-5} M.

Absorption spectrum measurements were done using a Shimadzu UV-360 spectrophotometer. In this case, the temperature of the sample solutions in 1.00 cm cells was controlled in a range from 0 to 80 °C by means of a Shimadzu SPR-5 variable temperature apparatus. The sample solutions of XA were irradiated by the light source (380 nm) of an Ushio 500 W xenon lamp attached with a Toshiba V-C1A glass filter and an infrared cutting filter. In necessary cases the ultraviolet (UV) sample solutions were degassed and sealed under a vacuum. To determine the quantum yield of the photochemical reaction,[5)] the exciting light intensity was measured by means of a potassium ferrioxalate actinometer.

Electron spin resonance (ESR) measurements were carried out on a JEOL JES-3BSX spectrometer. In this case the sample solutions in 5 mm sample tubes were always degassed and sealed under a vacuum. The degassed sample solutions were irradiated by the same light source described above.



10-(9*H*-xanthenylidene)-9(10*H*)-anthracenone

oxapenenone

Chart 1

Results and Discussion

From the absorption spectrum change shown in Fig. 1, we find that as the photoirradiation to the degassed sample solution is continued, a new peak at 348 nm (assigned to the XA radical) remarkably appears along with another peak at 482 nm (assigned to oxapenenone), and the original peak at 384 nm (XA) decreases. As described in the previous paper,[3)] an ESR signal of the XA radical was also found to be generated by photoirradiation to the degassed sample solution. These findings indicate that in the degassed sample solution which might contain a trace amount of oxygen, the XA radical, as well as oxapenenone, is produced from XA photochemically. When the seal of the sample tube was

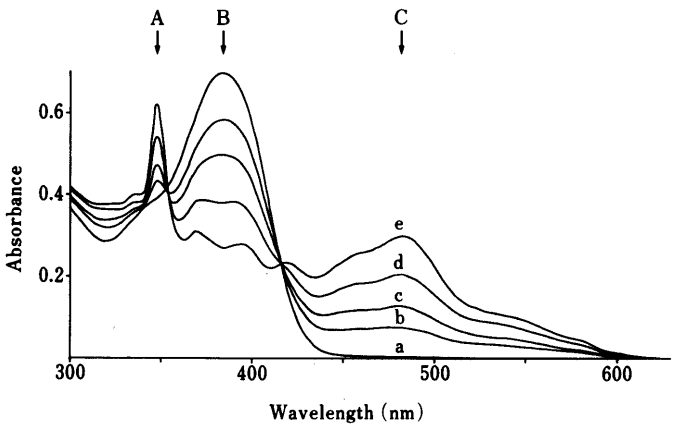
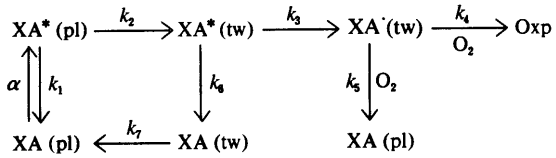


Fig. 1. The Absorption Spectra of the Irradiated Solution

The 4.5×10^{-5} M solution of XA in tetrahydrofuran was degassed under a vacuum and irradiated at −10 °C. Curve "a" refers to the initial absorption spectrum of XA. Taking the course of a→b→..., it shifts to curve "e" during the irradiation in a few minutes at room temperature. Peaks A, B and C are assigned to the XA radical, XA and oxapenenone respectively.

broken after photoirradiation, we observed an interesting phenomenon: the XA radical peak at 348 nm disappeared while the oxapenenone peak at 482 nm increased. This suggests that the cyclization reaction which produces oxapenenone may proceed *via* the formation of the XA radical. Moreover, by taking into consideration the additional fact that oxapenenone is the dominant photochemical product in the undegassed sample solution, we can presume that the disappearance of the XA radical followed by the generation of oxapenenone is highly accelerated by the existence of oxygen solved in the solution, even if the XA radical is formed during the primary photochemical reaction processes.

The scheme of the overall photochemical reactions in an aerated solution may thus be represented as follows:



where k_1, k_2, $\cdots$ k_7 are the rate constants, XA* is the excited state of XA, XA˙ is the radical of XA, the symbols (pl) and (tw) refer to planar and twisted conformations, respectively, and Oxp is the abbreviation of oxapenenone.

According to the overall reaction scheme proposed above, the reaction rate equations for XA* (pl), XA* (tw), XA˙ (tw) and Oxp can be formulated as follows:

$$\frac{d[XA^*(pl)]}{dt}=\alpha I-(k_1+k_2)[XA^*(pl)] \tag{1}$$

$$\frac{d[XA^*(tw)]}{dt}=k_2[XA^*(pl)]-(k_3+k_6)[XA^*(tw)] \tag{2}$$

$$\frac{d[XA^{\cdot}(tw)]}{dt}=k_3[XA^*(tw)]-(k_4+k_5)[XA^{\cdot}(tw)][O_2] \tag{3}$$

$$\frac{d[Oxp]}{dt}=k_4[XA^{\cdot}(tw)][O_2] \tag{4}$$

where α is the quantum yield for producing the XA*(pl), and I is the intensity of the irradiation light. By applying the stationary state approximation to the reaction rates of the transient species XA*(pl), XA*(tw) and XA˙(tw), we have:

$$[XA^*(pl)]=\frac{\alpha I}{(k_1+k_2)} \tag{5}$$

$$[XA^*(tw)]=\frac{k_2}{(k_3+k_6)}[XA^*(pl)] \tag{6}$$

$$[XA^{\cdot}(tw)]=\frac{k_3}{(k_4+k_5)[O_2]}[XA^*(tw)] \tag{7}$$

Substituting Eqs. 5—7 into Eq. 4, we have

$$\frac{d[Oxp]}{dt}=\frac{\alpha I k_2k_3k_4}{(k_1+k_2)(k_3+k_6)(k_4+k_5)} \tag{8}$$

In the degassed sample solution, the concentration of the radical [XA˙(tw)] was found to increase remarkably with photoirradiation.[3] This may be because the radical-scavenging action of oxygen has been largely suppressed. In this case, we may then omit the second term of the right-hand side of Eq. 3. This leads to the following equations which may be valid at the initial reaction stage.

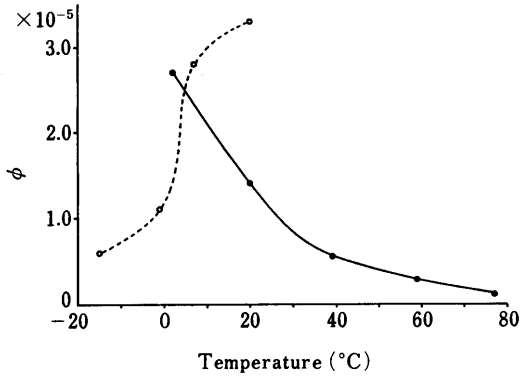


Fig. 2. The Temperature Dependence of Quantum Yields of the Radical Formation and the Cyclization Reaction

The closed circle refers to the photocyclization of XA and the open circle refers to the radical formation. The scale on the vertical axis is for photocyclization. The quantum yield is shown as relative values for the radical formation because the radical concentration was not determined. (This figure is quoted from the previous paper.[2])

$$\frac{d[XA^{\cdot}(tw)]}{dt}=k_3[KA^*(tw)] \tag{9}$$

$$\frac{d[XA^{\cdot}(tw)]}{dt}=\frac{\alpha I k_2k_3}{(k_1+k_2)(k_3+k_6)} \tag{10}$$

The reaction rates of XA˙(tw) and Oxp can be expressed as follows by using the quantum yields ϕ and ϕ', both of which could actually be measured as shown by Fig. 2.

$$\frac{d[XA^{\cdot}(tw)]}{dt}=\phi I \tag{11}$$

$$\frac{d[Oxp]}{dt}=\phi' I \tag{12}$$

The relationships between the quantum yields and the rate constants can be expressed as follows by comparing Eq. 10 with Eq. 11, and Eq. 8 with Eq. 12, respectively.

$$\phi=\frac{\alpha k_2k_3}{(k_1+k_2)(k_3+k_6)} \tag{13}$$

$$\phi'=\frac{\alpha k_2k_3k_4}{(k_1+k_2)(k_3+k_6)(k_4+k_5)}=\phi\frac{k_4}{k_4+k_5} \tag{14}$$

By taking the logarithm of Eqs. 13 and 14, we have:

$$\ln\phi=\ln\alpha+\ln\frac{k_2k_3}{(k_1+k_2)(k_3+k_6)} \tag{15}$$

$$\ln\phi'=\ln\phi+\ln\frac{k_4}{k_4+k_5} \tag{16}$$

Equation 15 can be rewritten as follows:

$$\ln\phi=\ln\frac{k_2}{k_1+k_2}+\ln\frac{k_3}{k_3+k_6}+\ln\alpha \tag{17}$$

In the first term of the right-hand side, k_1 and k_2 are the rate constants for the quenching and twisting reactions of the planar excited species, respectively. It is generally known that the quenching reaction of the excited species proceeds rapidly by the Franck–Condon process free from nuclear

displacements. On the other hand, the conformational change of the excited species is to be rate-determined by the potential barrier along the intramolecular reaction coordinate. This physically allows us to assume $k_1 \gg k_2$.

The second term of the right-hand side of Eq. 17 is related to the quenching and radical formation of the twisted excited species. The mechanisms of the radical formation have already been discussed in our previous work in terms of the preference of the twisted conformation in the excited species.[2] Since these processes are not directly accompanied by temperature-dependent conformational changes, Eq. 15 is thus simplified as follows by introducing the temperature-independent constants c and d.

$$\ln \phi = \ln \frac{k_2}{k_1} + c + d \tag{18}$$

From this equation we can evaluate the activation energy (E_a) of the radical formation reaction according to the Arrhenius method.

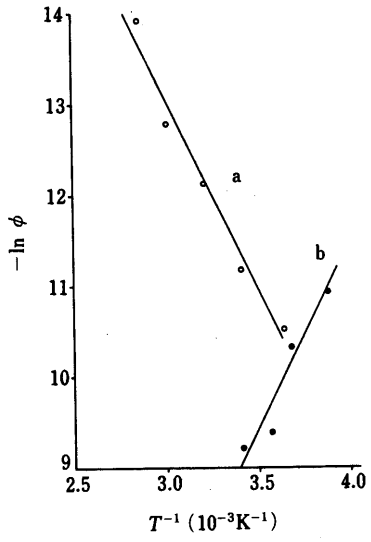


Fig. 3. The Arrhenius Plot for the Quantum Yield of the Photochemical Reaction of XA and the Temperature

The open circle refers to the photocyclization, and the closed circle refers to the radical formation.

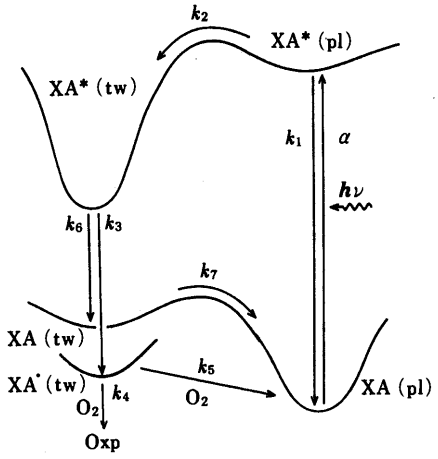


Fig. 4. A Conceptional Energy Diagram of the Photochemical Reaction of XA

XA* indicate the excited species and XA· indicates the radical.

$$\ln \frac{k_2}{k_1} = -E_a/RT + \ln A \tag{19}$$

$$\ln \phi = -E_a/RT + \ln A + c + d \tag{20}$$

Moreover, by taking into account the predominant radical-scavenging effect of oxygen as widely accepted, we may use an approximate relation of $k_5 \gg k_4$, and thus we have from Eqs. 16 and 20:

$$\ln \frac{k_4}{k_5} = -E_a'/RT + \ln A' \tag{21}$$

$$\ln \phi' = -(E_a + E_a')/RT + \ln A' + \ln A + c + d \tag{22}$$

where $(E_a + E_a')$ corresponds to the apparent activation energy for the cyclization reaction.

From the Arrhenius plots for Eqs. 20 and 22, we can estimate the activation energies of E_a and $E_a + E_a'$. The Arrhenius plots as stated above are shown in Fig. 3, from which we can find $E_a = 7.9$ kcal/mol and $E_a + E_a' = -8.5$ kcal/mol. The activation energy of the radical formation estimated here is only about half of the activation energy of the twisting reaction of bianthrone (BA).[6] This suggests that the excited species associated with the anti-bonding orbital character plays an important role in twisting of the molecule.

E_a' must be negative since the apparent activation energy $(E_a + E_a')$ is negative and E_a itself is positive. E_a' corresponds to the difference in the activation energies of the radical scavenging and the radical cyclization reactions, both of which are to be affected by oxygen solved in the sample solution. Hence, the negative value of E_a' suggests that the radical scavenging effect of oxygen under consideration may be accompanied by the conformational change (possibly from twisted to planar states) which occurs with a larger activation energy compared to the intramolecular cyclization reaction. According to this argument, the larger rate of the radical scavenging relative to the cyclization reaction may be explained as a result of the radical scavenging proceeding with a smaller steric restriction compared to the hydrogen abstraction by oxygen which would be required for the cyclization reaction. It is thus highly possible that the relative reaction rates of the radical scavenging and cyclization reaction are dominated by the compensation effect of the steric factor (entropy term) and the activation energy (enthalpy term) which has been recognized in various chemical reactions.

A conceptional energy diagram is shown in Fig. 4 in order to give a brief depiction of the overall reaction scheme proposed in this work.

References and Notes

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High Correlation between Hydrophobic Free Energy and Molecular Surface Area Characterized by Electrostatic Potential

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For 41 hydrocarbons including paraffins, olefins, acetylenes and aromatics, the solvent accessible surface areas partitioned according to the electrostatic molecular potential levels have been computed and correlated with the hydrophobic free energies derived from their aqueous solubilities. The results indicate that the free energy is a linear function of the areas of two parts of the solvent accessible surface, one of which has an electrostatic potential above −1 kcal/mol and the other has a potential of −1 kcal/mol or below. The coefficients of the two parameters are about 25 cal/mol/Å^2 and 5 cal/mol/Å^2 at 25 °C, respectively, and the multiple correlation coefficient is 0.987.

Keywords hydrophobic free energy; electrostatic potential; molecular surface area; hydrocarbon; QSAR

Introduction

Hydrophobic free energy, or hydrophobic Gibbs energy change ΔG° has been defined in terms of the unitary free energy of transfer of a hydrocarbon molecule from a pure hydrocarbon liquid phase to an aqueous phase by Tanford and others.[1-3] It can be determined from the solubility (X_s in mole fraction units) of the hydrocarbon in water. If μ_{HC}° and μ_W° are the hydrocarbon's standard chemical potentials in mole fraction units in hydrocarbon and water, respectively, the hydrophobic free energy is given as

$$\Delta G^\circ = \mu_W^\circ - \mu_{HC}^\circ = -RT \ln X_s \quad (1)$$

It is well established that ΔG° defined by Eq. 1 is a linear function of the solvent accessible surface area,[4] alias solvent cavity surface area[5] (*i.e.* area measured at the distance of closest approach of water molecules) of the saturated aliphatic or the aromatic hydrocarbon molecule.[1,2,5,6] The slopes of their lines, however, are different as reported by Tanford *et al.* (ΔG° per unit area amounts to about 25 cal/mol/Å^2 for paraffins and about 20 cal/mol/Å^2 for aromatics).[1,2] So, every given family of hydrocarbon needs its own equation to represent hydrophobic free energy by the above method. The analysis presented in this paper gives a new method for representing the ΔG° of miscellaneous hydrocarbons (paraffins, olefins, acetylenes and aromatics) by one equation. The hydrophobic free energy arises from contacts between hydrocarbon and water at the solute–solvent interface and thus, the number of water molecules that can be packed around the hydrocarbon molecule is an important quantity in hydrocarbon solubility. Hermann's idea was that the number of water molecules is related to the solvent accessible surface area (solvent cavity surface area) of the hydrocarbon molecule.[5] He treated the surface of a molecule as uniform. The solvent accessible surface of a molecule, however, is not uniform from the electrostatic point of view, as shown in Fig. 1,[7] especially, if the molecule has some unsaturated bonds. In Fig. 1, cyclohexane, 1,3-butadiene, heptadiyne and toluene are representative of paraffins, olefins, acetylenes and aromatics, respectively, of which the electrostatic potential levels are symbolized as depicted in the figure. It is understandable that the solvent accessible surface of a molecule can be divided into some electrostatic potential regions. Our method is to treat the accessible surface as electrostatically irregular (not uniform) and to correlate the hydrophobic free energy (ΔG°) of a hydrocarbon molecule with the solvent accessible surface areas divided according to the electrostatic potential values calculated on the surface of the molecule.

Calculation of Electrostatic Potential (*EP*) and Divided Solvent Accessible Surface Area According to *EP* Value As the simple classical formula reproduces the quantum mechanically calculated *EP* quite well at van der Waals distances and beyond,[8-11] we used the former formula to calculate the molecular potential on the solvent accessible surface. We used the value of a vacuum (1.0) as the dielectric constant to calculate the electrostatic potential. The classical representation of *EP* at a point x, $V(x)$, for a system of charges q_i at points r_i in a vacuum is given by

$$V(x) = \sum q_i / |r_i - x| \quad (2)$$

We used Mulliken net atomic charges obtained by a semiempirical molecular orbital method, MNDO[12] to represent q_i. The three-dimensional coordinates of the hydrocarbon molecules used here were obtained by the geometrical optimization method of MNDO using extended forms as the confomations. As most of the molecules used were small, the surface area can be thought to be little affected by the conformational change of the molecule. All calculations were carried out using the computer (HITAC M-680H) at the Computer Center of the University of Tokyo. The solvent accessible surface (SAS) of a molecule is defined as a curved surface traced out by the center of the sphere of rational radius representing a solvent molecule, as it slides over the van der Waals surface of the solute molecule.[4,5] We used 1.7 Å and 1.0 Å as the van der Waals radii of a carbon and a hydrogen atom, respectively, and 1.5 Å as the radius of a solvent (water) molecule as Hermann had done.

The SAS area divided according to *EP* values was calculated by a Monte Carlo method. To be more concrete, a number of points are uniformly generated on the SAS using three uniform random numbers[13] with a density of the points, 250 dots/Å (0.004 Å^2/dot). At each of those points, the *EP* value was calculated by Eq. 2 and the points on the SAS are classified into fourteen categories according to their *EP* values. That is to say, the 14 categories were separated by 13 boundaries of electrostatic potentials from −6 kcal/mol to +6 kcal/mol at an interval of 1 kcal/mol. The divided solvent accessible surface (DSAS) areas can be estimated by multiplying the number

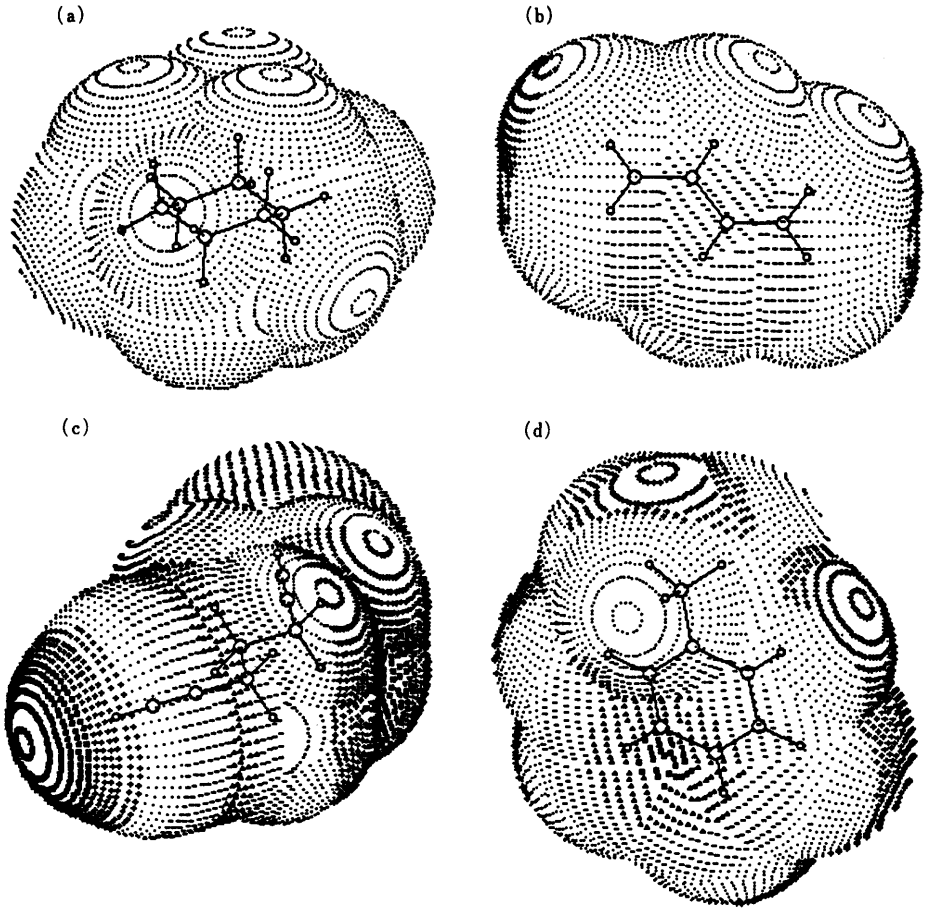

Fig. 1. Electrostatic Potential Images on the Solvent Accessible Surface of Hydrocarbons (a) Cyclohexane, (b) 1,3-Butadiene, (c) Heptadiyne, (d) Toluene

The meanings of the symbols are as follows, *, $+3 \leq EP$; +, $+1 \leq EP < +3$; ·, $-1 < EP < +1$; −, $-3 < EP \leq -1$; △, $-5 < EP \leq -3$; □, $EP \leq -5$. The EP means the electrostatic potential (kcal/mol).

TABLE I. Divided Solvent Accessible Surface (DSAS) Areas According to the Electrostatic Potential Values

Notation of DSAS area (Å²)	Electrostatic potential (kcal/mol)
$S_{HC}(+6 \leq EP)$	$+6 \leq EP$
$S_{HC}(+5 \leq EP < +6)$	$+5 \leq EP < +6$
$S_{HC}(+4 \leq EP < +5)$	$+4 \leq EP < +5$
$S_{HC}(+3 \leq EP < +4)$	$+3 \leq EP < +4$
$S_{HC}(+2 \leq EP < +3)$	$+2 \leq EP < +3$
$S_{HC}(+1 \leq EP < +2)$	$+1 \leq EP < +2$
$S_{HC}(0 \leq EP < +1)$	$0 \leq EP < +1$
$S_{HC}(-1 < EP < 0)$	$-1 < EP < 0$
$S_{HC}(-2 < EP \leq -1)$	$-2 < EP \leq -1$
$S_{HC}(-3 < EP \leq -2)$	$-3 < EP \leq -2$
$S_{HC}(-4 < EP \leq -3)$	$-4 < EP \leq -3$
$S_{HC}(-5 < EP \leq -4)$	$-5 < EP \leq -4$
$S_{HC}(-6 < EP \leq -5)$	$-6 < EP \leq -5$
$S_{HC}(EP \leq -6)$	$EP \leq -6$

$$S_{HC}(-1 < EP) = S_{HC}(-1 < EP < 0) + S_{HC}(0 \leq EP < +1) + S_{HC}(+1 \leq EP < +2) + S_{HC}(+2 \leq EP < +3) + S_{HC}(+3 \leq EP < +4) + S_{HC}(+4 \leq EP < +5) + S_{HC}(+5 \leq EP < +6) + S_{HC}(+6 \leq EP)$$

$$S_{HC}(EP \leq -1) = S_{HC}(-2 < EP \leq -1) + S_{HC}(-3 < EP \leq -2) + S_{HC}(-4 < EP \leq -3) + S_{HC}(-5 < EP \leq -4) + S_{HC}(-6 < EP \leq -5) + S_{HC}(EP \leq -6)$$

of the points belonging to each category by 0.004. The 14 areas are shown in Table I.

Results

The experimental data used for the analysis were the solubilities of hydrocarbons in water measured by McAuliffe.[14] Those solubility data were used to calculate $\Delta G°$ at 25 °C using Eq. 1 for a variety of hydrocarbon compounds having carbon numbers of four or above. Table II gives a list of the 41 compounds with their SAS areas (S_{HC}(total)) and two DSAS areas ($S_{HC}(-1 < EP)$, $S_{CH}(EP \leq -1)$) and $\Delta G°$ values calculated by the above method. The electrostatic potentials on the SAS of these molecules were in the range of −7 kcal/mol to +5 kcal/mol. 90% of the surface areas of the molecules used here belonged to the range of −5—+1 kcal/mol potential, 3% of the areas to the −7—−5 kcal/mol potential range and 2% to the +1—+5 kcal/mol range. The values of $\Delta G°$ for these compounds are plotted against S_{HC}(total) in Fig. 2. Figure 2 shows that $\Delta G°$ values of every family of hydrocarbon (paraffins, olefins, acetylenes and aromatics) may follow a straight line with a similar slope but the intercepts of these lines are respectively different. It can also be seen from Fig. 2 that the diynes (1,6-heptadiyne and 1,8-nonadiyne) follow a different line from the other acetylenes (monoynes). In order to represent $\Delta G°$ of the above hydrocarbons by one equation, we examined a correlation between $\Delta G°$ and various combinations of the DSAS areas stated above and we have found that $\Delta G°$ is a linear function of a SAS area

TABLE II. Hydrophobic Free Energies and Electrostatic Potential Surface Areas for 41 Hydrocarbons

Hydrocarbon	$\Delta G^{\circ a)}$	S_{HC} (total)	S_{HC} $(-1<EP)^{b)}$	S_{HC} $(EP\leq -1)^{b)}$
Paraffins				
Isobutane	5.85	243.5	243.5	0.0
n-Butane	5.97	248.4	248.4	0.0
Cyclopentane	6.00	249.5	249.5	0.0
Isopentane	6.72	270.6	270.6	0.0
Cyclohexane	6.72	272.1	272.1	0.0
n-Pentane	6.84	278.8	278.8	0.0
Methylcyclopentane	6.88	277.0	277.0	0.0
2,2-Dimethylbutane	7.39	288.3	288.3	0.0
2-Methylpentane	7.56	302.2	302.1	0.0
Methylcyclohexane	7.63	299.6	299.6	0.0
n-Hexane	7.78	309.8	309.8	0.0
n-Heptane	8.57	344.1	344.2	0.0
2,2,4-Trimethylpentane	8.75	339.9	339.9	0.0
n-Octane	9.53	337.7	337.7	0.0
Olefins				
1,3-Butadiene	4.34	229.5	181.5	48.0
1-Butene	5.05	240.2	216.8	23.4
1,4-Cyclohexadiene	5.19	258.4	202.1	56.3
1,4-Pentadiene	5.23	263.1	214.2	48.9
Cyclopentene	5.25	242.9	211.5	31.4
2-Pentene	5.84	275.0	245.3	29.7
Cyclohexene	5.91	265.8	235.6	30.2
1-Pentene	6.03	270.7	246.7	24.0
1-Hexene	6.78	302.9	279.7	23.2
1-Methylcyclohexene	6.84	295.2	261.0	34.2
4-Vinylcyclohexene	6.93	316.3	281.4	34.9
Acetylenes				
1-Butyne	4.12	232.0	149.1	82.9
1-Pentyne	4.61	263.3	179.3	84.0
1,6-Heptadiyne	4.76	311.1	164.6	146.5
1-Hexyne	5.60	294.8	211.6	83.2
1,8-Nonadiyne	6.45	376.6	219.3	157.3
1-Heptyne	6.49	327.8	243.0	84.8
1-Octyne	7.38	361.6	278.2	83.4
Aromatics				
Benzene	4.62	247.0	181.1	65.9
Toluene	5.45	277.6	207.6	70.0
p-Xylene	6.10	307.1	232.2	74.9
m-Xylene	6.11	307.9	233.1	74.8
o-Xylene	6.18	301.2	227.4	73.8
Ethylbenzene	6.26	307.6	237.5	70.1
Propylbenzene	6.88	339.6	268.0	71.0
1,2,4-Trimethylbenzene	6.91	330.5	252.0	78.5
Isopropylbenzene	6.99	326.4	253.9	72.5

a) Hydrophobic free energy (kcal/mol) calculated by Eq. 1 from McAuliffe's solubility data, except for the values of *m*-xylene and *p*-xylene which are taken from ref. 2. *b*) Surface area (Å^2) referred to Table I.

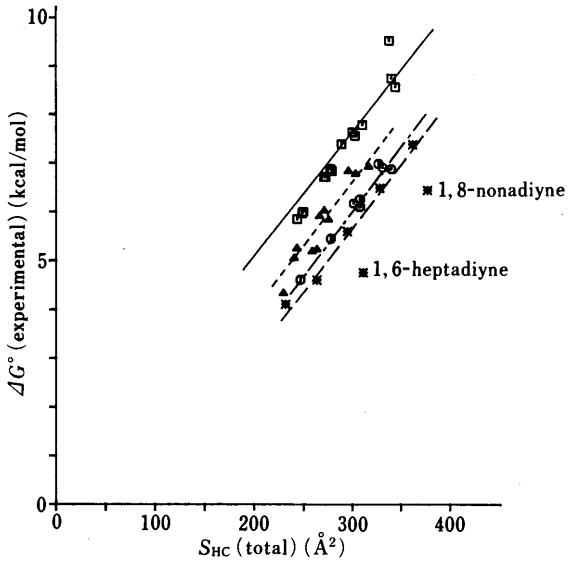

Fig. 2. Unitary Free Energies of Transfer of Hydrocarbons from the Pure Liquid to Aqueous Solution at 25°C from the Data Given in Table II against Solvent Accessible Surface Area

—□—, paraffins; --△--, olefins; --*--, acetylenes; —○—, aromatics.

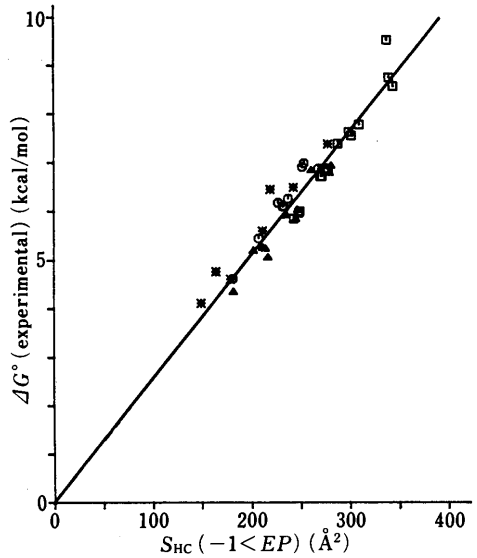

Fig. 3. The Same Data as in Fig. 2, Plotted as a Function of Solvent Accessible Surface Area with $EP>-1$ kcal/mol

□, paraffins; △, olefins; *, acetylenes; ○, aromatics.

having EP above -1 kcal/mol, $S_{HC}(-1<EP)$ (see Table I) as shown in Fig. 3. The following best-fit equation is found using linear regression analysis through an origin.

$$\Delta G^\circ = 0.025 S_{CH}(-1<EP) \quad (3)$$
$$N=41,\ r=0.967,\ s=0.302$$

where the unit of ΔG° is kcal/mol and that of $S_{HC}(-1<EP)$ is Å^2. As the maximum potential value of the molecules used here is less than $+5$ kcal/mol, $S_{HC}(-1<EP)$ equals to $S_{HC}(-1<EP\leq +5)$. Furthermore, since DSAS areas between $+1$ and $+5$ kcal/mol were only 2% of the total SAS areas, $S_{HC}(-1<EP)\approx S_{HC}(-1<EP<+1)$.

The contribution of the rest DSAS area, $S_{HC}(EP\leq -1)$ (see Table I) to ΔG° is evaluated using multivariate regression analysis through an origin and an equation giving a better correlation than Eq. 3 is obtained as follows,

$$\Delta G^\circ = 0.025 S_{HC}(-1<EP) + 0.005 S_{HC}(EP\leq -1) \quad (4)$$
$$N=41,\ r=0.986,\ s=0.203$$

In Fig. 1, the surface area represented by the symbols (·, +, *), corresponds to $S_{HC}(-1<EP)$ and that of moiety represented by (-, △, □) corresponds to $S_{HC}(EP\leq -1)$. As the minimum potential value of the molecules used here is greater than -7 kcal/mol, $S_{HC}(EP\leq -1)$ equals to $S_{HC}(-7<EP\leq -1)$. Furthermore, since DSAS areas between -7 and -5 kcal/mol were only 3% of the total SAS areas, $S_{HC}(EP\leq -1)\approx S_{HC}(-5<EP\leq -1)$.

The values of ΔG° calculated by Eq. 4 are plotted against those estimated from McAuliffe's solubility data

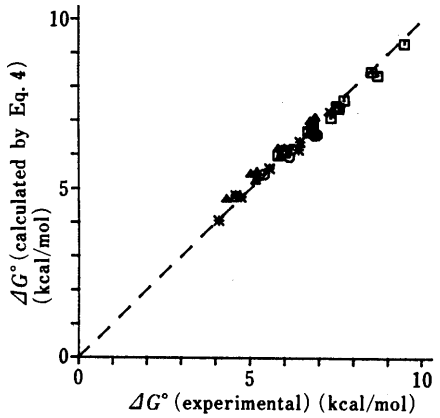

Fig. 4. Calculated (by Eq. 4) *versus* Observed Hydrophobic Free Energies from the Data Given in Table II

□, paraffins; △, olefins; ∗, acetylenes; ○, aromatics.

in Fig. 4.

Discussion

Tanford *et al.* concluded that the hydrophobic free energy per unit area of the SAS amounts to about 25 cal/mol/Å^2 at 25°C for saturated aliphatic hydrocarbons and to 20 cal/mol/Å^2 for aromatic hydrocarbons.[1] As can be seen from Fig. 1 and Table II, the electrostatic potential values on the SAS are absolutely small (limited to the range of $-1 < EP < +1$ kcal/mol) for paraffins to result in $S_{HC}(EP \leq -1)$ being zero, while the other families (olefins, acetylenes and aromatics) have regions with bigger and smaller electrostatic potential values. The surface areas belonged to the range of -5—$+1$ kcal/mol potential, -7—-5 kcal/mol and $+1$—$+5$ kcal/mol were 90, 3 and 2% of the total surface areas, respectively. Thus, the Eq. 4 can be regarded approximately the same as follows.

$$\Delta G^\circ \approx 0.025 S_{HC}(-1 < EP < 1) + 0.005 S_{HC}(-5 < EP \leq -1) + 0.000 S_{HC}(-7 < EP \leq -5) + 0.000 S_{HC}(+1 \leq EP < +5) \quad (5)$$

We conclude from Eqs. 3, 4 and 5 that the hydrophobic repulsive free energy between water and various hydrocarbons per unit area on the SAS having the electrostatic potential above -1 kcal/mol is 25 cal/mol/Å^2 at 25°C, is the same as Tanford's conclusion for paraffins. In addition, it is concluded that the hydrophobic free energy per unit area on the SAS with the electrostatic potential of -1 or below amounts to 5 cal/mol/Å^2, which means the surface of $EP \leq -1$ kcal/mol is less hydrophobic by a factor of about 5 than that of $EP > -1$ kcal/mol. It can be seen from Table II that $S_{HC}(-1 < EP)$ and $S_{HC}(EP \leq -1)$ are in the ratio of about three to one for aromatics. The average free energy per unit area of the two moiety of the SAS for aromatics, thus, is calculated to be 20 cal/mol/Å^2 (*i.e.* $(3 \times 25 + 5)/4 = 20$), which is consistent with Tanford's apparent value for aromatics. It is also understandable from the larger area of $EP \leq -1$ kcal/mol that the diynes are different from the other acetylenes. Consequently, our method of ΔG° estimation by the DSAS areas is thought to be a more general method applying to miscellaneous hydrocarbons. The idea that the hydrophobic free energy is proportional to the solvent accessible surface (S_{HC}(total)) of a solute molecule is correspondent to that the contribution of the enthalpy term to the hydrophobic free energy is regarded as almost zero for hydrocarbons. That is true of paraffins, but with regard to unsaturated hydrocarbons the solvent accessible surfaces are electrostatically not uniform as shown in Fig. 1, and hence the contribution of the enthalpy term to ΔG° might not be negligible. It might be thought that the contribution of the enthalpy term is taken into account by the use of the DSAS areas. It is also interesting from the standpoint of understanding hydration of a solute molecule that the hydrophobic free energy is little affected by the plus potential region on the SAS (25 cal/mol/Å for the SAS of $EP > -1$ kcal/mol) but is affected by the minus potential region (5 cal/mol/Å^2 for the SAS of $EP \leq -1$ kcal/mol), which suggests that the hydrogen atoms of water work more predominantly on the interaction of water molecules with a solute than the oxygen atom. The DSAS area parameter is expected to be applied to a variety of molecules including heteroatoms as well as hydrocarbons and to be a new measure of hydrophobicity of compounds.

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An INDO/S (Intermediate Neglect of Differential Overlap/Spectrum) Study of Photochemical Reactivity of 2,4,6,8(1*H*,3*H*,7*H*,9*H*)-Pyrimido[5,4-*g*]pteridinetetrone 5-Oxide

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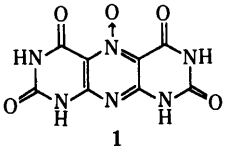
The specific photochemical reactivity of 2,4,6,8(1*H*,3*H*,7*H*,9*H*)-pyrimido[5,4-*g*]pteridinetetrone 5-oxide (1), an effective photochemical oxygen-atom transfer agent, was discussed based on the results of molecular orbital (INDO/S-CI (intermediate neglect of differential overlap/spectrum-configuration interaction)) calculations of the excited states, in comparison with several simple aromatic *N*-oxides, pyridine 1-oxide (2), pyrazine 1-oxide (3), acridine 10-oxide (4), and phenazine 5-oxide (5). The lowest π–π^* excited singlet ($S_1^{\pi-\pi^*}$) state of 1 was most characterized by the high proportion of the $\psi_{a_2} \rightarrow \psi^*_{b_2}$ transition for the CI (configuration interaction) state and by the marked decrease of the N–O bond order, distinct from those of 2—5. The low reactivity in photorearrangement of 1 was interpreted to result from the decrease of the N–O bond order in the $S_1^{\pi-\pi^*}$ state. Also, the high photochemical oxygen-atom transfer reactivity of 1 was well correlated to the special features of the lowest unoccupied molecular orbital in the ground state.

Keywords 2,4,6,8(1*H*,3*H*,7*H*,9*H*)-pyrimido[5,4-*g*]pteridinetetrone 5-oxide; aromatic *N*-oxide; molecular orbital; INDO/S-CI; photo-oxidation; photorearrangement

In the photochemistry of *N*-oxides the photorearrangement and photodeoxygenation have been extensively studied experimentally and theoretically.[1] It has been well established that the photorearrangements of aromatic *N*-oxides are responsible for the lowest π–π^* excited singlet ($S_1^{\pi-\pi^*}$) states and that the photochemical intermediates are oxaziridines.[2] The formation of oxaziridines in the excited state and the regioselectivity have also been well explained based on the molecular orbital consideration by HMO and PPP-CI methods.[3] Much experimental data, on the other hand, has suggested that the triplet state is responsible for the photodeoxygenation of aromatic *N*-oxides.[4] The efficiencies in photodeoxygenation for most *N*-oxides were generally low because of the occurrence of photorearrangement as the side reaction. Maki and his colleagues recently reported, however, that 2,4,6,8(1*H*,3*H*,7*H*,9*H*)-pyrimido-[5,4-*g*]pteridinetetrone 5-oxide (**1**), unlike most *N*-oxide derivatives, acts as an efficient oxygen-atom transfer agent to various substrates without any appreciable side reaction.[5]

The photoreaction of **1** is quite different from those of most *N*-oxides in the nature of the excited state responsible for the photodeoxygenation and in the reactivity for the photorearrangement. It has been clarified that the photodeoxygenations of **1** proceed through a single electron transfer (SET) in the $S_1^{\pi-\pi^*}$ state or in the $S_1^{\pi-\pi^*}$ state of the charge transfer (CT) complex with a substrate.[5] It has also been shown that the photoreactions of **1** gave no product other than those by oxygen-atom transfer to substrates and that **1** is quite stable on the ultraviolet (UV) irradiation in the absence of substrates.[5]



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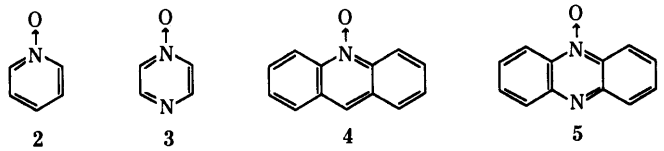

2 **3** **4** **5**

Chart 1

Thus, with a view to gaining insight into the nature of the electronic structure of **1** in the excited state, we have carried out molecular orbital calculations of the excited states for **1** and several structurally related aromatic *N*-oxides, such as pyridine 1-oxide (**2**), pyrazine 1-oxide (**3**), acridine 10-oxide (**4**), and phenazine 5-oxide (**5**), using the INDO/S-CI (intermediate neglect of differential overlap/spectrum-configuration interaction) method.

Calculations

The geometries of the *N*-oxides (**1**—**5**) were estimated by the full geometry optimization in the MNDO method.[6] Standard bond lengths and bond angles[7] were used as the starting geometric parameters for the calculations. The equilibrium structures thus calculated for all the *N*-oxides (**1**—**5**) were of nearly planar C_{2v} symmetry and were employed for the calculations of the excited states.

The CNDO/S-CI method with standard parametrization[8] and use of the Mataga–Nishimoto formula[9] for two-center two-electron repulsion integrals did not reproduce the observed UV spectrum of **1**[5]; the INDO/S-CI method was therefore applied for calculations of the excited state. The INDO/S-CI calculations were performed by the method and parameters given by Ridley and Zerner.[10] In the CI procedure, the number of configurations to be included was the 60 lowest singly excited states. The calculated transition energies reproduced acceptably the UV spectra of **1**—**5**.[5,11]

Calculations were carried out on a FACOM M-780/20 computer at the Computation Center of Nagoya University and on a HITAC M680-H computer at the Computer Center of the Institute of Molecular Science.

Results and Discussion

It is plausible that the difference in the photochemical

behavior of **1** from most *N*-oxides is due to the geometrical distortion of the *N*-oxide moiety from the planar structure which results from the steric hindrance by the carbonyl oxygen atoms at both the *peri*-positions. However, the structures optimized by the MNDO method were nearly planar for all the *N*-oxides examined and no anomalous feature in the geometry was shown for **1**.

The calculated singlet–singlet transition energies by the INDO/S-CI method are shown in Table I, together with the observed data.[5,11]

For all the *N*-oxides examined, the calculated lowest excited singlet states were the n–π^* states, and the $S_1^{\pi-\pi^*}$ states associated with the photoreactions of *N*-oxides were the second lowest.

The configuration mixings in the $S_1^{\pi-\pi^*}$ states of **1**—**5** are shown in Fig. 1 together with the coefficient distributions and nodal properties of the relevant molecular orbitals. The $S_1^{\pi-\pi^*}$ states of **1**—**5** were mainly constructed (over 95%) from the transitions between two higher occupied (ψ_{a_2} and ψ_{b_2}) and two lower unoccupied π-molecular orbitals ($\psi^*_{a_2}$ and $\psi^*_{b_2}$) in the ground state. The ψ_{b_2}'s and $\psi^*_{b_2}$'s have large orbital polarization on the *N*-oxide moiety, and the ψ_{a_2}'s and $\psi^*_{a_2}$'s on carbon atoms of a ring involving the *N*-oxide moiety. The ψ_{b_2}'s and $\psi^*_{b_2}$'s for **2**—**5** are the highest occupied molecular orbitals (HOMO's) and the lowest unoccupied molecular orbitals (LUMO's), respectively, in the ground state. The ψ_{b_2} orbital of **1**, however, is the next highest occupied molecular orbital (NHOMO) in the ground state.

TABLE I. Calculated Transition Energies (ΔE in eV) and Oscillator Strengths (f)

1				**2**				**3**				**4**				**5**			
Sym.	Calc. ΔE	Calc. f	Obs.[a] ΔE	Sym.	Calc. ΔE	Calc. f	Obs.[b] ΔE	Sym.	Calc. ΔE	Calc. f	Obs.[b] ΔE	Sym.	Calc. ΔE	Calc. f	Obs.[b] ΔE	Sym.	Calc. ΔE	Calc. f	Obs.[b] ΔE
A_2	2.47			A_2	3.07			A_2	2.94			A_2	2.82			A_2	2.73		
B_1	3.43	0.098	3.35	B_1	3.81	0.016	3.81	B_1	3.99	0.004	4.03—4.22	A_1	3.18	0.392	2.74≦	A_1	3.25	0.351	2.94≦
B_2	3.84			A_1	4.20	0.449	4.40	B_2	4.25			B_1	3.20	0.034	{3.22, 3.37}	B_1	3.30	0.021	{3.26, 3.42}
A_1	4.06	0.040		B_2	5.14			A_1	4.36	0.471	4.53	B_1	4.27	0.043		B_2	3.89		
A_2	4.21			B_2	5.26			B_1	5.37	0.416	5.58	A_1	4.40	0.000		B_1	4.26	0.117	
B_1	4.46	0.772	4.63	B_1	5.47	0.373	5.72	A_2	5.38			B_1	4.53	1.026	4.50	A_1	4.42	0.063	
A_1	4.72	0.583		A_2	6.00			B_2	5.52			A_1	4.63	0.017		B_1	4.57	1.839	4.58

a) Ref. 5. b) Ref. 11.

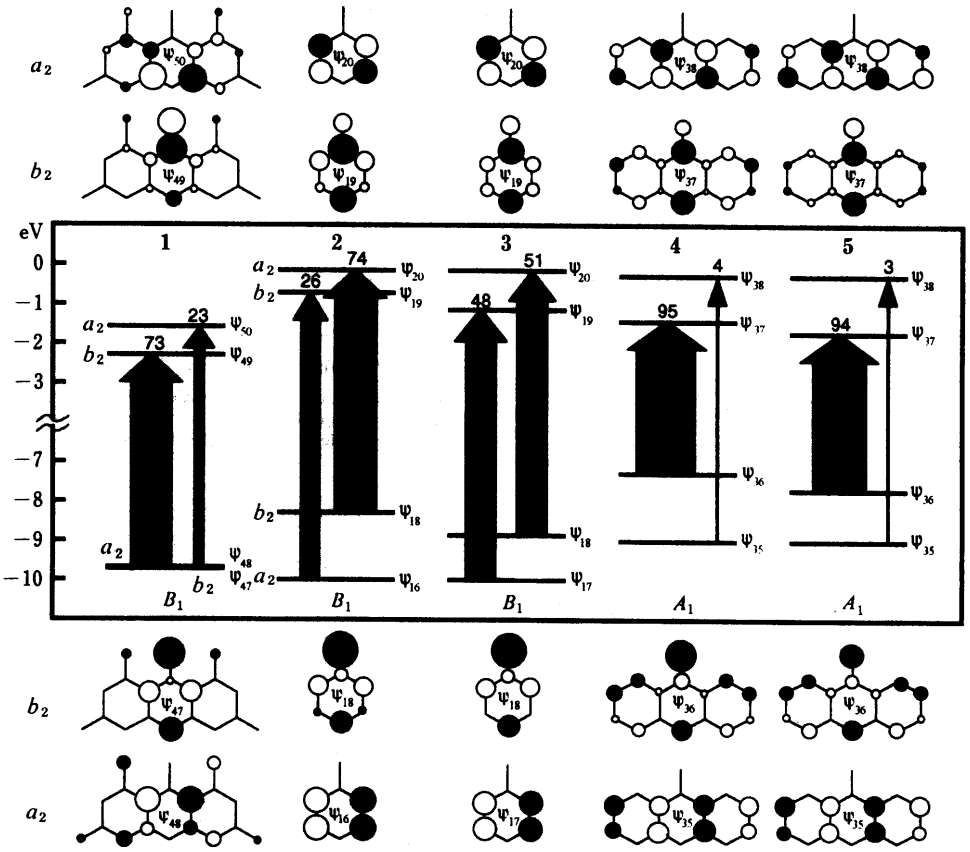

Fig. 1. Configuration Mixing in the $S_1^{\pi-\pi^*}$ States and the Relevant Molecular Orbitals

The proportion (%) of each configuration to the total is shown above the arrow indicating the transition.

The photoreactions in the *N*-oxide moiety are apparently associated with ψ_{b_2} and $\psi^*_{b_2}$; and the nodal properties of those orbitals on the hypothetical C···O bond between the oxygen and the *ortho*-carbon atoms and on the C–N bond showed that both the electronic transitions from ψ_{b_2} and those to $\psi^*_{b_2}$ are favorable for the formation of oxaziridine. Examination of Fig. 1 reveals that the configuration mixings in the $S_1^{\pi-\pi^*}$ state vary greatly with the compounds. The S_0–$S_1^{\pi-\pi^*}$ transitions in **1**, **2**, and **3** were of B_1 character and it was shown that the proportions of the $\psi_{a_2}\rightarrow\psi^*_{b_2}$ transition for the CI state of the $S_1^{\pi-\pi^*}$ state increase in the order of **2**<**3**≪**1**. The high proportion of the $\psi_{a_2}\rightarrow\psi^*_{b_2}$ transition for **1** is due to the small energy separation between ψ_{a_2} and $\psi^*_{b_2}$ and the large energy separation between ψ_{b_2} and $\psi^*_{a_2}$. These features in the energy separation of the orbitals for **1** result from the lowering in energy of the b_2 orbitals due to the electroinductive uracil rings. On the other hand, the S_0–$S_1^{\pi-\pi^*}$transitions for **4** and **5** are of A_1 character and are mainly due to the $\psi_{b_2}\rightarrow\psi^*_{b_2}$ transition. This is thought to be due to the small energy separations between ψ_{b_2} and $\psi^*_{b_2}$ caused by the electronic conjugation with fused benzene rings.

A quantum chemical study of the reactivity in photorearrangement of *N*-oxides has been reported by Koyano *et al.*, who showed that for a series of nitrones an increase in the quantum yield is correlated to an increase in the C···O bond order and a decrease in the C–N bond order associated with the excitation.[12] The changes (ΔP) of the C···O, C–N and N–O bond orders associated with the excitation to the $S_1^{\pi-\pi^*}$ state were thus calculated for the *N*-oxides (**1**—**5**) based on the configuration mixings and the orbital coefficients. The results are shown in Fig. 2.

The ΔP_{CO} and ΔP_{CN} values for **1** were not much different from those for **2**—**5**. However, the ΔP_{NO} value for **1** was obviously different from those for **2**—**5** and was largely negative. This marked decrease in the N–O bond order of **1** is considered to be due to the high proportion of the $\psi_{a_2}\rightarrow\psi^*_{b_2}$ transition for the configuration and the property of the $\psi^*_{b_2}$ orbital which has large orbital coefficients and a node on the N–O bond. Taking into account that the changes of the N–O bond order on the excitation are small for the *N*-oxides (**2**—**5**) as well as for a series of nitrones investigated by Koyano *et al.*,[12] the marked decrease for **1** is noteworthy and may be related to the distinct difference in the photoreactivity of **1** from other *N*-oxides.[13]

The decrease in the N–O bond order will lead to an increase of the N–O bond length, and this might lower the formation of oxaziridine owing to the change of the electronic structure induced by the geometric change. Thus, we have examined the change in N–O bond length associated with the excitation to the $S_1^{\pi-\pi^*}$ state and the effects of this length change on the N–O, C···O and C–N bond orders in the $S_1^{\pi-\pi^*}$ state for **1**. The examinations were also performed for **2**, which showed an increase of the N–O bond order on the excitation to the $S_1^{\pi-\pi^*}$ state.

The change in N–O bond length on excitation to the $S_1^{\pi-\pi^*}$ state was examined on the basis of variations in the energy of the ground (S_0) state and in the S_0–$S_1^{\pi-\pi^*}$ transition energy which were accompanied by an increase of the N–O bond length. As shown in Fig. 3, the potential curve of the $S_1^{\pi-\pi^*}$ state for **1** had a minimum at an N–O

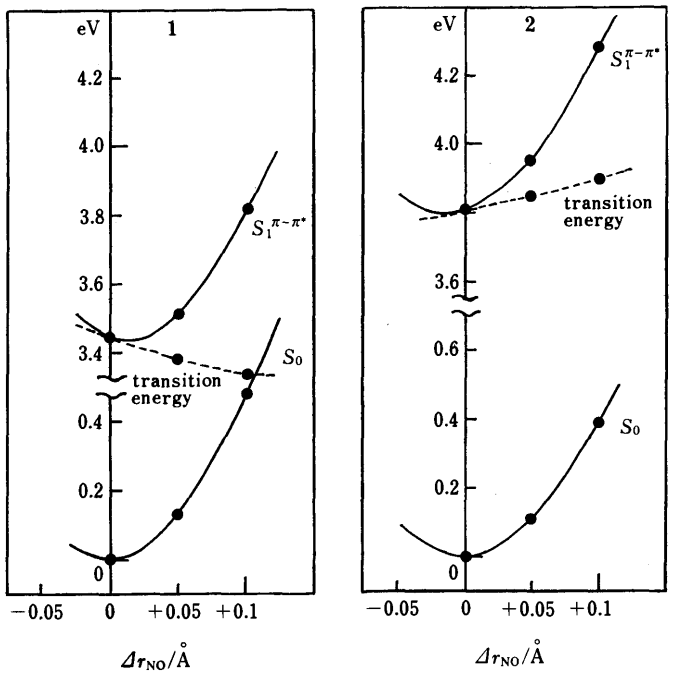

Fig. 3. Potential Curves in the Change of N–O Bond Length

Δr_{NO} represents the change of N–O bond length from its equilibrium bond length in the ground state. Energies in the ground state were calculated by the MNDO method. Dashed lines represent the changes in S_0–$S_1^{\pi-\pi^*}$ transition energy.

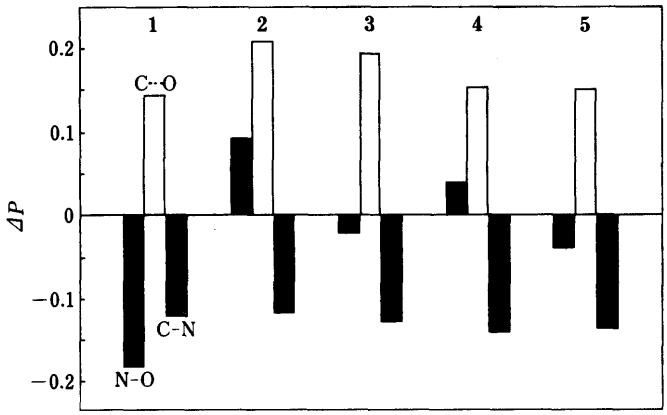

Fig. 2. Changes (ΔP) in the Bond Order Associated with the Excitation to the $S_1^{\pi-\pi^*}$ State

ΔP values were calculated as $\Delta P_{rs}=\sum d^2_{k,(i-j)}(C^j_r C^j_s - C^i_r C^i_s)$, in which $d_{k,(i-j)}$ is the coefficient of k-th configuration and C^j_r is the coefficient on r-th atom of j-th molecular orbital.

TABLE II. Effects of an Increase in N–O Bond Length on the Bond Orders

Compound	Bond	ΔP_0[a]	$\Delta P_{0.1}$[b]	$\Delta\Delta P$[c]	ΔP_G[d]
1	N–O	−0.181	−0.177	+0.004	−0.064
	C···O	+0.144	+0.140	−0.004	−0.004
	C–N	−0.122	−0.141	−0.019	+0.039
2	N–O	+0.093	+0.098	+0.005	−0.086
	C···O	+0.208	+0.191	−0.017	+0.027
	C–N	−0.116	−0.111	+0.005	+0.029

a) Change of the bond order associated with the excitation in ground state equilibrium geometry. *b*) Change of the bond order associated with the excitation in the geometry with N–O bond length stretched by 0.1 Å from its equilibrium length in the ground state. *c*) $\Delta\Delta P=\Delta P_{0.1}-\Delta P_0$. *d*) Change of the bond order in the ground state accompanied by an increase of N–O bond length by 0.1 Å. Values were calculated by the MNDO method.

distance longer than that of the ground state. This is due to a decrease in S_0–$S_1^{\pi-\pi^*}$ transition energy with an increase in N–O distance. Thus, it was confirmed that the N–O bond length in **1** increases upon excitation to the $S_1^{\pi-\pi^*}$ state. On the other hand, the potential curve of the $S_1^{\pi-\pi^*}$ state for **2** had a minimum at a shorter N–O distance than that of the ground state, due to an increase in S_0–$S_1^{\pi-\pi^*}$ transition energy with an increase in N–O distance.

The effects of increased N–O bond length on the formation of oxaziridine were investigated on the basis of the effects of that length on the bond orders in the $S_1^{\pi-\pi^*}$ state. That is, changes of the N–O, C···O and C–N bond orders in the $S_1^{\pi-\pi^*}$ state with an increase in N–O bond length by 0.1 Å were evaluated by both changes ($\Delta\Delta P$) of the ΔP values and changes (ΔP_G) of the bond orders in the ground state. The results are shown in Table II. We note that the positive changes for the N–O and C···O bond orders in Table II do not necessarily correspond to an increase in strength of the bond, since a comparison in strength between bonds of different lengths cannot directly be made on the bond orders (P values). However, a decrease of bond order accompanied by an increase of bond length may be correlated to the weakening of the bond. Since both the $\Delta\Delta P$ and the ΔP_G values for the C···O bond of **1** are negative, it is certain that at least in **1** the increase of N–O bond length in the $S_1^{\pi-\pi^*}$ state leads to the weakening of the C···O bond, *i.e.* reduction in the formation of oxaziridine.

From the results described above, it may be concluded that the marked decrease of the N–O bond order on the excitation for **1** is responsible for the noticeable stability of **1** towards the UV light, although other factors such as lifetime of the excited state are also important for photochemical reactions.

We next discuss the reactivity of the SET which is the initial step in the photochemical oxygen-atom transfer reaction of **1**. According to the Rehm–Weller treatment[14] for the free energy change of the SET process in the excited state, the feasibility of the SET from a substrate to the *N*-oxides in the excited state is evaluated by the excitation energy (ΔE_{ex}), the reduction potential (ΔE_{red}) in the ground state for the *N*-oxides and the oxidation potential (ΔE_{ox}) for a substrate. Thus, the greater the sum of the ΔE_{ex} and ΔE_{red} values for the *N*-oxides is, the more feasible the SET is. The ΔE_{ex} value for the $S_1^{\pi-\pi^*}$ state of **1** is smaller than those of **2** and **3** by *ca.* 0.6 eV and greater than those of **4** and **5** by *ca.* 0.2 eV, as shown in Table I. However, the ΔE_{red} value of **1**[5] is greatest among the examined *N*-oxides[15] and the differences from **2**, **3**, **4** and **5** are 1.33, 0.84, 0.33, and 0.002 V, respectively. These differences in ΔE_{ex} and ΔE_{red} values among **1**—**5** indicate that **1** is the most feasible for the SET in the $S_1^{\pi-\pi^*}$ state, and this is attributed to the high ΔE_{red} value of **1**. The correlation of the ΔE_{red} value with the LUMO energy is well known; in fact, the ΔE_{red} values of **1**—**5** were well correlated to the LUMO ($\psi_{b_2}^*$) energies. Also, since the orbital polarization and the nodal property in the LUMO's ($\psi_{b_2}^*$) of *N*-oxides are dissociative for the N–O bond, the introduction of an electron to the LUMO by the SET is considered favorable for the subsequent deoxygenation. The orbital polarization on the N–O bond in the LUMO for **1** is greatest among those for **1**—**5**. Thus, the high photochemical oxygen transfer reactivity of **1** is well correlated to the features of the LUMO in the ground state.

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Release Kinetics of Nicotinamide from Fatty Acid–Nicotinamide Equimolar Complexes. II.[1] Activation Thermodynamic Quantities

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The rates of release of nicotinamide (NAA) from fatty acid (FA)–NAA complexes, FA–NAA, were determined at various temperatures, and the thermodynamic quantities for the release of NAA were estimated. The results were compared with the previous results obtained for FA–thiamine disulfide (TDS) complexes, $(FA)_6$(TDS).

The values of activation enthalpy (ΔH^*) and activation entropy (ΔS^*) for the release of NAA from FA–NAA were positive and negative, respectively, indicating that the release of NAA is disadvantageous from not only enthalpic but also entropic viewpoints. The plots of ΔH^* against the carbon number (n) in the constituent FA showed a zig-zag line with an upward convex at an odd-numbered position and the polts of the absolute values of $|-\Delta S^*|$ showed a zig-zag line with a downward convex at an odd-numbered position, though the positive value of ΔH^* increases and the negative value of ΔS^* decreases with an increasing n for either even-numbered or odd-numbered FA. It was found that the release of NAA from FA–NAA formed with odd-numbered FA is more disadvantageous enthalpically but more advantageous entropically as compared with that from FA–NAA formed with even-numbered FA. This phenomenon was similar to that observed for $(FA)_6$(TDS). Furthermore, it is suggested that FA–NAA is formed at least by van der Waals forces and hydrophobic interactions and that van der Waals forces are dominant for the formation of FA–NAA formed with odd-numbered FA and that hydrophobic interactions are dominant for the formation of FA–NAA formed with even-numbered FA.

Keywords nicotinamide; complex; fatty acid; release; release rate; kinetics; activation energy; activation enthalpy; activation entropy; odd–even effect

It has been found[2] that nicotinamide (NAA) forms the crystalline complex with higher saturated fatty acid (FA) whose molar ratio of FA to NAA is 1 : 1, FA–NAA. The release behavior of NAA from FA–NAA at 37 °C has already been determined.[1,3] In the study,[3] it was suggested that FA–NAA might be applicable to the preparation of sustained-release drug formulation and that FA–NAA is clinically useful from the viewpoint of little side effects. Furthermore, interesting phenomenon were found[1] from the physicochemical viewpoint: namely (1) the release rate of NAA from FA–NAA formed with odd-numbered FA is slower than that formed with even-numbered FA whose alkyl chain length is one more carbon number longer; (2) the relationship between the release rate constant (k) and the carbon number (n) of the constituent FA is a zig-zag one with a downward convex at an odd-numbered position.

On the other hand, FA–drug complexes have been found for thiamine disulfide (TDS), $(FA)_6$(TDS), and the release kinetics of TDS from $(FA)_6$(TDS) has already been carried out and the activation thermodynamic quantities for the release of TDS from $(FA)_6$(TDS) have been obtained.[4]

In the previous paper,[1] it was found that the plots of k *vs.* n at 37 °C indicate similar patterns for both FA–NAA and $(FA)_6$(TDS). In this paper, the values of activation energy, activation enthalpy and activation entropy will be estimated by the measurements of the release rates of NAA from FA–NAA at various temperatures, and the results will be compared with the results[4] obtained for $(FA)_6$(TDS).

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.[1-3] FA–NAA were prepared as follows: FA and NAA were dissolved in warm 1,2-dichloroethane, and the solution was set aside to crystallize.[1-3] The purity of each FA–NAA was examined by measuring the melting point of FA–NAA.[3] 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[3] 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 papers.[1,3] About 29—33 mg of each FA–NAA was used in the test. Experiments were carried out not only at 37 °C[1] but also at 32, 42 and 47 ± 0.2 °C. All experiments were carried out in triplicate and the results were highly reproducible.

Quantitative Analysis of NAA The concentration of released NAA was determined spectrophotometrically as previously described.[1,3]

Results

Release Behavior of NAA from FA–NAA The release behaviors of NAA from C14–NAA, C15–NAA, C16–NAA, C17–NAA and C18–NAA at four temperatures are shown as a relationship between the percentage of released NAA and time in Figs. 1—5, respectively. The percentages of released NAA were calculated with respect to the theoretical total concentration of NAA which is contained

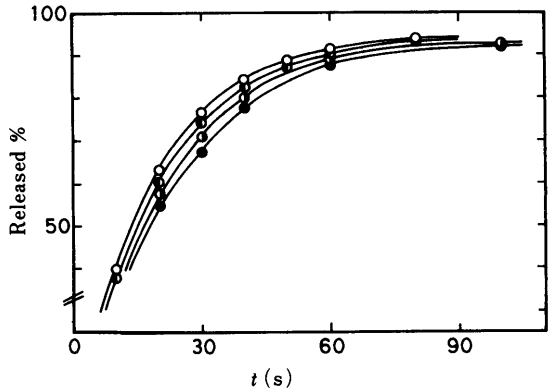

Fig. 1. Effect of Temperature on the Release Behavior of NAA from C14–NAA

Temperature: ●, 32 °C; ◑, 37 °C; ◐, 42 °C; ○, 47 °C. Particle size: 48—60 mesh.

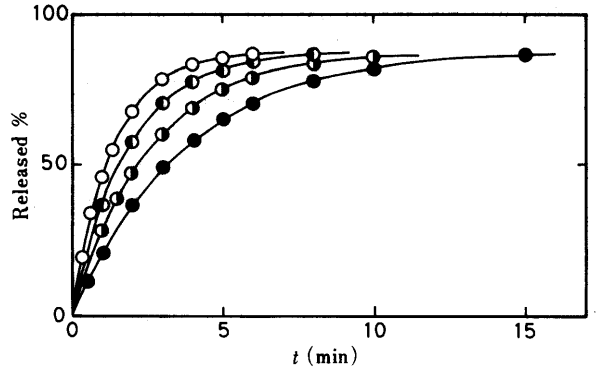

Fig. 2. Effect of Temperature on the Release Behavior of NAA from C15–NAA

Symbols are the same as in Fig. 1.

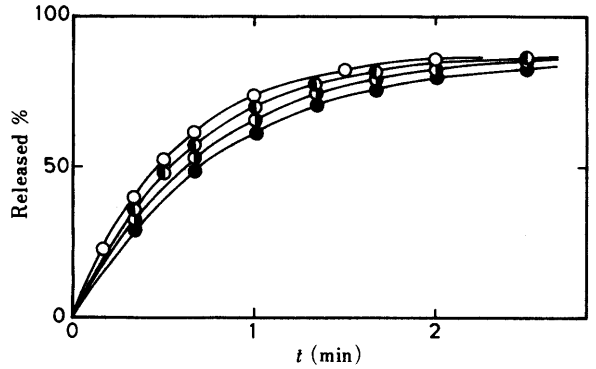

Fig. 3. Effect of Temperature on the Release Behavior of NAA from C16–NAA.

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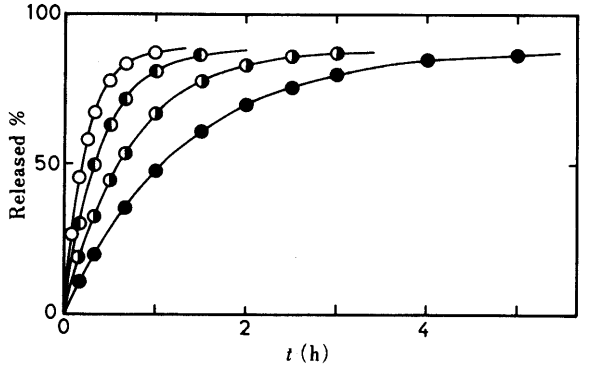

Fig. 4. Effect of Temperature on the Release Behavior of NAA from C17–NAA

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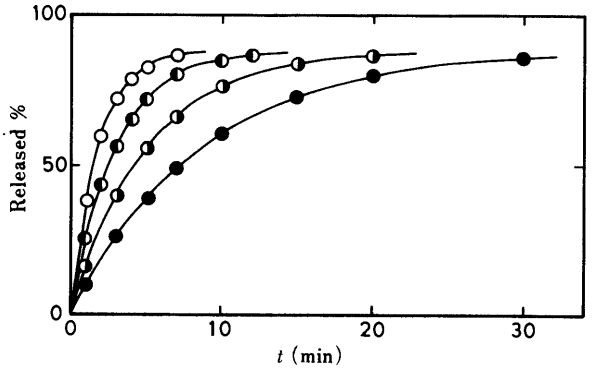

Fig. 5. Effect of Temperature on the Release Behavior of NAA from C18–NAA

Symbols are the same as in Fig. 1.

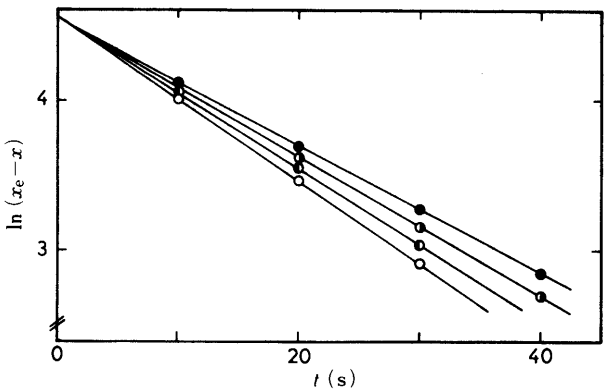

Fig. 6. Effect of Temperature on the Release of NAA from C14–NAA, $\ln(x_e-x)$ *vs.* Time

Symbols are the same as in Fig. 1.

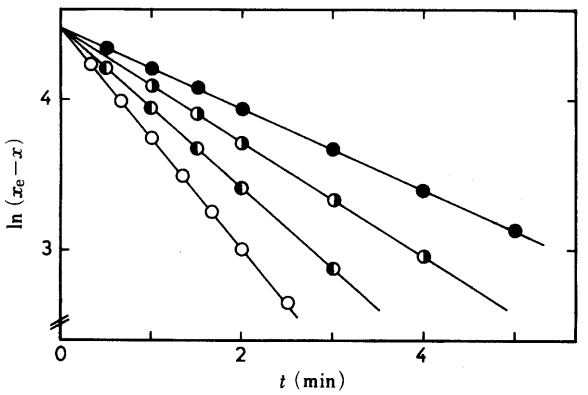

Fig. 7. Effect of Temperature on the Release of NAA from C15–NAA, $\ln(x_e-x)$ *vs.* Time

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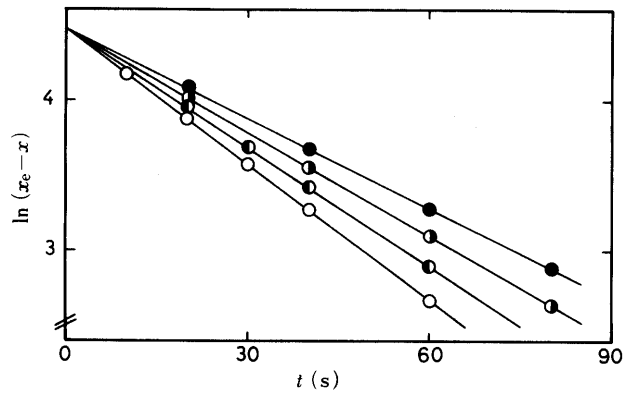

Fig. 8. Effect of Temperature on the Release of NAA from C16–NAA, $\ln(x_e-x)$ *vs.* Time

Symbols are the same as in Fig. 1.

in the 1 : 1 complex, FA–NAA. The equilibrium percent of released NAA was 90—95 under various conditions. The release rate of NAA is faster under the condition of higher temperature.

Rate Constants for the Release of NAA from FA–NAA It was confirmed[1] that the release of NAA from FA–NAA under the experimental conditions can be treated as a pseudo first-order reaction. The rate constant for the release of NAA is, therefore, defined as follows:

$$\ln(x_e-x)=\ln x_e-kt \quad (1)$$

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, calculated from the values shown in Figs. 1—5, are presented in Figs. 6—10. As can be seen in Figs. 6—10, good linear relationships were obtained in all cases. The values of k were obtained from the slopes shown in Figs. 6—10. The values of log k obtained for C14–NAA, C15–NAA, C16–NAA, C17–NAA and C18–NAA at three temperatures are plotted against n of the constituent FA in Fig. 11. As can be seen in Fig. 11, the plots of log k against n showed zig-zag lines with a downward convex at odd-numbered positions.

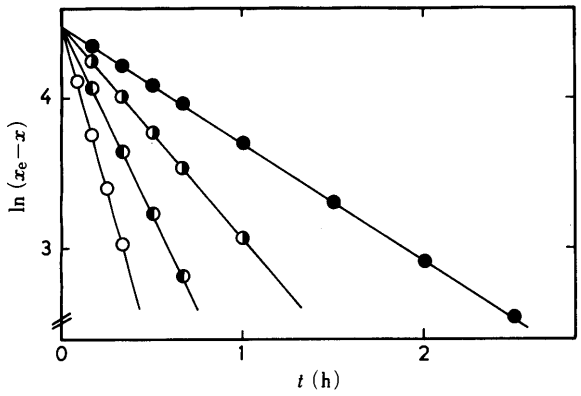

Fig. 9. Effect of Temperature on the Release of NAA from C17–NAA, $\ln(x_e-x)$ *vs.* Time

Symbols are the same as in Fig. 1.

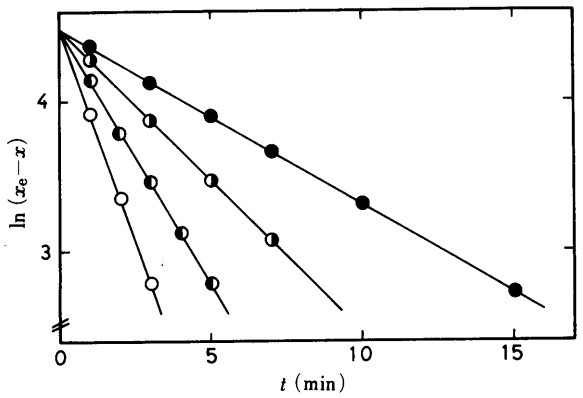

Fig. 10. Effect of Temperature on the Release of NAA from C18–NAA, $\ln(x_e-x)$ *vs.* Time

Symbols are the same as in Fig. 1.

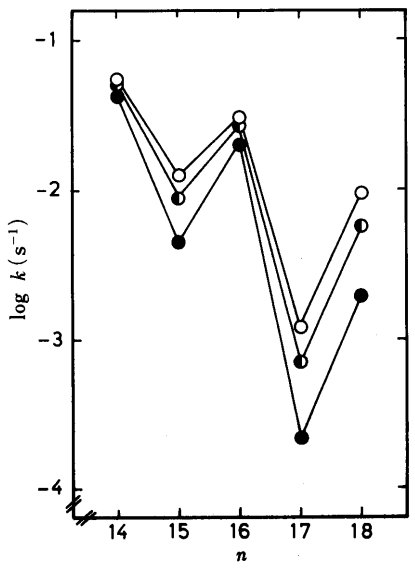

Fig. 11. Effect of FA on the Release Rate Constants (k) of NAA from FA–NAA

Symbols are the same as in Fig. 1.

Discussion

Activation Energy for the Release of NAA from FA–NAA The values of k were found to depend on the temperature. The activation energy (E^*) for the release can, therefore, be calculated from the values of k. According to the theory of Arrhenius, the relationship between k and the absolute temperature (T) is represented as follows:

$$\ln k = -\frac{E^*}{R}\cdot\frac{1}{T}+\ln A \qquad (2)$$

where R is the gas constant and A is a constant which is called frequency factor. Plots of ln k *vs.* $1/T$ based on Eq. 2 are shown in Fig. 12. As is clear in Fig. 12, the relationship between ln k and $1/T$ can be represented by a single line which depends on the alkyl chain length of FA. The values of E^* were, therefore, obtained from the values of the slopes, and the results were summarized in Table I. The positive value of E^* for FA–NAA formed with odd-numbered FA was larger than that for FA–NAA formed with even-numbered FA whose alkyl chain length is one more carbon number longer, though E^* increased rather regularly witn an increase of n for either even-numbered or odd-numbered FA. The slower release rate of NAA from FA–NAA formed with odd-numbered FA, which is shown in Fig. 11, could be explained by the relationship between E^* and n.

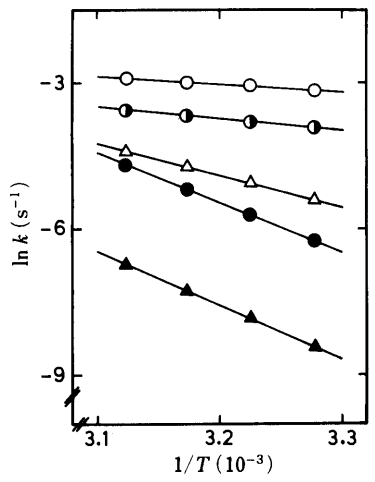

Fig. 12. Arrhenius Plots

Carbon numbers in FA: ○, 14; △, 15; ◑, 16; ▲, 17; ●, 18.

TABLE I. Activation Energies for the Release of NAA from FA–NAA

	C14–NAA	C15–NAA	C16–NAA	C17–NAA	C18–NAA
E^* (kJ mol^{-1})	13.9	54.4	22.2	92.7	85.2

Activation Thermodynamic Quantities for the Release of NAA from FA–NAA The values of activation Gibbs energy (ΔG^*) for the release of NAA from FA–NAA were estimated from the values of k as previously described.[4] The values of ΔG^* were graduated at ordinate in Fig. 13. All the values of ΔG^* are positive.

Next, we investigated which activation thermodynamic parameter contributes to the positive value of ΔG^*. ΔG^* is related to activation enthalpy (ΔH^*) and activation entropy (ΔS^*) as follows:

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (3)$$

According to Eq. 3, the values of ΔG^* were plotted against T, and the relationship is shown in Fig. 13.

As can be seen in Fig. 13, the relationship between ΔG^* and T can be represented by a single line for each complex with a different chain length of FA. The values of ΔS^* were, therefore, obtained from the values of the slope. ΔH^* was approximately estimated from the intercept, which agreed well with the value from the slope of $\Delta G^*/T$ *vs.* $1/T$ and nearly with the approximate evaluation, $E^* - RT$. The results are summarized in Table II. As can be seen in Table II, the value of ΔH^* is positive and the value of ΔS^* is negative. This indicates that the release of NAA from FA–NAA is disadvantageous not only enthalpically but also entropically. These are the same tendencies as the results obtained for $(FA)_6$(TDS).[4]

Effect of FA on the Activation Thermodynamic Quantities The values of ΔG^* at 310.15 K, ΔH^* and ΔS^* for the release of NAA from FA–NAA are represented graphically against n in Fig. 14. As can be seen in Fig. 14, the plots of positive values of ΔH^* *vs.* n indicate a zig-zag line with an upward convex at an odd-numbered position, while the plots of negative values of ΔS^* *vs.* n indicate a zig-zag line with a downward convex at an odd-numbered position. This indicates that the release of NAA from FA–NAA formed with odd-numbered FA is more disadvantageous enthalpically but more advantageous entropically as compared with that formed with even-numbered FA. Furthermore, the positive value of ΔH^* increases and the negative value of ΔS^* decreases with an increasing n for either even-numbered or odd-numbered FA. This indicates that the release of NAA from FA–NAA with a longer alkyl chain of FA is more disadvantageous enthalpically but more advantageous entropically. However, the positive value of ΔG^* increases with an increasing n for either even-numbered or odd-numbered FA, and the plots of ΔG^* *vs.* n indicate a zig-zag line with an upward convex at an odd-numbered position.

The comparison of the magnitude of $|\Delta H^*|$ with $|T\Delta S^*|$ were summarized in Table III. $|\Delta H^*|$ was larger than $|T\Delta S^*|$ for FA–NAA formed with odd-numbered FA. In addition, $|\Delta H^*|$ was larger than $|T\Delta S^*|$ for FA–NAA formed with even-numbered FA whose alkyl chain length is longer, while $|\Delta H^*|$ was smaller than $|T\Delta S^*|$ for FA–NAA formed with even-numbered FA whose alkyl chain length is shorter. This is the only difference from the results[4] that $|\Delta H^*|$ is larger than $|T\Delta S^*|$ for all of $(FA)_6$-(TDS). One reason for this cause is considered to be related to the extremely small values of ΔH^* for C14–NAA and C16–NAA as compared with the value of ΔH^* for $(FA)_6$-

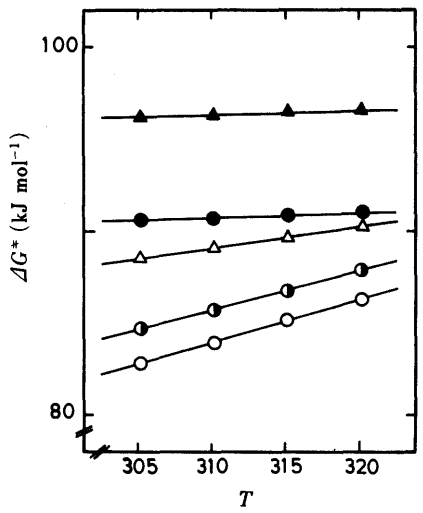

Fig. 13. Relationship between Activation Gibbs Energy and Temperature

Symbols are the same as in Fig. 12.

TABLE II. Activation Thermodynamic Quantities for the Release of NAA from FA–NAA

	ΔG^* (kJ mol^{-1})[a]	ΔH^* (kJ mol^{-1})	ΔS^* (J K^{-1} mol^{-1})
C14–NAA	84.0	11.6	−233
C15–NAA	89.1	51.9	−120
C16–NAA	85.8	19.3	−214
C17–NAA	96.3	90.1	−20.0
C18–NAA	90.8	83.0	−25.0

a) At 310.15 K.

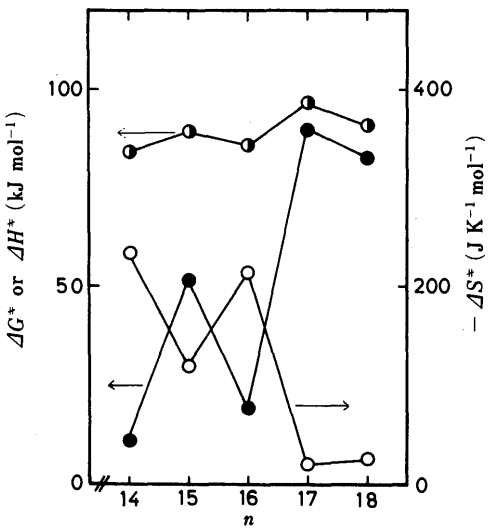

Fig. 14. Effect of FA on the Activation Thermodynamic Quantities for the Release of NAA from FA–NAA

Thermodynamic parameter: ◑, ΔG^*; ●, ΔH^*; ○, ΔS^*.

TABLE III. Comparison of the Magnitude of $|\Delta H^*|$ with $|T\Delta S^*|$

C14–NAA	$\|\Delta H^*\| < \|T\Delta S^*\|$
C15–NAA	$\|\Delta H^*\| > \|T\Delta S^*\|$
C16–NAA	$\|\Delta H^*\| < \|T\Delta S^*\|$
C17–NAA	$\|\Delta H^*\| > \|T\Delta S^*\|$
C18–NAA	$\|\Delta H^*\| > \|T\Delta S^*\|$

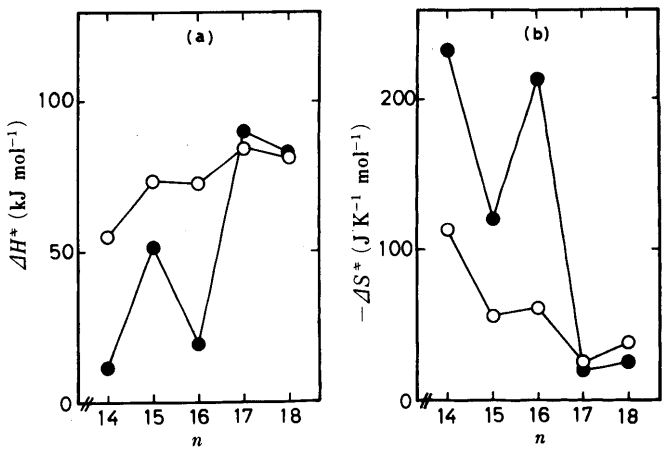

Fig. 15. Comparison of ΔH^* and ΔS^* between FA–NAA and $(FA)_6$-(TDS)

(a) Plots of ΔH^* *vs. n*. (b) Plots of ΔS^* *vs. n*. Complex: ●, FA–NAA; ○, $(FA)_6$-(TDS).

(TDS). Taking into account the change in the magnitude of $|\Delta H^*|$ and $|T\Delta S^*|$ ($|\Delta H^*| > |T\Delta S^*|$ or $|\Delta H^*| < |T\Delta S^*|$), it is reasonable to regard that FA–NAA is composed of *m* molecules of FA and *m* molecules of NAA, where *m* is not unity ($m > 1$).

Regarding the formation of complexes, it has been reported[5] that complexes formed by hydrophobic interactions have an approximate zero of ΔH^* and a positive value of ΔS^* and that complexes formed by van der Waals forces have a negative value of ΔH^* and a negative value of ΔS^*. According to the report,[5] it is suggested that hydrophobic interactions are dominant for the formation of FA–NAA composed of even-numbered FA whose alkyl chain length is shorter and that van der Waals forces are dominant for the formation of FA–NAA composed of even-numbered FA whose alkyl chain length is longer or composed of odd-numbered FA. This tendency for FA–NAA is similar to $(FA)_6$(TDS).

Comparison of the Activation Thermodynamic Quantities between FA–NAA and $(FA)_6$(TDS) In order to make a comparison between the release characteristics of NAA from FA–NAA and TDS from $(FA)_6$(TDS), the values of ΔH^* and $-\Delta S^*$ for FA–NAA were shown by closed circles in Fig. 15 together with the values[4] for $(FA)_6$-(TDS) which were shown by open circles. The plots of ΔH^* *vs. n* show zig-zag lines with an upward convex at odd-numbered positions. It is found that the relationship between ΔH^* and *n* for FA–NAA is similar to that for $(FA)_6$(TDS). However, the variation of ΔH^* for FA–NAA owing to the difference in *n* is larger than that for $(FA)_6$(TDS). Namely, the positive value of ΔH^* for FA–NAA is evidently smaller compared with that for $(FA)_6$(TDS) in the case of $14 \leqq n \leqq 16$, while the value of ΔH^* for FA–NAA becomes slightly larger than that for $(FA)_6$(TDS) in the case of $n \geqq 17$.

On the other hand, the polts of ΔS^* *vs. n* show zig-zag lines with a downward convex at odd-numbered positions. The relationship between ΔS^* and *n* for FA–NAA is similar to that for $(FA)_6$(TDS). The variation of ΔS^* for FA–NAA owing to the difference in *n* is larger than that for $(FA)_6$(TDS). Namely, the negative value of ΔS^* for FA–NAA is evidently larger as compared with that for $(FA)_6$(TDS) in the case of $14 \leqq n \leqq 16$, while the negative value of ΔS^* for FA–NAA becomes slightly smaller than that for $(FA)_6$(TDS) in the case of $n \geqq 17$. As described above, it is evident that FA–NAA has a similar physicochemical property to $(FA)_6$(TDS) and that the variation of physicochemical property of FA–NAA owing to the difference in *n* is larger than that of $(FA)_6$(TDS). This may be considered to the due to that FA–NAA is composed of *m* molecules of NAA and $(FA)_m$ in contrast to that $(FA)_6$(TDS) is composed of one molecule of TDS and $(FA)_6$. It is considered that FA–NAA with a longer alkyl chain of FA is more greatly stabilized than that with a shorter alkyl chain of FA as compared with the stability of $(FA)_6$(TDS) if the *m* molecules of NAA are included in $(FA)_m$. Recently, it was found that the molecular formula of FA–NAA is $(FA)_6(NAA)_6$. The content was promptly reported.[6]

$(FA)_6$(TDS) seems to be an inclusion compound.[4,7] The similar physicochemical properties between FA–NAA and $(FA)_6$(TDS) suggest that FA–NAA also may be an inclusion compound.

The structure formulas of NAA and TDS were shown in the previous paper.[1] NAA and TDS have the structure of a six-membered ring. NAA has a pyridine-ring, while TDS has two pyrimidine-rings which are linked by long side chains. It is therefore suggested that one molecule of TDS may be included axially in the $(FA)_6$ host structure, whereas six molecules of NAA may be included equatorially in $(FA)_6$. This may reflect the larger variation of ΔS^* and ΔH^* for FA–NAA owing to the difference in *n*.

Regarding the effect of polar groups, it has been found[7b] from the measurement of the heat of dissolution of $(FA)_6$-(TDS) that the polar groups ($-NH_2$, -OH and -CHO) do not contribute much to the binding force between $(FA)_6$ host and guest molecule. It is thought that the polar groups of the guest molecule contribute as a driving force for inclusion in the $(FA)_6$ host structure from the bulk phase, 1,2-dichloroethane solution.

Conclusion

The values of ΔH^* and ΔS^* for the release of NAA from FA–NAA were positive and negative, respectively. The plots of ΔH^* *vs. n* showed a zig-zag line with an upward convex at an odd-numbered position, while the plots of ΔS^* *vs. n* showed a zig-zag line with a downward convex at an odd-numbered position. These phenomena were similar to those observed for $(FA)_6$(TDS).

It is suggested by comparison of ΔH^* and ΔS^* among FA–NAA with various alkyl chains that FA–NAA is formed at least by van der Waals forces and hydrophobic interactions and that van der Waals forces are dominant for the formation of FA–NAA formed with odd-numbered FA and hydrophobic interactions are dominant for the formation of FA–NAA formed with even-numbered FA. These are also the same tendencies as estimated[4] for the formation of $(FA)_6$(TDS). It is suggested that FA–NAA may be an inclusion compound as well as $(FA)_6$(TDS).

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Synthetic Studies for Novel Structure of α-Nitrogenously Functionalized α-Fluorocarboxylic Acids. II.[1] Synthesis and Some Reactions of α-Fluoro-α-nitrocarboxylic Ester and Carboxamide Derivatives

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The first synthesis of α-fluoro-α-nitrocarboxamides 11b, c has been achieved by ammonolysis of the corresponding ethyl esters 6b, c. However, α-fluoro-α-nitrocarboxylic acids 8a—c derived from the corresponding esters 6a—c were found to decarboxylate readily to form 1-fluoro-1-nitroalkanes 9a—c. Reduction of the α-fluoro-α-nitrocarboxamide derivatives 11b, c produced the defluorination products 12b, c instead of the desired novel α-fluoro-α-aminocarboxamide 2.

Keywords α-fluoro-α-amino acid; fluoroglycine; fluorination; perchloryl fluoride; α-fluoro-α-nitrocarboxamide; α-nitrocarboxamide; reductive defluorination

α-Fluoro-α-amino acids **1** and the amides **2** have aroused much interest from both biological[2] and structural[3] chemistry viewpoints. However, the interest has been theoretical, since no report of success in the construction of such α-halogenated amino acid structures exists in the literature. We have recently reported[1] the first synthesis of some *N*-protected α-fluoroglycine derivatives, **3** and **4**, and the attempted conversion of them into α-fluoroglycine **1** (R = H) itself. However, we could not find conditions mild enough for complete removal of the *N*-protecting groups in **3** or **4** without damaging the C–F bond. We therefore attempted the synthesis of novel α-fluoro-α-nitrocarboxylic acid and α-fluoro-α-nitrocarboxamide precursors and the conversion of these new candidates into the α-fluoro-α-amino acid derivatives. The chemical behavior of the geminally functionalized molecules toward hydrogenolysis was also a point of interest.

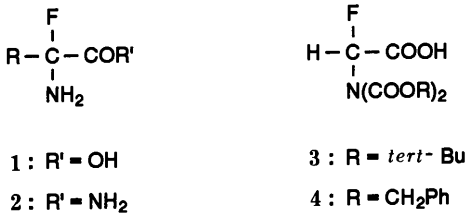

Fig. 1

Results and Discussion

In our previous work,[1] we selected bis(alkoxycarbonyl)-amino groups as an amino group precursor, as shown by the structures **3** and **4**. However, the carbamate structure generated at the final deprotecting stage easily formed an oxazolone with a free carboxylic acid, ultimately producing unwanted products instead of the free amine. We have therefore focused on the nitro group as an amino functionality precursor, since a nitro group can usually be reduced easily into an amino group under neutral conditions. Furthermore, fluorination of the position α to the nitro group seemed to proceed smoothly.[4]

The direct introduction of a nitro group into fluorocarboxylic esters proved to be difficult.[5] Therefore, some α-fluoro-α-nitrocarboxylic acid esters **6a**—**c** were prepared by direct fluorination of ethyl nitroacetate **5a** with perchloryl fluoride[6] or by fluorination of alkylated products **5b**, **c** which were prepared from **5a**.[7] Reduction of **6a**—**c** with H_2/Pd–C, H_2/Raney Ni T-1,[8] or H_2/Pd–$BaSO_4$,[9] however,

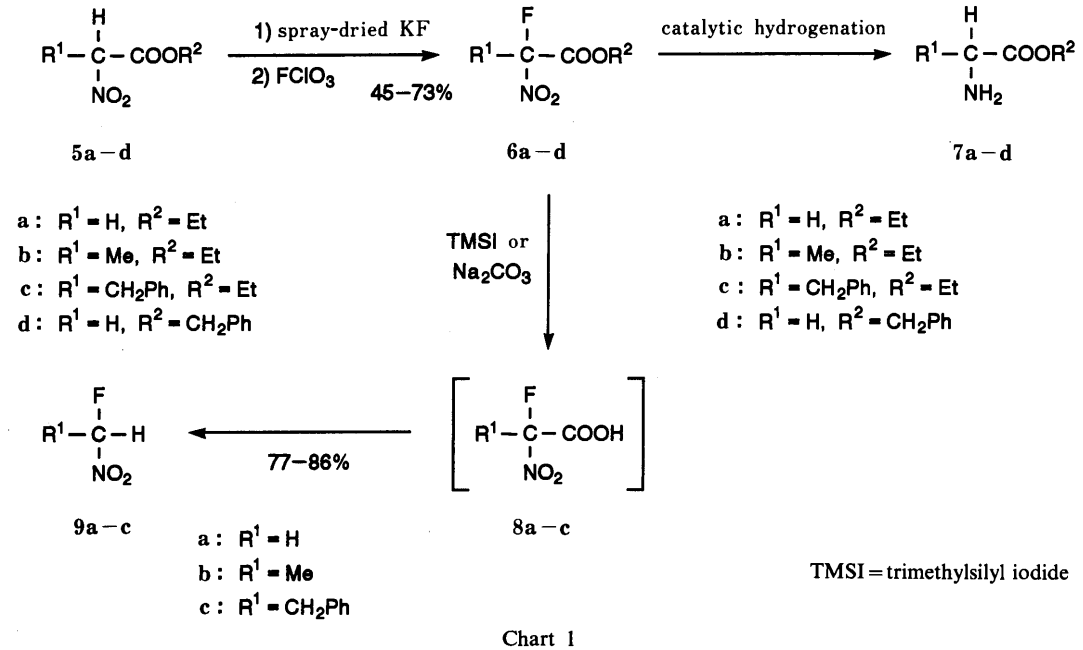

Chart 1

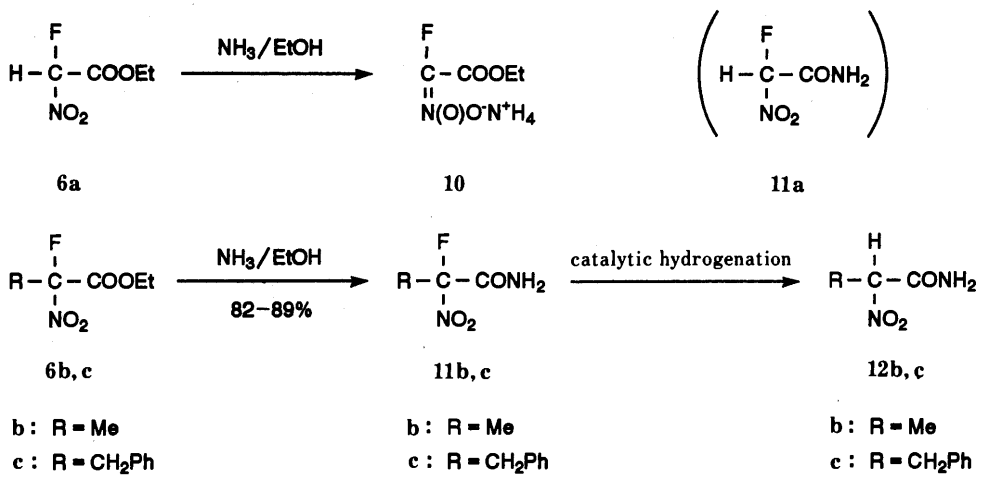

Chart 2

did not afford the desired products, usually giving the corresponding amino acid ethyl esters **7a—c** in good yield.

We thought that the free carboxylate form, which is deactivated as compared with the ester, would allow us to avoid the problematic step of hydrogenative defluorination. However, attempted conversion of the ethyl esters **6a—c** into the corresponding carboxylic acids **8a—c** by saponificative[10] and nonsaponificative[11] methods resulted in the formation of the decarboxylated[12] products **9a—c** instead of the desired **8a—c**.

We next focused on the benzyl ester **6d**, an analogous compound to the ethyl esters **6a—c**. Hydrogenative debenzylation and concomitant reduction of the nitro group should certainly form the zwitterion of the target structure **1**.[1] Thus, benzyl α-fluoro-α-nitroacetate **6d** prepared from benzyl nitroacetate **5d**[13] was subjected to hydrogenation using Pd–C or Raney Ni T-1[8] as a catalyst. However, only glycine benzyl ester **7d** was obtained, which suggested that both cleavage of the fluorine atom and reduction of the nitro group had proceeded prior to debenzylation (Chart 1).

The second carbonyl candidate chosen as an alternative to the ester group was the corresponding carboxamide structure, which would be expected to suppress the removal of fluorine under conditions of hydrogenolysis. However, the synthesis of α-fluoro-α-nitrocarboxamides has not yet been reported. Reaction of **6a** with ethanolic ammonia did not give the corresponding amide **11a**, yielding instead simply the salt **10**, presumably due to the presence of a proton α to the nitro group. We then investigated alkylated analogues having no acidic protons. Treatment of **6b, c** with ethanolic ammonia produced successfully the desired amides **11b, c** (82—89%).

The key compounds **11b, c** now being in hand, we started to investigate their possible conversion into the novel α-amino-α-fluorocarboxamide structure **2**. α-Fluoro-α-nitrocarboxamides **11b, c** were submitted to reduction under various conditions.[8,9,14–16] To our extreme disappointment again no amine compounds were obtained and the only detectable products were the defluorinated amides **12b, c**. Therefore, direct defluorination occurred before nitro group reduction even for the amide structure (Chart 2).

In the course of our study, we showed that α-fluoro-α-nitrocarboxamides can be obtained as stable molecules, although we were unable to achieve the construction of an α-amino-α-fluorocarboxamide structure. We are now investigating the use of an azido group as an amino group precursor, since an azido group can generally be reduced much more easily than the corresponding nitro group.[17] However, the synthesis of α-azido-α-fluorocarboxylic acid derivatives seems at this time to be very challenging.

Experimental

Infrared (IR) spectra were recorded on a JASCO A-102 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured in $CDCl_3$, unless otherwise noted, with Me_4Si as an internal standard and recorded on JEOL PMX-60 (60 MHz), Varian XL-200 (200 MHz), and JEOL GX-270 (270 MHz) spectrometers. ^{19}F-NMR spectra were measured in $CDCl_3$ with $CFCl_3$ as an internal standard and taken with a JEOL GX-270 (254 MHz) spectrometer. Upfield shifts are quoted as negative. Electron impact mass spectra (EI-MS) were taken with a JEOL JMS-D300 spectrometer. Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Elemental analyses were performed with a Hitachi 026 elemental analyzer. Column chromatography and preparative thin layer chromatography were performed using Kieselgel 60 (Merck, Art. 9385 and Art. 7748, respectively). α-Fluoro-α-nitrocarboxylic esters **6a—c** were prepared according to our method.[18]

Benzyl Fluoronitroacetate (6d) Spray-dried KF[19] (1.16 g, 20 mmol) was added to a solution of benzyl nitroacetate **5d** (1.95 g, 10 mmol) in dry MeOH (30 ml), and the mixture was stirred at room temperature for 0.5 h. The solvent was evaporated off, and the residual salt was dissolved in dry tetrahydrofuran (THF) (50 ml). Into this solution was introduced diluted perchloryl fluoride generated from $KClO_4$ (4 g) and FSO_3H (40 g) at 0 °C for 1 h. Evaporation of the solvent gave a solid, which was dissolved in AcOEt (50 ml) and washed with water (10 ml). The organic layer was dried on $MgSO_4$ and concentrated to give an oil. Purification by silica gel chromatography gave **6d** as a colorless oil in 54% yield (1.15 g). IR ν_{max}(neat) cm^{-1}: 1770 (CO), 1590 (NO_2). ^{1}H-NMR δ: 5.3 (2H, s, CH_2), 6.0 (1H, d, J=48 Hz, CH), 7.3 (5H, s, Ph). MS m/z: 213 (M^+), 267 (M^+-NO_2), 266 (M^+-HNO_2). High MS: Calcd for $C_9H_8FNO_4$ (mol. weight 213.0435), Found: 213.0397 (M^+). Calcd for $C_9H_8FO_2$ (mol. weight 167.0508), Found: 167.0478 (M^+-NO_2). Calcd for $C_9H_7FO_2$ (mol. weight 166.0428), Found: 166.0416 (M^+-HNO_2).

General Procedure for Catalytic Hydrogenation of α-Fluoro-α-nitrocarboxylic Acid Esters (6a—d) A solution of one of **6a—d** (2.5 mmol) in EtOH (50 ml) was hydrogenated over Pd–C, Raney Ni T-1,[8] or Pd–$BaSO_4$[9] using a Parr apparatus (4 kg/cm^2) for 20—24 h. Removal of the catalyst and evaporation of the solvent afforded the corresponding amino acid ethyl or benzyl ester **7a—d** as a colorless oil in 68—81% yield. The products were characterized through the spectral data.[20]

Hydrolysis of α-Fluoro-α-nitrocarboxylic Acid Esters (6a—d) The esters **6a—d** were hydrolyzed by a saponificative[10] or non-saponificative[11] method according to the literature. After usual work-up, 1-fluoro-1-nitroalkanes **9a—c** were obtained as colorless oils in 77—86% yields. The products were characterized through the spectral data.[18]

General Procedure for Preparation of α-Fluoro-α-nitrocarboxamides (11b, c) A solution of an ethyl ester **6b** or **c** (2 mmol) in EtOH (5 ml) was added to ammonia-saturated EtOH (15 ml) in an ice-bath and the whole was stirred at 0 °C for 2 h. Evaporation of the solvent under reduced pressure afforded colorless crystals, which were collected on a filter and dried.

2-Fluoro-2-nitropropionamide (11b) Colorless leaflets in 82% yield (224 mg). mp 50.0—50.5 °C (CCl_4/hexane). IR ν_{max} (KBr) cm^{-1}: 3400 (NH), 1690 (CO), 1570 (NO_2). ^{1}H-NMR δ: 2.13 (3H, d, J(H-F) = 21.0 Hz, Me), 6.57 (2H, br s, NH_2). ^{19}F-NMR δ: −123.78 (q, J(F-H) = 20.8 Hz). MS *m/z*: 136 (M^+), 90 ($M^+ - NO_2$), 46 ($M^+ - NO_2 - CONH_2$). *Anal.* Calcd for $C_3H_5FN_2O_3$: C, 26.48; H, 3.70; N, 20.59. Found: C, 26.27; H, 3.72; N, 20.47.

2-Fluoro-2-nitro-3-phenylpropionamide (11c) Colorless needles in 89% yield (377 mg). mp 116.5—118.5 °C ($CHCl_3$). IR ν_{max} (KBr) cm^{-1}: 3450 (NH), 1690 (CO), 1570 (NO_2). ^{1}H-NMR δ: 3.72 (1H, dd, J(H_a-F) = 21.5, J(H_a-H_b) = 14.9 Hz, CH_aH_b), 3.89 (1H, dd, J(H_b-F) = 26.5, J(H_b-H_a) = 14.9 Hz, CH_aH_b), 5.83 (1H, br s, NH_aH_b), 6.23 (1H, br s, NH_aH_b), 7.30—7.34 (5H, m, Ph). ^{19}F-NMR δ: −131.86 (dd, J(F-H_b) = 26.5, J(F-H_a) = 21.5 Hz). MS *m/z*: 212 (M^+), 166 ($M^+ - NO_2$), 122 ($M^+ - NO_2 - CONH_2$), 103 ($M^+ - NO_2 - CONH_2 - F$), 91 ($PhCH_2^+$). *Anal.* Calcd for $C_9H_9FN_2O_3$: C, 50.95; H, 4.28; N, 13.20. Found: C, 51.13; H, 4.36; N, 13.32.

General Procedure for Reduction of α-Fluoro-α-nitrocarboxamides (11b, c) A solution of **11b** or **c** (2 mmol) in EtOH (40 ml) was hydrogenated over Raney Ni T-1,[8] Raney Ni T-4,[14] Pd–$BaSO_4$,[9] or PtO_2[15] using a Parr apparatus (4 kg/cm^2) for 10—36 h. Removal of the catalyst and evaporation of the solvent under reduced pressure afforded the corresponding α-nitrocarboxamide derivative **12b** or **c** as a colorless solid in 68—77% yield. These products were characterized through spectral data.[20]

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A Regioselective Lithiation of *ortho*-Cresols Using the Bis(dimethylamino)phosphoryl Group as a Directing Group: General Synthesis of 2,3-Dihydrobenzo[*b*]furans Including Naturally Occurring Neolignans

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A general synthetic method was developed for 2-aryl-2,3-dihydrobenzo[*b*]furans *via* regioselective lithiation of *ortho*-tolyl tetramethylphosphorodiamidates followed by addition of aromatic aldehydes as a key step. *ortho*-Tolyl tetramethylphosphorodiamidates were regioselectively lithiated with *sec*-BuLi in tetrahydrofuran at −105 °C to give benzylic lithio species. The resulting lithio species were reacted with aromatic aldehydes to provide 1,2-diarylethanol derivatives. Reductive removal of the phosphoryl group with lithium aluminum hydride followed by acidic treatment led to 2-aryl-2,3-dihydrobenzo[*b*]furans in good overall yields. The utility of this strategy was demonstrated in regioselective syntheses of highly substituted neolignan natural products, such as (±)-licarin B and (±)-carinatol, starting from *O*-bis(dimethylamino)phosphorylated eugenol or isoeugenol.

Keywords lithiation; *ortho*-cresol; bis(dimethylamino)phosphoryl group; *trans*-2-aryl-2,3-dihydro-3-methylbenzo[*b*]furan; *cis*-2-aryl-2,3-dihydro-3-methylbenzo[*b*]furan; *threo*-1,2-diaryl-1-propanol; *erythro*-1,2-diaryl-1-propanol; neolignan; (±)-licarin B; (±)-carinatol

Several 2-aryl-2,3-dihydrobenzo[*b*]furans have been found among the lignans and neolignans.[1] Moreover, a number of 2,3-dihydrobenzo[*b*]furans exhibit many interesting pharmacological properties such as adrenergic α-blocking and diuretic activities.[2] Many synthetic methods have been developed and used for their construction.[2,3] Thermal transformation of *ortho*-allylphenols, which were prepared by Claisen rearrangement of allylethers of phenols, in *N*,*N*-diethylaniline at 225 °C has been widely employed for the synthesis of 2,3-dihydrobenzo[*b*]furans.[2,3] In this paper, we describe a new and convenient synthetic method for 2-aryl-2,3-dihydrobenzo[*b*]furans *via* regioselective lithiation[4] of *ortho*-cresols using the bis(dimethylamino)-phosphoryl group as a directing group.[5] An application of the new methodology to a total synthesis of naturally occurring neolignans[1] is also described.

Recently, we developed a general method for the synthesis of 2-arylbenzo[*b*]furans *via* regioselective lithiation of *ortho*-tolyl *N*,*N*,*N*′,*N*′-tetramethylphosphorodiamidates followed by reaction with aromatic esters as a key step.[5] This method was successfully applied to total syntheses of highly substituted neolignan natural products[5b,d] and phytoalexins.[5e] In order to extend the utility of this regioselective lithiation, we became interested in the syntheses of 2-aryl-2,3-dihydrobenzo[*b*]furans, especially neolignan natural products having a 2-aryl-2,3-dihydro-3-methylbenzo[*b*]furan[1] skeleton.

Synthesis of 2-Aryl-2,3-dihydrobenzo[*b*]furans Lithiation[5b,d] of *ortho*-tolyl *N*,*N*,*N*′,*N*′-tetramethylphosphorodiamidate (**1a**) with 1.2 eq of *sec*-BuLi in tetrahydrofuran (THF) at −105 °C for 1 h resulted in the formation of the yellow benzylic anion, which upon treatment with benzaldehyde (**2a**) at −105 °C followed by quenching with saturated NH_4Cl solution at −90 °C gave the 1,2-diarylethanol derivative[6] (**3a**)[5d] in 43% yield. When this reaction was carried out in the presence of 1.2 eq of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA), the yield of **3a** was improved to 69% yield. Treatment of **3a** with 1.1 eq of $LiAlH_4$ in refluxing THF for 1 h removed the bis(dimethylamino)phosphoryl group to give 2-(2′-hydroxy-

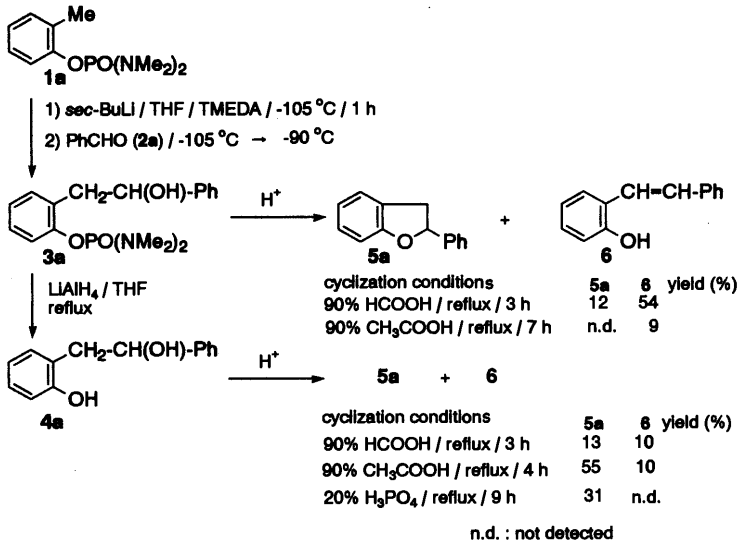
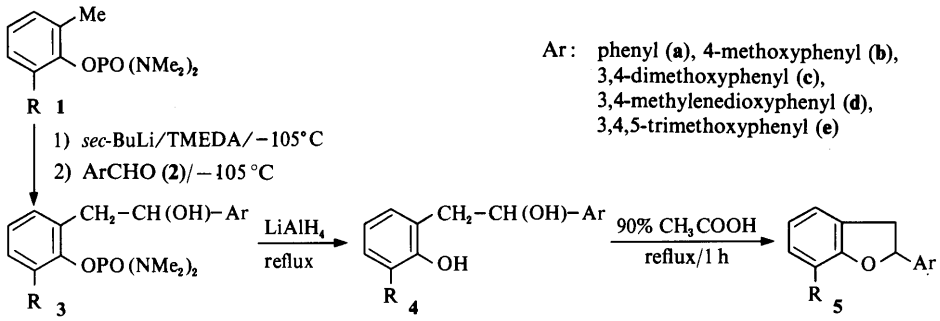


Chart 1

phenyl)-1-phenylethanol (**4a**) in 67% yield.[5d] For the synthesis of 2,3-dihydrobenzo[*b*]furans, acidic cyclization of **3a** and **4a** was investigated under various conditions (Chart 1). Treatment of **3a** with 90% formic acid under reflux for 3 h gave the desired 2,3-dihydro-2-phenylbenzo[*b*]furan (**5a**) in 12% yield along with the stilbene derivative (**6**) in 54% yield as a major product, which resulted from dephosphorylation and dehydration. When **3a** was refluxed in 90% acetic acid for 7 h, **6** was obtained in only 9% yield and no **5a** was detected. On the other hand, treatment of **4a** with 90% formic acid gave **5a** in 13% yield accompanied with **6** in 10% yield. The above results clearly suggested that dehydration was faster than dephosphorylation in **3a**. Finally, **5a** was obtained in satisfactory yield by the cyclodehydration of **4a** using 90% acetic acid or 20% phosphoric acid (Chart 1). As shown in Table I, various 2-aryl-2,3-dihydrobenzo[*b*]furan derivatives (**5**) were synthesized starting from **1a**, **b** and **2a**—**e**

TABLE I. Synthesis of 2-Aryl-2,3-dihydrobenzo[*b*]furans (**5**)



Ar: phenyl (**a**), 4-methoxyphenyl (**b**), 3,4-dimethoxyphenyl (**c**), 3,4-methylenedioxyphenyl (**d**), 3,4,5-trimethoxyphenyl (**e**)

Entry	1	R	2	3	Yield (%)	4	Yield (%)	5	R	Ar	Yield (%)
1	**1a**	H	**2a**	**3a**	69 (43)[a]	**4a**	67	**5a**	H	**a**	55
2	**1a**	H	**2b**	**3b**	77 (54)[a]	**4b**	77	**5b**	H	**b**	87
3	**1a**	H	**2c**	**3c**	89 (52)[a]	**4c**	58	**5c**	H	**c**	88
4	**1a**	H	**2d**	**3d**	77 (44)[a]	**4d**	62	**5d**	H	**d**	94
5	**1a**	H	**2e**	**3e**	88 (80)[a]	**4e**	54	**5e**	H	**e**	94
6	**1b**	Me	**2d**	**3f**	69	**4f**	89	**5f**	Me	**d**	99

a) Yields were obtained in the absence of TMEDA.

TABLE II. Physical Properties and Spectral Data of 1,2-Diarylethanol Derivatives (**3** and **4**)

Compound No.	mp (°C) (Recryst. solv.)	Formula (MS, *m/z*: M^+)	Analysis (%) Calcd (Found)	UV λ_{max}^{EtOH} nm (log ε)	IR ν_{max}^{KBr} cm^{-1}	^{1}H-NMR[a] δ ($CDCl_3$, *J*=Hz)
3c	Oil	$C_{20}H_{29}N_2O_5P$ (408)	C: 57.96 (58.15)[b] H: 7.21 (7.22) N: 6.76 (6.67)	229 (s) (3.98), 276 (3.51), 285 (s) (3.43)	3360, 2940, 1590, 1510, 1490, 1460, 1305, 1260, 1225, 1175, 1100, 1000[c]	2.67 (12H, d, *J*=10.2), 2.98—3.12 (2H, m), 3.77 (3H, s), 3.79 (3H, s), 4.33 (1H, br s), 4.75—4.84 (1H, m), 6.79—7.12 (7H, m)
3d	156—158 (CH_2Cl_2–hexane)	$C_{19}H_{25}N_2O_5P$ (392)	C: 58.15 (57.88) H: 6.42 (6.34) N: 7.14 (7.10)	236.5 (3.65), 267 (s) (3.11), 275 (s) (3.37), 287 (3.57)	3350, 2920, 2880, 1485, 1435, 1305, 1230, 1170, 1090, 1040, 990	2.74 (12H, d, *J*=9.6), 3.00—3.14 (2H, m), 3.52—3.70 (1H, m), 4.81—5.03 (1H, m), 5.94 (2H, s), 6.79—7.27 (7H, m)
3e	Oil	$C_{21}H_{31}N_2O_6P$ (438)	C: 56.74 (56.82)[d] H: 7.18 (7.15) N: 6.30 (6.11)	230 (s) (4.02), 268 (3.20), 271.5 (s) (3.20)	3360, 2970, 1590, 1490, 1460, 1230, 1130, 1000[c]	2.70 (12H, d, *J*=10.2), 2.97—3.26 (2H, m), 3.77 (6H, s), 3.79 (3H, s), 4.58—4.85 (1H, m), 6.56 (2H, br s), 7.14 (5H, br s)
3f	111—112 (Ether–CH_2Cl_2)	$C_{20}H_{27}N_2O_5P$ (406)	C: 59.10 (58.91) H: 6.70 (6.62) N: 6.89 (6.86)	236 (3.69), 287 (3.62)	3400, 2940, 2880, 1490, 1465, 1305, 1260, 1225, 1175, 1000	2.29 (3H, s), 2.69 (6H, d, *J*=9.6), 2.71 (6H, d, *J*=9.6), 2.89—3.19 (2H, m), 4.78—5.01 (1H, m), 5.87 (2H, s), 6.62—6.79 (2H, m), 6.96—7.18 (5H, m)
4a	84—86 (Ether–pentane)	$C_{14}H_{14}O_2$ (214)	C: 78.48 (78.20) H: 6.59 (6.63)	275.5 (3.42), 280 (3.39)	3350, 3025, 1585, 1490, 1425, 1240, 1105, 1045, 1025	2.93—3.03 (2H, m), 3.72 (1H, br s), 4.75—4.94 (1H, m), 6.76—6.95 (3H, m), 7.25 (6H, br s), 8.40 (1H, br s)
4b	51—53 (Ether–pentane)	$C_{15}H_{16}O_3$ (244)	C: 73.75 (73.53) H: 6.60 (6.74)	225 (4.30), 276 (3.64), 281 (s) (3.58)	3175, 1610, 1510, 1460, 1420, 1300, 1250, 1175, 1100, 1040	2.87—3.04 (2H, m), 3.73 (3H, s), 4.76—4.95 (1H, m), 6.72—7.28 (9H, m), 8.48 (1H, br s)
4c	85—88 (Ether–pentane)	$C_{16}H_{18}O_4$ (274)	C: 70.05 (70.11) H: 6.61 (6.65)	221 (s) (4.16), 277.5 (s) (3.76)	3450, 3220, 1580, 1510, 1485, 1460, 1260, 1250, 1140, 1120	2.90—3.03 (2H, m), 3.67 (3H, s), 3.75 (3H, s), 4.15 (1H, br s), 4.77—4.95 (1H, m), 6.61—7.11 (7H, m), 8.63 (1H, br s)
4d	110—113 (Ether–pentane)	$C_{15}H_{14}O_4$ (258)	C: 69.75 (69.62) H: 5.46 (5.53)	234 (s) (3.78), 275 (s) (3.74), 282.5 (3.82)	3190, 1590, 1505, 1495, 1445, 1260, 1240, 1100, 1040, 1000	2.88—3.06 (2H, m), 3.30—3.53 (1H, m), 4.78—4.97 (1H, m), 5.91 (2H, s), 6.76—7.16 (7H, m), 8.17 (1H, br s)
4e	144—147 (Ether–pentane)	$C_{17}H_{20}O_5$ (304)	C: 67.09 (66.91) H: 6.62 (6.53)	274 (4.31), 278 (s) (4.27)	3325, 2960, 1595, 1495, 1460, 1425, 1325, 1240, 1125, 1010	2.90—3.02 (3H, m), 3.70 (6H, s), 3.72 (3H, s), 4.76—4.94 (1H, m), 6.44 (2H, s), 6.71—7.11 (5H, m)
4f	65—67 (Ether–pentane)	$C_{16}H_{16}O_4$ (272)	C: 70.57 (70.64) H: 5.92 (6.01)	234 (s) (3.81), 282.5 (s) (3.78)	3550, 3350, 2900, 1500, 1480, 1435, 1235, 1035, 935	2.22 (3H, s), 2.80—3.02 (2H, m), 4.56 (1H, br s), 4.81 (1H, dd, *J*=8.4, 2.4), 5.88 (2H, s), 6.66—7.08 (6H, m)

Physical properties and spectral data of **3a** and **3b** have been published in ref. 5*d*. *a*) Listed as chemical shifts (number of protons, multiplicity, coupling constant in Hz). *b*) For $C_{21}H_{29}N_2O_5P \cdot 1/3H_2O$. *c*) Neat. *d*) For $C_{21}H_{31}N_2O_6P \cdot 1/3H_2O$.

TABLE III. Physical Properties and Spectral Data of 2-Aryl-2,3-dihydrobenzo[*b*]furan Derivatives (**5**)

Compound No.	mp (bp) (°C) (Recryst. solv.)	Formula (MS, *m/z*: M^+)	Analysis (%) Calcd (Found)	UV λ_{max}^{EtOH} nm (log ε)	IR ν_{max}^{KBr} cm^{-1}	^{1}H-NMR[a] δ ($CDCl_3$, *J*=Hz)
5a	170 (30)	$C_{14}H_{12}O$ (196)	C: 85.68 (85.69) H: 6.16 (6.35)	225(s) (3.85), 281.5 (3.56), 287(s) (3.51)	3040, 2910, 1600, 1480, 1460, 1230, 1170, 1015, 975[b]	3.14 (1H, dd, *J*=15.6, 8.4), 3.63 (1H, dd, *J*=15.6, 8.4), 5.73 (1H, t, *J*=8.4), 6.79—6.95 (2H, m), 7.04—7.35 (2H, m), 7.35 (5H, s)
5b	51—52 (Ether–hexane)	$C_{15}H_{14}O_2$ (226)	C: 79.62 (79.51) H: 6.24 (6.47)	228.5 (4.28), 279(s) (3.69), 282 (3.70), 288(s) (3.57)	2910, 1610, 1520, 1480, 1300, 1250, 1235, 1175, 1025	3.10 (1H, dd, *J*=15.6, 9.0), 3.50 (1H, dd, *J*=15.6, 9.0), 3.71 (3H, s), 5.63 (1H, t, *J*=9.0), 6.84 (2H, d, *J*=9.6), 6.80—7.06 (4H, m), 7.30 (2H, d, *J*=9.6)
5c	145 (0.25)	$C_{16}H_{16}O_3$ (256)	C: 74.98 (74.70) H: 6.29 (6.33)	230 (4.15), 281 (3.83), 285(s) (3.80), 314 (3.07), 327(s) (2.97)	2940, 1600, 1520, 1480, 1460, 1260, 1235, 1160, 1140, 1025[b]	3.04—3.56 (2H, m), 3.78 (3H, s), 5.60 (1H, t, *J*=9.0), 6.66—7.14 (7H, m)
5d	41—42 (Ether–pentane)	$C_{15}H_{12}O_3$ (240)	C: 74.99 (74.85) H: 5.03 (5.23)	228 (3.96), 285(s) (3.88), 288 (3.88)	2900, 1595, 1500, 1480, 1440, 1395, 1245, 1040	3.09 (1H, dd, *J*=15.0, 8.4), 3.55 (1H, dd, *J*=15.0, 8.4), 5.62 (1H, t, *J*=8.4), 5.87 (2H, s), 6.63—7.16 (4H, m)
5e	150 (0.3)	$C_{17}H_{18}O_4$ (286)	C: 71.31 (71.16) H: 6.34 (6.34)	225(s) (4.97), 280 (4.39), 287(s) (4.29), 310 (3.52), 325(s) (3.38)	2940, 1590, 1510, 1480, 1460, 1420, 1230, 1125, 1000[b]	3.12—3.55 (2H, m), 3.83 (9H, s), 5.66 (1H, t, *J*=9.0), 6.63 (2H, s), 6.79—7.25 (4H, m)
5f	72—75 (Ether)	$C_{16}H_{14}O_3$ (254)	C: 75.57 (75.80) H: 5.55 (5.64)	231(s) (3.91), 287 (3.85)	2880, 1595, 1500, 1490, 1445, 1250, 1190, 1035, 930	2.19 (3H, s), 3.00 (1H, dd, *J*=15.0, 9.0), 3.47 (1H, dd, *J*=15.0, 9.0), 5.54 (1H, t, *J*=9.0), 5.79 (2H, s), 6.57—6.87 (6H, m)

a) Listed as chemical shifts (number of protons, multiplicity, coupling constant in Hz). *b*) Neat.

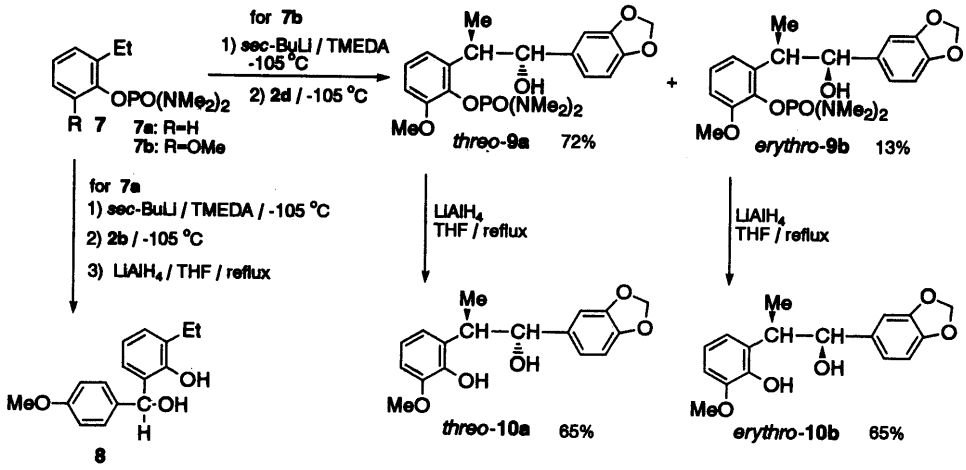


Chart 2

under the reaction conditions described above *via* **3**→**4**→**5** reaction sequences, which provide a general and efficient route to 2,3-dihydrobenzo[*b*]furans. The structures of all compounds were established by infrared (IR), ultraviolet (UV), proton nuclear magnetic resonance (^{1}H-NMR), mass spectral (MS) data and elemental analyses as summarized in Tables II and III.

Syntheses of 2-Aryl-2,3-dihydro-3-methylbenzo[*b*]furans and Naturally Occurring Neolignans For the application of the above method to the synthesis of 2-aryl-2,3-dihydro-3-methylbenzo[*b*]furan derivatives including naturally occurring neolignans, lithiations of 2-ethylphenyl- (**7a**) and 2-ethyl-6-methoxyphenyl *N,N,N',N'*-tetramethylphosphorodiamidate (**7b**) were examined. The lithiation behavior of these 2-ethyl-substituted compounds[7] in connection with 2-aryl-3-methylbenzo[*b*]furan synthesis has already been reported by us.[5d] Lithiation of **7a** under the standard conditions (*sec*-BuLi/TMEDA/−105 °C/1 h) followed by addition of *p*-anisaldehyde (**2b**) and dephosphorylation ($LiAlH_4$/THF) gave **8**, formed from the ring deprotonation species,[5d] in 25% overall yield (Chart 2). Previous results indicated that when the *ortho*-position of the phosphorodiamidate group was substituted with a methoxy group such as in **7b**, ring deprotonation was prevented and side chain deprotonation from the *ortho*-ethyl group took place.[5d] When a THF solution of piperonal (**2d**) was injected into a solution of lithiated **7b**, two diastereomers of the 1,2-diaryl-1-propanol[8] derivative (**9a** and **b**) were obtained after silica-gel column chromatography in 72% and 13% yield, respectively (Chart 2): **9a** (mp 107—110 °C) and **9b** (mp 131—132 °C). Characteristic differences between **9a** and **9b** were observed in their ^{1}H-NMR (400 MHz) spectra. Thus, the proton absorptions of the propanol protons for these compounds appeared at different chemical shifts with different coupling constants [**9a**: δ 0.92 (3H, d, *J*=6.96 Hz), δ 3.56 (1H, dq, *J*=10.62, 6.96 Hz), δ 4.49 (1H, d, *J*=10.62 Hz); **9b**: δ 1.21 (3H, d, *J*=7.33 Hz), δ 3.75 (1H, dq, *J*=7.33, 4.76 Hz), δ 4.90 (1H, d, *J*=4.76 Hz)]. From these observations, especially the coupling constants of C1-H (**9a**, *J*=10.62 Hz; **9b**, *J*=4.76 Hz), **9a** was assigned as the *threo*-[9] (*anti*-)[8d] diastereomer and **9b** as the *erythro*-[9] (*syn*-)[8d] diastereomer. This stereoselectivity (**9a**:**9b**=5.5:1) may be rationalized in terms of the transition states (A and B in Chart 3), in which approach of the lithiated **7b** to aromatic aldehyde (**2d**) favors the transition state A to minimize steric interaction between the methyl group of **7b**

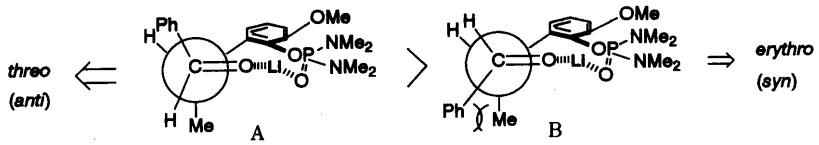


Chart 3

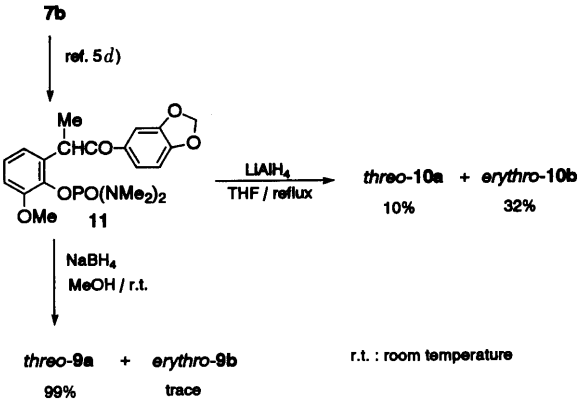


Chart 4

and the aromatic group of **2d** (Chart 3).[7b] Dephosphorylation of **9a** and **9b** with $LiAlH_4$ in refluxing THF gave *threo*-**10a** (mp 125—126 °C) and *erythro*-**10b** (mp 90—93 °C), each in 65% yield. As determined by ^{1}H-NMR analysis, the products (**10a** and **10b**) obtained in each reaction were single diastereomers.

Lithiation of **7b** in the presence of TMEDA followed by reaction with methyl piperonylate was reported to give the deoxybenzoin derivative (**11**) in 58% yield.[5d] We examined the reduction of this compound in an attempt to find an alternative route to **9** and **10** (Chart 4). Treatment of **11** with $LiAlH_4$ in refluxing THF for 3 h gave a mixture of *threo*-**10a** and *erythro*-**10b**, resulting from dephosphorylation and reduction of the ketone, in 10% and 32% yields, respectively. On the other hand, when **11** was treated with $NaBH_4$ in MeOH at room temperature for 3 h *threo*-**9a** was obtained in almost quantitative yield accompanied with a trace amount of *erythro*-**9b**. In the case where the *threo*-diastereomer is desired, this method using $NaBH_4$ can be adopted because *threo*-**9b** can be easily converted into *threo*-**10b** as shown before. The diastereoselectivity of the above reductions may be explained as follows.[8d] Reduction using $NaBH_4$ in a protic solvent proceeds *via* the basic model of Cram's rule. On the other hand, reduction using $LiAlH_4$ in an aprotic solvent predominantly proceeds *via* the chelation-controlled cyclic model.

The synthesis of 2,3-dihydro-7-methoxy-3-methyl-2-piperonylbenzo[*b*]furan (**12**) was accomplished by two different cyclization routes (Chart 5). Acidic treatment of *threo*-**10a** in refluxing 90% acetic acid for 1 h gave *trans*-**12a** in 97% yield as the sole stereoisomer. The *trans*-stereochemistry of **12a** was determined from its ^{1}H-NMR spectrum. Determination of the stereochemistry of *cis*- and *trans*-isomers of the series of 2,3-dihydro-2,3-disubstituted-benzo[*b*]furans has been achieved on the basis of the chemical shifts of C3-Me and C2-H, because the vicinal coupling constants of C2-H and C3-H are almost the same in each isomer.[10] In general, the C3-Me protons in the *cis*-isomer are shielded by the C2-aromatic group and appear at δ 0.7—0.8 (δ 1.3—1.4 in the *trans*-isomer). In addition, the C2-H protons in the *cis*-isomer and *trans*-isomer are observed at δ 5.7—5.8 and near δ 5.1, respectively.[10] In our case, the C3-Me and C2-H in **12a** appeared at δ 1.38 (J=6.96 Hz) and δ 5.10 (J=9.15 Hz), respectively. From this observation, **12a** was identified as the *trans*-isomer. When *erythro*-**10b** was refluxed in 90% acetic acid for 1 h, the same *trans*-**12a** was obtained in quantitative yield. These results indicate that the cyclizations of *threo*-**10a** and *erythro*-**10b** in acetic acid proceed *via* the same carbocation intermediate.

Next, the cyclization of **10** under the Mitsunobu reaction conditions[11] was investigated. A mixture of 1.0 eq of *erythro*-**10b**, 1.5 eq of triphenylphosphine, and 1.5 eq of diethyl azodicarboxylate in THF was stirred at room temperature for 32 h. After usual work-up and chromatographic purification, the expected *trans*-**12a** was obtained in almost quantitative yield. We envisaged that if *threo*-**10a** was employed in this reaction, one would obtain *cis*-**12b**, which is quite difficult to synthesize and which has previously been obtained only as a minor product in the thermal cyclization of *ortho*-allylphenols.[3b] However, when *threo*-**10a** was submitted to the Mitsunobu reaction, a mixture of *cis*-**12b** and *trans*-**12a** were obtained in 59% yield, and they were inseparable by column chromatography. The ^{1}H-NMR spectrum of the mixture showed a ratio of *cis*-**12b**: *trans*-**12a**=2:1 by integration of the C3-Me and C2-H signals of the *cis*-isomer (δ 0.87, J=7.33 Hz and δ 5.76, J=8.79 Hz)[3b] and those of the *trans*-isomer. The stereoselective synthesis of *cis*-**12b** from *threo*-**10a** under the Mitsunobu reaction condition probably fails because the corresponding benzylic carbocation is rapidly formed during the cyclization process, which leads to the themodynamically stable *trans*-isomer. Nevertheless, this method using the Mitsunobu reaction should find general application for the synthesis of 2,3-dihydro-2,3-*cis*-disubstituted-benzo[*b*]furan natural products which have not been synthesized so far.[1]

On the basis of these model studies, total synthesis of some benzofuranoid neolignans and related compounds was investigated. Licarin B (**19**) is a 2,3-dihydrobenzo[*b*]furan natural product isolated from the trunk wood of *Licaria aritu* DUCKE (Lauraceae) in 1973 and identified as (2*S*,3*S*)-2,3-dihydro-7-methoxy-3-methyl-2-piperonyl-5-(*E*)-propenylbenzo[*b*]furan.[12] The synthesis of (±)-licarin B (**19**) was reported in 1975 by Aiba and Gottlieb using the pyrolysis[3b] of 2-hydroxy-3-piperonyl-1-propyl[2-methoxy-4-(*E*)-propenyl]phenyl ether as a key step, in 2.6% overall yield.[12b] Before this total synthesis, Taylor *et al.* had synthesized (±)-**19** starting from dehydrodiisoeugenol, which was readily obtained by $FeCl_3$ oxidation of isoeugenol, in 11% overall yield during their investigation of eupomatene synthesis.[13] The neolignan **21** was isolated from *Krameria cystisoides* in 1987 and identified as (2*R*,3*R*)-2,3-dihydro-2-(4-hydroxyphenyl)-7-methoxy-

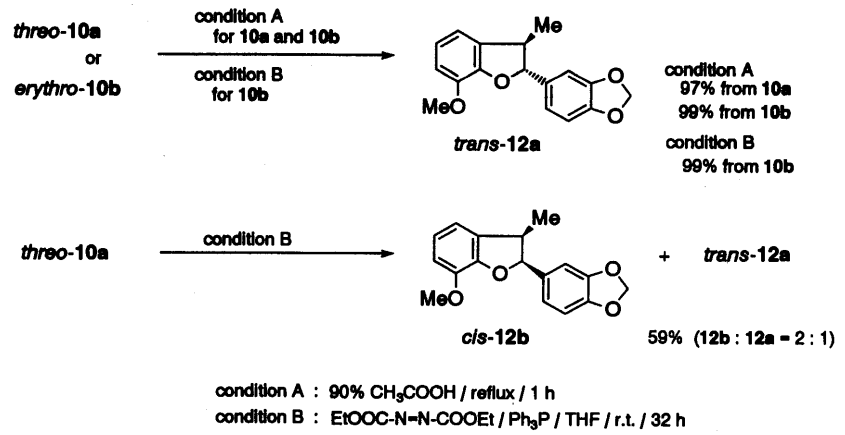


Chart 5

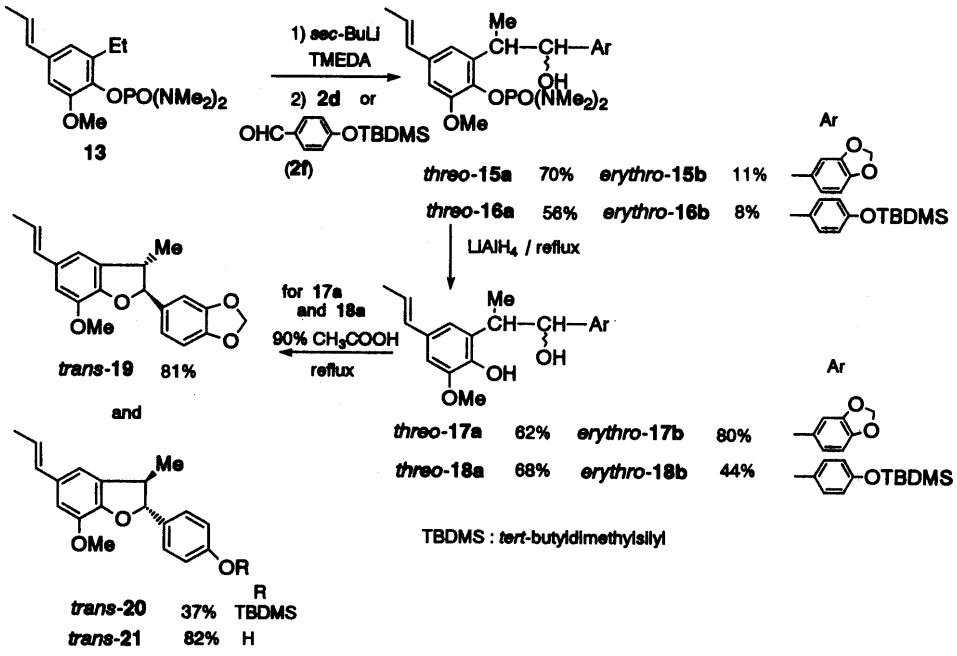


Chart 6

3-methyl-5-(*E*)-propenylbenzo[*b*]furan by spectroscopic analysis.[14] The 1,2-diaryl-1-propanol derivative carinatol (**23**) was isolated from the bark of *Virola carinata* and identified as (1*S*,2*S*)-1-(3′,4′-dimethoxyphenyl)-2-(3″-allyl-5″-methoxy-6″-hydroxyphenyl)-1-propanol, which was identical with the major product derived by the reduction of carinatone.[15] The structure of carinatol (**23**) was assigned on the basis of its conversion using phosphoric acid into dihydrocarinatin (**24**).[15a]

The syntheses of **13** and **14** had been reported by us[5d] using a tandem lithiation strategy[16] of *O*-bis(dimethylamino)phosphorylated isoeugenol and eugenol, respectively. Compound **13** was lithiated with *sec*-BuLi at −105 °C in the presence of TMEDA followed by addition of **2d** to give two diastereomers of the 1,2-diaryl-1-propanol derivative. After silica-gel column chromatography, *threo*-**15a** (mp 164—166 °C) and *erythro*-**15b** (mp 110—112 °C) were isolated in 70% and 11% yields, respectively (Chart 6). When 4-(*tert*-butyldimethylsilyloxy)benzaldehyde (**2f**) instead of **2d** was employed in the above reaction, *threo*-**16a** (a viscous oil) and *erythro*-**16b** (a viscous oil) were obtained in 56% and 8% yields, respectively, after column chromatography. Each of them (*threo*-**15a**, *erythro*-**15b**, *threo*-**16a**, and *erythro*-**16b**) was treated with $LiAlH_4$ to provide *threo*-**17a**, *erythro*-**17b**, *threo*-**18a**, and *erythro*-**18b** in 62%, 80%, 68%, and 44% yields, respectively. Acidic cyclization of *threo*-**17a** in 90% acetic acid for 1 h gave *trans*-**19** (a viscous oil) in 81% yield. When *threo*-**17a** was cyclized with 20% phosphoric acid for 5 h, *trans*-**19** was obtained in 57% yield. On the other hand, acidic treatment of *threo*-**18a** in 90% acetic acid gave *trans*-**20** in 37% yield and this product, without purification, was desilylated with tetra-*n*-butylammonium fluoride (TBAF) in THF for 10 min at room temperature to furnish *trans*-**21** (mp 103—105 °C) in 82% yield (30% overall yield from *trans*-**18a**). Synthetic *trans*-**19** was shown to be identical with (±)-licarin B on the basis of spectroscopic comparisons with the reported data.[12a] The structure of synthetic *trans*-**21** was confirmed by spectroscopic (^{1}H-NMR, UV, and IR) comparisons with an authentic sample of the neolignan **21**.[14a]

Neolignans (±)-carinatol and (±)-dihydrocarinatin were synthesized as follows (Chart 7). The reaction of

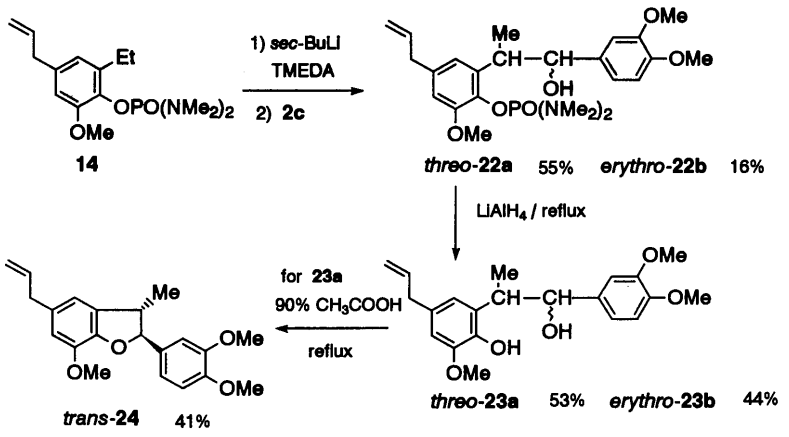


Chart 7

lithiated **14** with **2c** gave *threo*-**22a** and *erythro*-**22b** in 55% and 16% yields, respectively, after silica-gel column chromatographic separation. Dephosphorylation of *threo*-**22a** and *erythro*-**22b** under the standard conditions gave *threo*-**23a** (a viscous oil) and *erythro*-**23b** (a viscous oil) in 53% and 44% yields, respectively. (±)-Dihydrocarinatin was obtained by the cyclization of *threo*-**23a** with 90% acetic acid in 41% yield. The structures of synthetic *threo*-**23a** and **24** were confirmed by spectroscopic (^{1}H-NMR, UV, and IR) comparisons with authentic samples[15] of carinatol and (±)-dihydrocarinatin, respectively.

In conclusion, we have shown that lithiation of *ortho*-tolyl *N,N,N',N'*-tetramethylphosphorodiamidates gives the corresponding benzylic anions, which, upon reaction with various aromatic aldehydes, give 1,2-diarylethanol derivatives. Dephosphorylation followed by acidic cyclization of these compounds gave 2-aryl-2,3-dihydrobenzo[*b*]furans in good overall yields. The present methodology was applied for the stereoselective synthesis of 2-aryl-2,3-dihydro-3-methylbenzo[*b*]furan derivatives starting from 2-ethyl-6-methoxyphenyl *N,N,N',N'*-tetramethylphosphorodiamidate. During this investigation, diastereomers of 1,2-diaryl-1-propanol derivatives, and *trans*- and *cis*-2-aryl-2,3-dihydro-3-methylbenzo[*b*]furans were characterized. Finally, total syntheses of highly substituted naturally occurring neolignans and related compounds were achieved.

Experimental

All melting points are uncorrected. The IR spectra were measured neat on an NaCl plate or in a KBr disk with a JASCO 810 spectrophotometer. The UV spectra were recorded in 95% ethanol on a Hitachi 323 spectrophotometer. The ^{1}H-NMR spectra were obtained with Hitachi R 600 (60 MHz), JEOL FX 90Q (90 MHz), and JEOL GX-400 (400 MHz) spectrometers using $CDCl_3$ or $(CD_3)_2CO$ as a solvent and tetramethylsilane as an internal reference. The MS and high-resolution MS (HRMS) were determined on a JEOL DX-303 mass spectrometer. Elemental analyses were performed at the Microanalytical Laboratory of the Center for Instrumental Analysis in Nagasaki University. All solvents used for lithiation reaction were freshly distilled from sodium benzophenone ketyl before use. All metalation reactions were carried out in a nitrogen atmosphere by using the septum cap techniques. Conventional column or flash chromatography was carried out on a column of Kieselgel 60 (230—400 mesh).

***O*-Bis(dimethylamino)phosphorylated Phenol Derivatives** The preparations of compounds **1a**, **1b**, **7a**, **7b**, **13**, and **14** have already been reported by us.[5]

Lithiation Reaction of 1a, b with Aromatic Aldehydes (2). Synthesis of 3 The following procedure for the synthesis of 2-[2'-bis(dimethylamino)phosphoryloxy]phenyl-1-phenylethanol (**3a**) is representative; the other compounds (**3b**—**f**) were obtained similarly.

2-[2'-Bis(dimethylamino)phosphoryloxy]phenyl-1-phenylethanol (**3a**): A solution of *sec*-BuLi (0.98 M in cyclohexane, 7.89 ml, 7.50 mmol) was injected into a stirred solution of *ortho*-tolyl tetramethylphosphorodiamidate (**1a**, 1.45 g, 6.00 mmol) in the presence of TMEDA (1.13 ml, 7.50 mmol) in THF (50 ml) at −105 °C (liquid nitrogen–ethanol bath) under a nitrogen atmosphere. The mixture was stirred at −105 °C for 1 h, and then a solution of benzaldehyde (**2a**, 0.85 g, 8.00 mmol) in THF (10 ml) was injected into the yellow lithiated solution at −105 °C. Stirring was continued for an additional 1 h at −105 °C. The reaction was quenched with saturated NH_4Cl solution at −90 °C and the solution was allowed to warm to room temperature. THF was removed under reduced pressure. The residue was extracted with CH_2Cl_2 and the CH_2Cl_2 layer was dried over Na_2SO_4, and then evaporated to give a viscous oil. Crystallization with hexane and recrystallization from ether–pentane gave pure **3a** (1.45 g, 69%), mp 61 °C, see Table II. When TMEDA was not added in this reaction, **3a** was obtained in 43% yield.[5d] Compounds **3a** and **3b** have already been synthesized and characterized by us.[5d]

Dephosphorylation of 3. Synthesis of 4 The following procedure for the synthesis of 2-(2'-hydroxy)phenyl-1-phenylethanol (**4a**) is representative; the other compounds (**4b**—**f**) were obtained similarly.

2-(2'-Hydroxy)phenyl-1-phenylethanol (**4a**): A solution of **3a** (1.04 g, 3.00 mmol) and $LiAlH_4$ (0.17 g, 3.00 mmol) in THF (50 ml) was refluxed for 4 h, then allowed to cool. Water (5 ml) was added to the solution. Standard work-up and chromatographic purification (CH_2Cl_2 : acetone = 9 : 1 as an eluent) gave **4a** (0.43 g, 67%), mp 84—86 °C (ether–pentane), see Table II.

Acidic Cyclization of 4. Synthesis of 5 The following procedure for the synthesis of 2,3-dihydro-2-phenylbenzo[*b*]furan (**5a**) is representative; the other compounds (**5b**—**f**) were obtained similarly.

2,3-Dihydro-2-phenylbenzo[*b*]furan (**5a**): A solution of **4a** (0.10 g, 0.47 mmol) in 90% CH_3COOH (10 ml) was refluxed for 4 h. Acetic acid was removed under reduced pressure to give the residue. Then 5% $NaHCO_3$ solution was added to neutralize the residue and the mixture was extracted with CH_2Cl_2. The CH_2Cl_2 layer was dried over Na_2SO_4, and evaporated to give a residue, which was chromatographed with CH_2Cl_2 as an eluent to give 2,3-dihydro-2-phenylbenzo[*b*]furan (**5a**, 0.05 g, 55%), see Table III, and *ortho*-styrylphenol (**6**, 0.01 g, 10%).

ortho-Styrylphenol (**6**): mp 139—142 °C (ether–pentane). MS *m/z*: 196 (M^+). IR (KBr): 3570, 1580, 1500, 1450, 1330, 1250, 1195, 1095, 975 cm^{-1}. UV nm (log ε): 223.5 (4.14), 229 (s) (4.13), 288 (4.22), 298.5 (s) (4.17), 326 (4.14). ^{1}H-NMR (acetone-d_6) δ: 6.77—7.06 (3H, m), 7.20—7.61 (6H, m). *Anal.* Calcd for $C_{14}H_{12}O$: C, 85.68; H, 6.16. Found: C, 85.37; H, 6.20. When the cyclization of **3a** or **4a** was carried out in 90% formic acid or 20% phosphoric acid, the results shown in Chart 1 were obtained after usual work-up as above.

Lithiation Reaction of 7a with 2b A solution of *p*-anisaldehyde (**2b**, 0.41 g, 3.00 mmol) in THF (10 ml) was injected into a lithiated solution of **7a** (0.51 g, 2.00 mmol) in THF (50 ml), generated with *sec*-BuLi (0.95 M, 2.63 ml, 2.5 mmol) at −105 °C, with stirring at −105 °C. Stirring was continued for an additional 1h and standard work-up gave 1-[2'-bis(dimethylamino)phosphoryloxy-3'-ethylphenyl]-1-(4'-methoxyphenyl)-methanol (0.44 g, 56%) after silica-gel column chromatography (CH_2Cl_2 : acetone = 9 : 1 as an eluent). A viscous oil. ^{1}H-NMR δ: 1.14 (3H, d, J = 7.8 Hz), 2.48—2.78 (2H, m), 2.58 (6H, d, J = 9.6 Hz), 2.70 (6H, d,

J=9.6 Hz), 3.64 (3H, s), 5.72 (1H, br s), 6.07 (1H, br s), 6.77 (2H, d, J=10.2 Hz), 6.81—7.01 (3H, m), 7.26 (2H, d, J=10.2 Hz). Without further purification, the above product was used in the next step. A mixture of the above product (0.44 g, 1.12 mmol) and $LiAlH_4$ (0.043 g, 1.12 mmol) in THF (20 ml) was refluxed for 4 h. Standard work-up and chromatographic purification (CH_2Cl_2 : acetone = 9 : 1 as an eluent) gave 1-(3'-ethyl-2'-hydroxyphenyl)-1-(4-methoxyphenyl)methanol (**8**, 0.13 g, 45%), mp 57 °C (ether). MS m/z: 258 (M^+). IR (KBr): 3390, 3230, 2950, 1610, 1510, 1460, 1400, 1240, 1170, 1030, 990 cm^{-1}. UV nm (log ε): 226.5 (4.25), 276.5 (3.63), 283 (s) (3.41). ^{1}H-NMR δ: 1.15 (3H, t, J=7.8 Hz), 2.61 (2H, q, J=7.8 Hz), 3.90 (3H, s), 5.81 (1H, s), 6.59—7.30 (9H, m). *Anal.* Calcd for $C_{16}H_{18}O_3$: C, 74.39; H, 7.02: Found: C, 74.71; H, 7.06.

Lithiation Reaction of 7b with 2d. Synthesis of *threo*-9a and *erythro*-9b A solution of piperonal (**2d**, 1.31 g, 8.75 mmol) in THF (10 ml) was injected into a lithiated solution of **7b** (2.00 g, 7.00 mmol) in THF (50 ml), generated with *sec*-BuLi (0.95 M, 8.85 ml, 8.40 mmol) in the presence of TMEDA (1.28 ml, 8.40 mmol) at −105 °C for 1 h, with stirring at −105 °C. Standard work-up and chromatographic separation gave *threo*-**9a** (2.19 g, 72%) (CH_2Cl_2 : acetone = 9 : 1 as an eluent) and *erythro*-**9b** (0.40 g, 13%) (CH_2Cl_2 : acetone = 1 : 1 as an eluent).

threo-2-[2'-Bis(dimethylamino)phosphoryloxy-3'-methoxy]phenyl-1-piperonylpropanol (**9a**): mp 107—110 °C (ether–pentane). MS m/z: 436 (M^+). IR (KBr): 3275, 2880, 1605, 1580, 1480, 1440, 1300, 1245,1205, 1035, 1000 cm^{-1}. UV nm (log ε): 237 (s) (3.84), 280 (3.71), 287 (s) (3.70). ^{1}H-NMR (400 MHz) δ : 0.92 (3H, d, J=6.93 Hz), 2.71 (6H, d, J=10.25 Hz), 2.83 (6H, d, J=10.25 Hz), 3.56 (1H, dd, J=10.62, 6.96 Hz), 3.87 (3H, s), 4.49 (1H, d, J=10.62 Hz), 5.94 (2H, dd, J=1.45, 6.60 Hz), 6.76—6.80 (2H, m), 6.92 (1H, dd, J=1.66, 7.88 Hz), 7.08 (1H, dd, J=1.09, 7.70 Hz), 7.11 (1H, d, J=1.83 Hz), 7.19 (1H, dt, J=1.09, 8.06 Hz). *Anal.* Calcd for $C_{21}H_{29}N_2O_6P$: C, 57.79; H, 6.70; N, 6.42. Found: C, 58.29; H, 6.72; N, 6.40.

erythro-2-[2'-Bis(dimethylamino)phosphoryloxy-3'-methoxy]phenyl-1-piperonylpropanol (**9b**): mp 131—132 °C (ether–hexane). MS m/z: 436 (M^+). IR (KBr): 3325, 2940, 1605, 1580, 1480, 1440, 1295, 1250, 1220, 1170, 1000 cm^{-1}. UV nm (log ε): 237.5 (s) (3.73), 280 (3.68), 286 (s) (3.64). ^{1}H-NMR (400 MHz) δ: 1.21 (3H, d, J=7.33 Hz), 2.74 (12H, d, J=9.90 Hz), 3.74—3.77 (1H, m), 3.82 (3H, s), 4.90 (1H, d, J=4.76 Hz), 5.90—5.92 (2H, m), 6.67—6.71 (2H, m), 6.75—6.79 (2H, m), 6.87 (1H, d, J=1.47 Hz), 6.99 (1H, dt, J=1.10, 8.06 Hz). *Anal.* Calcd for $C_{21}H_{29}N_2O_6P$: C, 57.79; H, 6.70; N, 6.42. Found: C, 57.40; H, 6.55; N, 6.37.

Dephosphorylation of *threo*-9a and *erythro*-9b. Synthesis of *threo*-10a and *erythro*-10b A mixture of *threo*-**9a** (1.09 g, 2.00 mmol) and $LiAlH_4$ (0.10 g, 2.50 mmol) in THF (50 ml) was refluxed for 4 h. Standard work-up and chromatographic purification (CH_2Cl_2 : acetone = 9 : 1 as an eluent) gave *threo*-**10a** (0.39 g, 65%).

threo-2-(2'-Hydroxy-3'-methoxyphenyl)-1-piperonylpropanol (**10a**): mp 125—126 °C (ether–pentane). MS m/z: 302 (M^+). IR (KBr): 3510, 3300, 1610, 1490, 1445, 1280, 1250, 1220, 1090, 1030 cm^{-1}. UV nm (log ε): 235 (s) (4.06), 283 (3.79). ^{1}H-NMR (400 MHz) δ: 1.05 (3H, d, J=6.96 Hz), 2.25 (1H, br s), 3.37—3.45 (1H, m), 3.89 (3H, s), 4.70 (1H, d, J=9.16 Hz), 5.95 (2H, s), 6.12 (1H, br s), 6.75—6.86 (5H, m), 6.92 (1H, d, J=1.83 Hz). *Anal.* Calcd for $C_{17}H_{18}O_5$: C, 67.54; H, 6.00. Found: C, 67.39; H, 6.03. Similarly, *erythro*-**10b** was obtained in 65% yield by the reaction of *erythro*-**9b** (0.38 g, 0.87 mmol) with $LiAlH_4$ (0.04 g, 1.04 mmol).

erythro-2-(2'-Hydroxy-3'-methoxyphenyl)-1-piperonylpropanol (**10b**): mp 90—93 °C (CH_2Cl_2–pentane). MS m/z: 302 (M^+). IR (KBr): 3370, 1610, 1480, 1440, 1280, 1260, 1220, 1090, 1040 cm^{-1}. UV nm (log ε): 225 (s) (4.00), 283 (3.71). ^{1}H-NMR (400 MHz) δ: 1.21 (3H, d, J=4.40 Hz), 2.30 (1H, br s), 3.50—3.53 (1H, m), 3.89 (3H, s), 4.97 (1H, d, J=4.40 Hz), 5.91—5.92 (2H, m), 6.15 (1H, br s), 6.67—6.82 (6H, m). *Anal.* Calcd for $C_{17}H_{18}O_5$: C, 67.54; H, 6.00. Found: C, 67.22; H, 6.36.

Reaction of the Deoxybenzoin Derivative 11 with $LiAlH_4$ A mixture of **11** (1.04 g, 2.40 mmol) and $LiAlH_4$ (0.11 g, 2.88 mmol) in THF (50 ml) was refluxed for 4 h. Standard work-up and chromatographic separation (benzene : acetone = 9 : 1 as an eluent) gave *threo*-**10a** (0.07 g, 10%) and *erythro*-**10b** (0.23 g, 32%).

Reaction of the Deoxybenzoin Derivative 11 with $NaBH_4$ A mixture of **11** (1.50 g, 3.40 mmol) and $NaBH_4$ (0.16 g, 4.25 mmol) in MeOH (50 ml) was stirred for 3 h at room temperature. Standard work-up and chromatographic purification (CH_2Cl_2 : acetone = 4 : 1 as an eluent) gave *threo*-**9a** (1.47 g, 99%) and *erythro*-**9b** (0.008 g, 0.5%).

Cyclization of *threo*-10a and *erythro*-10b. Synthesis of *trans*-12a and *cis*-12b i) Using Acetic Acid: A solution of *threo*-**10a** (0.12 g, 0.40 mmol) in 90% CH_3COOH (20 ml) was refluxed for 1 h. Acetic acid was removed under reduced pressure. Standard work-up and chromatographic purification (CH_2Cl_2 as an eluent) gave *trans*-2,3-dihydro-7-methoxy-3-methyl-2-piperonylbenzo[*b*]furan (**12a**, 0.11 g, 97%). **12a**: bp 145 °C (0.3 mmHg). MS m/z: 284 (M^+). IR (neat): 2960, 2900, 1620, 1590, 1490, 1440, 1280, 1250, 1195, 1100, 1040, 935 cm^{-1}. UV nm (log ε): 232.5 (s) (4.03), 286 (3.80). ^{1}H-NMR (400 MHz) δ: 1.38 (3H, d, J=6.96 Hz), 3.44 (1H, dq, J=6.96, 8.79 Hz), 3.88 (3H, s), 5.10 (1H, d, J=9.15 Hz), 5.94 (2H, s), 6.75—6.80 (3H, m), 6.85—6.89 (2H, m), 6.94 (1H, d, J=1.83 Hz). *Anal.* Calcd for $C_{17}H_{16}O_4$: C, 71.82; H, 5.76. Found: C, 71.66; H, 5.70. Similarly, when *erythro*-**10b** (0.09 g, 0.30 mmol) was employed, *trans*-**12a** was obtained in 99% yield.

ii) Using Mitsunobu Reaction Conditions: A solution of diethyl azodicarboxylate (0.083 g, 0.48 mmol) in THF (2 ml) was added to a solution of *erythro*-**10b** (0.08 g, 0.265 mmol) and triphenylphosphine (0.13 g, 0.48 mmol) in THF (10 ml) at room temperature with stirring. The mixture was stirred for 32 h at room temperature. THF was removed to give a residue, which was chromatographed with CH_2Cl_2 as an eluent to give *trans*-**12a** (0.08 g, 99%). Similarly, a solution of *threo*-**10a** (0.29 g, 0.96 mmol), diethyl azodicarboxylate (0.30 g, 1.73 mmol), and triphenylphosphine (0.47 g, 1.73 mmol) was stirred for 32 h at room temperature. Standard work-up gave a mixture of *trans*-**12a** and *cis*-**12b** (0.16 g, 59%, 1 : 2).

cis-2,3-Dihydro-7-methoxy-3-methyl-2-piperonylbenzo[*b*]furan (**12b**): ^{1}H-NMR (400 MHz) δ: 0.87 (3H, d, J=7.33 Hz), 3.61—3.68 (1H, m), 3.91 (3H, s), 5.76 (1H, d, J=8.79 Hz), 5.94 (2H, s), 6.75—6.80 (3H, m), 6.85—6.89 (2H, m), 6.94 (1H, d, J=1.83 Hz). These ^{1}H-NMR data for **12b** were determined from the ^{1}H-NMR spectrum of the mixture of *trans*-**12a** and *cis*-**12b**.

Syntheseis of (±)-Licarin B (19) A solution of **2d** (0.90 g, 6.00 mmol) in THF (10 ml) was injected into a lithiated solution of **13**[5d] (1.30 g, 4.00 mmol), generated with *sec*-BuLi in the presence of TMEDA under the standard conditions, at −105 °C. Stirring was continued for an additional 1 h and standard work-up gave *threo*-**15a** (1.34 g, 70%) and *erythro*-**15b** (0.21 g, 11%) after silica-gel column chromatography (CH_2Cl_2 : acetone = 9 : 1 to 1 : 1 as an eluent).

threo-2-[2'-Bis(dimethylamino)phosphoryloxy-3'-methoxy-5'-(1-propenyl)]phenyl-1-piperonylpropanol (**15a**): mp 164—166 °C (ether). MS m/z: 476 (M^+). IR (KBr): 3330, 2940, 2880, 1585, 1485, 1460, 1440, 1305, 1245, 1225, 1190, 1145, 990, 930 cm^{-1}. UV nm (log ε): 219 (4.56), 257 (4.22), 287 (3.89), 308 (s) (3.42). ^{1}H-NMR (400 MHz) δ: 0.91 (3H, d, J=6.96 Hz), 1.89 (3H, dd, J=6.60, 1.47 Hz), 2.71 (6H, d, J=10.26 Hz), 2.82 (6H, d, J=10.26 Hz), 3.53 (1H, dq, J=10.26, 6.96 Hz), 3.88 (3H, s), 4.50 (1H, d, J=10.26 Hz), 5.79 (1H, dd, J=9.16, 1.83 Hz), 5.94 (2H, s), 6.20 (1H, dq, J=15.75, 6.60 Hz), 6.39 (1H, d, J=15.75 Hz), 6.77 (1H, d, J=7.88 Hz), 6.78 (1H, s), 6.93 (1H, dd, J=7.88, 1.46 Hz), 6.99 (1H, d, J=1.83 Hz), 7.11 (1H, d, J=1.83 Hz). *Anal.* Calcd for $C_{24}H_{33}N_2O_6P$: C, 60.50; H, 6.98; N, 5.88. Found: C, 60.40; H, 6.99; N, 5.81.

erythro-2-[2'-Bis(dimethylamino)phosphoryloxy-3'-methoxy-5'-(1-propenyl)]phenyl-1-piperonylpropanol (**15b**): mp 110—112 °C (ether–pentane). MS m/z: 476 (M^+). IR (KBr): 3299, 2930, 1585, 1490, 1460, 1445, 1310, 1250, 1220, 1195, 1140, 1040, 1000, 920, 900 cm^{-1}. UV nm (log ε): 216 (s) (4.51), 257 (4.17), 287 (3.86), 306 (s) (3.37). ^{1}H-NMR (90 MHz) δ: 1.19 (3H, d, J=7.29 Hz), 1.84 (3H, d, J=5.50 Hz), 2.72 (6H, d, J=9.89 Hz), 2.73 (6H, d, J=9.89 Hz), 3.55 (1H, br s), 3.72—3.82 (1H, m), 3.82 (3H, s), 4.90 (1H, d, J=4.62 Hz), 5.90 (2H, s), 6.04—6.18 (2H, m), 6.57—6.86 (5H, m). *Anal.* Calcd for $C_{24}H_{23}N_2O_6P \cdot 1/3H_2O$: C, 59.73; H, 7.03; N, 5.81. Found: C, 59.72; H, 6.75; N, 6.17. A mixture of *threo*-**15a** (0.85 g, 1.70 mmol) and $LiAlH_4$ (0.08 g, 2.00 mmol) in THF (50 ml) was refluxed for 4 h. Standard work-up gave *threo*-**17a** (0.36 g, 62%).

threo-2-[2'-Hydroxy-3'-methoxy-5'-(1-propenyl)]phenyl-1-piperonylpropanol (**17a**): A viscous oil. MS m/z: 342 (M^+). IR (KBr): 3425, 2975, 2880, 1600, 1490, 1440, 1300, 1250, 1150, 1095, 1040, 935 cm^{-1}. UV nm (log ε): 272 (4.09), 285 (s) (3.98), 307 (s) (3.54). ^{1}H-NMR δ: 1.00 (3H, d, J=7.2 Hz), 1.81 (3H, d, J=6.0 Hz), 2.42 (1H, br s), 3.19—3.45 (1H, m), 3.84 (3H, s), 4.65 (1H, d, J=8.4 Hz), 5.89 (2H, s), 6.06—6.22 (2H, m), 6.73—6.89 (6H, m). *Anal.* Calcd for $C_{20}H_{22}O_5 \cdot 1/3H_2O$: C, 68.92; H, 6.57. Found: C, 68.85; H, 6.58. Similarly, *erythro*-**17b** was obtained in 80% yield by the reaction of *erythro*-**15b** (0.07 g, 0.147 mmol) with $LiAlH_4$ (0.001 g, 0.190 mmol).

erythro-2-[2'-Hydroxy-3'-methoxy-5'-(1-propenyl)]phenyl-1-piperonylpropanol (**17b**): A viscous oil. MS m/z: 342 (M^+). IR (neat): 3460, 2970, 2940, 1710, 1600, 1490, 1445, 1370, 1300, 1240, 1155, 1100, 1040, 940 cm^{-1}. UV nm (log ε): 265 (4.11), 285 (s) (3.91). ^{1}H-NMR δ: 1.20 (3H, d, J=6.8 Hz), 1.84 (3H, d, J=5.4 Hz), 2.45 (1H, br s), 3.22—3.61 (1H, m), 3.87 (3H, s), 4.94 (1H, d, J=3.6 Hz), 5.90 (2H, s), 6.06—6.21 (2H, m), 6.66—6.82 (6H, m). *Anal.* Calcd for $C_{20}H_{22}O_5$: C, 70.16; H, 6.48. Found:

C, 69.85; H, 6.97. A solution of *threo*-**17a** (0.34 g, 0.99 mmol) in 90% CH_3COOH (20 ml) was refluxed for 1 h. Standard work-up and chromatographic purification (CH_2Cl_2 as an eluent) gave (±)-licarin B (**19**, 0.26 g, 81%).

(±)-Licarin B (**19**): An oil (lit.[12a] mp 91—92 °C, lit.[12b] an oil, lit.[13] a gum). MS *m/z*: 324 (M^+). IR (neat): 1600, 1490, 1450, 1335, 1250, 1210, 1145, 1040, 965, 940 cm^{-1}. UV nm (log ε): 216 (s) (4.47), 273 (4.21). ^{1}H-NMR (400 MHz) δ: 1.37 (3H, d, *J*=6.96 Hz), 1.86 (3H, dd, *J*=6.60, 1.47 Hz), 3.38—3.48 (1H, m), 3.88 (3H, s), 5.09 (1H, d, *J*=8.79 Hz), 5.94 (2H, s), 6.09 (1H, dq, *J*=15.57, 6.60 Hz), 6.35 (1H, dd, *J*=15.57, 1.83 Hz), 6.72—6.81 (3H, m), 6.87 (1H, dd, *J*=8.43, 1.83 Hz), 6.93 (1H, d, *J*=1.84 Hz). When 20% phosphoric acid was used in this cyclization, **19** was obtained in 57% yield. The above ^{1}H-NMR, IR, and UV data for **19** were essentially identical with the reported data.[12]

Synthesis of the Neolignan (±)-21 The neolignan **21** was synthesized *via* the sequence shown in Chart 6 under the conditions described above.

4-(*tert*-Butyldimethylsilyloxy)benzaldehyde (**2f**): Compound **2f** was prepared by the reaction of 4-hydroxybenzaldehyde with *tert*-butyldimethylsilyl chloride in the presence of imidazole in dimethyl formamide (DMF) at room temperature. 82% yield. bp 100 °C (0.4 mmHg). MS *m/z*: 236 (M^+). IR (neat): 2940, 2860, 1700, 1600, 1580, 1510, 1215, 1160, 910 cm^{-1}. UV nm (log ε): 217.5 (4.09), 272 (4.20). ^{1}H-NMR δ: 0.25 (6H, s), 0.99 (9H, s), 6.94 (2H, d, *J*=8.9 Hz), 7.79 (2H, d, *J*=8.9 Hz), 9.88 (1H, s). *Anal.* Calcd for $C_{13}H_{20}O_2Si$: C, 66.06; H, 8.53. Found: C, 65.82; H, 8.37.

1-(4-*tert*-Butyldimethylsilyloxyphenyl)-2-[2'-bis(dimethylamino)phosphoryloxy-3'-methoxy-5'-(1-propenyl)]phenylpropanol (**16**): *threo*-**16a**: 56% yield. A viscous oil. ^{1}H-NMR δ: 0.19 (6H, s), 0.89 (3H, d, *J*=7.2 Hz), 0.99 (9H, s), 1.88 (3H, d, *J*=5.4 Hz), 2.05 (1H, br s), 2.70 (6H, d, *J*=10.2 Hz), 2.81 (6H, d, *J*=10.2 Hz), 3.56—3.68 (1H, m), 3.84 (3H, s), 4.43 (1H, d, *J*=9.0 Hz), 6.21—6.32 (2H, m), 6.74—7.00 (4H, m), 7.38 (2H, d, *J*=9.0 Hz). *erythro*-**16b**: 8% yield. A viscous oil. ^{1}H-NMR δ: 0.20 (6H, s), 1.02 (9H, s), 1.28 (3H, d, *J*=5.4 Hz), 1.87 (3H, d, *J*=4.8 Hz), 2.73 (12H, d, *J*=10.2 Hz), 3.40—3.70 (1H, m), 3.80 (3H, s), 4.85 (1H, d, *J*=7.8 Hz), 6.12—6.23 (2H, m), 6.66—6.79 (4H, m), 7.18 (2H, d, *J*=8.4 Hz). Without further purification, each of these compounds was used in the next dephosphorylation reaction.

1-(4-*tert*-Butyldimethylsilyloxyphenyl)-2-[2'-hydroxy-3'-methoxy-5'-(1-propenyl)]phenylpropanol (**18**): *threo*-**18a**: 68% yield. A viscous oil. MS *m/z*: 428 (M^+). IR (neat): 3400, 2940, 1605, 1510, 1460, 1250, 1150, 910 cm^{-1}. UV nm (log ε): 224 (4.36), 272 (3.77). ^{1}H-NMR δ: 0.18 (6H, s), 0.99 (9H, s), 1.04 (3H, d, *J*=6.0 Hz), 1.85 (3H, d, *J*=5.4 Hz), 2.70 (1H, br s), 3.34—3.50 (1H, m), 3.84 (3H, s), 4.69 (1H, d, *J*=12.0 Hz), 6.16—6.36 (2H, m), 6.71—6.85 (4H, m), 7.13—7.28 (3H, m). HRMS *m/z*: M^+ Calcd for $C_{25}H_{36}O_4Si$ 428.2383. Found: 428.2394. *erythro*-**18b**: 44% yield. A viscous oil. MS *m/z*: 428 (M^+). IR (neat): 3420, 2940, 1610, 1510, 1460, 1295, 1255, 915 cm^{-1}. UV nm (log ε): 223 (s) (4.29), 278 (3.80), 309 (s) (3.52). ^{1}H-NMR δ: 0.15 (6H, s), 0.96 (9H, s), 1.18 (3H, d, *J*=7.8 Hz), 1.81 (3H, d, *J*=4.8 Hz), 2.60 (1H, br s), 3.36—3.71 (1H, m), 3.81 (3H, s), 4.94 (1H, d, *J*=4.2 Hz), 6.01—6.15 (2H, m), 6.43—6.78 (5H, m), 7.12 (2H, d, *J*=9.0 Hz). HRMS *m/z*: M^+ Calcd for $C_{25}H_{36}O_4Si$ 428.2383. Found: 428.2364. A solution of *threo*-**18a** (0.48 g, 1.12 mmol) in 90% CH_3COOH (20 ml) was refluxed for 1 h. Standard work-up gave *trans*-**20** in 37% yield.

2-(4'-*tert*-Butyldimethylsilyloxyphenyl)-7-methoxy-3-methyl-5-(1-propenyl)benzo[*b*]furan (**20**): A viscous oil. MS *m/z*: 410 (M^+). ^{1}H-NMR δ: 0.18 (6H, s), 0.98 (9H, s), 1.34 (3H, d, *J*=7.2 Hz), 1.84 (3H, d, *J*=4.8 Hz), 3.28—3.55 (1H, m), 3.85 (3H, s), 5.10 (1H, d, *J*=9.0 Hz), 6.09—6.25 (2H, m), 6.73—7.35 (6H, m). Without further purification, this product was used for the next desilylation reaction. A solution of *trans*-**20** (0.15 g, 0.37 mmol) and TBAF (1.0 M in THF, 0.44 ml, 0.44 mmol) in THF (10 ml) was stirred for 10 min. THF was removed to give a residue, which was chromatographed with CH_2Cl_2 as an eluent to give *trans*-**21** (0.09 g, 82%).

2-(4'-Hydroxyphenyl)-7-methoxy-3-methyl-5-(1-propenyl)benzo[*b*]-furan (**21**): mp 103—105 °C (hexane). (lit.[14a] mp 100—103 °C). MS *m/z*: 296 (M^+). IR (KBr): 3400, 1610, 1600 cm^{-1}. UV nm (log ε): 222.5 (4.49), 271 (4.19), 304 (s) (3.50). ^{1}H-NMR (400 MHz) δ: 1.37 (3H, d, *J*=6.59 Hz), 1.87 (3H, dd, *J*=6.60, 1.84 Hz), 3.40—3.45 (1H, m), 3.88 (3H, s), 4.95 (1H, br s), 5.11 (1H, d, *J*=8.79 Hz), 6.10 (1H, dq, *J*=15.39, 6.60 Hz), 6.39 (1H, dd, *J*=15.39, 1.84 Hz), 6.73—6.84 (4H, m), 7.29 (2H, d, *J*=8.43 Hz). The above ^{1}H-NMR, IR, and UV data of **21** agreed well with those of an authentic sample of the neolignan **21**.[14a]

Synthesis of (±)-Carinatol (*threo*-23a) and (±)-Dihydrocarinatine (24) The neolignan (±)-carinatol (**23a**) and (±)-dihydrocarinatine (**24**) were synthesized *via* the sequence shown in Chart 7 under the conditions described above.

2-[5'-Allyl-2'-bis(dimethylamino)phosphoryloxy-3'-methoxy]phenyl-1-(3,4-dimethoxy)phenylpropanol (**22**): *threo*-**22a**: 55% yield. A viscous oil. ^{1}H-NMR δ: 0.88 (3H, d, *J*=7.2 Hz), 2.70 (6H, d, *J*=10.2 Hz), 2.81 (6H, d, *J*=10.2 Hz), 3.36 (2H, d, *J*=6.6 Hz), 3.41—3.80 (1H, m), 3.80 (6H, s), 3.88 (3H, s), 4.40—4.97 (2H, m), 5.17 (1H, d, *J*=3.6 Hz), 5.67—5.90 (1H, m), 6.59—7.14 (5H, m). *erythro*-**22b**: 16% yield. A viscous oil. ^{1}H-NMR δ: 1.16 (3H, d, *J*=7.2 Hz), 2.71 (6H, d, *J*=10.2 Hz), 2.73 (6H, d, *J*=10.2 Hz), 3.47 (2H, d, *J*=7.0 Hz), 3.63—3.93 (1H, m), 3.82 (3H, s), 3.86 (6H, s), 4.60—4.95 (2H, m), 4.95—5.10 (1H, m), 5.70—6.10 (1H, m), 6.59—6.94 (5H, m).

2-(5'-Allyl-2'-hydroxy-3'-methoxy)phenyl-1-(3,4-dimethoxy)phenylpropanol (**23**): (±)-Carinatol (*threo*-**23a**): 53% yield. A viscous oil. (lit.[15] a viscous oil). MS *m/z*: 358 (M^+). IR (KBr): 3430, 2940, 1600, 1515, 1500, 1460, 1265, 1235, 1225, 1140, 1025 cm^{-1}. UV nm (log ε): 229 (4.30), 280 (3.87). ^{1}H-NMR (400 MHz) δ: 1.05 (3H, d, *J*=6.96 Hz), 2.61 (1H, br s), 3.29 (2H, dd, *J*=6.59, 13.56 Hz), 3.38 (1H, dq, *J*=1.47, 6.96 Hz), 3.83 (3H, s), 3.84 (3H, s), 3.85 (3H, s), 4.73 (1H, d, *J*=8.79 Hz), 5.00—5.09 (2H, m), 5.85—5.99 (1H, m), 6.39 (1H, s), 6.60 (1H, d, *J*=2.93 Hz), 6.61 (1H, s), 6.77—6.81 (1H, m), 6.88 (1H, dt, *J*=6.96, 1.84 Hz), 7.00 (1H, d, *J*=1.84 Hz). *Anal.* Calcd for $C_{21}H_{26}O_5 \cdot 1/2H_2O$: C, 68.46; H, 7.40. Found: C, 68.72; H, 7.21. The above ^{1}H-NMR, IR, and UV data for **23a** agreed well with those of an authentic sample of carinatol.[15] *erythro*-**23b**: 44% yield. A viscous oil. IR (neat): 3500, 2940, 2840, 1600, 1515, 1495, 1460, 1440, 1290, 1265, 1230, 1140, 1025, 915 cm^{-1}. UV nm (log ε): 202.5 (4.77), 226 (s) (4.27), 277.5 (3.82), 282.5 (s) (3.78), ^{1}H-NMR δ: 1.20 (3H, d, *J*=6.6 Hz), 2.93 (1H, br s), 3.24 (2H, d, *J*=6.6 Hz), 3.29—3.70 (1H, m), 3.73 (3H, s), 3.80 (6H, s), 4.87—4.94 (1H, m), 4.94—5.10 (2H, m), 5.58—5.83 (1H, m), 6.43—6.54 (3H, m), 6.96 (3H, br s). HRMS *m/z*: M^+ Calcd for $C_{21}H_{26}O_5$ 358.1780. Found: 358.1765.

(±)-Dihydrocarinatin (**24**): 41% yield from *threo*-**23a**. mp 73—74 °C (ether). (lit.[15] resinous product). MS *m/z*: 340 (M^+). IR (KBr): 2950, 2870, 1600, 1520, 1480, 1450, 1270, 1220, 1140, 1120, 1020, 960 cm^{-1}. UV nm (log ε): 232 (s) (4.24), 282 (3.78), 286 (s) (3.76). ^{1}H-NMR (90 MHz) δ: 1.37 (3H, d, *J*=6.81 Hz), 3.35 (2H, d, *J*=6.81 Hz), 3.40—3.54 (1H, m), 3.88 (9H, s), 4.98—5.10 (1H, m), 5.10 (1H, d, *J*=9.70 Hz), 5.11—5.20 (1H, m), 5.83—6.13 (1H, m), 6.61 (2H, br s), 6.77—6.93 (2H, m), 6.99 (1H, d, *J*=1.97 Hz). The above ^{1}H-NMR, IR, and UV data for **24** agreed well with those of an authentic sample of dihydrocarinatin.[15]

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Three New Flavonoids, 3′-Methoxylupinifolin, Laxifolin, and Isolaxifolin from the Roots of *Derris laxiflora* BENTH

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The following constituents were isolated from the roots of *Derris laxiflora* BENTH: flemichapparin-B (1), 3′-methoxylupinifolin (2a), lupinifolin (2b), β-amyrin (3), lupeol (4), prunetin (5), laxifolin (6a), and isolaxifolin (7a). Compounds 2a, 6a, and 7a are new flavonoids, and their structures were determined on the basis of spectral and chemical evidence.

Keywords *Derris laxiflora*; root; flavonoid; 3′-methoxylupinifolin; laxifolin, isolaxifolin

Extensive chemical studies on the constituents of the species of *Derris* (*D.*) have been reported,[1] and many interesting components including flavones, flavonols, chalcones, dihydrochalcones, isoflavans, rotenones, stilbenes, coumarins, aurones, pterocarpans, coumestans, triterpenes, and glycosides have been isolated. There are only three species of genus *Derris* (Leguminosae) indigenous to Taiwan: *D. trifoliata*, *D. laxiflora*, and *D. oblonga*. The chemical constituents of the first species have been investigated.[2] The latter two species are climbers and are distributed in forests at low altitudes in southern parts of Taiwan. The roots of both plants have been reported to possess insecticidal and pisicidal activities. In connection with our interest in flavonoids and in view of the biological activity of the root, chemical studies on *D. laxiflora* were undertaken in our laboratory. This paper deals with the chemical constituents of an ethanol extract of the root.

The air-dried roots of *D. laxiflora* were repeatedly extracted with ethanol. The ethanol extract was evaporated *in vacuo* to give a residue which was partitioned with ether and water. After repeated purification by silica gel chromatography, four components, flemichapparin-B (**1**),[3] 3′-methoxylupinifolin (**2a**) (a new prenylated flavanone), β-amyrin (**3**),[4] lupeol (**4**),[5] and lupinifolin (**2b**)[6] were isolated from the ether layer. The water layer was subsequently partitioned with butanol. The butanol layer was purified repeatedly by silica gel and Sephadex LH-20 chromatography and yielded three components, prunetin (**5**),[7] laxifolin (**6a**), and isolaxifolin (**7a**). Four known compounds were identified by the comparison of their physical data with those reported in the literature. This paper deals with the structural elucidation of the three new compounds **2a**, **6a**, and **7a**.

3′-Methoxylupinifolin (**2a**), mp 82—83 °C, yellow needles from methanol, was formulated as $C_{26}H_{28}O_6$ on the basis of elemental analysis, and gave a positive Mg–HCl test. The infrared (IR) spectum shows strong absorptions at 3220 (OH) and 1635 cm^{-1} (chelated CO). Its ultraviolet (UV) spectrum (λ_{max}^{MeOH} 268, 276, 299, 315 and 367 nm) and signals of an ABX system at δ 2.77 (1H, dd, J=17.1, 3.0 Hz), 3.01 (1H, dd, J=17.1, 12.7 Hz), 5.29 (1H, dd, J=12.7, 3.0 Hz) in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum (Table I) indicated that **2a** is a flavanone.[8] The addition of sodium acetate caused no bathochromic shift of band II (240—280 nm) in the UV spectrum.[8] The evidence suggests that the C-7 phenolic hydroxyl group is not free. The doublets at δ 5.48 and 6.62 (each 1H, J=9.9 Hz), and two singlets at δ 1.42 and 1.43 (each 3H) are characteristic of a *cis* double bond and *gem*-dimethyl group of the 2,2-dimethylchromene moiety.[9] The presence of a C-γ,γ-dimethylallyl group was inferred from the singlets at δ 1.64 and 1.65 (each 3H, br s), the doublet at δ 3.28 (2H, d, J=7.3 Hz), and the triplet at δ 5.17 (1H, t, J=7.3 Hz).[10] Signals due to three aromatic protons were discernible at δ 6.89 (1H, d, J=8.9 Hz), 6.92 (1H, d, J=8.9 Hz), and 6.97 (1H, s), and these could be readily assigned to a 1,3,4-

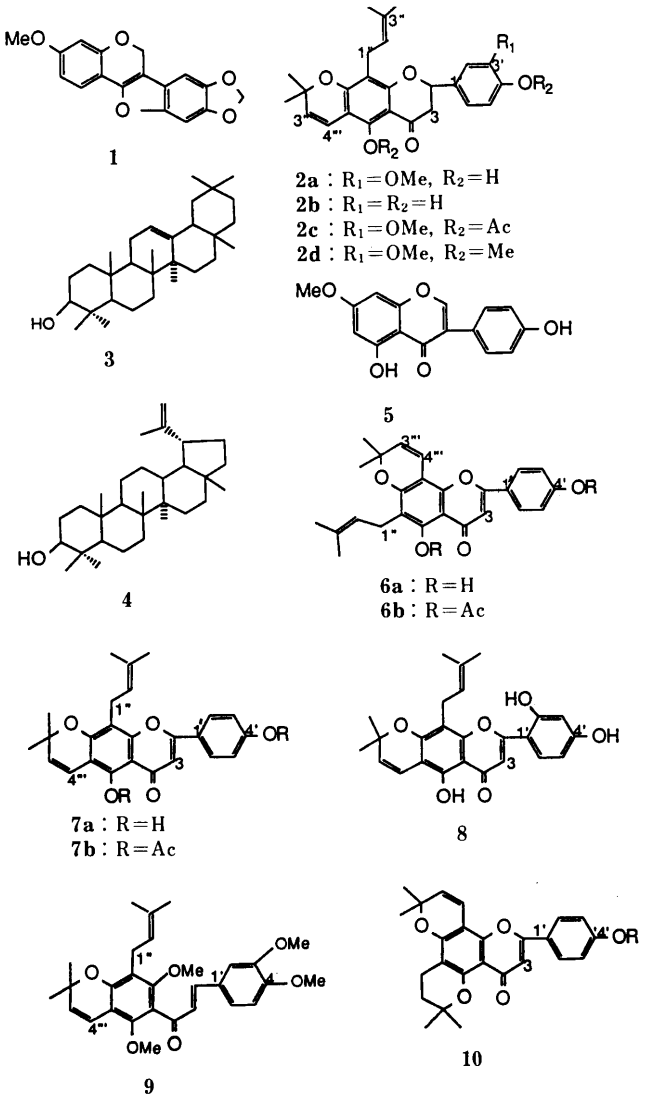

TABLE I. ^{1}H-NMR Data for 3′-Methoxylupinifolin (**2a**), Lupinifolin (**2b**), Laxifolin (**6a**) and Isolaxifolin (**7a**)

H	2a	2b	6a	7a
2	5.29 dd (12.7, 3.0)[a]	5.31 dd (12.9, 3.0)		
3	2.77 dd (17.1, 3.0) 3.01 dd (17.1, 12.7)	2.77 dd (17.0, 3.0) 3.00 dd (17.0, 12.9)	6.49 s	6.49 s
2′	6.97 s	7.30 d (9.0)	7.72 d (8.5)	7.72 d (8.7)
3′		6.61 d (9.0)	6.89 d (8.5)	6.89 d (8.7)
5′	6.89 d (8.9)	6.61 d (9.0)	6.89 d (8.5)	6.89 d (8.7)
6′	6.92 d (8.9)	7.30 d (9.0)	7.72 d (8.5)	7.72 d (8.7)
1″	3.28 d (7.3)	3.10 d (7.0)	3.28 d (7.1)	3.47 d (6.9)
2″	5.17 t (7.3)	5.12 t (7.0)	5.16 t (7.1)	5.17 t (6.9)
3″-CH_3	1.64, 1.65 s	1.63 s	1.61, 1.75 s	1.62, 1.76 s
2‴-CH_3	1.42, 1.43 s	1.42, 1.43 s	1.42 s	1.41 s
3‴	5.48 d (9.9)	5.48 d (9.9)	5.59 d (10.0)	5.57 d (10.0)
4‴	6.62 d (9.9)	6.61 d (9.9)	6.73 d (10.0)	6.70 d (10.0)
O-CH_3	3.89 s			

300 MHz in $CDCl_3$ (TMS as internal standard). *a*) Figures in parentheses are coupling constants.

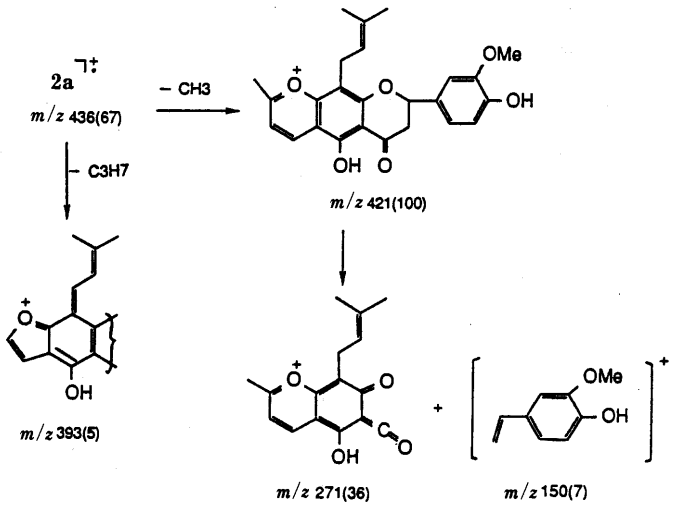

Chart 1

trisubstituted benzene ring. A phenolic methyl ether signal was seen at δ 3.89 (3H, s). The singlets at δ 5.81 and 12.25 (each 1H, s), which both disappeared upon addition of D_2O, were assigned to the two phenolic protons.

The substitution pattern of 3′-methoxylupinifolin (**2a**) was determined from ^{1}H-NMR and mass spectral data. The presence of a chelated C-5 hydroxyl was evident, as one of the phenolic protons resonated at low field (δ 12.25). The non-chelated OH group (δ 5.81) could thus be located either in the A- or B-ring. The mass spectrum (MS) of **2a** (Chart 1) showed fragments at *m/z* (%) 271 (36) and 150 (7), which can be rationalized only when non-chelated OH and –OMe groups are present in the B-ring, and γ,γ-dimethylallyl and 2,2-dimethylchromene side-chains in the A-ring. The non-chelated OH was assigned to C-4′ on the basis of a nuclear Overhauser effect (NOE) experiment, in which NOEs were observed between the –OMe and a signal at δ 6.97 (1H, s, H-2′, 20.8% enhancement), showing that –OMe is located at C-3′. Cardillo *et al.*[11] have shown that acid cyclization of *ortho*-γ,γ-dimethylallylphenol gives the corresponding chroman. Treatment of **2a** with formic acid

TABLE II. ^{13}C-NMR Data (δ Values) for 3′-Methoxylupinifolin (**2a**), Lupinifolin (**2b**), Laxifolin (**6a**) and Isolaxifolin (**7a**)

C	2a	2b	6a	7a
2	78.8 d	78.5 d	163.7 s	163.0 s
3	43.1 t	43.1 t	103.4 d	103.5 d
4	196.3 s	196.7 s	182.5 s	182.9 s
5	159.2 s	159.4 s	160.8 s	157.0 s
6	102.7 s	102.8 s	100.9 s	105.0 s
7	159.6 s	160.0 s	160.7 s	159.4 s
8	108.4 s	108.7 s	112.6 s	107.6 s
9	156.5 s	156.5 s	158.7 s	154.5 s
10	102.5 s	102.6 s	103.2 s	103.6 s
1′	125.8 s	126.0 s	127.0 s	123.7 s
2′	108.6 d	127.7 d	128.0 d	128.3 d
3′	146.7 s	115.5 d	116.2 d	116.2 d
4′	145.8 s	156.0 s	156.0 s	154.3 s
5′	115.5 d	115.5 d	116.2 d	116.2 d
6′	119.1 d	127.7 d	128.0 d	128.3 d
1″	21.3 t	21.4 t	21.7 t	21.6 t
2″	122.4 d	122.4 d	122.2 d	122.2 d
3″	130.6 s	130.7 s	128.3 s	131.7 s
4″	25.6 q	25.7 q	25.8 q	25.7 q
5″	17.7 q	17.6 q	18.1 q	18.0 q
2‴	78.0 s	78.1 s	77.8 s	78.6 s
3‴	130.8 d	131.1 d	131.4 d	131.9 d
4‴	114.4 d	115.6 d	115.6 d	115.8 d
5‴	28.1 q	28.4 q	28.1 q	28.0 q
6‴	28.2 q	28.3 q	28.2 q	28.1 q
OCH_3	55.8 q			

75 MHz in $CDCl_3$ (TMS as internal standard). Assignments established by off-resonance and DEPT methods. s, singlet; d, doublet; t, triplet; q, quartet.

failed to give chroman and only the starting materials was recovered. This result suggested the presence of a C-8 γ,γ-dimethylallyl side-chain in **2a**. Recently, Fukai *et al.*[12] reported that the MS base peaks of 8-prenylated flavanone and 6-prenylated flavanone are $M^+ - CH_3$ and $M^+ - C_3H_7$, respectively. Due to the fragment in the MS, the γ,γ-dimethylallyl side-chain was therefore determined to be at C-8 and the structure of **2a** is assigned as 3′-methoxylupinifolin. The structure of **2a** was further confirmed by carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy (Table II).

The reaction of **2a** with methyl iodide and potassium carbonate in dry acetone under reflux gave two products, a trimethoxyflavanone (**2d**) and a chalcone (**9**) [δ 6.91 and 7.34 (each 1H, J=16 Hz), δ 3.67, 3.70, 3.88, and 3.88 (each 3H, s)]. Compound **2d**, an amorphous solid, shows three methoxyl signals [δ 3.81 (3H, s) and 3.89 (6H, s)] and an ABX system [δ 2.79 (1H, dd, J=17.0, 3.1 Hz), 2.87 (1H, dd, J=17.0, 13.2 Hz), and 5.33 (1H, dd, J=13.2, 3.1 Hz)] characteristic of the flavanone moiety in its ^{1}H-NMR spectrum. In an NOE experiment, NOEs were observed between the chromene olefinic proton (δ 6.61) and 5-OMe (δ 3.89) and between H-2′ [δ 6.99 (1H, d, J=2.0 Hz)] and one methoxyl group [δ 3.81 (3H, s)] in **2d**. The results supported the proposed structure of **2a**.

Laxifolin (**6a**) and isolaxifolin (**7a**) were separated by medium-pressure liquid chromatography (MPLC). The former melted at 259—260 °C. It shows peaks at *m/z* (%) 404 (M^+, 70), 389 ($M^+ - CH_3$, 49), 361 ($M^+ - C_3H_7$, 100) and 349 ($M^+ - C_4H_9$, 49) in the MS, was formulated as $C_{25}H_{24}O_5$ on the basis of elemental analysis, and gave a positive Mg–HCl test. The UV absorption of **6a** at λ_{max}^{MeOH}

(log ε): 236 (4.12), 279 (4.14), 314 (4.18), and 337 (4.06) nm indicated it to be a flavone. The ^{1}H-NMR spectrum (Table I) of laxifolin (**6a**) showed the presence of one 2,2-dimethylchromene, one γ,γ-dimethylallyl group, one olefinic proton, an A_2B_2 system of four aromatic protons, and two phenolic protons (δ 5.50 and 12.36). Similarly isolaxifolin (**7a**), yellow needles, mp 230—232 °C, was formulated at $C_{25}H_{24}O_5$ based on its elemental analysis. It also gave a positive Mg–HCl test. The ^{1}H-NNR data (Table I) for isolaxifolin indicated it to possess one 2,2-dimethylchromene, one γ,γ-dimethylallyl group, one olefinic proton, an A_2B_2 system of four aromatic protons, and two phenolic protons (δ 5.96 and 12.47). The UV absorption of isolaxifolin (**7a**) is similar to that of laxifolin (**6a**). The fragment peaks assignable to M^+-CH_3, $M^+-C_3H_7$ and $M^+-C_4H_9$ in the MS also confirmed the presence of a dimethylchromene and a γ,γ-dimethylallyl group in both compounds.[13] From the above evidence, laxifolin and isolaxifolin are considered to be isomers. The base peaks, M^+-CH_3 (isolaxifolin) and $M^+-C_3H_7$ (laxifolin), proved the presence of the 8-prenyl group for isolaxifolin and the 6-prenyl group for laxifolin.[12] Acetylation of **6a** and **7a** with acetic anhydride in pyridine at 60 °C overnight afforded the diacetates, **6b** [mp 125—126 °C; ν_{max}^{KBr} 1750 cm^{-1}; δ 2.33 and 2.45 (each 3H, s)] and **7b** [mp 105—107 °C; ν_{max}^{KBr} 1755 cm^{-1}; δ 2.32 and 2.45 (each 3H, s)], respectively. In the ^{1}H-NMR spectrum of **7b**, the signal of H-4‴ at δ 6.48 (d) is shifted upfield (0.22 ppm) compared with that in **7a** (δ 6.70, d). On the other hand, the chemical shifts of H-4‴ of **6a** and **6b** exhibit opposite shifts. This result provides additional proof that the γ,γ-dimethylallyl side-chain is located at C-6 for laxifolin (**6a**) and at C-8 for isolaxifolin (**7a**).[14,15] Finally, the location of the γ,γ-dimethylallyl group was determined by chemical transformation of **6a** to chroman (**10**) by heating in acetic acid and sulfonic acid[15] [(**10**), mp 272—274 °C; ν_{max}^{KBr} 3300 cm^{-1}; MS *m/z* 404 (M^+); δ 1.74 and 2.55 (each 2H, t, J=7.0 Hz)]. Under the same conditions, isolaxifolin (**7a**) did not react at all. Thus, the structures of laxifolin and isolaxifolin were established as **6a** and **7a**, respectively. The ^{13}C-NMR data (Table I) also confirm these structures.

Experimental

Melting points were determined on a Yanagimoto melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 781 spectrometer. ^{1}H- and ^{13}C-NMR spectra were run on a Brucker AM 300 at 300 MHz and 75 MHz in the indicated solvent with tetramethylsilane (TMS) as an internal standard. Chemical shifts are given in δ-values and coupling constants (J) are given in hertz (Hz). Electron impact mass spectra (EI-MS) and UV spectra were taken on JEOL JMS-100 and Hitachi U-3200 instruments, respectively.

Extraction and Isolation The roots of *Derris laxiflora* were crushed into small pieces and dried at 50 °C to give 5.7 kg of raw material, which was extracted with 95% ethanol (50 l) three times (8 h each time) at 60 °C. The combined extracts were evaporated *in vacuo* to give a residue, which was subsequently subjected to partition with ethyl acetate and H_2O (each 1 l). The upper layer was purified by silica gel chromatography with gradient (hexane–$CHCl_3$) system to afford flemichapparin-B (**1**) (87 mg), 3′-methoxylupinifolin (**2a**) (156 mg), β-amyrin (**3**) (283 mg) and lupeol (**4**) (165 mg) (a crystalline mixture of **3** and **4** was separated on silica gel impregnated with 10% $AgNO_3$), and lupinifolin (**2b**) (4.6 g). The aqueous layer was subsequently partitioned with BuOH (3 l), and the BuOH layer was purified by silica gel chromatography ($CHCl_3$–MeOH gradient) to yield prunetin (**5**) (143 mg) and a yellow crystalline mixture, which was separated by Sephadex LH-20 column chromatography (MeOH) and then silica gel MPLC (hexane : ethyl acetate = 3 : 1) to give laxifolin (**6a**) (120 mg) and isolaxifolin (**7a**) (128 mg).

Flemichapparin-B (**1**)[3]: mp 182—184 °C. UV λ_{max}^{MeOH} nm (log ε): 250 (4.08), 328sh (4.35), 338 (4.48), 357 (4.46). IR ν_{max}^{KBr} cm^{-1}: 3080, 1640, 1610, 1600, 1510, 1320, 1035, 1015, 935. MS *m/z* (%): 296 (100). ^{1}H-NMR ($CDCl_3$) δ: 3.78 (3H, s), 5.50, 5.97 (each 2H, s), 6.48 (1H, d, J=2.4 Hz), 6.52 (1H, dd, J=8.3, 2.4 Hz), 6.71 (1H, s), 7.00 (1H, s), 7.35 (1H, d, J=8.3 Hz).

3′-Methoxylupinifolin (**2a**): mp 82—83 °C. $[\alpha]_D^{20}$ −250° (c=0.5, $CHCl_3$). MS *m/z* (%): 436 (M^+, 67), 421 (M^+-CH_3, 100), 271 (36), 243 (17), 231 (11), 215 (61). UV λ_{max}^{MeOH} nm (log ε): 228 (4.34), 268 (4.62), 276 (4.66), 297 (4.11), 315 (4.12), 367 (3.54). UV $\lambda_{max}^{MeOH+NaOAc}$ nm (log ε): 226 (4.34), 268 (4.62), 276 (4.66), 314 (4.11), 366 (3.51). IR ν_{max}^{KBr} cm^{-1}: 3220, 1635, 1610, 1590, 1510, 1370. ^{1}H-NMR: Table I. ^{13}C-NMR: Table II. *Anal.* Calcd for $C_{26}H_{28}O_6$: C, 71.54; H, 6.47. Found: C, 71.89; H, 6.55.

β-Amyrin (**3**)[4]: mp 196—197 °C. IR ν_{max}^{KBr} cm^{-1}: 3280, 1380, 1370, 1035, 995, 875, 810. MS *m/z* (%): 426 (M^+, 2), 411 (1), 218 (100), 203 (62), 189 (26). ^{1}H-NMR ($CDCl_3$) δ: 0.77, 0.81, 0.85, 0.85, 0.92, 0.95, 0.98, 1.11 (each 3H, s), 3.20 (1H, dd, J=10.2, 5.7 Hz), 5.16 (1H, t, J=3.3 Hz).

Lupeol (**4**)[5]: mp 206—208 °C. IR ν_{max}^{KBr} cm^{-1}: 3420, 3040, 1635, 1010, 980, 870. MS *m/z* (%): 426 (M^+, 12), 411 (6), 218 (42), 207 (60), 203 (38), 189 (100), 175 (37), 135 (60), 121 (60). ^{1}H-NMR ($CDCl_3$) δ: 0.74, 0.77, 0.81, 0.92, 0.94, 1.01 (each 3H, s), 1.66 (3H, br s), 2.34 (1H, dt, J=11.0, 5.6 Hz), 3.16 (1H, dd, J=11.0, 5.6 Hz), 4.56, 4.66 (each 1H, br s).

Prunetin (**5**)[7]: mp 178—179 °C. UV λ_{max}^{MeOH} nm (log ε): 262 (4.69), 327 (3.79). IR ν_{max}^{KBr} cm^{-1}: 3360, 1660, 1610, 1560, 1510, 1050, 945, 820, 745. MS *m/z* (%): 284 (M^+, 100), 166 (14), 138 (5), 118 (2). ^{1}H-NMR (DMSO-d_6) δ: 3.80 (3H, s), 6.26, 6.40 (each 1H, d, J=2.1 Hz), 6.78, 7.28 (each 2H, d, J=8.5 Hz), 8.04 (1H, s), 9.28, 12.82 (each 1H, s, –OH).

Lupinifolin (**2b**)[6]: mp 178—179 °C. MS *m/z* (%): 406 (M^+, 82), 391 (100), 271 (27), 243 (10), 215 (29). UV λ_{max}^{MeOH} nm (log ε): 266 (4.52), 275 (4.56), 299 (3.95), 314 (3.99), 366 (3.39). IR ν_{max}^{KBr} cm^{-1}: 3240, 1640, 1610, 1590, 1520. ^{1}H-NMR: Table I. ^{13}C-NMR: Table II.

Laxifolin (**6a**): mp 259—260 °C. IR ν_{max}^{KBr} cm^{-1}: 3430, 1645, 1605, 1580, 1510, 1175, 1125, 835. MS *m/z* (%): 404 (M^+, 70), 389 (49), 361 (100), 349 (49). ^{1}H-NMR: Table I. ^{13}C-NMR: Table II. *Anal.* Calcd for $C_{25}H_{24}O_5$: C, 74.25; H, 5.94. Found: C, 74.20; H, 5.97.

Isolaxifolin (**7a**): mp 230—232 °C. UV λ_{max}^{MeOH} nm (log ε): 234 (4.25), 282 (4.15), 311 (4.13), 341 (4.10). IR ν_{max}^{KBr} cm^{-1}: 3400, 1655, 1605, 1560, 1510, 1205, 1170, 1125, 830. MS *m/z* (%): 404 (26), 389 (100), 361 (26), 349 (13). ^{1}H-NMR: Table I. ^{13}C-NMR: Table II. *Anal.* Calcd for $C_{25}H_{24}O_5$. Found: C, 74.19; H, 5.98.

Acetylation of 2a with Acetic Anhydride and Pyridine 3′-Methoxylupinifolin (**2a**) (5 mg) was allowed to react with Ac_2O (1 ml) in pyridine (0.5 ml) at 60°C overnight. Usual work-up gave a diacetate (**2c**) (5 mg). IR ν_{max}^{KBr} cm^{-1}: 1750, 1670, 1600, 1500, 1200, 1155. ^{1}H-NMR ($CDCl_3$) δ: 1.42, 1.43, 1.63, 1.69, 2.31, 2.39, 3.84 (each 3H, s), 2.71 (1H, dd, J=16.8, 2.5 Hz), 2.93 (1H, dd, J=16.8, 13.2 Hz), 3.28 (2H, d, J=7.0 Hz), 5.17 (1H, t, J=7.0 Hz), 5.40 (1H, dd, J=13.2, 2.5 Hz), 5.63, 6.36 (1H, d, J=10.0 Hz), 6.97 (1H, dd, J=8.0, 1.2 Hz), 7.05 (1H, d, J=8.0 Hz), 7.07 (1H, d, J=1.2 Hz).

Methylation of 2a A mixture of 3′-methoxylupinifolin (**2a**) (20 mg), methyl iodide (1 ml), dry acetone (5 ml), anhydrous potassium carbonate (1 g), and a few drops of dimethyl sulfoxide (DMSO) was heated under reflux for 24 h. The mixture was filtered, and the filtrate was evaporated to dryness. The residue was subsequently purified by silica gel chromatography ($CHCl_3$) to afforded two amorphous compounds, a trimethoxyflavanone (**2d**) (10 mg) [IR ν_{max}^{KBr} cm^{-1}: 1670, 1580, 1510, 1150, 1110. ^{1}H-NMR ($CDCl_3$) δ: 1.41, 1.43, 1.64, 1.65, 3.81, 3.89, 3.89 (each 3H, s), 2.79 (1H, dd, J=17.1, 3.1 Hz), 2.97 (1H, dd, J=17.1, 13.0 Hz), 3.28 (2H, d, J=7.2 Hz), 5.19 (1H, t, J=7.2 Hz), 5.33 (1H, dd, J=13.0, 3.1 Hz), 5.57, 6.61 (each 1H, d, J=10.1 Hz), 6.87 (1H, d, J=8.0 Hz), 6.97 (1H, dd, J=8.0, 2.0 Hz), 6.99 (1H, d, J=2.0 Hz)] and a chalcone (**9**) (9 mg) [IR ν_{max}^{KBr} cm^{-1}: 1660, 1635, 1580, 1500, 1130. ^{1}H-NMR ($CDCl_3$) δ: 1.43, 1.43, 1.67, 1.76, 3.67, 3.70, 3.88, 3.88 (each 3H, s), 3.27 (2H, d, J=7.1 Hz), 5.18 (1H, t, J=7.1 Hz), 5.59, 6.52 (each 1H, d, J=10.0 Hz), 6.83 (1H, d, J=8.4 Hz), 6.91, 7.34 (each 1H, d, J=16.0 Hz), 7.05 (1H, d, J=1.9 Hz), 7.10 (1H, dd, J=8.4, 1.9 Hz)].

Acetylation of Laxifolin (6a) and Isolaxifolin (7a) Acetylation of **6a** (5 mg) and **7a** (5 mg) in the same was as mentioned above yielded **6b** (5 mg) and **7b** (5 mg) respectively. **6b**; IR ν_{max}^{KBr} cm^{-1}: 1750, 1645, 1635, 1590, 1500, 1190. ^{1}H-NMR ($CDCl_3$) δ: 1.45, 1.45, 1.65, 1.65, 2.33, 2.45 (each 3H, s), 3.25 (2H, d, J=7.1 Hz), 5.08 (1H, t, J=7.1 Hz), 5.71, 6.84 (each 1H, d, J=10.0 Hz), 6.53 (1H, s), 7.23, 7.84 (each 2H, d, J=8.5 Hz). **7b**; IR ν_{max}^{KBr} cm^{-1}: 1755, 1645, 1630, 1590, 1500, 1195. ^{1}H-NMR ($CDCl_3$) δ: 1.46, 1.46, 1.67, 1.81, 2.32, 2.45 (each 3H, s), 3.56 (2H, d, J=6.8 Hz), 5.21 (1H,

t, $J=6.8$ Hz), 5.74, 6.48 (each 1H, d, $J=10.0$ Hz), 6.53 (1H, s), 7.22, 7.85 (each 2H, d, $J=8.5$ Hz).

The Chroman 10 from 6a by Acidic Cyclization A mixture of laxifolin (**6a**) (5 mg), acetic acid (1 ml), and concentrated H_2SO_4 (0.2 ml) was heated at 100 °C overnight and then allowed to stand at room temperature for 36 h. The reaction mixture was poured into excess ice water. The organic compounds were extracted with ethyl acetate and purified by silica gel column chromatography (MeOH–$CHCl_3$, 5:95) to give the chroman (**10**) (3 mg) [mp 272—274 °C. IR ν_{max}^{KBr} cm^{-1}: 3300, 1650, 1160, 1565, 1500. MS *m/z*(%): 404 (50), 389 (100), 361 (35), 349 (31). ^{1}H-NMR (DMSO-d_6) δ: 1.29, 1.43 (each 6H, s), 1.74, 2.55 (each 2H, t, $J=7.0$ Hz), 5.74, 6.84 (each 1H, d, $J=10.1$ Hz). 6.31 (1H, s), 6.64, 7.66 (each 2H, d, $J=8.5$ Hz)].

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Highly Diastereoselective Reaction of Chiral *o*-[2-(1,3-Oxazolidinyl)]benzaldehydes with Alkylmetallic Reagents: Synthesis of Chiral 3-Substituted Phthalides

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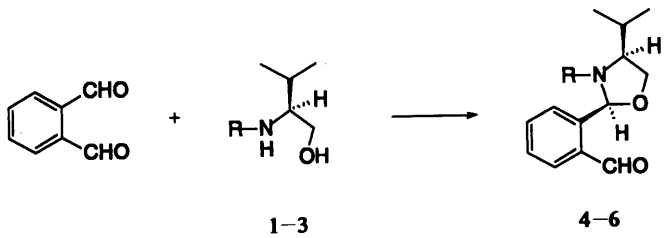
Highly diastereoselective reaction of chiral *o*-[2-(1,3-oxazolidinyl)]benzaldehydes (4—6) with alkylmetallic reagents provides a new synthetic method for chiral 3-alkylphthalides.

Keywords asymmetric synthesis; butylmagnesium chloride; (*S*)-3-butylphthalide; diethylzinc; (*S*)-3-ethylphthalide; (*S*)-*N*-isopropylvalinol; highly diastereoselective reaction; *o*-[2-(1,3-oxazolidinyl)]benzaldehyde; pyridinium chlorochromate

Many papers[1-3] and reviews[4-6] have been published on the synthesis of phthalides. In particular, asymmetric synthesis of chiral phthalides has recently been achieved by diastereoselective reaction of *ortho*-substituted benzaldehydes and lithiobenzenes bearing a chiral heterocyclic group, *e.g.*, 2,6a-diazaperhydropentalenyl,[7] 1,3-oxazolinyl,[8] imidazolidinyl,[9] and 1,3-dioxolanyl[9,10] groups. We have also reported highly diastereoselective reactions of chiral 1,3-oxazolidines,[11] 5-oxa-7,8a-diazaperhydroazulen-8-ones,[12] and 4-oxa-7,7a-diazaperhydroindans,[13] prepared from easily available enantiomerically pure (*S*)- and (*R*)-ethanolamines. In this paper, we wish to report a diastereoselective reaction of *o*-[2-(1,3-oxazolidinyl)]-benzaldehydes with alkylmetallic reagents, resulting the asymmetric synthesis of 3-alkylphthalides having a chiral center at the 3-position of the ring.

Chiral *o*-[2-(1,3-oxazolidinyl)]benzaldehydes (**4—6**) were synthesized by condensation of phthalaldehyde with (*S*)-*N*-methyl,[14,15] (*S*)-*N*-ethyl, and (*S*)-*N*-isopropyl[11,16] valinols (**1—3**) in 56—65% yields. These products were obtained as mixtures of two isomers, and the ratio of the major to the minor components was estimated as 93 : 7, 93 : 7, and 92 : 8, respectively, by proton nuclear magnetic resonance (^{1}H-NMR) spectral analysis. Diastereomerically pure compounds could not be isolated from the mixtures by column chromatography. The diastereomers may have equilibrated during column chromatography. The aryl group linked to the newly created asymmetric carbon atom at the 2-position of 1,3-oxazolidine ring is assumed to be in a *cis* relationship to the isopropyl group at the 4-position.[11,14]



	R
1, 4	CH_3
2, 5	C_2H_5
3, 6	iso-C_3H_7

Chart 1

The reaction of chiral *o*-[2-(1,3-oxazolidinyl)]benzaldehydes (**4—6**) with dibutylcupriolithium, diethylzinc, and alkylmagnesium halides gave oily products (**7—9**). These products were considered to be *o*-[2-(1,3-oxazolidinyl)]-benzylalcohols and/or 1-(2-hydroxyethyl)amino-2-oxaindans, but the structures were difficult to elucidate. The carbon–nitrogen bond of these products was easily cleaved by hydrolysis using *p*-toluenesulfonic acid monohydrate to give the corresponding 3-alkyl-1-hydroxy-2-oxaindans (**10a—c**). The chiral auxiliary reagents, *i.e.*, (*S*)-*N*-alkylvalinols (**1—3**), were recovered in good yields with no loss of optical purity. The products (**10a—c**) were confirmed to consist of two sets of diastereomers, *i.e.*, [(1*R*,3*R*)- and (1*S*,3*R*)-compounds] and [(1*S*,3*S*)- and (1*R*,3*S*)-compounds], by ^{1}H-NMR spectral analysis. The specific rotation of 3-ethyl-1-hydroxy-2-oxaindan (**10b**) thus obtained was consistent with that reported by Asami and Mukaiyama.[7]

The diastereomeric mixtures of 3-alkyl-1-hydroxy-2-oxaindans (**10a—c**) were oxidized with pyridinium chlorochromate (PCC) to give chiral 3-alkylphthalides (**11a—c**). The enantiomeric excesses (ee) of **11a—c** were estimated from the peak areas in a high performance liquid chromatography (HPLC) using a chiral packed column. Further, the absolute configurations were determined by comparison of the specific rotations with those of known 3-alkylphthalides [**11a**[8] and **11c**[17]] and 3-ethyl-1-hydroxy-2-oxaindan (**10b**).[7] These experimental data are summarized in Table I together with the total yields based on the originally used **4—6**.

The reactions of *o*-[2-(1,3-oxazolidinyl)]benzaldehydes (**4** and **6**) with diethylzinc in dichloromethane were highly diastereoselective (entries 4 and 6). The reaction of **6** with alkylmagnesium halides in dioxane (entry 10) and in a mixed solvent of tetrahydrofuran (THF)–dioxane (5 : 1) (entries 18 and 20) gave the corresponding chiral 3-alkylphthalides (**11a** and **11c**) of 85—90% ee However, the absolute configurations of the products depended on the solvents used and the bulkiness of the substituents, as shown in Table I.

In conclusion, this reaction proceeds with high diastereoselectivity, and the stereoselectivity is influenced by the bulkiness of substituents and the solvent used. Similar results have been reported in the diastereoselective reaction of *ortho*-substituted benzaldehydes with chiral heterocyclic groups, *e.g.*, 1,3-oxazolinyl,[8] imidazolidinyl,[9] and 1,3-dioxolanyl[9,10] groups. We propose a mechanism in which the oxygen atom of the 1,3-oxazolidine ring is nearly in the

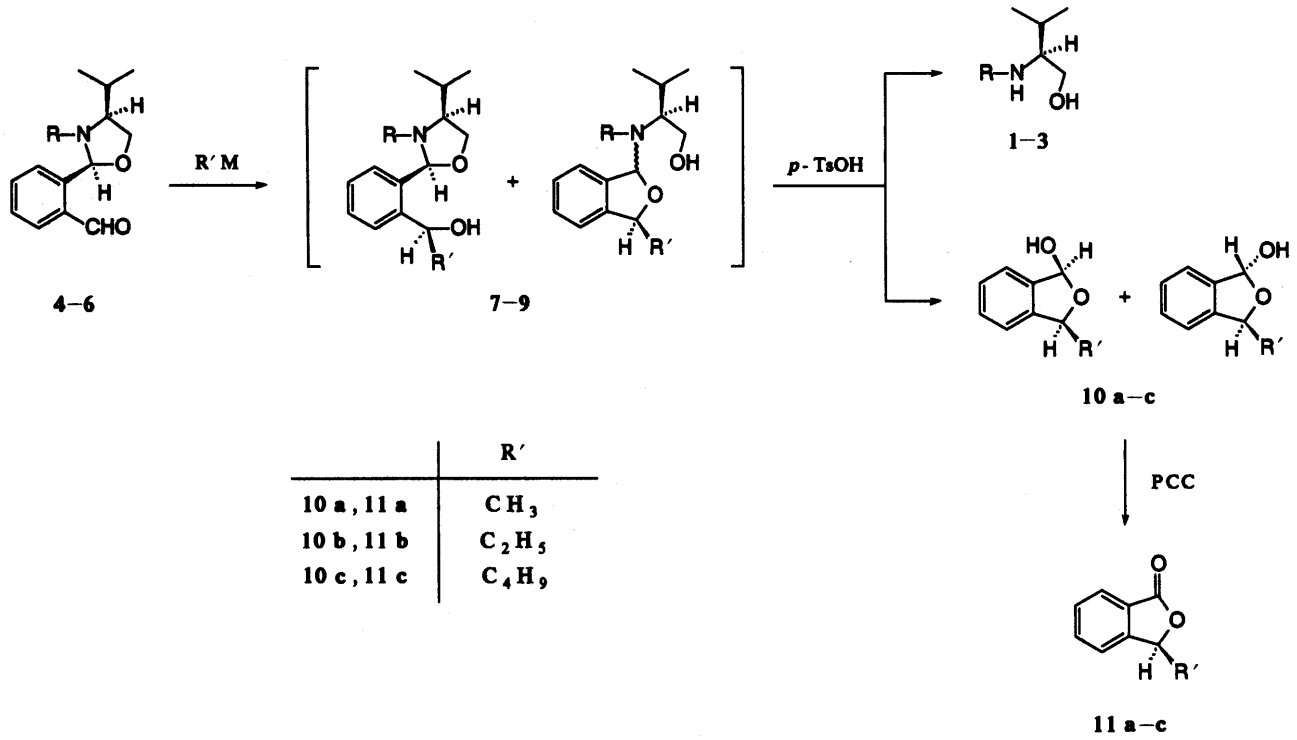


Chart 2

TABLE I. Diastereoselective Reaction of Chiral *o*-[2-(1,3-Oxazolidinyl)]benzaldehydes (**4**—**6**) with Alkylmetallic Reagents

Entry	Reactant	Reagent	Solvent	Temp (°C)	Time (h)	Product	Yield[a] (%)	ee[b] (%)	Configuration[c]
1	**6**	$(C_4H_9)_2CuLi$	Et_2O	−50	16	**11c**	82	28	*R*
2	**6**	$(C_4H_9)_2CuLi$	THF	−50	16	**11c**	80	58	*S*
3	**4**	$(C_2H_5)_2Zn$	THF	rt[d]	44	**11b**	56	7	*R*
4	**4**	$(C_2H_5)_2Zn$	CH_2Cl_2	rt[d]	16	**11b**	63	87	*R*
5	**5**	$(C_2H_5)_2Zn$	CH_2Cl_2	rt[d]	42	**11b**	82	76	*S*
6	**6**	$(C_2H_5)_2Zn$	CH_2Cl_2	rt[d]	18	**11b**	70	96	*S*
7	**6**	CH_3MgBr	Toluene	−50	16	**11a**	62	12	*R*
8	**6**	CH_3MgBr	Et_2O	−50	16	**11a**	65	33	*R*
9	**6**	CH_3MgBr	THF	−50	16	**11a**	72	73	*S*
10	**6**	CH_3MgBr	Dioxane	rt[d]	16	**11a**	53	85	*S*
11	**6**	CH_3MgBr	Mixed[e]	0	16	**11a**	62	80	*S*
12	**4**	CH_3MgBr	Mixed[f]	−50	16	**11a**	57	46	*S*
13	**4**	C_2H_5MgBr	Mixed[f]	−50	16	**11b**	43	47	*S*
14	**4**	C_4H_9MgCl	Mixed[f]	−50	16	**11c**	41	58	*S*
15	**5**	CH_3MgBr	Mixed[f]	−50	20	**11a**	52	66	*S*
16	**5**	C_2H_5MgBr	Mixed[f]	−50	19	**11b**	42	57	*S*
17	**5**	C_4H_9MgCl	Mixed[f]	−50	42	**11c**	65	70	*S*
18	**6**	CH_3MgBr	Mixed[f]	−50	16	**11a**	69	86	*S*
19	**6**	C_2H_5MgBr	Mixed[f]	−50	16	**11b**	63	77	*S*
20	**6**	C_4H_9MgCl	Mixed[f]	−50	20	**11c**	73	90	*S*

a) The yield was the isolated yield from *o*-[2-(1,3-oxazolidinyl)]benzaldehyde (**4**—**6**). *b*) The enantiomeric excess was estimated by HPLC using a chiral packed column. *c*) The absolute configuration was determined by comparison of the specific rotation with that of the known compound. *d*) Room temperature. *e*) Mixed solvent of ether–dioxane (5:1). *f*) Mixed solvent of THF–dioxane (5:1).

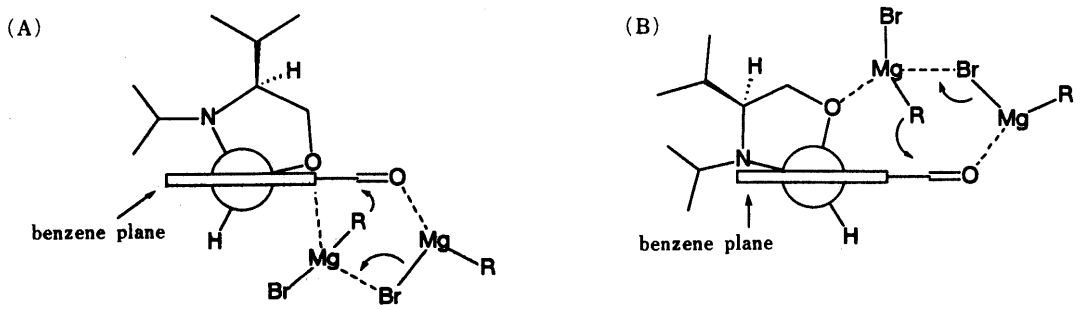


Fig. 1

plane of the benzene ring, forming a favorable intermediate (A) which is attacked by the alkylmetallic reagent from the *si*-face of the carbonyl carbon atom. If the nitrogen atom at the 1,3-oxazolidine ring is nearly in the plane of the benzene ring (B), attack of the reagent occurs from the *re*-face, as shown in Fig. 1.

Experimental

The ^{1}H-NMR spectra were obtained with a JEOL JNM-GSX270 spectrometer and the mass spectra (MS) were recorded with a JEOL JMS-D300 spectrometer by using the electron impact (EI) method. The melting points were measured with a Yanagimoto micromelting point apparatus and are uncorrected. The optical rotations were measured with a JASCO DIP-370 digital polarimeter.

(*S*)-*N*-Ethylvalinol (2) Acetyl chloride (9.42 g, 120 mmol) was added dropwise to a stirred solution of (*S*)-valinol (10.3 g, 100 mmol) and triethylamine (13.2 g, 130 mmol) in CH_2Cl_2 (150 ml) at 0 °C. After being stirred at room temperature for 3 h, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in THF (70 ml) and the solution was added dropwise to a stirred suspension of $LiAlH_4$ (7.7 g, 203 mmol) in THF (120 ml). After refluxing for 3 h, the reaction mixture was made alkaline. The resultant precipitate was filtered off and the filtrate was evaporated. The oily residue was distilled *in vacuo* to give a colorless oil (11.2 g, 85%), bp 83 °C (22 mmHg). $[\alpha]_D^{24}$ +17.1° (c=8.25, ethanol). MS *m/z*: 132 (M^++1, 100%). ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, d, J=6.71 Hz, $CHCH_3$), 0.96 (3H, d, J=6.71 Hz, $CHCH_3$), 1.11 (3H, t, J=7.32 Hz, CH_2CH_3), 1.79 (1H, octet, J=6.71 Hz, $CH(CH_3)_2$), 2.14 (2H, br, NH and OH), 2.38 (1H, dt, J=4.27, 6.71 Hz, $NCHCH_2O$), 2.60 (1H, dq, J=11.59, 7.32 Hz, CH_2CH_3), 2.71 (1H, dq, J=11.59, 7.32 Hz, CH_2CH_3), 3.30 (1H, dd, J=6.71, 10.38 Hz, $NCHCH_2O$), 3.59 (1H, dd, J=4.27, 10.38 Hz, $NCHCH_2O$).

(2*S*,4*S*)-*o*-[2-(4-Isopropyl-*N*-methyl-1,3-oxazolidinyl)]benzaldehyde (4) (*S*)-*N*-Methylvalinol (4.69 g, 40 mmol) was added dropwise to a solution of phthalaldehyde (5.37 g, 40 mmol) in CH_2Cl_2 (100 ml) in the presence of anhydrous Na_2SO_4 (10 g), and the reaction mixture was stirred at room temperature for 16 h. The precipitate was filtered off and the filtrate was evaporated under reduced pressure. The residue was distilled *in vacuo* to give **4** as a colorless oil (5.00 g, 56%), bp 143—145 °C (3.5 mmHg) (93 : 7 mixture). $[\alpha]_D^{24}$ −65.6° (c=2.89, hexane). *Anal.* Calcd for $C_{14}H_{19}NO_2$: C, 72.07; H, 8.21; N, 6.00. Found: C, 71.98; H, 8.34; N, 6.03. IR ($CHCl_3$): 1682 (C=O) cm^{-1}. MS *m/z*: 233 (M^+, 48%), 218 (M^+-CH_3, 23%), 190 ($M^+-C_3H_7$, 87%), 133 (86%). ^{1}H-NMR ($CDCl_3$) δ: major component; 0.97 (6H, d, J=7.02 Hz, $CH(CH_3)_2$), 1.96 (1H, d and septet, J=5.19, 7.02 Hz, $CH(CH_3)_2$), 2.16 (3H, s, NCH_3), 2.67 (1H, dt, J=5.19, 7.94 Hz, $NCHCH_2O$), 3.91 (1H, t, J=7.94 Hz, $NCHCH_2O$), 4.04 (1H, t, J=7.94 Hz, $NCHCH_2O$), 5.06 (1H, s, NCHO), 7.45—7.62 (3H, m, aromatic H), 7.90—7.94 (1H, m, aromatic H), 10.69 (1H, s, CHO).

(2*S*,4*S*)-*o*-[2-(*N*-Ethyl-4-isopropyl-1,3-oxazolidinyl)]benzaldehyde (5) Condensation of (*S*)-*N*-ethylvalinol (5.25 g, 40 mmol) with phthalaldehyde (5.37 g, 40 mmol) in CH_2Cl_2 (100 ml) was achieved in a similar manner to that described for the preparation of **4** to give **5** (6.13 g, 62%) as a colorless oil, bp 149—151 °C (3 mmHg) (93 : 7 mixture). $[\alpha]_D^{24}$ −93.5° (c=3.09, hexane). *Anal.* Calcd for $C_{15}H_{21}NO_2$: C, 72.84; H, 8.56; N, 5.66. Found: C, 72.61; H, 8.70; N, 5.66. IR ($CHCl_3$): 1680 (C=O) cm^{-1}. MS *m/z*: 247 (M^+, 20%), 218 ($M^+-C_2H_5$, 69%), 204 ($M^+-C_3H_7$, 71%), 133 (100%). ^{1}H-NMR ($CDCl_3$) δ: major component; 0.90 (3H, d, J=6.72 Hz, $CH(CH_3)_2$), 0.91 (3H, t, J=7.32 Hz, NCH_2CH_3), 0.97 (3H, d, J=6.72 Hz, $CH(CH_3)_2$), 1.72 (1H, octet, J=6.72 Hz, $CH(CH_3)_2$), 2.65—2.76 (2H, m, NCH_2CH_3), 2.83 (1H, q, J=6.72 Hz, $NCHCH_2O$), 3.77 (1H, dd, J=6.72, 8.54 Hz, $NCHCH_2O$), 4.00 (1H, dd, J=6.72, 8.54 Hz, $NCHCH_2O$), 5.58 (1H, s, NCHO), 7.43—7.91 (4H, m, aromatic H), 10.64 (1H, s, CHO).

