# Cryoprotective Effect of Saccharides on Denaturation of Catalase by Freeze-Drying

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**A mechanism of the cryoprotective effect of saccharides on the denaturation of catalase in the freeze-drying process was studied. Denaturation percents (*D*%) were measured by changing concentrations of saccharides and catalase. Dependence of *D*% on a weight ratio of saccharide to catalase suggested that they interact directly with each other. The mode of interaction is probably hydrogen-bonding. An effective number of hydrogen-bonding sites for several saccharides on the surface of the catalase molecule was evaluated by reference to the X-ray analysis data. Glucose, maltose and maltotriose showed a similar protective effect compared on the basis of their weights, and a glucoside group of those saccharides occupied about 5 hydrogen-bonding sites on a catalase molecule at the maximum protection. Saccharides with longer glucoside chains have less protection effect depending on their molecular weights. Judging from the above results, saccharides are bound to catalase as a monomolecular layer and this layer protects catalase from being denatured instead of the hydration monolayer.**

**Keywords** freeze-drying; catalase; denaturation; cryoprotection; saccharide; hydrogen-bonding

## Introduction

The freeze-drying process causes a change in conformation of a protein, accompanied with a change in biological activity, *i.e.*, denaturation.[1] For example phosphofructokinase is completely inactivated during freeze-drying. Carbonic anhydrase[2] and myosin[3] undergo some degree of denaturation in the course of freeze-drying. Catalase dissociates into a subunit accompanied with a decrease in the helix content.[4,5]

To reduce the degree of denaturation, stabilizing agents such as saccharides and amino acids are added to a protein solution prior to freeze-drying. These compounds also stabilize living cells[6] and liposome[7,8] during freeze-thawing and freeze-drying. Despite many practical uses, the mechanism of the stabilization of a water-soluble protein during freeze-drying is still obscure. Hanafusa[9] measured both residual water contents after freeze-drying and amounts of unfreezing water by nuclear magnetic resonance (NMR) for some proteins, suggesting that the stabilization resulted from the hydrogen-bond formation between protein molecules and additives in place of water molecules. Carpenter and Crowe[10] proposed a similar model and called it "the water substitute theory" based on their infrared (IR) studies of a freeze-dried mixture of lysozome and some carbohydrates. On the other hand, Hellman[11] *et al.* reported that stabilizing effects of various additives on the dissociation of L-asparaginase during freeze-drying are related to water ordering influences of additives rather than direct interactions such as hydrogen-bonding.

Although detailed information is required to discuss the stabilization mechanism, quantitative research is rather rare. In the present study, the degree of denaturation of catalase was measured by changing the concentrations of saccharides and protein, respectively. Several saccharides such as monosaccharide (glucose), oligosaccharides (maltose, maltotriose, maltopentaose, maltohexaose and maltoheptaose), and dextrans (molecular weight (Mw)= 19800 and 100000—200000) were used for the investigation. Polyalcohols were also examined for comparison. The stabilization effects of saccharides have been discussed based on these results. The change of the hydration state in a catalase molecule during freeze-drying was also evaluated.

## Experimental

**Materials** A suspension of twice-crystallized beef liver catalase with a specific activity of 40000—50000 units/mg was obtained from Sigma Chemical Company. The suspension was dialyzed against 5 mM phosphate buffer to remove thymol. After incubation for 45 min at 37 °C, the dialyzed suspension was centrifuged to remove insoluble residues, that is, denatured catalase molecules. The catalase solution thus obtained was stored in a refrigerator and was used within 3 weeks. Oligosaccharides were obtained from Nihon Shokuhin Kako Co., Ltd. Their purities were more than 99.5%. Dextrans (Mw=19800 and 100000—200000) were supplied from Wako Chemical Co., Ltd. All other compounds were of reagent grade. All saccharides were desiccated at 60 °C and at 0.1 Torr on $P_2O_5$ for 1 d before use. Water was purified by deionization and distillation.

**Enzyme Assay** Catalase was assayed at 25 °C by the method of Beers and Sizer,[12] in which the first-order decay of hydrogen peroxide was followed by a decrease in absorbance at 240 nm. At the beginning, 50 $\mu$l of the enzyme solution was added to 3 ml of buffer solution containing $4.5 \times 10^{-3}$% hydrogen peroxide (50 mM $KH_2PO_4$–$Na_2HPO_4$ buffer, pH: 7.0). The final concentration of catalase was 1.0 $\mu$g/ml. Catalase concentrations were determined by measuring the absorbance at 405 nm. Denaturation percentage (*D*%) was calculated as the ratio of initial velocity of the reaction before freeze-drying to that after freeze-drying. The velocity before freeze-drying was defined as 100.

**Freeze-Drying** Normally 100 $\mu$l-aliquots of buffer solution (5 mM $KH_2PO_4$–$Na_2HPO_4$) containing both catalase and additives described in the text were frozen in methanol–dry ice (−70 °C) and dried for at least 18 h at room temperature and at 0.003 Torr. The samples were rehydrated to their original volume with distilled water, and assayed after dilution with 50 mM buffer. Residual water contents of freeze-dried samples were determined gravimetrically. Samples in a freeze-dry holder were brought to atmospheric pressure with dried $N_2$ and immediately weighed. Weight loss upon drying at 60 °C and at 0.1 Torr on $P_2O_5$ for 1 d was also measured.

## Results

The denaturation percentage of catalase (*D*%) after freeze-drying is determined at various catalase concentrations ranging from 1 to 5000 $\mu$g/ml in the absence of a stabilizing agent. It is about 65%, which is independent of concentration. *D*% *vs.* maltose concentration plots are obtained at various catalase concentrations and Fig. 1 shows the plot at the catalase concentration of 1000 $\mu$g/ml. Fig. 2 summarizes the relations between maltose and catalase concentrations at certain constant values of *D*%. At the catalase concentration over about 500 $\mu$g/ml, there

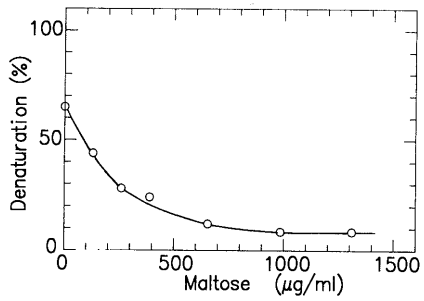


Fig. 1. Denaturation % *vs.* the Concentration of Maltose (μg/ml) Curve

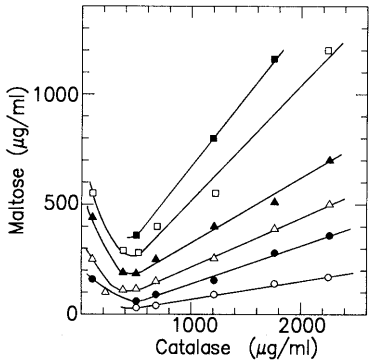


Fig. 2. Relation between Maltose Concentration and Catalase Concentration at $x$% Denaturation

■: $x=10$%; □: $x=15$%; ▲: $x=20$%; △: $x=30$%; ●: $x=40$%; ○: $x=50$%.

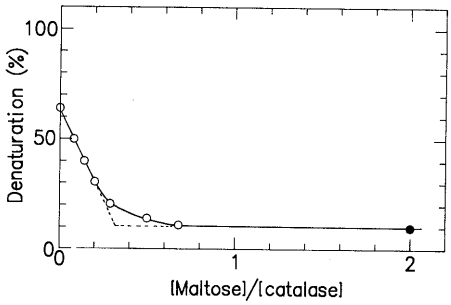


Fig. 3. Relation between $D$% and the Weight Ratio ($R$) of Maltose to Catalase

○: plots from the data in Fig. 2.; ●: experimental plot at $R=2$.

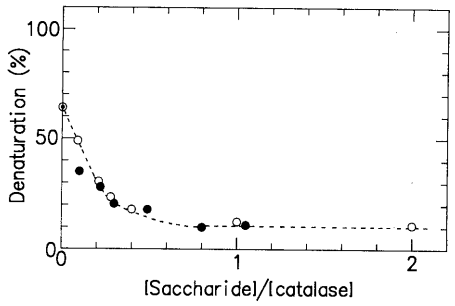


Fig. 4. $D$% *vs.* $R$ Plot

○: glucose; ●: maltotriose; A dotted curve represents the relation for maltose shown in Fig. 3.

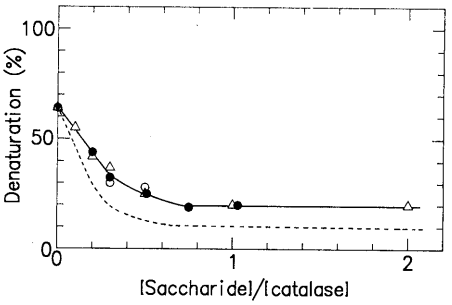


Fig. 5. $D$% *vs.* $R$ Plot

○: maltopentaose; ●: maltohexaose; △: maltoheptaose; A dotted curve represents the relation for maltose shown in Fig. 3.

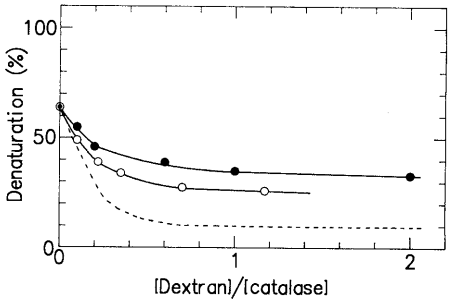


Fig. 6. $D$% *vs.* $R$ Plot

○: dextran (Mw = 19800); ●: dextran (Mw = 100000—200000); A dotted curve represents the relation for maltose shown in Fig. 3.

is a linear relationship between maltose and catalase concentrations at each $D$%. All the extrapolated lines pass through the zero point, indicating that $D$% depends not on bulk concentration of maltose, but on the weight ratio of maltose to catalase, $R$. Maltose concentration required for the same $D$% increases with decreasing catalase concentration in the low concentration range ($<500$ μg/ml). The reason why these deviations occur is not obvious at the present stage. All experiments were hereafter performed in the linear region ($>500$ μg/ml of catalase). In Fig. 3, $D$% are plotted against $R$ which has been obtained from the slopes of lines shown in Fig. 2. The $D$% values obtained from experimental data at $R=2$ using various catalase concentrations are also shown in Fig. 3 (closed circle). They converged to $D\%=10$ which is designated as $D_m$. The value of $D$% initially decreases linearly with an increase of $R$, and approaches to $D_m$. The $R$ value at which the initial line intersects the horizontal line ($D\%=D_m$) is designated as $R_m$. $R_m$ can be regarded as a minimum value of $R$ where the protective effect of maltose reaches the maximum.

Next, the effect of the length of glucoside chains of a saccharide molecule on its cryoprotective efficiency was investigated. $D$% is measured by changing saccharide concentrations in the presence of 1000 μg/ml of catalase. Fig. 4 shows the $D$% *vs.* $R$ plots for glucose and maltotriose. A dotted cruve represents the relation for maltose obtained in Fig. 3. There are no significant differences among three saccharides. Glucose, maltose and maltotriose show similar protective activities on the basis of their weights. Fig. 5 shows the $D$% *vs.* $R$ plots for maltopentaose, maltohexaose and maltoheptaose. They show similar behavior as maltose, except for the higher $D_m$ values at the same $R$ value. These results show that the protective activities on the basis of their weights decrease when glucoside chains of saccharide are longer than 3. Such molecular weight dependence is also observed in the case of dextran as shown in Fig. 6. The $D_m$

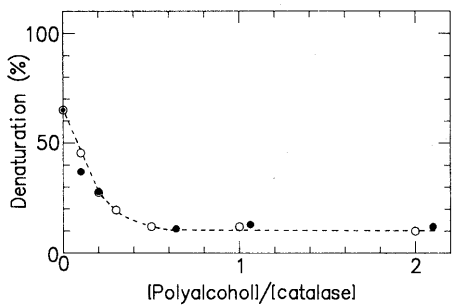


Fig. 7. $D\%$ *vs.* $R$ Plot

○: mannitol; ●: sorbitol; A dotted curve represents the relation for maltose shown in Fig. 3.

value increases with the molecular weight of dextran. Fig. 7 shows $D\%$ *vs.* $R$ plots for polyalcohols such as mannitol and sorbitol. Their activities are similar to that of maltose.

Amounts of residual water in the freeze-dried samples are examined for catalase and a mixture of catalase and maltose. The amounts were $0.7—1.3 \times 10^{-2}$ g of water per gram of catalase and $0.3—0.8 \times 10^{-2}$ g of water per gram of the mixture.

## Discussion

Water molecules cover the surface of the protein molecule as a monolayer and this monolayer plays an important role in the maintenance of the three-dimensional structure of the protein.[13,14] In this connection, we evaluated the change in the hydrated state of catalase by freeze-drying. At first, the amount of the hydration water in an aqueous protein solution was determined according to two proposed methods. Kunz[15] estimated amounts of hydration water of several proteins by summing up those of the amino acid residues exposed to the solvent (method 1). The amounts of hydration water of each amino acid in the respective polypeptide had been examined by the NMR method. The calculated values showed fairly good agreement with the experimental values by NMR. On the other hand, Lumry[16] estimated the amount of hydration water of chymotrypsin by counting numbers of nitrogen and oxygen atoms, which can be bound to water molecules on the surface of chymotrypsin molecule (method 2). In the present study, the amount of hydration water of catalase is estimated by these two methods. An X-ray study of beef liver catalase had been reported by Murthy *et al.*[17] Catalase contains four identical subunits and the monomer consists of 505 amino acid residues. They listed all external residues exposed to solvent and also the residues which contact with the neighboring subnuits. The amount of the hydration water of catalase thus obtained is 0.17—0.18 g per gram of catalase or $2.3 \times 10^3$—$2.6 \times 10^3$ molecules per one catalase molecule, as shown in Table I. At the final drying state in this freeze-drying process, the amount of residual water is $0.7—1.3 \times 10^{-2}$ g water per gram of catalase, which corresponds to about 5% of total hydration waters of catalase (0.17—0.18 g of water per gram of catalase). That means about 95% of hydration waters are removed by freeze-drying, which is a highly desiccated situation. This high desiccation probably results in the denaturation of catalase.

For the protection mechanism of saccharide, two mechanisms had been proposed as mentioned in the introduction. One is that saccharides directly interact with proteins. Carpenter and Crowe[10] investigated the mechanism by Fourier-transform infrared spectroscopy. They suggested that saccharides stabilize proteins, because these solutes are bound to proteins through hydrogen-bonding. The other is that saccharides affect the ordering degree of water around proteins (solvent-mediated interaction) as proposed by Hellman *et al.*[11] As shown in Fig. 2, denaturation percentage ($D\%$) depends not on bulk concentration of saccharide, but on the weight ratio of saccharide to catalase ($R$). This fact strongly suggests that the direct interaction of saccharides with catalase is mainly responsible for the protection effect. If the solvent-mediated interaction were dominant, $D\%$ should be absolutely dependent on the bulk concentration of saccharide. In the second model, the stabilizing effect should strongly depend on the ordering degree of water affected by additives. There is a large difference between the ordering degree of water affected by polyalcohol and saccharide. The position of oxygen atoms of equatorial -OH group in the hexopyranose ring of saccharide fits that of the oxygen atom in the tridymite-like structure of water.[18] However, those of polyalcohols such as mannitol and sorbitol do not. This fact would reflect a difference in the ordering effect of water by these two kinds of solutes. Polyalcohols such as mannitol showed a much lower stabilizing effect on the dissociation of L-asparaginase compared with saccharides such as glucose and sucrose.[11] On the contrary, in this study, mannitol and sorbitol show a similar effect as maltose on the stabilization of catalase. Therefore, in the case of catalase, the direct interaction must be the dominant one. L-Asparaginase shows solvent-mediated interaction,[11] probably because its dissociation occurs when a fairly large amount of water remains in the system during freeze-drying. It is reported that the denaturation of catalase by freeze-drying is also accompanied by the dissociation.[5] But, the protection mechanism in catalase was found to be the direct interaction. This fact suggests that the dissociation of catalase takes place after a large amount of water has been removed from the system.

Based on the above discussion, an interaction mechanism between saccharide and catalase can be proposed. The direct binding of saccharide to catalase reaches saturation at $R_m$. As described below, the saccharide may be bound to the catalase as a monomolecular layer at a point of $R_m$. The number of hydrogen-bonding sites per glucose residue of each saccharide at $R_m$, $n$, can be obtained by

$$n=(N/M_c)/(R_m/M_s)n_c \quad (1)$$

where $N$ is the total number of the hydrogen-bonding site. $M_c$ and $M_s$ are molecular weights of catalase and saccharide, respectively. $n_c$ is the number of glucose residues of each

Table I. Amounts of Hydration Water Molecules of Catalase Calculated by Two Different Methods

| Method | $N(\times 10^3)^{a)}$ | $H_2O$ (g)/catalase (g) |
|---|---|---|
| 1 | 2.6 | 0.18 |
| 2 | 2.3 | 0.17 |

*a*) Numbers of the hydration water molecules per one catalase molecule.

TABLE II. Values of $n$ and $D_m$ for Each Saccharide

| Saccharide | $n$ | $D_m$ |
|---|---|---|
| Glucose | 4.9 | 10 |
| Maltose | 4.8 | 10 |
| Maltotriose | 4.7 | 10 |
| Maltopentaose | 3.8 | 20 |
| Maltoheptaose | 3.7 | 20 |

saccharide. $N$ is assumed to be identical to the number of hydration water molecules per catalase molecule, $2.4 \times 10^3$, which is an average value by the two methods (see Table I). The residual water content of a mixture of protein and saccharide after freeze-drying should be taken into account, because saccharide can not be bound to the sites already occupied by water. The residual water content in this experimental condition is $0.3—0.8 \times 10^{-2}$ g of water per gram of the mixture of catalase and saccharide, which corresponds to only 3% of the total hydration water. This effect is negligible. $n$ and $D_m$ are summarized in Table II. For glucose, maltose and maltotriose, $n$ of each saccharide is about 5. The numbers of hydration water of saccharides obtained as unfreezable water by the differential scanning calorimeter are for example 4 and 6.5 for glucose and maltose, respectively.[19] The number of the hydrogen-bond per saccharide molecule actually formed on the surface of catalase may be less than that of the hydration water of the saccharide molecule. On the other hand, the $n$ value obtained by Eq. 1 must be greater than the number of the actual hydrogen-bond by two reasons. The first reason is that not all hydration sites on the surface of catalase are available for hydrogen-bonding with saccharide, since the surface of the catalase molecule is not smooth and a molecular size of water is far smaller than that of saccharide. The second is that a steric hindrance exists between saccharide molecules on the surface of catalase. By taking the above into account, the $n$ value obtained is consistent with the monomolecular layer mode. This monomolecular layer prevents catalase from being denatured instead of a hydration monolayer.

Agreement of the $D_m$ values of glucose, maltose and maltotriose suggests that the protection effect of these three saccharides are the same. $n$ is found to be clearly smaller when glucoside chains are longer than 3. It may be ascribed to configurational incompatibility between donor and acceptor groups of hydrogen bonding. In order to form hydrogen-bonding, an adequate bond angle and length must be required for donor and acceptor groups, that is, hydrogen bonding is the bond with orientation. When the glucoside chain is longer than 3, the chain tends to bend at ether linkage and to form intramolecular hydrogen-bonding.[20,21] As a result, the degree of incompatibility between saccharide molecules and amino acid residues exposed to the solvent increases. Therefore, the number of -OH groups free from hydrogen-bonding increases, and $n$ becomes smaller. The degree of the protection effect represented by $D_m$ also shows a clear change between $n=3$ and 5. This may be also explained by the same reasons. The protective layer must become loose, when the degree of incompatibility increases. Dextran in the present experiment shows a far less protection effect depending on molecular weight as shown in Fig. 6. This result may be related to the large size and configuration of the dextran, which makes it difficult to form the protective layer.

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# Thermochemical Aspects of Partition of Methyl- and Halogen-Substituted Alcohols in 1-Octanol/Water and a Novel Regression Analysis of Alcohol Toxicities

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**Thermodynamic parameters have been measured for alcohol partitioning in the widely used 1-octanol/water system in order to examine thermochemical aspects of partitioning quantitatively. A titration calorimetry is adopted for the experimental procedure, which can afford the partition coefficient and enthalpy ($\Delta H_p^0$) of partition, and hence the entropy term ($\Delta S_p^0$) as well, without recourse to the van't Hoff plot after variable temperature experiments. Linear correlations are observed between the observed values of $\Delta G_p^0$, $\Delta H_p^0$, and $\Delta S_p^0$ and molar volumes of alcohols. In these correlations, $\Delta H_p^0$ and $\Delta S_p^0$ can clearly discriminate between methyl- and halogen-substituted alcohols whereas $\Delta G_p^0$ cannot. The thermodynamic parameters have also been examined by novel quantitative structure–activity relationship (QSAR) analysis. The enthalpy term $\Delta H_p^0$ is found to afford simple and straightforward correlations with reported biological activities. The origin of this finding is discussed.**

**Keywords** partition; 1-octanol/water; free energy; enthalpy; entropy; alcohol; toxicity; QSAR analysis

Solute partitioning between immiscible phases is an important process in chemistry: it relates directly to the solvent extraction in analytical chemistry and also to the transport phenomena in medicinal chemistry. Hitherto, the partition coefficient has been studied for a variety of solutes in different partitioning systems, and its logarithm, $\log P$, has been utilized as a valuable parameter in the analysis of quantitative structure–activity relationships (QSAR).[1–3]

Recently, attention has been directed to the thermochemical aspects of partition. Fuchs *et al.* examined the $\Delta G_p^0$, $\Delta H_p^0$, and $T\Delta S_p^0$ data in an isooctane/water system upon their correlation with intrinsic volumes and solvatochromic parameters.[4] Studies of the thermodynamic data of some solutes in different partitioning systems have revealed that different partitioning systems such as liposome/water, micelle/water, 1-octanol/water, and alkane/water systems behave differently in giving thermodynamic parameters.[5–7] It is also suggested regarding the method of experiment that use of the popular van't Hoff plot may give erroneous results when the two partitioning solvents have high mutual solubility.[8] In spite of these interesting findings, systematic studies of the thermochemical aspects of partition are very rare: such studies are essential and urged for the full understanding of partition phenomena.

In the present study the $\log P$ term which corresponds to the Gibbs free energy change of partition is separated into two parts, enthalpy ($\Delta H_p^0$) and entropy ($\Delta S_p^0$), by means of titration calorimetry. The results are discussed on the basis of solution chemistry and in relation to QSAR analysis.

## Experimental

Alcohols were all distilled in a glass vessel, and water was distilled after desalting. The titration calorimetry was performed on a TRONAC 558 Calorimeter under an isothermal mode at 25 °C. The procedure follows that reported for the same type of calorimeter.[9] A typical run was as follows. A 0.01—0.03 M solute alcohol was dissolved in 40 ml water in a reaction vessel which was buffered at pH 7.0 by 1/15 M monopotassium phosphate plus 1/15 M disodium phosphate (3 : 2). About 10 ml of 1-octanol was added titrimetrically in 10 steps from a buret. Such titration and the following data collection were performed automatically with the aid of a CCP930 Programmer. The titration data was first analyzed by a graph method as described in ref. 9, and the partition coefficient and enthalpy change were obtained. Then, these parameters were refined by means of a computer program which minimizes the sum of squares of deviation between the observed and calculated heats of titration. The standard state adopted is 25 °C and 1 M in solution. These calculations were done on a NEAC S-2000 Computer at the Computation Center, Osaka University.

## Results

Calorimetric measurements are summarized in Table I.

TABLE I. Thermodynamic Parameters of Partition in 1-Octanol/Water (pH 7) System at 25 °C and Biological Activities of Alcohols

| No. | Alcohols | $\log P$ | | $\Delta G_p^0$ (kJ/mol) | $\Delta H_p^0$ (kJ/mol) | $\Delta S_p^0$ (J/K mol) | Biological activities[13] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | This work | Ref. 2 | | | | $ID_{50}$ (M) | $T_i$ (%/mol) | $H_{50}$ (M) | $LD_{50}$ (M) |
| 1 | Ethanol | −0.25±0.07 | −0.30 | 1.4±0.9 | 13.5±0.6 | 40.6±2.2 | 0.15540 | −304.6 | 2.160 | 0.01208 |
| 2 | 1-Propanol | 0.24±0.10 | 0.30 | −1.4±1.2 | 12.0±0.8 | 44.8±2.8 | 0.05581 | −950.9 | 0.698 | 0.00363 |
| 3 | 1-Butanol | 0.82±0.05 | 0.88 | −4.7±0.5 | 10.2±0.3 | 50.0±1.2 | — | — | — | — |
| 4 | 1-Pentanol | 1.41±0.06 | 1.40 | −8.1±0.2 | 8.4±0.1 | 55.2±0.6 | — | — | — | — |
| 5 | 1-Hexanol | 2.11±0.03 | 2.03 | −12.1±0.4 | 6.6±0.3 | 62.4±1.0 | — | — | — | — |
| 6 | 1-Heptanol | 2.72±0.07 | — | −15.6±0.2 | 2.7±0.1 | 61.3±0.7 | — | — | — | — |
| 7 | 2-Propanol | 0.11±0.05 | 0.05 | −0.7±1.0 | 14.5±0.7 | 50.8±2.4 | 0.09843 | −508.3 | 1.178 | 0.00608 |
| 8 | 2-Methyl-1-propanol | 0.74±0.05 | 0.76 | −4.2±0.3 | 10.5±0.2 | 49.4±0.7 | 0.02025 | −2366.9 | 0.270 | 0.004208 |
| 9 | 2,2-Dimethyl-1-propanol | 1.29±0.03 | 1.32 | −7.4±0.3 | 10.0±0.2 | 58.3±0.7 | 0.01078 | −4770.7 | 0.128 | 0.00505 |
| 10 | 2-Butanol | 0.62±0.09 | 0.61 | −3.6±1.0 | 14.2±0.7 | 59.5±2.5 | 0.04300 | −1130.0 | — | 0.008345 |
| 11 | 2-Chloroethanol | −0.08±0.14 | 0.03 | 0.4±1.0 | 6.5±0.6 | 20.3±2.4 | 0.03193 | −1649.8 | 0.593 | 0.00168 |
| 12 | 2,2-Dichloroethanol | 0.71±0.09 | 0.37 | −4.1±0.4 | 2.9±0.3 | 23.3±1.2 | 0.00345 | −9116.5 | — | 0.000746 |
| 13 | 2,2,2-Tricholoroethanol | 1.59±0.11 | 1.35 | −9.1±0.3 | 0.9±0.0 | 33.4±0.9 | 0.00084 | −69789.8 | 0.0072 | 0.000536 |
| 14 | 2-Bromoethanol | 0.29±0.12 | 0.23 | −1.6±0.8 | 4.9±0.6 | 22.0±2.1 | 0.00253 | −41174.6 | 0.048 | 0.0009416 |
| 15 | 2-Fluoroethanol | −0.68±0.06 | −0.92 | 3.9±0.7 | 9.5±0.5 | 19.0±1.7 | 0.02522 | −1997.3 | — | 0.0032877 |

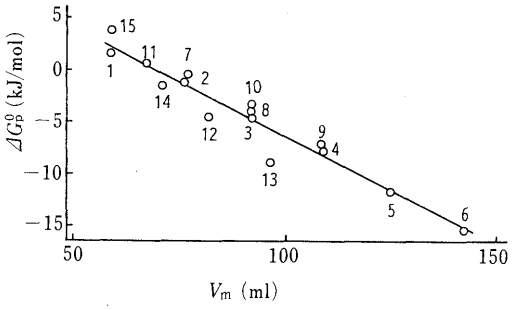


Fig. 1. Plots of $\Delta G_p^0$ against Molar Volume ($V_m$)
The numbering corresponds to that in Table I.

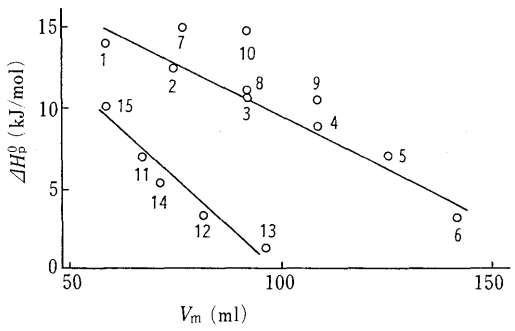


Fig. 2. Plots of $\Delta H_p^0$ against Molar Volume ($V_m$)
The numbering corresponds to that in Table I.

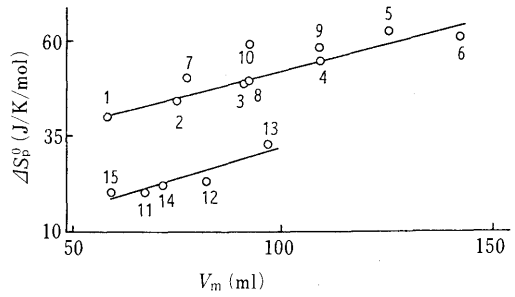


Fig. 3. Plots of $\Delta S_p^0$ against Molar Volume ($V_m$)
The numbering corresponds to that in Table I.

Standard deviations are derived from repeated experiments (3 to 5 times). When the experimental values are plotted against the molar volume ($V_m$) of solute, straight lines are observed for $\Delta G_p^0$, $\Delta H_p^0$, and $\Delta S_p^0$ (Figs. 1 to 3). $\Delta G_p^0$ changes linearly with $V_m$ for all the alcohols treated here, whereas $\Delta H_p^0$ and $\Delta S_p^0$ give rise to linear relations for the methyl- and halogen-substituted alcohols separately. These trends can be interpreted to show that the characteristic substitution of halogens is reflected in both the $\Delta H_p^0$ and $\Delta S_p^0$ terms, but that contributions to these terms almost cancel each other in yielding the Gibbs free energy term $\Delta G_p^0$.

## Discussion

Thermodynamic parameters measured above could be discussed by calculation based on the scaled particle theory which is reported by the authors[10] for the gas-liquid chromatographic process of several alkanes. According to this theory, the thermodynamic parameters of solution are related to the terms of cavity formation, solute-solvent interactions, and subsidiary corrections regarding the standard states in gas and liquid states. The cavity term is composed of the surface energy and PV work when a solute is dissolved in solvent, and hence it is closely connected with the molar volume (or surface area) of the solute. The molar volume dependency observed in Figs. 1 to 3 may be due to this term. The solute-solvent interaction term is expressed, for example, by means of the Kihara potential.[10] This term may be responsible for the discrimination between the halogenated and non-halogenated alcohols in $\Delta H_p^0$ and $\Delta S_p^0$.[11] It is not clear why halogenated alcohols are not discriminated on the $\Delta G_p^0$ scale. Further studies would be needed with various types of solutes, but it is evident that the enthalpy and entropy terms partly cancel each other in yielding the free energy term, and that the dependency on the molar volume observed separately for these two components is almost canceled on the $\Delta G_p^0$ scale.[12]

In the present study, theoretical consideration of the thermodynamic parameters themselves is not extended any more because of the lack of reliable potential parameters of the alcohols. Instead, the importance of these parameters in QSAR analysis is discussed.

Hitherto, biological activities have been examined for their dependency on partition coefficients and the role of the transport process in the manifestation of these activities judged. In order to estimate the role of transportation more widely, the thermodynamic properties of partition are tested in this study as independent parameters for QSAR analysis. The regression analyses of alcohol toxicities[13] using these properties are summarized in Table II. The toxicity data (Table I) cited are the tissue culture toxicity ($ID_{50}$, the concentration required to produce 50% inhibition of growth), the hemolytic activity ($H_{50}$, the concentration required to produce 50% hemolysis in saline, 37 °C), the product of intrinsic toxicity ($T_i$, the slope of the dose–

TABLE II. Linear Regression Analyses of Alcohol Toxicities Based on Thermodynamic Parameters[a]

| Equations | $r$[b] | $F$[c] | S.D.[d] |
|---|---|---|---|
| $-\log ID_{50}=0.677(0.615)\log P+1.475(0.466)$[e] | 0.639 | 6.20 | 0.571 |
| $-\log ID_{50}=-0.137(0.048)\Delta H_p^0+2.999(0.482)$ | 0.908 | 42.2 | 0.311 |
| $-\log ID_{50}=-0.018(0.032)\Delta S_p^0+2.440(1.298)$ | 0.391 | 1.63 | 0.683 |
| $-\log ID_{50}=-0.176(0.051)\Delta H_p^0$ $-0.017(0.015)\Delta S_p^0+2.682$ (0.476) | 0.951 | 38.0 | 0.243 |
| $-\log LD_{50}=0.328(0.594)\log P+2.380(0.455)$ | 0.410 | 1.62 | 0.453 |
| $-\log LD_{50}=-0.092(0.021)\Delta H_p^0+3.379(0.212)$ | 0.963 | 102.0 | 0.134 |
| $-\log LD_{50}=-0.024(0.017)\Delta S_p^0+3.517(0.714)$ | 0.759 | 10.9 | 0.323 |
| $-\log LD_{50}=-0.086(0.035)\Delta H_p^0$ $-0.003(0.012)\Delta S_p^0+0.3440(0.326)$ | 0.965 | 47.2 | 0.139 |
| $-\log H_{50}=1.006(0.721)\log P+0.119(0.567)$ | 0.813 | 11.7 | 0.513 |
| $-\log H_{50}=-0.155(0.081)\Delta H_p^0+2.025(0.816)$ | 0.887 | 22.1 | 0.407 |
| $-\log H_{50}=-0.016(0.057)\Delta S_p^0+1.238(2.402)$ | 0.264 | 0.45 | 0.848 |
| $-\log H_{50}=-0.212(0.064)\Delta H_p^0$ $-0.030(0.022)\Delta S_p^0+1.333(0.700)$ | 0.970 | 39.5 | 0.235 |
| $-\log 1/T_i=-0.675(0.680)\log P-1.806(0.515)$ | 0.599 | 5.04 | 0.631 |
| $-\log 1/T_i=-0.142(0.058)\Delta H_p^0-0.244(0.580)$ | 0.880 | 30.9 | 0.374 |
| $-\log 1/T_i=-0.019(0.034)\Delta S_p^0-0.790(1.377)$ | 0.396 | 1.67 | 0.724 |
| $-\log 1/T_i=-0.178(0.071)\Delta H_p^0$ $+0.016(0.021)\Delta S_p^0-0.545(0.658)$ | 0.916 | 20.8 | 0.336 |

*a*) The log $P$ data used are determined in the present study. Number of data: 11 ($ID_{50}$, $1/T_i$), 10 ($LD_{50}$, 2-fluoroethanol excluded), 8 ($H_{50}$, see Table I). *b*) Correlation coefficient. *c*) Variance ratio. *d*) Standard deviation. *e*) 95% confidence intervals are listed in parentheses.

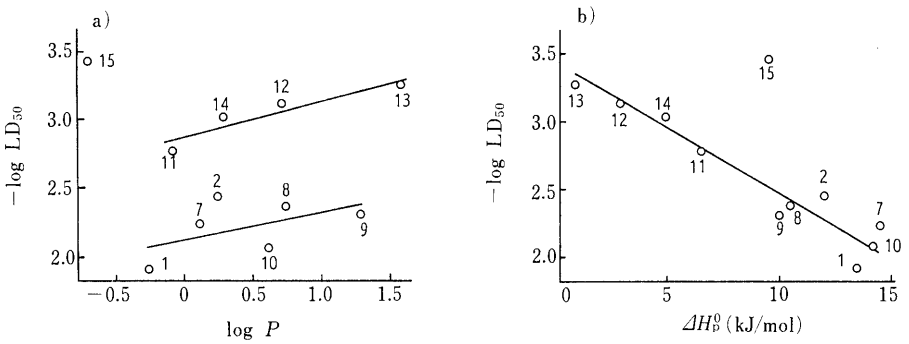


Fig. 4. Plots of $-\log LD_{50}$ against log $P$ (Left, a) and $\Delta H_p^0$ (Right, b)
The numbering corresponds to that in Table I.

TABLE III. Linear Regression Analyses of Alcohol Toxicities Based on Conventional Physical Parameters[a]

| Equations | $r$[b] | $F$[c] | S.D.[d] |
|---|---|---|---|
| $-\log ID_{50} = -0.850E_s(R_2) + 1.302$ | 0.83 | 20.23 | 0.41 |
| $-\log ID_{50} = 0.557\log P + 1.565$ | 0.53 | 3.44 | 0.63 |
| $-\log ID_{50} = 0.025\log P - 0.835E_s(R_2) + 1.301$ | 0.83 | 9.01 | 0.44 |
| $-\log LD_{50} = -0.116\log P + 2.678$ | 0.15 | 0.20 | 0.55 |
| $-\log LD_{50} = -0.402E_s(R_2) + 2.423$ | 0.53 | 3.43 | 0.47 |
| $-\log LD_{50} = 0.212(\log P)^2 - 0.158\log P + 6.647Q_C + 29.785Q(R_2) + 1.164$ | 0.95 | 15.24 | 0.20 |

*a*) Number of data: 11 ($ID_{50}$), 10 ($LD_{50}$, 2-fluoroethanol excluded). See ref. 13 for the parameters used. *b*) Correlation coefficient. *c*) Variance ratio. *d*) Standard deviation.

response curve in tissue culture), and the acute *in vivo* toxicity ($LD_{50}$, the single intraperitoneal dose required to kill 50% of the mice in 7 d). In the present study, the enthalpy term $\Delta H_p^0$ is found to reproduce well the whole data of toxicities from *in vitro* to *in vivo* (Table II).[14] On the contrary, conventional analyses using log $P$ give only worse fittings,[15] so other terms such as steric ($E_s$) or electronic ($Q$) must be included to obtain improved fittings as reported in ref. 13. Table III lists such an analysis using the same data treated in Table II. The simple correlation with $\Delta H_p^0$ (Fig. 4b) observed over the whole methyl- and halogen-substituted alcohols suggests that the two series of alcohols are working through a common mechanism controlled by transportation. This is in clear contrast to a conclusion reached from the analysis using log $P$ only,[13] where halogenated and non-halogenated alcohols had to be treated separately (Fig. 4a). It is seen from Fig. 4b that the halogenated alcohols are more toxic because of their lower $\Delta H_p^0$ values. Since the $\Delta H_p^0$ observed is positive for all alcohols treated here, the partition process itself is driven by the entropy term $\Delta S_p^0$. However, substituent dependency of the biological toxicity is dominated by the $\Delta H_p^0$ term as shown in Fig. 4 and in Table II. The halogen substituents reduce the $\Delta H_p^0$ value and enhance the activity. The biological activity $T_i$ which is an intrinsic toxicity and is considered[13] to express the toxicant–receptor interaction is also better correlated with $\Delta H_p^0$. At first sight, this might seem inconsistent with the determination that the $\Delta H_p^0$ term reflects the tendency of partition and not that of molecular interaction. However, this inconsistency may be solved when the following case is considered, where the toxicants interact with a receptor through penetrating into the hydrophobic moiety of the receptor by menas of molecular or hydrophobic interactions, just as they are partitioned into a hydrophobic moiety of the 1-octanol/water system.

It is evidenced from a model kinetic calculation[16] that biological activities can be approximated by an appropriate function of log $P$ if the manifestation of the activities is dominated by the transport process. Therefore, it is worth mentioning why $\Delta H_p^0$ correlates with the biological data much better than log $P$ does in the present case. It should be noted that the physical data are obtained in a model system of 1-octanol/water whereas the biological data are obtained *in vivo* or *in vitro*. Use of the model system enables us to obtain the physical data of drugs more easily and with better reproducibility. But it is also recognized that the 1-octanol/water system does not always simulate well the characteristics of biophase. For example, it is reported for the partition of *n*-hexane that an isotropic hydrocarbon liquid is not a good model for the interior of liquid bilayers.[5,17] It is also reported that the partition into a bilayer phase is prefered enthalpically and not entropically when compared with that into an isotropic phase.[9] It may well be understood that the relative contribution of the enthalpy and entropy of partition differs between the model and the biophase systems. Therefore, the Gibbs free energy term log $P$ in the biophase, where biological activities are tested, needs to be substituted with an appropriate combination of the enthalpy and entropy terms measured in a model system. In this sense, both $\Delta H_p^0$ and $\Delta S_p^0$ are candidates for the QSAR parameters. In the present case, however, inclusion of both terms did not improve the regression analysis appreciably (Table II). This fact is interpreted to show either that the entropy term observed in the model system does not successfully reflect the term in the biophase or that the entropy term does not play a dominant role in transportation in the biophase systems where the toxicants are tested. Probably the latter case holds in the present study, since the enthalpy term observed in the model system by itself succeeds in reproducing well the biological data (Table II).

In conclusion, the thermochemical aspects of partition are evidenced to be important in the QSAR analysis of alcohol toxicities. Such studies will enable us to discuss from a larger viewpoint the role of the transport process in the manifestation of biological activities.

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11) Other factors such as the physical properties, *e.g.*, thermal expansion coefficient, may also contribute to such a discrimination since the entropy term arising from the molecular interaction is small and usually neglected.
12) That the enthalpy and entropy do not exactly cancel each other is suggested from a finding that the halogenated alcohols (No. 11—15) give rise to an alternative slightly steep line in Fig. 1.
13) E. O. Dillingham, R. W. Mast, G. E. Bass, and J. Autian, *J. Pharm. Sci.*, **62**, 23 (1973).
14) Fluoroethanol is excluded from the *in vivo* data $LD_{50}$ since this compound is known to be converted to a more active intermediate *in vivo*.[13)]
15) This situation does not change whether the log *P* data determined in the present study or those given in ref. 13 are used (Tables II and III) and whether all the alcohols for which toxicities are measured or those for which thermodynamic properties are measured in the present study are used (ref. 13 and Table III).
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17) H. Fujiwara, to be published.

# A Reagent, Ethyl 2-(2-*tert*-Butyl-2*H*-tetrazol-5-yl)-3-(dimethylamino)acrylate (DMTE), for Facile Synthesis of 2,3-(Ring Fused)-5-(5-tetratzolyl)-4*H*-pyrimidin-4-one Derivatives

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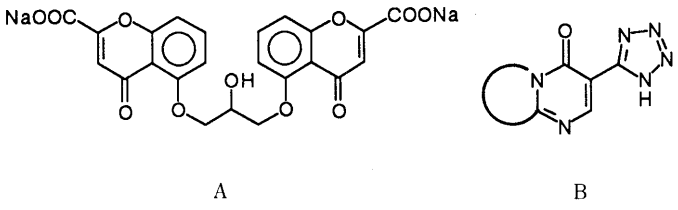
**A method for synthesizing 2,3-(ring fused)-5-(5-tetrazolyl)-4*H*-pyrimidin-4-one derivatives from ethyl 2-(2-*tert*-butyl-2*H*-tetrazol-5-yl)-3-(dimethylamino)acrylate (DMTE) (4a) and amino-heterocycles is described. The structure of DMTE, which was prepared from ethyl (2-*tert*-butyl-2*H*-tetrazol-5-yl)acetate (3a) with dimethylformamide diethylacetal, was determined by X-ray analysis to be *Z* form. The reaction of 2-amino-5-methyloxazole (6) with DMTE in acetic acid gave the oxazolo[3,2-*a*]pyrimidine derivative (8), heating of which in concentrated sulfuric acid afforded the desired tetrazole derivative (20). Pyrimido[2,1-*b*]benzothiazole (21), pyrazolo[1,5-*a*]pyrimidine (22 and 23) and [1,2,4]triazolo[1,5-*a*]pyrimidine (24) derivatives were prepared in a similar manner.**

**Keywords** ethyl 2-(2-*tert*-butyl-2*H*-tetrazol-5-yl)-3-(dimethylamino)acrylate; DMTE; fused pyrimidine; tetrazole derivative; cyclization; X-ray analysis

Disodium cromoglycate (DSCG),[1] which acts mainly as an inhibitor of chemical mediator release from sensitized mast cells, is used clinically for the prophylactic treatment of bronchial asthma. Many 5-(5-tetrazolyl)-4*H*-pyrimidin-4-ones fused at the 2,3 positions with a heterocycle have been reported to possess DSCG-like antiallergic activity.[2,3] During our search for new antiallergic drugs, we became interested in these tetrazolylpyrimidines, which, with respect to the carbonyl and acidic groups, have different chemical properties from those of DSCG.

Synthetic methods for the desired compounds has not been fully explored, affording only a limited number of analogues. The tetrazolyl derivatives were generally synthesized from the corresponding nitriles.[2] In the present cases, however, the synthesis of cyano derivatives sometimes presents difficult problems. For example, some ring systems (*e.g.*, pyrimido[2,1-*b*]benzoxazole) are so unstable under acidic or basic conditions, because of their facile ring-opening,[2] that the conversion from esters into nitriles *via* the amides is difficult. Also, the cyclization reaction of (3-pyrazolyl)aminomethylene cyanoacetate does not give 6-cyanopyrazolo[1,5-*a*]pyrimidin-5-one but instead yields 5-aminopyrazolo[1,5-*a*]pyrimidine-6-carboxylate.[4,5] Wade *et al.*[2] and Yevich *et al.*[3] reported methods for the synthesis of some 5-(5-tetrazolyl)-4*H*-pyrimidin-4-ones, but there still seems to be much room for improvement, since the yields and ranges of applicability were unsatisfactory.



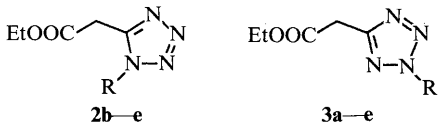

A B

Fig. 1. Chemical Structures of DSCG (A) and 2,3-(Ring Fused)-5-(5-tetrazolyl)-4*H*-pyrimidin-4-one (B)

In the present paper, we report a new reagent, ethyl (*Z*)-2-(2-*tert*-butyl)-2*H*-tetrazol-5-yl)-3-(dimethylamino)-acrylate (DMTE) (**4a**) for the efficient construction of ring systems which contain the 2,3-(ring fused)-5-(5-tetrazolyl)-4*H*-pyrimidin-4-one component.

**Preparation and Structure of DMTE Derivatives** Ethyl (1- or 2-substituted-1*H* or 2*H*-tetrazol-5-yl)acetates (**2b—e**

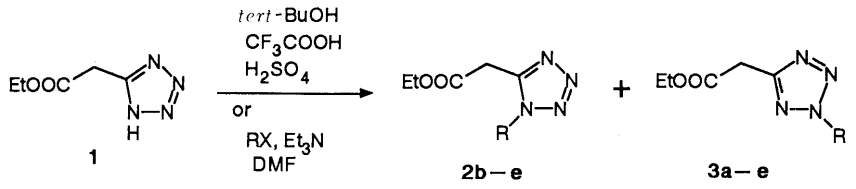
TABLE I. Ethyl (1- or 2-Substituted tetrazol-5-yl)acetates




**2b—e** **3a—e**

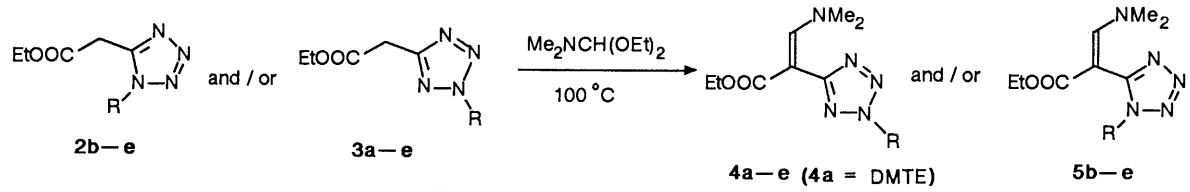
| R | Reaction conditions | 1-Substituted derivatives | | | 2-Substituted derivatives | | |
|---|---|---|---|---|---|---|---|
| | | Compd. | Yield (%) | mp or bp (°C) (Reported) | Compd. | Yield (%) | mp or bp (°C) (Reported) |
| *tert*-Bu | *tert*-BuOH, $CF_3COOH$, conc. $H_2SO_4$, r.t., 17 h | | | | **3a** | 44 | bp 113—120 (3 mmHg) (Oil)[7] |
| Me | MeI, $Et_3N$, DMF, r.t., 26 h | **2b** | 16 | mp 67—69[b] (mp 67—68)[8] | **3b** | 24 | bp 125—135 (3 mmHg) (bp 103—104 (0.8 mmHg))[8] |
| Et | EtI, $Et_3N$, DMF, r.t., 16 h | **2c** | 13[a] | bp 160—180 (3 mmHg) (Oil)[7] | **3c** | 41[a] | bp 117—120 (3 mmHg) (Oil)[7] |
| Benzyl | $C_6H_5CH_2Cl$, $Et_3N$, DMF, r.t., 16 h | **2d** | 15[a] | Oil | **3d** | 23[a] | Oil |
| *p*-Methoxy benzyl | *p*-$(MeO)C_6H_4CH_2Cl$, $Et_3N$, DMF, r.t., 93 h | **2e** | 33[a] | Oil | **3e** | 26[a] | Oil |

*a*) Determined by comparison of integration ratios of $^1$H-NMR signals. *b*) Recrystallized from benzene–*n*-hexane.




a : R=*tert*-Bu b : R=Me c : R=Et d : R=benzyl e : R=*p*-methoxybenzyl

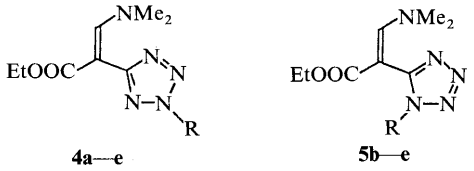
Chart 1




a : R=*tert*-Bu b : R=Me c : R=Et d : R=benzyl e : R=*p*-methoxybenzyl

Chart 2

TABLE II. Ethyl 3-(Dimethylamino)-2-(1- or 2-Substituted tetrazol-5-yl)acrylates




| Compd. | R | Yield (%) | mp (°C) | $(CH_3)_2N$– | >NCH=C< | $-CH_2$-Tet[g] | $^1$H-NMR ($CDCl_3$): $\delta$ ppm | | Miscellaneous |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | $CH_3CH_2O$ | $CH_3CH_2O$ | |
| **4a** | *tert*-Bu | 69 | 73—75[d] | 2.74 (s) | 7.78 (s) | | 1.15 (t, $J$=7.0) | 4.13 (q, $J$=7.0) | 1.73 (9H, s, $C(CH_3)_3$) |
| **4b** | Me | 55 | 101—103[e] | 2.76 (s) | 7.84 (s) | 4.40 (s) | 1.20 (t, $J$=7.0) | 4.16 (q, $J$=7.0) | |
| **5b** | Me | 56 | 94—95[f] | 2.25—3.25 (brs) | 7.93 (s) | 3.98 (s) | 1.21 (t, $J$=7.0) | 4.18 (q, $J$=7.0) | |
| **4c** | Et | 84 | Oil | 2.77 (s) | 7.82 (s) | 4.70 (q, $J$=7.0) | 1.19 (t, $J$=7.0) | 4.15 (q, $J$=7.0) | 1.66 (3H, t, $J$=7.0, $CH_3CH_2$-Tet) |
| **5c** | Et | 40[a] | Oil | 2.1— 3.5 (brs) | 7.91 (s) | 4.29 (q, $J$=7.0) | 1.20 (t, $J$=7.0) | 4.16 (q, $J$=7.0) | 1.56 (3H, t, $J$=7.0, $CH_3CH_2$-Tet) |
| **4d** | Benzyl | 46[b] | Oil | 2.65 (s) | 7.76 (s) | 5.79 (s) | 1.13 (t, $J$=7.0) | 4.11 (q, $J$=7.0) | 7.37 (5H, s, Ar-H) |
| **5d** | Benzyl | 34[b] | Oil | 1.5— 3.4 (brs) | 7.79 (s) | 5.46 (s) | 1.16 (t, $J$=7.0) | 4.14 (q, $J$=7.0) | 7.37 (5H, s, Ar-H) |
| **4e** | *p*-Methoxy benzyl | 43[c] | Oil | 2.65 (s) | 7.77 (s) | 5.73 (s) | 1.13 (t, $J$=7.0) | 4.12 (q, $J$=7.0) | 3.81 (3H, s, $CH_3O$) 6.89 (2H, d, $J$=9.0, Ar-H) 7.34 (2H, d, $J$=9.0, Ar-H) |
| **5e** | *p*-Methoxy benzyl | 49[c] | 100—103[f] | 1.7— 3.4 (brs) | 7.80 (s) | 5.39 (s) | 1.17 (t, $J$=7.0) | 4.15 (q, $J$=7.0) | 3.82 (3H, s, $CH_3O$) 6.83 (2H, d, $J$=8.0, Ar-H) 7.29 (2H, d, $J$=8.0, Ar-H) |

*a*) Prepared from a mixture of **2c** and **3c** (**2c**/**3c**=5/3). *b*) Prepared from a mixture of **2d** and **3d** (**2d**/**3d**=2/3). *c*) Prepared from a mixture of **2e** and **3e** (**2e**/**3e**=5/4). *d*) Recrystallized from ether–petroleum ether. *e*) Recrystallized from benzene–*n*-hexane. *f*) Recrystallized from benzene–petroleum ether. *g*) Tet=tetrazole.

and **3a—e**), listed in Table I, were prepared from ethyl (5-tetrazolyl) acetate (**1**)[6] according to the known method. Alkylation[7] of **1** yielded a mixture of 1- and 2-substituted derivatives (**2b—e** and **3b—e**), except that *tert*-butylation[8] of **1** yielded only the 2-substituted product (**3a**). The structures of the products were assigned on the basis of the proton nuclear magnetic resonance ($^1$H-NMR) spectra. It is known that the signals of *N*-methyl or *N*-methylene protons of 2-substituted tetrazole derivatives appear at lower field than those of 1-substituted ones,[9-12] and hence the substituted position in the products could be unambiguously determined.

We attempted to introduce a formyl or its equivalent group at the methylene moiety of **3a**. Neither ethoxymethylation of **3a** using triethyl orthoformate in acetic anhydride nor formylation using sodium methoxide in ethyl formate succeeded. The synthesis of DMTE from **3a** was accomplished in satisfactory yield by reacting **3a** with excess *N*,*N*-dimethylformamide (DMF) diethylacetal at 100 °C. The other derivatives (**4b—e** and **5b—e**) listed in Table II were prepared in a similar manner.

The structure of DMTE was estimated by examination of the $^{13}$C-NMR spectrum. Measurement of the vicinal $^{13}$C, H coupling constant $^3J$(CO, H) is a useful tool for the configurational assignment of C=C double bonds. Braun[13] reported that the *trans* $^3J$(CO, H) value (12 Hz) is characteristically greater than the *cis* $^3J$(CO, H) one (7 Hz). The $^3J$ (CO, H) value (6.9 Hz) of DMTE indicates that the ester moiety and the olefin proton have a *cis* configuration. Finally, to elucidate the stereostructure of DMTE, an X-ray crystallographic analysis was carried out. DMTE was found to be present in the *Z* form, and a computer-generated drawing of the structure is shown in Fig. 2. Selected X-ray data are listed in Table III. On the basis of the above results,

TABLE III. Bond Distances and Bond Angles for **4a**[a] with Their Estimated Standard Deviations in Parentheses

Bond distances (Å)[b]

| Atom 1 | Atom 2 | Distance | Atom 1 | Atom 2 | Distance | Atom 1 | Atom 2 | Distance |
|---|---|---|---|---|---|---|---|---|
| O1 | C4 | 1.209 (5) | N5 | C7 | 1.441 (6) | C2 | C4 | 1.447 (6) |
| O2 | C4 | 1.358 (5) | N5 | C8 | 1.457 (6) | C5 | C6 | 1.430 (8) |
| O2 | C5 | 1.468 (6) | N6 | N7 | 1.318 (5) | C9 | C10 | 1.474 (9) |
| O3 | C15 | 1.207 (5) | N6 | C13 | 1.327 (5) | C9 | C11 | 1.477 (9) |
| O4 | C15 | 1.346 (6) | N7 | N8 | 1.315 (5) | C9 | C12 | 1.445 (8) |
| O4 | C19 | 1.461 (8) | N7 | C21 | 1.493 (5) | C13 | C14 | 1.460 (6) |
| N1 | N2 | 1.330 (5) | N8 | N9 | 1.315 (5) | C14 | C15 | 1.447 (6) |
| N1 | C1 | 1.319 (5) | N9 | C13 | 1.350 (6) | C14 | C16 | 1.362 (7) |
| N2 | N3 | 1.316 (5) | N10 | C16 | 1.321 (6) | C19 | C20 | 1.42 (1) |
| N2 | C9 | 1.475 (5) | N10 | C17 | 1.461 (7) | C21 | C22 | 1.501 (8) |
| N3 | N4 | 1.318 (5) | N10 | C18 | 1.449 (7) | C21 | C23 | 1.475 (8) |
| N4 | C1 | 1.348 (5) | C1 | C2 | 1.461 (6) | C21 | C24 | 1.496 (8) |
| N5 | C3 | 1.326 (6) | C2 | C3 | 1.370 (6) | | | |

Bond angles (°)[b]

| Atom 1 | Atom 2 | Atom 3 | Angle | Atom 1 | Atom 2 | Atom 3 | Angle | Atom 1 | Atom 2 | Atom 3 | Angle |
|---|---|---|---|---|---|---|---|---|---|---|---|
| C4 | O2 | C5 | 116.9 (4) | C16 | N10 | C18 | 120.6 (5) | C11 | C9 | C12 | 111.1 (6) |
| C15 | O4 | C19 | 118.1 (5) | C17 | N10 | C18 | 116.0 (5) | N6 | C13 | N9 | 111.4 (4) |
| N2 | N1 | C1 | 103.0 (3) | N1 | C1 | N4 | 111.6 (4) | N6 | C13 | C14 | 124.0 (4) |
| N1 | N2 | N3 | 112.5 (3) | N1 | C1 | C2 | 124.6 (4) | N9 | C13 | C14 | 124.6 (4) |
| N1 | N2 | C9 | 123.3 (4) | N4 | C1 | C2 | 123.8 (4) | C13 | C14 | C15 | 120.0 (5) |
| N3 | N2 | C9 | 123.9 (4) | C1 | C2 | C3 | 125.3 (4) | C13 | C14 | C16 | 125.5 (5) |
| N2 | N3 | N4 | 106.8 (4) | C1 | C2 | C4 | 120.2 (4) | C15 | C14 | C16 | 114.5 (5) |
| N3 | N4 | C1 | 106.1 (3) | C3 | C2 | C4 | 114.6 (4) | O3 | C15 | O4 | 121.8 (5) |
| C3 | N5 | C7 | 121.3 (4) | N5 | C3 | C2 | 132.2 (4) | O3 | C15 | C14 | 126.3 (5) |
| C3 | N5 | C8 | 124.4 (4) | O1 | C4 | O2 | 121.4 (4) | O4 | C15 | C14 | 111.9 (4) |
| C7 | N5 | C8 | 114.3 (4) | O1 | C4 | C2 | 126.8 (4) | N10 | C16 | C14 | 133.6 (6) |
| N7 | N6 | C13 | 102.4 (4) | O2 | C4 | C2 | 111.8 (4) | O4 | C19 | C20 | 108.3 (7) |
| N6 | N7 | N8 | 113.6 (4) | O2 | C5 | C6 | 108.4 (6) | N7 | C21 | C22 | 109.2 (4) |
| N6 | N7 | C21 | 122.0 (4) | N2 | C9 | C10 | 108.4 (5) | N7 | C21 | C23 | 107.9 (5) |
| N8 | N7 | C21 | 124.1 (4) | N2 | C9 | C11 | 106.8 (4) | N7 | C21 | C24 | 106.3 (4) |
| N7 | N8 | N9 | 106.2 (4) | N2 | C9 | C12 | 111.0 (4) | C22 | C21 | C23 | 111.3 (6) |
| N8 | N9 | C13 | 106.3 (4) | C10 | C9 | C11 | 109.4 (6) | C22 | C21 | C24 | 110.4 (6) |
| C16 | N10 | C17 | 123.4 (5) | C10 | C9 | C12 | 110.0 (7) | C23 | C21 | C24 | 111.4 (7) |

*a*) Crystal data: Single-crystal diffractometry, graphite-monochromated Cu$K_\alpha$, $\lambda = 1.54178$ Å. Monoclinic cell parameters and calculated volume: $a = 15.606(5)$, $b = 11.701(3)$, $c = 18.224(7)$ Å, $\beta = 115.01(4)°$, $V = 3016(3)$ Å$^3$. For $z = 8$ and $M_r = 267.33$, the calculated density is 1.178 g/cm$^3$. Space group $P2_1/n$ (#14). *b*) Between the nonhydrogen atoms.

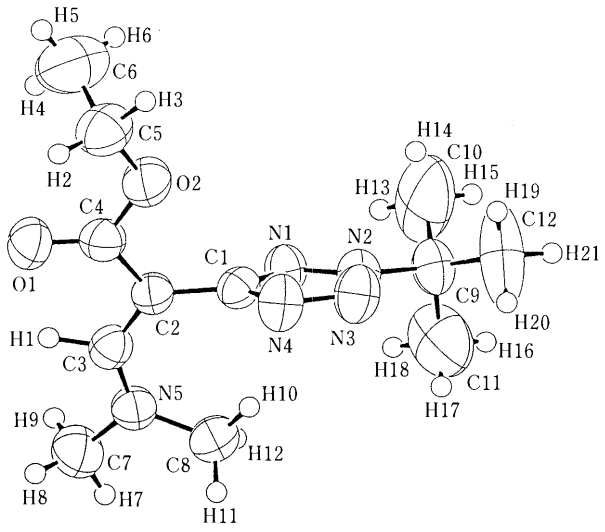



Fig. 2. Stereostructure of **4a**

we concluded from the $^1$H-NMR spectra that the other derivatives (**4b**—**e**) also have the *Z* configuration. In the case of 1-substituted derivatives (**5b**—**e**), we considered that they also have the *Z* configuration, because **5b** exhibited a $^3J$ (CO, H) value of 3.0 Hz in its $^{13}$C-NMR spectrum and **5b**—**e** showed similar patterns in their $^1$H-NMR spectra.

**Reactions of DMTE with Amino-heterocycles** We next examined the reaction of DMTE with amino-heterocycles, which contained a cyclic amidine component, to obtain the fused pyrimidine derivatives. Heating a mixture of DMTE and 2-amino-5-methyloxazole (**6**)[14] in acetic acid at reflux temperature gave a mixture of regioisomers (**7**) whose $^1$H-NMR spectrum showed signals due to the olefin and NH protons with a coupling constant of 15 Hz. The subsequent heating of **7** in acetic acid caused cyclization to the oxazolo[3,2-*a*]pyrimidine derivative (**8**) (procedure A). The structure of **8** was assigned on the basis of the $^1$H-NMR spectra: the proton on the oxazole ring was shifted further downfield in comparison with that of **7** (7.64 ppm for **8**, 6.60 ppm for **7**) because of the additional deshielding effect of the carbonyl group. The direct conversion of **6** into **8** was accomplished by reacting DMTE with **6** in refluxing acetic acid for a longer time (procedure B).

The conversions of 2-aminobenzothiazole (**9**), 3-aminopyrazole (**10**),[15] ethyl 3-aminopyrazole-4-carboxylate (**11**)[15] and 3-amino-1,2,4-triazole (**12**) into pyrimido[2,1-

*b*]benzothiazole derivative (**16**), pyrazolo[1,5-*a*]pyrimidine derivatives (**17**, **18**) and [1,2,4]triazolo[1,5-*a*]pyrimidine derivative (**19**), respectively, were carried out in a similar manner to the above, with the results shown in Tables IV and V. The one-pot reactions (procedure B) were always superior to the method *via* intermediates (**7** and **13**—**15**) in terms of total yield.

In the cyclization reaction of **12** with DMTE, the direction of cyclization was examined on the basis of the $^1$H-NMR spectra. In the $^1$H-NMR spectrum of **19**, the signal of the proton on the triazole ring appeared at 8.27 ppm, which was about the same value as that for **15** (8.05 ppm), and did not suffer the deshielding effect of the carbonyl group. This clearly showed that the cyclization of **15** occurred at the 2-position of the triazole ring as shown in Chart 4.

Removal of the *tert*-butyl moiety of **8** and **16**—**19** by heating at 100—140 °C in concentrated sulfuric acid gave the desired tetrazoles (**20**—**24**) in satisfactory yields, and the results are shown in Table VI. In the case of the pyrazolo[1,5-*a*]pyrimidine derivative (**18**) with an ethoxycarbonyl group, not only removal of the *tert*-butyl moiety but also hydrolysis of the ester group occurred at the same time to give **23** under these conditions. Later, the removal of the *tert*-butyl group was achieved by several methods, such as the use of *p*-toluenesulfonic acid in sulfolane, trifluoroacetic acid or boron trifluoride. Infrared (IR) spectra of the tetrazoles (**17**—**19** and **22**—**24**) showed that they contained a carbonyl group. As we did not identify the position of NH hydrogen of the compounds (**17**—**24**), they are tentatively depicted as 1*H*-pyrimidin-4-one (**17**—**19** and **22**—**24**) or 1*H*-tertazole (**20**—**24**) derivatives.

The reactions of DMTE and cyclic amidine type amino-heterocycles thus provide a general method for synthesizing a variety of fused pyrimidine derivatives containing the tetrazole moiety. This method not only provides a very simple, two-step conversion from amino-heterocycles to 2,3-(ring fused)-5-(5-tetrazolyl)-4*H*-pyrimidin-4-ones under mild conditions, but should also be applicable to the conversion of many heterocycles, of which only a few could be directly converted to the tetrazoles by other methods.

When the tetrazoles (**20**—**24**) were tested in the rat passive cutaneous anaphylaxis (PCA) assay, **21** showed activity when given by oral administration and **23** showed activity by intravenous administration. Details of syntheses of other ring systems and their biological activities will be reported elsewhere.

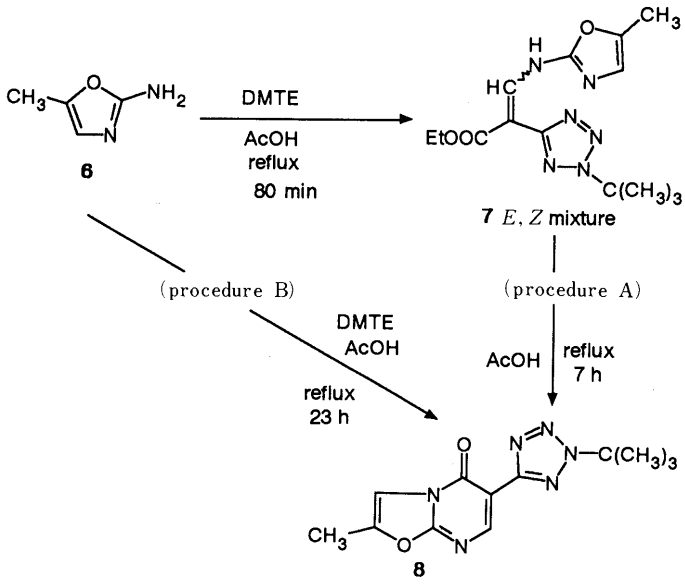



Chart 3

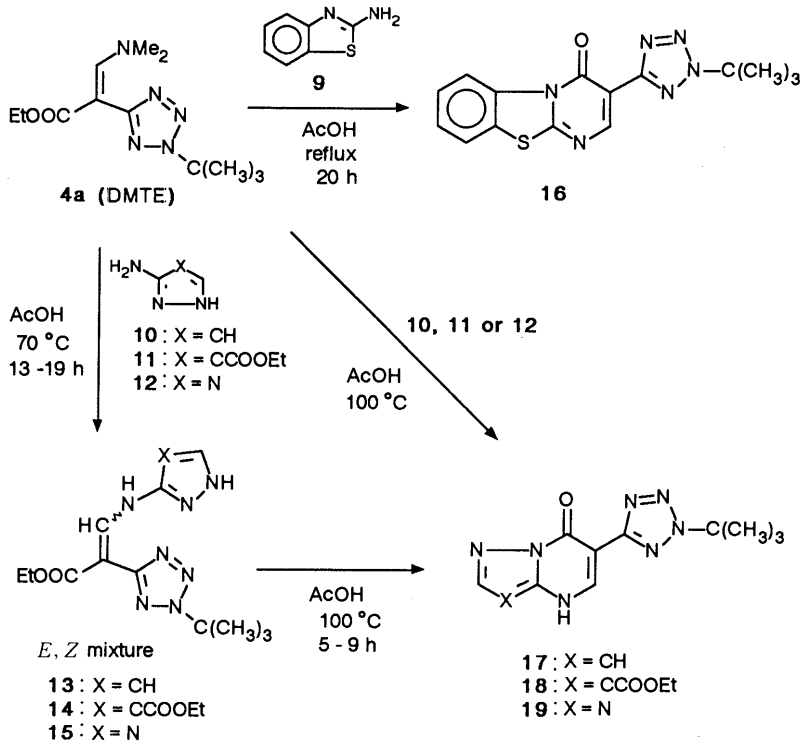
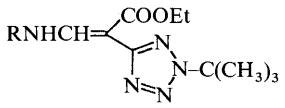
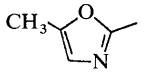
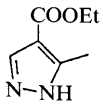



Chart 4

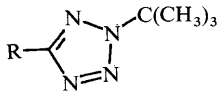
TABLE IV. Ethyl 2-(*tert*-Butyl-2*H*-tetrazol-5-yl)-3-(substituted amino)acrylates (A mixture of *E* and *Z* isomers)

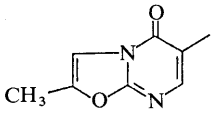
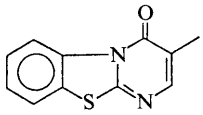
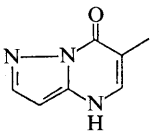
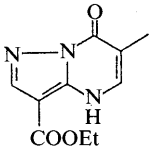
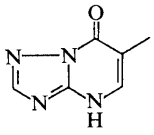


| Compd. | R | Reaction conditions | Yield (%) | $^1$H-NMR ($CDCl_3$): $\delta$ ppm |
|---|---|---|---|---|
| **7** |  | Reflux 80 min | 56 | 1.33, 1.37 (total 3H, each t, $J$=7.0 Hz), 1.77, 1.82 (total 9H, each s), 2.28, 2.30 (total 3H, each d, $J$=2.0 Hz), 4.35, 4.38 (total 2H, each q, $J$=7.0 Hz), 6.60 (1H, m), 8.20, 8.57 (total 1H, each d, $J$=13 Hz) |
| **13** |  | 70 °C 12.5 h | 73 | 1.32, 1.35 (total 3H, each t, $J$=7.0 Hz), 1.76, 1.80 (total 9H, each s), 4.34, 4.36 (total 2H, each q, $J$=7.0 Hz), 6.05, 6.12 (total 1H, each d, $J$=2.0 Hz), 7.57, 7.21 (total 1H, each d, $J$=2.0 Hz), 8.26, 8.60 (total 1H, each d, $J$=13 Hz), 10.24, 10.32 (total 1H, each d, $J$=13 Hz) |
| **14** |  | 70 °C 18.5 h | 53 (20)[a] | 1.37 (3H, t, $J$=7.0 Hz), 1.43 (3H, t, $J$=7.0 Hz), 1.77, 1.85 (total 9H, each s), 4.38 (2H, q, $J$=7.0 Hz), 4.43 (2H, q, $J$=7.0 Hz), 8.05, 8.08 (total 1H, each s), 8.57, 8.90 (total 1H, each d, $J$=13 Hz), 11.24, 11.49 (total 1H, each d, $J$=13 Hz) |
| **15** |  | 70 °C 16.5 h | 36[b] | 1.37 (3H, t, $J$=7.0 Hz), 1.81 (9H, s), 4.36 (2H, q, $J$=7.0 Hz), 8.05 (1H, s), 8.83 (1H, d, $J$=13 Hz), 10.55 (1H, d, $J$=13 Hz) |

*a*) The cyclized product (**18**) was obtained in 20% yield. *b*) Isolated as a single isomer. mp 208—211 °C ($CHCl_3$). *Anal.* Calcd for $C_{12}H_{18}N_8O_2$: C, 47.05; H, 5.92; N, 36.58. Found: C, 46.80; H, 5.97; N, 36.31.

TABLE V. 5-(2-*tert*-Butyl-2*H*-tetrazol-5-yl)pyrimidin-4-one Derivatives



| Compd. | R | Procedure[a] | Yield (%) | mp (°C) (Recryst. solvent) | Formula | Analysis (%) Calcd (Found) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | C | H | N |
| **8** |  | A<br>B | 26<br>39 | 202—203.5<br>(MeOH) | $C_{12}H_{14}N_6O_2$ | 52.55<br>(52.51 | 5.15<br>5.32 | 30.64<br>30.50) |
| **16** |  | B | 61 | 226—227<br>(EtOH) | $C_{15}H_{14}N_6OS$ | 55.20<br>(55.30 | 4.32<br>4.35 | 25.75<br>25.75) |
| **17** |  | A<br>B | 63<br>76 | Over 300<br>(DMF–MeOH) | $C_{11}H_{13}N_7O$ | 50.96<br>(50.75 | 5.05<br>5.26 | 37.82<br>37.57) |
| **18** |  | A<br>B | 61<br>81 | 264—267<br>($CHCl_3$–MeOH) | $C_{14}H_{17}N_7O_3$ | 50.75<br>(50.55 | 5.17<br>5.27 | 29.59<br>29.40) |
| **19** |  | A<br>B | 41<br>65 | 195—198<br>(AcOEt–MeOH) | $C_{10}H_{12}N_8O$ | 46.15<br>(45.71 | 4.65<br>5.14 | 43.05<br>42.60) |

*a*) See text.

## Experimental

All melting points were determined on a Yanagimoto micromelting point apparatus, and are uncorrected. $^1$H-NMR spectra were obtained at 60 MHz with a Varian EM-360 spectrometer, and $^{13}$C-NMR spectra, at 500 MHz with a JEOL GSX 500 spectrometer. Chemical shifts are expressed in $\delta$ (ppm) values with tetramethylsilane as the internal standard. The abbreviations used are follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. IR spectra were recorded on a Hitachi 260-30 spectrometer

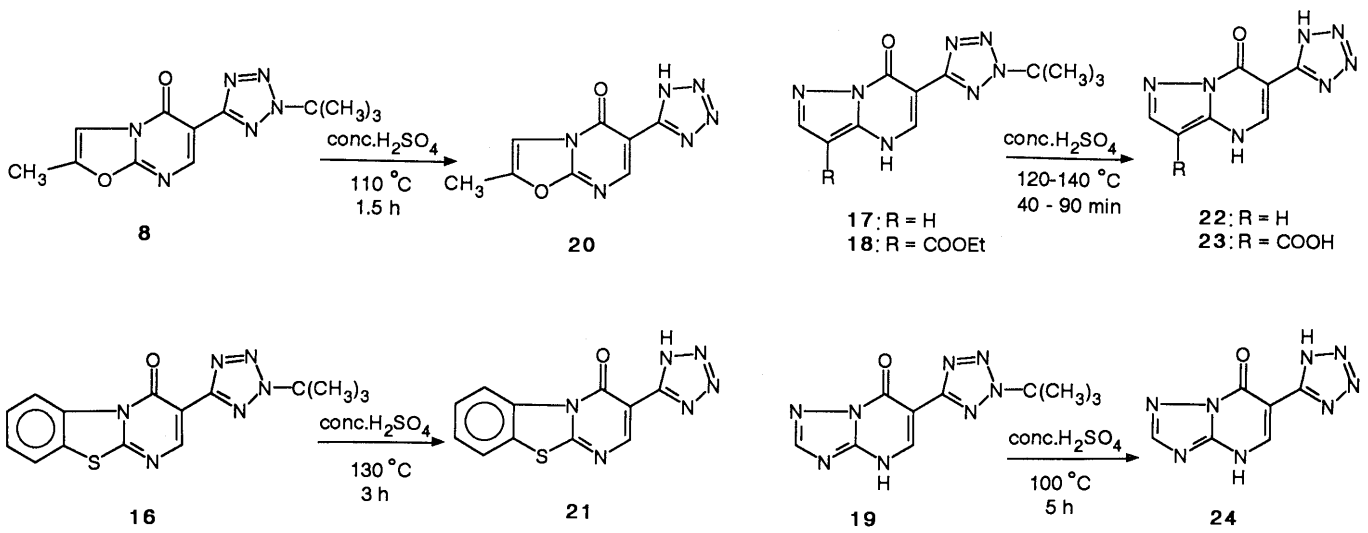
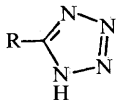
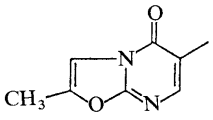
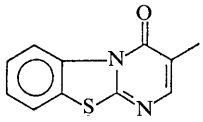
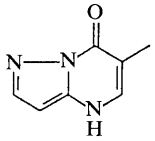
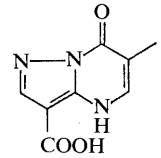
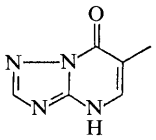



Chart 5

TABLE VI. 5-(Tetrazol-5-yl)-4*H*-pyrimidin-4-one Derivatives



| Compd. | R | Yield (%) | mp (°C) (Recryst. solvent) | Formula | Analysis (%) Calcd (Found) | | |
|---|---|---|---|---|---|---|---|
| | | | | | C | H | N |
| **20** |  | 72 | Over 300 (DMF) | $C_8H_6N_6O_2$ | 44.04 (44.41 | 2.77 2.95 | 38.52 38.21) |
| **21**[2,3] |  | 64 | Over 300 (DMF–EtOH) | $C_{11}H_6N_6OS$ | 48.98 (48.99 | 2.24 2.49 | 31.10 30.90) |
| **22** |  | 99 | Over 300 (DMF) | $C_7H_5N_7O$ | 41.38 (41.58 | 2.48 2.79 | 48.26 47.98) |
| **23** |  | 83 | Over 300 (DMF) | $C_8H_5N_7O_3$ DMF | 41.25 (41.29 | 3.78 3.78 | 34.99 35.26) |
| **24** |  | 75 | Over 300 (DMF) | $C_6H_4N_8O$ | 35.30 (35.66 | 1.98 2.34 | 54.89 54.58) |

using KBr disks. Silica gel 60 (E. Merck, 0.063—0.200 mm) was used for column chromatography, unless otherwise noted. Organic extracts were dried over anhydrous $Na_2SO_4$.

**Ethyl (Substituted tetrazol-5-yl)acetates (Table I). Ethyl (1-Benzyl-1*H*-tetrazol-5-yl)acetate (2d) and Ethyl (2-Benzyl-2*H*-tetrazol-5-yl)acetate (3d)** Benzyl chloride (3.17 g, 25 mmol) was added to a mixture of ethyl (5-tetrazolyl)acetate (**1**) (3.12 g, 20 mmol) and $Et_3N$ (9.10 g, 90 mmol). The mixture was stirred at room temperature for 16 h and concentrated *in vacuo*. The residue was taken up in 50 ml of water, and the aqueous mixture was extracted with EtOAc. The extract was washed successively with 14% $NH_4OH$ and brine, then the organic layer was dried. Removal of the solvent gave a mixture of **2d** and **3d** (1.87 g, total 37%, **2d**/**3d** = 2/3) as an oil. The product was used in the next step without further purification. The following $^1$H-NMR data are based on the spectrum of the mixture **2d**: $^1$H-NMR ($CDCl_3$) $\delta$: 1.21 (3H, t, $J$ = 7.0 Hz, $C\underline{H}_3CH_2O$), 3.83 (2H, s, $C\underline{H}_2COO$), 4.14 (2H, q, $J$ = 7.0 Hz, $CH_3C\underline{H}_2O$), 5.63 (2H, s, $PhC\underline{H}_2$),

7.38 (5H, s, Ar-H). **3d**: $^1$H-NMR ($CDCl_3$) $\delta$: 1.24 (3H, t, $J$=7.0 Hz, $CH_3CH_2O$), 3.94 (2H, s, $CH_2COO$), 4.19 (2H, q, $J$=7.0 Hz, $CH_3CH_2O$), 5,76 (2H, s, $PhCH_2$), 7.38 (5H, s, Ar-H).

A mixture of **2e** and **3e** (**2e**/**3e**=5/4) was prepared in a similar manner. **2e**: $^1$H-NMR ($CDCl_3$) $\delta$: 1.24 (3H, t, $J$=7.0 Hz, $CH_3CH_2O$), 3.80 (3H, s, $OCH_3$), 3.83 (2H, s, $CH_2COO$), 4.15 (2H, q, $J$=7.0 Hz, $CH_3CH_2O$), 5.55 (2H, s, $PhCH_2$), 6.88 (2H, d, $J$=8.8 Hz, Ar-H), 7.18 (2H, d, $J$=8.8 Hz, Ar-H). **3e**: $^1$H-NMR ($CDCl_3$) $\delta$: 1.24 (3H, t, $J$=7.0 Hz, $CH_3CH_2O$), 3.80 (3H, s, $OCH_3$), 3.93 (2H, s, $CH_2COO$), 4.19 (2H, q, $J$=7.0 Hz, $CH_3CH_2O$), 5.68 (2H, s, $PhCH_2$), 6.88 (2H, d, $J$=9 Hz, Ar-H), 7.33 (2H, d, $J$=9 Hz, Ar-H).

**Ethyl 3-(Dimethylamino)-2-(substituted tetrazol-5-yl)acrylates (Table II). Ethyl 2-(2-*tert*-Butyl-2*H*-tetrazol-5-yl)-3-(dimethylamino)acrylate (4a)** A mixture of **3a** (58.7 g, 0.28 mol) and DMF diethylacetal (75% purity, 59.7 g, 0.31 mol) was heated at 100 °C for 8 h, then concentrated *in vacuo*. The residue was dissolved in ether, and petroleum ether was added and then left standing. The precipitated prisms were collected by filtration to give 31.1 g (42%) of **4a**. The mother liquor was concentrated and then diluted with benzene, washed with water, dried and concentrated *in vacuo*. The resultant crystals were recrystallized from ether–petroleum ether to give a further 19.6 g (27%) of **4a**, which was identical with the first crop IR: 1695 cm$^{-1}$ (C=O). $^{13}$C-NMR ($CDCl_3$)$\delta$: 14.4 ($CH_2CH_3$), 29.3 ($C(CH_3)_3$), 35—50 (br, N ($CH_3)_2$), 59.7 ($OCH_2CH_3$), 63.4 ($C(CH_3)_3$), 85.1 (=CCOOEt), 152.4 ($CHNMe_2$), 161.3 (NC=N), 168.6 (COOEt). *Anal.* Calcd for $C_{12}H_{21}N_5O_2$: C, 53.92; H, 7.92; N, 26.20. Found: C, 53.83; H, 7.85; N, 26.55.

Compounds **4b**, **4c** and **5b** were prepared in a similar manner. **4b**: IR 1685 cm$^{-1}$ (C=O). $^{13}$C-NMR ($CDCl_3$) $\delta$: 14.5 ($CH_2CH_3$), 39.5 ($NCH_3$), 35—50 (broad, N ($CH_3)_2$), 59.9 ($OCH_2CH_3$), 85.2 (=CCOOEt), 152.7 ($CHNMe_2$), 162.1 (NC=N), 168.5 (COOEt). *Anal.* Calcd for $C_9H_{15}N_5O_2$: C, 47.99; H, 6.71; N, 31.09. Found: C, 47.94; H, 6.59; N, 31.87. **5b**: $^{13}$C-NMR ($CDCl_3$) $\delta$: 14.6 ($CH_2CH_3$), 34.1 ($NCH_3$), 35—50 (br, N ($CH_3)_2$), 60.2 ($OCH_2CH_3$), 78.9 (=CCOOEt), 151.7 (NC=N), 154.3 ($CHNMe_2$), 166.9 (COOEt). *Anal.* Calcd for $C_9H_{15}N_5O_2$: C, 47.99; H, 6.71; N, 31.09. Found: C, 48.06; H, 6.67; N, 30.99. **4c**: This compound was purified by column chromatography with benzene–acetone (20:1). IR: 1690 cm$^{-1}$ (C=O).

**Ethyl 2-(2-Benzyl-2*H*-tetrazol-5-yl)-3-(dimethylamino)acrylate (4d) and Ethyl 2-(1-Benzyl-1*H*-tetrazol-5-yl)-3-(dimethylamino)acrylate (5d)** DMF diethylacetal (94% purity, 1.43 g, 9.1 mmol) was added to a mixture of **2d** and **3d** (**2d**/**3d**=2/3, 1.87 g, 7.6 mmol). The mixture was heated at 100 °C for 20 h and concentrated *in vacuo*. The residue was chromatographed with benzene–acetone (20:1) to give 1.04 g (46%) of **4d** as an oily product from the first eluate. IR: 1695 cm$^{-1}$(C=O). The second eluate gave 0.78 g (34%) of **5d** as an oily product. IR: 1690 cm$^{-1}$ (C=O).

Compound **5c**, **4e** and **5e** were prepared in a similar manner. **5c**: IR; 1690 cm$^{-1}$ (C=O). **4e**: IR: 1690 cm$^{-1}$ (C=O). **5e**: IR 1690 cm$^{-1}$ (C=O). *Anal.* Calcd for $C_{16}H_{21}N_5O_3$: C, 57.99; H, 6.39; N, 21.14. Found: C, 58.11; H, 6.45; N, 21.00.

**Ethyl 2-(*tert*-Butyl-2*H*-tetrazol-5-yl)-3-(substituted amino)acrylates (Table IV). Ethyl 2-(2-*tert*-Butyl-2*H*-tetrazol-5-yl)-3-[(5-methyloxazol-2-yl)-amino]acrylate (7)** A mixture of 2-amino-5-methyloxazole (**6**) (100 mg, 1.0 mmol) and DMTE (267 mg, 1.0 mmol) in AcOH (5 ml) was refluxed for 80 min. The reaction mixture was concentrated *in vacuo*, and the residue was dissolved in $CHCl_3$ and water. The organic layer was separated, washed with saturated $NaHCO_3$ solution, dried and then concentrated *in vacuo*. The residue was purified by preparative thin layer chromatography (E. Merck, Silica gel 60 $F_{254}$) to give 180 mg (56%) of **7** as a colorless oil.

Compound **13**—**15** were prepared in a similar manner. **15**: mp 208—211 °C (recrystallized from benzene). IR: 1710 cm$^{-1}$ (C=O).

**2,3-(Ring Fused)-5-(2-*tert*-butyl-2*H*-tertazol-5-yl)-4*H*-pyrimidin-4-one Derivatives (Table V)** Procedure A, 6-(2-*tert*-Butyl-2*H*-tetrazol-5-yl)-4,7-dihydropyrazolo[1,5-*a*]pyrimidin-7-one (**17**): A solution of **13** (1.79 g, 5.9 mmol) in AcOH (20 ml) was refluxed for 7 h. The reaction mixture was concentrated *in vacuo*, and MeOH was added to the residue to give 440 mg (63%) of **17** as colorless crystals. mp over 300 °C.

Compounds **8**, **18** and **19** were prepared in a similar manner. The IR and $^1$H-NMR spectra of these products coincided with those of the products obtained in procedure B.

Procedure B, 6-(2-*tert*-Butyl-2*H*-tetrazol-5-yl)-2-methyl-5*H*-oxazolo-[3,2-*a*]pyrimidin-5-one (**8**): A mixture of **6** (1.47 g, 15 mmol) and DMTE (4.01 g, 15 mmol) in AcOH (30 ml) was refluxed for 30 h. The reaction mixture was concentrated *in vacuo*, and MeOH (3 ml) was added to the residue to give 1.60 g (39%) of **8** as pale yellow crystals. A part of the crystals was recrystallized from MeOH to give colorless needles, mp 202—203.5 °C. IR: 1700 cm$^{-1}$ (C=O). $^1$H-NMR ($CDCl_3$) $\delta$: 1.83 (9H, s, C ($CH_3)_3$), 2.50 (3H, d, $J$=2.0 Hz, $CH_3$), 7.64 (1H, d, $J$=2.0 Hz, $C_3$-H), 8.86 (1H, s, $C_7$-H).

Compounds **16**—**19** were prepared in a similar manner. **16**: IR: 1705 cm$^{-1}$ (C=O). $^1$H-NMR ($CDCl_3$) $\delta$: 1.82 (9H, s, $C(CH_3)_3$), 7.40—7.85 (3H, m, $C_7$-H, $C_8$-H and $C_9$-H), 8.85 (1H, s, $C_2$-H), 9.15—9.40 (1H, m, $C_6$-H). **17**: IR: 1690 cm$^{-1}$ (C=O). $^1$H-NMR ($CDCl_3$–$CF_3COOH$) $\delta$: 1.92 (9H, s, C ($CH_3)_3$), 7.04 (1H, d, $J$=3.0 Hz, $C_3$-H), 8.52 (1H, d, $J$=3.0 Hz, $C_2$-H), 9.23 (1H, s, $C_5$-H). **18**: IR: 1700 cm$^{-1}$ (C=O). $^1$H-NMR ($CDCl_3$–$CF_3COOH$) $\delta$: 1.44 (3H, t, $J$=7.0 Hz, $CH_3CH_2O$), 1.93 (9H, s, C ($CH_3)_3$), 4.58 (2H, q, $J$=7.0 Hz, $CH_3CH_2O$), 8.64 (1H, s, $C_2$-H), 9.07 (1H, s, $C_5$-H). **19**: IR: 1710 cm$^{-1}$ (C=O). $^1$H-NMR ($CDCl_3$–$CD_3OD$) $\delta$: 1.83 (9H, s, $C(CH_3)_3$), 8.27 (1H, s, $C_2$-H), 8.88 (1H, s, $C_5$-H).

**2,3-(Ring Fused)-5-(5-tetrazolyl)-4*H*-pyrimidin-4-one Derivatives (Table VI). 2-Methyl-6-(5-tetrazolyl)-5*H*-oxazolo[3,2-*a*]pyrimidin-5-one (20)** A mixture of **8** (1.60 g, 5.8 mmol) and concentrated $H_2SO_4$ (4.5 ml) was heated at 110 °C and stirred for 90 min. The reaction mixture was cooled, and then poured into ice water to yield a solid. The resulting solid was collected by filtration, washed thoroughly with water, and recrystallized from DMF to give 0.91 g (72%) of **20** as colorless prisms, mp over 300 °C. IR: 1690 cm$^{-1}$ (C=O). $^1$H-NMR ($CDCl_3$–$CF_3COOH$) $\delta$: 2.63 (3H, d, $J$=2.0 Hz, $CH_3$), 7.93 (1H, d, $J$=2.0 Hz, $C_3$-H), 9.32 (1H, s, $C_7$-H).

Compounds **21**—**24** were prepared in a similar manner. **21**: IR: 1670 cm$^{-1}$ (C=O). $^1$H-NMR ($CF_3COOH$) $\delta$: 7.90—8.30 (3H, m, $C_7$-H, $C_8$-H and $C_9$-H), 9.63 (1H, m, $C_2$-H), 9.30—9.70 (1H, m, $C_6$-H). **22**: IR: 1660 cm$^{-1}$ (C=O). $^1$H-NMR ($CDCl_3$–$CF_3COOH$) $\delta$: 6.99 (1H, d, $J$=3.0 Hz, $C_3$-H), 8.63 (1H, d, $J$=3.0 Hz, $C_2$-H), 9.56 (1H, s, $C_5$-H). **23**: $^1$H-NMR ($CF_3COOH$) $\delta$: 8.80 (1H, s, $C_2$-H), 9.61 (1H, s, $C_5$-H). **24**: IR: 1665 cm$^{-1}$ (C=O). $^1$H-NMR ($CDCl_3$–$CF_3COOH$) $\delta$: 8.70 (1H, s, $C_2$-H), 9.52 (1H, s, $C_5$-H).

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# Prostanoids and Related Compounds. IV.[1] Total Synthesis of Clavulones

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**An efficient and stereoselective synthesis of optically active clavulones has been accomplished by the use of the unnatural type dichloro Corey lactone 1 as a chiral pool.**

**Keywords** marine prostanoid; clavulone I; clavulone II; clavulone III; clavulone IV; unnatural type dichloro Corey lactone; iodolactonization; alkylation; Wittig reaction

Clavulones, novel marine prostanoids isolated from the Okinawan soft coral *Clavularia viridis*,[2] have attracted much attention, because of their unique structure and remarkably strong antitumor and antiinflammatory activities. Corey and Mehrotra[3] reported the first synthesis of clavulones in racemic form, and Yamada *et al.*,[4] Shibasaki and Ogawa[5] and Hashimoto *et al.*[6] obtained them in optically active form.

We wish to report an efficient and stereoselective synthesis of optically active clavulones by the use of the unnatural-type dichloro Corey lactone **1**[7] as a chiral pool. Chart 1 shows our strategy for construction of the clavulone skeleton by linking the α- and ω-carbon appendages to the key intermediate **8**.

We envisaged that the stereoselective introduction of the ω-carbon appendage onto the carbonyl carbon would result in the formation of the β-hydroxy group at C-12 (prostagrandin (PG) numbering), which is lacking in primary PG structures. The condensation of the lactol with the ylide from the phosphonium salt followed by dehydrochlorination would introduce the α-carbon appendage with the carbon–carbon double bonds between C-5/C-6 (PG numbering) and C-7/C-8 (PG numbering). The starting optically pure enone **8** was synthesized in accordance with the reaction sequence shown in Chart 2.

Treatment of the unnatural-type dichloro Corey lactone **1** with Zn powder (1.1 eq) in acetic acid resulted in the formation of the diastereomers **2a** (as crystals) and **2b** (as an oil) in 92% total yield. Their separation was effected by recrystallization and silica gel column chromatography. Their ratio was determined to be 65 : 35 after the separation. The configuration of the vicinal hydrogens between C-4 and C-5 of **2a** and **2b** was confirmed by proton nuclear magnetic resonance ($^1$H-NMR) analysis. In the $^1$H-NMR spectrum of **2a**, the appearance of a doublet signal due to the diastereotopic hydrogen of C-4 at 4.86 ppm with a coupling constant of 8.8 Hz supported the *cis* configuration of this predominant product. The *trans* configuration of **2b** was demonstrated by the fact that the signal of the C-4 diastereotopic hydrogen appeared at 4.29 ppm as a singlet. In the clavulone synthesis, **2a** can be led to clavulones III and IV, and **2b** to clavulones I and II. The 4-*exo*-monochloro Corey lactone **2a** was chosen for the subsequent conversion because of its predominant formation and easy purification. The iodolactonization[1a] of **2a** with $KI_3$ (room temperature for 3 h) afforded a *ca.* 75 : 25 mixture of regioisomers **3a** and **3b** in 83.6% total yield. The crystalline monochloroiodolactone **3a** was separated from oily **3b** in 62% yield by recrystallization. The predominant isomer **3a**, the formation of which had been thought to occur by recyclization of the lactone ring, had *cis* geometry of the vicinal hydrogens between C-4 and C-5, because the C-4 proton signal appeared as a doublet at 4.68 ppm with a coupling constant of 8.6 Hz in its $^1$H-NMR spectrum, while the appearance of C-4 proton signal of **3b** as a doublet at 4.75 ppm with a coupling constant of 1.2 Hz was consistent with *trans* geometry of the vicinal hydrogens between C-4 and C-5. The monochloroiodolactone **3a** was then protected with 3,4-dihydro-α-pyran (DHP, 2.5 eq) in the presence of a catalytic amount of *p*-toluenesulfonic acid in methylene chloride to afford the *O*-tetrahydropyranyl iodolactone **4**, an epimeric mixture at C-2 of the tetrahydropyranyl ring. The crude product **4** was used without further purification. The treatment of crude **4** with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 1.2 eq) in dry tetrahydrofuran (THF) gave the epimeric dehydroiodinated compound **5** in 92.2% yield (from **3a**). Compound **5** was then reduced to the lactol **6** (95.9% yield) by treatment with diisobutylaluminum hydride (DIBAL) in toluene at −78 °C. The deprotection of **6** with boron trifluoride diethyl etherate (0.2 eq) in MeOH

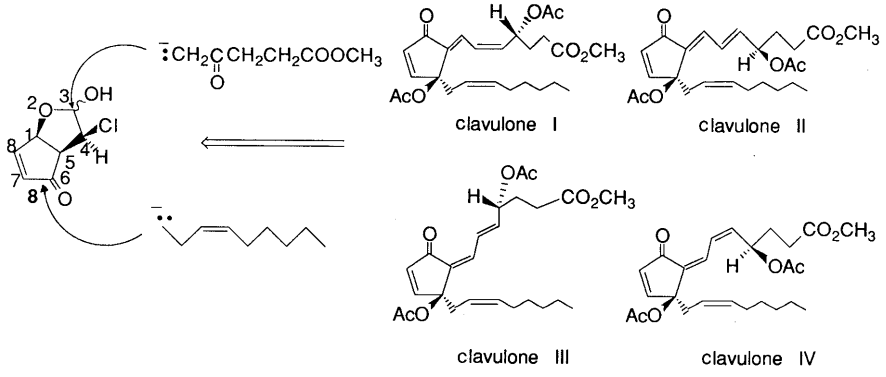


Chart 1

at room temperature afforded the hydroxy lactol **7** in 89.3% yield. Jones oxidation[8] of **7** at room temperature gave the key intermediate enone **8** in 82.5% yield. In this oxidation, the hydroxy group at C-3 remained unchanged. This is considered to have been due to the steric hindrance of the adjacent chlorine atom.

As shown in Chart 3, the stereoselective alkylation of the enone **8** with 2-octynyllithium in the presence of *N,N,N',N'*-tetramethylethylenediamine in dry THF–ether (1 : 1) gave the acetylene **9a** (39.4%) together with the allene **9b** (26.7%).

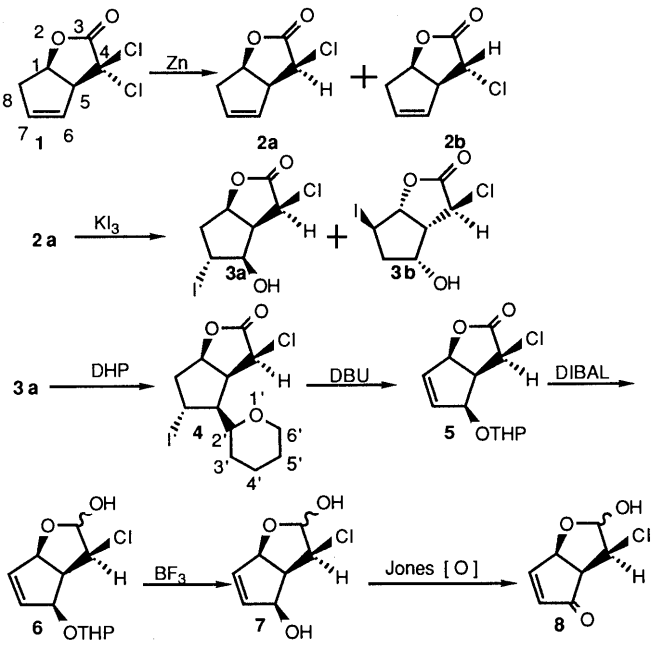


Chart 2

It is thought that the attack of 2-octynyllithium stereoselectively occurred at the α-face of the cyclopentenone ring due to the steric influence of the lactol ring orientated to the β-face, and thus formed the β-hydroxy function at the C-6 position. The partial hydrogenation of the acetylene **9a** over a Lindlar catalyst in benzene–cyclohexane in the presence of quinoline led to the *cis*-olefin **10** quantitatively.[9] The *cis*-configuration of **10** was deduced from its $^1$H-NMR spectrum, in which the vinylic proton signals appeared as double triplets at 5.46 ppm for $C_{2'}$-H of the 2-octenyl group and 5.51 ppm for $C_{3'}$-H of the 2-octenyl group with a vinylic coupling of 14.2 Hz.[10] The Wittig reaction of the *cis*-olefin **10** with dimethyl (4-carbomethoxy-2-oxobutyl)phosphonate[11] in dimethoxyethane (DME) did not proceed and **10** remained unchanged. We presumed the non-reactivity of **10** to the Wittig reaction is caused by the steric hindrance of the adjacent chlorine atom. Then, conversion of **10** to the ketoaldehyde **11** (44.4%) by Jones oxidation was conducted under more drastic conditions (80 °C, 20 min).

The Wittig reaction of **11** with dimethyl (4-carbomethoxy-

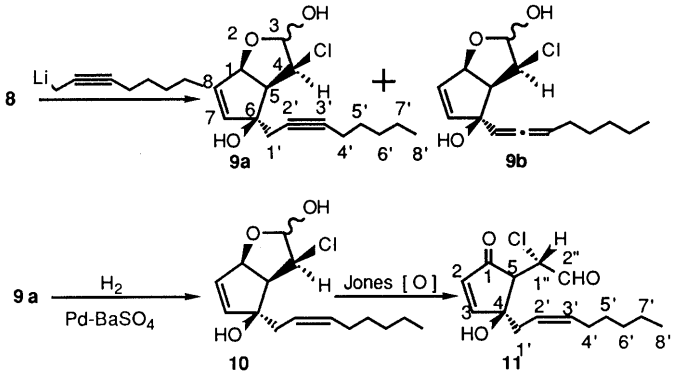


Chart 3

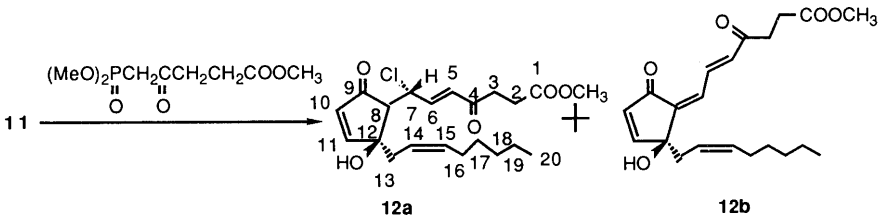


Chart 4

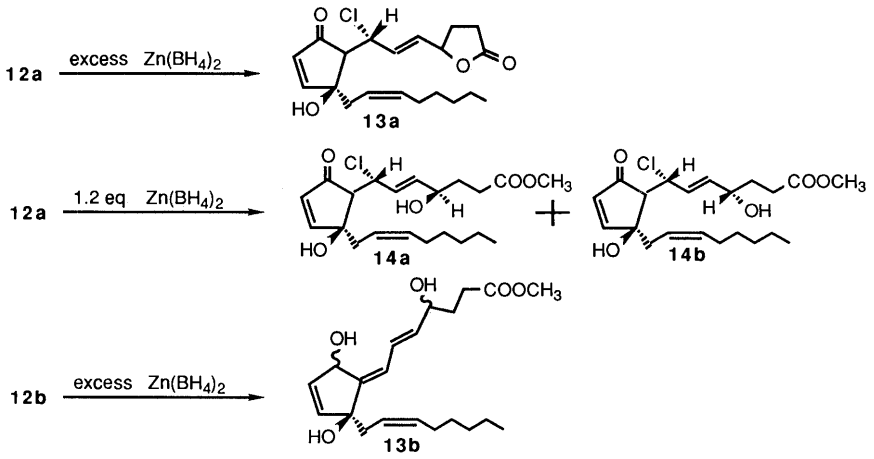


Chart 5

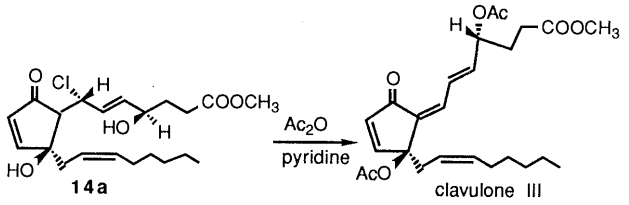


Chart 6

2-oxobutyl)phosphonate in DME smoothly proceeded to afford the chloroenone **12a** (61%) together with the dehydrochlorinated dienone **12b** (16%) (Chart 4).

As shown in Chart 5, the reduction of **12b** with excess zinc borohydride in DME at 25 °C afforded the diol **13b**, in which the keto functionalities at C-4 and C-9 were both reduced. Compound **12a** was regioselectively reduced with excess zinc borohydride[12] to form the lactone **13a**, in which the carbonyl group of C-4 was converted to the corresponding alcohol and that of C-9 remained intact. This selectivity was considered to be due to the steric hindrance of the chlorine atom. On this basis, the regioselective reduction of **12a** with 1.2 eq of zinc borohydride was conducted to afford an epimeric mixture of **14a** and **14b**. Separation by silica gel column chromatography with benzene–AcOEt (4 : 1) as the eluant gave **14a** (52%) as a less polar isomer (thin layer chromatography (TLC) $Rf=0.58$) and **14b** (11%) as a more polar isomer (TLC $Rf=0.55$). At this stage, the stereochemistry of the epimers **14a** and **14b** remained equivocal, but the subsequent conversions of the isomers served to elucidate the configuration of the epimers as follows: less polar **14a** is the 4$\beta$-isomer and more polar **14b** is the 4$\alpha$-isomer.

Thus, the treatment of **14a** with acetic anhydride–pyridine at 80 °C effected acetylation of the hydroxy functions at C-4 and C-12, and the *trans* elimination of hydrochloride to afford clavulone III as a sole product (Chart 6).

This synthetic clavulone III showed $[\alpha]_D^{20}$ +44.2° ($c=0.10$, $CHCl_3$) [lit.[2a] +45.5° ($c=0.22$, $CHCl_3$)] and was identical with natural clavulone III[2] (nuclear magnetic resonance (NMR), infrared (IR), ultraviolet (UV), mass spectrum (MS) and TLC). The product obtained from **14b** by the same treatment was not consistent with any clavulone.

Since clavulone III has been correlated to clavulones I, II and IV,[2] the formal total syntheses of these clavulones have been accomplished. We have demonstrated that optically active clavulones can be synthesized rapidly and efficiently from the unnatural-type dichloro Corey lactone **1**.

## 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. IR spectra were taken on JASCO A-202 and JASCO IR-810 infrared spectrophotometers and are given in $cm^{-1}$. $^1$H-NMR spectra were recorded on a JEOL JNM-FX90Q (90 MHz) spectrophotometer in $CDCl_3$; a JEOL JNM-270GSX (270 MHz) was also used in the case of clavulone III. Chemical shifts are given in $\delta$ (ppm) downfield from tetramethylsilane, and the abbreviations of signal patterns are as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. TLC was performed on silica gel (Kiesel $60F_{254}$ on aluminium sheets, Merck). All compounds were located by spraying the TLC plate with sulfuric acid and heating it on a hot plate. Preparative TLC was performed on a preparative layer chromatography plate (Kiesel gel $60F_{254}$ 2 and 0.5 mm, Merck). Column chromatography was performed on silica gel (Kiesel gel 60, 70—230 mesh, Merck).

**Preparation of (1*R*,4*S*,5*S*)-4-Chloro-2-oxabicyclo[3.3.0]oct-6-en-3-one (2a)** Zinc powder (36.0 g, 0.55 mol) was added in portions to a stirred solution of (1*R*,5*S*)-4,4-dichloro-2-oxabicyclo[3.3.0]oct-6-en-3-one (**1**, 96.5 g, 0.50 mol) in 360 ml of acetic acid at 15—20 °C. The mixture was stirred for 0.5 h at the same temperature, then poured into brine (2000 ml) and extracted with $Et_2O$ (400 ml × 4). The extract was washed with brine and saturated $NaHCO_3$ aqueous solution, dried over $MgSO_4$ and concentrated *in vacuo*. The solidified residue was recrystallized from diisopropyl ether (IPE) to give **2a** (44.8 g, 56.0%) as colorless prisms, mp 67—70 °C. The mother liquor was concentrated *in vacuo* and the residue was subjected to column chromatography on $SiO_2$ using benzene as the eluant to give additional **2a** (17.9 g, 22.4%, TLC $Rf=0.46$, benzene), **2b** (10.5 g, 13.1%, TLC $Rf=0.25$, benzene) and **1** (3.86 g, 4.0%, TLC $Rf=0.10$, benzene). The total yield of **2a** was 78.4% (62.7g). **2a**: $[\alpha]_D^{20}$ +77.6° ($c=2.76$, $CHCl_3$). IR (KBr): 1772 (C=O). $^1$H-NMR: 2.7–2.8 (2H, m, H-8), 3.8—4.0 (1H, m, H-5), 4.86 (1H, d, $J_{4,5}=8.8$ Hz, H-4), 5.1—5.2 (1H, m, H-1), 5.7—5.8 (1H, m, H-6 or H-7), 5.8—6.0 (1H, m, H-6 or H-7). $^{13}$C-NMR: 40.3 (t, C-8), 51.4 (d, C-5), 54.9 (d, C-4), 81.1 (d, C-1), 127.0, 131.7 (d and d, C-6 and C-7), 171.7 (s, C-3). *Anal.* Calcd for $C_7H_7ClO_2$: C, 53.02; H, 4.45. Found: C, 52.74; H, 4.41. **2b**: $[\alpha]_D^{20}$ +7.9° ($c=3.97$, $CHCl_3$). $^1$H-NMR: 2.7—2.8 (2H, m, H-8), 3.6—3.7 (1H, m, H-5), 4.29 (1H, s, H-4), 5.3—5.4 (1H, m, H-1), 5.6—5.7 (1H, m, H-6 or H-7), 5.8—6.0 (1H, m, H-6 or H-7). $^{13}$C-NMR: 38.9 (t, C-8), 52.8 (d, C-4), 56.8 (d, C-5), 82.4 (d, C-1), 127.5, 132.5 (d and d, C-6 and C-7), 171.9 (s, C-3).

**Preparation of (1*R*,4*S*,5*R*,6*R*,7*R*)-4-Chloro-6-hydroxy-7-iodo-2-oxabicyclo[3.3.0]octan-3-one (3a) (Iodolactonization of 2a)** An 8.81 N NaOH aqueous solution (35.4 ml) was added dropwise to a suspension of **2a** (44.6 g, 281 mmol) in $H_2O$ (180 ml) at room temperature. After **2a** had completely dissolved, 5 N acetic acid (52.8 ml) was added so as to adjust the pH value of the solution to 5. To this solution, $KI_3$ aqueous solution prepared from KI (94.2 g, 0.567 mol), $I_2$ (93.6 g, 0.369 mol) and $H_2O$ (145 ml) was added and the solution was stirred for 3 h at room temperature. Excess iodine was quenched with $Na_2SO_3$ (22.7 g). A part of **3a** (48.1 g, 56.6%) was precipitated as a solid by the salting-out technique and collected by suction. The filtrate was extracted with $CH_2Cl_2$ (100 ml × 5) and the extract was washed with brine (100 ml × 2) and aqueous $NaHCO_3$ (100 ml × 2), dried over $MgSO_4$ and concentrated *in vacuo*. The resulting residue was dissolved in hot benzene, from which additional **3a** (4.75 g, 5.60%) crystallized as colorless prisms on standing and was collected by suction. The viscous oily concentrate (22.9 g) of the filtrate was composed of **3a** and (1*R*,4*S*,5*R*,6*R*,8*R*)-4-chloro-6-hydroxy-8-iodo-2-oxabicyclo[3.3.0]octan-3-one (**3b**) in *ca.* 5 : 95 ratio, as determined by its $^1$H-NMR analysis and could not be further separated. The yield of isolated **3a** was 62.2% (52.9 g). **3a**: mp 142—144 °C, $[\alpha]_D^{20}$ −69.6° ($c=1.30$, MeOH). IR (KBr): 1796 (C=O). *Anal.* Calcd for $C_7H_8ClIO_3$: C, 27.80; H, 2.66. Found: C, 28.17; H, 2.63. $^1$H-NMR: 2.4—3.0 (2H, m, H-8), 3.40 (1H, ddd, $J_{4,5}=7.0$, $J_{1,5}=6.5$, $J_{5,6}=6.0$ Hz, H-5), 4.0—4.1 (1H, m, H-7), 4.5—4.6 (1H, m, H-6), 4.68 (1H, d, $J_{4,5}=7.0$ Hz, H-4), 5.21 (1H, ddd, $J_{1,8}=2.7$, $J_{1,8}=5.6$, $J_{1,5}=6.5$ Hz, H-1). $^{13}$C-NMR: 25.8 (d, C-7), 41.8 (t, C-8), 50.1 (d, C-5), 51.5 (d, C-4), 80.0 (d, C-6), 82.4 (d, C-1), 172.7 (s, C-3).

**Preparation of (1*R*,4*S*,5*S*,6*R*)-4-Chloro-6-(tetrahydropyran-2-yl)oxy-2-oxabicyclo[3.3.0]oct-7-en-3-one (5)** *p*-Toluenesulfonic acid monohydrate (0.1 g) was added to a stirred solution of **3a** (17.3 g, 57.2 mmol) and DHP (12.0 g, 143 mmol) in $CH_2Cl_2$ (114 ml) at 0 °C. Stirring was continued for 1.5 h at 20—25 °C, then the reaction solution was washed with saturated $NaHCO_3$ aqueous solution (40 ml × 2) and brine (40 ml × 2), dried over $MgSO_4$ and concentrated *in vacuo*. The residual viscous oil (29.2 g), which consisted mainly of **4**, was dissolved in 230 ml of THF. To the stirred THF solution, DBU (13.8 g, 75 mmol) was added at 0 °C and stirring was continued for 10 h at room temperature. The deposited precipitates were removed by suction and washed with a small quantity of THF. The combined filtrate and washings were concentrated under reduced pressure. The residual oily material was dissolved in benzene and the insoluble solids were again removed by suction and washed with benzene (50 ml × 5). The benzene filtrate and washings were washed with water (50 ml × 3), dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on $SiO_2$ using a 9 : 1 mixture of benzene–AcOEt as the eluant to give **5** as colorless crystals (20.4 g, 92.2% from **3a**). **5**: mp 46—48 °C (colorless prisms from IPE), $[\alpha]_D^{20}$ +78.9° ($c=2.80$, $CHCl_3$). IR (KBr): 1792 (C=O). *Anal.* Calcd for $C_{12}H_{15}ClO_4$: C, 55.71; H, 5.84. Found: C, 55.60; H, 6.02. $^1$H-NMR: 1.1—2.0 (6H, br m, H-4′, 5′, 6′), 3.4—3.7 (2H, m, H-5, $H_A$-6′), 3.7—4.1 (1H, m, $H_B$-6′), 4.67 (1H, d, $J_{4,5}=6.6$ Hz, H-4), 4.7—4.9 (1H, m, H-6), 4.93 (1H, t, $J_{2',3'}=7.3$ Hz, H-2′), 5.36 (1H, dd, $J_{1,5}=7.0$, $J_{1,8}=1.8$ Hz, H-1), 6.0—6.2 (2H, m, H-7, 8). $^{13}$C-NMR: 19.1, 19.4 (t, C-4′), 25.3 (t, C-5′), 30.3, 30.6 (t, C-3′),

50.7, 50.9 (d, C-5), 51.4, 51.5 (d, C-4), 62.2, 62.8 (t, C-6'), 76.6, 78.3 (d, C-6), 83.8, 84.2 (d, C-1), 97.7, 98.7 (d, C-2'), 130.3 and 131.1, 137.7 and 139.1 (d and d, d and d, C-7 and 8), 172.3, 172.6 (s, C-3).

In another run, crude **4** obtained by the procedures described above was subjected to column chromatography on silica gel using benzene as the eluant. The less polar epimeric isomer **4a** (TLC $Rf=0.66$, benzene) and more polar **4b** (TLC $Rf=0.59$, benzene) were obtained in 85 : 15 ratio. Physical data are given below. Both epimers were subjected to DIBAl treatment followed by deprotection of THP and Jones oxidation to afford the enone **8**, which was identical with the one from the epimeric mixture. **4a**: mp 119—120 °C (colorless prisms from IPE), $[\alpha]_D^{20}$ −117.4° ($c=2.54$, $CHCl_3$). $^1$H-NMR: 1.2—1.8 (6H, m, H-4', 5', 6'), 2.9—3.1 (2H, m, H-6'), 3.6—4.3 (3H, m, H-5, 8), 4.38 (1H, d, $J=3.9$ Hz, H-1), 4.6—4.8 (2H, m, H-6, H-1'), 4.80 (1H, d, $J_{4,5}=8.1$ Hz, H-4), 5.1 (1H, dt, $J_{6,7}=3.4$, $J_{7,8}=6.4$ Hz, H-7). $^{13}$C-NMR: 19.9 (t, C-4'), 25.5 (t, C-5'), 27.0 (d, C-7), 30.2 (t, C-3'), 44.4 (t, C-8), 47.5 (d, C-5), 52.0 (d, C-4), 63.2 (t, C-6'), 80.8 (d, C-1), 87.2 (d, C-6), 102.5 (d, C-2'), 170.6 (s, C-3). **4b**: mp 154.0—155.5 °C (colorless prisms from MeOH), $[\alpha]_D^{20}$ +24.8° ($c=3.05$, $CHCl_3$). $^1$H-NMR: 1.1—2.0 (6H, m, H-3', 4', 5'), 2.8—3.0 (2H, m, H-6'), 3.5—3.6 (1H, $H_A$-8), 3.7—4.1 (2H, m, H-5, $H_B$-8), 4.71 (1H, d, $J_{4,5}=10.0$ Hz, H-4), 4.6—4.9 (3H, m, H-1, 6, H-2'), 5.09 (1H, dt, $J_{6,7}=4.2$ Hz, $J_{7,8}=5.7$ Hz, H-7). $^{13}$C-NMR: 17.9 (t, C-4'), 23.8 (d, C-7), 25.1 (t, C-5'), 30.2 (t, C-3'), 43.6 (t, C-8), 47.8 (d, C-5), 51.9 (d, C-4), 62.4 (t, C-6'), 80.4 (d, C-1), 80.8 (d, C-6), 94.4 (d, C-2'), 170.6 (s, C-3).

**Preparation of (1*R*,4*S*,5*S*,6*S*)-4-Chloro-3-hydroxy-6-(tetrahydropyran-2-yl)oxy-2-oxabicyclo[3.3.0]oct-7-ene (6)** DIBAL (1.5 M toluene solution, 29.3 ml, 43.9 mmol) was added dropwise to a stirred solution of **5** (9.46 g, 36.6 mmol) in dry toluene (95 ml) at −78 °C under an argon atmosphere. After being stirred for 0.5 h at the same temperature, the reaction solution was quenched with saturated $NH_4Cl$ aqueous solution (9.5 ml). Deposited precipitates were filtered off over Celite and washed with toluene (15 ml × 3). The filtrate and washings were dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on $SiO_2$ using a 2 : 1 mixture of benzene–AcOEt as the eluant to give **6** (9.14 g, 95.9%) as a colorless oil. **6**: $[\alpha]_D^{20}$ −12.7° ($c=3.12$, $CHCl_3$). IR (neat): 3428 (OH), 1115, 1028 (C–O–C). *Anal.* Calcd for $C_{12}H_{17}ClO_4$: C, 55.28; H, 6.57; Cl, 13.60. Found: C, 55.07; H, 6.51; Cl, 13.87. $^1$H-NMR: 1.1—2.0 (6H, br m, H-3', 4', 5'), 3.2—3.6 (2H, m, H-5, $H_A$−6'), 3.6—4.0 (1H, m, $H_B$-6'), 4.1—4.6 (1H, m, H-4), 4.6—4.9 (3H, m, H-6, OH, H-2'), 4.9—5.4 (2H, m, H-1, 3), 5.9—6.3 (2H, m, H-7, 8). $^{13}$C-NMR: 19.0, 19.2 (t, C-4'), 25.1, 25.4 (t, C-5'), 30.3 (t, C-3'), 49.7 and 52.8 (d, C-5), 59.1 and 61.2 (d, C-4), 62.2 (t, C-6'), 78.2, 79.9 (d, C-6), 84.7, 86.2 (d, C-1), 98.9, 99.9 (d, C-2'), 98.1, 105.9 (d, C-3), 131.9 (d, C-7 or C-8), 134.8, 135.6 (d, C-7 or C-8).

**Preparation of (1*R*,4*S*,5*S*,6*S*)-4-Chloro-3,6-dihydroxy-2-oxabicyclo-[3.3.0]oct-7-ene (7)** Boron trifluoride diethyl etherate (2.18 g, 15.3 mmol) was added to a stirred solution of **6** (8.00 g, 30.7 mmol) in absolute MeOH (80 ml) at −78 °C under an argon atmosphere and stirring was continued for 1 h at room temperature. To the ice-cooled reaction solution, saturated $NaHCO_3$ aqueous solution (68 ml) was added and the mixture was concentrated under reduced pressure in order to remove MeOH. The resulting residue was partitioned between water and ether, and the aqueous solution was extracted with ether (50 ml × 4). The ether layer was dried over $MgSO_4$ and concentrated *in vacuo*. The resulting residue was subjected to column chromatography on $SiO_2$ using a 2 : 1 mixture of benzene–AcOEt as the eluant to give **7** (4.84 g, 89.3%) as colorless needles. **7**: mp 66—69°, $[\alpha]_D^{20}$ −74.2° ($c=5.83$, $CHCl_3$). IR (KBr): 3392 (OH), 1019 (C–O–C). *Anal.* Calcd for $C_7H_9ClO_3$: C, 47.61; H, 5.14. Found: C, 47.60; H, 5.09. $^1$H-NMR: 3.28 (1H, dd, $J_{1,5}=6.8$, $J_{5,6}=7.3$ Hz, H-5), 3.48 (1H, d, $J_{H,OH}=10.7$ Hz, $C_6$-OH), 4.09 (1H, d, $J_{H,OH}=3.7$ Hz, $C_3$-OH), 4.55 (1H, s, H-4), 4.76 (1H, ddd, $J_{6,7}=2.2$, $J_{5,6}=7.3$, $J_{H,OH}=10.7$ Hz, H-6), 5.26 (1H, dd, $J_{1,8}=2.2$, $J_{1,5}=6.8$ Hz, H-1), 5.52 (1H, d, $J_{H,OH}=3.7$ Hz, H-3), 5.93 (1H, dd, $J_{6,7}$ or $J_{1,8}=2.2$, $J_{7,8}=5.6$ Hz, H-7 or H-8), 6.19 (1H, dd, $J_{1,8}$ or $J_{6,7}=2.2$, $J_{7,8}=5.6$ Hz, H-8 or H-7). $^{13}$C-NMR: 53.1 (d, C-5), 60.8 (d, C-4), 74.6 (d, C-6), 87.9 (d, C-1), 104.8 (d, C-3), 134.9 (d, C-8), 136.4 (d, C-7).

**Preparation of (1*R*,4*S*,5*R*)-4-Chloro-3-hydroxy-2-oxabicyclo[3.3.0]oct-7-en-6-one (8)** An oxidizing agent solution produced from chromium trioxide (1.89 g, 18.9 mmol), $H_2O$ (8 ml) and 5 N $H_2SO_4$ (11.3 ml) was added dropwise to a stirred solution of **7** (4.55 g, 25.8 mmol) in reagent-grade acetone (75 ml) at 0 °C. The reaction mixture was stirred for 1 h at room temperature, then ether (75 ml) was added, and the separated aqueous layer was extracted with ether (25 ml × 5). The ether layer was once washed with brine (30 ml), dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on $SiO_2$ using a 1 : 1 mixture of benzene–AcOEt as the eluant to give **8** (3.71 g, 82.5%) as colorless needles. **8**: mp 84—88 °C, $[\alpha]_D^{20}$ −189.3° ($c=3.63$, $CHCl_3$). IR (KBr): 3401 (OH), 1716 (C=O), 1019 (C–O–C). *Anal.* Calcd for $C_7H_7ClO_3$: C, 48.16; H, 4.04; Cl, 20.30. Found: C, 48.02; H, 3.99; Cl, 20.54. $^1$H-NMR ($CDCl_3$): 3.18 (1H, dd, $J_{1,5}=2.7$, $J_{4,5}=5.7$ Hz, H-5), 4.28 (1H, s, H-3), 4.78 (1H, d, $J_{4,5}=5.7$ Hz, H-4), 5.10 (1H, br s, OH), 5.33 (1H, dd, $J_{1,8}=2.7$, $J_{1,5}=2.7$ Hz, H-1), 6.20 (1H, d, $J_{7,8}=5.9$ Hz, H-7), 7.72 (1H, dd, $J_{1,8}=2.7$, $J_{7,8}=5.9$ Hz, H-8). $^{13}$C-NMR: 56.4 (d, C-5), 61.8 (d, C-4), 82.0 (d, C-1), 105.7 (d, C-3), 134.8 (d, C-7), 162.0 (d, C-8), 204.4 (s, C-6).

**Preparation of (1*R*,4*S*,5*R*,6*S*)-4-Chloro-3,6-dihydroxy-6-(2-octynyl)-2-oxabicyclo[3.3.0]oct-7-ene (9a)** A dry THF–ether (1 : 1, 20 ml) solution of 2-octyne (2.18 g, 19.8 mmol) and *N*,*N*,*N'*,*N'*-tetramethylethylenediamine (1.53 g, 13.2 mmol) was placed in a two-necked flask under an argon atmosphere and cooled to −78 °C. *sec*-Butyllithium (1 M hexane solution, 20 ml, 20 mmol) was then added dropwise with stirring. Stirring was continued for 5 min, then a THF–ether (1 : 1, 10 ml) solution of **8** (1.05 g, 6.00 mmol) was added and the mixture was stirred for 15 min. Saturated $NH_4Cl$ solution (30 ml) was added to the mixture at the same temperature and the whole was allowed to stand at room temperature. The organic layer was separated and the aqueous layer was extracted with $CH_2Cl_2$ (30 ml × 5). The organic layer and extracts were washed with brine (10 ml × 2), dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to careful column chromatography on $SiO_2$ using a 4 : 1 mixture of hexane–AcOEt as the eluant to give **9a** (0.66 g, 39.4%; TLC $Rf=0.40$, hexane : AcOET = 4 : 1) and **9b** (0.46 g, 26.7%; TLC $Rf=0.48$, hexane : AcOEt = 4 : 1), each as a light yellow oil. **9a**: $[\alpha]_D^{20}$ −89.8° ($c=0.87$, $CHCl_3$). IR (KBr): 3392 (OH), 1019 (C–O–C). $^1$H-NMR: 0.7—1.1 (3H, t, $J_{7',8'}=7.1$ Hz, H-8'), 1.1—1.8 (6H, m, H-5', 6', 7'), 2.0—2.5 (2H, m, H-4'), 2.5—2.7 (2H, m, H-1'), 3.19 (1H, d, $J_{1,5}=7.0$ Hz, H-5), 3.74 (1H, s, $C_6$-OH), 4.04 (1H, d, $J_{H,OH}=4.9$ Hz, $C_3$-OH), 4.50 (1H, s, H-4), 5.29 (1H, dd, $J_{1,8}=2.4$, $J_{1,5}=7.0$ Hz, H-1), 5.52 (1H, d, $J_{H,OH}=4.9$ Hz, H-3), 5.75 (1H, d, $J_{7,8}=5.7$ Hz, H-7), 6.06 (1H, dd, $J_{1,8}=2.4$, $J_{7,8}=5.7$ Hz, H-8). $^{13}$C-NMR: 14.0 (q, C-8'), 18.6 (t, C-4'), 22.2 (t, C-7'), 28.1 (t, C-6'), 31.1 (t, C-5'), 31.5 (t, C-1'), 57.7 (d, C-5), 61.2 (d, C-4), 74.9 (s, C-6), 78.5 (s, C-2', C-3'), 88.5 (d, C-1), 105.2 (d, C-3), 134.1 (d, C-8), 139.3 (d, C-7). **9b**: IR (KBr): 3392 (OH), 1963 (–CH=C=CH–), 1020 (C–O–C). $^1$H-NMR: 0.90 (3H, t, $J_{7',8'}=5.9$ Hz, H-8'), 1.1—1.6 (6H, m, H-5', 6', 7'), 1.7—2.2 (2H, m, H-4'), 3.25 (1H, d, $J_{1,5}=7.1$ Hz, H-5), 4.13 (1H, br s, $C_6$-OH), 4.54 (1H, s, H-4), 4.6—5.2 (3H, m, H-1', 3', $C_3$-OH), 5.30 (1H, dd, $J_{1,8}=2.4$, $J_{1,5}=7.1$ Hz, H-1), 5.48 (1H, s, H-3), 5.85 (1H, d, $J_{7,8}=5.6$ Hz, H-7), 6.11 (1H, dd, $J_{1,8}=2.4$, $J_{7,8}=5.6$ Hz, H-8).

**Preparation of (1*R*,4*S*,5*R*,6*S*)-4-Chloro-3,6-dihydroxy-6-(2-octenyl)-2-oxabicyclo[3.3.0]oct-7-ene (10)** A solution of **9a** (142 mg, 0.5 mmol) in a 1 : 1 mixture of cyclohexane–benzene (10 ml) containing quinoline (3.2 mg, 0.025 mmol) was added to a flask containing 142 mg of Lindlar catalyst (5% Pd–$BaSO_4$) immersed in cyclohexane–benzene (1 : 1, 5 ml). Hydrogen was charged at ordinary pressure and the reaction mixture was vigorously stirred for 5 min. Removal of the catalyst by suction and concentration of the filtrate under reduced pressure gave almost pure **10** in quantitative yield as a light yellow oil. The product was used without further purification. **10**: $[\alpha]_D^{20}$ −85.1° ($c=2.32$, $CHCl_3$). IR (neat): 3392 (OH), 1017 (C–O–C). MS *m/z*: 268 ($M^+-H_2O$), 270 ($(M+2)^+-H_2O$). $^1$H-NMR: 0.88 (3H, t, $J_{7',8'}=5.7$ Hz, H-8'), 1.30 (6H, br s, H-5', 6', 7'), 1.9—2.2 (2H, m, H-4'), 2.47 (2H, dd, $J_{1',2'}=6.8$, $J_{gem}=4.2$ Hz, H-1'), 2.92 (1H, d, $J_{1,5}=6.8$ Hz, H-5), 3.82 (1H, s, $C_6$-OH), 4.51 (1H, d, $J_{H,OH}=4.4$ Hz, $C_3$-OH), 4.53 (1H, s, H-4), 5.26 (1H, dd, $J_{1,8}=2.2$ Hz, $J_{1,5}=6.8$ Hz, H-1), 5.46 (1H, dt, $J_{1',2'}=6.8$, $J_{2',3'}=14.2$ Hz, H-2'), 5.48 (1H, d, $J_{H,OH}=4.4$ Hz, H-3), 5.51 (1H, dt, $J_{2',3'}=14.2$ Hz, $J_{3',4'}=6.6$ Hz, H-3'), 5.79 (1H, d, $J_{7,8}=5.6$ Hz, H-7), 6.08 (1H, dd, $J_{1,8}=2.2$ Hz, $J_{7,8}=5.6$ Hz, H-8). $^{13}$C-NMR: 14.0 (q, C-8'), 22.5 (t, C-7'), 27.5 (t, C-6'), 29.2 (t, C-5'), 31.5 (t, C-4'), 38.4 (t, C-1'), 57.6 (d, C-5), 61.3 (d, C-4), 83.8 (s, C-6), 88.7 (d, C-1), 105.3 (d, C-3), 123.0 (d, C-3'), 133.6 (d, C-8), 134.4 (d, C-2'), 140.0 (d, C-7).

**Preparation of (4*S*,5*S*)-5-[(*S*)-1-Chloro-2-oxoethyl]-4-hydroxy-4-(2-octenyl)-2-cyclopentenone (11)** An oxidizing agent solution prepared from $CrO_3$ (42 mg, 0.42 mmol), $H_2O$ (0.2 ml) and 5 N $H_2SO_4$ (0.3 ml) was added dropwise to a stirred solution of **10** (40 mg, 0.14 mmol) in reagent-grade acetone (5 ml) at 0 °C. The reaction solution was heated to 80 °C for 20 min, cooled, and extracted with ether (15 ml × 5). The ethereal extracts were dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on $SiO_2$ using a 1 : 1 mixture of benzene–AcOEt as the eluant to give **11** (18 mg, 44.4%) as a light yellow oil. **11**: $[\alpha]_D^{20}$ +13.8° ($c=0.56$, $CHCl_3$). IR (neat): 3400 (OH), 1720 (C=O). MS *m/z*: 284 ($M^+$), 286 ($(M+2)^+$). $^1$H-NMR: 0.89 (3H, br t, $J_{7',8'}=6.3$ Hz,

H-8′), 1.26 (6H, br s, H-5′, 6′, 7′), 2.5—3.0 (4H, m, H-1′, H-4′), 3.18 (1H, d, $J_{1'',5}$=8.3 Hz, H-5), 4.42 (1H, d, $J_{1'',5}$=8.3 Hz, H-1″), 5.2—5.8 (2H, m, H-2′, 3′), 6.14 (1H, d, $J_{2,3}$=5.7 Hz, H-2), 7.45 (1H, d, $J_{2,3}$=5.7 Hz, H-3). $^{13}$C-NNR: 13.8 (q, C-8′), 22.3 (t, C-7′), 27.2 (t, C-6′), 28.9 (t, C-5′), 31.3 (t, C-4′), 36.5 (t, C-1′), 54.7 (d, C-5), 59.7 (d, C-1″), 92.4 (s, C-4), 121.9 and 133.3 (d, d, C-2′, 3′), 134.3 (d, C-2), 162.3 (d, C-3), 202.1 (d, C-2″), 203.1 (s, C-1).

**Preparation of Dimethyl (4-Carbomethoxy-2-oxobutyl)phosphonate** A solution of dimethyl methylphosphonate (6.82 g, 55 mmol) in THF (2 ml) was added to a solution of *n*-butyllithium (1.5 M hexane solution, 37 ml, 0.055 mmol) in THF (40 ml) at −78 °C under an argon atmosphere and the mixture was stirred for 10 min. To this was added copper iodide (10.5 g, 55 mmol), and the reaction mixture was stirred for 1 h at −30 °C. Then a solution of methyl succinyl chloride in ether (35 ml) at −40 °C was added and stirring was continued for 18 h at −30 °C and for an additional 3 h at room temperature. The insoluble solids deposited by addition of water (30 ml) to the solution were removed by suction and the filtrate was extracted with $CH_2Cl_2$ (30 ml × 4), dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on $SiO_2$ using AcOEt as the eluant to give dimethyl (4-carbomethoxy-2-oxobutyl) phosphonate (1.42 g, 11%) as a colorless oil.

**Reaction of 11 with Dimethyl (4-Carbomethoxy-2-oxobutyl)phosphate** A suspension of sodio dimethyl (4-carbomethoxy-2-oxobutyl)phosphonate was prepared under an argon atmosphere from NaH (17 mg as 60% dispersion in mineral oil, *ca.* 0.35 mmol) and dimethyl (4-carbomethoxy-2-oxobutyl)phosphonate (50 mg, 0.21 mmol) in 10 ml of DME at 0 °C with stirring for 0.5 h. To the suspension was added a solution of **11** (50 mg, 0.18 mmol) in DME (5 ml), and the whole was stirred for 5 min at 0 °C. The reaction mixture was poured onto an excess of ice and extracted with $CH_2Cl_2$ (15 ml × 5). The extracts were dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on $SiO_2$ using a 1 : 1 mixture of hexane–AcOEt as the eluant to give (7*S*,8*S*,12*R*)-7-chloro-12-hydroxy-4-oxo-7,8-dihydroclavulone (**12a**, 34 mg, 61%; TLC *Rf*=0.50, hexane : AcOEt = 2 : 1) and 4-oxo-12*R*-12-hydroxyclavulone (**12b**, 10 mg, 16%; TLC *Rf*=0.10, hexane : AcOEt = 2 : 1), each as a colorless oil. **12a**: $[\alpha]_D^{20}$ −3.8° (*c*=1.21, $CHCl_3$). IR (neat): 3404 (OH), 1739 ($COOCH_3$), 1720 (C–O). MS *m/z*: 396 ($M^+$), 398 ($(M+2)^+$). $^1$H-NMR: 0.88 (3H, br t, $J_{19,20}$=6.3 Hz, H-20), 1.25 (6H, br s, H-17, 18, 19), 1.6—2.3 (4H, m, H-13, 16), 2.5—2.9 (5H, m, H-2, 3, 8), 3.68 (3H, s, $COOCH_3$), 3.98 (1H, dd, $J_{7,8}$=6.6, $J_{6,7}$=10.0 Hz, H-7), 5.1—5.8 (2H, m, H-14, 15), 6.11 (1H, d, $J_{5,6}$=5.6 Hz, H-5), 6.32 (1H, d, $J_{10,11}$=6.1 Hz, H-10), 7.30 (1H, d, $J_{10,11}$=6.1 Hz, H-11), 7.54 (1H, dd, $J_{5,6}$=5.6, $J_{6,7}$=10.0 Hz, H-6). $^{13}$C-NMR: 14.0 (q, C-20), 22.5 (t, C-19), 27.6 (t, C-18), 29.2 (t, C-17), 31.5 (t, C-16), 33.9 (t, C-2), 38.1 (t, C-13), 43.7 (t, C-3), 51.9 (q, $COOCH_3$), 53.7 (d, C-8), 57.8 (d, C-7), 91.2 (s, C-12), 106.0 (d, C-5), 121.6 (d, C-15), 134.7 (d, C-6, C-4), 136.5 (d, C-10), 161.2 (d, C-11), 173.0 (s, C-1), 201.9, 205.3 (s and s, C-4 and C-9). **12b**: IR (neat): 1721 (C=O), 1740 ($COOCH_3$), 3400 (OH). MS *m/z*: 360 ($M^+$). $^1$H-NMR: 0.87 (3H, br t, $J_{19,20}$=6.5 Hz, H-20), 1.25 (6H, br s, H-17, 18, 19), 1.7—2.3 (4H, m, H-13, 16), 2.4—3.1 (4H, m, H-2, 3), 3.69 (3H, s, $COOCH_3$), 5.2—5.7 (2H, m, H-14, 15), 6.40 (1H, d, $J_{10,11}$=5.1 Hz, H-10), 6.68 (1H, d, $J_{5,6}$=6.7 Hz, H-5), 6.95 (1H, d, $J_{6,7}$=12.0 Hz, H-7), 7.43 (1H, d, $J_{10,11}$=5.1 Hz, H-11), 7.89 (1H, dd, $J_{5,6}$=6.7, $J_{6,7}$=12.0 Hz, H-6). $^{13}$C-NMR: 14.0 (q, C-20), 22.5 (t, C-19), 27.9 (t, C-18), 29.2 (t, C-17), 29.8 (t, C-16), 31.5 (t, C-2), 35.5 (t, C-13), 37.5 (t, C-3), 51.9 (q, $COOCH_3$), 100.0 (s, C-12), 121.7 (d, C-5, C-15), 128.5 (d, C-10), 134.9 (d, C-15), 135.2 (d, C-7), 135.7 (d, C-6), 136.1 (d, C-8), 161.6 (d, C-11), 172.1 (s, C-1), 191.6, 201.3 (s and s, C-4 and C-9).

**Reaction of 12a with Excess Zinc Borohydride** Freshly fused zinc chloride (68 mg) was added to sodium borohydride (40 mg) in redistilled DME (10 ml). The mixture was stirred overnight at 0—5 °C. After filtration under argon, the clear solution was used immediately. To a solution of **12a** (11 mg, 0.0277 mmol) in anhydrous DME (2 ml) was added 0.3 ml of the above solution of zinc borohydride. The mixture was stirred at room temperature until the reduction was complete (30 min), 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 subjected to column chromatography on $SiO_2$ using a 4 : 1 mixture of benzene–AcOEt as the eluant to give (7*S*,8*S*,12*R*)-7-chloro-12-hydroxy-7,8-dihydro-1,4-lactoylclavulone (**13a** 10 mg) in quantitative yield. **13a**: IR (neat): 3425 (OH), 1780 (O–C=O), 1722 (C=O), 1182 (C–O). MS *m/z*: 366 ($M^+$), 368 ($(M+2)^+$). $^1$H-NMR: 0.89 (3H, br t, $J_{19,20}$=6.5 Hz, H-20), 1.2—1.4 (6H, m, H-17, 18, 19), 1.5—2.4 (4H, m, H-13, 16), 2.6—2.8 (4H, m, H-2, 3), 2.95 (1H, s, $C_{12}$-OH), 2.97 (1H, d, $J_{7,8}$=6.7 Hz, H-8), 3.49 (1H, s, H-4), 4.42 (1H, d, $J_{7,8}$=6.7 Hz, H-7), 5.3—5.4 (2H, m, H-5, 14), 5.5—5.6 (2H, m, H-6, 15), 6.12 (1H, d, $J_{10,11}$=5.9 Hz, H-10), 7.54 (1H, d, $J_{10,11}$=5.9 Hz, H-11).

**Reaction of 12b with Excess Zinc Borohydride** By using the same procedure as described above, **12b** (11 mg, 0.031 mmol) was reduced with zinc borohydride (0.3 ml of the solution) in anhydrous DME (2 ml) to afford **13b** (10 mg) in quantitative yield. **13b**: $^1$H-NMR: 0.86 (3H, br t, $J_{19,20}$=6.5 Hz, H-20), 1.2—1.3 (6H, m, H-17, 18, 19), 1.8—2.1 (6H, m, H-3, 13, 16), 2.47 (2H, t, $J_{2,3}$=6.6 Hz, H-2), 2.8—3.0 (1H, m, H-9), 3.3—3.4 (1H, m, H-4), 3.66 (3H, s, $COOCH_3$), 4.24 (3H, s, 3 × OH), 5.1—5.8 (2H, m, H-14, 15), 6.2—6.6 (5H, m, H-5, 6, 7, 10, 11).

**Reaction of 12a with 1.2 eq of Zinc Borohydride** By using the same procedure as described for the reaction of **12a** with excess zinc borohydride, the reaction of **12a** (11 mg, 0.0277 mmol) with 1.2 eq of zinc borohydride (0.08 ml of the solution, *ca.* 0.033 mmol) was conducted in anhydrous DME (2 ml). Separation of the crude product by column chromatography on silica gel using a 4 : 1 mixture of benzene–AcOEt as the eluant gave the less polar isomer (**14a**, 5.0 mg, 45%; TLC *Rf*=0.47) and the more polar isomer (**14b**, 3.9 mg, 35%; TLC *Rf*=0.42), each as a colorless oil. **14a**: IR (neat): 3412 (OH), 1745 (O–C=O), 1720 (C=O). MS *m/z*: 396 ($M^+$), 398 ($(M+2)^+$). $^1$H-NMR: 0.83 (3H, br t, $J_{19,20}$=6.5 Hz, H-20), 1.2—1.3 (6H, m, H-17, 18, 19), 1.9—2.2 (4H, m, H-13, 16), 2.3—2.9 (5H, m, H-2, 3, 8), 3.62 (3H, s, $COOCH_3$), 3.9—4.0 (1H, m, H-4), 4.39 (1H, d, $J_{7,8}$=6.6 Hz, H-7), 5.23 (2H, s, 2 × OH), 5.4—5.6 (2H, m, H-14, 15), 6.2—6.4 (2H, m, H-5.6), 6.84 (1H, d, $J_{10,11}$=5.4 Hz, H-10), 7.43 (1H, d, $J_{10,11}$=5.4 Hz, H-11). **14b**: IR (neat): 3410 (OH), 1745 (O–C=O), 1720 (C=O). MS *m/z*: 396 ($M^+$), 398 ($(M+2)^+$). $^1$H-NMR: 0.83 (3H, br t, $J_{19,20}$=6.5 Hz, H-20), 1.2—1.3 (6H, m, H-17, 18, 19), 1.9—2.2 (4H, m, H-13, 16), 2.3—2.9 (5H, m, H-2, 3, 8), 3.62 (3H, s, $COOCH_3$), 3.9—4.0 (1H, m, H-4), 4.42 (1H, d, $J_{7,8}$=6.6 Hz, H-7), 5.23 (2H, s, 2 × OH), 5.4—5.6 (2H, m, H-14, 15), 6.2—6.4 (2H, m, H-5, 6), 6.84 (1H, d, $J_{10,11}$=5.4 Hz, H-10), 7.43 (1H, d, $J_{10,11}$=5.4 Hz, H-11).

**Preparation of Clavulone III** Compound **14a** (15 mg, 0.0377 mmol) was added to a stirred solution of acetic anhydride (3 ml) and pyridine (7 ml) in $CH_2Cl_2$ (10 ml), and the reaction solution was warmed to 70 °C for 17 h. The reaction solution was concentrated *in vacuo* and the residue was subjected to column chromatography on $SiO_2$ using a 4 : 1 mixture of benzene–AcOEt as the eluant to give clavulone III (7.6 mg, 45%). IR (neat): 1735, 1690, 1640 (C=O), 1230 (C–O). $[\alpha]_D^{20}$ +44.2° (*c*=0.10, $CHCl_3$). MS *m/z*: 446 ($M^+$). High-resolution MS Calcd for $C_{25}H_{34}O_7$ $M^+$: 446.2315. Found: 446.2305. $^1$H-NMR: 0.88 (3H, br t, H-20), 1.21—1.32 (6H, m, H-17, 18, 19), 1.94 (2H, dt, $J_{15,16}$=7.5, $J_{16,17}$=7.5 Hz, H-16), 2.02 (3H, s, OAc), 2.03 (2H, m, H-3), 2.10 (3H, s, OAc), 2.39 (2H, t, $J_{2,3}$=7.8 Hz, H-2), 2.67, 2.82 (2H, AB in ABX, $J_{AB}$=14.4, $J_{AX}$=7.0, $J_{BX}$=7.5 Hz, 13H), 5.21 (1H, X in ABX, dt-like, $J_{14,15}$=11.0, $J_{13,14}$=7.3 Hz, 14-H), 5.44 (1H, dt, $J_{4,5}$=6.1, $J_{3,4}$=5.5 Hz, H-4), 5.52 (1H, dt, $J_{14,15}$=11.0, $J_{15,16}$=7.3 Hz, 15-H), 6.02 (1H, dd, $J_{5,6}$=15.4, $J_{5,4}$=6.1 Hz, H-5), 6.36 (1H, d, $J_{10,11}$=6.4 Hz, H-10), 6.51 (1H, d, $J_{6,7}$=11.2 Hz, H-7), 7.51 (1H, d, $J_{10,11}$=6.4 Hz, H-11), 7.74 (1H, dd, $J_{5,6}$=15.4, $J_{6,7}$=11.2 Hz, H-6).

**Acknowledgement** The authors are greatly indebted to the staff of the central analysis room of this university for elemental analysis and mass spectral measurement. Thanks are also due to Mr. T. Ichida, Mr. T. Maeda, Mr. M. Eda, and Miss. H. Hasegawa, for their assistance.

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# Synthesis of Carbocyclic Nucleosides from 2-Azabicyclo[2.2.1]hept-5-en-3-ones: Sodium Borohydride-Mediated Carbon–Nitrogen Bond Cleavage of Five- and Six-Membered Lactams[1)]

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**Various carbocyclic ribofuranosyl nucleosides were stereoselectively synthesized through a small number of steps from 2-azabicyclo[2.2.1]hept-5-en-3-ones by the use of sodium borohydride-mediated C–N bond cleavage as a key step. Ready availability of a novel synthetic precursor, (±)-4β-hydroxymethyl-1β-ureidocyclopentane-2α,3α-diol [(±)-carbocyclic ribofuranosylurea], provides not only facile routes to carbocyclic ribofuranosylpyrimidines, but also another route to the corresponding cyclopentylamine, (±)-1β-amino-4β-hydroxymethylcyclopentane-2α,3α-diol [(±)-carbocyclic ribofuranosylamine], which is useful for the synthesis of the corresponding purine nucleosides.**

**Keywords** reductive amido bond cleavage; sodium borohydride; carbocyclic nucleoside; 2-azabicyclo[2.2.1]hept-5-en-3-one; chloroacetyl isocyanate; 2,2-dimethyl-1,3-dioxin-4-one; (±)-carbocyclic ribofuranosylurea; (±)-carbocyclic ribofuranosylamine

Carbocyclic nucleosides (B)[2)] are a group of compounds structurally analogous to nucleosides (A) in which the furanose oxygen is replaced by a methylene group.

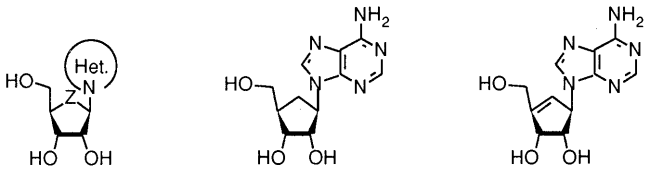
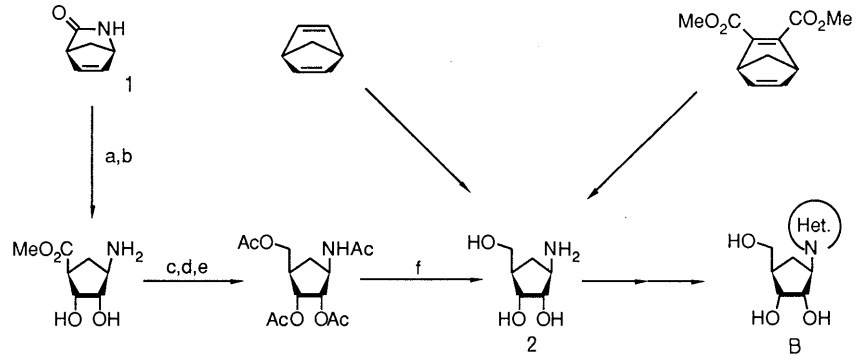



Fig. 1

Owing to the presence of the carbocyclic ring, they are not susceptible to the action of nucleoside phosphorylases and hydrolases that cleave normal nucleosides. However, the similarity in bond lengths and bond angles between the tetrahydrofuran (THF) and cyclopentane rings allows these analogues to behave as substrates or inhibitors of the enzymes that activate and interconvert nucleosides and nucleotides in living cells. As a result of this likeness, many of these compounds are endowed with an interesting range of biological activities, especially in the areas of anticancer and antiviral chemotherapy.[2,3)] The majority of carbocyclic nucleosides known to date are of synthetic origin but nature has provided two of the most active compounds, aristeromycin (C)[4)] and neplanocin A (D).[5)]




reagents and conditions: a, $OsO_4$ or $KMnO_4$; b, $H^+$/MeOH; c, $Ac_2O$/pyridine; d, $Ca(BH_4)_2$/MeOH; e, $Ac_2O$; f, aq. HCl

Chart 1

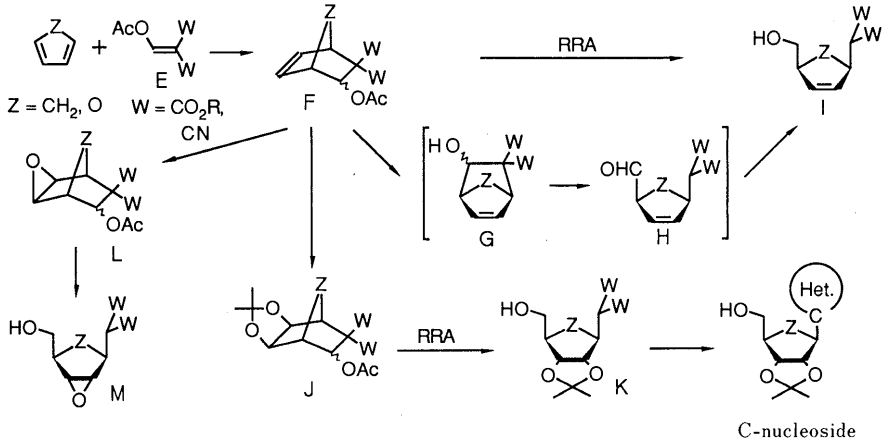



Chart 2

Carbocyclic ribofuranosylamine (**2**) has been used as a key precursor for the synthesis of carbocyclic nucleosides (B) and has been synthesized from norbornadiene[6] or the corresponding dicarboxylate.[7] An alternative synthesis of **2** developed originally by Cermak and Vince[8] and then modified by Kam and Oppenheimer.[9] has an advantage due to ready availability of 2-azabicyclo[2.2.1]hept-5-en-3-one (**1**), having all the functions of **2**, by Diels–Alder reaction of cyclopentadiene either with chlorosulfonyl isocyanate[10] or with tosyl cyanide.[11,12] However, its conversion to the key intermediate (**2**) needed not only a rather longer procedure (six steps) but also saturation of the C–C double bond (*e.g.* dihydroxylation) of **1** before the carbon–nitrogen bond cleavage (Chart 1). The latter requisite suggests that two important intermediates (Q and R, see Chart 3) can not be synthesized readily by the same route.

We have previously reported the synthesis of the adducts (F) by Diels–Alder reaction of 3-acetoxyacrylates (E) having an electron-withdrawing group at the 2-position with either furan[13] or cyclopentadiene.[14] The carbon–carbon bond originated from the dienophiles of the bicyclic system thus formed can be cleaved stereospecifically by reductive retrograde aldol reaction (RRA reaction: $NaBH_4$ in MeOH containing $K_2CO_3$), through the sequential reactions F→G→H→I. Hence the adduct (F) and its derivatives (*e.g.* J and L[15]) serve as versatile precursors (I, K, and M) for C-nucleosides, carbocyclic C-nucleosides, and their derivatives.[16]

If the amide bond in an appropriate derivative of **1** (N-N″) can be cleaved under the same conditions, it would provide the shortest route to **2** and its derivatives (Q-Q″). Thus, we have examined sodium borohydride mediated reductive amido bond cleavage of suitably manipulated 2-azabicyclo[2.2.1]hept-5-en-3-ones (N: W as an electron-withdrawing group). As a result, it was found that if an appropriate electron-withdrawing substituent was introduced at the amide nitrogen atom of **1**, the desired reductive C–N bond cleavage reaction proceeded smoothly. Also, use of N-carbamoyl derivatives of **1** permitted ready access to a series of the ureidocyclopentanes (Q-Q″: W= CONHR), novel precursors of pyrimidine-type nucleosides (*e.g.*, R). An alternative synthesis of (±)-carbocyclic ribofuranosylamine (**2**) from the ureido derivative (Q: W= $CONH_2$) was also achieved. Here, we report these results in detail.[17]

For the sake of clarity and brevity, we will report the results of three sections.

## Results and Discussion

**Sodium Borohydride Mediated Carbon–Nitrogen Bond Cleavage: Scope and Limitation** Knowing that the C–N bond cleavage of cyclic amido derivatives by $NaBH_4$ has been restricted to five-membered imides,[18] we first investigated the C–N bond cleavage of 2-pyrrolidinone (**3**) by sodium borohydride. As expected, the amide bond of 2-pyrrolidinone (**3**) did not suffer reduction, but its *N*-tosylate (**4a**) was found to give the corresponding amino alcohol (**5a**) when treated with sodium borohydride in methanol. It is clear that the tosyl group in **4a** not only activates the amide carbonyl by its electron-withdrawing nature to accept the nucleophile (hydride), giving **6a**, but also facilitates ring opening to give the aldehyde (**7a**), which is finally reduced to the alcohol (**5a**).

Next, in order to see if a much weaker electron-withdrawing group could still take the same role in the above reductive amido cleavage reaction, a carbamoyl group was selected as the *N*-substituent. *N*-Carbamoyl-2-pyrrolidinone (**4c**) was prepared *via* the corresponding *N*-chloroacetylcarbamoyl derivative (**4b**), which was formed from **3** by treatment with chloroacetyl isocyanate. Though removal of the chloroacetyl group of **4b** was expected to proceed on treatment with thiourea,[19] simple treatment of **4b** with triethylamine in methanol was also found to give **4c**. Treatment of **4c** with sodium borohydride in methanol followed by usual work-up, however, gave **5c** only in a poor yield (*ca.* 10%). The yield of **5c** was improved to 80% when the reaction was carried out in the presence of potassium carbonate. This fact implies that without the basic additive ($K_2CO_3$) the amino alcohol (**6c**: $CONH_2$ instead of Ts of **6a**) formed as the primary product did not undergo ring-opening, due to the much weaker electron-withdrawing nature of the carbamoyl group, to the corresponding acyclic

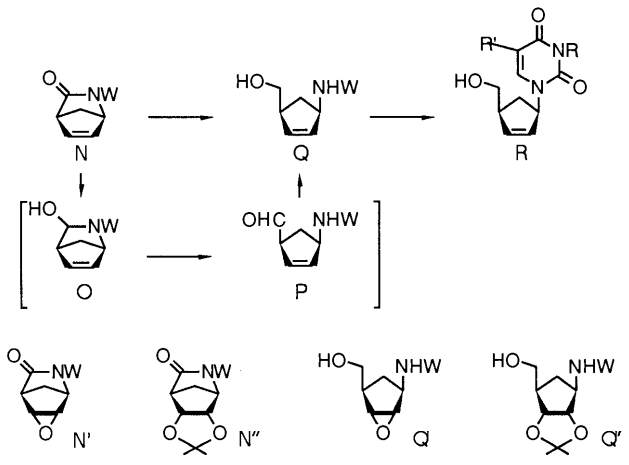



Chart 3

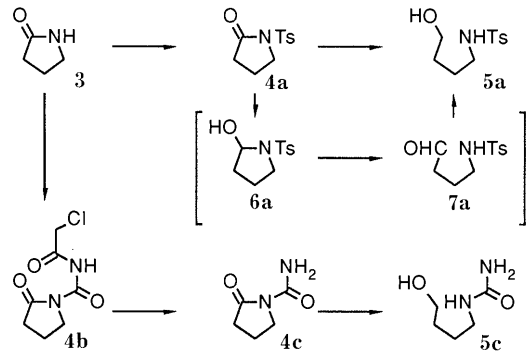



Chart 4

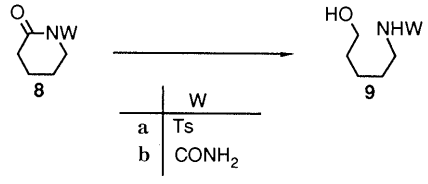
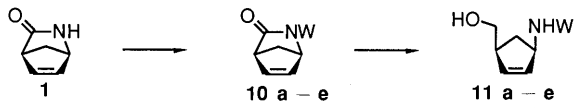



Chart 5

aminal (**7c**: $CONH_2$ instead of Ts of **7a**, probably an unstable species).

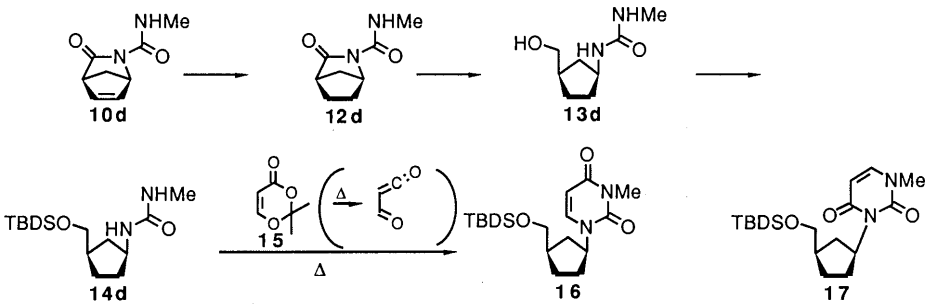
Next, in order to see how the ring size (and hence the ring strain) affects the above reaction, the corresponding piperidinones were used as the substrates in the above reaction. Though the tosylate (**8a**) did not give the desired product (**9a**) on treatment with sodium borohydride in methanol, **9a** was obtained in an almost quantitative yield when potassium carbonate was included in the reaction medium. Under the same conditions ($NaBH_4$–$K_2CO_3$/MeOH), the corresponding carbamoyl derivative (**8b**) was recovered and none of **9b** was obtained. These facts imply that not only is the amide carbonyl group in **8b** more stable than that in **8a** to sodium borohydride reduction, but also the putative intermediary six-membered aminal (*cf.* **6a**) would open less readily than the corresponding five-membered compounds to the aldehyde (*cf.* **7a**) due to the lower strain the six-membered ring.

**Synthesis of *cis*-4-Hydroxymethyl-1-ureidocyclopent-2-ene, a Versatile Synthetic Precursor for Carbocyclic Ribofuranosylpyrimidines** As mentioned above, 2-pyrrolidinones having an electron-withdrawing group at the 1-position were found to be reduced by sodium borohydride in methanol to give the corresponding 4-aminobutan-1-ols. This method was then extended to 2-azabicyclo[2.2.1]hept-5-en-3-ones (**10**) having an electron-withdrawing substituent at the 2-position and their dihydro derivatives (**12**). Though the *N*-unsubstituted compound (**1**) is stable to sodium borohydride reduction, all of them gave under the same reaction conditions the corresponding ring-opened amino alcohols (**11** and **13**). The fact that the *N*-carbamoyl derivatives (**10c—e**) readily afforded the desired products (**11c—e**) even in the absence of potassium carbonate indicates that the extra ring strain in these bicyclic amides accelerates the reactions (the reduction of the amides to the aminals and their subsequent ring-opening reaction). The relative configuration between hydroxymethyl and amino groups in the products should be *cis*, because in each reaction, the ring-opened product was obtained as a sole product. Even in the unsaturated amide series (**10a—e**), neither the double bond isomerization product nor the epimerization product at the hydroxymethyl group was



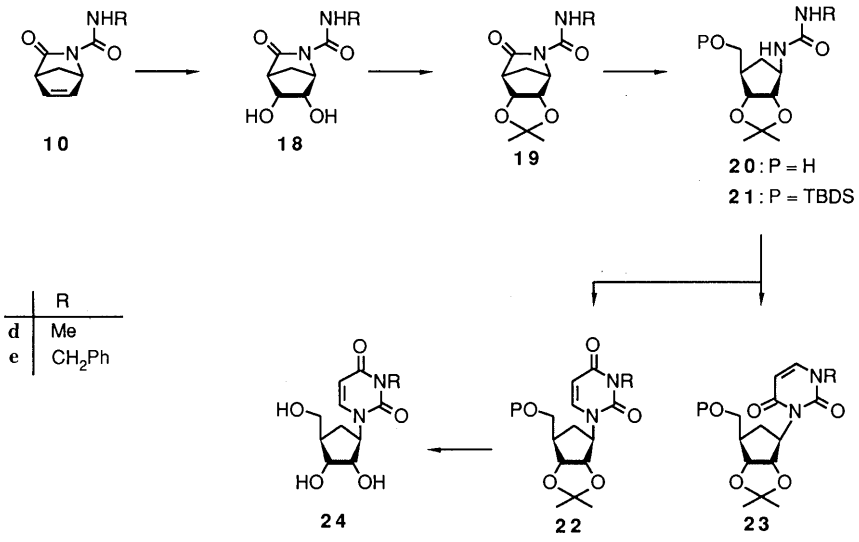

| | W |
|---|---|
| a | Ts |
| b | $CONHCOCH_2Cl$ |
| c | $CONH_2$ |
| d | CONHMe |
| e | $CONHCH_2Ph$ |

Chart 6




TBDS = *tert*-Bu(Me)$_2$Si

Chart 7




| | R |
|---|---|
| d | Me |
| e | $CH_2Ph$ |

Chart 8

formed.

Based on above data, we chose the bicyclic amides (**10c—e**) having a carbamoyl group at the 2-position as the most suitable synthetic precursors for the pyrimidine-type nucleosides. This is because the urea function is known to be readily manipulatable to a variety of pyrimidine derivatives.[16,20]

First, the synthesis of the carbocyclic 2′,3′-dideoxyuridine derivative (**16**) from 2-(*N*-methylcarbamoyl)-2-azabicyclo-[2.2.1]heptan-3-one (**12b**) was carried out.

Silylation of the hydroxyl group of ring-opened *N*-methyl ureido derivative (**13d**) to **14d** followed by refluxing in benzene containing 2,2-dimethyl-1,3-dioxin-4-one (**15**)[21,22] gave an 87% yield of uracils as a mixture of **16** and **17** (67 : 17), which were separable by silica gel column chromatography. The structures of these two uracils were determined by means of a proton nuclear magnetic resonance ($^1$H-NMR) study: 1) the signal due to $C_{1'}$-H of the major product (**16**) appeared at $\delta$ 4.95, and that of the minor one (**17**) at $\delta$ 5.30. In the corresponding nucleosides, the chemical shift of $C_{1'}$-H is lower in **17**-type nucleosides (note two C=O groups close to the methine proton) than in **16**-type ones[23] and 2) nuclear Overhauser effect (9%) between methyl and $C_6$-protons was observed only in the minor product (**17**).

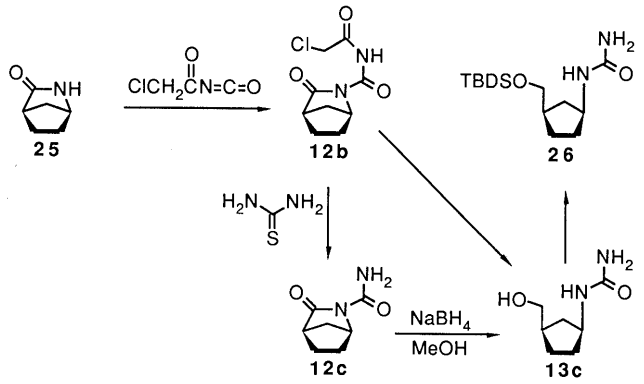



Chart 9

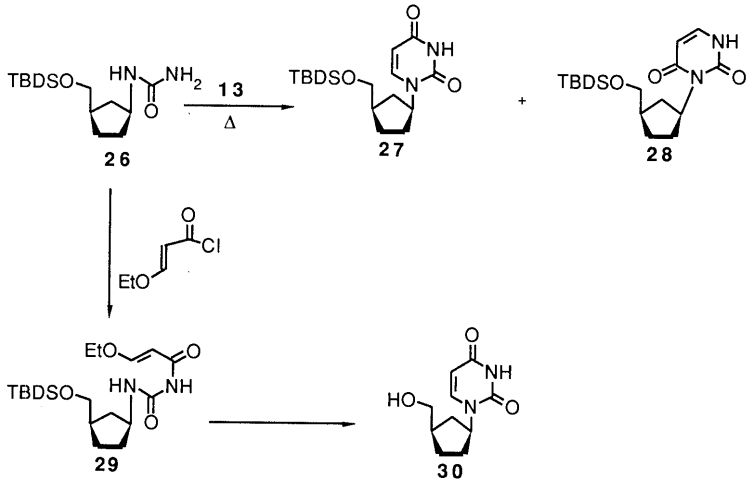



Chart 10

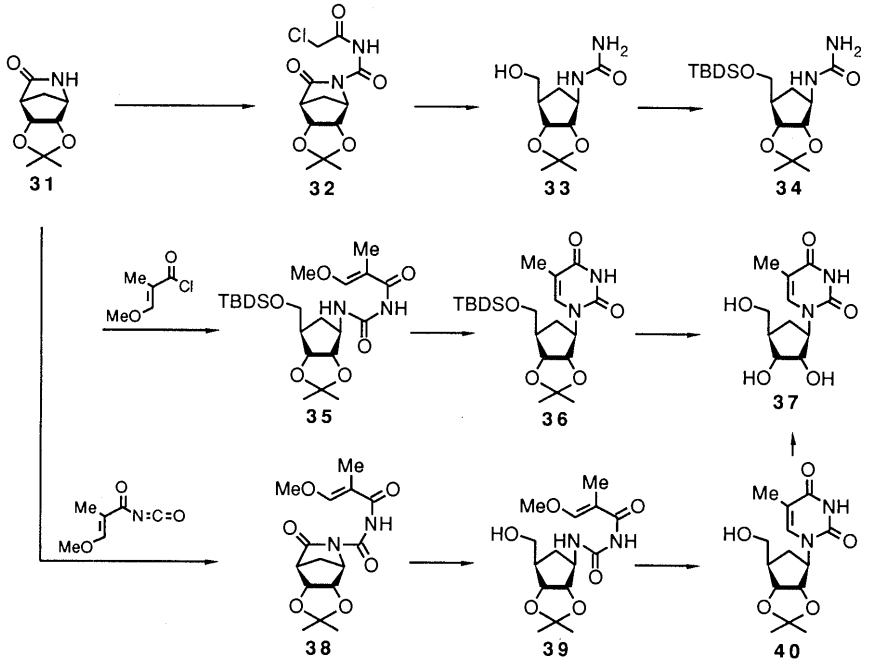



Chart 11

In the same manner, carbocyclic 3-methyluridine (**24d**) was synthesized using the dihydroxylated bicyclic amide (**18**). As noted already, the advantageous feature of bicyclic enamides (**1** and **10**) as synthetic precursors of the nucleosides is that the attack of reagents on the double bond always occurs from the less hindered *exo*-side.[16] Thus, the enamide (**10d**) when subjected to the usual dihydroxylation gave the *exo*-diol (**18d**), exclusively. The acetonide (**19d**) derived from **18d** was subjected to sodium borohydride-mediated reductive amido bond cleavage to give the more direct precursor (**20d**). After the silylation of the primary alcohol of **20d** to **21d**, the uracil formation reaction of the latter with the dioxinone (**15**) as above was examined.

Two uracils (**22d** and **23d**) were obtained from **21d** in 71 and 17% yields, respectively. The 3-benzyluridine (**22e**) was also synthesized from **10e** in the same manner.[24] The fact that in both reactions the desired uracils were obtained as the major products shows that the method has wide applicability for the synthesis of carbocyclic uridines having an alkyl group at the 3-position. It should be noted that deblocking of the silyl group is achieved readily by treatment with trifluoroacetic acid (TFA). Thus, the unprotected uridine (**24e**) was obtained by TFA mediated deblocking reaction of **22e**.

This methodology was then extended to the synthesis of the 3-unsubstituted uridine derivatives. As a model study, the synthesis of carbocyclic 2′,3′-dideoxyuridine (**30**) from the urea derivative (**13c**) was investigated first. The starting urea derivative (**13c**) was prepared from 2-(*N*-chloroacetylcarbamoyl)-2-azabicyclo[2.2.1]hyptan-3-one (**12b**) as in the case of **4c** from **4b** (Chart 4).

Though use of the dioxinone (**15**) in the uracil ring formation step for the silyl derivative (**26**) gave the uracils as a mixture of the two isomers (**27** and **28**) in poor yield (less than 10%), treatment with 3-ethoxyacryloyl chloride[25] followed by cyclization with an acid gave the desired product (**30**) as a sole product in satisfactory yield.

The corresponding ribothymidine derivative (**37**) was also synthesized from the acetonide (**31**) using 3-methoxymethacryloyl chloride instead of ethoxyacryloyl chloride. The starting acetonide was readily prepared from **1** *via* the corresponding diol.[8] Thus, a six-step synthesis of **37** from **31** was accomplished. The initial manipulation of **31** to **38** by 3-methoxymethacryloyl isocyanate treatment[26] followed by reductive amide bond cleavage has provided an alternative synthetic route to **37**. Since all of the steps proceed with complete regio- and stereoselectivity without the use of any protecting group, this method provides the shortest route (four steps) to carbocyclic ribothymidine (**37**) in high overall yield.

**An Alternative Synthetic Route to (±)-Carbocyclic Ribofuranosylamine: Utilization of (±)-4β-Hydroxymethyl-1β-ureidocyclopentane-2α,3α-diol in the Synthesis of Carbocyclic Purine-Type Nucleosides** As already mentioned, carbocyclic ribofuranosylamine (**2**) is a useful synthetic intermediate and so far has been used successfully for the synthesis of a variety of adenosine- and guanosine-type carbocyclic nucleosides. Since we have established an effective synthetic method for the 4-hydroxymethyl-1-ureidocyclopentane-2,3-diol (**33**), conversion of this compound to **2** was then examined. It was found that treatment of **33** with sodium nitrite in an acidic medium followed by acetylation gave the desired tetraacetate (**41**) in 64% yield. In the same manner, **11c** was transformed to the corresponding amino alcohol (**42**) in 65% yield, the structure of **42** was determined by its conversion to the mono- and diacetates (**43** and **44**).

Quite recently, Vince and Hua[27] synthesized several carbocyclic guanosine derivatives from **42** and examined their biological activity. As a result, carbovir (**45**) was found to show a significant antiviral activity against HIV (human immunodeficiency virus). The present method of synthesis of *cis*-4-hydroxymethylcyclopent-2-enylamine (**42**) would

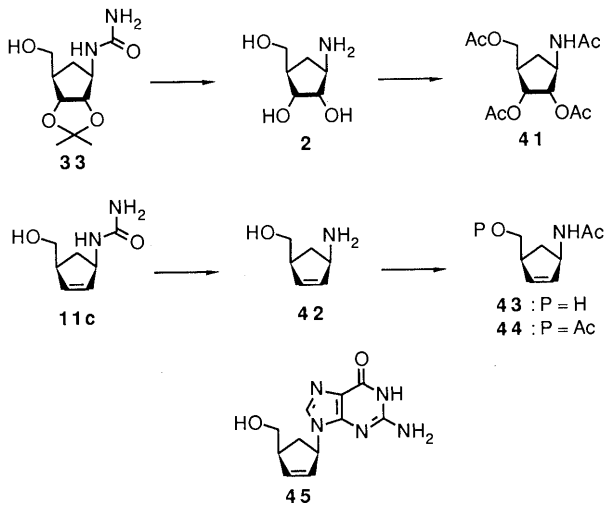



Chart 12

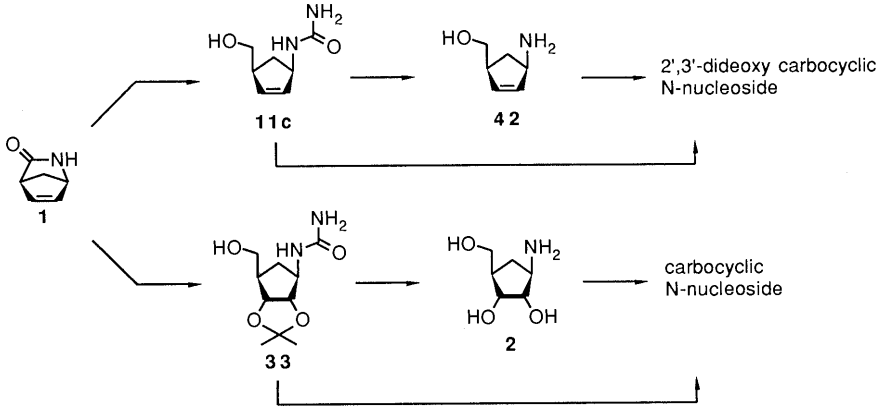



Chart 13

provide a variety of carbocyclic nucleosides having appropriate groups at the 2'- and/or 3'-positions, because manipulation of the C=C bond could be done even at the stage of the bicyclic amides (*e.g.* N in Chart 3).

## Conclusion

The bicyclic amide (**1**), readily available from cyclopentadiene, was converted by sodium borohydride mediated C–N bond cleavage as a key step to the corresponding monocyclic ureido derivatives (**11**, **13**, **20**, and **33**). Conversion of the ureido group to the amino group by the usual diazotization procedure led to carbocyclic ribofuranosylamine (**2**) and a related compound (**42**).[28]

The former compounds are useful for the synthesis of pyrimidine-type carbocyclic nucleosides and the latter, for purine-type ones. Since none of the above transformations required severe conditions, the present method should have wide applicability for the preparation of new analogues of carbocyclic nucleosides.

### Experimental

All melting points were determined on a Yanagimoto microhot stage and are uncorrected. Infrared (IR) spectra were measured on a JASCO A-102 spectrometer. $^1$H-NMR spectra were recorded with a JEOL JNM-PMX 60 or JEOL JNM-GX 500 spectrometer with tetramethylsilane (TMS) as an internal standard, and the abbreviations of signal patterns are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. High-resolution mass spectra (MS) were obtained on a JEOL JMS-DX-303 mass spectrometer. Wakogel (C-200) and Merck Kiesel-gel 60F 254 were employed for silica gel column and thin layer chromatography (TLC), respectively. The ratios of mixtures of solvents for chromatography are shown as volume/volume.

**1-*p*-Toluenesulfonyl-2-pyrrolidinone (4a)** 2-Pyrrolidinone (**3**: 340 mg, 4 mmol) was added to a mixture of sodium hydride (NaH) [about 60% in oil (192 mg, 4.8 mmol)] and dry ether (20 ml). The mixture was then stirred at room temperature for 1 h. To the resulting suspension, *p*-toluenesulfonyl chloride (915 mg, 4.8 mmol) was added and the whole was stirred overnight at room temperature. After removal of the precipitate by filtration, the filtrate was evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with hexane–AcOEt (1 : 1) gave 574 mg (60%) of the product (**4a**) as needles (from hexane–ether), mp 141—142 °C (lit.[29] mp 142—143 °C). *Anal.* Calcd for $C_{11}H_{13}NO_3S$: C, 55.22; H, 5.48; N, 5.86; S, 13.37. Found: C, 54.99; H, 5.24; N, 5.83; S, 13.41. IR ($CHCl_3$): 1740 cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 2.07 (2H, tt, $J$=7 Hz, $C_4$-H), 2.33 (2H, t, $J$=7 Hz, $C_3$-H), 2.52 (3H, s, Me), 3.90 (2H, t, $J$=7 Hz, $C_5$-H), 7.32 (2H, d, $J$=8 Hz, Ph), 7.93 (2H, d, $J$=8 Hz, Ph).

**1-(*N*-Chloroacetylcarbamoyl)-2-pyrrolidinone (4b)** Chloroacetyl isocyanate (598 mg, 5 mmol) was added to a solution of 2-pyrrolidinone (**3**, 340 mg, 4 mmol) in dry benzene (10 ml). The mixture was then stirred at room temperature for 3 h. The solvent was evaporated off under reduced pressure. The residue was recrystallized from $CH_2Cl_2$–hexane to give 498 mg (61%) of the product (**4b**), mp 118—120 °C. High-resolution MS *m/z* Calcd for $C_7H_9ClN_2O_3$ ($M^+$): 204.0301. Found: 204.0312. IR ($CHCl_3$): 3525, 1770, 1720, 1700 cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 2.14 (2H, tt, $J$=7 Hz, $C_4$-H), 2.72 (2H, t, $J$=7 Hz, $C_3$-H), 3.98 (2H, t, $J$=7 Hz, $C_5$-H), 4.47 (2H, s, $CH_2Cl$), 11.50 (1H, br s, NH).

**4-*p*-Toluenesulfonylaminobutan-1-ol (5a)** Sodium borohydride (57 mg, 1.5 mmol) was added to a stirred, ice-cooled solution of **4a** (32 mg, 0.12 mmol) in MeOH (2 ml). The mixture was stirred at room temperature for 30 min, and neutralized with AcOH–MeOH (1 : 10). The solvent was evaporated off under reduced pressure, and water was added to the residue. The mixture was extracted with $CH_2Cl_2$. The extract was dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue was subjected to silica gel column chromatography, and elution with hexane–AcOEt (1 : 1) gave 20 mg (62%) of the product (**5a**) as an oil. High-resolution MS *m/z* Calcd for $C_{11}H_{17}NO_3S$ ($M^+$): 243.0928. Found: 243.0962. IR ($CHCl_3$): 3530, 3400 cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.53 (4H, m, $C_2$-H and $C_3$-H), 2.38 (3H, s, Me), 2.87 (2H, m, $C_4$-H), 3.29 (1H, s, OH), 3.51 (2H, t, $J$=6 Hz, $C_1$-H), 5.87 (1H, br t, $J$=8 Hz, NH).

**1-Carbamoyl-2-pyrrolidinone (4c)** Triethylamine (5 drops) was added to a solution of **4b** (380 mg, 1.86 mmol) in dry MeOH (5 ml). The mixture was then stirred at room temperature for 2 h. The solvent was evaporated off under reduced pressure. The residue was recrystallized from $CH_2Cl_2$–hexane to give 100 mg (quant.) of the product (**4c**), mp 145—146 °C. High-resolution MS *m/z* Calcd for $C_5H_8N_2O_2$ ($M^+$): 128.0585. Found: 128.0567. IR ($CDCl_3$): 3425, 3350, 1720, 1698 cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 2.03 (2H, tt, $J$=7 Hz, $C_4$-H), 2.61 (2H, t, $J$=7 Hz, $C_3$-H), 3.85 (2H, t, $J$=7 Hz, $C_5$-H), 5.33 (1H, br s, NH), 8.17 (1H, br s, NH).

***N*-(4-Acetoxybutyl)urea and *N*-(4-Acetoxybutyl)-*N'*-acetylurea (the Acetylated Derivatives of 5c)** Sodium borohydride (168 mg, 4.45 mmol) and $K_2CO_3$ (185 mg, 1.34 mmol) were added to a stirred, ice-cooled solution of **4c** (57 mg, 0.45 mmol) in MeOH (3 ml). The mixture was stirred at room temperature for 6 h, and neutralized with AcOH–MeOH (1 : 10). The solvent was evaporated off under reduced pressure. Acetic anhydride (10 ml) and pyridine (0.5 ml) were added to the residue, and the mixture was stirred at room temperature for 2 d. The solvent was evaporated off under reduced pressure. The residue was extracted with $CHCl_3$. The extract was dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with AcOEt gave 32 mg (33%) of the *O*,*N*-diacetyl compound as needles, mp 88—89 °C (AcOEt). *Anal.* Calcd for $C_9H_{16}N_2O_4$: C, 49.97; H, 7.46; N, 12.96. Found: C, 49.84; H, 7.16; N, 13.06. IR ($CHCl_3$): 3450, 3325, 1720, 1705, 1670 cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.67 (4H, m, $C_2$-H and $C_3$-H), 2.03 (3H, s, OAc), 2.10 (3H, s, NAc), 3.35 (2H, t, $J$=6 Hz, $C_4$-H), 4.10 (2H, t, $J$=6 Hz, $C_1$-H), 8.55 (1H, br s, NH), 10.06 (1H, br s, NHAc).

Further elution with AcOEt–MeOH (5 : 1) gave 39 mg (50%) of the *O*-acetyl compound, mp 57—58 °C (AcOEt). *Anal.* Calcd for $C_7H_{14}N_2O_3$: C, 48.25; H, 8.10; N, 16.09. Found: C, 48.00; H, 7.87; N, 16.27. IR ($CHCl_3$): 3540, 3450, 3400, 1730, 1670 cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.63 (4H, m, $C_2$-H and $C_3$-H), 2.04 (3H, s, OAc), 3.18 (2H, t, $J$=6 Hz, $C_4$-H), 4.07 (2H, t, $J$=6 Hz, $C_1$-H), 5.10 (2H, br s, $NH_2$), 8.53 (1H, br s, NH).

**1-*p*-Toluenesulfonyl-2-piperidinone (8a)** This compound was obtained from 2-piperidinone by a procedure similar to that used for the preparation of **4a** from 2-pyrrolidone (**3**). **8a**: Yield, 60%, mp 140—142 °C (hexane–ether) (lit.[29] mp 143—144 °C). High-resolution MS *m/z* Calcd for $C_{12}H_{16}NO_3S$ ($M^+$+H): 254.0850. Found: 254.0837. IR ($CHCl_3$): 1715, 1600 cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.60—2.10 (4H, m, $C_4$-H and $C_5$-H), 2.23—2.63 (2H, m, $C_3$-H), 2.43 (3H, s, Me), 3.80—4.07 (2H, m, $C_6$-H), 7.33 (2H, d, $J$=8 Hz, Ph), 7.95 (2H, d, $J$=8 Hz, Ph).

**5-*p*-Toluenesulfonylaminohexan-1-ol (9a)** This compound was obtained from **8a** by the same procedure as that used for the preparation of **5a** from **4a**. **9a**: Yield, quantitative, oil. High-resolution MS *m/z* Calcd for $C_{12}H_{20}NO_3S$ ($M^+$+H): 258.1163. Found 258.1158. IR ($CHCl_3$): 3540, 3300, 1600 cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.07—1.67 (6H, m, $C_2$-H, $C_3$-H, and $C_4$-H), 2.42 (3H, s, Me), 2.50—3.20 (3H, m, $C_5$-H and OH), 3.37—3.83 (2H, m, $C_1$-H), 5.43 (1H, br s, NH), 7.37 (2H, d, $J$=8 Hz, Ph), 7.37 (2H, d, $J$=8 Hz, Ph).

**1-Carbamoyl-2-piperidinone (8b)** a) Preparation of 1-(*N*-Chloroacetylcarbamoyl)-2-piperidinone: This compound was obtained from 2-piperidinone by the same procedure as that used to synthesize **4b** from 2-pyrrolidinone (**3**). Yield, 78%, mp 66—67 °C ($CH_2Cl_2$–hexane). High-resolution MS *m/z* Calcd for $C_8H_{11}ClN_2O_3$ ($M^+$): 218.0457. Found: 218.0477. IR ($CHCl_3$); 1740, 1715, 1675 cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.70—2.20 (4H, m, $C_4$-H and $C_5$-H), 2.50—2.97 (2H, m, $C_3$-H), 3.70—4.10 (2H, m, $C_6$-H), 4.50 (2H, s, $CH_2Cl$), 12.63 (1H, br s, NH).

b) Triethylamine (3 drops) was added to a solution of the above product (656 mg, 3.0 mmol) in dry methanol. The mixture was then stirred under ice-cooling for 2 h. The residue obtained after evaporation of the solvent under reduced pressure was chromatographed over silica gel. Elution with hexane–ethyl acetate (1 : 1) gave 61 mg (8%) of the ring-opened product: methyl 5-(*N'*-chloroacetyl)ureidopentanoate. mp 79—80 °C ($CH_2Cl_2$–hexane). High-resolution MS *m/z* Calcd for $C_9H_{15}ClN_2O_4$ ($M^+$); 250.0719. Found: 250.0696. IR ($CHCl_3$): 3420, 3350, 1720 cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.33—1.95 (4H, m, $C_3$-H and $C_4$-H), 2.10—2.70 (2H, m, $C_2$-H), 3.10—3.53 (2H, m, $C_5$-H), 3.63 (3H, s, Me), 4.07 (2H, s, $CH_2Cl$), 8.20 (1H, br s, N'H), 9.52 (1H, br s, NH).

Further elution with the same solvent then afforded 247 mg (58%) of **8b**, mp 83—85 °C ($CH_2Cl_2$–hexane). High-resolution MS *m/z* Calcd for $C_6H_{10}N_2O_2$ ($M^+$): 142.0742. Found: 142.0748. IR ($CHCl_3$): 3510, 3420, 1705, 1665 cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.70—2.13 (4H, m, $C_4$-H and $C_5$-H), 2.37—2.85 (2H, m, $C_3$-H), 3.70—4.07 (2H, m, $C_6$-H), 5.70 and 9.07 (each 1H, br s, $NH_2$, one proton of which is hydrogen-bonded to the amide carbonyl).

When **8b** was treated with sodium borohydride in methanol (either in

the presence or absence of potassium carbonate), the starting material was recovered quantitatively.

**2-(*N*-Methylcarbamoyl)-2-azabicyclo[2.2.1]hept-5-en-3-one (10d)** 2-Azabicyclo[2.2.1]hept-5-en-3-one (**1**) (545 mg, 5 mmol) was added to a mixture of NaH (240 mg, 6 mmol) and dry ether (50 ml). The mixture was then stirred at room temperature for 1 h. To this suspension, methyl isocyanate (342 mg, 0.6 mmol) was added and the whole was stirred overnight at room temperature. After removal of the precipitate by filtration, the filtrate was evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with hexane–AcOEt (2:1) gave 451 mg (54%) of the product (**10d**), mp 90—91 °C (hexane–ether). *Anal.* Calcd for $C_8H_{10}N_2O_2$: C, 57.82; H, 6.07; N, 16.86. Found: C, 57.98; H, 6.06; N, 16.99. IR ($CHCl_3$): 3402, 1727, 1686 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 2.28 (2H, m, $CH_2$), 2.83 (3H, d, $J$=5 Hz, NHMe), 3.48 (1H, m, $C_4$-H), 5.28 (1H, m, $C_1$-H), 6.68 (1H, m, $C_5$-H), 7.03 (1H, m, $C_6$-H), 7.49 (1H, br s, NH).

**2-Azabicyclo[2.2.1]hept-5-en-3-ones Having a *p*-Toluenesulfonyl (10a) or Benzylcarbamoyl Group (10e) at the 2-Position** These two compounds were prepared by a procedure similar to that used for the preparation of **10d**.

**10a**: Yield, 46%, mp 110—111 °C (hexane–ether). *Anal.* Calcd for $C_{13}H_{13}NO_3S$: C, 59.30; H, 4.98; N, 5.32; S, 12.15. Found: C, 59.45; H, 4.94; N, 5.37; S, 12.42. IR ($CHCl_3$): 1760 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 2.06 (5H, m, $CH_2$ and Me), 3.37 (1H, m, $C_4$-H), 5.05 (1H, m, $C_1$-H), 6.33 (1H, m, $C_5$-H), 6.62 (1H, dd, $J$=6, 2 Hz, $C_6$-H), 7.25 (2H, d, $J$=8 Hz, Ph), 7.78 (2H, d, $J$=8 Hz, Ph).

**10e**: Yield, 66%, oil. High-resolution MS $m/z$ Calcd for $C_{14}H_{14}N_2O_2$ ($M^+$): 242.1054. Found: 242.1062. IR ($CHCl_3$): 3333, 1720, 1685 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 2.25 (2H, m, $CH_2$), 3.43 (1H, m, $C_4$-H), 4.45 (2H, d, $J$=6 Hz, $CH_2$Ph), 5.27 (1H, m, $C_1$-H), 6.63 (1H, ddd, $J$=6, 4, 2 Hz, $C_5$-H or $C_6$-H), 6.97 (1H, dd, $J$=6, 2 Hz, $C_5$-H or $C_6$-H), 7.32 (5H, s, Ph), 7.90 (1H, br t, $J$=6 Hz, NH).

**2-(*N*-Chloroacetylcarbamoyl)-2-azabicyclo[2.2.1]hept-5-en-3-one (10b)** Chloroacetyl isocyanate (920 mg, 7.7 mmol) was added to a solution of **1** (763 mg, 7 mmol) in dry benzene (10 ml). The mixture was then stirred at room temperature for 1.5 h. The solvent was evaporated off under reduced pressure. The crystalline residue was recrystallized from ether to give 1.10 g (69%) of the product (**10b**), mp 96—97 °C. *Anal.* Calcd for $C_9H_9ClN_2O_3$: C, 47.26; H, 3.97; Cl, 15.52; N, 12.25. Found: C, 47.20; H, 3.91; Cl, 15.59; N, 12.18. IR ($CHCl_3$): 3450, 1770, 1735, 1715 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 2.33 (2H, m, $CH_2$), 3.57 (1H, m, $C_4$-H), 4.43 (2H, s, $CH_2Cl$), 5.27 (1H, m, $C_1$-H), 6.67 (1H, m, $C_5$-H), 7.00 (1H, dd, $J$=6, 2 Hz, $C_6$-H), 10.33 (1H, br s, NH).

**2-Carbamoyl-2-azabicyclo[2.2.1]hept-5-en-3-one (10c)** Thiourea (72 mg, 0.93 mmol) was added to a solution of **10b** (86 mg, 0.38 mmol) in dry THF (4 ml). The mixture was then stirred at room temperature for 4 h. The solvent was evaporated off under reduced pressure. The residue thus obtained was subjected to silica gel column chromatography. Elution with hexane–AcOEt (1:1) gave 48 mg (84%) of the product (**10c**), mp 117—118 °C. *Anal.* Calcd for $C_7H_8N_2O_2$: C, 55.24; H, 5.30; N, 18.42. Found: C, 55.14; H, 5.39; N, 18.45. IR ($CHCl_3$): 3540, 1730, 1700 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 2.03 (2H, m, $CH_2$), 3.48 (1H, m, $C_4$-H), 5.20 (1H, m, $C_1$-H), 6.67 (1H, m, $C_5$-H), 6.97 (1H, m, $C_6$-H), 5.50—7.67 (2H, br s, $NH_2$).

***cis*-4-Hydroxymethyl-1-(*N'*-methylureido)cyclopent-2-ene (11d)** Sodium borohydride (259 mg, 7 mmol) was added to a stirred, ice-cooled solution of **10d** (117 mg, 0.7 mmol) in MeOH (7 ml). The mixture was stirred at room temperature for 30 min, and neutralized with AcOH–MeOH (1:10). The solvent was evaporated off under reduced pressure, and AcOEt was added to the residue. After removal of the precipitate by filtration, the filtrate was evaporated off under reduced pressure. The crystalline residue was purified by recrystallization from AcOEt–MeOH to give 133 mg (quant.) of the product (**11d**), mp 143—144 °C. *Anal.* Calcd for $C_8H_{14}N_2O_2$: C, 56.44; H, 8.27; N, 16.46. Found: C, 56.31; H, 8.21; N, 16.40. IR ($CHCl_3$): 3350, 1710, 1660 $cm^{-1}$. $^1$H-NMR ($CDCl_3$–$CD_3OD$) $\delta$: 1.43 (1H, m, CH-H), 2.00—3.00 (5H, m, CH-H, Me, and $C_4$-H), 3.60 (2H, m, $CH_2OH$), 4.80 (1H, m, $C_1$-H), 5.53 (2H, m, $C_2$-H and $C_3$-H).

***cis*-4-*p*-Toluenesulfonylaminocyclopent-2-enylmethanol (11a) and *cis*-4-Hydroxymethyl-1-ureidocyclopent-2-ene (11c)** These two compounds were prepared by a procedure similar to that used for the preparation of **11d**.

**11a**: Yield, 62%, mp 77—79 °C (hexane–ether). High-resolution MS $m/z$ Calcd for $C_{13}H_{17}NO_3S$ ($M^+$): 267.0945. Found: 267.0936. IR ($CHCl_3$): 3565, 3415 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.40 (1H, m, CH-H), 2.00—2.42 (5H, m, CH-H, Me, and OH), 2.77 (1H, m, $C_1$-H), 3.57 (2H, m, $CH_2OH$), 4.40 (1H, m, $C_4$-H), 5.43 (1H, br s, NH), 5.53 (2H, m, $C_2$-H and $C_3$-H), 7.33 (2H, d, $J$=8 Hz, Ph), 7.73 (2H, d, $J$=8 Hz, Ph).

**11c**: Yield, 64%, mp 138—139 °C. High-resolution MS $m/z$ Calcd for $C_7H_{12}N_2O_2$ ($M^+$): 156.0898. Found: 156.0868. IR (Nujol): 1640 $cm^{-1}$. $^1$H-NMR ($CDCl_3$–$CD_3OD$) $\delta$: 1.30 (1H, m, CH-H), 2.01—3.01 (2H, m, $C_4$-H and CH-H), 3.23 (2H, d, $J$=6 Hz, $CH_2OH$), 4.70 (1H, m, $C_1$-H), 5.77 (2H, m, $C_2$-H and $C_3$-H).

The same compound (**11c**) was obtained directly from **10b** in 59% yield.

**2-(*N*-Methylcarbamoyl)-2-azabicyclo[2.2.1]heptan-3-one (12d)** A mixture of **10d** (327 mg, 1.97 mmol) and 10% Pd–C (50 mg) in AcOEt (15 ml) was shaken under a hydrogen atmosphere (1 atm) at room temperature until the absorption of hydrogen ceased. The catalyst was filtered off, and the filtrate was concentrated under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with hexane–AcOEt (1:1) gave 313 mg (95%) of the product as an oil. High-resolution MS $m/z$ Calcd for $C_8H_{12}N_2O_2$ ($M^+$): 168.0890. Found: 168.0893. IR ($CHCl_3$): 3371, 1720, 1686 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.33—2.16 (6H, m, 3 × $CH_2$), 2.75 (3H, d, $J$=5 Hz, NHMe), 2.75 (1H, m, $C_4$-H), 4.87 (1H, m, $C_1$-H), 7.76 (1H, br s, NH).

***cis*-3-Hydroxymethyl-1-(*N'*-methylureido)cyclopentane (13d)** Sodium borohydride (703 mg, 18.6 mmol) was added to a stirred, ice-cooled solution of **12d** (313 mg, 1.86 mmol) in MeOH (20 ml). The mixture was stirred at room temperature for 30 min, and neutralized with AcOH–MeOH (1:10). The solvent was evaporated off under reduced pressure, and AcOEt was added to the residue. After removal of the precipitate by filtration, the filtrate was evaporated to dryness under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with 10% MeOH–AcOEt gave 355 mg (quant.) of the product (**13d**), mp 90—91 °C (AcOEt–MeOH). *Anal.* Calcd for $C_8H_{16}N_2O_2$: C, 55.77; H, 9.37; N, 16.27. Found: C, 55.86; H, 9.33; N, 16.54. IR ($CHCl_3$): 3385, 3300, 1660 $cm^{-1}$. $^1$H-NMR ($CDCl_3$–$CD_3OD$) $\delta$: 0.67—3.00 (7H, m, 3 × $CH_2$ and $C_3$-H), 2.68 (3H, s, Me), 3.52 (2H, d, $J$=6 Hz, $CH_2OH$), 4.00 (1H, m, $C_1$-H), 5.60 (2H, br s, 2 × NH).