(2*S*,4*S*)-*o*-[2-(4,*N*-Diisopropyl-1,3-oxazolidinyl)]benzaldehyde (6) A mixture of (*S*)-*N*-isopropylvalinol (5.81 g, 40 mmol) and phthalaldehyde (5.37 g, 40 mmol) in toluene (20 ml) was refluxed for 40 h using a Dean–Stark trap. The mixture was concentrated under reduced pressure and the residue was distilled *in vacuo* to give **6** as a colorless oil (6.8 g, 65%), bp 134—136 °C (0.45 mmHg) (92 : 8 mixture). $[\alpha]_D^{27}$ −93.7° (c=2.39, hexane). *Anal.* Calcd for $C_{16}H_{23}NO_2$: C, 73.53; H, 8.87; N, 5.36. Found: C, 73.74; H, 8.97; N, 5.39. IR ($CHCl_3$): 1680 (C=O) cm^{-1}. MS *m/z*: 261 (M^+, 2.5%), 246 (M^+-CH_3, 1.3%), 218 ($M^+-C_3H_7$, 100%), 133 (66%). ^{1}H-NMR ($CDCl_3$) δ: major component; 0.79 (3H, d, J=6.71 Hz, $CH(CH_3)_2$), 0.99 (3H, d, J=6.71 Hz, $CH(CH_3)_2$), 1.05 (3H, d, J=6.71 Hz, $CH(CH_3)_2$), 1.11 (3H, d, J=6.71 Hz, $CH(CH_3)_2$), 1.42 (1H, d and septet, J=8.39, 6.71 Hz, $CH(CH_3)_2$), 2.88 (1H, ddd, J=5.34, 7.32, 8.39 Hz, $NCHCH_2O$), 3.13 (1H, septet, J=6.71 Hz, $NCH(CH_3)_2$), 3.56 (1H, dd, J=5.34, 8.39 Hz, $NCHCH_2O$), 3.94 (1H, dd, J=7.32, 8.39 Hz, $NCHCH_2O$), 6.05 (1H, s, NCHO), 7.39—7.90 (4H, m, aromatic H), 10.58 (1H, s, CHO).

General Procedure for the Reaction of 4—6 with Alkylmetallic Reagent An alkylmetallic reagent [6 mmol; $(C_4H_9)_2CuLi$ (prepared from C_4H_9Li and CuI), $(C_2H_5)_2Zn$ (6 ml of a 1 M solution in hexane), CH_3MgBr (2 ml of a 3 M solution in ether), C_2H_5MgBr (2 ml of a 3 M solution in ether), or C_4H_9MgCl (3 ml of a 2 M solution in THF)] was added dropwise to a stirred solution of **4—6** (3 mmol) in the appropriate solvent (15 ml) under a nitrogen atmosphere. After being stirred at −50 °C or at room temperature for 16—42 h, the reaction mixture was treated with a saturated NH_4Cl aqueous solution (1 ml). The resulting precipitate was filtered off, and the filtrate was concentrated under reduced pressure to give a colorless oil (**7a—c**, **8a—c**, and **9a—c**). The product was used for the following reaction without purification.

General Procedure for the Cleavage of 7—9 with *p*-Toluenesulfonic Acid: Preparation of 3-Alkyl-1-hydroxy-2-oxaindanes (10a—c) and (*S*)-*N*-Alkylvalinols (1—3) *p*-Toluenesulfonic acid monohydrate (628 mg, 3.3 mmol) was added to a solution of an above product (**7a—c**, **8a—c**, or **9a—c**) in THF (10 ml) and H_2O (2 ml), and the refluxing was continued for 30 min. The reaction mixture was extracted with ether, and the organic layer was washed with water and dried over Na_2SO_4. Removal of the solvent gave a 3-alkyl-1-hydroxy-2-oxaindane (**10a—c**). On the other hand, the aqueous layer was saturated with K_2CO_3 and extracted with ether. The solution was washed with a saline solution and dried over Na_2SO_4. Removal of the solvent gave the corresponding (*S*)-*N*-alkylvalinol (**1—3**) in almost quantitative yield.

1-Hydroxy-3-methyl-2-oxaindan (**10a**): This product was confirmed to consist of an equimolar mixture of two diastereomers by ^{1}H-NMR spectral analysis. ^{1}H-NMR ($CDCl_3$) δ: 1.50 (3H, d, J=6.71 Hz, $CHCH_3$) and 1.58 (3H, d, J=6.71 Hz, $CHCH_3$), 2.88 (1H, d, J=8.51 Hz, OH) and 2.89 (1H, d, J=8.54 Hz, OH), 5.27 (1H, q, J=6.71 Hz, $CHCH_3$) and 5.51 (1H, dq, J=1.83, 6.71 Hz, $CHCH_3$), 6.41 (1H, d, J=8.54 Hz, CHOH) and 6.49 (1H, dd, J=1.83, 8.54 Hz, CHOH), 7.19—7.45 (4H, m, aromatic H).

2-Ethyl-1-hydroxy-2-oxaindan (**10b**): This product was confirmed to consist of an equimolar mixture of two diastereomers by ^{1}H-NMR spectral analysis. Specific rotation of the product was $[\alpha]_D^{27}$ −46.5° (c=2.01, benzene); lit.,[7] $[\alpha]_D$ −42.6° (c=5.35, benzene), 88% ee ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, t, J=7.32 Hz, CH_2CH_3) and 1.00 (3H, t, J=7.32 Hz, CH_2CH_3), 1.60—2.03 (2H, m, CH_2CH_3), 3.52 (1H, d, J=7.93 Hz, OH) and 3.62 (1H, d, J=7.93 Hz, OH), 5.13 (1H, dd, J=4.27, 6.71 Hz, $CHCH_2$) and 5.39 (1H, ddd, J=2.44, 4.27, 6.71 Hz, $CHCH_2$), 6.42 (1H, d, J=7.93 Hz, CHOH) and 6.48 (1H, dd, J=2.44, 7.93 Hz, CHOH), 7.18—7.43 (4H, m, aromatic H).

3-Butyl-1-hydroxy-2-oxaindan (**10c**): This product was confirmed to consist of an equimolar mixture of two diastereomers by ^{1}H-NMR spectral analysis. ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, t, J=7.32 Hz, $(CH_2)_3CH_3$) and 0.91 (3H, t, J=7.32 Hz, $(CH_2)_3CH_3$), 1.20—2.00 (6H, m, $(CH_2)_3CH_3$), 3.62 (1H, d, J=7.32 Hz, OH) and 3.75 (1H, d, J=7.32 Hz, OH), 5.14 (1H, dd, J=4.27, 7.32 Hz, $CH(CH_2)_3$) and 5.40 (1H, ddd, J=2.44, 4.27, 7.32 Hz, $CH(CH_2)_3$), 6.40 (1H, d, J=7.32 Hz, CHOH) and 6.46 (1H, dd, J=2.44, 7.32 Hz, CHOH), 7.18—7.43 (4H, m, aromatic H).

General Procedure for the Oxidation of 10a—c with Pyridinium Chlorochromate: Preparation of 3-Alkylphthalides (11a—c) PCC[18] (1.29 g, 6 mmol) was added to a stirred solution of **10a—c** in CH_2Cl_2 (5 ml). The mixture was stirred at room temperature for 30 min, then ether (10 ml) was added and the supernatant liquid was decanted from a black gum. The insoluble residue was washed with ether (3 ml × 5), leaving a black granular solid. The combined organic solution was passed through a short column of silica gel, and removal of the solvent gave **11a—c**. The ee of **11a—c** was estimated by HPLC using a chiral packed column "Chiralcel OB" (Daisel Chemical Industries, Ltd.), and the absolute configuration of **11a—c** was determined by comparison with known compounds. The crude product was subjected to column chromatography on silica gel. The values of ee and the absolute configuration are summarized in Table I together with the total isolated yields.

(*S*)-3-Methylphthalide (**11a**) Eluent: HPLC, hexane–isopropanol (80 : 20); column chromatography, hexane–ether (2 : 1). Optically pure **11a** was isolated from the isomeric mixture by recrystallization. Colorless needles, mp 41.5—42.5 °C (pentane–ether). The specific rotation of

100% ee compound, $[\alpha]_D^{24}$ −43.2° (c=5.11, $CHCl_3$) and $[\alpha]_D^{25}$ −43.5° (c=0.51, methanol); lit.,[8] (*S*)-3-methylphthalide $[\alpha]_D$ −13.2° (c=4.8, methanol), 44% ee ^{1}H-NMR ($CDCl_3$) δ: 1.65 (3H, d, J=6.72 Hz, $CHCH_3$), 5.57 (1H, q, J=6.72 Hz, $CHCH_3$), 7.46 (1H, d, J=7.33 Hz, aromatic H), 7.53 (1H, t, J=7.33 Hz, aromatic H), 7.69 (1H, t, J=7.33 Hz, aromatic H), 7.88 (1H, d, J=7.33 Hz, aromatic H).

(*S*)-3-Ethylphthalide (**11b**) Eluent: HPLC, hexane–isopropanol (95:5); column chromatography, hexane–THF (8:1). Colorless oil, bp 160°C (3 mmHg) (bulb-to-bulb distillation); Racemate, lit.,[19] bp 94°C (0.028 mmHg). The specific rotation of 96% ee compound, $[\alpha]_D^{25}$ −73.5° (c=3.60, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.01 (3H, t, J=7.32 Hz, CH_2CH_3), 1.83 (1H, d and quintet, J=14.64, 7.32 Hz, $CHCH_2CH_3$), 2.13 (1H, ddq, J=4.27, 14.64, 7.32 Hz, $CHCH_2CH_3$), 5.46 (1H, dd, J=4.27, 7.32 Hz, $CHCH_2CH_3$), 7.45 (1H, d, J=7.93 Hz, aromatic H), 7.52 (1H, t, J=7.93 Hz, aromatic H), 7.68 (1H, t, J=7.93 Hz, aromatic H), 7.90 (1H, d, J=7.93 Hz, aromatic H).

(*S*)-3-Butylphthalide (**11c**) Eluent: HPLC, hexane–isopropanol (95:5); column chromatography, hexane–ether (3:1). Colorless oil, bp 160—165°C (2 mmHg) (bulb-to-bulb distillation). The specific rotation of 73% ee compound $[\alpha]_D^{24}$ −51.7° (c=6.71, $CHCl_3$); lit.,[17] $[\alpha]_D$ −57° ($CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, t, J=7.32 Hz, CH_2CH_3), 1.31—1.53 (4H, m, $CHCH_2CH_2CH_2CH_3$), 1.70—1.83 (1H, m, $CHCH_2CH_2CH_2CH_3$), 1.99—2.12 (1H, m, $CHCH_2CH_2CH_2CH_3$), 5.48 (1H, dd, J=4.27, 7.93 Hz, CHC_4H_9), 7.44 (1H, d, J=7.32 Hz, aromatic H), 7.52 (1H, t, J=7.32 Hz, aromatic H), 7.67 (1H, t, J=7.32 Hz, aromatic H), 7.89 (1H, d, J=7.32 Hz, aromatic H).

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Studies on Sialic Acids. XXV. Synthesis of the α- and β-*N*-Glycosides of 3-Deoxy-D-*glycero*-D-*galacto*-2-nonulosonic Acid (KDN)

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Several α- and β-*N*-glycosides of 3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (1, KDN) were synthesized under Vorbrüggen and Williamson reaction conditions. Two hexa-*O*-acetyl derivatives of 1 were treated with trimethylsilyl derivatives of pyrimidine, 5-fluoropyrimidine and 5-methylpyrimidine, and azidotrimethylsilane to give mixtures of α- and -β-*N*-glycoside derivatives of 1. Two penta-*O*-acetyl-2-chloro derivatives of 1 were treated with the sodium salts of 2,4(1*H*,3*H*)-pyrimidinedione, 5-fluoro-2,4(1*H*,3*H*)-pyrimidinedione and 5-methyl-2,4(1*H*,3*H*)-pyrimidinedione to give only the α-*N*-glycosides. The anomeric configurations of these compounds could be elucidated on the basis of the coupling pattern of C-1 in ^{13}C-nuclear magnetic resonance spectral analysis.

Keywords 3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (KDN); glycosylation; *N*-glycoside; ^{13}C-NMR; sialic acid; 3-deoxy-D-nonulosonic acid

We have synthesized 3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (**1**, KDN) and several *O*-glycosides of **1** under Koenigs–Knorr and Williamson reaction conditions, using 2-halogenous derivatives as glycosyl donors.[1,2] The anomeric configurations of these *O*-glycosides could be elucidated by means of proton nuclear magnetic resonance (^{1}H-NMR) and circular dichroism (CD) spectral analysis. In studies on *N*-acetylneuraminic acid, several *N*-glycosides were synthesized[3] and biological activities of these derivatives were reported. As part of a program on the synthesis of glycoconjugates binding **1**, we wish to report the synthesis of several α- and β-*N*-glycosides of **1** under Vorbrüggen and Williamson reaction conditions. The anomeric configurations of these *N*-glycosides could be elucidated on the basis of the coupling pattern in gated proton-decoupled and selective proton-decoupled ^{13}C-NMR spectral analyses.

Syntheses of **1** and the glycosyl donors (**2**, **3**, **4**, **5**) were performed on the basis of reported procedures[1,2,4,5] (Chart 1). A synthetic modification of a carbohydrate needs a large quantity of starting material because the overall yield is usually low. We developed a facile procedure which reproducibly yields hundred gram quantities of **1** in high purity and high yield by utilizing the aldol condensation reaction. The obtained **1** was utilized for the synthesis of the glycosyl donors (**2**, **3**, **4**, **5**). Treatment of **1** with cesium carbonate and benzyl bromide or iodomethane in *N*,*N*-dimethylformamide (DMF) and subsequent acetylation with acetic anhydride gave benzyl and methyl 2,4,5,7,8,9-hexa-*O*-acetyl-3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonates (**2**, **4**) in 47 and 41% yields, respectively. These derivatives (**2**, **4**) were treated with hydrogen chloride in acetic acid to give benzyl and methyl 4,5,7,8,9-penta-*O*-acetyl-2-chloro-2,3-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonates (**3**, **5**) in 94 and 91% yields, respectively. The physical properties of **2**, **3** and **4** were in good agreement with published values.[1,2,5] The structure of **5** was elucidated by ^{1}H-NMR comparison with **3**. The ^{1}H-NMR data are summarized in Table I.

Glycosylation reactions of **2** or **4** with trimethylsilyl derivatives of pyrimidine, 5-fluoropyrimidine and 5-methyl pyrimidine in the presence of tin(IV) chloride under Vorbrüggen reaction conditions gave benzyl 4,5,7,8,9-penta-*O*-acetyl-2,3-dideoxy-2-(2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)-D-*glycero*-β-D-*galacto*-2-nonulopyranosonate (**7a**), **7b** and **7c** in 11, 18 and 6% yields, respectively. However, the α-anomers (**6a**—**c**) were obtained in poor yields (0—1%). The reaction of **5** and azidotrimethylsilane gave methyl 4,5,7,8,9-penta-*O*-acetyl-2-azido-2,3-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (**6d**) in 12% yield and the β-anomer (**7d**) in 14% yield. On the other hand, the condensation reactions of **3** or **5** with the sodium salts of 2,4(1*H*,3*H*)-pyrimidinedione, 5-fluoro-2,4(1*H*,3*H*)-pyrimidinedione and 5-methyl-2,4(1*H*,3*H*)-pyrimidinedione

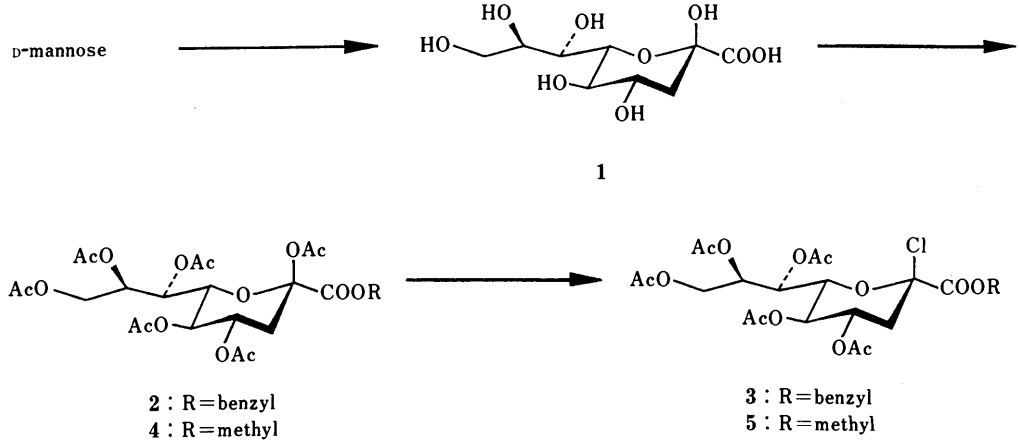
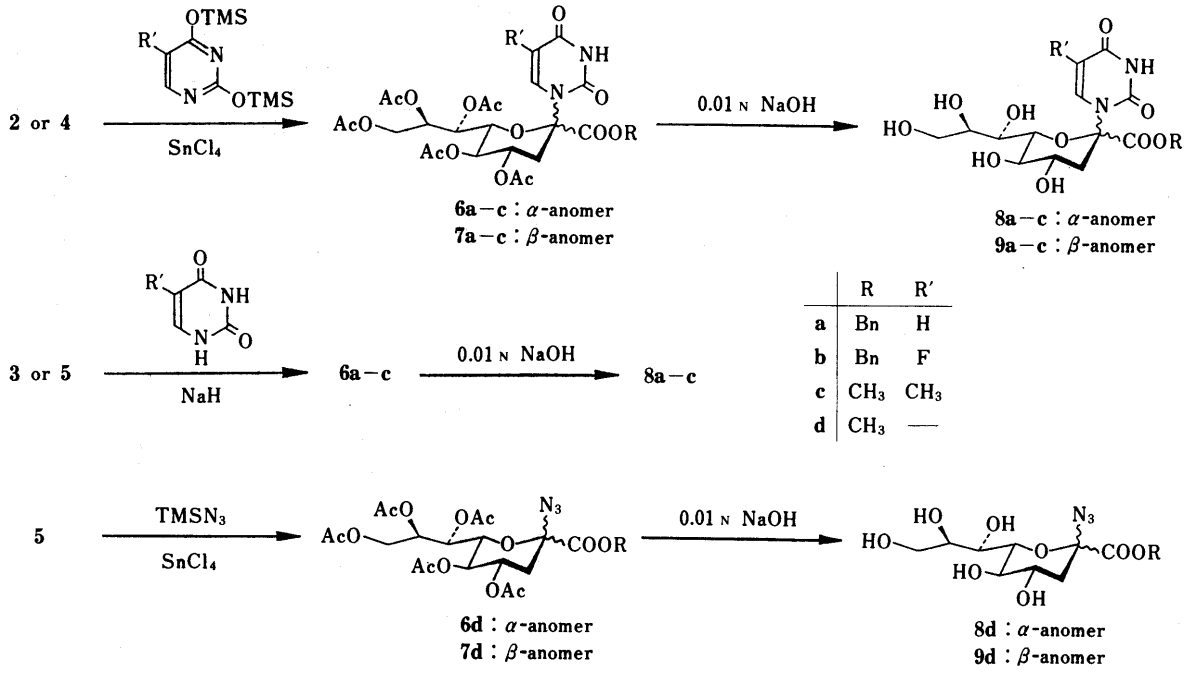


Chart 1



	R	R′
a	Bn	H
b	Bn	F
c	CH_3	CH_3
d	CH_3	—

Chart 2

TABLE I. Proton Chemical-Shift and Spin-Coupling Data at 300 MHz for **2**, **3**, **4**, **5**, **6a—d**, **7a—d**, **8a—d** and **9a—d**

Compound No.	Chemical shifts (δ)													Spin couplings (Hz)								
	H-3ax	H-3eq	H-4	H-5	H-6	H-7	H-8	H-9	H-9′	$COOCH_2Ph$	$COOCH_3$	$COCH_3$	Olefine	$J_{3ax,3eq}$	$J_{3ax,4}$	$J_{3eq,4}$	$J_{4,5}$	$J_{5,6}$	$J_{6,7}$	$J_{7,8}$	$J_{8,9}$	$J_{8,9'}$	$J_{9,9'}$
2	2.08	2.26	5.26	4.96	4.19	5.39	5.16	4.15	4.40	5.15, 5.23		1.99—2.11		13.5	11.4	5.2	9.8	10.2	2.2	6.1	5.9	2.6	12.5
3	2.24	2.84	5.44	4.95	4.48	5.47	5.22	4.10	4.34	5.22, 5.34		2.00—2.07		14.0	11.3	5.0	10.0	10.5	2.3	8.0	5.0	2.5	12.5
4	2.06	2.60	5.24	4.95	4.17	5.37	5.13	4.12	4.42		3.77	1.99—2.14		13.8	11.8	5.2	9.7	10.0	2.4	6.3	5.8	2.7	12.5
5	2.24	2.86	5.45	4.96	4.48	5.48	5.23	4.10	4.36		3.87	2.01—2.10		13.9	11.1	5.0	9.6	10.3	2.4	8.0	4.9	2.6	12.5
6a	1.90	3.36	5.56	4.92	3.89	5.35	5.17	4.08	4.22	5.16, 5.30		1.95—2.15	5.80, 7.65	13.8	10.0	5.3	8.5	10.3	2.0	7.9	5.0	2.8	12.5
7a	2.25	3.30	5.30	5.09	3.97	5.45	5.06	4.08	4.24	5.16, 5.21		2.02—2.09	5.73, 7.65	13.8	9.6	5.5	8.2	9.5	3.0	8.2	5.0	2.8	12.5
6b	1.98	3.35	5.54	4.92	4.00	5.35	5.21	4.09	4.23	5.17, 5.35		1.96—2.17	7.78	13.8	9.5	5.3	8.7	10.3	2.0	8.0	5.0	2.8	12.5
7b	2.29	3.27	5.07	5.03	3.89	5.35	5.18	4.12	4.49	5.24		1.99—2.18	7.64	14.5	11.0	3.9	9.6	9.6	2.0	6.2	5.9	2.5	12.5
6c	1.91	3.30	5.54	4.93	3.99	5.39	5.25	4.12	4.29		3.82	1.98—2.15	7.46	13.8	10.2	5.6	8.6	10.2	1.9	8.8	5.0	2.5	12.5
7c	2.21	3.16	5.08	5.07	3.83	5.36	5.12	4.13	4.58		3.81	2.00—2.12	7.45	14.5	11.5	3.2	—	9.8	2.0	5.5	6.0	2.3	12.5
6d	1.82	2.63	5.05	4.90	3.92	5.37	5.37	4.16	4.32		3.89	2.01—2.15		13.0	11.6	5.0	9.4	10.2	1.5	—	4.1	2.0	12.6
7d	2.07	2.31	4.89	5.22	4.27	5.43	5.24	4.10	4.46		3.88	1.99—2.09		13.3	—	5.0	9.8	10.0	2.0	7.0	5.1	2.2	12.5
8a	1.63	2.97	4.13	3.55	3.61	3.83	—	—	3.71	5.10, 5.29			5.77, 7.96	13.5	11.2	5.2	8.8	10.0	1.1	9.0	—	1.8	12.0
9a	2.01	2.98	4.53	3.67	3.91	3.59	3.65	3.57	3.75	5.10, 5.19			5.71, 7.87	14.7	9.0	5.6	8.3	9.0	1.1	8.5	6.0	2.8	11.6
8b	1.62	2.97	4.13	3.55	3.60	3.83	—	—	3.72	5.10, 5.30			8.15	13.5	11.0	5.3	8.5	10.0	1.1	9.0	—	2.0	11.2
9b	2.00	2.95	—	3.63	3.53	—	—	—	—	5.14, 5.24			8.01	15.0	9.8	4.8	8.9	10.0	0.5	4.2	—	—	—
8c	1.60	2.93	4.16	—	—	3.87	3.66	—	—		3.74		7.85	13.4	11.0	5.3	8.7	—	0.5	8.7	—	—	—
9c	2.06	2.89	3.92	3.63	3.46	3.78	3.63	3.57	3.72		3.72		7.65	14.0	11.8	5.0	9.0	9.0	0.8	9.0	5.0	2.2	11.5
8d	1.60	2.53	—	—	—	—	—	3.60	—		3.81			13.0	11.8	4.8	—	—	—	—	5.0	—	12.0
9d	1.80	2.18	3.85	3.50	3.95	3.84	3.73	3.60	3.76		3.79			14.5	11.5	5.0	8.9	10.1	1.0	9.3	5.5	2.8	12.0

Data for **2**, **3**, **4**, **5**, **6a—d** and **7a—d** were recorded in $CDCl_3$. Data for **8a—d** and **9a—d** were recorded in D_2O.

gave the corresponding α-anomers (**6a—c**) in 36, 28 and 20% yields, respectively. In this reaction, the β-anomer could not be obtained. These compounds were deprotected with 0.01 N sodium hydroxide solution to give the corresponding esters (**8a—d**, **9a—d**) in 35—68% yields.

We first attempted to confirm the stereochemistry at the anomeric configurations of these *N*-glycosyl derivatives by examination of the ^{1}H-NMR and CD spectra in comparison with those of the *O*-glycosyl derivatives.[1,2] Figure 1 shows the CD spectra of the α-anomers (**8a—d**) and the β-anomers (**9a—d**). Based on the CD spectra of the *O*-glycosyl derivatives, the peak around 220—230 nm is assigned to the n-π* Cotton effect of the carboxyl group and the negative Cotton effect was assigned to the α-anomer and the positive one to the β-anomer. But this method is not suitable for these *N*-glycosyl derivatives (**8a—c**, **9a—c**) which contain a chromophore such as a pyrimidine group. The spectra of the azido derivatives (**8d**, **9d**) were in accordance with the above results. Table I shows the ^{1}H-NMR data of the α-anomers (**6a—d**, **8a—d**) and the β-anomers (**7a—d**, **9a—d**). Empirical studies of **1** and *N*-acetylneuraminic acid indicated that the H-3(eq) signal of the α-anomer is usually observed at lower field than that of the β-anomer. The differences between the chemical shifts of H-3(eq) of the α-anomers (**6a—d**, **8a—d**) and those of the β-anomers (**7a—d**, **9a—d**) were +0.06, +0.08, +0.14, +0.32, −0.01, +0.02, +0.04, and +0.35 ppm, respectively. The values except those of the azido derivatives (**6d**, **7d**, **8d**, **9d**) are smaller than those of the *O*-glycosyl derivatives owing to the anisotropy of the

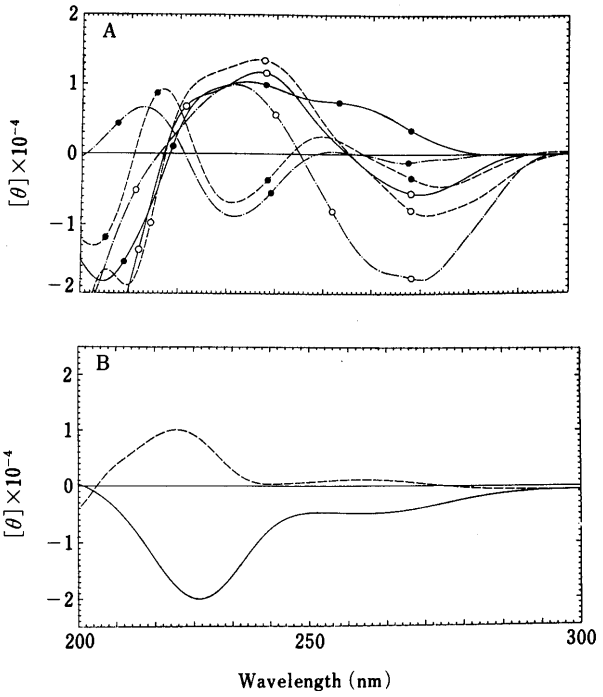


Fig. 1. CD Curves of **8a**—**d** and **9a**—**d** in MeOH at 20 °C

A. —○—, **8a**; —●—, **9a**; ---○---, **8b**; ---●---, **9b**; —·-○-·—, **8c**; —·-●-·—, **9c**. B. ——, **8d**; – – –, **9d**.

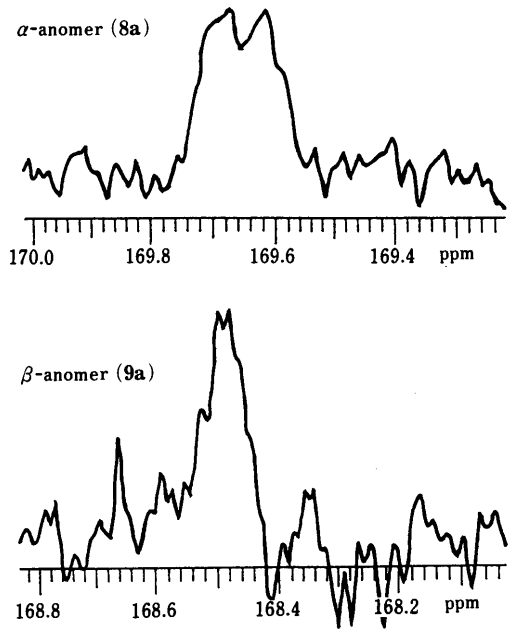


Fig. 2. C-1 Coupling Pattern of **8a** and **9a**

pyrimidine group. In conclusion, it was not possible to elucidate the stereochemistry at the anomeric position of the pyrimidine derivatives (**6a**—**c**, **7a**—**c**, **8a**—**c**, **9a**—**c**) from the CD and ^{1}H-NMR spectra. The stereochemistry at the anomeric position of the azido derivatives (**6d**, **7d**, **8d**, **9d**) could be elucidated by those methods, as could that of the *O*-glycosyl derivatives of **1**.

In studies on *N*-acetylneuraminic acid, Haverkamp *et al.*[6] and Hori *et al.*[7] have developed a method to deduce the anomeric configuration on the basis of the coupling pattern of C-1 in gated proton-decoupled or selective proton-decoupled ^{13}C-NMR spectra. We attempted to apply this method to elucidate the anomeric configuration of the *N*-glycosides of **1**. Figure 2 shows the C-1 coupling patterns of **8a** and **9a** in the gated proton-decoupled ^{13}C-NMR in D_2O at 100 MHz. The values of $J_{Cl,3ax}$ (α-anomer (**8a**): 6 Hz, β-anomer (**9a**): 1 Hz) were confirmed by selective decoupling of H-3eq and ester benzyl protons. Similar measurements of the other derivatives (**8b**—**d**, **9b**—**d**) were carried out and the same results were obtained. Therefore the anomeric configurations of the *N*-glycosides could be elucidated by this method.

In conclusion, we have synthesized the α- and β-*N*-glycosyl derivatives of **1** under Vorbrüggen and Williamson reaction conditions. The anomeric configuration of these compounds could be elucidated on the basis of the coupling pattern of C-1 in the ^{13}C-NMR spectra.

Experimental

Melting points were measured with a Yamato melting point apparatus and the results are uncorrected. Optical rotations were measured with a JASCO JIP-4 digital polarimeter. Thin layer chromatography (TLC) was performed on Silica gel GF-254 (Merck) plates, and spots were detected by ultraviolet (UV) irradiation and by spraying with 5% sulfuric acid solution. Fast atom bombardment mass spectra (FAB-MS), and infrared (IR) spectra were measured with JEOL JMS-DX300 and JASCO FT/IR-7300 instruments, respectively. CD spectra were measured in a 0.1 cm cell with a JASCO J-720 spectropolarimeter. The ^{1}H-NMR spectra were measured with Varian VXR-300 and XL-400 spectrometers. Tetramethylsilane (TMS) in $CDCl_3$ or sodium 3-(trimethylsilyl)-1-propanesulfonate (DDS) in D_2O was used as an internal reference. Column chromatography was conducted on Silica gel 60 (70—230 mesh).

3-Deoxy-D-*glycero*-D-*galacto*-2-nonulosonic Acid (1) Oxalacetic acid (73.3 g, 0.555 mol) was added to water (440 ml) at 5 °C, and its solution was adjusted to pH 11 with 10 M sodium hydroxide. D-Mannose (300 g, 1.66 mol) was added, and the mixture was stirred for 2 h at room temperature (the pH was maintained at 11 by adding 10 M sodium hydroxide). The solution was acidified (pH 6) with Dowex-50(H^+) resin in the presence of nickel(II) chloride (1.71 g, 13.2 mmol) at 50 °C, stirred for 1 h (the solution was kept at pH 6 with Dowex-50(H^+) resin), then filtered and evaporated. Purification of **1** was achieved by column chromatography on Dowex-50(H^+) resin with 0.3 N formic acid, and the eluate was concentrated under reduced pressure to give **1** (109 g, 73% from oxalacetic acid) as a colorless amorphous solid. $[\alpha]_D^{23}$ −40.7° (c=2.4, H_2O). FAB-MS m/z: 269 (M^++1). *Anal.* Calcd for $C_9H_{16}O_9 \cdot 2H_2O$: C, 35.53; H, 6.63. Found: C, 35.24; H, 6.51. IR ν_{max}^{KBr} cm^{-1}: 3385, 1745. ^{1}H-NMR (300 MHz, D_2O) δ: 1.69 (1H, dd, J=12.9, 11.8 Hz, 3_{ax}-H), 2.12 (1H, dd, J=12.9, 5.0 Hz, 3_{eq}-H), 3.45 (1H, dd, J=9.2, 9.8 Hz, 5-H), 3.52 (1H, dd, J=11.5, 6.3 Hz, 9-H), 3.62 (1H, ddd, J=9.0, 6.3, 2.4 Hz, 8-H), 3.70—3.76 (2H, m, 7 and 9-H), 3.85 (1H, dd, J=10.0, 9.8 Hz, 6-H), 3.86 (1H, ddd, J=11.8, 9.2, 5.0 Hz, 4-H).

Benzyl 2,4,5,7,8,9-Hexa-*O*-acetyl-3-deoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosonate (2) Anhydrous cesium carbonate (6.07 g, 18.6 mmol) was added to a solution of **1** (10.0 g, 37.2 mmol) in water (50 ml) at room temperature, and the solution was evaporated to dryness. The amorphous residue was suspended in DMF (50 ml), and benzyl bromide (12.7 g, 74.4 mmol) was added. The mixture was stirred for 18 h, then acetic anhydride (68.3 g, 0.669 mol), pyridine (52.9 g, 0.669 mol) and 4-dimethylaminopyridine (0.454 g, 3.72 mmol) were added at 5 °C. The whole was stirred for 18 h at room temperature, poured into 0.5 N HCl (1400 ml) and extracted with ethyl acetate (300 ml × 3). This extract was washed with sodium hydrogen carbonate solution and brine, dried and concentrated. The residual syrup was purified on a column of silica gel with ether–hexane (1 : 1) to yield **2** (10.7 g, 47%) as an amorphous powder. $[\alpha]_D^{25}$ −17.4° (c=1.0, $CHCl_3$). FAB-MS m/z: 611 (M^++1). *Anal.* Calcd for $C_{28}H_{34}O_{15}$: C, 55.08; H, 5.61. Found: C, 55.29; H, 5.78. IR ν_{max}^{KBr} cm^{-1}: 2960, 1745.

Benzyl 4,5,7,8,9-Penta-*O*-acetyl-2-chloro-2,3-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosonate (3) A solution of **2** (4.0 g, 6.55 mmol) in acetic acid (40 ml) and acetyl chloride (4 ml) was saturated with dry hydrogen chloride at 5 °C. After being stirred for 8 h at room temperature, the solution was evaporated, and the residue was purified

on a column of silica gel with ether–hexane (1 : 1) to yield **3** (3.61 g, 94%) as colorless plates, mp 108—110 °C (ether–hexane). $[\alpha]_D^{25}$ −61.9° (c=0.32, $CHCl_3$). Electron impact mass spectra (EI-MS) m/z: 586 (M^+). *Anal.* Calcd for $C_{26}H_{31}ClO_{13}$: C, 53.20; H, 5.32. Found: C, 53.12; H, 5.42. IR ν_{max}^{KBr} cm^{-1}: 2950, 1430, 1360.

Methyl 2,4,5,7,8,9-Hexa-*O*-acetyl-3-deoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosonate (4) Anhydrous cesium carbonate (3.64 g, 11.1 mmol) was added to a solution of **1** (6.00 g, 22.3 mmol) in water (30 ml) at room temperature, and the solution was evaporated to dryness. The amorphous residue was suspended in DMF (30 ml), and iodomethane (6.33 g, 44.6 mmol) was added. The mixture was stirred for 18 h, then acetic anhydride (40.9 g, 0.401 mol), pyridine (31.7 g, 0.401 mol) and 4-dimethylaminopyridine (0.272 g, 2.23 mmol) were added at 5 °C. The whole was stirred for 18 h at room temperature, poured into 0.5 N HCl (840 ml) and extracted with ethyl acetate (180 ml × 3). This extract was washed with sodium hydrogen carbonate solution and brine, dried and concentrated. The residual syrup was purified on a column of silica gel with ether–hexane (1 : 1) to yield **4** (4.90 g, 41%) as colorless prisms, mp 104—105 °C (ether–hexane). $[\alpha]_D^{19}$ −20.4° (c=0.71, $CHCl_3$). EI-MS m/z: 534 (M^+). *Anal.* Calcd for $C_{22}H_{30}O_{15}$: C, 49.44; H, 5.66. Found: C, 49.49; H, 5.70. IR ν_{max}^{KBr} cm^{-1}: 2950, 1750, 1440, 1370, 1230.

Methyl 4,5,7,8,9-Penta-*O*-acetyl-2-chloro-2,3-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosonate (5) A solution of **4** (2.00 g, 3.74 mmol) in acetic acid (20 ml) and acetyl chloride (2 ml) was saturated with dry hydrogen chloride at 5 °C. After being stirred for 8 h at room temperature, the solution was evaporated to dryness, and the residue was purified on a column of silica gel with ether–hexane (1 : 1) to yield **5** (1.74 g, 91%) as an amorphous powder. $[\alpha]_D^{22}$ −95.3° (c=0.44, $CHCl_3$). FAB-MS m/z: 511 (M^+ + 1). *Anal.* Calcd for $C_{20}H_{27}ClO_{13}$: C, 47.02; H, 5.33. Found: C, 47.31; H, 5.45. IR ν_{max}^{KBr} cm^{-1}: 2962, 1752, 1236.

Benzyl 4,5,7,8,9-Penta-*O*-acetyl-2,3-dideoxy-2-(2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)-D-*glycero*-α- and -β-D-*galacto*-2-nonulopyranosonates (6a, 7a) a) Tin(IV) chloride (1.72 g, 6.60 mmol) was added to a solution of **2** (2.00 g, 3.30 mmol) and trimethylsilylpyrimidine (1.69 g, 6.60 mmol) in acetonitrile (66 ml). The mixture was stirred for 24 h at room temperature, then sodium hydrogen carbonate (2.21 g, 26.4 mmol) in water (10 ml) was added under stirring. After evaporation of the solution, the residue was extracted with dichloromethane (50 ml × 3). The extract was washed with brine, dried and concentrated. The residual syrup was purified on a column of silica gel with ether to yield the α-anomer (**6a**) (18 mg, 1%) and the β-anomer (**7a**) (238 mg, 11%), each as an amorphous powder.

b) Sodium hydride (68.0 mg, 1.70 mmol) was added to a solution of 2,4(1*H*,3*H*)-pyrimidinedione (190 mg, 1.70 mmol) in DMF at room temperature. The mixture was stirred for 1 h, then **3** (1.00 g, 1.70 mmol) was added at room temperature, and stirring was continued for 5 h. Then the solution was poured into water (200 ml) and extracted with ethyl acetate (50 ml × 3). This extract was washed with brine, dried and concentrated. The residual syrup was purified on a column of silica gel with ether to yield the α-anomer (**6a**) (406 mg, 36%) as an amorphous powder.

α-Anomer (**6a**): $[\alpha]_D^{24}$ −36.8° (c=0.29, $CHCl_3$). FAB-MS m/z: 663 (M^+ + 1). *Anal.* Calcd for $C_{30}H_{34}N_2O_{15}$: C, 54.38; H, 5.17; N, 4.23. Found: C, 54.59; H, 5.33; N, 4.09. IR ν_{max}^{KBr} cm^{-1}: 3481, 1751, 1698, 1233.

β-Anomer (**7a**): $[\alpha]_D^{24}$ −6.4° (c=0.31, $CHCl_3$). FAB-MS m/z: 663 (M^+ + 1). *Anal.* Calcd for $C_{30}H_{34}N_2O_{15}$: C, 54.38; H, 5.17; N, 4.23. Found: C, 54.63; H, 5.26; N, 4.11. IR ν_{max}^{KBr} cm^{-1}: 1757, 1700, 1225.

Benzyl 4,5,7,8,9-Penta-*O*-acetyl-2,3-dideoxy-2-(2,4-dioxo-5-fluoro-1,2,3,4-tetrahydropyrimidin-1-yl)-D-*glycero*-α- and -β-D-*galacto*-2-nonulopyranosonates (6b, 7b) a) Tin(IV) chloride (1.72 g, 6.60 mmol) was added to a solution of **2** (2.00 g, 3.30 mmol) and 5-fluorotrimethylsilylpyrimidine (1.80 g, 6.60 mmol) in acetonitrile (66 ml). The solution was processed as described for **6a** and **7a** to give the α-anomer (**6b**) (24 mg, 1%) as colorless needles and the β-anomer (**7b**) (401 mg, 18%) as an amorphous powder.

b) Sodium hydride (68.0 mg, 1.70 mmol) was added to a solution of 5-fluoro-2,4(1*H*,3*H*)-pyrimidinedione (220 mg, 1.70 mmol) in DMF at room temperature. The mixture was stirring for 1 h, then **3** (1.00 g, 1.70 mmol) was added at room temperature. The solution was processed as described for **6a** to give the α-anomer (**6b**) (324 mg, 28%) as colorless needles.

α-Anomer (**6b**): mp 157—160 °C (ether). $[\alpha]_D^{24}$ −26.6° (c=0.35, $CHCl_3$). FAB-MS m/z: 681 (M^+ + 1). *Anal.* Calcd for $C_{30}H_{33}FN_2O_{15}\cdot H_2O$: C, 51.58; H, 5.05; N, 4.01. Found: C, 51.83; H, 4.85; N, 3.88. IR ν_{max}^{KBr} cm^{-1}: 3229, 1754, 1711, 1225.

β-Anomer (**7b**): $[\alpha]_D^{24}$ −44.3° (c=0.27, $CHCl_3$). FAB-MS m/z: 681 (M^+ + 1). *Anal.* Calcd for $C_{30}H_{33}FN_2O_{15}$: C, 52.94; H, 4.88; N, 4.11. Found: C, 53.01; H, 5.02; N, 3.92. IR ν_{max}^{KBr} cm^{-1}: 3231, 1751, 1236.

Methyl 4,5,7,8,9-Penta-*O*-acetyl-2,3-dideoxy-2-(2,4-dioxo-5-methyl-1,2,3,4-tetrahydropyrimidin-1-yl)-D-*glycero*-α- and -β-D-*galacto*-2-nonulopyranosonates (6c, 7c) a) Tin(IV) chloride (1.95 g, 7.48 mmol) was added to a solution of **4** (2.00 g, 3.74 mmol) and 5-methyltrimethylsilylpyrimidine (2.02 g, 7.48 mmol) in acetonitrile (75 ml). The solution was processed as described for **6a** and **7a** to give the β-anomer (**7c**) (135 mg, 6%) as an amorphous powder.

b) Sodium hydride (78.0 mg, 1.96 mmol) was added to a solution of 5-methyl-2,4(1*H*,3*H*)-pyrimidinedione (247 mg, 1.96 mmol) in DMF at room temperature. The mixture was stirred for 1 h, then **5** (1.00 g, 1.96 mmol) was added at room temperature. The solution was processed as described for **6a** to give the α-anomer (**6c**) (240 mg, 20%) as an amorphous powder.

α-Anomer (**6c**): $[\alpha]_D^{20}$ −53.9° (c=0.29, $CHCl_3$). FAB-MS m/z: 601 (M^+ + 1). *Anal.* Calcd for $C_{25}H_{32}N_2O_{15}$: C, 50.00; H, 5.37; N, 4.66. Found: C, 50.12; H, 5.44; N, 4.39. IR ν_{max}^{KBr} cm^{-1}: 1752, 1698, 1236.

β-Anomer (**7c**): $[\alpha]_D^{20}$ −71.6° (c=0.21, $CHCl_3$). FAB-MS m/z: 601 (M^+ + 1). *Anal.* Calcd for $C_{25}H_{32}N_2O_{15}$: C, 50.00; H, 5.37; N, 4.66. Found: C, 50.02; H, 5.65; N, 4.41. IR ν_{max}^{KBr} cm^{-1}: 1751, 1698, 1234.

Methyl 4,5,7,8,9-Penta-*O*-acetyl-2-azido-2,3-dideoxy-D-*glycero*-α- and -β-D-*galacto*-2-nonulopyranosonates (6d, 7d) Tin(IV) chloride (1.95 g, 7.48 mmol) was added to a solution of **4** (2.00 g, 3.74 mmol) and azidotrimethylsilane (860 mg, 7.48 mmol) in acetonitrile (75 ml). The solution was processed as described for **6a** and **7a** to give the α-anomer (**6d**) (232 mg, 12%) and the β-anomer (**7d**) (271 mg, 14%), each as an amorphous powder.

α-Anomer (**6d**): $[\alpha]_D^{20}$ −46.7° (c=0.33, $CHCl_3$). FAB-MS m/z: 518 (M^+ + 1). *Anal.* Calcd for $C_{20}H_{27}N_3O_{13}$: C, 46.42; H, 5.26; N, 8.12. Found: C, 46.71; H, 5.50; N, 8.03. IR ν_{max}^{KBr} cm^{-1}: 2123, 1749, 1218.

β-Anomer (**7d**): $[\alpha]_D^{20}$ −41.5° (c=0.21, $CHCl_3$). FAB-MS m/z: 518 (M^+ + 1). *Anal.* Calcd for $C_{20}H_{27}N_3O_{13}\cdot 1/2H_2O$: C, 45.63; H, 5.36; N, 7.98. Found: C, 45.58; H, 5.30; N, 7.85. IR ν_{max}^{KBr} cm^{-1}: 2125, 1747, 1220.

Benzyl 2,3-Dideoxy-2-(2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)-D-*glycero*-α- and -β-D-*galacto*-2-nonulopyranosonates (8a, 9a) A solution of **6a** or **7a** (100 mg, 0.151 mmol) in 0.01 N NaOH (76 ml) was stirred for 1 h at room temperature. The solution was neutralized with acetic acid and evaporated to dryness. The residual syrup was purified on a column of silica gel with CH_2Cl_2–EtOH (10 : 1) to yield the α-anomer (**8a**) (42 mg, 61%) or the β-anomer (**9a**) (33 mg, 48%) as an amorphous powder.

α-Anomer (**8a**): $[\alpha]_D^{24}$ −83.1° (c=0.17, EtOH). FAB-MS m/z: 453 (M^+ + 1). *Anal.* Calcd for $C_{20}H_{24}N_2O_{10}$: C, 53.10; H, 5.35; N, 6.19. Found: C, 52.94; H, 5.09; N, 6.36. IR ν_{max}^{KBr} cm^{-1}: 3395, 1745, 1695.

β-Anomer (**9a**): $[\alpha]_D^{20}$ −35.2° (c=0.19, EtOH). FAB-MS m/z: 453 (M^+ + 1). *Anal.* Calcd for $C_{20}H_{24}N_2O_{10}$: C, 53.10; H, 5.35; N, 6.19. Found: C, 52.96; H, 5.51; N, 5.82. IR ν_{max}^{KBr} cm^{-1}: 3393, 1750, 1684.

Benzyl 2,3-Dideoxy-2-(2,4-dioxo-5-fluoro-1,2,3,4-tetrahydropyrimidin-1-yl)-D-*glycero*-α- and -β-D-*galacto*-2-nonulopyranosonates (8b,9b) A solution of **6b** and **7b** (100 mg, 0.147 mmol) in 0.01 N NaOH (74 ml) was stirred for 1 h at room temperature. The solution was processed as described for **8a** and **9a** to give the α-anomer (**8b**) (43 mg, 63%) or the β-anomer (**9b**) (24 mg, 35%) as an amorphous powder.

α-Anomer (**8d**): $[\alpha]_D^{20}$ −97.7° (c=0.23, EtOH). FAB-MS m/z: 471 (M^+ + 1). *Anal.* Calcd for $C_{20}H_{23}FN_2O_{10}$: C, 51.07; H, 4.93; N, 5.96. Found: C, 50.83; H, 4.81; N, 5.71. IR ν_{max}^{KBr} cm^{-1}: 3394, 1701.

β-Anomer (**9d**): $[\alpha]_D^{20}$ −65.9° (c=0.18, MeOH). FAB-MS m/z: 471 (M^+ + 1). *Anal.* Calcd for $C_{20}H_{23}FN_2O_{10}$: C, 51.07; H, 4.93; N, 5.96. Found: C, 50.91; H, 4.99; N, 5.75. IR ν_{max}^{KBr} cm^{-1}: 3395, 1705.

Methyl 2,3-Dideoxy-2-(2,4-dioxo-5-methyl-1,2,3,4-tetrahydropyrimidin-1-yl)-D-*glycero*-α- and -β-D-*galacto*-2-nonulopyranosonates (8c, 9c) A solution of **6c** or **7c** (100 mg, 0.166 mmol) in 0.01 N NaOH (83 ml) was stirred for 1 h at room temperature. The solution was processed as described for **8a** and **9a** to give the α-anomer (**8c**) (44 mg, 68%) or the β-anomer (**9c**) (26 mg, 40%) as an amorphous powder.

α-Anomer (**8c**): $[\alpha]_D^{20}$ −89.6° (c=0.42, MeOH). FAB-MS m/z: 391 (M^+ + 1). *Anal.* Calcd for $C_{15}H_{22}N_2O_{10}\cdot 1/2H_2O$: C, 45.11; H, 5.80; N, 7.01. Found: C, 45.38; H, 5.81; N, 6.84. IR ν_{max}^{KBr} cm^{-1}: 3400, 1699.

β-Anomer (**9c**): $[\alpha]_D^{20}$ −104.8° (c=0.25, MeOH). FAB-MS m/z: 391 (M^+ + 1). *Anal.* Calcd for $C_{15}H_{22}N_2O_{10}\cdot H_2O$: C, 44.12; H, 5.92; N, 6.86. Found: C, 44.39; H, 6.07; N, 6.59. IR ν_{max}^{KBr} cm^{-1}: 3396, 1701.

Methyl 2-Azido-2,3-dideoxy-D-*glycero*-α- and -β-D-*galacto*-2-nonulopyranosonates (8d, 9d) A solution of **6d** or **7d** (100 mg, 0.193 mmol) in 0.01 N NaOH (96 ml) was stirred for 1 h at room temperature. The

solution was processed as described for **8a** and **9a** to give the α-anomer (**8d**) (31 mg, 52%) or the β-anomer (**9d**) (40 mg, 68%) as an amorphous powder.

α-Anomer (**8d**): $[\alpha]_D^{20}$ −92.6° (c=0.49, MeOH). FAB-MS m/z: 308 ($M^+ + 1$). *Anal.* Calcd for $C_{10}H_{17}N_3O_8 \cdot 1/2H_2O$: C, 37.98; H, 5.74; N, 13.29. Found: C, 38.17; H, 5.69; N, 13.53. IR ν_{max}^{KBr} cm^{-1}: 3383, 2939, 2122, 1741.

β-Anomer (**9d**): $[\alpha]_D^{20}$ −81.7° (c=0.31, MeOH). FAB-MS m/z: 308 ($M^+ + 1$). *Anal.* Calcd for $C_{10}H_{17}N_3O_8 \cdot 2/3H_2O$: C, 37.62; H, 5.58; N, 13.16. Found: C, 37.62; H, 5.56; N, 12.87. IR ν_{max}^{KBr} cm^{-1}: 3238, 2109, 1745.

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Synthetic Studies on Indoles and Related Compounds. XXIX.[1] Attempted Syntheses of Benz[*f*]indoles by Cyclization Reactions

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Syntheses of benz[*f*]indoles from 1,2-disubstituted naphthalene derivatives by means of cyclization reactions were attempted. The Fischer indolization of 1-methyl-(5a), 1-chloro-(5b), or 1-nitro-(5c)-2-naphthylhydrazones gave only benz[*e*]indole derivative or decomposed products, and the desired 9-substituted benz[*f*]indole (3) was not produced. On the other hand, the Fischer indolization of 2-methoxy-1-naphthylhydrazone (17) gave ethyl 5-chlorobenz[*g*]indole-2-carboxylate (19). The Hemetsberger reaction of 1-methoxy-2-naphthylazido acrylate (26a) gave an azirine (28a) and a nitrile (29a), whereas the same reaction of 1-chloro-2-naphthylazido acrylate (26b) gave the desired 4-chlorobenz[*f*]indole in a poor yield together with large amounts of by-products, the azirine (28b), the nitrile (29b) and the benz[*g*]indole (20). These results show that cyclization reactions of a 2-substituent toward the 3-position in naphthalene derivative are not suitable for preparing benz[*f*]indoles.

Keywords benz[*f*]indole; benz[*e*]indole; benz[*g*]indole; Fischer indolization; 2-naphthylhydrazone; Hemetsberger reaction; azido acrylate

Synthesis of benz[*f*]indole (**1**) derivatives is not easy,[2] though Fischer indolization has been employed for this purpose. For example, Goldsmith and Lindwall[3] reported the formation of ethyl 9-methoxy-1*H*-benz[*f*]indole-2-carboxylate (**3a**) by Fischer indolization of ethyl pyruvate 2-(1-methoxy-2-naphthyl)hydrazone (**2**) with ethanolic hydrogen chloride. However, we found[4] that the product they[2] had obtained was not the benz[*f*]indole (**3a**) but ethyl 5-chlorobenz[*e*]indole-2-carboxylate (**4**), on the basis of spectral and chemical evidence, and later, by authentic synthesis of the 9-methoxybenz[*f*]indole[5] (**3a**). The formation of the abnormal product (**4**) was explained in terms of cyclization toward the substituted 1-position of the naphthalene nucleus. Although the Fischer indolization did not take place at the desired 3-position in the 1-methoxy-2-naphthylhydrazone (**2**), cyclization reactions still seemed attractive for the synthesis of benz[*f*]indole derivatives because of easy preparation of the starting arylhydrazones and simple reaction conditions. In this paper we report the results of our trials for the synthesis of benz[*f*]indoles by Fischer indolization of various 1-substituted 2-naphthylhydrazones (**5**) and by Hemetsberger reaction[6] of 1-substituted 2-naphthylazido acrylates (**26**).

Fischer Indolization In Fischer indolization of *o*-substituted phenylhydrazones,[7] the kind of *ortho*-substituent determines the site at which the cyclization occurs, and it has been suggested[7,8] that phenylhydrazones (generally speaking, arylhydrazones) with a more electron-withdrawing group on one side of two *ortho*-positions tend to cyclize at the vacant side. Thus, as we expected that a more electron-withdrawing substituent than the methoxy group would serve as a blocking group against cyclization to the substituted 1-position, we tried the Fischer indolization of ethyl pyruvate 2-(1-methyl, 1-chloro-, and 1-nitro-2-naphthyl)hydrazones (**5a—c**).

The starting naphthylhydrazones (**5**) were prepared from the corresponding known amines, 1-methyl-,[9] 1-chloro-,[10] and 1-nitro-[11] 2-naphthylamines (**6a—c**), by Japp-Klingemann reaction with ethyl α-methylacetoacetate. Of the three naphthylhydrazones (**5**), the 1-methyl and 1-chloro compounds (**5a, b**) were prepared as usual. The Japp-Klingemann reaction of 1-nitro-2-naphthylamine (**6c**) was conducted with isoamyl nitrite and hydrochloric acid, followed by the addition of the resultant diazonium salt solution to a solution of ethyl α-methylacetoacetate in a basic medium. However, a product obtained in a good yield (77%) was not the desired hydrazone (**5c**). The elemental analysis and the mass spectrum (MS) (*m/z* 290, M^+) showed its molecular formula to be $C_{15}H_{15}ClN_2O_2$. The ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrum proved the presence of an ethoxy, a C-methyl (δ 2.15, singlet), and six aromatic protons, which indicated a naphthylhydrazone structure. The infrared (IR) spectrum showed no nitro group. Thus, the product was suggested to be the 1-chloro-2-naphthylhydrazone (**5b**), and it was confirmed

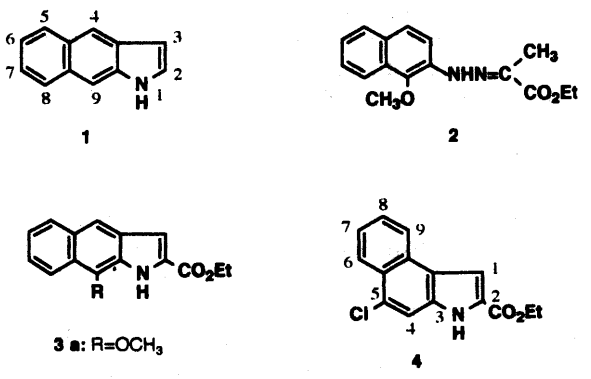


Fig. 1

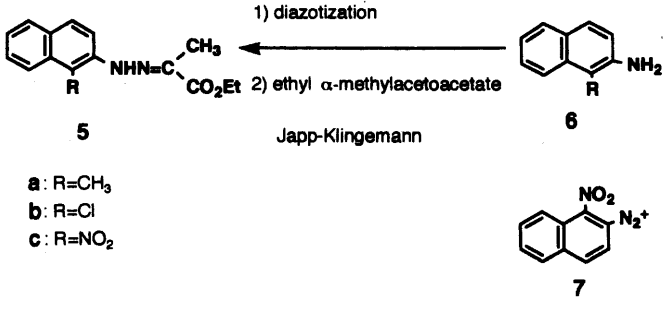


Chart 1

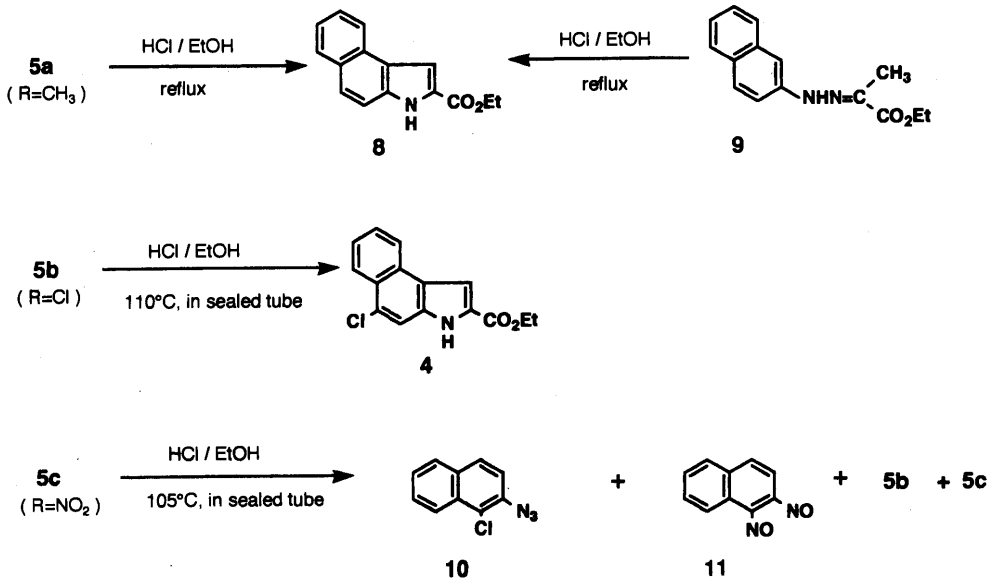


Chart 2

to be identical with a sample prepared from 1-chloro-2-naphthylamine (**6b**). This abnormal reaction could be explained on the basis of the fact[12] that the nitro group is easily replaced with a chlorine atom in the 1-nitro-2-naphthyldiazonium salt (**7**). Then, Japp-Klingemann reaction was carried out using *p*-toluenesulfonic acid, because *p*-toluenesulfonate is a much weaker nucleophile than hydrogen chloride. Although the yield was low (below 28%), the reaction was successful in giving the desired 1-nitro-2-naphthylhydrazone (**5c**) accompanied with 1-nitronaphthalene (8.7%) as a by-product. The Fischer indolization of the 1-methyl-2-naphthylhydrazone (**5a**) in refluxing ethanolic hydrogen chloride gave only one indolic compound[13] (**8**) in low yield (14.8%). This compound, mp 162—167 °C, was found to have the formula $C_{15}H_{13}NO_2$, by elemental analysis and MS (*m/z* 239, M^+). In the ^{1}H-NMR spectrum there appeared the signals of an ethoxy group [δ 1.43 (3H, t, $CH_2C\underline{H}_3$) and 4.43 (2H, q, $OC\underline{H}_2CH_3$)], an NH [δ 9.67 (1H, br s)], and seven aromatic protons (δ 7.20—8.30, m), but no methyl group. These data suggested that the product (**8**) is unsubstituted benz[*e*]indole. The structure was confirmed by comparison with an authentic sample (mp 164—165 °C) prepared from 2-naphthylhydrazone (**9**) according to the reported method.[3]

The Fischer indolization of the 1-chloro-2-naphthylhydrazone (**5b**) with ethanolic hydrogen chloride did not proceed well under the usual reflux conditions but proceeded at 110 °C in a sealed tube. The reaction gave only one indolic compound (**4**), mp 233—234 °C. The molecular formula was found to be $C_{15}H_{12}ClNO_2$ by elemental analysis and MS [*m/z* 273 (M^+), 275 ($M^+ + 2$, 35% intensity of M^+)]. As this product contained a chlorine atom, it should be a chlorobenzindole. The product (**4**) was identical with the 5-chlorobenz[*e*]indole (**4**), mp 232—233 °C, which we had obtained by the Fischer indolization of the 1-methoxy-2-naphthylhydrazone[4] (**2**).

The Fischer indolization of the 1-nitro-2-phenylhydrazone (**5c**) took place with ethanolic hydrogen chloride at 105 °C in a sealed tube to give a mixture of many products. The reaction did not occur at refluxing temperature under atmospheric pressure. Among the products, two main compounds (**10** and **11**) were isolated, besides the recovery of the hydrazone (**5c**) (22%) and a small amount of the chlorohydrazone (**5b**) (5.4%). Easy replacement of the nitro group with chlorine in this system had already been observed in the formation of the diazonium salt (**7**). The first product (**10**) had the molecular formula $C_{10}H_6ClN_3$ by elemental analysis, and MS [*m/z* 203 (M^+) and 205 (20% intensity of M^+, $M^+ + 2$)]. The ^{1}H-NMR spectrum showed six aromatic protons at δ 7.10—8.30 as multiplets and the IR spectrum showed an azido group absorption at 2115 cm^{-1}. These data showed that **11** was chloro-azidonaphthalene. The position and the origin of the substituents were rationalized as follows: the 1-nitro group could be replaced by a chlorine atom as in the diazonium salt (**7**), and the azido group might be derived from the 2-hydrozono group *via* an unknown route. Although we have no further structural data, we suggest that this compound is probably 1-chloro-2-azidonaphthalene.

The second product (**11**), mp 128—129 °C, had the molecular formula $C_{10}H_6N_2O_2$ by elemental analysis and MS [*m/z* 186 (M^+, 78% intensity of the base peak) and 126 ($C_{10}H_6$, 100%)]. The ^{1}H-NMR spectrum showed only six aromatic protons at δ 6.95—8.70 as multiplets and the IR spectrum showed no characteristic absorption. On the basis of these results we suggest that this compound (**11**) is 1,2-dinitrosonaphthalene. At the present time we cannot propose a reasonable mechanism for the formation of such curious products (**10** and **11**).

As described above, we failed to obtain any benz[*f*]indole by Fischer indolization of 1-substituted 2-naphthylhydrazones (**5**). This may be explained by a much greater tendency of **5** to cyclize toward the substituted 1-position than toward the unsubstituted 3-position. We can explain this fact on the basis of Robinson's concerted mechanism[14] of Fischer indolization as follows (Chart 3). In the key transformation step shown in Chart 3, aromaticity of the naphthalene nucleus in **13a** is forced to be broken to form **13a'**. On the other hand, transformation from **13b** to **13b'** does not involve such an energetically unfavorable transition state.

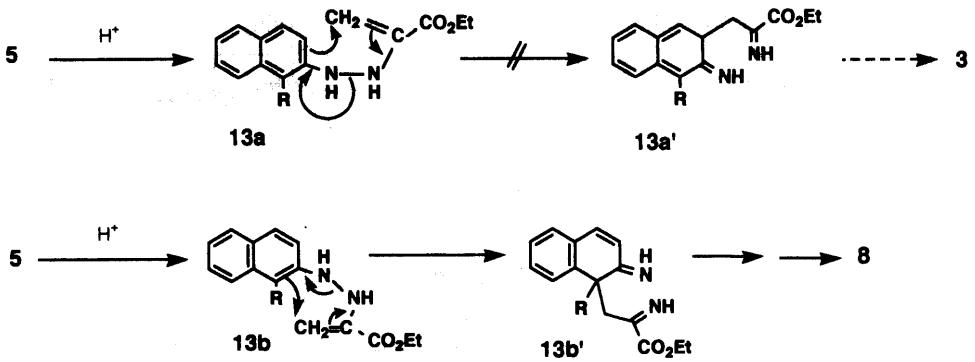


Chart 3

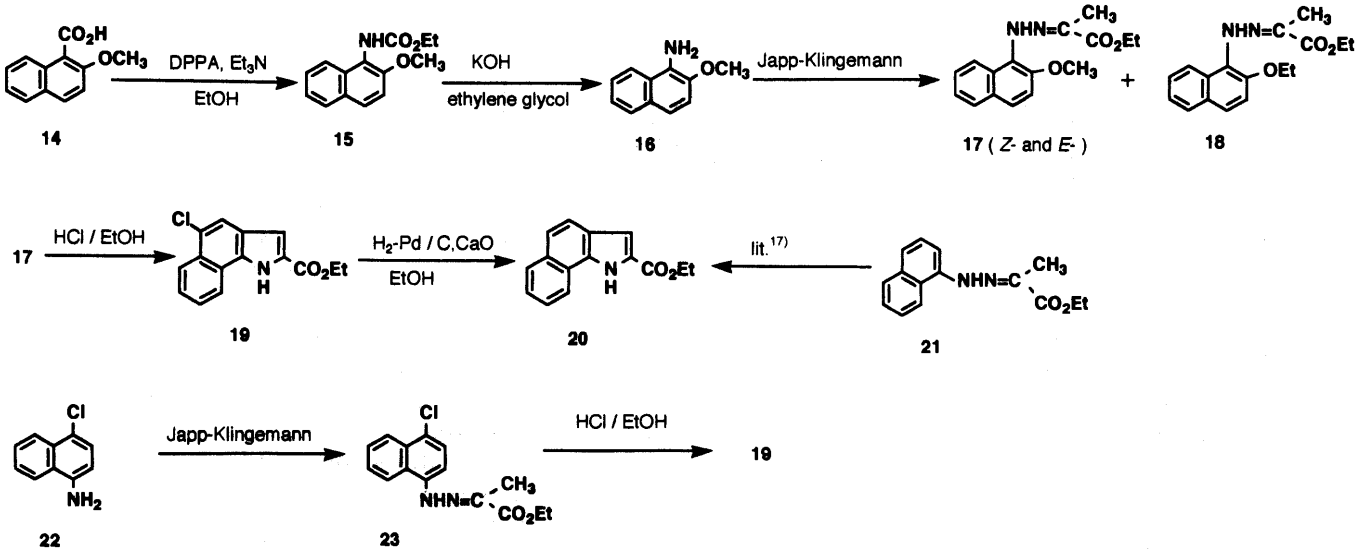


Chart 4

The difference of activation energy between the two routes would cause the exclusive formation of the benz[*e*]indole (**8**). This energy barrier might not be overcome by any 1-substituent group of the hydrazone (**5**).

In relation to the above result that Fischer indolization of 2-naphthylhydrazone toward the 3-position (*β*-position) did not take place at all, we were interested in the cyclization of 2-substituted 1-naphthylhydrazone toward the substituted 2-position (*β*-position). Thus the 2-methoxy-1-naphthylhydrazone (**17**) was selected as a substrate and prepared as shown in Chart 4. 2-Methoxy-1-naphthoic acid (**14**) was converted to 2-methoxy-1-napthylamine[15] (**16**) by modified Curtius reaction using diphenylphosphorazidate[16] (DPPA) followed by hydrolysis in a good yield; the Hofmann reaction of the corresponding amide of the carboxylic acid (**14**) gave a poorer yield. The amine (**16**) was converted to an *E*- and *Z*-mixture of the 2-methoxy-1-naphthylhydrazone (**17**) by Japp-Klingemann reaction with a small amount of the 2-ethoxy-1-napthylhydrazone (**18**) as a by-product.

The Fischer indolization of the 2-methoxy-1-naphthylhydrazone (**17**) with ethanolic hydrogen chloride proceeded smoothly at room temperature and gave an indolic product (**19**, 44%) as a main product, whereas the same reaction at 60 °C for a shorter reaction time gave a complex mixture of products, from which only 4.4% of **19** was isolated. This product (**19**) had the molecular formula $C_{15}H_{12}ClNO_2$ by elemental analysis and MS [*m*/*z* 273 (M^+) and 275 ($M^+ + 2$, 35% intensity of M^+)], suggesting that it should be chlorobenz[*g*]indole. The skeleton was confirmed by hydrogenolysis of **19** with H_2/Pd–C, leading to ethyl benz[*g*]indole-2-carboxylate (**20**), which was identical with an authentic sample[17] prepared from the 1-naphthylhydrazone (**21**). The position of the chlorine atom was determined as follows. The mechanism of the Fischer indolization of the hydrazone (**17**) can be visualized as in Chart 5 according to the previous paper.[18] Two positions (**19** or **24**) for chlorine can be envisaged, as shown in the intermediate d or f. However, the intermediate d leading to **19** should be thermodynamically more stable than the intermediate f leading to **24**. Thus, authentic 5-chlorobenz[*g*]indole (**19**) was prepared from the known 4-chloro-1-naphthylamine (**22**) as shown in Chart 4 and was found to be identical with the Fischer product. This result shows that in Fischer indolization of naphthylhydrazones the cyclization from the *α*- to the *β*-direction can occur even if the *β*-position is substituted, whereas *β* to *β*′-cyclization (namely, from C_2- to C_3-position) is impossible. This is due to the absence of a Kekulé formula favorable to *β*- to *β*′-cyclization in the naphthalene nucleus.

Hemetsberger Reaction Next we tried another cyclization reaction for preparation of benz[*f*]indole. Since Hemetsberger *et al.*[19] reported a new preparative method of indole-2-carboxylates by thermolysis of azido cinnamates

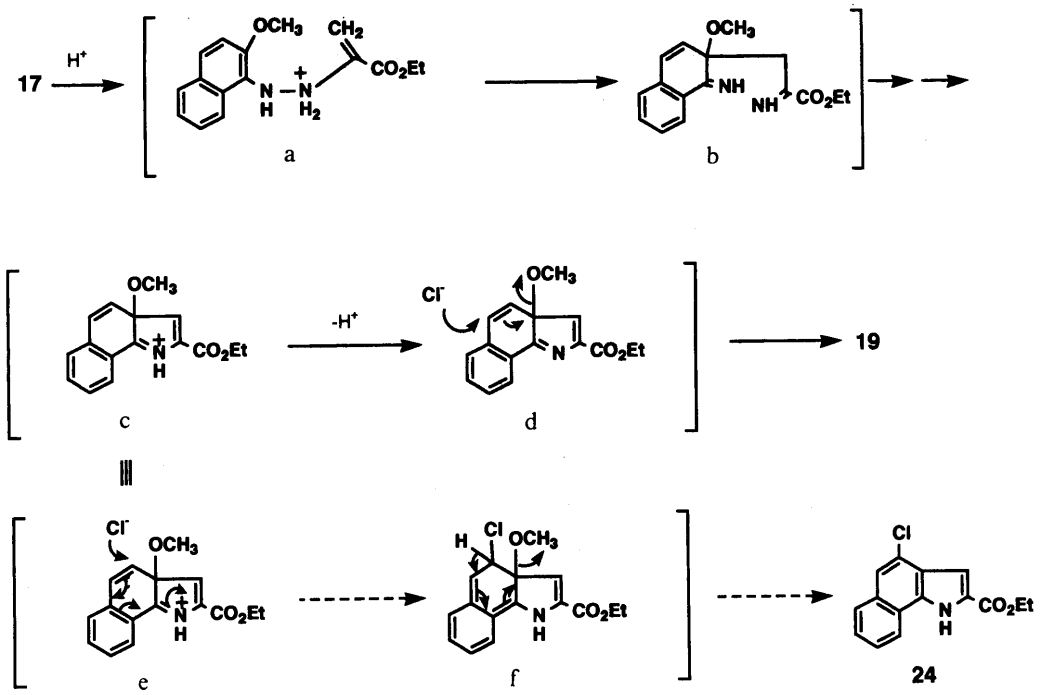


Chart 5

in 1970, this method has become widely used. It is characteristic[19] of this Hemetsberger reaction that *o*-substituted azido cinnamates can give the corresponding 4-substituted indoles without any abnormal reaction such as is found in Fischer indolization. Thus, we can expect that α-azido-β-(1-substituted-2-naphthyl)acrylate (**26**) would give 4-substituted benz[*f*]indoles (**30**), as the C_1-substituent (X in **26**) works as a blocking group against cyclization in that direction.

The azido esters (**26**) were prepared as usual[20] from 1-substituted 2-naphthalenecarbaldehyde (**25**) and ethyl azido acetate. This reaction sometimes gave the intermediate azido alcohol (**27**) as a by-product, especially in the case of preparation of the chloroazido esters (**26b**) under colder reaction conditions. This intermediate (**27**) thus formed was unexpectedly stable even in acidic media but was converted to the azido ester (**26b**) in quantitative yield ultimately by the use of thionyl chloride in pyridine. Thermolysis of the methoxy-azido ester (**26a**) in refluxing xylene, however, failed to give benz[*f*]indole (**30a**) but provided only an azirine derivative (**28a**), a usual intermediate to indole,[19] as an oily compound. Although having the same molecular formula $C_{16}H_{15}NO_3$ [MS; *m/z* 269 (M^+)] as the desired benz[*f*]indole (**30a**), this compound (**29a**) could be distinguished from the indole (**30a**) by observing the signal of the azirine ring proton as a singlet at δ 3.84 in the ^{1}H-NMR spectrum.

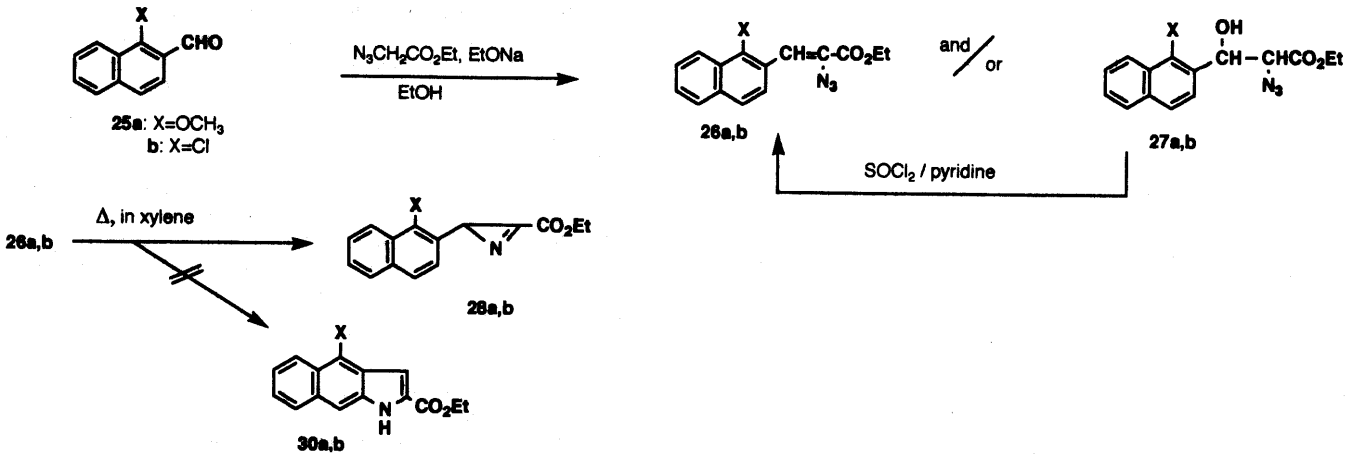
The azido compound (**26a**) was then heated at 230 °C in Dowtherm A[21] to give three compounds, **25a**, **20**, and **29a** in 1.1, 1.5, and 61% yields, respectively. The first product was found to be the aldehyde (**25a**), the decomposition product of **26a**. The second product (**20**) was suggested to be an indolic compound (Ehrlich reagent-positive), but has no methoxy signal in the ^{1}H-NMR spectrum. Thus, it was suggested to be the benz[*g*]indole (**20**), and was shown to be identical with an authentic sample (see Chart 4). The third product (**29a**) had the same molecular formula, $C_{16}H_{15}NO_3$, as the azirine (**28a**) and benz[*f*]indole (**30a**) in MS. However, the IR spectrum of this product showed an absorption at 2250 cm^{-1} attributed to a nitrile group. This suggested to us that it was the cyano ester (**29a**), such a compound is sometimes formed in the thermolysis of azido esters that do not cyclize to indole compounds.[22]

Next, the chloroazido ester (**26b**) was submitted to thermolysis as well as **26a**. At lower temperature (in refluxing xylene), the azirine (**28b**) was the sole product, similarly to the case of the methoxyazido ester (**26a**). At 240 °C in Dowtherm A three compounds (**25b**, **29b**, and **30b**) were formed in 4.5, 61, and 2.6% yields, respectively. The former two products were the aldehyde (**25b**) and the nitrile (**29b**), whose structures were determined in a similar way to those of the products from **26a**.

The third product had the molecular formula $C_{15}H_{12}ClNO_2$ by MS and showed a proton signal pattern characteristic of the benz[*f*]indole nucleus (δ 7.49, 1H, m, C_3-H and δ 7.78, 1H, s, C_9-H) in the ^{1}H-NMR spectrum. These data suggested that the third product was the desired 4-chlorobenz[*f*]indole (**30b**). To confirm the structure, the product (**30b**) was dechlorinated by using H_2/Pd–C to form the benz[*f*]indole[23] (**12**). Thus, this is the first example of the preparation of benz[*f*]indole by means of a cyclization reaction. Although the formation of a benz[*f*]indole (**30b**) was mechanistically valuable, the yield was so low that this Hemetsberger reaction is not practical for synthesis of benz[*f*]indoles.

Conclusion

A benz[*f*]indole was prepared by Hemetsberger reaction in low yield, but could not be obtained by Fischer indolization at all. This fact may show that cylization by Fischer indolization proceeds by a concerted mechanism, because Hemetsberger cyclization apparently proceeds through a one-centered mechanism (a kind of aromatic



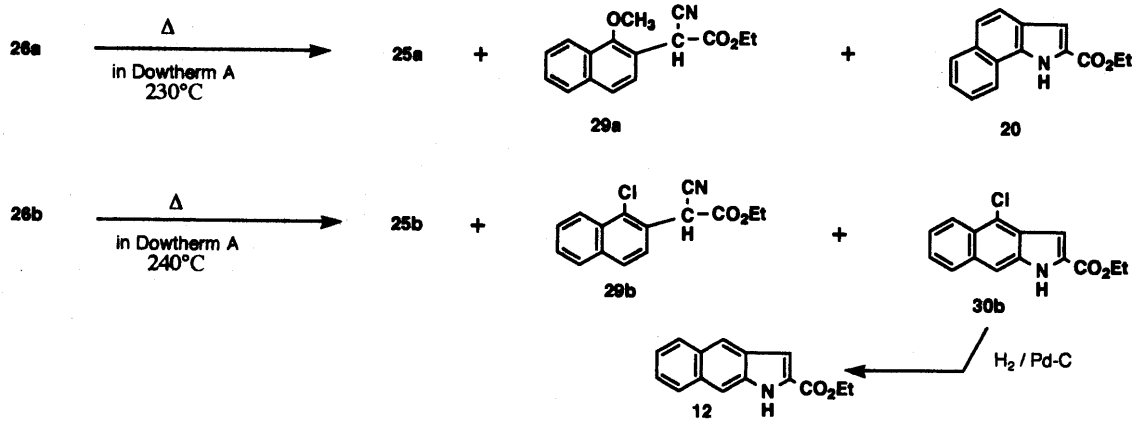


Chart 6

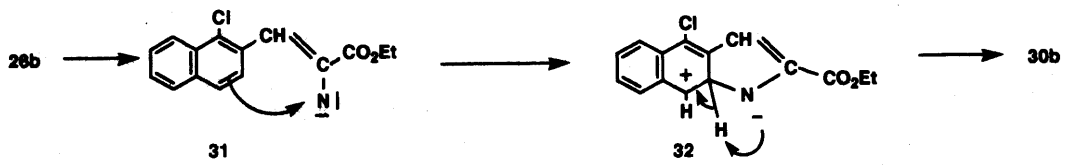


Chart 7

electrophilic substitution) by generation of nitrene. The mechanism involving intermediates **31** and **32** is illustrated in Chart 7. It is well known that electrophilic substitution reaction can occur at the β-position of the naphthalene nucleus. However, from the viewpoint of practical preparation, these cyclization methods are not suitable for preparing benz[*f*]indoles. It is clear that our previously reported method[5] for synthesis of benz[*f*]indoles starting from pyrrole derivatives is superior to the above cyclization methods based on naphthalene derivatives.

Experimental

All melting points were measured on a micro melting point hot stage (Yanagimoto) and are uncorrected. IR spectra were recorded in nujol mulls (unless otherwise stated) on a Shimadzu IR 400. ^{1}H-NMR spectra were recorded in $CDCl_3$ (unless otherwise stated) on Hitachi R-24B (60 MHz, unless otherwise stated) and JEOL GX-400 (400 MHz) spectrometers. In the ^{1}H-NMR spectra, chemical shifts are given in δ-values referred to internal tetramethylsilane, and the assignment of all NH and OH signals was confirmed by the disappearance of their signals after addition of D_2O. MS were measured by using the direct inlet system on a JEOL JMS-01-SG-2 spectrometer. For column chromatography, silica gel (Kiesel gel 60, 70—230 mesh, Merck), and for thin layer chromatography (TLC), Kiesel gel GF_{254}, Merck, were used. The abbreviations used are as follows: s, singlet; d, doublet; dd, double doublet; t, triplet; q, quartet; m, multiplet; dif, diffused; br, broad; arom, aromatic.

(*Z*)-Ethyl Pyruvate 2-(1-Methyl-2-naphthyl)hydrazone (5a) 1-Methyl-2-naphthylamine (**6a**) (6.179 g, 39.3 mmol) was diazotized with $NaNO_2$ (2.71 g, 39.3 mmol) and concentrated HCl (15 ml, 17.0 mmol) in water (45 ml) below 5 °C for 1 h. A 50 (w/w) % KOH solution (20 ml) and then the above solution containing diazonium salt were added to a solution of ethyl α-methylacetoacetate (5.62 g, 39.3 mmol) in EtOH (50 ml) under ice cooling at 0—5 °C, during which time, the reaction mixture was kept basic. The whole was stirred for 1 h, then poured into water (100 ml) and extracted with ether. The organic layer was washed with water, dried over $MgSO_4$, and evaporated to dryness. A solution of phosphoric acid (9 ml) in EtOH (120 ml) was added to this residue and the mixture was refluxed for 30 min, then poured into water (200 ml) and extracted with ether. The organic layer was washed with water, dried over $MgSO_4$ and evaporated to dryness. The residue was chromatographed over SiO_2 using benzene–AcOEt (10 : 1) to give the hydrazone (3.98 g, 38%). Recrystallization from hexane gave yellow needles, mp 108—109 °C. *Anal.* Calcd for $C_{16}H_{18}N_2O_2$: C, 71.09; H, 6.71; N, 10.36. Found: C, 71.03, H, 6.68, N, 10.38. IR ν_{max} cm^{-1}: 3250 (NH) and 1680 (CO). ^{1}H-NMR δ: 1.35 (3H, t, J=7 Hz, $CH_2\underline{CH}_3$), 2.20 (3H, s, arom-CH_3), 2.41 (3H, s, =C-CH_3), 4.28 (2H, q, J=7 Hz, $O\underline{CH}_2CH_3$), 7.10—8.10 (6H, m, arom H), 12.38 (1H, br s, NH). MS *m/z*: 155 (base peak), 270 (M^+, 98%).

Ethyl Pyruvate 2-(1-Chloro-2-naphthyl)hydrazone (5b) Concentrated

HCl (2.75 ml, 31 mmol) and isoamyl nitrite (1.44 ml, 10 mmol) were added to a solution of 1-chloro-2-naphthylamine (**6b**) (1.776 g, 10 mmol) in EtOH (20 ml) under ice-cooling. Ice (18 g), 50% KOH (8 ml, 60 mmol), and the above solution containing diazonium salt were successively added to a solution of ethyl α-methylacetoacetate (1.44 ml, 10 mmol) in EtOH (8 ml). The mixture was stirred at room temperature for 90 min, then poured into water and extracted with ether. The organic layer was washed with water, dried over $MgSO_4$ and evaporated to dryness. The residue was chromatographed over SiO_2 using benzene to give two fractions.

i) The (*Z*)-Hydrazone [(*Z*)-**5b**]: The product from the first eluate was recrystallized from hexane–benzene to give orange needles (0.566 g, 20%), mp 114—116 °C. *Anal.* Calcd for $C_{15}H_{15}ClN_2O_2$: C, 61.97, H, 5.20; N, 9.63. Found: C, 62.05; H, 5.26; N, 9.50. IR ν_{max} cm^{-1}: 3225 (NH), 1690 (CO). ^{1}H-NMR δ: 1.35 (3H, t, *J*=7 Hz, $CH_2C\underline{H}_3$), 2.19 (3H, s, =C–CH_3), 4.31 (2H, q, *J*=7 Hz, $OC\underline{H}_2CH_3$), 7.19—8.23 (6H, m, arom H), 12.50 (1H, br s, NH). MS *m/z*: 149 (100%), 290 (M^+, 72%), 292 (M^++2, 32%).

ii) The (*E*)-Hydrazone [(*E*)-**5b**]: The product from the second eluate was recrystallized from hexane–AcOEt to give wine-red plates (0.673 g, 23%), mp 132—134 °C. *Anal.* Calcd for $C_{15}H_{15}ClN_2O_2$: C, 61.97; H, 5.20; N, 9.63. Found: C, 61.58; H, 5.24; N, 9.41. IR ν_{max} cm^{-1}: 3310 (NH), 1670 (CO). ^{1}H-NMR δ: 1.38 (3H, t, *J*=8 Hz, $CH_2C\underline{H}_3$), 2.15 (3H, s, =C–CH_3), 4.32 (2H, q, *J*=8 Hz, $OC\underline{H}_2CH_3$), 7.18—8.16 (6H, m, arom H), 8.35 (1H, br s, NH). MS *m/z*: 290 (M^+), 292 (M^++2).

(*E*)-Ethyl Pyruvate 2-(1-Nitro-2-naphthyl)hydrazone (5c) 1-Nitro-2-naphthylamine (**6c**) (0.565 g, 3.0 mmol) solved in CH_3CN–H_2O (2 : 1, v/v) (40 ml) was diazotized with $NaNO_2$ (0.217 g, 3.0 mmol) and *p*-TsOH (2.381 g, 12.5 mmol) under ice-cooling. A 50% KOH solution (0.25 ml), ice (2.7 g) and the above diazonium salt solution was successively added to a solution of ethyl α-methyl acetoacetate (0.42 ml, 3.0 mmol) in CH_3CN–water (2 : 1) (2.3 ml), during which time the mixture was kept neutral or slightly basic. The reaction mixture was stirred for 90 min under ice-cooling, poured into water (200 ml) and extracted with ether. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated to dryness. The residue was chromatographed over SiO_2 using hexane–AcOEt (10 : 1) to give two fractions.

i) 1-Nitronaphthalene: The first fraction (0.045 g 8.7%) gave yellow needles, mp 55—56 °C. This product was identical with commercially available 1-nitronaphthalene.

ii) The 1-nitro-2-naphthylhydrazone (**5c**): The second fraction (0.253 g, 28%) was recrystallized from hexane–AcOEt (mp 150—153 °C) to give yellow needles. *Anal.* Calcd for $C_{15}H_{15}N_3O_4$: C, 59.80; H, 5.02; N, 13.95. Found: C, 59.46; H, 4.93; N, 13.71. IR ν_{max} cm^{-1}: 3340 (NH), 1690 (C=O). ^{1}H-NMR δ: 1.40 (3H, t, *J*=8 Hz, $CH_2C\underline{H}_3$), 2.21 (3H, s, =C–CH_3), 4.36 (2H, q, *J*=8 Hz, $OC\underline{H}_2CH_3$), 7.15—8.63 (6H, m, arom H), 10.85 (1H, br s, NH). MS *m/z*: 301 (M^+).

(*E*)-Ethyl Pyruvate (1-Chloro-2-naphthyl)hydrazone (5b) 1-Nitro-2-naphthylamine (**6c**) (0.565 g, 3 mmol) in EtOH (5 ml) was diazotized with concentrated HCl (1.1 ml, 12 mmol) and iso-amyl nitrite (0.42 ml, 3 mmol) for 1 h under ice-cooling. A 50% KOH solution (0.25 ml, 3.6 mmol), ice (3.7 g) and the above diazonium salt solution were successively added to a solution of ethyl α-methylacetoacetate (0.42 ml, 3 mmol) under ice-cooling for 90 min, during which time the mixture was kept slightly basic. The reaction mixture was poured into water and extracted with Et_2O. The organic layer was washed with water, dried over $MgSO_4$ and evaporated to dryness. The residue was chromatographed over SiO_2 using benzene to give fine brown plates (0.670 g, 77%). Recrystallization from hexane–AcOEt gave brown plates, mp 133—136 °C. This sample was identical with the hydrazone (**5b**) derived from 1-chloro-2-naphthylamine (**6b**). *Anal.* Calcd for $C_{15}H_{15}N_2O_2Cl$: C, 61.97; H, 5.20; N, 9.63. Found: C, 61.58; H, 5.24; N, 9.41. IR ν_{max} cm^{-1}: 3310 (NH), 1670 (C=O). ^{1}H-NMR δ: 1.38 (3H, t, *J*=8 Hz, $CH_2C\underline{H}_3$), 2.15 (3H, s, =C–CH_3), 4.32 (2H, q, *J*=8 Hz, $OC\underline{H}_2CH_3$), 7.18—8.16 (6H, m, arom H), 8.35 (1H, br s, NH). MS *m/z*: 290 (M^+), 292 (M^++2).

Fischer Indolization of Ethyl Pyruvate 2-(1-Methyl-2-naphthyl)hydrazone (5a). Ethyl 3*H*-Benz[*e*]indole-2-carboxylate (8) A solution of the hydrazone (**5a**) (299 mg, 1.1 mmol) in EtOH (50 ml) was saturated with HCl gas and refluxed for 1 h. The solvent was removed, then the residue was poured into water and extracted with ether. The ether solution was washed with brine, dried over $MgSO_4$, and evaporated to dryness. The residue was chromatographed over SiO_2 using benzene–AcOEt (10 : 1) to give a pale brown solid (39 mg, 15%), mp 163—167 °C (lit.[3] mp 164—165 °C).

This compound was identical with an authentic sample prepared from the 1-naphthyl hydrazone (**9**).

Fischer Indolization of Ethyl Pyruvate 2-(1-Chloro-2-naphthyl)hydrazone (5b). Ethyl 5-Chloro-3*H*-benz[*e*]indole-2-carboxylate (4) A solution of the hydrazone (**5b**) (0.407 g, 1.4 mmol) in anhydrous EtOH (30 ml) was saturated with HCl gas and was heated at 110 °C for 8.5 h in a sealed tube. The solvent was removed *in vacuo*, then the residue was poured into water and extracted with ether. The organic layer was washed with saturated Na_2CO_3 and brine, dried over $MgSO_4$, and evaporated to dryness. The residue was chromatographed over SiO_2 using benzene to give crystals (0.0944 g, 25%). Recrystallization from benzene gave colorless needles, mp 233—234 °C (lit.[4] mp 233—234 °C). *Anal.* Calcd for $C_{15}H_{12}ClNO_2$: C, 65.82; H, 4.42; N, 5.12. Found: C, 65.37; H, 4.32; N, 5.15. IR ν_{max} cm^{-1}: 3275 (NH), 1675 (C=O). ^{1}H-NMR (DMSO-d_6) δ: 1.40 (3H, t, *J*=7 Hz, $CH_2C\underline{H}_3$), 4.37 (2H, q, *J*=7 Hz, $OC\underline{H}_2CH_3$), 7.32—8.62 (6H, m, arom H), 12.29 (1H, br s, NH). This sample was identical with an authentic sample prepared from 1-methoxy-2-naphthylhydrazone (**2**).[4]

This reaction resulted only in recovery of the starting material (70%) under milder conditions (reflux for 1.5 h under atmospheric pressure).

Fischer Indolization of Ethyl Pyruvate 2-(1-Nitro-2-naphthyl)hydrazone (5c) The hydrazone (0.301 g, 1.0 mmol) in EtOH (30 ml) was saturated with HCl gas and stirred at 105 °C for 4 h in a sealed tube. The solvent was evaporated off *in vacuo*. The residue was dissolved in AcOEt and the solution was washed with saturated $NaHCO_3$ and brine, dried over $MgSO_4$, and evaporated to dryness. The residue was chromatographed over SiO_2 using hexane–AcOEt (10 : 1) to give four fractions. In order of elution, they yielded the following products.

i) 1-Chloro-2-azidonaphthalene (**10**): Recrystallization from hexane–benzene gave pale brown needles (158 mg, 8%), mp 114—116 °C. *Anal.* Calcd for $C_{10}H_6ClN_3$: C, 58.98; H, 2.97; N, 20.64. Found: C, 59.13; H, 2.86; N, 20.23. IR ν_{max} cm^{-1}: 2115 (N_3). ^{1}H-NMR δ: 7.10—8.30 (6H, m, arom H). MS *m/z*: 140 (100%), 203 (M^+, 29%), 205 (M^++2, 11%).

ii) (*Z*)-Ethyl 2-(1-Chloro-2-naphthyl)hydrazone (**5b**): Yield was 157 mg (5.4%). Recrystallization from hexane–benzene gave pale red needles, mp 113—116 °C. IR ν_{max} cm^{-1}: 3225 (NH), 1690 (C=O). ^{1}H-NMR δ: 1.35 (3H, t, *J*=7 Hz, $CH_2C\underline{H}_3$), 2.19 (3H, s, =CCH_3), 4.31 (2H, q, *J*=7 Hz, $OC\underline{H}_2CH_3$), 7.19—8.23 (6H, m, arom H), 12.50 (1H, br s, NH). MS *m/z*: 149 (100%), 290 (M^+, 92%), 292 (M^++2, 32%). The sample was identical with an authentic sample prepared from 1-chloro-2-naphthylamine (**6b**).

iii) 1-Nitronaphthylhydrazone [A mixture of (*E*)- and (*Z*)-**5c**]: Yellow solid (0.047 g, 22%). This sample was identified as the starting material.

iv) 1,2-Dinitrosonaphthalene (**11**): Yield was 0.0165 g (8.9%). Recrystallization from hexane–benzene gave pale red plates, mp 128—129 °C. *Anal.* Calcd for $C_{10}H_6N_2O_2$: C, 64.52; H, 3.25; N, 15.05. Found: C, 64.56; H, 3.20: N, 15.02. IR: ν_{max} cm^{-1}: no characteristic absorption. ^{1}H-NMR δ: 6.95—8.70 (6H, m, arom H). MS *m/z*: 126 (100%), 186 (M^+, 78%).

Ethyl Methoxy-1-naphthalene Carbamate (15) DPPA[16] (8.08 ml, 37.5 mmol) and Et_3N (6.97 ml, 50 mmol) were added successively to a solution of 2-methoxy-1-napththoic acid (**14**) (5.056 g, 25 mmol) in dioxane (25 ml) under ice-cooling. The mixture was refluxed for 30 min and allowed to stand at room temperature for 1 h. Anhydrous EtOH (10 ml) was added to this mixture and the whole was refluxed for 1.5 h. The solvent was removed *in vacuo*. The residue was dissolved in ether and the resulting precipitates were filtered off. The organic layer was washed with 5% citric acid, water, 5% $NaHCO_3$ and brine, dried over $MgSO_4$, and evaporated to dryness. The residue was chromatographed over SiO_2 using hexane–AcOEt (5 : 1) to give colorless prisms (4.50 g, 73.4%), mp 116—117 °C, which were recrystallized from benzene–hexane. *Anal.* Calcd for $C_{14}H_{15}NO_3$: C, 68.56; H, 6.16; N, 5.71. Found: C, 68.51; H, 6.11; N, 5.80. IR ν_{max} cm^{-1}: 3220 (NH), 1690 (C=O). ^{1}H-NMR δ: 1.28 (3H, t, *J*=7 Hz, $CH_2C\underline{H}_3$), 3.91 (3H, s, OCH_3), 4.23 (2H, q, *J*=7 Hz, $OC\underline{H}_2CH_3$), 6.37 (1H, br s, NH), 7.12—8.05 (6H, m, arom H). MS *m/z*: 245 (M^+, base peak).

2-Methoxy-1-naphthylamine (16) A mixture of the carbamate (**15**) (3.00 g, 12.2 mmol) and 85% KOH (46 g, 67 mmol) in ethylene glycol (30 ml) was stirred at 150 °C for 1 h under an Ar atmosphere. The reaction mixture was poured into water and extracted with ether. The organic layer was washed with 5% $NaHCO_3$ and water, dried over $MgSO_4$, and evaporated to dryness. The residue was chromatographed over SiO_2 using hexane–AcOEt (10 : 1) to give pale orange needles (2.018 g, 95%), mp 53—54 °C (lit.[15] mp 53—54 °C). IR ν_{max} cm^{-1}: 3450 and 3360 (NH). ^{1}H-NMR δ: 3.91 (3H, s, OCH_3), 4.05 (2H, br s, NH_2), 7.05—7.95 (6H, m, arom H).

Ethyl Pyruvate 2-(2-Methoxy-1-naphthyl)hydrazone (17) 2-Methoxy-1-naphthylamine (**16**) (2.018 g, 11.6 mmol) was diazotized with concentrated HCl (4.12 ml, 46.6 mmol), and $NaNO_2$ (0.816 g, 11.6 mmol) in water (10 ml) and EtOH (15 ml) under ice-cooling. A 50% KOH solution (5 ml) and

the above diazonium salt solution were added to a solution of ethyl α-methylacetoacetate (1.68 ml, 11.6 mmol) in EtOH (20 ml) under ice-cooling. The reaction mixture was stirred for 10 min at 5 °C, poured into water and extracted with ether. The organic layer was washed with 10% HCl, saturated $NaHCO_3$, and brine, dried over $MgSO_4$, and evaporated to dryness. The oily residue was chromatographed over SiO_2 using hexane–AcOEt (10 : 1) to give three fractions.

i) The (*Z*)-Hydrazone [(*Z*)-**17**]: The first eluate gave yellow needles (0.138 g, 4%), mp 104—106 °C, which were recrystallized from benzene–hexane. *Anal.* Calcd for $C_{16}H_{18}N_2O_3$: C, 67.12; H, 6.34; N, 9.78. Found: C, 67.24; H, 6.34; N, 9.62. IR ν_{max} cm^{-1}: 3180 (NH) and 1665 (C=O). ^{1}H-NMR δ: 1.41 (3H, t, *J*=7 Hz, CH_2CH_3), 2.23 (3H, s, =C–CH_3), 3.99 (3H, s, OCH_3), 4.35 (2H, q, *J*=7 Hz, OCH_2CH_3), 7.14—7.88 (5H, m, arom H), 8.81 (1H, dif d, *J*=9 Hz, C_8-H), 12.22 (1H, br s, NH). MS *m/z*: 172 (100%), 286 (M^+, 86%).

ii) Ethyl Pyruvate 2-(2-ethoxy-1-naphthyl)hydrazone (**18**): The second eluate gave yellow needles (0.0542 g, 1.5%), mp 80—81 °C, which were recrystallized from benzene–hexane. *Anal.* Calcd for $C_{17}H_{20}N_2O_3$: C, 67.98; H, 6.71; N, 9.33. Found: C, 67.72; H, 6.65; N, 9.34. IR ν_{max} cm^{-1}: 3320 (NH) and 1678 (CO). ^{1}H-NMR δ: 1.37 and 1.45 (each 3H, t, *J*=7 Hz, CH_2CH_3), 2.19 (3H, s, =C–CH_3), 4.18 and 4.30 (each 2H, q, *J*=7 Hz, OCH_2CH_3), 7.08—7.82 (5H, m, arom H), 8.42 (1H, br s, NH), 8.92 (1H, dif d, *J*=9 Hz, C_8-H). MS *m/z*: 158 (100%), 300 (M^+, 80%).

iii) The (*E*)-Hydrazone [(*E*)-**17**]: The third fraction gave yellow needles (1.463 g, 44%), mp 68—69 °C, which were recrystallized from benzne–hexane. *Anal.* Calcd for $C_{16}H_{18}N_2O_3$: C, 67.12; H, 6.34; N, 9.78. Found: C, 67.18; H, 6.19; N, 9.70. IR ν_{max} cm^{-1}: 3340 (NH), 1695 (C=O). ^{1}H-NMR δ: 1.37 (3H, t, *J*=7 Hz, CH_2CH_3), 2.19 (3H, s, =C–CH_3), 3.93 (3H, s, OCH_3), 4.29 (2H, q, *J*=7 Hz, OCH_2CH_3), 7.08—7.87 (5H, m, arom H), 8.34 (1H, br s, NH), 8.91 (1H, dif d, *J*=9 Hz, C_8-H). MS *m/z*: 286 (M^+, 100%).

Fischer Indolization of 2-Methoxy-1-naphthylhydrazone (17). Ethyl 5-Chloro-1*H*-benz[*g*]indole-2-carboxylate (19) A solution of the 2-methoxy-1-naphthylhydrazone (**17**) (0.821 g, 1.9 mmol) in anhydrous EtOH (40 ml) was saturated with HCl gas under ice-cooling. The reaction mixture was stirred for 1.5 h at room temperature. After the removal of EtOH and HCl gas *in vacuo*, the residue was poured into water and extracted with ether. The organic layer was washed with saturated $NaHCO_3$ and brine, dried over $MgSO_4$ and evaporated to dryness. The residue was recrystallized from benzene to give a yellow solid (0.1995 g). The mother liquor was chromatographed over SiO_2 using hexane–AcOEt (10 : 1) to give the same compound as a colorless solid (0.1482 g): totally 0.348 g (44%). Recrystallization from benzene–AcOEt gave colorless prisms, mp 208—211 °C. *Anal.* Calcd for $C_{15}H_{12}ClNO_2$: C, 65.82; H, 4.42; N, 5.12. Found: C, 65.85; H, 4.36; N, 5.13. IR ν_{max} cm^{-1}: 3310 (NH) and 1680 (C=O). ^{1}H-NMR (DMSO-d_6) δ: 1.48 (3H, t, *J*=7 Hz, CH_2CH_3), 4.48 (2H, q, *J*=7 Hz, OCH_2CH_3), 7.34 (1H, dif d, *J*=1 Hz, C_3-H), 7.64—7.91 (2H, m, $C_{7,8}$-H), 7.98 (1H, s, C_4-H), 8.28—8.42 (1H, m, C_6-H), 8.76—9.07 (1H, m, C_9-H), 12.88 (1H, br s, NH). MS *m/z*: 227 (100%), 273 (M^+, 54%), 275 (M^++2, 19%).

Dechlorination of the 5-Chlorobenz[*g*]indole (19). Ethyl 1*H*-Benz[*g*]-indole-2-carboxylate (20) A mixture of the 5-chlorobenz[*g*]indole (**19**) (62 mg, 0.227 mmol), 10% Pd–C (68 mg) and CaO (85 mg, 1.52 mmol) in EtOH (30 ml) was treated with H_2 gas for 4 h at atmospheric pressure and room temperature. The catalyst was filtered off and washed with AcOEt. The filtrate was evaporated to dryness. The residue was chromatographed over SiO_2 using ether–hexane (1 : 1) to give colorless needles (54 mg, 100%), mp 176—177.5 °C (lit.[17] mp 170 °C), which were recrystallized from CH_2Cl_2–hexane. IR ν_{max} cm^{-1}: 3300 (NH), 1680 (C=O). ^{1}H-NMR δ: 1.45 (3H, t, *J*=7 Hz, CH_2CH_3), 4.48 (2H, q, *J*=7 Hz, OCH_2CH_3), 7.18—8.45 (7H, m, arom H), 10.08 (1H, br s, NH). This sample was identified as **20** by comparison with an authentic sample[17] (**20**) prepared from 1-naphthylhydrazone (**21**).

Ethyl Pyruvate 2-(4-Chloro-1-naphthyl)hydrazone (23) 4-Chloro-1-naphthylamine (**22**) (0.807 g, 4.5 mmol) was diazotized with concentrated HCl (1.56 ml, 17.1 mmol), water (10 ml), $NaNO_2$ (0.381 g, 4.5 mmol), and EtOH (5 ml) under ice cooling. Aqueous 50% KOH (2 ml) and then the above diazonium salt solution were added to a solution of ethyl α-methylacetoacetate (0.66 ml, 4.7 mmol) in EtOH (10 ml) over 10 min, during which time the mixture was kept basic to neutral under ice-cooling. The reaction mixture was poured into water and extracted with ether. The organic layer was washed with 10% HCl, saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and evaporated to dryness. The residue was chromatographed over SiO_2 using benzene–hexane (1 : 1) to give brown prisms (0.832 g, 63%) 132—134.5 °C, which were recrystallized from hexane–AcOEt. *Anal.* Calcd for $C_{15}H_{15}ClN_2O_2$: C, 61.97; H, 5.20; N, 9.63. Found: C, 62.12; H, 5.16; N, 9.26. ^{1}H-NMR δ: 1.39 (3H, t, *J*=7 Hz, CH_2CH_3), 2.22 (3H, s, =C–CH_3), 4.35 (2H, q, *J*=7 Hz, OCH_2CH_3), 7.20—8.45 (7H, m, arom H and NH). MS *m/z*: 176 (100%), 290 (M^+, 83%), 292 (M^++2, 28%).

Ethyl 5-Chloro-1*H*-benz[*g*]indole-2-carboxylate (19) A solution of the hydrazone (**23**) (0.251 g, 0.86 mmol) in anhydrous EtOH (30 ml) was saturated with HCl gas under ice-cooling. The mixture was stirred for 5.5 h at room temperature. After removal of the solvent *in vacuo*, the residue was poured into water and extracted with ether. The organic layer was washed with saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and evaporated to dryness. The residue was chromatographed over SiO_2 using hexane–AcOEt (10 : 1) to give colorless needles (0.209 g, 88%), mp 210.5—212 °C, which were recrystallized from benzene–AcOEt. *Anal.* Calcd for $C_{15}H_{12}ClNO_2$: C, 65.82; H, 4.42; N, 5.12. Found: C, 65.90; H, 4.35; N, 5.16. IR ν_{max} cm^{-1}: 3300 (NH), 1680 (C=O). ^{1}H-NMR (DMSO-d_6) δ: 1.39 (3H, t, *J*=7 Hz, CH_2CH_3), 4.39 (2H, q, *J*=7 Hz, OCH_2CH_3), 7.26 (1H, d, *J*=2 Hz, C_3-H), 7.48—7.88 (2H, m, C_7 and C_8-H), 7.90 (1H, s, C_4-H), 8.10—8.38 (1H, m, C_6-H), 8.72—8.98 (1H, m, C_9-H), 12.78 (1H, br s, NH). MS *m/z*: 227 (100%), 273 (M^+, 57%), 275 (M^++2, 23%).

This compound was identical with the indole (**19**) prepared from the Fischer indolization of ethyl 2-(2-methoxy-1-naphthyl)hydrazone (**17**).

Ethyl 1-Azido-2-(1-methoxy-2-naphthyl)acrylate (26a) A solution of 1-methoxy-2-naphthalenecarbaldehyde[24] (**25a**) (1.862 g, 10 mmol) and ethyl azidoacetate (5.165 g, 40 mmol) in anhydrous EtOH (40 ml) was added dropwise to an ethanolic solution of sodium ethoxide prepared from Na (920 mg, 0.04 mol) in EtOH (50 ml) at −15 °C. The reaction mixture was stirred for 7 h at the same temperature, poured into ice-water, saturated with NH_4Cl, and extracted with AcOEt. The organic layer was washed with saturated NaCl, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue (3.45 g) was chromatographed over SiO_2 using benzene–hexane to give three compounds, listed below in the order of elution. The first compound (47 mg, 2.5%) was identified as the starting aldehyde (**25a**).

Ethyl 1-Azido-2-(1-methoxy-2-naphthyl)acrylate (**26a**): The second compound (1.656 g, 56%) was recrystallized from AcOEt–hexane to give the target compound (**26a**) as colorless needles, mp 78.5—80.5 °C. *Anal.* Calcd for $C_{16}H_{15}N_3O_3$: C, 64.64; H, 5.09; N, 14.13. Found: C; 64.88; H, 5.07; N, 14.12. IR ν_{max} cm^{-1}: 2100 (N_3), 1695 (C=O). ^{1}H-NMR δ: 1.40 (3H, t, *J*=7 Hz, CH_2CH_3), 3.93 (3H, s, OMe), 4.37 (2H, q, *J*=7 Hz, OCH_2CH_3), 7.35—8.40 (7H, m, arom H and –CH=C). MS *m/z*: 156 (100%), 297 (M^+, 20%).

Ethyl 1-Azido-2-hydroxy-2-(1-methoxy-2-naphthyl)propionate (**27a**): The third compound (0.700 g, 22%) was recrystallized from AcOEt–hexane to give colorless needles, mp 123—125 °C. *Anal.* Calcd for $C_{16}H_{17}N_3O_4$: C, 60.94; H, 5.43; N, 13.33. Found: C, 61.12; H, 5.43; N, 13.36. IR ν_{max} cm^{-1}: 3450 (OH), 2100 (N_3), 1730 (C=O). ^{1}H-NMR δ: 1.28 (3H, t, *J*=7 Hz, CH_2CH_3), 3.17—3.50 (1H, m, OH), 4.02 (3H, s, OMe), 4.28 (2H, q, *J*=7 Hz, OCH_2CH_3), 4.25—4.50 (1H, m, –CHN_3), 5.40—5.85 [1H, m, –CH(OH)], 7.35—8.30 (6H, m, arom H). MS *m/z*: 315 (M^+, trace), 187 (100%).

Ethyl 2-(1-Methoxy-2-naphthyl)-2*H*-azirine-3-carboxylate (28a) A solution of ethyl 1-azido-2-(1-methoxy-2-naphthyl)acrylate (**26a**) (147 mg) in *p*-xylene (15 ml) was heated at 105—120 °C for 1.5 h. The reaction mixture was evaporated *in vacuo* to give a yellow oil. Column chromatography over SiO_2 gave the title compound (**28a**) as a pale yellow oil. (131 mg, 99%). IR ν_{max} cm^{-1}: 1750 (C=O). ^{1}H-NMR (400 MHz) δ: 1.43 (3H, t, *J*=7 Hz, CH_2CH_3), 4.51 (2H, dq, *J*=7 and 1.6 Hz, OCH_2CH_3), 3.84 (1H, s, azirine-H), 4.00 (3H, s, OMe), 7.03 (1H, d, *J*=8 Hz, arom H), 7.56 (1H, d, *J*=8.6 Hz, arom H), 7.81 (1H, d, *J*=7.6 Hz, arom H), 8.10 (1H, d, *J*=8 Hz, arom H), 7.45—7.53 (2H, m, C_6- and C_7-H). MS *m/z*: 269 (M^+, 17%), 156 (100%). High resolution MS *m/z*: Calcd for $C_{16}H_{15}NO_3$ (M^+): 269.1048. Found: 269.1057.

Thermolysis of the Azidoacrylate (26a) in Dowtherm A A solution of the azidoacrylate (**26a**) (149 mg, 0.5 mmol) in Dowtherm A[21] (10 ml) was heated at 230 °C for 10 min under Ar. The solvent was removed *in vacuo* and the residue was purified by flash column chromatography over SiO_2 (Merck, Art. 9385) using AcOEt–hexane to give three fractions.

The first fraction gave 1-methoxy-2-naphthalenecarbaldehyde (**25a**) (2.0 mg, 1.1%) and the second fraction gave ethyl 1*H*-benz[*g*]indole-2-carboxylate (**20**) (2.0 mg, 1.5%), both of which were identical with authentic samples, (**25a** and **20**, respectively).

Ethyl α-Cyano-α-(1-methoxy-2-naphthyl)acetate (**29a**): The third fraction provided a brown oil (**29a**) (82 mg, 61%). IR ν_{max}^{neat} cm^{-1}: 2250 (CN), 1740 (C=O). ^{1}H-NMR δ: 1.26 (3H, t, *J*=7 Hz, CH_2CH_3), 3.99

(3H, s, OMe), 4.25 (2H, q, J=7 Hz, $OC\underline{H}_2CH_3$), 5.31 [1H, s, ArCH(CN)], 7.30—8.20 (6H, m, arom H). MS m/z: 269 (M^+, 94%), 196 (100%). High resolution MS m/z: Calcd for $C_{16}H_{15}NO_3$ (M^+): 269.1048. Found: 269.1066.

Ethyl α-Azido-β-hydroxy-β-(1-chloro-2-naphthyl)propionate (27b) A solution of 1-chloro-2-naphthalenealdehyde[25] (**25b**) (1.907 g, 10 mmol) and ethyl azidoacetate (5.159 g, 40 mmol) in a mixture of tetrahydrofuran (THF) (20 ml) and EtOH (30 ml) was added dropwise to a solution of NaOEt in EtOH [prepared from Na (928 mg, 40 mmol) and EtOH (40 ml)] at −25 °C during 40 min under an Ar atmosphere. The reaction mixture was stirred at 0—5 °C for 1 h, then poured into ice-water containing NH_4Cl, and extracted with AcOEt. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue (4.70 g) was chromatographed over SiO_2 using hexane–AcOEt (gradient from 20 : 1 to 5 : 1) to give **27b** as colorless prisms (1.567 g, 49%; a mixture of diastereoisomers). The product was recrystallized from hexane–AcOEt and melted in two stages at 81—85 and 90—96 °C. *Anal.* Calcd for $C_{15}H_{14}ClN_3O_3$: C, 56.35; H, 4.41; N, 13.14. Found: C, 56.46; H, 4.36; N, 13.18. IR ν_{max} cm^{-1}: 3540 and 3400 (OH), 2120 (N_3), 1740, 1720 (C=O). ^{1}H-NMR δ: 1.05 and 1.23 (totally 3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 3.52 (1H, br d like, OH), 3.8—4.5 [3H, m, $OC\underline{H}_2CH_3$ and $CH(OH)C\underline{H}N_3$], 5.75 and 5.93 (totally 1H, br s, ArCH), 7.3—8.45 (6H, m, arom H). MS m/z: 319 (M^+, 0.3%), 321 (M^++2, 0.14%).

This reaction sometimes produces ethyl α-azido-β-(1-chloro-2-naphthyl)acrylate (**26b**) directly, or gives a mixture of **26b** and **27b**.

Ethyl α-Azido-β-(1-chloro-2-naphthyl)acrylate (26b) from Ethyl α-Azido-α-hydroxy-β-(1-chloro-2-naphthyl)propionate (27b) Thionyl chloride (0.022 ml, 0.28 mmol) was added to a solution of the alcohol (**27b**) (31.9 mg, 0.1 mmol) in pyridine (0.7 ml) under ice-cooling. The whole mixture was stirred at room temperature for 2 h. The reaction mixture was poured into ice-water, and extracted with AcOEt.The organic layer was washed with 5% aqueous $CuSO_4$ and brine, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue was purified by column chromatography over SiO_2 using hexane–AcOEt (5 : 1) to afford a yellow solid (29 mg, 95%), mp 100—103 °C, which was recrystallized from AcOEt–hexane to give pale yellow needles, mp 105—107 °C (dec.). *Anal.* Calcd for $C_{15}H_{12}ClN_3O_2$: C, 59.71; H, 4.01; N, 13.93. Found: C, 59.53; H, 3.94; N, 13.83. IR ν_{max} cm^{-1}: 2130 (N_3), 1705 (C=O). ^{1}H-NMR(DMSO-d_6) δ: 1.34 (2H, t, J=7 Hz, $CH_2C\underline{H}_3$), 4.35 (2H, q, J=7 Hz, $OC\underline{H}_2CH_3$), 7.31 (1H, s, ArCH=), 7.40—8.40 (6H, m, arom H). MS m/z: 200 (100%), 301 (M^+, 6%), 303 (M^++2, 2%).

Ethyl 2-(1-Chloro-2-naphthyl)-2*H*-azirine-3-carboxylate (28b) A solution of ethyl α-azido-β-(1-chloro-2-naphthyl)acrylate (**22b**) (51 mg, 0.17 mmol) in *p*-xylene (5 ml) was heated at 140 °C for 5 min in an Ar atmosphere. Removal of the solvent *in vacuo* gave a pale brown oil (**28b**) (46 mg, 98%) as a single compound on thin layer chromatography (TLC). IR ν_{max}^{neat} cm^{-1}: 1720, 1750 (C=O). ^{1}H-NMR (400 MHz) δ: 1.44 (3H, t, J=7.2 Hz, $CH_2C\underline{H}_3$), 4.12 (1H, s, azirine-H), 4.51 (2H, q, J=7.2 Hz, $C\underline{H}_2CH_3$), 6.90 (1H, d, J=8.6 Hz, C_5-H), 7.71 and 7.82 (each 1H, d, J=7.5 Hz, C_3 and C_4-H), 8.34 (1H, d, J=8.6 Hz, C_8-H). MS m/z: 201 (100%), 273 (M^+, 16%), and 275 (M^++2, 7%). High resolution MS m/z: Calcd for $C_{15}H_{12}ClNO_2$ (M^+): 273.0554. Found: 273.0521.

Thermolysis of the Azidoacrylate (26b) in Dowtherm A A solution of the azidoacrylate (**26b**) (106 mg, 0.35 mmol) in Dowtherm A (10 ml) was heated at 240 °C for 10 min under Ar. The solvent was removed *in vacuo* and the residue was purified by flash column chromatography over SiO_2 using benzene–hexane to give three fractions.

The first fraction gave 1-chloro-2-naphthalenecarbaldehyde (**25b**) (3.0 mg, 4.5%), which was identical with an authentic sample (**25b**).

Ethyl α-Cyano-α-(1-chloro-2-naphthyl)acetate (**29b**): The second fraction provided colorless prisms (67 mg, 61%), which were recrystallized from ether, mp 72—76 °C. *Anal.* Calcd for $C_{15}H_{12}ClNO_2$: C, 65.82; H, 4.42; N, 5.12. Found: C, 65.76; H, 4.42; N, 5.15. IR ν_{max} cm^{-1}: 2250 (CN, weak), 1740 (CO). ^{1}H-NMR (400 MHz) δ: 1.30 (3H, t, J=7.2 Hz, $CH_2C\underline{H}_3$), 4.30 (2H, m, $OC\underline{H}_2CH_3$), 5.51 (1H, s, $Ar\underline{C}HCN$), 7.58—7.68 (3H, m, arom H), 7.86 (1H, d, J=7.9 Hz, arom H), 7.88 (1H, d, J=7.3 Hz, arom H), 8.32 (1H, d, J=8.5 Hz, C_8-H). MS m/z: 200 (100%), 273 (M^+, 42%), 275 (M^++2, 14%).

Ethyl 4-Chloro-1*H*-benz[*f*]indole-2-carboxylate (**30b**): The third fraction gave yellow needles (4.1 mg, 2.6%), which were recrystallized from AcOEt–hexane, mp 163.5—166.5 °C. *Anal.* Calcd for $C_{15}H_{12}ClNO_2$: C, 65.82; H, 4.42; N, 5.12. Found: C, 65.76; H, 4.42; N, 5.19. IR ν_{max} cm^{-1}: 3320 (NH), 1700 (CO). ^{1}H-NMR (400 MHz) δ: 1.46 (3H, t, J=7.2 Hz, $CH_2C\underline{H}_3$), 4.47 (2H, q, J=7.2 Hz, $OC\underline{H}_2CH_3$), 7.41—7.48 (2H, m, C_6 and C_7-H), 7.49 (1H, m, C_3-H), 7.78 (1H, s, C_9-H), 7.88 and 8.34 (each 1H, dd, J=9.2 and 1.6 Hz, C_5 and C_8-H), 8.90 (1H, br s, NH). MS m/z: 227 (100%), 273 (M^+, 60%), 275 (M^++2, 21%).

Hydrogenolysis of Ethyl 4-Chloro-1*H*-benz[*f*]indole-2-carboxylate (30b) to Ethyl 1*H*-Benz[*f*]indole-2-carboxylate (12) A mixture of the chloroindole (**30b**) (25.1 mg, 0.09 mmol), 10% Pd–C (15 mg), CaO (27 mg, 0.48 mmol), and EtOH (1 ml) was stirred at room temperature and atmospheric pressure under H_2 gas for 24 h. The reaction mixture was filtered and the filtrate was evaporated to dryness *in vacuo*. This procedure was repeated three times in order to complete the reaction. The residue (12 mg) was chromatographed with SiO_2 using cyclohexane–AcOEt (10 : 1) to give two fractions.

Ethyl 1*H*-Benz[*f*]indole-2-carboxylate[23] (**12**): The first fraction provided a yellow solid (3.4 mg, 16%), mp 178—183 °C. ^{1}H-NMR (400 MHz) δ: 1.45 (3H, t, J=7.1 Hz, $CH_2C\underline{H}_3$), 4.45 (2H, q, J=7.1 Hz, $OC\underline{H}_2CH_3$), 7.38 (1H, d, J=2.0 Hz, C_3-H), 7.33 and 7.40 (each 1H, dt, J=7.0 and 2.0 Hz, C_6 and C_7-H), 7.84 (1H, s, C_9-H), 7.88 and 7.93 (each 1H, d, J=7.4 Hz, C_5 and C_8-H), 8.24 (1H, s, C_4-H), 8.76 (1H, br s, NH). MS m/z: 193 (100%), 239 (M^+, 78%). High-resolution MS m/z: Calcd for $C_{15}H_{13}N_2O$ (M^+): 239.0940. Found: 239.0963.

The second fraction gave a yellow solid (4.2 mg, 17%), which was identical with the starting material (**30b**).

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Study on the Bile Salt, Sodium Scymnol Sulfate, from *Rhizoprionodon acutus*. II. The Structures of Scymnol, Anhydroscymnol and Sodium Scymnol Sulfate[1)]

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The crystal structures of anhydroscymnol (I) and scymnol (II), which were prepared from sodium scymnol sulfate (III) isolated from the bile of *Rhizoprionodon acutus*, have been determined by means of X-ray diffraction analyses. The crystals of I are orthorhombic, space group $P2_12_12_1$ with $Z=4$; unit-cell dimensions: $a=13.562(2)$, $b=21.636(2)$, $c=8.735(2)$ Å; II orthorhombic, space group $P2_12_12$, with $Z=4$; unit-cell dimensions $a=18.553(2)$, $b=19.887(2)$, $c=7.986(2)$ Å. Both structures, (24*R*,25*S*)-(+)-24,26-epoxy-5β-cholestane-3α,7α,12α,27-tetrol (I) and (24*R*)-(+)-5β-cholestane-3α,7α,12α,24,26,27-hexol (II), were solved from diffractometric data by direct methods and refined by least-squares calculations to $R=0.073$ (I) and $R=0.062$ (II) (2044 (I) and 2250 (II) observed independent significant reflections ($I>3\sigma(I)$), respectively. All the hydroxyl groups of both compounds are involved in a hydrogen-bonding network. The structure of III was determined to be (24*R*,25*S*)-(+)-3α,7α,12α,24,26-pentahydroxy-5β-cholestan-27-yl sodium sulfate, based on the chemical data that alkaline degradation of III with aqueous potassium hydroxide gives only I.

Keywords bile salt; sodium scymnol sulfate; *Rhizoprionodon acutus*; scymnol; anhydroscymnol

In the preceding paper,[1)] we reported the isolation of sodium scymnol sulfate (III) from *Rhizoprionodon acutus* and the structural elucidations of III, anhydroscymnol (24, 26-epoxy-5β-cholestane-3α,7α,12α,27-tetrol) (I) and scymnol (5β-cholestane-3α,7α,12α,24,26,27-hexol) (II), prepared from III by following the reported procedures,[2,3)] with the use of modern spectral techniques. But the stereochemistry at C(24) and/or C(25) of these compounds remained unestablished.

The structure of the bile alcohol 5β-ranol, the major bile constituent of the bull frog, *Rana catesbeiana*, has been proven by synthesis to be (24*R*)-27-nor-5β-cholestane-3α,7α,12α,24,26-pentol.[4)] We recently found that II influences bile secretion in mammals, and we also showed that there are equal quantities of III and another sodium scymnol sulfate in the bile of *Lamna ditropis*.[5)] The structures and stereochemistry of I—III are of interest both phylogenetically and pharmacologically, so an investigation was conducted to determine their absolute configurations by use of both crystallographic and spectroscopic methods. The results are presented here.

Experimental

The melting points were determined with a Yanaco micro melting point apparatus and are uncorrected. Proton and carbon-13 nuclear magntic resonance (^{1}H- and ^{13}C-NMR) spectra were recorded on JEOL JNM-GX270 and JNM-GX500 spectrometers.

Isolation of Sodium Scymnol Sulfate (III) As reported previously,[1)] it was isolated from the bile of *Rhizoprionodon acutus*.

Preparation of Anhydroscymnol (I) and Scymnol (II) As reported,[1)] I was prepared by alkaline degradation of III with aqueous potassium hydroxide. And II was prepared by alkaline hydrolysis of the product, which was obtained by trichloroacetic acid treatment of the *O*-acetylated derivative of III in a nonaqueous medium, with aqueous potassium hydroxide.

X-Ray Crystallographic Analyses Compounds I and II were carefully recrystallized from ethyl acetate–chloroform and methanol–ethyl acetate, respectively, to afford colorless plate-shaped crystals, mp 196—197 (I) and 183—184 °C (II), which were used for the following X-ray crystallographic analyses.

Crystals with dimensions of 0.3×0.3×0.2 mm (I) and 0.2×0.2×0.4 mm (II) were used for the measurements of cell constants and for data collections. In the case of II, solvent of crystallization was readily lost in air, so all data for the crystallographic analysis were obtained from the crystal sealed in a glasscapillary. The cell parameters, shown below, and orientation matrixes of both compounds were obtained by the least-squares procedure applied to 20 reflections carefully measured on a Rigaku four-circle diffractometer equipped with a rotation anode (graphite-monochromated Mo K_α (I) and Cu K_α (II) radiations ($\lambda=0.7107$ and 1.5418 Å), respectively. Intensities, on a relative scale, were measured by using the ω-2θ scan technique with the same instrument in the ranges of $2.5<2\theta<55$ (I) and $5<2\theta<140$ (II) (scan speed 4° min^{-1} in ω, scan range in ω $(1.3+0.14\tan\theta)°$. Three standards (monitored every 50 reflections) showed no significant change during data collection. Finally, 2044 and 2250 independent data with $I>3\sigma$ (I) remained after averaging the 2092 and 3344 symmetry-related reflections of compounds I and II, respectively, and were used in the analyses. Lorenz and polarization, but not absorption, corrections were applied. The space groups of the compounds, $P2_12_12_1$ (I) and $P2_12_12$ (II), were determined from the systematic absences. The correctness of the choices of the space groups was demonstrated by the successful structure determinations.

Crystal Data: I: $C_{27}H_{46}O_5$, $M_r=450.66$. Orthorhombic, $a=13.562(2)$, $b=21.636(2)$, $c=8.735(2)$ Å, $V=2563.1$ Å^3, $D_o=1.16$ g/cm^3, $D_c=1.17$ g/cm^3, $Z=4$. Space group $P2_12_12_1$ from systematic absences.

II: $C_{27}H_{48}O_6\cdot CH_3OH\cdot H_2O$, $M_r=518.76$. Orthorhombic, $a=18.553(2)$, $b=19.887(2)$, $c=7.986(2)$ Å, $V=2946.3$ Å^3, $D_o=1.22$ g/cm^3, $D_c=1.19$ g/cm^3, $Z=4$. Space group $P2_12_12$ from systematic absences.

Structure Determinations and Refinements The structures of I and II were solved by direct methods using normalized structure factors with $E>1.2$, and their refinements were carried out as follows.

I: The 10-th E map, in the total of 16 phase sets generated, gave the positions of all non-hydrogen atoms. Block-diagonal refinement, with isotropic thermal parameters of these atoms, converged to a residual (R) of 0.135. Further refinement of atomic coordinates with anisotropic thermal parameters reduced R to 0.111. At this stage all the hydrogen atoms except the hydrogens bound to oxygens and methyl functions were put in calculated positions and hydrogens bound to oxygens and methyl functions were located in the positions from a subsequent difference map. Further full matrix least-squares refinement of the position parameters and temperature factor coefficients, anisotropic for the non-hydrogen atoms and isotropic for the hydrogens, led to the values ($R=0.062$ ($R\omega=0.061$)) listed in Table I.

II: The 22-nd map, in the total of 32 phase sets generated, revealed 26 heavy atoms (C and O) of the structure. The successive difference electron density map located the remaining 8 non-hydrogen atoms ($R=0.146$). The structure was improved by block-diagonal least-squares refinement of the positional and the anisotropic thermal parameters of the non-hydrogen atoms to $R=0.108$. The hydrogen atoms bound to carbons were put in chemically reasonable positions with reference to the peaks in a

TABLE I. Positional Parameters ($\times 10^4$) and Thermal Parameters of I and II with Their Estimated Standard Deviation in Parentheses, Denoting the Least Significant Digits

I					II				
Atom	x	y	z	B_{eq}	Atom	x	y	z	B_{eq}
C(1)	−696 (5)	5876 (3)	−1192 (7)	5.4 (2)	C(1)	8568 (7)	7830 (7)	618 (17)	6.1 (4)
C(2)	−819 (4)	5202 (3)	−840 (7)	5.4 (2)	C(2)	8913 (6)	8523 (7)	488 (17)	5.8 (4)
C(3)	−1817 (4)	5079 (3)	−133 (7)	5.2 (2)	C(3)	9419 (5)	8631 (7)	1930 (18)	6.5 (4)
C(4)	−1963 (4)	5486 (3)	1260 (7)	4.5 (1)	C(4)	9034 (6)	8556 (6)	3600 (16)	5.2 (3)
C(5)	−1819 (4)	6162 (3)	948 (7)	5.1 (2)	C(5)	8649 (6)	7860 (6)	3773 (14)	4.8 (3)
C(6)	−1990 (4)	6540 (3)	2402 (8)	5.0 (2)	C(6)	8273 (6)	7793 (6)	5458 (15)	4.8 (3)
C(7)	−1158 (3)	6494 (3)	3564 (7)	4.4 (1)	C(7)	7558 (5)	8169 (6)	5579 (14)	4.2 (3)
C(8)	−159 (3)	6632 (2)	2830 (6)	3.9 (1)	C(8)	7060 (5)	8009 (5)	4065 (12)	3.3 (2)
C(9)	23 (3)	6231 (3)	1391 (6)	3.6 (1)	C(9)	7467 (5)	8145 (5)	2399 (13)	3.8 (3)
C(10)	−813 (3)	6315 (3)	179 (6)	4.9 (1)	C(10)	8151 (6)	7695 (5)	2282 (14)	4.2 (3)
C(11)	1063 (3)	6338 (3)	733 (6)	4.3 (1)	C(11)	6973 (6)	8097 (6)	880 (15)	4.8 (3)
C(12)	1882 (3)	6268 (2)	1915 (6)	3.7 (1)	C(12)	6280 (5)	8496 (5)	1025 (13)	3.7 (3)
C(13)	1711 (3)	6680 (2)	3313 (6)	3.5 (1)	C(13)	5856 (5)	8308 (4)	2607 (12)	3.4 (2)
C(14)	686 (3)	6527 (2)	3953 (5)	3.4 (1)	C(14)	6364 (4)	8408 (4)	4111 (12)	3.2 (2)
C(15)	647 (4)	6847 (3)	5487 (7)	5.1 (2)	C(15)	5862 (5)	8314 (6)	5647 (14)	4.3 (3)
C(16)	1721 (5)	6798 (3)	6085 (8)	5.5 (2)	C(16)	5131 (5)	8608 (6)	5026 (13)	4.3 (3)
C(17)	2350 (4)	6541 (3)	4741 (6)	4.0 (1)	C(17)	5214 (4)	8764 (5)	3135 (13)	3.2 (2)
C(18)	1802 (5)	7366 (3)	2850 (9)	5.1 (2)	C(18)	5610 (6)	7567 (5)	2427 (17)	4.8 (3)
C(19)	−812 (6)	6983 (4)	−431 (9)	6.5 (2)	C(19)	7942 (8)	6947 (6)	2278 (24)	6.3 (4)
C(20)	3436 (4)	6785 (3)	4824 (7)	4.9 (2)	C(20)	4507 (5)	8710 (5)	2142 (14)	3.6 (3)
C(21)	4043 (4)	6599 (3)	3436 (9)	5.5 (2)	C(21)	4614 (6)	8920 (8)	290 (16)	5.3 (4)
C(22)	3916 (5)	6568 (3)	6297 (8)	5.4 (2)	C(22)	3913 (5)	9133 (5)	2967 (15)	4.0 (3)
C(23)	3976 (5)	5875 (3)	6422 (8)	5.9 (2)	C(23)	3177 (5)	9075 (5)	2118 (15)	4.2 (3)
C(24)	4639 (4)	5616 (3)	7680 (7)	4.9 (1)	C(24)	2568 (6)	9378 (9)	3143 (16)	5.5 (4)
C(25A)	4477 (4)	5795 (3)	9361 (8)	4.7 (2)	C(25A)	1839 (10)	9014 (13)	2458 (27)	3.4 (5)
C(26A)	5577 (5)	5954 (4)	9334 (12)	7.5 (3)	C(26A)	1650 (12)	9291 (15)	697 (37)	3.5 (6)
C(27A)	4161 (4)	5278 (3)	10372 (7)	5.2 (2)	C(27A)	1262 (13)	9050 (14)	3751 (32)	4.9 (7)
O(3)	−1966 (3)	4451 (2)	206 (6)	6.0 (1)	C(25B)	1774 (11)	9401 (11)	2479 (33)	4.1 (6)
O(7)	−1110 (3)	5906 (2)	4293 (5)	4.5 (1)	C(26B)	1710 (18)	9627 (14)	699 (48)	4.6 (7)
O(12)	1983 (2)	5636 (2)	2379 (4)	3.9 (1)	C(27B)	1518 (13)	8658 (13)	2693 (39)	5.3 (8)
O(24)	5634 (3)	5882 (2)	7735 (6)	6.8 (1)	C(M)	6389 (14)	9998 (8)	−2842 (19)	10.2 (7)
O(27A)	3144 (3)	5105 (2)	10073 (5)	5.7 (1)	O(3)	9740 (5)	9279 (6)	1821 (17)	9.5 (4)
					O(7)	7702 (4)	8879 (4)	5705 (10)	4.7 (2)
					O(12)	6417 (3)	9209 (3)	905 (9)	4.2 (2)
					O(24)	2673 (4)	10089 (4)	3106 (11)	5.8 (2)
					O(26A)	1067 (9)	8934 (9)	2 (21)	5.9 (5)
					O(27A)	1111 (9)	9729 (9)	4093 (25)	6.1 (5)
					O(26B)	978 (8)	9722 (11)	208 (24)	5.6 (5)
					O(27B)	1522 (12)	8501 (10)	4395 (35)	9.1 (8)
					O(A)	1114 (12)	10253 (12)	7209 (30)	9.1 (7)
					O(B)	747 (41)	9497 (75)	6571 (38)	17.1 (10)
					O(M)	6880 (5)	9574 (4)	−2110 (12)	7.5 (3)

three-dimensional difference map and hydrogens bound to oxygens except for O(24), O(26), and O(27) were located in a difference map, obtaind by using the coordinates of $R=0.108$. Further block-diagonal least-squares refinement of atomic coordinates with anisotropic thermal parameters for the non-hydrogen atoms and isotropic ones for the hydrogens gave $R=0.080$. The final refinement was carried out, after location of the hydrogens bound to side chain oxygens from a subsequent difference map, by the full-matrix least-squares method, yielding the values ($R=0.73$ ($R\omega=0.076$)) listed in Table I for 2054 observed reflections with $I>3\sigma$ (I). The multiplicity of C(25), C(26), C(27), O(26) and O(27) of the side chain and O of H_2O was 0.5 in each case.

Structures were solved by direct methods using programs MULTAN80 and SAPI85[6,7] with the scattering factors from volume 4 of reference 8. The block-diagonal program empolyed for the refinement does not employ a weighting system. The function minimized was $\Sigma w[(F_o)^2-(F_c)^2]^2$ with $w=1/[\sigma^2(F_o)+0.02(F_o)^2]$, $\sigma(F_o)$ determined from counting statistics. The molecular structure was drawn with the aid of the ORTEP program.[9] All calculations were made on PANAFACOM computers (U-1200 and A-70) and a Hitachi M-280H computer at the Tokyo University Computer Center.

Results and Discussion

Crystal Structures of I and II Stereoscopic views of the molecules of I and II, with atom numberings, are depicted in Fig. 1. The intramolecular bond lengths and angles and other details of the molecular geometry for both molecules in the asymmetric are normal. In both molecules the geometry of the steroid nucleus is that expected for a cholane derivative.[10] The geometry of the rings was as expected, with the A/B-ring juncture *cis* while the B/C- and C/D-ring junctures are *trans*, as shown in Fig. 1. And it was also shown that rings A, B and C have normal chair conformations.

The torsion angles are given in Table II. The conformations of cyclopentane rings can be described by the parameters P and ψ_m where P is the phase angle and ψ_m is the maximum torsional angle attainable in this conformation ($P=+72°$, β-envelope: $P=0°$, half chair; $P=-72°$, α-envelope).[11] The D-rings of both molecules have a β-envelope conformation, defined by parameters $P=26°$ and $\psi_m=46.9$ for I and $P=34°$ and $\psi_m=47.5$ for II.

The molecular packings are characterized by an assembly

of wavy bilayers, extending into the *ac* plane in I and into the *bc* plane in II. All the O atoms of both compounds are involved in hydrogen bonding. The hydrogen bonds in the crystals of both molecules are described in Table III. The bilayer, composed of two monolayers, is generated from adjacent molecules by the 2_1 axis in I and by the 2 axis in II. The bilayer structures of both compounds are mainly stabilized by an efficient network of hydrogen bonds involved all the oxygen atoms in the crystals, whose repeat unit lies along *a* in I and along *c* in II, and are characterized by non-polar outer surfaces with protruding methyl groups. In II, two rows in a monolayer are connected by hydrogen bonds in a different way from by means of compound I's, belonging to methanol and water molecules. And in I, a monolayer is composed of rows of molecules, linked in each row by hydrogen bonds involving O(12) and O(27) of two consecutive molecules, while it is formed by head-to-tail hydrogen bonds *via* O(3) and O(26) of two corresponding molecules of II.

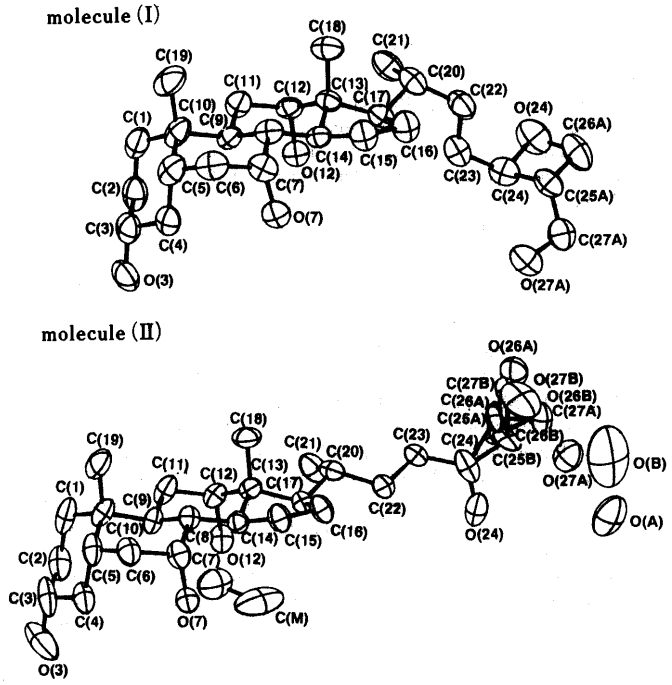


Fig. 1. ORTEP Drawings of (24*R*,25*S*)-(+)-24,26-Epoxy-5β-Cholestane-3α,7α,12α,27-tetrol (I) and (24*R*)-(+)-5β-Cholestane-3α,7α,-12α,24,26,27-hexol (II) with Atom Numberings Used in Tables I—III

In the side-chains the H-atoms at C(17) and C(20) are in an antiperiplanar conformation, and C(22) is antiperiplanar with respect to the C(13)–C(17) bond, whereas C(21) is synclinal; the relevant torsion angles are given in Table III. The orientation of C(23) is different in the two molecules; in I this group adopts the synclinal conformation with respect to C(17)–C(20) whereas in II it is antiperiplanar. It is worth noting that the conformation of the side chain in cholanic acids is related to the biological activity. Interestingly, the side chain conformation of II, about C(20)–C(22)–C(23), is similar to that of chenodeoxycholic acid or ursodeoxycholic acid, where all the oxygen atoms are involved in hydrogen bonding.[5,10f,k,12]

Chemical Structures of I, II and III From the above analyses, the chemical structures of I and II are concluded to be (24*R*,25*S*)-(+)-24,26-epoxy-5β-cholestane-3α,7α,-12α,27-tetrol and (24*R*)-(+)-5β-cholestane-3α,7α,12α,24,-26,27-hexol. The NMR [^{1}H and ^{13}C noise decoupled, insensitive nuclei enhanced by polarization transfer (INEPT), two dimensional correlation spectroscopy (2D-COSY), and C–H–shift COSY] spectra were measured, and it was found that the proton spin–spin coupling constants in the epoxy ring of I, $J_{24,25}$ (6.1 Hz), is close to the reported J_{trans} value (6.9 Hz) in oxetane derivative.[13] It is biologically and physiologically interesting that the configuration at C(24) of both natural 5β-ranol and 5β-scymnol (II) are the same.[3,5a]

As reported previously,[1] the alkaline degradation of III with potassium hydroxide gives only I not together with 25*R*-anhydroscymnol. This indicated that the structure of III is to be (24*R*,25*S*)-(+)-3α,7α,12α,24,26-pentahydroxy-5β-cholestan-27-yl sodium sulfate, being able to be explained the transformation of I from III followingly. The formation of the oxetane ring of I from III should be accounted for by elimination of OSO_3Na group due to the nucleophilic attack of hydroxy oxygen at C(24) during the

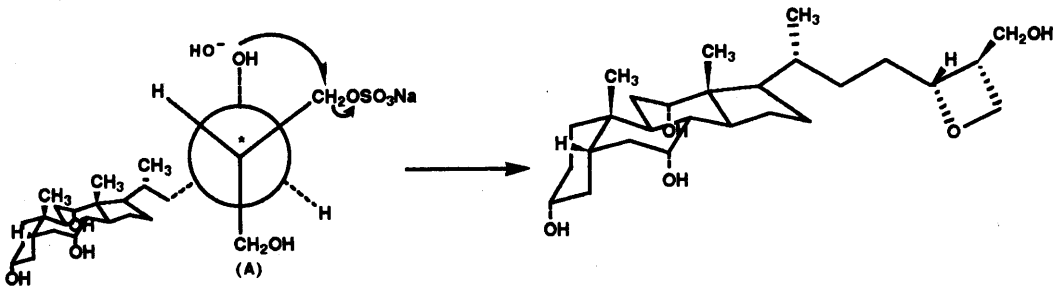
Table II. Selected Torsion Angles (°) for I and II

Ring D	I	II	17β Side-chain	I	II	17β Side-chain	I	II
ψ_1C(13)–C(14)–C(15)–C(16)	−35	−34	C(13)–C(17)–C(20)–C(21)	−53	−63	C(21)–C(20)–C(22)–C(23)	−64	59
ψ_2C(14)–C(15)–C(16)–C(17)	9	7	C(16)–C(17)–C(20)–C(21)	−174	176	C(20)–C(22)–C(23)–C(24)	167	168
ψ_3C(15)–C(16)–C(17)–C(13)	20	21	C(13)–C(17)–C(20)–C(22)	−178	173	C(22)–C(23)–C(24)–O(24)	−49	70
ψ_4C(16)–C(17)–C(13)–C(14)	−40	−42	C(16)–C(17)–C(20)–C(22)	62	52			
ψ_5C(17)–C(13)–C(14)–C(15)	47	47	C(17)–C(20)–C(22)–C(23)	61	−177			

Table III. O–O Distances (Å) of the Hydrogen Bonds in a Bilayers of I and II

Molecule (I)				Molecule (II)			
O(3)–O(24^{i})	2.954 (7)	O(3)–O(26A^{i})	2.94 (2)	O(3)–O(27A^{ii})	3.11 (2)	O(3)–O(26B^{i})	2.72 (2)
O(12)–O(27^{i})	2.864 (6)	O(3)–O(26B^{iii})	2.78 (2)	O(3)–O(3^{iv})	3.03 (2)	O(7)–O(M^{v})	2.70 (1)
O(3)–O(7^{ii})	2.840 (5)	O(7)–O(24^{ii})	3.00 (1)	O(7)–O(A^{i})	3.04 (2)	O(12)–O(24^{ii})	2.81 (1)
O(12)–O(27^{iii})	2.809 (6)	O(12)–O(M^{vi})	2.66 (1)	O(27A)–O(A^{i})	2.71 (3)	O(26B)–O(B^{vii})	2.98 (4)
		O(27B)–O(B^{vi})	3.00 (1)				

Symmetry code. I: i) 1/2−*x*, −*y*+1, −1/2+*z*; ii) 1/2−*x*, −*y*+1, 1/2−*z*; iii) *x*, *y*, *z*+1. II: i) *x*−1, *y*, *z*; ii) −*x*+1, −*y*+2, *z*; iii) *x*+1, *y*, *z*; iv) −*x*+2, −*y*+2, *z*; v) *x*, *y*, *z*+1; vi) *x*, *y*, *z*; vii) *x*, *y*, *z*−1.



III (24*R*, 25*S*)-(+)-3*α*, 7*α*, 12*α*, 24, 26-pentahydroxy-5*β*-cholestan-27-yl sodium sulfate

I (24*R*, 25*S*)-(+)-24, 26-epoxy-5*β*-cholestane-3*α*, 7*α*, 12*α*, 27-tetrol

Chart 1. Proposed Mechanism of Formation of 25*S*-Anhydroscymnol (I) from Sodium Scymnol Sulfate (III)

alkaline degradation and proceed normally, *via* a stable state (A), to give only I, as shown in the Chart 1. The detailed structure determination of sodium scymnol sulfate was discussed in the preceding paper.[5]

In summary, we have elucidated the side chain stereochemistry of anhydroscymnol (I), scymnol (II) and sodium scymnol sulfate (III) in the bile of *Rhizoprionodon acutus*. This is, to our knowledge, the first X-ray crystallographic study on the stereochemistry of bile alcohols obtained from fish biles.

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Chemistry of Tetrahydro-1,3-oxazin-2-one: New Method for the Synthesis of Indoloquinolizidine Derivatives

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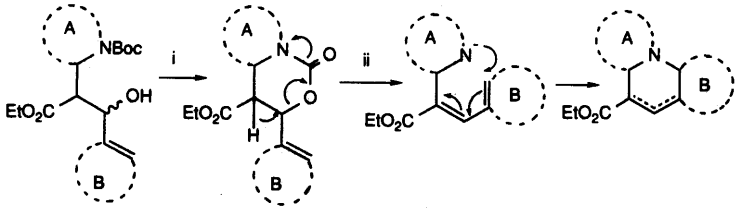
The aldol condensation of the *N*-Boc-tetrahydro-β-carboline-1-acetate (4) with acrolein in the presence of lithium diisopropylamide (LDA) gave an allyl alcohol (6), which was then treated with methanesulfonyl chloride and triethylamine to give a mixture of the mesylate (8) (55%) and the indolopyrido-3,5-oxazin-4-one (10) (14%). When 8 was treated with 1,8-diazabicyclo[5.4.0.]-7-undecene (DBU) in dimethylsulfoxide (DMSO) at room temperature, the azetopyridoindoles (12 and 13) were obtained unexpectedly. Alternative preparation of the indolopyrido-3,5-oxazin-4-ones (15 and 16), which are stereoisomers of 10, from 6 followed by heating with DBU in DMSO gave several indoloquinolizidines (18, 19 and 20), which are key intermediates for the synthesis of the indole alkaloids vindolosine and vindoline.

Keywords tetrahydro-1,3-oxazin-2-one; tetrahydro-β-carboline; azetidine; indolopyrido-3,5-oxazin-4-one; azetopyridoindole; indoloquinolizidine; 1,8-diazabicyclo[5.4.0.]-7-undecene; X-ray analysis

Cyclic carbamates such as oxazolidin-2-ones or tetrahydro-1,3-oxazin-2-ones, which are generally prepared from allylic[1] or homoallylic[2] acyclic carbamates by halolactonization, are versatile intermediates in organic synthesis, since hydrolytic cleavage of the heterocyclic rings readily provides the 1,2- or 1,3-amino alcohol moieties found in a number of biologically important compounds. Another cyclocarbamation, involving treatment of *N*-benzyloxycarbonyl 1,2-amino alcohols with thionyl chloride, has also been reported to give oxazolidin-2-one.[3]

Recently, we have reported[4] a novel cyclocarbamation of *N-tert*-butoxycarbonyl (Boc)-1,3-amino alcohol (I) by treatment with methanesulfonyl chloride (MsCl) and triethylamine (TEA) to give tetrahydro-1,3-oxazin-2-one (II) *via* S_N1 type C–O bond formation of the N–COO^- group, and also its efficient transformation to the tetrahydropyridine skeleton (IV) by treatment with 1,8-diazabicyclo[5.4.-0]-7-undecene (DBU) in dimethylsulfoxide (DMSO) *via* the diene intermediate (III).[5] This methodology was applied to the synthesis of some natural products.[6] We report here new syntheses of indoloquinolizidine derivatives by application of our methodology to compounds having a tetrahydro-β-carboline moiety in the A-part of I.

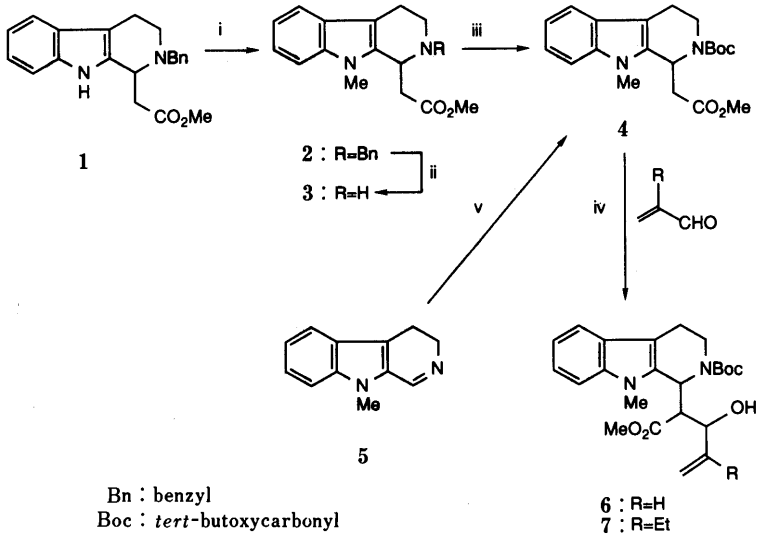
Treatment of the tetrahydro-β-carbolineacetate (**1**),[7] which was prepared by Pictet–Spengler condensation of *N*-benzyltryptamine with dimethyl acetylenedicarboxylate, with methyl iodide and sodium hydride (NaH) followed by catalytic debenzylation of the resulting **2** afforded **3** in 81% overall yield. Reaction of **3** with di-*tert*-butyl dicarbonate (Boc_2O) in tetrahydrofuran (THF) gave the carbamate (**4**) in 97% yield (method A). Compound **4** was alternatively obtained by the following one-pot operation based on the method of Nakagawa *et al.*[8] *N*-Methyl-3,4-dihydro-β-carboline (**5**)[9] was alkylated with lithio methyl acetate in the presence of boron trifluoride etherate ($BF_3 \cdot OEt_2$) followed by quenching with an excess of Boc_2O to give **4** in 90% overall yield (method B). However, method A was preferable to obtained **4** in a large quantity. Aldol condensation of **4** with acrolein or 2-ethylacrolein in the presence of lithium diisopropylamide (LDA) in THF at −78 °C gave the allyl alcohol (**6** or **7**) as an oily mixture of diastereomers, in good yield, after purification by column chromatography on SiO_2. The product (**6**) was treated with MsCl and TEA in dichloromethane (CH_2Cl_2) at room temperature to give a mixture of the mesylate (**8**) (less polar) and the indolopyrido-3,5-oxazin-4-one (**10**) (more polar), which were, without isolation, submitted to the usual cleavage of the Boc group by 2.3 N HCl–EtOAc. Cyclization of the resulting hydrochloride was attempted by treatment with DBU (2 eq) in DMSO at room temperature according to our previous method[5] in expectation of the formation of the indoloquinolizidine. The crude oil finally obtained was purified by column chromatography to give **12** [mass spectrum (MS) *m/z*: 296 (M^+)] (50%), **10** [MS *m/z*: 340 (M^+)] (9.5%), and **13** [MS *m/z*: 296 (M^+)] (6.5%). The proton nuclear magnetic resonance (^{1}H-NMR) spectra of **12** (and **13**) showed the presence of terminal methylene protons at δ 5.22 (5.08), 5.39 (5.21), and 5.91 (5.93) as well as the protons of the tetrahydro-β-carboline skeleton. Selected ^{1}H-NMR and carbon-13 nuclear magnetic



I II III IV

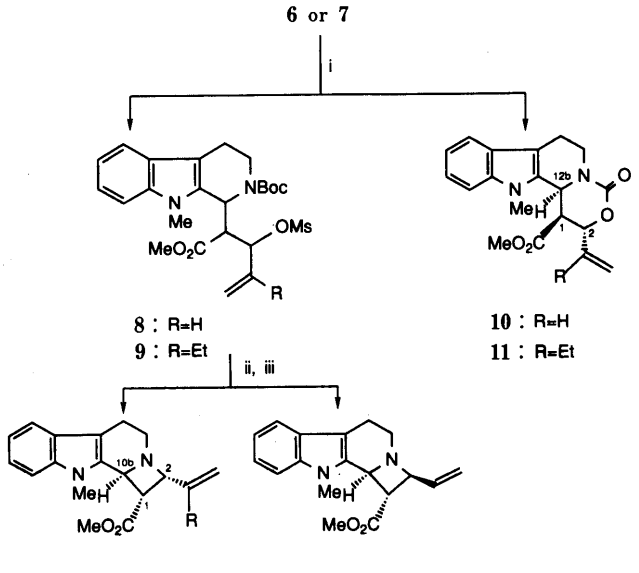
reagents: i, MsCl/TEA; ii, DBU/DMSO B=aryl or ethenyl and cycloalkenyl

Chart 1



reagents : i, NaH/MeI; ii, 10% Pd-C/H_2; iii, (*tert*-BuOCO)$_2$O; iv, LDA; v, $MeCO_2Me$/LDA/BF_3·OEt_2 then (*tert*-BuOCO)$_2$O

Chart 2

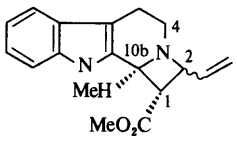


reagents: i, MsCl/Et_3N; ii, 2.3 N HCl/EtOAc; iii, DBU/DMSO/r.t.

Chart 3

resonance (^{13}C-NMR) data for both compounds are summarized in Table I. The ^{13}C-NMR spectra indicated the presence of three tertiary carbon atoms in each case. On the basis of these results, the structures of **12** and **13** were deduced to be methyl 2-ethenyl-9-methyl-1,4,5,10b-tetrahydro-2*H*-azeto[1′,2′ : 1,2]pyrido[3,4-*b*]indole-1-carboxylates, which are interesting substrates for Meisenheimer rearrangement of the corresponding *N*-oxides.[10] The stereochemistries of **12** (1,2-*cis*) and **13** (1,2-*trans*) were determined from the ^{1}H-NMR spectral data, based on the general rule developed for azeto[2,1-*a*]isoquinolines,[11] in which the vicinal coupling constants of the azetidines $^3J_{(H,H)}$*cis* (7—8 Hz) are larger than $^3J_{(H,H)}$*trans* (2—3 Hz). The structure of the third component (**10**) was readily

TABLE I. Selected ^{13}C- and ^{1}H-NMR Data for Compounds **12** and **13** (δ $CDCl_3$)



Number	Carbon		Proton (*J*, Hz)	
	12	**13**	**12**	**13**
1	48.6 (d)	47.7 (d)	3.13 (dd, *J*=8, 3)	2.88 (t, *J*=4)
2	68.5 (d)	61.2 (d)	4.30 (t, *J*=8)	4.54 (dd, *J*=10, 4)
4	41.4 (t)	41.1 (t)		
10b	54.1 (d)	56.5 (d)	5.10 (br s)	5.02 (br s)

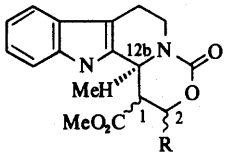
determined as 2-ethenyl-1-methoxycarbonyl-12-methyl-1,6,7,12b-tetrahydro-2*H*,4*H*-indolo[2,3-*c*][1,3]oxazin-4-one on the basis of the spectral data (see Experimental). Stereochemical assignment of **10** will be discussed later. After usual mesylation of **6**, the crude product was purified by column chromatography to give the mesylate (**8**) as an oily mixture of diastereomers in 55% yield, together with **10** in 14% yield. De-*tert*-butoxycarbonylation of **8** followed by DBU treatment gave a mixture of **12** (54%) and **13** (6.5%). Similarly, usual mesylation of **7** followed by column-chromatographic purification gave a mixture of the mesylate (**9**, 33%) and the indolopyrido-3,5-oxazin-4-one (**11**, 11%). De-*tert*-butoxycarbonylation of **9** followed by DBU treatment gave the azetidine **14** in 43% yield; its ^{1}H-NMR spectrum was very similar to that of **12**. Although the mechanistic details have not been established, it is suggested that the indole moiety may participate in the formation of azetidines.

Another approach to the indoloquinolizidine derivatives was investigated. De-*tert*-butoxycarbonylation of **6** followed by condensation of the resulting amino alcohol with carbonyl diimidazole (Im_2CO) in refluxing benzene afforded

oxazinones (**15** and **16**) in 54 and 23% yields, respectively. Their infrared (IR) and ^{1}H-NMR spectra were similar to those of **10**. Table II shows selected ^{1}H-NMR spectral data for **10**, **11**, **15**, and **16**. The coupling constants of the neighboring three methine protons, however, did not give the necessary information for determining the stereochemistry. But, the stereochemistry of **16** alone could be established to be as depicted in Chart 4 on the basis of the nuclear Overhauser effect (NOE) between H-1, H-2, and H-12b of the corresponding dihydro derivative (**17**), which was obtained by catalytic hydrogenation of **16** with 5% palladium on carbon (Pd–C). Irradiation of H-2 at δ 4.40 caused distinct enhancements of the signals at δ 3.55 and 5.15 due to H-1 and H-12b, respectively. However, stereochemistry of the other compounds (**10** and **15**) could not be resolved clearly by the same method. Thus, X-ray crystallographic analyses of compounds **10** and **15** were carried out and the molecular structures determined are shown in Fig. 1.

The mixture of tetrahydro-1,3-oxazin-2-ones (**15** and **16**)[12] thus obtained was heated with DBU (1.1 eq) in DMSO at 120 °C for 4 h, giving a mixture of three products (total yield 47.4%), **18**, **19**, and **20**, *via* successive decarboxylation–recyclization sequences. These compounds are key intermediates for the synthesis of indole alkaloids vindolosine and vindoline.[13] The spectroscopic data (IR, MS and ^{1}H-NMR) were in good agreement with reported values.[13]

TABLE II. Selected ^{1}H-NMR Data for Compounds **10**, **11**, **15**, **16**, and **17** [δ $CDCl_3$, J(Hz)]

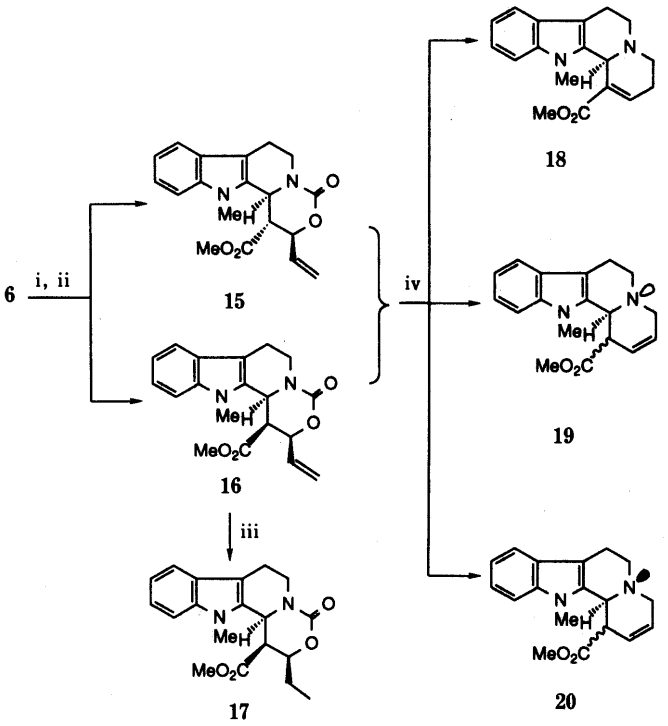


R = CH=CH$_2$, C(Et)=CH$_2$, Et

Compd.	H-1	H-2	H-12b
10	3.41 (dd, J=4.4, 1.8)	5.18 (dd, J=4.0, 1.0)	5.11 (brd, J=4.4)
11	3.47 (dd, J=4.5, 2.5)	5.05 (brs)	5.05 (brs)
15	3.10 (t, J=5.6)	4.96 (t, J=5.6)	5.39 (d, J=5.6)
16	3.62 (dd, J=5.8, 2.8)	5.04 (brs)	5.23 (d, J=5.8)
17	3.55 (1H, dd, J=5.3, 3.0)	4.40 (m)	5.15 (d, J=6.0)

Experimental

All melting points were measured on a Yanagimoto apparatus and are uncorrected. IR absorption spectra were recorded on a Shimadzu IR-435 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were obtained with a Varian Gemini-200 spectrometer; signals are given in ppm. Low-resolution (MS) and high-resolution mass spectra (HRMS) were obtained on a Hitachi M-80 instrument. All reactions were carried out under a nitrogen



reagents : i, 2.3 N HCl/EtOAc; ii, Im_2CO; iii, 5% Pd-C/H_2; iv, DBU/DMSO/120 °C

Chart 4

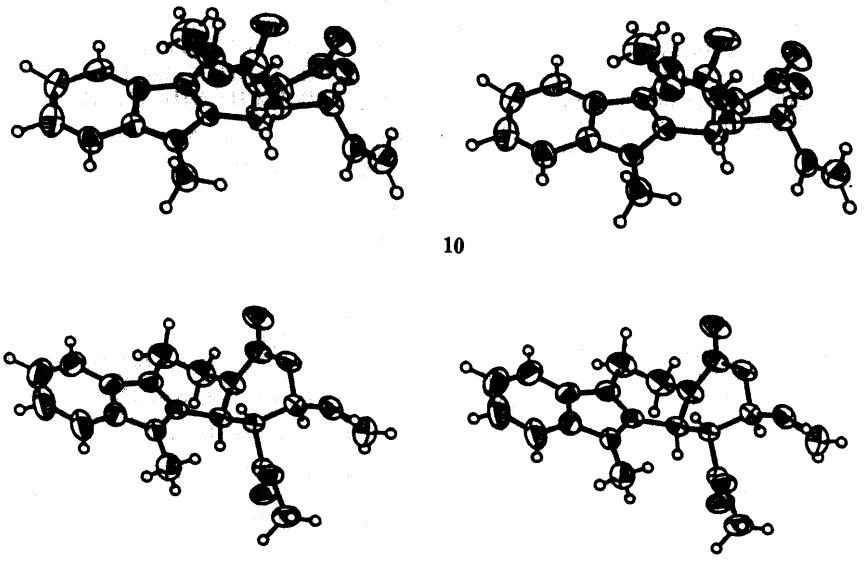


Fig. 1. Stereoscopic View of the Molecules **10** and **15**

atmosphere. For column chromatography, SiO_2 (Merck 9385) was used.

2-Benzyl-1-carbomethoxymethyl-9-methyl-1,2,3,4-tetrahydro-β-carboline (2) A solution of **1** (9.72 g, 29.1 mM) in dimethylformamide (DMF) (25 ml) was added dropwise to a suspension of 60% NaH (1.51 g, 37.8 mM) in DMF (15 ml) with stirring at room temperature. The mixture was stirred for 1 h, then a solution of methyl iodide (3.62 ml, 58.2 mM) in DMF (13 ml) was added dropwise with external cooling with water, and the whole was stirred for 2 h. The reaction mixture was poured into ice-water (500 ml), and extracted with EtOAc (100 ml × 3). The extract was washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was agitated with a mixture of hexane–EtOH (3 : 1), and the resulting precipitate was collected by filtration to give **2** (8.39 g, 83%), which was recrystallized from EtOH to give pale yellow crystals, mp 103—105 °C. IR ν_{max} (Nujol) cm^{-1}: 1735 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.5—3.3 (6H, m, $CH_2 \times 3$), 3.62, 3.70 (each 3H, each s, NCH_3 and/or $COOCH_3$), 3.69, 3.74 (each 1H, each d, J=13 Hz, NCH_2Ar), 4.33 (1H, dd, J=10.5, 3.5 Hz, 1-H), 7.1—7.4 (3H, m, ArH), 7.57 (1H, d, J=7.5 Hz, 8-H). MS *m/z* 348 (M^+). *Anal.* Calcd for $C_{22}H_{24}N_2O_2$: C, 75.83; H, 6.94; N, 8.04. Found: C, 75.87; H, 6.99; N, 8.00.

1-Carbomethoxymethyl-9-methyl-1,2,3,4-tetrahydro-β-carboline (3) A solution of **2** (18.0 g, 51.7 mM) in MeOH (400 ml) containing concentrated HCl (18 ml) was hydrogenated using a Skita apparatus (initial pressure: 5.0 kg/cm^2) with 10% Pd–C (5.8 g) for 10 h. The catalyst was removed by filtration through a celite pad, and the filtrate was concentrated *in vacuo*. The residue was neutralized with saturated $NaHCO_3$ solution, and extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residual oil (12.9 g, 97%) was found to be almost pure **3**. IR ν_{max} ($CHCl_3$) cm^{-1}: 3330 (NH), 1720 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.60—2.80 (4H, m, $CH_2 \times 2$), 3.15 (2H, m, CH_2COO), 3.62, 3.75 (each 3H, each s, NCH_3 and/or $COOCH_3$), 4.59 (1H, dd, J=10.0, 2.5 Hz, 1-H), 7.05—7.29 (3H, m, ArH), 7.50 (1H, d, J=7.7 Hz, 8-H). MS *m/z*: 258 (M^+). HRMS Calcd for $C_{15}H_{18}N_2O_2$: 258.1367. Found: 285.1368.

2-*tert*-Butoxycarbonyl-1-carbomethoxymethyl-9-methyl-1,2,3,4-tetrahydro-β-carboline (4). Method A: A solution of Boc_2O (11.3 g, 50.2 mM) in THF (10 ml) was added dropwise to a solution of **3** (12.9 g, 50.2 mM) in THF (60 ml) under ice cooling and the whole was stirred at room temperature for 1.5 h. Removal of the solvent gave a solid, which was recrystallized from EtOH to give **4** (17.6 g, 97%) as colorless crystals, mp 116—118 °C. IR ν_{max} (Nujol) cm^{-1}: 1740, 1680 (CO). ^{1}H-NMR ($CDCl_3$) δ: 1.49 [9H, s, $C(CH_3)_3$], 2.78—2.95 (4H, m, $CH_2 \times 2$), 3.70, 3.72 (each 3H, each s, NCH_3 and/or $COOCH_3$), 4.32—4.52 (2H, m, CH_2COO), 5.79 (1H, m, 1-H), 7.10—7.40 (3H, m, ArH), 7.48 (1H, d, J=7.5 Hz, 8-H). MS *m/z*: 358 (M^+). *Anal.* Calcd for $C_{20}H_{26}N_2O_4$: C, 67.02; H, 7.31; N, 7.82. Found: C, 66.91; H, 7.30; N, 7.75.

Method B: $BF_3 \cdot OEt_2$ (0.14 ml, 1.1 mM) was added to a solution of **5** (202 mg, 1 mM) in THF (7 ml) at room temperature, and the mixture was cooled to −23 °C. Methyl acetate (0.28 ml, 3.5 mM) was added to a solution of LDA, prepared from diisopropylamine (0.56 ml, 4 mM) and *n*-butyl lithium (*n*-BuLi) (15% hexane solution, 2.52 ml, 4 mM), in THF (7 ml) at −78 °C to prepare lithio methyl acetate. This solution was added to the mixture and the whole was stirred at −23 °C for 30 min. The reaction was quenched by the addition of a solution of Boc_2O (1.09 g, 5 mM) in THF (7 ml) at −23 °C. After the mixture had been stirred for 2 h, the solvent was removed by evaporation. The residue was agitated with a mixture of EtOH–hexane (1 : 1) and the resulting solid was collected by filtration, washed with cold EtOH, and dried to give **4** (213 mg, 60%). The filtrate was concentrated, and the residue was purified by column chromatography (20% EtOAc in hexane) to give **4** (109 mg, 30%) (total yield 90%). This was identical with the sample of **4** obtained by method A, based on comparison of their IR and ^{1}H-NMR spectra.

Methyl 2-(2-*tert*-Butoxycarbonyl-9-methyl-1,2,3,4-tetrahydro-β-carbolin-1-yl)-3-hydroxy-4-pentenoate (6) A solution of **4** (7.16 g, 20 mM) in THF (50 ml) was added dropwise to a solution of LDA [prepared from diisopropylamine (3.4 ml, 24 mM) and *n*-BuLi (15% hexane solution, 15.4 ml, 24 mM)] in THF (50 ml) at −78 °C, and the mixture was stirred at this temperature for 20 min. Then, 90% acrolein (pre-dried for 30 min with molecular sieves 4A) (2.7 ml, 40 mM) was added at once to this solution, and the whole was stirred at −78 °C for 20 min. The reaction mixture was quenched with water, and THF was removed by evaporation. The residue was extracted with EtOAc–benzene (1 : 1), and the extract was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residual oil was purified by column chromatography (15% EtOAc in hexane) to give pure **6** (8.11 g, 98%) as an oil. IR ν_{max} (neat) cm^{-1}: 3430 (OH), 1725, 1680 (CO). The ^{1}H-NMR spectrum was not sufficiently well resolved to permit assignment of the signals all. Selected signals were as follows: ^{1}H-NMR ($CDCl_3$) δ: 1.45 [9H, s, $C(CH_3)_3$], 2.7—3.2 (4H, m, $CH_2 \times 2$), 3.30 (3H, s, NCH_3), 3.62 (3H, s, $COOCH_3$), 7.18—7.35 (3H, m, ArH), 7.47 (1H, d, J=7.5 Hz, ArH). MS *m/z*: 414 (M^+). HRMS Calcd for $C_{23}H_{30}N_2O_5$: 414.2153. Found: 414.2161.

Methyl 2-(2-*tert*-Butoxycarbonyl-9-methyl-1,2,3,4-tetrahydro-β-carbolin-1-yl)-3-hydroxy-4-methylenehexanoate (7) By a similar procedure to that described for the preparation of **6**, the crude product which was obtained from **4** (1.08 g, 3 mM), LDA (3.3 mM), and 2-ethylacrolein (0.44 ml, 6 mM) was purified by column chromatography (15% EtOAc in hexane) to give pure **7** (928 mg, 70%) as an oil. IR ν_{max} (neat) cm^{-1}: 3450 (OH), 1730, 1685 (CO). ^{1}H-NMR ($CDCl_3$) δ: 1.15 (3H, t, J=8.0 Hz, $CH_2\underline{CH}_3$), 1.45 [9H, s, $C(CH_3)_3$], 1.97—2.20 (2H, m, $C\underline{H}_2CH_3$), 2.75—2.90 (2H, m, 4-CH_2), 3.19 (3H, s, NCH_3), 3.44—3.59 (2H, m, 3′-H_2), 3.68 (3H, s, $COOCH_3$), 4.20 (1H, d, J=6.0 Hz, $C\underline{H}OH$), 4.67 (1H, td, J=7.5, 6.0, CHCOO), 4.89, 5.09 (each 1H, each s, $=CH_2$), 5.76 (1H, d, J=6.0 Hz, 1-H), 7.0-7.29 (3H, m, ArH), 7.44 (1H, d, J=7.5 Hz, 8-H). MS *m/z*: 442 (M^+). HRMS Calcd for $C_{25}H_{34}N_2O_5$: 442.2466. Found: 442.2458.

2-Ethenyl-1-methoxycarbonyl-12-methyl-1,6,7,12b-tetrahydro-2*H*,4*H*-indolo[2,3-*c*]pyrido[1,2-*c*][1,3]oxazin-4-one (10), *cis*-Methyl 2-Ethenyl-10-methyl-1,4,5,10b-tetrahydro-2*H*-azeto[1′,2′ : 1,2]pyrido[3,4-*b*]indole-1-carboxylate (12), and *trans*-Methyl 2-Ethenyl-10-methyl-1,4,5,10b-tetrahydro-2*H*-azeto[1′,2′ : 1,2]-pyrido[3,4-*b*]indole-1-carboxylate (13) A solution of MsCl (1.6 ml, 20.6 mM) in CH_2Cl_2 (3 ml) was added dropwise to a solution of **6** (5.69 g, 13.7 mM) and triethylamine (5.8 ml, 41 mM) in CH_2Cl_2 (40 ml) at 0 °C, and the mixture was stirred at room temperature for 1 h, The reaction mixture was quenched with water, and extracted with $CHCl_3$. The extract was washed with water, and brine, dried over Na_2SO_4, and concentrated. The residue, which is a mixture of mesylate and oxazinone, was, without purification, dissolved in 2.3 N HCl in EtOAc (65 ml) and the mixture was stirred at room temperature for 2.5 h. After removal of the solvent by evaporation *in vacuo*, the residue was dissolved in DMSO (20 ml) containing DBU (4.1 ml, 27.4 mM). The mixture was allowed to stand for 2.5 h, diluted with water (200 ml), then extracted with EtOAc. The extract was washed with water and brine, dried over Na_2SO_4, and concentrated. The residue was subjected on column chromatography (40% EtOAc in hexane) to give **12** (2.04 g, 50% overall yield from **6**) from the first eluate. Recrystallization from EtOH gave colorless crystals, mp 97—99 °C. IR ν_{max} (Nujol) cm^{-1}: 1720 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.7—3.1 (4H, m, $CH_2 \times 2$), 3.13 (1H, dd, J=8.0, 3.0 Hz, 1-H), 3.55 (3H, s, NCH_3), 3.80 (3H, s, $COOCH_3$), 4.30 (1H, t, J=8.0 Hz, 2-H), 5.10 (1H, br s, 10b-H), 5.22 (1H, d, J=10.0 Hz, $H\!\!>\!\!\underline{H}$), 5.39 (1H, d, J=17.0 Hz, $H\!\!>\!\!\underline{H}$), 5.91 (1H, ddd, J=17.0, 10.0, 8.0 Hz, =CH), 7.20 (3H, m, ArH), 7.55 (1H, d, J=7.5 Hz, 8-H). ^{13}C-NMR ($CDCl_3$) δ: 16.5 (t), 29.8 (q), 41.1 (t), 48.6 (d), 52.5 (q), 54.1 (d), 61.2 (d), 108.1 (s), 109.3 (d), 118.8 (d), 119.0 (d), 121.0 (d), 127.3 (s), 134.0 (s), 136.4 (s), 137.3 (d), 172.0 (s). MS *m/z*: 296 (M^+). *Anal.* Calcd for $C_{18}H_{20}N_2O_2$: C, 72.95; H, 6.80; N, 9.45. Found: C, 72.93; H, 6.82; N, 9.42. The second eluate with the same solvent gave **10** (0.44 g, 9.5% overall yield from **6**). Recrystallization from EtOH gave colorless crystals, mp 170—172 °C. IR ν_{max} (Nujol) cm^{-1}: 1720, 1680 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.7—3.1 (3H, m, 7-CH_2, 6-H), 3.33 (3H, s, NCH_3), 3.41 (1H, dd, J=4.0, 2.0 Hz, 1-H), 4.68 (3H, s, $COOCH_3$), 4.76 (1H, br d, J=12.0 Hz, 6-H), 5.11 (1H, br d, J=4.0 Hz, 12b-H), 5.18 (1H, br q, J=2.0 Hz, 2-H), 5.49 (1H, d, J=10.0 Hz, $H\!\!>\!\!\underline{H}$), 5.55 (1H, d, J=16.0 Hz, $H\!\!>\!\!\underline{H}$), 5.98 (1H, ddd, J=16.0, 10.0, 4.0 Hz, =CH), 7.19 (3H, m, ArH), 7.50 (1H, d, J=7.5 Hz, 8-H). MS *m/z* 340 (M^+). *Anal.* Calcd for $C_{19}H_{20}N_2O_4$: C, 67.04; H, 5.92; N, 8.23. Found: C, 67.08; H, 5.91; N, 8.01. The third eluate with the same solvent gave **13** (263 mg, 6.5% overall yield from **6**). Recrystallization from EtOH–hexane (3 : 1) gave colorless crystals, 117—119 °C. IR ν_{max} (Nujol) cm^{-1}: 1720 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.70—3.10 (4H, m, $CH_2 \times 2$), 2.88 (1H, t, J=4.0 Hz, 2-H), 3.59 (3H, s, NCH_3), 3.78 (3H, s, $COOCH_3$), 4.54 (1H, dd, J=10.0, 4.0 Hz, 2-H), 5.02 (1H, br s, 12b-H), 5.08 (1H, d, J=10.0 Hz, $H\!\!>\!\!\underline{H}$), 5.21 (1H, d, J=17.0 Hz, $H\!\!>\!\!\underline{H}$), 5.93 (1H, dt, J=17.0, 10.0 Hz, =CH), 7.15 (3H, m, ArH), and 7.49 (1H, d, J=7.5 Hz, 8-H). ^{13}C-NMR ($CDCl_3$) δ: 21.8 (t), 30.0 (q), 41.1 (t), 47.7 (d), 52.7 (q), 56.5 (d), 68.5 (d), 108.0 (s), 109.3 (d), 119.0 (d), 119.6 (d), 120.6 (t), 122.3 (d), 127.6 (s), 135.1 (s), 137.7 (s), 138.2 (d), 173.7 (s). MS *m/z*: 296 (M^+). *Anal.* Calcd for $C_{18}H_{20}N_2O_2$: C, 72.95; H, 6.80; N, 9.45. Found: C, 72.82; H, 6.84; N, 9.36.

Reaction of 6 with MsCl and TEA A solution of MsCl (0.1 ml, 1.29 mM)

in CH_2Cl_2 (3 ml) was added dropwise to a solution of **6** (385 mg, 0.86 mM) and TEA (0.36 ml, 2.58 mM) in CH_2Cl_2 (2 ml) at 0 °C, and the mixture was stirred at room temperature for 1 h. Work-up gave a crude oil, which was submitted to column chromatography (20% EtOAc in hexane) to give methyl 2-(2-*tert*-butoxycarbonyl-9-methyl-1,2,3,4-tetrahydro-β-carbolin-1-yl)-3-methanesulfonyloxy-4-pentenoate (**8**) (234 mg, 55%) as an oily mixture of diastereomers from the first fraction. IR ν_{max} (neat) cm^{-1}: 1720, 1690 (CO), 1350, 1145 (SO_2). The ^{1}H-NMR spectrum was not sufficiently well resolved to permit assignment of the signals. Selected ^{1}H-NMR data were as follows: δ ($CDCl_3$) 1.40 [$C(CH_3)_3$], 2.85 (OSO_2CH_3), 3.40, 3.50 (NCH_3 and/or $COOCH_3$), 4.35 (1-H), 5.4—5.7 (=CH_2, $CHOSO_2$), 6.35 (=CH), 7.1—7.5 (ArH). MS *m/z*: 492 (M^+). HRMS Calcd for $C_{24}H_{31}N_2O_7S$: 492.1927. Found: 492.1896. The second fraction eluted with 40% EtOAc in hexane gave **10** (42 mg, 14%), which was identical with an anthentic sample (**10**), based on comparison of their IR and ^{1}H-NMR spectra.

Synthesis of 12 (and 13) from 8 The mesylate (**8**) (234 mg, 0.48 mM) was dissolved in 2.3 N HCl/EtOAc (2 ml), and the mixture was stirred for 1 h. After removal of the solvent by evaporation, the residue was dissolved in DMSO (1 ml) containing DBU (80 mg, 0.53 mM). The mixture was allowed to stand for 3 h, diluted with water (10 ml), then extracted with EtOAc. The extract was washed with water and brine, dried over Na_2SO_4, and concentrated. The residue was purified by column chromatography (40% EtOAc in hexane) to give **12** (77 mg, 54%) and **13** (9.2 mg, 6.5%), which were identical with an authentic samples (**12** and **13**), based on comparison of the IR and ^{1}H-NMR spectra.

Reaction of 7 with MsCl and TEA By a similar procedure to that described for the reaction of **6** with MsCl and TEA, a crude product was obtained from **7** (5.67 g, 12.8 mM), MsCl (1.48 ml, 19.2 mM), and TEA (5.39 ml, 38.4 mM). It was subjected to column chromatography. The first fraction eluted with 20% EtOAc in hexane gave methyl 2-(*tert*-butoxycarbonyl-9-methyl-1,2,3,4-tetrahydro-β-carbolin-1-yl)-3-methanesulfonyloxy-4-methylenehexanoate (**9**) (2.25 g, 34%) as a semi-solid. Recrystallization from EtOH gave colorless crystals, mp 155—157 °C. IR ν_{max} (Nujol) cm^{-1}: 1730, 1685 (CO), 1350, 1165 (SO_2). ^{1}H-NMR ($CDCl_3$) δ: 1.06 (3H, t, *J*=7.5 Hz, $CH_2C\underline{H}_3$), 1.53 [9H, s, $C(CH_3)_3$], 1.97—2.24 (2H, m, $C\underline{H}_2CH_3$), 2.58 (3H, s, SCH_3), 2.66—2.87 (3H, m, 4'-H_2, 3'-H), 3.38 (3H, s, NCH_3), 3.50 (1H, dd, *J*=10.0, 5.0 Hz, CHCOO), 3.72 (3H, s, $COOCH_3$), 4.37 (1H, dd, *J*=14.0, 5.0 Hz, 2-H), 5.12, 5.32 (each 1H, each s, =CH_2), 5.63 (1H, d, *J*=10.0 Hz, $CHOSO_2$), 5.87 (1H, br d, *J*=4.0 Hz, ArCH), 7.16 (3H, m, ArH), 7.43 (1H, d, *J*=7.5 Hz, 8-H). MS *m/z*: 520 (M^+). *Anal.* Calcd for $C_{26}H_{36}N_2O_7S$: C, 59.98; H, 6.97; N, 5.38. Found: C, 59.95; H, 6.98; N, 5.45. The second fraction eluted with 40% EtOAc in hexane gave 2-(1-buten-1-yl)-1-methoxycarbonyl-12-methyl-1,6,7,12b-tetrahydro-2*H*,4*H*-indolo[2,3-*c*]pyrido[1,2-*c*][1,3]-oxazin-2-one (**11**) (498 mg, 11%). Recrystallization from EtOH gave colorless crystals, mp 173—174 °C. IR ν_{max} (Nujol) cm^{-1}: 1720, 1680 (CO). ^{1}H-NMR ($CDCl_3$) δ: 1.20 (3H, t, *J*=7.5 Hz, $CH_2C\underline{H}_3$), 1.96—2.29 (2H, m, $C\underline{H}_2CH_3$), 2.70—3.05 (3H, m, 6-H and 7-H_2), 3.30 (3H, s, NCH_3), 3.47 (1H, dd, *J*=4.5, 2.5 Hz, 1-H), 3.64 (3H, s, $COOCH_3$), 4.73 (1H, m, 6-H), 5.05 (2H, br s, 12b-H, 2-H), 5.20, 5.27 (each 1H, each s, =CH_2), 7.17 (3H, m, ArH), 7.49 (1H, d, *J*=7.5 Hz, 11-H). MS *m/z*: 368 (M^+). *Anal.* Calcd for $C_{21}H_{24}N_2O_4$: C, 68.46; H, 6.57; N, 7.60. Found: C, 68.38; H, 6.55; N, 7.54.

***cis*-Methyl 2-(1-Buten-2-yl)-10-methyl-1,4,5,10b-tetrahydro-2*H*-azeto-[1',2':1,2]pyrido[3,4-*b*]indole-1-carboxylate (14)** By a similar procedure to that described for the preparation of **12** (and **13**), a crude product was obtained from **9** (539 mg, 1 mM). It was purified by column chromatography (40% EtOAc in hexane) to give **14** (139 mg, 43%). Recrystalization from EtOH gave colorless crystals, mp 132—134 °C. IR ν_{max} (Nujol) cm^{-1}: 1720 (CO). ^{1}H-NMR ($CDCl_3$) δ: 1.0 (3H, t, *J*=7.5 Hz, $CH_2C\underline{H}_3$), 1.70—2.12 (2H, m, $C\underline{H}_2CH_3$), 2.66—3.11 (4H, m, CH_2 × 2), 3.20 (1H, dd, *J*=7.5, 2.5 Hz, 1-H), 3.56 (3H, s, NCH_3), 3.72 (3H, s, $COOCH_3$), 4.27 (1H, d, *J*=7.5 Hz, 2-H), 5.0, 5.42 (each 1H, each s, =CH_2), 5.10 (1H, d, *J*=2.5 Hz, 12b-H), 7.1—7.34 (3H, m, ArH), 7.55 (1H, d, *J*=7.5 Hz, 9-H). MS *m/z*: 324 (M^+). *Anal.* Calcd for $C_{20}H_{24}N_2O_2$: C, 74.07; H, 7.46; N, 8.64. Found: C, 73.97; H, 7.46; N, 8.51.

2-Ethenyl-1-methoxycarbonyl-11-methyl-1,6,7,12b-tetrahydro-2*H*,4*H*-indolo[2,3-*c*]pyrido[1,2-*c*][1,3]oxazin-4-ones (15 and 16) A solution of **6** (1.17 g, 2.8 mM) in 2.3 N HCl in EtOAc solution (6.5 ml) was stirred at room temperature for 1 h. After removal of the solvent by evaporation, the residue was neutralized with saturated $NaHCO_3$ solution and extracted with EtOAt. The extract was washed with brine, dried over Na_2SO_4, and concentrated to give the de-Boc derivative (873 mg, 99%) of **6** as a viscous oil. A solution of the de-Boc derivative (873 mg, 2.78 mM) thus obtained and Im_2CO (541 mg, 3.34 mM) in benzene (6 ml) was refluxed for 1 h. The reaction mixture was diluted with EtOAc (20 m), washed with water, dried over Na_2SO_4, and concentrated. The residue was subjected to column chromatography (15% EtOAc in benzene) to give **15** (482 mg, 54%) from the first eluate. Recrystallization from EtOH gave colorless crystals, mp 167—169 °C. IR ν_{max} (Nujol) cm^{-1}: 1735, 1680 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.6—3.0 (3H, m, 7-H_2, 6-H), 3.10 (1H, t, *J*=5.6 Hz, 1-H), 3.53 (3H, s, NCH_3), 3.76 (3H, s, $COOCH_3$), 4.63 (1H, q, *J*=10.0 Hz, 6-H), 4.96 (1H, t, *J*=5.6 Hz, 2-H), 5.20 (1H, d, *J*=10.5 Hz, H⟩=⟨H H), 5.35 (1H, d, *J*=17.0 Hz, H⟩=⟨H H), 5.39 (1H, d, *J*=5.6 Hz, 12b-H), 5.72 (1H, ddd, *J*=17.0, 10.5, 5.6 Hz, =CH), 7.16 (3H, m, ArH), 7.49 (1H, d, *J*=7.5 Hz, 11-H). MS *m/z*: 340 (M^+). *Anal.* Calcd for $C_{19}H_{20}N_2O_4$: C, 67.04; H, 5.92; N, 8.23. Found: C, 66.74; H, 5.92; N, 8.13. The second eluate with the same solvent gave **16** (211 mg, 23%), which was recrystallized from EtOH to give colorless crystals, mp 198—200 °C. IR ν_{max} (Nujol) cm^{-1}: 1740, 1680 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.85 (3H, m, 7-H_2, 6-H), 3.26 (3H, s, NCH_3), 3.71 (3H, s, $COOCH_3$), 3.62 (1H, dd, *J*=5.8, 2.8 Hz, 1-H), 4.82 (1H, m, 6-H), 5.04 (1H, br s, 2-H), 5.23 (1H, d, *J*=5.8 Hz, 12b-H), 5.32 (1H, d, *J*=10.0 Hz, H⟩=⟨H H), 5.54 (1H, d, *J*=17.5 Hz, H⟩=⟨H H), 5.86 (1H, ddd, *J*=17.5, 10.0, 5.8 Hz, =CH), 7.17 (3H, m, ArH), 7.49 (1H, d, *J*=7.5 Hz, 11-H). MS *m/z*: 340 (M^+). *Anal.* Calcd for $C_{19}H_{20}N_2O_4$: C, 67.04; H, 5.92; N, 8.23. Found: C, 67.03; H, 5.98; N, 8.16.

Methyl 2-Ethyl-1-methoxycarbonyl-11-methyl-1,6,7,12b-tetrahydro-2*H*,4*H*-indolo[2,3-*c*]pyrido[1,2-*c*][1,3]oxazin-4-one (17) A solution of **16** (0.1 g, 0.29 mM) in EtOAc (20 ml) was hydrogenated under atmospheric pressure with 5% Pd–C (50 mg) for 3 h. The catalyst was removed by filtration, and the filtrate was concentrated. The residue was purified by column chromatography (15% EtOAc in hexane) to give **17** (75 mg, 75%) as an oil. IR ν_{max} (neat) cm^{-1}: 1730, 1685 (CO). ^{1}H-NMR ($CDCl_3$) δ: 1.09 (3H, t, *J*=7.5 Hz, $CH_2C\underline{H}_3$), 1.5—1.9 (2H, m, $C\underline{H}_2CH_3$), 2.6—3.0 (3H, m, 7-H_2, 6-H), 3.30 (3H, s, NCH_3), 3.70 (3H, s, $COOCH_3$), 3.55 (1H, dd, *J*=5.3, 3.0 Hz, 1-H), 4.40 (1H, m, 2-H), 4.81 (1H, m, 6-H), 5.15 (1H, d, *J*=5.3 Hz, 12b-H), 7.1—7.3 (3H, m, ArH), 7.48 (1H, d, *J*=7.5 Hz, 11-H). MS *m/z*: 342 (M^+). HRMS Calcd for $C_{19}H_{22}N_2O_4$: 342.1578. Found: 342.1574.

Preparation of Indoloquinolizidines (18, 19, and 20). Methyl 12-Methyl-3,6,7,12b-tetrahydro-4*H*-indolo[2,3-*a*]quinolizine-1-carboxylate (18) and Methyl 12-Methyl-1,6,7,12b-tetrahydro-4*H*-indolo[2,3-*a*]quinolizine-1-carboxylates (19 and 20) A solution of **15** (204 mg, 0.6 mM) and DBU (100 mg, 0.66 mM) in DMSO (3 ml) was heated at 120 °C for 4 h. The reaction mixture was diluted with EtOAc (40 ml), washed with water and brine, dried over Na_2SO_4, and concentrated. Purification of the residue by column chromatography afforded **20** (6 mg, 3.4%) (less polar), **19** (24 mg, 14%) (intermediate polar) by elution with 50% EtOAc in hexane, and **18** (54 mg, 30%) (more polar) by elution with EtOAc. The spectral data were in fair agreement with the reported values.[12]

18: IR ν_{max} ($CHCl_3$) cm^{-1}: 1700 (CO). ^{1}H-NMR ($CDCl_3$) δ: 3.54 (3H, 3H, NCH_3), 3.81 (3H, s, $COOCH_3$), 5.33 (1H, s, 2-H), 7.0—7.35 (3H, m, ArH), 7.58 (1H, d, *J*=7.5 Hz, 11-H). MS *m/z*: 296 (M^+). HRMS Calcd for $C_{18}H_{20}N_2O_2$: 296.1523, Found: 296.1541.

19: IR ν_{max} ($CHCl_3$) cm^{-1}: 1720 (CO). ^{1}H-NMR ($CDCl_3$) δ: 3.48 (3H, s, NCH_3), 3.71 (3H, s, $COOCH_3$), 3.95 (1H, br d, *J*=17.5 Hz, 4-H_{ax}), 4.5 (1H, d, *J*=9.5 Hz, 12b-H), 5.70, 5.95 (each 1H, each br d, *J*=10.5 Hz, 2-H, 3-H), 7.1—7.3 (3H, m, ArH), 7.45 (1H, d, *J*=7.5 Hz, 11-H). MS *m/z*: 296 (M^+). HRMS Calcd for $C_{18}H_{20}N_2O_2$: 296.1523. Found: 296.1497.

TABLE III. Crystal Data for Two Isomeric Tetrahydro-3,5-oxazin-4-ones (**10** and **15**)

	10	**15**
Molecular formula	$C_{19}H_{20}N_2O_4$	$C_{19}H_{20}N_2O_4$
Molecular weight	340.379	340.379
Crystal system	Orthorhombic	Orthorhombic
Space group	$P2_12_12_1$	*Pbca*
Cell constant (Å)		
a	9.235 (2)	13.594 (3)
b	23.328 (3)	8.768 (1)
c	7.799 (1)	28.402 (6)
Volume (Å^3)	1680.2 (5)	3342 (1)
Z	4	8
D_x (g·cm^{-3})	1.3456	1.3529

20: IR ν_{max} ($CHCl_3$) cm^{-1}: 2800—2750 (Bohlmann bands), 1720 (CO). ^{1}H-NMR ($CDCl_3$) δ: 3.26 (3H, s, NCH_3), 3.73 (3H, s, $COOCH_3$), 3.98 (1H, br s, 12b-H), 5.95, 6.03 (each 1H, each br d, J=10.5 Hz, 2-H, 3-H), 7.0—7.3 (3H, m, ArH), 7.46 (1H, d, J=7.5 Hz, 11-H). MS m/z: 296 (M^+). HRMS Calcd for $C_{18}H_{20}N_2O_2$: 296.1523. Found: 296.1536.

X-Ray Determinations of Compounds 10 and 15 A single crystal (colorless needles) of **10** or **15**, recrystallized from EtOH, was used for the X-ray studies. The crystal data are summarized in Table III. Unit-cell dimensions were determined on a Rigaku four-circle diffractometer using high-angle reflections (2θ) by empolying graphite-monochromated Cu K_α radiation and were refined by the least-squares method. A total of 1570 (**10**) or 2670 (**15**) independent reflections ($2\theta_{max} < 130°$) was measured using the ω–2θ scan mode and a scan rate of 4°/min. Both structures were solved by the direct method using the MULTAN program[14] and refined by the least-squares method to R=0.0895 (**10**) and 0.0745 (**15**).

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Synthesis of (±)-D-Homocephalotaxine, an Unnatural Analogue of Cephalotaxine

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(±)-D-Homocephalotaxine (2), an unnatural analogue of cephalotaxine, has been synthesized by a synthetic sequence involving an acid-catalyzed cyclization of the α-sulfinylacetamide 8 as a key step.

Keywords cationic cyclization; Pummerer reaction; sulfoxide; α-chlorosulfide; α-thiocarbocation; cephalotaxine analogue; D-homocephalotaxine

Cephalotaxine (**1**), the predominant alkaloid of *Cephalotaxus* species, has attracted much attention from synthetic chemists due to its unique structural features as well as the antileukemic activities of its ester derivatives, harringtonine and homoharringtonine.[1-3] So far, six total syntheses of **1**, including ours, have been reported.[4-9] In addition, considerable efforts to synthesize a variety of structural analogues of cephalotaxine have been made.[6,10-15]

As a part of our studies on carbon–carbon bond-forming reactions by using α-thiocarbocations,[16] we have recently reported a new entry to 3-methyl-1,3,4,5-tetrahydro-2*H*-3-benzazepin-2-ones which involves the acid-catalyzed cyclization of *N*-(2-arylethyl)-*N*-methyl-α-sulfinylacetamides.[17] As an extension of this work, it appeared to be of interest to see if the method could be applied to more complex systems such as **8**, because it has been reported that attempts to cyclize a variety of *N*-substituted derivatives of either the amine **3** or lactam **4** to the benzazepine ring system failed.[10-12,18] We describe here a successful application of our method to the synthesis of (±)-D-homocephalotaxine (**2**), an unnatural analogue of cephalotaxine (**1**).

The sulfoxide **8** required to test the feasibility of the cyclization was prepared by acylation of the readily available amine **3**[10-12] with (methylthio)acetic acid in the presence of 1,3-dicyclohexylcarbodiimide (DCC) followed by oxidation of the resulting sulfide **7** with sodium metaperiodate ($NaIO_4$) in aqueous methanol.

Cyclization of the sulfoxide **8** to the benzazepinone **10** was conducted under the Pummerer reaction conditions described previously.[17] Thus, treatment of **8** with trifluoroacetic anhydride (TFAA) in dichloromethane at room temperature for 24 h (procedure A) gave the benzazepinone **10** as an inseparable mixture of two diastereomers (*ca.* 10:1) due to the methylthio group in 63% yield. The benzazepinone **10** was also obtained in 65% yield, when **8** was treated with 2 eq of anhydrous *p*-toluenesulfonic acid (*p*-TsOH) in boiling 1,2-dichloroethane for 15 min (procedure B). Evidence that cyclization had occurred was given by the ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrum of the major isomer, which showed signals due to only two aromatic protons at δ 6.63 (s) and 6.83 (s) as well as a methine proton (H-12) at δ 4.48 (s) (the corresponding signals for the minor product appeared at δ 6.91, 7.47, and 5.30). Desulfurization of **10** with zinc in acetic acid gave the benzazepinone **11** in quantitative yield.

For comparison, a Lewis acid-catalyzed cyclization of the α-chlorosulfide **9** was also examined. Thus, treatment of **9**, prepared by direct acylation of **3** with chloro(methylthio)-acetyl chloride, with stannic chloride ($SnCl_4$) in dichloromethane at 0 °C (procedure C) gave the same benzazepinone **10**, but in lower yield (34%).

Introduction of the oxygen functionalities into the D-ring essentially followed the method of Kuehne and coworkers.[8] The benzazepinone **11** was oxidized with a catalytic amount of osmium tetroxide (OsO_4) in the presence of *N*-methylmorpholine *N*-oxide to give the diol **12** as a diastereomeric mixture (*ca.* 7:3). Oxidation of the crude diol **12** with Corey–Kim reagent[19] [*N*-chlorosuccinimide (NCS) and dimethyl sulfide] gave the α-diketone **13** in 77% overall yield (from **11**). The ^{1}H-NMR spectrum of **13** showed a singlet at δ 5.96 due to the olefinic proton at C_1, indicating that the α-diketone exists in the enolic form, as shown in Chart 2. The α-diketone **13** was then methylated by using Ponaras' procedure[20] [methoxytrimethylsilane (TMSOMe) in the presence of trifluoromethanesulfonic acid in dichloromethane] to give two isomeric methyl ethers **14a** (63%) and **14b** (16%), along with a small amount of the starting material **13**. The structures of **14a, b** were apparent from an examination of the ^{1}H-NMR spectra: the olefinic proton (H-1) signal of **14a** appeared at δ 5.68 as a singlet, whereas the olefinic proton (H-4) signal of **14b** appeared at δ 5.72 as a doublet (J=4.0 Hz) due to coupling with the C_5 proton at δ 4.27 (d, J=4.0 Hz).

Reduction of the resultant enone **14a** with sodium borohydride in methanol at 0 °C resulted in the formation of a mixture of two alcohols, **15a** (63%) and **15b** (36%). The stereochemical assignments of **15a, b** were made by

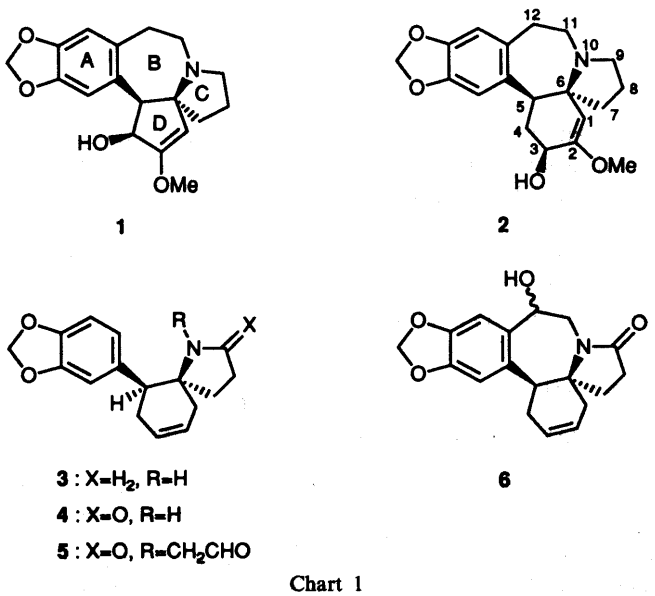

Chart 1

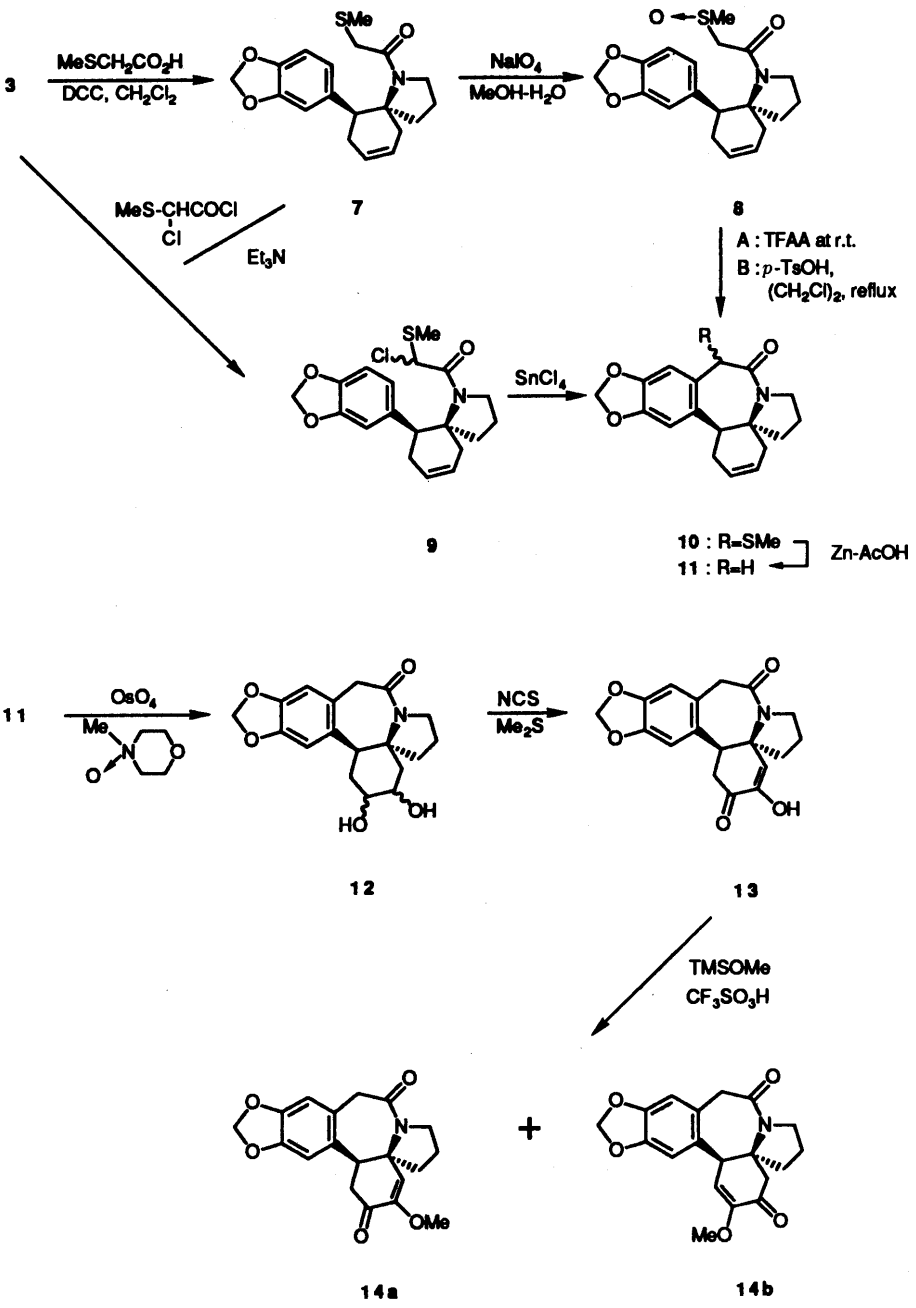

Chart 2

examining the ^{1}H-NMR spectra. The distinguishing feature of the ^{1}H-NMR spectrum of **15a** is the C_3 proton signal which displays relatively small couplings of 4.6 and 2.7 Hz. In contrast, the same proton of **15b** exhibits greater splittings (J = 10.4 and 5.1 Hz). These coupling patterns, along with the fact that the C_5 proton signal in both compounds appears as a triplet with J = 3.9 Hz, are consistent with the assigned conformations in which the hydroxyl group is axial in **15a** and equatorial in **15b**. The preferential formation of **15a** from **14a** can be rationalized by assuming the conformation of **14a** to be as shown in Chart 3, in which hydride attacks the convex face of the carbonyl group. In fact, when **14a** was reduced with bulkier lithium tri-*sec*-butylborohydride (L-Selectride), **15a** was obtained exclusively.

Finally, reduction of **15a** with sodium bis(2-methoxyethoxy)aluminum hydride (Red-Al) gave the desired (±)-D-homocephalotaxine (**2**) in 60% yield. The structure of **2** was fully supported by the spectroscopic data (see Experimental).

In summary, the acid-catalyzed aromatic cyclization of the α-sulfinylacetamides opens up a new route to cephalotaxine and its analogues.

Experimental

Melting points are uncorrected. Infrared (IR) spectra were recorded with a JASCO IRA-1 spectrophotometer. ^{1}H-NMR spectra were determined with a JEOL JNM-PMX 60 (60 MHz) or a Varian XL-300 (300 MHz) spectrometer and C-13 nuclear magnetic resonance (^{13}C-NMR) spectra with a Varian XL-300 (75 MHz) spectrometer, using tetramethylsilane as an internal standard. High resolution mass spectra (MS) were obtained with a Hitachi M-80 instrument at 20 eV. Column chromatography was performed on Silica gel 60 PF_{254} (Merck) under

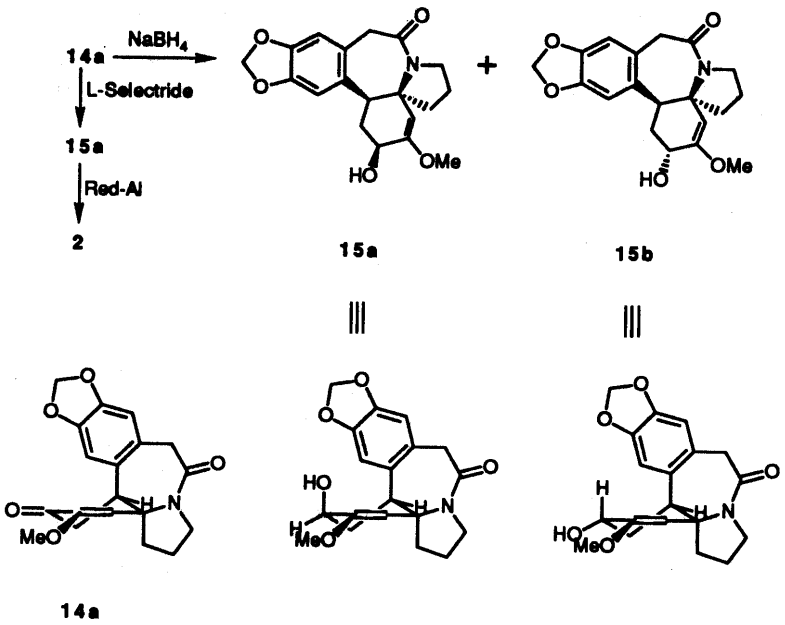

Chart 3

pressure.

(5*S,10*S**)-10-(1,3-Benzodioxol-5-yl)-1-[(methylthio)acetyl]-1-azaspiro-[4.5]dec-7-ene (7)** The Red-Al reduction was done essentially according to the procedure of Bryce *et al.*[10] Red-Al (70% in toluene, 9 ml, 30 mmol) was added dropwise to a solution of **4** (1.36 g, 5.00 mmol) in dry benzene (50 ml) and the mixture was heated under reflux overnight. Water (30 ml) was then added under ice-cooling to the mixture to destroy the excess reagent and the whole was made alkaline with saturated $NaHCO_3$ solution. The precipitates were filtered off and the organic layer was separated. The aqueous layer was further extracted with dichloromethane. The combined extracts were dried (Na_2SO_4) and concentrated to give the amine **3** (1.19 g) as an oil, which was used in the next stage without further purification.

(Methylthio)acetic acid (542 mg, 4.92 mmol) and DCC (1.04 g, 4.92 mmol) were added to a solution of **3** (1.15 g, 4.45 mmol) in dry dichloromethane (35 ml) at 0 °C and the mixture was stirred at room temperature for 48 h. Precipitated dicyclohexylurea was filtered off and the filtrate was concentrated. The residual oil was chromatographed on silica gel (hexane–AcOEt, 1 : 1) to give **7** (1.47 g, 90% from **3**) as an oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1645. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.5—3.8 (13H, m), 2.10 (3H, s, SMe), 5.7—5.9 (2H, m, CH=CH), 5.84 (2H, s, OCH_2O), 6.6—6.8 (3H, m, aromatic protons). Exact MS *m/z*: Calcd for $C_{19}H_{23}NO_3S$: 345.1396. Found: 345.1367.

(5*S,10*S**)-10-(1,3-Benzodioxol-5-yl)-1-[(methylsulfinyl)acetyl]-1-azaspiro[4.5]dec-7-ene (8)** A solution of $NaIO_4$ (451 mg, 2.11 mmol) in water (12 ml) was added dropwise to an ice-cooled solution of **7** (665 mg, 1.92 mmol) in methanol (6.0 ml) and the mixture was stirred at room temperature overnight. The precipitated inorganic material was removed by filtration and water (10 ml) was added. The aqueous layer was extracted with dichloromethane and the extract was washed with brine, dried ($MgSO_4$), and concentrated. The residue was chromatographed on silica gel (CH_2Cl_2–MeOH, 10 : 1) to give the sulfoxide **8** (607 mg, 87%), which solidified on standing. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1650. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.6—4.0 (13H, m), 2.60 (3H, br s, SMe), 5.7—6.0 (2H, m, CH=CH), 5.82 (2H, s, OCH_2O), 6.5—6.8 (3H, m, aromatic protons). The sulfoxide **8** was used in the next stage without further purification.

(5*S,6*S**)-12-Methylthio-4,5,8,9,11,12-hexahydro-1*H*,7*H*-[1,3]benzodioxolo[5,6-*d*]pyrrolo[2,1-*k*][1]benzazepin-11-one (10)** Procedure A: Trifluoroacetic anhydride (0.064 ml, 0.28 mmol) was added dropwise to a solution of the sulfoxide **8** (100 mg, 0.28 mmol) in dry dichloromethane (1.5 ml) at 0 °C under nitrogen and the mixture was stirred at room temperature for 24 h. Dichloromethane (10 ml) was added and the whole mixture was washed with saturated $NaHCO_3$ solution and brine, dried ($MgSO_4$), and concentrated. The residue was chromatographed on silica gel (hexane–AcOEt, 2 : 1) to give **10** (60 mg, 63%) as an inseparable mixture of two diastereomers (*ca.* 10 : 1) due to the methylthio group: mp 192—194 °C (from MeOH). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1610. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ (only the signals for the major isomer are recorded): 1.45—2.08 (6H, m), 2.27 (3H, s, SMe), 2.58 (2H, br), 3.31—3.41 (1H, m), 4.08—4.25 (2H, m), 4.48 (1H, s, H-12), 5.53—5.62, 5.84—5.92 (1H each, 2×m, CH=CH), 5.94 (2H, s, OCH_2O), 6.63, 6.83 (1H each, 2×s, aromatic protons). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ (only the signals for the major isomer are recorded): 17.58 (SMe), 18.69, 30.10, 30.99, 36.35, 40.11 (C-5), 46.41, 57.61 (C-12), 66.94 (C-6), 101.23 (OCH_2O), 103.37, 110.31, 125.54, 127.11, 128.24, 132.83, 145.99, 147.36, 166.41 (C-11). *Anal.* Calcd for $C_{19}H_{21}NO_3S$: C, 66.45; H, 6.16; N, 4.08. Found: C, 66.56; H, 6.12; N, 4.18.

Procedure B: A solution of *p*-toluenesulfonic acid monohydrate (700 mg, 3.68 mmol) in 1,2-dichloroethane (10 ml) was heated under reflux with azeotropic removal of water for 2 h, then cooled under a nitrogen atmosphere. A solution of the sulfoxide **8** (664 mg, 1.84 mmol) in 1,2-dichloroethane (4 ml) was added to the above solution containing anhydrous *p*-toluenesulfonic acid, and the mixture was heated again under reflux with azeotropic removal of water for 15 min. The reaction mixture was washed with saturated $NaHCO_3$ solution and brine, dried ($MgSO_4$), and concentrated. The residue was chromatographed on silica gel (hexane–AcOEt, 2 : 1) to give **10** (407 mg, 65%).

Preparation and Cyclization of 10-(1,3-Benzodioxol-5-yl)-1-[chloro-(methylthio)acetyl]-1-azaspiro[4.5]dec-7-ene (9) Procedure C: A solution of chloro(methylthio)acetyl chloride (610 mg, 3.84 mmol) in dry ether (7.5 ml) was added to a solution of the crude amine **3** (758 mg, 2.95 mmol) and triethylamine (0.63 ml, 4.43 mmol) in dry ether (10 ml) at 0 °C and the mixture was stirred for 30 min. The precipitates were filtered off and the filtrate was concentrated to give crude **9** as an oil, which was dissolved in dry dichloromethane (10 ml). $SnCl_4$ (0.52 ml, 4.43 mmol) was added to the above solution at 0 °C and the mixture was stirred at the same temperature for 2 h. The reaction was quenched with water and the organic layer was separated, washed with brine, dried ($MgSO_4$), and concentrated. The residue was chromatographed on silica gel (hexane–AcOEt, 2 : 1) to give **10** (345 mg, 34% based on **3**).

(5*S,6*S**)-4,5,8,9,11,12-Hexahydro-1*H*,7*H*-[1,3]benzodioxolo[5,6-*d*]-pyrrolo[2,1-*k*][1]benzazepin-11-one (11)** Zinc dust (458 mg, 7.00 mmol) was added to a solution of **10** (240 mg, 0.700 mmol) in acetic acid (1 ml) and the mixture was heated with vigorous stirring at 100—110 °C overnight, then cooled. Dichloromethane (10 ml) was added and the inorganic material was filtered off. The mother liquor was concentrated *in vacuo* and the residue was chromatographed on silica gel (hexane–AcOEt, 1 : 1) to give **11** (208 mg, quant.), mp 223—224 °C (from MeOH). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1600. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.4—2.7 (8H, m), 2.8—3.6 (2H, m), 3.49, 4.00 (1H each, ABq, J=16 Hz, H-12), 3.8—4.5 (1H, m), 5.3—6.1 (2H, m, CH=CH), 5.88 (2H, s, OCH_2O), 6.64, 6.82 (1H each, 2×s, aromatic protons). *Anal.* Calcd for $C_{18}H_{19}NO_3$: C, 72.71; H, 6.44; N, 4.71. Found: C, 72.72; H, 6.30; N, 4.78.

(5*S,6*R**)-2-Hydroxy-4,5,8,9,11,12-hexahydro-3*H*,7*H*-[1,3]benzodioxolo[5,6-*d*]pyrrolo[2,1-*k*][1]benzazepine-3,11-dione (13)** *N*-Methyl-

morpholine *N*-oxide (1.38 g, 11.8 mmol) and a catalytic amount of OsO_4 was added to a solution of **11** (700 mg, 2.35 mmol) in *tert*-butanol (32 ml), tetrahydrofuran (THF) (36 ml), and water (4 ml) and the mixture was stirred overnight. Saturated $NaHSO_3$ solution (15 ml) was added, and the whole was stirred for 20 min. The organic layer was separated and the aqueous layer was extracted with ethyl acetate. The combined organic layers were concentrated *in vacuo* and the residue was chromatographed on silica gel (CH_2Cl_2–MeOH, 15:1) to give the crude diol **12** (723 mg, 93%) as a mixture of two diastereomers (*ca.* 7:3 from the ^{1}H-NMR spectrum), which was used in the next step without further purification.

A solution of the crude diol **12** (375 mg, 1.13 mmol) in dry dichloromethane (30 ml) was added dropwise to a solution of Corey–Kim reagent[19] [prepared from *N*-chlorosuccinimide (754 mg, 5.65 mmol) and dimethyl sulfide (0.414 ml, 5.65 mmol) in dichloromethane (14 ml)] at −42 °C and the mixture was stirred for 2 h. Triethylamine (1.26 ml, 9.04 mmol) was added and the whole was stirred at room temperature overnight. The reaction mixture was poured into brine (50 ml) and extracted with dichloromethane. The extract was dried ($MgSO_4$) and concentrated, and the residue was chromatographed on silica gel (AcOEt) to give **13** (306 mg, 83%), mp 239—240 °C (from MeOH). IR ν_{max}^{KBr} cm^{-1}: 3450, 1680, 1640, 1610. ^{1}H-NMR (DMSO-d_6, 300 MHz) δ: 1.75—1.93 (2H, m, H-8), 2.12 (1H, ddd, J=12.9, 11.0, 6.7 Hz, one of H-7), 2.61 (1H, ddd, J=12.9, 5.2, 3.4 Hz, one of H-7), 2.82 (1H, dd, J=16.7, 2.3 Hz, one of H-4), 3.11 (1H, d, J=14.7 Hz, one of H-12), 3.24 (1H, dd, J=16.6, 5.2 Hz, one of H-4), 3.47 (1H, dt, J=10.5, 4.0 Hz, one of H-9), 3.50—3.56 (1H, m, H-5), 4.12 (1H, dd, J=10.5, 5.2 Hz, one of H-9), 4.28 (1H, d, J=14.7 Hz, one of H-12), 5.96 (3H, s, OCH_2O, H-1), 6.68, 6.90 (1H each, 2×s, aromatic protons), 8.83 (1H, s, OH). ^{13}C-NMR (DMSO-d_6, 75 MHz) δ: 21.44, 39.27, 41.96, 45.16, 46.87 (C-5), 47.05, 62.57 (C-6), 100.92 (OCH_2O), 110.07, 110.90, 123.52 (C-1), 123.60, 131.62, 145.64, 146.69, 146.81, 169.37 (C-11), 193.69 (C-4). Exact MS *m/z*: Calcd for $C_{18}H_{17}NO_5$: 327.1105. Found: 327.1107. *Anal.* Calcd for $C_{18}H_{17}NO_5 \cdot CH_3OH$: C, 63.50; H, 5.89; N, 3.90. Found: C, 63.35: H, 5.95; N, 3.98.

(5*S,6*R**)-2-Methoxy-4,5,8,9,11,12-hexahydro-3*H*,7*H*-[1,3]benzodioxolo[5,6-*d*]pyrrolo[2,1-*k*][1]benzazepine-3,11-dione (14a) and (5*S**,6*R**)-3-Methoxy-1,2,8,9,11,12-hexahydro-5*H*,7*H*-[1,3]benzodioxolo[5,6-*d*]pyrrolo[2,1-*k*][1]benzazepine-2,11-dione (14b)** Methylation of **13** was conducted essentially according to the procedure of Ponaras and Meah.[20] Methoxytrimethylsilane (0.051 ml, 0.376 mmol) and trifluoromethanesulfonic acid (0.132 ml, 1.50 mmol) were added dropwise to a solution of **13** (123 mg, 0.376 mmol) in dry dichloromethane (20 ml) at 0 °C, and the mixture was stirred at room temperature for 72 h. The reaction mixture was washed with saturated $NaHCO_3$ solution and brine, dried ($MgSO_4$), and concentrated. The residue was submitted to preparative thin layer chromatography (silica gel, AcOEt) to give **14b** (20 mg, 16%), **14a** (80 mg, 63%), and the unreacted starting material **13** (25 mg).

Compound **14a** had mp 249—251 °C (from MeOH). IR ν_{max}^{KBr} cm^{-1}: 1685, 1635, 1620. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.85—2.10 (2H, m, H-8), 2.25 (1H, ddd, J=13.0, 11.3, 6.6 Hz, one of H-7), 2.61 (1H, ddd, J=13.0, 5.8, 3.0 Hz, one of H-7), 3.01 (1H, dd, J=16.4, 2.7 Hz, one of H-4), 3.13 (1H, dd, J=16.4, 5.4 Hz, one of H-4), 3.19 (1H, d, J=15.0 Hz, one of H-12), 3.51—3.56 (1H, br, H-5), 3.61 (1H, ddd, J=11.8, 9.8, 6.8 Hz, one of H-9), 3.66 (3H, s, OMe), 3.77 (1H, ddd, J=11.2, 8.0, 3.0 Hz, one of H-9), 4.42 (1H, d, J=15.0 Hz, one of H-12), 5.68 (1H, s, H-1), 5.90, 5.91 (1H each, ABq, J=1.4 Hz, OCH_2O), 6.51, 6.62 (1H each, 2×s, aromatic protons). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 22.06, 41.19, 43.19, 46.02, 47.62 (C-5), 47.67, 55.16 (OMe), 63.25 (C-6), 101.27 (OCH_2O), 110.54, 110.74, 120.73 (C-1), 123.47, 130.25, 146.64, 147.57, 150.12 (C-2), 170.83 (C-11), 191.61 (C-3). Exact MS *m/z*: Calcd for $C_{19}H_{19}NO_5$: 341.1262. Found: 341.1266.

Compound **14b** had mp 212—213 °C (from CH_2Cl_2–AcOEt). IR ν_{max}^{KBr} cm^{-1}: 1700, 1625. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.75—1.96 (4H, m, H-7, H-8), 2.61, 2.92 (1H each, ABq, J=16.1 Hz, H-1), 3.28 (1H, ddd, J=12.0, 8.4, 6.5 Hz, one of H-9), 3.45 (1H, d, J=15.3 Hz, one of H-12), 3.68 (3H, s, OMe), 4.06 (1H, ddd, J=12.0, 8.3, 7.6 Hz, one of H-9), 4.17 (1H, d, J=15.3 Hz, one of H-12), 4.27 (1H, d, J=4.0 Hz, H-5), 5.72 (1H, d, J=4.0 Hz, H-4), 5.97, 5.98 (1H each, ABq, J=1.4 Hz, OCH_2O), 6.72, 6.76 (1H each, 2×s, aromatic protons). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 18.65, 38.35, 41.92, 44.87, 46.54 (C-5), 46.67, 55.35 (OMe), 67.86 (C-6), 101.40 (OCH_2O), 110.10, 110.60, 115.83, 128.64, 128.78, 146.97, 147.44, 151.94, 167.67 (C-11), 191.07 (C-2). Exact MS *m/z*: Calcd for $C_{19}H_{19}NO_5$: 341.1261. Found: 341.1247.

Reduction of Compound 14a With $NaBH_4$: $NaBH_4$ (44 mg, 1.17 mmol) was added to a solution of **14a** (40 mg, 0.117 mmol) in MeOH (5 ml) and dichloromethane (2.5 ml) at 0 °C and the mixture was stirred at room temperature for 2 h. The mixture was diluted with water (5 ml) and extracted with dichloromethane, then the extract was dried ($MgSO_4$) and concentrated. The residue was chromatographed on silica gel. The first eluate gave (3*S**,5*S**,6*R**)-3-hydroxy-2-methoxy-4,5,8,9,11,12-hexahydro-3*H*,7*H*-[1,3]benzodioxolo[5,6-*d*]pyrrolo[2,1-*k*][1]benzazepin-11-one (**15a**) (25 mg, 63%) and the second eluate gave the isomeric alcohol **15b** (15 mg, 36%).

Compound **15a** had mp 217—218 °C (from CH_2Cl_2–AcOEt). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3570, 1660, 1625. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.26 (1H, d, J=8.3 Hz, OH), 1.70—1.96 (2H, m, H-8), 2.04 (1H, ddd, J=12.8, 11.1, 6.2 Hz, one of H-7), 2.25 (1H, ddd, J=12.8, 5.8, 3.5 Hz, one of H-7), 2.35 (1H, dt, J=14.8, 4.7 Hz, one of H-4), 2.50 (1H, ddd, J=14.8, 3.5, 2.7 Hz, one of H-4), 3.15 (1H, d, J=14.4 Hz, one of H-12), 3.16 (1H, t, J=3.9 Hz, H-5), 3.52 (1H, ddd, J=11.7, 9.8, 6.6 Hz, one of H-9), 3.63 (3H, s, OMe), 3.65—3.75 (1H, m, one of H-9), 4.05 (1H, ddd, J=8.3, 4.6, 2.7 Hz, H-3), 4.64 (1H, d, J=14.4 Hz, one of H-12), 4.69 (1H, s, H-1), 5.91, 5.92 (1H each, ABq, J=1.4 Hz, OCH_2O), 6.58, 6.74 (1H each, 2×s, aromatic protons). ^{13}C-NMR ($CDCl_3$, 75 Hz) δ: 21.63, 37.95, 42.95, 43.49, 43.68 (C-5), 47.52, 54.78 (OMe), 63.87 (C-6), 65.52 (C-3), 101.23 (OCH_2O), 103.85 (C-1), 110.88, 110.95, 124.40, 132.72, 146.48, 147.34, 157.29 (C-2), 171.41 (C-11). Exact MS *m/z*: Calcd for $C_{19}H_{21}NO_5$: 343.1419. Found: 343.1424. *Anal.* Calcd for $C_{19}H_{21}NO_5 \cdot 1/2H_2O$: C, 64.76; H, 6.29; N, 3.98. Found: C, 65.18; H, 6.20; N, 4.11.

Compound **15b** had mp 179—180 °C (from AcOEt–hexane). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 1660, 1625. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.70—1.96 (2H, m, H-8), 2.09—2.20 (1H, m, one of H-7), 2.14 (1H, ddd, J=13.0, 10.4, 3.9 Hz, one of H-4), 2.30—2.39 (1H, m, one of H-7), 2.46 (1H, ddd, J=13.0, 5.1, 3.9 Hz, one of H-4), 2.57 (1H, d, J=1.9 Hz, OH), 3.08 (1H, d, J=14.4 Hz, one of H-12), 3.24 (1H, t, J=3.9 Hz, H-5), 3.48 (1H, ddd, J=11.5, 10.3, 6.6 Hz, one of H-9), 3.62 (3H, s, OMe), 3.67—3.76 (1H, m, one of H-9), 4.06 (1H, br dd, J=10.4, 5.1 Hz, H-3), 4.57 (1H, d, J=14.4 Hz, one of H-12), 4.57 (1H, s, H-1), 5.90, 5.92 (1H each, ABq, J=1.4 Hz, OCH_2O), 6.54, 6.70 (1H each, 2×s, aromatic protons). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 21.92, 39.17, 42.85, 44.72, 46.39 (C-5), 47.64, 54.89 (OMe), 63.01 (C-3), 63.89 (C-6), 101.08 (OCH_2O), 103.10 (C-1), 110.35, 110.42, 125.07, 132.03, 146.07, 147.08, 157.60 (C-2), 171.43 (C-11). Exact MS *m/z*: Calcd for $C_{19}H_{21}NO_5$: 343.1418. Found: 343.1401.

With L-Selectride: L-Selectride (1.0 M solution in THF, 0.112 ml, 0.528 mmol) was added to a solution of **14a** (60 mg, 0.176 mmol) in dry THF (20 ml) and dry dichloromethane (4 ml) at −78 °C and the mixture was stirred at the same temperature for 3 h. The solvent was evaporated off and the residue was diluted with dichloromethane (20 ml) and 5% HCl (10 ml). The organic layler was separated, washed with brine, dried ($MgSO_4$), and concentrated. The residue was chromatographed on silica gel (AcOEt) to give **15a** (60 mg, quant.).

(3*S,5*S**,6*R**)-3-Hydroxy-2-methoxy-4,5,8,9,11,12-hexahydro-3*H*,7*H*-[1,3]benzodioxolo[5,6-*d*]pyrrolo[2,1-*k*][1]benzazepine [(±)-D-Homocephalotaxine (2)** The procedure was essentially the same as that employed for the preparation of the amine **3**. A solution of **15a** (60 mg, 0.175 mmol) in dry benzene (15 ml) and dry dichloromethane (2 ml) was treated with Red-Al (70% in toluene, 2.02 ml, 7.00 mmol) to afford, after work-up, **2** (35 mg, 60%), mp 214—216 °C (from CH_2Cl_2–AcOEt). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3350, 1650. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.80—2.10 (3H, m), 2.10—2.25 (1H, m), 2.31 (1H, ddd, J=13.5, 8.0, 3.4 Hz, one of H-4), 2.70 (1H, ddd, J=16.6, 6.0, 2.7 Hz, one of H-12), 2.80—2.95 (2H, m), 2.98—3.09 (2H, m), 3.52 (1H, ddd, J=13.4, 6.0, 3.1 Hz, one of H-11), 3.59—3.79 (2H, m), 3.68 (3H, s, OMe), 4.32 (1H, t, J=8.1 Hz, H-3), 4.76 (1H, s, H-1), 5.95 (2H, s, OCH_2O), 6.58, 6.59 (1H each, 2×s, aromatic protons). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 19.08, 31.31, 34.31, 34.87, 48.07 (C-5), 50.32, 53.29, 53.41 (C-6), 55.19 (OMe), 66.87 (C-3), 97.09 (C-1), 101.22 (OCH_2O), 110.60, 111.34, 130.83, 131.63, 146.60, 146.87, 161.23 (C-2). Exact MS *m/z*: Calcd for $C_{19}H_{23}NO_4$: 329.1625. Found: 329.1613.

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Direct Photolysis of Halopyridines in Solutions; Generation of the 2-Pyridyl Cation

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Direct photolyses of 2-halopyridine in various solvents afforded the corresponding ionic products, as well as the radical product pyridine, while 3- and 4-halopyridines produced pyridine exclusively. The formation of the ionic products may occur *via* the 2-pyridyl cation generated through the initial photo-induced homolytic cleavage of the C–X bond followed by electron transfer within the resulting radical pair. The participation of the unshared pair of electrons adjacent to the radical carbon is suggested to be important for releasing the unpaired electron.

Keywords halopyridine; 2-halopyridine; *N*-methyl-2-iodopyridinium iodide; solvent; photolysis; 2-pyridyl radical; 2-pyridyl cation; ionic product; radical product

Since the photolysis of alkyl halides has been reported to generate the cationic species as an important reaction intermediate *via* homolytic cleavage of the C–halogen bond followed by electron transfer within the resulting radical pair (Chart 1),[1] it is necessary to reconsider previous studies, wherein the reaction mechanism of the direct photolysis of halides had been discussed in terms of homolysis.[2] Recently Inoue *et al.* reported that direct ultraviolet (UV) irradiation of halohexynes afforded no ionic product, while the photolysis of iodobenzene afforded the ionic product, anisole, in methanol albeit in very low ratio to the radical product, benzene (2:90—95).[3] However, very little has been reported about the direct photolysis of aryl halides in a liquid phase, wherein aryl or heteroaryl cations participate as important reaction intermediates.[4] In the present paper, we describe our findings that among 2-, 3-, and 4-halopyridines (2-, 3-, and 4-XPy's; X=I, Br, Cl), photolyses of the 2-isomers (2-XPy's) in various solvents afforded the ionic products in fairly high ratio with respect to the radical product (ion-to-radical ratio), and that the unshared pair of electrons of the adjacent nitrogen is important for the formation of the 2-pyridyl cation.

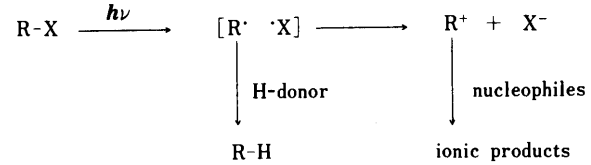

Chart 1

TABLE I. Photolyses of 2-Halopyridines (2-XPy's) (1) in Various Solvents

Solvent	1	Yields (%) of 2	Yields (%) of 3	Consumed 1 (%)
		2a		
CH_3OH	**1a**	27	15	49
	1b	29	28	64
	1c	7.5	3	18
		2b		
C_2H_5OH	**1a**	17	14	37
	1b	17	14	59
	1c	4	2	9.5
		2c		
CH_3CN+H_2O	**1a**	32	2	36
	1b	47	11	71
	1c	8	0.6	13
		2d		
Ethyl acetate	**1a**	34	1	35
	1b	39	2	56
	1c	7	0.7	13
		2b		
Ether	**1a**	3	22	35
	1a[a)]	3	6	13
	1b	20	42	88
	1c	3	2	16

a) At −16 °C.

Direct UV irradiation of 2-IPy (**1a**) in methanol afforded the ionic product (**2**), methoxypyridine (**2a**), as a major product (55% based on **1a** consumed) together with the radical-derived product, pyridine (**3**) (31%), while the photolyses of 3- and 4-IPy's gave **3** exclusively and no methoxypyridine was detected. Similarly, the photolyses of 2-BrPy (**1b**) and 2-ClPy (**1c**) were performed in methanol and in ethanol (Table I). Although some changes in the ion-to-radical ratio were observed, the ionic products, **2a** and 2-ethoxypyridine (**2b**), were produced preferentially over pyridine in each reaction. Furthermore, the photolyses of **1a**—**c** in acetonitrile containing 1% water[1d] and ethyl acetate, both of which are poorly nucleophilic, afforded the ionic products, 2-acetamidopyridine (**2c**) and 2-hydroxypyridine (**2d**),[5] in high ion-to-radical ratio, respectively (Table I),[6] suggesting that cationic species may be involved as the most important intermediate in the photolysis of **1a**—**c**. The photolyses of **1a**—**c** in ether also produced **2b** as well as the radical product **3**, albeit in low ion-to-radical ratio, especially for 2-IPy.[7] However, the ratio was significantly improved when the reaction (**1a**) was conducted at low temperature (−16 °C) (Table I). This finding is suggestive of the mechanism reported for the generation of the alkyl and vinyl cations in the photolyses of the corresponding halides (Chart 1).[1c] Similar temperature dependencies were observed in the photolyses of **1** in ethanol (Table II): the ion-to-radical

TABLE II. The Ion-to-Radical Ratio for the Photolyses of **1** in Ethanol at Various Temperatures

1	Ion-to-radical ratio at 1 °C	22 °C	55 °C
1a	2.1	1.2	0.6
1b	1.6	1.2	1.1
1c	3.0	2.0	1.6

TABLE III. Photolyses of 2-XPy (1) in Methanol in the Presence of Additives

1	Additive	Yield (%) of 2a	1b	3	Ratio of 2a/3	Consumed 1 (%)
1a	$Cu(CH_3CO_2)_2$[a]	7.7		1.7	4.5 (1.8)[c]	10
1b		9.1		0.7	13.0 (1.0)[c]	10
1c		0.8		0.1	8.0 (2.5)[c]	1
1a	$CuBr_2$[b]	3.3	15	0.6	5.5 (1.8)[c]	25
1c		1.0	0.5	0.2	5.0 (2.5)[c]	2

a) $Cu(CH_3CO_2)_2$ (0.66 mmol) in MeOH (10 ml) and H_2O (1 ml). *b*) $CuBr_2$ (1 mmol) in MeOH (10 ml). *c*) The ratios obtained in the absence of additives are given in parentheses.

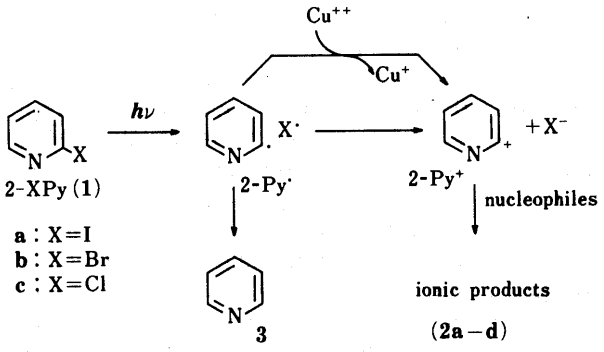

Chart 2

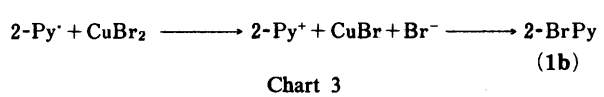

Chart 3

ratio apparently increased as the reaction temperature was lowered. Furthermore, the addition of Cu(II) ions (cupric acetate, cupric bromide)[8] to the reaction mixture with **1** in methanol gave a remarkably improved ion-to-radical ratio, though the reactions were retarded appreciably (Table III).[9] These findings suggest that the present reaction may proceed by the mechanism involving initial homolytic cleavage of the C–halogen bond, followed by electron transfer within the resulting radical pair (Chart 2). The added cupric salts would work as an electron acceptor from the initially produced 2-pyridyl radical (2-Py·) in competition with the halogen radical (X·). In the reaction with cupric bromide, considerable amounts of 2-bromo pyridine (**1b**) were produced, presumably *via* a coupling of the 2-pyridyl cation (2-Py^+) and the bromide anion, generated by electron transfer from the 2-pyridyl radical (2-Py·) to cupric bromide (Chart 3).[10]

Thus it was seen that, among 2-, 3-, and 4-halopyridines, the formation of the pyridyl cation occurred specifically in the photolyses of **1**. This suggests that the unshared pair of electrons of the nitrogen atom adjacent to the radical carbon may be important for the electron transfer from 2-Py·. In view of the electron spin resonance (ESR) study of the 2-Py·, showing that the unpaired electron in an sp^2 orbital on carbon, by overlap with the unshared pair of electrons in the exocyclic orbital on the adjacent nitrogen, would be accommodated in the antibonding orbital of the resulting pseudo-π-orbital,[11] it can be understood that the 2-Py·, with lowering its ionization potential, would release an electron to an appropriate electron acceptor. In fact, the photolysis of a methanolic solution of *N*-methyl-2-iodopyridinium iodide (**4**) (10 mM) under the same conditions as employed above, wherein the unshared pair of electrons was protected, afforded the radical-derived product, *N*-methylpyridinium iodide, exclusively (100%, based on 60% of **4** consumed) and no ionic product was detected.

The present work has thus opened up a novel aspect of the photoreaction of heteroaryl halides.

Experimental

UV irradiation was carried out externally with a 60 W low-pressure mercury lamp (Eiko-sha) in a quartz tube (low-pressure mercury lamp) under an argon atmosphere using a merry-go-round apparatus (Eiko-sha) at room temperature (*ca.* 22 °C), unless otherwise noted, and the yields of the products given in the tables were determined by gas-liquid chromatography (GLC). GLC was performed with a capillary column (CBP20-M25-025, Shimadzu) on a Shimadzu GC-7A gas chromatograph equipped with a hydrogen flame-ionization detector, using helium as a carrier gas.

Materials 2-Methoxypyridine (**2a**) and 2-hydroxypyridine (**2d**) are commercially available (Aldrich Chemical Company Inc.). 2-Ethoxypyridine (**2b**),[12] 2-acetamidopyridine (**2c**),[13] and *N*-methyl-2-iodopyridinium iodide[14] were prepared according to the reported procedures.

General Procedure for the Photolyses of 1 in Various Solvents A solution of **1** (0.1 mmol) in a solvent (methanol, ethanol, acetonitrile containing 1% H_2O, ethyl acetate, or ether) (10 ml) was irradiated for 1 h. After being neutralized with 30% aqueous K_2CO_3 and dried over anhydrous Na_2SO_4, the reaction mixture was submitted to GLC analysis with naphthalene (1 mg) as an internal standard.

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5) The formation of **2d** is presumed to occur *via* the following process.

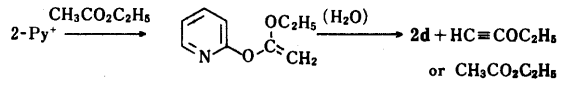

Chart 4

6) Similar photolyses of 3-IPy gave pyridine as the only detectable product.
7) The significantly reduced yield of **2b** in the photolysis of **1a** in ether may be accounted for by the preferential recombination of the iodide anion (I^-) and 2-Py^+, generated according to the reaction process depicted in Chart 2, in competition with the formation of **2b** due to the lower efficiency of solvation by ether of I^- than Br^-. The appreciably higher yield of the radical product (**3**) from **1b** could be explained by the fast diffusion of Br· in ether (with its low viscosity), resulting in suppression of the recombination of the radical pair (Br· and 2-Py·), initially produced by the mechanism shown in Chart 2.
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9) Under these conditions, the incident light was significantly absorbed by the additives (85% by $CuBr_2$ and 86.5% by $Cu(OCOCH_3)_2$).
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A Synthesis of (−)-Bursatellin

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A synthesis of (−)-bursatellin has been accomplished by an application of a new procedure for oxidation at a benzylic position with $K_2S_2O_8$ to 1-acetoxy-2(*S*)-*N*-Boc-amino-3-(4-benzyloxyphenyl) propane as a key step.

Keywords (−)-bursatellin; sea hare; *N*-Boc-tyrosine; potassium persulfate; oxidation; formylation; cyanation

(−)-Bursatellin (**1**)[1] has been isolated from the sea hare, *Bursatella* sp., as a metabolite and its synthesis has been completed by an Italian group.[2] We wish to report here an alternative synthesis of this compound using a new oxidation reaction at a benzylic position with potassium persulfate ($K_2S_2O_8$).[3]

Reduction of *N*-Boc-*O*-benzyltyrosine methyl ester (**3**), obtained from *N*-Boc-*O*-benzyltyrosine (**2**) by treatment with diazomethane, with lithium aluminum hydride (LAH) in tetrahydrofuran (THF) gave the alcohol (**4**) in 76% yield. Oxidation of its *O*-acetate (**5**) with $K_2S_2O_8$ in aqueous acetonitrile gave the cyclic carbamate (**6**) and *p*-benzyloxybenzaldehyde in 40% and 23% yields, respectively. No detectable amount of the stereoisomeric compound was isolated. This fact was consistent with the result of our preliminary investigation concerning the oxidation reaction of tyrosine derivatives with potassium persulfate.[3] Hydrolysis of the cyclic carbamate with 2% aqueous potassium hydroxide and toluene (two phases) under reflux overnight followed by *N*-formylation with ethyl formate furnished the *N*-formate (**7**) in 87% yield. Removal of the benzyl group under hydrogen in the presence of palladium on carbon gave the phenol (**8**) in quantitative yield. Treatment of **8** with dimethoxypropane in acetone in the presence of camphorsulfonic acid at room temperature afforded the acetonide (**9**) in 67% yield. The spectroscopic properties of this compound showed good agreement with the data reported by the Italian group, confirming a formal synthesis of (−)-bursatellin at this stage.

Attempts at cyanoethylation of the phenolic hydroxyl group in the acetonide (**9**) with acrylonitrile using several kinds of bases and solvents were unsuccessful, as mentioned by the Italian group. Thus, we tested a two-step procedure to introduce the cyanoethyl moiety. Heating the acetonide (**9**) with 1,2-dibromoethane and potassium carbonate in ethanol with efficient stirring overnight gave the bromide (**10**) in 75% yield. Treatment of the bromide (**10**) with sodium cyanide in dimethylsulfoxide (DMSO) or some other solvents gave rise to regeneration of the phenol (**9**) as a result of the retro-Michael fragmentation reaction due to sodium hydroxide present in commercial sodium cyanide. Eventually, we found that addition of sodium dihydrogenphosphate (NaH_2PO_4) as a buffer to the cyanation reaction mixture resulted in formation of the cyanide (**11**) in 73% yield. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of the synthetic compound exhibited good agreement with the data reported by the Italian group. Hydrolysis of the acetonide group with aqueous acetic acid gave (−)-bursatellin (**1**). Its spectroscopic properties and $[\alpha]_D$ value ($[\alpha]_D$ −8.0° (c=0.29, MeOH)) were also in good agreement with those of (−)-bursatellin ($[\alpha]_D$ −8.8°), indicating the accomplishment of the synthesis of **1** in 6.3% overall yield starting from *N*-Boc-*O*-benzyltyrosine (**2**).

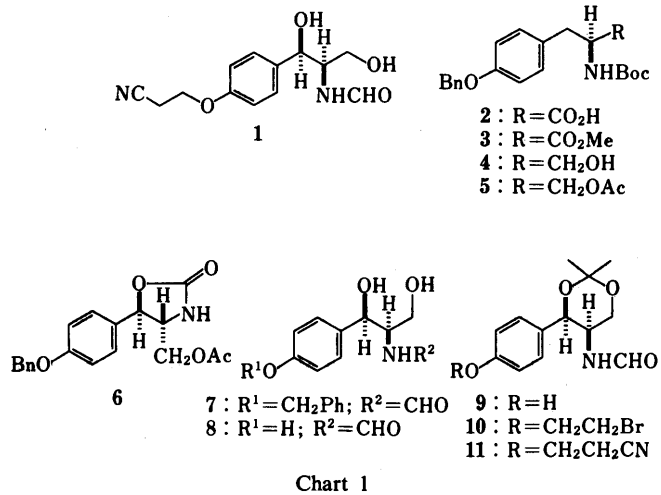

Chart 1

Experimental

Melting points were determined with a Yanagimoto micro-melting point apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on Varian EM 360 (60 MHz) and JEOL FX90Q (90 MHz) spectrometers with tetramethylsilane as an internal standard. Mass spectra (MS) were recorded on a JEOL 303A spectrometer. Infrared (IR) spectra were recorded with a Shimadzu IR-408 spectrometer. Optical rotations were measured at 19—25 °C with a JASCO DIP-181 digital polarimeter. Merck Kieselgel Art. 7731 was used for flash column chromatography, and Merck Kieselgel precoated Silica gel 60 F-254 plates were used for preparative thin layer chromatography (TLC).

(2*S*)-2-*N*-Boc-amino-3-(4-benzyloxyphenyl)propanol (4) A mixture of excess ethereal diazomethane and *N*-Boc-*O*-benzyl-L-tyrosine (5.0 g, 13.5 mmol) in ether (50 ml) was kept at room temperature for a few minutes, and a few drops of acetic acid were added to the reaction mixture. The solution was washed with 2% aqueous $NaHCO_3$ and water, dried with $MgSO_4$ and evaporated. A solution of the residue in anhydrous THF (150 ml) was added dropwise to a suspension of $LiAlH_4$ (740 mg, 20.3 mmol) in anhydrous THF (150 ml) at 0 °C. The mixture was stirred for 1 h. After addition of a few drops of water to the reaction mixture, the resulting precipitates were filtered off. The filtrate was concentrated *in vacuo* to give a residue which was taken up in ether. The ethereal solution was washed with 2% aqueous HCl and water, dried with $MgSO_4$ and evaporated to dryness to give the alcohol (**4**) (3.67 g, 76%) which was recrystallized from ether–hexane as colorless needles, $[\alpha]_D$ −17.38° (c=1.63, MeOH), mp 102—104 °C. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3650, 3430, 1700. ^{1}H-NMR (60 MHz in $CDCl_3$) δ: 1.47 (9H, s), 2.56 (1H, br s), 2.83 (2H, d, J=7.2 Hz), 3.65 (2H, d, J=3.6 Hz), 3.54—3.94 (1H, m), 4.94 (1H, d, J=7.8 Hz), 5.13 (2H, s), 7.02 (2H, d, J=9.0 Hz), 7.28 (2H, d, J=9.0 Hz), 7.53 (5H, s). *Anal.* Calcd for $C_{21}H_{27}NO_4$: C, 70.56; H, 7.61; N, 3.92. Found: C, 70.37; H, 7.59; N, 3.89.

(2*S*)-2-*N*-Boc-amino-3-(4-benzyloxyphenyl)propyl Acetate (5) Acetic anhydride (6 ml) was added to a solution of the alcohol (**4**) (3.11 g, 8.72 mmol) in pyridine (30 ml). The reaction mixture was stirred at room temperature overnight, then MeOH (6 ml) was added to it. The solvent was removed *in vacuo* and the residue was extracted with ether. The organic extract was washed with 2% aqueous HCl and water, dried with $MgSO_4$ and evaporated to afford the acetate (**5**) (3.14 g, 90%), which was recrystallized from ether–hexane as white needles, $[\alpha]_D$ −6.62° (c=0.89, MeOH), mp, 95—96 °C. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3430, 1730, 1710. ^{1}H-NMR (60 MHz in $CDCl_3$) δ: 1.47 (9H, s), 2.13 (3H, s), 2.83 (2H, d, J=6.6 Hz), 3.90—4.25 (1H, m), 4.12 (2H, d, J=1.8 Hz), 4.74 (1H, d, J=7.8 Hz), 5.15 (2H, s), 7.02 (2H, d, J=9.6 Hz), 7.28 (2H, d, J=9.6 Hz), 7.57 (5H, s). *Anal.* Calcd for $C_{23}H_{29}NO_5$: C, 69.15; H, 7.32; N, 3.51. Found: C, 69.02; H, 7.12; N, 3.59.

(4*R*,5*R*)-4-Acetoxymethyl-5-(4-benzyloxyphenyl)-1,3-oxazolidine-2-one (6) Solutions of $K_2S_2O_8$ (2.26 g, 8.36 mmol) in water (30 ml) and $CuSO_4$ (134 mg, 0.84 mmol) in water (5 ml) were added successively to a solution of the acetate (**5**) (1.67 g, 4.18 mmol) in CH_3CN (35 ml) under argon. After being stirred at 110 °C (bath temperature) for 100 min, the mixture was concentrated *in vacuo*. The residue was thoroughly extracted with $CHCl_3$ and the organic layer was washed with 2% aqueous $NaHCO_3$ and water, dried with $MgSO_4$ and concentrated *in vacuo* to give a residue, which was subjected to a flash chromatography. Elution with ethyl acetate–hexane (2:3) afforded the carbamate (**6**) (567 mg, 40%) as white needles, $[\alpha]_D$ +35.21° (c=0.85, $CHCl_3$), mp, 151—154 °C. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3430, 1760, 1745. MS m/z: 341 (M^+). ^{1}H-NMR (90 MHz in $CDCl_3$) δ: 2.09 (3H, s), 3.64—4.30 (2H, m), 5.05 (2H, s), 5.29 (2H, d, J=5.4 Hz), 6.36 (1H, br s), 6.97 (2H, d, J=8.1 Hz), 7.30 (2H, d, J=8.1 Hz), 7.37 (5H, s). *Anal.* Calcd for $C_{19}H_{19}NO_5$: C, 66.85; H, 5.61; N, 4.10. Found: C, 66.68; H, 5.67; N, 4.07.

(2*R*,3*R*)-3-(4-Benzyloxyphenyl)-2-formylamino-1,3-propanediol (7) A mixture of the carbamate (**6**) (513 mg, 1.50 mmol), 3% aqueous KOH (15 ml) and toluene (15 ml) was refluxed overnight under an argon atmosphere, then concentrated *in vacuo*. The residue was suspended in a few drops of water and the resulting mixture was treated with a few pieces of dry ice to reduce its basicity. The mixture was filtered, and the precipitate was washed with ether and dried under reduced pressure. The solid obtained was refluxed in HCO_2Et (10 ml) for 9 h under an argon atmosphere. After removal of HCO_2Et *in vacuo*, the residue was washed with $CHCl_3$. The washings were filtered, and the filtrate was concentrated *in vacuo* to afford the *N*-formate (**7**) (390 mg, 87%) as a white powder. IR ν_{max}^{Nujol} cm^{-1}: 3150—3450, 1645.

(2*R*,3*R*)-2-Formylamino-3-(4-hydroxyphenyl)-1,3-propanediol (8) A mixture of the *N*-formate (**7**) (423 mg, 1.41 mmol), 10% palladium on carbon (330 mg), and ethanol (20 ml) was stirred under H_2 for 1 h. The catalyst was filtered off, and the filtrate was evaporated *in vacuo* to afford the phenol (**8**) as crystals in quantitative yield, $[\alpha]_D$ −34.20° (c=0.80, MeOH), mp, 137—139 °C. IR ν_{max}^{film} cm^{-1}: 3200—3400, 1655. MS m/z: 211 (M^+). ^{1}H-NMR (90 MHz in CD_3OD) δ: 3.52 (1H, d, J=9.2 Hz), 3.58 (1H, d, J=9.2 Hz), 3.78 (1H, s), 4.00 (1H, s), 4.09 (1H, m), 4.66 (1H, d, J=4.0 Hz), 4.80 (1H, s), 6.73 (2H, d, J=8.1 Hz), 7.20 (2H, d, J=8.1 Hz), 7.72 (1H, s), 8.02 (1H, s). *Anal.* Calcd for $C_{10}H_{13}NO_4$: C, 56.86; H, 6.20; N, 6.63. Found: C, 56.53; H, 6.17; N, 6.65.

(4*R*,5*R*)-2,2-Dimethyl-4-(4-hydroxyphenyl)-5-formylamino-1,3-dioxolane (9) A solution of the phenol (**8**) (208 mg, 0.99 mmol), 2,2-dimethoxypropane (4 ml), and *dl*-camphorsulfonic acid (12 mg, 0.05 mmol) in acetone (4 ml) was stirred at room temperature for 3 h. Then 2% aqueous Na_2CO_3 (40 ml) was added to the reaction mixture, and the whole was concentrated *in vacuo* to give a residue, which was extracted with $CHCl_3$. The $CHCl_3$ extract was washed with brine, dried over K_2CO_3, and evaporated. The residue was purified by flash chromatography (ethyl acetate–hexane (12:5)) to afford the acetonide (**9**) (165 mg, 67%) as a colorless glass, $[\alpha]_D$ +6.62° (c=1.56, MeOH). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3250, 1675. MS m/z: 251 (M^+). ^{1}H-NMR (90 MHz in $CDCl_3$) δ: 1.54 (3H, s), 1.58 (3H, s), 3.83 (1H, dd, J=2.0, 12.3 Hz), 4.24 (2H, m), 5.14 (1H, br s), 6.47 (1H, d, J=8.1 Hz), 6.69 (2H, d, J=8.7 Hz), 7.14 (2H, d, J=8.7 Hz), 7.52 (1H, s), 7.69 (1H, s).

(4*R*,5*R*)-2,2-Dimethyl-4-[4-(2-bromoethyloxyphenyl)]-5-formylamino-1,3-dioxolane (10) A mixture of the acetonide (**9**) (50 mg, 0.20 mmol), 1,2-dibromoethane (172 μl, 2.0 mmol), potassium carbonate (138 mg, 1.0 mmol), and ethanol (3 ml) was stirred at 90 °C under argon for 40 h. The reaction mixture was diluted with ethyl acetate. The solution was washed with water, dried over $MgSO_4$ and evaporated *in vacuo* to afford an oil, which was purified by flash chromatography. Elution with ethyl acetate–hexane (4:1) gave the bromide (**10**) (54 mg, 75%) as a colorless oil, $[\alpha]_D$ +5.45° (c=0.88, MeOH). IR ν_{max}^{film} cm^{-1}: 3430, 1680. MS m/z: 357 (M^+). ^{1}H-NMR (90 MHz in $CDCl_3$) δ: 1.54 (3H, s), 1.58 (3H, s), 3.61 (2H, t, J=6.2 Hz), 3.85 (1H, dd, J=2.3, 12.5 Hz), 4.20 (2H, m), 4.25 (2H, t, J=6.2 Hz), 5.15 (1H, br s), 6.25 (1H, d, J=9.0 Hz), 6.86 (2H, d, J=9.0 Hz), 7.24 (2H, d, J=9.0 Hz), 7.97 (1H, s).

***O*,*O*-Isopropylidene-(−)-bursatellin (11)** A solution of the bromide (**10**) (54 mg, 0.15 mmol) in DMSO (2 ml) was treated with NaCN (11 mg, 0.22 mmol) and NaH_2PO_4 (5.4 mg, 0.05 mmol), and the whole was stirred overnight under an argon atmosphere. The reaction mixture was diluted with ethyl acetate, and the solution was washed three times with aqueous NaCl, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was purified by preparative TLC (ethyl acetate–hexane (10:1)) to give the cyanide (**11**) (33 mg, 73%) as a colorless oil, $[\alpha]_D$ +1.49° (c=0.97, $CHCl_3$). IR ν_{max}^{film} cm^{-1}: 3430, 2260, 1680. MS m/z: 304 (M^+). ^{1}H-NMR (90 MHz in $CDCl_3$) δ: 1.55 (3H, s), 1.58 (3H, s), 2.81 (2H, t, J=6.4 Hz), 3.86 (1H, dd, J=2.2, 12.5 Hz), 4.17 (2H, t, J=6.4 Hz), 4.28 (2H, m), 5.16 (1H, br s), 6.17 (1H, d, J=9.0 Hz), 6.86 (2H, d, J=8.9 Hz), 7.26 (2H, d, J=8.9 Hz), 7.99 (1H, s).

(−)-Bursatellin (1) A solution of the foregoing cyanide (**11**) (32 mg, 0.11 mmol) in 80% aqueous acetic acid (4 ml) was stirred at 100 °C for 30 min. After the solvent had been removed *in vacuo*, the residue was purified by preparative TLC ($CHCl_3$–MeOH (10:1)) to afford (−)-bursatellin (**1**) (20 mg, 72%) as a colorless glass, $[\alpha]_D$ −8.0° (c=0.29, MeOH). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3200—3400, 2250, 1660. ^{1}H-NMR (90 MHz in CD_3OD) δ: 2.90 (2H, t, J=5.9 Hz), 3.52 (1H, m), 3.63 (1H, dd, J=5.8, 10.8 Hz), 4.10 (1H, m), 4.17 (2H, t, J=5.9 Hz), 4.95 (1H, d, J=4.2 Hz), 6.91 (2H, d, J=8.7 Hz), 7.33 (2H, d, J=8.7 Hz), 8.00 (1H, s).

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Solubilization of Steviolbioside and Steviolmonoside with γ-Cyclodextrin and Its Application to Selective Syntheses of Better Sweet Glycosides from Stevioside and Rubusoside

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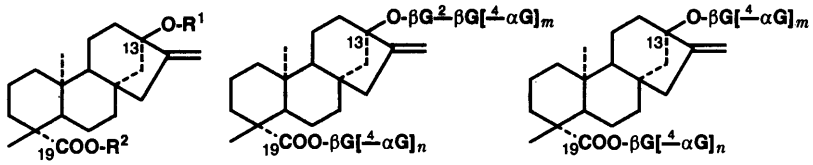
1,4-α-Glucosylation at the 13-*O*-glycosyl moiety of stevioside (S) and rubusoside (RU) results in a significant increase of sweetness. Saponification of the 19-COO-β-glucosyl linkage of S and RU yielded steviolbioside (SB) (=13-*O*-β-sophorosyl-steviol) and steviolmonoside (SM) (=13-*O*-β-glucosyl-steviol), respectively, both of which are poorly soluble in an acetate buffer. It was found that the solubilities of SM and SB in the buffer solution were remarkably increased in the presence of γ-cyclodextrin (γ-CD). SB was solubilized in the buffer solution with the aid of γ-CD, and the solution was subjected to 1,4-α-transglucosylation by using a cyclodextrin glucanotransferase–starch system to give a mixture of products which were glucosylated at the 13-*O*-glycosyl moiety. This mixture was acetylated, and the acetate was subjected to chemical β-glucosylation of 19-COOH followed by deacetylation to afford compounds which have superior sweetness to S. In the same way, derivatives with superior sweetness were selectively prepared from RU through SM.

Keywords sweetener; stevioside; rubusoside; steviolbioside; steviolmonoside; cyclodextrin; cyclodextrin glucanotransferase; solubilization

Stevioside (S),[1] a glycoside of steviol (ST) was isolated from *Stevia rebaudiana* BERTONI (Compositae) as the major sweet principle. Rebaudioside A (RA),[2] which is one of the congeners of S and has a subjectively better sweetness than S, was also isolated from this herb. Both S and RA are currently used as low calorie sweeteners in Japan. Rubusoside (RU), another sweet congener of S was isolated from leaves of a Chinese rosaceous shrub, *Rubus suavissimus* S. LEE in a yield of more than 5%.[3-5]

In order to improve the intensity and character of sweetness, enzymic transglycosylation of these glycosides has been extensively investigated.[6-12] Cyclodextrin glucanotransferase (CGTase) catalyzes regio- and stereo-selective α-D-glucosylation from starch or cyclodextrin (CD) to the 4-hydroxyl group of a glucopyranoside moiety. On treatment of S with this CGTase system, 1,4-α-mono-, di-, tri-, and greater glucosylation occurred at both the 13-*O*-glycosyl and 19-COO-β-glucosyl moieties, affording a complex mixture of glucosylated products (S-1a, -1b, -2a, -2b, -2c, -3a, -3b, -3c, -3d, *etc.*: structures, see Chart 1). This mixture derived from S, commercially named "glucosyl stevioside," is regarded as a better sweetener than S. The transglucosylation of RU under the same condition also yielded a mixture of similar 1,4-α-glucosylated products (RG-1a, -1b, -2a, -2b, -2c, -3a, -3b, -3c, -3d, -4a, -4b, -4c, -4d, -4e, *etc.*: structures, see Chart 1). Recently, isolation and identification of these products (Chart 1) from the complex mixtures of the glucosylated S and RU have been achieved (RG-4c and -4d were obtained as a mixture.[9,12] The sweetness evaluation of each product revealed that in the case of the 1,4-α-transglucosylation of S and RU with the CGTase system, glucosylation at the 13-*O*-glycosyl moiety (totally three or four glucosyl units at 13-OH) resulted in a great improvement of sweetness, while more glucosylation at this glycosyl moiety (totally more than five glucosyl units at 13-OH) or the glucosylation at the 19-COO-glycosyl moiety led to a decrease of the sweetness[7-9,12] (see Chart



	R^1	R^2
ST	H	H
S	βG²-βG	βG
RA	βG²-βG (3βG)	βG
RU	βG	βG
SM	βG	H
SB	βG²-βG	H

	m	*n*
S-1a	1	0
S-1b	0	1
S-2a	2	0
S-2b	1	1
S-2c	0	2
S-3a	3	0
S-3b	2	1
S-3c	1	2
S-3d	0	3

	m	*n*
RG-1a	1	0
RG-1b	0	1
RG-2a	2	0
RG-2b	0	2
RG-2c	1	1
RG-3a	3	0
RG-3b	2	1
RG-3c	1	2
RG-3d	0	3
RG-4a	4	0
RG-4b	3	1
RG-4c	2	2
RG-4d	1	3
RG-4e	0	4

G; D-glucopyranosyl

Chart 1

TABLE I. Relative Sweetness of Glucosylated Steviosides with Respect to Sucrose

Compound	$m^{a)}$	$n^{b)}$	RS[c]
S	2	1	160
S-1a	3	1	180
S-1b	2	2	133
S-2a	4	1	205
S-2b	3	2	136
S-2c	2	3	136
S-3a	5	1	117
S-3b	4	2	146
S-3c	3	3	150
S-3d	2	4	121

a) Number of glucose residues at 13-*O*-position. *b*) Number of glucose residues at 19-*O*-position. *c*) Relative sweetness. B/A; A, concentration (w/v%) of aqueous solution of sample (0.025%); B, concentration (w/v%) of aqueous solution of sucrose with the same sweetness as the sample solution.

TABLE II. Relative Sweetness of Glucosylated Rubusosides with Respect to Sucrose

Compound	$m^{a)}$	$n^{b)}$	RS[c]
R	1	1	114
RG-1a	2	1	132
RG-1b	1	2	102
RG-2a	3	1	278
RG-2b	1	3	99
RG-2c	2	2	95
RG-3a	4	1	214
RG-3b	3	2	182
RG-3c	2	3	110
RG-3d	1	4	58
RG-4a	5	1	115
RG-4b	4	2	202
RG-4c	3	3	184
RG-4d	2	4	
RG-4e	1	5	49

a) Number of glucose residues at 13-*O*-position. *b*) Number of glucose residues at 19-*O*-position. *c*) Relative sweetness. B/A; A, concentration (w/v%) of aqueous solution of sample (0.025%); B, concentration (w/v%) of aqueous solution of sucrose with the same sweetness as the sample solution.

1 and Tables I and II). The products S-1a and -2a from S as well as RG-1a, -2a and -3a from RU, all of which are 1,4-α-glucosylated exclusively at the 13-*O*-glycosyl moiety, have intense sweetness, and mixtures which are mainly composed of these glycosides, have attracted much attention as more desirable sweeteners than RA, S, RU and "glucosyl stevioside." Recently, we reported the regioselective syntheses of RG-1a, -2a and -3a from RU by means of enzymic protection of the 19-COO-glucosyl group against the CGTase glucosylation.[11,12] The present study deals with another route for selective syntheses of the better sweeteners such as S-1a and -2a or RG-1a, -2a and -3a.

On saponification of the 19-COO-glucosyl linkage with alkali[6] or with an LiI-2,6-lutidine–methanol system,[13] S and RU yielded steviolbioside (SB) and steviolmonoside (SM), respectively. It seems to be possible that transglucosylation of SB or SM by using the CGTase system followed by chemical β-glucosylation of the 19-COOH group affords better sweeteners which are glucosylated exclusively at the 13-*O*-glycosyl moiety. However, as shown in Fig. 1, the yield of this *trans*-glucosylation is inadequate because of the low solubility of SB and SM in

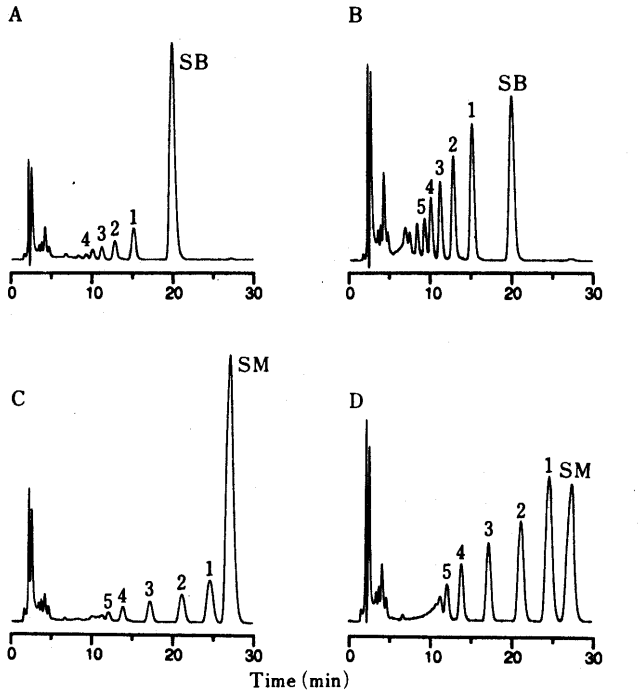


Fig. 1. High-Performance Liquid Chromatograms of Transglucosylated Products from SB and SM

A, SB without γ-CD; B, SB with γ-CD; C, SM without γ-CD; D, SM with γ-CD. Peak Nos. 1—5 in each chromatogram indicate the number of transferred glucosyl units. Conditions: column, YMC pack ODS-AM302 (4.6 mm × 150 mm); mobile phase, MeOH–0.05% TFA (60 : 40); flow rate, 0.8 ml/min; column temperature, 60 °C; detection, UV 210 nm.

TABLE III. Solubilizing Effect of CDs on SM or SB in 50 mM Acetate Buffer (pH 5.4)

	CD	Concentration of CD (mg/ml)	Solubility of SM or SB (mg/ml)
SM	None		0.69
	α-CD	15.1	1.26
	β-CD	17.7	2.48
	γ-CD	20.2	7.01
SB	None		0.21
	α-CD	20.3	0.28
	β-CD	23.6	0.90
	γ-CD	27.0	> 10.0

The amount of SM or SB added was 10 mg/ml.

an acetate buffer. We have looked for a solubilizing agent which would increase the solubilities of SB and SM in 50 mM acetate buffer solution (pH 5.4) and which can be used safely in food-additive production. Investigation of the well-known inclusion agents, cyclodextrins (CDs) disclosed that γ-CD greatly increased the solubility of both SB and SM. As shown in Table III, the solubility of SB in the buffer solution is 0.21 mg/ml at 37 °C, while equimolar addition of γ-CD to a suspension of SB [10 mg ($=1.5\times10^{-5}$ mol)/ml] led to an increase of the solubility to more than 10 mg/ml. In a similar manner, the solubility of SM was remarkably increased with γ-CD, as shown in Table III. This effect is presumably due to the formation of an inclusion complex with γ-CD. Such a remarkable solubilizing effect was not observed with α- and β-CDs, which have a smaller hole size than γ-CD and can not form an inclusion complex with SB or SM.

Subsequently, the 1,4-α-transglucosylation of SB and SM by the CGTase system in the presence of γ-CD was investigated. High performance liquid chromatography (HPLC) of both the reaction mixtures (Fig. 1) demonstrated the evident promotion of the transglucosylation by the addition of γ-CD.

Each mixture, after removal of the enzyme and saccharides, was acetylated and subjected to β-glucosylation of 19-COOH with acetobromoglucose in the presence of silver carbonate-Celite[6] followed by deacetylation with mild alkali. The reaction mixtures from SB and SM were compared by HPLC with the direct 1,4-α-transglucosylation mixtures of S[9] and RU[12] by the same enzyme system. It was demonstrated that the mixture from SB in the present experiment mainly consisted of the superior sweeteners, S-1a and -2a, together with S and more glucosylated products such as S-3a. Similarly, the mixture from SM mainly consisted of good sweeteners, RG-1a, -2a and -3a, along with RU and a small amount of more glucosylated products such as RG-4a. The products, S-1a, -2a and -3a from SB and RG-1a, -2a, -3a and -4a from SM were respectively isolated and identified by comparison of the ^{1}H- and ^{13}C-nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra with those of corresponding authentic samples.

Experimental

Solubilization of SB and SM A suspension of SB [10 mg (= 1.5×10^{-5} mol)/ml] and (or without) equimolar CD [α-CD: 20.3 mg, β-CD: 23.6 mg or γ-CD: 27.0 mg] in 50 mM acetate buffer (pH 5.4, 1 ml) was heated at 100 °C for 10 min and then incubated at 37 °C for 1 h. The mixture was filtered through an HPLC disposable filter (Ekikurodisk 25, 0.25 μm for aqueous solution, Gelman Science Japan Ltd., Tokyo). The filtrate (500 μl) was mixed with 500 μl of a 0.5% aqueous solution of RU (internal standard), and 10 μl of the solution was subjected to HPLC analysis on a ODS column, YMC pack ODS AM302 (4.6 mm × 150 mm, YMC Co., Ltd., Kyoto); mobile phase, MeOH–0.05% aqueous trifluoroacetic acid (TFA) (60 : 40); flow rate, 0.8 ml/min; detection, UV 210 nm. Concentration of SB was determined from the peak area ratio to the internal standard.

The solubility of SM was determined for a suspension of SM [10 mg (= 2.1×10^{-5} mol)/ml] and (or without) equimolar CD (α-CD: 15.1 mg, β-CD: 17.7 mg or γ-CD: 20.2 mg) in the same way as above.

Transglucosylation of SB and SM without γ-CD A suspension of SB (10 mg) and soluble starch (30 mg) in 50 mM acetate buffer (pH 5.4, 1 ml) was heated at 100 °C for 10 min. After cooling of the mixture, CGTase solution (125 μl, from *Bacillus circulans*, 2000 unit/ml,[14] supplied by Dr. S. Kitahata, Osaka Municipal Technical Research Institute) was added and the mixture was incubated at 40 °C for 8 h. The reaction mixture was heated at 100 °C, and after cooling, was extracted with 1-BuOH saturated with H_2O. The BuOH layer was concentrated to dryness and a methanolic solution of the residue was subjected to HPLC analysis on a ODS column, YMC pack ODS-AM302 (4.6 mm × 150 mm); mobile phase, MeOH–0.05% aqueous TFA (60 : 40); flow rate, 0.8 ml/min; detection, UV 210 nm. The transglucosylation of SM and analysis of the products were also carried out in the same way as above.

Transglucosylation of SB and SM in the Presence of γ-CD and Subsequent Chemical Glucosylation of the 19-COOH A suspension of SB (1.0 g), soluble starch (2.7 g) and γ-CD (2.7 g) in 50 mM acetate buffer (pH 5.4, 20 ml) was heated at 100 °C for 10 min to give a clear solution. After cooling and subsequent addition of CGTase (1.25 ml, from *B. circulans*, 2000 unit/ml), the solution was incubated at 40 °C for 8 h. After heating at 100 °C for 30 min and dilution with H_2O, the solution was extracted with 1-BuOH saturated with H_2O. The BuOH layer was concentrated to dryness. [An aliquot of the residue was subjected to HPLC analysis on an ODS column, YMC-pack ODS AM302 (4.6 mm × 150 mm, YMC Co., Ltd., Kyoto); mobile phase, MeOH–0.05% aqueous TFA (60 : 40); flow rate, 0.8 ml/min; detection, UV 210 nm.] The above residue was acetylated by refluxing with anhydrous Ac_2O–C_5H_5N (1 : 1, 20 ml) for 3 h and the solution was concentrated to dryness. A mixture of the residue and Ag_2CO_3–Celite (6 g) in anhydrous dichloromethane was partly distilled to remove moisture. Then, acetobromoglucose (0.5 g) was added and the mixture was refluxed for 1.5 h. Further acetobromoglucose (0.5 g) was added and the mixture was further refluxed for 1.5 h. The mixture was filtered through a column of silica gel and the column was washed with $CHCl_3$. The filtrate and washing were concentrated to dryness. The residue was deacetylated by treatment with 0.2 M BaO in anhydrous MeOH at room temperature for 30 min under stirring. The reaction mixture, after neutralization with Amberlite MB-3, was concentrated to dryness. [An aliquot of the residue was analyzed by HPLC on an ODS column of YMC-pack ODS AM302 (4.6 mm × 150 mm); mobile phase, MeOH–0.05% aqueous TFA (60 : 40); flow rate, 0.8 ml/min; detection, UV 210 nm.] The remaining residue was separated by HPLC on a column of YMC-pack D-ODS-5 (20 mm × 250 mm); mobile phase, 65% MeOH; flow rate, 6.0 ml/min; detection, UV 210 nm, to give S (78 mg), S-1a (62 mg), S-2a (56 mg) and S-3a (42 mg).

A mixture of SM (950 mg), γ-CD (1.92 g) and soluble starch (3.0 g) in 50 mM acetate buffer (pH 5.4, 100 ml) was heated at 100 °C for 10 min and then cooled to room temperature, affording a clear solution. CGTase (1.19 ml, 2000 U/ml) was added to the solution and the mixture was incubated at 40 °C for 8 h. After work up in the same manner as for SB, an aliquot of the reaction mixture was analyzed by HPLC under the same conditions as for SB. The remaining reaction mixture was glucosylated as in the case of SB and an aliquot of the products was analyzed by HPLC under the same conditions as for SB. The remaining products were separated by preparative HPLC in the same manner as the products from SB to give RU (136 mg), RG-1a (90 mg), RG-2a (71 mg), RG-3a (43 mg) and RG-4a (24 mg).

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Prostanoids and Related Compounds. V.[1] Synthesis of 6-Aza-5-oxo-2,3,4-trinor-1,5-inter-*m*-phenylene Prostacyclin Derivatives

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6-Aza-5-oxo-2,3,4-trinor-1,5-inter-*m*-phenylene prostacyclin derivatives were synthesized by use of 1,3-dipolar cycloaddition as a key step.

Keywords 6-aza-5-oxo-2,3,4-trinor-1,5-inter-*m*-phenylene prostacycline derivative; 1,3-dipolar cycloaddition; Wittig reaction; prostacycline; inhibitory activity; blood platelet aggregation; Prins reaction; Pfitzer–Moffat oxidation

Prostacyclin (PGI_2) displays a wide range of biological properties,[2] including inhibition of human blood platelet aggregation,[3,4] deaggregation of platelet thrombi,[5] vasodilation,[3] and inhibition of gastric secretion.[6] However, PGI_2 is a rather unstable molecule, whose instability arises from the presence of an enol ether functionality. Therefore, a wide range of stable analogues of prostacyclin have been prepared.

Prostacyclin analogues prepared to date may be regarded as involving formally the following general structural modifications of the natural compound[7]: (1) stabilization of the acid-labile enol ether 5,6-double bond by substitution at the 5-, 7-, or 10-positions; (2) modification of the tetrahydrofuran ring by replacement of the enol ether oxygen with a different atom, by introduction of an aromatic ring or by ring expansion; (3) manipulation of the 5,6-double bond by changing its position or altering the oxidation level.

Of these analogues, we have been interested in carbacyclines and 6-azacarboprostacyclin analogues as potential antithrombotic agents. H. Nakai *et al.*[8] have reported the synthesis of *dl*-6-aza-6,9-methano PGI_1 with weak inhibitory activity in blood platelet aggregation, and B. Horst *et al.*[9] have reported the synthesis of CG 4305.

On the other hand, K. Achiwa and his co-workers[10] have applied the 1,3-dipolar cycloaddition of azomethine ylides to some pyrrolizidine alkaloids.

Thus, we have designed *dl*-6-aza-5-oxo-2,3,4-trinor-1,5-inter-*m*-phenylene prostacyclin derivatives, **1a** and **1b**, whose skeletons can be constructed by 1,3-dipolar cycloaddition developed in our laboratory.[10] In this paper, we wish to report the synthesis of **1a** and **1b**.

As shown in Chart 1, we planned to construct the 6-azaprostacyclin skeleton by 1,3-dipolar cycloaddition developed in our laboratory. Retrosynthetically, **1a** and **1b** might be derived from aldehyde **2** by the Wittig reaction and benzoylation. The aldehyde **2** should be easily synthesized from diol **3** which, in turn, might be obtained by the Prins reaction of **4** followed by hydrolysis. The compound **4** should be obtained by reduction of the carbonyl group of *N*-benzyl-3-azabicyclo[3.3.0]octa-8-one (**5**) and following dehydrogenation, which should be obtained by 1,3-dipolar cycloaddition of *N*-benzyl-*N*-(methoxymethyl)trimethylsilylmethylamine (**6**) and 2-cyclopenten-1-one (**7**).

The actual synthetic route was shown in Chart 2. The compound **5** was synthesized by the 1,3-dipolar cycloaddition of an intermediary iminium ylide formed from **6** to **7** in the presence of a catalytic amount of trifluoroacetic acid in 79% yield. In order to introduce the C_2-double bond of the compound **5**, reduction of **5** to the alcohol compound by lithium aluminum hydride ($LiAlH_4$) was first attempted, but the dehydrogenation of the alcohol compound by $POCl_3$ did not proceed. On the other hand, protection of the hydroxyl group (the alcohol compound) with *p*-toluenesulfonyl chloride, followed by elimination under various reaction conditions was performed, but the reaction resulted in the recovery of the alcohol compound. Then, compound **5** was treated with *p*-toluenesulfonylhydrazide in ethanol containing a catalytic amount of trifluoroacetic acid to give tosylhydrazone (**8**), which was decomposed by disodium

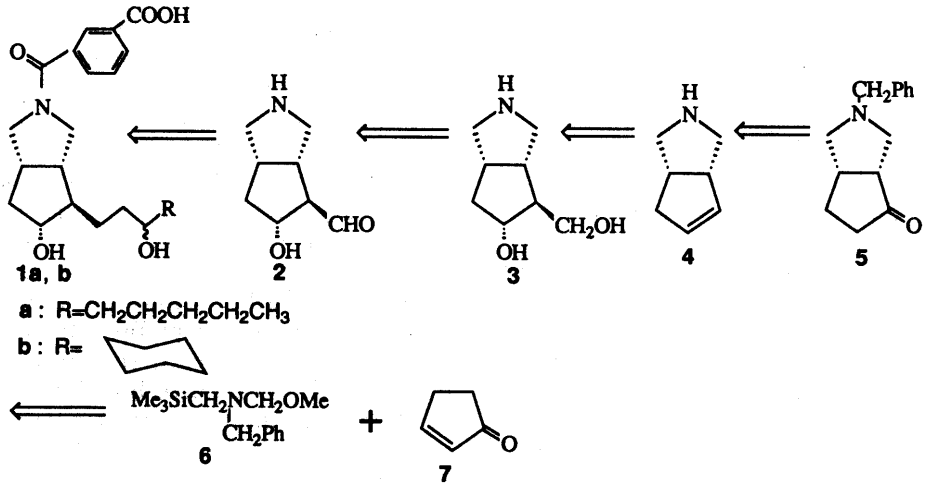

Chart 1

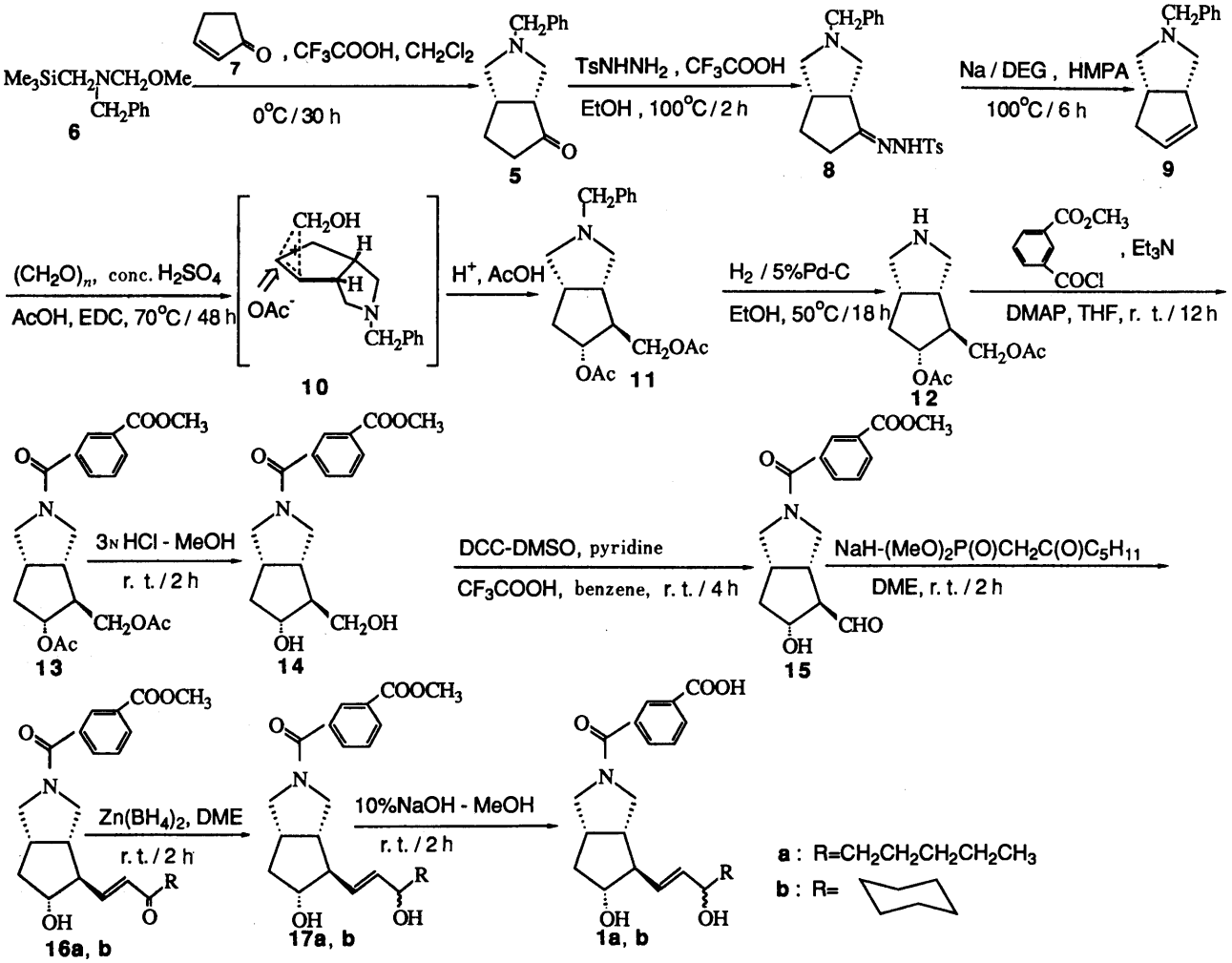

Chart 2

ethylene glycolate in diethylene glycol in the presence of a catalytic amount of hexamethylphosphoramide (HMPA) under reflux[11,12] to afford *N*-benzyl-3-azabicyclo[3.3.0]-oct-7-ene (**9**) (57% from **5**). Decomposition of **8** was initially performed under various reaction conditions, but no reaction occured with *n*-butyl lithium or *tert*-butyl lithium. On the other hand, treatment with NaH or sodium alkoxide in HMPA afforded **9** in poor yield.

The diacetate **11** was obtained by a regio- and stereospecific addition of formaldehyde on the olefinic double bond of **9**, followed by an attack of the acetate anion under the conditions of the Prins reaction.[13] The reaction of the compound **9** with excess paraformaldehyde monomerized *in situ* with sulfuric acid in glacial acetic acid took place at 70 °C to afford **11** in a 57% yield. The structure of **11** was confirmed by the mechanism of the Prins reaction and proton nuclear magnetic resonance (^{1}H-NMR) spectral data. No other regio- or stereoisomeric product could be detected in the reaction mixture. It is thought that the formation of a cyclopropenium cation (**10**) occurred at the less hindered face due to the steric influence of a bicyclo ring, and the following opening of the alleged three-center carbonium ion (**10**) by an attack of an acetate anion occured at the sterically less hindered position, *i.e.* the α-face at the C-7 position, which seems to account for both regio- and stereospecificity.

N-Debenzylation of the diacetate **11** was performed by hydrogenation with palladium on carbon in ethanol to afford key intermediate compound **12**. The carbon appendage to the compound **12** was introduced by acylation.

Treatment of **12** with isophthalic acid monomethyl ester chloride in the presence of triethylamine and 4-dimethylaminopyridine (DMAP) in tetrahydrofuran (THF) afforded **13** (86% from **11**). Isophthalic acid monomethyl ester chloride was prepared in two steps from dimethylisophthalate. Dimethylisophthalate was hydrolyzed with 1 N barium hydroxide in methanol to afford isophthalic acid monomethyl ester, which was halogenated with thionyl chloride to give isophthalic acid monomethyl ester chloride.

Methanolysis of **13** with 3 N hydrochloric acid in methanol gave the diol **14** in 80% yield. The diol **14** posseses prim- and *sec*-hydroxyl groups. Then, in order to selectively oxidate a primary alcohol, **14** was treated with Pfitzer–Moffatt[14] reagent to give the corresponding aldehyde **15**, which was used without further purification.The Wittig reaction of **15** with dimethyl (2-oxoheptyl)phosphonate[15] in dimethoxyethane (DME) proceeded smoothly to afford **16a** (67% from **15**). The reduction of **16a** with 1.5 eq zinc borohydride in DME gave the C-15 (prostanoic numbering) epimeric mixture **17a** in 85% yield. Hydrolysis of **17a** with 10% NaOH aq. in methanol afforded **1a** in 80% yield.

In the same route, the compound **1b** was synthesiz-

ed by using cyclohexyl(triphenylphosphoranyliden)methyl ketone[16] in place of dimethyl(2-oxoheptyl)phosphonate. The Wittig reaction of **15** with cyclohexyl(triphenylphosphoranylidene)methyl ketone in THF afforded the compound **16b** (33% from **14**). Reduction of **16b** with zinc borohydride in DME gave the C-15 epimeric mixture **17b** in 83% yield. Hydrolysis of **17b** with 10% NaOH aq. in methanol afforded **1b** in 73% yield.

The structures of **1a** and **1b** were supported by elemental analysis and confirmed by analysis of the IR, ^{1}H-NMR and fast atom bombardment-mass spectrometry (FAB-MS) spectra. These compounds **1a** and **1b** showed weak inhibitory activities in blood platelet aggregation.

Experimental

Melting points were determined on a micro-melting point apparatus (Yanagimoto) and are uncorrected. Optical rotations were measured on a JASCO DIP-140 digital polarimeter. Infrared (IR) spectra were taken on JASCO A-202 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 (Kieselgel $60F_{254}$ on aluminium sheets, Merck). All compounds were located by splaying the TLC plate with sulfuric acid and heating it on a hot plate. Preparative TLC (PTLC) was performed on a preparative layer chromatography plate (Kieselgel $60F_{254}$ 2 mm and 0.5 mm, Merck). Column chromatography was performed on silica gel (Kieselgel 60, 70—230 mesh, Merck).

Preparation of (1*R,5*S**)-*N*-Benzyl-3-azabicyclo[3.3.0]octan-8-one (5)** A solution of trifluoroacetic acid in CH_2Cl_2 (0.8 ml, 1 mol/l) was added to a stirred solution of *N*-benzyl-*N*-(methoxymethyl)trimethylsilylmethylamine (**6**) (2.4 g, 10 mmol) and 2-cyclopenten-1-one (**7**) (0.7 g, 8.3 mmol) in CH_2Cl_2 (20 ml) at 0 °C. The mixture was stirred for 3 h at the same temperature. The reaction mixture was washed with a saturated $NaHCO_3$ aqueous solution (20 ml × 3) and brine (20 ml × 2) and dried over $MgSO_4$. The CH_2Cl_2 solution was concentrated *in vacuo* and the residue was subjected to column chromatography on SiO_2 using a 9:1 mixture of hexane–AcOEt as the eluent to give **5** as a yellow oil (1.4 g, 78.6%). bp 117—118 °C (0.5 mmHg). *Anal.* Calcd for $C_{14}H_{17}NO$: C, 78.10; H, 7.96; N, 6.51. Found: C, 77.81; H, 7.96; N, 6.44. ^{1}H-NMR: 1.94—3.06 (10H, m, H-1, H-2, H-4, H-5, H-6, H-7), 3.60 (2H, s, C$\underline{H}_2$Ph), 7.13 (5H, s, $CH_2\underline{Ph}$). ^{13}C-NMR: 27.4 (t, C-6), 38.6 (d, C-7), 39.0 (d, C-5), 50.6 (d, C-1), 58.0, 59.5 (each t, C-2, C-4), 61.4 (t, $\underline{C}H_2$Ph), 126.9, 128.2, 128.5 (each d, Ph), 138.9 (s, Ph), 193.0 (s, C=O).

Preparation of (1*R,5*S**)-*N*-Benzyl-3-azabicyclo[3.3.0]oct-7-ene (9)** A catalytic amount of trifluoroacetic acid was added in portions to a solution of **5** (0.13 g, 1 mmol) and *p*-toluenesulfonylhydrazide (0.19 g, 1 mmol) in 5 ml of ethanol. The mixture was refluxed for 2 h. After being cooled, the mixture was concentrated *in vacuo* to afford a yellow residue (**8**), which was used without further purification. Na (0.084 g, 3.6 mmol) was added in 2.4 ml of diethylene glycol and the mixture was refluxed for 2 h. **8** (0.46 g, 1.2 mmol) was added to the mixture in the presence of HMPA (a catalytic amount). The reaction mixture was refluxed for 6 h. After being cooled, H_2O (30 ml) was added to the mixture. The reaction mixture was extracted with CH_2Cl_2 (20 ml × 10). The CH_2Cl_2 layer was washed with brine (20 ml × 2), dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on Al_2O_3 using a 4:1 mixture of hexane–AcOEt as the eluent to give **9** (0.10 g, 57% from **5**), bp 47—50 °C (2 mmHg). *Anal.* Calcd for $C_{14}H_{17}N$: C, 84.56; H, 8.75; N, 6.82. Found: C, 84.37; H, 8.60; N, 7.03. ^{1}H-NMR: 1.91—3.06 (8H, m, H-1, H-2, H-4, H-5, H-6), 3.60 (2H, s, C$\underline{H}_2$Ph), 5.62 (2H, m, H-7, H-8), 7.30 (5H, s, $CH_2\underline{Ph}$). ^{13}C-NMR: 39.0 (t, C-6), 39.9 (d, C-5), 50.3 (d, C-1), 59.9, 62.2 (t, t, C-2, C-4), 60.2 (t, $\underline{C}H_2$Ph), 126.8, 128.1, 128.8 (d, d, d, Ph), 130.1, 132.8 (d, d, C-7, C-8).

Preparation of (1*S,5*S**,7*R**,8*S**)-8-{7-acetoxy-*N*-benzyl-3-azabicyclo-[3.3.0]oct-8-yl}methyl Acetate (11)** A mixture of monomerized paraformaldehyde (0.27 g, 8.6 mmol), ethylene dichloride (EDC) (8 ml) and sulfuric acid (0.46 ml, 8.6 mmol) was stirred for 20 min at 70 °C in a sealed tube. After being cooled to 20 °C, **9** (0.68 g, 3.4 mmol) and dioxane (a catalytic amount) in EDC (2 ml) was added and the mixture was stirred for 48 h at 70 °C in a sealed tube. The reaction mixture was cooled to 10 °C and sodium acetate (0.70 g, 8.6 mmol) in H_2O was added. The reaction mixture was extracted with EDC (20 ml × 2). The EDC extracts were washed with 10% $NaHCO_3$ aq. (40 ml × 2) and H_2O (30 ml × 2), dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on Al_2O_3 using a 4:1 mixture of hexane–AcOEt as the eluent to give **11** (0.64 g, 57%) as a white crystal, mp 75—80 °C. IR (KBr): 1740 (C=O). *Anal.* Calcd for $C_{19}H_{25}NO_4$: C, 68.85; H, 7.64; N, 4.30. Found: C, 68.86; H, 7.60; N, 4.23. ^{1}H-NMR: 2.01 (3H, s, $OCOCH_3$), 2.04 (3H, s, $OCOCH_3$), 2.08—2.77 (9H, m, H-1, H-2, H-4, H-5, H-6, H-8), 3.59 (2H, s, C$\underline{H}_2$Ph), 4.06 (2H, d, J=5 Hz, C$\underline{H}_2$OAc), 4.52—5.03 (1H, m, H-7), 7.29 (5H, s, $CH_2\underline{Ph}$). ^{13}C-NMR: 20.8, 21.0 (q, q, OCO$\underline{C}H_3$ × 2), 37.3 (d, C-5), 37.8, (t, C-6), 42.3 (d, C-1), 49.0 (d, C-8), 59.3 (t, $\underline{C}H_2$OAc), 60.1 (t, CH_2Ph), 59.2, 64.4 (t, t, C-2, C-4), 76.0 (d, C-7), 126.9, 128.2, 128.6, 138.9 (d, d, d, s, Ph), 170.5, 170.8 (s, s, O$\underline{C}$OCH$_3$).

Preparation of Isophthalic Acid Monomethyl Ester Chloride In a flask fitted with a NaOH tube were placed dimethyl isophthalate (7.8 g, 0.02 mmol) and 1 N $Ba(OH)_2$ in methanol (20 ml, 0.02 mmol) and the mixture was stirred for 15 h. The solvent was removed under reduced pressure and the residue was washed with ether. The filtrate and washings were concentrated *in vacuo* to afford the residue, which was washed with ether. The residue was put together. To a solution of the residue in CH_2Cl_2 (20 ml), 3 N HCl aq. was added to create acidity. The CH_2Cl_2 layer was separated and the H_2O layer was extracted with CH_2Cl_2 (20 ml × 2). The CH_2Cl_2 layer and extracts were washed with H_2O (20 ml × 2) and brine (20 ml × 2), and dried over $MgSO_4$. Removal of the solvent *in vacuo* afforded isophthalic acid monomethyl ester (3.6 g), which was used without further purification. Thionyl chloride (4.2 g, 36 mmol) was added to isophthalic acid monomethyl ester (3.6 g, 20 mmol) in $CHCl_3$ (20 ml) and the mixture was refluxed for 2 h at 80 °C. The solvent was concentrated *in vacuo* to afford a residue, which was recrystallized from petr. ether to give a white powder isophthalic acid monomethyl ester chloride (3.1 g, 80%), mp 43—45 °C. IR (KBr): 1768 (COCl), 1731 ($COOCH_3$). ^{1}H-NMR: 3.98 (3H, s, CH_3), 7.50—8.89 (4H, m, $\underline{Ph}$). ^{13}C-NMR: 52.61 (q, $COOCH_3$), 129.3, 132.4, 135.0, 135.9 (d, Ph), 133.8, 137.0 (s, s, Ph) 165.46 (s, COCl), 167.74 (s, $COOCH_3$). MS *m/z*: 198 (M^+), 200 $(M+2)^+$.

Preparation of (1*S,5*S**,7*R**,8*S**)-8-{7-Acetoxy-*N*-(*m*-methoxycarbonylbenzoyl-3-azabicyclo[3.3.0]oct-8-yl}methylacetate (13)** 5% Pd–C (0.12 g) was added to a solution of **11** (0.24 g, 0.72 mmol) in ethanol (10 ml). Hydrogen was charged at ordinary pressure and the reaction mixture was vigorously stirred for 18 h at 50 °C. Removal of the catalyst by suction filtration and concentration of the filtrate under reduced pressure gave oily **12** (0.16 g, 93%), which was used without further purification. Isophthalic acid monomethyl ester chloride (1.3 g, 6.7 mmol) was added to **12** (0.91 g, 3.8 mmol) in dry THF (20 ml) at 0 °C. Triethylamine (0.46 ml, 4.5 mmol) and DMAP (0.46 g, 3.8 mmol) was added to the reaction mixture, which was stirred for 18 h at room temperature. After removal of the solvent *in vacuo*, the residue was extracted with CH_2Cl_2 (20 ml × 2). The CH_2Cl_2 layer was washed with aq. $NaHCO_3$ (30 ml × 2) and brine (15 ml × 2), dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on SiO_2 using a 4:1 mixture of AcOEt–hexane as the eluent to give **13** (1.3 g, 86%). IR (neat): 1629 (N–C=O), 1723 (C=O). High-resolution MS Calcd for $C_{21}H_{25}NO_7$ (M^+): 403.1600. Found: 403.1606. ^{1}H-NMR: 2.03 (3H, s, OCOC$\underline{H}_3$), 2.05 (3H, s, OCOC$\underline{H}_3$), 2.12—3.07 (9H, m, H-1, H-2, H-4, H-5, H-6, H-8), 3.92 (3H, s, COOC$\underline{H}_3$), 4.09 (2H, d, J=5 Hz, C$\underline{H}_2$OAc), 4.77—5.11 (1H, m, H-7), 7.36—8.24 (4H, m, Ph). ^{13}C-NMR: 20.8, 21.1 (q × 2, OCO$\underline{C}H_3$), 36.9 (t, C-6), 49.4 (d, C-8), 52.3 (q, COO$\underline{C}H_3$), 64.3 (t, $\underline{C}H_2$OAc), 77.1 (d, C-7), 128.3, 128.7, 130.4, 131.0, 131.7, 137.1 (d, d, s, d, d, s, Ph), 166.3 (s, N–$\underline{C}$=O), 168.6 (s, $\underline{C}$OOCH$_3$), 170.5, 170.8 (d, d, O$\underline{C}$OCH$_3$).

Preparation of (1*S,5*S**,7*R**,8*S**)-3-{7-Hydroxy-8-hydroxymethyl-3-azabicyclo[3.3.0]octanyl}-*m*-methoxycarbonylphenylketone (14) 13** (0.17 g, 0.42 mmol) was added to 20 ml of 3 N HCl in methanol. The mixture was stirred for 2 h at room temperature and concentrated *in vacuo*. The residue was subjected to column chromatography on SiO_2 using a 10:1 mixture of $CHCl_3/CH_3OH$ as the eluent to give **14** (0.11 g, 80%). IR (neat): 1630 (N–C=O), 1723 (C=O), 3390 (OH). High-resolution MS Calcd for $C_{17}H_{21}NO_5$ (M^+): 319.1389. Found: 319.1386. ^{1}H-NMR: 1.99—2.82 (9H, m, H-1, H-2, H-4, H-5, H-6, H-8), 3.17—4.21 (5H, m, C$\underline{H}$–O$\underline{H}$, C$\underline{H}_2$O$\underline{H}$), 3.92 (3H, s, $COOCH_3$), 7.43—8.22 (4H, m, Ph). ^{13}C-NMR: 39.1 (t, C-6), 50.9 (d, C-8), 52.2 (q, COO$\underline{C}H_3$), 63.8 (t, $\underline{C}H_2$OAc), 76.2 (d, C-7), 128.1, 128.5, 130.1, 130.8, 131.4, 136.8 (d, d, s, d, d, s, Ph), 166.2 (s, N–$\underline{C}$–O), 168.7 (s, O$\underline{C}$OCH$_3$).

Preparation of (1*S,5*S**,7*R**,8*R**)-8-{7-Hydroxy-*N*-(*m*-methoxycarbonylbenzoyl)-3-azabicyclo[3.3.0]octanyl}carbaldehyde (15) 14** (0.099 g, 0.31 mmol) was dissolved in anhydrous dimethylsulfoxide (DMSO) (0.47

ml) and benzene (2 ml) containing pyridine (0.025 ml, 0.31 mmol) and trifluoroacetic acid (0.012 ml, 0.31 mmol), and the mixture was stirred for 20 min. After the addition of *N,N'*-dicyclohexylcarbodiimide (0.19 g, 0.93 mmol), the sealed reaction was stirred for 30 min at 0 °C and for 4 h at room temperature. AcOEt (20 ml) was added followed by a solution of oxalic acid (0.12 g, 0.93 mmol) in methanol (0.8 ml). After gas evolution had ceased (about 30 min), H_2O (10 ml) was added and the insoluble dicyclohexylurea was removed by filtration. The organic layer was washed with 5% aq. Na_2CO_3 (20 ml × 2), 1% HCl aq. (20 ml × 1) and brine (20 ml × 2), dried over $MgSO_4$ and concentrated *in vacuo* to afford **15** (0.098 g), which was used without further purification.

Preparation of (1*S,5*S**,7*R**,8*R**)-7-Hydroxy-*N*-*m*-methoxycarbonylbenzoyl-8-(3-oxo-1-octenyl)-3-azabicyclo[3.3.0]octane (16a)** A suspension of sodio dimethyl (2-oxoheptyl)phosphonate (0.12 g, 0.56 mmol) was prepared under an argon atmosphere from NaH (23 mg as 60% dispersion in mineral oil, *ca.* 0.56 mmol) and dimethyl 2-oxoheptyl phosphonate (0.12 g, 0.56 mmol) in 2 ml of DME at room temperature for 0.5 h. To the suspension was added a solution of **15** (90 mg, 0.28 mmol) in DME (1 ml) at 0 °C, and the whole was stirred for 2 h at room temperature. After dilution of the suspension with brine (10 ml), the mixture was extracted with CH_2Cl_2 (15 ml × 3), dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on SiO_2 using a 4:1 mixture of AcOEt–hexane as the eluent to afford **16a** (57 mg, 49%). IR (neat): 1630 (N–C=O), 1728 (C=O), 3390 (OH). FAB-MS *m/z*: $(M)^+$ 413. ^{1}H-NMR: 0.88 (3H, br t, J=6.3 Hz, CH_3), 1.24 (6H, br s, –CH=CH–C(O)CH_2C$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$$CH_3$), 1.88—2.81 (11H, m, H-1, H-2, H-4, H-5, H-6, H-8, –CH=CH–C(O)C$\underline{H}_2$–), 3.60—4.23 (2H, m, H-7, OH), 3.92 (3H, s, COOC$\underline{H}_3$), 6.02—6.32 (2H, m, –CH=CH–), 7.32—8.21 (4H, m, ph). ^{13}C-NMR: 13.9 (q, CH_3), 22.5, 24.3, 29.7, 31.5 (t, CH=CHC(O)$\underline{C}H_2\underline{C}H_2\underline{C}H_2\underline{C}H_2CH_3$), 34.35 (t, C-6), 49.1 (d, C-8), 52.3 (q, COO$\underline{C}H_3$), 77.1 (d, C-7), 127.1, 128.5 (d, d, $\underline{C}H=\underline{C}H$), 128.1, 128.5, 130.3, 130.8, 131.5, 136.9 (d, d, s, d, d, s, Ph), 166.2 (s, N–C=O), 168.4 (s, $\underline{C}OOCH_3$), 173.23 (s, CH=CH–$\underline{C}$O–).

Preparation of (1*S,5*S**,7*R**,8*R**)-8-(3-Hydroxy-1-octenyl)-7-hydroxy-*N*-*m*-methoxycarbonylbenzoyl-3-azabicyclo[3.3.0]octane (17a)** Freshly fused zinc chloride (0.31 g) was added to sodium borohydride (0.18 g) in redistilled DME (4.1 ml) under argon. The mixture was stirred overnight at 0—5 °C. To a solution of **16a** (0.057 g, 0.14 mmol) in anhydrous DME (0.1 ml) was added 0.42 ml of the above solution of zinc borohydride. The mixture was stirred at room temperature for 2 h, then saturated sodium tartrate solution was added dropwise until no further evolution of gas was observed. Methylene chloride was then added and the solution was dried over $MgSO_4$, filtered and concentrated *in vacuo* to dryness. The residue was purified by PTLC with a solvent system ($CHCl_3$–methanol, 10:1) to give **17a** (49 mg, 85%, colorless oil). IR (neat): 1625 (N–C=O), 1723 (C=O), 3380 (OH). FAB-MS *m/z*: $(M)^+$ 415. ^{1}H-NMR: 0.88 (3H, br t, J=6.3 Hz, CH_3), 1.25 (8H, br s, –CH=CHCH(OH)C$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$$CH_3$), 1.86—2.95 (9H, m, H-1, H-2, H-4, H-5, H-6, H-8), 3.30—4.69 (4H, m, H-7, OH, –C$\underline{H}$–O$\underline{H}$), 3.92 (3H, s, $COOCH_3$), 5.54—5.78 (2H, m, –CH=CH–), 7.38—8.21 (4H, m, Ph). ^{13}C-NMR: 14.0 (q, CH_3), 22.6, 24.3, 29.7, 31.8 (each t, –CH=CHCH(OH)$\underline{C}H_2\underline{C}H_2\underline{C}H_2\underline{C}H_2CH_3$), 35.6 (t, C-6), 49.1 (d, C-8), 52.3 (q, COO$\underline{C}H_3$), 72.8 (d, C–OH), 76.6 (d, C-7), 129.1, 131.1 (each d, –$\underline{C}$H=$\underline{C}$H–), 128.2, 128.6, 130.5, 130.9, 131.7, 137.2 (d, d, s, d, d, s, Ph), 166.3 (s, N–$\underline{C}$=O), 168.5 (s, $\underline{C}OOCH_3$).

Preparation of (1*S,5*S**,7*R**,8*R**)-*N*-*m*-Carboxybenzoyl-7-hydroxy-8-(3-hydroxy-1-octenyl)-3-azabicyclo[3.3.0]octane (1a)** A solution of **17a** (20 mg, 0.048 mmol) in methanol (1 ml) and 10% aq. KOH (0.8 ml) was stirred at room temperature for 2 h. The reaction mixture was acidified by the addition of 10% aq. HCl followed by extraction with CH_2Cl_2 (15 ml × 2). The CH_2Cl_2 extracts were washed with brine (20 ml × 2), and dried over $MgSO_4$. Removal of the solvent *in vacuo* afforded a residue, which was purified by PTLC with a solvent ($CHCl_3$–methanol, 4:1) to afford a colorless oil **1a** (15 mg, 80%). IR (neat): 1626 (N–C=O), 1724 (C=O), 3382 (OH). FAB-MS *m/z*: $(M)^+$ 401. ^{1}H-NMR: 0.88 (3H, br t, J=6.3 Hz, CH_3), 1.26 (8H, br s, –CH=CHC(OH)C$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$$CH_3$), 1.86—2.95 (9H, m, H-1, H-2, H-4, H-5, H-6, H-8), 3.36—4.65 (4H, m, H-7, OH, C$\underline{H}$–O$\underline{H}$), 5.54—5.78 (2H, m, –CH=CH–), 7.36—8.21 (4H, m, Ph). ^{13}C-NMR: 14.0 (q, CH_3), 22.7, 25.3, 29.7, 31.9 (each t, CH=CHC(OH)$\underline{C}H_2\underline{C}H_2\underline{C}H_2\underline{C}H_2CH_3$), 36.9 (t, C-6), 49.7 (d, C-8), 72.0 (d, C–OH), 77.4 (d, C-7), 129.3, 131.7 (each d, –CH=CH–), 128.5, 128.9, 130.6, 131.4, 132.1, 135.1 (d, d, s, d, d, s, Ph), 166.8 (s, N–C=O), 169.9 (s, COOH).

Preparation of Cyclohexyl(triphenylphosphoranylidene)methylketone 6.7 ml of 1.5 mol/l *n*-butyl lithium (0.01 mol) was added to a solution of triphenylphosphine bromide (3.57 g, 0.01 mol) in dry toluene (20 ml) at 0 °C under argon. The mixture was stirred for 30 min at 0 °C and for 4 h at room temperature. To the mixture, ethyl thiolcyclohexanoate (77.4 mg) was added. The reaction mixture was stirred under reflux for 18 h, then extracted with CH_2Cl_2. The CH_2Cl_2 extract was dried over $MgSO_4$ and concentrated *in vacuo* to give the desired product.

Preparation of (1*S,5*S**,7*R**,8*R**)-8-(3-Cyclohexyl-3-oxo-1-propenyl)-7-hydroxy-*N*-*m*-methoxycarbonylbenzoyl-3-azabicyclo[3.3.0]octane (16b)** The compound **15** (0.18 g, 0.58 mmol) was added to a solution of cyclohexyl (triphenylphosphoranylidene) methyl ketone (0.89 g, 2.30 mmol) in dry THF (15 ml) at room temperature under argon. The mixture was refluxed at 80 °C for 3 h, then concentrated *in vacuo*. To the residue, petr. ether was added. Deposited triphenyl-phosphine oxide was filtered off and washed with petr. ether. The filtrate and washings were dried over $MgSO_4$, concentrated *in vacuo* and the residue was subjected to PTLC (silica gel) with a mixture of hexane and AcOEt (1:4) to give **16b** (0.088 g, 33%). IR (neat): 3330 (OH), 1724 (C=O), 1629 (N–C=O). FAB-MS *m/z*: $(M)^+$ 425. ^{1}H-NMR: 0.73—2.18 (11H, m, cyclohexyl), 2.18—3.23 (10H, m, H-1, H-2, H-4, H-5, H-6, H-8, H-12), 3.23—4.05 (2H, m, H-7, OH), 3.93 (3H, s, $COOCH_3$), 6.07—6.40 (2H, m, –CH=CH–), 7.34—8.24 (4H, m, Ph), ^{13}C-NMR: 25.7, 25.8, 28.7, 29.6, 29.9 (each t, cyclohexyl), 33.9 (t, C-6), 49.0 (d, C-8), 52.3 (q, COO$\underline{C}H_3$), 77.6 (d, C-7), 125.6, 133.1 (each d, –CH=CH–), 128.2, 128.6, 130.3, 130.9, 131.6, 136.9 (d, d, s, d, d, s, Ph), 166.3 (s, N–C=O), 168.4 (s, $\underline{C}OOCH_3$), 179.4 (s, CH=CH–$\underline{C}$O).

Preparation of (1*S,5*S**,7*R**,8*R**)-8-(3-Cyclohexyl-3-hydroxy-1-propenyl)-7-hydroxy-*N*-*m*-methoxycarbonylbenzoyl-3-azabicyclo[3.3.0]-octane (17b)** Freshly fused zinc chloride (0.31 g) was added to sodium borohydride (0.18 g) in redistilled DME (4.1 ml) under argon. The mixture was stirred overnight at 0—5 °C. To a solution of **16a** (0.03 g, 0.071 mmol) in anhydrous DME (1 ml) was added 0.5 ml (0.11 mmol) of the above solution of zinc borohydride at 0 °C. The mixture was stirred at room temperature for 2 h, then a saturated sodium tartrate solution was added dropwise until no further evolution of gas was observed. Methylene chloride was then added and the solution was dried over $MgSO_4$, filtered and concentrated *in vacuo* to dryness. The residue was purified by PTLC with a solvent system (hexane–AcOEt, 1:4) to give **17b** (24 mg, 80%, colorless oil). IR (neat): 1623 (N–C=O), 1730 (C=O), 3334 (OH). FAB-MS *m/z*: $(M)^+$ 427. ^{1}H-NMR: 0.71—2.21 (11H, m, cyclohexyl), 2.21—3.15 (9H, m, H-1, H-2, H-4, H-5, H-7, H-8), 3.91 (3H, s, $COOCH_3$), 3.15—4.84 (4H, m, H-7, OH, C$\underline{H}$–O$\underline{H}$), 5.26—5.76 (2H, m, –CH=CH–), 7.32—8.21 (4H, m, Ph). ^{13}C-NMR: 25.0, 25.7, 28.2, 29.4, 29.8 (each t, cyclohexyl), 34.0 (t, C-6), 49.1 (d, C-8), 53.3 (q, COO$\underline{C}H_3$), 69.4 (d, CH–OH), 75.8 (d, C-7), 126.5, 131.2 (each d, –CH=CH–), 128.3, 128.7, 130.4, 130.9, 131.7, 137.4 (d, d, s, d, d, s, Ph), 166.5 (s, N–C=O), 168.1 (s, $COOCH_3$).

Preparation of (1*S,5*S**,7*R**,8*R**)-*N*-*m*-Carboxybenzoyl-8-(3-cyclohexyl-3-oxo-1-propenyl)-7-hydroxy-3-azabicyclo[3.3.0]octane (1b)** A solution of **17b** (0.016 g, 0.037 mmol) in methanol (1 ml) and 10% aq. KOH (1 ml) was stirred at room temperature for 2 h. The reaction mixture was acidified by the addition of 10% aq. HCl followed by extraction with CH_2Cl_2 (15 ml × 3). The CH_2Cl_2 extract was washed with brine (20 ml × 2), and dried over $MgSO_4$. Removal of the solvent *in vacuo* afforded a residue, which was purified by PTLC with a solvent ($CHCl_3$–methanol, 10:1) to afford colorless oil **1b** (12 mg, 73%). IR (neat): 1621 (N–C=O), 1719 (C=O), 3400 (OH). FAB-MS *m/z*: $(M+Na)^+$ 435. ^{1}H-NMR: 0.72—2.11 (11H, m, cyclohexyl), 2.11—3.35 (9H, m, H-1, H-2, H-4, H-5, H-6, H-8), 3.35—4.38 (4H, m, H-7, OH, C$\underline{H}$–O$\underline{H}$), 5.25—5.72 (2H, m, –CH=CH–), 7.46—8.25 (4H, m, Ph). ^{13}C-NMR: 25.2, 25.7, 28.2, 29.4, 29.9 (each t, cyclohexyl), 34.2 (t, C-6), 49.7 (d, C-8), 69.5 (d, –CH–OH), 77.0 (d, C-7), 126.7, 131.3 (each d, –CH=CH–), 128.2, 128.6, 130.4, 130.8, 131.8, 137.2 (d, d, s, d, d, s, Ph), 167.4 (s, N–C=O), 169.9 (s, COOH).

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Synthesis and Protective Effect of 1,3,5-Triazine Derivatives, Leukotriene C_4 Antagonist, on HCl·Ethanol-Induced Gastric Lesions in Rats

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2,4-Diamino-[*E*]-6-[2-(3-pyridyl)ethenyl]-1,3,5-triazine (3a), leukotriene C_4 (LTC_4) antagonist, was found to possess a protective effect on HCl·ethanol-induced gastric lesions. Analogues of 3a were synthesized and evaluated for the effect as well as antagonistic activity against LTC_4-induced contraction. Seven compounds (3a—d, f—h) exhibited a potent protective effect on gastric lesions which was considered to be based on the antagonistic activity against LTC_4. The structure–activity relationships of the derivatives (3a—k) are discussed.

Keywords 1,3,5-triazine; leukotriene C_4 antagonist; HCl·ethanol-induced gastric lesions; structure–activity relationship

Antiinflammatory activity has been reported for 1,3,5-triazine derivatives.[1] On the other hand, antiallergic activity has been reported for substituted ethenylpyridine derivatives.[2] And so, 2,4-diamino-[*E*]-6-[2-(3-pyridyl)-ethenyl]-1,3,5-triazine (**3a**, Chart 1), which was originally designed as an antiallergic agent, was recently prepared. In the biological screening, compound **3a** had low activity *in vivo*.[3] However, compound **3a** potently antagonized the contraction of leukotriene (LT) C_4 (LTC_4) on isolated rat gastric fundus strips.

Recently, Whittle and co-workers[4] have reported that the potent vasoconstrictor action of gastric submucosal LTC_4 that was observed in ethanol and HCl induced gastric mucosal damage model was identified as potential endogenous proulcerogenic agent. We therefore investigated the protective effect of compound **3a** against HCl·ethanol-induced gastric lesions in rats and found it to possess the potent protective effect. These facts prompted us to synthesize and test novel analogues of **3a**. This paper describes the synthesis of substituted ethenyl-1,3,5-triazine derivatives and the determination of their structure–activity relationships for antagonistic activity against LTC_4-induced contraction and for protective effect against HCl·ethanol-induced gastric lesions.

Chemistry All compounds were prepared by two general methods according to Chart 2.

In method A, **1** was condensed with substituted benzaldehyde derivatives (**2b—j**) by the use of methanesulfonic acid or formic acid without a solvent. Pyridine derivatives (**3a, l**) were also prepared in the same manner as described above. In method B, **1** was condensed with **2k** in the presence of potassium hydroxide in 2-methoxyethanol. All compounds prepared were purified by recrystallization.

Biological Results and Discussion Compounds **3a** and **3l** with a hydroxy substituent in place of the amino group in the 2 position of **3a** were listed in Table I. They were tested for antagonistic activity against LTC_4- and LTD_4-induced contraction. Compound **3a** inhibited the LTC_4-induced contraction of gastric fundus strips at 1×10^{-6} M, thereby indicating antagonistic activity against LTC_4. This anti-LTC_4 activity of **3a** in the gastric preparation was about 100 times more potent than its anti-LTD_4 activity in the ileal preparation. However, the anti-LTC_4 and -LTD_4 activity of compound **3l** were rather feeble compared with that of **3a**.

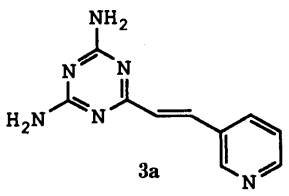
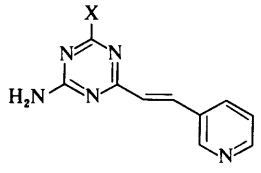


Chart 1

The listed compounds were tested for the protective effect at a dose of 5 mg/kg against HCl·ethanol-induced gastric lesions, using cimetidine as a standard. In the case of this test compound **3a** also showed substantial effect (>40% inhibition), but compound **3l** was not effective (<40% inhibition). These results suggested that the diamino substituents of **3a** were requisite for both the antagonistic activity and the protective effect.

Compounds **3b—k** with a phenyl group in place of the pyridyl group of **3a** were listed in Table II, and the effects of the substituent on the benzene ring were studied. These compounds showed remarkable variations in the activity as the substituent on the benzene ring was changed. The compounds with 2,4-dichloro (**3c**), 3,5-dichloro (**3d**), 2-trifluoromethyl (**3f**), 3-trifluoromethyl (**3g**), or 4-cyano

TABLE I. Biological Activity of 2-Amino-4-substituted-[*E*]-6-[2-(3-pyridyl)ethenyl]-1,3,5-triazine (**3a, l**)



Compd. No.	X	Antagonistic activity % inhibition against LTC_4-induced contraction at 1×10^{-5} M	1×10^{-6} M	LTD_4-induced contraction at 1×10^{-4} M	HCl·ethanol-induced gastric lesions % inhibition[a] at 5 mg/kg *p.o.*
3a	NH_2	60	43	36	75
3l	OH	21		5	31
Cimetidine		NT[b]		NT[b]	68[c]

a) Treatments in which inhibition values were more than 40% were evaluated as significantly effective. *b*) NT = not tested. *c*) Dose: 200 mg/kg.

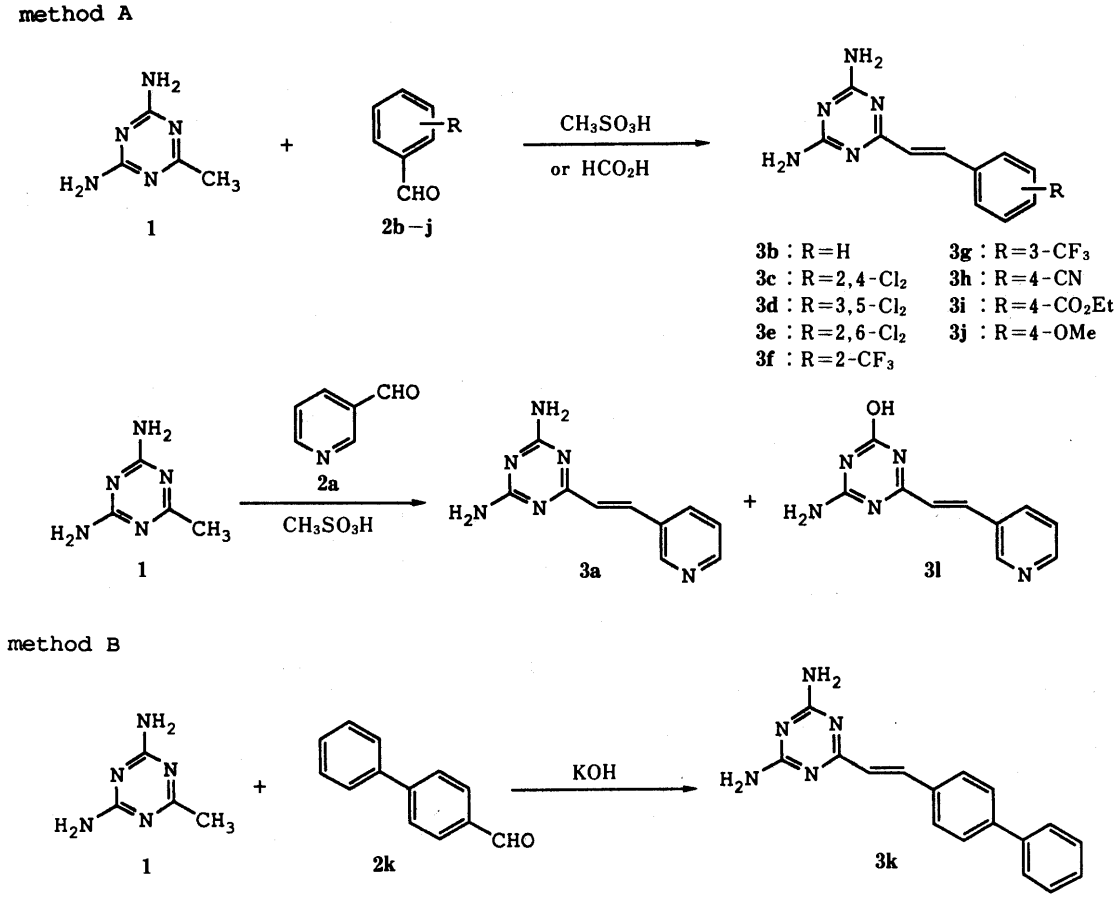
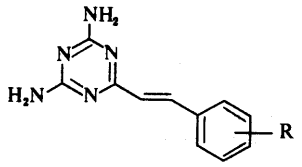


Chart 2

TABLE II. Biological Activity of 2,4-Diamino-[*E*]-6-[2-(substitutedphenyl)ethenyl]-1,3,5-triazine (**3b—k**)



Compd. No.	R	Antagonistic activity % inhibition against LTC_4-induced contraction at 1×10^{-5} M	HCl·ethanol-induced gastric lesions % inhibition[a] at 5 mg/kg *p.o.*
3b	H	53	44
3c	2,4-Cl_2	76	49
3d	3,5-Cl_2	41	67
3e	2,6-Cl_2	43	1
3f	2-CF_3	69	64
3g	3-CF_3	76	58
3h	4-CN	49	42
3i	4-CO_2Et	25	29
3j	4-OMe	16	8
3k	4-Ph	3	1

a) See footnotes *a*) in Table I.

(**3h**) group and the compound without substituents on the benzene ring (**3b**) showed both the antagonistic activity against LTC_4 and the protective effect on gastric lesions. Meanwhile, in these assays compounds with 4-ethoxycarbonyl (**3i**), 4-methoxy (**3j**) or 4-phenyl (**3k**) group were inactive. However, the compound with a 2,6-dichloro (**3e**) group showed only antagonistic activity against LTC_4.

These results suggested electron withdrawing substituents on the benzene ring except the 4-ethoxycarbonyl compound (**3i**) seemed to be preferable for both the anti-LTC_4 activity and the protective effect on gastric lesions. In the case of the 2,6-dichloro compound (**3e**), the results suggested that steric properties on the benzene ring might modify the protective effect on gastric lesions.

The results in Tables I and II suggested that the selectively potent antagonistic activity against a LTC_4-induced contraction of type 3 compounds, except compound **3e**, was closely correlated to the protective effect against HCl·ethanol-induced gastric lesions.

In conclusion, the present results demonstrate that **3a** and **3f** are suitable as lead compounds for the development of a new type of antiulcer drug.

Experimental

Melting points were determined on a Mettler FP62 apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were determined in dimethylsulfoxide (DMSO)-d_6 on a JEOL FX-200 NMR spectrometer using tetramethylsilane (TMS) as an internal standard. Mass spectra (MS) were measured with a JEOL JMS-DX 300 instrument. Infrared (IR) spectra were recorded on a Hitachi 270-30 spectrometer.

Typical Procedures of Method A 2,4-Diamino-[*E*]-6-styryl-1,3,5-triazine (**3b**): Benzaldehyde (**2b**, 4.4 ml) was added to a solution of **1** (5.25 g) in methanesulfonic acid (28 ml). The mixture was stirred for 2.5 h at 110 °C. It was cooled to room temperature and made basic with NaOH aqueous solution, with ice cooling. The precipitated solid was collected by filtration and recrystallized from 2-methoxyethanol to give **3b** (3.38 g, 38%), mp 273—274 °C. NMR δ: 6.65 (4H, br s), 6.74 (1H, d, J=15.9 Hz), 7.30—7.70 (5H, m), 7.80 (1H, d, J=15.9 Hz). IR ν (KBr):

3468, 3308, 3136, 1634, 1536, 1452 cm^{-1}. MS *m/z* (%): 213 (M^+, 54), 212 (M^+-1, 100), 170 (42), 144 (17), 128 (26). High resolution mass spectrum (HRMS) *m/z* Calcd for $C_{11}H_{11}N_5$ (M^+): 213.1013. Found: 213.1002. *Anal.* Calcd for $C_{11}H_{11}N_5$: C, 61.95; H, 5.20; N, 32.85. Found: C, 61.83; H, 5.43; N, 32.74.

Compounds **3a**, **3c**—**g** and **3j** were obtained in the same manner as described for **3b**.

2,4-Diamino-[*E*]-6-[2-(3-pyridyl)ethenyl]-1,3,5-triazine (**3a**): Yield, 51%. mp 287—289 °C (dec.) (2-methoxyethanol). NMR δ: 6.71 (4H, br s), 6.88 (1H, d, J=16.1 Hz), 7.43 (1H, dd, J=8.1, 4.6 Hz), 7.79 (1H, d, J=16.1 Hz), 8.12 (1H, br d, J=8.1 Hz), 8.55 (1H, d, J=4.6 Hz), 8.79 (1H, br s). IR ν (KBr): 3324, 3136, 1650, 1530 cm^{-1}. MS *m/z* (%): 215 (M^++1, 87), 214 (M^+, 100), 172 (50), 131 (32), 129 (23), 104 (17). HRMS *m/z* Calcd for $C_{10}H_{10}N_6$ (M^+): 214.0966. Found: 214.0966. *Anal.* Calcd for $C_{10}H_{10}N_6$: C, 56.06; H, 4.71; N, 39.23. Found: C, 56.39; H, 4.71; N, 38.90.

2,4-Diamino-[*E*]-6-[2-(2,4-dichlorophenyl)ethenyl]-1,3,5-triazine (**3c**): Yield, 66%. mp 264—265 °C (dec.) (2-methoxyethanol–MeOH). NMR δ: 6.75 (4H, br s), 6.82 (1H, d, J=15.9 Hz), 7.46 (1H, dd, J=8.6, 2.2 Hz), 7.70 (1H, d, J=2.2 Hz), 7.96 (1H, d, J=8.6 Hz), 8.08 (1H, d, J=15.9 Hz). IR ν (KBr): 3492, 3340, 3172, 1630, 1536, 976, 818 cm^{-1}. MS *m/z* (%): 285 (M^+), 283 (M^+, 19), 281 (M^+, 29), 248 (34), 246 (100), 206 (28), 204 (87), 162 (35). HRMS *m/z* Calcd for $C_{11}H_9Cl{}^*ClN_5$ (M^+): 283.0205. Found: 283.0197; Calcd for $C_{11}H_9{}^*Cl_2N_5$ (M^+): 285.0176. Found: 285.0176. *Anal.* Calcd for $C_{11}H_9Cl_2N_5$: C, 46.83; H, 3.22; N, 24.82. Found: C, 46.85; H, 3.37; N, 24.48.

2,4-Diamino-[*E*]-6-[2-(3,5-dichlorophenyl)ethenyl]-1,3,5-triazine (**3d**): Yield, 59%. mp 284—287 °C (dec.) (2-methoxyethanol–MeOH). NMR δ: 6.70 (4H, br s), 6.89 (1H, d, J=15.9 Hz), 7.60 (1H, d, J=15.9 Hz), 7.70—7.80 (3H, m). IR ν (KBr): 3432, 3344, 3148, 1644, 1532, 850 cm^{-1}. MS *m/z* (%): 285 (M^+), 283 (M^+, 66), 281 (M^+, 100), 240 (26), 238 (38), 212 (29), 162 (21). HRMS *m/z* Calcd for $C_{11}H_9Cl{}^*ClN_5$ (M^+): 283.0205. Found: 283.0210; Calcd for $C_{11}H_9{}^*Cl_2N_5$ (M^+): 285.0176. Found: 285.0179.

2,4-Diamino-[*E*]-6-[2-(2,6-dichlorophenyl)ethenyl]-1,3,5-triazine (**3e**): Yield, 48%. mp 251—252 °C (2-methoxyethanol–MeOH). NMR δ: 6.72 (1H, d, J=16.4 Hz), 6.76 (4H, br s), 7.37 (1H, dd, J=9.0, 7.1 Hz), 7.56 (1H, d, J=7.1 Hz), 7.56 (1H, d, J=9.0 Hz), 7.83 (1H, d, J=16.4 Hz). IR ν (KBr): 3492, 3472, 3316, 3112, 1666, 1648, 1548, 1430, 968, 808, 774 cm^{-1}. MS *m/z* (%): 285 (M^+), 283 (M^+, 33), 281 (M^+, 50), 248 (25), 246 (76), 206 (33), 204 (100), 162 (47). HRMS *m/z* Calcd for $C_{11}H_9Cl{}^*ClN_5$ (M^+): 283.0205. Found: 283.0196; Calcd for $C_{11}H_9{}^*Cl_2N_5$ (M^+): 285.0176. Found: 285.0168.

2,4-Diamino-[*E*]-6-[2-(2-trifluoromethylphenyl)ethenyl]-1,3,5-triazine (**3f**): Yield, 54%. mp 250—251 °C (EtOH). NMR δ: 6.72 (4H, br s), 6.78 (1H, d, J=15.9 Hz), 7.50—8.10 (4H, m), 8.11 (1H, br d, J=15.9 Hz). IR ν (KBr): 3496, 3344, 3168, 3072, 1672, 1640, 1532, 1312, 1164 cm^{-1}. MS *m/z* (%): 281 (M^+, 100), 212 (76), 170 (53), 111 (32). HRMS *m/z* Calcd for $C_{12}H_{10}F_3N_5$ (M^+): 281.0887. Found: 281.0886. *Anal.* Calcd for $C_{12}H_{10}F_3N_5$: C, 51.25; H, 3.58; N, 24.90. Found: C, 51.34; H, 3.81; N, 24.70.

2,4-Diamino-[*E*]-6-[2-(3-trifluoromethylphenyl)ethenyl]-1,3,5-triazine (**3g**): Yield, 39%. mp 171—173 °C (EtOH). NMR δ: 6.67 (4H, br s), 6.89 (1H, d, J=15.9 Hz), 7.60—7.80 (2H, m), 7.83 (1H, d, J=15.9 Hz), 7.90—8.00 (2H, m). IR ν (KBr): 3504, 3408, 3200, 1632, 1546, 1404, 1332, 1124 cm^{-1}. MS *m/z* (%): 282 (M^++1, 95), 281 (M^+, 100), 239 (50), 213 (34), 112 (40). HRMS *m/z* Calcd for $C_{12}H_{10}F_3N_5$ (M^+): 281.0888. Found: 281.0884.

2,4-Diamino-[*E*]-6-[2-(4-methoxyphenyl)ethenyl]-1,3,5-triazine (**3j**): Yield, 27%. mp 244 °C (dec.) (MeOH). NMR δ: 3.79 (3H, s), 6.59 (4H, br s), 6.59 (1H, d, J=15.9 Hz), 6.96 (2H, d, J=8.8 Hz), 7.56 (2H, d, J=8.8 Hz), 7.75 (1H, d, J=15.9 Hz). IR ν (KBr): 3324, 3168, 1636, 1546, 1510 cm^{-1}. MS *m/z* (%): 243 (M^+, 88), 242 (M^+-1, 100), 200 (32), 158 (19). HRMS *m/z* Calcd for $C_{12}H_{13}N_5O$ (M^+): 243.1019. Found: 243.1106.

2,4-Diamino-[*E*]-6-[2-(4-cyanophenyl)ethenyl]-1,3,5-triazine (**3h**): 4-Cyanobenzaldehyde (**2h**, 0.53 g) was added to a solution of **1** (0.5 g) in formic acid (10 ml). The mixture was refluxed for 57 h, and formic acid was evaporated off *in vacuo*. The residue was made basic with $NaHCO_3$ aqueous solution, with ice cooling. The precipitated solid was collected by filtration and recrystallized from 2-methoxyethanol to give **3h** (0.17 g, 18%), mp > 300 °C. NMR δ: 6.70 (4H, br s), 6.90 (1H, d, J=15.9 Hz), 7.80 (1H, d, J=15.9 Hz), 7.83 (4H, s). IR ν (KBr): 3504, 3440, 3328, 3176, 2220, 1640 cm^{-1}. MS *m/z* (%): 238 (M^+, 75), 237 (M^+-1, 100), 195 (41), 169 (25). HRMS *m/z* Calcd for $C_{12}H_{10}N_6$ (M^+): 238.0967. Found: 238.0974.

Compound **3i** was obtained in the same manner as described for **3h**.

2,4-Diamino-[*E*]-6-[2-(4-ethoxycarbonylphenyl)ethenyl]-1,3,5-triazine (**3i**): Yield, 15%. mp 230—231 °C (EtOH). NMR δ: 1.34 (3H, t, J=7.1 Hz), 4.33 (2H, q, J=7.1 Hz), 6.68 (4H, br s), 6.87 (1H, d, J=16.1 Hz), 7.76 (2H, d, J=8.3 Hz), 7.82 (1H, d, J=16.1 Hz), 7.97 (2H, d, J=8.3 Hz). IR ν (KBr): 3492, 3332, 3136, 1700, 1640, 1536, 1396, 1280 cm^{-1}. MS *m/z* (%): 285 (M^+, 74), 284 (M^+-1, 100), 256 (25), 242 (22). HRMS *m/z* Calcd for $C_{14}H_{15}N_5O_2$ (M^+): 285.1225. Found: 285.1245.

2-Amino-4-hydroxy-[*E*]-6-[2-(3-pyridyl)ethenyl]-1,3,5-triazine (**3l**): 3-Pyridinecarboxaldehyde (**2a**, 20.28 g) was added to a solution of **1** (19.75 g) in methanesulfonic acid (90 ml). The mixture was stirred for 2 h at 110 °C. The mixture was cooled to room temperature and made neutral with NaOH aqueous solution, with ice cooling. The precipitated solid was collected by filtration and recrystallized from DMSO–EtOH–MeOH to give **3l** (3.94 g, 12%), mp > 300 °C. NMR (D_2O–K_2CO_3) δ: 6.74 (1H, d, J=16.1 Hz), 7.43 (1H, dd, J=8.3, 4.9 Hz), 7.64 (1H, d, J=16.1 Hz), 8.05 (1H, br d, J=8.3 Hz), 8.44 (1H, dd, J=4.9, 1.5 Hz), 8.65 (1H, d, J=2.0 Hz). IR ν (KBr): 3368, 3128, 1690, 1588 cm^{-1}. MS *m/z* (%): 216 (M^++1, 85), 215 (M^+, 100), 172 (32), 147 (41), 137 (36), 132 (31), 130 (29), 104 (23). HRMS *m/z* Calcd for $C_{10}H_9N_5O$ (M^+): 215.0806. Found: 215.0796. *Anal.* Calcd for $C_{10}H_9N_5O$: C, 55.81; H, 4.22; N, 32.54. Found: C, 55.45; H, 4.16; N, 32.15.

Typical Procedure of Method B 2,4-Diamino-[*E*]-6-[2-(4-biphenylyl)-ethenyl]-1,3,5-triazine (**3k**): A mixture of **1** (1.25 g), KOH (0.66 g), 4-biphenylcarboxaldehyde (**2k**, 2.91 g) and 2-methoxyethanol (30 ml) was stirred for 16 h at 80 °C. The mixture was cooled to room temperature and the precipitated solid was collected by filtration and recrystallized from 2-methoxyethanol–MeOH to give **3k** (0.6 g, 21%), mp 276 °C (dec.). NMR δ: 6.66 (4H, br s), 6.79 (1H, d, J=15.9 Hz), 7.35—7.75 (5H, m), 7.72 (4H, s), 7.84 (1H, d, J=15.9 Hz). IR ν (KBr): 3324, 3112, 1632, 1538, 1530 cm^{-1}. MS *m/z* (%): 289 (M^+, 82), 288 (M^+-1, 100), 246 (34), 220 (13), 205 (16), 204 (31). HRMS *m/z* Calcd for $C_{17}H_{15}N_5$ (M^+): 289.1327. Found: 289.1306.

HCl·Ethanol-Induced Gastric Lesions[5] Male Wistar rats weighing 160—200 g (Charles-River Japan), 8 rats per group, were fasted but allowed free access to water for 24 h. One ml/200 g body weight of 60% ethanol (v/v) in 150 mM HCl (HCl·ethanol) was administered *p.o.* and the rats were sacrificed 1 h later. The sum of the length of each lesion (mm) was used as a lesion index. Percent inhibition was calculated as follows: [(lesion index of control-lesion index of test compound)/lesion index of control] × 100. The statistical significance of the difference between the mean ulcer index of the drug-treated group and that of the control group was calculated by using the Students' *t* test. Drugs were given *p.o.* 30 min before the HCl·ethanol administration suspended in 0.5% sodium carboxymethyl cellulose (CMC).

Bioassay of LTC_4 and LTD_4 LTs were performed on the isolated male Hartley guinea pigs (BW: *ca.* 300 g, Kiwa Lab. animals Co., Ltd.) ileum or male Sprague Dawley rats (BW: *ca.* 250 g, Charles-River Japan) fundus strips which were mounted at a load of 1.0 g in a 20 ml organ bath filled with the Tyrodes' solution and bubbled with 5% CO_2 in O_2. The contractile activities of 10^{-8} M LTC_4 and LTD_4 were assayed in the presence or absence of drugs. Drugs were given 10 min before the addition of LTs. To know the responsiveness of the tissue, histamine or acetylcoline (10^{-5} M) were added to the bath. Following the histamine or acetylcoline challenge, the tissues were washed and allowed 20 min to restabilize to base-line tension before LTs. Each data represents a mean of 4—5 animals.

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Synthesis and Antitumor Activity of 20(*S*)-Camptothecin Derivatives: A-Ring Modified and 7,10-Disubstituted Camptothecins[1a)]

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20(*S*)-Camptothecin derivatives having nitro, amino, chloro, bromo, hydroxyl and methoxyl groups in the A-ring were synthesized. B-Ring hydrogenated camptothecin (2a) was converted into 10-hydroxycamptothecin (6e) by treatment with lead tetraacetate in trifluoroacetic aicd. 10-Substituted derivatives (6) were obtained by a photoreaction of N-oxides (9). The cytotoxicity of the A-ring modified camptothecins was evaluated against KB cells *in vitro* and leukemia L1210 in mice. 7-Ethyl-10-hydroxycamptothecin (6i) was identified as a potential derivative for further modification.

Keywords 20(*S*)-camptothecin; nitrocamptothecin; aminocamptothecin; chlorocamptothecin; bromocamptothecin; hydroxycamptothecin; 7-ethyl-10-hydroxycamptothecin; lead tetraacetate; photoreaction; KB cell; L1210

20(*S*)-Camptothecin (**1a**) is an antitumor alkaloid which was first isolated from *Camptotheca acuminata* (Nyssaceae) by Wall and co-workers in 1966.[2a)] A-Ring substituted camptothecins were obtained by chemical modifications of **1a** or total synthetic routes. In 1975, Pei-Chuang and co-workers reported that they introduced hydroxyl, methoxyl and chloro substituents at the C-12 position of **1a** by nitration, followed by reduction and diazotization. They also announced that these derivatives showed higher activity than **1a** against leukemia L615 in mice.[3)] Wall and co-workers reported the synthesis of racemic 9- or 10-substituted camptothecins and demonstrated that they have significant antitumor activity.[4)] Naturally-occurring A-ring-oxygenated camptotheca alkaloids such as 10-, 11-hydroxy- or 9-, 10- and 11-methoxycamptothecins have been detected as minor components in *C. acuminata* and related plants.[5)] 10-Hydroxycamptothecin (**6e**) was reported to be effective for some human cancers in clinical trials in China.[6)]

We wish to report here the synthesis of derivatives having nitro, amino, chloro, bromo, hydroxyl and methoxyl groups in the A-ring. The activity of each derivative was evaluated against KB cells *in vitro* to understand the structure-activity relationships. We also report here effective hydroxylation methods of the 10-position of **1a**.

Chemistry A-Ring modified 20(*S*)-camptothecins were obtained starting from corresponding nitrocampotothecins. **1a** was treated with 61% nitric acid in concentrated sulfuric at room temperature for 3 d and the resulting mixture was purified through silica gel column chromatography to give 9- and 12-nitrocamptothecins (**5a**, 31.1% and **8a**, 57.8% yields).[1a,3,4a)] To obtain 10- or 11-nitrocamptothecins, **1a** was converted into the B-ring hydrogenated compound (**2a**),[7)] which contains *o*-toluidine grouping. A suspension of **1a** in a solution of acetic acid–dioxane was shaken with a platinum catalyst under an ambient hydrogen pressure to afford two products [*Rf* 0.33 (46.2% yield), *Rf* 0.28 (12.3% yield), with AcOEt]. In the proton nuclear magnetic resonance (^{1}H-NMR) spectra, each C_2-H signal was observed as a doublet at δ4.91 ppm (J=6 Hz) and δ 4.93 ppm (J=6 Hz), respectively. These products were stereoisomers having *cis* at the B–C ring junction. Tetrahydrocamptothecin (**2a**) was nitrated in sulfuric acid, *via* an anilinium salt, to give **3c** (51.4% yield) together with **1a** (16%) as an oxidized product. Aromatization of **3c** with 2,3-dichloro-4,5-dicyano-*p*-benzoquinone (DDQ) gave 11-nitrocamptothecin (**7a**, 78% yield), in an overall yield of 23% from **1a**.

Compound **2a** was acetylated to give *N*,20-*O*-diacetyl compound **2b** in 98% yield. The anilide was smoothly nitrated at −15 °C to give mononitro compound **3a** (96% yield), which was hydrolyzed by heating in 20% sulfuric acid to give **3b**, and then the product was oxidized with DDQ to afford 10-nitrocamptothecin (**6a**, 96% yield), in an overall yield of 42% from **1a**. The structures of the four nitrocamptothecins were confirmed by 400 MHz ^{1}H-NMR spectroscopy and the results are presented in Experimental.

The nitrocamptothecins were reduced to amino compounds (**5b**, **6b**, **7b** and **8b**) by catalytic hydrogenation. The amino derivatives were converted into chloro (**5c**, **6c**, **7c** and **8c**), bromo (**5d**, **7d** and **8d**) and hydroxyl derivatives (**5e**, **6e** and **7e**) *via* diazonium salts. Bromination of **2b** with tetrabromocyclohexadienone[8)] to give **3d** followed by hydrolysis and aromatization afforded 10-bromocamptothecin (**6d**) in good yield. 12-Hydroxycamptothecin (**8e**) was obtained in good yield by empolying Cohen's method.[9)] The methoxyl compounds (**5f**, **6f**, **7f** and **8f**) were obtained by alkylation of the corresponding phenols with diazomethane.

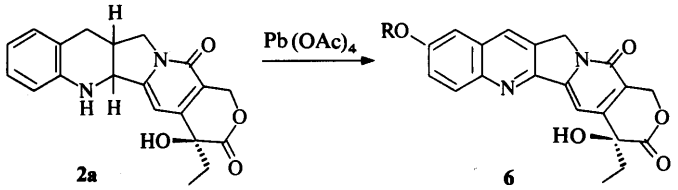
10-Hydroxy- and 9-methoxycamptothecins (**6e** and **5f**) were identified directly with authentic samples isolated from *C. acuminata* and *Nothapodytes foetida*, respectively.[5a)]

The E-lactone ring of the alkaloid was said to be an essential moiety of antitumor activity.[2)] To enable synthesis of water-soluble derivatives with the E-lactone ring intact, a hydroxyl group at 10 position of the alkaloid was a desirable foothold for further modification since 10-hydroxycamptothecin (**6e**) is known as a less toxic, potent derivative. We tried oxidative introduction of the hydroxyl group at the 10-position of **2a** with concomitant aromatization (Table I). Three molar amounts of lead tetraacetate (LTA) in trifluoroacetic acid (TFA) was the most effective for converting **2a** into **6e**. When acetic acid was used as a solvent, 10-acetoxycamptothecin (**6g**, 20.8% yield) was obtained along with **6e** (39.9% yield). Only dehydrogenation of **2a** to **1a** was observed when chromium trioxide, ferric chloride, ceric ammonium nitrate or Fremy salt was used as oxidant.

Quinoline 1-oxides having an electron withdrawing group at the α-position were converted photochemically into 6-hydroxyquinolines.[10] Camptothecin and 7-substituted compounds (**1b**—**f**) were conveniently oxidized to N-oxides (**9**) in good yields. The photoreaction of camptothecin 1-oxide (**9a**) under the reported conditions resulted only in deoxygenation. Examination of the reaction conditions led us to employ dioxane in the presence of 1 N sulfuric acid. Irradiation (450 W Hg high-pressure lamp with a Pyrex filter) of the mixture of **9a** for 30 min gave **6e** (75.0% yield) and **1a** (11.5% yield) as a deoxygenated product. In this reaction, 12-hydroxyl isomer was detected as a minor product (about 5% yield, in the reaction of **9b**). When the reaction was conducted with methanol, acetic acid and ethyl thioglycolate, **6f** (38.5% yield), **6g** (30.5% yield) and **6h** (11.9% yield) were obtained together with **6e** (Table II).

Thus, starting from 20(*S*)-camptothecin (**1a**), we obtained four possible nitrocamptothecins through direct or indirect method. These compounds were converted into the desired A-ring-modified derivatives in an ordinary manner. We also empolyed two routes to 10-hydroxyl

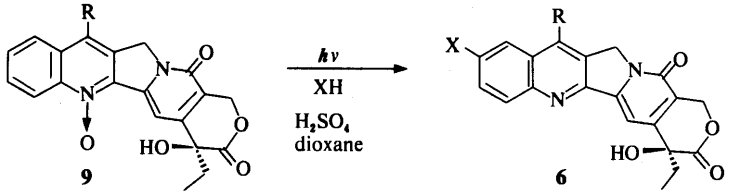
TABLE I. Preparation of 10-Hydroxycamptothecin (**6e**)



2a **6**

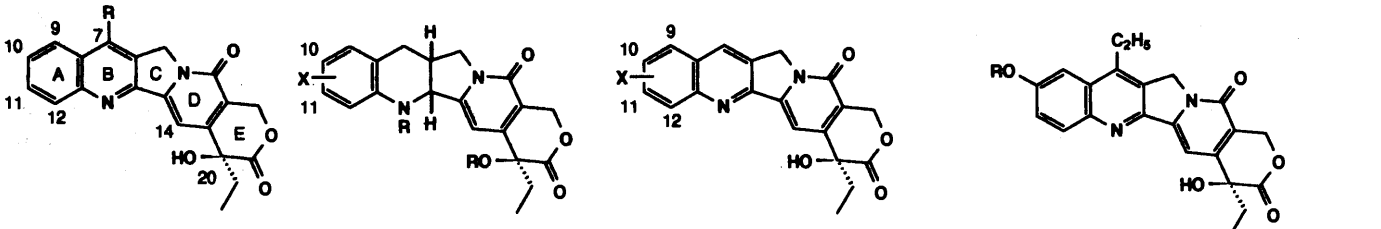
Solvent	$Pb(OAc)_4$ Molar ratio	Yield (%) 6e (R=H)	6g (R=Ac)	1a
AcOH	1.0	34.4	8.0	10.1
	3.0	30.9	20.8	8.1
CF_3CO_2H	1.0	48.4	—	8.1
	3.0	73.5	—	12.1

TABLE II. Synthesis of 10-Substituted Camptothecins (**6**) by the Photoreaction of N-Oxides (**9**)



9 **6**

N-Oxide Compd. No.	R	XH	6 Compd. No.	R	X	Yield (%)
9a	H	H_2O	**6e**	H	OH	75.0
		MeOH	**6f**	H	OMe	38.5
			6e	H	OH	22.5
		AcOH	**6g**	H	OAc	30.5
			6e	H	OH	25.0
		$HSCH_2CO_2Et$	**6h**	H	SCH_2CO_2Et	11.9
			6e	H	OH	58.0
9b	Et	H_2O	**6i**	Et	OH	49.1
9c	Pr	H_2O	**6j**	Pr	OH	60.5
9d	CH_2Ph	H_2O	**6k**	CH_2Ph	OH	59.8
9e	CH_2OAc	H_2O	**6l**	CH_2OAc	OH	32.0
9f	CH_2OH	H_2O	**6m**	CH_2OH	OH	40.0



1a : R=H, 20(*S*)-camptothecin
1b : R=Et
1c : R=Pr
1d : R=CH_2Ph
1e : R=CH_2OAc
1f : R=CH_2OH

2a : R=H, X=H
2b : R=Ac, X=H
3a : R=Ac, X=10-NO_2
3b : R=H, X=10-NO_2
3c : R=H, X=11-NO_2
3d : R=Ac, X=10-Br

5a : X=9-NO_2 **6a** : X=10-NO_2
5b : X=9-NH_2 **6b** : X=10-NH_2
5c : X=9-Cl **6c** : X=10-Cl
5d : X=9-Br **6d** : X=10-Br
5e : X=9-OH **6e** : X=10-OH
5f : X=9-OMe **6f** : X=10-OMe
7a : X=11-NO_2 **8a** : X=12-NO_2
7b : X=11-NH_2 **8b** : X=12-NH_2
7c : X=11-Cl **8c** : X=12-Cl
7d : X=11-Br **8d** : X=12-Br
7e : X=11-OH **8e** : X=12-OH
7f : X=11-OMe **8f** : X=12-OMe

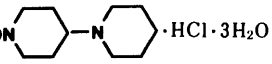
6i : R=H
CPT-11 : R= 

Chart 1

derivatives by oxidation of the tetrahydro compound **2a** with LTA and by photorearrangement of the N-oxides **9**. The latter was convenient method for the preparation of 7,10-disubstituted camptothecins.

Biological Results and Discussion

The cytotoxicity of each A-ring-modified camptothecins was examined *in vitro* (Table III). Significant cytotoxicity was observed in the derivatives having electron withdrawing chloro and bromo substituents at the 9 position and electron donating hydroxyl and amino groups at the 10 position. Contrary to the report of Pei-Chuang, all the 12-substituted derivatives were far less active than **1a** in this system. Methoxycamptothecins (**5f**,**6f** and **7f**) showed activity at least as potent as that of **1a**, while all the nitrocamptothecins were less potent than **1a**. Tetrahydrocamptothecin (**2a**) was not active, probably due to large changes in its geometric and electronic structures. The effect of substituents on cytotoxicity decreases in the sequence of 9=10>11≫12-position as pointed out by Wall *et al.*[4]

Derivatives with substituents fill in the concave region of the alkaloid, for example, 12-substituted camptothecins, N-oxide (**9a**) and 14-chlorocamptothecin,[11] showed low cytotoxicity. These substituents seemed to interfere with the critical role of the 20-hydroxyl group.[12]

Table IV shows the antitumor activities of 7,10-disubstituted camptothecins administered as sodium salts having a cleaved lactone grouping, compared with the activities of **1a**, **6e** and 7-ethylcamptothecin (**1b**).[1c,13] All the compounds tested exhibited the antitumor activity, with higher activities being observed in the suspension administrations. The sodium salt of 7-ethylcamptothecin (**1b**-Na) showed higher *T*/*C*% that **1a**-Na. In the corresponding 10-hydroxyl derivatives, 7-ethyl-10-hydroxycamptothecin (**6i**-Na, 0.4, ED_{50}/ED_{50} of **1a**-Na, KB cells *in vitro*) also showed higher activity than **6e**-Na, and its *TI* value was the largest (*TI*: therapeutic index; maximum tolerance dose/minimum effective dose, calculated from the dose responce curves; **1a**-Na, 3.1; **1b**-Na, 12.5; **6e**-Na, 8.96; **6i**-Na, 50.0).

Systematic modification and evaluation of the A-ring-modified camptothecins gave a highly potent derivative, **6i**, which has a 7-ethyl and an additional hydroxyl group. The 7-ethyl group tended to increase the antitumor activity, while the hydroxyl group reduced it. The chemical conversion of **1a** into **6i** enables us to prepare water soluble-derivatives without modifying the E-lactone ring. CPT-11, one of the water-soluble derivatives synthesized from **6i**, showed good solubility and significant activity in various murine tumor systems and it is now being tested in a Phase II clinical trial in Japan.[14]

TABLE III. Cytotoxicity of A-Ring Modified Camptothecins (KB Cells *in Vitro*)

Substituted position	Compd. No. (ED_{50} of the test sample/ED_{50} of **1a**-Na) Substituent					
	NO_2	NH_2	Cl	Br	OH	OMe
9	**5a** (1.7)	**5b** (1.0)	**5c** (0.5)	**5d** (0.5)	**5e** (0.9)	**5f** (0.7)
10	**6a** (1.6)	**6b** (0.3)	**6c** (0.8)	**6d** (0.6)	**6e** (0.7)	**6f** (0.8)
11	**7a** (10.3)	**7b** (1.3)	**7c** (1.0)	**7d** (0.9)	**7e** (0.5)	**7f** (0.8)
12	**8a** (150.0)	**8b** (125.0)	**8c** (18.9)	**8d** (17.1)	**8e** (58.3)	**8f** (5.9)

TABLE IV. Antitumor Activity of 20(*S*)-Camptothecin Derivatives (L1210 *in Vivo*)

Compd. No.	Substituent		*T*/*C*%[a] Total dose (mg/kg)							
	R	X	2.5	5	10	25	50	100	150	200
1a	H	H	—	112	127	168	84	—	—	—
1a[b]			—	—	180	313 (4/10)[c]	120	—	—	—
1b	Et	H	111	147	179	249 (2/6)	87	—	—	—
1b[b]			—	—	182	453 (9/10)	81	—	—	—
5f	H	9-OMe	163	229	155	—	—	—	—	—
5c	H	9-Cl	—	—	—	129	185	—	—	—
6e	H	10-OH	123	130	152	145	99	—	—	—
6f	H	10-OMe	168	215	147	—	—	—	—	—
6i	Et	10-OH	120	128	138	158	158	169	180	111
6i[b]			—	—	—	176	304 (2/6)	—	—	—
6j	Pr	10-OH	—	—	—	140	146	—	—	—
6k	CH_2Ph	10-OH	—	—	141	148	147	87	—	—

a) *T*/*C*% = (the mean survival time of tested mice)/(the mean survival time of control mice) × 100. *b*) Administered as a suspension. *c*) Number of cured mice/tested mice. —: not tested.

Experimental

The melting points (with decomposition) are uncorrected. Ultraviolet (UV) spectra were determined with a Shimadzu UV-240 spectrophotometer. ^{1}H-NMR spectra were recorded on a JEOL JMN-FX 100 (99.6 MHz) and a JEOL GX-400 (400 MHz) spectrometer with tetramethylsilane as the internal standard. Mass (MS) spectra were recorded on a JEOL JMS-D300 and a Hitachi M 80B spectrometer. Infrared (IR) spectra were measured with a JASCO A-102 spectrometer. Optical rotation values were determined with a JASCO DIP-140 apparatus.

Direct Nitration of 1a To a stirred solution of **1a** (3.00 g, 8.6 mmol) in conc. H_2SO_4 (150 ml) held in an ice-bath, 61% HNO_3 (d=1.38, 3.23 ml, 43.1 mmol) was added dropwise. The mixture was stirred at room temperature for 3 d, diluted with ice-water (1.5 l), and the solution was extracted with $CHCl_3$ (1.5 l × 3). The combined extracts were dried, filtered and then evaporated to dryness. The residue was mixed with Celite 545 and the mixture was purified through silica gel column chromatography with 1% MeOH–$CHCl_3$ to give 9- and 12-nitrocamptothecins (**5a** and **6a**).

5a: Yield 31.3%. Yellow needles, mp 190—192 °C (AcOEt) (182—186 °C).[4a] IR (KBr): 1740, 1655, 1600, 1525 cm^{-1}. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 5.36 (2H, s), 5.45 (2H, s), 6.53 (1H, s, 20-OH), 7.40 (1H, s, C_{14}-H), 8.05 (1H, t, J=8 Hz, C_{11}-H), 8.52 (1H, d, J=8 Hz, C_{10}- and/or C_{12}-H), 8.57 (1H, d, J=8 Hz, C_{12}- and/or C_{10}-H), 9.17 (1H, s, C_7-H). UV λ_{max}^{EtOH} nm: 210, 220, 239, 318, 334, 377. MS *m*/*z*: 393 (M^+). *Anal.* Calcd for $C_{20}H_{15}N_3O_6 \cdot 1/2H_2O$: C, 59.70; H, 4.00; N, 10.44. Found: C, 59.87; H, 4.08; N, 10.68. $[\alpha]_D^{25}$ −117.1° (c=0.100, pyridine).

6a: Yield 57.8%. Pale yellow needles, mp 272 °C (CH_2Cl_2–MeOH) (268—270 °C).[3] IR (KBr): 1750, 1655, 1605 cm^{-1}. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 5.34 (2H, br s), 5.44 (2H, s), 6.56 (1H, s, 20-OH), 7.30 (1H, s), 7.86 (1H, t, J=9 Hz, C_{10}-H), 8.37 (1H, dd, J=2, 9 Hz, C_{11}-H), 8.87 (1H, dd, J=2, 9 Hz, C_9-H, assigned by DIF NOE 2 method), 8.90 (1H, s, C_7-H). UV λ_{max}^{EtOH} nm (ε): 218 (34400), 243 (33500), 292 (7900), 368 (19500). MS *m*/*z*: 393 (M^+). *Anal.* Calcd for $C_{20}H_{15}N_3O_6$: C, 61.07; H, 3.84; N, 10.68. Found: C, 61.09; H, 3.68; N, 10.72. $[\alpha]_D^{25}$ −151.0° (c=0.100, pyridine).

1,2,6,7-Tetrahydrocamptothecin (2a) A suspension of **1a** (500 mg, 1.43 mmol) and Pt (PtO_2, 100 mg) in a 1 : 1 mixture of acetic acid–dioxane (100 ml) was shaken in a hydrogenation apparatus under an ambient H_2 pressure until the mixture had absorbed 2 eq of H_2 gas. The catalyst was filtered out on a celite pad by suction and the filtrate was evaporated to dryness. The residue was purified through silica gel column chromatography with 1% MeOH–$CHCl_3$. **2a**-1: Yield 46.2%. Colorless needles, mp 240 °C (AcOEt). ^{1}H-NMR (DMSO-d_6) δ: 0.75 (3H, t, J=7 Hz), 1.72 (2H, q, J=7 Hz), 1.99—2.28 (1H, m, C_6-H), 2.71—2.87 (2H, m, C_5-H_2), 3.91 (1H, d, J=1 Hz, C_7-H), 4.13 (1H, dd, J=5, 12 Hz, C_7-H), 4.91 (1H, d, J=6 Hz, C_2-H), 5.23 (2H, s, C_{17}-H), 6.28—6.95 (5H, m, C_{14}-H, arom). UV λ_{max}^{MeOH} nm (ε): 208 (30000), 240 (14600), 305 (11100). MS *m*/*z*: 352 (M^+). *Anal.* Calcd for $C_{20}H_{20}N_2O_4$: C, 68.17; H, 5.72; N, 7.95. Found: C, 68.05;

H, 5.87; N, 8.04. **2a**-2: Yield 12.3%. Colorless prisms, mp 235 °C (AcOEt). ^{1}H-NMR (DMSO-d_6) δ: 0.83 (3H, t, J=7 Hz), 1.76 (2H, q, J=7 Hz), 1.99—2.19 (1H, m), 2.68—2.88 (2H, m), 3.92 (1H, d, J=12 Hz), 4.13 (1H, dd, J=4, 12 Hz), 4.93 (1H, d, J=6 Hz, C_2-H), 5.24 (2H, s), 6.27 (1H, s, 20-OH), 6.40—6.94 (5H, m). UV λ_{max}^{MeOH} nm: 207, 241, 304. MS m/z: 352 (M^+).

10-Nitrocamptothecin (6a) To a solution of **2a** (1.00 g, 2.99 mmol) in pyridine, acetic anhydride (10 ml) was added dropwise and the mixture was heated at 70 °C for 1 h. The mixture was evaporated to dryness and the residue was purified through silica gel column chromatography with 1% MeOH–$CHCl_3$. **2b**: Yield 98%. Pale yellow needles. mp 251—253 °C ($CHCl_3$). MS m/z: 436 (M^+).

To a cold solution of **2b** (100 mg, 0.23 mmol) in conc. H_2SO_4 (5 ml) held in an ice-salt bath, HNO_3 (d=1.38, 20 μl, 0.27 mmol) was added dropwise with stirring. The stirring was continued for 1 h, and then the mixture was diluted with ice-water. The precipitate was extracted with $CHCl_3$ (100 ml × 2). The combined extracts were dried and evaporated. The residue was purified through silical gel column chromatography to give **3a** (97 mg, 96.3% yield) as pale yellow needles, mp 271—273 °C ($CHCl_3$). ^{1}H-NMR (DMSO-d_6) δ: 0.63 (3H, t, J=7 Hz), 1.63 (2H, q, J=7 Hz), 2.23 (3H, s), 2.8—3.0 (2H, m), 3.4—3.8 (3H, m), 4.0—4.2 (1H, m), 5.10 (2H, s), 6.26 (1H, s, 20-OH), 6.43 (1H, s), 7.53 (1H, d, J=9 Hz), 8.03 (1H, dd, J=2, 9 Hz), 8.22 (1H, d, J=2 Hz). MS m/z: 439 (M^+).

3a (500 mg, 1.14 mmol) was refluxed for 2 h in 20% H_2SO_4 (30 ml). The mixture was diluted with ice-water (500 ml) and the solution was extracted with $CHCl_3$ (500 ml × 2). The combined extracts were dried and evaporated. The residue was purified through silica gel column chromatography with 1% MeOH–$CHCl_3$ to give **3b** (372 mg in 82.3% yield). mp 222—223 °C (n-C_6H_{14}–$CHCl_3$). MS m/z: 379 (M^+).

3b was refluxed in dioxane (30 ml) for 2 h in the presence of DDQ (426 mg, 1.88 mmol). The mixture was condensed and the residue was dissolved in $CHCl_3$ (1 l). The solution was washed with water, dried, filtered and evaporated. The residue was purified through silica gel chromatography with 1% MeOH–$CHCl_3$ to give **6a** as yellow needles, mp 243—245 °C ($CHCl_3$) (297—300 °C for racemate).[4a] IR (KBr): 1740, 1655, 1620, 1590, 1530 cm^{-1}. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.88 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 5.37 (2H, br s), 5.45 (2H, s), 6.54 (1H, s, 20-OH), 7.43 (1H, s), 8.41 (1H, d, J=7 Hz, C_{12}-H), 8.51 (1H, dd, J=2, 9 Hz, C_{11}-H), 9.01 (1H, s, C_7-H), 9.16 (1H, d, J=2 Hz, C_9-H). UV λ_{max}^{EtOH} nm: 248, 260, 381. MS m/z: 393 (M^+). *Anal.* Calcd for $C_{20}H_{15}N_3O_6 \cdot H_2O$: C, 58.39; H, 4.17; N, 10.22. Found: C, 58.09; H, 4.01; N, 9.94. $[\alpha]_D^{25}$ −138.5° (c=0.094, pyridine).

11-Nitorocamptotecin (7a) To a solution of **2a** (100 mg, 0.25 mmol) in conc. H_2SO_4 (5 ml) held in an ice-salt bath, HNO_3 (d=1.38, 25.5 μl, 0.31 mmol) was added dropwise with stirring. The mixture was stirred at the same temperature for 1 h and worked up as described above to give **3c** (58 mg, 51.4% yield) as yellow prisms. mp 246 °C (n-C_6H_{14}–$CHCl_3$). ^{1}H-NMR (DMSO-d_6) δ: 0.75 (3H, t, J=7 Hz), 1.72 (2H, q, J=7 Hz), 2.36 (1H, m), 2.70—2.87 (2H, m), 3.99—4.11 (2H, m), 4.99 (1H, m), 5.22 (2H, s), 6.29 (1H, s, 20-OH), 6.52 (1H, s), 7.13—7.46 (4H, m). MS m/z: 397 (M^+).

A solution of **3c** (100 mg, 0.25 mmol) in dioxane (10 ml) was refluxed for 2 h in the presence of DDQ (126 mg, 0.55 mmol). The mixture was worked up as described above to give **7a** (77.5 mg, 78.3% yield) as yellow needles. mp 246 °C ($CHCl_3$) (176—180 °C for racemate).[4b] IR (KBr): 1740, 1655, 1600, 1525 cm^{-1}. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.90 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 5.37 (2H, s, C_5-H_2), 5.45 (2H, s, C_{17}-H_2), 6.51 (1H, s, 20-OH), 7.43 (1H, s, C_{14}-H), 8.40 (1H, s, C_9-H), 8.41 (1H, ABX q, J=9 Hz, C_{10}-H), 8.86 (1H, br s, C_7-H), 8.96 (1H, ABX q, J=2 Hz, C_{12}-H). UV λ_{max}^{MeOH} nm: 210, 220, 239, 318, 334, 377. MS m/z: 393 (M^+). *Anal.* Calcd for $C_{20}H_{15}N_3O_6$: C, 61.07; H, 3.84; N, 10.68. Found: C, 61.10; H, 3.75; N, 10.43. $[\alpha]_D^{25}$ −151.5° (c=0.104, pyridine).

Aminocamptothecins (General Procedure) Nitrocamptothecin (100 mg, 0.35 mmol) was dissolved in a 1 : 1 mixture of EtOH–dioxane (40 ml) and the mixture was shaken with Pt (PtO_2, 40 mg) under an ambient H_2 pressure in a hydrogenation flask for 2 h. The mixture was filtered by suction and the filtrate was condensed. The residue was triturated with EtOH and the solid was filtered by suction and washed with cold EtOH. The solid was mixed with Celite 545 and the mixture was purified through silica gel column chromatography with 2% MeOH–$CHCl_3$.

5b: Yield 95.2%. Orange needles, mp > 300 °C (MeOH–$CHCl_3$) (300 °C).[4a] IR (KBr): 1745, 1650, 1605, 1590, 1575 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), 6.78—6.85 (1H, m), 7.30 (1H, s), 7.36—7.61 (2H, m), 8.84 (1H, s). MS m/z: 363.1204 (M^+) for $C_{20}H_{17}N_3O_4$=363.1219.

6b: Yield 96.2%. Orange powder, mp 236—238 °C (MeOH–$CHCl_3$) (135 °C for racemate).[4a] IR (KBr): 1740, 1655, 1615, 1595 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.88 (3H, t, J=7 Hz), 1.86 (2H, q, J=7 Hz), 5.17 (2H, s), 5.39 (2H, s), 6.90 (1H, d, J=2 Hz), 7.19 (1H, s), 7.19 (1, dd, J=2, 9 Hz), 7.84 (1H, d, J=9 Hz), 8.20 (1H, s). MS m/z: 363.1183 (M^+) for $C_{20}H_{17}N_3O_4$=363.1219.

7b: Yield 95.7% Orange needles, mp > 300 °C (n-C_6H_{14}–MeOH–$CHCl_3$) (186—190 °C for racemate).[4b] IR (KBr): 1735, 1645, 1590, 1505 cm^{-1}. ^{1}H-NMR (400 MHz, DMSO-d_6–D_2O) δ: 0.86 (3H, t, J=7 Hz), 1.85 (2H, q, J=7 Hz), 5.19 (2H, s), 5.40 (2H, s), 7.16 (1H, dd, J=2, 10 Hz, C_{10}-H), 7.33 (1H, s, C_{14}-H), 7.38 (1H, d, J=2 Hz, C_{12}-H), 7.85 (1H, d, J=10 Hz, C_9-H), 8.54 (1H, s, C_7-H). MS m/z: 363.1200 (M^+) for $C_{20}H_{17}N_3O_4$=363.1219.

8b: Yield 93.1%. Orange powder, mp 266—268 °C (MeOH–$CHCl_3$) (278 °C).[3] IR (KBr): 1730, 1655, 1600, 1560 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.90 (3H, t, J=7 Hz), 1.87 (2H, q, J=7 Hz), 5.23 (2H, s), 5.41 (2H, s), 6.49 (1H, s), 6.88—7.44 (4H, m), 8.45 (1H, s). MS m/z: 363.1210 (M^+) for $C_{20}H_{17}N_3O_4$=363.1219.

Halogenocamptothecins (General Procedure) To a solution of aminocamptothecin (92 mg, 0.25 mmol) in 14% HCl (8 ml) held in an ice-salt bath, using 15% HBr instead of HCl for the preparation of bromocamptothecins, an aqueous solution of $NaNO_2$ (19 mg, 0.28 mmol) was added dropwise with stirring for 15 min. The mixture was added dropwise to a stirred solution of CuCl and/or CuBr (1.27 mmol) in 18% HCl or 24% HBr (10 ml) at 60—70 °C. After stirring for an additional hour, the mixture was diluted with ice-water (200 ml). The aqueous solution was extracted with $CHCl_3$ (150 ml × 3) and the combined extracts were dried, filtered and evaporated. The residue was mixed with Celite 545 and the mixture was purified through silica gel column chromatography with 1% MeOH–$CHCl_3$.

9-Chlorocamptothecin (**5c**): Yield 38.5%. Pale yellow needles, mp 257—260 °C ($CHCl_3$). IR (KBr): 1735, 1655, 1605, 1585 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.88 (3H, t, J=7 Hz), 1.87 (2H, q, J=7 Hz), 5.32 (2H, s), 5.43 (2H, s), 6.51 (1H, s, 20-OH), 7.37 (1H, s), 7.83—7.89 (2H, m), 8.12—8.24 (1H, m), 8.93 (1H, s). UV λ_{max}^{EtOH} nm (ε): 259 (30500), 296 (6000), 360 (sh, 18500), 373 (19000). MS m/z: 382 (M^+). *Anal.* Calcd for $C_{20}H_{15}ClN_2O_4$: C, 62.75; H, 3.95; N, 7.32. Found: C, 62.63, H, 4.45; N, 7.20.

10-Chlorocamptothecin (**6c**): Yield 58.1%. Colorless needles, mp 279—280 °C ($CHCl_3$) (270 °C for racemate).[4b] IR (KBr): 1745, 1650, 1585 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.87 (2H, q, J=7 Hz), 5.28 (2H, s), 5.42 (2H, s), 6.51 (1H, s, 20-OH), 7.33 (1H, s), 7.83 (1H, dd, J=2, 9 Hz), 8.16 (1H, d, J=9 Hz), 8.25 (1H, d, J=2 Hz), 8.64 (1H, s). UV λ_{max}^{EtOH} nm: 257, 294, 332, 363, 373. MS m/z: 382.0690 (M^+) for $C_{20}H_{15}ClN_2O_4$=382.0720.

11-Chlorocamptothecin (**7c**): Yield 56.0%. Pale yellow needles, mp 257 °C ($CHCl_3$). IR (KBr): 1745, 1655, 1605, 1590 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.87 (2H, q, J=7 Hz), 5.27 (2H, s), 5.42 (2H, s), 6.50 (1H, s, 20-OH), 7.35 (1H, s), 7.71 (1H, dd, J=2, 9 Hz), 8.12—8.19 (2H, m), 8.71 (1H, s). UV λ_{max}^{EtOH} nm (ε); 244 (31600), 254 (34600), 290 (6800), 364 (20600), 375 (22100). MS m/z: 382 (M^+). *Anal.* Calcd for $C_{20}H_{15}ClN_2O_4$: C, 62.75; H, 3.95; N, 7.32; Cl, 9.26. Found: C, 62.52; H, 3.89; N, 7.02; Cl, 8.98.

12-Chlorocamptothecin (**8c**): Yield 42.1%. Pale yellow needles, mp 269—271 °C (AcOEt) (258 °C).[3] IR (KBr): 1755, 1740, 1650, 1605, 1585 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.90 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 5.30 (2H, s), 5.43 (2H, s), 6.55 (1H, s, 20-OH), 7.38 (1H, s), 7.66 (1H, t, J=8 Hz), 8.00—8.14 (2H, m), 8.76 (1H, s). UV λ_{max}^{EtOH} nm (ε): 252 (sh, 31900), 258 (34800), 361 (22100), 372 (sh, 21400). MS m/z: 382 (M^+). *Anal.* Calcd for $C_{20}H_{15}ClN_2O_4$: C, 62.75; H, 3.95; N, 7.32. Found: C, 63.11; H, 3.99; N, 7.32.

9-Bromocamptothecin (**5d**): Yield 58.0%. Pale yellow needles, mp 260—262 °C (AcOEt). IR (KBr): 1735, 1655, 1605, 1585 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.87 (2H, q, J=7 Hz), 5.33 (2H, s), 5.43 (2H, s), 6.52 (1H, s, 20-OH), 7.37 (1H, s), 7.78 (1H, t, J=8 Hz), 8.03—8.87 (2H, m), 8.88 (1H, s). UV λ_{max}^{EtOH} nm (ε): 260 (34100), 294 (6600), 315 (7700), 363 (20700), 373 (21000). MS m/z: 426 (M^+). *Anal.* Calcd for $C_{20}H_{15}BrN_2O_4$: C, 56.22; H, 3.54; N, 6.56. Found: C, 56.02; H, 3.86; N, 6.05.

11-Bromocamptothecin (**7d**): Yield 40.8%. Colorless needles, mp 260—261 °C ($CHCl_3$). IR (KBr): 1745, 1655, 1600 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.87 (2H, q, J=7 Hz), 5.25 (2H, s), 5.41 (2H, s), 6.48 (1H, s, 20-OH), 7.34 (1H, s), 7.79 (1H, dd, J=2, 9 Hz), 8.08 (1H, d, J=9 Hz), 8.35 (1H, d, J=2 Hz), 8.69 (1H, s). UV λ_{max}^{EtOH} nm (ε): 256 (37900), 291 (7000), 362 (21100), 366 (22900). MS m/z: 426 (M^+). *Anal.* Calcd for $C_{20}H_{15}BrN_2O_4$: C, 56.22; H, 3.54; N, 6.56. Found: C,

55.97; H, 3.48; N, 6.85.

12-Bromocamptothecin (**8d**): Yield 42.3%. Pale yellow needles, mp 261—263 °C (AcOEt) (239 °C).[3] IR (KBr): 1750, 1655, 1625, 1605 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.90 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 5.32 (2H, s), 5.44 (2H, s), 6.56 (1H, s, 20-OH), 7.39 (1H, s), 7.6 (1H, t, J=8 Hz), 8.13—8.28 (2H, m), 8.75 (1H, s). UV λ_{max}^{EtOH} nm: 259, 290, 317, 362, 373. MS m/z: 426.0235 (M^+) for $C_{20}H_{15}BrN_2O_4$=426.0211.

10-Bromocamptothecin (6d) To a solution of **2a** (100 mg, 0.28 mmol) in CH_2Cl_2 (25 ml), 2,4,6-tetrabromocyclohexa-2,5-dienone[9] (116 mg, 0.28 mmol) in CH_2Cl_2 (5 ml) was added dropwise with stirring at −15 °C. The mixture was stirred at −15 °C for 30 min and then at room temperature for 30 min. The reaction mixture was washed with 5% $NaHCO_3$ and then with brine, dried, filtered and evaporated. The residue was passed through silica gel column chromatography with 1% MeOH–$CHCl_3$. **3d**: Yield 91.5%. Colorless needles, mp 250—251 °C. ^{1}H-NMR (DMSO-d_6) δ: 0.75 (3H, t, J=7 Hz), 1.72 (2H, q, J=7 Hz), 2.08—2.20 (1H, m), 2.67—2.84 (2H, m), 3.76—4.12 (2H, m), 4.91 (1H, d, J=5 Hz), 5.23 (2H, s), 6.52—6.63 (2H, m), 7.05—7.12 (2H, m).

3d (containing about 5% of **6d**) was stirred with ceric ammonium nitrate (446 mg, 0.817 mmol) in a 1 : 1 mixture of MeOH–dioxane (50 ml) at room temperature for 15 min. The mixture was evaporated to dryness and the residue was dissolved in $CHCl_3$ (100 ml). The solution was washed with brine, dried, filtered and then evaporated. The residue was purified through silica gel column chromatography to give **6d** (58 mg, overall 73.2% yield) as colorless needles, mp 273—275 °C ($CHCl_3$). IR (KBr): 1750, 1655, 1590 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.87 (2H, q, J=7 Hz), 5.28 (2H, s), 5.41 (2H, s), 6.48 (1H, s), 7.34 (1H, s), 7.93 (1H, dd, J=2, 9 Hz), 8.09 (1H, d, J=9 Hz), 8.41 (1H, d, J=2 Hz), 8.63 (1H, s). UV λ_{max}^{EtOH} nm: 257, 294, 333, 364, 375. MS m/z: 426.0239 (M^+) for $C_{20}H_{15}BrN_2O_4$=426.0215.

Hydroxycamptothecins (General Procedure) To a solution of an aminocamptothecin (92 mg, 0.25 mmol) in 20% H_2SO_4 (10 ml) held in an ice-salt bath, an aqueous solution of $NaNO_2$ (20 mg, 0.29 mmol) was added dropwise with stirring. The mixture was stirred for 15 min, added to hot water (100 ml) and heated under reflux for 1 h. The mixture was diluted with ice-water (100 ml) and shaken with $CHCl_3$ (100 ml). The precipitate was filtered out on a Celite pad by suction, and the pad was eluted with MeOH–$CHCl_3$. The eluate was evaporated and the residue was purified through silica gel column chromatography.

9-Hydroxycamptothecin (**5e**): Yield 34.3%. Pale yellow needles, mp 225 °C ($CHCl_3$–MeOH). IR (KBr): 1735, 1650, 1580, 1555 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.87 (2H, q, J=7 Hz), 5.27 (2H, s), 5.42 (2H, s), 6.49 (1H, s, 20-OH), 6.98—7.06 (1H, m), 7.32 (1H, s), 7.60—7.65 (2H, m), 8.81 (1H, s), 10.69 (1H, s, 10-OH), MS m/z: 364.1044 (M^+) for $C_{20}H_{16}N_2O_5$=364.1059.

10-Hydroxycamptothecin (**6e**): Yield 66.5%. Pale yellow needles, mp 270—272 °C ($CHCl_3$–MeOH) (268—270 °C).[5c] IR (KBr): 1720, 1655, 1590, 1505 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ:0.88 (3H, t, J=7 Hz), 1.86 (2H, q, J=7 Hz), 5.22 (2H, s), 5.40 (2H, s), 6.47 (1H, s, 20-OH), 7.26—7.28 (2H, m), 7.41 (1H, dd, J=2, 9 Hz), 8.01 (1H, d, J=9 Hz), 8.43 (1H, s), 10.31 (1H, s, 10-OH). UV λ_{max}^{MeOH} nm (ε): 222 (41200), 266 (239000), 293 (5700), 314 (8200), 330 (10700), 368 (22100), 382 (25500). MS m/z: 364 (M^+). *Anal.* Calcd for $C_{20}H_{16}N_2O_5 \cdot H_2O$: C, 62.82; H, 4.75; N, 7.33. Found: C, 63.11; H, 4.71; N, 7.32.

11-Hydroxycamptothecin (**7e**): Yield 27.0% Colorless needdles, mp > 300 °C (pyridine–MeOH) (327—330 °C).[5d] IR (KBr): 1720, 1655, 1590, 1560 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.90 (3H, t, J=7 Hz), 1.86 (2H, q, J=7 Hz), 5.22 (2H, s), 5.40 (2H, s), 6.47 (1H, s, 20-OH), 7.20—7.31 (1H, m), 7.36 (1H, d, J=2 Hz), 8.01 (1H, d, J=9 Hz), 8.43 (1H, s), 10.42 (1H, s, 10-OH). MS m/z: 364.1064 (M^+) for $C_{20}H_{16}N_2O_5$=364.1059.

12-Hydroxycamptothecin (8e)[10] To a solution of **8b** (100 mg, 0.28 mmol) in 35% H_2SO_4 (10 ml) held in an ice-salt bath, an aqueous solution of $NaNO_2$ (200 mg, 2.9 mmol) was added dropwise with stirring. The mixture was stirred for 5 min. Urea (100 mg) was added to the mixture, followed by further stirring for 5 min. The mixture was added to an aqueous solution of $CuNO_3$ (8.0 g in 70 ml), and then Cu_2O (300 mg) was added to the solution. The resulting mixture was stirred at room temperature for 30 min, extracted with $CHCl_3$ (200 ml × 10) and the combined extracts were dried, filtered and evaporated to dryness. A chromatographic work-up of the residue with 2% MeOH–$CHCl_3$ gave **8e**. Pale yellow needles (65 mg, 64.9% yield), mp 245—247 °C ($CHCl_3$–MeOH) (261 °C).[3] IR (KBr): 1735, 1655, 1590, 1560 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.90 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 5.28 (2H, s), 5.43 (2H, s), 6.52 (1H, s, 20-OH), 7.13—7.22 (1H, m), 7.50—7.55 (3H, m), 8.60 (1H, s), 10.04 (1H, s, 12-OH). MS m/z: 364.1064 (M^+) for $C_{20}H_{16}N_2O_5$=364.1059.

Preparation of 6e by Oxidation of 2a with LTA A mixture of **2a** (100 mg, 0.28 mmol) and 90% Pb $(OAc)_4$ (420 mg, 0.85 mmol) in CF_3COOH (3 ml) was stirred at room temperature for 15 min. The mixture was evaporated and the residue was purified through silica gel column chromatography with 2% MeOH–$CHCl_3$ to give **1a** (12 mg, 12% yield) and **6e** (76 mg, 73.5% yield).

Methoxycamptothecins (General Procedure) To a solution of a hydroxycamptothecin (100 mg, 0.27 mmol) in a 1 : 1 mixture of MeOH–dioxane (20 ml), diazomethane (2 mmol) in ether (10 ml) was added, and the mixture was stirred at room temperature for 1 h. Acetic acid (1 ml) was added to the mixture, followed by evaporation. The residue was purified through silica gel column chromatography with 1% MeOH–$CHCl_3$.

9-Methoxycamptothecin (**5f**): Yield 95.5%. Yellow needles, mp 225—226 °C (CH_2Cl_2–MeOH) (258—259 °C).[5b] IR (KBr): 1750, 1655, 1610, 1590 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.87 (2H, q, J=7 Hz), 4.04 (3H, s), 5.25 (2H, s), 5.42 (2H, s), 6.49 (1H, s), 7.10—7.19 (1H, m), 7.32 (1H, s), 7.73—7.76 (2H, m), 8.83 (1H, s). UV λ_{max}^{EtOH} nm (ε): 264 (25700), 306 (7500), 320 (11600), 358 (19900). MS m/z: 378 (M^+). *Anal.* Calcd for $C_{21}H_{18}N_2O_5$: C, 66.66; H, 4.80; N, 7.40. Found: C, 66.69; H, 4.81; N, 7.45. $[\alpha]_D^{25}$ −77.0° (c=0.20, pyridine) (−77.5°, c=1.0).[5b]

10-Methoxycamptothecin (**6f**): Yield 87.5%. Pale yellow needles, mp 255—256 °C ($CHCl_3$) (254—255 °C).[5c] IR (KBr): 1740, 1655, 1620, 1540 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.87 (2H, q, J=7 Hz), 3.95 (3H, s), 5.27 (2H, s), 5.42 (2H, s), 6.49 (1H, s, 20-OH), 7.28 (1H, s), 7.44—7.54 (2H, m), 8.07 (1H, d, J=10 Hz), 8.54 (1H, s). UV λ_{max}^{MeOH} nm: 266, 259, 313, 330, 367, 381. MS m/z: 378 (M^+). *Anal.* Calcd for $C_{21}H_{18}N_2O_5 \cdot H_2O$: C, 63.63; H, 5.09; N, 7.07. Found: C, 64.00; H, 5.09; N, 6.92. $[\alpha]_D^{25}$ −138.3° (c=1.0, pyridine).

11-Methoxycamptothecin (**7f**): Yield 99.5%. Pale yellow needles, mp 277—279 °C ($CHCl_3$) (264—265 °C).[5e] IR (KBr): 1740, 1655, 1615, 1505 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.87 (2H, q, J=7 Hz), 3.97 (3H, s), 5.24 (2H, s), 5.42 (2H, s), 6.48 (1H, s, 20-OH), 7.29—7.40 (2H, m), 7.55 (1H, d, J=2 Hz), 8.02 (1H, d, J=9 Hz), 8.59 (1H, s). UV λ_{max}^{MeOH} nm (ε): 259 (29200), 350 (14700), 367 (18900), 384 (21200). MS m/z: 378 (M^+). *Anal.* Calcd for $C_{21}H_{18}N_2O_5$: C, 66.66; H, 4.80; N, 7.40. Found: C, 66.52; H, 4.67; N, 7.16. $[\alpha]_D^{25}$ −143.3° (c=0.1, pyridine).

12-Methoxycamptothecin (**8f**): Yield 97.5%. Yellow needles, mp 248—249 °C (CH_2Cl_2–MeOH) (250 °C).[3] IR (KBr): 1740, 1660, 1650, 1600, 1580 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.90 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 4.04 (3H, s), 5.28 (2H, s), 5.43 (2H, s), 6.54 (1H, s, 20-OH), 7.24—7.28 (1H, m), 7.33 (1H, s), 7.60—7.65 (2H, m), 8.62 (1H, s). UV λ_{max}^{MeOH} nm: 264, 292, 308, 322, 362. MS m/z: 378.1217 (M^+) for $C_{21}H_{18}N_2O_5$=378.1216.

10-Substituted Camptothecins by the Photoreaction of 9 (General Procedure) **9** (1.0 g) was dissolved in dioxane (1 l) containing, 1 N H_2SO_4 (1 eq), and the mixture was degassed by bubbling N_2 gas for 20 min. The mixture was irradiated by a high pressure Hg lamp (450 W, Usio UM-452) with a Pyrex filter for 20—30 min with stirring. The reaction mixture was evaporated to dryness, and the residue was dissolved in 10% MeOH–$CHCl_3$ (50 to 100 ml). The organic solution was washed with water (500 ml), and the insoluble material in both phases was collected on a celite pad by suction; the celite pad was washed with 10% MeOH–$CHCl_3$ (200 ml × 3). The eluents were condensed, and the residue was washed with MeOH and then recrystallized.

6g: Colorless needles obtained by irradiation of **9a** in acetic acid, mp 258—259 °C (n-C_6H_{14}–$CHCl_3$) (255—257 °C).[5c] IR (KBr): 1750, 1655, 1605, 1585 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.88 (2H, q, J=7 Hz), 3.31 (1H, s), 5.29 (2H, s), 5.43 (2H, s), 6.52 (1H, s, 20-OH), 7.67 (1H, dd, J=2, 9 Hz), 7.90 (1H, d, J=2 Hz), 8.20 (1H, d, J=9 Hz), 8.67 (1H, s). MS m/z: 406 (M^+).

6h: Yellow needles, mp 228—231 °C (n-C_6H_{14}–$CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=8 Hz), 1.25 (3H, t, J=7 Hz), 1.90 (2H, q, J=8 Hz), 3.81 (2H, s), 4.21 (2H, q, J=7 Hz), 5.28 (1H, d, J=16 Hz), 5.75 (1H, d, J=16 Hz), 5.27 (2H, s), 7.64 (1H, s), 7.70 (2H, m), 8.17 (1H, d, J=9 Hz), 8.26 (1H, br s). MS m/z: 466 (M^+).

7-Ethyl-10-hydroxycamptothecin (**6i**): Pale yellow needles, mp 224 °C (EtOH). IR (KBr): 1738, 1655, 1636, 1586, 1570 cm^{-1}. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.90 (3H, t, J=7 Hz), 1.31 (3H, t, J=7 Hz), 1.85 (2H, q, J=7 Hz), 3.06 (2H, q, J=7 Hz), 5.21 (2H, s), 5.38 (1H, d, J=16 Hz), 5.43 (1H, d, J=16 Hz), 6.39 (1H, s, 20-OH), 7.25 (1H, s, C_{14}-H), 7.37 (1H, d, J=2 Hz, C_9-H), 7.39 (1H, dd, J=2, 8 Hz, C_{11}-H), 7.99 (1H, d, J=8 Hz, C_{12}-H), 10.20 (1H, s, 10-OH). UV λ_{max}^{MeOH} nm (ε): 223 (50000), 266 (293000), 292 (6000), 313 (10000), 329 (13300), 366 (26300), 381 (30500).

MS m/z: 392 (M^+). *Anal.* Calcd for $C_{22}H_{20}N_2O_5 \cdot H_2O$: C, 64.38; H, 5.40; N, 6.83. Found: C, 64.57; H, 5.24; N, 6.84. $[\alpha]_D^{25}$ +32.6° (c=0.216, $CHCl_3$: MeOH, 4:1).

7-Ethyl-12-hydroxycamptothecin: Yield 5%. Pale yellow needles, mp 294—295 °C (EtOH). IR (KBr): 1740, 1655, 1599, 1512 cm^{-1}. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.26 (3H, t, J=7 Hz), 1.83 (2H, q, J=7 Hz), 3.19 (2H, q, J=7 Hz), 5.27 (2H, s), 5.39 (2H, s), 6.48 (1H, s, 20-OH), 7.12 (1H, d, J=6 Hz, C_{11}-H), 7.50 (1H, t, J=6 Hz, C_{10}-H), 7.51 (1H, s, C_{14}-H), 7.62 (1H, d, J=6 Hz, C_9-H, assigned by DIF NOE 2 method), 9.90 (1H, 12-OH). MS m/z: 392 (M^+).

6j: Pale yellow needles, mp 237 °C (toluene). ^{1}H-NMR (DMSO-d_6) δ: 0.89 (3H, t, J=7 Hz), 1.06 (3H, t, J=7 Hz), 1.60—1.90 (4H, m), 3.00 (2H, t, J=7 Hz), 5.24 (2H, s), 5.40 (2H, s), 6.24 (1H, s, 20-OH), 7.30—7.38 (2H, m), 7.99 (1H, d, J=9 Hz). MS m/z: 406.1523 (M^+) for $C_{23}H_{22}N_2O_5$=406.1528.

6k: Colorless needles, mp 236 °C (n-C_6H_{14}–$CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, t, J=7 Hz), 1.85 (2H, q, J=7 Hz), 4.52 (2H, s), 5.11 (2H, s), 5.26 (1H, d, J=16 Hz), 5.73 (1H, d, J=16 Hz), 7.10 (5H, m), 7.53 (1H, dd, J=3, 9 Hz), 7.65 (1H, s), 7.89 (1H, d, J=3 Hz), 8.27 (1H, d, J=9 Hz). MS m/z: 454 (M^+). *Anal.* Calcd for $C_{27}H_{22}N_2O_5$: C, 68.63; H, 5.12; N, 5.93. Found; C, 68.91; H, 5.07; N, 6.03.

6l: Pale yellow needles, mp 257 °C (n-C_6H_{14}–$CHCl_3$). ^{1}H-NMR (DMSO-d_6) δ: 0.98 (3H, t, J=7 Hz), 1.90 (2H, q, J=7 Hz), 2.38 (3H, s), 5.17 (2H, s), 5.23 (2H, s), 5.54 (2H, s), 6.34 (1H, s, 20-OH), 7.34—7.40 (2H, m), 7.39 (1H, s), 7.92 (1H, d, J=9 Hz). MS m/z: 436 (M^+).

6m: Orange yellow powder, mp 260—263 °C (n-C_6H_{14}–$CHCl_3$). IR (KBr): 1735, 1650, 1590 cm^{-1}. MS m/z: 394 (M^+).

N-Oxides (9, General Procedure) **1** (3.0 g, **a** to **f**) was dissolved in acetic acid (800 ml), and 30% H_2O_2 (50 ml) was added. The mixture was heated at 70—80 °C for 3.5 h, condensed to about one-third at 45—55 °C, and the residue was poured into ice-water (3 l). The precipitated material in the aqueous solution was collected by suction. The filtered material was purified by recrystallization.

9a: Yield 90.6%. Yellow-orange needles, mp 254 °C (n-C_6H_{14}–$CHCl_3$). IR (KBr): 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^+).

9b: Yield 77.7%. Yellow-orange needles, mp 255 °C (n-C_6H_{14}–$CHCl_3$). ^{1}H-NMR (DMSO-d_6) δ: 0.87 (3H, t, J=7 Hz), 1.28 (3H, t, J=7 Hz), 1.84 (2H, q, J=7 Hz), 3.10 (2H, q, J=7 Hz), 5.26 (2H, s), 5.36 (2H, s), 6.24 (1H, s), 7.80 (3H, m), 8.19 (1H, s), 8.35 (1H, m). MS m/z: 392 (M^+).

9c: Yield 68.4%. Yellow-orange needles, mp 238 °C (n-C_6H_{14}–$CHCl_3$). MS m/z: 406 (M^+).

9d: Yield 63.5%. Yellow-orange needles, mp 220 °C (n-C_6H_{14}–$CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.09 (3H, t, J=8 Hz), 1.87 (2H, q, J=8 Hz), 4.48 (2H, s), 5.16 (2H, s), 5.20 (1H, d, J=16 Hz), 5.64 (1H, d, J=16 Hz), 7.05—8.12 (8H, m), 8.32 (1H, s), 8.80 (1H, m). MS m/z: 454 (M^+).

9e: Yield 65.9%. Yellow needles, mp 250 °C (n-C_6H_{14}–$CHCl_3$). ^{1}H-NMR (DMSO-d_6) δ: 0.87 (3H, t, J=7 Hz), 1.83 (2H, q, J=7 Hz), 2.05 (3H, s), 5.42 (4H, br s), 5.51 (2H, s), 6.42 (1H, s), 7.80 (2H, m), 7.91 (1H, s), 8.20 (1H, m), 8.63 (1H, m). MS m/z: 436 (M^+).

9f: Yield 45.4%. Yellow needles, mp 255—260 °C (MeOH–$CHCl_3$). IR (KBr): 1755, 1650, 1600, 1460 cm^{-1}. MS m/z: 394 (M^+).

Preparation of Sodium Salts Camptothecin or a derivative (1.0 g) was suspended in water (100 ml) containing an equimolar amount of 1 N NaOH (for the sodium salts of the hydroxycamptothecins, 2 eq of 1 N NaOH was used), and the suspension was shaken at an ambient temperature for 14 h under protection from light. The mixture was filtered through membrane filter (0.45 μm), and the filtrate was lyophilized. In the case of the sodium salts of the hydroxycamptothecins, the filtrate was neutralized with 0.1 N HCl before lyophilization.

Cytotoxicity KB cells were grown at 37 °C in Eagle's minimal essential medium with 10% calf serum. Cells (6×10^4) were placed in a plastic Petri dish on day 1 and incubated overnight. On day 0, the medium was discarded and replaced with sample-containing fresh medium and the cells were incubated for more 2 d. An Na salt form of a derivative was dissolved in water (1 mg/ml) and then diluted with the culture medium. On day 3, the cells were washed with trypsin, and then trypan blue-excluded cells were counted with a hemocytometer. **1a**-Na was used as the control for each test. ED_{50} values were calculated from the dose response curves.

Antitumor Activity L1210 leukemia cells (10^5) were implanted intraperitoneally (i.p.) in 7 week old BDF_1 female mice on day 0. Ten mice were used for each dose. The sample was dissolved in saline and was administered i.p. on days 1 to 5. Control mice were injected with saline and cured mice were counted on day 40.

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Studies on Nilvadipine. I. Synthesis and Structure–Activity Relationships of 1,4-Dihydropyridines Containing Novel Substituents at the 2-Position

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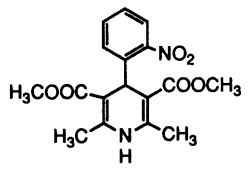
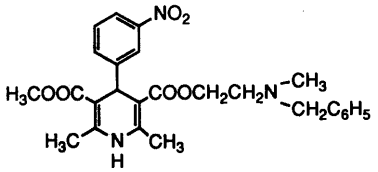
The synthesis of new 1,4-dihydropyridine derivatives containing novel substituent at the 2-position of the nucleus *via* the key intermediate 2-formyl-1,4-dihydropyridines (X), is described. The aldehydes (X) were prepared by hydrolysis of the acetals (IX) which were obtained from aryl aldehyde (V) and alkyl 4,4-dialkoxyacetoacetate (VI) by the Knoevenagel reaction and treatment with alkyl 3-aminocrotonate (VIII) according to the modified Hantzsch method. The formyl group of the aldehydes (X) was reactive enough to be converted to a variety of functional groups such as hydroxymethyl, cyano, substituted iminomethyl, carbamoyl, semicarbazone, substituted vinyl, ethynyl, and so on. In all of the novel compounds we prepared, 2-hydroxymethyl- and 2-cyano-1,4-dihydropyridines (IV and XXII) were found to possess potent activities in preliminary biological evaluations on hypotension in normotensive rats and on an increase in coronary blood flow in pentobarbital-anesthetized dogs. Optimization research in order to obtain a more potent compound was accomplished in the 2-hydroxymethyl- and 2-cyano-1,4-dihydropyridine series. We selected isopropyl 2-cyano-3-methoxycarbonyl-4-(3-nitrophenyl)-6-methyl-1,4-dihydropyridine-5-carboxylate (XXIIj) as a candidate compound for further biological evaluation studies. Fortunately, XXIIj (nilvadipine) has been accepted in clinical use for the treatment of hypertension.

Keywords 2-substituted-1,4-dihydropyridine; 2-hydroxymethyl-1,4-dihydropyridine; 2-cyano-1,4-dihydropyridine; calcium antagonist; antihypertension; structure–activity relationship

In the last decade, synthetic studies on Hantzsch-type 1,4-dihydropyridine derivatives[1] have been carried out in many research institutes all over the world because of their attractive biological activities as calcium antagonists. At the time that this work was initiated, nifedipine (I) was the only known compound used clinically for the treatment of angina pectoris,[2] and it was also known that nicardipine (II) had been developed in preclinical studies for the treatment of hypertension.[3] From the results of structure–activity relationships of the Hantzsch-type 1,4-dihydropyridines which had been published,[4] the desired structural characteristics of the substituents at the 2- and the 6-positions of the 1,4-dihydropyridine nucleus had been thought to be lower alkyl groups, especially a methyl group, although there had been some exceptions as Bayer's chemists had reported the synthesis and the structure–activity relationships on 2-amino and 2-alkoxymethyl derivatives.[5,6] In order to obtain novel 1,4-dihydropyridines having excellent biological activities, we intended to discover novel appropriate substituents at the 2-position of the 1,4-dihydropyridine nucleus. The information concerning the metabolism of nifedipine was a great hint in achieving this objective. As reported,[7] nifedipine is transformed in rats into pyridine derivatives (III). Both metabolites appear to be derived through the process of hydroxylation of the methyl group. We speculated that a hydroxymethyl group could be one of the candidates for new substituents at the 2-position. Therefore, we originally synthesized 2-hydroxymethyl-1,4-dihydropyridine derivatives (IV).[8] Alker[9] recently reported the synthesis of 2-hydroxymethyl-1,4-dihydropyridines for preparing new 2-substituted-1,4-dihydropyridines in accordance with our method described in the U.S. patent.[10]

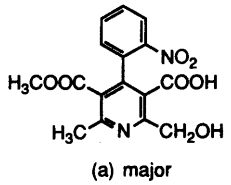


nifedipine (I)

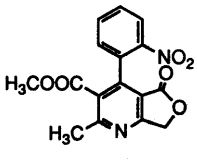


nicardipine (II)

Fig. 1

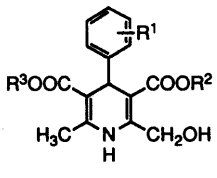


(a) major



(b) minor

metabolites of nifedipine (III)



IV

Fig. 2

In the course of our research for the first target, 2-hydroxymethyl-1,4-dihydropyridines, it was found that diethyl 2-formyl-4-(3-nitrophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (Xd) possessed potent activity in comparison with the corresponding 2-hydroxymethyl derivative (IVd). Their ED_{50} values in the percentage of increase on coronary blood flow in pentobarbital-anesthetized dogs after intravenous administration were 4.0 and 4.8 μg/kg, respectively. This finding implied that not only electron-donating substituents such as methyl, amino, alkoxymethyl and our hydroxymethyl, but also electron-withdrawing substituents at the 2-position, might play an important role in affecting biological activities. Therefore, we focused on the synthesis of other 1,4-dihydropyridine derivatives which have electron-withdrawing substituents such as an acetyl, cyano, iminomethyl, carbamoyl and so forth. Consequently, it turned out that a cyano group was the best in terms of potent activities on coronary blood flow increase in anesthetized dogs and also on hypotension in normotensive rats.

Corresponding vinylogous analogues (XXVII) were synthesized with the expectation of a similar effect.

We were also interested in preparing the 2-ethynyl-1,4-dihydropyridine derivative (XXX), because the structural feature of the ethynyl group seems to be similar to that of

a nitrile group in terms of both electronic and steric characteristics.

Among the novel substituents at the 2-position of the 1,4-dihydro-pyridine nucleus which we prepared, a hydroxymethyl and a cyano group were found to be suitable substituents. Thus we synthesized a number of 2-hydroxymethyl and 2-cyano-1,4-dihydropyridines by modification of the ester parts at the 3- and the 5-positions and the substituents on the phenyl ring at the 4-position.

In the present paper we describe the synthesis and the structure–activity relationships of these 1,4-dihydropyridines possessing novel substituents at the 2-position of the 1,4-dihydropyridine nucleus.

Chemistry The synthesis of 2-hydroxymethyl-1,4-dihydropyridines (IV) *via* 2-formyl-1,4-dihydropyridines (X) is illustrated in Chart 1. X was afforded by the hydrolysis of acetals (IX) which were obtained from α,β-unsaturated esters (VII) and alkyl 3-aminocrotonate (VIII) according to the modified Hantzsch method.[11] VII was prepared through the Knoevenagel reaction of aryl aldehydes (V) and alkyl 4,4-dialkoxyacetoacetate (VI).

The alcohol (IV) was obtained by reduction of X with sodium borohydride, accompanied with the lactonized by-product (XI). However, lactonization was found to be avoidable, if the reaction was carried out under cooling in an ice-bath. XI was alternatively prepared by treating the corresponding IV with a catalytic amount of *p*-toluenesulfonic acid. The 2-hydroxymethyl-1,4-dihydropyr-

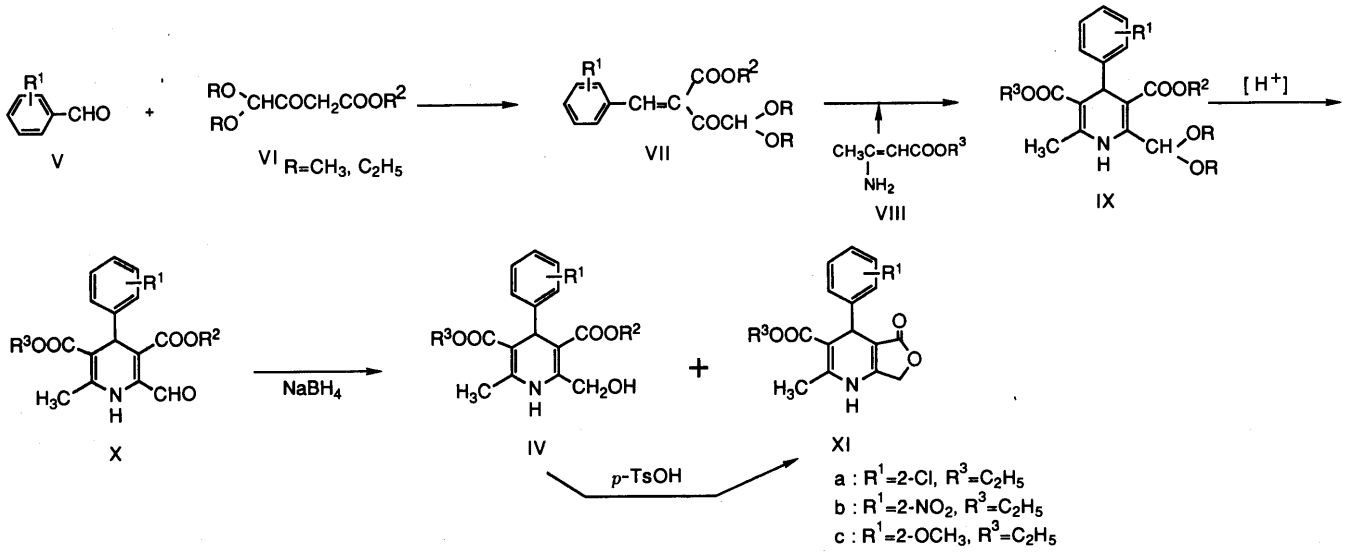


Chart 1

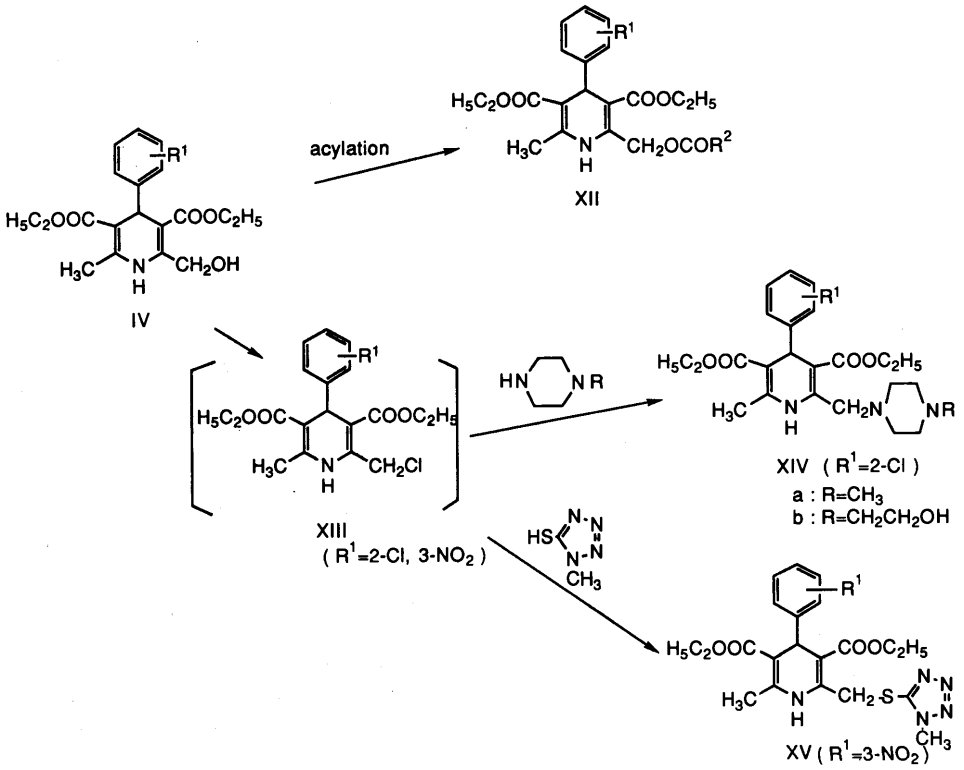
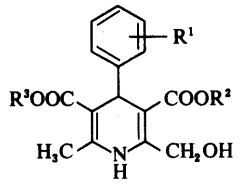


Chart 2

TABLE I. 2-Hydroxymethyl-1,4-dihydropyridines (IV)



Compd. No.	R^1	R^2	R^3	Yield (%)	mp (°C) (Recry. sol.)	NMR δ value ($CDCl_3$) C_4-H	NMR δ value ($CDCl_3$) C_6-CH_3	Formula	Analysis (%) Calcd (Found) C	H	N
IVa	2-NO_2	CH_3	CH_3	75.1	164.5—165.5 (AcOEt)	5.75	2.33	$C_{17}H_{18}N_2O_7$	56.33 (56.44	5.01 5.01	7.73 7.66)
IVb	3-NO_2	CH_3	CH_3	71.0	145—146 (MeOH)	5.11	2.40	$C_{17}H_{18}N_2O_7$	56.33 (56.35	5.01 4.93	7.73 7.65)
IVc	2-NO_2	C_2H_5	C_2H_5	80.5	112—113 (Et_2O-*n*-hexane)	5.91	2.36	$C_{19}H_{22}N_2O_7$	58.45 (58.35	5.68 5.65	7.18 7.21)
IVd	3-NO_2	C_2H_5	C_2H_5	65.0	141—141.5 (Et_2O-*n*-hexane)	5.14	2.43	$C_{19}H_{22}N_2O_7$	58.45 (58.33	5.68 5.71	7.18 6.96)
IVe	2-CN	CH_3	CH_3	72.3	177—178 (MeOH)	5.35	2.40	$C_{18}H_{18}N_2O_5$	63.13 (62.90	5.30 5.14	8.19 8.14)
IVf	2-Cl	C_2H_5	C_2H_5	16.8	143 (Et_2O)	5.41	2.33	$C_{19}H_{22}ClNO_5$	60.08 (59.78	5.84 5.89	3.69 3.61)
IVg	2-CF_3	C_2H_5	C_2H_5	79.8	147—148 (Et_2O-*n*-hexane)	5.58	2.30	$C_{20}H_{22}F_3NO_5$	58.11 (58.05	5.36 5.47	3.39 3.23)
IVh	3-OH	C_2H_5	C_2H_5	57.3	161.5—163 (Et_2O-AcOEt)	4.70	2.30	$C_{19}H_{23}NO_6$	63.15 (63.24	6.42 6.40	3.88 3.77)
IVi	2-OCH_3	C_2H_5	C_2H_5	33.1	125—126 (Et_2O)	5.26	2.30	$C_{20}H_{25}NO_6$	63.98 (64.14	6.71 6.81	3.73 3.70)
IVj	2-$COOCH_3$	C_2H_5	C_2H_5	61.0	106—108 (Et_2O-*n*-hexane)	5.98	2.25	$C_{21}H_{25}NO_7$	62.50 (62.75	6.25 6.32	3.47 3.43)
IVk	2-NO_2	CH_3	iso-C_3H_7	96.2	136.5—138 (AcOEt)	5.83	2.38	$C_{19}H_{22}N_2O_7$	58.45 (58.51	5.68 5.72	7.18 7.09)
IVl	3-NO_2	CH_3	iso-C_3H_7	92.2	164—166 (EtOH)	5.07	2.38	$C_{19}H_{22}N_2O_7$	58.45 (58.26	5.68 5.71	7.18 6.98)
IVm	2-Cl	CH_3	iso-C_3H_7	99.1	122—123 ((iso-Pr)$_2$O)	5.40	2.35	$C_{19}H_{22}ClNO_5$	60.08 (60.21	5.84 5.79	3.69 3.63)
IVn	2-CF_3	CH_3	iso-C_3H_7	Quant.	123—125 ((iso-Pr)$_2$O)	5.61	2.33	$C_{19}H_{22}F_3NO_5$	58.11 (57.95	5.36 5.38	3.39 3.39)
IVo	2-$COOCH_3$	CH_3	iso-C_3H_7	89.4	144—145 (MeOH)	5.98	2.33	$C_{21}H_{25}NO_7$	62.52 (62.47	6.25 6.30	3.47 3.57)
IVp	2-CN	CH_3	*n*-C_3H_7	66.6	149—152.5 (aqueous MeOH)	5.31	2.38	$C_{20}H_{22}N_2O_5$	64.85 (65.11	5.99 5.89	7.56 7.59)
IVq	2-CN	CH_3	iso-C_3H_7	99.1	135—137 ((iso-Pr)$_2$O–MeOH)	5.32	2.41	$C_{20}H_{22}N_2O_5$	64.85 (64.88	5.99 5.74	7.56 7.53)
IVr	2-CN	CH_3	iso-C_4H_9	74.1	134.5—137 (aqueous MeOH)	5.35	2.39	$C_{20}H_{24}N_2O_5$	65.61 (65.53	6.29 6.16	7.29 7.16)
IVs	2-CN	CH_3	neo-C_5H_{11}	76.6	178—179.5 (aqueous MeOH)	5.33	2.38	$C_{21}H_{26}N_2O_5$	66.31 (66.08	6.58 6.52	7.03 6.94)

idines (IV) prepared are summarized in Table I with their physical data.

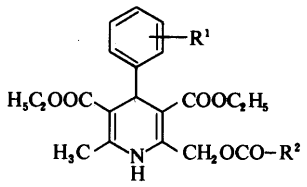
The hydroxymethyl group of IV ($R^2=R^3=C_2H_5$) was converted to variety kinds of novel substituents (Chart 2). Acylated compounds (XII) shown in Table II were prepared by treating IV with acylating reagents such as acid anhydrides or acid halides. Chloromethyl derivatives (XIII) prepared from IV under neutral conditions (Ph_3P-CCl_4)[12] were converted to 4-substituted piperazinomethyl (XIVa, b) or (1-methyltetrazol-5-yl)-thiomethyl (XV) by treatment with piperazine derivatives or 1-methyl-5-mercaptotetrazole in the presence of a base.

Other novel 1,4-dihydropyridine derivatives possessing an acetyl or 1-hydroxyethyl group at the 2-position of the nucleus (XIX and XX) were prepared according to the modified method described in the formation of the 2-formyl derivatives (X) and followed by reduction with sodium borohydride (Chart 3). Both the alcohol (XX) and the accompaning by-product lactone (XXI) obtained in this reduction reaction comprised of 1 : 1 mixtures of each diastereoisomer from the analysis of their nuclear magnetic resonance (NMR) spectra.

The preparation of 2-cyano-1,4-dihydropyridines (XXII) was carried out through the reaction of the corresponding aldehydes (X) with hydroxylamine at an ambient temperature, followed by treatment with acetic anhydride (Chart 4). XXII prepared according to this procedure are summarized in Table III with their physical data.

Other substituents at the 2-position such as iminomethyl (XXIV), carbamoyl (XXVI), vinyl (XXVII), and ethynyl (XXX) were derived from 2-formyl derivatives (Charts 4 and 5). In the course of our studies on the synthesis

TABLE II. 2-Acyloxymethyl-1,4-dihydropyridines (XII)



Compd. No.	R^1	R^2	Yield (%)	mp (°C) (Recry. sol.)	NMR δ value ($CDCl_3$)		Formula	Analysis (%) Calcd (Found)			
					C_4-H	C_6-CH_3		C	H	N	Cl
XIIa	2-NO_2	CH_3	68.6	89—90 (Et_2O)	5.90	2.36	$C_{21}H_{24}N_2O_8$	58.33 (58.21)	5.59 5.61	6.48 6.40)	
XIIb	3-NO_2	CH_3	67.0	133—135 (Et_2O–*n*-hexane)	5.14	2.36	$C_{21}H_{24}N_2O_8$	58.33 (57.93	5.59 5.71	6.48 6.15)	
XIIc	2-Cl	CH_3	53.6	98 (Et_2O–*n*-hexane)	5.33	2.10	$C_{21}H_{24}ClNO_6$	59.78 (59.82	5.73 5.68	3.32 3.34	8.42 8.20)
XIId	2-NO_2	C_6H_5	89.1	156—157 (AcOEt)	*a)*		$C_{26}H_{26}N_2O_8$	63.15 (63.27	5.30 5.26	5.67 5.49)	
XIIe	3-NO_2	3-Py	49.5	139—140 (MeOH)	5.15	2.34	$C_{25}H_{25}N_3O_8$	60.60 (60.48	5.09 4.96	8.48 8.51)	
XIIf	2-Cl	CH_2CH_2COOH	83.9	130—131 (aqueous MeOH)	5.40	2.32	$C_{23}H_{26}ClNO_8$	57.56 (57.65	5.46 5.43	2.92 3.00	7.39 7.77)

a) Not measured.

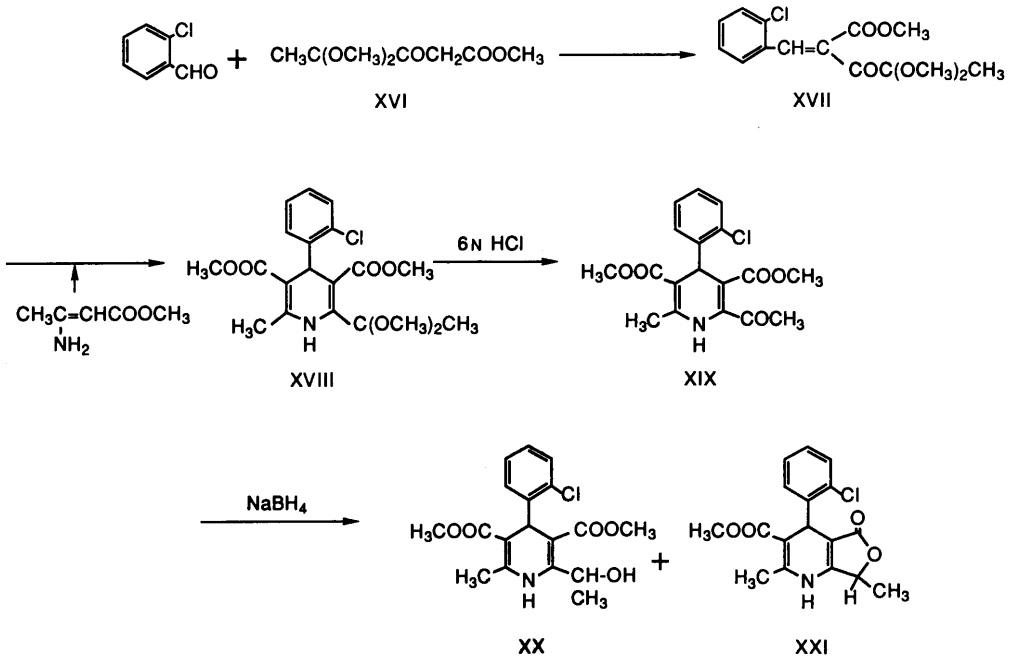


Chart 3

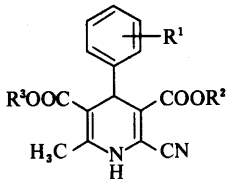
of 2-cyano-1,4-dihydropyridines (XXII), we found that 2-formyl derivatives (X) could react very smoothly with hydroxylamine. Therefore, we intended to synthesize various kinds of 2-iminomethyl derivatives (XXIV) through the reaction of X with primary amines. The obtained XXIV are shown in Table IV with their physical data. 2-(*N*-Methylcarbamoyl)-1,4-dihydropyridine (XXVI) was prepared by treating nitron (XXV) with acetic anhydride and subsequently with refluxing 6 N HCl. The nitron (XXV) was prepared from Xd and *N*-methylhydroxylamine.

2-(Tetrazol-5-yl)-1,4-dihydropyridine (XXIII) was prepared by heating 2-cyano derivative (XXIId) with sodium azide in the presence of ammonium acetate.

Some 1,4-dihydropyridines substituted at the 2-position with a vinylogous cyano, acetyl or formyl group (XXVIIa—c) were prepared by the Wittig reaction of 2-formyl-1,4-dihydropyridine (Xm) with the respective triphenylphosphorane as a single isomer, respectively. The 2-(3-hydroxyl-1-propenyl) derivative (XXVIId) was obtained by reducing XXVIIc with sodium borohydride. The geometry of the disubstituted vinyl group in these compounds (XXVII) turned out to be *E*-form from their coupling constants of the respective vinyl protons in their NMR spectra as shown in Table V.

2-Ethynyl-1,4-dihydropyridine (XXX) was synthesized from 2-formyl-1,4-dihydropyridine (Xm) according to the

TABLE III. 2-Cyano-1,4-dihydropyridines (XXII)



Compd. No.	R^1	R^2	R^3	Yield (%)	mp (°C) (Recry. sol.)	NMR δ value ($CDCl_3$)		Formula	Analysis (%) Calcd (Found)		
						C_4-H	C_6-CH_3		C	H	N
XXIIa	2-NO_2	CH_3	CH_3	57.4	170.5—171.5 (AcOEt–*n*-hexane)	5.90	2.37	$C_{17}H_{15}N_3O_6$	57.12 (56.86)	4.23 (4.13)	11.77 (11.61)
XXIIb	3-NO_2	CH_3	CH_3	52.5	206—207 (AcOEt–MeOH)	5.12	2.40	$C_{17}H_{15}N_3O_6$	57.12 (57.37)	4.23 (4.22)	11.77 (11.62)
XXIIc	2-NO_2	C_2H_5	C_2H_5	61.2	126—127.5 (Et_2O–*n*-hexane)	6.03	2.31	$C_{19}H_{19}N_3O_6$	59.22 (59.35)	4.97 (4.71)	10.90 (10.85)
XXIId	3-NO_2	C_2H_5	C_2H_5	52.8	174—177 (C_6H_6–Et_2O)	5.22	2.40	$C_{19}H_{19}N_3O_6$	59.22 (59.40)	4.97 (4.97)	10.90 (10.86)
XXIIe	2-CN	CH_3	CH_3	50.6	166—166.5 (MeOH)	5.42	2.40	$C_{18}H_{15}N_2O_4$	67.48 (67.51)	4.68 (4.82)	8.67 (8.55)
XXIIf	2-Cl	C_2H_5	C_2H_5	71.3	136—137 (Et_2O–*n*-hexane)	5.50	2.31	$C_{19}H_{19}ClN_2O_4$	60.88 (60.76)	5.11 (5.17)	7.46 (7.37)
XXIIg	2-CF_3	C_2H_5	C_2H_5	10.2[a]	140—143 (Et_2O–*n*-hexane)	5.68	2.30	$C_{20}H_{19}F_3N_2O_4$	58.82 (58.68)	4.69 (4.71)	6.86 (6.77)
XXIIh	2-$COOCH_3$	C_2H_5	C_2H_5	50.4	126—127 ((iso-Pr)$_2$O–EtOH)	6.22	2.33	$C_{21}H_{22}N_2O_6$	63.29 (63.10	5.57 5.58	7.03 6.99)
XXIIi	2-NO_2	CH_3	iso-C_3H_7	51.9	175.5—176.5 (MeOH)	5.95	2.33	$C_{19}H_{19}N_3O_6$	59.21 (59.05	4.97 4.86	10.90 10.86)
XXIIj (Nilvadipine)	3-NO_2	CH_3	iso-C_3H_7	67.0	148—150 (EtOH)	5.21	2.42	$C_{19}H_{19}N_3O_6$	59.21 (59.23	4.97 4.92	10.90 10.90)
XXIIk	2-CF_3	CH_3	iso-C_3H_7	53.0	172—173 (MeOH)	5.68	2.35	$C_{20}H_{19}F_3N_2O_4$	58.82 (58.83	4.69 4.62	6.86 6.83)
XXIIl	2-CN	CH_3	iso-C_3H_7	83.2	198—200 (MeOH)	5.44	2.43	$C_{20}H_{19}N_3O_4$	65.74 (65.51	5.24 5.00	11.50 11.34)
XXIIm	2-Cl	CH_3	iso-C_3H_7	80.8	176—177 (MeOH)	5.50	2.36	$C_{19}H_{19}ClN_2O_4$	60.88 (60.59	5.11 5.17	7.46 7.38)
XXIIn	2-$COOCH_3$	CH_3	iso-C_3H_7	69.1	121.5—123 (MeOH)	6.18	2.34	$C_{21}H_{22}N_2O_6$	63.30 (63.17	5.57 5.50	7.03 6.92)
XXIIo	2-CN	CH_3	*n*-C_3H_7	53.9	150—151 (EtOH)	5.38	2.37	$C_{20}H_{19}N_3O_4$	65.74 (65.77	5.24 5.13	11.50 11.40)
XXIIp	2-CN	CH_3	iso-C_4H_9	53.8	196—197.5 (EtOH)	5.41	2.40	$C_{21}H_{21}N_3O_4$	66.48 (66.66	5.58 5.43	11.08 11.94)
XXIIq	2-CN	CH_3	neo-C_5H_{11}	45.1	173—174 (EtOH)	5.44	2.41	$C_{22}H_{23}N_3O_4$	67.17 (67.09	5.58 5.87	10.18 10.02)

a) Dehydrated with thionyl chloride.

method developed by Corey.[13] 2-(2,2-Dibromovinyl)-1,4-dihydropyridine (XXVIII) accompanying the oxidized pyridine derivative (XXIX) was obtained by treating Xm with triphenylphosphine and carbon tetrabromide. When XXVIII was treated with *n*-butyl lithium under cooling at −20 °C and subsequently quenched with water, the desired 2-ethynyl-1,4-dihydropyridine (XXX) was obtained in 83.6% yield. The 2-(1-hexynyl)-1,4-dihydropyridine derivative was, however, concomitantly produced in a small amount. This suggested that the dilithium salt of XXX reacted with *n*-butyl bromide formed in the process of this reaction.

Structure–Activity Relationships and Discussion

The biological activities of the compounds prepared in this paper were evaluated in the following two tests; (i) increase of coronary blood flow in pentobarbital-anesthetized dogs and (ii) hypotensive effect in normotensive rats.

Dimethyl 2-hydroxymethyl-4-(2-nitrophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (IVa), which was the original compound designed on the basis of the information[7] concerning metabolites of nifedipine, showed potent activity for increase in coronary blood flow as shown in Table VI. This finding prompted us to discover other potent 2-hydroxymethyl-1,4-dihydropyridines. The strategy employed for this purpose was to modify the ester parts and also the substituent on the phenyl ring of the compound (IVa). With regard to the contribution of a nitro group on the phenyl ring, the 2-position is found to be superior to the 3-position in the series of symmetrical esters (IVa *vs.* IVb and IVc *vs.* IVd) in terms of the increase of coronary blood flow shown in Table VI. However, 2-nitro (IVk) and 3-nitro (IVl) having unsymmetrical esters exhibit almost equipotent activities for the increase of in coronary blood flow and for the hypotensive effect. Although the introduction of electron-donating substituents on the phenyl ring such as 3-hydroxy

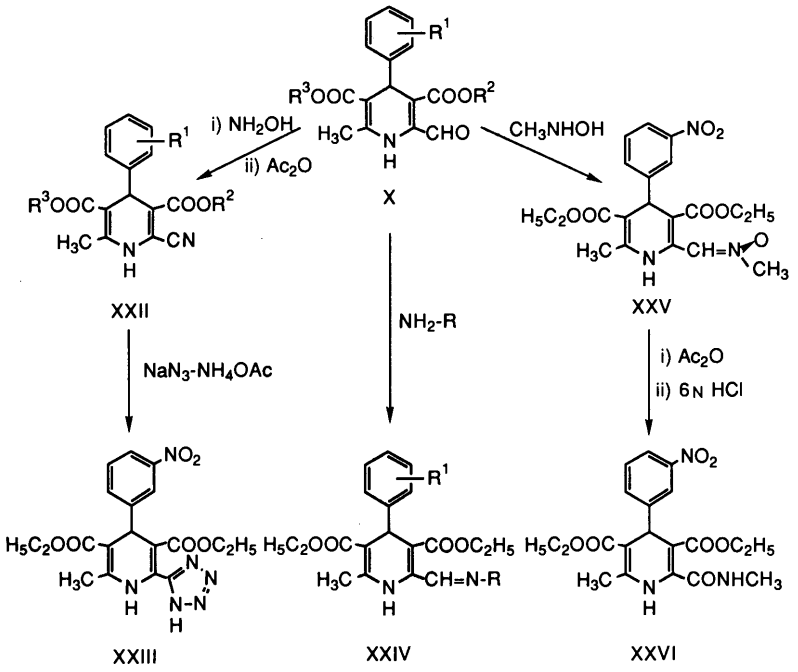
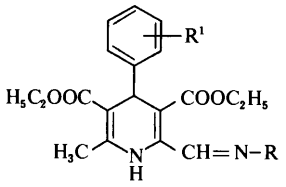
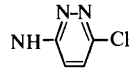
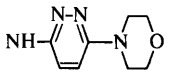
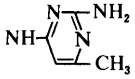


Chart 4

TABLE IV. 2-Substituted Iminomethyl-1,4-dihydropyridines (XXIV)



Compd. No.	R^1	R	Yield (%)	mp (°C) (Recry. solv.)	NMR δ value C_4-H	Formula	Analysis (%) Calcd (Found)		
							C	H	N
XXIVa	2-Cl	OCH_3	53.3	110—112 (Et_2O–*n*-hexane)	5.51[a]	$C_{20}H_{23}ClN_2O_5$	59.04 (59.06	5.70 5.71	6.89 6.89)
XXIVb	2-Cl	NH_2	91.8	107—110 (C_6H_6–Et_2O)	5.50[a]	$C_{19}H_{22}ClN_3O_4$	58.21 (58.17	5.66 5.63	10.73 10.56)
XXIVc	2-NO_2	CH_2CH_2OH	58.5	Oil	5.96[a]			[c]	
XXIVd	3-NO_2	$NHCSNH_2$	98.3	228—230 (dec.) (DMF–Et_2O)	5.10[b]	$C_{20}H_{23}N_5O_6S$	52.05 (52.27	5.02 4.88	15.18 15.24)
XXIVe	3-NO_2		97.1	>290 (DMF–Et_2O)	5.12[b]	$C_{21}H_{22}N_6O_6S$	51.84 (51.98	4.56 4.36	17.28 17.32)
XXIVf	3-NO_2		68.5	257 (dec.) (aqueous DMF–Et_2O)	5.07[b]	$C_{23}H_{23}ClN_6O_6$	53.65 (53.65	4.50 4.45	16.32 16.29)
XXIVg	3-NO_2		62.7	247—248 (aqueous DMF–Et_2O)	5.10[b]	$C_{27}H_{31}N_7O_7$	57.33 (57.57	5.53 5.62	17.34 17.16)
XXIVh	3-NO_2		85.8	248—249 (dec.) (aqueous DMF–Et_2O)	5.08[b]	$C_{24}H_{24}N_7O_6$	56.91 (56.91	4.78 5.08	19.36 19.39)

a) Measured in $CDCl_3$. *b*) Measured in DMSO-d_6. *c*) Not carried out.

(IVh) and 2-methoxy (IVi) results in a loss of coronary blood flow increase, the electron-withdrawing substituents appear to contribute to maintaining the activities. 2-Trifluoromethyl (IVg), 2-cyano (IVe), 2-methoxycarbonyl (IVj), and 2-chloro (IVf) turned out to be effective in this order in the series of 2-hydroxymethyl-1,4-dihydropyridines having two identical ethyl esters at the 3- and the 5-positions. In the unsymmetrical ester series, 2-trifluoromethyl (IVn), 2-methoxycarbonyl (IVo), and 2-cyano (IVq) are potent for the hypotensive effect but the 2-chloro (IVm) derivative slightly diminishes the activity. In comparison with each pair, the compounds having unsymmetrical esters

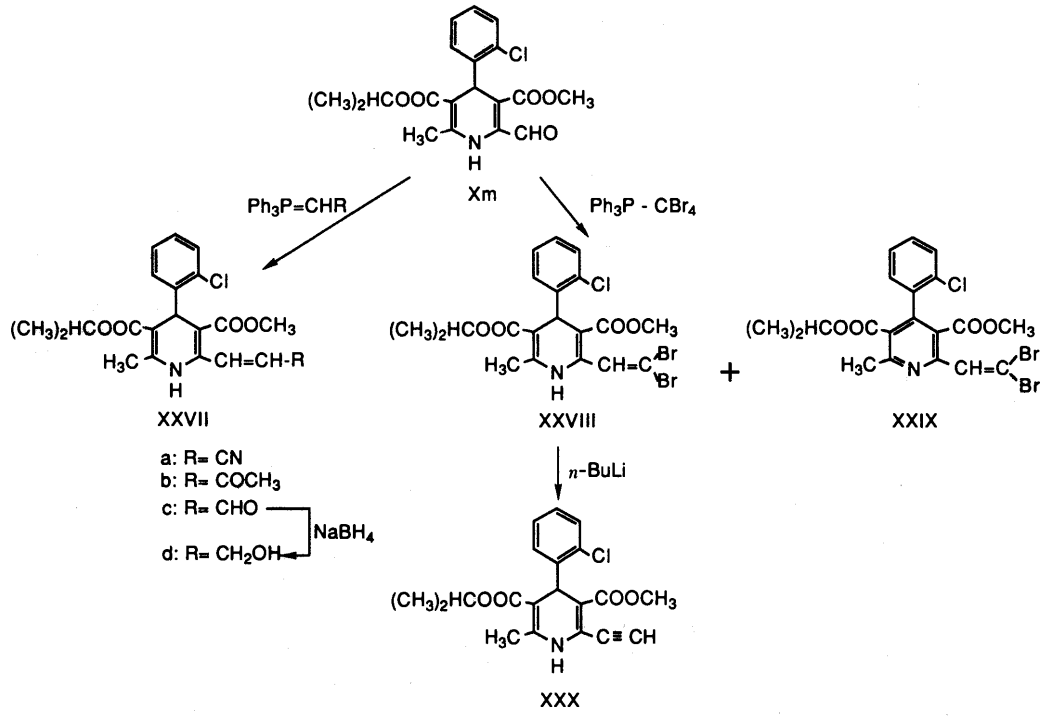
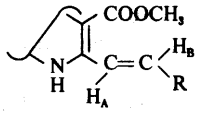


Chart 5

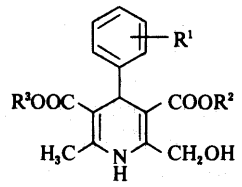
TABLE V. Chemical Shifts and Coupling Constants of the Olefinic Protons of 2-Substituted Vinyl Compounds (XXVII)



Compd. No.	R	Chemical shifts		Coupling constant J_{AB} (Hz)
		H_A	H_B	
XXVIIa	CN	7.79	5.53	12.5
XXVIIb	$COCH_3$	8.28	6.43	17.0
XXVIIc	CHO	8.29	6.52	16.0
XXVIId	CH_2OH	a)	6.18	16.5

a) Unable to be assigned because of overlapping with other proton's signals.

TABLE VI. Biological Activities of 2-Hydroxymethyl-1,4-dihydropyridines (IV)



Compd. No.	R^1	R^2	R^3	Increase of coronary blood flow[a] ED_{50} (μg/kg)	Hypotensive effect[b] 1 mg/kg	10 mg/kg
IVa	2-NO_2	CH_3	CH_3	2.6	NT	NT
IVb	3-NO_2	CH_3	CH_3	14.5	NT	NT
IVc	2-NO_2	C_2H_5	C_2H_5	2.2	NT	NT
IVd	3-NO_2	C_2H_5	C_2H_5	4.8	−20.7	−38.2
IVe	2-CN	CH_3	CH_3	11.0	−6.2	−31.8
IVf	2-Cl	C_2H_5	C_2H_5	21.0	−5.6	−28.2
IVg	2-CF_3	C_2H_5	C_2H_5	4.8	−20.6	−38.4
IVh	3-OH	C_2H_5	C_2H_5	>64.0	NT	NT
IVi	2-OCH_3	C_2H_5	C_2H_5	>64.0	NT	NT
IVj	2-$COOCH_3$	C_2H_5	C_2H_5	15.0	NT	NT
IVk	2-NO_2	CH_3	iso-C_3H_7	4.0	−17.2	−42.2
IVl	3-NO_2	CH_3	iso-C_3H_7	2.6	−12.2	−41.2
IVm	2-Cl	CH_3	iso-C_3H_7	NT	−3.0	−29.0
IVn	2-CF_3	CH_3	iso-C_3H_7	NT	−18.0	−46.6
IVo	2-$COOCH_3$	CH_3	iso-C_3H_7	NT	−12.2	−47.2
IVp	2-CN	CH_3	n-C_3H_7	NT	−12.2	−41.8
IVq	2-CN	CH_3	iso-C_3H_7	NT	−19.2	−46.8
IVr	2-CN	CH_3	iso-C_4H_9	NT	−10.6	−43.0
IVs	2-CN	CH_3	neo-C_5H_{11}	NT	−0.8	−16.8

a) % increase of coronary blood flow in pentobarbital-anesthetized dogs after i.v. administration. b) Δ_{max} (mmHg) on blood pressure in normotensive rats after *p.o.* administration. NT: not tested.

seem to be somewhat superior to those having symmetrical esters in their activities. The most suitable size of the alkyl group of the ester at the 3-position in 2-hydroxymethyl-4-(2-cyanophenyl)-3-methoxycarbonyl-1,4-dihydropyridines was elucidated. Isopropyl (IVq) is found to be best. A smaller group such as methyl (IVe) and a bigger group, namely neopentyl (IVs), dramatically diminish in their hypotensive effects.

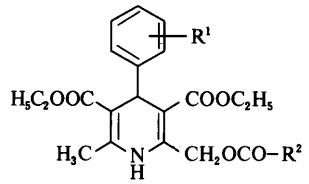
2-Acetoxymethyl-1,4-dihydropyridines (XIIa—c), which are thought to be pro-drugs of the corresponding 2-hydroxymethyl-1,4-dihydropyridines (IV), did not exhibit augmentation of the activity shown in Table VII. Benzoylation (XIId), nicotination (XIIe) and succination (XIIf) resulted in a dramatic decrease of the activity.

2-Piperazinomethyl (XIVa, b) and 2-(1-methyltetrazol-5-yl)-thiomethyl (XV), which are the novel substituents at the 2-position of 1,4-dihydropyridine nucleus derived from the corresponding 2-hydroxymethyl group, were also found to have very weak effectiveness to coronary blood flow.

As the 2-formyl-1,4-dihydropyridine (Xd) was found to have potent activity comparable to the corresponding 2-hydroxymethyl derivative (IVd), we hoped to discover another new electron-withdrawing substituent at the 2-position of the 1,4-dihydropyridine nucleus.

An acetyl group was found to be useful somehow as a

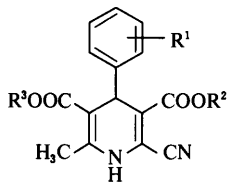
TABLE VII. Biological Activity of 2-Acyloxymethyl-1,4-dihydropyridines (XII)



Compound No.	R^1	R^2	Increase of coronary blood flow[a] ED_{50} (μg/kg)
XIIa	2-NO_2	CH_3	4.0
XIIb	3-NO_2	CH_3	3.6
XIIc	2-Cl	CH_3	16.0
XIId	2-NO_2	C_6H_5	>64.0
XIIe	3-NO_2	3-C_5H_4N	−19.4
XIIf	2-Cl	CH_2CH_2COOH	360

a) See footnote a) in Table VI.

TABLE VIII. Biological Activities of 2-Cyano-1,4-dihydropyridines (XXII)



Compd. No.	R^1	R^2	R^3	Increase of coronary blood flow[a] ED_{50} (μg/kg)	Hypotensive effect[b] 1 mg/kg	10 mg/kg
XXIIa	2-NO_2	CH_3	CH_3	<4.0	−23.0	−47.8
XXIIb	3-NO_2	CH_3	CH_3	9.6	NT	NT
XXIIc	2-NO_2	C_2H_5	C_2H_5	1.1	NT	NT
XXIId	3-NO_2	C_2H_5	C_2H_5	2.9	−15.3	−36.0
XXIIe	2-CN	CH_3	CH_3	2.6	NT	NT
XXIIf	2-Cl	C_2H_5	C_2H_5	6.0	−5.0	−22.0
XXIIg	2-CF_3	C_2H_5	C_2H_5	1.3	−30.0	−48.4
XXIIh	2-$COOCH_3$	C_2H_5	C_2H_5	18.0	−8.2	−40.6
XXIIi	2-NO_2	CH_3	iso-C_3H_7	2.7	−42.2	−56.8
XXIIj	3-NO_2	CH_3	iso-C_3H_7	1.4	−44.6	−50.0
XXIIk	2-CF_3	CH_3	iso-C_3H_7	NT	−34.6	−47.4
XXIIl	2-CN	CH_3	iso-C_3H_7	2.1	−39.6	−50.2
XXIIm	2-Cl	CH_3	iso-C_3H_7	NT	−29.2	−40.6
XXIIn	2-$COOCH_3$	CH_3	iso-C_3H_7	NT	−25.4	−49.4
XXIIo	2-CN	CH_3	n-C_3H_7	NT	−37.4	−50.6
XXIIp	2-CN	CH_3	iso-C_4H_9	NT	−20.2	−40.6
XXIIq	2-CN	CH_3	neo-C_5H_{11}	NT	−3.4	−3.8

a) See footnote a) in Table VI. b) See footnote b) in Table VI. NT: not tested.

substituent at the 2-position, although only the 4-(2-chlorophenyl)-1,4-dihydropyridine derivative (XIX) was tested. The ED_{50} value for the increase of coronary blood flow was shown to be 27 μg/kg. However, its reduced product, 2-(1-hydroxyethyl) derivative (XX), showed weak activity. The other electron-withdrawing substituents, iminomethyl (XXIV), methylcarbamoyl (XXVI) and tetrazoyl (XXIII), exhibited little activity.

From the results of modification of the electron-withdrawing substituents at the 2-position in 1,4-dihydropyridine nucleus, the compound having a cyano group, *e.g.* dimethyl 2-cyano-4-(2-nitrophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (XXIIa), is promising in terms of potent activities in both coronary blood flow increase and hypotensive effect. Therefore, the modification of XXIIa was carried out in detail according to the strategy employed in the optimization research in the 2-hydroxymethyl-1,4-dihydropyridine series. Almost the same tendency to structure–activity relationships as that described above for 2-hydroxymethyl-1,4-dihydropyridines was observed (Table VIII).

The 2-nitro substituent on the phenyl ring is more effective than 3-nitro and ethyl ester is superior to methyl ester in the case of two identical esters (XXIIa—d). On the contrary, 2-nitro (XXIIi) and 3-nitro (XXIIj) having methyl and isopropyl esters at the 3- and the 5-positions, respectively, show close activities. The other electron-withdrawing substituents on the phenyl ring such as 2-trifluoromethyl (XXIIg, k) and 2-cyano (XXIIe, l) exhibit comparable activities to a nitro group, although 2-methoxycarbonyl (XXIIh, n) and 2-chloro (XXIIf, m) diminish somewhat in their activities. The most suitable alkyl group of the ester at the 5-position in 2-cyano-3-methoxycarbonyl-4-(2-cyanophenyl)-1,4-dihydropyridines is found to be isopropyl (XXIII).

The structure–activity relationships on the ester parts and the substituents on the phenyl ring observed in 2-hydroxymethyl and 2-cyano-1,4-dihydropyridines closely coincide with that reported by Meyer[5] in a series of 2,6-dimethyl-1,4-dihydropyridines. And in comparison with each pair of 2-hydroxymethyl and 2-cyano-1,4-dihydropyridines containing the same substituents at the other positions of the nucleus, 2-cyano derivatives are found to be somewhat superior to 2-hydroxymethyl derivatives in terms of their biological evaluations.

The vinylogous derivatives (XXVII) of the 2-hydroxymethyl, 2-cyano-, 2-acetyl- and 2-formyl-1,4-dihydropyridines were all inactive contrary to our expectation. Furthermore, 2-ethynyl-1,4-dihydropyridine (XXX), whose substituent at the 2-position resembles a nitrile group in terms of electronic structure and also in sterical bulkiness, had unexpectedly little activity for coronary blood flow.

In all of the compounds evaluated in this paper, diethyl 2-cyano-4-(2-nitrophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (XXIIc) was the most potent in increasing coronary blood flow in pentobarbital-anesthetized dogs and isopropyl 2-cyano-3-methoxycarbonyl-4-(2-nitrophenyl)-6-methyl-1,4-dihydropyridine-5-carboxylate (XXIIi) was the most active for the hypotensive effect in normotensive rats. However, as both compounds have a 2-nitrophenyl group as a substituent at the 4-position of 1,4-dihydropyridine nucleus, they were found to be unstable in sun-light and to be easily converted by irradiation with 366 nm into 4-(2-nitrosophenyl)pyridines *via* an intramolecular oxidation–reduction process, as reported.[14] From high potent compounds in the biological evaluation results such as XXIIg, XXIIj or XXIIl, which possesses 2-trifluoromethyl, 3-nitro or 2-cyano substituted phenyl group at the 4-position of the 1,4-dihydropyridine nucleus, we selected isopropyl 2-cyano-3-methoxycarbonyl-4-(3-nitrophenyl)-6-methyl-1,4-dihydropyridine-5-carboxylate (XXIIj) as a candidate compound for further evaluations because of its potency of the activities, its stability in sun-light, and the ready availability of the starting material, *m*-nitrobenzaldehyde in comparison to *o*-trifluoromethyl- or *o*-cyanobenzaldehyde.

XXIIj (nilvadipine) has already been accepted in clinical use for the treatment of hypertension.

Experimental

Melting points were determined using a Thomas–Hoover capillary melting point apparatus and were uncorrected. NMR spectra were recorded on a JNN-PMR spectrometer using tetramethylsilane as an internal standard. Infrared (IR) spectra were taken on either a Hitachi 260-10 spectrophotometer or a Shimazu IR-420 spectrophotometer. Column chromatography was performed on silica gel (Merck Kieselgel 60, 230—400 mesh).

Diethyl 2-Diethoxymethyl-6-methyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (IXd) A solution of 3-nitrobenzaldehyde (2.27 g), ethyl 4,4-diethoxyacetoacetate[15] (3.28 g) and piperidine (0.2 ml) in C_6H_6 (15 ml) was refluxed under azeotropic dehydration for 3 h. The resulting solution was washed with H_2O and dried over $MgSO_4$. The solvent was removed to give an oily mixture of *E* and *Z* forms of ethyl 2-(3-nitrobenzylidene)-4,4-diethoxyacetoacetate (6.0 g). The mixture obtained and ethyl 3-aminocrotonate[16] (1.9 g) was heated at 95 °C to 100 °C for 7 h and then at 120 °C for 1.5 h with stirring. After cooling, the resultant oil was dissolved in AcOEt, washed with H_2O and dried. The solvent was removed to afford an oil (7.8 g), which was subjected to column chromatography on silica gel with an eluent (C_6H_6 : AcOEt = 20 : 1). The objective fragments were combined and evaporated. The residue (4.65 g) was triturated in a mixture of isopropyl ether (IPE) and *n*-hexane (1 : 3) to afford IXd (4.5 g, 64.8%) as a yellow powder, which was used in the following reaction without further purification. NMR ($CDCl_3$) δ: 1.23, 1.26 (12H, tt, J=7 Hz, $CH_2\underline{CH_3} \times 4$), 2.4 (3H, s, C_6-CH_3), 3.50—3.86 (4H, m, $OC\underline{H_2}CH_3 \times 2$), 4.11 (4H, q, J=7 Hz, $COOC\underline{H_2}CH_3 \times 2$), 5.16 (1H, s, C_4-H), 6.20 (1H, s, $CH(OR)_2$), 6.82 (1H, br s, NH), 7.25—8.16 (4H, m, aromatic protons).

The other 2-dialkoxymethyl-1,4-dihydropyridines (IX) were synthesized in a similar manner to that of IXd and their yields and physical data are listed in Table IX.

Diethyl 2-Formyl-6-methyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (Xd) To a solution of IXd (462.5 mg) in acetone (4 ml) was added 6 N HCl (0.4 ml) and the mixture was stirred for 1 h at room temperature. After the reaction was completed, the solvent was removed to give a residue, which was pulverized by adding H_2O. The powder was collected by filtration, washed with H_2O and dried. Recrystallization from EtOH afforded Xd (360 mg, 92.8%), mp 130—133 °C. *Anal.* Calcd for $C_{19}H_{20}N_2O_7$: C, 58.76; H, 5.19; N, 7.21. Found: C, 58.88; H, 5.17; N, 7.24. NMR ($CDCl_3$) δ: 1.28 (6H, t, J=7 Hz, $COOCH_2\underline{CH_3} \times 2$), 2.48 (3H, s, C_6-CH_3), 4.10, 4.33 (4H, each q, J=7 Hz, $COO\underline{CH_2}CH_3 \times 2$), 5.28 (1H, s, C_4-H), 7.11 (1H, br s, NH), 7.3—8.15 (4H, m, aromatic protons), 10.55 (1H, s, CHO).

The other 2-formyl-1,4-dihydropyridines (X) were synthesized in a similar manner to that of Xd and their yields and physical data are listed in Table X.

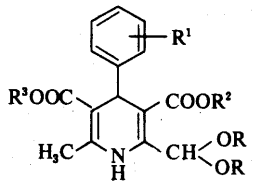
Diethyl 2-Hydroxymethyl-6-methyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (IVd) To a suspension of Xd (233 mg) in EtOH (10 ml) was added $NaBH_4$ (22.7 mg) at 0—5 °C under stirring and the resultant mixture was stirred for an additional 1 h under the same conditions. The mixture was adjusted to pH 6 with 50% aqueous AcOH, and then the solvent was removed under reduced pressure. The residual mixture was extracted with AcOEt and the extract was washed twice with H_2O. The dried solution was concentrated to give a residue, which was crystallized by treating it with a mixture of Et_2O and *n*-hexane. The precipitates were collected by filtration and dried. Recrystallization from a mixture of Et_2O and *n*-hexane afforded IVd (152 mg, 65.0%), mp 141—141.5 °C. Analytical data are listed in Table I. NMR ($CDCl_3$) δ: 1.23 (6H, t, J=7 Hz, $COOCH_2\underline{CH_3} \times 2$), 2.43 (3H, s, C_6-CH_3), 3.60 (1H, s, OH), 4.13 (4H, q, J=7 Hz, $COO\underline{CH_2}CH_3 \times 2$), 4.83 (2H, s, CH_2OH), 5.14 (1H, s, C_4-H), 7.23—8.13 (5H, m, aromatic protons and NH).

The other 2-hydroxymethyl-1,4-dihydropyridines (IV) were synthesized in similar manners to that of IVd and their yields and physical data are listed in Table I.

Ethyl 4-(2-Chlorophenyl)-2-methyl-5-oxo-1,4,5,7-tetrahydrofuro[3,4-*b*]-pyridine-3-carboxylate (XIa) A solution of IVf (592.5 mg) and a catalytic amount of *p*-TsOH in EtOH (6 ml) was refluxed for 1 h. Removal of the solvent and addition of Et_2O afforded crystals (317.5 mg), which were recrystallized from AcOEt to afford XIa (210.3 mg, 40.9%) as colorless needles, mp 211—212 °C. *Anal.* Calcd for $C_{17}H_{16}ClNO_4$: C, 61.17; H, 4.83; Cl, 10.62; N, 4.20. Found: C, 61.26; H, 4.87; Cl, 10.72; N, 4.16. NMR (DMSO-d_6) δ: 0.97 (3H, t, J=7.5 Hz, $CH_2\underline{CH_3}$), 2.31 (3H, s, C_2-CH_3), 3.88 (2H, q, J=7.5 Hz, $C\underline{H_2}CH_3$), 4.83 (2H, s, C_7-H_2), 5.21 (1H, s, C_4-H), 7.2 (4H, br, aromatic protons), 9.81 (1H, s, NH).

The other lactone compounds (XIb, c) were synthesized in a similar manner to that of XIa.

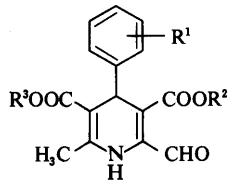
TABLE IX. Yields and Physical Data of 2-Dialkoxymethyl-1,4-dihydropyridines (IX)



Compd. No.	R^1	R^2	R^3	R	Yield (%)	mp (°C) (Recry. solv.)	δ ($CDCl_3$) C_4-H
IXa	2-NO_2	CH_3	CH_3	CH_3	63.6	132—133 (MeOH)	5.76
IXb	3-NO_2	CH_3	CH_3	CH_3	51.5	99—100 (IPE)	5.25
IXc	2-NO_2	C_2H_5	C_2H_5	C_2H_5	69.7	80—81.5 (Et_2O–*n*-hexane)	5.92
IXd	3-NO_2	C_2H_5	C_2H_5	C_2H_5	a)	a)	a)
IXe	2-CN	CH_3	CH_3	CH_3	70.4	133—134.5 (IPE–MeOH)	4.83
IXf	2-Cl	C_2H_5	C_2H_5	C_2H_5	44.8	75—77 (*n*-hexane)	5.60
IXg	2-CF_3	C_2H_5	C_2H_5	C_2H_5	57.6	82—83 (Et_2O–*n*-hexane)	5.60
IXh	3-OH	C_2H_5	C_2H_5	C_2H_5	56.3	107—108 (Et_2O–*n*-hexane)	5.00
IXi	2-OCH_3	C_2H_5	C_2H_5	C_2H_5	56.8	105—107 (*n*-hexane)	5.33
IXj	2-$COOCH_3$	C_2H_5	C_2H_5	C_2H_5	38.1	94—95 (*n*-hexane)	6.04
IXk	2-NO_2	CH_3	iso-C_3H_7	CH_3	56.8	143—145 (MeOH)	5.88
IXl	3-NO_2	CH_3	iso-C_3H_7	CH_3	51.1	Oil	5.17
IXm	2-Cl	CH_3	iso-C_3H_7	CH_3	59.3	86—87.5 (IPE–*n*-hexane)	5.57
IXn	2-CF_3	CH_3	iso-C_3H_7	CH_3	63.2	92—94 (IPE)	5.63
IXo	2-$COOCH_3$	CH_3	iso-C_3H_7	CH_3	52.3	Oil	5.93
IXp	2-CN	CH_3	*n*-C_3H_7	CH_3	39.2	139—141 (IPE)	5.42
IXq	2-CN	CH_3	iso-C_3H_7	CH_3	47.1	171—172.5 (IPE)	5.40
IXr	2-CN	CH_3	iso-C_4H_9	CH_3	35.6	126—128 (IPE)	5.42
IXs	2-CN	CH_3	neo-C_5H_{11}	CH_3	31.5	130—131 (IPE)	5.37

a) See Experimental.