***cis*-3-(*tert*-Butyldimethylsilyloxy)methyl-1-(*N*-methylureido)cyclopentane (14d)** A solution of **13d** (172 mg, 1 mmol), TBDSCl (540 mg, 3.6 mmol), and imidazole (480 mg, 6 mmol) in dimethylformamide (DMF) (10 ml) was kept at room temperature for 12 h. The reaction mixture was poured into ice-water, and extracted with ether. The extract was dried over anhydrous magnesium sulfate, and the solvent was evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with AcOEt gave 241 mg (84%) of the product (**14d**), mp 84—87 °C. High-resolution MS $m/z$ Calcd for $C_{13}H_{27}N_2O_2Si$ ($M^+$ − Me): 271.1840. Found: 271.1825. IR ($CHCl_3$): 1670 $cm^{-1}$. $^1$H-NMR ($CDCl_3$–$CD_3OD$) $\delta$: 0.87 (9H, s, *tert*-Bu), 1.10—2.43 (7H, m, 3 × $CH_2$ and $C_3$-H), 2.73 (3H, d, $J$=5 Hz, Me), 3.53 (2H, d, $J$=5 Hz, C<u>H</u>$_2$O), 4.00 (1H, m, $C_1$-H), 5.60 (2H, br s, 2 × NH).

**(±)-1-[3'*β*-(*tert*-Butyldimethylsilyloxymethyl)cyclopent-1'*β*-yl]-3-methyl-2,4(1*H*,3*H*)-pyrimidinedione (16) and (±)-3-[3'*β*-(*tert*-Butyldimethylsilyloxymethyl)cyclopent-1'*β*-yl]-1-methyl-2,4(1*H*,3*H*)-pyrimidinedione (17)** A solution of 2,2-dimethyl-1,3-dioxin-4-one (**15**) (128 mg, 1 mmol) in dry benzene (1 ml) was added dropwise to a refluxing solution of **14d** (143 mg, 0.5 mmol) in dry benzene (5 ml) over 10 min. The solvent was evaporated off under reduced pressure and the residue was subjected to silica gel column chromatography. Elution with AcOEt–hexane (1:1) gave 113 mg (69%) of the product (**16**) as an oil. High-resolution MS $m/z$ Calcd for $C_{16}H_{27}N_2O_3Si$ ($M^+$ − Me): 323.1819. Found: 323.1816. IR ($CHCl_3$): 1720, 1660 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.88 (9H, s, *tert*-Bu), 1.17—2.67 (7H, m, 3 × $CH_2$ and $C_{3'}$-H), 3.28 (3H, s, NMe), 3.62 (2H, d, $J$=5 Hz, C<u>H</u>$_2$O), 4.95 (1H, m, $C_{1'}$-H), 5.73 (1H, d, $J$=8 Hz, $C_5$-H), 7.30 (1H, d, $J$=8 Hz, $C_6$-H). UV $\lambda_{max}^{MeCN}$ nm: 266, 207, $\lambda_{min}^{MeCN}$ nm: 233.

Further elution with AcOEt–hexane (1:1) gave 29 mg (17%) of the product (**17**) as an oil. High-resolution MS $m/z$ Calcd for $C_{16}H_{27}N_2O_3Si$ ($M^+$ − Me): 323.1791. Found: 323.1819. IR ($CHCl_3$): 1702, 1660 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.90 (9H, s, *tert*-Bu), 1.50—2.50 (7H, m, 3 × $CH_2$ and $C_{3'}$-H), 3.33 (3H, s, NMe), 3.63 (2H, d, $J$=5 Hz, C<u>H</u>$_2$O), 5.30 (1H, m, $C_{1'}$-H), 5.67 (1H, d, $J$=8 Hz, $C_5$-H), 7.07 (1H, d, $J$=8 Hz, $C_6$-H). UV $\lambda_{max}^{MeCN}$ nm: 265, 205, $\lambda_{min}^{MeCN}$ nm: 233.

**5,6-*exo*-Dihydroxy-2-(*N*-methylcarbamoyl)-2-azabicyclo[2.2.1]heptan-3-one (18d)** A mixture of 0.7 ml of osmium tetroxide-*tert*-BuOH solution [prepared from $OsO_4$ (2 g), *tert*-BuOH (200 ml), and $H_2O_2$ (3 drops)] and 60% aqueous 4-methylmorpholine *N*-oxide (0.5 ml) was added to a solution of **10d** (415 mg, 2.5 mmol) in acetone (7 ml) under stirring at 0 °C. After being stirred at room temperature for 30 min, the reaction mixture was poured into ice-water and extracted with AcOEt. The organic layer was dried over anhydrous sodium sulfate, and concentrated under reduced

pressure. The residue was subjected to silica gel column chromatography. Elution with AcOEt gave 420 mg (quant.) of the product (**18d**), mp 124—126 °C. High-resolution MS *m/z* Calcd for $C_8H_{12}N_2O_4$ ($M^+$): 200.0796. Found: 200.0803. IR ($CHCl_3$): 3550, 1730, 1680 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 2.00 (2H, m, $CH_2$), 2.78 (3H, d, $J$=5 Hz, NHMe), 3.67—5.00 (6H, m, 2×OH, $C_1$-H, $C_4$-H, $C_5$-H, and $C_6$-H), 7.67 (1H, br s, NH).

**2-(*N*-Benzylcarbamoyl)-5,6-*exo*-dihydroxy-2-azabicyclo[2.2.1]heptan-3-one (18e)** This compound was prepared from **10e** by a procedure similar to that used for the preparation of **18d**. **18e**: Yield, 87%, oil. High-resolution MS *m/z* Calcd for $C_{14}H_{16}N_2O_4$ ($M^+$): 276.1109. Found: 276.1100. IR ($CHCl_3$): 3400, 3340, 1725, 1678 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 2.02 (2H, m, $CH_2$), 2.83 (1H, m, $C_4$-H), 4.10 (2H, t, $J$=6 Hz, $C_5$-H and $C_6$-H), 4.10 (1H, br s, OH), 4.43 (2H, d, $J$=6 Hz, C$\underline{H}_2$Ph), 4.53 (1H, m, $C_1$-H), 4.98 (1H, br s, OH), 7.30 (5H, s, Ph), 8.18 (1H, br t, $J$=6 Hz, NH).

**5,6-*exo*-Dimethylmethylenedioxy-2-(*N*-methylcarbamoyl)-2-azabicyclo-[2.2.1]heptan-3-one (19d)** 2,2-Dimethoxypropane (3.5 ml) and *p*-TsOH·$H_2O$ (56 mg) were added to a solution of **18d** (303 mg, 1.5 mmol) in acetone (5 ml) with stirring under ice-cooling. After being stirred at room temperature for 40 min, the reaction mixture was neutralized with sodium hydrogen carbonate and filtered. The filtrate was concentrated under reduced pressure, and the residue was subjected to silica gel column chromatography. Elution with hexane–AcOEt (1 : 1) gave 420 mg (quant.) of the product (**19d**) as needles, mp 111—112 °C ($CH_2Cl_2$–hexane). *Anal.* Calcd for $C_{11}H_{16}N_2O_4$: C, 54.97; H, 6.72; N, 11.66. Found: C, 55.24; H, 6.68; N, 11.64. IR ($CHCl_3$): 3350, 1730, 1680 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.33, 1.46 (6H, 2×s, isopropylidene-Me), 2.10 (2H, m, $CH_2$), 2.85 (3H, d, $J$=5 Hz, NMe), 2.85 (1H, m, $C_4$-H), 4.56 (2H, m, $C_5$-H and $C_6$-H), 4.73 (1H, m, $C_1$-H), 7.60 (1H, br s, NH).

**2-(*N*-Benzylcarbamoyl)-5,6-*exo*-dimethylmethylenedioxy-2-azabicyclo-[2.2.1]heptan-3-one (19e)** This compound was obtained by a procedure similar to that used for the preparation of **19d**. **19e**: Yield, 72%, mp 121—123 °C (ether). *Anal.* Calcd for $C_{17}H_{20}N_2O_4$: C, 64.53; H, 6.38; N, 8.86. Found: C, 64.24; H, 6.31; N, 8.80. IR ($CHCl_3$): 3350, 1730, 1690 $cm^{-1}$. $^1$H-NMR ($CDCl_3$, 500 MHz) $\delta$: 1.33, 1.46 (6H, 2×s, isopropylidene-Me), 2.01 (1H, dq, $J$=10.6, 1.4 Hz, CH-H), 2.16 (1H, dt, $J$=10.6, 1.25 Hz, CH-H), 2.93 (1H, m, $C_4$-H), 4.40 (1H, dd, $J$=14, 6 Hz, CHHPh), 4.50 (1H, dd, $J$=14, 6 Hz, CHHPh), 4.54 (1H, dt, $J$=5.0, 1.4 Hz, $C_5$-H or $C_6$-H), 4.57 (1H, dt, $J$=5.0, 1.4 Hz, $C_5$-H or $C_6$-H), 4.71 (1H, m, $C_1$-H), 7.33 (5H, s, Ph), 8.07 (1H, br t, $J$=6 Hz, NH).

**(±)-2α,3α-Dimethylmethylenedioxy-4β-hydroxymethyl-1β-(*N'*-methylureido)cyclopentane (20d)** Sodium borohydride (57 mg, 1.5 mmol) was added to a stirred, ice-cooled solution of **19d** (36 mg, 0.15 mmol) in MeOH (1 ml). The mixture was stirred at room temperature for 1 h, and neutralized with AcOH–MeOH (1 : 10). The solvent was evaporated off under reduced pressure, and AcOEt was added to the residue. After removal of the precipitate by filtration, the filtrate was evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with 10% MeOH–AcOEt gave 36 mg (98%) of the product (**20d**) as an oil. High-resolution MS *m/z* Calcd for $C_{10}H_{17}N_2O_4$ ($M^+$ − Me): 229.1225. Found: 229.1207. IR ($CHCl_3$): 3490, 3415, 3400, 1660 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.25 (1H, m, CH-H), 1.28, 1.48 (6H, 2×s, isopropylidene-Me), 2.33 (2H, m, $C_4$-H and CH-H), 2.73 (3H, s, Me), 3.67 (2H, d, $J$=4 Hz, C$\underline{H}_2$OH), 3.88 (1H, m, $C_1$-H), 4.31 (1H, dd, $J$=7, 2 Hz, $C_2$-H or $C_3$-H), 4.52 (1H, dd, $J$=7, 2 Hz, $C_2$-H or $C_3$-H), 5.00—6.40 (3H, br s, 2×NH and OH).

**(±)-1β-(*N'*-Benzylureido)-2α,3α-dimethylmethylenedioxy-4β-hydroxymethylcyclopentane (20e)** This compound was obtained from **19e** by a procedure similar to that used for preparation of **19d**. **20e**: Yield, 95%, oil. High-resolution MS *m/z* Calcd for $C_{17}H_{24}N_2O_4$ ($M^+$): 320.1735. Found: 320.1726. IR ($CHCl_3$): 3630, 3460, 3380, 1660 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.27, 1.40 (6H, 2×s, isopropylidene-Me), 1.50 (1H, m, CH-H), 2.27 (2H, m, CH-H and $C_4$-H), 3.60 (2H, d, $J$=4 Hz, C$\underline{H}_2$OH), 4.20—4.60 (2H, m, $C_1$-H and OH), 5.80 (1H, br t, $J$=6 Hz, NH), 6.02 (1H, br d, $J$=6 Hz, NH), 7.30 (5H, s, Ph).

**(±)-4β-(*tert*-Butyldimethylsilyloxy)methyl-2α,3α-dimethylmethylenedioxy-1β-(*N'*-methyl)ureidocyclopentane (21d)** A solution of **20d** (200 mg, 0.82 mmol), TBDSCl (540 mg, 2.95 mmol), and imidazole (335 mmol, 4.92 mmol) in DMF (10 ml) was kept at room temperature for 12 h. The reaction mixture was poured into ice-water, and extracted with ether. The extract was dried over anhydrous magnesium sulfate, and the solvent was evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography, and elution with AcOEt gave 215 mg (73%) of the product (**21d**) as an oil. High-resolution MS *m/z* Calcd for $C_{16}H_{31}N_2O_4Si$ ($M^+$ − Me): 343.2053. Found: 343.2080. IR ($CHCl_3$): 1670 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.84 (9H, s, *tert*-Bu), 1.22, 1.43 (6H, 2×s, isopropylidene-Me), 1.28 (1H, m, CH-H), 2.13 (2H, m, $C_4$-H and CH-H), 2.68 (3H, d, $J$=4 Hz, Me), 3.60 (2H, d, $J$=5 Hz, $CH_2O$), 3.77 (1H, m, $C_1$-H), 4.12 (1H, d, $J$=6 Hz, $C_2$-H or $C_3$-H), 4.35 (1H, d, $J$=6 Hz, $C_2$-H or $C_3$-H), 5.00—5.67 (2H, br s, 2×NH).

**(±)-1β-(*N'*-Benzyl)ureido-4β-(*tert*-butyldimethylsilyloxy)methyl-2α,3α-dimethylmethylenedioxycyclopentane (21e)** This compound was obtained by a procedure similar to that used for the preparation of **21d**. **21e**: Yield, 70%, oil. High-resolution MS *m/z* Calcd for $C_{23}H_{38}N_2O_4Si$ ($M^+$): 434.2599. Found: 434.2581. IR ($CHCl_3$): 3475, 3380, 1660 $cm^{-1}$. $^1$H-NMR ($CDCl_3$, 500 MHz) $\delta$: 0.99 (9H, s, *tert*-Bu), 1.25, 1.35 (6H, 2×s, isopropylidene-Me), 1.56 (1H, td, $J$=10, 3.75 Hz, CH-H), 2.25 (2H, m, CH-H and $C_4$-H), 3.63 (1H, dd, $J$=10, 5 Hz, CHHOSi), 3.72 (1H, dd, $J$=10, 5 Hz, CHHOSi), 4.26 (1H, dd, $J$=6.25, 3.75 Hz, $C_1$-H), 4.39 (1H, dd, $J$=15, 6 Hz, CHHPh), 4.44 (1H, dd, $J$=15, 6 Hz, CHHPh), 4.46 (1H, d, $J$=6.25 Hz, $C_2$-H or $C_3$-H), 4.47 (1H, d, $J$=6.25 Hz, $C_2$-H or $C_3$-H), 5.23 (1H, br d, $J$=6 Hz, NH), 5.70 (1H, br d, $J$=6 Hz, NH).

**(±)-1-[4'β-(*tert*-Butyldimethylsilyloxy)methyl-2'α,3'α-dimethylmethylenedioxycyclopent-1'β-yl]-3-methyl-2,4(1*H*,3*H*)-pyrimidinedione (22d) and (±)-3-[4'β-(*tert*-Butyldimethylsilyloxy)methyl-2'α,3'α-dimethylmethylenedioxycyclopent-1'β-yl]-1-methyl-2,4(1*H*,3*H*)-pyrimidinedione (23d)** A solution of 2,2-dimethyl-1,3-dioxin-4-one (**15**) (119 mg, 0.92 mmol) in dry benzene (1 ml) was added dropwise to a refluxing solution of **21d** (166 mg, 0.46 mmol) in dry benzene (10 ml) over 10 min. The solvent was evaporated off under reduced pressure and the residue was subjected to silica gel column chromatography. Elution with AcOEt–hexane (1 : 1) gave 135 mg (71%) of the product (**22d**) as an oil. High-resolution MS *m/z* Calcd for $C_{19}H_{31}N_2O_5Si$ ($M^+$ − Me): 395.2002. Found: 395.2020. IR ($CHCl_3$): 1712, 1670 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.83 (9H, s, *tert*-Bu), 1.21, 1.44 (6H, 2×s, isopropylidene-Me), 1.67—2.33 (3H, m, $C_{4'}$-H and $CH_2$), 3.23 (3H, s, NMe), 3.63 (2H, d, $J$=4 Hz, $CH_2$-O), 4.17—4.83 (3H, m, $C_{1'}$-H, $C_{2'}$-H, and $C_{3'}$-H), 5.65 (1H, d, $J$=8 Hz, $C_5$-H), 7.12 (1H, d, $J$=8 Hz, $C_6$-H). UV $\lambda_{max}^{MeCN}$ nm: 264, $\lambda_{min}^{MeCN}$ nm: 233.

Further elution with AcOEt–hexane (1 : 1) gave 22 mg (17%) of the product (**23d**) as an oil. High-resolution MS *m/z* Calcd for $C_{19}H_{31}N_2O_5Si$ ($M^+$ − Me): 395.2002. Found: 395.2018. IR ($CHCl_3$): 1715, 1660 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.87 (9H, s, *tert*-Bu), 1.25, 1.48 (6H, 2×s, isopropylidene-Me), 1.83—2.33 (3H, m, $CH_2$ and $C_{4'}$-H), 3.33 (3H, s, NMe), 3.72 (2H, t, $J$=4 Hz, $CH_2$-O), 4.51 (1H, m, $C_{2'}$-H or $C_{3'}$-H), 5.02 (1H, m, $C_{2'}$-H or $C_{3'}$-H), 5.33 (1H, m, $C_{1'}$-H), 5.66 (1H, d, $J$=8 Hz, $C_5$-H), 7.07 (1H, d, $J$=8 Hz, $C_6$-H). UV $\lambda_{max}^{MeCN}$ nm: 266, $\lambda_{min}^{MeCN}$ nm: 234.

**(±)-3-Benzyl-1-[4'β-(*tert*-butyldimethylsilyloxy)methyl-2'α,3'α-dimethylmethylenedioxycyclopent-1'β-yl]-2,4(1*H*,3*H*)-pyrimidinedione (22e) and (±)-1-Benzyl-3-[4'β-*tert*-butyldimethylsilyloxy)methyl-2'α,3'α-dimethylmethylenedioxycyclopent-1'β-yl]-2,4(1*H*,3*H*)-pyrimidinedione (23e)** These compounds were obtained from **21e** by a procedure similar to that used for the preparation of **22d** and **23d**. **22e**: Yield, 56%, oil. High-resolution MS *m/z* Calcd for $C_{25}H_{35}N_2O_5Si$ ($M^+$ − Me): 471.2313. Found: 471.2350. IR ($CHCl_3$): 1705, 1665 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.90 (9H, s, *tert*-Bu), 1.30, 1.56 (6H, 2×s, isopropylidene-Me), 2.20 (3H, m, $CH_2$ and $C_{4'}$-H), 3.73 (2H, d, $J$=4 Hz, $CH_2$OSi), 4.63 (3H, m, $C_{1'}$-H, $C_{2'}$-H, and $C_{3'}$-H), 5.13 (2H, s, $CH_2$Ph), 5.77 (1H, d, $J$=8 Hz, $C_5$-H), 7.15 (1H, d, $J$=8 Hz, $C_6$-H), 7.33 (5H, m, Ph), UV $\lambda_{max}^{MeCN}$ nm: 265, $\lambda_{min}^{MeCN}$ nm: 234.

**23e**: Yield, 20%, oil. High-resolution MS *m/z* Calcd for $C_{25}H_{35}N_2O_5Si$ ($M^+$ − Me): 471.2313. Found: 471.2320. IR ($CHCl_3$): 1705, 1665 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.90 (9H, s, *tert*-Bu), 1.27, 1.50 (6H, 2×s, isopropylidene-Me), 2.20 (3H, m, $CH_2$ and $C_{4'}$-H), 3.73 (2H, d, $J$=4 Hz, $CH_2$OSi), 4.57 (1H, br t, $J$=7 Hz, $C_{2'}$-H or $C_{3'}$-H), 4.87 (2H, s, $CH_2$Ph), 5.07 (1H, dd, $J$=7, 4 Hz, $C_{2'}$-H or $C_{3'}$-H), 5.40 (1H, m, $C_{1'}$-H), 5.67 (1H, d, $J$=8 Hz, $C_5$-H), 7.07 (1H, d, $J$=8 Hz, $C_6$-H), 7.33 (5H, m, Ph). UV $\lambda_{max}^{MeCN}$ nm: 265, $\lambda_{min}^{MeCN}$ nm: 235.

**(±)-3-Benzyl-1-(2'α,3'α-dihydroxy-4'β-hydroxymethylcyclopent-1'β-yl)-2,4-(1*H*,3*H*)-pyrimidinedione (24e)** A solution of **22e** (134 mg, 0.28 mmol) in TFA (1.5 ml) was stirred at room temperature for 15 min. The solvent was evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with hexane–AcOEt (2 : 1) gave 71 mg (77%) of the product (**24e**) as an oil. High-resolution MS *m/z* Calcd for $C_{17}H_{20}N_2O_5$ ($M^+$): 332.1371. Found: 332.1331. IR ($CHCl_3$): 3410, 1710, 1660 $cm^{-1}$. $^1$H-NMR ($CDCl_3$–$CD_3OD$) $\delta$: 1.20—2.30 (3H, m, $CH_2$ and $C_{4'}$-H), 3.60 (2H, d, $J$=4 Hz, C$\underline{H}_2$OH), 3.90 (1H, dd, $J$=6, 4 Hz, $C_{2'}$-H or $C_{3'}$-H), 4.20 (1H, br d, $J$=6 Hz, $C_{2'}$-H or $C_{3'}$-H), 4.65 (1H, br t, $J$=7 Hz, $C_{1'}$-H), 5.77 (1H, d, $J$=8 Hz, $C_5$-H), 7.30 (5H, s, Ph), 7.44 (1H, d, $J$=8 Hz, $C_6$-H). UV $\lambda_{max}^{MeCN}$ nm: 268, $\lambda_{min}^{MeCN}$ nm: 233.

**2-Azabicyclo[2.2.1]heptan-3-one (25)** This compound was prepared by catalytic hydrogenation of **1** by a procedure similar to that used for the

preparation of **12d** and used for subsequent reactions without further purification.

**2-(*N*-Chloroacetylcarbamoyl)-2-azabicyclo[2.2.1]heptan-3-one (12b)** Chloroacetyl isocyanate (655 mg, 5.5 mmol) was added to a solution of **25** (555 mg of the crude product obtained from **1**, 5 mmol) in dry benzene (10 ml). The mixture was then stirred at room temperature for 3 h. The solvent was evaporated off under reduced pressure, and the residue was recrystallized from ether to give 816 mg (71%) of the product (**12b**), mp 122—124 °C. *Anal.* Calcd for $C_9H_{11}ClN_2O_3$: C, 46.84; H, 4.81; Cl, 15.38; N, 12.14. Found: C, 46.92; H, 4.79; Cl, 15.42; N, 12.33. IR ($CHCl_3$): 3210, 1735, 1718, 1700 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.40—2.33 (6H, m, 3 × $CH_2$), 3.05 (1H, m, $C_4$-H), 4.50 (2H, s, $CH_2Cl$), 4.86 (1H, m, $C_1$-H), 10.20 (1H, br s, NH).

**2-Carbamoyl-2-azabicyclo[2.2.1]heptan-3-one (12c)** Thiourea (72 mg, 0.93 mmol) was added to a solution of **12b** (86 mg, 0.38 mmol) in dry THF (4 ml). The mixture was then stirred at room temperature for 4 h. The solvent was evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with hexane–AcOEt (1 : 1) gave 48 mg (84%) of the product (**12c**), mp 124—125 °C. *Anal.* Calcd for $C_7H_{10}N_2O_2$: C, 54.53; H, 6.54; N, 18.17. Found: C, 54.14; H, 6.77; N, 18.13. IR ($CHCl_3$): 3520, 3410, 3350, 1725, 1695 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.33—2.33 (6H, m, 3 × $CH_2$), 2.97 (1H, m, $C_4$-H), 4.80 (1H, m, $C_1$-H), 6.10 (1H, br s, NH), 7.67 (1H, br s, NH).

***cis*-3-Hydroxymethyl-1-ureidocyclopentane (13c)** a) Sodium borohydride (370 mg, 10 mmol) was added under stirring to an ice-cooled solution of **12c** (154 mg, 1 mmol) in MeOH (10 ml). The mixture was stirred at room temperature for 30 min, and neutralized with AcOH–MeOH (1 : 10). The solvent was evaporated off under reduced pressure, and AcOEt was added to the residue. After removal of the precipitate by filtration, the filtrate was evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with MeOH–AcOEt (1 : 10) gave 134 mg (85%) of the product (**13c**), mp 138—139 °C. High-resolution MS *m/z* Calcd for $C_7H_{14}N_2O_2$ ($M^+$): 158.1054. Found: 158.1075. IR (Nujol): 1640 $cm^{-1}$. $^1$H-NMR ($CDCl_3$–$CD_3OD$) $\delta$: 1.00—2.50 (7H, m, $C_3$-H and 3 × $CH_2$), 3.57 (2H, d, $J$=6 Hz, C$\underline{H}_2$OH), 4.10 (1H, m, $C_1$-H).

b) By the same procedure as described above, **12b** (460 mg, 2 mmol) afforded 193 mg (61%) of **13c**.

***cis*-3-(*tert*-Butyldimethylsilyloxy)methyl-1-ureidocyclopentane (26)** A solution of **13c** (100 mg, 0.63 mmol), TBDSCl (190 mg, 1.28 mmol), and imidazole (218 mmol, 3.15 mmol) in DMF (87 ml) was kept at room temperature for 12 h. The reaction mixture was poured into ice-water, and extracted with ether. The extract was dried over anhydrous magnesium sulfate, and the solvent was evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with AcOEt gave 138 mg (81%) of the product (**26**), mp 102—104 °C. High-resolution MS *m/z* Calcd for $C_{12}H_{25}N_2O_2Si$ ($M^+$): 257.1685. Found: 257.1692. IR ($CHCl_3$): 3530, 3430, 3375, 1670 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.83 (9H, s, *tert*-Bu), 1.00—2.36 (7H, m, 3 × $CH_2$ and $C_3$-H), 3.50 (2H, d, $J$=5 Hz, $CH_2O$), 3.93 (1H, m, $C_1$-H), 4.61 (2H, br s, $NH_2$), 5.26 (1H, br s, NH).

***cis*-3-(*tert*-Butyldimethylsilyloxy)methyl-1-[*N'*-(3-ethoxyacryloyl)-ureido]cyclopentane (29)** 3-Ethoxyacryloyl chloride (370 mg, 2.76 mmol) was added to a solution of **26** (500 mg, 1.84 mmol) and pyridine (291 mg, 3.68 mmol) in dry $CH_2Cl_2$ (8 ml). The mixture was then stirred at room temperature for 12 h. The solvent was evaporated off under reduced pressure, and the residue was subjected to silica gel column chromatography. Elution with hexane–AcOEt (5 : 1) gave 526 mg (78%) of the product (**29**) as an oil. High-resolution MS *m/z* Calcd for $C_{17}H_{31}N_2O_4Si$ ($M^+$): 355.2053. Found: 355.2074. IR ($CHCl_3$): 3450, 3310, 3240, 1700, 1678 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.83 (9H, s, *tert*-Bu), 1.10—2.40 (7H, m, 3 × $CH_2$ and $C_3$-H), 1.26 (3H, t, $J$=6 Hz, $CH_2\underline{Me}$), 3.45 (2H, d, $J$=6 Hz, $CH_2OSi$), 3.88 (1H, q, $J$=6 Hz, $OC\underline{H}_2Me$), 4.03 (1H, m, $C_1$-H), 5.32 (1H, d, $J$=10 Hz, acryloyl $C_2$-H), 7.50 (1H, d, $J$=10 Hz, acryloyl $C_3$-H), 8.61 (1H, br d, $J$=7 Hz, NH), 10.00 (1H, br s, NH).

**(±)-1-[3'β-Hydroxymethylcyclopent-1'β-yl]-2,4(1*H*,3*H*)-pyrimidinedione (30)** A solution of **29** (370 mg, 1 mmol) in 2 N sulfuric acid (12 ml) was heated under reflux for 1.5 h, cooled, and neutralized with aqueous sodium hydroxide. The aqueous solution was saturated with sodium chloride and extracted with 10% MeOH–AcOEt. The extract was dried over anhydrous magnesium sulfate, and the solvent was evaporated off under reduced pressure. The crystalline residue was recrystallized from MeOH–AcOEt to give 183 mg (87%) of the product (**30**), mp 138—140 °C (MeOH–AcOEt). High-resolution MS *m/z* Calcd for $C_{10}H_{14}N_2O_3$ ($M^+$): 210.1004. Found: 210.1032. IR (Nujol): 3375, 1712 $cm^{-1}$. $^1$H-NMR ($CDCl_3$–$CD_3OD$) $\delta$: 1.13—2.50 (7H, m, 3 × $CH_2$ and $C_3$-H), 3.55 (2H, d, $J$=6 Hz, $CH_2O$), 4.88 (1H, m, $C_1$-H), 5.68 (1H, d, $J$=8 Hz, $C_5$-H), 7.37 (1H, d, $J$=8 Hz, $C_6$-H). UV $\lambda_{max}^{MeCN}$ nm: 268, $\lambda_{min}^{MeCN}$ nm: 232.

**(±)-5,6-*exo*-Dimethylmethylenedioxy-2-azabicyclo[2.2.1]heptan-3-one (31)** A solution of 5,6-*exo*-dihydroxy-2-azabicyclo[2.2.1]heptan-3-one (1.43 g, 10 mmol), *p*-toluenesulfonic acid monohydrate (95 mg, 0.5 mmol), and 2,2-dimethoxypropane (2 ml) in acetone (5 ml) was allowed to stand at room temperature for 3 h. The mixture was then neutralized with sodium bicarbonate (42 mg, 0.5 mmol), and concentrated *in vacuo*. The residue was extracted with chloroform. The extract was concentrated *in vacuo* to give a crystalline residue, which was recrystallized from hexane–ethyl acetate to give the acetonide (**31**), mp 152—153 °C. *Anal.* Calcd for $C_9H_{13}NO_3$: C, 59.00; H, 7.15; N, 7.65. Found: C, 58.92; H, 7.20; N, 7.60. IR ($CHCl_3$): 3360, 1720 $cm^{-1}$.

**2-(*N*-Chloroacetylcarbamoyl)-5,6-*exo*-dimethylmethylenedioxy-2-azabicyclo[2.2.1]heptan-3-one (32)** Following the procedure given for the preparation of **12b**, chloroacetyl isocyanate (1.05 g, 8.79 mmol) and 5,6-*exo*-dihydroxy-di-*O*-isopropylidene-2-azabicyclo[2.2.1]heptan-3-one (**31**) afforded 1.69 g (95%) of **32**, mp 158—159 °C. *Anal.* Calcd for $C_{12}H_{15}ClN_2O_5$: C, 47.59; H, 5.00; Cl, 11.72; N, 9.27. Found: C, 47.30; H, 4.99; Cl, 11.92; N, 9.09. IR ($CHCl_3$): 3250, 1740, 1720 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.37, 1.50 (6H, 2 × s, isopropylidene-Me), 2.13 (2H, m, $CH_2$), 3.07 (1H, m, $C_4$-H), 4.43 (2H, s, $CH_2Cl$), 6.67 (1H, m, $C_5$-H), 7.00 (1H, dd, $J$=6, 2 Hz, $C_6$-H), 11.70 (1H, br s, NH).

**(±)-4β-Hydroxymethyl-2α,3α-dimethylmethylenedioxy-1β-ureidocyclopentane (33)** Sodium borohydride (390 mg, 10 mmol) was added to a stirred, ice-cooled solution of **32** (624 mg, 2.06 mmol) in MeOH (20 ml). The mixture was stirred at room temperature for 3 h, and neutralized with AcOH–MeOH (1 : 10). The solvent was evaporated off under reduced pressure, and AcOEt was added to he residue. After removal of the precipitate by filtration, the filtrate was evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with MeOH–AcOEt (1 : 10) gave 419 mg (88%) of the product (**33**) as an oil. High-resolution MS *m/z* Calcd for $C_{10}H_{19}N_2O_4$ ($M^+$ + H): 231.1344. Found: 231.1361. IR ($CHCl_3$): 3600, 3520, 3460, 3355, 1670 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.30 (1H, m, CH-H), 1.30, 1.50 (6H, 2 × s, isopropylidene-Me), 2.38 (2H, m, $C_4$-H and CH-H), 3.67 (2H, d, $J$=4 Hz, C$\underline{H}_2$OH), 3.80 (1H, m, $C_1$-H), 4.37 (1H, dd, $J$=6, 3 Hz, $C_2$-H or $C_3$-H), 4.55 (1H, dd, $J$=6, 3 Hz, $C_2$-H or $C_3$-H), 6.70 (4H, br, NH, $NH_2$, and OH).

**(±)-4β-(*tert*-Butyldimethylsilyloxy)methyl-2α,3α-dimethylmethylenedioxy-1β-ureidocyclopentane (34)** A solution of **33** (367 mg, 1.60 mmol), TBDSCl (577 mg, 3.84 mmol), and imidazole (434 mg, 6.40 mmol) in DMF (16 ml) was kept at room temperature for 12 h. The reaction mixture was poured into ice-water, and extracted with ether. The extract was dried over anhydrous magnesium sulfate, and the solvent was evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with AcOEt gave 399 mg (72%) of the product (**34**) as an oil. High-resolution MS *m/z* Calcd for $C_{15}H_{29}N_2O_4Si$ ($M^+$ − Me): 329.1895. Found: 329.1913. IR ($CHCl_3$): 1670 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.90 (9H, s, *tert*-Bu), 1.13 (1H, m, CH-H), 1.30, 1.47 (6H, 2 × s, isopropylidene-Me), 2.22 (2H, m, $C_4$-H and CH-H), 3.67 (2H, d, $J$=4 Hz, $CH_2OSi$), 3.83 (1H, m, $C_1$-H), 4.26 (1H, dd, $J$=6, 3 Hz, $C_2$-H or $C_3$-H), 4.47 (1H, dd, $J$=6, 3 Hz, $C_2$-H or $C_3$-H), 5.20 (2H, br s, $NH_2$), 5.62 (1H, d, $J$=6 Hz, NH).

**(±)-4β-(*tert*-Butyldimethylsilyloxy)methyl-2α,3α-dimethylmethylenedioxy-1β-[*N'*-(3-methoxymethacryloyl)-ureido]cyclopentane (35)** 3-Methoxymethacryloyl chloride (190 mg, 1.41 mmol) was added to a solution of **34** (323 mg, 0.94 mmol) and pyridine (223 mg, 2.82 mmol) in dry $CH_2Cl_2$ (10 ml), and the mixture was stirred at room temperature for 12 h. The solvent was then evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with AcOEt gave 266 mg (64%) of the product (**35**) as an oil. High-resolution MS *m/z* Calcd for $C_{20}H_{35}N_2O_6Si$ ($M^+$ − Me): 427.2262. Found: 427.2250. IR ($CHCl_3$): 3530, 3470, 3300, 1700, 1660 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.86 (9H, s, *tert*-Bu), 1.23, 1.43 (6H, 2 × s, isopropylidene-Me), 1.43 (1H, m, CH-H), 1.73 (3H, d, $J$=1 Hz, Me), 2.20 (2H, m, $C_4$-H and CH-H), 3.62 (2H, d, $J$=5 Hz, $CH_2OSi$), 3.83 (3H, s, OMe), 4.17 (1H, m, $C_1$-H), 4.38 (1H, d, $J$=7 Hz, $C_2$-H or $C_3$-H), 4.48 (1H, d, $J$=7 Hz, $C_2$-H or $C_3$-H), 7.35 (1H, d, $J$=1 Hz, $C_5$-H), 8.93 (2H, br d, $J$=6 Hz, NH), 9.00 (1H, br s, NH).

**(±)-1-[4'β-(*tert*-Butyldimethylsilyloxy)methyl-2'α,3'α-dimethylmethylenedioxycyclopent-1'β-yl]-5-methyl-2,4-(1*H*,3*H*)-pyrimidinedione (36)** A solution of **35** (98 mg, 0.22 mmol) in a mixture of MeOH (5 ml) and

concentrated $NH_4OH$ (2 ml) was heated at 50 °C in a sealed tube for 12 h. The solvent was evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography, and elution with AcOEt gave 42 mg (46%) of the product (**36**) as an oil. High-resolution MS *m/z* Calcd for $C_{19}H_{31}N_2O_5Si$ ($M^+ - Me$): 395.2000. Found: 395.1984. IR ($CHCl_3$): 3325, 1710, 1695, 1660 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.83 (9H, s, *tert*-Bu), 1.22, 1.45 (6H, 2 × s, isopropylidene-Me), 1.68 (1H, m, CH-H), 1.85 (3H, d, $J$=1 Hz, Me), 2.15 (2H, m, $C_{4'}$-H and CH-H), 3.65 (2H, d, $J$=4 Hz, $CH_2OSi$), 4.47 (1H, m, $C_{1'}$-H), 4.50 (1H, dd, $J$=7, 2 Hz, $C_{2'}$-H or $C_{3'}$-H), 4.61 (1H, dd, $J$=7, 2 Hz, $C_{2'}$-H or $C_{3'}$-H), 6.93 (1H, d, $J$=1 Hz, $C_6$-H), 8.77 (1H, br s, NH). UV $\lambda_{max}^{MeCN}$ nm: 268, $\lambda_{min}^{MeCN}$ nm: 235.

**5,6-*exo*-Dimethylmethylenedioxy-2-(3-methoxymethacryloyl)carbamoyl-2-azabicyclo[2.2.1]heptan-3-one (38)** a) Preparation of 3-Methoxymethacryloyl Isocyanate: A suspension of 3-methoxymethacryloyl chloride (2.17 g, 16.1 mmol) and silver cyanide (2.42 g, 16.1 mmol) in dry benzene (54 ml) was refluxed under an argon atmosphere for 1 h. The isocyanate thus prepared was used for the subsequent reaction.

b) Compound **31** (1.97 g, 10.8 mmol) was added to the above solution. The mixture was then stirred at 50 °C for 3 h. After removal of the precipitate by filtration, the filtrate was evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography, and elution with hexane–AcOEt (1 : 2) gave 2.01 g (58%) of the product (**38**), mp 184—185 °C ($CH_2Cl_2$–hexane). *Anal.* Calcd for $C_{15}H_{20}N_2O_6$: C, 55.53; H, 6.22; N, 8.64. Found: C, 55.61; H, 6.17; N, 8.71. IR ($CHCl_3$): 1762, 1730, 1690 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.35, 1.50 (6H, 2 × s, isopropylidene-Me), 1.80 (3H, d, $J$=1 Hz, C-Me), 2.12 (2H, m, $CH_2$), 2.98 (1H, m, $C_4$-H), 3.87 (3H, s, O-Me), 4.58 (2H, s, $C_5$-H and $C_6$-H), 4.80 (1H, m, $C_1$-H), 7.30 (1H, d, $J$=1 Hz, acryloyl $C_3$-H), 11.60 (1H, br s, NH).

**(±)-2α,3α-Dimethylmethylenedioxy-4β-hydroxymethyl-1β-[*N'*-(3-methoxymethacryloyl)ureido]cyclopentane (39)** Sodium borohydride (340 mg, 9 mmol) was added to a stirred, ice-cooled solution of **38** (583 mg, 1.8 mmol) in MeOH (20 ml). The mixture was stirred at room temperature for 1 h, and neutralized with AcOH–MeOH (1 : 10). The solvent was evaporated off under reduced pressure, and water was added to the residue. The mixture was extracted with AcOEt. The extract was dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with AcOEt gave 504 mg (85%) of the product (**39**) as an oil. High-resolution MS *m/z* Calcd for $C_{15}H_{24}N_2O_6$ ($M^+$): 328.1633. Found: 328.1658. IR ($CHCl_3$): 3640, 3350, 1690, 1660 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.30, 1.48 (6H, 2 × s, isopropylidene-Me), 1.38 (1H, m, CH-H), 1.78 (3H, d, $J$=1 Hz, C-Me), 2.42 (3H, m, $C_4$-H, CH-H, and OH), 3.73 (2H, d, $J$=5 Hz, $CH_2OH$), 3.90 (3H, s, O-Me), 4.27 (1H, m, $C_1$-H), 4.49 (1H, d, $J$=7 Hz, $C_2$-H or $C_3$-H), 4.61 (2H, d, $J$=7 Hz, $C_2$-H or $C_3$-H), 7.40 (1H, d, $J$=1 Hz, acryloyl $C_3$-H), 8.98 (1H, br s, NH), 9.11 (1H, br d, $J$=7 Hz, NH).

**(±)-1-[2'α,3'α-Dimethylmethylenedioxy-4'β-hydroxymethylcyclopent-1'β-yl]-5-methyl-2,4(1*H*,3*H*)-pyrimidinedione (40)** A solution of **39** (449 mg, 1.37 mmol) in a mixture of MeOH (30 ml) and concentrated $NH_4OH$ (12 ml) was heated at 80 °C in a sealed tube for 17 h. The solvent was evaporated off under reduced pressure. The residue was subjected to silica gel column chromatography. Elution with MeOH–AcOEt (1 : 10) gave 330 mg (82%) of the product (**40**) as an oil. High-resolution MS *m/z* Calcd for $C_{14}H_{20}N_2O_5$ ($M^+$): 296.1371. Found: 296.1409. IR ($CHCl_3$): 3305, 1700, 1685 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.33, 1.53 (6H, 3 × s, isopropylidene-Me), 1.33 (1H, m, CH-H), 1.90 (3H, d, $J$=1 Hz, Me), 2.22 (3H, m, $C_{4'}$-H, CH-H and OH), 3.73 (2H, br t, $J$=4 Hz, $CH_2OH$), 4.60 (3H, m, $C_{1'}$-H, $C_{2'}$-H, and $C_{3'}$-H). UV $\lambda_{max}^{water}$ nm: 271, $\lambda_{min}^{water}$ nm: 236.

**(±)-1-(2'α,3'α-Dihydroxy-4'β-hydroxymethylcyclopent-1'-yl)-5-methyl-2,4(1*H*,3*H*)-pyrimidinedione (37)** A solution of **40** (281 mg, 0.95 mmol) in TFA (5 ml) was stirred at room temperature for 15 min. The solvent was evaporated off under reduced pressure. The residue was recrystallized from MeOH–AcOEt to give 187 mg (77%) of the product (**37**), mp 219—220 °C (lit.[30] mp 212—216 °C). UV $\lambda_{max}^{MeCN}$ nm: 265, $\lambda_{min}^{MeCN}$ nm: 235.

**(±)-Carbocyclic Ribofuranosylamine (2) and Its Tetraacetate (41)** Sodium nitrate (110 mg, 1.6 mmol) was added under ice-cooling to a solution of **33** (184 mg, 0.8 mmol) in 10% hydrochloric acid and the whole was stirred at 0 °C for 90 min. After neutralization by adding sodium bicarbonate, the solvent was evaporated off under reduced pressure. The residue was dissolved in acetic anhydride (10 ml) containing pyridine (1 ml) and the whole was stirred at room temperature for 2 d. After addition of 5 ml of water to the residue obtained after evaporation, the product was taken up ethyl acetate and dried over $MgSO_4$. The crude product thus obtained was chromatographed over silica gel. Elution with ethyl acetate afforded 161 mg (64%) of the tetraacetate (**41**), mp 94—95 °C ($CH_2Cl_2$) (lit.[8] mp 94—95 °C). High-resolution MS *m/z* Calcd for $C_{14}H_{22}NO_7$ ($M^+ + H$): 316.1395. Found: 316.1400. IR ($CHCl_3$): 3450, 1740, 1675 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.20 (1H, m, $C_5$-H), 1.93 (3H, s, NAc), 2.02 (9H, s, OAc × 3), 2.23—2.93 (2H, m, $C_4$-H and $C_5$-H), 3.87—4.67 (1H, m, $C_1$-H), 4.04 (2H, d, $J$=5 Hz, $CH_2OAc$), 4.83—5.20 (2H, m, $C_2$-H and $C_3$-H), 6.29 (1H, br d, $J$=7 Hz, NH).

***cis*-4-Aminocyclopent-2-enemethanol (42) and Its Mono- (43) and Diacetates (44)** In the same manner as described above, 203 mg (1.3 mmol) of **11c** afforded 96 mg (65%) of **42** as an oil. In order to confirm the structure of **42**, it was acetylated under mild conditions. Thus, a solution of 23 mg (0.2 mmol) of the amine (**42**) in acetic anhydride (1 ml) containing pyridine (0.5 ml) was stirred at room temperature for 3 h. The residue after evaporation of the solvent was chromatographed over silica gel. Elution with ethyl acetate afforded first the diacetate (**44**) and then the monoacetate (**43**). **42**: Oil. High-resolution MS *m/z* Calcd for $C_6H_{11}NO$ ($M^+$): 113.0840. Found: 113.0841. IR ($CHCl_3$): 3380—3150 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.28 (1H, dt, $J$=13, 3 Hz, $C_5$-H), 1.81—3.21 (2H, m, $C_1$- and $C_5$-H), 2.94 (3H, br, OH and $NH_2$), 3.53 (2H, d, $J$=4 Hz, $CH_2O$), 3.92 (1H, dd, $J$=8, 3 Hz, $C_4$-H), 5.73 (1H, d, $J$=6 Hz, $C_2$-H), 5.83 (1H, d, $J$=6 Hz, $C_3$-H). **44**: Yield, 7 mg (18%), oil. High-resolution MS *m/z* Calcd for $C_{10}H_{15}NO_3$ ($M^+$): 197.1051. Found: 197.1029. IR ($CHCl_3$): 3450, 1740, 1665 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.18 (1H, m, $C_5$-H), 1.90 (3H, s, NAc), 2.00 (3H, s, OAc), 2.15—3.15 (2H, m, $C_1$-H and $C_5$-H), 3.88—4.12 (2H, m, $CH_2OAc$), 4.45—5.15 (1H, m, $C_4$-H), 5.22—5.65 (1H, br, NH), 5.74 (2H, s, $C_2$-H and $C_3$-H); **43**: Yield, 25 mg (80%), oil. High-resolution MS *m/z* Calcd for $C_8H_{13}NO_2$ ($M^+$): 155.0946. Found: 155.0945. IR ($CHCl_3$): 3450, 3400, 1660 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.10—1.60 (1H, m, $C_5$-H), 1.87 (3H, s, Me), 1.97—3.10 (2H, m, $C_1$-H and $C_5$-H), 2.15 (1H, br s, OH), 3.53 (2H, d, $J$=4 Hz, $CH_2OH$), 4.60—5.10 (1H, m, $C_4$-H), 5.70 (2H, s, $C_2$-H and $C_3$-H), 5.87—6.30 (1H, br, NH).

**Acknowledgement** This work was supported in part by a Grant-in-Aid for Cancer Research (No. 02152013) from the Ministry of Education, Science and Culture, Japan.

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# (−)-Camoensidine *N*-Oxide; A New Alkaloid from *Maackia tashiroi*[1)]

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**A new alkaloid (3) was isolated from the stems of *Maackia tashiroi* (Leguminosae), together with (−)-camoensidine, tashiromine, ammodendrine and six known lupin (quinolizidine) alkaloids. The structure of 3 was characterized as the $N_{15}$-oxide of (−)-camoensidine, possessing an indolizidine-quinolizidine ring system, by a combination of spectroscopic and chemical methods.**

**Keywords** *Maackia tashiroi*; Leguminosae; lupin alkaloid; quinolizidine alkaloid; quinolizidine-indolizidine alkaloid; (−)-camoensidine; (−)-camoensidine *N*-oxide; (−)-cytisine; (+)-epilupinine; tashiromine

In the course of our studies on lupin alkaloids in leguminous plants native to Japan, we have recently reported the isolation and structural determination of the new alkaloid named tashiromine from the stems of *Maackia tashiroi* (Leguminosae).[2)] Tashiromine **1** possesses an indolizidine ring system as a structural unit and is similar to the typical lupin (quinolizidine) alkaloid epilupinine (**2**) coexisting in the same plant. Further examination of alkaloid constituents in the plant led to the isolation of a new alkaloid (**3**), together with (−)-camoensidine (**4**), tashiromine (**1**), ammodendrine and six known lupin alkaloids. In this paper we wish to report the structural elucidation of **3** as the $N_{15}$-oxide of an indolizidine-quinolizidine alkaloid, (−)-camoensidine (**4**).

The basic fraction (5.9 g) obtained from the 75% MeOH extract of the dry stems (1.2 kg) of *M. tashiroi*, collected in Kumamoto prefecture in August, was subjected to repeated column chromatography on silica gel to yield the new alkaloid (**3**), together with the known alkaloids, (−)-camoensidine (**4**), (−)-cytisine, (−)-*N*-methylcytisine, (−)-rhombifoline, (−)-*N*-formylcytisine, (−)-lusitanine, (+)-epilupinine, tashiromine and ammodendrine, which were identified by direct comparison with authentic samples except for **4**.

The new alkaloid (**3**) was obtained as hygroscopic colorless crystals, $[\alpha]_D^{27}$ −57° ($c$=0.79, EtOH). The molecular formula, $C_{14}H_{22}N_2O_2$, was determined from the $M^+$ ion peak at $m/z$ 250 in the in-beam mass spectrum (MS), the highest mass peak at $m/z$ 234.1729 ($C_{14}H_{22}N_2O$) in the high-resolution electron impact (HR-EI) MS, and the carbon-13 nuclear magnetic resonance ($^{13}$C-NMR) spectrum (Table I), showing the presence of 14 carbons and 22 hydrogens. The EI-MS of **3** showed fragment peaks at $m/z$ 234 ($M^+$ −O), 233 ($M^+$ −OH), and 232 ($M^+$ −$H_2O$), characteristic of aliphatic amine *N*-oxides.[3)] Catalytic hydrogenation of **3** with Pd–C in MeOH gave the free base, which was identical with (−)-camoensidine (**4**), an oil, $[\alpha]_D^{27}$ −73° ($c$=1.13, EtOH), isolated from the same source. Treatment of **4** with $H_2O_2$ regenerated **3**. Accordingly, the

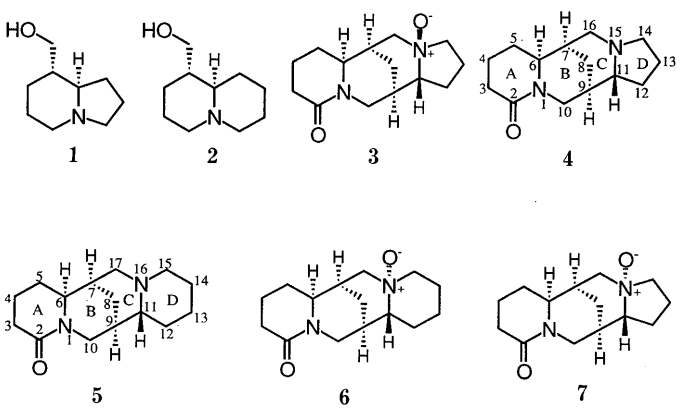



Chart 1

TABLE I. $^{13}$C-NMR Data for **3**—**7** ($\delta$ ppm)

| C | 3 | 4 | 5[6)] | 6[6)] | 7 |
|---|---|---|---|---|---|
| 2 | 170.1 | 171.0 | 171.3 | 172.1 | 172.1 |
| 3 | 32.8 | 32.9 | 33.1 | 33.0 | 33.0 |
| 4 | 20.0 | 19.8 | 19.7 | 19.4 | 19.3 |
| 5 | 27.6 | 28.4 | 26.8 | 27.7 | 27.7 |
| 6 | 58.6 | 59.8 | 60.9 | 61.8 | 61.7 |
| 7 | 32.6 | 33.1 | 35.0 | 33.6 | 31.7 |
| 8 | 25.1 | 27.3 | 27.4 | 22.7 | 26.5 |
| 9 | 30.8 | 30.9 | 32.5 | 31.7 | 30.9 |
| 10 | 45.4 | 47.4 | 46.8 | 47.0 | 47.4 |
| 11 | 78.9 | 64.2 | 64.0 | 71.4 | 72.7 |
| 12 | 27.6 | 27.4 | 33.6 | 27.7 | 26.4 |
| 13 | 20.9 | 20.8 | 24.5 | 25.7 | 20.3 |
| (14)[a)] | | | 25.3 | 20.3 | |
| 14 (15)[a)] | 72.2 | 54.2 | 55.4 | 69.6 | 69.6 |
| 16 (17)[a)] | 61.7 | 49.1 | 52.9 | 65.2 | 61.7 |

Spectra were run in $CDCl_3$. *a*) Numbers in brackets refer to the extra carbon in the D-ring of **5** and **6**.

TABLE II. $^1$H-NMR Data for **3**, **4** and **7** ($\delta$ ppm, $J$=Hz)

| H | 3 | 4 | 7 |
|---|---|---|---|
| 6 | 3.54, ddd, $J$=11.5, 3.2, 3.2 | 3.39, ddd, $J$=10.3, 4.9, 2.5 | 3.43, ddd, $J$=12.0, 4.8, 2.0 |
| 8α | 1.78, m | 1.46, dm, $J$=12.4 | 1.38, dm, $J$=12.5 |
| 8β | 2.02, m | 2.08, dddd, $J$=12.4, 3.9, 3.9, 2.4 | 3.90, dddd, $J$=12.5, 2.5, 2.5, 3.0 |
| 10α | 2.85, dd, $J$=14.0, 3.7 | 2.67, dd, $J$=13.2, 2.7 | 2.60. dm, $J$=13.0 |
| 10β | 4.87, d like, $J$=14.0 | 4.63, ddd, $J$=13.2, 2.4, 2.4 | 4.54, ddd, $J$=13.0, 3.0, 3.0 |
| 11 | 3.87, dd, $J$=9.3, 9.3 | 2.47, m | 2.65, m |
| 14α | 3.66, m | 2.40, dm, $J$=9.1 | 3.63, ddm, $J$=9.0, 9.0 |
| 14β | 3.66, m | 2.85, ddm, $J$=9.1, 9.1 | 3.14, ddd, $J$=9.0, 9.0, 9.0 |
| 16α | 3.43, dd, $J$=13.4, 4.6 | 2.80, dd, $J$=11.9, 7.0 | 3.90, dd, $J$=12.5, 10.5 |
| 16β | 3.78, dm, $J$=13.4 | 2.56, dm, $J$=11.9 | 2.98, dd, $J$=12.5, 2.5 |

Spectra were run in $CDCl_3$.

new base **3** was presumed to be an *N*-oxide of **4**.

The molecular formula of **4**, $C_{14}H_{22}N_2O$, determined from the HR-EI-MS, is one methylene unit less than that of a typical lupin alkaloid lupanine (**5**). The proton nuclear magnetic resonance ($^1$H-NMR) spectrum of **4** showed signals at $\delta$ 4.63 (1H, ddd, $J$=13.2, 2.4 and 2.4 Hz, 10$\beta$-H), 2.67 (1H, dd, $J$=13.2 and 2.7 Hz, 10$\alpha$-H) and 3.39 (1H, ddd, $J$=10.3, 4.9 and 2.5 Hz, 6-H), which correspond to the signals due to the C-10 methylene and the C-6 methine protons in the 2-quinolizidone moiety of lupanine-type lupin alkaloids.[4-6] The $^{13}$C-NMR spectrum of **4** resembles that of **5**, especially the signals due to 2-C through 11-C (Table I). The base peak at *m/z* 122 and significant peaks at *m/z* 135 and 136 in the EI-MS of **4** were 14 mass units less than those (*m/z* 136, 149 and 150, respectively) of **5** which arise from the C/D ring.[7] These results indicate that 4 might be a homolog of lupanine (**5**), the D-ring of which is a pyrrolidine ring. The new alkaloid (**3**) was, therefore, presumed to be an $N_{15}$-oxide of **4**.

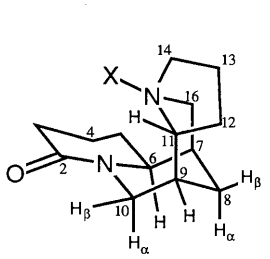
The relative stereochemistry at C-6, C-7, C-9 and C-11 of **4**, which is the same as that of **3**, was confirmed by measurements of the difference nuclear Overhauser effect (NOE) spectra of **3** and **4**. The $^1$H- and $^{13}$C-NMR spectra of **3** and **4** were assigned by means of $^1$H–$^1$H and $^1$H–$^{13}$C correlation spectroscopy (COSY), taking into account the assignments of lupanine (**5**)[5] and its *N*-oxide (**6**)[6] (Tables I and II). In the NOE spectrum of **4**, irradiation of the signal due to 6-H ($\delta$ 3.39) resulted in enhancements of the signals of 8$\alpha$-H ($\delta$ 1.46) and 10$\alpha$-H ($\delta$ 2.67), indicating a *trans* ring junction of the A/B ring (2-quinolizidone moiety) system, namely the $\alpha$-configuration of 6-H, 7-H and 9-H of **4** (and **3**), as illustrated in **4a** or **4b** (and **3a** or **7a**) (Chart 2). The $\beta$-configuration of 11-H in **3** (and **4**) was revealed by the NOE experiment on **3**. Irradiation of the signal due to 10$\beta$-H ($\delta$ 4.87) induced enhancements of the signals corresponding to 11-H ($\delta$ 3.87) and 10$\alpha$-H ($\delta$ 2.85) (Chart 2). The above results indicate that **4** has the same relative stereochemistry as that of lupanine (**5**) (Chart 1) and hence **4** was characterized as (−)-camoensidine,[8] isolated from *Camoensia maxima*[9] and *C. brevicalyx*[10] (Leguminosae). Therefore, it was concluded that the new alkaloid (**3**) was an $N_{15}$-oxide of (−)-camoensidine (**4**).

Two diastereomers (**3** and **7**) are possible for camoensidine *N*-oxide, taking into account the configuration of the *N*-oxide nitrogen: one (**3a**) involving a *cis*-indolizidine (C/D ring) with a chair piperidine ring (C-ring) and the other (**7a**) a *trans* indolizidine with a boat piperidine ring.

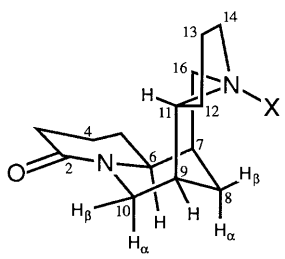
In fact, the two *N*-oxides (**3** and **7**) were obtained in 60% and 26% yields, respectively, on oxidation of **4** with *m*-chloroperbenzoic acid (MCPBA) in $CH_2Cl_2$, though oxidation of **4** with $H_2O_2$ gave only the natural *N*-oxide (**3**), as described above. The major product was identical with the natural *N*-oxide (**3**). The minor one (**7**), mp 111—113 °C, $[\alpha]_D^{27}$ −76°, was assigned as the other *N*-oxide of **4** from the $M^+$ peak at *m/z* 250.1683 and the similarity of the EI-MS of **7** to that of **3**.

The structural assignment of the two *N*-oxides **3** and **7** was confirmed by comparison of chemical shift differences between 8$\alpha$-H and 8$\beta$-H in the $^1$H-NMR spectra. The $^1$H-NMR assignment of **7** was determined by analysis of the $^1$H–$^1$H-COSY and $^1$H–$^{13}$C-COSY spectra (Table II). The difference (2.52 ppm) in **7** was much larger than that (*ca.* 0.24 ppm) in **3**, which was attributable to the unusual down-field shift of 8$\beta$-H ($\delta$ 3.90) in **7**. This could be explained by a through-space deshielding effect of the *N*-oxide oxygen. Accordingly, the structure of the synthetic *N*-oxide (**7**) could be assigned as **7a** in which the 8$\beta$-H is very close to the *N*-oxide oxygen. Consequently, the structure of the new alkaloid **3** was determined as **3a**, in which the 8-$H_2$ is very far from the *N*-oxide oxygen.

Further investigation on the absolute stereochemistry of (−)-camoensidine (**4**) is being undertaken in our laboratories.



**3a** : N - X = $N^+$ - $O^-$
**4a** : X = lone pair



**4b** : X = lone pair
**7a** : N - X = $N^+$ - $O^-$

Chart 2

## Experimental

Melting points were determined on a Yanagimoto micro melting apparatus and are uncorrected. The following equipments were used to obtain physical data: infrared (IR) spectra, Hitachi 215 grating infrared spectrometer; $^1$H- and $^{13}$C-NMR spectra, JEOL JNM-GX 400 ($^1$H, 399.78 MHz; $^{13}$C, 100.43 MHz) spectrometer with tetramethylsilane (TMS) as an internal standard; optical rotations, Jasco DIP-181 polarimeter; EI-MS and HR-EI-MS, JEOL D-300 spectrometer, in-beam MS; Hitachi RMU 7H spectrometer. Column chromatography was carried out with Kiesel gel 60 (70—230 mesh or 230—400 mesh, Merck). Thin-layer chromatography (TLC) was performed on 0.25 mm precoated silica gel (60 $F_{254}$, Merck), and spots were detected by exposure to $I_2$ vapor or spraying with Dragendorff's reagent. Preparative TLC was conducted with 1.0 mm precoated silica gel (Merck). Analytical high-performance liquid chromatography (HPLC) was carried out as described previously.[11]

**Plant Material** *M. tashiroi* was collected early in August in Kumamoto prefecture, Japan.

**Extraction and Isolation of Alkaloids** The dried stems (1.2 kg) of *M. tashiroi* were extracted three times with 75% MeOH at room temperature. The combined extracts were concentrated *in vacuo*, acidified with dilute HCl and filtered. The acid filtrate was extracted twice with ether, made strongly alkaline with $K_2CO_3$ under ice-cooling, and then extracted with $CH_2Cl_2$ several times. The $CH_2Cl_2$ extracts were combined, dried over anhydrous $K_2CO_3$ and evaporated to dryness to give 5.7 g (0.49%/dry material) of a crude alkaloid fraction. The alkaloid fraction was applied to a silica gel column (500 g) and eluted successively with $CH_2Cl_2$, 1%, 2%, 3%, 4%, 5%, 10%, 15% and 20% MeOH in $CHCl_3$, and MeOH, monitoring with TLC, to give seven fractions. Fraction 2 (170 mg) was purified on a silica gel column with $C_6H_6$–MeOH (20 : 1) to yield (−)-rhombifoline (61 mg, a colorless oil, $[\alpha]_D^{25}$ −175° ($c$=0.41, EtOH)). Fraction 3 (255 mg) consisted of (−)-anagyrine and (−)-*N*-methylcytisine, which were identified by gas liquid chromatography (GLC)-EI-MS and HPLC. Fraction 4 (2.15 g) was rechromatographed on a silica gel column using $CH_2Cl_2$–MeOH–28% $NH_4OH$ (90 : 9 : 1) to give (−)-*N*-formylcytisine (72 mg, mp 172 °C, $[\alpha]_D^{25}$ −230° ($c$=0.30, EtOH)) and (−)-cytisine (1.95 g, mp 155 °C, $[\alpha]_D^{25}$ −110° ($c$=0.26, EtOH)). Fraction 5 (2.4 g) was fractionated through a silica gel column with $CH_2Cl_2$–MeOH–28% $NH_4OH$ (95 : 5 : 0.5) to yield (+)-epilupinine (1.53 g, mp 77–78 °C, $[\alpha]_D^{25}$ +33° ($c$=0.50, EtOH), (−)-cytisine (0.75 g), ammodendrine (10 mg, a colorless oil), baptifoline (6 mg, mp 210 °C) and (−)-lusitanine (120 mg, mp 185—187 °C, $[\alpha]_D^{25}$ −7.7° ($c$=0.31, EtOH). Fraction 6 (0.6 g) was subjected to column chromatography on silica gel using $CH_2Cl_2$–MeOH–28% $NH_4OH$ (90 : 9 : 1). The tashiromine (**1**)-rich fraction obtained was purified by preparative TLC with

$Et_2O$–MeOH–28%$NH_4OH$ (17:2:1) to give **1**.[2] Fraction 7 (130 mg) was rechromatographed over a silica gel column with $Et_2O$–MeOH–25% $NH_4OH$ (40:2:1) to give (−)-camoensidine (**4**, 7 mg) and (−)-camoensidine *N*-oxide (**3**, 15 mg). **4**: A colorless oil, $[\alpha]_D^{27}$ −73° ($c$=1.13, EtOH). EI-MS (70 eV) *m/z*: 234.1735 ($M^+$, 234.1733 for $C_{14}H_{22}N_2O$, 71), 233 (62), 136 (82), 135 (47), 122 (100), 84 (53). **3**: Hygroscopic colorless crystals, $[\alpha]_D^{27}$ −57° ($c$=0.79, EtOH). In-beam MS (70 eV) *m/z*: 250 ($M^+$, 25), 234 ($M^+$ −O, 61), 233 ($M^+$ −OH, 100), 232 ($M^+$ $-H_2O$, 24), 84 (69). EI-MS (70 eV) *m/z*: 234.1729 ($M^+$ −O, 234.1733 for $C_{14}H_{22}N_2O$, 50), 233 ($M^+$ −OH, 53), 232 ($M^+$ $-H_2O$, 88), 136 (50), 135 (55), 134 (60), 122 (82), 120 (100). All the known alkaloids except for **4** were identified by direct comparison with authentic compounds by co-TLC, co-HPLC, MS, IR, and $^1$H-NMR.

**Oxidation of (−)-Camoensidine (4) with $H_2O_2$** A solution of **4** (11.7 mg, 0.05 mmol) in 30% $H_2O_2$ (0.5 ml) was stirred for 15 h at ambient temperature. After decomposition of the excess $H_2O_2$ with a catalytic amount of $MnO_2$, the reaction mixture was made strongly alkaline with $K_2CO_3$ and extracted with $CH_2Cl_2$ several times. The combined extracts were dried over anhydrous $K_2CO_3$ and concentrated to dryness *in vacuo*. The residue was purified on a silica gel column using $CH_2Cl_2$–MeOH–28% $NH_4OH$ (90:9:1) to give **3** as a colorless oily product (11.9 mg, 95% yield), which was identical with the natural *N*-oxide (**3**) (co-TLC, co-HPLC, MS and $^1$H-NMR).

**Oxidation of (−)-Camoensidine (4) with MCPBA** MCPBA (85%, 22.3 mg, 0.11 mmol) was added to a solution of **4** (26.4 mg, 0.11 mmol) in 1 ml of $CH_2Cl_2$. After being stirred for 3 h at ambient temperature, the reaction mixture was washed with a saturated solution of $K_2CO_3$ (1 ml). The organic layer was dried ($Na_2SO_4$) and concentrated under reduced pressure. The residue was subjected to column chromatography on silica gel with $CH_2Cl_2$–$CH_3OH$–28% $NH_4OH$ (90:9:1) to give the *N*-oxides **7** (7.2 mg, 26% yield) and **3** (16.9 mg, 60% yield), in that order of elution. **7**: Colorless crystals from $C_6H_6$–EtOH, mp 111—113 °C, $[\alpha]_D^{27}$ −76° ($c$=0.41, EtOH). EI-MS (70 eV) *m/z*: 250.1683 ($M^+$, 250.1682 for $C_{14}H_{22}N_2O_2$, 32), 234 ($M^+$ −O, 25), 233 ($M^+$ −OH, 38), 232 ($M^+$ $-H_2O$, 37), 134 (100), 122 (52), 120 (53).

**Hydrogenation of (−)-Camoensidine *N*-Oxide (3)** A solution of **3** (0.9 mg) in MeOH (1 ml) was hydrogenated over 10%Pd–C at atmospheric pressure and ambient temperature. The catalyst was filtered off and the filtrate was evaporated to dryness *in vacuo*. The residue was purified by column chromatography on silica gel with $CH_2Cl_2$–MeOH–28%$NH_4OH$ (90:9:1) to give an oily product (0.7 mg), which was identical with (−)-camoensidine (**4**) (co-TLC, co-HPLC, co-GLC, MS).

**Acknowledgement** The authors are grateful to Mr. M. Otomasu, Tsukigi Middle School, Kumamoto, and Mr. S. Shiiba, Minamata High School, Kumamoto, for providing the material of *M. tashiroi*. Thanks are also due to Mrs. M. Yuyama and Miss Y. Takahashi for their help in the MS and NMR measurements.

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# Asymmetric Synthesis of 1-Benzyltetrahydroisoquinolines Using Chiral Oxazolo[2,3-*a*]tetrahydroisoquinolines

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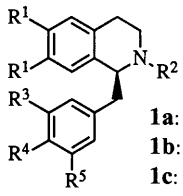
**A novel synthetic route to enantiomerically pure 1-benzyltetrahydroisoquinolines (1) was developed *via* the reaction of oxazolo[2,3-*a*]tetrahydroisoquinoline (5) with benzyltriisopropoxytitanium compounds (10). This method was applied to the synthesis of (*S*)-trimetoquinol (1c; a bronchodilator).**

**Keywords** oxazolo[2,3-*a*]tetrahydroisoquinoline; 1-benzyltetrahydroisoquinoline; 1-chloroisochroman; phenylglycinol; (*S*)-norarmepavine; (*S*)-tetrahydropapaverine; (*S*)-trimetoquinol; asymmetric synthesis

Chiral 1-benzyltetrahydroisoquinoline alkaloids are widely distributed among plants, and have interesting biological activities.[1] Fujii *et al.*[1a] reported that *dl*-1-(*p*-alkoxybenzyl)-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolines inhibit the collagen-induced activation (aggregation and adenosine triphosphate (ATP) secretion) of rabbit platelets. Akasu *et al.*[1b] found that *dl*-1-benzyloxybenzyl-1,2,3,4-tetrahydroisoquinoline derivatives have antitumor activity, while Fujiya *et al.*[1c] reported that armepavine (**1a**), laudanosine (**1b**), and related compounds have inhibitory activity against co-carcinorgens. In addition, (*S*)-trimetoquinol [**1c**; (*S*)-6,7-dihydroxy-1-(3,4,5-trimethoxybenzyl)-1,2,3,4-tetrahydroisoquinoline][2] has been used as a bronchodilator in Japan (Fig. 1).



**1a**: (*S*)-armepavine ($R^1$=OMe, $R^2$=Me, $R^3$, $R^5$=H, $R^4$=OH)
**1b**: (*S*)-laudanosine ($R^1$, $R^3$, $R^4$=OMe, $R^2$=Me, $R^5$=H)
**1c**: (*S*)-trimetoquinol ($R^1$=OH, $R^2$=H, $R^3$, $R^4$, $R^5$=OMe)

Fig. 1

Since 1-benzyltetrahydroisoquinolines (**1**) are also useful as intermediates for the synthesis of isoquinoline alkaloids, the development of a convenient method for the asymmetric synthesis of **1** is very important. Recently, several methods[3] for the highly stereoselective synthesis of 1-alkyltetrahydroisoquinolines have been developed and applied to the asymmetric synthesis of 1-benzyltetrahydroisoquinolines (**1**).

We have previously succeeded in the synthesis of enantiomerically pure (*S*)-1-alkyltetrahydroisoquinolines [(*S*)-**1**] *via* the stereoselective alkylation of chiral oxazolo-[2,3-*a*]tetrahydroisoquinolines (**5**) (Chart 1). The chiral key intermediate (**5**) was synthesized by the diastereoselective cyclization of the chiral isoquinolinium salt (**4**) prepared by the reaction of 2-(2-haloethyl)benzaldehyde (**3**) with (*R*)-phenylglycinol.[4] Compound **5**, which is regarded as a cyclic *N*,*O*-acetal, undergoes diastereoselective nucleophilic substitution by a variety of Grignard reagents (**7**), except benzylmagnesium halide, to give the corresponding (*S*)-1-alkylated tetrahydroisoquinoline derivatives (**8**). Removal of the chiral auxiliary moiety of **8** by hydrogenolysis gives

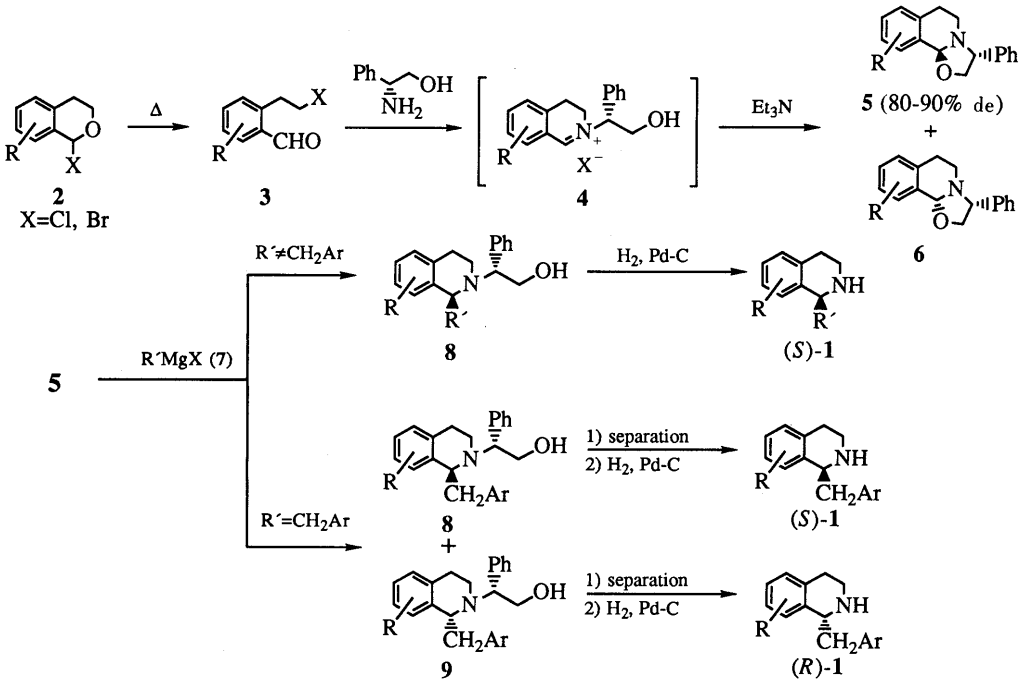


Chart 1

(*S*)-1-alkyltetrahydroisoquinolines (**1**). This method has been applied to the asymmetric syntheses of some natural products, *e.g.* salsolidine, homolaudanosine, and cryptostyline II.[4b]

At that time, we found that the reaction of **5** with benzylmagnesium halide (**7**) proceeds with exceptionally low diastereoselectivity, affording a separable mixture of (1*S*)- and (1*R*)-1-benzyl derivatives (**8** and **9**). As an extension of this work, we describe here an improved method for the synthesis of enantiomerically pure 1-benzyltetrahydroisoquinolines (**1**) *via* the reaction of **5** with benzyltriisopropoxytitanium compounds (**10**) and its application to the asymmetric synthesis of (*S*)-trimetoquinol (**1c**).

## Results and Discussion

The chiral (3*R*,10b*S*)-oxazolo[2,3-*a*]tetrahydroisoquinolines, **5a** (R=H) and **5b** (R=OMe), were prepared with 84 and 100% de, respectively, according to the method previously reported (Chart 1). In general, the cyclization of the isoquinolinium salt **4** to **5** proceeds with 80—90% de. When the cyclized product **5** and its diastereoisomer (**6**) are oils, the separation of **5** from **6** by column chromatography is difficult. On the other hand, when they are solids, pure **5** can be obtained by recrystallization of the mixture of **5** and **6** from ethanol.[4b]

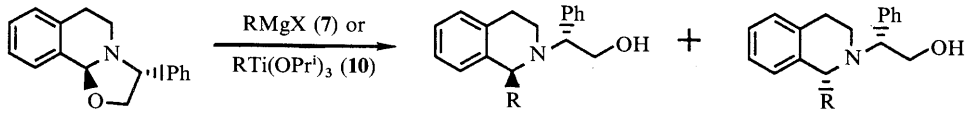
In earlier work,[4b] we examined asymmetric alkylation of **5a**, which had the diastereoisomeric purity of 82% de, with methyl-, butyl-, phenyl-, benzyl-, and phenethylmagnesium halides at −78 °C and found that the diastereoselectivity of the reaction of **5a** with benzylmagnesium chloride was exceptionally lower than the diastereoisomeric purity of **5a**. We have now reinvestigated the reaction of benzylmagnesium chloride (**7d**) with **5a** at −78 °C (Table I, run 8). The reproducibility was found to be poor with respect to the diastereoselectivity of the Grignard reaction of **5a** with **7d**. We therefore investigated stereoselective benzylation of **5** with another benzyl metal reagent. Benzyltriisopropoxytitanium compound (**10d**) was selected as a benzylating agent, because alkyltriisopropoxytitanium compounds (**10**) are known to react highly selectively with a carbonyl group.[5]

First, we examined the reaction of **5a** (84% de) with a variety of alkyltriisopropoxytitanium compounds (**10a—d**) (Table I). In order to compare the diastereoselectivity, the reaction of **5a** with the corresponding Grignard reagent under the same reaction condition was undertaken. The reaction (run 3) of **5a** with methyltriisopropoxytitanium (**10a**) occurred at room temperature to give a 59 : 41 mixture of **8a** and **9a**. The diastereoisomeric excess of **8a** was found to be 18% de, which is low as compared with that of the reaction (run 2) of **5a** with methylmagnesium iodide (**7a**). The reaction (run 5 or 7) of **5a** with butyltriisopropoxytitanium (**10b**) or phenyltriisopropoxytitanium (**10c**) did not take place even in refluxing benzene. In contrast with those reactions, the reaction (run 10) of **5a** with benzyltriisopropoxytitanium (**10d**) occurred at −5 °C to give an 18 : 82 mixture of **8d** and **9d**. The diastereoisomeric excess of **8d** was found to be 63% de. Although the major product was the reverse of that in the reaction of **5a** with **7d**, the diastereoisomeric excess was relatively high.

Since the benzylation of **5a** with benzyltriisopropoxytitanium (**10d**) smoothly proceeded with good diastereoselectivity, we then examined the generality of this method using diastereoisomerically pure **5b** and a variety of benzyltitanium reagents (**10d, f—i**) (Table II, method B). In order to compare the diastereoselectivity, the reactions of **5b** with the corresponding Grignard reagents (**7d, f—i**) were undertaken (method A). In method A, the diastereoisomeric excess in each reaction was found to be low. On the other hand, in method B, the benzylation proceeded with relatively high diastereoselectivity, affording **9** with approximately 65—90% de. Compound **8** accompanied with **9** was easily purified by column chromatography and subsequently converted to enantiomerically pure **9** by hydrogenolysis on palladium–carbon (Pd–C).

In the above reactions, the diastereoisomeric excess was determined by high performance liquid chromatography (HPLC) analysis. The structures of the major products, **8g** and **8h**, in the reaction of **5b** with Grignard reagent (method

TABLE I. Reaction of **5a** with Alkylmetal Reagents

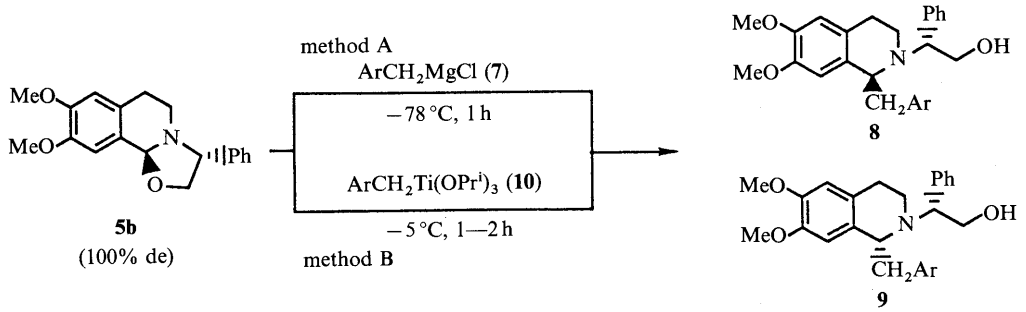



**5a** (84% de) **8** **9**

| Run | Alkylating agent | | Reaction condition | | | Product (8+9) | | |
|---|---|---|---|---|---|---|---|---|
| | No. | R | Temperature (°C) | Time (h) | Solvent | Yield (%) | Major product | % de |
| 1[a] | **7a** | Me | −78 | 1 | $Et_2O$ | 98 | **8a** | 82 |
| 2 | **7a** | Me | r.t. | 1 | $Et_2O$ | 84 | **8a** | 65 |
| 3 | **10a** | Me | r.t. | 16 | $Et_2O$ | 69 | **8a** | 18 |
| 4[a] | **7b** | Bu | −78 | 1 | $Et_2O$ | 81 | **8b** | 100 |
| 5 | **10b** | Bu | Reflux | 10 | Benzene | No reaction | | |
| 6[a] | **7c** | Ph | −78 | 1 | $Et_2O$ | 80 | **8c** | 100 |
| 7 | **10c** | Ph | Reflux | 6 | Benzene | No reaction | | |
| 8 | **7d** | $PhCH_2$ | −78 | 1 | $Et_2O$ | 85—95 | **8d** | 5—35 |
| 9 | **7d** | $PhCH_2$ | −5 | 1 | THF | 92 | **9d** | 8 |
| 10 | **10d** | $PhCH_2$ | −5 | 2 | $Et_2O$–THF | 95 | **9d** | 63 |

*a*) See reference 4*b*. r.t. = room temperature.

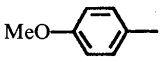
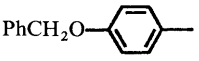
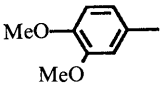
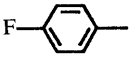
TABLE II. Benzylation of **5b**



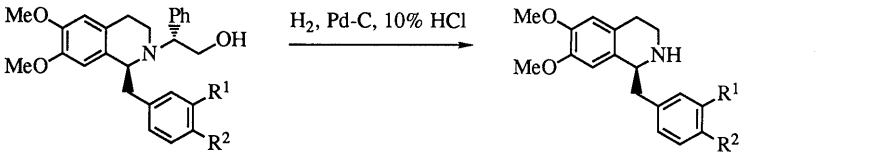

**5b** (100% de)

**8**

**9**

| Alkylating agent | | Method | Product (**8**+**9**) | | |
|---|---|---|---|---|---|
| No. | Ar | | Yield (%) | Major product | % de |
| **7d** |  | A | 84 | **8e** | 15 |
| **10d** | | B | 90 | **9e** | 77 (74)[a] |
| **7f** |  | A | 90 | **8f** | 18 |
| **10f** | | B | 91 | **9f** | 65 (72)[a] |
| **7g** |  | A | 92 | **8g** | 24 |
| **10g** | | B | 86 | **9g** | 90 (75)[a] |
| **7h** |  | A | 83 | **8h** | 21 |
| **10h** | | B | 73 | **9h** | 79 (54)[a] |
| **7i** |  | A | 78 | **8i** | 13 |
| **10i** | | B | 79 | **9i** | 77 (70)[a] |

*a*) Isolated yield of **9**.




**8g** : $R^1$ = H, $R^2$ = $OCH_2Ph$
**8h** : $R^1$, $R^2$ = OMe

(*S*)-norarmepavine (**1g**) : $R^1$ = H, $R^2$ = OH
(*S*)-tetrahydropapaverine (**1h**) : $R^1$, $R^2$ = OMe

Chart 2

A) were assigned after their conversion to known alkaloids, (*S*)-norarmepavine[6] (**1g**) and (*S*)-tetrahydropapaverine[7] (**1h**) (Chart 2). The structures of other compounds, **8e**, **8f**, and **8i**, were identified based on the similarities of the thin layer chromatography (TLC), HPLC, and nuclear magnetic resonance (NMR) spectral patterns not to those of **9g** and **9h**, but to those of **7g** and **7h**.

Since the above results seemed to indicate that method B is effective, we next attempted asymmetric synthesis of (*S*)-trimetoquinol (**1c**), which is currently obtained by optical resolution on an industrial scale. In order to obtain **1c** according to method B, (3*S*,10b*R*)-oxazolo[2,3-*a*]tetrahydroisoquinoline (**16**) was required as a chiral intermediate. Compound **16** was synthesized as follows. 3,4-Dibenzyloxyphenethyl alcohol[8] (**11**), on treatment with triethyl orthoformate in the presence of boron trifluoride etherate ($BF_3 \cdot Et_2O$) at room temperature, gave 6,7-dibenzyloxy-1-ethoxyisochroman (**12**) accompanied with several by-products, purification of which by recrystallization from hexane afforded pure **12** in 40% yield. Compound **12** was allowed to reflux in acetyl chloride for 2 h to give 2-(2-chloroethyl)benzaldehyde (**14**) in 72% yield. Treatment of **14** with (*S*)-phenylglycinol in ethanol at room temperature gave crude isoquinolium chloride (**15**), which, on treatment with triethylamine in methylene chloride at −5 °C, afforded **16** in 93% yield as an oily product. The diastereoisomeric excess of **16** was determined to be 85% by 500 MHz NMR spectral analysis. Compound **16** was, without further separation, treated with (3,4,5-trimethoxybenzyl)triisopropoxytitanium (**17**) at −5 °C in tetrahydrofuran (THF) to give an 80:20 (60% de) mixture of **18** and **19**. The major product **18** was separated from **19** by column chromatography on silica gel in 50% yield based on **16**. Hydrogenolysis of **18** in the presence of Pd–C in acidic ethanol gave enantiomerically pure (*S*)-trimetoquinol hydrochloride[9] (**1c**·HCl).

The synthetic strategy *via* the reaction of chiral oxazolo-[2,3-*a*]tetrahydroisoquinolines, easily prepared using (*R*)- and (*S*)-phenylglycinol, with benzyltriisopropoxytitaniums (**10**) provides a general and useful method for the synthesis of enantiomerically pure (*R*)- and (*S*)-1-benzyltetrahydroisoquinolines (**1**). Investigations on further applications of

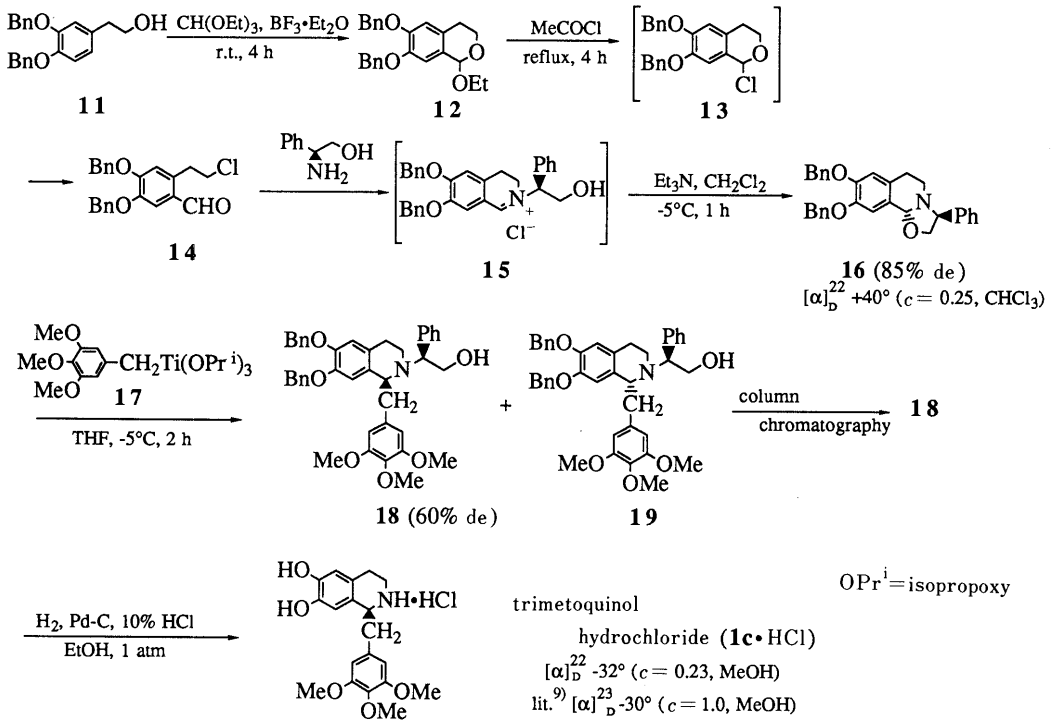



Chart 3

this strategy and on the reaction mechanism of stereoselective alkylation of the chiral intermediate **4** are in progress.

## 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 spectrometer or on a Varian VXR-500 instrument. 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 Chemcosorb 5Si-U (Chemco) fitted with an ultraviolet (254 nm) detector. Merck Silica gel 60 (230—400 mesh) and Wako activated alumina (300 mesh) were employed for column chromatography. Extracts were dried over anhydrous $MgSO_4$.

**Reaction of 5a with MeMgI (7a) at Room Temperature** A solution of **5a** (0.15 g, 0.6 mmol) in dry $Et_2O$ (5 ml) was added dropwise to a solution of MeMgI (2.4 mmol) in dry $Et_2O$ (20 ml). After being stirred for an additional 1 h at room temperature, the reaction mixture was quenched with aqueous $NH_4Cl$ solution 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 purified by flash chromatography on silica gel (hexane : AcOEt = 1 : 1) to give 0.13 g of a mixture of (1*S*,1′*R*)-2-(2-hydroxy-1-phenylethyl)-1-methyl-1,2,3,4-tetrahydroisoquinoline[4b] (**8a**) and (1*R*,1′*R*)-2-(2-hydroxy-1-phenylethyl)-1-methyl-1,2,3,4-tetrahydroisoquinoline (**9a**) in a combined chemical yield of 84%. The diastereomeric excess of **8a** was determined to be 65% by HPLC analysis.

**Reaction of 5a with Triisopropoxymethyltitanium (10a)** A solution of 95% $ClTi(OPr^i)_3$ (4.8 mmol) in dry hexane (10 ml) was added dropwise to a solution of MeMgI (4.8 mmol) in dry $Et_2O$ (20 ml) at −5 °C and the mixture was stirred for 2 h at −5 °C. After a precipitate was formed, the supernatant was added dropwise to a solution of **5a** (0.3 g, 1.2 mmol) in dry $Et_2O$ (10 ml) at −5 °C. The reaction mixture was stirred at room temperature for 16 h and then quenched with aqueous $NH_4Cl$ solution. The resulting precipitate was filtered off and the filtrate was extracted with $Et_2O$. The $Et_2O$ layer was washed with $H_2O$ and dried. The solvent was evaporated off and the residue was purified by flash chromatography on silica gel (hexane : AcOEt = 1 : 1) to give 0.22 g of a mixture of **8a** and **9a** in a combined chemical yield of 69%. The diastereomeric excess of **8a** was determined to be 18% by HPLC analysis.

**(1*R*,1′*R*)-2-(2-Hydroxy-1-phenylethyl)-1-methyl-1,2,3,4-tetrahydroisoquinoline (9a)** Viscous oil; IR (neat): 3430 cm$^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.48 (3H, d, $J$=8 Hz), 2.24—3.36 (4H, m), 2.66 (1H, s), 3.36—4.25 (4H, m), 6.87—7.67 (4H, m), 7.26 (5H, s). FAB-MS (positive ion mode) $m/z$: 268 ($M^+ + 1$). $[\alpha]_D^{17}$ −9.7° ($c$=0.7, $CHCl_3$).

**Reaction of 5a with $PhCH_2MgCl$ (7d) at −78 °C** Mg turnings (192 mg, 8 mmol) and dry $Et_2O$ (30 ml) were placed in a 3-necked flask, fitted with a reflux condenser and septum rubber and continuously maintained with a flow of argon. A trace of iodine was added and stirring was continued until the iodine color disappeared. Benzyl chloride (0.92 ml, 8 mmol) was then added dropwise at room temperature. Stirring was continued at room temperature for 1 h. Finally the mixture was diluted with $Et_2O$ to get the Grignard reagent in 0.1 molar concentration. The flask was cooled to −78 °C and 500 mg (2 mmol) of **5a** (84% de), dissolved in dry THF (10 ml), was added dropwise at −78 °C. After being stirred for an additional 1 h at −78 °C, the reaction mixture was quenched with aqueous $NH_4Cl$ 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 purified by flash chromatography on silica gel (hexane : AcOEt = 1 : 1) to give 0.58 g of a mixture of (1*S*,1′*R*)-1-benzyl-2-(2-hydroxy-1-phenylethyl)-1,2,3,4-tetrahydroisoquinoline[4b] (**8d**) and (1*R*,1′*R*)-1-benzyl-2-(2-hydroxy-1-phenylethyl)-1,2,3,4-tetrahydroisoquinoline (**9d**) in a combined chemical yield of 84%. The diastereomeric excess of **8d** was determined to be 34% by HPLC analysis. The mixture of **8d** and **9d** was chromatographed on an open column of silica gel (hexane : AcOEt = 2 : 1) to give pure **8d** and **9d**.

**(1*R*,1′*R*)-1-Benzyl-2-(2-hydroxy-1-phenylethyl)-1,2,3,4-tetrahydroisoquinoline (8d)** Oil. *Anal.* Calcd for $C_{24}H_{25}NO$: C, 83.92; H, 7.34; N, 4.08. Found: C, 84.12; H, 7.40; N, 4.11. IR (neat): 3450 cm$^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.98 (1H, s), 2.21—3.38 (6H, m), 3.41—4.34 (4H, m), 6.65—7.35 (4H, m), 7.10 (5H, s). FAB-MS (positive ion mode) $m/z$: 344 ($M^+ + 1$). $[\alpha]_D^{24}$ −21.50° ($c$=0.2, EtOH).

**Reaction of 5a with $PhCH_2MgCl$ (7d) at −5 °C** A solution of **4a** (0.2 g, 0.8 mmol) in dry THF (20 ml) was added dropwise to a solution of $PhCH_2MgCl$ (3.2 mmol) in dry THF (10 ml) at −5 °C. After being stirred for an additional 1 h at room temperature, the reaction mixture was quenched with aqueous $NH_4Cl$ solution 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 purified by flash chromatography on silica gel (hexane : AcOEt = 1 : 1) to give 0.25 g of a mixture of **7d** and **8d** in a combined chemical yield of 92%. The diastereomeric excess of **8d** was determined to be 8% by HPLC analysis.

**Reaction of 5a with Benzyltriisopropoxytitanium (10d)** A solution of 95% $ClTi(OPr^i)_3$ (4.8 mmol) in dry hexane (10 ml) was added dropwise to a solution of $PhCH_2MgCl$ (4.8 mmol) in dry $Et_2O$ (20 ml) at −5 °C, and the mixture was stirred for an additional 2 h at −5 °C. After a precipitate was formed, the supernatant was added dropwise to a solution of **4a** (0.3 g,

1.2 mmol) in dry $Et_2O$ (10 ml) at −5 °C. The reaction mixture was then stirred at −5 °C for 2 h. Work-up as described above and purification by chromatography on a column of silica gel (hexane : AcOEt = 1 : 1) gave 0.39 g of a mixture of **7d** and **8d** in a combined chemical yield of 95%. The diastereomeric excess of **8d** was determined to be 63% by HPLC analysis.

**General Procedure for Reaction of 5b with Benzylmagnesium Halide (7d, f—i) (Method A)** Grignard reagent, **7i**, was prepared as described for the reaction of **5a** with **7i**. The other Grignard reagent, **7f**, **7g**, or **7h**, was prepared as follows. Mg turnings (1.2 g, 50 mmol) were placed in a 3-necked flask fitted with a reflux condenser and septum rubber and continuously maintained with a flow of argon. Dry THF (20 ml) was added to cover the magnesium surface. A few crystals of iodine were added and the flask was kept undisturbed for 10 min. Stirring was started and a few drops of 1,2-dibromoethane were added. Stirring was continued until the iodine color disappeared. The flask was cooled to −10 °C and the corresponding benzyl chloride (13 mmol) dissolved in dry THF (50 ml) was added dropwise over a period of 1 h with vigorous stirring. After completion of the addition, dry THF (40 ml) was added. The reaction mixture was stirred for a further 30 min at −10 °C.

Next, 0.2 g (0.64 mmol) of **5b** was added dropwise at −78 °C to the flask containing Grignard reagent (**7**; 2.56 mmol) prepared by the above procedure. After being stirred for an additional 1 h at −78 °C, the reaction mixture was quenched with aqueous $NH_4Cl$ 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 purified by flash chromatography on silica gel (hexane : AcOEt = 1 : 1) to give a mixture of **8e—i** and **9e—i** in the combined chemical yield as shown in Table II. The diastereomeric excess of **8e—i** (Table II) was determined by HPLC analysis. The mixture of **8e—i** and **9e—i** was separated by chromatography on an open column of silica gel (hexane : AcOEt = 2 : 1) to give pure **8e—i** and **9e—i**.

**(1*S*,1′*R*)-1-Benzyl-2-(2-hydroxy-1-phenylethyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (8e)** Hygroscopic solid. IR (neat): 3540 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.02 (1H, s), 2.47—3.38 (6H, m), 3.44—4.02 (4H, m), 3.51, 3.81 (3H × 2, each s), 5.86, 6.50 (1H × 2, each s), 6.84—7.37 (10H, m). FAB-MS (positive ion mode) *m/z*: 404 ($M^+ + 1$). $[\alpha]_D^{22}$ +27° (*c* = 0.3, EtOH).

**(1*R*,1′*R*)-1-Benzyl-2-(2-hydroxy-1-phenylethyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (9e)** Hygroscopic solid. IR (neat): 3500 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.04 (1H, s), 2.24—3.39 (6H, m), 3.43—3.85 (4H, m), 3.60, 3.79 (3H × 2, each s), 6.10, 6.40 (1H × 2, each s), 6.85—7.31 (4H, m), 7.15 (5H, s). FAB-MS (positive ion mode) *m/z*: 404 ($M^+ + 1$). $[\alpha]_D^{22}$ −89° (*c* = 0.1, EtOH).

**(1*S*,1′*R*)-2-(2-Hydroxy-1-phenylethyl)-6,7-dimethoxy-1-(4-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline (8f)** Hygroscopic solid. IR (neat): 3540 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.19—3.41 (6H, m), 3.24 (1H, s), 3.45—4.12 (4H, m), 3.54, 3.77, 3.79 (3H × 3, each s), 5.90, 6.50 (1H × 2, each s) 6.62—7.40 (4H, m), 7.12 (5H, s). FAB-MS (positive ion mode) *m/z*: 434 ($M^+ + 1$). $[\alpha]_D^{22}$ +44.9° (*c* = 0.21, EtOH).

**(1*R*,1′*R*)-2-(2-Hydroxy-1-phenylethyl)-6,7-dimethoxy-1-(4-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline (9f)** Hygroscopic solid. IR (neat): 3540 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.27—3.40 (6H, m), 2.29 (1H, s), 3.56—3.95 (4H, m), 3.65, 3.75, 3.78 (3H × 3, each s), 6.22, 6.48 (1H × 2, each s), 6.66—7.10 (4H, m), 7.23 (5H, s). FAB-MS (positive ion mode) *m/z*: 434 ($M^+ + 1$). $[\alpha]_D^{22}$ −48.5° (*c* = 0.24, EtOH).

**(1*S*,1′*R*)-1-(4-Benzyloxybenzyl)-2-(2-hydroxy-1-phenylethyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (8g)** Hygroscopic solid. IR (neat): 3450 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.35 (1H, s), 2.45—3.41 (6H, m), 3.50—4.09 (4H, m), 3.53, 3.82 (3H × 2, each s), 5.04 (2H, s), 5.90, 6.58 (1H × 2, each s), 6.90 (5H, s), 7.03—7.60 (4H, m), 7.40 (5H, s). FAB-MS (positive ion mode) *m/z*: 510 ($M^+ + 1$). $[\alpha]_D^{18}$ +66° (*c* = 0.3, $CHCl_3$).

**(1*S*,1′*R*)-1-(4-Benzyloxybenzyl)-2-(2-hydroxy-1-phenylethyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (9g)** Hygroscopic solid. IR (neat): 3460 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.21—3.45 (6H, m), 2.49 (1H, s), 3.59—4.20 (4H, m), 3.62, 3.81 (3H × 2, each s), 5.09 (2H, s), 6.20, 6.50 (1H × 2, each s), 6.98 (4H, s), 7.31 (5H, s), 7.42 (5H, s). FAB-MS (positive ion mode) *m/z*: 510 ($M^+ + 1$). $[\alpha]_D^{18}$ −96° (*c* = 0.1, $CHCl_3$).

**(1*S*,1′*R*)-1-(3,4-Dimethoxybenzyl)-2-(2-hydroxy-1-phenylethyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (8h)** Hygroscopic solid. IR (neat): 3525 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.10—3.40 (6H, m), 2.47 (1H, s), 3.41—4.02 (4H, m), 3.56, 3.71, 3.79, 3.80 (3H × 4, each s), 5.90, 6.43 (1H × 2, each s), 6.55—6.91 (3H, m), 7.15 (5H, br s). FAB-MS (positive ion mode) *m/z*: 464 ($M^+ + 1$). $[\alpha]_D^{22}$ +28° (*c* = 0.27, $CHCl_3$).

**(1*R*,1′*R*)-1-(3,4-Dimethoxybenzyl)-2-(2-hydroxy-1-phenylethyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (9h)** Hygroscopic solid. IR (neat): 3530 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.19—3.30 (6H, m), 2.42 (1H, s), 3.32—4.39 (4H, m), 3.63, 3.70, 3.76, 3.80 (3H × 4, each s), 6.19, 6.46 (1H × 2, each s), 6.32—6.86 (3H, m), 7.26 (5H, s). FAB-MS (positive ion mode) *m/z*: 464 ($M^+ + 1$). $[\alpha]_D^{22}$ −77° (*c* = 0.12, $CHCl_3$).

**(1*S*,1′*R*)-1-(4-Fluorobenzyl)-2-(2-hydroxy-1-phenylethyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (8i)** Oil. IR (neat): 3540 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.45—3.42 (6H, m), 2.85 (1H, s), 3.50—4.20 (4H, m), 3.58, 3.82 (3H × 2, each s), 5.90, 6.50 (1H × 2, each s), 6.61—7.52 (9H, m). FAB-MS (positive ion mode) *m/z*: 422 ($M^+ + 1$). $[\alpha]_D^{23}$ +23.4° (*c* = 0.20, EtOH).

**(1*R*,1′*R*)-1-(4-Fluorobenzyl)-2-(2-hydroxy-1-phenylethyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (9i)** Oil. IR (neat): 3540 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.24—3.18 (6H, m), 2.39 (1H, s), 3.39—4.29 (4H, m), 3.64, 3.77 (3H × 2, each s), 6.19, 6.47 (1H × 2, each s), 6.75—7.19 (4H, m), 7.23 (5H, s). FAB-MS (positive ion mode) *m/z*: 422 ($M^+ + 1$). $[\alpha]_D^{23}$ −41.5° (*c* = 0.21, EtOH).

**General Procedure for Reaction of 5b with Benzyltriisopropoxytitanium Compounds (10d, f—i) (Method B)** A solution of 95% $ClTi(OPr^i)_3$ (1 ml, 4 mmol) in dry hexane (10 ml) was added dropwise at −5 °C to a solution of $ArCH_2MgCl$ (4 mmol) prepared as described above (method A) in dry THF (20 ml), and the mixture was stirred for 2 h at −5 °C. After a precipitate was formed the supernatant was added dropwise to a solution of **5b** (0.3 g, 0.96 mmol) in dry THF (10 ml) at −5 °C. The reaction mixture was then stirred at −5 °C for 1—2 h, until **5b** disappeared. Work-up as described above and subsequent purification by flash chromatography on silica gel (hexane : AcOEt = 1 : 1) gave a mixture of **8e—i** and **9e—i**. The diastereomeric excess of **9e—i** was determined by HPLC analysis. Rechromatography of the mixture of **8e—i** and **9e—i** gave pure **9e—i**. The chemical yield, diastereomeric excess of **8e—i**, and isolated yield of **8e—i** are given in Table II.

**(*S*)-1-(4-Hydroxybenzyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline [(*S*)-Norarmepavine; 1g]** A solution of **8g** (0.3 g, 0.87 mmol) in EtOH was hydrogenated in the presence of 10% Pd–C (0.3 g) and 10% HCl (2 ml). After the completion of hydrogen absorption, the solvent was evaporated off and 10% HCl (20 ml) was added to the residue. The acidic solution was washed with $Et_2O$, made basic with saturated $KHCO_3$ solution, and extracted with $CH_2Cl_2$. The organic layer was washed with $H_2O$ and dried, then the solvent was evaporated off. The resulting crude product was crystallized from $Me_2CO$ to give 0.24 g (80%) of **1g**. Spectral data were in good agreement with the literature values.[6] mp 155—157 °C (lit.[6] mp 152—153 °C). $[\alpha]_D^{24}$ −24° (*c* = 0.2, $CHCl_3$) (lit.[6] $[\alpha]_D^{25}$ −23° (*c* = 1.53, $CHCl_3$)).

**(*S*)-6,7-Dimethoxy-1-(3,4-dimethoxybenzyl)-1,2,3,4-tetrahydroisoquinoline [(*S*)-Tetrahydropapaverine; 1h]** Compound **8h** was hydrogenated as described for **1g** to **1h** in 85% yield. Spectral data were in good agreement with the literature values.[7] $[\alpha]_D^{24}$ −24° (*c* = 0.22, $CHCl_3$) [lit.[7] $[\alpha]_D$ −21° (*c* = 1.0, $CHCl_3$)]. **1h**·HCl: mp 162—164 °C (lit.[7] mp 167 °C).

**6,7-Dibenzyloxy-1-ethoxyisochroman (12)** $BF_3 \cdot Et_2O$ (5 ml, 41 mmol) was added quickly to a solution of 3,4-dibenzyloxyphenethyl alcohol[8] (**11**, 9 g, 27 mmol) in triethyl orthoformate (45 ml, 270 mmol) at 0 °C. The reaction mixture was stirred for 4 h at room temperature, made basic with saturated $KHCO_3$ solution, and extracted with $CH_2Cl_2$. The organic layer was washed with water and dried. Evaporation of the solvent gave a brown oily mass, which was crystallized from hexane to give **12** (4.2 g, 40%). mp 99—102 °C. *Anal.* Calcd for $C_{25}H_{26}O_4$: C, 76.90; H, 6.71. Found C, 76.94; H, 6.76. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.28 (3H, t, *J* = 6.4 Hz), 2.22—2.97 (2H, m), 3.50—4.19 (4H, m), 5.10 (4H, s), 5.46 (1H, s), 6.70 (1H, s), 6.85 (1H, s), 7.37 (10H, br s). EI-MS *m/z*: 390 ($M^+$).

**2-(2-Chloroethyl)-4,5-dimethoxybenzaldehyde (14)** A solution of **13** (1.7 g, 4.36 mmol) in acetyl chloride (3.5 ml, 49.2 mmol) was refluxed for 2.5 h. The excess acetyl chloride was distilled off and the residue was heated at 90—100 °C for 1 h. The volatiles were removed under reduced pressure and the residue was chromatographed on silica gel (hexane : AcOEt = 8 : 1) to give **14** (1.2 g, 72%). mp 75—77 °C (from $Et_2O$). *Anal.* Calcd for $C_{23}H_{21}ClO_3$: C, 72.59; H, 5.56. Found: C, 72.62; H, 5.61. IR (Nujol): 1668 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 3.15—3.78 (4H, m), 5.11 (2H, s), 5.19 (2H, s), 6.77 (1H, s), 7.30 (1H, s), 10.05 (1H, s). EI-MS *m/z*: 382 ($M^+ + 2$), 380 ($M^+$).

**(3*S*,10b*R*)-8,9-Dibenzyloxy-3-phenyl-2,3,5,6-tetrahydro-10b*H*-oxazolo-[2,3-*a*]isoquinoline (16)** A solution of **14** (1.0 g, 2.6 mmol), (*S*)-phenylglycinol (0.43 g, 3.2 mmol) and AcOH (2.6 mmol) in EtOH (50 ml) was stirred for 10 h at room temperature. The solvent was evaporated off under reduced pressure and the residue was dissolved in $CH_2Cl_2$ (50 ml). Triethylamine (0.45 g, 3.2 mmol) was added dropwise to the resulting solution at −5 °C. After being stirred at −5 °C for an additional 1 h,

the reaction mixture was washed with water and dried. The solvent was chromatographed on alumina (hexane : AcOEt = 3 : 1) to give an inseparable mixture (1.15 g, 94%) of **16** (85% de) and (3*S*,10b*S*)-8,9-dibenzyloxy-3-phenyl-2,3,5,6-tetrahydro-10b*H*-oxazolo[2,3-*a*]isoquinoline (**17**) as a viscous oil. The diastereoisomeric excess of **16** was determined from the 500 MHz NMR spectrum of the oily mixture. *Anal.* Calcd for $C_{31}H_{29}NO_3$: C, 80.82; H, 6.31; N, 3.02. Found: C, 80.86; H, 6.35; N, 3.06. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.59—3.11 (4H, m), 3.61—3.91 (1H, m), 4.10—4.45 (2H, m), 5.12 (4H, s), 5.36 (1H, s), 6.77 (1H, s), 7.06 (1H, s), 7.40 (15H, br s). FAB-MS (positive ion mode) *m/z*: 464 ($M^+ + 1$). $[\alpha]_D^{22}$ +40° ($c$ = 0.25, $CHCl_3$).

**(1*S*,1′*S*)-6,7-Dibenzyloxy-2-(2-hydroxy-1-phenylethyl)-1-(3,4,5-trimethoxybenzyl)-1,2,3,4-tetrahydroisoquinoline (18)** A solution of 95% $ClTi(OPr^i)_3$ (2.4 ml, 9.6 mmol) in dry hexane (10 ml) was added dropwise at −5 °C to a solution of 3,4,5-trimethoxybenzylmagnesium chloride (8 mmol) prepared as described for **7f**—**h** (method A) in dry THF (50 ml), and the mixture was stirred for 2 h at −5 °C. A solution of **4b** (0.92 g, 2 mmol) in dry THF (10 ml) was added dropwise to the mixture at −5 °C. The reaction mixture was then stirred at −5 °C for 1.5 h. Work-up as described above and subsequent purification by flash chromatography on silica gel (hexane : AcOEt = 2 : 1) gave 1.23 g (95%) as a mixture of **18** and (1*R*,1′*S*)-6,7-dibenzyloxy-2-(2-hydroxy-1-phenylethyl)-1-(3,4,5-trimethoxybenzyl)-1,2,3,4-tetrahydroisoquinoline (**19**). The diastereoisomeric excess of **18** was determined to be 60% by HPLC analysis. The mixture was separated by chromatography on an open column of silica gel (hexane : AcOEt = 3 : 1) to give 0.65 g (50%) of **18**. Hygroscopic solid. IR (Nujol): 3480 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.19—3.09 (6H, m), 2.42 (1H, s), 3.65, 3.75, 3.79 (3H × 3, each s), 3.56—4.20 (4H, m), 4.89, 5.05 (2H × 2, each s), 6.08 (2H, s), 6.30, 6.58 (1H × 2, each s), 7.10—7.59 (15H, m). FAB-MS (positive ion mode) *m/z*: 646 ($M^+ + 1$). $[\alpha]_D^{18}$ +26.4° ($c$ = 0.21, EtOH).

**(*S*)-(−)-Trimetoquinol Hydrochloride (1c·HCl)** Hydrogenolysis of **18** was carried out as described for **1g**. After absorption of hydrogen was complete, the catalyst was filtered off. The filtrate was concentrated to give the hydrochloride of (*S*)-trimetoquinol (**1c**·HCl, yield 86%). Spectral data of **1c**·HCl were in good agreement with literature values.[9] **1c**·HCl: mp 155—156 °C (lit.[9] mp 151—153 °C). $[\alpha]_D^{22}$ −32° ($c$ = 0.23, MeOH) [lit.[9] $[\alpha]_D^{23}$ −30° ($c$ = 1.0, MeOH)].

**Acknowledgement** We are grateful to the SC-NMR Laboratory of Okayama University for 500 MHz $^1$H-NMR experiments.

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# A New Entry to the Synthesis of 1,2-Benzenediol Congeners

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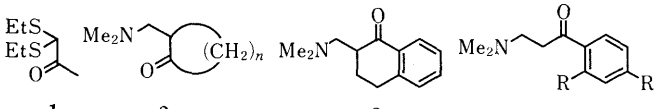
**1,2-Benzenediols were synthesized *via* 1,1-bis(ethylthio)-3-cyclohexen-2-one derivatives, which were prepared by condensation of 1,1-bis(ethylthio)-2-propanone with Mannich bases. Regioselective preparation of their monoethers was also achieved.**

**Keywords** annelation; aromatic synthesis; 1,2-benzenediol; pyrocatechol; 1,1-bis(ethylthio)-2-propanone; [3C+3C]annelation; condensation; Mannich base; monoether

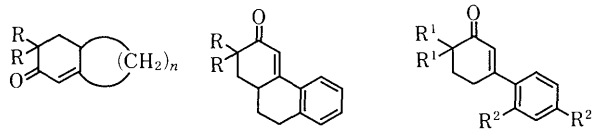
Construction of phenolic compounds is an important area of organic chemistry because of their utility in various fields. Derivation of aromatic systems from aliphatic sources has been studied extensively owing to its synthetic advantages.[2] However, there has been no report on the approach to pyrocatechols and their monoethers *via* annelation. The 1,2-benzenediol system is contained in a large number of naturally occurring and biologically active compounds.[3]



1 2 3 4

**a** : $n=3$
**b** : $n=4$
**c** : $n=5$

**a** : R=H
**b** : R=$OCH_2Ph$



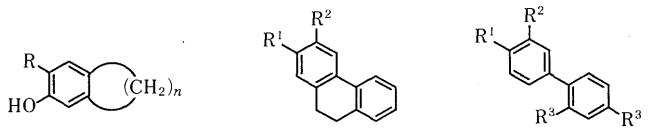
5 6 7

**a** : $n=3$, R=SEt
**b** : $n=4$, R=SEt
**c** : $n=5$, R=SEt
**d** : $n=3$, R=OMe
**e** : $n=4$, R=OMe
**f** : $n=5$, R=OMe
**g** : $n=3$, R=OEt
**h** : $n=4$, R=$OCH_2Ph$

**a** : R=SEt
**b** : R=OMe
**c** : R=$OCH_2Ph$
**d** : R=$OCH_2CHCH_2$

**a** : $R^1$=SEt, $R^2$=H
**b** : $R^1$=OMe, $R^2$=H
**c** : $R^1$=$OCH_2Ph$, $R^2$=H
**d** : $R^1$=$OCH_2CHCH_2$, $R^2$=H
**e** : $R^1$=SEt, $R^2$=$OCH_2Ph$

Chart 1



8 9 10

**a** : $n=3$, R=OH
**b** : $n=4$, R=OH
**c** : $n=5$, R=OH
**d** : $n=3$, R=OMe
**e** : $n=4$, R=OMe
**f** : $n=5$, R=OMe
**g** : $n=3$, R=OEt
**h** : $n=4$, R=$OCH_2Ph$

**a** : $R^1$=$R^2$=OH
**b** : $R^1$=OMe, $R^2$=OH
**c** : $R^1$=$OCH_2Ph$, $R^2$=OH
**d** : $R^1$=$OCH_2CHCH_2$, $R^2$=OH
**e** : $R^1$=OH, $R^2$=OMe
**f** : $R^1$=$OCH_2Ph$, $R^2$=OMe

**a** : $R^1$=$R^2$=OH, $R^3$=H
**b** : $R^1$=OMe, $R^2$=OH, $R^3$=H
**c** : $R^1$=$OCH_2Ph$, $R^2$=OH, $R^3$=H
**d** : $R^1$=$OCH_2CHCH_2$, $R^2$=OH, $R^3$=H
**e** : $R^1$=OH, $R^2$=OMe, $R^3$=H
**f** : $R^1$=$OCH_2Ph$, $R^2$=OMe, $R^3$=H
**g** : $R^1$=$R^2$=$R^3$=OH
**h** : $R^1$=$R^2$=OH, $R^3$=$OCH_2Ph$

Chart 2

Most synthetic approaches to 1,2-benzenediol derivatives have been performed by utilizing aromatic compounds bearing a 1,2-benzenediol moiety, *i.e.*, veratrol aldehyde, iso-veratrol aldehyde, and their derivatives. In the course of our investigations on the synthesis of phenolic compounds,[4] successful formation of 1,2-benzenediols was achieved. We here described in detail a new regiocontrolled route to the 1,2-benzenediols and their monoethers.[5]

In previous papers, we have used 1,1-bis(ethylthio)-2-propanone (**1**) as a component of the aromatic ring in a synthesis of 1,3,4-benzenetriol derivatives.[4b,c] Compound **1** and the Mannich bases (**2a—c**, **3** and **4a**) were proposed as 3C materials for [3C+3C] annelation to prepare 1,2-benzenediols, respectively. The former was prepared according to the literature procedure,[6] and the latter compounds were obtained easily from corresponding ketones by means of the Mannich reaction.[7] The condensation of **1** and the amines (**2a—c**, **3** and **4a**) was promoted by NaH in refluxing dimethoxyethane (DME) to give the cyclic compounds (**5a—c**, **6a** and **7a**). Their enone structures were confirmed by the observation of olefinic proton signals in their proton nuclear magnetic reconance ($^1$H-NMR) spectra and carbonyl absorption in their infrared (IR) spectra. These compounds with distinctive structures are synthetic precursors of 1,2-benzenediols.

The enones (**5a—c**, **6a** and **7a**) bearing thioacetalized 6-membered ring structures were transformed into the target aromatics by hydrolysis and isomerization. Dethioacetalization of **5a—c**, **6a** and **7a** with mercury (II) perchlorate trihydrate [MPC: $Hg(ClO_4)_2 \cdot 3H_2O$],[8] in tetrahydrofuran (THF)/$CHCl_3$ followed by treatment with refluxing acetic acid gave the 1,2-benzenediols (**8a—c**, **9a** and **10a**). Aromatic proton signals observed in their $^1$H-NMR spectra confirmed the successful formation of the benzene rings.

The enones (**5a—c**, **6a** and **7a**) were available for the preparation of 1,2-benzenediol monoethers. Modification of the reaction conditions used for the above dethioacetalization gave the desired intermediates (**5d—h**, **6b—d** and **7b—d**) suitable for the formation of the 1,2-benzenediol monoethers (**8d—h**, **9b—d** and **10b—d**). For methylethers, the cyclohexenones (**5a—c**, **6a** and **7a**) were treated with MPC in the presence of methyl alcohol to give the acetals (**5d—f**, **6b** and **7b**) as a result of transacetalization. Treatment of **5d—f**, **6b** and **7b** in refluxing acetic acid for 1 h gave the 1,2-benzenediol monomethyl ethers (**8d—f**, **9b** and **10b**). Several monoethers (**8g**, **h**, **9c**, **d**, and **10c**, **d**) were prepared *via* the acetals (**5g**, **h**, **6c**, **d**, and **7c**, **d**), which were obtained from the thioacetals (**5a—c**, **6a** and **7a**) using the corresponding alcohols in the same manner as noted for

the monomethyl ethers (**8d—f**, **9b** and **10d**). The formation of these monoethers demonstrated that the alcohol employed in the reaction was incorporated in the ether group on the aromatic nucleus.

In these preparations of ethers, compounds **9b** and **9e** could provide regioisomeric monomethyl ethers of the diol (**9a**) as follows. Compound **9e** was synthesized from the monobenzyl ether (**9c**). Methylation of **9c** with dimethyl sulfate gave the monobenzyl monomethyl ether (**9f**). Debenzylation of **9f** on treatment with $H_2$/Pd–C afforded the monomethyl ether (**9e**). Another isomeric pair of monomethyl ethers (**10b** and **10e**) was also obtained. The methylether (**10e**) was obtained from the benzyl ether (**10c**) *via* **10f** in a similar manner. Thus, the regioselective preparation of the isomeric monomethyl ethers (**9b**/**9e** and **10b**/**10e**) was unambiguously accomplished.

Our new aromatic synthesis was applied to the preparation of sappanin (**10g**),[9] which was isolated from sappan lignum. This compound (**10g**) has already been derived from 1,4,3′,4′-tetramethoxybiphenyl, which is one of three isomeric biphenyls assembled by the Ullmann reaction of 1,2-dimethoxy-4-iodobenzene and 1,3-dimethoxy-4-iodobenzene.[9c] The above new annelation method led to the objective compound without isomer formation, as follows. The cyclic enone (**7e**), a synthetic intermediate for **10g**, was obtained by the condensation of **1** with the Mannich base (**4b**), which was obtained from 2′,4′-dibenzyloxyacetophenone[10] in the usual way.[7f] Hydrolysis and isomerization of **7e** gave one biphenyl derivative (**10h**), which was converted into sappanin (**10g**) by debenzylation.

In conclusion, the 1,2-benzenediols (**8a—c**, **9a** and **10a**) were prepared from the cyclohexenones (**5a—c**, **6a** and **7a**), which were obtained from [3C+3C] annelation involving 1,1-bis(ethylthio)-2-propanone (**1**) and the Mannich bases (**2a—c**, **3** and **4a**). Several kinds of 1,2-benzenediol monoethers (**8d—h**, **9b—e** and **10b—e**) were also constructed from enones (**5a—c**, **6a** and **7a**) and alcohols. Sappanin (**10g**) was synthesized as a practical application. The present method is the first example of 1,2-benzenediol formation *via* [3C+3C] annelation.

## Experimental

IR spectra were recorded on a Hitachi 270-30 infrared spectrometer. $^1$H-NMR spectra were recorded with a JEOL JNM-PMX 60si spectrometer (60 MHz) or a JNM-GX270 FT spectrometer (270 MHz) using tetramethylsilane as an internal standard. Melting points were measured on a Yanaco model MP micro melting point apparatus and are uncorrected. High-resolution mass spectra (HRMS) were obtained on a JEOL JMS-DX300 mass spectrometer. NaH refers to sodium hydride suspended in mineral oil (60%). All organic extracts were dried over anhydrous $MgSO_4$. Column chromatography was performed with Kieselgel 60 (70—230 mesh).

**6,6-Bis(ethylthio)-1,2,3,6,7,7a-hexahydro-5(5*H*)-indenone (5a)** A solution of 1,1-bis(ethylthio)-2-propanone (**1**) (1.78 g, 0.01 mol) in DME (20 ml) was added to a suspension of NaH (120 mg, 0.3 eq) in DME (20 ml) with stirring at 0 °C. After 15 min at room temperature, a solution of 2-[(dimethylamino)methyl]cyclopentanone (**2a**)[7a] (1.73 g, 0.01 mol) in DME (10 ml) was added to the resultant clear solution at room temperature. The reaction mixture was refluxed for 15 min and the solvent was evaporated off under reduced pressure. The residue was taken up in water, acidified with diluted HCl and extracted with $Et_2O$. The extract was washed with brine, dried and then evaporated under reduced pressure. The residue was chromatographed on silica gel with $CH_2Cl_2$ to give title compound (**5a**) as an oil (1.26 g, 49%). IR (film): 1685 cm$^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.16, 1.23 (each 3H, t, $J$=10 Hz, $SCH_2C\underline{H}_3 \times 2$), 1.5—3.0 (m), 5.80 (1H, br s, $C_4$-H). HRMS: Calcd for $C_{13}H_{20}OS_2$ (256.0956). Found: *m/z* 256.0966 ($M^+$). *Anal.* Calcd for $C_{13}H_{20}OS_2$: C, 60.89; H, 7.86. Found: C, 61.15; H, 7.76.

**3,3-Bis(ethylthio)-4,4a,5,6,7,8-hexahydro-2(3*H*)-naphthalenone (5b)** Compound **5b** was obtained from **1** and 2-[(dimethylamino)methyl]cyclohexanone (**2b**)[7b] in the same manner as described for **5a** in 76% yield as an oil. IR (film): 1668 cm$^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.15, 1.23 (each 3H, t, $J$=10 Hz, $SCH_2C\underline{H}_3 \times 2$), 1.5—2.9 (m), 5.73 (1H, br s, $C_1$-H). HRMS: Calcd for $C_{14}H_{22}OS_2$ (270.1112). Found: *m/z* 270.1124 ($M^+$). *Anal.* Calcd for $C_{14}H_{22}OS_2$: C, 62.17; H, 8.20. Found: C, 62.10; H, 8.02.

**3,3-Bis(ethylthio)-3,4,4a,5,6,7,8,9-octahydro-2(2*H*)-benzocycloheptenone (5c)** Compound **5c** was obtained from **1** and 2-[(dimethylamino)-methyl]cycloheptanone (**2c**)[7c] in the same manner as described for **5a** in 78% yield as an oil. IR (film): 1664 cm$^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.16, 1.25 (each 3H, t, $J$=10 Hz, $SCH_2C\underline{H}_3 \times 2$), 1.3—2.8 (m), 5.79 (1H, d-like, $C_1$-H). HRMS: Calcd for $C_{15}H_{24}OS_2$ (284.1269). Found: *m/z* 284.1261 ($M^+$). *Anal.* Calcd for $C_{15}H_{24}OS_2$: C, 63.33; H, 8.50. Found: C, 63.25; H, 8.38.

**6,6-Dimethoxy-1,2,3,6,7,7a-hexahydro-5(5*H*)-indenone (5d)** A solution of MPC (3.54 g, 7.8 mmol) in THF (10 ml) was added to a solution of **5a** (1 g, 3.9 mmol) in $CHCl_3$ (5 ml) and MeOH (5 ml) with stirring at 0 °C. Stirring was continued for 30 min at room temperature and the precipitates formed were fitered off. Saturated aqueous $NaHCO_3$ was added to the filtrate and the product was extracted with $Et_2O$. The organic extract was washed with brine, dried, and then evaporated under reduced pressure. The residue was subjected to column chromatography on silica gel with $CH_2Cl_2$ to give the title compound (**5d**) as an oil (421 mg, 55%). IR (film): 1686, 1648 cm$^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.1—3.0 (m), 3.20, 3.33 (each 3H, s, OMe × 2), 5.85 (1H, br s, $C_4$-H). HRMS: Calcd for $C_{11}H_{16}O_3$ (196.1100). Found: *m/z* 196.1108 ($M^+$).

**3,3-Dimethoxy-4,4a,5,6,7,8-hexahydro-2(3*H*)-naphthalenone (5e)** Compound **5e** was obtained from **5b** in the same manner as described for **5d** in 72% yield as an oil. IR (film): 1690, 1630 cm$^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.1—2.8 (m), 3.16, 3.34 (each 3H, s, OMe × 2), 5.77 (1H, br s, $C_1$-H). HRMS: Calcd for $C_{12}H_{18}O_3$ (210.1256). Found: *m/z* 210.1242 ($M^+$).

**3,3-Dimethoxy-3,4,4a,5,6,7,8,9-octahydro-2(2*H*)-benzocycloheptenone (5f)** Compound **5f** was obtained from **5c** in the same manner as described for **5d** in 57% yield as an oil. IR (film): 1674, 1620 cm$^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.4—2.8 (m), 3.16, 3.33 (each 3H, s, OMe × 2), 5.80 (1H, d-like, $C_1$-H). HRMS: Calcd for $C_{13}H_{20}O_3$ (224.1413). Found: *m/z* 224.1407 ($M^+$).

**6,6-Diethoxy-1,2,3,6,7,7a-hexahydro-5(5*H*)-indenone (5g)** A solution of MPC (3.54 g) in THF (10 ml) was added to a solution of **5a** (1 g) in $CHCl_3$ (5 ml) and EtOH (5 ml) with stirring at 0 °C. Stirring was continued for 30 min at room temperature and the precipitates formed were filtered off. Saturated aqueous $NaHCO_3$ was added to the filtrate and the product was extracted with $Et_2O$. The organic extract was washed with brine, dried, and then evaporated under reduced pressure. The residue was subjected to column chromatography on silica gel with $CH_2Cl_2$ to give the title compound (**5g**) as pale yellow crystals (374 mg, 43%), mp 80—83 °C. IR (Nujol): 1646 cm$^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.10, 1.22 (each 3H, t, $J$=10 Hz, $OCH_2C\underline{H}_3 \times 2$), 1.5—3.8 (m), 5.80 (1H, br s, $C_4$-H). HRMS: Calcd for $C_{13}H_{20}O_3$ (224.1413). Found: *m/z* 224.1416 ($M^+$).

**2,2-Bis(ethylthio)-1,9,10,10a-tetrahydro-3(2*H*)-phenanthrenone (6a)** Compound **6a** was obtained from **1** and 2-[(dimethylamino)methyl]-3,4-dihydro-1(2*H*)-naphthalenone (**3**)[7d] in the same manner as described for **5a** in 62% yield as pale yellow crystals, mp 113—114 °C (EtOH). IR (Nujol): 1646, 1592 cm$^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.16 and 1.26 (6H, each t, $J$=8 Hz, $SCH_2C\underline{H}_3 \times 2$), 1.5—3.2 (m), 6.52 (1H, d, $J$=2 Hz, $C_4$-H), 7.0—7.9 (4H, m, aromatic H). *Anal.* Calcd for $C_{18}H_{22}OS_2$: C 67.88; H, 6.96. Found: C, 67.76; H, 6.89.

**2,2-Dimethoxy-1,9,10,10a-tetrahydro-3(2*H*)-phenanthrenone (6b)** Compound **6b** was obtained from **6a** in the same manner as described for **5d** in 35% yield as an oil. IR (film): 1678, 1610, 1594 cm$^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.6—3.1 (m), 3.20 and 3.36 (each 3H, s, OMe × 2), 6.53 (1H, d, $J$=2 Hz, $C_4$-H), 7.1—7.9 (4H, m, aromatic H). HRMS: Calcd for $C_{16}H_{18}O_3$ (258.1256). Found: *m/z* 258.1281 ($M^+$).

**2,2-Diallyloxy-1,9,10,10a-tetrahydro-3(2*H*)-phenanthrenone (6d)** A solution of MPC (2.80 g) in THF (10 ml) was added to a solution of **6a** (1 g) in $CHCl_3$ (5 ml) and allyl alcohol (0.75 g) with stirring at 0 °C. Stirring was continued for 30 min at room temperature and the precipitates formed were filtered off. Saturated aqueous $NaHCO_3$ was added to the filtrate and the product was extracted with $Et_2O$. The organic extract was washed with brine, dried, and then evaporated under reduced pressure. The residue

was subjected to column chromatography on silica gel with $CH_2Cl_2$ to give the title compound (**6d**) as an oil (448 mg, 46%). IR (film): 1678, 1615, 1592 $cm^{-1}$. $^1$H-NMR (270 MHz, $CDCl_3$) $\delta$: 1.5—2.1 (m), 2.6—3.1 (m), 3.8—4.2 (4H, m, $OC\underline{H}_2$–CH=$CH_2$ × 2), 5.05—5.40 (4H, m, $OCH_2$–CH=$C\underline{H}_2$ × 2), 5.7—6.1 (2H, m, $OCH_2$–$C\underline{H}$=$CH_2$ × 2), 6.59 (1H, d, $J$=2.2 Hz, $C_4$-H), 7.15—7.40 (3H, m), 7.79 (1H, d, $J$=8.1 Hz, $C_5$-H). HRMS: Calcd for $C_{20}H_{22}O_3$ (310.1569). Found: $m/z$ 310.1581 ($M^+$).

**1,1-Bis(ethylthio)-4-phenyl-3-cyclohexen-2-one (7a)** Compound **7a** was obtained from **1** and 3-dimethylaminopropiophenone (**4a**)[7e] in the same manner as described for **5a** in 69% yield as pale yellow crystals, mp 52—54 °C (EtOH). IR (Nujol): 1658 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.23 (6H, t, $J$=10 Hz, $SCH_2C\underline{H}_3$ × 2), 2.3—3.0 (m), 6.35 (1H, br d, $C_3$-H), 7.36 (5H, m, aromatic H). HRMS: Calcd for $C_{16}H_{20}OS_2$ (292.0956). Found: $m/z$ 292.0964 ($M^+$). *Anal.* Calcd for $C_{16}H_{20}OS_2$: C, 65.71; H, 6.89. Found: C, 65.45; H, 6.89.

**1,1-Dimethoxy-4-phenyl-3-cyclohexen-2-one (7b)** Compound **7b** was obtained from **7a** in the same manner as described for **5d** in 82% yield as an oil. IR (film): 1682, 1612 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.28 (2H, t-like, $C_6$-$H_2$), 2.90 (2H, t-like, $C_5$-$H_2$), 3.28 (6H, s, OMe × 2), 6.33 (1H, br s, $C_3$-H), 7.40 (5H, m, aromatic H). HRMS: Calcd for $C_{14}H_{16}O_3$ (232.1100). Found: $m/z$ 232.1121 ($M^+$).

**1,1-Diallyloxy-4-phenyl-3-cyclohexen-2-one (7d)** Compound **7d** was obtained from **7a** in the same manner as described for **6d** in 26% yield as an oil. IR (film): 1684, 1612 $cm^{-1}$. $^1$H-NMR (270 MHz, $CDCl_3$) $\delta$: 2.34 (2H, t-like, $C_6$-$H_2$), 2.92 (2H, t-like, $C_5$-$H_2$), 4.08 (4H, d-like, $OC\underline{H}_2$–CH=$CH_2$ × 2), 5.0—5.4 (4H, m, $OCH_2$–CH=$C\underline{H}_2$ × 2), 5.7—6.3 (2H, m, $OCH_2$–$C\underline{H}$=$CH_2$ × 2), 6.33 (1H, br s, $C_3$-H), 7.40 (5H, s-like, aromatic H). HRMS: Calcd for $C_{18}H_{20}O_3$ (284.1413). Found: $m/z$ 284.1414 ($M^+$).

**1,1-Bis(ethylthio)-4-(2,4-dibenzyloxy)phenyl-3-cyclohexen-2-one (7e)** A mixture of 2′,4′-dibenzyloxyacetophenone[10] (3.4 g), paraformaldehyde (0.62 g), dimethylamine hydrochloride (0.84 g), concentrated HCl (0.1 ml) and 2-propanol (5.7 ml) was refluxed for 7 h. The solvent was evaporated off under reduced pressure and water was added to the residue. The solution was washed with $Et_2O$ and made basic to litmus with 10% $Na_2CO_3$ solution. The product was extracted with $Et_2O$, and the organic was washed with brine, and then dried. The solvent was evaporated off under reduced pressure to give 2′,4′-dibenzyloxy-3-(dimethylamino)-propiophenone (**4b**) as an oil (3.73 g, 97%). IR (Nujol): 2860, 2820, 2760, 1659, 1606 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.10 (6H, s, $NMe_2$), 2.60 (2H, t-like, $CH_2$–N), 3.10 (2H, t-like, CO–$CH_2$), 5.05 (4H, s, $OC\underline{H}_2Ph$ × 2), 6.57 (2H, m, $C_{3'}$-H and $C_{5'}$-H), 7.33 (10H, s, $OCH_2\underline{Ph}$ × 2), 7.81 (1H, d, $J$=8 Hz, $C_{6'}$-H). Compound **7e** was obtained from **1** and **4b** in the same manner as described for **5a** in 62% yield as pale yellow crystals, mp 100—101 °C (EtOH). IR (Nujol): 1652, 1606 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.25 (6H, t, $J$=7 Hz, $SCH_2C\underline{H}_3$ × 2), 2.3—2.9 (5H, m), 5.05 (4H, s, $OC\underline{H}_2Ph$ × 2), 6.20 (1H, br s, $C_3$-H), 6.60 (2H, m, $C_{3'}$-H and $C_{5'}$-H), 7.1—7.3 (aromatic H). *Anal.* Calcd for $C_{30}H_{32}O_3S_2$: C, 71.39, H, 6.39. Found: C, 71.28; H, 6.36.

**2,3-Dihydro-1*H*-indene-5,6-diol (8a)** A solution of MPC (1.77 g) in THF (5 ml) was added to a solution of **5a** (500 mg) in $CHCl_3$ (3 ml) with stirring at room temperature. Stirring was continued for 30 min at room temperature and the precipitates formed were filtered off. Water was added to the filtrate and the product was extracted with $Et_2O$. The organic extract was washed with brine, dried, and then evaporated under reduced pressure. The resulting residue was taken up in AcOH (5 ml) and the mixture was refluxed for 1 h. The solvent was evaporated off under reduced pressure and the residue was subjected to column chromatography with 2% (v/v) MeOH–$CH_2Cl_2$ to give the title compound (**8a**) as pale yellow crystals (117 mg, 20%), mp 114—116 °C (petroleum ether), (lit.[11] mp 116 °C). IR (Nujol): 3272, 1614 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.10 (2H, q-like, $C_2$-$H_2$), 2.80 (4H, t-like, $C_1$-$H_2$ and $C_3$-$H_2$), 4.50 (2H, s, OH × 2), 6.66 (2H, s, $C_4$-H and $C_7$-H). HRMS; Calcd for $C_9H_{10}O_2$ (150.0681). Found: $m/z$ 150.0670 ($M^+$).

**5,6,7,8-Tetrahydro-2,3-naphthalenediol (8b)** Compound **8b** was obtained from **5b** in the same manner as described for **8a** in 27% yield as pale yellow crystals, mp 127—130 °C, (lit.[12] mp 125—128 °C). IR (Nujol): 3480, 3332, 1612 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.71 (4H, m, $C_6$-$H_2$ and $C_7$-$H_2$), 2.61 (4H, m, $C_5$-$H_2$ and $C_8$-$H_2$), 4.89 (2H, s, OH × 2), 6.53 (2H, s, $C_1$-H and $C_4$-H). HRMS: Calcd for $C_{10}H_{12}O_2$ (164.0837). Found: $m/z$ 164.0862 ($M^+$).

**6,7,8,9-Tetrahydro-5*H*-benzocycloheptene-2,3-diol (8c)** Compound **8c** was obtained from **5c** in the same manner as described for **8a** in 41% yield as colorless needles, mp 108—109 °C (pertroleum ether), (lit. mp 115—116 °C,[13] mp 121 °C[14]). IR (Nujol): 3240, 1620, 1600 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.69 (6H, m, $C_6$-$H_2$, $C_7$-$H_2$ and $C_8$-$H_2$), 2.68 (4H, m, $C_5$-$H_2$ and $C_9$-$H_2$), 3.80 (2H, br, OH × 2), 6.53 (2H, s, $C_1$-H and $C_4$-H). HRMS: Calcd for $C_{11}H_{14}O_2$ (178.0994). Found: $m/z$ 178.0977 ($M^+$). Diacetate of **8c**, mp 120—123 °C (EtOH), (lit.[15] mp 116—117 °C). HRMS: Calcd for $C_{15}H_{18}O_4$ (262.1205). Found: $m/z$ 262.1223 ($M^+$).

**2,3-Dihydro-6-methoxy-1*H*-inden-5-ol (8d)** A solution of **5d** (200 mg) in AcOH (3 ml) was refluxed for 1 h. The solvent was evaporated off under reduced pressure and the residue was subjected to column chromatography with 2% (v/v) MeOH–$CH_2Cl_2$ to yield **8d** as pale yellow crystals (88 mg, 53%), mp 62—64 °C. IR (Nujol): 3384, 1625, 1602 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.05 (2H, q-like, $C_2$-$H_2$), 2.83 (4H, t-like, $C_1$-$H_2$ and $C_3$-$H_2$), 3.85 (3H, s, OMe), 5.50 (1H, s, OH), 6.75, 6.80 (each 1H, s, $C_4$-H and $C_7$-H). HRMS: Calcd for $C_{10}H_{12}O_2$ (164.0837). Found: $m/z$ 164.0826 ($M^+$). Benzoate of **8d**, mp 152—154 °C (MeOH). *Anal.* Calcd for $C_{17}H_{16}O_3$: C, 76.10; H, 6.01. Found: C, 75.91; H, 6.13.

**3-Methoxy-5,6,7,8-tetrahydro-2-naphthol (8e)** Compound **8e** was obtained from **5e** in the same manner as described for **8d** in 52% yield as pale yellow crystals, mp 59—62 °C (petroleum ether). IR (Nujol): 3388, 1630, 1600 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.72 (4H, m, $C_6$-$H_2$ and $C_7$-$H_2$), 2.63 (4H, m, $C_5$-$H_2$ and $C_8$-$H_2$), 3.79 (3H, s, OMe), 5.33 (1H, s, OH), 6.46, 6.56 (each 1H, s, $C_1$-H and $C_4$-H). HRMS: Calcd for $C_{11}H_{14}O_2$ (178.0994). Found: $m/z$ 178.1012 ($M^+$). Benzoate of **8e**, mp 108—109 °C (MeOH). *Anal.* Calcd for $C_{18}H_{18}O_3$: C, 76.57; H, 6.43. Found: C, 76.38; H, 6.41.

**3-Methoxy-6,7,8,9-tetrahydro-5*H*-benzocyclohepten-2-ol (8f)** Compound **8f** was obtained from **5f** in the same manner as described for **8d** in 77% yield as pale yellow crystals, mp 84—86 °C, (lit.[14] mp 110 °C). IR (Nujol): 3436, 1620, 1594 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.70 (6H, m, $C_6$-$H_2$, $C_7$-$H_2$ and $C_8$-$H_2$), 2.66 (4H, m, $C_5$-$H_2$ and $C_9$-$H_2$), 3.83 (3H, s, Me), 5.33 (1H, s, OH), 6.60, 6.66 (each 1H, s, $C_1$-H and $C_4$-H). HRMS: Calcd for $C_{12}H_{16}O_2$ (192.1150). Found: $m/z$ 192.1148 ($M^+$). Benzoate of **8f**, mp 112—113 °C (EtOH). *Anal.* Calcd for $C_{19}H_{20}O_3$: C, 77.00; H, 6.80. Found: C, 76.72; H, 6.76. Acetate of **8f**, mp 63—65 °C (EtOH). HRMS: Calcd for $C_{14}H_{18}O_3$ (234.1256). Found: $m/z$ 234.1246 ($M^+$).

**2,3-Dihydro-6-ethoxy-1*H*-inden-5-ol (8g)** Compound **8g** was obtained from **5g** in the same manner as described for **8d** in 57% yield as pale yellow crystals, mp 37—38 °C. IR (Nujol): 3564, 3308, 1614, 1598 $cm^{-1}$. $^1$H-NMR (270 MHz, $CDCl_3$) $\delta$: 1.40 (3H, t, $J$=10 Hz, $OCH_2C\underline{H}_3$), 2.10 (2H, q-like, $C_2$-$H_2$), 2.81 (4H, t-like, $C_1$-$H_2$ and $C_3$-$H_2$), 4.04 (2H, q, $J$=10 Hz, ($OC\underline{H}_2CH_3$), 5.50 (1H, s, OH), 6.69, 6.78 (each 1H, $C_4$-H and $C_7$-H). HRMS: Calcd for $C_{11}H_{14}O_2$ (178.0994). Found: $m/z$ 178.0998 ($M^+$). 3,5-Dinitrobenzoate of **8g**, mp 140—142 °C (MeOH). *Anal.* Calcd for $C_{18}H_{16}N_2O_7$: C, 58.07; H, 4.33; N, 7.52. Found: C, 57.78; H, 4.33; N, 7.46.

**3-Benzyloxy-5,6,7,8-tetrahydro-2-naphthol (8h)** A solution of MPC (2.83 g) in THF (10 ml) was added to a solution of **5b** (1 g) in $CHCl_3$ (5 ml) and benzyl alcohol (1.6 g) with stirring at 0 °C. Stirring was continued for 30 min at room temperature and the precipitates formed were filtered off. Saturated aqueous $NaHCO_3$ was added to the filtrate and the product was extracted with $Et_2O$. The organic extract was washed with brine, dried, and then evaporated under reduced pressure. The residue was subjected to column chromatography on silica gel with $CH_2Cl_2$ to give 3,3-dibenzyloxy-4,4a,5,6,7,8-hexahydro-2(3*H*)-naphthalenone (**5h**) as an oil (455 mg, 34%). IR (film): 1690 $cm^{-1}$. $^1$H-NMR (270 MHz, $CDCl_3$) $\delta$: 1.1—2.8 (m), 4.66 (4H, m, $OC\underline{H}_2Ph$ × 2), 1.74 (1H, br s, $C_1$-H), 7.21, 7.29 (10H each s-like, $OCH_2\underline{Ph}$ × 2). Compound **8h** was obtained from **5h** in the same manner as described for **8d** in 67% yield as pale yellow crystals, mp 49—54 °C. IR (Nujol): 3440, 1600 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.72 (4H, m, $C_6$-$H_2$ and $C_7$-$H_2$), 2.62 (4H, m, $C_5$-$H_2$ and $C_8$-$H_2$), 4.98 (2H, s, $OC\underline{H}_2Ph$), 6.89 (2H, s-like, $C_1$-H and $C_4$-H), 7.30 (5H, s-like, $OCH_2\underline{Ph}$). HRMS: Calcd for $C_{17}H_{18}O_2$ (254.1307). Found: $m/z$ 254.1331 ($M^+$). Methylether of **8h**, mp 74—75 °C (diisopropylether). IR (Nujol): 1608 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 1.73 (4H, m, $C_6$-$H_2$ and $C_7$-$H_2$), 2.65 (4H, m, $C_5$-$H_2$ and $C_8$-$H_2$), 3.80 (3H, s, OMe), 5.03 (2H, s, $OC\underline{H}_2Ph$), 6.53 (2H, br s, $C_1$-H and $C_4$-H), 7.30 (5H, m, $OCH_2\underline{Ph}$). HRMS: Calcd for $C_{18}H_{20}O_2$ (268.1463). Found: $m/z$ 268.1445 ($M^+$). *Anal.* Calcd for $C_{18}H_{20}O_2$: C, 80.56; H, 7.51. Found: C, 80.47; H, 7.66.

**9,10-Dihydro-2,3-phenanthrenediol (9a)** Compound **9a** was obtained from **6a** in the same manner as described for **8a** in 67% yield as pale yellow crystals, mp 104—106 °C (cyclohexane). IR (Nujol): 3304, 1618 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.65 (4H, br s, $C_9$-$H_2$ and $C_{10}$-$H_2$), 6.60 (1H, br s, $C_1$-H), 7.0—7.5 (5H, m). HRMS: Calcd for $C_{14}H_{12}O_2$ (212.0837). Found: $m/z$ 212.0823 ($M^+$). Diacetate of **9a**, mp 172—173 °C (EtOH). HRMS: Calcd for $C_{18}H_{16}O_4$ (296.1049). Found: $m/z$ 296.1064 ($M^+$). *Anal.* Calcd for: $C_{18}H_{16}O_4$: C, 72.96, H, 5.44. Found: C,

73.01; H, 5.48.

**2-Methoxy-9,10-dihydro-3-phenanthrenol (9b)** Compound **9b** was obtained from **6b** in the same manner as described for **8d** in 48% yield as pale yellow crystals, mp 130—131 °C (cyclohexane). IR (Nujol): 3550, 3400, 1630, 1605, 1580 $cm^{-1}$. $^1$H-NMR (270 MHz, $CDCl_3$) $\delta$: 2.80 (4H, m, $C_9$-$H_2$ and $C_{10}$-$H_2$), 3.89 (3H, s, OMe), 6.71 (1H, s-like, $C_1$-H), 7.1—7.3 (3H, m), 7.34 (1H, br s, $C_4$-H), 7.61 (1H, d, $J$=7.4 Hz, $C_5$-H). HRMS: Calcd for $C_{15}H_{14}O_2$ (226.0994). Found: *m/z* 226.1018 ($M^+$). *Anal.* Calcd for $C_{15}H_{12}O_2$: C, 79.62; H, 6.24. Found: C, 79.71; H. 6.34.

**2-Benzyloxy-9,10-dihydro-3-phenanthrenol (9c)** Compound **6a** was converted into 2,2-dibenzyloxy-1,9,10,10a-tetrahydro-3(2*H*)-phenanthrenone (**6c**) in the same manner as described for **5h** in 33% yield as an oil. IR (film): 1680, 1615, 1595 $cm^{-1}$. $^1$H-NMR (270 MHz, $CDCl_3$) $\delta$: 1.5—2.1 (m), 2.6—3.1 (m), 4.42 (1H, d, $J$=11.7 Hz, OCHPh), 4.73 (1H, d, $J$=11.7 Hz, OCHPh), 4.75 (1H, d, $J$=12.1 Hz, OCHPh), 4.84 (1H, d, $J$=12.1 Hz, OCHPh), 6.62 (1H, d, $J$=2.6 Hz, $C_4$-H), 7.1—7.5 (13H, m, aromatic H), 7.76 (1H, d, $J$=7.7 Hz, $C_5$-H). Compound **9c** was obtained from **6c** in the same manner as described for **8d** in 88% yield as pale yellow crystals, mp 99—102 °C. IR (Nujol): 3560, 1605, 1575 $cm^{-1}$. $^1$H-NMR (270 MHz, $CDCl_3$) $\delta$: 2.79 (4H, m, $C_9$-$H_2$ and $C_{10}$-$H_2$), 5.10 (2H, s, $OCH_2Ph$), 6.79 (1H, br s, $C_1$-H), 7.1—7.4 (4H, m), 7.61 (1H, d, $J$=7.3 Hz, $C_5$-H). HRMS: Calcd for $C_{21}H_{18}O_2$ (302.1307). Found: *m/z* 302.1297 ($M^+$).

**2-Allyloxy-9,10-dihydro-3-phenanthrenol (9d)** Compound **9d** was obtained from **6d** in the same manner as described for **8d** in 77% yield as pale yellow crystals, mp 67—69 °C. IR (Nujol): 3440, 1605 $cm^{-1}$. $^1$H-NMR (270 MHz, $CDCl_3$) $\delta$: 2.80 (4H, m, $C_9$-$H_2$ and $C_{10}$-$H_2$), 4.61 (2H, ddd, $J$=5.9, 1.4, 1.4 Hz, $OCH_2CH=CH_2$), 5.32 (1H, ddd, $J$=11.0, 1.4, 1.4 Hz, $OCH_2CH=CH$), 5.42 (1H, ddd, $J$=17.2, 1.4, 1.4 Hz, $OCH_2CH=CH$), 6.07 (1H, m, $OCH_2CH=CH_2$), 6.72 (1H, s, $C_1$-H), 7.1—7.3 (4H, m), 7.62 (1H, d, $J$=7.5 Hz, $C_5$-H). HRMS: Calcd for $C_{17}H_{16}O_2$ (252.1150). Found: *m/z* 252.1177 ($M^+$). Benzoate of **9d**, mp 78—80 °C (MeOH). *Anal.* Calcd for $C_{24}H_{20}O_3$: C, 80.88; H, 5.66. Found: C, 80.78; H, 5.77.

**3-Methoxy-9,10-dihydro-2-phenonthrenol (9e)** A mixture of **9f** (100 mg), 10%Pd–C (30 mg) and AcOEt (10 ml) was stirred under a hydrogen atmosphere for 7 h at room temperature. After filtration, the filtrate was evaporated under reduced pressure. The residue was subjected to column chromatography with 1% (v/v) MeOH–$CH_2Cl_2$ to give the title compound (**9e**) as colorless crystals (42 mg, 59%), mp 89—90 °C (petroleum ether). IR (Nujol): 3500, 1625, 1595, 1570 $cm^{-1}$. $^1$H-NMR (270 MHz, $CDCl_3$) $\delta$: 2.79 (4H, m, $C_9$-$H_2$ and $C_{10}$-$H_2$), 3.95 (3H, s, OMe), 6.80 (1H, s, $C_1$-H), 7.1—7.3 (4H, m, aromatic H), 7.62 (1H, d, $J$=7.7 Hz, $C_5$-H). HRMS: Calcd for $C_{15}H_{14}O_2$ (226.0994). Found: *m/z* 226.1022 ($M^+$). *Anal.* Calcd for $C_{15}H_{14}O_2$: C, 79.62; H, 6.24. Found: C, 79.63; H, 6.26.

**2-Benzyloxy-3-methoxy-9,10-dihydro-3-phenanthrene (9f)** A mixture of compound **9c** (150 mg), potassium carbonate (100 mg), dimethyl sulfate (100 mg) and acetone (5 ml) was refluxed for 8 h. After filtration, the filtrate was evaporated under reduced pressure. The residue was subjected to column chromatography with $CH_2Cl_2$ to yield the title compound (**9f**) as colorless crystals (119 mg, 76%), mp 114—116 °C (MeOH). IR (Nujol): 1605 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.77 (4H, br s, $C_9$-$H_2$ and $C_{10}$-$H_2$), 4.90 (3H, s, OMe), 5.13 (2H, s, $OCH_2Ph$), 6.73 (1H, s, $C_1$-H), 7.0—7.7 (5H, m, aromatic H). HRMS: Calcd for $C_{22}H_{20}O_2$ (316.1463). Found: *m/z* 316.1477 ($M^+$). *Anal.* Calcd for $C_{22}H_{20}O_2$: C, 83.41; H, 6.58. Found: C, 83.51; H, 6.37.

**3,4-Biphenyldiol (10a)** Compound **10a** was obtained from **7a** in the same manner as described for **8a** in 54% yield as pale yellow crystals, mp 137—139 °C, (lit.[16] mp 136—137.5 °C). IR (Nujol): 3744, 3492, 1604 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 3.60 (2H, br, OH×2), 6.9—7.8 (8H, m, aromatic H). HRMS: Calcd for $C_{12}H_{10}O_2$ (186.0681). Found: *m/z* 186.0685 ($M^+$). Diacetate of **10a**, mp 75—76 °C (EtOH). HRMS: Calcd for $C_{16}H_{14}O_4$ (270.0892). Found: *m/z* 270.0883 ($M^+$).

**4-Methoxy-3-biphenylol (10b)** Compound **10b** was obtained from **7b** in the same manner as described for **8d** in 51% yield as pale yellow crystals, mp 117—118 °C, (lit. mp 111 °C,[17] mp 118 °C,[18] mp 114—115 °C[19]). IR (Nujol): 3396, 1595 $cm^{-1}$. $^1$H-NMR (270 MHz, $CDCl_3$) $\delta$: 3.89 (3H, s, Me), 5.69 (1H, s, OH), 6.89 (1H, d, $J$=8.4 Hz, $C_5$-H), 7.08 (1H, dd, $J$=2.2, 8.4 Hz, $C_6$-H), 7.19 (1H, d, $J$=2.2 Hz, $C_2$-H), 7.29 (1H, d-like, $C_{4'}$-H), 7.40 (2H, t-like, $C_{3'}$-H and $C_{5'}$-H), 7.54 (2H, d-like, $C_{2'}$-H and $C_{6'}$-H). HRMS: Calcd for $C_{13}H_{12}O_2$ (200.0837). Found: *m/z* 200.0834 ($M^+$).

**4-Benzyloxy-3-biphenylol (10c)** Compound **7a** was converted into 1,1-dibenzyloxy-4-phenyl-3-cyclohexen-2-one (**7c**) in the same manner as described for **5h** in 33% yield as an oil. IR (film): 1684, 1615 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 2.42 (2H, t-like, $C_6$-$H_2$), 2.96 (2H, t-like, $C_5$-$H_2$), 4.66 (4H, s, $OCH_2Ph$×2), 6.36 (1H, br s, $C_3$-H), 7.25 (15H, s-like, aromatic H). Compound **10c** was obtained from **7c** in the same manner as described for **8d** in 79% yield as pale yellow crystals, mp 112—113 °C (cyclohexane). IR (Nujol): 3544, 1595 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 5.06 (2H, s, $OCH_2Ph$), 5.60 (1H, s, OH), 6.9—7.8 (13H, m, aromatic H). HRMS: Calcd for $C_{19}H_{16}O_2$ (276.1150). Found: *m/z* 276.1200 ($M^+$). *Anal.* Calcd for $C_{19}H_{16}O_2$: C, 82.58; H, 5.84. Found: C, 82.42; H, 5.79.

**4-Allyloxy-3-biphenylol (10d)** Compound **10d** was obtained from **7d** in the same manner as described for **8d** in 68% yield as pale yellow crystals, mp 55—57 °C. IR (Nujol): 3500, 3336, 1615, 1595 $cm^{-1}$. $^1$H-NMR (270 MHz, $CDCl_3$) $\delta$: 4.63 (2H, ddd, $J$=5.5, 1.5, 1.5 Hz, $OCH_2CH=CH_2$), 5.33 (1H, ddd, $J$=10.3, 1.5, 1.5 Hz, $OCH_2CH=CH$), 5.41 (1H, ddd, $J$=17.2, 1.5, 1.5 Hz, $OCH_2CH=CH$), 5.73 (1H, s, OH), 6.06 (1H, m, $OCH_2CH=CH_2$), 6.91 (1H, d, $J$=8.4 Hz, $C_5$-H), 7.06 (1H, dd, $J$=8.4, 2.2 Hz, $C_6$-H), 7.21 (1H, d, $J$=2.2 Hz, $C_1$-H). HRMS: Calcd for $C_{15}H_{14}O_2$ (226.0994). Found: *m/z* 226.1022 ($M^+$). Benzoate of **10d**, mp 68—69 °C (MeOH). *Anal.* Calcd for $C_{22}H_{18}O_3$: C, 79.98; H, 5.49. Found: C, 79.83; H, 5.63.

**3-Methoxy-4-biphenylol (10e)** Compound **10e** was obtained from **10f** in the same manner as described for **9e** in 66% yield as colorless crystals, mp 60—63 °C, (lit.[19] mp 73—74 °C). IR (Nujol): 3532, 1620 $cm^{-1}$. $^1$H-NMR (270 MHz, $CDCl_3$) $\delta$: 3.94 (3H, s, OMe), 6.98 (1H, d, $J$=8 Hz, $C_5$-H), 7.07 (1H, d, $J$=2 Hz, $C_2$-H), 7.10 (1H, dd, $J$=2, 8 Hz, $C_6$-H), 7.3—7.5 (5H, m, aromatic H). HRMS: Calcd for $C_{13}H_{12}O_2$ (200.0837). Found: *m/z* 200.0844 ($M^+$).

**4-Benzyloxy-3-methoxybiphenyl (10f)** Compound **10f** was obtained from **10c** in the same manner as described for **9f** in 67% yield as colorless crystals, mp 82—83 °C (MeOH). IR (Nujol): 1605, 1595 $cm^{-1}$. $^1$H-NMR (270 MHz, $CDCl_3$) $\delta$: 3.94 (3H, s, OMe), 5.19 (2H, s, $OCH_2Ph$), 6.94 (1H, d, $J$=8 Hz, $C_5$-H), 7.07 (1H, dd, $J$=2, 8 Hz, $C_6$-H), 7.12 (1H, d, $J$=2 Hz, $C_2$-H), 7.2—7.6 (10H, m, $OCH_2Ph$×2). *Anal.* Calcd for $C_{20}H_{18}O_2$: C, 82.73; H, 6.25. Found: C, 82.49; H, 6.40.

**2,4,3′,4′-Biphenyltetraol (Sappanin) (10g)** A mixture of **10h** (199 mg), EtSH (2 ml) and $BF_3 \cdot Et_2O$ (1.14 g) was stirred for 1 h at room temperature. The solution was poured into water and extracted with ether. The organic extract was washed with brine, dried, and evaporated under reduced pressure. The residue was subjected to column chromatography with 8% (v/v) MeOH–$CH_2Cl_2$ to yield the title compound (**10g**) (40 mg, 37%) as colorless crystals, mp 202—203 °C, (lit. mp 201—202 °C,[9b] mp 210—211 °C,[9c] mp 202—203 °C[9d]). IR (Nujol): 3420, 1624, 1604 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 4.0 (2H, br s, OH), 6.3—7.3 (6H, m, aromatic H). HRMS: Calcd for $C_{12}H_{10}O_4$ (218.0579). Found: *m/z* 218.0574 ($M^+$).

**2′,4′-Dibenzyloxy-3,4-biphenyldiol (10h)** Compound **10h** was obtained from **7e** in the same manner as described for **8a** in 41% yield as a pale yellow oil. IR ($CHCl_3$): 3580 $cm^{-1}$. $^1$H-NMR (60 MHz, $CDCl_3$) $\delta$: 5.0 (4H, s-like, $OCH_2Ph$×2), 6.6—7.4 (6H, m, aromatic H). Dibenzoate of **10h**, mp 152—154 °C (EtOH). *Anal.* Calcd for $C_{40}H_{30}O_6$: C, 79.19; H, 4.98. Found: C, 78.96; H, 5.09.

**Acknowledgments** We thank Messrs. Y. Ishibashi and Y. Mabuchi and Misses M. Shirota and M. Hisamatsu for their technical assistance.

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# Tannins and Related Polyphenols of Euphorbiaceous Plants. VII.[1] Tirucallins A, B and Euphorbin F, Monomeric and Dimeric Ellagitannins from *Euphorbia tirucalli* L.

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**A new monomeric hydrolyzable tannin, tirucallin A (7), and two new dimers, tirucallin B (11) and euphorbin F (14), have been isolated, together with six known polyphenols including euphorbin A, from the stems of *Euphorbia tirucalli* L., and their structures were elucidated by spectral and chemical methods. Euphorbin F (14) possesses dehydrohexahydroxydiphenoyl and valoneoyl groups, and tirucallin A (7) has a dilactonized valoneoyl group. The orientation of the valoneoyl group of euphorbins A and B was revised.**

**Keywords** *Euphorbia tirucalli*; Euphorbiaceae; tannin; hydrolyzable tannin; tirucallin A; tirucallin B; euphorbin F; euphorbin A; structure revision

The stems and roots of *Euphorbia tirucalli* L. (Euphorbiaceae) have been used as a poultice applicable to broken bones, ulceration of the nose, swellings, hemorrhoids, and painful parts of the human body, in Indonesia, the Philippines and Malaysia.[2] The latex of this plant is known to be poisonous, and more than fifteen phorbol-type diterpenoids[3] have been isolated. Triterpenes such as euphorbol[4] are also known. However, no chemical study on the polyphenolic constituents of this plant has been reported. We have recently reported that *E. hirta* L. produces unique dimeric hydrolyzable tannins, euphorbins A—E,[5] which possess a dehydrohexahydroxydiphenoyl group in the molecule, whereas some other *Euphorbia* species contain rugosins D, E or G, which are oligomers of a type different from euphorbins.[1,6] In our continuing studies on tannins and related polyphenols of Euphorbiaceous plants, we have isolated from the stems of *E. tirucalli* nine polyphenols, including three new hydrolyzable tannins named tirucallins A and B and euphorbin F.

The ethyl acetate- and 1-butanol-soluble fractions of the aqueous acetone extractive of the stems were separately subjected to a combination of Toyopearl HW-40 and MCI-gel CHP 20P column chromatography, developing with aqueous alcohol, to afford nine compounds. Among them, six compounds were identified as quercitrin (**1**), 5-*O*-caffeoylquinic acid (**2**),[7] 3,3′,4-tri-*O*-methyl-4′-*O*-rutinosylellagic acid (**3**),[8] tellimagrandin II (**4**),[9] geraniin (**5**),[10] and euphorbin A.[5a]

Tirucallin A (**7**) was obtained as a pale yellow amorphous powder, $[\alpha]_D$ +2.8° (acetone), and its molecular formula was indicated to be $C_{55}H_{34}O_{34}$ by the elemental analysis and fast-atom bombardment mass spectrum (FAB-MS), which showed an $(M+Na)^+$ ion peak at *m/z* 1261. The proton nuclear magnetic resonance ($^1$H-NMR) spectrum of **7** showed the presence of two galloyl groups [$\delta$ 6.65 and 6.83 (each 2H, s)] and a hexahydroxydiphenoyl (HHDP) group [$\delta$ 6.49 and 6.58 (each 1H, s)]. Three 1H singlets at $\delta$ 6.79, 7.11 and 7.61, which are characteristic of a dilactonized valoneoyl group, were also observed. The presence of a $^4C_1$ glucopyranose core in **7** was indicated by the coupling pattern of a seven-spin system in the aliphatic region (see Experimental). These spectral features, along with the ultraviolet (UV) (218, 266 and 355 nm) and circular dichroism (CD) ($[\theta]_{237}$ +12.0 × 10$^4$) spectral data, were similar to those of prostratin A (**8**), which was recently isolated from *Euphorbia prostrata*.[1] A remarkable difference between **7** and **8** is an upfield shift of the anomeric proton signal of the former ($\delta$ 5.69, d, $J$=8 Hz) from that of the latter ($\delta$ 6.02, d, $J$=8.5 Hz). This upfield shift of H-1 in **7**, relative to the acylated anomeric proton ($\delta$ 6.0—6.2) of the hydrolyzable tannins having a galloyl group of O-2 on a $^4C_1$ glucopyranose core such as **8** and **4**, is analogous to that of cornusiins A, B (**9**) and others,[11] which have a valoneoyl group at O-2 of the glucose core. This can be interpreted in terms of an anisotropic effect of the biphenyl moiety of the valoneoyl group.[11] Tirucallin A was thus characterized as 1,3-di-*O*-galloyl-2-*O*-dilactonized valoneoyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-$\beta$-D-glucose. This structure was substantiated by enzymatic hydrolysis of **7** to yield cornusiin B (**9**),[11] oenothein C (**10**)[11] and gallic acid.

Tirucallin B (**11**), $[\alpha]_D$ −31° (acetone), was obtained as a light brown amorphous powder. Acid hydrolysis of **11** yielded gallic acid, ellagic acid, valoneic acid dilactone and glucose. This tannin is regarded as a hydrolyzable tannin dimer consisting of three galloyl groups, an HHDP group and a valoneoyl group, as revealed by three 2H singlets ($\delta$ 7.02, 7.05, 7.13), five 1H singlets ($\delta$ 6.37, 6.75, 6.79, 6.98, 7.13), and two sugar anomeric proton signals [$\delta$ 6.30 (d, $J$=3 Hz), 6.36 (d, $J$=2 Hz)] in the $^1$H-NMR spectrum. Both glucose cores were shown to adopt $^1C_4$ conformation by the small $J_{1,2}$ and $J_{3,4}$ values (2—3 Hz) and the absence of coupling between H-2 and H-3, which are similar features to those of geraniin (**5**).[10] The hydroxyl groups at C-2′ and C-4′ in one of the glucose cores were shown to be free, by upfield shifts of H-2′ [$\delta$ 4.01 (br s)] and H-4′ [$\delta$ 4.36 (br s)], while the other glucose core is fully acylated (see Experimental). The chemical shifts of these glucose signals are in accordance with those of corilagin (**12**)[12] and punicafolin (**13**).[13] These spectral data, including the FAB-MS data [*m/z* 1593 $(M+Na)^+$], are identical with those reported for the dimer **11**, which was recently obtained as a degradation product of excoecarianin.[14] The present isolation of **11** is the first report of this compound as a natural product.

Euphorbin F (**14**), a pale yellow amorphous powder, $[\alpha]_D$ −28° (acetone), gave a positive coloration of ellagitannin with the sodium nitrite reagent[15] in the spot test on paper. It was shown to be a dimer by an $[M+Na]^+$ ion peak at *m/z* 1911 in the FAB-MS. Acid hydrolysis of **14** yielded the same products (glucose, gallic acid, ellagic acid and valoneic acid dilactone) as those from **11**. In the $^1$H-NMR spectrum

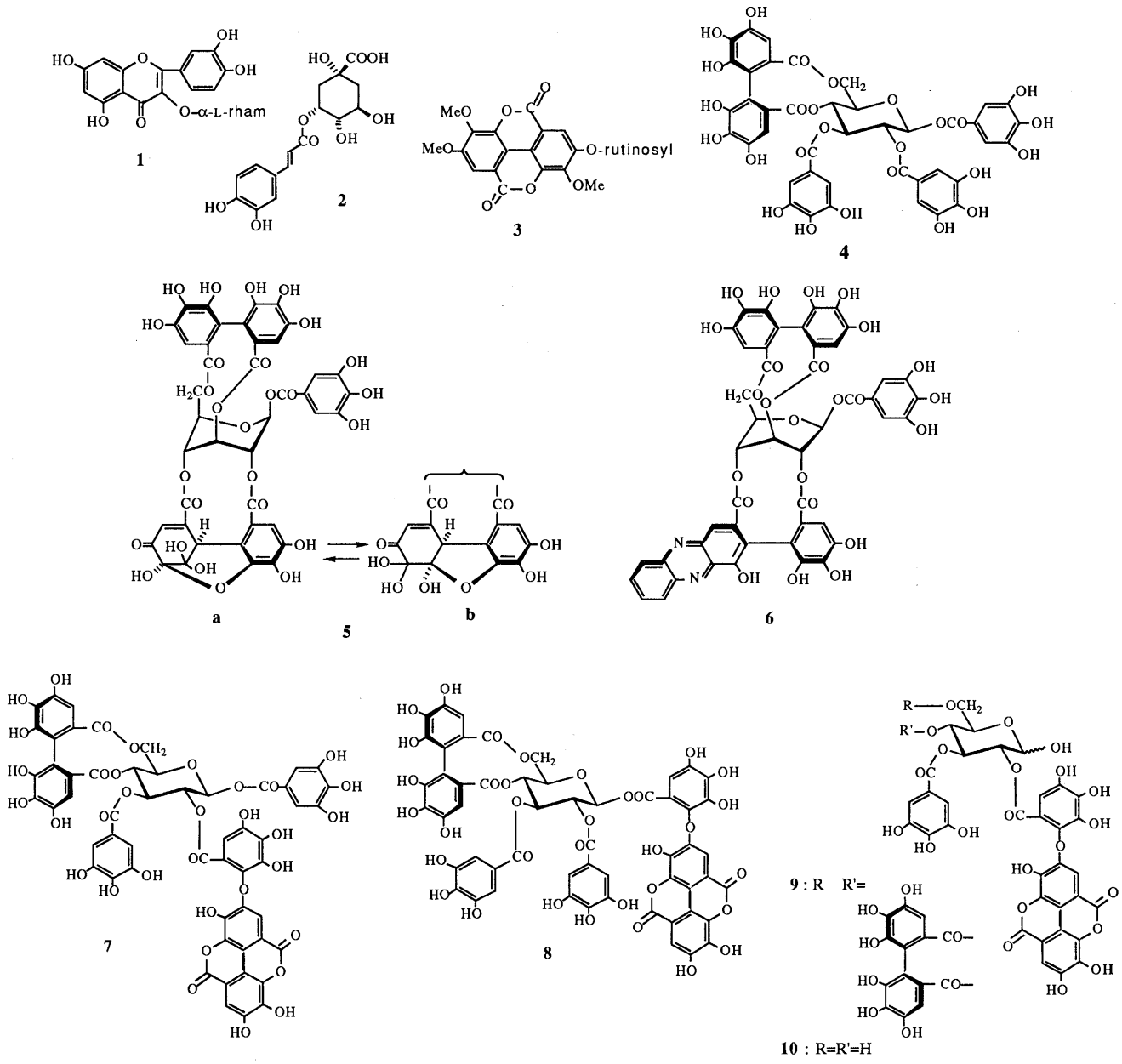
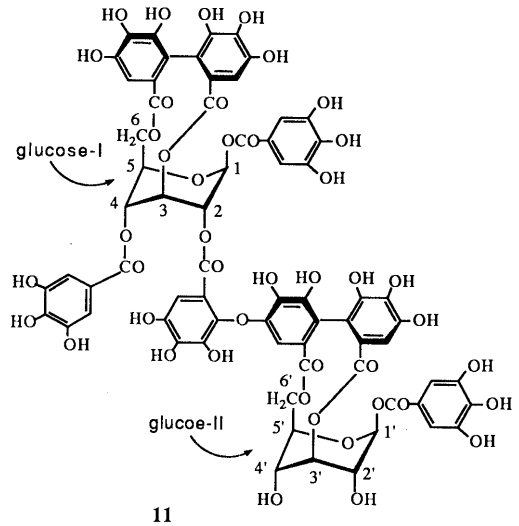



Chart 1

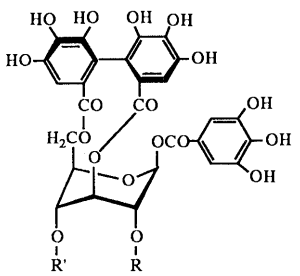
of **14**, the presence of a dehydrohexahydroxydiphenoyl (DHHDP) group equilibrating between five- and six-membered hemiacetal forms (**14a**⇄**14b**) was shown by the signal pairs of methine [$\delta$ 5.11 (s, **14a**) and 4.89 (d, $J$=1.5 Hz, **14b**)] and vinyl protons [$\delta$ 6.46 (s, **14a**) and 6.07 (br s, **14b**)]. The presence of an HHDP group, a valoneoyl group and three galloyl groups was also indicated by five 1H singlets and three 2H singlets, each duplicated in a ratio of *ca.* 1 : 1 (see Experimental). The conformations of the two glucose cores were shown to be $^4C_1$ (glucose-I) and $^1C_4$ (glucose-II) by the coupling patterns of their protons. The chemical shifts of the glucose-I signals are analogous to those of tellimagrandin II (**4**), except for the upfield shifts of H-1, H-3 and H-5 in **14**, while the chemical shifts of the glucose-II protons are closely similar to those of geraniin (**5**) (Table I). The glucose carbon resonances in the $^{13}$C-NMR spectrum of **14** also showed close similarity to the sum of those of **4** and **5** (Table II). The presence of the geraniin moiety in **14** was confirmed as follows. Upon treatment with *o*-phenylenediamine in an acidic medium, **14** gave a phenazine derivative (**15**) and a partial hydrolyzate (**16**). The $^1$H-NMR spectrum of **15** indicated a conformational change of glucose-II from $^1C_4$ to the skew-boat form, as revealed by the change of the coupling constants among its protons, H-1′—H-6′ (Table I). An upfield shift of H-1′ and a downfield shift of H-5′ from the corresponding signals of **14** were also observed. These spectral changes were analogous to those observed upon the conversion of **5** into its phenazine derivative (**6**),[10] and support the identity of the position, orientation and absolute stereostructure of the DHHDP group in **14** with those in geraniin (**5**). The location of the DHHDP group at O-2′—O-4′ in **14** was further confirmed by the formation of **16**, whose $^1$H-NMR spectrum showed upfield shifts of H-2′ ($\delta$ 4.07, br s) and H-4′ ($\delta$ 4.38, br s) in the $^1C_4$ glucose core (II), induced by the loss of the phenylphenazine moiety from **15**. Therefore,

TABLE I. $^1$H-NMR Data for the Glucose Moieties of **4**, **5**, **14** and **15** (Acetone-$d_6$ + $D_2O$, $J$ in Hz)

| | **4** | **5a** | **5b** | **14a** | **14b** | **15** |
|---|---|---|---|---|---|---|
| Glucose-I | | | | | | |
| H-1 | 6.20 (d, $J$=8) | | | 6.14 (d, $J$=8) | 6.01 | 5.95 (d, $J$=9) |
| H-2 | 5.58 (dd, $J$=8, 9.5) | | | 5.63 (dd, $J$=8, 10) | 5.65 | 5.60 (t, $J$=9) |
| H-3 | 5.83 (t, $J$=9.5) | | | 5.30 (t, $J$=10) | | 5.31 (t, $J$=9) |
| H-4 | 5.20 (t, $J$=9.5) | | | 5.09 (t, $J$=10) | | 5.09 (t, $J$=9) |
| H-5 | 4.54 (dd, $J$=6, 9.5) | | | 4.34 (dd, $J$=7, 10) | 4.38 | 4.31 (dd, $J$=6.5, 10) |
| H-6 | 5.36 (dd, $J$=6, 3) | | | 5.36 (dd, $J$=7, 13) | 5.32 | 5.24 (dd, $J$=6.5, 14) |
| | 3.87 (d, $J$=13) | | | 3.87 (d, $J$=13) | 3.86 | 3.80 (d, $J$=14) |
| Glucose-II | | | | | | |
| H-1′ | | 6.60 (br s) | | 6.53 (br s) | 6.60 | 6.11 (d, $J$=6) |
| H-2′ | | 5.60 (br s) | | 5.56 (br s) | 5.48 | 5.64 (d, $J$=6) |
| H-3′ | | 5.60 (br s) | 5.50 | 5.60 (br s) | 5.44 | 5.51 (d, $J$=4) |
| H-4′ | | 5.56 (br s) | 5.46 | 5.60 (br s) | | 5.47 (br d, $J$=4) |
| H-5′ | | 4.81 (m) | | 4.73 (m) | | 4.92 (dd, $J$=4, 8) |
| H-6′ | | 4.93 (t, $J$=11) | 4.78 (m) | 4.79 (t, $J$=10) | | 4.73 (dd, $J$=8, 12) |
| | | 4.33 (dd, $J$=8, 11) | 4.45 (dd, $J$=6, 9) | 4.24 (dd, $J$=7, 10) | 4.40 (dd, $J$=8, 10) | 4.02 (dd, $J$=4, 12) |




**11**



**12** : R=R′=H

**13** : R=R′= -OC-

Chart 2

TABLE II. $^{13}$C-NMR Data for the Glucose Moieties of **4**, **5** and **14** (Acetone-$d_6$ + $D_2O$)

| | | **4** | **5a** | **5b** | **14a** | **14b** |
|---|---|---|---|---|---|---|
| Glucose-I | C-1 | 93.8 | | | 93.6 | |
| | C-2 | 71.8 | | | 71.6 | |
| | C-3 | 73.3 | | | 73.9 | |
| | C-4 | 70.8 | | | 70.4 | |
| | C-5 | 73.1 | | | 72.7 | |
| | C-6 | 63.1 | | | 62.8 | |
| Glucose-II | C-1′ | | 90.8 | 91.8 | 91.0 | 91.9 |
| | C-2′ | | 69.9 | 70.4 | 69.9 | 70.8 |
| | C-3′ | | 63.3 | 62.3 | 63.3 | 62.5 |
| | C-4′ | | 65.9 | 66.8 | 65.9 | 66.9 |
| | C-5′ | | 72.6 | 73.1 | 72.5 | 73.4 |
| | C-6′ | | 63.6 | 63.8 | 63.8 | 64.0 |

euphorbin F was concluded to be a dimer having the tellimagrandin II and geraniin moieties as partial structures.

Conclusive proof of the structure of euphorbin F, including the position of the valoneoyl group in the molecule, was obtained by chemically correlating **14** with euphorbin E (**17**), which has recently been isolated from *E. hirta*.[5c] In this experiment, the catalytic hydrogenation of euphorbin E (**17**) yielded two products, one of which was characterized as **18**, which was produced *via* hydrogenolysis of the allylic system in the D-ring of the dehydroeuphorbinoyl group. This compound was also obtained by similar hydrogenation of **14**.

Since the structures **19** and **20**[5a] were previously assigned to euphorbin A and euphorbin B, which are isomers concerning the orientation of the valoneoyl group attached

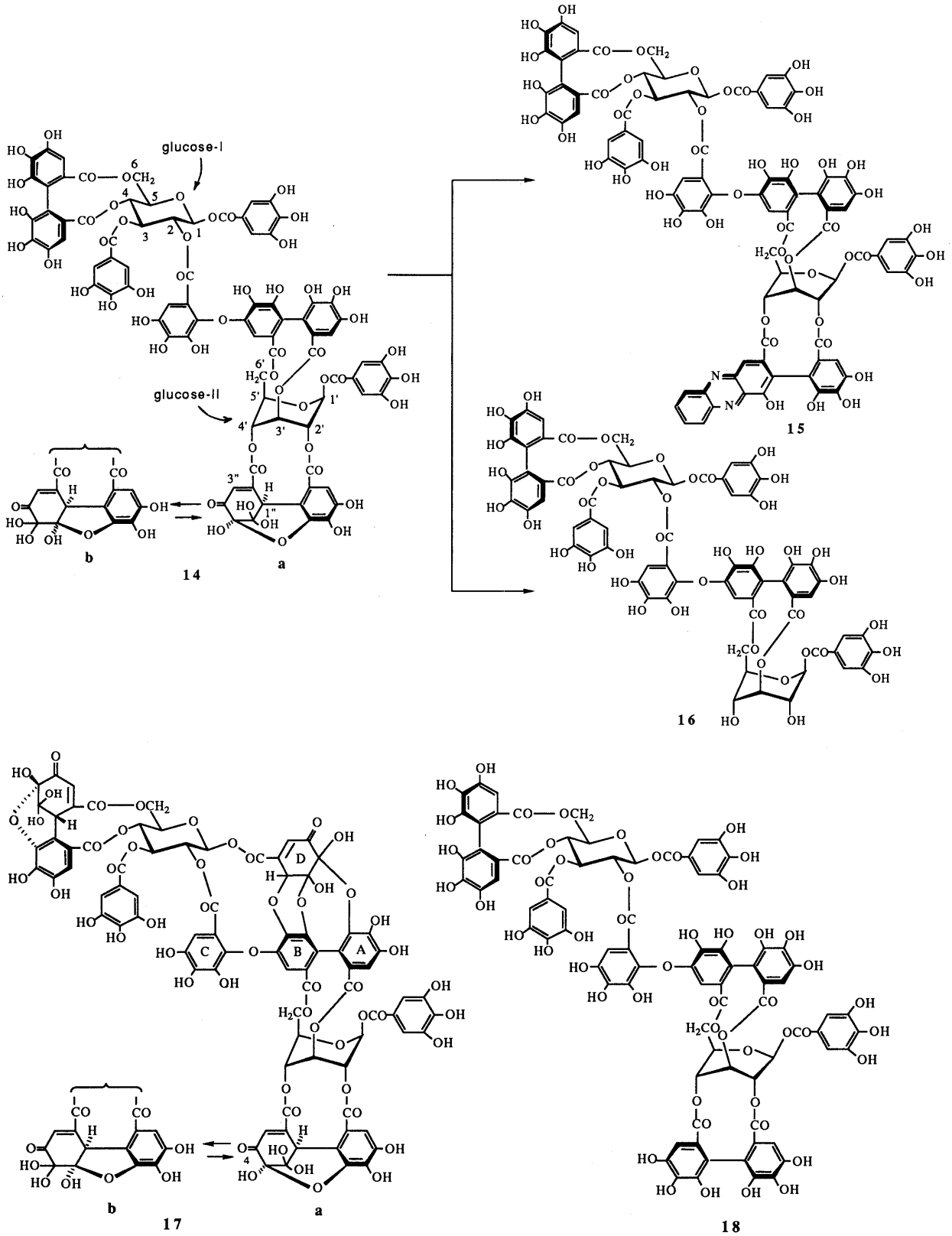



Chart 3

to O-3′/O-6′ of the glucose core-II, euphorbin F (**14**) may be characterized as an oxidative metabolite of euphorbin B. However, euphorbin B was not detected even in a trace amount in the extract of *E. tirucalli*. Therefore, the co-occurrence of euphorbins F and A prompted us to re-examine the previously proposed structures of euphorbins A and B.

The assignment of the orientations of the valoneoyl groups in euphorbins A and B was based on $^1$H-NMR spectral comparison, with the inspection of stereomodels.[5a] This comparison revealed that the chemical shifts of H-1, H-3 and H-5 of the glucose core-I were noticeably different

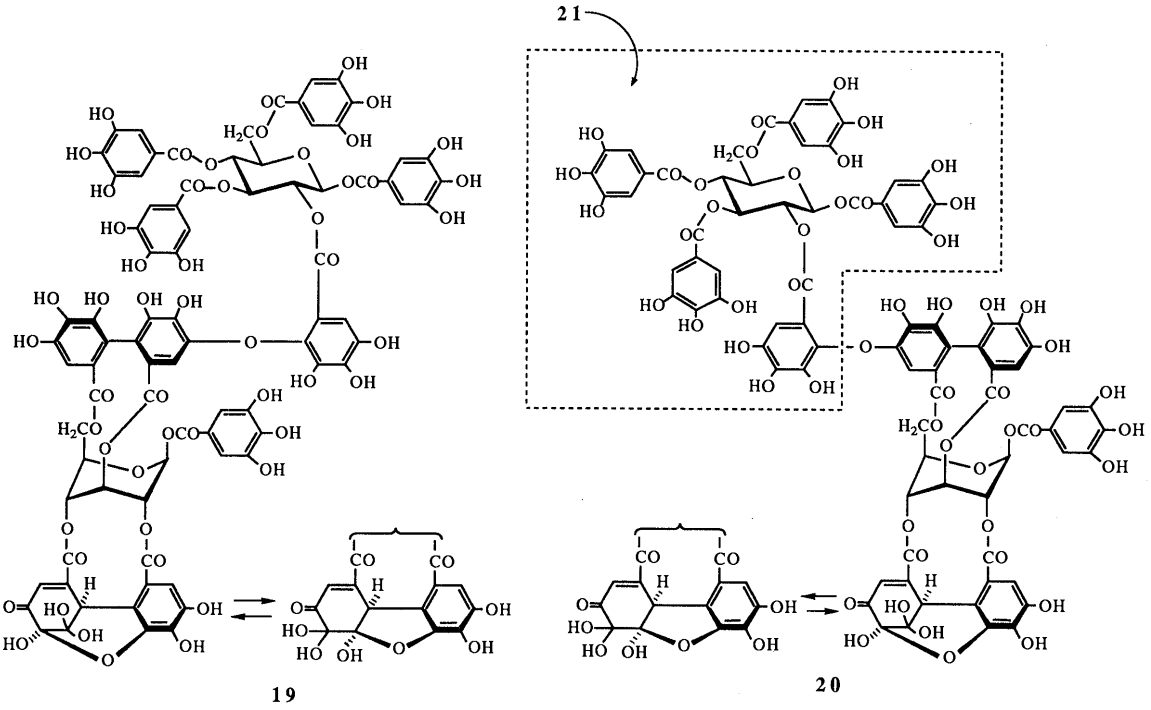



Chart 4

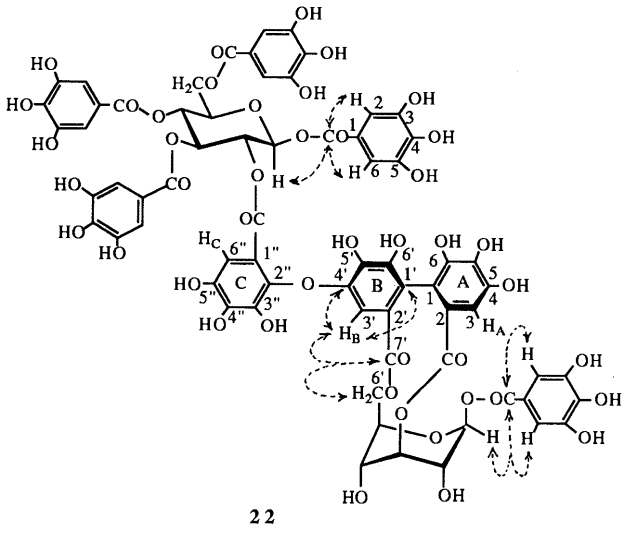



Chart 5

between euphorbins A and B. These signals in the former were shifted upfield by *ca.* 0.2—0.5 ppm from the corresponding signals of 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (**21**), while those in the latter are almost identical with those of **21**. The upfield shifts in the former were attributed to the anisotropic effect of the valoneoyl aromatic ring A, in the conformation arising from the restricted rotation around the ether linkage of the valoneoyl group.[5a)]

However, as mentioned earlier, H-1, H-3 and H-5 of glucose-I in **14** are shifted upfield from the corresponding signals of tellimagrandin II (**4**). This phenomenon is analogous to that observed in euphorbin A, rather than in euphorbin B, indicating the same orientation of the valoneoyl group in euphorbins A and F (**14**).

The measurement of the $^{1}H$–$^{13}C$ long-range shift correlation spectrum of the partial hydrolyzate (**22**)[5a)] from

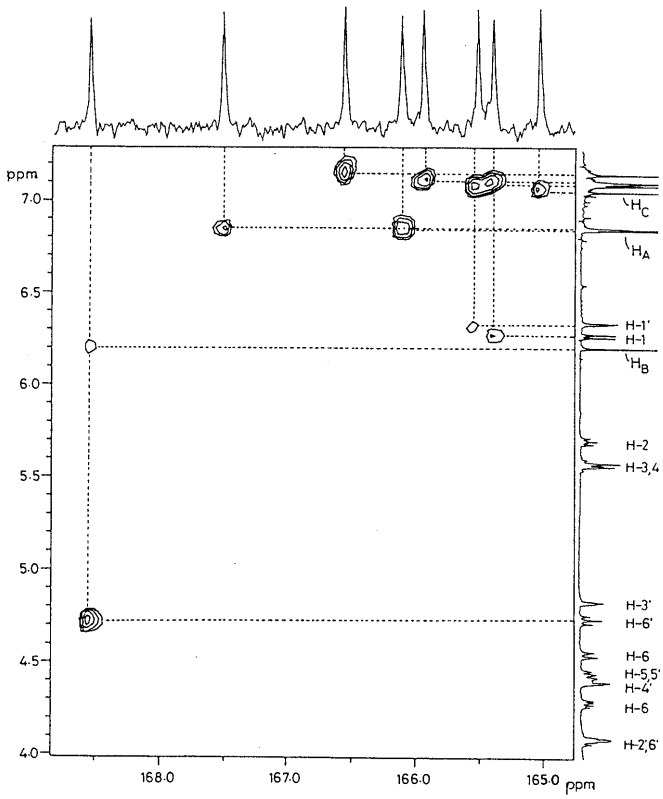



Fig. 1. $^{1}H$–$^{13}C$ Long-Range Shift Correlation Spectrum ($\delta_C$ 165.0—166.8) of **22** in Acetone-$d_6$+$D_2O$

Average $J_{CH}$ was set at 6 Hz.

the condensate of euphorbin A with *o*-phenylenediamine has now been carried out. This spectrum showed a clear cross peak by three-bond coupling between the H-6′ signal of the glucose core at $\delta$ 4.74 (t, $J$=10 Hz) and the ester cabonyl carbon resonance at $\delta$ 168.6, which is correlated with the aromatic proton signal at $\delta$ 6.21 (Fig. 1). This

aromatic signal was assigned to $H_B$ of the valoneoyl group, based on its correlation by two-bond coupling with the signal attributed to C-4′ ($\delta$ 146.7) of the valoneoyl B-ring, and also by three-bond coupling with C-1′ ($\delta$ 116.8). The connectivity between the valoneoyl B-ring and H-6′ of the glucose core-II was thus established, leading to the revised structure **20** for euphorbin A, and consequently structure **19** for euphorbin B.

## Experimental

**General** $^1$H- (500 MHz) and $^{13}$C-NMR (126 MHz) spectra were measured on a Varian VXR 500 instrument and chemical shifts are given in $\delta$ (ppm) values relative to acetone-$d_6$ (2.04 ppm for $^1$H and 29.8 ppm for $^{13}$C). High-performance liquid chromatography (HPLC) was conducted on Superspher Si 60 (4 mm × 119 mm) and LiChrospher RP-18 (4 mm × 250 mm) columns, using the following solvent systems: (A) hexane–MeOH–tetrahydrofuran (THF)–HCOOH (60 : 45 : 15 : 1) and oxalic acid (500 mg/1.2 l), (B) 0.05 M phosphate buffer–EtOH–EtOAc (85 : 10 : 5), (C) 0.05 M phosphate buffer–$CH_3CN$ (85 : 15), (D) 0.05 M phosphate buffer–EtOH–EtOAc (87 : 8 : 5), (E) 0.05 M phosphate buffer–$CH_3CN$ (87 : 13), (F) 0.05 M phosphate buffer–EtOH–EtOAc (83 : 12 : 5). Column chromatography was carried out on Toyopearl HW-40 (coarse and fine grades, Tosoh) and MCI-gel CHP-20P (Mitsubishi Chemical Industry Co., Ltd.).

**Isolation of Tannins and Other Polyphenols** The dried stems (900 g) of *E. tirucalli*, collected in a greenhouse at Okayama University, were homogenized in 70% aqueous acetone (13 l), and filtered. The filtrate was concentrated under reduced pressure, and extracted with $Et_2O$, EtOAc and 1-BuOH (pre-saturated with water), successively. The EtOAc extract (4.8 g) was subjected to column chromatography over Toyopearl HW-40 (coarse) (2.2 cm i.d. × 50 cm) developing with 50% MeOH→60% MeOH→70% MeOH→MeOH–$H_2O$–acetone (7 : 2 : 1). The eluate with 50% MeOH gave quercitrin (**1**) (96 mg). The 70% MeOH eluate yielded tellimagrandin II (**4**) (31 mg) and geraniin (**5**) (267 mg). Euphorbin F (**14**) (366 mg) was obtained from the MeOH–$H_2O$–acetone (7 : 2 : 1) eluate. The fraction (187 mg) eluted prior to **14** was rechromatographed over MCI-gel CHP-20P (30% MeOH→40% MeOH) to give tirucallin A (**7**) (16 mg) and euphorbin A (**20**) (38 mg). The BuOH extract was similarly chromatographed over Toyopearl HW-40, using the same solvents as those for the EtOAc extract mentioned above, to give 5-*O*-caffeoylquinic acid (**2**) (15 mg) and 3,3′,4-tri-*O*-methyl-4′-*O*-rutinosylellagic acid (**3**) (20 mg) from the 60% MeOH eluate. The 70% MeOH eluate was further purified by column chromatography over MCI-gel CHP-20P to give tirucallin B (**11**) (8 mg).

**3,3′,4-Tri-*O*-methyl-4′-*O*-rutinosylellagic Acid (3)** White needles, mp 260—263 °C. FAB-MS *m/z*: 675 $(M+Na)^+$. $^1$H-NMR (pyridine-$d_5$) $\delta$: 7.59, 8.29 (each 1H, s, aromatic), 3.63, 3.89, 4.05 (each 3H, s, OMe), 5.61 (d, $J$=7 Hz, glucose H-1), 5.25 (s, rhamnose H-1), 3.95—4.16 (8H, m, glucose H-2—H-6, rhamnose H-2—H-5), 1.32 (3H, d, $J$=6 Hz, rhamnose Me). $^{13}$C-NMR (pyridine-$d_5$) $\delta$: 18.7, 67.4, 69.8, 71.4, 72.2, 72.8, 74.0, 74.9, 77.8, 78.5, 102.4, 103.3 (rutinosyl), 56.7, 61.6, 62.1 (OMe), 108.2, 113.5, 113.6 (2C), 113.7, 114.5, 142.0, 142.1, 142.2, 143.1, 153.2, 155.1 (aromatic), 159.0, 159.2 (carbonyl).

Hexaacetate. mp 132—135 °C. $^1$H-NMR ($CDCl_3$) $\delta$: 7.89 (1H, s), 7.69 (1H, s) (aromatic), 5.0—5.4 (7H, glucose H-1, H-2, H-3, H-4, rhamnose H-2, H-3, H-4), 4.69 (1H, d, $J$=1 Hz, rhamnose H-1), 3.6—3.9 (4H, glucose H-5, H-6, H-6′, rhamnose H-5), 1.17 (3H, d, $J$=6 Hz, rhamnose H-6), 4.03, 4.16, 4.20 (each 3H, s, OMe), 1.94, 2.03, 2.05, 2.06, 2.08, 2.11 (each 3H, s, OAc).

**Tirucallin A (7)** A pale yellow amorphous powder, $[\alpha]_D$ +2.8° ($c$=0.5, acetone). UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$): 218 (4.90), 266 (4.74), 355 (3.78). CD (MeOH) [$\theta$] (nm): $+12.0\times10^4$ (237), $-10.0\times10^4$ (265). *Anal.* Calcd for $C_{55}H_{34}O_{34}\cdot10H_2O$: C, 46.54; H, 3.81. Found: C, 46.28; H, 3.45. FAB-MS *m/z*: 1261 $(M+Na)^+$. $^1$H-NMR (acetone-$d_6$ + $D_2O$) $\delta$: 5.69 (d, $J$=8 Hz, H-1), 5.57 (dd, $J$=8, 10 Hz, H-2), 5.63 (t, $J$=10 Hz, H-3), 5.08 (t, $J$=10 Hz, H-4), 4.31 (dd, $J$=6, 10 Hz, H-5), 5.30 (dd, $J$=6, 13 Hz, H-6), 3.76 (d, $J$=13 Hz, H-6), aromatic protons, see text. $^{13}$C-NMR (acetone-$d_6$ + $D_2O$) $\delta$: 93.4 (C-1), 71.4 (C-2), 73.6 (C-3), 70.7 (C-4), 72.9 (C-5), 62.9 (C-6) (glucose), 159.6, 160.5, 164.4, 164.8, 166.5, 167.7, 168.1 (ester carbonyl).

**Enzymatic Hydrolysis of 7** A mixture of **7** (0.5 mg) and tannase[16] (1 dr.) in $H_2O$ (1 ml) was left standing at 37 °C for 1 h. The concentrated solution was directly analyzed by reversed-phase HPLC (solvent B) to detect gallic acid ($t_R$ 3.10 min), **9** ($t_R$ 4.87, 5.99 min) and **10** ($t_R$ 3.48, 3.83 min). The identities of the products were further confirmed by reversed-phase HPLC using three other solvent systems (C—E), and by normal phase HPLC (solvent A).

**Tirucallin B (11)** A light brown amorphous powder, $[\alpha]_D$ −31° ($c$=0.5, acetone). UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$): 220 (5.11), 277 (4.76). FAB-MS *m/z*: 1593 $(M+Na)^+$. $^1$H-NMR (acetone-$d_6$ + $D_2O$) $\delta$: 6.36 (d, $J$=3 Hz, H-1), 5.28 (br d, $J$=3 Hz, H-2), 5.08 (br s, H-3), 5.71 (br d, $J$=3 Hz, H-4), 4.56 (t, $J$=8 Hz, H-5), 4.68 (dd, $J$=8, 11 Hz, H-6), 4.32 (dd, $J$=8, 11 Hz, H-6), 6.30 (d, $J$=2 Hz, H-1′), 4.01 (br s, H-2′), 4.74 (br s, H-3′), 4.36 (br s, H-4′), 4.32 (dd, $J$=8, 11 Hz, H-5′), 4.63 (t, $J$=11 Hz, H-6′), 3.83 (dd, $J$=8, 11 Hz, H-6′), aromatic protons, see text.