TABLE X. Yields and Physical Data of 2-Formyl-1,4-dihydropyridines (X)



Compd. No.	R^1	R^2	R^3	Yield (%)	mp (°C) (Recry. solv.)	δ ($CDCl_3$)	
						C_4-H	CHO
Xa	2-NO_2	CH_3	CH_3	98.4	a)	5.88	10.43
Xb	3-NO_2	CH_3	CH_3	91.7	157—157.5 (MeOH)	5.30	10.50
Xc	2-NO_2	C_2H_5	C_2H_5	96.9	101—103 (Et_2O–*n*-hexane)	5.98	10.39
Xd	3-NO_2	C_2H_5	C_2H_5	c)	c)	c)	
Xe	2-CN	CH_3	CH_3	98.3	a)	5.50	10.53
Xf	2-Cl	C_2H_5	C_2H_5	93.5	87—88 (EtOH–*n*-hexane)	5.63	10.47
Xg	2-CF_3	C_2H_5	C_2H_5	95.4	Oil	5.72	10.28
Xh	2-OH	C_2H_5	C_2H_5	82.7	141.5—142.5 (EtOH–*n*-hexane)	5.13	10.06
Xi	2-OCH_3	C_2H_5	C_2H_5	99.8	111—112 (EtOH–*n*-hexane)	5.48	b)
Xj	2-$COOCH_3$	C_2H_5	C_2H_5	Quant.	Oil	6.20	10.20
Xk	2-NO_2	CH_3	iso-C_3H_7	Quant.	a)	6.00	10.38
Xl	3-NO_2	CH_3	iso-C_3H_7	95.4	a)	5.27	10.60
Xm	2-Cl	CH_3	iso-C_3H_7	76.1	102—103 (*n*-hexane)	5.55	10.31
Xn	2-CF_3	CH_3	iso-C_3H_7	86.3	83—85 (*n*-hexane)	5.73	10.27
Xo	2-$COOCH_3$	CH_3	iso-C_3H_7	62.8	Oil	6.21	10.23
Xp	2-CN	CH_3	*n*-C_3H_7	54.5	137.5—141 (IPE)	5.45	10.51
Xq	2-CN	CH_3	iso-C_3H_7	92.4	132—134 (IPE)	5.50	10.51
Xr	2-CN	CH_3	*n*-C_4H_9	33.3	152.5—154.5 (IPE)	5.53	10.55
Xs	2-CN	CH_3	neo-C_5H_{11}	36.9	142—143 (IPE)	5.49	10.50

a) Powder which was used in a following reaction without further purification. *b*) Not measured in offset region. *c*) See Experimental.

XIb: mp 221—223 °C (recryst. from AcOEt). NMR ($CDCl_3$) δ: 2.23 (3H, s, C_2-CH_3), 5.63 (1H, s, C_4-H), 4.80 (2H, s, C_7-H_2). *Anal.* Calcd for $C_{17}H_{16}N_2O_6$: C, 59.30; H, 4.68; N, 8.14. Found: C, 59.55; H, 4.59; N, 8.10.

XIc: mp 219—220 °C (recryst. from AcOEt). NMR ($CDCl_3$) δ: 2.26 (3H, s, C_2-CH_3), 5.05 (1H, s, C_4-H), 4.75 (2H, s, C_7-H_2). *Anal.* Calcd for $C_{18}H_{19}NO_5$: C, 65.64; H, 5.82; N, 4.25. Found: C, 65.39; H, 5.83; N, 4.22.

Diethyl 2-Acetoxymethyl-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (XIIc) To a solution of IVf (759.7 mg) in dried pyridine (10 ml) was added dropwise a solution of AcCl (235.5 mg, 1.5 eq mol) in CH_2Cl_2 (5 ml) under cooling in an ice-bath. After the addition was completed, the ice-bath was removed and the reaction was continued at room temperature for 4.5 h. The solvent was removed and H_2O was added thereto. The mixture was acidified with dil. HCl and extracted with Et_2O. The extract was washed with H_2O twice and dried. Removal of the solvent afforded a viscous oil (0.99 g), which crystallized on standing. Recrystallization from a mixture of Et_2O and *n*-hexane afforded XIIc (452.0 mg, 53.6%) as colorless needles, mp 98 °C. Analytical data are listed in Table II. NMR (DMSO-d_6) δ: 1.13 (6H, t, J=7 Hz, CH_2CH_3 × 2), 2.10 (3H, s, C_6-CH_3), 2.30 (3H, s, $COCH_3$), 4.00 (4H, q, J=7 Hz, CH_2CH_3 × 2), 5.03 (2H, ABq, C_2-CH_2). 5.33 (1H, s, C_4-H), 7.0—7.4 (4H, m, aromatic protons), 8.9 (1H, br s, NH).

The other 2-acyloxymethyl-1,4-dihydropyridines (XIIa—e) were prepared in a similar manner to that of XIIc. Their yields and physical data are listed in Table II.

Diethyl 2-(3-Carboxypropionyl)oxymethyl-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (XIIf) A solution of IVf (379.8 mg), succinic anhydride (150.1 mg) and pyridine (791 mg) in dioxane (5 ml) was refluxed for 4.5 h. The resultant solution was concentrated, acidified with dil. HCl and extracted with Et_2O. The ethereal layer was washed with H_2O and then extracted with an aqueous solution of $NaHCO_3$. The combined aqueous extract was washed with Et_2O and acidified with dil. HCl to afford precipitates, which were collected by filtration, washed with H_2O and dried. Recrystallization from 50% aqueous EtOH afforded XIIf (402.1 mg, 83.9%), mp 130—131 °C. Analytical data are listed in Table II. NMR ($CDCl_3$) δ: 1.18 (6H, t, J=7 Hz, CH_2CH_3 × 2), 2.32 (3H, s, C_6-CH_3), 2.72 (4H, s, CH_2CH_2), 4.08 (4H, q, J=7 Hz, CH_2CH_3 × 2), 5.37 (2H, s, C_2-CH_2O), 5.40 (1H, s, C_4-H), 6.82 (1H, br s, NH), 7.0—7.5 (4H, m, aromatic protons).

Diethyl 4-(2-Chlorophenyl)-6-methyl-2-(4-methylpiperazin-1-yl)methyl-1,4-dihydropyridine-3,5-dicarboxylate (XIVa) To a solution of IVf (379.8 mg) in CCl_4 (40 ml) was added PPh_3 (314.7 mg) at room temperature. The mixture was refluxed for 2.5 h. After cooling, the mixture was concentrated and chromatographed on silica gel with an eluent (C_6H_6 : AcOEt = 10 : 1). The fractions containing the desired intermediate, 2-chloromethyl derivative (XIII), were combined and evaporated to give an oil, which was dissolved in EtOH (10 ml). To the solution was added 1-methylpiperazine (0.20 g) and the mixture was stirred for 50 h at room temperature. EtOH was removed and the residue obtained was diluted with H_2O. The aqueous solution was extracted with AcOEt. The extract was washed with H_2O and then with dil. HCl. The combined aqueous extract was washed with Et_2O, made alkaline with an aqueous solution of $NaHCO_3$, and then extracted with AcOEt. The extract was washed with H_2O, dried and evaporated to give crude crystals (115.2 mg, 25.0%), which were recrystallized from a mixture of EtOH and Et_2O to afford XIVa as colorless needles, mp 179—180 °C. *Anal.* Calcd for $C_{24}H_{32}ClN_3O_4$: C, 62.39; H, 6.98; Cl, 7.68; N, 9.10. Found: C, 62.22; H, 6.91; Cl, 7.71; N, 9.02. NMR ($CDCl_3$) δ: 1.18 (6H, t, J=7 Hz, CH_2CH_3 × 2), 2.30 (3H, s, C_6-CH_3), 2.34 (3H, s, NCH_3), 2.3—2.7 (8H, br s, piperazine ring protons), 3.76 (2H, ABq, C_2-CH_2N), 4.08 (4H, q, J=7 Hz, CH_2CH_3 × 2), 5.44 (1H, s, C_4-H), 6.9—7.4 (4H, m, aromatic protons), 7.99 (1H, br s, NH).

Diethyl 4-(2-Chlorophenyl)-2-[4-(2-hydroxyethyl)piperazin-1-yl]methyl-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (XIVb) XIVb was prepared in a similar manner to that of XIVa. Yield 37.8%, mp 145—147 °C. *Anal.* Calcd for $C_{25}H_{34}ClN_3O_5$: C, 61.01; H, 6.97; N, 8.54. Found: C, 60.49; H, 6.96; N, 8.54. NMR ($CDCl_3$) δ: 2.37 (3H, s, C_6-CH_3), 5.45 (1H, s, C_4-H).

Diethyl 6-Methyl-2-(1-methyltetrazol-5-yl)thiomethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (XV) A solution of IVd (3.90 g) and PPh_3 (3.93 g) in CCl_4 (40 ml) was refluxed for 2.5 h. After cooling, the mixture was concentrated and the residue was chromatographed on silica gel eluting with an eluent (C_6H_6 : AcOEt = 10 : 1). The desired fractions were combined and evaporated to give yellow powder (2.41 g) of 2-chloromethyl derivative (XIII) [NMR ($CDCl_3$) δ: 2.43 (3H, s, C_6-CH_3), 4.92 (2H, ABq, CH_2Cl), 5.18 (1H, s, C_4-H)]. To a solution of the powder obtained above in EtOH (70 ml) were added 1-methyl-5-mercaptotetrazole (1.31 g) and pyridine (0.8 g). The mixture was warmed at 50 °C for 7 h. After removal of the solvent, the residual oil was

dissolved in AcOEt. The solution was washed with H_2O twice and dried. Evaporation of the solvent afforded a yellow oil, which was subjected to column chromatography on silica gel with an eluent (C_6H_6 : AcOEt = 5 : 1). The desired fractions were combined and evaporated to give a yellow oil (2.39 g), which was crystallized by triturating with Et_2O. Collection by filtration and recrystallization from a mixture of MeOH and Et_2O afforded XV (1.89 g, 38.7%) as light yellow granules, mp 118—120 °C. *Anal.* Calcd for $C_{21}H_{24}N_6O_6S$: C, 51.63; H, 4.96; N, 17.21; S. 6.56. Found: C, 51.68; H, 4.84; N, 16.75; S, 6.85. NMR ($CDCl_3$) δ: 1.21, 1.22 (6H, each t, J=7.5 Hz, $CH_2\underline{CH_3}\times2$), 2.28 (3H, s, C_6-CH_3), 3.95 (3H, s, NCH_3), 4.09, 4.20 (4H, each q, J=7.5 Hz, $\underline{CH_2}CH_3\times2$), 4.63 (2H, ABq, CH_2S), 5.11 (1H, s, C_4-H), 7.2—8.16 (4H, m, aromatic protons), 8.37 (1H, br s, NH).

Dimethyl 4-(2-Chlorophenyl)-2-(1,1-dimethoxyethyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (XVIII) A mixture of methyl 3-aminocrotonate (2.33 g) and a crude oil of methyl 4,4-dimethoxy-2-(2-chlorobenzylidene)-3-oxopentanoate (XVII, 6.39 g), which was prepared by the Knoevenagel reaction of 2-chlorobenzaldehyde and methyl 4,4-dimethoxy-3-oxopentanoate, was heated in a neat state at 130 °C for 3.5 h. After cooling, the reaction mixture was dissolved in AcOEt, washed with H_2O and dried. Removal of the solvent gave a brown oil (8.28 g), which was subjected to column chromatography on silica gel with an eluent (C_6H_6 : AcOEt = 10 : 1). The fractions containing the desired compound were combined and concentrated to give crystals (5.26 g, 62.8%), which were recrystallized from a mixture of Et_2O and *n*-hexane (1 : 1) to afford XVIII as yellow granules, mp 145—146 °C. *Anal.* Calcd for $C_{20}H_{24}ClNO_6$: C, 58.61; H, 5.90; Cl, 8.65; N, 3.42. Found: C, 58.52; H, 5.92; Cl, 8.62; N, 3.42. NMR ($CDCl_3$) δ: 1.57 (3H, s, $C(OR)_2\underline{CH_3}$), 2.35 (3H, s, C_6-CH_3), 3.10, 3.12 (6H, each s, $OCH_3\times2$), 3.50, 3.57 (6H, each s, $COOCH_3\times2$), 5.29 (1H, s, C_4-H), 6.29 (1H, br s, NH), 6.9—7.4 (4H, m, aromatic protons).

Dimethyl 2-Acetyl-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (XIX) A solution of XVIII (409.9 mg) and 6 N HCl (0.5 ml) in acetone (5 ml) was stirred at room temperature for 20 min. After neutralization with an aqueous solution of $NaHCO_3$, acetone was removed under reduced pressure. To the residue was added H_2O and the mixture was allowed to stand at room temperature. The crystals formed were collected by filtration and dried (350.2 mg, 96.3%). Recrystallization from a mixture of AcOEt and *n*-hexane afforded XIX as yellow granules, mp 161—162 °C. *Anal.* Calcd for $C_{18}H_{18}ClNO_5$: C, 59.42; H, 4.99; Cl, 9.75; N, 3.85. Found: C, 59.67; H, 4.95; Cl, 9.62; N, 3.82. NMR ($CDCl_3$) δ: 2.30, 2.35 (6H, each s, $COCH_3$, C_6-CH_3), 3.56, 3.61 (6H, each s, $COOCH_3\times2$), 5.37 (1H, s, C_4-H), 6.91 (1H, br s, NH), 6.95—7.44 (4H, m, aromatic protons).

Dimethyl 4-(2-Chlorophenyl)-2-(1-hydroxyethyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (XX) and Methyl 4-(2-Chlorophenyl)-2,7-dimethyl-5-oxo-1,4,5,7-tetrahydrofuro[3,4-*b*]pyridine-3-carboxylate (XXI) To a solution of XIX (450 mg) in MeOH (15 ml) was added portionwise $NaBH_4$ (46.8 mg) under stirring and cooling in an ice-bath. The mixture was stirred for 35 min under the same conditions. The reaction mixture was neutralized with 2 N HCl under cooling. MeOH was removed at a temperature below 30 °C under reduced pressure. The residue was diluted with H_2O and allowed to stand. Colorless precipitates were collected by filtration and washed with Et_2O to afford XXI (264.2 mg, 64.0%) as a mixture of diastereoisomers, mp 228—233 °C. NMR (DMSO-d_6) δ: 1.40, 1.45 (3H, each d, J=7 Hz, C_7-CH_3), 2.31 (3H, s, C_2-CH_3), 3.41 (3H, s, $COOCH_3$), 5.02, 5.06 (1H, each q, J=7 Hz, C_7-H), 5.19 (1H, s, C_4-H), 7.0—7.5 (4H, m, aromatic protons), 9.55—10.0 (1H, br s, NH). The filtrate obtained after cropping XXI was concentrated at the temperature below 30 °C under reduced pressure to give crystals, which were triturated with *n*-hexane containing a small amount of Et_2O and filtered to afford XX (100.5 mg, 22.2%) as a mixture of diastereoisomers, mp 145—147 °C. NMR ($CDCl_3$) δ: 1.42, 1.44 (3H, each d, J=7 Hz, $CH(OH)\underline{CH_3}$), 2.33, 2.34 (3H, each s, C_6-CH_3), 3.2—3.22 (1H, m, OH), 3.60 (6H, s, $COOCH_3\times2$), 5.41, 5.43 (1H, each s, C_4-H), 5.56, 5.60 (1H, each q, J=7 Hz, $C\underline{H}(OH)CH_3$), 6.8—7.5 (5H, m, aromatic protons and NH).

Isopropyl 2-Cyano-3-methoxycarbonyl-6-methyl-4-(3-nitrophenyl)-1,4-dihydropyridine-5-carboxylate (XXIIj, Nilvadipine) To a solution of XI (4.5 g) in AcOH (35 ml) were added $NH_2OH\cdot HCl$ (0.97 g) and NaOAc (1.43 g), and the mixture was stirred at room temperature for 2.5 h. After Ac_2O (4.14 g) was added thereto, the mixture was stirred at the same temperature for 1.5 h and at 95 °C to 100 °C for an additional 4 h. AcOH and the excess Ac_2O were removed *in vacuo*, followed by the addition of H_2O to the residue and neutralized with an aqueous solution of $NaHCO_3$. This aqueous suspension was extracted twice with AcOEt and the combined extract was washed with H_2O, dried over $MgSO_4$. Removal of the solvent gave a reddish-brown oil (4.88 g), which was chromatographed on silica gel with an eluent (C_6H_6 : AcOEt = 10 : 1) to give XXIIj (2.99 g, 67.0%) as crystals. An analytical sample was given by recrystallization from EtOH as yellow granules, mp 148—150 °C. Analytical data are listed in Table III. IR (Nujol) cm^{-1}: 2260 (C≡N). NMR ($CDCl_3$) δ: 1.11, 1.27 (6H, each d, J=6 Hz, $CH(CH_3)_2$), 2.42 (3H, s, C_6-CH_3), 3.80 (3H, s, $COOCH_3$), 4.99 (1H, septet, J=6 Hz, $C\underline{H}(CH_3)_2$), 5.21 (1H, s, C_4-H), 7.17 (1H, s, NH), 7.3—8.3 (4H, m, aromatic protons).

The other 2-cyano-1,4-dihydropyridines (XXII) were synthesized in a similar manner to that of XXIIj and their yields and physical data are summarized in Table III.

Diethyl 6-Methyl-4-(3-nitrophenyl)-2-(tetrazol-5-yl)-1,4-dihydropyridine-3,5-dicarboxylate (XXIII) A mixture of XXIId (770.8 mg), sodium azide (143.0 mg) and ammonium acetate (169.6 mg) in *N*,*N*-dimethylformamide (DMF) (2 ml) was heated at 120 °C to 125 °C for 4.5 h under stirring. The solvent was removed under reduced pressure and the residue was poured into H_2O. The aqueous mixture was acidified with 6 N HCl and extracted with AcOEt. The extract was washed with H_2O twice and dried. Removal of the solvent gave an oil (1.21 g), which was pulverized in Et_2O to afford XXIII (0.98 g, 97.7%) as yellow powder. An analytical sample was given by recrystallization from Et_2O as yellow needles, mp 180—182 °C (dec.). *Anal.* Calcd for $C_{19}H_{20}N_6O_6$: C, 53.27; H, 4.71; N, 19.62. Found: C, 53.27; H, 4.67; N, 19.92. NMR (DMSO-d_6) δ: 0.87, 1.18 (6H, each t, J=7 Hz, $CH_2\underline{CH_3}\times2$), 2.38 (3H, s, C_6-CH_3), 3.87, 4.12 (4H, each q, J=7 Hz, $C\underline{H_2}CH_3\times2$), 5.20 (1H, s, C_4-H), 7.5—8.3 (5H, m, aromatic protons and NH), 9.81 (1H, s, NH).

Diethyl 4-(2-Chlorophenyl)-2-(methoxyiminomethyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (XXIVa) To a solution of Xf (525.3 mg) and $MeONH_2\cdot HCl$ (139.3 mg) in EtOH (10 ml) was gradually added an aqueous solution of Na_2CO_3 (88.5 mg in 1 ml of H_2O) under stirring at room temperature. After the addition was completed, the mixture was stirred for an additional 20 min under the same conditions. From the reaction mixture, EtOH was removed under reduced pressure and to the residue were added H_2O and Et_2O with stirring. The separated organic layer was washed with H_2O and dried. Removal of the solvent gave a yellow oil, which was triturated in *n*-hexane to afford yellow powder. Recrystallization from a mixture of Et_2O and *n*-hexane gave XXIVa (301.5 mg, 53.3%) as yellow granules, mp 110—112 °C. Analytical data are listed in Table IV. NMR ($CDCl_3$) δ: 1.20, 1.21 (6H, each t, J=8 Hz, $CH_2\underline{CH_3}\times2$), 2.38 (3H, s, C_6-CH_3), 3.97 (3H, s, OCH_3), 3.9—4.3 (4H, m, $C\underline{H_2}CH_3\times2$), 5.51 (1H, s, C_4-H), 6.96 (1H, br s, NH), 7.0—7.5 (4H, m, aromatic protons), 8.85 (1H, s, CH=N).

XXIVb and XXIVc were obtained in a similar manner to that of XXIVa and their physical data are shown in Table IV.

Diethyl 6-Methyl-2-[3-(1-morpholino)pyridazin-6-yl-hydrazono]-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (XXIVg) To a solution of Xd (2.31 g) in EtOH (50 ml) was added 3-(1-morpholino)-5-hydrazinopyridazine (1.16 g), which was prepared from 3-chloro-5-hydrazinopyridazine and morpholine. The mixture was stirred at room temperature for 10 min and then a catalytic amount of *p*-TsOH was added. The mixture was stirred at room temperature overnight. The yellow precipitates which formed were collected by filtration. The reprecipitates were dissolved in a small amount of DMF and then diluted with Et_2O to afford XXIVg (2.11 g, 62.7%), mp 247—248 °C. Analytical data are listed in Table IV. NMR (DMSO-d_6) δ: 1.18, 1.23 (6H, each t, J=7 Hz, $CH_2\underline{CH_3}\times2$), 3.4—4.0 (8H, br m, morpholino methylene), 4.07, 4.13 (4H, each q, J=7 Hz, $C\underline{H_2}CH_3\times2$), 5.10 (1H, s, C_4-H), 7.3—8.3 (7H, m, aromatic protons and NH). The signal of C_6-CH_3 was overlapped with the signals of DMSO in solvent DMSO-d_6.

XXIVd, XXIVe, XXIVf and XXIVh were prepared in a similar manner to that of XXIVg and their physical data are shown in Table IV.

***N*-[3,5-Diethoxycarbonyl-6-methyl-4-(3-nitrophenyl)-1,4-dihydropyridine-2-yl-methylidene]-methylamine-*N*-oxide (XXV)** A solution of Xd (3.88 g), $CH_3NHOH\cdot HCl$ (1.67 g) and pyridine (2.37 g) in EtOH (400 ml) was refluxed for 4 h and the reaction mixture was allowed to stand at room temperature overnight. Precipitated orange plates were collected by filtration and the filtrate was concentrated to afford the additional crop of orange powder by trituration. Recrystallization from a mixture of AcOEt and MeOH to give XXV (3.41 g, 81.8%) as orange plates, mp 192—194 °C. *Anal.* Calcd for $C_{20}H_{23}N_3O_7$: C, 57.55; H, 5.55; N, 10.07. Found: C, 57.35; H, 5.48; N, 9.96. NMR (DMSO-d_6) δ: 1.23 (6H, t, J=7 Hz, $CH_2\underline{CH_3}\times2$), 2.40 (3H, s, C_6-CH_3), 3.91 (3H, s, NCH_3), 4.10, 4.17 (4H, each q, J=7 Hz, $C\underline{H_2}CH_3\times2$), 5.20 (1H, s, C_4-H), 7.5—8.3

(5H, m, aromatic protons and NH), 8.70 (1H, s, CH=N).

Diethyl 6-Methyl-2-(*N*-methylcarbamoyl)-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (XXVI) A mixture of XXV (3.19 g), Ac_2O (60 ml) and AcOH (1.5 ml) was refluxed for 1 h. After cooling, the reaction mixture was concentrated under reduced pressure and the residue was diluted with H_2O. The resultant mixture was extracted with AcOEt and the extract was washed with H_2O, aqueous $NaHCO_3$ and H_2O in turn and dried. Removal of the solvent gave an oil, to whose solution in EtOH (40 ml) was added 6 N HCl (10 ml) and the mixture was stirred at room temperature overnight. After neutralization with aqueous $NaHCO_3$, EtOH was removed and the residual mixture was extracted with AcOEt. The extract was washed with H_2O and dried. The solvent was removed to afford a brown oil, which was subjected to column chromatography on silica gel with an eluent (C_6H_6 : AcOEt = 2 : 1). The fractions containing the desired compound were combined and evaporated to give a light yellow oil, which crystallized immediatelly. Recrystallization from 50% aqueous EtOH afforded XXVI (2.77 g, 88.2%) as colorless crystals, mp 179—182 °C. *Anal.* Calcd for $C_{20}H_{23}N_3O_7$: C, 57.55; H, 5.55; N, 10.07. Found: C, 57.70; H, 5.56; N, 9.97. NMR ($CDCl_3$) δ: 1.22, 1.32 (6H, each t, J=7.2 Hz, $CH_2\underline{CH_3}\times 2$), 2.45 (3H, s, C_6-CH_3), 2.98 (3H, d, J=5.5 Hz, NCH_3), 4.18, 4.24 (4H, each q, J=7.2 Hz, $\underline{CH_2}CH_3\times 2$), 5.38 (1H, s, C_4-H), 7.4—8.5 (5H, m, aromatic protons and NH), 10.60 (1H, br q, $N\underline{H}CH_3$).

Isopropyl 4-(2-Chlorophenyl)-2-(2-cyanovinyl)-3-methoxycarbonyl-6-methyl-1,4-dihydropyridine-5-carboxylate (XXVIIa) To a solution of $Ph_3P{=}CHCN$[17] (1.56 g) in CH_2Cl_2 (15 ml) was added dropwise a solution of Xm (1.30 g) in CH_2Cl_2 (15 ml) under stirring over a period of 50 min and the stirring was continued for 1 h at room temperature. After removal of the solvent, the residue was subjected to column chromatography on silica gel with an eluent (C_6H_6 : AcOEt = 20 : 1). The fractions containing the desired compound were combined and evaporated to dryness to give a red oil, which was crystallized by triturating in a mixture of isopropyl ether and *n*-hexane. Collection by filtration and recrystallization from a mixture of isopropyl ether and MeOH afforded XXVIIa (1.14 g, 84.6%) as yellow granules, mp 159—161.5 °C. *Anal.* Calcd for $C_{21}H_{21}ClN_2O_4$: C, 62.92; H, 5.28; Cl, 8.85; N, 6.99. Found: C, 62.84; H, 5.37; Cl, 9.12; N, 7.10. NMR ($CDCl_3$) δ: 1.05, 1.23 (6H, each d, J=6 Hz, $CH(\underline{CH_3})_2$), 2.22 (3H, s, C_6-CH_3), 3.63 (3H, s, $COOCH_3$), 4.98 (1H, septet, J=6 Hz, $C\underline{H}(CH_3)_2$), 5.49 (1H, s, C_4-H), 5.53 (1H, d, J=12.5 Hz, C=CHCN), 7.79 (1H, d, J=12.5 Hz, CH=CCN), 6.60 (1H, br s, NH), 6.9—7.55 (4H, m, aromatic protons).

XXVIIb and XXVIIc were obtained in a similar manner to that of XXVIIa.

XXVIIb: Yield, 75.0%, mp 154—156 °C. *Anal.* Calcd for $C_{22}H_{24}ClNO_5$: C, 63.23; H, 5.79; N, 3.35. Found: C, 63.08; H, 5.78; N, 3.32. NMR ($CDCl_3$) δ: 2.46 (3H, s, C_6-CH_3), 5.52 (1H, s, C_4-H).

XXVIIc: Yield, 27.9%, mp 179—180.5 °C. *Anal.* Calcd for $C_{21}H_{22}ClNO_5$: C, 62.45; H, 5.49; N, 3.47. Found: C, 62.16; H, 5.45; N, 3.48. NMR ($CDCl_3$) δ: 2.42 (3H, s, C_6-CH_3), 5.52 (1H, s, C_4-H).

Isopropyl 4-(2-Chlorophenyl)-2-(3-hydroxy-1-propenyl)-3-methoxycarbonyl-6-methyl-1,4-dihydropyridine-5-carboxylate (XXVIId) To a solution of XXVIIc (787.3 mg) in EtOH (60 ml) was added portionwise $NaBH_4$ (40.6 mg) under cooling at 0 °C over a period of 10 min with stirring. The mixture was stirred for additional 30 min under the same conditions. The reaction mixture was neutralized with AcOH and evaporated to give a residue, to which was added H_2O and extracted with AcOEt. The extract was washed with H_2O, aqueous $NaHCO_3$ and H_2O in turn. Removal of the solvent gave an oil, which was chromatographed on silica gel eluting with an eluent (C_6H_6 : AcOEt = 2 : 1) to give a yellow oil (0.86 g). Trituration of the oil in a mixture of *n*-hexane and Et_2O afforded crystals which were collected by filtration. Recrystallization from a mixture of *n*-hexane and Et_2O (1 : 2) afforded XXVIId (0.48 g, 60.7%) as yellow plates, mp 107—110 °C. *Anal.* Calcd for $C_{21}H_{24}ClNO_5$: C, 62.14; H, 5.96; Cl, 8.74; N, 3.45. Found: C, 62.14; H, 5.83; Cl, 9.02; N, 3.39. NMR ($CDCl_3$) δ: 1.05, 1.26 (6H, each d, J=6.5 Hz, $CH(CH_3)_2$), 2.34 (3H, s, C_6-CH_3), 3.12 (1H, br t, J=6 Hz, OH), 3.63 (3H, s, $COOCH_3$), 4.27 (2H, br s, turned to doublet (J=5.0 Hz) by addition of D_2O, CH_2OH; decoupling this region made a signal at 6.18 to doublet (J=16.5 Hz)), 5.42 (1H, s, C_4-H), 6.18 (1H, dt, J=16.5, 5.0 Hz, C=$\underline{CH}CH_2$), 6.26 (1H, s, NH), 6.8—7.4 (5H, m, aromatic protons and $\overline{CH}$=C, decoupling this region made a signal at 6.18 to triplet (J=5.0 Hz)).

Isopropyl 2-(2-Chlorophenyl)-2-(2,2-dibromovinyl)-3-methoxycarbonyl-6-methyl-1,4-dihydropyridine-5-carboxylate (XXVIII) A mixture of Ph_3P (1.31 g), CBr_4 (0.83 g) in CH_2Cl_2 (10 ml) was stirred under cooling at 0 °C for 10 min. To the mixture was added Xm (0.47 g) at once under the same conditions. The reaction mixture was stirred for 10 min at −3 °C and quenched with H_2O at the same temperature. The organic layer was washed with H_2O three times and dried over $MgSO_4$. After removal of the solvent, a viscous oil (2.66 g) obtained was diluted with Et_2O and insoluble mass was filtered off. The filtrate was concentrated to give an oil, which was subjected to column chromatography on silica gel with an eluent (C_6H_6 : AcOEt = 20 : 1). The fractions containing the minor product were combined and concentrated to afford 60 mg of isopropyl 4-(2-chlorophenyl)-2-(2,2-dibromovinyl)-3-methoxycarbonyl-6-methylpyridine-5-carboxylate (XXIX) as a brown oil. NMR ($CDCl_3$) δ: 0.91, 1.05 (6H, each d, J=7 Hz, $CH(CH_3)_2$), 2.69 (3H, s, C_6-CH_3), 3.55 (3H, s, $COOCH_3$), 4.92 (1H, septet, J=7 Hz, $C\underline{H}(CH_3)_2$), 7.0—7.5 (4H, m, aromatic protons), 7.72 (1H, s, CH=C). The fractions containing the desired compound were combined and removal of the solvent gave a yellow oil (0.61 g), which was crystallized by scratching and recrystallized from isopropyl ether to give XXVIII (403.0 mg, 60.1%) as light yellow needles, mp 155—157 °C. *Anal.* Calcd for $C_{20}H_{20}Br_2ClNO_4$: C, 45.01; H, 3.78; N, 2.63. Found: C, 44.82; H, 3.81; N, 2.85. NMR ($CDCl_3$) δ: 1.05, 1.16 (6H, each d, J=6 Hz, $CH(\underline{CH_3})_2$), 2.34 (3H, s, C_6-CH_3), 3.63 (3H, s, $COOCH_3$), 4.97 (1H, septet, J=6 Hz, $C\underline{H}(CH_3)_2$), 5.43 (1H, s, C_4-H), 6.36 (1H, s, NH), 6.9—7.5 (4H, m, aromatic protons), 7.61 (1H, s, CH=C).

Isopropyl 4-(2-Chlorophenyl)-2-ethynyl-3-methoxycarbonyl-6-methyl-1,4-dihydropyridine-5-carboxylate (XXX) To a solution of XXVIII (1.65 g) in dry tetrahydrofuran (THF) (30 ml) was added dropwise a solution of *n*-BuLi (5.95 ml of 10% solution in *n*-hexane) in THF (8 ml) under cooling at −27 °C with stirring over a period of 20 min. To the reaction mixture was added dil. HCl and then the solvent was removed under reduced pressure. The residue was dissolved in AcOEt. The solution was washed with H_2O three times and dried over $MgSO_4$. Removal of the solvent *in vacuo* gave a reddish oil, which was triturated in isopropyl ether and allowed to stand for 1 h to provide a yellowish orange powder (0.55 g). From the filtrate a second crop of the powder (0.42 g) was recovered. The combined powder was recrystallized from MeOH to afford XXX (0.58 g, 83.6%) as yellowish orange crystals, mp 172—173.5 °C. *Anal.* Calcd for $C_{20}H_{20}ClNO_4$: C, 64.26; H, 5.39; Cl, 9.49; N, 3.75. Found: C, 63.68; H, 5.47; Cl, 9.64; N, 3.76. NMR ($CDCl_3$) δ: 1.03, 1.25 (6H, each d, J=6.5 Hz, $CH(\underline{CH_3})_2$), 2.35 (3H, s, C_6-CH_3), 3.35 (1H, s, C≡CH), 3.67 (3H, s, $COOCH_3$), 4.97 (1H, septet, J=6.5 Hz, $C\underline{H}(CH_3)_2$), 5.54 (1H, s, C_4-H), 6.15 (1H, s, NH), 6.9—7.5 (4H, m, aromatic protons).

Biological Evaluation Procedures i) Hypotensive Effect: Five Wister rats were used per group. Each animal was immobilized in a cage sized to the body. Blood pressure was measured at the femoral artery by means of a pressure transduser and recorded as electrically integrated values of mean arterial pressure. Operation for the catheterization was performed under light anesthesia with ether. The test compound was administered intraveneously 3 h after completion of the operation.

ii) Increasing Effect of Coronary Blood Flow: This test was carried out according to the method described in the literature.[18]

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Synthesis and Platelet Aggregation Inhibitory Activities of 3-(2-Oxopropylidene)azetidin-2-one Derivatives. II[1,2]

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A series of 3-acylidene-4-methylazetidin-2-one derivatives bearing various substituents at the 1-position of the azetidin-2-one ring was synthesized. These compounds were evaluated for platelet aggregation inhibitory activities. Most of the compounds synthesized showed potent inhibitory activities against rabbit platelet aggregation induced by adenosine diphosphate or collagen *in vitro*. Structure–activity relationships are also discussed.

Keywords 3-(acylidene)azetidin-2-one; azetidin-2-one; platelet aggregation inhibition; adenosine diphosphate; collagen; structure–activity relationship

In the previous paper,[1] we showed that (*E* or *Z*)-3-(2-oxopropylidene)-4-methyl-1-phenylazetidin-2-ones inhibit rabbit platelet aggregation induced by adenosine diphosphate (ADP) or collagen *in vitro*, and the 3-acylideneazetidin-2-one skeleton is essential for the activity. In the next stage of the evaluation of this series of compounds, we have examined the synthesis of the azetidin-2-one derivatives bearing various acylidene moieties at the 3-position and various alkyl or substituted phenyl moieties at the 1-position of the azetidin-2-one ring. Synthesis of these compounds and the results of biological evaluations are described in this paper.

Chemistry The synthesis of 3-(acylidene)azetidin-2-ones (**10a—y**, **11a—p**) was accomplished by the methods shown in Chart 1.

Lithiation of 1,4-disubstituted-azetidin-2-ones (**1a—p**)[3] with lithium diisopropylamide (LDA) followed by condensation with the esters (**2**,[3] **3**[4] or **4**[5]) in tetrahydrofuran (THF) at −78 °C gave 3-acylazetidin-2-one derivatives (**5a—y**) as a single isomer in good yields. The stereochemistry of azetidin-2-one ring of (**5a—y**) was determined to be 3,4-*trans* based on the coupling constant (3—4 Hz) between C_3-H and C_4-H of (**5a—y**). Reduction of the ketone moiety of **5a—y** by $NaBH_4$ in MeOH at −78 °C proceeded in a stereoselective manner through a sodium cation chelated intermediate[1] to give corresponding α alcohols (**6a—y**) in excellent yields. The configuration of the hydroxy group of **6a—y** was determined by the stereochemistry of the product of elimination reaction described below. Compounds **6a—y** were treated with methanesulfonyl chloride (MsCl) in pyridine and triethylamine to give the corresponding mesylates (**7a—y**) in quantitative yields, which were treated with an excess of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in benzene under reflux to give a mixture of the *E* form olefins (**8**) and the *Z* form olefins (**9**). The geometry of the enone moiety of **8** was determined to be *E* and **9** was to be *Z* based on the characteristic olefinic proton signals observed in their ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrum. The olefinic proton of **8** resonated at a lower field than that of **9** because of the deshielding effect of the carbonyl group of the azetidin-2-one ring. The ratio of the formation of the *E* isomer (**8**) and the *Z* isomer (**9**)

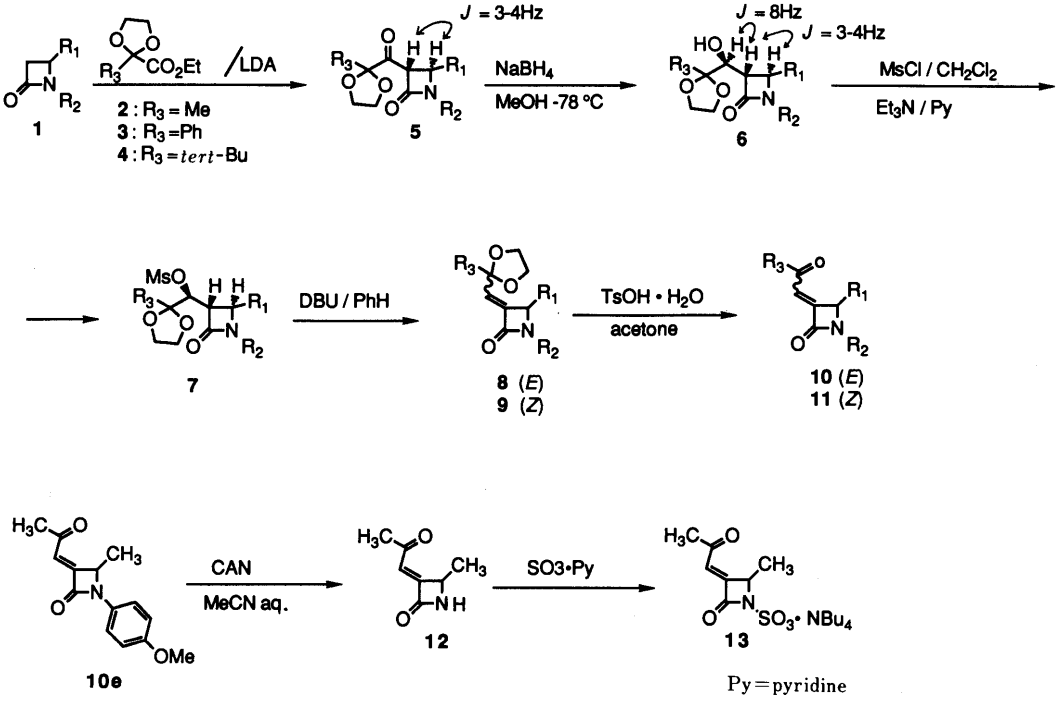


Chart 1

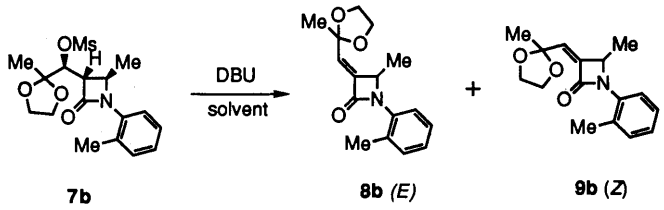
TABLE I. 3-Acylideneazetidin-2-one Derivatives and Their Inhibition of Platelet Aggregation

	R_1	R_2	R_3	Yield (%)	mp (Recryst. solvent)	Formula	Analysis (%) Calcd (Found)			*In vitro*[a] IC_{50} (μM)	
							C	H	N	ADP	Collagen
10a	Me	Ph	Me	86	120—122 (EtOH)	$C_{13}H_{13}NO_2$	72.54 (72.33	6.09 5.96	6.51 6.43)	29.0	27.0[b]
10b	Me	2-Me-Ph	Me	65	74—76 (EtOH)	$C_{14}H_{15}NO_2$	73.34 (73.12	6.60 6.58	6.11 6.08)	18.4	8.6
10c	Me	3-Me-Ph	Me	47	59.5—61.5 (EtOH)	$C_{14}H_{15}NO_2$	73.34 (73.62	6.60 6.62	6.11 6.14)	25.0	17.0
10d	Me	2-MeO-Ph	Me	74	79.5—81 (Ether)	$C_{14}H_{15}NO_3$	68.56 (68.64	6.16 6.21	5.71 5.94)	33.0	24.0
10e	Me	4-MeO-Ph	Me	55	107.5—109 (EtOH)	$C_{14}H_{15}NO_3$	68.56 (68.44	6.16 6.13	5.71 5.76)	14.0	19.0
10f	Me	2-F-Ph	Me	56	73—75.5 (EtOH)	$C_{13}H_{12}FNO_2$	66.94 (66.82	5.18 5.27	6.01 5.96)	27.0	20.0
10g	Me	4-F-Ph	Me	87	123—124 (EtOH)	$C_{13}H_{12}FNO_2$	66.94 (66.97	5.18 5.35	6.01 5.99)	14.0	13.0
10h	Me	2-Cl-Ph	Me	90	Oil	$C_{13}H_{12}ClNO_2$		265.0557[c] (265.0527)		19.0	15.0
10i	Me	3-CF_3-Ph	Me	50	54—57 (EtOH)	$C_{14}H_{12}F_3NO_2$	59.37 (59.16	4.27 4.20	4.95 4.79)	29.0	18.0
10j	Me	3-Py	Me	56	105—107 (EtOH)	$C_{12}H_{12}N_2O_2$	66.65 (66.69	5.59 5.62	12.95 12.95)	15.2	10.5
10k	H	Ph	Me	60	154.5—156.5 (EtOH–hexane)	$C_{12}H_{11}NO_2$	71.63 (71.50	5.51 5.61	6.96 6.82)	35.0	26.0
11a	Me	Ph	Me	84	114—115.5 (Ether)	$C_{13}H_{13}NO_2$	72.54 (72.38	6.09 6.11	6.51 6.52)	52.0	19.0[b]
11b	Me	2-Me-Ph	Me	7	78—79.5 (Ether–hexane)	$C_{14}H_{15}NO_2$	73.34 (73.45	6.60 6.69	6.11 5.87)	15.7	6.1
11c	Me	3-Me-Ph	Me	19	95—96 (EtOH)	$C_{14}H_{15}NO_2$	73.34 (73.06	6.60 6.55	6.11 6.22)	9.9	8.2
11d	Me	2-MeO-Ph	Me	50	100—101 (Ether)	$C_{14}H_{15}NO_3$	68.56 (68.40	6.16 6.16	5.71 5.71)	24.0	24.0
11e	Me	4-MeO-Ph	Me	2	158.5—160.5 (EtOH)	$C_{14}H_{15}NO_3$	68.56 (68.25	6.16 6.09	5.71 5.51)	16.7	8.3
11g	Me	4-F-Ph	Me	56	113—114 (EtOH)	$C_{13}H_{12}FNO_2$	66.94 (67.08	5.18 5.45	6.01 6.04)	14.0	18.0
11j	Me	3-Py	Me	55	121.5—123.5 (EtOH)	$C_{12}H_{12}N_2O_2$	66.65 (66.78	5.59 5.63	12.95 13.13)	23.5	11.7
12	Me	H	Me	29	117.5—120.5 (Ether)	$C_7H_9NO_2$	60.41 (60.34	6.53 6.52	10.07 10.14)	43.7	38.6
10l	Me	Me	Me	20	Oil	$C_8H_{11}NO_2$		153.0790[c] (153.0820)		80.8	29.1
10m	Me	Pr	Me	37	Oil	$C_{10}H_{15}NO_2$		181.1103[c] (181.1079)		102.2	24.0
10n	Me	iso-Bu	Me	37	Oil	$C_{11}H_{17}NO_2$		195.1259[c] (195.1254)		65.0	21.0
10o	Me	cyclo-Hex	Me	55	Oil	$C_{13}H_{19}NO_2$		221.1416[c] (221.1386)		17.1	15.1
10p	Me	Bz	Me	6	Oil	$C_{14}H_{15}NO_2$		229.1103[c] (229.1122)		63.0	35.0
11l	Me	Me	Me	34	Oil	$C_8H_{11}NO_2$		153.0790[c] (153.0799)		218.1	75.3
11m	Me	Pr	Me	45	Oil	$C_{10}H_{15}NO_2$		181.1103[c] (181.1128)		215.6	47.7
11n	Me	iso-Bu	Me	18	Oil	$C_{11}H_{17}NO_2$		195.1259[c] (195.1278)		79.0	40.0
11o	Me	cyclo-Hex	Me	22	Oil	$C_{13}H_{19}NO_2$		221.1416[c] (221.1378)		24.4	13.6
11p	Me	Bz	Me	3	Oil	$C_{14}H_{15}NO_2$		229.1103[c] (229.1094)		44.0	36.0
10q	Me	Ph	Ph	31	134—135 (EtOH)	$C_{18}H_{15}NO_2$	77.96 (77.66	5.45 5.76	5.05 4.90)	9.4	9.6
10r	Me	2-Me-Ph	Ph	71	73.5—75.5 (EtOH)	$C_{19}H_{17}NO_2$	78.33 (78.35	5.88 5.92	4.81 4.76)	6.4	6.0
10s	Me	3-Me-Ph	Ph	31	121—121.5 (EtOH)	$C_{19}H_{17}NO_2$	78.33 (78.01	5.88 5.95	4.81 4.89)	9.9	12.0
10t	Me	4-MeO-Ph	Ph	66	185—186 (EtOH)	$C_{19}H_{17}NO_3$	74.25 (74.27	5.57 5.74	4.56 4.58)	7.8	6.2

TABLE I. (continued)

	R_1	R_2	R_3	Yield (%)	mp (Recryst. solvent)	Formula	Analysis (%) Calcd (Found) C	H	N	*In vitro*[a] IC_{50} (μM) ADP	Collagen
10u	Me	2-F-Ph	Ph	54	114—115 (EtOH)	$C_{18}H_{14}FNO_2$	73.21 (72.97	4.78 4.71	4.74 4.95)	13.6	29.5
10v	Me	3-Py	Ph	44	151—153 (EtOH)	$C_{17}H_{14}N_2O_2$	73.37 (73.21	5.07 5.11	10.66 10.16)	2.1	4.0
10w	Me	iso-Pr	Ph	52	63—65 (EtOH)	$C_{15}H_{17}NO_2$	74.05 (74.31	7.04 7.21	5.76 5.65)	7.4	8.4
10x	Me	iso-Bu	Ph	24	71.5—73 (EtOH)	$C_{16}H_{19}NO_2$	74.68 (74.63	7.44 7.39	5.44 5.39)	6.8	6.4
10y	Me	2-Me-Ph	*tert*-Bu	50	71—72 (Ether)	$C_{17}H_{21}NO_2$	75.25 (75.19	7.80 7.58	5.16 5.19)	21.0	13.0
13	Me	SO_3NBu_4	Me		101.5—104.5 (EtOH)	$C_{23}H_{44}N_2O_5S$	59.95 (60.16	6.53 6.64	6.08 6.21)	>300	>300
Aspirine										>300	35.0
Ticlopidine										>300	>300

a) Micromolar condensation of test compound for 50% inhibition of rabbit platelet agregation induced by ADP (5 μM) or collagen (5 μg/ml). *b*) Ref. 1. *c*) High resolution MS (*m/z*).



7b **8b** *(E)* **9b** *(Z)*

solvent	condition	8b : 9b
benzene	reflux 2 h	4.7 : 1
$CHCl_3$	reflux 10 h	no reaction
CH_3CN	reflux 1 h	1 : 1.8
MeOH	reflux 2 h	1 : 1.8
DMSO	80 °C 2 h	1 : 5.6

Chart 2

in the elimination reactions was varied according to the substituents of R_3 and by the reaction solvent used. The ratio of *E* and *Z* isomer was about 5 : 1 in the case where R_3 was the methyl group, about 10 : 1 in the phenyl group, and 1 : 0 in the *tert*-butyl group, when the elimination reaction was carried out in benzene under reflux. Thus, it was obvious that the bulky substituent at R_3 tended to increase the formation of *E* form olefins (**8**). The next time, the effect of the solvents used in the elimination reaction was examined (Chart 2).

Although the formation of the *E* isomer was dominant in the less polar solvent such as benzene, the formation of the *Z* isomer has become dominant in polar solvents such as methanol or dimethyl sulfoxide (DMSO). The above result indicates that the *E* form olefins were formed from α form alcohols through *E*2 elimination in the less polar solvents. The mechanism of the formation of the *Z* form olefins is not clear but it seems to be formed through E_{CB}1-like elimination reactions. In the last stage of the synthesis, deprotection of the acetal moiety of **8** and **9** was accomplished by heating with a catalytic amount of *p*-toluenesulfonic acid in acetone to give the desired enone derivatives (**10**) and (**11**) in good yields (Chart 1). Compound (**10e**) was treated with ceric ammonium nitrate (CAN) to give **12**, which was further treated with sulfur trioxide to give sulfo derivative (**13**). In addition, the physiological and spectral data of **10a** and **11a** were identical with the data described in the previous paper.[1]

Pharmacological Results and Discussion

The platelet aggregation inhibitory activities of the compounds synthesized were tested on rabbit platelet-rich plasma (PRP) *in vitro* by the method shown in the previous paper.[1]

Almost all of the compounds tested showed potent platelet aggregation inhibitory activities *in vitro*, and the following structure activity relationships were investigated.

At first, the effect of the substituent at the acylidene moiety (R_3) was investigated. Although the substitution of the methyl group (**10b**) at the acylidene moiety with the *tert*-butyl group (**10y**) did not affect the activities, substitution with the phenyl group (**10r**) increased the activities 4-fold. Thus, the electron withdrawing group at the acylidene moiety seems to enhance the activities.

Next, the effect of the substituents at the 1 position of the azetidinone ring was investigated. Substitution of the phenyl group of (**10a**) with the cyclohexyl group (**10o**) did not affect the activities, but substitution with the hydrogen (**12**), methyl group (**10l**), propyl group (**10m**), isobutyl group (**10n**) and sulfo group (**13**) decreased the activities. Substitution of the phenyl group of (**10a**) with the 3-pyridyl group (**10j**) increased the activities 2 to 4-fold, and the introduction of a substituent to the *ortho* position of the phenyl group tends to increase the activities. Those results indicate that the introduction of the relative bulky group at the 1 position of the azetidinone ring increases the activities 1.5 to 2-fold.

At last, the effect of the geometry at the acylidene moiety was investigaged, and there were no differences between the activities of the *E* isomers and the *Z* isomers.

Experimental

Melting points were determined by Mettler FP-60 melting point apparatus. Infrared (IR) spectra were taken on a Jasco X-1A spectrometer. ^{1}H-NMR spectra were recorded with a Varian XL-200 spectrometer (Me_4Si as an internal standard, δ ppm value), and the following abbreviations are used: singlet (s), broad singlet (br s), doublet (d), double

TABLE II. Spectral Data for the Compounds in Table I

	IR	MS (*m/z*)	NMR (δ, $CDCl_3$)
10b	1745 1660	229 (M^+)	1.46 (3H, d, J=6 Hz), 2.36 (3H, s), 2.38 (3H, s), 5.06 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.70 (1H, d, J=2 Hz), 7.2—7.35 (4H, m)
10c	1725 1650	229 (M^+)	1.63 (3H, d, J=6 Hz), 2.36 (6H, s), 4.97 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.67 (1H, d, J=2 Hz), 6.98 (1H, m), 7.15—7.4 (3H, m)
10d	1740 1650	245 (M^+)	1.47 (3H, d, J=6 Hz), 2.35 (3H, s), 3.86 (3H, s), 5.25 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.62 (1H, d, J=2 Hz), 6.93 (1H, m), 6.99 (1H, m), 7.16 (1H, m), 7.93 (1H, m)
10e	1725 1650	245 (M^+)	1.62 (3H, d, J=6 Hz), 2.35 (3H, s), 3.80 (3H, s), 4.95 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.64 (1H, d, J=2 Hz), 6.92 (2H, m), 7.41 (2H, m)
10f	1750 1660	233 (M^+)	1.58 (1H, dd, J=6, 1 Hz), 2.36 (3H, s), 5.17 (1H, m), 6.66 (1H, d, J=2 Hz), 7.05—7.2 (3H, m), 8.05 (1H, m)
10g	1740 1665	233 (M^+)	1.62 (3H, d, J=6 Hz), 2.38 (3H, s), 4.96 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.60 (1H, d, J=2 Hz), 7.08 (2H, m), 7.42 (2H, m)
10h	1750 1660	249 (M^+)	1.52 (3H, d, J=6 Hz), 2.38 (3H, s), 5.46 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.70 (1H, d, J=2 Hz), 7.20 (1H, m), 7.31 (1H, m), 7.43 (1H, m), 7.85 (1H, m)
10i	1750 1660	283 (M^+)	1.66 (3H, d, J=6 Hz), 2.39 (3H, s), 5.03 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.72 (1H, d, J=2 Hz), 7.4—7.7 (4H, m)
10j	1755 1655	216 (M^+)	1.68 (3H, d, J=6 Hz), 2.40 (3H, s), 5.05 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.71 (1H, d, J=2 Hz), 7.34 (1H, dd, J=8, 6 Hz), 7.94 (1H, dt, J_d=8 Hz, J_t=2 Hz), 8.43 (1H, dd, J=6, 2 Hz), 8.65 (1H, d, J=2 Hz)
10k	1740 1680	201 (M^+)	2.38 (3H, s), 4.49 (2H, d, J=2 Hz), 6.72 (1H, t, J=2 Hz), 7.18 (1H, m), 7.3—7.5 (4H, m)
11b	1720 1685	229 (M^+)	1.44 (3H, d, J=6 Hz), 2.38 (3H, s), 2.76 (3H, s), 4.76 (1H, dq, J_d=1 Hz, J_q=6 Hz), 5.99 (1H, d, J=1 Hz), 7.2—7.4 (4H, m)
11c	1730 1660	229 (M^+)	1.60 (3H, d, J=6 Hz), 2.38 (3H, s), 2.74 (3H, s), 4.67 (1H, dq, J_d=1 Hz, J_q=6 Hz), 5.97 (1H, d, J=1 Hz), 7.01 (1H, m), 7.2—7.4 (3H, m)
11d	1730 1665	245 (M^+)	1.44 (3H, d, J=6 Hz), 2.74 (3H, s), 3.87 (3H, s), 4.98 (1H, dq, J_d=1 Hz, J_q=6 Hz), 5.91 (1H, d, J=1 Hz), 6.96 (1H, m), 7.03 (1H, m), 7.21 (1H, m), 7.93 (1H, m)
11e	1730 1665	245 (M^+)	1.59 (3H, d, J=6 Hz), 2.75 (3H, s), 3.82 (3H, s), 4.64 (1H, dq, J_d=1 Hz, J_q=6 Hz), 5.95 (1H, d, J=1 Hz), 6.95 (2H, m), 7.43 (2H, m)
11g	1740 1665	233 (M^+)	1.60 (3H, d, J=6 Hz), 2.72 (3H, s), 4.65 (1H, dq, J_d=1 Hz, J_q=6 Hz), 5.98 (1H, d, J=1 Hz), 7.10 (2H, m), 7.45 (2H, m)
11j	1740 1665	216 (M^+)	1.65 (3H, d, J=6 Hz), 2.73 (3H, s), 4.76 (1H, dq, J_d=1 Hz, J_q=6 Hz), 6.02 (1H, d, J=1 Hz), 7.36 (1H, dd, J=6, 8 Hz), 8.00 (1H, dt, J_d=1 Hz, J_q=6 Hz), 8.45 (1H, dd, J=2, 6 Hz), 8.63 (1H, d, J=2 Hz)
10l	1750 1660	153 (M^+)	1.43 (3H, d, J=6 Hz), 2.32 (3H, s), 2.95 (3H, s), 4.39 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.52 (1H, d, J=2 Hz)
10m	1740 1660	181 (M^+)	0.96 (3H, t, J=8 Hz), 1.45 (3H, d, J=6 Hz), 1.65 (2H, m), 2.33 (3H, s), 3.15 (1H, dt, J_d=14 Hz, J_t=8 Hz), 3.46 (1H, dt, J_d=14 Hz, J_t=8 Hz), 4.46 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.53 (1H, d, J=2 Hz)
10n	1740 1655	195 (M^+)	0.96 (3H, d, J=6 Hz), 0.98 (3H, d, J=6 Hz), 1.45 (3H, d, J=6 Hz), 1.94 (1H, m), 2.32 (3H, s), 2.96 (1H, dd, J=14, 6 Hz), 3.32 (1H, dd, J=14, 8 Hz), 4.46 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.55 (1H, d, J=2 Hz)
10o	1745 1665	221 (M^+)	1.05—2.1 (10H, m), 1.47 (3H, d, J=6 Hz), 2.31 (3H, s), 4.50 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.51 (1H, d, J=2 Hz)
10p	1745 1660	229 (M^+)	1.35 (3H, d, J=6 Hz), 2.31 (3H, s), 4.24 (1H, d, J=14 Hz), 4.35 (1H, dq, J_d=2 Hz, J_q=6 Hz), 4.79 (1H, d, J=14 Hz), 6.56 (1H, d, J=2 Hz), 7.2—7.5 (5H, m)
11l	1750 1655	153 (M^+)	1.39 (3H, d, J=7 Hz), 2.68 (3H, s), 2.96 (3H, s), 4.13 (1H, dq, J_d=1 Hz, J_q=7 Hz), 5.81 (1H, d, J=1 Hz)
11m	1740 1670	181 (M^+)	0.98 (3H, t, J=8 Hz), 1.40 (3H, d, J=6 Hz), 1.66 (2H, m), 2.68 (3H, s), 3.20 (1H, dt, J_d=14 Hz, J_t=8 Hz), 3.46 (1H, dt, J_d=14 Hz, J_t=8 Hz), 4.18 (1H, dq, J_d=1 Hz, J_q=6 Hz), 5.81 (1H, d, J=1 Hz)
11n	1740 1670	195 (M^+)	0.97 (3H, d, J=6 Hz), 1.00 (3H, d, J=6 Hz), 1.40 (3H, d, J=6 Hz), 1.96 (1H, m), 2.69 (3H, s), 3.00 (1H, dd, J=14, 6 Hz), 3.30 (1H, dd, J=14, 8 Hz), 4.19 (1H, dq, J_d=1 Hz, J_q=6 Hz), 5.81 (1H, d, J=1 Hz)
11o	1740 1675	221 (M^+)	1.1—2.1 (10H, m), 1.43 (3H, d, J=6 Hz), 2.38 (3H, s), 3.62 (1H, m), 4.24 (1H, dq, J_d=1 Hz, J_q=6 Hz), 5.81 (1H, d, J=1 Hz)
11p	1740 1670	229 (M^+)	1.29 (3H, d, J=6 Hz), 2.71 (3H, s), 4.08 (1H, dq, J_d=1 Hz, J_q=6 Hz), 4.28 (1H, d, J=14 Hz), 4.78 (1H, d, J=14 Hz), 5.81 (1H, d, J=1 Hz), 7.3—7.5 (5H, m)
10q	1740 1635	277 (M^+)	1.71 (3H, d, J=6 Hz), 5.13 (1H, dq, J_d=2 Hz, J_q=6 Hz), 7.08 (1H, m), 7.4—7.7 (7H, m), 7.50 (1H, d, J=2 Hz), 8.05 (2H, m)
10r	1740 1630	291 (M^+)	1.51 (3H, d, J=6 Hz), 2.38 (3H, s), 5.19 (1H, dq, J_d=2 Hz, J_q=2 Hz), 7.2—7.4 (4H, m), 7.5—7.7 (3H, m), 7.51 (1H, d, J=2 Hz), 8.05 (2H, m)
10s	1730 1630	291 (M^+)	1.70 (3H, d, J=6 Hz), 2.39 (3H, s), 5.12 (1H, dq, J_d=2 Hz, J_q=6 Hz), 7.00 (1H, m), 7.2—7.4 (3H, m), 7.49 (1H, d, J=2 Hz), 7.5—7.7 (3H, m), 8.06 (1H, m)
10t	1722 1630	307 (M^+)	1.68 (3H, d, J=2 Hz), 3.81 (3H, s), 5.06 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.94 (2H, m), 7.45 (1H, d, J=2 Hz), 7.46 (2H, m), 7.60 (3H, m), 8.04 (2H, m)
10u	1745 1635	295 (M^+)	1.65 (3H, dd, J=1, 6 Hz), 5.34 (1H, m), 7.1—7.25 (3H, m), 7.49 (1H, d, J=2 Hz), 7.5—7.7 (3H, m), 8.0—8.15 (3H, m)
10v	1730 1635	278 (M^+)	1.73 (3H, d, J=6 Hz), 5.20 (1H, dq, J_d=2 Hz, J_q=6 Hz), 7.36 (1H, dd, J=6, 8 Hz), 7.5—7.7 (3H, m), 7.98 (1H, m), 8.05 (2H, m), 8.45 (1H, dd, J=6, 2 Hz), 8.69 (1H, d, J=2 Hz)
10w	1730 1635	243 (M^+)	1.32 (3H, d, J=6 Hz), 1.37 (3H, d, J=6 Hz), 1.54 (3H, d, J=6 Hz), 4.02 (1H, m), 4.66 (1H, dq, J_d=2 Hz, J_q=6 Hz), 7.31 (1H, d, J=2 Hz), 7.5—7.7 (3H, m), 8.0 (2H, m)
10x	1735 1635	257 (M^+)	0.96 (3H, d, J=6 Hz), 1.00 (3H, d, J=6 Hz), 1.49 (3H, d, J=6 Hz), 2.00 (1H, m), 3.00 (1H, dd, J=14, 6 Hz), 3.37 (1H, dd, J=14, 8 Hz), 4.61 (1H, dq, J_d=2 Hz, J_q=2 Hz), 7.35 (1H, d, J=2 Hz), 7.5—7.7 (3H, m), 8.01 (2H, m)
10y	1745 1655	271 (M^+)	1.23 (9H, s), 1.46 (3H, d, J=6 Hz), 2.37 (3H, s), 5.07 (1H, dq, J_d=2 Hz, J_q=6 Hz), 7.01 (1H, d, J=2 Hz), 7.2—7.4 (4H, m)

doublet (dd), double quartet (dq), double triple (dt), multiplet (m). Mass spectra (MS) were taken on a Hitachi M-80A spectrometer. Microanalytical data were obtained by using a Carlo Elba 1106R or a Perkin-Elmer 240C elemental analyzer. For column chromatography, Wakogel 200 (Wako Pure Chemical) was used, and thin layer chromatography (TLC) was performed on silica gel pre-coated plates (Merck, Kieselgel 60F-254).

3-(2,2-Ethylenedioxypropionyl)-4-methyl-1-phenylazetidin-2-one (5a) A solution of 4-methyl-1-phenylazetidin-2-one (**1a**) (7.65 g, 52 mmol) in THF (52 ml) was added to a solution of LDA which was prepared from diisopropylamine (7.4 ml, 52 mmol) and *n*-BuLi (32.5 ml of 1.6 M *n*-hexane solution, 52 mmol) in THF (80 ml) at −78 °C over 30 min and stirred at the same temperature for 5 min. And then, a solution of ethyl 2,2-ethylenedioxypropionate (**2**) (7.6 g, 48 mmol) in THF (40 ml) was added dropwise to the reaction mixture over 1 h and stirred for an additional 1 h. The reaction mixture was poured into 5% HCl (60 ml) and extracted with $CHCl_3$. The organic layer was washed with water, aqueous $NaHCO_3$ and brine, successively, dried ($MgSO_4$) and evaporated *in vacuo* to give **5a** (8.9 g, yield, 68%), which was recrystallized from EtOH to give colorless needles, mp 74.5—76.5 °C. IR (KBr) *v*: 1750, 1715 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.54 (3H, s), 1.56 (3H, d, J=6 Hz), 3.9—4.2 (4H, m), 4.38 (1H, d, J=3 Hz), 4.50 (1H, m), 7.12 (1H, m), 7.38 (4H, m). MS *m/z*: 275 (M^+). *Anal.* Calcd for $C_{15}H_{17}NO_4$: C, 65.44; H, 6.22; N, 5.09. Found: C, 65.43; H, 6.22; N, 5.06.

3-(2,2-Ethylenedioxy-1-hydroxypropyl)-4-methyl-1-phenylazetidin-2-one (6a) A solution of **5a** (16.4 g, 59.6 mmol) in MeOH (150 ml) was added dropwise to a solution of $NaBH_4$ (4.06 g, 107 mmol) in MeOH (300 ml) at −78 °C over 1 h, and stirred for 1 h. Then acetic acid (18 ml) was added dropwise to the reaction mixture which was poured into water (500 ml), and extracted with $CHCl_3$. The extract was washed with aqueous $NaHCO_3$ and brine successively, dried ($MgSO_4$), and evaporated *in vacuo* to give **6a** (13.0 g, yield, 79%), which was recrystallized from EtOH to give colorless needles, mp 94.5—96 °C. IR (KBr) *v*: 3400, 1735, 1595 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.42 (3H, s), 1.52 (3H, d, J=6 Hz), 2.85 (1H, d, J=4 Hz), 3.07 (1H, dd, J=8, 3 Hz), 3.91 (1H, dd, J=8, 4 Hz), 4.01 (4H, br s), 4.12 (1H, dq, J_d=3 Hz, J_q=6 Hz), 7.08 (1H, m), 7.38 (4H, m). MS *m/z*: 277 (M^+). *Anal.* Calcd for $C_{15}H_{19}NO_4$: C, 64.97; H, 6.91; N, 5.05. Found: C, 64.93; H, 6.88; N, 4.98.

3-(2,2-Ethylenedioxy-1-methysulfonyloxypropyl)-4-methyl-1-phenylazetidin-2-one (7a) A solution of methanesulfonyl chloride (6.24 g, 56 mmol) in $CHCl_3$ (13 ml) was added dropwise to a mixture of **6a** (13.0 g, 46.9 mmol), triethylamine (11.4 g, 0.11 mol) and pyridine (130 ml) at 0 °C over 15 min and stirred for 1 h. Then the reaction mixture was poured into 10% HCl solution (700 ml) and extracted with $CHCl_3$. The organic layer was washed with water, aqueous $NaHCO_3$ solution and brine successively, dried over $MgSO_4$, and evaporated *in vacuo* to give **7a** (14.5 g, yield, 87%), which was recrystallized from EtOH to give a colorless amorphous solid, mp 135—139 °C. IR (KBr) *v*: 1745, 1595 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.54 (3H, s), 1.59 (3H, d, J=6 Hz), 2.21 (3H, s), 2.27 (1H, dd, J=8, 3 Hz), 4.08 (4H, m), 4.20 (1H, dq, J_d=6 Hz, J_q=3 Hz), 4.87 (1H, d, J=8 Hz), 7.12 (1H, m), 7.38 (4H, m). MS *m/z*: 355 (M^+). *Anal.* Calcd for $C_{16}H_{21}NO_6S$: C, 54.07; H, 5.96; N, 3.94. Found: C, 54.11; H, 5.01; N, 4.21.

(*E*)-3-(2,2-Ethylenedioxypropylidene)-4-methyl-1-phenylazetidin-2-one (8a) and (*Z*)-3-(2,2-Ethylenedioxypropylidene)-4-methyl-1-phenylazetidin-2-one (9a) A solution of DBU (26.3 g, 170 mmol) in benzene (140 ml) was added dropwise to a solution of **7a** (20.4 g, 57.4 mmol) in benzene (280 ml) at 5 °C over 30 min, and the reaction mixture was heated under reflux for 1 h. Then the reaction mixture was evaporated *in vacuo* and extracted with $CHCl_3$. The organic layer was washed with 5% HCl, water and brine successively, dried ($MgSO_4$), and evaporated *in vacuo*, and the residue was separated on silica gel column chromatography (CH_2Cl_2) to give **8a** (10.7 g, yield, 71.8%) and **9a** (2.1 g, 14%). **8a**: colorless prisms from EtOH, mp 96—101 °C. IR (KBr) *v*: 1735 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.55 (3H, s), 1.65 (3H, d, J=6 Hz), 3.85—4.05 (4H, m), 4.73 (1H, dq, J_q=2 Hz, J_q=6 Hz), 6.18 (1H, d, J=2 Hz), 7.10 (1H, m), 7.40 (4H, m). MS *m/z*: 259 (M^+). *Anal.* Calcd for $C_{15}H_{17}NO_3$: C, 69.48; H, 6.61; N, 5.40. Found: C, 69.32; H, 6.62; N, 5.61. **9a**: a colorless viscous oil. IR (neat) *v*: 1730 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.53 (3H, d, J=6 Hz), 1.62 (3H, s), 3.85—4.05 (4H, m), 4.48 (1H, dq, J_d=1 Hz, J_q=6 Hz), 5.75 (1H, d, J=1 Hz), 7.10 (1H, m), 7.40 (4H, m). MS *m/z*: 259 (M^+).

(*E*)-4-Methyl-3-(2-oxopropylidene)-1-phenylazetidin-2-one (10a) A mixture of **8a** (10.5 g, 40.5 mmol), *p*-TsOH·H_2O (1.09 g, 5.7 mmol) and acetone (500 ml) was heated under reflux for 3 h. Then the reaction mixture was evaporated *in vacuo*, and extracted with $CHCl_3$. The organic layer was washed with water, aqueous $NaHCO_3$ solution and brine successively, dried ($MgSO_4$), and evaporated *in vacuo* to give **10a** (8.5 g, yield, 97.9%), which was recrystallized from EtOH to give yellow prisms, mp 120—122 °C. *Anal.* Calcd for $C_{13}H_{13}NO_3$: C, 72.54; H, 6.09; N, 6.51. Found: C, 72.55; H, 6.09; N, 6.53. IR (KBr) *v*: 1745, 1650, 1590 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.63 (3H, d, J=6 Hz), 2.37 (3H, s), 4.98 (1H, dq, J_q=2 Hz, J_q=6 Hz), 6.67 (1H, d, J=2 Hz), 7.15 (1H, m), 7.42 (4H, m). MS *m/z*: 215 (M^+).

(*Z*)-4-Methyl-3-(2-oxopropylidene)-1-phenylazetidin-2-one (11a) Prepared from **9a** in the same manner as described above. Colorless needles from ether, mp 114—115.5 °C. *Anal.* Calcd for $C_{13}H_{13}NO_3$: C, 72.54; H, 6.09; N, 6.51. Found: C, 72.64; H, 5.98; N, 6.48. IR (KBr) *v*: 1740, 1660, 1590 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.61 (3H, d, J=6 Hz), 2.74 (3H, s), 4.68 (1H, dq, J_d=1 Hz, J_q=6 Hz), 5.98 (1H, d, J=1 Hz), 7.20 (1H, m), 7.45 (4H, m). MS *m/z*: 215 (M^+).

The spectral data of **10b**—**y** and **11b**—**p** were shown in Table II.

(*E*)-4-Methyl-3-(2-oxopropylidene)azetidin-2-one (12) A solution of CAN (15.4 g, 28.1 mmol) in 50% aqueous MeCN (80 ml) was added dropwise to a solution of **10e** (2.0 g, 8.16 mmol) in MeCN (40 ml) at 0 °C, and stirred for 2 h at the same temperature. Then the reaction mixture was extracted with AcOEt and the extract was washed with aqueous $NaHCO_3$ solution, saturated Na_2SO_3 solution and brine successively, dried ($MgSO_4$), and evaporated *in vacuo*, and the residue was purified on silica gel column chromatography to give **12** (0.24 g, yield, 29%), which was recrystallized from ether to give yellow plates, mp 117.5—120.5 °C. *Anal.* Calcd for $C_7H_9NO_2$: C, 60.41; H, 6.53; N, 10.07. Found: C, 60.34; H, 6.52; N, 10.14. IR (KBr) *v*: 3160, 1720, 1655 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.49 (3H, d, J=6 Hz), 2.34 (3H, s), 4.57 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.59 (1H, d, J=2 Hz), 6.72 (1H, br d). MS *m/z*: 139 (M^+).

(*E*)-Tetrabutylammonium 4-Methyl-3-(2-oxopropylidene)-1-sulfoazetidin-2-one (13) A mixture of **12** (101 mg, 0.73 mmol), pyridine–sulfur trioxide (247 mg, 1.55 mmol) and DMF (1 ml) was stirred at room temperature for 2 d. The reaction mixture was diluted with CH_2Cl_2 and extracted with 0.5 N KH_2PO_4 solution, then *n*-Bu_4NHSO_4 (247 mg, 0.73 mM) was added to the aqueous layer which was extracted twice with CH_2Cl_2, and the organic layer was dried ($MgSO_4$), evaporated *in vacuo* and the residue was purified on silica gel column chromatography (AcOEt : MeOH = 8 : 1) to give **13** (140 mg, yield, 47%), which was recrystallized from EtOH to give a colorless amorphous solid, mp 101.5—104.5 °C. *Anal.* Calcd for $C_{23}H_{44}N_2O_5S$: C, 59.95; H, 9.65; N, 6.08. Found: C, 60.28; H, 9.68; N, 5.98. IR (KBr) *v*: 1750, 1660 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.99 (12H, t, J=7 Hz), 1.94 (8H, m), 1.65 (8H, m), 1.65 (3H, d, J=6 Hz), 2.30 (3H, s), 3.28 (8H, m), 4.81 (1H, dq, J_d=2 Hz, J_q=6 Hz), 6.55 (1H, d, J=2 Hz). MS (SIMS) *m/z*: 461 (M+H).

Preparation of PRP Blood was taken from the carotid artery of male New Zealand white rabbit into a plastic syringe containing a 10% volume of 3.2% sodium citrate dihydrate solution under ether anesthesia. The citrated blood was centrifuged at 150 g for 15 min at room temperature to obtain PRP. The sediment was further centrifuged at 1500 *g* for 10 min to obtain platelet-poor plasma (PPP). The platelet count was adjusted to approximately 5×10^5—6×10^5/μl by adding PPP.

Platelet Aggregation Test *in Vitro* Platelet aggregation was measured by a Aggrecoder PA-3210 (Kyoto Daiichi Kagaku) at 37 °C under stirring at 1000 rpm. The agents used were ADP (Sigma Chemical Co., final concentration; 5 μM), and collagen (Kyoto Daiichi Kagaku Co., Ltd., final concentration; 5 μg/ml). The compound to be tested was dissolved in DMSO and diluted by saline, which was added in the volume of 25 to 275 μl of PRP 3 min before the addition of the aggregating agents. Platelet aggregation was measured for 5 min and the IC_{50} value was calculated by the maximum decrease in absorbancy of PRP from comparison of the vehicle treated PRP.

References and Notes

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Studies on the Synthesis of Compounds Related to Adenosine 3′,5′-Cyclic Phosphate. IX.[1] Synthesis and Cytotoxic Effect of Adenosine 3′,5′-Cyclic Alkylphosphoramidates

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A series of adenosine 3′,5′-cyclic monophosphoramidates (3, cAMP amidates), including long-chain alkyl amidates, were synthesized from adenosine 3′,5′-cyclic monophosphate (1, cAMP) by means of a one-pot reaction. This reaction proceeded by the treatment of cAMP tributylammonium salt (2) with phosphorus pentachloride (PCl_5) and alkylamine in *N,N*-dimethylformamide (DMF). Compounds 3 synthesized were investigated to determine their cytotoxic activities on the growth of mouse mastocytoma P-815 cells, mouse mammary tumor FM3A cells, and human mammary tumor ZR-75 cells in culture. It was found that compounds 3h—m showed significant cytotoxic activities against these cell lines, and that cAMP decylamidate (3j) was the most cytotoxic compound (the concentration required for 50% inhibition of cell growth, ID_{50} = 6.0, 15.0, 2.2 μM, respectively); the antitumor effect on P-815 cells by a total packed cell volume method showed 81.8% inhibition. The cytotoxic activity of 3 increased with the increase in alkyl chain length up to 10 carbon atoms and decreased in compounds having longer alkyl chain.

Keywords cAMP phosphoramidate; cytotoxicity; phosphorus pentachloride; alkylamine; one-pot reaction; mastocytoma P-815 cell

Introduction

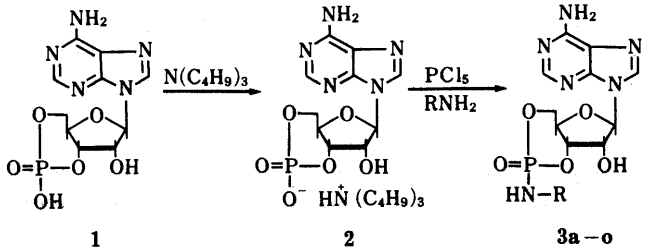
Adenosine 3′,5′-cyclic monophosphate (1, cAMP) is an important intracellular "second messenger" mediating various hormonal actions. In addition to its well-known mediating activity, 1 is assumed to have a role in cell proliferation.[2,3] The cellular levels of 1 vary as the cell division cycle proceeds, and it generally accumulates at the G_1 or G_2 phases of growing cells, which result in the inhibition of deoxyribonucleic acid (DNA) synthesis[4,5] or cell division,[6] respectively. Furthermore, N^6,2′-*O*-dibutyryl cAMP[2,7] has been shown to have a weak cytostatic activity against the growth of several tumor cell lines in culture. On the basis of these observations, various derivatives of 1 have been synthesized and examined for cytotoxic activity against the growth of cultured cancerous cells, and many effective compounds such as 8-amino cAMP and N^6-benzyl cAMP were reported.[2,8] We previously synthesized many cAMP derivatives[9-11] to examine their biological activities[10,11] and found that cAMP alkyltriester strongly inhibited the growth of mouse mastocytoma P-815 cells and mammary FM3A cells.[11]

Basically, the chemical modification of 1 is focused on the improvement of its poor cellular permeability, which is mostly derived from a single negative charge on the phosphate moiety of 1. Along with triesterification,[9d,12] the introduction of alkylamines to the phosphate is another appropriate way to increase the cell permeability of 1. A few cAMP amidates such as cAMP dimethylamidate and cAMP methylamidate have already been synthesized[13-15] but nothing is known about their cytotoxic effect on the growth of tumor cells. We found that long-alkyl chain derivatives of cAMP alkyltriester were more potent in cell cytotoxicity than the short-alkyl chain derivatives.[11] Such circumstances prompted us to investigate the effect of a series of cAMP amidates with long-alkyl chain on cell growth. In this paper, we report the synthesis of a series of cAMP amidates using a newly developed one-pot reaction from 1, and the examination of their cytotoxic activities against the growth of cultured mastocytoma P-815 cells.

Results and Discussion

Chemistry The synthetic route for the preparation of cAMP amidates (3) is shown in Chart 1. Treatment of the tri-*n*-butylammonium salt (2) of 1 with phosphorus pentachloride and alkylamine in absolute *N,N*-dimethylformamide (DMF) gave 3. Physical properties of 3 are summarized in Table I. The *Rf* values of 3 by paper chromatography and those by thin-layer chromatography (TLC) became larger according to the increase of alkyl chain length. All these compounds showed the maximal ultraviolet (UV) absorption at 259 nm in EtOH and the similar infrared (IR) spectral pattern, except for the intensity of the absorption by methylene in the vicinity of 2950 cm^{-1}. Though the yields of compound 3 were in the range from 18 to 48% (Table I), the formation of the by-product was almost negligible on TLC ($CHCl_3$: MeOH = 75 : 25) and the starting material was recovered.

We noticed in our preliminary experiment that the long-chain alkyl-substituted derivatives (3b—o) were hard to synthesize by other methods[13] as described in various published papers which indicated the need for the protection for 2′-hydroxyl and 6-amino groups. The present method is advantageous in its wide applicability for large-scale preparation and simplicity of operation in the



1 2 3a—o

a : R=CH_3 f : R=*n*-C_6H_{13} k : R=*n*-$C_{11}H_{23}$
b : R=C_2H_5 g : R=*n*-C_7H_{15} l : R=*n*-$C_{12}H_{25}$
c : R=*n*-C_3H_7 h : R=*n*-C_8H_{17} m : R=*n*-$C_{14}H_{29}$
d : R=*n*-C_4H_9 i : R=*n*-C_9H_{19} n : R=*n*-$C_{16}H_{33}$
e : R=*n*-C_5H_{11} j : R=*n*-$C_{10}H_{21}$ o : R=*n*-$C_{18}H_{37}$

Chart 1

TABLE I. Physical Constants of cAMP Amidates

Compd. No.	R	Yield (%)	Rf_1[a]	Rf_2[b]	Formula[c]	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N
3a	Methyl	26.2	0.44	0.51	$C_{11}H_{15}N_6O_5P \cdot 3/2H_2O$	35.78	4.91	22.76	35.68	4.75	22.62
3b	Ethyl	48.3	0.53	0.57	$C_{12}H_{17}N_6O_5P \cdot 2/3H_2O$	39.14	5.02	22.82	38.91	4.72	22.64
3c	Propyl	41.3	0.58	0.66	$C_{13}H_{19}N_6O_5P \cdot 7/9H_2O$	40.63	5.39	21.87	40.40	5.11	21.54
3d	Butyl	28.8	0.63	0.68	$C_{14}H_{21}N_6O_5P \cdot 1/4H_2O$	43.25	5.57	21.61	43.20	5.64	21.37
3e	Pentyl	24.9	0.66	0.70	$C_{15}H_{23}N_6O_5P \cdot 1/2H_2O$	44.23	5.94	20.63	44.44	5.82	20.30
3f	Hexyl	26.2	0.69	0.71	$C_{16}H_{25}N_6O_5P \cdot 1/4H_2O$	46.10	6.17	20.16	46.34	6.29	19.88
3g	Heptyl	25.4	0.73	0.74	$C_{17}H_{27}N_6O_5P \cdot 1/2H_2O$	46.89	6.48	19.30	46.73	6.40	19.03
3h	Octyl	27.4	0.75	0.75	$C_{18}H_{29}N_6O_5P \cdot 3/4H_2O$	47.63	6.77	18.51	47.90	6.61	18.22
3i	Nonyl	29.7	0.77	0.76	$C_{19}H_{31}N_6O_5P \cdot H_2O$	48.30	7.04	17.79	48.43	6.75	17.83
3j	Decyl	32.0	0.78	0.77	$C_{20}H_{33}N_6O_5P \cdot 1/2H_2O$	50.31	7.18	17.60	50.58	7.26	17.25
3k	Undecyl	24.6	0.78	0.80	$C_{21}H_{35}N_6O_5P \cdot 1/3H_2O$	51.63	7.36	17.20	51.79	7.20	17.24
3l	Dodecyl	29.5	0.78	0.84	$C_{22}H_{37}N_6O_5P \cdot 1/5H_2O$	52.83	7.54	16.80	53.02	7.58	16.93
3m	Tetradecyl	36.2	0.78	0.87	$C_{24}H_{41}N_6O_5P \cdot 1/4H_2O$	54.48	7.91	15.88	54.68	7.88	15.68
3n	Cetyl	33.3	0.78	0.87	$C_{26}H_{45}N_6O_5P \cdot 1/4H_2O$	56.05	8.23	15.08	56.20	8.36	14.73
3o	Stearyl	18.5	0.77	0.87	$C_{28}H_{47}N_6O_5P \cdot 3/4H_2O$	56.60	8.57	14.14	56.43	8.36	14.13

a) *Rf* by paper chromatography(0.5 M ammonium acetate/EtOH = 2/5). *b*) *Rf* by TLC(25% methanol in chloroform). *c*) Samples were dried over P_2O_5 at 50 °C at 3 mmHg for 3—5 h.

TABLE II. Effect of cAMP Amidates on the Growth of Mastocytoma P-815 Cells

Compd. No.	R	Cytotoxic activity (%)[a] P815[b] 1 μM	P815[b] 10 μM	P815[b] 20 μM	FM3A[c] 20 μM	ZR-75[d] 20 μM
1	—	0	0	0	0	0
3a	Methyl	1.7	0			
3b	Ethyl	1.7	0.8	5.2	3.0	18.6
3c	Propyl	0	0			
3d	Butyl	0	0	22.4	2.2	35.6
3e	Pentyl	0	0			
3f	Hexyl	0	0			
3g	Heptyl	0	9.3	45.2	4.8	62.0
3h	Octyl	0	43.2			
3i	Nonyl	4.7	50.9			
3j	Decyl	7.6	54.2	90.0	45.0	85.2
3k	Undecyl	0	34.0			
3l	Dodecyl	0	33.0	42.3	36.5	62.4
3m	Tetradecyl		22.0		26.4	43.4
3n	Cetyl		17.9			
3o	Stearyl		1.4	15.9	31.2	27.5

a) Percent inhibition of the growth of tumor cells treated with compounds to untreated controls. Values are the mean of duplicate assays. *b*) Mastocytoma P-815 cell. *c*) Mouse mammary tumor FM3A cells. *d*) Human mammary tumor ZR-75 cells.

synthesis of a series of cAMP amidates, especially those with long-alkyl chain. Similar reaction was recently reported by Bottka and Tomasz,[15] who synthesized only methyl and dimethyl cAMP amidate with $POCl_3$ and alkylamines in trimethyl phosphate.

Biological Studies The compounds (**3a—o**) listed in Table I were tested for growth inhibition against mouse mastocytoma P-815 cells, mouse mammary tumor FM3A cells, and human mammary tumor ZR-75 cells in culture. As shown in Table II, **3j** was the most active compound in inhibiting cell proliferation of these cell lines. ID_{50} values (the concentration required for 50% inhibition of cell growth) of **3j** were 6, 15.0, and 2.2 μM, respectively. The ID_{50} of 6-mercaptopurine (6-MP) and 5-fluorouracil (5-FU) against FM3A cells was 13 and 1.9 μM in our experiment. Comparing these results, the cytotoxicity of compound **3j** was similar to 6-MP. Then, the compound (**3j**) was tested for antitumor effect on P-815 cells *in vivo* by the total packed cell volume method and showed potent antitumor activity of 81.8% growth inhibition on day 6. Body weight of the animal did not decrease during this experiment. In a preliminary experiment, compound **3j** did not show toxicity after a single i.p. administration of 1 g/kg into ICR female mice.

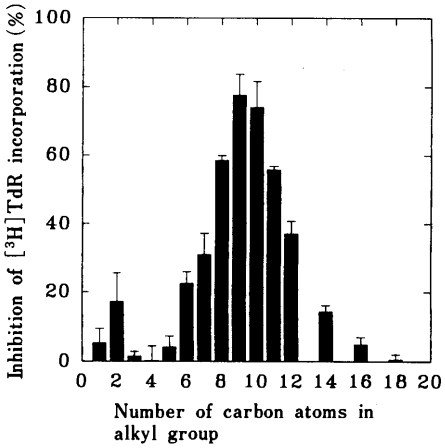


Fig. 1. Inhibition of [^{3}H]TdR Incorporation by **3** in Mastocytoma P-815 Cells

Details for this experiment are described in Experimental. The final concentration of **3** was 20 μM. The percent inhibition of the [^{3}H]TdR incorporation into DNA was the ratio of the radioactivity in a treated group to that in the control (924800 ± 33640 dpm).

Effects of **3** on the biosynthesis of DNA in P-815 cells were determined by measuring the incorporation of radioactivity into an acid-insoluble fraction. Among various cAMP amidates tested at 20 μM, the derivatives **3i** and **3j** showed the most potent inhibition of [^{3}H]thymidine (TdR) incorporation into macromolecules (Fig. 1). It was found that the cytotoxic activity of a series of cAMP amidates increased with the increasing length of the side chain from 6 to 10 carbon atoms and decreased with

compounds having longer alkyl residues. This inhibition related to the number of carbon atoms was comparable to the growth inhibition of P-815 cells.

Further studies are needed to clarify the detailed mechanism of the inhibition of growth of mastocytoma P-815 cells by **3j**.

Experimental

UV absorption spectra were recorded with a Hitachi 557 spectrophotometer. IR spectra were taken on a JASCO A-202 spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken at 200 MHz on a JEOL JNM-FX200 NMR spectrometer in dimethylsulfoxide (DMSO)-d_6. All ^{1}H-NMR data are reported in ppm downfield from tetramethylsilane as an internal standard. All melting points were determined on a micro-hot stage (Yanagimoto) and are uncorrected. Paper chromatograms were run on Toyo No. 51A filter paper (40 × 40 cm) with the developing solvent system of 0.5 M ammonium acetate–EtOH (2/5, v/v). Analytical TLC were performed on Kiesel gel 60F_{254} (Merck) plates. Column chromatography was carried out on YMC octadecyl silica (ODS) AQ-120 gel (YMC Co., Japan). Mass spectra (MS) were measured by the method of Lawson *et al.*[16] on a Hitachi RMU-7M spectrometer.

General Procedure for Preparation of cAMP Amidates[17] A solution of the tributylammonium salt (**2**)[18] of **1** (2 g, 3.89 mmol) in 50 ml of absolute DMF was cooled at 0 °C, and phosphorus pentachloride (1 g, 1.2 mol eq) was added to the solution with stirring. After 20 min, alkylamine·HCl or alkylamine (10 mol eq) and 10 ml of triethylamine were added to the solution and stirred for 20 min. Then, the mixture was evaporated *in vacuo*.

Method of Purification The residue was dissolved in an appropriate amount of water. The solution was directly poured onto an ODS column (3.5 × 30 cm). After washing with 500 ml of water, compound **3** was eluted from the column with a solution of acetonitrile (stepwise increase from 0 to 80%) in water. Each fraction was checked on TLC ($CHCl_3$: MeOH = 75 : 25) and *Rf* values are shown in Table I. Fractions containing **3** were collected and evaporated to dryness *in vacuo*. The residue was recrystallized from MeOH or EtOH–acetone to give a product.

3a: A white powder, mp 151—155 °C (dec.) IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 2.45 (3H, d, CH_3), 5.10—5.35 (1H, m, P–NH), 6.01 (1H, s, H-1′), 7.25 (2H, s, NH_2), 8.18, 8.35 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (13400). MS *m/z*: 558 $(M(TMS)_3)^+$.

3b: A white powder, mp 140—147 °C. IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 1.06 (3H, t, CH_3), 2.81—2.94 (2H, m, P–N–CH_2), 5.35—5.44 (1H, m, P–NH), 6.01 (1H, s, H-1′), 7.25 (2H, s, NH_2), 8.17, 8.35 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (14400). MS *m/z*: 572 $(M(TMS)_3)^+$.

3c: A white powder, mp 144—147 °C. IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 0.80 (3H, t, CH_3), 1.22—1.58 (2H, m, CH_2), 2.60—2.85 (2H, m, P–N–CH_2), 5.30—5.64 (1H, m, P–NH), 6.06 (1H, s, H-1′), 7.32 (2H, s, NH_2), 8.20, 8.35 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (15900). MS *m/z*: 586 $(M(TMS)_3)^+$.

3d: A white powder, mp 134—138 °C. IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 0.84 (3H, t, CH_3), 1.16—1.60 (4H, m, $(CH_2)_2$), 2.73—2.85 (2H, m, P–N–CH_2), 5.24—5.43 (1H, m, P–NH), 6.01 (1H, s, H-1′), 7.24 (2H, s, NH_2), 8.17, 8.35 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (13900). MS *m/z*: 600 $(M(TMS)_3)^+$.

3e: A white powder, mp 132—135 °C. IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 0.87 (3H, t, CH_3), 1.15—1.55 (6H, m, $(CH_2)_3$), 2.72—2.90 (2H, m, P–N–CH_2), 5.28—5.44 (1H, m, P–NH), 6.00 (1H, s, H-1′), 7.25 (2H, s, NH_2), 8.18, 8.35 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (14500). MS *m/z*: 614 $(M(TMS)_3)^+$.

3f: A white powder, mp 119—124 °C. IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 0.87 (3H, t, CH_3), 1.16—1.57 (8H, m, $(CH_2)_4$), 2.72—2.85 (2H, m, P–N–CH_2), 5.26—5.44 (1H, m, P–NH), 6.00 (1H, s, H-1′), 7.25 (2H, s, NH_2), 8.18, 8.34 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (14200). MS *m/z*: 628 $(M(TMS)_3)^+$.

3g: A white powder, mp 168—173 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 0.87 (3H, t, CH_3), 1.25—1.56 (10H, m, $(CH_2)_5$), 2.76—2.89 (2H, m, P–N–CH_2), 5.28—5.42 (1H, m, P–NH), 6.01 (1H, s, H-1′), 7.25 (2H, s, NH_2), 8.18, 8.35 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (14100). MS *m/z*: 642 $(M(TMS)_3)^+$.

3h: A white powder, mp 160—162 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 0.85 (3H, t, CH_3), 1.15—1.60 (12H, m, $(CH_2)_6$), 2.70—2.90 (2H, m, P–N–CH_2), 5.24—5.43 (1H, m, P–NH), 6.01 (1H, s, H-1′), 7.24 (2H, s, NH_2), 8.17, 8.34 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (14500). MS *m/z*: 656 $(M(TMS)_3)^+$.

3i: A white powder, mp 143—149 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 0.83 (3H, t, CH_3), 1.20—1.60 (14H, m, $(CH_2)_7$), 2.70—2.90 (2H, m, P–N–CH_2), 5.24—5.43 (1H, m, P–NH), 6.01 (1H, s, H-1′), 7.24 (2H, s, NH_2), 8.18, 8.34 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (14500). MS *m/z*: 670 $(M(TMS)_3)^+$.

3j: A white powder, mp 160—165 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 0.80 (3H, t, CH_3), 1.20—1.58 (16H, m, $(CH_2)_8$), 2.60—2.85 (2H, m, P–N–CH_2), 5.30—5.64 (1H, m, P–NH), 6.00 (1H, s, H-1′), 7.32 (2H, s, NH_2), 8.22, 8.35 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (14200). MS *m/z*: 684 $(M(TMS)_3)^+$.

3k: A white powder, mp 162—165 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 0.83 (3H, t, CH_3), 1.15—1.55 (18H, m, $(CH_2)_9$), 2.73—2.88 (2H, m, P–N–CH_2), 5.26—5.44 (1H, m, P–NH), 6.01 (1H, s, H-1′), 7.24 (2H, s, NH_2), 8.17, 8.35 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (14800). MS *m/z*: 698 $(M(TMS)_3)^+$.

3l: A white powder, mp 162—167 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 0.84 (3H, t, CH_3), 1.16—1.60 (20H, m, $(CH_2)_{10}$), 2.70—2.90 (2H, m, P–N–CH_2), 5.24—5.43 (1H, m, P–NH), 6.00 (1H, s, H-1′), 7.25 (2H, s, NH_2), 8.17, 8.35 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (13600). MS *m/z*: 712 $(M(TMS)_3)^+$.

3m: A white powder, mp 157—162 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 0.85 (3H, t, CH_3), 1.15—1.60 (24H, m, $(CH_2)_{12}$), 2.70—2.90 (2H, m, P–N–CH_2), 5.26—5.42 (1H, m, P–NH), 6.00 (1H, s, H-1′), 7.24 (2H, s, NH_2), 8.17, 8.34 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (14900). MS *m/z*: 740 $(M(TMS)_3)^+$.

3n: A white powder, mp 161—164 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 0.85 (3H, t, CH_3), 1.15—1.60 (28H, m, $(CH_2)_{14}$), 2.72—2.88 (2H, m, P–N–CH_2), 5.26—5.44 (1H, m, P–NH), 6.01 (1H, s, H-1′), 7.24 (2H, s, NH_2), 8.17, 8.34 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (13900). MS *m/z*: 768 $(M(TMS)_3)^+$.

3o: A white powder, mp 176—179 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3120, 2950, 1660. ^{1}H-NMR δ: 0.87 (3H, t, CH_3), 1.16—1.58 (32H, m, $(CH_2)_{16}$), 2.73—2.90 (2H, m, P–N–CH_2), 5.24—5.44 (1H, m, P–NH), 6.00 (1H, s, H-1′), 7.25 (2H, s, NH_2), 8.17, 8.35 (1H each, s, purine H's). UV λ_{max}^{EtOH} nm (ε): 259 (14700). MS *m/z*: 796 $(M(TMS)_3)^+$.

Measurement of Cytotoxicity Mastocytoma P-815 cells were grown as suspension culture in Fischer's medium supplemented with 10% fetal bovine serum (FBS). Mouse mammary tumor FM3A cells were maintained as suspension culture in RPMI 1640 medium supplemented with 10% FBS and human mammary tumor ZR-75 cells were maintained as monolayer culture in the same medium. All tumor cell lines were cultured in a 5% carbon dioxide humidified incubator at 37 °C. For determination of cell growth inhibition, cells (2×10^5) were seeded in a Falcon 6 well plate (3 ml/well). Cells were grown in the presence of compounds (**3**) at concentration of 1, 10 or 20 μM. After incubation for 48 h at 37 °C, number of P-815 cells and FM3A cells was determined using a Coulter cell counter. ZR-75 cells which were cultured for 7 d at 37 °C were counted using a Coulter counter after treatment with 0.25% trypsin.

Stock solutions of the drugs were made in DMSO and added to duplicate wells of the above cell lines. DMSO without drugs added to duplicate wells served as the control.

Antitumor Effect of cAMP Decyl Amidate (3j) on P-815 Cells Male BDF_1 mice of 20—30 g were used to test the antitumor activity of **3j** against mastocytoma P-815 cells. Groups of six mice were inoculated with P-815 cells (2×10^6) intraperitoneally. From the following day, **3j** at a dose of 0.2 mg/mouse was administered intraperitoneally once daily for 5 d; the animals were then killed by exsanguination under deep Nembutal anesthesia and the packed volume of P-815 cells in ascitic fluid was measured to determine antitumor activity.

Measurement of [^{3}H]TdR Incorporation into Macromolecules After the preincubation of P-815 cells (2×10^6) in phosphate-buffered saline (PBS) solution for 10 min with test compound (**3**), the cells were incubated for 1 h at 37 °C with radiolabeled precursor (0.2 μCi/ml). They were then collected on Whatman GF/C filter paper and washed three times with 3 ml each of ice-cold PBS and 10% $HClO_4$. The radioactivity was counted using a toluene based scintillation solution.

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New Fluorine-Substituted Analogue of Eticlopride with High Affinity toward Dopamine D2 Receptors

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Aiming at the development of positron-emitting ligands with specific and high affinity toward dopamine D2 receptors in the central nervous system, we synthesized a new fluorinated eticlopride derivative. A fluorine atom was introduced at the C-4 position of the pyrrolidine ring of eticlopride, a dopamine D2 antagonist of the benzamide series. The *in vitro* binding affinity of this ligand toward the D2 receptor was found to be as potent as eticlopride, suggesting that the corresponding ^{18}F-labelled compound may be useful as an *in vivo* radioligand for positron emission tomography.

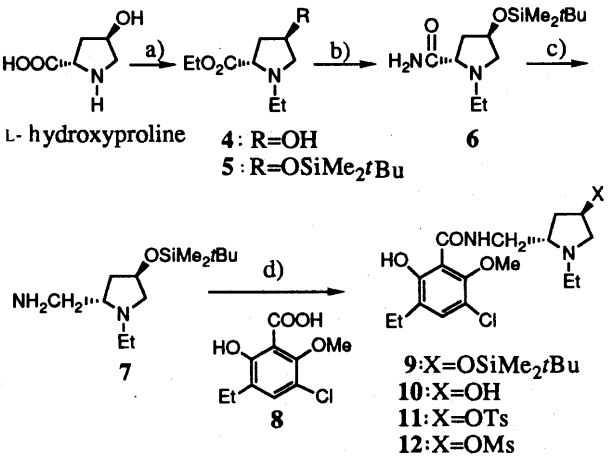
Keywords fluoroeticlopride; antagonist; dopamine D2 receptor; *in vitro* binding affinity

Much effort has been pursued to develop positron-emitting ligands for *in vivo* imaging of the D2 dopamine receptors in the central nervous system (CNS), expecting such radioligands to be clinically useful for monitoring the function of the dopaminergic system in the living human brain by positron emission tomography (PET).[1] Recently, benzamide neuroleptics, *i.e.* D2 antagonists have been chosen as parent compounds in designing a radioligand,[2] since they have been known to show specific, high affinity and reversible binding to D2 dopamine receptors.[3] In addition, among the positron-emitting nuclides, fluorine-18 with a relatively long half-life (110 min) has been accepted as a favorable nuclide for PET studies of slow ligand-receptor binding.[4] However, previous studies on fluorinated ligands by us and other groups have pointed out some problems, for example, eticlopride (**1**)[5] or raclopride (**2**)[6] derivatives with *N*-fluoroalkylated pyrrolidine showed only a low affinity towards D2 receptors. In the present study we wish to report that a new fluorinated eticlopride (**3**), in which a fluorine atom is introduced at the C-4 position of the pyrrolidine skeleton, has retained a high affinity toward D2 receptors as evidenced by *in vitro* binding experimentation.

The synthetic route for the new eticlopride analogue having the fluorinated pyrrolidine ring is summarized in Charts 1 and 2. The substituted pyrrolidine moiety was prepared from commercially available (*S*)-hydroxyproline (Chart 1). Esterification of (*S*)-hydroxyproline with ethanol–HCl, and subsequent *N*-ethylation using ethyl iodide in dioxane–water in the presence of triethylamine gave *N*-ethyl-4-hydroxy-(*S*)-proline ethyl ester **4** in 53% yield. The hydroxy group was protected by *tert*-butyldimethylsilyl group to give **5** (99% yield), then the ethoxycarbonyl group was transformed to the carboxamide (**6**) by the reaction with NH_3 in anhydrous methanol in the presence of a catalytic amount of KI, in 92% overall yield. The reduction of **6** with $LiAlH_4$ in ether afforded the diamine **7** in 82% yield.

Coupling between the amine **7** and the carboxylic acid **8** was performed using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (WSC) in the presence of 1-hydroxybenzotriazole as a catalyst to give the desired eticlopride derivative **9** in 84% yield. Deprotection of **9** with *n*-Bu_4NF in tetrahydrofuran (THF) (94% yield), and following sulfonylation of the resulting alcohol **10** with *p*-toluenesulfonyl chloride or methanesulfonyl chloride yielded the sulfonate precursors **11** (29% yield) or **12** (84% yield).

The fluoroeticlopride (**3**) was synthesized by three



(a) i) HCl–EtOH; ii) EtI, Et_3N, in dioxane–H_2O; iii) *tert*-butyldimethylchlorosilane (TBDMSCl), imidazole, in dimethylformamide (DMF). (b) NH_3–MeOH, KI. (c) $LiAlH_4$ in ether. (d) **9**; **8**, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, 1-hydroxybenzotriazole, in CH_2Cl_2, **10**: *n*-Bu_4NF in THF; **11**: TsCl in pyridine; **12**: MsCl, Et_3N in CH_2Cl_2. *t*Bu = *tert*-butyl.

Chart 1. Synthesis of (2*S*,4*R*)-(−)-2-Aminomethyl-1-ethyl-4-*tert*-butyldimethylsilyloxypyrrolidine Unit and the Intermediates for Fluorination

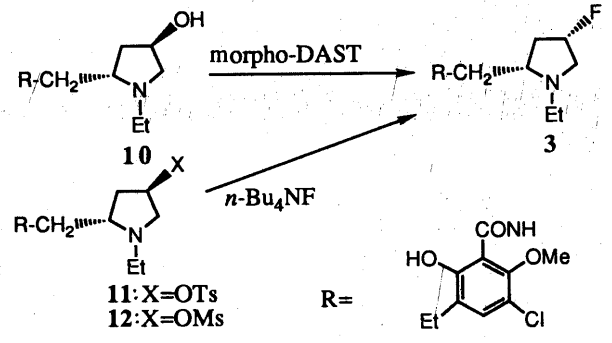
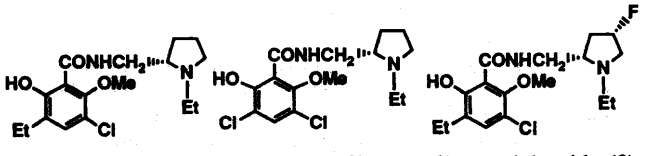


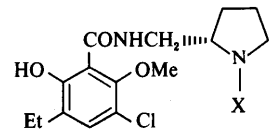
Chart 2. Synthesis of Fluoroeticlopride



eticlopride (**1**) raclopride (**2**) fluoroeticlopride (**3**)

TABLE I. Inhibition of [^{3}H]Spiperone Binding in Striatal Homogenates of Rat Brain[a]

Run	Compound	[^{3}H]Spiperone binding IC_{50} (nM)
1	Eticlopride	2.9
2	**9**	35
3	**10**	4.5
4	**3**	1.9
5[b]	**13**	23
6[b]	**14**	17
7[b]	**15**	4800



	X
13	CH_2CH_2F
14	$CH_2CH_2CH_2F$
15	$CH_2C_6H_4$-F(*p*)

a) Homogenates of the rat striatal tissue were incubated with 0.2 nM [^{3}H]spiperone and various concentrations of the compounds. Nonspecific binding was determined in the presence of 10^{-4} M sulpiride. IC_{50} represents the concentration of the compound required to inhibit 50% of specifically bound [^{3}H]spiperone. Values represent means of three determinations. *b*) Data taken from the literature in which homogenates of the bovine striatal tissue *in vitro* were used.[5]

approaches as shown in Chart 2. Fluorination of **11** by treatment with *n*-Bu_4NF in refluxing THF (25 min) gave **3** in 47% yield, whereas a longer reaction time (6.5 h) was needed in the case of the reaction using the mesylate **12** as a substrate (18% yield). The fluorination reaction could also be achieved by using the alcohol **10** and morpholinosulfur trifluoride (morpho DAST)[7] in 16% yield. The fluorinated compounds obtained by the above three approaches were identical in their spectral data, indicating that all the fluorinations proceeded in a manner of SN_2 inversion, and that the configuration of the fluoroeticlopride (**3**) should be as shown in Chart 2.

In order to evaluate the binding affinity of new eticlopride derivatives toward D2 receptors, IC_{50} values against [^{3}H]-spiperone binding in rat striatal tissue preparation were measured.[8] Table I summarizes the results, together with the reported data for comparison. It should be noted that the affinity of the fluoroeticlopride to the D2 receptor is as high as the parent eticlopride (run 1 *vs.* 4). It was also found that the hydroxy group had almost no effect in the binding affinity (run 3), whereas the bulky protecting group caused a slight decrease in the affinity (run 2).

Recent studies on the structure–affinity relationship of benzamide antagonists toward D2 receptors have suggested that the hydrogen bonding between the protonated pyrrolidine and the complementary receptor site plays an important role in the ligand affinity.[9] It is known that substitution by fluorine atoms of the methylene hydrogens in the β or γ-position of aliphatic amines causes a marked decrease in the amine basicity.[10] The fact that the benzamide ligands with the *N*-fluoroalkyl-substituted pyrrolidine showed only diminished affinities toward the D2 receptor (run 5 and 6) was explained partly on the basis of a large electronegativity of the fluorine atom.[6a] That is, the electron-withdrawing effect of the fluorine introduced may decrease protonation of the pyrrolidine nitrogen, and therefore, the hydrogen bonding with the receptor may be weakened. Interestingly, despite the fluorine atom introduced at the β position to the pyrrolidine nitrogen, the fluoroeticlopride **3** has retained a high affinity toward the

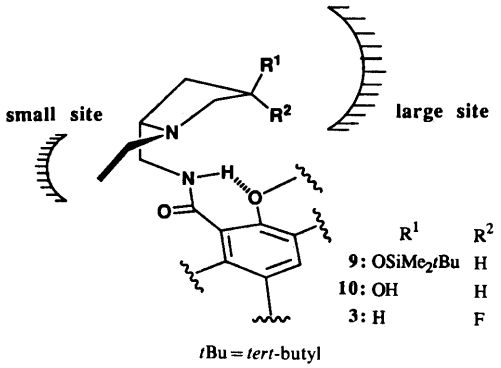


Fig. 1. A Model for the Binding of the Pyrrolidine Moiety of the Eticlopride Derivatives

D2 receptors. These findings may be of help in better understanding of the electronic effect in the ligand-receptor binding and the influence of the fluorine atom introduced.

Steric interactions between the ligands and the receptor surface are another important aspect in determining the affinity. The slight influence of the C-4 substituents (run 2, 3, and 4) on the affinity to the receptor, together with the affinity-reducing effect of *N*-fluorobenzyl derivative (**15**) (run 7), lead us to assume a receptor model for the binding sites in which pyrrolidine *N*-substituents are thought to be directed to a sterically hindered site, whereas a relatively large cleft appears to be accommodated to the C-4 substituents. Independently of us, a new spatial model for the D2 binding sites has been recently proposed based on the difference in affinities of enantiomeric benzamide derivatives, in which the D2 binding sites are thought to involve both a small and a large site for the interaction of the pyrrolidine moiety.[11] Figure 1 was drawn so that the new eticlopride derivatives described here can be applied to such a model, indicating that the substituents at the C-4 position of the pyrrolidine ring seem to be located in the larger site.

Thus, this study indicates that the new fluoroeticlopride **3** with a fluorine atom at the pyrrolidine C-4 position is a promising ligand for the investigation of dopamine D2 receptors. *In vivo* biodistribution in rats using the ^{18}F-labelled **3** showed a specific uptake in the striatum, and the detailed *in vivo* results will soon be reported.

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and are not corrected. Optical rotations were determined by a JASCO DIP-360 digital polarimeter. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken at either 100 MHz (JEOL FX-100) or at 270 MHz (JEOL GX-270), and chemical shifts are reported in ppm (δ) downfield from tetramethylsilane (δ 0.0). Infrared (IR) spectra were taken on a JASCO IRA-1 spectrometer. Low resolution electron impact (EI), field desorption (FD), and fast atom bombardment (FAB) mass spectra (respective abbreviations are as follows: EIMS, FDMS, and FABMS) as well as high resolution EI mass spectra (HRMS) were obtained on a JEOL TMS-D300 spectrometer. Low resolution secondary ion (SI) and high resolution SI mass spectra (SIMS and HRSIMS) were obtained on a HITACHI M-2000 spectrometer. Kieselgel 60 (70—230 mesh, Merck) was used for column chromatography. Elemental analyses were performed by the staff of the microanalytical section of Kyushu University. [^{3}H]-Spiperone (1.48 TBq/mmol) was purchased from New England Nuclear. Eticlopride, (*S*)-(−)-5-chloro-3-ethyl-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-6-methoxysalicylamide, was prepared by the published procedure.[12]

(2*S*,4*R*)-(−)-1-Ethyl-4-*tert*-butyldimethylsilyloxy-2-ethoxycarbonylpyrrolidine (5) A solution of 4-hydroxy-L-proline ethyl ester hydro-

chloride (1.5 g, 7.69 mmol), triethylamine (1.8 g, 17.8 mmol), and ethyl iodide (0.195 g, 12.5 mmol) in water–dioxane (7.5 ml–7.5 ml) was stirred at room temperature for 5 d. The reaction mixture was extracted with chloroform, and the extract was dried over anhydrous Na_2SO_4 and filtered, then evaporated. The residue was purified by column chromatography (silica gel, chloroform : methanol = 15 : 1) to give a colorless oil (0.77 g, 54%). This oil was used for the next reaction without further purification. IR (neat): 3400, 1740 cm^{-1}. ^{1}H-NMR ($CDCl_3$) (100 MHz) δ: 4.50 (1H, m), 4.19 (2H, q, J = 7.0 Hz), 3.50 (1H, dd, J = 10.0, 7.8 Hz), 3.46 (1H, dd, J = 10.0, 5.8 Hz), 2.66 (2H, q, J = 7.0 Hz), 2.41 (1H, m), 2.14 (2H, m), 1.27 (3H, t, J = 7.1 Hz), 1.10 (3H, t, J = 7.3 Hz).

A solution of the above oil (0.49 g, 2.60 mmol), *tert*-butyldimethylsilyl chloride (0.56 g, 3.72 mmol), and imidazole (0.46 g, 6.76 mmol) was stirred at room temperature for 3 d. The reaction mixture was extracted with pentane (65 ml), and the extract was washed successively with water and brine, then dried over anhydrous Na_2SO_4. Filtration and evaporation of the solvent gave an oil (0.78 g, 99%). IR (neat): 1740 cm^{-1}. ^{1}H-NMR ($CDCl_3$) (100 MHz) δ: 4.44 (1H, qui, J = 5.6 Hz), 4.19 (2H, q, J = 7.2 Hz), 3.41 (1H, dd, J = 9.5, 5.8 Hz), 3.38 (1H, dd, J = 8.3, 7.4 Hz), 2.77 (1H, dq, J = 12.0, 7.2 Hz), 2.52 (1H, dq, J = 12.0, 7.2 Hz), 2.29 (1H, dd, J = 9.5, 5.3 Hz), 2.08 (2H, m), 1.27 (3H, t, J = 7.2 Hz), 1.08 (3H, t, J = 7.2 Hz), 0.88 (9H, s), 0.04 (6H, s). EIMS m/z: 301 (M^+). HRMS m/z: Calcd for $C_{15}H_{31}NO_3Si$: 301.2071. Found: 301.2074.

(2*S*,4*R*)-(−)-1-Ethyl-4-*tert*-butyldimethylsilyloxy-2-pyrrolidinecarboxamide (6) A solution of **5** (0.47 g, 1.56 mmol) and KI (30 mg) in methanol saturated with NH_3 (20 ml) was heated at 40 °C for 7 d. Evaporation of the solvent gave a residue, which was purified by column chromatography (silica gel, chloroform) to yield colorless needles (0.40 g, 93%). mp 56 °C. $[\alpha]_D^{26} = -68.3°$ (c = 1.2, $CHCl_3$). IR (Nujol): 3400, 1680 cm^{-1}. ^{1}H-NMR ($CDCl_3$) (100 MHz) δ: 7.21 (2H, br), 4.31 (1H, qui, J = 5.5 Hz), 3.31 (1H, dd, J = 9.5, 4.8 Hz), 3.26 (1H, dd, J = 7.3, 7.3 Hz), 2.59 (2H, q, J = 7.1 Hz), 2.30 (1H, ddd, J = 9.5, 5.5, 4.7 Hz), 2.06 (2H, m), 1.07 (3H, t, J = 7.1 Hz), 0.87 (9H, s), 0.05 (6H, s). FABMS m/z: 273 (MH^+). *Anal.* Calcd for $C_{13}H_{28}N_2O_2Si$: C, 57.31; H, 10.36; N, 10.28. Found: C, 57.54; H, 10.30; N, 10.22.

(2*S*,4*R*)-(−)-2-Aminomethyl-1-ethyl-4-*tert*-butyldimethylsilyloxypyrrolidine(7) A mixture of **6** (1.00 g, 3.68 mmol) and $LiAlH_4$ (0.60 g, 15.5 mmol) in ether (10 ml) was heated under reflux for 2 d. The reaction mixture was quenched with aqueous saturated Na_2SO_4, and extracted with ether. The extract was dried over anhydrous Na_2SO_4, and evaporated to give a crude oil, which was purified by column chromatography (silica gel, chloroform : methanol = 5 : 1—methanol) to yield a pure oil (0.78 g, 82%). $[\alpha]_D^{30} = -41.3°$ (c = 0.8, $CHCl_3$). IR (neat): 3350 cm^{-1}. ^{1}H-NMR ($CDCl_3$) (100 MHz) δ: 4.32 (1H, qui, J = 6.0 Hz), 3.33 (1H, dd, J = 9.3, 6.1 Hz), 2.82 (1H, q, J = 7.1 Hz), 2.50—2.80 (3H, m), 2.29 (1H, q, J = 7.1 Hz), 2.20 (1H, dd, J = 9.5, 6.0 Hz), 1.90—1.75 (4H, m), 1.07 (3H, t, J = 7.2 Hz), 0.88 (9H, s), 0.05 (6H, s). FDMS m/z: 259 (MH^+). HRSIMS m/z: Calcd for $C_{13}H_{31}N_2OSi$: 259.2204. Found: 259.2270.

(2′*S*,4′*R*)-(−)-5-Chloro-3-ethyl-*N*-[(1-ethyl-4-*tert*-butyldimethylsilyloxy-2-pyrrolidinyl)methyl]-6-methoxysalicylamide (9) 1-Hydroxybenzotriazole (56 mg, 0.415 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (78 mg, 0.405 mmol) were added to a solution of 5-chloro-3-ethyl-6-methoxysalicylic acid (**8**)[12] (89 mg, 0.39 mmol) in anhydrous dichloromethane (2 ml) at −10 °C, and the whole was stirred for 100 min at −10 °C and for 30 min at room temperature. A solution of **7** (96 mg, 0.371 mmol) in dichloromethane (2 ml) was added dropwise to the above mixture, and the mixture was stirred overnight at room temperature. The solvent was removed and the residue was purified by column chromatography (silica gel, chloroform : methanol = 15 : 1) to give a pure oil (0.15 g, 84%). $[\alpha]_D^{25} = -34.2°$ (c = 0.95, $CHCl_3$). IR (neat): 3350, 1635 cm^{-1}. ^{1}H-NMR ($CDCl_3$) (270 MHz) δ: 13.8 (1H, br), 8.83 (1H, br), 7.22 (1H, s), 4.30 (1H, qui, J = 5.5 Hz), 3.87 (3H, s), 3.75 (1H, ddd, J = 14.1, 7.6, 2.3 Hz), 3.40 (1H, dd, J = 9.8, 5.9 Hz), 3.30 (1H, dd, J = 14.1, 2.6 Hz), 3.01 (1H, br), 2.86 (1H, dq, J = 12.1, 7.3 Hz), 2.62 (2H, q, J = 7.6 Hz), 2.37 (1H, dq, J = 12.1, 7.3 Hz), 2.27 (1H, dd, J = 9.8, 5.5 Hz), 1.83 (2H, m), 1.20 (3H, t, J = 7.6 Hz), 1.11 (3H, t, J = 7.3 Hz), 0.87 (9H, s), 0.04 (6H, s). SIMS m/z: 471 (MH^+). HRSIMS m/z: Calcd for $C_{23}H_{40}ClN_2O_4Si$: 471.2469. Found: 471.2472.

(2′*S*,4′*R*)-(−)-5-Chloro-3-ethyl-*N*-[(1-ethyl-4-hydroxy-2-pyrrolidinyl)methyl]-6-methoxysalicylamide (10) A solution of n-Bu_4NF (1.3 mmol) and **9** (0.19 g, 0.403 mmol) in THF (2.8 ml) was stirred at room temperature for 3 d. The reaction mixture was quenched with aqueous saturated NH_4Cl, and extracted with ether, then the extract was dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a crude product, which was purified by column chromatography (silica gel, chloroform : methanol = 10 : 1 or ethyl acetate : n-hexane = 3 : 1) to afford a pure oil (0.14 g, 94%). $[\alpha]_D^{28} = -52.0°$ (c = 1.0, $CHCl_3$). IR (neat): 3350, 1635 cm^{-1}. ^{1}H-NMR ($CDCl_3$) (270 MHz) δ: 13.7 (1H, br), 8.85 (1H, br), 7.22 (1H, s), 4.40 (1H, br), 3.87 (3H, s), 3.79 (1H, ddd, J = 14.2, 7.6, 2.0 Hz), 3.54 (1H, dd, J = 9.9, 5.6 Hz), 3.33 (1H, ddd, J = 14.2, 4.6, 2.6 Hz), 3.06 (1H, br), 2.89 (1H, dq, J = 12.1, 7.3 Hz), 2.62 (2H, q, J = 7.5 Hz), 2.37 (1H, dq, J = 12.1, 7.3 Hz), 2.31 (1H, dd, J = 9.9, 4.6 Hz), 1.91 (2H, m), 1.20 (3H, t, J = 7.5 Hz), 1.13 (3H, t, J = 7.3 Hz). FABMS m/z: 357 (MH^+). HRSIMS m/z: Calcd for $C_{17}H_{26}ClN_2O_4$: 357.1580. Found: 357.1579.

(2′*S*,4′*R*)-(−)-5-Chloro-3-ethyl-*N*-[(1-ethyl-4-*p*-toluenesulfonyloxy-2-pyrrolidinyl)methyl]-6-methoxysalicylamide (11) *p*-Toluenesulfonyl chloride (57 mg, 0.295 mmol) was added to a solution of **10** (105 mg, 0.295 mmol) in pyridine (0.76 g, 9.5 mmol) at 0 °C, and the whole was stirred for 4 h at 0 °C and for 26 h at room temperature. The reaction mixture was quenched with ice-water, then extracted with ether. The water layer was separated and alkalinized with concentrated aqueous NH_3, then again extracted with ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4, and evaporated to give a crude oil, which was purified by column chromatography (silica gel, chloroform : methanol = 10 : 1) to afford a pure oil (44 mg, 29%). $[\alpha]_D^{25} = -9.3°$ (c = 1.0, $CHCl_3$). IR (neat): 3350, 1635 cm^{-1}: ^{1}H-NMR ($CDCl_3$) (270 MHz) δ: 13.67 (1H, s), 8.72 (1H, br d, J = 6.3 Hz), 7.76 (2H, dt, J = 8.5, 1.98 Hz), 7.33 (2H, dd, J = 8.5, 0.7 Hz), 7.23 (1H, s), 4.87 (1H, m), 3.82 (3H, s), 3.75 (1H, ddd, J = 14.5, 7.9, 1.98 Hz), 3.51 (1H, dd, J = 10.9, 5.9 Hz), 3.27 (1H, dm, J = 14.2 Hz), 2.97 (1H, br), 2.85 (1H, dq, J = 12.2, 7.5 Hz), 2.61 (2H, q, J = 7.3 Hz), 2.55 (1H, dd, J = 10.9, 5.0 Hz), 2.44 (3H, s), 2.37 (1H, dq, J = 12.2, 7.5 Hz), 1.99 (1H, ddd, J = 14.5, 7.3, 3.3 Hz), 1.83 (1H, ddd, J = 14.5, 9.2, 7.3 Hz), 1.19 (3H, t, J = 7.5 Hz), 1.08 (3H, t, J = 7.3 Hz). FDMS m/z: 510 (M^+), 512 ($M+2$)$^+$. HRSIMS m/z: Calcd for $C_{24}H_{32}ClN_2O_6S$: 511.1695. Found: 511.1667.

(2′*S*,4′*R*)-(−)-5-Chloro-3-ethyl-*N*-[(1-ethyl-4-methanesulfonyloxy-2-pyrrolidinyl)methyl]-6-methoxysalicylamide (12) Methanesulfonyl chloride (45 mg, 0.389 mmol) and triethylamine (41 mg, 0.405 mmol) were added to a solution of **10** (97 mg, 0.27 mmol) in dichloromethane at 0 °C, and the whole was stirred for 2 h at 0 °C. The reaction mixture was quenched with ice-water, then extracted with dichloromethane. The extract was dried over anhydrous Na_2SO_4, and evaporated to give a crude oil, which was purified by column chromatography (silica gel, chloroform : methanol = 10 : 1) to afford a pure oil (99 mg, 84%). $[\alpha]_D^{24} = -20.7°$ (c = 0.9, $CHCl_3$). IR (neat): 3350, 1635 cm^{-1}. ^{1}H-NMR ($CDCl_3$) (100 MHz) δ: 13.67 (1H, br), 8.77 (1H, br), 7.23 (1H, s), 5.08 (1H, br), 3.87 (3H, s), 3.81—3.13 (3H, m), 3.01 (3H, s), 2.97—1.96 (8H, m), 1.20 (3H, t, J = 7.3 Hz), 1.12 (3H, t, J = 7.1 Hz). FABMS m/z: 435 (MH^+). HRSIMS m/z: Calcd for $C_{18}H_{28}ClN_2O_6S$: 435.1332. Found: 435.1354.

(2′*S*,4′*S*)-(−)-5-Chloro-3-ethyl-*N*-[(1-ethyl-4-fluoro-2-pyrrolidinyl)methyl]-6-methoxysalicylamide (3). The Synthesis Using 11 A solution of n-Bu_4NF (0.11 mmol) and **11** (14 mg, 0.027 mmol) in THF (0.6 ml) was heated under reflux for 25 min. The solvent was removed, and the residue was shaken with brine and ether. The ether layer was separated, and dried over anhydrous Na_2SO_4. Evaporation of the solvent gave a crude product, which was purified by column chromatography (silica gel, ethyl acetate : n-hexane = 3 : 1—5 : 1), then by HPLC (Whatman Partisil 5PAC, 9.4 mm × 10 cm, n-hexane : ethyl acetate = 4 : 1, flow rate = 2 ml/min) to afford a pure oil (4.6 mg, 47%). $[\alpha]_D^{24} = -20.9°$ (c = 1,3, $CHCl_3$). IR (neat): 3350, 1635 cm^{-1}. ^{1}H-NMR ($CDCl_3$) (270 MHz) δ: 13.9 (1H, s), 8.99 (1H, br), 7.21, (1H, s), 5.11 (1H, dddd, J = 55.1, 9.5, 4.6, 2.4 Hz), 3.92 (3H, s), 3.84 (1H, ddd, J = 14.4, 7.3, 2.0 Hz), 3.47 (1H, ddd, J = 20.1, 11.5, 1.4 Hz), 3.37 (1H, ddd, J = 14.4, 4.3, 2.6 Hz), 2.91 (1H, dq, J = 12.2, 7.0 Hz), 2.68 (1H, br), 2.62 (2H, q, J = 7.0 Hz), 2.48—2.15 (3H, m), 1.94 (1H, ddddd, J = 33.3, 14.9, 7.6, 1.7, 1.7 Hz), 1.20 (3H, t, J = 7.0 Hz), 1.15 (3H, t, J = 7.0 Hz). FDMS m/z: 359 (MH^+). HRSIMS m/z: Calcd for $C_{17}H_{25}ClFN_2O_3$: 359.1588, Found 359.1563.

The Synthesis Using 12 A solution of n-Bu_4NF (0.6 mmol) and **12** (66 mg, 0.15 mmol) in THF (1.6 ml) was heated under reflux for 6.5 h. The solvent was removed, and the residue was purified by column chromatography (silica gel, chloroform : methanol = 99 : 1) to afford a pure oil (9.6 mg, 18%). The IR, ^{1}H-NMR, and MS spectra of the product were identical with those of the compound obtained using **11**.

The Synthesis Using 10 A solution of morpholinosulfur trifluoride[7] (87 mg, 0.498 mmol) in dichloromethane (1 ml) was added dropwise to a solution of **10** (89 mg, 0.249 mmol) in dichloromethane (1 ml) at −78 °C. The reaction mixture was stirred for 2 h, while the reaction temperature was raised gradually from −78 °C to room temperature. The reaction mixture was poured into aqueous saturated $NaHCO_3$, and extracted with dichloromethane. The extract was washed with brine, dried over anhy-

drous Na_2SO_4, and then evaporated. The residue was purified by column chromatography (silica gel, *n*-hexane : ethyl acetate = 3 : 1) to give a pure oil (15 mg, 16%). The IR, ^{1}H-NMR, and MS spectra of the product were identical with those of the compound obtained using **11**.

***In Vitro* Receptor Binding Assay** The assays were performed in the rat striatal membranes using a previously described method.[8] Briefly, rat striata were homogenized in 100 volumes of ice-cold Tris–HCl buffer (50 mM, pH 7.7) and centrifuged (500 *g*, 10 min, 0 °C). The supernatant was centrifuged at 50000 *g* for 15 min. The pellet was suspended in 100 volumes of ice-cold Tris–HCl buffer (50 mM, pH 7.7) and recentrifuged (50000 *g*, 15 min, 0 °C). The final pellet was resuspended in 150 volumes of ice-cold Tris–HCl buffer (50 mM, pH 7.1) containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.1 mM ascorbic acid, and 10 mM pargyline and incubated at 37 °C for 10 min. A portion of this membrane suspension (900 μl) was placed in a tube, and 50 μl of either test compound or vehicle solution was added, followed by 50 μl of [^{3}H]spiperone (1.48 TBq/mmol) at a final concentration of 0.2 nM. The tubes were incubated at 37 °C for 20 min and filtered through Whatman GF/B glass filters, which were then washed three times with 3 ml of the Tris–HCl buffer (50 mM, pH 7.7). The nonspecific binding was determined in the presence of 10^{-4} M of (±)-sulpiride. The radioactivity trapped on the filters was measured by liquid-scintillation spectrometry. The IC_{50} values to displace specific [^{3}H]spiperone binding were determined from concentration-inhibition curves.

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Structure–Activity Studies on Triazolothienodiazepine Derivatives as Platelet-Activating Factor Antagonists

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A series of triazolodiazepines was synthesized and evaluated for anti-platelet activating factor (PAF) activities. Structure–activity relationship (SAR) studies on this series revealed that the introduction of a methyl group into the 8-position of the thienodiazepine nucleus can lead to a lengthening of the duration of action. Introduction of a methyl group produced an asymmetric center and the enantiomers so formed were separated with an optical resolving column. In the *in vitro* assay system, the (+)-isomers displayed 50—200 times more potent anti-PAF activity than the (−)-isomers. After comparison of toxicology and pharmacokinetics, (+)-6-(2-chlorophenyl)-3-cyclopropanecarbonyl-8,11-dimethyl-2,3,4,5-tetrahydro-8*H*-pyrido[4′,3′:4,5]thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine (35(+)-isomer, E6123) was selected from among the compounds synthesized as a candidate for clinical study.

Keywords platelet activating factor (PAF); PAF antagonist; triazolothienodiazepine; E6123

Platelet activating factor (PAF) is a phospholipid that appears to be involved in pathophysiological processes such as asthma, allergic inflammation, DIC (disseminated intravascular coagulation) and shock.[1] In particular, the following facts suggest that PAF plays an important role in asthma; 1) Inhaled PAF induced bronchoconstriction and hyperresponsiveness in man.[2] 2) An increase in the bronchoalveolar lavage fluid PAF level was found in asthmatic patients.[3] 3) PAF antagonist BN-52021 has effects on PAF and antigen-induced bronchial hyperreactivity and eosinophil accumulation in guinea-pigs.[4] In view of these facts, we have developed PAF receptor antagonists for the treatment of asthma. In 1984, E. Kornecki *et al.*, found that triazolobenzodiazepines such as alprazolam, triazolam showed weak anti-PAF activities,[5] and based on their finding, WEB2086, WEB2170, STY2108, WEB2347[6] and Y-24180[7] were developed as thieno-triazolo-1,4-diazepine (hetrazepines) PAF antagonists (Fig. 1).

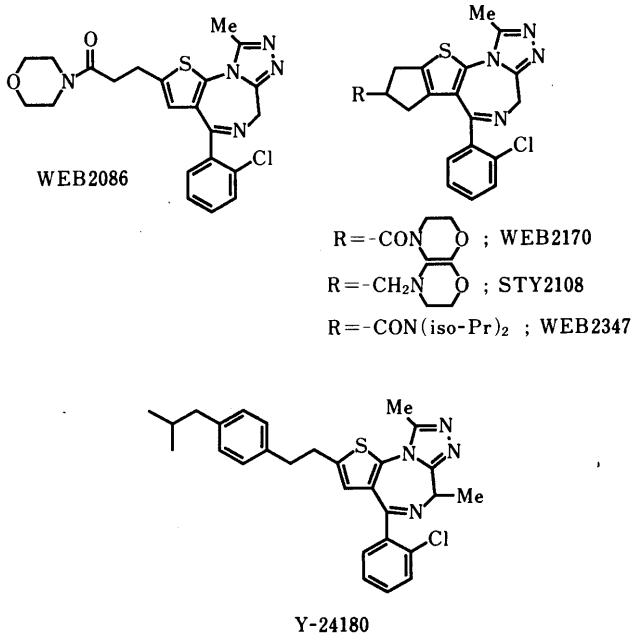


Fig. 1. Structural Formulas of WEB2086, WEB2170, STY2108, WEB2347 and Y-24180

We, too, have been interested in this class of compounds and have carried out structural modification in attempts to obtain compounds with a long duration of action and with good pharmacokinetics. During our study, we discovered that the introduction of a methyl group into the 8-position of the thienodiazepine nucleus can lead to a lengthening of the duration of action. Introduction of a methyl group produced an asymmetric center and the enantiomers so formed were separated with an optical resolving column. In the *in vitro* assay system, the (+)-isomers displayed 50—200 times more potent anti-PAF activities than the (−)-isomers. After comparison of toxicology and pharmacokinetics, (+)-6-(2-chlorophenyl)-3-cyclopropanecarbonyl-8,11-dimethyl-2,3,4,5-tetrahydro-8*H*-pyrido[4′,3′:4,5]-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine (**35**(+)-isomer, E6123)[8] was selected from the compounds synthesized as a candidate for clinical study. We herein report the synthesis and structure–activity studies on triazolothienodiazepines as novel and potent PAF receptor antagonists.

Chemistry The mother skeleton, 6-(2-chlorophenyl)-11-methyl-2,3,4,5-tetrahydro-8*H*-pyrido[4′,3′:4,5]thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine (**1**) was prepared from 2-chlorocyanoacetophenone, *N*-acetylpiperidone and sulfur based on previous literature.[9] The synthetic method for introducing the side chain was chosen according to the type of junction (Chart 1). To introduce an alkyl side chain, compound **1** was deprotonated with a base such as sodium hydride and then reacted with the corresponding alkylating agent (method A). To form ureas, an amine was converted into its phenylcarbamate with phenyl chloroformate, and this was then heated with amine **1** to give urea type compounds **3** (method B). Carbamate type compounds **4** were prepared in a similar fashion (method C). Namely, an alcohol was activated in the form of its phenyl carbonate and this was coupled with amine **1**. Two different methods were used to prepare the amides **5** depending on the availability of the carboxylic acid com-

ponent. If carboxylic acid chloride was available, method D was applied; otherwise, the carboxylic acid and amine **1** were coupled in the presence of a condensing agent such as dicyclohexylcarbodiimide (method E). To introduce alkyl substituents at the 8-position, the unsubstituted compound was reacted with the corresponding alkyl halide in the presence of sodium hydride as a base (Table II). Among these compounds, mono methyl-substituted compound **22** showed higher biological activity than the mother compound **10**. Therefore, we synthesized 6-(2-chlorophenyl)-8,11-dimethyl-2,3,4,5-tetrahydro-8*H*-pyrido[4′,3′:4,5]thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]-dioazepine (**33**) as the new mother skeleton (Chart 2). 2-Amino-3-benzoyl thiophene **27** was prepared by the reaction of 2-chlorocyanoacetophenone with *N*-acetylpiperidone **26** and sulfur in the presence of triethylamine. Compound **27** was then converted into haloacetoamide intermediate **28** with 2-bromopropionyl bromide under Schotten–Baumann reaction conditions. Compound **28** was ammonoloyzed by passing ammonia gas into a solution of the compound in dichloromethane–ethylacetate, giving amine **29**. The ring closure reaction of **29** to **30** was carried out by refluxing a mixture of **29** and a molar equivalent of acetic acid in a pyridine–toluene solution with azeotropic removal of water. Conversion of **30** to triazole **32** was accomplished by thioamidation (P_2S_5–$NaHCO_3$/1,2-dimethoxyethane, reflux) and successive cyclization with acetic hydrazide. The thioacetamide of **32** was cleaved under basic conditions to give **33**. The mono methyl-substitution at the 8-position of its triazolodiazepine skeleton introduces an assymmetric center into molecule **32**. These enantiomers were separated with an optical resolving column; ChiraSpher.

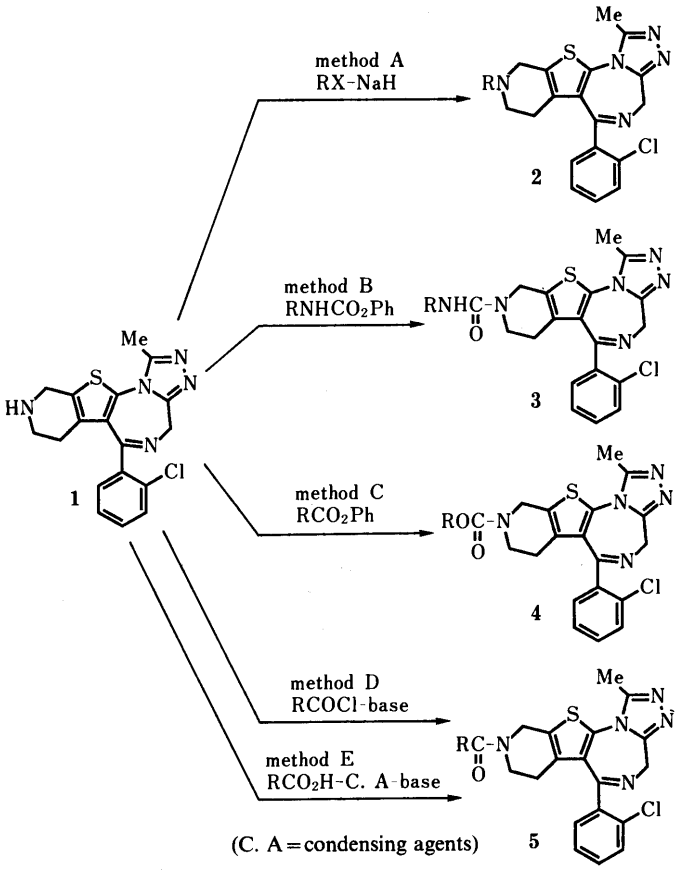
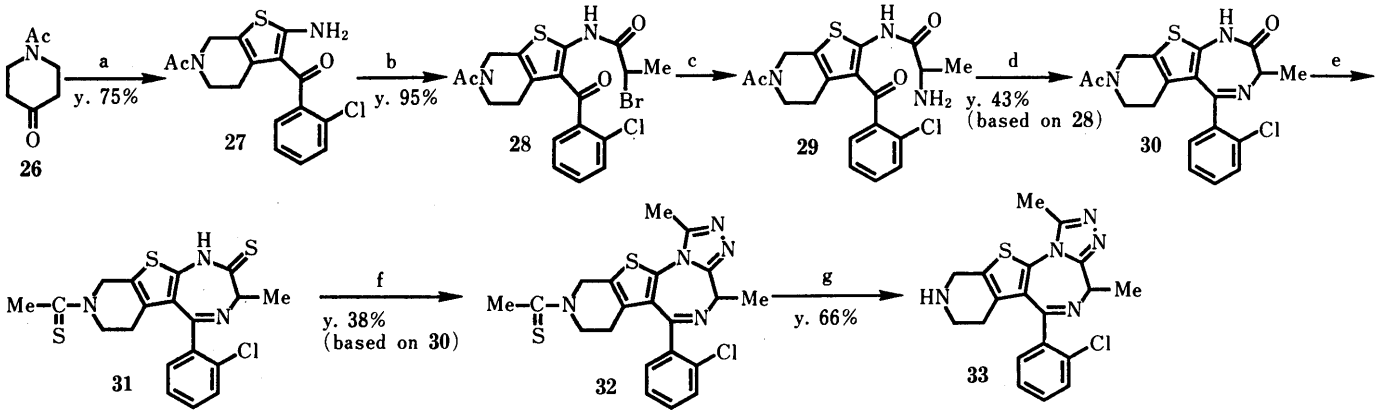


Chart 1

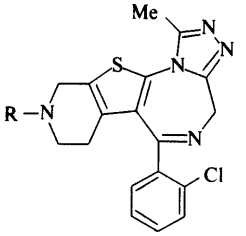
Structure–Activity Relationships Initial evaluation of the compounds was performed with two *in vitro* assay systems; 1) Inhibition of PAF-induced human platelet aggregation[10] and 2) [^{3}H]-PAF receptor binding assay.[11] Compounds with strong PAF antagonistic activity *in vitro* were further evaluated by measuring their inhibitory effect on PAF-induced bronchoconstriction in guinea pigs,[12] in which we employed two different dosages and time points to examine the potency and the duration of action of each compound. We first prepared a series of 8-unsubstituted thienotriazolodiazepines. The results are expressed in Table I. The alkyl-substituted derivative **6** showed very low activity in both of the *in vitro* assay systems, while compounds such as **8** and **9** having a urea junction group showed more potent activity than WEB2086, or WEB2170 in the receptor binding assay system. However compounds **8** and **9** were not so effective in PAF-induced bronchoconstriction at a dose of 0.05 mg/kg *p.o.* when given 90 min before challenge. Next we examined the biological activities of compounds such as **10**—**15** having a carbamate junction. All of them showed more potent activity than WEB2086, or WEB2170

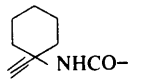
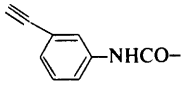
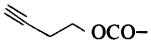
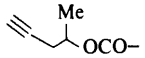
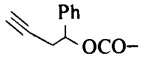
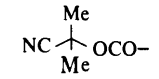
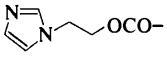
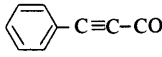
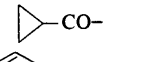
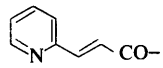
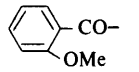
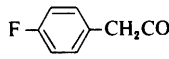


y., yield. a, 2-chlorocyanoacetophenone, S, NEt_3/DMF, 60°C; b, 2-bromopropionyl bromide-$NaHCO_3$/toluene-H_2O, 60°C; c, NH_3/AcOEt-dichloroethane, 100°C; d, AcOH/toluene-pyridine, reflux; e, P_2S_5-$NaHCO_3$/DME, reflux; f, $CH_3CONHNH_2$/dioxane, reflux; g, NaOH/aq. MeOH, reflux

Chart 2

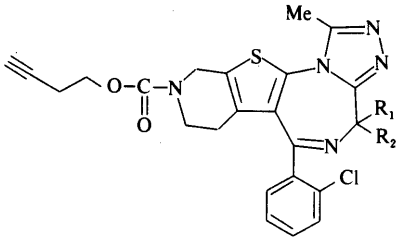
TABLE I. PAF Antagonist Property in Triazolothienodiazepine Series: Structure–Activity Relationships



No.	R	*In vitro* P.A.	*In vitro* R.B.A.	*In vivo* 0.05 mg/kg 90 min	*In vivo* 0.1 mg/kg 8 h
6	$HC\equiv C-CH_2-$	3.0	5.8	—	—
7	NC-⟨⟩-N(Me)CO-	0.3	0.019	43.8±24.3	—
8		0.14	0.0066	17.7	—
9		0.71	0.006	40.8±21.4	—
10		0.031	0.0035	93.0± 1.3	80.1± 1.0
11	$MeC\equiv C$-⟨⟩-OCO-	0.029	0.00074	100± 0	16.9±22.7
12		0.034	0.0019	97.4± 0.9	44.8±13.1
13		0.03	0.0011	0	—
14		0.076	0.0033	99.0± 1.0	91.5± 2.9
15		0.034	0.0088	7.3± 9.7	—
16	NC-⟨⟩-CO-	0.14	0.022	45.6±20.2	—
17		0.015	0.00056	0	—
18		0.15	0.0038	100	90.3± 7.2
19		0.016	0.0105	0	—
20		0.066	0.0058	48.3±33.6	—
21		0.31	0.0056	12.6±25.4	—
	WEB2086	0.03	0.046	7.9± 7.7	12.4± 5.6
	WEB2170	0.017	0.017	98.3± 1.7	72.4± 6.2

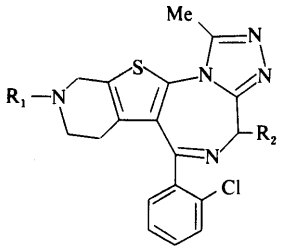
In vitro:P.A. = inhibition of human platelet aggregation induced by PAF IC_{50} (μM), R.B.A. = [^{3}H]-PAF receptor binding assay IC_{50} (μM). *In vivo*:Inhibitory effect on PAF-induced bronchoconstriction at 90 min and 8 h after oral administration of each dose. The inhibition rate was calculated by the following equation: inhibition (%) = 100 − (% peak bronchoconstriction in the test group/% peak bronchoconstriction in the vehicle group) × 100.

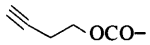
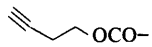
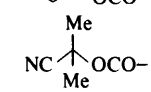
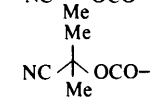
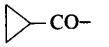
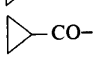
TABLE II. PAF Antagonist Property in Triazolothienodiazepine Series: Structure–Activity Relationships



No.	R_1	R_2	*In vitro* P.A.	*In vitro* R.B.A.	*In vivo* 0.05 mg/kg 90 min	*In vivo* 0.1 mg/kg 8 h
10	H	H	0.031	0.0035	93.0±1.3	80.1±1.0
22	Me	H	0.013	0.0015	97.9±0.6	95.9±0.4
23	Me	Me	0.68	0.16	—	—
24	Et	H	0.031	0.022	—	—
25	Et	Et	0.12	0.029	42.0±5.2	—
WEB2170			0.017	0.017	98.3±1.7	72.4±6.2

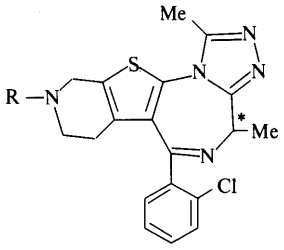
TABLE III. PAF Antagonist Property in Triazolothienodiazepine Series: Structure–Activity Relationships

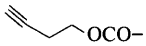
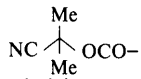
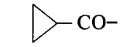


No.	R_1	R_2	*In vitro* P.A.	*In vitro* R.B.A	*In vivo* 0.01 mg/kg 90 min	*In vivo* 0.05 mg/kg 8 h
22		Me	0.013	0.0015	65.0± 6.1	93.9± 2.4
10		H	0.031	0.0035	59.9±16.5	—
34		Me	0.03	0.011	90.2± 6.9	92.6± 1.1
14		H	0.076	0.0033	95.8± 2.4	83.7± 7.0
35		Me	0.0072	0.03	98.8± 1.2	100± 0
18		H	0.15	0.0038	70.0±15.5	53.6±11.9
WEB2170			0.017	0.017	59.8±17.9	58.3± 6.8

in the receptor binding assay system. Compounds **10**—**12** and **14** showed the same potency as WEB2170 in the *in vivo* assay system I (0.05 mg/kg, *p.o.* given 90 min before challenge), but only compounds **10** and **14** showed approximately equivalent potency to WEB2170 in the *in vivo* assay system II (0.1 mg/kg, *p.o.* given 8 h before challenge). Compounds **16**—**21** bearing an amido junction had varied biological activity in the inhibition of PAF-induced platelet aggregation and, excepting compound **16**, most of them showed significantly higher activity than WEB2170 in the receptor binding assay system. But in the *in vivo* assay system I (0.05 mg/kg, *p.o.* given 90 min before challenge), only compound **18** showed the same potency as WEB2170. Next we introduced an alkyl substituent into the 8-position in order to induce the higher activity, and especially to prolong the duration of action. From the metabolic study of triazolodiazepines, psychotropic agents such as brotizolam, hydroxylation of the 8-position is known to be one of the main metabolic pathways in dogs and man.[13] Also, the introduction of an alkyl substituent into the 8-position seems to lead to a decrease in the effect on the central nervous system according to previous

TABLE IV. PAF-Antagonist Property in Triazolothienodiazepine Series: Structure–Activity Relationships



No.	R	*In vitro* R.A.	*In vitro* R.B.A.	*In vivo* 0.01 mg/kg 90 min	*In vivo* 0.05 mg/kg 8 h
22					
	(−)-isomer	0.69	0.082	—	—
	(+)-isomer	0.0034	0.00034	97.2	92.9±2.9
34					
	(−)-isomer	0.34	0.5	—	—
	(+)-isomer	0.007	0.0031	97.7± 0.4	99.5±0.5
35					
	(−)-isomer	1.4	0.42	—	—
	(+)-isomer	0.013	0.0028	100± 0	99.8±0.2
	WEB2170	0.017	0.017	59.8±17.9	58.3±6.8

structure activity relationship studies of the benzodiazepines.[14] Considering these facts, we prepared alkyl-substituted derivatives such as **22**—**25** based on compound **10** (Table II). Among them, the mono-methylated compound **22** showed more potent PAF antagonistic activity than the mother compound **10** in both of the *in vitro* assay systems, and it showed a greater effect against PAF-induced bronchoconstriction in guinea-pigs at a preadministered dose of 0.1 mg/kg. Next, we investigated the effect of a monomethyl substituent in the 8-position of the triazolodiazepine skeleton when introduced into other active compounds (**14**, **18**). These results are expressed in Table III. As we expected, each compound showed more potent PAF antagonistic activity than its mother compound, especially in the *in vivo* assay system. Mono methyl-substitution at the 8-position results in a new asymmetric center in the skeleton, so we investigated separation of the enantiomers by an optical resolving column, ChiraSpher, and we examined their biological activities with *in vitro* and *in vivo* assay systems. These results are expressed in Table IV. In each case the (+)-isomer showed more potent activity than the (−)-isomer. From the results of the biological evaluation, and comparison of the toxicity and pharmacokinetics of these three compounds, (+)-6-(2-chlorophenyl)-3-cyclopropanecarbonyl-8,11-dimethyl-2,3,4,5-tetrahydro-8*H*-pyrido[4′,3′:4,5]thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*]-[1,4]diazepine (E6123) was selected as a candidate for clinical study.

Experimental

General Methods Reagents and solvents were purchased from usual commercial sources. Silicagel (Kiesel gel 60, Merck) was used for column chromatography and silicagel (Kiesel gel 60 F_{254}, Merck) for analytical thin layer chromatography (TLC). Melting points were measured on a Yanagimoto micro melting apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL FX-100 (100 MHz) or Varian Unity 400 (400 MHz) spectrometer, and chemical shifts are expressed in ppm downfield from tetramethylsilane (TMS) as an internal reference. Abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad peak. Infrared (IR) spectra were obtained on a Hitachi 260-30 IR spectrometer. Mass spectra (MS) were obtained on a JEOL JMS-HX100 mass spectrometer. Optical rotations were measured with a JASCO DIP-140 digital polarimeter.

6-(2-Chlorophenyl)-11-methyl-3-(2-propynyl)-2,3,4,5-tetrahydro-8*H*-pyrido[4′,3′:4,5]thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine (6) Sodium hydride (60% content) (30 mg, 0.8 mmol) was added to a dimethylformamide (DMF, 20 ml) solution of 6-(2-chlorophenyl)-11-methyl-2,3,4,5-tetrahydro-8*H*-pyrido[4′,3′:4,5]thieno[3,2-*f*][1,2,4]-triazolo[4,3-*a*][1,4]diazepine (**1**, 0.12 g, 0.3 mmol), and this solution was heated at 60 °C for 1 h. The reaction mixture was cooled to room temperature and propargyl bromide (60 mg, 0.5 mmol) was added. The reaction mixture was heated at 60 °C for 1 h again. The reaction mixture was poured into water and extracted with AcOEt. The extract was dried over $MgSO_4$, filtered and concentrated. The residue was purified by column chromatography (1.5%MeOH/$CHCl_3$) to give **6** (20 mg, 7%) as a yellow-colored amorphous solid. **6**: MS (Pos, Fab) *m/z*: 407 (M^+). IR (Nujol): 1640, 1595, 1540 cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 1.52—2.12 (m, 2H), 2.25 (t, J=2 Hz, 1H), 2.16—2.84 (m, 2H), 2.66 (s, 3H), 3.45 (d, J=2 Hz, 2H), 3.74 (m, 2H), 3.90—4.40 and 5.20—5.76 (m, 2H), 7.27 (m, 4H).

6-(2-Chlorophenyl)-3-(1-cyano-1-methylethoxycarbonyl)-11-methyl-2,3,4,5-tetrahydro-8*H*-pyrido[4′,3′:4,5]thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine (14) a) 1-Cyano-1-methylethylphenylcarbonate. To a solution of acetone cyanohydrin (0.85 g, 10 mmol) in pyridine (20 ml) was added phenyl chloroformate (1.40 g, 8.9 mmol) at 0 °C. The reaction mixture was stirred for 30 min. The reaction mixture was concentrated in a vacuum and the residue was dissolved in $CHCl_3$ and then washed successively with 1 N-HCl and sat. $NaHCO_3$. The extract was dried over $MgSO_4$, filtered and concentrated. The residue was purified by column chromatography (1% AcOEt/*n*-hexane) to give 1-cyano-1-methylethyl-phenylcarbonate (1.83 g, quant.) as a white, waxy solid.

b) A mixture of 1-cyano-1-methylethylphenylcarbonate (0.15 g, 0.7 mmol) and **1** (0.15 g, 0.4 mmol) in $CHCl_3$ was heated at 120 °C for 1 h, boiling off the solvent. The residue was purified by column chromatography (1% MeOH/$CHCl_3$) to give **14** (0.18 g, 92%) as a pale yellow-colored amorphous solid. **14**: MS (Pos, Fab) *m/z*: 481 $(M+H)^+$. IR (Nujol): 1700, 1595, 1450 cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 1.77 (s, 6H), 1.80—2.20 (m, 2H), 2.68 (s, 3H), 3.10—3.60 (m, 2H), 4.22 (m, 1H), 4.50—4.88 (m, 2H), 5.60 (m, 1H), 7.35 (m, 4H).

6-(2-Chlorophenyl)-3-cyclopropanecarbonyl-11-methyl-2,3,4,5-tetrahydro-8*H*-pyrido[4′,3′:4,5]thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine (18) Cyclopropanecarbonyl chloride (90 mg, 0.9 mmol) was added to a solution of **1** (150 mg, 0.4 mmol) and triethylamine (210 mg, 2.1 mmol) in DMF (6 ml) at −60 °C and the reaction mixture was stirred for 30 min at this temperature. The reaction mixture was poured into sat. $NaHCO_3$ and extracted with $CHCl_3$. The extract was dried over $MgSO_4$, filtered and concentrated. The residue was purified by column chromatography (1% MeOH/$CHCl_3$) to give **18** (140 mg, 79%) as a yellow-colored amorphous solid. **18**: MS (Pos, Fab) *m/z*: 438 $(M+H)^+$. IR (Nujol): 1635, 1590, 1540 cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 0.4—1.3 (m, 4H), 1.4—2.7 (m, 3H), 2.67 (s, 3H), 2.8—5.8 (m, 6H), 7.1—7.6 (m, 4H).

6-(2-Chlorophenyl)-3-(4-fluorophenyl)acethyl-11-methyl-2,3,4,5-tetrahydro-8*H*-pyrido[4′,3′:4,5]thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]-diazepine (21) *N*,*N*′-Dicyclohexylcarbodiimide (80 mg, 0.4 mmol) was added to a solution of **1** (120 mg, 0.3 mmol), 4-fluorophenylacetic acid (60 mg, 0.4 mmol) and 1-hydroxybenzotriazole hydrate (60 mg, 0.4 mmol) in DMF (8 ml). The reaction mixture was stirred at 4 °C fof 12 h and then stirred at room temperature for 1 h. The reaction mixture was poured into sat. $NaHCO_3$ and extracted with $CHCl_3$. The extract was dried over $MgSO_4$, filtered and concentrated. The residue was purified with column chromatography (1% MeOH/CH_2Cl_2) to give **21** (80 mg, 49%) as a yellow colored amorphous solid. **21**: MS (Pos, Fab) *m/z*: 506 $(M+H)^+$. IR: 1610, 1590 cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 1.0—2.4 (m, 2H), 2.66 (s, 3H), 2.8—5.9 (m, 6H), 3.65 (br s, 2H), 6.65—7.6 (m, 8H).

3-(3-Butynyloxycarbonyl)-6-(2-chlorophenyl)-8,11-dimethyl-2,3,4,5-tetrahydro-8*H*-pyrido[4′,3′:4,5]thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]-diazepine (22) To a solution of 3-(3-butynyloxycarbonyl)-6-(2-chloro-phenyl)-11-methyl-2,3,4,5-tetrahydro-8*H*-pyrido[4′,3′:4,5]thieno[3,2-*f*]-

[1,2,4]triazolo[4,3-*a*][1,4]diazepine (**10**, 57 mg, 0.12 mmol) in DMF (2 ml) were added successively sodium hydride (55% content) (28 mg, 0.64 mmol) and iodomethane (0.2 ml, 3.2 mmol), and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was neutralized with AcOH and concentrated. The residue was extracted with CH_2Cl_2 and the extract was dried over $MgSO_4$, filtered and concentrated. The residue was purified by column chromatography (1% MeOH/CH_2Cl_2) to give **22** (24 mg, 32%) as a yellow-colored amorphous solid. **22**: MS (Pos, Fab) *m/z*: 480 (M^+). IR (Nujol): 1690, 1590, 1520 cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 2.0—3.0 (m, 5H), 2.1 (d, J=7 Hz, 3H), 2.5 (dt, J=1, 7 Hz, 2H), 2.7 (s, 3H), 4.1 (t, J=8 Hz, 2H), 4.2 (m, 1H), 4.5 (d, J=18 Hz, 1H), 4.9 (d, J=18 Hz, 1H), 7.4 (s, 5H).

3-(3-Butynyloxycarbonyl)-6-(2-chlorophenyl)-8,8,11-trimethyl-2,3,4,5-tetrahydro-8*H*-pyrido[4',3':4,5]thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]-diazepine (23) Compound **10** (0.68 g, 1.5 mmol) was dissolved in DMF (15 ml) and the solution was cooled to 0 °C. Sodium hydride (60% content) (0.153 g, 3.8 mmol) and iodomethane (0.45 ml, 7.2 mmol) were added. The reaction was quenched with water, then neutralized with sodium dihydrogen phosphate. The solvent was evaporated off, and the residue was portioned between CH_2Cl_2 (50 ml) and H_2O (50 ml). The aqueous layer was extracted with CH_2Cl_2 (50 ml), and combined organic layers were dried over $MgSO_4$, filtered and evaporated under reduced pressure. The residue was purified by column chromatography on silicagel, using CH_2Cl_2–1%MeOH/CH_2Cl_2 as an eluent while the fractions were analyzed by high performance liquid chromatography (HPLC). HPLC conditions: column Nucleosil $_5C_{18}$ 4.6 i.d. × 250 mm, mobile phase CH_3CN–H_2O–H-NEt_2 (40:60:0.01), flow rate 1.0 ml/min, detector UV 240 nm. The mono-methylated compound **22** (0.173 g, 25%) and the dimethylated compound **23** (0.050 g, 7%) were obtained, along with fractions containing a mixture of the two. **23**: MS (Pos, Fab) *m/z*: 494 (M^+). ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 2.0—3.1 (m, 5H), 2.5 (dt, J=1, 8 Hz, 2H), 2.7 (s, 3H), 3.1 (s, 3H), 3.7 (s, 3H), 4.2 (t, J=8 Hz, 2H), 4.5 (d, J=17 Hz, 1H), 4.9 (d, J=17 Hz, 1H), 7.4 (s, 4H).

6-Acetyl-2-amino-3-(2-chlorobenzoyl)-4,5,6,7-tetrahydro-thieno[2,3-*c*]-pyridine (27) To a mixture of *N*-acetylpiperidone **26** (440 g, 3.11 mol), sulfur (100 g, 3.11 mol) and 2-chlorocyanoacetophenone (559 g, 3.11 mol) in DMF (1 l) was added triethylamine (290 ml, 2.08 mol), and the mixture was heated at 60 °C for 1 h. The mixture was concentrated by vacuum pump and MeOH was added. The yellow powder that formed was filtered and washed with a small amount of MeOH to give **27** (712.4 g, 75%). **27**: mp 205 °C. MS (Pos, Fab) *m/z*: 335 ($M+H)^+$. IR (Nujol): 2900, 1620, 1585 cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.7—1.9 (m, 2H), 2.08, 2.12 (each s, total 3H), 3.4, 3.58 (each t like, total 2H), 4.37, 4.50 (br s, total 2H), 7.2—7.42 (m, 4H).

6-Acetyl-2-(2-bromopropionylamino)-3-(2-chlorobenzoyl)-4,5,6,7-tetra-hydro-thieno[2,3-*c*]pyridine (28) To a heated (60 °C) solution of **27** (600 g, 1.79 mol) and $NaHCO_3$ (301 g, 3.58 mol) in toluene (13.3 l) and H_2O (3.7 l) was added dropwise 2-bromopropionyl bromide (301 ml, 2.87 mol). A further portion of $NaHCO_3$ (170 g, 2.02 mol) and 2-bromopropionyl bromide (170 ml, 1.62 mol) was added to the above solution to drive the reaction to completion. After cooling the reactin mixture down to r.t., $NaHCO_3$ (500 g) was added to the reaction residues and the organic layer was separated. The aqueous layer was extracted twice with AcOEt and the combined organic layers were washed with H_2O, dried over $MgSO_4$, filtered and concentrated. The crystals thus formed were filtered and washed with Et_2O to give **28** (800 g, 95%) as pale yellow crystals. **28**: mp 151—153 °C. MS (Pos, Fab) *m/z*: 469 (M^+). IR (Nujol): 1680, 1630, 1610, 1510 cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 1.7—2.4 (m, 2H), 1.99 (d, J=7.2 Hz, 3H), 2.06 and 2.12 (each s, total 3H), 3.25—3.7 (m, 2H), 4.41 (q, J=7.2 Hz, 1H), 4.4—4.8 (m, 2H), 7.0—7.5 (m, 4H).

8-Acetyl-5-(2-chlorophenyl)-3-methyl-6,7,8,9-tetrahydro-1*H*,3*H*-pyrido-[4',3':4,5]thieno[3,2-*f*][1,4]diazepine-2-one (30) To a cooled (−10 °C) solution of **28** (841 g, 1.79 mol) in 1,2-dichloroethane (0.72 l) and AcOEt (1.08 l) was added liquid NH_3, and then the above solution was heated at 100 °C for 1 h in a sealed tube. After the reaction was complete, excess NH_3 (gas) was removed and the resultant solution was poured into ice-cold 3 N-HCl and the aqueous layer was extracted with AcOEt. The aqueous layer was neutralized with $NaHCO_3$ and extracted repeatedly with $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated. The residue was used without further purification. A solution of **29** (636.8 g) in toluene (2.3 l), pyridine (637 ml) and AcOH (94.3 ml) was heated for 24 h with azeotropic removal of water. The mixture was concentrated and benzene was added to the residue. Filtration of the crystals formed gave **30** (300 g, 43% based on **28**) as a pale brown powder. **30**: mp 260—265 °C. MS (Pos, Fab) *m/z*: 388 ($M+H)^+$. IR (Nujol): 2900, 1690, 1630, 1590 cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 1.3—2.6 (m, 2H), 1.76 (d, J=6.8 Hz, 3H), 2.06 and 2.12 (each s, total 3H), 2.8—4.1 (m, 2H), 3.87 (q, J=6.8 Hz, 1H), 4.1—5.1 (m, 2H), 7.1—7.5 (m, 4H), 9.0—9.5 (br s, 1H).

6-(2-Chlorophenyl)-3-thioacetyl-8,11-dimethyl-2,3,4,5-tetrahydro-8*H*-pyrido[4',3':4,5]thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine (32) A mixture of compound **30** (643 g, 1.66 mol), $NaHCO_3$ (418 g, 4.89 mol) and phosphorus pentasulfide (812 g, 3.65 mol) in 1,2-dimethoxyethane (7 l) was heated at refluxing temperature for 4 h. The reaction mixture was filtered through celite and then concentrated under reduced pressure. The resulting residue was dissolved in a small amount of CH_2Cl_2–MeOH and purified by dry column chromatography (CH_2Cl_2→3% MeOH/CH_2Cl_2) to give **31** (871.2 g) as a brown cake containing inorganic salts. This product was used in the next step without further purification. The di-thioamide **31** (871.2 g) and acetic hydrazide (154 g, 2.08 mol) in 3 l of 1,4-dioxane were heated at 100 °C for 5 h. The reaction mixture was then concentrated under reduced pressure and the resulting residue was purified by column chromatography. Elution by 2% MeOH in CH_2Cl_2 gave **32** (281 g, 38% based on **30**) as a pale brown powder. **32**: MS (Pos, Fab) *m/z*: 442 ($M+H)^+$. IR (Nujol): 1680, 1585, 1520 cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.7—1.9, 2.2—2.4 (m, 2H), 2.1 (d, J=7 Hz, 3H), 2.66 (s, 3H), 2.62, 2.70 (each s, total 3H), 3.5—3.6, 4.1—4.9 (m, 2H), 4.3 (t, J=7 Hz, 1H), 4.8—5.05, 5.7—5.9 (m, 2H), 7.2—7.6 (br s, 4H).

6-(2-Chlorophenyl)-8,11-dimethyl-2,3,4,5-tetrahydro-8*H*-pyrido[4',3':4,5]-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine (33) A solution of **32** (103 g, 0.23 mol) in MeOH (800 ml) and 4 N-NaOH (500 ml) was heated at refluxing temperature for 5 h. The mixture was brine extracted with chloroform. The extract was concentrated and the residue was purified by column chromatography. Elution by 2%—5% MeOH in CH_2Cl_2 gave **33** (59.2 g, 66%) as an orange-colored amorphous solid. **33**: mp 210—213 °C. MS (Pos, Fab) *m/z*: 384 ($M+H)^+$. IR (Nujol): 3300, 1595, 1555, 1530 cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 1.1—2.3 (m, 3H), 2.10 (d, J=6.8 Hz, 3H), 2.45—3.3 (m, 2H), 2.66 (s, 3H), 3.85—4.1 (m, 2H), 4.26 (q, J=6.8 Hz, 1H), 7.1—7.6 (m, 4H).

(+)-6-(2-Chlorophenyl)-3-cyclopropanecarbonyl-8,11-dimethyl-2,3,4,5-tetrahydro-8*H*-pyrido[4',3':4,5]thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]-diazepine ((+)-35) To a cooled (ice bath) solution of **33** (1.58 g, 4.2 mmol) and pyridine (0.42 ml, 5.2 mmol) in CH_2Cl_2 (10 ml) was added dropwise cyclopropionyl chloride (0.43 ml, 4.8 mmol) and the resulting solution was stirred at room temperature for 1 h. The mixture was poured into sat. aqueous $NaHCO_3$ and extracted with CH_2Cl_2 dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography. Elution first with 40% AcOEt in *n*-hexane and then successively from 1% to 5% MeOH in CH_2Cl_2 gave **35** (1.75 g, 92%) as a pale, yellow-white powder. The racemic **35** thus obtained was optically resolved on a ChiraSpher column packed by Sensyu Kagaku (Tokyo Japan) to give the two enantiomers. Preparative HPLC conditions: column ChiraSpher 25 μm 20 i.d. × 500 mm, mobile phase tetrahydrofurane–*n*-hexane (40:60), flow rate 20 ml/min, detector UV 254 nm. (+)-**35**: $[\alpha]_D$ +16.5—18.5° (c=0.1, $CHCl_3$). mp 150 °C. MS (Pos, Fab) *m/z*: 452 ($M+H)^+$. IR (Nujol): 1635, 1590, 1530 cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 0.55—1.15 (m, 4H), 1.45—2.5 (m, 3H), 2.10 (d, J=6.8 Hz, 3H), 2.66 (s, 3H), 2.8—4.8 (m, 3H), 4.26 (q, J=6.8 Hz, m, 1H), 4.8—5.2 (m, 1H), 7.05—7.65 (m, 4H).

***In Vitro* Experiments 1) Preparation of Platelet-Rich Plasma (PRP)** Blood samples were taken from human volunteers into syringes containing one-tenth volume of 3.8% sodium citrate, then centrifuged at 100 × *g* for 10 min. The upper phase was used as PRP in the platelet aggregation study.[10]

2) [^{3}H]-PAF Binding to Platelets Human PRP containing 15% ACD-A (anticoagulant acid citrate dextrose) solution (Terumo, Tokyo, Japan) was centrifuged at 800 × *g* for 15 min and the resulting pellet washed in a standard platelet buffer composed of 4 mmol/l KH_2PO_4, 6 mmol/l Na_2HPO_4, 100 mmol/l NaCl, 0.1% glucose and 20% (w/v) citrate which was adjusted to a final pH of 6.5 with NaOH. The PAF-receptor binding assay was performed using the method of Terashita *et al.*[11] The washed platelets were resuspended at a concentration of 2.2 × 10^5/μl in 10 mmol/l phosphate buffered saline, pH 7.0, containing 0.1% (w/v) bovine serum albumin (BSA) and 0.9 mmol/l $CaCl_2$. Platelets (10^8 cells) in 460 μl of the buffer were added to a polypropylene tube (Eiken, Tokyo, Japan) and preincubated with 20 μl of unlabeled PAF or test compound for 6 min at 37 °C. [^{3}H] PAF (20 μl, 0.6 nmol/l) was then added to the tube and the whole was incubated for 6 min. The binding reaction was stopped by adding 3 ml of ice-cold 0.1% BSA in saline. Platelets were isolated by vacuum filtration on glass filters (Whatman GF/C). Each tube or filter was rapidly washed 3 times with 3 ml of ice-cold 0.1% BSA in saline. The

radioactivity on the glass filter was measured in 5 ml of ACS-II (Amersham) with a scintillation counter (Aloka LSC 900). Each experiment was done in duplicate.

3) Platelet Aggregation Study Anti-platelet aggregation effects of the test compounds were determined by Born's turbidimetric method[10] using an aggregometer (Hematracer; Nicho Bioscience Co., Ltd., Tokyo, Japan). A 200 μl aliquot of PRP and 25 μl of a test compound in saline were placed in a cuvette of the aggregometer, and incubated at 37 °C for 3 min. After incubation, platelet aggregation was induced by the addition of 25 μl of a solution of PAF at a final concentration of 50 ng/ml for humans. The inhibitory effect of the compounds or the degree of platelet aggregation was quantified by the method of Stegmeier *et al.*[10,15]

***In Vivo* Experiments PAF-Induced Bronchoconstriction in the Guinea Pig** Guinea pigs were anesthetized by intraperitoneal injection of urethane at a dose of 1.25 g/kg and prepared for the recording of bronchospasms by the method of Konzett and Rössler.[12] PAF at a dose of 100 ng/kg was intravenously injected twice with a 30 min interval in between. Since bronchospasms induced by the second PAF injection were more stable in each animal than those induced by the first PAF injection, the effects of the test compounds were evaluated on the basis of the second bronchospasm response. The bronchospasm induced by the PAF injection was 66.2±2.8% of the maximal overflow volume obtained by tracheal clamping at the end of the experiment.

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Synthetic Studies of Vitamin D Analogues. IX.[1] Synthesis and Differentiation-Inducing Activity of 1α,25-Dihydroxy-23-oxa-, thia-, and azavitamin D_3[2]

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Three vitamin D_3 analogues, 1α,25-dihydroxy-23-oxavitamin D_3 (3), 1α,25-dihydroxy-23-thiavitamin D_3 (4) and 1α,25-dihydroxy-23-azavitamin D_3 (5) were synthesized. In the differentiation-inducing activity of human myeloid leukemia cells into macrophages *in vitro*, the 23-aza analogue (5) showed the least activity, while no remarkable differences were observed between the 23-oxa analogue (3) and the 23-thia analogue (4), which were less active than 1α,25-dihydroxyvitamin D_3 (1).

Keywords vitamin D_3 analogue; 1α,25-dihydroxy-23-oxavitamin D_3; 1α,25-dihydroxy-23-thiavitamin D_3; 1α,25-dihydroxy-23-azavitamin D_3; differentiation-inducing activity; 1α,25-dihydroxyvitamin D_3; 1α,25-dihydroxy-22-oxavitamin D_3; vitamin D_3 synthesis

In the course of our study of modification of the side chain of 1α,25-dihydroxyvitamin D_3 (**1**), with the aim of separating the differentiation-inducing activity and the potential hypercalcemic action of **1**, we recently found that an almost complete separation of these types of physiological actions was successfully realized with 1α,25-dihydroxy-22-oxavitamin D_3 (**2**). We found that **2** was about 10 times more active than **1** in suppressing proliferation and inducing differentiation of human myeloid leukemia cells (HL-60), and about 1/20 more active in inducing the release of ^{45}Ca from prelabeled fetal mouse calvaria.[1,3] These findings stimulated our interest in a compound oxygenated at a different position, namely 1α,25-dihydroxy-23-oxavitamin D_3 (**3**). From the point of view of bioisosterism,[4] we also focused our attention on compounds bearing other heteroatoms at the 23 position, 1α,25-dihydroxy-23-thiavitamin D_3 (**4**) and 1α,25-dihydroxy-23-azavitamin D_3 (**5**). Although these vitamin D_3 analogues (**3**, **4** and **5**) have already been synthesized by Hesse starting from a degradation product of vitamin D_2,[5] no biological information is yet available. This paper deals with an alternative synthesis of **3**, **4** and **5**, and the preliminary evaluation of their differentiation-inducing activities.

In our synthesis of **3**, **4** and **5**, the diene alcohol (**15**) was chosen as the common intermediate. For the synthesis of **15**, the readily available 17(*Z*)-ethylidene diacetate (**6**)[6] seemed to be a reasonable starting material. Thus, the ene reaction of **6** with paraformaldehyde in the presence of boron trifluoride etherate[7] stereoselectively gave the 20(*S*)-alcohol (**7**) in 89% yield. Subsequent catalytic hydrogenation of the C-16 double bond in **7** was quantitatively achieved from the less congested α-face to afford the alcohol (**8**). After protection of the hydroxy group in **8** as the tetrahydropyranyl ether (**9**), the 1,3-diacetoxy groups in **9** were converted to the 1,3-disilyl ethers in **11** *via* the diol (**10**) in 58% overall yield from **7**. The tetrahydropyranyl ether moiety in **11** was then selectively cleaved by dimethylaluminum chloride[8] to give the alcohol (**12**), in 70% yield, which was transformed into the diene alcohol (**15**) by the following procedure in 63% overall yield; i) acetylation giving **13**, ii) bromination with *N*-bromosuccinimide then dehydrobromination with γ-collidine giving **14**, iii) hydrolysis with potassium carbonate in aqueous ethanol giving **15**.

The synthesis of the 23-oxa analogue (**3**) from **15** was first carried out. The alkylation of the hydroxy part in **15** was effected with isobutylene oxide to give the ether (**17**) in 47% yield. Subsequent irradiation of **17** with a 400 W mercury lamp through a Vycor filter, followed by thermal isomerization in refluxing ethanol and elimination of the silyl group with tetrabutylammonium fluoride, gave rise to 1α,25-dihydroxy-23-oxavitamin D_3 (**3**) in 6% yield. Subsequently, two other analogues (**4** and **5**) were prepared from **15**. Treatment of the mesylate (**16**), prepared from **15** in the usual manner, with 1-mercapto-2-methyl-2-hydroxypropane or 1-amino-2-methyl-2-hydroxypropane, afforded the sulfide (**18**) or the amine (**19**) in 47 and 65% yields, respectively. Both **18** and **19** were irradiated, thermally isomerized and desilylated yielding 1α,25-dihydroxy-23-thia- and 23-azavitamin D_3 (**4** and **5**), in 10 and 13% yields, respectively.

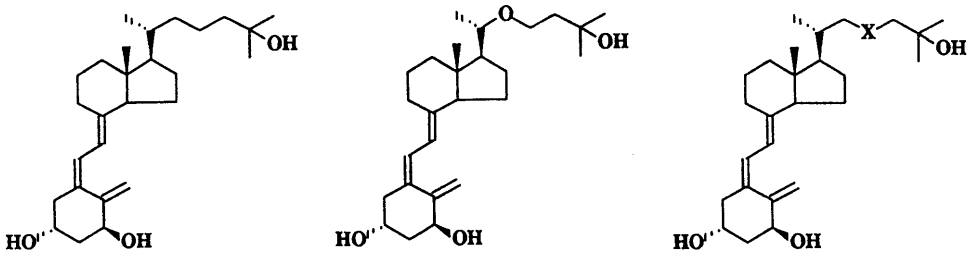

Chart 1

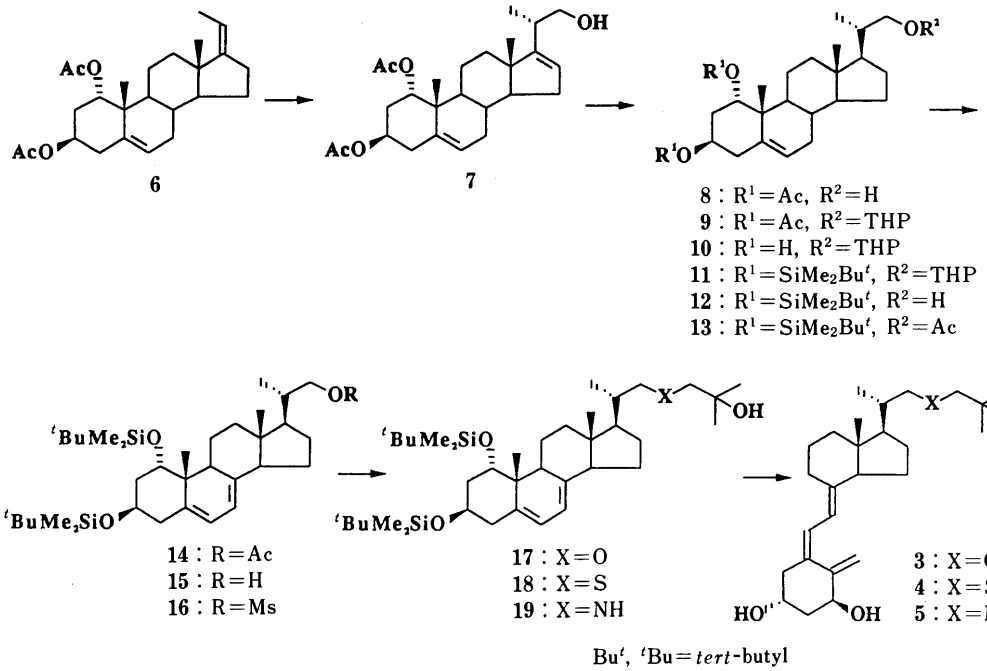

Chart 2

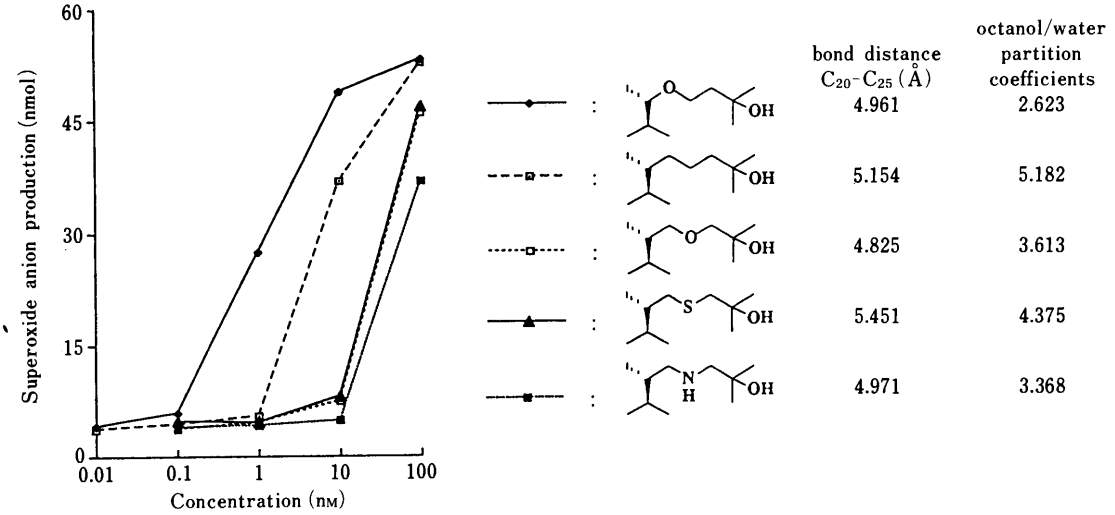

Fig. 1. Comparative Effects of Vitamin D_3 Analogues on the Induction of Superoxide Anion Production of HL-60

Figure 1 shows the preliminary results of the differentiation-inducing activity of HL-60 into macrophages *in vitro* estimated by superoxide anion production.[9] Among the three analogues synthesized, the 23-aza analogue (**5**) showed the least activity (about 1/8 of **1**), while no remarkable differences were observed between the 23-oxa analogue (**3**) and the 23-thia analogue (**4**), which had about 1/5 as much activity as 1α,25-dihydroxyvitamin D_3 (**1**). The calculated bond distance from C-20 to C-25 of each side chain,[10] and octanol/water partition coefficients,[11] are also shown in Fig. 1. It is suggestive that there are no strict relations between the differentiation-inducing activity and side chain length or hydrophilicity. The reason for the greatest potential for differentiation-inducing activity being in the 22-oxa analogue (**2**) (in this experiment, **2** was 6 times more active than **1**), is still ambiguous. Further pharmacological and physicochemical properties of these analogues are now under investigation, and will be reported elsewhere.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and uncorrected. Infrared (IR) spectra were recorded with a Hitachi 270-30 spectrometer, proton nuclear magnetic resonance (^{1}H-NMR) spectra with a JEOL FX-200 spectrometer in $CDCl_3$ with tetramethylsilane as an internal reference, mass spectra (MS) with a Shimadzu GCMS-QP 1000 spectrometer and ultraviolet (UV) spectra with a Shimadzu UV-240 spectrometer. All reactions with the exception of hydrogenation were carried out under an atmosphere of dry argon. Flash column chromatography was carried out with Merck Silica gel 60, 230—400 mesh ASTM. Thin layer chromatography (TLC) was carried out with Merck Silica gel 60 F254 (0.25 mm thickness) pre-coated TLC plates, and preparative TLC was performed on 20 × 20 cm plates coated with 0.5 mm thickness of Merck Silica gel 60 F254. The phrase "residue upon work-up" refers to the residue when the organic layer was separated, dried over $MgSO_4$ and the solvent was evaporated under reduced pressure. All new compounds described in this experimental section were homogeneous on TLC.

(20*S*)-20-Methyl-5,16-pregnadien-1α,3β,21-triol 1,3-Diacetate (7) BF_3·

OEt_2 (80 μl) was added dropwise to a stirred solution of the 17(*Z*)-ethylidene diacetate (**6**) (2.59 g, 6.5 mmol) and paraformaldehyde (0.32 g, 6.5 mmol) in CH_2Cl_2 (20 ml). The mixture was stirred at room temperature for 1.5 h and poured into saturated $NaHCO_3$ (20 ml). The separated organic layer was washed with saturated NaCl, and the residue upon work-up was chromatographed using *n*-hexane–AcOEt (3 : 1, v/v) as the eluent to afford the (20*S*)-alcohol (**7**) (2.48 g, 89%) as a colorless foam. IR (neat): 3460, 1730, 1240, 1030 cm^{-1}. ^{1}H-NMR δ: 0.83 (3H, s), 1.05 (3H, d, *J*=7.0 Hz), 1.15 (3H, s), 2.03 (3H, s), 2.07 (3H, s), 3.58 (2H, br d, *J*=6.5 Hz), 5.35—5.65 (2H, br). MS *m/z*: 310 ($M^+ - CH_3COOH \times 2$, 100%).

(20*S*)-20-Methyl-5-pregnen-1α,3β,21-triol 1,3-Diacetate (8) The (20*S*)-alcohol (**7**) (1.72 g, 4.0 mmol) in AcOEt (90 ml) was hydrogenated in the presence of 5% platinum on carbon (250 mg) at room temperature. After the absorption of equimolar hydrogen, the insoluble material was filtered off. The filtrate was concentrated under reduced pressure to give the practically pure alcohol (**8**) (1.72 g) as a colorless foam, which was used without further purification. Preparative TLC developed with CH_2Cl_2–AcOEt (9 : 1, v/v) gave the analytically pure (**8**). IR (neat): 3510, 1735, 1245, 1050 cm^{-1}. ^{1}H-NMR δ: 0.69 (3H, s), 1.04 (3H, d, *J*=6.6 Hz), 1.08 (3H, s), 2.02 (3H, s), 2.05 (3H, s), 3.27—3.41 (1H, br), 3.63 (1H, br d, *J*=9.0 Hz), 4.80—5.00 (1H, m), 5.03—5.09 (1H, br), 5.54 (1H, br d, *J*=6.0 Hz). MS *m/z*: 312 ($M^+ - CH_3COOH \times 2$), 118 (100%). *Anal.* Calcd for $C_{26}H_{40}O_5$: C, 72.19; H, 9.32. Found: C, 72.13; H, 9.22.

(20*S*)-20-Methyl-21-(tetrahydropyran-2-yl)oxy-5-pregnen-1α,3β-diol 1,3-Diacetate (9) A mixture of the crude alcohol (**8**) (1.45 g), 3,4-dihydropyrane (0.42 g, 5.0 mmol) and Amberlyst-15 (200 mg) in CH_2Cl_2 (30 ml) was stirred at room temperature for 15 h. The insoluble material was filtered off. The filtrate was concentrated under reduced pressure to give the practically pure tetrahydropyranyl ether (**9**) (1.70 g), as a colorless foam, which was used without further purification. Preparative TLC developed with CH_2Cl_2 gave the analytically pure (**9**). IR (neat): 1740, 1240 cm^{-1}. ^{1}H-NMR δ: 0.69 (3H, s), 1.04 (3H, s), 2.01 (3H, s), 2.04 (3H, s), 4.44—4.60 (1H, br), 4.80—4.96 (1H, br), 5.04 (1H, br s), 5.44—5.58 (1H, br). MS *m/z*: 457 ($M^+ - CH_3COO$), 85 (100%).

(20*S*)-20-Methyl-21-(tetrahydropyran-2-yl)oxy-5-pregnen-1α,3β-diol (10) A solution of the crude tetrahydropyranyl ether (**9**) (1.07 g) in tetrahydrofuran (THF) (50 ml) was added dropwise to a stirred suspension of $LiAlH_4$ (0.64 g, 16.8 mmol) in THF (50 ml). The mixture was refluxed for 1 h, quenched with 10% NaOH and the organic layer was separated. The aqueous layer was extracted with AcOEt. The combined organic layer was washed with saturated NaCl and the residue upon work-up was chromatographed using CH_2Cl_2–AcOEt (1 : 2, v/v) as the eluent to afford the diol (**10**) (1.13 g, 78% from **8**) as a colorless foam. IR (neat): 3400 cm^{-1}. ^{1}H-NMR δ: 0.69 (3H, s), 1.03 (3H, s), 1.10 (3H, d, *J*=7.0 Hz), 3.04 (1H, br t), 3.30—3.56 (2H, br), 3.72—4.08 (3H, br), 4.48—4.60 (1H, br d), 5.55—5.64 (1H, br d). MS *m/z*: 432 (M^+), 85 (100%).

(20*S*)-20-Methyl-1α,3β-bis(*tert*-butyldimethylsilyloxy)-21-(tetrahydropyran-2-yl)oxy-5-pregnene (11) A mixture of the diol (**10**) (1.13 g, 2.6 mmol), imidazole (3.56 g, 52.0 mmol), hydroxybenzotriazole (136 mg, 0.8 mmol) and *tert*-$BuMe_2SiCl$ (3.36 g, 15.6 mmol) in dimethylformamide (DMF) (35 ml) was stirred at 65—70 °C for 48 h. Then the mixture was poured into H_2O and extracted with ether. The extract was washed with saturated NaCl and the residue upon work-up was chromatographed using *n*-hexane–CH_2Cl_2 (3 : 1, v/v) as the eluent to afford the 1,3-disilyl ether (**11**) (1.27 g, 74%) as a colorless foam. ^{1}H-NMR δ: 0.04 (9H, s), 0.05 (3H, s), 0.69 (3H, s), 0.89 (18H, s), 0.95 (3H, s), 1.15 (3H, d, *J*=6.0 Hz), 4.45—4.65 (1H, br), 5.36—5.55 (1H, br). MS *m/z*: 660 (M^+), 85 (100%).

(20*S*)-20-Methyl-1α,3β-bis(*tert*-butyldimethylsilyloxy)-5-pregnen-21-ol (12) To a stirred solution of the 1,3-disilyl ether (**11**) (1.27 g, 1.9 mmol) in CH_2Cl_2 (20 ml), was added Me_2AlCl (1 mol/l in *n*-hexane, 3.84 ml, 3.8 mmol) dropwise at −5—−10 °C. The mixture was then stirred at room temperature for 15 h and quenched by saturated $NaHCO_3$. The separated organic layer was washed with saturated NaCl and the residue upon work-up was chromatographed using CH_2Cl_2 as the eluent to afford the alcohol (**12**) (0.79 g, 71%) as colorless glasses: mp 165—167 °C. IR (Nujol): 3250 cm^{-1}. ^{1}H-NMR δ: 0.05 (9H, s), 0.06 (3H, s), 0.70 (3H, s), 0.88 (18H, s), 0.96 (3H, s), 1.10 (3H, d, *J*=6.0 Hz), 5.30—5.55 (1H, br). MS *m/z*: 576 (M^+), 387 (100%). *Anal.* Calcd for $C_{34}H_{64}O_3Si_2$: C, 70.77; H, 11.18. Found: C, 70.24; H, 11.17.

(20*S*)-20-Methyl-1α,3β-bis(*tert*-butyldimethylsilyloxy)-5-pregnen-21-ol Acetate (13) A mixture of the alcohol (**12**) (785 mg, 1.36 mmol), 4,4-dimethylaminopyridine (100 mg), pyridine (20 ml) and acetic anhydride (20 ml) was stirred at room temperature for 20 h. Then the mixture was poured into H_2O and extracted with AcOEt. The extract was washed with 5% HCl, saturated $NaHCO_3$ and saturated NaCl, and the residue upon work-up was chromatographed using *n*-hexane–CH_2Cl_2 (2 : 1, v/v) as the eluent to afford the acetate (**13**) (702 mg, 84%) as colorless glasses: mp 104—105 °C. IR (Nujol): 1740, 1250 cm^{-1}. ^{1}H-NMR δ: 0.05 (9H, s), 0.06 (3H, s), 0.70 (3H, s), 0.88 (18H, s), 0.96 (3H, s), 1.05 (3H, d, *J*=6.0 Hz), 2.03 (3H, s), 5.22—5.50 (1H, br). MS *m/z*: 618 (M^+), 429 (100%). *Anal.* Calcd for $C_{36}H_{66}O_4Si_2$: C, 69.84; H, 10.75. Found: C, 69.86; H, 10.51.

(20*S*)-20-Methyl-1α,3β-bis(*tert*-butyldimethylsilyloxy)-5,7-pregnadien-21-ol Acetate (14) A mixture of the acetate (**13**) (702 mg, 1.13 mmol) and *N*-bromosuccinimide (262 mg, 1.47 mmol) in *n*-hexane (10 ml) was refluxed for 1.25 h. After cooling to room temperature, the precipitate was filtered out. The filtrate was concentrated under reduced pressure. The residue was dissolved in xylene (10 ml) and γ-collidine (1.5 ml). The resulting mixture was refluxed for 1.5 h, then cooled to room temperature, and diluted with toluene and H_2O. The organic layer was washed with saturated NaCl and the residue upon work-up was chromatographed using *n*-hexane–CH_2Cl_2 (3 : 2, v/v) as the eluent to afford the 5,7-diene (**14**) (590 mg, 87%) as a colorless semi-solid softening at 43—46 °C. IR (Nujol): 1745, 1250 cm^{-1}. ^{1}H-NMR δ: 0.05 (9H, s), 0.06 (3H, s), 0.64 (3H, s), 0.89 (18H, s), 0.96 (3H, s), 1.05 (3H, d, *J*=6.0 Hz), 2.02 (3H, s), 5.15—5.70 (2H, br). MS *m/z*: 616 (M^+), 427 (100%). UV λ_{max} nm: 293, 281, 270. *Anal.* Calcd for $C_{36}H_{64}O_4Si_2$: C, 70.07; H, 10.45. Found: C, 69.85; H, 10.69.

(20*S*)-20-Methyl-1α,3β-bis(*tert*-butyldimethylsilyloxy)-5,7-pregnadien-21-ol (15) A mixture of the 5,7-diene (**14**) (580 mg, 0.94 mmol) and K_2CO_3 (390 mg, 2.82 mmol) in EtOH (30 ml) was stirred at 30 °C for 21 h. The solvent was then evaporated under reduced pressure. The residue was taken up with CH_2Cl_2 and H_2O. The organic layer was washed with saturated NaCl and the residue upon work-up was chromatographed using CH_2Cl_2 as the eluent to afford the diene alcohol (**15**) as colorless glasses: mp 154—156 °C. IR (Nujol): 3270 cm^{-1}. ^{1}H-NMR δ: 0.05 (3H, s), 0.06 (3H, s), 0.11 (3H, s), 0.64 (3H, s), 0.89 (18H, s), 0.91 (3H, s), 1.09 (3H, d, *J*=6.0 Hz), 3.33—3.49 (1H, m), 3.59—3.76 (2H, br d), 3.94—4.14 (1H, br), 5.29—5.39 (1H, m), 5.60 (1H, d, *J*=6.0 Hz). MS *m/z*: 574 (M^+), 385 (100%). UV λ_{max} nm: 293, 281, 270. *Anal.* Calcd for $C_{34}H_{62}O_3Si_2$: C, 71.02; H, 10.87. Found: C, 71.20; H, 10.64.

1α,3β-Bis(*tert*-butyldimethylsilyloxy)-23-oxa-5,7-cholestadien-25-ol (17) To a stirred mixture of the diene alcohol (**15**) (65 mg, 0.11 mmol), dibenzo-18-crown-6 (25 mg) and isobutylene oxide (250 μl) in benzene (3 ml), was added *tert*-BuOK (139 mg, 1.24 mmol) at room temperature. The resulting mixture was then refluxed for 1 h and diluted with toluene. The mixture was washed with H_2O and saturated NaCl, and the residue upon work-up was chromatographed using *n*-hexane–AcOEt (9 : 1, v/v) as the eluent to afford the ether (**17**) (34 mg, 47%) as a colorless powder. ^{1}H-NMR δ: 0.07 (9H, s), 0.11 (3H, s), 0.65 (3H, s), 0.89 (21H, s), 1.06 (3H, d, *J*=6.0 Hz), 1.20 (6H, s), 5.15—5.39 (1H, m), 5.55 (1H, br d, *J*=6.0 Hz). MS *m/z*: 646 (M^+), 457 (100%). UV λ_{max} nm: 293, 282, 271.

1α,25-Dihydroxy-23-oxavitamin D_3 (3) A solution of the ether (**17**) (33.4 mg, 0.05 mmol) in EtOH (400 ml) was irradiated with a 400 W high pressure mercury lamp through a Vycor filter at 0 °C under argon bubbling for 3.5 min, then refluxed for 1.5 h. Removal of the solvent under reduced pressure gave an oil, which was dissolved in THF (5 ml) and *n*-Bu_4NF (1 mol/l in THF) (750 μl, 0.75 mmol). The resulting mixture was stirred at room temperature for 15 h, then diluted with AcOEt and washed with H_2O and saturated NaCl. The residue upon work-up was submitted to a two-stage purification: 1) flash column chromatography using CH_2Cl_2–EtOH (5 : 0.3, v/v) as the eluent, 2) preparative TLC developed twice with CH_2Cl_2–EtOH (10 : 1, v/v), to afford the 23-oxavitamin D_3 (**3**) (1.19 mg, 6%). ^{1}H-NMR δ: 0.57 (3H, s), 1.05 (3H, d, *J*=6.2 Hz), 1.21 (6H, s), 3.11—3.47 (4H, m), 4.03—4.27 (1H, br), 4.35—4.47 (1H, br), 4.99 (1H, s), 5.35 (1H, s), 6.00 (1H, d, *J*=11.4 Hz), 6.36 (1H, d, *J*=11.4 Hz). MS *m/z*: 418 (M^+), 59 (100%). UV λ_{max}: 263 nm, λ_{min}: 227 nm.

1α,3β-Bis(*tert*-butyldimethylsilyloxy)-23-thia-5,7-cholestadien-25-ol (18) To a stirred solution of the diene alcohol (**15**) (57.5 mg, 0.10 mmol) in pyridine (2 ml), was added MsCl (24 μl, 0.30 mmol) at 0 °C. The resulting mixture was stirred at room temperature for 45 min, then poured into H_2O and extracted with AcOEt. The extract was washed with saturated $CuSO_4$, H_2O, saturated $NaHCO_3$, H_2O and saturated NaCl, and the residue upon work-up was the practically pure mesylate (**16**) (67 mg) which was used without further purification. To a stirred mixture of the

above-mentioned mesylate (**16**) (67 mg; crude), 1-mercapto-2-methyl-2-hydroxypropane (300 μl) and hexamethylphosphoramide (HMPA, 300 μl) in THF (20 ml), was added NaH (60%) (180 mg, 4.50 mmol). The resulting mixture was stirred at room temperature for 4 h, then poured into H_2O and extracted with AcOEt. The extract was washed with saturated NaCl and the residue upon work-up was chromatographed using *n*-hexane–AcOEt (9:1, v/v) as the eluent to afford the sulfide (**18**) (31 mg, 47%) as a colorless oil. ^{1}H-NMR δ: 0.06 (9H, s), 0.11 (3H, s), 0.63 (3H, s), 0.87 (21H, s), 1.08 (3H, d, J=6.0 Hz), 1.26 (6H, s), 5.20—5.44 (1H, m), 5.48—5.75 (1H, br). MS *m/z*: 662 (M^+), 473 (100%). UV λ_{max} nm: 293, 282, 271.

1α,25-Dihydroxy-23-thiavitamin D_3 (4) A mixture of the sulfide (**18**) (40.6 mg, 0.06 mmol) in EtOH (400 ml) was irradiated with a 400 W high pressure mercury lamp through a Vycor filter at 0 °C under argon bubbling for 4.5 min, then refluxed for 1.5 h. Removal of the solvent under reduced pressure gave an oil, which was dissolved in THF (7 ml) and *n*-Bu_4NF (1 mol/l in THF) (1 ml, 1 mmol). The resulting mixture was stirred at room temperature for 16 h, then diluted with AcOEt and washed with H_2O and saturated NaCl. The residue upon work-up was submitted to a two-stage purification: 1) flash column chromatography using CH_2Cl_2–EtOH (5:0.3, v/v) as the eluent, 2) preparative TLC developed twice with CH_2Cl_2–EtOH (10:1, v/v), to afford the 23-thiavitamin D_3 (**4**) (2.67 mg, 10%). ^{1}H-NMR δ: 0.56 (3H, s), 1.10 (3H, d, J=6.2 Hz), 1.28 (6H, s), 2.53—2.89 (2H, m), 2.63 (2H, s), 4.09—4.29 (1H, br), 4.36—4.49 (1H, br), 4.99 (1H, s), 5.32 (1H, s), 6.01 (1H, d, J=11.4 Hz), 6.37 (1H, d, J=11.4 Hz). MS *m/z*: 434 (M^+), 134 (100%). UV λ_{max}: 263 nm, λ_{min}: 227 nm.

1α,3β-Bis(*tert*-butyldimethylsilyloxy)-23-aza-5,7-cholestadiene-25-ol (19) A mixture of the crude mesylate (**16**) [70 mg; prepared from the alcohol (**15**) (57.5 mg, 1 mmol) in the same manner described in the preparation of the sulfide (**18**)] and 1-amino-2-methyl-2-hydroxypropane (1 ml) was stirred at 60 °C for 3 h. The mixture was then taken up with H_2O and CH_2Cl_2. The organic layer was washed with saturated NaCl and the residue upon work-up was chromatographed using CH_2Cl_2–EtOH (10:1, v/v) as the eluent to afford the amine (**19**) (42 mg, 65%) as a pale yellow gum. ^{1}H-NMR δ: 0.05 (3H, s), 0.07 (3H, s), 0.66 (3H, s), 0.90 (18H, s), 0.91 (3H, s), 1.09 (3H, d, J=6.0 Hz), 1.23 (6H, s), 3.68—3.76 (1H, br), 3.96—4.16 (1H, br), 5.28—5.36 (1H, br), 5.59 (1H, br d, J=6.0 Hz). MS *m/z*: 645 (M^+), 43 (100%). UV λ_{max} nm: 293, 281, 270.

1α,25-Dihydroxy-23-azavitamin D_3 (5) A mixture of the amine (**19**) (38.2 mg, 0.059 mmol) in EtOH (400 ml) was irradiated with a 400 W high pressure mercury lamp through a Vycor filter at 0 °C under argon bubbling for 4 min, then refluxed for 1.5 h. Removal of the solvent under reduced pressure gave an oil, which was dissolved in THF (7 ml) and *n*-Bu_4NF (1 mol/l in THF) (1 ml, 1 mmol). The resulting mixture was stirred at room temperature for 15 h, then diluted with AcOEt and washed with H_2O and saturated NaCl. The residue upon work-up was purified by preparative TLC developed once with CH_2Cl_2–EtOH (3:1, v/v) to afford the 23-azavitamin D_3 (**5**) (3.2 mg, 13%). ^{1}H-NMR δ: 0.57 (3H, s), 1.02 (3H, d, J=6.2 Hz), 1.23 (6H, s), 2.51 (2H, s), 4.11—4.28 (1H, br), 4.31—4.50 (1H, br), 4.99 (1H, s), 5.32 (1H, s), 6.01 (1H, d, J=11.4 Hz), 6.37 (1H, d, J=11.4 Hz). MS *m/z*: 417 (M^+), 43 (100%). UV λ_{max}: 263 nm, λ_{min}: 227 nm.

Differentiation-Inducing Activity HL-60, kindly provided by Dr. T. Suda (Showa University, Tokyo, Japan), was cultured in an RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and 20 μg/ml gentamicin at 37 °C in a humidified atmosphere of 5% CO_2 in air. Induction of differentiation was estimated by the ability of the cell to generate superoxide anion. Vitamin D-induced cells were obtained by seeding HL-60 cells at 1×10^5/ml in a growth media and culturing it for 4 d in the presence of various concentrations of vitamin D_3 analogues. The cells were washed free of the compounds and suspended in a 1.5 ml reaction mixture containing 80 μM ferricytochrome c (Sigma Chemical Co., St. Louis, MO) and 500 ng/ml phorbol myristate acetate (Sigma) in a 0.1% gelatin Hanks' balanced salt solution without phenol red. The mixture was incubated at 37 °C for 60 min and centrifuged for 10 min at $400 \times g$ at 4 °C. The optical density of the supernates was determined with a Hitachi U-3200 dual-wavelength (550 *versus* 540 nm) spectrophotometer. The amount of superoxide anion generated was calculated with a molar extinction coefficient of 19.1×10^3/cm.

Acknowledgment We are grateful to Dr. M. Hamana, Professor Emeritus, Kyushu University for his encouragement.

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Central Cholinergic Agents. I. Potent Acetylcholinesterase Inhibitors, 2-[ω-[*N*-Alkyl-*N*-(ω-phenylalkyl)amino]alkyl]-1*H*-isoindole-1,3(2*H*)-diones, Based on a New Hypothesis of the Enzyme's Active Site

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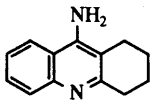
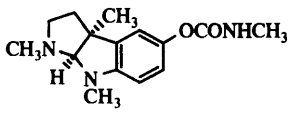
It has been suggested that the active site of acetylcholinesterase contains a hydrophobic binding site (HBS-1), which is closely adjacent to both the anionic and the esteratic sites. In this paper, we assumed that there exists another hydrophobic binding site (HBS-2), some distance removed from the anionic site. On this assumption, a new working hypothesis was proposed for the design of acetylcholinesterase inhibitors. A series of 2-[ω-[*N*-alkyl-*N*-(ω-phenylalkyl)amino]alkyl]-1*H*-isoindole-1,3(2*H*)-diones was designed based on this hypothesis and tested for its inhibitory activities on acetylcholinesterase. Some in this series were revealed to be more potent than physostigmine. Optimum activity was found to be associated with a five carbon chain length separating the benzylamino group from the 1*H*-isoindole-1,3(2*H*)-dione (phthalimide) moiety. Quantitative study of substitution effect on the phthalimide moiety revealed that hydrophilic and electron-withdrawing groups enhance the activity.

Keywords anticholinesterase; 1*H*-isoindole-1,3(2*H*)-dione; phthalimide; structure–activity relationship; Hansch–Fujita analysis; hydrophobic binding site

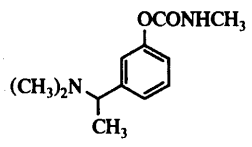
Senile dementia of the Alzheimer type (SDAT) has been shown to be closely associated with defects in the central cholinergic system. Many clinical and animal studies suggest that cholinergic dysfunction may be one of the causes of disturbances in learning and memory in SDAT patients.[1] Thus, pharmacological manipulation of the cholinergic system has been targeted as a viable approach for the treatment of SDAT. Cholinergic enhancement can be achieved by the use of acetylcholine precursors, muscarinic agonists, or acetylcholinesterase (AChE) inhibitors. Acetylcholine precursors such as choline or lecithin have failed to produce any obvious clinical effects.[2] Therapeutic benefits derived from muscarinic agonists such as arecoline or RS-86 are likely to be limited by peripheral cholinergic side effects.[3] On the other hand, clinical studies with various AChE inhibitors have shown the most success. After the report of clinical improvement with tetrahydroaminoacridine (THA),[4] much attention has been focused on AChE inhibitors such as THA, physostigmine, and their analogues (Chart 1),[5,6] which have been considered promising candidates as drugs to treat SDAT.



THA



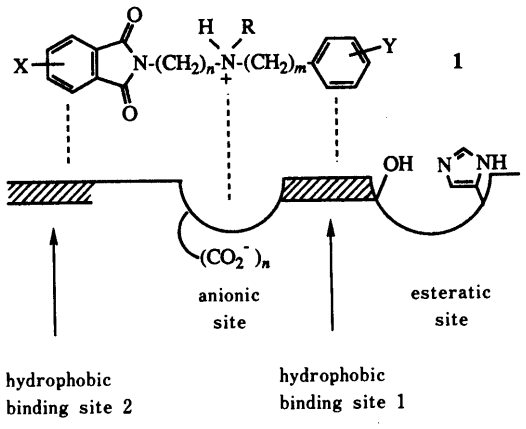
physostigmine



miotine

Chart 1

It has been shown that many AChE inhibitors interact with the active site of AChE, which contains an esteratic site and an anionic site. Physostigmine binds to the anionic site and carbamoylates the hydroxyl group of a serine residue in the esteratic site.[7] In addition, the existence of a hydrophobic binding site (HBS) has been suggested,[8] which is thought to be closely adjacent to both the anionic and the esteratic sites.[9,10] This will be referred to as HBS-1 in this paper. It has been suggested that THA binds at the anionic site as well as at HBS-1.[9] Quinn has given a stylized diagram of these subsites in his review,[11] which prompted us to assume that there may exist at least one more hydrophobic binding site (HBS-2) some distance away from the anionic site (Chart 2). Compounds which can interact with both hydrophobic binding sites as well as the anionic site seem to have provided us with a new type of AChE inhibitor. On the basis of this working hypothesis, we designed 2-[ω-[*N*-alkyl-*N*-(ω-phenylalkyl)amino]alkyl]-1*H*-isoindole-1,3(2*H*)-diones (**1**), some of which were revealed to be



a hypothetical diagram of the active site of AChE

Chart 2. Design of New AChE Inhibitors

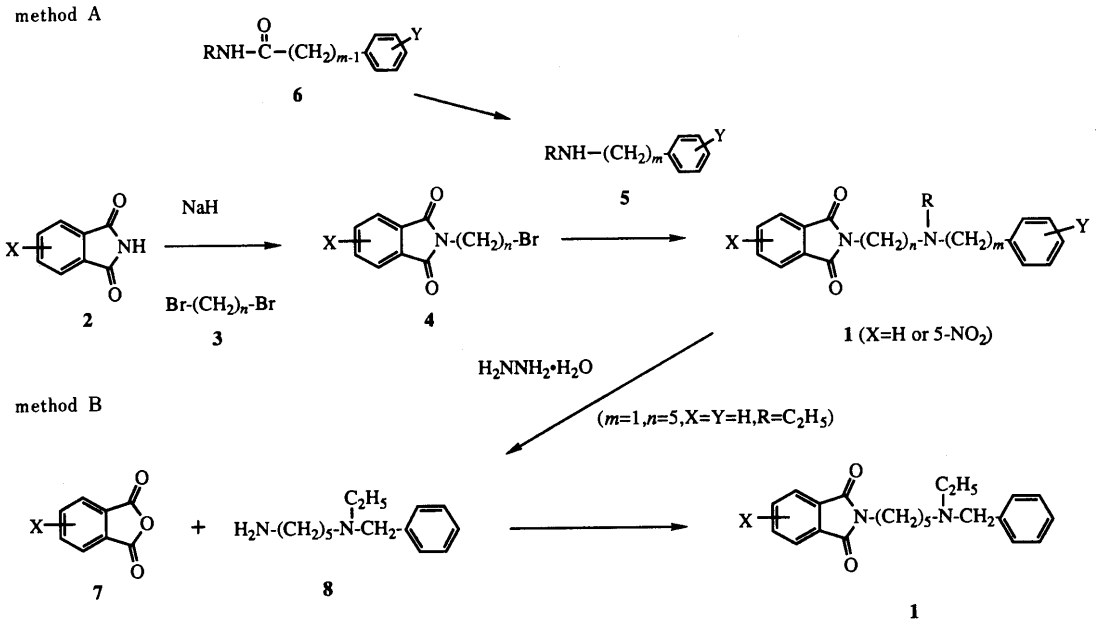


Chart 3

potent AChE inhibtors. For the following two reasons, 1*H*-isoindole-1,3(2*H*)-dione (phthalimide) was chosen as the moiety which could potentially interact with HBS-2. One is that the phthalimide moiety is useful for evaluating the working hypothesis since compounds having different carbon chain lengths (*n* in Chart 2) can be conveniently prepared. The other is that the phthalimide moiety has two carbonyl groups which may contribute to the enzyme inhibition by hydrogen bonding with the enzyme. The importance of the carbonyl groups is described in an accompanying paper[12] in this issue. The choice of an *N*-alkyl-*N*-(ω-phenylalkyl)amino moiety, especially the *N*-alkyl-*N*-benzylamino group, was based on its similarity to miotine which is known as a potent AChE inhibitor.[10] In this paper structure–activity relationships of the phthalimide derivatives (**1**) will be discussed in detail, demonstrating the effectiveness of the working hypothesis in the design of new AChE inhibitors.

Chemistry Synthesis of 2-[ω-[*N*-alkyl-*N*-(ω-phenylalkyl)amino]alkyl]-1*H*-isoindole-1,3(2*H*)-diones (**1**) was accomplished largely by methods A or B as described in the literature (Chart 3).[13] In method A, 1*H*-isoindole-1,3(2*H*)-dione (**2**) was first treated with sodium hydride, then allowed to react with 2 eq of 1,ω-dibromoalkanes (**3**) to afford 2-(ω-bromoalkyl)-1*H*-isoindole-1,3(2*H*)-diones (**4**). The desired compounds (**1**) were obtained by condensation of these bromides (**4**) with various *N*-(ω-phenylalkyl)-amines (**5**), which were prepared by $LiAlH_4$ reduction of the amides (**6**). The preparation of **1**, which has various substituents on the phthalimide moiety, was carried out chiefly by method B. Treatment of **1v** with hydrazine hydrate gave *N*-ethyl-*N*-(phenylmethyl)pentane-1,5-diamine (**8**), which led to **1** by reaction with 3- or 4-substituted phthalic anhydrides (**7**).

A variety of compounds were prepared by the usual transformations of substituents of compounds (**1**) obtained according to the procedure described above. Treatment of **1z** (X=5-OH) with sodium hydride, followed by reaction with methyl iodide or methanesulfonyl chloride yielded **1aa** (X=5-OMe) and **1bb** (X=5-OSO_2Me), respectively. Hydrogenation of a nitro group of **1j** over 10%Pd/C gave the amine (**1cc**), which was acylated to afford the acylamide (**1dd**). Sulfonamides (**1ee** and **1ff**) were obtained by reaction of **1cc** (X=5-NH_2) with sulfonyl chlorides. The acid (**1yy**: $m=1$, $n=5$, R=Et, X=5-COOH, Y=H) was converted into the esters (**1hh** and **1ii**) by treatment with a catalytic amount of concentrated HCl in the corresponding alcohol. Successive treatment of the acid (**1yy**) with thionyl chloride and amine gave the amides (**1jj** and **1kk**). The carbamate (**1uu**) was prepared by reaction of the phenol (**1zz**: $m=1$, $n=5$, R=Et, X=5-NO_2, Y=3-OH) with methyl isocyanate in the presence of triethylamine.

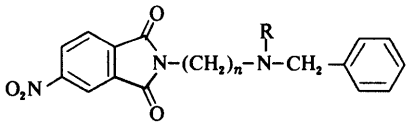
The *cis*-3a,4,5,6,7,7a-hexahydroisoindole-1,3(2*H*)-dione derivative (**9**) was prepared by condensation of *cis*-cyclohexane-1,2-dicarboxylic anhydride and the pentane-1,5-diamine (**8**). The bromide (**4c**) was allowed to react with *N*-(cyclohexylmethyl)-*N*-ethylamine (**10**) to give *N*-cyclohexylmethyl derivative (**11**). An *N*-acetyl derivative (**12**) was prepared by reaction of **1u** with acetic anhydride.

Biological Results and Discussion

The measurement of AChE inhibitory activity was carried out radiometrically *via* the method of Kleinberger and Yanai,[14] which is a slight modification of the method of Johnson and Russell.[15] The results were expressed as IC_{50} values (concentration required to inhibit control enzyme activity by 50%). The inhibitory activities of physostigmine and THA have been measured using the same technique: IC_{50}=220 nM (physostigmine), 300 nM (THA).

The effects on AChE inhibition of variation of the chain length (*n*) as well as the *N*-alkyl substituents (R) were examined, and the results are shown in Table I. Among the compounds having an *N*-Me group (**1a**—**g**), optimum activity was found to be associated with a chain length of 6 carbon atoms (**1d**). Figure 1 illustrates the relationship between enzyme inhibition and the chain length (*n*).

TABLE I. Physicochemical and Biological Properties of 2-[ω-[*N*-Alkyl-*N*-(phenylmethyl)amino]alkyl]-1*H*-isoindole-1,3(2*H*)-diones (**1a—u**)



1a—u

Compd. No.	*n*	R	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N	Inhibition of AChE (IC_{50}, nM)
1a	3	Me	64	156—159	$C_{19}H_{19}N_3O_4 \cdot HCl$	58.54	5.17	10.78	58.28	4.92	10.93	93600
1b	4	Me	61	188—192	$C_{20}H_{21}N_3O_4 \cdot HCl$	59.48	5.49	10.40	59.23	5.38	10.53	8320
1c	5	Me	65	199—202	$C_{21}H_{23}N_3O_4 \cdot HCl$	60.36	5.79	10.06	60.17	5.55	10.26	1360
1d	6	Me	69	161—163	$C_{22}H_{25}N_3O_4 \cdot HCl$	61.18	6.07	9.73	61.08	6.10	9.86	474
1e	7	Me	71	97—99	$C_{23}H_{27}N_3O_4 \cdot HCl$	61.95	6.33	9.42	61.80	6.48	9.33	1780
1f	8	Me	71	84—87	$C_{24}H_{29}N_3O_4 \cdot HCl$	62.67	6.57	9.14	62.51	6.69	9.01	8250
1g	9	Me	68	88—93	$C_{25}H_{31}N_3O_4 \cdot HCl$	63.35	6.80	8.87	63.29	6.72	8.84	27900
1h	3	Et	78	143—145	$C_{20}H_{21}N_3O_4 \cdot HCl$	59.48	5.49	10.40	59.39	5.31	10.20	12300
1i	4	Et	64	147—150	$C_{21}H_{23}N_3O_4 \cdot HCl$	60.36	5.79	10.06	60.09	5.56	10.35	252
1j	5	Et	67	126—129	$C_{22}H_{25}N_3O_4 \cdot HCl$	61.18	6.07	9.73	60.93	6.11	9.78	151
1k	6	Et	72	134—137	$C_{23}H_{27}N_3O_4 \cdot HCl$	61.95	6.33	9.42	61.88	6.09	9.28	607
1l	7	Et	70	86—89	$C_{24}H_{29}N_3O_4 \cdot HCl$	62.67	6.57	9.14	62.59	6.53	9.11	4530
1m	3	iso-Pr	26	199—202	$C_{21}H_{23}N_3O_4 \cdot HCl$	60.36	5.79	10.06	60.21	5.83	10.01	56200
1n	4	iso-Pr	57	154—157	$C_{22}H_{25}N_3O_4 \cdot HCl$	61.18	6.07	9.73	60.98	6.03	9.82	495
1o	5	iso-Pr	67	185—188	$C_{23}H_{27}N_3O_4 \cdot HCl$	61.95	6.33	9.42	61.88	6.30	9.37	824
1p	3	Pr	43	204—206	$C_{21}H_{23}N_3O_4 \cdot HCl$	60.36	5.79	10.06	60.08	5.89	9.93	44000
1q	4	Pr	53	Amorphous	$C_{22}H_{25}N_3O_4 \cdot HCl$	61.18	6.07	9.73	61.10	5.99	9.69	1870
1r	5	Pr	57	Amorphous	$C_{23}H_{27}N_3O_4 \cdot HCl$	61.95	6.33	9.42	61.69	6.17	9.32	2650
1s[a]	4	Bu	69	Amorphous	$C_{23}H_{27}N_3O_4 \cdot HCl$	61.95	6.33	9.42	61.32	6.27	9.17	19600
1t	5	Bu	61	128—130	$C_{24}H_{29}N_3O_4 \cdot HCl$	62.67	6.57	9.14	62.54	6.72	9.04	13300
1u	4	H	55	244—246	$C_{19}H_{19}N_3O_4 \cdot HCl$	58.54	5.17	10.78	58.26	4.88	10.60	13800

a) MS *m/z*: 409 [M^+].

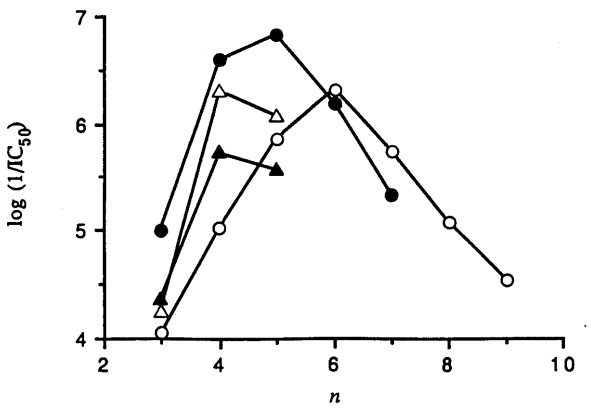


Fig. 1. Relationship between AChE Inhibition and Carbon Chain Length (*n*) of **1** with Various *N*-Alkyl Substituents (R) R=Me (○); Et (●); iso-Pr (△); Pr (▲)

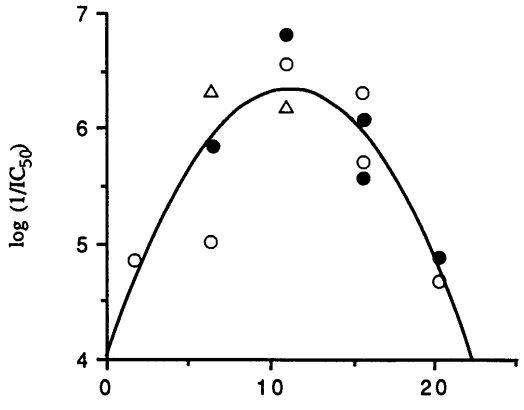


Fig. 2. Relationship between AChE Inhibition and MR Values of *N*-Alkyl Substituents (R) of **1** with Carbon Chain Lengths of 4 (○), 5 (●), and 6 (△)

The potency gradually decreased either by extending or reducing the chain length. A similar tendency was observed in the compounds having ethyl, propyl, or isopropyl groups on the benzylamino nitrogen. The number of carbon atoms (*n*) with optimum inhibitory activities were 5 (**1j**: R=Et) and 4 (**1q**: R=Pr or **1n**: R=iso-Pr). Interestingly, all these compounds have the same total of carbon atoms ($n+R=7$). This may suggest that a suitable size (and/or hydrophobicity) of molecule is required in order to access the catalytic surface of the enzyme.

The relationship between the potency of AChE inhibition and the size of *N*-alkyl substituents (R) is illustrated in Fig. 2. Molar refractivity (MR) of the substituent is used as a steric parameter. Quantitative analysis gave good correlation as shown in Eq. 1. The activity was related parabolically to the MR values. The optimum value was calculated to be 10.95, which corresponds to that of an ethyl group. Using a hydrophobic parameter (π), good correlation was also obtained (Eq. 2). Once again the activity was related parabolically to π with optimum value of 1.003. In each equation, *n*, *r*, *s*, and *F* represent the number of compounds used, correlation coefficient, standard deviation, and value in the *F* test, respectively. The number in parentheses is the 95%

confidence interval. The compounds used and their parameters are listed in Table II. Because of the known high collinearity between π and MR ($r=0.99$)[16] for the substituents (H, Me, Et, iso-Pr, Pr, and Bu), it is very hard to separate the hydrophobic and steric parameters definitely. Our data is not sufficient to determine which factor contributes more to the activity.[17]

$$\log(1/IC_{50}) = 0.416MR - 0.019MR^2 + 4.044 \quad (1)$$
$$(0.189) \quad (0.008) \quad (1.007)$$
$$(n=13,\ r=0.860,\ s=0.394,\ F_{10}^2=14.18)$$

$$\log(1/IC_{50}) = 3.205\pi - 1.597\pi^2 + 4.722 \quad (2)$$
$$(1.438) \quad (0.650) \quad (0.716)$$
$$(n=13,\ r=0.870,\ s=0.380,\ F_{10}^2=15.56)$$

TABLE II. Acetylcholinesterase Inhibitory Activity and Physicochemical Parameters[a] of 2-[ω-[*N*-Alkyl-*N*-(phenylmethyl)amino]alkyl]-5-nitro-1*H*-isoindole-1,3(2*H*)-diones (**1**)

Compd. No.	n	R	MR	π	log(1/IC$_{50}$) Obsd.	Eq. 1 Calcd (Δ)[b]	Eq. 2 Calcd (Δ)[b]
1u	4	H	1.68	0.00	4.86	4.69 (0.17)	4.72 (0.14)
1b	4	Me	6.34	0.50	5.08	5.93 (−0.85)	5.92 (−0.84)
1c	5	Me	6.34	0.50	5.87	5.93 (−0.06)	5.92 (−0.05)
1d	6	Me	6.34	0.50	6.32	5.93 (0.39)	5.92 (0.40)
1i	4	Et	11.00	1.00	6.60	6.36 (0.24)	6.33 (0.27)
1j	5	Et	11.00	1.00	6.82	6.36 (0.46)	6.33 (0.49)
1k	6	Et	11.00	1.00	6.22	6.36 (−0.14)	6.33 (−0.11)
1n	4	iso-Pr	15.66	1.37	6.31	5.98 (0.33)	6.11 (0.20)
1o	5	iso-Pr	15.66	1.37	6.08	5.98 (0.10)	6.11 (−0.03)
1q	4	Pr	15.66	1.50	5.73	5.98 (−0.25)	5.94 (−0.21)
1r	5	Pr	15.66	1.50	5.58	5.98 (−0.40)	5.94 (−0.36)
1s	4	Bu	20.32	2.00	4.71	4.79 (−0.08)	4.74 (−0.03)
1t	5	Bu	20.32	2.00	4.88	4.79 (0.09)	4.74 (0.14)

a) Taken from the literature.[16] b) Δ, the difference between observed and calculated values.

Among compounds (**1a—u**), **1j** ($n=5$, R=Et) showed the most potent activity, which was greater than that shown by either physostigmine or THA.

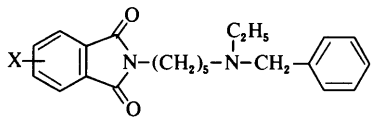
The effects of substituent (X) on the phthalimide moiety have been examined and the results are shown in Table III. A nitro group at the 5-position on the phthalimide moiety can be seen to enhance inhibitory activity (**1j** *vs.* **1v**). The 4-nitro derivative (**1w**) was less active than **1j**. The effects of substitution at the 5-position on the phthalimide moiety were quantitatively analyzed by the Hansch–Fujita method. Good correlation was obtained by regression analysis as shown in Eq. 3. The compounds used and their parameters are listed in Table IV. Correlations between the parameters used are insignificant, as shown in Table V.

$$\log(1/IC_{50}) = -0.279\pi + 0.926((\sigma_m+\sigma_p)/2) + 5.873 \quad (3)$$
$$(0.150) \quad (0.418) \quad (0.141)$$
$$(n=16,\ r=0.844,\ s=0.209,\ F_{13}^2=16.11)$$

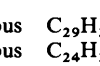
Initial studies using either σ_m or σ_p gave similar results (Eqs. 4 and 5). This indicates that the two carbonyls of the phthalimide moiety make almost equal contributions to AChE inhibition. Therefore, since substituents located meta to one carbonyl are also para to the other carbonyl of the phthalimide moiety, an average value of the electronic parameters, $(\sigma_m+\sigma_p)/2$, is used in Eq. 3. According to Eq. 3, hydrophilic and electron-withdrawing substituents enhance AChE inhibitory activity. In this study, satisfactory correlation was not obtained using steric parameters such as MR and Es.

$$\log(1/IC_{50}) = -0.244\pi + 1.240\sigma_m + 5.750 \quad (4)$$
$$(0.144) \quad (0.538) \quad (0.173)$$
$$(n=16,\ r=0.852,\ s=0.204,\ F_{13}^2=17.26)$$

TABLE III. Physicochemical and Biological Properties of 2-[5-[*N*-Ethyl-*N*-(phenylmethyl)amino]pentyl]-1*H*-isoindole-1,3(2*H*)-diones (**1v—kk**)



1v—kk

Compd. No.	X	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N	Inhibition of AChE (IC$_{50}$, nM)
1v	H	72	92—94	$C_{22}H_{26}N_2O_2\cdot HCl$	68.29	7.03	7.24	68.11	6.91	7.31	3370
1w[a]	4-NO_2	67	Amorphous	$C_{22}H_{25}N_3O_4\cdot HCl$	61.18	6.07	9.73	60.72	6.11	9.66	637
1x	5-Me	71	Amorphous	$C_{23}H_{28}N_2O_2\cdot HCl$	68.90	7.29	6.99	68.81	7.25	6.91	1750
1y	5-Cl	69	Amorphous	$C_{22}H_{25}ClN_2O_2\cdot HCl$	62.71	6.22	6.65	62.64	6.09	6.48	1330
1z[b]	5-OH	70	Amorphous	$C_{22}H_{26}N_2O_3\cdot HCl$	65.58	6.75	6.95	65.02	6.79	6.94	2450
1aa[c]	5-OMe	77	Amorphous	$C_{23}H_{28}N_2O_3\cdot HCl$	66.26	7.01	6.72	65.71	6.93	6.53	701
1bb	5-OSO_2Me	63	Amorphous	$C_{23}H_{28}N_2O_5S\cdot HCl$	57.43	6.08	5.82	57.33	6.01	5.75	379
1cc[d]	5-NH_2	76	Amorphous	$C_{22}H_{27}N_3O_2\cdot 2HCl$	60.28	6.67	9.59	59.82	6.79	9.43	1070
1dd	5-NHAc	81	120—121	$C_{24}H_{29}N_3O_3$	70.74	7.17	10.31	70.68	7.01	10.19	328
1ee	5-$NHSO_2Me$	63	94—96	$C_{23}H_{29}N_3O_4S$	62.28	6.59	9.47	62.03	6.44	9.41	467
1ff	5-$NHSO_2$-  -Me	68	109—112	$C_{29}H_{33}N_3O_4S$	67.03	6.40	8.09	66.93	6.33	7.92	2190
1gg	5-COPh	69	Amorphous	$C_{29}H_{30}N_2O_3\cdot HCl$	70.94	6.36	5.71	70.91	6.33	5.64	1070
1hh	5-CO_2Me	89	Amorphous	$C_{24}H_{28}N_2O_4\cdot HCl$	64.79	6.57	6.30	64.67	6.56	6.25	569
1ii	5-CO_2Et	49	Amorphous	$C_{25}H_{30}N_2O_4\cdot HCl$	65.42	6.81	6.10	65.31	6.63	6.03	679
1jj	5-CONHMe	48	93—95	$C_{24}H_{29}N_3O_3$	70.74	7.17	10.31	70.59	7.02	10.22	380
1kk[e]	5-$CONEt_2$	61	Amorphous	$C_{27}H_{35}N_3O_3\cdot HCl$	66.72	7.47	8.65	66.64	7.41	8.57	980

a) MS *m/z*: 395 [M$^+$]. b) MS *m/z*: 366 [M$^+$]. c) MS *m/z*:380 [M$^+$]. d) MS *m/z*: 365 [M$^+$]. e) MS *m/z*: 449 [M$^+$].

$$\log(1/IC_{50}) = -0.294\pi + 0.696\sigma_p + 5.953 \quad (5)$$
$$(0.162)\quad (0.347)\quad (0.132)$$
$$(n=16,\ r=0.822,\ s=0.222,\ F_{13}^2=13.53)$$

The effects of substitution on the phenyl ring of the benzylamino moiety are shown in Table VI. In general, inhibitory activity decreased with the position of substituent in the order *ortho*>*meta*>*para*. Among the substituents examined, a methoxy group at either *meta* or *ortho* position (**1pp** and **1qq**) increased the activity, whereas 2,3-dimethoxy substitution (**1tt**) resulted in a reduction of inhibitory potency. Incorporation of a $MeNHCO_2$-group at the *meta* position (**1uu**), which is expected to contribute to the activity by carbamoylating the esteratic site, resulted in a sharp enhancement of inhibitory potency. The IC_{50} value has been measured as about 1000 times stronger than that of physostigmine and THA.