**Euphorbin F (14)** A pale yellow amorphous powder, $[\alpha]_D$ −28° ($c$=0.8, acetone). UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$): 220 (5.13), 279 (4.76). CD (MeOH) [$\theta$] (nm): $+14.0\times10^4$ (236), $-7.0\times10^4$ (288). *Anal.* Calcd for $C_{82}H_{56}O_{53}\cdot6H_2O$: C, 49.30; H, 3.41. Found: C, 49.06; H, 3.78. FAB-MS *m/z*: 1911 $(M+Na)^+$. $^1$H-NMR (acetone-$d_6$ + $D_2O$) $\delta$: 6.15, 6.18, 6.62, 6.63, 6.73, 6.78, 6.99, 7.03, 7.09, 7.15, 7.17, 7.23 (each 1/2H, s, HHDP, valoneoyl, H-3‴ of DHHDP), 6.46 (1/2H, s), 6.07 (1/2H, br s) (H-3″ of DHHDP), 6.84, 6.87, 7.10, 7.11, 7.16, 7.17 (each 1H, s, galloyl), glucose protons, see text. $^{13}$C-NMR (acetone-$d_6$ + $D_2O$): 45.9 [DHHDP (a) C-1″], 51.8 [DHHDP (b) C-1″], 154.2 [DHHDP (a) C-2″], 147.6 [DHHDP (b) C-2″], 128.6 [DHHDP (a) C-3″], 124.7 [DHHDP (b) C-3″], 191.8 [DHHDP (a) C-4″], 194.5 [DHHDP (b) C-4″], 96.1 [DHHDP (a) C-5″], 92.2 [DHHDP (b) C-5″], 92.3 [DHHDP (a) C-6″]; 164.7 (1/2C), 165.1 (3/2C), 165.2 (3/2C), 165.3 (1/2C), 165.6 (1/2C), 165.7 (1/2C), 166.5 (3/2C), 166.6 (1/2C), 167.6 (1C), 168.1 (1C), 168.2 (1/2C), 168.3 (1/2C) (ester carbonyl); glucose carbons, see text.

**Acid Hydrolysis of 14** A solution of **14** (5 mg) in 5% $H_2SO_4$ (1 ml) was heated on a boiling water-bath for 5 h, and after cooling, extracted with EtOAc. The EtOAc extract was analyzed by HPLC (LiChrospher RP-18, solvent C) to detect gallic acid ($t_R$ 2.95 min), ellagic acid ($t_R$ 10.4 min) and valoneic acid dilactone ($t_R$ 7.12 min). The aqueous layer was neutralized with Amberlite IRA-410 (OH form), and evaporated. The syrupy residue was trimethylsilylated and analyzed by gas-liquid chromatography (GLC) to detect glucose ($t_R$ 3.5 and 5.0 min).

GLC: capillary column, G-250 (1.2 mm × 40 m); oven temperature, 170 °C; detection, flame ionization detector.

**Preparation of the Phenazine Derivative (15) from Euphorbin F (14)** A solution of *o*-phenylenediamine (40 mg) in 15% AcOH (10 ml) was added to a solution of **14** (100 mg) in MeOH (30 ml), and the reaction mixture was left standing at room temperature for 5 h. After removal of the solvent, the residue was suspended in $H_2O$ and the insoluble materials were collected by suction filtration, and washed with $H_2O$ and $Et_2O$. Reprecipitation of the crude product from MeOH–$CHCl_3$ gave **15** (31 mg). The mother liquor was chromatographed over MCI-gel CHP 20P developing with 30% MeOH→50% MeOH to yield **15** (5 mg) and **16** (9 mg).

**15**: An orange amorphous powder, $[\alpha]_D$ +26° ($c$=0.2, acetone). UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$): 219 (5.22), 281 (4.99). CD (MeOH) [$\theta$] (nm): $-7.5\times10^4$ (215), $+19\times10^4$ (236), $-11\times10^4$ (280). *Anal.* Calcd for $C_{88}H_{58}N_2O_{50}\cdot12H_2O$: C, 48.93; H, 3.80; N, 1.30. Found: C, 48.61; H, 3.15; N, 1.23. $^1$H-NMR (acetone-$d_6$ + $D_2O$) $\delta$: 6.92, 6.96, 7.18 (each 2H, s, galloyl), 6.28, 6.61, 6.69, 6.99, 7.00 (each 1H, s, HHDP and valoneoyl), 7.46 (1H, s), 8.21 (1H, s), 8.3—8.0 (4H, m) (phenylphenazine), glucose protons, see text.

**16**: A light brown amorphous powder, $[\alpha]_D$ +17° ($c$=1.0, acetone). UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$): 220 (5.14), 275 (4.79). FAB-MS *m/z*: 1593 $(M+Na)^+$. $^1$H-NMR (acetone-$d_6$ + $D_2O$) $\delta$: 6.82, 7.09, 7.13 (each 2H, s, galloyl), 6.17, 6.63, 6.78, 6.84, 7.06 (each 1H, s, HHDP, valoneoyl), 6.13 (d, $J$=8 Hz, H-1), 5.65 (dd, $J$=8, 10 Hz, H-2), 5.29 (t, $J$=10 Hz, H-3), 5.09 (t, $J$=10 Hz, H-4), 4.38 (m, H-5), 5.38 (dd, $J$=6, 13 Hz, H-6), 3.88 (d, $J$=13 Hz, H-6), 6.32 (d, $J$=2 Hz, H-1′), 4.07 (br s, H-2′), 4.80 (dd, $J$=2, 3 Hz, H-3′), 4.38 (d, $J$=3 Hz, H-4′), 4.43 (t, $J$=10 Hz, H-5′), 4.73 (t, $J$=10 Hz, H-6′), 4.07 (t, $J$=10 Hz, H-6′).

**Hydrogenation of Euphorbins E (17) and F (14)** A solution of **17** (100 mg) in EtOAc (10 ml) was hydrogenated over 5% Pd–C for 26 h at room temperature. The catalyst was removed by filtration, the solvent was evaporated off *in vacuo*, and the residue was chromatographed on Toyopearl HW-40 (superfine) (1.1 × 18 cm) [MeOH→MeOH–acetone (9 : 1)]. The eluate with MeOH–acetone (9 : 1) was further purified by column chromatography over Sephadex LH-20 (MeOH) to give two fractions, A and B, which showed single peaks at $t_R$ 5.88 min and 9.03 min, respectively, in reversed-phase HPLC (solvent F). Fraction A yielded the reduction product (**18**) (2.6 mg), as an off-white amorphous powder, $[\alpha]_D$ +43° ($c$=0.9, MeOH). UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$): 217 (5.17), 272 (4.83).

$^1$H-NMR (acetone-$d_6$ + $D_2O$) $\delta$: 6.22, 6.60, 6.69, 6.92, 6.97, 7.00, 7.37 (each 1H, s, HHDP and valoneoyl), 6.86, 7.11, 7.12 (each 2H, s, galloyl), 6.04 (d, $J$=8 Hz, H-1), 5.61 (dd, $J$=8, 10 Hz, H-2), 5.30 (t, $J$=10 Hz, H-3), 5.10 (t, $J$=10 Hz, H-4), 4.33 (dd, $J$=6.5, 10 Hz, H-5), 5.30 (dd, $J$=6.5, 14 Hz, H-6), 3.83 (d, $J$=14 Hz, H-6), 6.12 (d, $J$=6 Hz, H-1′), 5.56 (d, $J$=6 Hz, H-2′), 5.15 (br d, $J$=4 Hz, H-3′), 5.44 (d, $J$=4 Hz, H-4′), 4.75 (dd, $J$=4, 8.5 Hz, H-5′), 4.62 (dd, $J$=8.5, 12 Hz, H-6′), 3.99 (dd, $J$=4, 12 Hz, H-6′).

Euphorbin F (**14**) (5 mg) was hydrogenated in a similar way to that described above to yield the product (4.5 mg), $[\alpha]_D$ +32° ($c$=0.4, acetone), which was identical with **18**. Their identity was confirmed by $^1$H-NMR spectral comparison and co-chromatography on HPLC.

**$^{13}$C-NMR Spectrum of Partial Hydrolyzate (22)**[5a] $\delta$: 93.4 (C-1), 94.3 (C-1′), 71.4 (C-2), 68.9 (C-2′), 73.3 (C-3), 70.9 (C-3′), 69.4 (C-4), 62.1 (C-4′), 73.8 (C-5), 75.4 (C-5′), 62.8 (C-6), 64.6 (C-6′), 109.9, 110.1, 110.3 (each 2C), 110.5 (4C) (galloyl C-2, 6), 119.2, 119.5, 119.8, 120.5, 120.9 (galloyl C-1), 139.0, 139.40, 139.44 (2C), 139.9 (galloyl C-4), 145.66, 145.7, 145.9 (each 2C), 146.0 (4C) (galloyl C-3, 5), 165.4, 165.5, 165.9, 166.1, 166.6 (galloyl C-7), 116.3 [valoneoyl (Val) C-1], 110.0 (Val C-3), 144.7 (Val C-4), 137.2 (Val C-5), 167.5 (Val C-7), 116.8 (Val C-1′), 105.4 (Val C-3′), 146.7 (Val C-4′), 136.9 (Val C-5′), 168.6 (Val C-7′), 113.3 (Val C-1″), 136.6 (Val C-2″), 140.5 (Val C-3″), 140.6 (Val C-4″), 143.4 (Val C-5″), 110.5 (Val C-6″), 165.0 (Val C-7″), 124.8, 125.7 (Val C-2, 2′), 144.5, 145.6 (Val C-6, 6′).

**Acknowledgements** The authors are grateful to Dr. N. Toh, Faculty of Engineering, Kyushu Kyoritsu University, for the CD measurements, and to the SC-NMR Laboratory of Okayama University for the NMR experiments.

**References and Notes**

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# Tannins and Related Compounds. CVIII.[1] Isolation and Characterization of Novel Complex Tannins (Flavano-ellagitannins), Anogeissinin and Anogeissusins A and B, from *Anogeissus acuminata* (ROXB *ex* DC.) GUILL. *et* PERR. var. *lanceolata* WALL. *ex* CLARKE

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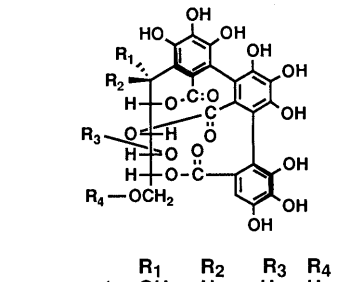
**Three novel complex tannins (flavano-ellagitannins), anogeissinin (9) and anogeissusins A (10) and B (11), have been isolated from the bark of *Anogeissus acuminata* (ROXB. *ex* DC.) GUILL. *et* PERR. var. *lanceolata* WALL. *ex* CLARKE (Combretaceae), together with eight known *C*-glycosidic hydrolyzable tannins. On the basis of spectroscopic and chemical evidence, anogeissinin (9) was shown to have two *C*-glycosidic ellagitannin (vescalagin) moieties connected to the C-6 and C-8 positions in the (+)-catechin moiety, while anogeissusins A (10) and B (11) have structures in which the dimeric *C*-glycosidic ellagitannin, castamollinin (8), is attached to the C-8 position of the (+)-catechin and (+)-gallocatechin moieties, respectively.**

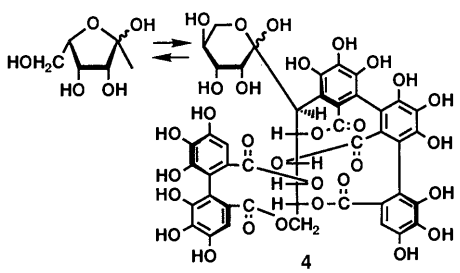
**Keywords** *Anogeissus acuminata* var. *lanceolata*; Combretaceae; anogeissinin; anogeissusin A; anogeissusin B; complex tannin; flavano-ellagitannin; *C*-glycosidic hydrolyzable tannin; dimeric ellagitannin; tannin

In the course of systematic chemical studies on tannins of Combretaceous plants, we reported the isolation and characterization of hydrolyzable tannins containing esters of triphenic acids (tergallic acid and flavogallonic acid) and tetraphenic acids (terchebulic acid and gallagic acid) from *Terminalia catappa* L.[2] and *T. chebula* RETZ.,[3] and of dimeric and trimeric ellagitannins from *T. calamansanai* (BLANCO) ROLFE.[4] Furthermore, we have found that almost all the *Terminalia* species so far examined contain surprisingly large amounts of punicalin and punicalagin,[2-5] which were originally isolated from the pomegranate (*Punica granatum* L.) and were reported to inhibit human immunodeficiency virus (HIV) reverse transcriptase (RT), while showing little cytotoxicity.[6] In continuing our chemical studies on tannins in the plants of this family, we have examined the bark of *Anogeissus acuminata* (ROXB. *ex* DC.) GUILL *et* PERR. var. *lanceolata* WALL. *ex* CLARKE, which is distributed in south China (Yunnan), Thailand, Laos, Vietnam and Cambodia, and is known as a rich source of tannins. As a result, we have isolated and structurally elucidated three novel complex tannins (flavano-ellagitannins) named anogeissinin (**9**) and anogeissusins A (**10**) and B (**11**), together with eight known *C*-glycosidic and complex tannins. This paper describes the isolation and characterization of these compounds.

The aqueous acetone extract of the dried bark, collected in Yunnan, China, was subjected to Sephadex LH-20 column chromatography with a solvent system of water–methanol–acetone to give four fractions. Each fraction was repeatedly chromatographed over Sephadex LH-20 (EtOH, $H_2O$–MeOH), MCI-gel CHP 20P ($H_2O$–MeOH) and various reversed-phase gels, such as Fuji gel ODS G3 and



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| 1 : | OH | H | H | H |
| 2 : | OH | H | HHDP | |
| 3 : | H | $CO_2H$ | HHDP | |



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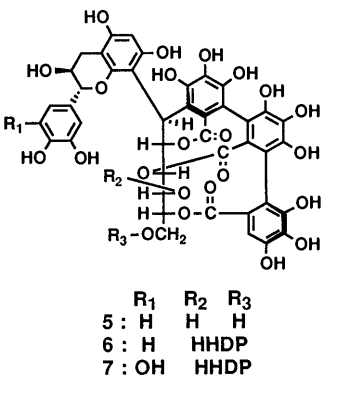
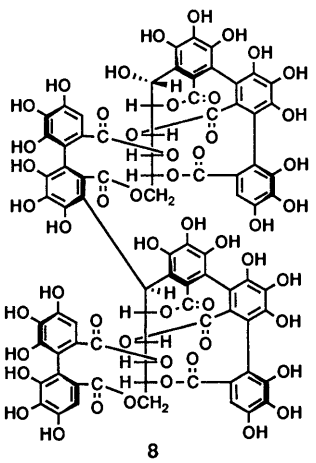


| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| 5 : | H | H | H |
| 6 : | H | HHDP | |
| 7 : | OH | HHDP | |



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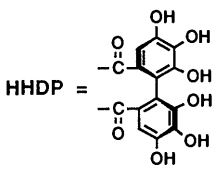


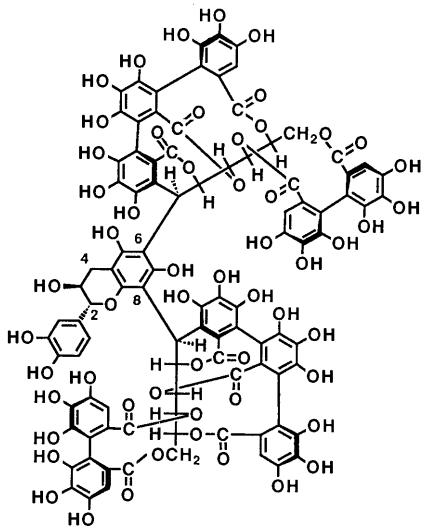
Bondapak $C_{18}$/Porasil B ($H_2O$–MeOH), to afford eleven compounds (**1**—**11**). Among them, compounds **1**—**8** were found to be known hydrolyzable tannins which were identified as castalin (**1**),[7,8] castalagin (**2**),[8,9] vescalagin carboxylic acid (**3**),[8,10] grandinin (**4**),[8,11] acutissimins C (**5**)[8,10] and A (**6**),[8,12] eugenigrandin A (**7**)[8,13] and castamollinin (**8**)[8,14] by direct comparisons of their physical and spectral data with those of authentic samples.

Anogeissinin (**9**) gave a dark blue coloration with the ferric chloride reagent and a reddish-brown coloration with the sodium nitrate–acetic acid reagent,[15] indicating an ellagitannin, while a pink coloration obtained on treatment with the anisaldehyde–sulfuric acid reagent[16] suggested the presence of a flavan-3-ol skeleton in the molecule. The presence of the flavan-3-ol moiety was deduced from the appearance of C-4, C-3 and C-2 signals at $\delta$ 25.0, 67.7 and 80.4, respectively, in the carbon-13 nuclear magnetic resonance ($^{13}$C-NMR) spectrum, and the lowfield aromatic carbon signals at $\delta$ 151.9, 154.6 and 155.8 were attributable to carbons bearing an oxygen function on the flavan A-ring. In the proton nuclear magnetic resonance ($^1$H-NMR) spectrum, the appearance of ABX-type signals at $\delta$ 6.75

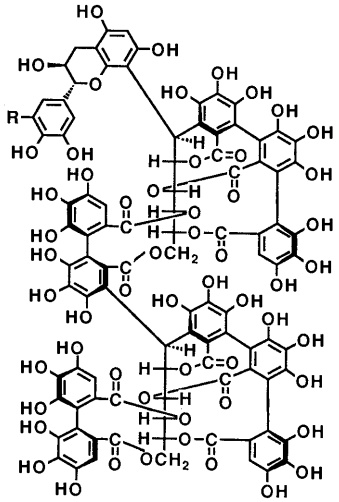
TABLE I. $^1$H-NMR Data for Compounds **9**, **10**, **11**, **8**, **6** and **13** ($\delta$ Value, $J$ Value in Hz)

| | | **9**[a] | **10**[a] | **11**[a] | **8**[a] | **6**[b] | **13**[b] |
|---|---|---|---|---|---|---|---|
| Flavan-3-ol | | | | | | | |
| C-ring | 2 | 5.52 (br d, $J$=2) | 5.50 (br s) | 5 42 (br s) | | 5.50 (br s) | 4.57 (d, $J$=9) |
| | 3 | 4.61 (d, $J$=2) | 4.46 (br s) | 4.40 (br s) | | 4.56 (br s) | 4.09 (ddd, $J$=9, 9, 6) |
| | 4 | 2.70 (m) | 2.46 (br d, $J$=16) | 2.52 (br d, $J$=16) | | 2.43 (br d, $J$=16) | 2.65 (2H, dd, $J$=15, 9) |
| | | 2.97 (br d, $J$=16) | 2.89 (br d, $J$=16) | 2.89 (br d, $J$=16) | | 2.89 (br d, $J$=16) | |
| A-ring | 6 | — | 6.01 (br s) | 6.03 (br s) | | 6.07 (br s) | — |
| | 8 | — | — | — | | — | 6.12 (br s) |
| B-ring | 2′ | 6.89 (br s) | 6.93 (br s) | 6.56 (2H, s) | | 6.89 (br s) | 6.97 (d, $J$=2) |
| | 5′ | 6.75 (d, $J$=8) | 6.80 (d, $J$=8) | — | | 6.79 (d, $J$=8) | 6.81 (d-like, $J$=11) |
| | 6′ | 6.95 (br d, $J$=8) | 6.98 (br d, $J$=8) | 6.56 (2H, s) | | 6.94 (br d, $J$=8) | 6.83 (dd-like, $J$=2, 11) |
| Polyalcohol | | | | | | | |
| | 1 | 4.95 (s) | 4.85 (d, $J$=1) | 4.84 (s) | 5.71 (d, $J$=5) | 4.82 (br s) | 4.72 (s) |
| | 1′ | 5.10 (s) | 4.76—4.80 | 4.76—4.80 | 4.89 (d, $J$=1) | | |
| | 2 | 5.28 (br s) | 5.11 (br s) | 5.08 (br s) | 5.00 (d, $J$=5) | 5.20 (s) | 5.14 (s) |
| | 2′ | 5.37 (br s) | 5.28 (br s) | 5.28 (br s) | 5.22 (d, $J$=1) | | |
| | 3 | 4.69 (d, $J$=7) | 4.76—4.80 | 4.76—4.80 | 5.03 (d, $J$=8) | 4.76 (br d, $J$=7) | 4.86 (d, $J$=8) |
| | 3′ | 4.71 (d, $J$=7) | 4.76—4.80 | 4.76—4.80 | 4.79 (d, $J$=8) | | |
| | 4 | 5.28 (t, $J$=7) | 5.27 (t, $J$=7) | 5.26 (t, $J$=7) | 5.21 (t, $J$=8) | 5.27 (dd, $J$=7, 8) | 5.27 (t, $J$=8) |
| | 4′ | 5.30 (t, $J$=7) | 5.30 (t, $J$=7) | 5.30 (t, $J$=7) | 5.30 (t, $J$=7) | | |
| | 5 | 5.63 (d, $J$=7) | 5.48 (d, $J$=7) | 5.47 (d, $J$=7) | 5.41 (br d, $J$=7) | 5.64 (br d, $J$=8) | 5.66 (br d, $J$=8) |
| | 5′ | 5.69 (d, $J$=7) | 5.53 (d, $J$=7) | 5.52 (d, $J$=7) | 5.52 (d, $J$=7) | | |
| | 6 | 4.17 (2H, br d, $J$=12) | 2.95 (d, $J$=12) | 2.95 (d, $J$=12) | 2.84 (d, $J$=13) | 4.11 (d, $J$=12) | 4.05 (d, $J$=13) |
| | | | 4.21 (d, $J$=12) | 4.20 (d, $J$=12) | 4.35 (dd, $J$=3, 13) | 4.69 (br d, $J$=12) | 4.89 (dd, $J$=2, 13) |
| | 6′ | 4.82 (d, $J$=12) | 4.03 (d, $J$=12) | 4.02 (d, $J$=12) | 4.02 (d, $J$=12) | | |
| | | 4.85 (d, $J$=12) | 4.76—4.80 | 4.76—4.80 | 4.75 (d, $J$=12) | | |
| Aromatic proton | | | | | | | |
| | | 6.59, 6.65, 6.73, 6.78, 6.79, 7.15 (each 1H, s) | 6.62, 6.71, 6.93, 7.38, 7.43 (each 1H, s) | 6.62, 6.71, 6.90, 7.38, 7.43 (each 1H, s) | 6.61, 6.71, 6.77, 7.36, 7.39 (each 1H, s) | 6.62, 6.78, 7.09 (each 1H, s) | 6.63, 6.79, 7.11 (each 1H, s) |

a) Measured at 270 MHz. b) Measured in acetone-$d_6$ with TMS as an internal standard.



**9**



R
10 : H
11 : OH

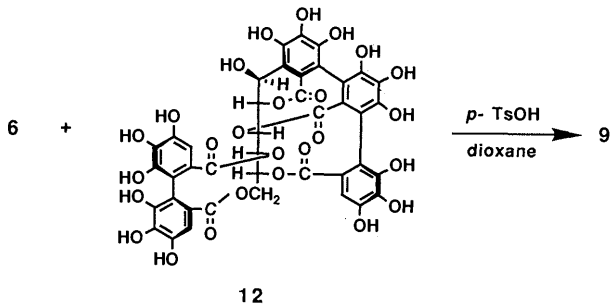
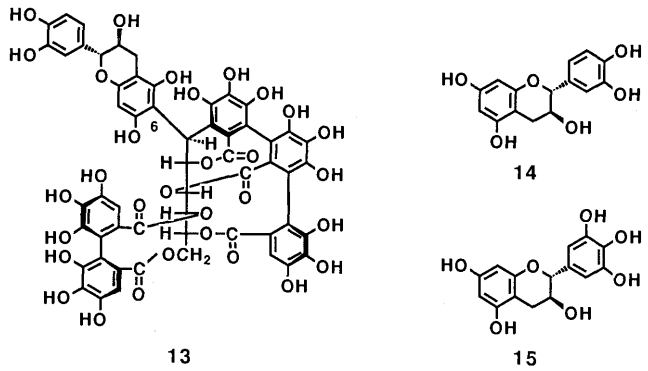



Chart 1




(1H, d, $J=8$ Hz), 6.89 (1H, br s) and 6.93 (1H, br d, $J=8$ Hz) indicated that the B-ring of the flavan-3-ol is of catechol type, while the absence of the signals due to the A-ring protons suggested that the C-6 and C-8 positions are both substituted (Table I). This was further supported by the absence of a doublet carbon signal between $\delta$ 90—100 in the off-resonance spectrum [acutissimin A (**6**), $\delta$ 97.8 (d, C-6); acutissimin B (**13**),[12] $\delta$ 96.7 (d, C-8)].

In the $^1$H-NMR spectrum, the aliphatic signal pattern, including the flavan-3-ol C-ring signal pattern, was closely related to that of acutissimin A (**6**), except for the appearance of additional signals due to an extra *C*-glycoside moiety, whose chemical shifts and coupling constants were similar to those of the signals arising from the *C*-glycoside moiety (vescalagin)[17] in **6** (Table I). These observations suggested that an additional vescalagin (**12**) unit is attached to the C-6 position of the catechin unit in the acutissimin A moiety through a carbon-to-carbon bond. The negative fast atom bombardment mass spectrum (FAB-MS) of **9** showed the $[M-H]^-$ ion peak at *m/z* 2121, which is consistent with the proposed structure **9**.

To confirm the structure, condensation of vescalagin (**12**) and acutissimin A (**6**) was attempted.[10,12,13] Refluxing of the mixture in dry dioxane containing *p*-toluenesulfonic acid successfully yielded anogeissinin (Chart 1). On the basis of these results, anogeissinin was unequivocally characterized as having the structure **9**.

Anogeissusins A (**10**) and B (**11**) showed, with the ferric chloride, nitrous acid and anisaldehyde–sulfuric acid reagents, colorations similar to those of **9**, suggesting that these compounds also have flavan-3-ol and ellagitannin moieties in the molecule. The $^1$H-NMR spectra (Table I) of **10** and **11** were closely related to each other, and showed signals due to the flavan-3-ol moiety and signals arising from two polyalcohol moieties whose chemical shifts and coupling constants were similar to those of acutissimin A (**6**), indicating the presence of two vescalagin units in each molecule. The marked difference in the spectra of **10** and **11** was in the signal pattern arising from the flavan-3-ol moiety. Namely, in the spectrum of **10**, the appearance of ABX-type signals indicated that the flavan-3-ol is of catechin type, while the two-proton singlet resonances at $\delta$ 6.56 in the case of **11** clearly showed it to be of gallocatechin type. This was supported by negative FAB-MS analysis, which displayed the $[M-H]^-$ ion peak at *m/z* 2137 in **11**, 16 mass units larger than that of **10** (*m/z* 2121). Furthermore, the 2,3-*trans* configuration of the flavan-3-ol unit in each molecule was confirmed by the chemical shifts of the C-ring carbon signals [**10**; $\delta$ 80.7 (C-2), 68.0 (C-3) and 24.5 (C-4), **11**; $\delta$ 80.8 (C-2), 68.1 (C-3) and 24.3 (C-4)], which were similar to those of acutissimin A (**6**) [$\delta$ 80.6 (C-2), 68.2 (C-3) and 24.1 (C-4)].

In the $^1$H-NMR spectra (Table I) of **10** and **11**, a significant upfield shift ($\delta$ 2.95) of one of the *C*-glycosidic H-6 signals was observed. This characteristic upfield shift was similarly observed in the spectrum of castamollinin (**8**), and was considered to be caused by a strong shielding effect of the lower vescalagin unit.[14] This observation, combined with the appearance of five one-proton aromatic singlet signals, suggested that the location and mode of the linkage between the two *C*-glycosidic units are the same as those of **8**. This was supported by the $^{13}$C-NMR chemical shifts of the C-1 signals in the two vescalagin units (**10**; $\delta$ 37.9 and 40.5, **11**; $\delta$ 37.9 and 40.6).

The location of the ellagitannin moiety in the flavan-3-ol nucleus was concluded to be at the C-8 position in each case on the basis of comparisons of the chemical shifts and coupling constant of the C-ring proton signals[18] (Table I) and C-4a carbon signal[19] (**10** and **11**; $\delta$ 98.4) with those of acutissimins A (**6**) ($\delta$ 98.4) and B (**13**) ($\delta$ 101.7).

Unequivocal structural assignments of **10** and **11** were achieved by condensation of castamollinin (**8**) and the corresponding flavan-3-ols, (+)-catechin (**14**) and (+)-gallocatechin (**15**), which yielded anogeissusins A and B, respectively. On the basis of these findings, the structures of anogeissusins A and B were established to be as shown by the formulae **10** and **11**, respectively.

Anogeissinin (**9**) and anogeissusins A (**10**) and B (**11**) represent complex tannins of a novel type consisting of a flavan-3-ol and two *C*-glycosidic ellagitannin moieties. The fact that, unlike other Combretaceous plants so far examined, *Anogeissus acuminata* var. *lanceolata* characteristically contains large quantities of *C*-glycosidic and complex tannins suggests that this species is chemotaxonomically related to the plants of Myrtaceae[20] and Fagaceae,[12] rather than the Combretaceous species.

## Experimental

Optical rotations were measured with a JASCO DIP-4 digital polarimeter. FAB-MS were taken with a JEOL JMS DX-300 instrument. $^1$H- and $^{13}$C-NMR spectra were recorded on JEOL FX-100 and JEOL GX-270 spectrometers, with tetramethylsilane (TMS) as an internal standard, and chemical shifts are given in $\delta$ (ppm). Column chromatography was performed with Sephadex LH-20 (25—100 $\mu$m, Pharmacia Fine Chemical Co., Ltd.), MCI-gel CHP 20P (75—150 $\mu$m, Mitsubishi Chemical Industries, Ltd.), Fuji-gel ODS-G3 (43 $\mu$m, Fuji-gel Hanbai Co., Ltd.) and Bandapak $C_{18}$/Porasil B (37—75 mesh, Waters Associates, Inc.). Thin-layer chromatography (TLC) was performed on precoated Kieselgel 60 $F_{254}$ plates (0.2 mm, Merck) and precoated cellulose $F_{254}$ plates (0.10 mm, Merck), and spots were detected by their blue

fluorescence under ultraviolet (UV) light (Manaslu light, 2536 Å) and with ferric chloride, anisaldehyde–sulfuric acid or 10% sulfuric acid reagent spray.

**Isolation of Tannins** The dried bark (1.6 kg) of *A. acuminata* var. *lanceolata*, collected in July, 1989, in Yunnan, China, was extracted four times with 60% aqueous acetone at room temperature. The extract was concentrated under reduced pressure and the resulting brown precipitates were removed by filtration. The filtrate was concentrated and subjected to Sephadex LH-20 column chromatography with water containing increasing amounts of MeOH and finally with a mixture of water–acetone (1 : 1) to give four fractions; fr. I (90 g), fr. II (55 g), fr. III (55 g) and fr. IV (54 g). Fraction I was chromatographed over MCI-gel CHP 20P ($H_2O$–MeOH) and Sephadex LH-20 ($H_2O$–MeOH) to yield castalin (**1**) (226 mg). Repeated chromatography of fr. II on MCI-gel CHP 20P, Fuji-gel ODS G3, Bondapak $C_{18}$/Porasil B ($H_2O$–MeOH) and Sephadex LH-20 (EtOH) yielded castalagin (**2**) (2.4 g), vescalagin carboxylic acid (**3**) (63 mg), grandinin (**4**) (3.1 g), acutissimins C (**5**) (26 mg) and A (**6**) (767 mg) and eugenigrandin A (**7**) (1.7 g). Fractions III and IV consisted of higher-molecular-weight tannins and were subjected to chromatographies over MCI-gel CHP 20P, Fuji-gel ODS G3, Bandapak $C_{18}$/Porasil B ($H_2O$–MeOH) and Sephadex LH-20 (60% MeOH and EtOH) to give castamollinin (**8**) and anogeissusin B (**11**) (404 mg) from fr. III, and anogeissinin (**9**) (225 mg) and anogeissusin A (**10**) (142 mg) from fr. IV. Compounds **1**—**8** were identified by direct comparisons of their physical and spectral data with those of authentic samples.

**Anogeissinin (9)** A tan amorphous powder, $[\alpha]_D^{20}$ +6.5° ($c$=1.1, MeOH). *Anal.* Calcd for $C_{97}H_{62}O_{56}\cdot 3H_2O$: C, 53.50; H, 3.15. Found: C, 53.48; H, 3.16. Negative FAB-MS $m/z$: 2121 $(M-H)^-$, 1206. $^1$H-NMR (acetone-$d_6$+$D_2O$, 270 MHz): Table I. $^{13}$C-NMR (acetone-$d_6$+$D_2O$, 25.05 MHz) $\delta$: 25.0 [catechin (cat.) C-4], 38.0 (2C) (C-1, 1′), 65.5 (2C) (C-6, 6′), 67.7 (cat. C-3), 70.1—72.5 (6C) (C-3, 3′, 4, 4′, 5, 5′), 77.6 (2C) (C-2), 80.4 (cat. C-2), 100.7 (cat. C-4a), 107.5—110.6 (cat. C-6, 8), 131.3 (cat. C-1′), 151.9, 154.6, 155.8 (cat. C-5, 7, 8a), 165.6 (2C), 167.1 (2C), 167.4, 167.5 (2C), 169.2, 169.4 (2C) (COO).

**Preparation of 9** A mixture of acutissimin A (**6**) (500 mg) and vescalagin (**12**) (500 mg) in dry dioxane (15 ml) containing *p*-toluenesulfonic acid (50 mg) was heated under reflux for 2.5 h with stirring. The solvent was evaporated off under reduced pressure, and the residue was chromatographed over Sephadex LH-20 with EtOH containing increasing amount of water–acetone (1 : 1) and then over Bondapak $C_{18}$/Porasil B with water containing increasing amounts of MeOH, to yield a condensation product (195 mg), which was shown to be identical with **9** by $[\alpha]_D$ and $^1$H- and $^{13}$C-NMR comparisons.

**Anogeissusin A (10)** A tan amorphous powder, $[\alpha]_D^{20}$ +15.3° ($c$=1.0, MeOH). *Anal.* Calcd for $C_{97}H_{62}O_{56}\cdot 3H_2O$: C, 53.50; H, 3.15. Found: C, 53.61; H, 3.22. Negative FAB-MS $m/z$: 2121 $(M-H)^-$, 915. $^1$H-NMR (acetone-$d_6$+$D_2O$, 270 MHz): Table I. $^{13}$C-NMR (acetone-$d_6$+$D_2O$, 25.05 MHz) $\delta$: 24.5 (cat. C-4), 37.9 (C-1), 40.7 (C-1′), 66.0 (2C) (C-6, 6′), 68.3 (cat. C-3), 70.6—73.0 (6C) (C-3. 3′, 4, 4′, 5, 5′), 77.8 (2C) (C-2, 2′), 80.5 (cat. C-2), 96.9 (cat. C-6), 98.4 (cat. C-4a), 131.9 (cat. C-1′), 153.0, 155.9, 156.3 (cat. C-5, 7, 8a), 165.4, 165.7, 166.8, 167.1 (3C), 168.0, 168.7, 169.4 (2C) (COO).

**Preparation of 10** A mixture of castamolinin (**8**) (250 mg) and (+)-catechin (**14**) (300 mg) in dry dioxane (15 ml) containing *p*-toluenesulfonic acid (30 mg) was heated under reflux for 2.5 h with stirring. After concentration under reduced pressure, the product was separated in a manner similar to that described for **9** to yield a condensation product (10 mg), which was shown to be identical with **10** by $[\alpha]_D$ and $^1$H- and $^{13}$C-NMR comparisons.

**Anogeissusin B (11)** A tan amorphous powder, $[\alpha]_D^{21}$ +10.1° ($c$=1.0, MeOH). *Anal.* Calcd for $C_{97}H_{62}O_{57}\cdot 3H_2O$: C, 53.11; H, 3.12. Found: C, 52.92; H, 3.31. Negative FAB-MS $m/z$: 2137 $(M-H)^-$. $^1$H-NMR (acetone-$d_6$+$D_2O$, 270 MHz): Table I. $^{13}$C-NMR (acetone-$d_6$+$D_2O$, 25.05 MHz) $\delta$: 24.3 [gallocatechin (g.c.) C-4], 37.9 (C-1), 40.5 (C-1′), 65.6 (2C) (C-6, 6′), 68.3 (g.c. C-3), 70.6—72.5 (6C) (C-3, 3′, 4, 4′, 5, 5′), 77.9 (2C) (C-2, 2′), 81.0 (g.c. C-2), 96.9 (g.c. C-6), 98.4 (g.c. C-4a), 131.6 (g.c. C-1′), 153.0, 155.8, 157.1 (g.c. C-5, 7, 8a), 165.3, 165.7, 166.8, 167.0 (3C), 168.1, 168.7, 169.3 (2C) (COO).

**Preparation of 11** A mixture of castamolinin (**8**) (250 mg) and (+)-gallocatechin (**15**) (300 mg) in dry dioxane (15 ml) containing *p*-toluenesulfonic acid (30 mg) was refluxed for 2.5 h with stirring. The reaction mixture was concentration to dryness under reduced pressure, and the residue was treated in a manner similar to that described for **9** to yield a product (12 mg), which was shown to be identical with **11** by $[\alpha]_D$ and $^1$H- and $^{13}$C-NMR comparisons.

**Acknowledgements** The authors are grateful to Mr. Y. Tanaka, Miss Y. Soeda and Mr. R. Isobe for $^1$H- and $^{13}$C-NMR and MS measurements, and to the staff of the Central Analysis Room of this university for elemental analyses.

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# Lewis Acid-Promoted Alkylations of Arenes and 1-Trimethylsilylalkynes with β-Chloro-β-thiopropanoic Esters

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**Ethyl 3-chloro-3-(3,4-dichlorophenylthio)propanoate (8) reacted with electron-rich arenes in the presence of titanium tetrachloride to give the Friedel–Crafts products 10a—13a. Reactions of 8 with 1-trimethylsilylalkynes 14a—d were effected with aluminum chloride to afford the substitution products 15a—d. Some chemical transformations of the products are also described.**

**Keywords** α-chlorosulfide; Friedel–Crafts reaction; 1-trimethylsilylalkyne; titanium tetrachloride; aluminum chloride; desulfurization; tributyltin hydride; alka-2,4-dienoic ester; alk-2-en-4-ynoic ester; pear ester

In previous papers,[1] we reported that ethyl α-chloro-α-(methylthio)acetate (**1a**: R = COOEt) reacted with arenes in the presence of a Lewis acid to give the Friedel–Crafts (F–C) products **2a** (R = COOEt) in excellent yields. This result is of particular interest in view of the fact that methylthiomethyl chloride (**1b**: R = H) gives only minute amounts of the desired products **2b** (R = H).[2] Thus, the effect of introducing an electron-withdrawing group into the α-position of the α-chloromethyl sulfides is significant. Our interest has now been focused on the reactivity of α-chlorosulfides containing an α-substituent into which methylene chains have been introduced between the reactive carbon atom and the ethoxycarbonyl group. We employed a series of α-chlorosulfides **4a—c** ($n$ = 1—3). In the present paper we wish to report that ethyl 3-chloro-3-(4-chlorophenylthio)propanoate (**4a**) and its 3,4-dichlorophenylthio congener **8** react smoothly with electron-rich arenes or 1-trimethylsilylalkynes in the presence of Lewis acid to give the respective substitution products.

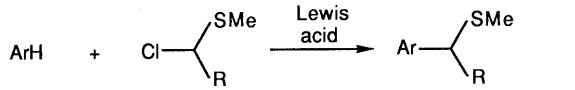
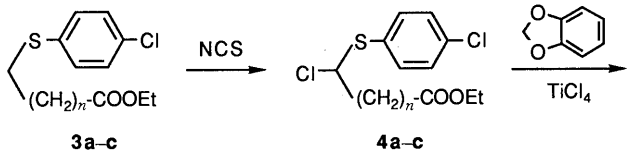
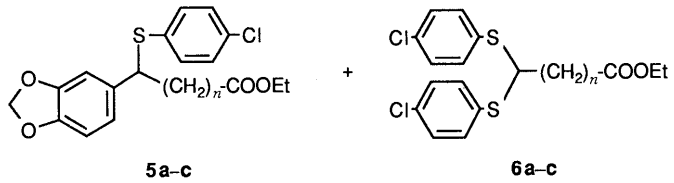



Chart 1




a : $n$=1 b : $n$=2 c : $n$=3

Chart 2

We began our investigation by examining the F–C reactions of 1,3-benzodioxole with the α-chlorosulfides **4a—c** bearing a 4-chlorophenyl group as the substituent on the sulfur atom. The chlorides **4a—c** were prepared from the corresponding ω-thioalkanoic esters **3a—c** by treatment with *N*-chlorosuccinimide (NCS). When a mixture of 1,3-benzodioxole and **4a** was treated with titanium tetrachloride ($TiCl_4$) in $CH_2Cl_2$ at −20 °C, the F–C product **5a** was obtained in 41% yield along with the dithioacetal **6a** (10%). Formation of the by-product **6a** may be a result of a Lewis acid-mediated disproportionation reaction of the starting chloride **4a**. A similar reaction with **4b** led to a complex mixture of products, from which the desired product **5b** was obtained in 24% yield. The proton nuclear magnetic resonance ($^1$H-NMR) spectrum of the crude reaction mixture showed it to contain a considerable amount of the dithioacetal **6b** [δ: 1.23 (3H, t, $J$=7 Hz), 2.19 (2H, d, $J$=7 Hz), 2.63 (2H, d, $J$=7 Hz), 4.11 (2H, q, $J$=7 Hz), 4.43 (1H, t, $J$=7 Hz), 7.24, 7.36 (4H each, AB q, $J$=9 Hz)]. The reaction with **4c** also proceeded poorly to give a 26% yield of the product **5c** together with **6c**. Thus, the β-chloro-β-thiopropanoic ester **4a** is a promising agent for carbon–carbon bond-forming reactions. Further experimentation revealed that the 3,4-dichlorophenylthio derivative **8** had advantages over **4a** as an alkylating agent in terms of the yield and the ease of chromatographic separation of the products.

The chloride **8** was prepared by exposure of the cor-

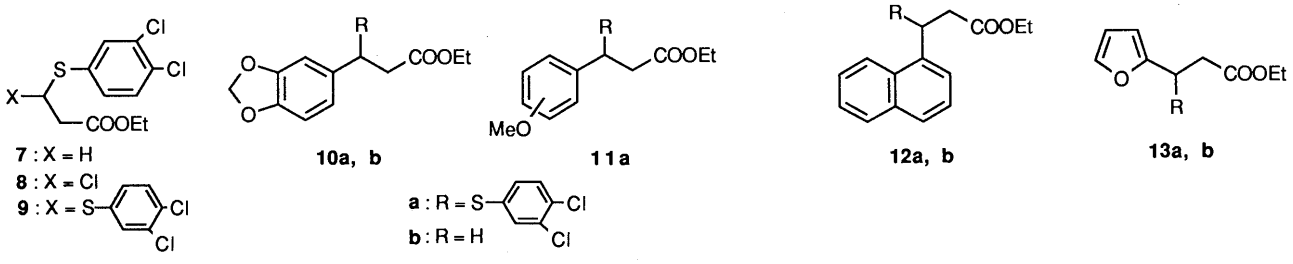
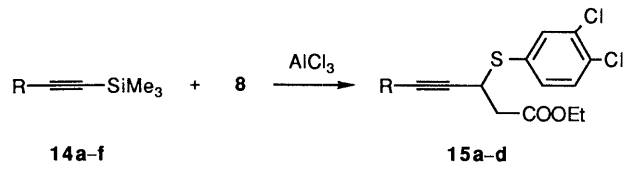



Chart 3



14a–f 15a–d

a : R = $CH_3$ b : R = $C_5H_{11}$ c : R = $C_6H_{13}$ d : R = $SiMe_3$
e : R = H f : R = Ph

Chart 4

responding sulfide **7** to sulfuryl chloride. Treatment of a mixture of equimolar amounts of 1,3-benzodioxole and **8** with $TiCl_4$ at $-20\,^{\circ}C$ gave the F–C product **10a** in 69% yield. When 2 molar eq of **8** and $TiCl_4$ were used in this reaction, **10a** was obtained in an almost quantitative yield (based on 1,3-benzodioxole). Similarly, the electron-rich arenes such as anisole, naphthalene, and furan reacted smoothly with **8** (2 eq) to afford the products **11a** (*o*/*p* = *ca.* 1 : 2), **12a**, and **13a** in 98, 98 and 86% yields, respectively. Benzene or toluene, however, gave no satisfactory result even when the reaction was carried out by using the arene itself as the solvent: the only isolated product was the dithioacetal **9**.

It is well known that 1-trimethylsilylalkynes undergo substitution reaction at the carbon carrying the silyl group with various electrophiles such as acyl chlorides in the presence of a Lewis acid.[3] The α-chlorosulfide **8** was found to react also with 1-trimethylsilylpropyne (**14a**) (1 eq) in the presence of 2 molar eq of aluminum chloride ($AlCl_3$), giving the substitution product **15a** in 61% yield.[4] when a stoichiometric amount of $AlCl_3$ was used, the yield of **15a** was lowered to 26%.[5] Similarly, the reactions with **14b** and **14c** gave **15b** and **15c** in 67 and 66% yields, respectively. The reaction of **8** with bis(trimethylsilyl)acetylene (**14d**) afforded **15d** in 63% yield. (Trimethylsilyl)acetylene (**14e**) or 1-phenyl-2-(trimethylsilyl)acetylene (**14f**), however, gave no satisfactory result.

Some chemical transformations of the products thus obtained were next examined. Desulfurization of the F–C products **10a**, **12a**, and **13a** with tributyltin hydride ($Bu_3SnH$)[6] afforded the corresponding arylpropanoic esters **10b**, **12b**, and **13b** in 65, 83, and 32% yields, respectively. Treatment of the alkyne **15a** with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in boiling acetonitrile afforded the 5-arylthio-2,4-hexadienoic ester **17** in 70% yield. The $^1$H-NMR spectrum of **17** showed it to be a mixture of (2*E*,4*E*)- and (2*E*,4*Z*)-isomers in a ratio of *ca.* 1 : 1. This reaction may proceed by a base-promoted elimination of the thiolate anion from **15a** to give ethyl hex-2-en-4-ynoate (**16**). This step is then followed by an attack of the thiolate anion on **16** in a 1,6-addition mode, leading to **17**. The use of triethylamine as a base instead of DBU resulted in recovery of the starting material. Treatment of **15d** with tetrabutylammonium fluoride (TBAF) in acetonitrile at room temperature gave, in 74% yield, the 5-arylthio-2,4-pentadienoic ester **20**, which was shown to be a mixture of (2*E*,4*E*)- and (2*E*,4*Z*)-isomers in a ratio of 1.6 : 1 (determined by $^1$H-NMR spectroscopy). A mechanistic rationalization of the formation of **20** involves an attack of the fluoride anion on the silyl center followed by protonation of the resulting acetylenic anion

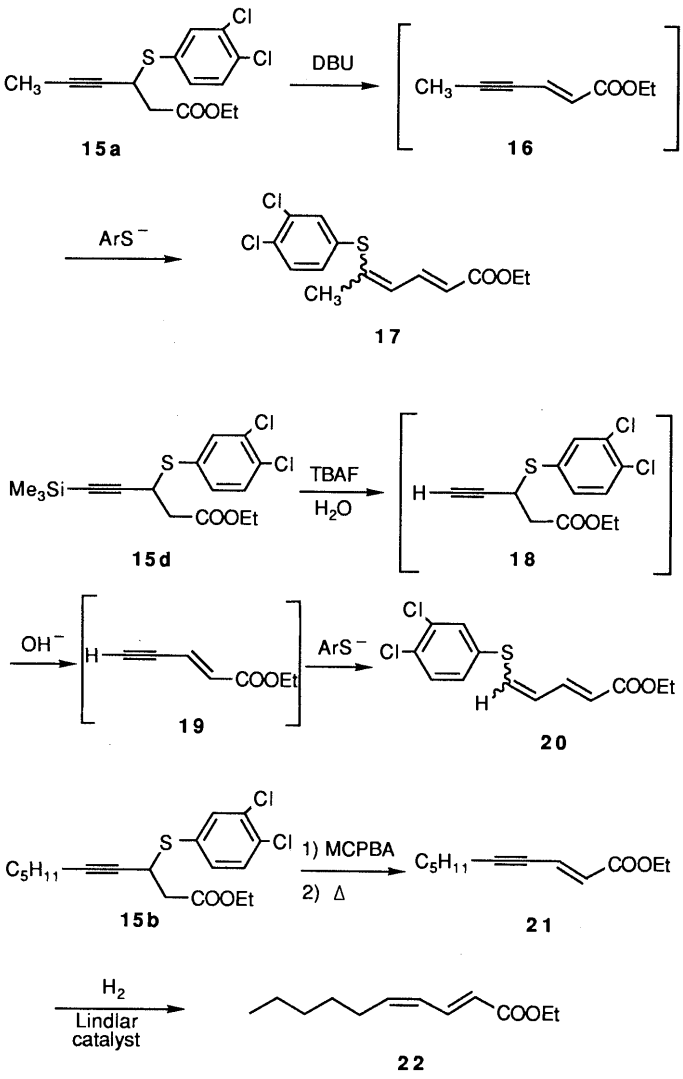


Chart 5

with water to give the desilylated alkyne **18**. Thus formed tetrabutylammonium hydroxide (a strong base) releases a thiolate anion from **18** to give the enyne **19**, which is then attacked by the thiolate anion to give **20**.

On the other hand, oxidation of **15b** with *m*-chloroperbenzoic acid followed by heating of the resulting sulfoxide in $CH_2Cl_2$ brought about elimination of sulfenic acid to give the enyne ester **21**, whose transformation into ethyl (*E*,*Z*)-2,4-decadienoate (**22**), one of the flavor constituents of Bartlett pears, by hydrogenolysis over Lindlar's catalyst has already been described in the literature.[7]

In summary, the Lewis acid-promoted reactions of a series of ω-chloro-ω-thioalkanoic esters with unsaturated compounds have been examined, and the β-chloro-β-thiopropanoic esters **4a** and **8** were found to react smoothly with arenes or 1-trimethylsilylalkynes. Taking into account the reactivity observed for the α-chlorosulfide **1a**, the effectiveness of the reactions of **4a** and **8** seems to be ascribable to the presence of an electron-withdrawing ethoxycarbonylmethyl group ($CH_2COOEt$) at the α-position of the chlorosulfides: the coordination of a Lewis acid to the ester carbonyl group would serve to enhance the inductive effect of the ester group. Such an electron-withdrawing effect of the ester group, however, would not

be exerted on the reactive carbon atom of the other homologous systems **4b** and **4c**.

## Experimental

Melting points are uncorrected. Infrared (IR) spectra were recorded with a JASCO A-100 spectrophotometer. $^1$H-NMR spectra were determined with a JEOL JNM-PMX 60 or a Varian XL-300 (300 MHz) spectrometer (60 MHz), and $\delta$ values are quoted relative to tetramethylsilane. Exact mass (MS) determinations were obtained on a Hitachi M-80 instrument operating at 20 eV. Chromatographic separations of the products were performed with Silica gel 60 $PF_{254}$ (Merck) under pressure.

**Ethyl 3-(4-Chlorophenylthio)propanoate (3a)** Triethylamine (0.7 g, 6.9 mmol) was added to a solution of *p*-chlorobenzenethiol (10 g, 69.2 mmol) and ethyl acrylate (6.9 g, 69.2 mmol) in $CHCl_3$ (50 ml) at 0 °C, and the mixture was stirred at room temperature for 2 h. After completion of the reaction, the mixture was washed successively with 5% NaOH solution and brine, then dried over $MgSO_4$. The solvent was evaporated off to give quantitatively the sulfide **3a**, which crystallized on standing, mp < 30 °C. An analytical sample was prepared by chromatographic purification (benzene–hexane, 1 : 1). IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1740. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.23 (3H, t, $J$=7 Hz), 2.56 (2H, t with splitting), 3.11 (2H, t with splitting), 4.10 (2H, q, $J$=7 Hz), 7.24 (4H, s). *Anal.* Calcd for $C_{11}H_{13}ClO_2S$: C, 53.99; H, 5.35. Found: C, 53.87; H, 5.48.

**Ethyl 4-(4-Chlorophenylthio)butanoate (3b)** *p*-Chlorobenzenethiol (7.2 g, 50 mmol) and ethyl 4-bromobutanoate (9.75 g, 50 mmol) were added successively to a solution of sodium ethoxide (55 mmol) in dry ethanol (50 ml) at 0 °C, and the mixture was heated under reflux for 2 h, then cooled. After removal of the solvent, water (30 ml) was added to the residue and the whole was extracted with diethyl ether. The solvent was evaporated off and the residue was distilled *in vacuo* to give the sulfide **3b** (10.6 g, 82%), bp 139—140 °C (2 mmHg). IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1740. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.24 (3H, t, $J$=7 Hz), 1.65—2.25 (2H, m), 2.46 (2H, t, $J$=7 Hz), 2.95 (2H, t, $J$=7 Hz), 4.13 (2H, q, $J$=7 Hz), 7.23 (4H, s). *Anal.* Calcd for $C_{12}H_{15}ClO_2S$: C, 55.70; H, 5.84. Found: C, 55.34; H, 5.99.

**Ethyl 5-(4-Chlorophenylthio)pentanoate (3c)** By using a procedure similar to that described above for the preparation of **3b**, *p*-chlorobenzenethiol was allowed to react with ethyl 5-bromopentanoate in the presence of sodium ethoxide. After usual work-up, the crude product was chromatographed on silica gel (benzene) to give quantitatively the sulfide **3c**, which crystallized on standing, mp 31.5 °C. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1740. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.24 (3H, t, $J$=7 Hz), 1.75 (4H, br s), 2.2—2.5 (2H, m), 2.8—3.1 (2H, m), 4.13 (2H, q, $J$=7 Hz), 7.24 (4H, s). *Anal.* Calcd for $C_{13}H_{17}ClO_2S$: C, 57.24; H, 6.28. Found: C, 56.89; H, 6.09.

**General Procedure for the Preparation of α-Chlorosulfides 4a—c** *N*-Chlorosuccinimide (267 mg, 2 mmol) was added in portions to a stirred solution of one of the sulfides **3a—c** in $CCl_4$ (10 ml) at 0 °C, and stirring was continued at room temperature overnight. The precipitated succinimide was filtered off and the filtrate was concentrated *in vacuo* to give quantitatively the corresponding chlorosulfide **4a—c**, which, without further purification, was used in the next stage. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$ for **4a**: 1.30 (3H, t, $J$=7 Hz), 3.05 (2H, d, $J$=7 Hz), 4.25 (2H, q, $J$=7 Hz), 5.58 (1H, t, $J$=7 Hz), 7.37, 7.53 (2H each, AB q, $J$=8 Hz); $\delta$ for **4b**: 1.30 (3H, t, $J$=7 Hz), 2.0—2.9 (4H, m), 4.17 (2H, q, $J$=7 Hz), 5.31 (1H, t, $J$=7 Hz), 7.31, 7.50 (2H each, AB q, $J$=8 Hz); $\delta$ for **4c**: 1.30 (3H, t, $J$=7 Hz), 1.6—2.9 (6H, m), 4.18 (2H, q, $J$=7 Hz), 5.23 (1H, t, $J$=7 Hz), 7.33, 7.50 (2H each, AB q, $J$=8 Hz).

**General Procedure for the Reactions of 4a—c with 1,3-Benzodioxole** $TiCl_4$ (417 mg, 2.2 mmol) was added to a solution of 1,3-benzodioxole (244 mg, 2 mmol) and one of the α-chlorosulfides **4a—c** (2 mmol) in $CH_2Cl_2$ (10 ml) at −20 °C and the mixture was stirred at the same temperature for 1 h. The reaction was quenched by the addition of water (10 ml) and the whole was extracted with $CH_2Cl_2$. The solvent was evaporated off and the residue was chromatographed on silica gel (benzene) to give the following compounds. Ethyl (1,3-benzodioxol-5-yl)-3-(4-chlorophenylthio)propanoate (**5a**), an oil, 41% yield from **4a**. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1735. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.16 (3H, t, $J$=7 Hz), 2.85 (2H, d, $J$=7 Hz), 4.06 (2H, q, $J$=7 Hz), 4.53 (1H, t, $J$=7 Hz), 5.90 (2H, s), 6.63 (2H, s), 6.79 (1H, br s), 7.19 (4H, s). Exact MS *m/z*: Calcd for $C_{18}H_{17}ClO_4S$: 364.0535. Found: 364.0562. Ethyl 3,3-bis(4-chlorophenylthio)propanoate (**6a**), an oil, 10% yield from **4a**. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1735. $^1$H-NMR ($CDCl_3$) $\delta$: 1.26 (3H, t, $J$=7 Hz), 2.75 (2H, d, $J$=7.5 Hz), 4.15 (2H, q, $J$=7 Hz), 4.71 (1H, d, $J$=7.5 Hz), 7.25, 7.39 (4H each, AB q, $J$=9 Hz). Exact MS *m/z*: Calcd for $C_{17}H_{16}Cl_2O_2S_2$: 385.9967. Found: 385.9941. Ethyl 4-(1,3-benzodioxol-5-yl)-4-(4-chlorophenylthio)butanoate (**5b**), an oil, 24% from **4b**. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1735. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.20 (3H, t, $J$=7 Hz), 2.0—2.6 (4H, m), 3.9—4.3 (3H, m), 5.87 (2H, s), 6.60 (2H, s), 6.75 (1H, br s), 7.15 (4H, s). Exact MS *m/z*: Calcd for $C_{19}H_{19}ClO_4S$: 378.0691. Found: 378.0702. Ethyl 5-(1,3-benzodioxol-5-yl)-5-(4-chlorophenylthio)pentanoate (**5c**), an oil, 26% yield from **4c**. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1735. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.23 (3H, t, $J$=7 Hz), 1.5—2.1 (4H, m), 2.28 (2H, t, $J$=7 Hz), 4.04 (1H, t, $J$=7 Hz), 4.10 (2H, q, $J$=7 Hz), 5.92 (2H, s), 5.62 (2H, s), 5.78 (1H, br s), 7.15 (4H, s). Exact MS *m/z*: Calcd for $C_{20}H_{21}ClO_4S$: 392.0847. Found: 392.0823.

**Ethyl 3-(3,4-Dichlorophenylthio)propanoate (7)** By using a procedure similar to that described above for the preparation of **3a**, compound **7**, an oil, was prepared quantitatively from 3,4-dichlorobenzenethiol and ethyl acrylate. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1740. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.26 (3H, t, $J$=7 Hz), 2.63 (2H, t with splitting), 3.19 (2H, t with splitting), 4.17 (2H, q, $J$=7 Hz), 7.0—7.5 (3H, m). *Anal.* Calcd for $C_{11}H_{12}Cl_2O_2S$: C, 47.32; H, 4.33. Found: C, 47.25; H, 4.04.

**Ethyl 3-Chloro-3-(3,4-dichlorophenylthio)propanoate (8)** Sulfuryl chloride (0.96 g, 0.58 ml, 7.16 mmol) was added in portions to a stirred solution of **7** (2 g, 7.16 mmol) in $CH_2Cl_2$ at 0 °C and stirring was continued at the same temperature for 1 h. After completion of the reaction, the solvent was evaporated off to give quantitatively the chloride **8** as an oil, which, without further purification, was used in the next stage. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.30 (3H, t, $J$=7 Hz), 3.05 (2H, d, $J$=7 Hz), 4.22 (2H, q, $J$=7 Hz), 5.59 (1H, t, $J$=7 Hz), 7.44 (2H, s), 7.67 (1H, br s).

**Ethyl 3,3-Bis(3,4-dichlorophenylthio)propanoate (9)** $TiCl_4$ (189 mg, 0.95 mmol) was added to a solution of **8** (300 mg, 0.95 mmol) in benzene (5 ml) at 10 °C and the mixture was stirred at the same temperature for 1 h. Water (10 ml) was added to the reaction mixture and the whole was extracted with benzene. The solvent was evaporated off and the residue was chromatographed on silica gel (benzene–hexane, 1 : 1) to give **9** (115 mg, 53%) as an oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1735. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.28 (3H, t, $J$=7 Hz), 2.80 (2H, d, $J$=7 Hz), 4.20 (2H, q, $J$=7 Hz), 4.78 (1H, t, $J$=7 Hz), 7.33 (4H, s), 7.50 (2H, br s). Exact MS *m/z*: Calcd for $C_{17}H_{14}Cl_4O_2S_2$: 453.9188. Found: 453.9169.

**General Procedure for the Preparation of 10a—13a** By using a procedure similar to that described above for the preparation of **5a—c**, 1,3-benzodioxole, anisole, naphthalene, or furan (1 mmol each) was allowed to react with **8** (627 mg, 2 mmol) in $CH_2Cl_2$ at −20 °C in the presence of $TiCl_4$ (398 mg, 2.1 mmol). Usual work-up gave the following compounds. Ethyl 3-(1,3-benzodioxol-5-yl)-3-(3,4-dichlorophenylthio)propanoate (**10a**), 98% yield, mp 54—55 °C (hexane). IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1740. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.15 (3H, t, $J$=7 Hz), 2.82 (2H, d, $J$=7.5 Hz), 4.05 (2H, q, $J$=7 Hz), 4.54 (1H, t, $J$=7.5 Hz), 5.88 (2H, s), 6.62 (2H, s), 6.75 (1H, br s), 6.9—7.4 (3H, m). *Anal.* Calcd for $C_{18}H_{16}Cl_2O_4S$: C, 54.15; H, 4.04. Found: C, 53.88; H, 4.05. Ethyl 3-(3,4-dichlorophenylthio)-3-(2-methoxy- and 4-methoxy-phenyl)propanoates (**11a**), 98% yield, an oil. The $^1$H-NMR spectrum of **11a** showed it to be a mixture of *ortho*- and *para*-isomers in a ratio of *ca.* 1 : 2. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1735. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$ for *o*-isomer: 1.17 (3H, t, $J$=7 Hz), 2.95 (2H, d, $J$=7.5 Hz), 3.82 (3H, s), 4.08 (2H, q, $J$=7 Hz), 5.10 (1H, t, $J$=7.5 Hz), 6.7—7.5 (7H, m); $\delta$ for *p*-isomer: 1.16 (3H, t, $J$=7 Hz), 2.90 (2H, d, $J$=7.5 Hz), 3.80 (3H, s), 4.10 (2H, q, $J$=7 Hz), 4.64 (1H, t, $J$=7.5 Hz), 6.80 (2H, d, $J$=8 Hz), 7.0—7.5 (5H, m). Exact MS *m/z*: Calcd for $C_{18}H_{18}Cl_2O_3S$: 384.0352. Found: 384.0332. Ethyl 3-(3,4-dichlorophenylthio)-3-(1-naphthyl)propanoate (**12a**), 98% yield, an oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1740. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.07 (3H, t, $J$=7 Hz), 3.03 (2H, d, $J$=7 Hz), 4.02 (2H, q, $J$=7 Hz), 5.45 (1H, d, $J$=7.5 Hz), 6.85—8.3 (10H, m). *Anal.* Calcd for $C_{21}H_{18}Cl_2O_2S$: C, 62.23; H, 4.48. Found: C, 61.81; H, 4.73. Ethyl 3-(3,4-dichlorophenylthio)-3-(2-furyl)propanoate (**13a**), 86% yield, an oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1740. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.23 (3H, t, $J$=7 Hz), 2.92 (2H, d, $J$=7.5 Hz), 4.14 (2H, q, $J$=7 Hz), 4.67 (1H, t, $J$=7.5 Hz), 6.01 (1H, d, $J$=3.5 Hz), 6.25 (1H, dd, $J$=3.5, 2 Hz), 7.13 (1H, d, $J$=2 Hz), 7.2—7.5 (3H, m). *Anal.* Calcd for $C_{15}H_{14}Cl_2O_3S$: C, 52.19; H, 4.09. Found: C, 52.27; H, 4.15.

**General Procedure for the Preparation of 3-Arylpropanoic Esters 10b, 12b, and 13b** A mixture of $Bu_3SnH$ (0.55—0.75 mmol) and azobisisobutyronitrile (0.055 mmol) in benzene (5 ml) was added dropwise to a boiling solution of **10a**, **12a**, or **13a** (0.5 mmol) in benzene (13 ml), and the mixture was further heated under reflux for 2—4 h. Usual work-up gave the following compounds. Ethyl 3-(1,3-benzodioxol-5-yl)propanoate (**10b**),[8] 65% yield, an oil. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.23 (3H, t, $J$=7 Hz), 2.4—3.1 (4H, m), 4.13 (2H, q, $J$=7 Hz), 5.88 (2H, s), 6.68 (3H, s). Ethyl 3-(1-naphthyl)propanoate (**12b**),[9] 83% yield, an oil. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.20 (3H, t, $J$=7 Hz), 1.5—1.9 (2H, m), 3.25—3.6 (2H, m),

4.10 (2H, q, $J$=7 Hz), 7.27—8.15 (7H, m). Ethyl 3-(2-furyl)propanoate (**13b**),[10] 32% yield, an oil. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.26 (3H, t, $J$=7 Hz), 2.4—3.1 (4H, m), 4.12 (2H, q, $J$=7 Hz), 5.98 (1H, d, $J$=3 Hz), 6.23 (1H, dd, $J$=3, 2 Hz), 7.2—7.3 (1H, m).

**General Procedure for the Preparation of 15a—d** $AlCl_3$ (467 mg, 3.5 mmol) was added to a solution of **8** (550 mg, 1.75 mmol) and one of **14a—d** (1.75 mmol) in $CH_2Cl_2$ (10 ml) at −20 °C, and the mixture was stirred vigorously at the same temperature for 1 h. The reaction was quenched by the addition of water (10 ml) and the whole was extracted with $CH_2Cl_2$. The solvent was evaporated off and the residue was chromatographed on silica gel (benzene–hexane, 1 : 1) to give the corresponding product. Ethyl 3-(3,4-dichlorophenylthio)hex-4-ynoate (**15a**), 61% yield, an oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 2240, 1740. $^1$H-NMR ($CDCl_3$) $\delta$: 1.24 (3H, t, $J$=7 Hz), 1.76 (3H, d, $J$=2 Hz), 3.66 (2H, d, $J$=7.5 Hz), 4.13 (1H, t, $J$=7.5 Hz), 4.15 (2H, q, $J$=7 Hz), 7.32 (2H, s), 7.60 (1H, br s). *Anal.* Calcd for $C_{14}H_{14}Cl_2O_2S$: C, 53.01; H, 4.45. Found: C, 52.70; H, 4.49. Ethyl 3-(3,4-dichlorophenylthio)dec-4-ynoate (**15b**), 67% yield, an oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 2240, 1740. $^1$H-NMR ($CDCl_3$) $\delta$: 0.7—1.7 (9H, m), 1.28 (3H, t, $J$=7 Hz), 1.9—2.4 (2H, m), 2.70 (2H, d, $J$=7.5 Hz), 4.0—4.4 (3H, m), 7.35 (2H, br s), 7.65 (1H, br s). *Anal.* Calcd for $C_{18}H_{22}Cl_2O_2S$: C, 57.91; H, 5.94. Found: C, 57.65; H, 5.90. Ethyl 3-(3,4-dichlorophenylthio)undec-4-ynoate (**15c**), 66% yield, an oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 2240, 1740. $^1$H-NMR ($CDCl_3$) $\delta$: 0.7—1.7 (11H, m), 1.28 (3H, t, $J$=7 Hz), 1.9—2.4 (2H, m), 2.74 (2H, d, $J$=7.5 Hz), 4.0—4.4 (3H, m), 7.40 (2H, s), 7.67 (1H, br s). Exact MS $m/z$: Calcd for $C_{19}H_{24}Cl_2O_2S$: 386.0872. Found: 386.0859. Ethyl 3-(3,4-dichlorophenylthio)-5-trimethylsilylpent-4-ynoate (**15d**), 63%, an oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 2180, 1740. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 0.13 (9H, s), 1.25 (3H, t, $J$=7 Hz), 2.66 (2H, d, $J$=7.5 Hz), 4.12 (2H, q, $J$=7 Hz), 4.14 (1H, t, $J$=7.5 Hz), 7.30 (2H, s), 7.62 (1H, br s). Exact MS $m/z$: Calcd for $C_{16}H_{20}ClOSSi$: 374.0329. Found: 374.0330.

**Ethyl 5-(3,4-Dichlorophenylthio)hexa-2,4-dienoate (17)** DBU (58 mg, 0.37 mmol) was added to a solution of **15a** (105 mg, 0.37 mmol) in acetonitrile (2 ml) and the mixture was heated under reflux for 3 h, then cooled. Water (10 ml) was added to the reaction mixture and the whole was extracted with $CH_2Cl_2$. The solvent was evaporated off and the residue was chromatographed on silica gel (hexane–benzene, 2 : 1) to give oily **17** (74 mg, 70%) as a mixture of the (2*E*,4*E*)- and (2*E*,4*Z*)-isomers in a ratio of about 1 : 1 (determined by $^1$H-NMR spectroscopy). IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1720, 1620. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 1.28, 1.31 (total 3H, both t, $J$=7 Hz), 2.03, 2.13 (total 3H, both s), 4.21, 4.24 (total 2H, both q, $J$=7 Hz), 5.75 (0.5H, d, $J$=15 Hz), 5.93 (0.5H, d, $J$=15 Hz), 6.03 (0.5H, d, $J$=11 Hz), 6.43 (0.5H, d, $J$=10 Hz), 7.0—7.7 (3.5H, m), 7.85 (0.5H, dd, $J$=15, 11 Hz). Exact MS $m/z$: Calcd for $C_{14}H_{14}Cl_2O_2S$: 316.0090. Found: 316.0065.

**Ethyl 5-(3,4-Dichlorophenylthio)penta-2,4-dienoate (20)** Tetrabutylammonium fluoride (139 mg, 0.53 mmol) was added to a solution of **15d** (200 mg, 0.53 mmol) in acetonitrile (3 ml) and the mixture was stirred at room temperature for 1.5 h. The solvent was evaporated off and the residue was chromatographed on silica gel (benzene–hexane, 2 : 1) to give **20** (162 mg, 74%) as a mixture of the (2*E*,4*E*)- and (2*E*,4*Z*)-isomers in a ratio of 1.6 : 1 (determined by $^1$H-NMR spectroscopy), mp 66—67 °C (from hexane–ethyl acetate). IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1720, 1630. $^1$H-NMR ($CDCl_3$, 300 MHz) $\delta$ for (2*E*,4*E*)-isomer: 1.29 (3H, t, $J$=7.1 Hz), 4.20 (2H, q, $J$=7.1 Hz), 5.79 (1H, d, $J$=14.6 Hz, H-2), 6.35 (1H, ddd, $J$=14.9, 11.2, 0.7 Hz, H-4), 6.78 (1H, d, $J$=14.9 Hz, H-5), 7.2—7.5 (3H, m), 7.27 (1H, ddd, $J$=14.7, 11.2, 0.7 Hz, H-3); $\delta$ for (2*E*,4*Z*)-isomer: 1.32 (3H, t, $J$=7.1 Hz), 4.23 (2H, q, $J$=7.1 Hz), 5.98 (1H, d, $J$=15.3 Hz, H-2), 6.43 (1H, ddd, $J$=11.2, 9.0, 0.7 Hz, H-4), 6.56 (1H, d, $J$=9.0 Hz, H-5), 7.2—7.5 (3H, m), 7.64 (1H, ddd, $J$=15.3, 11.2, 0.8 Hz, H-3). *Anal.* Calcd for $C_{13}H_{12}Cl_2O_2S$: C, 51.50; H, 3.99. Found: C, 51.33; H, 4.51.

**Ethyl (*E*)-Dec-2-en-4-ynoate (21)** *m*-Chloroperbenzoic acid (80%, 101 mg, 0.47 mmol) was added in portions to a stirred solution of **15b** (176 mg, 0.47 mmol) in $CH_2Cl_2$ (10 ml) at 0 °C, and stirring was continued at 0 °C for 30 min, then at room temperature for 5 min. Powdered $NaHCO_3$ (100 mg) was added to the resultant solution containing the crude sulfoxide, and the whole was heated under reflux for 1 h, then cooled. The inorganic materials were filtered off, the filtrate was concentrated *in vacuo*, and the residue was chromatographed on silica gel (hexane–ethyl acetate, 15 : 1) to give **21**[7] (35 mg, 38%) as an oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1720, 1630. $^1$H-NMR ($CDCl_3$, 60 MHz) $\delta$: 0.7—1.9 (9H, m), 1.28 (3H, t, $J$=7 Hz), 2.2—2.5 (2H, m), 4.20 (2H, q, $J$=7 Hz), 6.10 (1H, d, $J$=16 Hz, H-2), 6.75 (1H, dt, $J$=16, 2 Hz, H-3).

## References and Notes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# Synthetic Studies on Indoles and Related Compounds. XXVI.[1] The Debenzylation of Protected Indole Nitrogen with Aluminum Chloride. (2)[2]

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**A new debenzylation method using aluminum chloride in benzene or anisole, which had been developed by us for *N*-benzyl-2-acyl- and -2-ethoxycarbonylindoles, was applied to benzyl derivatives of other types of indoles and related compounds. Among them, *N*-benzyl derivatives of fully aromatized indoles, carbazoles and *β*-carbolines, and some benzamides were debenzylated successfully, whereas those of oxindoles and heterocyclic amides were not. As to the effect of a *p*-substituent on the benzyl group, it was found that an electron-donating substituent accelerates deprotection, whereas an electron-attracting substituent delays or prevents deprotection.**

**Keywords** debenzylation; aluminum chloride; indole; benzamide; benzene; anisole

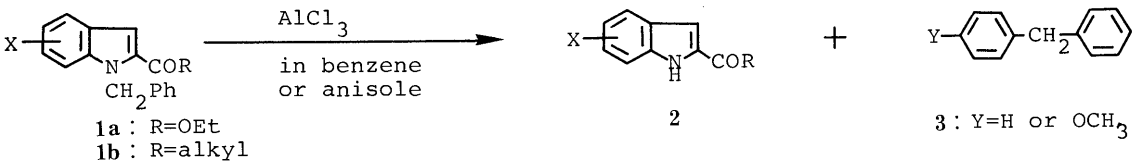
Some years ago we reported[2] a novel method for the debenzylation of protected nitrogen of indoles using aluminum chloride in benzene or anisole. In this reaction *N*-benzylindoles (**1a**, **b**) were easily converted to the corresponding NH-indoles (**2a**, **b**) under mild conditions, and the benzyl cation thus formed was trapped with benzene or anisole to form diarylmethane derivatives (**3**). The mechanism proposed in the case of ethyl 1-benzyl-1*H*-indole-2-carboxylate is shown in Chart 1, and is reminiscent of the Fries rearrangement. However, this method was applied only to the *N*-benzyl derivatives of ethyl indole-2-carboxylates (**2a**) and 2-acylindoles (**2b**). In this paper we describe the application of this method to other types of indoles and related compounds to examine its scope and limitations. If the expected debenzylation proceeds, the method is expected to be practically useful, because it has been reported that removal of the *N*-benzyl group from an amide by catalytic hydrogenolysis is rather difficult.[3]

The required unknown *N*-benzyl derivatives (**5**) were prepared by benzylation of the corresponding NH compounds (**4**) or by other methods, as shown in Table I, and their characteristics are listed in Table II.

Debenzylation reaction was carried out under the conditions used for the previous experiment[2]; that is, *N*-benzyl compounds (**5**) (1 eq) were allowed to react with aluminum chloride (4—6 eq) in benzene, or anisole at 0—50 °C. The results are summarized in Table III, and represent the highest yield obtained for each benzyl compound after many trials. The products, NH-compounds (**4**), were identical with authentic samples in terms of infrared (IR) and $^1$H-nuclear magnetic resonance ($^1$H-NMR) spectra, and melting points. The compounds which did not undergo debenzylation are given below Table III.

Run 1 shows that a 3-acyl group in the ethyl indole-2-carboxylate skeleton does not prevent debenzylation. However, the reaction was accompanied with formation of 3-acetyl-1*H*-indole-2-carboxylic acid (**9**) (9%) by hydrolysis, and some starting material (**5a**) was recovered (8%). Run 2 shows that the 2-amide group in place of the 2-ethoxycarbonyl group was also effective for debenzylation, although anisole gave a better result than benzene as a solvent. Successful debenzylation of the 3-acyl-2-unsubstituted indoles (**5c**) (run 3) shows that the presence of a 2-acyl group is not essential for debenzylation.

It is noteworthy that *N*-benzyl derivatives of fully aromatized indoles, carbazole and *β*-carboline (**5d** and **5e** in runs 4 and 5) were debenzylated easily in almost the same manner as 2-alkoxycarbonyl- and 2-acylindoles[2] (**1a**, **b**). On the basis of this knowledge, this method was successfully applied to the synthesis of crenatine,[4] a new kind of



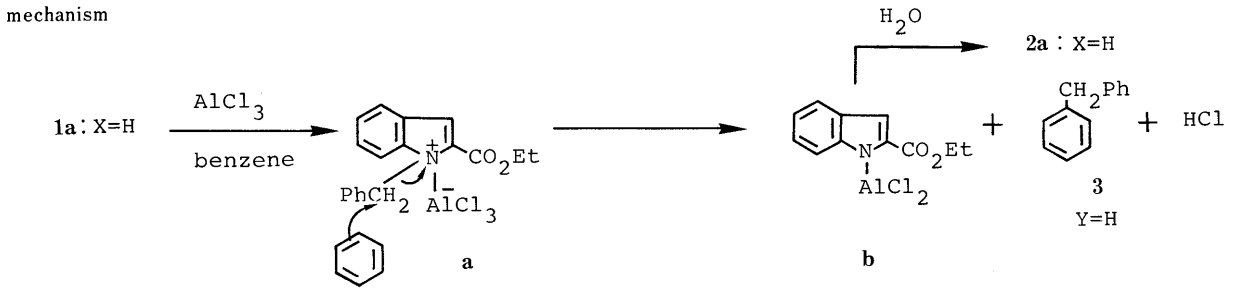
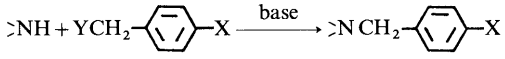



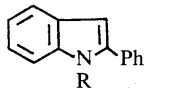
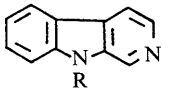
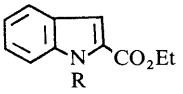
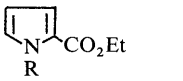
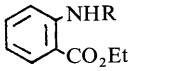

Chart 1

TABLE I. Preparation of *N*-Benzylindoles

A. *N*-Benzylation of NH Derivatives

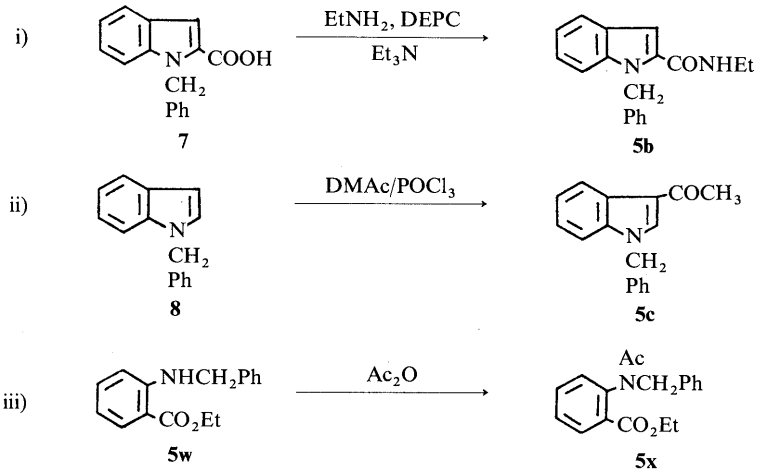



4 6 5

| Run | Starting material 4 (R=H) | X | Y | Base | Solvent | Temperature | Time | Yield (%) of 5 (R=CH$_2$-C$_6$H$_4$-X) |
|---|---|---|---|---|---|---|---|---|
| 1 |  (4n) | H | Cl | NaH | DMF | r.t. | 2 h | 75 |
| 2 |  (4e) | H | Cl | NaH | DMSO | 50 °C | 0.5 h | 69 |
| 3 |  (2a) | OMe | Cl | NaH | DMSO | 50 °C | 0.5 h | 57 |
| 4 | **2a** | $NO_2$ | Br | $K_2CO_3$ | Acetone | Reflux | 14 h | 71[a] |
| 5 |  (4g) | H | Cl | NaH | DMSO | 50 °C | 20 min | 58 |
| 6 |  (4w) | H | Cl | — | EtOH | Reflux | 12.5 h | 70 |

*a*) Yield was low (41%) when the reagent used was NaH in DMSO.

B. By other methods




β-carboline alkaloid, and would also be applicable to our synthesis[5] of ellipticine, in which the debenzylation step was carried out by means of the troublesome Birch reduction. In the present debenzylation the product was sometimes contaminated with a small amount of "$C_x$-benzyl-NH compounds," which would be formed by the attack of benzyl cation at the carbon moiety of the debenzylated NH-product. This was observed by mass spectrometry (MS, same molecular weight as starting material), $^1$H-NMR spectroscopy (the presence of an isolated $-CH_2-$ and an NH group), and IR spectroscopy (the presence of an NH group) in the case of **5g**.

*N*-Benzyl-1,2,3,4-tetrahydrocarbazole (**5f**, run 6) seemed to undergo debenzylation but gave a mixture of many products, when checked by thin layer chromatography (TLC), probably because **5f** and the expected product (NH-compound), which has similar reactivity to usual indoles (indoles which are not stabilized by an electronegative substituent), are susceptible to acid (aluminum chloride) to give a mixture of products. Ethyl 1-benzyl-1*H*-pyrrole-2-carboxylate (**5g**, run 7) showed a similar reactivity to **5f**; that is, the reaction in benzene gave a mixture of several products. From the mixture, only a C-benzyl-NH product was isolated in low yield, but the position of the benzyl group was uncertain. *N*-Benzylisatin (**5h**, run 8) did not undergo debenzylation but two equivalents of benzene reacted at the 3-position to give 1-benzyl-3,3-diphenyloxindole in 88% yield. The reaction of isatin with aluminum chloride in benzene was reported to give the same kind of compound, 3,3-diphenyloxindole.[6] Other indolic com-

TABLE II. Characterization of *N*-Benzyl Compounds

| Compound | Melting point or boiling point (°C) | Recrystallization solvent (Crystal form) | Formula | Analysis (%) Calcd (Found) | | | $^1$H-NMR ($NCH_2Ar$) $\delta$ ppm (in $CDCl_3$) |
|---|---|---|---|---|---|---|---|
| | | | | C | H | N | |
| **5c** | 115—116 | Benzene–hexane (Colorless needles) | $C_{17}H_{15}NO$ | 81.90 (81.72 | 6.06 6.18 | 5.62 5.55) | 5.30 (s) |
| **5n** | 96.5—98.5 | Ethyl acetate–hexane (Colorless prisms) | $C_{21}H_{17}N$ | 89.01 (89.23 | 6.05 6.13 | 4.94 4.90) | 5.33 (s) |
| **5b** | 124—125 | Benzene (Colorless needles) | $C_{18}H_{18}N_2O$ | 77.67 (77.90 | 6.52 6.51 | 10.06 10.04) | 5.77 (s) |
| **5e** | 118—120 | Benzene–hexane (Colorless needles) | $C_{18}H_{14}N_2$ | 83.69 (83.47 | 5.46 5.50 | 10.84 11.09) | 5.48 (s) |
| **5l** | 45—47.5 | Ethyl acetate–pentane (Colorless needles) | $C_{19}H_{19}NO_3$ | 73.77 (73.53 | 6.19 6.16 | 4.53 4.66) | 5.72 (s) |
| **5m** | 102—104 | Benzene–hexane (Colorless needles) | $C_{18}H_{16}N_2O_4$ | 66.66 (66.69 | 4.97 4.98 | 8.64 8.58) | 5.91 (s) |
| **5g** | bp 155 °C/17 mmHg | Colorless oil | $C_{14}H_{15}NO_2$ | | 229.1099[a] (229.1093) | | 5.50 (s) |
| **5w** | — | Colorless oil | $C_{16}H_{17}NO_2$ | | 255.1255[a] (255.1251) | | 4.42 (d) |
| **5x** | 54.5—56 | Benzene–hexane (Yellow plates) | $C_{18}H_{19}NO_3$ | 72.71 (72.82 | 6.44 6.48 | 4.71 4.71) | 4.17 and 5.35 (d) |

*a*) High-resolution mass spectral data.

pounds, *N*-benzyl-2-phenyl-1*H*-indole (**5n**), *N*-benzyloxindole (**5o**), and related heterocycles (**5p** and **5q**) did not undergo debenzylation at all. On the other hand *N*-benzylbenzamide derivatives (**5i**, **5j**, and **5k**, runs 9—11) underwent debenzylation successfully. In them, a more electronegative group on the benzene ring accelerated debenzylation. However, the *N*-methyl derivative of **5i** (**5u**), phthalimide (**5r**), benzylanilides (**5s** and **5t**), *N*-benzyldiphenylamine (**5v**), ethyl *N*-benzylanthranilate (**5w**), and the *N*-acetyl derivative of **5w** (**5x**) did not undergo debenzylation.

The effect of Lewis acids other than aluminum chloride, that is, boron trifluoride etherate, titanium chloride, stannic chloride, and ferric chloride, was examined. The reaction of ethyl 1-benzyl-1*H*-indole-2-carboxylate (**1a**, X=H) with the former three Lewis acids gave only recovered starting material, while the reaction with ferric chloride gave only a mixture of C-benzyl-NH products.

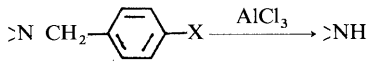
Finally, we examined the effect of a substituent on the benzyl group. Two kinds of ethyl 1-(*p*-substituted)benzylindole-2-carboxylate (**5l** and **5m**), were allowed to react under the same debenzylation conditions. Although the *p*-nitrobenzyl group was not removed at all, the *p*-methoxybenzyl group was removed easily to the same extent as the benzyl group.[2] Apparently a big substituent effect exists, suggesting that the stability of the benzyl cation ($X\text{-}C_6H_4CH_2^+$) determines the rate of deprotection. However, as the extent of removal of *p*-methoxybenzyl and benzyl groups is at the same level, the benzyl group is better for practical use.

In conclusion, the present method can be reliably applied to benzyl derivatives of 2-acyl, 2-alkoxycarbonyl, and fully aromatized indoles, as well as some benzamides. Before we started the present examination, we had suspected that our method might be applicable only to 2-carbonylindoles, in which aluminum chloride could react easily with indolic nitrogen owing to assistance of the neighboring carbonyl group. The present result shows that the debenzylation does not require 2-carbonyl group assistance and thus is not restricted to 2-acylindoles. Though no assistance of the 2-carbonyl group was required for debenzylation, the presence of this group is expected to favor debenzylation to some extent by its weak coordination with aluminum chloride. The reaction seems to be influenced by the basicity and steric crowdedness of nitrogen, because basic compounds (**5q**, **5v**, and **5w**) did not undergo debenzylation, and *N*-alkyl-*N*-benzylbenzamides (**5u**) did not, while corresponding *N*-benzyl-NH-benzamides (**5i**, **5j**, and **5k**) did. This steric crowdedness would be responsible for the non-reactivity of 1-benzyl-2-phenyl-1*H*-indole (**5n**). Usual indoles (not stabilized indoles) and oxindoles are not suitable substrates for this debenzylation. Although *N*-benzyl derivatives of other heterocycles and other amides are usually hard to debenzylate, a trial of the debenzylation process is advisable for every *N*-debenzylation, as the reactions of some benzylbenzamides were successful. As to the solvent (which has a trapping ability for the benzyl cation formed), the relative merits of benzene and anisole cannot be explained straightforwardly. Anisole seems to be better than benzene for trapping the benzyl cation, whereas anisole would combine weakly with aluminum chloride in an acid–base interaction to weaken the reactivity of aluminum chloride. Generally speaking, benzene is recommended due to its general applicability and easy handling. It may be worth trying both in any particular case.

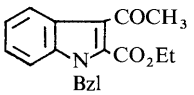
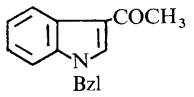
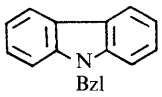
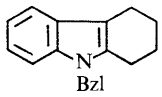
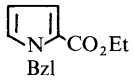
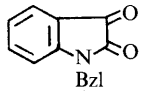
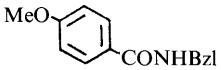
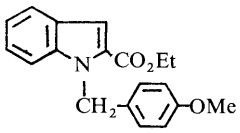
**Experimental**

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 instrument. $^1$H-NMR spectra were recorded in $CDCl_3$ (unless otherwise stated) on a Hitachi R-24B spectrometer (60 MHz). In the $^1$H-NMR spectra, chemical shifts are given in $\delta$ 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 $D_2O$. Mass spectra (MS) were measured by the direct inlet system on JEOL JMS-01-GS-2 spectrometer. For column

TABLE III. Results of Debenzylation Reaction

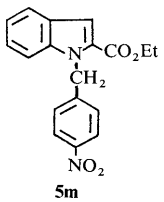



5 4

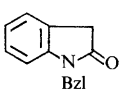
| Run | *N*-Benzyl compound 5 | | Temperature | Time | $AlCl_3$(eq)/ solvent[a] | Yield (%) of 4 | Melting point (°C) (Reported) | Starting material recovered (%) | Other products |
|---|---|---|---|---|---|---|---|---|---|
| 1 |  | (5a)[7] | r.t. | 24 h | 6/B | 84 | 97—102 (96—97.5)[7] | 8 | (9, 9%) |
| 2 |  | (5b) | 0 °C<br>r.t. | 30 min<br>2 h | 4/B<br>4/A | 25<br>70 | 185—188.5 (183)[21] | 70<br>11 | |
| 3 |  | (5c) | r.t. | 15 h | 4/B | 90 | 191—194 (188—192)[b] | | |
| 4 |  | (5d)[8] | r.t.<br>r.t. | 4 h<br>3 h | 4/B<br>4/A | —<br>85 | 218—220 (245—247)[b] | — | Many spots on TLC |
| 5 |  | (5e) | r.t.<br>r.t. | 40 min<br>24 h | 4/B<br>4/A | 61<br>— | 193—195 (198—200)[b] | <br>60 | |
| 6 |  | (5f)[9] | r.t. | 24 h | 4/B | — | — | — | Many spots on TLC |
| 7 |  | (5g) | r.t.<br>r.t. | 4 h<br>24 h | 4/B<br>4/A | —<br>— | —<br>— | —<br>65 | (10, 18%) |
| 8 |  | (5h)[10] | r.t. | 30 min | 4/B | — | — | — | (11, 88%) |
| 9 |  | (5i)[b] | r.t. | 3.5 h | 4/B | 74 | 129—130 (128—129)[b] | | |
| 10 |  | (5j)[11] | 50 °C | 6 h | 4/B | 40 | 169—170 (164—167)[b] | — | (12, 25%) |
| 11 |  | (5k)[12] | 50 °C | 2 h | 4/B | 91 | 181.5—183 (172—176)[b] | | |
| 12 |  | (5l) | 0 °C | 10 min | 4/A | 74 (Yield of 2a) | 117—121 (122—125)[b] | | |

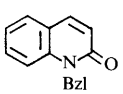
*a*) A = anisole, B = benzene. *b*) Commercially available.

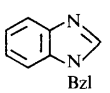
Compounds which did not undergo debenzylation were as follows.

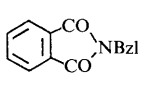

5m

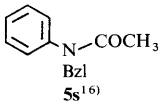

5n

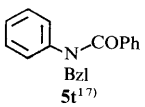

5o[10]

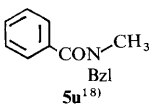

5p[13]


5q[14]

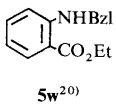

5r[15]

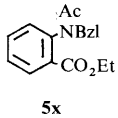

5s[16]


5t[17]

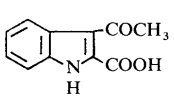

5u[18]


5v[19]


5w[20]


5x

By-products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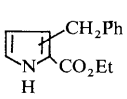
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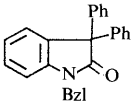
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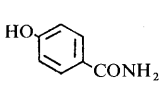
12[b]

chromatography, silica gel (Kiesel gel 60, 70—230 mesh, Merck), and for 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.

**General Procedure for *N*-Benzylation as Exemplified by Benzylation of 2-Phenylindole (5n)** A solution of 2-phenylindole (1.544 g, 8 mmol) in dimethylformamide (DMF) (15 ml) was added to a suspension of 60% NaH (0.32 g, 8 mmol) in DMF (10 ml) under ice-cooling and under an Ar atmosphere. The mixture was stirred for 0.5 h at 0 °C, and then benzyl chloride (0.92 ml, 8 mmol) was added. The whole mixture was stirred for 2 h at room temperature, poured into ice-water, and extracted with AcOEt. The organic layer was washed with 10% HCl, saturated $NaHCO_3$, and brine, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue was chromatographed on $SiO_2$ with benzene–hexane (1 : 5) to afford colorless prisms (1.706 g, 75%), mp 96.5—98.5 °C, which were recrystallized from AcOEt–hexane.

**General Procedure for Debenzylation of *N*-Benzyl Compounds** A solution of a benzyl compound (1.0 mmol) in benzene or anisole (1—14 ml) was added to a suspension of $AlCl_3$ (4—6 eq relative to the benzyl compound) in benzene or anisole (0.5—4.5 ml) under ice-cooling. The mixture was stirred under the conditions shown in Table III. The reaction mixture was poured into water and extracted with benzene or ethyl acetate. The organic layer was washed successively with 5% $NaHCO_3$ and brine, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue was chromatographed on $SiO_2$ to give the NH-compound; a by-product was also obtained in some cases as shown in Table III.

***N*-Ethyl-1-benzyl-1*H*-indole-2-carboxamide (5b)** A solution of ethylamine hydrochloride (1.469 g, 18 mmol) in DMF (5 ml) was added to a solution of 1-benzyl-1*H*-indole-2-carboxylic acid (**7**) (3.027 g, 12 mmol) in DMF (4 ml). Then triethylamine (5.018 ml, 36 mmol) and diethylphosphorocyanidate (DEPC) (3.262 g, 18 mmol) were added. The whole mixture was stirred at room temperature for 2 h, then poured into ice-water and extracted with AcOEt. The organic layer was washed with water, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue was chromatographed on $SiO_2$ with benzene–AcOEt to give colorless needles (2.49 g, 75%), which were recrystallized from benzene, mp 142—143.5 °C. *Anal.* Calcd for $C_{18}H_{18}N_2O$: C, 77.67; H, 6.52; N, 10.06. Found: C, 77.90; H, 6.51; N, 10.04. IR $\nu_{max}$ $cm^{-1}$: 3440 (NH), 1660 (C=O). $^1$H-NMR $\delta$: 1.17 (3H, t, $J$=8 Hz, $CH_2\underline{CH}_3$), 3.16—3.61 (2H, m, $NC\underline{H}_2CH_3$), 5.77 (2H, s, $NCH_2Ph$), 6.10 (1H, br s, NH), 6.80 (1H, s, $C_3$-H), 6.90—7.70 (9H, m, arom H). MS *m/z* (%): 91 (100), 278 ($M^+$, 52).

**3-Acetyl-1-benzyl-1*H*-indole (5c)** Phosphorus oxychloride (1.4 ml, 15 mmol) was added dropwise to dimethylacetamide (DMAc, 6 ml) under ice-cooling. A solution of *N*-benzylindole (**8**) (1.036 g, 5 mmol) in DMAc (4 ml) was added to this solution, and the whole was stirred at 95 °C for 2 h. A solution of NaOH (3.5 g) in water (10 ml) was added, and the reaction mixture was stirred at 95 °C for 10 min, then extracted with AcOEt. The organic layer was washed with saturated NaCl, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue was chromatographed on $SiO_2$ with benzene–ethyl acetate (10 : 1) to give colorless needles (1.06 g, 85.1%), which were recrystallized from benzene–hexane. *Anal.* Calcd for $C_{17}H_{15}NO$: C, 81.90; H, 6.06; N, 5.62. Found: C, 81.72; H, 6.18; N, 5.55. IR $\nu_{max}$ $cm^{-1}$: 1635 (C=O). $^1$H-NMR $\delta$: 2.49 (3H, s, $COCH_3$), 5.30 (2H, s, $NCH_2Ph$), 7.00—7.50 (8H, m, arom H), 7.70 (1H, s, $C_2$-H), 8.20—8.53 (1H, m, $C_4$-H). MS *m/z* (%): 91 (100), 249 ($M^+$, 45).

**Ethyl *N*-Acetyl-*N*-benzylanthranilate (5x)** A mixture of ethyl-*N*-benzylanthranilate (**5w**) (510 mg, 2 mmol), acetic anhydride (0.462 ml, 4.2 mmol) and pyridine (5 ml) was refluxed for 9 h. After removal of the solvent *in vacuo*, the residue was dissolved in AcOEt. This solution was washed with 10% HCl, saturated $NaHCO_3$, and saturated NaCl, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue was chromatographed on $SiO_2$ with AcOEt–hexane to give yellow plates (219 mg, 37%), mp 54.5—56 °C, which were recrystallized from benzene–hexane. *Anal.* Calcd for $C_{18}H_{19}NO_3$: C, 72.71; H, 6.44; N, 4.71. Found: C, 72.82; H, 6.48; N, 4.71. IR $\nu_{max}$ $cm^{-1}$: 1730, 1670 (C=O). $^1$H-NMR $\delta$: 1.32 (3H, t, $J$=7 Hz, $CH_2\underline{CH}_3$), 1.80 (3H, s, $COCH_3$), 4.23 (2H, q, $J$=7 Hz, $OC\underline{H}_2CH_3$), 4.17 and 5.35 (each 1H, d, $J$=15 Hz, $NC\underline{H}_2Ph$), 6.70—8.02 (9H, m, arom H). MS *m/z* (%): 254 (100), 297 ($M^+$, 5).

**Spectral Data for *N*-Benzyl Derivatives** 1-Benzyl-2-phenyl-1*H*-indole (**5n**): IR $\nu_{max}$ $cm^{-1}$: no characteristic absorption. $^1$H-NMR $\delta$: 5.33 (2H, s, $NCH_2Ph$), 6.62 (1H, s, $C_3$-H), 6.88—7.80 (14H, m, arom H). MS *m/z* (%): 283 ($M^+$, 100).

9-Benzyl-9*H*-pyrido[3,4-*b*]indole (**5e**): IR $\nu_{max}$ $cm^{-1}$: no characteristic absorption. $^1$H-NMR $\delta$: 5.48 (2H, s, $NCH_2Ph$), 6.89—7.57 (8H, m, arom H), 7.89 (1H, d, $J$=6 Hz, $C_4$-H), 8.10 (1H, d, $J$=7 Hz, $C_5$-H), 8.42 (1H, d, $J$=6 Hz, $C_3$-H), 8.79 (1H, s, $C_1$-H). MS *m/z* (%): 91 (100), 258 ($M^+$, 70).

Ethyl 1-(4-Methoxybenzyl)-1*H*-indole-2-carboxylate (**5l**): IR $\nu_{max}$ $cm^{-1}$: 1710 (C=O). $^1$H-NMR $\delta$: 1.34 (3H, t, $J$=8 Hz, $CH_2\underline{CH}_3$), 3.68 (3H, s, $OCH_3$), 4.30 (2H, q, $J$=8 Hz, $OC\underline{H}_2CH_3$), 5.72 (2H, s, $NCH_2Ph$), 6.50—7.75 (9H, m, arom H). MS *m/z* (%): 121 (100), 309 ($M^+$, 20).

Ethyl 1-(4-Nitrobenzyl)-1*H*-indole-2-carboxylate (**5m**): IR $\nu_{max}$ $cm^{-1}$: 1700 (C=O). $^1$H-NMR $\delta$: 1.35 (3H, t, $J$=7 Hz, $CH_2\underline{CH}_3$), 4.32 (2H, q, $J$=7 Hz, $OC\underline{H}_2CH_3$), 5.91 (2H, s, $NCH_2Ph$), 7.09—8.20 (9H, m, arom H). MS *m/z* (%): 144 (100), 324 ($M^+$, 32).

Ethyl 1-Benzyl-1*H*-pyrrole-2-carboxylate (**5g**): IR $\nu_{max}^{neat}$ $cm^{-1}$: 1690 (C=O). $^1$H-NMR $\delta$: 1.25 (3H, t, $J$=7 Hz, $CH_2\underline{CH}_3$), 4.18 (2H, q, $J$=7 Hz, $OC\underline{H}_2CH_3$), 5.50 (2H, s, $NCH_2Ph$), 6.05—6.25 (1H, m, arom H), 6.75—7.40 (7H, m, arom H). MS *m/z* (%): 229 ($M^+$, 100).

Ethyl *N*-Benzylanthranilate (**5w**): IR $\nu_{max}^{neat}$ $cm^{-1}$: 3340 (NH), 1670 (C=O). $^1$H-NMR $\delta$: 1.34 (3H, t, $J$=7 Hz, $CH_2\underline{CH}_3$), 4.30 (2H, q, $J$=7 Hz, $OC\underline{H}_2CH_3$), 4.42 (2H, d, $J$=7 Hz, $CH_2Ph$), 6.38—8.02 (9H, m, arom H), 8.12 (1H, br s, NH). MS *m/z* (%): 208 (100), 255 ($M^+$, 57).

**Ethyl *x*-Benzyl-1*H*-pyrrole-2-carboxylate (10)** According to the general procedure for debenzylation, ethyl 1-benzyl-1*H*-pyrrole-2-carboxylate (**5g**) (0.161 g, 0.7 mmol) was treated with $AlCl_3$ (0.414 g, 3.1 mmol) in benzene (3 ml). After work-up, the residue was chromatographed on $SiO_2$ with benzene to give a pale brown solid (0.0294 g, 18%), mp 63—65 °C. IR $\nu_{max}$ $cm^{-1}$: 3280 (NH), 1680 (C=O). $^1$H-NMR $\delta$: 1.30 (3H, t, $J$=7 Hz, $CH_2\underline{CH}_3$), 3.97 (2H, s, $CC\underline{H}_2Ph$), 4.25 (2H, q, $J$=7 Hz, $OC\underline{H}_2CH_3$), 6.73—6.98 (1H, br s, NH), 7.22 (7H, s, arom H). MS *m/z* (%): 229 ($M^+$, 100).

**1-Benzyl-3,3-diphenyloxindole (11)** A solution of 1-benzylisatin (**5h**) (237 mg, 1 mmol) in benzene (4 ml) was added dropwise to a suspension of $AlCl_3$ (533 mg, 4 mmol) in benzene (1 ml). The mixture was stirred at 0 °C for 30 min, then at room temperature for 30 min. The reaction mixture was poured into ice-water, and extracted with benzene. The organic layer was washed with 10% HCl, saturated $NaHCO_3$, and saturated NaCl, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The crude solid was recrystallized from hexane–$CHCl_3$ to give colorless prisms (328 mg, 88%), mp 166—168 °C. *Anal.* Calcd for $C_{27}N_{21}NO$: C, 86.37; H, 5.64; N, 3,73, Found: C, 86.13; H, 5.55; N, 3.64. IR$\nu_{max}$ $cm^{-1}$: 1700 (C=O). $^1$H-NMR $\delta$: 4.95 (2H, s, $NCH_2Ph$), 6.63—7.45 (19H, m, arom H). MS *m/z* (%): 284 (100), 375 ($M^+$, 43).

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# Woodfordin D and Oenothein A, Trimeric Hydrolyzable Tannins of Macro-Ring Structure with Antitumor Activity[1)]

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**Two new antitumor trimeric hydrolyzable tannins, woodfordin D (5) and oenothein A (13), were isolated from the dried flowers of *Woodfordia fruticosa*, and their macrocyclic structures, which have a novel constituent unit (woodfordinoyl group) connecting the monomers, have been elucidated on the basis of spectral and chemical evidence. Oenothein A (13) was also isolated from the leaves of *Oenothera biennis*.**

**Keywords** *Woodfordia fruticosa*; Lythraceae; *Oenothera biennis*; Onagraceae; woodfordin D; oenothein A; tannin; trimeric ellagitannin; woodfordinoyl group

The isolation and characterization of several dimeric hydrolyzable tannins, woodfordins A (**1**), B (**2**) and C (**3**), and oenothein B (**4**), from the dried flowers of *Woodfordia fruticosa* (L.) KURTZ. (Lythraceae), a popular crude drug called "Sidowaya" or "Sidawayah" in Indonesia and Malaysia,[2)] were reported previously.[3)] The dimers **3** and **4**, which belong to the class of macrocyclic hydrolyzable tannins,[3,4)] exhibited a potent host-mediated antitumor activity against sarcoma 180 in mice.[3,5)] Further investigation of this crude drug has led to the isolation of two new trimeric hydrolyzable tannins, named woodfordin D and oenothein A, which exhibited antitumor activity analogous to that of **3** and **4**. The latter trimer has also been isolated from *Oenothera biennis* L. (Onagraceae). This paper deals with the structure elucidation of these new trimers.

Woodfordin D (**5**), $[\alpha]_D$ +105° (acetone), was obtained as a light brown amorphous powder, and showed the coloration characteristic of ellagitannins with the sodium nitrite–acetic acid reagent.[6)] Its trimeric structure was suggested by an *m/z* 2527 $[M+Na]^+$ peak in the fast atom bombardment mass spectrum (FAB-MS).

The proton nuclear magnetic resonance ($^1$H-NMR) spectrum (500 MHz, acetone-$d_6$ + $D_2O$) of **5** did not give

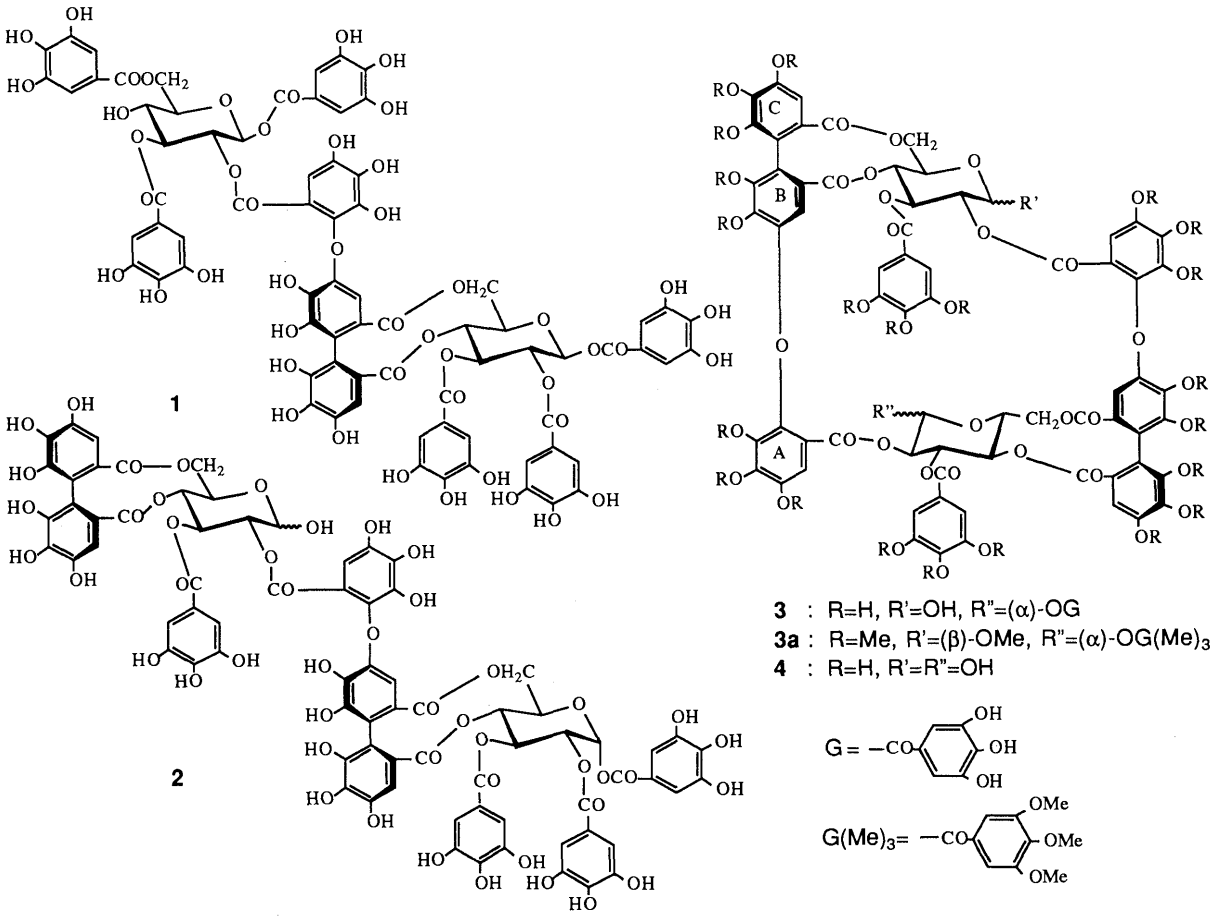
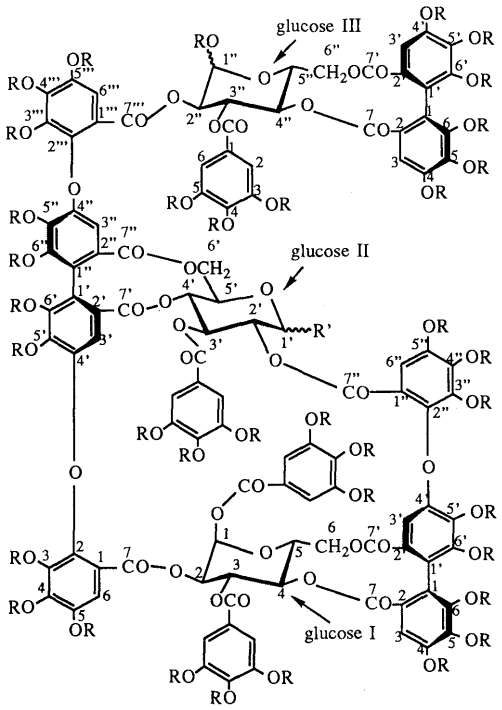
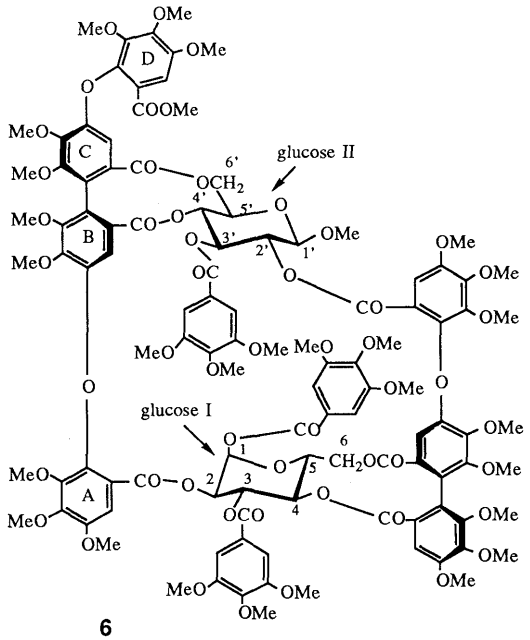



Chart 1

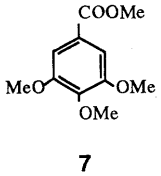
much structural information because of the complexity of the signals induced by equilibration between anomers, and by extensive broadening of most signals. The latter spectral feature is considered to reflect the poor flexibility of the molecule caused by the macro-ring structure, as observed in **3** and **4**.[3,4] However, a clue for the structure elucidation of **5** was obtained from its methylation with dimethyl sulfate and potassium carbonate, which provided a permethylated product (**5a**) and a partially degraded derivative (**6**). The latter product (**6**) was shown to be a nonacosamethylate by an $[M+Na]^+$ ion peak at *m/z* 2317 in the FAB-MS, and by fairly well-resolved methoxyl signals in the $^1$H-NMR spectrum. Methanolysis of **6** yielded, in addition to glucose and methyl β-D-glucoside, three methanolyzates, of which two were identified as methyl tri-*O*-methylgallate (**7**) and trimethyl octa-*O*-methylvaloneate (**9**).[3] The FAB-MS of the third methanolyzate (**10a**) showed an $[M+Na]^+$ ion peak at *m/z* 893, which is consistent with the molecular formula $C_{42}H_{46}O_{20}$. Its $^1$H-NMR spectrum (acetone-$d_6$) exhibited two 2H singlets at δ 6.90 and 7.31, and seven singlets arising from fourteen methoxyl groups at δ 4.02—3.42. The $^{13}$C-NMR spectrum of **10a** showed twenty-one carbon resonances (twelve $sp^2$ carbon peaks, and two ester carbonyl and seven methoxyl carbon signals). These spectral features clearly suggested that **10a** is a methylate of a gallic acid tetramer with a symmetrical structure. The two-dimensional nuclear Overhauser enhancement correlation spectrum (NOESY) displayed a cross peak due to NOE between only one aromatic singlet (δ 7.31, ring B- and C-H) and a methoxyl signal at δ 3.42 (6H, s). The structure **10a** was thus assigned to this methanolyzate, and the structure **10** to the parent acid, which was named woodfordinic acid. The circular dichroism (CD) spectrum of **10a** exhibited positive Cotton effects at 213 nm ($[\theta]+5.33\times10^4$) and 226 nm ($[\theta]+3.33\times10^4$), and a negative one at 250 nm ($[\theta]-3.07\times10^4$). This CD spectrum



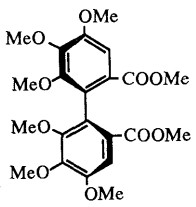

**5** : R=H, R'=OH
**5a** : R=Me, R'=(β)-OMe



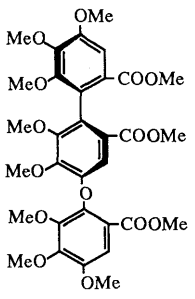

**6**



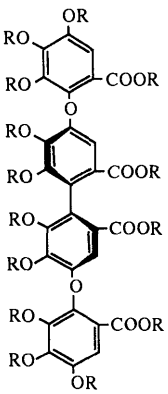
**7**



**8**



**9**



**10** : R=H
**10a** : R=Me

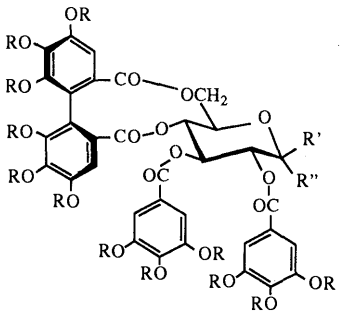
Chart 2

was analogous to those of dimethyl (*S*)-hexamethoxydiphenate (**8**)[7] and **9**,[3] although the amplitudes of the positive Cotton effects of **10a** were somewhat smaller than those of the latter two compounds. Therefore, the (*S*)-configuration was assigned to the biphenyl part of **10a**, and consequently to that of the woodfordinoyl group in **5**.

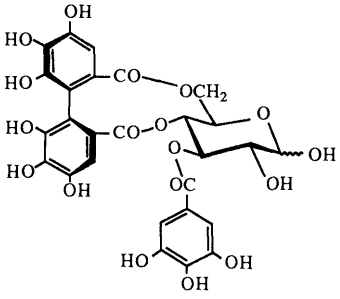
The $^1$H-NMR spectrum of **6** exhibited in the aromatic region three two-proton singlets due to the galloyl groups, and seven one-proton singlets attributable to a woodfordinoyl group and a valoneoyl group. It also showed the proton signals due to two $^4C_1$ glucopyranose cores, which were unequivocally assigned on the basis of the $^1$H–$^1$H shift correlation spectrum (COSY). The chemical shifts and coupling pattern of these signals were almost identical with those of hexacosamethylwoodfordin C (**3a**), except for the chemical shift of H-4′ in the glucose core-II (Table I), suggesting their structural analogy. The H-4′ signal of **6** appeared at $\delta$ 4.87 (t, $J$=10 Hz), being shifted upfield by 0.13 ppm from the corresponding signal of **3a**. This upfield shift can be interpreted in terms of an anisotropic effect of an additional ethereal bezene ring (D-ring) attached to the valoneoyl group at C-4′ and C-6′ of the glucose-II of **3a**. Therefore, the structure of this nonacosamethyl derivative was formulated as **6**, which shows the location of the woodfordinoyl group in both **6** and **5** on O-2 of glucose-I and O-4′—O-6′ of glucose-II.

The permethylated derivative (**5a**), FAB-MS *m/z* 3059 $(M+Na)^+$, obtained from **5**, was revealed to be a mixture (*ca.* 3:1) of $\alpha$- and $\beta$-anomers by the duplicate signals in the $^1$H-NMR spectrum. However, the $^1$H-NMR spectrum of **5a**, unlike that of **5**, showed fairly well-resolved signals without broadening of any proton signal. The presence of a valoneoyl group, a hexahydroxydiphenoyl (HHDP) group and a woodfordinoyl group in **5a** was clealy indicated by nine pairs of one-proton singlets in the aromatic region (see Experimental). The presence of four galloyl groups was also indicated by four pairs of two-proton singlets at $\delta$ 7.00—7.32. Among the glucose proton signals assigned by $^1$H–$^1$H COSY, those due to glucose-I and II, excluding H-2′—H-4′ of glucose-II, showed chemical shifts almost identical with those of **3a** and **6** (Table I). The differences observed here also support the above-mentioned location of the woodfordinoyl group in **5**. The anomeric proton signals of the third glucose core in **5**, which is regarded as being esterified with an HHDP group and a galloyl group, were observed as a pair of doublets at $\delta$ 5.18 ($J$=4 Hz) and 4.66 ($J$=8 Hz), indicating anomerization at this glucose core (glucose III). In fact, the chemical shifts of the glucose-III proton signals (H-1″—H-6″) showed close similarity to those of the $\alpha$-(**11a**) and $\beta$-anomer (**11b**) of the permethylated derivative of tellimagrandin I (**11**)[8] (Table I). The H-1″ proton ($\delta$ 4.66) signal of the $\beta$-anomer of **6** is shifted upfield from the anomeric proton signal of **11b**. This shift is analogous to that of H-1′ of **3**, **4** or other dimers possessing a galloyl part of the valoneoyl group on C-2′.[9] The D-ring of the woodfordinoyl group in **5** is therefore on O-2″, and consequently the galloyl group is on O-3″.

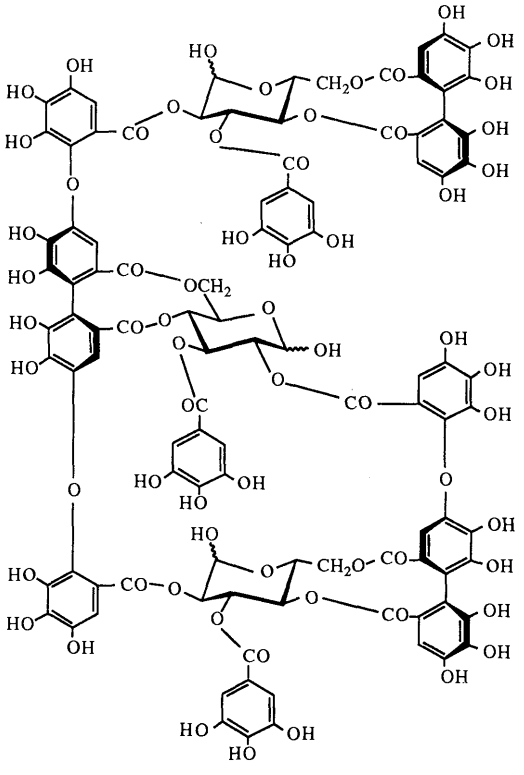
These structural aspects were confirmed by partial hydrolysis of **5** in boiling water, which gave woodfordin C (**3**) and gemin D (**12**).[10] The former is regarded as a product of the cleavage of an ether linkage of the woodfordinoyl group in **5**, occurring in an analogous way to the ether cleavage of the valoneoyl group, frequently observed upon the partial hydrolysis of oligomeric hydrolyzable tannins.[11]



**11** : R=H, R′,R″=H,OH
**11a** : R=Me, R′=H, R″=OMe
**11b** : R=Me, R′=OMe, R″=H



**12**



**13**

Chart 3

TABLE I. $^1$H-NMR Data for the Glucose Moieties of **3a**, **5a**, **6**, **11a** and **11b** (500 MHz, Acetone-$d_6$–$D_2O$, $J$ in Hz)

| | | **3a** | **5a** α-Anomer | **5a** β-Anomer | **6** | **11a** | **11b** |
|---|---|---|---|---|---|---|---|
| Glu-I | H-1 | 7.23 d ($J$=4) | | 7.13 d ($J$=4) | 7.13 d ($J$=4) | | |
| | H-2 | 5.93 dd ($J$=4, 10) | | 5.86 dd ($J$=4, 10) | 5.88 dd ($J$=4, 10) | | |
| | H-3 | 6.17 t ($J$=10) | | 6.18 t ($J$=10) | 6.18 t ($J$=10) | | |
| | H-4 | 6.06 t ($J$=10) | | 6.09 t ($J$=10) | 6.08 t ($J$=10) | | |
| | H-5 | 4.76 m | | 4.77 m | 4.76 m | | |
| | H-6 | 5.26 dd ($J$=7, 14) | | 5.25 dd ($J$=7, 14) | 5.25 dd ($J$=7, 14) | | |
| | H-6[a] | 3.57 | | 3.67 | 3.62 | | |
| Glu-II | H-1′ | 4.37 d ($J$=8) | | 4.29 d ($J$=8) | 4.32 d ($J$=8) | | |
| | H-2′ | 5.51 dd ($J$=8, 10) | | 5.35 dd ($J$=8, 10) | 5.44 dd ($J$=8, 10) | | |
| | H-3′ | 5.40 t ($J$=10) | | 5.55 t ($J$=10) | 5.40 t ($J$=10) | | |
| | H-4′ | 5.00 t ($J$=10) | | 5.17 t ($J$=10) | 4.87 t ($J$=10) | | |
| | H-5′[a] | 4.09 | | 4.09 | 4.06 | | |
| | H-6′ | 4.78 dd ($J$=7, 13) | | 4.78 dd ($J$=7, 13) | 4.72 dd ($J$=7, 13) | | |
| | H-6′[a] | 3.95 | | 3.92 | 3.93 | | |
| Glu-III | H-1″ | | 5.15 d ($J$=4) | 4.66 d ($J$=8) | | 5.28 d ($J$=4) | 4.93 d ($J$=8) |
| | H-2″ | | 5.30 dd ($J$=4, 10) | 5.37 dd ($J$=8, 10) | | 5.19 dd ($J$=4, 10) | 5.39 dd ($J$=8, 10) |
| | H-3″ | | 5.71 t ($J$=10) | 5.56 t ($J$=10) | | 5.84 t ($J$=10) | 5.80 t ($J$=10) |
| | H-4″ | | 5.18 t ($J$=10) | 5.14 t ($J$=10) | | 5.22 t ($J$=10) | 5.16 t ($J$=10) |
| | H-5″ | | 4.45 m | 4.73 m | | 4.50 m | 4.50 m |
| | H-6″ | | 5.25 dd ($J$=7, 13) | 5.24 dd ($J$=7, 13) | | 5.25 dd ($J$=7, 13) | 5.30 dd ($J$=7, 13) |
| | H-6″ | | *ca.* 3.90[a] | *ca.* 3.93[a] | | 3.96 d ($J$=13) | 4.00 d ($J$=13) |

a) Detected by $^1$H–$^1$H COSY as a signal that overlapped with OMe signals.

The molecular formula of woodfordin D, $C_{109}H_{76}O_{70}$, calculated based on these data, coincides with the FAB-MS data, and consequently the structure **5** was assigned to woodfordin D. Biogenetically this molecule should have been formed through intermolecular C–O oxidative coupling[11,12] between the galloyl group at O-2 of tellimagrandin I (**11**) and the C-ring of the valoneoyl group in woodfordin C (**3**).

Oenothein A (**13**), $[\alpha]_D$ +113° (MeOH), a light brown amorphous powder, was also considered to be a trimeric ellagitannin, based on the retention time being similar to that of **5** in normal-phase high performance liquid chromatography (HPLC),[13] and a positive coloration with the sodium nitrite reagent on the thin layer chromatography (TLC) plate. Methylation of **13**, followed by methanolysis with sodium methoxide, yielded **7**, **8**, **9**, and **10a**, in a molar ratio of 3 : 1 : 1 : 1. The sugar constituent liberated upon acid hydrolysis of **13** was identified as glucose by gas liquid chromatography (GLC) after trimethylsilylation. Therefore, oenothein A consists of three galloyl groups and one each of the HHDP, valoneoyl and woodfordinoyl groups, and three glucose cores. Upon partial degradation with hot water, **13** gave tellimagrandin I (**11**), gamin D (**12**) and oenothein B (**4**). This behavior of **13** upon partial hydrolysis is comparable to that of woodfordin D (**5**), which yielded **12** and **3** as mentioned above. Oenothein A was thus presumed to be a macrocyclic trimer, lacking a galloyl group at C-1 of the glucose-I of woodfordin D (**5**). The proposed structure (**13**) for oenothein A was substantiated by a prominent peak at $m/z$ 2375 due to $[M+Na]^+$ in the FAB-MS, which is consistent with the molecular formula $C_{102}H_{72}O_{66}$, and by enzymatic partial degalloylation of woodfordin D (**5**) yielding oenothein A.

Woodfordin D (**5**) and oenothein A (**13**) exhibited a significant host-mediated antitumor activity[14] in a screening test similar to that reported previously for **3**, **4** and other tannins.[3b,5]

**Experimental**

NMR spectra were recorded on Varian VXR 500 (500 MHz for $^1$H and 125.6 MHz for $^{13}$C) and JEOL GX 200 (200 MHz for $^1$H and 50.1 MHz for $^{13}$C) instruments, and chemical shifts are given in $\delta$ (ppm) from $Me_4Si$. FAB-MS were measured on a VG Analytical 70-SE mass spectrometer fitted with a standard FAB ion source. The samples for FAB-MS measurements were prepared by dissolving the compounds in MeOH or acetone, and mixing the solutions with *m*-nitrobenzyl alcohol and NaCl. The other instruments and chromatographic conditions (HPLC, TLC and column chromatography) used in this work are the same as those described in the preceding paper.[3b]

**Isolation of Woodfordin D (5) and Oenothein A (13) from *Woodfordia fruticosa* Flowers** The *n*-BuOH soluble portion (9 g), previously obtained[3b] from the homogenates of dried flowers (1 kg) of *W. fruticosa* (Sidowayah), was chromatographed over Toyopearl HW-40 (fine grade, 2.2 cm i.d. × 35 cm) developing with aqueous MeOH (60% MeOH→70% MeOH) and MeOH–acetone–$H_2O$ [(7:2:1)→(6:2:2)→(5:3:2)] in a stepwise gradient mode. The fraction eluted with MeOH–acetone–$H_2O$ (6:2:2) gave oenothein A (**13**) (588 mg) and woodfordin D (**5**) (834 mg).

**Isolation of Oenothein A (13) from *Oenothera biennis*** Fresh leaves (75 g) of *O. biennis* were homogenized in 70% acetone (150 ml × 3), and the homogenate was centrifuged (3000 rpm, 10 min). The supernatant was concentrated to 35 ml, and extracted with diethyl ether (35 ml × 10), ethyl acetate (35 ml × 10) and *n*-BuOH (pre-saturated with water, 23 ml × 3), successively. The aqueous layer was further concentrated, and then MeOH was added to the solution. After removal of the precipitate by centrifugation, the supernatant was evaporated to give a brown residue (2.6 g). A portion (986 mg) of the residue was chromatographed over Sephadex LH-20 developing with 80% EtOH and then with MeOH. Oenothein A (**13**) (90 mg) was obtained from the MeOH eluate. Analogous separation of the BuOH extract (766 mg) also afforded **13** (63 mg).

**Woodfordin D (5)** A light brown amorphous powder, $[\alpha]_D$ +105° ($c$=1.0, acetone). *Anal.* Calcd for $C_{109}H_{76}O_{70}\cdot 21H_2O$: C, 45.30; H, 4.09. Found: C, 45.15; H, 4.09. FAB-MS $m/z$: 2527 $(M+Na)^+$. UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 218 (5.30), 270 (4.95). CD (MeOH) [$\theta$] (nm): $+46.4\times10^4$ (218), $+14.5\times10^4$ (237), $-1.14\times10^4$ (259), $+16.6\times10^4$ (283). $^{13}$C-NMR (acetone-$d_6$+$D_2O$) $\delta$: 96.3, 95.5, 91.2, 91.1 [glucose (glu) C-1], 74.4, 74.1, 74.0, 73.9, 73.1, 72.9, 72.1, 71.9, 71.8, 71.7, 71.6, 71.5, 71.3, 71.1, 71.0, 70.9, 70.8, 69.3, 66.9 (glu C-2—C-5), 63.5, 62.6 (glu C-6), 103.6, 104.3, 105.6, 105.7 [valoneoyl (Val) C-3′, woodfordinoyl (Wf) C-3′, 3″], 107.6, 107.7, 107.8, 108.0, 108.2, 108.9, 109.0, 109.1, 110.1, 110.7 [Val C-3, 6″, Wf C-6, 6‴, HHDP C-3, 3′, galloyl (G) C-2, 6], 113.6, 114.2 (br), 115.5 (br), 117.1, 117.3 (br), 117.5 (br), 117.9 (br), 118.7 (Val C-1, 1′, 1″, Wf C-1, 1′, 1″, 1‴, HHDP C-1, 1′), 120.2, 120.3, 120.6, 121.1, 121.2, 121.7 (G C-1), 124.6, 125.0, 125.3, 125.4, 125.8, 125.9, 126.0, 126.4, 126.5, 126.7 (Val C-2, 2′, WF C-2′, 2″, HHDP C-2, 2′), 133.7, 135.5, 136.2, 136.4 (br), 136.5, 136.7, 136.9, 138.0 (br), 139.0 (br), 139.6, 140.0, 140.1, 140.2, 140.4, 140.7, 140.9, 141.2, 144.3, 144.7, 144.8, 144.9, 145.1 (br), 145.2, 145.5, 145.7, 146.1, 146.6, 146.7, 146.8, 146.9, 147.6, 147.7, 142.9, 143.3, 143.7 (Val C-4—6, C-4′—6′, C-2″—5″, Wf C-3—5, C-4′—6′, C-4″—6″, C-2″—5‴, HHDP C-4—6, C-4′—6′, G C-3—5), 164.1, 164.3, 165.0, 166.1, 166.2, 166.7, 166.8, 166.9, 167.5, 167.6, 167.8, 167.9, 168.1, 168.2, 169.4 (Val C-7, 7′, 7″, Wf C-7, 7′, 7″, 7‴, HHDP C-7, 7′, G C-7).

**Methylation of Woodfordin D (5)** A mixture of **5** (100 mg), anhydrous $K_2CO_3$ (300 mg) and $Me_2SO_4$ (0.15 ml) in dry acetone (10 ml) was stirred overnight at room temperature, and then refluxed for 3 h. After filtration, followed by removal of the solvent, the products were purified by preparative TLC [$SiO_2$, benzene–acetone (4:1). double development] to afford a nonacosamethyl derivative (**6**) ($Rf$ 0.52, 8.5 mg) and an octatriacontamethyl derivative (**5a**) ($Rf$ 0.47, 8.8 mg).

**Nonacosamethyl Derivative (6)** A white amorphous powder, $[\alpha]_D$ +90° ($c$=0.5, acetone). *Anal.* Calcd for $C_{111}H_{114}O_{53}\cdot 4H_2O$: C, 56.29; H, 5.15. Found: C, 56.12; H, 5.08. FAB-MS $m/z$: 2317 $(M+Na)^+$. UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 223 (4.76), 250 (4.52), 295 (4.08). $^1$H-NMR (acetone-$d_6$) $\delta$: 7.47, 7.34, 7.33 (each 2H, s, galloyl), 7.32, 7.29, 7.08, 6.65, 6.43, 6.42, 6.37 (each 1H, s, Wf and Val), 4.10, 3.96, 3.91, 3.87, 3.84, 3.81, 3.80, 3.78, 3.76, 3.73, 3.68, 3.67, 3.63, 3.60, 3.45, 3.37 (each 3H, s, 16 × OMe), 4.00, 3.94, 3.86, 3.74, 3.71 (each 6H, s, 10 × OMe), 3.82 (9H, s, 3 × OMe), glucose protons, see Table I.

**Octatriacontamethyl Derivative (5a)** A pale yellow amorphous powder, $[\alpha]_D$ +55° ($c$=0.8, acetone). *Anal.* Calcd for $C_{147}H_{152}O_{70}\cdot 10H_2O$: C, 54.85; H, 5.34. Found: C, 54.70; H, 5.08. FAB-MS $m/z$: 3059 $(M+Na)^+$. UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 223 (4.75), 255 (4.51), 293 (4.08). $^1$H-NMR (acetone-$d_6$) $\alpha$- and $\beta$-anomer (3:1) $\delta$: 7.46 (6/4H), 7.47 (2/4H), 7.34 (6/4H), 7.334 (2/4H), 7.329 (2H), 7.29 (6/4H), 7.28 (2/4H) (each s, galloyl), 7.33 (3/4H), 7.28 (1/4H), 7.18 (1H), 7.08 (1H), 6.93 (3/4H), 6.94 (1/4H), 6.76 (3/4H), 6.78 (1/4H), 6.65 (1H), 6.49 (3/4H), 6.50 (1/4H), 6.42 (3/4H), 6.43 (1/4H), 6.37 (1H) [each s, Wf, Val and HHDP], glucose protons, see Table I.

**Preparation of Tridecamethyltellimagrandin I** A mixture of tellimagrandin I (**11**) (20 mg), anhydrous $K_2CO_3$ (100 mg) and $Me_2SO_4$ (0.05 ml) in dry accetone (3 ml) was stirred overnight at room temperature, and then refluxed for 2.5 h. The reaction mixture was filtered, evaporated and purified by preparative TLC [$SiO_2$, benzene–acetone (6:1), double development] to give the $\alpha$-anomer (**11a**) (1 mg) and $\beta$-anomer (**11b**) (1 mg) of tridecamethyltellimagrandin I.

**Methanolysis of 6 and 5a** A solution of **6** (5 mg) in absolute MeOH (2 ml) containing 1% NaOMe (0.1 ml) was left standing overnight at room temperature. After acidification with AcOH, the solvent was evaporated off, and the residue was partitioned between water and EtOAc. The EtOAc soluble material was purified by preparative TLC ($SiO_2$, benzene–acetone 4:1) to give methyl tri-*O*-methylgallate (**7**) (2 mg), trimethyl octa-*O*-methylvaloneate (**9**) (0.8 mg) and tetramethyl deca-*O*-methylwoodfordinate (**10a**) (1.0 mg). The syrupy residue obtained from the aqueous layer was trimethylsilylated and subjected to GLC analysis (2.5% OV-17, column temperature 170 °C), by which procedure the sugar components were identified as glucose and methyl $\beta$-D-glucoside. Similar methanolysis of **5a** (8 mg) afforded **7** (3 mg), **8** (1.1 mg), **9** (1.5 mg) and **10a** (0.8 mg).

**Tetramethyl Deca-*O*-methylwoodfordinate (10a)** A white amorphous powder, FAB-MS $m/z$: 893 $(M+Na)^+$. UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 233 (4.67), 252 sh (4.56), 304 (4.10). CD (MeOH) [$\theta$] (nm): $+5.33\times10^4$ (213), $+3.33\times10^4$ (226), $-3.07\times10^4$ (250), $+0.53\times10^4$ (275), $+1.6\times10^4$ (310). $^1$H-NMR (acetone-$d_6$) $\delta$: 7.31, 6.90 (each 2H, s), 4.02, 3.94, 3.93, 3.76, 3.74, 3.63, 3.42 (each 6H, s) (14 × OMe). $^{13}$C-NMR (acetone-$d_6$) $\delta$: 120.5 (C-1, 1‴), 128.1 (C-1′, 1″), 142.7 (C-2, 2‴), 125.9 (C-2′, 2″), 147.9 (C-3, 3‴), 112.0 (C-3′, 3″), 148.1 (C-4, 4‴), 152.6 (C-4′, 4″), 151.5 (C-5, 5‴), 146.1 (C-5′, 5″), 109.5 (C-6, 6‴), 152.5 (C-6′, 6″), 165.6 (C-7, 7‴), 166.9 (C-7′, 7″).

**Partial Hydrolysis of Woodfordin D (5)** A solution of woodfordin D (**5**) (80 mg) in $H_2O$ (80 ml) was heated in a boiling-water bath for 25 h. The insoluble materials were filtered off, and the filtrate was concentrated and subjected to column chromatography over MCI gel CHP-20P (1.1 cm i.d. × 18 cm) with water containing increasing amounts of MeOH, to give 3-*O*-galloylglucose (3.1 mg) and gallic acid (3.0 mg) from the 10% MeOH eluate, and gemin D (**12**) (4.5 mg), $[\alpha]_D$ +45° ($c$=0.9, acetone) from the 20% MeOH eluate. Woodfordin C (**3**) (1.5 mg), $[\alpha]_D$ +200° ($c$=1.0, MeOH), was obtained from the 30% MeOH eluate. Identities of these products were confirmed by HPLC (normal- and reversed-phase) and $^1$H-NMR spectra.

**Oenothein A (13)** A light brown amorphous powder, $[\alpha]_D$ +113° ($c$=1.0, MeOH). *Anal.* Calcd for $C_{102}H_{72}O_{66}\cdot 23H_2O$. C, 44.25; H, 4.27. Found: C, 44.22; H, 4.40. FAB-MS $m/z$: 2375 $(M+Na)^+$. UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 218 (5.44), 272 (5.12). CD (MeOH) [$\theta$] (nm): $+62.5\times10^4$ (220), $+21.0\times10^4$ (238), $-11.5\times10^4$ (259), $+19.1\times10^4$ (283). $^{13}$C-NMR (50.1 MHz, acetone-$d_6$) $\delta$: 63.2, 63.7 [glu C-6, $\alpha$- and $\beta$-anomer], 65.1, 65.5, 67.2, 68.9, 70.4, 70.5, 71.0, 71.3, 71.4, 71.7, 72.1, 73.0, 73.3, 74.1, 74.8 (glu C-2—C-5, $\alpha$- and $\beta$-anomer), 90.7, 90.8, 90.9, 91.2, 91.5 (glu C-1, $\alpha$-anomer), 95.6, 95.9, 96.5, 96.6 (glu C-1, $\beta$-anomer), 104.0, 104.6, 104.8, 105.7 (Val C-3′, Wf C-3′, 3″), 107.8, 107.9, 108.3, 108.4, 109.0, 109.4, 110.1, 110.3, 110.5, 110.9, (Val C-3, 6″, Wf C-6, 6‴, HHDP C-3, 3′, G C-2, 6), 114.0, 114.5, 114.7, 115.5, 115.7, 116.5, 116.8, 117.3, 117.5, 117.8, 118.4, 119.1 (Val C-1, 1′, 1″, Wf C-1, 1′, 1″, 1‴, HHDP C-1, 1′), 120.6, 120.9, 121.2, 121.5, 121.7, 122.0 (G C-1), 124.8, 125.2, 125.6, 125.7, 126.2, 126.3, 126,4, 126.5, 126.7, 127.0 (Val C-2, 2′, Wf C-2, 2″, HHDP C-2, 2′), 133.8, 135.4, 136.3, 136.4, 136.9, 137.1, 137.6, 138.2, 138.5, 138.8, 139.0, 139.1, 139.2, 139.4, 140.0, 140.2, 140.3, 140.7, 141.1, 141.3, 142.8, 143.3, 143.6, 143,8, 144.3, 144.7, 144.8, 145.0, 145.1, 145.2, 145.3, 145.4, 145.6, 145.7, 146.8, 147.1, 147.6, 147.8 (Val C-4—6, C-4′—6′, C-2″—5″, Wf C-2—5, C-4′—6′, C-4″—6″, C-2‴—5‴, HHDP C-4—6, C-4′—6′, G C-3—5), 164.2, 164.3, 165.9, 166.8, 166.9, 167.5, 167.6, 167.9, 168.0, 168.1, 168.2, 168.3, 168.4, 169.2, 169.4 (Val C-7, 7′, 7″, Wf C-7, 7′, 7″, 7‴, HHDP C-7, 7′, G C-7).

**Treatment of Oenothein A (13) with Dimethyl Sulfate** Dimethyl sulfate (0.15 ml) and potassium carbonate (320 mg) were added to a solution of oenothein A (**13**) (63 mg) in acetone (3.8 ml), and the mixture was stirred for 12 h at room temperature, then refluxed for 2 h. After removal of the inorganic materials by filtration, the filtrate was evaporated and the residue was separated by preparative TLC [Kieselgel $PF_{254}$, light petroleum (bp 85—120 °C)–$CH_2Cl_2$–acetone 6:3:1)] to give methyl tri-*O*-methylgallate (**7**) [8.8 mg, $Rf$ 0.54, EI-MS $m/z$ 226 $(M^+)$]. dimethyl hexamethoxydiphenate (**8**) [6.6 mg $Rf$ 0.37, EI-MS $m/z$ 450 $(M^+)$], trimethyl octa-*O*-methylvaloneate (**9**) [14.8 mg, $Rf$ 0.27, EI-MS $m/z$ 660 $(M^+)$], and tetramethyl deca-*O*-methylwoodfordinate (**10a**) [1.9 mg, $Rf$ 0.19, EI-MS $m/z$ 870 $(M^+)$]. Methanolysis of the materials (mixture of methylated derivatives of **13**) present in the zone of $Rf$ 0—0.1 on TLC also afforded these four products.

**Acid Hydrolysis of Oenothein A (13)** A solution of **13** (8 mg) in 5% sulfuric acid (1.1 ml) in a sealed tube was heated in a boiling-water bath for 20 h. After neutralization with ion exchange resin (Amberlite IRA-410), the solvent was evaporated off. Glucose in the residue was identified by GLC (column, 2.5% OV-17 on Chromosorb W, oven temperature 170 °C)

after trimethylsilylation.

**Partial Hydrolysis of Oenothein A (13)** An aqueous solution (100 ml) of **13** (200 mg) was heated in a boiling-water bath for 15 h. After removal of the solvent, the reaction mixture was chromatographed over Sephadex LH-20 with EtOH and then with EtOH–MeOH (1 : 1), to afford gemin D (**12**) (14.5 mg), tellimagrandin I (**11**) (0.5 mg) and oenothein B (**4**) (2.4 mg).

**Transformation of Woodfordin D (5) into Oenothein A (13)** An aqueous solution of woodfordin D (**5**) (20 mg) was incubated with tannase at 37°C for 7 h. The reaction mixture was acidified with 1% HCl and passed through a Sep-pak C18 cartridge. After washing of the cartridge with water, the 20% MeOH eluate gave a degalloylated derivative (4 mg), which was identified as oenothein A (**13**) by HPLC, $[\alpha]_D$ and $^1$H-NMR spectral comparison.

**Acknowledgements** We are grateful to Dr. Y. Takeda and Mr. K. Yoshioka, Faculty of Pharmaceutical Sciences, Tokushima University, for measuring the NMR spectra (200 MHz), and Dr. N. Toh, Faculty of Engineering, Kyushu Kyoritsu University, for CD measurements. We thank Prof. K. Miyamoto, School of Pharmacy, Hokuriku University for the antitumor test. We are also indebted to the SC-NMR Laboratory of Okayama University for NMR experiments using the Varian VXR-500 instrument.

**References and Notes**

1) Part 2 in the series of "Tannins and Related Polyphenols of Lythraceous Plants." For Part 1, see ref. 3*b*; this work also constitutes Part 2 in the series of "Tannins of Onagraceous Plants". For Part 1, see ref. 4*b*.
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# Chemical Transformation of Protoberberines. XVII.[1] Biomimetic Introduction of an Oxy Functionality at the C-10 Position in the Benzo[*c*]phenanthridine Skeleton: Synthesis of 2,3,7,8,10-Pentaoxygenated Benzo[*c*]phenanthridine Alkaloids, Chelilutine and Sanguilutine[2]

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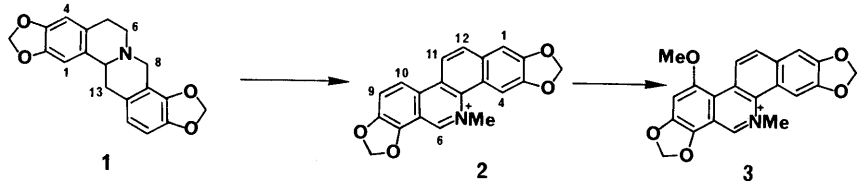
**An efficient and biomimetic introduction of an oxy functionality at the C-10 position in the benzo[*c*]phenanthridine skeleton was developed by regioselective oxygenation with salcomine-oxygen. This biomimetic procedure was successfully applied to a synthesis of 2,3,7,8,10-pentaoxygenated benzo[*c*]phenanthridine alkaloids, chelilutine (8) and sanguilutine (18), from the corresponding 2,3,7,8-tetraoxygenated benzo[*c*]phenanthridines.**

**Keywords** benzo[*c*]phenanthridine alkaloid; protoberberine; regioselective oxygenation; biomimetic transformation; salcomine; chelilutine; sanguilutine; *p*-quinone

Recent biosynthetic studies[3] on chelirubine (**3**), a representative 2,3,7,8,10-pentaoxygenated benzo[*c*]phenanthridine alkaloid[4] have disclosed that the methoxy group at the C-10 position in **3** is introduced after formation of the corresponding 2,3,7,8-tetraoxygenated benzo[*c*]phenanthridine alkaloid, sanguinarine (**2**), which is biosynthesized from the protoberberine alkaloid stylopine (**1**)[3,5] through $C_6$–N bond fission and recyclization between the C-6 and C-13 positions. This intriguing biogenetic pathway has led us to explore a direct method for the introduction of the methoxy group at the C-10 position in the 2,3,7,8-tetraoxygenated benzo[*c*]phenanthridine skeleton.

In connection with our recent results[1,6-8] on biomimetic synthesis of benzo[*c*]phenanthridine alkaloids from their biogenetic precursors, protoberberine alkaloids, we thought that a convenient transformation of 2,3,7,8-tetraoxygenated benzo[*c*]phenanthridines into the corresponding 2,3,7,8,10-pentaoxygenated ones would open up a new aspect of the chemistry of benzo[*c*]phenanthridine alkaloids, even though some syntheses of the pentaoxygenated compounds have already been reported.[8-11] We chose 7-*O*-demethyldihydrochelerythrine (**4**)[6b] as a suitable substrate for our purpose because the $C_7$-hydroxy group in **4** would be expected to make oxidation at the C-10 position much easier through the *p*-quinone derivative, which should be convertible into the desired trimethoxy compound. We describe here a novel and biomimetic synthesis of chelilutine (**8**) and sanguilutine (**18**) from the 2,3,7,8-tetraoxygenated benzo[*c*]phenanthridines by the use of salcomine-oxygen oxidation as a key step.

Several oxidants, including cerium ammonium nitrate (CAN),[12] iodosobenzene,[13] Fremy's salt,[14] and salcomine-oxygen,[15] are able to convert a phenol group into a *p*-quinone derivative. However, the former two reagents oxidized the tertiary amine moiety of **4** in preference to the phenol group to produce the quaternary salt rather than the *p*-quinone compound. Fremy's salt did not work in this case. Finally salcomine-oxygen was found to be the best oxidant for our purpose. Thus, **4** was treated with salcomine in tetrahydrofuran (THF) at room temperature in a stream of oxygen to afford the *p*-quinone derivative (**5**) in 94% yield. The structure of **5** was easily elucidated on the basis of spectral evidence. The proton nuclear magnetic resonance ($^1$H-NMR) spectrum of **5** showed the downfield-shifted C-11 proton signal at $\delta$ 8.15 ppm (the C-11 proton signal of the starting **4** appeared at $\delta$ 7.75 ppm[6b]) as a doublet, as well as the C-9 proton signal at $\delta$ 5.97 ppm as a singlet. Furthermore, **5** showed infrared (IR) absorptions at 1650 and 1620 $cm^{-1}$ due to the *p*-quinone moiety. It is noteworthy that no reaction took place when 7-*O*-demethyloxychelerythrine (**4'**) instead of 7-*O*-demethyldihydrochelerythrine (**4**) was exposed to salcomine-oxygen. The *p*-quinone moiety in **5** was then reduced with sodium hydrosulfite in aqueous acetone at room temperature. The reaction was monitored by thin-layer chromatography (TLC), which revealed consumption of the starting material and the appearance of a new product, presumably the 7,10-dihydroxy-8-methoxy derivative (**6**). However, isolation of **6** was troublesome, in spite of its easy formation. The dihydroxy compound (**6**) could not be purified because it was extremely susceptible to oxidation during work-up to regenerate partially the starting *p*-quinone (**5**). Therefore, the crude product was immediately methylated with diazomethane to afford dihydrochelilutine (**7**) in 53% yield from **5** (method A). Although **7** could thus be obtained from **5**, the yield was not satisfactory. Accordingly an alternative and more efficient procedure (method B) was developed. The *p*-quinone (**5**) was catalytically hydrogenated over palladium on carbon (Pd–C) in the presence of dimethyl sulfate[16] in an alkaline solution to provide **7** in 81% yield. The latter procedure (method B) was much easier and more efficient than the former one (method A). Dihydrochelilutine was finally converted into chelilutine (**8**)




1 2 3

Chart 1

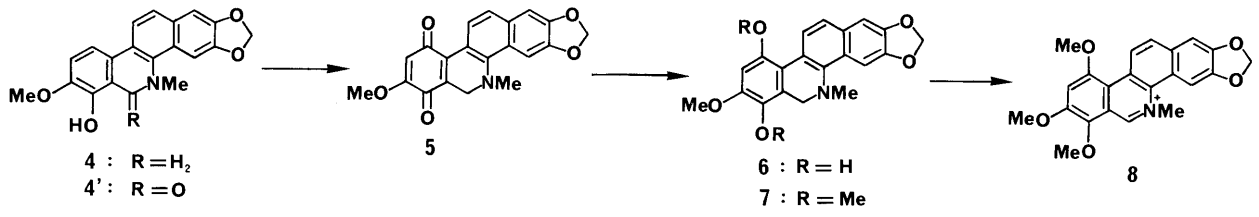
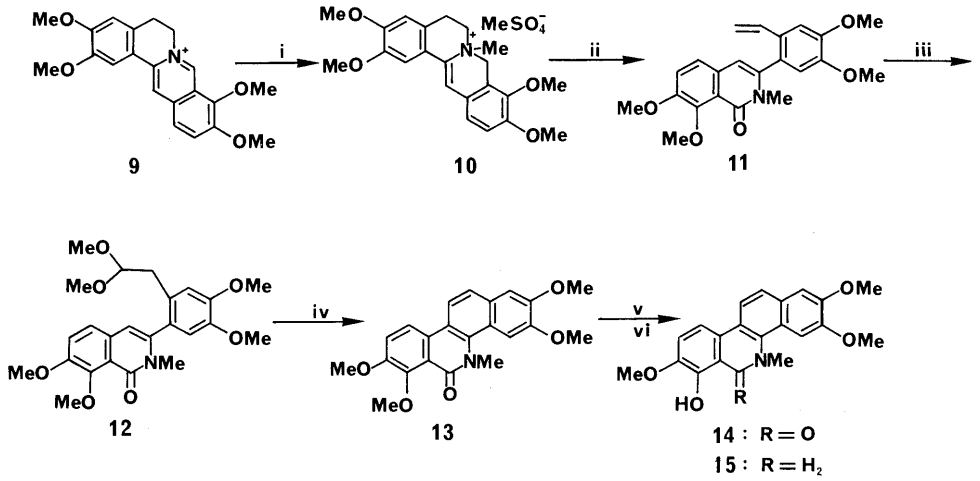



Chart 2




i) a: LAH–THF, b: $Me_2SO_4$–$C_6H_6$ (94%); ii) a: 25% KOH–MeOH, b: DDQ–$CHCl_3$ (31%); iii) $Tl(NO_3)_3$–MeOH (91%); iv) 10% HCl–MeOH (96%); v) *p*-TsOH–toluene (90%); vi) a: LAH–THF, b: $NaBH_4$–MeOH (86%).

Chart 3

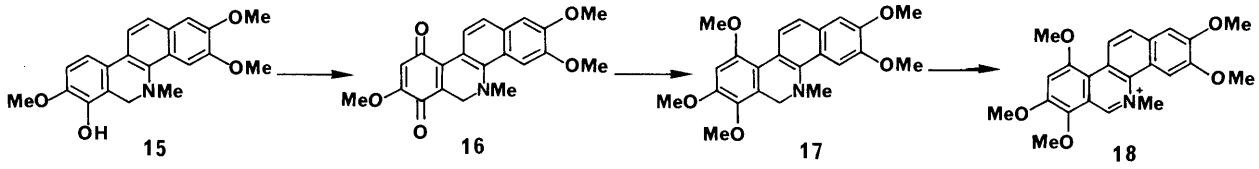



Chart 4

by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) oxidation. The synthetic chelilutine was identical with natural chelilutine by spectral comparison. Thus, we succeeded in synthesizing chelilutine (**8**) from **4** *via* a biomimetic procedure.

The next step in this investigation was to examine the generality of the above reaction. We therefore applied it to a biomimetic synthesis of sanguilutine (**18**),[8] a 2,3,7,8,10-pentamethoxybenzo[*c*]phenanthridine alkaloid. The required 7-hydroxybenzo[*c*]phenanthridine (**15**) was prepared according to our procedure[6] described previously for the synthesis of **4**. Palmatine (**9**) was converted to the 3-arylisoquinolone (**11**) *via* the methosulfate (**10**). Successive exposure of **11** to thallium trinitrate (TTN) and 10% hydrochloric acid afforded the benzo[*c*]phenanthridine (**13**). The regioselective demethylation of the $C_7$-methoxy group in **13** was achieved by heating in toluene in the presence of *p*-toluenesulfonic acid (*p*-TsOH) to furnish the phenol (**14**), reduction[6a] of which yielded the 7-hydroxy derivative (**15**). The results are summarized in Chart 3.

Application of salcomine-oxygen oxidation to **15** effected regioselective introduction of an oxy functionality at the C-10 position to produce the *p*-quinone (**16**) in 94% yield. Consecutive hydrogenation of **16** and methylation gave dihydrosanguilutine (**17**, 90%), which was identical with an authentic specimen[8] by spectral comparison. Since dihydrosanguilutine has already been converted into sanguilutine (**18**),[8] the present synthesis amounts to a formal synthesis of **18**.

Thus, we have completed a synthesis of chelilutine and sanguilutine from the corresponding 2,3,7,8-tetraoxygenated benzo[*c*]phenanthridines through the putative biosynthetic pathway. Although we had previously explored a method for the synthesis of 2,3,7,8,10-pentaoxygenated benzo[*c*]phenanthridine alkaloids[1,8] from the corresponding 2,3,8,9,10-pentaoxygenated protoberberines, the present convenient conversion provides an alternative and effective method for the preparation of 2,3,7,8,10-pentaoxygenated benzo[*c*]phenanthridine alkaloids. In the previous paper,[1] we described the regioselective oxygenation at C-12 of 2,3,7,8,10-pentaoxygenated alkaloid, therefore, the present conversion implies that we could accomplish a biomimetic transformation of the protoberberine alkaloids into all types of benzo[*c*]phenanthridine alkaloids according to the following sequences: protoberberine→2,3,7,8-tetraoxygenated[6]→2,3,7,8,10-pentaoxygenated→2,3,7,8,10,12-hexaoxygenated benzo[*c*]phenanthridine alkaloid.[1]

## Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Alumina (Aluminiumoxid 90, Aktivitätsstufe II—III, 70—230 mesh, Merck) and silica gel (Kieselgel 60, 70—230 mesh, Merck) were used for column chromatography. Organic extracts were dried over anhydrous $Na_2SO_4$. IR spectra were measured with a JASCO A-102 spectrometer in $CHCl_3$, mass spectra (MS) with a Hitachi M-80 mass spectrometer, ultraviolet (UV) spectra with a Hitachi U-3200 spectrophotometer, and $^1$H-NMR spectra with a JEOL FX-100 spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard, unless otherwise stated.

**5,6,7,10-Tetrahydro-8-methoxy-5-methyl-2,3-methylenedioxy-7,10-dioxobenzo[*c*]phenanthridine (5)** Salcomine (101 mg, 0.31 mmol) was added to a solution of 7-*O*-demethyldihydrochelerythrine (**4**)[6b] (104 mg, 0.31 mmol) in dry THF (20 ml) in a stream of oxygen at room temperature. After being stirred for 2 h at room temperature, the reaction mixture was concentrated and the residue was passed through Florisil with $CH_2Cl_2$–MeOH (100 : 3) to afford **5** (102 mg, 94%), dark brown needles, mp 198—205 °C (dec.) ($CH_2Cl_2$–hexane). IR $\nu_{max}$ cm$^{-1}$: 1650, 1620 (*p*-quinone). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 347 (3.83), 327 (3.89), 265 (4.49), 237 (4.50). $^1$H-NMR $\delta$: 8.15, 7.46 (each 1H, ABq, $J$=9.0 Hz, $C_{11}$-H, $C_{12}$-H), 7.57, 7.27 (each 1H, each s, $C_4$-H, $C_1$-H), 6.08 (2H, s, $OCH_2O$), 5.97 (1H, s, $C_9$-H), 4.14 (2H, s, $C_6$-H), 3.87 (3H, s, OMe), 2.69 (3H, s, NMe). MS *m/z* (%): 351 ($M^+$ +2,[17] 67), 350 ($M^+$ +1, 50), 349 ($M^+$, 70), 84 (45), 49 (100). *Anal.* Calcd for $C_{20}H_{15}NO_5$: C, 68.76; H, 4.33; N, 4.01. Found: C, 68.56; H, 4.37; N, 3.99.

**Dihydrochelilutine (7)** Method A: A saturated aqueous $Na_2S_2O_4$ solution (5 ml) was added to a stirred solution of **5** (148 mg, 0.42 mmol) in acetone (15 ml) at room temperature and stirring was continued for an additional hour. The reaction mixture was diluted with $CH_2Cl_2$ and the $CH_2Cl_2$ layer was washed with water and brine, dried, and concentrated. The residue was immediately dissolved in MeOH (10 ml) under an argon atmosphere. A solution of diazomethane in ether was added to the MeOH solution at −20 °C. The reaction mixture was allowed to stand at the same temperature for 3 h, then quenched by addition of a small amount of acetic acid. The solvent was evaporated off to leave the residue, which was chromatographed on silica gel with $CH_2Cl_2$ to give **7** (85 mg, 53%), mp 145—147 °C (MeOH) (lit.[11] 139—142 °C). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 327 (4.25), 279 (4.60), 230 (4.56). $^1$H-NMR $\delta$: 8.29, 7.45 (each 1H, ABq, $J$=8.7 Hz, $C_{11}$-H, $C_{12}$-H), 7.69, 7.10, 6.57 (each 1H, each s, $C_4$-H, $C_1$-H, $C_9$-H), 6.03 (2H, s, $OCH_2O$), 4.21 (2H, s, $C_6$-H), 3.95, 3.92, 3.82 (each 3H, each s, OMe × 3), 2.56 (3H, s, NMe). MS *m/z* (%): 379 ($M^+$, 100), 348 (47), 306 (27). *Anal.* Calcd for $C_{22}H_{21}NO_5$: C, 69.64; H, 5.58; N, 3.69. Found: C, 69.71; H, 5.52; N, 3.66.

Method B: A solution of **5** (128 mg, 0.37 mmol) in dimethylformamide (15 ml) was vigorously stirred with 5% Pd–C (61 mg) under 1 atm pressure of hydrogen at room temperature for 1 h, then $Ba(OH)_2 \cdot 8H_2O$ (579 mg, 1.84 mmol) and dimethyl sulfate (0.17 ml, 1.84 mmol) were added at once to the reaction mixture. Hydrogenation was continued for an additional 2 h. The solvent was evaporated off to leave the residual oil, to which a small amount of water and aqueous ammonia were added, and the mixture was extracted with AcOEt. The extract was washed with water and brine, dried, and concentrated to dryness. Chromatography of the residue furnished **7** (112 mg, 81%).

**Chelilutine Chloride (8)** A solution of DDQ (54 mg, 0.24 mmol) was added to a vigorously stirred mixture of **7** (60 mg, 0.16 mmol) and 5% aqueous sodium hydroxide (1 ml) in benzene (5 ml) at room temperature for 3 h. The benzene layer was separated and the aqueous layer was extracted with AcOEt. The combined organic layers were washed with water and brine, dried, and concentrated to dryness. The residue was dissolved in a small amount of acetone, and concentrated hydrochloric acid was added. The resulting precipitates were collected by filtration and recrystallized from MeOH to give **8** (59 mg, 90%), mp 194—195 °C (lit. 184—186 °C,[11] 197—198 °C (dec.)[9]). IR $\nu_{max}$ cm$^{-1}$: 1620 (N=C). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 334 (4.27), 278 (4.57), 230 (4.50). $^1$H-NMR ($(CD_3)_2SO$) $\delta$: 10.04 (1H, s, $C_6$-H), 9.39, 8.23 (each 1H, ABq, $J$=9.0 Hz, $C_{11}$-H, $C_{12}$-H), 8.21, 7.77, 7.73 (each 1H, each s, $C_4$-H, $C_1$-H, $C_9$-H), 6.34 (2H, s, $OCH_2O$), 4.95 (3H, s, NMe), 4.27, 4.17, 4.09 (each 3H, each s, OMe × 3). *Anal.* Calcd for $C_{22}H_{20}ClNO_5 \cdot 5/2H_2O$: C, 57.58; H, 5.49; N, 3.05. Found: C, 57.62; H, 5.18; N, 3.11.

**5,6,7,8-Tetrahydro-2,3,9,10-tetramethoxy-7-methyldibenzo[*a*,*g*]quinolizinium Monomethylsulfate (10)** Palmatine iodide (**9**, 7.0 g, 14.6 mmol) was added portionwise to a stirred suspension of lithium aluminum hydride (LAH) (1.67 g, 43.8 mmol) in dry THF (150 ml) over a period of 15 min in a stream of nitrogen at 0 °C. The suspension was stirred for 2 h at room temperature, then water was added to the suspension and the whole was filtered. The filtrate was concentrated to leave the crude dihydro derivative, which was dissolved in hot benzene (150 ml). Dimethyl sulfate (4.15 ml, 43.8 mmol) was added dropwise to the refluxing benzene solution and refluxing was continued for 2 h. After cooling of the reaction mixture to room temperature, the resulting precipitates were collected by filtration and dried to provide **10** (6.6 g, 94%), pale yellow cubes, mp 247—249 °C (MeOH). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 350 (4.54), 250 (4.13), 214 (4.42). $^1$H-NMR ($(CD_3)_2SO$) $\delta$: 7.78, 7.43, 6.93 (each 1H, each s, $C_4$-H, $C_1$-H, $C_{13}$-H), 7.31, 7.23 (each 1H, ABq, $J$=8.5 Hz, $C_{12}$-H, $C_{11}$-H), 5.11, 4.88 (each 1H, ABq, $J$=15 Hz, $C_8$-H), 4.27—3.95 (2H, m, $C_6$-H), 3.91, 3.86, 3.84, 3.83 (each 3H, each s, OMe × 4), 3.40—3.10 (2H, m, $C_5$-H), 3.38 (3H, s, $MeSO_4$), 3.06 (3H, s, NMe). *Anal.* Calcd for $C_{23}H_{29}NO_8S \cdot H_2O$: C, 55.52; H, 6.28; N, 2.82. Found: C, 55.87; H, 6.03; N, 2.81.

**7,8-Dimethoxy-3-(4,5-dimethoxy-2-vinylphenyl)-2-methylisoquinolin-1(2*H*)-one (11)** The methosulfate (**10**) (3.0 g, 6.24 mmol) was added in one portion to refluxing 25% methanolic potassium hydroxide (20 ml) and the mixture was heated under reflux for 10 min, then poured into ice-water (40 ml) and extracted with chloroform (150 ml). The extract was washed with water and brine, and dried. DDQ (2.0 g, 8.73 mmol) was added to the above solution and the mixture was stirred for 17 h at room temperature. The chloroform solution was washed with water and brine, dried, and concentrated to dryness. Chromatography of the residue on silica gel with $CH_2Cl_2$–MeOH (100 : 1) afforded **11** (740 mg, 31%), pale yellow pillars, mp 165.5—166.5 °C ($CH_2Cl_2$–iso-$Pr_2O$). IR $\nu_{max}$ cm$^{-1}$: 1650 (amide). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 354 (3.94), 299 (4.30), 267 (4.27), 224 (4.72). $^1$H-NMR $\delta$: 7.35, 7.18 (each 1H, ABq, $J$=8.8 Hz, aromatic H), 7.15, 6.75, 6.31 (each 1H, each s, aromatic H), 6.50 (1H, dd, $J$=17, 11 Hz, vinylic H), 5.62 (1H, dd, $J$=17, 1 Hz, vinylic H), 5.15 (1H, dd, $J$=11, 1 Hz, vinylic H), 4.03, 3.98, 3.96, 3.89 (each 3H, each s, OMe × 4), 3.24 (3H, s, NMe). MS *m/z* (%): 381 ($M^+$, 100), 366 (61), 352 (30). *Anal.* Calcd for $C_{22}H_{23}NO_5$: C, 69.27; H, 6.08; N, 3.67. Found: C, 69.31; H, 6.12; N, 3.86.

**7,8-Dimethoxy-3-[4,5-dimethoxy-2-(2,2-dimethoxyethyl)phenyl]-2-methylisoquinolin-1(2*H*)-one (12)** A solution of TTN trihydrate (187 mg, 0.42 mmol) in MeOH (10 ml) was added dropwise to a solution of **11** (133 mg, 0.35 mmol) in MeOH (20 ml) at room temperature and the reaction mixture was stirred for 15 min, then filtered. A saturated aqueous sodium bicarbonate solution (10 ml) was added to the filtrate and the mixture was extracted with $CH_2Cl_2$. The extract was washed with water and brine, dried, and concentrated to dryness. Chromatography of the residue on silica gel with $CH_2Cl_2$–MeOH (100 : 1) gave **12** (140 mg, 91%) as an oil. IR $\nu_{max}$ cm$^{-1}$: 1650 (amide). $^1$H-NMR $\delta$: 7.34, 7.22 (each 1H, ABq, $J$= 9.0 Hz, aromatic H), 6.96, 6.73, 6.31 (each 1H, each s, aromatic H), 4.42 (1H, dd, $J$=6.0, 5.0 Hz, C<u>H</u>$CH_2$), 4.03, 3.96, 3.92, 3.87 (each 3H, each s, OMe × 4), 3.26, 3.25 (each 3H, each s, OMe × 2), 3.21 (3H, s, NMe), 2.90 (1H, dd, $J$=14, 6.0 Hz, CHC<u>H</u>$_2$), 2.66 (1H, dd, $J$=14, 5.0 Hz, CHC<u>H</u>$_2$). MS *m/z* (%): 443 ($M^+$, 41%), 368 (23), 75 (100). High-resolution MS calcd for $C_{24}H_{29}NO_7$: 443.1942. Found: 443.1942.

**2,3,7,8-Tetramethoxy-5-methylbenzo[*c*]phenanthridin-6(5*H*)-one (13)** A solution of **12** (245 mg, 0.55 mmol) and 10% hydrochloric acid (5 ml) in MeOH (20 ml) was refluxed for 30 min, then MeOH was evaporated off and the residue was taken up in $CH_2Cl_2$. The solution was washed with water and brine, dried, and concentrated to leave the residue, which was chromatographed on silica gel with $CH_2Cl_2$–MeOH (100 : 1) to give **13** (202 mg, 96%), colorless needles, mp 221—223 °C (MeOH). IR $\nu_{max}$ cm$^{-1}$: 1640 (amide). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 323 (4.20), 286 (4.81), 277 (4.70), 241 (4.61). $^1$H-NMR $\delta$: 8.00, 7.56 (each 1H, ABq, $J$=9.0 Hz, $C_{11}$-H, $C_{12}$-H), 7.99, 7.39 (each 1H, ABq, $J$=9.0 Hz, $C_{10}$-H, $C_9$-H), 7.51, 7.16 (each 1H, each s, $C_4$-H, $C_1$-H), 4.09, 4.08, 4.03, 3.98 (each 3H, each s, OMe × 4), 3.96 (3H, s, NMe). MS *m/z* (%): 379 ($M^+$, 100), 364 (37), 350 (20). *Anal.* Calcd for $C_{22}H_{21}NO_5$: C, 69.64; H, 5.58; N, 3.69. Found: C, 69.71; H, 5.55; N, 3.77.

**7-Hydroxy-2,3,8-trimethoxy-5-methylbenzo[*c*]phenanthridin-6(5*H*)-one (14)** A solution of **13** (283 mg, 0.75 mmol) and *p*-TsOH (213 mg, 1.12 mmol) in toluene (30 ml) was heated under reflux for 3.5 h. After cooling, the solvent was evaporated off and the residue was taken up in $CH_2Cl_2$. The solution was washed with water and brine, dried, and concentrated to dryness. Chromatography of the residue on silica gel with $CH_2Cl_2$–MeOH (100 : 1) provided **14** (245 mg, 90%), pale yellow needles, mp 220—221 °C (MeOH). IR $\nu_{max}$ cm$^{-1}$: 3545 (OH), 1640 (amide). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 344 (4.21), 331 (4.17), 285 (4.73), 244 (4.67). $^1$H-NMR $\delta$: 8.00, 7.58 (each 1H, ABq, $J$=9.0 Hz, $C_{11}$-H, $C_{12}$-H), 7.64, 7.33 (each 1H, ABq, $J$=9.0 Hz, $C_{10}$-H, $C_9$-H), 7.51, 7.16 (each 1H, each s, $C_4$-H, $C_1$-H), 4.04 (3H, s, OMe), 4.00 (6H, s, OMe × 2), 3.99 (3H, s, NMe), 3.35 (1H, s, OH).

MS *m/z* (%): 365 ($M^+$, 100), 350 (43), 322 (20). *Anal.* Calcd for $C_{21}H_{19}NO_5$: C, 69.03; H, 5.24; N, 3.83. Found: C, 68.94; H, 5.23; N, 3.90.

**5,6-Dihydro-7-hydroxy-2,3,8-trimethoxy-5-methylbenzo[*c*]phenanthridine (15)** LAH (121 mg, 3.18 mmol) was added to a stirred solution of **14** (116 mg, 0.32 mmol) in dry THF (20 ml) in a stream of nitrogen at 0 °C. After being stirred for 1 h at room temperature, the reaction mixture was diluted with water and filtered. The filtrate was concentrated and the residue was dissolved in MeOH (20 ml). Sodium borohydride (121 mg, 3.18 mmol) was added portionwise to the above solution. The reaction mixture was stirred at room temperature for 30 min, then MeOH was evaporated off. The residue was taken up in $CH_2Cl_2$. The solution was washed with water and brine, dried, and concentrated to dryness. Chromatography of the residue on alumina with $CH_2Cl_2$ gave **15** (96 mg, 86%), colorless needles, mp 98—100 °C (MeOH). IR $\nu_{max}$ $cm^{-1}$: 3550 (OH). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 321 (4.21), 281 (4.64), 234 (4.53). $^1$H-NMR $\delta$: 7.72, 7.50 (each 1H, ABq, $J$=9.0 Hz, $C_{11}$-H, $C_{12}$-H), 7.67, 7.12 (each 1H, each s, $C_4$-H, $C_1$-H), 7.21, 6.99 (each 1H, ABq, $J$=8.0 Hz, $C_{10}$-H, $C_9$-H), 5.70 (1H, s, OH), 4.33 (2H, s, $C_6$-H), 4.07, 4.01, 3.93 (each 3H, each s, OMe × 3), 2.64 (3H, s, NMe). MS *m/z* (%): 351 ($M^+$, 100), 350 (67). *Anal.* Calcd for $C_{21}H_{21}NO_4 \cdot 1/2$MeOH: C, 69.79; H, 6.42; N, 3.88. Found: C, 69.79; H, 6.42; N, 3.87.

**5,6,7,10-Tetrahydro-2,3,8-trimethoxy-5-methyl-7,10-dioxobenzo[*c*]phenanthridine (16)** According to the procedure described for **4**, the hydroxy derivative (**15**) (79 mg, 0.23 mmol) was treated with salcomine (73 mg, 0.23 mmol) in a stream of oxygen to give, after passage through Florisil with $CH_2Cl_2$–MeOH (100:3), **16** (77 mg, 94%), dark brown needles, mp 197—203 °C (dec.) ($CH_2Cl_2$–hexane). IR $\nu_{max}$ $cm^{-1}$: 1650, 1620 (*p*-quinone). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 347 (3.84), 326 (3.86), 263 (4.56), 240 (4.52). $^1$H-NMR $\delta$: 8.17, 7.50 (each 1H, ABq, $J$=9.0 Hz, $C_{11}$-H, $C_{12}$-H), 7.54, 7.11, 5.96 (each 1H, each s, $C_4$-H, $C_1$-H, $C_9$-H), 4.16 (2H, s, $C_6$-H), 4.05, 4.02, 3.87 (each 3H, each s, OMe × 3), 2.72 (3H, s, NMe). MS *m/z* (%): 367 ($M^+$ +2,[17] 56), 366 ($M^+$ +1, 46), 365 ($M^+$, 100), 350 (32), 322 (30). *Anal.* Calcd for $C_{21}H_{19}NO_5$: C, 69.03; H, 5.24; N, 3.83. Found: C, 69.11; H, 5.48; N, 3.61.

**Dihydrosanguilutine (17)** According to the procedure (method B) described for **5**, the *p*-quinone (**16**) (61 mg, 0.17 mmol) was hydrogenated on 5% Pd–C (29 mg) in the presence of $Ba(OH)_2 \cdot 8H_2O$ (274 mg, 0.83 mmol) and dimethyl sulfate (0.08 ml, 0.83 mmol) in dimethylformamide (7 ml). Work-up and chromatography on silica gel with $CH_2Cl_2$ afforded **17** (59 mg, 90%), colorless cubes, mp 152—154 °C (MeOH) (lit.[8] 154—155 °C). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 326 (4.28), 277 (4.64), 228 (4.57). $^1$H-NMR $\delta$: 8.32, 7.48 (each 1H, ABq, $J$=9.0 Hz, $C_{11}$-H, $C_{12}$-H), 7.68, 7.12, 6.57 (each 1H, each s, $C_4$-H, $C_1$-H, $C_9$-H), 4.24 (2H, s, $C_6$-H), 4.07, 4.01, 3.95, 3.92, 3.82 (each 3H, each s, OMe × 5), 2.60 (3H, s, NMe). MS *m/z* (%): 395 ($M^+$, 100), 364 (17). *Anal.* Calcd for $C_{23}H_{25}NO_5$: C, 69.85; H, 6.37; N, 3.54. Found: C, 69.84; H, 6.57; N, 3.63.

**Acknowledgement** We are indebted to Professor J. Slavík, J. E. Purkyne University, Czechoslovakia, for a generous supply of natural chelilutine.

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# Reduction of Five-Membered α,β-Unsaturated Lactones and Related Compounds with the $Ni^{2+}/BH_4^-$ System

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**The reductions of an α,β-unsaturated lactone (5) and lactam (7) with the $Ni^{2+}/BH_4^-$ system resulted in the formation of *cis*-hydrogenated products (8a and 9a) with high stereoselectivity. The *cis* products (8a and 9a) were easily isomerized to the corresponding *trans* isomers (8b and 9b, respectively) by refluxing with sodium methoxide in anhydrous methanol. Isotope labeling studies with methyl cinnamate as the substrate indicated that the reduction with this reducing system poceeds stepwise *via* a carbon–nickel intermediate.**

**Keywords** $Ni^{2+}/BH_4^-$; reduction; hydrogenation; α,β-unsaturated 5-membered lactone; α,β-unsaturated lactam; isotope labeling study; methyl cinnamate

Reducing systems consisting of sodium borohydride ($NaBH_4$) with a transition metal halide such as $CoCl_2$, $NiCl_2$, or $CuCl_2$ have been employed in hydrogenation of various organic functional groups.[1-7] However, to our knowledge, no application of this reducing system to the stereoselective *cis* hydrogenation of C=C functionalities has been reported so far.[8] Quite recently, we have found that the reaction of the α,β-unsaturated 5-membered lactone (**1**) with $Ni^{2+}/BH_4^-$ in MeOH affords only the *cis*-hydrogenated compound (**2**) in good yield[9] (Chart 1). It seems likely that the nature of the reducing system is responsible for such high stereoselectivity.

In order to investigate further the above reducing system, we carried out some experiments with the five-membered α,β-unsaturated lactone (**5**) and lactam (**7**), as well as an isotope labeling study with methyl cinnamate.

We wish to report here that the $Ni^{2+}/BH_4^-$ reducing system possesses a high stereoselectivity in the hydrogenation of the C=C bond in a five-membered α,β-unsaturated lactone/lactam system. A homogeneous borohydride-containing complex[10,11] was suggested as the most probable reducing species in these reductions.

## Results and Discussion

The starting α,β-unsaturated lactone (**5**) and lactam (**7**) were prepared by the procedures described previously and their structures were confirmed by spectroscopic and elemental analysis (see Chart 2 and Experimental).

The reduction of the lactone (**5**) with the $Ni^{2+}/BH_4^-$ system in MeOH gave a stereoisomeric mixture [*cis*/*trans* (**8a**/**8b**) = 3/1] separable by high-performance liquid chromatography (HPLC). However, the reduction of the lactam (**7**) with this system resulted in the formation of the *cis* hydrogenated derivative (**9a**) with no formation of the *trans* isomer (**9b**) (Chart 3). The stereochemistry of these products was established from the $^{13}C$-nuclear magnetic resonance ($^{13}C$-NMR) spectroscopic data, compared with the $^{13}C$-resonance behavior of known 1,2-dimethylcyclopentanes.[12] Thus, the two methyl signals at higher magnetic field (2.5—3.8 ppm) were assigned to the isomers having *cis* configuration (**8a** and **9a**). Both *cis* products (**8a** and **9a**) were isomerized to the corresponding *trans* isomers (**8b** and

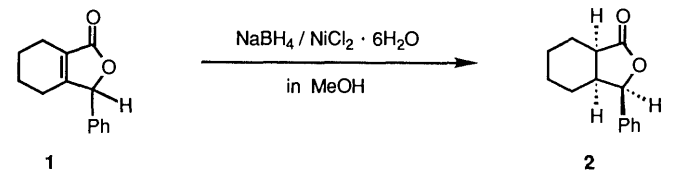


Chart 1

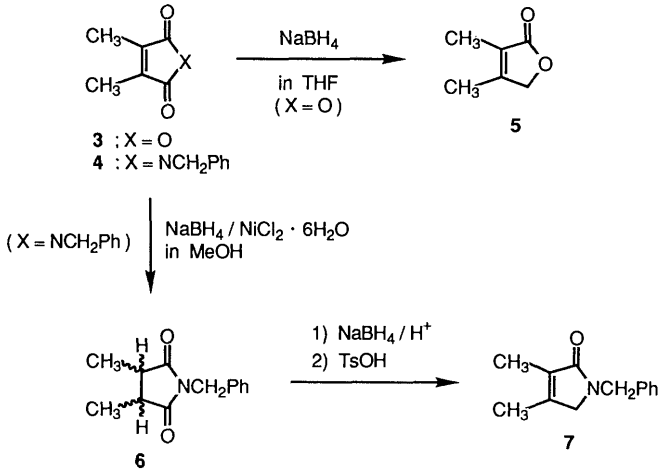


Chart 2

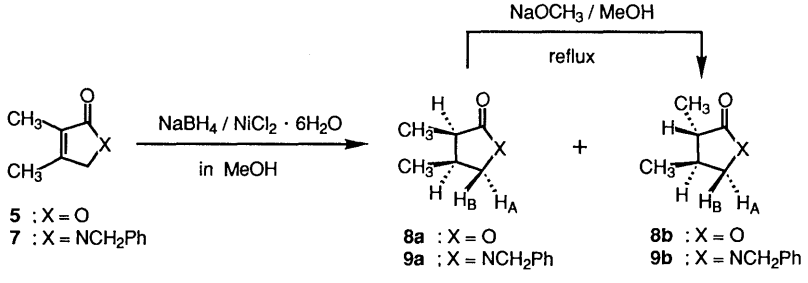


Chart 3

Table I. Isotope Labeling Study with Methyl Cinnamate

| | Reagent/solvent | Metal | $t_R$ (min) | Number of deuterium atoms per mole of methyl 3-phenylpropionate | | | |
|---|---|---|---|---|---|---|---|
| | | | | β Position | | α Position[c] | |
| | | | | MS[a] | $^1$H-NMR[b] | MS | $^1$H-NMR |
| Run 1 | $NaBH_4$/MeOD | $Ni_2B$ | 90 | 0.07 | 0.0 | 0.8 | 0.96 |
| Run 2 | $NaBH_4$/MeOD | $NiCl_2$ | 20 | 0.20 | 0.17 | 0.26 | 0.31 |
| Run 3 | $NaBD_4$/MeOH | $NiCl_2$ | 30 | 0.84 | 0.9 | 0.54 | 0.47 |

*a*) Calculated from the relative abundances of the benzyl fragment ion and the molecular ion. Relative abundances were corrected, using the $(M+1)^+$ ion in the authentic undeuterated sample. *b*) The deuterium incorporation was evaluated from the result of integration of the signals due to the α and β methylene units (δ 2.71—2.52 and 3.06—2.87 ppm) relative to that of the methyl group (δ 3.663 ppm). *c*) No significant H/D exchange was observed at the α position in methyl 3-phenylpropionate.

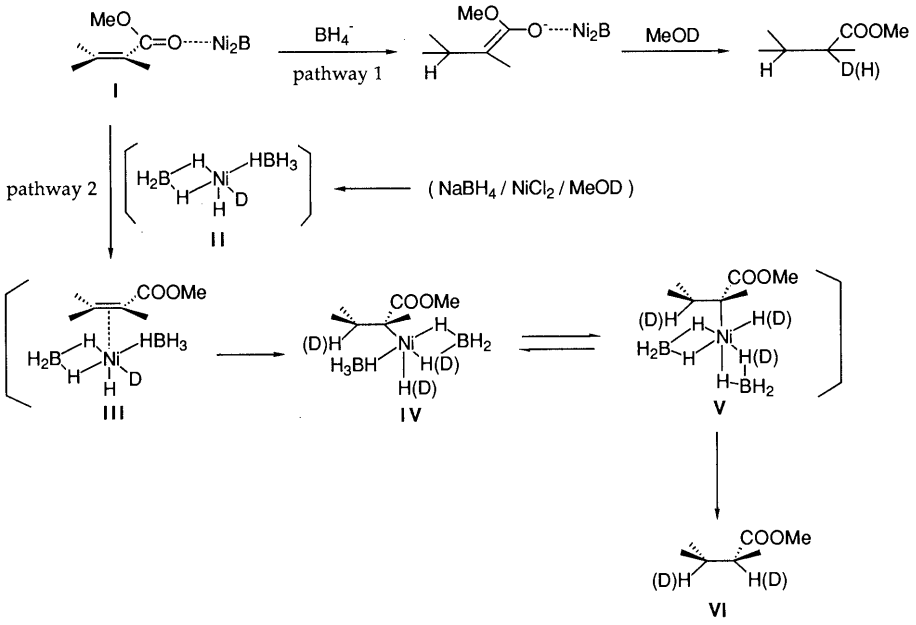


Chart 4

**9b**, respectively) in good yields by refluxing with sodium methoxide in anhydrous methanol, which also supports the above assignments. The fact of the formation of the thermodynamically unstable *cis* isomer (**8a** or **9a**) apparently indicates that these reductions take place in a *cis* fashion.

In order to elucidate the reduction mechanism,[2,5-7,13,14] isotope labeling studies with methyl cinnamate as the substrate were carried out. The ratios of incorporation determined by mass and $^1$H-NMR spectral techniques are summarized in the table.

The reaction under heterogeneous conditions with preformed $Ni_2B$ (run 1 in Table I)[6,15] resulted in the exclusive incorporation of a deuterium atom at the α position. This apparently indicates hydride addition from uncoordinated $BH_4^-$ [14] (pathway 1 in Chart 4) and little contribution of a heterogeneous catalytic hydrogenation process. On the other hand, under the usual conditions, where the formation of a black precipitate ($Ni_2B$) occurred *in situ* (run 2 in Table I), no significant specificity was observed regarding the incorporation of a deuterium atom at the α position. These results require that homogeneous species play some significant part in the reduction of methyl cinnamate with this system. The parallel experiment employing $NaBD_4$ and $NiCl_2$ in MeOH under the usual conditions led to methyl 3-phenylpropionate with about 84—90% deuterium at the β position and only about 47—54% deuterium at the α position (run 3 in Table I). The results obtained from runs 2 and 3 suggest that the hydrogen added to the β position arises directly from the starting sodium borohydride. From the outcome of the isotope labeling studies shown in the table, we consider that the overall hydrogenation with this reducing system proceeds stepwise through the following two stages: (a) formation of the carbon–nickel intermediate from the reaction of the olefin I and illustrated five-coordinate Ni complex II as the active species (I→III→IV), and then (b) reductive cleavage of the carbon–nickel bond in this intermediate (IV⇄V) yielding the product (V→VI). These stages are illustrated in Chart 4.

In the reduction of **5** or **7** with this reducing system, the *cis* stereoselectivities may be explained through the mechanism of pathway 2 shown in Chart 4. The nickel boride ($Ni_2B$) formed *in situ* would contribute to an initial activation of α,β-unsaturated carbonyl group in the substrates.[16]

Further synthetic applications of this mild reducing system are being examined and the details will be reported in separate papers.

**Experimental**

Infrared (IR) spectra were obtained on a Hitachi 250 spectrometer. $^1$H- and $^{13}$C-NMR spectra were recorded on a JEOL GX-400 spectrometer with a 5 mm probe and tetramethylsilane (TMS) as an internal standard

at 25 °C in $CDCl_3$ equipped with a G MHD 80R (JEOL) computer system. $^1$H-NMR spectra in isotope labeling studies were obtained with a Hitachi R-90H spectrometer using TMS as an internal standard. High-resolution mass spectra (MS) were measured on a JEOL D-300 spectrometer with electron impact ionization at an ionizing potential of 70 eV. High-performance liquid chromatographic analyses were performed by using a Waters M-45 or 501 solvent delivery system equipped with a U-6K injector and R-401 differential refractometer (Waters $\mu$-Porasil, 3.9 × 300 mm or YMC-pack S-043 I-15 sil. 20 × 250 mm).

3,4-Dimethyl-2(5*H*)-furanone (**5**)[17] was prepared from 2,3-dimethylmaleic anhydride according to the method reported previously.[18] The preparation of the lactam (**7**), according to the route shown in Chart 2, is described below.

**1-Benzyl-3,4-dimethyl-1*H*-pyrrole-2,5-dione (4)** This compound was prepared from 2,3-dimethylmaleic anhydride and benzylamine by the method described previously.[19] The yield was 90%, mp 44—45 °C (hexane). *Anal.* Calcd for $C_{13}H_{13}NO_2$: C, 72.54; H, 6.09; N, 6.51. Found: C, 72.72; H, 6.10; N, 6.21. IR (KBr): 1774, 1710 (C=O) cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 7.27 (5H, m, aromatic protons), 4.61 (2H, s, $-CH_2-$Ph), and 1.94 (6H, s, 2 × $CH_3$). $^{13}$C-NMR ($CDCl_3$) $\delta$: 171.5 [C(2) and C(5)], 137.1 and 136.6 [C(3) and C(4)], 128.3, 127.5 (aromatic carbons), 41.3 (a benzylic carbon), 8.5 (methyl carbons).

**1-Benzyl-1,5-dihydro-3,4-dimethyl-2*H*-pyrrol-2-one (7)** $NaBH_4$ (0.38 g, 0.01 mol) was added portionwise to a stirred solutions of 1-benzyl-3,4-dimethyl-1*H*-pyrrole-2,5-dione (**4**) (2.15 g, 0.01 mol) and $NiCl_2 \cdot 6H_2O$ (0.24 g, 0.001 mol) in methanol at −10 °C. After neutralization of the reaction mixture with aqueous HCl (5%), the solvent was removed under reduced pressure. The resulting residue was dissolved in $H_2O$ (10 ml) and the solution was extracted with dichloromethane. The extract was dried over anhydrous magnesium sulfate. Evaporation of the solvent gave an oily mixture of two stereoisomers of 1-benzyl-3,4-dimethyl-2,5-pyrrolidinedione (**6**) (2.12 g, 97.7%). This mixture was used in the next step. For the transformation to the $\alpha,\beta$-unsaturated lactam (**7**) from the 2,5-pyrrolidinedione ring system, the procedure reported by Hubert *et al.*[20] was employed. The oily product was obtained in 89% yield, and was purified by column chromatography on alumina with diethyl ether and then ethyl acetate as eluants. The purified oily material (**7**) was extremely hygroscopic. MS *m/z*: 201 ($M^+$). IR (KBr): 1675 (C=O) cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 7.26 (5H, m, aromatic protons), 4.60 (2H, s, $=NCH_2$Ph), 3.61 [2H, s, C(5)-H], 1.90 [3H, s, C(3)-$CH_3$], and 1.83 [3H, s, C(4)-$CH_3$]. $^{13}$C-NMR ($CDCl_3$) $\delta$: 172.38 (C-2), 128.3, 127.7, 127.2 (aromatic carbons), 145.7 (C-4), 137.5 (C-3), 53.5 (C-5), 45.9 (a benzylic carbon), 12.9 [C(4)-$CH_3$], 8.6 [C(3)-$CH_3$]. *Anal.* Calcd for $C_{13}H_{15}NO \cdot 0.35H_2O$: C, 75.22; H, 7.62; N, 6.75. Found: C, 75.26; H, 7.51; N, 6.55.

**Reduction of the $\alpha,\beta$-Unsaturated Lactone (5)** $NaBH_4$ (0.81 g, 21.4 mmol) was added portionwise to a stirred solution of $\alpha,\beta$-unsaturated lactone (**5**) (0.6 g, 5.4 mmol) and $NiCl_2 \cdot 6H_2O$ (0.63 g, 2.7 mmol) in methanol at 15 °C. The black precipitate was filtered off and the filtrate was acidified with aqueous HCl (10%). The solvent was removed under reduced pressure and the resulting residue was diluted with $H_2O$. The mixture was extracted with $Et_2O$ and the extract was washed with brine and dried over anhydrous magnesium sulfate. Evaporation of the solvent gave an oily mixture of **8a** and **8b**, in 91% yield. The isomeric ratio of the products [**8a**/**8b**] was 3/1 as determined by HPLC using 60% ether/hexane as the eluant. The retention times for the two isomers (**8a** and **8b**) were 4.0 and 5.1 min, respectively. *Anal.* Calcd for $C_6H_{10}O_2$: C, 63.14; H, 8.83. Found: C, 62.99; H, 9.11. Purification of this mixture by preparative HPLC afforded an analytical sample of *cis*-dihydro-3,4-dimethyl-2(3*H*)-furanone (**8a**). MS *m/z*: 114 ($M^+$). IR (KBr): 1775 (C=O) cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 4.30 [1H, dd, $J_{C(5)\text{-H}(gem)}$ = 9.0 Hz, $J_{C(4)\text{-H},C(5)\text{-H}_A}$ = 6.0 Hz, C(5)-$H_A$], 3.93 [1H, dd, $J_{C(5)\text{-H}(gem)}$ = 9.0 Hz, $J_{C(4)\text{-H},C(5)\text{-H}_B}$ = 3.0 Hz, C(5)-$H_B$], 2.68 [qd, 1H, $J_{C(3)\text{-CH}_3,C(3)\text{-H}}$ = 7.0 Hz, $J_{C(4)\text{-H},C(3)\text{-H}}$ = 8.0 Hz, C(3)-H], 2.62 [1H, m, C(4)-H], 1.17 [3H, d, $J_{C(3)\text{-H},C(3)\text{-CH}_3}$ = 7.0 Hz, C(3)-$CH_3$], 1.02 [3H, dd, $J_{C(4)\text{-H},C(4)\text{-CH}_3}$ = 7.0 Hz, $J_{C(5)\text{-H}_A,C(4)\text{-CH}_3}$ = 1.0 Hz, C(4)-$CH_3$]. $^{13}$C-NMR ($CDCl_3$) $\delta$: 179.686 (C-2), 72.948 (C-5), 38.254 (C-3), 33.837 (C-4), 13.349 [C(4)-$CH_3$], 9.798 [C(3)-$CH_3$].

**Isomerization of the *cis* Lactone (8a)** A mixture of the *cis* lactone (**8a**) with 100% isomeric purity (0.14 g, 1.25 mmol) and sodium methoxide (0.12 g, 2 mmol) in anhydrous methanol was refluxed for 3.5 h. The resulting solution was concentrated under reduced pressure, diluted with water, and acidified with HCl (10%). The mixture was extracted with ether, and the extract was washed with aqueous sodium carbonate, and dried over anhydrous magnesium sulfate. Evaporation of the solvent afforded the *trans* lactone (**8b**) with 95% isomeric purity [by HPLC using 60% ether/hexane as the eluant]. Purification of the product by preparative HPLC gave a pure sample of *trans*-4,5-dihydro-3,4-dimethyl-2(3*H*)-furanone (**8b**). MS *m/z*: 114 ($M^+$). IR (KBr): 1778 (C=O) cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 4.37 [1H, ddd, $J_{C(5)\text{-H}(gem)}$ = 9.0 Hz, $J_{C(4)\text{-H},C(5)\text{-H}_B}$ = 7.5 Hz, C(5)-$H_B$], 3.72 [1H, ddd, $J_{C(5)\text{-H}(gem)}$ = 9.0 Hz, $J_{C(4)\text{-H},C(5)\text{-H}_A}$ = 10.0 Hz, $J_{C(4)\text{-CH}_3,C(5)\text{-H}_A}$ = 0.5 Hz, C(5)-$H_A$], 2.18 [1H, m, C(4)-H], 2.11 [1H, qd, $J_{C(3)\text{-CH}_3,C(3)\text{-H}}$ = 6.5 Hz, $J_{C(4)\text{-H},C(3)\text{-H}}$ = 11.0 Hz, C(3)-H], 1.24 [3H, d, $J_{C(3)\text{-H},C(3)\text{-CH}_3}$ = 6.0 Hz, C(3)-$CH_3$], 1.15 [3H, dd, $J_{C(4)\text{-H},C(4)\text{-CH}_3}$ = 6.0 Hz, $J_{C(5)\text{-H}_A,C(4)\text{-CH}_3}$ = 0.5 Hz, C(4)-$CH_3$]. $^{13}$C-NMR ($CDCl_3$) $\delta$: 179.579 (C-2), 72.432 (C-5), 41.820 (C-3), 38.709 (C-4), 15.550 [C(4)-$CH_3$], 13.061 [C(3)-$CH_3$].