Table VI also shows the effects of chain length (*m*) of the *N*-(ω-phenylalkyl)amino moiety. Among the compounds examined (**1j** and **1vv**—**xx**), **1j** (*m*=1) was the most potent inhibitor. Extension of the chain length caused a gradual decrease in the activity. Surprisingly, **1ww** was as potent as **1j**. The reason is not clear, but one explanation could be that, by bending the propyl chain, the phenyl ring and the nitrogen of the (3-phenylpropyl)amino moiety could be

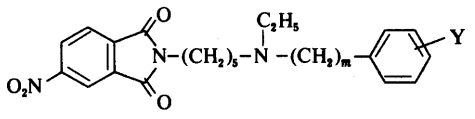
TABLE IV. Acetylcholinesterase Inhibitory Activity and Physicochemical Parameters[a] of 5-Substituted-2-[5-[*N*-ethyl-*N*-(phenylmethyl)amino]pentyl]-1*H*-isoindole-1,3(2*H*)-diones (**1**)

Compd. No.	X	π	$(\sigma_m+\sigma_p)/2$	log(1/IC50) Obsd.	Calcd[b]	(Δ)[c]
1v	H	0.00	0.00	5.47	5.87	(−0.40)
1j	NO_2	−0.28	0.745	6.82	6.64	(0.18)
1x	Me	0.56	−0.12	5.76	5.61	(0.15)
1y	Cl	0.71	0.30	5.88	5.95	(−0.07)
1z	OH	−0.67	−0.125	5.61	5.94	(−0.33)
1aa	OMe	−0.02	−0.075	6.15	5.81	(0.34)
1bb	OSO_2Me	−0.88	0.375	6.42	6.47	(−0.05)
1cc	NH_2	−1.23	−0.41	5.97	5.84	(0.13)
1dd	NHAc	−0.97	0.105	6.48	6.24	(0.24)
1ee	$NHSO_2Me$	−1.18	0.115	6.33	6.31	(0.02)
1ff	$NHSO_2$-C6H4-Me	0.95[d]	0.085[d]	5.66	5.69	(−0.03)
1gg	COPh	1.05	0.385	5.97	5.94	(0.03)
1hh	CO_2Me	−0.01	0.41	6.24	6.26	(−0.02)
1ii	CO_2Et	0.51	0.41	6.17	6.11	(0.06)
1jj	CONHMe	−1.27	0.355	6.42	6.56	(−0.14)
1kk	$CONEt_2$	0.23[d]	0.355[d]	6.01	6.14	(−0.13)

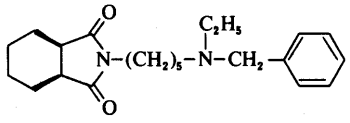
a) Taken from the literature.[20] *b*) Calculated from Eq. 3. *c*) Δ, the difference between observed and calculated values. *d*) Estimated from data for closely related substituents.

TABLE V. Simple Correlation Matrix for the Parameters of Eq. 3

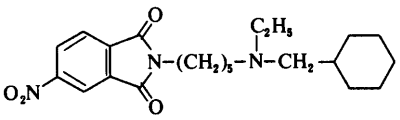
	π	$(\sigma_m+\sigma_p)/2$
π	1.000	
$(\sigma_m+\sigma_p)/2$	0.214	1.000



1ll—**xx**



9



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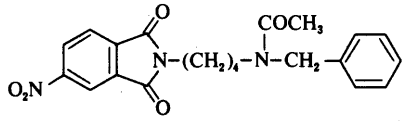

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TABLE VI. Physicochemical and Biological Properties of 2-[5-[*N*-Ethyl-*N*-(ω-phenylalkyl)amino]pentyl]-1*H*-isoindole-1,3(2*H*)-diones (**1ll**—**xx**) and Their Analogues (**9**, **11**, **12**)

Compd. No.	*m*	Y	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N	Inhibition of AChE (IC_{50}, nM)
1ll	1	4-Cl	55	138—142	$C_{22}H_{24}ClN_3O_4\cdot HCl$	56.66	5.40	9.01	56.59	5.32	8.97	240
1mm	1	3-Cl	55	148—152	$C_{22}H_{24}ClN_3O_4\cdot HCl$	56.66	5.40	9.01	56.61	5.21	8.88	244
1nn	1	2-Cl	57	Amorphous	$C_{22}H_{24}ClN_3O_4\cdot HCl$	56.66	5.40	9.01	56.45	5.36	8.92	129
1oo	1	4-OMe	60	Amorphous	$C_{23}H_{27}N_3O_5\cdot HCl$	59.80	6.11	9.10	59.68	6.01	9.06	269
1pp[a]	1	3-OMe	61	113—114	$C_{23}H_{27}N_3O_5\cdot HCl$	59.80	6.11	9.10	58.39	6.09	8.97	44.8
1qq	1	2-OMe	58	84—87	$C_{23}H_{27}N_3O_5\cdot HCl$	59.80	6.11	9.10	59.77	6.05	9.02	23.9
1rr[b]	1	2-F	57	Amorphous	$C_{22}H_{24}FN_3O_4\cdot HCl$	58.73	5.60	9.34	58.34	5.63	9.21	282
1ss[c]	1	2-Me	60	Amorphous	$C_{23}H_{27}N_3O_4\cdot HCl$	61.95	6.33	9.42	61.49	6.31	9.36	98.8
1tt	1	2,3-diOMe	57	Amorphous	$C_{24}H_{29}N_3O_6\cdot HCl$	58.59	6.15	8.54	58.41	6.02	8.44	274
1uu	1	3-OCONHMe	49	Amorphous	$C_{24}H_{28}N_4O_6\cdot HCl$	57.09	5.79	11.10	56.91	5.77	11.07	0.380
1vv	2	H	48	152—154	$C_{23}H_{27}N_3O_4\cdot HCl$	61.95	6.33	9.42	61.92	6.10	9.29	770
1ww	3	H	48	156—158	$C_{24}H_{29}N_3O_4\cdot HCl$	62.67	6.57	9.14	62.39	6.54	9.01	161
1xx	4	H	62	123—124	$C_{25}H_{31}N_3O_4\cdot HCl$	63.35	6.80	8.87	63.32	6.78	8.61	1750
9	—	—	46	135—137	$C_{22}H_{32}N_2O_2\cdot HCl$	67.24	8.46	7.13	67.11	8.52	6.98	18500
11[d]	—	—	43	Amorphous	$C_{22}H_{31}N_3O_4\cdot HCl$	60.33	7.36	9.59	60.09	7.43	9.56	1090
12[e]	—	—	95	Oil	$C_{21}H_{21}N_3O_5$	—	—	—	—	—	—	>100000

a) MS *m/z*: 425 [M^+]. *b*) MS *m/z*: 413 [M^+]. *c*) MS *m/z*: 409 [M^+]. *d*) MS *m/z*: 401 [M^+]. *e*) The structure was confirmed by IR, NMR and MS spectra (see Experimental).

placed in a similar position to the position they occupy in the benzylamino moiety.

Saturation of the benzene ring of the phthalimide or the phenyl ring of the benzylamino moiety decreased inhibitory activity 5- (**9** *vs.* **1v**) and 7-fold (**11** *vs.* **1j**), respectively. Replacement of the *N*-ethyl group of **1i** with an *N*-acetyl (**12**) reduced the potency remarkably.

The results obtained in this work can be summarized as follows.

1. Optimum activity was closely associated with a five carbon chain length ($n=5$) separating the phthalimide moiety from the nitrogen atom of the benzylamino moiety. 2. The most potent activity was observed when the *N*-alkyl substituent (R) was an ethyl group. 3. The compounds (**1d, j, n, q**) showed optimum activities of the *N*-Me, Et, iso-Pr, and Pr derivatives, each having the same total of carbon atoms ($n+R=7$). 4. Saturation of the benzene ring of the phthalimide moiety caused an approximately 5-fold decrease in activity. 5. Quantitative study of the substituents (X) on the phthalimide moiety revealed that hydrophilic and electron-withdrawing groups enhance the potency. 6. Reduction of the basicity of the benzylamino nitrogen significantly decreased the activity. 7. As an *N*-(ω-phenylalkyl)amino moiety, the benzylamino group was best for potent inhibition. 8. Methoxy substitution on the phenyl ring of the benzylamino moiety increased the activity. 9. Incorporation of a 3-$MeNHCO_2$-group on the phenyl ring of the benzylamino moiety greatly enhanced the inhibitory potency. 10. Saturation of the phenyl ring of the benzylamino moiety led to an approximately 7-fold loss in enzyme inhibition.

The above results seem to support the working hypothesis which has been the basis of this work. This hypothesis includes the following two points. The first, which may be supported by results 6, 9, and 10, is that the benzylamino moiety of **1** binds to the anionic site as well as to the hydrophobic binding site (HBS-1) which is closely adjacent to the anionic and esteratic sites. These interactions correspond to those postulated for binding in the case of miotine.[10] Results 1 and 4 may support the second point, which is that the phthalimide moiety interacts with another hydrophobic binding site (HBS-2) some distance removed from the anionic site. The distance can be estimated at a seven carbon chain length or less.[18] Hydrophobic interactions with both HBS-1 and HBS-2 seem to be mainly π–π interactions.[19] In addition, result 5 may provide us with some information about the nature of HBS-2. The enzyme may demand particular hydrophobic requirements with regard to the substituents (X). It is not clear how the electron-withdrawing character of the substituents (X) contributes to an enhancement of activity. One explanation may be that, after withdrawing the electron, the substituents interact with the enzyme electrostatically.

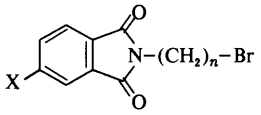
In conclusion, the working hypothesis proposed in this paper led to the discovery of several new potent AChE inhibitors, demonstrating its effectiveness in the design of such inhibitors. Detailed studies of structure–activity relationships may confirm the existence of a hydrophobic binding site (HBS-2) along with its location relative to the anionic site. Further studies concerning the nature of HBS-2 are described in an accompanying paper[12] in this issue.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Infrared (IR) spectra were taken on a Hitachi 260-10 spectrophotometer using KBr disks for solids and liquid films for oils. Mass spectra (MS) were measured on a JOEL JMS-01SC spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Varian EM-390 NMR spectrometer with tetramethylsilane as an internal standard. The following abbreviations are used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad. Chromatographic purifications were carried out on silica gel columns (Kieselgel 60, 0.063—0.200 mm, Merck).

Preparation of 2-[ω-[*N*-Alkyl-*N*-(ω-phenylalkyl)amino]alkyl]-1*H*-isoindole-1,3(2*H*)-diones (1). Method A. 2-(5-Bromopentyl)-5-nitro-1*H*-isoindole-1,3(2*H*)-dione (4c) Sodium hydride (1.38 g, oil free) was added portionwise to a solution of 5-nitro-1*H*-isoindole-1,3(2*H*)-dione (10.5 g) in *N*,*N*-dimethylformamide (DMF, 50 ml). The mixture was stirred at 60 °C for 30 min, cooled to 0 °C, and a solution of 1,5-dibromopentane (25.0 g) in acetone (50 ml) was added. The mixture was refluxed for 16 h, cooled to room temperature, and the resulting precipitate was removed by filtration. The filtrate was concentrated to give colorless crystals, which were recrystallized from methanol–ether to afford colorless cubes (17.3 g). IR (KBr): 3466, 3110, 3056, 2942, 2868, 1772, 1711, 1624, 1543 cm^{-1}.

TABLE VII. Physicochemical Properties of 2-(ω-Bromoalkyl)-1*H*-isoindole-1,3(2*H*)-diones (**4a—h**)



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Compd. No.	X	*n*	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N
4a	NO_2	3	94	103—104	$C_{11}H_9BrN_2O_4$	42.20	2.90	8.95	42.05	2.79	8.81
4b	NO_2	4	90	95—96	$C_{12}H_{11}BrN_2O_4$	44.06	3.39	8.56	44.01	3.20	8.42
4c	NO_2	5	93	78—79	$C_{13}H_{13}BrN_2O_4$	45.77	3.84	8.21	45.58	3.78	7.99
4d	NO_2	6	92	83—85	$C_{14}H_{15}BrN_2O_4$	47.34	4.26	7.89	47.09	4.22	7.63
4e	NO_2	7	94	73—74	$C_{15}H_{17}BrN_2O_4$	48.80	4.64	7.59	48.96	4.60	7.76
4f	NO_2	8	90	84—85	$C_{16}H_{19}BrN_2O_4$	50.14	5.00	7.31	50.08	4.93	7.31
4g	NO_2	9	94	81—83	$C_{17}H_{21}BrN_2O_4$	51.40	5.33	7.05	51.66	5.22	7.10
4h	H	5	79	60—61[a]	$C_{13}H_{14}BrNO_2$	52.72	4.76	4.73	52.84	4.68	4.89

a) Lit.[21] mp 58—60 °C.

TABLE VIII. Spectral Data of **1a—i, k—t, v, x—z, bb, ee—gg, ii, kk—tt, vv—xx, 4a, b, d—h, 9,** and **11**

Compd.	IR (KBr) cm^{-1}	^{1}H-NMR (DMSO-d_6) δ[a]
1a	3444, 1778, 1716, 1624, 1539	1.89—2.33 (2H, m), 2.58 (3H, br s), 2.88—3.29 (2H, m), 3.67 (2H, t, J=6 Hz), 4.00—4.50 (2H, m), 7.23—7.75 (5H, m), 8.15 (1H, d, J=9 Hz), 8.51 (1H, d, J=1.5 Hz), 8.67 (1H, dd, J=1.5, 9 Hz), 11.4 (1H, br s)
1b	3436, 1775, 1717, 1543	1.37—2.03 (4H, m), 2.58 (3H, d, J=5 Hz), 2.80—3.77 (4H, m), 3.98—4.37 (2H, m), 7.30—7.73 (5H, m), 8.12 (1H, d, J=9 Hz), 8.46 (1H, d, J=1.5 Hz), 8.62 (1H, dd, J=1.5, 9 Hz), 11.4 (1H, br s)
1c	3446, 1773, 1716, 1625, 1544	1.05—2.00 (6H, m), 2.59 (3H, br s), 2.78—3.76 (4H, m), 4.03—4.47 (2H, m), 7.30—7.76 (5H, m), 8.12 (1H, d, J=9 Hz), 8.48 (1H, d, J=1.5 Hz), 8.63 (1H, dd, J=1.5, 9 Hz), 11.45 (1H, br s)
1d	3436, 1774, 1717, 1624, 1543	1.15—1.93 (8H, m), 2.58 (3H, d, J=5 Hz), 2.75—3.13 (2H, m), 3.59 (2H, t, J=6 Hz), 4.05—4.45 (2H, m), 7.32—7.75 (5H, m), 8.13 (1H, d, J=8 Hz), 8.48 (1H, d, J=1.5 Hz), 8.64 (1H, dd, J=1.5, 8 Hz), 11.4 (1H, br s)
1e	3430, 2938, 1777, 1717, 1624, 1541	1.00—1.97 (10H, m), 2.59 (3H, d, J=5 Hz), 2.75—3.13 (2H, m), 3.60 (2H, t, J=6.5 Hz), 4.00—4.50 (2H, m), 7.26—7.80 (5H, m), 8.13 (1H, d, J=8 Hz), 8.48 (1H, d, J=1.5 Hz), 8.66 (1H, dd, J=1.5, 8 Hz), 11.45 (1H, br s)
1f	3436, 2934, 1777, 1718, 1624, 1540	1.03—1.97 (12H, m), 2.63 (3H, d, J=5 Hz), 2.75—3.13 (2H, m), 3.60 (2H, t, J=6.5 Hz), 4.00—4.50 (2H, m), 7.30—7.77 (5H, m), 8.14 (1H, d, J=8 Hz), 8.49 (1H, d, J=1.5 Hz), 8.66 (1H, dd, J=1.5, 8 Hz), 11.43 (1H, br s)
1g	3430, 2932, 1776, 1716, 1624, 1541	1.03—2.00 (14H, m), 2.62 (3H, d, J=5 Hz), 2.73—3.12 (2H, m), 3.60 (2H, t J=6.5 Hz), 4.05—4.50 (2H, m), 7.27—7.77 (5H, m), 8.11 (1H, d, J=8 Hz), 8.46 (1H, d, J=1.5 Hz), 8.63 (1H, dd, J=1.5, 8 Hz), 11.26 (1H, br s)
1h	3436, 2948, 1782, 1716, 1625, 1540	1.26 (3H, t, J=6 Hz), 1.85—2.30 (2H, m), 2.70—3.20 (4H, m), 3.64 (2H, t, J=6 Hz), 4.26 (2H, d, J=5 Hz), 7.22—7.74 (5H, m), 8.13 (1H, d, J=8 Hz), 8.47 (1H, d, J=1.5 Hz), 8.66 (1H, dd, J=1.5, 8 Hz), 11.43 (1H, br s)
1i	3432, 1778, 1714, 1624, 1540	1.26 (3H, t, J=7.5 Hz), 1.43—2.00 (4H, m), 2.70—3.20 (4H, m), 3.50—3.85 (2H, m), 4.25 (2H, d, J=5 Hz), 7.30—7.80 (5H, m), 8.12 (1H, d, J=9 Hz), 8.47 (1H, d, J=1.5 Hz), 8.63 (1H, dd, J=1.5, 9 Hz), 9.55 (1H, br s)
1k	3432, 2946, 1775, 1715, 1623, 1543	1.10—1.93 (11H, m), 2.66–3.20 (4H, m), 3.60 (2H, t, J=6.5 Hz), 4.26 (2H, d, J=5 Hz), 7.26—7.83 (5H, m), 8.14 (1H, d, J=8 Hz), 8.49 (1H, d, J=1.5 Hz), 8.66 (1H, dd, J=1.5, 8 Hz), 11.37 (1H, br s)
1l	3386, 2938, 1778, 1721, 1623, 1541	1.10—1.95 (13H, m), 2.70—3.26 (4H, m), 3.60 (2H, t, J=6 Hz), 4.30 (2H, d, J=6 Hz), 7.30—7.80 (5H, m), 8.12 (1H, d, J=8 Hz), 8.46 (1H, d, J=1.5 Hz), 8.63 (1H, dd, J=1.5, 8 Hz), 10.87 (1H, br s)
1m[b]	2966, 2822, 1778, 1718, 1620, 1542[c]	1.03 (6H, d, J=6.5 Hz), 1.70—1.87 (2H, m), 2.50 (2H, t, J=6.5 Hz), 2.91—3.06 (1H, m), 3.54 (2H, s), 3.73 (2H, t, J=7.5 Hz), 7.12—7.41 (5H, m), 8.01 (1H, d, J=8 Hz), 8.58 (1H, dd, J=2, 8 Hz), 8.63 (1H, d, J=2 Hz)[d]
1n	3444, 1782, 1709, 1621, 1544	1.32 (6H, d, J=6 Hz), 1.45—1.93 (4H, m), 2.78—3.77 (4H, m), 3.98—4.40 (3H, m), 7.26—7.85 (5H, m), 8.12 (1H, d, J=8 Hz), 8.47 (1H, d, J=1.5 Hz), 8.65 (1H, dd, J=1.5, 8 Hz), 10.83 (1H, br s)
1o	3436, 1777, 1718, 1623, 1541	1.15—1.93 (12H, m), 2.67—3.10 (2H, m), 3.57 (2H, t, J=6 Hz), 3.95—4.46 (3H, m), 7.30—7.87 (5H, m), 8.11 (1H, d, J=8 Hz), 8.46 (1H, d, J=1.5 Hz), 8.63 (1H, dd, J=1.5, 8 Hz), 11.4 (1H, br s)
1p[b]	2958, 2802, 1779, 1719, 1623, 1540[c]	0.88 (3H, t, J=7 Hz), 1.42—1.57 (2H, m), 1.77—1.93 (2H, m), 2.39 (2H, t, J=7 Hz), 2.49 (2H, t, J=7 Hz), 3.54 (2H, s), 3.76 (2H, t, J=7 Hz), 7.13—7.36 (5H, m), 8.02 (1H, d, J=8 Hz), 8.59 (1H, dd, J=2, 8 Hz), 8.64 (1H, d, J=2 Hz)[d]
1q	3432, 1778, 1714, 1624, 1540	0.90 (3H, t, J=6 Hz), 1.45—2.03 (6H, m), 2.55—3.17 (4H, m), 3.63 (2H, t, J=6 Hz), 4.15—4.40 (2H, m), 7.23—7.80 (5H, m), 8.12 (1H, d, J=8 Hz), 8.48 (1H, d, J=1.5 Hz), 8.65 (1H, dd, J=1.5, 8 Hz), 9.60 (1H, br s)
1r	3436, 1778, 1718, 1623, 1540	0.87 (3H, t, J=7 Hz), 1.18—1.38 (2H, m), 1.53—1.87 (6H, m), 2.82—3.08 (4H, m), 3.63 (2H, t, J=7.5 Hz), 4.31 (2H, d, J=5 Hz), 7.40—7.63 (5H, m), 8.13 (1H, d, J=8 Hz), 8.50 (1H, d, J=2 Hz), 8.64 (1H, dd, J=2, 8 Hz), 9.95 (1H, br s)
1s	3440, 1779, 1719, 1623, 1540	0.85 (3H, t, J=6 Hz), 0.97—2.00 (8H, m), 2.64—3.18 (4H, m), 3.61 (2H, t, J=6 Hz), 4.26 (2H, br d, J=4.5 Hz), 7.28—7.80 (5H, m), 8.12 (1H, d, J=8 Hz), 8.46 (1H, d, J=1.5 Hz), 8.63 (1H, dd, J=1.5, 8 Hz), 11.2 (1H, br s)
1t	3428, 2948, 1777, 1722, 1617, 1537	0.88 (3H, t, J=7 Hz), 1.14—1.37 (4H, m), 1.53—1.85 (6H, m), 2.83—3.07 (4H, m), 3.63 (2H, t, J=7.5 Hz), 4.30 (2H, d, J=5 Hz), 7.40—7.52 (3H, m), 7.55—7.68 (2H, m), 8.13 (1H, d, J=8 Hz), 8.50 (1H, d, J=2 Hz), 8.64 (1H, dd, J=2, 8 Hz), 10.37 (1H, br s)
1v[b]	2936, 1772, 1712[c]	1.00 (3H, t, J=7 Hz), 1.12—1.84 (6H, m), 2.23—2.61 (4H, m), 3.51 (2H, s), 3.64 (2H, t, J=7 Hz), 7.27 (5H, s), 7.60—7.92 (4H, m)[d]
1x[b]	2936, 1769, 1709,[c] 1618	1.00 (3H, t, J=7.5 Hz), 1.13—1.85 (6H, m), 2.28—2.64 (4H, m), 2.49 (3H, s), 3.52 (2H, s), 3.63 (2H, t, J=6 Hz), 7.24 (5H, s), 7.45 (1H, d, J=8 Hz), 7.60 (1H, s), 7.68 (1H, d, J=8 Hz)[d]
1y	3424, 2930, 2612, 1771, 1712, 1610	1.07—2.00 (9H, m), 2.76—3.23 (4H, m), 3.57 (2H, t, J=6.5 Hz), 4.26 (2H, d, J=5 Hz), 7.32—7.97 (8H, m), 11.05 (1H, br s)
1z[b]	3450, 2942, 2626, 1764, 1709, 1605[c]	0.96—1.96 (9H, m), 2.53—3.06 (4H, m), 3.54 (2H, t, J=6 Hz), 3.94 (2H, s), 6.86—7.70 (8H, m), 9.55 (1H, s)[d]
1bb[b]	2936, 1774, 1716, 1396, 1370, 1182[c]	1.03—1.95 (9H, m), 2.47—3.00 (4H, m), 3.25 (3H, s), 3.66 (2H, t, J=7 Hz), 3.85 (2H, s), 7.10—8.03 (8H, m)[d]
1ee	3290, 1764, 1700, 1623, 1340, 1160	1.03 (3H, t, J=7 Hz), 1.10—1.85 (6H, m), 2.30—2.70 (4H, m), 3.07 (3H, s), 3.47—3.76 (4H, m), 6.13 (1H, br s), 7.25 (5H, s), 7.40—7.82 (3H, m)[d]
1ff	3438, 2934, 1759, 1700, 1610, 1300, 1125	0.75—1.70 (9H, m), 1.97 (3H, s), 2.13—2.60 (4H, m), 3.13—3.65 (4H, m), 4.25 (1H, br s), 6.57—7.63 (12H, m)[d]
1gg[b]	2938, 1774, 1717, 1666[c]	0.99 (3H, t, J=7.5 Hz), 1.14—1.87 (6H, m), 2.23—2.63 (4H, m), 3.51 (2H, s), 3.66 (2H, t, J=6 Hz), 7.93—8.22 (13H, m)[d]
1ii[b]	2938, 1775, 1721[c]	1.02 (3H, t, J=7 Hz), 1.13—1.85 (6H, m), 1.43 (3H, t, J=7.5 Hz), 2.30—2.64 (4H, m), 3.56 (2H, s), 3.66 (2H, t, J=7 Hz), 4.43 (2H, q, J=7.5 Hz), 7.28 (5H, s), 7.88 (1H, d, J=8 Hz), 8.39 (1H, d, J=8 Hz), 8.46 (1H, s)[d]
1kk[b]	2972, 2938, 1774, 1713, 1638[c]	0.90—1.87 (15H, m), 2.33—2.73 (4H, m), 3.03—3.83 (8H, m), 7.30 (5H, s), 7.61—7.94 (3H, m)[d]
1ll[b]	2938, 1778, 1720, 1541[c]	1.00 (3H, t, J=7 Hz), 1.10—1.84 (6H, m), 2.27—2.61 (4H, m), 3.47 (2H, s), 3.70 (2H, t, J=7 Hz), 7.27 (4H, m), 8.01 (1H, d, J=8 Hz), 8.58 (1H, d, J=8 Hz), 8.64 (1H, s)[d]

TABLE VIII. (continued)

Compd.	IR (KBr) cm^{-1}	^{1}H-NMR (DMSO-d_6) δ[a]
1mm[b]	2966, 2938, 1779, 1720, 1541[c]	1.00 (3H, t, J=7 Hz), 1.13—1.87 (6H, m), 2.23—2.62 (4H, m), 3.48 (2H, s), 3.71 (2H, t, J=6.5 Hz), 7.03—7.33 (4H, m), 8.00 (1H, d, J=8 Hz), 8.58 (1H, d, J=8 Hz), 8.61 (1H, s)[d]
1nn[b]	2936, 1778, 1719, 1541[c]	1.01 (3H, t, J=7 Hz), 1.13—1.90 (6H, m), 2.30—2.67 (4H, m), 3.61 (2H, s), 3.70 (2H, t, J=6 Hz), 7.03—7.57 (4H, m), 8.01 (1H, d, J=9 Hz), 8.60 (1H, d, J=9 Hz), 8.64 (1H, s)[d]
1oo	2936, 1778, 1720, 1541	1.25 (3H, t, J=7.5 Hz), 1.10—2.00 (6H, m), 2.68—3.17 (4H, m), 3.60 (2H, t, J=6 Hz), 3.75 (3H, s), 4.18 (2H, d, J=5 Hz), 6.93 (2H, d, J=9 Hz), 7.58 (2H, d, J=9 Hz), 8.10 (1H, d, J=8 Hz), 8.45 (1H, d, J=1.5 Hz), 8.62 (1H, dd, J=1.5, 8 Hz), 11.03 (1H, br s)
1pp[b]	2938, 1778, 1719, 1541[c]	1.00 (3H, t, J=7 Hz), 1.13—1.90 (6H, m), 2.25—2.63 (4H, m), 3.49 (2H, s), 3.72 (2H, t, J=7 Hz), 3.78 (3H, s), 6.62—6.96 (3H, m), 7.17 (1H, dd, J=8, 8 Hz), 8.01 (1H, d, J=8 Hz), 8.58 (1H, d, J=8 Hz), 8.63 (1H, s)[d]
1qq[b]	2940, 1778, 1717, 1539[c]	1.01 (3H, t, J=7 Hz), 1.16—1.90 (6H, m), 2.33—2.65 (4H, m), 3.54 (2H, s), 3.62 (2H, br t, J=6 Hz), 3.80 (3H, s), 6.73—7.44 (4H, m), 8.00 (1H, d, J=8 Hz), 8.58 (1H, d, J=8 Hz), 8.63 (1H, s)[d]
1rr[b]	2938, 1778, 1720, 1541[c]	1.01 (3H, t, J=7 Hz), 1.13—1.90 (6H, m), 2.30—2.64 (4H, m), 3.57 (2H, s), 3.71 (2H, t, J=7 Hz), 6.80—7.50 (4H, m), 8.01 (1H, d, J=8 Hz), 8.58 (1H, d, J=8 Hz), 8.62 (1H, s)[d]
1ss[b]	2938, 1779, 1719, 1541[c]	0.99 (3H, t, J=7 Hz), 1.13—1.87 (6H, m), 2.31 (3H, s), 2.26—2.61 (4H, m), 3.46 (2H, s), 3.67 (2H, t, J=7 Hz), 6.98—7.40 (4H, m), 7.98 (1H, d, J=8 Hz), 8.56 (1H, d, J=8 Hz), 8.60 (1H, s)[d]
1tt[b]	2936, 1778, 1719, 1541[c]	1.00 (3H, t, J=7 Hz), 1.13—1.96 (6H. m), 2.30—2.63 (4H, m), 3.54 (2H, s), 3.71 (2H, t, J=7 Hz), 3.80 (3H, s), 3.84 (3H, s), 6.67—7.07 (3H, m), 8.02 (1H, d, J=8 Hz), 8.60 (1H, d, J=8 Hz), 8.65 (1H, s)[d]
1vv[b]	2936, 1777, 1719, 1541[c]	1.03 (3H, t, J=7 Hz), 1.16—2.10 (6H, m), 2.33—2.93 (8H, m), 3.73 (2H, t, J=7 Hz), 7.24 (5H, s), 8.05 (1H, d, J=9 Hz), 8.53—8.77 (2H, m)[d]
1ww	3472, 2966, 2476, 1778, 1708, 1626, 1540	1.10—1.41 (5H, m), 1.52—1.73 (4H, m), 1.85—2.05 (2H, m), 2.64 (2H, t, J=7.5 Hz), 2.88—3.07 (6H, m), 3.63 (2H, t, J=7 Hz), 7.20—7.36 (5H, m), 8.13 (1H, d, J=8 Hz), 8.49 (1H, d, J=2 Hz), 8.64 (1H, dd, J=2, 8 Hz), 10.1 (1H, br s)
1xx[b]	2934, 1778, 1719, 1619, 1540[c]	0.98 (3H, t, J=7 Hz), 1.24—1.87 (10H, m), 2.33—2.67 (8H, m), 3.73 (2H, t, J=7.5 Hz), 7.11—7.33 (5H, m), 8.02 (1H, d, J=8 Hz), 8.59 (1H, dd, J=2, 8 Hz), 8.65 (1H, d, J=2 Hz)[d]
4a	3470, 2950, 1773, 1712, 1623, 1538	2.13—2.50 (2H, m), 3.43 (2H, t, J=6 Hz), 3.92 (2H, t, J=6 Hz), 8.08 (1H, d, J=9 Hz), 8.67 (1H, d, J=9 Hz), 8.72 (1H, s)[d]
4b	3460, 2954, 1779, 1711, 1625, 1541	1.53—2.10 (4H, m), 3.43—3.80 (4H, m), 8.12 (1H, d, J=9 Hz), 8.46 (1H, d, J=1.5 Hz), 8.63 (1H, dd, J=1.5, 9 Hz)
4d	3452, 1774, 1706, 1625, 1543	1.20—2.04 (8H, m), 3.37 (2H, t, J=6 Hz), 3.73 (2H, t, J=6 Hz), 8.05 (1H, d, J=8 Hz), 8.62 (1H, d, J=8 Hz), 8.67 (1H, s)[d]
4e	3450, 2934, 2860, 1772, 1708, 1624, 1542	1.15—2.06 (10H, m), 3.38 (2H, t, J=7 Hz), 3.73 (2H, t, J=7 Hz), 8.06 (1H, d, J=8 Hz), 8.63 (1H, d, J=8 Hz), 8.70 (1H, s)[d]
4f	3466, 2932, 2856, 1773, 1708, 1625, 1544	1.16—2.05 (12H, m), 3.37 (2H, t, J=7 Hz), 3.73 (2H, t, J=7 Hz), 8.05 (1H, d, J=8 Hz), 8.63 (1H, d, J=8 Hz), 8.68 (1H, s)[d]
4g	3466, 2924, 2854, 1772, 1709, 1624, 1543	1.07—2.03 (14H, m), 3.37 (2H, t, J=7 Hz), 3.72 (2H, t, J=7 Hz), 8.05 (1H, d, J=8 Hz), 8.62 (1H, d, J=8 Hz), 8.67 (1H, s)[d]
4h	2942, 1772, 1714, 1616	1.34—2.10 (6H, m), 3.37 (2H, t, J=6.5 Hz), 3.67 (2H, t, J=6.5 Hz), 7.62—7.95 (4H, m)[d]
9[b]	2936, 2858, 1773, 1702, 1644[c]	0.90—2.03 (17H, m), 2.28—2.90 (6H, m), 3.44 (2H, t, J=7 Hz), 3.66 (2H, s), 7.33 (5H, s)[d]
11[b]	2924, 1779, 1720, 1622, 1542[c]	0.69—0.90 (2H, m), 0.96 (3H, t, J=7 Hz), 1.04—1.55 (7H, m), 1.56—1.83 (8H, m), 2.12 (2H, d, J=7 Hz), 2.35 (2H, t, J=7.5 Hz), 2.44 (2H, q, J=7 Hz), 3.75 (2H, t, J=7.5 Hz), 8.05 (1H, d, J=8 Hz), 8.59 (1H, dd, J=2, 8 Hz), 8.66 (1H, d, J=2 Hz)[d]

a) Chemical shifts are given with proton numbers, absorption patterns and coupling constants in parentheses. *b*) Spectral data of free base were measured. *c*) Taken as liquid film. *d*) Measured in $CDCl_3$.

^{1}H-NMR ($CDCl_3$) δ: 1.30—2.13 (6H, m), 3.40 (2H, t, J=6 Hz), 3.77 (2H, t, J=6 Hz), 8.08 (1H, d, J=9 Hz), 8.66 (1H, d, J=9 Hz), 8.71 (1H, s). The yield, melting point, and analytical data of this sample are shown in Table VII.

The following compounds (**4a, b, d—h**) listed in Tables VII and VIII were prepared in the same manner as described above.

***N*-Ethyl-*N*-[(2-methoxyphenyl)methyl]amine (5f)** A mixture of 70% ethylamine solution (5 ml) and $NaHCO_3$ (4.9 g) in dioxane–water (50/50 ml) was stirred for 20 min at room temperature. A solution of 2-methoxybenzoyl chloride (6.6 g) in dioxane (5 ml) was added dropwise to the mixture, which was stirred for an additional 1 h. Water (100 ml) and CH_2Cl_2 (100 ml) were added to the mixture and the organic phase was separated. Aqueous phase was extracted with CH_2Cl_2, and the combined organic phase was washed with water and dried over Na_2SO_4. The solvent was removed *in vacuo* to give a residue, which was dissolved in tetrahydrofuran (THF, 100 ml). $LiAlH_4$ (2.2 g) was added portionwise to the solution. The mixture was refluxed for 30 min, cooled to room temperature, and treated successively with water (4.4 ml) and 10% NaOH (3.6 ml). The resulting solid was removed by filtration and the filtrates were concentrated to give a residue. The residue was taken up in 5% HCl, washed with ether twice, made basic with 10% NaOH, and extracted with CH_2Cl_2. The extracts were dried over Na_2SO_4, and concentrated to give a pale yellow oil (5.63 g), which was used in the next reaction without further purification. The yield, IR and NMR data of this sample are given in Table IX.

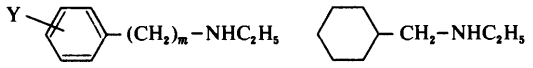
N-Ethyl-*N*-(ω-phenylalkyl)amines (**5a—e, g—l**) and *N*-(cyclohexylmethyl)-*N*-ethylamine (**10**) listed in Table IX were obtained in the same manner as described for **5f**.

***N*-Ethyl-*N*-[(3-hydroxyphenyl)methyl]amine (5m)** Ethylamine solution (70%, 10 ml) was added rapidly to a solution of 3-hydroxybenzaldehyde (12.2 g) in ethanol (12 ml) at about 40 °C. The mixture was allowed to stand at 0—5 °C for 2 h. The resulting precipitate was collected by filtration, washed successively with 40% ethanol and water, and dried *in vacuo* to give the Schiff base (13.1 g, 88%) as colorless needles, mp 127—129 °C.

Sodium borohydride (4.8 g) was added portionwise to a solution of the Schiff base (12.5 g) in methanol (190 ml) at 0—5 °C. The mixture was stirred at 0—5 °C until gas evolution stopped (about 30 min), then refluxed for 15 min, cooled, and quenched with water. Methanol was evaporated and the remaining aqueous solution was washed with CH_2Cl_2. The aqueous solution was made acidic with 10% HCl, washed with ether, neutralized with 10% NaOH, and extracted with 1-butanol. Removal of the solvent *in vacuo* gave a residue, which was dissolved in CH_2Cl_2. The solution was dried over Na_2SO_4 and the solvent was evaporated to afford a viscous oil. Crystallization from ether gave colorless cubes (11.7 g, 77% from the starting aldehyde), mp 107—111 °C. Spectral data of this sample are given in Table IX.

2-[5-[*N*-Ethyl-*N*-(phenylmethyl)amino]pentyl]-5-nitro-1*H*-isoindole-1,3(2*H*)-dione Hydrochloride (1j) A mixture of **4c** (1.0 g) and *N*-ethylbenzylamine (0.79 g) in toluene (15 ml) was refluxed for 6 h, cooled to room temperature, and the resulting precipitate was removed by

TABLE IX. Physicochemical Properties of *N*-Ethyl-*N*-(ω-phenylalkyl)amines (**5**) and *N*-(Cyclohexylmethyl)-*N*-ethylamine (**10**)



5 **10**

Compd. No.	*m*	Y	Yield (%)	IR (film) cm^{-1}	^1H-NMR ($CDCl_3$) δ[a]
5a	1	4-Cl	91	3316, 2966, 2820, 1491, 1458, 1089, 1014, 800	1.12 (3H, t, *J*=7 Hz), 1.31 (1H, br s), 2.66 (2H, q, *J*=7 Hz), 3.75 (2H, s), 7.27 (4H, s)
5b	1	3-Cl	90	3282, 2966, 2818, 1597, 1574, 1121, 1074, 777	1.13 (3H, t, *J*=7 Hz), 1.34 (1H, br s), 2.67 (2H, q, *J*=7 Hz), 3.77 (2H, s), 7.13—7.35 (4H, m)
5c	1	2-Cl	92	3314, 2964, 2818, 1470, 1442, 1124, 1049, 1035, 749	1.14 (3H, t, *J*=7 Hz), 1.55 (1H, br s), 2.68 (2H, q, *J*=7 Hz), 3.89 (2H, s), 7.14—7.28 (2H, m), 7.30—7.41 (2H, m)
5d	1	4-OMe	91	3308, 2962, 2834, 1612, 1512, 1245, 1174, 1035, 820	1.12 (3H, t, *J*=7 Hz), 1.45 (1H, br s), 2.67 (2H, q, *J*=7 Hz), 3.72 (2H, s), 3.79 (3H, s), 6.86 (2H, d, *J*=8.5 Hz), 7.23 (2H, d, *J*=8.5 Hz)
5e	1	3-OMe	89	3306, 2962, 2832, 1599, 1488, 1457, 1261, 1153, 1048, 779	1.13 (3H, t, *J*=7 Hz), 1.39 (1H, br s), 2.68 (2H, q, *J*=7 Hz), 3.77 (2H, s), 3.81 (3H, s), 6.79 (1H, dd, *J*=2, 8 Hz), 6.86—6.92 (2H, m), 7.22 (1H, dd, *J*=8, 8 Hz)
5f	1	2-OMe	88	3354, 2962, 2836, 1600, 1588, 1491, 1463, 1240, 1048, 1029, 752	1.12 (3H, t, *J*=7 Hz), 1.90 (1H, br s), 2.65 (2H, q, *J*=7 Hz), 3.79 (2H, s), 3.84 (3H, s), 6.83—6.96 (2H, m), 7.18—7.28 (2H, m)
5g	1	2-F	76	3300, 2968, 2870, 1594, 1539, 1490, 1454, 1227, 757	1.13 (3H, t, *J*=7 Hz), 1.55 (1H, br s), 2.67 (2H, q, *J*=7 Hz), 3.85 (2H, s), 6.97—7.46 (4H, m)
5h	1	2-Me	85	3318, 2964, 2814, 1456, 1377, 1127, 1104, 741	1.15 (3H, t, *J*=7 Hz), 1.30 (1H, br s), 2.35 (3H, s), 2.72, (2H, q, *J*=7 Hz), 3.77 (2H, s), 7.12—7.34 (4H, m)
5i	1	2,3-diOMe	67	3306, 2936, 2810, 1587, 1485, 1274, 1221, 1075, 1007, 748	1.03 (3H, t, *J*=7 Hz), 1.45 (1H, br s), 2.53 (2H, q, *J*=7 Hz), 3.67 (2H, s), 3.72 (3H, s), 3.78 (3H, s), 6.94 (3H, s)
5j	2	H	93	3298, 2932, 1635, 1495, 1454, 697	1.07 (3H, t, *J*=7 Hz), 2.00 (1H, br s), 2.67 (2H, q, *J*=7 Hz), 2.75—2.93 (4H, m), 7.23 (5H, s)
5k	3	H	92	3300, 2932, 1653, 1495, 1132, 746, 698	1.10 (3H, t, *J*=7 Hz), 1.27 (1H, br s), 1.82 (2H, tt, *J*=7, 7 Hz), 2.55—2.72 (6H, m), 7.13—7.34 (5H, m)
5l	4	H	90	3288, 2930, 1495, 1453, 1132, 746, 697	1.09 (3H, t, *J*=7 Hz), 1.13 (1H, br s), 1.45—1.75 (4H, m), 2.57—2.71 (6H, m), 7.12—7.33 (5H, m)
5m	1	3-OH	77	3420, 3280, 2898, 2562, 1609, 1582	1.13 (3H, t, *J*=7 Hz), 2.70 (2H, q, *J*=7 Hz), 3.67 (2H, s), 6.16 (2H, br s), 6.60—6.87 (3H, m), 7.10 (1H, dd, *J*=8, 8 Hz)
10	—	—	73	3294, 2922, 2852, 1651, 1539, 1447, 1132	0.78—1.57 (8H, m), 1.10 (3H, t, *J*=7 Hz), 1.59—1.82 (4H, m), 2.44 (2H, d, *J*=6.5 Hz), 2.62 (2H, q, *J*=7 Hz)

a) Chemical shifts are given with proton numbers, absorption patterns and coupling constants in parentheses.

filtration. The filtrate was concentrated to give an oily residue, which was chromatographed on silica gel eluting with ethyl acetate to afford an oil. Treatment of the oil with ethanolic HCl (1 eq) gave colorless cubes (0.85 g). IR (KBr): 3434, 2946, 1778, 1717, 1625, 1541 cm^{-1}. ^1H-NMR (dimethylsulfoxide-d_6 (DMSO-d_6)) δ: 1.10—2.00 (9H, m), 2.65—3.45 (4H, m), 3.60 (2H, t, *J*=6 Hz), 4.24 (2H, br d, *J*=4 Hz), 7.20—7.80 (5H, m), 8.10 (1H, d, *J*=8 Hz), 8.45 (1H, s), 8.61 (1H, d, *J*=8 Hz), 11.17 (1H, br s). The yield, melting point, and analytical data of this sample are shown in Table I.

The following compounds (**1a—i, k—t, v, ll—tt, vv—xx**, and **11**) listed in Tables I, III, VI, and VIII were similarly prepared by reaction of the bromides (**4a—h**) with the amines (**5a—l** or **10**).

Method B. *N*-Ethyl-*N*-(phenylmethyl)pentane-1,5-diamine (8) A mixture of **1v** (18 g, free base) and hydrazine hydrate (10 ml) in ethanol (300 ml) was refluxed for 1 h, cooled to room temperature, and the resulting precipitate was filtered off. The filtrate was concentrated and the resulting precipitate was removed again. This procedure was repeated until no precipitate appeared, giving an oil (10.35 g, 91%), which was used in the next reaction without further purification. IR (film): 3350, 2932, 2858, 2798, 1494, 1452, 1369, 734, 697 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.04 (3H, t, *J*=7 Hz), 1.21—1.58 (6H, m), 1.88 (2H, br s), 2.44 (2H, t, *J*=7.5 Hz), 2.52 (2H, q, *J*=7 Hz), 2.68 (2H, t, *J*=7 Hz), 3.57 (2H, s), 7.26 (5H, s). MS *m/z*: 220 [M^+].

2-[5-[*N*-Ethyl-*N*-(phenylmethyl)amino]pentyl]-4-nitro-1*H*-isoindole-1,3(2*H*)-dione Hydrochloride (1w) A mixture of **8** (0.5 g) and 3-nitrophthalic anhydride (0.44 g) in dioxane (15 ml) was refluxed for 8 h, cooled to 0 °C, diluted with water, and extracted with CH_2Cl_2. The extracts were dried over Na_2SO_4 and the solvent was removed *in vacuo* to give a residue, which was chromatographed on silica gel eluting with CH_2Cl_2–ethanol (20:1) to afford an oil. Treatment of the oil with ethanolic HCl (1 eq) gave a hygroscopic powder (0.66 g). IR (KBr): 3434, 2946, 2620, 1779, 1717, 1619, 1541 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 1.06—2.00 (9H, m), 2.72—3.20 (4H, m), 3.56 (2H, t, *J*=6 Hz), 4.26 (2H, d, *J*=5 Hz), 7.30—7.53 (3H, m), 7.53—7.80 (2H, m), 7.95—8.36 (3H, m), 11.13 (1H, br s). The yield and analytical data of this sample are shown in Table III.

The following compounds (**1x—z, gg**, and **9**) listed in Tables III, VI, and VIII were prepared in the same manner as described for **1w** by reaction of **8** with commercially available substituted phthalic anhydrides or *cis*-cyclohexane-1,2-dicarboxylic anhydride.

5-Nitro-2-[4-[*N*-(phenylmethyl)amino]butyl]-1*H*-isoindole-1,3(2*H*)-dione Hydrochloride (1u) A mixture of 4-chlorobutyronitrile (4.0 g), benzylamine (4.14 g), KI (7.1 g), and K_2CO_3 (5.9 g) in 1-butanol (40 ml) was refluxed for 8 h, cooled to room temperature, and insoluble salts were filtered off. Removal of the solvent *in vacuo* gave an oil, which was treated with ethanolic HCl (1 eq) to afford colorless crystals. Recrystallization from ethanol gave *N*-(3-cyanopropyl)benzylamine hydrochloride as colorless plates (3.5 g, 43%), mp 165—168 °C. IR (KBr): 3434, 2940, 2778, 2420, 2244, 1439, 746, 697 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 2.03 (2H, tt, *J*=7.5, 7.5 Hz), 2.67 (2H, t, *J*=7.5 Hz), 2.95 (2H, t, *J*=7.5 Hz), 4.11 (2H, s), 7.30—7.73 (5H, m), 9.80 (2H, br s). *Anal.* Calcd for $C_{11}H_{14}N_2 \cdot HCl$: C, 62.70; H, 7.18; N, 13.30. Found: C, 62.44; H, 6.96; N, 13.24.

A solution of the amine (2.9 g) in THF (50 ml) was added dropwise to a suspension of $LiAlH_4$ (1.05 g) in THF (50 ml). The mixture was refluxed for 15 min, cooled to 0 °C, and the resulting mixture was treated successively with water (2.2 ml) and 10% NaOH (1.8 ml). The precipitated solid was removed by filtration and the filtrate was concentrated to give

a residue. The residue was dissolved in 5% HCl, washed with ether twice, made basic with 10% NaOH, and extracted with CH_2Cl_2. The extracts were dried over Na_2SO_4 and the solvent was removed *in vacuo* to give *N*-(phenylmethyl)butane-1,4-diamine (1.7 g, 69%) as a pale yellow oil, which was used in the next step without further purification.

The diamine was allowed to react with 4-nitrophthalic anhydride in the same manner as described for **1w** to give the desired product **1u** as colorless cubes. IR (KBr): 3438, 1778, 1710, 1626, 1546 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.53—1.85 (4H, m), 2.70—3.07 (2H, m), 3.46—3.76 (2H, m), 3.94—4.16 (2H, m), 7.30—7.70 (5H, m), 8.13 (1H, d, J=8 Hz), 8.49 (1H, d, J=1.5 Hz), 8.65 (1H, dd, J=1.5, 8 Hz), 9.5 (2H, br s). The yield, melting point and analytical data of this sample are given in Table I.

Transformation of Substituents of 1. 2-[5-[*N*-Ethyl-*N*-(phenylmethyl)-amino]pentyl]-5-methoxy-1*H*-isoindole-1,3(2*H*)-dione Hydrochloride (1aa) A mixture of sodium hydride (20 mg) and **1z** (0.25 g) in DMF (5 ml) was stirred at room temperature for 2 h. Methyl iodide (0.1 g) was added to the mixture, which was then stirred at room temperature for 1 h, quenched with water, and extracted with CH_2Cl_2. The extracts were dried over Na_2SO_4 and the solvent was removed *in vacuo* to afford a residue, which was chromatographed on silica gel eluting with CH_2Cl_2–ethanol (20 : 1) to give an oil. IR (film): 2938, 2800, 1769, 1709, 1618, 1491 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.02 (3H, t, J=7.5 Hz), 1.10—1.86 (6H, m), 2.25—2.66 (4H, m), 3.55 (2H, s), 3.62 (2H, t, J=6 Hz), 3.89 (3H, s), 7.00—7.47 (7H, m), 7.71 (1H, d, J=8 Hz).

Treatment of the oil with ethanolic HCl (1 eq) gave a hygroscopic powder (0.22 g). The yield and analytical data of this sample are shown in Table III.

The compound (**1bb**) was prepared as described for **1aa** (Tables III and VIII).

5-Amino-2-[5-[*N*-ethyl-*N*-(phenylmethyl)amino]pentyl]-1*H*-isoindole-1,3(2*H*)-dione Dihydrochloride (1cc) A suspension of **1j** (4.4 g) and concentrated HCl (2 ml) in ethanol (150 ml) was hydrogenated over 10% Pd/C (0.3 g) under atmospheric pressure at room temperature. After removal of the catalyst by filtration, the filtrate was concentrated to give a residue, which was dissolved in 10% NaOH and extracted with CH_2Cl_2. The extracts were dried over Na_2SO_4 and the solvent was evaporated to afford a residue. The residue was chromatographed on silica gel eluting with ethyl acetate to give an oil, which was treated with ethanolic HCl (2 eq) to afford a hygroscopic powder (3.1 g). IR (KBr): 3438, 2942, 1774, 1757, 1709, 1617 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.00—2.00 (6H, m), 1.26 (3H, t, J=7.5 Hz), 2.70—3.20 (4H, m), 3.48 (2H, t, J=6 Hz), 4.26 (2H, d, J=5 Hz), 6.67—7.05 (4H, m), 7.30—7.80 (6H, m), 11.2 (2H, br s). The yield and analytical data of this sample are shown in Table III.

5-Acetylamino-2-[5-[*N*-ethyl-*N*-(phenylmethyl)amino]pentyl]-1*H*-isoindole-1,3(2*H*)-dione (1dd) A mixture of **1cc** (0.4 g) and acetyl chloride (0.09 g) in pyridine (4 ml) was stirred at room temperature for 1 h, diluted with water, and extracted with CH_2Cl_2. The extracts were dried over Na_2SO_4 and the solvent was removed *in vacuo* to give a viscous oil, which was triturated with hexane to give a powder. Recrystallization from CH_2Cl_2–hexane gave colorless cubes (0.33 g). IR (KBr): 3444, 3358, 2934, 1770, 1696, 1627, 1612, 1557 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.99 (3H, t, J=7 Hz), 1.10—1.86 (6H, m), 2.05—2.63 (4H, m), 2.24 (3H, s), 3.51 (2H, s), 3.63 (2H, t, J=6 Hz), 7.25 (5H, s), 7.76 (1H, d, J=9 Hz), 7.97 (1H, d, J=1.5 Hz), 8.07 (1H, dd, J=1.5, 9 Hz), 8.25 (1H, s). The yield, melting point, and analytical data of this sample are shown in Table III.

The sulfonamides (**1ee** and **1ff**) listed in Tables III and VIII were prepared in the same manner as described for **1dd**.

Methyl 2-[5-[*N*-Ethyl-*N*-(phenylmethyl)amino]pentyl]-1,3(2*H*)-dioxo-1*H*-isoindole-5-carboxylate Hydrochloride (1hh) 2-[5-[*N*-Ethyl-*N*-(phenylmethyl)amino]pentyl]-1,3-dioxo-1*H*-isoindole-5-carboxylic acid hydrochloride (**1yy**, a very hygroscopic powder) was prepared by reaction of trimellitic anhydride with **8** in the same manner as described for **1w** in 74% yield. IR (KBr): 3422, 2400—3200, 2946, 1774, 1716, 1656 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.27 (3H, t, J=7 Hz), 1.07—2.03 (6H, m), 2.67—3.23 (4H, m), 3.60 (2H, t, J=6 Hz), 4.28 (2H, d, J=5 Hz), 7.27—7.82 (5H, m), 7.95 (1H, d, J=8 Hz), 8.22 (1H, s), 8.35 (1H, d, J=8 Hz), 11.33 (2H, br s).

A mixture of the acid (**1yy**, 0.7 g) and concentrated HCl (2 drops) in methanol (30 ml) was refluxed for 30 min. The mixture was concentrated, diluted with CH_2Cl_2, washed successively with 10% NaOH and water, and dried over Na_2SO_4. The solvent was removed *in vacuo* to give a residue. Chromatographic purification of the residue on silica gel eluting with CH_2Cl_2–ethanol (20 : 1) gave an oil. IR (film): 2940, 1776, 1721 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.02 (3H, t, J=7 Hz), 1.10—1.87 (6H, m), 2.23—2.65 (4H, m), 3.54 (2H, s), 3.68 (2H, t, J=7 Hz), 4.00 (3H, s), 7.30 (5H, s), 7.90 (1H, d, J=7.5 Hz), 8.40 (1H, d, J=7.5 Hz), 8.47 (1H, s).

Treatment of the oil with ethanolic HCl (1 eq) afforded **1hh** (0.64 g) as an amorphous powder. The yield and analytical data of this sample are shown in Table III.

The ethyl ester (**1ii**) was prepared in the same manner as described above (Tables III and VIII).

2-[5-[*N*-Ethyl-*N*-(phenylmethyl)amino]pentyl]-5-methylaminocarbonyl-1*H*-isoindole-1,3(2*H*)-dione (1jj) A mixture of **1yy** (0.3 g) and thionyl chloride (0.55 ml) was heated at 70 °C for 30 min. After excess thionyl chloride was removed *in vacuo* the remaining residue was dissolved in CH_2Cl_2. A mixture of methylamine hydrochloride (53 mg) and Et_3N (0.22 ml) in CH_2Cl_2 (8 ml) was added to the solution. The resulting mixture was stirred at room temperature for 30 min. The mixture was concentrated and extracted with CH_2Cl_2. The extracts were washed successively with 5% NaOH and water, dried over Na_2SO_4, and the solvent was evaporated to give a residue. The residue was chromatographed on silica gel eluting with CH_2Cl_2–ethanol (20 : 1) to afford crystals. Recrystallization from CH_2Cl_2–ether gave colorless cubes (0.15 g). IR (KBr): 3428, 2932, 1773, 1702, 1677, 1534 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.00 (3H, t, J=7 Hz), 1.13—1.84 (6H, m), 2.27—2.65 (4H, m), 3.01 (3H, d, J=5 Hz), 3.52 (2H, s), 3.63 (2H, t, J=7 Hz), 7.08—7.40 (6H, m), 7.81 (1H, d, J=8 Hz), 8.10—8.30 (2H, m). The yield, melting point, and analytical data of this sample are given in Table III.

N,N-Diethylamide (**1kk**) was prepared in the same manner as described for **1jj** (Tables III and VIII).

2-[5-[*N*-Ethyl-*N*-[[3-(methylaminocarbonyloxy)phenyl]methyl]amino]-pentyl]-5-nitro-1*H*-isoindole-1,3(2*H*)-dione Hydrochloride (1uu) In the same manner as described for **1j**, **4c** (0.9 g) was allowed to react with **5m** (0.8 g) to give 2-[5-[*N*-ethyl-*N*-[(3-hydroxyphenyl)methyl]amino]pentyl]-5-nitro-1*H*-isoindole-1,3(2*H*)-dione (**1zz**, 0.6 g, 55%) as an oil, which was used in the next step without further purification. IR (film): 3200—3600, 3418, 2940, 1777, 1719, 1588, 1540 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.98 (3H, t, J=7 Hz), 1.20—1.93 (6H, m), 2.23—2.66 (4H, m), 3.47 (2H, s), 3.74 (2H, t, J=7 Hz), 5.54 (1H, br s), 6.55—7.22 (4H, m), 8.04 (1H, d, J=8 Hz), 8.51—8.70 (2H, m).

Methyl isocyanate (0.06 ml) and Et_3N (1 drop) were added successively to a solution of **1zz** (0.35 g) in CH_2Cl_2 (3 ml) at 0—5 °C. The mixture was stirred at room temperature for 10 min, quenched with water, and extracted with CH_2Cl_2. The extracts were washed with water, dried over Na_2SO_4, and the solvent was removed *in vacuo* to give a residue. Chromatographic purification of the residue on silica gel eluting with ethyl acetate–methanol (20 : 1) afforded a colorless oil, which was treated with ethanolic HCl (1 eq) to give a hygroscopic powder (0.38 g). IR (KBr): 3420, 2940, 1777, 1716 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.10—2.00 (9H, m), 2.59—3.23 (7H, m), 3.62 (2H, t, J=7 Hz), 3.81 (1H, br s), 4.27 (2H, d, J=*ca.* 5 Hz), 7.07—7.77 (5H, m), 8.14 (1H, d, J=8 Hz), 8.50 (1H, d, J=1.5 Hz), 8.67 (1H, dd, J=1.5, 8 Hz). The yield and analytical data of this sample are shown in Table VI.

2-[4-[*N*-Acetyl-*N*-(phenylmethyl)amino]butyl]-5-nitro-1*H*-isoindole-1,3(2*H*)-dione (12) A mixture of **1u** (0.5 g, free base) and acetic anhydride (0.15 g) in CH_2Cl_2 was refluxed for 10 min, cooled to room temperature, washed successively with 5% NaOH and water, dried over Na_2SO_4, and the solvent was removed *in vacuo* to give an oil. The oil was chromatographed on silica gel eluting with ethyl acetate to afford a colorless oil (0.53 g, 95%). IR (film): 2940, 1778, 1716, 1642, 1540 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.49—1.85 (4H, m), 2.11, 2.18 (3H, each s), 3.26, 3.40 (2H, each t, J=7 Hz), 3.72, 3.74 (2H, each t, J=6.5 Hz), 4.53, 4.59 (2H, each s), 7.14—7.42 (5H, m), 8.04, 8.06 (1H, each d, J=8 Hz), 8.58—8.68 (2H, m). MS *m/z*: 395 [M^+].

Biological Method and Materials The cerebral cortex of male Wistar rats was homogenized with 20 volumes of ice-cooled 0.32 M sucrose, and centrifuged at 1000 × *g* for 10 min. The supernatant (S1) was preincubated in a scintillation vial with test compound for 15 min at room temperature, and then [acetyl-^{3}H]-acetylcholine (final 200 μM) was added and the incubation was continued for 30 min. The reaction was terminated by adding a 1 M solution of chloroacetic acid, followed by a toluene-based scintillant, and the vials were capped and shaken to transfer the produced [^{3}H]-acetic acid to the toluene phase. Radioactivity in the toluene phase was then counted by liquid scintillation spectrometry (Aloka LSC-903 or LSC-1000). The inhibitory activities were expressed as the 50%-inhibitory concentration (IC_{50}), which was calculated by probit analysis. The inhibitory activities of physostigmine and THA were measured using the same technique.

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18) In potent AChE inhibitors such as **1pp**, **1qq**, or **1uu**, the benzylamino nitrogen is separated from the benzene ring of the phthalimide moiety by a five carbon chain length ($n=5$) and the –CON– part of the phthalimide. Therefore, a seven carbon chain length may be estimated as the distance between HBS-2 and the anionic site. However, since these molecules are very flexible, it is probable that they bend somewhat in order to better fit the enzyme. This possibility would make the estimation of the distance less than that above.
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Central Cholinergic Agents. II.[1] Synthesis and Acetylcholinesterase Inhibitory Activities of *N*-[ω-[*N*-Alkyl-*N*-(phenylmethyl)amino]alkyl]-3-arylpropenamides

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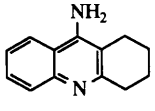
A series of *N*-[ω-[*N*-alkyl-*N*-(phenylmethyl)amino]alkyl]-3-arylpropenamides was prepared and tested for its inhibitory activities on acetylcholinesterase. Some in the series were found to be potent inhibitors. The structure–activity relationships were discussed in detail.

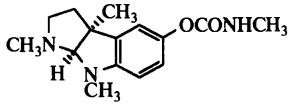
Keywords anticholinesterase; propenamide; structure–activity relationship; Hansch–Fujita analysis: hydrophobic binding site

The active site of acetylcholinesterase (AChE) is known to contain an anionic as well as an esteratic subsite and closely adjacent to these a hydrophobic binding site (HBS-1).[2] Inhibitors of AChE, such as physostigmine and tetrahydroaminoacridine (THA), bind to these sites, thus exerting their inhibitory effect on the enzyme (Chart 1).[2b,3] In a preceding paper,[1] we proposed that there may exist another hydrophobic binding site (HBS-2) some distance away from the anionic site. Investigation into compounds which interact with both hydrophobic binding sites as well as the anionic site was expected to reveal new types of AChE inhibitors. Based on this working hypothesis, 2-[ω-[*N*-alkyl-*N*-(ω-phenylalkyl)amino]alkyl]-1*H*-isoindole-1,3(2*H*)-diones (**1**) were designed and found to be potent AChE inhibitors. It was hoped to confirm the existence of HBS-2 by detailed study of the structure–activity relationships.

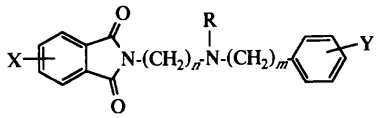
In order to find new inhibitors of AChE as well as to clarify the nature of HBS-2, we studied transformation of the phthalimide moiety of **1**, which is thought to interact with HBS-2 (Chart 2). The benzamide derivative (**2b**) was first prepared as a ring-opened analogue of **1a** which has 50%-inhibitory concentration (IC_{50}) of 151 nM. Replacement of the benzoyl group of **2b** with a cinnamoyl group led to **3b**, which showed stronger inhibitory activity than that of **2b** [IC_{50} = 1590 nM (**2b**), 539 nM (**3b**)]. *N*-[ω-[*N*-Alkyl-*N*-(phenylmethyl)amino]alkyl]-3-arylpropenamide derivatives (**3**) have two more positions for substitution than the benzamides (**2**). These prompted us to prepare a variety of 3-arylpropenamide derivatives (**3**) and examine their AChE inhibitory activities. This paper describes full details of the structure–activity relationships of the 3-arylpropenamide derivatives (**3**).



THA



physostigmine



1

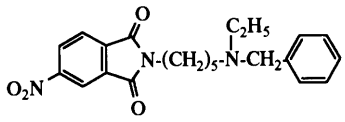
Chart 1

Chemistry Preparation of *N*-[ω-[*N*-alkyl-*N*-(phenylmethyl)amino]alkyl]-3-arylpropenamides (**3**) is illustrated in Chart 3. 2-[ω-[*N*-Alkyl-*N*-(phenylmethyl)amino]alkyl]-isoindole-1,3(2*H*)-diones (**1**)[1] were treated with hydrazine hydrate to give *N*-alkyl-*N*-(phenylmethyl)alkane-1,ω-diamines (**4**). Condensation of the 1,ω-diamines (**4**) with various 3-arylpropenoic acids (**5**) was carried out using diethyl phosphorocyanidate to yield the desired amides (**3**: R^3 = H). Reaction of the amide with acetic anhydride in the presence of *p*-toluenesulfonic acid gave *N*-acetyl derivatives (**3**: R^3 = Ac). Catalytic hydrogenation of the nitro group of **3a** over 10% Pd/C gave the amine (**3aa**), which was acylated to afford the acylamide (**3bb**).

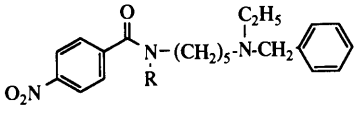
Butenamide (**6**) and 3-phenylpropionamide (**7**) were prepared in a similar manner to that described for **3** (R^3 = H). 1,5-Pentanediamine (**8**) was obtained by benzylation of **4c**.

Biological Results and Discussion

The measurement of AChE inhibitory activity was performed radiometrically according to the method of Klein-

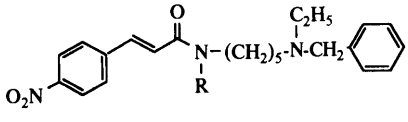


1a



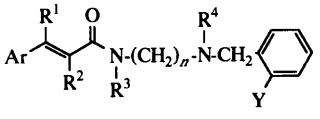
2a : R=H

2b : R=Ac



3a : R=H

3b : R=Ac



3

Chart 2

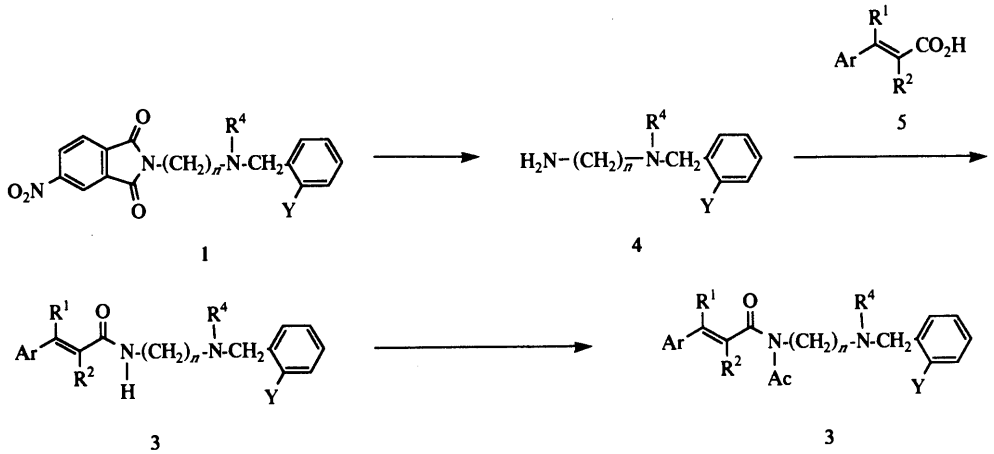
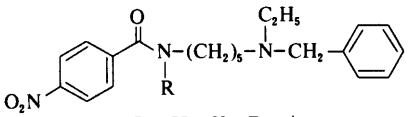
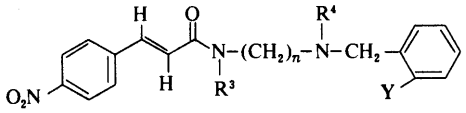


Chart 3

TABLE I. Physical and Biological Properties of *N*-[5-[*N*-Ethyl-*N*-(phenylmethyl)amino]pentyl]-4-nitrobenzamides (**2a**, **b**) and *N*-[ω-[*N*-Alkyl-*N*-(phenylmethyl)amino]alkyl]-3-arylpropenamides (**3a—l**)



2a: R=H **2b**: R=Ac



3a—k: Y=H **3l**: Y=OMe

Compd.[a] No.	*n*	R^3	R^4	Yield (%)	Formula	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N	Inhibition of AChE (IC_{50}, nM)
2a	—	—	—	91	$C_{21}H_{27}N_3O_3 \cdot HCl$	62.14	6.95	10.35	61.92	7.07	10.21	5280
2b[b,c]	—	—	—	80	$C_{23}H_{29}N_3O_4$	—	—	—	—	—	—	1590
3a	5	H	Et	86	$C_{23}H_{29}N_3O_3 \cdot HCl$	63.95	7.00	9.73	63.81	6.91	9.63	3000
3b[b,d]	5	Ac	Et	83	$C_{25}H_{31}N_3O_4$	—	—	—	—	—	—	539
3c	3	H	Et	82	$C_{21}H_{25}N_3O_3$	68.64	6.86	11.44	68.51	6.79	11.34	11000
3d	4	H	Et	82	$C_{22}H_{27}N_3O_3 \cdot HCl$	63.23	6.75	10.05	63.14	6.59	10.03	7290
3e	6	H	Et	87	$C_{24}H_{31}N_3O_3 \cdot HCl$	64.64	7.23	9.42	64.63	7.17	9.40	4780
3f	7	H	Et	84	$C_{25}H_{33}N_3O_3 \cdot HCl$	65.28	7.45	9.14	65.03	7.19	9.06	9440
3g[b,e]	4	Ac	Et	77	$C_{24}H_{29}N_3O_4$	—	—	—	—	—	—	5520
3h[b,f]	6	Ac	Et	75	$C_{26}H_{33}N_3O_4$	—	—	—	—	—	—	2950
3i	5	H	Me	82	$C_{22}H_{27}N_3O_3 \cdot HCl$	63.23	6.75	10.05	63.17	6.71	9.98	16000
3j	5	H	iso-Pr	85	$C_{24}H_{31}N_3O_3 \cdot HCl$	64.64	7.23	9.42	64.47	7.11	9.31	7800
3k	5	H	Pr	87	$C_{24}H_{31}N_3O_3 \cdot HCl$	64.64	7.23	9.42	64.61	7.22	9.38	27000
3l	5	H	Et	90	$C_{24}H_{31}N_3O_4 \cdot HCl$	62.40	6.98	9.10	62.25	7.06	9.02	286

a) Amorphous powder unless otherwise noted. *b*) Oil. The structure was confirmed by IR, ^{1}H-NMR (see Experimental or Table VI), and MS spectra. *c*) MS *m/z*: 411 [M^+]. *d*) MS *m/z*: 437 [M^+]. *e*) MS *m/z*: 423 [M^+]. *f*) MS *m/z*: 451 [M^+].

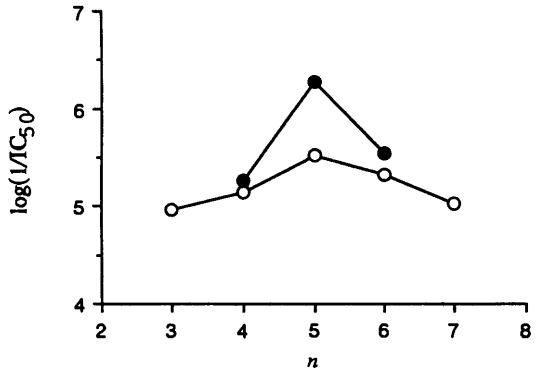


Fig. 1. Relationship between AChE Inhibition and Carbon Chain Length (*n*) of **3**

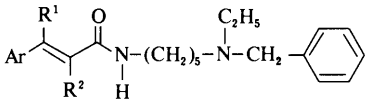
R^3=H (○); Ac (●).

berger and Yanai,[4] which is a slight modification of the method of Johnson and Russell.[5] The results were expressed as IC_{50} values. The IC_{50} values of physostigmine and THA were measured at 220 and 300 nM, respectively.

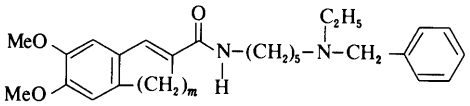
The effects of variation of the chain length (*n*) of **3** as well as the *N*-substituents (R^4) on AChE inhibition were examined first and the results are shown in Table I and Fig. 1. The figure clearly illustrates that optimum activities are associated with a chain length of 5 carbon atoms in both the *N*-H and *N*-Ac derivatives (**3a,c—f** and **3b,g,h**). The *N*-acetyl derivatives showed more potent activities than the *N*-H derivatives. Among the compounds bearing various *N*-alkyl substituents (**3a** and **3i—k**), **3a** (R^4=Et) was the most potent inhibitor. 2-Methoxy substitution on the phenyl ring of the benzylamino moiety, which we presume interacts with the anionic site as well as HBS-1,[1] increased the activity 10-fold (**3l** *vs.* **3a**). All these results are consistent with those obtained in the previous work,[1] thus supporting the existence of HBS-2.

The effects on AChE inhibition of variation of the aromatic ring (Ar) as well as substituents R^1 and R^2 were examined next and the results are shown in Table II. As an

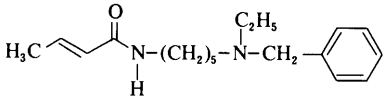
TABLE II. Physical and Biological Properties of *N*-[5-[*N*-Ethyl-*N*-(phenylmethyl)amino]pentyl]-3-arylpropenamides (**3m**—**w**) and Their Related Compounds (**6** and **7**)



3m—**t**



3u: $m=1$, **3v**: $m=2$, **3w**: $m=3$



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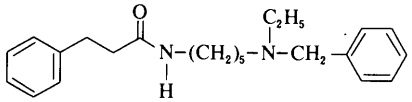
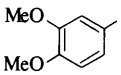


7

Compd.[a] No.	Ar	R^1	R^2	Yield (%)	Formula	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N	Inhibition of AChE (IC_{50}, nM)
3m	Ph	H	H	87	$C_{23}H_{30}N_2O\cdot HCl$	71.39	8.07	7.24	71.33	8.00	7.17	11800
3n		H	H	81	$C_{22}H_{29}N_2O\cdot HCl$	68.11	7.79	10.83	68.05	7.68	10.71	4.210
3o[b]		H	H	86	$C_{21}H_{28}N_2O_2\cdot HCl$	66.92	7.76	7.43	66.54	7.55	7.40	19400
3p[c]		H	H	84	$C_{21}H_{28}N_2OS\cdot HCl$	64.18	7.44	7.13	63.74	7.25	7.06	15400
3q	Ph	H	Me	88	$C_{24}H_{32}N_2O\cdot HCl$	71.89	8.30	6.99	71.76	8.09	6.83	11600
3r[d]	Ph	H	Ph	86	$C_{29}H_{34}N_2O\cdot HCl$	75.22	7.62	6.05	74.87	7.43	5.89	7320
3s	Ph	Ph	H	87	$C_{29}H_{34}N_2O\cdot HCl$	75.22	7.62	6.05	75.11	7.47	5.97	5400
3t		H	H	87	$C_{25}H_{34}N_2O_3\cdot HCl$	67.17	7.89	6.27	67.08	7.76	6.05	5350
3u	—	—	—	86	$C_{26}H_{34}N_2O_3\cdot HCl$	68.03	7.69	6.10	67.89	7.54	5.96	1950
3v[e]	—	—	—	87	$C_{27}H_{36}N_2O_3\cdot HCl$	68.55	7.88	5.92	68.11	7.73	5.81	2520
3w	—	—	—	92	$C_{28}H_{38}N_2O_3\cdot HCl$	69.05	8.07	5.75	68.88	7.92	5.63	7950
6	—	—	—	90	$C_{18}H_{28}N_2O\cdot HCl$	66.54	9.00	8.62	66.35	8.98	8.52	52000
7	—	—	—	91	$C_{23}H_{32}N_2O\cdot HCl$	71.02	8.55	7.20	70.89	8.56	7.08	26000

a) Amorphous powder. *b*) MS *m/z*: 340 [M^+]. *c*) MS *m/z*: 356 [M^+]. *d*) MS *m/z*: 426 [M^+]. *e*) MS *m/z*: 436 [M^+].

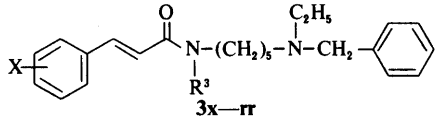
aromatic ring (Ar), the 3-pyridyl derivative (**3n**) was more potent than the phenyl derivative (**3m**), whereas the 2-furyl and 2-thienyl derivatives (**3o** and **3p**) were less potent inhibitors than **3m**. 2-Methyl-3-phenylpropenamide (**3q**) showed the same potency as **3m**. Both the 2,3-diphenylpropenamide (**3r**) and 3,3-diphenylpropenamides (**3s**) exerted more potency than **3m**. Ring-closed analogues of 3-(3,4-dimethoxyphenyl)propenamide (**3t**) were prepared in order to test the effect on enzyme inhibition of fixing the phenyl ring conformation. Among compounds (**3u**—**w**), both the indene and dihydronaphthalene derivatives (**3u** and **3v**) were found to be more potent inhibitors than **3t**, however, the dihydrobenzocycloheptene derivative (**3w**) was less potent than **3t**. This might be explained by their conformational differences: the benzene ring and the double bond of both indene and dihydronaphthalene have a coplanar conformation, whereas the olefinic group of the dihydrobenzocycloheptene ring is twisted (*ca.* 45°) out of plane.[7] Table II also demonstrates that replacement of the phenyl ring with a methyl group decreases the activity 4-fold (**6** *vs.* **3m**). Saturation of the double bond of **3m** resulted in a decrease of inhibitory potency (**7** *vs.* **3m**). From the above results the following conclusion may be drawn: (1) multiple hydrophobic interactions enhance the inhibitory activity; (2) the 3-phenyl ring and olefin portion should be linked together in a coplanar conformation in order to increase the potency. These suggested to us that there might be a similarity between HBS-2 and HBS-1, which has previously been described as a large area that is conformationally flexible and tends to assume a near planar form.[2b,6]

The effects of substituents (X) on the phenyl ring (Ar = Ph) were examined and analyzed quantitatively using the Hansch–Fujita method (Tables III and IV). Good correlation was obtained as shown in Eq. 1. The compounds used and their parameters are listed in Table IV. Correlations between the parameters used are insignificant, as shown in Table V.

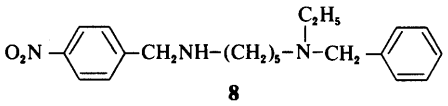
$$\log(1/IC_{50}) = -0.174\pi + 0.611\sigma + 0.734I + 4.991 \quad (1)$$
$$(0.149)\ (0.249)\ (0.236)\ (0.137)$$
$$(r=0.919,\ n=25,\ s=0.245,\ F^3_{21}=37.93)$$

In Eq. 1, π represents a hydrophobic parameter and σ is used as an electronic parameter. It was found that an indicator variable term I, which takes the value of one for the *N*-Ac and zero for the *N*-H derivatives, is effective in the combined analysis of the *N*-H and *N*-Ac derivatives. Equation 1 shows that hydrophilic and electron-withdrawing groups enhance AChE inhibitory activity. This tendency was similar to that observed for substituent effects of the phthalimide derivatives (**1**).[1] In this quantitative study, satisfactory correlation was obtained without using steric parameters such as molecular refractivity (MR) and Es. Contribution of I to the activity seems to indicate that

TABLE III. Physical and Biological Properties of *N*-[5-[*N*-Ethyl-*N*-(phenylmethyl)amino]pentyl]-3-arylpropenamides (**3x—rr**) and Pentane-1,5-diamine (**8**)



3x—rr



8

Compd. No.[a]	X	R^3	Yield (%)	Formula	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N	Inhibition of AChE (IC_{50}, nM)
3x[b,c]	H	Ac	72	$C_{25}H_{32}N_2O_2$	—	—	—	—	—	—	2300
3y	3-NO_2	H	89	$C_{23}H_{29}N_3O_3 \cdot HCl$	63.95	7.00	9.73	63.77	6.83	9.62	2930
3z[b,d]	3-NO_2	Ac	78	$C_{25}H_{31}N_3O_4$	—	—	—	—	—	—	525
3aa[e]	4-NH_2	H	74	$C_{23}H_{31}N_3O \cdot 2HCl$	63.01	7.59	9.58	62.97	7.52	9.44	10200
3bb[f]	4-NHAc	H	68	$C_{25}H_{33}N_3O_2 \cdot HCl$	67.63	7.72	9.46	67.58	7.61	9.29	5670
3cc	4-Cl	H	91	$C_{23}H_{29}ClN_2O \cdot HCl$	65.56	7.18	6.65	65.29	7.01	6.45	15500
3dd[b,g]	4-Cl	Ac	76	$C_{25}H_{31}ClN_2O_2$	—	—	—	—	—	—	2140
3ee	4-Me	H	91	$C_{24}H_{32}N_2O \cdot HCl$	71.89	8.30	6.99	71.66	8.09	6.85	15800
3ff	4-CN	H	89	$C_{24}H_{29}N_3O \cdot HCl$	69.97	7.34	10.20	69.74	7.17	10.03	4950
3gg[b,h]	4-CN	Ac	76	$C_{26}H_{31}N_3O_2$	—	—	—	—	—	—	454
3hh	4-OH	H	91	$C_{23}H_{30}N_2O_2 \cdot HCl$	68.56	7.75	6.95	68.33	7.58	6.71	12600
3ii	4-OMe	H	92	$C_{24}H_{32}N_2O_2 \cdot HCl$	69.13	7.98	6.72	69.00	7.83	6.59	16300
3jj	3-OMe	H	94	$C_{24}H_{32}N_2O_2 \cdot HCl$	69.13	7.98	6.72	69.04	7.94	6.67	9310
3kk[b,i]	3-OMe	Ac	73	$C_{26}H_{34}N_2O_3$	—	—	—	—	—	—	5740
3ll	3,4,5-$(OMe)_3$	H	92	$C_{26}H_{36}N_2O_4 \cdot HCl$	65.46	7.82	5.87	65.33	7.99	5.78	4080
3mm	4-SMe	H	90	$C_{24}H_{32}N_2OS \cdot HCl$	66.57	7.68	6.47	66.47	7.81	6.39	21000
3nn	4-SOMe	H	89	$C_{24}H_{32}N_2O_2S \cdot HCl$	64.19	7.41	6.24	64.05	7.48	6.11	7500
3oo	4-SO_2Me	H	92	$C_{24}H_{32}N_2O_3S \cdot HCl$	61.99	7.15	6.02	61.85	7.03	5.97	3150
3pp[b,j]	4-SO_2Me	Ac	60	$C_{26}H_{34}N_2O_4S$	—	—	—	—	—	—	164
3qq	3-NO_2, 4-Cl	H	88	$C_{23}H_{28}ClN_3O_3 \cdot HCl$	59.23	6.27	9.01	59.00	6.05	8.93	1880
3rr[b,k]	3-NO_2, 4-Cl	Ac	69	$C_{25}H_{30}ClN_3O_4$	—	—	—	—	—	—	408
8[l]	—	—	38	$C_{21}H_{29}N_3O_2 \cdot 2HCl$	58.88	7.29	9.81	58.48	7.49	9.36	10700

a) Amorphous powder unless otherwise noted. *b*) Oil. The structure was confirmed by IR, ^{1}H-NMR (see Table VII), and MS spectra. *c*) MS *m/z*: 392 [M^+]. *d*) MS *m/z*: 437 [M^+]. *e*) MS *m/z*: 365 [M^+]. *f*) MS *m/z*: 407 [M^+]. *g*) MS *m/z*: 428 [M+2], 426 [M^+]. *h*) MS *m/z*: 417 [M^+]. *i*) MS *m/z*: 422 [M^+]. *j*) MS *m/z*: 470 [M^+]. *k*) MS *m/z*: 473 [M+2], 471 [M^+]. *l*) MS *m/z*: 355 [M^+].

TABLE IV. Acetylcholinesterase Inhibitory Activity and Physicochemical Parameters of *N*-[5-[*N*-Ethyl-*N*-(phenylmethyl)amino]pentyl]-3-arylpropenamides (**3**)

Compd. No.	X	R^3	π[a]	σ[a]	I[b]	Inhibition of AChE log(1/IC_{50}) Obsd.	Calcd[c]	Δ[d]
3m	H	H	0.00	0.00	0	4.93	4.99	−0.06
3x	H	Ac	0.00	0.00	1	5.64	5.72	−0.08
3a	4-NO_2	H	−0.28	0.78	0	5.52	5.52	0.00
3b	4-NO_2	Ac	−0.28	0.78	1	6.27	6.25	0.02
3y	3-NO_2	H	−0.28	0.71	0	5.53	5.47	0.06
3z	3-NO_2	Ac	−0.28	0.71	1	6.28	6.21	0.07
3aa	4-NH_2	H	−1.23	−0.66	0	4.99	4.80	0.19
3bb	4-NHAc	H	−0.97	0.00	0	5.25	5.16	0.09
3cc	4-Cl	H	0.71	0.23	0	4.81	5.01	−0.20
3dd	4-Cl	Ac	0.71	0.23	1	5.67	5.74	−0.07
3ee	4-Me	H	0.56	−0.17	0	4.80	4.79	0.01
3ff	4-CN	H	−0.57	0.66	0	5.31	5.49	−0.18
3gg	4-CN	Ac	−0.57	0.66	1	6.34	6.23	0.11
3hh	4-OH	H	−0.67	−0.37	0	4.90	4.88	0.02
3ii	4-OMe	H	−0.02	−0.27	0	4.79	4.83	−0.04
3jj	3-OMe	H	−0.02	0.12	0	5.03	5.07	−0.04
3kk	3-OMe	Ac	−0.02	0.12	1	5.24	5.80	−0.56
3t	3,4-$(OMe)_2$	H	−0.04[e]	−0.15[e]	0	5.27	4.91	0.36
3ll	3,4,5-$(OMe)_3$	H	−0.06[e]	−0.03[e]	0	5.39	4.98	0.41
3mm	4-SMe	H	0.61	0.00	0	4.68	4.88	−0.20
3nn	4-SOMe	H	−1.58	0.49	0	5.12	5.57	−0.45
3oo	4-SO_2Me	H	−1.63	0.72	0	5.50	5.71	−0.21
3pp	4-SO_2Me	Ac	−1.63	0.72	1	6.79	6.45	0.34
3qq	3-NO_2, 4-Cl	H	0.43[e]	0.94[e]	0	5.73	5.49	0.24
3rr	3-NO_2, 4-Cl	Ac	0.43[e]	0.94[e]	1	6.39	6.22	0.17

a) Taken from the literature.[11] *b*) Indicator variable which takes the value of one for the NAc (R^3 = Ac) and zero for the NH (R^3 = H) derivatives. *c*) Calculated by Eq. 1. *d*) Δ, difference between observed and calcd values. *e*) Values summed for component substituents.

the interaction between the carbonyl group and the enzyme occurs in the case of the *N*-Ac derivatives. Furthermore, the importance of the carbonyl groups of both the amide portion and the *N*-acetyl group is shown by comparison of compounds **2a**, **b** and **8**: the *N*-acetyl derivative (**2b**) was more potent than the amide derivative (**2a**), which in turn was more potent than the 1,5-diamine (**8**). These results and the above quantitative study suggest that these carbonyl groups interact with the enzyme presumably by hydrogen bondings. In other words, there may exist at least one hydrogen bonding subsite in or near HBS-2.

In conclusion, some of the *N*-[ω-[*N*-alkyl-*N*-(phenylmethyl)amino]alkyl]-3-arylpropenamides (**3**) were found to be potent AChE inhibitors. The results of structure–activity relationships obtained in this study support the existence of HBS-2 and also allow speculation as to the nature of this site. Thus it appears that HBS-2 may contain at least one hydrogen bonding subsite in or close to the site. In order to confirm the hypothesis proposed in this and preceding papers as well as to search for new types of AChE inhibitors, further studies are now being undertaken.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Infrared (IR) spectra were taken on a Hitachi 260-10 spectrophotometer in KBr disks for solids and liquid films for oils. Mass spectra (MS) were measured on a JOEL JMS-01SC spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Varian EM-390 NMR spectrometer with tetramethylsilane as an internal standard. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Chromatographic purifications were carried out on silica gel column (Kieselgel 60, 0.063—0.200 mm, Merck).

TABLE V. Simple Correlation Matrix for the Parameters of Eq. 1

	π	σ	I
π	1.000		
σ	0.089	1.000	
I	0.063	0.361	1.000

Preparation of *N*-Alkyl-*N*-(phenylmethyl)alkane-1,ω-diamines (4) 2-[ω-[*N*-Alkyl-*N*-(phenylmethyl)amino]alkyl]isoindole-1,3(2*H*)-diones (**1**) were treated with hydrazine hydrate to give **4** according to the method described earlier.[1] The yields and spectral data are listed in Table VI.

Preparation of 3-Arylpropenoic Acids (5) Commercially available 3-arylpropenoic acids (**5**) were used in condensation with **4** with the exception of certain acids, which were prepared in the following manner.

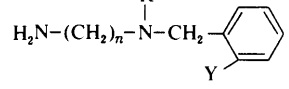
(*E*)-3-[4-(Methylthio)phenyl]propenoic Acid (5a) A mixture of 4-(methylthio)benzaldehyde (7.6 g) and ethoxycarbonylmethylidene triphenylphosphorane (20.9 g) in toluene (100 ml) was refluxed for 1 h and the solvent was removed *in vacuo*. Hexane was added to the residue and the resulting precipitate was removed by filtration. The filtrate was concentrated, diluted with CH_2Cl_2, passed through a plug of silica gel, and the solvent was evaporated off to give a residue. The residue was crystallized from hexane to afford ethyl (*E*)-3-[4-(methylthio)phenyl]propenoate (10.7 g, 96%) as colorless cubes, mp 47—48 °C. IR (KBr): 2978, 1706, 1628, 1590, 1492, 1437, 1365, 1307 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.30 (3H, t, J=7 Hz), 2.43 (3H, s), 4.23 (2H, q, J=7 Hz), 6.33 (1H, d, J=16 Hz), 7.15 (2H, d, J=8 Hz), 7.38 (2H, d, J=8 Hz), 7.59 (1H, d, J=16 Hz).

A mixture of the ester (10.5 g) and K_2CO_3 (8.0 g) in methanol–water (200/40 ml) was refluxed for 1 h and the solvents were removed under reduced pressure to give a residue. An aqueous solution of the residue was made acidic (pH = 5—6) with 10% HCl. The resulting precipitate was collected by filtration, washed with water three times and dried *in vacuo* to give colorless cubes (9.1 g, 99%), mp 175—176 °C (lit.[8] 170—171 °C). IR (KBr): 2960, 2916, 2550, 1687, 1624, 1591 cm^{-1}. ^{1}H-NMR (dimethylsulfoxide-d_6 (DMSO-d_6)) δ: 2.47 (3H, s), 6.45 (1H, d, J=16 Hz), 7.24 (2H, d, J=8 Hz), 7.51 (1H, d, J=16 Hz), 7.58 (2H, d, J=8 Hz), 8.00 (1H, br s).

(*E*)-3-[4-(Methylsulfinyl)phenyl]propenoic Acid (5b) A mixture of **5a** (2.0 g) and 30% H_2O_2 (0.7 ml) in acetic acid (8 ml) was heated at 50 °C for 1 h, cooled to room temperature and water was added to the mixture. The resulting precipitate was collected by filtration, washed with water three times and dried *in vacuo* to give colorless cubes (1.5 g, 69%), mp 217—218 °C. IR (KBr): 3422, 2914, 2566, 1692, 1639, 1016 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.73 (3H, s), 6.59 (1H, d, J=16 Hz), 7.62 (1H, d, J=16 Hz), 7.68 (2H, d, J=8 Hz), 7.87 (2H, d, J=8 Hz), 12.4 (1H, br s).

(*E*)-3-[4-(Methylsulfonyl)phenyl]propenoic Acid (5c) A mixture of **5a** (2.0 g) and 30% H_2O_2 (2 ml) in acetic acid (8 ml) was heated at 90—95 °C for 1 h, cooled to room temperature and water was added to the mixture.

TABLE VI. Spectral Data of *N*-Alkyl-*N*-(phenylmethyl)alkane-1,ω-diamines (4)



4

Compd.	n	R	Y	Yield (%)	IR (film) cm^{-1}	^{1}H-NMR ($CDCl_3$) δ[a]
4a	3	Et	H	95	3354, 2966, 2934	1.05 (3H, t, J=7 Hz), 1.64 (2H, tt, J=7, 7 Hz), 2.38—2.88 (6H, m), 3.17 (2H, br s), 3.53 (2H, s), 7.32 (5H, s)
4b	4	Et	H	93	3362, 2934	1.01 (3H, t, J=7.5 Hz), 1.30—1.70 (4H, m), 2.13 (2H, br s), 2.30—2.88 (6H, m), 3.53 (2H, s), 7.27 (5H, s)
4c	5	Et	H	91	3350, 2932	1.04 (3H, t, J=7 Hz), 1.21—1.58 (6H, m), 1.88 (2H, br s), 2.44 (2H, t, J=7.5 Hz), 2.52 (2H, q, J=7 Hz), 2.68 (2H, t, J=7 Hz), 3.57 (2H, s), 7.26 (5H, s)
4d	6	Et	H	93	3310, 2930	1.00 (3H, t, J=7 Hz), 1.10—1.67 (8H, m), 1.82 (2H, br s), 2.27—2.82 (6H, m), 3.52 (2H, s), 7.27 (5H, s)
4e	7	Et	H	96	3350, 2930	0.90—1.77 (10H, m), 1.02 (3H, t, J=7 Hz), 2.28—2.87 (6H, m), 3.55 (2H, s), 3.92 (2H, br s), 7.27 (5H, s)
4f	5	Me	H	94	3354, 2940	1.08—1.80 (8H, m), 2.18 (3H, s), 2.38 (2H, t, J=7 Hz), 2.68 (2H, t, J=6.5 Hz), 3.48 (2H, s), 7.30 (5H, s)
4g	5	Pr	H	91	3350, 2932	0.83 (3H, t, J=7 Hz), 1.07—1.75 (8H, m), 2.13—2.87 (8H, m), 3.53 (2H, s), 7.30 (5H, s)
4h	5	iso-Pr	H	95	3350, 2930	0.99 (6H, d, J=7 Hz), 1.10—1.77 (8H, m), 2.10—2.55 (4H, m), 2.90 (1H, q of q, J=7, 7 Hz), 3.53 (2H, s), 7.25 (5H, s)
4i	5	Et	OMe	93	3358, 2932	1.03 (3H, t, J=7 Hz), 1.20—2.07 (8H, m), 2.32—2.80 (6H, m), 3.57 (2H, s), 3.80 (3H, s), 6.73—7.03 (2H, m), 7.18 (1H, dd, J=7, 7 Hz), 7.40 (1H, d, J=7 Hz)

a) Chemical shifts are given with proton numbers, absorption patterns and coupling constants in parentheses.

TABLE VII. Spectral Data[a] of **2a**, **b**, **3c**—**z**, **cc**—**rr**, **6**, and **7**

Compd.	IR (film) cm^{-1}	^1H-NMR ($CDCl_3$) δ[b]
2a	3304, 2934, 1647, 1600, 1526	1.05 (3H, t, J=7 Hz), 1.25—1.68 (6H, m), 2.42—2.61 (4H, m), 3.46 (2H, dt, J=6, 7 Hz), 3.58 (2H, s), 6.31 (1H, br s), 7.18—7.37 (5H, m), 7.91 (2H, d, J=8.5 Hz), 8.28 (2H, d, J=8.5 Hz)
2b	2936, 1690, 1664, 1526	1.01 (3H, t, J=7 Hz), 1.21—1.68 (6H, m), 2.28 (3H, s), 2.38 (2H, t, J=7.5 Hz), 2.48 (2H, q, J=7 Hz), 3.53 (2H, s), 3.71 (2H, t, J=7.5 Hz), 7.29 (5H, s), 7.68 (2H, d, J=9 Hz), 8.29 (2H, d, J=9 Hz)
3c[c]	3276, 2934, 1656, 1619, 1579	1.08 (3H, t, J=7 Hz), 1.33—1.90 (2H, m), 2.17—2.73 (4H, m), 3.43 (2H, dt, J=5, 6 Hz), 3.53 (2H, s), 6.21 (1H, d, J=15 Hz), 7.00—7.67 (9H, m), 8.20 (2H, d, J=9 Hz)
3d	3280, 2934, 1660, 1623, 1519	1.03 (3H, t, J=7 Hz), 1.47—1.83 (4H, m), 2.37—2.70 (4H, m), 3.36 (2H, dt, J=5, 6 Hz), 3.57 (2H, s), 6.37 (1H, d, J=15 Hz), 6.93 (1H, br t, J=5 Hz), 7.27 (5H, s), 7.48 (2H, d, J=8 Hz), 7.59 (1H, d, J=15 Hz), 8.15 (2H, d, J=8 Hz)
3e	3286, 2932, 1660, 1622, 1519	1.00 (3H, t, J=7 Hz), 1.10—1.77 (8H, m), 2.20—2.64 (4H, m), 3.35 (2H, dt, J=5, 6 Hz), 3.52 (2H, s), 5.97 (1H, br), 6.50 (1H, d, J=15 Hz), 7.25 (5H, s), 7.58 (2H, d, J=8 Hz), 7.63 (1H, d, J=15 Hz), 8.17 (2H, d, J=8 Hz)
3f	3290, 2930, 1661, 1622, 1520	1.00 (3H, t, J=7 Hz), 1.15—1.77 (10H, m), 2.27—2.65 (4H, m), 3.36 (2H, dt, J=5, 6 Hz), 3.53 (2H, s), 6.08 (1H, br), 6.53 (1H, d, J=15 Hz), 7.26 (5H, s), 7.60 (2H, d, J=9 Hz), 7.65 (1H, d, J=15 Hz), 8.18 (2H, d, J=9 Hz)
3g	2938, 1698, 1680, 1624, 1596, 1519	1.03 (3H, t, J=7.5 Hz), 1.36–1.83 (4H, m), 2.33—2.67 (4H, m), 2.40 (3H, s), 3.57 (2H, s), 3.71 (2H, t, J=7.5 Hz), 7.10—7.40 (6H, m), 7.65 (2H, d, J=9 Hz), 7.70 (1H, d, J=15 Hz), 8.21 (2H, d, J=9 Hz)
3h	2934, 1698, 1680, 1519	1.03 (3H, t, J=7 Hz), 1.16—1.83 (8H, m), 2.30—2.70 (4H, m), 2.40 (3H, s), 3.57 (2H, s), 3.73 (2H, t, J=7.5 Hz), 7.20 (1H, d, J=15 Hz), 7.27 (5H, s), 7.65 (2H, d, J=9 Hz), 7.70 (1H, d, J=15 Hz), 8.22 (2H, d, J=9 Hz)
3i	3286, 2932, 1660, 1622, 1519	1.13—2.03 (6H, m), 2.62 (3H, d, J=5 Hz), 2.83—3.35 (4H, m), 4.03—4.45 (2H, m), 6.85 (1H, d, J=15 Hz), 7.36—7.90 (8H, m), 8.16—8.47 (3H, m), 11.30 (br s)[d]
3j	3296, 2932, 1662, 1623, 1519	0.99 (6H, d, J=7 Hz), 1.10—1.77 (6H, m), 2.40 (2H, t, J=6 Hz), 2.73—3.13 (1H, m), 3.13—3.50 (2H, m), 3.53 (2H, s), 5.97 (1H, br), 6.48 (1H, d, J=15 Hz), 7.25 (5H, s), 7.58 (2H, d, J=9 Hz), 7.78 (1H, d, J=15 Hz), 8.17 (2H, d, J=9 Hz)
3k	3280, 2934, 1660, 1623, 1519	0.83 (3H, t, J=7 Hz), 1.10—1.75 (8H, m), 2.16—2.55 (4H, m), 3.33 (2H, dt, J=5, 6 Hz), 3.50 (2H, s), 6.03 (1H, br t , J=5 Hz), 6.49 (1H, d, J=15 Hz), 7.25 (5H, s), 7.57 (2H, d, J=8 Hz), 7.62 (1H, d, J=15 Hz), 8.17 (2H, d, J=8 Hz)
3l	3280, 2934, 1661, 1623, 1599, 1519	1.01 (3H, t, J=7 Hz), 1.16—1.76 (6H, m), 2.27—2.67 (4H, m), 3.34 (2H, dt, J=5, 6 Hz), 3.54 (2H, s), 3.80 (3H, s), 6.28 (1H, br), 6.44 (1H, d, J=16 Hz), 6.72—7.76 (7H, m), 8.18 (2H, d, J=8 Hz)
3m	3276, 2934, 1656, 1619, 1579	1.00 (3H, t, J=7.5 Hz), 1.15—1.70 (6H, m), 2.27—2.63 (4H, m), 3.33 (2H, dt, J=5, 6 Hz), 3.50 (2H, s), 5.88 (1H, br), 6.35 (1H, d, J=15 Hz), 7.10—7.60 (10H, m), 7.58 (1H, d, J=15 Hz)
3n	3442, 2922, 1669, 1630, 1562	1.07—1.95 (9H, m), 2.73—3.35 (6H, m), 4.27 (2H, d, J=5 Hz), 7.00 (1H, d, J=16 Hz), 7.33—7.80 (6H, m), 7.93 (1H, dd, J=5, 8 Hz), 8.40—8.65 (2H, m), 8.81 (1H, d, J=5 Hz), 9.05 (1H, s), 11.30 (1H, br s)[d]
3o	3278, 2934, 1658, 1620, 1566	1.00 (3H, t, J=7.5 Hz), 1.10—1.70 (6H, m), 2.23—2.63 (4H, m), 3.32 (2H, dt, J=5, 6 Hz), 3.53 (2H, s), 5.67 (1H, br), 6.26 (1H, d, J=15 Hz), 6.33—6.57 (2H, m), 7.05—7.52 (7H, m)
3p	3280, 2934, 1649, 1613, 1557	1.00 (3H, t, J=7.5 Hz), 1.22—1.74 (6H, m), 2.26—2.64 (4H, m), 3.31 (2H, dt, J=5, 6 Hz), 3.51 (2H, s), 5.73 (1H, br), 6.16 (1H, d, J=15 Hz), 6.90—7.37 (8H, m), 7.70 (1H, d, J=15 Hz)
3q	3326, 2934. 1650, 1616, 1533	1.01 (3H, t, J=7.5 Hz), 1.23—1.70 (6H, m), 2.07 (3H, s), 2.30—2.63 (4H, m), 3.32 (2H, dt, J=5, 6 Hz), 3.52 (2H, s), 5.83 (1H, br), 7.10—7.46 (11H, m)
3r	3326, 2934, 1665, 1616, 1509	1.00 (3H, t, J=7.5 Hz), 1.10—1.60 (6H, m), 2.26—2.60 (4H, m), 3.24 (2H, dt, J=5, 6 Hz), 3.50 (2H, s), 5.43 (1H, br t, J=6 Hz), 6.87—7.51 (15H, m), 7.86 (1H, s)
3s	3294, 2934, 1643, 1630, 1576	0.95—1.50 (6H, m), 1.00 (3H, t, J=7.5 Hz), 2.22—2.62 (4H, m), 3.04 (2H, dt, J=5, 6 Hz), 3.50 (2H, s), 5.12 (1H, br), 6.36 (1H, s), 7.26 (15H, m)
3t	3284, 2932, 1654, 1617, 1599, 1514	1.00 (3H, t, J=7 Hz), 1.20—1.70 (6H, m), 2.29—2.63 (4H, m), 3.32 (2H, dt, J=5, 6 Hz), 3.51 (2H, s), 3.87 (6H, s), 5.74 (1H, br t, J=5 Hz), 6.23 (1H, d, J=15 Hz), 6.80 (1H, d, J=9 Hz), 6.96—7.13 (2H, m), 7.28 (5H, s), 7.53 (1H, d, J=15 Hz)
3u	3314, 2934, 1627, 1561, 1532	1.01 (3H, t, J=7 Hz), 1.15—1.73 (6H, m), 2.29—2.66 (4H, m), 3.35 (2H, dt, J=5, 6 Hz), 3.52 (2H, s), 3.57 (2H, br s), 3.87 (6H, s), 5.95 (1H, br t, J=5 Hz), 6.93 (1H, s), 7.00 (1H, s), 7.26 (5H, s), 7.32 (1H, s)
3v	3334, 2932, 1643, 1606, 1518	1.00 (3H, t, J=7 Hz), 1.16—1.70 (6H, m), 2.28—2.97 (8H, m), 3.32 (2H, dt, J=5, 6 Hz), 3.52 (2H, s), 3.83 (3H, s), 3.86 (3H, s), 5.85 (1H, br t, J=5 Hz), 6.67 (2H, s), 7.08 (1H, s), 7.26 (5H, s)
3w	3326, 2930, 1645, 1604, 1519	1.01 (3H, t, J=7 Hz), 1.18—1.76 (6H, m), 1.84—2.20 (2H, m), 2.23—2.94 (8H, m), 3.31 (2H, dt, J=5, 6 Hz), 3.52 (2H, s), 3.83 (3H, s), 3.86 (3H, s), 5.89 (1H, br t, J=5 Hz), 6.63 (1H, s), 6.77 (1H, s), 7,22 (1H, s), 7.28 (5H, s)
3x	2934, 1685, 1619	1.00 (3H, t, J=7 Hz), 1.13—1.82 (6H, m), 2.27—2.63 (4H, m), 2.41 (3H, s), 3.52 (2H, s), 3.73 (2H, t, J=7 Hz), 6.99 (1H, d, J=15 Hz), 7.10—7.63 (10H, m), 7.74 (1H, d, J=15 Hz)
3y	3282, 2932, 1662, 1623, 1530	1.01 (3H, t, J=7 Hz), 1.13—1.73 (6H, m), 2.20—2.65 (4H, m), 3.36 (2H, dt, J=5, 6 Hz), 3.53 (2H, s), 5.98 (1H, br), 6.48 (1H, d, J=15 Hz), 7.26 (5H, s), 7.36—7.80 (3H, m), 8.15 (1H, d, J=8 Hz), 8.32 (1H, s)
3z	2936, 1683, 1625, 1531	1.03 (3H, t, J=7 Hz), 1.15—1.83 (6H, m), 2.33—2.70 (4H, m), 2.40 (3H, s), 3.57 (2H, s), 3.73 (2H, br t, J=7 Hz), 7.18 (1H, d, J=15 Hz), 7.26 (5H, s), 7.53 (1H, dd, J=8, 8 Hz), 7.70 (1H, d, J=15 Hz), 7.82 (1H, d, J=8 Hz), 8.20 (1H, d, J=8 Hz), 8.35 (1H, s)
3cc	3284, 2936, 1655, 1618, 1554	1.00 (3H, t, J=7 Hz), 1.18—1.73 (6H, m), 2.16—2.62 (4H, m), 3.31 (2H, dt, J=5, 6 Hz), 3.51 (2H, s), 6.03 (1H, br t, J=5 Hz), 6.35 (1H, d, J=15 Hz), 7.13—7.47 (9H, m), 7.54 (1H, d, J=15 Hz)
3dd	2936, 1686, 1620	1.00 (3H, t, J=7 Hz), 1.13—1.80 (6H, m), 2.30—2.63 (4H, m), 2.42 (3H, s), 3.52 (2H, s), 3.71 (2H, t, J=7 Hz), 7.02 (1H, d, J=15 Hz), 7.13—7.57 (9H, m), 7.68 (1H, d, J=15 Hz)
3ee	3280, 2932, 1655, 1618,	1.00 (3H, t, J=7 Hz), 1.15—1.76 (6H, m), 2.25—2.63 (4H, m), 2.33 (3H, s), 3.33 (2H, dt,

TABLE VII. (continued)

Compd.	IR (film) cm^{-1}	^{1}H-NMR ($CDCl_3$) δ[b]
	1544	J=5, 6 Hz), 3.51 (2H, s), 5.93 (1H, br t, J=5 Hz), 6.32 (1H, d, J=15 Hz), 7.00—7.45 (9H, m), 7.56 (1H, d, J=15 Hz)
3ff	3280, 2934, 2226, 1659, 1622, 1556	1.00 (3H, t, J=7 Hz), 1.20—1.73 (6H, m), 2.28—2.63 (4H, m), 3.33 (2H, dt, J=5, 6 Hz), 3.50 (2H, s), 6.25 (1H, br t, J=5 Hz), 6.48 (1H, d, J=15 Hz), 7.26 (5H, s), 7.41—7.70 (5H, m)
3gg	2936, 2228, 1698, 1623, 1531	1.01 (3H, t, J=7 Hz), 1.13—1.94 (6H, m), 2.27—2.65 (4H, m), 2.40 (3H, s), 3.53 (2H, s), 3.72 (2H, br t, J=7 Hz), 7.05—7.38 (6H, m), 7.49—7.77 (5H, m)
3hh	3295, 2935, 1661, 1622, 1520	1.00 (3H, t, J=7 Hz), 1.16—1.70 (6H, m), 2.28—2.63 (4H, m), 3.31 (2H, dt, J=5, 6 Hz), 3.52 (2H, s), 5.25 (1H, br), 6.04—6.43 (2H, m), 6.71 (2H, d, J=9 Hz), 7.05—7.67 (8H, m)
3ii	3284, 2932, 1655, 1604, 1551, 1512	1.01 (3H, t, J=7 Hz), 1.21—1.70 (6H, m), 2.28—2.63 (4H, m), 3.32 (2H, dt, J=5, 6 Hz), 3.53 (2H, s), 3.81 (3H, s), 5.85 (1H, br t, J=5 Hz), 6.25 (1H, d, J=15 Hz), 6.84 (2H, d, J=9 Hz), 7.30 (5H, s), 7.42 (2H, d, J=9 Hz), 7.57 (1H, d, J=15 Hz)
3jj	3280, 2934, 1656, 1620, 1579, 1554	1.00 (3H, t, J=7 Hz), 1.20—1.73 (6H, m), 2.27—2.64 (4H, m), 3.33 (2H, dt, J=5, 6 Hz), 3.52 (2H, s), 3.77 (3H, s), 6.02 (1H, br), 6.37 (1H, d, J=15 Hz), 6.74—7.40 (4H, m), 7.27 (5H, s), 7.55 (1H, d, J=15 Hz)
3kk	2936, 1691, 1619	1.09 (3H, t, J=7 Hz), 1.16—1.83 (6H, m), 2.33—2.80 (4H, m), 2.40 (3H, s), 3.73 (2H, t, J=7 Hz), 3.72 (2H, s), 3.80 (3H, s), 6.76—7.50 (10H, m), 7.69 (1H, d, J=15 Hz)
3ll	3284, 2936, 1656, 1619, 1583, 1545, 1505	1.00 (3H, t, J=7 Hz), 1.20—1.70 (6H, m), 2.28—2.63 (4H, m), 3.33 (2H, dt, J=5, 6 Hz), 3.52 (2H, s), 3.86 (9H, s), 5.90 (1H, br t, J=5 Hz), 6.30 (1H, d, J=15 Hz), 6.70 (2H, s), 7.28 (5H, s), 7.52 (1H, d, J=15 Hz)
3mm	3280, 2932, 1656, 1618, 1556	1.00 (3H, t, J=7 Hz), 1.13—1.77 (6H, m), 2.27—2.63 (4H, m), 2.43 (3H, s), 3.32 (2H, dt, J=5, 6 Hz), 3.50 (2H, s), 6.10 (1H, br t, J=5 Hz), 6.33 (1H, q, J=16 Hz), 7.04—7.43 (9H, m), 7.53 (1H, d, J=16 Hz)
3nn	3450, 3286, 2934, 1661, 1621, 1552, 1041	1.00 (3H, t, J=7 Hz), 1.20—1.76 (6H, m), 2.23—2.63 (4H, m), 2.71 (3H, s), 3.33 (2H, dt, J=5, 6 Hz), 3.52 (2H, s), 6.46 (1H, br t, J=5 Hz), 6.49 (1H, d, J=16 Hz), 7.28 (5H, s), 7.59 (1H, d, J=16 Hz), 7.60 (4H, s)
3oo	3288, 2932, 1661, 1623, 1547, 1306, 1149	1.00 (3H, t, J=7 Hz), 1.25—2.07 (6H, m), 2.29—2.63 (4H, m), 3.02 (3H, s), 3.34 (2H, dt, J=5, 6 Hz), 3.51 (2H, s), 6.17 (1H, br t, J=5 Hz), 6.48 (1H, d, J=16 Hz), 7.25 (5H, s), 7.55 (2H, d, J=8 Hz), 7.58 (1H, d, J=16 Hz), 7.86 (2H, d, J=8 Hz)
3pp	2932, 1687, 1623, 1308, 1149	1.07 (3H, t, J=7 Hz), 1.23—1.82 (6H, m), 2.35—2.76 (4H, m), 2.41 (3H, s), 3.04 (3H, s), 3.67 (2H, s), 3.73 (2H, t, J=7 Hz), 7.19 (1H, d, J=16 Hz), 7.30 (5H, s), 7.68 (2H, d, J=8 Hz), 7.69 (1H, d, J=16 Hz), 7.94 (2H, d, J=8 Hz)
3qq	3284, 2934, 1663, 1626, 1537	1.01 (3H, t, J=7 Hz), 1.16—1.73 (6H, m), 2.27—2.63 (4H, m), 3.34 (2H, dt, J=5, 6 Hz), 3.53 (2H, s), 6.28 (1H, br t, J=5 Hz), 6.47 (1H, d, J=16 Hz), 7.29 (5H, s), 7.53 (2H, s), 7.55 (1H, d, J=16 Hz), 7.95 (1H, s)
3rr	2936, 1685, 1626, 1537	1.03 (3H, t, J=7 Hz), 1.13—1.80 (6H, m), 2.32—2.68 (4H, m), 2.39 (3H, s), 3.56 (2H, s), 3.71 (2H, br t, J=7 Hz), 7.15 (1H, d, J=15 Hz), 7.27 (5H, s), 7.43—7.72 (3H, m), 7.98 (1H, s)
6	3284, 2934, 1671, 1629, 1551	1.01 (3H, t, J=7 Hz), 1.10—1.67 (6H, m), 1.82 (3H, dd, J=1.5, 7 Hz), 2.24—2.64 (4H, m), 3.25 (2H, dt, J=6, 6 Hz), 3.53 (2H, s), 5.60 (1H, br), 5.75 (1H, dq, J=1.5, 15 Hz), 6.81 (1H, dq, J=7, 15 Hz), 7.30 (5H, s)
7	3290, 2932, 1643, 1552	1.01 (3H, t, J=7.5 Hz), 1.05—1.62 (6H, m), 2.23—2.63 (6H, m), 2.82—3.28 (4H, m), 3.53 (2H, s), 5.36 (1H, br s), 7.23 (5H, s), 7.30 (5H, s)

a) Spectral data of free bases were measured. b) Chemical shifts are given with proton numbers, absorption patterns and coupling constants in parentheses. c) mp 70—72 °C. d) HCl salt was measured in DMSO-d_6.

The resulting precipitate was collected by filtration, washed with water three times and dried *in vacuo* to give colorless cubes (1.8 g, 77%), mp 298—299 °C (lit.[8] 276—277 °C). IR (KBr): 3424, 2922, 2550, 1687, 1638, 1309, 1149 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.20 (3H, s), 6.66 (1H, d, J=16 Hz), 7.65 (1H, d, J=16 Hz), 7.93 (4H, s), 12.55 (1H, br s).

3,3-Diphenylpropenoic Acid (5d) Compound **5d** was prepared from 1,1-diphenylethylene and oxalyl chloride according to the method described in the literature.[9]

5,6-Dimethoxy-1*H*-indene-2-carboxylic Acid (5e), 3,4-Dihydro-6,7-dimethoxy-2-naphthalenecarboxylic Acid (5f), and 6,7-Dihydro-2,3-dimethoxy-5*H*-benzocycloheptene-8-carboxylic Acid (5g) Compound **5e—g** were prepared according to the method described in the literature.[10]

5e: Colorless cubes, mp 251—252 °C. Yield: 29% (from 5,6-dimethoxy-2,3-dihydro-1*H*-inden-1-one). IR (KBr): 3600—2300, 1665, 1610, 1555 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.67 (2H, s), 3.93 (3H, s), 3.95 (3H, s), 7.07 (1H, s), 7.10 (1H, s), 7.81 (1H, s), 9.6 (1H, br s). *Anal.* Calcd for $C_{12}H_{12}O_4$: C, 65.45; H, 5.49. Found: C, 65.19; H, 5.52.

5f: Colorless cubes, mp 190—193 °C. Yield: 74% (from 6,7-dimethoxy-1,2,3,4-tetrahydronaphthalen-1-one). IR (KBr): 3650—2400, 1660, 1604, 1568, 1518 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.59 (2H, t, J=8 Hz), 2.84 (2H, t, J=8 Hz), 3.88 (3H, s), 3.91 (3H, s), 6.73 (1H, s), 6.77 (1H, s), 7.57 (1H, s), 9.55 (br s). *Anal.* Calcd for $C_{13}H_{14}O_4$: C, 66.66; H, 6.02. Found: C, 66.54; H, 6.13.

5g: Colorless cubes, mp 158—159 °C. Yield: 64% (from 2,3-dimethoxy-5*H*-6,7,8,9-tetrahydrobenzocyclohepten-5-one). IR (KBr): 3600—2000, 1670, 1600, 1570, 1520 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.90—2.95 (6H, m), 3.87 (3H, s), 3.90 (3H, s), 6.68 (1H, s), 6.86 (1H, s), 7.80 (1H, s), 9.60 (1H, br s). *Anal.* Calcd for $C_{14}H_{16}O_4$: C, 67.73; H, 6.50. Found: C, 67.97; H, 6.55.

(*E*)-*N*-[5-[*N*-Ethyl-*N*-(phenylmethyl)amino]pentyl]-3-(4-nitrophenyl)-2-propenamide Hydrochloride (3a) Diethyl phosphorocyanidate (1.1 ml) and Et_3N (0.76 ml) were added successively to a suspension of 4-nitrocinnamic acid (0.88 g) and *N*-ethyl-*N*-(phenylmethyl)pentane-1,5-diamine (**4c**, 1.0 g) in *N*,*N*-dimethylformamide (DMF, 10 ml) at 0—5 °C. The mixture was stirred at 0—5 °C for 20 min, warmed to room temperature, diluted with water, and extracted with CH_2Cl_2. The extracts were dried over Na_2SO_4 and the solvent was removed *in vacuo* to give a residue, which was chromatographed on silica gel eluting with ethyl acetate–methanol (20 : 1) to afford an oil as a main fraction. IR (film): 3288, 2936, 1736, 1661, 1624, 1599, 1520 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.00 (3H, t, J=7 Hz), 1.10—2.04 (6H, m), 2.20—2.66 (4H, m), 3.36 (2H, dt, J=5, 6 Hz), 3.53 (2H, s), 6.13 (1H, br), 6.50 (1H, d, J=15 Hz), 7.27 (5H, s), 7.62 (1H, d, J=15 Hz), 7.58 (2H, d, J=9 Hz), 8.18 (2H, d, J=9 Hz).

The oil was treated with ethanolic HCl (1 eq) and the resulting solid was triturated in ether, collected by filtration, and dried *in vacuo* to give a hygroscopic amorphous powder (1.7 g). The yield and analytical data of **3a** are shown in Table I.

The following compounds (**2a**, **3c—f**, **i—w**, **y**, **aa—cc**, **ee**, **ff**, **hh—jj**, **ll—oo**, **qq**, **6**, and **7**) listed in Tables I, II, III and VII were prepared in the same manner as described for **3a**.

(*E*)-*N*-Acetyl-*N*-[5-[*N*-ethyl-*N*-(phenylmethyl)amino]pentyl]-3-(4-nitrophenyl)-2-propenamide (3b) A mixture of **3a** (0.34 g) and a catalytic amount (30 mg) of *p*-toluenesulfonic acid in acetic anhydride (5 ml) was heated at 80 °C for 6 h and cooled to room temperature. The mixture was diluted with water, made basic with 10% NaOH, and extracted with CH_2Cl_2. The extracts were dried over Na_2SO_4 and the solvent was evaporated to afford an oil, which was chromatographed on silica gel eluting with ethyl acetate–methanol (20 : 1) to give an oil (0.29 g). IR (film): 2936, 1697, 1682, 1624, 1597, 1520 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.02 (3H, t, J=7 Hz), 1.20—1.82 (6H, m), 2.27—2.67 (4H, m), 2.42 (3H, s), 3.54 (2H, s), 3.73 (2H, br t, J=6 Hz), 7.18 (1H, d, J=15 Hz), 7.31 (5H,

s), 7.67 (2H, d, J=9 Hz), 7.71 (1H, d, J=15 Hz), 8.23 (2H, d, J=9 Hz). The yield and mass spectrum of this sample are shown in Table I.

The following compounds (**2b**, **3g**, **h**, **x**, **z**, **dd**, **gg**, **kk**, **pp**, **rr**) listed in Tables I, III and VII were prepared in the same manner as described for **3b**.

(*E*)-3-(4-Aminophenyl)-*N*-[5-[*N*-ethyl-*N*-(phenylmethyl)amino]-pentyl]-2-propenamide Dihydrochloride (3aa) A suspension of **3a** (0.70 g) and concentrated HCl (0.4 ml) in ethanol (30 ml) was hydrogenated over 10% Pd/C (50 mg) under an atmospheric pressure at room temperature. The catalyst was removed by filtration, and the filtrate was concentrated to give a residue. The residue was dissolved in 10% NaOH and extracted with CH_2Cl_2. The extracts were dried over Na_2SO_4 and the solvent was removed *in vacuo* to give a residue. Chromatographic purification of the residue on silica gel eluting with ethyl acetate–methanol (20 : 1) gave an oil. IR (film): 3462, 3370, 2934, 1687, 1618 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.00 (3H, t, J=7 Hz), 1.13—1.83 (6H, m), 2.24—2.65 (4H, m), 3.43—3.83 (4H, m), 3.51 (2H, s), 6.04—7.37 (6H, m), 7.27 (5H, s), 7.65 (1H, d, J=15 Hz).

The oil was treated with ethanolic HCl (2 eq) and the resulting solid was triturated in ether, collected by filtration, and dried *in vacuo* to afford a hygroscopic amorphous powder (0.55 g). The yield, mass spectrum, and analytical data are given in Table III.

(*E*)-3-(4-Acetylaminophenyl)-*N*-[5-[*N*-ethyl-*N*-(phenylmethyl)amino]-pentyl]-2-propenamide Hydrochloride (3bb) A mixture of **3aa** (0.13 g) and acetyl chloride (53 mg) in pyridine (3 ml) was stirred at room temperature for 1 h, diluted with water, and extracted with CH_2Cl_2. The extracts were dried over Na_2SO_4 and the solvent was removed under reduced pressure to afford a residue. The residue was chromatographed on silica gel eluting with ethyl acetate–methanol (10 : 1) to give an oil (0.11 g). IR (film): 3318, 2936, 1678, 1618, 1589, 1552 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.00 (3H, t, J=7 Hz), 1.20—1.83 (6H, m), 2.13 (3H, s), 2.30—3.43 (6H, m), 3.52 (2H, s), 5.73 (1H, t, J=6 Hz), 6.28 (1H, d, J=15 Hz), 7.13—7.80 (11H, m).

The oil was treated with ethanolic HCl (1 eq) to afford a hygroscopic amorphous powder (0.09 g). The yield, mass spectrum, and analytical data of **3bb** are given in Table III.

***N*-(Ethyl-*N'*-[(4-nitrophenyl)methyl]-*N*-(phenylmethyl)pentane-1,5-diamine Dihydrochloride (8)** 4-Nitrobenzylbromide (0.5 g) was added portionwise to a mixture of *N*-ethyl-*N*-(phenylmethyl)pentane-1,5-diamine (**4c**, 1.0 g) and K_2CO_3 (0.45 g) in ethanol (20 ml) at room temperature. The resulting mixture was stirred for 16 h, and the solvent was removed *in vacuo* to give a residue. Water (100 ml) was added to the residue and the mixture was extracted with CH_2Cl_2. The extracts were dried over Na_2SO_4 and the solvent was evaporated to give an oil, which was chromatographed on silica gel eluting with ethyl acetate–methanol (5 : 1) to afford a pale yellow oil (0.37 g). IR (film): 3306, 2932, 2858, 2802, 1669, 1602, 1518 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.22—1.60 (6H, m), 2.20 (1H, br s), 2.39—2.65 (6H, m), 3.58 (2H, s), 3.88 (2H, s), 7.18—7.37 (5H, m), 7.49 (2H, d, J=8.5 Hz), 8.17 (2H, d, J=8.5 Hz).

The oil was treated with ethanolic HCl (2 eq) and the resulting solid was triturated in ether, collected by filtration, and dried *in vacuo* to give a hygroscopic amorphous powder (0.38 g). IR (KBr): 3418, 2948, 2774, 1635, 1607, 1523 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.20—1.43 (5H, m), 1.63—1.86 (4H, m), 2.82—3.14 (6H, m), 4.22—4.40 (4H, m), 7.37—7.53 (3H, m), 7.60—7.78 (2H, m), 7.93 (2H, d, J=8.7 Hz), 8.28 (2H, d, J=8.7 Hz), 9.82 (2H, br s), 11.0 (1H, br s). The yield and analytical data of this sample are given in Table III.

Biological Method and Materials The cerebral cortex of male Wistar rats was homogenized with 20 volumes of ice-coold 0.32 M sucrose, and centrifuged at 1000 × *g* for 10 min. The supernatant (Sl) was preincubated in a scintillation vial with a test compound for 15 min at room temperature, then [acetyl-^{3}H]-acetylcholine (final 200 μM) was added and the incubation was continued for 30 min. The reaction was terminated by adding a 1 M solution of chloroacetic acid, followed by a toluene-based scintillant, and the vials were capped and shaken to transfer the produced [^{3}H]-acetic acid to the toluene phase. Radioactivity in the toluene phase was then counted by liquid scintillation spectrometry (Aloka LSC-903 or LSC-1000). The inhibitory activities were expressed as the 50%-inhibitory concentration (IC_{50}), which was calculated by probit analysis. The inhibitory activities of physostigmine and THA were measured using the same technique.

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Synthesis and Biological Activities of Lipid A Analogs: Modification of a Glycosidically Bound Group with Chemically Stable Polar Acidic Groups and Lipophilic Groups on the Disaccharide Backbone with Tetradecanoyl or *N*-Dodecanoylglycyl Groups

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Six novel lipid A analogs were synthesized. The first two analogs, 4 and 5, have an α-glycosidically bound carboxymethyl or 1,3-dicarboxyisopropyl group on the disaccharide backbone with four tetradecanoyl groups. The next three analogs, 6, 7 and 8, have two or four *N*-dodecanoylglycyl groups on the 1-α-*O*-phosphonooxyethylated disaccharide backbone. Analog 6 bears *N*-dodecanoylglycyl groups on the hydroxyl functions at positions 3 and 3′, and tetradecanoyl groups on the amino functions at positions 2 and 2′. Analog 7 is a 2, 3, 2′ and 3′-tetrakis(*N*-dodecanoylglycyl) derivative, and analog 8 resembles compound 6, but the binding of the *N*-dodecanoylglycyl and tetradecanoyl groups at positions 2, 2′ and 3, 3′ are reversed. The third analog, 9, has the same acyl group configuration as compound 6, but has a 1,3-dicarboxyisopropyl group at position C-1.

Compounds 4 and 5 exhibited definite antitumor activity against Meth A fibrosarcoma, indicating that the phosphate group at the C-1 position in lipid A could be replaced by the carboxylic acid without reducing the antitumor activity. In rabbits, compounds 6 and 9 exhibited potent antitumor activity, but their toxicity was extremely low. On the other hand, compounds 7 and 8 showed no antitumor activity. The levels of antitumor activity of 6 and 9 were similar to those of the natural-type lipid A. The antitumor activities of analogs with a *N*-dodecanoylglycyl group on the disaccharide backbone depended on the connecting sites of the acyl groups.

Keywords lipid A analog; *N*-dodecanoylglycyl group; phosphate group; phosphonooxyethyl group; carboxymethyl group; 1,3-dicarboxyisopropyl group; antitumor activity; Meth A; toxicity; structure–activity; rabbit

The complete structure of natural *Escherichia coli* (*E. coli*) lipid A was deduced[1] and unequivocally confirmed by total synthesis.[2] This chemically synthesized *E. coli* lipid A showed biological activities identical to natural type, which possesses not only undesirable lethal toxicity but also beneficial characteristics such as its antitumor activity.[3]

However, we found that **1**, a novel synthetic analog of lipid A with an α-glycosidically bound phosphonooxyethyl group instead of the α-glycosyl phosphate group of natural lipid A, exhibited antitumor activity at the same level as synthetic *E. coli* lipid A.[4] It was proven that the α-glycosyl phosphate group is not essential for antitumor activity, and is replaceable, without loss of the activity, with other acidic groups such as chemically stable polar acidic groups.

We furthermore reported that compound **2**, which has four (*R*)-3-hydroxytetradecanoyl groups as acyl groups on the disaccharide backbone and an α-glycosidically bound phosphonooxyethyl group, exhibited distinctly less toxicity in rabbits than 1-α-*O*-phosphonooxyethylated compound **3**, which has four tetradecanoyl groups, and that, nevertheless, the difference of antitumor potency between these two compounds was not remarkable[5] (Fig. 1).

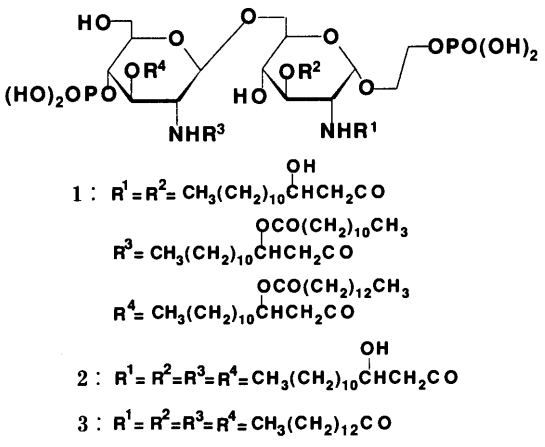

Fig. 1

The above results suggested the possibility of separating the antitumor activity from the toxicity of lipid A. Because the only structural difference between these two compounds was the presence or absence of hydroxyl groups in the fatty acid moiety, it was presumed that the effect of hydroxyl groups in decreasing the toxicity resulted from the capacity for hydrogen bond formation and an increase of polarity.

We therefore directed our effort to synthesize new antitumor compounds toward investigating the possibility of converting the phosphate group at the C-1 position into other chemically stable polar acidic groups, such as carboxylic acids. We also believed that the conversion of the hydroxyl group in (*R*)-3-hydroxytetradecanoyl groups into other functional groups, likely to have the same effect as the hydroxyl group, might lead to the separation of the beneficial antitumor activity and the lethal toxicity of lipid A.

As regards the acidic groups other than phosphoric

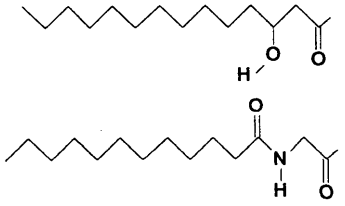

Fig. 2

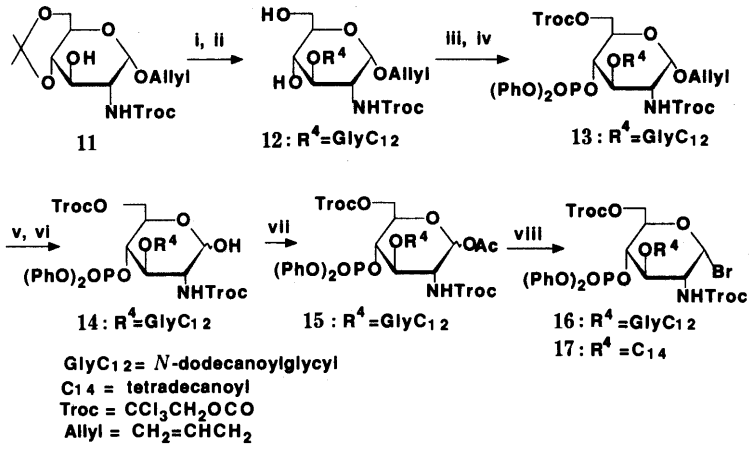

Chart 1

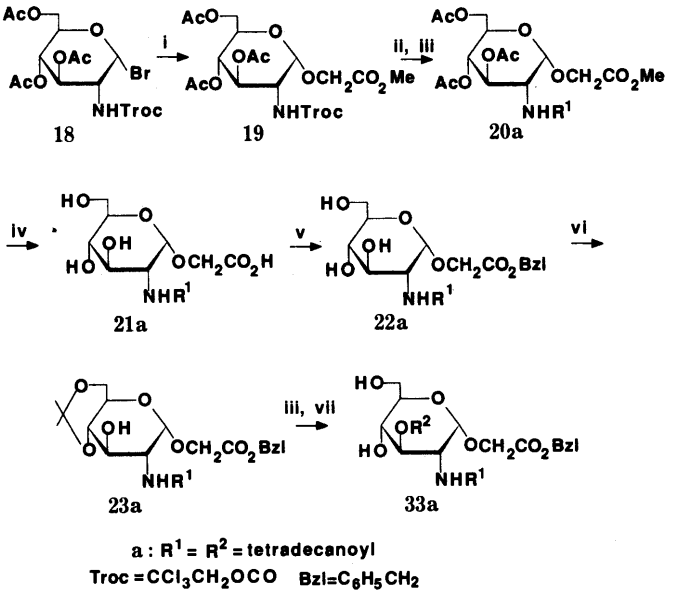

Chart 2

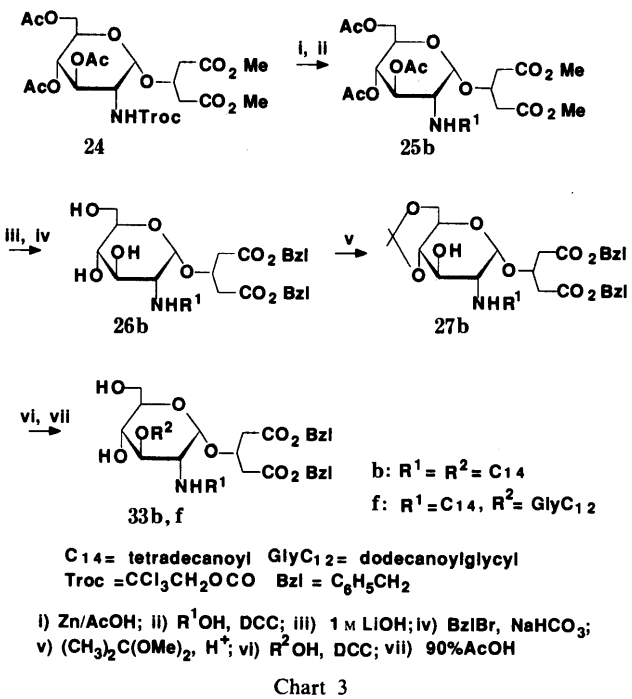

Chart 3

acid, we tried introducing carboxylic acid derivatives to the C-1 position. We chose glycolic acid as a monobasic acid and 3-hydroxyglutaric acid as a dibasic acid. Accordingly, we synthesized compounds **4** and **5** by glycosylation so that these carboxylic acid substituents were attached to the disaccharide backbone bearing four tetradecanoyl groups without a hydroxyl.

We were also interested in replacing the hydroxyl groups of the fatty acid residues of compound **2** with NH amides which can be expected to exert an effect similar to that of hydroxyl on the capacity for hydrogen bond formation and on the increase of polarity. We considered that the amide bond "CONH", which can confer properties similar to those described above without having an asymmetric carbon and without basicity, would be a suitable functional group for our purpose. Since the –CONH– sites of the acyl groups should be located at nearly the same positions as the hydroxyl groups in the (*R*)-3-hydroxytetradecanoyl group, and since the chain length should be similar to the length of the hydroxytetradecanoyl group, the *N*-dodecanoylglycyl group was chosen for introduction to the disaccaride backbone (Fig. 2).

Thus, compounds **6**, **7**, and **8**, substituted totally or partially with *N*-dodecanoylglycyl groups, were synthesized (Chart 5). The results of the biological activity evaluation of the synthesized compounds described above made it desirable to synthesize compound **9**, which has the same composition of acyl groups as compound **6**, as well as a 1,3-dicarboxyisopropyl group at the C-1 position.

This paper describes the synthesis and biological activities of compound **4**—**9**.

Chemistry The glycosyl donor **16** was synthesized from **11** by a method similar to that used for synthesizing the

glycosyl bromide **17** in our previous study,[5] as shown in Chart 1.

After 3-*O*-acylation of acetonide **11** with *N*-dodecanoylglycine in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP), the isopropylidene group was removed to give 3-*O*-acylated **12**. Selective *O*-trichloroethoxycarbonylation at position C-6, followed by phosphorylation with diphenyl chlorophosphate at the C-4 position, yielded the 4-*O*-diphenylphosphorylated compound **13**. After deallylation and acetylation of the C-1 position, the resulting glycosyl acetate **15** was brominated with 25% HBr–acetic acid to give the glycosyl bromide **16**.

Routes for the synthesis of glucosamine derivatives **33a**, **33b** and **33f**, glycosyl acceptors, are shown in Charts 2 and 3.

The selective glycosylation of bromide **18** with methyl-glycolate was employed in a similar manner, using zinc bromide, as reported for the synthesis of **24**,[6] to give α-glycoside **19**. The 2,2,2-trichloroethoxycarbonyl (Troc) group of compounds **19** and **24** were cleaved by treatment with zinc powder in acetic acid (AcOH), and a tetradecanoyl group was introduced into the resulting amino group by the acid chloride method or the DCC method to give *N*-tetradecanoyl derivatives **20a** and **25b**. Thereafter, all acetyl and methyl groups of **20a** were removed by treatment with a NaOH solution, and the resulting carboxyl group of **21a** was protected by a benzyl group to give **22a**. On the other hand, compound **25b** was deprotected by treatment with a LiOH solution, and without isolation of the deprotected compound, was immediately esterified with benzyl bromide and sodium hydrogen carbonate to give **26b**. Hydroxyl groups at the 4 and 6 positions of **22a** and **26b** were protected by isopropylidene fromation, and the residual hydroxyl groups of **23a** and **27b** were acylated with tetradecanoic acid or *N*-dodecanoylglycine (**10**) by the same method as described for **20a** and **25b**. Deprotection of isopropylidene groups with 90% AcOH afforded the glycosyl acceptors **33a**, **33b** and **33f**.

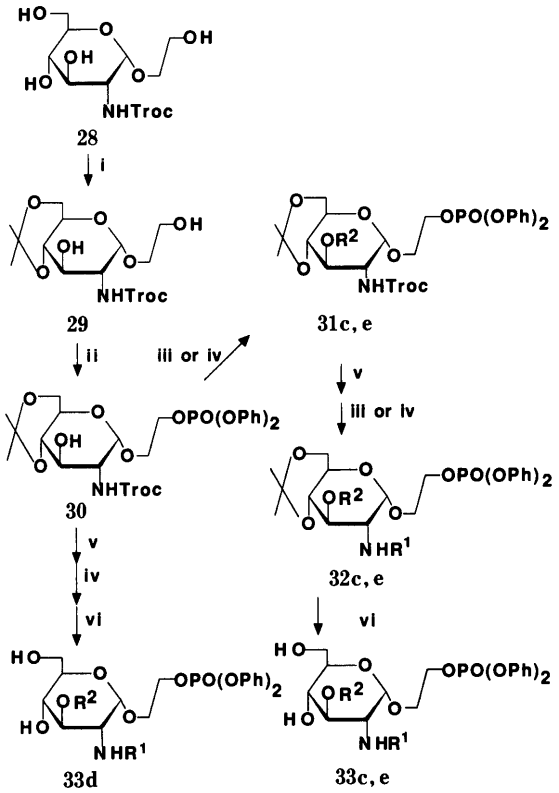

Chart 4

The other glucosamine components, **33c—e**, were prepared from α-glycoside **28** as shown in Chart 4.

After acetonide formation at the 4 and 6 hydroxyl groups of **28**, the remaining primary hydroxyl group was phosphorylated by treatment with diphenyl chlorophosphate to give the phosphonate **30**. Acylation of **30** with tetradecanoyl chloride or *N*-dodecanoylglycine in the presence of DCC and DMAP gave **31c** or **31e**. After removal of the Troc group of **31c** and **31e** with zinc powder in AcOH, the products were acylated as described for **31c** and **31e** to yield **32c** and **32e**, respectively. Subsequent hydrolysis with 90% AcOH produced the glycosyl acceptors **33c** and **33e**. Glycosyl acceptor **33d** was prepared from **30**. After removal of the Troc group of **30**, introduction of *N*-dodecanoylglycine to both of the resulting 2-amino and 3-hydroxyl functions, followed by hydrolysis in 90% AcOH, gave **33d**.

The basic strategy for the synthesis of compounds **4—9** was similar to that employed in our previous synthesis[5] of the 1-*O*-phosphonooxyethylated compound **3**, as shown in Chart 5.

Coupling of glycosyl bromides **16** and **17** with the acceptors **33a—f** yielded the disaccharides **34a—f**. After cleavage of the Troc groups, the resultant free amino groups were acylated with tetradecanoic acid by the 1-hydroxybenzotriazole (HOBt) active ester method to give compounds **35a—f**. Finally, hydrogenolytic deprotection of **35a—f** afforded the desired compounds **4—9**.

Antitumor Activity The antitumor activity of synthetic compounds was tested in BALB/c mice as described earlier.[5] Briefly, a group of 7 or 8 mice were inoculated intradermally with Meth A syngeneic fibrosarcoma cells (2×10^5). The triethylamine (Et_3N) salt of each compound was dissolved in an aqueous solution containing 5% glucose and 0.1% Et_3N. The resulting solution was then administered to the mice at a dose of 100 μg/mouse through the tail vein on the 7th, 12th and 17th days after implantation. The percentage antitumor effect on the growth of Meth A was determined by dividing the average tumor weight of the treated group on the 21st day by the average tumor weight of the control group, then multiplying the quotient by 100. Tables I and II show the results.

Toxicity The Et_3N salts of each of the compounds were dissolved in 5% (v/v) glucose containing 0.1% Et_3N to prepare a 100 μg/ml solution. This solution was administered through the ear vein to three NZW rabbits per group at a dose of 50 or 500 μg/kg-body weight for 3

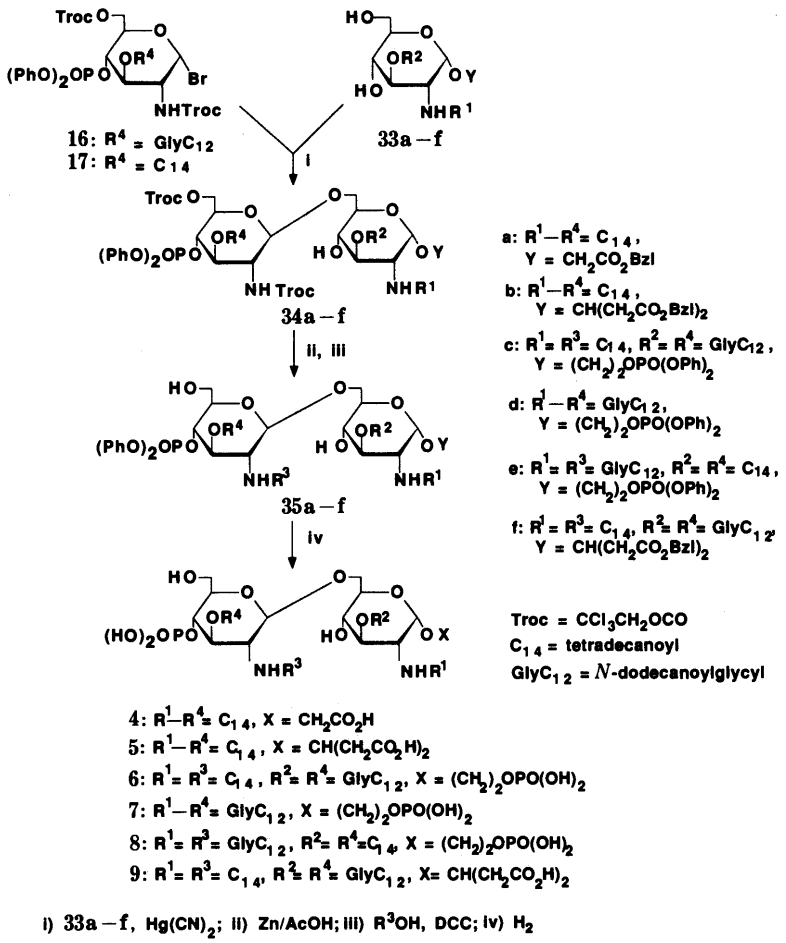

Chart 5

TABLE I. Antitumor Activity of Lipid A Analogs against Meth A Fibrosarcoma

Compound No.	Dose (μg/mouse)	T/C (%)[a]	Cured mice/ treated mice[b]
4	100 × 3	45[c]	1/7
5	100 × 3	24[d]	1/8
6	100 × 3	21[d]	1/8
7	100 × 3	80	0/7
8	100 × 3	77	0/7
9	100 × 3	20[d]	0/7
Control	—	100	0/8

a) (Mean tumor weight in tested group/that in control group) × 100. Results given are at 21 d after tumor inoculation. *b*) Number of tumor-free mice/number of mice tested. *c*) $p<0.01$ *vs.* control (Student's test). *d*) $p<0.001$ *vs.* control (Student's test).

TABLE II. Antitumor Activity of Lipid A and Compound **6** against Meth A Fibrosarcoma

Compound No.	Dose (μg/mouse)	T/C (%)[a]	Cured mice/ treated mice[b]
6	100 × 3	21[d]	1/8
Synthetic *E. coli* Lipid A[c]	100 × 3	18[d]	0/8
Control	—	100	0/8

a) (Mean tumor weight in tested group/that in control group) × 100. Results given are at 21 d after tumor inoculation. *b*) Number of tumor-free mice/number of mice tested. *c*) Synthetic *E. coli* lipid A (No. 506) was purchased from Daiichi Pure Chemicals Co., Ltd., Tokyo. *d*) $p<0.001$ *vs.* control (Student's test).

consecutive days. Toxicity was evaluated by mortality, clinical signs, decrease in body weight, decrease in platelets (%), blood chemistry and pathology (Table III).

Discussion

Compounds **4** and **5**, which have the same acyl composition and an α-glycosidically bound carboxymethyl group or 1,3-dicarboxyisopropyl group, exhibited definite antitumor activity. It becomes more clear that the conversion of substituents possessing carboxylic acid as an acidic group at the C-1 position, as well as those having glycosidically bound phosphoric acid or a phosphonooxyethyl group, retained the antitumor activity (Table I). The acidic polar moiety at the C-1 position of the dibasic acid analog **5** (which has greater acidity) demonstrated stronger antitumor activity than did monobasic acid analog **4**. On the other hand, concerning toxicity in rabbits, dicarboxylic acid analog **5** showed a tendency to be more toxic than monocarboxylic acid analog **4**, whereas the conversion of a phosphoric acid type group to a carboxylic acid type group at the C-1 position did not cause a decrease in toxicity compared with the phosphate type.

The antitumor activity of compound **6**, which has two *N*-dodecanoylglycyl groups at the C-3 and 3′ positions, was comparable to that of synthetic natural-type lipid A (Table II). However, the antitumor activity of **8**, which is

TABLE III. Toxicity of Lipid A Analogs in Rabbits

Observations	Compound							
	4	**5**	**6**	**7**	**8**	**9**	**6**	**9**
	Dose: 50	50	50	50	50	50	500	500 μg/kg
Mortality[a]	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3
Clinical signs[b]	HE, RD	HE, AD, PT, LY	HE	NO[i]	HE	NO	HE	NO
Decrease in body weight (g)[c]	100—400	350	NO	NO	NO	NO	NO	NO
Hematological examination[d]	GPT↑, UN↑, CRE↑, CPK↑, TG↑	GPT↑, UN↑, CRE↑, TP↓, ALB↓	NO	NO	UN↑, CRE↑, CHO↑	NO	NO	NO
Pathology[a]								
Thrombus	3/3 (3+)[h]	1/3 (+)	NO	NO	NO	NO	NO	NO
Change in liver[e]	1/3 (3+)	2/3 (+)	NO	NO	NO	NO	NO	NO
Change in kidney[f]	1/3 (+)	3/3 (+)	NO	NO	1/3 (+)	NO	NO	NO
Change in heart[g]	2/3 (+, 3+)	1/3 (3+)	NO	NO	NO	NO	NO	NO

a) Mortality and pathology: number of rabbits changed/number of rabbits tested. *b*) HE, hyperemia of eye; RD, respiratory depression; AD, decrease in locomotor activity; PT, ptosis; LY, lying on side. *c*) 24 h after final injection. *d*) GPT, glutamate pyruvate transaminase; UN, urea nitrogen; CRE, creatinine; CPK, creatine phosphokinase; TG, triglyceride; TP, total protein; ALB, albumin; CHO, total cholesterol. *e*) Liver change: degeneration and necrosis of liver cells. *f*) Kidney change: degeneration and necrosis of epithelium of uriniferous tubules. *g*) Heart change: degeneration and necrosis of muscle fiber. *h*) +, slight; 2+, moderate; 3+, severe. *i*) NO: no change.

a positional isomer of compound **6** or **7** having four *N*-dodecanoylglycyl groups, was extremely weak. On the other hand, compounds **6**, **7** and **8** did not show any lipid A toxicity on treatment with 50 μg/kg-body weight dose for 3 consecutive days in rabbits.

Since compound **6** showed both strong antitumor activity and low toxicity, it was compared to the activities of compound **9** in which only the C-1 substituent was different. As a result, **9** was found to have the same strong activity and low toxicity as the original compound **6**, thus suggesting that, with the same acyl composition on the disaccharide backbone, the biological effects of the 1,3-dicarboxyisopropyl and phosphonooxyethyl groups were nearly the same. These results indicate that the structural requirements for the antitumor activities of analogs with *N*-dodecanoylglycyl groups on a disaccharide backbone are very strict, and the binding position on the disaccharide backbone of this group is very important for the expression of activity. For example, compounds **6** and **9**, having a high antitumor activity, showed almost no toxicity on treatment with a dose of 500 μg/kg-body weight (Table III), and these compounds showed a clear separation between the antitumor activity and the undesired toxicity of lipid A. Since compound **3**, which has four (*R*)-3-hydroxytetradecanoyl groups, exhibited a definite antitumor activity, as reported in previous paper,[5] and although compound **8**, with four *N*-dodecanoylglycyl groups, did not show any such activity, the influence of the –CONH– of *N*-dodecanoylglycyl groups on antitumor activity differed considerably from that of the hydroxyl of 3-hydroxytetradecanoyl groups. This difference suggested that the *N*-dodecanoylglycyl group has another relevant property in addition to its capacity for hydrogen bond formation and polarity, probably due to the restricted rotation of the amide bond in a *N*-dodecanoylglycyl group.

In conclusion, compounds **6** and **9** represent the first successful examples of separating toxicity in natural-type lipid A from its significant antitumor activity, while retaining this activity. We hope that these findings will open the way for the clinical application of lipid A derivatives, and that these compounds will be useful for the investigation of a toxicological mode of action of natural-type lipid A as an endotoxin.

Experimental

All melting points are uncorrected. ^{1}H-Nuclear magnetic resonance (^{1}H-NMR) spectra were determined on a Varian XL-200 (200 MHz) or JEOL-GSX 500 (500 MHz) spectrometer in deuteriochloroform solution unless otherwise noted. The chemical shifts are given in δ values with tetramethylsilane (TMS) as the internal standard. Optical rotations were measured with a Horiba SEDA-200 polarimeter at 25 °C. Mass spectra (MS) were obtained on a JMS-HX 110 or JMX-300 instrument. Precoated Silica gel 150 A PLK5F plates (1.0 mm thickness; Whatmann) were used for preparative thin layer chromatography (TLC). Organic solution were dried over sodium sulfate before concentration.

***N*-Dodecanoylglycine (10)** Dodecanoyl chloride (10.9 g, 50 mmol) and an aqueous solution (30 ml) of NaOH (2.00 g, 50 mmol) were added to a mixture of glycine (4.13 g, 55 mmol) and an aqueous solution (30 ml) of NaOH (2.20 g, 55 mmol) under ice-cooling maintained at pH 9. The mixture was stirred for 30 min, and then neutralized with 35% HCl with ice-cooling to about at pH 1. After the mixture was extracted with ethyl acetate (EtOAc), the organic layer was washed with H_2O, saturated aqueous NaCl, dried and concentrated. The residue was crystallized from EtOAc–hexane to give **10** (11.3 g, 88%), mp 118—119 °C (118—119 °C).[7] NMR ($CDCl_3+CD_3OD$) δ: 0.88 (3H, t, J=6 Hz, CH_3), 1.27 (16H, s, CH_2), 1.60 (2H, br, CH_2CH_2CO), 2.26 (2H, t, J=8 Hz, CH_2CON), 3.95 (2H, s, NCH_2CO_2).

Allyl 2-Deoxy-3-*O*-(*N*-dodecanoylglycyl)-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranoside (12) *N*-Dodecanoylglycine (**10**) (1.42 g, 5.52 mmol), DMAP 0.28 g (5.52 mmol) and DCC (1.14 g, 5.53 mmol) were added to a solution of **11** (2.00 g, 4.60 mmol) in CH_2Cl_2 (60 ml) with ice-cooling, and the mixture was stirred for 15 h at room temperature. The precipitate was filtered off, and the filtrate was concentrated *in vacuo*. The residue in 90% AcOH (50 ml) was heated at 90 °C for 20 min. Evaporation of the solvent gave a viscous oil, which was purified by silica gel column chromatography ($CHCl_3$–MeOH, 19:1) to give **12** as a colorless oil (2.60 g, 89%), $[\alpha]_D$ +54.8° (c=1.0, $CHCl_3$). NMR δ: 0.89 (3H, t, J=7 Hz, CH_3), 1.28 (16H, s, CH_2), 1.64 (2H, br, CH_2CH_2CO), 2.26 (2H, t, J=8 Hz, CH_2CON), 4.69 and 4.84 (each 1H, AB type d, J=12 Hz, CH_2CCl_3), 4.97 (1H, d, J=4 Hz, H-1), 5.2—5.5 (4H, m, H-3, $CH=CH_2$ and NHTroc), 5.96 (1H, m, $CH=CH_2$), 6.24 (1H, m, CH_2CONH). MS m/z: 633 $[(M+H)^+]$.

Allyl 2-Deoxy-4-*O*-diphenylphosphono-3-*O*-(*N*-dodecanoylglycyl)-6-*O*-(2,2,2-trichloroethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-

α-D-glucopyranoside (13) Troc-Cl (0.77 ml, 5.62 mmol) was gradually added to a solution of **12** (2.55 g, 4.02 mmol) in pyridine (20 ml) with ice-cooling. After the mixture was stirred for 1.5 h, H_2O was added and the solvent was evaporated. The residue was purified by silica gel column chromatography ($CHCl_3$–acetone, 9:1) to give a colorless oil (2.44 g, 75%). Diphenyl chlorophosphate (1.20 g, 4.47 mmol), DMAP (0.55 g, 4.47 mmol) and pyridine (0.35 ml, 4.47 mmol) were added to a solution of the above oil (2.41 g, 2.98 mmol) in CH_2Cl_2 (30 ml). After stirring for 1 h at room temperature, the mixture was diluted with $CHCl_3$, and washed successively with 1 M HCl, H_2O, 5% aqueous $NaHCO_3$ and saturated aqueous NaCl, and dried. After the solvent was evaporated, hexane was added to the residual oil to give **13** as a white powder (2.71 g, 87%), mp 80—82 °C, $[\alpha]_D$ +46.4° (c=1.1, $CHCl_3$). *Anal.* Calcd for $C_{41}H_{53}Cl_6N_2O_{14}P$: C, 47.28; H, 5.13; N, 2.69. Found: C, 47.34; H, 5.04; N, 2.74. NMR δ: 0.89 (3H, t, J=7 Hz, CH_3), 1.28 (16H, s, CH_2), 1.58 (2H, br, CH_2CH_2CO), 2.10 (2H, t, J=8 Hz, CH_2CON), 3.80 (1H, dd, J=18, 4 Hz, NCH_2CO_2), 4.00 (1H, dd, J=18, 4 Hz, NCH_2CO_2), 4.67 and 4.82 (each 1H, AB type d, J=12 Hz, CH_2CCl_3), 4.80 (3H, m, H-4 and CH_2CCl_3), 5.01 (1H, d, J=4 Hz, H-1), 5.3—5.5 (4H, m, H-3, NHTroc, $CH=CH_2$), 5.96 (1H, m, $CH=CH_2$), 6.27 (1H, m, CH_2CONH), 7.2—7.4 (10H, m, arom. H).

2-Deoxy-4-*O*-diphenylphosphono-3-*O*-(*N*-dodecanoylglycyl)-6-*O*-(2,2,2-trichloroethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-D-glucose (14) Compound **13** (2.64 g, 2.53 mmol) and 1,5-cyclooctadienebis(methyldiphenylphosphine)iridium hexafluorophosphate (50 mg) were dissolved in tetrahydrofuran (THF, 40 ml) in a nitrogen atmosphere. After activation of the catalyst with a hydrogen atmosphere for 1 min, the mixture was heated at 50 °C in the nitrogen atmosphere for 2 h. After cooling, iodine (1.28 g, 5.06 mmol) and H_2O (4 ml) were added to the solution and the mixture was stirred for 30 min. The solution was neutralized with 5% Na_2SO_3 and extracted with $CHCl_3$. The solution was washed with saturated NaCl solution, dried, and concentrated. The residue was purified by silica gel column chromatography ($CHCl_3$–acetone, 9:1) to give **14** (2.34 g, 92%) as a yellow oil, $[\alpha]_D$ +31.0° (c=1.0, $CHCl_3$). NMR δ: 0.88 (3H, t, J=7 Hz, CH_3), 1.28 (16H, s, CH_2), 1.57 (2H, br, CH_2CH_2CO), 2.12 (2H, t, J=8 Hz, CH_2CON), 3.80 (1H, dd, J=18, 4 Hz, NCH_2CO_2), 3.98 (1H, dd, J=18, 4 Hz, NCH_2CO_2), 4.50—4.90 (4H, m, CH_2CCl_3), 5.38 (1H, m, H-1), 5.58 (2H, m, H-3 and NHTroc), 6.30 (1H, m, CH_2CONH), 7.1—7.4 (10H, m, arom. H).

1-*O*-Acetyl-2-deoxy-4-*O*-diphenylphosphono-3-*O*-(*N*-dodecanoylglycyl)-6-*O*-(2,2,2-trichloroethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-D-glucopyranose (15) Acetic anhydride (1.18 g, 11.6 mmol) and pyridine (0.93 ml, 11.6 mmol) were added to a solution of **14** (2.31 g, 2.31 mmol) in CH_2Cl_2 (40 ml). After being stirred for 19 h, the mixture was diluted with $CHCl_3$. The solution was washed with 1 M HCl, H_2O and 5% $NaHCO_3$, dried, and concentrated. The residue was purified by silica gel column chromatography ($CHCl_3$–acetone, 19:1) to give **15** (2.25 g, 93%) as a pale yellow oil, $[\alpha]_D$ +43.8° (c=1.2, $CHCl_3$). NMR δ: 0.88 (3H, t, J=7 Hz, CH_3), 1.28 (16H, s, CH_2), 1.58 (2H, CH_2CH_2CO), 2.11 (2H, t, J=8 Hz, CH_2CON), 2.25 (3H, s, OAc), 3.77 (1H, dd, J=18, 4 Hz, NCH_2CO_2), 4.07 (1H, dd, J=18, 4 Hz, NCH_2CO_2), 4.57 and 4.74 (each 1H, AB type d, J=12 Hz, CH_2CCl_3), 4.86 and 4.98 (each 1H, AB type d, J=12 Hz, CH_2CCl_3), 4.92 (1H, q, J=8 Hz, H-4), 5.18 (1H, d, J=10 Hz, NHTroc), 5.50 (1H, t, J=10 Hz, H-3), 6.31 (2H, m, H-1 and CH_2CONH), 7.2—7.4 (10H, m, arom. H).

Methoxycarbonylmethyl 3,4,6-Tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranoside (19) Zinc bromide (140 mg, 0.62 mmol) was added to a suspension of **18**[7] (330 mg, 0.61 mmol), methyl glycolate (65 mg, 0.73 mmol) and $CaSO_4$ (100 mg) in CH_2Cl_2 (5 ml) at room temperature, and the mixture was refluxed for 5 h, then diluted with $CHCl_3$, and filtered. The filtrate was washed with 5% aqueous $NaHCO_3$, dried, and concentrated. The residue was purified by silica gel column chromatography ($CHCl_3$–acetone, 50:1) to give **19** (204 mg, 61%) as a powder, mp 110—111 °C, $[\alpha]_D$ +85.6° (c=0.9, $CHCl_3$). *Anal.* Calcd for $C_{18}H_{24}Cl_3NO_{12}$: C, 39.11; H, 4.38; N, 2.53. Found: C, 39.13; H, 4.37; N, 2.48. NMR δ: 2.01, 2.03 and 2.10 (each 3H, s, OAc), 3.78 (3H, s, OCH_3), 4.1—4.3 (6H, m), 4.63 and 4.86 (each 1H, AB type d, J=12 Hz, CH_2CCl_3), 4.97 (1H, d, J=4 Hz, H-1), 5.13 (1H, t, J=10 Hz, H-4), 5.31 (1H, t, J=10 Hz, H-3), 5.63 (1H, d, J=10 Hz). MS m/z: 552 $[(M+H)^+]$.

Methoxycarbonylmethyl 3,4,6-Tri-*O*-acetyl-2-deoxy-2-tetradecanoylamino-α-D-glucopyranoside (20a) Zinc powder (2.0 g) was added to a solution of **19** (1.60 g, 2.89 mmol) in AcOH (20 ml), and the mixture was vigorously stirred for 3 h at room temperature. The insoluble materials were removed by filtration, and the filtrate was concentrated *in vacuo*. The resulting oily product was dissolved in EtOAc, and this solution was washed with 5% aqueous $NaHCO_3$, and dried. After evaporation of the solvent, tetradecanoyl chloride (1.07 g, 4.34 mmol) and *N*-methylmorpholine (0.48 ml, 4.34 mmol) were added to a solution of the resulting oily product in CH_2Cl_2 (20 ml). The mixture was vigorously stirred for 1 h. MeOH (5 ml) was added to the reaction mixture. After 1 h of stirring, the mixture was diluted with $CHCl_3$, washed with 1 M HCl, saturated aqueous NaCl, dried, and concentrated. The residue was purified by silica gel column chromatography ($CHCl_3$–acetone, 50:1) to give **20a** (1.68 g, 99%) as a wax, $[\alpha]_D$ +68.6° (c=1.2, $CHCl_3$). NMR δ: 0.89 (3H, t, J=7 Hz, CH_3), 1.26 (20H, s, CH_2), 1.63 (2H, br, CH_2CH_2CO), 2.02, 2.04 and 2.11 (each 3H, s, OAc), 2.20 (2H, m, CH_2CO), 3.78 (3H, s, CO_2CH_3), 4.1—4.5 (4H, m), 4.26 (2H, s, OCH_2CO_2), 4.90 (1H, d, J=4 Hz, H-1), 5.18 (1H, t, J=10 Hz, H-4), 5.32 (1H, t, J=10 Hz, H-3), 6.15 (1H, d, J=10 Hz, NH). MS m/z: 588 $[(M+H)^+]$.

Carboxymethyl 2-Deoxy-2-tetradecanoylamino-α-D-glucopyranoside (21a) A solution of 1 M NaOH (12.2 ml) was added to a solution of **20a** (1.63 g, 2.77 mmol) in THF (20 ml) with ice-cooling, and the mixture was stirred for 20 min at the same temperature, then neutralized with 10% citric acid. The insoluble material was collected by filtration, washed with H_2O and dried *in vacuo* to give **21a** (1.16 g, 93%) as a white solid, mp 153—156 °C, $[\alpha]_D$ +93.5° (c=1.0, DMF). NMR (CD_3OD–$CDCl_3$, 1:1) δ: 0.88 (3H, t, J=7 Hz, CH_3), 1.26 (20H, s, CH_2), 1.60 (2H, br, CH_2CH_2CO), 2.26 (2H, t, J=7 Hz), 4.20 (2H, d, J=5.4 Hz, OCH_2CO_2). MS m/z: 448 $[(M+H)^+]$.

Benzyloxycarbonylmethyl 2-Deoxy-2-tetradecanoylamino-α-D-glucopyranoside (22a) Benzyl bromide (0.86 g, 5.00 mmol) and Et_3N (0.70 ml, 5.00 mmol) were added to a solution of **21a** (1.12 g, 2.50 mmol) in dimethylformamide (DMF, 20 ml), and the mixture was heated at 60 °C for 2 h. The mixture was diluted with EtOAc, and washed with H_2O and 1 M HCl. The organic layer was concentrated *in vacuo*, and the residue was purified by silica gel column chromatography ($CHCl_3$–MeOH, 9:1) to give **22a** (1.18 g, 88%) as a white powder, mp 268—269 °C, $[\alpha]_D$ +82.0° (c=1.1, DMF). *Anal.* Calcd for $C_{29}H_{47}NO_8$: C, 64.78; H, 8.81; N, 2.61. Found: C, 64.63; H, 8.78; N, 2.52. NMR δ: 0.87 (3H, t, J=7 Hz, CH_3), 1.24 (20H, s, CH_2), 1.50 (2H, br, CH_2CH_2CO), 2.10 (2H, t, J=7 Hz, CH_2CO), 4.26 (2H, s, OCH_2CO_2), 4.83 (1H, d, J=4 Hz, H-1), 5.18 (2H, s, $CH_2C_6H_5$), 7.42 (5H, s, arom. H), 7.64 (1H, d, J=8 Hz, NH). MS m/z: 538 $[(M+H)^+]$.

Benzyloxycarbonylmethyl 2-Deoxy-4,6-*O*-isopropylidene-2-tetradecanoylamino-α-D-glucopyranoside (23a) Dimethoxypropane (0.45 g, 4.28 mmol) was added to a solution of **22a** (1.15 g, 2.14 mmol) in DMF (40 ml) in the presence of *p*-toluenesulfonic acid (*p*-TsOH·H_2O, 38 mg, 0.2 mmol), and the solution was stirred for 3.5 h. After evaporation of the solvent, the residue was purified by silica gel column chromatography ($CHCl_3$–acetone, 20:1) to give **23a** (1.10 g, 89%) as a white powder, mp 72—73 °C, $[\alpha]_D$ +35.0° (c=1.1, $CHCl_3$). *Anal.* Calcd for $C_{32}H_{51}NO_8$: C, 66.52; H, 8.90; N, 2.42. Found: C, 66.42; H, 8.87; N, 2.51. NMR δ: 0.88 (3H, t, J=6 Hz), 1.26 (20H, s, CH_2), 1.45 and 1.56 (each 3H, s, CCH_3), 1.66 (2H, br, CH_2CH_2CO), 2.30 (2H, t, J=8 Hz, CH_2CO), 4.28 (2H, s, OCH_2CO_2), 4.82 (1H, d, J=4 Hz, H-1), 5.25 (2H, s, $CH_2C_6H_5$), 7.42 (5H, s, arom. H). MS m/z: 578 $[(M+H)^+]$.

Benzyloxycarbonylmethyl 2-Deoxy-3-*O*-tetradecanoyl-2-tetradecanoylamino-α-D-glucopyranoside (33a) As described for **20a**, compound **23a** (1.07 g, 1.85 mmol) was reacted with tetradecanoyl chloride (640 mg, 2.60 mmol) in CH_2Cl_2 (20 ml) in the presence of pyridine (0.73 ml, 2.60 mmol) and DMAP (40 mg) with ice-cooling. After the usual work-up, the resulting oily residue was dissolved in 90% AcOH (40 ml), and the mixture was heated at 90 °C for 30 min. Evaporation of the solvent gave an oil, and the oily product was purified by silica gel column chromatography ($CHCl_3$–MeOH, 19:1) to give **33a** (1.24 g, 90%) as a colorless solid, mp 54—55 °C, $[\alpha]_D$ +46.9° (c=1.1, $CHCl_3$). *Anal.* Calcd for $C_{43}H_{73}NO_9$: C, 69.04; H, 9.84; N, 1.87. Found: C, 68.75; H, 9.67; N, 1.82. NMR δ: 0.89 (6H, t, J=7 Hz), 1.28 (40H, s, CH_2), 1.60 (4H, br, CH_2CH_2CO × 2), 2.15 and 2.35 (each 2H, m, CH_2CO), 3.8—3.9 (4H, m), 4.27 (2H, s, OCH_2CO_2), 4.33 (1H, m, H-2), 4.89 (1H, d, J=4 Hz, H-1), 5.18 (1H, m, H-3), 5.22 (2H, s, $CH_2C_6H_5$), 6.17 (1H, d, J=9 Hz, NH), 7.41 (5H, s, arom. H). MS m/z: 748 $[(M+H)^+]$.

1,3-Di(methoxycarbonyl)isopropyl 2-Deoxy-3,4,6-tri-*O*-acetyl-2-tetradecanoylamino-α-D-glucopyranoside (25b) Zinc powder (20 g) was added to a solution of **24**[6] (23.2 g, 0.04 mmol) in AcOH (100 ml), and the mixture was vigorously stirred for 1 h at room temperature. The insoluble materials were filtered off, and the filtrate was concentrated *in vacuo*. The resulting oily substance was dissolved in EtOAc, and the

solution was washed with 5% aqueous $NaHCO_3$, and dried. The solvent was distilled off at reduced pressure, and the oily product was dissolved in CH_2Cl_2 (200 ml). Tetradecanoic acid (8.2 g, 0.04 mol) and DCC (7.50 g, 0.04 mol) were added to the solution with ice-cooling, and the mixture was stirred for 1 h at room temperature. The precipitate was filtered off, and the filtrate was concentrated *in vacuo*. The oily residue was purified by silica gel column chromatography ($CHCl_3$–acetone, 20:1) to give **25b** (15.6 g, 77%) as a colorless oil, $[\alpha]_D$ +49.3° (c=1.4, $CHCl_3$). NMR δ: 0.88 (3H, t, J=7 Hz), 1.25 (20H, s, CH_2), 1.58 (2H, m, C$\underline{H}_2$CH$_2$CO), 1.99, 2.02 and 2.09 (each 3H, s, OAc), 2.56—2.67 (5H, m, C$\underline{H}_2$CON, OCH(C$\underline{H}_2$CO$_2$Me)$_2$ × 3), 2.82 (1H, dd, J=16, 6 Hz, OCH(C$\underline{H}_2$CO$_2$Me)$_2$), 3.70 and 3.72 (each 3H, s, CO_2CH_3), 4.04 (1H, m, H-5), 4.10 (1H, dd, J=12, 2 Hz, H-6), 4.20 (1H, dd, J=12, 5 Hz, H-6), 4.36 (1H, td, J=10, 4 Hz), 4.45 (1H, m, OC$\underline{H}$(CH$_2$CO$_2$Me)$_2$), 4.96 (1H, d, J=4 Hz, H-1), 5.09 (1H, t, J=10 Hz, H-4), 5.14 (1H, t, J=10 Hz, H-3), 6.28 (1H, d, J=10 Hz, NH).

1,3-Di(benzyloxycarbonyl)isopropyl 2-Deoxy-2-tetradecanoylamino-α-D-glucopyranoside (26b) Compound **25b** (500 mg, 0.74 mmol) was dissolved in MeOH–H_2O (3:1, 20 ml), and LiOH·H_2O (472 mg, 11 mmol) was added to the mixture with ice-cooling. The mixture was stirred for 12 h at room temperature. 1 M HCl was added to the mixture until the pH of the solution became acidic, then 5% $NaHCO_3$ was added until the neutral pH was achieved. After the solution was concentrated *in vacuo*, the residue was dissolved in DMF (14 ml). $NaHCO_3$ (624 mg, 7.4 mmol) and benzyl bromide (2.2 ml, 18.5 mmol) were added to this solution. After stirring for 24 h at room temperature, the solvent was removed at reduced pressure. The residue was purified by silica gel column chromatography ($CHCl_3$–MeOH, 20:1) to give **26b** (337 mg, 65%) as a colorless wax, $[\alpha]_D$ +13.2° (c=0.5, $CHCl_3$). *Anal.* Calcd for $C_{39}H_{57}NO_{10}\cdot H_2O$: C, 65.25; H, 8.30; N, 1.95. Found: C, 65.20; H, 8.21; N, 2.12. NMR (500 MHz) δ: 0.88 (3H, t, J=7.3 Hz), 1.24 (20H, s, CH_2), 1.64 (2H, m, C$\underline{H}_2$CH$_2$CO), 2.25 (2H, m, CH$_2$CO), 2.59 (1H, dd, J=16.7, 5.5 Hz, OCH(C$\underline{H}_2$CO$_2$Bzl)$_2$), 2.65 (1H, dd, J=16.7, 4.0 Hz, OCH(C$\underline{H}_2$CO$_2$Bzl)$_2$), 2.72 (1H, d, J=16.7, 8.7 Hz, OCH(C$\underline{H}_2$CO$_2$Bzl)$_2$), 2.82 (1H, dd, J=16.7, 6.4 Hz, OCH(C$\underline{H}_2$CO$_2$Bzl)$_2$), 3.49 (1H, t, J=8.7 Hz), 3.58 (1H, t, J=10.3 Hz), 3.71 (2H, m), 3.81 (1H, m), 3.94 (1H, m), 4.46 (1H, m, OC$\underline{H}$(CH$_2$CO$_2$Bzl)$_2$), 4.92 (1H, d, J=4.0 Hz, H-1), 5.14 (2H, s, C$\underline{H}_2$C$_6$H$_5$), 5.15 and 5.18 (each 1H, AB type d, J=11.9 Hz, C$\underline{H}_2$C$_6$H$_5$), 7.12 (1H, m, NH), 7.3—7.4 (10H, m, arom. H). MS m/z: 700 [(M+H)$^+$].

1,3-Di(benzyloxycarbonyl)isopropyl 2-Deoxy-4,6-*O*-isopropylidene-2-tetradecanoylamino-α-D-glucopyranoside (27b) In a manner similar to that described for **23a**, compound **26b** (640 mg, 0.91 mmol) was treated with 2,2-dimethoxypropane (0.5 ml) in the presence of *p*-TsOH·H_2O (30 mg) in acetone (10 ml) to give **27b** (540 mg, 80%) as a colorless oil, $[\alpha]_D$ +3.3° (c=0.7, $CHCl_3$). *Anal.* Calcd for $C_{42}H_{61}NO_{10}\cdot 1/4H_2O$: C, 67.75; H, 8.34; N, 1.87. Found: C, 67.53; H, 8.09; N, 1.97. NMR (500 MHz) δ: 0.88 (3H, t, J=7.3 Hz), 1.24 (20H, s, CH_2), 1.43 and 1.52 (each 3H, s, CCH_3), 1.64 (2H, m, C$\underline{H}_2$CH$_2$CO), 2.25 (2H, td, J=7.3, 2.8 Hz, CH$_2$CO), 2.57 (1H, dd, J=15.6, 6.4 Hz, OCH(C$\underline{H}_2$CO$_2$Bzl)$_2$), 2.69 (2H, m, OCH(C$\underline{H}_2$CO$_2$Bzl)$_2$ × 2), 2.78 (1H, dd, J=15.6, 6.4 Hz, OCH(C$\underline{H}_2$CO$_2$Bzl)$_2$), 3.57—3.70 (4H, m), 3.80 (1H, m), 4.05 (1H, m), 4.43 (1H, m, OC$\underline{H}$(CH$_2$CO$_2$Bzl)$_2$), 4.89 (1H, d, J=3.7 Hz, H-1), 5.10 and 5.13 (each 1H, AB type d, J=12.8 Hz, C$\underline{H}_2$C$_6$H$_5$), 5.16 (2H, s, C$\underline{H}_2$C$_6$H$_5$), 6.96 (1H, d, J=7.3 Hz, NH), 7.3—7.4 (10H, m, arom. H). MS m/z: 741 [(M+2)$^+$].

1,3-Di(benzyloxycarbonyl)isopropyl 2-Deoxy-3-*O*-tetradecanoyl-2-tetradecanoylamino-α-D-glucopyranoside (33b) Compound **27b** (480 mg, 0.65 mmol) and tetradecanoic acid (190 mg, 0.85 mmol) in CH_2Cl_2 (10 ml) were added to DMAP (100 mg, 0.85 mmol) and DCC (170 mg, 0.85 mmol) with ice-cooling, and the mixture was stirred for 2 h at room temperature. The insoluble material was filtered off, and the filtrate was concentrated at reduced pressure. The residue was dissolved in 90% AcOH (40 ml), and the mixture was heated at 90 °C for 20 min. After evaporation of the solvent, the residue was purified by silica gel column chromatography ($CHCl_3$–acetone, 10:1) to give **33b** (530 mg, 89%) as a colorless wax, $[\alpha]_D$ +32.8° (c=0.9, MeOH). *Anal.* Calcd for $C_{53}H_{83}NO_{11}$: C, 69.94; H, 9.19; N, 1.54. Found: C, 69.73; H, 9.03; N, 1.60. NMR δ: 0.88 (6H, t, CH_3), 1.26 (40H, s, CH_2), 1.60 (4H, br, C$\underline{H}_2$CH$_2$CO), 2.16 (2H, m, CH$_2$CO), 2.34 (2H, m, CH$_2$CO), 4.94 (1H, s, H-1), 5.20 (4H, s, C$\underline{H}_2$C$_6$H$_5$ × 2), 7.40 (10H, s, arom. H). MS m/z: 911 [(M+2)$^+$].

1,3-Di(benzyloxycarbonyl)isopropyl 2-Deoxy-3-*O*-(*N*-dodecanoylglycyl)-2-tetradecanoylamino-α-D-glucopyranoside (33f) As described for **33b**, compound **27b** (598 mg, 0.81 mmol) was treated with **10** (250 mg, 0.97 mmol) in the presence of DMAP (49 mg, 0.40 mmol) and DCC (200 mg, 0.97 mmol), and the resulting oil was treated with 90% AcOH to give **33f** (632 mg, 83%) as a wax, $[\alpha]_D$ +36.9° (c=1.3, $CHCl_3$). NMR (500 MHz) δ: 0.88 (6H, t, J=7.3 Hz, CH_3), 1.28 (36H, m, CH_2), 1.55—1.67 (4H, m, C$\underline{H}_2$CH$_2$CO × 2), 2.16 (2H, m, CH$_2$CO), 2.26 (2H, t, J=7.3 Hz, CH$_2$CO), 2.60 (1H, dd, J=16.5, 6.4 Hz, OCH(C$\underline{H}_2$CO$_2$Bzl)$_2$), 2.65 (1H, dd, J=16.5, 3.7 Hz, OCH(C$\underline{H}_2$CO$_2$Bzl)$_2$), 2.72 (1H, dd, J=16.5, 8.2 Hz, OCH(C$\underline{H}_2$CO$_2$Bzl)$_2$), 2.81 (1H, dd, J=16.5, 5.5 Hz, OCH(C$\underline{H}_2$CO$_2$Bzl)$_2$), 3.66 (1H, t, J=9.2 Hz), 3.74—3.83 (4H, m), 4.12 (1H, dd, J=17.4, 5.5 Hz), 4.19 (1H, td, J=9.2, 3.7 Hz), 4.46 (1H, m, OC$\underline{H}$(CH$_2$CO$_2$Bzl)$_2$), 4.92 (1H, d, J=4.6 Hz, H-1), 5.03 (1H, t, J=9.2 Hz, H-3), 5.10—5.18 (4H, m, C$\underline{H}_2$C$_6$H$_5$), 6.43 (1H, br, NH), 6.65 (1H, d, J=9.2 Hz, NH), 7.32—7.40 (10H, m, arom. H). MS m/z: 939 [(M+H)$^+$].

2-Hydroxyethyl 2-Deoxy-4,6-*O*-isopropylidene-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranoside (29) As described for **23a**, compound **28** (3.58 g, 8.98 mmol) was treated with 2,2-dimethoxypropane (3.70 ml, 30.2 mmol) in the presence of *p*-TsOH·H_2O (170 mg, 0.89 mmol) to give **29** (2.78 g, 71%) as a white powder, mp 190—192 °C. *Anal.* Calcd for $C_{14}H_{22}Cl_3NO_8$: C, 38.33; H, 5.06; Cl, 24.25; N, 3.19. Found: C, 38.40; H, 5.02; Cl, 24.46; N, 3.59. NMR δ: 1.45 and 1.54 (each 3H, s, CCH_3), 4.75 and 4.86 (each 1H, AB type d, J=12 Hz, CH_2CCl_3), 4.95 (1H, d, J=4 Hz, H-1), 5.80 (1H, d, NH). MS m/z: 438 [(M+H)$^+$].

2-(Diphenylphosphonooxy)ethyl 2-Deoxy-4,6-*O*-isopropylidene-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranoside (30) Diphenyl chlorophosphate (0.80 ml, 3.86 mmol), pyridine (0.4 ml) and DMAP (0.46 g, 3.77 mmol) were added to a solution of **29** (1.12 g, 2.55 mmol) in CH_2Cl_2 (20 ml) with ice cooling. After the mixture was stirred for 3 h, diphenyl chlorophosphate (0.30 ml, 1.45 mmol) and DMAP (0.19 mmol, 1.51 mmol) were added with ice cooling. After the addition of MeOH (1 ml), the mixture was stirred for 1 h and diluted with $CHCl_3$. The solution was washed with 1 M HCl, with aqueous 5% $NaHCO_3$, then with H_2O, and dried. After evaporation of the solvent, the residue was purified by silica gel column chromatography ($CHCl_3$–acetone, 30:1) to give **30** (1.23 g, 71.8%) as a white solid, mp 121—124 °C, $[\alpha]_D$ +46.4° (c=1.0, $CHCl_3$). *Anal.* Calcd for $C_{26}H_{31}Cl_3NO_{11}P$: C, 46.55; H, 4.66; N, 2.09. Found: C, 46.28; H, 4.55; N, 2.13. NMR δ: 1.45 and 1.52 (each 3H, s, CCH_3), 4.45 (2H, m, CH$_2$OP), 4.73 and 4.80 (each 1H, AB type d, J=12 Hz, CH_2CCl_3), 4.85 (1H, d, H-1), 7.10—7.50 (10H, m, arom. H). MS m/z: 671 [(M+2)$^+$].

2-(Diphenylphosphonooxy)ethyl 2-Deoxy-4,6-*O*-isopropylidene-3-*O*-(*N*-dodecanoylglycyl)-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranoside (31c) Compound **30** (1.89 g, 2.82 mmol) was allowed to react with *N*-dodecanoylglycine (0.83 g, 3.22 mmol) in the presence of DCC (0.67 g, 3.23 mmol) and DMAP (0.17 g, 1.40 mmol), in a manner similar to that used for **33b**, to give **31c** (2.57 g, quant.) as a colorless oil, $[\alpha]_D$ +32.2° (c=0.8, $CHCl_3$). NMR δ: 0.88 (3H, t, J=6 Hz), 1.28 (16H, br, CH_2), 1.38 and 1.48 (each 3H, s, CCH_3), 2.20—2.35 (2H, t, CH$_2$CON), 4.20 (2H, m, CH$_2$OP), 5.25 (1H, m, H-3), 7.2—7.5 (10H, m, arom. H).

2-(Diphenylphosphonooxy)ethyl 2-Deoxy-4,6-*O*-isopropylidene-3-*O*-tetradecanoyl-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranoside (31e) As described for **33a**, compound **30** (0.50 g, 0.75 mmol) was treated with tetradecanoyl chloride (221 mg, 0.90 mmol) in the presence of pyridine (0.30 ml, 3.75 mmol) and DMAP (20 mg) in CH_2Cl_2 to give **31e** (0.49 g, 74%) as a colorless oil, $[\alpha]_D$ +36.1° (c=1.0, $CHCl_3$). *Anal.* Calcd for $C_{40}H_{57}Cl_3NO_{12}P$: C, 54.52; H, 6.52; N, 1.59. Found: C, 54.41; H, 6.81; N, 1.72. NMR δ: 0.88 (3H, t, J=7 Hz), 1.28 (16H, s, CH_2), 1.37 and 1.47 (each 3H, s, CCH_3), 1.60 (2H, br, C$\underline{H}_2$CH$_2$CO), 2.30 (2H, m), 4.42 (2H, m, CH$_2$OP), 4.58 and 4.80 (each 1H, AB type d, J=12 Hz, CH_2CCl_3), 4.86 (1H, d, J=4 Hz, H-1), 5.22 (1H, m, H-3), 5.65 (1H, d, J=10 Hz, NH), 7.2—7.5 (10H, m, arom. H). MS m/z: 881 [(M+2)$^+$].

2-(Diphenylphosphonooxy)ethyl 2-Deoxy-4,6-*O*-isopropylidene-3-*O*-(*N*-dodecanoylglycyl)-2-tetradecanoylamino-α-D-glucopyranoside (32c) As described for **20a**, compound **31c** (0.66 g, 0.73 mmol) was treated with zinc powder in AcOH (5 ml), and the resulting oil was treated with tetradecanoyl chloride (0.23 g, 0.93 mmol) in CH_2Cl_2 (5 ml) to give **32c** (0.67 g, 97%) as a colorless oil, $[\alpha]_D$ +30.1° (c=1.7, $CHCl_3$). NMR δ: 0.88 (6H, t, J=6 Hz), 1.28 (40H, br, CH_2), 1.38 and 1.48 (each 3H, s, CCH_3), 1.64 (5H, br), 2.09 (2H, m), 2.26 (2H, m), 2.35 (2H, m), 3.6—4.2 (6H, m), 4.3—4.5 (3H, m), 4.78 (1H, d, J=4 Hz, H-1), 5.21 (1H, m, H-3), 6.25 (1H, br, NH), 6.95 (1H, m, NH), 7.2—7.5 (10H, m, arom. H).

2-(Diphenylphosphonooxy)ethyl 2-Deoxy-2-(*N*-dodecanoylglycylamino)-4,6-*O*-isopropylidene-3-*O*-tetradecanoyl-α-D-glucopyranoside (32e) As described for **25b**, compound **31e** (0.47 mg, 0.53 mmol) was treated with zinc

powder (0.5 g), and the resulting oil was allowed to react with **10** (0.21 g, 0.8 mmol) to give **32e** (0.48 g, 94%) as a powder, mp 79—80 °C, $[\alpha]_D$ +28.1° (c=1.1, $CHCl_3$). NMR δ: 0.88 (6H, t, J=6 Hz), 1.28 (36H, s, CH_2), 1.37 and 1.63 (each 3H, s, CCH_3), 2.12 (2H, m, CH_2CO), 2.30 (2H, t, J=8 Hz, CH_2CO), 4.42 (2H, m, CH_2OP), 4.86 (1H, d, J=4 Hz, H-1), 5.20 (1H, t, J=10 Hz, H-3), 6.78 (1H, br, NH), 6.94 (1H, d, J=8 Hz, NH), 7.2—7.5 (10H, m, arom. H).

2-(Diphenylphosphonooxy)ethyl 2-Deoxy-3-*O*-(*N*-dodecanoylglycyl)-2-tetradecanoylamino-α-D-glucopyranoside (33c) A solution of **32c** (0.63 g, 0.67 mmol) in 90% AcOH (20 ml) was heated at 90 °C for 30 min. After evaporation of the solvent, the residue was purified by silica gel column chromatography ($CHCl_3$–acetone, 10:1) to give **33c** (0.45 g, 73%) as an oil, $[\alpha]_D$ +46.8° (c=0.9, $CHCl_3$). NMR δ: 0.88 (6H, t, J=6 Hz), 1.2—1.8 (40H, br, CH_2), 2.08 (2H, t), 2.27 (2H, t), 3.7—4.0 (8H, m), 4.05—4.50 (4H, m), 4.83 (1H, d, J=4 Hz, H-1), 5.18 (1H, m, H-3), 6.43 (1H, m, NH), 6.85 (1H, m, NH), 7.2—7.5 (10H, m, arom. H). MS m/z: 906 $[(M+2)^+]$.

2-(Diphenylphosphonooxy)ethyl 2-Deoxy-2-(*N*-dodecanoylglycylamino)-3-*O*-tetradecanoyl-α-D-glucopyranoside (33e) As described for **33c**, compound **32e** (0.46 g, 0.48 mmol) was treated with 90% AcOH (20 ml) to give **33e** (0.39 g, 90%) as a waxy solid, $[\alpha]_D$ +36.1° (c=1.1, $CHCl_3$). NMR δ: 0.90 (6H, t, J=6 Hz), 1.28 (36H, s, CH_2), 1.63 (4H, br, $CH_2CH_2CO \times 2$), 2.13 (2H, m, CH_2CO), 2.36 (2H, t, J=8 Hz, CH_2CO), 4.90 (1H, d, J=4 Hz, H-1), 5.10 (1H, m, H-3), 6.74 (1H, br, NH), 6.96 (1H, d, J=9 Hz, NH), 7.2—7.5 (10H, m, arom. H). MS m/z: 906 $[(M+2)^+]$.

2-(Diphenylphosphonooxy)ethyl 2-Deoxy-3-*O*-(*N*-dodecanoylglycyl)-2-(*N*-dodecanoylglycylamino)-α-D-glucopyranoside (33d) Compound **30** (0.60 g, 0.89 mmol) was treated with zinc powder (0.6 g) in AcOH (10 ml). After stirring for 1 h, the mixture was filtered off, and the filtrate was concentrated *in vacuo* to give an oil, which was then allowed to react with **10** (0.69 g, 2.67 mmol). The resulting oil was treated with 90% AcOH, in the same manner as described for **33f**, to give **33d** as a colorless oil (0.57 g, 93%), $[\alpha]_D$ +16.6° (c=0.8, $CHCl_3$). NMR δ: 0.90 (6H, t, J=6 Hz), 1.28 (32H, s, CH_2), 1.60 (4H, br, $CH_2CH_2CO \times 2$), 2.0—2.3 (4H, m, $CH_2CO \times 2$), 4.85 (1H, d, J=4 Hz, H-1), 5.24 (1H, t, J=10 Hz, H-3), 6.88 (1H, d, J=10 Hz, NH), 7.2—7.5 (12H, m, arom. H and NH × 2). MS m/z: 934 $[(M+H)^+]$.

Benzyloxycarbonylmethyl 2-Deoxy-6-*O*-[2-deoxy-4-*O*-diphenylphosphono-3-*O*-tetradecanoyl-6-*O*-(2,2,2-trichloroethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-3-*O*-tetradecanoyl-2-tetradecanoylamino-α-D-glucopyranoside (34a) Compounds **17** (515 mg, 0.51 mmol) and **33a** (380 mg, 0.51 mmol) were dissolved in CH_2Cl_2 (8 ml), and mercuric cyanide (253 mg, 1.0 mmol) and $CaSO_4$ (600 mg) were added to the mixture, which was then refluxed for 16 h. After the reaction mixture was diluted with $CHCl_3$, the mixture was filtered through Cerite 545, and the filtrate was washed with 5% aqueous potassium iodide and saturated aqueous NaCl, then dried. After evaporation of the solvent, the residue was purified by silica gel column chromatography ($CHCl_3$–acetone, 9:1) to give **34a** (717 mg, 83%) as a colorless oil, $[\alpha]_D$ +18.7° (c=1.1, $CHCl_3$). NMR δ: 0.89 (9H, t, J=6 Hz), 1.28 (60H, s, CH_2), 1.60 (6H, br, $CH_2CH_2O \times 3$), 2.16 (4H, m, $CH_2CO \times 2$), 2.36 (2H, t, J=8 Hz, CH_2CO), 4.64—4.86 (4H, m, $CH_2CCl_3 \times 2$), 5.13 (1H, t, J=10 Hz, H-3), 5.24 (2H, s, $CH_2C_6H_5$), 5.56 (1H, t, J=10 Hz, H-3'), 7.2—7.4 (15H, m, arom. H). MS m/z: 1698 (M^+), 1703 $[(M+5)^+]$.

1,3-Di(benzyloxycarbonyl)isopropyl 2-Deoxy-6-*O*-[2-deoxy-4-*O*-diphenylphosphono-3-*O*-tetradecanoyl-6-*O*-(2,2,2-trichloroethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-3-*O*-tetradecanoyl-2-tetradecanoylamino-α-D-glucopyranoside (34b) Similarly to the preparation of **34a**, compound **17** (500 mg, 0.49 mmol) was treated with **33b** (450 mg, 0.49 mmol) in CH_2Cl_2 (4 ml) in the presence of $CaSO_4$ (0.6 g) and mercuric cyanide (250 mg, 1.0 mmol) to give **34b** (790 mg, 86%) as a colorless oil, $[\alpha]_D$ +14.0° (c=0.8, $CHCl_3$). *Anal.* Calcd for $C_{91}H_{131}Cl_6N_2O_{23}P$: C, 58.61; H, 7.08; N, 1.50. Found: C, 58.54; H, 7.08; N, 1.47. NMR δ: 0.88 (9H, t, J=6 Hz), 1.26 (60H, s, CH_2), 4.70 (4H, m, $CH_2CCl_3 \times 2$), 5.18 (4H, d, $CH_2C_6H_5 \times 2$), 7.16—7.46 (20H, m, arom. H). MS m/z: 1860 (M^+), 1864 $[(M+4)^+]$.

2-(Diphenylphosphonooxy)ethyl 2-Deoxy-6-*O*-[2-deoxy-4-*O*-diphenylphosphono-3-*O*-(*N*-dodecanoylglycyl)-6-*O*-(2,2,2-trichloroethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-3-*O*-(*N*-dodecanoylglycyl)-2-tetradecanoylamino-α-D-glucopyranoside (34c) HBr-AcOH (25%, 16 ml) was added to a solution of **15** (2.54 g, 2.43 mmol) in CH_2Cl_2 (18 ml), and the mixture was stirred for 3 h. The reaction mixture was diluted with $CHCl_3$ and the solution was washed with ice-H_2O, 5% aqueous $NaHCO_3$ and saturated aqueous NaCl, and dried. After evaporation of the solvent, the residue (**16**) and **33c** (2.51 g, 2.77 mmol) were dissolved in CH_2Cl_2 (18 ml), then $CaSO_4$ (3.0 g) and mercuric cyanide (1.23 g, 4.86 mmol) were added to the solution. After being refluxed for 2.5 h, the reaction mixture was filtered through Celite 545, and the filtrate was washed with 5% aqueous potassium iodide and saturated aqueous NaCl, dried, and concentrated. The residue was purified by silica gel chromatography ($CHCl_3$–MeOH, 19:1) to give **34c** (4.06 g, 88%) as a colorless oil, $[\alpha]_D$ +24.0° (c=1.0, $CHCl_3$). NMR δ: 0.89 (9H, t, J=7 Hz), 1.26 (52H, s, CH_2), 1.60 (6H, br, $CH_2CH_2CO \times 3$), 2.10 (4H, m, $CH_2CO \times 2$), 2.27 (2H, t, J=8 Hz, CH_2CO), 5.00 (1H, d, J=8 Hz, H-1'), 5.11 (1H, t, J=10 Hz, H-3), 5.79 (1H, t, J=10 Hz, H-3'), 7.1—7.5 (20H, m, arom. H). MS m/z: 1884 (M^+), 1889 $[(M+5)^+]$.

2-(Diphenylphosphonooxy)ethyl 2-Deoxy-6-*O*-[2-deoxy-4-*O*-diphenylphosphono-3-*O*-(*N*-dodecanoylglycyl)-6-*O*-(2,2,2-trichloroethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-3-*O*-(*N*-dodecanoylglycyl)-2-(*N*-dodecanoylglycylamino)-α-D-glucopyranoside (34d) Compound **16**, obtained from **15** (0.28 g, 0.27 mmol) was allowed to react with **33d** (0.23 g, 0.24 mmol) in the presence of mercuric cyanide (0.14 g, 0.54 mmol), as described for **34c**, to give **34d** (0.41 g, 88%) as a colorless viscous oil, $[\alpha]_D$ +11.9° (c=1.0, $CHCl_3$). NMR δ: 0.90 (9H, t, J=6 Hz), 1.28 (48H, s, CH_2), 1.60 (6H, br, $CH_2CH_2CO \times 3$), 2.0—2.3 (6H, m, $CH_2CO \times 2$), 4.96 (1H, d, J=8 Hz, H-1'), 5.19 (1H, t, J=10 Hz, H-3), 5.58 (1H, t, J=10 Hz, H-3'), 6.1, 6.2, and 6.8 (each 1H, br, NH), 7.1—7.5 (22H, m, arom. H and NH). MS m/z: 1913 (M^+), 1916 $[(M+3)^+]$.

2-(Diphenylphosphonooxy)ethyl 2-Deoxy-6-*O*-[2-deoxy-4-*O*-diphenylphosphono-3-*O*-tetradecanoyl-6-*O*-(2,2,2-trichloroethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-3-*O*-tetradecanoyl-2-(*N*-dodecanoylglycylamino)-α-D-glucopyranoside (34e) Compound **17** was allowed to react with **33e** (0.32 g, 0.35 mmol) in the presence of mercuric cyanide (0.20 g, 0.78 mmol), as described for **34a**, to give **34e** (0.59 g, 90%) as a colorless viscous oil, $[\alpha]_D$ +14.8° (c=1.0, $CHCl_3$). NMR δ: 0.89 (9H, t, J=7 Hz), 1.28 (56H, s, CH_2), 1.60 (6H, br, $CH_2CH_2CO \times 3$), 2.14 (4H, m, $CH_2CO \times 2$), 2.35 (2H, t, J=8 Hz, CH_2CO), 5.04 (1H, d, J=10 Hz, H-3), 5.56 (2H, m, H-3' and NH), 6.66 and 6.84 (each 1H, br, NH), 7.1—7.5 (20H, m, arom. H). MS m/z: 1855 (M^+), 1859 $[(M+4)^+]$.

1,3-Di(benzyloxycarbonyl)isopropyl 2-Deoxy-6-*O*-[2-deoxy-4-*O*-diphenylphosphono-3-*O*-(*N*-dodecanoylglycyl)-6-*O*-(2,2,2-trichloroethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-3-*O*-(*N*-dodecanoylglycyl)-2-tetradecanoylamino-α-D-glucopyranoside (34f) As described for **34c**, compound **16** obtained from **15** (416 mg, 0.40 mmol), was treated with **33f** (376 mg, 0.40 mmol) to give **34f** (531 mg, 69%) as a colorless viscous oil, $[\alpha]_D$ +19.4° (c=1.1, $CHCl_3$). NMR (500 MHz) δ: 0.88 (9H, t, J=7.3 Hz, CH_3), 1.25 (52H, s, CH_2), 1.54 (2H, m, CH_2CH_2CO), 1.63 (2H, m, CH_2CH_2CO), 2.07 (2H, t, J=7.3 Hz, CH_2CO), 2.25 (2H, m, CH_2CO), 2.26 (2H, t, J=7.3 Hz, CH_2CO), 2.64—2.74 (3H, m, $OCH(CH_2CO_2Bzl)_2 \times 3$), 2.99 (1H, m, $OCH(CH_2CO_2Bzl)_2$), 3.29 (1H, m), 3.49 (1H, m), 3.62 (1H, m), 3.78—3.91 (m), 4.09 (1H, dd, J=17.4, 5.5 Hz), 4.17 (3H, m), 4.33 (1H, dd, J=11.9, 4.6 Hz), 4.46—4.56 (m), 4.68 (2H, m), 4.80 (1H, m), 4.89 (1H, d, J=3.4 Hz, H-1), 4.99 (2H, m), 5.09—5.18 (5H, m), 5.74 (1H, t, J=9.2 Hz, H-3'), 6.06 (1H, d, J=7.3 Hz, NH), 6.17 (1H, m, NH), 6.36 (1H, m, NH), 6.54 (1H, d, J=9.2 Hz, NH), 7.10—7.37 (20H, m, arom. H). MS m/z: 1918 (M^+), 1923 $[(M+5)^+]$.

Benzyloxycarbonylmethyl 2-Deoxy-6-*O*-(2-deoxy-4-*O*-diphenylphosphono-3-*O*-tetradecanoyl-2-tetradecanoylamino-β-D-glucopyranosyl)-3-*O*-tetradecanoyl-2-tetradecanoylamino-α-D-glucopyranoside (35a) Zinc powder (0.7 g) was added to a solution of **34a** (700 mg, 0.41 mmol), and the mixture was vigorously stirred for 1 h at room temperature. The insoluble materials were removed by filtration, and the filtrate was concentrated *in vacuo*. The residue was dissolved in benzene, and washed with 5% $NaHCO_3$ aqueous solution, and then with saturated aqueous NaCl, and dried. Evaporation of the solvent gave an oil.

Separately, DCC (134 mg, 0.65 mmol) was added to a solution of tetradecanoic acid (142 mg, 0.62 mmol) and HOBt (99 mg, 0.65 mmol) in THF (3 ml) with ice-cooling. The mixture was stirred for 3 h at room temperature, and the precipitate was filtered off to give an active ester solution. This solution was added to a solution of the above oil in CH_2Cl_2 (5 ml), then *N*-methylmorpholine (71 μl, 0.65 mmol) was added to the mixture with ice cooling, and the final mixture was stirred for 18 h at room temperature. After evaporation of the solvent, the residue was purified by silica gel column chromatography ($CHCl_3$–MeOH, 50:1) to give **35a** (400 mg, 62%) as a colorless oil, $[\alpha]_D$ +8.5° (c=0.9, $CHCl_3$). NMR δ: 0.90 (12H, t, J=6 Hz), 1.26 (80H, s, CH_2), 1.58 (8H, br,

$C\underline{H}_2CH_2CO\times4$), 2.1—2.4 (8H, m, $CH_2CO\times4$), 4.75 (1H, m, H-4'), 4.84 (1H, d, J=4 Hz, H-1), 4.96 (1H, d, J=8 Hz, H-1'), 5.16 (1H, t, J=10 Hz, H-3), 5.22 (2H, s, $C\underline{H}_2C_6H_5$), 5.51 (1H, t, J=10 Hz, H-3'), 7.2—7.4 (15H, m, arom. H). MS m/z: 1562 [$(M+2)^+$].

1,3-Di(benzyloxycarbonyl)isopropyl 2-Deoxy-6-*O*-(2-deoxy-4-*O*-diphenylphosphono-3-*O*-tetradecanoyl-2-tetradecanoylamino-β-D-glucopyranosyl)-3-*O*-tetradecanoyl-2-tetradecanoylamino-α-D-glucopyranoside (35b) As described for **35a**, compound **34b** (780 mg, 0.42 mmol) was treated with zinc powder (1.2 g), and the resulting oil was allowed to react with the HOBt ester of tetradecanoic acid (140 mg, 0.63 mmol) to give **35b** (550 mg, 76%) as a white powder, mp 78—80 °C, $[\alpha]_D$ +4.4° (c=1.3, $CHCl_3$). *Anal.* Calcd for $C_{99}H_{155}N_2O_{20}P$: C, 68.96; H, 9.06; N, 1.62. Found: C, 68.72; H, 8.95; N, 1.63. NMR δ: 0.90 (12H, t, J=6 Hz), 1.26 (80H, s, CH_2), 2.1—2.4 (8H, m, $CH_2CO\times4$), 2.64—2.98 (4H, m, $OCH(CH_2CO_2Bzl)_2$), 4.75 (1H, m, H-4'), 4.94 (1H, d, J=4 Hz, H-1), 5.00 (1H, d, J=8 Hz, H-1'), 5.16 (4H, s, $C\underline{H}_2C_6H_5\times2$), 5.58 (1H, t, J=10 Hz, H-3').

2-(Diphenylphosphonooxy)ethyl 2-Deoxy-6-*O*-[2-deoxy-4-*O*-diphenylphosphono-3-*O*-(*N*-dodecanoylglycyl)-2-tetradecanoylamino-β-D-glucopyranosyl]-3-*O*-(*N*-dodecanoylglycyl)-2-tetradecanoylamino-α-D-glucopyranoside (35c) As described for **35a**, compound **34c** (4.06 g, 2.15 mmol) was treated with zinc powder, and the resulting oil was allowed to react with the HOBt ester of tetradecanoic acid (0.74 g, 3.23 mmol) in THF (15 ml) to give **34c** (3.30 g, 88%) as a colorless oil, $[\alpha]_D$ +20.9° (c=1.1, $CHCl_3$). NMR δ: 0.88 (12H, t, J=6 Hz), 1.28 (76H, s, CH_2), 1.60 (8H, br, $C\underline{H}_2CH_2CO\times4$), 2.0—2.4 (8H, m, $CH_2CON\times4$), 4.70 (1H, q, J=10 Hz, H-4'), 4.90 (1H, d, J=4 Hz, H-1), 5.16 (1H, t, J=10 Hz, H-3, 5.28 (1H, d, J=8 Hz, H-1), 5.66 (1H, t, J=10 Hz, H-3'), 7.2—7.4 (20H, m, arom. H).

2-(Diphenylphosphonooxy)ethyl 2-Deoxy-6-*O*-[2-deoxy-4-*O*-diphenylphosphono-3-*O*-(*N*-dodecanoylglycyl)-2-(*N*-dodecanoylglycylamino)-β-D-glucopyranosyl]-3-*O*-(*N*-dodecanoylglycyl)-2-(*N*-dodecanoylglycylamino)-α-D-glucopyranoside (35d) As in the preparation of **35a**, compound **34d** (0.39 g, 0.20 mmol) was treated with zinc powder, and the resulting oil was allowed to react with the HOBt ester of **10** (0.10 g, 0.40 mmol) to give **34d** (0.20 g, 54%) as a colorless oil, $[\alpha]_D$ +16.7° (c=1.0, $CHCl_3$). NMR δ: 0.9 (12H, t, J=6 Hz), 1.28 (64H, s, CH_2), 1.64 (8H, br, $C\underline{H}_2CH_2CO\times4$), 2.0—2.3 (8H, m, $CH_2CO\times4$), 4.74 (1H, m, H-4'), 4.84 (1H, d, J=4 Hz, H-1), 5.01 (1H, d, J=8 Hz, H-1'), 5.21 (1H, m, H-3), 5.60 (1H, t, J=10 Hz, H-3'), 7.2—7.5 (m, arom. H and NH). MS m/z: 1806 [$(M+2)^+$].

2-(Diphenylphosphonooxy)ethyl 2-Deoxy-6-*O*-[2-deoxy-4-*O*-diphenylphosphono-2-(*N*-dodecanoylglycylamino)-3-*O*-tetradecanoyl-β-D-glucopyranosyl]-2-(*N*-dodecanoylglycylamino)-3-*O*-tetradecanoyl-α-D-glucopyranoside (35e) As described for **35a**, compound **34e** (0.58 g, 0.31 mmol) was treated with zinc powder, and the oily product was reacted with the HOBt ester of **10** to give **35e** (0.49 g, 89%) as a colorless oil, $[\alpha]_D$ +16.4° (c=1.0, $CHCl_3$). NMR δ: 0.90 (12H, t, J=7 Hz), 1.26 (72H, s, CH_2), 2.1—2.3 (8H, m, $CH_2CO\times4$), 4.73 (1H, m, H-4'), 4.88 (1H, d, J=4 Hz, H-1), 4.93 (1H, d, J=8 Hz, H-1'), 5.41 (1H, m, H-3), 5.65 (1H, t, J=10 Hz, H-3'), 6.42 (1H, br, NH), 6.66 (2H, m, NH×2), 6.88 (1H, d, J=9 Hz, NH), 7.2—7.5 (20H, m, arom. H). MS m/z: 1748 [$(M+2)^+$].

1,3-Di(benzyloxycarbonyl)isopropyl 2-Deoxy-6-*O*-[2-deoxy-3-*O*-(*N*-dodecanoylglycyl)-2-tetradecanoylamino-4-*O*-diphenylphosphono-β-D-glucopyranosyl]-3-*O*-(*N*-dodecanoylglycyl)-2-tetradecanoylamino-α-D-glucopyranoside (35f) As described for **35a**, compound **34f** (509 mg, 0.26 mmol) was treated with zinc dust, and the resulting oil was allowed to react with the HOBt ester to tetradecanoic acid (91 mg, 0.40 mmol) to give **35f** (376 mg, 80%) as a colorless powder, mp 135—139 °C, $[\alpha]_D$ +18.8° (c=0.5, $CHCl_3$). *Anal.* Calcd for $C_{99}H_{153}N_4O_{22}P$: C, 66.62; H, 8.65; N, 3.14. Found: C, 66.81; H, 8.54; N, 3.11. NMR (500 MHz) δ: 0.89 (12H, t, J=7.3 Hz), 1.25 (72H, s, CH_2), 1.54 (2H, m, $C\underline{H}_2CH_2CO$), 1.63 (2H, m, $C\underline{H}_2CH_2CO$), 2.12 (4H, m, $CH_2CO\times2$), 2.24 (2H, t, J=7.3 Hz, CH_2CO), 2.62 (1H, dd, J=16.5, 7.3 Hz, $OCH(C\underline{H}_2CO_2Bzl)_2$), 2.67 (2H, d, J=6.4 Hz, $OCH(C\underline{H}_2CO_2Bzl)_2\times2$), 2.90 (1H, dd, J=16.5, 4.6 Hz, $OCH(C\underline{H}_2CO_2Bzl)_2$), 3.28 (1H, m), 3.56—3.67 (4H, m), 3.74—3.90 (5H, m), 4.07 (2H, m), 4.36 (1H, m), 4.66 (1H, m, $O\underline{C}H(CH_2CO_2Bzl)_2$), 4.67 (1H, q, J=9.2 Hz, H-4'), 4.90 (1H, d, J=3.7 Hz, H-1), 5.03 (1H, t, J=10.1 Hz, H-3), 5.12 (4H, m, $C\underline{H}_2C_6H_5\times2$), 5.31 (1H, d, J=8.3 Hz, H-1'), 5.67 (1H, t, J=10.1 Hz, H-3'), 6.26 (1H, t, J=5.5 Hz, NH), 6.29 (1H, t, J=5.5 Hz, NH), 6.37 (1H, d, J=6.4 Hz, NH), 6.50 (1H, d, J=9.2 Hz, NH), 7.14—7.36 (20H, m, arom. H). MS m/z: 1782 [$(M+2)^+$].

Carboxymethyl 2-Deoxy-6-*O*-(2-deoxy-4-*O*-phosphono-3-*O*-tetradecanoyl-2-tetradecanoylamino-β-D-glucopyranosyl)-3-*O*-tetradecanoyl-2-tetradecanoylamino-α-D-glucopyranoside (4) Compound **35a** (379 mg, 0.24 mmol) was dissolved in THF (30 ml) and shaken with 5% palladium–carbon (400 mg) at room temperature for 1 h in H_2 at atmospheric pressure. Then, platinum dioxide (200 mg) was added to the mixture, and the mixture was further shaken at room temperature for 1.1 h in H_2 at atmospheric pressure. The catalyst was removed by filtration and washed with $CHCl_3$–MeOH–H_2O (8:3:1, lower layer). The combined filtrate washings were concentrated at reduced pressure. The residue was purified by preparative TLC ($CHCl_3$–MeOH–H_2O, 6:4:0.2). After extraction with $CHCl_3$–MeOH–H_2O–Et_3N (6:4:1:0.02), the solvent was evaporated *in vacuo*. The residue was dissolved in $CHCl_3$–MeOH–H_2O (6:4:0.5), and the solution was desalted with Dowex 50 (H^+ type). A portion of the desalted solution was concentrated, and the residue was lyophilized from a dioxane suspension to give **4** (249 mg, 78%) as a white powder, mp 145—148 °C (dec.), $[\alpha]_D$ +14.2° (c=0.5, $CHCl_3$–MeOH, 3:1). IR (KBr): 3450, 2925, 2855, 1740, 1640 cm^{-1}. NMR ($CDCl_3$–CD_3OD) δ: 0.90 (12H, t, J=6 Hz), 1.28 (80H, s, CH_2), 1.56 (8H, br, $C\underline{H}_2CH_2CO\times4$), 2.1—2.4 (8H, m, $CH_2CO\times4$), 4.80 (1H, d, J=4 Hz, H-1), 5.24 (2H, m, H-3, H-3').

The product (220 mg) in 0.1% aqueous Et_3N was lyophilized to give 250 mg of Et_3N salt as a white powder.

1,3-Dicarboxyisopropyl 2-Deoxy-6-*O*-(2-deoxy-4-*O*-phosphono-3-*O*-tetradecanoyl-2-tetradecanylamino-β-D-glucopyranosyl)-3-*O*-tetradecanoyl-2-tetradecanoylamino-α-D-glucopyranoside (5) In a manner similar to that described for **4**, compound **35b** (280 mg, 0.20 mmol) was hydrogenolyzed with palladium-black (200 mg) in dioxane (30 ml) for 2.5 h, and then with platinum dioxide (300 mg) for 18 h to give **5** (173 mg, 77%) as a white powder, mp 142—147 °C (dec.), $[\alpha]_D$ +11.7° (c=0.7, $CHCl_3$–MeOH, 3:1). NMR δ: 0.90 (12H, t, J=6 Hz), 1.30 (80H, s, CH_2), 2.20 (m), 2.36 (m), 2.70 (m), 4.98 (1H, d, H-1).

The Et_3N salt was prepared similarly to that of **4**.

2-Phosphonooxyethyl 2-Deoxy-6-*O*-[2-deoxy-3-*O*-(*N*-dodecanoylglycyl)-4-*O*-phosphono-2-tetradecanoylamino-β-D-glucopyranosyl]-3-*O*-(*N*-dodecanoylglycyl)-2-tetradecanoylamino-α-D-glucopyranoside (6) Platinum dioxide (0.40 g) was added to a solution of **35c** (0.42 g, 0.24 mmol) in THF (80 ml). The mixture was stirred in a hydrogen atmosphere for 25 h, then a mixture of $CHCl_3$–MeOH–H_2O (8:3:1, lower layer) was added. After the catalysts had been filtered off, the filtrate was concentrated, and the resulting residue was purified by preparative TLC ($CHCl_3$–MeOH–H_2O, 6:4:0.8). The extracted solution with $CHCl_3$–MeOH–H_2O–Et_3N (6:4:1:0.02) was concentrated by evaporation. After dissolution in $CHCl_3$–MeOH–H_2O (8:3:1, lower layer), the solution was desalted with Dowex 50 (H^+). The desalting solution was concentrated and the dioxane suspension was freeze-dried to give **4** (95 mg, 27%) as a white powder, mp 140—145 °C (dec.), $[\alpha]_D$ +13.3° (c=0.6, $CHCl_3$–MeOH, 3:1). IR (KBr): 3400, 2930, 2855, 1750, 1660, 1560 cm^{-1}. NMR ($CDCl_3$–CD_3OD, 1:1) δ: 0.90 (12H, t, J=7 Hz), 1.32 (72H, s, CH_2), 1.60 (8H, br, $C\underline{H}_2CH_2CO\times4$), 2.1—2.3 (8H, m, $CH_2CO\times4$), 5.10 (1H, t, J=10 Hz, H-3), 5.38 (1H, t, J=10 Hz, H-3').

The Et_3N salt was prepared in a manner similar to that described for **4**.

2-Phosphonooxyethyl 2-Deoxy-6-[2-deoxy-3-*O*-(*N*-dodecanoylglycyl)-2-(*N*-dodecanoylglycylamino)-4-*O*-phosphono-β-D-glucopyranosyl]-3-*O*-(*N*-dodecanoylglycyl)-2-(*N*-dodecanoylglycylamino)-α-D-glucopyranoside (7) As described for **6**, compound **35d** (0.42 g, 0.23 mmol) was hydrogenolyzed in the presence of platinum dioxide, and the resulting powder was purified by preparative TLC ($CHCl_3$–MeOH–H_2O, 6:4:0.9) and desalted. The desalting solution was concentrated and the dioxane suspension was freeze-dried to give **7** (0.14 g, 39%) as a white powder, mp 145—150 °C (dec.), $[\alpha]_D$ +7.6° (c=0.8, $CHCl_3$–MeOH, 3:1). IR (KBr): 3300, 1760, 1665, 1555 cm^{-1}. NMR ($CDCl_3$–CD_3OD, 1:1) δ: 0.90 (12H, t, J=6 Hz), 1.30 (64H, s, CH_2), 1.66 (8H, br, $C\underline{H}_2CH_2CO\times4$), 2.30 (8H, m, $CH_2CO\times4$), 4.84 (1H, d, J=4 Hz, H-1), 5.19 (1H, t, J=10 Hz, H-3), 5.33 (1H, t, J=10 Hz, H-3').

The Et_3N salt was prepared similarly to that of **4**.

2-Phosphonooxyethyl 2-Deoxy-6-*O*-[2-deoxy-2-(*N*-dodecanoylglycylamino)-4-*O*-phosphono-3-*O*-tetradecanoyl-β-D-glucopyranosyl]-2-(*N*-dodecanoylglycylamino)-3-*O*-tetradecanoyl-α-D-glucopyranoside (8) In the manner described for **6**, compound **35e** (0.46 g, 0.26 mmol) was hydrogenolyzed in the presence of platinum dioxide, and the resulting powder was purified by preparative TLC ($CHCl_3$–MeOH–H_2O, 6:4:0.8) and desalted. The desalting solution was concentrated, and the dioxane suspension was freeze-dried to give **8** (0.22 g, 59%) as a white powder, mp 148—153 °C (dec.), $[\alpha]_D$ +18.4° (c=0.9, $CHCl_3$–MeOH, 3:1). IR (KBr): 3300, 1745, 1645, 1555 cm^{-1}. NMR ($CDCl_3$–CD_3OD, 1:1) δ: 0.90 (12H, t, J=6 Hz), 1.30 (72H, s, CH_2), 1.6 (8H, br, $C\underline{H}_2CH_2CO\times4$), 2.3 (8H, m, $CH_2CO\times4$), 4.86 (1H, d, J=4 Hz, H-1), 5.16 (1H, t,

$J=10$ Hz, H-3), 5.34 (1H, t, $J=10$ Hz, H-3').

The Et_3N salt was prepared in a manner similar to that for **4**.

1,3-Dicarboxyisopropyl 2-Deoxy-6-*O*-[2-deoxy-3-*O*-(*N*-dodecanoylglycyl)-4-*O*-phosphono-2-tetradecanoylamino-β-D-glucopyranosyl]-3-*O*-(*N*-dodecanoylglycyl)-2-tetradecanoylamino-α-D-glucopyranoside (9) In a manner similar to that described for **4**, compound **35f** (361 mg, 0.20 mmol) was hydrogenolyzed with 5% palladium-carbon (400 mg) in 5% aqueous THF (32 ml) for 6 h, and then with platinum dioxide for 3 h. The resulting residue was purified by preparative TLC ($CHCl_3$–MeOH–H_2O, 6:4:0.5), then desalted with Dowex 50 (H^+ type). A portion of the desalted solution was concentrated, and the residue was lyophilized from dioxane suspension to give **9** (116 mg, 39%) as a white powder, mp 158—165 °C (dec.), $[\alpha]_D$ +18.6° ($c=0.6$, $CHCl_3$–MeOH, 3:1). *Anal.* Calcd for $C_{73}H_{133}N_4O_{22}P\cdot 0.5H_2O$: C, 60.10; H, 9.26; N, 3.84. Found: C, 60.11; H, 9.55; N, 3.87. IR (KBr): 3448, 2924, 2856, 1748, 1646, 1550, 1470 cm^{-1}. NMR (500 MHz, $CDCl_3$–CD_3OD, 1:1) δ: 0.88 (12H, t, $J=7.3$ Hz), 1.26 (72H, s, CH_2), 1.56 (4H, m, $C\underline{H}_2CH_2CO\times 2$), 1.63 (4H, m, $C\underline{H}_2CH_2CO\times 2$), 2.16 (2H, t, $J=7.1$ Hz, CH_2CO), 2.21 (2H, t, $J=7.1$ Hz, CH_2CO), 2.26 (4H, t, $J=7.1$ Hz, $CH_2CO\times 2$), 2.67 (3H, m, $OCH(C\underline{H}_2CO_2H)_2\times 3$), 2.83 (1H, dd, $J=16.5$, 5.5 Hz, $OCH(C\underline{H}_2CO_2H)_2$), 3.52 (2H, m, H-4 and H-5'), 3.70 (1H, t, $J=9.5$ Hz, H-2'), 3.77 (1H, dd, $J=11.2$, 5.6 Hz, H-6), 3.84 (1H, dd, $J=12.7$, 4.8 Hz, $COCH_2N$), 3.9—4.0 (7H, m, $COCH_2N\times 3$, H-5, H-6 and H-6'$\times 2$), 4.12 (1H, m, H-2), 4.30 (1H, q, $J=9.5$ Hz, H-4'), 4.40 (1H, m, $OC\underline{H}(CH_2CO_2H)_2$), 4.84 (1H, d, $J=7.9$ Hz, H-1'), 4.95 (1H, d, $J=4.0$ Hz, H-1), 5.05 (1H, t, $J=9.5$ Hz, H-3), 5.40 (1H, t, $J=9.5$ Hz, H-3').

The Et_3N salt was prepared similarly to that of **4**.

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Studies on the Constituents of the Leaves of *Cassia torosa* CAV. II.[1] The Structure of Two Novel Flavones, Torosaflavone C and D

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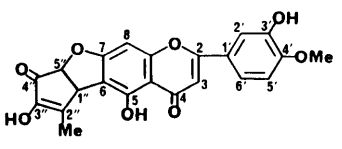
Two novel flavones, torosaflavone C (1) and D (2), were isolated from the leaves of *Cassia torosa* CAV. The structures of 1 and 2 were established based upon spectral studies. Compound 1 displayed cytotoxic activity toward KB cells.

Keywords *Cassia torosa*; Leguminosae; torosaflavone C; torosaflavone D; flavonoid; HMBC; cytotoxic activity

In a previous paper,[2] we reported the isolation of two new *C*-deoxyglycosylflavones from the leaves of *Cassia torosa* CAV. We also reported the isolation[3-11] of anthraquinones, an anthrone, a dimeric anthrone, hydroanthracenes, dimeric hydroanthracenes, naphthalenic lactones, and sterols, from the leaves, the ripe and unripe seeds, the seedlings, and the roots of this plant.

In our present study, as part of our continuing investigation of the ether-soluble fraction of the methanolic extract of the leaves, we isolated two novel flavones, named torosaflavone C and D. We determined their structures by means of spectral methods using a two-dimensional nuclear magnetic resonance (2D-NMR) spectra. In this paper, we wish to report the structure and cytotoxic activity of torosaflavones C and D.

Torosaflavone C (**1**), yellow needles, mp 305.5—306 °C, displayed a positive coloration in the Mg–HCl test and was optically inactive. The molecular formula of **1** was determined to be $C_{22}H_{16}O_8$ from mass spectrum (MS) and high-resolution MS (HR-MS). The ultraviolet (UV) spectrum showed maxima at 245, 253, 271 and 343 nm, and the infrared (IR) spectrum exhibited absorption bands due to the presence of hydroxyls (3430 cm^{-1}), a carbonyl (1720 cm^{-1}), and an α,β-unsaturated ketone (1661, 1614 cm^{-1}). The characteristic color reaction and spectral properties of **1** indicated that it is a flavone having a carbonyl group except for its nucleus. The bathochromic shifts[12] of the UV bands of **1** with aluminum chloride ($\lambda_1 = 24$ nm) suggested the presence of a free 5-hydroxyl, and as **1** did not show shifts with sodium acetate, the absence of a 7-hydroxyl is demonstrated. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** in dimethyl sulfoxide (DMSO)-d_6 indicated the following: an AMX system due to a 3′,4′-disubstitution in the flavone skeleton at δ 7.09 (d, J=8.5 Hz), 7.43 (d, J=2.3 Hz), and 7.55 (dd, J=8.5, 2.3 Hz); two singlets at δ 6.70 and 6.82 attributed respectively to an A-ring proton and H-3[4]; and an olefinic methyl (δ 2.16, br s), a methoxyl (δ 3.87), two vicinally coupled methines (δ4.49, br s, J=6.6 Hz; 5.27, d, J=6.6 Hz), and three hydroxyls (δ 9.48, 9.60, 13.61). A singlet at δ 13.61 was assigned to a chelated hydroxyl at C-5.



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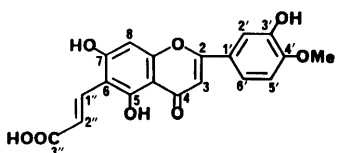

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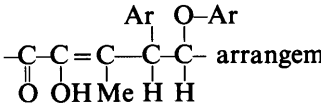
Chart 1

In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra of **1** there were twenty-one signals due to two carbonyls (δ 182.1, 195.3), sixteen sp^2 carbons (δ 90.0—165.8) and four sp^3 carbons [a methyl carbon (δ 14.2), a methoxyl carbon (δ 55.8), a methine carbon (δ 43.7), an oxymethine (δ 82.2)]. The ^{1}H detected multiple quantum coherence (HMQC)[13,14] and the ^{1}H-detected hetronuclear multiple bond connectivity (HMBC)[14] spectra were measured and the connectivity of quarternary carbons were investigated through the detection of ^{1}H–^{13}C long-range correlation as shown in Fig. 1.

In the HMBC spectrum, the chelated hydroxyl proton at δ 13.61 (5-OH) showed cross peaks because of long-range coupling with the three quaternary carbons at δ 105.2, 109.3 and 157.6 (C-5). The carbon at δ 105.2 showed cross peaks with H-3 (δ 6.82), so that the carbon signals at δ 105.2 and 109.2 are assigned to the C-4a and C-6 positions, respectively. The aromatic proton at δ 6.70 showed cross peaks with the carbon signals due to C-4a (δ 105.2), C-6 (δ 109.2), C-7 (δ 165.8) and C-8a (δ 157.1),[15] indicating that the proton at δ 6.70 should be assigned to the H-8 position. From ^{1}H–^{13}C long-range correlations, the methoxyl protons and the hydroxyl proton at δ 9.48 are located respectively at the C-4′ and C-3′ positions in the B-ring. The structure of the flavone nucleus was suggested as diosmetin (5,7,3′-trihydroxy-4′-methoxyflavone)[16] through to be analysis of the HMBC spectrum. In the ^{1}H-NMR spin-decoupling experiments, irradiation of the broad methyl at δ 2.16 (2″-Me) distinctly produced a change in a sharp doublet at δ 4.49 (H-1″), while irradiation of a doublet at δ 4.49 changed a doublet at δ 5.27 (H-5″) to a singlet. Therefore, these experiments revealed the presence of the partial structure, $>C=\underset{\text{Me}}{C}-\underset{\text{H}}{C}-\underset{\text{H}}{C}-$, in the molecule **1**. The methine proton at δ 4.49 (H-1″) showed long-range correlation with the carbons at δ 109.3 (C-6), 165.8 (C-7), 147.0 (C-2″), 149.7 (C-3″) and 195.3 (C-4″). On the other hand, the methine proton at δ 5.27 (H-5″) exhibited long-range correlation with carbons at δ 165.8 (C-7) and 147.0 (C-2″). Therefore, two methine carbons at δ 43.7 (C-1″) and 82.2 (C-5″) must be bonded at the C-6 position and at an oxygen linked at the C-7 position, respectively. According to the proton spin-decoupling experiments and the HMBC spectra, C-5″, C-1″, C-6, C-7 and an oxygen linked at C-7 should form a dihydrofuran ring. The methyl protons at δ 2.16 (2″-Me) exhibited long-range correlation with the

carbons at δ 147.0 (C-2″), 149.7 (C-3″) and 43.7 (C-1″), while the hydroxyl proton at δ 9.60 (3″-OH) exhibited long-range correlation with the carbon at δ 147.0 (C-2″) and the carbonyl carbon at δ 195.3 (C-4″). From the long-range correlations and the ^{1}H-NMR spin-decoupling experiments, the partial structure of a side chain is revealed as a



arrangement. According to fifteen degrees of unsaturation in the molecular formula, the carbonyl carbon (C-4″) at δ 195.3 and the oxygen-bearing methine carbon (C-5″) at δ 82.2 must be connected to each other. This results in a five-membered ring having an α,β-unsaturated ketone and a dihydrofuran ring which is linked by the two methine carbons due to C-1″ and C-5″, and the ring junction must have a *cis*-configuration.

Thus, the structure of **1** can be represented as a racemic compound as seen in formula **1**.

Torosaflavone D (**2**), yellow needles, mp 244.5—255.5 °C, $C_{19}H_{14}O_8$, produced a positive coloration in the Mg–HCl test and dissolved in sodium bicarbonate. The specific UV spectrum showed maxima at 303 (4.41), 317 (4.39) and 344 (4.34) nm, and the IR spectrum exhibited absorption bands due to the presence of hydroxyls (3390 cm^{-1}), chelated hydroxyls (3200—2400 cm^{-1}), a carboxylic acid (1695 cm^{-1}), α,β-unsaturated ketone (1643, 1612 cm^{-1}), and aromatic double bonds (1513, 1491 cm^{-1}). The UV and IR spectra suggested that **2** is a flavonoid having a conjugated functional group on the A-ring of the flavone nucleus. The ^{1}H-NMR spectrum of **2** in DMSO-d_6 indicated the following: a methoxyl at δ 3.89; two aromatic protons at δ 6.57 and 6.82 due to H-8, H-6 or H-3; a chelated hydroxyl proton at δ 14.39; an AMX system due to 3′,4′-disubstitution in the flavone skeleton at δ 7.09 (d, J=8.5 Hz, H-5′), 7.44 (d, J=2.5 Hz, H-2′) and 7.55 (dd, J=8.5, 2.5 Hz, H-6′); and two olefinic protons at δ 6.84 (d, J=16.1 Hz) and 7.90 (d, J=16.1 Hz) appearing low field. In the ^{1}H- and ^{13}C-NMR data, the chemical shifts on a B-ring are similar to those of **1**. Consequently, their NMR data suggested the same partial structure on a B-ring as a flavone nucleus. The chemical ionization (CI)-MS of **2** gave M^+-1 at m/z 369, M^++1-CO_2 at m/z 327 and M^+-CO_2 at m/z 326. From the spectral data and empirical formula, **2** was considered to be a flavone-bonding acrylic acid, –CH=CH–COOH, at the C-6 or C-8 position on the A-ring. This structure was confirmed by an analysis of the HMBC spectrum of **2**.

In the HMBC spectrum of **2**, the chelated hydroxyl proton at δ 14.39 (5-OH) showed cross peaks due to long-range coupling with the carbons at δ 161.8 (C-5), 103.3 (C-4a) and 105.8 (C-6). The aromatic methine proton at δ 6.57 (H-8) showed long-range correlation with the carbons at δ 103.3 (C-4a), 105.8 (C-6), 163.5 (C-7) and 157.2 (C-8a). On the other hand, the methoxyl proton signals at δ 3.87 also showed long-range correlation with the carbons at δ 151.3 (C-4′). Therefore, the structure of the flavone nucleus must be diosmetin. Two olefinic protons at δ 7.90 and 6.84 bonded to a side chain exhibited long-range correlations with the carbons at δ 161.8 (C-5), 120.0 (C-2″) and 168.6 (C-3″), and those at δ 105.8 (C-6) and 168.6 (C-3″), respectively. Therefore, the acryl acid moiety should be connected to C-6, and the double bond indicated at *E*-configuration

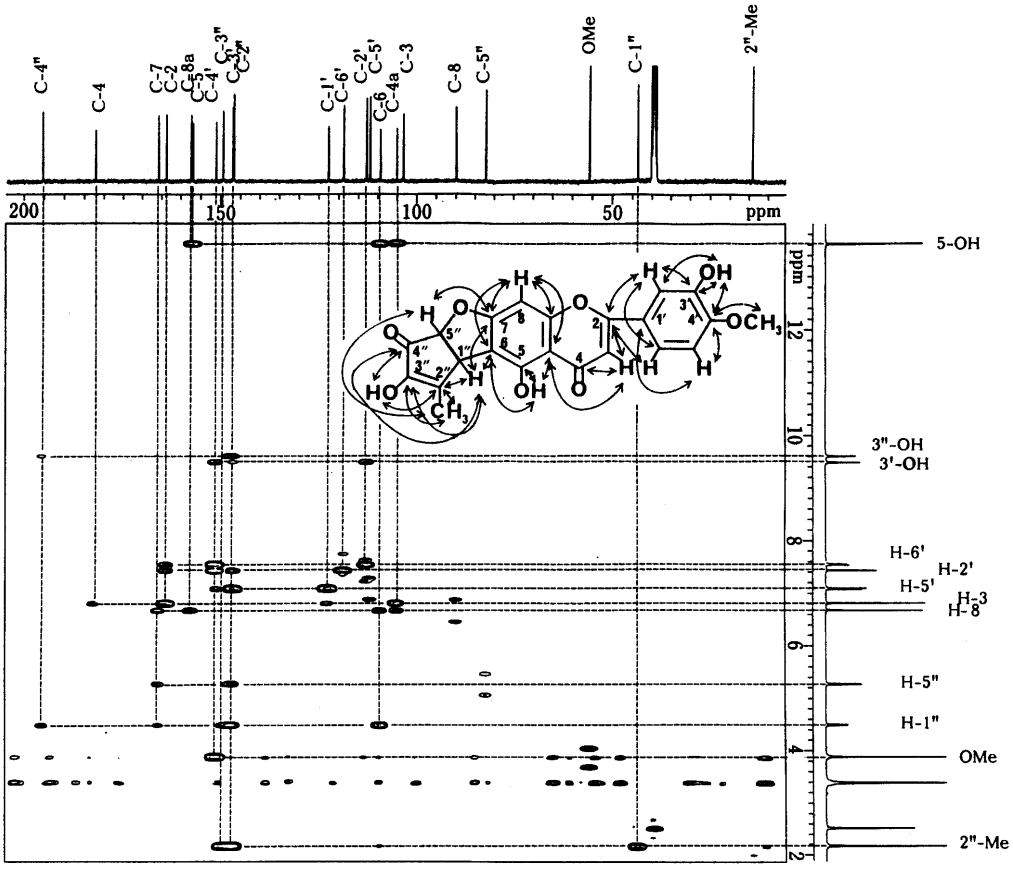


Fig. 1. HMBC Spectrum of Torosaflavone C (**1**) in DMSO-d_6

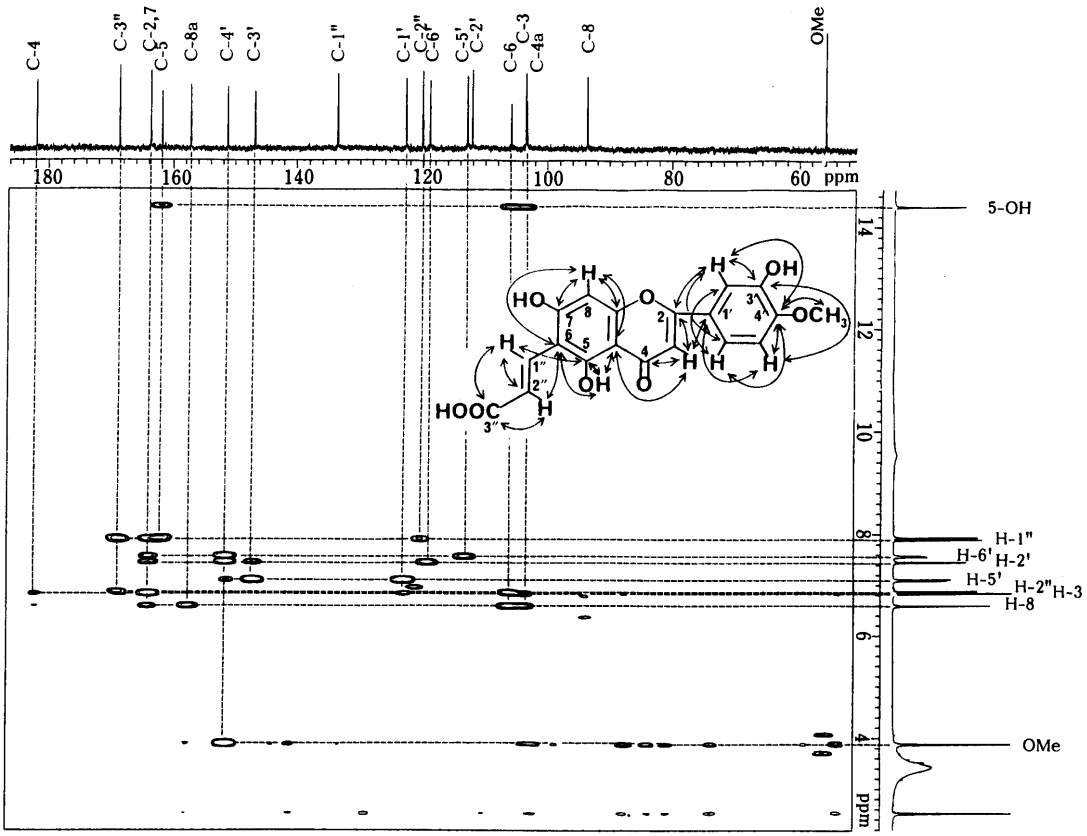


Fig. 2. HMBC Spectrum of Torosaflavone D (**2**) in DMSO-d_6

through their coupling constants in the ^{1}H-NMR spectrum.

Thus, the structure of **2** was determined as shown in Chart 1.

The effective doses for 50% inhibition (ED_{50}) of the *in vitro* KB cell line in torosaflavone C (**1**) was 2.74 μg/ml.

Experimental

All the melting points were taken on a Yanagimoto micromelting-point apparatus and are uncorrected. The UV spectrum was obtained in MeOH with a Hitachi 200-10 spectrophotometer, and the IR spectra were recorded on a JASCO IR A-2 spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra were taken on JEOL EX-90 and GX-400 spectrometers, respectively, using tetramethylsilane (TMS) as an internal standard. The MS were obtained on a Hitachi M-80B spectrometer. Column chromatography was carried out with silica gel (Wako gel C-200, Wako Pure Chemical Co., Ltd.).

Isolation Separation of an ether-soluble fraction of MeOH extract from *Cassia torosa* CAV. was described in a previous paper.[2] Fraction 4 ($CHCl_3$) (120 mg) was chromatographed on SiO_2 with $CHCl_3$ to give **1** (35 mg). Fractions 6—8 (3% MeOH–$CHCl_3$) (2.6 g) gave **2** (130 mg) from MeOH.

Torosaflavone C (1) Recrystallization (MeOH) gave pale yellow needles, mp 305.5—306 °C, $[\alpha]_D^{25}$ ±0° (c=0.98, pyridine). UV λ_{max}^{MeOH} nm (log ε): 219 (4.20), 245 (4.11), 253 (4.12), 271 (4.15), 343 (4.19); $\lambda_{max}^{MeOH+AcONa}$ nm: 223, 272, 342; $\lambda_{max}^{MeOH+AcONa+H_3BO_3}$: 226, 257 sh, 272, 345; $\lambda_{max}^{MeOH+AlCl_3}$: 220, 263, 280, 367; $\lambda_{max}^{MeOH+AlCl_3+HCl}$: 208 sh, 216, 263 sh, 281, 359. IR ν_{max}^{KBr} cm^{-1}: 3430, 1720, 1661, 1614, 1510—1332, 841, 812. EI-MS m/z: 408 (M^+, 100%), 380 (M^+-CO, 3.9), 365 ($M^+-CO-Me$, 3.7), 352 (M^+-2CO, 61.7), 337 ($M^+-2CO-Me$, 5.8), 325 ($M^+-2CO-C_2H_4$, 10.6), 309 ($M^+-3CO-Me$, 6.3). ^{1}H-NMR (DMSO-d_6) δ: 2.16 (3H, brs, 2″-Me), 3.87 (3H, s, OMe), 4.49 (1H, brd, J=6.6 Hz, H-1″), 5.27 (1H, d, J=6.6 Hz, H-5″), 6.70 (1H, s, H-8), 6.82 (1H, s, H-3), 7.09 (1H, d, J=8.5 Hz, H-5′), 7.43 (1H, d, J=2.3 Hz, H-2′), 7.55 (1H, dd, J=8.5, 2.3 Hz, H-6′), 9.48 (1H, brs, 3′-OH), 9.60 (1H, brs, 3″-OH), 13.61 (1H, s, 5-OH). ^{13}C-NMR (DMSO-d_6) δ: 14.2 (q, C-2″-Me), 43.7 (d, C-1″), 55.8 (q, OMe-4), 82.2 (d, C-5″), 90.0 (d, C-8), 103.5 (d, C-3), 105.2 (s, C-4a), 109.3 (s, C-6), 112.1 (d, C-2′), 113.0 (d, C-5′), 118.8 (d, C-6′), 122.7 (s, C-1′), 146.8 (s, C-3′), 147.0 (s, C-2″), 149.7 (s, C-3″), 151.3 (s, C-4′), 157.1 (s, C-8a), 157.6 (s, C-5), 163.8 (s, C-2), 165.8 (s, C-7), 182. 1 (s, C-4), 195.3 (s, C-4″). High-resolution MS m/z: Calcd for $C_{22}H_{16}O_8$: 408.0843, Found: 408.0823.

Torosaflavone D (2) Recrystallization (pyridine–hexane) gave yellow needles, mp 244.5—255.5 °C. *Anal.* Calcd for $C_{19}H_{14}O_8$: C, 61.63; H, 3.81. Found: C, 61.81; H, 3.60. UV λ_{max}^{MeOH} nm (log ε): 208 (4.32), 231 sh (4.14), 244 sh (4.10), 303 (4.41), 317 (4.39), 344 (4.34); $\lambda_{max}^{MeOH+AcONa}$ nm: 221, 273, 298, 392; $\lambda_{max}^{MeOH+AcONa+H_3BO_3}$ nm: 230, 300, 350 sh; $\lambda_{max}^{MeOH+AlCl_3}$ nm: 210, 250 sh, 331; $\lambda_{max}^{MeOH+AlCl_3+HCl}$ nm: 210, 250, 326. IR ν_{max}^{KBr} cm^{-1}: 3390, 3200—2400, 1695, 1643, 1612, 1513, 1491. CI-MS m/z: 369 (M^+-1, 8.9%), 327 (M^++1-CO_2, 100), 326 (M^+-CO_2, 94.8). ^{1}H-NMR (DMSO-d_6) δ: 3.89 (3H, s, OMe), 6.57 (1H, s, H-8), 6.82 (1H, s, H-3), 6.84 (1H, d, J=16.1 Hz, H-2″), 7.09 (1H, d, J=8.5 Hz, H-5′), 7.44 (1H, d, J=2.5 Hz, H-2′), 7.55 (1H, dd, J=8.5, 2.5 Hz, H-6′), 7.90 (1H, d, J=16.1 Hz, H-1″), 14.39 (1H, s, 5-OH). ^{13}C-NMR (DMSO-d_6) δ: 55.7 (q, OMe), 93.6 (d, C-8), 103.3 (s, C-4a), 103.3 (d, C-3), 105.8 (s, C-6), 112.1 (d, C-2′), 112.9 (d, C-5′), 118.8 (d, C-6′), 120.0 (d, C-2″), 122.7 (s, C-1′), 137.6 (d, C-1″), 146.8 (s, C-3′), 151.3 (s, C-4′), 157.2 (s, C-8a), 161.8 (s, C-5), 163.5 (s, C-7), 163.7 (s, C-2), 168.6 (s, C-3″), 181.8 (s, C-4).

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New Acyclic Diterpene Glycosides, Capsianosides VI, G and H from the Leaves and Stems of *Capsicum annuum* L.[1)]

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***Capsicum* species are very important plants used as vegetable foods, spices and external medicines. In the preceding paper, we disclosed the occurrence of novel acyclic diterpene glycosides from fruits of the *Capsicum* species. We now obtained three new acyclic diterpene glycosides, named capsianosides VI (1), G (2) and H (3), along with capsianosides II, A, B, C and D from the leaves and stems of the *Capsicum annuum* species, and their structures were elucidated by spectroscopic and chemical means.**

Keywords *Capsicum annuum* var. *fasciculatum*; *Capsicum annuum* var. *conoides*; Solanaceae; capsianoside; acyclic diterpene glycoside; geranyllinalool derivative

In the previous paper,[2)] the isolation and structure elucidation of acyclic diterpene glycosides designated as capsianosides (A—F and I—V) from fruits of the *Capsicum* species were reported. In a continuing study, the chemical examination of glycosides in the *Capsicum* species led to the isolation of a new monomeric, capsianoside VI (**1**), and two new dimeric diterpene glycosides, capsianosides G (**2**) and H (**3**), along with capsianosides II, A, B, C and D from the leaves and stems of *Capsicum annuum* L. var. *fasciculatum* BAILE and *C. annuum* L. var. *conoides* BAILE. We now wish to describe the characterization of those compounds. The isolation of **1**—**3** from the H_2O soluble portion from the methanolic extracts was achieved by combination chromatography on MCI gel CHP 20P (MeOH–H_2O), silica gel ($CHCl_3$–MeOH–H_2O) and Bondapak C_{18} (MeOH–H_2O). Capsianosides II, A, B, C and D were identified by direct comparison of their physical and spectral data.

Capsianoside VI (**1**), a white powder, $[\alpha]_D$ −36.2°, showed an infrared (IR) absoption band at 3450 cm^{-1} due to a hydroxyl group. The negative fast atom bombardment mass spectrum (FAB-MS) of **1** exhibited an intense molecular ion peak $[M-H]^-$ at *m/z* 921, together with minor peaks at *m/z* 775 (*m/z* 921 − deoxyhexose), 759 (*m/z* 921 − hexose), 692 (*m/z* 775 − deoxyhexose) and 613. Acid hydrolysis of **1** yielded glucose and rhamnose as sugar components. Carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **1** showed the occurrence of an acyclic diterpene, 17-hydroxygeranyllinalool, in **1** by the appearance of signals due to eight double bond carbons [δ 115.9 (t), 144.4 (d), 125.9 (d), 135.9 (s), 125.8 (d), 135. 4(s), 131.2 (d) and 132.4 (s)], methylene and quarternary carbons with an oxygen function [δ 67.7 (t) and 81.5 (s)], four methyl carbons [δ 21.9, 16.3, 16.1 and 23.2] and six methylene carbons [δ 42.6, 23.5, 40.7, 27.6, 40.9 and 27.2] along with two α-rhamnopyranosyl and two β-glucopyranosyl anomeric signals [δ 101.5 (J_{C-H} = 170 Hz), 102.6 (J_{C-H} = 170 Hz) and 99.5 (J_{C-H} = 155 Hz), 102. 1 (J_{C-H} = 158 Hz)]. These signals were similar to those of capsianoside II (**4**)[2)] except for a lack of signals due to the terminal glucosyl moiety attached to the C-3 position in the aglycone moiety. The ^{1}H-NMR spectrum of **1** displayed terminal vinyl protons [δ 5.21 (1H, d, *J* = 11 Hz), 5.23 (1H, d, *J* = 18 Hz), and 5.93 (1H, dd, *J* = 11, 18 Hz)] and a methyl proton [δ 1.38 (s)] on a quaternary carbon having an oxygen group whose chemical shifts were similar to those of geranyllinalool 3-*O*-β-D-glucopyranoside type.[2)] These ^{1}H- and ^{13}C-NMR signals were assigned by ^{1}H–^{1}H, ^{1}H–^{13}C and ^{1}H–^{13}C long range correlation spectroscopy (COSY) spectra. Therefore, the structure of **1** was expressed as 17-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl-(1→3)-α-L-rhamopyranosyl-6*E*,10*E*,14*Z*-3(*S*)-geranyllinalool-3-*O*-β-D-glucopyranoside.

Capsianoside G (**2**), a white powder, $[\alpha]_D$ −24.2°, showed IR absorption bands due to a hydroxy group (3440 cm^{-1}) and α,β-unsaturated ester carbonyl group (1716 and 1648 cm^{-1}). The negative FAB-MS of **2** exhibited a molecular ion peak $[M-H]^-$ at *m/z* 1401, together with prominent peaks at *m/z* 921 (originated from **1**) and 497 (from **5**). The ^{13}C-NMR spectrum of **2** also suggested the presence of an ester carbonyl group (δ 169.2), therefore **2** was saponified with alkali to give two compounds, **1** and **5**,[2)] which were identified in respect to thin layer chromatography (TLC) and ^{1}H-NMR spectrum. The location of the ester bond between **1** and **5** was determined by comparing the ^{1}H- and ^{13}C-NMR spectra of **2** with those of **1** and capsianoside A (**6**).[2)] Namely, signals due to H-1, H-2 and H-3 of the terminal rhamnosyl moiety in **1** appeared at δ 4.90 (1H, s), 4.98 (1H, d, *J* = 3 Hz) and 3.89 (1H, dd, *J* = 3, 10 Hz), respectively, which showed acylation shifts and whose chemical shifts were coincident with those of **6**. Moreover, a signal pattern due to the aglycone was the same as that of **6**. This meant that **2** possessed an ester linkage between the hydroxyl group at C-2 of the terminal rhamnosyl residue in **1** and the 16-oic acid group in **5**. The ^{13}C-NMR and ^{1}H–^{1}H, ^{1}H–^{13}C-COSY data were also consistent with the above result. Consequently, capsianoside G (**2**) could be represented as shown in the formula.

Capsianoside H (**3**), a white powder, $[\alpha]_D$ −22.0°, showed absorption bands at 3436 cm^{-1} (OH), 1716, 1646 cm^{-1} (α,β-unsaturated ester carbonyl group) in its IR spectrum and peaks at *m/z* 1401 $[M-H]^-$, 921, 775, 629 and 497 in the negative FAB-MS, whose pattern was superimposable on that of **2**, indicating **3** to be an isomer of **2** such as the relation between capsianoside A (**6**) and B (**7**).[2)] Saponification of **3** with alkali gave **1** and **5** similarly to the case of **2**. In comparing the ^{1}H-NMR spectrum of **3** with the spectra of **1**, **2** and **7**, signals due to the H-3 of the terminal rhamnosyl moiety in **3** showed an acylation shift to δ 4.99 (dd, *J* = 3, 10 Hz) together with shifts of H-1 (δ 4.92, s) and H-2 (δ 4.02, d, *J* = 3 Hz). This observation was quite similar to the case of **7**. Meanwhile signals due

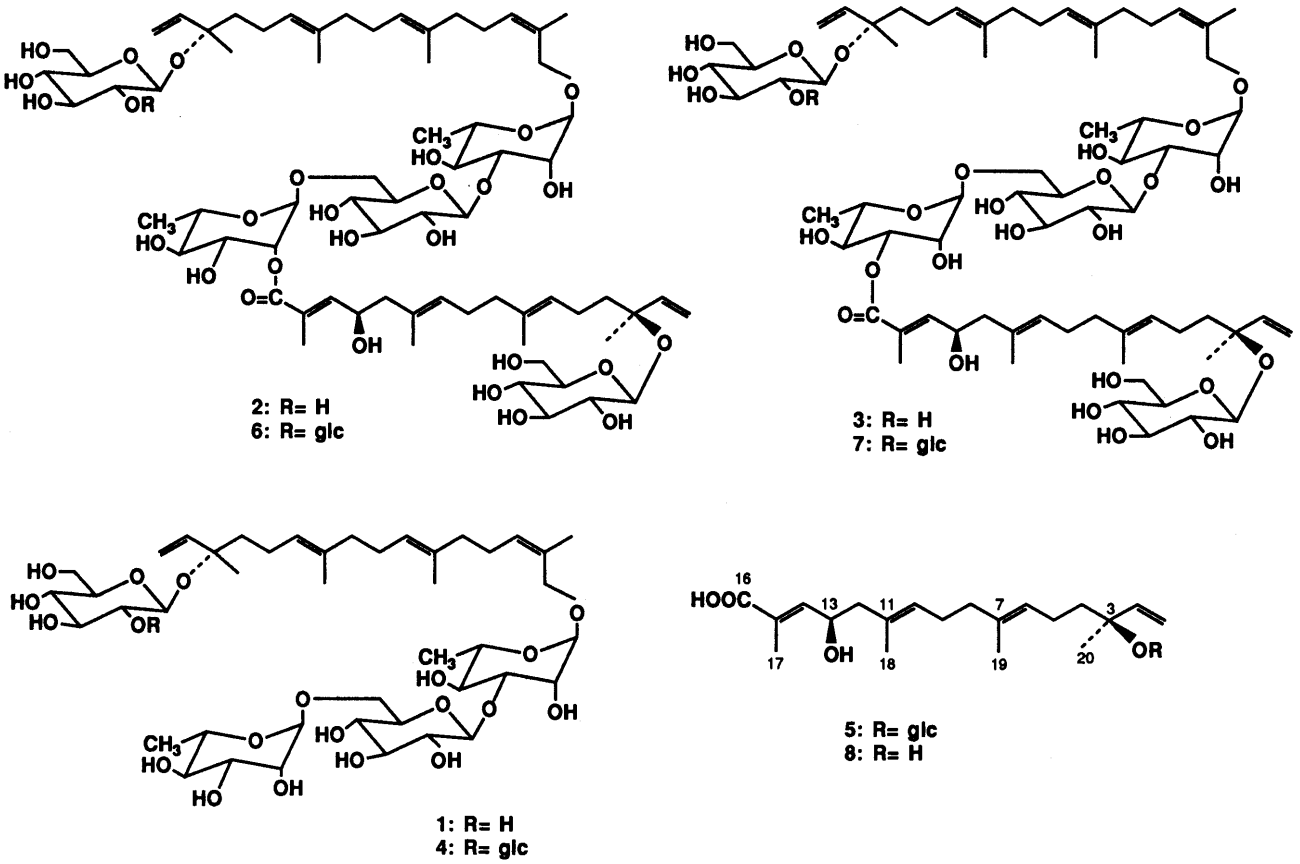

Fig. 1

to the aglycone were the same as those of **7**. From the above evidence, **3** was regarded to be a compound possessing an ester linkage between the C-3-OH group of the terminal rhamnosyl residue of **1** and the 16-oic acid group of **5**. The ^{13}C-NMR and ^{1}H–^{13}C COSY data also supported this structure. When a solution of **2** and **3** in dil. MeOH was heated at 70 °C for 1 h, an acyl migration took place to yield a mixture of **2** and **3** in each case. Therefore, capsianoside H (**3**) could be represented as shown in the formula.

The chirality at C-13 of the aglycone (**8**)[2] derived from **5** was elucidated by ^{1}H-NMR analysis of the optically active α-methoxy-α-(trifluoromethyl)phenylacetate (MTPA ester)[3] of **8**. The H-13, H-14 and H_3-17 proton signals had higher chemical shifts in the (*R*)-ester compound than those in the (*S*)-ester one, and the H-10, H_2-12 and H_3-18 signals had lower chemical shifts in the (*R*)-ester compound than those in the (*S*)-ester compound of **8** in the ^{1}H-NMR spectra, thus the C-13 configuration was determined as *R*.

These acyclic diterpene glycosides occur rarely in nature and these compounds were isolated from fruits, leaves and stems, but the glycosides were not isolated from roots and seeds.

Experimental

The instruments used to obtain physical and spectral data and the experimental conditions for chromatography was the same as described in our previous paper.[2]

Isolation *Capsicum annuum* L. var. *fasciculatum* BAILEY (Yatsubusa): The fresh leaves and stems (2 kg) were extracted with MeOH, and the extract was evaporated under reduced pressure to afford a residue (90 g). The H_2O soluble portion (52 g) of the MeOH extract was subjected to column chromatographies of MCI gel CHP 20P (eluted with H_2O, 20%, 40%, 60%, 80%, 100% MeOH gradiently), silica gel (eluted with $CHCl_3$: MeOH : H_2O = 7 : 3.5 : 0.5) and Bondapak C_{18} (eluted with 70% MeOH) to furnish capsianosides A (20 mg), B (11 mg), C (7 mg), D (5 mg), G (27 mg), H, (12 mg), II (47 mg) and VI (30 mg).

C. annuum L. var. *conoides* BAILEY (Takanotsume): The fresh leaves and stems (2.3 kg) were extracted with MeOH, and the extract (150 g) was separated similarly to above to afford capsianosides A (102 mg), B (56 mg), C (110 mg), D (70 mg), G (68 mg), H (46 mg) and II (41 mg).

Capsianoside VI (1) A white power, $[\alpha]_D^{18}$ −36.2° (*c* = 0.55, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3450 (OH). Positive FAB-MS *m/z*: 945.4660 ($[M+Na]^+$, $C_{44}H_{74}O_{20}Na$, requires: 945.4671). Negative FAB-MS *m/z*: 921 $[M-H]^-$, 775, 759, 629, 613. ^{1}H-NMR (CD_3OD) δ: 1.26, 1.27 (each 3H, d, *J* = 6 Hz, rha H_3-6), 1.38, 1.77 (each 3H, s, H_3-20, 16), 1.60 (6H, s, H_3-18, 19), 1.60 (2H, m, H_2-4), 1.95—2.20 (10H, m, H_2-5, 8, 9, 12, 13), 3.15—3.85 (m, sugar-H), 3.92 (1H, d, *J* = 10 Hz, glc H-6), 4.12, 4.33 (each 1H, d, *J* = 12 Hz, H_2-17), 4.20, 4.35 (each 1H, d, *J* = 8 Hz, glc H-1), 4.71, 4.82 (1H, s, rha H-1), 5.12 (2H, br t, *J* = 7 Hz, H-6, 10), 5.21 (1H, d, *J* = 11 Hz, H-1), 5.23 (1H, d, *J* = 18 Hz, H-1), 5.40 (1H, t, *J* = 7 Hz, H-14), 5.93 (1H, dd, *J* = 11, 18 Hz, H-2). ^{13}C-NMR (CD_3OD) δ: 115.9, 144.4, 81.5, 42.6, 23.5, 125.9, 135.9, 40.7, 27.6, 125.8, 135.4, 40.9, 27.2, 131.2, 132.4, 21.9, 67.7, 16.3, 16.1, 23.2 (C-1—20), 101.5, 72.2, 79.1, 73.7, 70.5, 17.9 (inner rha C-1—6), 102.1, 75.1, 76.7, 72.3, 75.3, 66.8 (glc C-1—6), 102.6, 72.2, 72.4, 73.8, 69.8, 18.2 (terminal rha C-1—6), 99.5, 75.2, 78.2, 71.6, 77.5, 62.8 (C-3 glc C-1—6).

Acid Hydrolysis of 1 A solution of **1** (5 mg) in 1 N HCl (1 ml) was heated at 80 °C for 2 h on a hot bath to furnish glucose and rhamnose detected by TLC ($CHCl_3$: MeOH : acetone : H_2O = 3 : 3 : 3 : 1).

Capsianoside G (2) A white powder, $[\alpha]_D^{20}$ −24.2° (*c* = 1.00, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3440 (OH), 1716, 1648 (α,β-unsaturated ester group). Positive FAB-MS *m/z*: 1425.7400 ($[M+Na]^+$, $C_{70}H_{114}O_{28}Na$, requires: 1425.7390). Negative FAB-MS *m/z*: 1401 $[M-H]^-$, 921, 775, 629, 497, 479. ^{1}H-NMR (CD_3OD) δ: 1.26, 1.28 (each 3H, d, *J* = 6 Hz, rha H_3-6), 1.38 (6H, s, H_3-20, 20'), 1.59, 1.60, 1.61, 1.66, 1.77, 1.89 (each 3H, s, H_3-19, 19', 18, 18', 16', 17), 1.60 (4H, m, H_2-4, 4'), 2.00—2.16 (17H, m, H_2-5, 8, 9, 5', 8', 9', 12', 13', H-12), 2.29 (1H, dd, *J* = 7, 13 Hz, H-12), 3.15—3.80 (m, sugar H), 3.89 (1H, dd, *J* = 3, 10 Hz, rha H-3), 3.96 (1H, d, *J* = 10 Hz, glc H-6), 4.11, 4.33 (each 1H, d, *J* = 11 Hz, H-17'), 4.20 (1H, d, *J* = 8 Hz, C-17'-O-glc H-1), 4.36 (2H, d, *J* = 8 Hz, C-3, 3'-O-glc H-1), 4.54 (1H, m, H-13), 4,81, 4.90 (each 1H, s, rha H-1), 4.98 (1H, d, *J* = 3 Hz, rha H-2),

5.12 (3H, br s, H-6, 6', 10'), 5.25 (5H, m, H_2-1, 1', H-10), 5.40 (1H, t, J=6 Hz, H-14'), 5.93 (2H, dd, J=11, 18 Hz, H-2, 2'), 6.71 (1H, dd, J=1.5, 9 Hz, H-14). ^{13}C-NMR (CD_3OD) δ: 115.9, 144.5, 81.5, 42.6, 23.6, 125.9, 135.9, 40.6, 24.8, 129.3, 131.8, 47.8, 68.0, 145.9, 128.9, 169.2, 13.1, 16.8, 16.2, 23.3 (C-1—20), 115.9, 144.5, 81.5, 42.7, 23.6, 125.9, 135.9, 40.8, 27.6, 125.8, 135.5, 40.9, 27.3, 131.3, 132.4, 21.9, 67.6, 16.3, 16.2, 23.3 (C-1'—20'), 99.5, 75.2, 78.3, 71.7, 77.6, 62.8 (each 2C, C-3, 3' glc C-1—6), 101.0, 72.3, 78.5, 74.1, 70.3, 18.2 (innar rha C-1—6), 102.2, 75.0, 76.5, 72.4, 75.5, 66.1 (glc C-1—6), 99.3, 75.0, 70.3, 74.4, 69.6, 18.9 (terminal rha C-1—6).

Alkaline Hydrolysis of 2 **2** (10 mg) was dissolved in 0.5 N sodium carbonate solution (1 ml) and the mixture was heated at 60 °C for 30 min. After cooling, the reaction mixture was passed through MCI-gel CHP 20P column and washed with H_2O until it was neutral, and then subsequently eluted with MeOH. The methanolic fraction was evaporated under reduced pressure to afford a residue, which was purified by silica gel column chromatography ($CHCl_3$: MeOH : H_2O = 7 : 3 : 0.5) to give two products identical to **1** (3 mg) and **5** (1 mg) by TLC and ^{1}H-NMR spectrum.

Capsianoside H (3) A white powder, $[\alpha]_D^{20}$ −22.0° (c=1.01, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3436 (OH), 1716, 1646 (α,β-unsaturated ester group). Positive FAB-MS m/z: 1425.7420 ($[M+Na]^+$, $C_{70}H_{114}O_{28}Na$, requires: 1425.7390). Negative FAB-MS m/z: 1401 $[M-H]^-$, 921, 775, 629, 497, 479. ^{1}H-NMR (CD_3OD) δ: 1.26, 1.29 (each 3H, d, J=6 Hz, rha H_3-6), 1.38, 1.59 (each 6H, s, H_3-20, 20' and 19, 19'), 1.60, 1.66, 1.77, 1.89 (each 3H, s, H_3-18, 18', 16', 17), 1.60 (4H, m, H_2-4, 4'), 1.97—2.20 (17H, m, H_2-5, 8, 9, 5', 8', 9', 12', 13', H-12), 2.27 (1H, dd, J=7, 13 Hz, H-12), 3.15—3.90 (m, sugar-H), 3.94 (1H, d, J=11 Hz, glc H-6), 4.02 (1H, d, J=3 Hz, rha H-2), 4.14, 4.32 (each 1H, d, J=11 Hz, H_2-17), 4.23 (1H, d, J=8 Hz, C-17-O-glc H-1), 4.35 (2H, d, J=8 Hz, C-3, 3'-O-glc H-1), 4.54 (1H, m, H-13), 4.81, 4.92 (each 1H, s, rha H-1), 4.99 (1H, dd, J=3, 10 Hz, rha H-3), 5.12 (3H, m, H-6, 6', 10'), 5.22 (5H, m, H_2-1, 1', H-10), 5.40 (1H, t, J=7 Hz, H-14'), 5.93 (2H, dd, J=11, 18 Hz, H-2, 2'), 6.74 (1H, dd, J=1.3, 9 Hz, H-14). ^{13}C-NMR (CD_3OD) δ: 115.8, 144.4, 81.5, 42.6, 23.6, 125.8, 135.4, 40.5, 27.6, 129.1, 131.8, 47.8, 68.0, 145.4, 128.9, 169.3, 13.1, 16.7, 16.2, 23.3 (C-1—20), 115.8, 144.4, 81.5, 42.7, 23.6, 125.9, 135.9, 40.8, 27.7, 125.8, 135.4, 40.9, 27.3, 131.2, 132.4, 21.9, 67.8, 16.3, 16.3, 23.3 (C-1'—20'), 99.5, 75.2, 78.2, 71.7, 77.5, 62.8 (each 2C, C-3, 3' glc C-1—6), 101.5, 72.2, 79.1, 74.0, 70.8, 17.9 (inner rha C-1—6), 102.2, 75.2, 76.7, 72.3, 75.3, 66.8 (glc C-1—6), 102.7, 70.2, 75.8, 71.7, 69.8, 18.2 (terminal rha C-1—6).

Alkaline Hydrolysis of 3 A solution (1 ml) of **3** (10 mg) in 0.5N sodium carbonate was heated at 60 °C for 30 min and passed through MCI-gel CHP 20P eluted with H_2O and MeOH successively. The solvent of the methanolic eluate was removed by reduced pressure to give a residue, which was subjected to silica gel ($CHCl_3$: MeOH : H_2O = 7 : 3 : 0.5) column chromatography to give two products identical to **1** (4 mg) and **5** (2 mg) by TLC and ^{1}H-NMR spectrum.

***(R)*-(+)-MTRA Ester of 8** **8** (50 mg) was treated with (+)-(*S*)-methoxy(trifluoromethyl)phenylacetyl chloride (100 mg) in dry pyridine (400 μl) overnight at room temperature. After removal of the solvent, the residue was purified by silica gel chromatography with $CHCl_3$–MeOH (25 : 1) to give (*R*)-(+)-MTPA ester (10 mg). ^{1}H-NMR ($CDCl_3$) δ: 1.29 (H_3-20), 1.55 (H_3-19), 1.58 (H_3-18), 1.95 (H_3-17), 2.25 (1H, dd, J=6, 14 Hz, H-12), 2.42 (1H, dd, J=7, 14 Hz, H-12), 3.52 (3H, s, OMe), 5.06 (1H, d, J=11 Hz, H-1), 5.08 (1H, br t, J=7 Hz, H-6), 5.21 (1H, d, J=18 Hz, H-1), 5.10 (1H, br t, J=7 Hz, H-10), 5.85 (1H, ddd, J=6, 7, 8 Hz, H-13), 5.94 (1H, dd, J=11, 18 Hz, H-2), 6.69 (1H, d, J=8 Hz, H-14), 7.48 (3H, br s, Ar-H), 7.49 (2H, br s, Ar-H).

***(S)*-(−)-MTPA Ester of 8** **8** (50 mg) was treated with (−)-(*R*)-methoxy-(trifluoromethyl)phenylacetyl chloride (100 mg) in dry pyridine (400 μl) under the same conditions as above to give (*S*)-(−)-MTPA ester (10 mg). ^{1}H-NMR ($CDCl_3$) δ: 1.29 (H_3-20), 1.56 (H_3-19), 1.66 (H_3-18), 1.94 (H_3-17), 2.30 (1H, dd, J=6, 14 Hz, H-12), 2.49 (1H, dd, J=7, 14 Hz, H-12), 3.53 (3H, s, OMe), 5.06 (1H, d, J=11 Hz, H-1), 5.08 (1H, br t, J=7 Hz, H-6), 5.19 (1H, br t, J=7 Hz, H-10), 5.22 (1H, d, J=18 Hz, H-1), 5.83 (1H, ddd, J=6, 7, 8 Hz, H-13), 5.91 (1H, dd, J=11, 18 Hz, H-2), 6.56 (1H, d, J=8 Hz, H-14), 7.46 (3H, br s, Ar-H), 7.48 (2H, br s, Ar-H).

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Iridoid Glycosides from *Verbascum thapsus* L.

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Five novel iridoid glycosides were isolated from the fresh whole plants of *Verbascum thapsus* L. (Scrophulariaceae). The structures of these iridoid glycosides were determined on the basis of spectral and chemical evidence. These compounds were classified into two types: one iridoid glycoside contains ajugol, and the others contain 6-*O*-(α-L-rhamnopyranosyl)-catalpol in the structures.

Keywords *Verbascum thapsus*; Scrophulariaceae; iridoid glycoside; ajugol; 6-*O*-(α-L-rhamnopyranosyl)-catalpol

Verbascum thapsus L. (Scrophulariaceae) is indigenous to Europe and ranges widely throughout Europe, Asia, Northern America and Northern Africa.[1] Since some phytochemical investigations have already been reported on this plant, we also decided to investigate its constituents in the course of our search for iridoid glycosides of Scrophulariaceae plants. This paper describes the isolation and structural determination of 5 new iridoid glycosides along with 18 known ones (**1**[1]; **2**[2]; **3**, **4**[3]; **6**[5]; **7**[6]; **8**[7]; **9**, **10**[8]; **11**[9]; **12**[10]; **13**[8]; **14**, **15**[11]; **17**, **18**[12]; **19**, **20**[13]).

Water extract from the fresh whole plant was passed through a Mitsubishi Diaion HP-20 column, and the absorbed material was eluted with methanol. The methanol eluate was rechromatographed on a Toyopearl HW-40C column, and secondly on a SiO_2 column to give iridoid glycoside fractions. Five new compounds were obtained from these fractions and they were classified into two groups: ajugol type (**5**) and catalpol type (**16** and **21**—**23**).

The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of compound **5** showed the signals of two carbinyl protons [δ 5.04 (1H, m), 5.51 (1H, d, J=2.5 Hz)], two *cis*-olefinic protons [δ 5.00 (1H, dd, J=6, 2 Hz), 6.24 (1H, dd, J=6, 2 Hz)] and two methylene protons [δ 2.06 (1H, dd, J=14, 3.5 Hz), 2.28 (1H, dd, J=14, 6 Hz)]. The carbon-13 nucelar magnetic resonance (^{13}C-NMR) spectrum also exhibited the signals of the carbinyl (δ 79.2, 80.8) and two olefinic carbons (δ 104.5, 141.2) in the aglycone moiety. The ^{1}H- and ^{13}C-NMR spectra were very similar to those of adjugol (**24**)[4] except for the signals arising from a benzoic acid derivative. In the ^{13}C-NMR spectrum of **5**, the signal due to C-6 of the aglycone moiety (δ 80.8) shifted downfield (Δ +2.6 ppm) and the signals due to C-5 and C-3 shifted upfield (Δ −1.8 ppm) and Δ −2.3 ppm, respectively) by comparison with those of ajugol (**24**). In the decoupling experiment, by irradiation of signals due to H-7 at δ 2.06 and 2.28, the signal due to H-6 was found to have shifted downfield to δ 5.04, which was considered an acylation shift. Thus, it is suggested that a benzoic acid derivative was attached to the C-6 position of ajugol (**24**). Regarding a benzoic acid derivative, the ^{1}H-NMR spectrum, showed two equivalent aromatic proton signals at δ 7.35 (2H, s) and a methoxyl proton signal at δ 3.89 (6H, s). The ^{13}C-NMR spectrum exhibited four aromatic carbon signals (δ 108.3, 121.6, 142.0, 148.9), a carbonyl carbon signal (δ 167.9) and a methoxyl group signal [δ 56.8 (2C)]. These explained that one benzoic acid derivative is syringic acid. Hydrolized with 0.2% sodium hydroxide to confirm the aglycone and the ester moiety, compound **5** afforded syringic acid and ajugol which were identified with authentic samples using high performance liquid chromatography (HPLC). Accordingly, the structure of **5** is determined to be 6-*O*-syringoyl-ajugol.

The ^{1}H- and ^{13}C-NMR spectra of compound **16** showed that **16** was an iridoid glycoside which had a cinnamic acid derivative in its structure. This cinnamic acid derivative was presumed to be caffeic acid, because two *trans*-olefinic proton signals [δ 6.32 (1H, d, J=16 Hz), 7.59 (1H, d, J=16 Hz)] and ABC-type proton signals [δ 6.79 (1H, d, J=8.5 Hz), 6.97 (1H, dd, J=8.5, 1.5 Hz), 7.07 (1H, d, J=1.5 Hz)] were in the ^{1}H-NMR spectrum of **16**. Moreover, the ^{13}C-NMR data of this ester moiety corresponded to that reported for caffeoyl ester.[9] Mild alkaline hydrolysis

TABLE I. ^{13}C-NMR Spectral Data of Compounds **5**, **16** and **21**—**23**

Carbon No.	5	16	21	22	23
Aglycone moiety					
1	93.4	95.1	95.1	95.1	95.1
3	141.2	142.3	142.3	142.2	142.2
4	104.5	103.4	103.4	103.4	103.5
5	39.5	37.2	37.3	37.2	37.1
6	80.8	84.1	84.1	84.3	83.7
7	47.7	59.5	59.4	59.4	59.3
8	79.2	66.5	66.5	66.5	66.5
9	51.7	43.2	43.2	43.2	43.2
10	26.2	61.4	61.4	61.4	61.4
Sugar moiety					
Glc 1	99.4	99.7	99.7	99.7	99.7
Glc 2	74.7	74.8	74.7	74.8	74.7
Glc 3	78.0	77.6	77.6	77.6	77.6
Glc 4	71.7	71.7	71.6	71.7	71.7
Glc 5	78.2	78.5	78.5	78.6	78.5
Glc 6	62.8	62.9	62.9	62.9	62.9
Rham 1		100.4	100.4	97.7	100.1
Rham 2		72.4	72.4	74.2	70.3
Rham 3		70.2	70.2	70.5[a]	75.3
Rham 4		75.2	75.3	74.2	71.3
Rham 5		68.3	68.2	70.3[a]	70.3
Rham 6		17.9	17.9	18.0	18.0
Ester moiety					
α	167.9	168.8	168.7	168.4	168.6
β		115.0[a]	116.0	116.1	116.6
γ		147.3	146.8	147.0	146.6
1	121.6	127.7	128.8	128.7	128.8
2	108.3	115.2[a]	112.4	111.4	111.5
3	148.9	149.5	151.5	152.9	152.7
4	142.0	146.7	147.9	150.7	150.6
5		116.5	114.7	112.5	112.6
6		123.1	122.9	124.2	123.9
OMe	56.8		56.4	56.5[b]	56.5[a]
OMe′				56.4[b]	56.4[a]

Run at 67.80 MHz in CD_3OD solution. *a, b*) Assignments may be interchanged in each column.

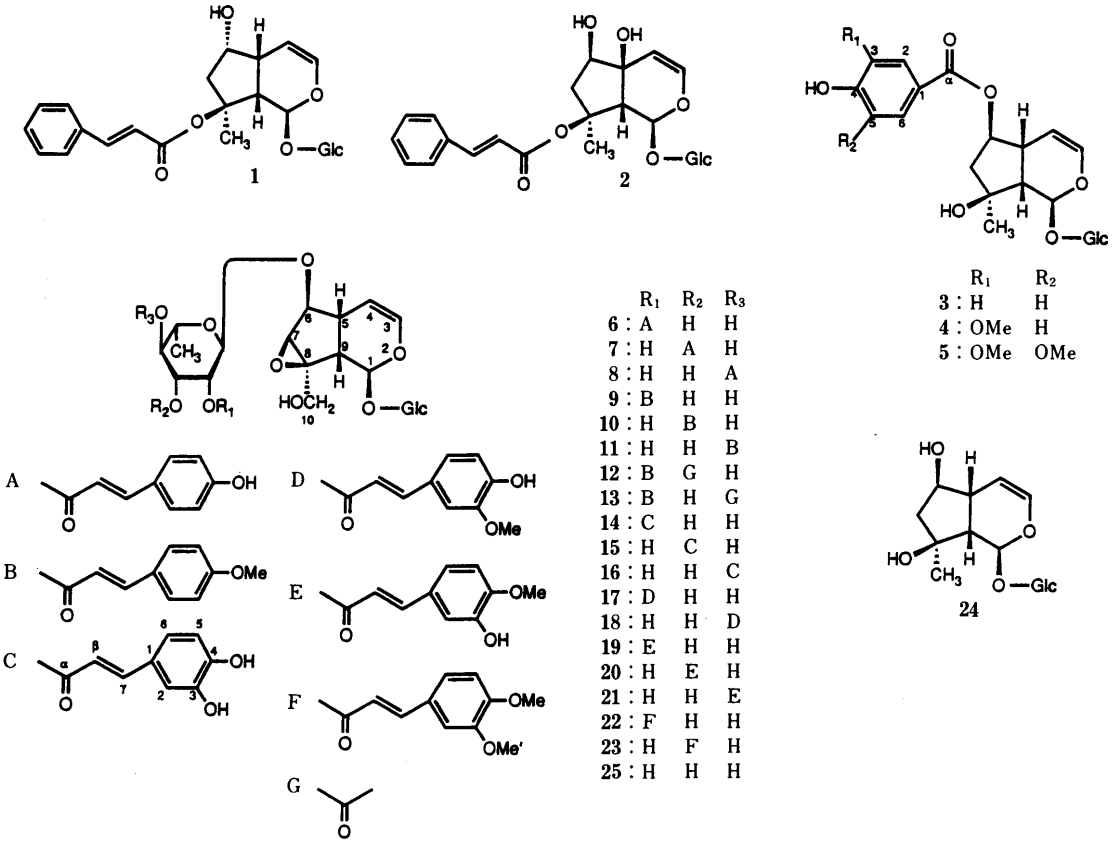

Chart 1

TABLE II. ^{1}H-NMR Spectral Data of Compounds **5**, **16**, **21**—**23** and **25**

Proton No.	5	16	21	22	23	25
Aglycone moiety						
1	5.51 (1H, d, J=2.5 Hz)	5.08 (1H, d, J=9.5 Hz)	5.09 (1H, d, J=10 Hz)	5.07 (1H, d, J=9.5 Hz)	5.09 (1H, d, J=9.5 Hz)	5.07 (1H, d, J=9.5 Hz)
3	6.24 (1H, dd, J=6, 2 Hz)	6.38 (1H, dd, J=6, 1.5 Hz)	6.38 (1H, dd, J=6, 1.5 Hz)	6.37 (1H, dd, J=6, 2 Hz)	6.38 (1H, dd, J=6, 2 Hz)	6.36 (1H, dd, J=6, 2 Hz)
4	5.00 (1H, dd, J=6, 2 Hz)	5.05 (1H, dd, J=6, 4 Hz)	5.06 (1H, dd, J=6, 4.5 Hz)	5.07 (1H, dd, J=6, 4.5 Hz)	5.02 (1H, dd, J=6, 4 Hz)	5.06 (1H, dd, J=6, 4.5 Hz)
5	3.00 (1H, m)	2.42 (1H, m)	2.42 (1H, m)	2.43 (1H, m)	2.46 (1H, m)	2.39 (1H, m)
6	5.04 (1H, m)	4.01 (1H, d, J=8 Hz)	4.02 (1H, d, J=8 Hz)	4.02 (1H, d, J=8 Hz)	4.04 (1H, d, J=8 Hz)	4.00 (1H, d, J=8 Hz)
7	2.06 (1H, dd, J=14, 3.5 Hz)	3.65 (1H, brs)	3.65 (1H, brs)	3.65 (1H, brs)	3.65 (1H, brs)	3.64 (1H, brs)
7′	2.28 (1H, dd, J=14, 6 Hz)					
9	2.62 (1H, dd, J=9, 2.5 Hz)	2.57 (1H, dd, J=9.5, 7.5 Hz)	2.56 (1H, dd, J=9.5, 8 Hz)	2.56 (1H, dd, J=9.5, 7.5 Hz)	2.57 (1H, dd, J=9.5, 7.5 Hz)	2.55 (1H, dd, J=9.5, 8 Hz)
10	1.41 (3H, s)	3.82 (1H, d, J=13 Hz)	3.81 (1H, d, J=13 Hz)	3.80 (1H, d, J=13 Hz)	3.82 (1H, d, J=13 Hz)	3.81 (1H, d, J=13.5 Hz)
10′		4.16 (1H, d, J=13 Hz)	4.15 (1H, d, J=13 Hz)	4.14 (1H, d, J=13 Hz)	4.15 (1H, d, J=13 Hz)	4.13 (1H, d, J=13.5 Hz)
Ester moiety						
β		6.32 (1H, d, J=16 Hz)	6.36 (1H, d, J=16 Hz)	6.48 (1H, d, J=16 Hz)	6.48 (1H, d, J=16 Hz)	
γ		7.59 (1H, d, J=16 Hz)	7.61 (1H, d, J=16 Hz)	7.66 (1H, d, J=16 Hz)	7.71 (1H, d, J=16 Hz)	
2	7.35 (2H, s)	7.07 (1H, d, J=1.5 Hz)	7.10 (1H, d, J=1.5 Hz)	7.22 (1H, d, J=1.5 Hz)	7.21 (1H, d, J=2 Hz)	
3						
5		6.79 (1H, d, J=8.5 Hz)	6.94 (1H, d, J=8 Hz)	6.96 (1H, d, J=8 Hz)	6.98 (1H, d, J=8.5 Hz)	
6		6.97 (1H, dd, J=8.5, 1.5 Hz)	7.07 (1H, dd, J=8, 1.5 Hz)	7.17 (1H, dd, J=8, 1.5 Hz)	7.18 (1H, dd, J=8.5, 2 Hz)	
OMe	3.89 (6H, s)		3.88 (3H, s)	3.86 (3H, s)	3.87 (3H, s)	
OMe′				3.86 (3H, s)	3.87 (3H, s)	

Run at 270.0 or 500.0 MHz in CD_3OD solution.

of **16** afforded 6-*O*-(α-L-rhamnopyranosyl)-catalpol (**25**) and caffeic acid, which were identified with authentic samples on HPLC. By comparison with the ^{13}C-NMR spectrum of 6-*O*-(α-L-rhamnopyranosyl)-catalpol (**25**), an acylation shift was observed at the C-3, C-4 and C-5 positions of the rhamnopyranose moiety in **16** (C-3; Δ −2.0 ppm, C-4; Δ +1.4 ppm, C-5; Δ −1.8 ppm). The signal due to H-4 of rhamnopyranose was shown at δ 5.07 (1H, t, J=9 Hz), which shifted downfield by comparison with that of **25**. These established that the position of a caffeoyl ester in **16** is at C-4 of rhamnopyranose.

Compound **21** showed an $(M+H)^+$ ion peak at m/z 685

TABLE III. ^{1}H-NMR Spectral Data of Compounds **5**, **16**, **21**—**23** and **25**

Proton No.	5	16	21	22	23	25
Sugar moiety						
Glc 1	4.67 (1H, d, J=8 Hz)	4.79 (1H, d, J=8 Hz)	4.78 (1H, d, J=8 Hz)	4.78 (1H, d, J=8 Hz)	4.78 (1H, d, J=8 Hz)	4.77 (1H, d, J=8 Hz)
Glc 2	3.19[a] (1H, t, J=8 Hz)	3.28 (1H, t, J=8 Hz)	3.36[a] (1H, t, J=8 Hz)	3.26 (1H, t, J=8 Hz)	3.25[a] (1H, t, J=8 Hz)	3.25 (1H, t, J=8 Hz)
Glc 3	3.37 (1H, t, J=8 Hz)	3.42 (1H, t, J=8 Hz)	3.40 (1H, t, J=8 Hz)	3.41 (1H, t, J=8 Hz)	3.40 (1H, t, J=8 Hz)	3.40 (1H, t, J=8 Hz)
Glc 4	3.22[a] (1H, t, J=8 Hz)	3.28 (1H, t, J=8 Hz)	3.37[a] (1H, t, J=8 Hz)	3.26 (1H, t, J=8 Hz)	3.26[a] (1H, t, J=8 Hz)	3.25 (1H, t, J=8 Hz)
Glc 5	3.30 ( )[c]	3.33 (1H, m)	3.32 ( )[c]	3.30 ( )[c]	3.33 ( )[c]	3.33 ( )[c]
Glc 6	3.64 (1H, dd. J=11.5, 5.5 Hz)	3.64 (1H, dd, J=11.5, 6 Hz)	3.63 (1H, dd, J=11.5, 6.5 Hz)	3.63 (1H, dd, J=12, 6 Hz)	3.63 (1H, dd, J=12, 6.5 Hz)	3.62 (1H, dd, J=12, 6.5 Hz)
Glc 6′	3.90 ( )[c]	3.90 ( )[c]	3.91 ( )[c]	3.87 (1H, dd, J=12, 2 Hz)	3.92 (1H, dd, J=12, 2 Hz)	3.91 (1H, dd, J=12, 2 Hz)
Rham 1		5.00 (1H, br s)	5.09 (1H, br s)	5.03 (1H, d, J=1.5 Hz)	4.97 (1H, d, J=1.5 Hz)	4.93 (1H, d, J=1.5 Hz)
Rham 2		3.94 (1H, br d, J=3.5 Hz)	3.92[b] ( )[c]	5.16 (1H, dd, J=3.5, 1.5 Hz)	4.09 (1H, dd, J=3.5, 1.5 Hz)	3.86 (1H, dd, J=4, 1.5 Hz)
Rham 3		3.93 (1H, dd, J=9, 3.5 Hz)	3.91[b] ( )[c]	3.93 (1H, dd, J=9.5, 3.5 Hz)	5.11 (1H, dd, J=9.5, 3.5 Hz)	3.68 (1H, dd, J=9.5, 3.5 Hz)
Rham 4		5.07 (1H, t, J=9 Hz)	5.08 (1H, t, J=9 Hz)	3.49 (1H, t, J=9.5 Hz)	3.68 (1H, t, J=9.5 Hz)	3.40 (1H, t, J=9.5 Hz)
Rham 5		3.89 (1H, m)	3.91[b] ( )[c]	3.76 (1H, m)	3.82 (1H, m)	3.66 (1H, m)
Rham 6		1.18 (3H, d, J=6 Hz)	1.16 (3H, d, J=6 Hz)	1.31 (3H, d, J=6 Hz)	1.31 (3H, d, J=6 Hz)	1.26 (3H, d, J=6 Hz)

Run at 270.0 or 500.0 MHz in CD_3OD solution. *a, b*) Assignment may be interchanged in each column. *c*) Obscured by other signals; therefore, the coupling could not be accurately determined.

in the positive ion fast atom bombardment mass (FAB-MS) spectrum, which was larger by 14 mass units than that of **16**. This difference derived from the ester moiety. The ^{1}H-NMR spectrum of the ester moiety in **21** exhibited two *trans*-olefinic proton signals [δ 6.36 (1H, d, J=16 Hz), 7.61 (1H, d, J=16 Hz)], ABC-type signals in the aromatic proton region [δ 6.94 (1H, d, J=8 Hz), 7.07 (1H, dd, J=8, 1.5 Hz), 7.10 (1H, d, J=1.5 Hz)] and a methoxyl proton signal [δ 3.88 (3H, s)]. Because this data identified with that of compounds **19** and **20**,[11] this ester moiety was considered to be isoferulic acid, which was confirmed by mild hydrolysis with authentic samples. The esterified position is determined to be at C-4 of rhamnopyranose, an acylation shift being observed at C-3, C-4 and C-5 of the rhamnopyranose moiety in the ^{13}C-NMR spectrum of **21** as in **16**.

Both compounds **22** and **23** showed an $(M+H)^+$ ion peak at m/z 699, which was larger by 28 mass units than that of **16**. In addition, because the ^{13}C-NMR spectra of compounds **22** and **23** were very similar each other except for the rhamnopyranose moiety, these compounds were positional isomers as to the ester function. According to the ^{1}H- and ^{13}C-NMR spectra of rhamnopyranose in **22** and **23** compared with those in **9** and **10**, the attached positions of the ester chain in **22** and **23** are determined to be at the C-2 and C-3 position of rhamnopyranose moiety, respectively. Regarding the ester moiety, the ^{13}C-NMR spectra of **22** and **23** exhibited two methoxyl group signals at δ 56.4 and 56.5. Thus, the ester moieties of **22** and **23** were deduced to be 3,4-dimethoxycinnamic acid, which was also supported by the ^{1}H-NMR and FAB-MS spectra. This ester was confirmed by mild alkaline hydrolysis.

Experimental

Optical rotations were determined with a JASCO-360 digital polarimeter. And ultraviolet (UV) spectra were taken on a Hitachi U-3410 spectrophotometer, FAB-MS spectra on a JEOL JMS-SX102 spectrometer. The ^{1}H- and ^{13}C-NMR were recorded on JEOL GSX-500 (500 and 125.65 MHz, respectively) and JEOL GSX-270 (270 and 68.70 MHz, respectively) spectrometers. Chemical shifts were given on the δ (ppm) scale with tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; m, multiplet; br, broad). Gas chromatography (GC) was run on a Hitachi G-3000 gas chromatograph. HPLC was run on a JASCO system 800 instrument.

Isolation The fresh whole plant of *Verbascum thapsus* L. (6.6 kg), harvested in August in Shizuoka, Japan, was extracted twice with water. The extract was passed through a Mitsubishi Diaion HP-20 column and the MeOH eluate was concentrated under reduce pressure. The residue (80.7 g) was rechromatographed on a Toyopearl HW-40C column using water and MeOH as an eluent. The water eluate consisted of three fractions and the last fraction (23.5 g) was rechromatographed on a SiO_2 column with a $CHCl_3$–MeOH system and semi-preparative HPLC [octadecyl silica (ODS) and PhA: MeOH–H_2O (3 : 7—2 : 3); CH_3CN–H_2O (3 : 17—11 : 29)] to give compound **1** (356 mg), **2** (588 mg), **3** (32 mg), **4** (127 mg), **5** (5 mg), **6** (143 mg), **7** (241 mg), **8** (109 mg), **9** (203 mg), **10** (372 mg), **11** (299 mg), **12** (129 mg), **13** (62 mg), **14** (38 mg), **15** (138 mg), **16** (58 mg), **17** (69 mg), **18** (55 mg), **19** (456 mg), **20** (260 mg), **21** (128 mg), **22** (35 mg) and **23** (57 mg).

6-*O*-Syringoyl-ajugol (**5**): Amorphous powder. $[\alpha]_D^{23}$ −140.7° (c=0.54, MeOH). *Anal.* Calcd for $C_{24}H_{32}O_{13}\cdot 3/2H_2O$: C, 51.89; H, 6.35. Found: C, 51.70; H, 6.28. UV λ_{max}^{MeOH} nm (log ε): 217 (4.54), 277 (4.10). FAB-MS m/z: 551 $(M+Na)^+$, 529 $(M+H)^+$. ^{1}H- and ^{13}C-NMR: Tables I—III.

6-*O*-[α-L-(4-Caffeoyl)-rhamnopyranosyl]-catalpol (**16**): Amorphous powder. $[\alpha]_D^{22}$ −162.8° (c=0.78, MeOH). *Anal.* Calcd for $C_{30}H_{38}O_{17}\cdot 3/2H_2O$: C, 51.65; H, 5.92. Found: C, 51.57; H, 6.03. UV λ_{max}^{MeOH} nm (log ε): 217 (4.23), 235 (sh), 244 (4.06), 301 (sh), 330 (4.30). FAB-MS m/z: 693 $(M+Na)^+$, 671 $(M+H)^+$, ^{1}H- and ^{13}C-NMR: Tables I—III.

6-*O*-[α-L-(4-Isoferuloyl)-rhamnopyranosyl]-catalpol (**21**): Amorphous powder. $[\alpha]_D^{22}$ −139.8° (c=0.54, MeOH), *Anal.* Calcd for $C_{31}H_{40}O_{17}\cdot 3/2H_2O$: C, 52.32; H, 6.09. Found: C, 52.28; H, 6.16. UV λ_{max}^{MeOH} nm (log ε): 216 (4.26), 233 (sh), 242 (4.10), 296 (4.21), 326 (4.27). FAB-MS m/z: 707 $(M+Na)^+$, 685 $(M+H)^+$, ^{1}H- and ^{13}C-NMR: Tables I—III.

6-*O*-[α-L-(2-3,4-Dimethoxycinnamoyl)-rhamnopyranosyl]-catalpol (**22**): Amorphous powder. $[\alpha]_D^{22}$ −126.2° (c=1.05, MeOH). *Anal.* Calcd for $C_{32}H_{42}O_{17}\cdot 5/2H_2O$: C, 51.68; H, 6.37. Found: C, 51.67; H, 6.30. UV λ_{max}^{MeOH} nm (log ε): 217 (sh), 234 (4.15), 297 (4.21), 323 (4.31). FAB-MS m/z: 721 $(M+Na)^+$, 699 $(M+H)^+$. ^{1}H- and ^{13}C-NMR: Tables I—III.

6-*O*-[α-L-(3-3,4-Dimethoxycinnamoyl)-rhamnopyranosyl]-catalpol (**23**): Amorphous power. $[\alpha]_D^{22}$ −94.8° (c=0.67, MeOH). *Anal.* Calcd for $C_{32}H_{42}O_{17}\cdot 3/2H_2O$: C, 52.96; H, 6.25. Found: C, 53.02; H, 6.33. UV λ_{max}^{MeOH} nm (log ε): 231 (sh), 294 (4.12), 321 (4.19). FAB-MS m/z: 721 $(M+Na)^+$, 699 $(M+H)^+$. ^{1}H- and ^{13}C-NMR: Tables I—III.

Mild Alkaline Hydrolysis of Compounds 5, 16 and 21—23 Compound **5** (*ca.* 0.1 mg) in 0.2% NaOH (2 drops) was treated for 5 h at room temperature with stirring under a N_2 gas atmosphere, and compounds **16** and **21**—**23** in 0.05% NaOH (2 drops) were reacted for 4 h under the same conditions. The reaction mixture was passed through an Amberlite IR-120 column, and the MeOH eluate was concentrated under reduced pressure and was partitioned between AcOEt and H_2O. The AcOEt layer and the H_2O layer were concentrated to dryness and the residues were analyzed by HPLC with authentic samples. (Condition: column; ODS. flow rate; 1.3 ml/min. 3% CH_3CN. t_R; 6-*O*-(α-L-rhanmopyranosyl)-catalpol, 8.4 min. 7% CH_3CN. t_R; ajugol, 7.0 min. 17.5% CH_3CN +0.05% trifluoroacetic acid. t_R: syringic acid, 7.2 min, caffeic acid, 6.4 min, isoferulic acid, 14.8 min, 3,4-dimethoxycinnamic acid, 13.8 min.)

Acid Hydrolysis of Compounds 5, 16 and 21—23 A solution of each compound (*ca.* 0.1 mg) in 5% H_2SO_4 (2 drops) was heated at 100 °C for

30 min. The solution was passed through an Amberlite IR-45 column and concentrated to give a residue, which was reduced with $NaBH_4$ (*ca.* 1.0 mg) for 1 h at room temperature. The reaction mixture was passed through an Amberlite IR-120 column and concentrated to dryness. Boric acid was removed by co-distillation with MeOH, and the residue was acetylated with Ac_2O (1 drop) and pyridine (1 drop) at 100 °C for 1 h. The reagents were removed *in vacuo*. Glucitol acetate and rhamnitol acetate were detected by GC from all glycosides. (GC conditions: column; Supelco SP-2380 capillary column (0.25 mm × 30 m), column temperature; 250 °C. carrier gas; N_2. t_R; glucitol acetate, 11.1 min, rhamnitol acetate, 5.0 min.)

Acknowledgment We thank the staff of the Central Analytical Laboratory of this university for elemental analysis and measurement of MS.

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Biologically Active Constituents of *Magnolia salicifolia*: Inhibitiors of Induced Histamine Release from Rat Mast Cells

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The extracts of the flower buds of *Magnolia salicifolia* showed remarkable anti-allergy effects in passive cutaneous anaphylaxis (PCA) test. The bioactive constituents of this medicinal drug were isolated by monitoring their activities with an *in vitro* bioassay system measuring inhibitory effects on induced histamine release from rat mast cells. Of the ten isolated compounds magnosalicin is a new compound of neolignan structure. In addition to the isolated compounds samples of coumarins and lignans were evaluated their biological activities with the *in vitro* bioassay.

Keywords *Magnolia salicifolia*; anti-allergy; PCA; histamine release; mast cell; magnosalicin; coumarin; lignan

Traditional Kampo and Chinese medicines lack the definition of immunology, however patients suffering from allergic diseases are treated with orally administered traditional medicinal drugs. This indicates that medicinal plants used for allergy treatment may contain anti-allergic compounds. Baicalein, a flavonoid, is a good example of an anti-allergic constituent which has been isolated from the roots of *Scutellaria baicalensis*.[1] Since voluminous clinical records have been published in China we made a literature survey to identify the medicinal drugs which might be responsible in anti-allergic action. Medicinal drugs selected by the survey were tested with rat homologous passive cutaneous anaphylaxis (PCA) and the results were reported in a previous paper.[2] We have also used an *in vitro* bioassay to test inhibitory activity against histamine release from mast cells induced by compound 48/80 or concanavalin A (Con A) for evaluating anti-allergy activity during separation and isolation procedure. This *in vitro* bioassay has a good correlation with *in vivo* PCA test.[2] In our studies on the identification and structural elucidation of anti-allergic substances, the active principle of *Citrus* fruit was identified as a polymethoxyflavone, nobiletin, whose mechanism of action was also discussed.[2] Another medicinal plant used for anti-allergic purpose, *Centipeda minima*, contained sesquiterpenes possessing an inhibitory effect against histamine release from mast cells as well as anti-allergy activity in PCA.[3]

The flower buds of *Magnolia salicifolia* (Japanese name, Shin-i) have been used for nose trouble in traditional Kampo medicine. In a preliminary screening study on traditional medicinal drugs the hot aqueous extract of this medicinal drug was highly active in a PCA test and inhibited anaphylaxis response almost 100% at a dose of 100 mg/kg in i.v. administration.[2] The extract also inhibited compound 48/80 induced histamine release from mast cells 45% at a concentration of 50 μg/ml.[2] Based on the results, we first investigated active compounds in the hot aqueous extract. In spite of extensive separation of the extract with HP-20 column using gradient elution of methanol and water, and also with preparative thin-layer chromatography (TLC), adenosine was the sole active compound isolated from the hot aqueous extract. Adenosine showed the maximum activity at a concentration of 10^{-8} M and IC_{50} (50% inhibitory concentration) was *ca.* 10^{-8} M. At higher concentrations the inhibitory activities of adenosine were less than the maximum value (data not shown). At this stage of study we changed our strategy and plant material was extracted successively with hexane, chloroform, methanol and water. The obtained extracts were submitted to a PCA test as well as an *in vitro* inhibition test on histamine release from rat peritoneal mast cells induced by compound 48/80 or Con A. As it appears in Tables I and II the chloroform, methanol and water extracts showed significant anti-allergic activities in the *in vivo* and *in vitro* bioassay systems.

A part of chloroform extract prepared from 5 kg material was separated repeatedly with silica-gel, Lobar RP-8 and

TABLE I. Anti-allergic Activities of *Magnolia salicifolia* Extracts in Rat Homologous Passive Cutaneous Anaphylaxis

Test material	Dose (mg/kg)	Inhibition % (Amount of pigment)	Inhibition % (Spot area of pigment)	$n^{a)}$
Hexane ext.	500 i.p.	10.7±5.8	1.5±6.0	6
Chlololform ext.	500 i.p.	42.2±4.3	28.1±7.8	6
Methanol ext.	500 i.p.	28.1±2.6	20.8±4.7	6
Water ext.	500 i.p.	33.7±8.4	49.5±8.2	8
Water ext.	100 i.p.	22.4±7.0	62.0±3.6	4
DSCG	20 i.p.	89.7±3.1	55.7±0.8	4
DSCG	2 i.v.	43.1±2.6	61.4±2.1	4

a) Number of animal. The results are expressed as mean±S.E.M. DSCG, disodium cromoglycate.

TABLE II. Inhibitory Effects of *Magnolia salicifolia* Extracts on Histamine Release from Rat Mast Cells Induced by Compound 48/80 or Con A

	Inhibition % of histamine release			
	Inducer			
	Compound 48/80		Con A	
	50 μg/ml	500 μg/ml	50 μg/ml	500 μg/ml
Hexane ext.	25	62	0	53
Chlololform ext.	41	90	18	72
Methanol ext.	41	88	53	88
Water ext.	60	95	47	94
DSCG	15	40	14	32
Tranilast	0	18	36	95

DSCG, disodium cromoglycate, Tranilast, 3,4-dimethoxycinnamoyl anthranilic acid.

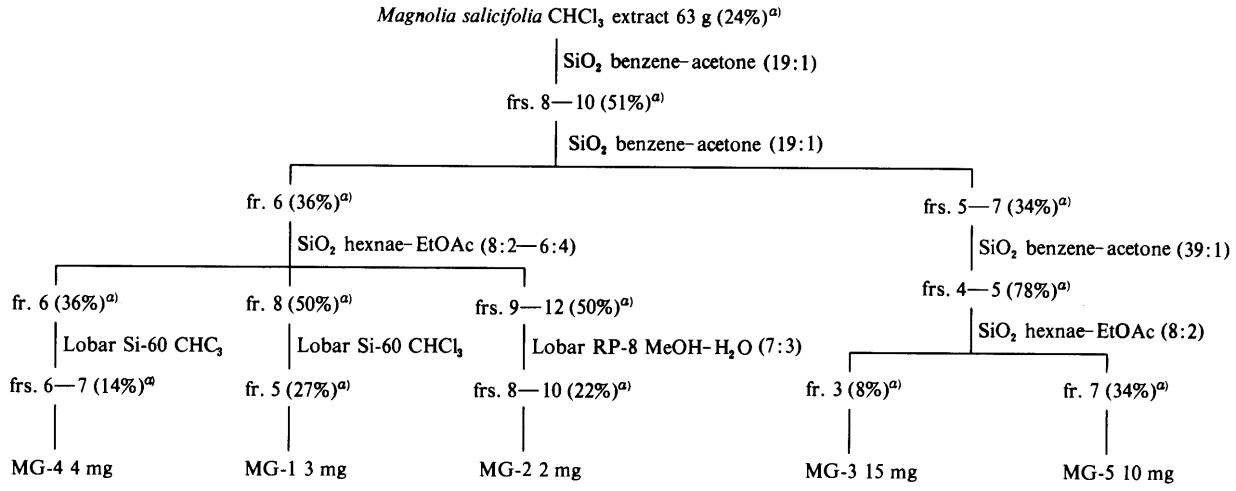
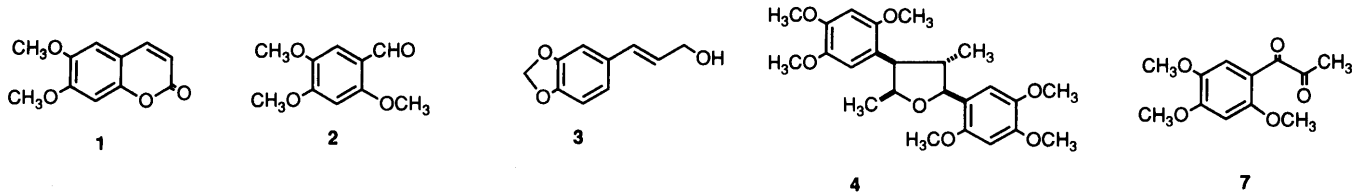


Chart 1. Fractionation, Separation and Isolation of *Magnolia salicifolia* Chloroform Extract

a) Inhibition% for histamine release from mast cells induced by compound 48/80 at a substrate concentration of 50 μg/ml.



Lobar Si-60 column chromatographies by monitoring the inhibitory activity on compound 48/80 induced histamine release from mast cells. Five compounds tentatively designated MG-1—MG-5 were so far isolated from the chloroform extract. (Chart 1) MG-1 was identified as scoparone (**1**) a coumarin.[4] MG-2 and MG-3 were identified as known constituents of *M. salicifolia*, asarylaldehyde (**2**)[5] and 3,4-methylenedioxycinnamyl alcohol (**3**),[5a,6] respectively. The spectral data of MG-4 suggested that it was a new compound named magnosalicin (**4**), whose structure was reported briefly in a previous communication.[7] The molecular formula of MG-4 was given by high resolution mass spectrum (HR-MS) as $C_{24}H_{32}O_7$. The ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrum of magnosalicin gave six singlet signals arising from six methoxy groups and four singlets of aromatic protons. The presence of six methoxy groups in magnosalicin indicated its close biogenetical relationship to known neolignans isolated from *M. salicifolia*, magnosalin (**5**) and magnoshinin (**6**).[8] The structure of magnosalicin however is different from the known neolignans because it contains extra oxygen as an ether, which was indicated by the ^{1}H-NMR signals at δ 4.61 (1H, qd, J=6.5 and 8.5 Hz) and δ 4.98 (1H, d, J=9 Hz). Another two methine signals appeared at δ 3.61 (1H, dd, J=8.5 and 10.5 Hz) and δ 2.32 (1H, m). The NMR data indicated that magnosalicin contained a tetrahydrofuran ring in its structure and a planar structure in Fig. 1 emerged from coupling relations among four methine and two methyl groups, indicating close biogenetical relationships with asarone (Fig. 1). The structure of magnosalicin (**4**) including stereochemistry was unambiguously established by X-ray analysis.[7] Magnosalicin (**4**) gave a crystal suitable for X-ray analysis from methanol with a space group of monoclinic $P2_1/a$ containing four molecules in a unit cell, indicating that magnosalicin (**4**) is a racemate.

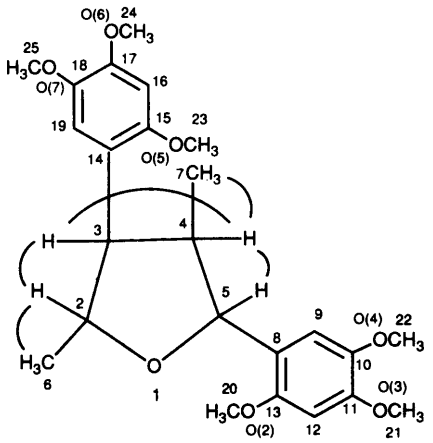


Fig. 1. Planar Structure for Magnosalicin (**4**) and Coupling Relations in ^{1}H-NMR Spectrum

The biogenesis of magnosalin (**5**) and magnoshinin (**6**) are readily explained by the dimerization of α- and β-asarone, respectively. On the contrary, the biogenesis of magnosalicin (**4**) should involve an α-asarone epoxide (**7**) and dimerization reaction is probably initiated by the cleavage of the epoxide as it appears in Chart 2.[7] Mori *et al.* has succeeded in the synthesis of magnosalicin (**4**) according to the proposed biogenetical scheme and the validity of the scheme has been proved by the synthesis of magnosalicin (**4**) from α-asarone by one pot reaction using peracetic acid.[9]

The ^{1}H-NMR spectrum of MG-5 revealed the presence of three methoxy groups, two non-coupled aromatic protons and a C-methyl group giving a singlet signal. ^{13}C-NMR indicated the presence of two carbonyls and it was finally identified as 1-(2,4,5-trimethoxyphenyl)-1,2-propanedione (**7**). **7** had been synthesized before but this was the first case

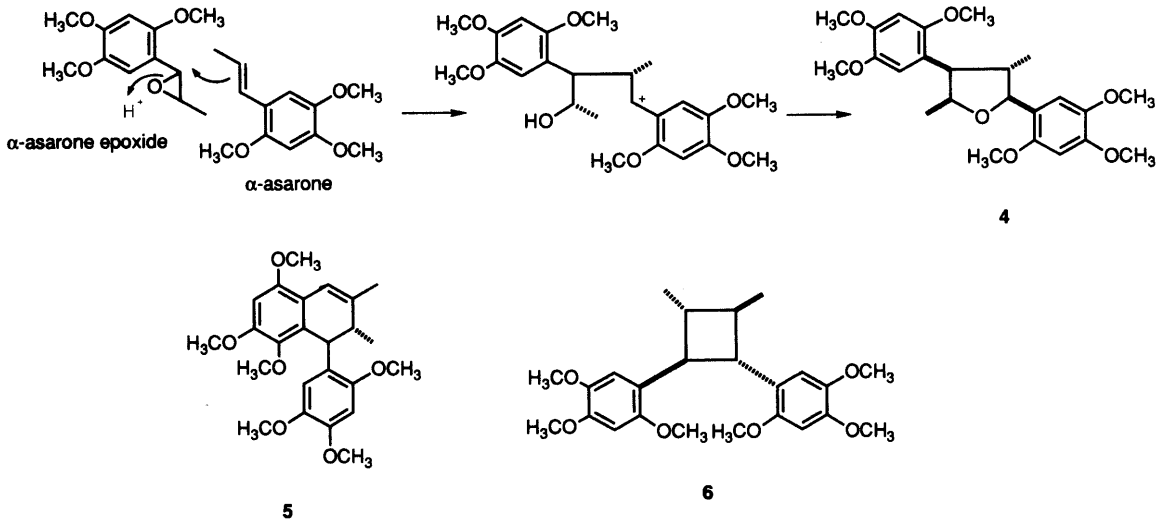


Chart 2. Biogenesis of Magnosalicin (**4**) and Structures of Magnoshinin (**6**) and Magnosalin (**7**)

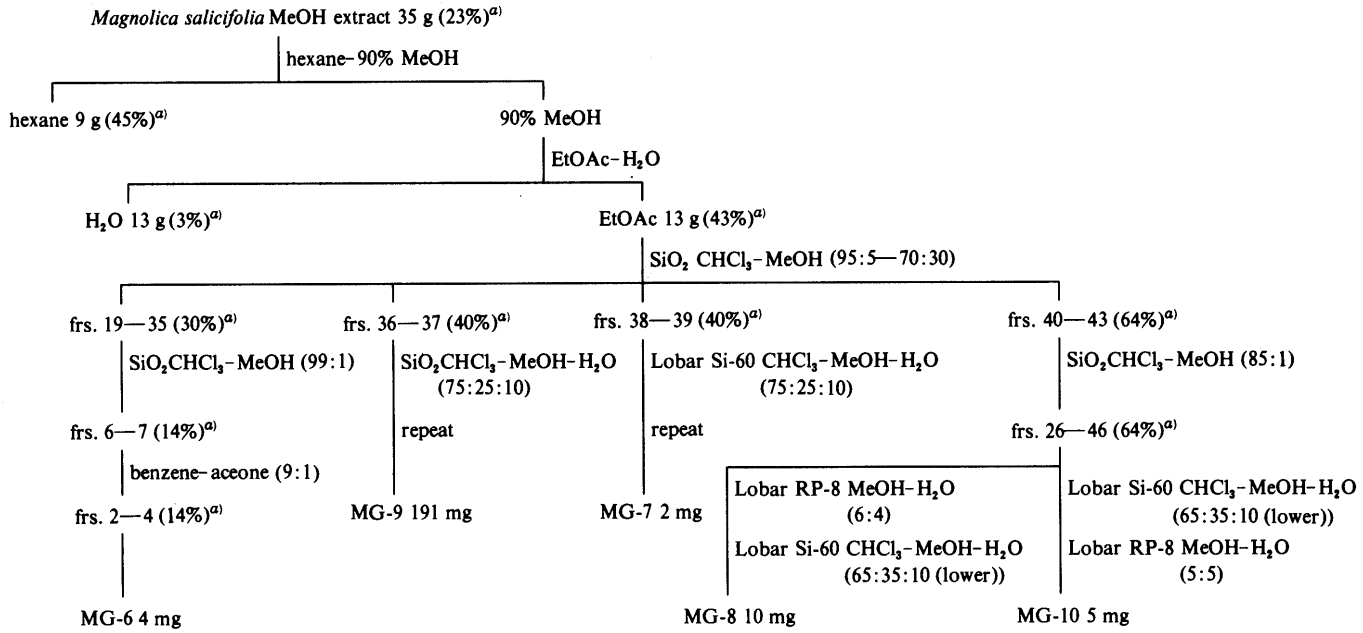
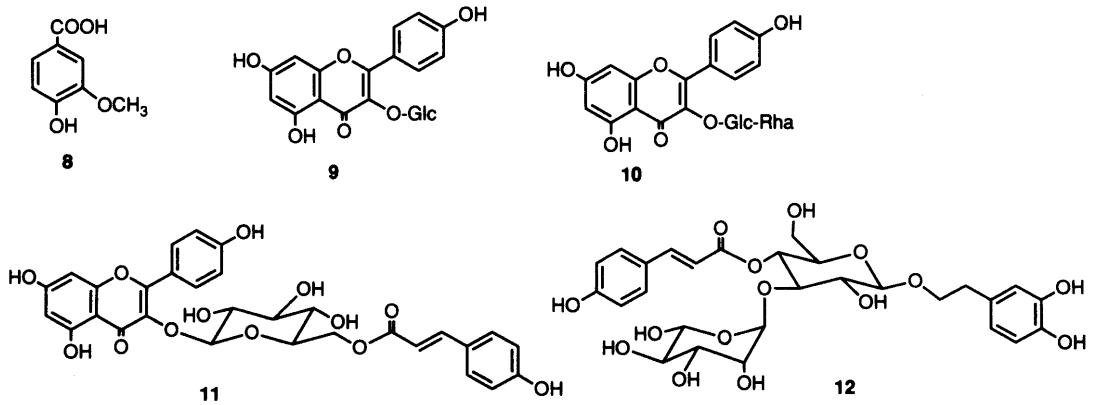


Chart 3. Fractionation, Separation and Isolation of *Magnolia salicifolia* Methanol Extract

a) Inhibition% for histamine release from mast cells induced by compound 48/80 at a substrate concentration of 50 μg/ml.



of it being isolated from plant.[10]

To isolate bioactive compounds contained in the methanol extract it was fractionated as shown in Chart 3. The ethyl acetate extract was further separated by repeated column chromatographies with silica-gel, Lobar RP-8 and Lobar Si-60 to give five compounds tentatively designated as MG-6—MG-10. MG-6 was identified as veratric acid (**8**).[5a] MG-7 and MG-8 were identified as astragalin

(kaempferol-3-*O*-glucoside) (**9**) and nicotoflorin (kaempferol-3-*O*-rutinoside) (**10**), respectively, by the comparison of spectral data.[11] MG-9 was identified as tiliroside (**11**)[12] and MG-10 as acteoside (**12**)[13] by direct comparison with authentic samples.

Table III summarizes the inhibitory activities of the isolated compounds on histamine release from rat mast cells induced by compound 48/80 or Con A. Asaryladehyde (**2**) and tiliroside (**11**) were the most effective in the experiments where compound 48/80 was used as an inducer. Scoparone (**1**), astragalin (**9**), tiliroside (**11**) and acteoside (**12**) were significantly effective on Con A induced histamine release at a concentration of 10^{-4} M. Since a significant effect was observed in scoparone (**1**) on histamine release from mast cells, we tested the samples of coumarins which were available in our laboratory and the results are summarized in Table IV. All coumarins tested inhibited histamine release more or less to the same degree, indicating that this activity is general for coumarins and is not affected by structural variation. Although magnosalicin (**4**) is not so active compound, a considerable number of samples of lignans was available, so we tested the effects of lignans of different skeletons on induced histamine release from mast cells. Of the lignans tested those having significant effects are listed in Table V. Active lignans are limited to bistetrahydrofuran and butanolide lignans. Tetrahydrofuran type lignans were not active. The most active class of lignans showed IC_{50} values less than 20 μM. However, no distinct structure activity relationship was found from the results obtained. It may worth noting that matairesinol and trachelogenin (IIIa and IIIe in Table V) have been shown to act as Ca^{2+}-antagonists and their hypotensive effects were confirmed in experiments using spontaneous hypertensive rats.[14] The inhibitory activity of lignans on histamine release may have a close relation with Ca^{+2}-antagonist activity.

The results so far obtained demonstrate that the flower buds of *M. salicifolia* contain anti-allergic compounds as shown in the PCA test of plant extracts (Table I). Isolation of active compounds possessing an inhibitory effect on histamine release from mast cells indicates that they may be responsible compounds exhibiting this effect, though the isolated compounds were not so active nor obtained in large quantities. The activity found in the original extract was not explained by the isolated compounds as it is clear from their activities and yields. Many other compounds not isolated in this study should contribute to the activities of the extracts.

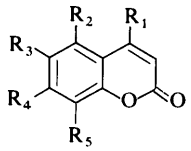
TABLE III. Inhibitory Effects of Isolated Compounds from *Magnolia salicifolia* on Histamine Release from Rat Mast Cells Induced by Compound 48/80 or Con A

	Inhibition % of histamine release			
	Inducer			
	Compound 48/80		Con A	
	10^{-3} M	10^{-4} M	10^{-3} M	10^{-4} M
Scoparone (**1**)	37	−6	71	22
Asarylaldehyde (**2**)	68	27	46	−8
3,4-Dimethoxycinnamoyl-alcohol (**3**)	0	−2	96	−1
Magnosalicine (**4**)	21	−4	5	5
1-(2,4,5-Trimethoxyphenyl)-1,2-propanedione (**7**)	46	4	1	−1
Veratric acid (**8**)	24	10	40	10
Astragalin. (**9**)	15	9	41	36
Nicotoflorin (**10**)	14	9	39	5
Tiriloside (**11**)	52	29	51	38
Acteoside (**12**)	48	8	81	24

Experimental

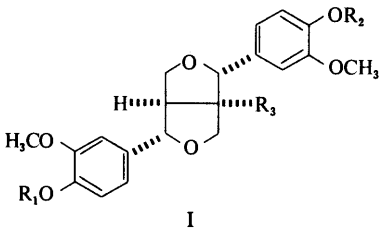
^{1}H- and ^{13}C-NMR spectra were measured on a JEOL JNM FX-100 or FX-400, MS on JEOL JMS-DX300, infrared (IR) spectrum on a JASCO model 701G, ultraviolet (UV) spectrum on a Hitachi Spectrophotometer model 100-60 and optical rotation on a JASCO DIPO-140 Digital polarimeter. Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Samples of coumarins are from the collection of this laboratory. A part of lignan samples is our

TABLE IV. Inhibitory Effects of Coumarins on Histamine Release from Rat Mast Cells Induced by Compound 48/80 or Con A



	R_1	R_2	R_3	R_4	R_5	Inhibition of histamine release			
						Inducer			
						Compound 48/80		Con A	
						10^{-3} M	10^{-4} M	10^{-3} M	10^{-4} M
Umbelliferone	H	H	H	OH	H	32	10	106	39
4-Hydroxycoumarin	OH	H	H	H	H	55	13	99	34
Esculetin	H	H	OH	OH	H	34	18	107	38
5,7-Dihydroxycoumarin	H	OH	H	OH	H	42	13	107	33
Daphnetin	H	H	H	OH	OH	67	47	106	17
Scopoletin	H	H	OMe	OH	H	28	14	103	15
Isoscopoletin	H	H	OH	OMe	H	33	15	105	31
Scoparone	H	H	OMe	OMe	H	44	26	102	58
Limettin	H	OMe	H	OMe	H	7	11	91	63

TABLE V. Inhibitory Effects of Lignans on Histamine Release from Rat Mast Cells Induced by Compound 48/80 or Con A



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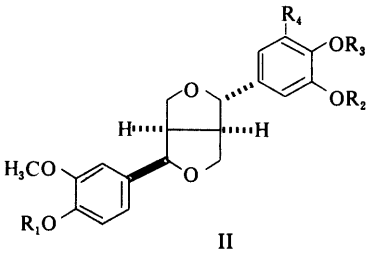

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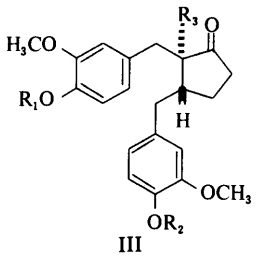

III

	R_1	R_2	R_3	R_4	Inhibition % and IC_{50} of histamine release				IC_{50} μM
					Inducer				
					Compound 48/80		Con A		
					10^{-3} M	10^{-4} M	10^{-3} M	10^{-4} M	
Ia	H	H	H	H	98	34	109	55	68
Ib	Me	Me	H	H	27	13	103	74	37
Ic	Me	Me	OAc	H	22	16	87	63	50
Id	Me	Me	OH	H	7	2	94	65	47
IIa	H	Me	H	H	99	59	97	71	39
IIb	Me	Me	H	H	16	3	92	59	59
IIc	Me	Me	H	H	16	10	79	60	52
IId	Me		$-CH_2-$	H	19	14	83	81	31
IIe	Me	Me	Me	MeO	98	40	114	54	70
IIIa	H	H	H		106	54	109	105	18
IIIb	H	Me	H		80	26	115	94	30
IIIc	Me	Me	H		60	27	108	91	32
IIId	H	Me	OH		98	32	107	100	19
IIIe	Me	Me	OH		98	37	111	89	25

Ia, (+) pinoresinol; Ib, (+) pinoresinol dimethyl ether; Ic, (+) acetoxypinoresinol dimethyl ether; Id, (+) isognerinol; IIa, (+) epipinoresinol; IIb, phyligenin; IIc, (+) epipinoresinol dimethyl ether; IId, fargasin; IIe, phyllyrin; IIIa, matairesinol; IIIb, arctigenin; IIIc, arctigenin methyl ether; IIId, trachelogenin; IIIe, trachelogenin methyl ether.

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Buffers Phosphate buffered saline (PBS, pH 7.2) was prepared as usual. Mast cell isolating buffer (pH 7.0) contains NaCl (150 mM), KCl (2.7 mM), $CaCl_2$ (10 mM), NaH_2PO_4 (1.0 mM) and human serum albumin (HSA) (1 mg/ml). Mast cell challenging buffer (pH 6.8) consists of NaCl (105 mM), KCl (3.5 mM), $CaCl_2$ (4.6 mM), heparin (35 ng/ml). $Al_2(SO_4)_3$ (0.1 mM), $MgCl_2$ (0.4 mM), *N*-hydroxyethylpiperazine-*N'*-2-ethansulfonate (HEPES, 10 mM), 2-(*N*-morpholino)ethanesulfonic acid (MES, 10 mM) and HSA (1 mg/ml). HSA, HEPES and MES were obtained from Sigma, others from Wako.

Animals Sprague–Dawley rats (SPE) weighing 180 to 250 g were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals. Rats were provided with a normal diet and water.

Antisera Rat antiserum for passive sensitization was prepared according to Mota.[15] Egg albumin (×5 recrystallized, Seikagaku Kogyo) (20 mg/ml in PBS) was mixed with the same volume of aluminum hydroxide (20 mg/ml in saline). To each animal a total of 1 ml of the mixture was injected intamuscularly into the four legs and at the same time 2×10^{10} *Bordetella pertussis* organisms (Chiba-prefecture Serum Research Institute) was injected intraperitoneally. After 12 to 14 d, blood was collected from the aorta to prepare anti-egg albumin antiserum.

PCA in Rats The interval between sensitization and challenge was 48 h in homologous PCA using rat antiserum. Male rats were sensitized by intradermal injections of 0.05 ml serum. After the appropriate latent period 1 ml of a solution containing 5 mg each of egg albumin and Evans blue were injected intravenously. The animals were sacrificed 30 min after challenge, the skin flayed, and the responses measured as long radius × short radius of blue spots. The area of blue spot was cut off and extracted with 2 N NaOH (1 ml) at 37 °C over night. The extract was neutralized by adding 5 N phosphoric acid (5 ml). To this solution acetone (3 ml) was added and centirufuged at 2500 rpm for 10 min. The amount of pigment was measured photometrically at 620 nm. Test materials (saline as blank control) were administered i.v. 30 s or i.p. 30 min before challenging. As references disodium cromoglycate (DSCG, Intal) was tested in parallel. DSCG was obtained from Fujisawa Pharmaceutical Co., Ltd.

Preparation of Rat Peritoneal Mast Cells Cells were kept at 4 °C throughout the operations. Male rats were killed by bleeding, 10 ml of isolation buffer were then injected intraperitoneally. After being massaged for 1 min the abdomen was opened and peritoneal fluid was collected. The fluid was then centrifuged at $160 \times g$ for 5 min. The cell pellet was washed once with 10 ml of challenge buffer and was diluted with the same buffer to make a cell suspension of about 1×10^6 cells/ml.

***In Vitro* Assay on Histamine Release**[16] All materials added to the assay system were dissolved in HSA-free challenge buffer. 80 μl of mast cell suspension was added to 20 μl of test materials and immediately challenged with 20 μl of compound 48/80 (Sigma, 0.8 μg/ml) or Con A (Wako, 10 μg/ml with phosphatidylserine from Avanti Polar Lipids of 25 μg/ml). After challenging for 10 min (compound 48/80) or 30 min (Con A) at 37 °C, the reaction was stopped by placing the incubation tubes into ice water. Incubation tubes were then centrifuged at 4 °C at $780 \times g$ for 5 min. 90 ml of supernatants were transferred into other tubes, heated at 95 °C for 5 min to precipitate the proteins, and centrifuged at 4 °C at $1200 \times g$. The contents in supernatants were then separated with high performance liquid chromatography (HPLC) using Toyosoda IEX-215 cationic ion-exchange column. The peaks were quantitated fluorometrically by post-column interaction with *o*-phthaldialdehyde (Wako). Total cell histamine release was achieved by heating the cell suspension at 95 °C for 5 min in the presence of 0.1% of Triton-X 100. DSCG and Tranilast were used as reference compounds. Tranilast was obtained from Kissei Pharmaceutical Co.

Small Scale Extraction and Testing of Biological Activities The buds of *Magnolia salicifolia* MAXIM. (200 g), purchased from Yamamoto Pharmaceutical Industry (Tokyo), were extracted successively with each 1 l of hexane, $CHCl_3$, MeOH and H_2O. Solvents were removed *in vacuo* and the obtained extracts were submitted to PCA and inhibition of histamine release from mast cells.

TABLE VI. Fractional Coordinates ($\times 10^4$), B_{eq} (Å^2), and U_{eq} (Å$^2 \times 10^4$) for Non Hydrogen Atoms with Estimated Standard Deviations in Parentheses

Atom	x	y	z	B_{eq}	U_{eq}
O(1)	1450 (1)	1442 (1)	2535 (3)	3.77 (0.04)	478 (5)
C(2)	1774 (2)	2132 (2)	3337 (4)	3.88 (0.05)	491 (7)
C(3)	886 (2)	2632 (2)	3113 (4)	3.33 (0.05)	21 (6)
C(4)	105 (2)	2043 (2)	2914 (3)	3.06 (0.05)	388 (6)
C(5)	443 (2)	1474 (2)	1905 (3)	3.21 (0.05)	407 (6)
C(6)	2281 (3)	1941 (2)	4963 (5)	5.53 (0.07)	701 (9)
C(7)	−872 (2)	2351 (2)	2235 (4)	3.99 (0.05)	506 (7)
C(8)	26 (2)	704 (2)	1896 (3)	3.14 (0.05)	398 (6)
C(9)	−803 (2)	525 (2)	829 (3)	3.30 (0.05)	417 (6)
C(10)	−1222 (2)	−177 (2)	844 (4)	3.56 (0.05)	451 (6)
C(11)	−808 (2)	−703 (2)	1917 (4)	3.66 (0.05)	464 (7)
C(12)	37 (2) 2	−539 (2)	2985 (4)	3.61 (0.05)	457 (6)
C(13)	430 (2) 2	163 (2)	2976 (3)	3.37 (0.05)	427 (6)
C(14)	909 (2)	3230 (2)	4303 (4)	3.34 (0.05)	423 (6)
C(15)	1206 (2)	3953 (2)	4083 (4)	3.72 (0.05)	471 (7)
C(16)	1211 (2)	4521 (2)	5123 (4)	4.10 (0.06)	519 (7)
C(17)	913 (2)	4379 (2)	6406 (4)	3.94 (0.05)	499 (7)
C(18)	597 (2)	3656 (2)	6658 (4)	4.04 (0.06)	511 (7)
C(19)	601 (2)	3100 (2)	5621 (4)	3.82 (0.05)	483 (7)
C(20)	−2060 (3)	960 (2)	−1234 (5)	5.78 (0.07)	732 (9)
C(21)	−2043 (3)	−1602 (2)	979 (5)	5.55 (0.07)	703 (9)
C(22)	1294 (3)	−952 (2)	5030 (4)	5.08 (0.07)	644 (8)
C(23)	1477 (4)	4802 (3)	2185 (6)	8.80 (0.12)	1115 (15)
C(24)	1216 (3)	5635 (2)	7328 (5)	5.81 (0.08)	735 (10)
C(25)	−260 (3) 3	2947 (3)	8114 (5)	6.45 (0.09)	817 (11)
C(2)	−1156 (3)	1075 (2)	−223 (3)	4.28 (0.04)	542 (5)
O(3)	−1163 (2)	−1412 (2)	2025 (3)	5.01 (0.04)	635 (6)
O(4)	414 (2) 2	−1110 (2)	3968 (3)	4.69 (0.04)	593 (5)
O(5)	1505 (2) 2	4066 (2)	2778 (3)	5.51 (0.05)	698 (7)
O(6)	904 (2)	4893 (2)	7530 (3)	4.90 (0.04)	621 (6)
O(7)	299 (2)	3570 (2)	7972 (3)	6.65 (0.06)	842 (8)

$U_{eq} = 1/3 \sum_i \sum_j U_{ij} a_i^* a_i^* a_i a_j$.

TABLE VII. Fractional Coordinates ($\times 10^4$), B_{eq} (Å^2) and U_{eq} (Å$^2 \times 10^4$) for Hydrogen Atoms with Estimated Standard Deviations in Parentheses

Atom	x	y	z	B_{eq}	U_{eq}
H(C2)	2287 (2)	2405 (2)	2818 (4)	6.0 (1)	760 (11)
H(C3)	793 (2)	2959 (2)	2059 (4)	5.0 (1)	660 (10)
H(C4)	97 (2)	1806 (2)	4027 (3)	5.0 (1)	570 (9)
H(C5)	263 (2)	1676 (2)	725 (3)	5.0 (1)	590 (10)
H(C6)	2822 (2)	1516 (2)	4963 (4)	8.0 (1)	960 (13)
H′(C6)	2613 (3)	2451 (2)	5542 (4)	7.0 (1)	930 (13)
H″(C6)	1776 (3)	1720 (2)	5562 (4)	6.0 (1)	810 (12)
H(C7)	−1091 (2)	2703 (2)	3079 (4)	6.0 (1)	770 (11)
H′(C7)	−863 (2)	2699 (2)	1240 (4)	6.0 (1)	760 (11)
H″(C7)	−1367 (2)	1882 (2)	1905 (4)	6.0 (1)	790 (11)
H(C10)	−1876 (2)	−311 (2)	−4 (4)	6.0 (1)	71 (11)
H(C13)	1080 (2)	307 (2)	3827 (4)	5.0 (1)	68 (11)
H(C16)	1451 (2)	5088 (2)	4916 (4)	6.0 (1)	71 (11)
H(C19)	356 (2)	2532 (2)	5807 (4)	5.0 (1)	67 (11)
H(C20)	−2241 (3)	1445 (2)	−1999 (4)	6.0 (1)	91 (13)
H′(C20)	−2047 (3)	448 (2)	−1907 (4)	7.0 (1)	89 (13)
H″(C20)	−2588 (2)	897 (2)	−574 (4)	7.0 (1)	82 (12)
H(C21)	−2238 (3)	−2181 (2)	1195 (4)	6.0 (1)	90 (12)
H′(C21)	−2592 (3)	−1213 (2)	1136 (4)	7.0 (1)	104 (14)
H″(C21)	−1971 (2)	−1558 (2)	−193 (4)	8.0 (1)	80 (12)
H(C22)	1522 (2)	−1448 (2)	5750 (4)	6.0 (1)	88 (12)
H′(C22)	1824 (2)	−816 (2)	4407 (4)	7.0 (1)	85 (12)
H″(C22)	1210 (2)	−472 (2)	5745 (4)	7.0 (1)	81 (12)
H(C23)	1553 (3)	4779 (2)	1014 (4)	6.0 (1)	106 (14)
H′(C23)	802 (3)	5066 (2)	2193 (4)	8.0 (1)	108 (14)
H″(C23)	2053 (3)	5136 (2)	2887 (5)	9.0 (1)	131 (16)
H(C24)	1173 (3)	5985 (2)	8305 (4)	10.0 (1)	99 (13)
H′(C24)	1947 (3)	5616 (2)	7239 (4)	8.0 (1)	90 (12)
H″(C24)	770 (2)	5878 (2)	6283 (4)	7.0 (1)	760 (11)
H(C25)	−495 (3)	2998 (2)	9166 (4)	8.0 (1)	102 (14)
H′(C25)	−875 (3)	2927 (2)	7136 (5)	10.0 (1)	130 (16)
H″(C25)	151 (3)	2428 (2)	8151 (4)	9.0 (1)	111 (14)

Large Scale Extraction, Fractionation and Separation The flower buds of *M. salicifolia* (5 kg) were extracted twice with 5 l per kg solvents for 5 h. Hexane extract (91 g), $CHCl_3$ extract (116 g), MeOH extract (227 g) and H_2O extract (496 g) were obtained on evaporation of solvents. A part of $CHCl_3$ extract was repeatedly separated with silica-gel, Lobar RP-8 and Lobar Si-60 columns. Each fraction was monitored by inhibitory activity on histamine release from mast cells as shown in Chart 1. Five compounds tentatively designated as MG-1—MG-5 were obtained as active constituents. A part of MeOH extract was fractionated between hexane and 90% MeOH, and the 90% MeOH fraction was further fractionated with EtOAc–H_2O. The activity was observed only in the EtOAc fraction, which was then subjected to further separation with silica-gel, Lobar RP-8 and Lobar Si-60 columns to afford five active compounds, MG-6—MG-10.

Scoparone (MG-1, 1) Colourless needles from hexane–$CHCl_3$, mp 143—144 °C. HR-MS *m/z*: Calcd for $C_{11}H_{10}O_4$ 206.0578, Found 206.0580. MG-1 was identified by direct comparison with an authentic sample of our collection.

Asarylaldehyde (MG-2, 2)[5] Colourless needles from 50% MeOH, mp 114—114.5 °C. HR-MS *m/z*: Calcd for $C_{10}H_{12}O_4$ 196.0735, Found 196.0739. ^{1}H-NMR ($CDCl_3$, 100 MHz) δ: 3.89, 3.94, 3.99 (each 3H, s), 6.50 (1H, s), 7.34 (1H, s), 10.32 (1H, s).

3,4-Methylenedioxycinnamyl Alcohol (MG-3, 3)[5a,6] Colourless needles from hexane–EtOAc, mp 66—67 °C. HR-MS *m/z*: Calcd for $C_{10}H_{10}O_3$ 173.0630, Found 173.0633. ^{1}H-NMR (100 MHz) δ: 4.30 (2H, d, J=5.5 Hz), 5.96 (2H, s), 6.19 (1H, dt, J=15.5, 5.5 Hz), 6.54 (1H, d, J=15.5 Hz), 6.74 (1H, d, J=8.5 Hz), 6.84 (1H, d, J=8.5 Hz), 6.94 (1H, br s).

Magnosalicin (MG-4, 4) Slightly red prisms from MeOH, mp 134.5—135 °C. HR-MS *m/z*: Calcd for $C_{24}H_{32}O_7$ 432.2146, Found 432.2170. MS *m/z* (rel. int.): 432 (M^+, 40), 388 ($M^+ - CH_3 - CHO$, 100), 220 (38), 205 (59). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.91 (3H. d, J=6.5 Hz, C-6 CH_3), 104 (3H, d, J=6.5 Hz, C-7 CH_3) 2.32 (1H, ddq, J=6.5, 9.0, 10.5 Hz, C-4 H), 3.61 (1H, dd, J=8.5, 10.5 Hz, C-3 H), 3.80, 3.82, 3.83, 3.88, 3.90, 3.91 (each 3H, s, OCH_3), 4.61 (1H, qd, J=6.5, 8.5 Hz, C-2 H), 4.98 (1H, d, J=9.0 Hz, C-5 H), 6.54, 6.55, 6.70, 7.15 (each 3H, s, Arom H).

X-Ray Analysis of Magnosalicin (4) Crystal suitable for X-ray analysis was monoclinic space group $P2_1/a$ with four molecules in a unit cell of dimensions, a=14.725(7), b=17.796(8), c=9.100(4) Å; b=107.47, D_x=1.34 g cm^{-3}, V=2309.03 Å^3. Reflection data was recorded on a Philips PW-1100 diffractometer with graphite monochromated CuK_α radiation. The cell parameters were determined by the least square method. A total of 3603 reflections were collected as above the $2\sigma(I)$ level within a range of 3—78° (θ). The structure was solved by the direct method (MULTAN) and refined by block diagonal least squares. A final R value was 0.0573 with anisotropic temperature factors for 24 carbon and 7 oxygen atoms, and isotropic temperature factors for 32 hydrogen atoms. Fractional coordinate, B_{eq} and U_{eq} of non-hydrogen atoms and hydrogen atoms are shown in Table VI and VII, respectively.

1-(2,4,5-Trimethoxyphenyl)-1,2-propanedione (MG-5, 7)[10] Colourless needles from hexane–EtOAc, mp 129—130 °C. HR-MS *m/z*: Calcd for $C_{12}H_{14}O_5$ 238.0840, Found 238.0845. MS *m/z* (rel. int.): 238 (M^+, 1), 195 ($M^+ - CH_3CO$, 100), 180 (7), 152 (8), 137 (7). UV λ_{max} MeOH nm (log ε): 342 (4.12), 273 (4.17), 237 (4.34). ^{1}H-NMR ($CDCl_3$, 100 MHz) δ: 2.39 (3H, s), 3.81, 3.88, 3.96 (each 3H, s), 6.48 (1H, s), 7.34 (1H, s). ^{13}C-NMR ($CDCl_3$, 25 MHz) δ: 25.1 (CH_3), 56.2 (CH_3), 56.7 ($CH_3 \times 2$), 96.5 (CH), 110.8 (CH), 114.3 (C), 144.2 (C), 156.0 (C), 156.7 (C), 193.0 (CO), 201.1 (CO).

Vetratric Acid (MG-6, 8) Colourless needles from H_2O–MeOH, mp 176—181 °C. MG-6 was identified by direct comparison with a commercial authentic sample.

Astragalin (MG-7, 9)[11] Amorphous powder. Fast atom bombardment mass spectrum (FAB—MS) *m/z*: 449 ($M+H^+$, $C_{21}H_{21}O_{11}$), 287 (aglycon).

Nicotoflorin (MG-8, 10)[11] Yellow powder. FAB-MS *m/z*: 617 ($M+Na^+$), 595 ($M+H^+$, $C_{27}H_{31}O_{15}$), 449, 287 (aglycon).

Tiliroside (MG-9, 11)[13] Yellow powder from MeOH, mp 244—246 °C. FAB-MS *m/z*: 595 ($M+H^+$, $C_{30}H_{27}O_{13}$). Acetylation with pyridine and acetic anhydride gave heptaacetate of mp 183.5—185 °C.

Acteoside (MG-10, 12)[14] Yellow amorphous powder. FAB-MS *m/z*: 625 ($M+H^+$, $C_{29}H_{37}O_{15}$).

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Biologically Active Constituents of *Centipeda minima*: Sesquiterpenes of Potential Anti-allergy Activity

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Ether, methanol and aqueous extracts of *Centipeda minima* (Compositae) herbs were found to have significant anti-allergy activities in passive cutaneous anaphylaxis (PCA) test. Three flavonoids, two sesquiterpene lactones and an amide were isolated from this plant material as inhibitors to induced histamine release from mast cells. The sesquiterpenes were identified as isobutyroylplenolin and senecioylplenolin by spectral investigations. The flavonoids and sesquiterpenes exhibited significant anti-allergy activity in PCA test with *p.o.* administration.

Keywords *Centipeda minima*; Compositae; sesquiterpene lactone; senecioylplenolin; isobutyroylplenolin; auratiamide acetate; quercetin methyl ether; anti-allergy; mast cell; PCA

In our search for potential anti-allergy compounds from traditional medicinal drugs, the *in vivo* passive cutaneous anaphylaxis (PCA) test has been used in combination with an *in vitro* mast cell bioassay testing inhibitory effect on histamine release.[1] The latter *in vitro* bioassy was employed throughout the separation procedure of plant constituents to monitor the biological activities of extracts, fractions and isolated compounds. Flavonoids, coumarins, lignans and stilbenes were found as potential anti-allergy compounds which significantly inhibited histamine release from rat mast cells induced by compound 48/80 or concanavalin A (Con A).[1]

In a preliminary screening study, the herbs of *Centipeda minima* L. A. BRAUN *et* ASCHERS (Compositae) were found to be highly active in the PCA test and significantly inhibited histamine release from mast cells.[1a] The hot aqueous extract of *C. minima* herbs inhibited histamine release 100% at concentrations higher than 20 μg/ml. This plant occurs widely in Asia and Oceania and has been known as a medicinal drug used for anti-allergy and anti-innflammatory purpose in Chinese medicine.[2] Phytochemical study reported taraxasteryl palmitae, taraxasteryl acetae, taraxaserol, stigmasterol, β-stitosterol and arnidiol as the constituents of this plant.[3]

We first investigated the hot aqueous extract of the herbs to clarify active constituents, but despite extensive fractionation and separation we failed to characterize responsible compounds for the biological activity of the hot aqueous extract. Repeated fractionations of the hot aqueous extract by ethanol precipitaion and column chromatography gave very active fractions which inhibited compound 48/80 induced histamine release 100% at a concentration of 33 μg/ml. Spectral investigation indicated that the fractions consisted of polysaccharides and nitrogen containing compounds presumably protein, however further characterization was not possible. At this stage of study we changed our strategy to investigate active compounds soluble in organic solvents since compounds that were extracted by chloroform from the aqueous extract showed significant activity in the mast cell bioassay. To clarify the chemical properties of active compounds soluble in organic solvents the herbs were successively extracted with ether, methanol and water, and the extracts were subjected to an anti-allergy test with PCA. The results in Table I indicated that the compounds soluble in organic solvents were also active in the PCA test.

Based on these observations *Centipeda minima* herbs (5 kg) were extracted with chloroform. After being concentrated *in vacuo* the chloroform extract was repeatedly fractionated with hexane and methanol as shown in Chart 1. The hexane soluble fractions (fr. CH and fr. MH) were rich in volatile compounds and gas chromatography–mass spectrometry (GC-MS) analysis using a data base revealed the fractions contained the following compounds: 2-heptanal, 2,4-heptadienol, iso-butyric acid, benzyl alcohol, *cis*-chrysanthenal, *cis*-chrysanthenyl acetate, methyl linoleate, β-gurjunene, methyl palmitate, ethyl palmitate, 2,4-decadienol, phytol, caryophyllane-2,6β-oxide and dihydroactinidiolide.

Fraction CM was further separated with Sephadex LH-20 to give four fractions (fr. 1—fr. 4), in which the most polar fr. 4 afforded three flavonoids, CM-1, CM-2 and CM-3, by chromatographic separation on LH-20 and silica-gel. They were identified as quercetin-3,3′-dimethyl ether,[4] quercetin-3-methyl ether[5] and apigenin, respectively, by spectroscopical investigations as well as by direct comparison with authentic samples.

Next we investigated fr. 1 (Chart 1) since it was the most active and was obtained in the highest yield. Fraction 1 contained non-polar compounds which were insoluble in buffer for the mast cell bioassay. This caused a serious problem since organic solvents such as acetone, ethanol and dimethylsulphoxide (DMSO), as well as normally used detergents, caused spontaneous release of histamine from mast cells. However, water soluble polyvinylpyrrolidone (mol. wt. 40000) was found to be an excellent co-solubilizing agent and we could overcome this problem.[6] Fraction 1 was repeatedly fractionated with Sephadex LH-20 and silica-gel to afford three compounds CM-4, CM-5 and CM-6. High resolution mass spectrum (HR-MS) gave

TABLE I. Anti-allergy Activities of *Centipeda minima* Extracts in PCA Test

Test material	Inhibition % 1:1[a]	Inhibition % 1:2[a]
Ether ext.	49.2±5.0	70.0±0.7
Methanol ext.	36.9±0.7	50.0±2.9
Water ext.	30.8±2.1	46.0±0.7
DSCG	56.9±2.1	46.0±2.9

a) Dilution ratios of antisera. Sprague–Dawley male rats were used. Test materials were administered i.p. 30 min except for DSCG i.v. 1 min before challenging with egg albumin (25 mg/kg). The dose of materials is 10 g of plant material/kg rat equivalent. The results are expressed as mean±S.E.M. DSCG: disodium cromoglycate.

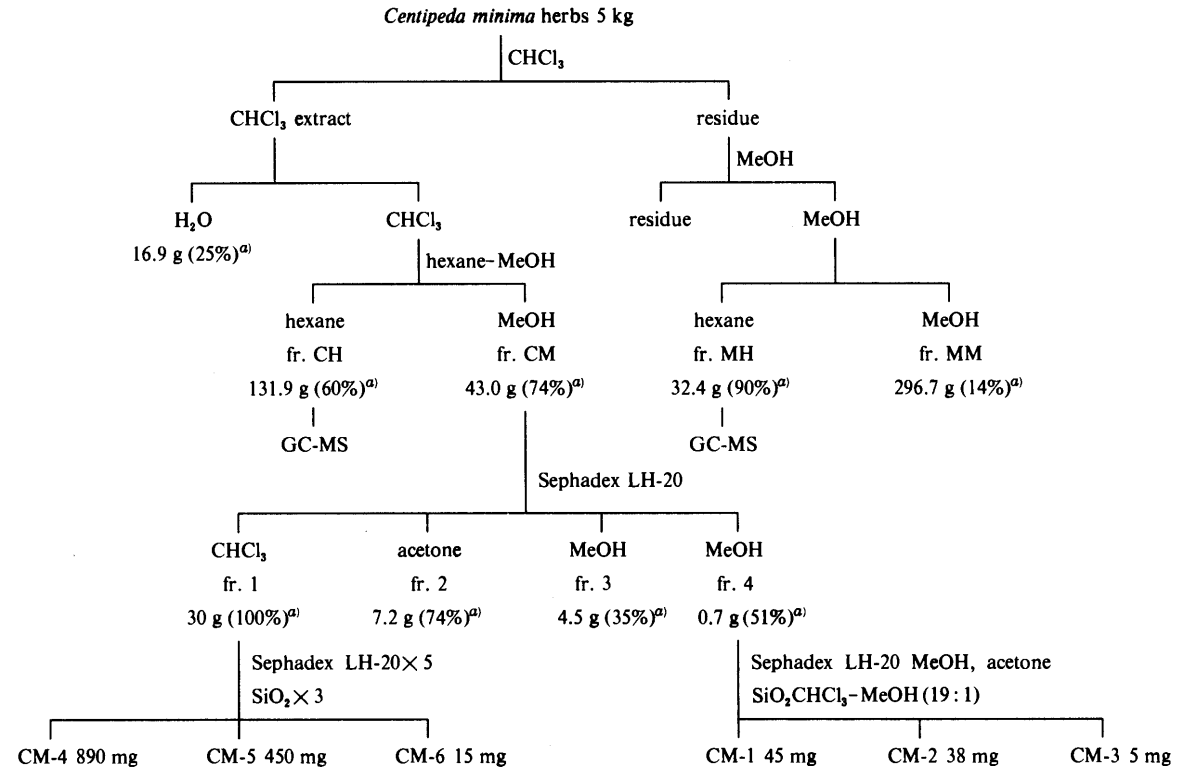
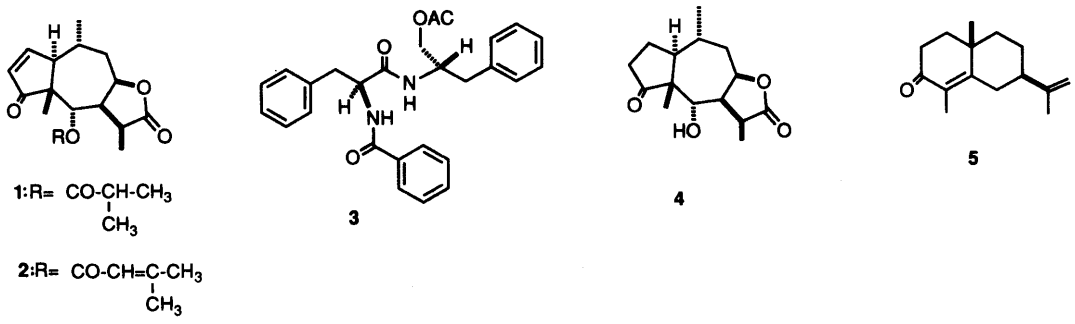


Chart 1. Extraction and Separation of Chloroform Extract of *Centipeda minma* Herbs

a) Inhibition % for histamine release from mast cells induced by compound 48/80 at a substrate concentration of 100 μg/ml.



a molecular formula, $C_{19}H_{26}O_5$, for CM-4. Infrared (IR) absorption at 1765, 1730 and 1718 cm^{-1} indicated the presence of three different carbonyls and ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrum showed signals of four doublet methyls and one singlet methyl. These spectral data suggested CM-4 to be a plenolin (13β-11,13- dihydrohelenalin) ester, a sesquiterpene lactone of pseudoguaianolide skeleton, which had been isolated from plants belonging to Compositae such as *Arnica montana*. CM-4 was identified as isobutyroylplenolin (arnicolide C) (**1**) by comparison of its spectral data with those reported.[7] The spectral data of CM-5 was very similar to those of CM-4 and they differ from one another in their ester groups. CM-5 was finally identified as a new plenolin, senecioylplenolin (**2**).[8] CM-6 contained nitrogen atom and a molecular formula $C_{27}H_{28}N_2O_4$ was given by elemental analysis and MS. The ^{1}H-NMR spectrum (correlation spectroscopy (COSY)) revealed the presence of phenylalanine and phenylalanol moieties and indicated that it is aurantiamide acetate (**3**), an amide having been isolated from *Piper auranticum*.[9] The IC_{50} value of aurantiamide acetate for Con A induced histamine release from rat mast cells was 23 mM and the least active among those isolated from this material.

The inhibitory activities of isolated and related compounds were summarized in Table II. Among flavonoids tested apigenin was the most active and IC_{50} values of other methylated flavonoids were in the order of 10 μM, whereas plenolin esters showed IC_{50} values one order less than quercetin methyl ether. As far as the present results on sesquiterpenes are concerned, the ester group seemed to be essential for the activities. In contrast to plenolin esters (**1**,**2**), guaianolide sesquterpenes which were isolated from the roots of *Thapsia garganica* L. were reported to act as a potent skin irritant and strongly induce histamine release from mast cells.[10] This may indicate that a rather small structural difference causes dramatic changes in biological activities. During the course of this study, isolation of thymol derivatives and plenolin type sesquiterpenes from *Centipeda minima* herbs was reported by Bohlmann and Chen.[11]

Table III summarizes the results of the PCA test of the

TABLE II. Inhibitory Effects of Isolated Compounds from *Centipeda minima* and Reference Compounds on Histamine Release from Rat Mast Cells Induced by Compound 48/80 or Con A

	Inhibition % of histamine release				
	Inducer				
	Compound 48/80		Con A		IC_{50} (μM)
	0.8×10^{-4} M	3.2×10^{-4} M	0.8×10^{-4} M	3.2×10^{-4} M	
Apigenin	40	68	72	100	9.8
Quercetin-3-Me-ether	17	34	67	65	18
Quercetin-3,3'-diMe-ether	17	34	39	89	45
Quercetin-3,7,3'-triMe-ether	20	24	16	58	26
Quercetin-3,7,3',4'-tetraMe-ether	8	18	14	35	—
Isobutyroylplenolin (**1**)	53	84	54	94	6.4
Senecioylplenolin (**2**)	67	73	35	98	9.6
Aurantiamide acetate (**3**)	—	—	—	—	230
Tetrahydrohelenalin (**4**)	37	43	0	37	—
α-Cyperone (**5**)	35	40	0	23	—

The suspension of mast cell (20 μl, 8×10^4 cells) was added to the solution of test materials in 80 μl challenge buffer and immediately challenged with compound 48/80 (0.8 μg/ml) or Con A (10 μg/ml with 25 μg/ml phosphatidylserine). After challenging for 10 min (compound 48/80) or 30 min (Con A) at 37 °C, the reaction mixture was subjected to the measurement of released histamine after removing mast cells by centrifugation.[1)]

TABLE III. Anti-allergy Activities of Isolated Compounds from *Centipeda minima* in PCA Test

Test sample	Inhibition % 1 : 8[a)]	Inhibition % 1 : 16[a)]
Apigenin	42.5	38.8
Quercetin-3-Me-ether	38.8	38.6
Quercetin-3,3'-diMe-ether	67.3	61.0
Isobutyroylplenolin (**1**)	61.6	44.4
Senecioylplenolin (**2**)	37.4	41.0
DSCG	94.6	89.7

a) Dilution ratios of antisera. Sprague–Dawley male rats weighing 170—180 g were used. Test samples (50 mg/kg) were administered *p.o.* 2 h before challenging with egg albumin (25 mg/kg) except for DSCG which was administered i.v. 1 min before challenging.[1)] DSCG: disodium cromoglycate.

isolated compounds, which demonstrated the anti-allergic activities of **1** and **2** in the PCA test with *p.o.* administration. The results are well in accord with the traditional use of the herb for anti-allergy and anti-immflamatory purpose and also give a reasonable account of the *Arnica montana* herb which has been used as a medicinal plant in Europe, since the *Arnica* herbs contains plenolin esters as their main constituents.[12)] The positive results so far obtained support our strategy. The combination of the *in vivo* and *in vitro* bioassay systems is particularly effective in searching for bio-active compounds from medicinal plants.

Experimental

^{1}H- and ^{13}C-NMR spectra were measured on a JEOL JNM FX-100 or FX-400, MS on JEOL JMS-DX300, IR on a JASCO model 701G, ultraviolet spectrum (UV) on a Hitachi Spectrophotometer model 100-60 and optical rotation on an JASCO DIPO-140 Digital polarimeter. Melting points were determined on a Yanagimoto micro-melting point apparatus or in a silicon bath with a glass capillary and are uncorrected. GC-MS analysis was carried out at the Institute of Takasago International Cooperation by using a Hitachi Mass Spectrometer M-80B and a GC HP-5790A GC with a PEG-20M (0.25 mm × 50 m) column. Takasago MS Private Data Base was used for reference. An authentic sample of quercetin-3-methylether was obtained from Prof. P. R. Jefferies of the University of Western Australia, quercetin-3,3'-dimethylether from Prof. E. Wollenwever of Technische Hochschule Darmstadt and tetrahydrohelenalin from Prof. W. Herz of Florida State University. α-Cyperone, apigenin, quercetin-3,7,3'-trimethylether and quercetin- 3,7,3',4'-tetramethylether were from our collection.

Bioassay Tests The methods of bioassay tests are basically the same as those reported in previous papers.[1)] Test solutions of water insoluble samples were prepared using a water soluble polyvinylpyrrolidone (PVP) of molecular weight 40000 as a co-solubilizing reagent. A water insoluble sample was dissolved in an appropriate organic solvent such as $CHCl_3$ or acetone with 5 times the weight of PVP. The solvent was evaporated and completely removed *in vacuo*. A buffer solution was added to the residue and sonicated to give a clear solution of the sample which can be kept for weeks as a clear solution.

A Preliminary Fractionation of Hot Aqueous Extract A hot aqueous extract of *Centipeda minima* herbs was fractionated with a different concentration of EtOH to give the most active 70% EtOH precipitate, 57% inhibition in the mast cell bioassay at 20 μg/ml concentration. The precipitate was separated on Toyopearl HW-40C with H_2O–EtOH as an eluent to give the most active fraction, 100% inhibition at 33 μg/ml concentration. The elemental analysis of the active fraction gave a composition of C, *ca.* 40%; H, *ca.* 4% and N, *ca.* 1.6%. ^{13}C-NMR spectral data of the active fraction indicated that it probably consisted of polysaccharides and proteins, however further characterization was not possible.

A Preliminary Extraction with Organic Solvents and Activity Testings *Centipeda* herbs (100 g) purchased in the Hongkong market were successively extracted with ether, MeOH and H_2O. Upon removal of solvents *in vacuo* ether extract (2.9 g), MeOH extract (10 g) and H_2O extract (2.1 g) were obtained. The extracts equivalent to 10 g herbs/kg dose were prepared in saline with Tween 80 and subjected to PCA test (Table I).

A Large Scale Extraction, Fractionation and Separation *Centipeda minia* herbs (700 g) were extracted with 5 l $CHCl_3$ for 5 times and then with 5 l MeOH for 5 times. The extraction was repeated for 7 times to extract 5 kg of material. $CHCl_3$ extracts were concentrated to 3 l *in vacuo* and 2 l H_2O was added to disperse into H_2O (fr. CW) and $CHCl_3$. $CHCl_3$ was removed from the $CHCl_3$ solution and the residue was dissolved in MeOH (3 l). H_2O (500 ml) was added to the MeOH solution and fractionated with hexane (2 l) to give frs. CH and CM. The MeOH extract of the material was concentrated to 3 l *in vacuo* and diluted with H_2O (500 ml). The solution was fractionated with hexane to give fr. MH (upper layer) and fr. MM (lower layer).

Separation of Fr. CM Fraction CM was fractionated with LH-20 column chromatography using $CHCl_3$, acetone and MeOH to give 4 fractions, frs. 1—4 as it appears in Chart 1. Further separation of fr. 4 with LH-20 gave two active fractions, fr. 4-3 and 4-4. The fr. 4-3 was further separated with LH-20 (acetone) to afford compound CM-1. The fr. 4-4 was also separated with LH-20 (acetone) followed by silica-gel ($CHCl_3$–MeOH, 19 : 1) to give two yellow compounds CM-2 and CM-3. The most active fr. 1 was subjected to LH-20 separation with EtOAc, $CHCl_3$ and MeOH. The EtOAc fraction was divided into two parts, one of which was further chromatographed twice with silica-gel ($CHCl_3$–acetone, 49 : 1; benzene–acetone, 24 : 1), three times with LH-20 (hex-

ane–$CHCl_3$, 3 : 2; MeOH × 2) to remove chlorophylls and finally with RP-8 (MeOH–H_2O, 65 : 35) to give CM-4, 890 mg. The other part was chromatographed first on LH-20 (hexane–CH_2Cl_2, 1 : 4), twice on silica-gel ($CHCl_3$; ether–benzene, 1 : 9), four times on LH-20 (H_2O–MeOH, 2 : 3, acetone, EtOAc, MeOH) and finally on silica-gel to give CM-5, 450 mg. The fraction eluted by $CHCl_3$ from LH-20 column chromatography of fr. 1 was further separated with LH-20 (MeOH), twice with silica-gel ($CHCl_3$–MeOH, 9 : 1 × 2) and three times with Lobar RP-8 (H_2O–MeOH, 1 : 4 × 3) to give CM-6, 15 mg.

Quercetin-3,3'-dimethyl Ether (CM-1) Yellow needles from benzene, mp 259—263 °C (lit. 256—260 °C).[4]

Quercetin-3-methyl Ether (CM-2) Yellow needles from EtOH–H_2O, mp 273—276 °C (lit. 173—175 °C).[5]

Isobutyroylplenolin (Isobutyroyl-11β-11,13,-dihydrohelenalin, Arnicolide C, CM-4, 1) Colourless needles from EtOH, mp 135—137 °C (lit. 137—138 °C).[6] $[\alpha]_D^{26}$ −80° (c = 1.0, EtOH) (lit. −91.1°) HR-MS m/z: Calcd for $C_{19}H_{26}O_5$ 334.1777, Found 334.1775. UV λ_{max}^{MeOH} (log ε): 277 (3.34). IR ν_{max}^{KBr} cm^{-1}: 1765, 1730, 1718, 1580. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.03 (3H, s, C-15), 1.04 (3H, d, J = 6.8 Hz, C-18 or 19), 1.08 (3H, d, J = 6.8 Hz, C-18 or 19), 1.24 (3H, d, J = 6.6 Hz, C-14), 1.51 (3H, d, J = 7.6 Hz, C-13), 1.65 (1H, ddd, J = 1.7, 11.0, 15.4 Hz, C-9a), 2.22 (1H, m, C-10), 2.41 (1H, sept, J = 7.1 Hz, C-17), 2.48 (1H, ddd, J = 2.2, 6.1, 15.4 Hz, C-9b), 2.83 (1H, dd, J = 6.4, 10.1 Hz, C-11), 3.02—3.10 (1H, m, C-1), 3.09 (1H, dq, J = 10.1, 7.6 Hz, C-11), 4.76 (1H, ddd, J = 1.7, 6.1, 6.4 Hz, C-8), 5.40 (1H, brs, C-6), 6.06 (1H, dd, J = 3.2, 6.0 Hz, C-3), 7.68 (1H, dd, J = 1.9, 6.0 Hz).

Senecioylplenolin (Senecioyl-11β-11,13-dihydrohelenalin; CM-5, 2)[8] Colourless prisms from MeOH, mp 187—190 °C. $[\alpha]_D^{24}$ −47.6° (c = 1.0 EtOH). HR-MS m/z: Calcd for $C_{20}H_{26}O_5$ 346.1777, Found 346.1740. UV λ_{max}^{EtOH} nm (log ε): 222 (4.0). IR $\nu_{max}^{chloroform}$ cm^{-1}: 1770, 1722, 1715, 1580. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.04 (3H, s, C-15), 1.24 (3H, d, J = 6.8 Hz, C-14), 1.54 (3H, d, J = 7.6 Hz, C-13), 1.68 (1H, ddd, J = 2.0, 11.0, 15.4, C-9a), 1.85 (3H, d, J = 1.6, C-20), 2.13 (3H, d, J = 1.6 Hz, C-19), 2.21 (1H, m, C-10), 2.46 (1H, ddd, J = 2.2, 6.1, 15.3, C-9b), 2.91 (1H, dd, J = 6.4, 10.3 Hz, C-7), 3.05—3.12 (1H, m, C-1), 3.09 (1H, dq, J = 10.3, 6.1 Hz, C-11), 4.75 (1H, ddd, J = 1.7, 6.1, 6.4 Hz, C-8), 5.46 (1H, brs, C-6), 5.49 (1H, sept, J = 1.5 Hz, C-17), 6.05 (1H, dd, J = 3.2, 6.1 Hz, C-3), 7.66 (1H, dd, J = 1.9, 6.0 Hz, C-2).

Aurantiamide Acetate (CM-6, 3) Colourless needles from EtOH, mp 188—190 °C (lit. 188 °C).[8] *Anal.* Calcd for $C_{27}H_{28}N_2O_4$: C, 72.95; H, 6.35; N, 6.30. Found: C, 72.76; H, 6.33; N, 6.20. MS m/z: 444 (M^+), 353, 252, 224, 105 (base peak), 91, 77. $[\alpha]_D^{30}$ −28.6° (lit. −23.6°).

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Biologically Active Constituents of *Melaleuca leucadendron*: Inhibitors of Induced Histamine Release from Rat Mast Cells

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Chloroform and methanol extracts of the fruits of *Melaleuca leucadendron* strongly inhibited histamine release from rat mast cells induced by compound 48/80 or concanavalin A. Ursolic acid, a triterpene, was the most active compound contained in the chloroform extract and two stilbenes, piceatannol and oxyresveratrol, were isolated as active compounds from the methanol extract. Several other stilbenes and related compounds were examined to obtain information on the structure activity relationships of stilbenes.

Keywords *Melaleuca leucadendron*; stilbene; piceatannol; oxyresveratrol; ursolic acid; anti-allergy; mast cell

Melaleuca leucadendron L. (Myrtaceae) is a large tree growing both wild and cultivated in tropical Asia. The leaves of the tree have been used to produce an essential oil, cayuput oil,[1] while the tiny fruits of this plant have been used as a medicinal drug in traditional Indonesian Jamu medicine.[2] The fruits are called Borong-Borong in Indonesia, which means 'many holes'. The Indonesian name derives from the hole of the tiny fruit. This Jamu drug is used as a tonic and is available in the herbal medicine shops in Java and Bahli islands where Jamu herbal drugs are used in daily health care. During a survey study of Indonesian Jamu medicinal drugs we purchased the material at Sukabumi, a city in east Java. The aqueous ethanol extract of the fruits that was prepared in small scale was subjected to random screening with *in vitro* bioassay systems which were routinely used in our studies on medicinal plants.[3] It was highly active in a bioassay testing the inhibitory effect on histamine release from rat mast cells. The mast cell bioassay system has been used to find potential anti-allergy compounds[4] and has good correlation with passive cutaneous anaphylaxis (PCA) test which is a standard bioassay to evaluate anti-allergy activity.[4a] The *in vitro* mast cell bioassay has been extensively used in our bioassay oriented phytochemical studies on medicinal plants. Flavonoids, coumarins, lignans and sesquiterpenes were found as potential anti-allergy compounds which were highly active in the mast cell bioassay as well as PCA test.[4] Since no reports were available on the constituents of the fruits of *M. leucadendron*, we investigated the active constituents of the medicinal drug with *in vitro* monitoring of the mast cell bioassay. To clarify the chemical properties of active constituents the fruits of *M. leucadendron* (200 g) were successively extracted with hexane, chloroform, acetone and methanol. The inhibitory effects of the extracts to the mast cell bioassay are shown in Table I. Except for the hexane extract all the other extracts exhibited significant inhibitory activities on histamine release. Following this observation, 5 kg of this plant material was successively extracted with chloroform, acetone and methanol to isolate and identify bio-active constituents in the extracts.

Extensive separation of the chloroform extract by column chromatographies afforded six known triterpenoids, betulinic acid,[5] acetyl betulinic acid,[5] betulinaldehyde,[6] pyracrenic acid,[7] ursolic acid and ursolaldehyde,[8] and one known sesquiterpene, globulol.[9] Ursolic acid was contained 5.6% in the chloroform extract and inhibited compound 48/80 induced histamine release 95% and 26% at concentrations of 10^{-3} and 10^{-4} M, respectively. Since the activities of the other terpenoids are not significant, ursolic acid can be regarded as the main active compound that contributes to the bioactivity of the chloroform extract.

The methanol extract was fractionated with silica-gel column chromatography and the biological activities of obtained fractions were monitored by the mast cell bioassay. (Chart 1). Five compounds were so far isolated from active fractions and tentatively named ML-1—ML-5. ML-1 and ML-5 were identified as quercetin and gallic acid, respectively. ML-2 was found to be a phenolic compound and identified as piceatannol (**1**), a stilbene by the comparison of spectral data.[10] The chemical and spectral properties of ML-3 were similar to those of piceatannol (**1**) and it was finally identified as oxyresveratrol (**2**) by the comparison of its spectral data with those reported.[11] The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of ML-4 suggested it to be a dimeric stilbene and finally identified it as scirpusin B (**3**).[10b]

Quercetin (ML-1) inhibited concanavalin A (Con A) induced histamine release nearly 100% at a concentration of 10^{-4} M and the result is well in accord with those reported elsewhere.[4,12] Monomeric stilbenes, **1** and **2**, were highly active and in particular the IC_{50} value of piceatannol (**1**) was 9.6 μM in an experiment using Con A as a histamine releaser. The activities of monomeric stilbenes are comparable to those of active flavonoids. To obtain information on structure activity relationships of stilbenes we investigated inhibitory activities of available stilbenes and related compounds. The results are summar-

TABLE I. Inhibitory Effects of *Melaleuca leucadendron* Extracts on Histamine Release from Rat Mast Cells Induced by Compound 48/80 or Con A

	Inhibition % of histamine release			
	Inducer			
	Compound 48/80		Con A	
	50 μg/ml	500 μg/ml	50 μg/ml	500 μg/ml
Hexane ext.	19	84	6	47
Chlololform ext.	30	100	5	88
Acetone ext.	40	100	2	95
Methanol ext.	28	70	14	81
DSCG	15	40	14	32

DSCG: disodium cromoglycate.

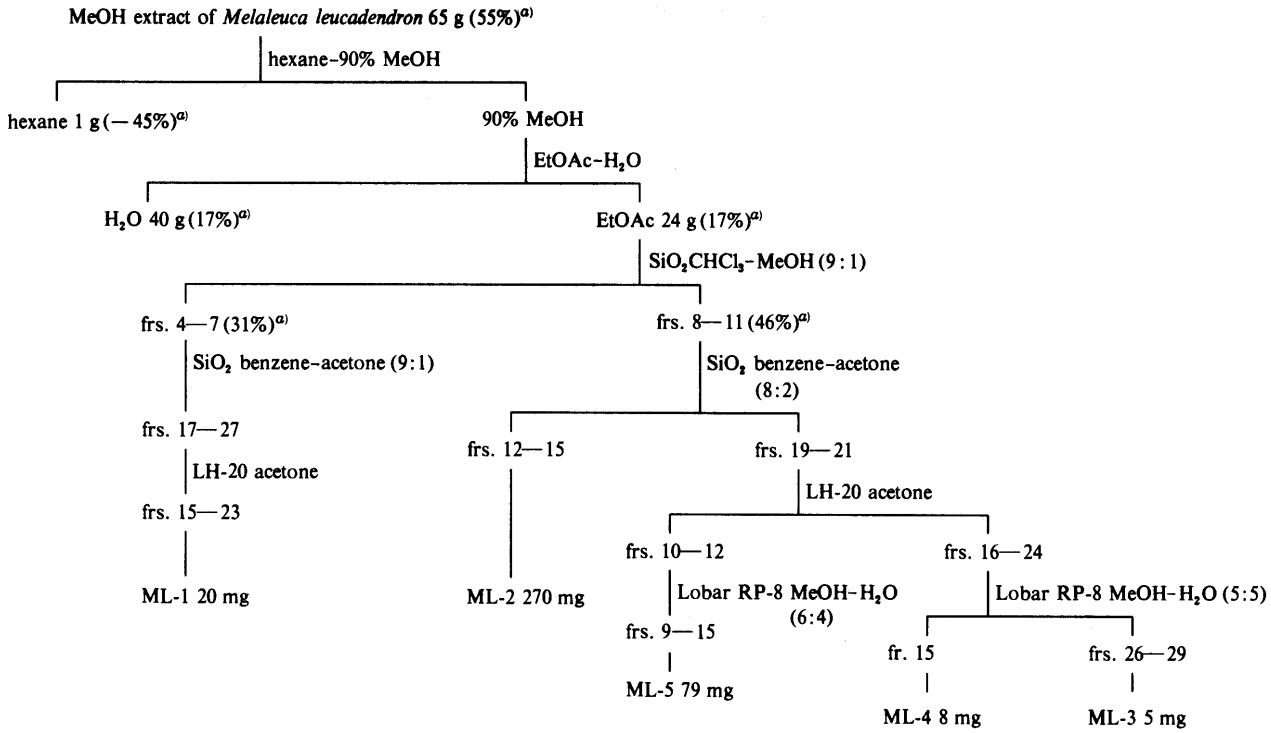


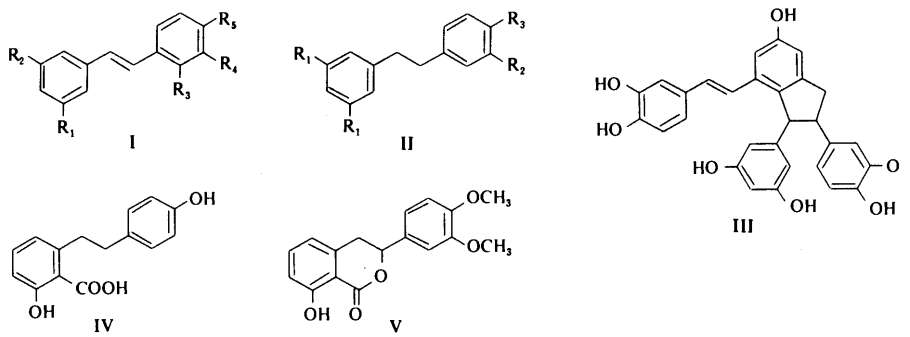
Chart 1. Fractionation of the MeOH Extract of *Melaleuca leucadendron*

a) Inhibition % for histamine release from mast cells induced by compound 48/80 at a substrate concentration of 50 μg/ml.

TABLE II. Inhibitory Effects of Stilbenes on Histamine Release from Rat Mast Cells Induced by Compound 48/80 or Con A

	R_1	R_2	R_3	R_4	R_5	Inhibition % of histamine release				IC_{50} μM
						Inducer				
						Compound 48/81		Con A		
						10^{-3} M	10^{-4} M	10^{-3} M	10^{-4} M	
Ia (**1**)	OH	OH	H	OH	OH	86	40	108	103	9.6
Ib (**2**)	OH	OH	OH	H	OH	31	26	103	84	22
Ic (**4**)	OH	OH	H	OH	OMe	74	28	108	108	22
Id (**5**)	H	H	H	OMe	OH	12	2	107	92	35
Ie (**6**)	OH	O-G	H	OH	OH	11	14	32	23	
If (**7**)	OH	O-G	H	OH	OMe	12	14	32	15	
IIa (**8**)	OH	OH	OH			76	14	107	97	27
IIb (**9**)	H	OH	OMe			−15	−6	49	25	
IIc (**10**)	H	OMe	OH			−46	−21	38	8	
III (**3**)						25	10	84	22	
IV (**11**)						90	27	105	60	53
V (**12**)						−8	−4	97	84	9.5

O-G = *O*-glucoside. Ia, piceatannol (**1**); Ib, oxy-resveratrol (*2*); Ic, rhapontigenin (**4**); Id, 4-hydroxy-3-methoxystilbene (**5**); Ie, piceatannol glucoside (**6**); If, rhaponticin (**7**); IIa, dihydropiceatannol (**8**); IIb, 3,4-dihydroxydihydrostilbene (**9**); IIc, 4-hydroxy-3-methoxydihydrostilbene (**10**); III, scirpusin B (**3**); IV, lunuralic acid (**11**); V, monomethylphylodulcin (**12**).



ized in Table II. In general, monomeric stilbenes (**1**, **2**, **4**, **5**) were highly active except for glucosides (**6**, **7**). A very interesting compound is monomethyl phylodulcin (**12**) which showed selective inhibition to Con A induced histamine release, but it did not inhibit histamine release induced by compound 48/80. The inhibitory effect of piceatannol (**1**) on *in vitro* anti-immunoglobulin E (IgE)-induced histamine release from human basophils was reported quite recently and the result is well in accord with our observation.[13] The two benzene rings of stilbene structure are somewhat similar to those of flavonoid whose anti-allergic activity has been firmly established.[12] This indicates that the presence of two benzene rings with an appropriate distance may be essential to their common inhibitory effect on induced histamine release from mast cells.

Experimental

^{1}H- and ^{13}C-NMR spectra were measured on a JEO JNM FX-100 or a FX-400, mass spectra (MS) on a JEOL JMS-DX300, infrared (IR) on a JASCO model 701G and ultraviolet (UV) on a Hitachi spectrophotometer model 100-60. Melting points were determined on a Yanagimoto micro-melting point apparatus and uncorrected. The fruits of *M. leucadendron* were purchased in a Jamu shop in Skabumi, Indonesia. Samples for reference and testing of bioactivity were obtained from the following sources. Betulinic acid: Prof. H. Ageta, Showa College of Pharmacy; betulinaldehyde: Dr. H. Miles, Mississippi State University; piceatannol (**1**), rhapontigenenin (**4**), piceatannol glucoside (**6**), rhapontici-n (**7**): Prof. I. Nishioka, Kyushu University; scirpusin B (**3**): Dr. H. Taguchi, Tsumura Co.; 4-hydroxy-3-methoxystirbene (**5**), 3,4-dihydroxy-dihydrostirbene (**9**), 4-hydroxy-3-methoxydihydrostirbene (**10**), mono-methylphylodulcin (**12**): Prof. M. Yamato, Okayama University. The mast cell bioassay was purformed as described in previous papers.[4]

Small Scale Extraction Dried fruits of *M. leucadendron* (200 g) were each extracted twice successively each twice with 500 ml hexane, $CHCl_3$, acetone and MeOH. The solvents were removed *in vacuo* to give hexane (6 g), $CHCl_3$ (13 g), acetone (12 g) and MeOH (7 g) extracts, which were submitted to the *in vitro* mast cell bioassay. (Table I)

Large Scale Extraction, Fractionation and Separation Dried fruits of *M. leucadendron* (5 kg) were extracted each twice with $CHCl_3$, acetone and MeOH. On evaporation of solvents *in vacuo*, $CHCl_3$, acetone and MeOH extracts were obtained in yields of 453, 287 and 179 g, respectively. A part of $CHCl_3$ extract (36 g) was chromatographed over silica-gel with benzene–acetone (99 : 1—90 : 10) and 106 eluted fractions were combined into four active fractions, frs. 20—29, 44—49, 50—55 and 56—66. Upon repeated fractionation with silica-gel frs. 20—29 gave globulol (570 mg), betulinaldehyde (83 mg), acetylbetulinic acid (32 mg) and ursolaldehyde (5 mg). Fractions 44—49 were further separated with silica-gel column to give pyracrenic acid (160 mg). From frs. 50—55 and 56—60 gave crystals of betulinic acid (2.1 g) and ursolic acid (2.0 g), respectively.

A part of MeOH extract (65 g) was subjected to fractionation as shown in Chart 1. The MeOH extract was partitioned between hexane and 90% aqueous MeOH, and after removing the solvent the MeOH fraction was again partitioned with EtOAc–H_2O. The EtOAc fraction was subjected to silica-gel column chromatography and the active fractions were combined into two fractions, frs. 4—7 and frs. 8—11, which were further separated with silica-gel, Sephadex LH-20 and Lobar RP-8 columns to give compounds ML-1—ML-5. ML-1 and ML-5 were readily identified as quercetin and gallic acid, respectively.

Piceatannol (ML-2, 1)[10] Slightly brown plate from MeOH–$CHCl_3$, mp 236—237 °C. MS *m/z* (rel. int.): 244 (M^+, $C_{14}H_{12}O_4$, 100), 243 (10). Upon acetylation with pyridine and acetone **1** gave a tetraacetate of mp 120—122 °C (H_2O–EtOH). ML-2 was identified as piceatannol by the comparison of spectral data of those of authentic samples of piceatannol and its acetate.

Dihydropiceatannol (8) **1** (50 mg) was hydrogenated with Pt–H_2 in AcOEt for 2 h at r.t. The product was purified with Lobar RP-8 column with MeOH–H_2O (5 : 5) and recrystallized from MeOH–H_2O to give dihydropiceatannol (18 mg) of mp 158—160 °C. MS *m/z* (rel. int.): 246 (M^+, $C_{14}H_{14}O_4$, 100), 245 (14). ^{1}H-NMR (MeOH-d_4, 100 MHz) δ: 2.67 (4H, s, $CH_2 \times 2$), 6.0—6.2 (3H, m, C-2′, C-4′ and C-6′), 6.47 (1H, dd, J=2.0, 7.5 Hz, C-6), 6.60 ((1H, d, J=2.0 Hz, C-2), 6.65 (1H, d, J=7.5 Hz, C-5).

Oxyresveratrol (ML-3, 2)[11] Pale brown powder. MS *m/z* (rel. int.): 244 (M^+, $C_{14}H_{12}O_4$, 100), 243 (12). ML-3 was identified by the comparison of spectral data with those reported.

Scirpusin B (ML-4, 3)[10b] Pale yellow powder. MS *m/z* (rel. int.): 486 (M^+, $C_{28}H_{22}O_8$, 100), 378 (85), 244 (16), 242 (27).

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Improved Method for Measurement of Rhodanese Activity Using Methanethiosulfonate as Sulfur Donor Substrate and Its Application to Human Serum

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We have developed a sensitive method for the measurement of rhodanese activity in human serum which is based on the colorimetric method for the determination of thiocyanate produced from methanethiosulfonate and cyanide as substrates. Thiocyanate gives a red complex with ferric ion in an acidic condition. The present method is about 70-fold more sensitive than the conventional method using cyanide and thiosulfate as substrates and correlates well ($r=0.997$) with the conventional method in bovine liver rhodanese. Within-run precision of the method is 0.91% for 420 units/l serum and the calibration curve is linear up to 1850 units/l. The normal value for human serum, determined by the present method on 31 healthy persons, was 20.9± 20.0 units/l (mean ±2S.D.). Rhodanese activity was clearly elevated in some serum samples which were observed at abnormal values in some biochemical diagnostic tests and showed significant positive correlations with guanase activity ($r=0.728$, $p<0.01$) and glutamic-oxalacetic transaminase activity ($r=0.625$, $p<0.01$).

Keywords rhodanese activity; colorimetric method; ferric thiocyanate complex; methanethiosulfonate; bovine liver rhodanese; human serum; thiosulfonates; cyanide

Rhodanese (thiosulfate: cyanide sulfurtransferase, EC 2.8.1.1) is widely distributed in the living organism as a mitochondrial enzyme and catalyzes the transfer of sulfane sulfur atom of thiosulfate to a nucleophilic acceptor such as cyanide to form thiocyanate.[1] In mammalian tissues, the highest activity is present in liver and kidney.[2] Biological functions of the enzyme are considered to be the detoxication of cyanide and sulfide[3] and the incorporation of sulfur in the Fe–S center of iron–sulfur proteins.[4] Several assay methods for rhodanese activity have been reported,[5] and the method of Sörbo[6] which measures the formation of thiocyanate from thiosulfate and cyanide is widely accepted. The produced thiocyanate is determined colorimetrically with ferric ion in the acidic condition. Recently, the enzyme activity has been demonstrated in low activity samples such as erythrocytes,[7] leukocytes,[8] and serum.[9] However, for the measurement of these low activity samples, the method of Sörbo is not sufficient in sensitivity. In the investigation of the mechanism of rhodanese reaction, Mintel and Westley[10] reported that some thiosulfonates had higher reaction velocity than that of thiosulfate as donor substrate of the enzyme and methanethiosulfonate was a good alternative sulfur-donating substrate for rhodanese. However, they did not investigate the assay method for the enzyme using methanethiosulfonate. In this study, we have developed a high sensitive method for the assay of low level rhodanese activity using methanethiosulfonate as the sulfur donor substrate and applied this method to measure rhodanese activity in human serum.

Experimental

Materials The following four thiosulfonates were synthesized as sodium salts from parent sulfonyl chlorides and sodium sulfide by the method of Mintel and Westley[10]: methanethiosulfonate (MTS), ethanethiosulfonate (ETS), benzenethiosulfonate (BTS), *p*-toluenethiosulfonate (TTS). Sodium thiosulfate pentahydrate was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Tris(hydroxymethyl)aminomethane (Tris) and potassium cyanide were obtained from Wako Pure Chemical, Ltd. (Osaka, Japan). Dithiothreitol (DTT), tris(hydroxymethyl)methylglycine (Tricine), *N*,*N*-bis(2-hydroxyethyl)glycine (Bicine), and 2-(cyclohexylamino)ethanesulfonic acid (CHES) were purchased from Dojin Kagaku (Kumamoto, Japan). Bovine liver rhodanese (type II, 90 units/mg), human transferrin, and human ceruloplasmin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine liver rhodanese was dissolved at an appropriate concentration in 0.1 M glycine-acetate (pH 5.0) containing 0.025% bovine serum albumin. Rhodanese solution was divided into aliquots and stored at −20 °C. Bilirubin control® was obtained from American Dade (Aguada, Puerto Rico). All other chemicals were of analytical grade. Acceptor substrate buffer containing 62 mM potassium cyanide, pH 8.6, was prepared as follows: buffer A, to a 40 ml of buffer containing Tris (0.454 g, 3.75 mmol) and phosphoric acid (0.245 g, 2.5 mmol), potassium cyanide (0.202 g, 3.1 mmol) was added and made up to 50 ml with distilled water; buffer B, to a 40 ml of buffer containing Tris (0.454 g, 3.75 mmol) and potassium dihydrogenphosphate (0.340 g, 2.5 mmol), potassium cyanide (0.202 g, 3.1 mmol) was added and made up to 50 ml with distilled water. The pH of buffer B (pH 8.9) was adjusted with buffer A (pH 8.1) to 8.6 at 25 °C. This buffer was stable for a week in the refrigerator. Each aqueous solution of 0.3 M DTT and 0.17 M MTS (donor substrate) was freshly prepared.

Assay Procedure To 0.8 ml of the acceptor substrate buffer, 0.1 ml of 0.3 M DTT and 0.1 ml of 0.17 M MTS are added. After preincubation for 1 min at 25 °C, 0.1 ml of sample is added, and the reaction mixture is incubated for 5 min at 25 °C to form thiocyanate. After the enzyme reaction is stopped by the addition of 0.2 ml of formaldehyde (38%), 0.1 ml of distilled water, 0.2 ml of 0.23 M thiosulfate, and 2.5 ml of 0.25 M ferric perchlorate in 28% perchloric acid are added serially to produce a red complex of thiocyanate with ferric ion. The mixture is centrifuged for 10 min at 10000 × *g* before the measurement. The sample blank is similarly constituted except that the sample is added, instead of distilled water, after the addition of formaldehyde. The absorbance of the supernatant is measured at 460 nm against a sample blank by using a Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan).

Calculations One unit (U) of rhodanese activity is defined as that amount of enzyme which produces 1 μmol of thiocyanate per minute at 25 °C under the present conditions. Enzyme activity per liter is calculated from

$$\text{rhodanese activity, U/l} = \frac{A/\text{min} \times 4.1 \times 10^6}{4320 \times 0.1 \times 1.0}$$

A/min : change value in absorbance per min
4320 : apparent molar absorptivity of thiocyanate complex with ferric ion
4.1 : total volume (ml)
0.1 : sample volume (ml)
1.0 : cell length (cm)

Other Procedures As an alternative procedure, the rhodanese activity was assayed also by the method of Sörbo.[6] Guanase activity was determined using a commercial kit of Toyobo (Osaka, Japan). Glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, and blood urea nitrogen were assayed by using commercial kits of Kainos Lab., Inc. (Tokyo, Japan).

Results

Optimization of Rhodanese Assay Conditions The assay conditions were investigated at 25 °C using bovine liver rhodanese. The effect of the donor substrate on rhodanese activity was examined by the use of thiosulfate and four thiosulfonates, MTS, ETS, BTS, and TTS. The highest reaction velocity, about 95 times that of thiosulfate, was obtained when MTS was used as a sulfur donor. However, in the spontaneous transfer reaction with cyanide, MTS was about 12 times greater than that of thiosulfate. The spontaneous reaction of the thiosulfonates with cyanide was greater in the order of ETS, MTS, BTS, and TTS. ETS showed less non-enzymatic reaction than that of MTS, but its reaction by rhodanese was not higher than MTS. Reaction velocities of thiosulfate and thiosulfonates for bovine liver rhodanese were examined by double reciprocal plots. From the data, the values of K_m (mM) and V_{max} (μmol/min) of the thiosulfonates and thiosulfate were 5.7 and 127 for MTS, 9.0 and 24.2 for ETS, 6.1 and 18.7 for BTS, 3.2 and 10.7 for TTS, 4.0 and 1.23 for thiosulfate, respectively. Figure 1 shows double reciprocal plots of four thiosulfonates. Considering the enzymatic and spontaneous reactions, MTS was chosen as a donor substrate. A K_m value of cyanide when MTS was used as a donor substrate, was 16 mM. Concerning the effect of pH on rhodanese activity, the experiments were carried out at 8.25—9.90 by adjusting the phosphate concentration of Tris–phosphate buffer. A maximum thiocyanate formation was observed at pH 9.0—9.5, but the spontaneous reaction also increased markedly at a higher pH (above 9.0). Therefore, the condition at pH 8.6, which showed a blank absorbance less than 0.3 and relatively high enzyme activity, was chosen. The effect of the buffer species on rhodanese activity was examined using glycine, Bicine, Tricine, and CHES instead of Tris, but good results were not obtained. Since the optimum concentration of MTS, donor substrate, was in the range of 8 to 15 mM in the reaction mixture and the substrate inhibition was observed at the concentrations above 15 mM, 15 mM was chosen as the optimum concentration. The optimum concentration of cyanide as an acceptor substrate was at concentrations above 70 mM, but too much spontaneous reaction also occurred at these conditions. Therefore, cyanide concentration was chosen as 45 mM in the reaction mixture. The enzyme activity of bovine liver rhodanese solution stored at −20 °C was gradually decreased. Since the enzyme activity was restored by the addition of DTT in the concentration of 5 mM or above in the reaction mixture and blank absorbance also decreased with increasing DTT concentrations, the DTT concentration was maintained at 27 mM. The data suggest that DTT contributes not only to stabilization of the enzyme but also to prevention of the spontaneous reaction between MTS and cyanide. When color development was achieved by the addition of ferric perchlorate in perchloric acid solution to the reaction mixture, a turbidity which was not protein denature gradually appeared. Since this turbidity was avoided by the addition of thiosulfate at concentrations over 5.5 mM, 11 mM thiosulfate was added to the reaction mixture to prevent the turbidity. Under the condition, color development was stable for at least 60 min.

Estimation of Present Method Figure 2 shows the time course of the enzyme catalyzed reaction by bovine liver rhodanese. The reaction proceeded linearly for at least 15 min. The present method was compared with the method of Sörbo[6] using appropriate dilutions of bovine liver rhodanese. In the assays, the present method used 100 times dilution samples of that of Sörbo's method with 0.1 M glycine-acetate (pH 5.0) containing 0.025% bovine serum albumin. Both methods were correlated well ($r=0.997$), and the present method showed about 95-fold higher activity than the method of Sörbo,[6] as shown in Fig. 3. The detection limit of the present method was defined as 7.6

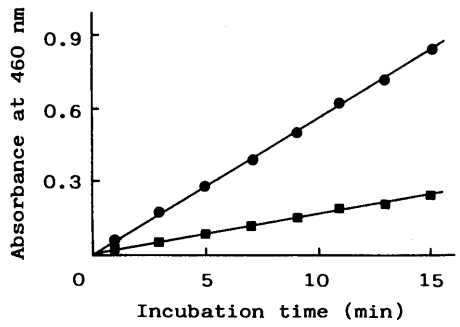

Fig. 2. Time Courses of Rhodanese Reaction

Bovine liver rhodanese activity: ●, 540 units/l; ■, 160 units/l. Assay conditions were as described under Experimental except for incubation time.

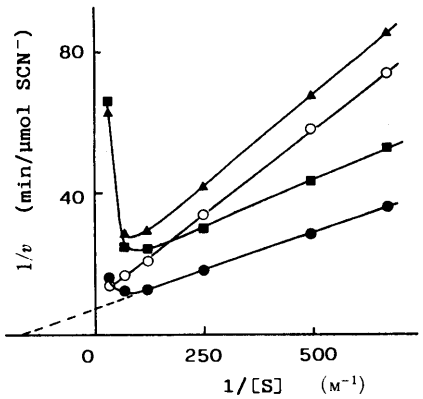

Fig. 1. Double Reciprocal Plots for Rhodanese Reaction of Thiosulfonates

●, MTS; ○, ETS; ■, TTS; ▲, BTS. Assay conditions were as described under Experimental except for species of donor substrate and its concentration.

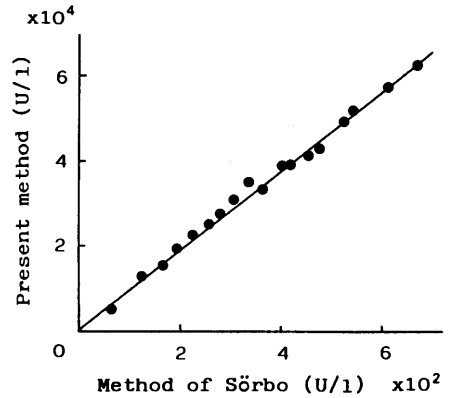

Fig. 3. Correlation between the Present Method and the Method of Sörbo in Bovine Liver Rhodanese Activity

Bovine liver rhodanese was dissolved at appropriate concentrations with 0.1 M glycine-acetate (pH 5.0) containing 0.025% bovine serum albumin. In the assays, the present method used 100 times dilution samples of that of Sörbo's method with the above glycine-acetate buffer. $Y=93.7x+551$, $r=0.997$, $n=18$.

units/l (absorbance = 0.004) by 2-fold of the standard deviation of sample blank absorbance.

Measurement of Rhodanese Activity in Human Serum The linearity of the calibration curve was examined by serial dilutions of two human sera having high rhodanese activities (2320 units/l, 1560 units/l). As shown in Fig. 4, the calibration curve is linear at least up to 1850 units/l, corresponding to the absorbance of 0.97. Rhodanese activity in human serum was measured on 31 normal subjects of both sexes. The enzyme activities in normal subjects are 20.9 ± 20.0 units/l (mean $\pm$ 2S.D.), as shown in Table I. Table II gives the results for within-run and between-run precision studies. The precision of the present method is satisfactory. The influence of the foreign substances was examined. Additions of Bilirubin control® (up to 20 mg/dl), hemolysate (up to 200 mg/dl as hemoglobin), human transferrin (up to 1000 mg/dl), and human ceruloplasmin (up to 100 mg/dl) to serum did not interfere with the assay.

Comparison with Other Certain Biochemical Diagnostic Parameters Using the serum which was observed at abnormal values in some diagnostic tests, rhodanese activity was compared with the activities of guanase (EC 3.5.4.3), glutamic-oxalacetic transaminase (EC 2.6.1.1, GOT), and glutamic-pyruvic transaminase (EC 2.6.1.2, GPT) and the concentration of blood urea nitrogen (BUN). Rhodanese activity showed significant positive correlations with guanase activity ($p<0.01$) and GOT activity ($p<0.01$), as shown in Fig. 5. It was also significantly correlated ($p<0.02$) with GPT activity. On the other hand, no significant correlation was observed between rhodanese activity and BUN.

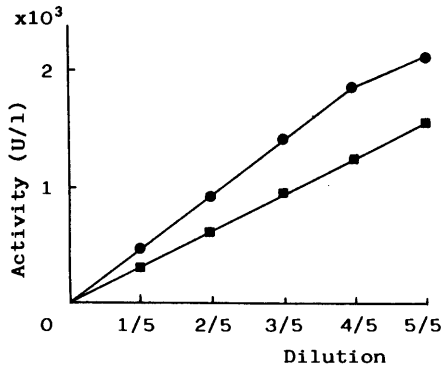

Fig. 4. Linearity of Calibration Curves in Human Serum

●, human serum (2320 units/l) was diluted serially with a saline. The value of 2320 units/l was calculated from that of 4/5 dilution, because the original serum rhodanese activity went up to over the linear range; ■, 1560 units/l serum was diluted serially with a saline.

Discussion

We have examined various assay conditions for the measurement of low rhodanese activity using thiosulfonates as sulfur donor substrates and established a highly sensitive method using MTS as a sulfur donor. In the study of donor substrate, we obtained similar results to those reported by Mintel and Westley[10] except that TTS showed a higher spontaneous reaction than BTS. We presume that this disagreement is due to the difference of reaction conditions. In the present method, the actual sensitivity is about 70-fold higher that of the conventional method reported by Sörbo.[6] Despite a slightly high blank absorbance (about 0.27) due to the spontaneous sulfur transfer reaction between the substrates, the present method is satisfactory in the

TABLE I. Rhodanese Activity in Normal Human Serum

	Rhodanese activity (U/l)			
	n	Mean	(Range)	2 S.D.
Male	15	21.5	(11.4—36.1)	12.7
Female	16	20.3	(ND.[a]—47.5)	25.4
Total	31	20.9	(ND.[a]—47.5)	19.1

a) ND.; below detection limit (<7.6 U/l).

TABLE II. Precision Data for the Present Method in Human Serum

	Within-run ($n=10$)[a]				Between-run ($n=10$)[b]			
	Absorbance at 460 nm				Absorbance at 460 nm			
	Mean	S.D.	CV[c] (%)		Mean	S.D.	CV[c] (%)
Sample 1	0.020	0.0014	7.3	Sample 4	0.020	0.0014	7.2
2	0.221	0.0020	0.91	5	0.143	0.0055	3.8
3	0.533	0.0075	1.4	6	0.455	0.0143	3.1

a) Ten preparations of three sera with different rhodanese activity were measured. The data were expressed as absorbance. *b*) Three sera containing different rhodanese activity were measured in ten separate runs. *c*) Coefficient of variation.

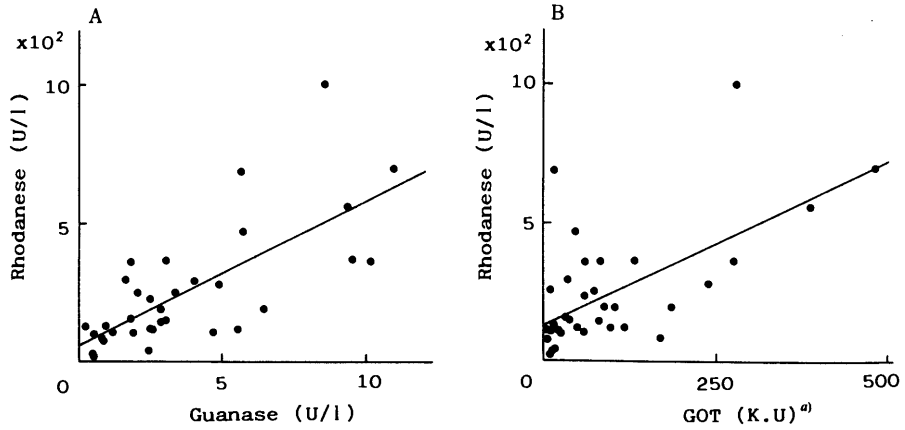

Fig. 5. Correlations between Rhodanese Activity and Guanase Activity (A) or GOT Activity (B) in Human Serum

Sera used were of observed abnormal values in some diagnostic tests. Assay conditions were described under Experimental. *a*) GOT activity was expressed as Karmen unit.[11] A, $Y=52.4x+58.9$, $r=0.728$ ($p<0.01$), $n=35$; B, $Y=1.19x+132$, $r=0.625$ ($p<0.01$), $n=35$.

precision and in the linearity of the calibration curve and is considered to be useful for the determination of the enzyme in low activity samples such as serum, erythrocytes, and leukocytes. Rhodanese activity was observed in the serum of healthy subjects, but the mean value was very low compared with liver and kidney.[1] Because of the existence of rhodanese activity in erythrocytes, serum must be prepared carefully to avoid hemolysis, but the activity is not interfered with up to 200 mg/dl as hemoglobin.

We used ferric perchlorate–perchloric acid in place of ferric nitrate–nitric acid used by the usual method as a color producing reagent for the produced thiocyanate. Under physiological conditions, serum thiocyanate is 40% protein-bound,[12] exclusively to albumin. It seems that the produced thiocyanate by rhodanese reaction also binds to albumin. Perchlorate is more excellent to displace thiocyanate from serum albumin than nitrate, although both anions are strong chaotropic agents.[13] Perchloric acid is also an effective deproteinizing agent for serum. The mean recovery of thiocyanate added to serum was quantitative (102%) with three different concentrations of thiocyanate (12, 23, and 115 μM). The apparent molar absorptivities of colored ferric thiocyanate complex obtained by the methods of us and Sörbo were almost same. After the addition of ferric perchlorate in perchloric acid for color development, formation of a turbidity which was not protein denature gradually appeared and this phenomenon was avoided by the addition of thiosulfate, but the mechanisms for appearance and protection of the turbidity are not yet clarified.

Rhodanese activities exceeding normal values were first found in some sera which were observed at abnormal values in some biochemical diagnostic tests. Since high rhodanese activities are observed in human liver and kidney,[14] we compared the results from the present method for rhodanese activities in abnormal sera with those obtained by four biochemical diagnostic tests of liver and kidney disorders, *i.e.*, guanase, GOT, GPT, and BUN. Rhodanese activity showed significant positive correlations with guanase ($r=0.728$), GOT ($r=0.625$), and GPT ($r=0.413$) levels, which are pathological indexes of liver disease. Although more detailed evaluation is needed, these results suggest that the high serum rhodanese activity may be derived from liver. In the present study, rhodanese activities did not correlate significantly with BUN concentrations as a functional index of kidney disease. Further detailed investigations on the relationship of serum rhodanese activity with various pathological conditions, including liver and kidney diseases, is desired.

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Chemical Conversion of Corticosteroids to 3α,5α-Tetrahydro Derivatives. Synthesis of 5α-Cortol 3-Glucuronides and 5α-Cortolone 3-Glucuronides

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The synthesis of the 3-glucuronides of 5α-cortol-20α, 5α-cortolone-20α and their 20β-epimers is described. The 5α-cortol 20,21-diacetates (12, 17) and 5α-cortolone 20,21-diacetates (14, 19) were the key intermediates. Sodium borohydride reduction of the carbonyl group at C-20 in 5α-tetrahydrocortisol 3-*tert*-butyldimethylsilyl ether 17,21-acetonide (8) gave the 20α-hydroxy-acetonide (9). Selective removal of the acetonide ring was successful when the 20α-acetoxy-17α,21-acetonide (10) was treated with 50% acetic acid. Subsequent acetylation with acetic anhydride in pyridine, followed by removal of the protecting group at C-3 in the silyl ether-acetate (11) gave the desired 20α-intermediate (12). The 11-ketone (14) was prepared from 11 by oxidation with pyridinium chlorochromate, followed by desilylation. The 20β-acetates (17, 19) were synthesized from 21-acetoxy-3α,11β,17α-trihydroxy-5α-pregnan-20-one 3-*tert*-butyldimethylsilyl ether (15). Introduction of the glucuronyl residue at C-3 was carried out by means of the Koenigs–Knorr reaction.

Keywords cortisol metabolite; 5α-cortol; 5α-cortolone; 5α-cortol-20α 3-glucuronide; 5α-cortol-20β 3-glucuronide; 5α-cortolone-20α 3-glucuronide; 5α-cortolone-20β 3-glucuronide; Koenigs–Knorr reaction

Cortisol metabolism in humans involves various transformations, for example, reduction of the Δ^4-3-keto group to form 3α-hydroxy products, oxidation at C-11, and reduction at C-20. Most of the metabolites, such as tetrahydrocortisol, tetrahydrocortisone and their 5α-isomers, are excreted in the urine largely as conjugates with glucuronic acid. Measurement of the urinary 17-hydroxycorticosteroids has long been an important index of adrenocortical activity. We have previously prepared the glucuronides of these metabolites for use in metabolic studies and immunoassays of corticosteroids.[1] Mattox *et al.* have also synthesized the 5β-steroid glucuronides.[2]

The existence of the 3-monoglucuronides of 5α-cortol-20α,[3] 5α-cortolone-20α and their 20β-isomers, in addition to the corresponding 5β-metabolites, in the urine[4] and plasma[5] has also been reported. The latter four 5β-compounds have been synthesized earlier.[6] The recent development of high-performance liquid chromatography/mass spectrometry may enable us to characterize individually these polar metabolites or to perform a steroid profile analysis without the enzymatic or acid hydrolysis of the conjugates. For this purpose, it is essential to have authentic steroid samples; this prompted us to prepare possibly rather minor metabolites of cortisol in normal subjects for use as standards in analytical studies. This paper deals with the synthesis of 5α-cortol-20α 3-glucuronide (**24**), 5α-cortolone-20α 3-glucuronide (**25**) and their 20β-isomers (**26**, **27**).

In the present work, the 20,21-diacetates of 5α-cortol and 5α-cortolone were the key intermediates for introduction of the glucuronyl residue at C-3 and cortisol (**1**) was selected as a starting material. Transformation of **1** into the target compounds involves the stereoselective reduction of the Δ^4-3-keto group and the carbonyl group at C-20. We previously developed a route *via* a 3,5-diene derivative, which yielded the desired 5α-corticosteroid with a satisfactory stereoselectivity.[7] Concerning the carbonyl group at C-20, it has been shown that reduction of 17α,21-dihydroxy-20-ketones with sodium borohydride yields predominantly 20β-alcohols.[8] On the other hand, when 17α,21-cyclic derivatives of corticosteroids are reduced with metal hydrides the 20α-ol is practically the sole product.[9] In previous work,[1b] the chemical conversion of **1** to various 5α-tetrahydrocortisol derivatives was undertaken, and some of these were used as starting materials.

First, 5α-cortol-20α 20,21-diacetate (**12**) and 5α-cortolone-20α 20,21-diacetate (**14**) were prepared. The route *via* 3,11β,17α,21-tetrahydroxy-3,5-pregnadien-20-one 3-methyl ether 17,21-acetonide (**2**) seemed attractive for this preparation in view of the combination of the 3,5-diene and cyclic side-chain systems. Therefore, this route was examined as follows: hydrogenation of the γ,δ-double bond in **2** over palladium, followed by reduction of the product (**3**) with sodium borohydride was carried out to give the 20α-hydroxy compound (**4**). Subsequent acid hydrolysis, followed by acetylation with acetic anhydride in pyridine, gave the 20α,21-diacetoxy-3-ketone (**5**), which in turn was treated with potassium tri-*sec*-butylborohydride (K-Selectride) to give the desired **12**. Preliminary experiments showed that this route was not necessarily practical owing to the instability of the intermediate **3** and to the difficulty in chromatographic purification of the product in each step. An alternative route was therefore explored. Silylation of 5α-tetrahydrocortisol 17,21-acetonide (**7**) with *tert*-butyldimethylsilyl chloride and imidazole in pyridine–dimethylformamide afforded the 3-silyl ether-17α,21-acetonide (**8**). On reduction with sodium borohydride in methanol, **8** was converted to the 20α-hydroxy-17α,21-acetonide (**9**). Selective removal of the acetonide ring in the presence of the silyl group at C-3 in **9** was not successful under the acidic conditions tested. However, when the 20α-acetoxy-17α,21-acetonide (**10**) was treated with 50% acetic acid under mild conditions, selective cleavage of the side-chain ring took place, giving a 20α-hydroxy-3-silyl ether[10] in good yield. Subsequent acetylation with acetic anhydride in pyridine, followed by removal of the protecting group at C-3 with 1% hydrofluoric acid in acetonitrile gave the desired **12** (58% yield from **7**). Meanwhile, **11** was oxidized with pyridinium chlorochromate in methylene chloride to give the 11-ketone (**13**). Desilylation of **13** yielded the desired **14**.

5α-Cortol-20β 20,21-diacetate (**17**) and 5α-cortolone-20β 20,21-diacetate (**19**) were then synthesized. Treatment of

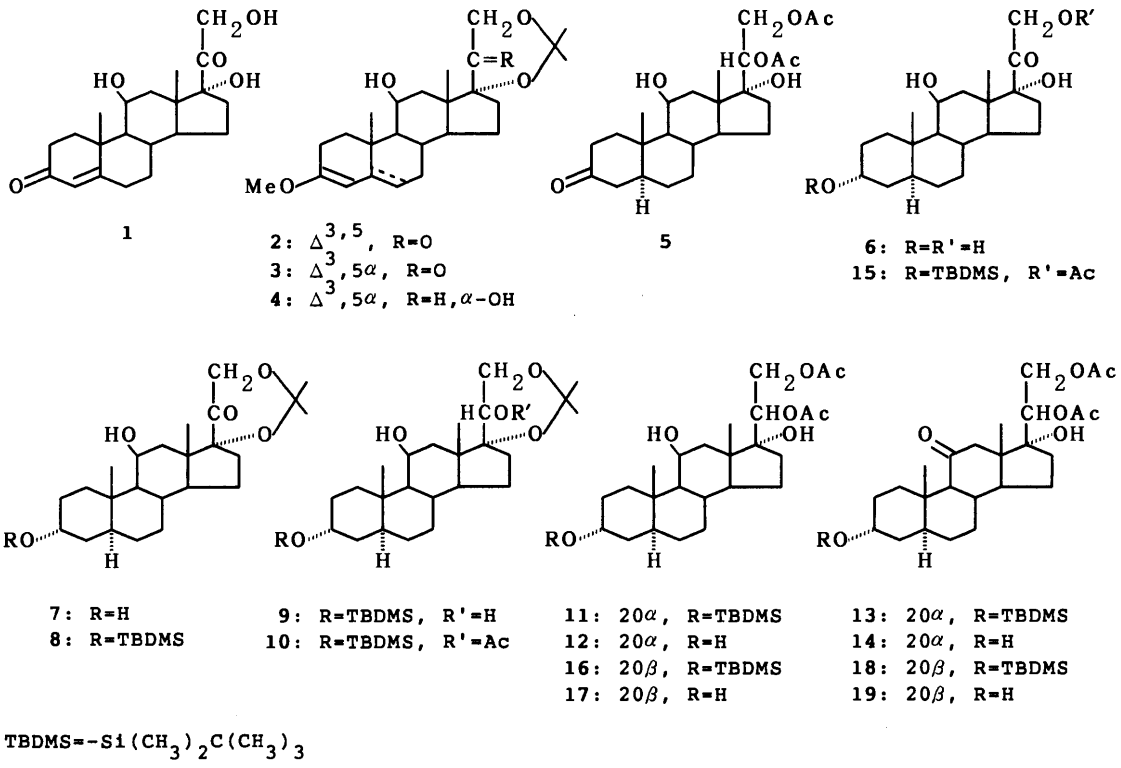

Chart 1

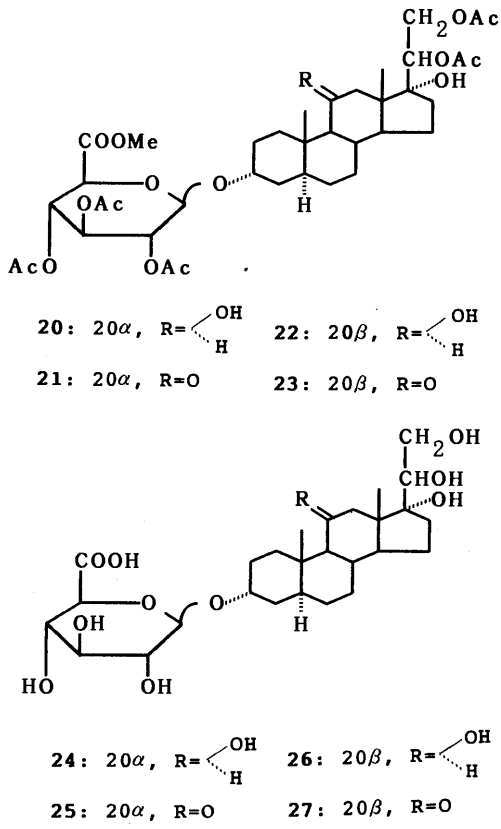

Chart 2

21-acetoxy-3α,11β,17α-trihydroxy-5α-pregnan-20-one 3-*tert*-butyldimethylsilyl ether (**15**) with sodium borohydride in methanol, followed by acetylation with acetic anhydride in pyridine gave the 5α-cortol-20β 3-silyl ether-20,21-diacetate (**16**). The formation of the 20α-isomer was less than 10%, as judged from the proton nuclear magnetic resonance (^{1}H-NMR) spectrum. Desilylation of **16** with hydrofluoric acid in acetonitrile gave **17** in good yield. Compound **16** was oxidized with pyridinium chlorochromate in methylene chloride to give the 11-ketone (**18**), which, on removal of the protecting group at C-3 with hydrofluoric acid yielded the desired compound (**19**).

The stereochemistry at C-20 was confirmed on the basis of ^{1}H-NMR spectral data. It has been reported that the C-18 proton signal of a 20α-acetate appears at lower field than that of the corresponding 20β-epimer.[6b,11] The C-18 protons of **12**, **17**, **14** and **19** resonate at δ 1.08, 0.95, 0.80 and 0.67, respectively, showing that the configuration at C-20 in **12** or **14** is α and in **17** or **19** is β.

Finally, the preparations of 5α-cortol-20α 3-glucuronide (**24**), 5α-cortolone-20α 3-glucuronide (**25**), 5α-cortol-20β 3-glucuronide (**26**), and 5α-cortolone-20β 3-glucuronide (**27**) were carried out. Introduction of the glucuronyl residue into **12**, **14**, **17** and **19** was achieved by using the Koenigs–Knorr reaction with methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate in toluene in the presence of silver carbonate, yielding the corresponding glucuronide acetate-methyl esters (**20**, **21**, **22**, **23**). Subsequent removal of the protecting groups with methanolic potassium hydroxide provided the desired compounds (**24**—**27**). The very polar glucuronides were isolated by the solid-phase extraction method using Amberlite XAD-2 as an adsorbent.

In the ^{1}H-NMR spectra of **20**, **21**, **22** and **23**, the anomeric proton of the sugar moiety resonates in the range of δ 4.54—4.56 as a doublet of J = 7 Hz, showing β-configuration of the anomeric center. In the case of the free glucuronides (**24**—**27**), the water-eliminated Fourier transform method

was employed on measurement of their ^{1}H-NMR spectra. The anomeric proton signal in each compound was observed at δ 4.33—4.35 as a doublet of $J=7$ Hz.

We have previously prepared the glucuronides of related cortisol[1,6b] and 11-deoxycortisol[7,12] metabolites. The glucuronides obtained here should also be useful as standard samples in the direct analysis of plasma and urinary corticosteroids by immunoassay or chromatography.

Experimental

All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 in $CHCl_3$ unless otherwise specified. ^{1}H-NMR spectra were taken with a JEOL JNM-FX-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard.

5α-Tetrahydrocortisol 3-*tert*-Butyldimethylsilyl Ether 17,21-Acetonide (8) A solution of 5α-tetrahydrocortisol (**6**) (1.13 g, 3.1 mmol), prepared from its 21-tetrahydropyranyl ether[1b] by acid hydrolysis, 2,2-dimethoxypropane (1.7 ml) and *p*-toluenesulfonic acid (50 mg) in dimethylformamide (6 ml) was stirred at 80 °C for 4 h. The resulting solution was diluted with AcOEt, washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The crude product obtained was chromatographed on silica gel (30 g) with hexane–AcOEt (2 : 1) as an eluent, yielding the acetonide (**7**) as semi-crystals. A solution of **7** (670 mg, 1.6 mmol), *tert*-butyldimethylsilyl chloride (615 mg, 4.1 mmol) and imidazole (560 mg, 8.2 mmol) in pyridine (1.3 ml)–dimethylformamide (2.6 ml) was allowed to stand at room temperature for 6 h. The resulting solution was diluted with AcOEt, washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The residue obtained was chromatographed on silica gel (30 g). Elution with hexane–AcOEt (10 : 1) and recrystallization of the product from $CHCl_3$ gave **8** (870 mg, 54% from **6**) as colorless needles. mp 156—158 °C. $[\alpha]_D^{25}$ +40° ($c=1.0$). ^{1}H-NMR ($CDCl_3$) δ: 0.02 (6H, s, 3-OSi$(CH_3)_2$), 0.82 (3H, s, 18-CH_3), 0.88 (9H, 3-OSi-*tert*-Bu), 0.99 (3H, s, 19-CH_3), 1.38, 1.48 (each 3H, s, isopropyl CH_3), 3.9—4.2 (3H, 3β-, 21-H), 4.40 (1H, m, 11α-H). *Anal.* Calcd for $C_{30}H_{52}O_5Si$: C, 69.18; H, 10.06. Found: C, 69.00; H, 10.10.

20α-Acetoxy-5α-pregnane-3α,11β,17α,21-tetrol 3-*tert*-Butyldimethylsilyl Ether 17,21-Acetonide (10) A solution of **8** (870 mg, 1.7 mmol) and $NaBH_4$ (150 mg, 4.0 mmol) in MeOH (10 ml) was stirred at room temperature for 1 h. After careful addition of AcOH, the mixture was extracted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. A solution of the product (**9**) (840 mg, 1.6 mmol) and acetic anhydride (4 ml) in pyridine (8 ml) was allowed to stand overnight at room temperature. After addition of H_2O, the mixture was extracted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The residue obtained was chromatographed on silica gel (30 g). Elution with hexane–AcOEt (4 : 1) as an eluent gave **10** (770 mg, 70% from **8**) as colorless semi-crystals. ^{1}H-NMR ($CDCl_3$) δ: 0.02 (6H, s, 3-OSi$(CH_3)_2$), 0.84 (3H, s, 18-CH_3), 0.89 (9H, 3-OSi-*tert*-Bu), 0.99 (3H, s, 19-CH_3), 1.33, 1.50 (each 3H, s, isopropyl CH_3), 2.04 (3H, s, 20-OCOCH_3), 3.54 (1H, dd, $J=6$, 12 Hz, one of 21-H), 3.8—4.0 (2H, 3β-H, one of 21-H), 4.40 (1H, m, 11α-H), 4.95 (1H, t, $J=6$ Hz, 20β-H).

5α-Cortol-20α 3-*tert*-Butyldimethylsilyl Ether 20,21-Diacetate (11) A solution of **10** (770 mg, 1.4 mmol) in 50% acetic acid (6 ml) was allowed to stand at room temperature for 1 h. The resulting solution was diluted with AcOEt, washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The product obtained was treated with acetic anhydride (2 ml) in pyridine (4 ml). Purification by chromatography on silica gel (30 g) using hexane–AcOEt (3 : 1) and recrystallization of the product from MeOH gave **11** (660 mg, 85% from **10**) as colorless leaflets. mp 179—181 °C. $[\alpha]_D^{25}$ −5° ($c=1.1$). ^{1}H-NMR ($CDCl_3$) δ: 0.02 (6H, s, 3-OSi$(CH_3)_2$), 0.88 (9H, s, 3-OSi-*tert*-Bu), 1.00 (3H, s, 19-CH_3), 1.08 (3H, s, 18-CH_3), 2.03, 2.11 (each 3H, s, –OCOCH_3), 3.9—4.7 (4H, 3β-, 11α-, 21-H), 5.34 (1H, dd, $J=3$, 9 Hz, 20β-H). *Anal.* Calcd for $C_{31}H_{54}O_7Si$: C, 65.69; H, 9.60. Found: C, 65.60; H, 9.63.

5α-Cortol-20α 20,21-Diacetate (12) i) A solution of **11** (260 mg, 0.46 mmol) and 47% HF (0.13 ml) in acetonitrile (6 ml) was allowed to stand at room temperature for 2 h. The resulting solution was diluted with AcOEt, washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated down. Recrystallization of the crude product from MeOH gave **12** (205 mg, 99%) as colorless prisms. mp 242—245 °C. $[\alpha]_D^{25}$ −18° ($c=1.0$). ^{1}H-NMR ($CDCl_3$) δ: 1.02 (3H, s, 19-CH_3), 1.08 (3H, s, 18-CH_3), 2.03, 2.11 (each 3H, s, –OCOCH_3), 3.9—4.7 (4H, 3β-, 11α-, 21-H), 5.32 (1H, dd, $J=3$, 9 Hz, 20β-H). *Anal.* Calcd for $C_{25}H_{40}O_7$: C, 66.34; H, 8.91. Found: C, 66.30; H, 8.82.

ii) A solution of 3,11β,17α,21-tetrahydroxy-3,5-pregnadien-20-one 3-methyl ether 17,21-acetonide[13] (**2**) (240 mg, 0.58 mmol) in AcOEt (5 ml)–EtOH (2 ml) was stirred under a hydrogen gas stream for 14 h at an atmospheric pressure in the presence of 5% Pd–C (30 mg). After addition of AcOEt (30 ml) followed by removal of the catalyst by filtration, the filtrate, which contained the enol ether (**3**), was treated with $NaBH_4$ (150 mg) in MeOH (5 ml) at 0 °C for 1 h. The crude product (**4**) obtained upon the usual workup was treated with 2 N HCl (1 ml) in MeOH (5 ml). The resulting solution was diluted with AcOEt, washed with 5% $NaHCO_3$, dried over anhydrous Na_2SO_4, and evaporated down. Acetylation of the crude product was then carried out with acetic anhydride (1 ml) in pyridine (2 ml). After the usual workup, the crude product obtained was chromatographed on silica gel (20 g) with hexane–AcOEt (1 : 1) as an eluent, yielding the 20α,21-diacetoxy-3-one (**5**). A solution of **5** (60 mg, 0.13 mmol) and K-Selectride (1 M in tetrahydrofuran, 0.2 ml) in dry tetrahydrofuran (3 ml) was stirred for 30 min at −78 °C under a nitrogen atmosphere. After addition of 30% H_2O_2 (0.3 ml), the resulting mixture was extracted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The residue obtained was chromatographed on silica gel (15 g). Elution with hexane–AcOEt (1 : 2) and recrystallization of the product from MeOH gave **12** (55 mg, 21% from **2**) as colorless prisms. mp 242—245 °C. Its infrared spectrum was identical with that of the sample obtained in i).

5α-Cortolone-20α 3-*tert*-Butyldimethylsilyl Ether 20,21-Diacetate (13) A mixture of **11** (400 mg, 0.71 mmol) and pyridinium chlorochromate (500 mg, 2.3 mmol) in CH_2Cl_2 (6 ml) was stirred at room temperature for 2.5 h. After addition of ether, the resulting solution was passed through Florisil on a sintered-glass funnel, and the filtrate was evaporated down. Recrystallization of the product from MeOH gave **13** (395 mg, 99%) as colorless leaflets. mp 203—205 °C. $[\alpha]_D^{25}$ +14° ($c=0.8$). ^{1}H-NMR ($CDCl_3$) δ: 0.01 (6H, s, 3-OSi$(CH_3)_2$), 0.80 (3H, s, 18-CH_3), 0.88 (9H, s, 3-OSi-*tert*-Bu), 0.99 (3H, s, 19-CH_3), 2.02, 2.11 (each 3H, s, –OCOCH_3), 3.9—4.2 (2H, 3β-H, one of 21-H), 4.38 (1H, dd, $J=3$, 12 Hz, one of 21-H), 5.26 (1H, dd, $J=3$, 9 Hz, 20β-H). *Anal.* Calcd for $C_{31}H_{52}O_7Si$: C, 65.92; H, 9.28. Found: C, 65.80; H, 9.45.

5α-Cortolone-20α 20,21-Diacetate (14) Hydrolysis of **13** (390 mg, 0.69 mmol) with HF was carried out in the manner described for **12**. Recrystallization of the product from MeOH gave **14** (300 mg, 96%) as colorless leaflets. mp 271—273 °C. $[\alpha]_D^{24}$ +5° ($c=0.5$). ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 2.02, 2.11 (each 3H, s, –OCOCH_3), 3.9—4.2 (2H, 3β-H, one of 21-H), 4.40 (1H, dd, $J=3$, 12 Hz, one of 21-H), 5.27 (1H, dd, $J=3$, 9 Hz, 20β-H). *Anal.* Calcd for $C_{25}H_{38}O_7$: C, 66.64; H, 8.50. Found: C, 66.53; H, 8.37.

5α-Cortol-20β 3-*tert*-Butyldimethylsilyl Ether 20,21-Diacetate (16) Sodium borohydride reduction of 21-acetoxy-3α,11β,17α-trihydroxy-5α-pregnan-20-one 3-*tert*-butyldimethylsilyl ether (**15**) (570 mg, 1.1 mmol) was carried out in the manner described for **10**. The product obtained was treated with acetic anhydride (2 ml) in pyridine (4 ml). Purification by chromatography on silica gel (20 g) using hexane–AcOEt (3 : 1) and recrystallization of the product from MeOH gave **16** (450 mg, 73% from **15**) as colorless leaflets. mp 178—180 °C. $[\alpha]_D^{24}$ +54° ($c=0.7$). ^{1}H-NMR ($CDCl_3$) δ: 0.02 (6H, s, 3-OSi$(CH_3)_2$), 0.88 (9H, s, 3-OSi-*tert*-Bu), 0.95 (3H, s, 18-CH_3), 0.99 (3H, s, 19-CH_3), 2.02, 2.11 (each 3H, s, –OCOCH_3), 3.94 (1H, m, 3β-H), 4.13 (1H, dd, $J=8$, 12 Hz, one of 21-H), 4.3—4.6 (2H, 11α-H, one of 21-H), 5.35 (1H, dd, $J=3$, 8 Hz, 20α-H). *Anal.* Calcd for $C_{31}H_{54}O_7Si$: C, 65.69; H, 9.60. Found: C, 65.58; H, 9.40.

5α-Cortol-20β 20,21-Diacetate (17) Hydrolysis of **16** (73 mg, 0.13 mmol) with HF was carried out in the manner described for **12**. Recrystallization of the product from MeOH gave **17** (57 mg, 98%) as colorless leaflets. mp 201—203 °C. $[\alpha]_D^{25}$ +74° ($c=0.5$). ^{1}H-NMR ($CDCl_3$) δ: 0.95 (3H, s, 18-CH_3), 1.01 (3H, s, 19-CH_3), 2.03, 2.12 (each 3H, s, –OCOCH_3), 3.9—4.6 (4H, 3β-, 11α-, 21-H), 5.36 (1H, dd, $J=3$, 8 Hz, 20α-H). *Anal.* Calcd for $C_{25}H_{40}O_7$: C, 66.34; H, 8.91. Found: C, 66.16; H, 8.93.

5α-Cortolone-20β 3-*tert*-Butyldimethylsilyl Ether 20,21-Diacetate (18) Oxidation of **16** (250 mg, 0.44 mmol) with pyridinium chlorochromate was carried out in the manner described for **13**. Purification by chromatography on silica gel (20 g) using hexane–AcOEt (5 : 1) and recrystallization of the product from MeOH gave **18** (245 mg, 98%) as colorless leaflets. mp 167—169 °C. $[\alpha]_D^{25}$ +56° ($c=1.1$). ^{1}H-NMR ($CDCl_3$) δ: 0.01 (6H, s, 3-OSi$(CH_3)_2$), 0.67 (3H, s, 18-CH_3), 0.88 (9H, s, 3-OSi-*tert*-Bu), 0.97 (3H, s, 19-CH_3), 2.02, 2.10 (each 3H, s, –OCOCH_3), 3.9—4.3 (2H, 3β-H, one

of 21-H), 4.44 (1H, dd, $J=3$, 12 Hz, one of 21-H), 5.26 (1H, dd, $J=3$, 8 Hz, 20α-H). *Anal.* Calcd for $C_{31}H_{52}O_7Si$: C, 65.92; H, 9.28. Found: C, 65.96; H, 9.34.

5α-Cortolone-20β 20,21-Diacetate (19) Hydrolysis of **18** (240 mg, 0.43 mmol) with HF was carried out in the manner described for **12**. Recrystallization of the product from MeOH gave **19** (190 mg, 99%) as colorless leaflets. mp 209—211 °C. $[\alpha]_D^{25}$ +77° ($c=0.6$). ^{1}H-NMR ($CDCl_3$) δ: 0.67 (3H, s, 18-CH_3), 0.99 (3H, s, 19-CH_3), 2.03, 2.11 (each 3H, s, -$OCOCH_3$), 3.9—4.3 (2H, 3β-H, one of 21-H), 4.46 (1H, dd, $J=3$, 12 Hz, one of 21-H), 5.27 (1H, dd, $J=3$, 8 Hz, 20α-H). *Anal.* Calcd for $C_{25}H_{38}O_7$: C, 66.64; H, 8.50. Found: C, 66.58; H, 8.49.

Methyl (20α,21-Diacetoxy-11β,17α-dihydroxy-5α-pregnan-3α-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid)uronate (20) Freshly prepared Ag_2CO_3 (240 mg) and methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate (490 mg, 1.23 mmol) were added to a solution of **12** (280 mg, 0.62 mmol) in toluene (10 ml), and the suspension was stirred at room temperature for 2 d. After addition of AcOEt, the resulting solution was passed through Celite (30 g) on a sintered-glass funnel, and the filtrate was evaporated down. The residue was chromatographed on silica gel (30 g) with hexane–AcOEt (1 : 1) as an eluent, yielding a mixture of **20** and a sugar derivative. Separation of these products was achieved after acetylation of the latter compound. Repurification by chromatography using hexane–AcOEt (1 : 1) yielded **20** (110 mg, 23%) as colorless semi-crystals. ^{1}H-NMR ($CDCl_3$) δ: 1.01 (3H, s, 19-CH_3), 1.07 (3H, s, 18-CH_3), 2.03, 2.11 (15H, -$OCOCH_3$), 3.73 (3H, s, -$COOCH_3$), 3.8—4.7 (6H, 3β-, 11α, 21-, 1′-, 5′-H), 4.8—5.4 (4H, 20β-, 2′-, 3′-, 4′-H).

Methyl (20α,21-Diacetoxy-17α-hydroxy-11-oxo-5α-pregnan-3α-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid)uronate (21) The Koenigs–Knorr reaction of **14** (150 mg, 0.33 mmol) and purification were carried out in the manner described for **20**, yielding **21** (60 mg, 24%) as colorless semi-crystals. ^{1}H-NMR ($CDCl_3$) δ: 0.79 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 2.03, 2.11 (15H, -$OCOCH_3$), 3.74 (3H, s, -$COOCH_3$), 3.8—4.7 (5H, 3β-, 21-, 1′-, 5′-H), 4.8—5.4 (4H, 20β-, 2′-, 3′-, 4′-H).

Methyl (20β,21-Diacetoxy-11β,17α-dihydroxy-5α-pregnan-3α-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid)uronate (22) The Koenigs–Knorr reaction of **17** (300 mg, 0.66 mmol) was carried out in the manner described for **20**. The crude product was purified by chromatography on silica gel (30 g) using hexane–AcOEt (1 : 1) and benzene–AcOEt (1 : 1) as eluents. Recrystallization of the product from ether–hexane gave **22** (110 mg, 22%) as colorless leaflets. mp 175—178 °C. $[\alpha]_D^{20}$ +25° ($c=0.5$). ^{1}H-NMR ($CDCl_3$) δ: 0.94 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 2.01, 2.11 (15H, -$OCOCH_3$), 3.74 (3H, s, -$COOCH_3$), 3.8—4.7 (6H, 3β-, 11α, 21-, 1′-, 5′-H), 4.8—5.4 (4H, 20α-, 2′-, 3′-, 4′-H). *Anal.* Calcd for $C_{38}H_{56}O_{16}\cdot 5/2H_2O$: C, 56.08; H, 7.55. Found: C, 56.30; H, 7.25.

Methyl (20β,21-Diacetoxy-17α-hydroxy-11-oxo-5α-pregnan-3α-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid)uronate (23) The Koenigs–Knorr reaction of **19** (400 mg, 0.89 mmol) and purification were carried out in the manner described for **22**. Recrystallization of the product from ether–hexane gave **23** (230 mg, 34%) as colorless leaflets. mp 128—130 °C. $[\alpha]_D^{20}$ +24° ($c=1.1$). ^{1}H-NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-CH_3), 0.97 (3H, s, 19-CH_3), 1.99, 2.03, 2.10 (15H, -$OCOCH_3$), 3.73 (3H, s, -$COOCH_3$), 3.8—4.7 (5H, 3β-, 21-, 1′-, 5′-H), 4.8—5.4 (4H, 20α-, 2′-, 3′-, 4′-H). *Anal.* Calcd for $C_{38}H_{54}O_{16}\cdot 3/2H_2O$: C, 57.49; H, 7.24. Found: C, 57.51; H, 6.82.

5α-Cortol-20α 3-Glucuronide (24) A solution of **20** (100 mg, 0.13 mmol) in 2% methanolic KOH (5 ml) was stirred at room temperature for 3 h. After addition of H_2O followed by neutralization with AcOH, the resulting solution was evaporated down under reduced pressure. The crude product obtained was subjected to column chromatography on Amberlite XAD-2. Elution with MeOH gave the crude product, which was chromatographed on silica gel (10 g) with $CHCl_3$–MeOH–H_2O–AcOH (100 : 30 : 1 : 0.5) as an eluent, and then on Amberlite XAD-2, yielding **24** (38 mg, 54%). mp 185 °C (dec.) (colorless leaflets from MeOH–AcOEt). $[\alpha]_D^{23}$ −9° ($c=0.5$, MeOH). ^{1}H-NMR (CD_3OD) δ: 0.97 (3H, s, 18-CH_3), 1.04 (3H, s, 19-CH_3), 3.93 (1H, m, 3β-H), 4.32 (1H, m, 11α-H), 4.35 (1H, d, $J=7$ Hz, 1′-H). *Anal.* Calcd for $C_{27}H_{44}O_{11}$: C, 59.54; H, 8.14. Found: C, 59.31; H, 8.36.

5α-Cortolone-20α 3-Glucuronide (25) Saponification of **21** (70 mg, 0.09 mmol) with 2% KOH and purification were carried out in the manner described for **24**, yielding **25** (31 mg, 63%). mp 196 °C (dec.) (colorless leaflets from MeOH–AcOEt). $[\alpha]_D^{18}$ +5° ($c=0.6$, MeOH). ^{1}H-NMR (CD_3OD) δ: 0.70 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 3.95 (1H, m, 3β-H), 4.33 (1H, d, $J=7$ Hz, 1′-H). *Anal.* Calcd for $C_{27}H_{42}O_{11}\cdot 3/4H_2O$: C, 58.31; H, 7.88. Found: C, 58.22; H, 7.94.

5α-Cortol-20β 3-Glucuronide (26) Saponification of **23** (100 mg, 0.13 mmol) with 2% KOH and purification were carried out in the manner described for **24**, yielding **26** (36 mg, 51%). mp 195 °C (dec.) (colorless leaflets from MeOH–AcOEt). $[\alpha]_D^{17}$ +6° ($c=0.5$, MeOH). ^{1}H-NMR (CD_3OD) δ: 1.04 (6H, s, 18-, 19-CH_3), 3.98 (1H, m, 3β-H), 4.31 (1H, m, 11α-H), 4.35 (1H, d, $J=7$ Hz, 1′-H). *Anal.* Calcd for $C_{27}H_{44}O_{11}\cdot 3/2H_2O$: C, 56.73; H, 8.29. Found: C, 56.97; H, 8.08.

5α-Cortolone-20β 3-Glucuronide (27) Saponification of **24** (230 mg, 0.30 mmol) with 2% KOH and purification were carried out in the manner described for **24**, yielding **27** (125 mg, 76%). mp 190 °C (dec.) (colorless leaflets from MeOH–AcOEt). $[\alpha]_D^{15}$ +14° ($c=0.4$, MeOH). ^{1}H-NMR (CD_3OD) δ: 0.75 (3H, s, 18-CH_3), 1.03 (3H, s, 19-CH_3), 3.92 (1H, m, 3β-H), 4.34 (1H, d, $J=7$ Hz, 1′-H). *Anal.* Calcd for $C_{27}H_{42}O_{11}\cdot 1/2H_2O$: C, 58.79; H, 7.86. Found: C, 58.66; H, 7.86.

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Vanadate-Stimulated Release of Hepatic Lipase Activity from Liver

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Vanadate stimulated the release of rat hepatic lipase activity from liver slices into an incubation medium in a time- and dose-dependent manner. Insulin, however, failed to have this stimulatory action, and the release by heparin was recognized, but was not additive to that by vanadate. Amiloride, an inhibitor of tyrosine kinase in some receptors and of the Na^+/H^+ exchange system suppressed the vanadate-stimulated release. Biochanin A, a different type of tyrosine kinase inhibitor than amiloride, also suppressed the effect of vanadate. The stimulation by vanadate was clearly preserved in Na^+-, K^+-, or Ca^{2+}-free medium, suggesting that neither the Na^+/H^+ exchange system, Na^+, K^+-adenosine triphosphatase, nor Ca^{2+}-influx into cells is involved in the action of this substance. These results suggest that vanadate-stimulated release of the enzyme activity is associated with the activation of the tyrosine kinase activity.

Keywords hepatic lipase; vanadate; amiloride; biochanin A; tyrosine kinase

Introduction

Hepatic lipase (HTGL; hepatic triacylglyceride lipase, EC 3.1.1.3), which is a salt-resistant and heparin-releasable enzyme, is thought to play an important role in high- and intermediate-density lipoprotein metabolisms,[1-11] but this has not been unequivocally established. It has also been reported that adrenalin, vasopressin, and calcium ionophores all reduce the release of HTGL activity from rat hepatic parenchymal cells into the medium.[12]

Vanadate, a substance capable of insulin-like action,[13-17] exerts various biological effects, such as the inhibition of Na^+, K^+-adenosine triphosphatase (ATPase), the activation of Na^+/H^+ exchange system, and the stimulatory phosphorylation of insulin receptor,[16,17] and has been considered to be an important probe in studies of the mechanism of insulin action. However, contrary to its reported insulin-like effects in adipocytes and skeletal muscle,[15,17] vanadate exerts glycogenolytic, noninsulin-like actions in rat hepatocyte.[18] In the present study, we show that vanadate, in contrast to insulin, stimulates the release of HTGL activity from rat liver.

Materials and Methods

Materials Sodium orthovanadate (vanadate), heparin (167 U/mg), and insulin (26 U/mg) were purchased from Wako Chemical Industries (Osaka, Japan). Amiloride and biochanin A were obtained from Sigma (Mo, U.S.A.). Intralipos, an emulsion composed of 1.2% yolk lecthin, 2.5% glycerol, and 10% soybean oil was from Green Cross Co. (Osaka). All other chemicals used were of analytical grade.

Animal Male Wistar rats weighing 250—300 g were kept on a commercial laboratory chow *ad libitum* for one week and starved for 24 h before the experiments.

Release of HTGL Activity from Liver Liver was quickly removed from rats killed under anaesthesia. The tissue was sliced with a razor into small pieces (100—150 mg).[19] Three slices (400 mg total) were incubated in 2 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) containing 5 mM glucose and 2% bovine serum albumin under 95% O_2–5% CO_2 in siliconized vials at 28 °C for 120 min. After incubation, the slices were removed by centrifugation and the obtained supernatant served as the preparation for the released enzyme activity.

Determination of HTGL Activity HTGL activity released into the medium was determined by the method using Intralipos as substrate.[6] The free fatty acids (FFA) produced were determined by Duncombe's method.[20] The HTGL activity was expressed as μmol FFA produced/h/g liver. Results are means ± S.E. of four or five observations for two separate experiments.

Results and Discussion

Stimulatory Release of HTGL Activity from Liver by Vanadate Figure 1 shows the release of HTGL activity from liver into the medium in the presence of vanadate (10 mM), heparin (10 U/ml) or insulin (30 nM) over a 150-min period. Vanadate stimulated the release in a time-dependent manner, and the stimulated release reached a maximum at 120 min. The heparin-stimulated release progressively increased up to 150 min. Insulin did not stimulate the release

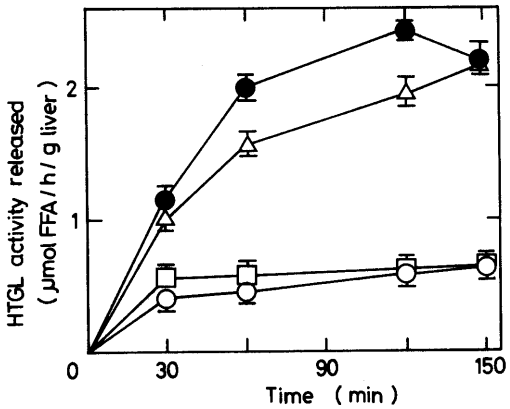

Fig. 1. Time Course of Release of HTGL Activity from the Liver by Vanadate, Heparin or Insulin

The liver was incubated for 0—150 min with vanadate (10 mM, ●), heparin (10 U/ml, △), insulin (30 nM, □) or without any of the three (○). HTGL activity released into the medium was determined as described in Materials and Methods.

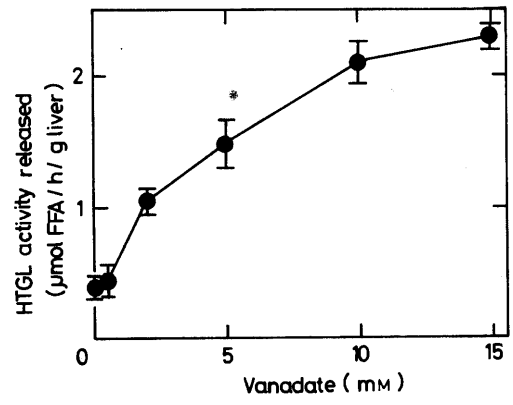

Fig. 2. Dependence of Release of HTGL Activity from the Liver on Vanadate Concentration

The liver was incubated for 120 min with various concentration of vanadate.

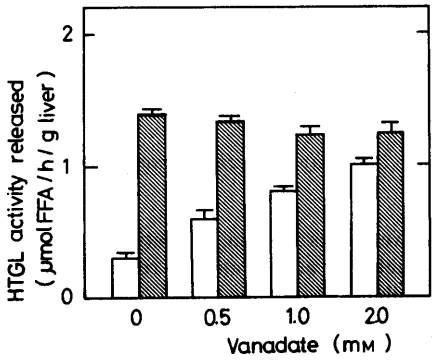

Fig. 3. Effect of Heparin on the Stimulated Release of HTGL Activity by Vanadate

The liver was incubated for 120 min with vanadate in the presence (hatched bar) or absence (white bar) of heparin (10 U/ml).

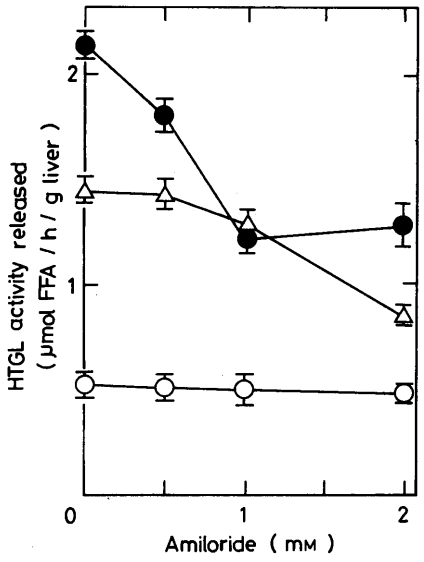

Fig. 4. Effect of Amiloride on the Stimulated Release of HTGL Activity by Vanadate or Heparin

The liver was incubated for 120 min with vanadate (10 mM, ●), heparin (10 U/ml, △) or without either one (○) in the presence of amiloride (0—2 mM).

of the enzyme activity at 30 nM or even at a high concentration of 90 nM (data not shown). When the liver was incubated with vanadate at various concentrations for 120 min, the release of HTGL activity was increased in a dose-dependent manner up to 15 mM (Fig. 2). The liver incubated with vanadate in the presence of heparin showed no additive action by this substance to incubation with vanadate alone (Fig. 3).

Effects of Tyrosine Kinase Inhibitors To determine whether the stimulation of tyrosine kinase activity of the insulin receptor and/or the activation of Na^+/H^+ exchange system are involved in the action of vanadate, the liver was incubated with vanadate or heparin in the presence of amiloride.[21-23] An appreciable decrease in the vanadate-stimulated release was observed with amiloride at 0.5 and 1 mM concentrations (Fig. 4). The heparin-stimulated release was also markedly decreased by amiloride at 2 mM concentration, but the reason for this inhibition is unknown.

Biochanin A, which is a tyrosine kinase inhibitor of a different type than amiloride, inhibited the vanadate-stimulated release but not the heparin-stimulated release (Fig. 5).

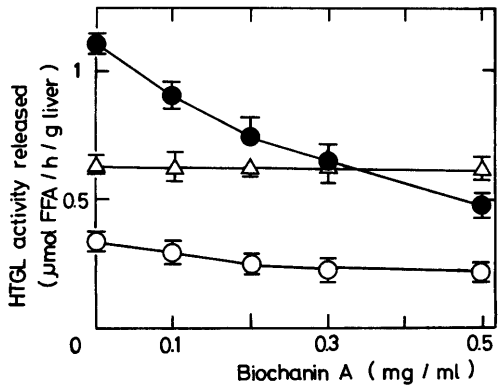

Fig. 5. Effect of Biochanin A on the Stimulated Release of HTGL Activity by Vanadate or Heparin

The liver was incubated for 120 min with vanadate (5 mM, ●), heparin (5 U/ml, △) or without either one (○) in the presence of biochanin A (0—0.5 mg/ml).

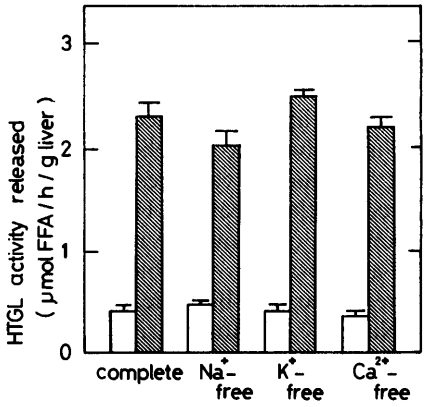

Fig. 6. Effects of Various Extracellular Ions on the Stimulated Release of HTGL Activity by Vanadate

The liver was incubated with (hatched bar) or without (white bar) vanadate (10 mM) in the following media. The Na^+-free buffer was prepared with choline ion instead of Na^+, the K^+-free buffer with Na^+ instead of K^+, and the Ca^{2+}-free buffer omitted $CaCl_2$ from the complete medium described from Materials and Methods.

TABLE I. Changes of HTGL Activity in the Liver by Vanadate

	HTGL activity	
	In medium	In liver
No addition	0.41 ± 0.05	13.3 ± 0.85
Vanadate (10 mM)	3.36 ± 0.04	6.62 ± 0.42
Heparin (10 U/ml)	1.62 ± 0.11	12.0 ± 1.50
Insulin (30 nM)	0.34 ± 0.04	14.8 ± 1.30

The liver was incubated for 120 min with vanadate (10 mM), heparin (10 U/ml), insulin (30 nM) or without any of these. After incubation, the liver and the medium were separated by centrifugation as described in Materials and Methods. The liver was homogenized in 3.6 ml of 50 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose with a Potter-Elvehjem homogenizer, and contrifuged at 1500 × *g* for 10 min. The resultant supernatant and the separated incubation medium served for the measurement of enzyme activity.

These findings suggest that the release of HTGL activity by vanadate is associated with the activation of a biochanin A-sensitive process, probably tyrosine kinase.

Requirement of Various Extracellular Cations The requirement of various extracellular cations for vanadate action was tested (Fig. 6). No appreciable decrease in the vanadate-stimulated release of HTGL activity was observed by replacement of Na^+ with choline ion in the medium,

suggesting that the Na^+/H^+ exchange system is not involved in the action of vanadate, though this substance is reported to be an activator of the system.[16,17] When the liver was incubated in K^+-free medium with vanadate, which is also known to be a potent inhibitor of Na^+, K^+-ATPase,[16] the vanadate-stimulated release of HTGL activity was well preserved. Thus, the inhibition of Na^+, K^+-ATPase by vanadate may not be related to the stimulatory release of HTGL activity by vanadate. Although vanadate was reported to activate the influx of Ca^{2+} and Na^+ into the carcinoma cells,[23] its release of HTGL activity was still possible even without Ca^{2+} in the medium, suggesting that the activation of Ca^{2+}-influx into cells by vanadate was not concerned in its enzyme activity release.

Changes of HTGL Activity in the Liver by Vanadate Table I shows the changes of HTGL activity in the liver and in the medium by the addition of vanadate, heparin or insulin. When HTGL activity was stimulated to release from the liver into the medium by vanadate, HTGL activity in the liver decreased notably. In the case of heparin, HTGL activity released into the medium was stimulated and the activity in the liver decreased slightly. Insulin did not change the activity in either the medium or the liver.

Recently, it was reported that the secretion of rat HTGL is blocked by the inhibition of oligosaccharide processing at the stage of glucosidase I.[24] In our experiments, the action of vanadate was not inhibited by tunicamycin, monencin or 2'-deoxyglucose (data not shown).

These results show that vanadate stimulates the release of HTGL activity from liver *via* an amiloride- and biochanin A-sensitive process, probably the tyrosine kinase activity of insulin receptor, and that the step of glycosylation of the enzyme is dissociated from the mechanisms of vanadate action.

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Enhancement of Murine Erythropoiesis *in Vitro* by a Porcine Kidney Extract

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We have examined the effect of porcine kidney extract (PKE) on the growth of erythroid progenitor-derived colonies in a methylcellulose culture. The addition of PKE resulted in an enhancement of burst-forming-unit-erythroid (BFU-E)-derived colonies, and the enhancement of the colony was also observed in a low concentration of erythropoietin (Epo) and fetal calf serum (FCS).

The activity of PKE on BFU-E was compared with the erythroid growth factors, such as recombinant murine interleukin-3 (IL-3), recombinant murine granulocyte/macrophage colony-stimulating factor (GM-CSF) and recombinant human granulocyte colony-stimulating factor (G-CSF) which are all well known to stimulate BFU-E growth. IL-3 showed a potent burst-promoting activity (BPA), but GM-CSF and G-CSF did not enhance BFU-E growth in our culture conditions.

In the cultures supplemented with Epo, the rapid loss of BFU-E was prevented with IL-3; however, PKE alone did not prevent the disappearance of BFU-E.

These results suggest that PKE possesses a BPA-like activity which is considered an enhancement of BFU-E.

Keywords kidney extract; burst-forming-unit-erythroid (BFU-E); burst-promoting activity (BPA); interleukin-3 (IL-3)

At least two major groups of erythroid progenitor cells, the burst-forming-unit-erythroid (BFU-E) and the colony-forming-unit-erythroid (CFU-E), have been recognized[1,2] in *in vitro* culture systems. The growth of CFU-E, relatively mature erythroid progenitors, requires the presence of erythropoietin (Epo). Epo is able to stimulate the proliferation of CFU-E and promotes maturation leading to the hemoglobinization of proerythroblast. Epo and its recombinant protein have been purified as a glycoprotein with a molecular weight of 39000.[3,4] On the other hand, the proliferation of BFU-E, an early erythroid progenitor, requires burst promoting activity (BPA) which is different from Epo.[1,2] BPA stimulates the proliferation of BFU-E and may be responsible for the differentiation of BFU-E into CFU-E with a high responsiveness to Epo. BPA has been found in media conditioned by some human monocyte cell lines,[5] various kinds of blood mononuclear cells,[6] surface membrane vesicles,[7] urine[8,9] and serum.[10]

Interleukin-3 (IL-3) has been purified from a WEHI-3 conditioned medium[11,12] and demonstrated to have all of the properties ascribed to BPA.[13] In addition, granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and IL-4 have also been demonstrated to have "BPA-like" activity.[1,14,15]

Previously, we have reported that porcine kidney extract (PKE) has colony promoting activity (CPA) on GM colony formation.[16–18] In those papers, we showed some biological and biochemical properties of the CPA and suggested that the CPA of PKE appears to be a factor different from IL-3, G-CSF, GM-CSF and IL-4.

In this study, we examined the effects of PKE on colony formation by erythroid progenitors from murine bone marrow cells (BMC) *in vitro*.

Experimental

Preparation of PKE PKE was prepared as previously described.[16] Briefly, porcine kidneys were homogenized with distilled water. The homogenate was centrifuged at 1×10^5 *g* and the clear supernatants thus obtained were lyophilized. The lyophilized material was dissolved in distilled water, and saturated ammonium sulfate solution was added to the solution. The precipitate from 50 to 65% saturation of ammonium sulfate was collected, dialyzed against distilled water, and lyophilized. The lyophilized material was dissolved in distilled water and insoluble material was removed by centrifugation. The clear supernatant obtained was dialyzed against phosphate-buffered saline (PBS), passed through a membrane filter (0.45 μm pore size, Fuji Photo Film Co., Ltd.), and stored at -20 °C until use. The same preparation was used throughout the experiment. The results of repeated experiments showed good reproducibility on CPA of the preparations, and no detectable inactivation of CPA was observed during several months under the above storage conditions.

Mice Male mice of the ddY strain (Sankyo Labo Service Co., Sapporo) aged 6—10 weeks were used.

Growth Factors Human recombinant Epo (ESPO Injection, Sankyo Co., Ltd.) was used in this study. Recombinant murine IL-3 (specific activity $>1 \times 10^5$ U/mg) and recombinant murine GM-CSF (specific activity 5×10^7 U/mg) were purchased from Genzyme, Inc. (Boston). Recombinant human G-CSF was kindly provided by Kirin Brewery Co. (Tokyo, Japan). Doses of these factors per culture dish were as follows: (1) IL-3, 100 units; (2) GM-CSF, 100 units (3) G-CSF, 1 μg.

CPA Assay The assay for CPA was carried out in methylcellulose cultures. 5×10^4 BMC from a mouse femur were cultured in 35 mm plastic Petri dishes (SH-S3512SW, Terumo, Tokyo) in 1 ml of Iscove's modified Dulbecco's medium (IMDM, Gibco) supplemented with 0.8% methylcellulose (Wako Chemicals, Tokyo), 20% horse serum, G-CSF, and test samples. Cultures were incubated at 37 °C in a humidified atmosphere of 5% O_2, 10% CO_2, and 85% N_2 for 7 d. Colonies consisting of 50 or more cells were scored using an inverted microscope. From a dose response study, 30 μl of PKE per dish was considered to be optimal for promoting activity in methylcellulose cultures. PKE alone did not support GM colony formation, even at 100 μl.

Erythroid-Burst (EB) Colony Assay Erythroid progenitors were assayed in a methylcellulose culture system as described by Iscove *et al.*[19] In routine experiments, 2×10^5 BMC were cultured in 35 mm plastic Petri dishes in 1 ml of IMDM supplemented with 0.8% methylcellulose, 1% deionized bovine serum albumin (BSA, Sigma), 30% fetal calf serum (FCS, Mitsubishi Kasei Ltd.), 1×10^{-4} M 2-mercaptoethanol (ME), 2 units of Epo, and test samples. Cultures were incubated at 37 °C in a humidified atmosphere of 5% O_2, 10% CO_2, and 85% N_2 for 8 d. EB colonies were stained with benzidine solution which was a freshly prepared mixture of 6 volumes of 0.3% diaminobenzidine in 0.05 M Tris–HCl buffer (pH 7.6) and 1 volume of 0.5% H_2O_2. The solution (0.5—1.0 ml) was gently overlaid on the cultures. After 20—60 min at room temperature, the colonies dyed brown-dark were scored using an inverted microscope. Benzidine positive colonies on 8 d cultures were defined as an EB colony. BFU-E-derived colonies contained 50 or more erythroblasts.

Trypsin Treatment 0.1 ml of PKE (13.3 mg protein/ml) was treated with 0.1 ml of trypsin solution (8 mg/ml, bovine pancreas, Sigma) at 37 °C for 2 h, then the treatment was stopped by the addition of 0.2 ml trypsin inhibitor (4 mg/ml, soybeans, Sigma) at 37 °C for 0.5 h. After

treatment, aliquots of 40 μl were applied to the EB colony assay.

Delayed Addition The cultures of 2×10^5 BMC prepared in 0.9 ml of a methylcellulose medium containing PKE (10 μl) or IL-3 (100 units) without Epo, or containing Epo (2 units) without PKE or IL-3, were incubated. The cultures on days 0, 2 and 4, were overlaid with 0.1 ml of Epo-solution (2 units), PKE or IL-3, and were incubated for another 8 d, then the EB colonies formed were scored.