**Reduction of the $\alpha,\beta$-Unsaturated Lactam (7)** $NaBH_4$ (0.95 g, 25 mmol) was added portionwise to a stirred solution of $\alpha,\beta$-unsaturated lactam (**7**) (1.11 g, 5 mmol) and $NiCl_2 \cdot 6H_2O$ (0.6 g, 2.5 mmol) in methanol at 15 °C. The black precipitate was filtered off and the filtrate was acidified with aqueous HCl (10%). The methanol was removed under reduced pressure and the resulting material was diluted with $H_2O$. After extraction with $Et_2O$, the ether extract was washed with brine, and dried over anhydrous magnesium sulfate. Evaporation of the solvent afforded the oily *cis*-1-benzyl-3,4-dimethyl-2-pyrrolidinone (**9a**) with 100% isomeric purity in 99.2% yield. An analytical sample was obtained by HPLC (AcOEt/hexane/$Et_2O$ = 1/4/5 as the solvent). The product was extremely hygroscopic. *Anal.* Calcd for $C_{13}H_{17}NO \cdot 0.25H_2O$: C, 75.14; H, 8.49; N, 6.74. Found: C, 75.11; H, 8.48; N, 6.61. MS *m/z*: 203 ($M^+$). IR (KBr): 1682 (C=O) cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 7.342—7.207 (5H, m, aromatic protons), 4.485 (1H, d, $J$ = 14.5 Hz, $NCH_2$Ph), 4.392 (1H, d, $J$ = 14.5 Hz, $NCH_2$Ph), 3.269 [1H, dd, $J_{C(5)\text{-H}(gem)}$ = 9.5 Hz, $J_{C(4)\text{-H},C(5)\text{-H}_A}$ = 7 Hz, C(5)-$H_A$], 2.766 [1H, dd, $J_{C(5)\text{-H}(gem)}$ = 9.5 Hz, $J_{C(4)\text{-H},C(5)\text{-H}_B}$ = 5 Hz, C(5)-$H_B$], 2.557 [1H, dq, $J_{C(4)\text{-H},C(3)\text{-H}}$ = 7.0 Hz, $J_{C(3)\text{-CH}_3,C(3)\text{-H}}$ = 7.5 Hz, C(3)-H], 2.419 [1H, dddq, $J_{C(3)\text{-H},C(4)\text{-H}}$ = 7 Hz, $J_{C(5)\text{-H}_A,C(4)\text{-H}}$ = 7.0 Hz, $J_{C(5)\text{-H}_B,C(4)\text{-H}}$ = 5.0 Hz, $J_{C(4)\text{-CH}_3,C(4)\text{-H}}$ = 7 Hz, C(4)-H], 1.113 [3H, d, $J_{C(3)\text{-H},C(3)\text{-CH}_3}$ = 7.5 Hz, C(3)-$CH_3$], 0.925 [3H, d, $J_{C(4)\text{-H},C(4)\text{-CH}_3}$ = 7.0 Hz, C(4)-$CH_3$]. $^{13}$C-NMR ($CDCl_3$) $\delta$: 177.014 (C-2), 136.781, 128.510, 128.252, 128.130, 127.356 (aromatic carbons), 52.065 (C-5), 46.419 ($NCH_2$Ph), 40.485 (C-3), 30.286 (C-4), 14.062 [C(4)-$CH_3$], 10.298 [C(3)-$CH_3$].

**Isomerization of the *cis* Lactam (9a)** A mixture of the *cis* lactam (**9a**) with 100% isomeric purity (0.2 g, 1 mmol) and sodium methoxide (0.12 g, 2 mmol) in anhydrous methanol was refluxed for 20 h. The resulting solution was concentrated under reduced pressure, diluted with water, and extracted with ether. The extract was washed with brine, and dried over anhydrous magnesium sulfate. Evaporation of the solvent afforded the *trans* lactam (**9b**) with 85% isomeric purity (by HPLC using AcOEt/hexane/$Et_2O$ = 1/4/5 as the eluant) in 75% yield. The retention times of the two isomers [**9a** and **9b**] were 9.8 and 12.4 min, respectively. Separation by HPLC afforded the pure *trans*-1-benzyl-3,4-dimethyl-2-pyrrolidinone (**9b**). MS *m/z*: 203 ($M^+$). IR (KBr): 1677 (C=O) cm$^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 7.338—7.206 (5H, m, aromatic protons), 4.462 (1H, d, $J$ = 14.5 Hz, $NCH_2$Ph), 4.413 (1H, d, $J$ = 14.5 Hz, $NCH_2$Ph), 3.242 [1H, dd, $J_{C(5)\text{-H}(gem)}$ = 8.0 Hz, $J_{C(4)\text{-H},C(5)\text{-H}_B}$ = 9.5 Hz, C(5)-$H_B$], 2.758 [1H, dd, $J_{C(5)\text{-H}(gem)}$ = 8.0 Hz, $J_{C(4)\text{-H},C(5)\text{-H}_A}$ = 9.0 Hz, C(5)-$H_A$], 2.042 [1H, dq, $J_{C(4)\text{-H},C(3)\text{-H}}$ = 10 Hz, $J_{C(3)\text{-CH}_3,C(3)\text{-H}}$ = 7 Hz, C(3)-H], 1.909 [1H, dddq, $J_{C(3)\text{-H},C(4)\text{-H}}$ = 10 Hz, $J_{C(5)\text{-H}_A,C(4)\text{-H}}$ = 9.0 Hz, $J_{C(5)\text{-H}_B,C(4)\text{-H}}$ = 9.5 Hz, $J_{C(4)\text{-CH}_3,C(4)\text{-H}}$ = 7 Hz, C(4)-H], 1.213 [3H, d, $J_{C(3)\text{-H},C(3)\text{-CH}_3}$ = 6.83 Hz, C(3)-$CH_3$], 1.077 [3H, d, $J_{C(4)\text{-H},C(4)\text{-CH}_3}$ = 6.83 Hz, C(4)-$CH_3$]. $^{13}$C-NMR ($CDCl_3$) $\delta$: 176.893 (C-2), 136.660, 128.555, 128.009, 127.371 (aromatic carbons), 52.034 (C-5), 46.525 ($NCH_2$Ph), 44.598 (C-3), 35.901 (C-4), 17.325 [C(4)-$CH_3$], 14.381 [C(3)-$CH_3$].

**Isotope Labeling Study with Methyl Cinnamate** Run 1: $NaBH_4$ (0.38 g, 10 mmol) was added in three portions to a stirred solution of $NiCl_2$ (0.32 g, 2.5 mmol) and $D_2O$ (0.4 g, 20 mmol) in MeOD (over 99.5%, 25 ml). Gas evolution, followed by the formation of a black precipitate of $Ni_2B$ was observed. After 10 min, the supernatant was decanted, and the black precipitate was washed with MeOD, until no $Ni^{2+}$ could be detected in the washings. Then $NaBH_4$ (0.188 g, 5 mmol) was added portionwise to a mixture of the black precipitate and methyl cinnamate (0.4 g, 2.5 mmol) in MeOD (25 ml) at 10 °C over a period of 90 min. The mixture was filtered and the filtrate was acidified with aqueous HCl (10%). The solvent was removed under reduced pressure and the residue was taken up in $H_2O$ (5 ml). The mixture was extracted with $Et_2O$ and the extract was washed with brine and dried over anhydrous magnesium sulfate. Evaporation of the solvent gave 0.37 g (91.4%) of methyl 3-phenylpropionate.

Run 2: $NaBH_4$ (0.2 g, 5.3 mmol) was added portionwise to a stirred solution of $NiCl_2 \cdot 6D_2O$ (0.6 g, 2.5 mmol) and methyl cinnamate (0.4 g, 2.5 mmol) in MeOD (25 ml) at 10 °C over a period of 20 min. The mixture was filtered and the filtrate was acidified with aqueous DCl (20%). The

solvent was removed under reduced pressure and the residue was taken up in $D_2O$ (5 ml). The mixture was extracted with $Et_2O$ and the extract was washed with brine and dried over anhydrous magnesium sulfate. Evaporation of the solvent gave 0.35 g (86.4%) of methyl 3-phenylpropionate.

Run 3: Reduction of methylcinnamate (0.4 g, 2.5 mmol) was carried out in the same manner as in run 2, using $NaBD_4$ (0.35 g, 9 mmol) and MeOH (25 ml) instead of $NaBH_4$ and MeOD (reaction time, 30 min). The results on the incorporation of deuterium into methyl 3-phenylpropionate are summarized in the table.

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16) We confirmed that the reduction of **5** did not take place under heterogeneous conditions [10 mol eq of $NaBH_4$ + 1 mol eq of preformed $Ni_2B$] or under homogeneous conditions in the absence of a black precipitate [at −10 °C, 3 mol eq of $NaBH_4$ + 1 mol eq of $NiCl_2 \cdot 6H_2O$]. In addition, we observed that under the latter homogeneous conditions the reduction was initiated by the formation of a black precipitate ($Ni_2B$).
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# Chemical Studies on Crude Drug Processing. VII.[1] On the Constituents of Rehmanniae Radix. (1): Absolute Stereostructures of Rehmaglutins A, B, and D Isolated from Chinese Rehmanniae Radix, the Dried Root of *Rehmannia glutinosa* LIBOSCH.

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**An iridoid alcohol, rehmaglutin A, and two chlorinated iridoids, rehmaglutins B and D, were isolated from the less polar fraction of Chinese Rehmanniae Radix [the dried root of *Rehmannia glutinosa* LIBOSCH. (Kan-jio in Japanese)], together with rehmaglutin C, rehmaionoside C, jio-cerebroside, and acteoside. The absolute configurations of rehmaglutins A, B, and D were established on the basis of chemical and spectral evidence which included the chemical derivations of rehmaglutins from the known iridoid glycoside catalpol and the application of the benzoate chirality method.**

**Keywords** *Rehmannia glutinosa*; Scrophulariaceae; crude drug processing; iridoid alcohol; iridoid chlorinated; rehmaglutin A; rehmaglutin B; rehmaglutin D

Rehmanniae Radix, which is a crude drug prepared from the roots of various *Rehmannia* spp. (Scrophulariaceae) is listed as an upper grade drug (上薬) in Shen Nung's Herbal (神農本草経) and is one of the most important traditional Chinese medicines. Depending upon the kind of processing method, Rehmanniae Radix is classified into three types named in Japanese as Shō-jiō (生地黄 fresh root), Kan-jiō (乾地黄 dried root), and Juku-jiō (熟地黄 variously treated root), which have quite distinct applications in herbal formulae of Chinese traditional medicine. In recent years, due to the poor supply of Japanese Rehmanniae Radix, which is prepared from the root of *Rehmannia glutinosa* LIBOSCH. var. *purpurea* MAKINO (Akaya-jiō in Japanese) or *R. glutinosa* LIBOSCH. forma *hueichingensis* HSIAO (Kaikei-jiō in Japanese), Chinese Rehmanniae Radix has been imported and commonly used in Chinese medicinal treatment in Japan.[2]

In regard to chemical studies on the constituents of Rehmanniae Radix, we first reported in 1971 the isolation of catalpol (**1**) as the major iridoid glycoside from the fresh root of *R. glutinosa* LIBOSCH. forma *hueichingensis* HSIAO.[3] Since then, several chemical investigations of Japanese Rehmanniae Radix have been carried out to discover more iridoid glycosides such as aucubin, leonuride, melittoside, rehmanniosides A, B, C, and D, and various carbohydrates and amino acids.[4] However, no work on the chemical constituents of Chinese Rehmanniae Radix has been reported.[5]

In our continuing chemical studies on the processing of crude drugs,[1,11] we have compared the chemical constituents of differently processed Japanese, Chinese, and Korean Rehmanniae Radices.[9] From Chinese Rehmanniae Radix (Chinese Kan-jiō), the botanical origin of which was identified as the dried root of *R. glutinosa* LIBOSCH.,[12] we have isolated various then-new constituents, namely four iridoids designated rehmaglutins A (**3**), B (**4**),[6] C,[7] and D (**5**),[6] a chlorinated iridoid glycoside, glutinoside,[7] three ionone glucosides, rehmaionosides A, B, and C,[8] a monoterpene glucoside, rehmapicroside,[8] and jio-cerebroside, together with a phenethylalcohol glycoside, acteoside (**2**), and six known iridoid glycosides, catalpol (**1**), leonuride, monomelittoside, melittoside, rehmannioside D, and dihydrocornin.[9]

In this paper, we present a full account of the structure elucidation of rehmaglutins A (**3**), B (**4**), and D (**5**), which were isolated from the less polar fraction of the constituents of Chinese Rehmanniae Radix.[13]

After some preliminary examinations to identify optimal extraction conditions, it was found that the extraction of the dried root with 50% aqueous acetone below 25 °C seemed a promising. The extract thus obtained was subjected to fractionation and purification procedures as shown

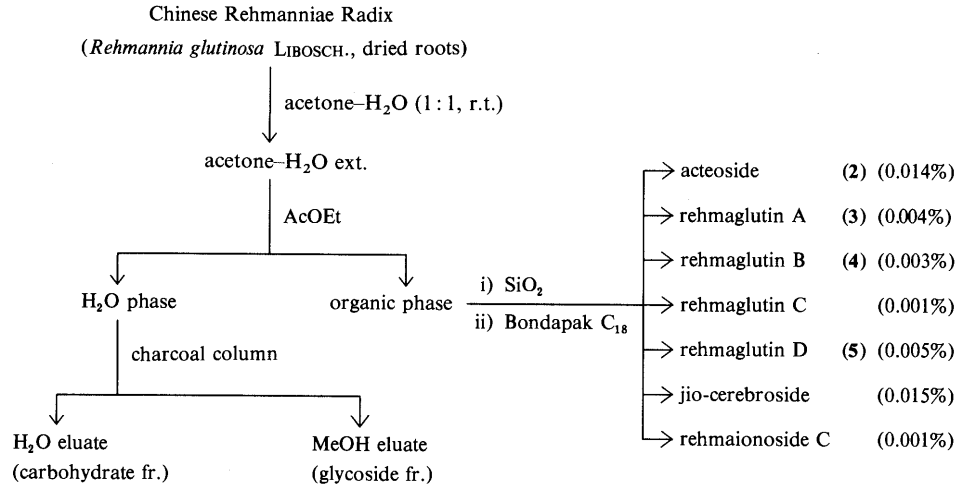


Chart 1

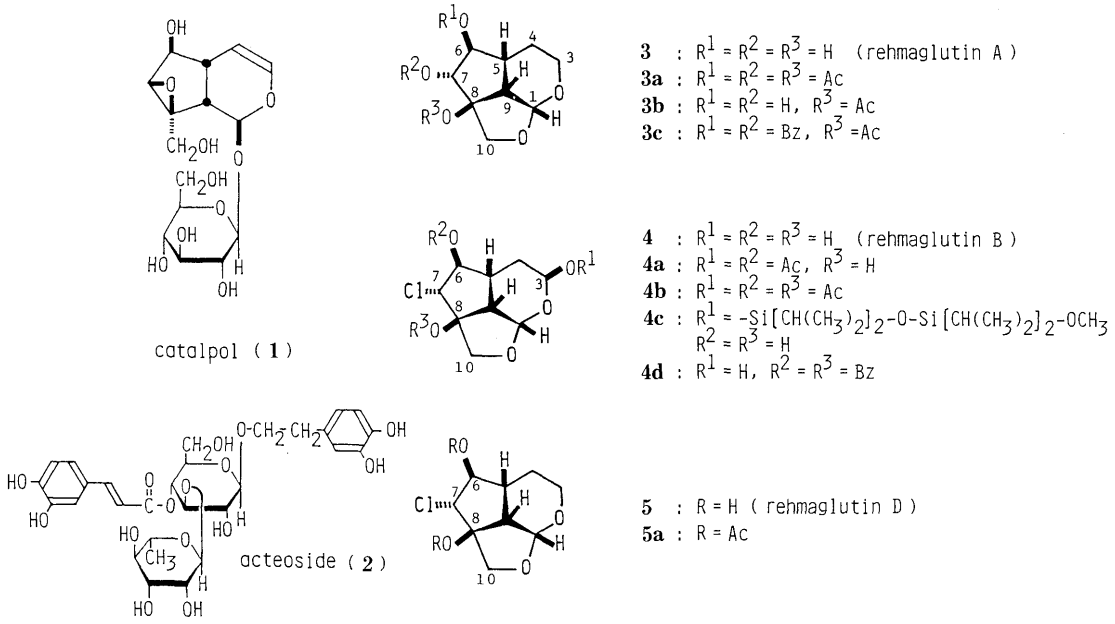


Chart 2

in Chart 1. Chromatographic purification of the ethyl acetate-soluble protion furnished acteoside (**2**),[14] rehmaglutins A (**3**), B (**4**), C,[7] and D (**5**), jio-cerebroside, and rehmaionoside C.[8] The water soluble portion, after charcoal column chromatography, provided six known iridoid glycosides as mentioned above, together with eight carbohydrates: fructose, glucose, galactose, mannitol, sucrose, manninotriose, raffinose, stachyose, and verbascose.

**Rehmaglutin A (3)** Rehmaglutin A (**3**) was obtained as colorless needles of mp 134—136 °C. The molecular formula $C_9H_{14}O_5$ was confirmed from the molecular ion peak in the mass spectrum (MS) and by high-resolution mass (high MS) measurement. The infrared (IR) spectrum of **3** showed a hydroxyl absorption band at 3450 $cm^{-1}$. The proton nuclear magnetic resonance ($^1$H-NMR) and the carbon-13 nuclear magnetic resonance ($^{13}$C-NMR) spectra of **3** showed signals ascribable to an acetal moiety [$\delta$ 5.19 (d, $J$=5 Hz); $\delta_C$ 101.0], two secondary hydroxyl groups [$\delta$ 3.79 (dd, $J$=10, 10 Hz), 3.91 (dd, $J$=1, 10 Hz); $\delta_C$ 75.4, 85.0] and a tertiary hydroxyl group [$\delta_C$ 85.2]. Acetylation of **3** with acetic anhydride and pyridine afforded the triacetate (**3a**), the $^1$H-NMR data of which were assigned as shown in Table I on the basis of detailed decoupling experiments. Comparisons of the $^1$H- and $^{13}$C-NMR data (Table II) for **3a** with those for **3** led us to presume the presence of 6-, 7-, and 8-hydroxyl groups and a 1,10-oxide ring in the tricyclic iridoid structure of **3**. Furthermore, the relative configuration of **3a** was clarified by nuclear Overhauser effect (NOE) experiments as depicted in Fig. 1 and also by comparison of the $^1$H–$^1$H coupling constants with those reported for related iridoids.[15]

In order to elucidate the absolute configuration of rehmaglutin A (**3**), the dibenzoate chirality method[16] was applied to **3c** which was prepared from **3**. Namely, **3** was first converted to the 8-*O*-acetate **3b** through the following procedures: i) silylation with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (TIPDSiCl$_2$) in pyridine to protect the 6,7-*trans* diol moiety, ii) acetylation of the 8-hydroxyl group with acetic anhydride in pyridine and dimethylaminopyridine (DMAP), and then iii) removal of the tetraisopropylsilyl group with tetra-*n*-butylammonium fluoride (*n*-Bu$_4$NF) in tetrahydrofuran (THF). The $^1$H-NMR spectrum of **3b** exhibited signals due to two hydroxy-bearing methines [$\delta$ 3.78 (dd, $J$=9, 10 Hz), 4.16 (dd, $J$=2, 9 Hz)], and an acetylation shift around C-8 was observed in the $^{13}$C-NMR spectrum of **3b** (Table II). The 8-*O*-acetate **3b** was then subjected to benzoylation with benzoyl chloride in pyridine to furnish **3c**. The $^1$H-NMR data for **3c** showed the presence of two benzoyloxy-bearing methines at C-6 and C-7 [$\delta$ 5.91 (dd, $J$=10, 10 Hz), 6.36 (br d, $J$=*ca.* 10 Hz)]. The circular dichroism (CD) spectrum of **3c** gave a split Cotton curve ([$\theta$]$_{237}$ +61600 and [$\theta$]$_{222}$ −24200), indicating the 6*S*,7*R* configurations of **3c**.

Finally, the absolute stereostructure of rehmaglutin A (**3**) was further confirmed by chemical derivation from catalpol (**1**). Thus, catalytic hydrogenation of **1** gave dihydrocatalpol (**6**)[17] which was converted to rehmaglutin A (**3**) in 15% overall yield, by alkaline treatment with 10% aqueous NaOH, cleaving the 7,8-epoxide ring to give the 7,8-diol derivative (presumably expressed as **7**), and by subsequent methanolysis of this derivative to construct the 1,10-oxide ring with concomitant removal of the glucosyloxy moiety.

**Rehmaglutin B (4)** Rehmaglutin B (**4**), obtained as colorless prisms of mp 152—153 °C, was shown to possess a chlorine atom by the positive Beilstein test. The chemical ionization mass spectrum (CI-MS) of **4** showed pairs of isotope ion peaks at *m/z* 237 (25%) and 239 (9%) due to a quasimolecular ion $(M+H)^+$ and at *m/z* 219 (100%) and 221 (33%) due to $(M+H-H_2O)^+$. The high MS measurement of **4** revealed the molecular formula to be $C_9H_{13}ClO_5$. The $^1$H- and $^{13}$C-NMR data for **4**, which resembled those for **3**, suggested the presence of two acetal moieties, two secondary alcohols and a tertiary alcohol in the tricyclic iridoid structure. Ordinary acetylation of **4** gave the 3,6-di-*O*-acetate (**4a**) and the 3,6,8-tri-*O*-acetate (**4b**).

Detailed $^1$H-NMR decoupling experiments enabled us to make complete assignments of the signals of **4a** and **4b** (Table I). In the $^1$H-NMR spectrum of **4b**, the signals of

TABLE I. $^1$H-NMR Data for Rehmaglutin Acetates[a]

| | 3a | 4a | 4b | 5a |
|---|---|---|---|---|
| H-1 | 5.34 (d, $J$=5.2) | 5.59 (d, $J$=4.9) | 5.55 (d, $J$=4.9) | 5.46 (d, $J$=5.2) |
| H-3 | 3.63 (dd, $J$=4.9, 11.9, β-H)<br>4.07 (ddd, $J$=2.4, 11.9, 12.8, α-H) | 6.41 (dd, $J$=6.4, 7.3) | 6.44 (dd, $J$=6.7, 7.9) | 3.62 (dd, $J$=5.2, 12.0, α-H)<br>4.06 (ddd, $J$=2.1, 12.0, 12.2, β-H) |
| H-4 | 1.46 (br d, $J$=*ca.* 14.6, α-H)<br>1.78 (dddd, $J$=4.9, 5.2, 12.8, 14.6, β-H) | 1.63 (ddd, $J$=4.9, 7.3, 14.7, α-H)<br>2.11 (ddd, $J$=3.8, 6.4, 14.7, β-H) | 1.58 (ddd, $J$=4.9, 7.9, 14.3, α-H)<br>2.12 (ddd, $J$=2.5, 6.7, 14.3, β-H) | 1.47 (br d, $J$=14.3, α-H)<br>1.77 (dddd, $J$=4.6, 5.2, 12.2, 14.3, β-H) |
| H-5 | 2.64 (ddd, $J$=5.2, 9.8, 11.0) | 2.47 (dddd, $J$=3.8, 4.9, 10.1, 10.5) | 2.77 (dddd, $J$=2.5, 4.9, 9.8, 10.4) | 2.56 (ddd, $J$=4.6, 9.8, 10.4) |
| H-6 | 5.44 (dd, $J$=9.5, 11.0) | 5.20 (dd, $J$=10.1, 10.1) | 5.25 (dd, $J$=9.8, 10.4) | 5.39 (dd, $J$=10.4, 10.4) |
| H-7 | 5.85 (dd, $J$=1.5, 9.5) | 4.25 (d, $J$=10.1) | 4.95 (d, $J$=9.8) | 4.81 (dd, $J$=1.5, 10.4) |
| H-9 | 2.74 (dd, $J$=5.2, 9.8) | 2.71 (dd, $J$=4.9, 10.5) | 3.16 (dd, $J$=4.9, 9.8) | 2.85 (dd, $J$=5.2, 9.8) |
| H-10 | 3.59 (dd, $J$=1.5, 10.5, β-H)<br>4.59 (d, $J$=10.5, α-H) | 3.86 (β-H), 4.34 (α-H) (both d, $J$=11.0) | 4.12 (β-H), 4.37 (α-H) (both d, $J$=11.0) | 3.74 (dd, $J$=1.5, 10.7, β-H)<br>4.61 (d, $J$=10.7, α-H) |

a) Measured at 500 MHz in $CDCl_3$. Chemical shifts are in $\delta$ and $J$ values are in Hz.

TABLE II. $^{13}$C-NMR Data for Rehmaglutins A (**3**), B (**4**), and D (**5**) and Their Derivatives (22.5 MHz, $\delta_C$)[a]

| | 3[b] | 3a[b] | 3a[c] | 3b[c] | 3c[c] | 4[b] | 4a[b] | 4a[c] | 4b[b] | 4b[c] | 5[b] | 5a[b] | 5a[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C-1 | 101.0 (d) | 100.5 | 99.1 (d) | 98.9 (d) | 99.5 (d) | 102.1 (d) | 100.6 | 98.9 (d) | 100.6 | 98.7 (d) | 101.3 (d) | 101.2 | 99.9 (d) |
| C-3 | 56.4 (t) | 56.4 | 55.5 (t) | 55.8 (t) | 56.0 (t) | 85.8 (d) | 86.2 | 85.9 (d) | 90.8 | 89.7 (d) | 56.3 (t) | 56.3 | 55.6 (t) |
| C-4 | 22.4 (t) | 22.2 | 21.1 (t) | 21.2 (t) | 21.6 (t) | 32.4 (t) | 26.7 | 25.8 (t) | 26.5 | 25.4 (t) | 22.3 (t) | 21.1 | 21.2 (t) |
| C-5 | 34.9 (d) | 33.8 | 32.5 (d) | 33.7 (d) | 33.1 (d) | 38.9 (d) | 36.4 | 35.1 (d) | 37.0 | 35.4 (d) | 37.0 (d) | 35.8 | 34.6 (d) |
| C-6 | 75.4 (d) | 74.2 | 73.1 (d) | 75.2 (d) | 73.6 (d) | 74.8 (d) | 79.0 | 78.1 (d) | 78.8 | 77.6 (d) | 73.0 (d) | 75.6 | 74.7 (d) |
| C-7 | 85.0 (d) | 79.1 | 78.0 (d) | 82.8 (d) | 78.3 (d) | 78.1 (d) | 70.1 | 67.7 (d) | 64.9 | 62.6 (d) | 75.3 (d) | 67.5 | 65.4 (d) |
| C-8 | 85.2 (s) | 89.7 | 88.6 (s) | 92.8 (s) | 88.9 (s) | 89.9 (s) | 90.8 | 89.9 (s) | 92.5 | 91.4 (s) | 85.5 (s) | 90.5 | 89.5 (s) |
| C-9 | 44.9 (d) | 42.6 | 41.3 (d) | 42.6 (d) | 41.6 (d) | 48.4 (d) | 52.8 | 51.4 (d) | 50.1 | 48.9 (d) | 46.2 (d) | 42.7 | 41.5 (d) |
| C-10 | 71.0 (t) | 68.4 | 67.5 (t) | 67.6 (t) | 67.8 (t) | 74.3 (t) | 76.6 | 76.3 (t) | 74.8 | 74.0 (t) | 76.4 (t) | 69.5 | 68.9 (t) |

a) The characterization of each carbon signal was made by INEPT (insensitive nuclei enhanced by polarization) and off-resonance experiments. b) Measured in $d_6$-acetone solution. c) Measured in $CDCl_3$ solution.

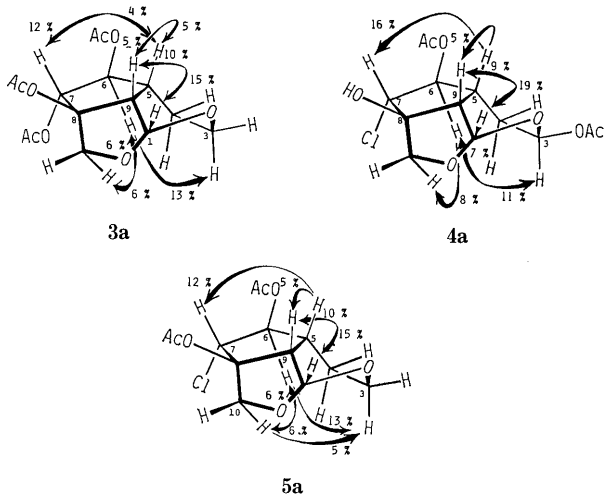


Fig. 1. NOE (%) of **3a**, **4a**, and **5a** [$^1$H-NMR (500 MHz, $CDCl_3$)]

the 7,9,10β-protons were observed with remarkable downfield shifts of 0.70, 0.45, and 0.26 ppm, respectively, as compared with those of **4a**. These downfield shifts were ascribable to the paramagnetic effect of the acetyl-carbonyl group attached to the 8-hydroxyl group which indicated a *cis*-relationship of the 7,9,10β-protons and the 8-acetoxyl group. The absolute configuration of rehmaglutin B (**4**) was determined as described for rehmaglutin A (**3**). First, the relative configuration of **4** was clarified by the NOE examinations as depicted in Fig. 1 and by comparison of $^1$H–$^1$H coupling constants. Then, the aromatic chirality method was applied. Silylation of **4** with $TIPDSiCl_2$ under the same conditions as for **3** and subsequent treatment with methanol gave an unstable 3-*O*-silylated product (**4c**) which was further subjected to bezoylation followed by desilylation finally to furnish the 6,8-di-*O*-benzoyl derivative (**4d**). The CD spectrum of **4d** showed a split Cotton curve with a small $[\theta]$ value ($[\theta]_{234}$ +50000, $[\theta]_{225}$ −10000), which indicated the presence of a long-range dibenzoate chirality in **4d**.[16]

Furthermore, the absolute stereostructure of rehmaglutin B (**4**) was substantiated by chemical correlation with catalpol (**1**). Thus, treatment of **1** with 0.6% HCl–dry methanol gave a chlorinated product (**8**)[18] formed *via* cleavage of the 7,8-epoxide ring and acetal-formation between C-3 and C-10. Subsequent hydrolysis of the product with 10% aqueous HCl provided rehmaglutin B (**4**) in 45% overall yield.

**Rehmaglutin D (5)** Rehmaglutin D (**5**) was also a chlorine-containing iridoid of mp 132—133 °C (colorless prisms) as shown from the Beilstein test and the isotope ion peaks [$m/z$ 221 (100%), 223 (33%) for $(M+H)^+$] observed in the CI-MS. Rehmaglutin D (**5**) was less polar than rehmaglutin B (**4**) and the molecular formula $C_9H_{13}ClO_4$ was determined by high-MS. The $^1$H- and $^{13}$C-NMR (Table II) spectra of **5** indicated a close similarity of its structure to those of rehmaglutins A (**3**) and B (**4**). Ordinary acetylation of **5** furnished the 6,8-di-*O*-acetate (**5a**) and the $^1$H- (Table I) and $^{13}$C- (Table II) NMR data for **5a** showed the presence of 6,8-acetoxyl and 7-chloro functions and also a 1,10-oxide moiety in its tricyclic iridoid structure. The

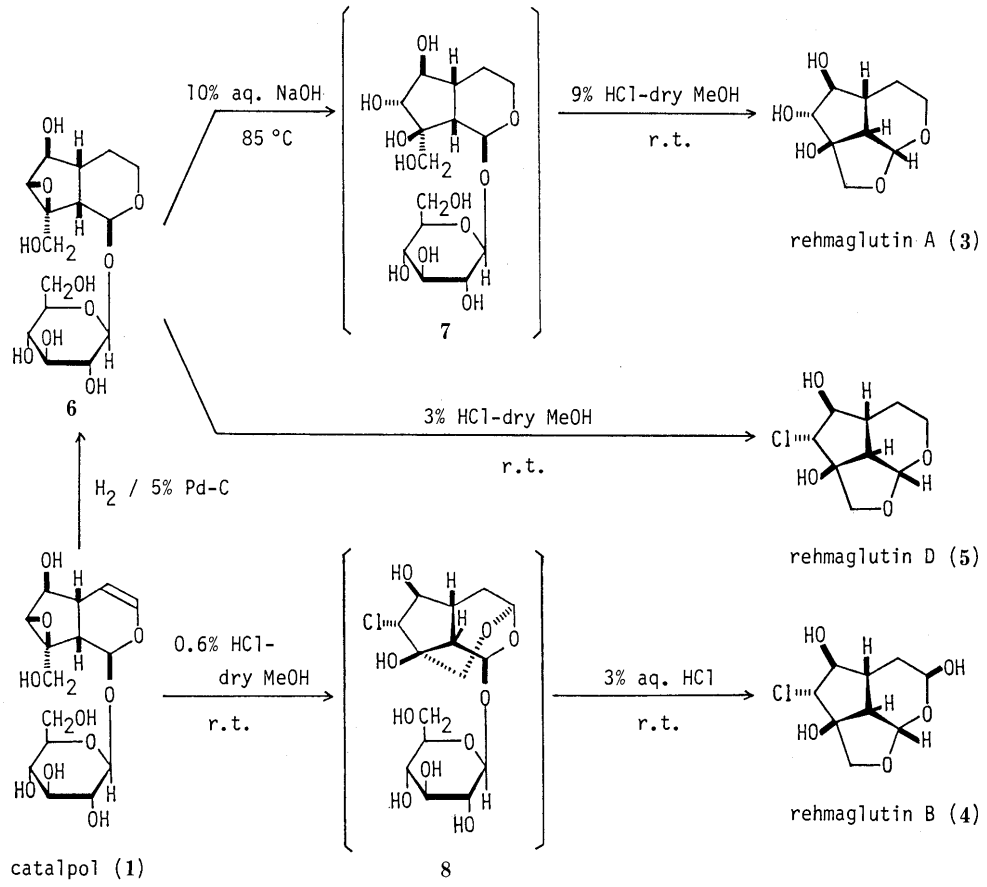


Chart 3

relative configuration of **5** was substantiated by detailed NOE examinations (Fig. 1) and comparisons of $^1H$–$^1H$ coupling constants as carried out for **3a** and **4a**. Finally, methanolysis of dihydrocatalpol (**6**) with 3% HCl in dry methanol furnished **5** in 53% yield. Thus, the absolute stereostructure of rehmaglutin D (**5**) was determined to be as shown.

## Experimental

The instruments used to obtain physical data and the experimental conditions for chromatography were the same as described in our previous paper.[1)]

**Isolation of Rehmaglutins A (3), B (4), C, and D (5), Rehmaionoside C, Jio-cerebroside, and Acteoside (2)** The air-dried roots of Chinese Rehmanniae Radix (3 kg, imported from China, purchased from Tochimoto-Tenkaido, Osaka) were cut finely and extracted with 50% aqueous acetone three times (15 l each) with occasional stirring at room temperature (below 25 °C). After removal of the organic solvent from the aqueous acetone extract under reduced pressure, the remaining aqueous solution was extracted with AcOEt. Removal of the solvent from the organic phase under reduced pressure gave the residue (10.1 g), which was fractionated by column chromatography [$SiO_2$ 200 g, $CHCl_3$–MeOH–$H_2O$ (10:3:1→7:3:1→65:35:10, using the lower phase in each case) as the eluent] to furnish four fractions. Evaporation of the solvent under reduced pressure gave fr. 1 (lipids *etc.*, 2.4 g), fr. 2 (1.8 g), fr. 3 (0.7 g), and fr. 4 (1.4 g).

Fraction 2 (1.8 g) was purified by reversed-phase silica gel column chromatography [Bondapak $C_{18}$ 200 g with $H_2O$–MeOH (3:1→2:1) gradient elution] to give rehmaglutin D (**5**, 147 mg) and jio-cerebroside (435 mg). Fraction 3 (0.7 g) was subjected successively to reversed-phase silica gel column chromatography [Bondapak $C_{18}$ 100 g, $H_2O$–MeOH (3:1)] and ordinary-phase column chromatography [$SiO_2$ 50 g, $CHCl_3$–*n*-BuOH (1:1)] to give rehmaglutins A (**3**, 108 mg), B (**4**, 87 mg), and C (27 mg). Fraction 4 (1.4 g) was purified by reversed-phase silica gel column chromatography [Bondapak $C_{18}$ 200 g, elution with $H_2O$ and $H_2O$–MeOH (10:1→2:1)] to furnish acteoside (**2**, 410 mg) and rehmaionoside C (25 mg). Acteoside (**2**) was obtained as a white powder and was identified by comparing its physical data, $[\alpha]_D^{20}$ −80° ($c$=1.1, MeOH), ultraviolet (UV), IR, secondary ion mass spectrometry (SIMS), $^1H$- and $^{13}C$-NMR, with those reported.[19)] The water-soluble portion (1.9 kg), obtained after removal of the solvent from the aqueous phase under reduced pressure, was subjected to active charcoal column chromatography [charcoal 2 kg–Celite 2 kg, elution with $H_2O$, $H_2O$–MeOH (1:1), and then MeOH] to give a glycoside mixture (43 g). The procedure for separation of the glycoside mixture will be reported in detail in our forthcoming paper.

Rehmaglutin A (**3**): mp 134—136 °C (colorless needles from MeOH), $[\alpha]_D^{19}$ +43.6° ($c$=0.28, MeOH). High MS: Found 202.085. Calcd for $C_9H_{14}O_5$ ($M^+$) 202.084. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3450, 2950, 1035. $^1H$-NMR (90 MHz, $d_6$-acetone) $\delta$: 1.48—1.61 (2H, m, 4-$H_2$), 2.00—2.18 (2H, m, 5, 9-H), 3.22 (1H, dd, $J$=1, 10 Hz, 10$\beta$-H), 3.60—3.82 (2H, m, 3-$H_2$), 3.79 (1H, dd, $J$=10, 10 Hz, 6-H), 3.91 (1H, dd, $J$=1, 10 Hz, 7-H), 4.35 (1H, d, $J$=10 Hz, 10$\alpha$-H), 5.19 (1H, d, $J$=5 Hz, 1-H). $^{13}C$-NMR: see Table II. CI-MS $m/z$ (%): 203 [$(M+H)^+$, 98], 185 [$(M+H-H_2O)^+$, 100], 167 (45).

Rehmaglutin B (**4**): mp 152—153 °C (colorless prisms from MeOH), $[\alpha]_D^{19}$ +33.8° ($c$=0.79, MeOH). High MS: Found 236.035. Calcd for $C_9H_{13}{}^{35}ClO_5$ ($M^+$) 236.035. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3280, 2920, 1049, 1031. $^1H$-NMR (90 MHz, $d_6$-acetone) $\delta$: 1.38—1.63 (1H, m, 4$\alpha$-H), 1.92—2.08 (1H, m, 4$\beta$-H), 2.23—2.37 (1H, m, 5-H), 2.43 (1H, dd, $J$=5, 10 Hz, 9-H), 3.42 (1H, dd, $J$=1, 10 Hz, 10$\beta$-H), 3.81 (1H, dd, $J$=9, 10 Hz, 6-H), 4.12 (1H, dd, $J$=1, 10 Hz, 7-H), 4.21 (1H, d, $J$=10 Hz, 10$\alpha$-H), 5.25 (1H, dd, $J$=4, 9 Hz, 3-H), 5.48 (1H, d, $J$=5 Hz, 1-H). $^{13}C$-NMR: see Table II. CI-MS $m/z$ (%): 239 (9), 237 (25) [$(M+H)^+$], 221 (34), 219 (100) [$(M+H-H_2O)^+$].

Rehmaglutin D (**10**): mp 132—133 °C (colorless prisms from $Et_2O$), $[\alpha]_D^{19}$ +60.6° ($c$=0.19, MeOH). High MS: Found 220.051. Calcd for $C_9H_{13}{}^{35}ClO_4$ ($M^+$) 220.050. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3400, 2920, 1045, 1025. $^1H$-NMR (90 MHz, $d_6$-acetone) $\delta$: 1.62—1.81 (2H, m, 4-$H_2$), 2.10—2.30 (1H, m, 5-H), 2.35 (1H, dd, $J$=5, 10 Hz, 9-H), 3.43 (1H, dd, $J$=2, 10 Hz, 10$\beta$-H), 3.60—4.00 (2H, m, 3-$H_2$), 3.81 (1H, dd, $J$=9, 10 Hz, 6-H), 4.18 (1H, dd, $J$=2, 10 Hz, 7-H), 4.36 (1H, d, $J$=10 Hz, 10$\alpha$-H), 5.28 (1H, d, $J$=5 Hz, 1-H). $^{13}C$-NMR: see Table II. CI-MS $m/z$ (%): 223 (33), 221 (100) [$(M+H)^+$], 205 (7), 207 (18) [$(M+H-H_2O)^+$], 187 (2), 185 (5).

SIMS *m/z* (%): 223 (20), 221 (66); 205 (10), 203 (31); 169 (10), 167 (30); 55 (100).

**Acetylation of Rehmaglutin A (3)** A solution of **3** (12 mg) in pyridine (1.0 ml) was treated with $Ac_2O$ (1.0 ml) and the mixture was stirred at room temperature (20 °C) for 8 h, then poured into ice-water. The whole was extracted with AcOEt. The AcOEt extract was washed with diluted aqueous HCl, water, aqueous saturated $NaHCO_3$, and brine, and then dried over $MgSO_4$. After removal of the solvent from the AcOEt extract under reduced pressure, the product was purified by column chromatography [$SiO_2$ 4 g, *n*-hexane–AcOEt (2:1)] to furnish the triacetate (**3a**, 19 mg).

**3a**: mp 128—130 °C (colorless needles from $Et_2O$), $[\alpha]_D^{19}$ +3.6° (*c*=0.35, MeOH). High MS: Found 328.117. Calcd for $C_{15}H_{28}O_8$ ($M^+$) 328.116. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 1740, 1240, 1035. $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$: 2.04, 2.06, 2.10 (3H each, all s, OAc × 3), and other signals as given in Table I. NOE (%): as shown in Fig. 1. $^{13}$C-NMR $\delta_C$: ($d_6$-acetone) 21.1 (2C), 21.8, 171.0, 171.3, 171.6; ($CDCl_3$) 20.8 (2C), 21.3, 170.2 (2C), 170.7, and other data as given in Table II. CI-MS *m/z* (%): 329 [(M+H)$^+$, 1], 269 [(M+H−AcOH)$^+$, 100], 209 (49).

**Conversion of 3 to 3b** A solution of **3** (14 mg) in pyridine (1.5 ml) was treated with $TIPDSiCl_2$ (30 mg) and the mixture was stirred at room temperature (20 °C) under an $N_2$ atmosphere for 5 h, then poured into ice-water. The whole was extracted with AcOEt. The AcOEt extract was washed successively with 2N HCl, aqueous saturated $NaHCO_3$, and brine, then dried over $MgSO_4$. Removal of the solvent under reduced pressure gave a product, which was dissolved in pyridine (1.0 ml), and the solution was treated with $Ac_2O$ (1.5 ml) and DMAP (a catalytic amount). The reaction mixture was stirred at room temperature (20 °C) under an $N_2$ atmosphere for 2 h and then poured into ice-water. The whole was extracted with AcOEt and the AcOEt extract was worked up in the same manner as described above to give the product, which was dissolved in THF (2.0 ml). The solution was treated with *n*-$Bu_4NF$ (104 mg) and the mixture was stirred at room temperature (20 °C) under an $N_2$ atmosphere for 2 h. The reaction mixture was poured into ice-water and the whole was extracted with AcOEt. The AcOEt extract was washed with aqueous saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Removal of the solvent under reduced pressure gave a product, which was purified by column chromatography [$SiO_2$ 1 g, *n*-hexane–acetone (2:1)] to furnish **3b** (9 mg).

**3b**: mp 134—136 °C (colorless prisms from MeOH), $[\alpha]_D^{20}$ +12.4° (*c*=0.34, MeOH). High MS: Found 244.095. Calcd for $C_{11}H_{16}O_6$ ($M^+$) 244.095. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3460, 1718, 1245, 1037. $^1$H-NMR (90 MHz, $d_6$-acetone) $\delta$: 1.58—1.73 (2H, m, 4-$H_2$), 2.02 (3H, s, OAc), 2.17—2.32 (1H, m, 5-H), 2.61 (1H, dd, *J*=6, 10 Hz, 9-H), 3.35—3.54 (1H, m, 3$\beta$-H), 3.48 (1H, dd, *J*=2, 10 Hz, 10$\beta$-H), 3.72—3.90 (1H, m, 3$\alpha$-H), 3.78 (1H, dd, *J*=9, 10 Hz, 6-H), 4.16 (1H, dd, *J*=2, 9 Hz, 7-H), 4.51 (1H, d, *J*=10 Hz, 10$\alpha$-H), 5.35 (1H, d, *J*=6 Hz, 1-H). $^{13}$C-NMR ($CDCl_3$) $\delta_C$: 20.9, 173.0, and others as given in Table II. CI-MS *m/z* (%): 245 [(M+H)$^+$, 4], 227 [(M+H−$H_2O$)$^+$, 3], 185 [(M+H−AcOH)$^+$, 100].

**Benzoylation of 3b Giving 3c** A solution of **3b** (9 mg) in pyridine (1.5 ml) was treated with benzoyl chloride (0.05 ml) and the mixture was stirred at room temperature (20 °C) for 3 h, then poured into ice-water. The whole was extracted with AcOEt. After work-up of the AcOEt extract in the usual manner, the product was purified by column chromatography [$SiO_2$ 1 g, *n*-hexane–AcOEt (4:1)] to furnish **3c** (11 mg).

**3c**: A colorless oil, $[\alpha]_D^{20}$ +53.2° (*c*=0.59, MeOH). High MS: Found 453.157. Calcd for $C_{25}H_{25}O_8$ (M+H)$^+$ 453.156. UV $\lambda_{max}^{MeOH}$ nm ($\varepsilon$): 229 (21000). CD (MeOH): $[\theta]_{237\,nm}$ +61600 (pos. max.), $[\theta]_{222\,nm}$ −24200 (neg. max.). IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 1720, 1600, 1278, 1091. $^1$H-NMR (90 MHz, $CDCl_3$) $\delta$: 1.58—1.72 (2H, m, 4-$H_2$), 2.10 (3H, s, OAc), 2.80—3.00 (2H, m, 5, 9-H), 3.60—3.80 (1H, m, 3$\beta$-H), 3.67 (1H, dd, *J*=1, 11 Hz, 10$\beta$-H), 4.09—4.41 (1H, m, 3$\alpha$-H), 4.73 (1H, d, *J*=11 Hz, 10$\alpha$-H), 5.42 (1H, d, *J*=4 Hz, 1-H), 5.91 (1H, dd, *J*=10, 10 Hz, 6-H), 6.36 (1H, br d, *J*=*ca.* 10 Hz, 7-H), 7.30—7.60 (6H), 7.95—8.09 (4H) (both m, aromatic protons). $^{13}$C-NMR ($CDCl_3$) $\delta_C$: 21.5, 128.5 (4C), 128.6 (4C), 130.0 (2C), 133.4 (2C), 165.8 (2C), 170.6, and others as given in Table II. CI-MS *m/z* (%): 453 [(M+H)$^+$, 2] 393 [(M+H−AcOH)$^+$, 100], 271 (74).

**Conversion of Catalpol (1) to Rehmaglutin A (3)** A solution of catalpol (**1**, 475 mg) in MeOH (15 ml) containing 5% palladium–carbon (252 mg) was stirred at room temperature under a hydrogen atmosphere (3 kg/cm$^2$) for 40 min. After removal of the catalyst by filtration, the solvent was evaporated off from the filtrate under reduced pressure to yield dihydrocatalpol (**6**, 478 mg) which was identical with an authentic sample[17] as judged from TLC [$CHCl_3$–MeOH–$H_2O$ (65:35:10, lower phase), *n*-BuOH–AcOH–$H_2O$ (4:1:5, upper phase)], IR (KBr), $^1$H- (pyridine-$d_5$) and $^{13}$C- (pyridine-$d_5$) NMR comparisons. A solution of **6** (36 mg) in $H_2O$ (1.0 ml) was treated with 20% aqueous NaOH (1.0 ml) and the mixture was stirred at 85 °C under an $N_2$ atmosphere for 2 h, then neutralized with Dowex 50W × 8 ($H^+$ form). The resin was removed by filtration. After removal of the solvent from the filtrate under reduced pressure, the product (crude **7**) was dissolved in 9% HCl–dry MeOH (1.0 ml) and the solution was stirred at room temperature (27 °C) for 30 min, then neutralized with $Ag_2CO_3$ and filtered. Removal of the solvent from the filtrate gave a product (31 mg), which was purified by column chromatography [$SiO_2$ 10 g, $CHCl_3$–MeOH–$H_2O$ (7:3:1, lower phase)] to furnish **3** (3 mg). **3** thus obtained was shown to be identical with authentic rehmaglutin A, which was isolated previously from Rehmanniae Radix, by TLC [$CHCl_3$–MeOH–$H_2O$ (7:3:1, lower phase), $CHCl_3$–MeOH (10:1), benzene–MeOH (3:1)], IR (KBr), and $^1$H-NMR ($d_6$-acetone) comparisons.

**Acetylation of Rehmaglutin B (4) Giving 4a and 4b** A solution of **4** (12 mg) in pyridine (1.0 ml) was treated with $Ac_2O$ (1.0 ml) and the whole was stirred at room temperature (22 °C) for 4 h. The reaction mixture was poured into ice-water and the whole was extracted with AcOEt. Work-up of the AcOEt extract in the usual manner gave the product, which was purified by column chromatography [$SiO_2$ 1 g, benzene–acetone (5:1)] to furnish **4a** (13 mg) and **4b** (2 mg).

**4a**: mp 147—148 °C (colorless needles from $Et_2O$), $[\alpha]_D^{19}$ +61.9° (*c*=0.54, $CHCl_3$). High MS: Found 320.064. Calcd for $C_{13}H_{17}{}^{35}ClO_7$ ($M^+$) 320.066. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3300, 1740, 1235, 1034. $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$: 2.07, 2.14 (3H, each, both s, OAc × 2), and others as given in Table I. NOE (%): as shown in Fig. 1. $^{13}$C-NMR $\delta_C$: ($d_6$-acetone) 21.1, 21.5, 170.2, 170.6; ($CDCl_3$) 20.8, 21.1, 169.5, 170.4, and others as given in Table II. CI-MS *m/z* (%): 323 (0.3), 321 (1) [(M+H)$^+$], 263 (35), 261 (100) [(M+H−AcOH)$^+$].

**4b**: A colorless oil, $[\alpha]_D^{19}$ +40.6° (*c*=0.72, $CHCl_3$). High MS: Found 362.077. Calcd for $C_{15}H_{19}{}^{35}ClO_8$ ($M^+$) 362.077. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 2928, 1735, 1230, 1036. $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$: 2.07, 2.12, 2.14 (3H each, all s, OAc × 3) and others as given in Table I. $^{13}$C-NMR $\delta_C$: ($d_6$-acetone) 21.1, 21.5, 22.2, 170.2, 171.5, 172.0; ($CDCl_3$) 20.4, 20.9, 21.6, 169.1, 170.1, 170.8, and others as given in Table II. CI-MS *m/z* (%): 365 (0.3), 363 (1) [(M+H)]$^+$], 305 (16), 303 (45) [(M+H−AcOH)$^+$], 245 (20), 243 (54) [(M+H−2AcOH)$^+$], 185 (34), 183 (100) [(M+H−3AcOH)$^+$].

**Preparation of 4d from 4** A solution of **4** (7 mg) in pyridine (1.5 ml) was treated with $TIPDSiCl_2$ (22 mg) and the mixture was stirred at room temperature (21 °C) under an $N_2$ atmosphere for 7 h, then treated with MeOH (2 ml). The reaction mixture was left to stand for 15 min, and poured into ice-water and the whole was extracted with AcOEt. The AcOEt extract was washed successively with 2N HCl, aqueous saturated $NaHCO_3$, and brine, then dried over $MgSO_4$. Evaporation of the solvent from the AcOEt extract under reduced pressure gave the product, which was purified by column chromatography [$SiO_2$ 1 g, benzene–acetone (8:1)] to furnish **4c** (9 mg).

**4c**: A colorless oil, $[\alpha]_D^{20}$ +32.1° (*c*=0.41, MeOH). IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3300, 2930, 1037. $^1$H-NMR (90 MHz, $CDCl_3$) $\delta$: 1.04—1.07 (24H, m, *sec*-$CH_3$ × 8), 3.51 (1H, dd, *J*=1, 10 Hz, 10$\beta$-H), 3.56 (3H, s, $OCH_3$), 3.77 (1H, dd, *J*=9, 10 Hz, 6-H), 4.08 (1H, dd, *J*=1, 10 Hz, 7-H), 4.19 (1H, d, *J*=10 Hz, 10$\alpha$-H), 5.48 (1H, d, *J*=4, 1-H), 5.49 (1H, dd, *J*=4, 8 Hz, 3-H). CI-MS *m/z* (%): 513 (2), 515 (5) [(M+H)$^+$], 222 (35), 220 (100). A solution of **4c** (9 mg) in pyridine (1.0 ml) was treated with benzoyl chloride (0.03 ml) and DMAP (a catalytic amount) and the mixture was stirred at room temperature (21 °C) under an $N_2$ atmosphere for 14 h, then poured into ice-water. The whole was extracted with AcOEt. After work-up of the AcOEt extract in the usual manner, the product was dissolved in THF (1.0 ml). This solution was treated with *n*-$Bu_4NF$ (28 mg), then the mixture was stirred at room temperature (20 °C) under an $N_2$ atmosphere for 1 h, and poured into ice-water. The whole was extracted with AcOEt. Work-up of the AcOEt extract in the usual manner gave the product which was purified by column chromatography [$SiO_2$ 1 g, *n*-hexane–AcOEt (2:1)] to furnish **4d** (6 mg).

**4d**: A colorless oil, $[\alpha]_D^{20}$ +14.6° (*c*=0.15, MeOH). High MS: Found 445.106. Calcd for $C_{23}H_{22}{}^{35}ClO_7$ (M+H)$^+$ 445.106. UV $\lambda_{max}^{MeOH}$ nm ($\varepsilon$): 231 (19500). CD (MeOH): $[\theta]_{238\,nm}$ +50000 (pos. max.), $[\theta]_{225\,nm}$ −10000 (neg. max.). $^1$H-NMR (90 MHz, $d_6$-acetone) $\delta$: 4.19 (1H, d, *J*=10 Hz, 10$\beta$-H), 4.56 (1H, d, *J*=10 Hz, 10$\alpha$-H), 4.98 (1H, dd, *J*=8, 8 Hz, 3-H), 5.35 (1H, d, *J*=10 Hz, 7-H), 5.65 (1H, dd, *J*=10, 10 Hz, 6-H), 5.75 (1H, d, *J*=5 Hz, 1-H), 7.55—7.70 (6H), 8.00—8.18 (4H) (both m, aromatic protons). CI-MS *m/z* (%): 447 (2), 445 (5) [(M+H)$^+$], 325 (16), 323 (49) [(M+H−BzOH)$^+$], 123 (100).

**Conversion of Catalpol (1) to Rehmaglutin B (4)** A solution of **1** (105 mg) in MeOH (14 ml) was treated with 9% HCl–dry MeOH (1.0 ml) and the

reaction mixture was stirred at room temperature (19 °C) for 14 h, then neutralized with Dowex 1 × 2 ($OH^-$ form) and filtered. Removal of the solvent from the filtrate under reduced pressure gave the product (crude **8**), which was dissolved in 3% aqueous HCl (5.0 ml). The solution was stirred at room temperature (19 °C) for 24 h, then neutralized with Dowex 1 × 2 ($OH^-$ form), and filtered. After removal of the solvent from the filtrate under reduced pressure, the product was purified by column chromatography [$SiO_2$ 10 g, $CHCl_3$–MeOH (10 : 1)] to furnish **4** (31 mg). **4** thus obtained was shown to be identical with authentic rehmaglutin B, which was isolated above from Rehmanniae Radix, by TLC [$CHCl_3$–MeOH–$H_2O$ (7 : 3 : 1, lower phase), $CHCl_3$–MeOH (10 : 1), benzene–acetone (2 : 1)], IR (KBr), and $^1$H-NMR ($d_6$-acetone) comparisons.

**Acetylation of Rehmaglutin D (5)** A solution of **5** (12 mg) in pyridine (1.0 ml) was treated with $Ac_2O$ (1.0 ml) and the mixture was stirred at room temperature (21 °C) for 3 h, then poured into ice-water. The whole was extracted with AcOEt. Work-up of the AcOEt extract in the usual manner gave the product, which was purified by column chromatography [$SiO_2$ 1 g, *n*-hexane–AcOEt (2 : 1)] to furnish **5a** (16 mg).

**5a**: mp 96—97 °C (colorless prisms from $Et_2O$), $[\alpha]_D^{13}$ +28.0° ($c$ = 0.48, MeOH). High MS: Found 304.071. Calcd for $C_{13}H_{17}{}^{35}ClO_6$ ($M^+$) 304.071. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 1733, 1235, 1039. $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$: 2.10, 2.13 (3H each, both s, OAc × 2), and others as given in Table I. NOE (%): as shown in Fig. 1. $^{13}$C-NMR $\delta_C$: ($d_6$-acetone) 22.1, 22.2, 171.3 (2C); ($CDCl_3$) 20.8, 21.7, 170.3, 170.4, and others as given in Table II. CI-MS $m/z$ (%): 307 (1), 305 (5) [$(M+H)^+$], 247 (13), 245 (40) [$(M+H-AcOH)^+$], 187 (32), 185 (100) [$(M+H-2AcOH)^+$].

**Conversion of Dihydrocatalpol (6) to Rehmaglutin D (5)** A solution of **6** (231 mg) in dry MeOH (2.0 ml) was treated with 9% HCl–dry MeOH (1.0 ml) and the mixture was stirred at 34 °C for 3 h, then neutralized with Dowex 1 × 2 ($OH^-$ form), and filtered. After removal of the solvent from the filtrate, the product was purified by column chromatography [$SiO_2$, 50 g, benzene–acetone (2 : 1)] to furnish **5** (74 mg). **5** thus obtained was shown to be identical with authentic rehmaglutin D, isolated above from Rehmanniae Radix, by mixed melting point determination, and by $[\alpha]_D^{20}$ (+60.0°), TLC [$CHCl_3$–MeOH–$H_2O$ (10 : 3 : 1, lower phase), $CHCl_3$–MeOH (10 : 1), benzene–acetone (2 : 1)], IR (KBr), and $^1$H-NMR ($d_6$-acetone) comparisons.

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# Marine Natural Products. XXVI.[1] Biologically Active Tridecapeptide Lactones from the Okinawan Marine Sponge *Theonella swinhoei* (Theonellidae). (2). Structures of Theonellapeptolides Ia, Ib, Ic, and Ie

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**Five tridecapeptide lactones, named theonellapeptolides Ia (1), Ib (2), Ic (3), Id (4), and Ie (5), were isolated from the Okinawan marine sponge *Theonella swinhoei*. Following the structure elucidation of theonellapeptolide Id (4), the structures of theonellapeptolides Ia (1), Ib (2), Ic (3), and Ie (5) were determined on the basis of chemical and physicochemical evidence including high performance liquid chromatography and circular dichroism combined analysis of the amino acid compositions. Theonellapeptolides Ib (2), Ic (3), Id (4), and Ie (5) exhibit moderate cytotoxic activity towards for L1210 *in vitro* ($IC_{50}$ 1.6, 1.3, 2.4, and 1.4 μg/ml, respectively), and theonellapeptolide Ie (5) exhibits ion-transport activities for $Na^+$ and $K^+$ ions.**

**Keywords** *Theonella swinhoei*; marine sponge; theonellapeptolide Ia; theonellapeptolide Ib; theonellapeptolide Ic; theonellapeptolide Ie; peptide lactone; D-amino acid; *N*-methyl amino acid; cytotoxicity

In the preceding papers, we reported the isolation of five peptide lactones, theonellapeptolides Ia, Ib, Ic, Id, and Ie, together with a significantly cytotoxic dimeric macrolide, swinholide A,[2] from the Okinawan marine sponge *Theonella swinhoei* (Theonellidae) and the structure elucidation of the major peptide lactone, theonellapeptolide Id (**4**).[1,3] These peptide lactones characteristically inhibit the development of the fertilized eggs of the sea urchin *Hemicentrotus pulcherrimus*. In a continuing study, we have elucidated the structures of the remaining minor peptide lactones, theonellapeptolides Ia (**1**), Ib (**2**), Ic (**3**), and Ie (**5**),[4] by using a newly developed analysis method for amino acid composition in which high-performance liquid chromatography (HPLC) and circular dichroism (CD) analysis are combined.[1,3,5] In this paper, we present a full account of the structure elucidation of those minor theonellapeptolides.[6]

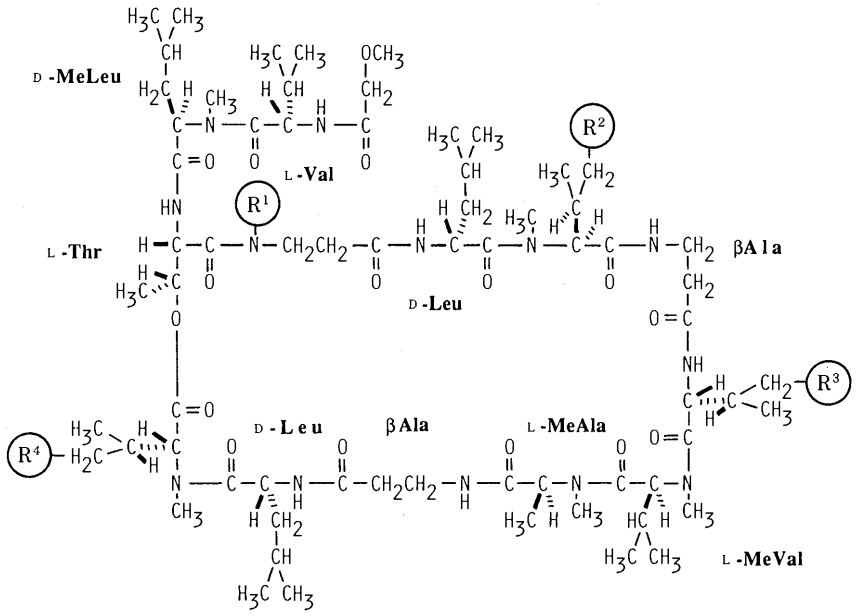
Theonellapeptolides Ia (**1**), Ib (**2**), Ic (**3**), and Ie (**5**) move with the same *Rf* value on a thin-layer chromatogram (TLC) as theonellapeptolide Id (**4**) and are also characterized by their lipophilic solubility, as is Id (**4**). The fast atom bombardment mass spectra (FAB-MS) of theonellapeptolides Ia (**1**), Ib (**2**), Ic (**3**), and Ie (**5**) showed quasimolecular $(M+H)^+$ ion peaks at *m/z* 1390, 1390, 1390, and 1418, respectively, whereas the $(M+H)^+$ ion peak of theonellapeptolide Id (**4**) was observed at *m/z* 1404. The infrared (IR) spectra of these theonellapeptolides showed the presence of amide groups and a lactone group. The proton nuclear magnetic resonance ($^1$H-NMR) spectra of theonellapeptolides Ia (**1**), Ib (**2**), and Ic (**3**) showed signals due to eight amide protons, five *N*-methyl groups, and a methoxyacetyl group, while that of theonellapeptolide Ie (**5**) showed signals due to seven amide protons, six *N*-methyl groups, and a methoxyacetyl group. The carbon-13 nuclear magnetic resonance ($^{13}$C-NMR) spectra of these four theonellapeptolides, which closely resembled that of theonellapeptolide Id (**4**), showed signals ascribable to thirteen amide carbons, a lactone carbonyl, a β-carbon in a threonine moiety ($\delta_C$ 70.7 for **1**, **2**, and **3** and $\delta_C$ 71.6 for **5**), and a methoxyacetyl group [$\delta_C$ 60.4 (q), 73.2 (t)].

Theonellapeptolides Ia (**1**), Ib (**2**), Ic (**3**), and Ie (**5**) were negative to the ninhydrin test and were unaffected by diazomethane treatment. Thus, it was presumed that theonellapeptolides Ia (**1**), Ib (**2**), Ic (**3**), and Ie (**5**) are tridecapeptide lactones in which the N-terminal is protected with a methoxyacetyl group and the C-terminal is connected through a lactone linkage to the β-hydroxyl group of threonine (Thr), as was found in theonellapeptolide Id (**4**). In order to clarify the amino acid compositions, theonellapeptolides Ia (**1**), Ib (**2**), Ic (**3**), and Ie (**5**) were subjected to complete hydrolysis with 6 N aqueous HCl by heating at 110 °C for 24 h in a sealed tube. The respective hydrolysates were then analyzed by HPLC as described in preceding papers[1,4]: the absolute configurations of component amino acids were determined by measuring the CD spectrum (taken in 0.5 N aqueous HCl) of each amino acid[7] eluted from the HPLC column. As summarized in Table I, theonellapeptolides Ia (**1**), Ib (**2**), Ic (**3**), and Ie (**5**) were found to have very similar amino acid compositions to that of theonellapeptolide Id (**4**).

The evidence described above has led us to presume that i) theonellapeptolide Ia (**1**) has a structure in which D-alloisoleucine (D-aIle) in theonellapeptolide Id (**4**) is replaced with D-valine (D-Val), ii) theonellapeptolide Ib (**2**) has a structure in which *N*-methyl-L-isoleucine (L-MeIle) in **4** is replaced with *N*-methyl-L-valine (L-MeVal), iii) theonellapeptolide Ic (**3**) has a structure in which *N*-methyl-D-alloisoleucine (D-MeaIle) in **4** is replaced with

TABLE I. Amino Acid Compositions of Theonellapeptolides Ia (**1**), Ib (**2**), Ic (**3**), Id (**4**), and Ie (**5**)

| Amino acid | Ia (1) Relative ratio | CD | Ib (2) Relative ratio | CD | Ic (3) Relative ratio | CD | Id (4) Relative ratio | CD | Ie (5) Relative ratio | CD |
|---|---|---|---|---|---|---|---|---|---|---|
| βAla | 3 | | 3 | | 3 | | 3 | | 2 | |
| L-Thr | 1 | + | 1 | + | 1 | + | 1 | + | 1 | + |
| MeβAla | 0 | | 0 | | 0 | | 0 | | 1 | |
| L-MeAla | 1 | + | 1 | + | 1 | + | 1 | + | 1 | + |
| L-Val | 1 | + | 1 | + | 1 | + | 1 | + | 1 | + |
| D-Val | 1 | − | 0 | | 0 | | 0 | | 0 | |
| L-MeVal | 1 | + | 2 | + | 1 | + | 1 | + | 1 | + |
| D-MeVal | 0 | | 0 | | 1 | − | 0 | | 0 | |
| D-aIle | 0 | | 1 | − | 1 | − | 1 | − | 1 | − |
| D-Leu | 2 | − | 2 | − | 2 | − | 2 | − | 2 | − |
| D-MeaIle | 1 | − | 1 | − | 0 | | 1 | − | 1 | − |
| L-MeIle | 1 | + | 0 | | 1 | + | 1 | + | 1 | + |
| D-MeLeu | 1 | − | 1 | − | 1 | − | 1 | − | 1 | − |




| | R¹ | R² | R³ | R⁴ |
|---|---|---|---|---|
| theonellapeptolide Ia (1) | H βAla | $CH_3$ L-Melle | H D-Val | $CH_3$ D-Mealle |
| theonellapeptolide Ib (2) | H βAla | H L-MeVal | $CH_3$ D-alle | $CH_3$ D-Mealle |
| theonellapeptolide Ic (3) | H βAla | $CH_3$ L-Melle | $CH_3$ D-alle | H D-MeVal |
| theonellapeptolide Id (4) | H βAla | $CH_3$ L-Melle | $CH_3$ D-alle | $CH_3$ D-Mealle |
| theonellapeptolide Ie (5) | $CH_3$ MeβAla | $CH_3$ L-Melle | $CH_3$ D-alle | $CH_3$ D-Mealle |

Chart 1

*N*-methyl-D-valine (D-MeVal), and iv) theonellapeptolide Ie (**5**) has a structure in which one of the three *β*-alanine (*β*Ala) moieties in **4** is replaced with *N*-methyl-*β*-alanine (Me*β*Ala).

In order to verify these presumptions, theonellapeptolides Ia (**1**), Ib (**2**), Ic (**3**), and Ie (**5**) were each subjected to partial acidic hydrolysis with 30% aqueous trifluoroacetic acid (TFA) at 110 °C for 40 min in a sealed tube, and the resulting hydrolysates were preparatively separated by HPLC (Zorbax ODS). The amino acid sequences of these hydrolysates were then examined by the combination of HPLC-CD analysis for amino acids,[1,4] N-terminal analysis by means of dansyl derivation, and FAB-MS analysis. The structures of theonellapeptolides Ia (**1**), Ib (**2**), Ic (**3**), and Ie (**5**) have been determined by examination of the partial hydrolysates as described below.

Theonellapeptolide Ia (**1**) comprises thirteen amino acids (molar ratios given in parentheses): *β*Ala (3), L-threonine (L-Thr) (1), *N*-methyl-L-alanine (L-MeAla) (1), L-Val (1), D-Val (1), L-MeVal (1), D-leucine (D-Leu) (2), D-MeIle (1), L-MeIle (1), and *N*-methyl-D-leucine (D-MeLeu) (1). Thirteen partial hydrolysates: fr. a2 (**16**), fr. a3 (**9**), fr. a4 (**17**), fr. a8 (**18**), fr. a11 (**10**), fr. a15 (**6**), fr. a16 (**11**), fr. a18 (**7**), fr. a21 (**12**), fr. a23 (**13**), fr. a26 (**14**), fr. a41 (**20**), and fr. a44 (**21**), which were obtained by mild acidic hydrolysis of **1**, were utilized to construct the structure of theonellapeptolide Ia (**1**) as shown in Charts 2 and 3.

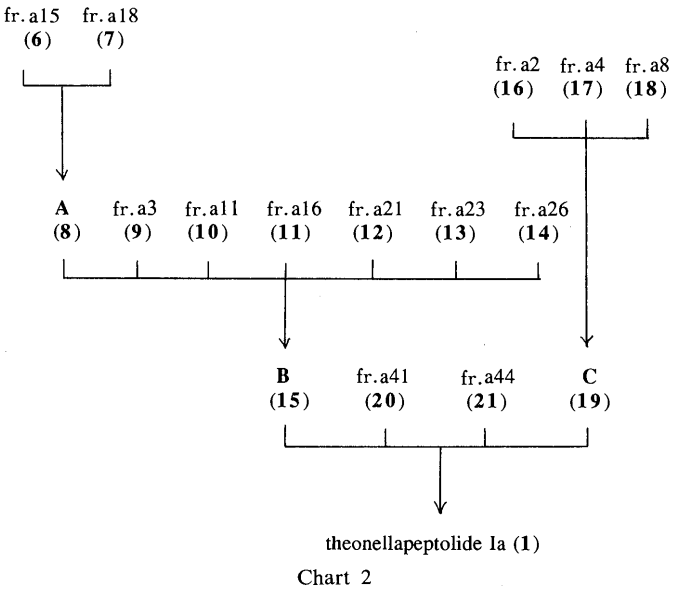



Chart 2

The combination of the elucidated structures for fr. a2 (**16**), fr. a4 (**17**), and fr. a8 (**18**) disclosed the amino acid sequence in a partial structure **C** (**19**). On the other hand, comparison of fr. a15 (**6**) and fr. a18 (**7**) showed the presence of a partial structure **A** (**8**) in **1**. The comparative analysis of the partial structure **A** (**8**), fr. a3 (**9**), fr. a11 (**10**), fr.

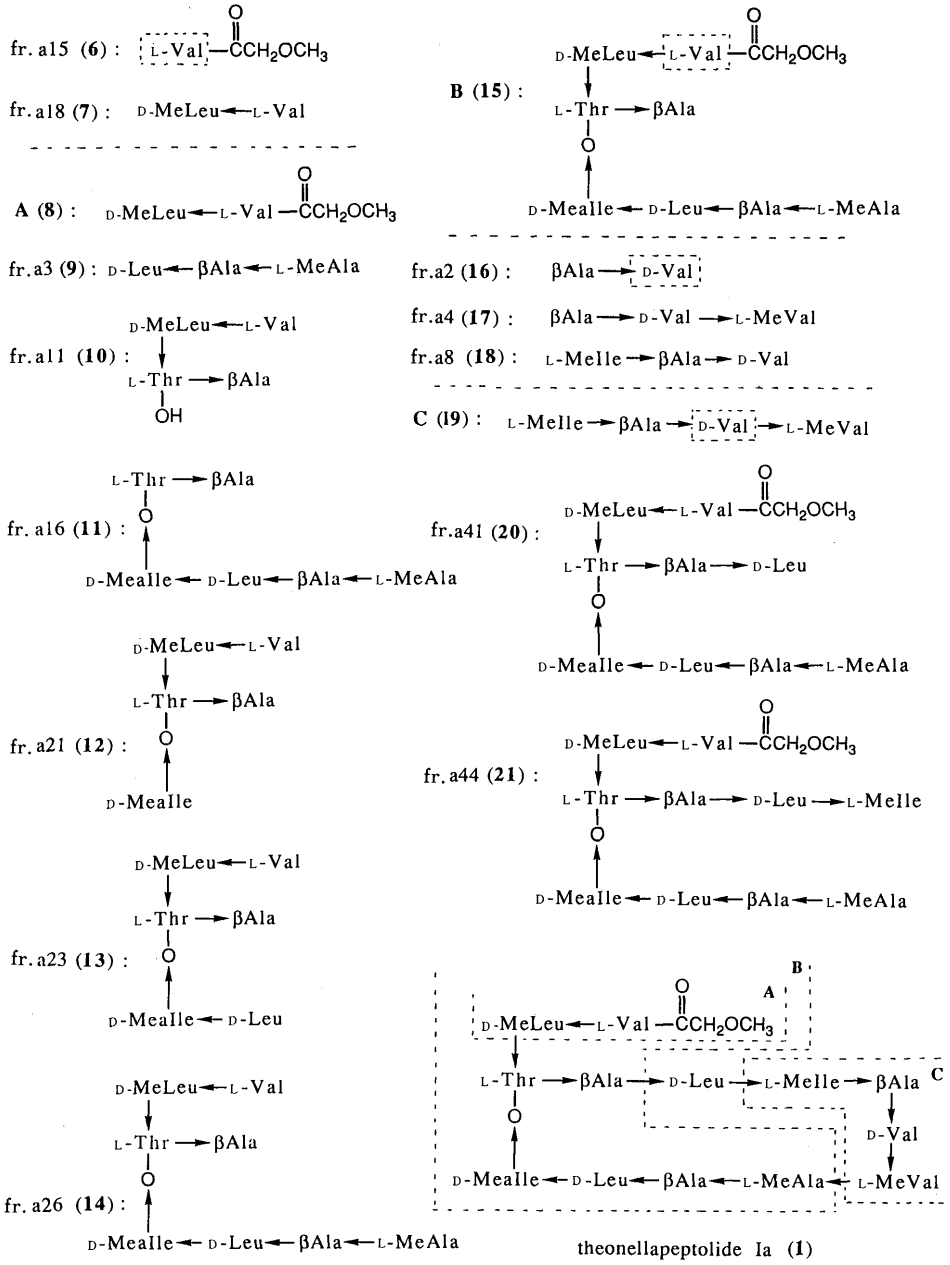



Chart 3

a16 (**11**), fr. a21 (**12**), fr. a23 (**13**), and fr. a26 (**14**), led us to construct another partial structure **B** (**15**). Finally, comparison of the partial structures **B** (**15**) and **C** (**19**), fr. a41 (**20**), and fr. a44 (**21**) gave rise to the amino acid sequence in theonellapeptolide Ia (**1**). Theonellapeptolide Ia (**1**) contains one mole each of D-valine and L-valine in the molecule. The positions of the D- and L-valine moieties in **1** were confirmed by HPLC-CD combined analysis of the amino acid compositions in fr. a2 (**16**) and fr. a15 (**6**), which contain 1 mol each of D-valine and L-valine, respectively. From the accumulated evidence mentioned above, the structure of theonellapeptolide Ia has been determined as **1**.

Theonellapeptolide Ib (**2**) also contains thirteen amino acids: βAla (3), L-Thr (1), L-MeAla (1), L-Val (1), L-MeVal (2), D-aIle (1), D-Leu (2), D-MeAlle (1), and D-MeLeu (1), in the molecule. As illustrated in Charts 4 and 5, the structure of theonellapeptolide Ib (**2**) has been determined by combination of the elucidated structures of ten partial

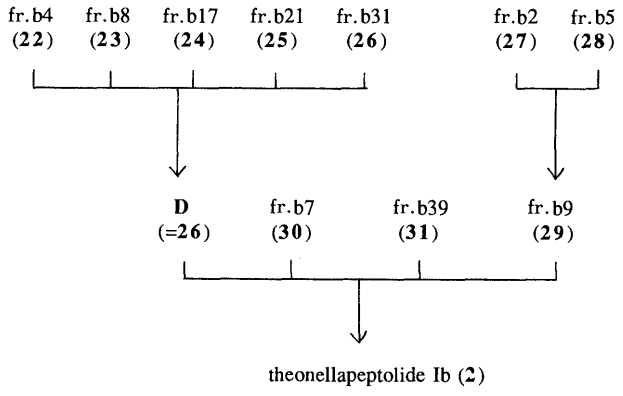



Chart 4

hydrolysates: fr. b2 (**27**), fr. b4 (**22**), fr. b5 (**28**), fr. b7 (**30**), fr. b8 (**23**), fr. b9 (**29**), fr. b17 (**24**), fr. b21 (**25**), fr. b31 (**26**), and fr. b39 (**31**), which were obtained by mild acidic

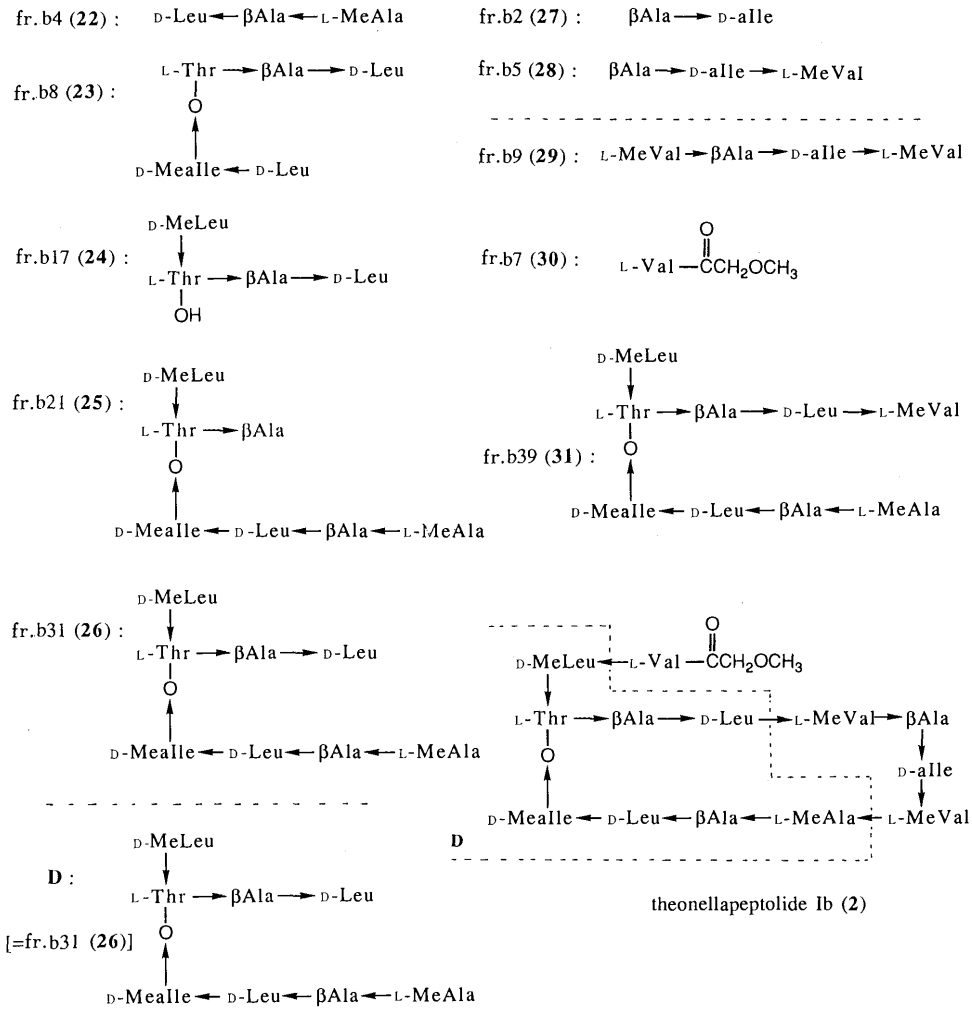



Chart 5

hydrolysis of **2**. The comparative structure analysis of fr. b4 (**22**), fr. b8 (**23**), fr. b17 (**24**), fr. b21 (**25**), and fr. b31 (**26**), led us to presume fr. b31 (**26**) being simultaneously the partial structure **D** (**26**), while the combination of fr. b2 (**27**) and fr. b5 (**28**) revealed the amino acid sequence of fr. b9 (**29**). Finally, the combination of the partial structure **D** (**26**) with the elucidated structures of fr. b9 (**29**), fr. b7 (**30**), and fr. b39 (**31**), led to a formulation of the whole structure of theonellapeptolide Ib (**2**) as shown.

Theonellapeptolide Ic (**3**) contains thirteen amino acids as well: βAla (3), L-Thr (1), L-MeAla (1), L-Val (1), L-MeVal (1), D-MeVal (1), D-aIle (1), D-Leu (2), L-MeIle (1), and D-MeLeu (1). As illustrated in Charts 6 and 7, the structure of theonellapeptolide Ic (**3**) has been clarified by combining the elucidated structures of fifteen partial hydrolysates: fr. c4 (**42**), fr. c6 (**32**), fr. c7 (**33**), fr. c8 (**34**), fr. c9 (**43**), fr. c10 (**44**), fr. c11 (**35**), fr. c12 (**36**), fr. c13 (**37**), fr. c19 (**38**), fr. c22 (**39**), fr. c24 (**40**), fr. c36 (**41**), fr. c44 (**46**), and fr. c48 (**47**), which were obtained by mild acidic hydrolysis of **3**. The structure analysis of fr. c6 (**32**), fr. c7 (**33**), fr. c8 (**34**), fr. c11 (**35**), fr. c12 (**36**), fr. c13 (**37**), fr. c19 (**38**), fr. c22 (**39**), and fr. c24 (**40**) allowed us to construct the amino acid sequence of fr. c36 (**41**). On the other hand, comparison of the structures of fr. c4 (**42**), fr. c9 (**43**), and fr. c10 (**44**) gave rise to a partial structure **E** (**45**). Furthermore, combination of the elucidated structures of fr. c36 (**41**) with fr. c44 (**46**), fr. c48 (**47**), and the partial structure **E** (**45**), led us to the amino acid sequence of theonellapeptolide Ic (**3**). Theonellapeptolide Ic (**3**) contains both enantiomers of *N*-methylvaline, *i.e.*, *N*-methyl-D-valine and *N*-methyl-L-valine, in the molecule. The position of each enantiomer was confirmed by HPLC-CD combined analysis of fr. c36

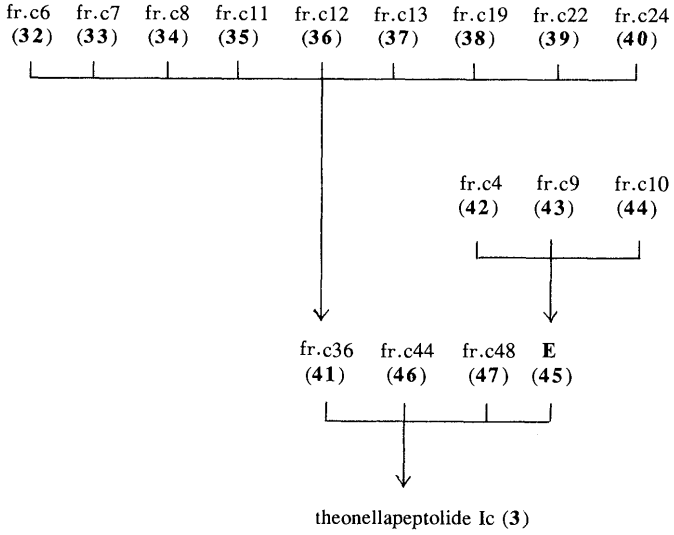



Chart 6

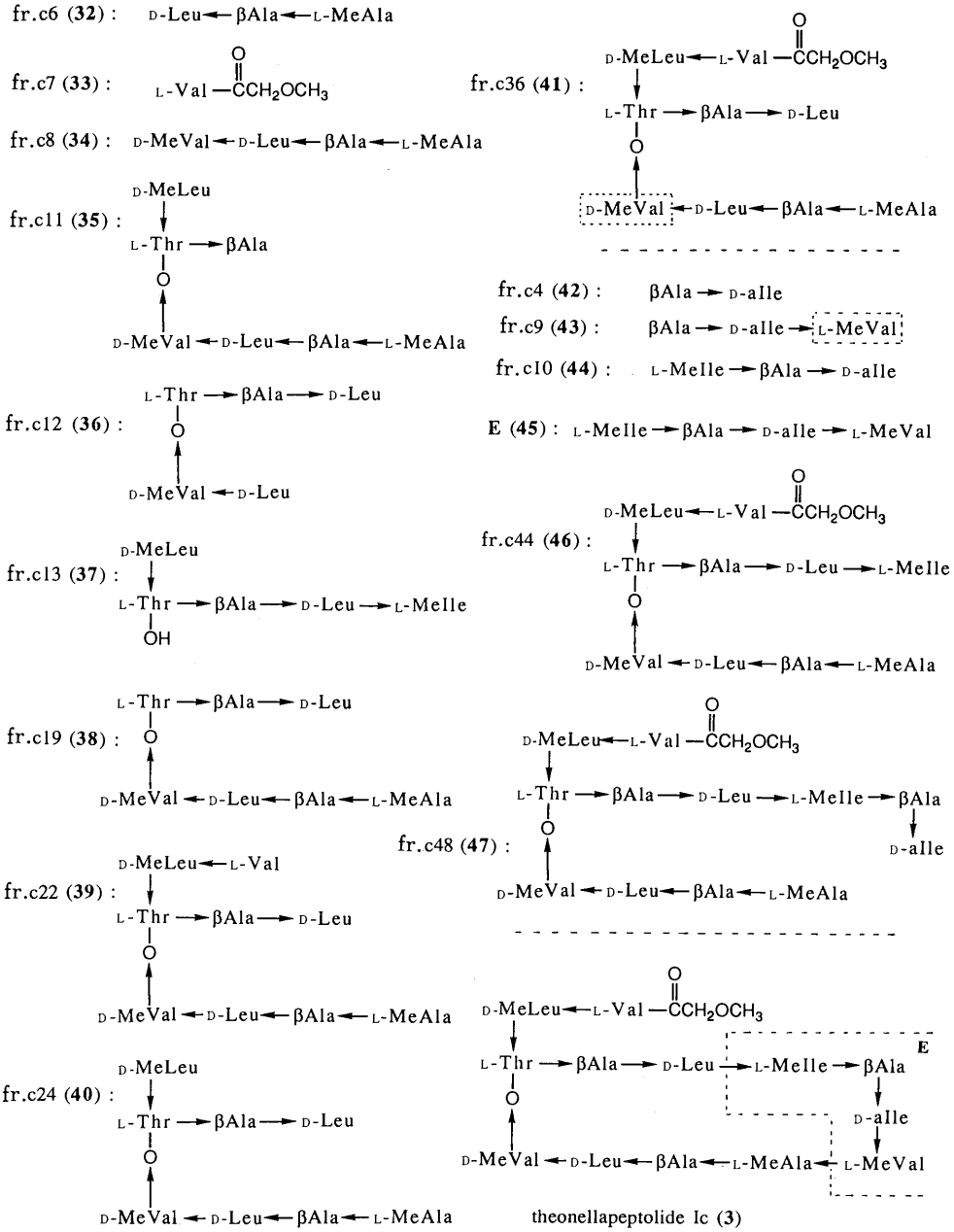



Chart 7

(**41**) and fr. c9 (**43**), which contain each *N*-methylvaline independently. Consequently, the structure of theonellapeptolide Ic has been determined as **3**.

Theonellapeptolide Ie (**5**) contains thirteen amino acids: βAla (2), L-Thr (1), MeβAla (1), L-MeAla (1), L-Val (1), L-MeVal (1), D-aIle (1), D-Leu (2), D-MeaIle (1), L-MeIle (1), and D-MeLeu (1). As shown in Charts 8 and 9, the structure of theonellapeptolide Ie (**5**) has been determined by combination of the results for eleven partial hydrolysates: fr. e8 (**48**), fr. e9 (**52**), fr. e10 (**49**), fr. e16 (**50**), fr. e21 (**58**), fr. e24 (**55**), fr. e26 (**59**), fr. e29 (**60**), fr. e32 (**56**), fr. e36 (**53**), and fr. e39 (**54**), which were obtained by mild acidic hydrolysis of **5**. Comparison of the elucidated amino acid sequences of fr. e8 (**48**), fr. e10 (**49**), and fr. e16 (**50**) disclosed the presence of a partial structure **F** (**51**) in **5**. On the other hand, comparison of the structures of fr. e9 (**52**), fr. e36 (**53**), and fr. e39 (**54**) revealed the presence of another partial structure **G** (**52**), which corresponded

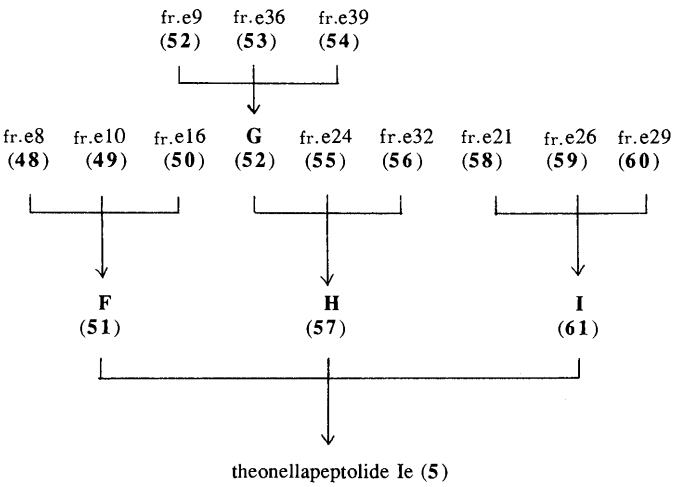



Chart 8

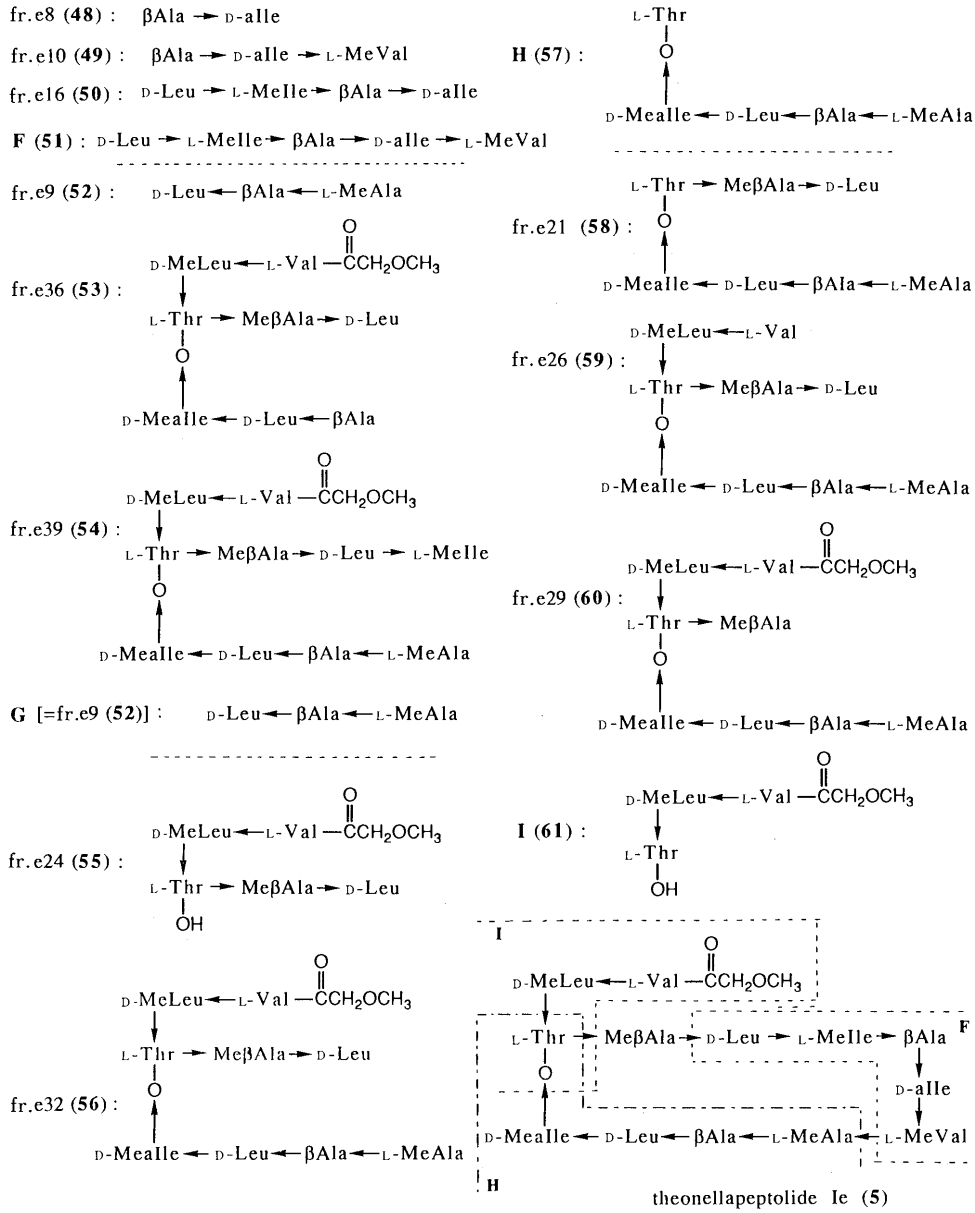



Chart 9

to fr. e9. The combination of the partial structure **G** (**52**) with fr. e24 (**55**) and fr. e32 (**56**), led us to construct an extended partial structure **H** (**57**). Furthermore, comparison of the structures of fr. e21 (**58**), fr. e26 (**59**), and fr. e29 (**60**) disclosed the presence of another partial structure **I** (**61**). Finally, the combination of partial structures **F** (**51**), **H** (**57**), and **I** (**61**) disclosed the whole structure of theonellapeptolide Ie (**5**) as shown.[4]

Treatment of theonellapeptolides Ia (**1**), Ib (**2**), Ic (**3**), and Ie (**5**) with sodium methoxide in methanol furnished methyl esters of the respective seco acids: **62**, **63**, **64**, and **65**. The $^1$H-NMR spectra of these methyl esters showed one-proton multiplet signals around $\delta$ *ca.* 4.1—4.3 which were assignable to the $\beta$-proton geminal to the hydroxyl group in the threonine moiety. As illustrated in Chart 10, the FAB-MS of these methyl esters (**62**, **63**, **64**, **65**) gave fragment ion peaks which were consistent with the amino acid sequences elucidated above for theonellapeptolides Ia (**1**), Ib (**2**), Ic (**3**), and Ie (**5**), respectively.

As was found in the case of theonellapeptolide Id (**4**),[1,3] theonellapeptolides Ia (**1**), Ib (**2**), Ic (**3**), and Ie (**5**) characteristically contain *N*-methyl amino acid and D-amino acid in high ratios. Among the five theonellapeptolides, it has been found that theonellapeptolides Ib (**2**), Ic (**3**), Id (**4**), and Ie (**5**) exhibit moderate cytotoxic activity towards L1210 *in vitro* with $IC_{50}$ values of 1.6, 1.3, 2.4, and 1.4 $\mu$g/ml, respectively. As reported in previous papers,[1,3] theonellapeptolide Id (**4**) has ion-transport activities for $Na^+$, $K^+$, and $Ca^{2+}$ ions. The four theonellapeptolides described in the present paper were also examined, and theonellapeptolide Ie (**5**) exhibited ion-transporting activities for $Na^+$ and $K^+$ ions across human erythrocyte membranes.[8]

## Experimental

Instruments for obtaining physical data and experimental conditions for chromatography were the same as described in the previous paper.[1]

**Isolation of Theonellapeptolides Ia (1), Ib (2), Ic (3), and Ie (5)** Isolation

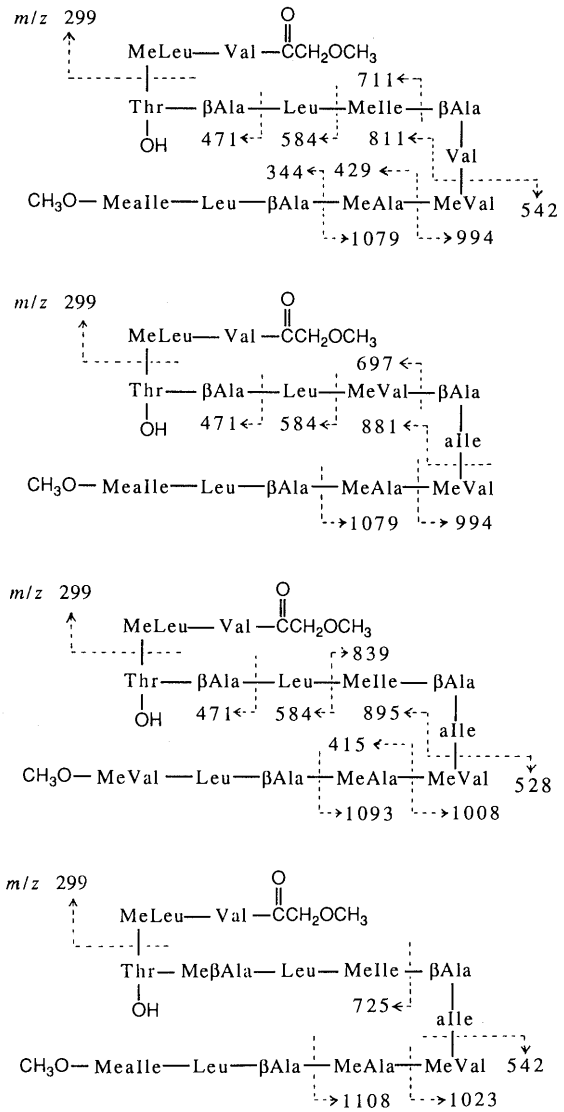


Chart 10

procedures for theonellapeptolides Ia (**1**), Ib (**2**), Ic (**3**), and Ie (**5**) were as described in our previous paper.[1] Theonellapeptolide Ia (**1**), colorless needles, mp 156—157 °C (MeOH–$H_2O$), $[\alpha]_D$ −58° (MeOH, $c$=1.4, 20 °C). FAB-MS $m/z$: 1390 $(M+H)^+$. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3337, 1733, 1678, 1543. $^1$H-NMR (500 MHz, $CDCl_3$:$CD_3OD$=10:1) $\delta$: 8.35 (1H, br s), 8.25 (1H, d, $J$=8.9 Hz), 8.15 (1H, d, $J$=8.9 Hz), 8.04 (1H, d, $J$=7.3 Hz), 7.25 (1H, d, $J$=8.9 Hz), 7.08 (1H, m), 6.93 (2H, br s) (eight amide protons), 3.29, 3.24, 3.18, 3.16, 2.74 (each 3H, s) (five $N$-methyl protons), 3.40 (3H, s), 3.95, 3.88 (both 1H, d, $J$=15.0 Hz) (a methoxyacetyl). $^{13}$C-NMR (125 MHz, $CD_3OD$) $\delta_C$: 176.5 (2C), 176.2, 175.8, 175.1, 174.7, 174.3, 173.7, 173.3, 173.2, 172.5 (2C), 172.2, 171.1, 73.2, 70.7, 60.4. Theonellapeptolide Ib (**2**), colorless needles, mp 159 °C (MeOH–$H_2O$), $[\alpha]_D$ −54° (MeOH, $c$=1.8, 20 °C). FAB-MS $m/z$: 1390 $(M+H)^+$. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3339, 1735, 1675, 1550. $^1$H-NMR (500 MHz, $CDCl_3$:$CD_3OD$=10:1) $\delta$: 8.33 (1H, br s), 8.21 (2H, t-like), 8.09 (1H, d, $J$=8.5 Hz), 7.27 (1H, d, $J$=9.2 Hz), 7.12 (1H, m), 6.92 (2H, m) (eight amide protons), 3.29, 3.25, 3.18, 3.15, 2.92 (each 3H, s) (five $N$-methyl protons), 3.40 (3H, s), 3.95, 3.88 (both 1H, d, $J$=15.0 Hz) (a methoxyacetyl). $^{13}$C-NMR (125 MHz, $CD_3OD$) $\delta_C$: 176.8, 176.5, 176.2, 175.7, 175.2, 174.7, 174.3, 173.8, 173.2 (2C), 172.5, 172.4, 172.1, 171.3, 73.2, 70.7, 60.4. Theonellapeptolide Ic (**3**), colorless needles, mp 147 °C (MeOH–$H_2O$), $[\alpha]_D$ −50° (MeOH, $c$=1.1, 20 °C). FAB-MS $m/z$: 1390 $(M+H)^+$. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3337, 1734, 1671, 1544. $^1$H-NMR (500 MHz, $CDCl_3$:$CD_3OD$=10:1) $\delta$: 8.30 (1H, br s), 8.23 (1H, d, $J$=8.9 Hz), 8.16 (1H, d, $J$=9.0 Hz), 8.03 (1H, d, $J$=7.9 Hz), 7.26 (1H, d, $J$=8.9 Hz), 7.07 (1H, m), 6.92 (2H, m) (eight amide protons), 3.28, 3.25, 3.17, 3.15, 2.75 (each 3H, s) (five $N$-methyl protons), 3.40 (3H, s), 3.95, 3.88 (both 1H, d, $J$=15.0 Hz) (a methoxyacetyl). $^{13}$C-NMR (125 MHz, $CD_3OD$) $\delta_C$: 176.8, 176.5, 176.2, 175.8, 175.1, 174.7, 174.4, 173.8, 173.3, 173.2, 172.6, 172.5, 172.2, 171.2, 73.2, 70.7, 60.4. Theonellapeptolide Ie (**5**), colorless needles, mp 153—155 °C (MeOH–$H_2O$), $[\alpha]_D$ −62° (MeOH, $c$=0.2, 20 °C). FAB-MS $m/z$: 1418 $(M+H)^+$. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3350, 1735, 1675, 1545. $^1$H-NMR (500 MHz, $CDCl_3$:$CD_3OD$=10:1) $\delta$: 8.32 (1H, br d, $J$=8.9 Hz), 8.15 (1H, d, $J$=9.2 Hz), 8.00 (1H, d, $J$=8.9 Hz), 7.31 (1H, br d, $J$=8.9 Hz), 7.19 (2H, d, $J$=9.2 Hz), 6.93 (1H, d, $J$=6.1 Hz) (seven amide protons), 3.30, 3.26, 3.23, 3.16, 2.76, 2.74 (each 3H, s) (six $N$-methyl signals), 3.40 (3H, s), 3.95, 3.88 (both 1H, d, $J$=15.0 Hz) (a methoxyacetyl). $^{13}$C-NMR (125 MHz, $CD_3OD$) $\delta_C$: 176.8, 176.4, 176.1, 175.9, 174.4, 174.3, 173.8, 173.6, 173.3, 172.9, 172.4, 172.3, 172.1, 170.6, 73.2, 71.6, 60.4.

**Complete Acidic Hydrolysis of Theonellapeptolides Ia (1), Ib (2), Ic (3), and Ie (5) Followed by HPLC-CD Analysis** A sample of **1**, **2**, **3**, or **5** (5 mg) was treated with 6 N aqueous HCl (0.5 ml) and, after degassing, the reaction mixture was heated at 110 °C for 24 h in a sealed tube. After cooling, each reaction mixture was evaporated *in vacuo* to give an amino acid mixture. A small portion of the amino acid mixture was subjected to the HPLC analysis of amino acids as described in our previous paper.[1] The retention time of amino acids obtained were: βAla, $t_R$=12 min 44 s; threonine (Thr), $t_R$=13 min 10 s; MeβAla, $t_R$=13 min 30 s; $N$-methylalanine (MeAla), $t_R$=13 min 51 s; valine (Val), $t_R$=18 min 20 s; $N$-methylvaline (MeVal), $t_R$=21 min 09 s; alloisoleucine (aIle), $t_R$=32 min 22 s; leucine (Leu), $t_R$=35 min 25 s; $N$-methylalloisoleucine (MeaIle), $t_R$=41 min 00 s; $N$-methylisoleucine (MeIle), $t_R$=43 min 57 s; $N$-methylleucine (MeLeu), $t_R$=55 min 22 s. The absolute configurations of these amino acids were determined by the CD measurement of each amino acid (in 0.5 N aqueous HCl) which was collected repeatedly by HPLC separation. The results are summarized in Table I.

**N-Terminal Analysis by Dansyl Derivation** Each sample (*ca.* 5 nM) was treated with 0.2 M aqueous $NaHCO_3$ (10 μl) and the mixture was evaporated *in vacuo*. The residue was treated with 10 μl of water and a 10 mM dansyl chloride–acetone solution (10 μl) and after thorough mixing, the reaction mixture was left to stand at 37 °C for 1 h. Removal of the solvent from the reaction mixture under reduced pressure gave a crude product. Complete acidic hydrolysis (6 N aqueous HCl, 110 °C, 24 h, in a degassed, sealed tube) of the crude product furnished a dansyl derivative of the $N$-terminal amino acid, which was analyzed on a Polyamide TLC plate (polyamide pre-coated for thin-layer chromatography, aluminum sheet) developed in two dimensions with benzene–acetic acid (1:1) and 1.5% aqueous HCOOH.

**Partial Hydrolysis of Theonellapeptolide Ia (1)** A solution of **1** (100 mg) in 30% aqueous trifluoroacetic acid (TFA) was degassed and heated at 110 °C for 40 min in a sealed tube. The reaction mixture was evaporated under reduced pressure to give a mixture of partial hydrolysates. The partial hydrolysates were separated by HPLC [Zorbax ODS, eluted with a gradient of 0.1% aqueous TFA→2-propanol (PrOH)–acetonitrile ($CH_3CN$) (7:3) containing 0.1% TFA] to furnish fr. a2 (**16**), fr. a3 (**9**), fr. a4 (**17**), fr. a8 (**18**), fr. a11 (**10**), fr. a15 (**6**), fr. a16 (**11**), fr. a18 (**7**), fr. a21 (**12**), fr. a23 (**13**), fr. a26 (**14**), fr. a41 (**20**), and fr. a44 (**21**). Fr. a2 (**16**), FAB-MS $m/z$: 189 $(M+H)^+$, [amino acid composition: βAla (1), Val (1)], N-terminal amino acid: βAla. Fr. a3 (**9**), FAB-MS $m/z$: 288 $(M+H)^+$, [βAla (1), MeAla (1), Leu (1)], N-terminal: MeAla. Fr. a4 (**17**), FAB-MS $m/z$: 302 $(M+H)^+$, [βAla (1), Val (1), MeVal (1)], N-terminal: βAla. Fr. a8 (**18**), FAB-MS $m/z$: 316 $(M+H)^+$, [βAla (1), Val (1), MeIle (1)], N-terminal: MeIle. Fr. a11 (**10**), FAB-MS $m/z$: 417 $(M+H)^+$, [βAla (1), Thr (1), Val (1), MeLeu (1)], N-terminal: Val. Fr. a15 (**6**), FAB-MS $m/z$: 190 $(M+H)^+$, [Val (1)], N-terminal: none. Fr. a16 (**11**), FAB-MS $m/z$: 587 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), Leu (1), MeaIle (1)], N-terminal: Thr, MeAla. Fr. a18 (**7**), FAB-MS $m/z$: 245 $(M+H)^+$, [Val (1), MeLeu (1)], N-terminal: Val. Fr. a21 (**12**), FAB-MS $m/z$: 544 $(M+H)^+$, [βAla (1), Thr (1), Val (1), MeaIle (1), MeLeu (1)], N-terminal: Val, MeaIle. Fr. a23 (**13**), FAB-MS $m/z$: 657 $(M+H)^+$, [βAla (1), Thr (1), Val (1), Leu (1), MeaIle (1), MeLeu (1)], N-terminal: Val, Leu. Fr. a26 (**14**), FAB-MS $m/z$: 813 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), Val (1), Leu (1), MeaIle (1), MeLeu (1)], N-terminal: MeAla, Val. Fr. a41 (**20**), FAB-MS $m/z$: 998 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), Val (1), Leu (2), MeaIle (1), MeLeu (1)], N-terminal: MeAla. Fr. a44 (**21**), FAB-MS $m/z$: 1124 $(M-H)^-$, [βAla (2), Thr (1), MeAla (1), Val (1), Leu (2), MeaIle (1), MeIle (1),MeLeu (1)], N-terminal: MeAla.

**Acidic Hydrolysis of Fr. a2 (16) and Fr. a15 (6) Followed by CD Measurement** Samples (1 mg) of **16** and **6** were each hydrolyzed with 6 N aqueous HCl at 110 °C for 24 h in a sealed tube to furnish valine-containing hydrolysates, which were purified by HPLC (Cosmosil $5C_{18}$, eluted with water). Valine from fr. a2 (**16**) gave a negative CD maximum at 210 nm in 0.5 N aqueous HCl. Valine from fr. a15 (**6**) gave a positive

CD maximum at 210 nm in 0.5 N aqueous HCl.

**Partial Hydrolysis of Theonellapeptolide Ib (2)** A solution of **2** (100 mg) in 30% aqueous TFA was degassed and heated at 110 °C for 40 min in a sealed tube. The reaction mixture was evaporated under reduced pressure to give a mixture of partial hydrolysates. The partial hydrolysates were then subjected to HPLC separation as described above to furnish fr. b2 (**27**), fr. b4 (**22**), fr. b5 (**28**), fr. b7 (**30**), fr. b8 (**23**), fr. b9 (**29**), fr. b17 (**24**), fr. b21 (**25**), fr. b31 (**26**), and fr. b39 (**31**). Fr. b2 (**27**), FAB-MS *m/z* : 203 $(M+H)^+$, [βAla (1), aIle (1)], N-terminal: βAla. Fr. b4 (**22**), FAB-MS *m/z*: 288 $(M+H)^+$, [βAla (1), MeAla (1), Leu (1)], N-terminal: MeAla. Fr. b5 (**28**), FAB-MS *m/z*: 316 $(M+H)^+$, [βAla (1), MeVal (1), aIle (1)], N-terminal: βAla. Fr. b7 (**30**), FAB-MS *m/z*: 190 $(M+H)^+$, [Val], N-terminal: none. Fr. b8 (**23**), FAB-MS *m/z*: 544 $(M+H)^+$, [βAla (1), Thr (1), Leu (2), MeaIle (1)], N-terminal: Thr, Leu. Fr. b9 (**29**), FAB-MS *m/z*: 429 $(M+H)^+$, [βAla (1), MeVal (2), aIle (1)], N-terminal: MeVal. Fr. b17 (**24**), FAB-MS *m/z*: 431 $(M+H)^+$, [βAla (1), Thr (1), Leu (1), MeLeu (1)], N-terminal: MeLeu. Fr. b21 (**25**), FAB-MS *m/z*: 714 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), Leu (1), MeaIle (1), MeLeu (1)], N-terminal: MeAla, MeLeu. Fr. b31 (**26**), FAB-MS *m/z*: 827 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), Leu (2), MeaIle (1), MeLeu (1)], N-terminal: MeAla, MeLeu. Fr. b39 (**31**), FAB-MS *m/z*: 940 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), MeVal (1), Leu (2), MeaIle (1), MeLeu (1)], N-terminal: MeLeu, MeAla.

**Partial Hydrolysis of Theonellapeptolide Ic (3)** Compound **3** (100 mg) was hydrolyzed under the same reaction conditions as described in the case of theonellapeptolide Ia (**1**) to give fr. c4 (**42**), fr. c6 (**32**), fr. c7 (**33**), fr. c8 (**34**), fr. c9 (**43**), fr. c10 (**44**), fr. c11 (**35**), fr. c12 (**36**), fr. c13 (**37**), fr. c19 (**38**), fr. c22 (**39**), fr. c24 (**40**), fr. c36 (**41**), fr. c44 (**46**), and fr. c48 (**47**). Fr. c4 (**42**), FAB-MS *m/z*: 203 $(M+H)^+$, [βAla (1), aIle (1)], N-terminal: βAla. Fr. c6 (**32**), FAB-MS *m/z*: 288 $(M+H)^+$, [βAla (1), MeAla (1), Leu (1)], N-terminal: MeAla. Fr. c7 (**33**), FAB-MS *m/z*: 190 $(M+H)^+$, [Val], N-terminal: none. Fr. c8 (**34**), FAB-MS *m/z*: 401 $(M+H)^+$, [βAla (1), MeAla (1), MeVal (1), Leu (1)], N-terminal: MeAla. Fr. c9 (**43**), FAB-MS *m/z*: 315 $(M+H)^+$, [βAla (1), MeVal (1), aIle (1)], N-terminal: βAla. Fr. c10 (**44**), FAB-MS *m/z*: 330 $(M+H)^+$, [βAla (1), aIle (1), MeIle (1)], N-terminal: MeIle. Fr. c11 (**35**), FAB-MS *m/z*: 700 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), MeVal (1), Leu (1), MeLeu (1)], N-terminal: MeAla, MeLeu. Fr. c12 (**36**), FAB-MS *m/z*: 530 $(M+H)^+$, [βAla (1), Thr (1), MeVal (1), Leu (2)], N-terminal: Thr, Leu. Fr. c13 (**37**), FAB-MS *m/z*: 558 $(M+H)^+$, [βAla (1), Thr (1), Leu (1), MeIle (1), MeLeu (1)], N-terminal: MeLeu. Fr. c19 (**38**), FAB-MS *m/z*: 686 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), MeVal (1), Leu (2)], N-terminal: Thr, MeAla. Fr. c22 (**39**), FAB-MS *m/z*: 912 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), Val (1), MeVal (1), Leu (2), MeLeu (1)], N-terminal: MeAla, Val. Fr. c24 (**40**), FAB-MS *m/z*: 813 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), MeVal (1), Leu (2), MeLeu (1)], N-terminal: MeAla, MeLeu. Fr. c36 (**41**), FAB-MS *m/z*: 984 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), Val (1), MeVal (1), Leu (2), MeLeu (1)], N-terminal: MeAla. Fr. c44 (**46**), FAB-MS *m/z*: 1111 $(M+H)^+$, [βAla (2), Thr (1), MeAla (1), Val (1), MeVal (1), Leu (2), MeIle (1), MeLeu (1)], N-terminal: MeAla. Fr. c48 (**47**), FAB-MS *m/z*: 1295 $(M+H)^+$, [βAla (3), Thr (1), MeAla (1), Val (1), MeVal (1), aIle (1), Leu (2), MeIle (1), MeLeu (1)], N-terminal: MeAla.

**Acidic Hydrolysis of Fr. c9 (43) and Fr. c36 (41) Followed by CD Measurement** Samples (1 mg) of **43** and **41** were each hydrolyzed with 6 N aqueous HCl at 110 °C for 24 h in a sealed tube to furnish *N*-methylvaline-containing hydrolysates which were purified by HPLC (Cosmosil $5C_{18}$, eluted with water). *N*-Methylvaline obtained from fr. c9 (**43**) showed a positive CD maximum at 210 nm. *N*-Methylvaline from fr. c36 (**41**) showed a negative maximum at 210 nm.

**Partial Hydrolysis of Theonellapeptolide Ie (5)** Compound **5** (100 mg) was hydrolyzed under the same reaction conditions as described above to furnish fr. e8 (**48**), fr. e9 (**52**), fr. e10 (**49**), fr. e16 (**50**), fr. e21 (**58**), fr. e24 (**55**), fr. e26 (**59**), fr. e29 (**60**), fr. e32 (**56**), fr. e36 (**53**), and fr. e39 (**54**). Fr. e8 (**48**), FAB-MS *m/z*: 203 $(M+H)^+$, [βAla (1), aIle (1)], N-terminal: βAla. Fr. e9 (**52**), FAB-MS *m/z*: 288 $(M+H)^+$, [βAla (1), MeAla (1), Leu (1)], N-terminal: MeAla. Fr. e10 (**49**), FAB-MS *m/z*: 316 $(M+H)^+$, [βAla (1), MeVal (1), aIle (1)], N-terminal: βAla. Fr. e16 (**50**), FAB-MS *m/z*: 443 $(M+H)^+$, [βAla (1), aIle (1), Leu (1), MeIle (1)], N-terminal: Leu. Fr. e21 (**58**), FAB-MS *m/z*: 714 $(M+H)^+$, [βAla (1), Thr (1), MeβAla (1), MeAla (1), Leu (2), MeaIle (1)], N-terminal: Thr, MeAla. Fr. e24 (**55**), FAB-MS *m/z*: 616 $(M+H)^+$, [Thr (1), MeβAla (1), Val (1), Leu (1), MeLeu (1)], N-terminal: none. Fr. e 26 (**59**), FAB-MS *m/z*: 940 $(M+H)^+$, [βAla (1), Thr (1), MeβAla (1), MeAla (1), Val (1), Leu (2), MeaIle (1), MeLeu (1)], N-terminal: MeAla, Val. Fr. e29 (**60**), FAB-MS *m/z*: 899 $(M+H)^+$, [βAla (1), Thr (1), MeβAla (1), MeAla (1), Val (1), Leu (1), MeaIle (1), MeLeu (1)], N-terminal: MeAla. Fr. e32 (**56**), FAB-MS *m/z*: 940 $(M+H)^+$, [βAla (1), Thr (1), MeβAla (1), MeAla (1), Val (1), Leu (2), MeaIle (1), MeLeu (1)], N-terminal: MeAla. Fr. e36 (**53**), FAB-MS *m/z*: 925 $(M-H)^-$, [βAla (1), Thr (1), MeβAla (1), Val (1), Leu (2), MeaIle (1), MeLeu (1)], N-terminal: βAla. Fr. e39 (**54**), FAB-MS *m/z*: 1139 $(M+H)^+$, [βAla (1), Thr (1), MeβAla (1), MeAla (1), Val (1), Leu (2), MeaIle (1), MeIle (1), MeLeu (1)], N-terminal: MeAla.

**NaOMe Treatment of Theonellapeptolides Ia (1), Ib (2), Ic (3), and Ie (5)** A solution of **1** (10 mg) in MeOH (2 ml) was treated with 28% NaOMe in MeOH (0.2 ml) at room temperature for 2 h. The reaction mixture was poured into ice-water and the whole was extracted with ethyl acetate. The ethyl acetate solution was taken, washed with brine, and evaporated under reduced pressure to furnish a product (8 mg). The product was subjected to silica gel column chromatography ($CHCl_3$ : MeOH = 8 : 1) to give the methyl ester **62** (6 mg). A solution of **2**, **3**, or **5** (10 mg each) was treated with NaOMe–MeOH as described above to give **63** (5 mg), **64** (5 mg), or **65** (6 mg). **62**, FAB-MS: as shown in Chart 10. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3430, 3330, 1733, 1675, 1540. $^1$H-NMR (500 MHz, $CD_3OD$) $\delta$: 4.30 (1H, m, Thr β-H), 3.92 (2H, m, methoxyacetyl), 1.05 (3H, d, $J$ = 6.0 Hz, Thr β-$CH_3$). **63**, FAB-MS: as shown in Chart 10. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3430, 3330, 1735, 1670, 1540. $^1$H-NMR (500 MHz, $CD_3OD$) $\delta$: 4.27 (1H, m), 3.95 (2H, m), 1.13 (3H, d, $J$ = 6.0 Hz). **64**, FAB-MS: as shown in Chart 10. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3430, 3330, 1730, 1665, 1545. $^1$H-NMR (500 MHz, $CD_3OD$) $\delta$: 4.30 (1H, m), 3.95 (2H, m), 1.13 (3H, d, $J$ = 6.0 Hz). **65**, FAB-MS: as shown in Chart 10. IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3420, 3340, 1735, 1670, 1540. $^1$H-NMR (500 MHz, $CD_3OD$) $\delta$: 4.09 (1H, m), 3.96 (2H, m), 1.14 (3H, d, $J$ = 6.5 Hz).

**Acknowledgement** The authors are grateful to the Ministry of Education, Science, and Culture of Japan (Grant-in-Aid for Cancer Research No. 02151037) and Yamada Science Foundation for financial support.

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# Structures of Two New Fibrinolytic Saponins from the Seed of *Luffa cylindrica* ROEM.[1)]

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**Two new fibrinolytic saponins, lucyosides N and P, were isolated from the seeds of *Luffa cylindrica* ROEM. (Cucurbitaceae). On the basis of chemical and spectral evidence, lucyoside N was characterized as 3-*O*-*β*-D-galactopyranosyl-(1→2)-*β*-D-glucuronopyranosyl-28-*O*-*β*-D-xylopyranosyl-(1→4)-[*β*-D-glucopyranosyl-(1→3)]-*α*-L-rhamnopyranosyl-(1→2)-*α*-arabinopyranosyl quillaic acid. Lucyoside P was characterized as a gypsogenin glycoside with the same sugar moiety as lucyoside N.**

**Keywords** *Luffa cylindrica*; Cucurbitaceae; triterpene saponin; lucyoside; quillaic acid; gypsogenin; fibrinolytic saponin; fibrin plate method

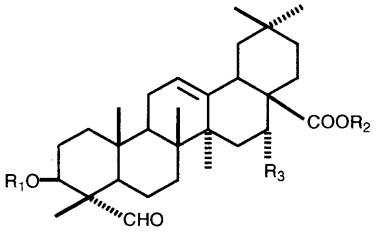
We have already reported the isolation and the structure determination of thirteen oleanane glycosides, lucyosides A-M, from the aerial parts and fruits of *Luffa cylindrica* ROEM. (Cucurbitaceae).[2)] In the course of our survey on the fibrinolytic activities of crude drugs,[3)] several parts (seed, aerial part, fruit, pericarp, luffa) of the title plant were tested for fibrinolytic activity by the fibrin plate method. The water extract of the seed showed the highest activity. In this paper, we describe the structure elucidation and activity of two active principles isolated from the water extract of the seed.

The water extract of the seed was chromatographed on an Amberlite XAD-2 column. The MeOH eluate was subjected to repeated reversed-phase and ordinary-phase silica gel column chromatography to give two very potent active principles, new saponins named lucyoside N(**1**) and lucyoside P(**2**).

Lucyoside N(**1**), the major constituent, showed an $(M-H)^-$ ion at *m/z* 1395 in the negative fast atom bombardment mass spectrum (FAB-MS), and the elemental analysis was consistent with $C_{64}H_{100}O_{33}\cdot 7/2H_2O$. On acid hydrolysis, **1** afforded glucuronic acid (glcUA), galactose (gal), arabinose (ara), rhamnose (rha), xylose (xyl) and glucose (glc) as sugar components. The $^1$H-nuclear magnetic resonance ($^1$H-NMR) spectrum of **1** showed signals of six tertiary methyl groups (*δ* 0.83, 1.01, 1.04, 1.14, 1.36 and 1.77), one secondary methyl group [*δ* 1.73 (d, *J*=5.5 Hz)], one trisubstituted olefinic proton [*δ* 5.58 (m)], one aldehydic proton [*δ* 9.90 (s)] and six anomeric protons [*δ* 4.93 (d, *J*=7.5 Hz), 5.20 (d, *J*=7.0 Hz), 5.23 (d, *J*=7.0 Hz), 5.42 (d, *J*=8.0 Hz), 5.58 (br s) and 6.45 (d, *J*=3.0 Hz)].

The $^{13}$C-NMR spectrum revealed signals of the corresponding six anomeric carbons (*δ* 103.4, 105.2, 106.5, 105.3, 101.0 and 93.8), two olefinic carbons (*δ* 122.6 and 144.6), one carboxylic carbon (*δ* 172.5), one ester carbon (*δ* 175.9) and one aldehydic carbon (*δ* 209.5). These spectral data, coupled with the results of FAB-MS and elemental analysis, suggested **1** to be an oleanane-type triterpene hexaglycoside in which one of the sugar moieties is linked to the aglycone by an ester bond.

Alkaline hydrolysis of **1** with 2% KOH in 50% EtOH yielded a prosapogenin and subsequent treatment with Dowex 50w×8 ($H^+$, form, 80% MeOH), furnished a monomethyl ester (**3**) of the prosapogenin. Compound **3** showed an $[M-H]^-$ ion at *m/z* 837 in its negative FAB-MS and the elemental analysis was consistent with $C_{43}H_{66}O_{16}\cdot 9/2H_2O$. It gave, on acid hydrolysis, glucuronic acid and galactose. The $^1$H-NMR spectrum of **3** showed two anomeric proton signals at *δ* 4.92 (d, *J*=7.5 Hz) and 5.23 (d, *J*=7.0 Hz), indicating *β*-glycosidic linkages. Methanolysis of **3** under mild conditions provided a monoglycoside (**4**) and an aglycone (**5**), which was identified as quillaic acid by comparison of its melting point, optical rotation and $^{13}$C-NMR spectrum with reported data.[4,5)] Compound **4** revealed an $[M-H]^-$ ion at *m/z* 675 in its negative FAB-MS, indicating the molecular formula $C_{37}H_{56}O_{11}$. On acid hydrolysis, **4** yielded glucuronic acid. The $^{13}$C-NMR spectrum of **4** indicated that the glucuronopyranosyl group is attached at C-3 of the aglycone (*δ* 82.4). Accordingly, **4** was formulated as quillaic acid 3-*O*-methyl-*β*-D-glucopyranuronate. In comparing the $^{13}$C-NMR spectrum of **3** with that of **4**, glycosylation shifts[6)] were observed at the signals of $C_2$ (+8.4 ppm) and $C_1$ (−2.2 ppm) of the glucuronopyranosyl group, indicating that the galactopyranosyl group is linked to $C_2$-OH of the glucuronosyl group. Consequently, **3** was formulated as quillaic acid 3-*O*-*β*-D-galactopyranosyl-




| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| 1 | -glcUA-(2)-gal | -ara-(2)-rha-(4)-xyl, (3)-glc | -OH |
| 2 | -glcUA-(2)-gal | -ara-(2)-rha-(4)-xyl, (3)-glc | -H |
| 3 | Me-(6)-glcUA-(2)-gal | -H | -OH |
| 4 | Me-(6)-glcUA | -H | -OH |
| 5 | -H | -H | -OH |
| 6 | -glcUA | -ara-(2)-rha | -OH |
| 7 | -glcUA-(2)-gal | -ara-(2)-rha-(4)-xyl | -OH |
| 8 | Me-(6)-glcUA-(2)-gal | -H | -H |

Chart 1

Table I. $^{13}$C-NMR Chemical Shifts of Aglycone Moieties in $C_5D_5N$

| C No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 38.3 | 38.0 | 38.1 | 38.3 | 38.1 | 38.2 | 38.2 | 38.9 | 38.9 |
| 2 | 25.1 | 24.9 | 24.8 | 25.3 | 26.5 | 24.8 | 25.0 | 24.8 | 26.0 |
| 3 | 82.6 | 82.3 | 82.3 | 82.4 | 71.7 | 82.1 | 82.5 | 82.4 | 82.5 |
| 4 | 55.2 | 55.0 | 55.1 | 55.7 | 56.2 | 55.4 | 55.2 | 55.0 | 43.6 |
| 5 | 48.6 | 48.5 | 48.4 | 48.0 | 48.1 | 47.8 | 48.6 | 48.4 | 48.3 |
| 6 | 20.6 | 20.4 | 20.4 | 20.7 | 20.7 | 20.6 | 20.5 | 20.4 | 18.3 |
| 7 | 33.0 | 32.7 | 32.8 | 32.9 | 32.3 | 32.7 | 32.9 | 32.5 | 33.0 |
| 8 | 40.4 | 40.1 | 40.1 | 40.3 | 40.0 | 40.3 | 40.3 | 40.0 | 39.9 |
| 9 | 47.2 | 47.3 | 47.0 | 47.3 | 46.7 | 47.1 | 47.1 | 47.8 | 47.9 |
| 10 | 36.4 | 36.3 | 36.3 | 36.3 | 35.9 | 36.1 | 36.3 | 36.3 | 37.0 |
| 11 | 23.9 | 23.2 | 23.8 | 23.9 | 23.2 | 23.8 | 23.8 | 23.7 | 23.8 |
| 12 | 122.6 | 122.6 | 122.0 | 123.7 | 123.8 | 123.0 | 122.6 | 122.2 | 122.7 |
| 13 | 144.6 | 144.2 | 145.2 | 145.5 | 145.0 | 144.4 | 144.6 | 144.9 | 145.1 |
| 14 | 42.2 | 42.1 | 42.1 | 42.4 | 42.3 | 42.1 | 42.2 | 42.2 | 42.4 |
| 15 | 36.2 | 28.1 | 36.2 | 36.1 | 35.4 | 36.1 | 36.1 | 28.3 | 28.5 |
| 16 | 74.6 | 23.7 | 74.7 | 74.8 | 74.7 | 73.8 | 74.1 | 23.7 | 24.0 |
| 17 | 49.6 | 47.8 | 48.8 | 49.1 | 48.7 | 49.5 | 49.6 | 46.6 | 46.7 |
| 18 | 41.4 | 41.6 | 41.4 | 41.7 | 41.9 | 41.2 | 41.3 | 42.0 | 42.2 |
| 19 | 47.3 | 46.2 | 47.3 | 47.5 | 46.4 | 47.1 | 47.3 | 46.4 | 46.6 |
| 20 | 31.1 | 30.9 | 31.1 | 31.2 | 30.5 | 30.9 | 31.0 | 31.0 | 31.1 |
| 21 | 36.3 | 34.1 | 36.2 | 36.3 | 36.3 | 36.1 | 36.2 | 34.2 | 34.4 |
| 22 | 32.2 | 32.5 | 32.8 | 32.9 | 32.3 | 32.0 | 32.2 | 33.2 | 33.4 |
| 23 | 209.5 | 209.7 | 209.3 | 207.4 | 207.2 | 207.1 | 209.5 | 209.5 | 64.5 |
| 24 | 11.1 | 11.0 | 10.9 | 10.6 | 19.6 | 10.4 | 10.9 | 10.9 | 13.8 |
| 25 | 16.0 | 15.7 | 15.7 | 15.9 | 15.7 | 15.8 | 15.9 | 15.9 | 16.2 |
| 26 | 17.7 | 17.4 | 17.4 | 17.6 | 16.9 | 17.5 | 17.6 | 17.3 | 17.7 |
| 27 | 27.4 | 26.0 | 27.2 | 27.4 | 27.0 | 27.2 | 27.2 | 26.2 | 26.4 |
| 28 | 175.9 | 176.2 | 180.1 | 180.6 | 180.9 | 175.8 | 175.9 | 180.1 | 180.0 |
| 29 | 33.4 | 33.2 | 33.4 | 33.6 | 33.6 | 33.3 | 33.4 | 33.3 | 33.5 |
| 30 | 24.9 | 23.7 | 24.8 | 25.0 | 25.0 | 24.8 | 24.8 | 23.8 | 24.0 |

Table II. $^{13}$C-NMR Chemical Shifts of Sugar Moieties in $C_5D_5N$

| C No. | | 1 | 2 | 3 | 4 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| C-3 sugars | | | | | | | | | |
| glcUA | 1 | 103.4 | 103.4 | 103.2 | 105.4 | 105.3 | 103.4 | 103.3 | 104.0 |
| or | 2 | 83.5 | 83.6 | 83.4 | 75.0 | 75.1 | 83.5 | 83.5 | 83.8 |
| glc | 3 | 77.5 | 77.4 | 77.5 | 77.7 | 77.9 | 77.5 | 77.5 | 78.0 |
| | 4 | 73.0 | 72.6 | 72.6 | 73.1 | 73.3 | 73.0 | 72.6 | 71.3 |
| | 5 | 77.7 | 77.7 | 76.8 | 77.3 | 77.8 | 77.7 | 76.8 | 78.5 |
| | 6 | 72.5 | 72.5 | 70.3 | 70.9 | 72.9 | 72.4 | 70.3 | 62.7 |
| COOMe | | | | 52.1 | 52.4 | | | 52.1 | |
| gal | 1 | 106.5 | 106.4 | 106.3 | | | 106.4 | 106.4 | 106.5 |
| | 2 | 74.6 | 74.5 | 74.5 | | | 74.6 | 74.5 | 74.3 |
| | 3 | 74.9 | 74.9 | 74.9 | | | 74.9 | 74.9 | 75.1 |
| | 4 | 70.2 | 70.1 | 70.2 | | | 70.2 | 70.2 | 69.7 |
| | 5 | 77.2 | 77.1 | 77.2 | | | 77.2 | 77.2 | 77.1 |
| | 6 | 62.2 | 62.1 | 62.2 | | | 62.2 | 62.2 | 61.6 |
| C-28 sugars | | | | | | | | | |
| ara | 1 | 93.8 | 93.5 | | | 93.7 | 93.8 | | |
| | 2 | 75.7 | 75.7 | | | 75.4 | 76.1 | | |
| | 3 | 69.6 | 69.3 | | | 70.3 | 70.4 | | |
| | 4 | 66.1 | 65.9 | | | 66.2 | 66.4 | | |
| | 5 | 63.1 | 62.8 | | | 63.2 | 63.5 | | |
| rha | 1 | 101.1 | 100.8 | | | 101.6 | 101.0 | | |
| | 2 | 71.7 | 71.6 | | | 72.3 | 72.7 | | |
| | 3 | 82.8 | 82.6 | | | 72.6 | 72.0 | | |
| | 4 | 79.0 | 79.0 | | | 73.8 | 83.5 | | |
| | 5 | 68.9 | 68.8 | | | 70.6 | 68.6 | | |
| | 6 | 18.8 | 18.7 | | | 18.7 | 18.5 | | |
| xyl | 1 | 105.2 | 105.0 | | | | 106.8 | | |
| | 2 | 75.5 | 75.4 | | | | 74.9 | | |
| | 3 | 78.4 | 78.3 | | | | 78.6 | | |
| | 4 | 71.3 | 71.2 | | | | 71.7 | | |
| | 5 | 67.3 | 67.2 | | | | 67.5 | | |
| glc | 1 | 105.3 | 105.3 | | | | | | |
| | 2 | 75.5 | 75.5 | | | | | | |
| | 3 | 78.4 | 78.5 | | | | | | |
| | 4 | 71.3 | 71.7 | | | | | | |
| | 5 | 78.1 | 78.1 | | | | | | |
| | 6 | 62.7 | 62.6 | | | | | | |

(1→2)-methyl-β-D-glucopyranuronate.

On cellulase hydrolysis, **1** provided prolucyoside N-1 (**6**) and prolucyoside N-2 (**7**) with **7** having a greater polarity than **6**. Compound **6** showed an $[M-H]^-$ ion at *m/z* 939 in the negative FAB-MS and yielded glucuronic acid, arabinose and rhamnose on acid hydrolysis. The $^1$H-NMR spectrum disclosed three anomeric protons $\delta$ 4.93 (d, $J$=7.5 Hz), 5.82 (br s) and 6.49 (d, $J$=3.0 Hz). The electron impact mass spectrum (EI-MS) spectrum of the peracetate of **6** exhibited fragment ions at *m/z* 273 $[rha(Ac)_3)]^+$ and 489 $[(rha\text{-}ara)Ac_5]^+$, which, coupled with signals of an anomeric proton ($\delta$ 6.49) and corresponding anomeric carbon ($\delta$ 93.7) in the NMR spectra of **4**, led to the formulation of **3** as the C-28-*O*-arabinose-rhamnose derivative of **4**. Regarding ester-linked sugar carbons, the signal of C-2 of the arabinopyranosyl group was seen at $\delta$ 75.4, indicating that the rhamnopyranosyl group is attached to $C_2$-OH of the arabinosyl group. The $^{13}$C-NMR chemical shifts of the ester-linked sugar moiety were in good agreement with those reported for 3-*O*-acetyl oleanolic acid-28-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside.[7] The configuration of the anomeric proton of the arabinopyranosyl group is considered to be α-based on the $J_{H1,H2}$ (3 Hz) and $J_{C1,H1}$ (170 Hz) values and the chemical shifts of C-3, -4 and -5 of the arabinosyl unit.[8] Therefore, **6** was formulated as 3-*O*-β-D-glucuronopyranosyl-28-*O*-α-L-rhamnopyranosyl-(1→2)-α-arabinopyranosyl quillaic acid.

In the negative FAB-MS, **7** revealed an $[M-H]^-$ ion at *m/z* 1233, 294 mass units more than that of **6**. Compound **7** gave glucuronic acid, galactose, arabinose, rhamnose and xylose on acid hydrolysis. The $^1$H-NMR spectrum showed five anomeric proton signals at $\delta$ 4.94 (d, $J$=7.5 Hz), 5.18 (d, $J$=7.0 Hz), 5.23 (d, $J$=7.0 Hz), 5.79 (br s) and 6.45 (d, $J$=2.5 Hz). The $^{13}$C-NMR signals due to the C-3-linked sugar moiety were almost the same as those of **3**. On the other hand, in the C-28-linked sugar moiety, glycosylation shifts were observed at the signals of $C_4$ (+9.7 ppm), $C_3$ (−0.6 ppm) and $C_5$ (−2.0 ppm) of the rhamnopyranosyl group compared with those of **6**, indicating that the xylopyranosyl group is attached to $C_4$-OH of the rhamnosyl group. Consequently, **7** was formulated as 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl-28-*O*-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-arabinopyranosyl quillaic acid.

The $[M-H]^-$ ion at *m/z* 1395, 162 mass units more than that of **7**, in the negative FAB-MS of **1**, coupled with result on the sugar components, revealed that **1** has one additional glucosyl unit in **7**. In a comparison of the $^{13}$C-NMR spectrum of **1** with that of **7**, the signal due to C-3 of the rhamnopyranosyl group was displaced downfield by 10.8 ppm, and the signals due to C-2 and C-4 were moved upfield by 1.0 and 4.5 ppm, respectively, while other signals remained almost unshifted. It follows that the glucopyranosyl group is attached to $C_3$-OH of the rhamnosyl group.

Based on the above evidence, **1** was concluded to be 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl-28-*O*-β-D-xylopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→3)]-

α-L-rhamnopyranosyl-(1→2)-α-arabinopyranosyl quillaic acid.

Lucyoside P(**2**) was obtained as colorless needles and elemental analysis was consistent with $C_{64}H_{100}O_{32}\cdot H_2O$. Its FAB-MS showed an $[M-H]^-$ ion at *m/z* 1379, 16 mass units less than that of **1**. On acid hydrolysis, **2** afforded glucuronic acid, galactose, arabinose, rhamnose, xylose and glucose, the same sugar components as **1**. In the $^{13}$C-NMR spectrum of **2**, the signals of the sugar moiety were almost superimposable on those of **1**, while the signals of the aglycone moiety were a little different.

In the same way as described for **1**, **2** was treated with 2% KOH in 50% EtOH to afford a prosapogenin, and treatment with Dowex 50w×8 ($H^+$, form, 80% MeOH) furnished a monomethyl ester (**8**) of the prosapogenin, which was hydrolyzed with acid to give glucuronic acid and galactose. However, the aglycone could not be identified. Since the NMR spectra of **8** suggested the aglycone to be gypsogenin, **8** was converted into a relatively stable saponin (**9**) by reduction with $NaBH_4$, and **9** was identified as 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranosyl-hederagenin by comparing the melting point, optical rotation and $^{13}$C-NMR spectrum with reported data.[9] Accordingly, **8** was formulated as gypsogenin 3-*O*-β-D-galactopyranosyl-(1→2)-methyl-β-D-glucopyranuronate.

Therefore, **2** was determined to be 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl-28-*O*-β-D-xylopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→2)-α-arabinopyranosylgypsogenin. The configuration of the esterified arabinosyl group was presumed to be α- because the arabinosyl group showed almost the same NMR signals as those of **1**.

In the test for fibrinolytic acitivity, lucyosides N and P showed strong activity (82% and 92% respectively), which was almost the same as that of ginsenoside $R_{g1}$ (88%)[10] used as the positive control, employing as an *in vitro* fibrinolysis system the fibrin plate method of Noren *et al.*[11]

## Experimental

Melting points were measured with a Yanagimoto micro melting point apparatus, and are uncorrected. Optical rotations were taken on a JASCO J-20A digital polarimeter. Infrared (IR) spectra were taken on a Hitachi IR-27G. NMR spectra were recorded on JEOL FX-100 and GX-400 spectrometers in $C_5D_5N$ solution using tetramethylsilane (TMS) as an internal standard. The following abbreviations are used: s=singlet, d=doublet, m=multiplet and br=broad. Coupling constants (*J* values) are given in hertz (Hz). $^1$H–$^1$H-COSY, $^{13}$C–$^1$H-COSY and distortionless enhancement by polarization transfer (DEPT) measurement were carried out to verify the assignments. The EI- and FAB-MS were measured on Shimadzu LKB-9000B and JEOL JMS-PX303 mass spectrometers. High performance liquid chromatography (HPLC) was carried out with a Waters ALC/GPC 244 instrument. For column chromatography, Kieselgel 60 (230—400 mesh, Merck) was used.

**Isolation of Saponin** The crushed seeds (1 kg) of *L. cylindrica* were extracted with hexane to remove the fatty oil. The defatted seeds were air-dried, and extracted with $H_2O$. The water extract was passed through an Amberlite XAD-2 column and eluted with MeOH. Crude saponins (45 g) obtained by evaporation of the MeOH eluate were chromatographed with Servachrome XAD-2 (eluted with 40—70% MeOH) to give four fractions, frs. I—IV in order of elution. Fraction III and IV were repeatedly chromatographed on silica gel with $CHCl_3$–MeOH–$H_2O$ [65:35:10 (lower layer)—65:40:10] followed by treatment with Dowex 50w×8 ($H^+$ form, 70% EtOH), and recrystallization from aqueous MeOH, to furnish **1** (4.5 g, 1% from the seeds) and **2** (1.5 g, 0.3%).

Lucyoside N(**1**): Colorless fine needles, mp 268—270 °C, $[\alpha]_D$ −36.1° (*c*=2.4, pyridine). IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3400, 1725, 1635, 1070, 1040. *Anal.* Calcd for $C_{64}H_{100}O_{33}\cdot 7/2H_2O$: C, 52.63; H, 7.38. Found: C, 52.55; H, 6.91. (−)FAB-MS *m/z*: 1395 $(M-H)^-$. $^1$H-NMR δ: aglycone moiety: $CH_3$; 0.83 ($C_{25}$-H), 1.01 ($C_{29}$-H), 1.04 ($C_{26}$-H), 1.14 ($C_{30}$-H), 1.36 ($C_{24}$-H), 1.77 ($C_{27}$-H). >CH–OH, 5.24 (br s, $C_{16}$-H). >C=CH–, 5.58 (m, $C_{12}$-H). ≥C–CHO, 9.90 (s, $C_{23}$-H). sugar moiety: 1.73 (d, *J*=5.5 Hz, $C_6$-$H_3$ of rha); anomeric H, 4.93 (d, *J*=7.5 Hz, glcUA), 5.20 (d, *J*=7.0 Hz, xyl), 5.23 (d, *J*=7.0 Hz, gal), 5.58 (br s, rha), 5.42 (d, *J*=8.0 Hz, glc), 6.45 (d, *J*=3.0 Hz, ara).

Lucyoside P(**2**): Colorless fine needles, mp 228—230 °C, $[\alpha]_D$ −12.2° (*c*=6.5, pyridine). IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3400, 1725, 1630, 1060, 1040. *Anal.* Calcd for $C_{64}H_{100}O_{32}\cdot 6H_2O$: C, 51.61; H, 7.58. Found: C, 51.25; H, 7.14. (−)FAB-MS *m/z*: 1379 $(M-H)^-$. $^1$H-NMR δ: aglycone moiety: –$CH_3$; 0.79 ($C_{25}$-H), 0.93 ($C_{29}$-H), 1.00 ($C_{26}$-H), 1.00 ($C_{30}$-H), 1.24 ($C_{27}$-H), 1.43 ($C_{24}$-H). >C=CH–, 5.40 (m, $C_{12}$-H). ≥C–CHO, 9.95 (s, $C_{23}$-H). sugar moiety: 1.75 (d, *J*=5.5 Hz, $C_6$-$H_3$ of rha); anomeric H, 4.93 (d, *J*=7.0 Hz, glcUA), 5.21 (d, *J*=7.0 Hz, xyl), 5.23 (d, *J*=7.0 Hz, gal), 5.58 (br s, rha), 5.42 (d, *J*=8.0 Hz, glc), 6.45 (d, *J*=3.0 Hz, ara).

**Identification of Component Sugars** Each compound (2—3 mg) was hydrolyzed with 2% $H_2SO_4$ (1 ml) at 100 °C for 1 h. The reaction mixture was diluted with water, neutralized with Amberlite IR-45 and evaporated *in vacuo* to dryness. The residue was checked by HPLC (Shodex RSpak DC-613, 75% $CH_3CN$, 1 ml/min, refraction index (RI), 70 °C) with authentic sugars as standards. These sugars gave the following peaks: glcUA, 2.4 min; rha, 4.8 min; xyl, 5.75 min; ara, 6.2 min; glc, 7.38 min; gal, 8.0 min.

**Alkaline Hydrolysis of 1 and 2** **1** (200 mg) was refluxed with 2% KOH in 50% EtOH (5 ml) for 7 h, then the reaction mixture was neutralized with Amberlite IRC-50 and filtered. The filtrate was subjected to silica gel column chromatography [$CHCl_3$–MeOH–$H_2O$ (25:8:0.5)] followed by treatment with Dowex 50w×8 ($H^+$ form, 80% MeOH), to furnish **3** (80 mg). The same treatment of **2** (100 mg) gave **8** (45 mg).

**3**: mp 220—222 °C, $[\alpha]_D$ +8.4° (*c*=1.3, MeOH). *Anal.* Calcd for $C_{43}H_{66}O_{16}\cdot 9/2H_2O$: C, 56.13; H, 8.21. Found: C, 55.98; H, 8.45. (−)FAB-MS *m/z*: 837 $(M-H)^-$. $^1$H-NMR δ: aglycone moiety: $CH_3$; 0.82 ($C_{25}$-H), 0.96 ($C_{26}$-H), 1.06 ($C_{29}$-H), 1.18 ($C_{30}$-H), 1.40 ($C_{24}$-H), 1.81 ($C_{27}$-H). >CH–OH, 5.24 (br s, $C_{16}$-H). >C=CH–, 5.62 (m, $C_{12}$-H). ≥C–CHO, 9.90 (s, $C_{23}$-H). sugar moiety: 3.75 (s, –COOMe of glcUA); anomeric H, 4.92 (d, *J*=7.5 Hz, glcUA), 5.23 (d, *J*=7.0 Hz, gal).

**8**: mp 155—157 °C, $[\alpha]_D$ −30.9° (*c*=1.2, MeOH). *Anal.* Calcd for $C_{43}H_{66}O_{15}\cdot H_2O$: C, 61.41; H, 8.15. Found: C, 61.82; H, 8.28. (−)FAB-MS *m/z*: 821 $(M-H)^-$. $^1$H-NMR δ: aglycone moiety: $CH_3$; 0.78 ($C_{25}$-H), 0.93 ($C_{26}$-H), 0.97 ($C_{29}$-H), 1.01 ($C_{30}$-H), 1.27 ($C_{27}$-H), 1.40 ($C_{24}$-H). >C=CH–, 5.46 (m, $C_{12}$-H). ≥C–CHO, 9.94 (s, $C_{23}$-H). sugar moiety: 3.75 (s, COOMe of glcUA); anomeric H, 4.91 (d, *J*=7.0 Hz, glcUA), 5.24 (d, *J*=7.0 Hz, gal).

**Mild Acid Hydrolysis of 3** **3** (60 mg) was dissolved in 7% HCl–MeOH (2 ml) and the solution was stirred at room temperature for 7 h. The acid was neutralized with $Ag_2CO_3$ and the mixture was filtered. The filtrate was concentrated to dryness and the residue was chromatographed on silica gel [$CHCl_3$–MeOH (25:1—10:1)] to provide **4** (20 mg) and **5** (10 mg).

**4**: mp 164—166 °C, $[\alpha]_D$ +11.2° (*c*=0.5, MeOH). *Anal.* Calcd for $C_{37}H_{56}O_{11}\cdot 3H_2O$: C, 60.80; H, 7.72. Found: C, 60.97; H, 7.81. (−)FAB-MS *m/z*: 675 $(M-H)^-$. $^1$H-NMR δ: 4.94 (d, *J*=7.5 Hz, anomeric H of glcUA).

**5**: mp 260—262 °C, $[\alpha]_D$ +50.5° (*c*=1.0, MeOH). (−)FAB-MS *m/z*: 485 $(M-H)^-$.

**Enzymatic Hydrolysis of 1 with Cellulase** A solution of **1** (1 g) and cellulase (1 g, Sigma) in 5 mM $NaH_2PO_4$ (pH 4.0) was incubated for 8 d at 37 °C. After cooling, the reaction mixture was concentrated to dryness. The residue was chromatographed on silica gel [$CHCl_3$–MeOH–$H_2O$=65:35:10 (lower layer)], followed by treatment with Dowex 50w×8 ($H^+$ form, 70% EtOH), to furnish **6** (30 mg) and **7** (55 mg).

**6**: mp 204—206 °C, $[\alpha]_D$ −37.2° (*c*=3.1, MeOH). *Anal.* Calcd for $C_{47}H_{72}O_{19}\cdot H_2O$: C, 58.86; H, 7.78. Found: C, 58.58; H, 7.67. (−)FAB-MS *m/z*: 939 $(M-H)^-$. $^1$H-NMR δ: aglycone moiety: $CH_3$; 0.85 ($C_{25}$-H), 1.03 ($C_{29}$-H), 1.03 ($C_{26}$-H), 1.14 ($C_{30}$-H), 1.30 ($C_{24}$-H), 1.79 ($C_{27}$-H). >CH–OH, 5.27 (br s, $C_{16}$-H). >C=CH–, 5.60 (m, $C_{12}$-H). ≥C–CHO, 9.76 (s, $C_{23}$-H). sugar moiety: 1.68 (d, *J*=6.0 Hz, $C_6$-$H_3$ of rha); anomeric H, 4.93 (d, *J*=7.5 Hz, glcUA), 5.82 (br s, rha), 6.49 (d, *J*=3.0 Hz, ara).

**7**: mp 170—172 °C, $[\alpha]_D$ −21.8° (*c*=5.2, MeOH). *Anal.* Calcd for $C_{58}H_{90}O_{28}\cdot 3H_2O$: C, 54.03; H, 7.50. Found: C, 53.97; H, 6.99. (−)FAB-MS *m/z*: 1233 $(M-H)^-$. $^1$H-NMR δ: aglycone moiety: $CH_3$; 0.82 ($C_{25}$-H), 1.02 ($C_{29}$-H), 1.02 ($C_{26}$-H), 1.14 ($C_{30}$-H), 1.42 ($C_{24}$-H), 1.77

($C_{27}$-H). >CH–OH, 5.27 (br s, $C_{16}$-H). >C=CH–, 5.60 (m, $C_{12}$-H). ≥C–CHO, 9.89 (s, $C_{23}$-H). sugar moiety: 1.73 (d, $J$=5.5 Hz, $C_6$-$H_3$ of rha); anomeric H, 4.94 (d, $J$=7.5 Hz, glcUA), 5.18 (d, $J$=7.0 Hz, xyl), 5.23 (d, $J$=7.0 Hz, gal), 5.79 (br s, rha), 6.45 (d, $J$=2.5 Hz, ara).

**$NaBH_4$ Reduction of 8** A solution of **8** (30 mg) in MeOH (3 ml), was treated with $NaBH_4$ (20 mg), and the reaction mixture was stirred for 6 h. After neutralization with AcOH and evaporation of the solvent, the residue was chromatographed on silica gel [$CHCl_3$–MeOH–$H_2O$ (25 : 6 : 0.5)] to give **9** (20 mg).

**9**: mp 248—250 °C, $[\alpha]_D$ +3.53° ($c$=1.7, MeOH). *Anal.* Calcd for $C_{42}H_{68}O_{14}\cdot 3H_2O$: C, 59.28; H, 8.76. Found: C, 59.15; H, 8.59. $^1$H-NMR $\delta$: aglycone moiety: $CH_3$, 0.89, 0.93, 1.00, 1.00, 1.05, 1.26; anomeric H, 5.08 (d, $J$=7.0 Hz), 5.30 (d, $J$=7.0 Hz). After acid hydrolysis of **9** with 2% $H_2SO_4$, the hydrolyzate was analyzed by TLC [$CHCl_3$–MeOH (20 : 1)] with authentic hederagenin as a standard. HPLC check revealed the presence of glucose and galactose.

**Fibrinolytic Activity** a) Preparation of Fibrin Plate (Modified Noren Method): A 0.3% fibrinogen solution in 5 ml of phosphate-buffered saline (pH 7.4, 0.1 M phosphate buffer in 0.15 M NaCl) was added to 5 ml of 1% agarose solution in the same buffer, and 0.1 ml of 100 NIH units/ml thrombin (Sigma) was added to this mixture with or without plasminogen. Then 7 wells of 4 mm diameter were made in each fibrin plate.

b) Measurement of Fibrinolytic Activity: A sample was dissolved in phosphate-buffered saline (2 mg/0.1 ml) to give a test solution. Then 10 $\mu$l aliquots of mixtures of 100 $\mu$l of test solution and 30 $\mu$l urokinase (600 U/ml) were placed in two wells in each of three plates. Phosphate-buffered saline with urokinase was used in place of the sample as a control. After incubation (37 °C for 18 h), the diameters of the sample- and control-lysed areas were measured. These procedures (a and b) were repeated at least 3 times. The value of activity (%) was calculated by use of the following equation:

$$\text{fibrinolytic activity (\%)} = [(\text{lysed area of test solution}/\text{lysed area of control}) - 1] \times 100$$

**References and Notes**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# Synthesis of Pyrido[4,3-*d*]pyrimidin-5(6*H*)-ones *via* Anionic Cycloaddition of Methyl 2,4-Dimethoxy-6-methyl-5-pyrimidinecarboxylate with Imines

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**A new route is described for the synthesis of pyrido[4,3-*d*]pyrimidin-5(6*H*)-ones. Treatment of methyl 2,4-dimethoxy-6-methyl-5-pyrimidinecarboxylate with lithium diisopropylamine in tetrahydrofuran at −70 °C under nitrogen followed by reaction with diaryl imines afforded cycloaddition products. The cycloadducts were aromatized to the corresponding pyrido[4,3-*d*]pyrimidin-5(6*H*)-ones by treatment with *N*-bromosuccinimide *via* a benzylic bromination-dehydrobromination sequence.**

**Keywords** anionic cycloaddition; methyl 2,4-dimethoxy-6-methyl-5-pyrimidinecarboxylate; diaryl imine; pyrido[4,3-*d*]pyrimidin-5(6*H*)-one; aromatization; *N*-bromosuccinimide

Methods for the preparation of pyrido[4,3-*d*]pyrimidin-5(6*H*)-ones are of considerable interest in view of the potentially significant biological activities, such as herbicidal, antihypertensive, and antiallergic activities, of these compounds.[1] Since Ismail and Wibberley[2] reported the first synthesis of pyrido[4,3-*d*]pyrimidine-5(6*H*)-one systems by the reaction of pyrano[4,3-*d*]pyrimidin-5-one with amines, several approaches have been reported by different groups.[1,3] During studies on a new synthesis of biologically useful heterocycles through anionic cyclization using benzylic carbanion species of heteroaromatics,[4] we succeeded in a synthesis of quinazolines[5] and pyrano[4,3-*d*]pyrimidines[6] *via* anionic cyclization of methyl 2,4-dimethoxy-6-methyl-5-pyrimidinecarboxylate (**1**) with olefins and aldehydes, respectively. We describe here a further application of this cyclization, using **1** as a 1,4-dipolar synthon, to a novel synthesis of pyrido[4,3-*d*]pyrimidin-5(6*H*)-ones.

**Cycloaddition of 1 with Imines** Treatment of the lithium salt of **1**, generated by the deprotonation with lithium diisopropylamido (LDA) in tetrahydrofuran (THF), with benzalaniline (**2a**) at −70 °C followed by work-up at 0 °C gave the cycloadduct (**3a**) in 30% yield along with the starting material (**1**) in 27% yield. The structure of **3a** was determined on the basis of the elemental analysis and spectral data. The $^1$H nuclear magnetic resonance ($^1$H-NMR) spectrum showed a methine proton signal at 5.23 (dd, 1H, $J$=6.5 Hz, 2.5 Hz) and two methylene proton signals at 3.79 (dd, 1H, $J$=17, 6.5 Hz) and 3.22 ppm (dd, 1H, $J$=17, 2.5 Hz).

In order to examine the generality of this cyclization, the reactions with various kinds of imine derivatives, such as diaryl imines (**2**), aryl alkyl imines (**4a**, **b**), aldoxime ether (**4c**), *N*-phenyl formimidate (**5**), lactim ether (**6**), and keto alkyl imine (**7**), were also investigated. The reactions with **2b**—**k** afforded the expected cycloadducts (**3b**—**k**) in modest yields accompanied with the recovery of **1** (Chart 1). The results with **2** are summarized in Table I. None of the other imines gave the desired cycloadducts and only the starting ester (**1**) was recovered.

It is noteworthy that in the reaction of *p*-nitrobenzalaniline (**2k**), the aromatized cycloadduct (**8k**) was obtained in 39% yield. This compound was presumably produced by oxidation of the initially formed cycloadduct (**3k**) under the reaction conditions. In fact, when the reaction was quenched immediately at −70 °C, the expected cycloadduct (**3k**) and the aromatized product (**8k**) were obtained in 24 and 7% yields, respectively.

A comparison of the present results with those for the reaction with olefins[5] and aldehydes[6] indicated that the reactivity of the anion from **1** with the imines (**2**) is rather low. Recently, Akiba *et al.* reported that boron trifluoride-etherate activates the imine and the yield in the addition reaction of the alkyl anion is dramatically improved.[7] We tried this procedure, but the desired cycloaddition did not occur, and the starting ester (**1**) and imine (**2**) were recovered. In addition, an attempt to modify the basicity of the lithio species through the use of anhydrous cerium (III) chloride[8] and dialkyl aluminum chloride[9] also failed to produce the cycloadduct.

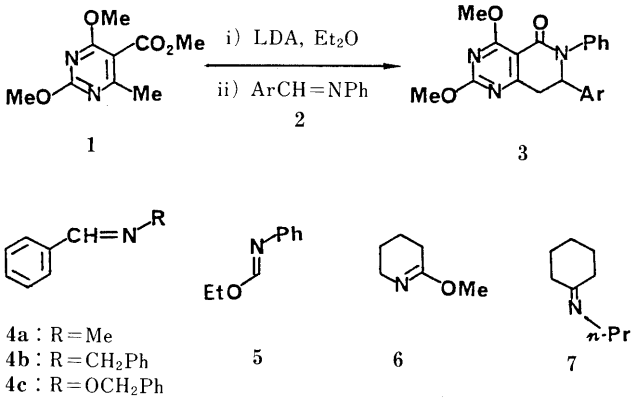
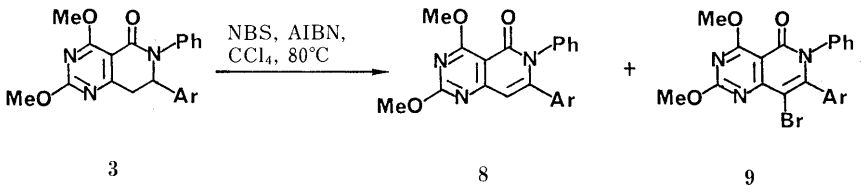



Chart 1

TABLE I. Reaction of **1** with Imines (**2**)

| Run | **2** Ar | | Yield **3** | Yield (%)[a] | Recovery yield **1** (%)[a] |
|---|---|---|---|---|---|
| 1 | $C_6H_5$ | (**2a**) | **3a** | 30 | 27 |
| 2 | 2-$MeOC_6H_4$ | (**2b**) | **3b** | 18 | 29 |
| 3 | 3-$MeOC_6H_4$ | (**2c**) | **3c** | 21 | 30 |
| 4 | 4-$MeOC_6H_4$ | (**2d**) | **3d** | 16 | 32 |
| 5 | 2-$ClC_6H_4$ | (**2e**) | **3e** | 27 | 25 |
| 6 | 3-$ClC_6H_4$ | (**2f**) | **3f** | 8 | 37 |
| 7 | 4-$ClC_6H_4$ | (**2g**) | **3g** | 12 | 35 |
| 8 | 2-$MeC_6H_4$ | (**2h**) | **3h** | 17 | 49 |
| 9 | 2-$NO_2C_6H_4$ | (**2i**) | **3i** | 11 | 61 |
| 10 | 3-$NO_2C_6H_4$ | (**2j**) | **3j** | 16 | 48 |
| 11 | 4-$NO_2C_6H_4$ | (**2k**) | **3k** | 24[b] | 47[b] |

*a*) Isolated yield. *b*) The reaction was quenched at −70 °C.




3 8 9

Chart 2

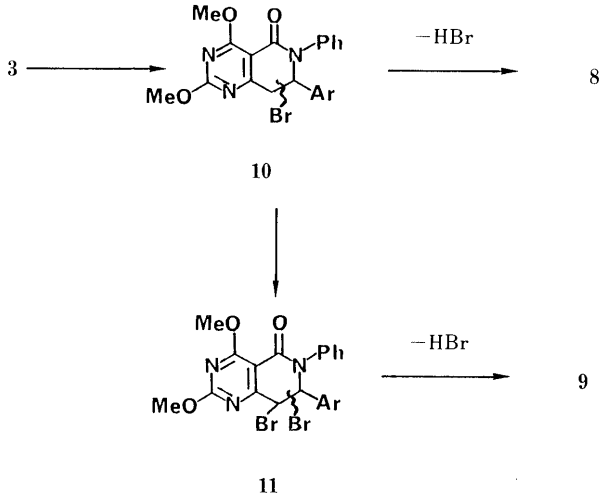



Chart 3

TABLE II. Aromatization of Cycloadducts (3)

| Run | Substrate | | Yield of product | | | |
|---|---|---|---|---|---|---|
| | 3 | Ar | 8 | (%)[a] | 9 | (%)[a] |
| 1 | **3a** | $C_6H_5$ | **8a** | 32 | **9a** | 41 |
| 2 | **3c** | 3-$MeOC_6H_4$ | **8c** | 44 | **9c** | 32 |
| 3 | **3d** | 4-$MeOC_6H_4$ | **8d** | 35 | **9d** | 29 |
| 4 | **3e** | 2-$ClC_6H_4$ | **8e** | 51 | **9e** | 29 |
| 5 | **3g** | 4-$ClC_6H_4$ | **8g** | 45 | **9g** | 11 |
| 6 | **3h** | 2-$MeC_6H_4$ | **8h** | 37 | **9h** | 0 |
| 7 | **3i** | 2-$NO_2C_6H_4$ | **8i** | 7 | **9i** | 0 |

a) Isolated yield.

**Aromatization of the Cycloadduct** Aromatization of the cycloadducts (**3**) to pyrido[4,3-*d*]pyrimidin-5(6*H*)-ones (**8**) was successfully achieved by means of a bromination-dehydrobromination sequence according to our previous work.[6a] In contrast to dihydropyrano[4,3-*d*]pyrimidin-5-ones,[6a] treatment of **3a** with *N*-bromosuccinimide (NBS) in the presence of a catalytic amount of 2,2′-azobisisobutyronitrile (AIBN) afforded the bromo compound (**9a**) in addition to the desired aromatized product (**8a**) in 41 and 32% yields, respectively. The former (**9a**) was readily converted to **8a** in 84% yield by hydrogenolysis over palladium on carbon (Chart 2).

The possibility of the formation of the bromide (**9a**) from the initially formed aromatized product (**8a**) was ruled out since the bromination of **8a** did not occur under the reaction conditions used. A plausible mechanism for the formation of these aromatized products (**8a** and **9a**) is shown in Chart 3. It involves initial bromination at the benzylic position of **3a** to give the monobromo intermediate (**10**), which was transformed to **8a** with the elimination of hydrogen bromide. On the other hand, further bromination of **8a**, followed by loss of hydrogen bromide from the dibromo intermediate (**11**) afforded **9a**.

In a similar fashion, on treatment of the other cycloadducts (**3**) with NBS, the aromatized pyrido[4,3-*d*]pyrimidin-5(6*H*)-ones (**8** and **9**) were obtained. These results are summarized in Table II. In the cases of **3h** and **3i**, the corresponding bromo derivatives could not be obtained, presumably due to the steric hindrance of the *ortho*-substituent to further bromination of the monobromo intermediate (**10**).

In summary, the lithium salt of **1** reacted with diaryl imines to afford cycloadducts (**3**) and this novel anionic cycloaddition reaction provides a new route for the preparation of pyrido[4,3-*d*]pyrimidin-5(6*H*)-one derivatives (**8**).

## Experimental

All melting points were determined on a Yanagimoto melting point apparatus and are uncorrected. Infrared (IR) spectra were measured in $CHCl_3$ with a JASCO A-102 spectrometer, $^1$H-NMR spectra with a JEOL FX-100 spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard, and mass spectra (MS) with a Hitachi M-80 spectrometer. Imines were prepared by removing water by azeotropic distillation from a benzene solution of aldehydes and aniline.

**General Procedure for the Reaction of 1 with Imines** A solution of *n*-BuLi (1.4 M in hexane solution, 1.5 ml, 2.1 mmol) was added dropwise to a stirred solution of diisopropylamine (210 mg, 2.1 mmol) at 0 °C under a nitrogen atmosphere. The mixture was stirred for 15 min and then THF (5 ml) was added. A solution of the ester (**1**, 400 mg, 1.9 mmol) in THF (5 ml) was added dropwise to the solution of LDA over a few minutes at −70 °C. Stirring was continued for 10 min, then a THF solution of the imine (**2**, 1.9 mmol) in THF (4 ml) was added slowly. The reaction mixture was kept at −70 °C for 30 min, then warmed slowly to 0 °C, and quenched with saturated aqueous ammonium chloride (3 ml). The organic layer was separated and the aqueous layer was extracted with dichloromethane (15 ml × 3). The combined organic layers were washed with brine, dried over $Na_2SO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with chloroform–ethyl acetate (9 : 1) to afford the cycloadduct.

**7,8-Dihydro-2,4-dimethoxy-6,7-diphenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (3a)** This was prepared from **1** (580 mg, 3.2 mmol) and benzalaniline (**2a**, 550 mg, 3.3 mmol) in 30% yield (295 mg), mp 119—121 °C (petroleum ether). IR: 1660, 1580, 1565 cm$^{-1}$. $^1$H-NMR $\delta$: 3.22 (1H, dd, $J$=2.5, 17 Hz, $C_8$-H), 3.79 (1H, dd, $J$=6.5, 17 Hz, $C_8$-H), 3.98 (3H, s, OMe), 4.11 (3H, s, OMe), 5.23 (1H, dd, $J$=2.5, 6.5 Hz, $C_7$-H), 7.22 (5H, s, Ph), 7.24 (5H, s, Ph). MS *m/z*: 361 ($M^+$). *Anal.* Calcd for $C_{21}H_{19}N_3O_3$: C, 69.79; H, 5.30; N, 11.63. Found: C, 69.84; H, 5.45; N, 11.49.

**7,8-Dihydro-2,4-dimethoxy-7-(2-methoxyphenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (3b)** This was prepared from **1** (200 mg, 0.94 mmol) and 2-methoxybenzalaniline (**2b**, 280 mg, 1.3 mmol) in 18% yield (65 mg). mp 154—155.5 °C (ether). IR: 1665, 1580, 1560 cm$^{-1}$. $^1$H-NMR $\delta$: 3.28 (1H, dd, $J$=2, 19 Hz, $C_8$-H), 3.68 (1H, dd, $J$=6, 19 Hz, $C_8$-H), 3.81 (3H, s, OMe), 3.96 (3H, s, OMe), 4.11 (3H, s, OMe), 5.52 (1H, dd, $J$=2, 6 Hz, $C_7$-H), 6.7—7.4 (4H, m, ArH), 7.28 (5H, s, Ph). MS *m/z*: 391 ($M^+$). *Anal.* Calcd for $C_{22}H_{21}N_3O_4$: C, 67.50; H, 5.41; N, 10.74. Found: C, 67.72; H, 5.28; N, 10.45.

**7,8-Dihydro-2,4-dimethoxy-7-(3-methoxyphenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (3c)** This was prepared from **1** (200 mg, 0.94 mmol) and 3-methoxybenzalaniline (**2c**, 220 mg, 1.1 mmol) in 21% yield

(77 mg). mp 85—87 °C (ether). IR: 1655, 1580, 1560 cm$^{-1}$. $^1$H-NMR $\delta$: 3.21 (1H, dd, $J$=2.5, 16.5 Hz, $C_8$-H), 3.72 (3H, s, OMe), 3.78 (1H, dd, $J$=6, 16.5 Hz, $C_8$-H), 3.98 (3H, s, OMe), 4.10 (3H, s, OMe), 5.24 (1H, dd, $J$=2.5, 6 Hz, $C_7$-H), 6.5—7.2 (4H, m, ArH), 7.28 (5H, s, Ph). MS $m/z$: 391 ($M^+$). *Anal.* Calcd for $C_{22}H_{21}N_3O_4$: C, 67.50; H, 5.41; N, 10.74. Found: C, 67.63; H, 5.34; N, 10.76.

**7,8-Dihydro-2,4-dimethoxy-7-(4-methoxyphenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (3d)** This was prepared from **1** (150 mg, 0.71 mmol) and 4-methoxybenzalaniline (**2d**, 300 mg, 1.4 mmol) in 16% yield (46 mg) as a pale yellow oil. IR: 1665, 1580, 1560 cm$^{-1}$. $^1$H-NMR $\delta$: 3.12 (1H, dd, $J$=2.5, 16 Hz, $C_8$-H), 3.74 (3H, s, OMe), 3.76 (1H, dd, $J$=6, 16 Hz, $C_8$-H), 3.97 (3H, s, OMe), 4.10 (3H, s, OMe), 5.17 (1H, dd, $J$=2.5, 6 Hz, $C_7$-H), 6.78 (2H, d, $J$=9 Hz, ArH), 7.14 (2H, d, $J$=9 Hz, ArH), 7.26 (5H, s, Ph). High-resolution MS Calcd for $C_{22}H_{21}N_3O_4$: 391.1530. Found: 391.1508.

**7,8-Dihydro-2,4-dimethoxy-7-(2-chlorophenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (3e)** This was prepared from **1** (200 mg, 0.94 mmol) and 2-chlorobenzalaniline (**2e**, 280 mg, 1.3 mmol) in 27% yield (101 mg). mp 158—159.5 °C (ether). IR: 1660, 1580, 1560 cm$^{-1}$. $^1$H-NMR $\delta$: 3.32 (1H, dd, $J$=2, 17 Hz, $C_8$-H), 3.78 (1H, dd, $J$=7, 17 Hz, $C_8$-H), 3.97 (3H, s, OMe), 4.13 (3H, s, OMe), 5.58 (1H, dd, $J$=2, 7 Hz, $C_7$-H), 7.0—7.4 (4H, m, ArH), 7.26 (5H, s, Ph). MS $m/z$: 395, 397 ($M^+$). *Anal.* Calcd for $C_{21}H_{18}ClN_3O_3$: C, 63.72; H, 4.58; N, 10.62. Found: C, 63.57; H, 4.49; N, 10.60.

**7,8-Dihydro-2,4-dimethoxy-7-(3-chlorophenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (3f)** This was prepared from **1** (200 mg, 0.94 mmol) and 3-chlorobenzalaniline (**2f**, 280 mg, 1.3 mmol) in 8% yield (30 mg) as a pale yellow oil. IR: 1660, 1580, 1560 cm$^{-1}$. $^1$H-NMR $\delta$: 3.18 (1H, dd, $J$=2.5, 17 Hz, $C_8$-H), 3.80 (1H, dd, $J$=6.5, 17 Hz, $C_8$-H), 3.99 (3H, s, OMe), 4.11 (3H, s, OMe), 5.19 (1H, dd, $J$=2.5, 6.5 Hz, $C_7$-H), 6.9—7.3 (9H, m, ArH). High-resolution MS Calcd for $C_{21}H_{18}ClN_3O_3$: 395.1034. Found: 395.1008.

**7,8-Dihydro-2,4-dimethoxy-7-(4-chlorophenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (3g)** This was prepared from **1** (200 mg, 0.94 mmol) and 4-chlorobenzalaniline (**2g**, 280 mg, 1.3 mmol) in 12% yield (43 mg) as a pale yellow oil. IR: 1660, 1580, 1560 cm$^{-1}$. $^1$H-NMR $\delta$: 3.16 (1H, dd, $J$=2.5, 17 Hz, $C_8$-H), 3.77 (1H, dd, $J$=6.5, 17 Hz, $C_8$-H), 3.98 (3H, s, OMe), 4.11 (3H, s, OMe), 5.20 (1H, dd, $J$=2.5, 6.5 Hz, $C_7$-H), 7.18 (2H, d, $J$=8 Hz, ArH), 7.24 (2H, d, $J$=8 Hz, ArH), 7.27 (5H, s, Ph). High-resolution MS Calcd for $C_{21}H_{18}ClN_3O_3$: 395.1034. Found: 395.1026.

**7,8-Dihydro-2,4-dimethoxy-7-(2-methylphenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (3h)** This was prepared from **1** (210 mg, 1.0 mmol) and 2-methylbenzalaniline (**2h**, 215 mg, 1.1 mmol) in 17% yield (51 mg). mp 97—99 °C (ether). IR: 1665, 1580, 1560 cm$^{-1}$. $^1$H-NMR $\delta$: 2.29 (3H, s, Me), 3.08 (1H, dd, $J$=2, 17 Hz, $C_8$-H), 3.76 (1H, dd, $J$=7, 17 Hz, $C_8$-H), 3.97 (3H, s, OMe), 4.13 (3H, s, OMe), 5.37 (1H, dd, $J$=2, 7 Hz, $C_7$-H), 6.9—7.4 (9H, m, ArH). High-resolution MS Calcd for $C_{22}H_{21}N_3O_3$: 375.1583. Found: 375.1577.

**7,8-Dihydro-2,4-dimethoxy-7-(2-nitrophenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (3i)** This was prepared from **1** (424 mg, 2.0 mmol) and 2-nitrobenzalaniline (**2i**, 498 mg, 2.2 mmol) in 11% yield (93 mg). mp 178—184 °C (AcOEt). IR: 1670, 1590, 1570 cm$^{-1}$. $^1$H-NMR $\delta$: 3.19 (1H, dd, $J$=2, 17 Hz, $C_8$-H), 3.92 (1H, dd, $J$=5.5, 17 Hz, $C_8$-H), 3.98 (3H, s, OMe), 4.13 (3H, s, OMe), 6.04 (1H, dd, $J$=2, 5.5 Hz, $C_7$-H), 7.0—7.7 (7H, m, ArH), 8.00 (1H, dd, $J$=8, 2 Hz, ArH). High-resolution MS Calcd for $C_{21}H_{18}N_4O_5$: 406.1277. Found: 406.1262.

**7,8-Dihydro-2,4-dimethoxy-7-(3-nitrophenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (3j)** This was prepared from **1** (106 mg, 0.5 mmol) and 3-nitrobenzalaniline (**2j**, 124 mg, 0.55 mmol) in 16% yield (33 mg). mp 96—99 °C (AcOEt). IR: 1660, 1580, 1560 cm$^{-1}$. $^1$H-NMR $\delta$: 3.24 (1H, dd, $J$=2.5, 17 Hz, $C_8$-H), 3.85 (1H, dd, $J$=6.5, 17 Hz, $C_8$-H), 3.99 (3H, s, OMe), 4.12 (3H, s, OMe), 5.37 (1H, dd, $J$=2.5, 6.5 Hz, $C_7$-H), 7.0—8.13 (9H, m, ArH). High-resolution MS Calcd for $C_{21}H_{18}N_4O_5$: 406.1277. Found: 406.1286.

**7,8-Dihydro-2,4-dimethoxy-7-(4-nitrophenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (3k) and 2,4-Dimethoxy-7-(4-nitrophenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (8k)** i) These were prepared from **1** (210 mg, 1.0 mmol) and 4-nitrobenzalaniline (**2k**, 249 mg, 1.1 mmol) by quenching at −70 °C.

**3k**: 24% yield (90 mg). mp 117—121 °C (AcOEt). IR: 1660, 1580, 1560 cm$^{-1}$. $^1$H-NMR $\delta$: 3.19 (1H, dd, $J$=2.5, 17 Hz, $C_8$-H), 3.82 (1H, dd, $J$=6, 17 Hz, $C_8$-H), 3.99 (3H, s, OMe), 4.12 (3H, s, OMe), 5.34 (1H, dd, $J$=2.5, 6 Hz, $C_7$-H), 7.1—7.3 (5H, m, ArH), 7.45 (2H, d, $J$=9 Hz, ArH), 8.16 (2H, d, $J$=9 Hz, ArH). High-resolution MS Calcd for $C_{21}H_{18}N_4O_5$: 406.1277. Found: 406.1281.

**8k**: 7% yield (26 mg). mp 117—121 °C (AcOEt–*n*-hexane). IR: 1670, 1620, 1590 cm$^{-1}$. $^1$H-NMR $\delta$: 4.10 (3H, s, OMe), 4.13 (3H, s, OMe), 6.51 (1H, s, $C_8$-H), 7.0—7.3 (5H, m, ArH), 7.37 (2H, d, $J$=8 Hz, ArH), 8.02 (2H, d, $J$=8 Hz, ArH). High-resolution MS Calcd for $C_{21}H_{16}N_4O_5$: 404.1120. Found: 404.1122.

ii) The aromatized cycloadduct (**8k**) was prepared from **1** (210 mg, 1.0 mmol) and 4-nitrobenzalaniline (**2k**, 250 mg, 1.1 mmol) by quenching at room temperature in 39% yield (165 mg). This was identical with the sample obtained in i).

**General Procedure for Aromatization of the Cycloadduct** A mixture of a cycloadduct (**3**, 0.50 mmol), NBS (90 mg, 0.55 mmol), and AIBN (5 mg, 0.03 mmol) in $CCl_4$ (15 ml) was refluxed for 2 h. After cooling, the reaction mixture was poured into ice-water (30 ml) and extracted with $CHCl_3$ (30 ml). The extract was successively washed with aqueous 10% $Na_2S_2O_3$ (40 ml), and brine (40 ml), and then dried over $Na_2SO_4$. This solvent was removed *in vacuo* and the residue was purified by column chromatography on silica gel with dichloromethane–ethyl acetate (19 : 1) to afford **9** and **8** from the first and second fractions, respectively.

**2,4-Dimethoxy-6,7-diphenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (8a) and 8-Bromo-2,4-dimethoxy-6,7-diphenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (9a)** These were prepared from **3a** (70 mg, 0.20 mmol) and NBS (46 mg, 0.28 mmol).

**8a**: 32% yield (22 mg). mp 228—229 °C (dichloromethane–petroleum ether). IR: 1670, 1620, 1570, 1545 cm$^{-1}$. $^1$H-NMR $\delta$: 4.03 (3H, s, OMe), 4.11 (3H, s, OMe), 6.47 (1H, s, $C_8$-H), 6.9—7.5 (10H, m, Ph × 2). MS $m/z$: 359 ($M^+$). *Anal.* Calcd for $C_{21}H_{17}N_3O_3$: C, 70.18; H, 4.77; N, 11.69. Found: C, 70.41; H, 4.65; N, 11.88.

**9a**: 41% yield (35 mg). mp 275—277 °C (petroleum ether). IR: 1670, 1620, 1580, 1540 cm$^{-1}$. $^1$H-NMR $\delta$: 4.12 (6H, s, OMe × 2) 6.7—7.4 (10H, m, Ph × 2). MS $m/z$: 437, 439 ($M^+$). *Anal.* Calcd for $C_{21}H_{16}BrN_3O_3$: C, 57.55; H, 3.68; N, 9.59. Found: C, 57.62; H, 3.65; N, 9.34.

**2,4-Dimethoxy-7-(3-methoxyphenyl)-6-phenylpyrido[4,3-*d*]-pyrimidin-5(6*H*)-one (8c) and 8-Bromo-2,4-dimethoxy-7-(3-methoxyphenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (9c)** These were prepared from **3c** (45 mg, 0.11 mmol) and NBS (24 mg, 0.13 mmol).

**8c**: 44% yield (19 mg). mp 179—181 °C (dichloromethane–petroleum ether). IR: 1660, 1620, 1575, 1540 cm$^{-1}$. $^1$H-NMR $\delta$: 3.64 (3H, s, OMe), 4.09 (3H, s, OMe), 4.16 (3H, s, OMe), 6.56 (1H, s, $C_8$-H), 6.6—7.4 (9H, m, ArH). MS $m/z$: 389 ($M^+$). *Anal.* Calcd for $C_{22}H_{19}N_3O_4$: C, 67.85; H, 4.92; N, 10.79. Found: C, 67.71; H, 4.81; N, 10.77.

**9c**: 32% yield (17 mg). mp 153—155 °C (petroleum ether). IR: 1660, 1610, 1570, 1535 cm$^{-1}$. $^1$H-NMR $\delta$: 3.73 (3H, s, OMe), 4.15 (3H, s, OMe), 4.17 (3H, s, OMe), 6.7—7.3 (9H, m, ArH). MS $m/z$: 467, 469 ($M^+$). *Anal.* Calcd for $C_{22}H_{18}BrN_3O_4$: C, 56.42; H, 3.87; N, 8.97. Found: C, 56.39; H, 3.91; N, 8.76.

**2,4-Dimethoxy-7-(4-methoxyphenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (8d) and 8-Bromo-2,4-dimethoxy-7-(4-methoxyphenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (9d)** These were prepared from **3d** (22 mg, 0.056 mmol) and NBS (12 mg, 0.067 mmol).

**8d**: 35% yield (8 mg). mp 53—54 °C (dichloromethane–petroleum ether). IR: 1665, 1615, 1570, 1540 cm$^{-1}$. $^1$H-NMR $\delta$; 3.73 (3H, s, OMe), 4.09 (3H, s, OMe), 4.15 (3H, s, OMe), 6.52 (1H, s, $C_8$-H), 6.68 (2H, d, $J$=8.5 Hz, ArH), 6.9—7.3 (5H, m, ArH), 7.07 (2H, d, $J$=8.5 Hz, ArH). High-resolution MS Calcd for $C_{22}H_{19}N_3O_4$: 389.1374. Found: 389.1374.

**9d**: 29% yield (7.5 mg). mp 201—203 °C (petroleum ether). IR: 1665, 1610, 1575, 1535 cm$^{-1}$. $^1$H-NMR $\delta$: 3.73 (3H, s, OMe), 3.99 (3H, s, OMe), 4.16 (3H, s, OMe), 6.72 (2H, d, $J$=9 Hz, ArH), 6.9—7.4 (7H, m, ArH). High-resolution MS Calcd for $C_{22}H_{18}BrN_3O_4$: 467.0481. Found: 467.0470.

**2,4-Dimethoxy-7-(2-chlorophenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (8e) and 8-Bromo-2,4-dimethoxy-7-(2-chlorophenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (9e)** These were prepared from **3e** (33 mg, 0.08 mmol) and NBS (18 mg, 0.10 mmol).

**8e**: 51% yield (17 mg). mp 216—218 °C (dichloromethane–petroleum ether). IR: 1665, 1620, 1575, 1545 cm$^{-1}$. $^1$H-NMR $\delta$: 4.09 (3H, s, OMe), 4.16 (3H, s, OMe), 6.49 (1H, s, $C_8$-H), 7.0—7.4 (9H, m, ArH). MS $m/z$: 393, 395 ($M^+$). *Anal.* Calcd for $C_{21}H_{16}ClN_3O_3$: C, 64.04; H, 4.10; N, 10.67. Found: C, 64.15; H, 4.11; N, 10.71.

**9e**: 29% yield (12 mg). mp 263—265 °C (petroleum ether). IR: 1665, 1605, 1570, 1535 cm$^{-1}$. $^1$H-NMR $\delta$: 4.17 (6H, s, OMe × 2), 6.9—7.4 (9H, m, ArH). MS $m/z$: 471, 473 ($M^+$). *Anal.* Calcd for $C_{21}H_{15}BrClN_3O_3$: C, 53.35; H, 3.20; N, 8.89. Found: C, 53.39; H, 3.37; N, 8.71.

**2,4-Dimethoxy-7-(4-chlorophenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (8g) and 8-Bromo-2,4-dimethoxy-7-(4-chlorophenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (9g)** These were prepared from **3g**

(25 mg, 0.06 mmol) and NBS (14 mg, 0.07 mmol).

**8g**: 45% yield (11 mg). mp 188—190 °C (dichloromethane–petroleum ether). IR: 1665, 1620, 1575, 1540 $cm^{-1}$. $^1$H-NMR $\delta$: 4.08 (3H, s, OMe), 4.15 (3H, s, OMe), 6.51 (1H, s, $C_8$-H), 6.9—7.4 (9H, m, ArH). High-resolution MS Calcd for $C_{21}H_{16}ClN_3O_3$: 393.0881. Found: 393.0879.

**9g**: 11% yield (3.3 mg). mp 242—246 °C (petroleum ether). IR: 1665, 1600, 1575, 1535 $cm^{-1}$. $^1$H-NMR $\delta$: 4.16 (3H, s, OMe), 4.18 (3H, s, OMe), 6.9—7.4 (9H, m, ArH). High-resolution MS Calcd for $C_{21}H_{15}BrClN_3O_3$: 470.9984. Found: 470.9978.

**2,4-Dimethoxy-7-(2-methylphenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (8h)** This was prepared from **3h** (41 mg, 0.11 mmol) and NBS (23 mg, 0.13 mmol) in 37% yield (15 mg). mp 111—114 °C (dichloromethane–petroleum ether). IR: 1665, 1620, 1575, 1540 $cm^{-1}$. $^1$H-NMR $\delta$: 2.20 (3H, s, Me), 4.09 (3H, s, OMe), 4.16 (3H, s, OMe), 6.42 (1H, s, $C_8$-H), 6.9—7.4 (9H, m, ArH). High-resolution MS Calcd for $C_{22}H_{19}ClN_3O_3$: 373.1424. Found: 373.1418.

**2,4-Dimethoxy-7-(2-nitrophenyl)-6-phenylpyrido[4,3-*d*]pyrimidin-5(6*H*)-one (8i)** This was prepared from **3i** (24 mg, 0.06 mmol) and NBS (21 mg, 0.12 mmol) in 7% yield (2 mg). mp 114—116 °C (dichloromethane–petroleum ether). IR: 1665, 1620, 1575, 1540 $cm^{-1}$. $^1$H-NMR $\delta$: 4.08 (3H, s, OMe), 4.17 (3H, s, OMe), 6.46 (1H, s, $C_8$-H), 6.8—7.4 (8H, m, ArH), 7.89 (1H, d, $J$=8 Hz, ArH). High-resolution MS Calcd for $C_{21}H_{16}N_4O_5$: 404.1119. Found: 404.1097.

**Hydrogenolysis of 9a** A mixture of **9a** (4.6 mg, 0.01 mmol) and 10% Pd–C (15 mg) in EtOH (4 ml) was stirred for 2 h under $H_2$. After removal of Pd–C by filtration, the filtrate was concentrated *in vacuo* and the residue was purified by column chromatography on silica gel with ethyl acetate–*n*-hexane (1 : 2) to afford **8a** (3.2 mg, 84%). This was identical with an authentic sample obtained by the aromatization of **3a**.

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# New Hypocholesterolemic Abietamide Derivatives. II. Synthesis and Hypocholesterolemic Activity of *N*-Phenyl-$\Delta^8$-dihydroabietamides

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**A series of *N*-phenyl-$\Delta^8$-dihydroabietamide analogs were prepared and tested for hypocholesterolemic activity. The effects of substituents of the phenyl moiety on the activities were quantitatively analyzed by using various substituent parameters. The activities were enhanced by the electron-donating effect of *ortho* and *para* substituents and the bulkiness of *ortho* substituents. A combination of 2,6-dimethylaniline with resin acids other than $\Delta^8$-dihydroabietic acid produced lower activities than *N*-(2,6-dimethylphenyl)-$\Delta^8$-dihydroabietamide, abietane-type carboxamides being somewhat stronger than pimarane-type carboxamides. The conversion of the carboxamide group to other groups resulted in more or less of a decrease in activity, giving evidence that the carboxamide group is important to hypocholesterolemic activity.**

**Keywords** *N*-phenyl-$\Delta^8$-dihydroabietamide; resin acid; secondary carboxamide; hypocholesterolemic activity; quantitative structure–activity relationship; regression analysis; cholesterol-fed rats

The etiological cause of atherosclerosis remains obscure although a considerable amount of research has been done on it. Therapeutic approaches to this disease have therefore focused on the minimization of epidemiologically defined risk factors, primarily on the reduction of serum cholesterol levels.

In a previous paper,[1] we reported that the coexistence of a secondary carboxamide group in resin acid arylmethylamides and a phenyl ring was a necessary constituent of the amine moiety in abietamide derivatives for hypocholesterolemic activity and that tetrahydroabietic (I) or $\Delta^8$-dihydroabietic acid (II) was superior in terms of the activity to their less saturated analogs (*e.g.* abietic (III) or dehydroabietic acid (IV)) as an abietane-type acid moiety. The activity of *N*-benzyl-$\Delta^8$-dihydroabietamide (V) was remarkably intensified by substitution in the phenyl ring or at the benzyl position in the amine moiety, as represented by *N*-(4-methoxybenzyl)-(VI) and *N*-(α-benzylbenzyl)-$\Delta^8$-dihydroabietamide (VII).

Further investigation revealed that some $\Delta^8$-dihydroabietanilide derivatives had potent hypocholesterolemic activity. We here report the synthesis of arylamides of $\Delta^8$-dihydroabietic acid, some other abietane-type acids, and some pimarane-type acids. To elucidate the factors that affected the activities, quantitative structure–activity relationship (QSAR) analysis was done for $\Delta^8$-dihydroabietic acid derivatives with various substituents of the phenyl moiety.

**Structure–Activity Relationship** Many of the $\Delta^8$-dihydroabietanilide derivatives shown in Table I have hypocholesterolemic properties. Various modifications were made in the substitution pattern of the phenyl ring of the aniline moiety in order to obtain more potent cholesterol-lowering activity than that of the parent carboxamide (**1**). These data were analyzed quantitatively by using the substituent parameters listed in Table II.

Equation 1 is given for the unsubstituted and 17 mono-*ortho*-, mono-*meta*-, and mono-*para*-substituted compounds in Table I.

$$\mathrm{pID}_{50} = -1.516(\pm 0.707)\sigma - 0.353(\pm 0.276)\mathrm{E_s(AMD)} - 2.372(\pm 0.252) \quad (1)$$

$$n=18,\ s=0.394,\ r=0.790,\ F=12.5$$

In these and the following equations, $n$ is the number of

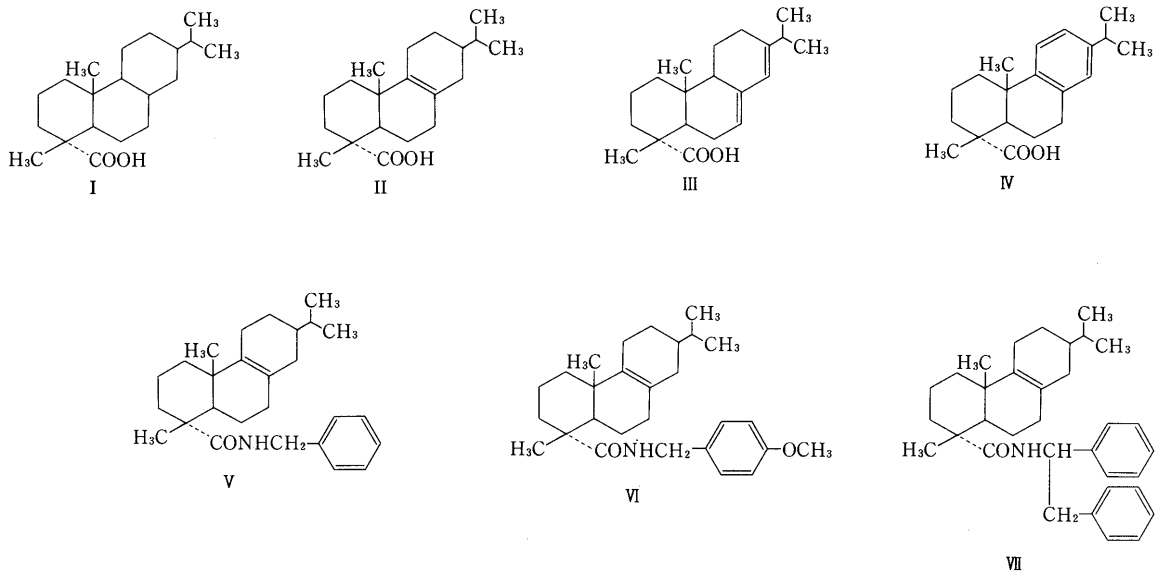
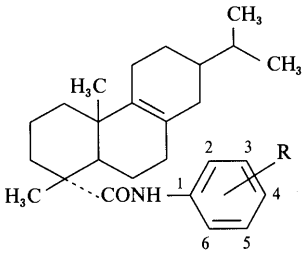



Chart 1

TABLE I. Hypocholesterolemic Activity of $\Delta^8$-Dihydroabietanilide Derivatives




| No. | R[a] | Formula | Yield (%) | mp (°C) | Crystn. solvent[b] | % inhibition 3 | 10 | 30 | 100 | 300 | 1000 | 3000 ppm[c] | $ID_{50}$ (ppm) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | H | $C_{26}H_{37}NO$ | 89 | 151—152 | E | — | — | — | 18 | 67 | 97 | — | 175 |
| **2** | 2-Me | $C_{27}H_{39}NO$ | 74 | 146—148 | A | 3 | 19 | 55 | 83 | 98 | — | — | 26 |
| **3** | 2-Et | $C_{28}H_{41}NO$ | 83 | 167—169 | EA | 13 | 43 | 78 | 98 | — | — | — | 12 |
| **4** | 2-Ph | $C_{32}H_{41}NO$ | 65 | 45—46 | PE | 5 | 11 | 41 | 103 | — | — | — | 37 |
| **5** | 2-$CO_2$Et | $C_{29}H_{41}NO_3$ | 58 | Oil | — | — | — | — | — | 23 | — | — | — |
| **6** | 2-Cl | $C_{26}H_{36}NOCl$ | 82 | 74—76 | A | — | — | — | 9 | 22 | 87 | 98 | 540 |
| **7** | 2-Br | $C_{26}H_{36}NOBr$ | 48 | 89—90 | E | — | — | — | 3 | 36 | 87 | 103 | 420 |
| **8** | 2-I | $C_{26}H_{36}NOI$ | 67 | 108—110 | H | — | — | — | 16 | 32 | 94 | 102 | 430 |
| **9** | 2-$NO_2$ | $C_{26}H_{36}N_2O_3$ | 70 | Oil | — | — | — | — | 11 | 19 | 72 | 98 | 680 |
| **10** | 3-Me | $C_{27}H_{39}NO$ | 78 | 112—115 | M | — | — | −36 | 8 | 85 | — | — | 195 |
| **11** | 3-$CF_3$ | $C_{27}H_{36}NOF_3$ | 69 | 137—138 | M | — | — | — | 9 | 34 | 64 | 95 | 580 |
| **12** | 3-OMe | $C_{27}H_{39}NO_2$ | 76 | 151—152 | M | — | −9 | 18 | 30 | 82 | — | — | 175 |
| **13** | 3-OPh | $C_{32}H_{41}NO_2$ | 63 | 92—94 | H | −3 | 2 | −10 | 12 | — | — | — | — |
| **14** | 3-Br | $C_{26}H_{36}NOBr$ | 85 | 98—101 | E | — | — | 4 | 10 | 61 | 94 | — | 250 |
| **15** | 4-Me | $C_{27}H_{39}NO$ | 71 | 84—86 | H–B | — | — | — | — | 22 | 66 | 91 | 480 |
| **16** | 4-OEt | $C_{28}H_{41}NO_2$ | 85 | 139—140 | M | — | 24 | 35 | 72 | 89 | — | — | 55 |
| **17** | 4-$CO_2$Me | $C_{28}H_{39}NO_3$ | 69 | 152—154 | M | — | — | — | — | 13 | — | 48 | 3000 |
| **18** | 4-Ph | $C_{32}H_{41}NO$ | 73 | 195—197 | A–C | — | — | — | 31 | 31 | 53 | — | 800 |
| **19** | 4-Cl | $C_{26}H_{36}NOCl$ | 61 | 90—92 | H | — | — | — | 15 | 43 | 87 | 88 | 360 |
| **20** | 4-OH | $C_{26}H_{37}NO_2$ | 72 | 180 | E | — | — | 10 | 61 | 76 | 110 | — | 70 |
| **21** | 2,3-$Me_2$ | $C_{28}H_{41}NO$ | 70 | 182—184 | A | 9 | 53 | 67 | 91 | 114 | — | — | 9 |
| **22** | 2,4-$Me_2$ | $C_{28}H_{41}NO$ | 68 | 148—150 | A | — | 42 | 58 | 88 | 95 | — | — | 20 |
| **23** | 2,5-$Me_2$ | $C_{28}H_{41}NO$ | 62 | 85—87 | B | 2 | 41 | 57 | 88 | 111 | — | | 17 |
| **24** | 2,6-$Me_2$ | $C_{28}H_{41}NO$ | 76 | 195—197 | MEK | 31 | 53 | 79 | 97 | 103 | — | — | 8.8 |
| **25** | 3,4-$Me_2$ | $C_{28}H_{41}NO$ | 74 | 113—115 | A | — | — | — | −27 | 22 | 37 | 64 | 1700 |
| **26** | 3,5-$Me_2$ | $C_{28}H_{41}NO$ | 70 | 151—154 | A | 9 | −11 | −8 | 46 | 98 | — | — | 108 |
| **27** | 2-Me-6-Et | $C_{29}H_{43}NO$ | 72 | 192—195 | A | 35 | 68 | 91 | 99 | 111 | — | — | 5.5 |
| **28** | 2,6-$Et_2$ | $C_{30}H_{45}NO$ | 79 | 219—221 | B–EA | 25 | 47 | 82 | 89 | — | — | — | 11 |
| **29** | 2,6-iso-$Pr_2$ | $C_{32}H_{49}NO$ | 69 | 255—258 | C–M | −2 | 18 | 38 | 52 | — | — | — | 84 |
| **30** | 2-Me-5-Cl | $C_{27}H_{38}NOCl$ | 82 | 160—161 | EA | — | 6 | 59 | 72 | 84 | — | — | 22 |
| **31** | 2-Me-6-Cl | $C_{27}H_{38}NOCl$ | 86 | 187—189 | EA | 26 | 23 | 63 | 96 | 109 | — | — | 20 |
| **32** | 2,5-$(MeO)_2$ | $C_{28}H_{41}NO_3$ | 65 | 120—123 | A | — | −1 | −4 | 39 | 76 | — | — | 140 |
| **33** | 2,4-$Cl_2$ | $C_{26}H_{35}NOCl_2$ | 72 | 102—104 | A | — | — | — | 4 | 48 | 78 | 89 | 310 |
| **34** | 2,6-$Cl_2$ | $C_{26}H_{35}NOCl_2$ | 63 | 190—192 | A–M | −22 | −22 | 52 | 84 | 100 | — | — | 28 |
| **35** | 3,4-$Cl_2$ | $C_{26}H_{35}NOCl_2$ | 82 | 110—111 | B | — | — | — | 3 | 27 | 62 | 85 | 660 |
| **36** | 3-OH-4-$CO_2$H | $C_{27}H_{37}NO_4$ | 74 | 260 | EA | — | — | — | — | 7 | 19 | 66 | 1800 |
| **37** | 2,4,5-$Me_3$ | $C_{29}H_{43}NO$ | 68 | 124—125 | A | 21 | 47 | 63 | 87 | — | — | — | 13 |
| **38** | 2,4,6-$Me_3$ | $C_{29}H_{43}NO$ | 82 | 215—218 | A | 27 | 55 | 85 | 100 | — | — | — | 8 |
| **39** | 2,4,6-$Me_3$-3-Br | $C_{29}H_{42}NOBr$ | 72 | 231—232 | MEK | 38 | 55 | 74 | 98 | — | — | — | 7 |
| **40** | 2,3,5,6-$F_4$ | $C_{26}H_{33}NOF_4$ | 53 | 215—217 | H | 0 | 0 | 25 | 45 | 92 | — | — | 115 |

*a*) Me: methyl, Et: ethyl, Ph: phenyl. *b*) A=acetone, B=benzene, C=chloroform, E=ethanol, EA=ethyl acetate, H=hexane, M=methanol, MEK=methylethylketone, PE=petroleum ether. *c*) Concentration of test compounds in the atherogenic diet containing 1% cholesterol and 0.25% cholate.

compounds, $s$ is the standard deviation, $r$ is the correlation coefficient, and $F$ is the value of the $F$-ratio between variances of the observed and calculated data. The figures in parentheses are the 95% confidence interval of the regression coefficients and intercept.