Preincubation of BMC 1×10^7 BMC were incubated in a 25 cm^2 culture flask (MS-20050, Sumitomo, Tokyo) with 10 ml of IMDM containing 1% BSA, 30% FCS, 1×10^{-4} M ME, and 20 units of Epo and/or 0.5 ml of PKE at 37 °C in a humidified atmosphere of 5% O_2, 10% CO_2, and 85% N_2 for 4 or 7 d beforehand. The nonadherent cells were harvested, washed three times with IMDM, and then assayed for EB colony formation.

Results

Effect of PKE Concentration on EB Colony Formation The results are shown in Fig. 1. The addition of PKE into the cultures containing Epo resulted in an increase of EB colonies in comparison with the absence of PKE. A maximal increase of 2.4-fold was induced at 10 μl of PKE. In contrast, no EB colony formation was observed at any PKE concentrations in the absence of Epo.

Effect of Epo and FCS Concentration on EB Colony Formation The influence of PKE on the requirements of Epo was tested. As shown in Fig. 2, no colonies were found in the presence of less than 0.2 units per dish of Epo alone, and a maximum stimulation was obtained at 2—4 units of Epo. The addition of PKE (10 μl) to the cultures containing 0.5 units of Epo resulted in nearly the same colony formation in comparison with the cultures containing 2—4 units of Epo alone, and the plating efficiency was significantly increased corresponding to the concentration of Epo.

The influence of PKE on the requirements of FCS was tested. As shown in Fig. 3, the EB colony formation reached a plateau at a concentration 15—30% of FCS. The addition of PKE (10 μl) to the cultures containing 5% of FCS resulted in nearly the same colony formation in comparison with the cultures containing 15—30% of FCS without PKE.

The Correlation between the Number of EB Colonies Formed and of BMC Plated We examined the plating efficiency of the EB colony with an inoculation of between 1×10^5 and 4×10^5 cells per dish. As shown in Fig. 4, a linear relationship between the cell number plated and the colony number formed was observed in the cases both Epo alone and in a combination of PKE and Epo, showing of correlation coefficients of 0.9969 and 0.9952, respectively.

Stability of BPA in PKE PKE was treated by heat and trypsin. As shown in Table I, the reduction of BPA was

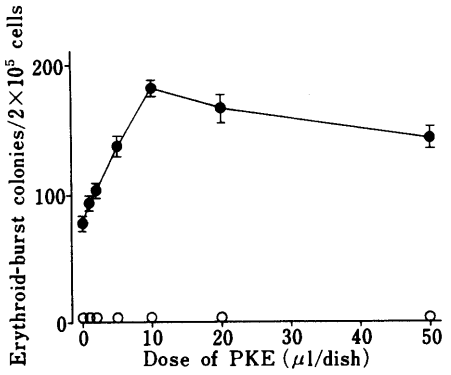

Fig. 1. Effect of PKE Concentration on Erythroid-Burst Colony Formation

EB cultures were incubated in the absence (○) or the presence of 2 units of Epo (●). Numbers represent the mean ± S.E. of 9 dishes from three separate experiments. ○, Epo (−); ●, Epo (+).

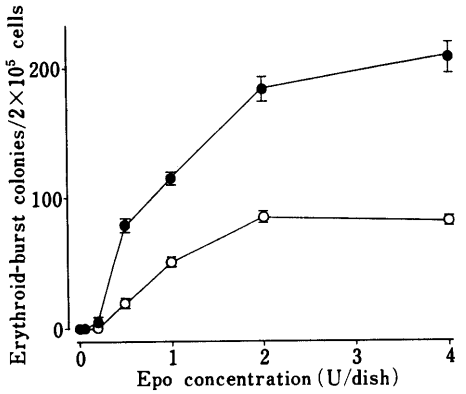

Fig. 2. Effect of Epo Concentration on Erythroid-Burst Colony Formation

EB cultures were incubated in the absence (○) or the presence of PKE (●). Numbers represent the mean ± S.E. of 9 dishes from three separate experiments. ○, PKE (−); ●, PKE (+).

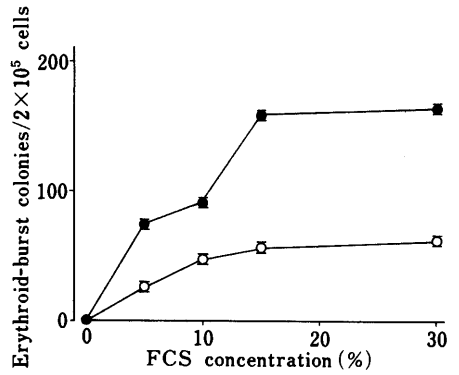

Fig. 3. Effect of FCS Concentration on Erythroid-Burst Colony Formation

EB cultures were incubated in the absence (○) or the presence of PKE (●) with 2 units of Epo. Numbers represent the mean ± S.E. of 9 dishes from three separate experiments. ○, PKE (−); ●, PKE (+).

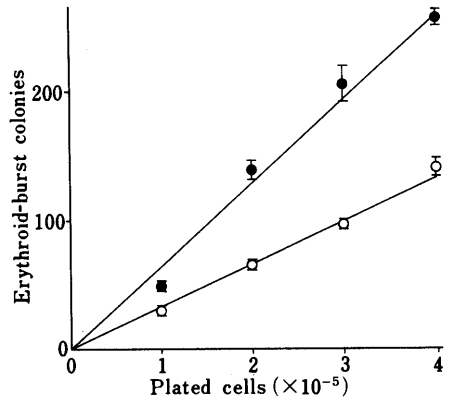

Fig. 4. Correlation between the Number of Erythroid-Burst Colony and BMC Plated

EB cultures were incubated in the absence (○) or the presence of PKE (●) with 2 units of Epo. Each value indicated a coefficient of correlation. Numbers represent the mean ± S.E. of 9 dishes from three separate experiments. ○, PKE (−), $r = 0.9969$; ●, PKE (+), $r = 0.9952$.

observed from 2.1 to 1.66 and 1.65 at 56 °C, 30 min and 70 °C, 30 min, respectively. And the BPA of PKE was completely inactivated by the trypsin treatment. Similar inactivation of the CPA of PKE on GM lineage CSFs has also been observed as shown in previous papers.[16] In addition, a mixture of trypsin and trypsin inhibitor did not affect the CPA or the BPA of PKE (data not shown).

Effect of IL-3, GM-CSF and G-CSF on EB Colony Formation The BPA of PKE was compared with that of IL-3, GM-CSF, and G-CSF. As shown Fig. 5, IL-3 enhanced EB colony formation, which was equivalent to PKE. However, GM-CSF and G-CSF did not enhance EB colony formation in our culture conditions.

Effect of PKE on the Survival of BFU-E In order to investigate the supporting function of PKE on the survival of BFU-E, the delayed addition of Epo was examined. As shown in Fig. 6, in the control cultures (PBS+Epo), EB colonies decreased with a lag of Epo addition (PKE+Epo), showing a decrease in the survival of BFU-E. In the case of PKE, a rapid loss of BFU-E was also observed. In contrast with PKE, IL-3 could support the survival of BFU-E, since the number of EB colonies on day 4 of the addition of Epo were found to be 72% of day 0 of the addition, indicating the difference between PKE and IL-3 on their supporting activity.

The addition of PKE or IL-3 to Epo-containing cultures was also examined. In regard to the addition of PKE on day 4, the EB colonies were found to be 153% of the day 0 addition in the control cultures (PBS+Epo). When IL-3 was added on day 2, the number of EB colonies was nearly equal to that of the day 0 addition. However, the addition of IL-3 on day 4 resulted in a decrease in colony number. When the cultures were overlaid with PBS instead of PKE or IL-3 to Epo-containing cultures for 2 or 4 d, no difference in the number of EB colonies was observed. Furthermore, IL-3 alone did not support EB colony

TABLE I. Effect of Various Treatments of PKE

Treatments	CFU-GM[a]	Ratio	BFU-E[b]	Ratio
Control	120.4± 4.7	1.00	59.6±1.6	1.00
Untreated PKE	216.1± 8.4	1.79	125.4±6.3	2.10
56 °C, 30 min PKE	172.9± 3.2[d]	1.44	98.8±5.4[c]	1.66
70 °C, 30 min PKE	166.8± 7.7[c]	1.39	98.1±3.0[c]	1.65
Trypsinized PKE	106.1± 4.2	0.88	61.4±4.4	1.03
37 °C, 2.5 h PKE	206.1±10.1	1.71	117.0±9.0	1.96

a) The number of CFU-GM colonies per 5×10^4 BMC stimulated by 1 μg of G-CSF. *b*) The number of erythroid-burst colonies per 2×10^5 BMC stimulated by 2 units of Epo. Data exhibits mean±S.E. of 9 dishes from three separate experiments. *c*) $p<0.01$ *vs.* untreated PKE. *d*) $p<0.001$ *vs.* untreated PKE.

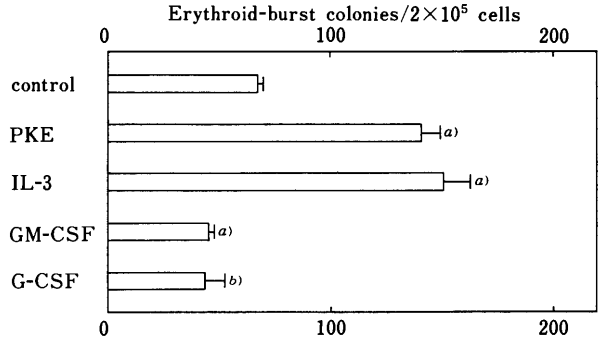

Fig. 5. Effect of IL-3, GM-CSF and G-CSF on Erythroid-Burst Colony Formation

Cultures were carried out in the presence of 2 units of Epo and each factor. Numbers represent the mean±S.E. of 6 dishes from two separate experiments. *a*) $p<0.001$, *b*) $p<0.01$.

TABLE II. Preincubation of BMC with Epo and/or PKE

Incubation period	Additive	Number of cells ($\times10^{-4}$)	Number of BFU-E	BFU-E recovery rate (%)
0 time		1,000	2725±141	100.0
4 d	PBS	257	381± 36	14.0
	Epo	208	1949±258	71.7
	PKE	220	672± 52	24.6
	PKE+Epo	363	3137±162	115.2
7 d	PBS	115	46± 20	1.7
	Epo	144	171± 34	6.3
	PKE	247	56± 13	2.1
	PKE+Epo	301	1660±143	61.0

Mean±S.E. of 6 dishes from two separate experiments.

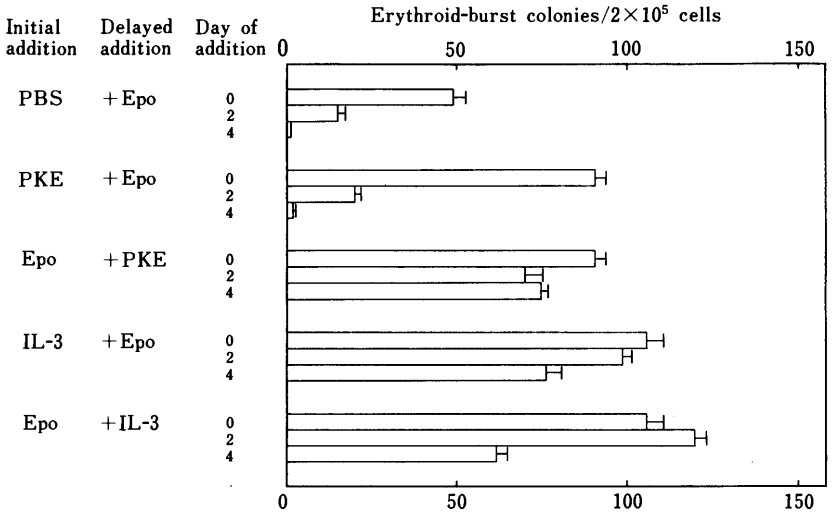

Fig. 6. Effect of Delayed Addition of Epo, PKE or IL-3 on Erythroid-Burst Colony Formation

Cultures were started containing an "initial addition," to which was added Epo, PKE or IL-3 on different days. Each culture was incubated for 8 more days. Numbers represent the mean±S.E. of 6 dishes from two separate experiments.

formation (data not shown).

Since PKE alone did not possess a supporting function for the survival of BFU-E, we studied the effect of the combination of Epo plus PKE on the ability to maintain BFU-E under liquid culture conditions. The results are shown in Table II. The number of BFU-E was reduced to 72 and 6% by day 4 or day 7 of preincubation with Epo, respectively. In the case of PKE, the recovery rates of BFU-E on days 4 and 7 were 25 and 2%. In the combination of Epo and PKE, 115 and 61% of BFU-E were recovered on those days, suggesting the synergistic action of Epo and PKE on maintenance of erythroid progenitors.

Discussion

The addition of PKE to the erythroid cultures containing Epo enhanced EB colony formation, and was able to decrease the requirement of both Epo and FCS concentrations for the growth of EB colony (Figs. 1—3). Epo was not detected in PKE, because PKE alone did not support the erythroid colony formation. A linear relationship between cell numbers plated and colony numbers was observed in the presence of PKE, and the enhancement ratios of each point by PKE were nearly constant (Fig. 4). These results suggested that the enhancing activity of PKE correlated directly with the number of BFU-E and the stimulation of Epo. From these results, it could be concluded that PKE contains the activity capable of potentiating the growth of murine BFU-E.

Ihle *et al.*[11,12] purified IL-3 from a medium conditioned by a mouse myelomonocytic leukemia cell line WEHI-3. And Goldwasser *et al.*[13] indicated that IL-3 had all of the properties ascribed to BPA, and presumed that IL-3 was identical to BPA. In addition, they reported that IL-3 alone does not support EB colony formation of murine BMC in the absence of Epo. It indicates that IL-3 is able to stimulate both the differentiation and the proliferation of the early stage of progenitors such as CFU-S and BFU-E, and then differentiation into a late stage of progenitors such as CFU-E. However, following terminal differentiation of CFU-E requires the presence of Epo. Additionally, some reports have demonstrated that IL-4, G-CSF and GM-CSF also have a potency of BPA-like activity.[1,14,15]

BPA has the following biological characteristics[13]: (1) It greatly diminishes the requirement of Epo or FCS to stimulate EB growth. (2) It prevents the rapid loss of BFU-E *in vitro* in the absence of Epo.

In this study, IL-3 prevented the rapid loss of BFU-E, but, PKE alone could not prevent it (Fig. 6). Additionally, when PKE was combined with Epo in the preincubation of BMC, about 61% of BFU-E was recovered compared to that of the zero time control after 7 d of incubation (Table II). These results suggest that PKE acts synergistically with Epo in supporting the survival, proliferation and differentiation of erythroid progenitors. Thus, the factor(s) detected in PKE appear to have "BPA-like" activity.

We have already demonstrated that PKE stimulates the differentiation of immature hematopoietic stem cells.[16,17] The CPA in PKE stimulates primitive hematopoietic stem cells in the presence of GM-CSF, but does not stimulate colony formation on murine BMC by itself.

The combination of PKE with IL-3, GM-CSF, or G-CSF was examined in the presence of one of these factors such as CSF in agar cultures.[18] It was found that PKE acts synergistically with these factors. We have suggested that the CPA of PKE appears to be a factor different from IL-3, GM-CSF, and G-CSF because of its functional properties and chemical characteristics. Furthermore, since IL-4 does not enhance GM colony formation in the presence of GM-CSF or IL-3, the factor(s) in PKE would be different from IL-4.

Stability of CPA and the BPA-like activity of PKE were observed to have similar properties regarding heat stability and trypsin sensitivity (Table. I). Furthermore, partial purification of the factor(s) in PKE were carried out by hydrophobic column chromatography, ion exchange column chromatography, and gelfiltration high-performance liquid chromatography. The partially purified factor(s) possessed a potent BPA-like activity as did CPA (data not shown). These results suggest that the key factor in PKE appears to be a multipotent regulator in hematopoiesis.

Most recently, Royet *et al.*[20] reported erythropoietic activity, termed mature-BPA (MBPA), in murine kidney cell extracts, that predominantly stimulates the proliferation of mature BFU-E progenitors giving rise to small bursts. They have also showed that MBPA is different from IL-3, IL-4, GM-CSF, and Epo. The activities detected in PKE are possibly different from MBPA, because PKE seems to act also on immature BFU-E and to stimulate large EB colony formation.

It has been well known that kidneys are one of the most important tissues in hematopoiesis by reason of the regulation of Epo and CSF production.[21-24] It is suggested that the kidney possibly contains certain factors which regulate hematopoiesis, and the factor in PKE is one of those factors.

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Changes in Surface Charge Density of Lecithin Liposomes by Lipid Peroxidation. A Fluorescence Study with 8-Anilino-1-naphthalenesulfonate

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Treatment of lecithin liposomes with 100 μM ascorbic acid and 10 μM ferrous ion resulted in the formation of fluorescent products exhibiting an emission maximum at 430 nm and a decrease in the fluorescence intensity of 8-anilino-1-naphthalenesulfonate (ANS) bound to the liposomes without change in the emission maximum. The degree of ascorbic acid/Fe^{2+}-induced decrease in the ANS fluorescence was dependent on the extent of fluorescent product formation. The results of kinetic studies on ANS-binding to the liposomes showed that treatment of the liposomes with ascorbic acid/Fe^{2+} causes an increase of the apparent dissociation constant (K_d) of ANS–liposome complex. This indicates that lipid peroxidation of the liposomes by treatment with ascorbic acid/Fe^{2+} decreases the binding affinity of ANS to the liposomes. In addition, it was also found that there is a good correlation between degrees of the K_d value and the formation of fluorescent products. The fluorescence properties, *i.e.* emission maximum and response of the fluorescence intensity for borohydride reduction, of the products formed by lipid peroxidation of the liposomes were similar to those derived from modification of the liposomes with monofunctional aldehydes such as acetaldehyde and heptaldehyde. From these results, it is suggested that the decrease of ANS-binding affinity to the liposomes by treatment with ascorbic acid/Fe^{2+} may be due to changes in the surface charge density of the liposomes relating to the formation of fluorescent products.

Keywords lipid peroxidation; surface charge density; aldehyde modification; ANS fluorescence; lecithin liposome

The peroxidation of polyunsaturated fatty acids of biological membrane lipids is a highly destructive process through various free-radical-mediated chain reactions.[1,2]

Recently, the peroxidation reaction has been regarded as being involved in the damage to membrane structure and in the pathological events including aging of cells.[3,4] In fact, it has been reported by several investigators that lipid peroxidation of biological membranes results in changes in their lipid organization such as a decrease of the lipid fluidity.[5-7] In addition, several lines of evidence showing the damage of membrane proteins by lipid peroxidation have been reported, *i.e.* protein fragmentation,[8-10] intermolecular cross-links formation[11] and amino acid modification.[12] We have also recently reported[13,14] that the conformation of the proteins in the porcine intestinal brush-border membranes is sensitively modified by lipid peroxidation of the membranes.

Lipid hydroperoxides formed during lipid peroxidation reaction are degraded into a complex mixture of secondary oxidative products such as aldehydes in the presence of metal ions.[15-17]

As is well known,[18] these aldehydes are able to react with primary amino groups in proteins and phospholipids in biological membranes to form fluorescent conjugated products. Previously, we have reported[19] that ascorbic acid/Fe^{2+}/*tert*-butyl hydroperoxide-induced lipid peroxidation of the porcine intestinal brush-border membranes causes the formation of fluorescent products due to the interaction of the membrane components with monofunctional aldehydes, rather than malondialdehyde. Therefore, it is of interest to study whether the interaction of membrane components with monofunctional aldehydes induces modification in the membrane structure.

In the present study, we examined using a fluorescence dye, 8-anilino-1-naphthalenesulfonate (ANS), the effects of lipid peroxidation and of aldehyde modification on the membrane structure of lecithin liposomes.

Experimental

Materials ANS (magnesium salt) was purchased from Nakarai Chemical Co. Phosphatidylcholine (egg yolk) was obtained from Sigma Co. 2-Thiobarbituric acid, 3(2)-*tert*-butyl-4-hydroxyanisole (BHA), acetaldehyde and heptaldehyde were obtained from Wako Pure Chemical Co. All other chemicals were of the purest grade commercially obtainable.

Preparation of Lecithin Liposomes A chloroform solution of egg yolk phosphatidylcholine (30 mg/ml) was evaporated to dryness with a stream of nitrogen gas. After the residual solvent was completely removed under vacuum, the appropriate amount of 10 mM Tris–HCl buffer (pH 7.4) was added and then sonicated with an Ultra Sonic Disruptor UR-200 P (Tomy Seiko Co., Tokyo) until the dispersion became clear. This clear solution was centrifuged at 25000 × *g* for 20 min and the supernatant was used in the present study.

Lipid Peroxidation of Lecithin Liposomes The lecithin liposomal suspension (3 mg/ml) was incubated with 100 μM ascorbic acid and 10 μM $FeSO_4$ in 30 mM Tris–HCl buffer (pH 7.4) for 10 min at 37 °C, unless otherwise specified. After termination of the reaction by the addition of 5 mM BHA (as a final concentration), the extent of lipid peroxidation was checked by measurement of the formation of conjugated diene as described in our previous paper.[20] The control liposomes were treated in a similar manner in the presence of 5 mM BHA, but without ascorbic acid/Fe^{2+}.

Fluorescence Measurements Fluorescence measurements were performed using a Hitachi spectrofluorometer MPF-4 equipped with a rhodamine B quantum counter at 25 °C. The excitation and emission wavelengths used in the measurement of ANS fluorescence were 340 and 480 nm, respectively. On the other hand, the formation of fluorescent products associated with lipid peroxidation of the liposomes was measured by monitoring the fluorescence intensity at 430 nm using the excitation wavelength at 355 nm as previously reported.[19] In all experiments, the 390 nm cutoff filter was used to prevent error due to light scattering of the sample emission.

Determination of ANS-Binding Parameters The apparent dissociation constant of the ANS–liposome complex was estimated by the fluorescence titration procedure as described in our previous paper.[21] In this experiment, the ANS concentration was varied from 6.62 to 62.5 μM at a fixed concentration of the liposomes (0.1 mg/ml) in 10 mM Tris–HCl buffer (pH 7.4) at 25 °C.

Preparation of Aldehyde-Modified Liposomes The lecithin liposomes (3 mg/ml) were incubated with 0.5 mM acetaldehyde or heptaldehyde (as a final concentration) for 60 min at 37 °C in 10 mM phosphate buffer (pH 7.1) in the presence of 5 mM BHA. The reaction of the liposomes with these aldehydes was checked by the measurement of fluorescence

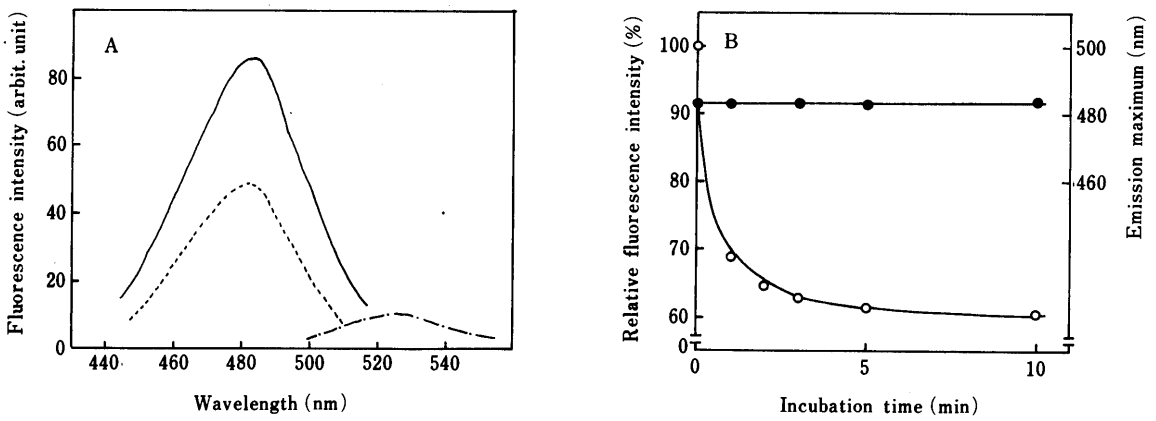

Fig. 1. Changes in Fluorescence Characteristics of Bound ANS to Liposomes

(A) Fluorescence spectra of ANS-probed liposomes. The fluorescence spectra were recorded 10 min after the addition of 10 μM ANS (as a final concentration) to control and peroxidized liposomes (0.1 mg/ml) in 10 mM Tris–HCl buffer (pH 7.4). The procedure and conditions of lipid peroxidation of the liposomes are described under Experimental. ——, control liposomes; – – –, peroxidized liposomes; –·–·–, ANS alone. (B) Changes in ANS fluorescence by lipid peroxidation. The experimental conditions were the same as those described in (A), except variation of the incubation time of the liposomes with ascorbic acid/Fe^{2+} (1—10 min). The fluorescence intensity was represented as relative to that of control liposomes. Symbols: ○, fluorescence intensity; ●, emission maximum. Values are expressed as means of triplicate determinations.

development at 430 nm using the excitation wavelength at 355 nm as described in our previous paper.[19]

Treatment of Liposomes with Sodium Borohydride Sodium borohydride reduction was performed by incubation of the liposomes (0.1 mg/ml) with 10 mM $NaBH_4$ (as a final concentration) in 10 mM phosphate buffer (pH 7.1) for 60 min at 37 °C.

Results and Discussion

Fluorescence Spectra of ANS-Probed Control and Peroxidized Liposomes As previously reported,[20] treatment of lecithin liposomes with 100 μM ascorbic acid/10 μM Fe^{2+} at 37 °C results in the formation of conjugated diene and thiobarbituric acid-reactive substances, depending on the incubation time.

Figure 1A shows the fluorescence spectra of ANS-probed control and peroxidized liposomes at 25 °C.

When 10 μM ANS was added to the liposomal suspension, the fluorescence intensity of the dye markedly increased and the emission maximum shifted from 526 to 483 nm. On the other hand, treatment of the liposomes with ascorbic acid/Fe^{2+} for 10 min at 37 °C resulted in a decrease in the fluorescence intensity by approximately 40%, but the emission maximum did not change by the treatment. Under these conditions, the amount of conjugated diene formed during the reaction was 54.6± 1.7 nmol/mg liposome ($n=3$, S.D.).

In addition, as shown in Fig. 1B, the extent of decrease in the fluorescence intensity of ANS-probed liposomes by treatment with ascorbic acid/Fe^{2+} was dependent on the incubation time. In this experiment, the fluorescence intensity of the complex did not change by treatment of the liposomes with ascorbic acid or Fe^{2+} alone (data not shown).

From these results, it is clear that a change in the fluorescence intensity of ANS-probed liposomes by treatment with ascorbic acid/Fe^{2+} is due to lipid peroxidation of the liposomes. As is well known,[22] the emission maximum of ANS sensitively changes depending upon the solvent polarity. In the present study, the emission maximum of ANS-probed liposomes did not change by treatment with ascorbic acid/Fe^{2+}. This indicates that the polarity around ANS bound to the liposomes did not change by lipid peroxidation of the liposomes.

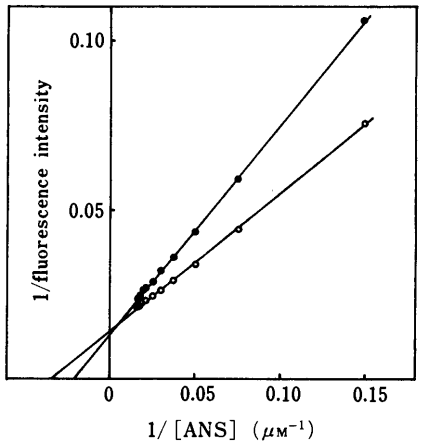

Fig. 2. Double Reciprocal Plots of Fluorescence Intensity against ANS Concentration

The concentration of ANS was varied from 6.62 to 62.5 μM. Other experimental conditions were the same as those described in the legend to Fig. 1A. Symbols: ○, control liposomes; ●, peroxidized liposomes (10 min).

Effects of Lipid Peroxidation on ANS-Binding Parameter To investigate what kind of factors are related to change in the fluorescence intensity of ANS-probed liposomes by lipid peroxidation, the effect of ascorbic acid/Fe^{2+} treatment on the binding affinity of the dye to the liposomes was examined by the fluorescence titration procedure.

As shown in Fig. 2, the titration curves with ANS at a fixed concentration of the liposomes followed simple saturation kinetics in both control and peroxidized liposomes, because the double reciprocal plots of the fluorescence intensity against the concentration of ANS were linear over the ANS concentration tested. From these plots, the apparent dissociation constants (K_d) of ANS for the control and peroxidized liposomes were determined to be 29.2 and 43.0 μM, respectively. On the other hand, treatment of the liposomes with 100 μM ascorbic acid or 10 μM Fe^{2+} alone did not have an affect on the ANS-binding affinity (30.4 and 27.9 μM for ascorbic acid and Fe^{2+} treatment, respectively).

From these results, it is concluded that the decrease in

the fluorescence intensity of ANS-probed liposomes by lipid peroxidation is mainly due to the decrease in the binding affinity of the dye to the liposomes.

Fluorescence Product Formation and ANS-Binding Affinity As is well known,[18] lipid peroxidation of biological membranes results in the formation of fluorescent products which are derived from interaction of the membrane components with secondary peroxidation products such as aldehydes. Therefore, we examined the contribution of the formation of fluorescent products on peroxidation-induced change in the ANS fluorescence in the liposomes.

As shown in Figs. 3 and 4, incubation of the liposomes with 100 μM ascorbic acid/10 μM Fe^{2+} resulted in the formation of fluorescent products with the emission maximum around 430 nm, depending on the incubation time. In addition, this emission maximum was very close to that of acetaldehyde- or heptaldehyde-modified liposomes. On the other hand, the incubation of the liposomes with ascorbic acid or Fe^{2+} alone did not induce the formation of fluorescent products (data not shown). In addition, it was found that there is a good correlation between the extents of fluorescent product formation and the ANS-binding affinity (Fig. 4).

Next, we examined by the fluorescence titration procedure the effects of aldehyde modification of the liposomes on the ANS-binding affinity. The results are summarized in Table I.

It is clear that the K_d value for ANS is markedly increased by modification of the liposomes with acetaldehyde or heptaldehyde.

Effect of Sodium Borohydride Reduction Table II shows the effect of $NaBH_4$ reduction on the intensity of the fluorescent products formed in peroxidized and aldehyde-modified liposomes. The fluorescence intensity of acetaldehyde- or heptaldehyde-modified liposomes increased (approximately 35—40%) by incubation with 10 mM $NaBH_4$. A similar response of the fluorescence intensity for $NaBH_4$ reduction was also observed in peroxidized liposomes. In addition, these results are consistent with those obtained previously in the experiment with the porcine intestinal brush-border membranes.[19]

Recently, it has been proposed that monofunctional aldehydes are possibly involved in the formation of fluorescent products in biological membranes by lipid peroxidation.[19,23,24] In the present study, it was found that the emission maximum (430 nm) of fluorescent products formed in the liposomes by treatment with ascorbic acid/Fe^{2+} was very close to that of the products derived from the interaction of lysine[19] or polylysine[25] with monofunctional aldehydes. In addition, the fluorescence characteristics of the products formed in peroxidized liposomes resembled those of the products derived by

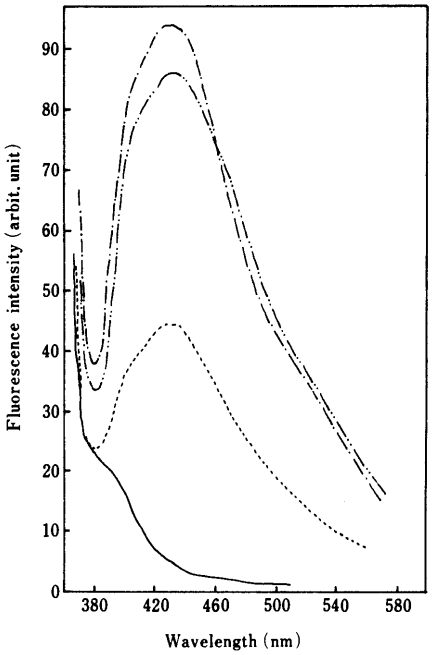

Fig. 3. Fluorescence Spectra of Peroxidized and Aldehyde-Modified Liposomes in 10 mM Phosphate Buffer (pH 7.1)

The concentrations of liposomes employed in the fluorescence measurement were 0.1 mg/ml. The procedure and conditions of lipid peroxidation of the liposomes are described under Experimental. ——, control liposomes; – – –, peroxidized liposomes; —·—, acetaldehyde-modified liposomes; ——·——, heptaldehyde-modified liposomes.

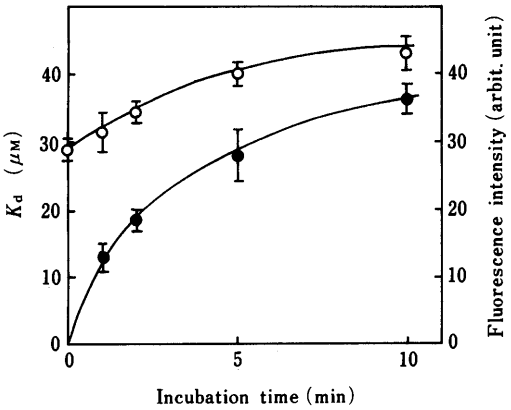

Fig. 4. Relationship between Degrees in Fluorescent Product Formation and ANS-Binding Affinity

The experimental conditions were the same as those described in the legends to Figs. 1B, 2 and 3, except for the use of 10 mM Tris–HCl buffer (pH 7.4) in all experiments. Symbols: ○, K_d value; ●, fluorescence intensity. Values are expressed as means ± S.D. for triplicate determinations.

TABLE I. Effects of Aldehyde Modification of Liposomes on ANS-Binding Parameter

System	K_d (μM)
Control	25.1 ± 2.4
Acetaldehyde-modified	35.8 ± 2.0
Heptaldehyde-modified	37.4 ± 0.6

The experimental conditions and procedure are the same as those described in the legend to Fig. 2. Values are expressed as means ± S.D. for triplicate determinations.

TABLE II. Response of Fluorescence Intensity for Sodium Borohydride Reduction in 10 mM Phosphate Buffer (pH 7.1)

System	Relative fluorescence intensity (%)
Control	101
Peroxidized	131
Acetaldehyde-modified	142
Heptaldehyde-modified	135

The procedure and conditions of peroxidized and aldehyde-modified liposomes are described under Experimental. The fluorescence intensity was represented as relative to that in the absence of $NaBH_4$ in each system. Values are expressed as means of triplicate determinations.

modification of the liposomes with monofunctional aldehydes, *i.e.* emission maximum (Fig. 3) and response of the fluorescence intensity for $NaBH_4$ reduction (Table II). In addition, it was found that the fluorescence intensity at 480 nm of the products which are derived by the interaction of the liposomes with monofunctional aldehydes were negligible as compared to that of ANS-probed liposomes (4.8, 4.5 and 95.0 in the arbitrary units for acetaldehyde-, heptaldehyde-modified and ANS-probed liposomes, respectively). This result indicates that a decrease in the fluorescence intensity at 480 nm of ANS-probed liposomes by lipid peroxidation is not due to an inner filter effect of the fluorescent products formed in the liposomes.

It is of interest that the fluorescence intensities of peroxidized and aldehyde-modified liposomes increased by treatment with $NaBH_4$, because it has been known[25] that the intensity of the adduct of polylysine with acetaldehyde or heptaldehyde is reduced by $NaBH_4$ reduction. This discrepancy may be explained as due to a possible difference in the products formed in the liposomes.

It is concluded, from these findings, that the fluorescence change in the ANS fluorescence in the liposomes by treatment with ascorbic acid/Fe^{2+} primarily reflects alterations in the electrostatic properties, *i.e.* surface charge density, influencing the binding of ANS anion to the liposomal surface. In addition, the present study proposed the possibility that the formation of fluorescent products in the liposomes is involved in changes in the electrostatic properties of the liposomes, although the exact mechanism of fluorescent product formation in the liposomes associated with lipid peroxidation is unclear at present.

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Internalization Model Using Rat Liver Plasma Membrane for Receptor-Mediated Endocytosis of Epidermal Growth Factor

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We have demonstrated the internalization of epidermal growth factor (EGF) using membrane isolated from rat liver. The isolated membrane exhibited a saturation curve of the binding of ^{125}I-EGF. Furthermore, competition between the binding of ^{125}I-EGF and unlabeled EGF to the isolated membrane was observed. These results were similar to those obtained from whole hepatocytes. In order to confirm whether or not the present experimental system using the isolated membrane can be used for the study of receptor-mediated endocytosis, the internalization of pre-bound EGF in the isolated membrane was assessed by two different methods. First, acid-insensitive ^{125}I-EGF time-dependently increased following incubation at 37 °C. Secondly, EGF became inaccessible to the exogenous ^{125}I-anti-EGF antibody when under the same condition. These processes were dependent on adenosine triphosphate, but independent of Ca^{2+} and stimulated by guanosine triphosphate. These results demonstrate that receptor-mediated endocytosis occurred in the isolated membrane.

Keywords endocytosis; liver plasma membrane; cell-free assay; EGF-internalization; ATP; GTP

Introduction

Receptor-mediated endocytosis has been observed in almost all eukaryotic cells.[1,2] This event appears to have been a general mechanism for the internalization of many polypeptide hormones.[3-5] Morphological studies have indicated that ligand to receptor binding mediated first receptor-clustering into the coated pit of the cell surface.[6,7] These coated pit regions invaginate and pinch-off to form coated vesicles, then bound ligands are taken up into the cell.[6,7] Biochemical studies have shown that the major component of the coat is clathrin and that the assembly polypeptides are integral components of the clathrin-coated vesicles.[7,8] In spite of this structural and biochemical information in receptor-mediated endocytosis, the detailed mechanism by which the endocytosis is induced is unclear.

It is difficult to directly approach the mechanism because the components involved in endocytosis are located on the cytoplasmic face of the plasma membrane. Therefore, the demonstration of endocytosis in a cell-free system serves to provide useful information of its mechanisms. It has been demonstrated that the fusion of endosomes has occurred in a cell-free system.[9,10] From these studies, a preliminary characterization of the later stage of endocytosis has been performed. In their study of the early stage of endocytosis, Smythe *et al.*[11] studied the budding of coated vesicles in A431 cells. They demonstrated that the internalization of pre-bound transferrin occurred in broken A431 cells. Similarly, Heuser[12] studied endocytosis in fibroblast cells broken by sonication. However, these broken cells do not seem to be adequate tools for approaching to the mechanism in the early stage of endocytosis because those broken cells contain both the plasma membrane and cytosol proteins.

In this study, we attempted to employ the isolated plasma membranes from rat liver to demonstrate the early stage of endocytosis in the internalization of epidermal growth factor (EGF).

Materials and Methods

Animals Male Wistar rats (180—200 g body wt.) were used. Animals were given food and water *ad libitum*.

Materials Materials were obtained as follows: Mouse EGF (receptor grade) from Collaborative Research. Iodine-125 labeled mouse EGF (^{125}I-EGF, 6.78 MBq/μg) and iodine-125 (^{125}I) from New England Nuclear. Rabbit anti-mouse EGF antibody (anti-EGF Ab) from Biomedical Technologies. Anti-EGF Ab was labeled with ^{125}I by the method of Markwell.[13]

Preparation of Liver Plasma Membrane Liver plasma membrane was prepared by the method of Tanaka *et al.*[14] with minor modification. Rat liver was perfused with STE buffer (10 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 2 mM ethylene glycol bis(2-aminoethyl ether)-*N,N,N'N'*-tetraacetic acid (EGTA). The perfused liver was homogenized in a 4-fold volume of STE buffer with a Dounce homogenizer (pestle B). The homogenates were centrifuged at 1000 × *g* for 30 s and the pellets were resuspended in an STE buffer, homogenized, and centrifuged. The two supernatants were combined and centrifuged at 5000 × *g* for 10 min. The pellet was resuspended in an equal volume of STE buffer containing 2 mM $MgCl_2$ (STEM buffer) with Dounce homogenizer. The suspension was dispersed in a 4-fold volume of iso-osmotic Percoll solution composed of 7 volumes of Percoll (Pharmacia), 1 volume of conc. STEM buffer (80 mM Tris–HCl (pH 7.5), 2 M sucrose, 8 mM EGTA, 16 mM $MgCl_2$) and 32 volumes of STE buffer. The mixture was centrifuged at 10000 × *g* for 60 min. The clear band of membranes just below the surface was collected and washed with a 5-fold volume of 50 mM Tris–HCl buffer (pH 7.5) and centrifuged at 1000 × *g* for 10 min. The pellet was washed twice, suspended in 50 mM of Tris–HCl buffer and stored at −80 °C until use.

Preparation of Rat Hepatocytes Preparation of rat hepatocytes was performed essentially according to Tanaka *et al.*[15] as follows. Rat liver was perfused *in situ* at 37 °C *via* the portal vein with 300 ml of calcium-free Hanks' balanced salt solution containing 5 mM EGTA and 10 mM *N*-hydroxyethylpiperazine-*N'*-2-ethansulfonate (Hepes, pH 7.4). The perfusion was continued with 200 ml of Hanks' balanced salt solution containing 5 mM $CaCl_2$ and 0.05% (w/v) collagenase. After the perfusion, the digested liver was suspended in 20 ml of Williams' medium E (Flow Laboratories) containing 10% fetal calf serum (Flow Laboratories), 100 IU/l potassium penicillin G and 100 μg/l streptomycin sulfate, and was filtered through coarse gauze. The filtrate was centrifuged for 1 min at 20 × *g* at 4 °C and the resulting pellet was washed twice with serum-free Williams' medium E. The final pellet was suspended in a medium containing serum to give a cell density of 5×10^5 cells/ml. Isolated hepatocytes were plated in 24-well collagen-coated culture dishes (Corning). After 3 h of incubation, the culture medium was replaced to a serum-free medium containing 10^{-9} M insulin and 10^{-9} M dexamethasone. The EGF binding assay was performed after 16 h of incubation.

EGF Binding Assay EGF binding to the receptor of the primary cultured hepatocytes was measured as follows. The culture medium was removed from monolayer cells and the cells were then washed twice with a binding buffer containing 20 mM Hepes, 150 mM NaCl and 2 mg/ml bovine serum albumin, pH 7.4. The cells were incubated for 2 h at 4 °C in the binding buffer with ^{125}I-EGF at the indicated concentrations. After the incubation, the cells were washed twice with the binding buffer and then lysed by the addition of 2 N NaOH. The radioactivity of the lysate was determined using a NaI scintillation counter. Non-specific binding was determined in the presence of a 200-fold concentration of unlabeled EGF, and amounted to less than 7% of the total binding.

Regarding the EGF binding to the receptor of the liver plasma membrane, the binding assay mixture (100 μl) contained various concentrations of ^{125}I-EGF, 50 mM Tris–HCl buffer (pH 7.4), 2 mg/ml bovine serum albumin (BSA), 1 mM EGTA, 1 mM $MgCl_2$, 20 μg/mg leupeptin, 20 μg/ml aprotinin, and liver membranes (0.5 mg/ml). Assay at 4 °C was started by the addition of liver membranes. After 2 h of incubation, the assay was terminated by the addition of 1 ml of ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 2 mg/ml BSA. Membranes were sedimented by centrifugation at 10000 × *g* for 1 min and the supernatant was removed. The pellets were washed twice with 50 mM Tris–HCl buffer and the radioactivity of the pellets was determined using a NaI scintillation counter. Non-pecific binding was determined in the presence of a 200-fold concentration of unlabeled EGF, and amounted to less than 9% of the total binding.

Assay for Internalization of EGF in Hepatocytes Primary cultured rat hepatocytes with pre-bound ^{125}I-EGF (prepared as mentioned above) were incubated in the binding buffer at 37 °C for the indicated time periods. After incubation, the cells were washed twice with the binding buffer and rinsed with 300 μl of 0.5 M NaCl–0.2 M acetic acid for 5 min at 4 °C. Radioactivity of ^{125}I-EGF released from or remaining in the cells by this treatment was expressed as cell surface bound or internalized ^{125}I-EGF, respectively.

Assay for Internalization of EGF in the Liver Membrane Internalization of EGF in the membrane was assessed by two different methods as follows. 1) Acid-resistance assay; the membrane pre-bound ^{125}I-EGF (prepared as mentioned above) was suspended in a 100 μl volume of 50 mM Tris–HCl buffer (pH 7.4) containing 2 mg/ml BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM adenosine triphosphate (ATP) to give a final concentration of 0.5 mg protein/ml. The membrane suspension was incubated at 37 °C for the indicated time periods. Incubation was followed by treatment with a 100 μl volume of ice-cold stopping buffer (3 mM Tris (pH 7.4) containing 1 mM ethylenediaminetetra acetic acid (EDTA), 0.5 mM dithiothreitol and 1 mg/ml BSA) which was added to the suspension, then the membrane was washed twice with 50 mM Tris–HCl buffer. The membrane was rinsed with a 100 μl volume of 0.5 M NaCl–0.2 M acetic acid at 4 °C for 5 min. After this acid treatment, the membrane was washed and radioactivity was measured as mentioned above. 2) Antibody-inaccesibility assay; this assay was performed by the method of Smythe *et al.*[11] with some modifications. Briefly, the membrane pre-bound unlabeled EGF was prepared, suspended, and incubated as mentioned above. After the addition of a stopping buffer, 10 μl of ^{125}I-labeled anti-EGF Ab (1.85 MBq/μg, 4 kBq/10 μl) was added to the membrane. After incubation for 90 min at 4 °C, unbound antibodies were removed by centrifugation at 10000 × *g* for 30 s and filtrated through a 5% BSA-soaked glass filter (GF/C, Whatman). Radioactivity remaining on the filter was determined using a NaI scintillation counter. Non-specific binding was determined in the presence of a 200-fold concentration of unlabeled anti-EGF Ab, and amounted to less than 5% of the total binding.

Results and Discussion

The early stage of receptor-mediated endocytosis occurs on the surface of the plasma membrane of cells. This event seems to be induced by ligand-receptor binding on the cell surface. Since the surface of hepatocytes expresses a large number of receptors for EGF, hepatocytes are used as a general model for receptor-mediated endocytosis.[3,16] Therefore, we chose the liver membrane as an experimental material to approach the mechanism of this early stage of receptor-mediated endocytosis.

We studied the properties of EGF-receptors of isolated membranes from hepatocytes by two binding assay systems as follows. First, in order to investigate whether or not the EGF-receptor of the isolated membrane has a similar EGF-binding pattern to that of the hepatocytes, we demonstrated the saturable process of ^{125}I-EGF binding to the isolated membranes. A maximal binding at 4 °C was obtained at 10—15 nM of ^{125}I-EGF (Fig. 1A). This result was similar to that obtained from the whole hepatocytes (Fig. 1B). Secondly, in order to determine whether or not ^{125}I-EGF binds to a specific EGF receptor of the isolated

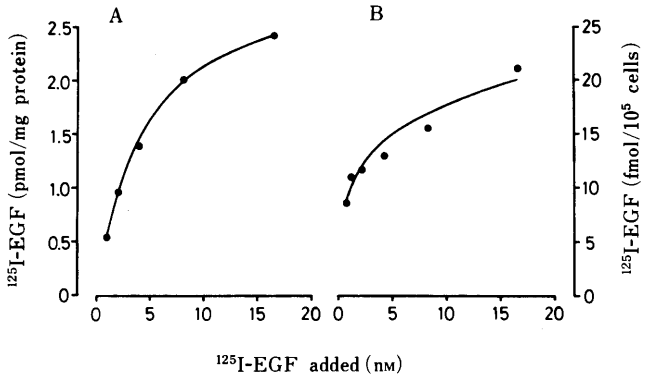

Fig. 1. The Saturation Curves of ^{125}I-EGF Binding to Its Receptors (A) of the Isolated Membrane or (B) in the Primary Cultured Hepatocytes

The membranes (50 μg) or hepatocytes (1.5 × 10^5 cells) were incubated with various concentrations of ^{125}I-EGF for 2 h at 4 °C. Bound ^{125}I-EGF was measured as described in Materials and Methods.

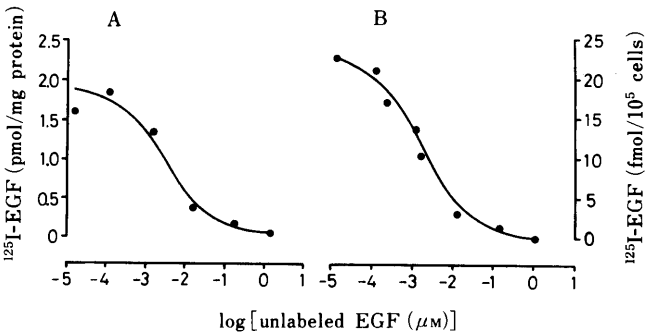

Fig. 2. The Inhibition Curves of ^{125}I-EGF Binding to Its Receptors (A) of the Isolated Membrane or (B) in the Primary Cultured Hepatocytes by Unlabeled EGF

The membranes (50 μg) or hepatocytes (1.5 × 10^5 cells) were incubated with 16.5 nM ^{125}I-EGF in the presence of various concentrations of unlabeled EGF for 2 h at 4 °C. Bound ^{125}I-EGF was measured as described in Materials and Methods.

membrane, we studied the competition between the binding of ^{125}I-EGF to the isolated membrane and that of unlabeled EGF. The results showed that the amount of bound ^{125}I-EGF proportionally decreased to the amount of unlabeled EGF added (Fig. 2A). A similar competition curve was also obtained from whole hepatocytes (Fig. 2B). Furthermore, the competition between the binding of ^{125}I-EGF to the isolated membrane and that of transferrin did not occur (data not shown). A Scatchard plot of the binding data for ^{125}I-EGF in the isolated membrane was linear and yielded apparent dissociation constants (K_D) of 4.1×10^{-9} M. The number of binding sites (B_{max}) was 2.47 pmol/mg protein. The values of K_D for ^{125}I-EGF from hepatocytes was similar to that obtained from the membrane (K_D; 3.4×10^{-9} M, B_{max}; 21.5 fmol/10^5 cells). These results coincide with the results of a previous report by Moriarity and Savage.[16] These findings suggest that EGF binds to a specific receptor on the liver membrane, and that its binding pattern closely resembles the pattern in primary cultured hepatocytes.

The processes of receptor-mediated endocytosis have been studied using acetic acid treatment. It is known that the sensitivity of a pre-bound ligand to acetic acid indicates its ligand location on the cell surface receptor.[17,18] We studied the time courses of internalized or remaining pre-bound EGF on the cell surface by incubation at 37 °C in primary

cultured hepatocytes. The results showed that pre-bound EGF to the cell surface receptor was internalized within 5—10 min (Fig. 3). This observation concurs with the results of the previous report,[16] and is similar to the uptake of EGF by fibroblasts[18] and WB 344 cells.[19] Using this technique, we investigated whether or not the internalization of pre-bound EGF to the receptor occurred in the isolated membrane. We found that acid-insensitive EGF time-dependently increased during incubation at 37 °C and was saturated within 4 min (Fig. 4). This result suggests that the internalization of EGF occurs in the isolated membrane at 37 °C. Furthermore, in order to confirm this suggestion, we performed an antibody-inaccessible assay using the method of Smythe *et al.*[11] with some modifications. The result of this assay showed that pre-bound EGF to the isolated membrane became inaccessible to exogenous ^{125}I-anti-EGF antibodies during incubation at 37 °C (Fig. 5). No antibody bound to the isolated membrane when pre-binding to the membrane had not occurred (data not shown). These observations support the ability of the isolated membrane to internalize EGF.

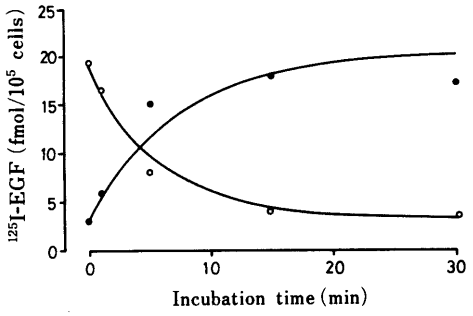

Fig. 3. Time Courses of Cell-Surface Binding or Internalization of Pre-bound ^{125}I-EGF in the Primary Cultured Hepatocytes

Hepatocytes (1.5×10^5 cells) were incubated with 16.5 nM ^{125}I-EGF for 2 h at 4 °C. After unbound ligands were removed, hepatocytes were further incubated for the indicated time periods at 37 °C. (○) Cell surface bound or (●) internalized ligands were determined as described in Materials and Methods. Similar results were obtained in three separate experiments.

In order to investigate whether or not this assay system is adequate to approach the mechanism of receptor-mediated endocytosis, we studied the effects of various reagents which affect the endocytosis on the internalization of EGF. Mozhayeva *et al.*[20] reported that the binding of EGF to the receptor of A431 cells induced a Ca^{2+}-influx into the cells by activation of a Ca^{2+}-channel. Furthermore, it has been suggested that extracellular Ca^{2+} is required for receptor-mediated endocytosis of transferrin in reticulocytes.[21,22] In this study, the internalization of EGF was inhibited by treatment with EDTA (Table I). No effect on internalization, however, was observed by treatment with EGTA, a specific Ca^{2+}-chelating agent (Table I). These results suggest that the internalization of EGF in the present assay system requires a metal ion, such as Mg^{2+}, but not Ca^{2+}. Beckers and Balch[23] reported that Ca^{2+} was provided for the transport of intracellular vesicles from endoplasmic reticulum to the Golgi compartment in

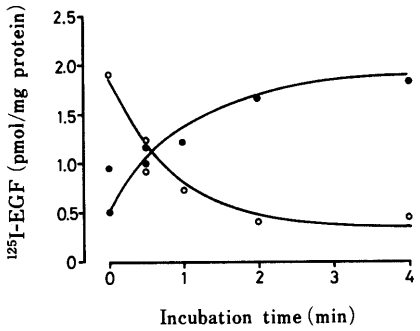

Fig. 4. Time Courses of Surface Binding or Internalization of Pre-bound ^{125}I-EGF as Assessed by the Acid-Resistance Assay in the Isolated Membrane During Incubation at 37 °C

The membrane (50 μg) was incubated with 16.5 nM ^{125}I-EGF for 2 h at 4 °C. After unbound ligands were removed, the membrane was incubated in the presence of 1 mM ATP for the indicated time periods at 37 °C. (○) Surface bound or (●) internalized ligands were determined as described in Materials and Methods. Similar results were obtained in three separate experiments.

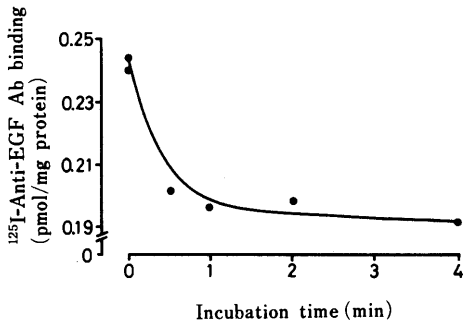

Fig. 5. Time Course of Exogenous ^{125}I-Anti-EGF Antibody-Inaccessibility to Pre-bound Unlabeled EGF as Assessed by the Antibody-Inaccessibility Assay in the Isolated Membrane during Incubation at 37 °C

The membrane (50 μg) was incubated with 16.5 nM EGF for 2 h at 4 °C. After unbound ligands were removed, the membrane was incubated in the presence of 1 mM ATP at 37 °C. At the indicated time periods, the membrane was incubated with ^{125}I-anti-EGF Ab for 90 min at 4 °C. Binding of ^{125}I-anti-EGF Ab to the membrane was determined as described in Materials and Methods. Similar results were obtained in three separate experiments.

TABLE I. Effects of EDTA or EGTA on the Internalization of Pre-bound ^{125}I-EGF in the Liver Membrane

Incubation time (min)	Assay for internalization					
	Acid-resistance			Antibody-inaccessibility		
	^{125}I-EGF binding (pmol/mg protein)			^{125}I-anti-EGF Ab binding (fmol/mg protein)		
	Control	EDTA	EGTA	Control	EDTA	EGTA
0	0.58±0.03	0.64±0.06	0.60±0.04	240±5	237±6	236±5
1	0.95±0.15	0.79±0.03	0.89±0.11	205±4	224±1[a]	198±3
4	1.47±0.35	0.81±0.09[a]	1.37±0.35	196±4	219±1[b]	193±4

The isolated membrane (50 μg) was incubated with ^{125}I-EGF for 2 h at 4 °C. After unbound ligands were removed, the membrane was incubated in the presence of 1 mM ATP with or without 1 mM EDTA or EGTA for the indicated time periods at 37 °C. Assays for internalization were performed as described in Materials and Methods. Each value is the mean ± S.D. of two samples. Similar results were obtained in two separate experiments. Significantly different from control by Student's *t*-test (*a*) $p < 0.05$, *b*) $p < 0.01$).

TABLE II. Effects of ATP or GTP on the Internalization of Pre-bound ^{125}I-EGF in the Liver Membrane

Incubation time (min)	Acid-resistance assay ^{125}I-EGF binding (pmol/mg protein)				Antibody-inaccessibility assay ^{125}I-anti-EGF Ab binding (fmol/mg protein)				
ATP	(+)	(−)	(+)	(−)	ATP	(+)	(−)	(+)	(−)
GTP	(−)	(−)	(+)	(+)	GTP	(−)	(−)	(+)	(+)
0	0.59±0.02	0.64±0.15	0.57±0.01	0.68±0.03		236±5	243± 2	233±4	241±2
1	1.03±0.18	0.75±0.02	1.36±0.07	0.77±0.03		199±5	232±13	166±4[a]	235±5
4	1.46±0.03	0.77±0.02[b]	1.83±0.01[b]	0.79±0.04		195±6	240± 2[b]	148±2[b]	234±5

The isolated membrane (50 μg) was incubated with ^{125}I-EGF for 2 h at 4 °C. After unbound ligands were removed, the membrane was incubated in the presence or absence of 1 mM ATP and/or 100 μM GTP for the indicated time periods at 37 °C. Internalization of ^{125}I-EGF was assessed by the acid-resistance assay or the antibody inaccessibility assay. Each assay for internalization was performed as described in Materials and Methods. Each value is the mean±S.D. of two samples. Similar results were obtained in two separate experiments. Significantly different from "ATP (+) and GTP (−)" by Student's *t*-test (*a*) $p<0.05$, *b*) $p<0.01$).

the secretory pathway *in vitro*. Furthermore, it was reported that the endocytosed vesicle was transferred from endosomes to a trans-Golgi network in a cell-free assay system.[24] Therefore, the Ca^{2+}-independent stage which occurs prior to transport to the Golgi compartment seems to take place in the present assay system. However, we think that further study needs to be done regarding the role of Ca^{2+} in the present internalization model because it seems that 1 mM EGTA cannot entirely chelate 1 mM Ca^{2+}.

It has been suggested that receptor-mediated endocytosis requires cellular ATP.[25,26] Recently, Schmid and Carter[27] suggested that ATP was required for the formation of coated vesicles, but not required for the formation of invaginated pits in the endocytosis of transferrin in K562 cells and HeLa cells. In this study, the internalization of EGF on the isolated membrane was inhibited by the removal of ATP (Table II). These findings suggest that the formation of coated vesicles occurs on the isolated membrane in the present assay system. Furthermore, it is suggested that early endosome fusion occurs on the membrane in the present assay system because the fusion of endosome requires cellular ATP.[28] Chavrier *et al.*[29] reported that rab5, a small guanosine triphosphate (GTP)-binding protein, localized on the cytoplasmic face of the plasma membrane and early endosomes. They suggested that rab5 was involved in the process of early endosome fusion.[30] Therefore, in order to investigate whether or not early endosome fusion occurs in the present assay system, we studied the effect of 100 μM GTP on the internalization of EGF. The result was that GTP stimulated the internalization of EGF on the isolated membrane in the presence of ATP, but not in the absence of ATP (Table II). It was reported that the stimulation of early endosome fusion by GTP was observed in a cell-free system.[31] From these findings, we think that the early endosome fusion occurs on the membrane in the present assay system. Additionally, it is also suggested that Mg^{2+} contributes to the activation of GTP-binding protein since Mg^{2+} is required for the activity of this regulatory protein.[32] In order to understand the processes of receptor-mediated endocytosis, it is important to clarify the detailed mechanism by which the early stage of endocytosis is induced. The present experimental system using the isolated membrane is available for further studies on the mechanism of receptor-mediated endocytosis.

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Urethanase of *Bacillus licheniformis* sp. Isolated from Mouse Gastrointestine

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Trace levels of urethane, a cancer causing chemical, were detected in many kinds of wine, sherry, whisky, brandy and sake. Urethane formation from urea and ethanol in sake can be prevented by the treatment of acid urease, which is produced by *Lactobacillus fermentum*, but urethane, once formed, is very difficult to decompose. In order to keep the safety of alcoholic beverages, enzymatic removal of urethane has become an urgent problem. We found that *Bacillus licheniformis* sp., isolated from mouse gastrointestine, decomposed urethane to ethanol and ammonia. The enzyme showed higher urethanase activity at an acidic condition than at a neutral condition, and was resistant against ethyl alcohol of high concentrations. However, the enzyme had a low affinity to urethane for the industrial removal of the compound from alcoholic beverages.

Keywords ethylcarbamate; carcinogenic; mutagenic; teratogenic; alcoholic beverage; *Bacillus licheniformis*; gastrointestinal bacteria; urethanase

Introduction

Ethylcarbamate (urethane), which is known to be a cancer-causing chemical,[1,2] was reported to contaminate various kinds of alcoholic beverages.[3,4] Several years ago, trace levels of urethane were detected in many brands of wine, sherry, whisky, brandy and sake in Canada. Since this problem is world-wide and involves all fermented beverages, many studies on the mechanisms of urethane formation and methods for lowering urethane content have been performed.[5-8] It was reported that urethane is produced by the chemical reaction of urea with ethanol in wine[1,7,9] and sake.[6] Removal of urea, the major precursor, by an acid urease from wine[10,11] and sake[12,13] has been successful. However urethane, once formed by distillation or during long-term storage, could not be decomposed by urease. The fact that ^{14}C-labelled urethane orally administrated to mouse is decomposed to exhale [^{14}C]carbon dioxide had been reported in the 1960s.[2] Recently, Yamamoto *et al.*[14] reported the urethane hydrolysing activity in the homogenates of mouse liver. However, they did not confirm the hydrolysis of urethane to be an enzyme reaction owing to its low activity. We[15] found that *Citrobacter* sp. isolated from mouse feces decomposed urethane to ethanol and carbon dioxide at a neutral pH range and purified the enzyme partially from bacteria and named urethanase. In this report, we searched for further gastrointestinal bacteria producing urethane-hydrolyzing enzyme(s) in mouse and found that *Bacillus licheniformis* sp., isolated from bacterial flora, decomposed urethane at an acidic condition.

Materials and Methods

Screening of Urethanase-Producing Bacteria from Gastrointestinal Contents in Mouse The intestinal contents of mouse (ddY female, 4 weeks, SLC, Japan) were obtained 4 d after the oral administration of urethane solution (20 mg/kg body weight/d). After suspending the contents in sterile 0.9% NaCl, 1 ml of the suspensions (10 mg wet weight/ml) was inoculated into the following media. The screening media were composed of 0.238% HEPES: 2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 0.1% vitamin mixture (30 g/l choline HCl, 10 mg/l nicotinamide, 2.5 mg/l each of Ca pantothenate and thiamine HCl, 1.25 mg/l riboflavin, 0.75 mg/l pyridoxine HCl, 0.6 mg/l *p*-aminobenzoate, 0.5 mg/l folic acid and 0.1 mg/l biotin), 0.02% mineral mixture (Oriental Yeast Co., Tokyo, Japan), containing 0.5% urethane, 0.2% acetamide or 0.2% glutamine. The cultivation was performed aerobically at 37 °C for 4 d. After 4 d, bacteria were collected by centrifugation (5000*g*, 20 min) and sonicated. Urethane-hydrolyzing activity was measured both at pH 4.5 and 7.0. Three species of bacterium were isolated from agar plates of screening medium, pH 5.0, containing 0.2% acetamide, and these bacteria were found to hydrolyze urethane. The bacterium showing the highest urethanase activity among them was characterized according to the method of Cowan.[16]

Preparation of Crude Enzyme Solution from the Bacterium The bacterium, *Bacillus licheniformis* sp. 1013 which was isolated from mouse stomach contents, was aerobically grown in 10 l of the screening medium for 4 d. Cells harvested from the screening medium (10 l) were suspended in 9 ml of 10 mM phosphate buffer, pH 7.0, and disrupted by sonication (70 W, Heat Systems-Ultrasonics, U.S.A.) for 5 times at an interval of 1 min in an ice bath. After centrifugation (20000*g*, 15 min) the supernatant fluid was used as a crude enzyme solution.

Enzyme Assay Method Urethanase was assayed at 37 °C by measuring the amounts of ammonia or ethanol produced from urethane. The standard reaction mixture contained 13.3 mM urethane and 0.1 M 2-(*N*-morpholins)ethane sulfonic acid (MES) buffer, pH 4.5. The reaction was initiated by adding 100 μl of the crude enzyme solution to 300 μl of the standard reaction mixture. The reaction was terminated by adding 100 μl of 1 N sulfuric acid. After removing the precipitate by centrifugation, the supernatant was analyzed for ammonia or ethanol. The ammonia was determined spectrophotometrically at 630 nm by the method of indophenol,[17] and ethanol was measured by the enzymatic method using alcohol dehydrogenase.[18]

Protein Measurement Protein was measured by the Folin–Lowry method[19] using bovine serum albumin as the standard.

Results and Discussion

The urethanase activity of each gastrointestine content in mouse and the feces in mouse were shown in Table I.

TABLE I. Urethanase Activity of Intestinal Contents in Mouse

Origin	Urethanase activity (nmol/min/mg·protein)	
	At pH 4.5	At pH 7.0
Stomach	0.55±0.19	0.34±0.08
Upper small intestine	0.96±0.23	1.25±0.21
Lower small intestine	0.46±0.09	0.68±0.17
Caecum	0.34±0.05	0.63±0.12
Large intestine	0.14±0.11	0.36±0.06
Feces	0.61±0.20	0.91±0.26

Mouse (female) was anesthetized with ether. Contents of stomach, upper small intestine, lower small intestine, caecum, large intestine and feces were taken out separately. After washing the contents with 0.9% NaCl by centrifugation, the sediments were suspended and sonicated. Urethanase activity in the supernatant obtained by centrifugation (10000*g*, 20 min) was measured according to the methods as described in Materials and Methods. The values were mean ± S.D. of 4 experiments.

They were very low both at pH 4.5 and 7.0. They did not increase even by cultivation of each content at 37 °C for 4 d. However, higher urethanase activities were observed in the intestinal contents taken from the mouse orally administered urethane solution (20 mg/kg body weight/d) for 4 consecutive days and cultured aerobically at 37 °C for 4 d. As shown in Table II, urethanase activity was detected by the addition of acetamide or glutamine. The activity at pH 4.5 increased 6-fold in the case of stomach contents cultivated on the screening medium, pH 5.0, containing 0.2% acetamide. But bacteria did not grow at all with the addition of 0.5% urethane and therefore urethanase activity was not detected.

From these results, we attempted to isolate urethanase-producing bacteria from mouse stomach contents and by cultivation with an acetamide-containing medium.

Three species of bacterium, which hydrolyze urethane at acidic (pH 4.5) and neutral (pH 7.0) conditions, were isolated from agar plates of screening medium, pH 5.0, containing 0.2% acetamide. Among them, bacterium I showed the highest urethanase activity both at pH 4.5 and 7.0 (Table III). This bacterium was spore-forming, motile and facultative anaerobe. The cell grown on a nutrient agar plate was positive in Gram-staining, and was short rod-shaped. Some other biochemical characteristics were listed in Table IV. It differed from *Streptococcus* in the mobility test, *Lactobacillus* and *Micrococcus* in the VP

TABLE II. Urethanase Activity of Intestinal Bacteria Grown in Media Containing Amide Derivatives

Amide	Origin	Urethanase activity (nmol/min/mg·protein)	
		At pH 4.5	At pH 7.0
0.2% acetamide	Stomach	3.4 ±0.78	1.6 ±0.32
	Small intestine	2.5 ±0.44	3.7 ±0.57
	Caecum	1.1 ±0.31	2.1 ±0.24
	Large intestine	2.5 ±0.69	5.1 ±0.63
0.2% glutamine	Stomach	0	0.035±0.021
	Small intestine	0.26±0.08	0.90 ±0.21
	Caecum	0	0.15 ±0.11
	Large intestine	0	0.35 ±0.21

Urethanase activity was measured according to the methods as described in Materials and Methods. The values were mean±S.D. of 4 experiments.

TABLE III. Urethanase Activity of Bacteria Isolated from Cultured Stomach Contents

		Isolated bacteria		
		I	II	III
Colony		Rough transparent	Smooth transparent	Smooth opaque
Cell shape		Short-rod	Rod	Long-rod
Gram staining		+	−	−
Urethanase activity (nmol/min/mg·protein)	pH 4.5	19±2.71	4.9±0.87	5.3±0.93
	pH 7.0	14±1.32	3.5±0.62	3.1±0.51

The colony isolated from a screening medium agar plate was cultivated in 0.2% acetamide-screening medium, pH 5.0 at 37 °C for 4 d. Harvested cells were suspended in phosphate buffered saline in a final concentration of 4.0 mg·protein/ml. After sonication, the suspensions were centrifuged at 20000 *g* for 20 min. Urethanase activity in the supernatants was measured by the methods as described in Materials and Methods. The values were mean±S.D. of 4 experiments.

TABLE IV. Characteristics of the Isolated Bacterium I

	The isolated bacterium	*B. licheniformis*[16]
Catalase	+	+
Oxidase	+	+
Gas production	+	+
OF test	F	F
Citrate utilization	+	+
Carbohydrate; acid		
Glucose	+	+
Arabinose	+	+
Mannitol	+	+
Xylose	+	+
VP test	+	+
Nitrate reduction to nitrite	+	+
Indole test	−	−
Urease test	−	−

VP: Voges–Proskauer reaction. OF: oxidation and fermentation. −: negative, +: positive.

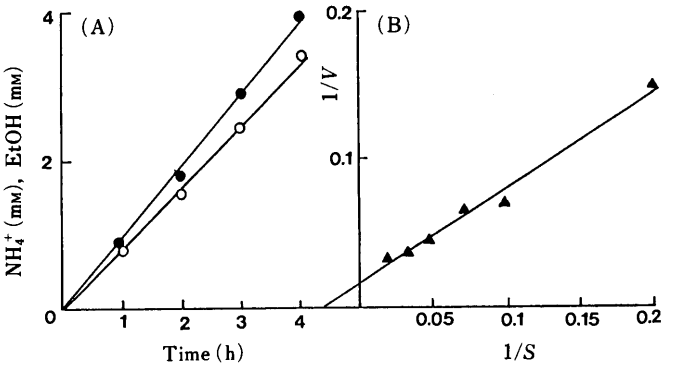

Fig. 1. Time Course and Lineweaver–Burk Plot of Urethanase of *Bacillus licheniformis* sp. 1013

(A) Time course; the enzyme preparation (100 μl) was incubated with 13.3 mM urethane (300 μl) in 0.1 M MES buffer, pH 4.5 at the indicated period of time at 37 °C. After incubation, the concentrations of ammonia (●), and ethanol (○) were measured by the methods described in Materials and Methods. (B) The relationship between substrate concentrations and reaction velocity; the enzyme preparation was incubated with urethane of various concentrations in 0.1 M MES buffer, pH 4.5 for 2 h at 37 °C.

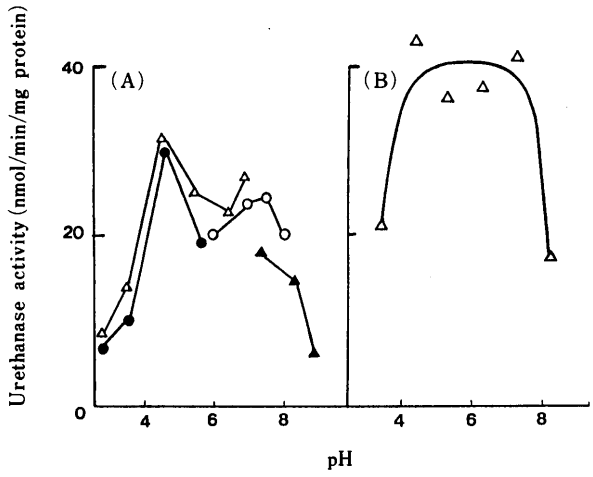

Fig. 2. Effect of pH on the Activity and Stability of Urethanase of *B. licheniformis* sp. 1013

(A) Effect of pH on the enzyme activity; the enzyme preparation (100 μl) was incubated with 13.3 mM urethane (300 μl) in 0.1 M acetate (●), phosphate (○), citrate (△) and Tris–HCl (▲) buffer for 2 h at 37 °C. (B) Effect of pH on the enzyme stability; the enzyme preparation was preincubated in 0.1 M citrate buffers at various pHs at 37 °C for 30 min. The residual enzyme activity was assayed after adjustment of the pH of the mixture to pH 4.5.

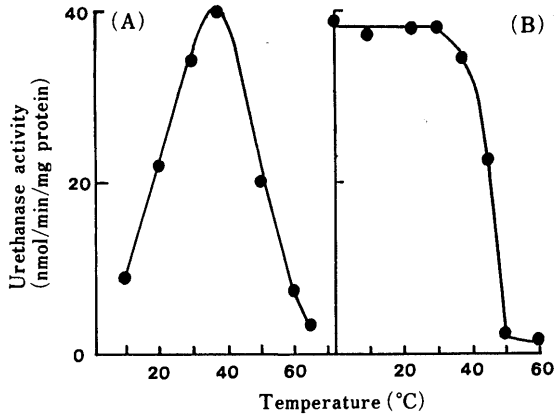

Fig. 3. Effects of Temperature on the Activity and Stability of Urethanase of *B. licheniformis* sp. 1013

(A) Effect of temperature on the enzyme activity; the enzyme preparation (100 μl) was incubated with 13.3 mM urethane (300 μl) in 0.1 M MES buffer, pH 4.5 for 2 h at the indicated temperatures. (B) Effect of temperature on the enzyme stability; the enzyme preparation was preincubated for 1 h at the indicated temperatures and then the whole reaction mixture was rapidly cooled in an ice bath. The residual enzyme activity was measured as described in Materials and Methods.

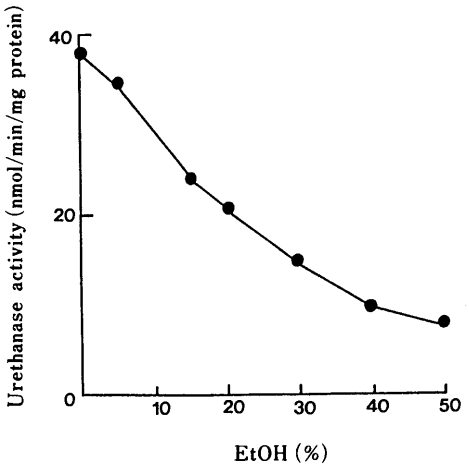

Fig. 4. Effect of Ethanol on the Urethanase Activity of *B. licheniformis* sp. 1013

The enzyme preparation (100 μl) was incubated with 13.3 mM urethane (300 μl) in 0.1 M MES buffer, pH 4.5, containing various concentrations of ethanol for 2 h at 37 °C. The residual enzyme activity was measured as described in Materials and Methods.

test, *Aerococcus* in the growth test on KCN-medium, *Nocardia* in the acid production test with mannitol, arabinose and xylose, *Clostridium* in the catalase test. From these data, the isolated bacterium I was identified to be *Bacillus* and named *B. licheniformis* sp. 1013. The urethanase activity of this bacterium was detected in the cells but not in the culture medium, and 70% of this activity was recovered in a cytosol fraction.

When the crude extract from *B. licheniformis* sp. 1013 was incubated with 13.3 mM urethane in 0.1 M MES buffer, pH 4.5, equimolar amounts of ammonia and ethanol were produced progressively with time (Fig. 1A). The K_m value of urethanase at pH 4.5 was calculated to be 42 mM from Lineweaver–Burk plots (Fig. 1B). The enzyme preparation was incubated with urethane in various buffers of different pH values for 2 h at 37 °C, and its optimum pH was found to be 4.5 (Fig. 2A). The enzyme preparation was preincubated at various pHs at 37 °C for 30 min, and the residual enzyme activity was assayed after adjustment of the pH of the mixture to pH 4.5. The results show that the enzyme was stable from pH 4.5 to 7.5 (Fig. 2B).

TABLE V. Properties of Bacterial Urethanases

Bacteria	Optimal pH	K_m (mM)	EtOH-resistance[a]
B. licheniformis sp. 1013	4.5	42	54
	7.5	56	—
B. thiaminolyticus IAM 1034	6—8	0.88	20
Citrobacter sp.[15]	6—8	1.6	8.9
P. lilacinum	7—8	50–	0
Bacterium from soil[b]	6—8	36	48

a) Residual activity (%) in 20% EtOH for 2 h at 37 °C at their optimal pHs. *b*) Personal communication from Mr. T. Kobayashi, Nagase Biochem. Ind., Japan.

The enzyme preparation was incubated with urethane at the indicated temperature for 2 h. Optimum temperature was shown at 37 °C under the conditions (Fig. 3A). The activity was completely lost after heating at 50 °C for 1 h (Fig. 3B). The enzyme activity was not affected by the addition of ethylenediaminetetraacetic acid (EDTA) or 2-mercaptoethanol at 1 mM concentration, suggesting that the enzyme does not require metal ions.

The enzyme activity was assayed at various concentrations of ethanol for 2 h at 37 °C. The activity decreased depending upon the ethanol concentrations and 54% activity remained at 20% ethanol corresponding to the ethanol concentration of sake (Fig. 4).

The urethanase of *Bacillus licheniformis* sp. 1013 reported in this study is quite different from those which were found in *Bacillus thiaminolyticus*, *Penicillium lilacinum* and bacterium from soil (unpublished data) and *Citrobacter* sp.,[15] as reported previously. Their optimal pH ranged from 6 to 8 and they were inactivated with ethyl alcohol of relatively low concentrations. The urethanase in this report showed higher activity at acidic condition pH 4.5 and was more resistant against ethyl alcohol of high concentrations than the other enzymes (Table V). However, the urethanase from *B. licheniformis* sp. had a higher K_m value which may restrict its practical use for the elimination of urethane from alcoholic beverages.

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Purification of a Rat Liver Phenol Sulphotransferase ($P\text{-}ST_G$) with the Aid of Guanidine Hydrochloride Treatment

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An isoenzyme of phenol sulphotransferase, designated $P\text{-}ST_G$, was purified 157-fold from male rat liver cytosol by diethylaminoethyl-cellulose (DEAE-cellulose) and agarose-hexane-adenosine-3',5'-bisphosphate affinity chromatography. The $P\text{-}ST_G$ fraction obtained after DEAE-cellulose chromatography rapidly lost its activity during storage at 4 °C, however, the activity was recovered by the addition of 1.6 M guanidine hydrochloride (Gndn HCl) followed by dialysis. Gndn HCl also substantially improved the yield of $P\text{-}ST_G$ in a subsequent purification step using affinity chromatography, while the specific activity of the purified $P\text{-}ST_G$ was not changed by Gndn HCl treatment. It is possible that the Gndn HCl treatment caused $P\text{-}ST_G$ recovery from an inactivated to an active form rather than reactivating it for increased activity.

Purified $P\text{-}ST_G$ is a homodimer with a native molecular mass of 67 kDa; the subunit molecular mass is 35 kDa. Immunoblot analysis carried out with antibodies raised against the purified enzyme indicated that male rat liver contains a higher level of the enzyme than female rat liver. This enzyme is also expressed in the kidney and the stomach. $P\text{-}ST_G$ reaches maximum activity when 1-naphthol, 2-naphthol and 4-nitrophenol are used as substrates at pH 5.5. Using dopamine as a substrate the pH optimum is about 9.0. $P\text{-}ST_G$ activity is markedly inhibited by the addition of sodium chloride to the reaction mixture.

Keywords phenol sulphotransferase; sulphotransferase; purification; sulfation; isoenzyme; homodimer; guanidine hydrochloride; rat liver

Introduction

Addition of a sulfate group is an important conjugation reaction in the metabolism of many xenobiotics and endogenous compounds such as drugs, food additives, steroids and neurotransmitters. Phenol sulphotransferases (P-ST) catalyze sulfate addition to a wide variety of phenolic compounds of endogenous as well as exogenous origin. Evidence has accumulated to indicate that P-ST constitutes a family of isoenzymes with partially overlapping substrate specificities. Four P-ST isoenzymes have been purified from rat liver cytosol,[1,2] and three isoenzyme from human liver[3,4] and platelets[5] have been purified or characterized. Cloning of complementary deoxyribonucleic acid (cDNAs) encoding P-ST isoenzymes is a useful approach to studying the structure, function and multiplicity of P-ST enzymes. As a first step in that direction, we have purified an isoform of P-ST from rat liver cytosol.

The purification of P-STs has been extensively detailed by Jakoby and coworkers.[2] At least four sulphotransferase (ST) isoforms (ST I—IV) were purified from male rat liver cytosol.[2] Using Affi-gel blue, anion exchange, gel filtration and adenosine triphosphate-agarose (ATP-agarose) affinity chromatography they obtained highly purified P-STs. Recently a ST isoenzyme was purified by a simple two-step procedure using anion exchange and an agarose-hexane-adenosine-3',5'-bisphosphate (PAP-agarose) affinity resin.[6] In this communication, we describe purification of an isoform of P-ST from male rat liver cytosol which takes advantage of unique features of the enzyme we discovered during our purification attempts. Some characteristics of the purified enzyme are also reported.

Materials and Methods

Materials Sephacryl S-300 superfine, PAP-agarose, type 2 and Mono P HR 5/5 were purchased from Pharmacia (Uppsala, Sweden). Diethylaminoethyl-cellulose (DEAE-cellulose) DE-52 was obtained from Whatman Biosystems, Ltd. (Maidstone, Kent, England). 1-Naphthol, 2-naphthol, 4-nitrophenol and guanidine hydrochloride (Gndn HCl) were obtained from Wako Chemical Ind. (Osaka, Japan). Dopamine (3-hydroxytyramine), 1-naphthol sulfate (potassium salt), adenosine 3',5'-bisphosphate (PAP), equine liver alcohol dehydrogenase and horseradish peroxidase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2-Naphthyl sulfate (potassium salt) was a product of Koch Light Lab., Ltd. (Colnbrook, Bucks, England). Dopamine-3-*O*-sulfate was generously provided by Dr. K. Imai, Univ. of Tokyo. Peroxidase-conjugated affinipure goat anti-rabbit immunoglobulin G (IgG) (H+L) was a product of Jackson Immunoresearch Laboratories Inc. (West Grove, PA, U.S.A.). Horse heart cytochrome c, bovine liver catalase, ovalbumin and bovine serum albumin (BSA) were purchased from Serva, Feinbiochemica GmbH. and Co. (Heidelberg, Germany). 3'-Phosphoadenosine 5'-phosphosulfate (PAPS) was prepared according to the method of Singer.[7] All other reagents were of the highest grade available.

Enzyme Assay P-ST activity using 2-naphthol as a substrate was assayed by the method of Arand *et al.*[8] with a slight modification. The standard incubation mixture (500 μl) contained the enzyme, 0.5 mM 2-naphthol, 0.1 mM PAPS and 100 mM sodium acetate, pH 5.5. A stock solution of 5 mM 2-naphthol was prepared by sonication in 50 μM ethylenediaminetetraacetic acid (EDTA) and 100 mM Tris/HCl, pH 7.4 and kept at 4 °C. A 50 μl aliquot of this stock was used for each assay within a week of stock preparation. The reaction mixture was incubated at 37 °C for 30 min and the reaction was stopped by placing the tube in boiling water for 2 min, followed by the addition of 2 ml of chloroform. After vigorous shaking, the aqueous and organic phases were separated by centrifugation at 3000 rpm for 15 min. A 300 μl aliquot of the aqueous phase was mixed with 2 ml of 1.6 M glycine buffer, pH 10.3. The fluorescence intensity of 2-naphthyl sulfate in the mixture was recorded using excitation and emission wavelengths of 286 and 341 nm, respectively.

ST activity against 1-naphthol was assayed in the same manner as 2-naphthol using excitation and emission wavelengths of 283 and 338 nm, respectively. Enzyme activity against 4-nitrophenol was assayed as previously described.[9] Enzyme activity using dopamine as a substrate was determined using 1 mM dopamine. The products were separated and quantified by high performance liquid chromatography (HPLC) according to the method of Young *et al.*[10] using the solvent system described by Sharpless *et al.*[11] and dopamine-3-*O*-sulfate as a standard.

Preparation of Rat Liver Cytosol Four to five male rats of 7 weeks of age were decapitated and a 30% (w/v) liver homogenate was prepared in ice-cold 250 mM sucrose, 3 mM 2-mercaptoethanol, 0.1 mM EDTA in 10 mM Tris/HCl, pH 7.4 (buffer A). The cytosolic fraction was obtained by differential centrifugation (2000*g* for 10 min, 16000*g* for 45 min and 105000*g* for 60 min). Protein was assayed by the method of Bradford[12] using BSA as a standard.

DEAE-Cellulose Column Chromatography The cytosolic fraction of rat liver was applied to a column of DE-52 (26.4 × 870 mm) which had

been equilibrated in buffer A. The column was washed with 3000 ml of buffer A and the ST activity was eluted with a 3000 ml linear gradient of 0—0.3 M NaCl in buffer A at a flow rate of 180 ml/h. Fractions (15 ml) were collected and aliquots of the fractions were assayed for P-ST activity at pH 5.5.

Treatment with Gndn HCl The freshly prepared DEAE-cellulose fractions containing P-ST activity were pooled and mixed with one-third volume of 6.4 M Gndn HCl in 250 mM sucrose, 3 mM 2-mercaptoethanol and 10 mM sodium phosphate, pH 7.4 (buffer B). The mixture was dialyzed twice overnight against 30 volumes of buffer B, followed by centrifugation at 1500*g* for 15 min to remove precipitates which formed during dialysis. The ST activity recovered in the supernatant was determined.

PAP-Agarose Affinity Column Chromatography PAP-agarose affinity column chromatography was performed in essentially the same manner as reported by Heroux and Roth.[5] The P-ST fraction after Gndn HCl treatment was applied to a column of PAP-agarose (10 × 128 mm) which had been previously equilibrated in buffer B. The P-ST fraction was applied so that its protein content did not exceed 16 mg. The column was washed at a flow rate of 50 ml/h sequentially with 50 ml each of buffer B, 1 mM adenosine 3'-monophosphate (3'-AMP) in buffer B and 1 mM adenosine 5'-monophosphate (5'-AMP) in buffer B. The ST activity was then eluted using a 30 ml linear gradient of 0—0.3 mM PAP in buffer B and 1 ml fractions were collected.

Gel Filtration Chromatography Gel filtration chromatography was used to estimate the molecular weight of the active form of P-ST. The DEAE-cellulose fractions which contained P-ST activity were pooled and concentrated about 8-fold by ultrafiltration on a Diaflo membrane PM-10, Amicon (Beverly, MA, U.S.A.). This enzyme preparation was subjected to Gndn HCl treatment as described above. The preparation thus obtained was applied to a column (26.4 × 915 mm) of Sephacryl S-300 that had been previously equilibrated in buffer B. The activity was eluted at a flow rate of 16 ml/h with the same buffer and fractions of 1 ml were collected. The column was calibrated with equine liver alcohol dehydrogenase (79.5 kDa), BSA (67 kDa), ovalbumin (45 kDa) and horse heart cytochrome c (12.3 kDa). Values for Stokes radii were obtained from a published source.[13]

Sucrose Density Gradient Centrifugation The molecular weight of the active form of P-ST was also determined by sucrose density gradient centrifugation according to the procedure of Heroux and Roth.[5] A 300 μl aliquot of the affinity-purified P-ST or standard marker proteins was loaded on a 12 ml linear sucrose gradient (5—20%) in buffer B containing 100 μM PAP. This was centrifuged at 4 °C for 24 h at 40000 rpm using a Hitachi RPS-40T rotor. From the top of the tube, fractions (approximately 300 μl/tube) were collected and the distance from the top of the gradient was measured for each fraction. Horse heart cytochrome c (12.3 kDa), horseradish peroxidase (40.2 kDa), equine liver alcohol dehydrogenase (79.5 kDa) and bovine liver catalase (240 kDa) were used as external standards. The standard marker proteins were detected by enzyme activity according to Heroux and Roth,[5] except for cytochrome c which was measured for absorbance at 515 nm. Values for S_{20w} were obtained from a published source.[13]

Chromatofocusing on a Mono P Column A portion of the purified P-ST was applied to a Mono P HR 5/5 column. The column was pre-equilibrated with a starting buffer (25 mM triethanolamine–iminodiacetic acid, pH 8.3) and P-ST was eluted at a flow rate of 0.5 ml/min with an elution buffer (pH 5.0) made of Polybuffer 96, Polybuffer 74 (Pharmacia) and iminodiacetic acid according to the instructions of the supplier. Liquid chromatography was performed as described previously.[9,14] The P-ST activity and the pH of each 0.5 ml fraction were determined.

Preparation of Antiserum One male New Zealand White rabbit weighing approximately 3.0 kg was used for preparation of antiserum against purified P-ST. Purified P-ST (100 μg of protein) was mixed with an equal volume of complete adjuvant H37 Ra, Difco Laboratories (Detroit, MI, U.S.A.) and injected subcutaneously along the back of the rabbit. Four weeks later, the rabbit received a booster injection containing the same amount of protein in Freund's incomplete adjuvant (Difco). Ten days after the final injection, the animal was bled for collection of the antiserum. Preimmune serum was used as a control serum.

Immunoblotting Proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were transferred from the gel to a nitrocellulose filter, Bio-Rad (Richmond, CA, U.S.A.) using a Bio-Rad Trans-Blot SD apparatus according to the manufacturer's instructions. The nitrocellulose filter was blocked overnight at 4 °C using Block-ace, a blocking reagent produced by Dainippon Seiyaku Co., Ltd. (Osaka, Japan). Incubation with primary and secondary antibodies were done in 10-fold dilutions of Block-ace for 1 h at room temperature. A 1 : 10000 dilution of rabbit antiserum and a 1 : 1000 dilution of peroxidase-conjugated goat antiserum were routinely used as primary and secondary antibodies, respectively. The immunocomplex was visualized using a color generating reaction, the oxidation of 3,3'-diaminobenzidine tetrachloride by hydrogen peroxide.

Other Methods SDS-PAGE was performed as described by Laemmli.[15] An electrophoresis calibration kit (Pharmacia) was used for SDS-PAGE molecular weight markers. This included phosphorylase B (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The proteins were visualized by Coomassie blue staining.

Results

Anion Exchange Chromatography The cytosolic fraction from male rat liver cells was subjected to DEAE-cellulose column chromatography for the initial purification of P-ST. Figure 1 illustrates the elution pattern of P-ST activity; several peaks corresponding to isoenzymes to P-ST were observed. This elution pattern is similar to that obtained by Sekura and Jakoby.[16] However, P-ST activity in each peak fraction was rapidly lost during storage at 4 °C. During our attempts to purify P-ST we learned that the activity taken from the second peak in this profile (fraction 88) has a unique characteristic.

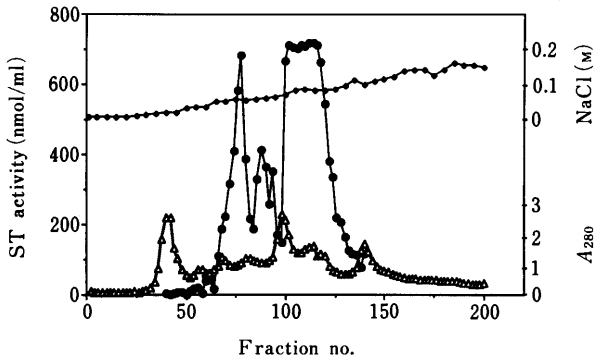

Fig. 1. DEAE-Cellulose Column Chromatography of Male Rat Liver Cytosol

The cytosolic fraction of male rat livers was loaded on a DEAE-cellulose column (2.64 × 87 cm) and the P-ST activity was eluted as described in Materials and Methods. P-ST activity (●) was assayed with 2-naphthol as the substrate in each fraction. Absorbance at 280 nm (△) and NaCl concentration (◆) were also determined.

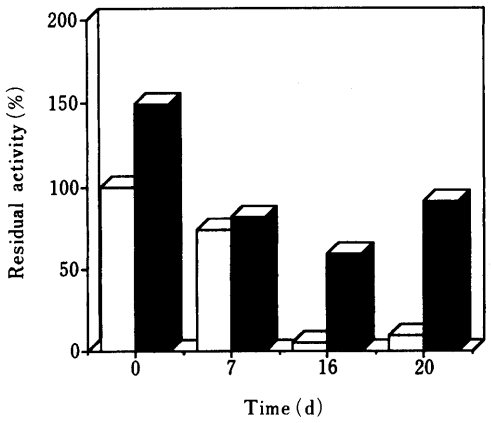

Fig. 2. Recovery of P-ST Activity by Gndn HCl Treatment

The fraction corresponding to the second peak (fraction 88 in Fig. 1) obtained from DEAE-cellulose chromatography was stored at 4 °C for the number of days indicated before determining its P-ST activity (white bars). A portion of the fraction was also treated with Gndn HCl as described in Materials and Methods, then the P-ST activity was measured (shaded bars). P-ST activity was assayed using 2-naphthol as the substrate. The activity at day 0 was 414 nmol/ml/30 min.

Figure 2 shows that the activity in the second peak rapidly diminished during storage at 4 °C, however, it was restored to its original level when it was mixed with 1.6 M Gndn HCl and dialyzed. Furthermore, P-ST activity in this peak increased 1.5 fold when the freshly prepared activity was treated with Gndn HCl (Fig. 2, day 0). Gndn HCl treatment caused some proteins in the preparation to denature and precipitate. When these proteins were removed by centrifugation, the specific activity of the P-ST preparation increased. These properties were only observed in the P-ST activity found in the second peak of this elution profile. It was designated as P-ST_G and was further purified.

PAP-Agarose Affinity Chromatography The DEAE-cellulose fractions containing P-ST_G were pooled, treated with Gndn HCl (Table I) and subjected to PAP-agarose affinity chromatography (Fig. 3). The recovery of P-ST_G activity in this step was approximately 35% and yielded a homogeneous preparation of P-ST_G. After this step, SDS-PAGE analysis revealed that the preparation contained a single protein band with a molecular mass of 35 kDa (Fig. 4). When the fractions pooled from DEAE-cellulose were not treated with Gndn HCl and applied to a PAP-agarose column, the final preparation had a single protein band with a molecular mass of 35 kDa and a similar specific activity. However, the recovery of activity through the affinity column was only 8%. Therefore, Gndn HCl treatment is an essential step in preparing large quantities of purified P-ST_G.

The specific activity of the purified preparation was 488 nmol/min/mg protein, representing a 157-fold purification over its activity in the cytosol (Table I). PAP is a product of sulfuric acid transfer from PAPS to various ST substrates and ST have very high affinity for PAP.[1] Its presence in the affinity chromatography elution buffer appears to stabilize purified P-ST_G since no significant loss of enzyme activity was observed during a month of storage in this buffer at 4 °C.

Characterization of Purified P-ST_G Since homogeneous P-ST was obtained from rat liver cytosol using modifications of previously reported methods,[16,17] some characteristics of the final preparation were determined in detail. Figure 5 shows the results of gel filtration chromatography with a Sephacryl S-300 column. The elution position of P-ST_G activity corresponds to a Stokes radius of 37.2 Å and an apparent molecular mass of 64.4 kDa, based on calculations using standard proteins. The minor peak at

TABLE I. Purification of P-ST_G from Male Rat Liver Cytosol

	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)
Cytosol	4423	13927	3.1	1
DEAE-cellulose	121.7	2637	21.7	7
Gndn HCl treatment	100.8	5242	52	16.8
PAP-agarose[a)]	0.45	219.4	487.5	157.3

ST activity was determined with 2-naphthol as the substrate. One unit represents 1 nmol of substrate conjugated/min. *a)* One-third of the Gndn HCl-treated fraction was subjected to PAP-agarose affinity chromatography.

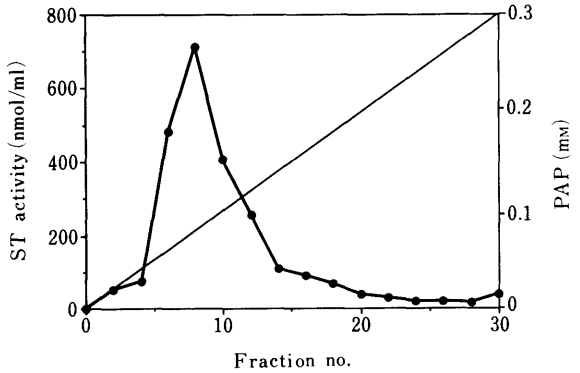

Fig. 3. PAP-Agarose Affinity Chromatography

The P-ST_G fractions obtained from DEAE-cellulose chromatography were pooled and treated with Gndn HCl as described in Materials and Methods. A portion of the resulting P-ST_G preparation (16 mg protein in 38 ml of buffer) was loaded onto a PAP-agarose column (1 × 12.8 cm). After washing the column with various buffers as described, the P-ST activity was eluted using a linear gradient of 0—0.3 mM PAP. P-ST was determined using 2-naphthol as a substrate.

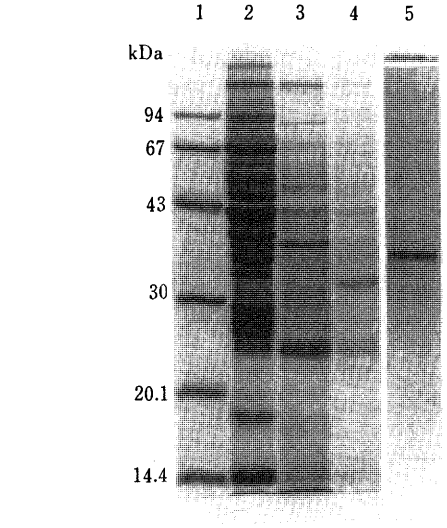

Fig. 4. SDS-PAGE of P-ST_G

Sample preparations of P-ST_G were analyzed at each step of purification by SDS-PAGE and visualized with Coomassie blue staining. Lane 1, standard size markers; lane 2, cytosolic fraction (39 μg protein); lane 3, pooled fractions after DEAE-cellulose chromatography (25 μg protein); lane 4, Gndn HCl treated preparation (7 μg protein); lane 5, purified P-ST_G after PAP-agarose chromatography (0.5 μg protein).

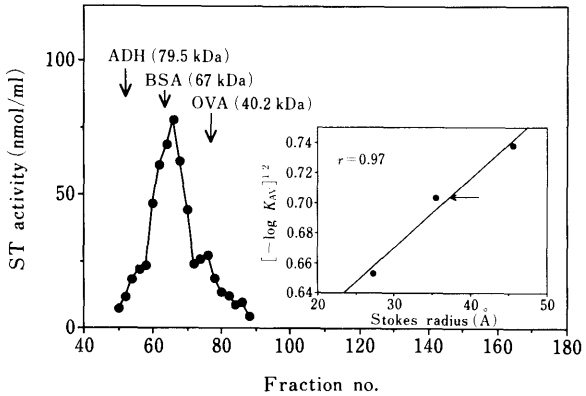

Fig. 5. Sephacryl S-300 Superfine Gel Filtration Chromatography

The P-ST activity (●) using 2-naphthol as a substrate was determined in each fraction. Arrows indicate the elution positions of standard proteins with various molecular weights: ADH, equine liver alcohol dehydrogenase, 79.5 kDa; BSA, bovine serum albumin, 67 kDa; OVA, ovalbumin, 40.2 kDa. The inset shows a calibration curve using standard proteins. The arrow corresponds to P-ST_G. K_{AV} represents the partition coefficient and r represents the regression coefficient.

around fraction No. 75 might be due to the contamination of other P-ST present in DEAE-cellulose fractions used in this experiment, but we did not pursue this further. The results of sucrose density gradient analysis illustrated in Fig. 6 yielded a S_{20w} value of 4.39 and an apparent molecular mass of 68.8 kDa. The fractions containing P-ST activity invariably contained a band with a subunit molecular mass of 35 kDa on SDS-PAGE (as in Fig. 4). The molecular mass as calculated by the method of Heroux and Roth[5] using the S_{20w} value and Stokes radius was 67.2 kDa. These data indicate that P-ST_G has a native molecular mass of 67 kDa, and probably exists as a homodimer with a subunit molecular mass of 35 kDa.

Application of purified P-ST_G to a Mono P column yielded a sharp and symmetrical peak of activity with the highest activity appearing at pH 6.4 (data not shown). The purified enzyme activity was markedly inhibited by the presence of sodium chloride in the reaction mixture (Fig. 7). Figure 8 shows the pH profile of the purified enzyme activity using several phenolic substrates. Maximal activities against 1-naphthol, 2-naphthol and 4-nitrophenol were observed at pH 5.5. However, the pH optimum was 9.0 when dopamine was used as the substrate. Determination of the N-terminal sequence by automated Edman degradation was unsuccessful, probably due to blockage of the N-terminus.

Immunoblot Analysis Rabbit antiserum raised against purified P-ST_G recognized the purified P-ST_G polypeptide on immunoblots and precipitated more than 90% of the

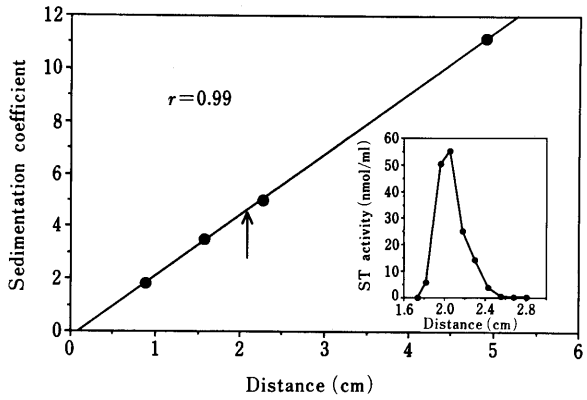

Fig. 6. Determination of Molecular Weight by Sucrose Density Gradient Centrifugation

A calibration curve was made using standard marker proteins, horse heart cytochrome c, 12.3 kDa; horseradish peroxidase 40.2 kDa; equine liver alcohol dehydrogenase, 79.5 kDa; and bovine liver catalase, 240 kDa. The horizontal axis indicates distance from the top of the gradient. An arrow indicates the elution position of P-ST_G; r represents the regression coefficient. This figure presents values from four independent experiments which were averaged. The inset shows a typical distribution of P-ST_G activity on a sucrose gradient.

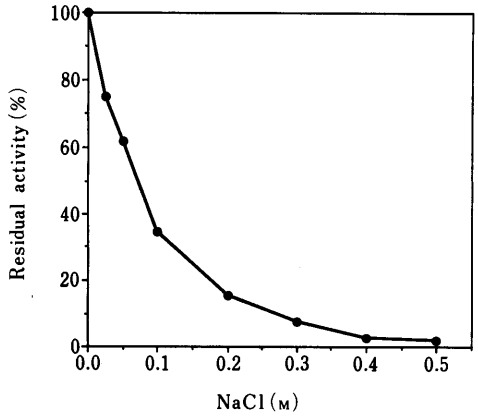

Fig. 7. Inhibition of Purified P-ST_G Activity by Sodium Chloride

Purified P-ST_G (1.5 μg) was incubated in the presence of various concentrations of sodium chloride using 2-naphthol as a substrate. The data are presented as the percentage of P-ST_G activity obtained when assayed without sodium chloride.

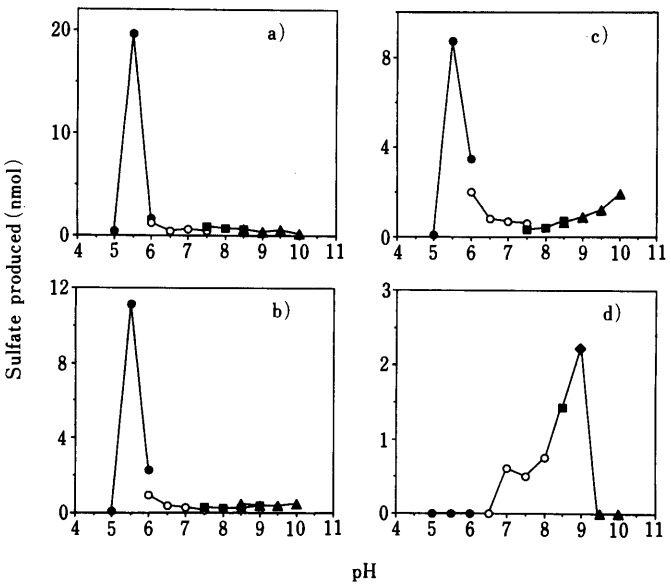

Fig. 8. pH Activity Profile of P-ST_G

Purified P-ST_G (0.75 μg) was incubated for 30 min with various substrates: a) 1-naphthol, b) 2-naphthol, c) 4-nitrophenol, d) dopamine. The assay buffers used were 0.2 M sodium acetate (●), 0.2 M sodium phosphate (○), 0.2 M Tris/phosphate (■), 0.2 M sodium borate (▲), 0.1 M Tris/HCl (◆).

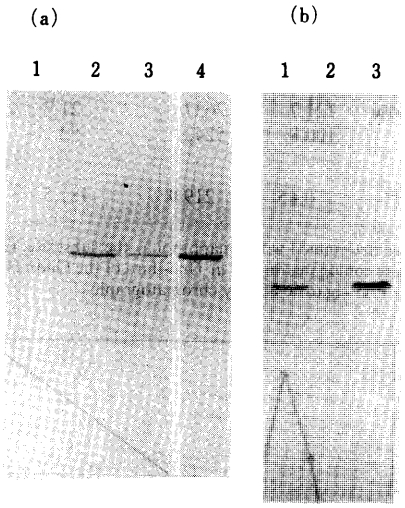

Fig. 9. Immunoblot Analysis of Various Rat Tissues

(a) Cytosolic fractions of male and female rat liver (7 weeks of age) were subjected to 12.5% SDS-PAGE and transferred to a nitrocellulose filter. This blot was probed with anti-P-ST_G antiserum and the immunoreactive proteins were visualized as described in Materials and Methods. Lanes 1 and 3 contain 0.5 and 1.0 μg, respectively, of female rat liver cytosolic protein. Lanes 2 and 4 contain 0.5 and 1.0 μg, respectively, of male rat liver cytosolic protein. (b) Cytosolic fractions of various tissues from a male rat (9 weeks of age) were prepared and immunoblot analysis was performed as in (a). Twenty micrograms of kidney cytosolic protein (lane 1), intestinal cytosolic protein (lane 2) and stomach cytosolic protein (lane 3) were loaded on the gel. P-ST activity on 2-naphthol was 7.0, 9.0 and 7.1 nmol/30 min/mg protein in the kidney, intestinal and stomach cytosolic fractions, respectively. The corresponding liver cytosolic activity was 143 nmol/30 min/mg protein. A similar result was obtained using separate cytosolic preparations from another male rat which was 7 weeks of age. No. immunoreactive band was revealed in blots treated with preimmune serum (data not shown).

purified enzyme activity in an immunoprecipitation reaction with protein A-Sepharose (data not shown). Figure 9a shows an immunoblot of cytosolic proteins of male and female rat livers using the rabbit antiserum raised against P-ST_G. The liver cytosol of both male and female rats contained a polypeptide with a molecular mass of 35 kDa, which corresponds to the molecular mass of the P-ST_G subunit. The amount of immunoreactive polypeptide was lower in females than in males, which is consistent with the observation that P-ST activity is lower in female than in male rat liver.[18,19] An immunoreactive polypeptide with a subunit molecular mass of approximately 35 kDa was also found in kidney and stomach cells (Fig. 9b). Consistent with immunoblot analysis, this P-ST activity was found in the kidney and the stomach. The cytosolic fraction of the intestine also contained P-ST activity despite the absence of immunoreactive polypeptide, suggesting the presence of a different form of P-ST in the intestine.

Discussion

We have purified an isoenzyme of P-ST from male rat liver cytosol by a simple two step column chromatography procedure. We took advantage of a unique feature of this isoenzyme and obtained a homogeneous preparation of a P-ST enzyme, which we have designated P-ST_G. The final preparation was purified 157-fold compared to the cytosolic activity with a yield of 5%.

Jakoby and his coworkers purified four isoenzymes from male rat liver.[2] They obtained a homogeneous protein with a yield of 0.7—1.0% using 4—6 steps of column chromatography. The method of Jakoby *et al.* provided a greater degree of purification than this procedure, however, the specific activity was lower than that reported here. The final preparation obtained with this procedure contains PAP, which was included in the elution buffer of the PAP-agarose affinity column. While PAP is very effective in stabilizing the purified enzyme, it is a competitive inhibitor of ST.[1] Thus the specific activity of the final preparation obtained with this method as well as the degree of purification should be considered minimal values.

P-ST_G activity rapidly diminished during storage at 4 °C after separation on a DEAE-cellulose column, however, this activity was recovered by the addition of Gndn HCl to the preparation. Addition of Gndn HCl also improved recovery of P-ST activity in the subsequent affinity chromatography step. The specific activity of the purified P-ST_G was not increased by Gndn HCl treatment, implying that this treatment does not increase the enzyme activity but rather restores P-ST_G from an inactive to an active form. The exact mechanism of P-ST_G reactivation by Gndn HCl remains unclear, however, it seems possible that some factor(s) in liver cytosol inactivates P-ST_G. Gndn HCl may inactivate the inhibitory factor(s) or may abolish its interaction with P-ST_G.

Alternatively, P-ST_G may itself be responsible for the inactivation and subsequent reactivation with Gndn HCl. Barford and Jones[20] partially purified P-ST from female rat liver cytosol by DEAE-cellulose chromatography and reported that P-ST activity decreased during storage at 4 °C. This decrease was accompanied by aggregation of the enzyme into units of greater molecular mass. It is possible that the molecular size of P-ST_G also changes during storage and that the enzyme aggregate was dissociated into an active form by Gndn HCl. It was reported that P-ST activity which diminished during storage could be restored by addition of dithiothreitol,[20] however, it did not reactivate diminished P-ST_G activity.

P-ST_G has a native molecular mass of 67 kDa, and probably exists as a homodimer with a subunit molecular mass of 35 kDa. P-ST I and P-ST II, which were purified from male rat liver cytosol,[17] have native molecular masses of 64 and 67 kDa, respectively. They are both homodimers, each with a subunit molecular mass of 35 kDa. P-ST_G was initially presumed to correspond to P-ST II according to its elution profile from DEAE-cellulose, however, several features are different from the corresponding features of P-ST II. P-ST_G has a pH optimum of 5.5 for simple phenolic substrates such as 2-naphthol and a pH optimum of 9.0 fo dopamine (Fig. 8). When 2-naphthol is used as a substrate, maximal P-ST II activity occurs at pH 6.5 and its activity minimum occurs at pH 5.5.[17] The pH profile of P-ST_G is similar to that of P-ST IV,[16] which also has a pH optimum of 5.5 for 2-naphthol and 9.0 for dopamine. However, they are clearly different species since P-ST IV has a native molecular mass of 61 kDa, and is a homodimer with a subunit molecular mass of 33 kDa.[16]

The effect of sodium chloride on P-ST_G is different from its effect on P-ST II and P-ST IV. P-ST_G is markedly inhibited by sodium chloride at concentrations above 0.05 M (Fig. 7), while P-ST II activity is enhanced by sodium chloride as well as potassium chloride at concentrations above 0.1 M.[17] P-ST IV activity is enhanced by sodium chloride below 0.1 M but is inhibited by both sodium and potassium chloride at concentrations above 0.2 M.[16]

It remains unclear at present whether P-ST_G is a distinct isoenzyme of P-ST or whether its unique features are due to Gndn HCl treatment during its purification procedure. It is possible that P-ST_G remained unnoticed in previous investigations, since it rapidly lost its activity after DEAE-cellulose chromatography. Very recently, paracetamol-sulfating ST and minoxidil-sulfating ST were purified from male rat liver cytosol.[21,22] These isoforms of ST are also active on small phenols and are specifically expressed in liver. They were shown to be unique isoenzymes distinct from ST I—IV, based on their physical and catalytic properties. P-ST_G is assumed to be a different isoform from the ST isoenzymes, since it is also present in the cytosol of kidney and stomach cells as shown by immunoblot analysis (Fig. 9b). Isolation of paracetamol- and minoxidil-STs suggest that there are a number of isoenzymes of P-ST in rat liver in addition to the STs characterized to date.

Characterization of cDNA and genomic clones encoding P-ST enzymes will help disclose the organization of this family of isoenzymes. Immunoscreening of cDNA clones of P-ST_G with anti-P-ST_G antiserum is now underway in our laboratory.

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Preferred Orientation of Crystallites in Tablets. II.[1] Evaluation of Preferred Orientation of Crystallites by X-Ray Powder Diffractometry[2]

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An X-ray powder diffraction method was presented to evaluate the preferred orientation of crystallites in tablets by using transmission and reflection techniques. In the present method, the X-ray diffraction intensities of the *hkl* reflections were measured with particular geometrical arrangements of the X-ray source, sample and detector so as to permit the measurement of diffraction intensities from the particular *hkl* planes oriented at various angles within the tablet. Thus, the preferred orientation plane was evaluated by comparing the observed intensities with the theoretical intensities calculated from the crystal structure factors.

Aspirin, salicylic acid, benzoic acid and nicotinic acid were used as samples. In all samples, it was found that the crystallites had a preferred orientation in the tablet. The 100 plane in aspirin, the 110 or 210 plane in salicylic acid, the 002 plane in benzoic acid and the 002 plane in nicotinic aicd, had a tendency to orient parallel to the upper surface of the tablet during compression.

Keywords preferred orientation; crystallite; tablet; X-ray powder diffraction; diffractometry; aspirin; salicylic acid; benzoic acid; nicotinic acid

In the previous paper,[1] the preferred orientation of crystallites in tablets was investigated by X-ray diffractometry and flat-film technique. In the diffractometry, the symmetrical-transmission and symmetrical-reflection methods were empolyed in order to measure the diffraction intensities from the *hkl* planes oriented perpendicularly to each other in the tablet. The preferred orientation of the crystallites in the tablets was evaluated by comparing the observed diffraction intensities with those calculated from the crystal structure factors. A flat-film technique was also used and the results were well consistent with those obtained by the diffractometric method.

In the present paper, a novel X-ray powder diffractometric method to evaluate the preferred orientation of the crystallites in the tablets was investigated. For some crystalline powders classified as molecular crystals, the preferred orientation of crystallites in the tablet was presented by the present method.

Experimental

Materials Aspirin (JP XI grade, Iwaki Pharmaceutical Co., Ltd.), salicylic acid (JP XI grade, Iwaki Pharmaceutical Co., Ltd.), benzoic acid (reagent grade, Koso Chemical Co., Ltd.) and nicotinic acid (reagent grade, Daiichi Pure Chemicals Co., Ltd.) were used without further purification. Crystal structure of these pharmaceuticals have been determined; aspirin,[3] salicylic acid,[4] benzoic acid[5] and nicotinic acid.[6] The crystal structures were identified as those reported in the literatures[3-6] by using X-ray powder diffraction patterns.

Tableting The tablets were prepared by a direct compression method using a flat-faced punch with a 1.30-cm in diameter under a pressure of 750 kg/cm^2. Fine powders, having to pass through a No. 200 sieve (75 μm), were used in order to avoid the effect of particle size and crystal habit of the crystalline particles on the X-ray diffraction intensities. The weight and thickness of each tablet were 200 mg and 1.1—1.3 mm, respectively.

X-Ray Diffraction (Powder Method) A Geigerflex RAD type diffractometer (Rigaku Denki Co., Ltd.) was used. The X-ray source was Ni-filtered Cu-K_α radiation and a scintillation counter was used as a detector. Other conditions were as follows: voltage, 35 kV; current, 10 mA; divergence and scatter slits, 0.5°; receiving slit, 0.15 mm. Scattering intensities were measured by the fixed-time step-scanning method between 5° and 35° (2θ) at an interval of 0.1° (2θ). The counting period was 10 s at each point. Control of the goniometer and the collection of scattering data (2θ and $I(2\theta)$) were carried out by using a personal computer HP-9816S (Hewlett-Packard Co., Ltd.) through a RS-232C interface. The sample tablet was set on a goniometer as described later.

The present method was based on the procedures of pole figure analysis[7] for the fibrous materials. The procedure was as follows:

1) Geometries of the Transmission and the Reflection Techniques for the Preferred Orientation Analysis: The geometrical arrangements of the transmission and the reflection techniques, relative to Cartesian axis, are shown in Figs. 1 and 2.[8,9] The Z axis coincides with a goniometer axis and XY is a plane involving the incident and diffracted beams with symbols of $\vec{s}_0$ and $\vec{s}$, respectively. The vector $(\vec{s}-\vec{s}_0)/\lambda$ is the scattering vector which coincides with the Y axis. If the Bragg condition is satisfied, the scattering vector is identical with the reciprocal lattice vector. α desig-

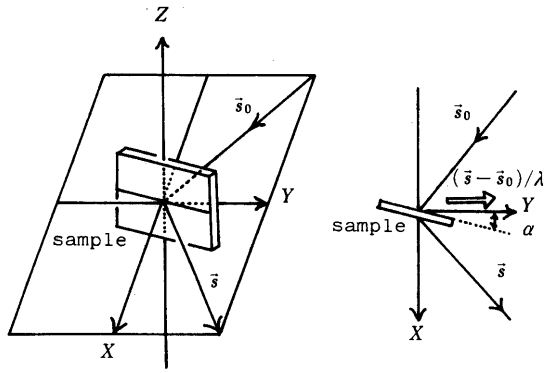

Fig. 1. Geometries of the Transmission Technique for Evaluating the Crystallite Orientation in Tablets

$\vec{s}_0$, $\vec{s}$, unit vectors of the incident and diffracted beams; $(\vec{s}-\vec{s}_0)/\lambda$, scattering vector.

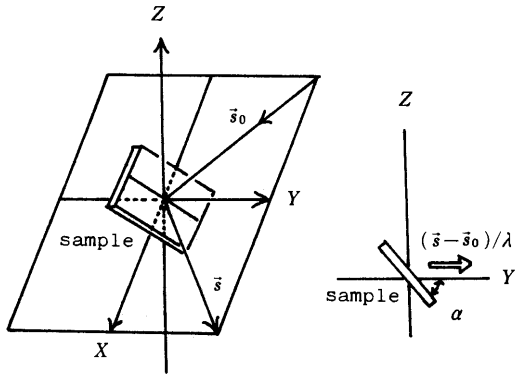

Fig. 2. Geometries of the Reflection Technique for Evaluating the Crystallite Orientation in Tablets

$\vec{s}_0$, $\vec{s}$, unit vectors of the incident and diffracted beams; $(\vec{s}-\vec{s}_0)/\lambda$, scattering vector.

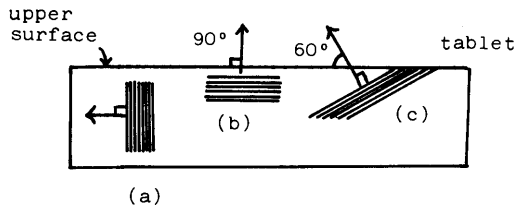

Fig. 3. Schematic Representation of Orientation of Diffracting *hkl* Planes Measured at $\alpha=0°$, 90° and 60°

(a), $\alpha=0°$; (b), $\alpha=90°$; (c), $\alpha=60°$. The arrows represent the direction of scattering vectors.

nates the angle between the scattering vector and the upper surface of the tablet and defines the orientation of diffracting *hkl* planes in the tablet. The diffraction of the X-ray occurs only from the particular *hkl* planes, both being perpendicular to the scattering vector and satisfying the Bragg condition. For example, diffraction intensities from the *hkl* planes perpendicular and parallel to the upper surface of the tablet can be measured at $\alpha=0°$ and 90°, respectively. Figure 3 shows the orientation of *hkl* planes in tablets measured at $\alpha=0°$, 90° and 60°. α can be varied with the rotation of the tablet about the Z axis in the transmission technique and about the X axis in the reflection technique. Thus, the scattering intensities only from the *hkl* planes oriented at a definite angle to the upper surface of the tablet are able to be measured by means of 2θ scans at fixed values of α.

X-Ray diffraction intensities were measured with the transmission technique at $\alpha=0°$—60° and with the reflection technique at $\alpha=60°$—90° for each 10°. At $\alpha=60°$, both the transmission and reflection techniques were employed in order to calculate the factor to scale the transmission intensities to the level of the reflection intensities. A sample tablet was mounted in a circular hole with a diameter of 1.4 cm in the aluminum plate to meet the above geometrical requirement. In the transmission technique and in the reflection technique at $\alpha=90°$, the tablet was mounted in the aluminum plate with the upper surface in line with the front of the plate. In the reflection technique at $\alpha=60$—80°, the tablet was mounted in the aluminum plate designed to meet the geometrical requirement as shown in Fig. 2.

Rotation about the compression axis of the tablet gave no influence on the observed scattering intensities for all the samples.

2) Correction for the Absorption of X-Ray: Observed scattering intensities were corrected for the absorption of X-ray by Eq. 1 for the transmission technique[8] and by Eq. 2 for the reflection technique.[9]

$$\frac{I_{0°}}{I_\alpha}=\frac{\mu t\exp(-\mu t/\cos\theta)}{\cos\theta}\cdot\frac{[\cos(\theta-\alpha)/\cos(\theta+\alpha)]-1}{\exp[-\mu t/\cos(\theta-\alpha)]-\exp[-\mu t/\cos(\theta+\alpha)]} \quad (1)$$

$$\frac{I_{90°}}{I_\alpha}=\frac{1-\exp(-2\mu t/\sin\theta)}{1-\exp(-2\mu t/\sin\theta\sin\alpha)} \quad (2)$$

where, $I_{0°}$, I_α and $I_{90°}$ are scattering intensities measured at $\alpha=0°$, $0°<\alpha<90°$ and $\alpha=90°$, respectively. θ is the Bragg angle. μ is a linear absorption coefficient and t is the thickness of the tablet. The value of μt was determined experimentally.

3) Scaling of the Diffraction Intensities: It was assumed that the diffraction peak profiles corrected for absorption consisted of Gaussian diffraction profiles and a linear part corresponding to background scattering. Individual parts were separated analytically by a non-linear least-squares procedure. The diffraction intensities were calculated by integrating the Gaussian part after subtracting a background scattering. The diffraction intensities of *hkl* reflections measured with transmission and reflection techniques were normalized on a common bases using the scaling factor. The scaling factors for the respective reflections were obtained by comparing the diffraction intensities measured with the reflection technique at $\alpha=60°$ to that measured with the transmission technique at $\alpha=60°$.

Calculation of Theoretical Diffraction Intensities Assuming random orientation of crystallites, theoretical diffraction intensities were calculated from the crystal structure factors as described in the previous paper.[1]

Results and Discussion

Aspirin Figure 4 shows the absorption corrected X-ray scattering intensities of aspirin tablet as a function of α.

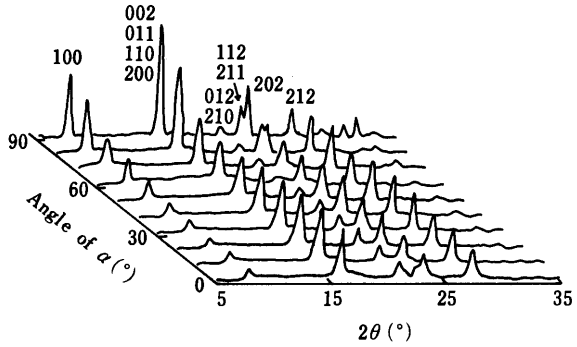

Fig. 4. Crystallite Orientation in Aspirin Tablet

The numbers represent the diffracting plane indices.

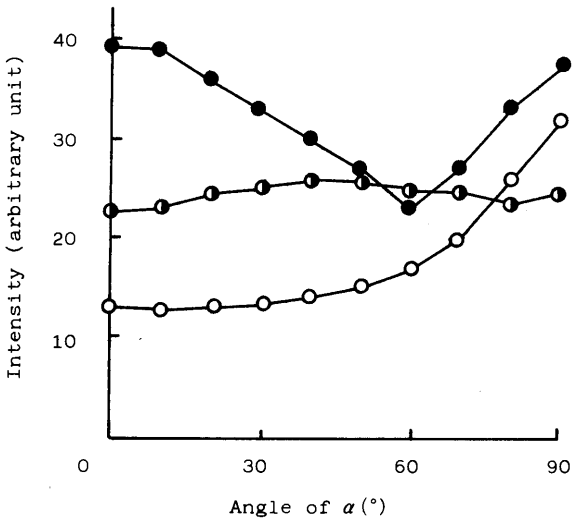

Fig. 5. Variation of Intensities of Some Reflections from Aspirin Tablet with the Angle α

○, 100 reflection; ●, (002, 011, 110, 200) reflections; ◑, 212 reflection.

TABLE I. Structure Factors and Multiplicity Factors of Four Reflections of Aspirin Crystal

hkl[a]	$2\theta_{hkl}$	Structure factor, *Fc*	Multiplicity factor
002	15.64	108.04	2
011	15.55	−31.19	4
110	15.53	−29.64	4
200	15.56	−46.09	2

a) The reflecting plane indices.

It was shown that the intensities of 100 and (002, 011, 110, 200) reflections varied with α, indicating that the crystallites in the tablet have a preferred orientation. The latter four reflections were observed as a single peak and could not be separated analytically because Bragg angles for these reflections were too close to separate as shown in Table I.

The normalized diffraction intensities of some selected reflections of aspirin tablet were plotted against α in Fig. 5. The intensities of 100 reflection increased with the increase of α. As described in the previous paper,[1] the relative diffraction intensities obtained at $\alpha=0°$ (symmetrical-transmission technique) were closest to those calculated from the crystal structure factors by assuming a random

orientation of crystallites.

These results indicate that the 100 plane of aspirin crystals has a tendency to orient parallel to the upper surface of the tablet during compression.

The intensity of (002, 011, 110, 200) reflections decreased with the increase of α up to 60° and then increased. This result was regarded as follows: If crystallites have random orientation in the tablet, the intensity of the observed diffraction peak may be attributed mainly to 002 reflection in view of the magnitude of the structure factor (Table I). Since the acute angle of the (100) and (002) planes is 84.45°, the 002 plane would orient almost perpendicular to the upper surface of the tablet as a consequence of the preferred orientation of the 100 plane. So, the intensity of 002 reflection should decrease with the increase of α. Furthermore, because the (200) plane was parallel to the (100) plane, the 200 plane of aspirin also has a tendency to orient parallel to the upper surface of the tablet. So the intensity of 200 reflection would increase with an increase of α. As a whole, the intensity of (002, 011, 110, 200) reflections had a minimum value at about $\alpha=60°$.

Hereafter, the plane showing a marked tendency to orient parallel to the upper surface of tablet, such as the 100 plane in aspirin, will be called a preferred orientation plane.

In the compressed tablet of micronized aspirin powder (ground powder using agate ball mill), aspirin crystallites had a preferred orientation in the same manner. Thus, it would be considered that the preferred orientation of crystallites in the tablet was little affected by the size and habit of the crystalline particles so long as fine powders smaller than 75 μm were used for tableting.

Salicylic Acid Figure 6 shows the absorption corrected X-ray scattering intensities of salicylic acid tablet as a function of α. The normalized diffraction intensities of some selected reflections were plotted against α in Fig. 7. The intensities of 110 and 210 reflections increased with the increase of α, though the intensity of (121, 211) reflections (individual reflection could not be separated: $2\theta_{121}$, 25.38°; $2\theta_{211}$, 25.26°) decreased with the increase of α.

The relative diffraction intensities obtained at $\alpha=0°$ were closest to those calculated from crystal structure factors.[1] This result and a marked increase in intensities of 110 and 210 reflections with an increase of α indicate that the crystallites in tablet have a preferred orientation and that these planes have a tendency to orient parallel to the upper surface of the tablet during compression. A marked decrease in diffraction intensity of (121, 211) reflections at about $\alpha>50°$ is considered as a consequence of preferred orientation of the 110 or 210 planes. Thus, the acute angles between the planes are as follows: angle between (110) and (121) is 48.1°, (110) and ($1\bar{2}1$) is 76.8°, (210)

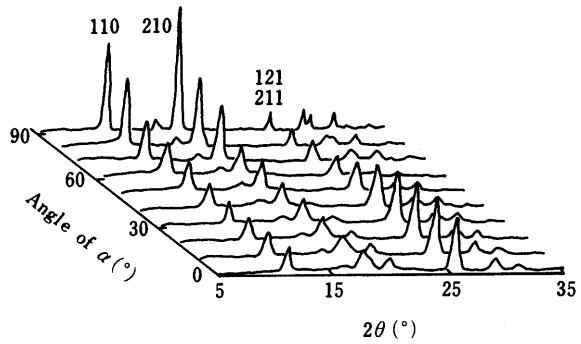

Fig. 6. Crystallite Orientation in Salicylic Acid Tablet
The numbers represent the diffracting plane indices.

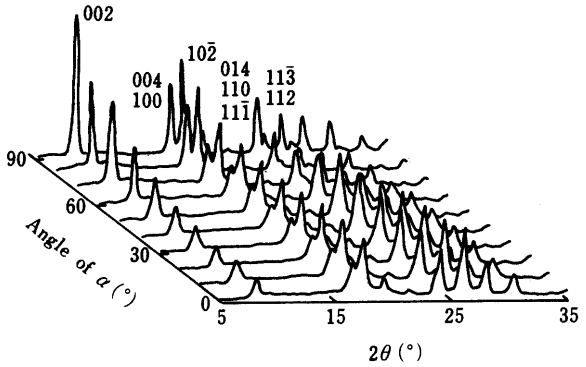

Fig. 8. Crystallite Orientation in Benzoic Acid Tablet
The numbers represent the diffracting plane indices.

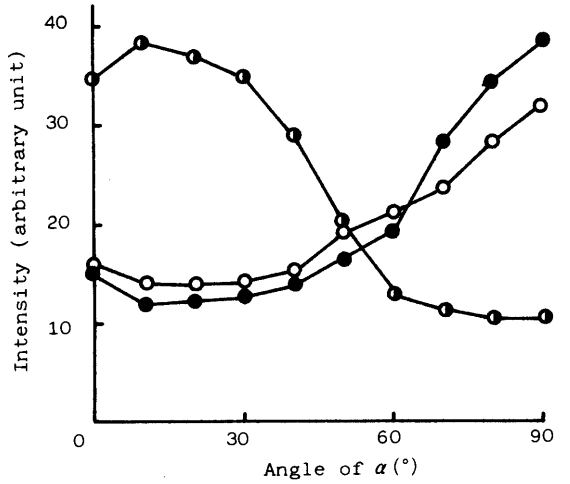

Fig. 7. Variation of Intensities of Some Reflections from Salicylic Acid Tablet with the Angle α

○, 110 reflection; ●, 210 reflection[a]; ◑, (121, 211) reflections. *a*) This peak is (120, 210) reflections. Because the contribution of 120 reflection to this peak is less than 10%, 120 reflection was ignored.

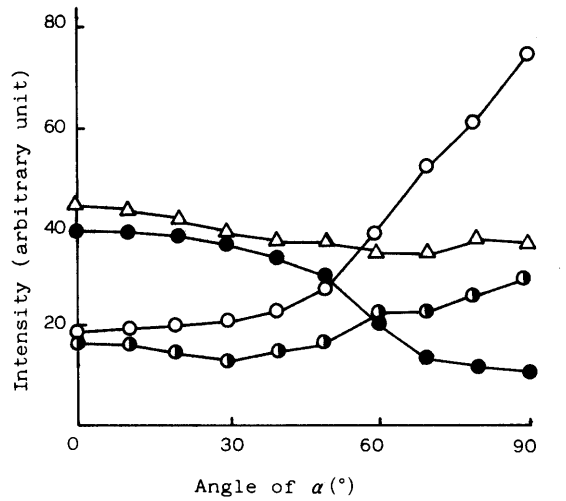

Fig. 9. Variation of Intensities of Some Reflections from Benzoic Acid Tablet with the Angle α

○, 002 reflection; ●, (112, 11$\bar{3}$) reflections; ◑, (004, 100) reflections; △, 10$\bar{2}$ reflection.

TABLE II. Relative Integrated Intensities of the Diffraction Lines of Benzoic Acid

$2\theta_{obs}$	hkl[a]	$2\theta_{cal}$	Structure factor, $\|Fc\|$	Multiplicity factor	Relative intensity
8.1	002	8.12	33.2	2	75
16.3	004	16.29	30.6	2	44
	100	16.25	41.2	2	
17.2	$10\bar{2}$	17.23	82.0	2	100
19.1	012	19.13	19.7	4	25
	102	19.12	36.6	2	
23.8	014	23.84	73.0	4	88
	$11\bar{1}$	23.81	18.4	4	
	110	23.82	10.4	4	
25.9	$11\bar{3}$	25.85	3.2	4	86
	112	25.88	81.8	4	
27.8	$10\bar{6}$	27.73	6.4	2	50
	$11\bar{4}$	27.75	8.5	4	
	113	27.80	66.6	4	
30.2	016	30.18	24.7	4	38
	$11\bar{5}$	30.12	18.9	4	
	114	30.18	56.2	4	

a) The reflecting plane indices.

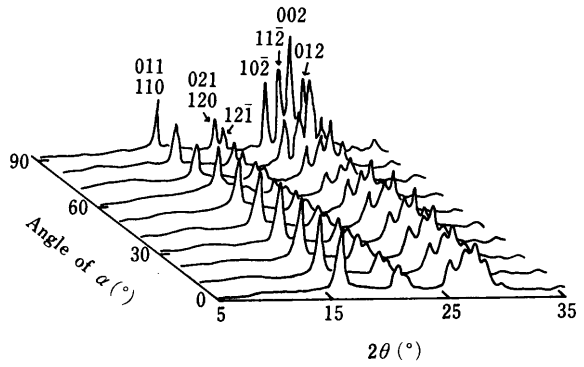

Fig. 10. Crystallite Orientation in Nicotinic Acid Tablet
The numbers represent the diffracting plane indices.

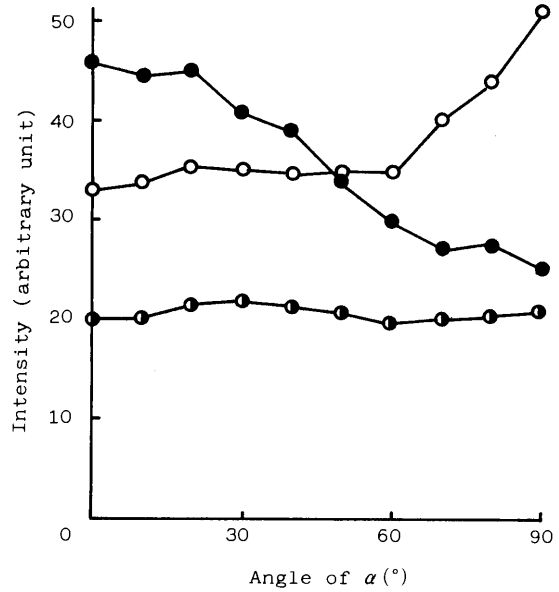

Fig. 11. Variation of Intensities of Some Reflections from Nicotinic Acid Tablet with the Angle α
○, 002 reflection; ●, (011, 110) reflections; ◑, $10\bar{2}$ reflection.

TABLE III. Relative Integrated Intensities of the Diffraction Lines of Nicotinic Acid

$2\theta_{obs}$	hkl[a]	$2\theta_{cal}$	Structure factor, Fc	Multiplicity factor	Relative intensity
15.4	011	15.37	−5.6	4	100
	110	15.44	−41.6	4	
20.3	021	20.28	5.7	4	36
	120	20.32	−33.1	4	
21.2	$12\bar{1}$	21.20	−26.5	4	21
24.8	$10\bar{2}$	24.83	63.4	2	43
26.0	$11\bar{2}$	25.99	−49.9	4	48
26.9	002	26.91	−82.2	2	61
28.0	012	28.00	−49.8	4	41

a) The reflecting plane indices.

and (121) is 55.5°, (210) and ($1\bar{2}1$) is 89.7°, (110) and (211) is 48.7°, (110) and ($2\bar{1}1$) is 77.9°, (210) and (211) is 45.8° and (210) and ($2\bar{1}1$) is 65.8°, respectively. So, if 110 and 210 planes orient parallel to the upper surface of the tablet, 121 and 211 planes would orient with more than about 50° to the upper surface of the tablet.

Thus, the 110 or 210 planes of salicylic acid seemed to be a preferred orientation plane.

Benzoic Acid Figure 8 shows the absorption corrected X-ray scattering intensities of benzoic acid tablet as a function of α. The normalized diffraction intensities of some reflections were plotted against α in Fig. 9. It was found that the intensities of 002 and (004, 100) reflections increased and that of (112, $11\bar{3}$) reflection decreased with the increase of α. The calculated relative diffraction intensities of benzoic acid crystals are listed in Table II.

The relative diffraction intensities obtained at $\alpha=0°$ were closest to those calculated from crystal structure factors. This result and a marked increase in intensity of 002 reflection with the increase of α indicate that the crystallites in the tablet have a preferred orientation and that 002 plane has a tendency to orient parallel to the upper surface of the tablet during compression. The preferred orientation of 002 plane would cause the increase in intensity of 004 reflection and decrease in intensity of 100 reflection with the increase of α, because the acute angle of (002) and (004) is 0° and that of (002) and (100) is 82.6°. It was considered that by means of both effects, the intensity of (004, 100) reflections slightly increased with the increase of α. Furthermore, because the intensity of (112, $11\bar{3}$) reflections is attributed mainly to 112 reflection (Table II) and the acute angle of (002) and (112) planes is large as 66.5°, the decrease in intensity of this peak at $\alpha>60°$ is explicable as a consequence of the preferred orientation of 002 plane.

Thus, the 002 plane of benzoic acid seemed to be a preferred orientation plane.

Nicotinic Acid Figure 10 shows the absorption corrected X-ray scattering intensities of nicotinic acid tablet as a function of α. In contrast to aspirin, salicylic acid and benzoic acid, nicotinic acid crystallites have only a slight tendency to orient during compression. The normalized diffraction intensities of some reflections are plotted against α in Fig. 11.

The calculated relative diffraction intensities of nicotinic acid crystals are listed in Table III.

The relative diffraction intensities obtained at $\alpha=0°$ were

closest to those calculated from crystal structure factors. This result and an increase in intensities of 002 reflection with an increase of α indicate that the crystallites in the tablets have a preferred orientation and that 002 plane has a tendency to orient parallel to the upper surface of the tablet during compression. The decrease in the intensity of (011, 110) reflections with the increase of α is considered as the preferred orientation of the 002 plane, because the intensity of this peak is attributed mainly to 110 reflection (Table III) and the acute angle between (002) and (110) is as large as 69.9°.

Thus, the 002 plane of nicotinic acid seemed to be a preferred orientation plane.

It is concluded that the present X-ray powder diffraction method can evaluate the preferred orientation of crystallites in tablet. In four crystalline powders, a preferred orientation plane having a tendency to orient parallel to the upper surface of the tablet is revealed by the present method. The feature of the preferred orientation plane will be discussed in the following paper.

Acknowledgment We are grateful to Dr. K. Terada, Chugai Pharmaceutical Co., Ltd., for his valuable suggestions.

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Influence of Compression Force on Consolidation Behavior and Drug Release Property of Wax Matrix Tablets

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In order to characterize the force-dependence of the consolidation behavior and drug release properties of wax matrix tablets, granules consisting of isoniazid and hydrogenated castor oil (80:20) were compressed at various compression force, then the compacts obtained were tested for various properties including tablet density, crushing force and dissolution rate. The packing fraction increased with increasing compression force and reached a constant level (0.973) at a force above 1273 kg/cm^2. The tensile strength of the compacts increased with the increase of the packing fraction, but it continued to increase slightly even after the packing fraction held at an almost constant value. Although the matrix structure became tighter with increasing compression force, the drug release rate from the tablet noticeably increased. Theoretical analysis of this seemingly extraordinary phenomenon provides a reasonable explanation in which the void space left after compression could not work as an effective water channel during dissolution due to the poor wettability of the matrix material. Also, the force-dependence of the disorder in the internal structure of the tablets was examined on the basis of the two-direction dissolution rate analysis. As a result, it was found that the internal disorder increased with increasing compression force, and when the compression force exceeded 1273 kg/cm^2, the disorder was considerably extended.

Keywords wax matrix tablet; isoniazid; hydrogenated castor oil; drug release; compression; porosity; tortuosity; wettability; internal structure

Introduction

A matrix system, in which drugs are homogeneously incorporated into inert matrix materials, has been frequently utilized to achieve a sustained drug release from a dosage form. Although many types of matrix forms including granules,[1] tablets,[2,3] microspheres,[4] *etc.* have been extensively studied over a long period of time, tablet formulation may be the most suitable for large-scale production at pharmaceutical industries because of a comparative ease in controlling the drug release rate and high reproducibility, and hence a fairly low cost for manufacturing.

The drug release from a matrix type tablet is considered to involve the following processes; the dissolution fluid enters into the drug-matrix phase through intergranular spaces; the drug dissolves slowly into the permeating fluid phase and diffuses from the system along the capillary channels filled with an extracting solvent. Higuchi[5] theoretically treated the matrix model and proved that the release amount of drug per a unit surface is proportional to the square root of time. As his equation implies, besides the solubility of drug, the matrix structure in terms of porosity and tortuosity can substantially influence the dissolution rate. In fact, it has been observed that the drug release rate from a matrix tablet varies depending upon a variety of factors[2c,3] such as the amount and type of the matrix material, crystal size, hydrophilic additives and preparation method. These factors should affect either the matrix structure or the wettability against dissolution fluid, and consequently, they should alter the kinetics of water penetration into the core matrix or the pore size distribution or tortuosity of the aqueous channels newly created during dissolution.

In a previous paper,[6] we demonstrated the fact that the drug release rate from the compressed surface of a wax matrix tablet consisting of isoniazid (INZ) and hydrogenated castor oil (HCO) was significantly slower than from the lateral surface of the same tablet. This was clear evidence showing that the internal matrix structure is not uniform within a tablet and that it can strongly influence the release rate. The applied compression force in the tabletting process, therefore, is thought to be a significant factor in achieving precise control of the drug release rate because it must significantly affect the matrix formation process.

The objective of the present study is to examine the effect of compression force on the properties of wax-matrix tablets. Various INZ–HCO tablets were prepared by varying the compression force over a wide range. The compacts were tested for dissolution and physical properties. The disorder of the internal structure occuring during compression will be also discussed on the basis of dissolution results.

Experimental

Materials Isoniazid JP (INZ) was obtained from Yukigosei Yakuhin Kogyo Co., and pulverized to about 7 μm prior to use. HCO was obtained from Kawaken Fine Chemical Co. (K_3wax®; mp 84—88 °C). Ethyl cellulose (EC) was obtained from Shin-Etsu Chemical Co. (grade: N-10-F), and both were used as received. Table I summarizes the properties of the raw materials used in this study.

Granulation The melt granulation method was applied. Pulverized INZ and HCO powder were mixed together at a ratio of 80:20. One hundred grams of the powder blend was melted in a vessel at 95—98 °C under continuous agitation. The homogeneous mass was cooled to room temperature and then pulverized using a mortar and pestle. The granules obtained were sized by passing them through a 20 mesh-sieve. An INZ–EC formula consisting of the drug and EC at a ratio of 80:20 was mixed together using a mixer.

TABLE I. Properties of Raw Materials Used in This Study

Parameters	INZ	HCO	EC
Solubility in water (g/ml)[a]	0.195	Insoluble	
Density (g/cm^3)[b]	1.42	1.03	1.13
Mean diameter (μm)[c]	7	28	5
Contact angle (°)[d]	ND	102	75

a) Determined at 37 °C. *b*) Determined with an air comparison pycnometer. *c*) Calculated from specific surface area. *d*) Determined with a contact angle meter (CA-A type: Kyowa Kaimenkagaku Co.). ND: not determined.

Tabletting Five hundred milligrams of the INZ–HCO granules was compressed by a reciprocating press (Autograph IS-5000, Shimadzu Seisakusyo) using a flat-faced punch and die with a diameter of 10 mm. The applied force was varied from 64 to 5093 kg/cm^2 and the punch velocity was 10 mm/min. For INZ–EC tablets, 500 mg of the powder blend of INZ and EC was compressed under identical conditions.

Dissolution Test Dissolution tests were conducted according to the paddle method described in JPXI. Nine hundred milliliters of distilled water thermostated at 37 °C was used as the dissolution fluid, and was stirred with a paddle at the rate of 100 rpm. A sinker was applied to prevent flotation of the tablet. The amount of drug released was spectrophotometrically assayed at 310 nm.

Determination of Tablet Properties Weight, diameter and thickness of each tablet were determined using an ordinary balance and gages. Initial porosity, the remaining void space after compression ε_i, was calculated from Eq. 1.

$$\varepsilon_i = 1 - \frac{Wf_I/\rho_I + Wf_H/\rho_H}{V} \quad (1)$$

where W and V are weight and the geometrical volume of the tablet, respectively. f is the fractional ratio of a component in formula, ρ is the true density, subscripts I and H represent INZ and HCO, respectively. The tensile strength of each tablet calculated by Eq. 2.

$$T = \frac{2H}{\pi DL} \quad (2)$$

where H, D, and L are crashing force, diameter and thickness of the tablet, respectively.

Calculation of the Dissolution Parameters Various parameters necessary for analysis of dissolution behavior were calculated from dissolution data according to Eq. 3,[6]

$$f_t = 1 - \frac{V_t}{V_0} = 1 - \frac{q}{r_0^3}(r_0 - K_{Lb}t^{1/2})^2(r_0/q - K_{Cb}t^{1/2}) \quad (3)$$

$$q = r_0/h_0$$

where f_t is the fraction of released drug at time t; r_0, h_0 and q are initial tablet radius, initial tablet half-thickness and a ratio factor (usually, $q > 1$); and K_{Cb} and K_{Lb} are the boundary retreat rate constants from the compressed and lateral surfaces, respectively. The determination of each parameter was done by computer calculation using the SIMPLEX method.

Results and Discussion

The physical characteristics of the INZ–HCO tablets prepared at various applied forces and the necessary parameters for the analysis of dissolution behavior are listed in Table II.

Effect of Compression Force on Compressibility and Compactibility To examine the consolidation behavior of a wax matrix tablet, the relation between the packing fraction and the applied force was shown in Fig. 1 as the Heckel plot. The packing fraction increased with increasing compression force until the value reached an almost constant level of about 0.973 at a force above 1273 kg/cm^2. When INZ or HCO alone were directly compressed under identical conditions, the packing fraction of the compacts were 0.881 for the INZ tablet and 0.958 for the HCO tablet, respectively. Therefore, the compressibility of INZ–HCO granules was thought to be improved by blending both powders.

In the range of lower applied force, the observed relation was not linear, so that a clear yield pressure was not obtained. According to Duberg and Nyström,[7] such non-linear behavior exhibited in a Heckel plot implied that the powders were consolidated mainly through particle rearrangement and/or fragmentation rather than plastic deformation. HCO is a typical plastic powder like fatty acids, and INZ is considered a brittle powder. It is known that the apparent consolidation behavior of a mixture of plastic powder and brittle powder should be governed by the compressibility of the major component.[8] Therefore, the above mentioned consolidation behavior of the wax matrix probably results from the compressibility of INZ, which occupies eighty percent of the total granules.

In Fig. 2, T values were plotted against ε_i according to Hasegawa *et al.*[9] A good linear relation was found. This behavior seems quite reasonable, when considering the fact that the void space between particles was effectively

TABLE II. Various Parameters of Wax Matrix Tablets Used in This Study

INZ : HCO	Applied force (kg/cm^2)	Weight (g)	Diameter (cm)	Thickness (cm)	A[a] (g/cm^3)	Initial porosity	T[b] (kg/cm^2)
80 : 20	64	0.495	1.002	0.673	0.745	0.293	1.66
	127	0.497	1.002	0.608	0.829	0.215	3.55
	318	0.497	1.002	0.537	0.939	0.110	9.08
	637	0.495	1.002	0.501	1.002	0.051	14.34
	955	0.494	1.002	0.492	1.019	0.035	17.93
	1273	0.498	1.002	0.492	1.026	0.027	19.18
	1910	0.497	1.002	0.490	1.029	0.025	19.75
	2546	0.496	1.002	0.490	1.027	0.027	21.04
	3820	0.496	1.002	0.490	1.027	0.027	22.42
	5093	0.496	1.002	0.490	1.027	0.027	23.25
90 : 10[c]	1273	0.501	1.003	0.484	1.179	0.042	17.57
85 : 15[c]	1273	0.497	1.003	0.485	1.107	0.034	18.97
75 : 25[c]	1273	0.500	1.002	0.501	0.949	0.024	19.21
70 : 30[c]	1273	0.501	1.002	0.511	0.870	0.025	19.21

a) Drug concentration in the matrix. *b*) Tensile strength. *c*) This data was represented in a previous paper.[6]

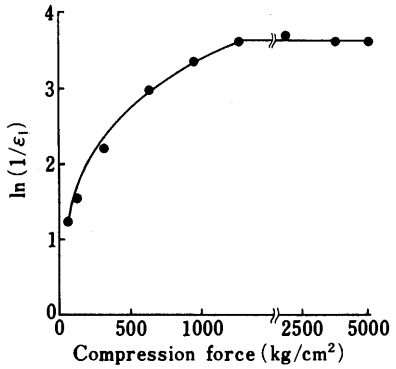

Fig. 1. Heckel Plot of Wax Matrix Tablet

Ordinate: logarithm of the reciprocal ε_i, abscissa: compression force (kg/cm^2). This figure is plotted with data from the INZ to HCO ratio of 80 : 20.

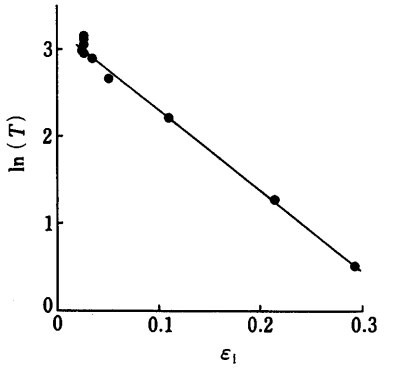

Fig. 2. Relationship between Tensile Strength and Porosity of Wax Matrix Tablets Prepared at Various Compression Forces

eliminated even at a lower force as shown in Fig. 1. T_0, the T value at which ε_i was extrapolated to zero, was an index representing the ultimate hardness of the compact. The estimated T_0 by linear regression at the force range under 2500 kg/cm^2 was about 26 kg/cm^2. This figure means that the wax matrix tablet has enough hardness for handling, even though it's a little lower than those determined for other direct compressible powders. However, it is noted that the T value slightly increased as the applied force increased, even though the ε_i value had become almost constant (Table II). This is probably caused by the partial fusion of HCO occuring during compression; thus, the contact area between particles more or less increased depending on applied force, even if the resultant void space remained the same.

Effect of Compression Force on Dissolution Figure 3 shows the dissolution profiles of the INZ–HCO tablets prepared at various applied forces. Unexpectedly, the drug release rate increased as the applied force increased. This result seems quite unusual, because it was generally considered that the higher applied force would form a tighter matrix, hence lowering the drug is diffusibility in the matrix.

The reason for this discrepancy was thought to be connected with the wettability of the matrix materials. To examine how much the wettability of the matrix materials affects drug release behavior, INZ–EC tablets in which the whole amount of HCO was replaced with EC, were prepared as a comparable example of more wettable matrix tablets, because EC is considered more wettable to water than HCO in terms of contact angle, as shown in Table I. Figure 4 shows the dissolution profiles of the INZ–EC matrix tablets prepared at various applied forces. It was found that the dissolution rate increased even faster than the INZ–HCO tablet, yet it was noted that the release rate decreased with an increase in applied force. Therefore, the wettability of the matrix material may influence not only the dissolution rate but also the rate's dependency on compression force. Foster and Parrott[3c] recently reported that the dissolution rate from a wax-matrix tablet decreased as the applied force increased. Even though their result was completely different from ours, this was probably brought about by the difference in the dissolution method applied. They conducted the dissolution test using a fluid containing surfactant to improve water penetration into the wax matrix tablet. Therefore, the observed discrepancy in the dissolution behavior suggests that the water wettability of matrix materials should be one of the most important factors influencing dissolution.

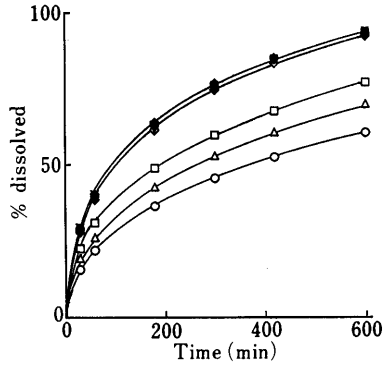

Fig. 3. Drug Release Profiles from HCO Matrix Tablets Prepared at various Compression Forces

Compression force (kg/cm^2): ○, 64; △, 127; □, 318; ◇, 637; ▽, 955; ●, 1273; ▲, 1910; ■, 2564; ◆, 3820; ▼, 5093.

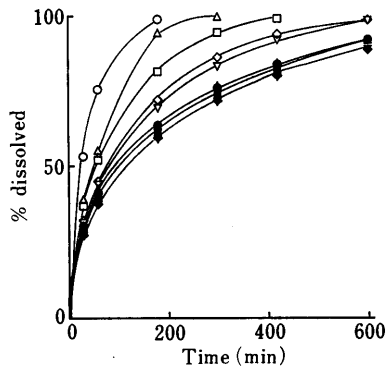

Fig. 4. Drug Release Profiles from EC Matrix Tablets, Prepared at Various Compression Forces

Compression force (kg/cm^2): ○, 64; △, 127; □, 318; ◇, 637; ▽, 955; ●, 1273; ▲, 1910; ■, 2564; ◆, 3820; ▼, 5093.

Theoretical Consideration of Compression Force Dependency of Dissolution Rate To understand more rationally the above mentioned compression force dependency of the dissolution rate, a theoretical consideration was made from the view point of the dissolution mechanism. The leaching type release kinetics from a planar matrix system is generally expressed by a diffusion equation Eq. 4, which was proposed by Higuchi.[5] In this case, the dissolution boundary distance retreating from the surface after time t, X_t, is expressed as Eq. 5,

$$Q=\sqrt{\frac{D\varepsilon}{\tau}(2A-\varepsilon C_s)C_s t} \tag{4}$$

$$X_t=\frac{2Q}{2A-\varepsilon C_s} \tag{5}$$

where Q is the amount of drug released per unit surface area, D is the diffusion coefficient of the drug in the permeation fluid, ε is the porosity of the matrix, τ is the tortuosity of the matrix, A is the concentration of solid drug in the matrix, C_s is the solubility of drug in the dissolution medium, and t is time. From Eqs. 4 and 5, X_t can be rewritten as:

$$X_t=K_b t^{1/2} \tag{6}$$

and,

$$K_b=2\sqrt{\frac{D\varepsilon C_s}{\tau(2A-\varepsilon C_s)}} \tag{7}$$

where K_b is the boundary retreat rate constant. Among those parameters, ε, τ, and A can vary depending on the matrix structure. The total porosity of matrix, ε_t, consists of the void space remained between particles after compression, ε_i, and the void space newly created after drug release, ε_d. That is:

$$\varepsilon_t=\varepsilon_i+\varepsilon_d \tag{8}$$

and

$$\varepsilon_d=A/\rho_t$$

As the compression force increased, ε_i clearly decreased but ε_d also increased at the same time, because A is raised with the reducing volume of the tablet. As shown in Table II, however, the increment of ε_d was considerably small compared with the decrement of ε_i, so finally, the increasing of compression force resulted in a significant decrease in ε_t. If all the void space, originally existing and newly created, acts effectively as the pathway of drug diffusion, the boundary retreat rate constant, k_b, prepared at the lower applied force becomes larger than at higher applied force. However, the force dependency of the dissolution behavior shown in Fig. 3 is completely opposite to the expectation. This implies that not all of the void space plays the role of water channel. Then, to examine how much of the void space in a wax matrix tablet is actually acting as a dissolution pathway, the dissolution data was analyzed for K_{Cb} according to Eq. 3, then τ was calculated from the K_{Cb} values according to Eq. 7. The relationship between ε_t and τ was shown in Fig. 5. The τ drastically increased as porosity increased. This result seems quite extraordinary, when considering the commonly held belief that the tortuosity decreases with increasing porosity.[10] The figure also shows the relation between ε_t and τ for the matrix tablets of various INZ : HCO ratios (data was referred from our previous paper). In this case, the calculated τ value surely increased as ε_t decreased. From the results, it is assumed that only the restricted void space can be utilized as water channels due to the poor wetting property of HCO.

In the case where the void space which originally existed between particles could not be utilized as effective water channels, Eq. 9 could be used instead of Eq. 7.

$$K_b = 2\sqrt{\frac{D\varepsilon_d C_s}{\tau_d(2A-\varepsilon_d C_s)}} \tag{9}$$

where τ_d is the tortuosity in connection with the void space created after drug release.

Figure 6 shows the relation between ε_d and τ_d. By this approach, which represents the relation between the tortousity and the porosity restricted the void space created after drug release, all data show the same tendency, in spite of the difference in the formulation and compression force. From these results, it was found that the Higuchi equation can not always be applied if the matrix substance shows adhesional wetting.

Assessment of the Disorder in the Internal Structure of Wax Matrix Tablet In the previous paper, we suggested that the disorder of the internal structure of a wax matrix tablet occurring during compression could be quantitatively evaluated by the ratio of the two dissolution parameters, K_{Cb} and K_{Lb} (two-direction dissolution rate). K_{Cb}/K_{Lb} can be rewritten from Eq. 9.

$$\frac{K_{Cb}}{K_{Lb}} = \sqrt{\frac{\tau_{dL}}{\tau_{dC}}} \tag{10}$$

where τ_{dL} and τ_{dC} are τ_d of the water channels from the

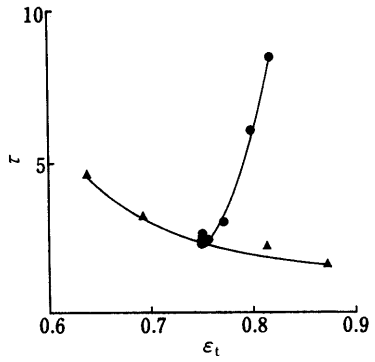

Fig. 5. Relationship between τ and ε_t

Key: ●, prepared at various compression forces; ▲, prepared with various INZ–HCO ratios compressed at 1273 kg/cm^2.

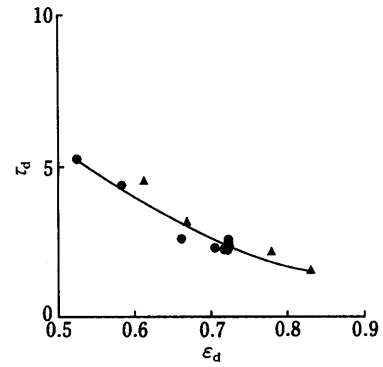

Fig. 6. Relationship between τ_d and ε_d

Key: ●, prepared at various applied forces; ▲, prepared with various INZ–HCO ratios compressed at 1273 kg/cm^2.

TABLE III. Calculated Various Parameters of Wax Matrix Tablets Used in This Study

INZ : HCO	Applied force (kg/cm^2)	K_{Cb}[a] ($\times 10^{-3}$ cm/min$^{1/2}$)	K_{Lb}[a]	τ_L/τ_C
80 : 20	64	5.56	6.00	0.86
	127	6.10	6.60	0.85
	318	7.93	8.69	0.83
	637	8.46	9.34	0.82
	955	8.55	9.56	0.80
	1273	8.46	9.39	0.81
	1910	8.35	9.33	0.80
	2546	8.12	9.35	0.75
	3820	8.17	9.60	0.72
	5093	7.98	9.46	0.71
90 : 10[b]	1273	10.28	14.14	0.53
85 : 15[b]	1273	8.69	9.91	0.77
75 : 25[b]	1273	7.18	8.14	0.78
70 : 30[b]	1273	5.99	6.63	0.81

a) Calculated by Eq. 2. *b*) This data was represented in a previous paper.[6]

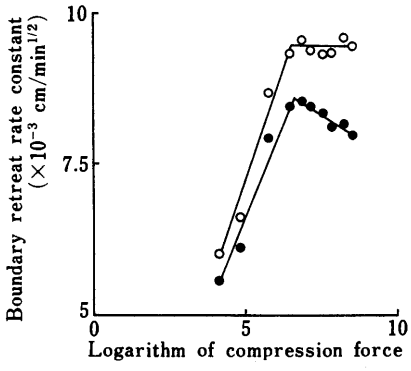

Fig. 7. Changes of K_{Cb} and K_{Lb} Values with Applied Force

Key: ●, K_{Cb}; ○, K_{Lb}. These tablets were prepared at an INZ–HCO ratio of 80 : 20.

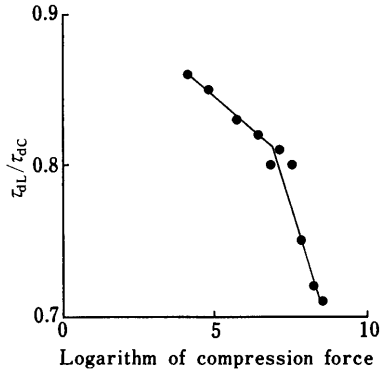

Fig. 8. Change of τ_{dL}/τ_{dC} Value with Compression Force
These tablets were prepared at an INZ–HCO ratio of 80:20.

dissolution boundary surface to compressed and lateral surfaces, respectively. Since the tortuosity is intimately related to the internal structure of the matrix, the ratio of τ_{dL}/τ_{dC} can be used for quantitative evaluation of the disorder in the internal structure of an individual tablet. Table III shows the two dissolution parameters K_{Cb} and K_{Lb} and the tortuosity ratio calculated from Eq. 10. Figure 7 shows the relation between compression force and K_{Cb} or K_{Lb} values. The values of both rate parameters increased with increasing the applied force, but K_{Lb} reached a constant level at a force over 1273 kg/cm^2, whereas K_{Cb} provided a maximum value and then decreased. This phenomenon may be caused by the following: when the granules were compressed by the compression force over 1273 kg/cm^2, HCO partially fused and then became elongated in a horizontal direction easier than in a vertical direction, so the horizontal surface of the drug particles might be easily covered with HCO after fragmentation.

In order to quantitatively examine the relation between compression force and the degree of the structural disorder of the tablet, τ_{dL}/τ_{dC} was plotted against compression force in Fig. 8. As is clear, the tortuosity ratio drastically decreased with an increasing compression force, meaning that the disorder increased as the compression force increased. It was also found that two lines different in slope bounded at 1273 kg/cm^2 as a flection point, and the slope of the line at the higher compression force was larger than that of the lower one. From this result, the excessive compression force was thought to be caused largely by the disorder in the internal structure of the tablet. The smaller value of τ_{dL}/τ_{dC} implies that the tablet has a more lameller structure and would have a greater lamination tendency. It is generally known that capping, the phenomenon of the stratiform segregation of the tablet, occurs when the local lamination extends over a wide range in a tablet. Therefore, the disorder in the internal structure of matrix tablet should be one of the major factors relating to the capping phenomena.

Although the critical τ_{dL}/τ_{dC} value which induces the actual capping phenomenon has not yet been established, the method presented here should be a convenient way to evaluate how much the structural defect of a tablet occurs during compression or to prejudge whether the tablet formulation currently examined essentially involved any possibility of suffering from a capping phenomenon in the manufacturing process. This is because τ_{dL}/τ_{dC} represents the tortousity ratio in connection with the void space created after drug release, hence this value is an index of the disorder of drug particles in the tablet. In the present study, no sign suggesting the capping phenomenon was observed under the tabletting condition we applied, where the minimum value of τ_{dL}/τ_{dC} was around 0.7. So the critical τ_{dL}/τ_{dC} value may actually be far lower than this value. However, it is possible that an internal structural defect can differ depending on the drug species incorporated due to the variety of powder property or the interaction between drug and matrix material. Further investigation will be conducted to find the critical τ_{dL}/τ_{dC} value and to elucidate the effect of drug species on the disorder in the internal structure.

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Kinetics and Mechanism of the Acid–Base Equilibrium and the Hydrolysis of Benzodiazepinooxazines[1)]

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Ring-opening and ring-closing (acid–base equilibrium) reactions of the six-membered oxazine of benzodiazepinooxazines (BZINs) and the subsequent hydrolyses of the diazepine ring have been investigated kinetically and compared with the reactions of benzodiazepinooxazoles (BZOLs) which have the five-membered oxazolidine ring. The ring-closing reaction for BZINs is slower than that for BZOLs due to the increase in the degree of freedom for the moving moiety of BZINs. The rates of the ring-opening reactions are almost independent of the substituents at 12b-position (H-(1), CH_3-(2), and C_6H_5-(3)), indicating that an attacking proton may approach equally (non-sterically) to the lone pair of N_5 atoms. Possible conformational aspects of BZINs in solution are proposed. Cleavages (hydrolyses) of the diazepine ring occur at the C_{12b}-N_5 (iminium) bond for 2 and 3 and are 10—100 times faster than those for the corresponding BZOLs. For 1, in contrast to 2 and 3, hydrolysis of the amide bond (N_8-C_7) of the diazepine ring takes place instead of the iminium bond, similar to the case of 11b-hydrogen BZOL.

Keywords benzodiazepinooxazine; benzodiazepinooxazole; acid–base equilibrium; kinetics; oxazine ring-opening, -closing; diazepine ring; hydrolysis; reaction pathway; iminium bond; amide bond

Introduction

From the standpoint of drug behavior after oral administration, the acid–base equilibrium (oxazolidine ring-opening and ring-closing reactions) of benzodiazepinooxazoles (BZOLs) and the subsequent hydrolysis of the diazepine ring have been studied kinetically.[2-6)] These reactivities of BZOLs which contain a five-membered oxazolidine ring fused to a seven-membered diazepine ring are found to be greatly influenced by the substituents at 11b-, 2-, 3-, and 7-positions.[2-6)] It is thus of interest to investigate the reactivities of benzodiazepinooxazines (BZINs, six-membered oxazine rather than the oxazolidine of BZOLs) in order to compare the differences of kinetic behavior in the various ring systems. In this paper we described the reactivities of BZINs in comparison to those of BZOLs (Chart 1).

Experimental

Materials and Instruments Compounds **1**—**5** were synthesized by methods similar to those reported by Deriege *et al.*,[7)] Miyadera *et al.*,[8)] and Lemke and Hanze.[9)] The chemical structures of these compounds were confirmed by elemental analyses as well as ^{1}H- and ^{13}C-nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) measurements. Melting points and the results of the elemental analyses of the compounds studied are listed in Table I. All other chemicals were purchased commercially and were of reagent grade, and used without further purification.

Ultraviolet (UV) spectra were measured by Shimadzu UV-260 and UV-2200 spectrophotometers. A stopped-flow spectrophotometer (Otsuka Denshi RA-401) was used for the measurement of the reaction rates. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-FX 100 spectrometer at 100 and 25 MHz, respectively. An NEC microcomputer (PC-9801E) was used for the analyses of pH-rate profiles.

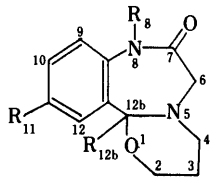
Kinetic Runs The buffer systems were the same as those empolyed in previous studies.[2-6)] The rates of the oxazine ring-opening and ring-closing were measured by the pH-jump method using the stopped-flow instruments as reported previously.[2-4,6)] The pseudo first-order rate constants (k_{obs}) for the reactions were determined by the ordinary first-order analysis and also by the Guggenheim method[10)] in the case where the reaction end point is unknown. These analyses were performed directly using a Sord microcomputer (M223 mark III) linked to the stopped-flow instruments.

The hydrolysis of **1** was carried out at 70 °C. Aliquots of the sample solution were withdrawn at appropriate intervals and cooled to room temperature (25 °C). Their UV spectra were measured and compared with spectra of the model compounds *N*-acetyl-2-aminobenzaldehyde (**9**) and 2-aminobenzaldehyde-ethylenediimine (**10**).[6,11,12)] All other reactions were carried out at 25 °C.

Determination of Equilibrium Constant The apparent equilibrium constants of BZINs were determined by a method similar to those reported previously.[2-4,6)]

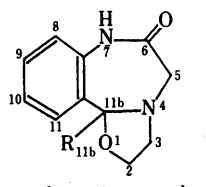
Results and Discussion

Oxazine Ring-Opening and -Closing Reactions Figure 1 shows the UV spectra of compound **1** in various pH buffer solutions. These spectra are attributed to equilibrium



benzodiazepinooxazines

compound	R_{12b}	R_8	R_{11}
1	H	H	H
2	CH_3	H	H
3	C_6H_5	H	H
4	C_6H_5	CH_3	H
5	C_6H_5	H	Cl



benzodiazepinooxazoles

compound	R_{11b}
6	H
7	CH_3
8	C_6H_5

Chart 1

TABLE I. Physical and Analytical Data for Benzodiazepinooxazines (BZINs)

Compd.	mp (°C) (lit.)	Recrystn. solvent	Formula	Analysis (%) Calcd (Found) C	H	N
1	176—178 (175—176)[8)]	Ethanol	$C_{12}H_{14}N_2O_2$	66.03 (66.24)	6.46 (6.35)	12.83 (12.82)
2	147—150	Ethanol	$C_{13}H_{16}N_2O_2$	67.22 (67.04)	6.94 (6.72)	12.06 (11.90)
3	220—222	Ether–ethanol	$C_{18}H_{18}N_2O_2$	73.45 (73.29)	6.16 (5.89)	9.52 (9.51)
4	150—152	Ethanol	$C_{19}H_{20}N_2O_2$	73.99 (74.28)	6.55 (6.64)	9.09 (9.11)
5	206—208 (220—222,[8)] 213—216)[9)]	Ethanol	$C_{18}H_{17}ClN_2O_2$	65.75 (65.49)	5.21 (5.18)	8.52 (8.58)

mixtures of the ring-opened iminium form (AF, acid free N_8 form) in acid solution and the ring-closed form (BF, basic free N_8 form) in weakly alkaline solution.[2-6] From the spectral data, similar to the case of BZOLs studied previously,[2-4,6] the apparent pk_{eq}^{UV} value ($-\log([BF][H^+]/[AF])$) was determined to be 5.95.

Figure 2 illustrates the pH-rate profile for the oxazine ring-opening and ring-closing reactions of compounds **1** and **4**. The shapes of the profiles are similar to those for BZOLs reported previously,[2-4,6] suggesting that the reaction scheme shown in Chart 2 is applicable. In Chart 2, AC and AA represent acid cationic N_8 form and acid anionic N_8 form, respectively, and basic cationic and anionic forms are represented by BC and BA, respectively. The superscripts and subscripts of the rate constants have the following meanings: The superscripts H^+, 0, and OH^- represent the hydrogen ion-catalyzed, water-catalyzed or unimolecular (intramolecular), and hydroxide ion-catalyzed reactions, respectively. The first subscript indicates whether ring-opening (Op) or ring-closing (Cl) occurs, and the second one represents the cationic form (C), the free form (F) or the anionic form (A) at the 8 nitrogen (N_8) atom of the compound. $K_{a,1}$, $K'_{a,1}$, $K_{a,2}$, and $K'_{a,2}$ are the dissociation constants of the respective forms, and the dissociation processes are, in general, much faster than the processes of the ring-opening and the ring-closing.[2,4]

The rate constants and dissociation constants in Chart 2 were determined by procedures similar to those empolyed previously.[2-4,6] The values obtained along with the pK_{eq}^{UV} values are summarized in Table II. For the sake of comparison, Table II also shows the values for compounds **6**—**8** obtained previosuly.[4,6] The solid curves in Fig. 2 were

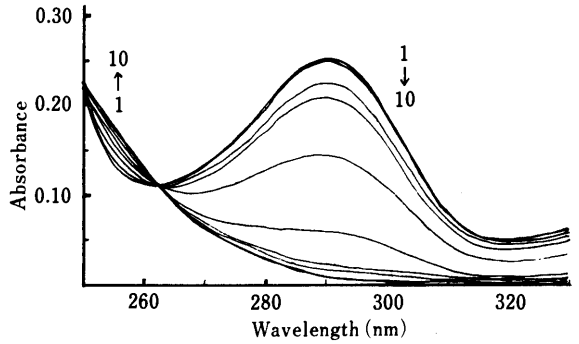


Fig. 1. UV Absorption Spectra of Compound **1** at Various pH Values

Concentration of **1** was 2.98×10^{-5} M. 1, pH 1.89; 2, 2.79; 3, 3.97; 4, 5.00; 5, 5.38; 6, 5.87; 7, 6,49; 8, 7.00; 9, 8.27; 10, 9.02.

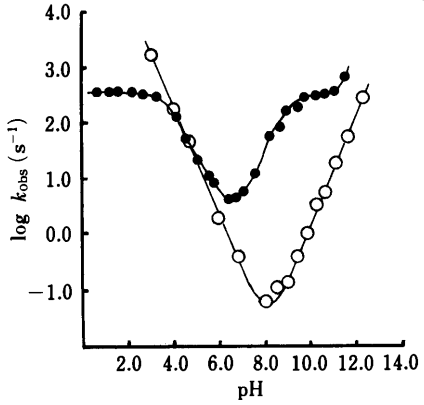


Fig. 2. The pH-Rate Profiles for Oxazine Ring-Opening and Ring-Closing Reactions of Compounds **1** and **4** at 25 °C

●, compound 1; ○, compound 4.

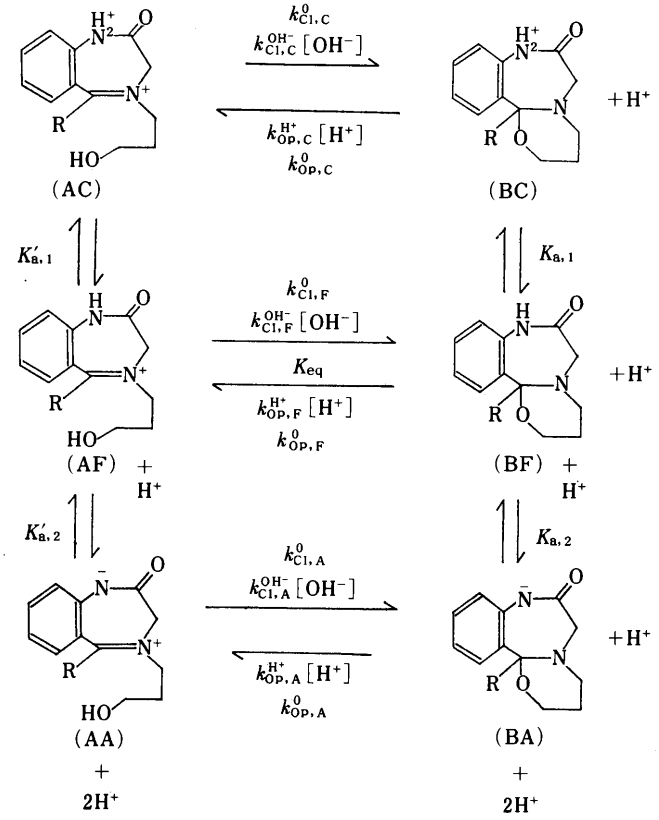


Chart 2

TABLE II. Estimated Rate Constants and Equilibrium Constants[a]

Compound	$k_{O_p,F}^{H^+}$ $s^{-1}M^{-1}$	$k_{O_p,F}^{0}+k_{Cl,F}^{0}$ s^{-1}	$k_{Cl,F}^{OH^-}$ $s^{-1}M^{-1}$	$k_{Cl,A}^{0}$ s^{-1}	$k_{Cl,A}^{OH^-}$ $s^{-1}M^{-1}$	$pK'_{a,2}$	$pK_{a,2}$	pK_{eq}^{UV}
1	2.23×10^{6}	1.87	3.72×10^{7}	0	1.43×10^{5}	8.79	13.33	5.95
2	c.n.d.[b]	c.n.d.[b]	2.04×10^{5}	0	2.80×10^{1}	9.58	c.n.d.[b]	8.37
3	2.10×10^{6}	0	1.40×10^{4}	0	1.20×10^{1}	9.60	c.n.d.[b]	8.59
4	2.14×10^{6}	0	1.39×10^{4}	—[c]	—[c]	—[c]	—[c]	8.51
5	7.33×10^{5}	0	1.85×10^{4}	0	2.93×10^{1}	8.95	c.n.d.[b]	8.00
6	1.45×10^{6}	7.16	5.87×10^{9}	c.n.m.[d]	c.n.m.[d]	c.n.m.[d]	c.n.m.[d]	6.23
7	c.n.d.[b]	c.n.d.[b]	1.81×10^{7}	0	6.34×10^{5}	9.76	c.n.d.[b]	7.5
8	7.90×10^{4}	5.83×10^{1}	5.42×10^{7}	0	1.20×10^{6}	9.38	>12[e]	7.2

a) At 25 °C; in the presence of 4% (v/v) ethanol. *b*) c.n.d. means "could not determine" because of relatively fast subsequent hydrolysis. *c*) The 8-nitrogen atom has no proton to be deprotonated. *d*) c.n.m. means "could not measure" because of fast reaction. *e*) The absorbance change due to the deprotonation of the 7-nitrogen atom was not observed.

calculated by applying the values in Table II to equations which were derived in a manner similar to that used for BZOLs.[2,4,6] The following equations were used:

a) pH < *ca.* 4.0

$$k_{obs}=\frac{[H^+]}{K_{a,1}+[H^+]}(k^0_{Op,C}+k^{H^+}_{Op,C}[H^+])\fallingdotseq\frac{k^0_{Op,C}[H^+]}{K_{a,1}+[H^+]} \quad (1)$$

b) *ca.* 4.0 < pH < *ca.* 8.0

$$k_{obs}=k^{H^+}_{Op,F}[H^+]+k^0_{Op,F}+k^0_{Cl,F}+k^{OH^-}_{Cl,F}[OH^-] \quad (2)$$

c) pH > *ca.* 8.0

$$k_{obs}=(k^0_{Cl,F}+k^{OH^-}_{Cl,F}[OH^-])f_{AF}+(k^0_{Cl,A}+k^{OH^-}_{Cl,A}[OH^-])f_{AA}$$
$$=\frac{1}{[H^+]+K'_{a,2}}\{k^0_{Cl,F}[H^+]+k^{OH^-}_{Cl,F}K_W+k^0_{Cl,A}K'_{a,2}$$
$$+k^{OH^-}_{Cl,A}(K_W/[H^+])K'_{a,2}\} \quad (3)$$

where f_{AF} and f_{AA} are the molar fractions of AF and AA, respectively. K_W is the ionic product of water and is assumed to be $1\times10^{-14}M^2$. The observed data points fit well to the calculated lines. The plateau portion found in the acid region for compound **1** suggests the involvement of 8 nitrogen atom protonation in the ring-opening rate. From the analysis of this region (from Eq. 1) the values of $k^0_{Op,C}$ and $pK_{a,1}$ were estimated to be 1.50×10 ($s^{-1}M^{-1}$) and 3.83, respectively. The following characteristics can be read from the determined kinetic constants in Table II: (1) The $k^{H^+}_{Op,F}$ value (ring-opening reaction) for **1** is similar to that for **6** and the $k^{OH^-}_{Cl,F}$ value (ring-closing reaction) for **1** is about 1/200 of that for **6**. (2) Although the $k^{H^+}_{Op,F}$ values for **1**—**4** are almost the same, the values of $k^{OH^-}_{Cl,F}$ decrease with the increase in the bulkiness of 12b-substituents. (3) The substituens at 8- and 11-positions do not influence the $k^{H^+}_{Op,F}$ and $k^{OH^-}_{Cl,F}$ values (compare the results among compounds **3**—**5**).

These observations may be interpreted as follows. The ring-closure to oxazine is considered to be more difficult than that to oxazolidine because of the increased freedom of the moiety (2 or 3 carbon chain containing terminal oxygen atom). Also the bulkiness of 12b-groups may prevent the ring-closure.

The ring-opening reaction ($k^{H^+}_{Op,F}$) occurs through an approach of a proton to the lone pair of N atoms at 5-position. The reaction is almost independent of the substituents at 12b-positions, indicating that the proton may approach equally to the lone pair of N_5 atoms among compounds **1**—**5**. Under the conditions that the diazepine ring is in a fixed conformation, that is, that the C_6 atom is not inverted, the oxazine ring of BZINs adopts 4 possible conformations (R_{I-IV}) as shown in Chart 3. Since the carbon atom at 12b-position is chiral, *i.e.*, BZIN is asymmetric, only R-enantiomer is considered here. Construction of the Dreiden molecular model for BZINs lead the following characteristics in each conformation: R_I form, the substituent at 12b-position and the lone pair of N_5 are *gauche*, and the oxazine ring and diazepine ring are skewed with each other; R_{II} form, the substituent at 12b-position and the lone pair of N_5 are *gauche*, and the oxazine ring and diazepine ring are skewed with each other; the conformation R_{III} cannot be formed for the diazepine ring, so this conformation is ignored; R_{IV} form, the substituent at 12b-position and the lone pair of N_5 is *anti*, and the oxazine ring and diazepine ring are approximately coplanar with each other. In the R_{IV} conformation the lone pair of N_5 is most sterically unhindered by 12b-substituent. The oxazine ring and diazepine ring in R_{IV} are almost coplanar and thus less crowded sterically in the vicinity of N_5. Therefore, the majority of the conformations may be R_{IV} in solution or, at least, the ring-opening reaction may occur through the conformation R_{IV}.

Hydrolyses of BZINs Similar to compounds **7** and **8**,[5,11] hydrolyses of compounds **2** and **3** proceed through cleavage of N_5–C_{12b} bond (cleavage of diazepine ring), and subsequent hydrolyses through cleavage of amide bond (C_7–N_8 bond of the original numbering). The cleavage of diazepine ring for **2** is about 10 times faster than that for **7** at neutral to alkaline regions.[5] The cleavage for **3** is also faster than that for **8** by a factor of 10—100.[5]

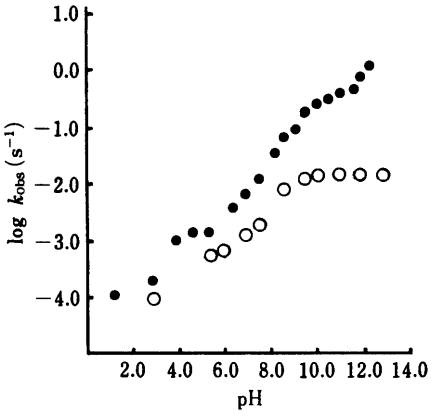


Fig. 3. The pH-Rate Profiles for the Hydrolyses of Compounds **3** and **4** at 25 °C

●, compound **3**; ○, compound **4**.

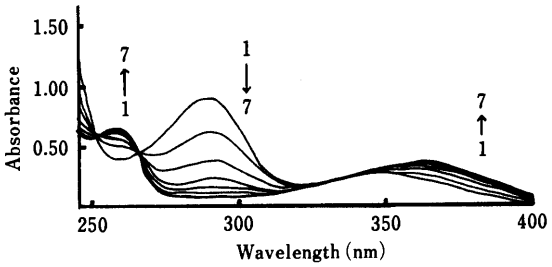


Fig. 4. Typical UV Spectral Changes for Hydrolysis of Compound **1** at pH 3.9 and 70 °C

Concentration of **1** was 1.00×10^{-4} M. 1, 0; 2, 2; 3, 5; 4, 9; 5, 15; 6, 21; 7, 47 d.

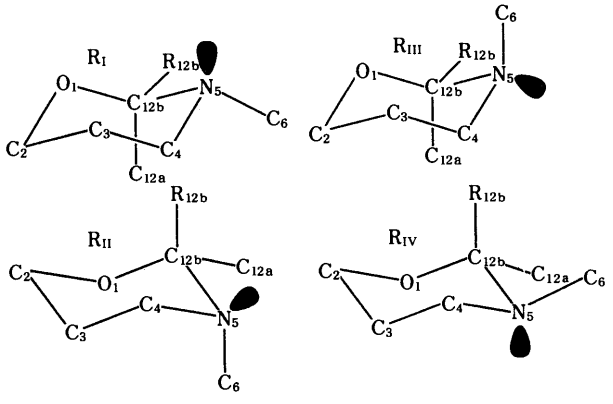


Chart 3

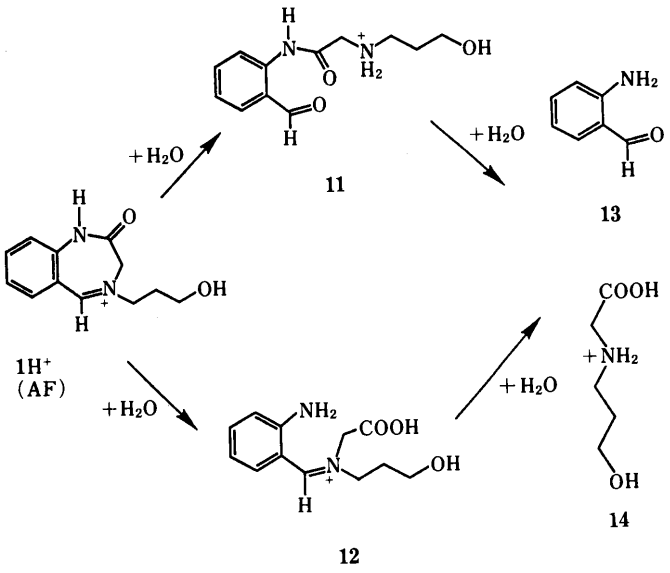


Chart 4

Figure 3 shows the pH-rate profiles for the cleavage of diazepine ring of compounds **3** and **4**. The shapes of the pH-profiles are very complicated. Although buffer concentration effects were not corrected, the shape of the profile may be a result of a reaction mechanism involving many molecular species, as shown in Chart 2, proton and hydroxide ion catalyses for each step, transition of the rate-determining step of the reaction,[13,14] and so on. The difference between the shapes at alkaline for compounds **3** and **4** may reflect the involvement of N_8 deprotonation of **3** in the cleavage of diazepine ring. Complete quantitative analyses of these pH-profiles are difficult at present because of the complexity of the system.

Since hydrolysis of compound **1** at 25 °C was very slow, the reaction was followed at 70 °C. Figure 4 shows the time course of the UV spectral changes for **1** in buffer solution. The initial and final pH of the reaction solution after cooling to 25 °C was the same at 3.9, *i.e.*, the pH of the solution seemed to be unchanged during the reaction. The pseudo first-order rate constant (k_{obs} in s^{-1}) of 2.32×10^{-6} was obtained from this spectral change. The spectral change shown in Fig. 4 is very similar to those for **6** in pH 2.0 at 70 °C, where the k_{obs} value for **6** was 4.97×10^{-6} s^{-1}.[6] The close values suggest that the reaction scheme for the hydrolysis of **1** is considered to be the same as that for **6**. As shown in Chart 4, two branching routes of hydrolysis of AF are now proposed.[6] The hydrolysis of **1**, therefore, proceeds *via* the intermediate **12** rather than **11** which is formed first in compounds **2**—**5**, and both result in the ultimate formation of **13** and **14**.

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Fibrinolytic Effect of Tissue Plasminogen Activator on Cerebral Embolism in Stroke-Prone Spontaneously Hypertensive Rats

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To study the thrombolytic effect of tissue plasminogen activator (t-PA) on cerebral emboli, we characterized cerebral embolization in stroke-prone spontaneously hypertensive rats (SHRSPs) and Wistar Kyoto rats (WKYs). [^{125}I]Fibrin clot particles (20—100 μm diameter) were injected twice at an interval of 90 min into the left internal carotid artery of WKYs and SHRSPs. After each injection, spontaneous embolus dissolution was monitored with a γ-ray detector placed on the head of the embolic rats. Embolus dissolution was spontaneously generated in 15 min after the injection of fibrin clots. In WKYs, 21% and 42% of the clots were dissolved 30 and 90 min after the second embolization, respectively. On the other hand, the spontaneous embolus dissolution in SHRSPs was significantly lower than that of WKYs, indicating that the endogenous fibrinolytic ability of SHRSPs is less potent than that of normotensive rats. The intravenous administration of t-PA at doses of 75, 250 and 750 μg/kg caused a dose-dependent embolus dissolution in SHRSPs. Furthermore, systemically applied t-PA produced embolus dissolution without causing systemic plasminogen activation, fibrinogen breakdown or bleeding.

In conclusion, the intravenous administration of t-PA produces selective embolus dissolution without systemic fibrino(geno)lysis in a cerebral embolic SHRSP.

Keywords cerebral embolism; stroke-prone spontaneously hypertensive rat; Wistar Kyoto rat; tissue plasminogen activator; fibrinolytic therapy

Introduction

Mammalian plasma contains an enzymatic system capable of dissolving the fibrin in blood clots. One component of this fibrinolytic system, the serine protease plasminogen activators, generates the active enzyme plasmin by limited proteolysis of the zymogen plasminogen. Plasmin subsequently dissolves the fibrin network of a clot to form soluble products.[1] Two major plasminogen activators are tissue plasminogen activator (t-PA), found in several tissue extracts, and urokinase plasminogen activator (u-PA) isolated from human urine. The t-PA has been extracted from a cultured human melanoma cell line[2] and produced by recombinant deoxyribonucleic acid (DNA) techniques in bacterial cells[3] or mammalian cells.[4] In this study we used the t-PA obtained from cultured human diploid fibroblasts.[5]

According to several studies *in vitro*, t-PA has a much higher affinity for fibrin compared with u-PA, and it increases plasminogen binding to a fibrin clot.[6,7] Therefore, it has been considered that the activation of plasminogen by fibrin-bound t-PA causes the efficient generation of plasmin on the thrombus without systemic plasminogen activation, which can produce indiscriminate digestion of coagulation proteins and significantly increase the risk of hemorrhage during treatment.

Atherothrombosis and thromboembolism are the main causes of acute stroke, and several thrombolytic agents such as u-PA and streptokinase (SK) have been routinely used for the therapy. However, there is criticism against the therapeutic use of thrombolytic agents because of the possibility of intracerebral hemorrhage during treatment.[8] Particularly, the intravenous infusion of large doses of u-PA or SK has generally been contraindicated to initiate the bleeding tendency because it can bring on an accerelation of systemic fibrino(geno)lysis.[9] On the other hand, if the early elimination of thrombi has not been achieved in acute stroke patients, permanent neurological deficits are difficult to avoid. Recently, intraarterial infusion with low doses of u-PA or SK has been used to demonstrate clinical improvement without intracerebral hemorrhagic complication.[10,11] Nevertheless, the intravenous application of a thrombolytic agent is clinically most desirable in order to avoid the burden of catheterization in patients and to practice rapid therapy. The t-PA is expected to possess "clot-selective" thrombolysis and may be systemically applicable for the therapy of acute stroke.

Previously, we reported that the intraarterial administration of t-PA produced embolus dissolution in an experimental cerebral embolic model of Wistar rats.[12] However, hypertension is one of the important systemic risk factors in the pathogenesis of stroke, and stroke-prone spontaneously hypertensive rat (SHRSP) is a good pathogenetic animal model for studies on stroke in humans as advocated by Yamori *et al.*[13] In the present study, we prepared a thrombo-embolic stroke model by injecting [^{125}I]fibrin clot particles into the left internal carotid artery in SHRSPs and normotensive Wistar Kyoto rats (WKYs), and characterized the deterioration of defensive fibrinolysis in SHRSPs. Furthermore, we demonstrated that t-PA dissolved cerebral emboli without causing systemic fibrino(geno)lysis in a cerebral embolic SHRSP.

Materials and Methods

Materials The t-PA (sigle chain form, molecular weight *ca.* 63000) was purified from a confluent culture of human diploid fibroblasts as previously described.[5] The enzymatic activity of t-PA was approximately 40000 U/mg protein, which formed the same lysis area with u-PA at a concentration of 10 IU/ml on a fibrin plate according to the method reported by Rijken *et al.*[14] Human fibrinogen (Fbg) was purchased from Sigma Co., Ltd., St. Louis. Tissue thromboplastin (Simplastin®, Warner–Lambert Co., Ltd., New Jersey) was used for plasma clot formation. The t-PA was dissolved with saline containing 0.02% Tween-80.

Iodination of Fbg Fbg was labelled with Na[^{125}I] (17.4 Ci/mg, NEN Products, Boston) using solid state lactoperoxidase and glucose oxidase (Enzymobeads®, Bio-Rad, Richmond) according to the method reported by David and Reisfeld.[15] The [^{125}I]Fbg showed 0.5×10^8 cpm/nmol specific radioactivity and approximately 90% clottability, and the same pattern as that of the respective native Fbg on sodium dodecyl sulfate–

polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of the Suspension with [^{125}I]Fibrin Clot Particles Plasma of (2 ml) obtained from a male Jla: Wistar rat was mixed with 200 μl of [^{125}I]Fbg (4×10^7 cpm) and 10 μl of tissue thromboplastin (11 mg/ml). After incubation at 37 °C for 20 min, the formed plasma clot was washed 3 times with 0.175 M NaCl (saline) and then powdered in a mortar under a freezing condition with liquid nitrogen. The powdered [^{125}I]fibrin clots were further homogenized using a Teflon homogenizer after an addition of 5 ml saline. The [^{125}I]fibrin clot particles (20—100 μm) had a specific activity of approximately 4×10^3 cpm/μg protein.

Preparation of Cerebral Embolic Model Male SHRSPs were maintained and breeded in our laboratories after transfer from the Department of Pathology, Kinki Medical University (Osaka, Japan). SHRSPs, weighing *ca.* 310 g (23—30 weeks of age), and Male WKYs, weighing *ca.* 400 g (23—30 weeks of age) were used. Tail systolic blood pressures measured prior to the experiments in both strains were 257 ± 3 mmHg (in SHRSPs, $n=23$) and 132 ± 4 mmHg (in WKYs, $n=5$), respectively. Preparation for the embolic model was based upon the previous paper.[12] The rats were anesthetized with an intraperitoneal injection of urethane/chloralose/water (500 mg/40 mg/ml, 1.5 ml/kg body weight). After a polyethylene tracheostomy tube was inserted, the left carotid artery was exposed and bifurcations (occipital and pterygopalatine arteries) of the left internal carotid artery were ligated. A temporary clip was then placed at the origin of the external carotid artery. The external carotid artery was cannulated retrogradely with PE-10 (Intramedic®) tubing, the clip removed and the tip was positioned near the origin of the internal carotid artery. Through this catheter, 50 U/50 μl heparin and 50 μl of 2% NaI were simultaneously injected. Then, the cerebral embolization was produced in the left hemisphere by an injection of 50 μl [^{125}I]fibrin clot suspension (3×10^4 cpm/7.5 μg protein) containing 1% bovine serum albumin (BSA) in saline *via* the same catheter.

Measurement of Cerebral Embolus Dissolution The suspension of [^{125}I]fibrin clot particles was injected twice consecutively at an interval of 90 min through the external carotid artery of the animals. Disappearance of radioactivity in the brain following the embolus dissolution was measured with a γ-ray detector (Aloka TCS-153, Japan) which was stereotaxically placed on the head of a cerebral embolic rat. The changes in radioactivity were continuously recorded through out the experimental period. The quantity of embolus dissolution was shown as the percentage of the initial value obtained from the embolus dissolution curve.

Administration of t-PA Thirty minutes after the second embolization in SHRSPs, t-PA was administered with a bolus (1 ml/kg for 20 s) into the caudal vein. The embolus dissolution was continuously recorded until 60 min after administration. A control group was treated with the same volume of vehicle (saline containing 0.02% Tween-80). The t-PA groups were studied with three doses of 75, 250 and 750 μg/kg.

Levels of Plasminogen and Fbg in Plasma after t-PA Administration Blood samples were taken from the abdominal vena cava just after the end of the experiments on embolus dissolution. The blood samples (9 volumes) were collected in plastic tubes containing 3.8% trisodium citrate (1 volume) and centrifuged at 4 °C. Plasma was frozen and stored at −70 °C for assay. Plasminogen plasma levels were measured with the chromogenic substrate S-2251 after activation with a 2000 IU of u-PA/200 μl euglobulin fraction of plasma at 37 °C for 5 min.[16] Fbg plasma levels were determined according to the method of Quick[17] after the addition of *trans*-4-aminomethyl cyclohexane carboxylic acid (*t*-AMCHA) to the plasma samples to prevent proteolysis.

Results

Spontaneous Embolus Dissolution in WKYs and SHRSPs [^{125}I]Fibrin clot suspension was consecutively injected twice at an interval of 90 min. The radioactivity measured with a γ-ray detector placed on the heads of the animals was accumulated by consecutive injection. The initial radioactivities in WKYs and SHRSPs were 6420 ± 670 (mean $\pm$ S.E.) and 7200 ± 560 cpm at the first injection, and 10030 ± 570 and 11990 ± 1560 cpm at the second injection, respectively. These values between WKYs and SHRSPs were not significantly different. After the injection of fibrin clots to WKYs and SHRSPs, the radioactivity spontaneously decreased. As previously reported,[12] the spontaneous decrease in radioactivity is the dissolution of fibrin clots produced by an endogenous fibrinolytic system. The time course of the spontaneous embolus dissolution in WKYs and SHRSPs was shown in Table I. The spontaneous dissolution after the second embolization was much smaller than that after the first embolization in both the WKYs and SHRSPs. Furthermore, SHRSPs showed the less potent dissolution when compared with WKYs. The dissolution in WKYs after the second embolization was 21% at 30 min and 42% at 90 min. On the other hand, the dissolution in SHRSPs was 10% at 30 min and 26% at 90 min. The values in SHRSP s were significantly different from those in WKYs.

Effect of t-PA on Cerebral Embolism in SHRSPs As mentioned above, the spontaneous dissolution after the second embolization was smaller than that after the first embolization. Therefore t-PA was expected to show more effective clotlysis after the second embolization than after the first. Thirty minutes after the second embolization in SHRSPs, t-PA and the vehicle were intravenously administered with a bolus. The typical dissolution curve of cerebral emboli in a t-PA-treated animal is shown in Fig. 1. The embolus dissolution was generated soon after the administration of t-PA. The dissolution of cerebral emboli was presented as a percentage of the initial value, which was radioactivity 30 min after the second injection of fibrin clots. The magnitudes of the embolus dissolution 30 and 60 min after t-PA administration are shown in Fig. 2. The t-PA in doses of 75, 250 and 750 μg/kg showed a dose-

TABLE I. Spontaneous Dissolution of Cerebral Emboli after the Repeated Injection of [^{125}I]Fibrin Clots into the Internal Carotid Artery in WKYs and SHRSPs

Animal	Dissolution of emboli (%)					
	1st embolization			2nd embolization		
	30 min	60 min	90 min	30 min	60 min	90 min
WKY	30.6±0.7	50.6±1.2	59.9±1.4	20.5±0.3	34.6±1.1	41.9±1.0
SHRSP	19.3±1.1[a)]	36.1±2.2[a)]	44.5±2.5[a)]	10.0±1.1[a)]	20.5±1.8[a)]	26.1±1.5[a)]

Values represent the mean ± S.E. of 5 animals. *a)* $p<0.01$ (significant difference compared to the WKY group by unpaired Student's *t*-test). Initial radioactivities after the 1st and 2nd injection of [^{125}I]fibrin clots were mentioned in text.

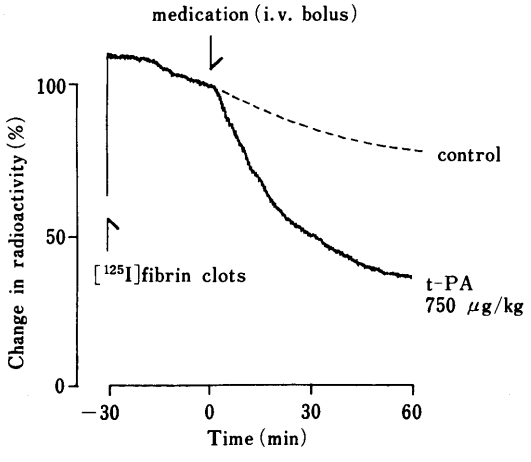

Fig. 1. Typical Dissolution Curve of Cerebral Emboli after Intravenous Administration of t-PA in SHRSPs

Decrease in radioactivity shows embolus dissolution. The radioactivity at 0 min, namely, 30 min after the second injection of fibrin clots, was 8640 cpm and represents 100%. Broken line shows the predicted dissolution curve of control.

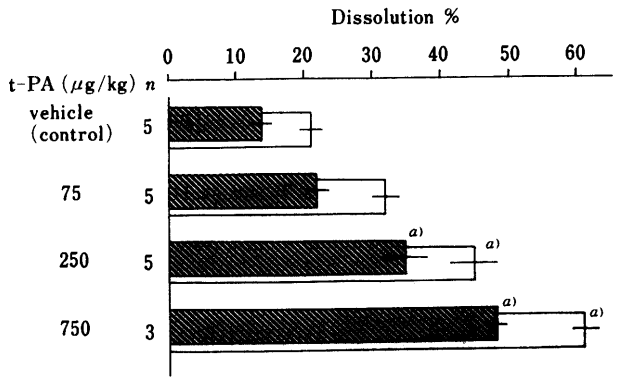

Fig. 2. Embolus Dissolution Effect of t-PA Intravenously Administered to Cerebral Embolic Model of SHRSPs

▨ embolus dissolution 30 min after administration; □ embolus dissolution 60 min after administration. Embolus dissolution (decrease in radioactivity) is represented as a percentage of initial radioactivity. Initial radioactivities (30 min after the second injection of fibrin clots) in the control, 75, 250 and 750 μg/kg t-PA groups were 9950±610, 9680±1180, 8160±880 and 9850±940 cpm, respectively. Column lengths represent the mean±S.E. *a)* $p<0.01$ (significant difference compared to the control group by Bartlett's test for comparison of variances and by Dunnett's method for evaluation).

TABLE II. Plasma Levels of Plasminogen and Fbg after Intravenous Administration of t-PA to Cerebral Embolic Model in SHRSPs

Drug	Dose	*n*	Plasma levels	
			Plasminogen (CU/ml)	Fbg (mg/dl)
Control		5	3.36±0.04	173±15
t-PA	75 μg/kg	5	3.32±0.05	161± 4
t-PA	250 μg/kg	5	3.44±0.04	155± 8
t-PA	750 μg/kg	3	3.41±0.01	150± 9

Values represent the mean±S.E.

dependent embolus dissolution. Approximately 60% of fibrin clots were dissolved 60 min after the administration of 750 μg/kg t-PA, while dissolution in the control group was less than 20%.

Systemic administration of t-PA did not cause evident bleeding from the surgical site as compared to the control rats. Plasma samples were obtained at the end of experiments. As shown in Table II, the plasminogen and Fbg levels in plasma of the groups administered with t-PA were not different from the control group levels.

Discussion

Previously we reported that fibrin clots injected to the left hemicerebrum vasculature in Wistar rats were spontaneously dissolved by an endogenous fibrinolytic system since the embolus dissolution is blocked by a plasmin inhibitor, *t*-AMCHA.[12] In the present study, the spontaneous embolus dissolution was observed in WKYs as well as SHRSPs after the injection of fibrin clots. In the WKYs, 21 and 42% of clots were dissolved 30 and 90 min after the second embolization, respectively. These values were approximately the same as those previously obtained in Wistar rats. However, the spontaneous embolus dissolution produced in SHRSPs was significantly lower than that of WKYs. These results indicate that the defensive fibrinolytic ability in SHRSPs is deteriorated and suggest that an abnormality of the fibrinolytic system may relate to the pathogenesis of stroke in SHRSPs. Coyle *et al.*[18] have reported that the sudden occlusion of the middle cerebral artery in SHRSPs invariably results in a large infarction. They suggested that genetic factors may contribute to the difference in the susceptibility to infarction between SHRSPs and Wistar rats. Tagami *et al.*[19] also showed the occurrence of arterial occlusion with monocyte adhesion and fibrin deposition in cerebral perforating arteries after symptoms of stroke in SHRSPs. As hypertension is one of the important systemic risk factors in the pathogenesis of stroke, the SHRSP is a good pathogenetic animal model for studies on stroke in humans.

Thrombolytic therapy of stroke with t-PA is potentially more attractive than that with u-PA or SK since t-PA causes clot-selective fibrinolysis through the activation of plasminogen. Although many investigators have reported the thrombolytic effects of t-PA in various animal models,[20-27] there are few studies on the direct proof of embolus dissolution with t-PA in the brain.[28] Previously we autoradiographically observed the presence of microthrombi in the hemicerebrum of Wistar rats after injection of [^{125}I]fibrin clots.[12] Although several investigators have also used radiolabelled materials to study thrombolysis,[29] the methods they used were not sufficient to quantify cerebral embolism or thrombosis. In the present study we used a γ-ray detector placed stereotaxically on the head of the cerebral embolic rat after the injection of [^{125}I]fibrin clots, and we continuously monitored the radioactivity. This method allowed for the quantitative analysis of embolus dissolution with t-PA. As mentioned above, the endogenous fibrinolytic ability of SHRSPs is less potent than that of normotensive rats. Therefore it is of worth to study the thrombolytic effect of t-PA on cerebral embolism produced in SHRSPs. In the present study, the bolus intravenous injection of t-PA showed a dose-dependent embolus dissolution in SHRSPs. In addition, embolus dissolution was observed through the experiment periods of 60 min. These results were noteworthy since the thrombolytic effect of t-PA was sustained beyound its time of clearance from circulation. The half life-time of t-PA in circulation is reportedly 1—2 min in rats.[30] A discrepancy between the effect and blood levels of t-PA has been observed in a jugular vein thrombosis model of rabbits.[31] We previously reported that t-PA is more preferably accumulated to thrombi than u-PA in thrombin-induced DIC (disseminated intravascular coagulation) rats.[32,33] The sustained thrombolytic effect of t-PA after the bolus injection is possibly due to the localization of t-PA on thrombi. The present study suggests that the bolus injection of t-PA could be effective for thrombosis therapy.

Although u-PA, SK and performed plasminogen–SK complex have been therapeutically used in clinical studies on arterial occlusion in stroke as exogenous plasminogen activators, the systemic thrombolytic states are generated because circulating plasminogen and Fbg are markedly decreased or depleted.[9] Consequently, the risk of hemorrhage is augmented secondarily by possible lysis of fibrin-stabilized hemostatic plugs which are protecting the sites of vascular disruption. Thrombolytic agents given systemically for treatment of stroke might produce intracerebral hemorrhage. However, in the present study, systemically applied t-PA produced extensive embolus dissolution

without causing systemic plasminogen activation, Fbg breakdown or bleeding in SHRSPs.

In conclusion, clot-selective t-PA may be systemically applicable for the therapy of acute stroke as a thrombolytic agent which does not induce intracerebral hemorrhagic complications.

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Smiles Rearrangement of 2-(1-Methyl-1*H*-tetrazol-5-ylthio)acetamides and Their Sulfonyl Derivatives

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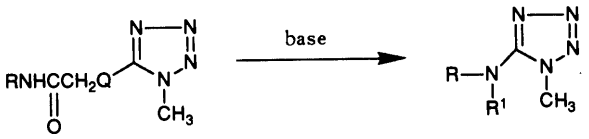
The Smiles rearrangement of 2-(1-methyl-1*H*-tetrazol-5-ylthio)acetamides and their sulfonyl derivatives occurred under basic conditions to yield 5-amino-1-methyl-1*H*-tetrazole derivatives in excellent yields.

Keywords Smiles rearrangement; 2-(1-methyl-1*H*-tetrazol-5-ylthio)acetamide; 5-amino-1-methyl-1*H*-tetrazole; 5-mercapto-1-methyl-1*H*-tetrazole

The Smiles rearrangement is generally considered to be an intramolecular nucleophilic aromatic substitution reaction resulting in the migration of an aromatic ring from one hetero atom to another. The scope of the reaction has been extended to diverse molecular systems.[1] However, little is known about Smiles rearrangement of migrating tetrazole groups.[2] This paper describes the base-promoted Smiles rearrangement of 2-(1-methyl-1*H*-tetrazol-5-ylthio)-acetamides and their sulfinyl and sulfonyl derivatives to 1-methyl-5-(*N*-substituted amino)-1*H*-tetrazoles (Chart 1).



1 : R = H, Q = S
2 : R = C_6H_5, Q = S
3 : R = C_6H_5, Q = SO
4 : R = C_6H_5, Q = SO_2
5 : R = $C_6H_5CH_2$, Q = S
6 : R = $C_6H_5CH_2CH_2$, Q = S
7 : R = A, Q = S
8 : R = A, Q = SO
9 : R = A, Q = SO_2

10 : R = R^1 = H,
11 : R = C_6H_5, R^1 = H
12 : R = C_6H_5, R^1 = $COCH_3$
13 : R = $C_6H_5CH_2$, R^1 = H
14 : R = $C_6H_5CH_2CH_2$, R^1 = H
15 : R = A, R^1 = H
16 : R = A, R^1 = $COCH_3$

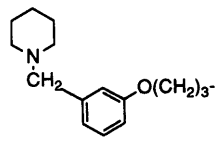
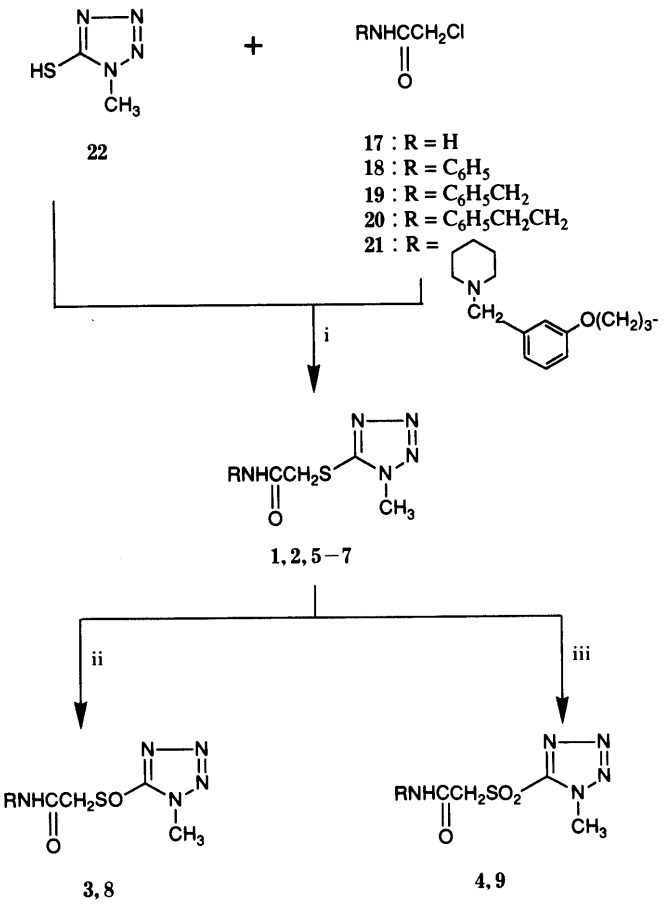
A = 

Chart 1

A series of 2-(1-methyl-1*H*-tetrazol-5-ylthio)acetamides and their sulfinyl and sulfonyl derivatives was prepared according to Chart 2. 2-(Tetrazol-5-ylthio)acetamides (**1**, **2**, **5**, **6** and **7**) were prepared in good yields by condensation of 5-mercapto-1-methyl-1*H*-tetrazole[3] (TzSH: **22**), using 1 eq of KOH in MeOH, with 2-chloroacetamides (**17**—**21**). Corresponding sulfinyl (**3** and **8**) and sulfonyl derivatives (**4** and **9**) were obtained by treating **2** and **7** with 1 eq of *m*-chloroperbenzoic acid (*m*CPBA) in CH_2Cl_2 at 20 °C or 2 eq of *m*CPBA in refluxing CH_2Cl_2. The elemental analysis and spectral data of compounds **1**—**9** are given in Tables I and III.



reagents and conditions : i, KOH, MeOH, 20°C; ii, 1 eq *m*CPBA, CH_2Cl_2, 20°C; iii, 2 eq *m*CPBA, CH_2Cl_2, reflux

Chart 2

TABLE I. 2-(1-Methyl-1*H*-tetrazol-5-ylthio)acetamides and Their Sulfinyl and Sulfonyl Derivatives

Compd.	Yield (%)	mp (°C) (Recrystn. solvent)[a]	Formula	Analysis (%) Calcd (Found)		
				C	H	N
1	88	187.0—188.0 (MeOH)	$C_4H_7N_5OS$	27.74 (27.76	4.07 3.98	40.44 40.16)
2	92	168.4—168.5 (AcOEt)	$C_{10}H_{11}N_5OS$	48.18 (47.96	4.45 4.24	28.09 27.92)
3	81	165.5—166.0 (dec.) (CH_2Cl_2–IPE)	$C_{10}H_{11}N_5O_2S$	45.27 (45.16	4.18 4.25	26.40 26.18)
4	88	126.0—126.5 (CH_2Cl_2–IPE)	$C_{10}H_{11}N_5O_3S$	42.72 (42.91	3.94 3.78	24.90 24.72)
5	86	105.5—106.0 (CH_2Cl_2–IPE)	$C_{11}H_{13}N_5OS$	50.18 (50.34	4.98 4.92	26.60 26.72)
6	87	81.5—82.5 (AcOEt–IPE)	$C_{12}H_{15}N_5OS \cdot 1/13H_2O$	51.71 (52.01	5.48 5.67	25.13 24.90)
7[b]	93	90.0—90.5 (IPE)	$C_{19}H_{28}N_6O_2S$			
8[b]	63	Oil	$C_{19}H_{28}N_6O_3S$			
9[b]	58	Oil	$C_{19}H_{28}N_6O_4S$			

a) IPE: isopropyl ether. *b*) Reference 8.

When compounds **1**, **2**, **5**, **6** and **7** were refluxed in ethanolic sodium hydroxide, corresponding rearrangement products (**10**, **11**, **13**, **14** and **15**) were obtained in excellent yields. Under mild reaction conditions such as ethanolic sodium hydroxide at room temperature and with *N*-methylpiperidine in refluxing toluene, compounds **1**, **2**, **5**, **6** and **7** remained unchanged. When the sulfinyl derivative (**3**) was treated in ethanolic sodium hydroxide at room temperature or with *N*-methylpiperidine in refluxing toluene, **3** was completely decomposed into unidentified polar materials. Two-phase reaction of **3** with $NaHCO_3$ in a mixture of H_2O and CH_2Cl_2 (1 : 1) at room temperature for 4 h gave **11** in 11% yield. Unchanged starting material **3** was recovered in 64% yield from the reaction mixture. Reaction of sulfonyl derivatives (**4** and **9**) in ethanolic sodium hydroxide at room temperature gave the corresponding rearrangement products (**11** and **15**) in 95% and 80% yields, respectively. When **4** was allowed to react with *N*-methylpiperidine in refluxing toluene for 30 min, 5-acetylamino-1-methyl-1*H*-tetrazole (**12**) was obtained in 89% yield. Reaction of **9** in refluxing toluene for 13 h gave **16** in 73% yield. The results are summarized in Table II.

The Smiles rearrangement of $RNHCOCH_2QTz$, in which Tz is the 1-methyl-1*H*-tetrazole moiety and Q represents S, SO and SO_2 functions, was shown to occur easily under basic conditions, giving two types of *N*-acetylamino and

Table II. Preparation of 5-Amino-1-methyl-1*H*-tetrazoles *via* the Smiles Rearrangement

Starting material	Method[a]	Time (min)	Rearrangement product: Compd.	mp (°C) (Recrystn. solvent)	Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	N
1	E	60	10	224 (H_2O) (lit.,[9] 223—225)	93	$C_2H_5N_5$	24.24 (24.30	5.09 5.00	70.68 70.35)
2	E	180	11	185.6—185.9 (AcOEt) (lit.,[10] 185.5—186.5)	97	$C_8H_9N_5$	54.84 (54.59	5.18 5.30	39.98 40.03)
3	C	240	11		11			[b]	
4	D	120	11		95			[b]	
4	B	30	12	Oil	89	$C_{10}H_{11}N_5O$	HRMS: 217.0963 (217.0964)		
5	E	35	13	133—134 (C_6H_6–hexane) (lit.,[10] 99)	95	$C_9H_{11}N_5$	57.12 (57.01	5.86 5.90	37.02 36.89)
6	E	40	14	153—154 (C_6H_6–hexane)	94	$C_{10}H_{13}N_5$	59.10 (59.20	6.45 6.44	34.46 34.41)
7	E	60	15	124.5 (IPE) (lit.,[11] 118—120)	97	$C_{17}H_{26}N_6O$	61.79 (61.96	7.93 8.03	25.44 25.17)
8	C	240	15		20			[b]	
9	D	120	15		80			[b]	
9	A	(13 h)	16	Oil	73	$C_{19}H_{28}N_6O_2$	HRMS: 372.2273 (372.2247)		

a) Conditions: A, without base in refluxing toluene; B, with *N*-methylpiperidine in refluxing toluene; C, with $NaHCO_3$ in a mixture of H_2O and CH_2Cl_2 (1 : 1); D, with NaOH in EtOH at room temperature; E, with NaOH in refluxing EtOH. *b*) The structure of each compound was confirmed by direct comparision with an authentic sample.

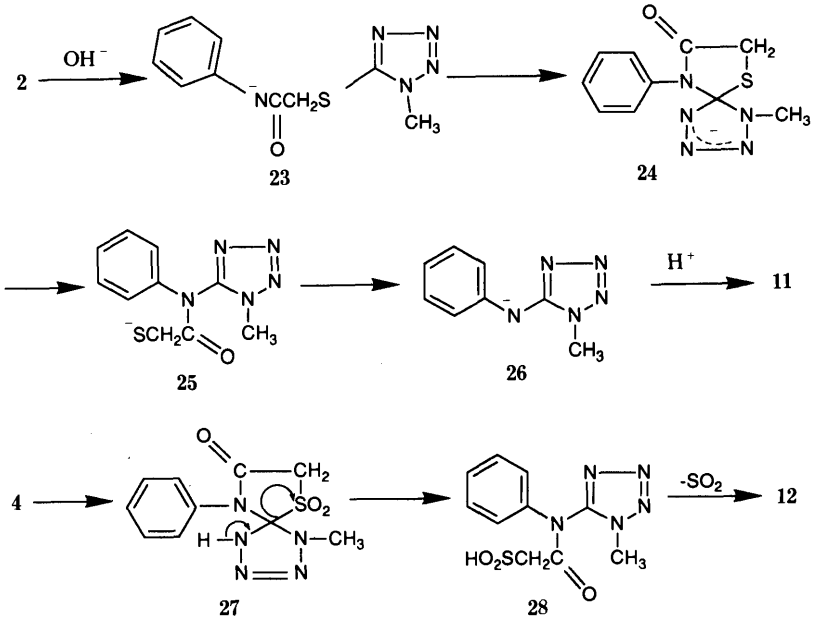


Chart 3

free amino derivatives. The ease of rearrangement was shown to depend on the leaving group in the order $SO_2 \gg SO > S$, which is the general order for base-catalyzed β-elimination of these groups in production of olefins from aliphatic sulfonyl, sulfinyl and sulfenyl compounds.[4] The rearrangement in this series may proceed through a stabilized intermediate **24**, as opposed to a transition state and an intermediate **27** (Chart 3). In the Smiles rearrangement of **2** in refluxing ethanolic sodium hydroxide the initial step of the reaction involves the removal of a proton from the $-NHCOCH_2-$ group of **2** to give an amide anion **23**, which then acts as the attacking nucleophile to give the anionic spiro-intermediate (**24**). The intermediate **24** is converted *via* **25** into an intermediate **26** and thence to the final product **11**. The rearrangement of **4** to **12** occurs through an intermediate **28** (not isolated) produced *via* the neutral spiro-intermediate **27**, followed by conversion into **12**, with loss of sulfur dioxide.

Experimental

Melting points were measured in a Gallenkamp melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Hitachi 260-10 infrared spectrophotometer and proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured on Hitachi R-90 (90 MHz) and Bruker AM 360 (360 MHz) spectrometers with tetramethylsilane as an internal standard. Chemical shifts are reported in ppm (δ) and signals are described as s (singlet), d (doublet), t (triplet), m (multiplet), q (quartet), qt (quintet) or br (broad). All spectra were consistent with the assigned structures. Mass spectra (MS) and high resolution mass spectra (HRMS) were obtained on a JMS-DX 300 spectrometer operating at an ionization potential of 70 eV. Combustion analyses were performed on a Perkin-Elmer Model 240C elemental analyzer.

2-Chloroacetamide (**17**) was commercially available. *N*-Substituted 2-chloroacetamides (**18**—**20** and **21**) were prepared by procedures described in the literature.[5-8]

Preparation of 2-(1-Methyl-1*H*-tetrazol-5-ylthio)acetamides (1, 2, 5, 6 and 7) 2-(1-Methyl-1*H*-tetrazol-5-ylthio)-*N*-phenylacetamide (**2**): A mixture of 2-chloro-*N*-phenylacetamide (**18**)[5] (1.19 g, 7 mmol), TzSH[3] (1.22 g, 10.5 mmol) and KOH (85% purity; 695 mg) in MeOH (20 ml) was stirred for 3 h at room temperature. After removal of the solvent, the residue was treated with H_2O to give a crystalline powder, which was recrystallized from AcOEt to give pure **2** (1.61 g). 2-(1-Methyl-1*H*-tetrazol-5-ylthio)acetamide (**1**), and 2-(1-methyl-1*H*-tetrazol-5-ylthio)-*N*-benzyl- and -*N*-phenethylacetamides (**5** and **6**) were prepared by a procedure similar to that used for **2**. 2-(1-Methyl-1*H*-tetrazol-5-ylthio)-*N*-[3-{3-(piperidinomethyl)phenoxy}propyl]acetamide (**7**) was prepared according to the procedure described in the literature.[8] The elemental analysis and spectral data are given in Tables I and III.

2-(1-Methyl-1*H*-tetrazol-5-ylsulfinyl)-*N*-phenylacetamide (**3**): A mixture of **2** (100 mg, 0.4 mmol) and *m*CPBA (80% purity; 86 mg, 0.4 mmol) in CH_2Cl_2 (25 ml) was stirred for 24 h at room temperature, washed with chilled saturated $NaHCO_3$ aqueous solution and brine successively, dried over $MgSO_4$ and evaporated *in vacuo* to give a crystalline powder. The powder was recrystallized from a mixed solvent of CH_2Cl_2 and isopropyl ether (IPE) to give pure **3** (86 mg).

2-(1-Methyl-1*H*-tetrazol-5-ylsulfinyl)-*N*-[3-{3-(piperidinomethyl)-phenoxy}propyl]acetamide (**8**) was prepared from **7** and 1 eq of *m*CPBA in the presence of methanesulfonic acid in CH_2Cl_2 according to the procedure described in the literature.[8] The elemental analysis and spectral data of **3** and **8** are given in Tables I and III.

2-(1-Methyl-1*H*-tetrazol-5-ylsulfonyl)-*N*-phenylacetamide (**4**): A mixture of **2** (100 mg, 0.4 mmol) and *m*CPBA (80% purity; 172 mg, 0.8 mmol) in CH_2Cl_2 (25 ml) was stirred for 3 h under reflux. Work-up by a procedure similar to that used for **3** gave pure **4** (99 mg).

2-(1-Methyl-1*H*-tetrazol-5-ylsulfonyl)-*N*-[3-{3-(piperidinomethyl)phenoxy}propyl]acetamide (**9**) was prepared from **7** and 2 eq of *m*CPBA in the presence of methanesulfonic acid in refluxing CH_2Cl_2 according to the procedure described in the literature.[8] The elemental analysis and spectral data of **4** and **9** are given in Tables I and III.

The Smiles Rearrangement of 2-(1-Methyl-1*H*-tetrazol-5-ylthio)acetamides and Their Sulfinyl and Sulfonyl Derivatives: General Procedures Method A: A solution of **9** (100 mg, 0.22 mmol) in toluene (10 ml) was refluxed for 13 h with stirring. After removal of the solvent, the residue was purified by column chromatography on silica gel with a mixture of $CHCl_3$ and MeOH (10 : 1) to give pure **16** as an oil (62 mg).

Method B: A mixture of **4** (450 mg, 1.6 mmol) and *N*-methylpiperidine (160 mg, 1.6 mmol) in toluene (50 ml) was refluxed for 30 min. After removal of the solvent, the residue was purified by column chromatography on silica gel with a mixture of $CHCl_3$ and MeOH (15 : 1) to give pure **12** as an oil (310 mg).

Method C: A solution of **3** (280 mg, 1.1 mmol) in a mixture of saturated $NaHCO_3$ aqueous solution and CH_2Cl_2 (50 ml) was vigorously stirred for 4 h. The reaction mixture was extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with H_2O, and brine successively, and dried over $MgSO_4$. After removal of the solvent, the oily residue was purified by column chromatography on silica gel with a mixture of $CHCl_3$ and MeOH (10 : 1) to give pure **11** (20 mg). Unchanged starting material **3** was recovered in 64% yield. Compound **8** was treated under conditions similar to those used for **3** to give **15**.

Method D: A solution of **4** (560 mg, 2.0 mmol) and NaOH (80 mg, 2.0 mmol) in EtOH (50 ml) was stirred at room temperature for 2 h. After removal of the solvent, the residue was dissolved in CH_2Cl_2. The CH_2Cl_2 solution was washed with H_2O and brine successively, dried over $MgSO_4$ and evaporated to give a crystalline material, which was recrystallized from AcOEt to give pure **11** (340 mg). Compound **9** was treated under conditions similar to those used for **4** to give **15**.

TABLE III. Spectral Data for 2-(1-Methyl-1*H*-tetrazol-5-ylthio)acetamides and Their Sulfinyl and Sulfonyl Derivatives

Compd.	IR ν cm^{-1} (KBr)	^{1}H-NMR δ	
		Solvent[a]	Chemical shift
1	3350 (NH), 1695 (C=O)	D	3.96 (3H, s), 4.03 (2H, s), 7.11—7.37 (1H, br), 7.46—7.72 (1H, br)
2	3290 (NH), 1675 (C=O)	C	3.96 (3H, s), 4.05 (2H, s), 6.98—7.59 (5H, m), 9.14—9.42 (1H, br)
3	3320 (NH), 1675 (C=O), 1050 (SO)	C	4.27 (3H, s), 4.57 (2H, dd, J=14 Hz), 6.98—7.58 (6H, m, Ar-H and NH)
4	3350 (NH), 1670 (C=O), 1350 and 1145 (SO_2)	D	4.34 (3H, s), 4.73 (2H, s), 7.13—7.56 (5H, m), 10.15—10.20 (1H, br)
5	3240 (NH), 1650 (C=O)	D	3.90 (3H, s), 3.97 (2H, s), 4.40 (2H, d, J=6 Hz), 7.23 (5H, m), 7.26—7.58 (1H, br)
6	3290 (NH), 1670 (C=O)	D	2.80 (2H, t, J=7 Hz), 3.54 (2H, q, J=7 Hz), 3.65 (1H, br), 3.86 (2H, s), 3.90 (3H, s), 7.10—7.30 (5H, m)
7	3300 (NH), 1640 (C=O)	C	1.39—1.47 (2H, m), 1.53—1.62 (4H, m), 1.98 (2H, qt, J=6.0 Hz), 2.33—2.41 (4H, m), 3.43 (2H, s), 3.47 (2H, q, J=6.0, 6.5 Hz), 3.90 (3H, s), 3.93 (2H, s), 3.98 (2H, t, J=6.0 Hz), 6.71—7.23 (5H, m)
8	1660 (C=O)	C	1.38—1.74 (6H, m), 1.74—2.13 (2H, m), 2.23—2.57 (4H, m), 3.27—3.73 (2H, m), 3.48 (2H, s), 3.99 (2H, t, J=6.0 Hz), 4.26 (3H, s), 4.37 (2H, dd, J=4.0 Hz), 6.62—7.53 (5H, m)
9	1690 (C=O), 1335 and 1260 (SO_2)	C	1.40—1.78 (6H, m), 2.01—2.27 (2H, m), 2.31—2.65 (4H, m), 3.38—3.67 (4H, m), 3.97 (3H, s), 4.05 (2H, t, J=6.0 Hz), 4.32 (2H, s), 6.63—7.58 (5H, m)

a) C, $CDCl_3$; D, dimethyl sulfoxide-d_6 (DMSO-d_6).