In Eq. 1, $\sigma$ is the Hammett electronic substituent constant.[2] The Hammett $\sigma$ constant for *ortho*-substituents is unavailable, and we put $\sigma_{ortho}=\sigma_{para}$.[3] $E_s$(AMD) is a set of steric parameter for *ortho*-substituents defined by us.[4] The $E_s$ (AMD) value for *meta*- and *para*-substituents is necessarily zero. Equation 1 means that the electron-donating substituents and bulky *ortho*-substituents favor the activity.

The variations in the activity of *meta* derivatives are small, showing relatively weak effects of *meta*-substituents. For this reason, we examined the significance of the electronic effect of *meta*-substituents by separating the $\sigma$ paramater set into $\sigma(m)$ for *meta*-substituents and $\sigma(op)$ for *ortho*- and *para*-substituents. These modified electronic parameter sets are listed in Table II.

$$\begin{aligned} pID_{50} = &-0.288(\pm 1.393)\sigma(m) - 1.861(\pm 0.715)\sigma(op) \\ &-0.456(\pm 0.266)E_s(AMD) - 2.473(\pm 0.246) \\ &n=18,\ s=0.350,\ r=0.850,\ F=12.2 \end{aligned} \quad (2)$$

In Eq. 2, the 95% confidence interval for $\sigma(m)$ term was very large, and $\sigma(m)$ was significant only above 26%. Exclusion of the $\sigma(m)$ term gave Eq. 3.

$$pID_{50} = -1.862(\pm 0.689)\sigma(op) - 0.468(\pm 0.247)E_s(AMD) - 2.490(\pm 0.214) \quad (3)$$
$$n=18,\ s=0.340,\ r=0.849,\ F=19.3$$

The quality of Eq. 3 is comparable to that of Eq. 2. The effects of *para*- and *ortho*-substituents are large, whereas those of *meta*-substituents are actually negligible. The correlation coefficient of Eq. 3 is not excellent, but the standard deviation is small enough.

Mono-substituted and poly-substituted analogs were then analyzed together, to give Eq. 4.

$$pID_{50} = -1.488(\pm 0.413)\sigma(op) - 0.471(\pm 0.136)E_s(AMD) - 2.416(\pm 0.174) \quad (4)$$
$$n=36,\ s=0.350,\ r=0.899,\ F=69.0$$

The addition of the $\sigma(m)$ term did not improve the correlation, as for the case of Eq. 3. Equation 4 shows that the electron-donating effect of *ortho*- and *para*-substituents and the bulkiness of *ortho*-substituents enhanced the hypocholesterolemic activities of $\Delta^8$-dihydroabietanilide derivatives. Although the structural variations are large for the 36 compounds of Eq. 4, only two substituent parameters are needed to explain their activity variations. The expected values according to Eq. 4 are summarized in Table II.

To get more active compounds, there are two ways. First is to increase the bulkiness of *ortho*-substituents. However, 2,6-diisopropyl derivative is less active than 2,6-dimethyl or 2-ethyl-6-methyl derivatives, as can be seen in Table II. This fact suggests that highly crowded substituents such as 2,6-di-*tert*-butyl are not favorable to the activities and that the 2,6-dimethyl or 2-ethyl-6-methyl are most desirable *ortho*-substituents. Second is to increase the electron-donating properties of *ortho*- and *para*-substituents. Alkyl groups at the *ortho* positions have electron-donating properties. Hence, substitution of electron-donating groups such as OH, OMe, $NMe_2$ at the *para* position of 2,6-dimethyl or 2-ethyl-6-methyl derivatives should increase the hypocholesterolemic activities.

In Eq. 4, the use of the Taft–Kutter–Hansch $E_s$ steric parameter[5] instead of $E_s(AMD)$ gave statistically less significant equations (correlation coefficient $r=0.814$ for the 18 compounds, and $r=0.896$ for the 36 compounds). We also examined the hydrophobic effect on the activities, but hydrophobic substituent parameters such as 1-octanol/water partition coefficient ($\pi$)[2] were insignificant in this set of compounds. Among the compounds listed in Table I, 3,4-$Me_2$ and 2,6-(iso-Pr)$_2$ derivatives were excluded from the analysis, because the calculated values for these compounds largely deviated from the correlation line. The reasons for these deviations remain unfortunately unclear.

In Eq. 4, the simple sum of steric parameters for 2- and 6-position substituents was used for 2,6-disubstituted derivatives. For these compounds, the two *ortho*-substituents were classified according to their bulkiness; the one with the more negative $E_s(AMD)$ value was defined as being the larger. Using $E_s(AMD)^S$ for the smaller and $E_s(AMD)^L$ for the larger substituents, we derived Eq. 5 for 36 compounds used in Eq. 4. For mono-*ortho*-substituted derivatives, the smaller *ortho*-substituent was taken to be hydrogen, and the $E_s(AMD)^S$ value was put zero, because

TABLE II. Substituent Parameters Used for Correlation Analyses[a] and Hypocholesterolemic Activity of *N*-Phenyl-$\Delta^8$-dihydroabietamides

| No. | R | $E_s(AMD)^b$ | $E_s^c$ | $\sigma^c$ | $\sigma(m)^d$ | $\sigma(op)^e$ | $pID_{50}$ Obsd. | $pID_{50}$ Calcd[f] |
|---|---|---|---|---|---|---|---|---|
| 1 | H | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | −2.243 | −2.416 |
| 2 | 2-Me | −1.16 | −1.24 | −0.17 | 0.00 | −0.17 | −1.415 | −1.617 |
| 3 | 2-Et | −1.33 | −1.31 | −0.15 | 0.00 | −0.15 | −1.079 | −1.566 |
| 4 | 2-Ph | −2.19 | −1.01 | −0.01 | 0.00 | −0.01 | −1.568 | −1.369 |
| 5 | 2-COOEt | —[g] | —[g] | 0.45 | 0.00 | 0.45 | | |
| 6 | 2-Cl | −0.98 | −0.97 | 0.23 | 0.00 | 0.23 | −2.732 | −2.296 |
| 7 | 2-Br | −1.12 | −1.16 | 0.23 | 0.00 | 0.23 | −2.623 | −2.231 |
| 8 | 2-I | −1.44 | −1.40 | 0.18 | 0.00 | 0.18 | −2.633 | −2.005 |
| 9 | 2-$NO_2$ | −1.65 | −1.01 | 0.78 | 0.00 | 0.78 | −2.833 | −2.799 |
| 10 | 3-Me | 0.00 | 0.00 | −0.07 | −0.07 | 0.00 | −2.290 | −2.416 |
| 11 | 3-$CF_3$ | 0.00 | 0.00 | 0.43 | 0.43 | 0.00 | −2.763 | −2.416 |
| 12 | 3-OMe | 0.00 | 0.00 | 0.12 | 0.12 | 0.00 | −2.243 | −2.416 |
| 13 | 3-OPh | 0.00 | 0.00 | 0.25 | 0.25 | 0.00 | | −2.416 |
| 14 | 3-Br | 0.00 | 0.00 | 0.39 | 0.39 | 0.00 | −2.398 | −2.416 |
| 15 | 4-Me | 0.00 | 0.00 | −0.17 | 0.00 | −0.17 | −2.681 | −2.163 |
| 16 | 4-OEt | 0.00 | 0.00 | −0.24 | 0.00 | −0.24 | −1.740 | −2.059 |
| 17 | 4-COOMe | 0.00 | 0.00 | 0.45 | 0.00 | 0.45 | −3.477 | −3.086 |
| 18 | 4-Ph | 0.00 | 0.00 | −0.01 | 0.00 | −0.01 | −2.903 | −2.401 |
| 19 | 4-Cl | 0.00 | 0.00 | 0.23 | 0.00 | 0.23 | −2.556 | −2.758 |
| 20 | 4-OH | 0.00 | 0.00 | −0.37 | 0.00 | −0.37 | −1.845 | −1.866 |
| 21 | 2,3-$Me_2$ | −1.16 | −1.24 | −0.24 | −0.07 | −0.17 | −0.954 | −1.617 |
| 22 | 2,4-$Me_2$ | −1.16 | −1.24 | −0.34 | 0.00 | −0.34 | −1.301 | −1.364 |
| 23 | 2,5-$Me_2$ | −1.16 | −1.24 | −0.24 | −0.07 | −0.17 | −1.230 | −1.617 |
| 24 | 2,6-$Me_2$ | −2.32 | −2.48 | −0.34 | 0.00 | −0.34 | −0.944 | −0.817 |
| 25 | 3,4-$Me_2$ | 0.00 | 0.00 | −0.24 | −0.07 | −0.17 | −3.230 | −2.163 |
| 26 | 3,5-$Me_2$ | 0.00 | 0.00 | −0.14 | −0.14 | 0.00 | −2.033 | −2.416 |
| 27 | 2-Me-6-Et | −2.49 | −2.55 | −0.32 | 0.00 | −0.32 | −0.740 | −0.767 |
| 28 | 2,6-$Et_2$ | −2.66 | −2.62 | −0.30 | 0.00 | −0.30 | −1.041 | −0.716 |
| 29 | 2,6-iso-$Pr_2$ | −3.32 | −3.42 | −0.30 | 0.00 | −0.30 | −1.924 | −0.405 |
| 30 | 2-Me-5-Cl | −1.16 | −1.24 | 0.20 | 0.37 | −0.17 | −1.342 | −1.617 |
| 31 | 2-Me-6-Cl | −2.14 | −2.21 | 0.06 | 0.00 | 0.06 | −1.301 | −1.497 |
| 32 | 2,5-$(OMe)_2$ | −0.40 | −0.55 | −0.15 | 0.12 | −0.27 | −2.146 | −1.826 |
| 33 | 2,4-$Cl_2$ | −0.98 | −0.97 | 0.46 | 0.00 | 0.46 | −2.491 | −2.639 |
| 34 | 2,6-$Cl_2$ | −1.96 | −1.94 | 0.46 | 0.00 | 0.46 | −1.447 | −2.177 |
| 35 | 3,4-$Cl_2$ | 0.00 | 0.00 | 0.60 | 0.37 | 0.23 | −2.820 | −2.758 |
| 36 | 3-OH-4-COOH | 0.00 | 0.00 | 0.57 | 0.12 | 0.45 | −3.255 | −3.086 |
| 37 | 2,4,5-$Me_3$ | −1.16 | −1.24 | −0.41 | −0.07 | −0.34 | −1.114 | −1.364 |
| 38 | 2,4,6-$Me_3$ | −2.32 | −2.48 | −0.51 | 0.00 | −0.51 | −0.903 | −0.564 |
| 39 | 2,4,6-$Me_3$-3-Br | −2.32 | −2.48 | −0.12 | 0.39 | −0.51 | −0.845 | −0.564 |
| 40 | 2,3,5,6-$F_4$ | −0.64 | −0.92 | 0.80 | 0.68 | 0.12 | −2.061 | −2.293 |

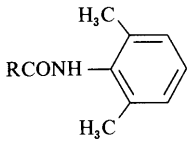
*a*) Compounds **5**, **13**, **25** and **29** were excluded from analysis. *b*) Taken from reference 4). *c*) Taken from reference 2). *d*) The values for *ortho* and *para* substituents were put at zero. *e*) The values for *meta* substituents were put at zero. *f*) Calculated according to Eq. 4. *g*) Unavailable parameter.

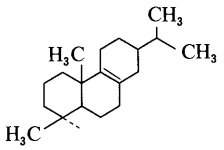
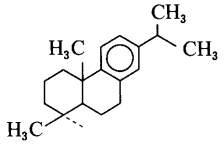
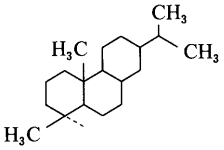
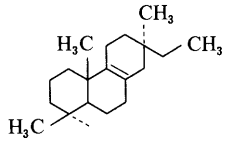
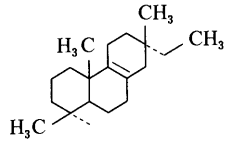
TABLE III. Relative Hypocholesterolemic Activity

| No. | $ID_{30}$ ppm | $ID_{30}$ Ratio[a] | $ID_{40}$ ppm | $ID_{40}$ Ratio[a] | $ID_{50}$ ppm | $ID_{50}$ Ratio[a] | $ID_{90}$ ppm | $ID_{90}$ Ratio[a] |
|---|---|---|---|---|---|---|---|---|
| **24** | 2.8 | 1.0 | 5.0 | 1.0 | 8.8 | 1.0 | 63 | 1.0 |
| **2** | 14 | 0.2 | 20 | 0.3 | 26 | 0.3 | 155 | 0.4 |
| **3** | 7 | 0.4 | 9 | 0.6 | 12 | 0.7 | 56 | 1.1 |
| **4** | 23 | 0.1 | 30 | 0.2 | 37 | 0.2 | 90 | 0.7 |
| **21** | 5 | 0.6 | 7 | 0.7 | 9 | 1.0 | 90 | 0.7 |
| **22** | — | — | 8 | 0.6 | 20 | 0.4 | 110 | 0.6 |
| **23** | 7 | 0.4 | 10 | 0.5 | 17 | 0.5 | 120 | 0.5 |
| **27** | 2.3 | 1.2 | 3.8 | 1.3 | 5.5 | 1.6 | 28 | 2.3 |
| **28** | 4 | 0.7 | 7 | 0.7 | 11 | 0.8 | 150 | 0.4 |
| **30** | 14 | 0.2 | 17 | 0.3 | 22 | 0.4 | 460 | 0.1 |
| **31** | 8 | 0.4 | 13 | 0.4 | 20 | 0.4 | 80 | 0.8 |
| **34** | 13 | 0.2 | 18 | 0.3 | 28 | 0.3 | 130 | 0.5 |
| **38** | 3.4 | 0.8 | 5.2 | 1.0 | 8 | 1.0 | 44 | 1.4 |
| **39** | 2.0 | 1.4 | 3.6 | 1.4 | 7 | 1.3 | 65 | 1.0 |

*a*) $ID_x$ of test compound/$ID_x$ of **24**.

TABLE IV. Variation in Hypocholesterolemic Activity with Change in the Acid Moiety



| No. | R | Formula | Yield (%) | mp (°C) | Crystn. solvent[a] | % inhibition | | | | $ID_{50}$ (ppm) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 10 | 30 | 100 | 300 ppm[b] | |
| **24** |  | $C_{28}H_{41}NO$ | 76 | 195—197 | MEK | 53 | 79 | 97 | 103 | 8.8 |
| **41** |  | $C_{28}H_{37}NO$ | 76 | 227—229 | EA | 8 | 24 | 66 | 92 | 68 |
| **42** |  | $C_{28}H_{43}NO$ | 82 | 218—220 | A | 17 | 39 | 72 | — | 48 |
| **43** |  | $C_{28}H_{41}NO$ | 54 | 187—189 | MEK | −4 | 17 | 46 | 74 | 120 |
| **44** |  | $C_{28}H_{41}NO$ | 58 | 173—176 | MEK | 7 | 6 | 36 | 60 | 180 |
| **45** |  | $C_{16}H_{23}NO$ | 76 | 198 | M | — | — | 7 | 17 | 1100 |

*a, b*) See Table I.

the $E_s$(AMD) for hydrogen is zero.

$$pID_{50} = -1.511(\pm 0.431)\sigma(op) - 0.409(\pm 0.303)E_s(AMD)^S - 0.505(\pm 0.203)E_s(AMD)^L - 2.427(\pm 0.182) \quad (5)$$
$$n=36,\ s=0.354,\ r=0.899,\ F=45.0$$

The coefficients with the $E_s(AMD)^S$ and $E_s(AMD)^L$ terms were very close to each other, meaning that the susceptibility of the activity to steric effect is almost identical for the two *ortho*-substituents. Thus, the overall steric effect was expressible by the simple sum of the two $E_s$(AMD) constants in this series of compounds.

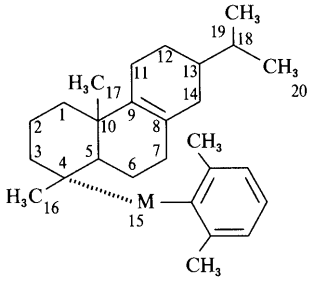
The relative hypocholesterolemic activities of those derivatives comparable to the activity of **24** are listed in Table III. The inhibitory doses at which serum cholesterol elevation was inhibited by 30, 40, 50 and 90% were calculated. Accurate relative activities for these derivatives could be estimated by comparing many inhibitory doses. When derivatives that had considerable hypocholesterolemic activity were compared in terms of activity at 30, 40, 50 and 90% inhibitory doses, four analogs (**24**, **27**, **38**, **39**) had about the same degree of activity.

**Hypocholesterolemic Activity of *N*-(2,6-Dimethylphenyl)-Analogs of Several Resin Acids** We compared the hypocholesterolemic activities of various amide derivatives of 2,6-dimethylaniline in order to assess the effect of the acid moiety.

Table IV shows that the abietane-type carboxamides (**41**, **42**) appear to be preferable to the pimarane-type analogs (**43**, **44**) in terms of activity. *N*-(2,6-Dimethylphenyl)-1-methylcyclohexanecarboxamide (**45**), having a structure that resembles ring A in the resin acid, was inactive.

**Variation in Hypocholesterolemic Activity Due to Conversion of the Carboxamide Group in *N*-(2,6-Dimethylphenyl)-$\Delta^8$-Dihydroabietamide** Table V shows the effect of the conversion of the carboxamide group in **24** on hypocholesterolemic action. The reversed-type carboxamide (**46**) of **24** was decreased markedly, to about 1/205 the activity of the parent carboxamide (**24**). The thioamide (**47**) had only about 1/18 the parent activity, and the ester (**48**) no activity. These results indicate the importance of the carboxamide group for hypocholesterolemic activity. On the derivatives synthesized, *N*-(2,6-dimethylphenyl)-$\Delta^8$-dihydroabietamide, **24**, was the most potent in the series. The structural

TABLE V. Variation in Hypocholesterolemic Activity Due to Conversion of the Carboxamide Group



| No. | M | Formula | Yield (%) | mp (°C) | Crystn. solvent[a)] | % inhibition | | | | $ID_{50}$ (ppm) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 100 | 300 | 1000 | 3000 ppm[b)] | |
| **46** | NHOC | $C_{28}H_{41}NO$ | 67 | 104—106 | C–M | 16 | 22 | 24 | 68 | 1800 |
| **47** | CSNH | $C_{28}H_{41}NS$ | 85 | 215—217 | C–M | 36 | 64 | 95 | — | 160 |
| **48** | $CO_2$ | $C_{28}H_{40}O_2$ | 58 | (wax) | — | — | — | 3 | 10 | — |

a, b) See Table I.

resemblance of $\Delta^8$-dihydroabietic acid to cholesterol is considered to be a factor affecting cholesterol absorption.

**Chemistry** The carboxamides (**1**—**45**) were prepared by the Schotten–Baumann reaction. The reversed type of carboxamide (**46**) of **24** was prepared as follows: $\Delta^8$-dihydroabietic acid was converted to 4-amino-15-nor-8-abietene by the Curtius reaction, after which the product was condensed with 2,6-dimethylbenzoyl chloride. The thioamide (**47**) was derived from **24** by treatment with phosphorous pentasulfide. The ester (**48**) was prepared by reacting $\Delta^8$-dihydroabietoyl chloride with sodium 2,6-dimethylphenoxide.

The method for the preparation of these compounds is described in detail in Experimental.

**Assay Method for Hypocholesterolemic Activity** As described elsewhere,[1)] 4-week-old male Wistar rats were fed synthetic basal and cholesterol-containing diets *ad libitum* for 3 d. They then were killed after overnight starvation and their serum cholesterol concentrations measured. The test compounds were added to the cholesterol diet. Hypocholesterolemic activity was expressed as the % of inhibition of serum cholesterol elevation induced by cholesterol feeding and the $ID_{50}$. The % of inhibition of each test compound at concentrations from $3 \times 10^{-4}$ to $3 \times 10^{-1}$% is shown in Table I. The $ID_{50}$ is the concentration of test compounds in the diet which produces 50% inhibition of the serum cholesterol elevation.

## Experimental

Melting points were measured in open capillary tubes with a Büchi melting point apparatus, and are uncorrected. The boiling points of oily compounds were not determined. Infrared (IR) absorption spectra were measured with a Hitachi 215 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded in deuterochloroform with a Varian A-60 or Varian XL-200 that had tetramethylsilane (TMS) as the internal standard. Chemical shifts are expressed in ppm downfield from TMS. Column chromatography was done on silica gel (Wakogel, C-200).

***N*-(2,6-Dimethylphenyl)-$\Delta^8$-dihydroabietamide (24)** A mixture of $\Delta^8$-dihydroabietic acid (6.10 g, 20 mmol), thionyl chloride (7.14 g, 60 mmol), and benzene (30 ml) was refluxed for 2 h. A pale brown resinous product was obtained by removal of the solvent and excess thionyl chloride under reduced pressure. The acid chloride (IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 1783) was used without further purification. It was first dissolved in toluene (30 ml), then at an ambient temperature, added by drops with stirring to a solution of 2,6-xylidine (2.54 g, 21 mmol), triethylamine (2.23 g, 22 mmol) and toluene (20 ml). After additional stirring for 4 h, the reaction mixture was washed with 5% HCl (20 ml), water (20 ml × 3), 5% NaOH (20 ml), and water (20 ml × 3) in that order. The organic layer was dried with anhydr. magnesium sulfate, filtered, and evaporated. The residual mass was crystallized from acetone. Recrystallization from methyl ethyl ketone gave colorless needles (6.21 g, 76%), mp 195—197 °C. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1635. NMR $\delta$: 0.88 (6H, d, $J$=5 Hz), 1.02 (3H, s), 1.28 (3H, s), 2.13 (6H, s), 7.02 (3H, s). *Anal.* Calcd for $C_{28}H_{41}NO$: C, 82.50; H, 10.14; N, 3.44. Found: C, 82.81; H, 10.27; N, 3.46.

***N*-(3-Bromo-2,4,6-trimethylphenyl)-$\Delta^8$-dihydroabietamide (39)** The acid chloride prepared from $\Delta^8$-dihydroabietic acid (6.10 g, 20 mmol) was dissolved in acetone (20 ml) then added by drops to a stirred and ice-cooled solution containing acetone (20 ml), triethylamine (2.23 g, 22 mmol) and 3-bromomesidine (4.71 g 22 mmol) as described by Adams and Dankert.[6)] After additional stirring for 3 h, the reaction mixture was poured into water (100 ml). The crystals that precipitated were collected, washed twice with 20 ml of water, then dried. Recrystallization from methyl ethyl ketone gave colorless needles (7.22 g, 72%), mp 231—232 °C. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1640. NMR $\delta$: 0.88 (6H, d, $J$=5 Hz), 1.00 (3H, s), 1.28 (3H, s), 2.00 (3H, s), 2.20 (3H, s), 2.34 (3H, s), 6.90 (1H, br), 7.32 (1H, br). *Anal.* Calcd for $C_{29}H_{42}BrNO$: C, 69.58; H, 8.46; N, 2.80. Found: C, 69.57; H. 8.58; N, 2.56.

***N*-(2,6-Dimethylphenyl)-$\Delta^8$-dihydropimaramide (43)** $\Delta^8$-Dihydropimaric acid was prepared from pimaric acid by partial reduction followed by isomerization in the presence of an acid catalyst according to the method of Edwards and Howe.[7)] The acid chloride prepared from $\Delta^8$-dihydropimaric acid (1.00 g, 3.3 mmol) and thionyl chloride (1.17 g, 99 mmol) was added to a solution of benzene (30 ml) containing 2,6-xylidine (0.49 g, 4.0 mmol) and triethylamine (0.46 g, 4.5 mmol). This reaction mixture was treated by the procedure described above. Recrystallization from methyl ethyl ketone gave colorless needles (0.72 g, 54%), mp 187—189 °C. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1638. NMR (200 MHz) $\delta$: 0.760 (3H, s), 0.819 (3H, t, $J$=7.4 Hz), 1.053 (3H, s), 1.357 (3H, s), 2.186 (6H, s), 7.048 (3H, s). *Anal.* Calcd for $C_{28}H_{41}NO$: C, 82.50; H, 10.14; N, 3.44. Found: C, 82.42; H, 10.20; N, 3.44.

***N*-(2,6-Dimethylphenyl)-$\Delta^8$-dihydroisopimaramide (44)** Isopimaric acid was purified from the resin of *Cryptomeria japonica* D. DON by silica gel column chromatography and recrystallized several times from methyl acetate as *n*-butanolamine salt.[8)] The amine salt was suspended in ethyl ether and decomposed with 10% sulfuric acid, the resulting solution was treated in the usual manner to give the acid. $\Delta^8$-Dihydroisopimaric acid was prepared from isopimaric acid by the method of Edwards and Howe.[7)] A solution of (chloroform, 10 ml) the acid chloride prepared from $\Delta^8$-dihydroisopimaric acid (2.0 g, 6.6 mmol) and thionyl chloride (2.34 g, 19.8 mmol) was added to a stirred solution of 2,6-xylidine (0.98 g, 8.0 mmol), triethylamine (0.92 g, 9.0 mmol) and chloroform (20 ml). After additional stirring for 4 h, this reaction mixture was worked up in the usual manner. Recrystallization from methyl ethyl ketone gave colorless needles (1.55 g, 58%), mp 173—176 °C. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1636. NMR (200 MHz) $\delta$: 0.782 (3H, t, $J$=7.4 Hz), 0.807 (3H, s), 1.052 (3H, s), 1.363 (3H, s), 2.195 (6H, s), 7.054 (3H, s). *Anal.* Calcd for $C_{28}H_{41}NO$: C, 82.50; H, 10.14; N, 3.44. Found: C, 82.54; H, 10.09; N, 3.46.

***N*-(15-Nor-8-abieten-4-yl)-2,6-dimethylbenzamide (46)** 4-Amino-15-nor-8-abietene was prepared from $\Delta^8$-dihydroabietic acid by the Curtius reaction.[9] A 20% aq. solution of sodium azide (38.8 g, 120 mmol) was added with vigorous stirring to a cooled (15 °C) solution in acetone (92 ml) of acid chloride prepared from $\Delta^8$-dihydroabietic acid (30.45 g, 100 mmol) and thionyl chloride (35.70 g, 300 mmol). After stirring for 30 min, water (300 ml) was added and the mixture was then treated twice with benzene (100 ml) (IR $\nu_{max}^{film}$ cm$^{-1}$: 1780). After the benzene solution had been refluxed for 5 h, the solvent was removed *in vacuo*, giving a brown oily product (IR $\nu_{max}^{film}$ cm$^{-1}$: 2250). This product was purified by column chromatography on silica gel (50 g) eluting with *n*-hexane (500 ml). Removal of the solvent gave a colorless oil (28.3 g). A mixture of conc. sulfuric acid (30 ml) and water (5 ml) was added dropwise during 30 min to a stirred and cooled solution of the oil obtained above in diethyl ether (300 ml). After additional stirring for 30 min, ice and water were added. The colorless crystals formed were filtered and dissolved in chloroform (300 ml). The chloroform solution was washed with 5% sodium hydroxide (300 ml) and with water, and was dried over anhydr. magnesium sulfate. The solvent was removed *in vacuo* giving a clear oil (27.4 g, 85%). NMR $\delta$: 0.88 (6H, d, $J$=6 Hz), 0.95 (3H, s), 1.02 (3H, s), 1.28 (2H, br). Hydrochloride monohydrate; mp 173—175 °C. *Anal.* Calcd for $C_{19}H_{33}N \cdot HCl \cdot H_2O$: C, 69.92; H, 11.60; N, 4.25. Found: C, 69.45; H, 11.52; N, 4.38. A solution of toluene (30 ml) and 2,6-dimethylbenzoyl chloride, prepared from the corresponding acid (3.00 g, 20 mmol) and thionyl chloride (7.14 g, 60 mmol), was treated with a mixture of 4-amino-15-nor-8-abietene (4.13 g, 15 mmol) and triethylamine (2.02 g, 20 mmol). After the reaction had continued for 4 h, the mixture was worked up as described above. The resulting product was crystallized from a small volume of acetone and then recrystallized from chloroform–methanol (1 : 1) to give colorless needles (4.09 g, 67%), mp 104—106 °C. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 1625. NMR $\delta$: 0.90 (6H, d, $J$=5 Hz), 1.00 (3H, s), 1.39 (3H, s), 2.36 (6H, s), 5.35 (1H br), 7.00 (3H, m). *Anal.* Calcd for $C_{28}H_{41}NO$: C, 82.50; H, 10.14; N, 3.44. Found: C, 82.71; H, 10.21; N, 3.48.

***N*-(2,6-Dimethylphenyl)-$\Delta^8$-dihydroabietenecarbothioamide (47)** A mixture of the carboxamide **24** (10.00 g, 24.5 mmol), phosphorus pentasulfide (3.00 g, 13.5 mmol) and anhydr. benzene (100 ml) was refluxed for 1 h. After cooling, 5% sodium hydroxide (100 ml) was added and the reaction mixture was stirred for 1 h. The benzene layer was separated, filtered, and dried over anhydr. magnesium sulfate. After removal of the drying agent, the solvent was evaporated under reduced pressure. The resulting residue crystallized from chloroform–methanol (10 : 7) gave pale yellow needles (8.83 g, 85%), mp 215—217 °C. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 1493, 1340, 1200. NMR $\delta$: 0.87 (6H, d, $J$=5 Hz), 1.07 (3H, s), 1.50 (3H, s), 2.12 (3H, s), 2.20 (3H, s), 7.05 (3H, s), *Anal.* Calcd for $C_{28}H_{41}NS$: C, 79.38; H, 9.76; N, 3.31; S, 7.55. Found: C, 79.21; H, 9.42; N, 3.48; S, 7.44.

**2,6-Dimethylphenyl $\Delta^8$-Dihydroabietate (48)** A mixture of $\Delta^8$-dihydroabietoyl chloride (6.10 g, 20 mmol) and sodium 2,6-dimethylphenoxide, prepared from 2,6-xylenol (3.67 g, 30 mmol) and sodium hydride (1.44 g, 30 mmol, 50% in oily suspension), was stirred in anhydr. benzene (20 ml) for 3 h. This reaction mixture was then washed, dried and filtered. Removal of the solvent gave a brown oil which was purified by column chromatography on silica gel (200 g). Elution with *n*-hexane–benzene (1 : 1, 300 ml), followed by removal of the solvent, gave an almost colorless oil (4.75 g, 58%) which solidified into a waxy material on standing for several days. IR $\nu_{max}^{film}$ cm$^{-1}$: 1735. NMR $\delta$: 0.86 (6H, d, $J$=5 Hz), 1.05 (3H, s), 1.35 (3H, s), 2.10 (6H, s), 6.95 (3H, s). *Anal.* Calcd for $C_{28}H_{37}O_2$: C, 82.92; H, 9.19. Found: C, 83.01; H, 9.23.

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# Synthesis and Biological Activity of (*S*)-2-Amino-3-(2,5-dihydro-5-oxo-4-isoxazolyl)propanoic Acid (TAN-950 A) Derivatives[1)]

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**(*S*)-2-Amino-3-(2,5-dihydro-5-oxo-4-isoxazolyl)propanoic acid (TAN-950 A (1)) is a novel amino acid antibiotic which shows a high affinity for glutamate receptors of the central nervous system. To improve the affinity for glutamate receptors, the structure–activity relationships of TAN-950 A derivatives 6a—o, 15a—o were investigated. Optically active TAN-950 A analogs 15a—h were synthesized starting with methyl (*S*)- and (*R*)-*N*-Boc-pyroglutamate (8) *via* acylation at the C-4 position followed by isoxazolone formation with hydroxylamine and subsequent deprotection reactions. The lactam 16, prepared from (*RS*)-aminoadipic acid, and dimethyl esters 19 of (*R*)- and (*S*)-aspartic acid were converted to (*RS*)-3-methyl-homo-TAN-950 A (15i) and optically active nor-TAN-950 A derivatives 15j—o, respectively, utilizing a similar sequence of reactions.**

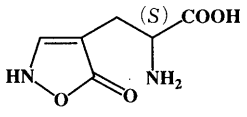
**Most of TAN-950 A derivatives 6a—o, 15a—o showed an affinity for glutamate receptors. The 3-alkyl derivatives 15b, d—g, especially, showed a high affinity for the quisqualate subtype-receptor and had a strong activating effect on the hippocampal neurons (glutamate agonistic activity). The (*R*)-enantiomer 15a of TAN-950 A had increased selectivity for the *N*-methyl-D-aspartate (NMDA) subtype-receptor. This selectivity was further enhanced by removal of the methylene group in the amino acid moiety of 15a. The most potent and selective NMDA agonistic activity was observed with (*R*)-3-methyl-nor-TAN-950 A (15m).**

**Keywords** TAN-950 A; isoxazolone; isoxazolyl-L-alanine; asymmetric synthesis; excitatory amino acid; glutamate receptor; kainate; quisqualate; *N*-methyl-D-aspartate; receptor binding

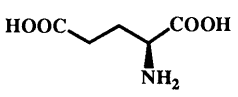
TAN-950 A (**1**), (*S*)-2-amino-3-(2,5-dihydro-5-oxo-4-isoxazolyl)propanoic acid,[2a)] is a new antifungal antibiotic isolated from the culture filtrate of *Streptomyces platensis* A-136.[2b)] The pharmacological studies of **1** have revealed that it has a high affinity for glutamate receptors of the central nervous system and produces agonistic activity for hippocampal neurons.[2b,3)]

Glutamate receptors, which exist widely in the brain, have been known as excitatory amino acid receptors which play an important role in learning and memory. Recently, glutamate receptors have been classified into at least three subtypes, quisqualate-, *N*-methyl-D-aspartate (NMDA)- and kainate-receptors, on the basis of their relative affinities for natural and synthetic excitatory amino acids. Each of the receptors has a unique pharmacological profile.[4)] The quisqualate and/or kainate-receptors are thought to endow synaptic transmission in the mammalian central nervous system with special properties, and the NMDA-receptor plays a key role in plasticity during development and learning. There is a particular interest in selective agonists and antagonists for these receptors to investigate the physiological function of the excitatory amino acid receptors and to modulate their function. Therefore, many works aiming at the more selective agonists or antagonists have been carried out.[5)] Chart 1 shows some of the important excitatory amino acids which have been known as potent agonists.

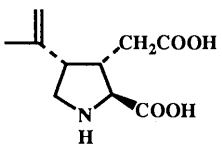
(*RS*)-α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) is a synthetic compound which is classified into the quisqualate-type agonist and has been used as



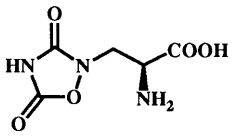
TAN-950 A
1



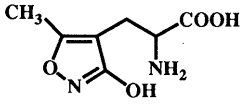
glutamic acid



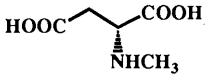
kainic acid



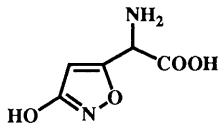
quisqualic acid



AMPA



NMDA



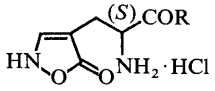
ibotenic acid

Chart 1

a potent neuronal activator. The structure–activity relationship (SAR) of AMPA analogs has been reported.[6] Naturally occurring ibotenic acid, (*RS*)-α-amino-3-hydroxy-5-isoxazolylacetic acid, has been known as a potent NMDA-type neuronal activator. The structural similarity between these isoxazole-containing glutamate agonists and TAN-950 A (**1**) prompted us to investigate the SAR of TAN-950 A congeners and to search selective and potent glutamate agonists. Thus, we carried out modification of the amino acid and isoxazolone moieties of **1**: *i.e.* condensation with amino acids to give dipeptide derivatives (**6**: Table I), introduction of a substituent at 3-position of the isoxazolone ring (3-substituted TAN-950 A, **15b—h**: Table II), and insertion and removal of the methylene group at the alanine moiety (homo-TAN-950 A and nor-TAN-950 A, **15i, j—o**: Table II). Furthermore, we planned to synthesize both enantiomers of TAN-950 A congeners, since the configuration of the asymmetric carbon in the amino acid moiety of glutamate agonists has been reported to be critical for the affinity and selectivity.[7]

**Chemistry** The dipeptide derivatives of TAN-950 A were synthesized as shown in Chart 2. The reaction of *N-tert*-butoxycarbonyl-TAN-950 A (*N*-Boc-TAN-950 A) (**2**)[8] and glycine ethyl ester in the presence of diethylphosphoryl cyanide (DEPC)[9] and triethylamine gave the *N*-Boc-TAN-950 A-glycine ethyl ester (**4a**) in a 29% yield, which was then deprotected to TAN-950 A-glycine (**6a**) by alkaline hydrolysis followed by treatment with hydrogen chloride in a 58% overall yield. As the dipeptide **6a** showed a moderate affinity for glutamate receptors as shown in Table III, we synthesized some dipeptide derivatives condensed with α-amino acids, ε-aminoalkanecarboxylic acids, β-aminoala-

TABLE I. Dipeptide Derivatives of TAN-950 A (**6a—o**)



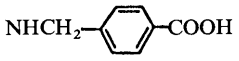
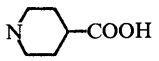
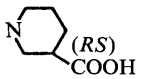
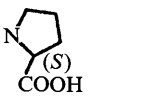
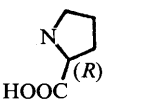
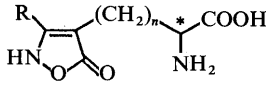
| No. | $R^3$ | Yield (%) | $^1$H-NMR δ (in $D_2O$) | SI-MS (*m/z*) $(M+H)^+$ |
|---|---|---|---|---|
| **6a** | $NHCH_2COOH$ | 67 | 2.91 (2H, d, $J$=6.2 Hz), 4.03 (2H, s), 4.24 (1H, t, $J$=6.2 Hz), 8.35 (1H, s) | 270 |
| **6b** | (*S*) $NHCHCOOH$ $(CH_2)_2CH_3$ | 96 | 0.85 (3H, t, $J$=7.2 Hz), 1.24—1.36 (2H, m), 1.65—1.80 (2H, m), 2.82—2.86 (2H, m), 4.12—4.18 (1H, m), 4.27—4.35 (1H, s), 8.26 (1H, s) | 272 |
| **6c** | (*S*) $NHCHCOOH$ $(CH_2)_3CH_3$ | 49 | 0.87—0.90 (3H, m), 1.27—1.33 (4H, m), 1.73—1.88 (2H, m), 2.78–2.84 (2H, m), 4.14 (1H, t, $J$=6.8 Hz), 4.38—4.47 (1H, m), 8.03 (1H, s) | 286 |
| **6d** | $NH(CH_2)_2COOH$ | 75 | 2.59 (2H, t, $J$=6.2 Hz), 2.85 (2H, d, $J$=6.6 Hz), 3.40—3.59 (2H, m), 4.11 (1H, t, $J$=6.6 Hz), 8.31 (1H, s) | 244 |
| **6e** | $NH(CH_2)_3COOH$ | 84 | 1.77 (2H, m), 2.36 (2H, t, $J$=6.2 Hz), 2.85 (2H, d, $J$=6.6 Hz), 3.18—3.39 (2H, m), 4.09 (1H, t, $J$=6.6 Hz), 8.32 (1H, s) | 258 |
| **6f** | $NH(CH_2)_4COOH$ | 92 | 1.42—1.68 (4H, m), 2.31—2.48 (2H, m), 2.88 (2H, d, $J$=6.6 Hz), 3.09—3.38 (2H, m), 4.08 (1H, t, $J$=6.6 Hz), 8.31 (1H, s) | 272 |
| **6g** | (*S*) $NHCH_2CHCOOH$ NHZ | 78 | 2.61—2.89 (2H, m), 3.31—3.85 (2H, m), 4.02—4.18 (1H, m), 4.31—4.50 (1H, m), 5.16 (2H, s), 7.42 (5H, s), 8.19 (1H, s) | 393 |
| **6h** | (*S*) $NHCH_2CHCOOH$ $NH_2\cdot HCl$ | 98 | 2.89 (2H, d, $J$=6.2 Hz), 3.69—3.85 (2H, m), 4.02—4.11 (1H, m), 4.18 (1H, t, $J$=6.2 Hz), 8.36 (1H, s) | 259 |
| **6i** | (*R*) $NHCH_2CHCOOH$ $NH_2\cdot HCl$ | 95 | 2.77 (2H, d, $J$=6.2 Hz), 3.58—3.59 (2H, m), 3.82—3.93 (1H, m), 4.08 (1H, t, $J$=6.2 Hz), 8.02 (1H, s) | 259 |
| **6j** |  | 89 | 2.76—2.87 (2H, m), 4.12—4.23 (1H, m), 4.35 (1H, d, $J$=15.4 Hz), 4.56 (1H, d, $J$=15.4 Hz), 7.37 (2H, d, $J$=8 Hz), 7.96—8.01 (3H, m) | 306 |
| **6k** |  | 69 | 1.31—1.71 (1H, m), 1.92—2.11 (2H, m), 2.61—3.35 (6H, m), 3.61—4.02 (2H, m), 4.21—4.35 (1H, m), 8.37 (1H, s) | 284 |
| **6l** |  | 75 | 1.28—1.81 (5H, m), 2.61—2.98 (3H, m), 3.31—4.21 (4H, m), 8.34 (1H, s) | 284 |
| **6m** |  | 89 | 1.28—1.81 (5H, m), 2.19—2.40 (1H, m), 2.61—2.99 (3H, m), 3.21—3.41 (1H, m), 3.58—3.89 (1H, m), 4.13—4.22 (1H, m), 8.33 (1H, s) | 284 |
| **6n** |  | 92 | 1.97—2.61 (4H, m), 2.92 (2H, d, $J$=6.2 Hz), 3.55—3.88 (2H, m), 4.41—4.66 (2H, m), 8.33 (1H, s) | 270 |
| **6o** |  | 82 | 1.88—2.62 (4H, m), 2.91 (2H, d, $J$=6.2 Hz), 3.52—3.88 (2H, m), 4.42—4.65 (2H, m), 8.36 (1H, s) | 270 |

TABLE II. TAN-950 A-Related Analogs (**15a—o**)



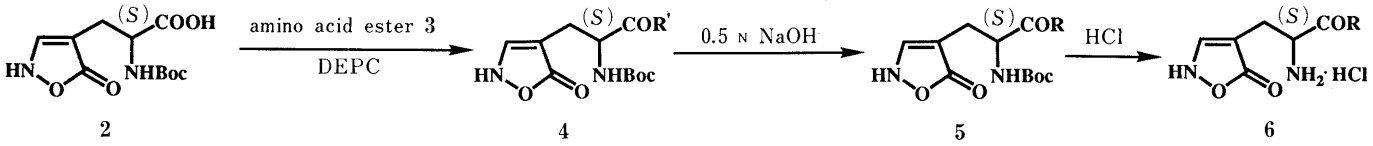
| Compound | Configuration of R | Configuration of *n* | Configuration of C* | Yield (%) | $^1$H-NMR $\delta$ (in $D_2O$) | IR $\nu_{max}^{KBr}$ ($cm^{-1}$) | $[\alpha]_D$ (*c*) {temp. °C} in $H_2O$ | SI-MS (*m/z*) $(M+H)^+$ |
|---|---|---|---|---|---|---|---|---|
| **15a**·HCl | H | 1 | *R* | 85 | 2.95 (2H, d, *J*=5.8 Hz), 4.23 (1H, t, *J*=5.8 Hz), 8.36 (1H, s) | 3400, 1640, 1510, 1410 | +9.3 (0.5) {23} | 173 |
| **15b**·HCl | $CH_3$ | 1 | *S* | 75 | 2.22 (3H, s), 2.91 (2H, d, *J*=6.2 Hz), 4.18 (1H, t, *J*=6.2 Hz) | 3400, 1640, 1500, 1420 | −9.5 (0.3) {25} | 187 |
| **15b** | $CH_3$ | 1 | *S* | — | 2.16 (3H, s), 2.84 (1H, dd, *J*=15.8, 5.2 Hz), 2.84 (1H, dd, *J*=15.8, 6.4 Hz), 3.90 (1H, dd, *J*=6.4, 5.2 Hz) | 3400, 3000, 1620, 1520, 1440, 1340 | −36.7 (0.15) {20} | 187 |
| **15c**·HCl | $CH_3$ | 1 | *R* | 88 | 2.22 (3H, s), 2.91 (2H, d, *J*=6.2 Hz), 4.18 (1H, t, *J*=6.2 Hz) | 3400, 1640, 1500, 1420 | +10.7 (0.5) {25} | 187 |
| **15d**·HCl | $CH_3CH_2$ | 1 | *S* | 81 | 1.20 (3H, t, *J*=7.6 Hz), 2.56 (2H, q, *J*=7.6 Hz), 2.92 (2H, d, *J*=6.2 Hz), 4.23 (1H, t, *J*=6.2 Hz) | 3410, 2970, 1720, 1680, 1580, 1500 | −7.9 (0.4) {25} | 201 |
| **15e**·HCl | $(CH_3)_2CH$ | 1 | *S* | 82 | 1.24 (6H, d, *J*=7 Hz), 2.93 (2H, d, *J*=6.2 Hz), 3.10 (1H, m, *J*=7 Hz), 4.21 (1H, t, *J*=6.2 Hz) | 3420, 2990, 1720, 1660, 1420, 1400 | −5.0 (0.6) {25} | 215 |
| **15f**·HCl |  | 1 | *S* | 83 | 0.85—1.10 (4H, m), 2.01 (1H, m), 3.03 (2H, d, *J*=6.2 Hz), 4.27 (1H, t, *J*=6.2 Hz) | 3450, 2950, 1740, 1680, 1570, 1510 | — | 213 |
| **15g**·HCl |  | 1 | *S* | 90 | 1.50—2.20 (8H, m), 2.94 (2H, d, *J*=6.4 Hz), 3.17 (1H, m), 4.22 (1H, t, *J*=6.4 Hz) | 3450, 2950, 1740, 1680, 1570, 1510, 1440 | — | 241 |
| **15h**·HCl | Ph | 1 | *S* | 67 | 3.11 (2H, d, *J*=6.4 Hz), 4.22 (1H, t, *J*=6.4 Hz), 7.62 (5H, s) | 3420, 2940, 1690, 1610, 1500, 1110 | +9.6 (0.4) {25} | 249 |
| **15i**·HCl | $CH_3$ | 2 | *RS* | 80 | 2.23 (3H, s), 2.15 (2H, m), 2.43 (2H, m), 4.05 (1H, t, *J*=6 Hz) | 3420, 2940, 1720, 1640, 1500, 1450 | — | 201 |
| **15j**·HCl | H | 0 | *S* | 62 | 4.91 (1H, s), 8.49 (1H, s) | 3400, 1630, 1520, 1400 | +17.9 (0.3) {25} | 159 |
| **15k**·HCl | H | 0 | *R* | 75 | 4.91 (1H, s), 8.49 (1H, s) | 3400, 1640, 1520, 1400 | −18.2 (0.5) {23} | 159 |
| **15l**·HCl | $CH_3$ | 0 | *S* | 93 | 2.32 (3H, s), 4.89 (1H, s) | 3430, 2900, 1740, 1710, 1580, 1500 | +28.3 (0.1) {26} | 173 |
| **15m**·HCl | $CH_3$ | 0 | *R* | 88 | 2.31 (3H, s), 4.89 (1H, s) | 3430, 2900, 1740, 1710, 1580, 1500 | −24.8 (0.8) {26} | 173 |
| **15m**·HCl | $CH_3$ | 0 | *R* | — | | | −23.7[a] (0.4) {30} | 173 |
| **15n**·HCl | $CH_3CH_2$ | 0 | *R* | 42 | 1.24 (3H, t, *J*=7.6 Hz), 2.74 (2H, q, *J*=7.6 Hz), 4.90 (1H, s) | 3400, 3000, 1740, 1720, 1700, 1580 | −23.0 (0.2) {23} | 187 |
| **15o**·HCl | $(CH_3)_2CH$ | 0 | *R* | 63 | 1.28 (3H, d, *J*=7.0 Hz), 1.30 (3H, d, *J*=7.0 Hz), 3.17 (1H, m), 4.90 (1H, s) | 3450, 3000, 1700, 1630, 1580, 1500 | −23.1 (0.13) {23} | 201 |

a) The lyophilization product of the above hydrochloride.

nine and cyclic amino acids, in a route similar to the synthesis of **6a**. The dipeptide derivatives **6a—o** prepared were shown in Table I.

The total synthesis of TAN-950 A (**1**) starting with methyl (*S*)-*N*-Boc-pyroglutamate [(*S*)-**8**] has been already accomplished in our research division.[2] Since this synthetic protocol seemed to be applicable to the preparation of the compounds having various substituents on the isoxazolone nucleus as well as the modification of the amino acid moiety, a similar route was adopted to prepare TAN-950 A derivatives **15** starting with lactam derivatives **8**, **16** of ε-carboxy-α-amino acids as shown in Chart 3. First, the synthesis of (*R*)-enantiomer **15a** of TAN-950 A was undertaken in order to see the influence of the configuration (*R* or *S*) on the binding affinity and selectivity. Thus, methyl (*R*)-*N*-Boc-pyroglutamate [(*R*)-**8**] was converted into the

*N*-Boc-4-formylpyroglutamate (**11a**) by formylation with isopropyl formate in the presence of lithium diisopropylamide (LDA) in a 29% yield. The proton nuclear magnetic resonance ($^1$H-NMR) spectrum showed that the compound **11a** in chloroform equilibrates with the enol form **12** (*ca.* 1 : 1). The reaction of **11a** with hydroxylamine effected the ring conversion and gave the isoxazolone derivative **13a** in a 75% yield. Hydrolysis followed by removal of the Boc

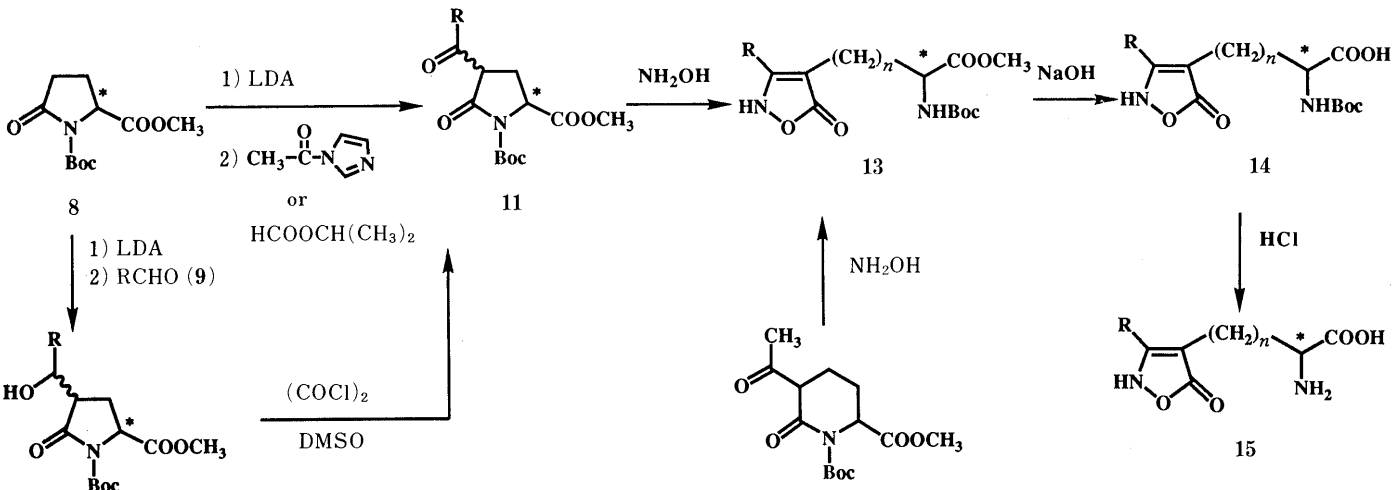



2 4 5 6

a : R=NHCH$_2$COOH
b : R=NH(*S*)CH(CH$_2$)$_2$CH$_3$COOH
c : R=NH(*S*)CH(CH$_2$)$_3$CH$_3$COOH
d : R=NH(CH$_2$)$_2$COOH
e : R=NH(CH$_2$)$_3$COOH
f : R=NH(CH$_2$)$_4$COOH
g : R=NHCH$_2$(*S*)CH(NHZ)COOH
h : R=NHCH$_2$(*S*)CH(NH$_2$·HCl)COOH
i : R=NHCH$_2$(*R*)CH(NH$_2$·HCl)COOH
j : R=NHCH$_2$-C$_6$H$_4$-COOH
k : R = N-C$_5$H$_9$-COOH
l : R = N-C$_5$H$_9$-(*RS*)-COOH
m : R = N-C$_5$H$_9$-(*RS*)-COOH
n : R = N-C$_5$H$_9$-(*S*)-COOH
o : R = N-C$_5$H$_9$-(*R*)-COOH

Z=benzyloxycarbonyl

Chart 2




8 11 13 14

10 17 16 15

TAN-950B, C, D, E

7 12

| | R | *n* | configuration of C* |
|---|---|---|---|
| 15a | H | 1 | *R* |
| 15b | $CH_3$ | 1 | *S* |
| 15c | $CH_3$ | 1 | *R* |
| 15d | $CH_3CH_2$ | 1 | *S* |
| 15e | $(CH_3)_2CH$ | 1 | *S* |
| 15f |  | 1 | *S* |
| 15g |  | 1 | *S* |
| 15h | Ph | 1 | *S* |
| 15i | $CH_3$ | 2 | *RS* |

Chart 3

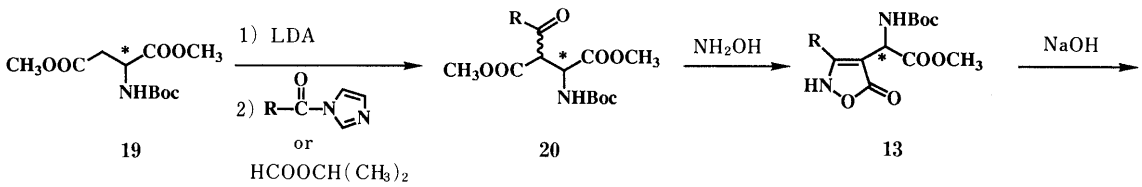
group with hydrogen chloride afforded the (*R*)-isomer **15a** as a hydrochloride salt in an 85% yield.

Next, we carried out the synthesis of 3-methyl-TAN-950 A (**15b**). Methyl (*S*)-*N*-Boc-pyroglutamate [(*S*)-**8**][10] was treated with LDA followed by the addition of *N*-acetylimidazole to afford 4-acetylpyroglutamate (**11b**) in a 72% yield. The $^1$H-NMR spectrum of the compound **11b** showed that **11b** is a mixture of two diastereomers (*ca.* 1 : 1) (Table VI). The compound **11b** was converted to **15b** *via* **13b** and **14b** in a similar manner as above (26% overall yield). The aqueous solution of **15b**·hydrochloride was adjusted to pH 3.5 with aqueous NaOH, and then submitted to chromatography on microcrystalline cellulose to form the inner salt **15b**. 3-Methyl-TAN-950 A (**15b**) and the hydrochloride **15b**·HCl were proved to be stable in an aqueous solution after standing for 4 weeks at room temperature. In the case of TAN-950 A (sodium salt), the formation of isomers **7** (*ca.* 40%)[2] was observed after 3 weeks. On the contrary, the hydrochloride salt of TAN-950 A in water decomposed (about 40% after 4 weeks) to give a variety of unknown products in place of isomers **7**. These observations indicate that the introduction of a methyl group on the isoxazolone ring influences not only the hydrophobicity of the product but also the stability of the isoxazolone moiety.[11] The binding study revealed that 3-methyl-TAN-950 A (**15b**) has a high affinity for the glutamate receptors as well as the strong excitatory activity on the central nervous neurons, therefore the synthetic work was further extended to the preparation of a variety of 3-substituted derivatives in order to investigate the SAR. The (*R*)-3-methyl-TAN-950 A (**15c**) was synthesized from (*R*)-**8** in the same route as that of **15b**. In the case of a series of 3-alkyl- and 3-aryl-TAN-950 A derivatives such as (*S*)-3-ethyl-, (*S*)-3-isopropyl-, (*S*)-3-cyclopropyl-, (*S*)-3-cyclopentyl- and (*S*)-3-phenyl-TAN-950 A (**15d—h**), the preparation of the intermediate **11d—h** was carried out *via* aldol condensation followed by Swern oxidation (**8**→**10**→**11**). The treatment of **11d—h** with hydroxylamine to give **13d—h** and the subsequent deprotection reactions afforded **15d—h**.

The synthesis of homo-TAN-950 A [(*RS*)-**15i**] was done in order to know the influence of the insertion of the methylene group on the affinity for the glutamate receptors. The lactam (*RS*)-**16** derived from (*RS*)-2-aminoadipic acid was allowed to react with LDA and *N*-acetylimidazole to give the acetyl derivative **17** in a 20% yield. The reaction of **17** with hydroxylamine gave the isoxazolone derivative **13i**, which was then deprotected to afford (*RS*)-3-methyl-homo-TAN-950 A (**15i**, a 78% overall yield).

The removal of the methylene group to give nor-TAN-950 A (**15j**) seemed to be quite interesting because of the structural similarity to ibotenic acid. The first attempted synthesis of **15j** starting with acylation of the β-lactam derivative **18** was unsuccessful, presumably due to the unstableness of **18** under the reaction conditions. The synthesis of (*S*)- and (*R*)-nor-TAN-950 A (**15j, k**) was accomplished by using dimethyl (*S*)- and (*R*)-*N*-Boc-aspartate [(*S*)- and (*R*)-**19**][12] as the starting materials for the acylation reaction as shown in Chart 4. Thus, (*S*)-**19** and (*R*)-**19** were formylated to dimethyl *N*-Boc-3-formylaspartate (**20j, k**) in a 20—28% yield with isopropyl formate in the presence of LDA. The reaction of **20j, k** with hydroxylamine gave the isoxazolone derivatives (**13j, k**), and subsequent alkaline hydrolysis followed by removal of the Boc group with hydrogen chloride afforded the (*S*)-nor-TAN-950 A (**15j**) and (*R*)-nor-TAN-950 A (**15k**) as the hydrochloride salts (a 28% overall yield).

Both (*S*)- and (*R*)-nor-TAN-950 A (**15j, k**) showed the selective affinity for the NMDA receptor (Table III), and the affinity of (*R*)-isomer **15k** was higher than that of (*S*)-isomer (**15j**). Therefore, we continued the synthesis of (*R*)-nor-TAN-950 A derivatives **15m—o** having an alkyl substituent at the C-3 position of the isoxazolone ring. (*R*)-3-Methyl derivative **15m** was synthesized *via* the acetylation of (*R*)-**19** with *N*-acetylimidazole and the subsequent reactions (**20m**→**13m**→**14m**→**15m**) in a 4% overall yield. The attempted introduction of a propionyl group into dimethyl (*R*)-*N*-Boc-aspartate [(*R*]-**19**] by aldol condensation followed by Swern oxidation, which was successfully applied in the case of **15d—h**, failed to proceed affording the ring-closure compound **21**. Alternatively, (*R*)-**19** was allowed to react with *N*-propionylimidazole in the presence of LDA to form **20n** in a 92% yield. The isobutyryl compound **20o** was also prepared in a similar way using *N*-isobutyrylimidazole. The propionyl derivative **20n** was converted to isoxazolone derivative **13n** under a condition (pH 3) similar to that of the synthesis of 3-methyl derivative **13j**. But the reaction of isobutyryl derivative **20o** and hydroxylamine needed a




19 20 13

14 15·HCl 18 21

| | R | configuration of C* |
|---|---|---|
| 15j | H | *S* |
| 15k | H | *R* |
| 15l | $CH_3$ | *S* |
| 15m | $CH_3$ | *R* |
| 15n | $CH_3CH_2$ | *R* |
| 15o | $(CH_3)_2CH$ | *R* |

Chart 4

more basic condition (pH 8) to give the ring-closure compound, which was isolated as the hydrolyzed product **14o** in a low yield (9%). Deprotection of the isoxazolone derivatives **14n, o** gave the desired (*R*)-products **15n, o**. (*S*)-3-Methyl derivative **15l** was also prepared from (*S*)-**19** in the same route as that of **15m**.

**Determination of the Enantiomer Excess** The first attempt to determine the enantiomer excess (e.e.) of **15** by high performance liquid chromatography (HPLC) using a chiral column was unsuccessful.[13] Therefore, we planned to determine the diastereomer excess of the diastereomeric amide **23** to see the e.e. of **14**. Compound **15** are considered to have the same level of optical purity as **14** because the removal of the Boc group is known to proceed without racemization.[14] Thus, the amides **23a—f** were synthesized by the condensation of **2** (derived from natural TAN-950 A), **14a—c, l**, and **14m** with (*R*)-1-(1-naphthyl)ethylamine (**22**) in the presence of DEPC as shown in Chart 5. The $^1$H-NMR (400 MHz) spectrum of TAN-950 A-amide **23a** showed a singlet of the isoxazolone ring proton at $\delta$ 8.21 in DMSO-$d_6$–$D_2O$, while that of **23b** ((*R*)-form) showed a singlet at $\delta$ 8.13 accompanying a small peak of a singlet at $\delta$ 8.21. From the intensity of these singlets (*S*)-*N*-Boc-TAN-950 A (**2**) and (*R*)-*N*-Boc-TAN-950 A (**14a**) were determined to have >99% e.e. and 87% e.e., respectively.

The $^1$H-NMR spectra of the amides **23c, d** showed singlets of the methyl groups on the C-3 isoxazolone ring at $\delta$ 1.88 and 1.93, respectively, in DMSO-$d_6$–$D_2O$. The enantiomer excess of both **14b** and **14c** was determined to be >99%. The high optical purity of these compounds was considered to be due to the purification of the intermediates **13b, c** by recrystallization.

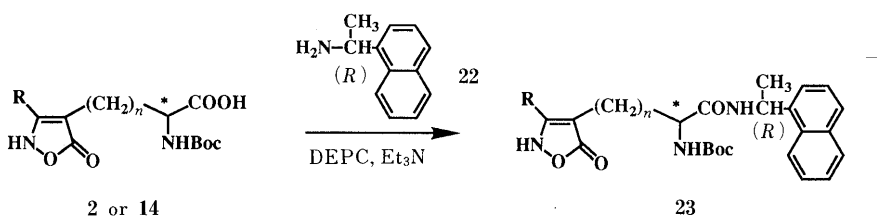
The $^1$H-NMR spectra of the (*S*)-form **23e** and (*R*)-form **23f** of 3-methyl-nor-TAN-950 A showed two singlets of the methyl groups on the C-3 at $\delta$ 2.10, and at $\delta$ 1.85 in DMSO-$d_6$–$D_2O$. From the intensity of each singlet, the enantiomer excess of the **14l** and **14m** was determined to be 45 and 21%, respectively. The decreased optical purity of nor-TAN-950 A derivatives is considered to be due to the proton on the chiral carbon adjacent to the isoxazolone ring being more acidic[15] than those of TAN-950 A-type compounds **14a—c**.

**Biological Activity** The binding assays were performed using $^3$H-kainic acid (racemate),[16] $^3$H-AMPA,[16] and $^3$H-CPP [(*RS*)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphoric acid][16] as labeled ligands to the three (kainate-, quisqualate- and NMDA-) types of receptors on rat brain membrane preparations.[3b] The affinities of the TAN-950 A congeners to each receptor are expressed in terms of $IC_{50}$ ($\mu$M: the concentration of the test compound giving a 50% inhibition of $^3$H-kainic acid-, $^3$H-AMPA-, and $^3$H-CPP-specific binding to the corresponding receptors). The results are shown in Tables III. The excitatory potency of the compound was also determined by the microelectrophysiological method by using a rat hippocampus *in vitro*,[3] and is expressed as the minimum effective concentration (MEC, $\mu$M) in Table III.

TAN-950 A derivatives showed unique structure–activity relations on their selectivities and excitatory potencies to the three types of excitatory amino acid receptors. Naturally occurring TAN-950 A (**1**) had high affinity for the glutamate receptors, but the excitatory potency in the hippocampus was as moderate (MEC, 300 $\mu$M) as L-glutamic acid. Dipeptide derivatives, *e.g.* TAN-950 A-$\beta$-alanine (**6d**), TAN-950 A-$\gamma$-aminobutanoic acid (**6e**), TAN-950 A-$\beta$-aminoalanine

Table III. Glutamate Receptor Binding of TAN-950A Derivatives (**6a—o** and **15a—o**)

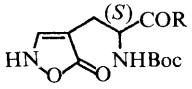
| No. | Glutamate receptor binding $IC_{50}$ ($\mu$M) K | Q | N | Excitation of neurons MEC ($\mu$M) |
|---|---|---|---|---|
| **6a** | 19 | 48 | 21 | — |
| **6b** | 51 | 20 | 10 | >1000 |
| **6c** | 57 | >100 | >100 | — |
| **6d** | 5.2 | 6.4 | 11 | 1000 |
| **6e** | 12 | 3.0 | 6.5 | 1000 |
| **6f** | 31 | 5.5 | 16 | 1000 |
| **6g** | 22 | 21 | 47 | — |
| **6h** | 29 | 13 | 16 | >1000 |
| **6i** | 24 | 21 | 11 | >1000 |
| **6j** | 37 | 59 | 27 | — |
| **6k** | 18 | 17 | 10 | — |
| **6l** | 36 | 13 | 10 | — |
| **6m** | 4.5 | 1.0 | 6.3 | 300 |
| **6n** | 6.8 | 61 | 20 | — |
| **6o** | 20 | 67 | 40 | — |
| **15a** | 37 | 14 | 6.2 | 100 |
| **15b** | 17 | 0.30 | 40 | 1 |
| **15c** | 98 | 3.9 | 68 | 300 |
| **15d** | 17 | 0.67 | 35 | 1 |
| **15e** | 67 | 3.8 | >100 | 3 |
| **15f** | 3.8 | 0.12 | 13 | 0.3 |
| **15g** | 57 | 0.31 | >100 | 0.3 |
| **15h** | 26 | 15 | 11 | 30 |
| **15i** | 89 | 100 | 20 | — |
| **15j** | >100 | >100 | 47 | — |
| **15k** | >100 | >100 | 5.8 | 30 |
| **15l** | >100 | >100 | 21 | — |
| **15m** | >100 | >100 | 8.3 | 10 |
| **15n** | >100 | >100 | 7.9 | 30 |
| **15o** | 33 | >100 | 2.1 | >300 |
| **1** TAN-950A | 3.6 | 0.28 | 19 | 300 |
| L-Glutamate | 0.11 | 0.24 | 0.98 | 300 |
| AMPA | 87 | 0.036 | >100 | 1 |
| NMDA | >100 | >100 | 14 | 10 |
| Ibotenate | 54 | 100 | 6.4 | 10 |

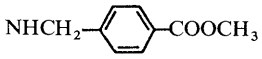
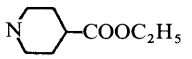
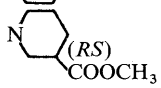
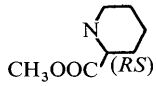
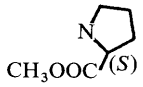
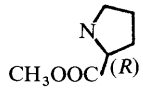



| | R | n | configuration of C* | R | $^1$H-NMR DMSO-$d_6$–$D_2O$ $\delta$ |
|---|---|---|---|---|---|
| **23a** | H | 1 | *S* | H | 8.21 |
| **23b** | H | 1 | *R* | H | 8.13 |
| **23c** | $CH_3$ | 1 | *S* | $CH_3$ | 1.88 |
| **23d** | $CH_3$ | 1 | *R* | $CH_3$ | 1.93 |
| **23e** | $CH_3$ | 0 | *S* | $CH_3$ | 2.10 |
| **23f** | $CH_3$ | 0 | *R* | $CH_3$ | 1.85 |

Chart 5

TABLE IV. Protected TAN-950 A Derivatives (**4a—o**)

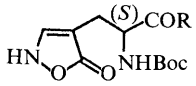


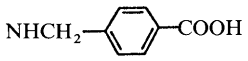
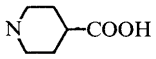
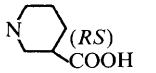
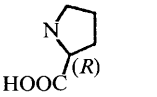
| No. | R | Yield (%) | $^1$H-NMR $\delta$ (in DMSO-$d_6$) |
|---|---|---|---|
| **4a** | $NHCH_2COOC_2H_5$ | 29 | 1.19 (3H, t, $J$=7 Hz), 1.37 (9H, s), 2.34—2.49 (2H, m), 3.78—3.93 (3H, m), 4.08 (2H, dd, $J$=7, 15 Hz), 7.17 (1H, d, $J$=7.4 Hz), 7.67 (1H, s), 8.10—8.19 (1H, m) |
| **4b** | (*S*) $NHCHCOOPNB$ $(CH_2)_2CH_3$ | 55 | 0.71—0.90 (3H, m), 1.12—1.70 (4H, m), 1.37 (9H, s), 2.42—2.60 (2H, m), 4.08—4.32 (1H, m), 4.43—4.60 (1H, m), 5.21 (2H, s), 7.48 (2H, d, $J$=8.4 Hz), 7.81 (1H, s), 8.21 (2H, d, $J$=8.4 Hz) |
| **4c** | (*S*) $NHCHCOOPNB$ $(CH_2)_3CH_3$ | 40 | 0.81—0.94 (3H, b), 1.16—1.46 (4H, m), 1.37 (9H, s), 1.64—1.80 (2H, m), 2.32—2.51 (2H, m), 4.06—4.15 (1H, m), 4.29—4.40 (1H, m), 5.28 (2H, s), 6.89 (1H, d, $J$=8.2 Hz), 7.63 (2H, d, $J$=8.4 Hz), 8.13 (1H, s), 8.23 (2H, d, $J$=8.4 Hz), 8.28—8.32 (1H, m) |
| **4d** | $NH(CH_2)_2COOC_2H_5$ | 30 | 1.20 (3H, t, $J$=7 Hz), 1.37 (9H, s), 2.32—2.41 (4H, m), 3.23—3.33 (2H, m), 3.79—3.89 (1H, m), 4.07 (2H, q, $J$=7.15 Hz), 7.36—7.39 (1H, m), 7.57 (1H, s), 7.67—7.81 (1H, m) |
| **4e** | $NH(CH_2)_3COOC_2H_5$ | 18 | 1.18 (3H, t, $J$=7 Hz), 1.37 (9H, s), 2.21—2.41 (4H, m), 3.02—3.21 (2H, m), 3.29—3.49 (2H, m), 3.74—3.89 (1H, m), 4.03 (2H, q, $J$=7 Hz), 7.21—7.29 (1H, m), 7.56 (1H, s), 7.68—7.81 (1H, m) |
| **4f** | $NH(CH_2)_4COOC_2H_5$ | 20 | 1.18 (3H, t, $J$=7 Hz), 1.36 (9H, s), 1.37—1.70 (2H, m), 2.21—2.42 (4H, m), 3.02—3.19 (2H, m), 3.74—3.88 (1H, m), 4.04 (2H, q, $J$=7 Hz), 7.04—7.08 (1H, m), 7.59 (1H, s), 7.62—7.64 (1H, m) |
| **4g** | (*S*) $NHCH_2CHCOOCH_3$ NHZ | 28 | 1.35 (9H, s), 2.29—2.48 (2H, m), 3.22—3.53 (2H, m), 3.62 (3H, s), 3.73—3.90 (1H, m), 4.07—4.21 (1H, m), 5.03 (2H, s), 7.36 (5H, s), 7.62 (1H, b), 7.89 (1H, b) |
| **4h** | (*S*) $NHCH_2CHCOOCH_3$ NHBoc | 42 | 1.38 (18H, s), 2.22—2.41 (2H, m), 3.22—3.52 (2H, m), 3.61 (3H, s), 3.70—3.81 (1H, m), 3.92—4.10 (1H, m), 7.08—7.18 (1H, m), 7.31—7.40 (1H, m), 7.58 (1H, s), 7.81—7.86 (1H, m) |
| **4i** | (*R*) $NHCH_2CHCOOCH_3$ NHBoc | 39 | 1.37 (18H, s), 2.22—2.41 (2H, m), 3.22—3.48 (2H, m), 3.62 (3H, s), 3.74—3.81 (1H, m), 3.95—4.10 (1H, m), 7.08 (1H, d, $J$=7.6 Hz), 7.19—7.25 (1H, m), 7.58 (1H, s), 7.80—7.86 (1H, m) |
| **4j** |  | 49 | 1.39 (9H, s), 2.44 (2H, d, $J$=6 Hz), 3.84 (3H, s), 3.88—3.97 (1H, m), 4.31, 4.34 (2H, m), 7.28 (2H, d, $J$=8 Hz), 7.56 (1H, s), 7.62—7.79 (1H, m), 7.85 (2H, d, $J$=8 Hz) |
| **4k** |  | 43 | 1.19 (3H, t, $J$=7 Hz), 1.35 (9H, s), 1.22—1.50 (1H, m), 1.69—1.89 (2H, m), 2.17—3.19 (6H, m), 3.99—4.43 (5H, m), 6.98 (1H, d, $J$=7.6 Hz), 7.60 (1H, s) |
| **4l** |  | 21 | 1.21—2.08 (5H, m), 1.35 (9H, s), 2.29—2.91 (3H, m), 3.25—3.48 (2H, m), 3.62 (3H, s), 3.82—4.03 (1H, m), 4.35—4.59 (1H, m), 6.97—7.09 (1H, m), 8.12 (1H, s) |
| **4m** |  | 11 | 1.32—1.69 (5H, m), 1.36 (9H, s), 2.07—2.63 (4H, m), 3.20—3.51 (1H, m), 3.62 (3H, s), 3.91—4.22 (2H, m), 6.71—6.86 (1H, m), 7.56 (1H, s) |
| **4n** |  | 34 | 1.34 (9H, s), 1.79—2.43 (6H, m), 3.68—3.91 (2H, m), 3.61 (3H, s), 4.01—4.42 (2H, m), 6.98 (1H, d, $J$=7 Hz), 7.73 (1H, s) |
| **4o** |  | 29 | 1.37 (9H, s), 1.71—2.42 (6H, m), 3.38—3.91 (3H, m), 3.58 (3H, s), 4.12—4.29 (1H, m), 6.86 (1H, d, $J$=7 Hz), 7.57 (1H, s) |

derivatives (**6g—i**), TAN-950 A-piperidinecarboxylic acids (**6k—m**) and TAN-950 A-proline derivatives (**6n, o**), had an affinity for glutamate receptors, and the excitatory action of these compounds were rather weaker than L-glutamic acid as shown in Table III. The synthetic derivatives **15b, d, f, g** having alkyl substituents (methyl, ethyl, cyclopropyl and cyclopentyl) at the C-3 position of the isoxazolone ring also showed a high affinity selective for the quisqualate-receptor. Among them, the compounds which have a cycloalkyl group such as a cyclopropyl group and a cyclopentyl group **15f, g** showed the strongest agonistic activity (*ca.* 1000 times as strong as L-glutamic acid) and were stronger than AMPA. 3-Methyl and ethyl derivatives **15b, d** had the same potency as AMPA in the excitatory activities (MEC, 1 $\mu$M). The introduction of a sterically hindered substituent (isopropyl) and an aromatic ring (phenyl) lowered the binding values to the quisqualate-receptor as well as the excitatory activities in the neurons as seen in compounds **15e, h**. These results indicate that the excitatory potency of the compounds (agonistic effect on the quisqualate-receptor) is not always parallel to the affinity value ($IC_{50}$),[17] and that a suitable bulkiness of the C-3 substituent is required to show strong agonistic activity.

(*R*)-TAN-950 A (**15a**), the enantiomer of TAN-950 A, decreased the affinity for kainate- and quisqualate-receptors, but showed some increased affinity for the NMDA-receptor compared with TAN-950 A. The introduction of methyl group [compound **15c**, (*R*)-3-methyl-TAN-950 A] lowered the affinity for the NMDA receptor. On the other hand, the removal of a methylene group [compound **15k**, (*R*)-nor-TAN-950 A[18]] increased the selectivity to the NMDA receptor as well as the excitatory activity. The NMDA-selectivity was unaffected by the introduction of an alkyl

TABLE V. Protected TAN-950 A Derivatives (**5a—o**)



| No. | $R^3$ | Yield (%) | $^1$H-NMR $\delta$ (in DMSO-$d_6$) |
|---|---|---|---|
| **5a** | $NHCH_2COOH$ | 87 | —[a] |
| **5b** | $NHCH(CH_2)_2CH_3$ (*S*)-COOH | 83 | —[a] |
| **5c** | $NHCH(CH_2)_3CH_3$ (*S*)-COOH | 54 | 0.78—0.93 (3H, b), 1.15—1.46 (4H, m), 1.36 (9H, s), 1.55—1.99 (2H, m), 2.30—2.51 (2H, m), 3.90—4.05 (1H, m), 4.19—4.30 (1H, m), 7.08 (1H, d, $J$=7.6 Hz), 7.91 (1H, s), 8.07 (1H, d, $J$=7.4 Hz) |
| **5d** | $NH(CH_2)_2COOH$ | 65 | —[a] |
| **5e** | $NH(CH_2)_3COOH$ | 83 | — SI-MS $m/z$: 358 $(M+H)^+$ |
| **5f** | $NH(CH_2)_4COOH$ | 90 | — SI-MS $m/z$: 372 $(M+H)^+$ |
| **5g** | $NHCH_2CH(NHZ)$ (*S*)-COOH | 82 | 1.35 (9H, s), 2.22—2.53 (2H, m), 3.22—3.59 (2H, m), 3.97—4.19 (2H, m), 5.03 (2H, s), 7.82 (1H, d, $J$=8 Hz), 7.35 (5H, s), 7.50 (1H, d, $J$=8 Hz), 7.08 (1H, b), 8.16 (1H, s) |
| **5h** | $NHCH_2CH(NHBoc)$ (*R*)-COOH | 83 | 1.36 (18H, s), 2.22—2.47 (2H, m), 3.22—3.57 (2H, m), 3.97—4.08 (2H, m), 6.82 (1H, d, $J$=7.8 Hz), 6.98 (1H, d, $J$=7.8 Hz), 7.86—7.99 (1H, m), 8.17 (1H, s) |
| **5i** | $NHCH_2CH(NHBoc)$ COOH | 92 | 1.36 (18H, s), 2.27—2.42 (2H, m), 3.22—3.51 (2H, m), 3.93—4.08 (2H, m), 6.82 (1H, d, $J$=7.8 Hz), 6.99 (1H, d, $J$=7.8 Hz), 7.91—8.01 (1H, m), 8.16 (1H, s) |
| **5j** |  | 92 | 1.38 (9H, s), 2.43—2.57 (2H, m), 3.97—4.18 (1H, m), 4.34 (2H, d, $J$=5.8 Hz), 7.05 (1H, d, $J$=7.4 Hz), 7.32 (2H, d, $J$=8 Hz), 7.87 (2H, d, $J$=8 Hz), 8.20 (1H, s), 8.47—8.52 (1H, m) |
| **5k** |  | 47 | 1.36 (9H, s), 1.18—1.50 (1H, m), 1.72—1.88 (2H, m), 2.31—2.84 (4H, m), 3.21—3.39 (2H, m), 3.81—4.52 (3H, m), 7.88—8.03 (1H, m), 8.30 (1H, s) |
| **5l** |  | 78 | 1.21—2.09 (5H, m), 1.35 (9H, s), 2.17—2.45 (3H, m), 2.63—3.53 (2H, m), 3.81—4.15 (1H, m), 4.31—4.59 (1H, m), 6.82—7.10 (1H, m), 8.22 (1H, s) |
| **5m** |  | 85 | 1.31—1.72 (5H, m), 1.37 (9H, s), 2.08—2.71 (4H, m), 3.17—3.42 (1H, m), 3.88—4.11 (2H, m), 7.04 (1H, d, $J$=7.6 Hz), 8.22 (1H, s) |
| **5n** |  | 56 | 1.38 (9H, s), 1.81—2.48 (6H, m), 3.57—3.82 (2H, m), 4.21—4.45 (2H, m), 6.98 (1H, m), 8.20 (1H, s) |
| **5o** |  | 65 | 1.36 (9H, s), 1.81—2.47 (6H, m), 3.37—3.79 (2H, m), 4.11—4.43 (2H, m), 6.81 (1H, d, $J$=7 Hz), 8.30 (1H, s) |

*a*) Used for the next step without purification.

group as seen in the binding values ($IC_{50}$) of 3-substituted nor-TAN-950 A derivatives **15m, n, o**, but the excitatory activities varied in the wide range as shown in Table III. The compound **15m** having a methyl group at the C-3 position was the strongest NMDA-activator and the 3-ethyl derivative **15n** was a little weaker than **15m**. The 3-isopropyl derivative **15o** was much less active in spite of a high affinity for the NMDA-receptor.

3-Methyl-nor-TAN-950 A derivatives **15l, m**, however, were proved to be partially racemized as described above, and the e.e. was considered to be 45 and 21%, respectively, as described above. Since (*R*)-rich compound **15m** showed a higher affinity than that of (*S*)-rich compound **15l**, the major part of the binding affinity of **15l** for the NMDA-receptor might be due to the (*R*)-isomer in **15l**. Compounds **15l, m** with higher enantiomeric purity are needed to elucidate the relationship between the stereochemistry and NMDA-affinity. The alternative synthetic route to **15l, m** with high enantiomer excess is under investigation.

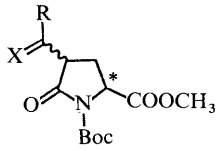
In contrast to the high activity of the above 3-methyl-TAN-950 A derivatives **15b, m**, 3-methyl-homo-TAN-950 A (**15i**) decreased not only the selectivity but also the affinity for glutamate receptors.

Thus, the derivatives of TAN-950 A, in particular **15b, f, g** (quisqualate-type agonists) and **15m** (NMDA-type agonist), revealed high affinities as well as strong excitatory potencies. These compounds will be good tools for investigating the pharmacological and physiological roles of the excitatory amino acid receptors.

**Experimental**

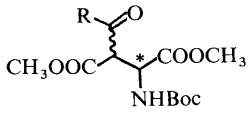
Infrared (IR) spectra were measured with a Hitachi 215 spectrophotometer. $^1$H-NMR spectra were taken on a Varian EM-390 (90 MHz), Varian Gemini-200 (200 MHz) and JEOL JNM-GX400FT (400 MHz) with tetramethylsilane as an internal standard. Abbreviations are as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. The optical rotations were recorded with a JASCO DIP-181 digital polarimeter. The secondary ion mass spectra (SI-MS) were measured with a Hitachi M-80A mass spectrometer. Extracted solutions were dried over

TABLE VI. Pyroglutamic Acid Derivatives (**10d—h** and **11a—h**)




| No. | Configuration of | | | Yield (%) | $^1$H-NMR $\delta$ (in $CDCl_3$) | IR (C=O) $\nu_{max}^{neat}$ ($cm^{-1}$) |
|---|---|---|---|---|---|---|
| | R | X | C* | | | |
| **10d** | $CH_3CH_2$ | OH, H | *S* | 40 | 0.99 (3H, t, $J$=7.2 Hz), 1.50 (2H, m), 1.51 (9H, s), 1.90—2.25 (2H, m), 3.69 (2H, m), 3.81 (3H, s), 4.61 (1H, dd, $J$=9.0, 1.8 Hz) | 1780, 1750, 1720, 1640 |
| **10e** | $(CH_3)_2CH$ | OH, H | *S* | 69 | 0.90 (3H, d, $J$=6.8 Hz), 1.04 (3H, d, $J$=6.8 Hz), 1.51 (9H, s), 1.69 (1H, m), 1.91—2.50 (2H, m), 2.72 (1H, m), 3.60 (1H, m), 3.81 (3H, s), 4.61 (1H, dd, $J$=8.8, 2.2 Hz) | 1780, 1750, 1720, 1640 |
| **10f** |  | OH, H | *S* | 99 | 0.20—0.80 (5H, m), 1.51 (9H, s), 1.95—3.10 (4H, m), 3.79 (3H, s), 4.64 (1H, m) | 1780, 1750, 1740, 1710 |
| **10g** |  | OH, H | *S* | 48 | 1.50 (9H, s), 1.41—1.90 (9H, m), 2.12—2.90 (4H, m), 3.80 (3H, s), 4.59 (1H, m) | 1780, 1750 |
| **10h** | Ph | OH, H | *S* | 65 | 1.46 (9H, s), 2.30—3.30 (3H, m), 3.66 (3H, s), 4.51 (1H, dd, $J$=10, 15 Hz), 5.40 (1H, dd, $J$=2.7, 2.0 Hz), 7.29 (5H, s) | 1790, 1750 |
| **11a** | H | O | *R* | 29 | 1.51, 1.50, 1.49 (9H, s each), 2.10—3.10 (2H, m), 3.61 (0.3H, dd, $J$=10, 4.0 Hz), 3.75—3.85 (0.7 H, m), 3.76, 3.79, 3.81 (3H, s each), 4.62 (0.5H, dd, $J$=10, 2.5 Hz), 4.66 (0.5H, dd, $J$=10.5, 3.6 Hz), 7.00 (0.33H, s), 9.80 (0.33H, s), 9.91 (0.33H, s) | 1790, 1755, 1730, 1650 |
| **11b** | $CH_3$ | O | *S* | 72 | 1.49 (9H, s), 2.1—2.9 (2H, m), 2.43 (1.5H, s), 2.47 (1.5H, s), 3.5—3.8 (1H, m), 3.79 (3H, s), 4.62 (1H, dd, $J$=9.0, 3.0 Hz) | 1800, 1755, 1730, 1640 |
| **11c** | $CH_3$ | O | *R* | 51 | 1.49 (9H, s), 2.1—2.9 (2H, m), 2.43 (1.5H, s), 2.47 (1.5H, s), 3.5—3.8 (1H, m), 3.79 (3H, s), 4.62 (1H, dd, $J$=9.0, 3.0 Hz) | 1800, 1755, 1730, 1640 |
| **11d** | $CH_3CH_2$ | O | *S* | 66 | 1.06 (1.5H, t, $J$=7.2 Hz), 1.09 (1.5H, t, $J$=7.2 Hz), 1.50 (9H, s), 1.90—3.20 (4H, m), 3.63 (1H, dd, $J$=9.6, 3.6 Hz), 3.78, 3.80 (3H, s each), 4.60 (0.5H, dd, $J$=7.2, 3.0 Hz), 4.66 (0.5H, dd, $J$=6.8, 3.2 Hz) | 1780, 1750, 1720, 1630 |
| **11e** | $(CH_3)_2CH$ | O | *S* | 48 | 1.13 (6H, d, $J$=7 Hz), 1.49 (9H, s), 1.90—3.41 (4H, m), 3.77, 3.80 (3H, s each), 4.61 (0.5H, dd, $J$=9.2, 3.0 Hz), 4.65 (0.5H, dd, $J$=9.6, 3.2 Hz) | 1790, 1750, 1720, 1630 |
| **11f** |  | O | *S* | 50 | 1.0—1.3 (4H, m), 1.47 (1H, m), 1.49 (9H, s), 2.2—2.8 (3H, m), 3.75 (3H, s), 4.65 (1H, m) | 1800, 1760, 1730, 1710 |
| **11g** |  | O | *S* | 99 | 1.49 (9H, s), 1.51—2.20 (9H, m), 2.20—3.10 (2H, m), 3.74 (3H, s), 3.74 (1H, m), 4.63 (1H, m) | 1790, 1745, 1720 |
| **11h** | Ph | O | *S* | 69 | 1.50 (9H, s), 2.01—3.30 (2H, m), 3.79 (3H, s), 4.35 (1H, m), 4.20—4.80 (1H, m), 7.20—7.70 (3H, m), 8.02—8.20 (3H, m) | 1790, 1750, 1685 |

TABLE VII. Aspartic Acid Derivatives (**20**)



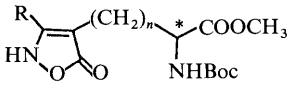

| No. | Configuration of | | Yield (%) | $^1$H-NMR $\delta$ (in $CDCl_3$) | IR (C=O) $\nu_{max}^{neat}$ ($cm^{-1}$) |
|---|---|---|---|---|---|
| | R | C* | | | |
| **20j** | H | *S* | 28 | 1.45 (9H, s), 3.60—4.00 (1H, m), 3.69, 3.74, 3.76, 3.79, 3.83 (6H, s each), 4.83 (0.5H, m), 5.08 (0.5H, m), 5.52 (1H, m), 7.72 (0.50H, s), 9.83 (0.25H, s), 9.84 (0.25H, s) | 1760—1660 |
| **20k** | H | *R* | 20 | 1.45 (9H, s), 3.60—4.00 (1H, m), 3.69, 3.74, 3.76, 3.79, 3.83 (6H, s each), 4.83 (0.5H, m), 5.08 (0.5H, m), 6.03 (1H, m), 7.72 (0.50H, s), 9.83 (0.25H, s), 9.84 (0.25H, s) | 1760—1660 |
| **20l** | $CH_3$ | *S* | 81 | 1.43 (9H, s), 2.31 (1.5H, s), 2.33 (1.5H, s), 3.75, 3.76 (6H, s each), 4.27 (1H, m), 4.97 (1H, m), 5.61 (1H, m) | 1760—1720 |
| **20m** | $CH_3$ | *R* | 38 | 1.43 (9H, s), 2.31 (1.5H, s), 2.33 (1.5H, s), 3.75, 3.76 (6H, s each), 4.27 (1H, m), 4.97 (1H, m), 5.61 (1H, m) | 1760—1720 |
| **20n** | $CH_3CH_2$ | *R* | 92 | 1.08 (1.5H, t, $J$=7.2 Hz), 1.09 (1.5H, t, $J$=7.2 Hz), 1.45 (9H, s), 2.65 (1H, q, $J$=7.2 Hz), 2.66 (1H, q, $J$=7.2 Hz), 3.76 (3H, s), 3.77 (3H, s), 4.28 (1H, d, $J$=4.6 Hz), 4.95 (1H, dd, $J$=9.2, 4.6 Hz), 5.6—5.7 (1H, m) | 1760—1720 |
| **20o** | $(CH_3)_2CH$ | *R* | 62 | 1.11 (3H, d, $J$=7.0 Hz), 1.13 (3H, d, $J$=7.0 Hz), 1.43 (4.5 H, s), 1.44 (4.5H, s), 2.84 (1H, m), 3.72 (1.5H, s) 3.75 (3H, s), 3.76 (1.5H, s), 4.49 (0.5H, d, $J$=4.4 Hz), 4.51 (0.5H, d, $J$=3.4 Hz), 4.92 (1H, m), 5.57 (0.5H, d, $J$=8.8 Hz), 5.70 (0.5H, d, $J$=4.5 Hz) | 1760—1720 |

anhydrous magnesium sulfate.

**Starting Materials** Glycine ethyl ester (**3a**), β-alanine ethyl ester (**3d**), ethyl isonipecotate (**3k**), (*S*)-proline methyl ester (**3n**) and (*R*)-proline methyl ester (**3o**) were commercially available.

Compound **3b**: *p*-Nitrobenzyl bromide (5.52 g, 25.6 mmol) was added to a mixture of *N*-Boc-(*S*)-norvaline (4.65 g, 21.4 mmol), $NaHCO_3$ (18 g,

TABLE VIII. Esters of *N*-Boc-TAN-950 A-Related Analogs (**13a—o**)



| No. | Configuration of | | | Yield (%) | $^1$H-NMR $\delta$ (in $CDCl_3$) | IR (C=O) $\nu_{max}^{neat}$ ($cm^{-1}$) |
|---|---|---|---|---|---|---|
| | R | *n* | C* | | | |
| **13a** | H | 1 | *R* | 75 | 1.44 (9H, s), 2.70 (1H, dd, $J$=15.8, 6.8 Hz), 2.83 (1H, dd, $J$=15.2, 5.6 Hz), 3.76 (3H, s), 4.44 (1H, m), 5.58 (1H, d, $J$=7.2 Hz), 7.98 (1H, s) | 1700, 1600 |
| **13b** | $CH_3$ | 1 | *S* | 45 | 1.43 (9H, s), 2.15 (3H, s), 2.74 (2H, d, $J$=5 Hz), 3.75 (3H, s), 4.44 (1H, m), 5.78 (1H, d, $J$=7 Hz) mp 134—136 °C, *Anal.* Calcd for $C_{13}H_{20}N_2O_6$: C, 51.99; H, 6.71; N, 9.33. Found: C, 51.73; H, 6.58; N, 9.23. $[\alpha]_D^{24}$ −16.5° ($c$=0.5, MeOH) | 1740, 1680, 1630 (KBr) |
| **13c** | $CH_3$ | 1 | *R* | 43 | 1.43 (9H, s), 2.15 (3H, s), 2.74 (2H, d, $J$=5 Hz), 3.75 (3H, s), 4.44 (1H, m), 5.73 (1H, d, $J$=7 Hz) mp 134—136 °C, *Anal.* Calcd for $C_{13}H_{20}N_2O_6$: C, 51.99; H, 6.71; N, 9.33. Found: C, 51.67; H, 6.63; N, 9.33. $[\alpha]_D^{24}$ +16.3° ($c$=0.3, MeOH) | 1740, 1680, 1630 (KBr) |
| **13d** | $CH_3CH_2$ | 1 | *S* | 69 | 1.23 (3H, t, $J$=7.4 Hz), 1.41 (9H, s), 2.55 (2H, d, $J$=7.4 Hz), 2.70 (2H, m), 3.72 (3H, s), 4.43 (1H, m), 5.95 (1H, d, $J$=7.6 Hz) | 1710, 1690, 1620 |
| **13e** | $(CH_3)_2CH$ | 1 | *S* | 24 | 1.24 (6H, d, $J$=7 Hz), 1.40 (9H, s), 2.65 (2H, m), 2.85 (1H, m), 3.69 (3H, s), 4.48 (1H, m), 6.02 (1H, m) | 1710, 1670, 1620 |
| **13f** |  | 1 | *S* | 13 | 0.8—1.2 (4H, m), 1.43 (9H, s), 1.85 (1H, m), 2.70—2.90 (2H, m), 3.75 (3H, s), 4.78 (1H, m), 5.82 (1H, m) | 1740, 1710, 1690 |
| **13g** |  | 1 | *S* | 54 | 1.42 (9H, s), 1.30—2.21 (8H, m), 2.73 (2H, m), 2.98 (1H, m), 3.73 (1H, s), 4.48 (1H, m), 5.93 (1H, m) | 1740, 1710, 1690, 1610 |
| **13h** | Ph | 1 | *S* | 75 | 1.33 (9H, s), 2.76 (2H, m), 3.48 (3H, s), 4.35 (1H, m), 7.40 (5H, s) | 1740, 1710, 1680, 1610 |
| **13i** | $CH_3$ | 2 | *RS* | 37 | 1.45 (9H, s), 2.15 (3H, s), 1.60—2.20 (4H, m), 3.77 (3H, s), 4.35 (1H, m), 5.20 (1H, b) | 1750, 1720, 1670, 1620 |
| **13j** | H | 0 | *S* | 54 | 1.37 (9H, s), 3.68 (3H, s), 5.07 (1H, m), 6.11 (1H, b), 7.08 (1H, s) | 1740—1680, 1630 |
| **13k** | H | 0 | *R* | 53 | 1.37 (9H, s), 3.68 (3H, s), 5.07 (1H, m), 6.10 (1H, b), 7.08 (1H, s) | 1740—1680, 1630 |
| **13l** | $CH_3$ | 0 | *S* | 30 | 1.39 (9H, s), 1.98 (3H, s), 3.65 (3H, s), 4.87 (1H, m), 6.30 (1H, m) | 1750, 1710, 1640 |
| **13m** | $CH_3$ | 0 | *R* | 32 | 1.39 (9H, s), 1.98 (3H, s), 3.65 (3H, s), 4.87 (1H, m), 6.26 (1H, m) | 1750, 1710, 1640 |
| **13n** | $CH_3CH_2$ | 0 | *R* | 12 | 1.12 (3H, t, $J$=7.2 Hz), 1.38 (9H, s), 2.38 (2H, q, $J$=7.2 Hz), 3.78 (3H, s), 4.88 (1H, m), 6.15 (1H, b) | 1740, 1720, 1710, 1620 |

214.3 mmol) and KI (4.24 g, 25.5 mmol) in *N*,*N*-dimethylformamide (DMF) (100 ml), and the mixture was stirred at room temperature for 15 h. Water was added, and the mixture was extracted with AcOEt. The extract was washed with water, and dried. After evaporation of the solvent, the residue was subjected to column chromatography on silica gel. Elution with AcOEt–$CHCl_3$–hexane (1 : 1 : 5) afforded *N*-Boc-(*S*)-norvaline *p*-nitrobenzyl ester (5.5 g, 73%) as pale yellow crystals, mp 45—46 °C. $^1$H-NMR ($CDCl_3$) $\delta$: 0.61—1.90 (7H, m, $CH_2CH_2CH_3$), 1.41 (9H, s, *tert*-Bu), 4.19—4.46 (1H, m, CH), 4.75—4.99 (1H, m, NH), 5.23 (2H, s, $CH_2Ar$), 7.51 (2H, d, $J$=8 Hz, aromatic protons), 8.24 (2H, d, $J$=8 Hz, aromatic proton). The ester (2.5 g, 7.1 mmol) dissolved in 4 N HCl–dioxane (20 ml), and the mixture was stirred for 1 h. After evaporation of the solvent, $Et_2O$ was added to the residue and the supernatant layer was removed by decantation (3 times). (*S*)-Norvaline *p*-nitrobenzyl ester hydrochloride (**3b**·HCl) (1.9 g, 80%) was obtained as pale yellow crystals.

Compound **3c**: (*S*)-Norleucine *p*-nitrobenzyl ester hydrochloride (**3c**·HCl) was prepared in a manner similar to that described for the synthesis of **3b**. Yield 68%, pale yellow crystals, mp 133—135 °C

The ethyl esters **3e, f** were prepared from corresponding amino acids by the HCl–EtOH method,[19] and used for the next step without purification.

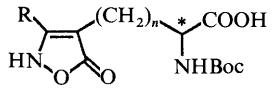
The methyl esters **3g, l, m** were prepared from corresponding amino acids by the $SOCl_2$–MeOH method.[20] **3g**·HCl: yield 88%, mp 131—133 °C. **3l**·HCl: yield 97%, mp 116—118 °C. **3m**·HCl: yield 97%, mp 195—196 °C.

Compound **3h**: A solution of benzyl chloroformate (6.9 ml, 48.3 mmol) in $Et_2O$ (100 ml) was added dropwise to a mixture of $N^{\alpha}$-Boc-(*S*)-$\beta$-aminoalanine[21] (8.16 g, 39.96 mmol) and $NaHCO_3$ (20 g, 238.1 mmol) in water (200 ml). After being stirred for 2 h, the mixture was extracted with $Et_2O$. The aqueous layer was made acidic (pH 2) with 5% aqueous phosphoric acid at 0 °C and extracted with AcOEt. The extract was washed with water and dried. The solution was concentrated under reduced pressure, and the concentrate was dissolved in DMF (150 ml). Sodium bicarbonate (34 g, 404.7 mmol) and $CH_3I$ (15 ml, 240.1 mmol) were added, and the mixture was stirred for 15 h. Water was added, and the mixture was extracted with AcOEt. The extract was washed with water and dried. After evaporation of the solvent, the residue was chromatographed on silica gel. Elution with AcOEt–hexane (2 : 3) followed by recrystallization from AcOEt–hexane gave $N^{\beta}$-benzyloxycarbonyl-$N^{\alpha}$-Boc-(*S*)-$\beta$-aminoalanine methyl ester (5.2 g, 38%) as a white powder, mp 42—43 °C. $^1$H-NMR ($CDCl_3$) $\delta$: 1.44 (9H, s, *tert*-Bu), 3.59 (2H, t, $J$=5.8 Hz, $CH_2N$), 3.74 (3H, s, $OCH_3$), 4.36—4.41 (1H, m, CH), 5.10 (2H, s, $CH_2Ar$), 7.35 (5H, s, aromatic protons), $[\alpha]_D^{24}$ −9.9° ($c$=1.0, MeOH). A mixture of this compound (1.3 g) and 10% Pd–C (0.3 g) in MeOH (40 ml) was stirred under a hydrogen atmosphere. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to afford $N^{\alpha}$-Boc-(*S*)-$\beta$-aminoalanine methyl ester (**3h**, 0.79 g, quantitative) as a colorless oil.

Compound **3i**: $N^{\alpha}$-Boc-(*R*)-$\beta$-aminoalanine methyl ester (**3i**) was prepared from $N^{\alpha}$-Boc-(*R*)-$\beta$-aminoalanine[21] in a manner similar to that described for the synthesis of **3h**. $N^{\beta}$-Benzyloxycarbonyl-$N^{\alpha}$-Boc-(*R*)-$\beta$-aminoalanine methyl ester (19%), mp 42—43 °C. The NMR spectrum was identical with that of the (*S*)-form, $[\alpha]_D^{24}$ +9.9° ($c$=1.8, MeOH). Compound **3i** (oil, quantitative) was used for the next step without purification.

Compound **3j**: Methyl *N*-Boc-*p*-aminomethylbenzoate (oil, 25%) was prepared from *N*-Boc-*p*-aminomethylbenzoic acid[22] by methylation in a manner similar to that described as above. $^1$H-NMR ($CDCl_3$) $\delta$: 1.41 (9H, s, *tert*-Bu), 3.82 (3H, s, $OCH_3$), 4.32 (2H, d, $J$=6 Hz, $CH_2NAr$), 4.73—5.02 (1H, br, NH), 7.30 (2H, d, $J$=8 Hz, aromatic protons), 7.98 (2H, d, $J$=8 Hz, aromatic protons). The ester (1.3 g, 4.9 mmol) was dissolved in 4 N HCl–dioxane (15 ml), and the solution was stirred for 1 h. After evaporation of the solvent, $Et_2O$ was added to the residue and the

TABLE IX. *N*-Boc-TAN-950 A-Related Analogs (**14a—o**)



| No. | Configuration of | | | Yield (%) | $^1$H-NMR $\delta$ (in $CDCl_3$) | IR (C=O) $\nu_{max}^{neat}$ ($cm^{-1}$) |
|---|---|---|---|---|---|---|
| | R | *n* | C* | | | |
| **14a** | H | 1 | *R* | 79 | 1.37 (9H, s), 2.43 (1H, dd, $J$=9.4, 14.6 Hz), 2.57 (1H, dd, $J$=5.1, 14.6 Hz), 4.02 (1H, ddd, $J$=5.1, 8.2, 9.4 Hz), 7.12 (1H, d, $J$=8 Hz), 8.24 (1H, s) (in DMSO-$d_6$) $[\alpha]_D^{23}$ +22.0° ($c$=0.5, MeOH) | 1740—1690 |
| **14b** | $CH_3$ | 1 | *S* | 78 | 1.43 (9H, s), 2.17 (3H, s), 2.60—2.90 (2H, m), 4.40 (1H, m) (+acetone-$d_6$) | 1740—1680 |
| **14c** | $CH_3$ | 1 | *R* | 80 | 1.40 (9H, s), 2.17 (3H, s), 2.60—2.90 (2H, m), 4.39 (1H, m) (+acetone-$d_6$) | 1740—1680 |
| **14d** | $CH_3CH_2$ | 1 | *S* | 98 | 1.21 (3H, t, $J$=7.4 Hz), 1.44 (9H, s), 2.59 (2H, q, $J$=7.4 Hz), 2.75 (2H, m), 4.42 (1H, m), 6.33 (1H, d, $J$=8.2 Hz) | 1720—1690 |
| **14e** | $(CH_3)_2CH$ | 1 | *S* | 99 | 1.24 (6H, d, $J$=7 Hz), 1.43 (9H, s), 2.71 (1H, dd, $J$=14, 4.6 Hz), 2.84 (1H, dd, $J$=16, 6 Hz), 3.04 (1H, m), 4.45 (1H, m), 6.34 (1H, m) | 1720—1660 |
| **14f** |  | 1 | *S* | 78 | 0.8—1.2 (4H, m), 1.44 (9H, s), 1.90 (1H, m), 2.83 (2H, m), 4.45 (1H, m), 6.16 (1H, d, $J$=7.0 Hz) | 1740, 1720, 1690, 1660 |
| **14g** |  | 1 | *S* | 92 | 1.43 (9H, s), 1.60—2.21 (8H, m), 2.76 (2H, m), 3.07 (1H, m), 4.44 (1H, m), 6.27 (1H, d, $J$=7.6 Hz) | 1730, 1710, 1690, 1670 (Neat) |
| **14h** | Ph | 1 | *S* | 98 | 1.33 (9H, s), 2.86 (2H, d, $J$=6 Hz), 4.42 (1H, m), 6.08 (1H, b), 7.48 (5H, s) | 1720, 1680 |
| **14i** | $CH_3$ | 2 | *RS* | 97 | 1.45 (9H, s), 2.18 (3H, s), 2.04 (2H, m), 2.37 (2H, m), 4.25 (1H, m), 5.60 (1H, d, $J$=7 Hz) | 1740—1670 |
| **14j** | H | 0 | *S* | 85 | 1.39 (9H, s), 4.76 (1H, d, $J$=8 Hz), 7.35 (1H, d, $J$=8 Hz), 8.38 (1H, s) (DMSO-$d_6$) | 1740—1690 |
| **14k** | H | 0 | *R* | 72 | 1.39 (9H, s), 4.76 (1H, d, $J$=8 Hz), 7.35 (1H, d, $J$=8 Hz), 8.38 (1H, s) (DMSO-$d_6$) | 1740—1690 |
| **14l** | $CH_3$ | 0 | *S* | 83 | 1.40 (9H, s), 2.12 (3H, s), 4.79 (1H, d, $J$=7.2 Hz), 7.15 (1H, d, $J$=7.2 Hz) (DMSO-$d_6$) | 1750, 1680, 1650 |
| **14m** | $CH_3$ | 0 | *R* | 35 | 1.39 (9H, s), 2.12 (3H, s), 4.79 (1H, d, $J$=7.2 Hz), 7.13 (1H, d, $J$=7.2 Hz) (DMSO-$d_6$) | 1750, 1680, 1650 |
| **14n** | $CH_3CH_2$ | 0 | *R* | 85 | 1.14 (3H, t, $J$=7.2 Hz), 1.43 (9H, s), 2.21 (2H, q, $J$=7.2 Hz), 5.12 (1H, d, $J$=7.4 Hz), 6.13 (1H, d, $J$=7.4 Hz) | 1740, 1720, 1680, 1660 |
| **14o** | $(CH_3)_2CH$ | 0 | *R* | 9[a] | 1.16 (6H, d, $J$=6.6 Hz), 1.38 (9H, s), 2.79 (1H, m), 4.57 (1H, d, $J$=7.6 Hz), 6.80 (1H, m) | 1740, 1720, 1710, 1620 |

*a*) The yield based on compound **20o**.

supernatant layer was removed by decantation (3 times). Methyl *p*-aminomethylbenzoate hydrochloride (**3j**·HCl) (0.65 g, 66%) was obtained as colorless crystals, mp 235—238 °C.

Methyl (*R*)-*N*-Boc-pyroglutamate [(*R*)-**8**][10] was obtained by the *tert*-butoxycarbonylation[23] of methyl (*R*)-pyroglutamate, mp 71—72 °C, $[\alpha]_D^{22}$ +44.4° ($c$=0.885, EtOH). Methyl (*S*)-*N*-Boc-pyroglutamate [(*S*)-**8**]: mp 71—72 °C, $[\alpha]_D^{22}$ −44.6° ($c$=0.625, EtOH).

Dimethyl (*S*)-*N*-*tert*-butoxycarbonylaspartate[12] ((*S*)-**19**): mp 70—71 °C, $[\alpha]_D^{20}$ −19.6° ($c$=0.945, MeOH). Dimethyl (*R*)-*N*-*tert*-butoxycarbonylaspartate [(*R*)-**19**]: mp 70—71 °C, $[\alpha]_D^{22}$ +20.4° ($c$=1.09, MeOH).

**Ethyl *N*-[(*S*)-2-*tert*-Butoxycarbonylamino-3-(2,5-dihydro-5-oxo-4-isoxazolyl)propionyl]glycinate (4a, Table IV)** DEPC (0.66 ml, 4.35 mmol) and $Et_3N$ (0.98 ml, 7.03 mmol) were added to a mixture of **2** (1.0 g, 3.67 mmol) and glycine ethyl ester (**3a**) (512 mg, 3.67 mmol) in DMF (10 ml) at 0 °C. After being stirred for 1 h, the mixture was diluted with AcOEt and washed successively with 5% aqueous phosphoric acid and water. The organic layer was separated and dried. After evaporation of the solvent, the residue was subjected to column chromatography on silica gel. Elution with $CH_2Cl_2$–acetone–MeOH (5:5:1) afforded **4a** (388 mg, 29%) as a pale yellow oil.

Compounds **4b—o** (Table IV)[24] were prepared in a similar manner using amino acid esters **3b—o** and **2**.

***N*-[(*S*)-2-*tert*-Butoxycarbonylamino-3-(2,5-dihydro-5-oxo-4-isoxazolyl)-propionyl]glycine (5a, Table V)** A 0.5 N solution of NaOH (6 ml) was added to a solution of **4a** (370 mg, 1.03 mmol) in MeOH (4 ml), and the mixture was stirred for 30 min. Water and AcOEt were added to the reaction mixture, and the aqueous layer was separated. The aqueous layer was made acidic (pH 3.5) with 5% aqueous phosphoric acid and extracted with AcOEt. The extract was washed with water and dried. The solvent was removed under reduced pressure to give **5a** (310 mg, 87%) as a colorless oil.

Compounds **5b—o** (Table V)[24] were prepared in a similar manner starting with **4b—o**.

***N*-[(*S*)-2-Amino-3-(2,5-dihydro-5-oxo-4-isoxazolyl)propionyl]glycine Hydrochloride (6a, Table I)** A 4 N HCl–dioxane solution (20 ml) was added to **5a** (310 mg, 0.90 mmol), and the resulting solution was stirred at room temperature for 1 h. After evaporation of the solvent, $Et_2O$ was added to the residue and the supernatant layer was removed by decantation (3 times). Compound **6a** (160 mg, 67%) was obtained as a white amorphous powder. *Anal.* Calcd for $C_8H_{11}N_3O_5 \cdot HCl \cdot 1/2dioxane \cdot 2H_2O$: C, 34.74; H, 5.83; N, 12.15. Found: C, 34.64; H, 5.63; N, 12.40.

Compounds **6b—o** (Table I)[24] were prepared in a similar manner starting with **5b—o**. *Anal.* Calcd for $C_{10}H_{15}N_3O_5 \cdot HCl$ (**6e**): C, 38.53; H, 5.82; N, 13.48. Found: C, 38.76; H, 5.75; N, 13.52. *Anal.* Calcd for $C_{11}H_{17}N_3O_5 \cdot HCl \cdot 1/2H_2O$ (**6f**): C, 41.71; H, 6.05; N, 13.27. Found: C, 41.57; H, 6.29; N, 13.06. *Anal.* Calcd for $C_{12}H_{17}N_3O_5 \cdot HCl \cdot 1/2dioxane \cdot 2H_2O$ (**6l**): C, 43.81; H, 6.83; N, 10.95. Found: C, 43.82; H, 6.36; N, 10.66. *Anal.* Calcd for $C_{11}H_{15}N_3O_5 \cdot HCl \cdot 1/2dioxane \cdot 3/2H_2O$ (**6n**): C, 41.44; H, 6.15; N, 11.15. Found: C, 41.10; H, 5.96; N, 11.20.

**Methyl (2*S*)-1-*tert*-Butoxycarbonyl-4-(1-hydroxypropyl)pyroglutamate (10d, Table VI)** Diisopropylamine (1.54 ml, 11 mmol) and *n*-butyllithium (6.9 ml of 1.6 M hexane solution, 11 mmol) were added to dry tetrahydrofuran (THF) (40 ml) at −78 °C under a nitrogen atmosphere, and the mixture was stirred for 10 min at −78 °C. A solution of methyl (*S*)-*N*-Boc-pyroglutamate [(*S*)-**8**][10] (2.43 g, 10 mmol) in dry THF (15 ml) was added dropwise to the mixture. After stirring at −78 °C for 10 min, the mixture was stirred at −40 °C for 20 min, and then cooled to −78 °C. A solution of propionaldehyde (0.8 ml, 11 mmol) in dry THF (4 ml) was added to the solution, and the mixture was stirred for 10 min. Saturated aqueous $NH_4Cl$ was added, and the reaction mixture was warmed to room temperature. The mixture was extracted with AcOEt, and the extract was washed successively with water and saturated aqueous NaCl. The extract was dried and evaporated *in vacuo*. The residue was subjected to chromatography on silica gel. Elution with AcOEt–hexane (1:3→1:2→1:1) gave **10d** (1.20 g, 40%) as a colorless oil.

The compounds **10e, f, g, h** (Table VI) were synthesized from (*S*)-**8** by reaction with the corresponding aldehyde.

**Methyl (2*R*)-1-*tert*-Butoxycarbonyl-4-formylpyroglutamate (11a, Table VI)** Lithium diisopropylamide (24.2% hexane solution, 24 mmol) was added dropwise to a solution of methyl (*R*)-*N*-Boc-pyroglutamate [(*R*)-**8**]

(4.86 g, 20 mmol) in dry THF (100 ml) at −78 °C under a nitrogen atmosphere, and the mixture was stirred for 10 min at −78 °C. The mixture was stirred at −40 °C for 20 min, and then cooled to −78 °C. A solution of isopropyl formate (2.4 ml, 24 mmol) in dry THF (5 ml) was added to the solution, and the mixture was stirred for 10 min at −78 °C. The mixture was then stirred at −40 °C and stirring was continued until the temperature reached −20 °C. Isopropyl alcohol was added to the reaction mixture and the whole was poured into the mixture of $Et_2O$ and water. The aqueous layer was separated and the organic layer was extracted with water. The aqueous layers were combined and made acidic (pH 2) with 1 N HCl. The aqueous solution was saturated with NaCl and extracted with AcOEt. The extract was washed with water and saturated aqueous NaCl, and dried. After removal of the solvent, the residue was subjected to chromatography on silica gel. Elution with AcOEt–hexane (2 : 1)→AcOEt→AcOEt–MeOH (9 : 1) gave **11a** (1.57 g, 29%) as a colorless oil.

**Methyl (2*S*)-4-Acetyl-1-*tert*-butoxycarbonylpyroglutamate (11b, Table VI)** Diisopropylamine (2.94 ml, 21 mmol) and *n*-butyllithium (14 ml of 1.6 M hexane solution, 22 mmol) were added to dry THF (40 ml) at −78 °C under a nitrogen atmosphere. The mixture was stirred for 10 min at −78 °C. A solution of methyl (*S*)-*N*-Boc-pyroglutamate [(*S*)-**8**][10] (2.43 g, 10 mmol) in dry THF (15 ml) was added dropwise to the mixture. After stirring at −78 °C for 10 min, the mixture was stirred at −40 °C for 20 min, and then cooled to −78 °C. A solution of *N*-acetylimidazole (1.32 g, 12 mmol) in dry THF (10 ml) was added to the solution, and the mixture was stirred for 10 min at −78 °C. The mixture was then stirred at −40 °C, and stirring was continued until the temperature reached −20 °C. Saturated aqueous $NH_4Cl$ was added and the reaction mixture was warmed to room temperature. The mixture was extracted with AcOEt. The extract was washed successively with water and saturated aqueous NaCl, and dried. After evaporation of the solvent, the residue was subjected to chromatography on silica gel. Elution with AcOEt–hexane (1 : 1→2 : 1) gave **11b** (2.04 g, 72%) as a colorless oil.

The compound **11c** (Table VI) was synthesized by the same procedure using (*R*)-**8** as the starting material.

**Methyl (2*RS*)-5-Acetyl-1-*tert*-butoxycarbonyl-6-oxo-2-piperidinecarboxylate (17)** Compound **17** was prepared from methyl (2*RS*)-1-*tert*-butoxycarbonyl-6-oxo-2-piperidinecarboxylate (**16**)[25] in a manner similar to that described for the preparation of **11b**. Yield 20%. IR $\nu_{max}^{neat}$ $cm^{-1}$: 2980, 1780, 1760, 1720, 1620, 1470, 1370. $^1$H-NMR ($CDCl_3$) $\delta$: 1.51 (9H, s, *tert*-Bu), 1.90—2.60 (5H, m, $CH_2 \times 2$, CH), 2.00 (3H, s, $COCH_3$), 3.77 (3H, s, $OCH_3$), 4.81 (1H, m, CHN).

**Methyl (2*S*)-1-*tert*-Butoxycarbonyl-4-propionylpyroglutamate (11d, Table VI)** A solution of dimethyl sulfoxide (0.52 ml, 7.33 mmol) in $CH_2Cl_2$ (2 ml) was added to a mixture of $CH_2Cl_2$ (8 ml) and oxalyl chloride (0.35 ml, 3.65 mmol). The resulting mixture was cooled to −60 °C and then stirred for 2 min under a nitrogen atmosphere. A solution of (*S*)-**10d** (1.01 g, 3.35 mmol) in $CH_2Cl_2$ (2 ml) was added dropwise during the period of 5 min, and stirring was continued for 15 min at −60 °C. Triethylamine (2.34 ml, 16.79 mmol) was added, and the reaction mixture was stirred for 5 min at −60 °C and then allowed to warm to room temperature. Water was added and the organic layer was separated. The aqueous layer was extracted with $CH_2Cl_2$. The organic layers were combined, washed with saturated aqueous NaCl and dried. After removal of the solvent, the residue was chromatographed on silica gel. Elution with AcOEt–hexane (1 : 3→1 : 2→1 : 1) gave **11d** (657 mg, 66%) as a colorless oil.

The compounds **11e, f, g, h** (Table VI) were synthesized by the same procedure from **10e, f, g, h**, respectively.

**Dimethyl (2*S*)-2-*tert*-Butoxycarbonyl-3-formylaspartate (20j, Table VII)** Diisopropylamine (1.54 ml, 11 mmol) and *n*-butyllithium (7.3 ml of 1.6 M hexane solution, 11 mmol) were added to dry THF (40 ml) at −78 °C under a nitrogen atmosphere, and the mixture was stirred for 10 min at −78 °C. A solution of dimethyl (*S*)-*N*-Boc-aspartate [(*S*)-**19**] (1.31 g, 5 mmol) in dry THF (5 ml) was added dropwise to the mixture. After being stirred at −78 °C for 10 min, the mixture was stirred at −40 °C for 20 min, and then cooled to −78 °C. A solution of isopropyl formate (0.6 ml, 6 mmol) in dry THF (1 ml) was added to the solution, and the mixture was stirred for 10 min at −78 °C. The mixture was then stirred at −40 °C, and stirring was continued until the temperature reached −20 °C. Isopropyl alcohol (2 ml) was added to the reaction mixture, and the mixture was warmed to room temperature. The mixture was poured into water–$Et_2O$ and extracted with water. The aqueous extract was adjusted to pH 2 with 2 N HCl and saturated with NaCl. The aqueous solution was extracted with AcOEt. The extract was washed with saturated aqueous NaCl and dried. After evaporation of the solvent, the residue was subjected to chromatography on silica gel. Elution with AcOEt–hexane (1 : 1) gave **20j** (407 mg, 28%) as a colorless oil.

The compound **20k** (Table VI) was synthesized by the same procedure starting with (*R*)-**19**.

**Dimethyl (2*S*)-3-Acetyl-2-*tert*-butoxycarbonylaspartate (20l, Table VII)** Lithium diisopropylamide (30.8% hexane solution, 15.8 ml, 33 mmol) was added dropwise to a solution of (*S*)-**19** (3.92 g, 15 mmol) in dry THF (70 ml) at −78 °C under a nitrogen atmosphere, and the mixture was stirred for 10 min at −78 °C. The mixture was stirred at −40 °C for 20 min, and then cooled to −78 °C. A solution of *N*-acetylimidazole (3.30 g, 30 mmol) in dry THF (30 ml) was added to the solution and the mixture was stirred for 10 min at −78 °C. The mixture was stirred at −40 °C, and stirring was continued until the temperature reached −20 °C. Saturated aqueous $NH_4Cl$ was added to the reaction mixture, and the mixture was stirred at room temperature for 10 min. AcOEt and water were added to the reaction mixture. The organic layer was separated, washed with water and saturated aqueous NaCl, and dried. The solvent was removed, and the residue was subjected to chromatography on silica gel. Elution with AcOEt–hexane (1 : 3) gave **20l** (3.68 g, 81%) as a colorless oil.

The compounds **20m—o**[26] (Table VII) were synthesized in a similar manner using (*R*)-**19** and corresponding acylimidazole.[27]

**Methyl (2*R*)-2-*tert*-Butoxycarbonylamino-3-(2,5-dihydro-5-oxo-4-isoxazolyl)propanoate (13a, Table VIII)** Hydroxylamine (403 mg, 5.80 mmol) was added to a mixture of **11a**, 1,4-dioxane (16 ml) and water (10 ml), and the mixture was stirred at room temperature for 1 h. AcOEt (50 ml) was added to the reaction mixture. The organic layer was separated, washed successively with water and saturated aqueous NaCl, and dried. After evaporation of the solvent, the residue was dissolved in MeOH (50 ml), and 0.5 N aqueous NaOH (12 ml) was added. The resulting mixture was stirred at room temperature for 2 h, and poured into water–$Et_2O$. The aqueous layer was separated, adjusted to pH 3 with 2 N aqueous HCl, saturated with NaCl and extracted with AcOEt. The extract was washed with saturated aqueous NaCl and dried. The solvent was evaporated off, and the residue was subjected to column chromatography on silica gel. Elution with AcOEt→AcOEt–MeOH (9 : 1) afforded **13a** (1.25 g, 75%) as a pale yellow oil.

**Methyl (*S*)-2-*tert*-Butoxycarbonylamino-3-(2,5-dihydro-3-methyl-5-oxo-4-isoxazolyl)propanoate (13b, Table VIII)** Hydroxylamine hydrochloride (497 mg, 7.15 mmol) was added to a mixture of **11b** (2.04 g, 7.15 mmol) in 1,4-dioxane (12 ml) and water (10 ml), and the mixture was stirred for 2 h. A 0.5 N aqueous solution of NaOH (14.2 ml) was added, and the reaction mixture was stirred for 1 h. The mixture was adjusted to pH 6.5 with 1 N aqueous HCl and extracted with AcOEt. The extract was washed successively with water and saturated aqueous NaCl, and dried. The solvent was removed, and the residue was subjected to column chromatography on silica gel. Elution with AcOEt→AcOEt–MeOH (9 : 1) followed by recrystallization from AcOEt–hexane gave **13b** (956 mg, 45%) as colorless prisms.

Compounds **13c—e, 13i—n** (Table VIII) were synthesized in a similar manner starting with **11c—e, 17, 20i—n**, respectively.

**Methyl (*S*)-2-*tert*-Butoxycarbonylamino-3-(3-cyclopropyl-2,5-dihydro-5-oxo-4-isoxazolyl)propanoate (13f, Table VIII)** A mixture of **11f** (641 mg, 2.1 mmol), hydroxylamine hydrochloride (161 mg, 2.3 mmol) in 1,4-dioxane (4 ml) and water (0.5 ml) was stirred at room temperature for 24 h. The mixture was adjusted to pH 2.5 with 1 N aqueous HCl, saturated with NaCl and extracted with AcOEt. The extract was washed with saturated aqueous NaCl and dried. After evaporation of the solvent, the residue was subjected to column chromatography on silica gel using AcOEt→AcOEt–MeOH (9 : 1) as an eluent. The compound **13f** (89 mg, 13%) was obtained as a pale yellow oil.

**Methyl (*S*)-2-*tert*-Butoxycarbonylamino-3-(2,5-dihydro-5-oxo-3-phenyl-4-isoxazolyl)propanoate (13h, Table VIII)** A mixture of **11h** (1.54 g, 4.44 mmol), hydroxylamine hydrochloride (309 mg, 4.45 mmol) and sodium carbonate (235 mg, 2.22 mmol) in EtOH (35 ml) was refluxed under an argon atmosphere for 1 h. Insoluble substance was filtered off, and the filtrate was concentrated under reduced pressure. The concentrate was subjected to chromatography on silica gel. Elution with AcOEt–hexane (2 : 1)→AcOEt→AcOEt–MeOH (9 : 1) afforded **13h** (1.21 g, 75%) as a pale yellow amorphous powder.

Compound **13g** (Table VIII) was prepared in a similar manner starting with **11g**.

**(*S*)-2-*tert*-Butoxycarbonylamino-3-(2,5-dihydro-3-methyl-5-oxo-4-isoxazolyl)propanoic Acid (14b, Table IX)** A 0.5 N aqueous solution of NaOH (6.4 ml) was added to **13b** (320 mg, 1.07 mmol), and the mixture was stirred at room temperature for 30 min. The mixture was extracted with AcOEt.

The aqueous layer was adjusted to pH 2.7 with 1 N aqueous HCl and saturated with NaCl. The aqueous layer was extracted with AcOEt. The extract was washed with saturated aqueous NaCl and dried. The solvent was evaporated off to give **14b** (238 mg, 78%) as a pale yellow amorphous powder.

Compounds **14a** and **14c—n** (Table IX) were prepared in a similar manner starting with **13a** and **13c—n**, respectively.

**(*S*)-2-*tert*-Butoxycarbonylamino-3-(2,5-dihydro-3-isopropyl-5-oxo-4-isoxazolyl)acetic Acid (14o, Table IX)** A mixture of **20o** (1.44 g, 4.35 mmol) and hydroxylamine hydrochloride (302 mg, 4.35 mmol) in 1,4-dioxane (8.5 ml) and water (3 ml) was adjusted to pH 8 with 0.5 N aqueous NaOH, and stirred at room temperature for 24 h. The mixture was adjusted to pH 2.5 with 1 N aqueous HCl and saturated with NaCl. The resulting mixture was extracted with AcOEt. The extract was washed with saturated aqueous NaCl and dried. After evaporation of the solvent, the residue was subjected to chromatography on silica gel. Elution with AcOEt→AcOEt–MeOH (9 : 1→8 : 1) afforded **14o** (115 mg, 9%) as a pale yellow amorphous powder.

**(*S*)-2-Amino-3-(2,5-dihydro-3-methyl-5-oxo-4-isoxazolyl)propanoic Acid Hydrochloride (15b·HCl, Table II)** A 4 N aqueous HCl–dioxane (20 ml) was added to **14b** (410 mg, 1.43 mmol). The mixture was stirred at room temperature for 1 h. After evaporation of the solvent, $Et_2O$ was added to the residue, and the supernatant layer was removed by decantation (3 times). The hydrochloride **15b**·HCl (351 mg, 75%) was obtained as a white powder. *Anal.* Calcd for $C_7H_{10}N_2O_4 \cdot HCl \cdot$dioxane$\cdot H_2O$: C, 40.19; H, 6.44; N, 8.52. Found: C, 39.91; H, 6.12; N, 8.70.

Compounds **15a**·HCl and **15c—o**·HCl (Table II) were prepared in a similar manner starting with **14a** and **14c—o**, respectively. *Anal.* Calcd for $C_6H_8N_2O_4 \cdot HCl \cdot 1/2$dioxane$\cdot H_2O$ (**15a**): C, 35.50; H, 5.59; N, 10.35. Found: C, 35.63; H, 5.73; N, 10.37. *Anal.* Calcd for $C_8H_{12}N_2O_4 \cdot HCl \cdot$dioxane$\cdot 1/2H_2O$ (**15d**): C, 43.18; H, 6.64; N, 8.39. Found: C, 43.62; H, 6.91; N, 8.22. *Anal.* Calcd for $C_{12}H_{12}N_2O_4 \cdot HCl \cdot$dioxane$\cdot H_2O$ (**15h**): C, 49.17; H, 5.93; N, 7.17. Found: C, 48.96; H, 5.91; N, 7.15. *Anal.* Calcd for $C_6H_8N_2O_4 \cdot HCl \cdot 1/2$dioxane$\cdot H_2O$ (**15l**): C, 35.50; H, 5.59; N, 10.35. Found: C, 35.64; H, 5.28; N, 10.05. *Anal.* Calcd for $C_6H_8N_2O_4 \cdot HCl \cdot 2/3H_2O$ (**15m**): C, 30.59; H, 5.13; N, 11.89. Found: C, 30.69; H, 5.29; N, 12.28.

**(*S*)-2-Amino-3-(2,5-dihydro-3-methyl-5-oxo-4-isoxazolyl)propanoic Acid (15b, Table II)** The hydrochloride of **15b**·HCl (1.89 g) was dissolved in water (22 ml), and the resulting aqueous solution was adjusted to pH 3.5 with 0.1 N aqueous NaOH. After concentration of the mixture, the concentrate was subjected to column chromatography on microcrystalline cellulose (*ca.* 3 l). Elution with $CH_3CN$–water (87 : 17) afforded NaCl and **15b** hydrochloride. Then, the fractions eluted with $CH_3CN$–water (8 : 2) was collected and lyophilized to afford **15b** (695 mg) as a white powder. *Anal.* Calcd for $C_7H_{10}N_2O_4 \cdot H_2O$: C, 41.18; H, 5.92; N, 13.72. Found: C, 41.41; H, 5.68; N, 13.56.

**(*S*)-2-*tert*-Butoxycarbonylamino-3-(2,5-dihydro-5-oxo-3-isoxazolyl)-*N*-[(*R*)-1-(1-naphthyl)ethyl]propanamide (23c)** (*R*)-1-(1-Naphthyl)ethylamine (0.063 ml, 0.39 mmol), DEPC (0.065 ml, 0.43 mmol) and $Et_3N$ (0.054 ml, 0.39 mmol) were added to a solution of **14b** (113 mg, 0.39 mmol) in DMF (1.2 ml) at 0 °C. After stirring for 1 h at 0 °C, AcOEt and 5% aqueous phosphoric acid were added, and the mixture was extracted with AcOEt. The extract was washed successively with water and saturated aqueous NaCl, and dried. The solvent was evaporated off, and the residue was subjected to column chromatography on silica gel. Elution with AcOEt–hexane (1 : 1) afforded **23c** (111 mg, 65%) as a pale yellow amorphous powder. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3320, 1720, 1700, 1650, 1510, 1440, 1390. $^1$H-NMR (DMSO-$d_6$–$D_2O$, 400 MHz) $\delta$: 1.35 (9H, s, *tert*-Bu), 1.43 (3H, d, $J$=6.8 Hz, $CHC\underline{H}_3$), 1.88 (3H, s, $CH_3$), 2.34 (1H, dd, $J$=14.4, 7.3 Hz, $CH\underline{H}$), 2.41 (1H, dd, $J$=14.4, 5.6 Hz, $CH\underline{H}$), 4.01 (1H, m, CHN), 5.62 (1H, q, $J$=6.8 Hz, $C\underline{H}Me$), 7.53 (3H, m, arom. H), 7.85 (1H, d, $J$=8 Hz, arom. H), 7.94 (1H, d, $J$=8 Hz, arom. H), 8.08 (1H, d, $J$=8 Hz, arom. H), 8.29 (1H, d, $J$=8 Hz, arom. H).

Compounds **23a**, **b**, **d**, **e**, **f** were prepared in a similar manner starting with **2**, **14a**, **c**, **l**, **m**, respectively.

Compound **23a**: A pale yellow amorphous powder, yield 36%. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3300, 2980, 1720, 1700, 1640, 1510, 1440, 1390. $^1$H-NMR (DMSO-$d_6$–$D_2O$, 400 MHz) $\delta$: 1.34 (9H, s, *tert*-Bu), 1.47 (3H, d, $J$=6.8 Hz, $CHC\underline{H}_3$), 2.42 (1H, dd, $J$=14.2, 8.3 Hz, $CH\underline{H}$), 2.49 (1H, dd, $J$=14.2, 6.6 Hz, $CH\underline{H}$), 4.13 (1H, m, CHN), 5.65 (1H, q, $J$=6.8 Hz, $C\underline{H}Me$), 7.53 (1H, m, arom. H), 7.57 (3H, m, arom. H), 7.83 (1H, d, $J$=8 Hz, arom. H), 7.95 (1H, d, $J$=8 Hz, arom. H), 8.07 (1H, d, $J$=8 Hz, arom. H), 8.21 (1H, s, isoxazolone H).

Compound **23b**: A pale yellow amorphous powder, yield 46%. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3300, 2980, 1720, 1700, 1640, 1510, 1440, 1390. $^1$H-NMR (DMSO-$d_6$–$D_2O$, 400 MHz) $\delta$: 1.35 (9H, s, *tert*-Bu), 1.49 (3H, d, $J$=6.8 Hz, $CHC\underline{H}_3$), 2.36 (1H, dd, $J$=13.7, 8.5 Hz, $CH\underline{H}$), 2.49 (1H, dd, $J$=13.7, 5.6 Hz, $CH\underline{H}$), 4.13 (1H, m, CHN), 5.67 (1H, q, $J$=6.8 Hz, $C\underline{H}Me$), 7.48 (1H, m, arom. H), 7.55 (3H, m, arom. H), 7.83 (1H, m, arom. H), 7.94 (1H, d, $J$=8 Hz, arom. H), 8.07 (1H, d, $J$=8 Hz, arom. H), 8.13 (0.934H, s, isoxazole H ((*R*)-form)), 8.21 (0.066H, s, isoxazolone H ((*S*)-form)).

Compound **23d**: A pale yellow amorphous powder, yield 50%. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3300, 1720, 1700, 1650, 1510, 1440, 1390. $^1$H-NMR (DMSO-$d_6$–$D_2O$, 400 MHz) $\delta$: 1.34 (9H, s, *tert*-Bu), 1.48 (3H, d, $J$=6.8 Hz, $CHC\underline{H}_3$), 1.93 (3H, s, $CH_3$), 2.33 (1H, dd, $J$=14.1, 8.3 Hz, $CH\underline{H}$), 2.46 (1H, dd, $J$=14.1, 6.1 Hz, $CH\underline{H}$), 4.10 (1H, m, CHN), 5.68 (1H, q, $J$=6.8 Hz, $C\underline{H}Me$), 7.51 (3H, m, arom. H), 7.82 (1H, m, arom. H), 7.93 (1H, d, $J$=8 Hz, arom. H), 8.08 (1H, d, $J$=8 Hz, arom. H), 8.41 (1H, d, $J$=8 Hz, arom. H).

Compound **23e**: A pale yellow amorphous powder, yield 70%. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3300, 2980, 1720, 1700, 1660, 1600, 1500, 1450. $^1$H-NMR (DMSO-$d_6$–$D_2O$, 400 MHz) $\delta$: 1.37 (9H, s, *tert*-Bu), 1.46 (2.17H, d, $J$=6.8 Hz, $CHC\underline{H}_3$), 1.50 (0.83H, d, $J$=6.8 Hz, $CHC\underline{H}_3$), 1.85 (0.83H, s, $CH_3$), 2.10 (2.17H, s, $CH_3$), 4.82 (1H, m, CHN), 5.71 (1H, q, $J$=6.8 Hz, $C\underline{H}Me$), 7.46—7.78 (3H, m, arom. H), 7.82 (1H, m, arom. H), 7.93 (1H, d, $J$=8 Hz, arom. H), 8.09 (1H, d, $J$=8 Hz, arom. H), 8.52 (1H, d, $J$=8 Hz, arom. H).

Compound **23f**: A pale yellow amorphous powder, yield 66%. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3300, 2980, 1720, 1700, 1660, 1600, 1500, 1450. $^1$H-NMR (DMSO-$d_6$–$D_2O$, 400 MHz) $\delta$: 1.37 (9H, s, *tert*-Bu), 1.46 (1.18H, d, $J$=6.8 Hz, $CHC\underline{H}_3$), 1.50 (1.82H, d, $J$=6.8 Hz, $CHC\underline{H}_3$), 1.85 (1.82H, s, $CH_3$), 2.10 (1.18H, s, $CH_3$), 4.82 (1H, m, CHN), 5.72 (1H, q, $J$=6.8 Hz, $C\underline{H}Me$), 7.46—7.78 (3H, m, arom. H), 7.83 (1H, m, arom. H), 7.92 (1H, d, $J$=8 Hz, arom. H), 8.09 (1H, d, $J$=8 Hz, arom. H), 8.53 (1H, d, $J$=8 Hz, arom. H).

**Acknowledgement** We thank Dr. S. Terao and Dr. A. Nagaoka for their encouragement and fruitful suggestions throughout this work.

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# Solid Phase Synthesis and Opioid Receptor Binding Properties of Presumable Dermorphin Precursor Derivatives[1)]

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**Presumable dermorphin precursor peptide derivatives comprized of 35 amino acids and their fragments, which are based on the amino acid sequence determined by recombinant deoxyribonucleic acid (DNA) techniques, were synthesized by the solid phase method. A 35-residue peptide amide containing L-Ala$^2$-dermorphin sequence at the N-terminus (1) as well as its D-Ala$^2$ isomer (2) and the C-terminal 20-residue peptide amide were found to be unexpectedly stable against aminopeptidase M digestion and in rat brain membrane fractions mixture, suggesting that the C-terminal Glu-rich moiety of 1 and 2 serves to protect from enzymatic breakdown. In the opioid receptor binding assay, 2 showed 40 and 25-fold higher affinities than 1 for $\mu$ and $\delta$-receptors, respectively. The N-terminal 15-residue peptide fragment of 2 showed greatly increased affinities for both receptors, being one half of those of dermorphin, whereas that of 1 showed low affinities. Opioid receptor binding properties of these synthetic peptides may be useful in investigation of the processing to dermorphin.**

**Keywords** dermorphin precursor peptide; 35-residue peptide amide; 15-residue peptide; solid phase synthesis; receptor binding assay; enzymatic stability

Dermorphin (Tyr–D-Ala–Phe–Gly–Tyr–Pro–Ser–$NH_2$) is a highly potent opioid peptide isolated from the South American frog.[2)] By using a complementary deoxyribonucleic acid (cDNA) library prepared from the skin of *Phyllomedusa sauvagei*, the amino acid sequence of several dermorphin precursor polypeptides were recently determined.[3)] Among these precursors, a polypeptide deduced from cDNA termed D-1/2 comprized 197 amino acid residues. As shown in Fig. 1, in the C-terminal fragment of this prepro-dermorphin, four copies containing dermorphin sequence are present. Three copies of these are 33-residue peptide flanked by the typical pro-hormone processing signal (Lys–Arg) at the amino and carboxyl ends and contain additional processing signals (Lys–Lys and Lys–Arg) in the polypeptide chain. A processing cascade including epimerization of Ala residue to D-Ala from the prepro-dermorphin to dermorphin is yet unknown.

The present paper deals with the synthesis and opioid receptor binding properties of presumable processing intermediate peptides or peptide derivatives. The peptides synthesized are shown in Fig. 2. The 35-residue peptides (**1** and **2**) and their C-terminal 20-residue peptide (**5**) were synthesized as the C-terminal amide form to enhance enzymatic stability during biological assays. In general, it is also well known that C-terminal amide derivatives of opioid peptide do not decrease the biological activity dramatically.

Peptides were synthesized by the solid phase method. Boc–amino acids with following side chain protecting groups were used: Tos for Arg, Cl–Z for Lys, cHex for Glu, Bzl for Ser, Br–Z for Tyr and DNP for His. For the synthesis of **1**, **2** and **5**, starting with benzhydrylamine resin, double coupling was performed at each residue with 4-fold excess symmetrical anhydride except for the incorporation of Boc–Asn which was effected with the HOBt/DIPCDI[5)] method. The peptide was cleaved from the resin, with simultaneous side chain deprotection, by treatment with a mixture of HF/*p*-cresol/*p*-thiocresol followed by treatments with dil. alkaline solution to reverse any N→O shift[6)] and with 20% $\beta$-mercaptoethanol to remove the DNP group from the His residue.[7)] Purification of peptides was performed first by gel filtration on Sephadex G-25 and then by preparative medium-pressured HPLC on Develosil LOP ODS. Analytical HPLC of crude 35-peptides (**1** and **2**) gave similar elution patterns. The HPLC profiles of crude and purified **2** are shown in Fig. 3. Peptides **3** and **4** were analogously synthesized starting with Boc–Arg(Tos)–Merrifield resin with some modifications that the peptide assembly was performed by single coupling after the incorporation of Lys$^{11}$ residue and the peptide was cleaved from the resin with a mixture of HF/anisole. Amino acid ratios of all synthetic peptides after acid hydrolysis agreed well with those of expected values. Interestingly, **1**, **3** and **5**, which did not contain D-amino acid in the mole-

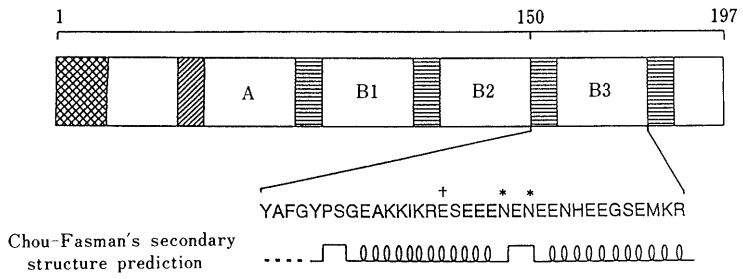


Fig. 1. Schematic Representation of a Dermorphin Precursor Polypeptide (D-1/2)

B1, B2 and B3 are highly homologous: only one or two residues of B3 region are substituted at respective positions of B1 (†, E$^{165}$—V) and B2 (*, N$^{170}$—K and N$^{172}$—I) regions. A region is preceded by deltorphin (or dermenkephalin, ref. 4) sequence (YMFHLMD). ▩, signal peptide; ▨, deltorphin sequence; ▤, dermorphin sequence. Chou–Fasman's secondary structure prediction: -----, random coil; ⎍, $\beta$-turn; ⌇⌇, $\alpha$-helix.

cule, are unexpectedly stable against aminopeptidase M (AP-M) digestion; only appreciable amounts (2%>) of N-terminal amino acids from **1** and **5** and 20% of Tyr of threoretical amounts from **3** were released after overnight incubation while Tyr–Ala–Phe–Gly was completely digested within 4 h of incubation under the same conditions (data not shown). In addition, **1** showed enhanced stability in a mixture of rat brain membrane fractions as compared with **3** as described below. Almost complete enzymatic digestion of **1**—**5** was accomplished by the combined use of protease V. 8 (*S. aureus*)[8] and AP-M (Table I). The 35-residue peptide is predicted to have a high degree of helical conformation according to the method of Chou–Fasman (Fig. 1). Since **5** itself did not show an inhibitory effect on AP-M activity (data not shown), these results suggest that the particular structural and/or ionic effects of C-terminal Glu residue-rich moiety in the 35-residue peptide molecules serves to protect from enzymatic degradation.

**1**: YAFGYPSGEAKKIKRESEEENENEENHEEGSEMKR–$NH_2$
**2**: Y<u>A</u>FGYPSGEAKKIKRESEEENENEENHEEGSEMKR–$NH_2$
**3**: YAFGYPSGEAKKIKR
**4**: Y<u>A</u>FGYPSGEAKKIKR
**5**: ESEEENENEENHEEGSEMKR–$NH_2$

<u>A</u> = D-Ala

Fig. 2. Synthetic Peptides

Opioid receptor binding affinities of peptides were derermined by competition experiments with rat brain membrane fractions using radioligands, [$^3$H]DAGO ($\mu$) and [$^3$H]DADLE ($\delta$), and the results are shown in Table II. **1** showed very weak affinities for $\mu$ and $\delta$-receptors. **5** showed virtually no affinities for both receptors. On the

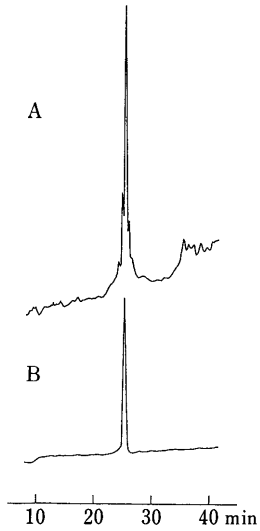


Fig. 3. HPLC Profiles of **2**
A: Crude peptide after gel filtration. B: Purified peptide.

TABLE I. Amino Acid Ratios of Synthetic Peptides

| Amino acid | **1** | | **2** | | Expected value[a] | **3** | | **4** | | **5** | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Acid | AP-M | Acid | AP-M | | Acid | AP-M | Acid | AP-M | Acid | AP-M |
| Tyr | 1.6 | 1.9 | 1.5 | 1.2 | 2 | 1.5 | 1.4 | 1.6 | 1.8 | — | — |
| Ala | 2.1 | 1.9 | 2.1 | 1.1 | 2 | 2.1 | 2.1 | 2.1 | 1.0 | — | — |
| Phe | 1.1 | 1.1 | 1.1 | 0.2 | 1 | 1.0 | 1.0 | 1.0 | 0.2 | — | — |
| Gly | 3.0 | 2.5 | 3.1 | 1.8 | 3 | 2.0 | 1.6 | 2.0 | 0.9 | 1.0 | 1.0 |
| Pro | 0.7 | 0.7 | 0.8 | 0.2 | 1 | 1.0 | 0.3 | 0.9 | 0.5 | — | — |
| Ser | 2.5 | 3.2[b] | 2.6 | 3.8[b] | 3 | 0.8 | 1.0 | 0.8 | 1.0 | 1.0 | 2.3[b] |
| Glu | 11.2 | 11.3 | 11.0 | 11.1 | 11 | 1.0 | 0.6 | 1.0 | 0.6 | 10.8 | 10.1 |
| Lys | 3.8 | 3.5 | 3.8 | 4.0 | 4 | 3.1 | 3.0 | 1.0 | 3.0 | 1.0 | 1.0 |
| Ile | 1.1 | 1.1 | 1.0 | 1.1 | 1 | 1.0 | 1.1 | 1.0 | 1.0 | — | — |
| Arg | 2.0 | 1.8 | 2.0 | 2.2 | 2 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.1 |
| Asn | — | —[b] | — | —[b] | 3 | — | — | — | — | — | —[b] |
| Asp | 3.1 | — | 3.1 | — | 0 | — | — | — | — | 3.4 | — |
| His | 1.0 | 0.8 | 1.0 | 1.3 | 1 | — | — | — | — | 0.95 | 0.86 |
| Met[c] | 0.7 | 0.8 | 0.7 | 1.0 | 1 | — | — | — | — | 0.84 | 0.86 |

*a*) Theoretical values for **1** and **2**. *b*) Asn was coeluted with Ser and calculated as Ser on amino acid analyzer. *c*) Met was partially oxydized to Met(O) during the hydrolyses. AP-M = aminopeptidase-M.

TABLE II. Opioid Receptor Binding Assays of Synthetic Peptides

| Peptides | [$^3$H]-DAGO | | [$^3$H]-DADLE | | $K_i(\delta)/K_i(\mu)$ |
|---|---|---|---|---|---|
| | $K_i(\mu)$, nM | R.P.[a] | $K_i(\delta)$, nM | R.P.[b] | |
| DAGO | 0.41± 0.11 | 100 | 3.54± 2.27 | 41.1 | 8.63 |
| DADLE | 4.09± 0.85 | 10.1 | 1.46± 0.29 | 100 | 0.36 |
| Dermorphin | 0.20± 0.03 | 211 | 3.77± 1.71 | 38.7 | 18.9 |
| **1** | 292.27±105.10 | 0.14 | 513.04± 73.39 | 0.28 | 1.76 |
| **2** | 7.78± 1.68 | 5.33 | 19.99± 6.32 | 7.28 | 2.57 |
| **3** | 250.56± 11.87 | 0.17 | 677.30± 85.40 | 0.21 | 2.70 |
| **4** | 0.45± 0.04 | 92.1 | 1.64± 0.30 | 88.9 | 3.64 |
| **5** | $IC_{50}$ > 10000 | — | $IC_{50}$ > 10000 | — | — |

The inhibition constants ($K_i$) were calculated from $IC_{50}$ values using the Cheng and Prusoff equation (ref. 13). *a*) Relative potencies to DAGO. *b*) Relative potencies to DADLE.

other hand, **2** showed 40 and 25-fold higher affinities than those of **1** for $\mu$ and $\delta$-receptor, respectively. Further, its N-terminal fragment peptide **4**, showed greatly increased $\mu$ and $\delta$-receptor affinities, being 500-fold those of **1** and **3**, while **1** did not changes affinities by lacking its C-terminal 20-residue peptide amide. It seems unlikely that the enzymatic breakdown of **1** and **3** during binding assay is responsible for the low affinity because the hydrolysis rates under conditions similar to the binding assay[9] are **1**: $<5\%$ and **3**: $<20\%$ when assessed by HPLC. The $K_i(\delta)/K_i(\mu)$ values of synthetic peptides (**1**—**4**) were 1—4, which indicate $\mu$-selectivity of these peptides though their $\mu$-affinities and $\mu$-selectivities were considerably lower than that of dermorphin. It should be noted that **3** had low but significant affinities for $\mu$ and $\delta$-receptors whereas L-Ala$^2$-dermorphin has been found to be devoid of biological activities[10] and rat brain membrane binding affinity.[11]

In conclusion, the present study demonstrate that a presumable dermorphin precursor 15-residue peptide extended at the C-terminus of dermorphin (**4**) had high affinities, being one half of those of dermorphin, for $\mu$ and $\delta$-receptors in rat brain. Several experimental data have been accumulated to date showing the presence of immunoreactive dermorphin or its higher molecular forms in mammalian tissues.[12] It is of interest to know the step of the processing cascade in which the epimerization of Ala$^2$ residue of dermorphin occurs. In this respect, the synthetic peptides reported here may be useful in investigation of the processing to dermorphin.

## Experimental

Optical rotations were measured by a JASCO DIP-40 polarimeter. Amino acid analysis was performed using a Hitachi 835 amino acid analyzer after 6 N HCl hydrolysis of the peptide in an evacuated sealed tube at 110 °C for 25 h. Analytical HPLC were performed on a YMC ODS column (AM-303-10, 250 × 4.6 mm) by gradient elution using the following solvent systems. A: 0.06% TFA and B: 80% acetonitrile containing 0.06% TFA. A linear gradient from 10% B to 45% B over 40 min at a flow rate of 1.2 ml/min was used and the eluate was monitored at 215 nm.

**Solid Phase Synthesis** Solid phase synthesis was carried out on benzhydrylamine resin hydrochloride (0.6 meq/g, 1% cross link) or Boc–Arg(Tos)–Merrifield resin (0.2 meq/g, 1% cross link) using a Biosearch 9600 peptide synthesizer according to a coupling schedule as shown in Table III. For the double coupling, steps 10—14 in Table III were repeated before the capping step (15).

**Isolation of Peptides** Peptides **1**, **2** and **5** were obtained in essentially the same way. For example, protected D-Ala$^2$-containing peptide resin (600 mg) was treated with a mixture of HF/*p*-cresol/*p*-thiocresol (90 : 5 : 5, 15 ml) at −20 °C for 30 min and then at 0 °C for 60 min. After evaporation of HF under reduced pressure the resulting residue was triturated with abs. ether and extracted with 10% AcOH. The extract was evaporated to dryness. The product was treated with dil. $NH_4OH$ (pH 8.0) at 0 °C for 60 min and then with 20% aqueous $\beta$-mercaptoethanol at room temperature for 2 h. The entire solution was concentrated to small volumes, when some insoluble materials were removed by centrifugation, and the resulting solution was applied to a column (2.5 × 90 cm) of Sephadex G-25. The column was eluted with 10% AcOH and Pauly reaction-positive eluent was collected and lyophilized to yield a colorless fluffy material, 290 mg. The product (150 mg) was dissolved in 1% TFA (1 ml), when some insoluble materials were removed by centrifugation, and applied to a column (3 × 30 cm) of Develosil LOP ODS which was eluted with a linear gradient from 12% to 28% acetonitrile in 0.06% TFA over 150 min at a flow rate of 3 ml/min. Fractions of 6 ml each were collected and the Nos. 55—57 were pooled, evaporated to dryness and lyophilized from $H_2O$ to yield **2** as a colorless fluffy material, 16.5 mg (7.8% based on the starting resin), $[\alpha]_D^{20}$ −50.2° ($c$=0.32, 1% AcOH), HPLC-$t_R$ 25.4 min.

**1**: Yield 8.0% based on the starting resin, $[\alpha]_D^{20}$ −58.6° ($c$=0.24, 1% AcOH), HPLC-$t_R$ 24.3 min.

TABLE III. Schedule for Solid Phase Peptide Synthesis

| Step | Operation | Time (min) |
|---|---|---|
| 1 | DCM (×4) | 0.5 |
| 2 | 45% TFA, 2% anisole/DCM (×1) | 1 |
| 3 | 45% TFA, 2% anisole/DCM (×1) | 20 |
| 4 | DCM (×2) | 0.5 |
| 5 | DMF (×2) | 0.5 |
| 6 | DCM (×2) | 0.5 |
| 7 | 10% DIPEA/DCM (×3) | 1 |
| 8 | DCM (×3) | 0.5 |
| 9 | DMF (×2) | 0.5 |
| 10 | Symmetrical anhydride (4 eq.)/ DCM–DMF (1 : 1) (×1) | 120 |
| 11 | DCM–DMF (1 : 1) (×2) | 0.5 |
| 12 | 10% DIPEA/DCM (×1) | 0.8 |
| 13 | DCM (×3) | 0.5 |
| 14 | DMF (×2) | 0.5 |
| 15 | 0.4 M Acetyl imidazole/DMF (×1) | 30 |
| 16 | DMF (×3) | 0.5 |

**5**: Yield 18.0% base on the starting resin; $[\alpha]_D^{20}$ −57.1° ($c$=0.28, 1% AcOH), HPLC-$t_R$ 12.3 min.

**3** and **4** were obtained in essentially the same way. For example, protected L-Ala$^2$-15 peptide resin (520 mg) was treated with a mixture of HF/anisole (90 : 10, 10 ml) at 0 °C for 60 min. After evaporation of HF under reduced pressure, the residue was triturated with abs. ether and the peptide was extracted with 10% AcOH. The extract was evaporated to dryness. The product was purified by preparative HPLC as described above with a linear gradient from 14% to 40% acetonitrile in 0.06% TFA over 150 min. Fractions of Nos. 28—29 were pooled and lyophilized to yield **3** as a fluffy material, 40 mg (35.1% based on the starting resin); $[\alpha]_D^{20}$ −54.0° ($c$=0.41, 1% AcOH); HPLC-$t_R$ 23.4 min.

**4**: Yield 49.5% based on the starting resin; $[\alpha]_D^{20}$ −38.4° ($c$=0.37, 1% AcOH); HPLC-$t_R$ 25.3 min.

**Binding Assay** The opioid receptor binding assay was performed by the same method described previously in detail.[9] [$^3$H]DAGO and [$^3$H]DADLE were used as radioligands for $\mu$ and $\delta$-receptor bindings, respectively. Inhibition constants ($K_i$) were calculated from $IC_{50}$ values using the relation of $K_i = IC_{50}/(1 + L/K_d)$,[13] where $L$ is a concentration of radioligand and $K_d$ is its equilibrium dissociation constant. The $IC_{50}$ values were determined from log dose-displacement curves. The $K_d$ values of [$^3$H]DAGO and [$^3$H]DADLE used are 0.46 and 1.74, respectively.

**Enzymatic Hydrolysis of Peptides by Protease V.8 and AP-M** Peptide (200 nmol) was incubated with 12 $\mu$g of protease V.8 (S/E=20) in 0.1 M Tris–HCl buffer at pH 7.50 (total volume of 1.2 ml) at 37 °C for 20 h. The reaction mixture was heated at 80 °C for 10 min and then incubated with 50 $\mu$g of AP-M at 37 °C for 20 h. The hydrolysis solution was directly injected to amino acid analyzer.

**Enzymatic Stability of Peptides in Rat Brain Membrane Fractions Mixture** Peptide (50 nmol) was incubated with 150 $\mu$l of crude rat brain synaptosomal membrane fractions (2 mg of protein/ml) in 50 mM Tris–HCl buffer (pH 7.40) containing 250 $\mu$g of BSA, 20 $\mu$g of bacitracin and 5 $\mu$g of bestatin (total volume 250 $\mu$l) at 25 °C for 2 h.[9] The reaction was stopped by the addition of 0.5 M HCl (50 $\mu$l). After centrifugation at 10000 rpm for 10 min, the supernatant (20 $\mu$l) was injected into HPLC. The hydrolysis rate was estimated by integrating peaks of intact peptide and catabolite(s). The hydrolysis rates are as follows: **1** = <5%, **2** = <2%, **3** = <20%, **4** = <15%, **5** = <2%.

## References and Notes

1) Amino acids and peptides are of L-configuration unless otherwise noted. Abbreviations used are: Boc = *tert*-butoxycarbonyl, Tos = tosyl, Cl–Z = 2-chlorobenzyloxycarbonyl, cHex = cyclohexyl, Bzl = benzyl, Br–Z = 2-bromobenzyloxycarbonyl, DNP = dinitrophenyl, HOBt = 1-hydroxybenztriazole, DIPCDI = *N*,*N*′-diisopropylcarbodiimide, DIPEA = *N*,*N*′-diisopropylethylamine, TFA = trifluoroacetic acid, DCM = dichloromethane, DMF = dimethylformamide, DAGO = [D-Ala$^2$, MePhe$^4$, Gly-ol$^5$]enkephalin, DADLE = [D-Ala$^2$, D-Leu$^5$]enkephalin, HPLC = high performance liquid chromatography, HPLC-$t_R$ = retention time on analytical HPLC, BSA = bovine serum albumin.

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# Hydrophobicity Parameters Determined by Reversed-Phase Liquid Chromatography. II.[1] Dependence of Retention Behavior of Pyrazines on Mobile Phase Composition

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**The retention behavior of monosubstituted pyrazines was investigated by comparing the hydrophobic contribution of each substituent ($\kappa$) to the capacity factor obtained from reversed-phase high-performance liquid chromatography (HPLC), using a $C_{18}$ capsule-type column and methanol–water eluents of various compositions. The mobile phase dependency of $\kappa$ showed that the retention mechanism varied with the substituent, especially in mobile phases with very high water content, due to the intervention of the selective solute–solvent interactions. Under such circumstances, the capacity factor (log $k'$) obtained from HPLC methods correlated very poorly with the octanol–water partition coefficient (log $P$). The log $k'$ for pyrazines with only alkyl and/or alkoxy groups was measured and the correlation with log $P$ was examined. An excellent linear correlation was obtained with the eluent containing about 30% methanol. However, at other methanol concentrations, the log $k'$ values of the alkyl and alkoxy derivatives correlated with log $P$ by two separated linear equations. The use of log $k_W$ (log $k'$ at 100% water), which has so far been considered to be the best index of hydrophobicity, failed to simulate log $P$.**

**Keywords** HPLC; capacity factor; partition coefficient; substituent effect; pyrazine; mobile phase; methanol; hydrophobicity; electronic interaction

The importance of the 1-octanol–water partition coefficient (log $P$), as a hydrophobicity parameter, has rapidly increased not only in quantitative structure–activity relationships (QSAR) but also in the field of environmental research.[2] Although the measurements of log $P$ value by the shake-flask method are conventional and standard, this method is sometimes time-consuming and laborious. An alternative technique using reversed-phase high performance liquid chromatography (RP-HPLC) has been developed in recent years to predict the log $P$ value.[3-5]

In HPLC methods, the logarithm of capacity factor (log $k'$), is used as an index of hydrophobicity. For structurally related compounds, the log $k'$ value obtained from the combinations of alkyl-bonded stationary-phases and the methanol–water mobile phase system is considered to correlate linearly with log $P$ by a Collander-type equation.[6] The correlation is often improved by introducing correction terms for hydrogen-bond effects as shown by Eq. 1,[3,7,8]

$$\log k' = a \log P + bHB_A + cHB_D + d \quad (1)$$

where, $HB_A = 1$ for H-acceptors and 0 for others, $HB_D = 1$ for H-donors and 0 for others, and $a$, $b$, $c$ and $d$ are constants.

In addition to the hydrogen-bond effects, another problem in predicting log $P$ values by HPLC methods is the possibility of peak inversion. The elution order of solutes is sometimes reversed depending on the methanol content in the eluent. For such a system, accuracy of the predicted log $P$ values is subject to the mobile phase composition used for the measurements.

To eliminate or reduce the uncertainties caused by these phenomena, recent investigators have proposed to use the log $k_W$ value (the extrapolated log $k'$ value at 100% water) rather than log $k'$ values at a single eluent composition.[4,7] In fact, many successful works supporting that log $k_W$ is more closely related to log $P$ have been reported.[4,7,9] However, such a conclusion has been drawn mostly from benzene derivatives, and it is still not completely clear whether the log $k_W$ approach can be regarded as a standard method for predicting log $P$, even in the system where polar functional groups exhibiting solute–solvent interactions are involved.

Recently, we undertook a systematic study of heteroaromatic partition coefficients.[10] In a previous paper,[10a] we analyzed the log $P$ value of monosubstituted diazines (pyrazines, pyrimidines and pyridazines) in terms of substituent effects, and demonstrated that the partitioning behavior of diazines in the octanol–water system showed features considerably different from the corresponding monosubstituted benzenes, indicating that the electronic interactions between the ring N-atom(s) and the substituent caused a modification in the hydrogen bonding behavior of the substituent as well as the ring N-atoms.

These results prompted us to examine how far the log $P$ value could be correctly predicted by HPLC methods in heteroaromatic systems where hydrogen-bonding effects are so important in governing the log $P$ value. As the first step, we previously examined the relationship between log $P$ and log $k'$ for monosubstituted pyrazines (2PR).[1] As expected, the relationship was much more complicated than that for the corresponding monosubstituted benzenes. The log $k'$ value for 2PR, measured using the Capcell pak $C_{18}$ column and eluents containing 15 to 70 volume percent of methanol (% MeOH), was formulated by the following general equation,[1]

$$\log k' = a \log P + bHB_{CO} + cHB_{AM} + \rho\sigma_I + \text{const.} \quad (2)$$

where $HB_{CO} = 1$ for ester and amide substituents and 0 for the others, $HB_{AM} = 1$ for amphiprotic substituents and 0 for the others, and $\sigma_I$ is Charton's electronic constant.[11] The coefficients $a$, $b$, $c$ and $\rho$, and the constant term are determined by regression analysis. It was observed that the relationship between log $P$ and log $k'$ became more complicated at higher water contents: the coefficients of $HB_{CO}$ and $\sigma_I$ terms became more positive and more negative, respectively, with decreasing methanol concentration in the eluents, while these terms were insignificant or of small significance around 50%MeOH. Thus, the log $k_W$ approach proved to be ineffective in this system.

To gain a closer insight into the retention mechanism of

2PR

Chart 1

pyrazine derivatives, and to look for reasonable HPLC conditions for reliable estimation of log $P$, we examined in this work the change in substituent contribution to the over-all log $k'$ value for monosubstituted pyrazines as a function of the mobile phase composition. Then, the experiments were extended to the retention behavior of various pyrazine derivatives having alkyl and/or alkoxy substituents.

### Materials and Methods

**Materials** The monosubstituted pyrazines used in this study are the same as those used in the previous paper[1] and listed in Table I. A variety of pyrazines with alkyl and/or alkoxy substituent(s) were also examined; their structures are shown in Table II. The preparation of the compounds has been described elsewhere.[10]

**Partition Coefficients** Most of the 1-octanol–water log $P$ values necessary for the discussion were taken from our previous papers.[10] Some were newly measured in this work by the conventional shake-flask method as was previously described.[10]

**HPLC Procedure** The apparatus used was previously described[1]: a Shimadzu LC5A liquid chromatograph with a Model 7125 valve loop injector (Rheodyne), and SPD-2A UV (Shimadzu) and Shodex SE-31 refractive index (Shoden) detectors were used. Retention times were measured using a C-R4A Chromatopac (Shimadzu). A Commercial Capcell pack $C_{18}$ column (4.6 mm × 15 cm, Shiseido) was used without further treatment (Capcell pak was demonstrated to be more effective in suppressing the silanol effects than trimethylsililation of octadecyl silica (ODS) phases).[12] Commercial HPLC grade methanol and water were used. Eluents were prepared by volume. Capacity factors were determined at 25 °C as previously described.[1] The log $k'$ values of 2PR obtained using methanol–0.01 M sodium borate (pH 9.2) as eluents over 15 to 70% MeOH (M15 to M70) were available from our previous study.[1] The buffer solutions of pH 9.2 had been used because of the necessity of measuring stronger bases (pyridines) simultaneously. In this work the log $k'$ value with a methanol–buffer (pH 9.2) (5:95, M5) was measured. Since preliminary examination showed that the use of buffered solutions was not needed in the case of pyrazines, measurements for the compounds in Table II were done using methanol–water mobile eluents. The capacity factor, $k'$, is defined by Eq. 3,

$$k' = (t_R - t_0)/t_0 \quad (3)$$

where $t_R$ is the retention time of the compound and $t_0$ is that of an unretained compound. The flow-rate was 0.5—2.0 ml/min and the peak of methanol was used to calculate the $t_0$ value.

### Results and Discussion

The plots of log $k'$ for 2PR against %MeOH for most substituents were approximately linear in the region of 15—70%MeOH. However, the log $k'$ value was generally seen to deviate upward from the linear line as the methanol content became very low. Schoenmakers and coworkers insisted that even a quadratic equation, being better than a linear approximation, was still insufficient to describe the relationship between experimental log $k'$ and %MeOH in these regions.[13]

Although the number of data for M5 was not enough because of the extremely long retention time for some compounds, the analysis of log $k'$ by Eq. 2 yielded Eq. 4.

$$\log k' = 0.879 \log P + 0.535 HB_{CO} - 0.287 HB_{AM} - 0.513 \rho\sigma_I + 0.693$$
$$n = 16, \quad r = 0.979, \quad s = 0.106, \quad F = 61.9 \quad (4)$$

Here and throughout this work, $n$ is the number of compounds used for calculations, $r$ is the correlation coefficient and $s$ is the standard deviation. $F$ is the value of the $F$ ratio between the variances of observed and calculated values. The correlation was improved by adding one more hydrogen-bonding indicator variable $HB_A$: $HB_A = 1$ for H-acceptor other than ester and amide groups, and $HB_A = 0$ for others (Eq. 5).

$$\log k' = 0.822 \log P + 0.638 HB_{CO} - 0.370 HB_{AM} - 0.647 \rho\sigma_I + 0.177 HB_A + 0.647$$
$$n = 16, \quad r = 0.990, \quad s = 0.076, \quad F = 98.0 \quad (5)$$

These parameters are included in Table I. The fact that the

Table I. The $\kappa$ Values and the Substituent Parameters for Monosubstituted Pyrazines

| Substituent | $\kappa$[a] | | | | | log $P$[c] | $\sigma_I$ | $HB_{CO}$ | $HB_{AM}$ | $HB_A$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | M5[b] | M15[b] | M30[b] | M50[b] | M70[b] | | | | | |
| H | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | −0.260 | 0.00 | 0.0 | 0.0 | 0.0 |
| F | 0.159 | 0.229 | 0.285 | 0.297 | 0.269 | 0.290 | 0.54 | 0.0 | 0.0 | 0.0 |
| Cl | 0.541 | 0.576 | 0.612 | 0.545 | 0.480 | 0.700 | 0.47 | 0.0 | 0.0 | 0.0 |
| Me | 0.452 | 0.371 | 0.313 | 0.169 | 0.114 | 0.210 | −0.01 | 0.0 | 0.0 | 0.0 |
| Et | 0.891 | 0.789 | 0.668 | 0.476 | 0.348 | 0.690 | −0.01 | 0.0 | 0.0 | 0.0 |
| OMe | 0.760 | 0.740 | 0.693 | 0.588 | 0.482 | 0.730 | 0.30 | 0.0 | 0.0 | 1.0 |
| OEt | 1.303 | 1.220 | 1.148 | 0.923 | 0.724 | 1.280 | 0.28 | 0.0 | 0.0 | 1.0 |
| OPr | —[d] | 1.745 | 1.582 | 1.280 | 0.972 | 1.840 | 0.28 | 0.0 | 0.0 | 1.0 |
| SMe | 1.120 | 1.038 | 0.963 | 0.795 | 0.644 | 1.170 | 0.30 | 0.0 | 0.0 | 1.0 |
| CN | 0.067 | 0.093 | 0.109 | 0.102 | 0.025 | −0.010 | 0.63 | 0.0 | 0.0 | 1.0 |
| Ac | 0.498 | 0.426 | 0.382 | 0.285 | 0.213 | 0.200 | 0.30 | 0.0 | 0.0 | 1.0 |
| $CO_2Me$ | 0.501 | 0.342 | 0.181 | 0.049 | −0.078 | −0.230 | 0.32 | 1.0 | 0.0 | 0.0 |
| $CO_2Et$ | 1.029 | 0.818 | 0.607 | 0.394 | 0.208 | 0.280 | 0.30 | 1.0 | 0.0 | 0.0 |
| $CONMe_2$ | —[d] | 0.036 | −0.138 | −0.300 | — | −0.800 | 0.28 | 1.0 | 0.0 | 0.0 |
| $NMe_2$ | —[d] | 1.062 | 0.843 | 0.573 | 0.445 | 0.930 | 0.17 | 0.0 | 0.0 | 1.0 |
| $NH_2$ | −0.113 | −0.117 | −0.162 | −0.317 | −0.320 | −0.050 | 0.17 | 0.0 | 1.0 | 1.0 |
| NHMe | 0.567 | 0.430 | 0.274 | 0.115 | 0.045 | 0.560 | 0.13 | 0.0 | 1.0 | 1.0 |
| NHAc | 0.321 | 0.187 | 0.067 | −0.146 | −0.322 | −0.030 | 0.28 | 1.0 | 1.0 | 0.0 |
| $CONH_2$ | −0.124 | −0.222 | −0.322 | −0.502 | −0.812 | −0.500 | 0.28 | 1.0 | 1.0 | 0.0 |

*a*) M5, this work; M15—M70, calculated from the log $k'$ values taken from ref. 1. *b*) Mobile phase; the figure represents the volume % of MeOH. *c*) Taken from ref. 10*a*. *d*) The data could not be obtained because the retention time was too long.

TABLE II. $\log k'$ Values and Related Parameters for Alkyl and/or Alkoxy Pyrazines

| Substituents | $\log k'$ | | | $\log P$[b] | *HB* | $\log k_W$[c] | $\log k_W$[d] |
|---|---|---|---|---|---|---|---|
| | M15[a] | M30[a] | M50[a] | | | | |
| Me | 0.413 | −0.059 | −0.468 | 0.21 | 0.0 | 0.75 | 1.03 |
| Et | 0.861 | 0.357 | −0.163 | 0.70 | 0.0 | 1.27 | 1.46 |
| Me, 3-Me | 0.726 | 0.226 | −0.295 | 0.54 | 0.0 | 1.14 | 1.32 |
| Me, 6-Me | — | 0.219 | −0.298 | 0.54 | 0.0 | 1.00 | — |
| Me, 3-Et | 1.209 | 0.633 | 0.023 | 1.07 | 0.0 | 1.69 | 1.89 |
| Me, 3-*n*-Pr | 1.670 | 1.019 | 0.332 | 1.57 | 0.0 | 2.21 | 2.44 |
| Me, 3-Bu | 2.242 | 1.474 | 0.672 | 2.10 | 0.0 | 2.88 | 3.15 |
| Me, 3-isoBu | — | 1.353 | 0.681 | 1.96[e] | 0.0 | 2.36 | — |
| Et, 3-Et | 1.656 | 1.005 | 0.337 | 1.51 | 0.0 | 2.19 | 2.44 |
| Tri-Me | 1.148 | 0.525 | −0.077 | 0.95 | 0.0 | 1.63 | 1.92 |
| Me, 3-Et, 5-Et | 1.923 | 1.256 | 0.525 | 1.95 | 0.0 | 2.49 | 2.69 |
| Tetra-Me | 1.553 | 0.822 | 0.125 | 1.28 | 0.0 | 2.12 | 2.46 |
| OMe | 0.787 | 0.397 | −0.046 | 0.73 | 1.0 | 1.13 | 1.23 |
| OEt | 1.289 | 0.833 | 0.288 | 1.28 | 1.0 | 1.71 | 1.79 |
| OPr | 1.803 | 1.299 | 0.643 | 1.84 | 1.0 | 2.30 | 2.32 |
| Me, 3-OMe | 1.283 | 0.772 | 0.223 | 1.24 | 1.0 | 1.71 | 1.88 |
| Me, 3-OEt | 1.808 | 1.227 | 0.577 | 1.82 | 1.0 | 2.31 | 2.47 |
| Me, 6-OPr | 2.290 | 1.683 | 0.931 | 2.38 | 1.0 | 2.86 | 2.93 |
| Me, 3-$OCHMe_2$ | — | 1.640 | 0.865 | 2.24 | 1.0 | 2.80 | — |
| OMe, 6-OMe | 1.517 | 1.019 | 0.414 | 1.58 | 1.0 | 1.98 | 2.05 |
| OEt, 6-OEt | — | 1.781 | 1.014 | 2.55 | 1.0 | 2.93 | — |

*a*) Mobile phase composition; the figure represents the volume % of MeOH. *b*) Taken from ref. 10*b* unless otherwise noted. *c*) $\log k_W$ value calculated by linear equation. *d*) $\log k_W$ value calculated by quadratic equation. *e*) This work.

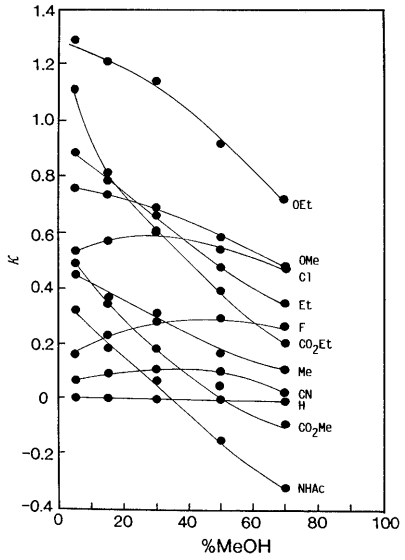


Fig. 1. Dependence of the Substituent Hydrophobic Contribution for Monosubstituted Pyrazines ($\kappa$) on the Mobile Phase Composition

$HB_A$ term became significant in the M5 eluent suggests that the mobile phase of very high water content is more discriminating between non-hydrogen bonders and hydrogen-acceptors, and hence the retention mechanism would become even more complex. We have found, in the previous[1] and our continuing studies, that the $HB_A$ term is also required in the correlations derived from the different water–organic modifier systems of very high water content. The possibility that the complexity of the retention mechanism is due to the so-called silanol effects was thought to be negligible under our conditions.[1]

In order to grasp the retention behavior of each substituent more visibly, we attempted to calculate the hydrophobic contribution of substituent, $\kappa$, at each mobile phase composition by taking the increment of $\log k'$ produced by an X-substitution on the parent pyrazine molecule, just as the $\pi$ value is derived from the $\log P$ value.

$$\kappa_X = \log k'(X) - \log k'(H) \quad (6)$$

The $\kappa$ values for 2PR are summarized in Table I, and the typical results are illustrated graphically in Fig. 1. If the solutes follow the Collander-type equation ($\log k' = a \log P + b$) at each mobile phase composition, each substituent should have a slope proportional to its $\pi$ value; the slope of the plot for a substituent of higher hydrohobicity should be more negative. Figure 1 shows, however, that the features are considerably different depending on the nature of the substituents. In most cases, except for halogens and CN, the $\kappa$ value increased continuously with different slopes as %MeOH dropped. In the case of alkoxy groups, we observed that the linearity declined and the slope became gradual as %MeOH became very low, while the plot for alkyls was almost linear up to 5% MeOH. We will discuss this phenomenon later in detail.

On the other hand, the esters and amide groups exhibited a curve with a steeper slope than other substituents. This corresponds to the fact that the contribution of the $HB_{CO}$ term became more positive with a higher water content.

The substituents like halogens and CN, which have a large $\sigma_I$ value, formed another group, where a horizontal curve with a maximum point was observed. Finding a maximum point in the $\kappa$–%MeOH plot would mean that a dual parameter should operate. The $\rho\sigma_I$ term, which makes a more negative contribution to $\log k'(\kappa)$ as %MeOH decreases, would be responsible for the appearance of a maximum point.

We considered that the electronic effects expressed by the $\rho\sigma_I$ term would be associated with the $pK_a$ of the solute, and represent selective solute-solvent interactions. The fact

that the $\rho\sigma_I$ term was significant in water-rich mobile phases demonstrates that the selective solute-solvent interactions become important in more polar mobile phases. Under such conditions, the capacity factor would not completely reflect the hydrophobic property of the solute. The major factor hampering the use of HPLC techniques for the estimation of log $P$ arises from the contribution of the $\rho\sigma_I$ term which breaks the log $P$ – log $k'$ linearity to a great extent since the $\sigma_I$ value varies with the substituent. Braumann reported that selective solute-solvent interactions can be eliminated by using log $k_W$.[4] However, in our case, the electronic effect would play an even more important role in pure water; hence, the failure of using log $k_W$ as an alternative hydrophobicity index could be explained partly on this basis.

The $\kappa$ values for 2-chloro-6-substituted pyrazines were calculated using the log $k'$ values from our previous work[14] and the $\kappa$–% MeOH plot was drawn; we observed features very similar to Fig. 1, though not shown.

We see in Fig. 1 that some plots intersect each other, especially in the regions of low methanol concentrations, indicating clearly that the relationship between log $P$ and log $k'$ ($\kappa$) is highly mobile phase dependent. For example, the substituents Et and OMe have $\pi$ values (0.95 and 0.99, respectively) which are very close to each other. Figure 1 shows that the two plots intersect around 30% MeOH. As far as these two compounds are concerned, the measurement with the M30 mobile phase would give the most reliable log $P$ values. To ascertain whether such retention behavior is common to other pyrazines with substituents of a similar nature to Et and OMe, we attempted to measure the log $k'$ values for various pyrazines having only alkyl and/or alkoxy groups at 15, 30 and 50% MeOH (M15, M30 and M50). The results are summarized in Table II with the corresponding log $P$ values. The plot of log $k'_{M15}$ to log $k'_{M50}$ (Fig. 2), showed that alkoxypyrazines were less sensitive to the change in mobile phase composition. The relationship between log $P$ and log $k'$ at each composition are plotted in Fig. 3. It was found that log $k'$ for M30 gave an excellent linear correlation with log $P$ as shown by Eq. 7,

$$\log k'_{M30} = 0.794 \log P - 0.207 \quad (7)$$
$$n=21,\ r=0.998,\ s=0.032,\ F=5298.8$$

For M15 and M50, however, it is seen in Fig. 3 that the compounds with OR and the ones with only alkyl(s) fall on two slightly separated parallel lines. Although the simple correlation between log $P$ and log $k'$ was sufficient, the correlation was significantly improved by introducing the indicator variable $HB$, which takes 1 for alkoxy pyrazines and 0 for the others. Equations 8 and 9 were yielded.

$$\log k'_{M15} = 0.922 \log P - 0.138HB + 0.246 \quad (8)$$
$$n=17,\ r=0.995,\ s=0.057,\ F=696.0$$

$$\log k'_{M50} = 0.611 \log P + 0.098HB - 0.616 \quad (9)$$
$$n=21,\ r=0.997,\ s=0.036,\ F=1417.6$$

It is of interest to find that the difference in the retention behavior between OR and alkyl substituents is universal, regardless of the structure of compounds. Alkyl and/or alkoxy pyrazines with the same log $P$ values have an equivalent hydrophobicity in the methanol–water (30 : 70) elutent. But alkyl pyrazines are apparently more hydrophobic than alkoxy pyrazines with the same log $P$ in mobile phases containing less than about 30% methanol. The opposite is true in the mobile phases with higher methanol content. It should be noted that the extrapolated log $k'_W$ values were much higher than the log $P$ values (Table II), presenting further evidence that the log $k_W$ approach is unsuitable for the prediction of log $P$.

The results given above demonstrate that each substituent exhibits different retention behavior and the difference is enhanced as the mobile phase composition approaches pure water. Strictly speaking, the linear relationship between log $P$ and log $k'$ appear to hold only within substituents of a similar nature. The log $k_W$ treatment, which has so far been considered the best index of hydrophobicity, failed in simulating log $P$, probably due to concomitant electronic polar effects. In this sense, the use of mobile phases containing rather high methanol concentrations would be desirable to predict the log $P$ for a series of compounds with different kinds of substituents. For example, as far as the 2PR series is concerned, the M50 eluent is thought to be preferable, provided that the amphiprotic derivatives are omitted (if we also exclude the ester and amide ones from consideration, M30 is as good as, or better than, M50). For the analytic system consisting of the solutes which exhibit

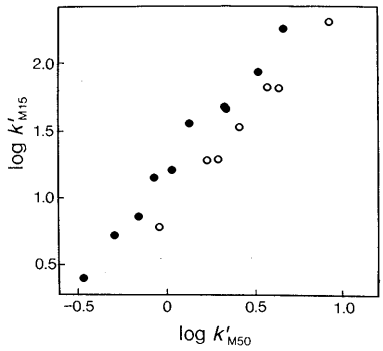


Fig. 2. Relationship between log $k'$ for M15 (Methanol : Water = 15 : 85) and M50 (Methanol : Water = 50 : 50) for Pyrazines Variously Substituted with Alkyl and/or Alkoxy Group(s)

○, alkoxypyrazine with and without alkyl groups; ●, alkylpyrazine.

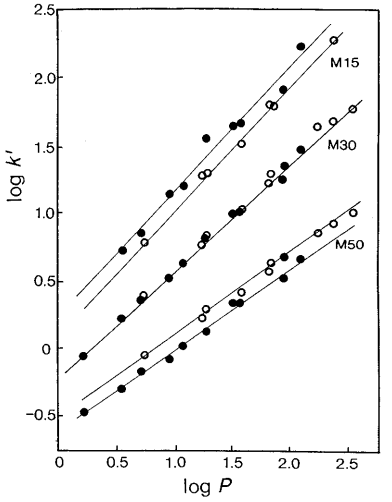


Fig. 3. Relationship between log $P$ and log $k'$ for Pyrazines Variously Substituted with Alkyl and/or Alkoxy Group(s)

○, alkoxypyrazine with and without alkyl groups; ●, alkylpyrazine.

$\kappa$–%MeOH plots of similar shape, the effort to look for mobile phase composition, where some model compounds with equivalent log $P$ values give almost identical log $k'$ values, would yield better results. Anyway, the log $k'_W$ approach should be avoided. Further investigation to establish potential HPLC conditions is now underway.

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# Reconstruction of Weight Matrices in Neural Networks—A Method of Correlating Outputs with Inputs[1)]

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**Introducing a forgetting process in the learning phase of the neural network resulted in localizing the connections in the weight matrices. Such weight matrices, reconstructed weight matrices, indicated the active neurons in the network and were used to analyze the relationships between the input and output data. The relationship between the adaptive least-squares (ALS) method and the neural network led to the conclusion that the operation of the ALS method is, indeed, a restricted operation of the neural network.**

**Keywords** neurocomputer; neural network; perceptron; reconstruction-learning; graded classification; ALS

We have investigated the operation of perceptron-type neural networks in an attempt to apply them to developing new drugs. So far the following characteristics have been revealed. (1) Since the neural networks are good at pattern recognition, such an ability can readily be applied to the structue–activity relationship (SAR) of drugs resulting in a better graded classification than any of the other conventional methods.[2)] (2) Usually, the number of neurons of the output layer equals that of the classification. We have discovered that if the number of neurons of the output layer is set to be one which is allowed to take an analog value, this type of neural network (named the MR-type neural network) operates as a generalized multiregression analysis. The MR-type neural network is easily applied to quantitative structure–activity relationship (QSAR) analysis. Because of its nonlinear operation, the results were remarkable in both fitting and prediction.[3)] (3) The operation of the neural network is basically carried out in a nonlinear manner.[4)] The nonlinearity makes it difficult to correlate the output strength with any input parameters, but this problem was solved by tracing the propagation of the infinitesimal change of the input values to the neurons in the output layer, *i.e.*, the divided difference of the output intensity with respect to the input parameter ($\delta O/\delta x$).[5)] Thus, we have shown that the neural network works better than any other method.

As we study the operation of such neural networks, we are surprised to see how the behavior of the neural network resembles that of the brain. As our experience tells us, we learn things repeatedly through the processes of recognizing and forgetting. The memory that is obtained through such processes is settled firmly in the mind. We considered that these processes may be incorporated in the perceptron-type neural networks, and when this is done, it is interesting to see what happens to the connections of the neurons.

In the neural network, the information which is accumulated in the learning phase is kept according to the strength of the connections between the neurons recorded in the weight matrices. As we stated in the previous paper,[3)] such matrices are not unique even if the network gives the same results. This indicates that various kinds of reconstructions of the weight matrices are possible. Therefore, we tried to introduce the procedures of both the learning and forgetting processes in the learning phase of the neural network. The weight matrices thus obtained are called "reconstructed matrices." The reconstructed matrices were surprising and suggestive. They were found to be widely applicable in finding active neurons of the network and could serve in the analysis of the relationship between the input and output data.

In the previous paper, we described the relationship between the multiregression analysis and the MR-type neural network.[3)] We also consider here the relationship between the adaptive least-squares (ALS) method[6)] and the classification by the perceptron-type network. In fact, this was well illustrated by the reconstructed weight matrices.

## Theory

**Introduction of the Forgetting Procedure in the Learning Phase** Shown in Fig. 1 is a perceptron-type three-layer neural network: the circles are neurons which are actually variables taking a value ranging from 0 to 1. The data are input to A and are output from B. The value of a neuron ($O_j$) at the second and third layers can be expressed by Eq. 1,

$$O_j = 1/[1+\exp(-\alpha y_j)] \equiv f(y_j) \tag{1}$$

$$y_j = \left(\sum_i W_{ij} x_i\right) - \theta_j$$

where $x_i$ is one of the values of the neurons at the first or second layer; $W_{ij}$, an element of the weight matrix, expresses the weight value between the neurons $i$ and $j$ and can take either a positive or a negative value; $\theta_j$ is a threshold value for neuron $j$; $\alpha$ is a parameter which expresses the nonlinearity of the neuron's operation.

Given $N$ neurons at the first layer, a set of the input data can be expressed by a vector with $N$ elements for $N$ neurons. This is called an "input pattern." Likewise, the output data can also be regarded as a vector and is called an "output pattern." The vector which is compared with an output

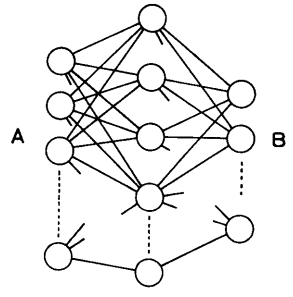


Fig. 1. Three-Layer Neural Network

pattern to obtain an appropriate $W_{ij}$ in the learning phase is called a "training pattern" ($t_j$). The training of the network is based on the following equations.

$$\delta W_{ij} = -d_j x_i \varepsilon \tag{2}$$

$$d_j = (O_j - t_j) f'(y_j) \tag{3a}$$

$$d_j = (W'_{jl} d'_l) f'(y_j) \tag{3b}$$

where $\varepsilon$ is a parameter which determines the shift for correction in recursive cycles. Equation 3a is used only for the correction between the second and third (output) layers and 3b for that between the first and second layers where $W'_{jl}$ and $d'_l$ are $W_{ij}$ and $d_j$ between the second and third layers, respectively. The function $f'$ in Eq. 3 is,

$$f'(y_j) = f(y_j)[1 - f(y_i)] \cdot \alpha \tag{4}$$

where both $\varepsilon$ and $\alpha$ can be set to be independent of the layer.

The training is carried out according to the above back-propagation algorithm[7] until

$$E = \sum_j (O_j - t_j)^2 \tag{5}$$

becomes small enough. Even when M sets of the input and training patterns are given, all of the output patterns can be made close enough to the training patterns by iteration through Eqs. 1 and 2. If convergence is attained, the neural network has the ability to classify the input patterns into M groups.

Here we consider the procedure in which the absolute values of weight matrices are lessened by the equation:

$$W_{ij} = W_{ij} - sgn(W_{ij}) \zeta \{1 - D(W_{ij})\} \tag{6}$$

where $D$ is a function that gives 1 at $|W_{ij}| < \zeta$ or 0 at $|W_{ij}| > \zeta$ and $\zeta$ is set to be *ca.* a tenth of $\varepsilon$ as an initial value and is varied so as not to greatly change $E$, *i.e.*, not to greatly change the decision by the network.

If this procedure is applied to the network, some of the information which is given by training is partly erased. This procedure corresponds to forgetting in memory and is termed "erasing." Suppose M sets of input data are given. We call here the training procedure for M sets of data a "training cycle". If after a training cycle is carried out, the erasing procedure is applied to the same network, then the information which is accumulated in the training cycle is partly lost from the network. Remarkably, we discovered that these contradictory procedures do not vary all connections equally. Some connections are affected by the training cycle more strongly than by the erasing procedure to give stronger connections and others are affected by the erasing procedure strongly to give weak or null connections. Therefore, the information accumulated between neurons can be reconstructed without changing the contents of the information originally embodied in the network. We term this series of procedures "reconstruction-learning."

Reconstruction-learning often reveals the role of each neuron and gives characteristic connections between the neurons; if one traces the connections between the input and output neurons, then one can understand the roles of the input parameters to a specific decision or the output intensity.

**The Relationship of the Operation in the ALS Method to the Perceptron-Type Neural Network** Now consider the relationship of the operations between the ALS method and the neural network. The discriminative function $L$ in the ALS method is expressed using an $m$ number of descriptors $x$ and weight coefficients $w$ as

$$L = w_0 + w_1 x^{(j)}{}_1 + w_2 x^{(j)}{}_2 + \cdots \equiv L^{(j)} = X W^{(j)} \tag{7}$$

The rule of discrimination is given according to the value of $L$. Thus if $a_n < L < a_{n+1}$ then the group is placed in class $n$.

The weight coefficient at cycle $j$, $w^{(j)}$ is obtained by,

$$W^{(j)} = (X^t X)^{-1} X^t S^{(j)} \tag{8}$$

Here using the correction term $C$, $S$ is given by,

$$S^{(j+1)} = L^{(j)}{}_i \quad \text{or} \quad = L^{(j)}{}_i - C^{(j)} \tag{9}$$

Here, consider the role of the term $S$ in Eq. 9. Since the dimension of $W$ is null, $S$ must have the same mathematical and physical characteristics with $x$. Therefore, the expression by Eq. 8 is appropriate and unique since there is no other quantity equivalent to $x$ in the ALS system. Equation 9 indicates that $S$ receives a feedback from the output and is, indeed, a kind of back-propagation procedure. It is, therefore, easy to simulate the ALS operation in the neural network by imposing the following

TABLE I. Relative $^{13}$C-NMR Chemical Shifts and Conformations in Norbornenes

| Compd. | $C_1$ | $C_2$ | $C_3$ | $C_4$ | $C_5$ | $C_6$ | $C_7$ | *exo/endo* |
|---|---|---|---|---|---|---|---|---|
| **1** | 6.7 | 6.7 | 10.1 | 0.5 | 0.2 | −1.1 | −3.7 | *exo* |
| **2** | 8.9 | 25.3 | 12.4 | −0.4 | −1.2 | −3.1 | −4.4 | *exo* |
| **3** | 7.7 | 44.3 | 12.3 | −1.0 | −1.3 | −5.2 | −4.4 | *exo* |
| **4** | 4.6 | 16.7 | 4.4 | −0.2 | −0.3 | −1.0 | −1.8 | *exo* |
| **5** | 1.8 | 15.1 | 4.4 | −0.2 | 0.2 | −0.7 | −3.3 | *exo* |
| **6** | 5.7 | 3.0 | 2.6 | −0.5 | −0.4 | 0.7 | −3.5 | *exo* |
| **7** | 6.1 | 5.9 | 10.6 | 0.6 | 0.2 | 0.2 | −3.7 | *exo* |
| **8** | 6.5 | 6.3 | 10.4 | 0.3 | −0.8 | −0.1 | −3.5 | *exo* |
| **9** | 6.5 | 7.5 | 9.5 | 0.5 | 1.7 | 0.7 | −3.8 | *exo* |
| **10** | 7.8 | 47.0 | 11.7 | −1.3 | 3.9 | −2.7 | −3.2 | *exo* |
| **11** | 6.9 | 6.4 | 10.1 | 0.7 | −1.2 | 0.1 | −3.9 | *exo* |
| **12** | 5.6 | 4.9 | 7.0 | 0.2 | −1.1 | 0.2 | −3.9 | *exo* |
| **13** | 2.5 | 42.5 | 11.9 | −0.8 | −1.1 | −2.4 | 1.4 | *exo* |
| **14** | 5.4 | 4.5 | 10.6 | 1.4 | 0.5 | −7.7 | 0.2 | *endo* |
| **15** | 6.8 | 23.3 | 10.5 | 1.2 | 0.6 | −9.5 | 0.3 | *endo* |
| **16** | 6.3 | 42.4 | 9.5 | 0.9 | 0.2 | −9.7 | −0.9 | *endo* |
| **17** | 4.2 | 16.2 | 2.1 | 0.9 | −0.6 | −4.8 | 1.9 | *endo* |
| **18** | 1.7 | 12.8 | 4.0 | 0.4 | 0.2 | −7.2 | 1.4 | *endo* |
| **19** | 4.7 | 3.1 | 2.2 | 0.3 | 1.3 | −6.5 | −0.6 | *endo* |
| **20** | 4.7 | 5.3 | 9.2 | 1.3 | −0.4 | −6.5 | 1.4 | *endo* |
| **21** | 4.6 | 11.5 | 8.9 | −0.1 | 0.8 | 0.4 | 1.8 | *endo* |
| **22** | 5.6 | 7.5 | 8.7 | 1.4 | 1.7 | −3.0 | 1.7 | *endo* |
| **23** | 7.1 | 47.8 | 13.3 | 2.2 | 3.6 | −3.4 | 0.3 | *endo* |
| **24** | 4.1 | 4.2 | 7.0 | 0.7 | 0.5 | −7.4 | 0.0 | *endo* |
| **25** | 3.2 | 40.2 | 10.4 | −0.5 | 0.0 | −10.3 | 3.1 | *endo* |
| **26** | 5.5 | 1.0 | 6.3 | −0.3 | −1.5 | −1.6 | −1.3 | *exo* |
| **27** | 3.4 | 0.1 | 5.5 | 0.2 | −0.7 | −4.9 | 0.0 | *endo* |
| **28** | 5.1 | 16.4 | 4.2 | −0.4 | −1.1 | −1.4 | −2.1 | *exo* |
| **29** | 4.0 | 15.9 | 2.2 | 0.7 | −0.7 | −5.0 | 1.7 | *endo* |
| **30** | 6.6 | 7.0 | 10.1 | 0.2 | −1.2 | 0.5 | −3.7 | *exo* |
| **31** | 6.0 | 8.4 | 11.2 | −0.1 | 0.7 | −1.5 | −1.6 | *endo* |
| **32** | 6.3 | 7.2 | 9.8 | 0.7 | −0.1 | 0.8 | −3.5 | *exo* |
| **33** | 5.1 | 4.8 | 8.4 | 1.1 | −0.1 | −7.3 | 1.6 | *endo* |
| **34** | 1.9 | 17.1 | 5.2 | −0.1 | 0.9 | 0.9 | −3.4 | *exo* |
| **35** | 2.3 | 18.3 | 5.0 | 0.3 | 1.3 | −2.9 | 1.4 | *endo* |
| **36** | 5.1 | 4.0 | 8.4 | 1.1 | 0.2 | −7.7 | 1.6 | *endo* |
| **37** | 2.9 | 30.3 | 13.4 | −0.5 | −2.1 | −0.7 | 2.0 | *exo* |
| **38** | 3.7 | 29.8 | 10.8 | −1.6 | −1.1 | −9.0 | 2.2 | *endo* |

restrictions on the network.

1. Use a two-layer neural network.
2. Use a linear output function for the neurons.
3. Set $\theta$ to be $a_n$.
4. Set $w_{ij}=w_{ik}$, where $j$ and $k$ represent any of the neurons in the second layer.
5. Give the training pattern that ignites $n$ number of output neurons for the $n$-graded classification.

It is, therefore, understood that the operation of the ALS method is simply a special case of the neural network. The numerical correlation between the ALS's and a three-layer neural network's results are shown in the next section.

## Results and Discussion

We use the expression, $N(l, m, n)$ to show the network structure, where $l$, $m$, and $n$ are the numbers of the neurons in the first, second, and third layers, respectively. All calculations were carried out at $\alpha=1$ and $\varepsilon=0.1$ and all input data are scaled between 0.1 and 1.

**The Relationship between Carbon-13 Nuclear Magnetic Resonance ($^{13}$C-NMR) Chemical Shift and the Conformation of Norbornene** Shown in Table I are the *endo*/*exo* conformations, the relative $^{13}$C-NMR chemical shifts in the derivatives of norbornene, quoted from the literature,[8] where the same compound numbers are used.[9] In accord with the former studies, we used 25 (No. 1 to 25) out of 38 data as training data and the others were used for prediction. The network structure was set to be $N(7, 14, 2)$. The reconstruction learning, which consists of one training cycle and one erasing procedure, was repeated 50 times. Table II shows the values of the connections obtained by the usual back-propagation and Table III, those by the reconstruction-learning, where the maximum value of the connection was scaled to be 0.999 to compare them in the same level of magnitude (the scale factor is given in each table).

We have shown in the previous paper that the predictions by the neural network were better than those by the linear learning machine and cluster analysis.[9] The results by the present method are the same. Without reconstruction, the information obtained through the learning phase is widely distributed among neurons. With reconstruction, however, connections are localized between the special neurons. It is also understood that the neurons other than 6 and 8 in the second layer have nothing to do with the resulting classification other than to optimize the $\theta$ values. Firing of neuron 1 in the last layer corresponds to the *exo* conformation and that of the other neuron, to the *endo* conformation. Therefore, the conformation is determined by the neurons 6 and 8 propagating the information to two neurons in the third layer with the types of connections $(a, -a)$ and $(-b, b)$. The neurons in the third layer make the decision by combining them.

The 6 and 8 neurons in the second layer have the strongest connections with the neurons 6 and 7 in the first layer. Since the order of the neurons in the first layer is made to correspond to that of the number of the carbon atoms, it is understood that the information on the chemical shifts at $C_6$ and $C_7$ plays the major role in deciding the *endo*/*exo* conformations of the derivatives of norbornene. This is consistent with the chemical idea that the $C_6$ and $C_7$ carbon atoms are located near the substituent and that the effect of the substituent on $C_6$ may be reversed on $C_7$. Note here that the neuron 8 in the second layer connects only with

Table II. Weight Matrices and Outputs in $^{13}$C-NMR Chemical Shifts in Norbornenes[a]

A[b]

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | −0.022 | 0.133 | −0.188 | −0.147 | 0.047 | 0.063 | −0.009 | 0.112 | −0.044 | −0.214 | −0.117 | −0.112 | 0.092 | −0.221 |
| 2 | 0.219 | 0.203 | 0.240 | −0.040 | 0.108 | 0.265 | −0.355 | −0.483 | 0.333 | −0.054 | −0.099 | 0.230 | −0.358 | 0.011 |
| 3 | 0.321 | −0.043 | −0.013 | −0.173 | −0.141 | 0.083 | −0.143 | −0.186 | 0.156 | −0.035 | −0.029 | 0.097 | −0.182 | 0.128 |
| 4 | −0.233 | −0.149 | −0.273 | −0.192 | 0.205 | −0.559 | 0.489 | 0.451 | −0.339 | −0.023 | 0.043 | −0.350 | 0.445 | −0.242 |
| 5 | −0.406 | −0.095 | −0.097 | 0.120 | 0.093 | −0.261 | 0.367 | 0.324 | −0.429 | −0.149 | 0.318 | −0.288 | 0.381 | 0.067 |
| 6 | 0.449 | 0.068 | 0.421 | 0.006 | −0.095 | 0.664 | −0.760 | −0.747 | 0.524 | 0.180 | −0.251 | 0.358 | −0.535 | 0.276 |
| 7 | −0.773 | −0.381 | −0.495 | −0.086 | −0.038 | −0.716 | 0.905 | 0.999 | −0.645 | −0.235 | 0.583 | −0.278 | 0.615 | −0.404 |

B[c]

| | 1 | 2 |
|---|---|---|
| 1 | 0.751 | −0.732 |
| 2 | 0.221 | −0.374 |
| 3 | 0.624 | −0.387 |
| 4 | 0.149 | 0.038 |
| 5 | −0.217 | −0.078 |
| 6 | 0.747 | −0.876 |
| 7 | −0.913 | 0.970 |
| 8 | −0.998 | 0.956 |
| 9 | 0.692 | −0.746 |
| 10 | 0.188 | −0.237 |
| 11 | −0.437 | 0.453 |
| 12 | 0.431 | −0.445 |
| 13 | −0.655 | 0.703 |
| 14 | 0.467 | −0.219 |

C[d]

| Compd. | *exo* | *endo* | Compd. | *exo* | *endo* |
|---|---|---|---|---|---|
| **1** | 0.913 | 0.084 | **14** | 0.014 | 0.985 |
| **2** | 0.989 | 0.009 | **15** | 0.015 | 0.983 |
| **3** | 0.994 | 0.005 | **16** | 0.088 | 0.900 |
| **4** | 0.844 | 0.155 | **17** | 0.020 | 0.979 |
| **5** | 0.955 | 0.049 | **18** | 0.017 | 0.984 |
| **6** | 0.961 | 0.039 | **19** | 0.025 | 0.975 |
| **7** | 0.948 | 0.051 | **20** | 0.014 | 0.986 |
| **8** | 0.964 | 0.034 | **21** | 0.220 | 0.788 |
| **9** | 0.926 | 0.073 | **22** | 0.015 | 0.984 |
| **10** | 0.968 | 0.030 | **23** | 0.067 | 0.926 |
| **11** | 0.970 | 0.028 | **24** | 0.021 | 0.979 |
| **12** | 0.974 | 0.026 | **25** | 0.019 | 0.981 |
| **13** | 0.841 | 0.161 | | | |

D[e]

| Compd. | *exo* | *endo* |
|---|---|---|
| **26** | 0.703 | 0.293 |
| **27** | 0.079 | 0.924 |
| **28** | 0.906 | 0.091 |
| **29** | 0.025 | 0.974 |
| **30** | 0.981 | 0.018 |
| **31** | 0.708 | 0.293[f] |
| **32** | 0.953 | 0.045 |
| **33** | 0.009 | 0.990 |
| **34** | 0.974 | 0.029 |
| **35** | 0.063 | 0.941 |
| **36** | 0.008 | 0.992 |
| **37** | 0.825 | 0.177 |
| **38** | 0.103 | 0.898 |

*a*) The sum of the squared errors ($E$)<0.110. *b*) Weight matrix between the first (column) and second (row) layers (scale factor=0.4176). *c*) Weight matrix between the second (column) and third (row) layers (scale factor=0.390). *d*) Outputs for the training data. *e*) Outputs for the untrained data. *f*) Error.

TABLE III. Weight Matrices and Outputs by Reconstruction Learning Method[a]

A[b]

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | −0.002 | 0.000 | 0.002 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 2 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.594 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 3 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.004 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 4 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | −0.515 | 0.000 | 0.006 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 5 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | −0.626 | 0.000 | 0.003 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 6 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.994 | 0.000 | −0.122 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 7 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | −0.998 | 0.000 | 0.744 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

B[c]

| | 1 | 2 |
|---|---|---|
| 1 | −0.001 | 0.001 |
| 2 | −0.001 | 0.001 |
| 3 | −0.001 | 0.001 |
| 4 | −0.001 | 0.001 |
| 5 | −0.001 | 0.001 |
| 6 | 0.998 | −0.998 |
| 7 | 0.001 | 0.001 |
| 8 | −0.577 | 0.577 |
| 9 | −0.001 | 0.001 |
| 10 | −0.001 | 0.001 |
| 11 | −0.001 | 0.001 |
| 12 | −0.001 | 0.001 |
| 13 | −0.001 | 0.001 |
| 14 | −0.001 | 0.001 |

C[d]

| Compd. | *exo* | *endo* | Compd. | *exo* | *endo* |
|---|---|---|---|---|---|
| **1** | 0.899 | 0.101 | **14** | 0.050 | 0.950 |
| **2** | 0.973 | 0.027 | **15** | 0.051 | 0.949 |
| **3** | 0.978 | 0.022 | **16** | 0.136 | 0.864 |
| **4** | 0.855 | 0.145 | **17** | 0.063 | 0.937 |
| **5** | 0.939 | 0.061 | **18** | 0.049 | 0.951 |
| **6** | 0.955 | 0.045 | **19** | 0.072 | 0.928 |
| **7** | 0.931 | 0.069 | **20** | 0.046 | 0.954 |
| **8** | 0.947 | 0.053 | **21** | 0.226 | 0.774 |
| **9** | 0.914 | 0.086 | **22** | 0.050 | 0.950 |
| **10** | 0.935 | 0.065 | **23** | 0.115 | 0.885 |
| **11** | 0.959 | 0.041 | **24** | 0.059 | 0.941 |
| **12** | 0.962 | 0.038 | **25** | 0.042 | 0.958 |
| **13** | 0.759 | 0.241 | | | |

D[e]

| Compd. | *exo* | *endo* |
|---|---|---|
| **26** | 0.707 | 0.293 |
| **27** | 0.115 | 0.885 |
| **28** | 0.904 | 0.096 |
| **29** | 0.070 | 0.930 |
| **30** | 0.966 | 0.034 |
| **31** | 0.629 | 0.371[f] |
| **32** | 0.941 | 0.059 |
| **33** | 0.041 | 0.959 |
| **34** | 0.958 | 0.042 |
| **35** | 0.100 | 0.900 |
| **36** | 0.038 | 0.962 |
| **37** | 0.738 | 0.262 |
| **38** | 0.093 | 0.907 |

*a*) The sum of the squared errors ($E$) < 0.153. *b*) The weight matrix between the first (column) and second (row) layers (scale factor = 0.3227). *c*) The weight matrix between the second (column) and third (row) layers (scale factor = 0.1681). *d*) Outputs for the trained data. *e*) Outputs for the untrained data. *f*) Error.

TABLE IV. Reconstructed Weight Matrices and Outputs in Mitomycins[a]

A[b]

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.000 | 0.000 | 0.000 | 0.001 | −0.064 | 0.001 | 0.000 | 0.001 | 0.000 | −0.355 | 0.000 | 0.000 |
| 2 | 0.000 | 0.682 | 0.000 | 0.001 | −0.044 | 0.000 | −0.073 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| 3 | 0.000 | 0.515 | 0.000 | 0.000 | 0.000 | −0.039 | 0.000 | 0.000 | 0.000 | −0.211 | 0.000 | 0.000 |
| 4 | 0.000 | 0.001 | 0.000 | −0.001 | 0.585 | −0.001 | 0.010 | 0.000 | −0.001 | −0.032 | 0.000 | 0.000 |
| 5 | 0.000 | 0.000 | 0.000 | 0.005 | 0.113 | 0.075 | −0.201 | 0.000 | 0.000 | −0.121 | 0.000 | 0.000 |
| 6 | 0.000 | −0.698 | 0.000 | 0.000 | 0.999 | 0.000 | 0.000 | 0.000 | 0.000 | 0.398 | 0.000 | 0.000 |
| 7 | 0.000 | 0.000 | 0.000 | 0.064 | −0.682 | 0.065 | 0.174 | 0.125 | 0.000 | −0.110 | 0.000 | 0.000 |

B[c]

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | −0.001 | 0.001 | −0.001 | 0.000 | 0.000 |
| 2 | −0.001 | −0.827 | 0.813 | 0.333 | 0.001 |
| 3 | −0.001 | 0.000 | −0.164 | −0.001 | 0.000 |
| 4 | −0.001 | 0.001 | −0.378 | 0.000 | 0.001 |
| 5 | 0.000 | 0.529 | 0.850 | −0.001 | −0.937 |
| 6 | 0.000 | 0.000 | −0.812 | −0.001 | 0.000 |
| 7 | −0.007 | −0.001 | 0.000 | −0.760 | 0.562 |
| 8 | −0.529 | 0.001 | −0.262 | −0.001 | 0.000 |
| 9 | −0.001 | 0.001 | 0.000 | −0.001 | 0.001 |
| 10 | 0.892 | −0.999 | 0.000 | −0.001 | 0.000 |
| 11 | −0.001 | 0.000 | 0.000 | 0.000 | 0.000 |
| 12 | 0.000 | 0.001 | −0.001 | 0.000 | 0.000 |

C[d]

| Compd. | 3+ | 2+ | + | +/− | − | Obs |
|---|---|---|---|---|---|---|
| **1** | 0.893 | 0.075 | 0.016 | 0.001 | 0.012 | 3+ |
| **2** | 0.942 | 0.037 | 0.018 | 0.001 | 0.014 | 3+ |
| **3** | 0.039 | 0.947 | 0.030 | 0.001 | 0.012 | 2+ |
| **4** | 0.032 | 0.951 | 0.034 | 0.001 | 0.012 | 2+ |
| **5** | 0.013 | 0.900 | 0.157 | 0.002 | 0.013 | 2+ |
| **6** | 0.011 | 0.916 | 0.000 | 0.080 | 0.013 | 2+ |
| **7** | 0.065 | 0.938 | 0.019 | 0.001 | 0.012 | 2+ |
| **8, 9** | 0.007 | 0.038 | 0.760 | 0.012 | 0.110 | + |
| **10** | 0.041 | 0.026 | 0.945 | 0.011 | 0.012 | + |
| **11** | 0.010 | 0.069 | 0.961 | 0.027 | 0.007 | + |
| **12** | 0.007 | 0.042 | 0.988 | 0.042 | 0.007 | + |
| **13** | 0.006 | 0.001 | 0.067 | 0.893 | 0.076 | +/− |
| **14** | 0.087 | 0.095 | 0.000 | 0.001 | 0.948 | − |
| **15** | 0.006 | 0.002 | 0.159 | 0.017 | 0.884 | − |
| **16** | 0.006 | 0.000 | 0.023 | 0.054 | 0.978 | − |

*a*) The sum of the erorrs ($E$) < 0.057. *b*) The weight matrix between the first (column) and second (row) layers (scale factor = 0.0631). *c*) The weight matrix between the second (column) and third (row) layers (scale factor = 0.0951). *d*) Outputs by the reconstruction-learning method.

TABLE V. The Reconstructed Weight Matrices and Outputs by ALS-Type Training Patterns in Mitomycins[a]

A[b]

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.000 | 0.043 | −0.065 | 0.000 | 0.499 | −0.001 | 0.000 | 0.001 | −0.001 | 0.000 | 0.001 | 0.000 |
| 2 | 0.000 | 0.479 | −0.027 | 0.001 | 0.001 | −0.001 | 0.000 | 0.000 | −0.002 | −0.001 | 0.000 | 0.000 |
| 3 | 0.000 | 0.403 | −0.001 | 0.000 | 0.155 | 0.000 | −0.001 | 0.001 | 0.000 | 0.000 | −0.001 | 0.000 |
| 4 | 0.000 | −0.119 | 0.633 | 0.001 | 0.002 | 0.000 | 0.000 | 0.000 | 0.060 | −0.001 | 0.000 | 0.001 |
| 5 | 0.001 | −0.165 | 0.268 | −0.002 | 0.003 | 0.001 | 0.001 | 0.001 | −0.005 | 0.002 | 0.000 | 0.001 |
| 6 | 0.000 | −0.293 | 0.999 | 0.001 | −0.389 | 0.001 | 0.001 | 0.001 | 0.000 | 0.001 | 0.001 | 0.001 |
| 7 | 0.001 | 0.002 | −0.816 | 0.000 | 0.051 | 0.000 | 0.002 | 0.000 | 0.149 | 0.000 | 0.000 | 0.000 |

B[c]

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 0.001 | 0.000 | 0.000 | 0.000 | 0.001 |
| 2 | 0.002 | 0.000 | −0.659 | −0.866 | −0.317 |
| 3 | 0.053 | 0.999 | 0.867 | 0.505 | 0.270 |
| 4 | 0.002 | −0.001 | 0.000 | 0.000 | 0.092 |
| 5 | 0.068 | −0.002 | −0.001 | 0.000 | −0.721 |
| 6 | 0.001 | −0.001 | −0.001 | 0.000 | 0.000 |
| 7 | 0.001 | −0.001 | 0.000 | 0.001 | 0.001 |
| 8 | 0.063 | −0.002 | 0.000 | 0.001 | 0.001 |
| 9 | 0.224 | −0.437 | −0.001 | 0.000 | 0.003 |
| 10 | 0.002 | −0.002 | −0.001 | 0.000 | 0.001 |
| 11 | 0.001 | 0.000 | 0.000 | 0.001 | 0.000 |
| 12 | 0.002 | −0.001 | 0.001 | 0.000 | 0.000 |

C[d]

| Compd. | − | +/− | + | 2+ | 3+ | Obs |
|---|---|---|---|---|---|---|
| **1** | 0.975 | 0.999 | 1.000 | 0.992 | 0.795 | 3+ |
| **2** | 0.973 | 0.999 | 1.000 | 0.987 | 0.908 | 3+ |
| **3** | 0.981 | 0.998 | 0.999 | 0.960 | 0.104 | 2+ |
| **4** | 0.981 | 0.998 | 0.999 | 0.954 | 0.085 | 2+ |
| **5** | 0.982 | 0.997 | 0.998 | 0.872 | 0.024 | 2+ |
| **6** | 0.972 | 0.966 | 0.993 | 0.883 | 0.050 | 2+ |
| **7** | 0.981 | 0.999 | 0.999 | 0.930 | 0.098 | 2+ |
| **8, 9** | 0.980 | 0.806 | 0.779 | 0.139 | 0.001 | + |
| **10** | 0.980 | 0.999 | 0.966 | 0.080 | 0.047 | + |
| **11** | 0.985 | 0.999 | 0.958 | 0.063 | 0.002 | + |
| **12** | 0.986 | 0.998 | 0.893 | 0.023 | 0.000 | + |
| **13** | 0.977 | 0.852 | 0.116 | 0.002 | 0.000 | +/− |
| **14** | 0.963 | 0.084 | 0.092 | 0.021 | 0.004 | − |
| **15** | 0.977 | 0.194 | 0.142 | 0.017 | 0.000 | − |
| **16** | 0.971 | 0.043 | 0.001 | 0.000 | 0.000 | − |

*a*) The maximum sum of errors ($E$) < 0.099. *b*) The weight matrix between the first (column) and second (row) layers (scale factor = 0.1066). *c*) The weight matrix between the second (column) and third (row) layers (scale factor = 0.0894). *d*) Outputs.

neurons 6 and 7 in the first layer, suggesting that the information of the *endo*/*exo* conformation entailed on the neuron 6 of the second layer is corrected by the neuron 8.

**Graded Classification of Activities in Mitomycins** Table IV shows the reconstructed weight matrices in mitomycins. The experimental data and structural parameters were taken from the literature[10,11] and the same compound numbers were used.[2,10] The scaled structural parameters, *i.e.*, $F_x$, $\sigma_{m-X}$, $V_{w-X}$, $Y_{OMe}$, $Y_{OH}$, and $E_{S-Z}$, are input using the network structure $N(7, 12, 5)$, where neurons 1—6 were given for the structural parameters and neuron 7 for the constant 1. Neurons 1, 2, 3, 4, and 5 in the third layer correspond to the biological activities 3+, 2+, +, +/−, and −, respectively.

Note the forth and sixth rows of Table IV, under heading A. They show the positively large weights to the neurons (5 and 10) which contribute positively to the mitomycin activity, *i.e.*, the neurons 1 and 2 in the output layer. From Table IV, under heading B, the neurons of the 3+ and 2+ activities connect to the neurons 10 and 5 in the second layer; these neurons do not have positive contributions to the +/− and − neurons in the third layer. Although there are no neurons in the second layer which strongly suppress the 3+ neuron in the output layer, those which suppress the 2+ neurons are neurons 2 and 10 in the second layer. It is also understood that to suppress the − neuron, neuron 5 in the second layer must be fired. This is identical to the activation of the 2+ and + neurons.

The factors in the first layer that ignite the 3+ neuron are, therefore, the large Taft's steric constant ($E_{S-Z}$) of the substituent Z and low $F_x$ and $V_{w-X}$ of the substituent X. This plus the fact that the parameter $\sigma_{m-X}$ has the least contribution supports the former results by the ALS method.[10,11] However, the present result that the dummy constant, $Y_{OMe}$, and $Y_{OH}$ do not contribute to the activity contradicts the results by the ALS method.

TABLE VI. Contributions of Input Parameters to Important Paths in Mitomycins

| Path | $F_x$ | $\sigma_{m-X}$ | $V_{w-X}$ | $Y_{OMe}$ | $Y_{OH}$ | $E_{S-Z}$ |
|---|---|---|---|---|---|---|
| 2 | −0.04 | −0.48 | −0.40 | 0.12 | 0.17 | 0.29 |
| 3 | −0.07 | −0.03 | 0.00 | 0.63 | 0.27 | 0.99 |
| 5 | −0.50 | 0.00 | −0.16 | 0.00 | 0.00 | 0.39 |

The 2+ neuron is ignited through neuron 5 in the second layer, which is controlled by $E_{S-Z}$ and $Y_{OMe}$. The existence of the substituent X slightly suppresses the 2+ neuron. But this does not contradict the conclusion by the ALS method.

The ALS method recognizes the X's and Y's parameters together as the smaller X is associated with the larger Y, while the neural network deals with them seperately. However, the fact that the contribution to the activity of OMe is larger than that of OH is obtained by both the ALS and neural network methods.

The negative factors for the 2+ neuron are shown forth by neurons 2 and 10 in the second layer. The weights between neuron 10 and the 2+ neuron, and neuron 10 and the +3 neuron are opposite in sign indicating that the network recognizes them seperately too. The information route to neuron 2 from the input layer is opposite to that to neuron 5. Namely, the contribution of the parameter $E_{S-Z}$ is small

TABLE VII. Reconstructed Weight Matrices and Outputs by ALS-Type Training Patterns in Arylacryloylpiperazines[a]

A[b]

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | −0.001 | 0.639 | 0.001 | 0.001 | 0.000 | 0.000 | 0.000 | 0.000 | 0.672 | −0.395 | 0.000 | 0.000 | −0.001 | −0.999 |
| 2 | 0.000 | 0.001 | 0.000 | 0.667 | 0.000 | −0.001 | 0.000 | −0.001 | 0.381 | 0.000 | −0.001 | 0.000 | 0.000 | 0.335 |
| 3 | 0.000 | −0.001 | 0.001 | −0.805 | 0.000 | 0.000 | 0.000 | 0.000 | −0.089 | −0.239 | 0.000 | 0.000 | −0.001 | −0.443 |
| 4 | 0.000 | 0.001 | 0.000 | −0.001 | 0.000 | 0.000 | 0.000 | 0.000 | 0.001 | 0.018 | 0.000 | 0.000 | 0.000 | −0.376 |
| 5 | 0.000 | 0.004 | 0.001 | −0.001 | 0.000 | 0.000 | 0.000 | 0.000 | −0.001 | −0.016 | 0.000 | 0.000 | 0.000 | 0.000 |
| 6 | 0.000 | −0.301 | 0.001 | −0.001 | 0.000 | 0.000 | 0.000 | 0.000 | −0.028 | 0.188 | 0.000 | 0.000 | −0.001 | −0.010 |
| 7 | 0.000 | −0.193 | 0.080 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | −0.290 | 0.000 | 0.000 | 0.000 | −0.001 | 0.635 |

B[c]

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | 0.000 | 0.000 | 0.000 | 0.000 |
| 2 | 0.001 | 0.001 | −0.162 | −0.555 |
| 3 | 0.242 | 0.000 | 0.000 | 0.000 |
| 4 | 0.001 | 0.377 | 0.263 | 0.395 |
| 5 | 0.001 | 0.000 | 0.000 | 0.000 |
| 6 | 0.000 | 0.000 | −0.001 | 0.000 |
| 7 | 0.000 | 0.000 | −0.001 | 0.000 |
| 8 | 0.001 | 0.000 | 0.000 | 0.000 |
| 9 | 0.001 | −0.487 | −0.696 | −0.276 |
| 10 | 0.000 | 0.001 | 0.000 | −0.615 |
| 11 | 0.001 | 0.000 | 0.000 | 0.000 |
| 12 | 0.000 | 0.000 | 0.000 | 0.000 |
| 13 | 0.001 | 0.000 | −0.001 | 0.000 |
| 14 | 0.001 | 0.665 | 0.998 | 0.327 |

C[d]

| Compd. | 1 | 2 | 3 | 4 | Obs | Compd. | 1 | 2 | 3 | 4 | Obs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 0.984 | 0.151 | 0.000 | 0.000 | 1 | **16** | 0.984 | 0.991 | 0.449 | 0.002 | 2 |
| **2** | 0.984 | 0.037 | 0.000 | 0.032 | 1 | **17** | 0.984 | 0.999 | 0.880 | 0.014 | 3 |
| **3** | 0.984 | 0.064 | 0.000 | 0.037 | 1 | **18** | 0.984 | 1.000 | 0.998 | 0.057 | 3 |
| **4** | 0.984 | 0.001 | 0.000 | 0.003 | 1 | **19** | 0.983 | 1.000 | 1.000 | 0.155 | 3 |
| **5** | 0.983 | 0.028 | 0.005 | 0.056 | 1 | **20** | 0.984 | 0.890 | 0.961 | 0.180 | 3 |
| **6** | 0.984 | 0.000 | 0.000 | 0.000 | 1 | **21** | 0.984 | 0.999 | 0.999 | 0.042 | 3 |
| **7** | 0.984 | 0.000 | 0.000 | 0.000 | 1 | **22** | 0.984 | 0.999 | 0.972 | 0.012 | 3 |
| **8** | 0.984 | 0.269 | 0.169 | 0.054 | 1 | **23** | 0.984 | 0.996 | 0.994 | 0.006 | 3 |
| **9** | 0.984 | 0.004 | 0.000 | 0.000 | 1 | **24** | 0.983 | 1.000 | 1.000 | 0.213 | 3 |
| **10** | 0.984 | 0.005 | 0.000 | 0.000 | 1 | **25** | 0.984 | 1.000 | 1.000 | 0.958 | 4 |
| **11** | 0.984 | 0.000 | 0.000 | 0.000 | 1 | **26** | 0.984 | 0.995 | 0.890 | 0.889 | 4 |
| **12** | 0.984 | 0.880 | 0.007 | 0.001 | 2 | **27** | 0.983 | 1.000 | 1.000 | 0.876 | 4 |
| **13** | 0.984 | 0.996 | 0.415 | 0.006 | 2 | **28** | 0.984 | 1.000 | 1.000 | 0.942 | 4 |
| **14** | 0.984 | 0.991 | 0.365 | 0.168 | 2 | **29** | 0.983 | 1.000 | 1.000 | 0.800 | 4 |
| **15** | 0.984 | 0.952 | 0.262 | 0.047 | 2 | | | | | | |

*a*) The sum of error ($E$)<0.142. *b*) The weight matrix between the first (column) and second (row) layers (scale factor=0.0616). *c*) The weight matrix between the second (column) and third (row) layers (scale factor=0.0484). *d*) Outputs.

while those for $\sigma_{m-X}$ and $V_{w-X}$ are large. This also agrees with the ALS method.

To correlate more clearly the results of the three-layer neural network with those of ALS, an $n$ number of the output neurons are forced to ignite for the $n$th grade; for example, for the ± and + grades, the training patterns (1, 1, 0, 0, 0) and (1, 1, 1, 0, 0) are given. (This type of training pattern is here called "ALS-type" training pattern). The weight matrices obtained through reconstruction-learning are given in Table V.

The second, third, and fifth neurons in the second layer are important ones regarding the biological activity. Table VI shows the connection values and the structural parameters, where −1 is multiplied by the coefficients connected with suppressive neurons. The ALS discrimination functions have been reported as,[10,11]

$$L = -4.10\sigma_{m-X} - 1.76V_{w-X} + 2.49Y_{OMe} + 2.29Y_{OH} + 0.79 \quad (10)$$

$$L = -4.33F_x - 2.48V_{w-X} + 2.47Y_{OMe} + 2.28Y_{OH} + 0.78E_{S-Z} + 1.42 \quad (11)$$

Note that Eq. 10 corresponds to path 2 while Eq. 11 expresses the addition of paths 3 and 5. The weight matrix between the first and second layers indicates that the relationship between the structural parameters and biological activities should be expressed in terms of three paths as shown in Table VI.

**Antihypertensive Activity in Arylacryloylpiperazines** The same method was applied to the antihypertensive activity in the derivatives of arylacryloylpiperazine. We have used the same biological data and structural parameters.[2,12,13] The input data are $\Delta RI$, $\sum\pi$, $\sum\sigma$, $I$(2-OR), $I(R^1)$, HB, and the constant 1. The ALS-type training patterns were given to the network to see the correlation with the ALS method. The results of reconstructed learning are shown in Table VII. The classification was correctly carried out, as it was in the usual back-propagation learning.

TABLE VIII. Contributions of Input Parameters to Important Paths in Arylacryloylpiperazines

| Path | $\Delta RI$ | $\pi$ | $\sigma$ | $I$(2-OR) | $I(R^1)$ | HB |
|---|---|---|---|---|---|---|
| 2 | −0.64 | 0.00 | 0.00 | 0.00 | 0.00 | 0.30 |
| 4 | 0.00 | 0.67 | −0.81 | 0.00 | 0.00 | 0.00 |
| 10 | 0.40 | 0.00 | 0.24 | −0.02 | 0.02 | −0.19 |

The reconstruction reveals that neurons 2, 3, 4, 9, 10, and 14 in the second layer play important roles (since the actual neurons' values are not important here, they are not shown). Among them, neurons 4 and 14 positively contribute to neuron 4 which is referred to as the strongest biological activity while neurons 2, 9, and 10 contribute negatively. Neurons 2, 4, and 10 contribute differently to neurons 3 and 4 in the last layer. This indicates that the neural network recognizes the igniting factor of neuron 4 being different from that of neuron 3.

Table VIII shows the connection values and the structural parameters, where, as a rule, −1 is multiplied by the coefficients connected with suppressive neurons. The discriminative functions of the ALS analysis were given as[12,13]

$$L = -1.018\Delta RI - 2.357\sum\sigma - 1.838I(2\text{-OR}) + 0.769 \quad (12)$$

$$L = -1.259\sum\pi - 2.166\sum\sigma - 1.777I(\text{2-OR}) + 1.169I(\text{R}^1) + 0.358 \quad (13)$$

by which the ratios of correct classification are only 76% and 65%, respectively. The neural network indicates that parameters, $\Delta RI$, $\sum\sigma$, and $\sum\pi$, are important for the strong activity while the ALS method shows, in addition, the contributions of $I$(2-OR) and $I(R^1)$: The roles regarding the common parameters are the same in both methods. However, since the ALS method treated the parameters $\Delta RI$ and $\sum\pi$ separately, one cannot compare the coefficients regarding $\sum\pi$. Further investigation of the correlation may be meaningless. Consequently, the major parameters contributing to the activity are consistent in both methods.

**Concluding Remarks** Simply from curiosity, we incorporated the forgetting process in the learning phase. The outcome was surprising: the connections between neurons are greatly simplified and one can easily correlated the output results with the input parameters. We understand that such a simplification is brought forth simply by the Hebbian learning rule.[14] So far, we have examined 15 cases of SAR data and found that in all cases the simplification of connections, as reported above took place without diminishing the network's resolution ability. Although by adopting the erasing procedure, the learning phase takes 10 to 50 times longer than the usual back-propagation learning does, the use of hardware-constructed neural networks or so-called neuro-chips may solve this problem. Besides, application of this method will be wide-spread, not only in the SAR study but also in any problem that seeks the relationship between the causes and the results.

As already stated, there are a number of possible ways to localize the distribution of connections. Recently, Tani *et al.*[15] reported a method of using the criterion function, Eq. 14, instead of Eq. 5,

$$E = \sum_j (O_j - t_j)^2 + c\sum_j \left|\prod_i W_{ji}\right| \quad (14)$$

where $c$ is a parameter. As Eq. 14 indicates, this method apparently diminishes the dispersion of connections. The difficulty of this method, however, is that there is no physical or physiological meaning in this procedure.

**Acknowledgment** This research was financially supported by the Ministry of Education, Science and Culture (Grant-in-Aid for Developmental Scientific Research No. 02807197).

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# Studies on *Balanites aegyptiaca* Fruits, an Antidiabetic Egyptian Folk Medicine

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**An aqueous extract of mesocarps of the fruits of *Balanites aegyptiaca* exhibited a prominent antidiabetic activity by oral administration in streptozotocin induced diabetic mice. From one of the active fractions of this extract, two new steroidal saponins were isolated, and their structures were determined as 26-*O*-β-D-glucopyranosyl-(25*R*)-furost-5-ene-3β,22,26-triol 3-*O*-[α-L-rhamnopyranosyl-(1→2)]-[β-D-xylopyranosyl-(1→3)]-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside and its 22-methyl ether. In addition, two known saponins, 26-*O*-β-D-glucopyranosyl-(25*R*)-furost-5-ene-3β,22,26-triol 3-*O*-(2,4-di-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside and its methyl ether were isolated and identified. It was revealed that the individual saponins did not show antidiabetic activity, while the recombination of these saponins resulted in significant activity. From an ethanolic extract of the epicarps, two known flavonol glycosides, isorhamnetin-3-*O*-robinobioside and isorhamnetin-3-*O*-rutinoside were isolated and identified.**

**Keywords** *Balanites aegyptiaca*; Balanitaceae; Egyptian medicinal plant; steroidal glycosides; flavonoidal glycoside; antidiabetic activity

*Balanites aegyptiaca* DEL. (Balanitaceae) is widely distributed in Africa and has a variety of uses as a folk medicine in many countries of Africa.[1] In Egyptian folk medicine, the fruits (after removal of the epicarp) are used as an oral antidiabetic drug. This paper describes the antidiabetic activities of an aqueous extract of the mesocarps and its fractions as well as isolation, characterization and biological activity of the steroidal glycosides from one of the active fractions. In addition, the isolation and identification of the major flavonol glycosides from an ethanolic extract of the epicarps is also reported.

The mesocarps were extracted with water. To an aqueous solution of the extract, was added an excess of ethanol to give precipitates and supernatant. The precipitate was dialyzed against water to yield a non-dialyzed fraction tentatively named polysaccharide fraction. The antidiabetic activity test in streptozotocin (STZ) induced diabetic mice (Table I) revealed that oral administration of the aqueous extract, as well as the polysaccharide fraction and the supernatant, exhibited prominent antidiabetic actions in 6 h; −33%, −53% and −53%, respectively. In order to identify the active principles, the supernatant was chromatographed on a highly porous synthetic resin (Diaion HP-20) by eluting with water, 40% methanol (MeOH), 80% MeOH, MeOH and acetone, successively. The activity of each eluate was tested orally in STZ diabetic mice. As shown in Table II, the most remarkable decrease (−57%) in the blood glucose levels was observed for the 80% MeOH eluate 6 h after administration.

The active 80% MeOH eluate was separated by chromatography on silica gel to give four fractions tentatively designated as F-1—4 in order of increasing polarity. Of these fractions, F-2 was proved to be remarkably active for its antidiabetic effect (Table II), so that this fraction was further chromatographed on a column of LiChroprep RP-8 and finally purified by high-performance liquid chromatography (HPLC) to give four compounds, **1**—**4** together with several minor compounds, **5**—**8**. Compounds **1**—**4** were assumed to be furostanol saponins on the basis of the positive coloration with Ehrlich reagent.[2] Acid hydrolysis of **1** yielded L-rhamnose, D-glucose and diosgenin. Methylation analysis of **1** revealed the presence of a 2,4-linked glucosyl unit together with terminal glucosyl and rhamnosyl units. Inspection of the carbon-13 and proton nuclear magnetic resonance ($^{13}$C- and $^{1}$H-NMR) spectra (Tables III, IV), as well as the negative fast atom bombardment mass spectrum (FAB-MS), led to the formulation of **1** as 26-*O*-β-D-glucopyranosyl-(25*R*)-furost-5-ene-3β,22,26-triol 3-*O*-(2,4-di-*O*-α-L-rhamnopy-

TABLE I. Antidiabetic Effect of Aqueous Extract, Polysaccharide Fraction and Supernatant on Blood Glucose Levels in STZ Diabetic Mice

| Sample[a] | 0 | | 4 h | | 6 h | |
|---|---|---|---|---|---|---|
| | m±S.E. (mg%) | % | m±S.E. (mg%) | % | m±S.E. (mg%) | % |
| Control | 299±21.1 | 100 | 299± 7.2 | 100 | 290±4.6 | 97 |
| Extract | 299± 5.1 | 100 | 268±11.4 | 90 | 194±7.2 | 65 |
| Polysaccharide fraction | 273± 2.7 | 100 | 192±16.0 | 70 | 137±3.5 | 50 |
| Supernatant | 223± 5.5[b] | 100 | 173± 6.7[c] | 78 | 137±5.0 | 61 |

*a*) Dose: 80 mg/kg, orally and $n=5$ (m, mean; S.E., standard error). *b*) $p<0.01$. *c*) $0.1>p>0.05$.

TABLE II. Antidiabetic Effect of Eluates and Fractions of Supernatant Chromatography (Diaion HP-20 and Silica Gel) on Blood Glucose Levels in STZ Diabetic Mice

| Sample[a] | 0 | | 4 h | | 6 h | |
|---|---|---|---|---|---|---|
| | m±S.E. (mg%) | % | m±S.E. (mg%) | % | m±S.E. (mg%) | % |
| Exp. 1 | | | | | | |
| Control | 286± 2.5 | 100 | 168± 4.9 | 59 | 194± 7.3 | 68 |
| 40% MeOH eluate | 303± 4.5[b] | 100 | 259± 5.5 | 85 | 169±21.1 | 56 |
| 80% MeOH eluate | 305±18.1[c] | 100 | 134± 5.9 | 44 | 83± 2.7 | 27 |
| MeOH eluate | 279±26.9 | 100 | 167± 8.2 | 60 | 128± 5.1 | 46 |
| Exp. 2 | | | | | | |
| Control | 366±18.0 | 100 | 395± 3.3 | 108 | 337± 4.4 | 103 |
| F-2 | 380±11.1 | 100 | 315± 4.6 | 83 | 107± 7.7[d] | 28 |
| F-3 | 383± 7.6 | 100 | 380±14.7 | 99 | 373± 7.6 | 97 |

*a*) Dose: 80 mg/kg, orally and $n=5$ (m, mean; S.E., standard error). *b*) $p<0.01$. *c*) $p<0.05$. *d*) $p<0.001$.

TABLE III. $^{13}$C-NMR Data of the Sugar Moieties of Compounds **1**—**4** (100 MHz, in $C_5D_5N$)

| Carbon No. | 1 | 2 | 3 | 4 | Carbon No. | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|---|---|---|
| 26-Glucosyl | | | | | 3 | 72.7 | 72.8 | 72.7 | 72.7 |
| 1 | 104.9[a] | 105.1[a] | 104.5[a] | 104.5[a] | 4 | 74.9[b] | 74.9[b] | 74.9[b] | 75.1[b] |
| 2 | 75.2[b] | 75.0[b] | 75.3[b] | 75.2[b] | 5 | 69.4 | 69.4 | 69.4 | 69.4 |
| 3 | 78.6[c] | 78.6[c] | 78.6[c] | 78.2[c] | 6 | 18.6 | 18.6 | 18.6 | 18.6 |
| 4 | 71.7 | 71.8 | 71.2 | 71.7 | 1′ | 100.1 | 100.0 | 99.9 | 99.9 |
| 5 | 78.3[c] | 78.2[c] | 78.2[c] | 78.1[c] | 2′ | 71.3 | 71.2 | 71.7 | 71.7 |
| 6 | 62.9 | 62.9 | 62.1 | 62.8 | 3′ | 72.4 | 72.4 | 72.5 | 72.4 |
| 3-Glucosyl | | | | | 4′ | 74.1 | 74.1 | 74.1 | 74.1 |
| 1 | 105.1[a] | 105.1[a] | 104.9[a] | 105.1[a] | 5′ | 69.4 | 69.4 | 69.0 | 69.0 |
| 2 | 82.0 | 82.0 | 82.5 | 81.5 | 6′ | 18.6 | 18.6 | 18.6 | 18.6 |
| 3 | 76.2 | 76.2 | 87.0 | 87.3 | Xylosyl | | | | |
| 4 | 78.4[c] | 77.7[c] | 78.8[c] | 78.6[c] | 1 | | | 106.2 | 106.2 |
| 5 | 77.4 | 77.4[c] | 77.6 | 77.6 | 2 | | | 75.4[b] | 75.2[b] |
| 6 | 63.8 | 63.8 | 62.8 | 62.8 | 3 | | | 78.0[c] | 78.0[c] |
| Rhamnosyl | | | | | 4 | | | 70.9 | 70.9 |
| 1 | 101.7 | 101.8 | 101.7 | 101.7 | 5 | | | 67.3 | 67.3 |
| 2 | 71.7 | 71.7 | 72.4 | 72.4 | | | | | |

*a*—*c*) Signals may be interchangeable in each column.

TABLE IV. $^1$H-NMR Spectral Data of Compounds **3**, **4** and **9** (400 MHz, in $C_5D_5N$)

| Proton No. | 3 | 9 | 4 |
|---|---|---|---|
| 3 | | 3.53, br t (8.9) | |
| 18 | 0.79, s | | 0.73, s |
| 19 | 1.02, s | | 1.02, s |
| 21 | 1.30, d (6.6) | | 1.22, d (6.5) |
| 27 | 0.88, d (7.0) | | 0.82, d (6.8) |
| Glc-1 | 4.82, d (6.8) | 4.12, d (8.1) | 4.80, d (7.1) |
| Glc-2 | | 4.32, dd (8.1, 9.7) | |
| Glc-3 | | 4.27, dd (9.7, 9.7) | |
| Glc-4 | | 4.15, dd (9.7, 9.8) | |
| Glc-5 | | 4.06, ddd (2.7, 6.5, 9.8) | |
| Glc-6a | | 4.40, dd (2.7, 9.8) | |
| Glc-6b | | 4.30, dd (6.5, 9.8) | |
| Glc-1′ | 5.09, d (6.4) | | 5.09, d (7.1) |
| Rha-1 | 6.20, br s | 4.95, br s | 6.18, br s |
| Rha-1′ | 6.20, br s | 5.03, br s | 6.18, br s |
| Xyl-1 | 5.20, d (7.2) | 4.47, d (8.0) | 5.20, d (7.7) |
| $OCH_3$ | | | 3.22, s |

Coupling constants (*J* in Hz) are in parenthesis.

ranosyl)-β-D-glucopyranoside.

Acid hydrolysis of **2** yielded the same sugars as those of **1**. Boiling **2** with aqueous acetone yielded **1**, and on boiling with methanol, **1** regenerated **2**.[3] The $^{13}$C- and $^1$H-NMR spectra (Tables IV, V) revealed the presence of a methoxyl group at C-22,[4] leading to the formulation of **2** as a 22-*O*-methyl derivative of **1**. Compounds **1** and **2** have already been isolated from rhizomes of *Dioscorea gracillima* by Kawasaki *et al.*[5] The identification was substantiated by comparison of the NMR spectra with the reported data.

Acid hydrolysis of **3** produced D-xylose, L-rhamnose and D-glucose together with aglycone, diosgenin. A peak at *m/z* 1179.1779 $[M-H]^-$ in the negative FAB-MS of **3** is consistent with the molecular formula $C_{56}H_{92}O_{26}$. The following significant FAB-MS fragment peaks were observed; $[M-H-Xyl]^-$ at *m/z* 1047, $[M-H-Xyl-Glc]^-$ at *m/z* 885 and $[M-H-Xyl-Glc-Rha]^-$ at *m/z* 739. The carbon signals due to the aglycone moeity of **3** appeared at almost the same positions as those of **1** (Table V), indicating that **3** is also a 3,26-*O*-bisdesmosidic furostanol saponin with the same aglycone as **1**.[6,7]

The $^{13}$C- and $^1$H-NMR signals due to the sugar moiety of **3** (Tables III, IV) revealed the presence of five sugar units; one terminal β-glucopyranosyl, one substituted β-glucopyranosyl, one terminal β-xylopyranosyl and two terminal α-rhamnopyranosyl units. The structure of the sugar moiety of **3** was established by $^1$H–$^1$H two-dimensional nuclear Overhauser effect (NOE) correlation spectroscopy (2D NOESY, Chart 1) of the acetate of **3** (**9**) based on the assignment of the sugar proton signals (Table IV) by two-dimensional correlation spectroscopy (2D COSY). The NOE were observed between H-1 of the xylosyl unit and H-3 of the glucosyl unit. The presence of NOE between one of two H-1 of rhamnosyl units and H-2 of the glucosyl unit, as well as between another H-1 of rhamnosyl units and H-4 of the same glucosyl unit, was also noticed. These results led to the formulation of the sugar moiety as illustrated in Chart 1. The location of this glycoside group at the 3β-hydroxyl group of the aglycone was established by the presence of the NOE between H-1 of the 2,3,4-trisubstituted glucosyl unit and H-3 of the aglycone moiety. Consequently, the structure of **3** was assigned as 26-*O*-β-D-glucopyranosyl-(25*R*)-furost-5-ene-3β,22,26-triol 3-*O*-[α-L-rhamnopyranosyl-(1→2)]-[β-D-xylopyranosyl-(1→3)]-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside.

The carbon signals due to the aglycone moiety of **4** (Table V) were very similar to that of **2**. Acid hydrolysis of **4** gave D-xylose, L-rhamnose and D-glucose in addition to diosgenin, and the $^{13}$C- and $^1$H-NMR signals of the sugar moeity of **4** are almost superimposable over those of **3**. As in the case of **1** and **2**, **3** and **4** are interconvertible by boiling with MeOH (from **3** to **4**) and with aqueous acetone (from **4** to **3**).[3] Accordingly, **4** was formulated as the 22-methyl ether of **3**.

Preliminary NMR inspection suggested that the other minor compounds, **5**—**8** may be tetrahydroxycholestadiene glycosides, biogenetic precursors of **1**—**4**. However, owing to the very low yields and exhaustion of the samples for the pharmacological test, chemical characterization of these

TABLE V. $^{13}$C-NMR Spectral Data of the Aglycone Moieties of Compounds **1**—**4** (100 MHz, in $C_5D_5N$)

| Carbon No. | 1 | 2 | 3 | 4 | Carbon No. | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 37.5 | 37.5 | 37.5 | 37.4 | 15 | 33.2[b] | 32.8[b] | 32.8[b] | 32.7[b] |
| 2 | 30.1 | 30.2 | 30.1 | 29.9 | 16 | 81.1 | 81.1 | 81.5 | 81.3 |
| 3 | 78.4 | 78.4 | 78.4 | 78.4 | 17 | 62.1 | 61.9 | 61.9 | 61.5 |
| 4 | 38.9[a] | 39.0[a] | 38.9[a] | 38.9[a] | 18 | 16.4 | 16.3 | 16.4 | 16.4 |
| 5 | 140.8 | 140.9 | 140.8 | 140.8 | 19 | 19.4 | 19.4 | 19.3 | 19.3 |
| 6 | 121.8 | 121.8 | 121.8 | 121.7 | 20 | 40.7 | 40.5 | 40.7 | 40.5 |
| 7 | 32.3[b] | 32.3[b] | 32.2[b] | 32.3[b] | 21 | 16.4 | 16.3 | 16.0 | 16.2 |
| 8 | 31.7 | 31.7 | 31.7 | 31.6 | 22 | 110.7 | 112.7 | 110.7 | 112.5 |
| 9 | 50.4 | 50.4 | 50.3 | 50.3 | 23 | 37.1 | 31.0 | 37.1 | 31.4 |
| 10 | 37.1 | 37.3 | 37.1 | 37.1 | 24 | 28.3 | 28.3 | 28.9 | 28.3 |
| 11 | 21.1 | 21.1 | 21.1 | 21.1 | 25 | 34.3 | 34.5 | 34.3 | 34.2 |
| 12 | 39.8[a] | 39.8[a] | 39.8[a] | 39.8[a] | 26 | 75.2 | 75.2 | 75.2 | 75.1 |
| 13 | 40.8 | 40.8 | 40.1 | 40.7 | 27 | 17.4 | 17.5 | 17.4 | 17.2 |
| 14 | 56.6 | 56.6 | 56.5 | 56.5 | $OCH_3$ | | 47.4 | | 47.2 |

*a*, *b*) Signals may be interchangeable in each column.

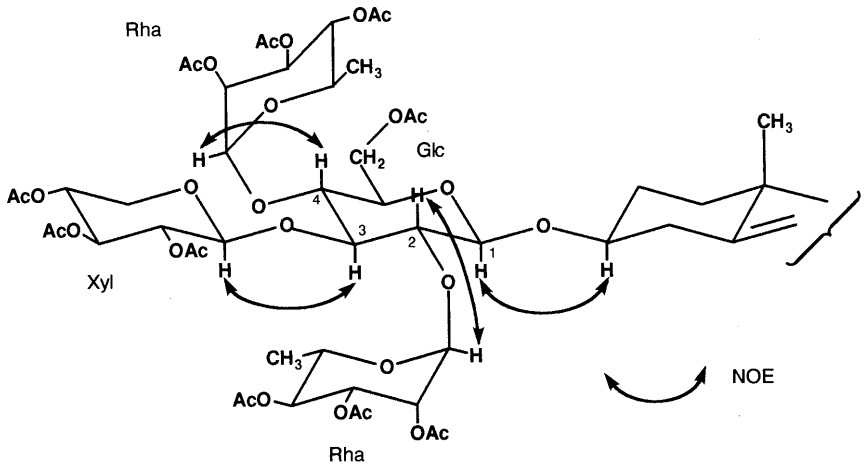


Chart 1. NOE Correlation of **9**

TABLE VI. Antidiabetic Effect of Isolated Compounds on Blood Glucose Levels in STZ Diabetic Mice

| Sample[a] | 0 | | 4 h | | 6 h | |
|---|---|---|---|---|---|---|
| | m±S.E. (mg%) | % | m±S.E. (mg%) | % | m±S.E. (mg%) | % |
| Exp. 1 | | | | | | |
| Control | 400±20.4 | 100 | 311±21.1 | 78 | 272± 5.4 | 68 |
| Compds**1**—**8** | 400± 4.1 | 100 | 333± 2.8 | 83 | 150± 9.1 | 38 |
| Exp. 2 | | | | | | |
| Control | 400± 8.2 | 100 | 400± 2.4 | 100 | 283± 5.3 | 71 |
| Compds **1**—**4** | 368±11.4[b] | 100 | 247± 3.9[c] | 67 | 196± 4.8 | 53 |
| Exp. 3 | | | | | | |
| Control | 389± 7.4 | 100 | 358± 2.0 | 92 | 395± 2.9 | 102 |
| Compds **1**, **2** | 370±23.0 | 100 | 389±12.9 | 105 | 390± 1.3 | 105 |
| Compd **1** | 388±11.0 | 100 | 393± 4.0[b] | 101 | 369±18.2 | 95 |
| Compd **2** | 340±24.5[b] | 100 | 377±13.3 | 111 | 391± 5.8 | 115 |
| Exp. 4 | | | | | | |
| Control | 400±10.8 | 100 | 363±15.9 | 91 | 293± 4.6 | 73 |
| Compds **3**, **4** | 400±12.4 | 100 | 350± 7.6 | 88 | 289± 2.5 | 72 |
| Compd **3** | 390± 5.4 | 100 | 372± 2.5 | 95 | 295±10.0 | 76 |
| Compd **4** | 400± 5.0 | 100 | 364±14.2 | 91 | 287±13.3 | 71 |

*a*) Doses: 80 mg/kg (orally), $n=5$ (m, mean; S.E., standard error). *b*) $0.1>p>0.05$. *c*) $p<0.05$.

compounds has not been achieved as yet. Properties and structures of these compounds will be reported in the near future.

TABLE VII. Effect of Polysaccharide Fraction and Saponin Mixture on Hexokinase Activity in Mouse Liver

| Sample[a] | Activity (IU/mg) | % |
|---|---|---|
| Control | $5.27\times10^{-5}\pm3.8$ | 100 |
| Polysaccharide fraction | $6.23\times10^{-5}\pm2.5$ | 118 |
| Saponin mixture[b] | $5.52\times10^{-5}\pm5.1$ | 105 |

*a*) Doses: 80 mg/kg (orally), $n=4$. The activity was measured 6 h after administration. *b*) Mixture of compounds **1**—**4** (1 : 1 : 1 : 1).

The biological test revealed that no antidiabetic activity was observed for individual saponins **1**—**4**, while it was disclosed that recombined mixture of **1**—**4** (1 : 1 : 1 : 1) and a recombined mixture of **1**—**8** (1 : 1 : 1 : 1 : 0.5 : 0.5 : 0.5 : 0.5) exhibited significant activity, as shown in Table VI. A mixture of **1**+**2** and of **3**+**4** showed no activity.

With regard to the mechanism of the antidiabetic action of the saponin mixture and polysaccharide fraction, the following tests were conducted. Liver hexokinase is an important enzyme in the metabolism of carbohydrates. It was found that the saponin mixture (compounds **1**—**4**) and polysaccharide fraction did not exhibit a significant effect on its activity (Table VII). No effect on glucose transport through membranes of Ehrlich ascites tumor cells was also observed for the saponin mixture and the polysaccharide fraction (Table VIII). As shown in Table II, the antidiabetic

TABLE VIII. Effect of Polysaccharide Fraction and Saponin Mixture on Glucose Transport through Membranes of Ehrlich Ascites Tumor Cells (EATC)

| Sample[a)] | Activity (pmol/$10^6$ cells) | % |
|---|---|---|
| Exp. 1 | | |
| Control | 310±13.0 | 100 |
| P. (oral) | 140± 7.7 | 45 |
| P. (i.p.) | 330±17.5 | 106 |
| Exp. 2 | | |
| Control | 6000± 5.9 | 100 |
| P. (*vitro*) | 2800± 4.6 | 47 |
| Exp. 3 | | |
| Control | 106±16.0 | 100 |
| S. (oral) | 99±22.8 | 93 |
| Exp.4 | | |
| Control | 5800± 9.1 | 100 |
| S. (i.p.) | 3800±10.9 | 66 |
| Exp. 5 | | |
| Control | 252±10.4 | 100 |
| S. (*vitro*) | 190± 5.2 | 75 |

P., polysaccharide fraction; S., saponin mixture (compds **1**—**4**, 1 : 1 : 1 : 1). Doses: 80 mg/kg (orally and i.p.), 0.4 mg/20 $\mu$l cell suspension (*vitro*) and $n=4$.

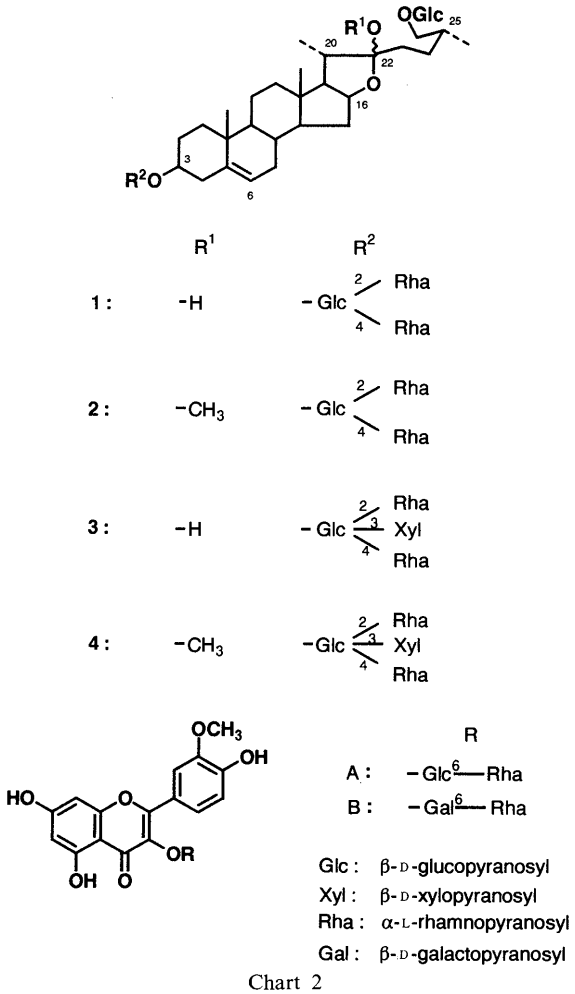


Chart 2

activity of F-2 is stronger than the saponin mixtures (*vide supra*). This suggests the presence of additional compounds which promote the activity in this active fraction.

The ethanolic extract of the epicarps was subjected to column chromatography on a Diaion HP-20 followed by chromatography on RP-8 and subsequent reverse phase HPLC, affording two flavonol glycosides A and B (Chart 2). Acid hydrolysis of A produced D-glucose and L-rhamnose in addition to isorhamnetin. Inspection of the $^{13}$C- and $^{1}$H-NMR spectra, as well as the FAB-MS, led to the structure of A as isorhamnetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (isorhamnetin-3-*O*-rutinoside), which has already been isolated from *Cucurbita pepo* L. by Itokawa *et al.*,[8] and the identification was confirmed by comparison of the $^{13}$C-NMR spectrum with that reported in literature.[9]

Acid hydrolysis of B yielded D-galactose, L-rhamnose and isorhamnetin. The inspection of the $^{13}$C- and $^{1}$H-NMR spectra, as well as the FAB-MS, revealed that B is isorhamnetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (isorhamnetin-3-*O*-robinobioside) previously isolated from *Gomphena martiana* by Pomilio *et al.*[10]

## Experimental

Optical rotations were taken on a Union PM-1 digital polarimeter. NMR spectra were recorded on a JEOL JNM GX 400 spectrometer (400 MHz for proton and 100 MHz for carbon-13) using tetramethylsilane (TMS) as an internal standard. FAB-MS were taken on a JEOL JMS-SX 102 spectrometer. For steroidal glycosides, preparative HPLC was carried out on a column of TSK-gel ODS-120T (21.5 mm i.d. × 30 cm) (detection: refraction index (RI) by a Tosoh RI-8 differential refractometer). For flavonol glycosides, preparative HPLC was carried out on a column of Amide-80 (21.5 mm i.d. × 30 cm) (detection: ultraviolet (UV) 254 nm by a Tosoh UV-8 detector). Flow rate of the mobile phase in both the cases: 6 ml/min.

Thin layer chromatography (TLC) was carried out on a precoated silica gel plate (Kieselgel 60 $F_{254}$, Merck). For column chromatography, Silica gel G (Merck), LiChroprep RP-8 (40—63 $\mu$m, Merck) and Diaion HP-20 (Mitsubishi Chem. Ind. Co., Ltd.) were used. Solvent systems: (a) $CHCl_3$–MeOH–$H_2O$ (65 : 35 : 7, homogeneous); (b) $CHCl_3$–MeOH–$H_2O$ (6 : 4 : 1, homogeneous); (c) 0.05% trifluoroacetic acid (TFA) in 67% MeOH; (d) 0.05% TFA in 70% MeOH; (e) 50% MeOH; (f) 90% $CH_3CN$ and (g) $CHCl_3$–MeOH–$H_2O$ (65 : 35 : 5, homogeneous). Spray reagents, 10% $H_2SO_4$ unless otherwise stated.

**Extraction and Isolation** The fruits (3 kg) of *B. aegyptiaca* were collected from the western desert of Egypt. The epicarps were removed and the mesocarps (2.9 kg) were extracted with hot water. After removal of water by evaporation, the extract (91 g) was dissolved in $H_2O$ (200 ml) and to the aqueous solution was added EtOH (1.5 l) to yield precipitates and supernatant. The precipitates were dissolved in $H_2O$ and dialyzed against running water through seamless cellulose tubing for 24 h. The non-dialysate, a brown powder after lyophilization (tentatively named polysaccharide fraction) was tested for antidiabetic activity. The supernatant was chromatographed on a column of Diaion HP-20 with $H_2O$, 40% MeOH, 80% MeOH, MeOH and $Me_2CO$. The 80% MeOH eluate (19.5 g) was chromatographed on a column of silica gel with solvent (a) to give four fractions. Fraction 2 was subjected to chromatography on LiChroprep RP-8 with solvents (c) and (d), and finally purified by preparative HPLC with solvent (c) to produce eight compounds **1**—**8**. Compound **1** (109 mg): A white powder from MeOH, $[\alpha]_D^{19}$ +60° ($c=0.22$, MeOH). TLC *Rf* 0.22 (solvent a). HPLC $t_R$ 36 min (solvent c). Acid hydrolysis yielded L-rhamnose, D-glucose and diosgenin. FAB-MS *m/z*: 1047 $[M-H]^-$, 885 $[M-H-Glc]^-$, 739 $[M-H-Glc-Rha]^-$, 593 $[M-H-Glc-2Rha]^-$, 415 $[M-H-Glc-2Rha-(O-Glc)]^-$. $^1$H-NMR ($C_5D_5N$): $\delta$ 0.87 (3H, s, H-18), 1.02 (3H, s, H-19), 0.96 (3H, d, $J=6.8$ Hz, H-27), 1.33 (3H, d, $J=6.8$ Hz, H-21), 1.75 (6H each d, $J=6.1$ Hz, H-6 and -6′ of Rha), 4.79 (1H, d, $J=7.7$ Hz, H-1 of Glc), 5.12 (1H, d, $J=7.7$ Hz, H-1′ of Glc), 6.21 (2H each, br s, H-1 and -1′ of Rha). Compound **2** (85 mg): A white powder from MeOH, $[\alpha]_D^{26}$ −70° ($c=0.35$, MeOH). TLC *Rf* 0.34 (solvent a). HPLC $t_R$ 45.5 min (solvent c). Acid hydrolysis produced L-rhamnose, D-glucose and diosgenin. $^1$H-NMR ($C_5D_5N$): $\delta$ 0.79 (3H, s, H-18), 1.01 (3H, s, H-19), 0.91 (3H, d, $J=7.5$ Hz, H-27), 1.14 (3H, d, $J=6.8$ Hz, H-21), 1.69 (6H each, d, $J=6.0$ Hz, H-6 and -6′ of Rha), 6.19 (2H each, br s, H-1 and -1′ of Rha), 3.22 (3H, s, $OCH_3$). Compound **3** (22 mg): A white powder from MeOH, $[\alpha]_D^{19}$ +25.2° ($c=0.15$, MeOH). TLC *Rf* 0.20 (solvent a). HPLC $t_R$ 33.5 min (solvent c). FAB-MS $[M-H]^-$ Calcd for $C_{56}H_{92}O_{26}-H$: 1179.1779. Found:

1179.1794. Acid hydrolysis produced D-xylose, L-rhamnose, D-glucose and diosgenin. **3** (15 mg) was acetylated with acetic anhydride and pyridine for 12 h at room temp. The solvent was removed and the acetylated sample (**9**) was subjected to 2D NMR analysis. Compound **4** (26 mg): A white powder from MeOH, $[\alpha]_D^{19}$ −14.6° ($c$=0.1, MeOH). TLC $Rf$ 0.3 (solvent a). HPLC $t_R$ 40.5 min (solvent c). Ehrlich reagent test: positive. Acid hydrolysis produced D-xylose, L-rhamnose and D-glucose as well as diosgenin. Compound **5** (11 mg): A white powder from MeOH, $[\alpha]_D^{20}$ −43° ($c$=0.3, MeOH). TLC $Rf$ 0.21 (solvent a). HPLC $t_R$ 64.0 min (solvent c). Ehrlich reagent test: negative. Acid hydrolysis produced L-rhamnose and D-glucose. FAB-MS $m/z$: 1047 $[M-H]^-$, 885 $[M-H-Glc]^-$. Compound **6** (10 mg): A white powder from MeOH. $[\alpha]_D^{20}$ −123° ($c$=0.07, MeOH). TLC $Rf$ 0.17 (solvent a). HPLC $t_R$ 61.5 min (solvent c). Ehrlich reagent test: negative. Acid hydrolysis produced L-rhamnose, D-glucose and D-xylose. FAB-MS $m/z$: 1179 $[M-H]^-$, 1047 $[M-H-Xyl]^-$, 885 $[M-H-Xyl-Glc]^-$ and 739 $[M-H-Xyl-Glc-Rha]^-$. Compound **7** (9 mg): A white powder from MeOH, $[\alpha]_D^{20}$ −49° ($c$=0.2, MeOH). TLC $Rf$ 0.33 (solvent a). HPLC $t_R$ 71 min (solvent c). Ehrlich reagent test: negative. Acid hydrolysis produced L-rhamnose and D-glucose. FAB-MS $m/z$: 1047 $[M-H]^-$, 885 $[M-H-Glc]^-$. Compound **8** (10 mg): A white powder from MeOH, $[\alpha]_D^{20}$ −93° ($c$=0.13, MeOH). TLC $Rf$ 0.28 (solvent a). HPLC $t_R$ 67 min (solvent c). Ehrlich reagent test: negative. Acid hydrolysis produced D-xylose, L-rhamnose and D-glucose. FAB-MS $m/z$: 1179 $[M-H]^-$, 1047 $[M-H-Xyl]^-$, 885 $[M-H-Xyl-Glc]^-$.

**Methylation Analysis of Compound 1** To a solution of compound **1** (5 mg) in dimethyl sulfoxide (DMSO 100 $\mu$l) was added a solution of NaH in DMSO (100 $\mu$l), and the solution was sonicated for 1 h. To this solution, $CH_3I$ (150 $\mu$l) was added. After sonication for another 1 h, the reaction mixture was diluted with water and extracted with $CHCl_3$. After evaporation of $CHCl_3$, 90% HCOOH (2 ml) was added to the residue and the solution was kept at 100 °C for 1 h. HCOOH was evaporated and the residue was treated with 2 M TFA (1 ml) at 100 °C for 2 h. TFA was evaporated and the residue was dissolved in water (2 ml) and then treated with $NaBH_4$ (25 mg). After standing at room temp. for 2 h, the mixture was acidified by Dowex 50W-X1 ($H^+$ form) and concentrated to dryness. The residue was acetylated with $Ac_2O$–$C_5H_5N$ (1 : 1, 1 ml) for 12 h at room temp. The reagent was removed by evaporation and the resulting mixture of methylated alditol acetates was subjected to gas chromatography–mass spectrum (GC-MS) analysis.[11] GC-MS was taken on a JEOL JMS-SX 102 spectrometer; on a Neutral Bond-1 column (0.25 mm i.d. × 25 m, Gasukuro Kogyo Inc.), injection temp. 200 °C, column temp. 150—250 °C (2 °C/min) and carrier gas He at 1 ml/min.

**Isolation of Flavonol Glycosides** The epicarps (100 g) were extracted with hot EtOH. After evaporation of the solvent, the extract (10 g) was chromatographed on a column of Diaion HP-20 with $H_2O$, MeOH and $Me_2CO$. The MeOH eluate was subjected to chromatography on RP-8 with a solvent (e) to give three fractions. Fraction 1 was subjected to HPLC with solvent (f) to produce two compounds (A and B). Compound A (22 mg): A yellow powder, $[\alpha]_D^{20}$ +54° ($c$=0.1, MeOH). TLC $Rf$ 0.36 (solvent g). HPLC $t_R$ 95 min (solvent f). Mg–HCl test: positive. Acid hydrolysis yielded L-rhamnose and D-glucose in addition to isorhamnetin. FAB-MS $m/z$: 625 $[M+H]^+$, 479 $[M+H-Rha]^+$, 317 $[M+H-Rha-Glc]^+$. Compound B (15 mg): A yellow powder, $[\alpha]_D^{20}$ +67° ($c$=0.07, MeOH). TLC $Rf$ 0.39 (solvent g). HPLC $t_R$ 81 min (solvent f). Mg–HCl test: positive. Acid hydrolysis gave L-rhamnose, D-galactose and isorhamnetin. FAB-MS $m/z$: 625 $[M+H]^+$, 479 $[M+H-Rha]^+$, 317 $[M+H-Rha-Gal]^+$.

**Acid Hydrolysis of Compounds 1—8, A and B** A sample (5 mg) was heated with 2 N HCl in $H_2O$–dioxane (1 : 1) in a sealed micro-tube at 80 °C for 3 h. The reaction mixture was diluted with $H_2O$ and extracted with $CHCl_3$. The aqueous layer was subjected to TLC on silica gel with the solvent (b) (detection: triphenyltetrazolium chloride reagent). $Rf$ values were 0.17, 0.32 and 0.25 for D-glucose, L-rhamnose and D-xylose, respectively. The aqueous layer was concentrated, trimethylsilylated and subjected to gas liquid chromatography (GLC) analysis with a Shimadzu GC-8A apparatus equipped with a dual flame ionization detector; carrier gas, He 3.1 ml/min, Neutra Bond-1 column, column temp. 150 °C and injection temperature 220 °C. $t_R$ of sugars were 6.1 min for L-rhamnose, 8.3 min for D-xylose and 22.1, 38.1 min for D-glucose. The identification of the aglycones (diosgenin and isorhamnetin) isolated from the $CHCl_3$ layer was based on $^{13}$C-NMR analysis and comparison with reported data.[4,9]

**Measurement of Antidiabetic Activity**[12] For measurement of antidiabetic activity, male mice (Std.: dd Y strain, 20—25 g) were used in groups of five. Experimental diabetes was induced by i.p. injection of streptozotocin (200 mg/kg body weight) and blood samples were taken from the orbital sinus with micro-hematocrit tubes periodically. The glucose was determined using Dextrostix strips and a glucometer (Sankyo Co.).

**Measurements of Hexokinase Activity and 2-Deoxyglucose Uptake** The hexokinase activity of mouse liver cytosol was measured spectrometrically on the basis of an increase in absorbance at 340 nm due to the formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) from $NADP^+$ in accordance with the formation of glucose-6-phosphate. The formation of 1 $\mu$mol of glucose-6-phosphate per minute was designated as 1 unit (U).

2-Deoxyglucose uptake by Ehrlich ascites tumor cells was measured by adding 20 $\mu$l of cell suspension (about $10^6$ cells) to 180 $\mu$l of Krebs Ringer phosphate buffer containing 0.25 mM [$^3$H] 2-deoxyglucose, which was incubated for 2.5 minutes at 37 °C. After incubations, samples were filtered through Whatman glass filters (GF/B).

**Acknowledgement** We are grateful to the Ministry of Education of Egyptian Government for financial support for one of the authors, M. S. Kamel, during study at Hiroshima University.

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# The Crystal and Molecular Structures of Hancokinol and Hancolupenone from *Cynanchum hancokianum* (MAXIM.) AL. ILJINSKI. (Asclepiadaceae)

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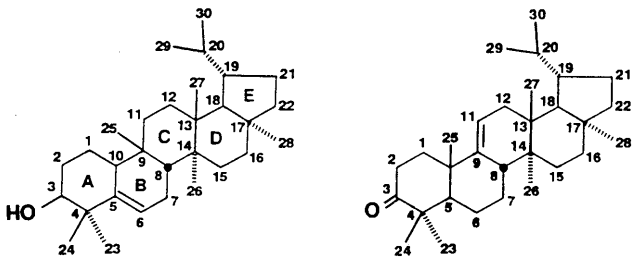
**The crystal and molecular structures of hancokinol and hancolupenone from *Cynanchum hancokianum* have been determined by X-ray analysis. In the case of hancokinol, among four molecules related on a 4-fold rotation axis, hydrogen bondings between the hydroxyl groups of neighboring molecules are observed.**

**Keywords** pentacyclic triterpene; *Cynanchum hancokianum*; hancokinol; hancolupenone; X-ray analysis; stereochemistry

*Cynanchum hancokianum* (MAXIM.) AL. ILJINSKI. (Asclepiadaceae), distributed in Inner Mongolia, is known as a Chinese folk medicine possessing antitumor activity. In the previous papers,[1] we have briefly reported the isolation and absolute stereochemistry of triterpenes, hancokinol, hancolupenone, and hancolupenol from this plant. In this paper, we describe the detailed crystal and molecular structures of hancokinol (**1**) and hancolupenone (**2**) obtained by X-ray analysis.



1 2

Chart 1

TABLE I. Crystal Data, Experimental Conditions and Refinement Details

| | Hancokinol (**1**) | Hancolupenone (**2**) |
|---|---|---|
| Chemical formula | $C_{30}H_{50}O$ | $C_{30}H_{48}O$ |
| Formula weight | 426.725 | 424.709 |
| Cell parameters | | |
| $a$ (Å) | 26.272 (4) | 12.280 (2) |
| $b$ (Å) | 26.272 (4) | 14.296 (2) |
| $c$ (Å) | 7.489 (8) | 8.314 (2) |
| $\alpha=(°)$ | | 101.20 (1) |
| $\beta=(°)$ | | 100.09 (2) |
| $\gamma=(°)$ | | 113.07 (1) |
| $V$ (Å$^3$) | 5169.2 | 1265.2 |
| Space group | $I4$ | $P1$ |
| $Z$ | 8 | 2 |
| $\mu$ (Cu$K_\alpha$)/cm$^{-1}$ | 0.589 | 0.602 |
| $D_x$/g cm$^{-3}$ | 1.096 | 1.115 |
| Size mm | $0.2\times0.2\times0.1$ | $0.2\times0.3\times0.1$ |
| Diffractometer | Rigaku AFC-4 | Rigaku AFC-4 |
| Radiation | Graphite-monochromated Cu$K_\alpha$ | |
| $\theta$ range | $2\theta_{max}=140°$ | |
| Scan mode | $\omega-2\theta$ | $\omega-2\theta$ |
| No. of unique reflections | 1285 | 3612 |
| No. of observed reflections | 1142 | 3509 |
| Criterion for observed reflections | $F_O>3\sigma(F_O)$ | $F_O>3\sigma(F_O)$ |
| Atomic scattering factors | International Tables for X-Ray Crystallography (1974) | |
| $R$ | 0.074 | 0.078 |

## Experimental

Crystals were obtained by slow evaporation from ethanol for **1** and from methanol for **2**. No absorption correction was applied for the collected reflections. The crystal data, experimental conditions and refinement procedures are summarized in Table I. The structures were solved by direct methods using the program MULTAN 78.[2] Each structure was refined by a block-diagonal least-squares method with anisotropic temperature factors for non-hydrogen atoms. All hydrogen atoms, except those attached to methyl groups, were located from the different Fourier maps. In the case of **2**, two crystallographically independent molecules existing in an asymmetric unit were represented by

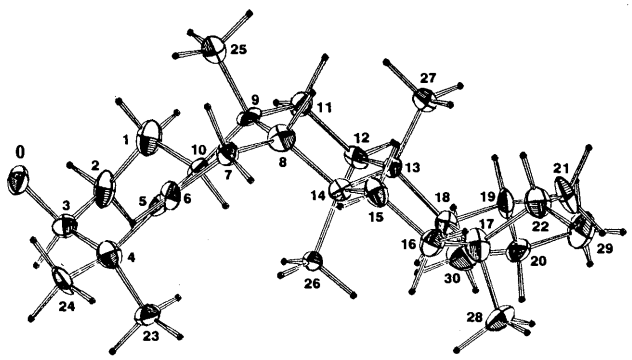
TABLE II. Fractional Coordinates ($\times10^4$) and Equivalent Isotropic Thermal Parameters ($B_{eq}$/Å$^2$) of Non-hydrogen Atoms, with Estimated Standard Deviations in Parentheses for Hancokinol (**1**)

| Atom | $x$ | $y$ | $z$ | $B_{eq}$ |
|---|---|---|---|---|
| O1 | 10024 (3) | 9261 (3) | 7830 (15) | 4.3 |
| C1 | 9638 (5) | 8330 (5) | 9594 (23) | 5.1 |
| C2 | 10183 (5) | 8442 (5) | 9076 (22) | 4.6 |
| C3 | 10211 (4) | 8765 (4) | 7376 (19) | 3.5 |
| C4 | 9893 (5) | 8534 (5) | 5797 (20) | 3.9 |
| C5 | 9363 (4) | 8366 (4) | 6398 (16) | 2.5 |
| C6 | 8960 (5) | 8471 (4) | 5473 (17) | 3.2 |
| C7 | 8427 (4) | 8249 (4) | 5841 (19) | 3.2 |
| C8 | 8433 (5) | 7787 (5) | 7061 (20) | 3.9 |
| C9 | 8803 (5) | 7880 (4) | 8699 (19) | 3.5 |
| C10 | 9359 (4) | 8046 (4) | 8123 (18) | 3.0 |
| C11 | 8846 (5) | 7420 (5) | 9910 (19) | 3.5 |
| C12 | 8919 (4) | 6911 (4) | 8917 (19) | 3.1 |
| C13 | 8484 (4) | 6814 (4) | 7524 (18) | 2.2 |
| C14 | 8496 (4) | 7250 (4) | 6133 (18) | 2.4 |
| C15 | 8026 (4) | 7172 (5) | 4863 (21) | 3.8 |
| C16 | 8058 (5) | 6669 (5) | 3818 (19) | 3.9 |
| C17 | 8242 (4) | 6194 (5) | 4884 (23) | 4.4 |
| C18 | 8596 (4) | 6293 (4) | 6519 (19) | 2.6 |
| C19 | 8536 (5) | 5812 (4) | 7721 (23) | 4.3 |
| C20 | 9013 (5) | 5467 (4) | 7789 (21) | 4.1 |
| C21 | 8045 (5) | 5529 (6) | 7061 (24) | 5.9 |
| C22 | 7802 (5) | 5894 (5) | 5634 (24) | 5.1 |
| C23 | 10192 (5) | 8057 (5) | 5122 (22) | 4.4 |
| C24 | 9886 (5) | 8930 (5) | 4306 (19) | 4.5 |
| C25 | 8554 (5) | 8313 (5) | 9832 (22) | 4.6 |
| C26 | 8982 (4) | 7244 (4) | 4897 (18) | 2.4 |
| C27 | 7976 (4) | 6791 (4) | 8578 (20) | 3.9 |
| C28 | 8532 (5) | 5849 (5) | 3496 (21) | 4.5 |
| C29 | 8896 (7) | 4985 (5) | 8871 (28) | 7.5 |
| C30 | 9482 (5) | 5715 (5) | 8549 (25) | 5.3 |

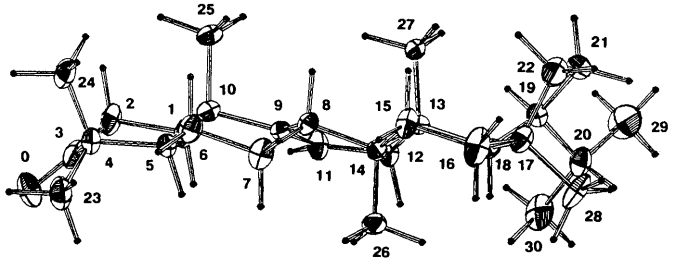
TABLE III. Fractional Coordinates ($\times 10^4$) and Equivalent Isotropic Thermal Parameters ($B_{eq}$/Å$^2$) of Non-hydrogen Atoms, with Estimated Standard Deviations in Parentheses for Hancolupenone (**2A** and **2B**)

| Atom | $x$ | $y$ | $z$ | $B_{eq}$ |
|---|---|---|---|---|
| O1A | 6392 (10) | 10668 ( 7) | −394 (12) | 7.4 |
| C1A | 5123 ( 9) | 7947 ( 7) | −905 (11) | 3.3 |
| C2A | 6245 ( 9) | 8925 ( 8) | −1019 (12) | 4.0 |
| C3A | 6364 ( 9) | 9935 ( 8) | 173 (13) | 3.9 |
| C4A | 6434 ( 9) | 9970 ( 7) | 2001 (13) | 3.5 |
| C5A | 5404 ( 8) | 8914 ( 7) | 2101 (11) | 3.0 |
| C6A | 5448 (10) | 8841 ( 8) | 3918 (12) | 4.3 |
| C7A | 4187 (10) | 7987 ( 7) | 3940 (12) | 4.0 |
| C8A | 3882 ( 8) | 6899 ( 6) | 2735 (10) | 2.5 |
| C9A | 4054 ( 8) | 6926 ( 6) | 1010 (10) | 2.5 |
| C10A | 5218 ( 8) | 7850 ( 7) | 901 (12) | 3.2 |
| C11A | 3292 ( 8) | 6151 ( 7) | −380 ( 9) | 2.8 |
| C12A | 2227 ( 8) | 5152 ( 7) | −410 (10) | 2.6 |
| C13A | 2290 ( 8) | 4943 ( 6) | 1341 (10) | 2.3 |
| C14A | 2592 ( 8) | 6024 ( 7) | 2699 (10) | 2.6 |
| C15A | 2639 (10) | 5862 ( 8) | 4473 (12) | 3.9 |
| C16A | 1359 (10) | 5066 ( 8) | 4483 (11) | 3.8 |
| C17A | 769 ( 8) | 3974 ( 7) | 3035 (10) | 2.7 |
| C18A | 1001 ( 8) | 4054 ( 6) | 1289 (10) | 2.3 |
| C19A | 788 ( 8) | 2908 ( 7) | 343 (11) | 2.8 |
| C20A | −464 ( 9) | 2269 ( 7) | −1088 (13) | 3.8 |
| C21A | 940 (11) | 2378 ( 8) | 1784 (14) | 4.3 |
| C22A | 1294 (11) | 3218 ( 8) | 3509 (13) | 4.2 |
| C23A | 6210 (10) | 10920 ( 8) | 2880 (15) | 4.8 |
| C24A | 7768 ( 9) | 10190 ( 8) | 2923 (13) | 4.1 |
| C25A | 6298 ( 9) | 7537 ( 8) | 1473 (15) | 4.2 |
| C26A | 1611 ( 9) | 6417 ( 7) | 2130 (12) | 3.3 |
| C27A | 3369 ( 9) | 4641 ( 7) | 1758 (12) | 3.2 |
| C28A | −605 (10) | 3489 ( 8) | 2898 (14) | 4.0 |
| C29A | −480 (10) | 2738 ( 8) | −2563 (13) | 4.2 |
| C30A | −697 (12) | 1102 ( 8) | −1790 (17) | 5.9 |
| O1B | −7284 ( 7) | 1647 ( 7) | −665 (12) | 6.1 |
| C1B | −4239 (10) | 3486 ( 7) | 52 (12) | 3.7 |
| C2B | −5112 (10) | 2375 ( 7) | 165 (12) | 4.0 |
| C3B | −6348 (10) | 1941 ( 7) | −1105 (14) | 4.2 |
| C4B | −6385 ( 9) | 1844 ( 7) | −2956 (13) | 3.7 |
| C5B | −5378 ( 8) | 2924 ( 7) | −3063 (13) | 3.4 |
| C6B | −5279 ( 9) | 2891 ( 7) | −4862 (12) | 3.8 |
| C7B | −4596 ( 9) | 4038 ( 8) | −4952 (11) | 3.7 |
| C8B | −3280 ( 8) | 4636 ( 7) | −3662 (11) | 2.6 |
| C9B | −3321 ( 8) | 4536 ( 6) | −1876 (11) | 2.5 |
| C10B | −4091 ( 8) | 3409 ( 7) | −1757 (10) | 2.6 |
| C11B | −2633 ( 9) | 5360 ( 7) | −498 (11) | 3.4 |
| C12B | −1741 ( 8) | 6412 ( 7) | −533 (10) | 2.7 |
| C13B | −1365 ( 7) | 6396 ( 6) | −2243 ( 9) | 2.1 |
| C14B | −2608 ( 8) | 5799 ( 7) | −3740 (11) | 2.7 |
| C15B | −2286 (10) | 5796 ( 7) | −5439 (11) | 3.8 |
| C16B | −1586 (12) | 6941 ( 8) | −5583 (12) | 5.1 |
| C17B | −493 (10) | 7713 ( 7) | −4006 (11) | 3.4 |
| C18B | −634 ( 8) | 7570 ( 6) | −2252 (10) | 2.4 |
| C19B | 696 ( 8) | 8127 ( 7) | −1055 (12) | 3.2 |
| C20B | 1082 (10) | 9250 ( 7) | 217 (13) | 4.0 |
| C21B | 1551 (10) | 8180 ( 9) | −2253 (16) | 5.1 |
| C22B | 726 (12) | 7642 ( 9) | −4058 (16) | 5.5 |
| C23B | −7654 (10) | 1678 ( 9) | −3968 (16) | 5.3 |
| C24B | −6161 (11) | 861 ( 8) | −3673 (16) | 5.0 |
| C25B | −3279 ( 9) | 2807 ( 8) | −2057 (14) | 3.8 |
| C26B | −3437 ( 8) | 6353 ( 7) | −3526 (13) | 3.5 |
| C27B | −585 ( 8) | 5754 ( 7) | −2332 (13) | 3.3 |
| C28B | −337 (12) | 8851 ( 7) | −4111 (14) | 4.9 |
| C29B | 196 (11) | 9218 ( 9) | 1327 (14) | 5.6 |
| C30B | 2432 (12) | 9714 (11) | 1324 (20) | 7.2 |

the suffix letters A and B. The atomic coordinates and equivalent isotropic temperature factors of non-hydrogen atoms of **1** and **2** are listed in Tables II and III, respectively. Computations were performed on Hitachi M-680H and M-682H in the Computer Center of the University of Tokyo, using a local version of the UNICS program.[3)]



hancokinol (**1**)



hancolupenone (**2**)

Fig. 1. Perspective Views of the Structures of Hancokinol (**1**) and Hancolupenone (**2A**) with the Atom Numbering Scheme

TABLE IV. Selected Dihedral Angles ($\phi$/°) of Hancokinol (**1**) and Hancolupenone (**2A** and **2B**)

| Compound | **1** | **2A** | **2B** |
|---|---|---|---|
| Ring A | | | |
| C10–C1–C2–C3 | 59 (1) | −57 (1) | −58 (1) |
| C1–C2–C3–C4 | −52 (1) | 54 (1) | 56 (1) |
| C2–C3–C4–C5 | 46 (1) | −47 (1) | −47 (1) |
| C3–C4–C5–C10 | −48 (1) | 45 (1) | 45 (1) |
| C4–C5–C10–C1 | 54 (1) | −49 (1) | −51 (1) |
| C2–C1–C10–C5 | −59 (1) | 55 (1) | 56 (1) |
| Ring B | | | |
| C10–C5–C6–C7 | −6 (2) | −64 (1) | −63 (1) |
| C5–C6–C7–C8 | −16 (2) | 58 (1) | 59 (1) |
| C6–C7–C8–C9 | 44 (1) | −47 (1) | −50 (1) |
| C7–C8–C9–C10 | −53 (1) | 42 (1) | 47 (1) |
| C8–C9–C10–C5 | 33 (1) | −45 (1) | −50 (1) |
| C6–C5–C10–C9 | −3 (2) | 55 (1) | 57 (1) |
| Ring C | | | |
| C14–C8–C9–C11 | −43 (1) | −13 (1) | −12 (1) |
| C9–C8–C14–C13 | 50 (1) | 45 (1) | 45 (1) |
| C12–C13–C14–C8 | −58 (1) | −61 (1) | −62 (1) |
| C11–C12–C13–C14 | 61 (1) | 47 (1) | 48 (1) |
| C9–C11–C12–C13 | −56 (1) | −16 (1) | −15 (1) |
| C8–C9–C11–C12 | 45 (1) | −3 (1) | −4 (1) |
| Ring D | | | |
| C13–C14–C15–C16 | −63 (1) | −64 (1) | −61 (1) |
| C14–C15–C16–C17 | 42 (1) | 53 (1) | 48 (1) |
| C15–C16–C17–C18 | −27 (2t) | −38 (1) | −34 (1) |
| C16–C17–C18–C13 | 32 (1) | 34 (1) | 35 (1) |
| C14–C13–C18–C17 | −53 (1) | −45 (1) | −48 (1) |
| C18–C13–C14–C15 | 68 (1) | 60 (1) | 60 (1) |
| Ring E | | | |
| C18–C17–C22–C21 | −38 (1) | −38 (1) | −36 (1) |
| C19–C21–C22–C17 | 27 (1) | 25 (1) | 23 (1) |
| C18–C19–C21–C22 | −6 (1) | −3 (1) | −2 (1) |
| C17–C18–C19–C21 | −16 (1) | −21 (1) | −21 (1) |
| C22–C17–C18–C19 | 33 (1) | 37 (1) | 35 (1) |

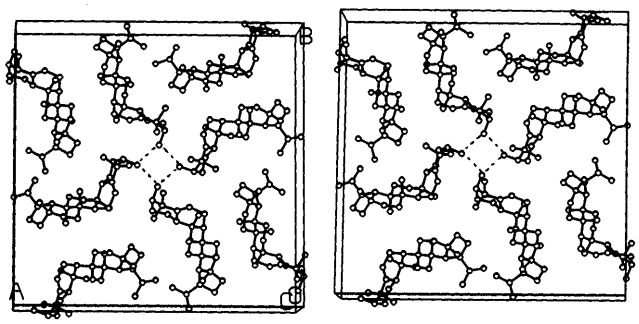


Fig. 2. Stereoscopic Drawing of the Crystal Structure for Hancokinol (**1**) along the **c** Axis

Dashed lines indicate the hydrogen bonds.

TABLE V. Bond Lengths (Å) of Hancokinol (**1**) and Hancolupenone (**2A** and **2B**)

| Compound | 1 | 2A | 2B |
|---|---|---|---|
| O–C3 | 1.43 (2) | 1.22 (2) | 1.21 (2) |
| C1–C2 | 1.51 (2) | 1.57 (1) | 1.56 (2) |
| C1–C10 | 1.52 (2) | 1.52 (1) | 1.53 (2) |
| C2–C3 | 1.53 (2) | 1.52 (2) | 1.50 (2) |
| C3–C4 | 1.57 (2) | 1.50 (2) | 1.51 (2) |
| C4–C5 | 1.53 (2) | 1.57 (1) | 1.59 (2) |
| C4–C23 | 1.56 (2) | 1.56 (2) | 1.54 (2) |
| C4–C24 | 1.53 (2) | 1.56 (2) | 1.56 (2) |
| C5–C6 | 1.29 (2) | 1.53 (1) | 1.52 (2) |
| C5–C10 | 1.54 (2) | 1.56 (1) | 1.55 (2) |
| C6–C7 | 1.54 (2) | 1.56 (1) | 1.55 (2) |
| C7–C8 | 1.52 (2) | 1.54 (1) | 1.57 (2) |
| C8–C9 | 1.59 (2) | 1.50 (1) | 1.53 (1) |
| C8–C14 | 1.58 (2) | 1.58 (1) | 1.56 (1) |
| C9–C10 | 1.58 (2) | 1.55 (1) | 1.55 (1) |
| C9–C11 | 1.52 (2) | 1.32 (1) | 1.33 (1) |
| C9–C25 | 1.56 (2) | | |
| C10–C25 | | 1.58 (2) | 1.57 (2) |
| C11–C12 | 1.54 (2) | 1.50 (1) | 1.50 (2) |
| C12–C13 | 1.57 (2) | 1.54 (1) | 1.55 (1) |
| C13–C14 | 1.55 (2) | 1.59 (1) | 1.58 (1) |
| C13–C18 | 1.59 (2) | 1.58 (1) | 1.56 (1) |
| C13–C27 | 1.55 (2) | 1.55 (2) | 1.57 (1) |
| C14–C15 | 1.57 (2) | 1.53 (1) | 1.53 (2) |
| C14–C26 | 1.58 (2) | 1.56 (2) | 1.53 (1) |
| C15–C16 | 1.54 (2) | 1.54 (1) | 1.56 (2) |
| C16–C17 | 1.56 (2) | 1.58 (1) | 1.55 (2) |
| C17–C18 | 1.56 (2) | 1.54 (1) | 1.54 (2) |
| C17–C22 | 1.51 (2) | 1.54 (2) | 1.55 (2) |
| C17–C28 | 1.57 (2) | 1.52 (1) | 1.58 (2) |
| C18–C19 | 1.56 (2) | 1.57 (1) | 1.55 (1) |
| C19–C20 | 1.55 (2) | 1.57 (1) | 1.58 (2) |
| C19–C21 | 1.57 (2) | 1.56 (2) | 1.56 (2) |
| C20–C29 | 1.53 (2) | 1.51 (2) | 1.54 (2) |
| C20–C30 | 1.51 (2) | 1.55 (2) | 1.55 (2) |
| C21–C22 | 1.57 (2) | 1.55 (1) | 1.51 (2) |

TABLE VI. Bond Angles (°) of Hancokinol (**1**) and Hancolupenone (**2A** and **2B**)

| Compound | 1 | 2A | 2B |
|---|---|---|---|
| C2–C1–C10 | 111.4 (12) | 113.1 ( 6) | 110.5 ( 9) |
| C1–C2–C3 | 111.4 (11) | 109.3 ( 9) | 110.5 (10) |
| O–C3–C2 | 106.9 (10) | 119.3 (10) | 121.0 (12) |
| O–C3–C4 | 110.4 ( 9) | 123.3 (10) | 121.2 (11) |
| C2–C3–C4 | 112.8 ( 9) | 117.4 (10) | 117.8 (10) |
| C3–C4–C5 | 111.9 (11) | 109.8 ( 6) | 108.3 ( 9) |
| C3–C4–C23 | 106.6 ( 9) | 109.2 (10) | 109.9 (10) |
| C3–C4–C24 | 107.1 ( 9) | 108.3 ( 9) | 107.9 (10) |
| C5–C4–C23 | 108.8 ( 9) | 108.4 ( 9) | 108.0 ( 9) |
| C5–C4–C24 | 113.6 (10) | 113.8 ( 9) | 113.2 ( 9) |
| C23–C4–C24 | 108.4 (11) | 107.3 ( 7) | 107.3 ( 9) |
| C4–C5–C6 | 121.7 (11) | 113.9 ( 6) | 112.8 ( 9) |
| C4–C5–C10 | 114.2 ( 9) | 117.4 ( 9) | 116.8 ( 9) |
| C6–C5–C10 | 124.1 (10) | 110.8 ( 9) | 111.6 ( 9) |
| C5–C6–C7 | 124.5 (10) | 109.7 ( 7) | 109.2 ( 9) |
| C6–C7–C8 | 113.6 ( 9) | 110.4 ( 9) | 111.6 ( 9) |
| C7–C8–C9 | 110.3 ( 9) | 114.4 ( 8) | 111.3 ( 8) |
| C8–C9–C10 | 113.5 (10) | 117.9 ( 6) | 116.5 ( 8) |
| C8–C9–C11 | 112.6 ( 9) | 121.5 ( 8) | 120.9 ( 9) |
| C8–C9–C25 | 106.0 ( 9) | | |
| C10–C9–C11 | 108.3 ( 9) | 120.5 ( 8) | 122.3 ( 9) |
| C10–C9–C25 | 109.6 ( 8) | | |
| C11–C9–C25 | 106.6 (11) | | |
| C1–C10–C5 | 109.7 ( 9) | 108.7 ( 8) | 109.5 ( 9) |
| C1–C10–C9 | 112.4 (11) | 112.3 ( 6) | 109.4 ( 8) |
| C1–C10–C25 | | 109.5 ( 9) | 108.6 ( 9) |
| C5–C10–C25 | | 112.9 ( 7) | 115.9 ( 9) |
| C9–C10–C25 | | 104.1 ( 8) | 104.5 ( 8) |
| C5–C10–C9 | 112.5 ( 9) | 109.3 ( 8) | 108.7 ( 8) |
| C9–C11–C12 | 114.4 (11) | 125.3 ( 8) | 124.4 (10) |
| C11–C12–C13 | 111.8 ( 9) | 113.4 ( 6) | 114.0 ( 8) |
| C12–C13–C14 | 108.2 ( 8) | 107.3 ( 7) | 107.4 ( 7) |
| C12–C13–C18 | 108.7 ( 8) | 109.4 ( 6) | 108.5 ( 7) |
| C12–C13–C27 | 107.1 (10) | 107.2 ( 8) | 106.4 ( 8) |
| C14–C13–C18 | 108.4 (10) | 109.8 ( 8) | 111.1 ( 7) |
| C14–C13–C27 | 112.8 ( 8) | 109.4 ( 6) | 110.3 ( 8) |
| C18–C13–C27 | 111.5 ( 8) | 113.6 ( 8) | 112.8 ( 8) |
| C8–C14–C13 | 111.3 (10) | 109.0 ( 8) | 108.3 ( 8) |
| C8–C14–C15 | 107.4 ( 8) | 109.4 ( 6) | 110.0 ( 8) |
| C8–C14–C26 | 110.7 ( 8) | 107.3 ( 8) | 109.3 ( 8) |
| C13–C14–C15 | 107.1 ( 8) | 109.9 ( 8) | 108.3 ( 8) |
| C13–C14–C26 | 113.8 ( 8) | 109.4 ( 6) | 110.2 ( 8) |
| C15–C14–C26 | 106.2 (10) | 111.8 ( 9) | 110.7 ( 9) |
| C14–C15–C16 | 112.1 ( 9) | 109.8 ( 7) | 112.6 (10) |
| C15–C16–C17 | 116.4 (11) | 115.0 ( 9) | 114.6 (11) |
| C16–C17–C18 | 116.9 (10) | 115.1 ( 7) | 116.8 (10) |
| C16–C17–C22 | 111.8 (10) | 111.4 ( 8) | 113.3 (11) |
| C16–C17–C28 | 105.8 (12) | 105.6 ( 9) | 103.7 (10) |
| C18–C17–C22 | 104.6 (12) | 104.3 ( 9) | 103.2 ( 9) |
| C18–C17–C28 | 109.1 ( 9) | 111.4 ( 7) | 111.2 (10) |
| C22–C17–C28 | 108.5 (10) | 109.0 ( 8) | 108.6 (10) |
| C13–C18–C17 | 114.0 ( 8) | 116.2 ( 6) | 115.3 ( 8) |
| C13–C18–C19 | 114.0 (11) | 112.7 ( 8) | 113.5 ( 8) |
| C17–C18–C19 | 104.9 ( 9) | 104.4 ( 7) | 105.0 ( 8) |
| C18–C19–C20 | 114.4 (10) | 113.2 ( 9) | 116.0 ( 8) |
| C18–C19–C21 | 106.5 (11) | 105.2 ( 7) | 105.6 ( 9) |
| C20–C19–C21 | 113.6 ( 9) | 114.5 ( 7) | 112.1 ( 9) |
| C19–C20–C29 | 109.7 (11) | 111.8 ( 8) | 112.4 (10) |
| C19–C20–C30 | 114.9 (10) | 110.3 (10) | 110.3 (10) |
| C29–C20–C30 | 108.8 (12) | 107.9 ( 9) | 111.6 (11) |
| C19–C21–C22 | 105.1 (11) | 107.5 ( 9) | 107.1 (11) |
| C17–C22–C21 | 105.1 (10) | 103.5 ( 9) | 105.2 (11) |

## Results and Discussion

ORTEP drawings with the atomic-numbering systems showing the overall molecular conformations of **1** and **2** are given in Figs. 1 and 2, respectively. The selected torsional angles are listed in Table IV. The bond distances and angles, none of which were unusual, are presented in Tables V and VI, respectively. It was found that **1** is a new pentacyclic triterpene with B/C/D/E *cis*-transoid-*trans*-transoid-*cis* and Me-9/H-10 *trans*. Rings A, C and D deviated slightly from a chair form. Ring B adopted an envelope-like form and ring E a puckered form. The 3-hydroxyl group in **1** was determined to be axial. Also, **2** was a new pentacyclic triterpene with A/B *trans*, C/D/E *trans*-transoid-*cis* and H-8/Me-10 *cis*. The values of the bond lengths, bond

angles and torsional angles of the two crystallographically independent molecules (**2A** and **2B**) were in good agreement, so the conformations of the two molecules were almost the same. Rings A, B and D adopted a distorted chair form. The ring C conformation was an envelope-like form which was less flattened than ring B in **1**. Ring E adopted the same puckered form as that in **1**.

There are no intermolecular contacts shorter than the sum of van der Waals radii in **2**. However, as shown in Fig. 3, an oxygen atom in each of four molecules related to the 4-fold rotation axis in **1** contained the bond lengths of 2.75 (1) and 3.88 (1) Å between oxygen atoms in neighboring and opposite molecules, respectively. Thus, intermolecular hydrogen bondings were observed between neighboring molecules.

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# Phenolic Constituents of Licorice. III.[1] Structures of Glicoricone and Licofuranone, and Inhibitory Effects of Licorice Constituents on Monoamine Oxidase

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**Two new phenolic compounds, glicoricone (3) and licofuranone (4), were isolated from a species of licorice brought from the northwestern region of China, and their structures were assigned.**

**Among the twelve licorice constituents examined for the inhibition of monoamine oxidase (MAO), six compounds, 3, 4, genistein (6), licopyranocoumarin (7), licocoumarone (14) and glycyrrhisoflavone (15), inhibited the enzyme with the $IC_{50}$ (concentration required for 50% inhibition of the enzyme activity) values of $6.0 \times 10^{-5}$—$1.4 \times 10^{-4}$ M. Glycyrrhizin (1) also inhibited MAO with the $IC_{50}$ value of $1.6 \times 10^{-4}$ M.**

**Keywords** glicoricone; licofuranone; genistein; licocoumarone; licopyranocoumarin; glycyrrhizin; *Glycyrrhiza*; licorice; monoamine oxidase; enzyme inhibitor

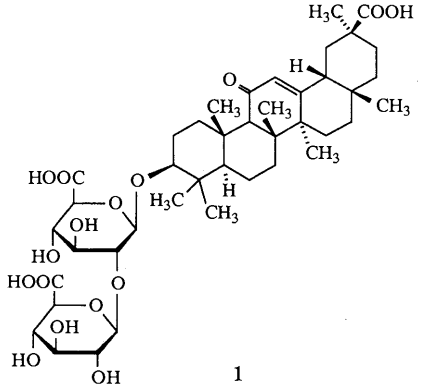
Although glycyrrhizin (**1**) is known to be the main constituent of licorice (root and rhizome of *Glycyrrhiza* spp.), a large number of phenolic constituents of root, rhizome and overground part of *Glycyrrhiza* spp., have been found recently.[1] In addition to the structures of these phenolic constituents, their pharmacological activities have also been investigated.[1-4] The inhibition of the lipoxygenase-dependent peroxidation in the arachidonate metabolism is one such activity of licorice phenolics.[4a] In a previous paper, we reported that several licorice phenolics showed inhibitory effects on xanthine oxidase (XOD) which catalyzes the oxidation of hypoxanthine and xanthine into uric acid.[1] This enzyme has been considered to participate in the oxidative damage of the living body through the generation of superoxide.[5] The participation of monoamine oxidase (MAO) in the oxidative stress in the nervous system has also been pointed out.[6] Synthetic MAO inhibitors, which have been used for depression, are accompanied by some adverse effects, and therefore attempts have been made to find natural MAO inhibitors[7-10]; among those tested has been isoliquiritigenin (**2**),[8] a licorice constituent. We report here the isolation and structure elucidation of two additional new phenolic constituents of licorice, and also the inhibitory effects of several licorice constituents on MAO.

## Results and Discussion

**Isolation of Two New Phenolics from Licorice** The licorice imported from the northwestern region of China (Seihokukanzo in the Japanese markets[11]) and frequently used for Japanese traditional Kampo medicine, was defatted with *n*-hexane and then extracted with EtOAc. The EtOAc extract was subjected to centrifugal partition chromatography (CPC)[12] and column chromatography to yield two new compounds, which we have named glicoricone (**3**) and licofuranone (**4**), together with echinatin (**5**),[13] genistein (**6**)[14] and licopyranocoumarin (**7**).[1]

**Structures of the Two New Compounds** Glicoricone (**3**) was obtained as colorless needles. The high-resolution electron-impact mass (EI-MS) spectrum of **3** indicated the molecular formula $C_{21}H_{20}O_6$. The ultraviolet (UV) spectrum of **3** [$\lambda_{max}$ nm ($\log\varepsilon$): 209 (4.55), 240 (sh, 4.27), 247 (4.24), 285 (4.02) and 305 (sh, 3.94)] is similar to the reported spectra of isoflavones, especially that of licoricone (**8**)[15] possessing a B-ring with the phloroglucinol-type substitution pattern. The proton nuclear magnetic resonance ($^1$H-NMR) spectrum of **3** showed a 1H singlet in the low field ($\delta$ 8.10), which is characteristic of H-2 of the isoflavone structure. The spectrum also showed the signals of a tri-substituted benzene ring [$\delta$ 6.95 (1H, d, $J$=2 Hz), 7.03 (1H, dd, $J$=2, 9 Hz), 8.07 (1H, d, $J$=9 Hz)], a penta-substituted benzene ring [$\delta$ 6.32 (1H, s)], a $\gamma,\gamma$-dimethylallyl group [$\delta$ 1.64, 1.74 (3H each, br s; *gem*-dimethyl at C-3″), 3.29 (2H, br d, $J$=7 Hz; H-1″), 5.25 (1H, br t, $J$=7 Hz; H-2″)] and a methoxyl group [$\delta$ 3.41 (3H, s)].

Methylation of **3** afforded licoricone dimethyl ether (**9**),[15]



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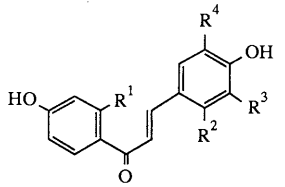
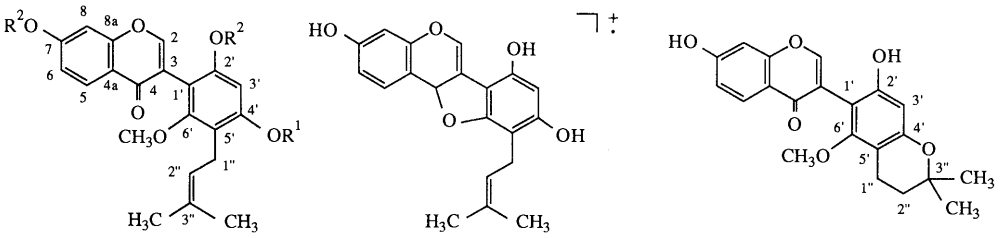


2 : $R^1$= OH, $R^2$= $R^3$= $R^4$= H
5 : $R^1$= H, $R^2$= $OCH_3$, $R^3$= $R^4$= H
16 : $R^1$= $R^3$= H, $R^2$= $OCH_3$, $R^4$= $-C(CH_3)_2-CH=CH_2$
17 : $R^1$= $R^4$= H, $R^2$= $OCH_3$, $R^3$= OH

Chart 1

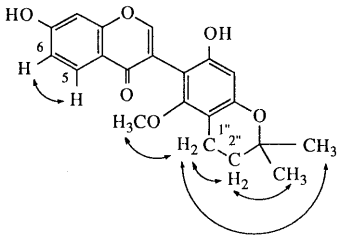


3 : $R^1 = R^2 = H$
8 : $R^1 = CH_3$, $R^2 = H$
9 : $R^1 = R^2 = CH_3$

3a

10

Chart 2



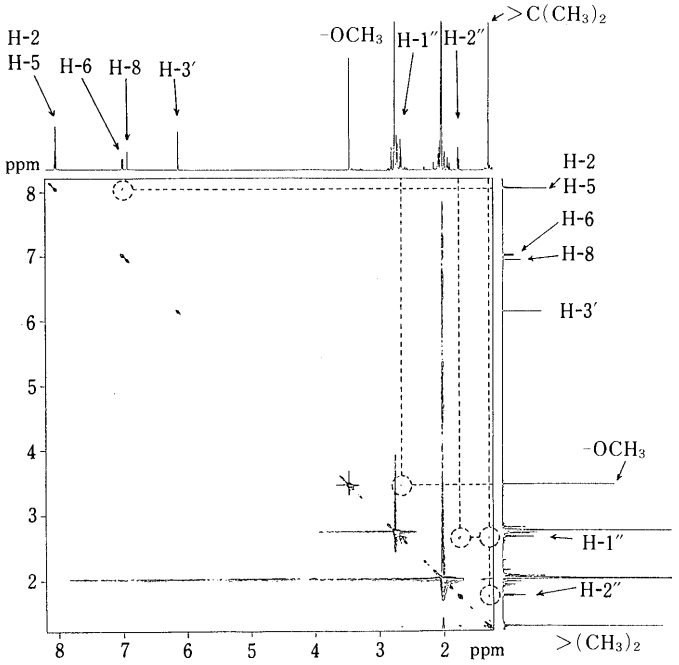



Fig. 1. The ROESY Spectrum of **10** (30 °C, in Acetone-$d_6$)

The dashed line circles indicate the cross peaks attributable to NOEs shown by the arrows in the structural formula. The signals at around $\delta$ 2.8 and $\delta$ 2.0 in the accompanying 1D spectrum are those of water and the solvent, respectively.

indicating that the structure of glicoricone has three hydroxyl groups in place of three of the four methoxyl groups of **9**.

The EI-MS spectrum of **3** showed an $[M-31]^+$ peak (relative intensity, 40%) ascribable to the fragment ion **3a**, and indicated that the location of the methoxyl group in **3** is *ortho* to C-1′ on the B-ring.[15,16]

Treatment of **3** with HCl caused cyclization between the $\gamma,\gamma$-dimethylallyl group and the adjacent hydroxyl group, to give **10**. The rotating flame Overhauser enhancement

TABLE I. One-Bond and Long-Range $^1H$–$^{13}C$ Correlation Data for Glicoricone (**3**)

| Carbon | $\delta_C$ | Correlated proton | |
|---|---|---|---|
| | | Proton coupled *via* one bond ($\delta_H$) | Proton coupled *via* two or three bonds[a] |
| C-2 | 157.0 | 8.10 | |
| C-3 | 119.6 | | H-2 |
| C-4 | 177.6 | | H-2/H-5[b] |
| C-4*a* | 118.2 | | H-6/H-8[c] |
| C-5 | 128.4 | 8.07 | |
| C-6 | 115.9 | 7.03 | H-8[d] |
| C-7 | 163.4 | | H-5 |
| C-8 | 103.2 | 6.95 | H-6[e] |
| C-8*a* | 158.8 | | H-5 |
| C-1′ | 106.2 | | H-2, H-3′ |
| C-2′ | 156.6 | | H-3′ |
| C-3′ | 100.9 | 6.32 | |
| C-4′ | 157.7 | | H-3′, H-1″ |
| C-5′ | 114.2 | | H-3′, H-1″ |
| C-6′ | 159.1 | | $OC\underline{H}_3$, H-1″ |
| C-1″ | 23.5 | 3.29 | |
| C-2″ | 125.1 | 5.25 | |
| C-3″ | 130.4 | | $=C(C\underline{H}_3)_2$ |
| $=C(C\underline{H}_3)_2$ | 17.9 | 1.74 | |
| | 25.8 | 1.64 | |
| $OCH_3$ | 61.0 | 3.41 | |

500 MHz for $^1H$-NMR and 125.7 MHz for $^{13}C$-NMR, in acetone-$d_6$. *a*) The average $J_{CH}$ value for the long-range couplings was set at 7 Hz. *b, c*) Unresolved cross peak ascribable to the couplings with the two protons. *d, e*) The cross peak due to the long-range coupling which is expected to be observed was overlapped by the nearby cross peak due to the one-bond coupling.

spectroscopy (ROESY) spectrum of **10** (Fig. 1) showed a cross peak, due to the nuclear Overhauser effect (NOE) between the methoxyl group and the signal of the methylene protons at C-1″ of the newly formed pyrane ring. On the other hand, no cross peak was observed between the methoxyl group and the aromatic protons. The location of the methoxyl group *ortho* to the aromatic protons is thus excluded, and structure **10**, in which the methoxyl group is at C-6′, is the only one which satisfies these findings.

Structure **3**, in which the methoxyl group is at C-6′, was thus assigned for glicoricone. The carbon-13 nuclear magnetic resonance ($^{13}C$-NMR) spectrum of **3** is also consistent with this structure. The assignments of the $^{13}C$ signals were confirmed by the one-bond and long-range $^1H$–$^{13}C$ correlation spectral data shown in Table I.

Licofuranone (**4**) was obtained as colorless needles. The $[M+H]^+$ ion peak in the high-resolution fast-atom bom-

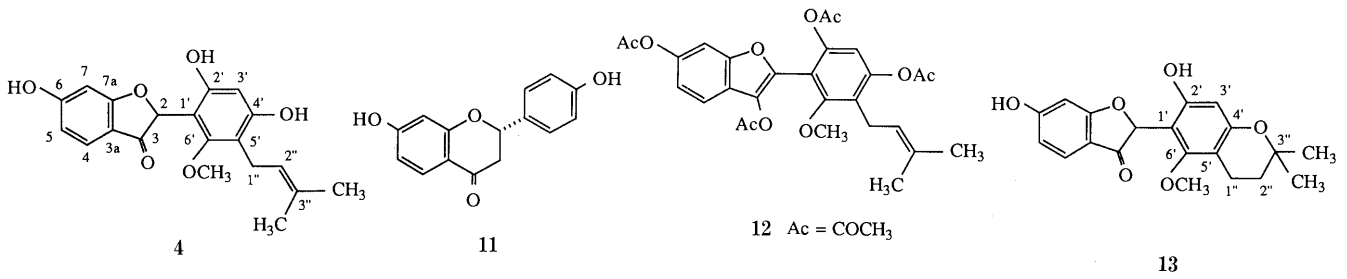



Chart 3

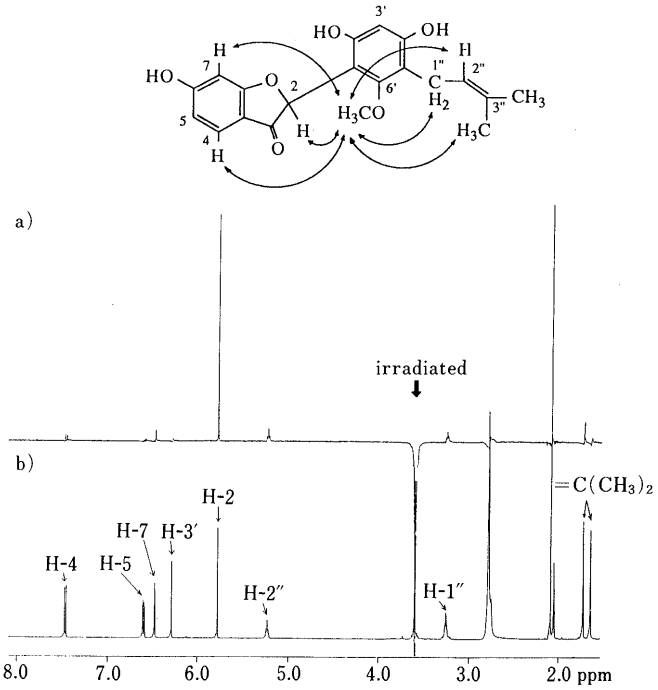



Fig. 2. The NOE Difference Spectrum of **4** (40 °C, in Acetone-$d_6$)

a) Difference spectrum with the irradiation of the methoxyl signal. b) Reference spectrum.

TABLE II. One-Bond and Long-Range $^1H$–$^{13}C$ Correlation Data for Licofuranone (**4**)

| Carbon | $\delta_C$ | Correlated proton | |
|---|---|---|---|
| | | Proton coupled *via* one bond ($\delta_H$) | Proton coupled *via* two or three bonds[a] |
| C-2 | 81.4 | 5.77 | |
| C-3 | 198.8 | | |
| C-3*a* | 115.7 | | H-5 |
| C-4 | 125.6 | 7.45 | |
| C-5 | 111.2 | 6.60 | H-7 |
| C-6 | 166.5 | | H-4 |
| C-7 | 99.0 | 6.47 | |
| C-7*a* | 174.9 | | H-4 |
| C-1′ | 108.3 | | H-3′ |
| C-2′ | 156.7 | | H-2 |
| C-3′ | 99.6 | 6.28 | |
| C-4′ | 158.6 | | |
| C-5′ | 113.8 | | H-3′ |
| C-6′ | 161.0 | | |
| C-1″ | 23.3 | 3.23 | |
| C-2″ | 124.9 | 5.21 | |
| C-3″ | 130.7 | | |
| $=C(CH_3)_2$ | 17.9 | 1.71 | |
| | 25.8 | 1.63 | |
| $OCH_3$ | 62.9 | 3.58 | |

500 MHz for $^1H$-NMR and 125.7 MHz for $^{13}C$-NMR, in acetone-$d_6$. *a*) The average $J_{CH}$ value for the long-range couplings was set at 7 Hz. Several cross peaks expected to be observed were lacking in the long-range $^1H$–$^{13}C$ correlation spectrum because of a limitation in quantity of the available sample.

bardment mass (FAB-MS) spectrum of **4** indicated its molecular formula $C_{20}H_{20}O_6$. The UV spectrum of **4** [$\lambda_{max}$ nm (log $\varepsilon$): 212 (4.69), 232 (sh, 4.32), 272 (4.12), 317 (3.92) and 331 (sh, 3.84)] resembles those of flavanones such as liquiritigenin (**11**).[17] The $^1H$-NMR spectrum of **4** also showed the presence of two aromatic rings [$\delta$ 6.47 (1H, d, $J$=2 Hz), 6.60 (1H, dd, $J$=2, 8.5 Hz), 7.45 (1H, d, $J$=8.5 Hz) (tri-substituted benzene ring), and 6.28 (1H, s; penta-substituted benzene ring)] and an aliphatic methine [$\delta$ 5.77 (1H, br s; H-2)], and also a $\gamma,\gamma$-dimethylallyl group [$\delta$ 1.63, 1.71 (3H each, br s; *gem*-dimethyl at C-3″), 3.23 (2H, m, H-1″), 5.21 (1H, br t, $J$=7 Hz; H-2″)] and a methoxyl group [$\delta$ 3.58 (3H, br s)]. However, this spectrum lacks the signals of the methylene protons at C-3 of flavanones.

The $^{13}C$-NMR spectrum of **4** showed a ketone carbon signal at $\delta$ 198.8, and also the signals of a methine carbon [$\delta$ 81.4 (C-2)], a $\gamma,\gamma$-dimethylallyl group [$\delta$ 17.9, 25.8 (*gem*-dimethyl at C-3″), 23.3 (C-1″), 124.9 (C-2″) and 130.7 (C-3″)], a methoxyl group ($\delta$ 62.9) and twelve carbons of the two aromatic rings.

Acetylation of **4** afforded a tetra-acetate **12**, as indicated by its molecular-ion peak (*m/z* 524) in the EI-MS spectrum. The $^1H$-NMR spectrum of **12** showed the signals of four acetyl groups [$\delta$ 2.12, 2.28, 2.29 and 2.31 (3H each, s)], along with those of two aromatic rings [$\delta$ 7.09 (1H, dd, $J$=2, 8.5 Hz), 7.41 (1H, d, $J$=2 Hz), 7.53 (1H, d, $J$=8.5 Hz); $\delta$ 6.89 (1H, s)], a methoxyl group [$\delta$ 3.50 (3H, s)] and a $\gamma,\gamma$-dimethylallyl group [$\delta$ 1.67, 1.77 (3H each, s; *gem*-dimethyl at C-3″), 3.33 (2H, d, $J$=6.5 Hz; H-1″), 5.12 (1H, br t, $J$=6.5 Hz; H-2″)]. The signal of the methine proton at C-2 was absent in this spectrum. Therefore, it is considered that the number of hydroxyl groups in **4** is three, and one of the four acetyl groups in **12** was introduced after tautomerization of the keto-form of **4** to the enol-form, as occurs in the acetylation of a 2-arylbenzofuranone.[18]

These findings indicate that **4** has a 2-arylbenzofuranone structure with three hydroxyl groups, a methoxyl group and a $\gamma,\gamma$-dimethylallyl group.

The treatment of **4** with HCl afforded **13**. The formation of a new pyrane ring in **13** indicates that the location of the $\gamma,\gamma$-dimethylallyl group in **4** is *ortho* to one of the hydroxyl groups on the same aromatic ring.

The NOE difference spectrum of **4** (Fig. 2) showed a distinctive NOE of H-2 upon the irradiation of the methoxyl

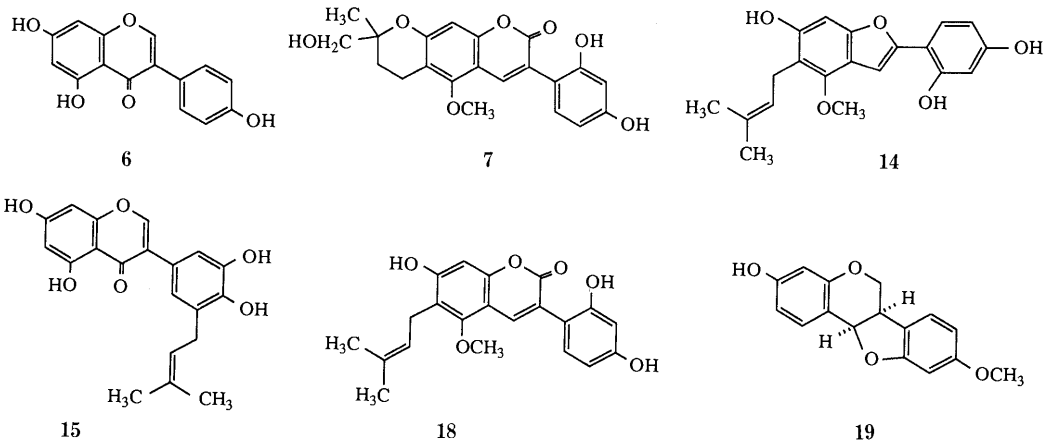


Chart 4

signal, indicating that the methoxyl group is *ortho* to C-1′ on the B-ring. This irradiation also enhanced NOEs of H-1″, H-2″ and a methyl signal of the γ,γ-dimethylallyl group, to indicate that the γ,γ-dimethylallyl group is *ortho* to the methoxyl group.

The $^{13}C$ chemical shifts of the B-ring carbons of **4** [δ 108.3 (C-1′), 156.7 (C-2′), 99.6 (C-3′), 158.6 (C-4′), 113.8 (C-5′), 161.0 (C-6′) (B-ring)] are closely similar to those of the B-ring carbons of **3** [δ 106.2 (C-1′), 156.6 (C-2′), 100.9 (C-3′), 157.7 (C-4′), 114.2 (C-5′), 159.1 (C-6′)].

These data indicate the identity of the substitution pattern on the B-ring in **4** with that in **3**.

The coupling pattern of the ABX protons (thus assigned to be the A-ring protons) in the $^1H$-NMR spectrum of **4** indicates that the one remaining hydroxyl group on the A-ring is at C-6 or at C-5 of the benzofuranone structure. Considering that an *O*-functional group on the A-ring in the reported licorice flavonoids is, without exception, at C-7 or the corresponding position, we assigned the hydroxyl group at C-6.

Structure **4** thus proposed for licofuranone was substantiated by the $^1H$–$^{13}C$ correlation spectral data shown in Table II. Each chemical shift of the C-ring carbons (C-2, C-3, C-3a, C-7a) in the $^{13}C$-NMR spectrum of **4**, especially the noticeable downfield shift of C-7a (δ 174.9), from the corresponding carbons of flavanones[19,20] or isoflavanones,[3d,21] is consistent with the constrained furanone structure.

In spite of the presence of an asymmetric center in the molecule, isolated **4** was optically inactive. This is attributable to the racemization caused by the tautomerization between the keto-form and the enol-form described above, although the $^1H$- and $^{13}C$-NMR spectra of **4** did not show duplication of the signals due to this tautomerization.

**Inhibitory Effects of Licorice Constituents on MAO** The inhibitory effects of twelve licorice constituents, **1**—**4**, **6**, **7**, licocoumarone (**14**),[22] glycyrrhisoflavone (**15**),[4b] licochalcones A (**16**)[23] and B (**17**),[23] glycycoumarin (**18**)[24] and (−)-medicarpin (**19**),[3e] on MAO were examined, and the results are summarized in Table III.

Among the tested compounds, six, **3**, **4**, **6**, **7**, **14** and **15** showed inhibition with the $IC_{50}$ values of $6.0\times10^{-5}$—$1.4\times10^{-4}$ M. Although the inhibitory activity of **1** against MAO was not strong ($IC_{50}$: $1.6\times10^{-4}$ M), it cannot be ignored because of its abundant presence in most species of licorice.[25]

It is noticeable that **14**, which was the strongest inhibitor of XOD among the tested compounds,[1] is again the most

Table III. Inhibitory Effects of Licorice Constituents on MAO

| Compound | $IC_{50}$[a] (M) |
|---|---|
| Isoflavone | |
| Glycyrrhisoflavone (**15**) | $9.5\times10^{-5}$ |
| Glicoricone (**3**) | $1.4\times10^{-4}$ |
| Genistein (**6**) | $9.5\times10^{-5}$ |
| Chalcone | |
| Licochalcone A (**16**) | $>2.0\times10^{-4}$ |
| Licochalcone B (**17**) | $>2.0\times10^{-4}$ |
| Isoliquiritigenin (**2**) | $>2.0\times10^{-4}$ |
| 3-Arylcoumarin | |
| Glycycoumarin (**18**) | $>2.0\times10^{-4}$ |
| Licopyranocoumarin (**7**) | $1.4\times10^{-4}$ |
| 2-Arylbenzofuran | |
| Licocoumarone (**14**) | $6.0\times10^{-5}$ |
| Licofuranone (**4**) | $8.7\times10^{-5}$ |
| Pterocarpan | |
| (−)-Medicarpin (**19**) | $>2.0\times10^{-4}$ |
| Saponin | |
| Glycyrrhizin (**1**) | $1.6\times10^{-4}$ |
| Others | |
| Harmane hydrochloride | $5.7\times10^{-5}$ |
| Quinine sulfate | $6.4\times10^{-5}$ |

a) Concentration required for 50% inhibition.

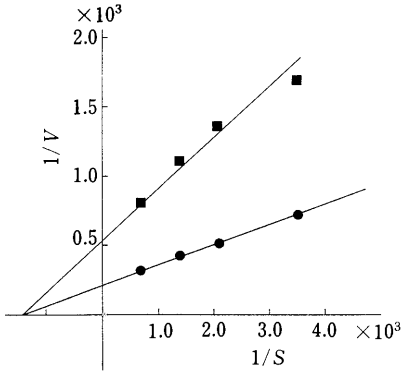



Fig. 3. Inhibitory Effects of **14** on MAO

Lineweaver–Burk plots in the absence (●—●) and in the presence (■—■, $6.0\times10^{-5}$ M) of **14**. *V*, μmol substrate metabolized/mg enzyme/min; *S*, molar concentration of substrate.

potent inhibitor of MAO among the compounds tested in the present study. The kinetic analysis using Lineweaver–Burk plots (Fig. 3) indicated that **14** inhibited MAO non-competitively at the concentration of $6.0 \times 10^{-5}$ M.

Chalcones **16** and **17**, which remarkably inhibit XOD,[1] showed only weak or practically no inhibition against MAO. Another chalcone, **2**, which was reported to be a stronger inhibitor of MAO than harmane hydrochloride,[8] showed only very weak inhibition ($IC_{50}$: $9.0 \times 10^{-4}$ M) in our experiment. This difference in the inhibitory activity of **2** may be due to the difference of the origin of the enzyme. The poor inhibitory activity of the chalcones suggests strongly that the mechanism of the inhibition by the phenolic constituents against MAO is different from the mechanism proposed for the inhibition of phenylalanine ammonia-lyase[26] by chalcones.

## Experimental

UV spectra were recorded on a Hitachi 200-10 spectrophotometer. EI-MS and FAB-MS spectra were recorded on a VG 70-SE instrument. $^1$H- and $^{13}$C-NMR spectra were recorded on a Varian VXR-500 instrument (500 MHz for $^1$H-NMR and 125.7 MHz for $^{13}$C-NMR); chemical shifts were based on those of the solvent signals (acetone-$d_6$: $\delta_H$ 2.04 and $\delta_C$ 29.8; chloroform-$d$: $\delta_H$ 7.24), and given in $\delta$ values (ppm) from tetramethylsilane. A JEOL GSX-500 spectrometer was also used for recording the $^1$H-NMR spectra. CPC was performed on a Sanki L-90 machine (Nagaoka-kyo, Kyoto) equipped with twelve 1000E cartridges. Analytical thin layer chromatography (TLC) was conducted on Kieselgel 60 F254 plates (0.2 mm thick) and preparative TLC (PTLC) on Kieselgel 60 PF254 plates (0.5 mm thick). Solvents for TLC were A ($CHCl_3$–MeOH, 10:1), B (toluene–acetone–HCOOH, 36:6:1), C (*n*-hexane–EtOAc, 2:1), D ($CHCl_3$–acetone–HCOOH, 16:2:1) and E ($CHCl_3$). High-performance liquid chromatography (HPLC) was performed on a Merck LiChrospher cartridge column with $CH_3CN$–$H_2O$–AcOH (70:25:5) at 40 °C, and the flow rate was set at 1.2 ml/min. An MCPD-350 instrument (Otsuka Electronics) was used for the HPLC detection.

**Isolation of Phenolic Constituents from Licorice** Licorice (2 kg) (Seihoku-kanzo; Tochimoto-tenkai-do, Osaka) was pulverized and then successively extracted with *n*-hexane (6 l × 3) and EtOAc (6 l × 3), and solvents were evaporated off. A portion (20 g) of the EtOAc extract (87 g) was subjected to CPC [$CHCl_3$–MeOH–$H_2O$ (7:13:8); reversed-phase development; flow rate, 3 ml/min], to separate fractions I—V. Fraction II (473 mg) was further separated by column chromatography on MCI gel CHP-20P (1.1 × 38 cm) with 60% MeOH and then by PTLC with solvent A, to give licopyranocoumarin (**7**) (19 mg) and licofuranone (**4**) (9 mg), and also two crude compounds. The latter two were respectively purified by PTLC with solvent B, and gave echinatin (**5**) (2 mg) and genistein (**6**) (2 mg). Fraction IV (717 mg) of CPC was purified by column chromatography on MCI gel CHP-20P (with 60% MeOH), and by PTLC with solvent B, to give glicoricone (**3**) (20 mg).

**Glicoricone (3)** Colorless needles, mp 192 °C. *Anal.* Calcd for $C_{21}H_{20}O_6 \cdot 1/2H_2O$: C, 66.83; H, 5.60. Found: C, 66.16; H, 5.35. EI-MS: *m/z* 368 {$[M]^+$, 100% (relative intensity)}, 353 ($[M-CH_3]^+$, 37%), 337 ($[M-OCH_3]^+$, 40%), 313 ($[M-(CH_3)_2C=CH]^+$, 63%). High-resolution EI-MS: *m/z* 368.1257 ($[M]^+$; Calcd for $C_{21}H_{20}O_6$, 368.1260). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 209 (4.55), 240 (sh, 4.27), 247 (4.24), 285 (4.02) and 305 (sh, 3.94). $^1$H-NMR (acetone-$d_6$) $\delta$: 1.64, 1.74 (3H each, br s; *gem*-dimethyl at C-3″), 3.29 (2H, br d, $J$=7 Hz; H-1″), 3.41 (3H, s, $OCH_3$), 5.25 (1H, br t, $J$=7 Hz; H-2″), 6.32 (1H, s; H-3′), 6.95 (1H, d, $J$=2 Hz; H-8), 7.03 (1H, dd, $J$=2, 9 Hz; H-6), 8.07 (1H, d, $J$=9 Hz, H-5), 8.10 (1H, s; H-2). $^{13}$C-NMR: See, Table I.

**Methylation of 3** Ethereal diazomethane (0.4 ml) was added to an EtOH solution (0.4 ml) of **3** (2.2 mg), and the mixture was left to stand for 12 h. The solvent was distilled off and the residue was purified by PTLC with solvent C, to give **9** (1.1 mg). EI-MS: *m/z* 410 ($[M]^+$). $^1$H-NMR (30 °C, acetone-$d_6$) $\delta$: 1.64, 1.73 (3H each, br s, *gem*-dimethyl at C-3″), 3.26, 3.29 (1H each, dd, $J$=7, 14 Hz), 3.46, 3.71, 3.89, 3.97 (3H each, s; 4 × $OCH_3$), 5.19 (1H, br t, $J$=7 Hz, H-2″), 6.54 (1H, s; H-3′), 7.05 (2H, m; H-6 and H-8), 7.88 (1H, s; H-2), 8.05 (1H, d, $J$=9 Hz; H-5). $^1$H-NMR (chloroform-$d$)[15] $\delta$: 1.65, 1.73 (3H each, br s; *gem*-dimethyl at C-3″), 3.25, 3.33 (1H each, dd, $J$=7, 14 Hz), 3.45, 3.74, 3.85, 3.90 (3H each, s; 4 × $OCH_3$), 5.18 (1H, br t, $J$=7 Hz; H-2″), 6.34 (1H, s; H-3′), 6.86 (1H, d, $J$=2.5 Hz; H-8), 6.96 (1H, dd, $J$=2.5, 8.5 Hz; H-6), 7.78 (1H, s, H-2), 8.19 (1H, d, $J$=8.5 Hz; H-5). This compound was identical with licoricone dimethyl ether (**9**)[15] prepared from licoricone (**8**)[15] [mixed mp, EI-MS, $^1$H-NMR, TLC (solvent C, *Rf* 0.20) and HPLC (retention time, 5.8 min). The UV spectra recorded using the MCPD detector were also identical].

**Treatment of 3 with HCl** Conc. HCl (0.1 ml) was added to an MeOH solution (0.5 ml) of **3** (0.7 mg), and the mixture was left to stand for 36 h. The solvent was evaporated, and the residue was purified by PTLC using solvent D, to give **10** (0.7 mg), mp 138 °C. EI-MS: *m/z*: 368 ($[M]^+$). $^1$H-NMR (acetone-$d_6$) $\delta$: 1.30 (6H, s; *gem*-dimethyl at C-3″), 1.77 (2H, t, $J$=7 Hz; H-2″ × 2), 2.68 (2H, t, $J$=7 Hz; H-1″ × 2), 3.48 (3H, s; $OCH_3$ at C-6′), 6.14 (1H, s; H-3′), 6.94 (1H, d, $J$=2 Hz; H-8), 7.02 (1H, dd, $J$=2, 9 Hz; H-6), 8.06 (1H, d, $J$=9 Hz; H-5).

**Licofuranone (4)** Colorless needles, mp 162 °C, $[\alpha]_D$ 0° ($c$=3, acetone). *Anal.* Calcd for $C_{20}H_{20}O_6 \cdot 1/2H_2O$: C, 65.74; H, 5.79. Found: C, 65.22; H, 5.62. High-resolution FAB-MS: *m/z* 357.1385 ($[M+H]^+$; Calcd for $C_{20}H_{20}O_6+H$, 357.1338). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 212 (4.69), 232 (sh, 4.32), 272 (4.12), 317 (3.92), 331 (sh, 3.84). $^1$H-NMR (acetone-$d_6$) $\delta$: 1.63, 1.71 (3H each, br s; *gem*-dimethyl at C-3″), 3.23 (2H, m, H-1″), 3.58 (3H, br s; $OCH_3$ at C-6′), 5.21 (1H, br t, $J$=7 Hz; H-2″), 5.77 (1H, br s; H-2), 6.28 (1H, s; H-3′), 6.47 (1H, d, $J$=2 Hz; H-7), 6.60 (1H, dd, $J$=2, 8.5 Hz; H-5), 7.45 (1H, d, $J$=8.5 Hz; H-4). $^{13}$C-NMR: See Table II.

**Acetylation of 4** A mixture of **4** (1 mg), acetic anhydride (0.2 ml) and pyridine (0.2 ml) was left to stand overnight at room temperature, and then the solvent was removed by evaporation. The residue was purified by PTLC with solvent E, to give a tetra-acetate, **12** (0.7 mg), mp 108 °C. EI-MS: *m/z* 524 ($[M]^+$). $^1$H-NMR (acetone-$d_6$) $\delta$: 1.67, 1.77 (3H each, s; *gem*-dimethyl at C-3″), 2.12, 2.28, 2.29, 2.31 (3H each, s; $OCOCH_3$ × 4), 3.33 (2H, d, $J$=6.5 Hz; H-1″), 3.50 (3H, s; $OCH_3$ at C-6′), 5.12 (1H, br t, $J$=6.5 Hz; H-2″), 6.89 (1H, s; H-3′), 7.09 (1H, dd, $J$=2, 8.5 Hz; H-5), 7.41 (1H, d, $J$=2 Hz; H-7), 7.53 (1H, d, $J$=8.5 Hz; H-4).

**Treatment of 4 with HCl** A mixture of **4** (0.7 mg) in MeOH (0.5 ml) and conc. HCl (0.1 ml) was left to stand for 36 h. The solvent was then evaporated, and the residue was purified by PTLC using solvent D, to give **13** (0.7 mg), mp 148 °C. High-resolution EI-MS: *m/z* 356.1282 ($[M]^+$; Calcd for $C_{20}H_{20}O_6$, 356.1260). $^1$H-NMR (acetone-$d_6$) $\delta$: 1.28, 1.29 (6H in total; *gem*-dimethyl at C-3″), 1.74 (2H, t, $J$=7 Hz; H-2″ × 2), 2.63 (2H, t, $J$=7 Hz; H-1″ × 2), 3.55 (3H, br s; $OCH_3$ at C-6′), 5.79 (1H, s; H-2), 6.10 (1H, s; H-3′), 6.47 (1H, d, $J$=2 Hz, H-7), 6.61 (1H, dd, $J$=2, 8.5 Hz; H-5), 7.47 (1H, d, $J$=8.5 Hz; H-4).

**Estimation of Inhibitory Effects of Licorice Phenolics on MAO** The reagents other than licorice constituents were purchased from the following companies: MAO (from bovine plasma), Sigma; benzylamine hydrochloride, Tokyo Kasei; harmane hydrochloride and quinine sulfate, Nacalai Tesque. The inhibitory activity of each compound was determined by the reported method[10] with a slight modification. A mixture consisting of an MAO solution [0.012 U/ml in 0.1 M phosphate buffer (pH 7.4); 25 $\mu$l], a tested compound solution [175 $\mu$l; in water–dimethylsulfoxide (8:2, by volume)], 0.1 M phosphate buffer (pH 7.4) (350 $\mu$l) and water (300 $\mu$l), was preincubated for 15 min at 37 °C. An aqueous solution (0.06 M; 25 $\mu$l) of benzylamine hydrochloride was then added to the mixture and the resulting solution was incubated for 30 min at 37 °C. The enzyme reaction was stopped by adding 1 N HCl (25 $\mu$l), and the absorbance at 250 nm of the reaction mixture was measured. The blank was prepared in an analogous way, except that the MAO solution was added after the addition of HCl. Inhibitory activity (%) was calculated using the following equation, and was given as the mean value of triplicate experiments.

$$\text{inhibitory activity } (\%) = (1 - B/A) \times 100$$

where $A$ is the enzyme activity in the absence of the tested compound, and $B$ is that in the presence of the compound.

**Acknowledgements** The Varian VXR-500 instrument used in this study is the property of the SC-NMR Laboratory of Okayama University. This study was supported in part by a Grant-in-Aid for Scietific Research from the Ministry of Education, Science and Culture of Japan.

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# The Complete Amino Acid Sequence of an Abortifacient Protein, Karasurin

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**The complete amino acid sequence of a new abortifacient protein, karasurin, was determined. Karasurin, which was isolated from fresh root tubers of *Trichosanthes kirilowii* MAXIMOWICZ var. *japonicum* KITAMURA (Cucurbitaceae), was a highly basic protein with pI 10.1 and molecular weight of 28000. Intact karasurin was cleaved with cyanogen bromide, lysyl endopeptidase, formic acid and 2-(2′-nitrophenyl-sulfenyl)-3-methyl-3-bromoindolenine (BNPS-skatole), respectively. Cleavages with *N*-bromosuccinimide (NBS), trypsin and pepsin were performed for the fragments. The resultant peptide fragments were separated by gel filtration chromatography, reversed-phase high performance liquid chromatography (HPLC) or gel filtration HPLC following sequence analyses by automated Edman methods. Karasurin consists of 246 or 247 amino acid residues with a calculated molecular weight of 27144 or 27215 differing only at the C-terminus with the addition of alanyl residue. Two C-terminal sequences were identified as Asn–Asn–Met–OH and Asn–Asn–Met–Ala–OH by sequence analyses and hydrazinolysis, but there was no micro-heterogeniety in other peptides analysed. The sequence of karasurin revealed a considerable similarity to that of trichosanthin and α-trichosanthin, which are known as abortifacient, ribosome-inactivating and anti human immunodeficiency virus (HIV) (the virus causing aquired immunodeficiency syndrome (AIDS)) proteins, with 93% and 98% identity, respectively.**

**Keywords** karasurin; *trichosanthes kirilowii* var. *Japonicum*; abortifacient protein; HPLC; amino acid sequence; cyanogen bromide; lysyl endopeptidase; BNPS-skatole

Karasurin, which was isolated from fresh root tubers of *Trichosanthes kirilowii* MAX. var. *japonicum* KITAM. (Cucurbitaceae), was a highly basic protein with pI 10.1, and its molecular weight was estimated as 28000 by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. It was shown to be potent in inducing mid-term abortion in pregnant mice.[1]

Karasurin shows a considerably high homology to trichosanthin (from the root tubers of *Trichosanthes kirilowii* MAX.),[2-7] trichokirin (from the seeds of *Trichosanthes kirilowii* MAX.)[8] and α- and β-momorcharin (from the seeds of *Momordica charantia*),[9,10] which are abortifacient or ribosome-inactivating proteins from other plants of the Cucurbitaceae family; however, their physicochemical properties (molecular weight, isoelectric point and amino acid composition) and amino acid sequence in the N-terminal region are not identical to each other.[1] Therefore, we determined here the complete amino acid sequence of karasurin and compared it with that of trichosanthin in order to study the homology between their primary structures.

## Materials and Methods

**Materials** Karasurin was purified as described previously.[1] Reagents used for chemical cleavage were as follows: cyanogen bromide (BrCN) (Wako Pure Chemical), 2-(2′-nitrophenyl-sulfenyl)-3-methyl-3-bromoindolenine (BNPS-skatole) (Pierce), N-bromosuccinimide (NBS) (Wako Pure Chemical) and anhydrous hydrazine (Pierce). Proteases were as follows: lysyl endopeptidase (Wako Pure Chemical), trypsin (Boehringer Mannheim) and pepsin (Boehringer Mannheim). All chemicals used for the sequence determination were of sequential grade and purchased from Applied Biosystems, Nacalai Tesque and Wako Pure Chemical.

**Chemical and Enzymatic Cleavage** Intact karasurin (68 mg) was cleaved with a 100-mol excess of BrCN in 70% formic acid at 37 °C in the dark for 24 h.[11] The reaction solution was diluted with 3-fold distilled water and then lyophilyzed. The lyophylizate was dissolved in 2.1% formic acid–8.7% acetic acid (v/v), and then fractionated by gel filtration chromatography. The fractions were further separated by reversed-phase high performance liquid chromatography (RP-HPLC).

Intact karasurin (10 mg) was also digested with lysyl endopeptidase (enzyme(E)/substrate(S) = 1/100, by weight) in 0.05 M Tris–HCl, pH 8.8, at 37 °C for 20 h. The digests were centrifuged at 10000 rpm for 10 min (Tomy MC-150 micro centrifuge) to separate insoluble peptides. The fragments in the supernatant were separated by RP-HPLC and gel filtration HPLC (GF-HPLC). The fragments in the precipitate were dissolved in 10% formic acid and then separated by GF-HPLC. A cleavage with NBS in 10% formic acid–50% acetic acid (v/v) was performed for some of the peptides.[12] The resulting fragments were separated by RP-HPLC.

Dilute acid hydrolysis at aspartic acid was performed essentially according to the method of Inglis[13] as follows: intact karasurin (3.2 mg) was hydrolyzed in 2% formic acid at 108 °C for 4 h *in vacuo*. The fragments were separated by RP-HPLC.

Intact karasurin (1.3 mg) was cleaved with a 100-mol excess of BNPS-skatole in 66% acetic acid at room temperature in the dark for 30 h.[14] The resulting peptides were separated by GF-HPLC. One of the fragments was digested with trypsin (E/S = 1/50, by weight) in 0.1 M Tris–HCl, pH 8.0, at 37 °C for 27 h or pepsin (E/S = 1/100, by weight) in 0.01 N HCl at 37 °C for 4 h followed by separation on RP-HPLC.

**Fractionation of Fragment Peptides** Gel filtration chromatography of the peptides, which were cleaved with BrCN, was performed on a Sephadex G-100 column (1.0 × 100 cm, superfine) in 2.1% formic acid–8.7% acetic acid (v/v) at a flow rate of 1.2 ml/h. Eluate was monitored by the absorption at 280 nm. Rechromatography of the preceding fraction by RP-HPLC was performed on an Aquapore RP-300 column (0.21 × 22 cm, 7 μm: Applied Biosystems) using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.2 ml/min. Eluate was monitored by the absorption at 215 nm.

Fractionations of the soluble lysyl endopeptidase fragments and the peptic digest of the BNPS-skatole fragment by RP-HPLC were performed on a Spheri-5 RP-18 column (0.46 × 22 cm, 5 μm: Applied Biosystems) using a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 0.5 ml/min. The other fractionations by RP-HPLC were performed on an Aquapore RP-300 column (0.46 × 22 cm, 7 μm: Applied Biosystems) using a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min. Eluate was monitored by the absorption at 215 nm.

Fractionations by GF-HPLC were performed exclusively on a TSK gel G3000PWXL column (0.78 × 60 cm; Tosoh) at 60 °C in 0.1% TFA–45% acetonitrile (v/v) at a flow rate of 0.3 ml/min. Eluate was monitored by the absorption at 215 nm.

**Sequence Analysis** The N-terminal sequence of intact karasurin and the sequences of the fragments obtained from various cleavages were determined by an automated Edman degradation using an Applied Biosystems model 470A and model 477A automated gas phase sequencer.

Determination of C-terminal residue was performed essentially according to the method of Akabori.[15] Intact karasurin (0.25 mg) was dissolved in anhydrous hydrazine and then hydrolyzed at 100 °C for 10 h *in vacuo*. The amino acid analysis of C-terminal residue was performed with a Hitachi amino acid analyzer, model 835 (Hitachi).

**Amino Acid Analysis** The amino acid composition of karasurin was

determined as described previously.[1)]

## Results and Discussion

**N-Terminal Sequence of Intact Karasurin** Automated Edman degradation was performed on intact karasurin and revealed the sequence of N-terminal 37-residues.[1)] The N-terminal 37-residues were found to be, Asp–Val–Ser–Phe–Arg–Leu–Ser–Gly–Ala–Thr–Ser–Ser–Ser–Tyr–Gly–Val–Phe–Ile–Ser–Asn–Leu–Arg–Lys–Ala–Leu–Pro–Tyr–Glu–Arg–Lys–Leu–Tyr–Asp–Ile–Pro–Leu–Leu.

**Amino Acid Sequence of BrCN Fragments** After cleavage of intact karasurin by BrCN, the reaction mixture was separated by gel filtration chromatography and gave two peaks (data not shown). The first peak corresponded to intact karasurin. The BrCN cleaved fragments (about 35% of the starting materials), the second peak, were not separated from each other, obtaining a molecular weight between 8000—10000, suggesting that each fragment consists of approximately 70—90 amino acid residues. The low yield of BrCN cleavage may due to the incomplete solubility of karasurin in 70% formic acid. Then, the BrCN fragments (CB) were separated by RP-HPLC (Fig. 1). Three peptides, CB-4, CB-11 and CB-12, were subjected to sequence analysis. The sequence of the N-terminal region of CB-4 was shown to be identical with that of intact karasurin at N-terminal 37-residues, indicating that CB-4 is located at the N-terminal of this protein. The direct sequencing of CB-11 and CB-12 were carried out for 59 and 30 cycles, respectively: for CB-11, $^{73}$Gly—$^{131}$Ala; for CB-12, $^{153}$Val—$^{182}$Ser.

**Amino Acid Sequence of Lysyl Endopeptidase Fragments** Lysyl endopeptidase fragments in the supernatant (LE-S) were separated by RP-HPLC (Fig. 2) or GF-HPLC (data not shown). Eight peptides by RP-HPLC were sequenced: for LE-S-1, $^{98}$Asp—$^{100}$Lys; for LE-S-2, $^{101}$Arg—$^{102}$Lys; for LE-S-3, $^{174}$Arg—$^{177}$Lys; for LE-S-4, $^{24}$Ala—$^{30}$Lys; for LE-S-5, $^{166}$Phe—$^{173}$Lys; for LE-S-6, $^{94}$Tyr—$^{97}$Lys; for LE-S-12, $^{103}$Val—$^{120}$Lys; for LE-S-15, $^{178}$Thr—$^{197}$Lys. One peptide by GF-HPLC (LE-S-G) was subjected to sequence analysis. The direct sequencing of LE-S-G-1 was carried out for 24 cycles: $^{198}$Gln—$^{221}$Gln.

Besides intact karasurin and the incomplete digests, three peptides were obtained by separation of lysyl endopeptidase fragments in the precipitate (LE-P) by GF-HPLC, and their molecular weights were estimated as *ca.* 6000 (LE-P-1), 4500 (LE-P-2) and 3500 (LE-P-3), respectively (Fig. 3). These peptides were subjected to sequence analysis. The direct sequencing of LE-P-1 and LE-P-2 was carried out for 40 and 36 cycles, respectively: for LE-P-1, $^{31}$Leu—$^{70}$Tyr; for LE-P-2, $^{121}$Ile—$^{156}$Gln. The sequence of the N-terminal region of LE-P-1 was shown to be identical with that of

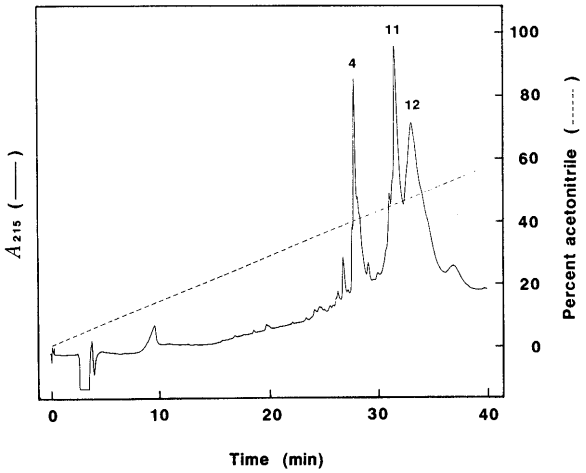


Fig. 1. HPLC of the Complete Cleavage Peptides with BrCN on an Aquapore RP-300 Column

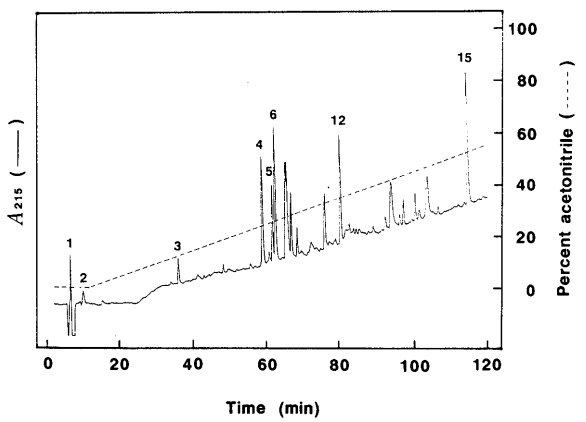


Fig. 2. HPLC of the Soluble Lysyl Endopeptidase Fragments on a Spheri-5 RP-18 Column

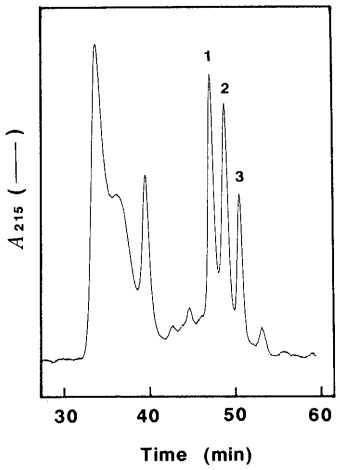


Fig. 3. HPLC of the Insoluble Lysyl Endopeptidase Fragments on a TSK Gel G3000PWXL Column

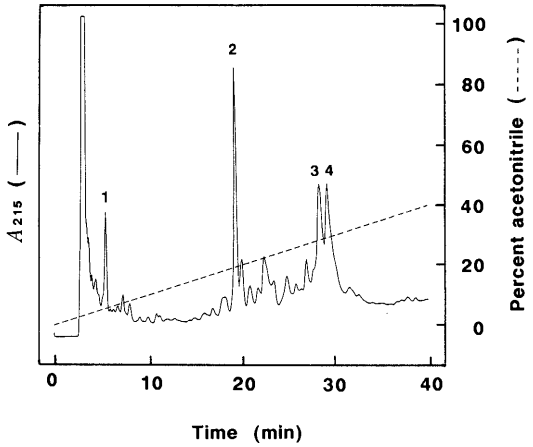


Fig. 4. HPLC of the Dilute Acid Hydrolysis Fragments on an Aquapore RP-300 Column

the C-terminal region of CB-4. Since LE-P-2 contained Met, the peptide joined the CB fragments, CB-11 and CB-12. The sequence of LE-P-3 was as follows: $^{1}$Asp–$^{23}$Lys.

LE-P-1 and LE-P-2 were further cleaved by NBS (LE-P-1-N, LE-P-2-N), and the resulting peptides were separated by RP-HPLC (data not shown). From the products of LE-P-1, three fragments were subjected to sequence analysis, whose results were as follows: for LE-P-1-N-1, $^{82}$Phe—$^{93}$Lys; for LE-P-1-N-3, $^{71}$Val—$^{81}$Tyr; for LE-P-1-N-4, $^{33}$Asp—$^{47}$Tyr. The sequences of two fragments obtained from LE-P-2 were as follows: for LE-P-2-N-1, $^{143}$Asn—$^{164}$Tyr; for LE-P-2-N-5, $^{121}$Ile—$^{141}$Tyr.

**Amino Acid Sequence of Dilute Acid Hydrolysis Fragments** Fractionation of karasurin after cleavage at aspartic acid by dilute acid hydrolysis (HF) resulted in the separation of four HF fragments (Fig. 4). These fractions were subjected to sequence analysis. The sequences of HF-1 and HF-2 were as follows: for HF-1, $^{66}$Val—$^{77}$Gly; for HF-2, $^{79}$Thr—$^{97}$Lys. HF-1 was found to contain Met; therefore, the connection between LE-P1 and CB-11 was confirmed. The direct sequencing of HF-3 and HF-4 was carried out for 23 and 12 cycles, respectively: for HF-3, $^{99}$Ala—$^{121}$Ile; for HF-4, $^{2}$Val—$^{13}$Ser.

**Amino Acid Sequence of BNPS-skatole Fragments** Fractionation of karasurin after cleavage by BNPS-skatole (BS) resulted in the separation of three fragments (Fig. 5). Their molecular weights were estimated as *ca.* 28000 (BS-1), 22000 (BS-2) and 6000 (BS-3), respectively. BS-1 and BS-2 were assigned as intact karasurin and a product from the N-terminal region, respectively. BS-3 was assigned as a product from the C-terminal region and was subjected to sequence analysis. The direct sequencing of BS-3 was carried out for 53 cycles: $^{193}$Ser—$^{245}$Asn.

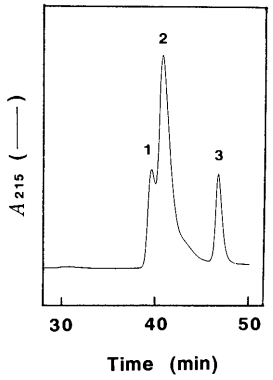


Fig. 5. HPLC of the BNPS-skatole Fragments on a TSK Gel G3000PWXL Column

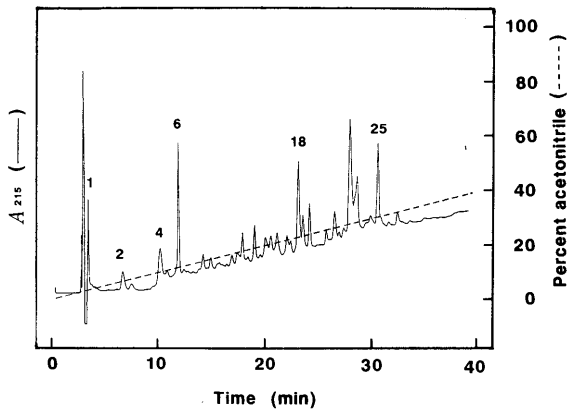


Fig. 6. HPLC of the Trypic Fragments of BN-3 on an Aquapore RP-300 Column

BS-3 was further digested by trypsin (BS-3-T) (Fig. 6) or pepsin (BS-3-P) (data not shown), and the resulting peptides were separated by RP-HPLC, respectively. From tryptic digest, six fragments were subjected to sequence analysis. The sequence of each fragment except BS-3-T-25 was as follows: for BS-3-T-1, $^{244}$Asn—$^{247}$Ala, whose yield of the PTH amino acids was shown in Table I and discussed below; for BS-3-T-2, $^{241}$Leu—$^{243}$Arg; for BS-3-T-4, $^{193}$Ser—$^{197}$Lys; for BS-3-T-6, $^{216}$Ile—$^{222}$Arg; for BS-3-T-18, $^{223}$Val—$^{236}$Asn. For BS-3-T-25, 13 amino acid residues of the N-terminal region were determined: $^{198}$Gln—$^{210}$Glu. Four peptides, except BS-3-T-1 and BS-3-T-4, may be derived from nonspecific hydrolysis with chymotryptic activity. From the peptic digest, one peptide was sequenced: for BS-3-P-10, $^{240}$Leu—$^{246}$Met.

**Determination of C-Terminal Residue** As a result of amino acid analysis after hydrazinolysis, karasurin was shown to have two distinct C-terminal residues in which were found two amino acids, Met and Ala, in a ratio of 4 : 1 (mol/mol).

**Primary Structure of Karasurin** The complete amino acid sequence of karasurin was determined by analyses of intact karasurin and its peptide fragments, prepared by cleavages with BrCN, lysyl endopeptidase, formic acid, BNPS-skatole, NBS, trypsin and pepsin as described above. Arrangement of these fragments was performed as follows.

Amino acid sequence analysis of intact karasurin allowed the sequence of 37 N-terminal residues. Since the peptide gave the same N-terminal residues as the intact protein, CB-4 was located at the N-terminal of the protein.

In order to determine the amino acid sequence of the middle portion of the protein, and to obtain overlapping peptides to connect CB fragments, the intact protein was cleaved with lysyl endopeptidase and dilute formic acid. Analysis of LE-P-1 provided the sequence that overlapped with that of CB-4 and thus extended the additional 33 residues up to 70th. HF-1 clarified connections between LE-P-1 and CB-11, since there were 5 overlapping residues in each fragment. The connecting peptide between CB-11 and CB-12 was found to be LE-P-2. On the other hand, the N-terminal sequence of LE-S-15 was identical to the C-terminal sequence of CB-12. Therefore, the additional 15 residues up to 197th were extended.

The amino acid sequence of the C-terminal region after the 197th residue was almost determined from the analysis of BS-3 obtained with BNPS-skatole. Analysis of BS-3 provided the sequence that overlapped with 5 residues after $^{192}$Trp of LE-S-15, and thus extended the additional 48

TABLE I. The Amino Acid Sequence of BS-3-T-1

| Cycle | Amino acid | Yield (pmol) |
|---|---|---|
| 1 | Asn | 171.3 |
| 2 | Asn | 222.7 |
| 3 | Met | 190.3 |
| 4 | Ala | 77.6 |

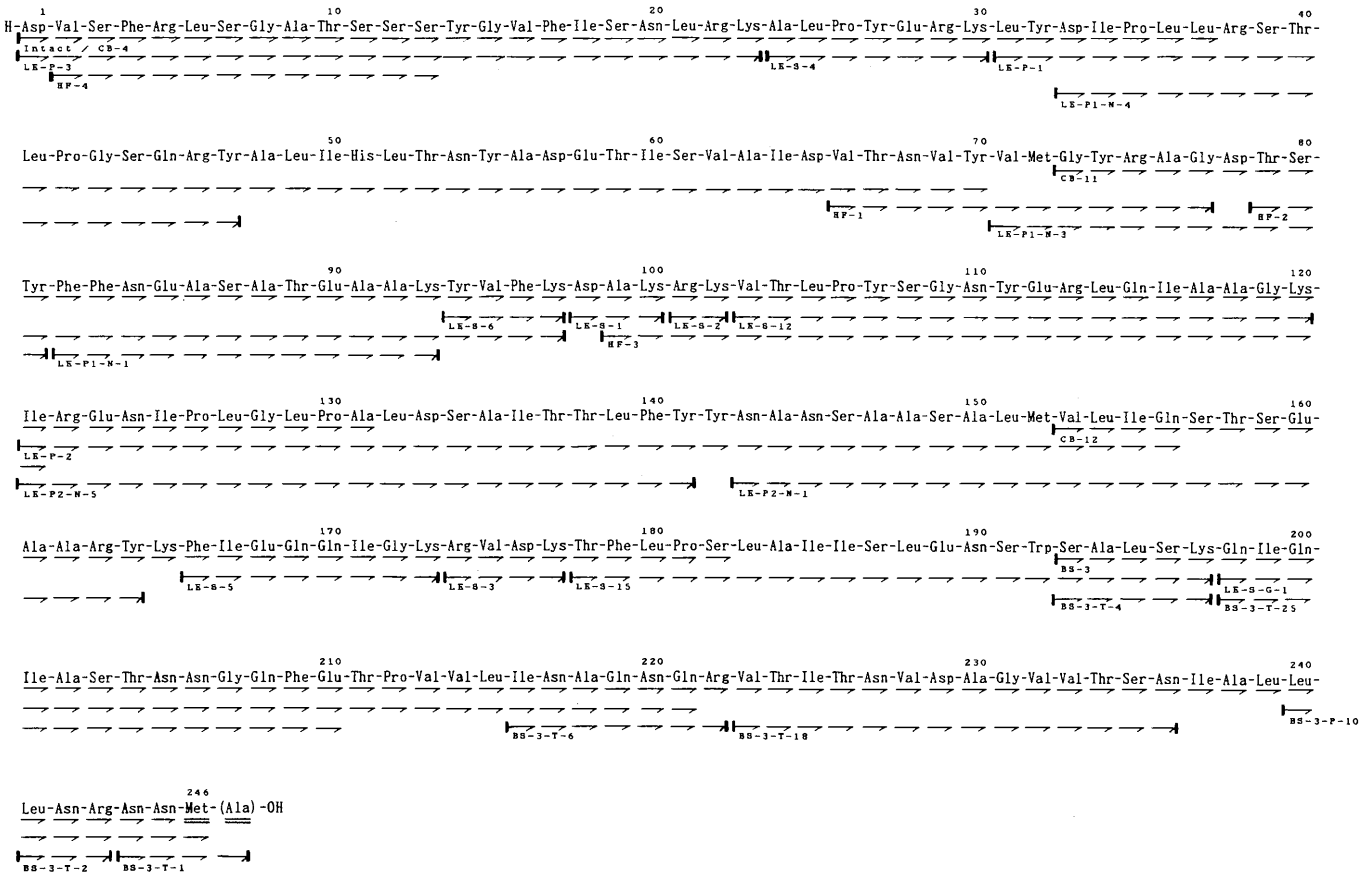


Fig. 7. The Complete Amino Acid Sequence of Karasurin

Arrowed residues were identified by automated Edman degradation. Vertical lines indicate an amino or carboxyl terminus of the peptides. Names of peptide fragments are shown under arrows. The double-underlines indicate the residues confirmed by hydrazinolysis.

residues up to $^{245}$Asn. On the other hand, the C-terminal residue of karasurin was determined by hydrazinolysis. Two amino acids, Met and Ala, were found and indicated a heterogeneous C-terminus. In order to determine the amino acid sequence of the C-terminal region of karasurin, BS-3 was further digested with trypsin or pepsin. In the resulting fragments, separated by RP-HPLC, a fragment containing Met was obtained from each digest: for BS-3-T-1, Asn–Asn–Met–Ala; for BS-3-P-10, Leu–Leu–Arg–Asn–Asn–Met. Since there were identical sequences of three amino acid residues between BS-3-T-1 and BS-3-P-10, two peptides are overlapping. Furthermore, the sequence of the N-terminal 5 residues of BS-3-P-10 was identical to that of the C-terminal region of BS-3. In particular, the result of sequence analysis of BS-3-T-1 was shown in Table I. The recovery of Ala at the 4th cycle decreased to 40% of Met at the 3rd cycle, indicating that BS-3-T-1 is a mixture of two peptides, Asn–Asn–Met and Asn–Asn–Met–Ala, derived from the heterogeneous C-terminal sequence consistently with the results of hydrazinolysis. Thus, BS-3-T-1 and BS-3-P-10 were estimated as C-terminal peptides of karasurin. However, there was no microheterogeniety in other peptides analysed. Therefore, karasurin is a protein of a single polypeptide chain, differing only in the C-terminus with or without the addition of an alanyl residue.

Other fragments obtained from various cleavages respectively confirmed the amino acid sequence as follows: LE-S-1, LE-S-2, LE-S-3, LE-S-4, LE-S-5, LE-S-6, LE-S-12, LE-S-G-1, LE-P-3, LE-P-1-N-1, LE-P-1-N-3, LE-P-1-N-4, LE-P-2-N-1, LE-P-2-N-5, HF-2, HF-3, HF-4, BS-3-T-2, BS-3-T-4, BS-3-T-6, BS-3-T-18, BS-3-T-25.

Thus, we determined the complete amino acid sequence

Table II. Amino Acid Composition of Karasurin

| | mol/mol[a] | Found[b] |
|---|---|---|
| Asp | 26.8 | 9 |
| Asn | — | 18 |
| Thr | 15.6 | 17 |
| Ser | 21.1 | 24 |
| Glu | 20.4 | 10 |
| Gln | — | 10 |
| Pro | 7.6 | 8 |
| Gly | 11.1 | 11 |
| Ala | 26.5 | 27/28 |
| Cys | 0.0 | 0 |
| Val | 14.4 | 16 |
| Met | 1.9 | 3 |
| Ile | 17.3 | 19 |
| Leu | 25.6 | 25 |
| Tyr | 14.0 | 14 |
| Phe | 9.0 | 9 |
| Lys | 11.4 | 11 |
| His | 0.7 | 1 |
| Arg | 13.5 | 13 |
| Trp | 1.0 | 1 |
| Total | 237.9 | 246/247 |

*a*) Composition was calculated from the data revealed with mol% in the corresponding paper. *b*) Composition was arranged from the sequence in this work.

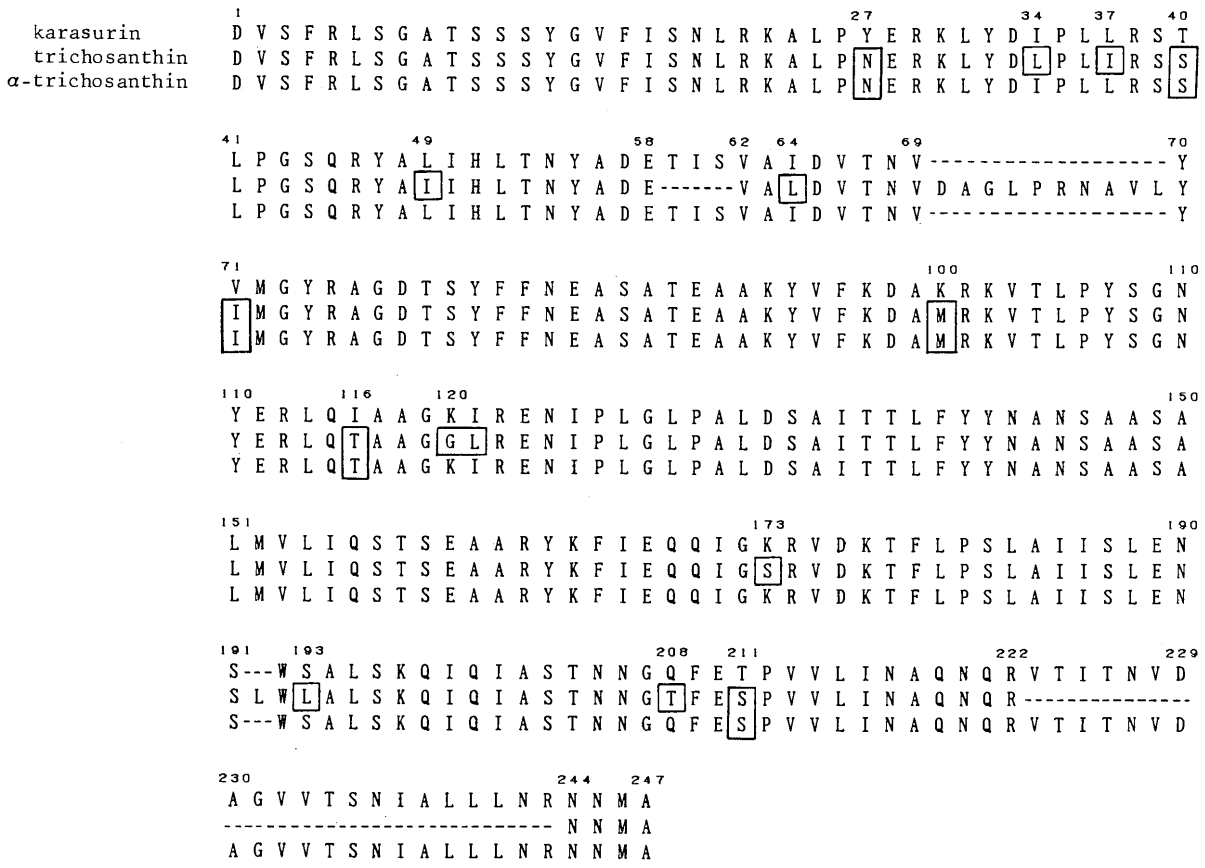


Fig. 8. Comparison of the Amino Acid Sequences of Karasurin, α-Trichosanthin and Trichosanthin

Differences in the sequences are boxed. The dotted line denotes the gap.

of 246 or 247 residues for karasurin as shown in Fig. 7. In the sequence of karasurin, two and three basic amino acid clusters were present at three and one positions, respectively. Hydrophobic clusters were also characteristically present at $^{16}$Val—$^{18}$Ile, $^{31}$Leu—$^{37}$Leu, $^{69}$Val—$^{71}$Val, $^{139}$Leu—$^{142}$Tyr, $^{153}$Val—$^{155}$Ile, $^{213}$Val—$^{216}$Ile. It is noteworthy that the two homologous sequences consisting of aromatic amino acids were present at $^{81}$Tyr—$^{84}$Asn and $^{140}$Phe—$^{143}$Asn, and the sequence of Thr(or Ser)–Glu–Ala–Ala–Lys-(or Arg)–Tyr–Val(or Lys)–Phe consisting of charged and aromatic amino acids was repeated at $^{89}$Thr—$^{96}$Phe and $^{159}$Ser—$^{166}$Phe.

Table II shows the amino acid number of karasurin from the determined sequence. This result was consistent with the amino acid number, which was estimated from the amino acid composition and the molecular weight as described previously.[1] Since sequence determinations for overlapping fragments were adequately obtained, all expected amino acid residues of karasurin were recovered in the sequence. Karasurin consisted of 246 or 247 amino acid residues with a calculated molecular weight of 27144 or 27215. Karasurin had an abundance of His, Arg and Lys (25 residues) compared with Asp and Glu (19 residues). Thus, karasurin is a basic protein, as expected previously.

**Comparison of the Amino Acid Sequence** We described previously that karasurin is a homologous protein to trichosanthin, which was purified from the root tubers of *Trichosanthes kirilowii* MAX. (collected in Southern China), a varietas of *Trichosanthes kirilowii* MAX. var. *japonicum* KITAM. of this study, and its amino acid sequence was determined by Wang *et al.*[5] Comparison of the complete amino acid sequence of karasurin with trichosanthin showed a considerable homology between them, with 93% identity. However, there were gaps at four positions. These are, the absence of 3 amino acids ($^{59}$Thr—$^{61}$Ser of karasurin) in trichosanthin, 10 amino acids ($^{69}$Val—$^{70}$Tyr) in karasurin, a single amino acid ($^{191}$Ser—$^{192}$Trp) in karasurin and 21 amino acids ($^{223}$Val—$^{243}$Arg) in trichosanthin, respectively (Fig. 8). Thus, karasurin and trichosanthin are different, but are closely related as previously expected.[1]

Most recently, α-trichosanthin was purified from the root tubers of *Trichosanthes kirilowii* MAX. (collected in Southern China), which is a ribosome-inactivating protein and is shown to have potent activity against human immunodeficiency virus (HIV)-1-infected T-cells and macrophage, and the amino acid sequence was determined.[16] The genomic sequence of α-trichosanthin, which was obtained from the leaves of *Trichosanthes kirilowii* MAX. (collected in South Korea), has been also determined and the amino acid sequence of α-trichosanthin confirmed except for two amino acid substitutions among two proteins from Southern China and South Korea.[17] Interestingly, the results of molecular cloning and the direct protein sequencing of α-trichosanthin revealed an extended carboxyl terminus and the processing of the terminus, which resulted in heterogeneous carboxyl terminus as Met and Ala, consistent with our results. These data showed that α-trichosanthin has the same amino acid length as karasurin, suggesting that karasurin and α-trichosanthin are highly homologous proteins, with 98% identity in the varietas of *Trichosanthes kirilowii* MAX. (Fig.

8). It is likely that karasurin–α-trichosanthin is present universally in *Trichosanthes kirilowii* MAX., even if the habitats are different. On the contrary, we have not yet obtained a protein identical to trichosanthin from *Trichosanthes kirilowii* MAX. var. *japonicum* KITAM. during this study (described in previous paper).[1] Nevertheless, the amino acid sequences of karasurin and α-trichosanthin showed differences at six positions, $^{27}$Tyr, $^{40}$Thr, $^{71}$Val, $^{100}$Lys, $^{116}$Ile and $^{211}$Thr of karasurin (Fig. 8). The divergence of their sequences is unexpectedly large in respect to their phylogenical distance, although these differences could be explained by a single nucleotide substitution. We expect that the ancestral gene of karasurin–α-trichosanthin diverged rapidly, expressing several structurally related proteins, and formed a family consisting of a large number of abortifacient proteins. In fact, we have isolated a protein which is more basic than karasurin and is potent in inducing mid-term abortion in pregnant mice similarly to karasurin, from the root tubers of *Trichosanthes kirilowii* MAX. var. *japonicum* KITAM. (in preparation).

**Acknowledgements** The authors are grateful to Dr. Y. Yamakawa of the National Institute of Human Health Sciences of Japan and Dr. K. Kitamura and Dr. Y. Sakamoto of the Saitama Medical College for their help in the sequence analysis and for valuable suggestions. The authors also thank Dr. K. Sakamoto and Mr. Y. Waki of the Tumura Research Institute for Pharmacology for their continuing interest and encouragement.

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*Chem. Pharm. Bull.* **39**(5) 1250—1252 (1991) Vol. 39, No. 5

# Solubilization of Saponins of Bupleuri Radix with Ginseng Saponins: Effect of Malonyl-ginsenosides on Water Solubility of Saikosaponin-$b_1$

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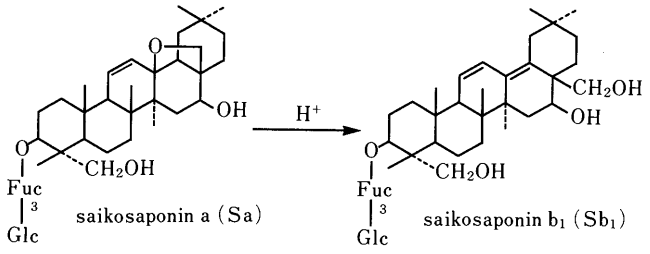
**Saikosaponins, the active principles of Bupleuri Radix, are generally sparingly soluble in water. The solubilizing effect of Ginseng saponins on saikosaponin-$b_1$ ($Sb_1$) which is formed from saikosaponin-a (Sa) by mild acid treatment, was investigated. It was revealed that the significant increase of the water-solubility of $Sb_1$ with Ginseng saponin mixture was due mainly to malonyl-ginsenosides. Some solubilizing effect was also observed with ginsenoside-Ro (Ro). No solubilizing effect was found with the neutral dammarane saponins, while the effects of malonyl-ginsenosides and Ro were remarkably potentiated in the presence of these neutral dammarane saponins. The water-solubility of Sa was also increased in the presence of malonyl-ginsenosides under cooperation with the neutral dammarane saponins.**

**Keywords** Ginseng saponin; solubilizing effect; malonyl-ginsenoside; ginsenoside; saikosaponin; Bupleuri Radix; dammarane saponin; cooperative solubilization

In our serial studies on surface-active properties of oligo-glycosides, it has been reported that the water solubility of saikosaponin-a (Sa), one of the active principles of the Bupleuri Radix, is greatly increased in the presence of glucuronide saponins of Ginseng[1,2] and licorice[3], which are sometimes coprescribed with Bupleuri Radix in oriental traditional medicines. Investigation of the structure–solubilization relationship revealed the important role of the carboxyl group of the glucuronide moiety for increasing the water solubility of Sa.

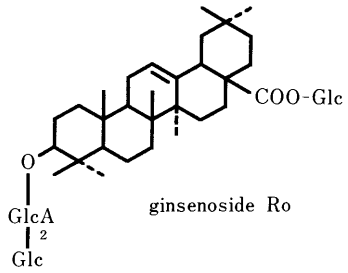
It has been known that the allyloxide moiety of Sa is very unstable and readily converted into the diene-saponin, saikosaponin-$b_1$ ($Sb_1$) on heating or by mild acid treatment.[4,5] Formation of some of $Sb_1$ from Sa was also observed during the decoction of Bupleuri Radix. Ogihara *et al.* reported on the pharmacological importance of diene-saponins of this type.[6] The present paper deals with the solubilization of $Sb_1$ with Ginseng saponins.

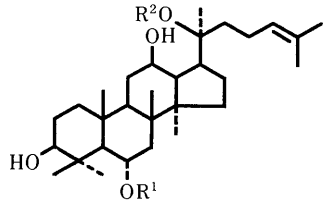
The results of the solubilizing effects are illustrated in Fig. 1. It was found that $Sb_1$ is much less soluble in water than Sa; saturated concentrations of Sa and $Sb_1$ in water at 37 °C are 0.1 and 0.02 mg/ml, respectively. An aqueous extract of Ginseng was subjected to chromatography on highly porous synthetic polymer resin with water, 40% methanol, and then methanol as eluates. An increase of the water solubility of $Sb_1$ was observed not with the water and 40% methanol



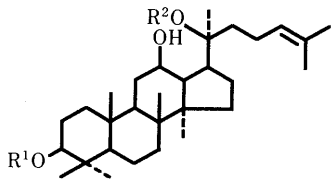

Fuc : β-D-fucopyranosyl
Glc : β-D-glucopyranosyl

Chart 1



| | R¹ | R² |
|---|---|---|
| ginsenoside $Rg_1$ | -Glc | -Glc |
| Rf | -Glc²—Glc | -H |
| Re | -Glc²—Rha | -Glc |



| | R¹ | R² |
|---|---|---|
| ginsenoside $Rb_1$ | -Glc²—Glc | -Glc⁶—Glc |
| $Rb_2$ | -Glc²—Glc | -Glc⁶—Ara (*p*) |
| Rc | -Glc²—Glc | -Glc⁶—Ara (*f*) |
| Rd | -Glc²—Glc | -Glc |
| malonyl-ginsenoside $Rb_1$ | -Glc²—Glc⁶—Ma | -Glc⁶—Glc |
| $Rb_2$ | -Glc²—Glc⁶—Ma | -Glc⁶—Ara (*p*) |
| Rc | -Glc²—Glc⁶—Ma | -Glc⁶—Ara (*f*) |
| Rd | -Glc²—Glc⁶—Ma | -Glc |

GlcA : β-D-glucuronopyranosyl
Glc : β-D-glucopyranosyl
Ara (*p*) : α-L-arabinopyranosyl
Ara (*f*) : α-L-arabinofuranosyl
Rha : α-L-rhamnopyranosyl
Ma : malonyl

Chart 2

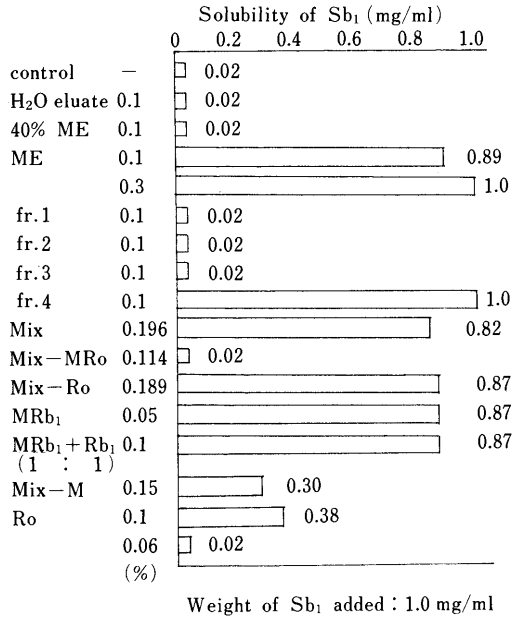



Fig. 1. Solubilizing Effects on $Sb_1$ (37 °C, 24 h, in $H_2O$)

eluates but with the methanol eluate (ME). This eluate (ME) was separated by chromatography on silica gel, affording four fractions tentatively named frs. 1—4 according to an increasing order of polarity. Fractions 1—3 showed no solubilizing effect, while the solubility of $Sb_1$ is greatly increased in the presence of fr. 4; 1 ml of 0.1% solution of fr. 4 dissolved more than 1 mg of $Sb_1$ at 37 °C. At thin layer chromatogram (TLC) of each fraction demonstrated that fr. 1 contained no saponin and fr. 2 consisted of neutral saponins of 20(*S*)-protopanaxatriol. Fraction 3 contained neutral saponins of both 20(*S*)-protopanaxatriol and 20(*S*)-protopanaxadiol, while fr. 4 was composed mainly of neutral saponins of 20(*S*)-protopanaxadiol and acidic saponins "Ro and malonyl-ginsenosides," the latter of which were recently isolated from Ginseng by Kitagawa *et al.*[7] This suggested that the acidic saponins play a major role in the solubilization of $Sb_1$.

Contents of the major saponins in ME determined by high performance liquid chromatography (HPLC)[8] were as follows: ginsenoside-$Rg_1$: 11.7%, -Rf; 12.2%, -Re: 3.6%, -Rd: 3.3%, -Rc: 3.4%, -$Rb_2$: 5.5% and $Rb_1$ ($Rb_1$): 8.2%, Ro: 1.9% and malonyl-ginsenosides-$Rb_1$ ($MRb_1$): 10.3%, -$Rb_2$: 2.8% and -Rc: 2.3%. Based on these analytical results, a mixture of neutral and acidic saponins in a ratio of contents in ME was prepared, being designated as Mix; 1.96 mg of Mix is equivalent to 3 mg of ME with respect to the composition of the major saponins. As shown in Fig. 1, a 0.196% aqueous solution of Mix exhibited almost the same solubilizing effect as that of 0.3% aqueous solution of ME. On the other hand, no solubilizing effect was observed with the neutral saponin mixture (Mix without Ro and malonyl-ginsenosides designated as Mix−MRo) at the concentration of 0.144% [=Mix 1.96 mg−acidic saponins 0.52 mg in 1 ml]. A 0.189% aqueous solution of a saponin mixture without Ro (Mix−Ro) [=Mix 1.96 mg−Ro 0.07 mg in 1 ml] showed a similar solubilizing effect to that of a 0.3% solution of ME. It was further noted that a 0.05% aqueous solution of $MRb_1$ which is approximately equivalent to the concentration of total malonyl-ginsenosides in a 0.3% solution of ME, exhibited the same solubilizing effect as a 0.3% solution of ME. These results indicated that the solubilization of $Sb_1$ with ME (0.3% solution) is mainly due to the presence of malonyl-ginsenosides.

In our study on the solubilization of Sa with Ro, it was found that the solubilizing effect of Ro on Sa was significantly potentiated in the presence of the neutral dammarane saponins, which themselves do not increase the water solubility of Sa.[2] A similar cooperation of the neutral dammarane saponin was also observed in the present study. A 0.025% aqueous solution of $MRb_1$ did not show a solubilizing effect on $Sb_1$, while a 0.05% aqueous solution of a mixture of $MRb_1$ and $Rb_1$ (1 : 1) dissolved $Sb_1$ at the same concentration as that in a 0.05% solution of $MRb_1$ (*vide supra*).

It was reported that malonyl-ginsenosides are unstable and partly or completely decomposed to give the parent neutral ginsenosides[7] during the extraction or separation process under vigorous conditions. Decomposition of malonyl-ginsenosides is observed also during the process of the preparation of Red-Ginseng (treatment with hot steam). As shown in Fig. 1, some solubilizing effect was observed also with a 0.15% aqueous solution of a mixture of the neutral saponins and Ro (Mix−M) [=Mix 1.96 mg−(malonyl-ginsenosides) 0.46 mg/ml]. This evidence indicates that an increase in solubility of $Sb_1$ can be expected for Ginseng extract or saponin fractions prepared even under the vigorous conditions which result in decomposition of malonyl-ginsenosides. The potentiation of the solubilizing effect by concurring neutral dammarane saponins is operative also in case of the solubilization of $Sb_1$ with Ro. It was found that 1 ml of 0.1% aqueous solution of Ro dissolved 0.38 mg of $Sb_1$, while no solubilizing effect was observed with a 0.006% aqueous solution of Ro which approximately corresponds to a 0.3% aqueous solution of Mix−M with respect to the concentration of Ro. However, as mentioned above, addition of the neutral dammarane saponins to this dilute solution of Ro (0.15% solution of Mix−M) resulted in the regeneration of the solubilizing effect.

In our previous study on the solubilization of Sa,[2] a saponin mixture was obtained by the extraction of the aqueous suspension of an aqueous extract of Ginseng with 1-butanol. It was found that in the case of this butanolic participation, most malonyl-ginsenosides remain in an aqueous layer. Accordingly the saponin mixture used in the previous study[2] contained only a trace of malonyl-ginsenosides. In the present study, an increase in the water solubility of Sa with Ginseng saponins was reinvestigated using the fraction (ME) which contains malonyl-ginsenosides. The result revealed the important role of both Ro and malonyl-ginsenosides.[10] The saturated concentration of Sa in water at 37 °C is 0.1 mg/ml. It was found that more than 5 mg of Sa were dissolved in 1.0 ml of 0.1 and 0.3% aqueous solutions of ME at 37 °C. Aqueous solutions of Mix exhibited a similar solubilizing effect on Sa at the corresponding concentration, 0.065 and 0.196%, respectively. Solubilizing effects of Mix−MRo, Mix−Ro and Mix−M at the concentrations which correspond to 0.1 and 0.3% solutions of ME were determined. No solubilizing effect was observed with 0.048 and 0.144% solutions of

Mix – MRo. A 0.15% aqueous solution of Mix – M showed a similar effect to that of a 0.3% aqueous solution of ME, while a 0.05% aqueous solution of Mix – M dissolved Sa only at the concentration of 1.0 mg/ml, which was less than that with a 0.1% aqueous solution of ME. On the other hand, with regard to Mix – Ro, 1 ml of its 0.064% aqueous solution dissolved more than 5.0 mg of Sa but 1 ml of its 0.189% aqueous solution dissolved only 2.1 mg of Sa. This result suggested that the participation of the solubilizing effect of Ro is more significant in a relatively higher concentration, while that of malonyl-ginsenosides is more remarkable in relatively lower concentrations in the case of Sa.

The present results in the surface-active interaction between the constituents of the crude drugs must be noteworthy in view of the pharmaceutical superiority of extracts of the oriental traditional medicines, which are prepared by coprescription of several crude drugs.

## Experimental

**Materials** All of the standard saponins in the present study were obtained in our serial studies.

**Extraction and Separation of the Saponin Mixture from Ginseng Roots** Commercial dried Ginseng roots without peeling (1 kg) were extracted with $H_2O$ at room temperature. The water extract was subjected to chromatography on a highly porous synthetic polymer resin (Diaion HP-20), Mitsubishi Chemical Industries Co., Ltd., Japan) with $H_2O$, 40% MeOH and MeOH successively. The fraction eluted with MeOH was further chromatographed on silica gel with solvent: $CHCl_3$–MeOH–$H_2O$ (6 : 4 : 1) to give four fractions, fr. 1 (yield from Ginseng 0.017%), fr. 2 (0.097%), fr. 3 (0.092%) and fr. 4 (2.77%).

**Quantitative Analysis of Ginsenosides in ME by HPLC** An HPLC 803D (Toyo Soda Co., Ltd., Japan), equipped with a variable wave length detector (UV 8000, Toyo Soda) and an Ultron N-18 column (Chromato Packings Center, Kyoto, Japan, particle size, 5 $\mu$m; 4.6 × 150 mm) was used; flow rate: 1.0 ml/min, detection: absorption at 203 nm.

For HPLC analysis of Re and $Rg_1$, $CH_3CN$–0.5% $H_3PO_4$ (20 : 80 v/v) was used as a mobile phase. A methanolic solution of $Rg_1$ was subjected to HPLC analysis. The calibration plots for peak area ratio *vs.* concentration were found to be linear in the range of 5—10 $\mu$g per injection of $Rg_1$, and the curves could be extrapolated through zero. 10 $\mu$l of 1.0 mg/ml methanolic solution of Re was subjected to HPLC analysis under the same conditions as for $Rg_1$. The relative ratio of the peak area of Re to that of the same concentration of $Rg_1$ was obtained, being used for the quantitative analysis of these saponins.

For HPLC analysis of $MRb_1$, $MRb_2$, MRc, $Rb_1$, $Rb_2$, Rc, Rd, Rf and Ro, 50 mM $KH_2PO_4$ in 31% (v/v) $CH_3CN$ was used as a mobile phase. The calibration plots for peak area ratio *vs.* concentration were found to be linear in the range of 5—15 $\mu$g per injection of Rc, and the curve could be extrapolated through zero. 10 or 15 $\mu$l of 1.0 mg/ml methanolic solutions of the other saponins to that of the same concentration of Rc were obtained, respectively, being used for the quantitative analysis of these saponins.

For analysis of saponins in ME, a solution of ME was prepared to 10 mg/ml, and 10 or 15 $\mu$l of this solution were subjected to HPLC analysis.

**Determination of Solubilizing Effects** A solution of an excess of $Sb_1$ in MeOH containing a Ginseng saponin (or a mixture), was concentrated to complete dryness. The residue was taken up in water, and after sonication for several minutes, the mixture was shaken at 37 °C for 24 h. The mixture was filtered to remove insoluble material. A measured volume of the $Sb_1$ content was determined by TLC-densitometry on a Shimadzu Dual wave length TLC-scanner, CS-930, equipped with a Shimadzu data-recorder DR-2 according to the methods reported in previous papers[1,2,9]; TLC plate: Kieselgel $60F_{254}$ (20 × 20 cm, Art. 11798, Merck), solvent: $CHCl_3$–MeOH–$H_2O$ (30 : 10 : 1, homogeneous), s, 256 nm; r, 370 nm.

A solution of an excess of Sa in MeOH containing a Ginseng saponin (or mixture), was concentrated to complete dryness. The residue was taken up in water and after sonication for several minutes, the mixture was shaken at 37 °C for 24 h. The mixture was filtered to remove insoluble material. A measured volume of an aliquot of the filtrate was lyophylized, and the residue was treated with a mixture of 1.5 N HCl and MeOH (1 : 1) at 25 °C for 16 h. The reaction mixture was neutralized with 1.5 N aqueous NaOH and diluted with MeOH to a definite volume. The content of Sa was determined by TLC densitometry of the resulting diene-saponin $Sb_1$ under the same conditions as described above.

**Acknowledgment** We are grateful to Wakunaga Pharmaceutical Co., Ltd, for supplying Ginseng roots and to Drs. H. Matsuura and H. Yamaguchi of this Company for their valuable advice on the analysis of Ginseng saponins.

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10) It should be noted that the exact comparison of the data on the solubilization in the present experiments with those of the previous study[2] is difficult, since the compositions of saponins in ME are somewhat different from those of FII in the previous study.

# High-Performance Liquid Chromatographic Determination of 2-Alkenals in Oxidized Lipid as Their 7-Amino-6-methylquinoline Derivatives

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**High-performance liquid chromatographic determination of several 2-alkenals, specially acrolein, crotonaldehyde, 2-pentenal, 2-hexenal, 2-heptenal, 2-octenal and 2-nonenal, were studied using the precolumn derivatization reagent 2,4-diaminotoluene. After the selective condensation of these 2-alkenals to form fluorescent 7-amino-6-methylquinoline derivatives, separation of these compounds was achieved on a ODS column (150 × 4 mm i.d.) with a linear gradient system of acetonitrile and 50 mM mono-ammonium phosphate containing 5 mM sodium 1-octanesulfonate (from 20% acetonitrile to 60% acetonitrile in 1 h), and the separated quinoline derivatives were detected with a fluorescence monitor (396 nm for excitation and 485 nm for emission). Three autooxidized unsaturated fatty acid methyl esters and five autooxidized edible oils were subsequently assayed for acrolein ($C_3$), crotonaldehyde ($C_4$), 2-pentenal ($C_5$), 2-heptenal ($C_7$), 2-octenal ($C_8$) and 2-nonenal ($C_9$). The peak of 2-hexenal was interfered with the unknown peak derived from oxidized lipids. Their detection limits ($S/N=2$) were 10, 10, 25, 25, 50, 50 pg for acrolein, crotonaldehyde, 2-pentenal, 2-heptenal, 2-octenal and 2-nonenal, respectively.**

**Keywords** 2-alkenal; autooxidized lipid; acrolein; 2-pentenal; 2-heptenal; 2-octenal; 2-nonenal; 2,4-diaminotoluene; 7-amino-6-methylquinoline derivative; high-performance liquid chromatography

## Introduction

Current emphasis on the problems of lipid peroxidation has stimulated research in food chemistry and biochemistry. Hydroperoxides and secondary decomposition products from peroxidized lipids have been recognized as health hazards.[1-4] The chemical characterization of these compounds has recently received much attention.[5,6]

It has also been generally accepted that malondialdehyde (MDA), which forms during lipid peroxidation *in vitro*, can react with a wide variety of amino-substituted compounds,[7-9] including nucleic acid.[10,11] MDA has been often reported as a weak mutagen against bacterial tester strains and mammalian cells.[12-15] Marnett and Tuttle[16] pointed out that the mutagenic potency in *Salmonella typhimurium* strains depended not only on MDA but also on by-products formed during MDA chemical synthesis. They also found that 3,3-dimethoxypropionaldehyde and 3-methoxyacrolein,[17] which were formed from 1,1,3,3-tetramethoxypropane by acid hydrolysis, were more potent mutagens than MDA in *S. typhimurium* strains.[16] The reactivities of the acrolein derivatives such as 3-methoxyacrolein were higher than those of MDA.