TABLE IV. Spectral Data for 5-Amino-1-methyl-1*H*-tetrazoles

Compd.		IR ν cm^{-1}	^{1}H-NMR δ	
			Solvent[a]	Chemical shift
10[b]	KBr	3320 (NH), 1665 (C=N)	D	3.71 (3H, s), 8.28 (1H, s), 8.29 (1H, s)
11[b]	KBr	3300 (NH), 1620 (C=N)	C	3.88 (3H, s), 6.20—6.38 (1H, br), 7.26—7.44 (5H, m)
12[b]	$CHCl_3$	1695 (C=O)	C	2.14 (3H, s), 3.93 (3H, s), 7.43 (5H, s)
13[b]	KBr	3350 (NH), 1620 (C=N)	C	3.74 (3H, s), 4.60 (3H, s), 7.35 (5H, s)
14[b]	KBr	3290 (NH), 1610 (C=N)	C	2.99 (2H, t, J=6.5 Hz), 3.65 (3H, s), 3.74 (2H, q, J=6.5 Hz), 3.90—4.09 (1H, br), 7.26 (5H, s)
15[c]	KBr	3280 (NH), 1630 (C=N)	C	1.42—1.46 (2H, m), 1.53—1.59 (4H, m), 2.16 (2H, qt, J=6 Hz), 2.31—2.41 (4H, m), 3.42 (2H, s), 3.67 (2H, q, J=6 Hz), 3.73 (3H, s), 4.09 (2H, t, J=6 Hz), 5.47 (1H, t, J=6 Hz), 6.70—7.20 (4H, m)
16[c]	Neat	1690 (C=O)	C	1.51—1.55 (2H, m), 1.62—1.69 (4H, m), 2.21 (2H, m), 2.41—2.49 (7H, m), 3.52 (2H, s), 3.92—4.03 (5H, br), 4.10 (2H, t, J=6 Hz), 6.79—7.31 (4H, m)

a) C, $CDCl_3$; D, DMSO-d_6. *b*) 90 MHz. *c*) 360 MHz.

Method E: A mixture of **1** (173 mg, 1 mmol) and NaOH (40 mg) in EtOH (10 ml) was stirred for 1 h under reflux. After removal of the solvent, the crude residue was washed with H_2O and recrystallized from H_2O to give pure **10** (92 mg). Compounds **2**, **5**, **6** and **7** were treated under conditions similar to those used for **1** to give corresponding rearrangement products (**11**, **13**, **14** and **15**).

The results and spectral data are given in Tables II and IV.

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Reaction of 19-Hydroxysteroids with Diethylaminosulfur Trifluoride

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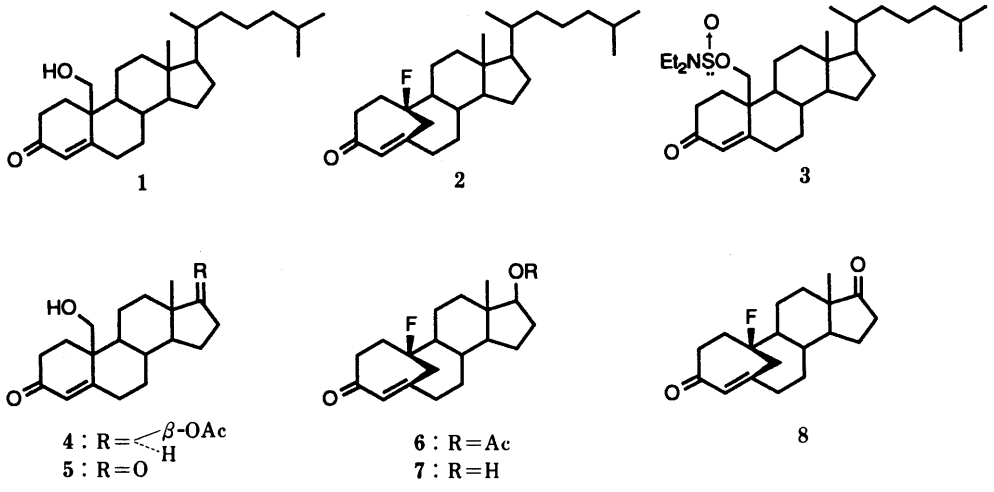
Reaction of 19-hydroxycholest-4-en-3-one (1) with diethylaminosulfur trifluoride (DAST) in acetonitrile at reflux gave 10β-fluoro-5,10-seco-5,19-cyclocholest-4-en-3-one (2) in 60% yield. Analogous reactions of androstane derivatives afforded the corresponding bicyclo[4.4.1]compounds 6 and 8. Under milder reaction conditions, the diethylaminosulfinate 3 was secured in 20% yield from 1.

Keywords 19-hydroxycholest-4-en-3-one; diethylaminosulfur trifluoride (DAST); bicyclo[4.4.1]system; 10β-fluoro-5,10-seco-5,19-cyclocholest-4-en-3-one; 10β-fluoro-5,10-seco-5,19-cycloandrost-4-ene-3,17-dione

In order to study the cholesterol structure–biological function relationship, we require 19-fluorinated cholesterol analogs. Previously 19,19-difluorocholesterol was prepared *via* reaction of a 19-oxocholesterol derivative with diethylaminosulfur trifluoride (DAST).[1] However, similar reaction of 19-hydroxycholesteryl acetate with DAST was reported[2] to give a complex mixture including 7-fluoro-B-homo-19-norcholest-5(10)-en-3β-ol acetate. The present paper deals with an investigation of the reactions of steroids bearing the 3-oxo-4-ene system in the A ring, such as 19-hydroxycholest-4-en-3-one (**1**),[3] 17β,19-dihydroxyandrost-4-en-3-one 17-yl acetate (**4**),[3] and 19-hydroxyandrost-4-ene-3,17-dione (**5**),[4] with DAST. Structural determination revealed that major products of the reactions contain the 1-fluoro-bicyclo[4.4.1]undec-5-en-4-one system.

The hydroxyenone (**1**) was treated with excess DAST in actonitrile at reflux, and column chromatography of the products gave a fluorine-containing compound (tentatively named X) in 60% yield. A similar yield of X was also obtained by reaction in CH_2Cl_2–CF_3Cl (1 : 1) at ambient temperature. On the other hand, treatment of **1** with DAST in CH_2Cl_2 at −15 °C for 15 min afforded the diethylaminosulfinate (**3**) in 20% yield, together with compound X (2%) and recovered **1** (20%). Compound X showed *m/z* 402 (M^+), indicating that the hydroxyl group of the starting compound **1** was substituted with fluorine. However, its proton nuclear magnetic resonance (^{1}H-NMR) exhibited no signal around 4—5 ppm due to $-CH_2F$. Thus, the fluorine should be located at a quaternary carbon atom, and this was supported by the ^{13}C-NMR signal (Table I) at 97.2 ppm (J_{C-F} 183 Hz). That the 3-oxo-4-ene system, the C, D-ring and side chain portions had remained intact during the reaction was evident from the ultraviolet (UV), λ_{max} 242 nm (ε 13000), and ^{1}H- and ^{13}C-NMR spectra (Experimental). The unassigned ^{13}C-NMR signals at this stage were one quaternary carbon bearing fluorine, two methines and five methylenes, corresponding to C-1, 2, 6, 7, 8, 9, 10, and 19. Taking together all of these data and the published observations concerning analogous reactions,[5] the structure **2** seemed possible for compound X. Final proof of the structure **2** was obtained by X-ray crystallography (Experimental). The ORTEP drawing is shown in Fig. 1.

The sulfinate **3** exhibited no molecular ion M^+ (519) in the electron impact mass spectrum (EI-MS), but *m/z* 520 ($M^+ + H$) was clearly observed in the fast atom bombardment (FAB)-MS. The presence of the diethylamino group in **3** was indicated by the triplet signal at 1.10 ppm and the multiplet around 3.0—3.2 ppm in the ^{1}H-NMR. These spectroscopic data, as well as the acid-lability of the compound, regenerating the starting alcohol **1**, strongly suggested the structure **3** for the sulfinate. That the C-19 methylene signal appeared at 3.8—4.3 ppm as a quartet of doublets and that the ^{13}C-NMR signals of C-1, 2, 4, 5, 10, and 11 appeared as twin peaks (Table I) indicated that **3** was a pair of *S*-atom isomers, as observed with the piperidine-sulfinate which was obtained on reaction of an 11β-hydroxy steroid with piperidinosulfur trifluoride.[6] The sulfinate **3** can be considered to be formed hydrolytically



1 2 3

4 : R = β-OAc / H
5 : R = O

6 : R = Ac
7 : R = H

8

Chart 1

TABLE I. Selected ^{13}C-NMR Chemical Shifts (ppm) of **1**,**2**,**3**,**7** and **8**

Position	1	3	2	7	8
C- 1	35.0	34.7, 34.8	37.0[a]	37.1 (27.5)[b]	37.0 (25.7)
C- 2	33.6	33.5, 33.7	40.1[c]	40.1	40.1
C- 3	200.3	199.6	203.6	203.5	203.3
C- 4	126.7	126.3, 126.7	126.8	127.0	127.2
C- 5	167.4	166.0, 166.5	157.6 (16.5)	157.2 (18.3)	156.6 (18.3)
C- 6	33.4	33.2	39.8	39.7	39.5
C- 7	32.2	32.1	37.0	35.9	35.8
C- 8	36.3	36.1, 36.2	38.4	38.3	37.6
C- 9	54.0	54.1	54.1 (20.2)	54.5 (20.2)	54.5 (20.2)
C-10	43.9	42.6, 42.7	97.2 (183.3)	97.1 (181.5)	96.9 (183.3)
C-11	21.6	21.5, 21.6	21.6 (18.3)	21.3 (18.3)	20.9 (18.3)
C-12	39.4	39.9	39.0	36.5	35.8
C-13	42.4	42.4	41.9	42.2	47.2
C-14	56.0	56.0	56.1	50.6	51.0
C-15	24.0	24.0	26.1	25.0	23.2
C-16	27.9	28.1	27.6	29.9	35.5
C-17	56.2	56.0	56.1	81.6	218.6
C-18	12.0	12.0	11.5	10.6	13.3
C-19	65.9	66.2	41.0 (29.3)	41.0 (29.3)	41.0 (31.2)

a) J_{C-F} was not estimated exactly due to overlapping with the C-7 signal. *b*) The values in parentheses are J_{C-F} in Hz. *c*) Vicinal couplings between F and C-2/C-8 were not observed, probably due to their torsional angles being approximately 90°. See H.-J. Schneider, W. Gschwendtner, D. Heiske, V. Hoppen and F. Thomas, *Tetrahedron*, **33**, 1769 (1977).

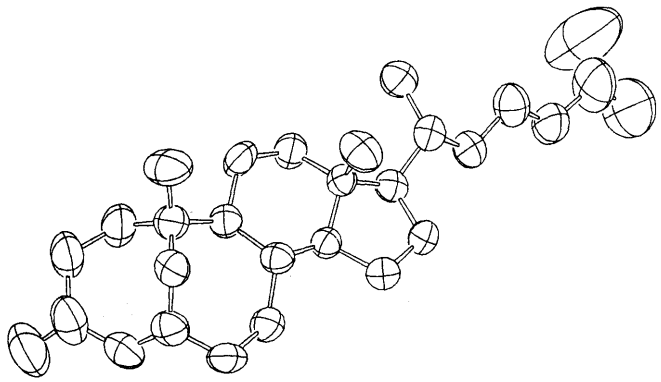


Fig. 1. ORTEP Drawing of **2**

from the postulated[7] early reaction intermediate, RO–SF_2–NEt_2.

With the unique fluorinated bicyclo[4.4.1]undecane structure **2** determined, we anticipated that the corresponding androstane analogs such as **7** and **8** might exhibit interesting biological activity *e.g.* aromatase inhibition and/or 5-reductase inhibition.[8] Thus, 17β,19-dihydroxyandrost-4-en-3-one 17-yl acetate (**4**)[3] and 19-hydroxyandrost-4-ene-3,17-dione (**5**)[4] were prepared, and subjected to reaction with DAST under the same conditions as described above. Compounds **6** and **8** having spectral data consistent with the bicyclo[4.4.1]undecane skeleton, were obtained in 26% and 16% yields, respectively. Saponification of **6** gave the 17β- ol **7**. Oxidation of **7** with pyridinium chlorochromate was found to be an alternative method for preparation of **8**. This compound **8** appeared to be identical with a product obtained by reaction of **4** with diethyl [2-chloro-1,1,2-trifluoroethyl]-amine, but no definite evidence for the proposed structure[5a,b] was presented.

Experimental

10β-Fluoro-5,10-seco-5,19-cyclocholest-4-en-3-one (2) (a) DAST (50 μl) was added at ambient temperature to a stirred solution of 19-hydroxycholest-4-en-3-one (**1**, 48 mg) in a mixture of dry CH_2Cl_2 (1 ml) and CF_3Cl (1 ml). The solution was stirred for 10 min, poured into saturated $NaHCO_3$ solution and extracted with CH_2Cl_2. The organic layer was washed with saturated $NaHCO_3$ solution and water. After the solution had been dried ($MgSO_4$) and the solvent removed, the residue was chromatographed on silica gel with a mixture of *n*-hexane–ethyl acetate (50 : 1) as an eluant to give **2** (30 mg), mp 128—129 °C (from *n*-hexane). MS *m/z* 402 (M^+): 289, 247, 125. UV λ_{max}: 242 nm (ε, 13000). ^{1}H-NMR δ: 0.71 (3H, s, 13-Me), 0.86 (6H, d, J=6.5 Hz, 25-Me_2), 0.92 (3H, d, J=6.5 Hz, 20-Me), 5.8 ppm (1H, s, 4-H). ^{13}C-NMR (Table I). (b) DAST (1.4 ml) was added to a refluxing solution of **1** (500 mg) in CH_3CN (25 ml). The solution was refluxed for 0.5 h under Ar, poured into saturated $NaHCO_3$ solution and extracted with *n*-hexane. The organic extract was chromatographed as described above to give **2** (270 mg).

19-Hydroxycholest-4-en-3-one Diethylaminosulfinate (3) DAST (0.2 ml) was added to a solution of **1** (210 mg) in CH_2Cl_2 (5 ml) at −15 °C. The solution was stirred at this temperature for 15 min, poured into ice/saturated $NaHCO_3$ solution and extracted with CH_2Cl_2. The organic layer was washed with saturated $NaHCO_3$ solution and water, dried over $MgSO_4$ and evaporated to dryness. The residue was chromatographed on silica gel. The fluoride (**2**, 5 mg) was obtained from the eluate with benzene–ethyl acetate (10 : 1). Further elution with benzene–ethyl acetate (5 : 1), and then with benzene–ethyl acetate (3 : 1) gave the sulfinate (**3**, 55 mg), and recovered **1** (42 mg), respectively. **3**: oil, FAB-MS *m/z*: 520 (M^+ + H), 401, 383 (base peak), 369. EI-MS *m/z*: 448, 398, 369, 120 (base peak). ^{1}H-NMR δ: 0.70 (3H, s, 13-Me), 0.86 (6H, d, J=6.5 Hz, 25-Me_2), 0.89 (3H, d, J=6.5 Hz, 20-Me), 1.10 (6H, t, J=7.5 Hz, $(CH_3CH_2)_2N$–), 3.0—3.2 (4H, m, $(CH_3CH_2)_2N$–), 3.88, 3.97, 4.08, 4.25 (2H, q of AB d, J=10 Hz, 19-H_2), 5.88 (1H, s, 4-H). ^{13}C-NMR (Table I).

X-Ray Crystallography of 2 Crystal data for **2** ($C_{27}H_{43}FO$, M=402): monoclinic; space group $P2_1$; unit cell a=17.018(4) Å, b=6.008(2) Å, c=11.933(2) Å, β=93.15(2); V=1218.3 Å^3; Z=2; Dc=1.10 g/cm^3; μ(Cu K_α)=5.4 cm^{-1}. Intensities were measured on a Rigaku AFC-5 diffractometer by using graphite-monochromated CuK_α radiation (λ=1.5418 Å). Of 2286 independent reflections with $0° < 2\theta < 130°$, 1475 with $F > 3\sigma(F)$ were used for structure determination. The structure was determined by direct methods (MULTAN 78) and successive Fourier syntheses, and refined by using the block-diagonal least-squares method with anisotropic temperature factors. The final *R* factor, based on the reflections used, was 0.080.

Acidic Treatment of 3 The sulfinate (**3**, 26 mg) was allowed to stand in a mixture of concentrated HCl (50 μl), acetone (4 ml) and water (0.2 ml) at ambient temperature for 0.5 h. Extraction with CH_2Cl_2, washing with water, drying ($MgSO_4$) and solvent evaporation gave **1** (15 mg).

10β-Fluoro-5,10-seco-5,19-cyclo-17β-acetoxyandrost-4-en-3-one (6) DAST (320 μl) was added to a solution of 17β,19-dihydroxyandrost-4-en-3-one 17-acetate (**4**, 100 mg)[3] in dry benzene (6 ml). The solution was stirred at ambient temperature for 17 h, and worked up as described above. Column chromatography on silica gel with *n*-hexane–ethyl acetate (15 : 1) gave **6** (32 mg). Alternatively, **6** was obtained in 26% yield by refluxing a solution of **4** (43 mg) in CH_3CN (3 ml) containing DAST (160 μl) for 15 min. **6**: mp 139—141 °C (from *n*-hexane–CH_2Cl_2,). MS *m/z*: 348 (M^+, base peak). ^{1}H-NMR δ: 0.84 (3H, s, 13-Me), 2.05 (3H, s, acetyl), 4.59 (1H, t, J=7.1 Hz, 17-H), 5.79 ppm (1H, s, 4-H).

10β-Fluoro-5,10-seco-5,19-cycloandrost-4-en-17β-ol-3-one (7) A solution of **6** (28 mg) in 5% KOH–methanol (7 ml) was stirred at ambient temperature for 10 min, extracted with ethyl acetate. The extract was washed with water, and dried over $MgSO_4$. The solvent was evaporated off and the residue was chromatographed on silica gel with *n*-hexane–ethyl acetate (5 : 1) to give **7** (17 mg): mp 142—145 °C (from *n*-hexane–CH_2Cl_2). ^{1}H-NMR δ: 0.78 (3H, s, 13-Me), 3.62 (1H, t, J=8.1 Hz, 17-H), 5.77 ppm (1H, s, 4-H). ^{13}C-NMR (Table I).

10β-Fluoro-5,10-seco-5,19-cycloandrost-4-ene-3,17-dione (8) (a) DAST (160 μl) was added to a solution of 19-hydroxyandrost-4-ene-3,17-dione (**5**, 56 mg)[4] in CH_3CN (3 ml). The solution was refluxed for 20 min, poured into saturated $NaHCO_3$ solution and extracted with *n*-hexane. The organic layer was washed with brine, dried over $MgSO_4$ and evaporated to dryness. The residue was chromatographed on silica gel with *n*-hexane–ethyl acetate (5 : 1) to give **8** (9 mg): mp 181—183 °C (from *n*-hexane–CH_2Cl_2, lit.[5a] mp 179—181 °C). ^{1}H-NMR δ: 0.92 (3H, s, 13-Me), 5.82 ppm (1H, s, 4-H). ^{13}C-NMR (Table I). (b) A solution of the 17β-ol (**7**, 11 mg) in CH_2Cl_2 (0.5 ml) was added to a stirred suspension of pyridinium chlorochromate (20 mg) in CH_2Cl_2 (1 ml). The mixture was stirred at ambient temperature for 1.6 h and filtered through a Florisil column with CH_2Cl_2–ethyl acetate

(5 : 1). The solvent was evaporated off and the residue was chromatographed on silica gel with *n*-hexane–ethyl acetate (5 : 1) to give **8** (7 mg).

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Asymmetric Syntheses of 1-Alkyltetrahydro-β-carbolines and a 9-Thio Analogue

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Tetrahydro-β-carbolines, (*S*)-tetrahydroharman (8a) and (*S*)-1-phenyltetrahydro-β-carboline (8b), were asymmetrically synthesized starting from (*R*)-phenylglycinol and 1-benzyl-3-(2-bromoethyl)indole (1). Asymmetric synthesis of the 9-thio analogue (15) of 8a was also achieved.

Keywords tetrahydro-β-carboline; (*S*)-tetrahydroharman; (*S*)-aryltetrahydro-β-carboline; tetrahydrobenzo[4,5]thieno[2,3-*c*]pyridine; phenylglycinol; asymmetric synthesis

Tetrahydro-β-carboline alkaloids are widely distributed among plants, and have interesting biological activities. The development of a convenient method for the asymmetric synthesis of 1-alkyltetrahydro-β-carbolines (IV) (Chart 1) is very important in connection with the asymmetric synthesis of tetrahydro-β-carboline alkaloids. Several reports on their highly stereoselective synthesis have recently appeared.[1)]

We have previously succeeded in the synthesis of enantiomerically pure 1-alkyltetrahydroisoquinolines (III) *via* the stereoselective alkylation of chiral oxazolo[2,3-*a*]tetrahydroisoquinolines (II) with Grignard reagents (Chart 1).[2)] This methodology seemed to be applicable to the asymmetric synthesis of IV. We therefore selected (*S*)-tetrahydroharman (**8a**) and (*S*)-1-phenyltetrahydro-β-carboline (**8b**) (Chart 2), simple indole alkaloids, as typical examples of IV and investigated their asymmetric syntheses. From the viewpoint of medicinal chemistry, thio analogues of tetrahydro-β-carboline alkaloids with biological activity are interesting target structures. Consequently, asymmetric synthesis of the 9-thio analogue (**15**) of tetrahydroharman (**8a**) was also attempted. This paper describes convenient asymmetric syntheses of **8a**, **8b** and **15**.

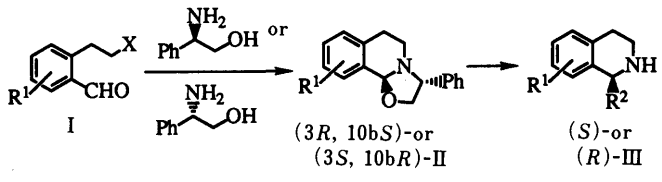

Chart 1

Based on the previous findings, asymmetric synthesis of **8a** was achieved as shown in Chart 2. The formyl intermediate (**2**) was easily prepared by the Vilsmeier–Haack reaction of **1** in 50% yield. Treatment of **2** with (*R*)-phenylglycinol at room temperature followed by azeotropic distillation with benzene gave the crude iminium salt (**3**), which was then cyclized to the chiral **4** with 85% de by treatment with Et_3N at −5 °C. The crude **4** accompanied with its diastereomer, on recrystallization from EtOH, afforded diastereomerically pure **4**, mp 117—118 °C, $[\alpha]_D^{25}$ −73° (c=0.4, $CHCl_3$), in 62% yield. The diastereomeric purity of **4** was confirmed by 500 MHz nuclear magnetic

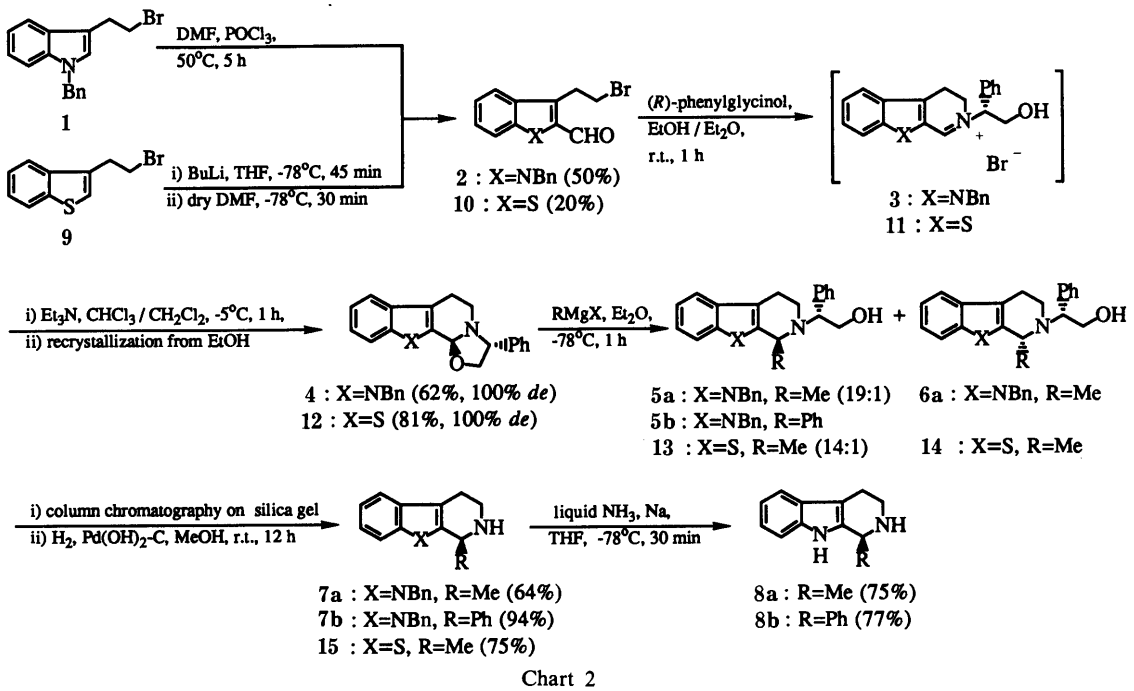

Chart 2

resonance (NMR) spectroscopy. The diastereomerically pure intermediate **4** was then asymmetrically methylated by reaction with MeMgI in Et_2O at −78 °C. The reaction gave a 19:1 (90% de) mixture of (*S*)- and (*R*)-1-methylated derivatives **5a** and **6a**. The major diastereomer **5a** was separated from **6a** by column chromatography on silica gel. Hydrogenolysis of **5a** on $Pd(OH)_2$–carbon gave **7a**. The benzyl group of the indole ring in **7a** was removed by treatment with sodium in liquid ammonia, affording enantiomerically pure **8a**.[3] The configuration of **8a** was determined to be *S* by comparison of its optical rotation, $[\alpha]_D^{25}$ −51.1° (*c*=0.5, EtOH), with the literature value, $[\alpha]_D^{25}$ −52° (*c*=2.0, EtOH).[3]

This methodology was applied to the asymmetric synthesis of (*S*)-1-aryltetrahydro-β-carboline (**8b**). Phenylmagnesium bromide in Et_2O was used to phenylate **4** at −78 °C. The thin layer chromatographic (TLC) and high performance liquid chromatographic (HPLC) analyses of the resulting compound (**5b**) showed that only one diastereomer was produced. The structure of **5b** was identified based on the great similarity of the NMR spectral pattern not to that of **6a** but to that of **5a**. Compound **5b** was also converted to enantiomerically pure **8b**, mp 195—196 °C, $[\alpha]_D^{21}$ −4.0° (*c*=0.5, $CHCl_3$).

The thio-analogue **15** was also synthesized by applying a synthetic approach similar to that used for **8a** (Chart 2). The synthesis of the key formyl intermediate **10** was first attempted through the Vilsmeier–Haack formylation of **9**. However, the reaction did not occur, and the starting material was recovered. Meanwhile, lithiation of **9** with BuLi followed by treatment of dimethylformamide (DMF) gave the desired **10** in 20% yield. Compound **10** was then converted to the diastereomerically pure **12**, mp 137—139 °C, $[\alpha]_D^{24}$ −112° (*c*=0.2, $CHCl_3$) in 81% yield by reaction with (*R*)-phenylglycinol followed by treatment with Et_3N. The reaction of **12** with MeMgI at −78 °C gave a 14:1 (87% de) diastereomeric mixture of 1-methylated derivatives (**13** and **14**). The major diastereomer, separated by column chromatography, could be assigned as the (*S*)-1-methyl derivative (**13**) based on the close similarities of the TLC, HPLC, and NMR spectral characteristics to those of the corresponding (*S*)-1-methylcarboline derivative (**5**). Hydrogenolysis of **13** on $Pd(OH)_2$–carbon gave enantiomerically pure **15**, $[\alpha]_D^{23}$ −37° (*c*=0.1, $CHCl_3$) in 75% yield.

The synthetic strategies shown in Chart 2 should provide general and useful methods for asymmetric syntheses of 1-alkyltetrahydro-β-carbolines and their 9-thio analogues.

Experimental

Melting points are uncorrected. Infrared (IR) spectra were recorded on a JASCO A-102 spectrometer. Mass spectra (MS) were recorded on a Shimadzu LKB 9000 spectrometer and fast atom bombardment mass spectra (FAB-MS) were recorded on a VG-70SE spectrometer. ^{1}H-NMR spectra were run on a Hitachi R-24 (60 MHz) spectrometer or on a Varian VXR-500 (500 MHz) spectrometer. Optical rotations were measured on a JASCO DIP-4 spectrometer. Analytic HPLC was performed with a Shimadzu SPD-6A instrument on a chiral phase column, Chiralcel OD (Daisel) or a silica gel column, Chemcosorb 5Si-U (Chemco). Preparative HPLC was performed with a Waters 510 instrument on a silica gel column, μ-Porasil (RCM Model, Waters). Merck silica gel 60 (230—400 mesh) and Wako activated alumina (300 mesh) were employed for column chromatography. Extracts were dried over anhydrous $MgSO_4$.

1-Benzyl-3-(2-bromoethyl)indole (1) PBr_3 (12.5 ml, 340 mmol) was added dropwise to a solution of 1-benzyl-3-(2-hydroxyethyl)indole (30 g, 120 mmol) in Et_2O (300 ml) at 0 °C. The reaction mixture was stirred at room temperature for 3 h, quenched with 10% $NaHCO_3$ solution, and extracted with Et_2O. The Et_2O layer was washed with saturated NaCl solution and dried. The residue was purified by column chromatography on silica gel (hexane:AcOEt=10:1) to give 32 g (84%) of **1** as a viscous oil. *Anal.* Calcd for $C_{17}H_{16}BrN$: C, 64.98; H, 5.13; N, 4.46. Found: C, 65.12; H, 5.23; N, 4.62. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 3.05—3.80 (4H, m), 5.19 (2H, s), 6.95 (1H, s), 6.82—7.40 (3H, m), 7.40—7.70 (1H, m). EI-MS *m/z*: 315 (M^++2), 313 (M^+).

1-Benzyl-3-(2-bromoethyl)-2-formylindole (2) Compound **1** (30 g, 96 mmol) dissolved in dry DMF (200 ml) was added under cooling with ice-water to a mixture of dry DMF (60 ml, 77 mmol) and $POCl_3$ (36 ml). The reaction mixture was then stirred for 5 h at 50 °C and quenched with 10% $NaHCO_3$ solution. The reaction mixture was extracted with Et_2O and, the Et_2O layer was washed with saturated NaCl solution and dried. Evaporation of the solvent gave an oily mass which was purified by column chromatography on silica gel (hexane:AcOEt=5:1) followed by recrystallization from Et_2O to give 16 g (49%) of **2**, mp 95—97 °C. *Anal.* Calcd for $C_{18}H_{16}BrNO$: C, 63.23; H, 4.71; N, 4.09. Found: C, 63.17; H, 4.72; N, 4.12. IR (Nujol): 1655 cm^{-1}. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 3.30—3.93 (4H, m), 5.76 (2H, s), 6.81—7.50 (8H, m), 7.72 (1H, dd, *J*=7 and 2 Hz), 10.02 (1H, s).

(3*R*,11b*S*)-11-Benzyl-3-phenyl-2,3,5,6-tetrahydro-11b*H*-oxazolo-[3′,2′:1,2]pyrido[3,4-*b*]indole (4) A mixture of (*R*)-phenylglycinol (4 g, 29 mmol) and **2** (10 g, 29 mmol) was stirred in a mixture of dry tetrahydrofuran (THF) (20 ml) and dry Et_2O (200 ml) at room temperature for 0.5 h. The solvent was then evaporated off under reduced pressure. Azeotropic distillation was done with dry benzene (3×100 ml) at 80 °C under reduced pressure to give crude **3** as a solid.

Et_3N (8.14 ml, 58 mmol) was added dropwise at 0 °C to a solution of the crude **3** in dry $CHCl_3$ (100 ml). The mixture was then stirred at 0 °C for 2 h, washed with H_2O, and concentrated. Crystallization of the residue from EtOH gave 2.5 g of optically pure **4** (62%), mp 117—118 °C. The diastereoisomeric purity was confirmed by examination of the 500 MHz NMR spectrum. *Anal.* Calcd for $C_{26}H_{24}N_2O$; C, 82.07; H, 6.36; N, 7.36. Found: C, 82.27; H, 6.61; N, 7.55. ^{1}H-NMR (60 MHz, acetone-d_6) δ: 2.73—3.00 (2H, m), 3.04—3.30 (2H, m), 3.78 (1H, t, *J*=2 Hz), 4.28—4.43 (2H, m), 5.49 (2H, s), 5.72 (1H, s), 6.98—7.78 (14H, m). FAB-MS (positive ion mode) *m/z*: 381 [$(M+1)^+$]. $[\alpha]_D^{25}$ −73.0° (*c*=0.4, $CHCl_3$).

(1*S*,1′*R*)-9-Benzyl-2-(2-hydroxy-1-phenylethyl)-1-methyl-1,2,3,4-tetrahydropyrido[3,4-*b*]indole (5a) A solution of **4** (1.5 g, 4.0 mmol) in dry Et_2O (100 ml) was added dropwise at −78 °C to a solution of MeMgI (15.8 mmol) in dry Et_2O (50 ml). The mixture was stirred at −78 °C for 3 h, then the reaction was quenched with NH_4Cl, and the mixture was extracted with Et_2O. The Et_2O layer was washed with H_2O, dried, and concentrated to give a 19:1 (90% de) mixture of **5a** and (1*R*,1′*R*)-9-benzyl-2-(2-hydroxy-1-phenylethyl)-1-methyl-1,2,3,4-tetrahydropyrido[3,4-*b*]-indole (**6a**). The mixture was separated by preparative HPLC to give 1.02 g (72%) of **5a** and 120 mg (8%) of **6a**, each as an amorphous powder.

5a: *Anal.* Calcd for $C_{27}H_{28}N_2O$: C, 81.78; H, 7.12; N, 7.06. Found: C, 82.01; H, 7.35; N, 7.14. IR (neat): 3450 cm^{-1}. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 1.25 (3H, d, *J*=6 Hz), 2.43—3.55 (5H, m), 3.62—4.02 (3H, m), 4.94 (2H, s), 6.60—6.95 (1H, m), 6.95—7.32 (7H, m), 7.15 (5H, s), 7.35—7.70 (1H, m). FAB-MS (positive ion mode) *m/z*: 397 [$(M+1)^+$]. $[\alpha]_D^{25}$ −52° (*c*=0.7, $CHCl_3$).

6a: *Anal.* Calcd for $C_{27}H_{28}N_2O$: C, 81.78; H, 7.12; N, 7.07. Found: C, 81.95; H, 7.28; N, 7.30. IR (neat): 3410 cm^{-1}. ^{1}N-NMR (60 MHz, $CDCl_3$) δ: 1.29 (3H, d, *J*=7 Hz), 2.12 (1H, s), 2.35—2.75 (2H, m), 2.75—3.24 (2H, m), 3.34—3.72 (3H, m), 4.10 (1H, q, *J*=7 Hz), 5.16 (2H, dd, *J*=4, 3 Hz), 6.74—7.49 (9H, m), 7.19 (5H, s). FAB-MS (positive ion mode) *m/z*: 397 [$(M+1)^+$]. $[\alpha]_D^{25}$ −103.5° (*c*=0.2, $CHCl_3$).

Similarly, (1*S*,1′*R*)-9-benzyl-2-(2-hydroxy-1-phenylethyl)-1-phenyl-1,2,-3,4-tetrahydropyrido[3,4-*b*]indole (**5b**) was prepared from **4** and phenylmagnesium bromide. The crude product was purified by flash chromatography on alumina (AcOEt:hexane=1:4) to give **5b** in 77% yield (100% de), mp 155—156 °C (from EtOH). The diastereoisomeric purity was confirmed by TLC and HPLC analyses. *Anal.* Calcd for $C_{32}H_{30}N_2O$: C, 83.81; H, 6.59; N, 6.11. Found: C, 83.72; H, 6.71; N, 6.31. IR (Nujol): 3590 cm^{-1}. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 1.76 (1H, br s), 2.52—3.42 (4H, m), 3.75—4.06 (3H, m), 4.43 (1H, d, *J*=16 Hz), 4.68 (1H, s), 4.94 (1H, d, *J*=16 Hz), 6.52—6.83 (2H, m), 7.14 (10H, s with shoulder), 7.44—7.75 (2H, m). FAB-MS (positive ion mode) *m/z*: 459 [$(M+1)^+$]. $[\alpha]_D^{26}$ +31.3° (*c*=1.0, $CHCl_3$).

(*S*)-(+)-9-Benzyl-1-methyl-1,2,3,4-tetrahydropyrido[3,4-*b*]indole (7a) A solution of **5** (0.47 g, 1.2 mmol, 100% de) in absolute MeOH (20 ml)

was hydrogenated with $Pd(OH)_2$–carbon (120 mg). After the completion of H_2 absorption, the catalyst was filtered off and the filtrate was evaporated. The residue was made basic with 10% $KHCO_3$ solution and extracted with $CHCl_3$. The $CHCl_3$ layer was dried and evaporated. The resulting crude mass was purified by column chromatography on silica gel ($CHCl_3$: MeOH = 9 : 1) to give 210 mg (64%) of **7a** as a viscous oil. *Anal.* Calcd for $C_{19}H_{20}N_2$: C, 82.57; H, 7.29; N, 10.14. Found: C, 82.76; H, 7.51; N, 10.28. IR (neat): 3330 cm^{-1}. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 1.28 (3H, d, J = 6 Hz), 1.70 (1H, br s), 2.51—3.40 (4H, m), 3.95 (1H, q, J = 6 Hz), 5.03 (2H, s), 6.70—7.50 (9H, m). FAB-MS (positive ion mode) m/z: 277 $[(M+1)^+]$. $[\alpha]_D^{25}$ +18.1° (c = 0.14, $CHCl_3$).

Similarly, (*S*)-(+)-9-benzyl-1-phenyl-1,2,3,4-tetrahydropyrido[3,4-*b*]-indole (**7b**) was prepared in 77% yield, mp 98—100 °C (from a mixture of hexane and Et_2O). *Anal.* Calcd for $C_{24}H_{22}N_2$: C, 85.17; H, 6.55; N, 8.28. Found: C, 85.42; H, 6.83; N, 8.45. IR (neat): 3310 cm^{-1}. ^{1}H-NMR (60 MHz, CCl_4) δ: 1.55 (1H, br s), 2.50—3.09 (4H, m), 4.48 (1H, d, J = 16 Hz), 4.84 (1H, s), 5.03 (1H, d, J = 16 Hz), 6.50—6.87 (2H, m), 7.05 (5H, s), 7.12 (5H, s). FAB-MS (positive ion mode) m/z: 339 $[(M+1)^+]$. $[\alpha]_D^{25}$ +65.7° (c = 0.7, EtOH).

(*S*)-(−)-Tetrahydroharman (8a) Sodium (0.167 g, 7.25 mmol) was added to liquid NH_3 (21 ml) at −78 °C. A solution of **7** (0.2 g, 0.725 mmol) in dry THF (5 ml) was then added to the solution dropwise at −78 °C. The reaction mixture was stirred at −78 °C for 30 min. The NH_3 and THF were evaporated off and the residue was extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with H_2O, dried, and evaporated to give 60 mg (75%) of **8a** as crystals. The optical purity was confirmed by chiral HPLC analysis (hexane and iso-PrOH (10 : 1) eluant; flow rate = 1 ml/min; wavelength = 254 nm; retention time = 35 min). The spectral properties were in good agreement with the literature values. mp 175—177 °C (lit.[3] mp 177—180 °C). $[\alpha]_D^{22}$ −51.1° (c = 0.5, EtOH) [lit.[3] $[\alpha]_D^{25}$ −52° (c = 2.0, EtOH)]. IR (Nujol): 3320, 3480 cm^{-1}. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 1.37 (3H, d, J = 6 Hz), 2.36 (1H, br s), 2.50—3.45 (4H, m), 4.10 (1H, q, J = 6 Hz), 6.90—7.30 (3H, m), 7.30—7.60 (1H, m), 8.18 (1H, br s).

Similarly, (*S*)-(+)-1-phenyl-1,2,3,4-tetrahydropyrido[3,4-*b*]indole (**8b**) was prepared in 78% yield, mp 195—196 °C (from EtOH). *Anal.* Calcd for $C_{17}H_{16}N_2$: C, 82.22; H, 6.49; N, 11.28. Found: C, 82.49; H, 6.71; N, 11.45. IR (neat): 3450, 3260 cm^{-1}. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 1.74 (1H, br s), 2.61—3.02 (2H, m), 3.02—3.44 (2H, m), 5.04 (1H, s), 6.87—7.64 (4H, m), 7.28 (5H, s), 7.64—7.89 (1H, br s). FAB-MS (positive ion mode) m/z: 249 $[(M+1)^+]$. $[\alpha]_D^{21}$ +40° (c = 0.5, $CHCl_3$).

2-Formyl-3-(2-bromoethyl)benzo[*b*]thiophene (10) BuLi (1.37 M in hexane, 18.1 ml, 24.8 mmol) was added dropwise to a solution of 3-(2-bromoethyl)benzo[*b*]thiophene (**9**) (4.6 g, 19.1 mmol) in dry THF (120 ml) under Ar at −78 °C. The reaction mixture was stirred at −78 °C for 45 min, then dry DMF (2.9 ml, 38.2 mmol) was added dropwise. After being stirred for a further 30 min, the reaction mixture was quenched with H_2O and extracted with Et_2O. The Et_2O layer was washed with H_2O and dried. The solvent was evaporated off and the residue was column-chromatographed on silica gel (hexane : AcOEt = 8 : 1) to give **10** (1 g, 20%), mp 101—103 °C (from a mixture of hexane and Et_2O). *Anal.* Calcd for $C_{11}H_9BrOS$: C, 49.08; H, 3.37. Found: C, 49.28; H, 3.57. IR (Nujol): 1660 cm^{-1}. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 3.71 (4H, s with shoulder), 7.31—7.60 (2H,m), 7.81—8.09 (2H, m), 10.30 (1H, s). FAB-MS (positive ion mode) m/z: 271 $[(M+1)^+ +2]$, 269 $[(M+1)^+]$.

(3*R*,11b*S*)-3-Phenyl-2,3,5,6-tetrahydro-11b*H*-benzo[4,5]thieno[2,3-*c*]-oxazolo[3,2-*a*]pyridine (12) A solution of **10** (1 g, 3.7 mmol), (*R*)-phenylglycinol (0.6 g, 4.4 mmol), and a catalytic amount of *p*-toluenesulfonic acid in a mixture of dry Et_2O (20 ml) and absolute EtOH (7 ml) was stirred for 12 h at room temperature. The solvent was evaporated off under reduced pressure. Azeotropic distillation was done with dry benzene (50 ml × 3) and finally with CCl_4 (50 ml) to give **11** as a solid, which was used in the following reaction without further purification.

The crude **11** was dissolved in a mixture of dry CH_2Cl_2 (60 ml) and dry $CHCl_3$ (30 ml), then Et_3N (0.44 g, 4.3 mmol) was added dropwise at −78 °C. The mixture was washed with H_2O, and concentrated. Crystallization of the residue from MeOH gave optically pure **12** (0.9 g, 81%). The diastereoisomeric purity was confirmed by examination of the 500 MHz NMR spectrum, mp 137—139 °C (from MeOH). *Anal.* Calcd for $C_{19}H_{17}NOS$: C, 74.23; H, 5.57; N, 4.56. Found: C, 74.46; H, 5.76; N, 4.72. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 2.53—3.00 (2H, m), 3.02—3.45 (2H, m), 3.82 (1H, t, J = 4 Hz), 4.13 (1H, dd, J = 12, 4 Hz), 4.40 (1H, dd, J = 12, 4 Hz), 5.82 (1H, s), 7.34 (5H, s), 7.05—8.00 (4H, m). FAB-MS (positive ion mode) m/z: 3.08 $[(M+1)^+]$. $[\alpha]_D^{24}$ −112° (c = 0.2, $CHCl_3$).

(1*S*,1′*R*)-2-(2-Hydroxy-1-phenylethyl)-1-methyl-1,2,3,4-tetrahydrobenzo[4,5]thieno[2,3-*c*]pyridine (13) The reaction of **12** (0.64 g, 2.0 mmol) with MeMgI (8 mmol) was carried out as described for the reaction of **4** with MeMgI. The reaction was quenched with NH_4Cl solution and the mixture was extracted with Et_2O. The Et_2O layer was extracted with 10% HCl. The aqueous layer was made basic with $KHCO_3$ and extracted with Et_2O. The Et_2O layer was washed with H_2O, dried, and concentrated to give a 14 : 1 (87% de) mixture of **13** and (1*R*,1′*R*)-2-(2-hydroxy-1-phenylethyl)-1-methyl-1,2,3,4-tetrahydrobenzo[4,5]thieno[2,3-*c*]pyridine (**14**) in 95% yield. The mixture was separated by open column chromatography on silica gel (hexane : AcOEt = 3 : 1) to give optically pure **13** as a hygroscopic solid (0.53 g, 1.6 mmol) in 80% yield. IR (neat): 3450 cm^{-1}. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 1.42 (3H, d, J = 7 Hz), 2.10 (1H, s), 2.45—3.54 (4H, m), 3.85 (3H, s), 4.05 (1H, q, J = 7 Hz), 7.30 (5H, s), 7.05—7.87 (4H, m). FAB-MS (positive ion mode) m/z: 324 $[(M+1)^+]$. $[\alpha]_D^{23}$ −73° (c = 0.1, $CHCl_3$).

(*S*)-1-Methyl-1,2,3,4-tetrahydrobenzo[4,5]thieno[2,3-*c*]pyridine (15) A solution of **10** (0.42 g, 1.3 mmol) in absolute MeOH (20 ml) was hydrogenated on $Pd(OH)_2$–carbon (195 mg). After the completion of H_2 absorption, the catalyst was filtered off and the filtrate was evaporated. The crude residue was purified by column chromatography on silica gel ($CHCl_3$: MeOH = 9 : 1) followed by molecular distillation (oil bath temp. 125—130 °C (0.09 mmHg)) to give **15** (0.2 g, 0.98 mmol) in 75% yield as an oil. The chiral HPLC analysis (hexane and iso-PrOH (10 : 1) eluant; flow rate = 1.0 ml/min; wavelength = 254 nm; retention time = 8 min) of the free base showed its enantiomeric purity to be 100% ee. IR (neat): 3300 cm^{-1}. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 1.42 (3H, d, J = 7 Hz), 1.70 (1H, s), 2.46—2.90 (2H, m), 2.95—3.55 (2H, m), 4.15 (1H, q, J = 7 Hz), 7.15—7.90 (4H, m). FAB-MS (positive ion mode) m/z: 204 $[(M+1)^+]$. $[\alpha]_D^{23}$ −37° (c = 0.1, $CHCl_3$). Hydrochloride salt of **15**: mp 240—242 °C (from a mixture of Et_2O and MeOH). *Anal.* Calcd for $C_{12}H_{13}NS \cdot HCl$: C, 60.11; H, 5.89; N, 5.84. Found: C, 60.39; H, 5.79; N, 6.09.

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Stereochemistry of Knoevenagel Condensation Products from Cyanoacetates and Aromatic Aldehydes

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The C-13 long-range selective proton decoupling method and X-ray crystallographic analysis were employed to determine the stereochemistry of the caffeic acid derivatives, extremely potent 12-lipoxygenase inhibitors, synthesized from cyanoacetates and aromatic aldehydes. The ester group of 1 and 2 was found to be *trans* to the phenyl group.

Keywords stereochemistry; Knoevenagel condensation; X-ray crystallographic analysis; caffeic acid derivative; 12-lipoxygenase; 12-lipoxygenase inhibitor; C-13 NMR long-range selective proton decoupling (LSPD); E-regioisomer; Z-regioisomer; regioselectivity

Little work has been done on the stereochemistry of Knoevenagel condensation products obtained from cyanoacetates and aldehydes. Hayashi *et al.* studied the stereochemistry of α-cyano-β-alkoxy-β-alkylacrylic esters by nuclear magnetic resonance (NMR) spectroscopy and concluded that they were mixtures of *cis* and *trans* isomers.[1] Schwarz reported the stereochemistry of ethyl alkylidenecyanoacetates.[2] On the other hand, Zabicky examined ethyl arylidenecyanoacetates by infrared (IR) spectroscopy and suggested that the cyano group should be *cis* to the phenyl group.[3]

In connection with a synthetic study of 12-lipoxygenase inhibitors,[4] we needed to confirm the stereochemistry of condensation products of cyanoacetates with 3,4-dihydroxybenzaldehyde. We employed the ^{13}C-NMR LSPD (^{13}C-nuclear magnetic resonance long-range selective proton decoupling) method to determine the chemical structure. From a Newman projection, it seemed likely that the large ester group should be opposite to the bulky phenyl group, and *trans* elimination of water would occur to give a caffeic acid derivative. However, confirmation of the stereochemistry was required by ^{13}C-NMR spectroscopy and X-ray crystallographic analysis, because compound **1** exhibited the most potent 12-lipoxygenase-inhibitory activity among compounds reported so far.[4] Thus, we chose two compounds **1** and **2**, synthesized by condensation to give regioselectively a single isomer, and took the ^{13}C-NMR spectra to compare the coupling constants between the cyano carbon or ester carbon and the olefinic proton. Upon irradiation of the olefinic proton, three-bond couplings were observed at those carbons. Thus, the coupling constant of carbon *trans* to the olefinic proton is about 13—14 Hz in both **3** and **4**, while that of the ester carbon *cis* to the olefinic proton is 3 Hz in compound **4** and that of the cyano carbon *cis* to the proton is 8 Hz in compound **3**. The long-range three-bond coupling constants of **1** and **2** between the olefinic proton and the ester carbon (δ 164.52 in compound **1** and δ 164.55 in compound **2**) and 3 and 6.3 Hz, respectively. Moreover, upon irradiation of the olefinic proton, 15% nuclear Overhauser effect (NOE) was observed at the carbon at δ 164.52 in compound **1**.

The ^{13}C-NMR LSPD technique described above to determine the stereochemistry of the three-substituted olefinic bond seems to be useful and should be generally applicable to amides, aldehydes, *etc.* These results suggested that the ester group should be *trans* to the phenyl group and the chemical structures of the caffeic acid derivatives can be drawn as **1** and **2**. A fine crystal of compound **2** was obtained and subjected to X-ray crystallographic analysis. The ORTEP drawing of **2** is shown in Fig. 2, confirming that the phenyl group is *trans* to the ester group.

Experimental

Synthesis of Phenethyl 2-Cyano-3-(3,4-dihydroxyphenyl)-2-propenoate (2) i) EDC Reaction: To a stirred solution of 3.68 g (30 mmol) of phen-

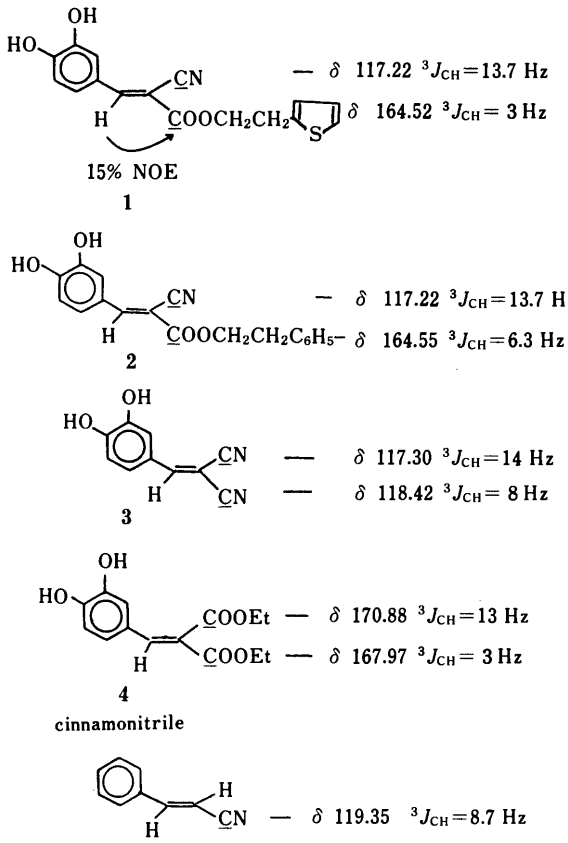

Fig. 1. The Chemical Shifts of Cyano and Ester Carbons and Three-Bond Coupling Constants between Olefinic Proton and Carbon

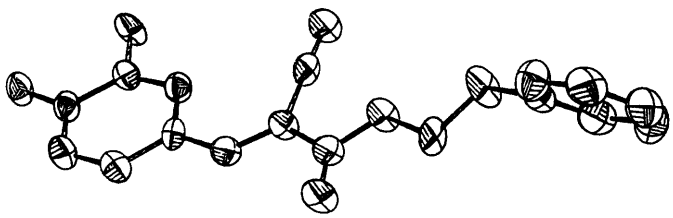

Fig. 2. ORTEP Drawing of Compound **2**

ethyl alcohol in 30 ml of *N,N*-dimethylformamide (DMF) was added a solution of 2.55 g (30 mmol) of cyanoacetic acid in 20 ml of DMF at 0 °C. To the stirred solution were added successively both solutions of 4.66 g (30 mmol) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in 5 ml of DMF and 0.37 g (3 mmol) of 4-dimethylaminopyridine (DMAP) in 10 ml of DMF at 0 °C. The solution was stirred for 18 h at room temperature. After DMF was removed *in vacuo*, the residue was quenched with brine and extracted with Et_2O. The organic layer was washed with brine, dried over anhyd. $MgSO_4$ and evaporated to leave the residue, which was purified by SiO_2 column chromatography (hexane : AcOEt = 4 : 1) to give 2.49 g (44%) of phenethyl cyanoacetate.

ii) Knoevenagel Reaction: To a solution of 1.73 g (12.5 mmol) of 3,4-dihydroxybenzaldehyde in 3 ml of DMF and 30 ml of benzene was added a solution of 2.49 g (13.2 mmol) of phenethyl cyanoacetate in 70 ml of benzene. After addition of 3 drops of piperidine, the solution was refluxed with a Dean–Stark apparatus for 2 h. After evaporation of the solvents under reduced pressure, water was added to the concentrated residue. After collection of the precipitated crude crystals, recrystallization from EtOH–H_2O afforded 3.5 g (91%) of the desired compound, mp 171—172 °C (yellow crystals). IR (Nujol): 2230, 1730 cm^{-1}. ^{1}H-NMR (270 Mz, CD_3OD) δ: 3.03 (2H, t, J=7 Hz), 4.45 (2H, t, J=7 Hz), 6.86 (1H, d, J=9 Hz), 7.15—7.39 (6H, m), 7.63 (1H, d, J=2 Hz), 8.05 (1H, s). *Anal.* Calcd for $C_{18}H_{15}NO_4$: C, 69.89; H, 4.89; N, 4.53. Found: C, 69.92; H, 4.93; N, 4.39. Similarly, 2-(2-thienyl)ethyl 2-cyano-3-(3,4-dihydroxyphenyl)-2-propenoate (**1**) was synthesized, mp 168—170 °C (yellow crystals). IR (Nujol): 2220, 1680 cm^{-1}. ^{1}H-NMR (270 MHz, CD_3OD) δ: 3.27 (2H, t, J=7 Hz), 4.45 (2H, t, J=7 Hz), 6.83 (1H, d, J=9 Hz), 6.68—6.99 (2H, m), 7.18—7.27 (1H, m), 7.35 (1H, dd, J=8, 2 Hz), 7.65 (1H, d, J=2 Hz), 8.08 (1H, s). *Anal.* Calcd for $C_{16}H_{13}NO_4S$: C, 60.94; H, 4.15; N, 4.44. Found: C, 60.99; H, 4.17; N, 4.40.

^{13}C-NMR Spectra ^{13}C-NMR spectra were measured on GN-300 instrument (75.5 MHz for ^{13}C). LSPD was obtained by selective decoupling of the olefinic proton during data acquisition. Steady-state NOE was observed by selective irradiation prior to data acquisition, with subtraction of a reference spectrum.[5]

X-Ray Crystallographic Analysis Crystal Data For Compound **2**: $C_{18}H_{15}NO_4$, MW = 309.32. The crystals were recrystallized from EtOH–H_2O, mp 171—172 °C. Yellow crystals, orthorhombic, space group $P2_12_12_1$ a=10.291 (1), b=32.323 (3), c=4.610 (1) Å, $\alpha=\beta=\gamma=90°$, U=1533.4(3) Å^3, Z=4, D_c=1.34 g/cm^3, F (000)=147, μ(Cu K_α)=5.28 mm^{-1}. X-Ray diffraction intensity data from the crystal (0.1 × 0.05 × 0.2 mm) were obtained on a Rigaku AFC diffractometer equipped with a rotating anode X-ray generator (50 kV—200 mÅ), using graphite-monochromated Cu K_α radiation (λ=1.5418 Å). A total of 1506 independent reflections with $2\theta < 126°$ were collected in the ω scanning mode ($2\theta < 45°$) and $\omega/2\theta$ scanning mode ($2\theta > 45°$). The structure was solved by the direct method using MULTAN 84.[6] Hydrogen atoms were not determined. The refinement was carried out by the block-diagonal, least-squares method with anisotropic thermal parameters. The *R*-factor was reduced to 0.080 using 1422 reflections. All calculations were performed on a PANAFACOM U-1200 II in the Rigaku RASA-5RP system.

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Synthesis of [^{125}I]Iodoclorgyline, a Selective Monoamine Oxidase A Inhibitor, and Its Biodistribution in Mice

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A new radioiodinated monoamine oxidase A (MAO-A) specific inhibitor, [^{125}I]iodoclorgyline, was synthesized from its tin precursor by iododestannylation reaction using sodium [^{125}I]iodide and hydrogen peroxide with high yield and site specificity. The product possessed a high radiochemical purity as well as high specific activity. The method can be readily applicable for labeling with ^{123}I, a very suitable radioisotope for *in vivo* imaging with single photon emission computer tomography (SPECT). Biodistribution studies of the [^{125}I]iodoclorgyline in mice showed high initial uptake in the brain, and brain radioactivity reached a constant level at 60 min after intravenous injection. The results suggested that [^{125}I]iodoclorgyline might have potential as a radiopharmaceutical for MAO-A studies in the brain with SPECT.

Keywords monoamine oxidase inhibitor; iodoclorgyline; radioiodination; iododestannylation; biodistribution

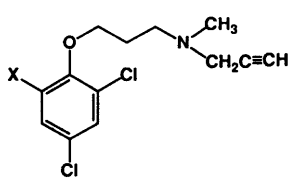
Monoamine oxidase (MAO) [E.C. 1.4.3.4] catalyzes the oxidative deamination of endogenous neurotransmitter amines as well as exogenous amines. It has been divided into two subtypes, MAO-A and MAO-B on the basis of substrate and inhibitor selectivity.[1] Clorgyline and *l*-deprenyl inhibit irreversibly and selectively MAO-A and MAO-B, respectively, by binding covalently to the enzyme itself.[2] Both subtypes, MAO-A and MAO-B, appear to be important for neurotransmitter regulation, and fluctuations in functional MAO activity may be associated with human diseases such as Parkinson's disease, depression and certain psychiatric disorders.[3]

The development of positron emission tomography (PET) and single photon emission computer tomography (SPECT) has made possible the studies of metabolism and physiological processes in the living human body utilizing organic molecules labeled with a positron emitter or a single photon emitter. For the direct and non-invasive mapping and functional studies of MAO activity in the living brain, the carbon-11 labeled suicide inhibitors, pargyline, clorgyline and *l*-deprenyl, have been investigated as positron ligands for PET studies.[4]

Despite attractive features associated with PET techniques, PET studies are still limited, since they usually require on-site cyclotrons. On the other hand, SPECT studies are more commonly used in nuclear medicine clinics. For SPECT imaging, iodine-123 possesses very suitable radiation properties, half-life of 13 h and gamma ray energy of 159 keV.

We have explored the feasibility of [^{123}I]radioiodinated MAO inhibitors as alternatives to clorgyline and *l*-deprenyl for functional MAO studies in the brain with SPECT. Previously, a series of novel iodinated clorgyline derivatives were prepared and evaluated as selective inhibitors for MAO-A.[5] *N*-[3-(2,4-Dichloro-6-iodophenoxy)propyl]-*N*-methyl-2-propynylamine (iodoclorgyline, **2**, Chart 1) was found to be relatively potent and selective toward MAO-A, comparable to clorgyline examined under the same conditions. We report here the synthesis of the radioiodinated counterpart, [^{125}I]iodoclorgyline (**3**, Chart 1). Preliminary biological studies on the *in vivo* inhibitory potency and selectivity for MAO-A and biodistribution in mice of this compound were also performed in order to evaluate it as a new ligand for *in vivo* MAO-A studies with SPECT.

Electrophilic iododestannylation reaction offers several distinct advantages for synthesis of radiopharmaceuticals.[6] The labeling procedure may be performed in the last step under very mild conditions and with very high site



1 : X=H clorgyline
2 : X=I iodoclorgyline
3 : X=^{125}I [^{125}I] iodoclorgyline

Chart 1

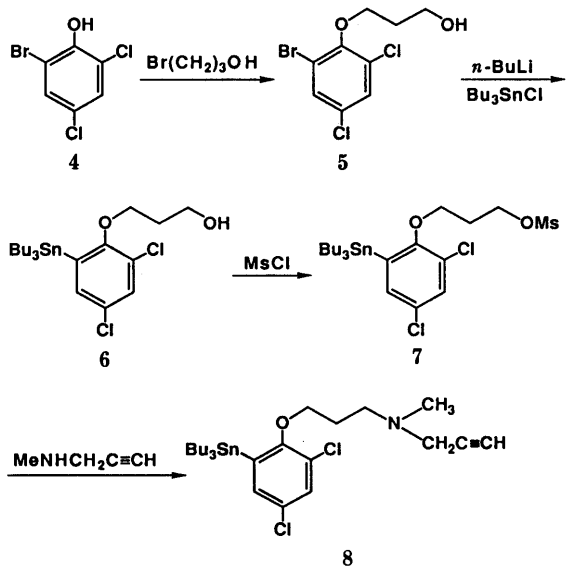


Chart 2

selectivity as well as high specific radioactivity. The requisite tin atom may be appended at any step in the synthesis. Moreover, the approach appears to be quite general with respect to the substrate. This paper describes a convenient method for the introduction of ^{125}I into clorgyline.

[^{125}I]Iodoclorgyline and its key tin precursor (**8**) were synthesized by the reactions outlined in Chart 2. The bromophenol derivative (**4**) was converted to its corresponding phenoxypropanol (**5**). Reaction of **5** with *n*-butyllithium and tri-*n*-butyltin chloride in tetrahydrofuran at $-78\,^{\circ}C$ yielded the stannyl phenoxypropanol (**6**), which was converted into a reactive mesylate (**7**) by treatment with methanesulfonyl chloride in dichloromethane. Alkylation of *N*-methylpropargylamine with **7** in the presence of potassium carbonate in acetonitrile gave the desired key intermediate (**8**).

Iodination of **8** was very easily accomplished in high yield (88%) by treatment with iodine in chloroform. Radioiodination of **8** was achieved using hydrogen peroxide[7] as an oxidant and sodium [^{125}I]iodide (specific activity 7.4 TBq/mmol) in 0.1 N HCl aqueous solution, followed by high performance liquid chromatography (HPLC) purification. The radiochemical purity of the product was higher than 99% as assessed by HPLC analysis, and the product comigrated on HPLC with authentic unlabeled material (Fig. 1). The radiochemical yield based on Na-^{125}I was 80%. The specific activity of the product was approximately 7.4 TBq/mmol.

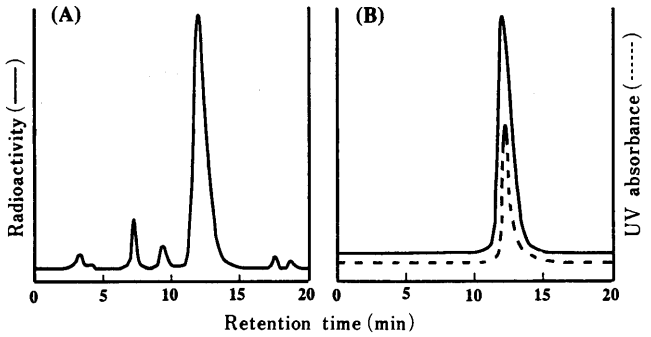


Fig. 1. HPLC Chromatograms of (A) Crude Radioiodination Reaction Mixture (Radioactivity), and (B) Purified [^{125}I]Iodoclorgyline (Radioactivity) and Authentic Unlabeled Iodoclorgyline (UV Absorbance)

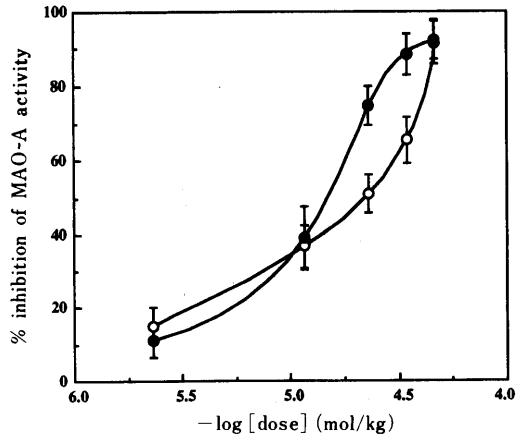


Fig. 2. Dose Response Curves for the Inhibition of Rat Brain (●) and Liver (○) MAO-A Activity by Iodoclorgyline Treatment *in Vivo*

Importantly, the tin precursor **8** is stable over six months when stored at $-20\,^{\circ}C$, and [^{125}I]iodoclorgyline can be readily generated.

The *in vivo* inhibition of MAO activity by iodoclorgyline was examined in the brain and liver homogenates from iodoclorgyline administered rats. The IC_{50} values calculated from the results presented in Fig. 2 were 1.4×10^{-5} and 2.2×10^{-5} mol/kg for MAO-A in the brain and liver homogenate, respectively. On the contrary, MAO-B activity was not effected by the iodoclorgyline treatment examined under the same conditions (dose: 2.3×10^{-6}—4.6×10^{-5} mol/kg). Thus, these results showed that iodoclorgyline had a specific inhibitory effect on MAO-A *in vivo* as well as *in vitro*.[5]

The *in vivo* biodistribution behavior of [^{125}I]iodoclorgyline was examined in male ddY mice. The total amount of iodoclorgyline present in a single injection dose of [^{125}I]iodoclorgyline is significantly lower than the IC_{50} value of iodoclorgyline required for *in vivo* MAO inhibition estimated by the specific activity. Therefore, the *in vivo* behavior of this compound can be assessed without causing an inhibitory effect against MAO. As summarized the results in Table I, the [^{125}I]iodoclorgyline was transported well into various organs and disappeared slowly from the blood. Initial brain uptake was high (2.8% dose/g at 5 min post injection) and the clearance was rapid for the first 60 min and then retained thereafter. The brain to blood activity ratio was, however, in a range of 0.5—0.75 at 15—120 min post injection. It is desirable to increase this ratio to obtain high quality neuronal images. In the liver, the highest uptake and the slowest clearance were observed. The kidney uptake pattern was similar to that of the liver.

In conclusion, the new radioiodinated clorgyline derivative, [^{125}I]iodoclorgyline, was expediently synthesized from its tin precursor by iododestannylation reaction using sodium [^{125}I]iodide and hydrogen peroxide with high yield and site specificity. The product possessed a high radiochemical purity as well as high specific activity. The method can be readily applicable for the labeling with ^{123}I, a very suitable radioisotope for *in vivo* imaging with SPECT. Iodoclorgyline possessed the desirable characteristics, high inhibitory potency and selectivity for MAO-A *in vivo* as well as *in vitro*. Biodistribution studies of the [^{125}I]iodoclorgyline in mice showed high initial uptake in the brain and the brain radioactivity reached a constant level at 60 min after intravenous injection. The results

Table I. Biodistribution of Radioactivity in Mice after Intravenous Injection of [^{125}I]Iodoclorgyline

	Mean% injected dose±s.d./g organ[a]				
	5 min	15 min	30 min	60 min	120 min
Blood	1.58±0.11	2.01±0.30	2.25±0.10	1.28±0.16	1.28±0.23
Pancreas	6.37±0.11	4.11±0.89	2.57±0.30	1.21±0.26	1.03±0.16
Spleen	2.15±0.43	1.60±0.30	1.29±0.19	0.81±0.20	0.79±0.17
Liver	10.20±0.88	10.07±1.83	9.72±0.45	5.62±1.50	5.67±2.09
Kidney	7.71±0.55	6.49±0.99	8.88±1.55	5.47±2.95	4.05±1.72
Heart	3.27±0.23	1.73±0.31	1.51±0.20	0.73±0.11	0.69±0.11
Lung	5.97±1.19	3.34±0.67	3.03±0.63	1.84±0.26	1.68±0.42
Brain	2.80±0.24	1.51±0.31	1.10±0.13	0.64±0.17	0.69±0.12

a) Four animals per each point.

suggested that [^{125}I]iodoclorgyline might have potential as a SPECT radiopharmaceutical for MAO-A studies in the brain. Further studies of this new agent are now in progress.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded on a JASCO IR-700 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Varian Gemini-200 (200 MHz) spectrometer and the chemical shifts are reported in ppm downfield from an internal tetramethylsilane standard. High resolution mass spectra (HRMS) were obtained on a Hitachi M-80 instrument. The HPLC system used included a Waters M 600 pump, a Lambda-Max 481 UV detector (254 nm), a Beckman 170 NaI radioactivity detector, and a Cosmosil 5C18-AR column (10 mm × 25 cm, Nacalai tesque).

3-(2-Bromo-4,6-dichlorophenoxy)-1-propanol (5) A solution of sodium hydroxide (1.2 g, 30 mmol) in water (5 ml) was added to a mixture of 2-bromo-4,6-dichlorophenol (6.0 g, 25 mmol) and 3-bromo-1-propanol (3.5 g, 25 mmol) in ethanol (20 ml), and the reaction mixture was stirred at reflux overnight. After removing the solvent *in vacuo*, the residue was taken up with chloroform (50 ml) and washed with water (30 ml × 3). The organic layer was dried over sodium sulfate and evaporated *in vacuo* to give crude crystals. Recrystallization from hexane afforded **5** (6.2 g, 83%), mp 60—62 °C. *Anal.* Calcd for $C_9H_9BrCl_2O_2$: C, 36.04; H, 3.02. Found: C, 36.18; H, 3.05. IR ($CHCl_3$): 3620, 3018, 2974, 1450, 1217, 1045 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.92 (1H, br, OH), 2.10 (2H, quintet, J=5.9 Hz, $CH_2C\underline{H}_2CH_2$), 3.97 (2H, q, J=5.9 Hz, $C\underline{H}_2OH$), 4.15 (2H, t, J=5.9 Hz, OCH_2), 7.36 (1H, d, J=2.4 Hz, aromatic), 7.48 (1H, d, J=2.4 Hz, aromatic). HRMS Calcd for $C_9H_9BrCl_2O_2$ m/z: 299.9143. Found: 299.9140.

3-(2,4-Dichloro-6-tributylstannylphenoxy)-1-propanol (6) A 2.0 M solution of *n*-butyllithium (9.0 ml, 18 mmol) in pentane was added to a solution of **5** (2.4 g, 8 mmol) in dry tetrahydrofuran (15 ml) at −78 °C under argon atmosphere. After 30 min, tributyltin chloride (5.9 g, 18 mmol) was added and the resulting solution was allowed to warm gradually to room temperature. The reaction was quenched with a 10% aqueous ammonium chloride solution (10 ml). The reaction mixture was extracted with ether (20 ml × 3). The combined organic extracts were washed with water (30 ml), dried over sodium sulfate and the solvent was removed *in vacuo* to give an oil. Column chromatography on silica gel eluting with chloroform afforded **6** (2.3 g, 56%) as a colorless liquid. IR ($CHCl_3$): 3016, 2958, 2926, 1421, 1375, 1217, 1045 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.86—1.57 (27H, m, $SnBu_3$), 1.94 (1H, t, J=5.9 Hz, OH), 2.06 (2H, quintet, J=5.9 Hz, $CH_2C\underline{H}_2CH_2$), 3.93 (2H, q, J=5.9 Hz, $C\underline{H}_2OH$), 4.06 (2H, t, J=5.9 Hz, OCH_2), 7.19 (1H, d, J=2.4 Hz, aromatic), 7.33 (1H, d, J=2.4 Hz, aromatic). CI-HRMS Calcd for $C_{21}H_{37}Cl_2O_2Sn$ (MH^+) m/z: 511.1192. Found: 511.1196.

3-(2,4-Dichloro-6-tributylstannylphenoxy)-1-(methanesulfonyloxy)-propane (7) A solution of methanesulfonyl chloride (0.27 g, 2.4 mmol) in dichloromethane (2 ml) was added slowly at 0 °C to a mixture of **6** (1.02 g, 2.0 mmol) and triethylamine (0.24 g, 2.4 mmol) in dichloromethane (20 ml). After stirring at 0 °C for 2 h, the reaction mixture was washed with water (10 ml × 3), dried over sodium sulfate and the solvent was removed *in vacuo* to give crude **7** (1.06 g, 90%) as an oil, which was used without further purification.

***N*-[3-(2,4-Dichloro-6-tributylstannylphenoxy)propyl]-*N*-methyl-2-propynylamine (8)** A solution of potassium carbonate (0.7 g, 5 mmol) in water (2 ml) was added to a solution of **7** (1.06 g, 1.8 mmol) in acetonitrile (5 ml) followed by *N*-methylpropargylamine (0.5 ml, 5.9 mmol) and the resulting solution was stirred at room temperature for 3 d. After dilution with ether (30 ml), the reaction mixture was washed with water (10 ml × 3) and dried over sodium sulfate. Removal of the solvent *in vacuo* gave an oil. Purification by column chromatography on silica gel using 10% ethyl acetate in chloroform as an eluent afforded **8** (0.60 g, 59%) as a colorless liquid. IR ($CHCl_3$): 2956, 2926, 2852, 1462, 1421, 1374, 1071 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.86—1.55 (27H, m, $SnBu_3$), 1.98 (2H, quintet, J=6.8 Hz, $CH_2C\underline{H}_2CH_2$), 2.22 (1H, t, J=2.3 Hz, C≡CH), 2.35 (3H, s, NCH_3), 2.64 (2H, t, J=6.8 Hz, $CH_2C\underline{H}_2N$), 3.38 (2H, d, J=2.3 Hz, $C\underline{H}_2C{\equiv}CH$), 3.96 (2H, t, J=6.8 Hz, OCH_2), 7.17 (1H, d, J=2.6 Hz, aromatic), 7.32 (1H, d, J=2.6 Hz, aromatic). CI-HRMS Calcd for $C_{25}H_{42}Cl_2NOSn$ (MH^+) m/z: 562.1665. Found: 562.1663.

***N*-[3-(2,4-Dichloro-6-iodophenoxy)propyl]-*N*-methyl-2-propynylamine (Iodoclorgyline, 2)** A 0.1 M solution of iodine in chloroform was added at room temperature to a solution of **8** (1.0 g, 1.8 mmol) in chloroform (2 ml) until a pink color remained. The reaction mixture was washed with 10% aqueous sodium thiosulfate solution (10 ml) and then with water (10 ml). The organic phase was then extracted with 1 N HCl solution (7 ml × 3). The combined aqueous layers were made basic with a 5 N NaOH solution and extracted with chloroform (10 ml × 3). The combined organic layers were washed with water (20 ml), dried over sodium sulfate and the solvent was removed *in vacuo* to give free base, which was then converted to hydrochloride salt. Recrystallization from ethanol–ether afforded iodoclorgyline hydrochloride (0.68 g, 88%), mp 177—178 °C. *Anal.* Calcd for $C_{13}H_{15}Cl_3INO$: C, 35.93; H, 3.48; N, 3.22. Found: C, 35.90; H, 3.34; N, 3.17. IR (KBr): 3208, 2934, 2628, 1467, 1442, 1375, 1244 cm^{-1}. ^{1}H-NMR (free base, $CDCl_3$) δ: 2.04 (2H, quintet, J=6.8 Hz, $CH_2C\underline{H}_2CH_2$), 2.23 (1H, t, J=2.3 Hz, C≡CH), 2.35 (3H, s, NCH_3), 2.69 (2H, t, J=6.8 Hz, $CH_2C\underline{H}_2N$), 3.39 (2H, d, J=2.3 Hz, $C\underline{H}_2C{\equiv}CH$), 4.03 (2H, t, J=6.8 Hz, OCH_2), 7.37 (1H, d, J=2.2 Hz, aromatic), 7.66 (1H, d, J=2.2 Hz, aromatic). HRMS Calcd for $C_{13}H_{14}Cl_2INO$ (free base) m/z: 396.9496. Found: 396.9498.

***N*-[3-(2,4-Dichloro-6-[^{125}I]iodophenoxy)propyl]-*N*-methyl-2-propynylamine ([^{125}I]Iodoclorgyline, 3)** Aqueous hydrogen peroxide (10 μl, 30%, w/v) was added to a mixture of **8** (10 μl, 1 mg/ml in ethanol), 0.1 N HCl (0.1 ml), and sodium [^{125}I]iodide (10 μl, 7.4 MBq, specific activity 7.4 TBq/mmol) in a sealed vial. The reaction was allowed to proceed for 30 min at room temperature, after which it was terminated by the addition of sodium bisulfite (0.1 ml, 100 mg/ml in water). The desired product was isolated by HPLC using 0.05 N ammonium formate–methanol (1 : 9, v/v) as an eluent at a flow rate of 3.0 ml/min. The product fractions were collected and the solvent was removed *in vacuo*. The final product, [^{125}I]iodoclorgyline, was taken up in an isotonic saline solution and passed through a 0.22 μm filter. The radiochemical yield was 80%. The radiochemical purity and the specific activity were higher than 99% and about 7.4 TBq/mmol, respectively, determined by HPLC.

Assay of MAO Activity in the Brain and Liver Male Wistar rats (200—250 g) were administered iodoclorgyline (2.3×10^{-6}—4.6×10^{-5} mol/kg, 0.1 ml in water) intraperitoneally. The animals were sacrificed 1 h post injection, and the brain and liver were excised and homogenized. The MAO activity was assayed fluorometrically using kynutramine as a substrate according to the previously reported method.[8]

Biodistribution of [^{125}I]Iodoclorgyline in Mice [^{125}I]Iodoclorgyline (37 kBq in 0.1 ml saline) was injected intravenously into male ddY mice (20—25 g) through a lateral tail vein. At the desired time interval after administration, the animals were sacrificed. Samples of blood and organs of interest were excised, weighed, and the radioactivity was measured with a NaI(Tl) gamma scintillation counter. The results were expressed as percent injected dose per gram of blood or organ.

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Studies on Aldose Reductase Inhibitors from Natural Products. IV.[1] Constituents and Aldose Reductase Inhibitory Effect of *Chrysanthemum morifolium*, *Bixa orellana* and *Ipomoea batatas*

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The hot water extracts of *Chrysanthemum morifolium*, *Bixa orellana* and *Ipomoea batatas*, were found to have potent inhibitory activity towards lens aldose reductase (AR). Ellagic acid (4) was isolated from *C. morifolium* and *I. batatas*, isoscutellarein (7) from *B. orellana* and 3,5-dicaffeoylquinic acid (10) from *I. batatas*, respectively, as potent inhibitors.

Keywords *Chrysanthemum morifolium*; *Bixa orellana*; *Ipomoea batatas*; aldose reductase inhibitor; ellagic acid; isoscutellarein; 3,5-dicaffeoylquinic acid

Aldose reductase (AR) is the enzyme which, along with the coenzyme nicotinamide adenine dinucleotide phosphate (NADPH), catalyzes the reduction of glucose to sorbitol. AR is likely to be responsible for the increased intracellular accumulation of sorbitol found in many tissues, such as lens, nerve, retina and kidney of diabetic animals. It has currently been expected that AR inhibitors will play an important role in the management of diabetic complications such as cataracts,[2] neuropathy,[3,4] retinopathy[5] and nephropathy.[6] Carboxylic acids,[7,8] flavonoids,[9-11] hydantoin analogues[12,13] and so forth have hitherto been examined as AR inhibitory drugs.

We have made screening tests on AR inhibitory effect with useful plants, for several years, among which *Chrysanthemum morifolium* (Compositae), *Bixa orellana* (Bixaceae) and *Ipomoea batatas* (Convolvulaceae) were found to show high inhibitory effects. In this paper, we report the isolation and identification of the active compounds inhibiting rat lens AR from these plants.

AR Inhibitors of *Chrysanthemum morifolium* The flower of *C. morifolium*, "kikuka," has traditionally been used for the treatment of eye disease in Japan and China and some flavonoids[14] were isolated without relation to the AR inhibitory effect. The hot water extract of this flower, which showed high inhibitory activity towards crude rat lens AR ($IC_{50} = 3.3 \times 10^{-6}$ g/ml), was partitioned between Et_2O and water to afford the Et_2O extract and water layer. The water layer was further extracted with AcOEt and *n*-BuOH successively to afford the AcOEt extract and *n*-BuOH extract. The AcOEt extract, which was most active (Table I), was applied to columns of silica gel and Sephadex LH-20 to give four compounds **1**—**4**. Compounds **1**—**3** and **4** were identified as acacetin 7-*O*-glucoside, diosmetine 7-*O*-glucoside, luteoline 7-*O*-glucoside and ellagic acid, respectively, by comparison with published spectral data[14] and their authentic samples.

TABLE I. Inhibitory Activity against Rat Lens AR by Extracts

	IC_{50} (μg)		
	C. morifolium	*B. orellana*	*I. batatas*
Crude ext.	3.3	3.0	1.0
Et_2O ext.	0.60	3.0	0.25
AcOEt ext.	0.40	0.15	0.20
n-BuOH ext.	5.5	2.0	2.0
H_2O ext.	>10	>10	>10

AR Inhibitors of *Bixa orellana* The leaf of *B. orellana* has traditionally been used for the treatment of an antipyretic in South America. The IC_{50} of the hot water extract was 3.3×10^{-6} g/ml. The extract was partitioned between preceding solvents. The AcOEt extract, which was most active (Table I), was applied to columns of silica gel to give three compounds **5**—**7**. Compounds **5** and **6** were identified as gallic acid and pyrogallol, respectively, by direct comparison with their authentic samples. Compound **7**, yellow needles, exhibited a positive reaction test for flavonoids. Infrared (IR) and ultraviolet (UV) spectra of **7** showed the characteristic absorption patterns of flavone type. At the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **7** and **7** acetate suggested the presence of four hydroxyl groups. The positions of four hydroxyl groups on **7** were thus considered to be 5, 7, 8 and 4′ by the addition of shift reagents in the UV spectrum[15] and comparison with published spectral data.[16] Consequently, **7** was concluded to be isoscutellarein.

AR Inhibitors of *Ipomoea batatas* *I. batatas*, a root called "satsumaimo," has been used for food. The hot water extract of this leaf showed high inhibitory activity towards crude rat lens AR ($IC_{50} = 1.0 \times 10^{-6}$ g/mg). The extract was partitioned between preceding solvents. The AcOEt extract, which was most active (Table I), was applied to columns of silica gel and preparative thin layer chromatography to give four compounds **4**, **8**—**10**. Compounds **8** and **9** were identified as scopoletin and caffeic acid by direct comparison with their authentic samples. Compound **10**, pale yellow amorphous powder, gave a positive color reaction to $FeCl_3$. The UV spectrum of **10** was very similar to **9**. In the IR spectrum of **10**, the absorption due to α,β-unsaturated carboxylic acid group found in **9** disappeared, but the presence of another carboxylic acid group was suggested. The ^{1}H-NMR spectrum and ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectrum of **10**, suggested the presence of two caffeoyl groups and one quinic acid. The position of two caffeoyl groups on quinic acid were thus considered to be 3 and 5 by comparing with published spectral data.[17] Consequently, **10** was determined to be 3,5-dicaffeoylquinic acid, which is the first isolation from this plant.

Results and Discussion

The isolated compounds **1**—**10** were tested for AR inhibitory activity (Table II). Among them, **4** exhibited the highest activity ($IC_{50} = 2.0 \times 10^{-7}$ M), which was previously reported as the high potent AR inhibitor from

TABLE II. Inhibitory Activity against Rat Lens AR by Compounds

Compd. No.	IC_{50} (μM)	Compd. No.	IC_{50} (μM)
1	>100	**7**	3.2
2	25	**8**	10
3	1.0	**9**	>100
4	0.20	**10**	0.46
5	>100	Quercitrin[a]	1.2
6	>100		

a) Quercitrin was assayed previously, and was tested again as a reference in this study.

Phyllanthus niruri.[18] Compound **10**, which has never been tested for inhibitory activity towards AR, also showed high effect ($IC_{50} = 4.6 \times 10^{-7}$ M). To determine the type of inhibition, the kinetics of inhibition of AR by **10** were plotted according to Lineweaver–Burk, and **10** was found to be a non-competitive inhibitor at the concentration of $0.1—1.0 \times 10^{-6}$ M. This type was also seen in the case of **4** and other inhibitors. The inhibitory effect of **10** on lens AR was also checked in the presence of a large amount of bovine serum albumin (BSA). Compound **10** showed almost the same degree of inhibition in the presence and absence of BSA. Comparison of the activity of **1**—**3** seems to suggest that the hydroxy group in the B-ring of flavonoid is potentialy concerned with AR inhibitory activity. This is in agreement with the structure–activity relationship of flavonoid in AR inhibitory activity previously reported.[6]

We concluded that compound **4** is a main AR inhibitor of *C. morifolium* and *I. batatas*. In addition, compounds **3**, **7** and especially **10** are also considered to play important roles as AR inhibitors.

Experimental

Melting points were measured with a Yanagimoto micromelting point apparatus and were uncorrected. Spectral data were obtained as follows: IR spectra with a Hitachi 260-0611 spectrophotometer; UV spectra with a Shimadzu UV-260 spectrophotometer; mass spectrum (MS) with a JEOL JMS-D 200 spectrometer (70 eV); ^{1}H-NMR spectra with a JEOL GX-270 spectrometer (270 MHz) and ^{13}C-NMR spectra with a JEOL XL-200 spectrometer (50.3 MHz) in dimethyl sulfoxide (DMSO)-d_6. Chemical shifts are given in δ (ppm) values referring to internal tetramethylsilane.

Plant Material *Chrysanthemum morifolium* extract was purchased from Nihon Funmatsu Co., Ltd. *Bixa orellana* has been cultivated in this medicinal plant garden. *Ipomoea batatas* was collected in Toyama, Japan.

Bioassay Crude AR was obtained from the supernatant fraction of the homogenate of rat lens according to the method of Kador and Sharpless,[19] and showed a specific activity of 22 unit/mg. One unit was defined as the amount catalyzing the oxidation of 1 μmol of reduced NADPH per minute. Inhibitory activity of the extracts and compounds on AR was assayed by the method previously reported.[20] Samples were dissolved in DMSO, which was found to have no effect on the enzyme activity at a concentration below 1%.

Extraction and Isolation Hot water extract of *C. morifolium* (500 g) was partitioned between Et_2O and distilled water to afford an Et_2O extract (4 g). The aqueous layer was successively extracted with AcOEt and *n*-BuOH to give AcOEt extract (16 g), *n*-BuOH extract (38 g) and water extract (435 g), respectively. The AcOEt extract was chromatographed on a silica gel column eluting first with $CHCl_3$ and then with an increasing amount of MeOH to yield compounds **3** (52 mg) and **4** (24 mg). 20% MeOH elute was purified with Sephadex LH-20 column eluting with MeOH to give **1** (27 mg) and **2** (8 mg).

Dried leaf of *B. orellane* (800 g) was extracted with hot distilled water (3 h × 2) to yield 190 g of the crude extract. The crude extract was partitioned with aforesaid method, to give Et_2O extract (4 g), AcOEt extract (4 g) *n*-BuOH extract (14 g) and water extract (127 g), respectively. The AcOEt extract was chromatographed on a silica gel column eluting first with $CHCl_3$ and then with an increasing amount of MeOH to yield compounds **5** (500 mg), **6** (20 mg) and **7** (25 mg).

Dried leaf of *I. batatas* (2 kg) was extracted with hot distilled water (2 h × 3) to yield 320 g of the crude extract. The crude extract (300 g) was partitioned with aforesaid method, to give Et_2O extract (4 g) AcOEt extract (33 g), *n*-BuOH extract (52 g) and water extract (200 g), respectively. The AcOEt extract was chromatographed on a silica gel column eluting first with $CHCl_3$ and then with an increasing amount of MeOH to yield compounds **4** (150 mg), **8** (80 mg), **9** (20 mg) and **10** (15 mg).

Acacetin 7-*O*-glucoside (**1**), diosmetin 7-*O*-glucoside (**2**), luteolin 7-*O*-glucoside (**3**), ellagic acid (**4**), gallic acid (**5**), pyrogallol (**6**), scopoletin (**8**) and caffeic acid (**9**) were identified by comparing (UV, ^{1}H-NMR, ^{13}C-NMR) with published spectral data and by direct comparison (UV, IR and MS) with authentic samples.

Isoscutellarein (7) Yellow needles, mp 298—300 °C ($CHCl_3$). Mg+HCl: orange. MS *m/z*: 286 (M^+), 168. UV λ_{max}^{MeOH} nm: 222, 278, 304. $\lambda_{max}^{MeOH+NaOMe}$ nm: 222, 290, 355. $\lambda_{max}^{MeOH+NaOAc}$ nm: 220, 290, 304. $\lambda_{max}^{MeOH+NaOAc+H_3BO_3}$ nm: 220, 280, 304. $\lambda_{max}^{MeOH+AlCl_3}$ nm: 228, 297, 324. $\lambda_{max}^{MeOH+AlCl_3+HCl}$ nm: 228, 283, 313, 342. IR ν_{max}^{KBr} cm^{-1}: 3350, 1650, 1615, 1580. ^{1}H-NMR (DMSO-d_6) δ ppm: 6.26, 6.71, 6.29, 8.00.

3,5-Dicaffeoylquinic Acid (10) Pale yellow amorphous powder, mp 169—172 °C (dec.), yellow-green to $FeCl_3$ reagent. UV λ_{max}^{MeOH} nm: 232, 242, 296, 325. ^{1}H-NMR (DMSO-d_6) δ ppm: 7.63, 7.59, 7.08, 6.98, 6.97, 6.793, 6.790, 6.37, 6.29, 5.50, 3.99, 2.20. ^{13}C-NMR (DMSO-d_6) δ ppm: 175.4, 166.0, 165.5, 148.3, 148.2, 145.5, 145.1, 144.5, 144.4, 125.7, 125.6, 151.3, 151.1, 115.9, 115.8, 115.0, 114.8, 114.6, 114.1, 72.5, 70.9, 70.8, 67.8, 36.7, 34.8.

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10-Hydroxypheophytins and a New Norlabdane Diterpene from the Leaves of *Cupressus funebris* ENDL.

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The leaves of *Cupressus funebris* (Cupressaceae) were shown to contain four chlorophyll-derived pigments (2—5a), together with a new natural norlabdane-type diterpene 1a. Compounds 1a and 5a were isolated as methyl esters 1b and 5b. The pigments were identified as pheophytin a (2), 10*S*- and 10*R*-hydroxypheophytin a (3 and 4), and methyl pheophorbide a (5b), from spectroscopic analyses.

Keywords *Cupressus funebris*; Cupressaceae; pheophytin a; 10*S*- and 10*R*-hydroxypheophytin a; methyl pheophorbide a; norlabdane diterpene

Cupressus funebris ENDL. is a Cupressaceae plant whose leaves are used in China as the source material of the crude drug. Its Japanese name is sokuhaku-yo.[1] Chemical investigations of *C. funebris* have been scarce, except for its flavones[2a] and volatile hydrocarbons,[2b] in contrast to the many regarding the Cupressaceae plant *Biota orientalis* L., [leaves,[3] heartwood,[4] seeds,[5] and pollen[6]], which is used also as sokuhaku-yo.[1] During the examination of *C. funebris* leaves, we noticed its prominent dark-green color, both in its dried material and its crude extract. Chlorophyll-derived compounds were naturally expected, but the extent of the darkness suggested a notably high content. Repeated column chromatography of the extract afforded four pigments (**2**, **3**, **4**, **5b**) together with a new norditerpene 15-norlabda-8 (20), 12*E*-diene-14-carboxaldehyde-19-oic acid methyl ester (**1b**). The diterpenes known as the Cupressaceae plant constituents, *cis*- and *trans*-communic acid,[7] 13-oxo-15,16-dinorlabda-8(20),-11*E*-dien-19-oic acid methyl ester,[5] 13-*R*/*S* mixture of 12*R*,13-dihydroxylabda-8(20),14-dien-19-oic acid methyl ester,[5] totarol,[8] and cupressic acid methyl ester,[9] were obtained simultaneously. These compounds, except for **2**, were isolated after treatment of the original components with diazomethane, but pigments **3** and **4** were unaffected by methylation.

The structure of **1b**, $[\alpha]_D^{31}$ +30°, was derived from the proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra which showed the presence of a 3-methyl-4-oxo-2*E*-butenyl side chain (^{1}H-NMR, δ 1.76, 3H, d, J=1.0 Hz; 6.42, 1H, br t, J=6.5 Hz; 9.34, 1H, s; ^{13}C-NMR, δ 9.3 (q), 138.9 (s), 155.9 (d), 194.9 (d)). The chemical shifts of the protons and carbons, regarding the decalin portion and its substituents, were virtually identical to those found in communic acids and other labdanoids isolated simultaneously. Seco-aldehyde ($[\alpha]_D$ +35.8°),[10a] recently reported as a trace by-product of the ozonolysis of *trans*-communic acid, shows identical ^{1}H-NMR chemical shifts to **1b**, while those of the *ent*-labdane nor-diterpene **6**,[10b] which has the same C-5 configuration, are different.

The amount of the pigment isolated was indeed significant, *i.e.* 150 (**2**), 50 (**3** and **4**) and 12.6 mg (**5b**) from 500 g of the dried material. Compounds **2** and **5b** were shown to be pheophytin a and methyl pheophorbide a, respectively, by comparison of their ^{1}H- and ^{13}C-NMR spectral data with those recorded in the literature.[11,12]

Compounds **3** and **4** were resistant to separation but the ^{1}H-NMR spectrum of the mixture showed close analogy with **2**. In ^{13}C-NMR, the signal due to C-10 (δ 64.8, d) in **2** was replaced with that of a quarternary carbon (δ 89.1) in the mixture, and those due to C-7, C-9, C-16 and C-17 showed small deviations. High-resolution fast atom bombardment mass spectrum (FAB-MS) showed a molecular formula $C_{55}H_{74}N_4O_6$, indicating the addition of one oxygen atom to that of **2**. Apparently, compounds **3** and **4** are 10-hydroxy derivatives of pheophytin a (**2**). The 10-hydroxy derivative of chlorophyll a has been known long since. It was originally reported as one of the "allomerization products,"[13] derived by the autoxidation of chlorophyll a. This hydroxylation reaction was later shown, by Pennington *et al.*,[14] to also occur enzymatically, Isolation of the demetallated 10-hydroxy derivatives of the chlorophyll a and b series, which were accumulated in the excreta of the silkworm *Bombyx mori*, was reported by Nakatani *et al.*[11] The derivatives were shown to be unresolved mixtures of C-10 diastereoisomeric pairs, and assignment of ^{1}H-NMR chemical shifts to each isomer was described. Endo *et al.*, apparently unaware of the precedent paper, reported methyl 10-hydroxypheophorbide a, supposedly also a 10*R*/*S* mixture, from the Chlorella cells exposed

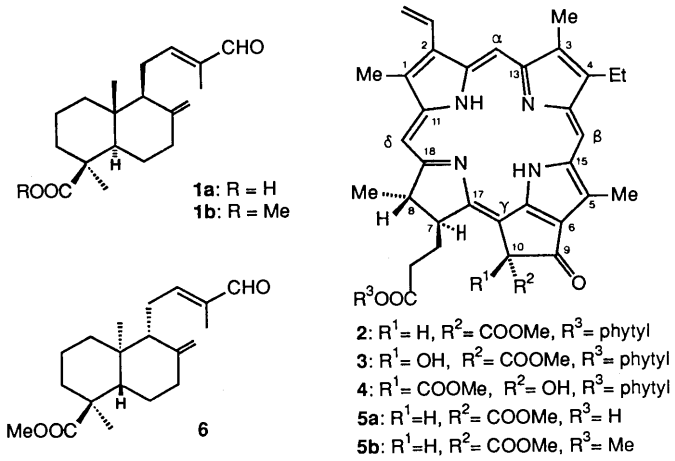

Chart 1

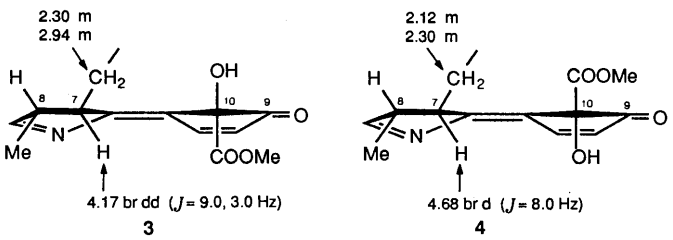

Chart 2. Partial ^{1}H-NMR Chemical Shifts of **3** and **4** (δ)

to air in aq EtOH.[15] Although they were later shown to be resolved by high-performance liquid chromatography,[16] isolation and characterization of the individual diastereoisomers has not been recorded. We found that simple silica gel column chromatography, using a $CHCl_3$–hexane (1 : 1) mixture containing 6% of acetone, separates the two diastereoisomers in a preparative scale. The ^{1}H-NMR spectra of **3** and **4** showed a prominent deshielding effect of the hydroxyl group on 7a-methylene protons or 7α-H, located in the same side of the porphyrin ring (Chart 2), in accordance with the report of Nakatani *et al.*,[11] using the 10*R*/*S* mixture. Compound **3**, having higher mobility on this chromatography, was shown to be the 10*S*-diastereoisomer, and **4** to be the 10*R*-diastereoisomer. To our knowledge, characterization of 10-hydroxypheophytins in crude drugs has not been reported. According to Nakatani *et al.*,[11] 10-hydroxypheophytins are cytostatic against hepatoma tissue culture. Endo *et al.*[15] showed that their 10-hydroxypheophorbide had stronger photodynamic activity than pheophytin a (**2**) and methyl pheophorbide a (**5b**). Although their formation process remains unsettled, the present discovery of the significant levels of **2** to **5a** in *C. funebris* leaves indicates that examination of such chlorophyll-derived substances is necessary for the crude drugs made from the plant leaves.

Experimental

Melting points were determined on a Kofler hot stage and are uncorrected. Optical rotations were determined in $CHCl_3$ on a JASCO DIP-370 digital polarimeter. NMR spectra were determined in a $CDCl_3$ solution on a JEOL JNM GX-270 spectrometer at 270 MHz (^{1}H) and on a JEOL JNM FX-90Q spectrometer at 22.5 MHz (^{13}C) with tetramethylsilane (δ 0) and $CDCl_3$ (δ 77.1) as internal standards. MS was determined on a JEOL JMS D300 mass spectrometer. Chromatography was done by flash column chromatography[17] using silica gel (Wako gel C-300, 200—300 mesh, Wako Pure Chemical Industries).

Isolation of the Diterpene 1b and the Pigments 2 to 5b The dried and pulverized leaves of *C. funebris* (500 g) were extracted thoroughly with hexane, a mixture of $CHCl_3$–MeOH (2 : 1), and MeOH. A portion (0.63 g) of the hexane extract (10 g) was subjected to column chromatography with $CHCl_3$–hexane (1 : 1) and 3% ethyl acetate in hexane, giving totarol (70 mg). A portion (1.0 g) of the hexane extract was methylated by an ethereal diazomethane solution and subjected to 7.5% $AgNO_3$-impregnated silica gel. Elution with ethyl acetate–hexane (3 : 11) gave *cis*-(100 mg) and *trans*-communic acid (110 mg). MeOH–$CHCl_3$ extract (36 g) and MeOH extract (8 g) were combined and subjected to column chromatography. Elution with ethyl acetate–hexane (3 : 7) gave fraction A (1.3 g) which contained **1a** and **2**. Elution with ethyl acetate–hexane (4 : 6) gave fraction B (1.4 g) which contained **2**, **3** and **4**, and fraction C (1.6 g) which contained pheophorbide a (**5a**). Fraction A was treated with an ethereal diazomethane solution. Column chromatography of the mixture with 5% acetone in hexane gave **2** (18 mg) and a mixture (330 mg) which contained **1b**. It was purified with 6% acetone in hexane–$CHCl_3$ (3 : 1) and hexane–$CHCl_3$ (2 : 1.2) giving **1b** (45 mg). Fraction B was eluted with ethyl acetate–hexane (1 : 9 to 3 : 7), giving subfraction B′ (300 mg) which contained **2**, and subfraction B″ (500 mg) which contained **3** and **4**. Subfraction B′ was passed through a column of alumina, eluting with ethyl acetate. Most of the acidic materials were removed by this treatment, giving 140 mg of crude **2**. It was purified by chromatography with acetone–hexane (1 : 9) giving pure **2** (90 mg). Subfraction B″ was methylated with ethereal diazomethane. Column chromatography of the mixture with ethyl acetate–hexane (1 : 4) gave 15,16-dinorlabda-8(20),11*E*-dien-13-one-19-oic acid methyl ester (56 mg), cupressic acid (200 mg), and a mixture of **3** and **4** (50 mg). Column chromatography of the mixture with $CHCl_3$–hexane (1 : 1) containing 6% of acetone gave **3** (24.6 mg) and **4** (21 mg). Fraction C was methylated with an ethereal diazomethane solution. Column chromatography of the mixture with acetone–hexane (1 : 4) and 5% acetone in hexane–$CHCl_3$ (1 : 1) gave **5b** (12.6 mg) and 13-*R*/*S* mixture of 12*R*-12,13-dihydroxylabda-8(20),14-diene 19-oic acid methyl ester (28 mg). Structures of the known diterpenes were confirmed by ^{1}H- and ^{13}C-NMR spectra, MS and high-resolution MS, and comparison of the specific rotations with those in the literature cited in the text.

15-Norlabda-8(20),12*E*-diene-14-carboxaldehyde-19-oic Acid Methyl Ester (1b) Oil, $[\alpha]_D^{31}$ +30° (*c*=0.48). ^{1}H-NMR δ: 0.59 (3H, s, 17-H_3), 1.21 (3H, s, 18-H_3), 1.76 (3H, d, *J*=1.0 Hz, 16-H_3), 3.63 (3H, s, OMe), 4.39 (1H, br s, 20-H), 4.87 (1H, q, *J*=1.5 Hz, 20-H), 6.42 (1H, br t, *J*=6.5 Hz, 12-H), 9.34 (1H, s, 14-H). ^{13}C-NMR δ: C-1 (39.3), C-2 (19.9), C-3, 7 (38.1, 38.3), C-4 (44.2), C-5, 9 (55.8, 56.1), C-6 (25.9), C-8 (147.6), C-10 (40.2), C-11 (24.4), C-12 (155.9), C-13 (138.9), C-14 (194.9), C-16 (9.3), C-17 (12.6), C-18 (28.8), C-19 (177.4), C-20 (107.8), -OMe (51.1). MS *m/z*: 318 (M^+), 300, 260, 121. High-resolution MS [Found (Calcd)] *m/z*: $C_{20}H_{30}O_3$ (M^+), 318.2181 (318.2195).

Pheophytin a (2) Amorphous solid. ^{1}H-NMR was identical as reported.[12] ^{13}C-NMR δ: phytyl moiety, q 16.4, 19.7 (2C), 22.8 (2C); t 24.4, 24.8, 25.0, 36.7, 37.3 (3C), 39.5, 39.8, 61.6; d 28.0, 32.7, 32.8, 117.9; s 142.8. Other signals were identical to those in **5b**,[12] within a deviation of 0.2 ppm. High-resolution FAB-MS [Found (Calcd)] *m/z*: $C_{55}H_{75}N_4O_5$ (MH^+), 871.5714 (871.5737).

10*S*-10-Hydroxypheophytin a (3) Amorphous solid. ^{1}H-NMR was identical as reported.[11] ^{13}C-NMR δ: C-6 (129.4), C-7 (52.0), C-7c, 10a, 18 (172.4, 172.8, 173.7), C-8 (50.4), C-8a (22.7), C-9 (192.1), C-10 (89.1), C-13 (155.3), C-16 (150.0), C-17 (162.6), C-α (98.0), C-γ (107.8), C-δ (93.6). Other signals are identical to those in **2** and **5b**,[12] within a deviation of 0.2 ppm. High-resolution FAB-MS (as a mixture of **3** and **4**) [Found (Calcd)] *m/z*: $C_{55}H_{75}N_4O_6$ (MH^+), 887.5733 (887.5680).

10*R*-10-Hydroxypheophytin a (4) Amorphous solid. ^{1}H-NMR was identical as reported.[11] ^{13}C-NMR δ: C-6 (129.4), C-7 (50.9), C-7c, 10a, 18 (173.1, 173.5, 174.0), C-8 (50.5), C-8a (22.7), C-9 (192.1), C-10 (89.1), C-13 (155.5), C-16 (150.3), C-17 (162.0), C-α (98.0), C-γ (107.8), C-δ (93.5). Other signals are identical to those in **2** and **5b**,[12] within a deviation of 0.2 ppm.

Methyl Pheophorbide a (5b) Amorphous solid. ^{1}H- and ^{13}C-NMR spectra were identical as reported.[11,12]

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Mitogenic Activity of *Tulipa gesneriana* Lectins on Mouse and Human Lymphocytes

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***Tulipa gesneriana* lectin-erythrocyte (TGL-E) which agglutinates mouse erythrocytes showed a potent mitogenic activity on mouse spleen cells and human peripheral blood lymphocytes, however, TGL-E had only slight mitogenic activity on mouse thymus cells. Its subunit α with a molecular weight (MW) of about 26000 showed a potent mitogenic activity as did that of native lectin, but subunit β with a MW of about 14000 showed no activity, indicating that the mitogenic activity of TGL-E originates from subunit α. TGL-E stimulated T cell enriched spleen cells which passed through a nylon column, but not spleen cells from a nude mouse or spleen cells treated with anti-Thy 1·2 antibody and complement. Thus, TGL-E stimulates only mouse T cells but not B cells. The other lectin in tulip bulbs, *Tulipa gesneriana* lectin-yeast showed no mitogenic activity on mouse spleen, thymus cells or human paripheral blood lymphocytes.**

Keywords mitogenic activity; *Tulipa gesneriana*; lectin; mouse spleen; mouse thymus; human peripheral blood lymphocyte; T cell; B cell

Introduction

Lectins are carbohydrate-binding proteins (glycoproteins) which agglutinate various cells. Some lectins are well known to stimulate quiescent, nondividing lymphocytes into a state of growth and proliferation. They have been widely used for studying transmembrane control of lymphocyte activation.

We have isolated two different lectins, *Tulipa gesneriana* lectin-erythrocyte (TGL-E)[1] and *Tulipa gesneriana* lectin-yeast (TGL-Y)[2] in tulip bulbs. TGL-Y is a D-mannose specific lectin and agglutinates the yeasts of the *Saccharomyces* genus. TGL-E agglutinates mouse erythrocytes and is thought to recognize complex carbohydrate structures.

In this study we examined mitogenic activities of the two lectins on mouse and human lymphocytes.

Experimental

Materials Concanavalin A (Con A), peanut agglutinin (PNA) and lipopolysaccharide (LPS, *E. coli* 055: B5) were purchased from Sigma, EY Laboratories and Difco Laboratories, respectively. Sugars and glycoproteins used were products of Sigma.

Preparation of TGL-Y, TGL-E and Its Subunits TGL-Y and TGL-E were purified by affinity chromatography as described previously.[1,2] Subunits of TGL-E were separated on a Sepharose CL-6B column equilibrated with 6 M guanidine·HCl.[2]

Animals BALB/c male mice (Shimizu Jitsuken Doubutsu) 5 to 10 weeks old were used.

Preparation of Lymphocytes Mouse spleen and thymus cells were prepared as described previously.[3] To obtain T cell-depleted spleen cells, spleen cells were treated with alloantiserum specific for Thy 1·2 and Low-tox rabbit complement, and viable cells were separated from dead cells using Lympholyte-M (all three materials were from Cedelane Laboratories). T cells were purified from spleen cells by passage on a nylon wool (Wako Pure Chemical Industries) column.[4] Fractionation of mouse thymus cells into mature and immature cells was carried out according to the methods of Reisner *et al.*[5] Human peripheral blood lymphocytes were obtained from heparinized blood samples of healthy volunteers.

Measurement of Mitogenic Activity Measurement of mitogenic activity was carried out as described previously.[3] Briefly, cultures were pulsed with 0.5 μCi of [^{3}H]thymidine (Amersham, 28.4 Ci/mmol) during the last 24 h of a 72-h culture period, were then harvested onto glass fiber filters, and their radioactive incorporation was determined. For measurement of mitogenic activity toward human peripheral lymphocytes, a mixture of human heparinized blood (0.1 ml) and RPMI 1640 medium containing mitogen (0.9 ml) was incubated and the incorporation of [^{3}H]thymidine was determined in the manner described above. Con A was used as a T cell mitogen. LPS and Pokeweek mitogen (PWM, EY Laboratories) were used as B cell mitogens for mouse and human lymphocytes, respectively. Data are represented as mean dpm of triplicate cultures.

Results

Mitogenic Activity of *Tulipa gesneriana* Lectins toward Mouse Spleen, Thymus Cells and Human Peripheral Blood Lymphocytes TGL-E showed a potent mitogenic activity which was comparable to Con A toward mouse spleen cells (Fig. 1) and human peripheral blood lymphocytes (Fig. 2). The optimal doses were 2 μg/ml in mouse spleen cells and 1 μg/ml in human periperal blood lymphocytes. TGL-E, on the contrary, exhibited only a weak activity toward mouse thymus cells, and a higher dose was required for stimulation of thymus cells than for spleen cells (Fig. 3). Subunit α had the same mitogenic activity as that of native lectin, but subunit β and TGL-Y had no activity on mouse spleen, thymus cells and human peripheral blood lymphocytes at a concentration of 1—100 μg/ml (data not shown).

Mitogenic Activity of TGL-E and Its Subunits toward Various Mouse Spleen Cells TGL-E showed no mitogenic activity on mouse spleen cells treated with anti-Thy 1·2 and complement which depleted T cells, or on spleen cells from nude mouse which contained only B cells (Table I). In contrast, it strongly stimulated T cell-rich spleen cells

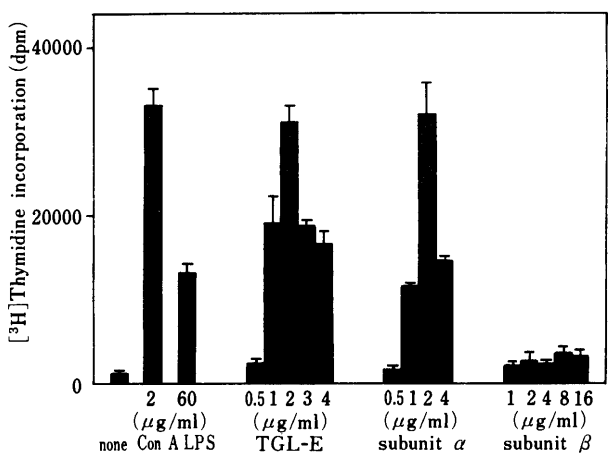

Fig. 1. Mitogenic Activities of TGL-E and Its Subunits on Mouse Spleen Cells

Con A and LPS were used as T cell and B cell mitogen, respectively.

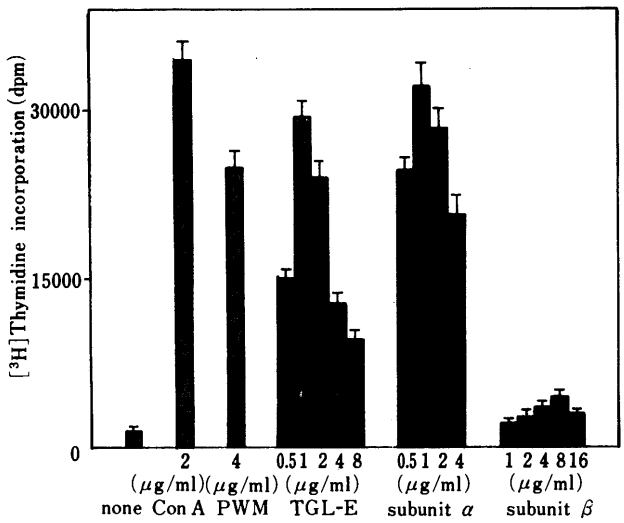

Fig. 2. Mitogenic Activities of TGL-E and Its Subunits on Human Peripheral Lymphocytes

PWM (Pokeweed Mitogen) was used as a B cell mitogen.

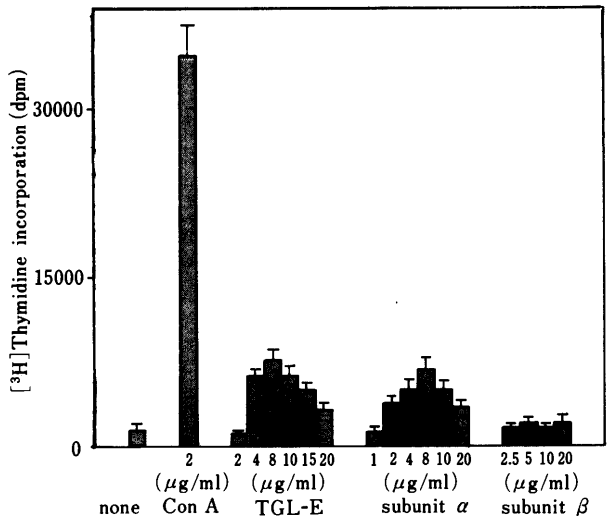

Fig. 3. Mitogenic Activities of TGL-E and Its Subunits on Mouse Thymus Cells

TABLE I. Mitogenic Activity of TGL-E on Various Mouse Spleen Cells

Mitogen	[3H]Thymidine incorporation (dpm)			
	None-treated spleen cells	Anti-Thy 1.2+ complement[a] treated spleen cells	Nude mouse spleen cells	Nylon- column passed[b] spleen cells
None	1373± 144	801±107	1375± 208	1391±204
Con A (1 μg/ml)	33315±1922	1602±162	1894± 350	19349±573
LPS (60 μg/ml)	13517± 752	24287±306	38315±1169	2209±103
TGL-E (2 μg/ml)	31304±2076	964± 76	1349± 95	15437±500

a) T cells were lysed with anti-Thy 1.2 and complement. *b*) To obtain T cells, B cells were removed by passing spleen cells through a nylon column.[4] Data are represented as mean±S.E. of triplicate cultures.

which had been passed through a nylon wool column. The same results were obtained in subunit α (data not shown). These results indicated that TGL-E and subunit α stimulated T cells but not B cells.

Mitogenic Activity of TGL-E toward Thymus Cells Fractionated with Peanut Agglutinin The mouse thymus

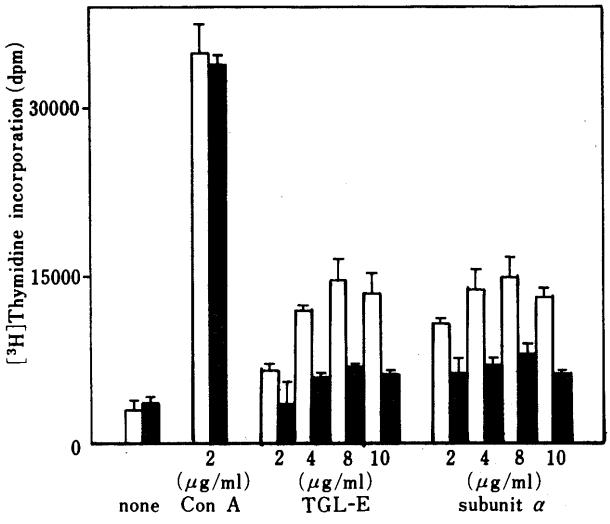

Fig. 4. Mitogenic Activities of TGL-E and Subunit α on Mouse Thymus Cells Fractionated by PNA

■, mouse thymus cells agglutinated by PNA; □, mouse thymus cells unagglutinated by PNA.

contains at least two different subpopulations of mature and immature lymphocytes. These can be readily separated by agglutination with PNA.[5] We examined the mitogenic activity of TGL-E on these two subpopulations. The results are shown in Fig. 4. Con A well stimulated both PNA-agglutinated and unagglutinated cells, while TGL-E and subunit α showed a more potent mitogenic activity toward PNA-unagglutinated (mature) cells than toward PNA-agglutinated (immature) cells.

Inhibitory Effect of Various Sugars and Glycoproteins on the Mitogenic Activity of TGL-E The inhibitory effect of various sugars and glycoproteins on the mitogenic activity of TGL-E toward mouse spleen cells was examined. Only thyroglobulin (bovine) which is a potent inhibitor of agglutinating activity of the lectin showed an inhibition: 50% inhibition at a concentration of 600 μg/ml (data not shown). The other sugars and glycoproteins listed below showed no inhibition at a concentration of 100 mM and 1000 μg/ml, respectively. Sugars: D-glucose, D-mannose, D-galactose, L-fucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine and *N*-acetylneuraminic acid. Glycoproteins: thyroglobulin (bovine), mucins (bovine), orosomucoid (human), fetuin (calf), transferrin (bovine), ovomucoid and ovalbumin.

Discussion

Subunit α of TGL-E showed potent mitogenic activity on mouse and human lymphocytes as well as native lectin, but the subunit β had no mitogenic activity indicating that the mitogenic activity originates from subunit α. In contrast, each subunit alone has only a very weak hemagglutinating activity and both subunits are required to exhibit the full hemagglutinating activity of the lectin.[2]

TGL-E was stable in 0.2 M acetic acid, but subunit α completely lost mitogenic activity when placed in 0.2 M acetic acid at room temperature for 2 h (data not shown), suggesting that subunit β stabilizes the structure of the lectin molecule.

As many lectins with mitogenic activity, TGL-E stimulated only T cells. In addition, TGL-E showed a potent

activity to PNA-nonagglutinated thymus cells, mouse spleen cells and human peripheral blood lymphocytes which are thought to contain functionally mature T cells; however, it had only weak activity on mouse thymus cells, about 90% of which are immature cells.[6,7] There was therefore an indication that TGL-E preferentially stimulates mature T cells. *Phaseolus vulgaris* agglutinin was reported to have a similar mitogenic activity.[5,6]

Con A, *Lens culinaris* lectin, and *Pisum sativum* lectin are known to be D-mannose specific lectins which agglutinate yeasts. All of them have mitogenic activity on lymphocytes.[8-10] TGL-Y is also a D-mannose specific lectin and agglutinates yeasts, however, TGL-Y showed no mitogenic activity. This is apparently due to the difference in sugar specificity of TGL-Y and those of the other D-mannose specific lectins. We are now studying the sugar specificity of TGL-Y in detail.

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Inhibitory Effects of 3,3′,4,5′-Tetrahydroxystilbene and 3,3′,4,5′-Tetrahydroxybibenzyl, the Constituents of *Cassia garrettiana* on Antigen-Induced Histamine Release *in Vitro*

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3,3′,4,5′-Tetrahydroxystilbene (I) and 3,3′,4,5′-tetrahydroxybibenzyl (II), isolated from the heartwood of *Cassia garrettiana* CRAIB (Leguminosae), showed inhibitory effects on antigen-induced histamine release from rat peritoneal mast cells *in vitro*. The inhibitory effect of I ($IC_{50}=30.2\,\mu M$) was much stronger than that of II ($>100\,\mu M$). Compound II, as well as I ($IC_{50}=7.3\,\mu M$) reported previously, also inhibited the histamine release from human peripheral basophils induced by anti-immunoglobulin E (IgE) *in vitro*, and its IC_{50}-value was 68.0 μM. These results suggest that the *trans*-olefin structure in the molecule may be necessary for I to have an inhibitory effect on histamine release. Considering that disodium cromoglycate did not show any significant inhibitory effect on anti-IgE-induced histamine release from human basophils, the strong inhibitory effects of I in both tests are of considerable interest.

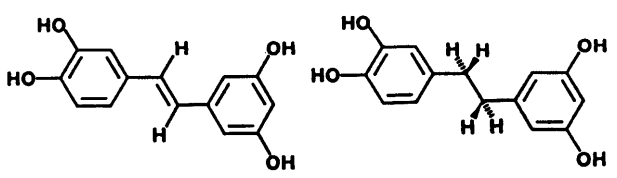
Keywords 3,3′,4,5′-tetrahydroxystilbene; 3,3′,4,5′-tetrahydroxybibenzyl; oxystilbene-related compound; *Cassia garrettiana*; histamine release; rat peritoneal mast cell; human peripheral basophil; disodium cromoglycate; theophylline

3,3′,4,5′-Tetrahydroxystilbene (I, Chart 1) and 3,3′,4,5′-tetrahydroxybibenzyl (II, Chart 1), constituents of *Cassia garrettiana* CRAIB (Leguminosae),[1] were already reported to demonstrate antimicrobial activity,[2] phytogrowth-inhibitory activity,[2] a hypotensive effect on rats[3] and coronary vasodilator action on isolated guinea-pig heart.[3] It has also been found that the above-mentioned activities of 3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene derived from I were much higher than those of I.[4,5] Recently, the authors reported that I showed strong inhibitory effect on the histamine release from human peripheral basophils.[6] However, no work has yet been done on the inhibitory effects of either compound against antigen-induced histamine release from rat peritoneal mast cells. The release of histamine from isolated mast cells is a useful *in vitro* model for studying allergic diseases. There has been no report on the inhibitory activity of II, which is the dihydro-form of I, on anti-immunoglobulin E (IgE)-induced histamine release from human peripheral basophils.

In this work, as a preliminary step toward developing anti-allergic agents, the inhibitory effects of I and II, and II on antigen-induced histamine release from rat peritoneal mast cells and human peripheral basophils, respectively, were examined. The correlation between the structures of I and II and their inhibitory effects is discussed.

Materials and Methods

Materials 3,3′,4,5′-Tetrahydroxystilbene (I) and 3,3′,4,5′-tetrahydroxybibenzyl (II)[1] (Chart 1) were isolated from the heartwood of *Cassia garrettiana* CRAIB (Leguminosae). Disodium cromoglycate (Funakoshi Chemical Co., Ltd.) and theophylline (Nacalai Tesque Inc.) were used as positive controls.



3,3′,4,5′-tetrahydroxystilbene (I) 3,3′,4,5′-tetrahydroxybibenzyl (II)

Chart 1

Animals Male Std: Wistar rats (Japan SLC, Inc.), weighing 150—300 g, were used.

Histamine Release from Rat Peritoneal Mast Cells The test was carried out using peritoneal mast cells. Mast cells were obtained from the peritoneal cavity according to the modified method of Kusner *et al.*[7] Rats were sensitized by intraperitoneal injection of 2 ml/animal 2:1 diluted rat egg-albumin anti-serum (the titer was 1:34). At 20 h after sensitization, Hank's solution (10 ml) containing human albumin (1 mg/ml) and heparin (2 units/ml) was intraperitoneally injected into these rats, then, peritoneal exudate cells (PEC) were collected. After the PEC were washed three times with Hank's solution, the mast cells were suspended in the same solution to yield 2×10^4 cells/ml. Mast cells were also determined by the toluidine blue staining test. A reaction mixture containing a cell suspension (1.7 ml) and a test compound solution (0.2 ml) was incubated for 5 min at 37 °C. Thereafter, egg-albumin (60 μg/100 μl) was added and incubation was continued for another 10 min. The reaction was terminated by cooling at 4 °C and the reaction mixture was centrifuged at 2000 rpm. After the removal of protein with 3% perchloric acid, the histamine in the supernatant and precipitate was measured using the fluorometric method.[8,9] The IC_{50}-value was calculated from the regression equation plotted for the mean inhibitory rate from three rats versus concentration. Neither compounds alone produced histamine release. Statistical analyses were performed with the Student's *t*-test.

Histamine Release from Human Peripheral Basophils A leukocyte fraction containing basophils (Alcian blue staining), separated from whole blood of healthy donors by the dextran sedimentation method, was used as the basophil sample. Leukocytes were suspended in Tris buffer (25 mM Tris, 120 mM KCl, 0.6 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.03% human albumin, pH 7.6) to yield concentrations of $1—10\times10^6$ cells/ml. These 1 ml suspensions were incubated with various concentrations of a test compound solution of 0.1 ml for 15 min at 37 °C. Thereafter, 0.1 ml of anti-human-IgE ($1:1.2\times10^4$; affinity purified from goat anti-serum; International Immunology Corporation, U.S.A.) was added and incubation was continued for another 45 min. Histamine was measured using the fluorometric method.[8,9] The IC_{50}-value was calculated from the regression equation plotted for the mean inhibitory rate from three donors versus concentration. The test compound alone did not produce histamine release.

Results

Effects of 3,3′,4,5′-Tetrahydroxystilbene (I) and 3,3′4,5′-Tetrahydroxybibenzyl (II) on Antigen-Induced Histamine Release from Rat Peritoneal Mast Cells The effects of I and II on antigen-induced histamine release from rat peritoneal mast cells were tested. As shown in Table I, I showed strong inhibitory activity on antigen-induced histamine release from cells. This compound exhibited over 90% inhibition of histamine release at a concentration of

TABLE I. Inhibitory Effects of 3,3',4,5'-Tetrahydroxystilbene (I) and 3,3',4,5'-Tetrahydroxybibenzyl (II) on Antigen-Induced Histamine Release from Rat Peritoneal Mast Cells *in Vitro*

Compound	Concentration (μM)	No. of[a] experiments	Histamine release		IC_{50}[b] (μM)
			Mean (%)±S.E.	Inhibition (%)	
Vehicle		3	26.7±1.2		
I	10	3	22.1±0.5[e]	17.2	
	30	3	15.0±0.6[e]	43.8	30.2
	100	3	0.9±0.3[e]	96.6	
Vehicle		3	26.7±1.2		
II	10	3	25.2±1.3	5.6	
	30	3	23.3±0.8[d]	12.7	>100
	100	3	17.2±1.5[e]	35.6	
Vehicle		3	42.0±2.2		
DSCG[c]	10	3	20.8±2.6[e]	50.5	*ca.*10

a) Each concentration consisted of 3 tubes per experiment. *b*) 50% inhibitory concentration. *c*) Disodium cromoglycate. *d*) $0.01<p<0.05$ and *e*) $p<0.01$, significantly different from the vehicle control.

TABLE II. Inhibitory Effects of 3,3',4,5'-Tetrahydroxybibenzyl (II) on Anti-IgE-Induced Histamine Release from Human Peripheral Basophils *in Vitro*

Compound	Concentration (μM)	No. of[a] donors	Histamine release		IC_{50}[b] (μM)
			Mean (%)±S.E.	Inhibition (%)	
Vehicle		3	51.5±2.8		
II	10	3	45.7±3.4	11.3	
	30	3	37.2±3.3[c]	27.8	68.0
Vehicle		4	53.2±2.6		
II	100	4	21.0±2.8[d]	60.5	
Vehicle		2	40.8±7.4		
Theophylline	300	2	12.2±4.0	70.1	<300

a) Each concentration consisted of 2 to 4 tubes per donor. *b*) 50% inhibitory concentration. *c*) $0.01<p<0.05$ and *d*) $p<0.01$, significantly different from the matched vehicle control.

100 μM, and its IC_{50}-value was 30.2 μM. The inhibitory effect of I was weaker than that of disodium cromoglycate (DSCG), which was used as a standard. On the other hand, the inhibitoy activity of II ($IC_{50}=>100$ μM) was much lower than that of I.

Effect of II on Anti-IgE-Induced Histamine Release from Human Peripheral Basophils *in Vitro* The effect of II on anti-IgE-induced histamine release from human basophils was examined. As shown in Table II, II, at a concentration of 100 μM, showed more than 60% inhibition of histamine release from this cell, and its IC_{50}-value was 68.0 μM. This inhibitory activity of II was much higher than that of theophylline as a standard. On the other hand, DSCG showed no inhibitory activity on histamine release even at the high concentration of 1000 μM.[6]

Discussion

It was found that 3,3',4,5'-tetrahydroxystilbene (I)[6] and 3,3',4,5'-tetrahydroxybibenzyl (II) had strong inhibitory effects on histamine release from human peripheral basophils induced by anti-IgE and from rat peritoneal mast cells induced by antigen. In particular, the inhibitory effect of I on histamine release from both the cells was strong and its IC_{50}-values were 7.3[6] and 30.0 μM, respectively. Considering that DSCG did not show any inhibitory effect on histamine release from human peripheral basophils,[6] such a strong inhibitory of I should be emphasized. Like other biological activities of the two compounds,[2-5] the inhibitory effect of I was again stronger than that of II. These suggest that the *trans*-olefin structure in the molecule may be necessary for I to show its biological activity. This consideration is also supported by the following findings; 1) tranilast, which has a partial structure in common with I, is clinically used as anti-allergic agent,[10] and 2) caffeic acid[11] and chlorogenic acid[11] containing phenolic hydroxyl groups and a *trans*-olefin structure in the molecule inhibit histamine release from rat peritoneal mast cells *in vitro*. However, the inhibitory effect of oxystilbene derivatives on histamine release from rat peritoneal mast cells *in vitro* is reported for the first time in this paper.

Studies of the mechanism of the inhibitory effects of both compounds on histamine release from both the cells are in progress, together with their chemical modification.

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Dissolution of Solid Dosage Form. III.[1] Equations for the Dissolution of a Nondisintegrating Single Component Tablet under a Nonsink Condition

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An equation was derived for the dissolution from the whole surface of a nondisintegrating single component tablet under a nonsink condition where the initial weight of a tablet is equal to the weight needed to saturate the solution. Also, equations for several dissolution manners of the tablet under the nonsink condition were derived in the postulation of a dominant dissolution rate constant which determines the dissolution manner. The applicability of these equations was examined by dissolution measurements with nondisintegrating single component tablets. Dissolution measurements of tablets prepared using the amount required to saturate the solution were conducted by masking their flat or side surface with adhesive tape in accordance with the conditions for the derivation of equations. These dissolution behaviors were treated by the use of derived equations to confirm the validity.

Among the derived equations, dissolution from the whole surface of a tablet was expressed in the same form as the negative two-thirds law equation for particles, and a nondisintegrating single component tablet compressed by the use of stuitable amount was thought to behave like a single crystal. Also, equations, *i.e.*, the negative two-thirds law and the negative square root law, thus derived were thought to be applicable for the dissolution of a nonspherical particle and crystal concerning the crystal's habit and its dissolution property.

Keywords tablet; dissolution; nonsink condition; cube root law; two-thirds law; square root law

Introduction

A rotating disk method is useful to determine an intrinsic dissolution rate constant. Also, the intrinsic dissolution rate constant can be obtained from the dissolution measurement of particles.[2] However, the value differs from method to method,[2a] and, as can be expected, the dissolution behavior of a whole tablet can not be predicted by these methods. Hence, basic equations under the sink condition for a nondisintegrating single component tablet were derived previously for practical use.[1] Dissolution measurements of a tablet under the sink condition may be useful for substances of relatively high solubility concerning the preparation of a tablet. However, these dissolution measurements may be difficult to apply to substances of relatively poor solubility, when preparing a tablet of a small amount. Thus, dissolution equations under a nonsink condition for a tablet was considered.

An equation for dissolution from the whole surface of a nondisintegrating single component tablet under a nonsink condition where the initial weight of the tablet is the weight required to saturate the solution was derived in the postulation of isotropic dissolution. Also, dissolution equations for various dissolution manners of the tablet under the nonsink condition were derived by assuming a dominant dissolution rate constant which defines the dissolution manner as reported previously.[1] Equations thus derived were examined by the dissolution measurement of a nondisintegrating single component tablet under conditions in accordance with the derivation of the equations.

Experimental

Materials Salicylic acid (guaranteed reagent grade, Wako Pure Chemical Ind., Ltd.: abbreviated as SA) was used.

Dissolution Measurement of Tablet A suitable amount of SA required to saturate the solution (1.70 g)[1] was compressed at a pressure of 2 t to make a model flat faced tablet of 16.0 mm diameter and 6.5 mm thickness. The dissolution test was carried out following the method of the previous paper.[1] The tablet used was masked with adhesive tape in accordance with the condition for the derivation of the dissolution equation. A dissolution apparatus, type NTR-VS3 (Toyama Sangyo Co., Ltd.) was used. As a solvent 1000 ml of 0.1 N HCl (pH 1.0) was used. The dissolution measurement was carried out at a paddle rotation speed of 250 rpm at 25 °C. The concentration was estimated from the absorbance at 302.5 nm by a flow cell set in a type 200-20 spectrophotometer (Hitachi Ind., Co.) where the concentration was relatively low. In a higher concentration range, 1 ml of the solution was sampled. After diluting the solution with the solvent, the absorbance was measured by the use of a type UV-160 spectrophotometer (Shimadzu Ind., Co.).

Results and Discussion

Equations for Several Dissolution Manners Usually, according to the film theory, the dissolution rate is expressed by[4]:

$$dC/dt=(k/V)S(C_s-C) \quad (1)$$

where k, S, V, C_s and C are the dissolution rate constant (= diffusion coefficient/the thickness of the diffusion layer), the effective surface area, the volume of solvent, the saturation concentration and the concentration at time t, respectively. The equation was applied to the dissolution of a nondisintegrating single component tablet.

Denoting by W_s the weight required to saturate the solution, by W_o the initial weight of tablet and by W the weight of undissolved tablet at time t, Eq. 1 can be rewritten as:

$$-dW/dt=(k/V)S\{W_s-(W_o-W)\} \quad (2)$$

When the effective surface area changes with dissolution time and W_o is equal to W_s, Eq. 2 can be simplified as:

$$-dW/dt=(k/V)S(t)W \quad (3)$$

where $S(t)$ is the effective surface area at time t. Introducing W_s to extinguish the solvent volume (V), Eq. 3 is transformed as:

$$-dW/dt=kC_s\{S(t)/W_s\}W \quad (4)$$

and it shows that dissolution can be estimated by the changes in the effective surface area.

Hence, equations for some simplified dissolution manners

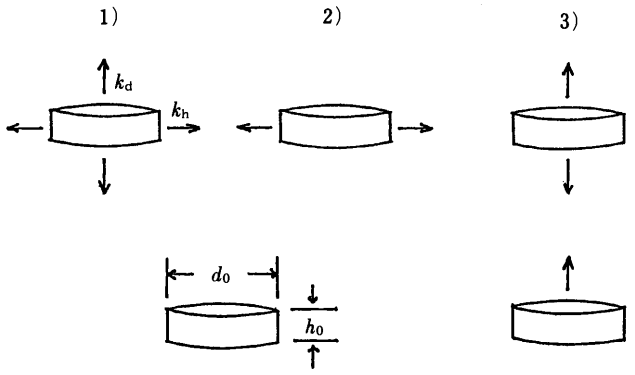

Fig. 1. Dissolution Manners of Tablet
1), $k=k_h=k_d$; 2), $k=k_h \gg k_d$; 3), $k=k_d \gg k_h$.

as illustrated in Fig. 1 were derived as follows. Here, h_0 and d_0 are the initial thickness and diameter of the tablet, respectively, and the initial surface areas are represented by $S_h=\pi d_0 h_0$ and $S_d=\pi d_0^2/4$ for the side and a single flat surface, respectively. Also, k_h and k_d are the dissolution rate constants, and are defined by the dissolution from the side surface and flat surface of the tablet, respectively. Hence, these illustrated dissolution manners were classified in terms of k_h and k_d, and equations were derived by estimating the effective surface area as follows.

1) $k=k_h=k_d$ In this case, dissolution of the tablet occurs isotropically from the whole surface, *i.e.*, the two flat and curved side surfaces, and the thickness and diameter of the tablet decrease with dissolution time. Since the size of the tablet was postulated to decrease isotropically, the thickness and diameter of the tablet kept the equation

$$h/h_0=d/d_0 \tag{5}$$

during dissolution. Here, h and d are the thickness and diameter of the tablet at time t, respectively. The effective surface area of the tablet at time t is expressed as[1]:

$$S(t)=2(\pi d^2/4)+\pi dh \tag{6}$$

$$S(t)=(2S_d+S_h)(W/W_s)^{2/3} \tag{7}$$

Here, $2S_d+S_h$ is the initial effective surface area (S_0) of the tablet. By inserting Eq. 7, Eq. 4 is transformed as:

$$-\mathrm{d}W/\mathrm{d}t=kC_s\{(2S_d+S_h)/W_s\}W_s^{-2/3}W^{5/3} \tag{8}$$

Integrating Eq. 8, the following two-thirds law equations are obtained.

$$(3/2)W^{-2/3}=(3/2)W_s^{-2/3}+kC_s\{(2S_d+S_h)/W_s\}W_s^{-2/3}t \tag{9}$$

$$(W_s/W)^{2/3}=1+(2/3)kC_s\{(2S_d+S_h)/W_s\}t \tag{10}$$

On the other hand, the two-thirds law equation applied for dissolution of particles can be expressed by[2c,5]:

$$(M_s/M)^{2/3}=1+(4kC_s/\rho fD)t \tag{11}$$

where M_s is the initial amount, M is the undissolved amount at time t, ρ is the density, f is Carman's shape factor and D is the initial particle diameter. By using specific surface area ($S_{sp}=6/\rho fD$), Eq. 11 can be transformed as:

$$(M_s/M)^{2/3}=1+(2/3)kC_sS_{sp}t \tag{12}$$

When the initial effective surface area $S_0(=S_{sp}M_s)$ is used in place of S_{sp}, Eq. 12 can be expressed as:

$$(M_s/M)^{2/3}=1+(2/3)kC_s(S_0/M_s)t \tag{13}$$

The equation is expressed by the initial surface area and amount of particles independent of the particle shape factor.

Thus, the dissolution process from the whole surface of the tablet is expressed by the same from as to the two-thirds law or negative two-thirds law equation for particles in terms of the initial effective surface area and the weight of the tablet.

2) $k=k_h \gg k_d$ In this case, dissolution occurs more dominantly from the curved side surface than the flat surface of the tablet, and the diameter decreases with dissolution time while the thickness (h_0) keeps constant during dissolution. Then $S(t)$ is expressed as follows:

$$S(t)=\pi dh_0 \tag{14}$$

By inserting Eq. 5, $S(t)$ is given as the following equation, since $W/W_s=(d/d_0)^2$.

$$S(t)=\pi(dd_0/d_0)h_0$$
$$=S_h(W/W_s)^{1/2} \tag{15}$$

Hence, by inserting Eq. 15, Eq. 4 is transformed as follows.

$$-\mathrm{d}W/\mathrm{d}t=kC_s(S_h/W_s)W_s^{-1/2}W^{3/2} \tag{16}$$

$$W^{-1/2}=W_s^{-1/2}+(1/2)kC_s(S_h/W_s)W_s^{-1/2}t \tag{17}$$

$$(W_s/W)^{1/2}=1+(1/2)kC_s(S_h/W_s)t \tag{18}$$

Thus, the dissolution process is expressed by the square root law or negative square root law equation in terms of the initial effective surface area ($S_0=S_h$) and the weight of the tablet.

3) $k=k_d \gg k_h$ (a) When dissolution occurs from both flat surfaces of the tablet, the thickness decreases with dissolution time while the diameter (d_0) of the tablet keeps constant. Then $S(t)$ remains the initial effective surface area during dissolution, and is given by:

$$S(t)=2(\pi d_0^2/4)=2S_d \tag{19}$$

and Eq. 4 can be transformed as:

$$-\mathrm{d}W/\mathrm{d}t=kC_s(2S_d/W_s)W \tag{20}$$

By integrating Eq. 20 and rearrangement, the following equations expressed in terms of the initial effective surface area ($S_0=2S_d$) and the weight of the tablet is obtained.

$$\ln W=\ln W_s-kC_s(2S_d/W_s)t \tag{21}$$

$$\ln(W_s/W)=kC_s(S_0/W_s)t \tag{22}$$

(b) When dissolution occurs from a single flat surface, $S(t)$ is equal to $S_d(=S_0)$, and dissolution equation can be expressed by the same form of Eq. 22.

Equation 22 derived in the same manner applied for derivation of Eqs. 10 and 18 can be rewritten as:

$$\ln\{C_s/(C_s-C)\}=(k/V)S_0t \tag{23}$$

and is a familiar equation for the dissolution by means of the rotating disk method.

Thus, various simplified dissolution manners for a tablet can be expressed by the two-thirds law, square root law and semilogarithmic equations in terms of the initial effective surface area and the weight of the tablet.

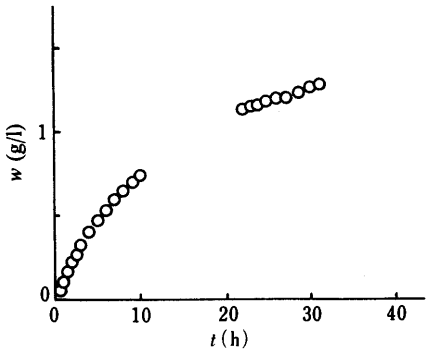

Fig. 2. Dissolution Curve from Whole Surface of Tablet

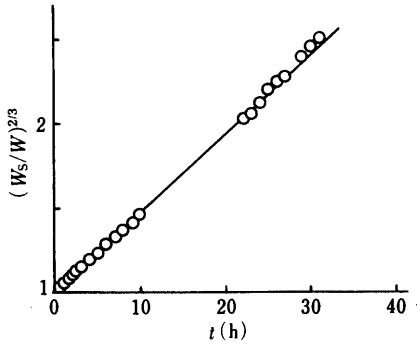

Fig. 3. Applicability of Eq. 10

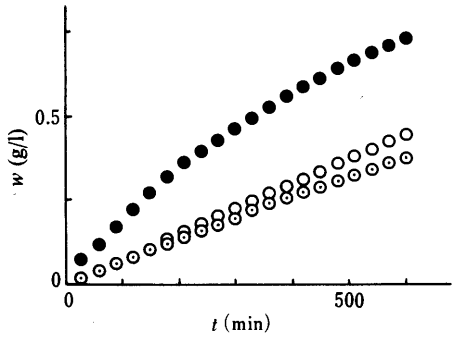

Fig. 4. Dissolution Curves for Various Dissolution Manners
●, $k=k_h=k_d$; ⊙, $k=k_h \gg k_d$; ○, $k=k_d \gg k_h$.

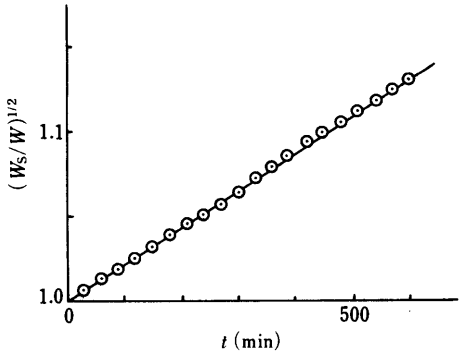

Fig. 5. Applicability of Eq. 18

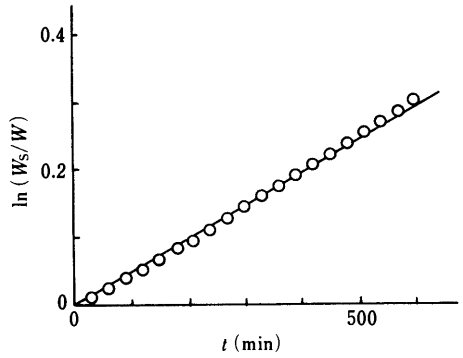

Fig. 6. Applicability of Eq. 22

Applicability of Equations Equations derived for various dissolution manners of a nondisintegrating single component tablet were examined by dissolution measurement.

1) $k=k_h=k_d$ Dissolution measurement was carried out by the use of a single component tablet without masking any surface in accordance with the derivation condition for Eq. 10, and dissolution occurs from the whole surface of the tablet. The result obtained was shown in Fig. 2, and the applicability of Eq. 10 was examined as shown in Fig. 3.

As can be seen in Fig. 3, the dissolution process of the single component tablet was expressed as a straight line up to 30 h. The amount dissolved at that time was about 75% of the initial amount of the tablet. Hence, it was suggested that Eq. 10 adequately expresses the dissolution behavior from the whole surface of a nondisintegrating single component tablet compressed with a suitable amount. The dissolution rate constant (k) estimated from the slope of the straight line was 0.159 cm/min for salicylic acid. The k-value was smaller than that obtained by dissolution under the sink condition with a small tablet, *i.e.*, 0.193 cm/min.[1] The difference in these k-values was supposed to be caused by the movement of the tablet or hydrodynamic effect, and it was confirmed that prediction or simulation of the dissolution process requires the dissolution rate constant estimated from a proper or reasonable comparable experimental method.

2) $k=k_h \gg k_d$ The dissolution measurement was carried out with a tablet in which both flat surfaces were masked. The result obtained was shown in Fig. 4, and the applicability of Eq. 18 was examined as shown in Fig. 5. The dissolution curves of different dissolution manners were also shown in Fig. 4 for comparison.

The dissolution process up to about 10 h can be well expressed by Eq. 18, and the dissolution rate constant was estimated as 0.135 cm/min.

3) $k=k_d \gg k_h$ A tablet whose side surfaces was masked was used for dissolution measurement. The dissolution results and the applicability of Eq. 22 were shown in Figs. 4 and 6, respectively. The dissolution process fitted well Eq. 22, and the dissolution rate constant estimated from the slope of the straight line was 0.115 cm/min.

The dissolution rate constants obtained by the various dissolution manners showed different values. Also, these values were smaller than those obtained from each corresponding dissolution manner under the sink conditions.[1] These differences were supposed to have arisen mainly from the difference in the motion of the tablet, because dissolution measurements by means of a rotating disk method and particle method gave different dissolution rate constants.[2a] Nevertheless, when the tablet dissolved in the defined manner, the dissolution process was well expressed by the corresponding equation.

Thus, the equations derived for several dissolution

manners could be expressed in terms of the initial effective surface area (S_0) and the weight (W_0) of the tablet, and were experimentally proven to be reasonable. When dissolution occurs from the whole surface of the tablet, the dissolution process can be explained by the two-thirds law equation, and a suitable amount of a single component tablet was revealed to behave as if it were a single crystal. Also, equations derived for the dissolution from a specific surface were thought to be useful for the examination of the crystal's habit and its dissolution property as an extended application, as were equations derived for dissolution under the sink condition.[1)]

When a crystal's habit and its dissolution property were treated with simplified or idealized crystal as described in the previous paper,[1)] the same dissolution equations could be obtained in accordance with the dissolution manner.

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Transport to Intestinal Lumen and Peritoneal Cavity of Intravenously Administered Aprindine in Rats

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Transfer of aprindine from the blood into the intestinal lumen or into the peritoneal cavity was examined after intravenous administration of the drug at a dose of 5 mg/kg in rats. The amount of the drug transferred from the blood into the intestinal lumen was much greater than into the peritoneal cavity. The average amounts of aprindine transported into the intestinal lumen and the peritoneal cavity were 0.12 and 0.03% of the dose (5 mg/kg) in 120 min, respectively. Thus, a notable difference in the clearance values of the drug was obtained between the intestinal lumen (14.8 ml/h) and the peritoneal cavity (4.94 ml/h). The net water flux showed that secretion predominated in the peritoneal transport while absorption overbalanced secretion in the intestinal transport. It seems likely that a solvent drag effect by water movement did not contribute much to the transport of aprindine from the blood to the intestinal lumen or the peritoneal cavity. The differences in transport across the two membranes could be due to differences in the surface area and other geometrical factors. Differences could also be due to a difference in the pharmacologic effects of the drug which causes a decrease in tissue splanchnic perfusion.

Keywords aprindine; intestinal lumen; peritoneal cavity; intestinal clearance; transfer rate; serum concentration; rat; solvent drag; water movement

Introduction

Aprindine is an antiarrhythmic drug with efficacy in the management of ventricular and supraventricular arrhythmia.[1] The drug exhibits a narrow therapeutic range and a marked interindividual variability in the steady state.[1] Moreover, it has displayed non-linear pharmacokinetic behavior.[2] Thus, it is a significant drug in view of overdoses.

In acute overdoses, orally administered activated charcoal has been employed to inhibit the absorption of excess drugs from the gastrointestinal (g.i.) lumen into the blood. Recently, it has been noted that the clearance of drugs which were parenterally administered or were already absorbed into the systemic circulation was accelerated by orally administered activated charcoal.[3-5] The g.i. mucous membranes have a large surface area when considered as a whole. In particular, the total absorptive and/or exsorptive area of the small intestine has been calculated to be about 200 m^2 in an adult human, and is far larger than that of the peritoneal membrane (about 2 m^2).[6] Accordingly, if the g.i. mucous membranes can act as a dialysis membrane, a considerable amount of drug would be expected to enter the g.i. lumen by passive diffusion in drug overdoses. However, g.i. dialysis has not yet been studied as extensively as peritoneal dialysis in spite of involvement of a similar mechanism *i.e.* diffusion according to concentration gradients between the blood (a region of a higher drug concentration) and the g.i. lumen or the peritoneal cavity (a region of a lower drug concentration).

The present study was thus undertaken to compare the removal efficacy of g.i. dialysis with that of peritoneal dialysis, which has been generally established as a valuable means of hemopurification in drug overdoses.

Experimental

Materials Aprindine hydrochloride was kindly supplied by Mitsui Pharmaceuticals, Inc. (Tokyo, Japan), and imipramine hydrochloride was purchased from Nakarai Tesque (Kyoto, Japan). Scintillation fluid (Instagel) was from United Technologies (Packard, Illinois, U.S.A.). All other chemicals used in this study were of analytical grade.

Transfer Experiments Male Wistar rats, weighing 220—290 g, were fasted overnight with free access to water. Intestinal exsorption experiments were performed using an *in situ* single-pass perfusion technique reported previously.[7] Lactated Ringer's solution (pH 6.5, 37 °C) was perfused at a rate of 1.3 ml/min from the duodenum through the small intestine to the ileocecal junction using a perfusion pump. Aprindine was injected over about 1 min at a dose of 5 mg/kg into the right femoral vein. After injection, blood samples and dialysate were collected periodically. In the case of peritoneal transfer experiments, a small incision was made in the abdomen and 20 ml of lactated Ringer's solution (37 °C) was injected into the peritoneal cavity. The dialysate was exchanged for a corresponding volume of new dialysate every 15 min. The procedure for administration of the drug and the collection time of samples were the same as in the *in situ* single-pass perfusion experiments.

Water Movement Water movement experiments were performed in the same manner as in the aprindine transfer experiments above. The rate of water movement from the blood into the g.i. lumen or peritoneal cavity and from the g.i. lumen or peritoneal cavity back to the blood was calculated from changes in concentrations in ^3H_2O (0.74 MBq) in the dialysate. Phenol red (10 μg/ml) was used as a nonabsorbable marker. The equations used in the calculation were as follows:

$$\mathrm{Flux}_{i,p\to b}=\frac{[\mathrm{PhR}]_0}{[\mathrm{PhR}]_t}\left(\frac{[^3H_2O]_0-[^3H_2O]_t}{[^3H_2O]_0}\right)Q$$

$$\mathrm{Flux}_N=\left(\frac{[\mathrm{PhR}]_0}{[\mathrm{PhR}]_t}-1\right)Q$$

$$\mathrm{Flux}_{b\to i,p}=Q+\mathrm{Flux}_N-\mathrm{Flux}_{i,p\to b}$$

where $\mathrm{Flux}_{i,p\to b}$ and $\mathrm{Flux}_{b\to i,p}$ are water flux from intestinal lumen or peritoneal cavity to the blood and water flux from the blood to intestinal lumen or peritoneal cavity, respectively. Flux_N is net water flux. $[\mathrm{PhR}]_0$ and $[\mathrm{PhR}]_t$ are concentrations of phenol red in the dialysates at time 0 and t, respectively. $[^3H_2O]_0$ and $[^3H_2O]_t$ are concentrations of tritiated water at time 0 and t, respectively. Q is the flow rate of the perfusate or the volume instilled in the peritoneal cavity per time t.

Analytical Methods Aprindine levels in the serum and dialysate were determined by sensitive gas chromatography according to the method of Kawano *et al.*[8] using imipramine as the internal standard. A Shimadzu GC-RIA gas chromatograph (Kyoto, Japan) equipped with a surface ionization detector and a flexible fused-silica megabore column (DB-17, film thickness 1.0 μm, J & S Scientific, U.S.A.) was used. Helium was used as a carrier and make-up gas. The operating temperatures were set as follows: injection port, 270 °C; column, 240 °C; detector, 270 °C. In the assessment of ^3H_2O concentrations, 5 ml of Instagel scintillation fluid was added to 200 μl of dialysate and then radioactivity was determined. Phenol red was determined spectrophotometrically by absorbance at a wavelength of 550 nm after addition of 4 ml of 1 M sodium hydroxide to a 0.5 ml aliquot of dialysate.

Pharmacokinetic Analysis The intestinal and peritoneal clearance values of aprindine were calculated by dividing the overall amount of the

drug transferred into both dialysates in 120 min by the appropriate value for an area under the serum concentration time curve of the drug obtained over the same period of time. The unpaired *t*-test was used to evaluate significant differences.

Results

Transport of Aprindine Figure 1 shows the transfer rate of aprindine from the blood into the g.i. lumen and the peritoneal cavity following intravenous administration at a dose of 5 mg/kg in rats. Aprindine was appreciably transported from the blood into both the intestinal lumen and peritoneal cavity with its transfer rates decreasing as the serum concentrations declined. As shown in Fig. 1, the transfer rate into the intestinal lumen was considerably higher than the rate into the peritoneal cavity. The amount of drug transferred into the intestinal and peritoneal dialysates was 0.12 and 0.03% of the total dose in 120 min, respectively.

To express the ability of each system to remove aprindine, intestinal and peritoneal clearances were calculated. A notable difference in the clearance values of the drug was observed between the intestinal lumen and the peritoneal cavity. The intestinal and peritoneal clearance of the drug were 14.8 and 4.94 ml/h, respectively, the former being significantly higher.

Water Movement Table I shows water movement into the g.i. lumen and peritoneal cavity after intravenous administration of aprindine at a dose of 5 mg/kg. Water movement from the blood into the g.i. lumen was less than that from the blood into the peritoneal cavity. The net water flux showed that secretion predominated in the peritoneal cavity, while absorption predominated in the intestinal lumen.

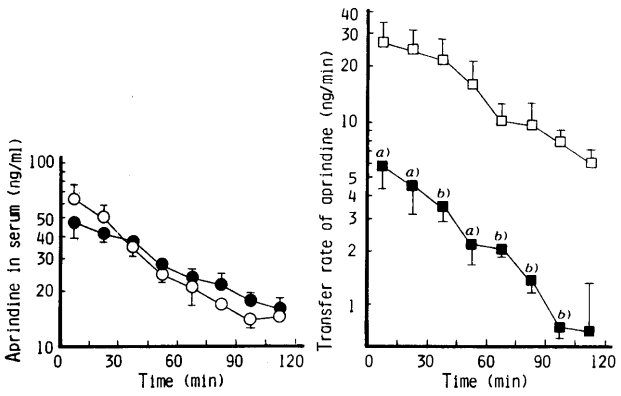

Fig. 1. The Transfer Rate of Aprindine from the Blood into the Intestinal Lumen (○, □) and the Peritoneal Cavity (●, ■) after Intravenous Administration at a Dose of 5 mg/kg to Rats

Each point represents the mean ± S.E.M. of 4 rats. *a*) $p<0.05$, *b*) $p<0.01$.

TABLE I. Water Flux into the Intestinal Lumen or the Peritoneal Cavity after Intravenous Administration of Aprindine (5 mg/kg) to Rats

	Water flux from blood to intestinal lumen or peritoneal cavity (ml/h)	Water flux from intestinal lumen or peritoneal cavity to blood (ml/h)	Net water flux[a] (ml/h)
Intestinal lumen	27.0 ± 1.19	31.5 ± 0.10	−4.51 ± 1.29
Peritoneal cavity	54.8 ± 1.71[b]	47.4 ± 1.46[b]	+7.38 ± 0.67[b]

Each value represents the mean ± S.E.M. of 4 rats. *a*) Positive (+) sign indicates secretion and negative (−) sign indicates absorption. *b*) $p<0.01$.

Discussion

Our results indicated that transport of aprindine into the intestine was greater than into the peritoneal cavity. Similar results have also been obtained using procainamide and *N*-acetylprocainamide, which have the same antiarrhythmic effect as aprindine.[9] We have previously shown that the amounts of both drugs transferred from the blood into the intestinal lumen were much greater than those into the peritoneal cavity. The average amounts of procainamide transferred in 2 h into the intestinal lumen and the peritoneal cavity were 12.7 and 1.7% of the total dose (10 mg/kg), respectively, while the same figures for *N*-acetylprocainamide were 3.5 and 1.4% of the total dose. Accordingly, it seems likely that in general, a smaller percentage of antiarrhythmic drugs are transferred into the peritoneal cavity than into the g.i. lumen. These results may support an early report that peritoneal dialysis is ineffective in enhancing the clearance of these drugs.[10]

In general, the rate of transfer across a membrane depends on geometrical factors of the membrane such as its surface area and thickness and the distribution of blood vessels within it or upon the pharmacological effects of a drug. The small intestine has a much larger surface area than the peritoneal membrane, as mentioned in the introduction. As far as area is concerned, the intestine is more suitable as a permeable or exsorbable organ than the peritoneum. The rate of transfer of a drug also depends upon physicochemical factors such as the extent of binding to serum proteins, distribution volume, lipophilicity and molecular size of the drug. The extent of binding of a drug to serum proteins is an important factor in permeation since it is well known that only an unbound drug can permeate through capillary walls into the g.i. tract. The reason why aprindine was exsorbed into the g.i. tract or the peritoneal cavity to a lesser extent than procainamide might be explained by aprindine's extensive binding to a serum protein (94—97%).[11] Furthermore, it is known that the apparent volume of distribution is considerably decreased in procainamide overdoses owing to a decrease in tissue splanchnic perfusion caused by hypotension.[12] Such a decrease in distribution volume in antiarrhythmic drugs may also reduce the transport of aprindine into the peritoneal cavity.

Several studies have demonstrated that the net water flux caused a drag effect on the permeability of some drugs.[13-17] For example, Kitazawa *et al.*[15] reported that absorption of sulfanilamide, sulfisoxazole, and metoclopramide increased with increasing transmucosal fluid movement from the lumen to the blood and decreased when the movement of water was directed from the blood to the lumen in rats. They have also reported that exsorption of sulfanilamide after intravenous administration was increased by increasing the tonicity of the perfusate, with the movement of water being directed from the blood to the lumen.[18]

During perfusion with lactated Ringer's solution in the present study, the net water fluxes were from the g.i. lumen to the blood and from the blood to the peritoneal cavity (Table I). This indicates that the effect of solvent drag

upon permeability from the blood to the intestinal lumen is less than on permeability from the blood to the peritoneal cavity, if transport from the blood across both membranes is in fact enhanced in each drug by solvent drag. More definitive experiments may be necessary to confirm this solvent drag effect of a drug by water movement.

In conclusion, it was shown that the intestinal lumen exhibits an exsorption/excretion function for aprindine in rats. The present findings confirm previous observations that antiarrhythmic drugs such as procainamide,[9] *N*-acetylprocainamide[9] and disopyramide[19] are greately transferred into the intestinal lumen. Consequently, it is expected that g.i. dialysis may be more useful than the peritoneal dialysis in the removal of antiarrhythmic drugs.

Acknowledgements This work was supported in part by Sankyo Life Science Research Foundation.

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Redispersible Dry Emulsion System as Novel Oral Dosage Form of Oily Drugs: *In Vivo* Studies in Beagle Dogs

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The absorption characteristics of vitamin E acetate (VEA) formulated into a dry emulsion system after its oral administration to beagle dogs were determined and compared to those of two different dosage forms (an oily mixture of the drug with cottonseed oil and an oil (drug)-in-water emulsion). The three dosage forms were administered in a crossover fashion to six nonfasting subjects, and the drug absorption was assessed from the plasma concentration of the major metabolite (free vitamin E). VEA formulated in the dry emulsion was rapidly absorbed, which suggested that a considerable amount was released as reformed emulsion droplets in the gastrointestinal tract as well as in water *in vitro*. Based on the analysis of variance, no significant differences in bioavailability parameters (AUC, C_{max} or T_{max}) were observed among the three dosage forms.

Keywords dry emulsion; vitamin E acetate; bioavailability; drug release; oral administration; beagle dog

Development of ideal dosage forms of oily drugs like lipophilic vitamins to improve bioavailability and patient compliance have been highly desired because of the increasing number of these drugs used in current therapies. In formulating the oily drugs in oral dosage form, much attention should be paid to ease of handling in both manufacturing and dosing and to the drug releasing property affecting the bioavailability after oral administration. The former may be achieved by transforming the oily drugs into solid dosage form, *e.g.*, powdered form.[1] The oily drug formulated into solid form should be easily released and dispersed in the gastrointestinal tract in order to ensure sufficient bioavailability.[2] The emulsion system of oily drugs, which is considered to lead to enhancement of their bioavailability, is thermodynamically unstable and bulky, and thus difficult to formulate into a final dosage form.

To overcome these difficulties in formulation we devised a powdered form of oily and waxy drugs with good redispersibility or drug releasing property in water, so-called dry emulsion.[3,4] The dry emulsion system of a model oily drug vitamine E acetate (VEA) with excellent flowability and drug releasing property was developed using a spray-drying technique.[5] The term "dry adsorbed emulsion" has been used for the powdered form of a water-in-oil emulsion with the drug dissolved in the water.[6] The system is expected to function as a sustained drug-release dosage form by keeping the powdered form after its administration. The dry emulsion system we developed has an oil releasing property to reform the original emulsion when rehydrated. We termed the system "redispersible dry emulsion" in order to discriminate it from other dry emulsion systems.

The aim of the present investigation is to confirm the rapid drug releasing function of the redispersible dry emulsion system *in vivo* by measuring the drug absorption pattern after its administration to beagle dogs.

Experimental

Dosage Forms Three dosage forms containing VEA were subjected to the bioavailability test. The dry emulsion was prepared by spray-drying the emulsified VEA with colloidal silica and a disintegrant (low-substituted hydroxypropylcellulose, L-HPC) as described previously.[5] The other two dosage forms were an oily mixture of the drug with cottonseed oil and an o/w (drug-in-water) type emulsion. The oily mixture was prepared by adding cottonseed oil to the drug at the ratio of 1:2 and subsequently mixing with a spatula, while the emulsion was produced by agitating the drug with an aqueous phase dissolving the same surfactant (polyoxyethylene-polyoxypropylene-blockcopolymer, Pluronic F-68) as used for the dry emulsion, using a high speed homogenizer at 20000 rpm for 5 min. The formulating ratio of drug: surfactant: water in this emulsion was 15:1:234. All the preparations were applied to the *in vivo* test at the dose of 300 mg as free vitamin E.

Absorption Studies in Beagle Dogs Six healthy, male beagle dogs weighing 9.4—11.9 kg (mean 10.3 kg), were used in this study with two subjects per group. The dry emulsion, oily mixture and o/w emulsion were put in hard gelatin capsules and administered orally to the six subjects in a crossover fashion 60 min after feeding (250 g of solid food). Washing out period between administrations was one week.

Each dosage form was ingested with 20 ml of water, and blood specimens (3 ml) were collected at 1, 2, 4, 6, 8, 10, 12 and 24 h after the drug administration. A time zero blood specimen (3 ml) was collected just prior to the drug administration. The amount of vitamin E absorbed was determined by considering the endogenous vitamin E concentration in plasma observed at time 0. The plasma fraction was separated from each specimen by centrifuging at 3000 rpm for 10 min, and an aliquot was stored in the frozen state until assayed for the content of free vitamin E.

Free vitamin E in the plasma was determined using a high performance liquid chromatograph (HPLC) system equipped with a stainless steel column (15 cm × 4-mm i.d.) packed with Finepak SIL C18S at 40 °C, after being deproteinized with an ethanol–water mixture (8:13) and extracted with *n*-hexane. In the HPLC analysis, 100% methanol and 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMC) were used as the mobile phase and internal standard, respectively. The concentration of vitamin E was detected spectrofluorometrically at the extinction of 298 nm and emission of 325 nm.

Differences among the three mean values were statistically evaluated by an analysis of variance, and differences between any two means were compared using the Dunnett test.

Results and Discussion

Figure 1 shows the mean plasma concentration of vitamin E as a function of time after oral administration of the dry emulsion, the intact o/w emulsion and the oily mixture to the six beagle dogs in a crossover fashion. The oily mixture of VEA with cottonseed oil in a hard gelatin capsule was tested as an alternative form of a soft gelatin capsule. The absorption patterns were similar for these three different dosage forms. There was no difference in the statistic analysis for C_{max}, T_{max} and AUC among the three dosage forms (Table I). As shown previously,[5] the

TABLE I. Bioavailability of Vitamin E after Oral Administration to Beagle Dogs in Three Dosage Forms

Subject	C_{max} (μg/ml)			T_{max} (h)			AUC (μg·h/ml)		
	DE	EM	OM	DE	EM	OM	DE	EM	OM
A	10.88	12.97	14.03	4.00	6.00	6.00	168.81	228.55	245.71
B	10.75	11.05	8.41	4.00	6.00	8.00	167.77	162.58	140.40
C	10.17	14.21	9.06	4.00	8.00	6.00	175.34	242.67	162.29
D	14.01	12.55	12.08	4.00	8.00	4.00	238.97	226.95	215.04
E	10.25	14.56	8.22	8.00	10.00	6.00	170.89	249.97	122.38
F	11.99	11.18	12.33	8.00	6.00	12.00	224.46	181.97	207.68
Mean	11.34	12.75	10.81	5.33	7.33	7.00	191.04	215.45	182.25
±S.D.	1.46	1.47	2.54	2.07	1.63	2.76	31.95	35.08	47.92
±S.E.	0.60	0.60	1.04	0.84	0.67	1.13	13.04	14.32	19.56

Statistical comparison I (analysis of variance, ANOVA)
Differences in C_{max} among three dosage forms: $p<0.05$ ($F=1.422$; $df=2, 8$)
Differences in T_{max} among three dosage forms: $p<0.05$ ($F=1.851$; $df=2, 8$)
Differences in AUC among three dosage forms: $p<0.05$ ($F=1.185$; $df=2, 8$)
(Critical F value for 2 and 8 df at the 5% level is 8.65)

Statistical comparison II (Dunnett test)
Differences in C_{max}, T_{max} and AUC between two dosage forms
C_{max}: Dunnett's allowable difference ($p=0.05$; $df=2, 8$)=3.182:
OM *vs.* EM= 1.945; not significant OM *vs.* DE= 0.533; not significant
T_{max}: Dunnett's allowable difference ($p=0.05$; $df=2, 8$)=2.974:
OM *vs.* EM= 0.333; not significant OM *vs.* DE= 1.667; not significant
AUC: Dunnett's allowable difference ($p=0.05$; $df=2, 8$)=59.672:
OM *vs.* EM=33.197; not significant OM *vs.* DE= 8.792; not significant

DE, dry emulsion. EM, o/w emulsion. OM, oily mixture.

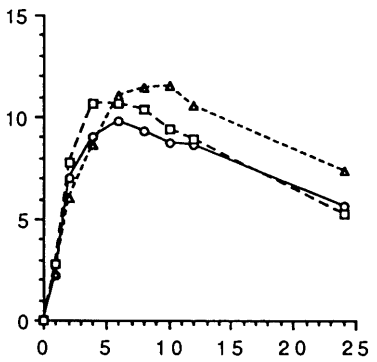

Fig. 1. Time Course of Mean Plasma Concentration of Vitamin E after Oral Administration of Redispersible Dry Emulsion (□), Intact o/w Emulsion (△) and Oily Mixture (○) to Beagle Dogs ($n=6$)

well-designed dry emulstion system can swell and instantly release VEA droplets when dispersed in water, and a stable o/w emulsion is resultantly formed in the medium. The present *in vivo* test suggests that the dry emulsion can redisperse the oil droplets to form the emulsion in the gastrointestinal tract as in the *in vitro* test.

Based on the average value of the vitamin E absorbed, we might discuss some different behaviors in the drug absorption pattern of the three dosage forms. The mean T_{max} value of the dry emulsion (5.33+0.84 h) was a little lower than that of the oily mixture (7.00+1.13 h). This might be attributable to the fact that the dry emulsion offers a good dispersion of oily ingredients. As the absorption of oily drugs in the gastrointestinal tract follows solubilization, the large surface area of the oily ingredient for the dispersed system is more desirable for absorption than the oily mass in the formulation. In dosing the o/w emulsion system a larger amount of water was administered than with the dry emulsion system, which may cause dilution of VEA droplet concentration in the gastrointestinal tract and thus lead to relatively larger value of T_{max} (7.33+0.64 h) than with the dry emulsion system. The higher values of C_{max} and AUC for the emulsion system than that for the oily mixture (Table I) suggest it is possible to attain higher bioavailability by applying the dispersion systems of oily ingredients.

This study confirmed that the oily drug formulated in the present dry emulsion system is rapidly *in vivo* as well as *in vitro*. The drug-releasing property of the system in water has been found to be as preserved as a freshly prepared one after three years storage.[5] Considering that emulsion systems are thermodynamically unstable, the dry system is a more acceptable form than the liquid system. It has been pointed out that the bioavailability of vitamin E from a conventional solid dosage form is relatively inferior to that for an oily solution form such as a soft gelatin capsule system.[7] The dry emulsion system is considered to be comparable to the oily solution system with respect to bioavailability. Recently Tarr and Yalkowsky[8] reported that the reduction of emulsion droplet size enhanced intestinal absorption of cyclosporine in rats. Kimura *et al.*[9] showed that a combination of lecithin liposomes with medium-chain triglycerides greatly improved the bioavailability of VEA. These reports suggest that the modification of physicochemical properties of oily droplets released from the dry emulsion system would lead to more acceptable bioavailability of the system.

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Oral Administration of Calcium Hydroxide Stimulates Bone Metabolism in the Femoral Diaphysis of Rats with Skeletal Unloading

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The preventive effect of calcium hydroxide on the disorder of bone metabolism caused by skeletal unloading was investigated. Skeletal unloading was designed using the model of hindlimb hang in rats. Skeletal unloading for 7 d caused a significant decrease of inorganic phosphorus concentration in the serum and of alkaline phosphatase activity and deoxyribonucleic acid (DNA) content in the femoral diaphysis of rats. Oral administration of calcium hydroxide (16 and 24 mg Ca/kg) caused a significant increase in serum inorganic phosphorus concentration and femoral-diaphyseal calcium content and alkaline phosphatase activity of rats with skeletal unloading. Bone DNA content was significantly increased by the dose of 24 mg Ca/kg. These results clearly indicate that skeletal unloading-induced disorder of bone metabolism is partly prevented by oral administration of calcium hydroxide. Calcium ingestion may be useful as a therapeutic tool in the disorder of bone metabolism caused by skeletal unloading.

Keywords calcium hydroxide; bone metabolism; skeletal unloading; rat femur

It is well known that calcium ion plays an important role in cellular functions. The requirement of calcium in a living body is greatest in the growing and aging stages, however, calcium deficiency is often seen. Calcium supply, therefore, is of pharmacological and nutritional importance. Among the many calcium compounds, calcium lactate and calcium gluconate are most used as nutrients. Meanwhile, calcium hydroxide may exist in non-ionic form in an aqueous solution in the gatsrointestinal tract, because the dissociation constant of the compound is so small. Since the non-ionized form of a substance penetrates membrances more easily than the ionized form,[1] one could expect a considerable amount of absorption of this substance when it is orally administered. This, however, has not been fully clarified. More recently, the bioavailability of calcium hydroxide has been examined in growing rats; calcium hydroxide orally administered caused increases in intestinal calcium absorption and bone calcium content more effectively than did calcium gluconate.[2] Therefore, the present study was undertaken to clarify the preventive effect of calcium hydroxide on the disorder of bone metabolism caused by skeletal unloading (immobilization). Findings suggest a therapeutic role of calcium hydroxide in the disorder of bone metabolism.

Materials and Methods

Weanling male Wistar rats (conventional), weighing 75—80 g (4 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 57.5% carbohydrate, 1.1% Ca and 1.1% P at a room temperature of 25 °C, and were given distilled water freely.

The method of skeletal unloading[3] comprised a number of steps. Rats were placed in a circular cage with a steel-wire floor and fed chow and distilled water freely. The hindlimbs were allowed to hang freely but were immobilized with filament tape. The forelimbs enabled the rat to move about in the cage. Hindlimbs were hung for up to 7 d. The animals were divided into two groups; one group of 5 normal rats was fed and allowed to move around the cage. The other group of 5 rats with hindlimb hang (skeletal unloading) were fed in the circular cage. Initiation of hindlimb hang was designed for each group so that all animals were the same age at the end of the experiment.

Rats with skeletal unloading received oral administration of a calcium hydroxide solution by a stomach tube for 7 d (twice a day). Calcium hydroxide was supplied by Tachikawa Penicillin Co., Ltd. (Tokyo, Japan). The solution was given to rats at 9:00 and 17:00 in individual doses of 8.0, 16, and 24 mg Ca per kg body weight. Rats were killed 17 h after the final administration of calcium hydroxide. Control rats with skeletal unloading received vehicle solution (distilled water) by mouth.

Rats were bled by cardiac puncture under light anesthesia with ether, and the blood and femur were removed immediately. Blood samples were centrifuged 30 min after collection. The serum was separated and analyzed immediately. Serum calcium was determined by the method of Willis.[4] Serum inorganic phosphorus was measured by the method of Taussky and Shon.[5]

The femur was removed after bleeding and soaked in ice-cold 0.25 M sucrose solution. The femur was cleaned of soft tissue and marrow, and the diaphysis and epiphysis (containing metaphyseal tissue) were separated and weighed. The diaphyseal tissues were ashed for 24 h at 640 °C, weighed, and then dissolved in 6.0 N HCl solution. Calcium was determined by atomic absorption spectrophotometry. The calcium content in bone was expressed as mg per g bone ash.

To assay alkaline phosphatase activity the diaphyseal tissues were immersed in 3.0 ml ice-cold 6.5 mM barbital buffer (pH 7.4), cut into small pieces, homogenized with a Physcotron homogenizer, and disrupted for 60 s with an ultrasonic device. The supernatant centrifuged at $600 \times g$ for 5 min was used for the measurement of enzyme activity. The enzyme assay described below was carried out under optimal conditions. Alkaline phosphatase activity was determined by the method of Walter and Schutt.[6] The enzyme activity was expressed as μmol of *p*-nitrophenol liberated per min per mg protein. Protein concentration was determined by the method of Lowry *et al.*[7]

The diaphyseal tissues were shaken with 4.0 ml ice-cold 0.1 N NaOH solution for 24 h after the homogenization of the bone tissue.[8] After alkali extraction, the samples were centrifuged at $10000 \times g$ for 5 min, and the supernatant was collected. Deoxyribonucleic acid (DNA) content in the supernatant was determined by the method of Ceriotti[9] and expressed as the amount of DNA (mg)/g wet weight of bone tissue.

Data are expressed as the mean ± S.E.M. Statistical differences were analyzed using Student's *t*-test. p values of less than 0.05 were considered to indicate statistically significant differences.

Results

The effect of skeletal unloading with hindlimb hang on bone metabolism in rats was investigated. The rats with skeletal unloading for 7 d had no significant change of food ingestion in comparison with that of animals without the unloading (data not shown). Alterations in calcium and inorganic phosphorus concentrations in the serum of rats with skeletal unloading are shown in Table I. Serum calcium concentration was not altered by skeletal unloading for 7 d, while serum inorganic phosphorus concentration was significantly decreased. This decrease was not seen when calcium hydroxide (16 and 24 mg Ca/kg) was orally

TABLE I. Effect of Calcium Hydroxide Administration on Calcium and Inorganic Phosphorus Concentrations in the Serum of Rats with Hindlimb Hang

Treatment	Serum concentration (mg/100 ml)	
	Calcium	Inorganic phosphorus
Normal	10.38±0.12	9.81±0.12
Skeletal unloading		
Control	10.11±0.15	9.22±0.15[a]
8 mg Ca/kg	10.15±0.09	9.39±0.17
16 mg Ca/kg	10.22±0.13	9.70±0.20[b]
24 mg Ca/kg	10.43±0.11	9.83±0.18[b]

Each value is the mean±S.E.M. of 5 rats. Rats were fed for 7 d with hindlimb hang (skeletal unloading). Calcium hydroxide was orally administered for 7 d (twice a day), and rats were killed 18 h after the final administration. *a*) $p<0.05$, as compared with the normal value. *b*) $p<0.01$, as compared with the control value (skeletal unloading).

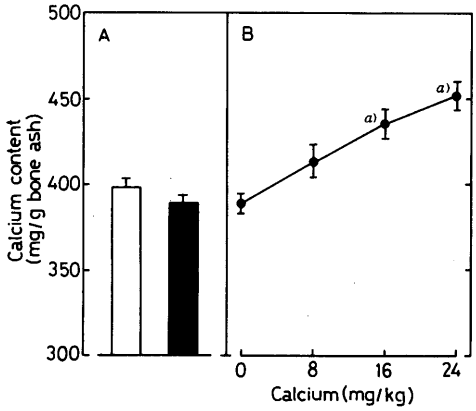

Fig. 1. Effect of Calcium Hydroxide on Calcium Content in the Femoral Diaphysis of Rats with Skeletal Unloading

Animals were fed for 7 d in the state of hindlimb hang (A). Calcium hydroxide (8.0, 16, and 24 mg Ca/kg) was orally administered twice a day to rats subjected to skeletal unloading for 7 d, and the animals were killed 17 h after the final administration (B). Each value represents the mean±S.E.M. for 5 rats. *a*) $p<0.01$, as compared with the value of normal group or without calcium administration. □, normal; ■, skeletal unloading.

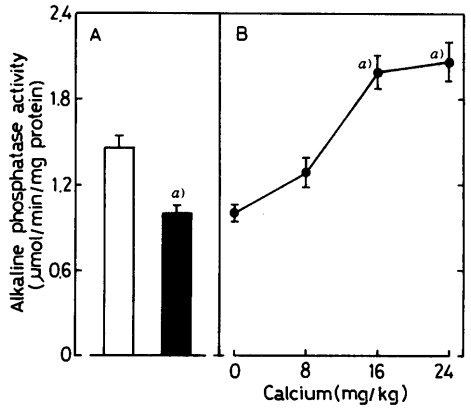

Fig. 2. Effect of Calcium Hydroxide on Alkaline Phosphatase Activity in the Femoral Diaphysis of Rats with Skeletal Unloading

Animals were fed for 7 d in hindlimb hang (A). Calcium hydroxide (8.0, 16, and 24 mg Ca/kg) was administered as described in Fig. 1. Each value represents the mean±S.E.M. for 5 rats. *a*) $p<0.01$, as compared with the value of normal group or without calcium administration. □, normal; ■, skeletal unloading.

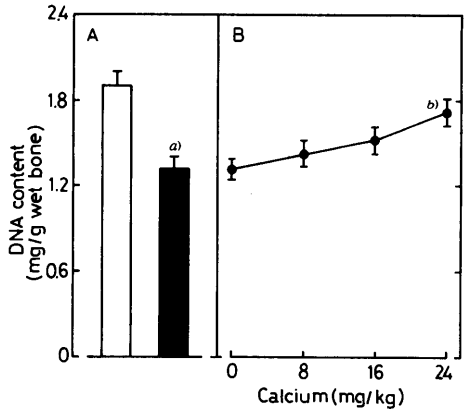

Fig. 3. Effect of Calcium Hydroxide on DNA Content in the Femoral Diaphysis of Rats with Skeletal Unloading

Animals were fed for 7 d in hindlimb hang (A). Calcium hydroxide (8.0, 16, and 24 mg Ca/kg) was administered as described in Fig. 1. Each value represents the mean±S.E.M. for 5 rats. *a*) $p<0.01$, as compared with the value of normal. *b*) $p<0.05$, as compared with the value without calcium administration. □, normal; ■, skeletal unloading.

administered twice a day for 7 d to rats with skeletal unloading.

The content of calcium in the femoral diaphysis of rats was not significantly reduced by skeletal unloading for 7 d, as compared with that of normal rats (Fig. 1A). Oral administration of calcium hydroxide (16 and 24 mg Ca/kg) produced a significant increase in calcium content in the femoral diaphysis of rats with skeletal unloading (Fig. 1B). Also, oral administration of calcium hydroxide (16 and 24 mg Ca/kg) for 7 d to normal rats produced a significant increase in calcium content in the femoral diaphysis (data not shown). This increase was to the same extent as that of skeletal unloading.

The effect of calcium hydroxide administration on alkaline phosphatase activity in the femoral diaphysis of rats with skeletal unloading is shown in Fig. 2. Bone alkaline phosphatase activity was clearly decreased by skeletal unloading (Fig. 2A). This decrease was prevented to a significant extent by the administration of calcium hydroxide (16 and 24 mg Ca/kg) (Fig. 2B). The effect was not seen at the dose of 8.0 mg Ca/kg.

DNA content in the femoral diaphysis of rats was markedly reduced by skeletal unloading (Fig. 3A). This reduction was significantly blocked by oral administration of calcium hydroxide (24 mg Ca/kg), although the doses of 8.0 and 16 mg Ca/kg did not have an appreciable effect (Fig. 3B).

Discussion

It is known that skeletal unloading caused by immobilization,[10] spaceflight,[11] bed rest,[12] or hindlimb elevation[13] results in osteopenia.[14] Recently, it has been demonstrated that inhibition of bone formation is induced using hindlimb hang as the method of skeletal unloading.[3,15] Inhibition of bone formation subsequent to skeletal unloading may be partly accompanied by falls in the serum level of 1,25-dihydroxyvitamin D_3[16] and in the bone content of zinc,[17,18] which are known to stimulate bone formation and mineralization. However, the factors preventing skeletal unloading-induced disorder of bone metabolism are not fully resolved.

The present study was undertaken to clarify whether oral administration of calcium hydroxide can influence the

decrease in bone formation and calcification caused by skeletal unloading with hindlimb hang in rats. Oral administration of calcium hydroxide produces a temporal elevation of serum calcium concentration, indicating that calcium is absorbed form intestine.[2] In the present study, oral administration of calcium hydroxide caused a significant increase of calcium content, alkaline phosphatase activity and DNA content in the femoral diaphysis of rats with skeletal unloading. These findings indicate that skeletal unloading-induced disorder of bone metabolism is prevented by oral administration of calcium hydroxide. This suggests that ingestion of a calcium hydroxide solution has preventive effect on the development of osteopenia during skeletal unloading.

Skeletal unloading also caused a significant decrease in serum inorganic phosphorus concentration; this decrease was completely blocked by oral administration of calcium hydroxide. Meanwhile, skeletal unloading with hindlimb hang did not have an appreciable effect on food ingestion.[3] It appears that skeletal unloading-induced decrease in serum inorganic phosphorus concentration may be involved in calcitropic hormones. It is reported that serum 1,25-dihydroxyvitamin D_3 level is decreased by skeletal unloading,[16] that the steroid produces an increase in serum inorganic phosphate concentration[19] and that the production of steroid hormone is elevated by augmentation of extracellular calcium.[20]

Oral administration of calcium hydroxide caused a significant increase of calcium content in the femoral diaphysis of rats with skeletal unloading, indicating that accumulation of calcium into the bone is stimulated by calcium administration. Furthermore, oral administration of calcium hydroxide produced a significant increase of alkaline phosphatase activity in the femoral diaphysis of rats with skeletal unloading. The enzyme is localized in osteoblastic cells related to bone mineralization.[21] Presumably the increase in bone calcium content by calcium hydroxide administration may be involved in the rise of bone alkaline phosphatase activity. Administration of calcium hydroxide also produced a significant increase of DNA content in the femoral diaphysis of rats with skeletal unloading, although the effect required a comparatively high dose of calcium hydroxide. These findings suggest that calcium hydroxide administration has a stimulative effect on the proliferation of bone cells. Thus, oral administration of calcium hydroxide had an anabolic effect on bone metabolism in rats with skeletal unloading. A previous study showed that oral administration of calcium hydroxide (16 mg Ca/kg) could produce an appreciable increase of calcium content in the femoral diaphysis of normal rats.[2] We do not yet know the mechanism by which oral administration of calcium hydroxide can produce an anabolic effect on bone metabolism in rats with skeletal unloading. It appears that calcium absorbed from intestine directly affects bone cells (osteoblastic cells), although the possibility of a calcitropic-hormonal effect cannot be excluded.

In conclusion, it has been demonstrated that oral administration of calcium hydroxide can prevent deterioration of bone metabolism in rats with skeletal unloading.

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Low Molecular Weight Heparin Enhances Prostacyclin Production by Cultured Human Endothelial Cells

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Confluent cultures of vascular endothelial cells derived from the human umbilical vein were incubated in a serum-free medium in the presence of low molecular weight heparin (LMWH) with molecular weights of 4000—6000 dalton (Da), or of unfractionated heparin (UFH) with average molecular weight 12000 Da, and prostacyclin production was determined by radioimmunoassay for 6-keto-prostaglandin $F_1\alpha$, the stable metabolite of prostacyclin. LMWH at 1 U/ml as anti-factor Xa activity significantly increased prostacyclin production after 6 h or longer; however, UFH at 1 USP U/ml did not induce such a significant change. The LMWH-induced increase in prostacyclin production occurred at 0.1 U/ml and above after 6 h of treatment. Since prostacyclin is both a potent inhibitor of platelet aggregation and a vasodilator, it was suggested that the increased endothelial cell prostacyclin production induced by LMWH may be a component of the anticoagulant activity of the drug.

Keywords endothelial cell; heparin; low molecular weight heparin; prostacyclin; prostaglandin

Heparin is an anticoagulant that has been used clinically for almost 50 years. The anticoagulant activity of heparin depends on its interaction with antithrombin III, an inhibitor of thrombin and most other enzymes in the clotting cascade. In particular, antithrombin III inactivates the coagulation factor Xa as well as thrombin. Heparin induces a configurational change of antithrombin III, resulting in an approximate 1000-fold increase in its activity in neutralizing the clotting enzymes.[1)]

The commercial preparations of heparin are heterogenous and contain heparin molecules with molecular weights from 5000 to 30000 dalton (Da).[2)] Recently, low molecular weight heparin (LMWH) with molecular weights of 3000 to 6000 has been attractive clinically, since its use is associated with less bleeding[3)] which is the major side effect of unfractionated heparin (UFH). Also, there are some differences between UFH and LMWH on the activity of the coagulation factors.[4,5)]

On the other hand, vascular endothelial cells have an anticoagulant activity which is mediated by the production of either prostacyclin,[6)] anticoagulant heparan sulfate,[7)] thrombomodulin[8)] or tissue plasminogen activator.[9)] Both UFH and LMWH enhance thrombin-stimulated prostacyclin production by cultured endothelial cells[10)]; UFH but not LMWH stimulates the production[11)] and release[12)] of heparan sulfate from the cells; UFH increases tissue plasminogen activator production by cultured microvascular endothelial cells.[13)]

The present study was undertaken to clarify whether LMWH is individually capable of inducing prostacyclin production by cultured endothelial cells.

Materials and Methods

Materials Dulbecco's modified Eagle's medium and ASF 301 medium were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan) and Ajinomoto Co., Ltd. (Tokyo, Japan), respectively. Fetal bovine serum (FBS) was from Bioproducts, Inc. (Walkersville, MD, U.S.A.). Endothelial cell growth supplement (ECGS) and UFH (average molecular weight 12000 Da) were obtained from Sigma Chemical Co. (St. Louis, MD, U.S.A.). LMWH (molecular weights 4000—6000 Da) was from KabiVitrum AB (Stockholm, Sweden). Tissue culture plates coated with type I collagen were purchased from Sumitomo Bakelite, Co., Ltd. (Tokyo, Japan). Vascular endothelial cells from the human umbilical vein were from Kurabo (Osaka, Japan). The 6-keto-prostaglandin $F_1\alpha$ (6-keto-$PGF_1\alpha$) [^{125}I]radioimmunoassay kit was obtained from Amersham Corp. (U.K.).

Cell Culture Endothelial cells were cultured in 24-well culture plates with Dulbecco's modified Eagle's medium supplemented with 10% FBS and 200 μg/ml ECGS at 37 °C in a humidified chamber with an atmosphere of 5% CO_2 in air until confluent. The medium was then discarded and the cell layer was washed twice with serum-free ASF 301 medium was harvested and the content of 6-keto-$PGF_1\alpha$, the stable 301 medium in the presence of UFH (1.0 USP U/ml) or LMWH (0.1, 1.0, 10 U/ml; anti-factor Xa activity) for 2, 6, or 8 h. After incubation, the medium was harvested and the content of 6-keto-$PGF_1\alpha$, the stable metabolite of prostacyclin, was measured by radioimmunoassay. The activity of UFH and LMWH were expressed as USP units and anti-factor Xa activity, respectively; however, the amount (μg) of UFH at 1 USP unit was almost equal to that at 1 anti-factor Xa activity.

Statistical Analysis The effect of LMWH was assessed by analysis of variance. p values less than 0.05 were considered to indicate a statistically significant difference.

Results

Since the blood concentration of heparin reaches about 1 U/ml in cases where the drug is used clinically, we

TABLE I. Comparison of the Effect of LMWH and UFH on the Production of Prostacyclin by Cultured Endothelial Cells

	6-Keto-$PGF_1\alpha$ (pg/well)		
	2 h	6 h	8 h
Control	4.21±0.97	3.99±0.79	11.19±1.56
LMWH	3.86±1.09	6.82±0.16	16.22±0.79
UFH	4.97±0.84	4.00±0.97	9.72±0.59

Confluent cultures of endothelial cells were treated with 1 U/ml LMWH or UFH for 2 or 6 or 8 h. Values are means±S.E. of 4 samples. The effect of LMWH on the prostacyclin production was significant ($p<0.01$).

TABLE II. Effect of LMWH on the Production of Prostacyclin by Cultured Endothelial Cells

	6-Keto-$PGF_1\alpha$ (pg/well)
Control	7.76±0.76
0.1 U/ml LMWH	13.48±1.51
1.0 U/ml LMWH	14.70±0.47
10 U/ml LMWH	22.58±2.54

Confluent cultures of endothelial cells were treated with 0.1 or 1.0 or 10 U/ml LMWH for 6 h. Values are means±S.E. of 6 samples. The effect of LMWH on the prostacyclin production was significant ($p<0.01$).

investigated the effect of 1.0 U/ml LMWH and UFH on the production of prostacyclin by cultured endothelial cells (Table I). LMWH significantly increased the prostacyclin production after 6 h. In the 6 h incubation, the prostacyclin production was unexpectedly insufficient for unknown reasons; however, LMWH-stimulated prostacyclin production was observed. UFH did not show such a significant change in the tested conditions. As shown in Table II, the LMWH-induced increase in prostacyclin production occurred at 0.1 U/ml and above.

Discussion

It has been proposed that heparin is capable of interacting with blood vessels. Heparin binds to the endothelium[14] and may exhibit an anticoagulant action on the surface. It is likely that the anticoagulant action of heparin is due not only to the anti-serine protease actions but also to a stimulation of the anticoagulant functions of endothelial cells. In the present study, we found that LMWH individually stimulates the production of prostacyclin by cultured human endothelial cells (Table II). Since prostacyclin is a potent inhibitor of platelet aggregation, this LMWH effect may contribute to the anticoagulant action of the drug.

We showed that UFH at 1.0 U/ml did not increase prostacyclin production (Table I); however, in another experiment, we observed that UFH at 10 U/ml did stimulate prostacyclin production. Although this UFH effect may be dependent on the content of LMWH in UFH, a possibility that high molecular weight heparin also can induce endothelial cell prostacyclin production is not excluded. In our preliminary experiment, exogenous heparan sulfate, dermatan sulfate and hyaluronic acid were each capable of inducing prostacyclin production (data not shown). Thrombin[10] and antithrombin III[15] each stimulate endothelial cell prostacyclin production; however, heparan sulfate on the cell surface mediates only the stimulation by antithrombin III.[16] Endothelial cells may have receptors for glycosaminoglycans which mediate prostacyclin production by the cells.

In conclusion, it was shown that LMWH enhances the production of prostacyclin by cultured endothelial cells. Although the potential activity of LMWH appears to be much lower than that of thrombin[6,10] (prostacyclin production at 1 NIH U/ml for 20 min is approximately 200 pg/well in our system) and antithrombin III,[15] this LMWH effect may contribute to its anticoagulant action. This stimulatory effect of LMWH is an appropriate one as an anticoagulant drug compared with UFH. The effect of heparin on endothelial cell functions must be investigated further.

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N-[(1-LONG ALKYL-2-PYRROLIDINYL)METHYL]BENZAMIDES WITH POTENT SEROTONIN 5-HT1A RECEPTOR AFFINITIES

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A series of *N*-(2-pyrrolidinylmethyl)-2-methoxy-5-sulfamoylbenzamide derivatives bearing a long alkyl chain at the 1-position of the pyrrolidine ring was synthesized and found to possess high affinities for serotonin 5-HT1A receptors.

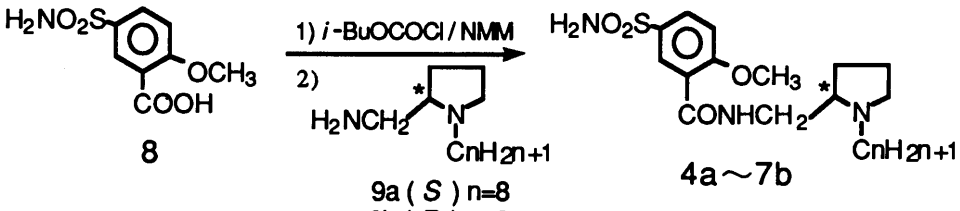
KEYWORDS benzamide; 5-HT1A receptor; long alkyl substituent; pyrrolidine; sulpiride

During the past decade there has been a marked increase in research on serotonin 5-HT1A receptors, as summarized in recent reviews.[1] To date, several different chemical classes of agents such as aminotetraline, arylpiperazine, and indole derivatives have been reported to interact potently with serotonin 5-HT1A receptors.[1]

In the course of the studies on sulpiride analogues previously reported,[2] we found that high affinities for serotonin 5-HT1A receptors resided in *N*-[(1-long alkyl-2-pyrrolidinyl) methyl]-2-methoxy-5-sulfamoylbenzamides retaining significant dopamine D2 receptor affinities. The present paper describes the synthesis of the benzamides and the results of serotonin 5-HT1A receptor binding assay *in vitro*.

Chemistry

The synthesis of the new benzamides (4a-7b) was carried out by use of an approach similar to that employed for the synthesis of the benzamides (1a-3b) reported previously (Chart 1).[2] The coupling reaction of the known 2-methoxy-5-sulfamoylbenzoic acid (8) with the enantiomers of the 1-alkyl-2-(aminomethyl)pyrrolidines (9a-12b) was performed by a mixed anhydride method as follows. Isobutyl chloroformate was added to a mixture of the acid (8) and *N*-methylmorpholine (NMM) in DMF and THF (−10℃, 30 min). The resulting mixture was treated with the enantiomers of the amines (9a-12b) to yield the benzamides (4a-7b) (−10℃→rt, 2h).



9a (*S*) n=8
9b (*R*) n=8
10a (*S*) n=9
10b (*R*) n=9
11a (*S*) n=10
11b (*R*) n=10
12a (*S*) n=12
12b (*R*) n=12

Chart 1

The requisite enantiomers of the 1-alkyl-2-(aminomethyl) pyrrolidines (9a-12b) were prepared by the following synthetic sequence according to the procedure reported in the previous paper[2]: (i) *N*-alkylation of 2-pyrrolidinone, (ii) treatment with nitromethane in the presence of dimethyl sulfate and sodium methoxide, (iii) reduction (H_2, Raney Ni), and (iv) resolution of the racemic amines through the formation of their L-and D-ditartrate salts. The absolute configuration of the resolved amines was established by comparison in optical rotation with the amines derived from L-prolinamide by a stereoconservative method.

The chemical data of (+)-(*R*)-*N*-[(1-nonyl-2-pyrrolidinyl) methyl]-2-methoxy-5-sulfamoylbenzamide (5b) are representative: mp 115-117℃(IPA/IPE);[α]$_D^{24}$ + 53.5° (c=1.0, MeOH); ee>98% ; MS m/z: 439 (M^+); ^{1}H-NMR($CDCl_3$, 400 MHz) δ : 0.85 (3H, t, *J*=7Hz), 1.1-2.0 (18 H), 2.1-2.3 (2H, m), 2.5-2.8 (2H, m), 3.1-3.4 (2H, m), 3.7-3.8 (1H, m), 4.0 (3H, s), 5.7 (2H, brs), 7.04 (1H, d, *J*=9Hz), 8.01 (1H, dd, *J*=2Hz, 9Hz), 8.3(1H, brs), 8.75(1H, d, *J*=2Hz); *Anal.* Calcd for $C_{22}H_{37}N_3O_4S$: C, 60.11; H, 8.48; N, 9.56. Found: C, 60.03; H, 8.34; N, 9.52.

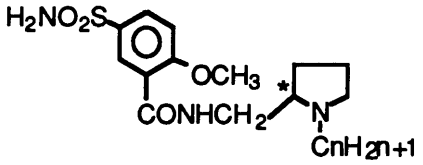
Results

The enatiomeric benzamides were evaluated for their abilities to displace [^{3}H]-8-OH-DPAT from serotonin 5-HT$_{1A}$ binding sites in rat hippocampal membranes *in vitro* and to displace [^{3}H]-spiperone from dopamine D_2 binding sites in rat striatal membranes *in vitro* (Table I). It is well known that the 1-ethyl substituted benzamide (sulpiride) interacts potently and selectively with dopamine D_2 receptors. In our experiments, both enantiomers of the 1-ethyl benzamides (1a, 1b) were virtually devoid of 5-HT$_{1A}$ receptor affinities (*Ki*=36000 and 18000 nM for 1a and 1b, respectively). However, it is of particular interest to note that the serotonin 5-HT$_{1A}$ receptor binding affinities gradually increased as the chain length of the *N*-alkyl function on the pyrrolidine nitrogen atom was made greater in both enantiomers series as shown in Table I. Thus, the 1-hexyl-substituted benzamides (3a, 3b) showed moderate affinities to the 5-HT$_{1A}$ receptors (*Ki* of 3a and 3b were 370 and 430 nM, respectively) and the 1-nonyl derivatives (5a, 5b) exhibited high affinities in the 5-HT$_{1A}$ receptor binding (*Ki* of 5a and 5b were 81 and 16 nM, respectively). Substitution with further long alkyl function, dodecyl group (7a, 7b), resulted in lower affinities than the nonyl substitution in each absolute configuration. Correlation between the 5-HT$_{1A}$ receptor binding affinities and the chain length showed a parabolic relationship, with nonyl group producing the most potent compounds for each enantiomer series. These results indicate that the steric bulk and/or lipophilicity of the long alkyl substituent may greatly contribute to the increased 5-HT$_{1A}$ receptor binding affinities rather than the stereochemistry, although there are some small differences in affinity between the enantiomers. Comparing the 5-HT$_{1A}$ receptor affinities with the D_2 receptor affinities, these 1-long alkylated benzamides (3a, 4a-7b) were found to be more potent at 5-HT$_{1A}$ receptor binding sites than at D_2 receptor binding sites, as shown in Table I (D_2/5-HT$_{1A}$ ratio >1).

In the case studied, the nonyl substituted *R*-enantiomer 5b was ranked as having the highest affinities for 5-HT$_{1A}$ receptors and was 3 orders of magnitude more potent than sulpiride, binding with significant affinities to D_2 receptor sites (*Ki*=92 nM). Such compounds, which act on 5-HT$_{1A}$ receptors more potently

than D_2 receptors, will represent a new class of substituted benzamides family and may have unique therapeutic potential (anxiolytics, neuroleptics, antidepressant). Extensive biochemical and pharmacological studies on the compounds with both 5-HT_{1A} and D_2 receptor affinities are ongoing.

TABLE I. Affinities of Benzamides to 5-HT_{1A} and D_2 Receptors



Compd.[a]	n	Isomer	Ki[b] (nM) 5-HT_{1A}[c]	Ki[b] (nM) D_2[d]	D_2/5-HT_{1A} ratio
1a	2	S	36000	29	<0.01
1b	2	R	18000	2700	0.15
2a	4	S	1200	51	0.04
2b	4	R	4500	230	0.05
3a	6	S	370	1400	3.8
3b	6	R	430	59	0.14
4a	8	S	120	310	2.6
4b	8	R	47	120	2.6
5a	9	S	81	180	2.2
5b	9	R	16	92	5.8
6a	10	S	120	230	1.9
6b	10	R	21	80	3.8
7a	12	S	1600	1700	1.1
7b	12	R	68	130	1.9

a) All compounds gave satisfactory IR, ^{1}H-NMR, MS, and elemental analysis. The enantiomeric purites of all compounds were confirmed to be >98 % ee by HPLC(column; CHIRALPAK AD DAICEL Chemical Industries, Ltd.).
b) Each value is the mean from triplicate assays in a single experiment.
c) Binding studies were conducted according to the method of M.D.Hall et al.[3]
d) [^{3}H]-spiperone binding.[4]

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All test compounds inhibited the specific binding of [^{3}H]-8-OH-DPAT in a concentration-dependent manner. The maximum effect of each compound was 100 %.
4) D_2 receptor affinities of compounds 1a-3b have previously been reported.[2] The newly synthesized compounds 4a-7b were evaluated according to a previously described method.[2]

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PREPARATION OF [ARG-GLY-ASP]-[AMINO-POLY(ETHYLENE GLYCOL)] HYBRIDS AND THEIR INHIBITORY EFFECT ON EXPERIMENTAL METASTASIS[1)]

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Hybrids of a fibronectin-related tripeptide (Arg-Gly-Asp) and amino-poly(ethylene glycol) were prepared and their inhibitory effect on experimental metastasis in mice was examined. The hybrids exhibited a potent inhibitory effect on the metastasis of B16 melanoma BL6.

KEYWORDS RGD; poly(ethylene glycol); peptide-poly(ethylene glycol) hybrid; drug-carrier; antitumor; fibronectin

The tripeptide Arg-Gly-Asp(RGD) was first found in the cell attachment domain of fibronectin[2)] and then in other adhesive proteins, such as vitronectin[3)] and collagens.[4)] RGD in fibronectin plays as crucial a role in cell attachment as Tyr-Ile-Gly-Ser-Arg (YIGSR) does in laminin.[5)] RGD- or YIGSR-containing peptides [Gly-Arg-Gly-Asp-Ser(GRGDS),[6)] YIGSR, Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg (CDPG-YIGSR),[5)] poly(RGD) and poly(YIGSR),[7)] etc.] have been reported to be inhibitors in experimental metastasis.

Since poly(ethylene glycol) is stable, low toxic, bioinert and low immunogenic, it will be a promising polymer for a drug-carrier. Recently, we found that the hybrid of YIGSRG and amino-poly(ethylene glycol) (APEG) was a potent inhibitor in experimental metastasis of B16 melanoma BL6.[8)] This paper describes the preparation of RGD-APEG hybrids and their inhibitory effect on experimental metastasis in mice.

Two different types of PEG, #4000(4K;MW,3000-3700) and #6000(6K;MW,7300-9000), were converted into APEG according to the procedure reported by Pillai and Mutter.[9)] APEGs 4K and 6K were purified by carboxymethyl cellurose column chromatography using ammonium acetate buffer as an eluent. The amino contents of APEGs(4K and 6K) were 0.51 meq/g and 0.27 meq/g respectively.

The protected RGD tripeptide, Boc-Arg(Tos)-Gly-Asp(OBzl)-OH, was prepared by the solution method as shown in Fig.1. $[\alpha]_D^{27}$+8.2°(c=1.0,dioxane). Amino acid ratios in an acid hydrolysate(6N HCl, 24h):Arg 0.99; Gly 1.00; Asp 0.97(average recovery 86%). The tripeptide was coupled with APEG by dicyclohexylcarbodiimide(DCC)/1-hydroxybenzotriazole(HOBt) method[10)] and the product was treated with hydrogen fluoride(HF) to remove the protecting groups. The resulting RGD-APEG hybrid was purified by sephadex G-25 column chromatography followed by high performance liquid chromatography (HPLC, YMC ODS-AQ column was used). Amino acid ratios in an acid hydrolysate (6N HCl,48h) of RGD-APEG(4K) and RGD-APEG(6K) were [Arg 0.91; Gly 1.00; Asp 0.94] and [Arg 0.87; Gly 1.00; Asp 0.84] respectively. RGD contents of the hybrid 4K and 6K were 278 μmol/g and 115 μmol/g respectively.

The inhibitory effect of the synthetic hybrids on experimental metastasis of B16 melanoma BL6 were examined in mice according to the method reported in the preceding paper.[8)] In advance, PEG and APEG were independently examined for inhibitory effect on experimental metastasis and were found to have no effect. As shown in Fig.2, the hybrid 4K and 6K inhibit metastasis. The inhibitory effect of the hybrid 6K was nearly equal to that of RGD, but not distinctive. Therefore, the effect of the hybrid 6K was further examined in its diluted concentrations. As shown in Fig.3, diluted hybrid 6K inhibited metastasis dose-

dependently. The inhibitory effect of 0.1 mg of the hybrid 6K nearly equaled that of 1 mg of RGD. Thus it can be said that the inhibitory effect of the hybrid 6K is 10 times and 230 times as potent as that of RGD in terms of weight and molar ratios respectively.

The effect of the mixture of RGD (25 μg) and APEG 6K (300 μg) was examined in the same procedure, and it was less active than that of 300 μg of RGD-APEG 6k hybrid in metastasis inhibition. 300 μg of RGD-APEG 6K hybrid contain 13 μg of RGD. This indicates that the presence of the covalent bond between the tripeptide and APEG is necessary for potentiation of the inhibitory effect. Thus the high inhibitory effect of the hybrid can be roughly explained by the hypothesis that the bulky APEG moiety might prevent enzymatic hydrolysis of RGD and stabilize the binding between RGD and its receptor. Recently Koyama reported that subcutaneously injected PEG in mice was distributed in tumor cells in high concentrations.[11] This fact also suggests a high inhibitory effect of the hybrids.

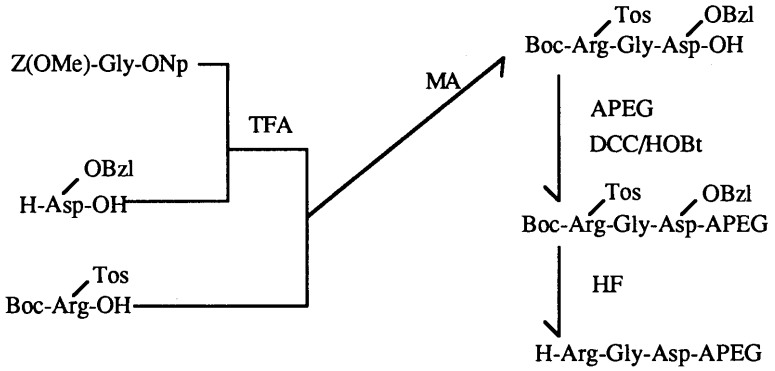

Fig. 1. Synthetic Scheme for RGD-APEG Hybrid
MA:Mixed anhydride method.
DCC:Dicyclohexylcarbodiimide.
HOBt:1-Hydroxybenzotriazole.

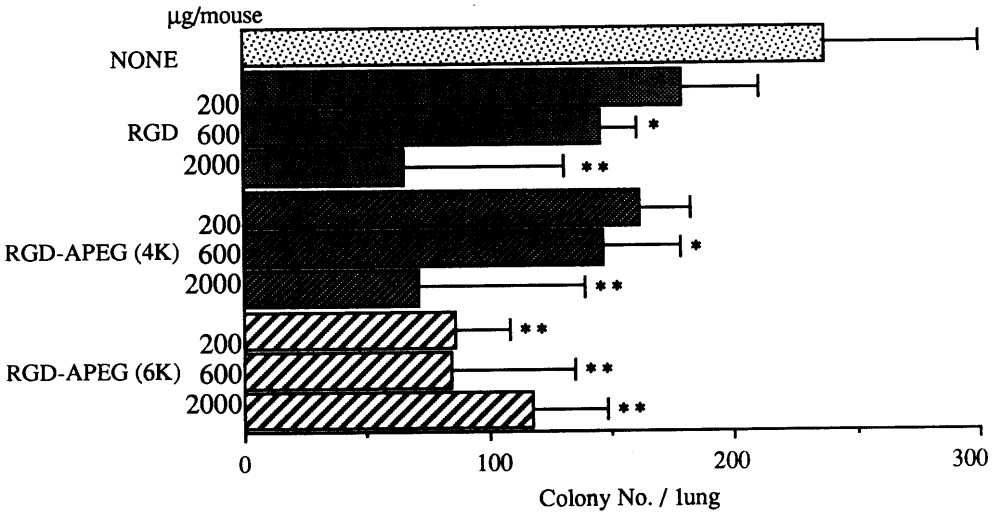

Fig. 2. Inhibitory Effect of RGD-APEG Conjugates on the Formation of Lung Metastasis
B16-BL6 cells ($1x10^5$/0.2ml) were injected i.v. with or without admixing with various concentrations of APEG-RGD into five mice per group. Lung tumor colonies were examined 21 days later. Values were the mean ± SD.
*;$p<0.05$, **;$p<0.01$ compared with untreated control by Student's t-test.

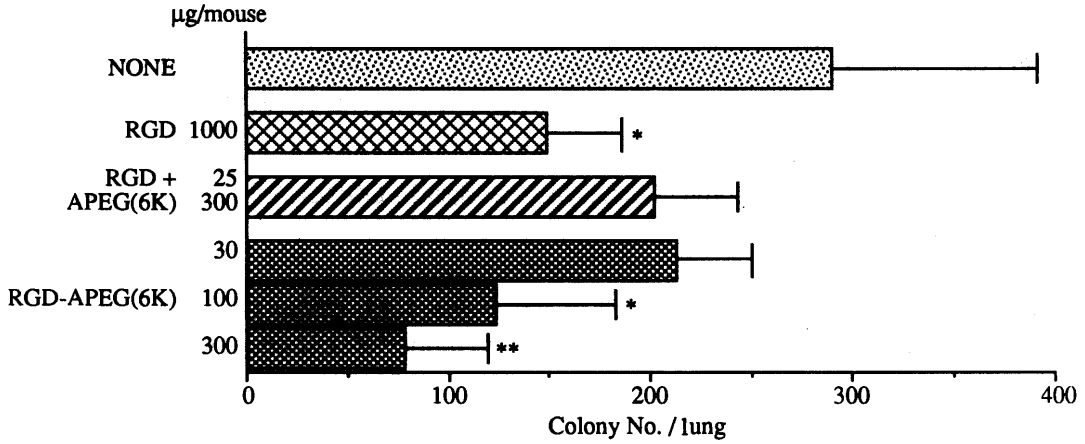

Fig. 3. Inhibition of Lung Colonization by RGD-APEG
The tumor colonization assay was carried out as described in Fig. 2 . Values were the mean ± SD.
*;$p<0.05$, **;$p<0.005$ compared with untreated control by Student's t-test.

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BIOSYNTHESIS OF β-(ISOXAZOLIN-5-ON-2-YL)ALANINE, THE PRECURSOR OF THE NEUROTOXIC AMINO ACID β-*N*-OXALYL-L-α,β-DIAMINOPROPIONIC ACID

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β-(Isoxazolin-5-on-2-yl)alanine (BIA), a biosynthetic precursor of the neurotoxic amino acid β-*N*-oxalyl-L-α,β-diaminopropionic acid (ODAP), was confirmed to be derived from *O*-acetyl-L-serine (OAS) and isoxazolin-5-one by cysteine synthase in higher plants. Some properties of this enzyme in the biosynthesis of BIA are described.

KEYWORDS *Lathyrus sativus*; *Pisum sativum*; Leguminosae; cysteine synthase; neurotoxin; β-*N*-oxalyl-L-α,β-diaminopropionic acid; β-(isoxazolin-5-on-2-yl)alanine; *O*-acetyl-L-serine; biosynthesis

A number of isoxazolinone derivatives have been isolated from legume seedlings belonging to the genera *Pisum, Lathyrus* and *Lens*.[1] β-(Isoxazolin-5-on-2-yl)alanine (BIA) is a prominent metabolite only during the seedling stage of the grass pea (*Lathyrus sativus*) and some other closely related legumes.[2] Recently, it has been demonstrated that BIA is the biosynthetic precursor of the neurotoxin β-*N*-oxalyl-L-α,β-diaminopropionic acid (ODAP) found in *L. sativus* (Chart 1).[3] The neurotoxin ODAP has been recognized as the cause of human lathyrism.[4] In our previous studies on the biosynthesis of heterocyclic β-substituted alanines in higher plants, we have demonstrated the formation of a number of β-substituted alanines such as L-quisqualic acid by crude enzyme preparations or cysteine synthases prepared from several higher plants.[5-9]

This paper reports the biosynthesis of BIA from *O*-acetyl-L-serine (OAS) and isoxazolin-5-one by cysteine synthases purified from the pea (*Pisum sativum*) and grass pea. The enzyme cysteine synthase was purified from the seedlings (cotyledons removed) of peas and grass peas grown in moistened vermiculite in the dark for 5-6 days at 26-28°C by methods described in our previous papers.[5-9] The purified cysteine synthase isoenzymes A and B were used directly as the source of enzyme activity for the formation of BIA. Isoxazolin-5-one was synthesized following the method of Sarlo,[10] and the structure was confirmed by spectroscopic methods.

Reaction mixtures used to demonstrate the formation of BIA contained 12.5 mM of OAS, 70 mM of isoxazolin-5-one and 100 μl enzyme preparation containing 2-4 μg of the protein and were incubated at 30°C in a final volume of 0.5 ml. The pH of the incubation mixtures was normally adjusted to pH 8 by 50 mM K-Pi buffer. Reactions were terminated by the addition of 10 μl of 1 N KOH. The resulting solution acidified with 6 N HCl to pH 1.7-1.8 was

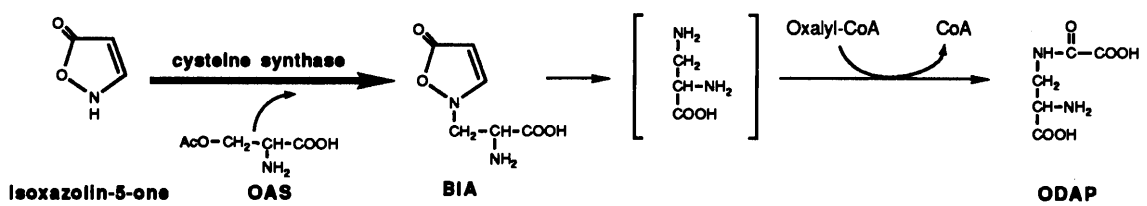

Chart 1. Proposed Biosynthetic Pathway for L-ODAP in *L. sativus*.

examined by using an automatic amino acid analyzer (Hitachi 835-10) coupled the UV detector (254 nm) for the formation of BIA : under standard operating conditions (2.6 x 250 mm column, 33-68°C, Li-citrate buffer system, pH 3.0-7.0, flow rate 0.275 ml min^{-1}),[11] BIA eluted at about 22 min from the column. The presence of BIA in final reaction mixtures was also established by HPLC (Wakosil 5C18 column) using 2% ammonium dihydrogen phosphate buffer (pH 2.4) following the modified method of Lowry.[12] These methods indicated clearly the formation of a product, reacting positively with ninhydrin reagent and showing the UV absorption at 265 nm, that was inseparable from added authentic BIA. The product was not formed in reaction mixtures lacking OAS or isoxazolin-5-one, nor was it formed when the enzyme preparation was pretreated at 100°C for 15 min. Quantitative determination of BIA was also made using an amino acid analyzer. The formation of L-cysteine was spectrophotometrically measured at 560 nm according to the method of Gaitonde.[13] The unit of enzyme activity used in this paper is equivalent to 1 μmol of L-cysteine or BIA produced per min. Protein was determined by a dye-binding method.[14] Under standard assay conditions, the enzymes purified from pea and grass pea seedlings clearly formed BIA, showing it to be specific for OAS as a donor for the alanyl-moiety. The purified enzymes showed no activity in the presence of *O*-acetyl-D-serine, *O*-phospho-L-serine or L-serine. The optimum pH for the enzymatic formation of BIA was 7.8-8.0 using 50 mM K-Pi buffer. The synthase activity for BIA was dependent upon the concentration of isoxazolin-5-one used. A relatively low final concentration (*ca* 70 mM) was sufficient to give a maximum rate of BIA formation in the presence of a fixed concentration (12.5 mM) of OAS.

Cysteine synthase isoenzyme B purified from peas and grass peas could synthesize more BIA than isoenzyme A : The relative rates of synthesis were 0.9-1.0 % by isoenzyme B and 0.2-0.3 % by isoenzyme A, compared with that of L-cysteine by each enzyme, respectively. These isoenzymes also could catalyze the formation of some heterocyclic β-substituted alanines such as β-(pyrazol-1-yl)-L-alanine and β-(1,2,4-triazol-1-yl)-L-alanine at low rates, in line with our previous findings.[6-9]

From the above results and from our earlier work it can be suggested that the plant cysteine synthases also catalyze the formation of BIA, the precursor of the neurolathyrotoxin ODAP, from OAS and isoxazolin-5-one. These results open a possible path towards a solution of the lathyrism problem : modification of the BIA synthesizing isoenzyme in order to stop the formation of BIA, would result in a toxin free *L. sativus* plant, while the other isoenzyme is still responsible for the primary L-cysteine formation.

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ISOLATION OF *BACILLUS STEAROTHERMOPHILUS* CONSTITUENT THAT SUPPRESSES MOUSE MIXED LEUKOCYTE REACTION *IN VITRO*

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A novel immunosuppressant, Fr.5-B, has been isolated from the debris of *Bacillus stearothermophilus* UK563 autolysate. Fr.5-B suppressed mouse mixed leukocyte reaction at doses ranging from 10^{-4} to 1 μg/ml, but not proliferation stimulated by concanavalin A and lipopolysaccharide.

KEYWORDS thermophile; *Bacillus stearothermophilus*; immunosuppressant; mixed leukocyte reaction; cyclosporin A; T cell proliferation

Cyclosporin A (CYA), a fungal metabolite, is an effective immunosuppressant with low myelotoxicity[1] and has been used successfully as the primary drug to suppress the rejection of transplants.[2] The immunosuppressive agents which would attack specific target sites of cells are expected to provide a useful prototype of drugs for immunotherapy. Accordingly, we focused on thermophile that contains a variety of components such as lipids and polyamines which are chemically different from those of mesophile.[3] We have tested products and constituents of *B. stearothermophilus* for specific inhibitory effects on mixed leukocyte reaction (MLR). As a result, the strain UK563 was found to contain the potent immunosuppressive compound designated as Fr. 5-B. In this paper, we describe the isolation of Fr. 5-B. Biological properties and structural elucidation of Fr. 5-B will be published elsewhere.

EXPERIMENTAL

Isolation *B. stearothermophilus* UK563 (Fermentation Research Institute, Deposit No. 7275) was cultured in a continuous fermenter at 60°C at a flow rate of 400 l/h in a medium (adjusted to pH 7.0) of the following composition: glucose (0.35%), $MgSO_4 \cdot 7H_2O$ (0.1%), $FeSO_4 \cdot 7H_2O$ (0.005%), $MnSO_4 \cdot H_2O$ (0.0001%) and Na_2MoO_4 (0.0001%).[4] After 4 days culture, the culture broth was separated into cells and fluid by centrifugation. The collected cells were autolyzed by incubation in 25 mM phosphate buffer (pH 8.0)-2 mM ethylenediaminetetraacetic acid at 40°C for 2 h. Supernatant was removed by centrifugation. The cell debris (equivalent to approximately 500 g wet weight of cells) was extracted with hot EtOH. After removal of the solvent, the extract was suspended in deionized water, and the suspension was mixed and extracted with CH_2Cl_2, three times. The prepared CH_2Cl_2 extract was dissolved in $CHCl_3$ and fractionated by silica gel column chromatography, which was successively developed with $CHCl_3$, $CHCl_3$-MeOH (100:1), $CHCl_3$-MeOH (50:1), $CHCl_3$-MeOH (10:1) and MeOH. Fraction 5 eluted with $CHCl_3$-MeOH (100:1) was collected and concentrated under reduced pressure to give a yellow oil (1.1 g) containing the substance that shows appreciable suppression of MLR. The active fraction was dissolved in n-hexane and refractionated by silica gel column chromatography, which was successively developed with n-hexane-AcOEt (2:1) and n-hexane-AcOEt (1:1). Fraction 5-B (Fr.5-B) eluted with n-hexane-AcOEt (2:1) was collected and concentrated under reduced pressure to give Fr.5-B in the form of white powder (91 mg) showing MLR suppression activity.

Drugs Cyclosporin A (CYA) was a generous gift from Sandoz Ltd., Biological and Medical Research (Basel Switzerland). Fr.5-B and CYA were dissolved in ethanol and further diluted in medium to the proper concentrations in culture.

Suppression of MLR The MLR test was performed in flat-bottomed microtiter plates, with each well containing $5x10^5$ C57BL/6 responder cells ($H\text{-}2^b$) and $5x10^5$ mitomycin C-treated (incubated with 25 μg/ml mitomycin C at 37°C for 30 min and washed 3 times with Hank's balance salt solution) BALB/C stimulater cells ($H\text{-}2^d$) in 0.15 ml PRMI 1640 medium (Nissui) supplemented with 10% fetal calf serum, $5x10^{-5}$ M 2-mercaptoethanol (2ME), 12 mM HEPES, 100 U/ml benzylpenicillin and 100 μg/ml streptomycin (RPMI 1640 complete medium). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 : 95% air for 112 h pulsed with [^{3}H] thymidine ([^{3}H] TdR, American Radiolabeled Chemicals, Inc., 0.5 μCi/well) 16 h before the end of culture, and harvested onto glass fiber filter papers. The papers were dried and processed for liquid scintillation counting.

Determination of Lymphocyte Proliferation *in Vitro* The splenocytes obtained from a BALB/C mouse were cultured in RPMI 1640 complete medium, at a concentration of $2.5x10^6$ cells/ml ($5x10^5$ cells/well) with or without 1.25 μg/ml concanavalin A (Con A) or 50 μg/ml lipopolysaccharide (LPS) and a test sample in flat-bottom microtiter plates. Cells were cultured for 64 h at 37°C in a humidified atmosphere of 5% CO_2 : 95% air and pulsed with [^{3}H] TdR (0.5 μCi/well) 16 h before the end of the culture. The incorporated [^{3}H] TdR was determined as described for suppression of MLR.

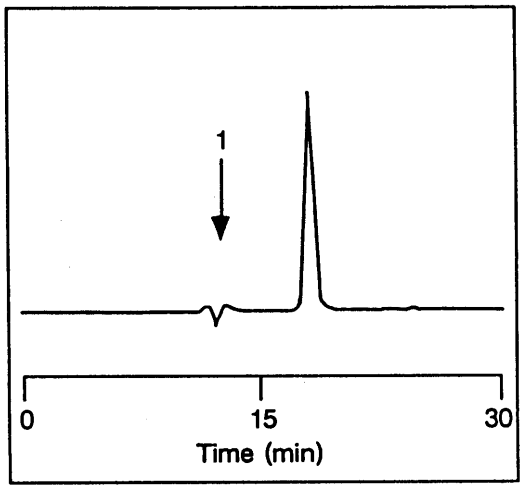

Fig.1. HPLC of Fr.5-B
Column,TSK gel silica - 150 (0.78 X 30 cm); eluant, n-hexane : AcOEt (4 : 1); flow rate, 1.0 ml/min; detection, refractive index. Fr. 5-B (25 μl) was dissolved in n-hexane : AcOEt (4 : 1). Peak 1 is due to the injected solution.

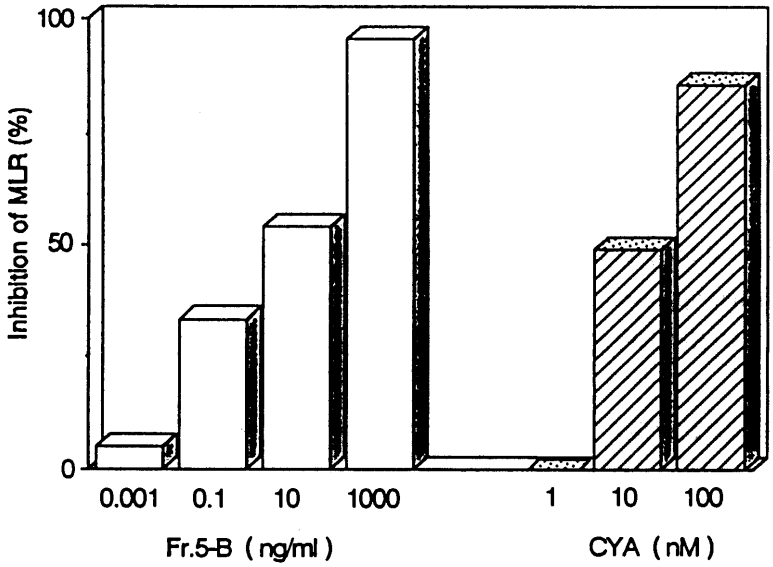

Fig.2. Effect of Fr. 5-B and CYA on Mouse MLR
The data are presented as the percentage of inhibition based on response in the control diluent. Mean cpm of [^{3}H]TdR uptake for mouse MLR was 28,832 ± 1646. Unstimulated lymphocytes average 2,726 cpm of [^{3}H]TdR.

RESULTS AND DISSCUSSION

In the course of our screening for immunomodulators, we found that *B. stearothermophilus* UK563 contained a T cell-specific immunosuppressant. An active component, Fr.5-B, in the debris of the strain UK563 autolysate for inhibition of the MLR was purified by successive silica gel column chromatographies. The homogeneity was confirmed by HPLC as shown in Fig.1. Fr. 5-B and CYA were tested for ability to suppress the *in vitro* proliferative response of mouse lymphocytes to alloantigen stimulation. The results of representative experiments in which various quantities of Fr. 5-B and CYA were added to mouse MLR at the initiation of culture are illustrated in Fig. 2. The IC_{50} values of Fr. 5-B and CYA were 1 ng/ml and 11 nM (13 ng/ml), respectively. Then we examined the suppressive effects of Fr. 5-B and CYA on mitogenic response. While Fr. 5-B did not inhibit Con A- and LPS-induced proliferative responses of splenocytes, CYA inhibited both mitogenic responses (Table I). These results suggest that the

TABLE I. Effect of Fr.5-B and CYA on Mitogenic Responses of Mouse Splenocytes

Sample	Concentration (ng/ml)	[^{3}H]TdR uptake (cpm, mean ± s.e., n = 4)			
		Con A		LPS	
Control	—	57963 ± 2632		32001 ± 1012	
Fr.5-B	0.001	60494 ± 3674	(0)[a]	32008 ± 893	(0)
	0.1	60494 ± 1941	(0)	31401 ± 1127	(2)
	10	60999 ± 973	(0)	33127 ± 1498	(0)
	1000	61361 ± 2447	(0)	27496 ± 991*	(14)
CYA	10[b]	40929 ± 2573**	(30)	24105 ± 1052**	(25)
	100	13660 ± 696***	(77)	15380 ± 1418***	(53)

a) % Inhibition. b) nM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; versus control.

immunosuppressive mechanism of Fr. 5-B was unique and different from those of CYA and FK506. CYA and FK506 affected mainly the stage of interleukin-2 production induced by alloantigen and Con A stimulation.[5] As far as the cytotoxicity tests are concerned, Fr. 5-B was non-toxic at less than 1 μg/ml against splenocytes used in the MLR (data not shown). Fr. 5-B specifically inhibited the proliferative response of lymphocytes to alloantigen stimulation at non-toxic concentrations. The action mode and immunosuppressant activity *in vivo* of Fr.5-B should be further studied.

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