Yoshioka and Kaneda found acrolein derivatives such as 4-hydroxy- and 4-hydroperoxy-2-alkenal in autooxidized methyl linoleate.[18] Furthermore, Benedetti *et al.* reported that 4-hydroxy-2,3-*trans*-nonenal, as the major cytotoxic substance, was isolated from the reaction mixture of the reduced nicotinamide adenine dinucleotide phosphate (NADPH)–Fe-induced oxidation of liver microsomal lipids.[19] They further reported the determination of alkanal and 2-alkenal as their 2,4-dinitrophenylhydrazone derivatives in the liver of bromobenzene-poisoned mice using high-performance liquid chromatography (HPLC).[20]

In a previous paper,[21] we investigated the quantification of acrolein in autooxidized unsaturated fatty acid methyl esters by using a modified fluorescence method based on the reaction of acrolein with *m*-aminophenol to yield a fluorescent derivative, 7-hydroxyquinoline. But instability of the fluorophore in wide ranges of pH was suspected and the HPLC analysis was not adopted for determination of 2-alkenals (acrolein derivatives) as a 7-hydroxyquinoline derivative.

The recent paper[22] reported the optimized conditions for fluorescent derivatization of 2-alkenals by using 2,4-diaminotoluene (DAT). The fluorometric determination of 2-alkenals in autooxidized lipids by use of 2,4-DAT was also carried out. Then, acrolein in autooxidized lipids was able to be determined by HPLC using the fluorometric method.

In the present paper, we examine the conditions for HPLC separation of seven 2-alkenals as 7-amino-6-methylquinoline derivatives ($C_3$—$C_9$). Finally, the fluorometric-HPLC determination of 2-alkenals in autooxidized lipids is carried out.

## Experimental

**Chemicals** All chemicals were of reagent grade. 2,4-Diaminotoluene (DAT) was obtained from Nakarai tesque (Kyoto, Japan), recrystallized from water before use and prepared its dihydrochloride salt with dry HCl gas in diethyl ether solution (Caution: 2,4-DAT is a carcinogen and must be handled with care.). Acrolein (2-propenal), crotonaldehyde (2-butenal), and methacrolein (2-methyl-2-propenal) were purchased from Aldrich (Milwaukee, WI) and were kept refrigerated. The other aldehydes, *e.g.*, 2-pentenal, 2-hexenal, 2-heptenal, 2-octenal, and 2-nonenal, were synthesized from formyl methyl triphenyl phosphorane with adequate aldehydes by the method of Trippett and Walker,[23] and were purified by distillation. Chemical purities were confirmed by gas chromatography. The 2-alkenals, whose purities are above 95%, were used as standard samples. Ethanol was refluxed with aluminum and potassium hydroxide for removal of aldehydes and ketones, and was distilled. Aldehyde stock solutions were prepared by weighing the cold aldehyde liquid previously pipetted into a flask on an analytical balance. To keep down evaporative losses, approximately 10 ml of ethanol was already contained in the tared flask. Because of the volatility and polymerizing capabilities of these unsaturated aldehydes, the stock solutions were kept refrigerated in the dark and used for no more than a week for analytical work. All analytical aldehyde standard solutions were pipetted fresh daily from the stock solutions. The 1.0% 2,4-DAT·2HCl solution (w/v) was prepared weekly with water. HPLC grade solvents were used for the chromatography. Methyl oleate, methyl linoleate, methyl linolenate and sodium 1-octanesulfonate were

purchased from Nakarai tesque (Kyoto, Japan). Five edible oils were obtained at a local market.

**Syntheses of 7-Amino-6-methylquinoline Derivatives** Acrolein (0.69 g, 11 mmol) was added to 2,4-DAT (1.5 g, 12 mmol) in 5 N HCl (5 ml) and ethanol (30 ml), and heated for 2 h in a boiling water bath.

To the residue obtained by concentrating the reaction mixture were added 50 ml of 10% NaOH, and the residue extracted with 30 ml portions of ethyl acetate twice. The ethyl acetate extracts were chromatographed on a silica gel column (Merck, Kiesel gel 60 $F_{254}$) with benzene–ethyl acetate (1 : 4). The second-eluting yellow compound was recrystallized from *n*-hexane to give 7-amino-6-methylquinoline (89 mg), mp 118—120 °C. The other physico-chemical data were listed in Table I. The other seven quinoline derivatives were synthesized as described above, and the physico-chemical data were also listed in Table I.

**Equipment** The HPLC system was composed of two Shimadzu (Shimadzu Seisakusho, Kyoto, Japan) model LC-6A pumps with a Shimadzu system controller SCL-6A, a Rheodyne (Berkeley, CA) 7010 injector with a 20 $\mu$l loop, a Shimadzu Techno-research Inc. (Kyoto, Japan) STR ODS-H column (150 × 4 mm i.d.) and a Gasukuro Kogyo Inc. (Tokyo, Japan) line filter (2 $\mu$m) insert, and a Shimadzu fluorescence HPLC monitor RF-535. The HPLC conditions were as follows; mobile phase: linear gradient system of acetonitrile + 50 mM mono-ammonium phosphate containing 5 mM sodium 1-octanesulfonate (from 20% acetonitrile to 60% acetonitrile in 1 h), flow rate: 0.8 ml/min, column temperature: at ambiance. The excitation and emission wavelengths were set at 396 and 485 nm, respectively, and the sensitivity: high, range: × 32. The detector signal was recorded by a Shimadzu Chromatopac C-R3A, and peak area integration was carried out.

**Procedure** The reaction conditions were essentially the same as those used by Hirayama *et al.*[22] A 1 ml volume of 1.0% 2,4-DAT·2HCl and 2 ml of 5 N HCl were added to each 0.5 ml of an alcoholic sample and 25 $\mu$g/ml methacrolein as the internal standard solution. There were placed in a 10 ml glass-stopper test tube marked at 4 ml and the mixture heated for 60 min in the boiling water bath. The reaction mixture was allowed to cool 10 min, and then water was added to the mark.

**Calibration Curves and Calculation** Calibration curves for 2-alkenal standard solutions containing 1.0, 2.0, 3.0 and 4.0 $\mu$g/ml were prepared by plotting ratios of 2-alkenal peak area to internal standard peak area against micrograms of 2-alkenal per milliliter. From the resulting ratios of 2-alkenal peak area to internal standard peak area from the sample chromatogram, the concentrations of 2-alkenals samples were read from calibration curves.

## Results and Discussion

**Synthesis of 7-Amino-6-methylquinoline Derivatives from 2-Alkenals** 7-Amino-6-methylquinoline was synthesized from acrolein and 2,4-DAT and its proton nuclear magnetic resonance ($^1$H-NMR) and mass spectral data are shown in Table I. 2-Alkyl-7-amino-6-methylquinolines were synthesized from linear 2-alkenals, whereas 7-amino-3,6-dimetylquinoline was formed from methacrolein (2-methyl-2-propenal).

**Optimization of Reaction and Chromatographic Conditions** Very few changes from the original work by Hirayama *et al.*[22] were necessary in the derivatization of 2-alkenals. 2,4-DAT was replaced by its hydrochloride salt, since free amino-compounds such as *m*-phenylenediamine derivative are oxidized by oxygen and sun-light as a reagent stock. In the previous paper, a hydroxylamine hydrochloride was used as an aldehyde trapping reagent, but $NH_2OH \cdot HCl$ was omitted from the reaction mixture in the present method, since Joshi and Jauhar[24] pointed out that the oximes of $\alpha,\beta$-unsaturated carbonyl compounds may form cyclization compounds such as isoxazoline derivatives. The excitation and emission maxima of the 7-amino-6-methylquinoline derivatives derived from 2-alkenals ($C_3$—$C_9$), measured in the HPLC eluent, are shown in Table II. The average of excitation and emission wavelengths of eight fluorophores were 395.9 and 484.8 nm, respectively. Therefore, 396 and 485 nm were selected to the maxima excitation and emission wavelengths, respectively, for the fluorescence detection.

As shown in Fig. 1, eight 2-alkenals could be separated perfectly on the ODS column which protected the silanol group by trimethylsilylation as their quinoline derivatives

TABLE I. Physico-chemical Data of 7-Amino-6-methylquinoline Derivatives for 2-Alkenals

| Starting material | mp (°C) | Formula | MS (*m/z*) | $^1$H-NMR ($\delta$ ppm, $CDCl_3$) Arom-H | $NH_2$ | $CH_3$ | $CH_2$ |
|---|---|---|---|---|---|---|---|
| Acrolein (2-Propenal) | 118—120 | $C_{10}H_{10}N_2$ | 158 (100%)<br>130 (17%) | 7.10 (dd, $J$=4.48, 8.15)<br>7.23, 7.46 (each s)<br>7.92 (dd, $J$=1.95, 8.30)<br>8.70 (dd, $J$=1.70, 4.30) | 3.80 (s) | 2.35 (s) | |
| Crotonaldehyde (2-Butenal) | 152—156 | $C_{11}H_{12}N_2$ | 172 (100%)<br>144 (15%) | 7.01 (d, $J$=3.28)<br>7.17, 7.43 (each s)<br>7.83 (d, $J$=8.30) | 4.01 (s) | 2.34 (s)<br>2.66 8s) | |
| Methacrolein | 87—89 | $C_{11}H_{12}N_2$ | 172 (100%)<br>144 (15%) | 7.21, 7.41 (each s)<br>7.71 (br s)<br>8.57 (d, $J$=2.00) | 3.96 (s) | 2.35 (s)<br>2.43 (s) | |
| 2-Pentenal | 115 | $C_{12}H_{14}N_2$ | 186 (100%)<br>158 (81%) | 7.04 (d, $J$=8.27)<br>7.19, 7.43 (each s)<br>7.85 (d, $J$=8.31) | 3.99 (s) | 1.36 (t, $J$=7.64)<br>2.34 (s) | 2.92 (2H, q, $J$=7.64) |
| 2-Hexenal | 79—81 | $C_{13}H_{16}N_2$ | 200 (100%)<br>185 (90%) | 7.02 (d, $J$=8.27)<br>7.20, 7.44 (each s)<br>7.84 (d, $J$=8.30) | 3.99 (s) | 1.00 (t, $J$=7.35)<br>2.34 (s) | 1.74—1.88 (2H, m)<br>2.87 (t-like) |
| 2-Heptenal | 83—84 | $C_{14}H_{18}N_2$ | 214 (5%)<br>199 (11%)<br>172 (100%) | 7.02 (d, $J$=8.27)<br>7.19, 7.43 (each s)<br>7.84 (d, $J$=8.30) | 3.98 (s) | 0.95 (t, $J$=7.68)<br>2.34 (s) | 1.35—1.49 (2H, m)<br>1.70—1.82 (2H, m)<br>2.88 (2H, t-like) |
| 2-Octenal | 81—82 | $C_{15}H_{20}N_2$ | 228 (11%)<br>199 (9%)<br>172 (100%) | 7.02 (d, $J$=8.30)<br>7.19, 7.43 (each s)<br>7.84 (d, $J$=8.06) | 3.98 (s) | 0.89 (t-like)<br>2.34 (s) | 1.30—1.45 (4H, m)<br>1.70—1.85 (2H, m)<br>2.88 (2H, t-like) |
| 2-Nonenal | 77—79 | $C_{16}H_{22}N_2$ | 242 (9%)<br>199 (6%)<br>172 (100%) | 7.02 (d, $J$=8.28)<br>7.20, 7.43 (each s)<br>7.84 (d, $J$=8.31) | 3.98 (s) | 0.88 (t-like)<br>2.34 (s) | 1.15—1.45 (6H, m)<br>1.70—1.85 (2H, m)<br>2.88 (2H, t-like) |

Abbreviation: s = singlet, d = doublet, dd = double doublet, t = triplet, q = quartet, m = multiplet, $J$ = coupling constant (Hz).

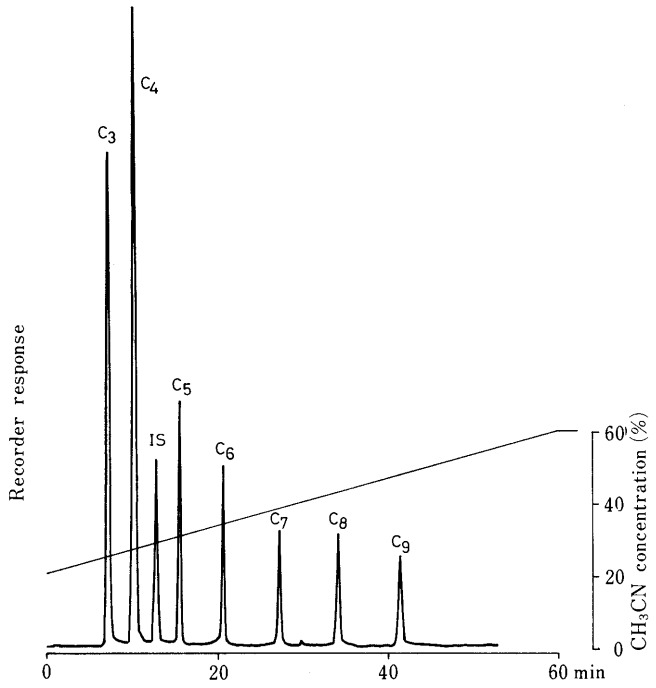


Fig. 1. High-Performance Liquid Chromatogram of Seven Standard 2-Alkenals as 7-Amino-6-methylquinoline Derivatives

IS (25 μg/ml, 0.5 ml) and seven standard 2-alkenals (containing each 4 μg/ml) solution (0.5 ml) were mixed and the mixture was reacted with 2,4-DAT reagent. IS = internal standard (methacrolein), $C_3$ = acrolein (2-propenal), $C_4$ = crotonaldehyde (2-butenal), $C_5$ = 2-pentenal, $C_6$ = 2-hexenal, $C_7$ = 2-heptenal, $C_8$ = 2-octenal, $C_9$ = 2-nonenal. Chromatographic conditions were described in experimental section. Detector sensitivity: high, range: 32, chart speed: 2.5 mm/min.

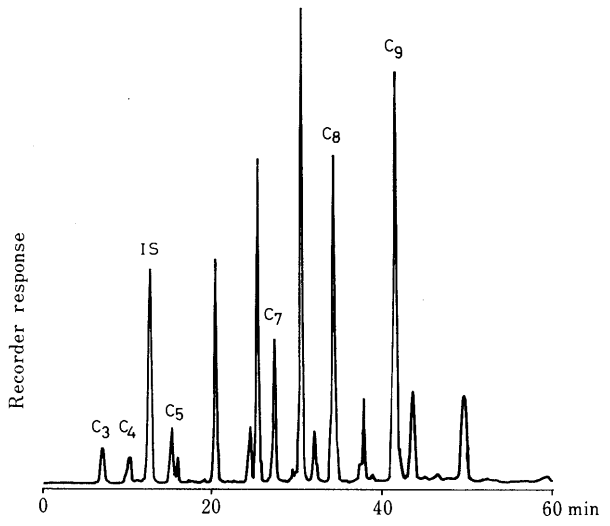


Fig. 2. Typical High-Performance Liquid Chromatogram of 2-Alkenals as 7-Amino-6-methylquinoline Derivatives in 20 h Autooxidized Methyl Linoleate

Sample solution (10 mg/ml, 0.5 ml) and IS (25 μg/ml, 0.5 ml) were mixed and the mixture was reacted with 2,4-DAT reagent. IS = internal standard (methacrolein), $C_3$ = acrolein, $C_4$ = crotonaldehyde, $C_5$ = 2-pentenal, $C_7$ = 2-heptenal, $C_8$ = 2-octenal, $C_9$ = 2-nonenal. Detector conditions were as described in Fig. 1.

by using a linear gradient elution system of acetonitrile and 50 mM $NH_4H_2PO_4$ containing 5 mM of sodium 1-octanesulfonate as an ion pair reagent. In the preliminary chromatographic studies on 2-alkenals in autooxidized unsaturated fatty acid methyl esters, methacrolein as 7-amino-3,6-dimethylquinoline was not observed in autooxidized lipid samples. Therefore, methacrolein was selected

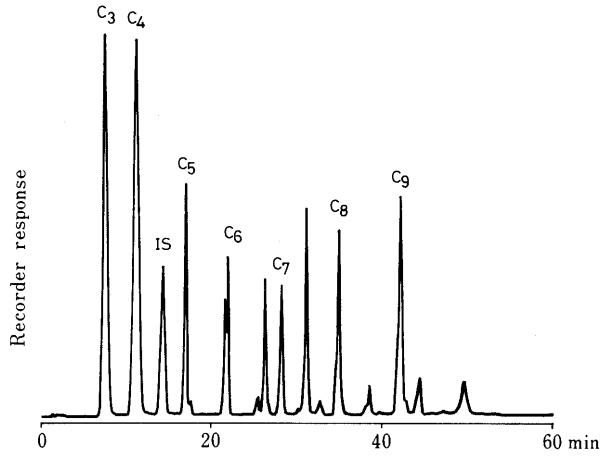


Fig. 3. High-Performance Liquid Chromatogram of 2-Alkenals in 20 h Autooxidized Methyl Linoleate Spiked with Seven Standard 2-Alkenals

Sample solution (10 mg/ml, 0.25 ml), 7 standard 2-alkenals (containing each 4 μg/ml) solution (0.25 ml) and IS (25 μg/ml, 0.5 ml) were mixed and the mixture was reacted with 2,4-DAT reagent. IS = internal standard (methacrolein), $C_3$ = acrolein, $C_4$ = crotonaldehyde, $C_5$ = 2-pentenal, $C_6$ = 2-hexenal, $C_7$ = 2-heptenal, $C_8$ = 2-octenal, $C_9$ = 2-nonenal. Detector conditions were as described in Fig. 1.

TABLE II. Excitation and Emission Maxima of 7-Amino-6-methylquinoline Derivatives from Standard 2-Alkenals (0.02 μg/ml) in 50 mM $NH_4H_2PO_4$ (Containing 5 mM Sodium 1-Octanesulfonate): $CH_3CN$ = 80 : 20

| Starting material | Excitation maximum (nm) | Emission maximum (nm) | Fluorescence intensity (%) |
|---|---|---|---|
| Acrolein (2-Propenal) | 398 | 490 | 43.8 |
| Crotonaldehyde (2-Butenal) | 396 | 482 | 100.0 |
| Methacrolein | 395 | 495 | 30.0 |
| 2-Pentenal | 395 | 481 | 89.0 |
| 2-Hexenal | 395 | 482 | 78.5 |
| 2-Heptenal | 396 | 483 | 65.2 |
| 2-Octenal | 396 | 483 | 59.8 |
| 2-Nonenal | 397 | 483 | 51.6 |

Fluorescence intensities were measured at 396 nm (Ex) and 485 nm (Em).

as an internal standard. As already shown in Table II, the fluorescence intensity of 7-amino-3,6-dimethylquinoline derived from methacrolein was weaker than those of 2-alkyl-7-amino-6-methylquinolines, and 0.5 ml of 25 μg/ml of methacrolein was selected for internal standard solution.

Further, the reaction yield (%) of 2-alkenals (each 5 μg/ml ethanol solution) with 2,4-DAT was measured by comparison of the amounts of formed quinoline derivatives with each standard quinoline derivatives.

The yields of quinoline derivatives [mean values and standard deviations (%, $n=3$)] were 63.69 ± 0.13 (2-propenal = acrolein), 57.39 ± 0.67 (2-butenal = crotonaldehyde), 19.92 ± 0.31 (methacrolein), 16.11 ± 0.34 (2-pentenal), 12.53 ± 0.29 (2-hexenal), 8.01 ± 0.24 (2-heptenal), 9.91 ± 0.35 (2-octenal) and 8.71 ± 0.19 (2-nonenal). The yields varied from 8.01% to 63.69%, but their standard deviations were satisfactorily small.

Figures 2 and 3 show the chromatograms of 2-alkenals in 20 h autooxidized methyl linoleate unspiked and spiked with seven standard 2-alkenals respectively. Although an unknown peak interfered the peak derived from 2-hexenal, the other peaks derived from 2-alkenals were separated from unknown peaks. The recoveries (%) of six 2-alkenals from

TABLE III. Recoveries of 2-Alkenals from 50 h Autooxidized Fatty Acid Methyl Esters

| Aldehyde | Added (μg) | Methyl linoleate[a] | | | Methyl linolenate[a] | | |
|---|---|---|---|---|---|---|---|
| | | Ratio of peak area[b] | Found (μg) | Recovery (%) | Ratio of peak area[b] | Found (μg) | Recovery (%) |
| Acrolein | 0 | 0.114±0.001 | 0.11 | — | 0.196±0.016 | 0.19 | — |
| (2-Propenal) | 2 | 1.891±0.062 | 1.83 | 86 | 2.257±0.027 | 1.86 | 84 |
| | 4 | 3.741±0.090 | 3.62 | 88 | 3.596±0.054 | 3.48 | 82 |
| Crotonaldehyde | 0 | 0.126±0.001 | 0.09 | — | 0.239±0.024 | 0.17 | — |
| (2-Butenal) | 2 | 2.374±0.086 | 1.69 | 80 | 2.346±0.056 | 1.67 | 75 |
| | 4 | 4.621±0.014 | 3.29 | 80 | 4.411±0.133 | 3.14 | 74 |
| 2-Pentenal | 0 | 0.069±0.033 | 0.21 | — | 0.467±0.015 | 1.42 | — |
| | 2 | 0.769±0.065 | 2.34 | 107 | 1.121±0.027 | 3.41 | 100 |
| | 4 | 1.430±0.018 | 4.35 | 103 | 1.706±0.042 | 5.19 | 94 |
| 2-Heptenal | 0 | 0.238±0.016 | 1.57 | — | 0.083±0.006 | 0.55 | — |
| | 2 | 0.553±0.025 | 3.64 | 104 | 0.404±0.013 | 2.66 | 106 |
| | 4 | 0.867±0.014 | 5.71 | 104 | 0.726±0.021 | 4.78 | 106 |
| 2-Octenal | 0 | 0.640±0.030 | 4.49 | — | 0.151±0.004 | 1.06 | — |
| | 2 | 0.913±0.052 | 6.40 | 96 | 0.438±0.015 | 3.07 | 101 |
| | 4 | 1.239±0.016 | 8.69 | 105 | 0.729±0.026 | 5.11 | 101 |
| 2-Nonenal | 0 | 0.993±0.084 | 7.48 | — | 0.259±0.009 | 1.95 | — |
| | 2 | 1.276±0.100 | 9.61 | 107 | 0.515±0.019 | 3.88 | 97 |
| | 4 | 1.552±0.062 | 11.69 | 105 | 0.758±0.029 | 5.71 | 94 |

*a*) 100 mg of 50 h autooxidized methyl linoleate and methyl linolenate were dissolved in ethyl alcohol (10 ml), separately, and sample solution (0.25 ml), standard 2-alkenals solution (0.25 ml) and internal standard solution (0.5 ml) were mixed and the mixture was reacted with 2,4-DAT reagent. *b*) Ratio of peak area [2-alkenal/internal standard (25 μg/ml methacrolein)]; average±standard deviation ($n=3$).

50 h autooxidized methyl linoleate and methyl linolenate at two fortification levels are summarized in Table III. The recoveries of aldehydes from these fatty acid methyl esters were satisfactory.

**Determination of 2-Alkenals by the 2,4-DAT-HPLC Method in 20 and 50 h Autooxidized Fatty Acid Methyl Esters** Table IV shows the amounts of six 2-alkenals in 20 and 50 h autooxidized unsaturated fatty acid methyl esters. In methyl oleate, 515.4 μg/g of 2-nonenal was determined in 20 h autooxidation sample, but five 2-alkenals except 2-pentenal decreased after prolonged oxidation (50 h). In methyl linoleate, 738.7 and 628.0 μg/g of 2-heptenal, 1016.0 and 1794.7 μg/g of 2-octenal, and 1912.4 and 2992.3 μg/g of 2-nonenal in 20 and 50 h autooxidized sample, respectively, were determined. The most abundant 2-alkenal was 2-nonenal in methylated oleic and linoleic acid, and about 0.3% of the aldehyde was determined in 50 h autooxidized methyl linoleate.

Katsuki *et al.*[25] reported the determination of volatile decomposition products in the oxidation mixtures of triolein, trilinolein, methyl oleate and methyl linoleate. In their paper, 2-decenal and 2-undecenal in oxidized methyl oleate, and 2-heptenal, 2-octenal and 2,4-decadienal in oxidized methyl linoleate were determined by gas chromatography-mass spectrometry (GC-MS) method, but 2-nonenal was detected from neither oxidized methyl oleate nor methyl linoleate. In the present study, 2-nonenal was detected in all of the autooxidized fatty acid methyl esters. In methyl linolenate, 668.0 and 563.9 μg/g of 2-pentenal were determined in 20 and 50 h autooxidized samples, respectively, and it is easily estimated that the formation of 2-pentenal from methyl linolenate is due to the cleavage of 13, 14 bond of methyl 13-hydroperoxy-9,11,15-octadecatrienoate by oxidation. In the present study, the amounts of lower molecular aldehydes such as acrolein and crotonaldehyde in 50 h autooxidized samples decreased as compare with those in 20 h autooxidation, since oils are oxidized at 80 °C under aeration and these aldehydes may vaporize and/or polymerize. Further, unknown large peaks at retention times of 21, 25 and 31 min were observed on the HPLC chromatograms of autooxidized methyl linoleate and methyl linolenate. These peaks, which are not observed in the chromatograms of autooxidized edible oils (data not shown), may be derived from methyl formylalkenoate as a cleavage fragment possessed a methyl ester end.

TABLE IV. Determination of 2-Alkenals (μg/g) in Autooxidized Fatty Acid Methyl Esters and Edible Oils

| Sample | Time[a] | $C_3$ | $C_4$ | $C_5$ | $C_7$ | $C_8$ | $C_9$ |
|---|---|---|---|---|---|---|---|
| Me oleate | 20 | 41.9 | 19.5 | 85.4 | 286.7 | 326.2 | 515.4 |
| | 50 | 23.6 | 17.6 | 105.6 | 145.2 | 145.2 | 290.4 |
| Me linoleate | 20 | 64.9 | 32.7 | 173.8 | 738.7 | 1016.0 | 1912.4 |
| | 50 | 43.2 | 34.0 | 83.3 | 628.0 | 1794.7 | 2992.3 |
| Me linolenate | 20 | 82.9 | 66.7 | 668.0 | 447.6 | 546.1 | 626.1 |
| | 50 | 74.3 | 64.5 | 563.9 | 219.0 | 423.5 | 780.5 |
| Corn oil | 20 | 34.7 | 14.5 | 96.8 | 80.5 | 235.5 | 622.4 |
| Olive oil | 20 | 11.9 | 3.4 | 12.9 | 26.3 | 76.3 | 136.5 |
| Rapeseed oil | 20 | 38.1 | 5.9 | 40.7 | 191.8 | 524.7 | 1969.6 |
| Soy bean oil | 20 | 32.9 | 8.8 | 69.8 | 157.4 | 457.2 | 1339.9 |
| Peanut oil | 20 | 25.0 | 6.3 | 34.4 | 149.3 | 487.4 | 918.9 |

*a*) Each oil was heated at 80 °C while being aerated (100 ml/min). Each 100 mg of 20 and 50 h oxidized fatty acid methyl esters and edible oils were dissolved in ethyl alcohol (10 ml), separately. Sample solution (0.5 ml) and internal standard solution (0.5 ml) were mixed and the mixture was reacted with 2,4-DAT reagent as described in the experimental section.

**Determination of 2-Alkenals by the 2,4-DAT-HPLC Method in 20 h Autooxidized Edible Oils** The amounts of six 2-alkenals in five 20 h autooxidized edible oils are also shown in Table IV. In the case of olive oil containing 88% oleate and 10% linoleate, the amount of each 2-alkenal was minimal in five edible oils. In rapeseed oil containing 37% of both oleate and linoleate and 23% linolenate, 1969.6 μg/g of 2-nonenal was detected. Regardless of the linolenate content, only 40.7 μg/g of 2-pentenal was detected. In soy bean oil containing 52% oleate and 47% linoleate,

1339.9 μg/g of 2-nonenal was detected in 20 h autooxidation sample.

From the above results, the content of 2-alkenals in the autooxidized oils were dependent not only on the content of highly unsaturated fatty acid but also on those of antioxidants in edible oils.

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# Isolation of Mitogenic Substance from Sclerotia of *Sclerotinia sclerotiorum* IFO 9395 Extracted with Phosphate Buffer

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**The buffer extracts (3S) of sclerotia of *Sclerotinia sclerotiorum* IFO 9395 contained mitogenic substance(s) to murine splenocytes (Shinohara *et al. Chem. Pharm. Bull.*, 38, 2219 (1990)). Although the native 3S was slightly mitogenic, heating of 3S induced significant mitogenic activity. To isolate the mitogen, we separated 3S by ion-exchange and gel filtration chromatographies. The isolated mitogen, named sclerogen, has a molecular mass of 32 kilodaltons (kDa) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the isoelectric point (pI) of 5.9 by chromatofocusing. Sclerogen was significantly mitogenic *in vitro* against murine splenocytes after heat denaturation, and also showed the augmentation of the primary mixed lymphocyte reaction (MLR) *in vitro*. However, sclerogen did not show the activation of an alternative pathway of complement and hemagglutination activity. These results suggest that sclerogen is a unique mitogen which differs from lectins and shows mitogenicity after heat denaturation.**

**Keywords** *Sclerotinia sclerotiorum*; sclerotia; mitogenic activity; fungal mitogen; phosphate buffer extract; heat denaturation; mixed lymphocyte reaction

Previously, we have demonstrated the existence of immunomodulator(s) in hot water extracts from sclerotia of *Sclerotinia sclerotiorum* IFO 9395.[1] The extracts, named TSHW, showed mitogenicity, polyclonal B cell activation, reticuloendothelial system stimulation, and antitumor activities.[1] In these activities, (1→3)-β-D-glucan parts and water insoluble-protein fractions played a part in the antitumor and mitogenic activities in TSHW, respectively.[2] Preliminary characterization of TSHW suggested the charge and molecular mass heterogeneities of the mitogen(s) in TSHW and resulted in difficulty in isolating the mitogenic substance. We concluded that this heterogeneity must result from some post-translational modifications during extraction.

Recently, we overcame this difficulty by extracting sclerotia with milder conditions. Interestingly, the phosphate buffer (P.B.) extracts, referred to as 3S, showed significant mitogenic activity after heat denaturation.[3] Treatment of native 3S for only 1 min in boiling water or 10 min at 70 °C generated an obvious mitogenicity. The activity was also observed against the splenocytes from C3H/HeJ, a lipopolysaccharide low-responder strain, and athymic (BALB/c Nu/Nu) mice. Accompanied with heat denaturation, the apparent physicochemical changes (probably polymerization) were observed in normal-polyacrylamide gel electrophoresis (PAGE).

In this study, we tried to isolate a mitogenic substance from 3S, and some biochemical characteristics were examined. The isolated mitogenic substance, named sclerogen, was a 32 kilodaltons (kDa) protein having no hemagglutinating activity against erythrocytes, showed mitogenicity after heat denaturation and augmented the primary mixed lymphocyte reaction (MLR) of splenocytes *in vitro*.

## Materials and Methods

**Mice** ICR, C3H/He, C3H/HeJ and C57BL/6 mice (6—12-wk-old, male) were from SLC, Japan, and were maintained under specific pathogen-free conditions for the duration of the experiments.

**Materials and Microorganisms** DEAE-Sephadex A-25, Polybuffer 74, and PBE 94 were obtained from Pharmacia Fine Chemicals, Sweden. Toyopearl TSK-gel HW-55F was from Tosoh, Japan. The prestained sodium dodecyl sulfate (SDS) molecular weight markers were from Sigma Chemical Co., Ltd., U.S.A. Silver stain kit Wako was from Wako Pure Chemical Industries, Japan. Tritiated thymidine ($^3$H-TdR), specific activity 38 Ci/mmol, was from ICN Biomedicals Inc., U.S.A. Sheep whole blood in Alsever's solution was from Japan Material Center, and human whole blood groups (A, B, O, and AB) were from healthy volunteers. Mouse whole blood was from C3H/HeJ. The mycelia of *Sclerotinia sclerotiorum* IFO 9395 were from the Institute for Fermentation, Osaka, Japan (IFO).

**Production of Sclerotia from *S. sclerotiorum* IFO 9395** The sclerotia were obtained by culturing on potato-sucrose agar media at 25 °C in our laboratory.[4] Sclerotia on the agar media were picked up after 3—4 weeks and lyophilized.

**Preparation of the Buffer Extracts from Sclerotia** The buffer extracts (3S) from sclerotia was prepared by the method previously described.[3]

**Assay for Mitogenic Activity** The measurement of mitogenic activity was carried out by the method previously described.[5] Briefly, the splenocytes from ICR or C3H/HeJ were prepared at a concentration of $5 \times 10^6$ cells/ml in ice cold RPMI 1640 medium with or without fetal calf serum (FCS) at a final concentration of 5%. Fifty μl of osmotic stabilized native sclerogen (sclerogen-M) or heat denatured-sclerogen (sclerogen-MB) were placed in a flat-bottomed 96-well microplate, and the cell suspension was added in 100 μl volumes. Cultures were incubated for 28 h at 37 °C in a 5% $CO_2$ incubator and pulsed with 0.5 μCi of $^3$H-TdR for a further 20 h. After harvesting cells, radioactivity incorporated into the cells was measured by a liquid scintillation counter. Data are expressed as mean cpm ± S.D. of triplicate cultures.

**Primary Mixed Lymphocyte Reaction (MLR)** Splenocytes from C3H/He were used as a responder and C57BL/6 spleen cells were used as a stimulator. Responder cells were prepared at $5 \times 10^6$ cells/50 μl in RPMI-1640 containing 10% FCS and $5 \times 10^{-5}$ M β-mercaptoethanol. Stimulators from C3H/He and C57BL/6 prepared at $3 \times 10^7$ cells/ml in Hanks' balanced salt solution (HBSS) were treated with mitomycin C (25 μg/ml final concentration) at 37 °C in a water bath for 30 min with gentle stirring every 10 min.[6] After washing with HBSS (three times), the cells were prepared at $5 \times 10^6$ cells/50 μl in RPMI-1640 containing 10% FCS and $5 \times 10^{-5}$ M β-mercaptoethanol. An osmotic stabilized-sample (sclerogen-M and -MB) in RPMI-1640 (50 μl) was added to each well in a flat-bottomed 96-well microplate, and then 50 μl of each prepared cell suspension was added. After 76 h of incubation at 37 °C in a 5% $CO_2$ incubator, 0.5 μCi of $^3$H-TdR was added and further incubated for 20 h. After harvesting cells, radioactivity incorporated into the cells was measured by a liquid scintillation counter. Data are expressed as mean cpm ± S.D. of quadruplicate cultures.

**Assay for Alternative Pathway of Complement** Twenty five μl of human serum from healthy volunteers were mixed with 5 μl of VBS–(EGTA)–$MgCl_2$ (VEM; 4.45 mM barbital sodium and 127 mM NaCl pH 7.4 (veronal buffered saline (VBS)) supplemented with 0.1% (w/v) gelatin, 2 mM $MgCl_2$, and 8 mM ethylene glycol bis(2-aminoethyl ether) tetraacetic acid (EGTA)), placed in a flat-bottomed 96-well microplate, and then incubated for 10 min at 37 °C. In the resulting mixture, 50 μl of sclerogen-M and -MB (82.3 μg/ml final concentration as bovine serum albumin (BSA)) were added and further incubated from 60 min at 37 °C. After incubation, the plates

were cooled on ice, and 50 $\mu$l of rabbit red blood cells (RRBC) suspended in VEM ($7.5 \times 10^8$ cells/ml) after washing with VBS–ethylenediaminetetraacetic acid (EDTA) (VBS containing 0.1% (w/v) gelatin and 10 mM EDTA) were added to each well. The turbidity of RRBC was measured during incubation at 25 °C every 15 min for 60 min by using a microplate reader at 630 nm.

**Hemagglutination Assay** Packed sheep red blood cells (SRBC), four gourps (A, B, O, and AB) of human red blood cells (HRBC), or mouse red blood cells from C3H/HeJ (MRBC) were washed with phosphate buffered saline (PBS) three times and then suspended to 1.5% (v/v) with the same buffer. Twenty five $\mu$l of sclerogen in saline (20.9 ng/ml to 42.8 $\mu$g/ml final concentration as BSA) were placed in each well of a round bottomed 96-well microplate, and 0.2% of gelatin in PBS (75 $\mu$l) and the prepared SRBC, HRBC, or MRBC (25 $\mu$l) were added. The plate was shaken for 30 s at r.t. and incubated for 1.5 h at 37 °C. Then, the plate was placed at r.t. overnight and assessed for hemagglutination ability. As a positive control, concanavalin A (Con A) was used in the range of 48.8 ng/ml to 100 $\mu$g/ml as a final concentration.

**Electrophoresis** Gel electrophoresis in the absence of SDS was carried out in 12.5% polyacrylamide slab gels according to the method of Ornstein[7] and Davis,[8] and in the presence of SDS by the method of Laemmli.[9] The details were given in a previous paper.[3]

**Chemical Analyses** Protein concentration was determined by the BCA protein assay reagent[10] (BSA as a standard). Amino acid analysis was performed in a Hitachi L-8500 amino acid analyzer after hydrolysis in 6 N HCl at 110 °C for each time.

## Results

### Conditions of Phosphate Buffer Extraction from Sclerotia

We have demonstrated that the P.B. extracts (3S) of sclerotia of *S. sclerotiorum* IFO 9395 showed significant mitogenic activity after heat denaturation. To determine the appropriate conditions of phosphate buffer extraction from sclerotia, we compared the efficiency of extraction at four kinds of pH (5.0, 6.0, 7.0, and 8.0) and for various periods (3, 6, 12, 24, and 48 h). Consequently, no significant difference was observed in each 3S extracted by the four kinds of pH, and the 3S have already been extracted within 3h (data not shown). Furthermore, the mitogenic activity of each 3S was similar to each other (data not shown). So, we determined to use the extraction condition for 3 h at 37 °C in 10 mM P.B. pH 6.8 for further analyses. The components of 3S extracted by this condition were carbohydrate (29%), protein (69%), and phosphate (19%).

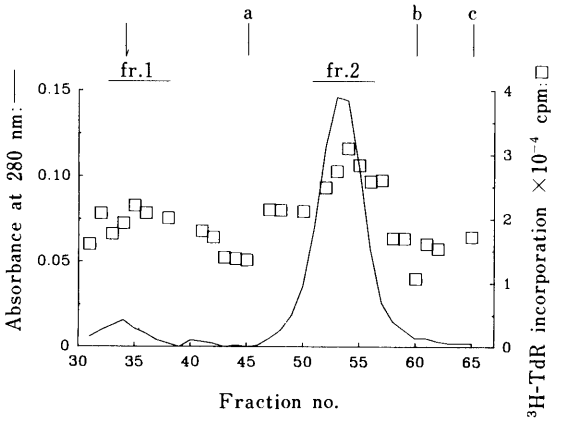


Fig. 1. Elution Profile of DEAE-Adsorbed 3S on TSK-gel HW-55F

DEAE-adsorbed 3S was applied to the column (1.7 × 50 cm) of TSK-gel HW-55F equilibrated with 10 mM P.B. pH 6.8. Fractions of 2.7 ml were collected at 7 °C and protein was monitored by UV absorption measurement at 280 nm (—). Mitogenicity of aliquots of fraction (□) were measured by using the splenocytes from C3H/HeJ ($5 \times 10^5$ cells/culture) under FCS free conditions described in Materials and Methods. Arrows indicate the void volume. a, bovine serum albumin (66 kDa); b, papain (23.4 kDa); c, cytochrome c (12.4 kDa). $V_t$ = fr. 75.

### Isolation and Characterization of the Mitogen, Sclerogen

To isolate the mitogenic substance, 3S was applied to the column of DEAE-Sephadex A-25 equilibrated with 10 mM P.B. pH 6.8. After being washed with the same buffer, the column was eluted with 1 M NaCl containing 10 mM P.B. pH 6.8. The first peak of the adsorbed fraction was collected, and then the fraction was applied to the column to TSK-gel HW-55 equilibrated with the same buffer. As shown in Fig. 1, only one peak corresponding to about 30 kDa monitored by ultraviolet (UV) absorption measurement was detected. From the results of the mitogenic activity of each fraction after autoclaving, two peaks (frs. 1 and 2, respectively) were detected (Fig. 1). However, on SDS-PAGE analysis, although fr. 2 showed a clear single band at 32 kDa under a reducing condition which corresponded to the main band

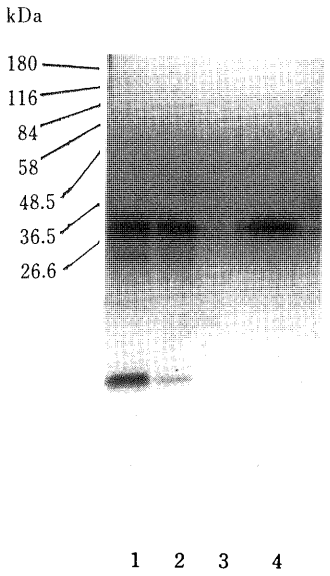


Fig. 2. SDS-PAGE Analysis of Sclerogen Following Isolation by Ion-Exchange Chromatography and Gel Filtration

Each sample was subjected to 12.5% SDS-PAGE under reducing conditions. Lane 1, crude 3S; lane 2, DEAE-adsorbed 3S; lane 3, fr. 1 from gel filtration on TSK-gel HW-55F (Fig. 3); lane 4, fr. 2 (sclerogen) from the gel filtration.

TABLE I. Amino Acid Composition of Sclerogen

| Amino acids | mol/mol |
|---|---|
| Asp (Asn) | 34.3 ± 2.2[a] |
| Thr | 20.1 ± 0.7 |
| Ser | 17.1 ± 3.4 |
| Glu (Gln) | 28.0 ± 1.2 |
| Gly | 25.2 ± 4.1 |
| Ala | 15.2 ± 1.6 |
| Cys | n.d. |
| Val | 14.4 ± 0.6 |
| Met | 4.5 ± 0.8 |
| Ile | 12.5 ± 1.0 |
| Leu | 17.9 ± 0.9 |
| Tyr | 7.5 ± 0.6 |
| Phe | 9.7 ± 0.5 |
| Lys | 19.2 ± 2.3 |
| His | 7.4 ± 1.5 |
| Trp | n.d. |
| Arg | 6.5 ± 0.4 |
| Pro | 12.4 ± 0.6 |

*a*) The values given are the mean ± S.D. of the three hydrolyses for 24, 48, and 72 h, expressed as each mol of amino acid per one mol of sclerogen. The values of Cys and Trp are not determined (expressed as n.d.).

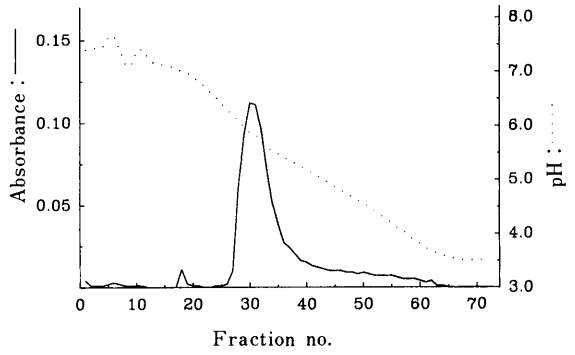


Fig. 3. Elution Profiles of Sclerogen on Chromatofocusing

Native sclerogen (sclerogen-M) was applied to the column of PBE 94 (10 ml) equilibrated with 0.025 M imidazole–HCl buffer pH 7.4. The column was eluted with a step gradient by using Polybuffer 74 adjusted to pH 4.0. Fractions of 1.6 ml were collected and protein (—) and pH (---) were monitored by ultraviolet absorption measurement and pH meter, respectively.

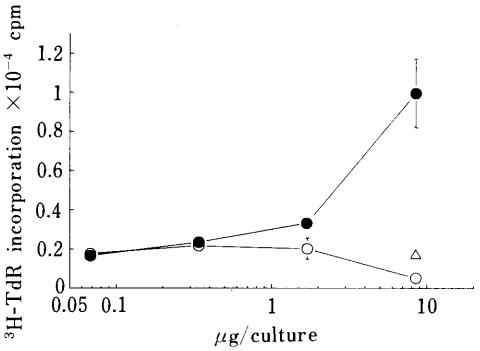


Fig. 4. Effect of Heat Denaturation on the Expression of Mitogenic Activity by Sclerogen

Splenocytes from C3H/HeJ ($5 \times 10^5$ cells/culture) were cultured with native sclerogen (sclerogen-M, ○) or heat denatured sclerogen (sclerogen-MB, ●) for 30 min in boiling water or in their absence (△) under FCS free conditions. The concentrations of sclerogen-M and -MB were expressed as BSA measured by the BCA protein assay reagent.[10]

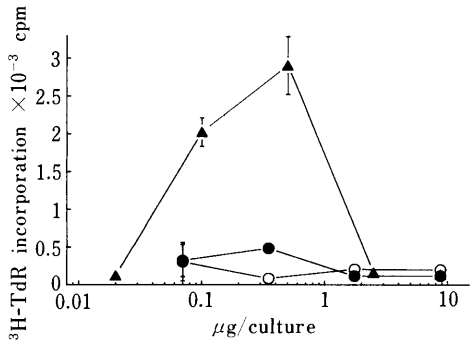


Fig. 5. Mitogenic Activity of Sclerogen to Thymocytes

Thymocytes ($5 \times 10^5$ cells/culture) from C3H/He were cultured with sclerogen-M (○) or -MB (●) or double distilled-water (△) in the presence of 5% FCS for 72 h. As a positive control, Con A (▲) was added to the culture. The concentrations of sclerogen-M and -MB were expressed as BSA, and expressed as the lyophilized-weight in the case of Con A.

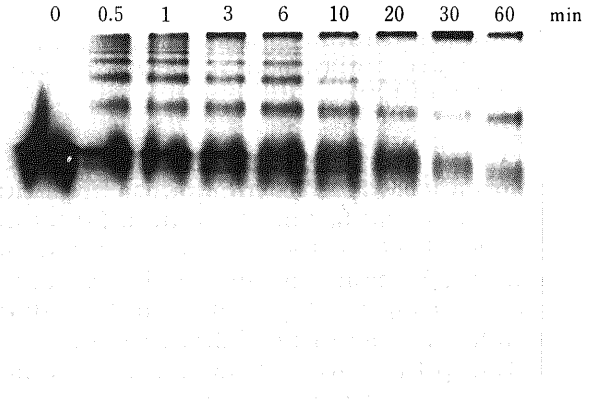


Fig. 6. Effect of the Heat Treatment Time on the Pattern of Sclerogen in Normal-PAGE

Each of sclerogen-M (8.75 μg) was treated in boiling water for the indicated time and subjected to 12.5% normal-PAGE.

in 3S, no bands were detected in fr. 1 (Fig. 2). The reason for this observation will be discussed later. Thus, we determined fr. 2 as the isolated mitogenic substance from 3S, and designated it sclerogen. The yield of sclerogen was 1.4% from sclerotia, determined by the UV absorption measurement. By amino acid analysis, sclerogen was found to mainly contain Glu (Gln), Asp (Asn), and Gly more than any other amino acids (Table I). Interestingly, sclerogen contained a small proportion of aromatic amino acids such as Tyr and Phe. The pI of sclerogen determined by chromatofocusing was approximately 5.9, and the elution profile gave a single peak (Fig. 3), suggesting that sclerogen is slightly anionic molecule.

**Sclerogen Requires Heat Denaturation to Express Mitogenicity** To evaluate whether sclerogen also requires heat denaturation to express mitogenic activity as 3S, we compared the mitogenicity of sclerogen before and after the heat denaturation. Both sclerogen sterilized with only a 0.45 μm membrane filter (sclerogen-M) and sclerogen-M further treated in boiling water for 30 min (sclerogen-MB) were prepared as testing samples. As shown in Fig. 4, although sclerogen-M showed no mitogenicity, sclerogen-MB showed significant activity against spleen cells similar to the case of 3S-MB from the previous results.[3] On the other hand, little or no mitogenicity of sclerogen were observed against thymus cells in comparison with that of Con A (Fig. 5), suggesting that sclerogen did not act on thymocytes.

When sclerogen was treated in boiling water for 0—60 min, new bands and the top of the gel in normal-PAGE were observed accompanied with the heat denaturation (Fig. 6). These results indicated that significant physicochemical changes of sclerogen proceeded to express mitogenicity against murine splenocytes. To evaluate whether these physicochemical changes were the result of the polymerization of sclerogen, we demonstrated the polymerization by using an ultrafiltration membrane which

Table II. Evaluation of the Polymerization of Sclerogen after Heat Denaturation[a] by 200 kDa Cut Off Ultrafiltration Membrane

| Form | Protein content[b] (μg) | |
|---|---|---|
| | Untreated | Pass-through fr. |
| Native | 216.0 | 173.8 (80.5%)[c] |
| Boiled | 204.2 | 14.4 ( 7.1%) |

*a*) Native sclerogen were treated in boiling water for 30 min, and then applied to the ultrafiltration membrane UK-200 which cut off 200 kDa. *b*) Protein concentrations of each fraction were measured by using BCA protein assay. *c*) Parentheses mean the percentage of the recovery of protein content in the pass-through fraction after ultrafiltrating.

TABLE III. Responses of Splenocytes to Allogeneic Splenocytes in Primary MLR[a)]

| Sample | Dose (μg/culture) | Responder only | Responder plus syngeneic stimulator | Responder plus allogeneic stimulator |
|---|---|---|---|---|
| Sclerogen-M | 0.35 | 30402±552 | 31900±3787 | 87853±22243 |
| | 1.75 | 25584±2500 | 22319±1539 | 59975±13062 |
| | 8.75 | 5761±451 | 2715±224 | 7254±790 |
| Sclerogen-MB | 0.35 | 35682±2761 | 36913±2108 | 76120±24800 |
| | 1.75 | 36535±4569 | 46304±1398 | 80561±23070 |
| | 8.75 | 46624±3492 | 42632±6117 | 138279±38098 |
| Control | — | 30003±4578 | 36288±4380 | 37312±11424 |

a) Splenocytes from C3H/He were cultured with mitomycin C-treated splenocytes from C3H/He or C57BL/6 (as syngeneic and allogeneic stimulator, respectively) in the absence or presence of sclerogen-M or -MB at the presented dose. The numbers of stimulator and responder cells were described in Materials and Methods. Results are presented as the mean±S.D. of the incorporation of $^3$H-TdR. Allogeneic stimulator only = 1024±425 cpm.

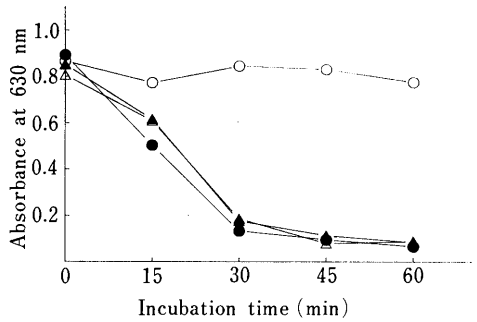


Fig. 7. Effect of Sclerogen to Alternative Pathway of Complement

Human serum was incubated with 10.7 μg/50 μl of sclerogen-M (△) or -MB (▲) or in their absence (●) for 60 min and the ability of the lysis of RRBC was measured, as described in Materials and Methods. To assess the spontaneous lysis of RRBC, no serum (○) was added to the well.

cut off 200 kDa. As shown in Table II, although the same protein concentration in the passed- and retained-fractions after ultrafiltration were observed in native sclerogen, apparent differences were observed in heat denatured sclerogen. These results suggested that sclerogen was clearly polymerized more than 200 kDa after heat denaturation.

**Biological Activities of Sclerogen** To evaluate the other biological effects of sclerogen *in vitro*, we examined whether sclerogen-M and/or -MB augment the primary MLR. Consequently, sclerogen-M as well as -MB augmented the primary MLR (Table III). Sclerogen-MB more effectively augmented the MLR response than sclerogen-M.

Furthermore, we examined the ability of the activation of an alternative pathway of complement by assessing the lysis of RRBC by human serum after incubation with sclerogen. As shown in Fig. 7, consumption of the complement components was not observed in either sclerogen-M and -MB, suggesting that sclerogen is not the activator of the alternative pathway of complement.

To assess whether sclerogen is a mitogenic lectin as Con A, hemagglutination activity of SRBC, HRBC, and MRBC were performed. Con A used as a positive control showed hemagglutination activity more than 195.3 ng/ml (in the case of SRBC), more than 4 μg/ml (in the case of all four groups of HRBC), and more than 160 ng/ml (in the case of MRBC). However, no agglutination of these RBC were observed by the addition of sclerogen-M and -MB within the concentration range of 20.9 ng/ml to 42.8 μg/ml. Considering the results that sclerogen did not show agglutination of splenic lymphocytes under microscopic observation (data not shown), the mitogenicity of sclerogen was not due to the lectin-like property.

## Discussion

We could isolate the fungal mitogen from the buffer extracts (3S) of sclerotia of *S. sclerotiorum* IFO 9395. The isolated mitogen, sclerogen, has a molecular mass of 32 kDa and showed unique characteristics as pro-mitogen which showed significant mitogenic activity after heat denaturation. Considering the physicochemical changes during heat denaturation, the condition to express mitogenicity by sclerogen may be due to the results from the homopolymerization. The reason why native 3S and sclerogen (3S-M and sclerogen-M, respectively) did not show mitogenicity at the highest dosage is probably due to the inhibition mechanism at a high dosage similar to the case of mitogenic lectin, such as Con A.

Although fr. 2 showed a clear single band at 32 kDa under the reducing condition which corresponded to the main band in 3S, no bands were detected in fr. 1 on SDS-PAGE (Fig. 2). The reason for this is due to the inability to permeate to the gel of SDS-PAGE, and may be from the results of polymerization. Previously, we have demonstrated the existence of mitogen in hot water extracts from the fruit bodies and mycelia of fungi.[1-3)] Mitogen was also found in hot water extracts from sclerotia of *S. sclerotiorum* IFO 9395.[4-5)] Since hot water extraction would subject these kinds of mitogenic substances to show the complicated physicochemical properties during extraction from a fungal source, these substances could not be detected by SDS-PAGE and reversed-phase high performance liquid chromatography (unpublished data). The purpose of our series of studies about fungal mitogenic substance is to get the mitogenic protein which is detectable on these analyses. So, we used fr. 2 as the isolated mitogen (named sclerogen) from the buffer extracts (3S) of sclerotia of *S. sclerotiorum* IFO 9395. Furthermore, the passed-through fraction of 3S on ion-exchange chromatography (DEAE-Sephadex A-25) also showed mitogenic activity as well as the adsorbed fraction (data not shown). Interestingly, the passed-through fraction showed significant mitogenicity without the heat treatment (data not shown). However, this fraction would be composed of nucleic acid from the results of UV spectrum (data not shown). Accordingly, we did not recover all the substances to show mitogenicity in 3S but isolated the mitogenic protein which could be analyzed by ordinary methods.

Recently, Kino *et al.* reported a novel immunomodulatory protein from a fungus.[11)] The protein, named Ling Zhi-8 (LZ-8), was extracted from the homogenized mycelia of an oriental medicinal fungus, Ling Zhi (*Ganoderma lucidium*), and showed blast-formation stimulatory activity resembling lectins. LZ-8 also agglutinated SRBC *in vitro* like as lectins. However, they concluded that LZ-8 is not a lectin since mono- or dimeric haptenic sugars they examined did not inhibit the activity. Whether the heat-denatured LZ-8 show mitogenicity and agglutinate SRBC, HRBC, or MRBC are not certain, sclergen did not agglutinate these RBC, suggesting that sclerogen is not a lectin and the action

pattern would be different from LZ-8.

What attracted us to sclerogen was the significant expression of mitogenic activity after heat denaturation. Only a few minutes of heat denaturation make sclerogen a potent mitogen, and drastic physicochemical changes occur in sclerogen during heat denaturation. To elucidate the changes generated during heat denaturation may be the important key to dissolving the problems as to what changes are needed to express mitogenic activity by sclerogen and why these post-translationally modified-substances should be recognized by murine splenocytes.

**Acknowledgments** We are very grateful to Ms. Yasuyo Aida, Messrs. Hideki Oka and Takahiro Kato, and Ms. Yuko Wanajo of our laboratory for their technical assistance.

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# Growth and Alkaloid Production in *Duboisia myoporoides* and *D. leichhardtii* Root Cultures

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**Root cultures of *Duboisia myoporoides* and *D. leichhardtii* have been established from granular tissues isolated from a culture line of callus. The granular tissues easily differentiated roots which vigorously grew in liquid Murashige–Skoog medium supplemented with indole-3-butyric acid (IBA) (2 mg/l) and gibberellic acid (1 mg/l).**

**These cultured roots produced atropine, scopolamine and nicotine. Furthermore, anabasine and nornicotine were detected in root cultures of *D. myoporoides*, and apoatropine in *D. leichhardtii* root cultures. A high concentration (7—10% (w/v)) of sucrose in the medium was effective in improving both root growth and tropane alkaloid production.**

**Keywords** *Duboisia*; *D. myoporoides*; *D. leichhardtii*; granular tissue; root culture; indole-3-butyric acid; gibberellic acid; tropane alkaloid

## Introduction

Tropane alkaloids are biosynthesized and decomposed in the roots of *Duboisia myoporoides*, although atropine esterase activity in adventitious roots from callus cultures was extremely low in comparison with that in roots of intact plants.[1] The intact roots would decompose tropane alkaloids which were transported back from the leaf,[2] but since the alkaloid content in the intact roots was not so different from that in adventitious roots the cause of biochemical differences between cultured roots and intact roots with respect to tropane alkaloid decomposition is not clear.

Adventitious roots were infrequently differentiated from our callus cultures and their growth was very poor,[3] although Endo and Yamada[4] successfully cultured adventitious roots from callus cultures of three *Duboisia* species. Reportedly, hairy roots of *Duboisia* infected with *Agrobacterium rhizogenes* grew better than non-transformed cultured roots,[5,6] but the effect of the gene transfer on atropine esterase was not examined. We have established root cultures from granular tissues isolated from the callus of *D. myoporoides* and *D. leichhardtii* for the purposes of tropane alkaloid production and of investigation of atropine esterase activity in comparison with intact roots.[7]

## Materials and Methods

**Plant Material and Culture Condition** Calli were induced from stem segments of 2-month-old seedlings of *D. myoporoides* R. BR. and *D. leichhardtii* F. MUELL on a Murashige and Skoog basal (MS) agar medium[8] containing 2,4-dichlorophenoxyacetic acid (2,4-D) (1 mg/l) and kinetin (0.01 mg/l) at 25±1 °C in the dark. These calli were subcultured monthly under the same conditions. During 10 months of subculturing, *D. myoporoides* and *D. leichhardtii* calli were separated into 8 and 6 culture lines, respectively, according to morphological characteristics.

Granular tissues isolated from a callus line of *D. myoporoides* and *D. leichhardtii*, respectively, were cultured in a liquid MS medium (25 ml) containing 2,4-D (1 mg/l) in 100-ml flask on a rotatory shaker (120 rpm) for 4 weeks. These tissues were used for differentiation experiments. Indole-3-butyric acid (IBA), 2,4-D, 6-benzylaminopurine (BA) and gibberellic acid were used as plant hormones; MS, B5[9] and Modified White's[10] medium as basal media; and sucrose, glucose, fructose, mannose, maltose, xylose and rhamnose as carbon sources.

**Histological Examination** Granular tissues were fixed in formalin–acetic acid–50% ethanol (1 : 1 : 18), dehydrated in dist. ethanol, and embedded in paraffin. Cross sections (50 μm in thickness) were stained with Delafield's hematoxylin solution, followed by eosine solution, before examination.

**Extraction and Identification of Alkaloids** Extraction and determination of alkaloids were performed by the methods described elsewhere.[11] Gas liquid chromatography-mass spectrometery (GC-MS) was also used for the identification of alkaloids. Commercially available atropine (Nakarai Chemicals), scopolamine-HBr (Nakarai Chemicals) and nicotine (Tokyo Kasei Co.) were used as standard samples. Anabasine and nornicotine were isolated from leaves of *D. myoporoides*.[12] Apoatropine was synthesized in our laboratory according to Maeda's method.[13] Chromatographic conditions for the analysis of atropine, scopolamine and apoatropine were as follows: a glass column (1 m × 3 mm) packed with 2% Silicon OV-1 coated with acid-washed and silanized Chromosorb W, column temperature 190 °C, injector temperature 240 °C, helium as the carrier gas at a flow rate of 30 ml/min, total ion chromatogram (TIC) detector and an ionizing energy 70 eV. For the analysis of nicotine, nornicotine and anabasine, a glass column (2 m × 3 mm) packed with 10% Silicon DC-550 was used at a column temperature of 180 °C and an injector temperature of 230 °C. Atropine *m/z* (rel. int.): 289 [$M^+$] (23), 140 (9), 124 (100), 94 (17), 83 (25), 82 (24). Scopolamine: 303 [$M^+$] (55), 154 (42), 138 (86), 136 (34), 108 (49), 94 (100). Apoatropine: 271 [$M^+$] (31), 124 (100), 103 (15), 96 (35), 83 (30), 82 (34). Nicotine: 162 [$M^+$] (23), 161 (14), 133 (22), 119 (6), 85 (6), 84 (100). Nornicotine: 148 [$M^+$] (39), 147 (36), 120 (36), 119 (100), 80 (32), 70 (82). Anabasine: 162 [$M^+$] (53), 133 (49), 119 (36), 106 (43), 105 (53), 84 (100).

## Results

**Isolation of Granular Tissue** Calli induced from stem segments of a *D. myoporoides* seedling and subcultured on a solid MS medium containing 2,4-D (1 mg/l) and kinetin (0.01 mg/l) were not uniform in appearance. Therefore, calli were separated into various culture lines during subculture according to such morphological characters as color, shape, and texture. After 10 months, 8 culture lines were established. A culture line having hard and compact callus produced granular tissues. When the callus was transferred to a liquid MS medium containing 2,4-D (1 mg/l) and cultured for 4 weeks on a shaker (80 rpm), granular tissues were separated from suspension cells, aggregating in the bottom of culture flasks since they were hard and heavy. About 2 g of callus tissues produced 15—20 granular tissues (1—3 mm in diameter) which existed as round to oval single bodies generally, although occasionally there were several in the medium (Fig. 1a). Granular tissues of *D. leichhardtii* were formed same as those of *D. myoporoides*. These granular tissues never differentiated roots as they were subcultured in the MS medium containing 1 mg/l 2,4-D.

Histological examination of the granular tissues showed that they were meristimatic and formed a root apex (Fig. 1b).

**Differentiation of Granular Tissue** In a previous paper, we showed that the callus of *D. myoporoides* regenerated shoots and roots on a MS medium supplemented with either BA (1—10 mg/l) or IBA (1—5 mg/l).[3] Accordingly, the effect of BA and IBA on morphogenesis in granular tissues

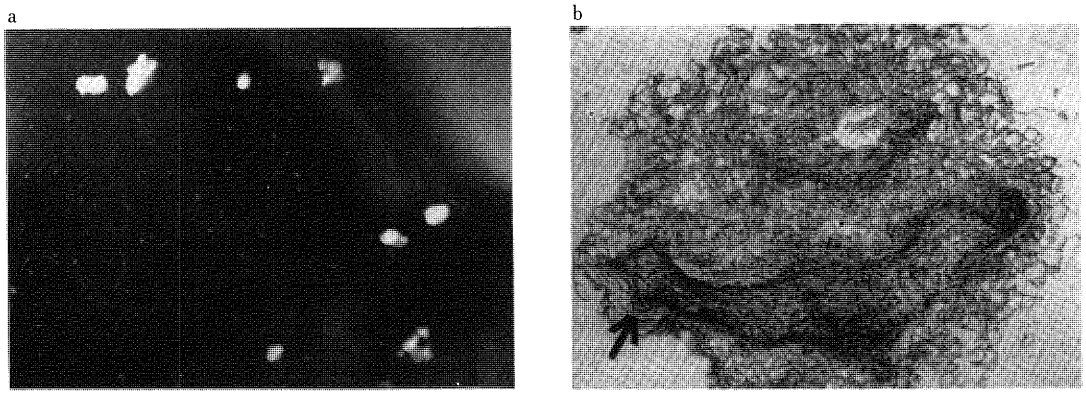

Fig. 1a. Granular Tissues Separated from Cell Suspension Cultures of *D. myoporoides* (×2)
1b. Cross Section of a Granular Tissue of *D. myoporoides* (×40)
Strong black parts are meristimatic tissue and an arrow shows a root apex.

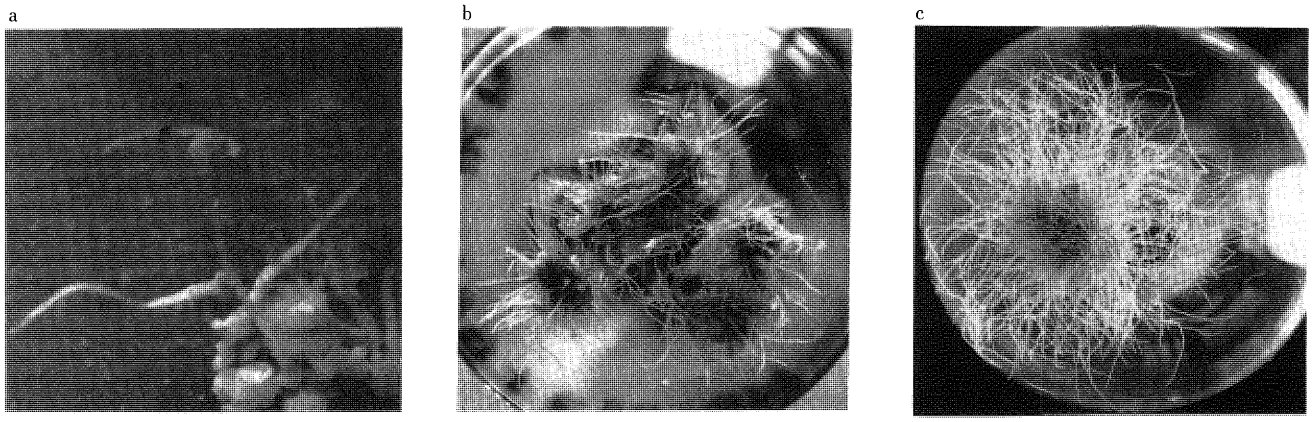

Fig. 2a. Roots Differentiated from Granular Tissues of *D. myoporoides* Cultured in a Medium Containing Gibberellic Acid for 2 Weeks (×3)
2b. Roots Differentiated from the Granular Tissues Cultured in a Medium Containing Gibberellic Acid and IBA for 4 Weeks (×1.5)
2c. Roots Subcultured under the Same Condition for 4 Weeks (×1)

TABLE I. Effect of IBA and BA on Organ Formation in Granular Tissue

| Morphogenesis | Plant growth hormones (mg/l) added to medium | | | | | |
|---|---|---|---|---|---|---|
| | IBA | | BA | | IBA + BA | None |
| | 0.5 | 2 | 0.5 | 2 | 0.5 + 0.5 | |
| No. of roots /No. of G.T. | 60/19 | 68/20 | 21/19 | 0/15 | 35/19 | 23/17 |
| No. of buds /No. of G.T. | 0/19 | 0/20 | 46/19 | 145/15 | 0/19 | 0/17 |

Granular tissues (G.T.) were cultured for 6 weeks in MS medium containing hormone and 3% sucrose.

TABLE II. Effect of 2,4-D and Gibberellic Acid on Root Formation in Granular Tissue

| Root formation | Plant growth hormones (mg/l) added to medium | | | | | | |
|---|---|---|---|---|---|---|---|
| | None | 2,4-D | | | Gibberellic acid | | |
| | | 0.001 | 0.01 | 0.1 < [a] | 0.1 | 1 | 10 |
| No. of roots /G.T. | 0–2 | 1–2 | 1–2 | 0 | 1–2 | 1–2 | 1–2 |
| Length (mm) | 20–30 | 20–30 | 30–40 | — | 50–60 | 50–60 | 30–40 |
| Diameter (mm) | 0.3–0.5 | 0.3–0.5 | 0.3–0.5 | — | 0.8–1.0 | 0.8–1.0 | 0.8–1.0 |

Granular tissues (G.T.) were cultured for 6 weeks in MS medium containing hormone and 3% sucrose. *a*) No root formation was observed.

was examined (Table I). Granular tissues formed adventitious roots (1—5 roots/granular tissue) on media containing no BA other than IBA (0—2 mg/l); the roots had a lot of hairs, but did not elongate (< 0.5 mm in length). Buds were formed only when BA alone was added to the medium. Both roots and buds were formed on a medium containing 0.5 mg/l BA. Adventitious roots also differentiated when granular tissues were treated with 2,4-D or gibberellic acid where root formation was poor (1—2/granular tissue) but root elongation was good (Table II) (Fig. 2a). Gibberellic acid at a concentration of 1 mg/l was most suitable for root growth.

These results showed that treatment with IBA caused an increase in root number, whereas gibberellic acid contributed to root elongation. Accordingly, a combination of gibberellic acid (1 mg/l) and IBA (2 mg/l) was added to the culture medium. The treated roots grew well for 8 weeks, forming a mass from which thin roots extended in every direction from the center (Fig. 2b, c). Root cultures from granular tissues of *D. leichhardtii* have been also established with a medium containing gibberellic acid and IBA. They could be subcultured in the same medium for more than 3 years.

TABLE III. Effect of Basal Medium on Root Growth and Alkaloid Production

| Basal medium | Increase in weight (final/initial) | | Alkaloid production (μg/flask) | |
|---|---|---|---|---|
| | Fresh wt. | Dry wt. | Atropine | Scopolamine |
| MS | 10.4±1.1 | 4.8±0.4 | 35±10 | 93± 6 |
| B5 | 8.3±0.9 | 3.9±0.7 | 33±10 | 44±12 |
| White | 4.8±3.6 | 2.0±0.5 | 25±18 | 30± 5 |

Average of three determinations (mean±S.E.). Initial fresh wt. and dry wt. of *D. leichhardtii* roots were 150 and 17 mg, respectively. The roots were cultured for 3 weeks in the basal medium containing IBA (2 mg/l), gibberellic acid (1 mg/l) and 3% sucrose.

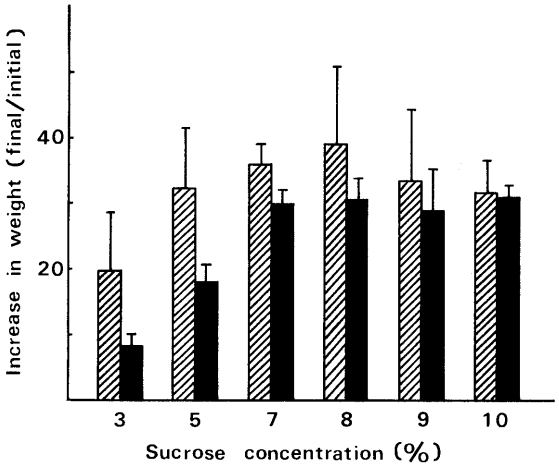


Fig. 3. Effect of Sucrose Concentration on the Root Growth of *D. leichhardtii*

Initial fresh weight, 150 mg; initial dry weight, 17 mg; culture periods, 4 weeks. $n=3$. ▨, fresh wt.; ■, dry wt.

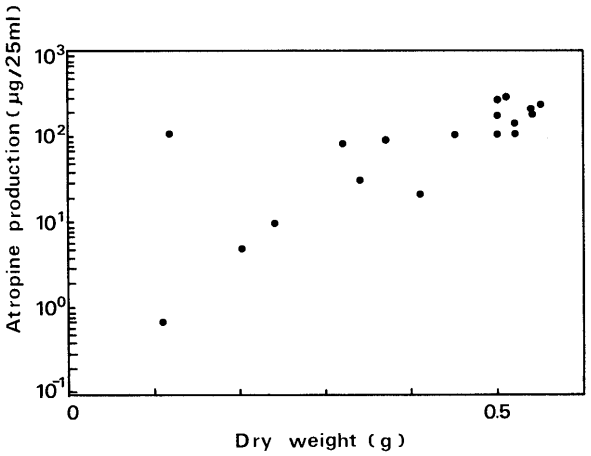


Fig. 4. Relationship between Yields of *D. leichhardtii* Roots Cultured with Various Sucrose Concentrations and Their Atropine Production

$n=18$, $r=0.778$.

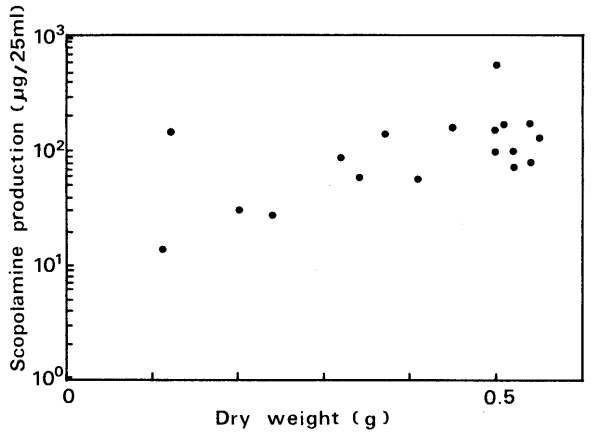


Fig. 5. Relationship between Yields of *D. leichhardtii* Roots Cultured with Various Sucrose Concentrations and Their Scopolamine Production

$n=18$, $r=0.603$.

**Alkaloid Production by Cultured Roots** Alkaloids in the cultured roots of *D. leichhardtii* and *D. myoporoides* were separated by TLC and confirmed by GC-MS. Both roots contained tropane and pyridine alkaloids. Atropine, scopolamine, and nicotine were the main alkaloids common to both. Apoatropine was found in the roots of *D. leichhardtii*, whereas anabasine and nornicotine were detected in the roots of *D. myoporoides* as minor alkaloids.

**Effects of Culture Conditions on Root Growth and Tropane Alkaloid Production** In *D. leichhardtii* root cultures, the total tropane alkaloid content was higher than that of *D. myoporoides*. Therefore, *D. leichhardtii* roots were used in the following experiments.

**(a) Basal Medium** Among MS, B5, and modified White's basal media tested, MS basal medium was more suitable for both root growth and scopolamine production (Table III).

Regarding the effect of the comparative strength of the MS basal medium (×1/3, ×1/2, ×1 and ×2) on root growth and alkaloid production, no remarkable difference was found (data not shown). However, a high concentration of mineral salts tended to inhibit alkaloid production in cultured roots.

**(b) Carbon Source** Sucrose was most suitable for root growth among 7 carbon sources tested, followed by fructose, maltose, and glucose. Mannose, xylose or rhamnose failed to support root growth. Cultured roots grew better at higher sucrose concentrations (5—10%) (Fig. 3). The yields of atropine and scopolamine tended to increase with increasing root growth (Figs. 4 and 5), showing a good correlation with growth ($r=0.778$ for atropine; $r=0.603$ for scopolamine).

**(c) Shaking Speed** Since aeration is known to be important for root growth and alkaloid production,[4] the effect of shaking speed on root growth was determined. Cultures were performed at 3 different shaking speeds (80, 100 and 120 rpm), but no significant difference was found among them.

## Discussion

We have established root cultures of *D. myoporoides* and *D. leichhardtii* from granular tissues isolated from callus cultures. There had been no previous report on isolation of such a granular tissue having meristimatic structures in *Duboisia*. These granular tissues were found in a culture line of callus subcultured for more than 10 months, and we were able to maintain the ability to differentiate among these roots for the following 15 months. Simultaneous addition of IBA and gibberellic acid were effective in promoting not only root differentiation from the granular tissue, but also subsequent growth. This combination was

also found to be useful in promoting cultured roots of other tropane alkaloid-producing plants, *Datura*, *Atropa*, *Scopolia* and *Hyoscyamus* (data not shown).

Root cultures of *D. myoporoides* and *D. leichhardtii* in the present study produced tropane and pyridine alkaloids, as reported by Endo and Yamada.[4] *D. leichhardtii* root cultures produced not only atropine and scopolamine but also apoatropine, which was detected in *Duboisia* cultured roots for the first time. Both growth and alkaloid production in the *D. leichhardtii* root could be improved in a MS medium containing a higher concentration (7—10%) of sucrose. Changes in the nitrogen source and its concentration did not affect the increase of tropane alkaloid production (data not shown).

Leaves of *D. leichhardtii* plants contain *ca.* 0.1% scopolamine and 0.3% atropine, whereas the roots cultured with 7% sucrose produce *ca.* 0.1% scopolamine and only 0.08% atropine. Cultured roots of *D. leichhardtii* established by Endo and Yamada produced *ca.* 0.9 mg scopolamine and 0.6 mg atropine with a 25 ml medium during 4 weeks when 0.20 g roots were used as the initial fresh weight. Since our cultured roots produced *ca.* 0.5 mg scopolamine and 0.4 mg atropine under the same conditions, but with 0.15 g roots at first, the alkaloid productivity of our roots is at a similar level to theirs. To increase the alkaloid productivity of our cultured roots, careful selection of the mother plant, granular tissue, and cultured roots is required.

**Acknowledgement** We thank Mr. N. Yamaguti for measurement of GC-MS, and Ms. Y. Yukizane for her technical assistance.

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# Characteristics of Peroxisome Proliferation: Co-induction of Peroxisomal Fatty Acid Oxidation-Related Enzymes with Microsomal Laurate Hydroxylase[1)]

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**The profile of the changes in the peroxisomal fatty acid oxidation activity in rat liver was compared with that in microsomal ω-oxidation under various conditions such as a 2-week administration of phenoxyacetic acid derivatives and perfluorinated compounds, short and long-term administration of clofibrate and bezafibrate, high-fat diet feeding, starvation and diabetes. The results were summarized as follows: 1) when phenoxyacetic acid derivatives and perfluorinated compounds were administered, there was a significant correlation in the increase of the activities between peroxisomal fatty acid oxidation and microsomal ω-oxidation. 2) On the long-term administration (79 weeks) of peroxisome proliferators the activities of the enzymes were significantly reduced, but the levels were still higher than the control level in a similar manner. 3) On high-fat diet feeding the patterns of the changes in the activities of peroxisomal fatty acid oxidation, carnitine acetyltransferase and microsomal ω-oxidation were similar to each other, differing from the changes in the activities of microsomal aminopyrin demethylase and mitochondrial carnitine palmitoyltransferase. 4) Under starved and diabetic conditions, co-induction of peroxisomal fatty acid oxidation and microsomal ω-oxidation was observed. From these results it is suggested that 1) the biosynthesis of these enzymes would be regulated on the gene expression of the nearby domain and 2) peroxisomal fatty acid oxidation and microsomal ω-oxidation were co-operatively regulated in order to achieve fatty acid metabolism smoothly.**

**Keywords** peroxisome proliferation; laurate hydroxylase; co-induction; fatty acid oxidation; enzyme activity

Induction and inhibition of enzyme activities by drugs are important to evaluate their efficacy and toxicity to humans. Clofibrate (CPIB), structurally related (*e.g.* nafenopin and ciprofibrate) and unrelated (*e.g.* Wy-14643 and BR-931) hypolipidemic agents, and certain plasticizers (*e.g.* di(2-ethylhexyl)phthalate) and herbicides (*e.g.* 2,4,5-trichlorophenoxyacetic acid) are classified as peroxisome proliferators.[1-4)] These compounds induce hepatomegaly, proliferation of hepatic peroxisomes, and induction of various peroxisomal and non-peroxisomal enzymes in rodents.[5,6)] Furthermore, these are classified as non-mutagenic hepatocarcinogens.[7,8)] Recently, the induction of the activities of microsomal laurate hydroxylase,[9-11)] epoxide hydrolase, and acyl-CoA hydrolase[12,13)] by peroxisome proliferators has been reported. However, it has not yet been clarified whether the changes in the peroxisomal fatty acid oxidation activity induced by peroxisome proliferators are linked with those in enzymes which localized in other subcellular compartments than peroxisomes.

The present paper describes the correlation of the induction of hepatic peroxisomal enzymes to that of lauric acid hydroxylase under the conditions with 1) treatment with various compounds (*e.g.* phenoxy acetic acid (PAA) derivatives and perfluoro compounds), 2) long term-administration of peroxisome proliferators, 3) high-fat feeding,[14,15)] 4) diabetes[16)] and 5) fasting,[17)] and discusses the mechanism of enzyme induction under these conditions and the presumed role of microsomal ω-oxidation in fatty acid metabolism in liver cells.

## Method

**Materials** PAA derivatives (include phenoxypropionic acid derivatives) were obtained from Wako Pure Chemicals Co., Ltd. (Japan). Perfluorinated compounds were kindly provided by Sankyo Co., Ltd. (Japan). l-Carnitine-HCl was kindly provided by Earth Pharmaceutical Co., Ltd. (Japan). Acetyl-CoA, nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide phosphate (NADPH), palmitoyl-CoA and bovine serum albumin (BSA, fatty acid free) were obtained from Sigma Chemical Co., Ltd., St. Louis, Mo., U.S.A. All other chemicals were of analytical grade from commercial sources.

**Animals and Treatments** Exp. I: The effects of various compounds on some hepatic enzymes. Male Wistar rats weighing around 180 g were used. These animals were obtained from Tokyo Laboratory Animals Co., Ltd. (Japan). The animals (5 animals/group) were orally administered compounds suspended in 1% methylcellulose at the dose level of 150 mg/kg body weight (b.w.)/d for 2 weeks except for perfluorinated compounds. Perfluorinated compounds other than perfluorooctanesulfonic acid PFOS were solubilized in corn oil and administered intraperitoneally to the rats at one time at the dose level of 50 mg/kg b.w. and the animals were maintained for 3 d. PFOS was solubilized in 0.9% NaCl and administered to the rats in the same manner as other perfluorinated compounds.

TABLE I. Fatty Acid Composition of Diets[a)]

| Fatty acid | Control | PHMO-diet |
|---|---|---|
| 12:0 | 0 | 0.3 |
| 14:0 | 0.4 | 7.8 |
| 14:1 | 0 | 0.1 |
| 15:0 | 0.1 | 0.6 |
| 16:0 | 16.5 | 20.7 |
| 16:1 | 0.7 | 11.4 |
| 17:0 | 0.2 | 0.6 |
| 17:1 | 0.2 | 1.2 |
| 16:4(w1) | 0 | 0.1 |
| 18:0 | 2.2 | 6.4 |
| 18:1(w9) | 0 | 16.0 |
| 18:1(w7) | 23.2 | 6.4 |
| 18:1(w5) | 0 | 1.0 |
| 18:2(w6) | 50.2 | 0.4 |
| 18:3(w3) | 4.1 | 0 |
| 20:0 | 0.2 | 1.8 |
| 20:1(w11) | 0.8 | 2.9 |
| 20:1(w9) | 0 | 10.1 |
| 22:0 | 0.4 | 0.8 |
| 22:1(w13+11) | 0 | 3.8 |
| 22:1(w9) | 0 | 4.2 |
| 24:0 | 0 | 0.8 |

a) Values are percentage of total fatty acids. PHMO, partially hydrogenated marine oil.

Exp. II: The short- and long-term effect of CPIB and bezafibrate (BF) on hepatic enzymes. Male Wistar rats were used and housed into 5 animals/group. For the short-term experiment, rats were orally administered with CPIB and BF at the dose levels of 250 and 100 mg/kg b.w. daily for 2 weeks, respectively. For the long-term experiment, rats initially weighing around 120 g were used and the animals housed into 5 animals/group were administered CPIB and BF in their diet at 0.25% and 0.1% for 79 weeks.

Exp. III: Changes in the activities of some hepatic enzymes in the high-fat feeding rat. Male Wistar rats weighing around 130 g were housed into 4 animals/group. The high-fat feeding group was administered a powdered chow (CE-2, Clea Japan Co., Ltd.) containing partially hydrogenated marine oil at a concentration of 30% and the control group was maintained with only the standard diet CE-2. The fatty acid composition of the diet is shown in Table I. After 2 weeks feeding, half of the animals were withdrawn from the diet and refed with a normal diet. Animals were sacrificed by decapitation at the time indicated in Fig. 3 and the enzyme activities of the livers were measured.

Exp. IV: The activities of some hepatic enzymes under diabetic conditions. Male Wistar rats weighing about 180 g were used. The rats of the treated groups (5 animals/group) were intravenously injected with streptozotocin in a 1% citrate buffer at the dose of 65 mg/kg b.w. and 3 d after the injection the liver homogenates were prepared for the assay of the enzymes. All animals of the treated group showed a blood sugar level of above 400 mg/dl, and then, these animals were used as the diabetic animals.

Exp. V: The activities of some hepatic enzymes under starvation. Male Wistar rats, weighing about 250 g (5 animals/group) were starved for 1 d and the liver homogenates were prepared for the assay of the enzymes.

**Assay Methods** The activity of peroxisomal cyanide-insensitive fatty acyl-CoA oxidizing system (FAOS) was determined by measuring the palmitoyl-CoA-dependent reduction of NAD at 340 nm with the method described by Lazarow and de Duve.[18] One unit of the activity was defined as the amount of the enzyme that reduced 1 nmol/min. The activities of CAT localized in peroxisomes and mitochondria, and carnitine palmitoyltransferase (CPT) localized in mitochondria were determined by measuring the amount of CoA–SH released during the reaction with 5,5′-dithio-bis(2-nitrobenzoic acid) at 412 nm.[19] One unit of the activity was defined as the amount of the enzyme that produced 1 nmol of CoA–SH from acetyl-CoA or palmitoyl-CoA per min. Fatty acyl-CoA dehydrogenase (FADH) in mitochondria was determined according to the method of Hryb and Hogg[20] using palmitoyl-CoA as the substrate, and one unit of the enzyme was the amount of the enzyme that reduced 1 nmol of 2,6-dichlorophenolindophenol per min. Catalase, a marker enzyme of peroxisomes was determined according to the previous report.[21] One unit of catalase activity was defined as the amount of the enzyme giving $K=1$, where $K$ is the rate constant of the enzyme. Microsomal enzymes and cytochrome P-450 content were assayed as follows: Aminopyrine *N*-demethylase (AD) and aniline hydroxylase (AH) activities were determined by the method of Mazel.[22] The activity of (LH) was determined by the method of Orton and Parker[9] using (1-$^{14}$C)lauric acid as the substrate. NADPH-dependent cytochrome c reductase (Cyt. C-R) activity was determined by Beaufay *et al.*[23] The cytochrome P-450 content was determined by the method of Omura and Sato.[24] The protein content was determined by the method of Lowry *et al.*[25] with BSA as a standard.

**Statistical Evaluation** The statistical significance of each value and correlation constant ($r$) were evaluated by the $t$-test.

## Results

**Effects of PAA-Derivatives and Perfluorinated Compounds on Biochemical Values and Some Enzyme Activities in Peroxisomes, Mitochondria and Microsomes** The effects of PAA-derivatives and perfluorinated compounds were summarized in Tables II and III, where the values showed the ratios to the control values. The difference in the control values of FAOS activity in Tables II and III might be due to the difference in the lot of the reaction mixture used. Thus, accumulative difference in the activity of FAOS was frequently observed according to the lot of the reagents involved in the reaction mixture. However, the difference is not an obstacle to the evaluation of the results. On the basis of these data, the correlation between the activity of FAOS and that of LH were examined (Fig. 1A, B). When PAA-derivatives were administered, the slope of the correlation was 41 and the correlation coefficient was 0.92. In the case of perfluorinated compounds, the values were 18 and 0.84, respectively.

**Changes in the Activities of Peroxisomal, Mitochondrial and Microsomal Enzymes in the Liver of the Rat after Short- and Long-Term Treatments** Table IV shows the changes in the activities of hepatic peroxisomal, mitochondrial and microsomal enzymes after short-term (2 weeks) and long-term (79 weeks) with CPIB and BF. The results are indicated as the relative values to the controls. After a 2-week administration of both drugs the activities of FAOS, carnitine acetyltransferase (CAT) and LH were markedly increased. Although the increases in the activities of those

TABLE II. Effects of PPA-Derivatives on the Liver Weight, Protein Content and Activities of Some Peroxisomal, Microsomal and Mitochondrial Enzymes of Rat Livers

| Parameters | Control value | Relative value (ratio) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PAA | *o*-CPA | *p*-CPA | 2,4-D | 2,4,5-T | MCPA | *p*-CPP | 2,4-DP | 2,4,5-TP | CPIB |
| Relative liver weight (% of b.w.) | 4.52±0.28 | 1.18 | 1.17 | 1.02 | 1.04 | 1.21 | 1.13 | 1.13[a] | 1.22[a] | 1.45[a] | 1.24[a] |
| Protein (mg/g liver) | 155±2 | 0.85 | 0.97 | 0.94 | 0.92 | 0.90 | 0.87 | 0.98 | 1.06 | 1.07 | 1.01 |
| Activities (U/g liver) | | | | | | | | | | | |
| Catalase | 39.9±5.3 | 0.71[a] | 0.74[a] | 0.66[a] | 0.68[a] | 0.80[a] | 0.74[a] | 1.06 | 1.34[a] | 1.68[a] | 1.44[a] |
| FAOS | 1194±229 | 1.06 | 0.82 | 1.04 | 1.69[a] | 2.46[a] | 2.85[a] | 2.37[a] | 6.63[a] | 12.47[a] | 4.54[a] |
| CAT | 911±101 | 0.99 | 0.82 | 1.23[a] | 2.22[a] | 8.07[a] | 2.59[a] | 4.40[a] | 50.94[a] | 83.20[a] | 39.60[a] |
| CPT | 2032±157 | 1.07 | 1.01 | 1.10 | 1.49[a] | 2.67[a] | 1.62[a] | 1.50[a] | 2.61[a] | 1.82[a] | 2.29[a] |
| FADH | 436±44 | 0.81 | 1.04 | 1.09 | 1.22[a] | 1.61[a] | 1.18 | 1.27 | 1.75[a] | 1.64[a] | 1.65[a] |
| AD | 119±14 | 1.12 | 1.13 | 0.99 | 1.02 | 1.47[a] | 0.96 | 0.69[a] | 0.81[a] | 0.89 | 0.88 |
| AH | 76.1±22.1 | 0.61[a] | 0.69[a] | 0.83 | 1.04 | 1.04 | 0.70[a] | 0.82 | 0.87 | 0.92 | 0.79[a] |
| LH | 22.8±6.9 | 1.13 | 0.82 | 1.12 | 1.47 | 2.19[a] | 1.52[a] | 4.45[a] | 6.37[a] | 11.02[a] | 6.03[a] |
| Cyt. C-R | 1837±422 | 1.00 | 1.05 | 1.03 | 1.39 | 1.33 | 1.05 | 1.26 | 1.83[a] | 2.52[a] | 1.59[a] |
| Cytochrome P-450 (nmol/g liver) | 27.1±11.9 | 1.12 | 1.00 | 1.01 | 1.29 | 1.45 | 0.95 | 1.18 | 1.51[a] | 1.33[a] | 1.14 |

Control values are expressed as means±S.D. of 5 rats, and other results are expressed as relative values to the controls. *a*) Statistical significance $p<0.05$. *o*-CPA, *o*-chlorophenoxyacetic acid; *p*-CPA, *p*-chlorophenoxyacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; MCPA, 2 methyl-4-chlorophenoxyacetic acid; *p*-CPP, *p*-chlorophenoxypropionic acid; 2,4-DP, 2,4-dichlorophenoxypropionic acid; 2,4,5-TP, 2,4,5-trichlorophenoxypropionic acid; CPIB, clofibrate.

TABLE III. Effects of Perfluorinated Compounds on the Liver Weight, Protein Content and Activities of Some Peroxisomal, Microsomal and Mitochondrial Enzymes of Rat Liver

| Parameters | Control | Relative value (ratio) | | | | |
|---|---|---|---|---|---|---|
| | | PFBA | PFOA | PFDA | PFOL | PFOS |
| Relative liver weight (% of b.w.) | 4.10±0.35 | 1.15 | 1.45[a)] | 1.16[a)] | 1.13[a)] | 1.16[a)] |
| Protein (mg/g liver) | 171±21 | 1.01 | 0.86 | 0.83[a)] | 1.01 | 0.96 |
| Activities (U/g liver) | | | | | | |
| Catalase | 32.4±6.9 | 1.10 | 0.84 | 1.01 | 0.82 | 1.13 |
| FAOS | 684±155 | 2.01 | 4.33[a)] | 3.11[a)] | 2.02[a)] | 1.14 |
| CAT | 522±53 | 6.75[a)] | 17.69[a)] | 16.40[a)] | 5.89[a)] | 2.64[a)] |
| CPT | 2208±268 | 1.40 | 1.55[a)] | 1.41[a)] | 1.40[a)] | 1.00 |
| FADH | 1066±156 | 1.37[a)] | 1.33[a)] | 1.21 | 1.38[a)] | 1.27[a)] |
| AD | 178±29 | 0.85 | 0.59[a)] | 0.49 | 0.93 | 1.13 |
| AH | 90.5±7.7 | 0.65[a)] | 0.64[a)] | 0.77[a)] | 0.74[a)] | 0.83 |
| LH | 15.8±3.3 | 5.93[a)] | 7.58[a)] | 7.25[a)] | 5.03[a)] | 1.75[a)] |
| Cyt. C-R | 2769±93 | 1.40[a)] | 1.69[a)] | 1.14 | 1.52[a)] | 1.51[a)] |
| Cytochrome p-450 (nmol/g liver) | 26.1±4.1 | 1.15 | 2.72[a)] | 1.37 | 2.24[a)] | 2.51[a)] |

Results are expressed as relative value to control and control values are expressed as means±S.D. of 5 rats. *a)* Statistical significance $p<0.05$. PFBA, perfluorobutyric acid; PFOA, perfluorooctanoic acid; PFDA, perfluorodecanoic acid; PFOL, 1-H,1-H-pentadecafluorooctanol; PFOS, perfluorooctanesulfonic acid.

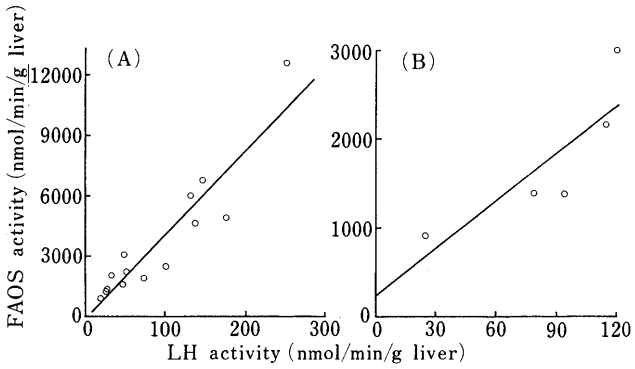


Fig. 1. Correlation between FAOS and LH Activities in the Liver of Rats Administered with PAA-Derivatives (A) and Perfluorinated Compounds (B)

(A) $Y=-174+14X$, $r=0.92$, (B) $Y=206+18X$, $r=0.84$.

TABLE IV. Effects of CPIB and BF on the Peroxisomal, Mitochondrial and Microsomal Enzymes in Rat Liver

| Enzymes | CPIB | | BF | |
|---|---|---|---|---|
| | 2 wks | 79 wks | 2 wks | 79 wks |
| FAOS | 7.76[a)] | 2.35[a)] | 13.61[a)] | 5.04[a)] |
| CAT | 26.40[a)] | 18.56[a)] | 54.41[a)] | 39.64[a)] |
| CPT | 1.21[a)] | 3.62[a)] | 1.84[a)] | 3.68[a)] |
| FADH | 1.54[a)] | 1.79[a)] | 1.72[a)] | 2.06[a)] |
| LH | 3.88[a)] | 2.33[a)] | 5.49[a)] | 3.55[a)] |
| Cyt. C-R | 1.10 | 1.12 | 1.07 | 1.34[a)] |

Data are expressed as relative activities (ratios) to control. *a)* Statistical significance $p<0.05$.

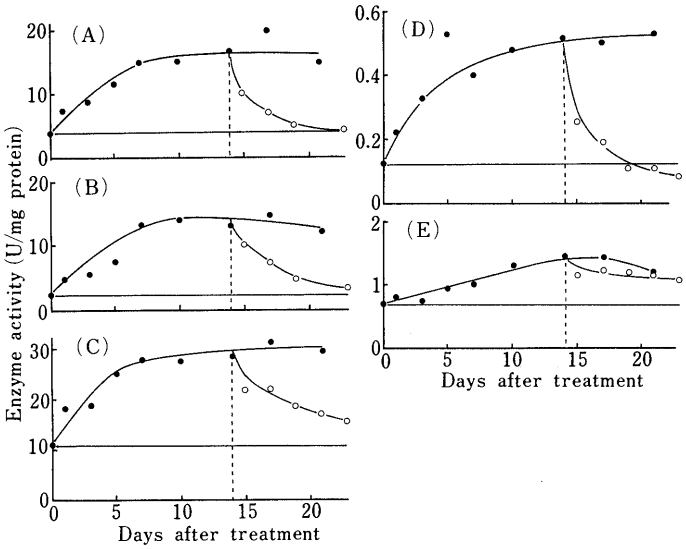


Fig. 2. Changes in the Enzyme Activities in the Livers of Rats Fed a High-Fat Diet

Animals were fed a high-fat diet for 3 weeks and the time dependent changes in the activities of FAOS (A), CAT (B), CPT (C), LH(D) and AD (E) were examined. ●, high-fat feeding; ○, withdraw from the high-fat diet after 2 weeks of the diet treatment and refed a normal diet.

enzymes after 79 weeks of treatment were also observed, the extent of the change was less than that observed in short-term treatment. On the contrary, the activities of CPT and FADH further increased after 79 weeks of treatment compared with those of 2 weeks of treatment. Except for a slight increase on the long-term administration of BF, no significant change in the activity of Cyt. C-R was observed in both short- and long-term administrations. When the correlation between FAOS and LH activities were examined from these results, the correlation coefficient was observed to be 1.00.

**Changes in the Activities of Peroxisomal, Mitochondrial and Microsomal Enzymes in the Liver of the Rats Fed High-Fat Diet and Refed Normal Diet** In Fig. 2 the changes in the hepatic activities of FAOS, CAT, CPT, LH and AD on high-fat diet feeding and after withdraw from the diet were shown. In 7- to 10-d feeding of a high-fat diet the activities of FAOS, CAT and LH reached the maximum level, and on 5 to 10 d after withdraw from the diet at 14-d of treatment, the increased levels of the activities were restored to the control levels. The increasing response of CPT was relatively rapid and on 5 d of treatment the level showed a maximum and steady state level through the time the diet was fed. The level was not restored to the control level even more than 10 d after withdraw from the diet. On the other hand, the response of AD activity to the high-fat diet and restoration of the increased activity to the control level after withdraw from the diet were extremely late.

**Changes in the Hepatic Activities of Peroxisomal, Mitochondrial and Microsomal Enzymes under High-Fat Feeding, Diabetic and Fasted Conditions** It has been well known that high-fat feeding, diabetes and fasting conditions induced an increase in the activities of hepatic peroxisomal fatty acid oxidation related enzymes. The changes in the activities of peroxisomal, mitochondrial and microsomal enzymes under these conditions were summarized in Table V. Streptozotocin (STZ) did not show any direct effect on the activities of the enzymes. The results were indicated as relative values to the controls, and were calculated based on the values 7 d after high-fat feeding, 3 d after the administration of streptozotocin, and 1 d on fasted condition, respectively. On high-fat feeding the activities of

TABLE V. Changes in Peroxisomal, Mitochondrial and Microsomal Enzyme Activities in the Liver of Rats under the Physiological and Pathological Conditions

| Enzymes | High-fat | Diabetes | Starvation |
|---|---|---|---|
| Catalase | 1.41[a)] | 0.95 | 0.85 |
| FAOS | 3.92[a)] | 1.62[a)] | 2.17[a)] |
| CAT | 5.91[a)] | 2.33[a)] | 2.36[a)] |
| CPT | 2.76[a)] | 1.38[a)] | 1.58[a)] |
| FADH | 1.47[a)] | 1.16[a)] | 1.13 |
| LH | 4.23[a)] | 1.36[a)] | 2.41[a)] |
| Cyt. C-R | 1.10 | 0.82 | 0.93 |
| AD | 1.33 | 0.40[a)] | 0.49[a)] |

Data are expressed as relative activities to control. High-fat diet, 30% PHMO diet for 1 week. Diabetes, 3 d after the injection of STZ (65 mg/kg b.w.). Starvation, removal of the diet for 1 d. *a)* Statistical significance $p<0.05$.

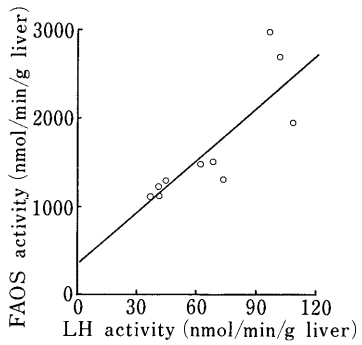


Fig. 3. Correlation between FAOS and LH Activities in the Livers of Rats under the Physiological and Pathological Conditions

The values at 1, 3, 5, 7, 10 d after high-fat diet feeding, at 1, 3, 5 d after injection of streptozotosin, and at 1 and 3 d after starvation were used. $Y=369+23X$, $r=0.82$.

catalase, FAOS, CAT and LH increased 1.4-, 3.9-, 5.9- and 4.2-fold, respectively with statistical significance. The activities of mitochondrial CPT and FADH increased 2.8- and 1.5-fold, respectively. Although the profiles of the changes in the activities on diabetic and fasted conditions were similar to each other, the extent of the change was relatively small compared with the case of high-fat feeding. The activity of FAOS increased 1.6- and 2.2-fold in diabetes and in the fasted condition, respectively. The activity of LH increased 1.4- and 2.4-fold, respectively. The activities of CAT, CPT and FADH increased 2.3-, 1.5- and 1.1-fold, respectively in both cases. There were decreasing tendencies in the activities of catalase, cytochrome c reductase and AD, and especially the activity of AD under diabetic and fasted conditions was 40—50% of the control. On the basis of the activities under these physiological and pathological conditions the interrelationship between FAOS and LH activities was examined (Fig. 3). The results gave the FAOS and LH activities was examined (Fig. 3). The results gave the following regression curve and correlation coefficient: $Y=369+23X$ and $r=0.82$, showing the correlation between both enzyme activities.

## Discussion

When administered peroxisome proliferators, induction of the activities of microsomal laurate hydroxylase,[9-10] cytosolic epoxide hydrolase[12] and acyl-CoA hydrolase[13] was observed in association with the induction of peroxisomal $\beta$-oxidation in the livers of rats and mice. The mechanism of the induction has not yet been clarified. These enzymes are related to lipid metabolism and if the enzyme activities would change in the same manner under many conditions, the co-operative changes in the activities might have a physiological meaning. Then we examined the changes in the activities of certain enzymes under various conditions in detail. In the present report, the change in the activity of microsomal lauric acid hydroxylase ($\omega$-oxidation), which might be useful for comparative study in lipid metabolism because the increase in the activity was comparable to that in peroxisomal $\beta$-oxidation, was mainly examined and the correlation between both enzyme activities was evaluated.

In the experiments using PAA derivatives and perfluorinated compounds, the activities of FAOS and LH showed a good correlation. When CPIB or BF, peroxisome proliferators, were administered for 2 weeks, the activities of both enzymes were also increased markedly. On 79-week administration under a similar condition the level of the induction of both enzymes were 50% of that of the 2-week administration, whereas the activities of the mitochondrial enzymes, CPT and FADH were further increased from the level of the 2 week administration. The changes in the activities of FAOS and LH were co-operative to each other and there was a good correlation. An enhancement of the activity of fatty acid oxidation was considered to relate to the hypolipidemic action of CPIB, and the peroxisomal fatty acid oxidation system responded faster than the mitochondrial system in terms of the enhancement of the activity of fatty acid oxidation. On the other hand, under all of the conditions examined the change in the LH activity correlated with that of peroxisomal $\beta$-oxidation activity, suggesting that the mechanisms of the regulation of synthesis and degradation of these two enzymes might be quite similar. There might be a regulatory gene and/or protein which is commonly involved in the expression of the structural genes of the two enzymes.

In the experiment when physiological conditions were varied, where the high-fat diet containing partially hydrogenated marine oil was administered for 2 weeks and thereafter withdrawn, the activities of peroxisomal, mitochondrial and microsomal enzymes were also examined. The patterns of the changes in FAOS, CAT and LH activities were similar to each other, differing clearly from those of CPT and AD. The ratios of the changes in FAOS, CAT and LH activities were less than those of CPT and AD, and when the diet was withdrawn the activities were restored to the control levels, whereas the activities of CPT and AD were not. On the other hand, in the cases of diabetes and fasted conditions the changes in the activity of peroxisomal FAOS correlated with that of microsomal LH activity, whereas little changes in the activities of mitochondrial CPT and FADH were observed. Under these conditions, energy production depends remarkably on fatty acid degradation, and the relationship between the increase in fatty acid supply and the increase in the activity of peroxisomal $\beta$-oxidation might be considered. In fact, under this condition peroxisomes allowed the transport of fatty acids independently to carnitine, and actively oxidized the fatty acids by their $\beta$-oxidation system, assisting the role of mitochondria.[26] However, excess fatty acids which could not be degraded by the $\beta$-oxidation system were esterified

to triglycerides, resulting in an accumulation of lipids in the liver. Simultaneously, the excess of fatty acids were oxidized to long chain dicarboxylic acids by the induced microsomal laurate hydroxylase, followed by oxidation by the peroxisomal $\beta$-oxidation system and then the produced short-chain dicarboxylic acids were excreted in urine. Concurrently, a large amount of short-chain dicarboxylic acids were excreted in urine.[27-29] Thus, the formation of dicarboxylic acids from fatty acids by $\omega$-oxidation and their excretion seems to be one of the mechanisms of removal of fatty acids supplied in excess in the liver under these conditions. Furthermore, a marked increase in the urinary excretion of dicarboxylic acids was observed under the condition in which the function of mitochondrial $\beta$-oxidation was impaired.[30,31] From these facts, in the metabolism of fatty acids peroxisomal $\beta$-oxidation and microsomal $\omega$-oxidation would concern a common function co-operatively. In conclusion, the activities of peroxisomal fatty acid oxidation and microsomal $\omega$-oxidation were induced simultaneously in order to achieve co-operative fatty acid metabolism smoothly in the liver, assisting the function of the mitochondrial $\beta$-oxidation system. The mechanism of the co-operative induction of peroxisomal and microsomal enzymes should be clarified in the molecular level in the near future.

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# Contribution of Glycation to Human Lens Coloration

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**To study the contribution of glycation or the Maillard reaction to the spontaneous coloration of human crystalline lens in aging, we determined 1-deoxyfructosyl adduct and the fluorescent material, which are produced in the early stage of glycation, in the proteins of normal and colored human lenses of different ages. The amount of both glycation products in the lens increased significantly in proportion to aging or the advance of lens coloration. The insolubility of lens protein also increased with the advance of glycation. In addition, the present study showed that glucose and glucose-6-phosphate have higher reactivities with human lens protein than fructose and glucose-1-phosphate.**

**This paper demonstrates that the deeper colored or older aged lens contains larger amounts of glycation products, and that glycation between lens protein and various sugars *in vivo* may be a serious factor in human lens coloration or insolubilization of lens protein.**

**Keywords** human lens; normal lens; cataractous lens; glycation; Maillard reaction; lens coloration; 1-deoxyfructosyl adduct; fluorescent material

It is well known that the crystalline lens coloration of white to brown is observed only in human. An understanding of the mechanism of lens coloration is desired because advanced coloration induces a loss of vision. Photooxidation of tryptophan residues in the lens protein has been suspected of being a serious factor affecting coloration.[1] Recently, glycation has also been proposed as one cause of human lens coloration, because lens protein is exceptionally long-lived in the lens and may be vulnerable to attack from glucose, its derivatives or other sugars.[2] Glycation is a nonenzymatic binding reaction between proteins and sugars; it is called the Maillard reaction or nonenzymatic browning reaction, and occurs widely throughout the natural world.[3] In the initial stage of the Maillard reaction, the condensation product, an aldosylamine, undergoes an Amadori rearrangement to form a 1-deoxyfructosyl adduct (DFA) or fructosamine.[4] In the later stage of the Maillard reaction, the Amadori product undergoes dehydration and rearrangement to form advanced glycosyl end products (AGE), and subsequently yellow-brown fluorescence and protein cross-links that decrease the protein solubility.[3c,5] It was also reported that AGE may be produced by the reaction of protein with 3-deoxyglucosone that is an intermediate in the Maillard reaction.[6] The proposed mechanism of the Maillard reaction is summarized in Fig. 1.[3-7]

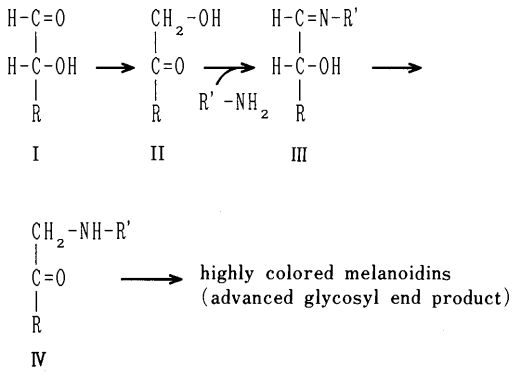


Fig. 1. The Process of the Maillard Reaction or Glycation

The ammono analogue of compound IV derived from glucose is called DFA or fructosamine.

Similar phenomena have been observed in senile cataractous human lenses.[8] These include protein aggregation with a decrease in its solubility, production of nondisulfide covalent cross-links between proteins, advanced coloration from white to brown, and a dramatic increase in protein-bound yellow-brown fluorescence not attributable to tryptophan. It is also well known that normal human lenses become yellow in aging. From studies on glycation in animal lens proteins or aged senile cataractous lenses,[9] the possibility of the involvement of glycation in human lens coloration has been considered, but no conclusion can yet be drawn. To clarify the relationship between glycation and lens coloration, we believed it indispensable to follow the extent of glycation in the normal human lens in aging. There are few reports of such studies, because it is hard to obtain a large number of normal human lenses of different ages. We were fortunate in obtaining several of these, and with them we attempted to clarify whether glycation occurs in the human lens.

The current paper reports the extent of glycation in the human lens in aging, or the advance of coloration by measuring DFA and fluorescence material that have been identified as products in the initial stage of the Maillard reaction.[3c,4a,5] The involvement of glycation in the lens protein insolubilization and reactivity of various sugars with human lens proteins were also examined in this study.

## Materials and Methods

**Lenses Used** Human normal and colored lenses (or senile cataractous ones) were kindly donated by Dr. Horwitz, Jules Stein Eye Institute, University of California, Los Angeles. Briefly, normal human lenses were obtained from autopsy eyes which were removed 4 to 8 h following death. Upon removal, the eye was stored at −80 °C until use. Cataractous lenses were also obtained during routine cataract surgery at the same institute. Bovine lenses were purchased from a local slaughterhouse and stored at −80 °C until use.

**Materials** D-[1-$^{14}$C]Glucose (2.4 GBq/mmol), D-[$^{14}$C(U)]glucose-1-phosphate (11.5 GBq/mmol), D-[1-$^{14}$C]glucose-6-phosphate (2.1 GBq/mmol) and D-[$^{14}$C(U)]fructose (12.7 GBq/mmol) were purchased from DuPont Company. 1-Deoxymorpholinofructose used as a standard material for DFA determination was from Sigma Chemical Co. Polylysine (> M.W. = 8000) was also from Peptide Institute, Inc., Japan.

**Preparation of Soluble and Insoluble Protein Fractions from Human and Bovine Lenses** The soluble and insoluble protein fractions of human and bovine lenses were prepared according to the method described previously.[10] Briefly, after a human lens was homogenized in 3.0 ml

of 0.2 M phosphate buffer, pH 7.4, the suspension was centrifuged at 17000 rpm for 20 min at 4 °C. The supernatant was used as a soluble protein fraction. The soluble proteins were exhaustively washed out of the resulting pellet by 7 repetitions of suspensions in the same buffer and centrifugation. After washing the pellet, the water soluble protein could no longer be detected in the pellet by measurement of protein content, by colorimetry with a Bio Rad assay kit or by the absorbance at 280 nm. The pellet was then dissolved in 3.0 ml of 6 M urea. A small amount of the insoluble material was removed by centrifugation at 27000 rpm for 20 min at 4 °C. The resulting supernatant was used as an insoluble protein fraction. One bovine lens was also homogenized in 7.0 ml of 0.2 M phosphate buffer, pH 7.4, and its soluble and insoluble protein fractions were treated by the same procedure. Except for centrifugation, all work was carried out on a clean bench using tools sterilized in an autoclave or by dry air sterilization.

**Glycation between Lens Protein and Sugars** This reaction was done according to the methods of other investigators.[8b,11] Regarding glycation between human lens protein and glucose, 0.1 μmol of [$^{14}$C]glucose plus 0.1 mmol unlabeled glucose was used to react with 10 mg of protein in 3 ml of 0.2 M phosphate buffer, pH 7.4. The reaction was undertaken at 37 °C under a sterilized condition for 28 d in a dark place to avoid the effect of light. An aliquot of the reactant was taken from the sample solution at regular intervals. In the experiment of incorporation of [$^{14}$C]-labeled sugars into human lens protein, 0.01 μmol of sugar plus 0.01 mmol of unlabeled sugar were added to 1 ml of the solution containing 2 mg of lens protein. The solution was then filtered into a sterilized glass tube through a 0.45 μm Millipore filter, and the filtrate was shaken at 37 °C for 28 d in a dark place. After the reaction had finished, the reactant was treated with 1 mg of $NaBH_4$ for 30 min.[11] The unreactive sugars were removed from the reactant by PD-10 and TSKG3000SW column chromatography followed using 0.2 M phosphate buffer containing 0.1 M NaCl and 0.2% sodium dodecyl sulfate (SDS), pH 6.8, as an eluent. The main peak was pooled and lyophilized. The reactant was dissolved in an appropriate volume of 0.1 M phosphate buffer, pH 6.8, containing 0.1 M NaCl and 0.2% SDS, and its radioactivity was measured.

**Reaction of Glucose with Polylysine** The detailed procedure was described elsewhere.[5] Briefly, 0.1 mmol glucose and 5 mg of polylysine was dissolved in 1 ml of 0.2 M phosphate buffer, pH 7.4, and filtered through a 0.45 μm Millipore filter into a sterilized glass tube with a sterilized stopper. Then, the solution was slowly shaken at 37 °C for 10 d in a dark place.

**Other Analyses** Amino acid composition was analyzed with a Hitachi 835 amino acid analyzer. Fluorescence intensity was determined with a Hitachi MPF-2 fluorescence spectrophotometer. The radioactivity of the incorporated [$^{14}$C]-labeled sugar into the protein was measured with a Packard TRI-CARB liquid scintillation counter using a conventional method. DFA was measured by the method of Baker *et al.*[4] Briefly, 0.1 ml of the sample solution was mixed with 1.0 ml of 0.25 mM nitrotetrazolium blue in 0.1 M bicarbonate buffer, pH 10.35. After the solution had been shaken at 37 °C for 15 min and then cooled, the amount of DFA was determined from the absorbance at 530 nm and 1-deoxymorpholinofructose was used as a standard material. The protein concentration was measured by the method of Bradford.[12]

## Results

**Identification of Reliable Indications of Glycation** Before investigating the extent of glycation in human lens protein, a preliminary experiment was done to confirm whether the DFA level and fluorescence intensity in the glycation product are directly proportional to the extent of glycation or the amount of sugar incorporated into protein, because there was no information on the determination of DFA in lens protein. This experiment was undertaken using [$^{14}$C]glucose and bovine lens protein that consists of crystallin species similar to those of human lens protein. Figure 2 reveals the correlation among the amount of incorporated glucose, the DFA level, and fluorescence intensity at 440 nm (Ex = 340 nm) in the time-course of glycation.

This finding shows that the DFA level and fluorescence intensity in the glycation product increased proportionally with an increase in the amount of incorporated sugar, and that these two factors are reliable indications by which to determine the extent of the glycation.

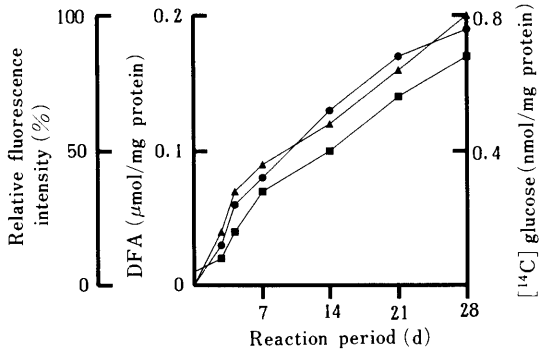


Fig. 2. Relationship among Incorporated [$^{14}$C]Glucose Levels, Produced DFA Levels and Fluorescence Intensity in the Time-Course of the reaction of Bovine Lens Protein with Glucose

Each value represents the mean of three separate experiments. Control data were omitted, because their values in the 28-d reactant showed were similar to those observed in the initial stage. ●, incorporated [$^{14}$C]glucose; ■, DFA levels; ▲, fluorescence intensity at 440 nm (Ex = 340 nm). Fluorescence intensity in the 28-d reactant was arbitrarily taken as 100%.

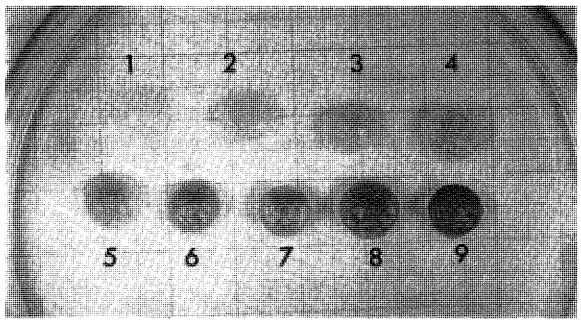


Fig. 3. Cataractous Human Lenses with Various Coloration, White to Brown

Normal lens with transparent and also pale yellow to yellow (nos. 2 to 5 lenses), in aging. Lenses of white, yellow and brown used in the present experiment corresponded to nos. 1, 5 and 9, respectively.

**Variation in Content of Maillard Reaction Products in Normal and Colored Human Lens Protein in Aging and with Advance of Coloration** Human crystalline lens has various colors from white to brown as shown in Fig. 3. Although there are many types of lenses of various colors, three types with typical colors of white, yellow and brown were picked out and used in the experiment.

The amount of glycation product in the lens protein was determined from the DFA level and fluorescence intensity at 440 nm (Ex = 340 nm), which the above experiment had confirmed to be indicative the extent of glycation.

Figures 4A and 4B show the DFA level in the soluble and insoluble protein fractions of normal and colored human lenses. The levels in both proteins increased with aging. In the soluble protein, however, there was no significant difference in levels between normal and colored lenses at a similar age, except for the yellow lens that was conspicuous for its higher level of DFA. In the insoluble proteins, on the other hand, the colored and aged yellow and brown lenses showed higher DFA levels than in normal and white ones.

The fluorescent material produced by the Maillard

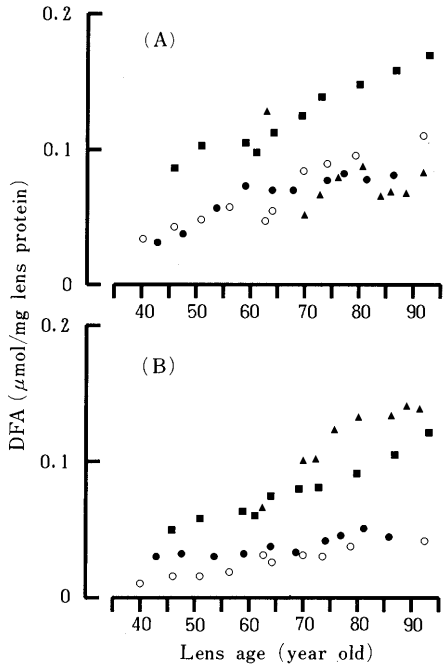


Fig. 4. DFA Levels in Soluble (A) and Insoluble (B) Protein of Normal and Cataractous Human Lenses in Aging or with Advance of Coloration

Each dot represents the value of one lens. ○, normal; ●, white; ■, yellow; ▲, brown lens, respectively.

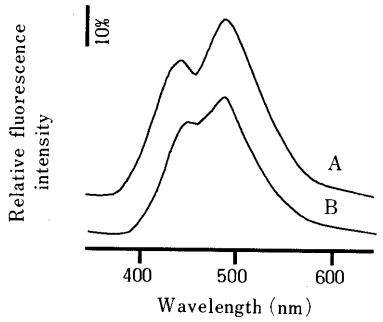


Fig. 5. Fluorescent Spectra of Reactant between Polylysine and Glucose (A), and of Soluble Protein of Normal or Cataractous Human Lens (B)

The spectra were obtained at an excitation wave number of 340 nm. Insoluble proteins of normal and cataractous lenses dissolved in 6 M urea showed similar spectra to those of soluble proteins (data not shown).

reaction in human lens protein was also determined from fluorescence intensity at 440 nm (Ex = 340 nm) as another indication of the reaction. Figure 5A shows the fluorescent spectrum of the reactant of polylysine with glucose in the 10-d reaction, which was used as a typical fluorescent spectrum of the Maillard reaction product. Figure 5B also shows the almost similar spectra of the soluble proteins in normal and colored lenses. The spectra of the insoluble lens proteins also showed a similar pattern to that of the polylysine–glucose reactant (data not shown). This indicates that the fluorescence of colored insoluble protein excited at 340 nm might be because it is a product of the Maillard reaction.

Then, using a portion of the lens protein prepared for determining DFA level, the correlation among aging, coloration and the content of the fluorescent material in the lens protein was determined from the fluorescence intensity at 440 nm (Ex = 340 nm). In both soluble (Fig. 6A)

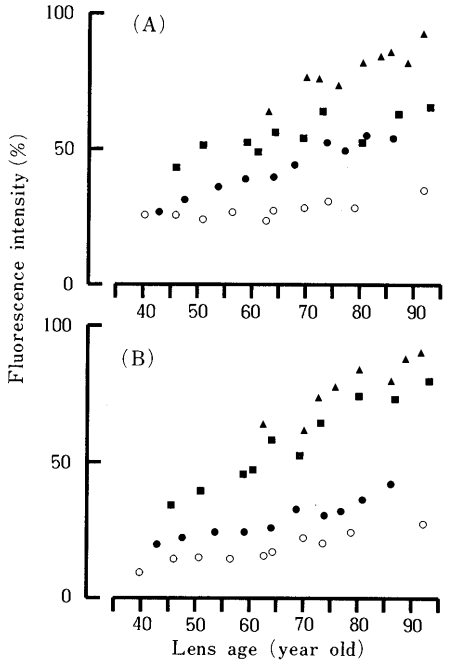


Fig. 6. Fluorescence Intensity of Soluble (A) and Insoluble (B) Proteins of Human Lenses in Aging or with Various Colors

Each dot represents the value of one lens. ○, normal; ●, white; ■, yellow; ▲, brown lens, respectively.

and insoluble (Fig. 6B) proteins, the fluorescent material increased with aging and the advance of coloration. Its contents in the proteins of deeper colored and aged lenses were always higher than those of the normal and white ones. The DFA levels in the soluble protein of yellow and brown lenses were approximately 3-fold that of other lenses.

This tendency was similar to that of DFA levels in the insoluble proteins. However, a discrepancy between the DFA and the fluorescent material levels in the soluble proteins of the brown lens was observed, because the DFA level was very low in spite of the high level of fluorescent materials. These facts show that the Maillard reaction or glycation has taken place in the human lens protein with aging and the advance of coloration. Furthermore, from the disappearance of DFA in the brown lens it is also suggested that the Maillard reaction might progress to a further stage in the deeper colored lenses, and that in the later stage of the reaction DFA may be converted to the advanced glycosyl end product.[4-7] These facts were also supported by an amino acid composition analysis of the soluble proteins as shown in Table I. Lysine and histidine residues in the lens protein decreased in proportion to the advance of lens coloration. Arginine residues in the brown lens protein in addition to those of both amino acid residues, reduced significantly as compared to those in other lenses. This fact also suggests the existence of the Maillard reaction in the lens and the progress of its reaction to a further stage with the advance of coloration. These assumptions were made after referring to many papers reporting the reactivity of various sugars with proteins and basic studies on the Maillard reaction or glycation.[4-7]

It was also observed that the content ratios of cysteine residues reduced to approximately one-half the value of normal lens. Many investigators have reported that this phenomenon might result from the formation of disulfide

TABLE I. Amino Acid Composition (%) of Soluble Protein of Human Lenses

| Amino acid | Normal[a] | Cataract[b] | | |
|---|---|---|---|---|
| | | White | Yellow | Brown |
| ASP | 9.76 | 9.82 | 9.85 | 9.93 |
| THR | 4.17 | 3.87 | 4.03 | 4.06 |
| SER | 7.59 | 7.77 | 8.16 | 8.30 |
| GLU | 14.58 | 14.63 | 14.85 | 15.06 |
| GLY | 9.22 | 9.24 | 9.28 | 9.44 |
| ALA | 5.36 | 5.40 | 5.48 | 5.81 |
| VAL | 5.38 | 5.55 | 5.65 | 5.84 |
| CYS | 0.56 | 0.25 | 0.24 | 0.21 |
| MET | 2.59 | 2.84 | 2.89 | 2.32 |
| ILE | 4.86 | 4.98 | 5.02 | 5.33 |
| LUE | 7.50 | 7.58 | 7.67 | 7.58 |
| TYR | 5.71 | 5.92 | 6.05 | 6.96 |
| PHE | 6.16 | 6.04 | 6.26 | 6.32 |
| LYS | 4.95 | 4.62 | 3.22 | 2.25 |
| HIS | 3.45 | 3.07 | 2.56 | 2.35 |
| ARG | 8.23 | 8.12 | 8.05 | 6.58 |
| PRO | 1.56 | 1.66 | 1.71 | 1.69 |
| Total | 101.63 | 101.36 | 100.97 | 100.03 |

The data represents the mean of two separate analyses. *a*) Ages of normal lenses were 48 to 50. *b*) Ages of white, yellow and brown cataractous lenses were 42 to 47, 52 to 56, and 72 to 80, respectively.

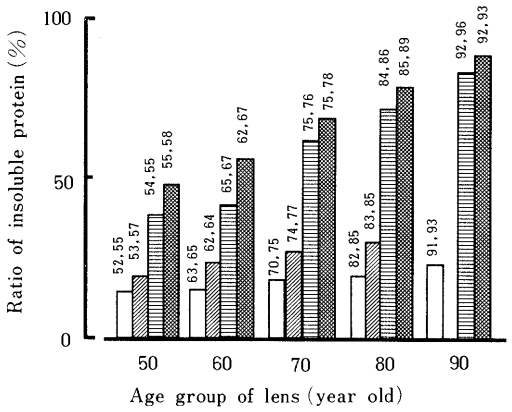


Fig. 7. Insoluble Protein Levels in Human Lenses in Aging or with Various Colors

Each value, which is the mean of two lenses of similar ages, represents a percentage of insoluble protein to the total lens protein in the whole lens. The amount of insoluble protein was determined by the method of Bradford, after the suspension of insoluble protein had been treated with 0.1 N NaOH. □, normal; ▨, white; ▤, yellow; ▩, brown lens. Figures at the top of each column indicate the lens age used in each experiment. There is no data on 90-age white lens because we were unable to obtain such lens.

bonds between lens proteins.[13]

**Insolubility of Lens Protein in Aging or with Advance of Its Coloration** In this experiment, the insolubility of lens protein in aging or with the advance of coloration was determined from the content ratios of insoluble protein in the lens. Figure 7 shows the percent ratios of the insoluble protein to the total protein of the whole lens. Findings indicate that an increase in insolubility of lens protein is directly proportional to aging and the advance of coloration.

**Reactivity of Sugar with Human Lens Protein** Using [$^{14}$C]-labeled sugars and the soluble protein fraction of human lens, this experiment was done to determine the

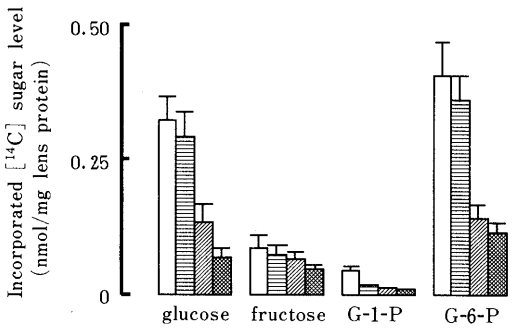


Fig. 8. Incorporation of [$^{14}$C]Labeled Sugars, Glucose, Fructose, Glucose-1-phosphate (G-1-P) and Glucose-6-phosphate (G-6-P), into the Soluble Proteins of Normal and Cataractous Human Lenses

Each bar represents the mean±S.D. of three separate experiments. Detailed experimental conditions were described in the text. □, normal (age 62 to 65); ▤, white (age 60 to 67); ▨, yellow (age 62 to 66); ▩, brown (age 66 and 68) lenses.

reactivity of various sugars with lens proteins. Figure 8 shows the incorporated [$^{14}$C]-labeled sugar levels in the 28-d reaction product. In all cases, normal lens protein incorporated larger amounts of sugars than seen in the colored ones, although there was considerable variety of the incorporated levels depending on the sugars. In colored lenses, the incorporated sugar levels decreased with the advance of coloration, yellow to brown.

These findings show that free glucose and glucose-6-phosphate have considerably higher reactivity with lens protein than fructose and glucose-1-phosphate.

## Discussion

Nonenzymatic glycation has been shown to occur with hemoglobin to form hemoglobin $A_{1c}$ and with a variety of other body proteins, *e.g.*, collagen and nerve protein.[14] Furthermore, nonenzymatic browning has been hypothesized to occur in long-lived proteins such as crystallin and collagen in diabetes.[15] Such coloration is also observed in human crystalline lens, which colors pale yellow in aging under normal conditions or white to brown in cataract. Glycation[2] and photooxidation of tryptophan residues in the lens protein[1] have been the main suspects as causes of lens coloration; however, not much corroborating evidence has been found for this.

Abraham *et al.* reported that nonenzymatic glycation does not progress in human lens in aging, nor contribute to the lens coloration.[2a] However, our study revealed that DFA and fluorescent substance in the human lens, which are products in the early stage of the Maillard reaction, apparently increase in aging or with the advance of coloration. This fact suggests that glycation may contribute to human lens coloration. Furthermore, the content of arginine residues, in addition to lysine and histidine ones, in the brown lens protein diminishes significantly as compared to those in white and yellow lens proteins. In the initial stage of the Maillard reaction, it was reported that lysine or histidine reacts predominantly with sugars.[7] From this point of view, a decrease of arginine residues in the protein of brown lenses suggests that the glycation may progress further in deeper colored lenses. It is necessary to determine the advanced glycosyl end product to clarify the exact contribution of glycation to human lens coloration, because it has been observed widely that the pigmentation

in the protein by glycation is produced in this products in the later stage of the Maillard reaction.[3c,5] At the present time, however, it is not possible to determine such material, because there is no method of analysis for the advanced glycosyl end product and no information on its detailed structure.

In conclusion, this investigation revealed that glycation between lens protein and glucose or its derivatives may be one of the causes of human lens coloration and of lens protein insolubilization in normal lens with aging. Further understanding of nonenzymatic browning *in vivo* is needed so that strategies for interfering with the process on long-lived proteins can be devised.

**Acknowledgment** We thank Dr. Horwitz, Jules Stein Eye Institute, University of California, Los Angeles, who provided normal and colored cataractous human lenses.

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# Particle Design of Enoxacin by Spherical Crystallization Technique. II. Characteristics of Agglomerated Crystals[1)]

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**The characteristics of agglomerated crystals of enoxacin prepared by a spherical crystallization technique with the acetone–ammonia water–dichloromethane solvent system were investigated. Enoxacin forms a sesquihydrate, but has two different pseudopolymorphs, *i.e.* anhydrate and trihydrate. The crystalline form of the resultant agglomerates could be controlled by selecting the composition ratio of the three solvents and their mixing procedure. In order to obtain the sesquihydrous agglomerates, the mixing of an ammonia water solution of enoxacin with acetone in the first stage was required. When ammonia concentration in ammonia water used as a bridging liquid was higher, agglomerated crystals became smaller in size and less spherical in shape. The average size of agglomerates decreased with an increase in agitation speed and with a decrease in the ammonia water fraction in the solvent system. Primary crystals composing the agglomerates grew larger in size in the solvent system where the crystallization rate was reduced, resulting in less spherical agglomerates. Spherically agglomerated crystals prepared with a low fraction of ammonia water improved their flowability and packability without much delay in their dissolution rate, as compared with the primary crystals.**

**Keywords** enoxacin; spherical crystallization; agglomerated crystal; pseudopolymorphism; ammonia water; acetone; micromeritic property; dissolution rate

The authors established a novel spherical crystallization technique using ADS (ammonia diffusion system) in a previous paper.[1)] This technique was applicable to amphoteric drug substances like enoxacin. In this process, as a crystallization solvent, a mixture of three partially miscible solvent, *i.e.* acetone–ammonia water–dichloromethane was used. By selecting a proper ratio, a small amount of ammonia water was liberated from the system. This ammonia water played two roles, a good solvent for enoxacin and a bridging liquid, which collected fine crystals precipitated into spherical agglomerates in one step. Enoxacin, which is widely prescribed for antibacterial properties, forms a sesquihydrate, having two different pseudopolymorphs, *i.e.* anhydrate and trihydrate. But the crystalline forms of enoxacin could be controlled by the composition of the solvent in this technique.

The aim of this study was to elucidate effects of the composition of the crystallization solvent on crystalline forms and the micromeritic characteristics of resultant agglomerates.

## Experimental

**Spherical Crystallization (SC) Process** The main procedure is shown in Chart 1. Mixtures (200 ml) of acetone and dichloromethane in various ratios (acetone/dichloromethane volume ratio; 4.17, 2.45, 1.26, 0.70) were used as crystallization solvents. Enoxacin (obtained from Dainippon Pharmaceutical Co.) was dissolved in ammonia water (concentration; 10—25% (w/v)) to 16% (w/v) at about 40 °C. The system was kept agitating at 250—600 rpm using a screw-type agitator in a cylindrical vessel (500 ml), and thermally controlled at 20 °C during the process. The procedures for adding the above solvents were performed in three ways as follows: (A) 1) acetone, 2) ammonia water solution of enoxacin, 3) dichloromethane, (B) 1) acetone, 2) dichloromethane, 3) ammonia water solution of enoxacin, and (C) 1) dichloromethane, 2) ammonia water solution of enoxacin, 3) acetone. Herein, the 1), 2), and 3) mean the addition order of solvents or the solution. After 20 min, the agglomerated crystals were separated, washed with dichloromethane and dried at 50 °C for 16 h.

As a reference, ordinary crystallization without agglomerating enoxacin was undertaken to get primary crystals by introducing the ammonia water solution of enoxacin (16% (w/v), 30 or 15 ml) into acetone (270 or 200 ml) under agitation (250 rpm).

**Measurement of Physicochemical and Micromeritic Properties of Agglomerates** The shape and surface topography of the resultant agglomerated crystals and primary crystals were determined with an optical microscope (Olympus Optical Co., Model VANOX) and a scanning electron microscope (Nihon Densi, Model JSM-T20). The particle size distributions of agglomerated crystals and primary crystals were measured by sieve analysis or by a photographic counting method (particle number: about 600 counted) using a particle size frequency analyzer (Shimadzu, SF-20). As a reference, the original enoxacin was comminuted by a Pulverizer (Hosokawa Micron Co.). The crystalline forms of agglomerates were analyzed by X-ray diffractometry (Rigaku Denki, Geigerflex).

The flowability of agglomerates was evaluated by the angle of repose (disk diameter: 30 mm) and the apparent density. The packability of agglomerates was investigated by using a tapping machine (Konishi Seisakusho Co.). The sphericity of agglomerates was evaluated by a shape index (minimum diameter/maximum diameter) measured by a photographic counting method in the same way as measurement of size distribution.

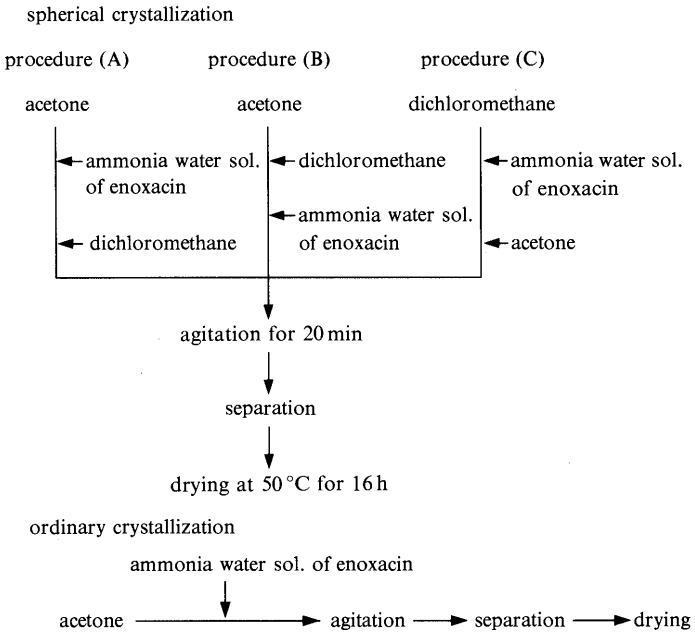


Chart 1. Process Flow Sheet of Spherical Crystallization and Ordinary Crystallization

The direct compressibility of the resultant agglomerates into the tablets was tested by using a single punch tablet machine (Kikusui, No. 2-B; tablet diameter 7.5 mm; tablet weight 150 mg).

**Measurement of Drug Dissolution Rate of Agglomerates** The dissolution tests of agglomerates were carried out at 50 rpm in distilled water by the paddle method specified in JP XI. Enoxacin was assayed spectrophotometrically at 268 nm after being diluted with 0.1 N hydrochloric acid aq. solution. The agglomerates (100 mg), 177—250 $\mu$m in diameter, were used. The average diameter of agglomerates used was estimated to be 214 $\mu$m. In addition, a physical mixture (200 mg) of primary crystals (average diameter 15 $\mu$m) and corn starch (1 : 1) was also used for the dissolution test as a reference. Dissolution experiments were triplicated and were closely reproducible.

## Results and Discussion

**Agglomeration Process of Enoxacin** The authors reported in a previous paper[1] that acetone was an indispensable solvent to obtain the spherical agglomerates of enoxacin as a sesquihydrous crystalline form. In addition, acetone provided a rapid crystallization rate of enoxacin and yielded the high recovery. Therefore, the agglomeration system containing acetone was focused in this study.

The region capable of agglomeration is shown by a shaded area in the triangular phase diagram for the acetone–ammonia water–dichloromethane system in Fig. 1. In the region (M) above the phase separation curve, enoxacin crystals were only dispersed without agglomeration because an ammonia water was freely miscible with the two other solvents; whereas, in the region (I) below the shaded area, the resultant agglomerates formed a big solid mass or a paste. Each (a), (b), (c) or (d) in Fig. 1 shows a zone of solvent compositions where SC was successfully carried out, based on 4.17, 2.45, 1.26 and 0.70 of acetone/dichloromethane volume ratio, respectively.

**Physicochemical and Micromeritic Properties of Agglomerated Crystals** Table I shows the effects of solvent compositions and mixing procedures of solvents in the region capable of agglomeration on pseudopolymorphs of agglomerates. The solvent compositions in Table I correspond to zones (a)—(d) in Fig. 1, respectively. The resultant agglomerates formed a sesquihydrate in all solvent compositions of zones (a)—(d) for procedure (A) where the ammonia water solution of enoxacin was mixed first with acetone. This indicates the sesquihydrous crystals are precipitated so long as ammonia water is mixed with acetone alone first, regardless of their mixed ratios. On the other hand, for procedures (B) and (C) such as the system where the ammonia water solution of enoxacin is mixed with the

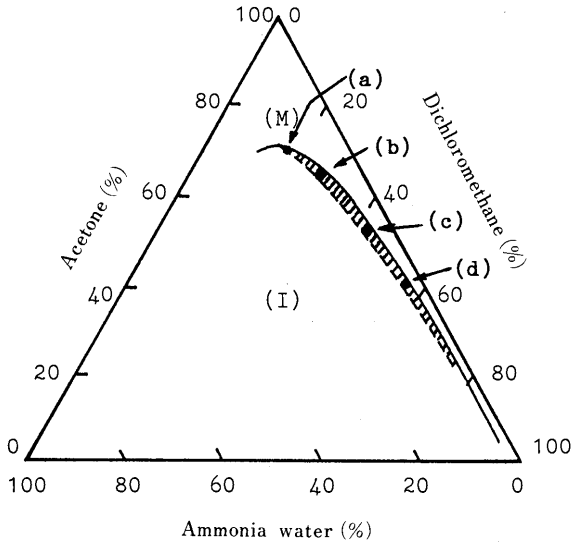


Fig. 1. Phase Diagram for Agglomeration of Enoxacin in the Acetone–Ammonia Water–Dichloromethane System

Ammonia water was miscible (M) in the above region and immiscible (I) in the region below the solid line. Each (a), (b), (c) or (d) shows the solvent fraction zone where SC was carried out.

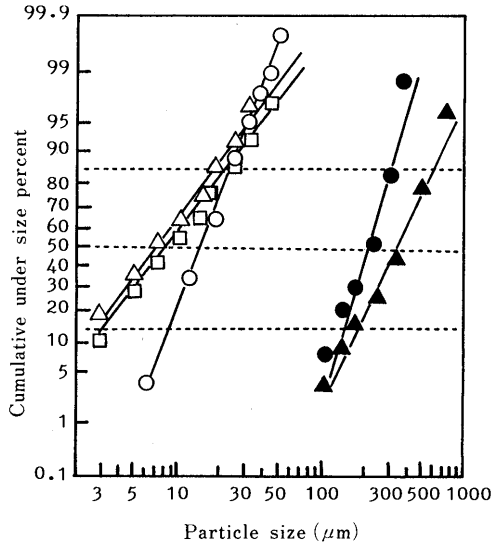


Fig. 2. Size Distribution of Agglomerated Crystals and Primary Crystals of Enoxacin

(○) ordinary comminuted primary crystals, (□) crystallized particles without agglomeration (ammonia water : acetone = 3 : 27), (△) crystallized particles without agglomeration (ammonia water : acetone = 3 : 40), (●) SC agglomerates prepared by procedure (A) (zone (a), ammonia water fraction 12.8%), (▲) SC agglomerates prepared by procedure (A) (zone (a), ammonia water fraction 14.6%).

Table I. Effects of Solvent Composition and Mixing Procedure of Solvents on Pseudopolymorphs of Resultant Agglomerates (Agglomeration Time: 20 min)

| Zone in Fig. 1 | Solvent composition (%) | | | Pseudopolymorphs | | |
|---|---|---|---|---|---|---|
| | | | | Mixing procedure of solvent[a] | | |
| | Acetone | Dichloromethane | Ammonia water | A | B | C |
| a | 69.6 | 16.7 | 13.7 | Sesqui. | Sesqui. + Tri. | Sesqui. + Tri. |
| b | 65.3 | 26.7 | 7.9 | Sesqui. | Sesqui. + Tri. | Sesqui. + Tri. |
| c | 53.5 | 42.6 | 4.0 | Sesqui. | Tri. | Tri. |
| d | 39.8 | 57.2 | 3.0 | Sesqui. | Tri. | Tri. |

a) A (1) acetone (2) ammonia water solution of enoxacin (3) dichloromethane. B) (1) acetone (2) dichloromethane (3) ammonia water solution of enoxacin. C) (1) dichloromethane (2) ammonia water solution of enoxacin (3) acetone. Each number represents the addition order. Tri., trihydrate; Sesqui., sesquihydrate.

mixture of acetone and dichloromethane, with an increasing acetone fraction, like the zones of (a) and (b), the resultant agglomerates consisted of a mixture of sesquihydrate and a small amount of trihydrate; whereas, in the zones of (c) and (d), where the acetone fraction is smaller than (a) and (b), the trihydrous agglomerates were predominantly obtained. These findings show the pseudopolymorphs of agglomerates depend upon the crystallization rate from the system. The precipitation from the system such as procedures (B) and (C) had an induction time (1 to 2.5 min) and became slower in the low fraction of acetone. Furthermore, from the preliminary experimental results, it was observed that in the binary system consisting of an acetone and ammonia water solution of enoxacin, they were mixed and simultaneously sesquihydrous crystals were precipitated at 25 °C, but the crystallization rate was delayed at 50 °C in the above same system, resulting in the yield of a small amount of trihydrate. Considering the facts, this delay seems to result in the formation of a part of trihydrous crystalline seeds. In this connection, similar results were recognized from the experiment undertaken by replacing dichloromethane with *n*-hexane.

The size distribution of agglomerates is shown in Fig. 2, together with primary crystals of enoxacin. The geometric average diameter of ordinary comminuted enoxacin crystals was about 15 $\mu$m, whereas that of enoxacin crystals prepared by ordinary crystallization (agitation time; 5 min) according to Chart 1 was smaller, *i.e.* 6 to 7 $\mu$m. The size of primary crystals increased with increasing the volume ratio of ammonia water to acetone. Agglomerated crystals prepared by procedure (A) were composed of these fine primary crystals. On the other hand, the sesquihydrous primary crystals composing agglomerates prepared by procedure (B) in zone (b) were remarkably large in size as shown in Fig. 3-II. As described before, the precipitation from the system where the acetone fraction was low had an induction time for procedure (B), resulting in sesquihydrous crystals growing slowly and large at the same time, though a part of trihydrous crystals occurred. It was therefore difficult to agglomerate their crystals spherically because of their large primary crystal sizes (see Fig. 3-II). Thus, it was found that the size and crystalline forms of primary crystals composing agglomerates could be controlled by selecting the fractions and mixing procedures of solvents.

The representative agglomerates obtained with 12.8% and 14.6% of an ammonia water fraction in zone (a) in Fig. 1 was about 209 and 395 $\mu$m in the average diameter, respectively. The agglomerates were spherical in shape. Original primary crystals of enoxacin were rectangular platen, and were collected and closely packed inside of agglomerates as well as on the surface (Figs. 3-I and 3-IV).

Influences of ammonia concentration in ammonia water on micromeritic properties of the resultant agglomerates prepared by procedure (A) were tabulated in Table II. When the ammonia concentration in ammonia water was less than 10%, the agglomeration could not be observed because the solubility of enoxacin in the ammonia water decreased. When the ammonia concentration in ammonia water was more than 20%, the sesquihydrous agglomerates could be obtained. The average geometric diameter of the agglomerates prepared with 25% of ammonia water was smaller than that with 20% of ammonia water, and the shape was less spherical. These findings suggest that the shape tends to become more irregular when the ammonia concentration in ammonia water is higher. As flocculation of particles are dominant when the cohesive forces of particles are stronger, it is not likely to yield compact agglomeration between

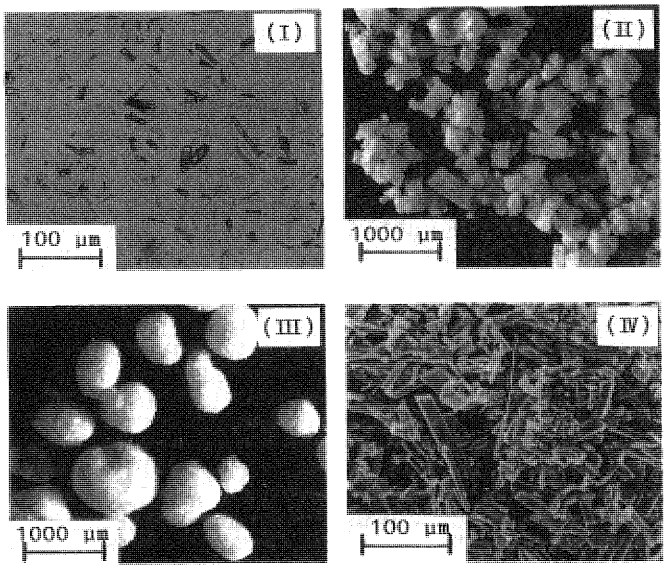


Fig. 3. Optical and Scanning Electron Microphotographs of Agglomerated Crystals and Primary Crystals of Enoxacin

Optical microphotographs: I) crystallized primary particles without agglomeration, II) primary crystals composing agglomerates (procedure (B), zone (b)), III) agglomerated crystals (procedure (A), zone (a)). Scanning electron microphotograph: IV) surface of agglomerated crystals (procedure (A), zone (b)).

TABLE II. Influences of Ammonia Concentration in Ammonia Water on Micromeritic Properties of Resultant Agglomerates (Procedure (A), Zone (b))

| Ammonia conc. in ammonia water (%(w/v)) | Shape index[a)] | Average diameter ($\mu$m) | $\sigma_g$[b)] | Pseudopolymorphs |
|---|---|---|---|---|
| 10 | 0.84 | 559 | 3.19 | Tri. |
| 15 | 0.83 | 359 | 1.52 | Tri. + Sesqui. |
| 20 | 0.77 | 409 | 2.28 | Sesqui. |
| 25 | 0.72 | 373 | 2.42 | Sesqui. |

*a*) Minimum diameter/maximum diameter. *b*) Geometric standard deviation. Tri., trihydrate; Sesqui., sesquihydrate.

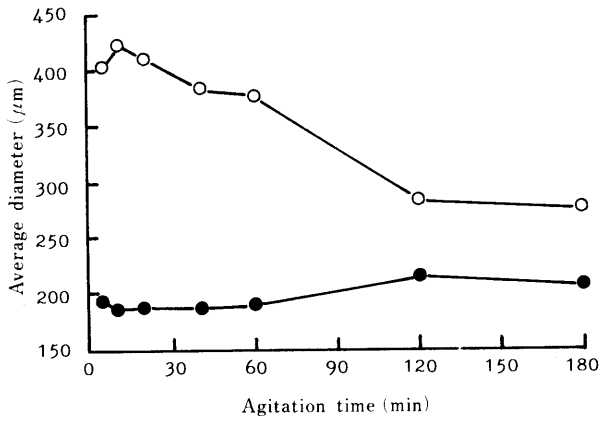


Fig. 4. Effect of Agitation Time on Average Diameter of Agglomerates Prepared in Zone (a)

(●) procedure (A), (○) procedure (B).

flocculated particles, resulting in the agglomerates irregular in shape. This phenomena coincided with results of the simulation[2] reported by Kawashima *et al.* Herein, they suggested the stronger probability of adhesion of particles at their collision sites made resultant agglomerates more irregular in shape and larger in size due to the generation of bulky agglomerates. The result which indicated the size enlargement of agglomerates was different from that of our experiments, which seemed to be caused by a destructive force such as agitation in the practical system. When the ammonia concentration in ammonia water was 10 to 15%, the agglomerates contained trihydrate and the average diameter of the agglomerates prepared in the region of high ammonia concentration in ammonia water decreased. The strength of agglomerates prepared with 10 to 15% of ammonia concentration in ammonia water was not enough for further processing, *e.g.* tableting or coating.

The average diameter of agglomerates is shown as a function of agitation time in Fig. 4. Trihydrous agglomerates prepared by procedure (B) (zone (a)) decreased in the average diameter gradually. This phenomena may be due to the fact that trihydrous agglomerates disintegrated into dispersing sesquihydrous particles during the process from the result on X-ray diffraction analysis of dispersing crystals and agglomerates. In the case of sesquihydrous crystals (procedure (A), zone (a)), the smaller average diameter was obtained within the initial 10 min. After that, no significant changes in the size were observed. In the conventional SC technique,[3,4] Kawashima and coworkers reported that a bridging liquid in the agglomerates moved onto the surface by the compaction of agglomerate, and introduced to the secondary agglomeration by adhesion of individual agglomerates or by layering agglomeration. In this experiment, however, the effect of agitation time on the average diameter was not recognized.

Other factors influencing the average diameter of agglomerates were tabulated in Table III. With increasing agitation speed, the average diameter of agglomerates decreased. Under an agitation speed slower than 250 rpm, the resultant agglomerate shape became more irregular and some of them adhered to the propeller and the vessel wall. On the other hand, the size of agglomerates increased with an increase in the ammonia water fraction in the solvent system. Moreover, with increasing acetone fraction, the size distribution of agglomerates became sharp.

TABLE III. Influences of Agitation Speed and Ammonia Water Fraction in Solvent on Average Diameter and Geometric Standard Deviation of Agglomerates (Procedure (A))

| Factors | Average diameter ($\mu$m) | $\sigma_g$[b] |
|---|---|---|
| Agitation speed (rpm)[a] | | |
| 250 | 522 | 2.63 |
| 350 | 522 | 2.06 |
| 500 | 409 | 2.28 |
| 600 | 373 | 2.42 |
| Ammonia water fraction in solvent (%)[c] | | |
| Acetone/dichloromethane[d]=4.17[e] | | |
| 12.9 | 158 | 1.46 |
| 13.7 | 209 | 1.42 |
| 14.6 | 395 | 1.63 |
| Acetone/dichloromethane[d]=2.45[a] | | |
| 7.0 | 263 | 2.94 |
| 7.5 | 373 | 2.42 |
| 7.9 | 472 | 2.42 |

*a*) Zone (b). *b*) Geometric standard deviation. *c*) Agitation speed 600 rpm. *d*) Volume ratio. *e*) Zone (a).

The micromeritic properties of agglomerated crystals (procedure (A), zone (a)) are shown in Table IV, including those of original crystals of enoxacin as a reference. The angle of repose of agglomerated crystals was 40.3°, while that of crystals without agglomeration was >50°. The data of the angle of repose and the apparent density suggested that the flowability of agglomerated crystals was much improved compared with those of the original crystals. The packability was investigated by tapping crystals. The data were evaluated by the Kawakita and Lüdde equation,[5] represented by Eq. 1

$$n/c = 1/ab + n/a \qquad c = (V_0 - V_n)/V_0 \tag{1}$$

where $a$ and $b$ are the constants, $n$ is the tap number, and $V_0$ and $V_n$ are the powder bed volumes at the initial and $n$th tapped state, respectively. The smaller value of parameter "$a$" for the agglomerates indicated their higher packabilities. Due to the good flowabilities and packabilities of agglomerated crystals, they were directly compressible with commonly used excipients such as lactose, corn starch or crystalline cellulose. The resultant tablet hardness was 6—7 kg and its disintegration time was about 20 s.

Agglomerated crystals were gradually dissolved without their disintegration. The dissolution rate of the spherically agglomerated crystals prepared with 12.9% ammonia water

TABLE IV. Comparison of Micromeritic Properties of Agglomerated Crystals with Those of Primary Crystals

| Micromeritic properties | Agglomerated crystals[b] | Primary crystals |
|---|---|---|
| Average diameter ($\mu$m) | 158 | 15 |
| Angle of repose (°) | 40 | >50 |
| Apparent density (g/cm$^3$) | 0.52 | 0.34 |
| Parameter $a$[a] | 0.21 | 0.62 |
| Parameter $1/b$[a] | 30.5 | 22.6 |

*a*) Parameters in Kawakita equation. *b*) Procedure (A), zone (a), ammonia water fraction 12.9%.

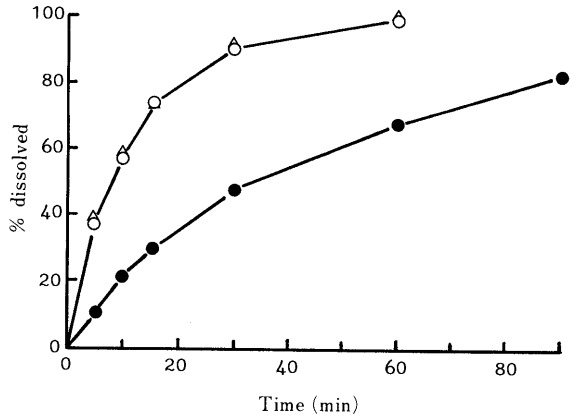


Fig. 5. Dissolution Profiles of Agglomerated and Primary Crystals in Distilled Water of 900 ml at 50 rpm According with JP XI Paddle Method

Agglomerated crystals: ammonia water fraction (○) 12.9%, (●) 14.6% in zone (a). Primary crystals: (△) physical mixture of enoxacin primary crystals and corn starch (1 : 1).

TABLE V. Ratio of Dissolution Percent per Apparent Unit Surface Area of Primary and Agglomerated Crystals

| | Average diameter (μm) | Dissolution percent after 10 min (%) | Ratio of dissolution percent per apparent unit surface area (−) |
|---|---|---|---|
| Primary crystals | 15 | 60 | 1 |
| Agglomerated crystals[a] I | 214 | 58 | 197 |
| II | 214 | 20 | 68 |

a) I and II were prepared with 12.9% and 14.6% ammonia water fraction, respectively.

fraction in zone (a) did not decrease as much as predicted by their low apparent specific surface area (see Fig. 5). These agglomerates have quite a lot of pores inside of the agglomerates because of a weak cohesive force, leading not to a decrease their surface area. Therefore, the agglomerates seem to have shown the same dissolution profiles as their primary particles. Agglomerated crystals prepared with a 14.6% ammonia water fraction consisted of large primary crystals in addition to forming stronger bridges between primary crystals, resulting in a slower dissolution rate. Moreover, from the data on dissolution rates (Fig. 5), and the average size of primary crystals and agglomerates (177—250 μm), the ratio of dissolution percent per the apparent unit specific surface area was calculated as shown in Table V. Supposing that the ratio of dissolution percent of primary crystals (average diameter; 15 μm) was 1, that of the agglomerates used (average diameter; 214 μm) prepared with 12.9 and 14.6% ammonia water fractions would be 197 and 68 μm, respectively. In order to achieve the successful direct tabletting, a drug substance should be generally required to have a suitable particle size from the viewpoint of their flowability and uniform mixing with other excipients. Table V suggested that even if enoxacin crystals were agglomerated, the dissolution percent per the surface area became fast. Thus, the spherical crystallization technique enabled the particle design to obtain the agglomerates for direct tabletting, having a fast dissolution rate different from that of merely large primary crystals.

## Conclusion

The composition ratio of three solvents (acetone–ammonia water–dichloromethane) and their mixing procedure were the main factors determining the crystalline forms of the resultant agglomerated crystals.

The average size of the resultant agglomerated crystals (sesquihydrate) was dependent on the agitation speed of the system or on the ammonia water fraction in the solvent.

The flowability and packability of spherically agglomerated crystals were improved without much delay in their dissolution rate.

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# Viscosity Study on the Self-Association of Doxorubicin in Aqueous Solution

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*Received November 5, 1990*

**The viscosity of doxorubicin aqueous solution as a function of pH, ionic strength, and temperature was studied in detail. The viscosity increased in the presence of NaCl and maximum viscosity was observed at pH 5 to 7 in the pH profiles. When NaCl was added to various concentrations of doxorubicin aqueous solution, the viscosity suddenly increased when the NaCl concentration exceeded a certain level. This concentration for critical gel formation was found to be inversely correlated with the logarithmic doxorubicin concentration.**

**The viscosity of doxorubicin aqueous solution was decreased with rising temperature. The analysis of viscosity on temperature-dependence revealed two gel structures at 20 to 30 °C. Activation energy of the viscous flow at lower temperatures was about 15 kcal/mol and that at higher temperatures was about 60 kcal/mol.**

**The viscosity of doxorubicin aqueous solution also increased in the presence of various cations or anions. This increase in the viscosity caused by monovalent cations or anions was in almost inverse correlation with the lyotropic series, which showed that those ions having a weaker dehydration ability induce a higher viscosity of doxorubicin aqueous solution and promote gel formation. In the viscosity increase caused by monovalent cations or anions, a mechanism involving hydration and self-association owing to $\pi$–$\pi$ stacking of doxorubicin molecules was strongly suggested.**

**In the case of bivalent or trivalent cations, those cations having a stronger chelation ability caused a larger increase in the viscosity of doxorubicin aqueous solution, which indicated that self-association due to intermolecular chelation could be the mechanism of gel formation.**

**Keywords** doxorubicin hydrochloride; viscosity; gelation; self-association; lyotropic series; chelation

## Introduction

Doxorubicin (Fig. 1) is a water-soluble molecule of low molecular weight, but its molecular structure consists of a hydrophobic anthracycline ring and a hydrophilic side chain ($C_{14}$–OH moiety, and sugar moiety).[1] Therefore, the interaction between doxorubicin and water seems to be derived from its hydrophobic/hydrophilic character.

We previously reported[2] that doxorubicin interacted with NaCl and various other metal salts in water, that the viscosity of its aqueous solution increased as a result of the formation of an associated product containing water, and that it was considered a gel microscopically.

The gel formation was thought to be responsible for the prolongation of dissolution time which occurs when a freeze-dried product of doxorubicin is reconstituted with isotonic sodium chloride solution. We further reported that methyl parahydroxybenzoate, urea, and other additives have the effect of shortening dissolution time and that this effect is correlated with the viscosity-decreasing effects of these additives. We also indicated,[1] by visible absorption spectra and proton nuclear magnetic resonance ($^1$H-NMR) analysis, that the increase in the viscosity of the aqueous solution of doxorubicin with the addition of NaCl is caused by the self-association of doxorubicin due to $\pi$–$\pi$ stacking of its molecules.

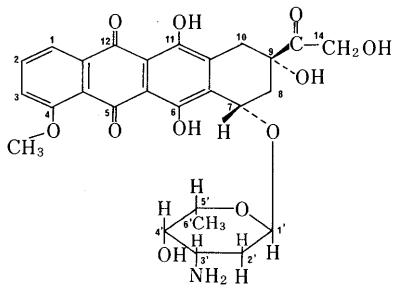

Fig. 1. Chemical Structure of Doxorubicin

Many reports have been published on the interaction of doxorubicin with various metal ions. These reports have related to binding sites,[3] stability constants,[4] intercalation with deoxyribonucleic acid (DNA),[5] generation of radical oxygen,[6] *etc.*, but few have related to gel formation. We have studied the interaction of doxorubicin with various electrolytes in aqueous solution and have investigated the gel structure in detail.

## Experimental

**Experimental Materials** Doxorubicin hydrochloride was provided by Farmitalia Carlo Erba Co. All other reagents employed were special grade products.

**Viscosity Measurements** Viscosity measurements were made by determining the efflux time, $t$ (s), in a thermostatic bath (model UB-7S type, Ikeda Rika Co.), using a Ubbelohde-type viscometer (Kusano Co.).

The viscosity was measured 5 times for each concentration of sample at each temperature, and the average of 3 values, eliminating the highest and the lowest, was regarded as the efflux time.

Kinematic viscosity, $v$ (cSt), was calculated according to the following formula:

$$v = Kt \quad (1)$$

where $K$ is a constant of the viscometer and is predetermined using a reference standard liquid with known kinematic viscosity.

To calculate absolute viscosity, $\eta$ (cP), the density $d$ (g/ml) of the sample at the same temperature was measured and the following formula was used:

$$\eta = vd \quad (2)$$

The density was measured using a Gay-Lussac type picnometer of about 25 ml volume in the above-described thermostatic bath. The samples were homogenously dissolved in distilled water at 40 °C, individually prepared for each ingredient, allowed to stand for 20 min at a given temperature and then used for measurement.

In the doxorubicin and NaSCN mixture, viscosity reached equilibrium in about 2 h, which indicated that gel formation was progressing gradually. In other test samples, however, the time-course change of viscosity was not observed.

In this study, viscosity was measured to make the following four determinations:

(1) Effects of pH on the Viscosity of Doxorubicin Aqueous Solution: Various metal chlorides were added to doxorubicin aqueous solution (3 mg/ml), and the mixture was adjusted for pH with diluted NaOH and HCl; the viscosity was measured at 25 °C. Porumb[7] reported that, when pH was adjusted with phosphate or other buffers, the addition of various metal chlorides precipitated doxorubicin and this disturbed viscosity measurement. Based on this finding, buffer solution was not used in this experiment. The pH was measured with a pH meter (model HM-5ES, Toa Co.).

(2) Relationship between Doxorubicin Concentration, NaCl Amount, and the Viscosity of Doxorubicin Aqueous Solution: NaCl at various concentrations was added to doxorubicin aqueous solution at concentrations of 1 to 10 mg/ml and the viscosity was measured at 25 °C. The pH ranged from 5 to 6 in all the samples. As the effect of pH on viscosity was considered to be negligible in this pH range (Fig. 2), in this experiment the viscosity was measured without pH adjustment.

(3) Temperature Dependence of the Viscosity of Doxorubicin Aqueous Solution: The addition of NaCl at various concentrations to doxorubicin aqueous solution (5 mg/ml) produced highly viscous solutions. The viscosity of these solutions was measured at 10 to 35 °C. The temperature-dependent change of viscosity was analyzed according to Andrade's formula:

$$\eta = Ae^{\Delta E/RT}$$

$$\log\eta = \log A + \frac{\Delta E}{2.303R}\cdot\frac{1}{T} \quad (3)$$

where $\Delta E$: activation energy of viscous flow, $R$: gas constant, $T$: absolute temperature, $A$: frequency factor.

(4) Effects of Various Cations or Anions on the Viscosity of Doxorubicin Aqueous Solution: Various concentrations of monovalent, bivalent, or trivalent cations, or monovalent anions were added to doxorubicin aqueous solution (5 mg/ml) and the viscosity was measured at 25 °C.

This experiment was conducted with and without pH adjustment for all the samples. Except for $AlCl_3$ and $FeCl_3$, the viscosity of the samples adjusted to pH 5.5 (with diluted NaOH and HCl) did not significantly differ from that of the samples without pH adjustment. The viscosity of the samples without pH adjustment is described in this report.

## Results and Discussion

**Effects of pH on the Viscosity of Doxorubicin Aqueous Solution Containing Metal Chloride** The effect of NaCl on the viscosity of doxorubicin aqueous solution at 25 °C is shown in Fig. 2 as a function of pH. In the presence of NaCl, the viscosity of the solution increased significantly in a pH-dependent manner, while in the case of doxorubicin alone or NaCl alone no increase in the viscosity was observed over a wide pH range.

In the presence of NaCl, the maximum viscosity of doxorubicin aqueous solution was observed at about pH 4.5 to 7.0. The $pK_a$ ($NH_2$) in the sugar moiety of doxorubicin is about 7.4, while the $pK_a$ (OH) in the phenolic group is about 10.[8] The sharp decrease in the viscosity at pH 7 to 8 is thought to be related to the $pK_a$ ($NH_2$). Therefore, the high viscosity could be attributed to the interaction of protonated doxorubicin with NaCl and $H_2O$.

We previously reported by visible absorption spectra and $^1$H-NMR analysis that the addition of NaCl shielded the positive charge of doxorubicin molecules and promoted self-association due to $\pi$–$\pi$ stacking by reducing the electrostatic repulsion force among these molecules at about pH 5 to 6, and that an increase in the viscosity of the aqueous solution of doxorubicin was observed as a result.[1] The decrease in viscosity below pH 4.5 shown in Fig. 2 is thought to be related to the inhibition of self-association by the elevated electrostatic repulsion force due to low pH values.

Figure 3 shows the pH profiles of the viscosity of

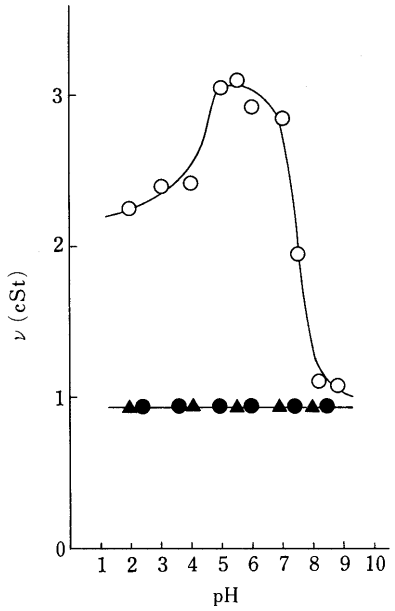


Fig. 2. pH Profiles of the Viscosity of Doxorubicin Aqueous Solution at 25 °C in the Presence or in the Absence of NaCl

○, [doxorubicin] = 3 mg/ml, [NaCl] = 0.3 M; ●, [doxorubicin] = 3 mg/ml; ▲, [NaCl] = 0.3 M.

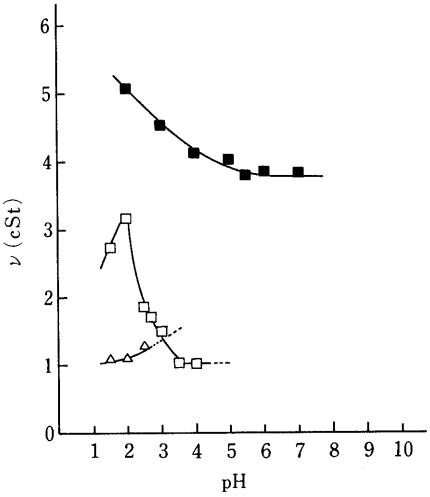


Fig. 3. pH Profiles of the Viscosity of Doxorubicin Aqueous Solution at 25 °C in the Presence of Various Metal Chloride Salts

■, [doxorubicin] = 3 mg/ml, [$CaCl_2$] = 0.2 M; □, [doxorubicin] = 3 mg/ml, [$AlCl_3$] = 0.15 M; △, [doxorubicin] = 3 mg/ml, [$FeCl_3$] = 0.2 M. Dotted lines mean the appearance of precipitation.

doxorubicin in the presence of bivalent or trivalent cation chloride. All the samples exhibited high viscosity compared with aqueous solution of doxorubicin alone, but in a manner largely different from the viscosity behavior in the presence of NaCl. $CaCl_2$ and $AlCl_3$ had a tendency to induce high viscosity at low pH values.

$FeCl_3$ increased the viscosity at higher pH values and produced precipitation at pH more than around 3. The doxorubicin and Fe ion complex is known as quelamycin and is obtained by neutralizing a mixed solution of doxorubicin and $FeCl_3$.[9] The structural formula of this complex has been presented in other papers.[4,9-11]

**Relationship between Doxorubicin Concentration, NaCl Amount, and the Viscosity of Doxorubicin Aqueous Solution** Figure 4 shows the relationship between doxorubicin con-

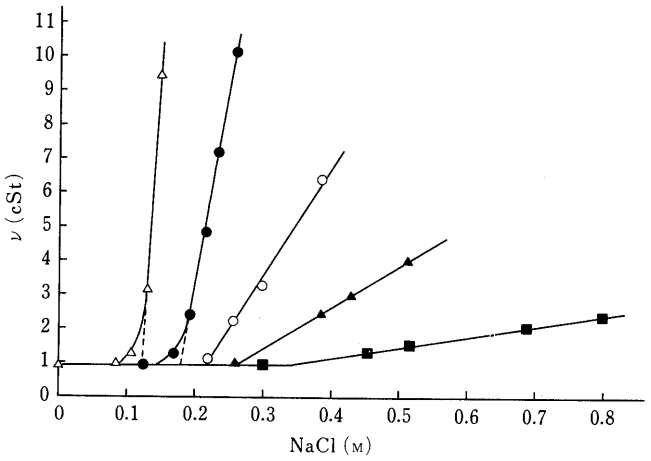


Fig. 4. Effect of NaCl Concentration on the Viscosity of Doxorubicin at 25 °C

[Doxorubicin]: ■, 1 mg/ml; ▲, 2 mg/ml; ○, 3 mg/ml; ●, 5 mg/ml; △, 10 mg/ml. Dotted lines represent extrapolation in order to obtain CGFC.

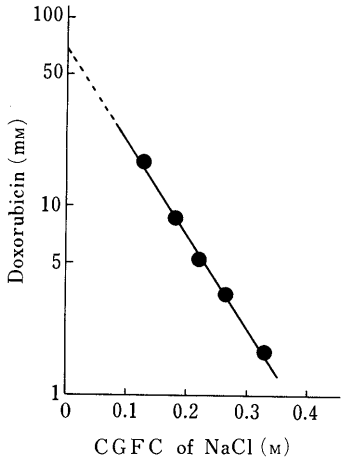


Fig. 5. Relationship between CGFC (Critical Gel Formation Concentration) of NaCl and Doxorubicin Concentration at 25 °C

Above the line the solution is a highly viscous liquid (gel formation), while below the line the solution shows low viscosity, almost the same as that of water.

centration, NaCl amount, and the viscosity of doxorubicin aqueous solution. It was shown in this study that the viscosity of doxorubicin aqueous solution increased suddenly when the NaCl amount exceeded a certain value. It was also demonstrated that, at higher doxorubicin concentrations, the amount of NaCl required to induce the sudden viscosity increase was less, *i.e.*, less NaCl was required to trigger viscosity increase, and the slope of viscosity increase became steeper.

The amount of NaCl required to induce the sudden viscosity increase was considered to be the critical gel formation concentration (CGFC); the relationship between CGFC and doxorubicin concentration is shown in Fig. 5. The CGFC was found to be inversely correlated with the logarithmic doxorubicin concentration.

**Temperature Dependence of the Viscosity of Doxorubicin Aqueous Solution** Figure 6 shows the temperature dependence of the viscosity of the solution in the temperature range of 10 to 35 °C when NaCl was added to doxorubicin (5 mg/ml) aqueous solution at various concentrations. The temperature dependence of the viscosity of doxorubicin aqueous solution without NaCl addition (dotted line)

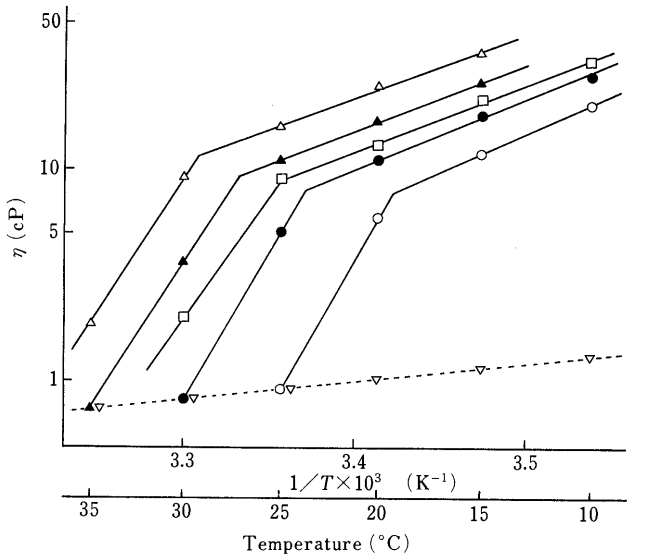


Fig. 6. Temperature Dependence of the Absolute Viscosity of Doxorubicin Aqueous Solution Containing Various Concentrations of NaCl

[Doxorubicin] = 5 mg/ml (8.6 mM). [NaCl]: ▽, 0 M; ○, 0.154 M; ●, 0.227 M; □, 0.257 M; ▲, 0.300 M; △, 0.350 M.

TABLE I. Activation Energy of Viscous Flow at pH 5.5

| Conc. of NaCl (M) | $\Delta E_1$ (kcal/mol) | $\Delta E_2$ (kcal/mol) |
|---|---|---|
| 0 | 3.92 | — |
| 0.154 | 16.88 | 65.01 |
| 0.227 | 14.69 | 64.88 |
| 0.257 | 14.51 | 53.94 |
| 0.300 | 17.46 | 59.45 |
| 0.350 | 15.74 | 58.89 |

[Doxorubicin] = 5 mg/ml (8.6 mM).

almost coincided with the viscosity behavior of water and followed Andrade's formula (Eq. 3). The activation energy of the viscous flow of this solution was 3.92 kcal/mol, slightly less than that of water at 25 °C, which is 4.01 kcal/mol.[12]

In the presence of NaCl, the viscosity of doxorubicin aqueous solution increased more, due to gel formation at higher NaCl concentrations. In all the samples, the viscosity was decreased with rising temperature. When the temperature was elevated gradually from a low point, the straight line refracted when the viscosity reached around 8 to 10 cP and the viscosity sharply turned into a decrease at any NaCl concentration. No hysteresis concerning the thermal behavior of the viscosity was observed.

Table I shows the activation energy of viscous flow in each part of the straight line obtained from Eq. 3.

$\Delta E_1 = ca.$ 15 kcal/mol at low temperatures,

$\Delta E_2 = ca.$ 60 kcal/mol at high temperatures

Doxorubicin aqueous solution presented two types of gel structure: one which had a lower temperature dependence at lower temperatures and one which had a higher temperature dependence at higher temperatures. The gel formed by the addition of NaCl to doxorubicin aqueous solution was found to change from a rigid structure to a flexible structure at the refraction point when the temperature of

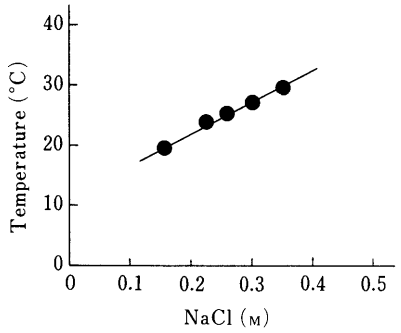


Fig. 7. Relationship between NaCl Concentration and the Temperature at the Refraction Point

[Doxorubicin] = 5 mg/ml.

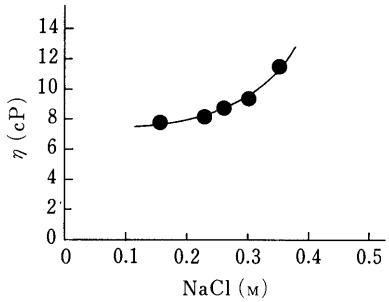


Fig. 8. Relationship between NaCl Concentration and the Viscosity at the Refraction Point

[Doxorubicin] = 5 mg/ml.

the solution was elevated.

The relationship between NaCl concentration and the temperature at the refraction point in each sample is shown in Fig. 7. Temperature at the refraction point of the viscosity of the doxorubicin solution increased with increasing NaCl concentration. The increase in NaCl concentration changed gel structure at higher temperatures, indicating that the structure became more rigid with increasing NaCl concentration.

Figure 8 shows the relationship between NaCl concentration and the viscosity at the refraction point. The viscosity increased with increasing NaCl concentration.

It was indicated that a qualitative change of gel structure was induced when the viscosity exceeded about 10 cP overall.

**Effects of Various Cations or Anions on the Viscosity of Doxorubicin Aqueous Solution** Figure 9 shows the relationship between the concentration of various cation chlorides added to doxorubicin aqueous solution and its viscosity. The effects of the Na salts of various anions are shown in Fig. 10.

Table II shows the relationship between CGFC and the lyotropic series for various ions, and the hydration numbers.[13,14] The viscosity of doxorubicin aqueous solution proved to be increased with the addition of various cations or anions, as well as with NaCl. The viscosity increase caused by monovalent cations or anions correlated inversely with the hydration numbers, *i.e.*, the lyotropic series as a whole.

The effects of various anions were studied in the presence of $Na^+$ as the common cation. In lyotropic series, NaI and NaSCN are known to be the electrolytes with small hydration numbers and weak dehydration ability.[15] Table

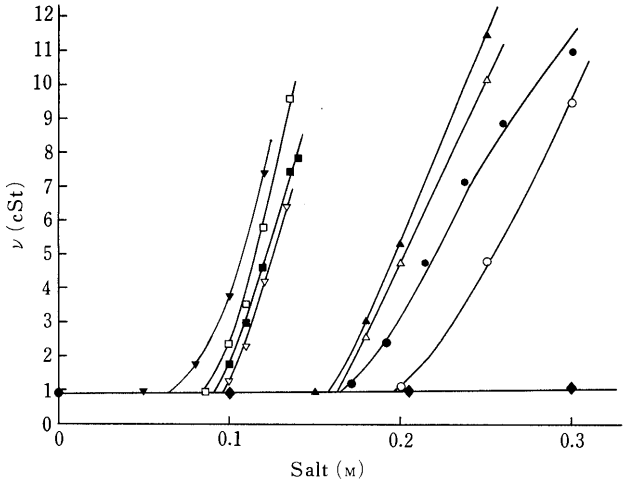


Fig. 9. Effect of Cations on the Viscosity of Doxorubicin at 25 °C

[Doxorubicin] = 5 mg/ml. Monovalent: ○, LiCl, (pH 5.1); ●, NaCl, (pH 5.5); △, KCl, (pH 5.0); ▲, $NH_4Cl$, (pH 4.7). Bivalent: □, $MgCl_2$, (pH 5.7); ■, $CaCl_2$, (pH 6.5); ▽, $BaCl_2$, (pH 5.3). Trivalent: ▼, $AlCl_3$, (pH 2.9); ◆, $FeCl_3$, (pH 1.8). Average pH is shown in parentheses.

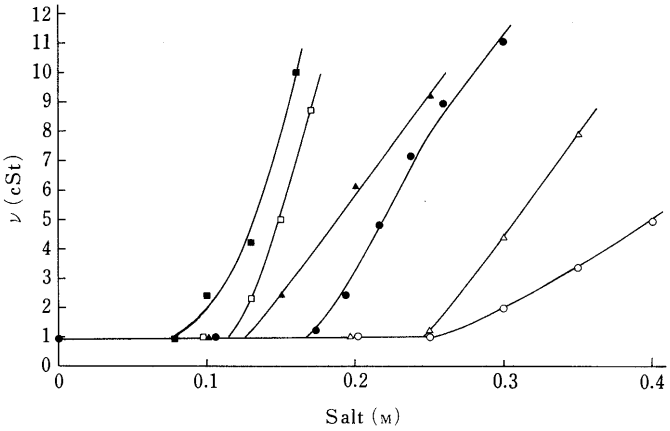


Fig. 10. Effect of Anions on the Viscosity of Doxorubicin at 25 °C

[Doxorubicin] = 5 mg/ml. ○, $CH_3COONa$, (pH 6.9); △, NaF, (pH 6.4); ●, NaCl, (pH 5.5); ▲, NaI, (pH 5.1); □, NaBr, (pH 5.1); ■, NaSCN, (pH 5.1). Average pH is shown in parentheses.

TABLE II. Relationship between CGFC and Lyotropic Series for Various Ions Added to Doxorubicin Aqueous Solution at 25 °C

| Ions added (order of lyotropic series) | Hydration numbers (mol $H_2O$/mol) | CGFC (M) |
|---|---|---|
| Divalent or trivalent cations | *a)* | |
| $AlCl_3$ | 14.6 | 0.064 |
| $MgCl_2$ | 6.5 | 0.084 |
| $CaCl_2$ | 5.9 | 0.092 |
| $BaCl_2$ | 4.2 | 0.097 |
| Monovalent cations | *a)* | |
| LiCl | 4.3 | 0.196 |
| NaCl | 2.7 | 0.168 |
| KCl | 1.7 | 0.164 |
| $NH_4Cl$ | 1.1 | 0.158 |
| Anions | *b)* | |
| NaF | 3.5 | 0.246 |
| $CH_3COONa$ | — | 0.254 |
| NaCl | 1.9 | 0.168 |
| NaBr | 1.5 | 0.115 |
| NaI | 0.6 | 0.126 |
| NaSCN | — | 0.080 |

*a)* Hydration numbers for salt.[13] *b)* Hydration numbers for anion.[14]

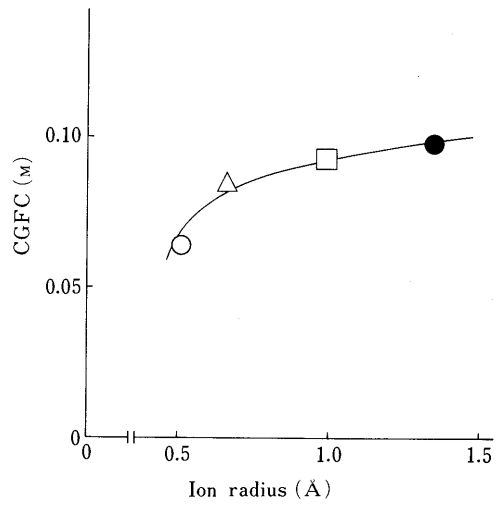


Fig. 11. Relationship between CGFC and Ion Radius
○, $Al^{3+}$; △, $Mg^{2+}$; □, $Ca^{2+}$; ●, $Ba^{2+}$.

II indicates that these electrolytes with weak dehydration ability induce higher viscosity of doxorubicin aqueous solution and promote gel formation. In contrast, electrolytes NaF and $CH_3COONa$, which have strong dehydration ability, remove water from the doxorubicin associated product that contains it, and we deduce that the gel structure shrinks and that viscosity increase is suppressed.

The same results were obtained when the effects of $Li^+$, $Na^+$, $K^+$ and $NH_4^+$ were investigated in the presence of $Cl^-$ as the common anion. Such relationships are similar to the effects of electrolytes on the viscosity of the water-soluble polymers, polyvinylpyrolidone (PVP) and polyvinylalcohol (PVA).[15]

We previously reported that the addition of NaCl shielded the electric charge of doxorubicin molecules and promoted self-association due to $\pi$–$\pi$ stacking.[1] The findings described above strongly suggest the involvement of water, *i.e.*, hydration of doxorubicin molecules, as well as their self-association, in the monovalent cation- or anion-induced mechanism of viscosity increase in aqueous solutions of this compound. Bivalent or trivalent cations exhibited different tendencies in increasing the viscosity of doxorubicin aqueous solution from the tendency observed in monovalent cations or anions. The Fe ion in particular behaved abnormally; this was attributed to the inhibition of gel formation because of extremely low pH values.

It has been generally reported that bivalent and trivalent cations form intra- and inter-molecular complexes with doxorubicin, and that the donor sites of doxorubicin are the two $O^-$ groups on the naphthacene rings, in positions 6 and 11.[3,4,9–11] Based on such reports, this complex formation is thought to be induced by ion binding.

As the energy for ion binding is equivalent to the electrostatic work required to transport the ligand close to the central metal ions, the complex is more stable when these ions have larger electric charge and smaller binding radius. Consequently, the formation constant of the complex is larger when the ion radius is smaller.[16] This argument coincides with the report of Kiraly and Martin on the formation constant of doxorubicin complex.[4]

The relationship between ion radius and CGFC for bivalent and trivalent cations is presented in Fig. 11. Cations of smaller ion radius, which are more likely to form a complex, induced higher viscosity in doxorubicin aqueous solution and promoted the formation of gel structure.

Doxorubicin is partially stacked in aqueous solution. Kiraly and Martin reported that because of this factor, the binding of a second doxorubicin ligand to a metal ion is more successful than the first.[4]

The findings described above indicate that, when bivalent or trivalent cations are added to doxorubicin aqueous solution, a reticulated gel structure is formed between the doxorubicin molecules, through the interaction of metal ions and water molecules.

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# Interaction between Polyethylene Films and Bromhexine·HCl in Solid Dosage Form. III. Prevention of Drug Sorption by Improving Manufacturing Processes

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**The interaction between polyethylene containers and bromhexine·HCl solid dosage forms prepared by different methods was studied. It was found that bromhexine·HCl tablets prepared by the wet method (kneading) had more drug remaining than those prepared by the dry method. The sorption of drug to polyethylene was influenced by the kneading solvents used, and the most effective solvent in preventing sorption was methanol, which has high solubility for bromhexine·HCl. A ground mixture of bromhexine·HCl with crystalline cellulose was effective inhibiting the sorption of bromhexine to polyethylene. This was explained in terms of the monomolecular dispersion of bromhexine molecules within the network of cellulose molecules in the ground mixture.**

**Keywords** bromhexine·HCl; polyethylene; sorption; ground mixture; kneading; content decrease; solid dosage form

In a previous paper,[1] we reported that the decrease of bromhexine·HCl content in granules and tablets was due to the sorption of bromhexine·HCl to the polyethylene film of packaging materials. In the present paper, the effect of the manufacturing method on the amount of bromhexine·HCl remaining in tablets was studied using a direct compression method and a wet granulation method.

It is known that the co-grinding method[2] of drugs with additives is useful to enhance the dissolution rate of drugs,[3] to prevent sublimation of the components,[4] and to accelerate the chemical reactions[5] by a mechanochemical effect.[6] In order to investigate the effect of co-grinding on the prevention of the sorption, comparisons were made between physical mixtures, ground mixtures, and kneaded granules.

## Experimental

**Materials** Bromhexine·HCl (JP XI) was obtained from Boehringer Ingelheim Pharmaceutical, Inc. Crystalline cellulose (JP XI) obtained from Asahi Chemical Industry Co., Ltd. was dried at 60 °C in a vacuum.[3-5] Solvents used were methanol, ethanol, and isopropanol, all special reagent grade from Kokusan Chemical Co., Ltd. A mixed solvent (weight ratio; water: isopropanol = 1 : 1) was also used. The packaging material of polyethylene film (Dainippon Printing Co., Ltd.) was the same as used previously.[1]

**Preparation of Physical Mixture Powder** The physical mixture powder was prepared by simple blending of bromhexine·HCl and crystalline cellulose in a mill (Yariya Type No. 1). The mixing ratio of bromhexine·HCl to crystalline cellulose was 1 : 249 in weight. The D-mannitol physical mixture was prepared by simple blending of bromhexine·HCl and D-mannitol (weight ratio = 1 : 249).

**Preparation of Kneading Powder** The kneading powder was prepared by kneading 100 g of the physical mixture powder with kneading solvent using a mortar and pestle. The kneading solvents were ethanol (10, 30, 60 g), distilled water (60 g), methanol (60 g), or the mixed solvent (60 g). After kneading, each sample was dried at 60 °C for 2 h. After here, this kind of powder is referred to as kneading powder (A). Other kneading powders (B) were prepared by mixing 60 g of bromhexine·HCl solution containing 400 mg bromhexine·HCl and 100 g of crystalline cellulose, kneading and drying at 60 °C for 2 h.

**Preparation of Ground Mixture** The ground mixture of bromhexine·HCl with crystalline cellulose was prepared by grinding the physical mixture with a mortar and pestle (Yamato, Labomill UT-21) for from 30 min to 20 h. The total weight of specimen was 20.0 g.

**Preparation of Tablets** The tablets (diameter: 2.0 cm, thickness: 4.0 mm) were compressed directly by the tableting machine as reported previously.[1] Formulations of tablets are listed in Tables I—IV.

**Storage** Each tablet was packed with polyethylene film and stored at 65 ± 1 °C.

**Measurement of Bromhexine·HCl** The remaining amount of bromhexine·HCl in each tablet was determined as a function of time using high performance liquid chromatography (HPLC) as described previously.[1]

**Solubility Study** An excess amount of bromhexine·HCl was added to 5 ml of each solvent at 25 °C, and the suspension was shaken constantly for 7 h. After filtration, the bromhexine·HCl concentrations were determined by HPLC.

**Powder X-Ray Diffractometry** Powder X-ray diffraction patterns were measured using a Rigaku Denki RAD-3C diffractometer using a scintillation counter. The measurement conditions were as follows: target, Cu; filter, Ni; voltage, 30 kV; current, 40 mA; scanning speed, 4°/min.

**Dissolution Test** The dissolution rates of bromhexine·HCl from the physical mixture powder, the kneading powders (A and B), and the ground mixture were tested by the JP XI dissolution test (method 2). Test conditions were as follows: rotating speed, 100 rpm; test fluids, methanol, isopropanol, or JP XI 1st fluid; volume of fluid, 400 ml; temperature, 37 ± 0.5 °C. At appropriate intervals, an aliquot solution was withdrawn and filtered by means of a membrane filter (0.45 μm). The concentrations of bromhexine·HCl were determined by HPLC.

## Results and Discussion

**Effect of Amount of Solvent Used in Kneading Powder (A) on the Sorption of Bromhexine·HCl to Polyethylene Film** The amounts of bromhexine·HCl remaining in tablets after packaging in polyethylene film were determined by using tablets which were prepared from the kneading powder (A). The formulations for kneading powder (A) are listed in Table I. The weight of ethanol was varied from 100 to 600 mg for each tablet.

The amount of bromhexine·HCl remaining in each tablet are shown in Fig. 1. The amount of bromhexine·HCl remaining in the tablet increased with an increase of ethanol. No degradation of bromhexine·HCl was found during storage as was reported in the previous paper.[1] It was concluded that the decrease of bromhexine·HCl from the tablet was due to the sorption to polyethylene film, and that the use of a large quantity of kneading solvent prevented the sorption of bromhexine·HCl to the film.

**Effect of Kneading Solvent on the Sorption of Bromhexine·HCl to Polyethylene Film** The effect of the kneading solvent on the sorption was studied for methanol, ethanol, distilled water, and mixed solvent (mixing weight ratio of water to isopropanol = 1 : 1).

The tablets, of which formulations are shown in Table II, were packaged in polyethylene film and stored at 65 °C. The remaining bromhexine·HCl was determined, and the

TABLE I. Formulaltion of Tablets

| | 1 | 2 | 3 |
|---|---|---|---|
| Bromhexine·HCl (mg) | 4 | 4 | 4 |
| Crystalline cellulose (mg) | 996 | 996 | 996 |
| Ethanol (mg) | 100 | 300 | 600 |
| Weight of tablet (mg) | | 1000 | |

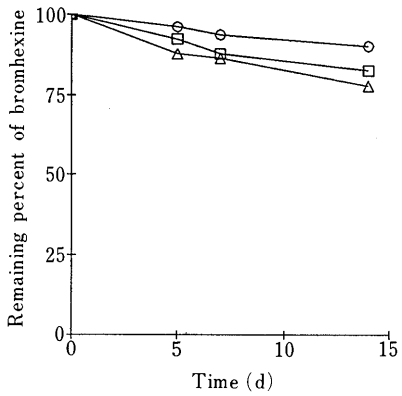


Fig. 1. Effect of Amount of Ethanol Used for Kneading on the Amount of Bromhexine·HCl Remaining in Tablets after Storage in a Polyethylene Package at 65 °C

Weight of ethanol: ○, 600 mg/tablet; □, 300 mg/tablet; △, 100 mg/tablet.

TABLE II. Formulation of Tablets

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| | Kneading solvent | | | |
| | MeOH | EtOH | Water | Mixed solvent[a)] |
| Bromhexine·HCl (mg) | 4 | 4 | 4 | 4 |
| Crystalline cellulose (mg) | 996 | 996 | 996 | 996 |
| Weight of solvent (mg) | 600 | 600 | 600 | 600 |
| Weight of tablet (mg) | | 1000 | | |

a) Water : isopropanol = 1 : 1.

results are shown in Fig. 2, with the result of physical mixture as the reference. Great variations in the remainder were observed among the tablets prepared from different kneading solvents. Those prepared from kneading powder (A) which were kneaded by methanol showed the highest remainder of bromhexine·HCl. The effect of the solvent on the remaining bromhexine·HCl was in decreasing order, methanol > the mixed solvent > ethanol > distilled water.

**Solubility of Bromhexine·HCl in Various Solvents** It was found that the amount and kind of solvents exerted influence on the sorption behavior of bromhexine·HCl to the polyethylene film. The solubility of bromhexine·HCl in each solvent was measured at 25 °C, and results are shown in Fig. 3. The greatest solubility of bromhexine·HCl was observed in methanol: 38.5 μg/ml at 25 °C. The order of solubility of the solvents was in decreasing order, methanol > the mixed solvent > ethanol > distilled water. A close relationship between the solubility of bromhexine·HCl in a solvent and the amount remaining in the tablets prepared from that solvent was observed; the powder

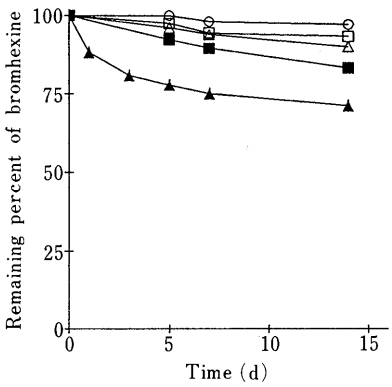


Fig. 2. Effect of Kneading Solvents on the Amount of Bromhexine·HCl Remaining in Tablets after Storage in a Polyethylene Package at 65 °C

▲, physical mixture. Kneading solvent: ○, methanol; □, mixed solvent; △, ethanol; ■, distilled water.

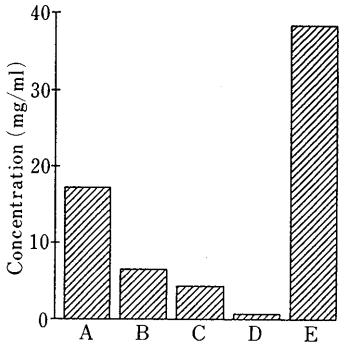


Fig. 3. Solubility of Bromhexine·HCl in Each Solvent at 25 °C

A, mixed solvent (water : isopropanol = 1 : 1); B, ethanol; C, distilled water; D, isopropanol; E, methanol.

TABLE III. Formulation of Tablets

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| | Solvent | | | |
| | MeOH | Mixed solvent[a)] | EtOH | Water |
| Bromhexine·HCl (mg) | 4 | 4 | 4 | 4 |
| Crystalline cellulose (mg) | 996 | 996 | 996 | 996 |
| Weight of solvent (mg) | 600 | 600 | 833 | 1111 |
| Weight of tablet (mg) | | 1000 | | |

a) Water : isopropanol = 1 : 1.

kneaded with a high solubility solvent showed strong prevention of the sorption of bromhexine·HCl to polyethylene film as shown in Fig. 2. The results indicate that the dissolution process of bromhexine·HCl to the kneading solvent during kneading has an important role in preventing the sorption of bromhexine·HCl to polyethylene film.

**Effect of Kneading Powder (B) on the Sorption of Bromhexine·HCl to Polyethylene Film** Another kneading method was used to investigate the effect of the dissolution step on the drug sorption. The formulation of kneading powders (B) is listed in Table III. The weight of each solvent was enough to dissolve the total amount of bromhexine·HCl contained. In method (B), bromhexine·HCl was first

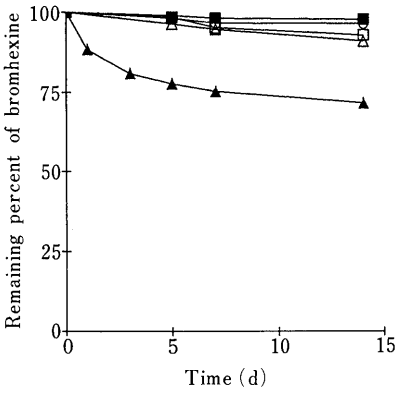


Fig. 4. Amount of Bromhexine·HCl Remaining in Tablets after Storage in a Polyethylene Package at 65 °C

▲, physical mixture. Kneading solvent: ○, methanol; □, mixed solvent; △, ethanol; ■, distilled water.

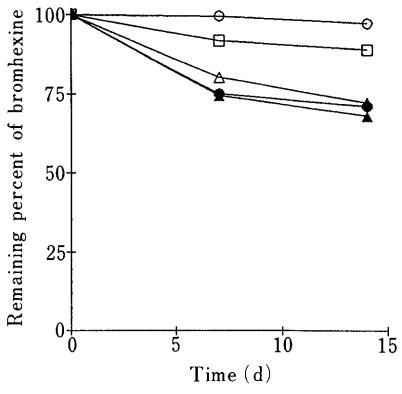


Fig. 5. Effect of Grinding Time on the Amount of Bromhexine·HCl Remaining in Tablets after Storage in a Polyethylene Package at 65 °C

Grinding time: ○, 20 h; □, 10 h; △, 2 h; ▲, 0.5 h; ●, without grinding.

dissolved in each solvent. The amount remaining in tablets was then determined after storage in polyethylene film at 65 °C. The results are shown in Fig. 4. Using kneading powders (B), the transfer of bromhexine·HCl to polyethylene film was prevented. The amount remaining in the tablets was especially significant for distilled water or ethanol, as compared with the results shown in Fig. 2, in which tablets were made of kneading powders (A). The remainder of bromhexine·HCl in the tablets kneaded by methanol or the mixed solvent was almost the same as that shown in Fig. 2.

From Figs. 2 and 4, it is apparent that the complete dissolution of bromhexine·HCl in the kneading solvents improved the drug sorption to the polyethylene film. This means that intact bromhexine·HCl crystals had a high trend for the change from a solid dosage form to polyethylene film, while most of that deposited on the additives remained in the tablets. In Fig. 4, however, there was a small variation among the samples in their ability to retain bromhexine·HCl in tablets. It was assumed that an other property of the kneading solvent could be related to this difference in retention ability.

**Effect of Grinding on the Sorption of Bromhexine·HCl**

Great sorption of bromhexine·HCl to polyethylene film took place with tablets made of the physical mixture powder (Fig. 2). In order to investigate the effect of the dispersing state of molecules within crystalline cellulose on this sorption, the co-grinding method was studied. The physical mixture powder of bromhexine·HCl (0.4%) with crystalline cellulose was ground in an automatic mortar for a definite time, and the ground mixture powder was compacted by direct compression method. The amount of bromhexine·HCl remaining in the tablets was determined after packaging in polyethylene film and storage at 65 °C (Fig. 5).

The remainder of bromhexine·HCl in the tablets increased with an increase of grinding time. The results indicate that co-grinding was effective to prevent the sorption of bromhexine·HCl to polyethylene film.

Figure 6 shows X-ray diffraction patterns of the ground mixture (ground for 20 h), the methanol kneading powder (A), and the physical mixture powder. The samples were prepared in various bromhexine·HCl concentrations from 0.4% to 5.0%. In the physical mixture of bromhexine·HCl concentration of 0.4%, no diffraction peaks due to

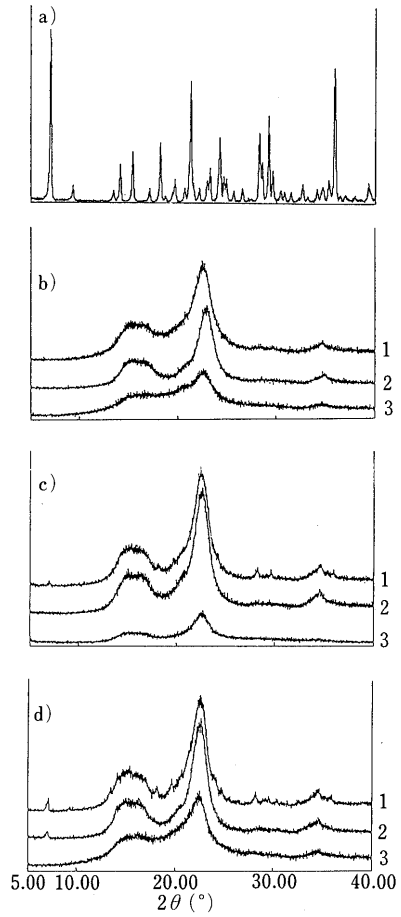


Fig. 6. Powder X-Ray Diffraction Patterns of Bromhexine·HCl in the Mixtures with Crystalline Cellulose

a) Bromhexine·HCl. Bromhexine·HCl concentration: b) 0.4% c) 2.5% d) 5.0%. 1, physical mixture; 2, kneading powder (A) with methanol; 3, ground mixture ground for 20 h.

bromhexine·HCl crystals were observed. At concentrations of 2.5% and 5.0%, however, X-ray diffraction peaks due to these crystals were observed. In the ground mixture, the peaks of bromhexine·HCl crystals were not observed, indicating that the drug molecules were dispersed monomolecularly in the matrix as reported previously.[2] In the case of methanol kneading powder (A), the peaks of these crystals disappeared in low concentration samples,

while small peaks were still observed in the 5.0% sample.

**Change of Sorption Behavior with the Addition of Sodium Bicarbonate to the Tablet** In a previous paper,[7] we reported the effects of the excipients in preparations on the sorption of bromhexine·HCl to polyethylene film. The addition of acids (citric acid and tartaric acid) prevented the sorption of bromhexine·HCl to polyethylene film, while the addition of sodium bicarbonate accelerated the sorption. In previous experiments, tablets were made by the physical mixture of bromhexine·HCl, crystalline cellulose, and additives, it was assumed that the additives dispersed within the tablets acted directly with bromhexine·HCl, and that the sorption behavior was affected. Sodium bicarbonate addition to the tablets was carried out and the amount of bromhexine·HCl remaining was measured for each sample to investigate the effect of the molecular state of bromhexine·HCl in the ground mixture and in methanol kneading powder (A) on the sorption behavior.

Table IV. Formulation of Tablets Containing Sodium Bicarbonate

| | | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| | | Kneading powder (A) | | Ground mixture | | Physical mixture | |
| Drug | BHCl (mg) | 4 | 4 | 4 | 4 | 4 | 4 |
| Excipient | C.C. (mg) | 996 | 996 | 996 | 996 | — | — |
| | D-Mannitol (mg) | — | — | — | — | 996 | 996 |
| Additives | S.B. (mg) | — | 50 | — | 50 | — | 50 |

BHCl bromhexine·HCl. C.C., crystalline cellulose. S.B., sodium bicarbonate.

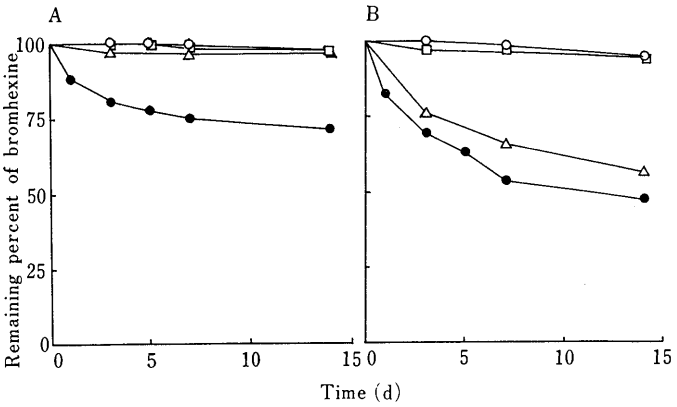


Fig. 7. Effect of Sodium Bicarbonate Addition on the Amount of Bromhexine·HCl Remaining in Tablets after Storage in a Polyethylene Package at 65 °C

A, without sodium bicarbonate; B, with sodium bicarbonate (●, physical mixture with crystalline cellulose; ○, ground mixture ground for 20 h; □, kneading powder (A) with methanol; △, physical mixture with D-mannitol).

Tablets were compacted from two kinds of powders: one prepared with 1000 mg of the ground mixture powder and 50 mg of sodium bicarbonate, and the other prepared with 1000 mg of methanol kneading powder (A) and 50 mg of sodium bicarbonate. The formulations are listed in Table IV (2 and 4). As a reference, tablets were prepared from the D-mannitol physical mixture powder as shown in Table IV (5 and 6). The amount of bromhexine·HCl remaining in the tablets after packaging in polyethylene film and storage at 65 °C is shown in Fig. 7. The remainder did not change before and after the addition of sodium bicarbonate, in the cases of the ground mixture and methanol kneading powder (A). However, in the physical mixture with crystalline cellulose, the remainder with sodium bicarbonate was smaller than that without. The addition of sodium bicarbonate accelerated the sorption of bromhexine·HCl to the polyethylene film as reported previously.[7] The D-mannitol physical mixture (Table IV-5) prevented the sorption of bromhexine·HCl: however, the addition of sodium bicarbonate to the D-mannitol physical mixture (Table IV-6) accelerated the sorption.

It was reasonable to consider that the sorption was accelerated by the direct effect of sodium bicarbonate on bromhexine·HCl in the physical mixture powders. On the other hand, in the ground mixture and methanol kneading powder (A), no effect of sodium bicarbonate addition on the sorption was observed. This fact was ascribed to the monomolecular dispersions of the bromhexine·HCl molecules within the network structure of crystalline cellulose molecules in both substances.

**Dissolution Test** The dissolution rates of bromhexine·HCl were measured for the ground mixture and the methanol kneading powder (A). Both samples prevented the sorption of bromhexine·HCl to polyethylene film. The test fluids used were JP XI 1st fluid, methanol, and isopropanol. As a reference, the physical mixture powder was studied (Fig. 8).

In JP XI 1st fluid, a high dissolution rate of bromhexine·HCl was observed for the physical mixture powder, the

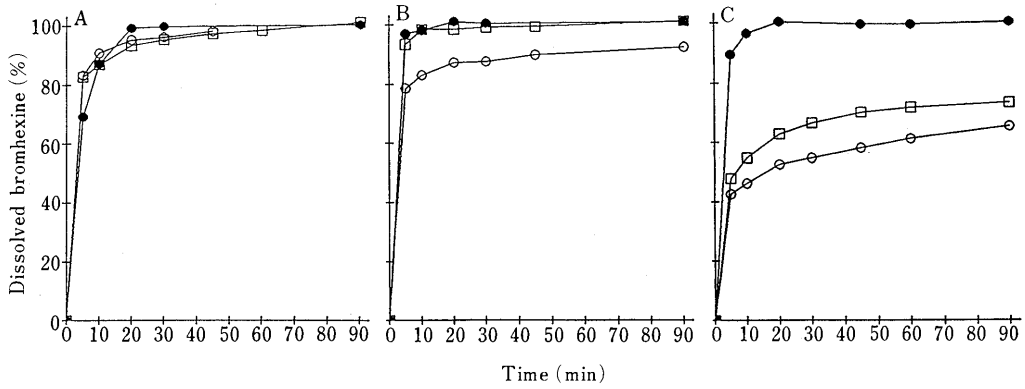


Fig. 8. Dissolution Rate of Bromhexine·HCl from Kneading Powder (A), Ground Mixture, and Physical Mixture at 37 °C

Dissolution media: A, JP XI 1st fluid; B, methanol; C, isopropanol (○, ground mixture ground for 20 h; ●, physical mixture with crystalline cellulose; □, kneading powder (A) with methanol).

methanol kneading powder (A) and the ground mixture. In methanol, the dissolution from the physical mixture powder and the methanol kneading powder (A) were fast, while it was slow from the ground mixture. The dissolution rate in isopropanol from the physical mixture powder remained fast but the rates from the ground mixture and the methanol kneading powder (A) were slow.

The low dissolution rate of bromhexine·HCl in isopropanol from the ground mixture and the methanol kneading powder (A) was attributed to the molecular state of bromhexine·HCl in the matrix, that is, each drug molecule was dispersed within the hydrogen bonding network structure of crystalline cellulose and the isopropanol was not polarized enough to break this structure. The dissolution of bromhexine·HCl from methanol kneading powder (A) in methanol media was rather fast. In this matrix, bromhexine·HCl molecules were thought to be dispersed within the network of the cellulose molecules in the amorphous region[8] which were extended by methanol at the kneading process, resulting in fast dissolution in the fluid of methanol. The high dissolution rate of bromhexine·HCl into water from all powders was due to the great polarity of water molecules.

## Conclusion

A manufacturing method in which bromhexine·HCl molecules are dispersed within the network of cellulose molecules effectively prevents the sorption of bromhexine·HCl to polyethylene film, even in the presence of sodium bicarbonate.

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# Esterase-like Activity of Human Serum Albumin. VII.[1] Reaction with *p*-Nitrophenyl 4-Guanidinobenzoate

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**The reaction of *p*-nitrophenyl 4-guanidinobenzoate (NPGB) with human serum albumin (HSA) was examined kinetically at various pH's and 25 °C. The Michaelis constant ($K_s$ in M) and the catalytic rate constant ($k_2$ in $s^{-1}$) were determined. The ratio of $k_2$ to $k_0$ (hydrolysis rate constant of NPGB in $s^{-1}$) at pH 7.4 was 75.6, indicating the esterase-like activity of HSA. The effects of the reversible binding of site-specific drugs and the chemical modification by site-specific reagents on the HSA activity indicated that HSA has multiple reactive sites towards NPGB. Results of the reaction in the presence of excess NPGB over HSA also suggested the existence of multiple active sites. The pH-profile for $k_2$ showed inflection points at about pH 6.0 and pH 10.0, suggesting the involvement of groups with p$K_a$'s of 6.0 and 10.0 in HSA.**

**Keywords** human serum albumin; esterase-like activity; *p*-nitrophenyl 4-guanidinobenzoate; kinetics; Michaelis constant; pH profile; drug binding; competitive inhibition; chemical modification; site-specific

It has been reported that human serum albumin (HSA) possesses esterase-like activity towards esters,[1-4] amides,[5] and phosphates.[6] 4'-Methylumbelliferyl 4-guanidinobenzoate (MUGB) and aryl guanidinobenzoates are used as active site titrant for many serine proteases (*e.g.*, trypsin) on the basis of the formation of stable 4-guanidinobenzoyl-linked enzymes.[7,8] These guanidinobenzoates also are candidates for contraceptive drugs because they are inhibitors of acrosin (a serine protease), which has an essential function in the fertilization process.[8,9] Schwartz demonstrated that MUGB is degraded in plasma and amniotic fluid and that albumin may be responsible for this degradation.[10] It has been reported recently[11] that HSA enhanced the hydrolysis rate of MUGB by a factor of 46.6 at pH 7.4 and 25 °C, and that only one histidine residue was critical for the activity among the 16 histidine residues in HSA. Since the mechanism involved still seems to be unclear, we examined further the reaction of *p*-nitrophenyl 4-guanidinobenzoate (NPGB) with HSA. NPGB was selected as a model substrate, since NPGB has higher solubility in water than MUGB and the reaction can be easily followed spectrophotometrically. The degradation of NPGB in a HSA solution was accelerated by a factor of 75.6 at pH 7.4, and HSA had multiple reactive sites towards NPGB. The kinetics and mechanism of the reactions are described herein.

## Experimental

**Materials** HSA (Sigma Chem. Co., Fraction V, lots 16F-9344 and 16F-9633) was used after purification by Chen's method.[12,13] The molecular weight of HSA was assumed to be 69000 and the concentration was determined by use of molar absorptivity ($\varepsilon = 3.66 \times 10^4\ M^{-1}\ cm^{-1}$) at 278 nm.[13,14] NPGB hydrochloride was initially synthesized by the dicyclohexylcarbodiimide (DCC)-assisted condensation of *p*-nitrophenol (NP) and 4-guanidinobenzoic acid (GB) hydrochloride in dry pyridine and dimethylformamide (1:1).[7,8] Later, NPGB·HCl was purchased from Sigma Chem. Co. Cinnamoylimidazole (CI), MUGB, and diethylpyrocarbonate (DEP) were obtained from Sigma Chem. Co. Phenylbutazone (PB) was purchased from Aldrich Chem. Co. Clofibric acid (CA) and 5-nitroaspirin (NA) were the same as used in the previous study.[15] All other chemicals obtained commercially were of a reagent grade.

Ultraviolet (UV) absorption spectroscopy was carried out with a Shimadzu UV-260 spectrophotometer and a Hitachi UV-124 spectrophotometer.

**Kinetic Runs** The buffer systems used were as follows: pH 5.0, 0.2 M acetate; pH 6.0—8.0, 0.067 M phosphate; pH 9.0, 0.1 M phosphate–0.05 M borate; pH 10.0—11.0, 0.05 M borate. Ionic strength was adjusted to 0.2 with NaCl. The reaction temperature was 25 °C.

The reactions of NPGB ($1.00 \times 10^{-5}$ M) with HSA (an excess concentration over NPGB) in the presence and absence of a drug were followed spectrophotometrically by monitoring the release of *p*-nitrophenol (NP) at 320 nm from pH 5.0 to 6.0 and at 400 nm from pH 7.0 to 11.0. The pseudo first-order rate constant ($k_{obs}$) was determined from a plot of log $(A_\infty - A_t)$ *versus* time, where $A_\infty$ and $A_t$ are the absorbances at the completion of the reaction and at time $t$, respectively.

The reactions in the presence of excess NPGB ($2.5 \times 10^{-5}$ M to $1.0 \times 10^{-4}$ M) over HSA ($5.0 \times 10^{-6}$ M) were also followed spectrophotometrically.

**Effects of Chemical Modification of HSA on the Reaction Rate with NPGB** HSA ($5.00 \times 10^{-5}$ M) was modified with equimolar CI, NA, and MUGB before the reaction with NPGB ($1.00 \times 10^{-5}$ M). The modification of HSA was followed spectrophotometrically at 330, 370, and 360 nm for CI,[5] NA,[15] and MUGB,[11] respectively. After the completion of the modification (no change in absorbance occurred), 15 $\mu$l of $2.00 \times 10^{-3}$ M NPGB in water was added to 3 ml of the reaction solution containing modified HSA and modifier. The rate of NP release was followed at 400 nm, and the pseudo first-order rate constant ($k^m_{obs}$) was calculated.

Ethoxycarbonylation of HSA with DEP was carried out by the method described previously.[6] The effects of the modification of HSA on the reaction rate with NPGB were examined in a way similar to that in the previous study.[6]

## Results and Discussion

Figure 1a shows the effects of HSA concentration on $k_{obs}$ in its reaction with NPGB. The concentration with subscript 0 in this paper always indicates the initial concentration. The $k_{obs}$ value increases asymptotically with the concentration of HSA, suggesting the reaction pathway shown in Chart 1. Figure 1b shows a typical Lineweaver–Burk (double reciprocal) plot[1,16] for the data shown in Fig. 1a. The $K_s$ and $k_2$ values are obtainable from the slope and intercept. The values of $k_2$, $K_s$, and $k_0$ at pH 7.4 and 25 °C were estimated to be $4.44 \times 10^{-4}\ s^{-1}$, $8.13 \times 10^{-4}$ M, and $5.87 \times 10^{-6}\ s^{-1}$, respectively. The ratio of $k_2$ to $k_0$ is 75.6, indicating an esterase-like activity of HSA towards NPGB.

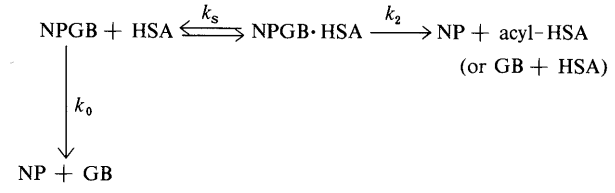


Chart 1

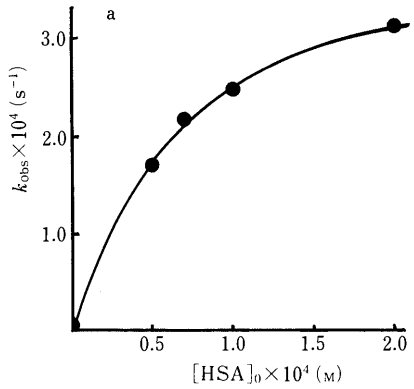


Fig. 1a. Effect of HSA Concentration on the Rate of *p*-Nitrophenol Release

pH 7.4, 0.067 M phosphate buffer ($\mu=0.2$ with NaCl) at 25 °C; $[NPGB]_0=1.00\times10^{-5}$ M.

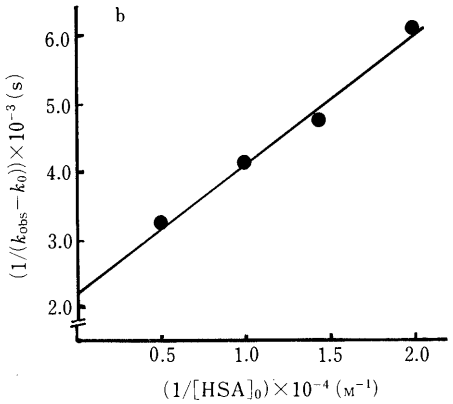


Fig. 1b. Plot of $1/(k_{obs}-k_0)$ *versus* $1/[HSA]_0$

Data from Fig. 1a.

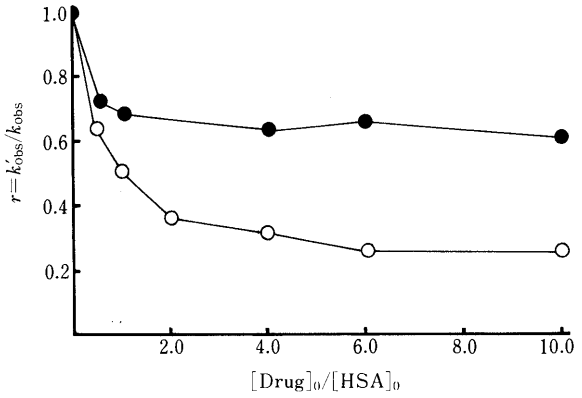


Fig. 2. Effect of CA and PB on the Reaction Rate of NPGB with HSA at pH 7.4 and 25 °C

●, CA; ○, PB; $[NPGB]_0=1.00\times10^{-5}$ M; $[HSA]_0=5.00\times10^{-5}$ M; $k_{obs}=1.69\times10^{-4}$ $s^{-1}$.

To localize the reactive site(s) towards NPGB, the effects of some drugs, whose binding sites on HSA are known already, on $k_{obs}$ were examined. Figure 2 shows the results for CA and PB. In this figure, $k'_{obs}$ on the ordinate represents the rate constant in the presence of the drug, and $r$ is the ratio of $k'_{obs}$ to $k'_{obs}$. Both CA and PB inhibit the reaction of NPGB with HSA. It is well known that CA strongly binds to the R site alone and PB binds primarily to the U site and secondarily to the R site.[15,17] These inhibitions shown in Fig. 2 could not be interpreted as simple competitive inhibition, because in the case of competitive inhibition, the $r$ value at a large excess of CA over HSA, for example, should be 0.0347 ($k_0/k_{obs}=5.87\times10^{-6}/1.69\times10^{-4}$). Two possibilities can be considered for the inhibition. One is non-competitive inhibition or mixed-type inhibition[18] by CA, that is, the complex CA·HSA still has reactivity towards NPGB less than that of the native (uncomplexed) HSA. The other is that HSA has multiple reactive sites towards NPGB and that the R site is only one site out of many (the contribution of the R site activity to the total activity of HSA may only be about 30% ($r=0.7$ in the excess CA over HSA)).

In order to determine which mechanism is proper, the effects of chemical modification of the reactive sites on the reaction rate with NPGB were examined, and further, the reaction in the presence of excess NPGB over HSA was carried out. It has been reported that CI and NA acylate tyrosine-411 residue of the R site[5] and lysine-199 residue of the U site,[15] respectively. MUGB[11] and DEP[6] modify the histidine residue. Table I shows the results of the effects of chemical modification. The residual activities listed in Table I imply the existence of multiple reactive sites on HSA.

Figure 3 shows the effect of ethoxycarbonylation by

TABLE I. Effects of Chemical Modification on the Reaction Rate of NPGB with HSA at 25 °C[a]

| Reagent | Modified site | Residual activity (%)[b] | Ref. |
|---|---|---|---|
| CI | Tyrosine-411 (R site) | 83.0 | 5 |
| NA | Lysine-199 (U site) | 106.5 | 15 |
| MUGB | Histidine residue | 67.9 | 11 |

*a*) HSA ($5.00\times10^{-5}$ M) was modified with the equimolar reagent before the reaction with NPGB ($1.00\times10^{-5}$ M). *b*) Residual activity (%) $=\{(k^m_{obs}-k_0)/(k_{obs}-k_0)\}\times100$, where $k^m_{obs}$ is the pseudo first-order rate constant for the reaction of NPGB with the modifier-treated HSA as described in the experimental section.

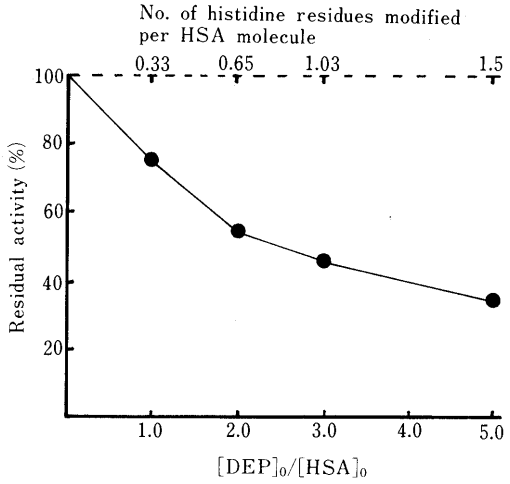


Fig. 3. Effects of Ethoxycarbonylation on the Reaction Rate of NPGB with HSA

pH 7.4 phosphate buffer containing 0.5% (v/v) ethanol at 25 °C; $[NPGB]_0=1.00\times10^{-5}$ M; $[HSA]_0=5.00\times10^{-5}$ M.

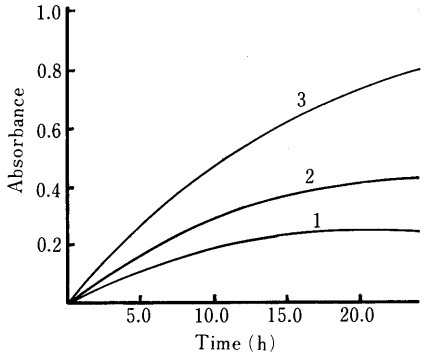


Fig. 4. Time Courses for the Reaction of Excessive NPGB with HSA at pH 7.4 and 25 °C

Concentration of HSA was $5.00 \times 10^{-6}$ M; 1, $2.5 \times 10^{-5}$ M of NPGB; 2, $5.00 \times 10^{-5}$ M of NPGB; 3, $1.00 \times 10^{-4}$ M of NPGB.

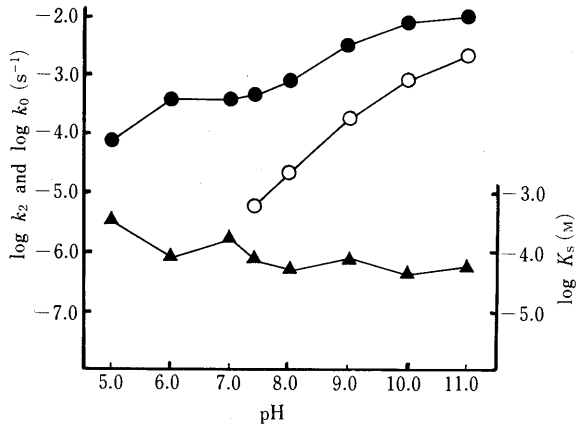


Fig. 5. The pH Profiles of the Kinetic Parameters for the Reactions of NPGB with HSA at 25 °C

●, $k_2$; ○, $k_0$; ▲, $K_s$.

DEP on the reaction rate with NPGB. The lower scale of abscissa shows the ratio of the initial concentration of DEP employed to that of HSA, and the upper scale is the number of histidine residues modified per HSA molecule, which was calculated according to the method described in the previous paper.[6] When about 1 and 1.5 mol of histidine residues per mol of HSA molecule were modified by DEP, the residual activities were about 45% and 35%, respectively. These results indicate that the imidazole group(s) of histidine residues also is (are) the catalytic group(s) towards NPGB, and that there should be at least two reactive histidine residues because of the existence of about 35% residual activity remaining after the modification of about 1.5 mol of histidine per mol of HSA.

Figure 4 shows the time courses for the reactions of excessive NPGB with HSA. The absorbance of about 0.05 on ordinate in Fig. 4 corresponds to one mol of *p*-nitrophenol (NP, $5.00 \times 10^{-6}$ M) released from the reaction with NPGB per mol of HSA ($5.00 \times 10^{-6}$ M). There seems to be no heterogeneity in the reactivities towards NPGB, that is, all sites on HSA appear to be homogeneous. These results again indicate that HSA has multiple reactive sites towards NPGB.[19]

The pH profiles of the kinetic parameters for the reaction are shown in Fig. 5. With the pH profile of $k_0$, the deviation from a slope of +1 in the alkaline region may be due to the deprotonation of the guanidino group of NPGB ($pK_a = 12$[11]). The pH profile of $k_2$ suggests the involvement of two ionizable catalytic groups with $pK_a$'s of about 6 and 10 in the reaction. It may be reasonable to consider that the catalytic group with a $pK_a$ of 6 would be the imidazole group of histidine residue and that the group with the $pK_a$ of 10 the hydroxy group of tyrosine or the amino group of lysine residue, because HSA has multiple reactive sites towards NPGB as described above. The $K_s$ value at pH 5 is larger than those at neutral and alkaline pH's; that is, binding of NPGB to the reactive site at pH 5 is weaker than those at the neutral regions. The imidazole group in the reactive site may play an important role in the binding of the substrate.

**Acknowledgements** We are thankful to Dr. Naoko Ohta and Mr. Ikuo Kushida for their discussions on the results of this study. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan, and also by funds from the Uehara Memorial Foundation.

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19) Similarly to the case of NPGB, except for an existence of 5% (v/v) methanol as the solubilizer of MUGB, we examined the time courses for the reactions of excess MUGB ($4.00 \times 10^{-5}$, $3.00 \times 10^{-5}$, and $2.00 \times 10^{-5}$ M) with HSA ($5.00 \times 10^{-6}$ M). The results indicated that HSA seems to possess at least 3 reactive sites towards MUGB.

# Inhibition of Endothelin (ET)-1- and ET-2-Induced Vasoconstriction by Anti-ET-1 Monoclonal Antibody

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**We produced a monoclonal antibody to endothelin (ET)-1, tested cross-reactivities with the related peptides by enzyme immunoassay, and investigated the effects of the antibody on ET-1- or ET-2-induced vasoconstriction of rat isolated thoracic aorta. The antibody recognized ET-1, ET-2 and ET-3, and the immunoreactive site proved to be the N-terminal region but not the C-terminal region of ET-1. Moreover, at an approximate molar-equivalent concentration, the antibody absorbed ET-1 and ET-2, and significantly inhibited ET-1- and ET-2-induced vasoconstriction notwithstanding the presence of the endothelin receptor.**

**Keywords** endothelin-1; endothelin-2; anti endothelin-1 monoclonal antibody; vasoconstriction; rat isolated aorta; cross-reactivity

## Introduction

Endothelin-1 (ET-1) is the most potent vasoconstrictor peptide in hitherto known constrictors, is produced by vascular endothelial cells, and consists of 21-amino acid residues containing two intramolecular disulfide linkages.[1] Recently, endothelin-related genes were found in human genomes, and three correspondent peptides designated as ET-1, ET-2 and ET-3. ET-2 and ET-3 are also potent constrictors and exhibit distinct pharmacological activities. Their structures are different from ET-1 in the second to the seventh amino acid positions of the N-terminal fragment.[2] In addition, it has been demonstrated that immunoreactive substances like endothelin exist in human plasma, and the plasma level is increased in patients with various disorders.[3] This paper describes the production of anti ET monoclonal antibody and the possibility of immunological therapy with the antibody.

## Materials and Methods

**Chemicals** Materials were obtained from the following commercial sources and used according to the procedures given by the respective suppliers: Sodium pentobarbital from Abbott Laboratories (North Chicago); Formyl-cellulofine and Protein A-cellulofine from Seikagaku Kogyo Co., Ltd. (Tokyo); Sepharose 6B from Pharmacia LKB Biotechnology (Uppsala); Freund's complete adjuvant (CFA) and incomplete adjuvant (IFA) from Difco Laboratories (Detroit); ET-1, ET-2 and big ET from Peptide Institute Inc. (Osaka); Porcine thyroglobulin (PTG) from Servo Feinbiochemica GMBH & Co. (New York); β-D-galactosidase from Boehringer Mannheim GMBH (Mannheim). The ET-1 isomer, as described by S. Kumagaye *et al.*,[4] was synthesized by peptide synthesizer (applied biosystem model 430). Lysylendopeptidase-digested ET-1 was obtained as described by S. Kimura *et al.*[5]

**Animals** Wistar rats and BALB/c mice from Japan SLC, Inc. (Shizuoka).

**Preparation of Murine Monoclonal Anti ET-1 Immunoglobulin G (IgG) and Murine Normal IgG** ET-1 (1.1 mg) was dissolved in 4.4 ml of 50 mM sodium phosphate buffer (pH 7.4) containing 4.4 mg of PTG, and 110 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide was added. The reaction mixture was stirred magnetically overnight at room temperature (r.t.). The reaction solution was dialized for 48 h against 3 l of 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl at 4 °C, and then 8.5 ml of ET-1–PTG conjugate solution (0.52 mg protein/ml) was obtained. The conjugated antigen (50 μg protein), emulsified with an equal volume of CFA, was administrated intraperitoneally in BALB/c mice. Two weeks later, the antigen (50 μg protein) emulsified with an equal volume of IFA was injected intraperitoneally in mice. Immunizations by this method were repeated 3 times at 3 week intervals. Four days after the last i.p. injection, splenocytes of immunized mice were taken out, and were fused with mouse myeloma cell line P3-X63 Ag8U. 1.[6] The mixture of cells was plated in 768 wells of 96 well plates. Hybridomas were screened for production of the monoclonal anti ET-1 antibodies by using the competitive enzyme immunoassay with ET-1–β-D-galactosidase conjugate. Selected hybridomas were cloned at least twice by limiting dilution. A monoclonal anti ET-1 antibody, ET-MA51 ($IgG_1$), was selected. The antibody was purified from ascites fluids produced in BALB/c by precipitation with ammonium sulfate, followed by Protein A-cellulofine column chromatography and dialization, and then was lyophylized.

Murine normal IgG was purified from sera in a similar manner.

**Enzyme Immunoassay** The enzyme immunoassay was performed by the competitive binding procedure. β-D-Galactosidase-conjugated ET-1 was synthesized in a similar way to the preparation of β-D-galactosidase-conjugated bradykinin by Ueno *et al.*[7] ET-MA51 solution (100 μl, 2 μg/ml in 50 mM sodium carbonate buffer, pH 9.6) was added to each well of microtitre plates. After incubation at 25 °C for 2 h, followed by washing 3 times with 250 μl of phosphate buffered saline (PBS) containing 0.05% (w/v) Tween 20 (PBS-T), and standard ET-1 (or sample, 100 μl) in PBS-T and 100 μl of β-D-galactosidase-conjugated ET-1 diluted 2000 times in PBS-T were added to each well. After incubation at 4 °C for 16 h, followed by washing 3 times with 250 μl of PBS-T, and 100 μl of 0.2 mM 4-methylumbelliferyl-β-D-galactoside, in 20 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 0.1% (w/v) $NaN_3$, 0.1% (w/v) bovine serum albumin and 2 mM $MgCl_2$ (buffer A), was added to each well. After incubation at 25 °C for 2 h, the reaction was terminated by the addition of 0.2 M sodium phosphate buffer (100 μl, pH 10.3). The amount of 4-methylumbelliferone was measured with a spectrofluorometer (FCA, Pandex) at 450 nm (emission wave length) and at 365 nm (excitation wave length) in duplicate.

**Characterization of ET-MA51** Isotiping of ET-MA51 was performed by the Ouchterlony technique. Crossreactivity experiments were performed by the enzyme immunoassay used following ET-1 related peptides, ET-2, ET-3, porcine big ET, ET-1 (16—21), ET-1 (4—10), porcine big ET (22—39), synthesized ET-1 isomer linked with $Cys^1$–$Cys^{11}$ and $Cys^3$–$Cys^{15}$ bonds (type B ET-1), and lysylendopeptidase-digested ET-like peptide containing 21 amino acid residue, of which $Lys^9$–$Glu^{10}$ bond was cleaved (Nicked ET-1).

**Absorption of ET-1 and ET-2 by ET-MA51** ET-MA51 was dissolved in PBS-T, and an absorption of ET-1 or ET-2 was examined by the enzyme immunoassay. Namely, 150 μl of ET-1 (8 pmol/ml) or ET-2 (8 pmol/ml) was added into 150 μl of the diluent of ET-MA51, and the mixture was incubated at 37 °C for 1 h, and then the amount of ET-1 or ET-2 was measured.

**Inhibition of ET-1- and ET-2-Induced Vasocontriction by ET-MA51 in Rat Thoracic Aorta** Wistar strain male rats, weighing 200 to 300 g, were stunned and killed by bleeding, and then the thoracic aortae were removed rapidly. Ring segments (3 mm width) were prepared and were mounted between hooks in a plastic organ bath soaked with 3 ml of Krebs–Henseleit solution aerated with a gas mixture of 95% $O_2$ and 5% $CO_2$, and maintained at 37 °C. The composition of Krebs–Henseleit solution was as follows (in mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; glucose, 10.0. The magnitude of vasoconstriction was measured isometrically with a force-displaced transducer (Orientic T7-30-240) and recorded on a thermal recticorder (NEC San-ei Instruments, Rectigraph 8K) through an amplifier (Nihon-kohden AR-621G). The resting tension

TABLE I. Inhibition of ET-1- and ET-2-Induced Vasoconstriction by ET-MA51 in Rat Thoracic Aorta

| Treatment | | N | ET-1-Induced vasoconstriction | | | N | ET-2-Induced vasoconstriction | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | S.E. (%) | Statistical analysis | | Mean | S.E. (%) | Statistical analysis |
| Non-treated | | 6 | 53.8 | 8.7 | Control | 6 | 37.0 | 5.9 | Control |
| Normal IgG | 1 μg/ml | 6 | 49.5 | 6.4 | N.S. | 6 | 34.3 | 6.6 | N.S. |
| Antibody | 0.3 μg/ml | 6 | 34.5 | 5.5 | N.S. | 6 | 14.7 | 5.4 | a) |
| Antibody | 1 μg/ml | 6 | 2.7 | 0.8 | b) | 6 | 0.5 | 0.5 | b) |

The greatest tension developed by endothelin (10 ng/ml) was expressed as a percent of the maximum response 80 mM $K^+$. Data express the mean ± S.E. of constriction in six experiments. N.S.: not significant. *a*) and *b*) were statistically significant from the control at $p<0.05$ and $p<0.01$, respectively.

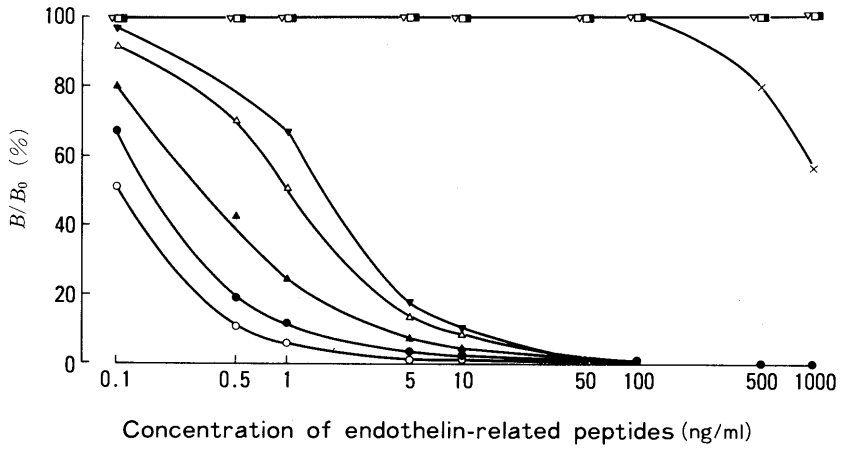


Fig. 1. Crossreactivity in the Enzyme Immunoassay Using ET-MA51 for ET-1 and Other ET-1 Related Peptides

Data show the dose–response curve for ET-1 (●), ET-2 (○), ET-3 (▲), porcine big ET (△), ET (16—21) (■), ET (4—10) (□), ET-1 type B (▼), porcine big ET (22—39) (▽) and Nicked ET-1 (×) in enzyme immunoassay using ET-MA51 and β-D-galactosidase-labeled ET-1. $B/B_0$: ($B_0$) enzyme activity bound to the solid phase without ET-1 or ET-1 related peptides ($B$) enzyme activity bound to the solid phase with added ET-1 or ET-1 related peptides.

was adjusted to 2 g and the equilibration for 2 h was followed by measurement of the maximum response to $K^+$ (80 mM) repeated at intervals of 30 min, until a steady response was obtained. ET-MA51 in Krebs–Henseleit solution was added into the organ bath. After preincubation with ET-MA51 for 10 min, ET-1 or ET-2 in PBS containing 0.1% (w/v) bovine serum albumin was added into the organ bath.

**Statistical Analysis** The greatest tension developed by ET-1 was expressed as a percent of the maximum response to 80 mM $K^+$. Statistical analysis was performed by means of analysis of variance with Student's or modified Student's $t$-test for repeated measures and a $p$-value of $<0.05$ was considered significant. All data are presented as means ± S.E.

## Results and Discussion

**Characterization of ET-MA51** The obtained ET-MA51 belonged to the $IgG_1$ subclass. The standard curves and crossreactivity experiments in the enzyme immunoassay, using ET-MA51, for ET-1 and the other ET-1 related peptides are shown in Fig. 1. In the assays, the minimal detectable concentration (at 90% $B/B_0$) was 40 pg/ml, and the 50% $B/B_0$ value of ET-1 was 230 pg/ml. The ET-MA51 showed 150% crossreactivity with ET-2; 60% with ET-3; 40% with porcine big ET; 10% with type B ET-1, respectively. On the other hand, ET-MA51 showed no crossreactivity with ET-1 (16—21), ET-1 (4—10), porcine big ET (22—39) and Nicked ET-1.

These facts, that not only type B ET-1, Nicked ET-1 but also ET (16—21) show a significant fall or a lack in immunoreactivity, implied that the steric restricted N-terminal region (ET(1—15)) possesing two disulfide bonds ($Cys^1$–$Cys^{15}$, $Cys^3$–$Cys^{11}$) but not the C-terminal region (ET(16—21)) plays an important role in the ET-1-related peptide being recognized by ET-MA51.

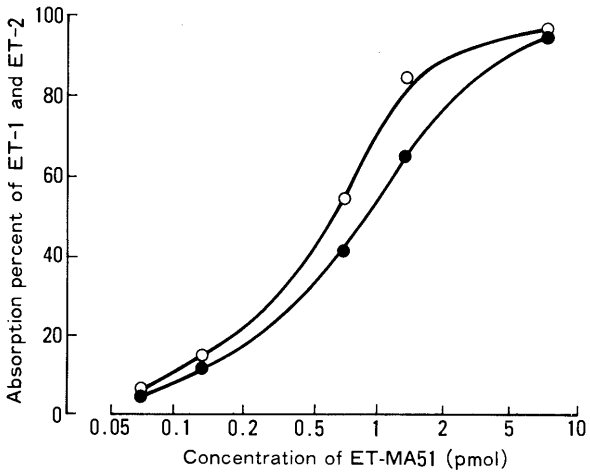


Fig. 2. Absorption of ET-1 and ET-2 by EM-MA51

150 μl of ET-1 (8 pmol/ml) or ET-2 was added to 150 μl of diluent ET-MA51, and the mixture was incubated at 37 °C for 1 h. The absorption percent of ET-1 (●) and ET-2 (○) with ET-MA51 were examined by the enzyme immunoassay.

**Absorption of ET-1 and ET-2 by ET-MA51** ET-MA51 absorbed ET-1 and ET-2 dose-dependently as shown in Fig. 2. Namely, 0.7 and 7.0 pmol of ET-MA51 absorbed, on one hand, 42% and 95% of ET-1 (1.2 pmol), and on the other hand, 55% and 96% of ET-2 (1.2 pmol), respectively. Besides, about 50% of 1.2 pmol of ET-1 and ET-2 were neutralized with 0.9 and 0.6 pmol of ET-MA51, respectively.

These results show not only correspondence with the fact that ET-2 exhibited a 1.5 fold potent cross-reactivity to ET-1, but also a high affinity of ET-MA51 for ET-1 and ET-2 at the equivalent molar concentrations.

**Inhibition of ET-1- and ET-2-Induced Vasoconstriction by ET-MA51 in Rat Thoracic Aorta** ET-1 induced a slow-developing and long-lasting constriction of the rat aorta at a concentration of 10 ng/ml. ET-2 was more potent (about 150%) than ET-1. The ET-1- or ET-2 (each 10 ng/ml)-induced constriction of the aorta was significantly ($p < 0.01$) inhibited with 1 $\mu$g/ml of ET-MA51 as shown in Table I. Table I, while the ET-MA51 pretreatment (10 $\mu$g/ml) did not affect $K^+$ ($2.5 \times 10^{-3}$ M)- and/or a *dl*-norepinephrine ($2 \times 10^{-7}$ M)-induced constriction comparable to that induced by ET-1 (10 ng/ml) (data not shown).

These results also show the high affinity of ET-MA51 for ET-1 and ET-2.

In addition, an antibody to toxins has been clinically used since the discovery of an antiserum therapy for tetanus. It has recently been found that the immunoreactive levels of ET related peptides rise in the human plasma of patients with acute myocardial infarction,[3a] uremia,[3b] subarachnoidal hemorrhage,[3c] and in bronchial exudate during acute asthmatic episodes.[3d] Moreover, ET-1 can also stimulate phospholipase $A_2$[8] and oxygen radical formation.[9] And so, endogenous ET-1 can be a candidate for the attack of various disorders caused by the constriction of smooth muscle cells, or by an increase of an oxygen radical, thromboxan $A_2$ and leukotorien. Immunological prophylactic and therapeutic applications of this antibody might be possible, since it has high cross-reactivities and high affinities, as it were, high neutralizing abilities, with ET-1 related peptides.

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# Strong Base-Induced Cycloaddition Reaction of Homophthalic Anhydride with Aldehydes

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**The reaction of homophthalic anhydride (1) and aldehydes in the presence of a strong base was studied. Reaction of 1 and benzaldehyde in the presence of NaH in anhydrous tetrahydrofuran (THF) at low temperature (0 °C—room temperature) followed by treatment with diazomethane gave the cycloadduct, *trans*-4-methoxycarbonyl-3-phenyl-3,4-dihydrocoumarin, and the reaction at 50 °C gave, after similar work-up, the C-4 methylene condensed product, methyl 2-(2-methoxycarbonylphenyl)-3-phenylacrylate, selectively. Treatment of homophthalic anhydride having a terminal aldehyde group in the side chain at the C-4 position with NaH in anhydrous THF at low temperature resulted in intramolecular cycloaddition in fair yield.**

**Keywords** homophthalic anhydride; cycloaddition; sodium hydride; aldehyde; hexahydro-6*H*-dibenzo[*b*,*d*]pyran-6-one

The fact that homophthalic anhydride (**1**) has two active sites (C-1 and C-3 positions) toward nucleophiles and one active site (C-4 position) toward electrophiles makes its chemical behavior of interest, and the anhydride is an important compound in organic synthesis. In view of these characteristics, **1** has been examined as a reactant for cycloaddition with polar double bonds (C=O[1] and C=N[2]), and used in the synthesis of natural products.[3] Previously, we reported[4] that heating of **1** with compounds containing carbon–carbon multiple bonds caused a cycloaddition reaction to give polycyclic *peri*-hydroxy aromatic compounds, and we also demonstrated[5] that a strong base such as sodium hydride (NaH) or lithium diisopropylamide (LDA) dramatically accelerated the cycloaddition reaction. The strong base-induced cycloaddition reaction of **1** with carbon–carbon multiple bonds was successively applied to syntheses of anthracyclines,[6] heteroanthracyclines,[7] SS-228R,[8] and other compounds.[9] We now report that the use of a strong base in the reaction of **1** with aldehydes is also effective for the cycloaddition reaction.

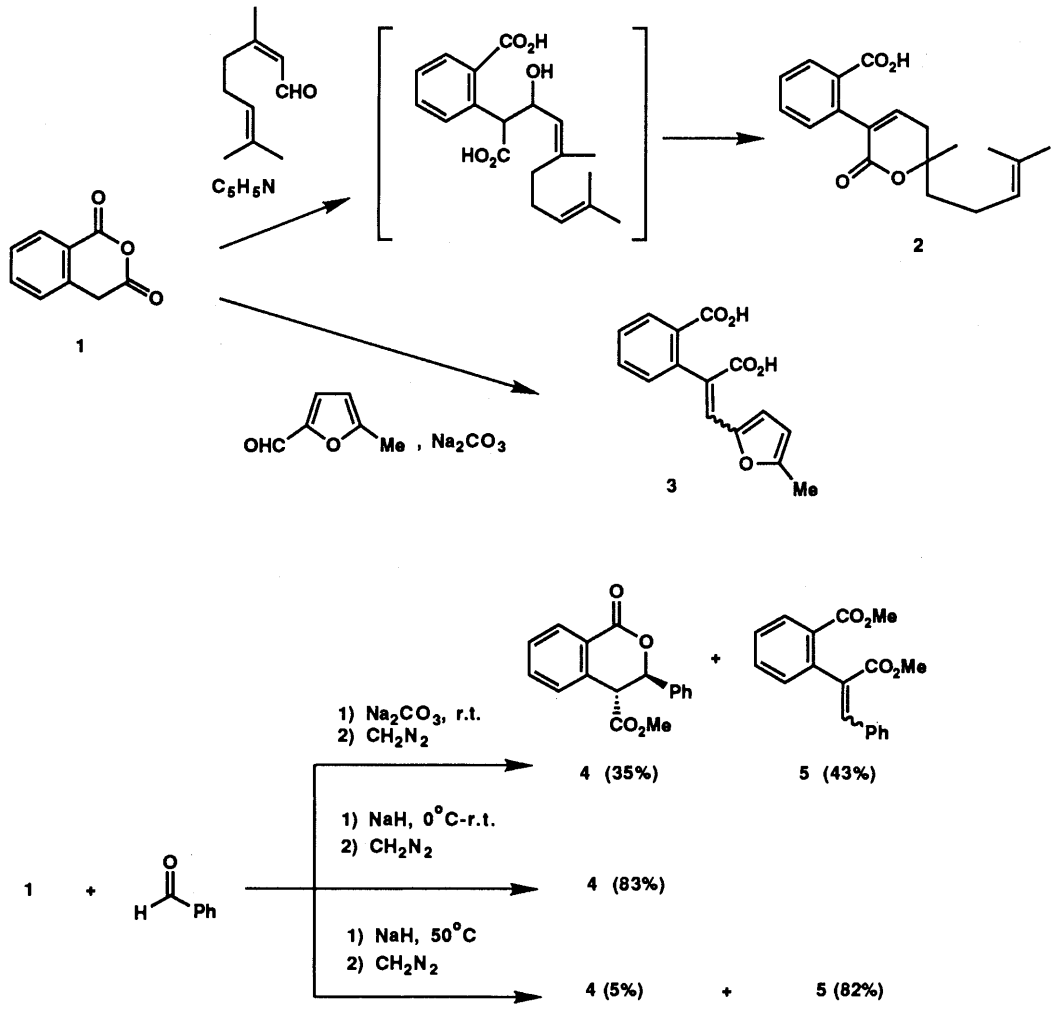



Chart 1

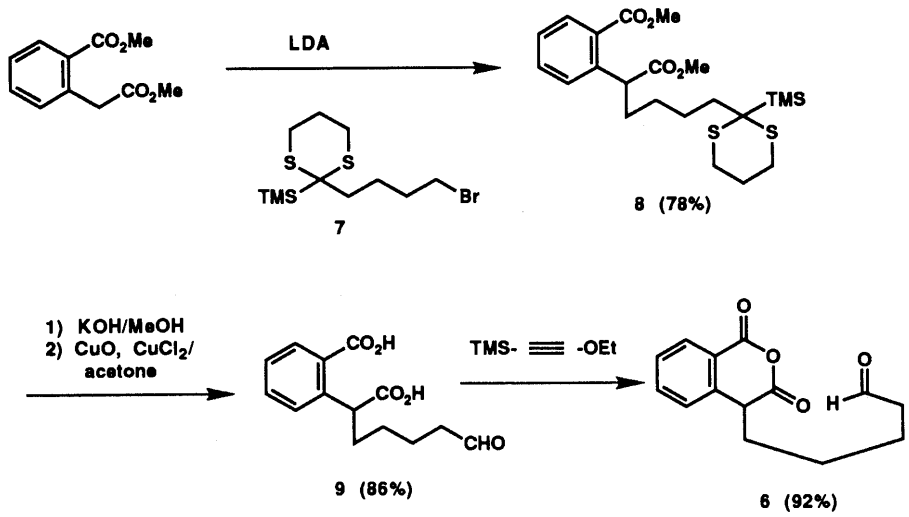


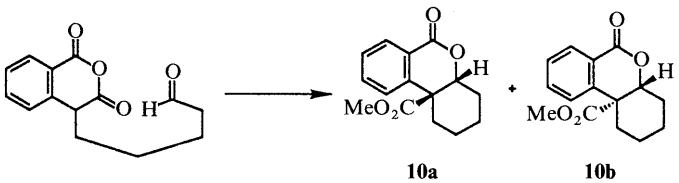

Chart 2

Reaction of **1** with some aldehydes, citral[1b] and furfural[1c] in the presence of pyridine and sodium carbonate ($Na_2CO_3$) is known to yield the C-4 methylene condensed products (**2** and **3**, respectively). 8-Hydroxy- and 8-methoxyhomophthalic anhydrides were reported to react with benzaldehyde in the presence of $Na_2CO_3$ to give the cycloadducts in moderate yield.[1d] Homophthalic anhydride (**1**), however, reacted with benzaldehyde under the same conditions to give the cycloadduct and the C-4 methylene condensed product, respectively, which were isolated as the methyl esters (**4** and **5**) after treatment with diazomethane. Although reaction of **1** with aldehydes under weakly basic conditions has been examined extensively, there has been no report on the reaction under strongly basic conditions utilizing NaH or LDA. We have now found that reaction of **1** and benzaldehyde in the presence of NaH in anhydrous tetrahydrofuran (THF) at low temperature (0 °C—room temperature) followed by treatment with diazomethane gave the cycloadduct (**4**) in 83% yield as a single product and the reaction at 50 °C gave, after similar work-up, the C-4 methylene condensed product (**5**) (82%), selectively. Therefore, it is presumed that the initially formed cycloadduct is a kinetically controlled product and the C-4 methylene condensed product is a thermodynamically controlled product.

Next, we investigated the intramolecular version of the reaction. The starting homophthalic anhydride (**6**) having a terminal aldehyde group in the side chain at the C-4 position was prepared as shown in Chart 2. Dimethyl homophthalate was treated with the bromothioacetal (**7**) to give the C-2 alkylated compound (**8**) in 78% yield. After hydrolysis of **8** with potassium hydroxide in methanol, the resulting dicarboxylic acid was dethioacetalized with copper(II) oxide-copper(II) chloride in acetone[10] to give the formyldiacid (**9**) in 86% yield. The diacid (**9**) was dehydrated with (trimethylsilyl)ethoxyacetylene[11] to give the anhydride (**6**) having a terminal aldehyde in the side chain.

Treatment of **6** with NaH in anhydrous THF at low temperature (0 °C—room temperature) for 10 min gave a 58% yield of the *cis*-cycloadduct (**10a**) and *trans*-cycloadduct (**10b**) in a 32:26 ratio. Although LDA gave the same cycloadducts (**10a** and **10b**) from **6** in approximately the same yield, the use of $Na_2CO_3$ or heating in a sealed tube provided the mixture of cycloproducts (**10a** and **10b**) in only 29 and 21% yields, respectively.

TABLE I.




**10a** **10b**

| Entry | Reaction conditions | Yield (%) | Ratio **10a : 10b** |
|---|---|---|---|
| 1 | NaH, 0 °C—r.t., 10 min | 58 | 32:26 |
| 2 | LDA, −78 °C, 1 h and r.t., 30 min | 53 | 26:27 |
| 3 | $Na_2CO_3$, r.t., 65 h | 29 | 14:15 |
| 4 | 200 °C in a sealed tube, 24 h | 21 | 14: 7 |

The strong base-induced cycloaddition of homophthalic anhydride was found to be quite useful to lead aldehydes to 4-carboxy-3,4-dihydrocoumarins.

## Experimental

All boiling and melting points are uncorrected. Infrared (IR) absorption spectra were recorded on a JASCO HPIR-102 spectrophotometer. Proton nuclear magnetic resonance ($^1$H-NMR) spectra were measured on Hitachi R-600 (60 MHz), R-22 (90 MHz), and JEOL JNM-GX500 (500 MHz) spectrometers with tetramethylsilane as an internal standard. Mass spectra (MS) and high-resolution MS were obtained on ESCO EMD-05A and JEOL JMS-D300 mass spectrometers. E. Merck Silica gel 60 (70—230 mesh ASTM) for column chromatography and E. Merck precoated TLC plates, Silica gel $F_{254}$ for preparative thin layer chromatography (TLC) were used. Organic layers were dried with anhydrous $MgSO_4$. THF was distilled from the sodium benzophenone dianion under nitrogen.

***trans*-4-Methoxycarbonyl-3-phenyl-3,4-dihydroisocoumarin (4) and Methyl 2-(2-Methoxycarbonylphenyl)-3-phenylacrylate (5)** [Entry 1]: A mixture of **1** (50 mg, 0.309 mmol), benzaldehyde (0.048 ml, 0.463 mmol), $Na_2CO_3$ (65.5 mg, 0.618 mmol), and dry benzene (5 ml) was stirred at room temperature for 24 h. The reaction mixture was quenched with aqueous $NH_4Cl$. The obtained carboxylic acid was extracted with 10% NaOH solution, and the aqueous layer was acidified (pH = 3) with 10% HCl and extracted with $Et_2O$. The organic layer was dried with $Na_2SO_4$ and concentrated under reduced pressure. The residue was diluted with AcOEt and treated with a $Et_2O$ solution of diazomethane, which was obtained from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide. The mixture was stirred

at 0 °C for 1 h. The excess diazomethane was trapped with acetic acid and the mixture was concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (hexane : AcOEt = 10 : 1) to give the esterified cycloproducts, **4** (30.8 mg, 35%) and **5** (39.0 mg, 43%). **4**: Colorless crystals, mp 129—132 °C (hexane–$CH_2Cl_2$). IR ($CHCl_3$) $\nu$: 3030, 3000, 2950, 1735, 1725, 1600 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 3.70 (s, 3H, $OCH_3$), 4.35 (d, 1H, $J$=8 Hz, CH), 5.86 (d, 1H, $J$=8 Hz, CH), 7.09—7.60 (m, 8H, ArH × 8), 8.17 (dd, 1H, $J$=7, 2 Hz, ArH). Exact MS Calcd for $C_{17}H_{14}O_4$: 282.0891. Found: 282.0891. *Anal.* Calcd for $C_{17}H_{14}O_4$: C, 72.33; H, 5.00. Found: C, 72.24; H, 4.88. **5**: A colorless viscous oil. IR ($CHCl_3$) $\nu$: 2950, 1720 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 3.75 (s, 3H, $OCH_3$), 3.87 (s, 3H, $OCH_3$), 6.84—7.47 (m, 8H, ArH and vinyl H), 7.78 (s, 1H, ArH), 8.04—8.15 (m, 1H, ArH). Exact MS Calcd for $C_{18}H_{16}O_4$: 296.1048. Found: 296.1048.

[Entry 2]: An anhydrous THF solution (3 ml) of **1** (100 mg, 0.617 mmol) was added dropwise to a suspension of NaH (60% in mineral oil, 27.2 mg, 0.679 mmol) in anhydrous THF (2 ml) at 0 °C under nitrogen. The mixture was stirred for 30 min, then a solution of benzaldehyde (0.0630 ml, 0.617 mmol) in anhydrous THF (3 ml) was added dropwise at the same temperature. The reaction mixture was stirred at room temperature for 24 h and then worked up in a similar manner to that described above. Purification by column chromatography on silica gel (hexane : AcOEt = 10 : 1) gave **4** (144 mg, 83%).

[Entry 3]: A mixture of **1** (100 mg, 0.617 mmol), benzaldehyde (0.0630 ml, 0.617 mmol), NaH (60% in mineral oil, 27.2 mg, 0.679 mmol), and anhydrous THF (9 ml) was stirred for 33 h at 50 °C. The reaction mixture was worked up in a similar manner to that described above. Purification by column chromatography on silica gel (hexane : AcOEt = 10 : 1) gave **4** (8.9 mg, 5%) and **5** (150.3 mg, 82%).

**2-(4-Bromobutyl)-2-trimethylsilyl-1,3-dithiane (7)** *n*-BuLi (1.6 N in hexane, 1.77 ml, 2.86 mmol) was added dropwise to a THF (7 ml) solution of 2-trimethylsilyl-1,3-dithiane (500 mg, 2.60 mmol) at −50 °C under nitrogen, and the solution was stirred for 30 min under the same conditions. The anion solution was added dropwise to a THF (17 ml) solution of 1,4-dibromobutane (1.56 ml, 13.0 mmol) at the same temperature, and the mixture was stirred at −50 °C for 2 h and then at room temperature for 1 h. The reaction was quenched with aqueous saturated $NH_4Cl$, and the mixture was extracted with $CH_2Cl_2$. The extract was washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (hexane : AcOEt = 10 : 1) to give **7** (569 mg, 67%) as a pale yellow oil. IR ($CHCl_3$) $\nu$: 2970, 2920, 1430, 1250, 850 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.20 (s, 9H, $Si(CH_3)_3$), 1.53—2.64 (m, 8H, $CH_2$ × 4), 2.82—3.22 (m, 4H, $CH_2$ × 2), 3.46 (t, 2H, $J$=7 Hz, $BrCH_2$). Exact MS Calcd for $C_{11}H_{23}BrS_2Si$: 326.0195. Found: 326.0201; and Calcd for $C_{11}H_{23}Br^*S_2Si$: 328.0175. Found: 328.0197.

**Dimethyl 2′-(5,5-Propylenedithio-5-trimethylsilyl)pentylhomophthalate (8)** A THF (5.5 ml) solution of dimethyl homophthalate (208 mg, 1.00 mmol) was added dropwise at −78 °C under nitrogen to a THF (5.5 ml) solution of LDA, which had been prepared from diisopropylamine (0.154 ml, 1.10 mmol) and *n*-BuLi (1.6 N in hexane, 0.680 ml, 1.10 mmol), and the mixture was stirred at the same temperature for 30 min. To this anion solution, hexamethylphosphoric triamide (HMPA) and a THF (5.5 ml) solution of **7** (327 mg, 1.00 mmol) were added continuously, and the mixture was stirred at −78 °C for 1.5 h and at room temperature for 2 h. The reaction was quenched with aqueous saturated $NH_4Cl$, and the mixture was extracted with $CH_2Cl_2$. The extract was washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (hexane : AcOEt = 10 : 1) to give **8** (355 mg, 78%) as a pale yellow oil. IR ($CHCl_3$) $\nu$: 2950, 1720 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.16 (s, 9H, $Si(CH_3)_3$), 1.25—2.41 (m, 8H, $CH_2$ × 4), 2.41—2.60 (m, 2H, $CH_2$), 2.80—3.20 (m, 4H, $CH_2$ × 2), 3.65 (s, 3H, $OCH_3$), 3.90 (s, 3H, $OCH_3$), 4.46 (t, 1H, $J$=7 Hz, COCH), 7.20—7.58 (m, 3H, ArH × 3), 7.89 (m, 1H, ArH). Exact MS Calcd for $C_{22}H_{34}O_4S_2Si$: 454.1667. Found: 454.1667. *Anal.* Calcd for $C_{22}H_{34}O_4S_2Si$: C, 58.11; H, 7.53. Found: C, 57.68; H, 7.41.

**2′-(4-Formylbutyl)homophthalic Acid (9)** A solution of **8** (198 mg, 0.436 mmol) and KOH (244 mg, 4.36 mmol) in MeOH (17 ml) and $H_2O$ (3.7 ml) was refluxed for 3 h. The solvent was evaporated under reduced pressure, then the residue was diluted with brine and washed with AcOEt. The aqueous layer was acidified (pH = 3) with 10% HCl and extracted with AcOEt; the extract was dried and concentrated under reduced pressure. Recrystallization of the crude product gave pure 2′-(5,5-propylenedithiopentyl)homophthalic acid as colorless crystals (147 mg, 96%), mp 137—139 °C (hexane–$CH_2Cl_2$). IR ($CHCl_3$) $\nu$: 2940, 1705 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.25—1.87 (m, 8H, $CH_2$ × 4), 1.94—2.11 (m, 2H, $CH_2$), 2.74—2.96 (m, 4H, $CH_2$ × 2), 4.07 (t, 1H, $J$=7 Hz, SSCH), 4.78 (t, 1H, $J$=7 Hz, COCH), 7.20—7.57 (m, 3H, ArH × 3), 7.98 (m, 1H, ArH). Exact MS Calcd for $C_{17}H_{22}O_4S_2 - H_2O$: 336.0852. Found: 336.0746.

A suspension of 2′-(5,5-propylenedithiopentyl)homophthalic acid (35.0 mg, 0.0990 mmol), $CuCl_2$ (27.0 mg, 0.198 mmol), CuO (31.0 mg, 0.396 mmol) and acetone (2 ml) was stirred at room temperature for 3 h. The mixture was filtered through Celite and the filtrate was concentrated under reduced pressure. The residue was diluted with aqueous saturated $NH_4Cl$ and washed with $Et_2O$. The aqueous layer was acidified (pH = 3) with 10% HCl and extracted with $Et_2O$; the extract was dried and concentrated under reduced pressure to give **9** (23.4 mg, 90%) as a colorless oil. IR ($CHCl_3$) $\nu$: 3020, 2940, 1720, 1705 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.25—2.82 (m, 8H, $CH_2$ × 4), 4.71 (t, 1H, $J$=7 Hz, COCH), 7.19—7.60 (m, 3H, ArH × 3), 8.01 (d, 1H, $J$=7 Hz, ArH), 9.75 (t, 1H, $J$=1 Hz, CHO), 10.57 (br s, 2H, OH). Exact MS Calcd for $C_{14}H_{16}O_5$: 264.0998. Found: 264.1020.

**2′-(4-Formylbutyl)homophthalic Anhydride (6)** The dicarboxylic acid **9** (81.0 mg, 0.307 mmol) was treated with (trimethylsilyl)ethoxyacetylene (0.0900 ml, 0.307 mmol) in dry $CH_2Cl_2$ (4 ml) at room temperature for 3 h. The reaction mixture was concentrated under reduced pressure to give **6** (69.5 mg, 92%), which was used for the next reaction without further purification. IR ($CHCl_3$) $\nu$: 3030, 2970, 1795, 1755, 1725, 1605 $cm^{-1}$.

***cis*-10*b*-Methoxycarbonyl-1,2,3,4,4*a*,10*b*-hexahydro-6*H*-dibenzo[*b*,*d*]-pyran-6-one (10a) and *trans*-10*b*-Methoxycarbonyl-1,2,3,4,4*a*,10*b*-hexahydro-6*H*-dibenzo[*b*,*d*]pyran-6-one (10b)** [Entry 1]: An anhydrous THF solution (1 ml) of **6** (25.2 mg, 0.102 mmol) was added dropwise at 0 °C under nitrogen to a suspension of NaH (60% in mineral oil, 4.50 mg, 0.113 mmol) in anhydrous THF (0.5 ml). After being stirred at room temperature for 10 min, the reaction mixture was worked up in a similar manner to that described above. Purification by preparative TLC (hexane : AcOEt = 5 : 1) gave **10a** (8.4 mg, 32%) and **10b** (6.9 mg, 26%). **10a**: Colorless crystals, mp 95—97 °C (hexane–$CH_2Cl_2$). IR ($CHCl_3$) $\nu$: 2960, 1730, 1720, 1605 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.55—2.35 (m, 8H, $CH_2$ × 4), 3.77 (s, 3H, $OCH_3$), 5.09 (dd, 1H, $J$=6, 2 Hz, CH), 7.25 (dd, 1H, $J$=7.9, 1.2 Hz, ArH), 7.44 (td, 1H, $J$=7.9, 1.2 Hz, ArH), 7.59 (td, 1H, $J$=7.9, 1.2 Hz, ArH), 8.17 (dd, 1H, $J$=7.9, 1.2 Hz, ArH). Exact MS Calcd for $C_{15}H_{16}O_4$: 260.1022. Found: 260.1034. *Anal.* Calcd for $C_{15}H_{16}O_4$: C, 69.21; H, 6.19. Found: C, 68.53; H, 6.38. **10b**: Colorless crystals, mp 148—150 °C (hexane–$CH_2Cl_2$). IR ($CHCl_3$) $\nu$: 2970, 1730, 1725, 1605 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 1.42—2.17 (m, 8H, $CH_2$ × 4), 3.63 (s, 3H, $OCH_3$), 4.34 (dd, 1H, $J$=12, 4 Hz, CH), 7.29 (dd, 1H, $J$=7.3, 1.2 Hz, ArH), 7.45 (td, 1H, $J$=7.3, 1.2 Hz, ArH), 7.59 (td, 1H, $J$=7.3, 1.2 Hz, ArH), 8.12 (dd, 1H, $J$=7.3, 1.2 Hz, ArH). Exact MS Calcd for $C_{15}H_{16}O_4$: 260.1022. Found: 260.1034. *Anal.* Calcd for $C_{15}H_{16}O_4$: C, 69.21; H, 6.19. Found: C, 68.88; H, 6.24.

[Entry 2]: An anhydrous THF (1.5 ml) solution of **6** (25.2 mg, 0.102 mmol) was added dropwise at −78 °C under nitrogen to an anhydrous THF (1 ml) solution of LDA, obtained from diisopropylamine (0.0160 ml, 0.112 mmol) and *n*-BuLi (0.0700 ml, 0.112 mmol). The mixture was stirred at −78 °C for 1 h and at room temperature for 30 min. The reaction mixture was worked up in a similar manner to that described above. Purification by preparative TLC (hexane : AcOEt = 5 : 1) gave **10a** (6.8 mg, 26%) and **10b** (7.3 mg, 27%).

[Entry 3]: A mixture of **6** (69.5 mg, 0.283 mmol) and anhydrous $Na_2CO_3$ (36.0 mg, 0.339 mmol) in dry benzene (8 ml) was stirred at room temperature for 65 h. The reaction mixture was worked up in a similar manner to that described above. Purification by preparative TLC (hexane : AcOEt = 5 : 1) gave **10a** (10.2 mg, 14%) and **10b** (11.2 mg, 15%).

[Entry 4]: A mixture of **6** (17.5 mg, 0.0711 mmol) and *o*-dichlorobenzene (2 ml) was heated in a sealed tube at 200 °C for 24 h. The reaction mixture was worked up in a similar manner to that described above. Purification by preparative TLC (hexane : AcOEt = 5 : 1) gave **10a** (2.6 mg, 14%) and **10b** (1.3 mg, 7%).

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# Chemical Transformation of Terpenoids. VIII.[1] Anodic Oxidation of Geranyl Acetate

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**Constant-current electrolysis of geranyl acetate (2) in $CH_3CN$–$H_2O$ afforded eight oxidation products (3—10) which were presumed to be formed through initial oxidation of the double bond at $C_6$–$C_7$. Based on the results of electrolysis of 2 in $CH_3CN$–$H_2{}^{18}O$, we have found that the oxygen atom(s) in the products (3—10) is(are) derived from water used as the reaction medium.**

**Keywords** anodic oxidation; geraniol; geranyl acetate; geranyl acetate 6,7-epoxide; water-$^{18}O$; constant-current electrolysis

Many chemical studies on the cyclization of acyclic terpenoids have been reported.[2] On the other hand, oxidation–reduction reactions have been shown to play important roles in the biogenesis of cyclic terpenoids. In this connection, we have been interested in the electrochemical modification of acyclic terpenoids, since only a limited number of studies on chemical transformation of acyclic terpenoids by means of electrochemical reactions have been reported.[3] As part of our continuing studies on chemical transformation of readily available acyclic terpenoids[4] and electrochemical modification of naturally abundant materials[5] leading to biologically active substances, we have been investigating the electrochemical modification of acyclic terpenoids. In this paper, we report the anodic oxidation of geranyl acetate (**2**), prepared from geraniol (**1**) by acetylation, yielding various oxidation products.[6]

After several preliminary examinations of anodic oxidation of geranyl acetate (**2**) under various reaction conditions, it was found that the constant-current electrolysis of **2** in $CH_3CN$–$H_2O$ (10 : 1) containing $NaClO_4$ as the supporting electrolyte afforded eight products with a total conversion yield of 53%. The product mixture was subjected to silica gel column chromatography and subsequently to high-performance liquid chromatography (HPLC) to furnish **3** (in 7% yield), **4** (42%), **5** (3%), **6** (8%), **7** (8%), **8** (2%), **9** (7%), and **10** (1%).

The proton nuclear magnetic resonance ($^1$H-NMR) spectrum of **3**, a colorless oil, $C_{12}H_{20}O_3$, showed signals due to one olefinic proton and one methine proton attached to a carbon bearing an oxygen function. In addition, it showed the presence of one acetoxyl group, one vinyl methyl group, two tertiary methyl groups on a carbon having an oxygen function, and one methylene proton attached to a carbon bearing an acetoxyl function. The infrared (IR) spectrum of **3** showed an ester carbonyl absorption band (1738 $cm^{-1}$), while a fragment ion peak [*m/z* 152 ($M^+$ – AcOH)] was observed in its mass spectrum (MS). Based on these spectral data, the structure of **3** was determined to be geranyl acetate 6,7-epoxide [*i.e.*, (2*E*)-1-*O*-acetyl-3,7-dimethyl-2-octen-1-ol 6,7-epoxide]. The structure was chemically substantiated by an alternative synthesis of **3** from **2** by *m*-chloroperbenzoic acid oxidation.

The IR spectrum of the major product **4**, a colorless oil, $C_{12}H_{20}O_4$, showed absorption bands ascribable to hydroxyl (3430 $cm^{-1}$) and ester (1726 $cm^{-1}$) groups. The $^1$H-NMR spectrum of **4** showed signals attributable to two tertiary methyl groups on a carbon bearing an oxygen function, one vinyl methyl group, one methine proton geminal to a hydroxyl function, and one olefinic proton. Thus, **4** was concluded to be 6,7-dihydroxygeranyl acetate [(2*E*)-1-*O*-acetyl-3,7-dimethyl-6,7-dihydroxy-2-octen-1-ol].

The IR spectrum of **5**, a colorless oil, $C_{12}H_{20}O_3$, showed two carbonyl absorption bands at 1739 and 1720 $cm^{-1}$ (an ester and a ketone). In its $^1$H-NMR spectrum, signals due to a methyl group on a carbonyl residue and a secondary methyl group were observed. The $^1$H-NMR spectrum also showed the presence of one vinyl methyl group and one acetoxyl group. From these findings, **5** was shown to be (2*E*)-1-*O*-acetyl-3,6-dimethyl-7-oxo-2-octen-1-ol, which was presumably formed through a 1,2-shift of a methyl group at C-7 to C-6 in the carbenium ion **i**.

The IR spectrum of **6**, a colorless oil, $C_{12}H_{20}O_3$, showed absorption bands due to hydroxyl (3614 $cm^{-1}$) and ester (1715 $cm^{-1}$) groups. The $^1$H-NMR spectrum of **6** showed signals assignable to three olefinic protons [$\delta$ 5.37 (t, $J$ = 6.5 Hz, 2-H); $\delta$ 5.61 (B in $ABM_2$, $J_{AB}$ = 15.5 Hz, $J_{BM}$ = 6.5 Hz, 5-H); $\delta$ 5.67 (A in $ABM_2$, $J_{AB}$ = 15.5 Hz, $J_{AM}$ = 0 Hz, 6-H)], two tertiary methyl groups on a carbon having a hydroxyl group, and one vinyl methyl group, as well as an acetoxyl group. Based on these data, the structure of **6** was determined to be (2*E*,5*E*)-1-*O*-acetyl-3,7-dimethyl-7-hydroxy-2,5-octadien-1-ol.

The IR spectrum of **7**, a colorless oil, $C_{12}H_{20}O_3$, exhibited two carbonyl absorption bands at 1741 and 1715 $cm^{-1}$ (an ester and a ketone). The $^1$H-NMR spectrum of **7** showed signals attributable to two secondary methyl groups,

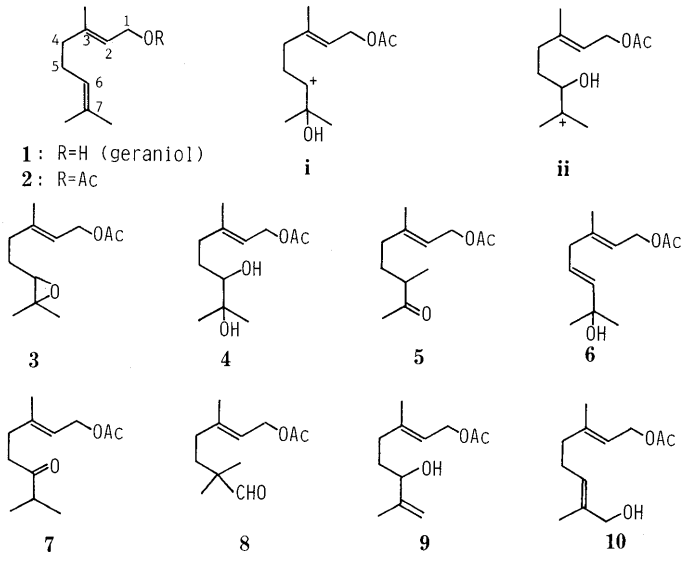
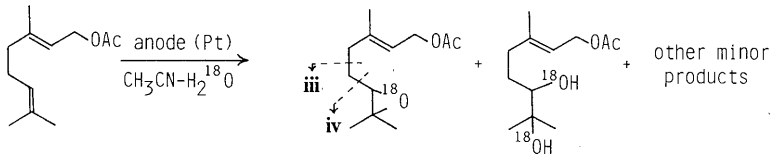



Chart 1




2 3a 4a

Chart 2

one vinyl methyl group, and one olefinic proton. From these findings, **7** was concluded to be (2*E*)-1-*O*-acetyl-3,7-dimethyl-6-oxo-2-octen-1-ol, which was presumably formed through a 1,2-hydride shift from C-6 to C-7 in the carbenium ion **ii**.

The $^1$H-NMR spectrum of **8**, a colorless oil, $C_{12}H_{20}O_3$, showed a signal due to a formyl proton besides signals due to two tertiary methyl groups, one vinyl methyl group, and one acetoxyl group. The IR spectrum of **8** exhibited two carbonyl absorption bands due to an ester function and an aldehyde function (1735, 1720 cm$^{-1}$). Thus, the structure of **8** was determined to be (2*E*)-1-*O*-acetyl-3,6-dimethyl-6-formyl-2-hepten-1-ol, which was assumed to be formed by a 1,2-shift of the $C_5$–$C_6$ bond from C-6 to C-7 in the carbenium ion **ii**.

The IR spectrum of **9**, a colorless oil, $C_{12}H_{20}O_3$, showed absorptions due to hydroxyl (3434 cm$^{-1}$), ester (1735 cm$^{-1}$), and exomethylene (903 cm$^{-1}$) groups. The $^1$H-NMR spectrum of **9** showed signals assignable to one methine proton geminal to hydroxyl function, two vinyl methyl groups, and one exomethylene group. Thus, the structure of **9** was concluded to be (2*E*)-1-*O*-acetyl-3,7-dimethyl-6-hydroxy-2,7-octadien-1-ol.

The structure of the final product **10** was assigned as $\omega$-hydroxygeranyl acetate [*i.e.*, (2*E*,6*E*)-1-*O*-acetyl-3,7-dimethyl-8-hydroxy-2,6-octadien-1-ol] on the basis of the physicochemical evidence. The assignment was corroborated by the direct comparison (IR, $^1$H-NMR, and MS) of **10** with an authentic sample.[4a]

In order to improve the selectivity of the reaction and obtain a better yield of the products, cyclic voltammetry of geranyl acetate (**2**) in $CH_3CN$–$H_2O$ containing $NaClO_4$ was carried out. It has been found that **2** possesses two oxidation potentials (1.65 and 2.16 V) *vs.* a standard calomel electrode (S.C.E.). In consequence, **2** was subjected to controlled potential electrolysis at 1.65 V *vs.* S.C.E. in $CH_3CN$–$H_2O$ (10 : 1) containing $NaClO_4$. However, the resulting products were similar to those obtained above under constant-current electrolytic conditions.

To shed light on the source of the oxygen atom(s) in the products obtained by the electrolysis of **2**, we next examined the constant-current electrolysis of **2** in $CH_3CN$–$H_2{}^{18}O$ (10 : 1) containing $NaClO_4$. The reaction mixture, which contained eight products, the same as those obtained above by electrolysis of **2** in $CH_3CN$–$H_2O$, as shown by thin layer chromatography (TLC) analysis, was purified by silica gel column chromatography to furnish **3a** (6%) and **4a** (35%) as the major products. The IR and $^1$H-NMR spectra of **3a** and **4a** were quite similar to those of **3** and **4**. In the MS of **3a**, characteristic fragment ion peaks were observed at *m/z* 154 ($M^+ - AcOH$), *m/z* 87 (**iii**), and *m/z* 73 (**iv**), which are larger by 2 mass units than those obtained in the MS of **3**. Furthermore, the high-resolution MS of **3a** revealed the following compositions of those ions: $C_{10}H_{16}{}^{18}O$ for the ion of *m/z* 154, $C_5H_9{}^{18}O$ for *m/z* 87, and $C_4H_7{}^{18}O$ for *m/z* 73, respectively. In the same manner as for **3a**, the elemental composition of a fragment ion peak at *m/z* 174 observed in the MS of **4a** was clarified to be $C_{10}H_{18}{}^{18}O_2$ ($M^+ - AcOH$). Based on these findings, it has been ascertained that the oxygen atom(s) in the anodic oxidation products, **3a** and **4a**, is(are) derived from water used as the reaction medium.

Furthermore, constant-current electrolysis of geranyl acetate 6,7-epoxide (**3**) under the same reaction conditions as for geranyl acetate (**2**) gave **4** (40%), **6** (4%), **9** (4%), and **10** (1%) in 52% conversion. It has been presumed therefore that, among the eight anodic oxidation products, four products (**4**, **6**, **9**, and **10**) are formed *via* the carbenium ions (**i**, **ii**) and/or **3**, whereas the other products (**5**, **7**, and **8**) may not be formed by way of **3**.

In conclusion, it has been found that anodic oxidation of geranyl acetate (**2**) results in the formation of eight kinds of products (**3**—**10**) which may be derived from the oxidation of the double bond at $C_6$–$C_7$ of **2**.

## Experimental

The following instruments were used to obtain physical data: IR spectra, a Hitachi 260-30 infrared spectrometer; $^1$H-NMR spectra, a JEOL JMN FX-90Q (90 MHz) NMR spectrometer or a JEOL JMN GX-500 (500 MHz) NMR spectrometer [in $CDCl_3$ solution with tetramethylsilane (TMS) as an internal standard unless otherwise specified]; MS and high-resolution MS, a JEOL JMS-D300 mass spectrometer or a JEOL JMN-01SG-2 mass spectrometer. For HPLC, a Shimadzu LC-5A chromatograph with an refraction index detector was used. Silica gel (Merck, Kieselgel 60, 70—230 mesh) and precoated TLC plates (Merck, Kieselgel $60F_{254}$) were used for column chromatography and TLC. Detection of spots on TLC was done by spraying 1% $Ce(SO_4)_2$–10% $H_2SO_4$ or 5% vanillin–concentrated $H_2SO_4$ with subsequent heating. Water-$^{18}$O (90% atom $^{18}$O) was purchased from Amersham Japan Co. Ltd. Cyclic voltammetry was carried out on a three-electrode system using a linear scanning unit (Hokuto Denko Co., Model HB-101) connected with a potentiostat (Hokuto Denko Co., Model PS-500B). As electrode systems, a glassy-carbon indicator electrode, a glassy-carbon electrode, and a S.C.E. were used. The indicator electrode was prepared from a cut of glassy carbon (GC-20, Tokai Carbon Co.), coated on one side with epoxy resin, in a glass cylinder. The S.C.E. was separated by an agar plug containing 0.1 M sodium perchlorate. Current-potential curves were recorded on a Toa XYR-2A XY recorder at a scanning rate of 50 mV·s$^{-1}$. For preparative oxidations, a potentiostat/galvanostat apparatus (Hokuto Denko Co., Model HA-105) was used.

**Constant-Current Electrolysis of 2** A solution of geranyl acetate (**2**, 250 mg) in $CH_3CN$ (116 ml)–$H_2O$ (11.6 ml) containing $NaClO_4 \cdot H_2O$ (1.57 g) was subjected to constant-current electrolysis (Pt electrode, 1 mA/cm$^2$) in an ice-cooling bath for 1 h. The reaction mixture was concentrated under reduced pressure to *ca.* 20 ml, then the whole was poured into water and extracted with EtOAc. The EtOAc extract was taken and washed with aqueous saturated NaCl, then dried over $MgSO_4$. Removal of the solvent under reduced pressure from the EtOAc extract gave a product, which was purified by column chromatography ($SiO_2$ 25 g, *n*-hexane : EtOAc = 15 : 1→8 : 1→3 : 2) to furnish **3** (9.5 mg, 7%), **4** (37.1 mg, 42%), a mixture of **5**, **7**, and **8**, and a mixture of **6**, **9**, and **10** along with recovered **2** (47% recovery). The mixture of **5**, **7**, and **8** was

further separated by HPLC (YMC-A014, *n*-hexane : EtOAc = 10 : 1) to give **5** (3.6 mg, 3%), **7** (10.7 mg, 8%), and **8** (2.6 mg, 2%). The mixture of **6**, **9**, and **10** was also separated by HPLC (YMC-A014, *n*-hexane : EtOAc = 5 : 1) to give **6** (10.5 mg, 8%), **9** (9.4 mg, 7%), and **10** (2.1 mg, 1%).

**3**: A colorless oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1738. $^1$H-NMR (90 MHz) $\delta$: 1.27, 1.31 (3H each, both s, 7-$CH_3$ × 2), 1.73 (3H, s, 3-$CH_3$), 2.06 (3H, s, OAc), 2.71 (1H, t, $J$ = 6 Hz, 6-H), 4.59 (2H, d, $J$ = 7 Hz, 1-$H_2$), 5.40 (1H, t, $J$ = 7 Hz, 2-H). MS *m/z* (%): 152 ($M^+$ − AcOH, 0.3), 85 (100), 71 (79). *Anal.* Calcd for $C_{12}H_{20}O_3$: C, 67.89; H, 9.50. Found: C, 67.61; H, 9.63.

**4**: A colorless oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 3430, 1726. $^1$H-NMR (90 MHz) $\delta$: 1.17, 1.21 (3H each, both s, 7-$CH_3$ × 2), 1.73 (3H, s, 3-$CH_3$), 2.06 (3H, s, OAc), 3.35 (1H, dd, $J$ = 3, 9 Hz, 6-H), 4.59 (2H, d, $J$ = 7 Hz, 1-$H_2$), 5.40 (1H, t-like, 2-H). MS *m/z* (%): 170 ($M^+$ − AcOH, 1), 59 (100). *Anal.* Calcd for $C_{12}H_{22}O_4$: C, 62.58; H, 9.63. Found: C, 62.48; H, 9.45.

**5**: A colorless oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1739, 1720. $^1$H-NMR (90 MHz) $\delta$: 1.10 (3H, d, $J$ = 6.5 Hz, 6-$CH_3$), 1.70 (3H, s, 3-$CH_3$), 2.06 (3H, s, OAc), 2.15 (3H, s, 7-$CH_3$), 4.58 (2H, d, $J$ = 7 Hz, 1-$H_2$), 5.31 (1H, t, $J$ = 7 Hz, 2-H). MS *m/z* (%): 152 ($M^+$ − AcOH, 1), 84 (100). *Anal.* Calcd for $C_{12}H_{20}O_3$: C, 67.89; H, 9.50. Found: C, 67.88; H, 9.46.

**6**: A colorless oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 3614, 1738. $^1$H-NMR (500 MHz) $\delta$: 1.33 (6H, s, 7-$CH_3$ × 2), 1.69 (3H, s, 3-$CH_3$), 2.06 (3H, s, OAc), 2.75 (2H, $M_2$ in $ABM_2$, $J_{AM}$ = 0 Hz, $J_{BM}$ = 6.5 Hz, 4-$H_2$), 4.60 (2H, d, $J$ = 6.5 Hz, 1-$H_2$), 5.37 (1H, t, $J$ = 6.5 Hz, 2-H), 5.61 (1H, B in $ABM_2$, $J_{AB}$ = 15.5 Hz, $J_{BM}$ = 6.5 Hz, 5-H), 5.67 (1H, A in $ABM_2$, $J_{AB}$ = 15.5 Hz, $J_{AM}$ = 0 Hz, 6-H). MS *m/z* (%): 152 ($M^+$ − AcOH, 14), 81 (100). *Anal.* Calcd for $C_{12}H_{20}O_3$: C, 67.89; H, 9.50. Found: C, 67.82; H, 9.56.

**7**: A colorless oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1741, 1715. $^1$H-NMR (90 MHz) $\delta$: 1.10 (6H, d, $J$ = 7 Hz, 6-$CH_3$ × 2), 1.71 (3H, s, 3-$CH_3$), 2.05 (3H, s, OAc), 4.57 (2H, d, $J$ = 7 Hz, 1-$H_2$), 5.34 (1H, t, $J$ = 7 Hz, 2-H). MS *m/z* (%): 152 ($M^+$ − AcOH, 4), 68 (100). *Anal.* Calcd for $C_{12}H_{20}O_3$: C, 67.89; H, 9.50. Found: C, 67.78; H, 9.73.

**8**: A colorless oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1735, 1720. $^1$H-NMR (90 MHz) $\delta$: 1.08 (6H, s, 6-$CH_3$ × 2), 1.72 (3H, s, 3-$CH_3$), 2.06 (3H, s, OAc), 4.59 (2H, d, $J$ = 7 Hz, 1-$H_2$), 5.36 (1H, t, $J$ = 7 Hz, 2-H), 9.46 (1H, s, CHO). MS *m/z* (%): 152 ($M^+$ − AcOH, 15), 71 (100). *Anal.* Calcd for $C_{12}H_{20}O_3$: C, 67.89; H, 9.50. Found: C, 67.78; H, 9.61.

**9**: A colorless oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 3618, 1735, 903. $^1$H-NMR (90 MHz) $\delta$: 1.73, 1.78 (3H each, both s, 3-$CH_3$, 7-$CH_3$), 2.05 (3H, s, OAc), 4.09 (1H, t-like, 6-H), 4.59 (2H, d, $J$ = 7 Hz, 1-$H_2$), 4.85, 4.95 (1H each, both br s, 8-$H_2$), 5.36 (1H, t, $J$ = 7 Hz, 2-H). MS *m/z* (%): 152 ($M^+$ − AcOH, 12), 84 (100). *Anal.* Calcd for $C_{12}H_{20}O_3$: C, 67.89; H, 9.50. Found: C, 67.84; H, 9.79.

**10**: A colorless oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 3434, 1732. $^1$H-NMR (90 MHz) $\delta$: 1.69, 1.73 (3H each, both s, 3-$CH_3$, 7-$CH_3$), 2.07 (3H, s, OAc), 4.02 (2H, s, 8-$H_2$), 4.60 (2H, d, $J$ = 7 Hz, 1-$H_2$), 5.36 (2H, m, 2-H, 6-H). MS *m/z* (%): 152 ($M^+$ − AcOH, 10), 68 (100). *Anal.* Calcd for $C_{12}H_{20}O_3$: C, 67.89; H, 9.50. Found: C, 67.88; H, 9.71.

**Oxidation of 2 with *m*-Chloroperbenzoic Acid** A solution of **2** (50 mg) in $CH_2Cl_2$ (10 ml) was treated with *m*-chloroperbenzoic acid (80%, 62 mg) at 0 °C for 10 min. After quenching of the reaction by adding aqueous saturated $Na_2SO_3$ (10 ml), the reaction mixture was poured into water and the whole was extracted with $CH_2Cl_2$. The $CH_2Cl_2$ extract was washed with aqueous saturated NaCl, then dried over $MgSO_4$. Removal of the solvent under reduced pressure from the $CH_2Cl_2$ extract gave a product, which was purified by column chromatography ($SiO_2$ 10 g, *n*-hexane : EtOAc = 5 : 1) to furnish **3** (51 mg). The product was identical with **3** obtained above by anodic oxidation of **2**, based on a comparison of physical data.

**Cyclic Voltammetry of 2** Cyclic voltammetry of **2** (25 mg) was carried out in $CH_3CN$ (12 ml)–$H_2O$ (1.2 ml) containing $NaClO_4 \cdot H_2O$ (157 mg) at 25 °C, and two potential peaks were observed at 1.65 and 2.16 V *vs.* S.C.E.

**Constant-Potential Electrolysis of 2** A solution of **2** (196 mg) in $CH_3CN$ (91 ml)–$H_2O$ (9.1 ml) was subjected to constant-potential electrolysis (1.65 V *vs.* S.C.E.) in an ice-cooling bath for 19 h. Work-up of the reaction mixture in the usual manner gave the same products as those obtained by the constant-current electrolysis of **2**. These products were identical with authentic samples (**3**—**10**) as judged from TLC and HPLC comparisons.

**Constant-Current Electrolysis of 2 in $CH_3CN$–$H_2{}^{18}O$** A solution of **2** (100 mg) in $CH_3CN$ (5 ml)–$H_2{}^{18}O$ (0.5 ml) containing $NaClO_4 \cdot H_2O$ (66 mg) was subjected to constant-current electrolysis (Pt electrode, 1 mA/cm$^2$) in an ice-cooling bath for 1 h. The reaction mixture was poured into water and the whole was extracted with EtOAc. Work-up of the EtOAc extract as described above gave a product, which was purified by column chromatography ($SiO_2$ 10 g, *n*-hexane : EtOAc = 8 : 1 → 3 : 2) to furnish **3a** (3.0 mg, 6%) and **4a** (19 mg, 35%) along with recovered **2** (54 mg, 54% recovery).

**3a**: A colorless oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 1738. $^1$H-NMR (90 MHz) $\delta$: 1.27, 1.31 (3H each, both s, 7-$CH_3$ × 2), 1.73 (3H, s, 3-$CH_3$), 2.06 (3H, s, OAc), 2.71 (1H, t, $J$ = 6 Hz, 6-H), 4.59 (2H, d, $J$ = 7 Hz, 1-$H_2$), 5.40 (1H, t, $J$ = 7 Hz, 2-H). MS *m/z* (%): 154 ($M^+$ − AcOH, 8), 87 (**iii**, 84), 73 (**iv**, 100). High-resolution MS *m/z*: Calcd for $C_{10}H_{16}{}^{18}O$: 154.124; Found: 154.125 ($M^+$ − AcOH), Calcd for $C_5H_9{}^{18}O$: 87.069; Found: 87.069 (**iii**), Calcd for $C_4H_7{}^{18}O$: 73.045; Found: 73.052 (**iv**).

**4a**: A colorless oil. IR $\nu_{max}^{CCl_4}$ cm$^{-1}$: 3430, 1726. $^1$H-NMR (90 MHz) $\delta$: 1.17, 1.21 (3H each, both s, 7-$CH_3$ × 2), 1.73 (3H, s, 3-$CH_3$), 3.35 (1H, dd, $J$ = 3, 9 Hz, 6-H), 4.59 (2H, d, $J$ = 7 Hz, 1-$H_2$), 5.40 (1H, t, $J$ = 7 Hz, 2-H). MS *m/z* (%): 174 ($M^+$ − AcOH, 0.6), 61 (100). High-resolution MS *m/z*: Calcd for $C_{10}H_{18}{}^{18}O_2$: 174.139; Found: 174.140 ($M^+$ − AcOH).

**Constant-Current Electrolysis of 3** A solution of **3** (200 mg) in $CH_3CN$ (86 ml)–$H_2O$ (8.6 ml) containing $NaClO_4 \cdot H_2O$ (1.15 g) was subjected to constant-current electrolysis (Pt electrode, 1 mA/cm$^2$) in an ice-cooling bath for 2 h. The reaction mixture was concentrated under reduced pressure to *ca.* 10 ml, then the whole was poured into water and extracted with EtOAc. Work-up of the EtOAc extract as described above gave a product, which was purified by column chromatography ($SiO_2$ 20 g, *n*-hexane : EtOAc = 8 : 1 → 3 : 2) to furnish **4** (44 mg, 40%) and a mixture of **6**, **9**, and **10** along with recovered **3** (90 mg, 45% recovery). The mixture of **6**, **9**, and **10** was separated by HPLC (YMC-A014, *n*-hexane : EtOAc = 5 : 1) to give **6** (4.4 mg, 4%), **9** (4.3 mg, 4%), and **10** (1.2 mg, 1%).

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# Lipid A and Related Compounds. XXVI.[1] Syntheses of Biologically Active Penta-*O*-acetyl-*N*-glycoloylneuraminyl- and Penta-*O*-acetyl-3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic Acid-(α 2→6)-D-glucosamine-4-phosphate Analogs of Lipid A

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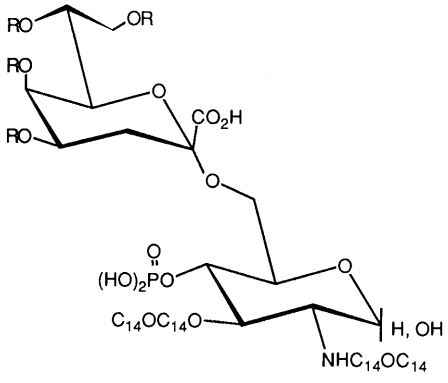
**The syntheses of novel penta-*O*-acetyl-*N*-glycoloylneuraminyl- and penta-*O*-acetyl-3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid-(α 2→6)-D-glucosamine-4-phosphate analogues of lipid A containing sialic acid in place of 3-deoxy-D-*manno*-2-octulosonic acid are described. Preliminary examination of the biological activity revealed that two synthetic disaccharides showed mitogenic activities and weak antitumor activities.**

**Keywords** *N*-acetylneuraminic acid; *N*-glycoloylneuraminyl-glucosamine-4-phosphate; KDN-glucosamine-4-phosphate; lipid A analog; mitogenic activity

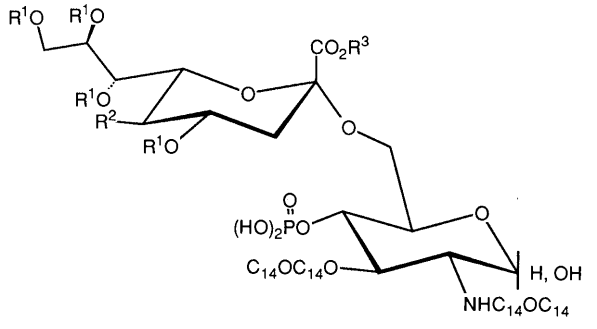
Lipid A is known to be responsible for the expression of many biological activities of lipopolysaccharide (LPS) of gram-negative bacteria.[2] In previous papers,[3] we and other groups demonstrated that acyloxyacyl glucosamine-4-phosphates as the nonreducing sugar moiety of lipid A is more important than the reducing sugar moiety (lipid X and lipid Y) for expressing the biological activities of lipid A. Furthermore, 2-keto-3-deoxyoctulosonic acid (KDO) linked-acylated 4-*O*-monophosphorylglucosamine derivatives (**1** and **2** in Chart 1) possessed mitogenic activity comparable to that of lipid A.[4] Recently, we prepared *N*-acetylneuraminyl-(α 2→6)-D-glucosamine-4-phosphate analogues[5] (**3** and **4** in Chart 1) of lipid A containing *N*-acetylneuraminic acid, whose structure is similar to KDO and whose function has attracted much interest.[6] Compounds **3** and **4** possessed weak mitogenic activities.[7] *N*-Glycoloylneuraminic acid[8] is of relatively widespread occurrence, more resistant to cleavage by neuraminidase than *N*-acylneuraminic acid. And 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (KDN)[9] bearing an hydroxyl group instead of the acetylamino group at C-5 of *N*-acetylneuraminic acid is an important terminal unit of the oligosaccharide chain of polysialoglycoproteins of the membranes of the rainbow trout.

As a synthetic approach to investigate the relationship between the chemical structure and the biological activity of the nonreducing subunit of lipid As, we now describe the syntheses of two new penta-*O*-acetyl-*N*-glycoloylneuraminyl- (**5**) and penta-*O*-acetyl-KDN-(α 2→6)-D-glucosamine-4-phosphate analogs (**6**) of lipid As.

First, we describe a synthetic sequence for the penta-*O*-acetyl *N*-glycoloylneuraminyl glucosamine-4-phosphate derivative (**5**). Treatment of 2-chloro per-*O*-acetylated methyl ester derivative (**7**) with benzyl alcohol in the presence of silver salicylate[10] gave stereospecificially the corresponding 2-*O*-α-benzyl compound (**8**) in a 96% yield. The proton nuclear magnetic resonance ($^1$H-NMR) spectrum of **8** showed signals of the equatorial proton H-$3_{eq}$ at 2.66 ppm, suggestive of an α-anomeric configuration (ranges for α-linked sialyl derivatives: H-$3_{eq}$ at 2.6—2.8 ppm).[11] The replacement of the *N*-acetyl group of **8** by an *N*-glycoloyl group was carried out first by alkylation with phosphorus pentachloride and methanol,[12] then reacylation by acetyl glycoloyl chloride to give the *N*-glycoloylamino compound (**9**) in a 51% yield. The presence of the acetoxymethyl group of **9** was suggested by the




**1**: R= Ac **2**: R= H




**3**: $R^1$= Ac, $R^2$= AcNH, $R^3$= H **4**: $R^1$= H, $R^2$= AcNH, $R^3$= H

**5**: $R^1$= Ac, $R^2$= $AcOCH_2CONH$, $R^3$= Me **6**: $R^1$= Ac, $R^2$= AcO, $R^3$= H

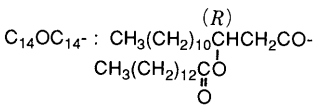
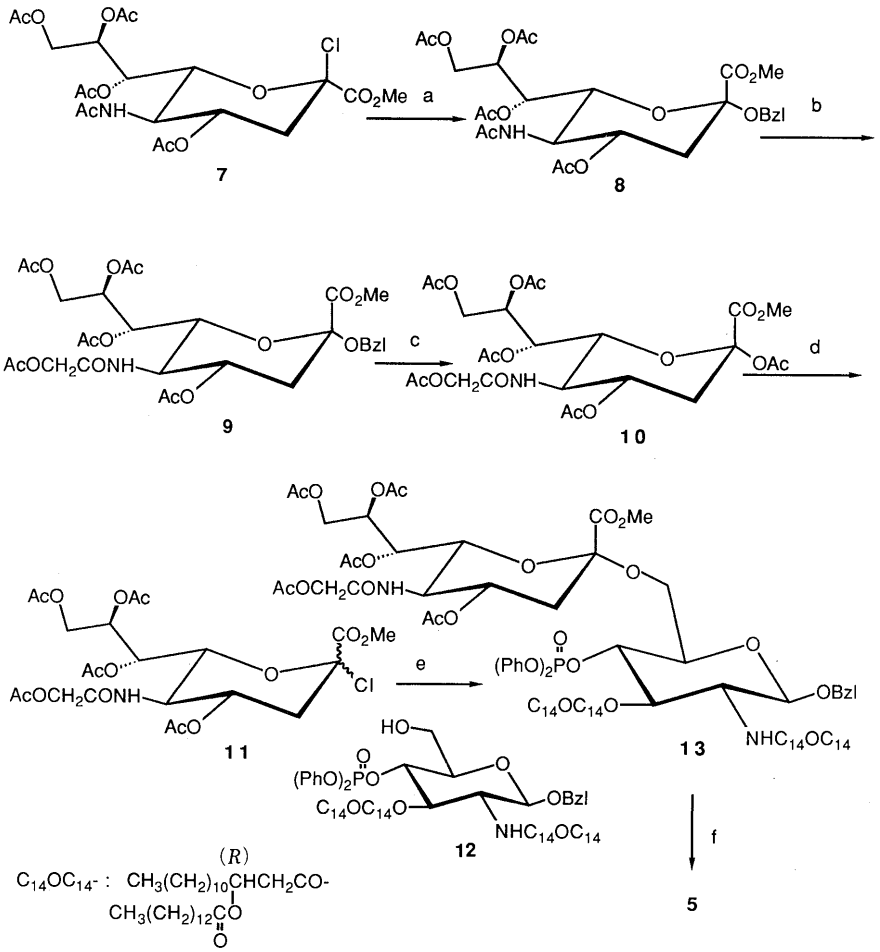



Chart 1

$^1$H-NMR spectrum [$\delta$ 2.19 (3H, s), 4.31, 4.60 (each 1H, d, $J$=15.4 Hz)]. Removal of the benzyl group of **9** was performed by hydrogenation in the presence of palladium–carbon followed by acylation with acetic anhydride and pyridine to give **10**[13] in a 57% overall yield. Treatment of **10** with acetyl chloride saturated with dry HCl at room temperature for 24 h gave the 2-chloro compound (**11**) in quantitative yield. The unstable compound (**11**) was used for subsequent glycosylation without further purification, because of its instability. In our previous investigations,[3e,f] 2-deoxy-4-*O*-phosphono-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-D-glucose showed some distinct biological activities, such as antitumor activity. Therefore, we used benzyl 2-deoxy-4-*O*-phosphono-[(*R*)-tetradecanoyloxytetradecanamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-$\beta$-D-glycopyranoside (**12**) carrying the tetradecanoyloxytetradecanoyl group at both N-2 and O-3 of the glucosamine residue, as the reducing part. Glycosylation of the glycosyl acceptor (**12**), easily prepared from benzyl 2-amino-2-deoxy-4,6-*O*-isopropylidene $\beta$-D-glucopyranoside[3d] in 5 steps, with the glycosyl donor (**11**) in the presence of $Hg(CN)_2$, $HgBr_2$ and Molecular Sieves (MA) 4A in $CH_2Cl_2$ at room temperature for 2 d gave the desired ($\alpha$ 2→6) linked disaccharide (**13**$\alpha$) in an 11% yield, and its $\beta$-anomer (**13**$\beta$) in a 6% yield, respectively, after separation by preparative thin layer chromatography. The signal due to the H-$3_{eq}$ proton of the sialosyl moiety of **13**$\alpha$ was observed at 2.70 ppm ($\alpha$-anomer). The IR spectrum of **13**$\alpha$ showed characteristic absorptions of P–O–Ph at 955 cm$^{-1}$. The protective benzyl and phenyl groups of **13**$\alpha$ were cleaved stepwise by hydrogenation by palladium–carbon and then platinum oxide as catalysts in MeOH to give the penta-*O*-acetyl *N*-glycoloylneuraminyl glucosamine-4-phosphate derivative (**5**) in a 17% yield. Compound **5** gave a positive test with the specific spray-reagent for phosphate.[14]

Next, the synthesis of penta-*O*-acetyl KDN-($\alpha$ 2→6)-glucosamine-4-phosphate derivative (**6**) was carried out as follows. Benzyl (4,5,7,8,9-penta-*O*-acetyl-3-deoxy-D-*glycero*-$\beta$-D-*galacto*-2-nonulopyranosyl bromid) onate (**17**)[15] was synthesized by the following procedures. The hydroxyl group and carboxyl group of **14** were successfully protected by acetylation with acetic anhydride/pyridine, then phenyldiazomethane, to give **16** in a 50% yield from **14**. The anomerical acetyl group of **16** was quantitatively converted into the 2-bromo derivative (**17**) with titanium(IV) bromide in ethyl acetate at room temperature for 24 h. Glycosylation of **12** with **17** in the presence

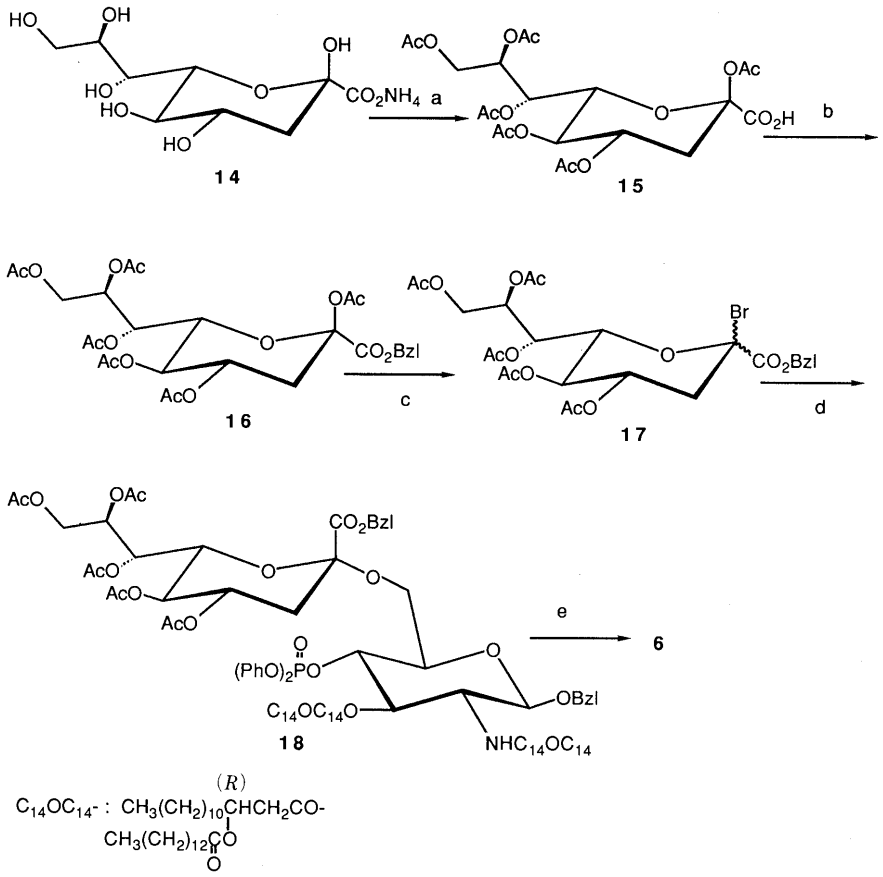



reagents : a) Ag salicylate, BzlOH ; b) i) $PCl_5$, then MeOH, ii) $AcOCH_2COCl$, DMAP, pyridine;

c) i) Pd / C, $H_2$, ii) $Ac_2O$, DMAP, pyridine; d) AcCl, HCl ; e) **12**, $Hg(CN)_2$, $HgBr_2$, MS4A ;

f) i) Pd / C, $H_2$, ii) $PtO_2$, $H_2$

Chart 2




reagents : a) $Ac_2O$, pyridine; b) $PhCHN_2$; c) $TiBr_4$; d) **12**, $Hg(CN)_2$, $HgBr_2$, MS4A ; e) i) Pd / C, $H_2$ , ii) $PtO_2$, $H_2$

Chart 3

of $Hg(CN)_2$, $HgBr_2$ and MS 4A in $CH_2Cl_2$ at room temperature for 2 d gave the α (2→6) linked disaccharide (**18**α) in a 20% yield, and its β-anomer (**18**β) in a 17% yield, respectively. The stereochemistry of **18**α was confirmed by $^1$H-NMR spectrum of the chemical shift of the H-$3_{eq}$ proton of the sialosyl moiety of **18**α at 2.68 ppm. Finally, deprotection of **18**α as described for the preparation of **5**, gave compound **6** in a 37% yield. The structures of all compounds were characterized by $^1$H-NMR and infrared (IR) spectroscopy and elemental analyses.

Preliminary examination of the biological activity showed that two chemically synthesizing disaccharides (**5**), (**6**) possessed mitogenic activities and weak antitumor activities.

## Experimental

All melting points are uncorrected. Optical rotations were measured with a JASCO DIP-140 digital polarimeter. IR spectra were recorded on a JASCO A-202 infrared spectrophotometer. $^1$H-NMR spectra were recorded on a JEOL JNM-FX 90 (90 MHz) and JEOL JNM-GX 270 (270 MHz) spectrometer with tetramethylsilane (in $CDCl_3$) as an internal standard, and the chemical shifts are given in δ values. The abbreviations of signal patterns are as follows: s, singlet; br s, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Column chromatography was carried out on Silica gel 60 (70—230 mesh, Merck). Thin layer chromatography (TLC) on Silica gel 60-$F_{254}$ (Merck) was used to monitor the reaction and to ascertain the purity of the reaction products. The spots were visualized by spraying with a 5% aqueous sulfuric acid solution and then heating.

**Methyl (Benzyl 5-Acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (8)** Silver salicylate (5.38 g, 22.1 mmol) was added to a stirred solution of compound **7**, freshly prepared from *N*-acetylneuraminic acid (4.75 g, 14.7 mmol) and benzyl alcohol (10 ml) in $CH_2Cl_2$ (90 ml) at room temperature in the dark under argon. After stirring for 5 h, the precipitate was filtered off and the filtrate was washed successively with ice-cold 5% aqueous $NaHCO_3$, 5% aqueous $Na_2S_2O_3$, brine and dried over anhydrous $MgSO_4$. After removal of the solvent, the residue was chromatographed on a column of silica gel with $CHCl_3$–acetone (5 : 1) to give **8** (8.23 g, 96.4%), mp 64—65 °C. $[\alpha]_D^{19}$ +2.38° ($c$ = 1.79, $CHCl_3$). IR (KBr): 1740 (ester), 1650 (amide) $cm^{-1}$. $^1$H-NMR ($CDCl_3$) δ: 1.89 (3H, s, AcN), 1.95 (1H, dd, $J_{3a,3e}$ = 12.8, $J_{3a,4}$ = 8.1 Hz, H-$3_{ax}$), 2.03, 2.04, 2.14, 2.17 (each 3H, s, OAc), 2.66 (1H, dd, $J_{3e,4}$ = 4.6 Hz, H-$3_{eq}$), 3.68 (3H, s, OMe), 4.03—4.17 (2H, m, H-5,6), 4.15 (1H, dd, $J_{9,9'}$ = 12.4, $J_{8,9'}$ = 2.7 Hz, H-9'), 4.34 (1H, dd, $J_{8,9}$ = 3.0 Hz, H-9), 4.44 (1H, d, $J_{gem}$ = 11.6 Hz, $OCH_2Ph$), 4.82 (1H, d, $OCH_2Ph$), 4.89 (1H, ddd, $J_{4,5}$ = 10.5 Hz, H-4), 5.14 (1H, br d, $J_{NH,5}$ = 9.2 Hz, NH), 5.35 (1H, dd, $J_{7,8}$ = 8.4, $J_{6,7}$ = 2.2 Hz, H-7), 5.46 (1H, ddd, H-8), 7.26—7.35 (5H, m, aromatic H). *Anal.* Calcd for $C_{27}H_{30}NO_{13}\cdot H_2O$: C, 54.55; H, 5.42; N, 2.36. Found; C, 54.18; H, 5.77; N, 2.47.

**Methyl (Benzyl 5-Acetoxyacetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (9)** Phosphorus pentachloride (2.17 g, 10.4 mmol) was added to a solution of **g** (3.03 g, 5.21 mmol) and *N*,*N*-dimethylaniline (2.52 g, 20.8 mmol) in dry $CH_2Cl_2$ (40 ml), and cooled at −35 °C with stirring. The mixture was stirred at the same temperature for 7 h, then MeOH (15 ml) was added to the mixture. After stirring for 2.5 h at −35 °C, $H_2O$ (10 ml) was added to the mixture, warmed at room temperature and stirred overnight. The resulting mixture was washed with aqueous saturated $NaHCO_3$ and brine. The organic phase was dried over anhydrous $MgSO_4$ and evaporated to dryness *in vacuo*. The residual oil was redissolved in dry $CH_2Cl_2$ (40 m), pyridine (1.24 g, 15.6 mmol) and 4-dimethylaminopyridine (1.27 g, 10.4 mmol), and acetyl glycoloyl chloride (2.13 g, 15.6 mmol) was added to the mixture which was cooled at 0 °C and kept at 0 °C for 0.5 h, then warmed at room temperature. After stirring overnight, the resulting mixture was washed with saturated aqueous $NaHCO_3$, and brine. The organic phase was dried over anhydrous $MgSO_4$ and evaporated to dryness. The residue was purified by a column of silica gel ($CHCl_3$–acetone = 10 : 1) to give **9** (1.68 g, 51%), mp 63—64 °C.

$[\alpha]_D^{20}$ −1.29° ($c$=1.40, $CHCl_3$). IR (KBr): 3354 (NH), 1749 (ester), 1650 (amide), 699 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 2.01, 2.04, 2.14, 2.18, 2.19 (each 3H, s, Ac), 2.03 (1H, t, $J_{3a,3e}=J_{3a,4}$=12.7 Hz, H-3$_{ax}$), 2.68 (1H, dd, $J_{3e,4}$=4.9 Hz, H-3$_{eq}$), 3.80 (3H, s, OMe), 4.08 (1H, ddd, $J_{4,5}=J_{NH,5}=J_{6,5}$=10.0 Hz, H-5), 4.12 (1H, dd, $J_{9,9'}$=12.4, $J_{8,9'}$=5.7 Hz, H-9'), 4.22 (1H, dd, $J_{6,7}$=2.2 Hz, H-6), 4.35 (1H, dd, $J_{8,9}$=2.7 Hz, H-9), 4.31 (1H, d, $J_{gem}$=15.4 Hz, OC$\underline{H}_2$OAc), 4.44 (1H, d, $J_{gem}$=11.9 Hz, OC$H_2$Ph), 4.60 (1H, d, OC$\underline{H}_2$OAc), 4.83 (1H, d, OC$H_2$Ph), 4.96 (1H, ddd, H-4), 5.30 (1H, dd, $J_{7,8}$=8.1 Hz, H-7), 5.47 (1H, ddd, H-8), 5.85 (1H, br d, NH), 7.28—7.34 (5H, m, aromatic H). *Anal.* Calcd for $C_{29}H_{32}NO_{15}\cdot 2H_2O$: C, 51.94; H, 5.41; N, 2.09. Found: C, 51.52; H, 5.89; N, 2.04.

**Methyl (5-Acetoxyacetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl)onate (10)** Compound **9** (0.43 g, 0.67 mmol) in methanol (2 ml) was hydrogenated in the presence of 10% Pd-on-carbon (0.05 g) at room temperature for 5 h. The catalyst was filtrated and the filtrate was concentrated to dryness *in vacuo*. The residue was redissolved in dry $CH_2Cl_2$ (2 ml), pyridine (0.08 g, 1.0 mmol) and 4-dimethylaminopyridine (DMAP) (0.061 g, 0.5 mmol), and $Ac_2O$ (0.10 g, 1.0 mmol) was added to the mixture cooled at 0 °C. After stirring at room temperature overnight, the resulting mixture was washed successively with ice-cold 1 N HCl, aqueous saturated $NaHCO_3$, and brine. The organic phase was dried over anhydrous $MgSO_4$ and evaporated to dryness *in vacuo*. The residue was purified by a column of silica gel ($CHCl_3$–acetone=5:1) to give **10** (0.225 g, 57%), as a syrup. $[\alpha]_D^{23}$ +1.83° ($c$=0.76, $CHCl_3$). IR (film): 3354 (NH), 1742 (ester), 1650 (amide) $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 2.02, 2.04, 2.07, 2.14, 2.16, 2.20 (each 3H, s, Ac), 2.10 (1H, dd, $J_{3a,3e}$=13.8, $J_{3a,4}$=11.1 Hz, H-3$_{ax}$), 2.56 (1H, dd, $J_{3e,4}$=5.1 Hz, H-3$_{eq}$), 3.80 (3H, s, OMe), 4.09 (1H, ddd, $J_{5,6}$=10.8, $J_{4,5}$=9.2, $J_{NH,5}$=9.5 Hz, H-5), 4.14 (1H, dd, $J_{9,9'}$=12.4, $J_{8,9'}$=6.2 Hz, H-9'), 4.18 (1H, dd, $J_{5,6}$=10.8, $J_{6,7}$=2.2 Hz, H-6), 4.30 (1H, d, $J_{gem}$=15.4 Hz, OC$\underline{H}_2$OAc), 4.49 (1H, dd, $J_{8,9}$=2.7 Hz, H-9), 4.61 (1H, d, OC$\underline{H}_2$OAc), 5.09 (1H, ddd, $J_{4,5}$=9.2 Hz, H-4), 5.32 (1H, dd, $J_{7,8}$=5.4 Hz, H-7), 5.34 (1H, ddd, H-8), 5.95 (1H, d, NH). *Anal.* Calcd for $C_{24}H_{28}NO_{16}\cdot H_2O$: C, 47.69; H, 5.00; N, 2.32. Found: C, 47.99; H, 5.32; N, 2.43.

**Methyl (5-Acetoxyacetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosyl chlorid)onate (11)** Compound **10** (0.067 g, 0.11 mmol) was dissolved in AcCl (5 ml) and cooled at −5—−10 °C. Dry hydrogen chloride gas was passed through the solution for 10 min. The mixture was kept at room temperature for 24 h and concentrated to syrup. After further coevaporations of the syrup from dry toluene (3×5 ml) and then dry ether (5×5 ml), the resulting residue was dried under reduced pressure to give a white powder **11** (quantitative). The unstable chloride (**11**) was used for the subsequent glycosylation without further purification.

**Benzyl [2-Deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-6-*O*-(methyl 5-acetoxyacetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α,β-D-*galacto*-2-nonulopyranosyl)-4-*O*-diphenylphosphono-β-D-glucopyranoside (13)** Compound **12** (0.36 g, 0.64 mmol), pulverized MS 4A (0.5 g), $Hg(CN)_2$ (0.11 g, 0.45 mmol), and $HgBr_2$ (0.069 g, 0.19 mmol) were dried by a high vacuum-pump for 3 h. The mixture was dissolved in dry $CH_2Cl_2$ (3 ml) and stirred at room temperature for 1 h under argon. The chloride **11** in dry $CH_2Cl_2$ (2 ml) was added to the mixture over a period of 1 h, and then the suspension was stirred at room temperature for 4 h under argon. The resulting mixture was filtered off and the filtrate was washed with 10% aqueous KI solution and brine. The organic phase was dried over anhydrous $MgSO_4$ and concentrated to dryness *in vacuo*. The residue was applied to a column of silica gel with ether to give **13**α and **13**β. The anomeric mixture (**13**α and **13**β) was further purified by preparative TLC ($CHCl_3$–acetone=20:1) to give (α-linked) (**13**α) (0.135 g, 11%) and (β-linked) (**13**β) (0.072 g, 6%). **13**α: Syrup, $[\alpha]_D^{22}$ −11.0° ($c$=0.14, $CHCl_3$). IR (film): 3352 (NH), 1749 (ester), 1650 (amide), 955 (PO), 700 (Ph) $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.88 (12H, t, $J$=5.9 Hz, –CH$_3$), 1.25 (80H, br s, –CH$_2$–), 1.99, 2.02, 2.14, 2.15, 2.19 (each 3H, s, Ac), 2.66 (1H, dd, $J$=12.8 Hz, H-3$_{eq}$), 3.72 (3H, s, OMe), 4.54 (2H, s, OCH$_2$O), 7.26—7.35 (15H, m, Ph). *Anal.* Calcd for $C_{103}H_{156}N_2O_{28}P\cdot H_2O$: C, 64.46; H, 8.30; N, 1.46. Found: C, 64.60; H, 8.33; N, 1.43. **13**β: Syrup, $[\alpha]_D^{22}$ −5.88° ($c$=0.10, $CHCl_3$). IR (film): 3352 (NH), 1751 (ester), 1654 (amide), 955 (PO), 700 (Ph) $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.89 (12H, t, $J$=5.8 Hz, –CH$_3$), 1.26 (80H, br s, –CH$_2$–), 1.99, 2.01, 2.02, 2.06, 2.13 (each 3H, Ac), 2.48 (1H, dd, $J$=5.1, 13.5 Hz, H-3$_{eq}$), 3.72 (3H, s, OMe), 4.56 (2H, s, OCH$_2$O), 7.29—7.36 (15H, m, Ph). *Anal.* Calcd for $C_{103}H_{156}N_2O_{28}P$: C, 65.07; H, 8.27; N, 1.47. Found: C, 65.33; H, 8.75; N, 1.55.

**2-Deoxy-2-[(*R*)-3-tetradecanoyloxytetradecanamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-6-*O*-(methyl 5-acetoxyacetamido-4,7,8,9-tetra-*O*-acetyl-3,5-didoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl)-4-*O*-phosphono-D-glucopyranose (5)** Compound **13**α (0.135 g, 0.07 mmol) in MeOH (2 ml) was hydrogenated in the presence of 10% Pd-on-carbon (0.07 g) at 30 °C for 4 h. The catalyst was filtered off and Adams' platinum catalyst (0.135 g) was added to the filtrate. Hydrogenolysis was continued at 30 °C for 18 h. The catalyst was filtered off and the filtrate was evaporated to dryness *in vacuo*. The residue was purified by preparative TLC ($CHCl_3$–MeOH=5:1) to give **5** (0.02 g, 17%), as a syrup. $[\alpha]_D^{18}$ +9.9° ($c$=0.38, $CHCl_3$), IR (film): 3405 (NH, OH), 1750 (ester), 1650 (amide), 1260 (HO–P) $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.89 (12H, t, $J$=5.9 Hz, –CH$_3$), 1.25 (80H, br s, –CH$_2$–), 1.93—2.20 (16H, m, OAc, H-3$_{ax}$), 3.72 (3H, s, OMe). *Anal.* Calcd for $C_{77}H_{136}N_2O_{28}P$: C, 58.95; H, 8.74; N, 1.79. Found: C, 58.62; H, 8.42; N, 1.69.

**2,4,5,7,8,9-Hexa-*O*-acetyl-3-deoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonic Acid (15)** Ammonium 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonate (**14**) (0.242 g, 0.85 mmol) was dissolved in pyridine (2.5 ml) and DMAP (0.01 g, 0.82 mmol), and then acetic anhydride (2.5 ml) was added to the mixture cooled first at 0 °C, and then at room temperature for 1 d. The reaction mixture was diluted with $CH_2Cl_2$ and washed with ice-cold 1 N HCl, saturated aqueous $NaHCO_3$, and brine. The organic phase was dried over anhydrous $MgSO_4$ and concentrated to dryness *in vacuo*. The residue was purified by a column of silica gel ($CHCl_3$–MeOH=5:1) to give **15** (0.20 g, 50%), mp 128—130 °C. $[\alpha]_D^{20}$ −19.5° ($c$=2.74, MeOH). IR (film): 1730 (ester), 1650 (amide) $cm^{-1}$. $^1$H-NMR ($CDCl_3$–$CD_3OD$) $\delta$: 2.00, 2.02, 2.04, 2.07, 2.09, 2.13 (each 3H, s, OAc), 2.58 (1H, dd, $J_{3e,3a}$=14.0, $J_{3e,4}$=5.4 Hz, H-3$_{eq}$), 4.07 (1H, dd, $J_{5,6}$=10.0, $J_{6,7}$=2.7 Hz, H-6), 4.10 (1H, dd, $J_{9,9'}$=12.1, $J_{8,9'}$=6.5 Hz, H-9'), 4.51 (1H, dd, $J_{8,9}$=2.2 Hz, H-9), 4.93 (1H, t, $J$=10.0 Hz, H-5), 5.14 (1H, ddd, H-8), 5.28 (1H, ddd, H-4), and 5.39 (1H, dd, $J_{7,8}$=7.0 Hz, H-7), *Anal.* Calcd for $C_{21}H_{28}O_{15}$: C, 48.46; H, 5.42. Found: C, 48.13, H, 5.08.

**Benzyl 2,4,5,7,8,9-Hexa-*O*-acetyl-3-deoxy-D-*glycero*-β-D-*galacto*-2-nonolopyranosonate (16)** Phenyldiazomethane was added to a suspension of **15** (0.22 g, 0.42 mmol) and methanol (2 ml) with stirring at room temperature. The mixture was stirred at room temperature overnight, then the excess phenyldiazomethane was decomposed with acetic acid until the red color disappeared. The resulting solution was evaporated to dryness *in vacuo*. The resulting residue was purified on a column of silica gel ($CHCl_3$–isopropyl ether (PE)=20:1) to give **16** (quantitative), as a syrup. $[\alpha]_D^{20}$ −15.4° ($c$=1.19, $CHCl_3$). IR (film): 1753 (ester), 700 (Ph) $cm^{-1}$. EI-MS $m/z$: 551 ($M^+-59$). $^1$H-NMR ($CDCl_3$) $\delta$: 2.00, 2.01, 2.02, 2.09, 2.11 (18H, s, OAc), 2.09 (1H, dd, $J_{3a,3e}$=14.3, $J_{3a,4}$=11.8 Hz, H-3$_{ax}$), 2.63 (1H, dd, $J_{3e,4}$=5.1 Hz, H-3$_{eq}$), 4.15 (1H, dd, $J_{9,9'}$=12.4, $J_{8,9'}$=4.9 Hz, H-9'), 4.20 (1H, dd, $J_{5,6}$=10.3 Hz, $J_{6,7}$=2.7 Hz, H-6), 4.42 (1H, dd, $J_{8,9}$=2.7 Hz, H-9), 4.97 (1H, t, $J$=10.0 Hz, H-5), 5.15 (1H, ddd, $J_{8,7}$=6.8 Hz, H-8), 5.16 (1H, d, $J_{gem}$=12.4 Hz, –OC$\underline{H}_2$Ph), 5.23 (1H, d, –OC$\underline{H}_2$Ph), 5.26 (1H, m, H-4), 5.39 (1H, dd, $J_{6,7}$=2.7 Hz, H-7), 7.34—7.36 (5H, m, aromatic H). *Anal.* Calcd for $C_{28}H_{34}O_{15}\cdot H_2O$: C, 53.50; H, 5.77. Found: C, 53.92; H, 5.48.

**Benzyl (4,5,7,8,9-Penta-*O*-acetyl-3-deoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosyl bromid)onate (17)** Compound **16** (0.12 g, 0.20 mmol) was dissolved in $CH_2Cl_2$–AcOEt (10:1) and MS 4A (0.10 g), and stirred at room temperature for 1 h. $TiBr_4$ (0.14 g, 0.37 mmol) was added to the mixture cooled at 0 °C, then stirred at the same temperature overnight. Acetonitrile (3 ml) and anhydrous NaOAc (1.0 g) was added to the reaction mixture, and stirred for 0.5 h, then toluene (5 ml) was added to the mixture and stirred a further 15 min. The insoluble materials were filtered off and the filtrate was evaporated to dryness *in vacuo*. The unstable bromide **17** was used for the subsequent glycosylation without further purification.

**Benzyl 2-Deoxy-2-[(*R*)-3-tetradecanoyloxytetradecamido]-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-6-*O*-(benzyl 4,5,7,8,9-penta-*O*-acetyl-3-deoxy-D-*glycero*-α,β-D-*galacto*-2-nonulopyranosyl)-4-*O*-diphenylphosphono-β-D-glucopyranoside (18)** Compound **12** (0.107 g, 0.18 mmol), $Hg(CN)_2$ (0.137 g, 0.54 mmol) and $HgBr_2$ (0.032 g, 0.09 mmol) was dried by the use of a high vacuum-pump for 3 h. The mixture was dissolved in dry $CH_2Cl_2$ (3 ml) and stirred at room temperature for 1 h under argon. The bromide **17** in dry $CH_2Cl_2$ (2 ml) was added at room temperature over a period of 1 h, and then the suspension was stirred at room temperature for 2 d. The resulting mixture was filtered off and the filtrate was washed with 10% aqueous KI solution and brine. The organic phase was concentrated to dryness *in vacuo* and the residue was purified by column chromatography on silica gel with $CHCl_3$–IPE=20:1 to give **18**α and **18**β. The anomeric mixture (**18**α and **18**β) was further purified by preparative TLC to give (α-linked) **18**α (0.026 g, 20%) and (β-linked) **18**β (0.022 g, 17%), respectively. **18**α: Syrup, $[\alpha]_D^{23}$ −3.60° ($c$=1.20, $CHCl_3$). IR (film): 3352 (NH), 1749 (ester), 1650 (amide), 955 (PhOP), 700 (Ph)

$cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.89 (12H, t, $J$=5.7 Hz, $-CH_3$), 1.26 (80H, br s, $-CH_2-$), 1.85—2.15 (16H, m, OAc, H-$3_{ax}$), 2.68 (1H, dd, $J$=4.4, 13.4 Hz, H-$3_{eq}$), 5.20 (2H, s, $PhCH_2$), and 7.30—7.35 (20H, m, Ph). *Anal.* Calcd for $C_{107}H_{157}N_2O_{27}P$: C, 66.44; H, 8.18; N, 1.45. Found: C, 66.69; H, 8.63; N, 1.70 **18**$\beta$: Syrup, $[\alpha]_D^{22}$ −2.91° ($c$=0.10, $CHCl_3$). IR (film): 3354 (NH), 1751 (ester), 1654 (amide), 955 (PhOP), 700 (Ph) $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.87 (12H, t, $J$=5.9 Hz, $-CH_3$), 1.25 (80H, br s, $-CH_2-$), 1.85—2.15 (16H, m, OAc, H-$3_{ax}$), 2.66 (1H, dd, $J$=4.4, 12.3 Hz, H-$3_{eq}$), 5.20 (2H, s, $PhCH_2$).

**2-Deoxy-2-[(*R*)-3-tetradecanoyloxytetradecamido]-3-*O*-[(*R*)-3-tetradecanoxyloxytetradecanoyl]-6-*O*-(4,5,7,8,9-penta-*O*-acetyl-3-deoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl)-4-*O*-phosphono-D-glucopyranose (6)** Compound **18**α (0.026 g, 0.013 mmol) in MeOH (1.0 ml) was hydrogenated in the presence of 10% Pd-on-carbon (0.013 g) at 30 °C for 4 h. The catalyst was filtered off and Adams' platinum catalyst (0.026 g) was added to the filtrate. Hydrogenolysis was continued at 30 °C for 18 h. The catalyst was filtered off and the filtrate was evaporated to an oil and applied to preparative TLC ($CHCl_3$–MeOH = 5 : 1) to give **6** (8 mg, 37%), as a syrup. $[\alpha]_D^{28}$ −7.6° ($c$=0.08, $CHCl_3$). IR (film): 3405 (NH, OH), 1750 (ester), 1650 (amide), 1260 (HOP) $cm^{-1}$. $^1$H-NMR ($CDCl_3$) $\delta$: 0.89 (12H, t, $J$=7.1 Hz, $-CH_3$), 1.27 (80H, br s, $-CH_2-$), 1.93—2.20 (16H, m, OAc, H-$3_{ax}$), 3.72 (3H, s, OMe). *Anal.* Calcd for $C_{81}H_{137}N_2O_{27}P$: C, 60.73; H, 8.62; N, 1.75. Found: C, 60.35; H, 8.17; N, 1.55.

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# Comparison of *Aristolochia* Species with Chemical Constituents

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**Chemical constituents, in particular, aristololactam derivatives of nine *Aristolochia* species (*A. shimadai*, *A. manshuriensis*, *A. cucurbitifolia*, *A. westlandii*, *A onoei*, *A. kaempferi*, *A. liukiuensis*, *A. debilis* and *A. tagara*) were analyzed by means of high performance liquid chromatography. The results chemotaxonomically suggested their subgenera, and sometimes their sections are restricted by the relative content of aristololactam derivatives.**

**Keywords** *Aristolochia* species; Aristolochiaceae; aristololactam derivative; aristolochic acid derivative; chemotaxonomy

## Introduction

Some *Aristolochia* species have been utilized as folklore medicine. In China, the roots of *A. debilis* or *A. contorta* (青木香), the leaves and twining stems of the same plant (天仙藤), and the matured fruits (馬兜鈴) were known for their bronchiectatic effect and for decreasing high blood-pressure. Other species of *Aristolochia*, including *A. manshuriensis*, *A. shukangii*, *A. mollissima*, *etc.*, have also been used for the same purposes. The roots of *Aristochia* species such as *A. indica* are used for emmenagogue or abortifacient in other countries,[1] and the activities were supported by experiments on female mice.[2] The antiviral activity of *A. elegans* and *A. frockelii* was also screened.[3] The principal biological activities of the *Aristolochia* species however, have not been clearly determined. The so-called aristolochic acid and aristololactam derivatives are recognized as special chemical constituents of this genus. The former has a phenanthrene skeleton with a nitro group and the latter has the same skeleton with a lactam structure. In a previous paper, the structure of a new aristolochic acid derivative in *A. liukiuensis* was described.[4] The present paper deals with quantitative analysis of the aristololactam derivatives by use of high performance liquid chromatography (HPLC) in order to differentiate the above species.

## Materials and Methods

**Plant Materials** The dates and localities of the present *Aristolochia* species are as follows: *A. shimadai* (March 1989, Nan-t'ou, Formosa), *A. manshuriensis* (June 1986, Mt. Sorak, Korea), *A. cucurbitafolia* (July 1988, Meishan, China), *A. kaempferi* (August 1987, Chiba pref., Japan), *A. liukiuensis* (July 1986, Okinawa pref., Japan), *A. onoei* (May 1988, Rokko-san, Japan) and *A. tagara* (July 1987, Doi Inthanon, Thailand). *A. westlandii* and *A. debilis* used in this experiment are cultivated in the Botanical Gardens, University of Tokyo. The voucher specimens are deposited in the Herbarium of Gifu Pharmaceutical University.

**Chemical Constituents** By repeated column chromatography, 30 known compounds including a new compound [4] were isolated from each methanolic extract of the leaves and stems of *A. cucurbitifolia*, *A. kaempferi*, *A. liukiuensis* and *A. tagara*, and the structures were confirmed by means of spectroscopic analysis: aristolochic acids, I, Ia, II, IV and IVa, aristololactam β-D-glucoside from *A. cucurbitifolia*; isorahmnetin-3-*O*-rhamnosyl-(1→6)-galactoside, 2-hydroxy-1-methoxy-4,5-dioxo-6a,7-dehydroaporphine, *N*-(*p*-hydroxyphenyl)-β-ethyl-*p*-hydroxycinnamic acid, aristolochic acid I, aristololactams AII, AIII, AIIIa, BII from *A. kaempferi*; β-sitosterol-β-D-*O*-glucoside, 2-hydroxy-1-methoxy-4,5-dioxo-6a,7-dehydroaporphine, *N*-(*p*-hydroxyphenyl)-β-ethyl-*p*-hydroxycinnamic acid, 3-hydroxy-4-methoxyaristolochic acid methylester, aristololactams AII, AIII, AIIIa and BII from *A. liukiuensis*; β-sitosterol, sesamin, cepharadione A, aristololactams I, II and IIIa from *A. tagara*.

**HPLC Conditions** The conditions are described in Fig. 1. The aristololactam derivatives analyzed in the present study are as follows: aristolactams II (**1**), I (**2**), IIIa (**3**), AII (**4**), AIII (**5**), BII (**6**), and aristololactam I *N*-glucoside (**7**). The structures are drawn in Chart 1. For fluorescent detection, the excitation (Ex) and emission (Em) wavelengths were measured and the appropriate wavelengths were determined; [EX (nm), Em (nm)]: **1** [399, 460], **2** [414, 473], **3** [386, 499], **4** [417, 505], **5** [373, 487], **6** [386, 496], **7** [400, 482].

**Sample Preparation and Quantitative Determination** About 2 g of powder of the dried stems was weighed accurately and extracted with MeOH (20 ml) for 1 h under reflux. After filtration, the residue was washed with MeOH until the volume of filtrate reached 20 ml. An aliquot of the MeOH was filtrated again with a mambrane filter (0.45 mm) and subjected to HPLC. The content of each aristololactam derivative was calculated by calibration curves prepared in advance, and is shown by the diagram in Fig. 2.

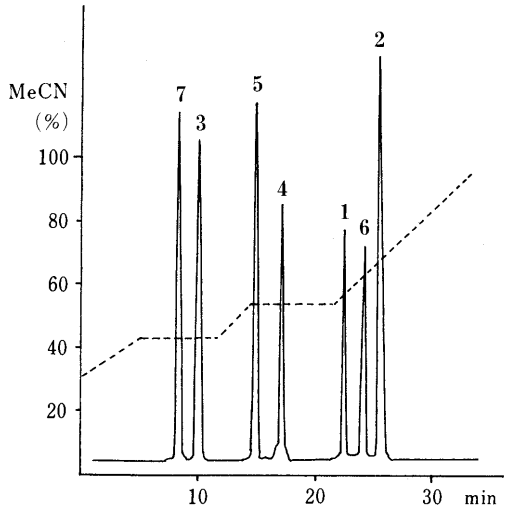
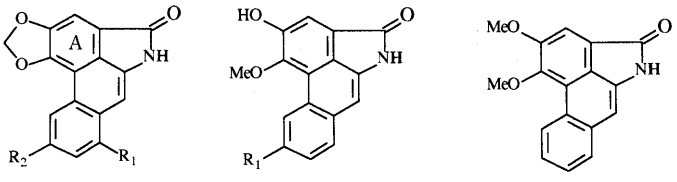



Fig. 1. HPLC Chromatogram of Aristololactam Derivatives (**1**—**7**)

Conditions: column, Cosmosil $5C_{18}$ 4.6 × 250 mm; mobile phase, MeCN, 0.01 mM $H_3PO_4$ buffer (pH 2.6), (30 : 70→85 : 15 (v/v)); flow rate, 1.0 ml/min; column temp., room temp.; detector, fluorescent detection, wavelength, Ex = 390 nm, Em = 480 nm.




**1** : $R_1 = R_2 = H$
**2** : $R_1$ = OMe, $R_2$ = H
(**7** : *N*-glucoside)
**3** : $R_1$ = H, $R_2$ = OH

**4** : $R_1$ = H
**5** : $R_1$ = OMe

**6**

Chart 1

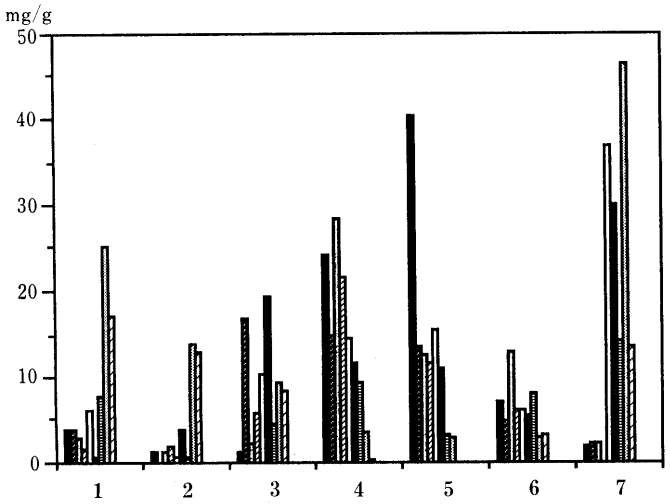



Fig. 2. Content of Aristololactum Derivatives (**1**—**7**) in Nine *Aristolochia* Species

■, *A. onoei*; ▨, *A. kaempferi*; ▩, *A. liukiuensis*; ▨, *A. shimadai*; □, *A. cucurbitiforia*; ■, *A. manshuriensis*; ▤, *A. westlandii*; □, *A. debilis*; ▨, *A. tagala*.

## Results and Discussion

The genus *Aristlochia* is composed of two subgenera, Siphisia and Aristolochia. In the present study, the aristololactam derivatives with methoxyl group(s) on the A ring were found abundantly in the subgenus Siphisia, such as *A. shimadai*, *A. manshuriensis* and *A. kaempferi*. On the contrary, the plants of subgenus Aristolochia (*A. debilis* and *A. tagara*) were rich in derivatives with a methylenedioxy group such as **1**—**3**. The plants of the Siphisia section (*A. shimadai*, *A. manshuriensis*, *A. cucurbitifolia* and *A. westlandii*) in the subgenus Siphisia were richer in aristololactam I *N*-β-D-glucoside than the plants in the section Hexodon (*A. onoei*, *A. kaempferi* and *A. liukiuensis*). The stems of *A. debilis* (天仙藤) were characterized by an abundant quantity of aristololactam derivatives within the methlenedioxy group.

These results suggest that the aristololactam derivatives are a good specific-marker for distinguishing the *Aristolochia* species.

**Acknowledgement** The authors sincerely thank Dr. Jin Murata in the Botanical Gardens, University of Tokyo for providing and identifying the *Aristolochia* species.

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# Gardenia Fruit Extract Does Not Stimulate the Proliferation of Cultured Vascular Smooth Muscle Cells, A10

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**We investigated the effect of a hot water extract from Gardenia fruit (*Gardenia jasminoides* ELLIS) (GFE), which has a stimulatory effect on endothelial cell proliferation, on the proliferation of A10 cells, an established cell line of vascular smooth muscle cell from murine aorta in a culture system. GFE did not change the number of A10 cells after a 48 h culture. GFE significantly increased the incorporation of [$^3$H]thymidine and [$^{14}$C]leucine into the acid-soluble fraction of bovine aortic endothelial cell layers, but significantly decreased that of A10 cells. These results suggested that GFE stimulates the proliferation of endothelial cells but not of A10 cells. In the endothelial cell culture, GFE significantly increased the accumulation of basic fibroblast growth factor, which is an autocrine for endothelial cell proliferation in medium and low-affinity (glycosaminoglycans-binding) fractions, while A10 cells did not produce a significant amount of the factor. Since it is postulated that a selective stimulation of endothelial cell proliferation by increasing the production of basic fibroblast growth factor is appropriate for prevention of arteriosclerosis and thrombosis, GFE may contain a beneficial component as a useful drug.**

**Keywords** basic fibroblast growth factor; endothelial cell; endothelium; *Gardenia jasminoides*; proliferation; smooth muscle cell

The healthy arterial wall consists of intimal endothelial cells, which line the lumen, and underlying smooth muscle cells which remain in a quiescent growth state. The proliferation of endothelial cells is importantly involved in the physiology and pathology of blood vessels. When the endothelium is injured slightly, endothelial cells will proliferate and cover the area and smooth muscle cell growth will cease. However, if endothelial cell injury is repeated and endothelial cell proliferation does not occur sufficiently, smooth muscle cell hyperplasia will occur with the eventual development of an atherosclerotic lesion.

We have studied the effect of traditional herbal drugs on the blood coagulation–fibrinolytic system. We found that a water-soluble extract from the Artemisia leaf inhibited both blood coagulation and fibrinolysis[2] and an extract from Gardenia fruit accelerated fibrinolysis[3] in human plasma *in vitro*. Recently, we found that a hot water extract from either the Artemisia leaf (AFE) or Gardenia fruit (GFE) stimulates the proliferation of cultured endothelial cells.[4,5] Furthermore, we revealed that AFE stimulation of endothelial cell proliferation may result from an enhanced production of basic fibroblast growth factor (bFGF) by the cells.[6]

To clarify whether or not GFE selectively stimulates endothelial cell proliferation will be important for its evaluation as a useful drug. In the present study, we investigated the effect of GFE on the proliferation of cultured A10 cells, an established cell line of vascular smooth muscle cells, from the murine aorta.

## Materials and Methods

**Materials** RPMI 1640 medium and fetal bovine serum was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan) and Filtron (Australia), respectively. Tissue culture plates and dishes were from Costar (Cambridge, MA, U.S.A.). [Methyl-$^3$H]thymidine (740 GBq/mmol) and L-[$^{14}$C]leucine (11.8 GBq/mmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Endothelial cells were isolated from bovine aorta by scraping the surface of the intima. An established cell line of vascular smooth muscle cells from the murine aorta, A10 cells, was obtained from American Type Culture Collection (Rockville, MD, U.S.A.). The bFGF [$^{125}$I]radioimmunoassay kit was from Amersham Corp. (U.K.).

**Preparation of GFE** A hot water extract from Gardenia fruit (*Gardenia jaminoides* ELLIS) was prepared as described previously.[4] The same GFE examined in that report was used in the present study. An aqueous solution of GFE was sterilized through a filter and added to the culture medium.

**Cell Culture and Cell Counting** A10 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humid atmosphere at 5% $CO_2$ in air in a 100 mm dish until confluent. They were then transferred into 24-well culture plates at $2 \times 10^4$ cells/well and cultured in the presence of GFE (10, 25, 50, or 100 μg/ml) for 48 h. After culturation, the medium was discarded and the cell layer was washed twice with Ca, Mg-free phosphate-buffered saline (CMF-PBS). The cell layer was dispersed with 0.25% trypsin–0.05% ethylenediaminetetraacetic acid in CMF-PBS. The cell suspension was well pipetted and the cell number was counted with a hematocytometer.

**Deoxyribonucleic Acid (DNA) and Protein Syntheses** In another experiment, the cells were cultured in 6-well culture plates for 72 h in the presence of 25 μg/ml GFE and labeled with both 4.81 kBq/ml [$^3$H]-thymidine and 3.33 kBq/ml [$^{14}$C]leucine during the last 3 h of the culture. At this time, endothelial cells were cultured and labeled in the same way. After culturation, the medium was discarded and the cell layer was washed twice with CMF-PBS; the cells were scraped off with a rubber policeman in the presence of CMF-PBS. The cell homogenate was prepared by sonication and an aliquot was used for the determination of DNA by the method of Kissane and Robins.[7] The incorporation of [$^3$H]thymidine and [$^{14}$C]leucine into the 5% trichloroacetic acid (TCA)-insoluble fraction of the cell homogenate was measured by a liquid scintillation counter using a portion of the homogenate.

**Accumulation of bFGF** Endothelial cells were cultured in the presence of 25 μg/ml GFE in 24-well culture plates for 72 h. After culturation, the medium was removed; cultures were washed with CMF-PBS (the wash was combined with the medium); twice with 0.5 ml of 2 M NaCl in 20 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes), pH 7.5; twice with 0.5 ml of 2 M NaCl in 20 mM sodium acetate, pH 4.0; and were extracted with 1 ml of 0.5% Triton X-100 in 0.1 M sodium phosphate, pH 8.1. The bFGF in the medium; bFGF eluted with 2 M NaCl at neutral pH (the low-affinity fraction), which represents bFGF bound to glycosaminoglycans; bFGF eluted with a subsequent wash with 2 M NaCl at pH 4.0 (the high-affinity fraction), which represents bFGF bount to receptors; and bFGF released by a subsequent extraction with 0.5% Triton X-100 (the cell lysate fraction), which represents internalized bFGF, were all measured by radioimmunoassay. The fractionation of bFGF was performed by the method of Moscatelli.[8]

**Statistical Analysis** Data were analyzed for statistical significance using the Student's *t* test.

## Results and Discussion

In the previous study, we demonstrated that GFE

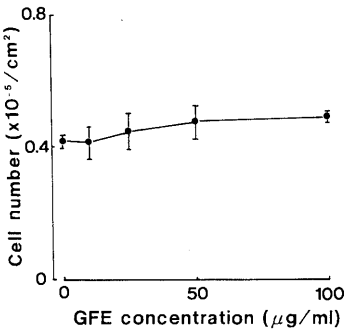


Fig. 1. Effect of GFE on the Number of Cultured A10 Cells

The cells were cultured in the presence of 10, 25, 50 or 100 μg/ml GFE for 48 h and the number was counted. Values are means ± S.E. of 4 samples.

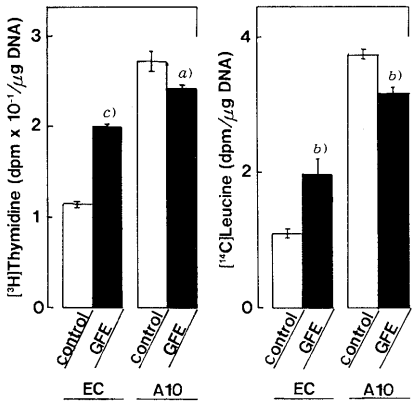


Fig. 2. Effect of 25 μg/ml GFE on the Incorporation of [$^3$H]Thymidine and [$^{14}$C]leucine into 5% TCA-Insoluble Fraction of the Layers of Endothelial Cells and A10 Cells

Endothelial cells or A10 cells were cultured for 72 h and labeled with [$^3$H]thymidine and [$^{14}$C]leucine during the last 3 h of the culture. Values are means ± S.E. of 5 samples. Significantly different from the corresponding control, *a*) $p<0.05$; *b*) $p<0.01$; *c*) $p<0.001$. EC, endothelial cells; A10, A10 cells.

stimulates the prolifer ation of cultured endothelial cells from the bovine aorta.[5] However, it is essential when evaluating GFE as a useful drug that GFE stimulates the proliferation of endothelial cells selectively, since an increase in the proliferation of vascular smooth muscle cells may induce an arteriosclerotic lesion. GFE increased the number of cultured endothelial cells after 48 h and above cultivation,[5] so that the effect of GFE on A10 cell proliferation was evaluated after 48 or 72 h of cultivation in the present study. Figure 1 shows the effect of GFE on the number of vascular smooth muscle A10 cells. It was revealed that GFE at 100 μg/ml and below did not enhance the number of A10 cells. Thus, it was suggested that GFE-stimulated proliferation may occur selectively on endothelial cells.

Figure 2 shows the effect of GFE on the incorporation of [$^3$H]thymidine and [$^{14}$C]leucine by either endothelial cells or A10 cells. GFE significantly increased both incorporations by endothelial cells, suggesting that GFE stimulated both DNA and protein syntheses by the cells. This result is consistent with our previous report,[5] and warranted the quality of GFE used in the present study. In contrast, GFE significantly decreased the incorporation of both [$^3$H]thymidine and [$^{14}$C]leucine by A10 cells. Although the mechanism by which GFE decreased them is unclear, this GFE effect may be desirable for the prevention

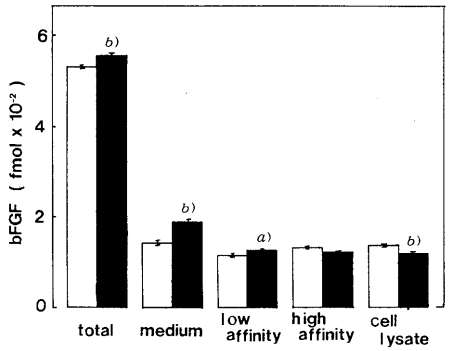


Fig. 3. Effect of 25 μg/ml GFE on the Content and Distribution of bFGF in Cultured Endothelial Cells

□, control; ■, GFE. Endothelial cells were cultured for 72 h in the presence of 25 μg/ml GFE. Values are means ± S.E. of 4 samples. Significantly different from the corresponding control, *a*) $p<0.05$; *b*) $p<0.01$.

of arteriosclerosis. At any rate, it was therefore postulated that GFE stimulates the proliferation of endothelial cells but does not enhance that of vascular smooth muscle cells.

It appears to be important to clarify the mechanism by which GFE stimulation occurred selectively on endothelial cell proliferation. Previously, we showed that AFE also selectively stimulated the proliferation of endothelial cells.[6] In that report, we showed that the stimulatory effect of AFE on endothelial cell proliferation may result from an increased production of basic fibroblast growth factor (bFGF) which is an autocrine of the cells.[9,10] However, we also described that A10 cells may have bFGF receptors[11] but did not produce a detectable amount of bFGF, so that the proliferation of the cells would fail to be stimulated by AFE. We examined the effect of GFE on bFGF production in the endothelial cell cultures, and found that GFE, as well as AFE,[6] significantly increased the production (Fig. 3): GFE significantly increased bFGF in both the medium and low-affinity fractions. Since the bFGF in these fractions can be a source of supply of the factor to the cells,[12] GFE-induced bFGF production could cause an enhanced proliferation of endothelial cells.

As shown in the present study, the effect of GFE on cell proliferation was almost similar to that of AFE.[6] Both GFE and AFE stimulated the proliferation of endothelial cells but not of A10 cells. The production of bFGF by endothelial cells was increased by either GFE or AFE. In the previous study, we showed that the active component of both GFE and AFE occurred in the low-molecular-weight fraction ($\leqq$10000 dalton).[4,5] In addition, both GFE and AFE are hot water extracts. We therefore speculate that the active component of GFE and AFE would be a heat-resistant and low-molecular-weight substance which has a similar structure, for instance, glycosaminoglycans. Heparin, a glycosaminoglycan, is known to potentiate the effect of endothelial cell growth factor[13] but inhibits the proliferation of vascular smooth muscle cells.[14] Thus, the structural elucidation is under-performing.

In conclusion, it was shown that GFE did not stimulate the proliferation of cultured vascular smooth muscle cells. In other words, GFE-stimulated proliferation occurred selectively on endothelial cells. This selective effect may be related to the bFGF production capacity of each cell line rather than to their original species. The effect of GFE on the proliferation of endothelial cells and A10 cells

was similar to that of AFE.[4,6] It was therefore suggested that GFE as well as AFE contains a beneficial component in treating arteriosclerosis and thrombosis.

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# The Measurement of *cis*-Platin and *trans*-Platin by Graphite Atomic Absorption Spectrophotometry

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*Received July 23, 1990*

**In order to determine a micro amount of *cis*- and *trans*-platin (*cis*- and *trans*-diamminedichloroplatinum(II)) separately, we have utilized the *trans* effect of $SCN^-$.**

***cis*-Platin reacted with potassium thiocyanate to give anionic $[Pt(SCN)_4]^{2-}$ which was extracted into nitroethane as an ion pair with zephiramine. On the other hand, *trans*-platin gave neutral *trans*-$[Pt(SCN)_2(NH_3)_2]$ under the same conditions, which was not extracted into nitroethane. Therefore, it is possible to determine *cis*- and *trans*-platin, separately, by the solvent extraction method. In the determination of the mixture of *cis*- and *trans*-platin ($4.00 \times 10^{-6}$ and $2.67 \times 10^{-6}$ M, respectively), the recovery of each platinum complex was $94 \pm 4$ and $101 \pm 10\%$ for *cis*- and *trans*-platin, respectively.**

**Keywords** graphite atomic absorption spectrophotometry; platinum(II) complex; separatory determination; solvent extraction; *cis*-platin; *trans*-platin

In the synthesis of *cis*-platin, there is a possibility that *trans*-platin contaminates *cis*-platin. It is very difficult to determine *cis*-platin from *trans*-platin because their physical and chemical characters are very similar to each other. Recently, the geometric isomers of platinum complexes were separated by thin-layer chromatography.[1] Therefore, we attempted to determine *cis*- and *trans*-platin, separately, by the solvent extraction method.

Various methods for the quantitative analyses of platinum complexes were reported. Graphite atomic absorption spectrophotometry (AAS) is considered to be one of the best tools for platinum determination. The solvent extractions of the $[PtI_6]^{2-}$,[2a,b] $[Pt(SCN)_6]^{4-}$ [2c] were reported, in the presence of zephiramine with chloroform, followed by plasma atomic emission spectrometry,[2a] graphite atomic absorption spectrometry[2b] and spectrophotometry.[2c] However, the extraction of $[PtCl_4]^{2-}$ with chloroform was unsuccessful, even in the presence of zephiramine. We attempted to establish a method to determine *cis*- and *trans*-platin, separately, based on the idea that *cis*- and *trans*-platin give different products, *i.e.*, $[Pt(SCN)_4]^{2-}$ and $[Pt(SCN)_2(NH_3)_2]$, respectively, in reaction with $SCN^-$.

## Materials and Methods

**Reagents** Potassium tetrachloroplatinate(II), zephiramine, and nitroethane were used without further purification. Nitroethane was presaturated with water to prevent a change in the volume of the organic phase in the extraction processes. *cis*-, *trans*-Platin (*cis*-, *trans*-$[PtCl_2(NH_3)_2]$) and potassium tetrathiocyanatoplatinate(II)($K_2[Pt(SCN)_4]$) were synthesized in our laboratory, according to the references.[3] *trans*-Diamminedithiocyanatoplatinum(II)(*trans*-$[Pt(SCN)_2(NH_3)_2]$) was synthesized by the following procedures. An aqueous solution of *trans*-platin ($1.2 \times 10^{-3}$ M: 10 ml) was added to 0.1 ml of 1.0 M KSCN solution and the mixture was left at 60 °C for 1 h. The solution was concentrated to 1/3 by a flash evaporator and allowed to stand overnight. The *trans*-$[Pt(SCN)_2(NH_3)_2]$ crystals were collected and recrystallized from water.

Phosphate–borate buffer solutions of pH 1.5—9.0 (0.3 M $KH_2PO_4$ and 0.15 M $Na_2B_4O_7 \cdot 10H_2O$) were used in the experiment.

**Apparatus** A Shimadzu atomic absorption spectrophotometer, AA-670G, equipped with a graphite furnace atomizer GFA-4A was used for the measurement of platinum. The measurement of graphite atomic absorption spectrophotometry was set up under the following conditions.

| | Temp. (°C) | Time (s) |
|---|---|---|
| stage 1 (dry) | 150 | 50 |
| stage 2 (ashing) | 600 | 40 |
| stage 3 (atomization) | 2700 | 5 |

**The Pretreatment of *cis*-Platin with KSCN** A half ml of platin solution ($10^{-3}$ M) and 0.1 ml of 1.0 M KSCN solution were added to 8.9 ml phosphate–borate buffer of pH 7.4 and the mixture was increased to 10 ml with water. The mixture was reacted on a water bath at 60 °C for 3 h. The reaction solution was diluted seven times by the phosphate–borate buffer and then was submitted to the extraction.

**The Extraction Procedure** To 35 ml of the aqueous solutions containing $[Pt(SCN)_4]^{2-}$ (0—$1.60 \times 10^{-5}$ M) or *cis*- and *trans*-platin (about $7.7 \times 10^{-6}$ M) pretreated by KSCN were added 10 ml of phosphate–borate buffer (pH 2.0) and 5 ml of 0.01 M zephiramine. The mixture was extracted with 10 ml of nitroethane for 10 min, followed by centrifugation. The lower organic layer was diluted twice with nitroethane and the platinum content in the organic phase was determined by means of graphite AAS. The platinum content in the aqueous phase was also measured by using $K_2[PtCl_6]$ (0—$1.60 \times 10^{-5}$ M in 0.1 N HCl) solution as the standard.

## Results and Discussion

**Effect of pH on the $K_2[Pt(SCN)_4]$ Extraction** Thirty-five mililiters of $K_2[Pt(SCN)_4]$ solution ($1.43 \times 10^{-5}$ M) at

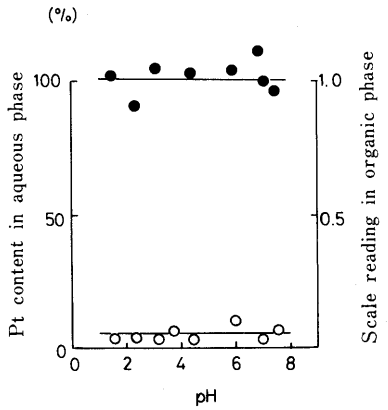


Fig. 1. The pH Dependence on the Extraction of $[Pt(SCN)_4]^{2-}$ with Zephiramine

$K_2[Pt(SCN)_4]$ solutions ($1.43 \times 10^{-5}$ M) in various pHs were extracted into nitroethane by zephiramine. ○, Pt content remained in aqueous phase; ●, Pt content extracted into nitroethane by zephiramine.

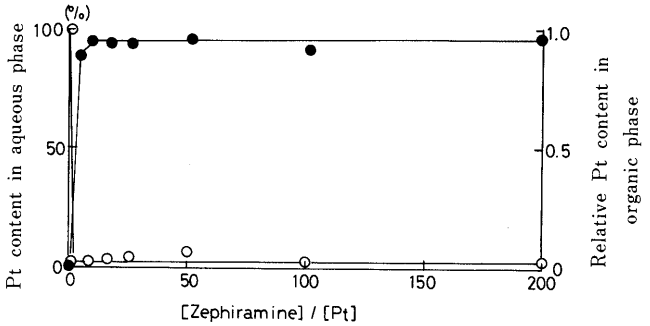


Fig. 2. Effect of Zephiramine Concentration on the $[Pt(SCN)_4]^{2-}$ Extraction

$K_2[Pt(SCN)_4]$ ($1.43 \times 10^{-5}$ M) solutions at pH 2.0 were extracted into nitroethane by various concentrations of zephiramine (0—$2.86 \times 10^{-3}$ M). ○, Pt content remained in aqueous phase; ●, Pt content extracted into nitroethane by zephiramine.

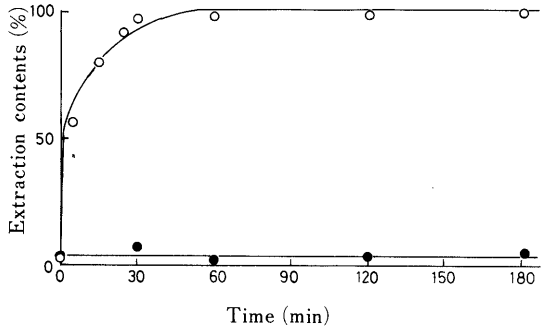


Fig. 3. Effect of Incubation Time on the Reaction of *cis*- and *trans*-Platin with KSCN at 60 °C

Platin derivatives ($10^{-4}$ M) were reacted with 0.01 M KSCN in phosphate buffer (pH 7.4). ○, *cis*-platin; ●, *trans*-platin.

various pHs (phosphate–borate buffer) were sumitted to the standard extraction procedure. Figure 1 shows the platinum contents in the organic and aqueous phases in a pH range from 1 to 8.

Only a small percent of platinum contents remained in the aqueous phases after the extraction. The results indicated that $[Pt(SCN)_4]^{2-}$ is nearly completely extractable into the organic phase as an ion pair with zephiramine. We decided to use pH 2.0 phosphate–borate buffer in this experiment.

**Effect of Zephiramine Concentration on the $K_2[Pt(SCN)_4]$ Extraction** Using phosphate–borate buffer of pH 2.0 and 35 ml of $K_2[Pt(SCN)_4]$ ($1.43 \times 10^{-5}$ M), zephiramine ratios to $K_2[Pt(SCN)_4]$ were changed from 0 to 200 fold. Figure 2 shows that the extraction was almost complete when zephiramine was present in more than a 10 fold excess to the platinum complex.

We decided to use 100 fold of zephiramine to the platinum complex.

**Calibration Curve of $K_2[Pt(SCN)_4]$ Determination** Various concentrations of $[Pt(SCN)_4]^{2-}$, *i.e.*, 0, $4 \times 10^{-6}$, $8 \times 10^{-6}$, $12 \times 10^{-6}$, and $16 \times 10^{-6}$ M in phosphate–borate buffers at pH 2.0 were submitted to the extraction procedure under the same conditions and the platinum contents in nitroethane were measured by graphite AAS. It showed a good linearity in the range of 0—$1.6 \times 10^{-5}$ M. This calibration curve was used for determination of the *cis*-platin.

*trans*-Platin in an aqueous phase was determined from the standard curve (0—$1.6 \times 10^{-5}$ M) prepared from a commercially available AAS standard solution of platium.

TABLE I. Separatory Determination of a Mixture of *cis*-Platin and *trans*-Platin ($n=5$)

| | *cis*-Platin | *trans*-Platin |
|---|---|---|
| Added Pt (M) | $4.00 \times 10^{-6}$ | $2.67 \times 10^{-6}$ |
| Found value (M) | $(3.74 \pm 0.15) \times 10^{-6}$ | $(2.66 \pm 0.25) \times 10^{-6}$ |
| Recovery (%) | $94 \pm 4$ | $101 \pm 10$ |

**Effect of Reaction Time of *cis*-Platin and *trans*-Platin with KSCN** The reaction was carried out at 60 °C and the reaction times were varied from 0 to 180 min (Fig. 3).

In the case of *cis*-platin, the extraction was almost complete after 30 min, while *trans*-platin was not extracted even after 180 min, as shown in Fig. 3. The explanation of this is that *cis*-platin was completely substituted with thiocyanate ions to afford $[Pt(SCN)_4]^{2-}$, which is extracted into nitroethane in the presence of a cationic surfactant, zephiramine, as an ion association complex. However, *trans*-platin was only partially substituted with thiocyanate ions to afford neutral *trans*-$[Pt(SCN)_2(NH_3)_2]$ which is not extractable with nitroethane. The difference in behavior between *cis*- and *trans*-platin can be explained by the *trans* effect and we are able to determine *cis*-platin and *trans*-platin, separately.

**Identification of Reaction Products of *cis*-Platin and *trans*-Platin with KSCN** In order to identify the formation of $[Pt(SCN)_4]^{2-}$ or $[Pt(SCN)_2(NH_3)_2]$ from *cis*- or *trans*-platin, respectively, in the presence of KSCN (the same condition as Fig. 3), the absorption spectra were measured with respect to time.

The spectra of *cis*- and *trans*-platin in the presence of KSCN were changed with time and their final spectra were completely consistent with those of authentic samples of $[Pt(SCN)_4]^{2-}$ and $[Pt(NH_3)_2(SCN)_2]$, respectively.

**Separatory Determination of a Mixture of *cis*-Platin and *trans*-Platin** The mixture of *cis*- and *trans*-platin was analyzed by the solvent extraction method to determine the geometrical isomers based on the measurments of platinum contents in nitroethane and aqueous phases after pretreatment with KSCN at 60 °C. These results are shown in Table I.

*trans*-Platin was not extractable with nitroethane and remained in an aqueous phase, while *cis*-platin was extracted with nitroethane. The recoveries of *cis*- and *trans*-platin were $94 \pm 4$ and $101 \pm 10$%, respectively.

**Acknowledgments** We would like to thank Misses Shinobu Kawashima, Yoko Shimizu, and Akiko Sugiyama for their helpful cooperation in this experiment. Thanks are also due to Grants-in-Aid from the Ministry of Education, Health and Welfare.

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# Chemiluminescence Assay of *N*-Acetyl-β-D-glucosaminidase in Urine Using *o*-Aminophthalylhydrazido-*N*-acetyl-β-D-glucosaminide

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**The chemiluminescence assay of urinary *N*-acetyl-β-D-glucosaminidase using *o*-aminophthalylhydrazido-*N*-acetyl-β-D-glucosaminide as the substrate was examined. Under optimized conditions, a linear chemiluminescence response to the standard *N*-acetyl-β-D-glucosaminidase (NAGase) of different concentrations was observed with a correlation coefficient of 0.999 over the range of 0.3 to 20 I.U./l. The present method was compared with the prevalent method using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in the assay of 20 urine samples from healthy subjects, and the correlation coefficient of 0.891 was obtained.**

**Keywords** *N*-acetyl-β-D-glucosaminidase; urine; *o*-aminophthalylhydrazido-*N*-acetyl-β-D-glucosaminide; chemiluminescence assay; chemiluminogenic substrate

Urinary *N*-acetyl-β-D-glucosaminidase (NAGase) is well known as a sensitive parameter for renal damage.[1] Synthetic substrates reported so far for the assay of this enzyme include chromogenic[2] and fluorogenic[3] compounds, with which the enzyme activity can be determined by monitoring a released aglycon spectrophotometrically. Since chemiluminescent enzyme assay has been receiving much attention in connection with enzyme immunoassay, we recently synthesized a luminol analogue, *o*-aminophthalylhydrazido-*N*-acetyl-β-D-glucosaminide[4] (**1**, Chart 1) and demonstrated that it could serve as a substrate for NAGase by liberating luminol upon an enzymatic hydrolysis, as outlined in Chart 1. NAGase was selected as an enzyme to demonstrate this enzyme-triggered chemiluminescence because the introduction of an enzyme-removable *N*-acetyl-D-glucosaminide group to the hydrazide portion of luminol was most readily achieved under basic conditions.

In order to examine the potential of compound **1** as a substrate for NAGase, we undertook the study of chemiluminescence assay of urinary NAGase using compound **1**, and wish to describe herein our preliminary results.

## Experimental

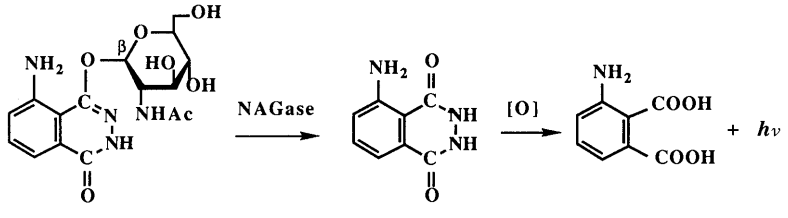
**Apparatus** Chemiluminescences were detected by a photoncounter (Hamamatsu Photonics, Model C-1230) equipped with a photomultiplier (Hamamatsu Photonics, Model R 464) which was jacketed with a cooling device (−20 °C) to minimize a dark current. Chemiluminescent reactions were performed on a micro slide that was placed close to and face to face to the photomultiplier in a dark box.

**Materials** Substrate **1** was synthesized as described in a previous paper.[4] NAGase (56 I.U./ml, from jack beans) was purchased from Sigma (St. Louis, MO, U.S.A.) and diluted with water before use. The activity was re-evaluated by the conventional method using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (*p*NP-NAG) as the substrate.[2d] Peroxidase (POD, from horseradish) was from Sigma as a crystalline solid (200 I.U./mg) and a solution of 1 mg/dl in water was freshly prepared prior to use. α,β,γ,δ-Tetraphenylporphine tetrasulfonic acid (TPPS) was obtained from Dojindo Laboratories (Kumamoto, Japan) and was complexed with Fe(III) (TPPS–Fe(III)) by refluxing TPPS with an equivalent of $FeCl_3$ hexahydrate in water for 3 h, which resulted in precipitation. Other chemicals were of reagent grade.

**Assay Procedure** Urine samples were collected from ostensibly healthy adults (age 23 to 38) and assayed within 2 h of collection. A 10 μl of NAGase of a given concentration or 100 μl of a urine sample was added to a pre-incubated 0.1 M citrate buffer solution (pH 6.2, 1 ml) containing substrate **1** (0.21 μmol), and the mixture was incubated at 37 °C for 10 min. A 0.1 M $H_3BO_3$–0.2 M KOH buffer (pH 12.8, 5 ml) was then added to terminate the enzymatic reaction. A 10-μl aliquot of this solution and 10 μl of TPPS–Fe(III) (0.4 μM $H_2O$) were placed in a single spot on a micro slide *via* a fixed microsyringe. Chemiluminescent reaction was initiated by adding 10 μl of hydrogen peroxide (0.01%) to this spot *via* the microsyringe, and the resulting chemiluminescence was measured for 2 min as integrated counts taken each 10 s.

## Results and Discussion

Chart 1 outlines the principle of chemiluminescent NAGase assay using substrate **1**. Assuming that the enzymatic reaction of substrate **1** with NAGase follows the Michaelis–Menten rate law and that the concentration of the substrate is much larger than its $K_m$ value, the rate of the luminol release (d[luminol]/d$t$) on the hydrolysis is the product of a catalytic rate constant ($k_{cat}$) and a total enzyme concentration ($[E]_t$), whereas a total light emission in the subsequent chemiluminescent reaction can be expressed as an initial concentration of luminol ([luminol]) multiplied by a chemiluminescence quantum yield ($\Phi_{CL}$). Then, the total light emission should be proportional to the product of $[E]_t$ and the incubation time. We therefore expected a linear relationship between the total chemiluminescence light emission and the enzyme activity to be determined. The enzymatic reaction of substrate **1** with NAGase




**1** **luminol** **3-aminophthalic acid**

Chart 1. Chemiluminescent NAGase Assay Using Substrate **1**

proceeds in a weakly acidic pH, whereas the subsequent chemiluminescent oxidation with the released luminol in the presence of hydrogen peroxide and a catalyst usually requires an alkaline condition. Thus, the assay with substrate **1** consists basically of two steps (an end-point assay procedure). After the enzymatic reaction, the pH needs to be adjusted upward (>pH 12) for the subsequent chemiluminescent oxidation, which simultaneously inactivates the enzyme. In order to optimize these reaction conditions, we first investigated the effect of pH on the activity in the enzymatic hydrolysis and the effect of the concentration of hydrogen peroxide and of TPPS–Fe(III) complex (used as a catalyst) in the subsequent chemiluminescent reaction.

Figure 1 shows a pH profile of the enzymatic reaction of substrate **1** with NAGase. Since a maximum chemiluminescence was observed at pH 6.2, which seemed slightly higher than the optimum pH of NAGase (pH 4—5), we fixed the pH of the enzymatic reactions at 6.2 thereafter. An optimum concentration of hydrogen peroxide was found to be 0.01% and that of TPPS–Fe(III) complex was $4.0 \times 10^{-7}$ M. Varying the substrate concentration under these conditions gave a Michaelis–Menten type kinetics curve, which led to a relatively small $K_m$ value of 0.20 mM from its Lineweaver–Burk plots.

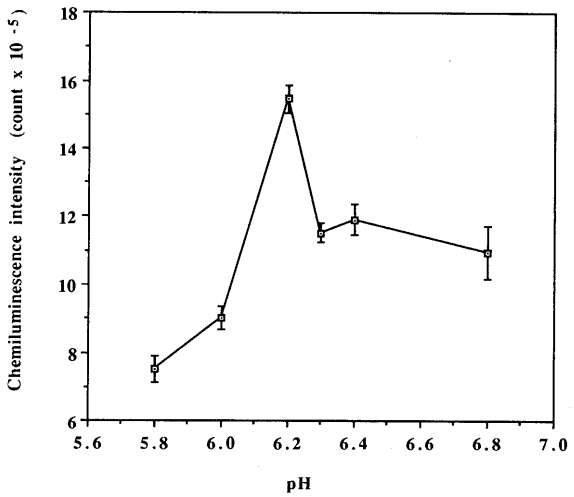



Fig. 1. pH Profile of the Enzymatic Reaction of Substrate **1** with NAGase

Reactions were carried out in the manner described in the Experimental section, except for the incubation pHs that were adjusted with 0.1 M citrate buffers. Each point and bar shows the mean and standard deviation ($n=5$).

Employing these optimum conditions, we intended to achieve a quantitative determination of standard NAGase (Fig. 2). A linear response of the chemiluminescence to the NAGase activity, determined independently by the *p*NP-NAG method, a prevalent colorimetric method for the NAGase assay,[2d] was observed over the range of 0.3 to 20 I.U./l. The detection limit of 0.3 I.U./l is the same order as that of *p*NP-NAG method[2d] under identical enzymatic reaction conditions. Linear regression analysis of the data yielded regression coefficients (for the equation $y=a+bx$) as follows: $a=0.131 \times 10^6$; $b=1.007 \times 10^6$. The correlation coefficient ($r$) was 0.999. Consequently, NAGase activity can be computed from the observed counts by the following equation:

$$\text{NAGase activity (I.U./l)} = \text{chemiluminescence (count)} \times 9.933 \times 10^{-7} - 0.131$$

The intercept is due to a chemiluminescence background which is presumably caused by a non-enzymatic hydrolysis. Since it was difficult to know the exact amount of the released luminol, which would enable us to calculate the activity (by the definition of the rate of the luminol release in μmole per minute), this empirical equation was accordingly employed for the determination of the NAGase activity in urine samples.

We then attempted to apply this method to urinary NAGase assay (20 urine samples). The results are shown in Fig. 3, in which the present method is compared with the pNP-NAG method. The addition of urine gave rise to a lower chemiluminescence intensity than the background chemiluminescence ($CL_{BKG}$) with only hydrogen peroxide and TPPS–Fe(III) complex, presumably due to decreased catalytic activity in the presence of urine.[5] All urine blanks observed ($CL_{UB}$) were therefore corrected with the background chemiluminescence ($CL_{UB}-CL_{BKG}<0$). The present method which determines the activity based on the

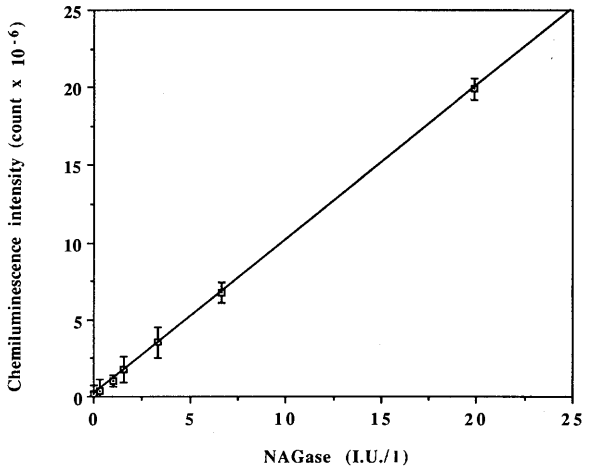



Fig. 2. Chemiluminescent Response of Substrate **1** to the Increasing Concentration of NAGase

Reactions were carried out as described in the Experimental section. Each point and bar shows the mean and standard deviation ($n=5$).

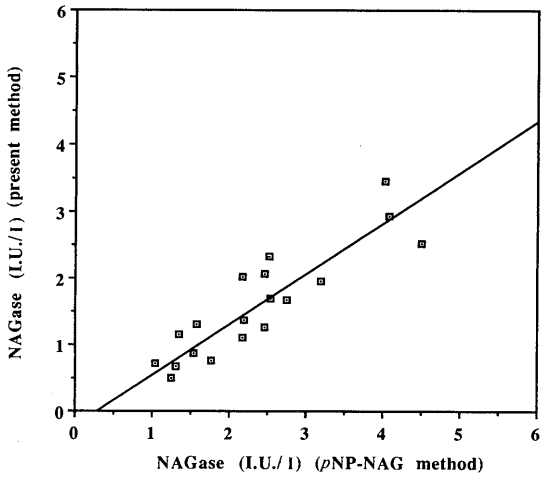



Fig. 3. Comparison of the Present Method with *p*NP-NAG Method in the Urinary NAGase Assay

Reactions were carried out in an identical fashion as described in the Experimental section. $y=-0.23821+0.76271x$ ($r=0.891$).

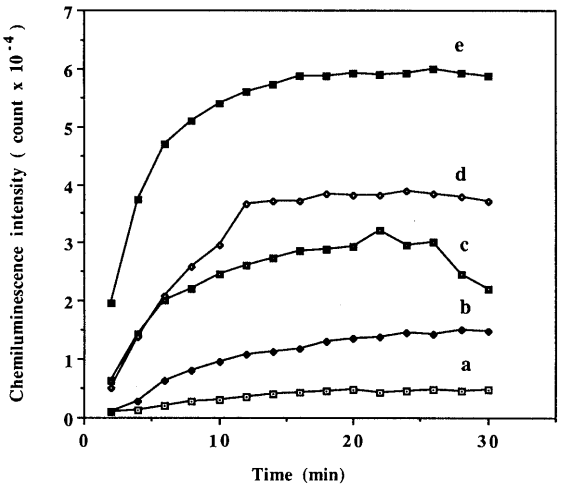



Fig. 4. Time Course of the Enzyme-Triggered Chemiluminescent Reaction with POD as a Catalyst at pH 6.2

The reactions were performed as described in the Experimental section with NAGase at various units. NAGase (I.U./l): a, 0.33; b, 0.99; c, 1.65; d, 3.31; e, 6.62.

above equation gave somewhat lower activity than that by *p*NP-NAG method. A linear correlation with a correlation coefficient of 0.891, however, appeared to suggest some validity of this method in urinary NAGase assay.

We next turned our attention to chemiluminescence assay of NAGase utilizing POD as a catalyst. With POD, the chemiluminescent reactions of the luminol system can be potentially performed at a neutral pH, which could minimize the problems of high background, autooxidation of luminol or spontaneous decomposition of peroxide that are encountered under basic conditions. The reactions were carried out as follows: Hydrogen peroxide (0.01%, 25 $\mu$l) was added to a 0.1 M citrate buffer solution (pH 6.2, 0.5 ml) containing substrate **1** (0.21 $\mu$mol), POD (1 mg) and NAGase (0—20 $\mu$l); the chemiluminescence, which occurred almost instantly, was measured for 30 min as integrated counts over a 10-s interval (Fig. 4). The chemiluminescence intensities were proportional to the amount of enzyme added. The linear regression analysis, however, gave a low value of correlation coefficient ($r=0.857$) as well as large variation in the data of the chemiluminescence intensity measured for 10 min; no higher $r$ value could be obtained even by varying the time range measured. This might be largely because of poor reproducibility of these data and complex reaction kinetics due to a possible interaction between NAGase and POD activities. Quantitative assay of urinary NAGase under these conditions was not further attempted.

In summary, feasibility of conducting urinary NAGase assay by chemiluminescence detection was demonstrated using chemiluminogenic substrate **1**. However, only an inferior correlation was obtained in the urinary assay, although the chemiluminescence correlated very closely with the standard NAGase. Increasing the chemiluminescence efficiency of the hydrazide aglycon could provide a solution to this problem.

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# Co-suppression by Nicardipine, a Calcium Antagonist, of Induction of Microsomal Lauric Acid Hydroxylation with Peroxisome Proliferation in Clofibrate-Treated Rat Liver

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**The *in vivo* effect of nicardipine, a well-known calcium antagonist, on microsomal ω-oxidation of laurate in clofibrate-treated rat liver was studied. The 15.3-fold induction of the activity by 2 weeks administration of 0.25% clofibrate in the diet was markedly suppressed to about 6-fold by co-administration of nicardipine at 100 mg/kg body weight. Similarly, the induction of peroxisomal β-oxidation and carnitine acetyltransferase activities were also suppressed by this simultaneous administration by more than 50%. Although clofibrate also induced the activity of reduced nicotineamide adenine dinucleotide phosphate (NADPH)–cytochrome c reductase and increased the hepatic content of cytochrome P-450, no suppressive effect of nicardipine was observed. Contrarily, nicardipine induced the reductase activity and increased the hepatic content of cytochromes P-450 and $b_5$. These results provide the first demonstration of a calcium antagonist, *e.g.* nicardipine acting as inhibitor of the induction of microsomal ω-oxidation, in association with the inhibition of peroxisome proliferation in animals. The suppression of drug-induced peroxisome proliferation and microsomal ω-oxidation by the calcium antagonist may help in elucidating the causal relationship of the induction mechanisms between peroxisomal and microsomal enzymes.**

**Keywords** peroxisome proliferation; ω-oxidation; laurate hydroxylase; fatty acid oxidation; co-suppression; calcium antagonist

The effect of hypolipidemic drugs on rat liver has been extensively studied in terms of morphometric and biochemical responses in peroxisomes.[1-4] Certain hypolipidemic drugs such as clofibrate (CL) and its derivatives induce hepatomegaly and the proliferation of hepatic peroxisomes, in association with the induction of peroxisomal fatty acid oxidation-related enzymes.[5-7] These are classified as non-mutagenic hepatocarcinogens.[8-10] Thus, induction or inhibition of enzymes by drugs is important to evaluate their efficacy and toxicity. Many recent studies showed that peroxisome proliferators induced microsomal ω-oxidation activity of fatty acids through the induction of a cytochrome P-450 isozyme termed P-450 IVA1 (formerly termed cytochrome P-452), which has an unusually narrow substrate specificity for the terminal (or ω) hydroxylation of fatty acids including lauric acid.[11-13] From the finding of co-induction of peroxisomal fatty acid oxidation activity with microsomal lauric acid hydroxylation activity, it has been postulated that these responses are based on a mechanistic interrelationship. In a previous report, we first reported that CL-induced peroxisome proliferation was suppressed by calcium antagonists such as nicardipine and suggested that a calcium-related mechanism might participate in the process of peroxisome proliferation.[14,15] In this report, we examined whether the induction of microsomal lauric acid hydroxylation activity by CL was suppressed by nicardipine (NC) in rat liver, in an attempt to clarify the mechanistic interrelationship between such induction and the peroxisome proliferation.

## Experimental

Male Wistar rats weighing about 120 g were divided into 4 groups (5 animals/group), fed standard laboratory diet (CE-2, Clea Japan) and served drinking water *ad lib.* Groups 2 and 3 were orally administered 0.25% clofibrate (CL) in the diet and nicardipine at 100 mg/kg body weight (b.w.) daily for 2 weeks suspended in 0.5% methylcellulose (1 ml/100 g b.w.), respectively. Group 4 was simultaneously administered the above quantities of clofibrate and nicardipine. The control group (group 1) received an equal volume of the same vehicles. After treatment the livers were removed and 10% (w/v) homogenates were prepared in 0.25 M sucrose–1 mM ethylenediamine tetraacetic acid (EDTA)–10 mM Tris–HCl (pH 7.4). The homogenate was centrifuged at $10000 \times g$ for 20 min at 4 °C, then at $105000 \times g$ for 20 min and the resulting pellet was suspended in 0.25 M sucrose at 0.2 g original tissue/ml. The suspension was used as a microsomal fraction for assay of the microsomal enzyme activities and the content of cytochromes P-450 and $b_5$ content. The activity of the cyanide-insensitive fatty acyl-CoA oxidizing system (FAOS) was determined using palmitoyl-CoA as substrate.[16] The activity of carnitine acetyltransferase (CAT) was determined using acetyl-CoA as substrate.[17] Unless otherwise stated, 1 unit of each enzyme activities was defined as the amount of the enzyme that produced 1 nmol of reaction product per min per g of liver. The activity of microsomal laurate hydroxylase (LH) was determined using (1-$^{14}$C)lauric acid as substrate.[18] Aminopyrine demethylase (AD) and aniline hydroxylase (AH) activities were determined by the method of Mazel.[19] Reduced nicotinamide adenine dinucleotide phosphate (NADPH)–cytochrome c reductase (Cyt. C-R) activity was determined by the method of Beaufay *et al.*[20] Cytochromes P-450 and $b_5$ contents were determined by the method of Omura and Sato,[21] and protein content was determined by the method of Lowry *et al.*[22] using bovine serum albumin as a standard.

## Results

The effects of *in vivo* administration of NC on body weight gain, liver weight and hepatic protein content of normal and CL treated rats are summarized in Table I. The body weight gain of the group administered both NC and CL decreased slightly compared with the control, whereas no significant alteration was observed in the groups treated with each drug separately. Hepatomegaly was observed in all of the treated groups. There was no significant difference in the liver protein among the groups. Table II shows the effects of drugs on microsomal enzyme activities. Although the activity of AD was decreased to 70% of the control by CL, no significant change was observed in the NC- and NC plus CL-treated groups. No significant change in AH activity was observed in any groups compared with the control. Cytochrome c reductase activity was increased 1.5-fold and 1.9-fold in NC- and CL-treated groups, respectively, and 2.11-fold in the group treated with both drugs. The hepatic content of total cytochrome P-450 species was increased 2-fold in NC- and CL-treated groups, and

TABLE I. *In Vivo* Effect of Nicardipine (NC) on Body Weight Gain, Liver Weight and Hepatic Protein Content

| | Control | CL | NC | CL+NC |
|---|---|---|---|---|
| Body weight gain (+g) | 103± 8 | 97± 8 | 82±22 | 83± 7 |
| Liver weight (% of body weight) | 4.3±0.1 | 5.6±0.6[a] | 5.2±0.5[a] | 5.7±0.3[a] |
| Liver protein (mg/g) | 205±15 | 203±15 | 197±23 | 223±11 |

Experimental conditions are described in Experimental. Each value is the mean±S.D. of 5 rats. Statistical evaluation was performed by Student's *t*-test: *a*) $p<0.05$. *vs.* control.

TABLE II. *In Vivo* Effect of Nicardipine (NC) on Some Peroxisomal and Microsomal Enzymes of Normal and Clofibrate (CL)-Treated Rat Liver

| | Control | CL | NC | CL+NC |
|---|---|---|---|---|
| Peroxisomal | | | | |
| FAOS | 728± 50 | 5871± 740[a] | 660± 80 | 2214± 384[a,b] |
| CAT | 299± 81 | 17882±3848[a] | 672± 66[a] | 7555±1387[a,b] |
| Microsomal | | | | |
| LH | 11± 4 | 169± 39[a] | 10± 1 | 65± 29[a,b] |
| AD | 129± 17 | 91± 16 | 109± 26 | 127± 37 |
| AH | 47± 5 | 37± 11 | 42± 8 | 47± 13 |
| NADPH-Cyt. C-R | 2328±255 | 3586± 302[a] | 4426±600[a] | 5578±766[a] |
| Cytochrome P-450 | 55± 16 | 110± 30[a] | 109± 18[a] | 158± 44[a] |
| Cytochrome $b_5$ | 41± 4 | 36± 8 | 53± 8[a] | 50± 7[b] |

Experimental conditions are described in Experimental. Each value is the mean±S.D. of 5 rats. Statistical evaluation was performed by Student's *t*-test: *a*) $p<0.05$ *vs.* control; *b*) $p<0.05$ *vs.* clofibrate. The activities of enzyme activities are expressed as nmol/min/g liver; cytochromes P-450 and $b_5$ contents, nmol/g liver.

the increase was enhanced 3-fold by the simultaneous administration of the two drugs. The ω-oxidation activity of the control group was 11.0±4.4 U/g liver. With CL administration the activity was increased by 15.3-fold, however, NC suppressed the increase to about 6-fold.

## Discussion

The administration of peroxisome proliferators induced the activities of microsomal LH,[11-13] cytosolic epoxidehydrolase[23] and acyl-CoA hydrolase[24] together with the peroxisomal β-oxidation in liver of rats and mice. Although these results suggest that a similar biochemical mechanism might participate in the induction process of these enzymes housed in different subcellular compartments, the detailed mechanism has not yet been clarified. Studies of the mechanism of ω-oxidation induction by drugs, such as hypolipidemic peroxisome proliferators reveal that agents with the ability to inhibit this induction must be a useful tool. Although some drugs are known to be inhibitors of peroxisomal β-oxidation,[25,26] to the author's there has to date been no report concerning drugs which can inhibit microsomal ω-oxidation induction *in vivo*. Our present experiments demonstrate for the first time that the calcium antagonist, NC, when administered simultaneously with CL *in vivo*, suppresses the induction of ω-oxidation activity by the latter drug (Table II). In the present experiments we chose a dose of 100 mg/kg b.w. However, in our previous report the suppression of peroxisomal enzyme induction by NC was dose dependent in the range of 20—100 mg/kg. A 2-week time course study of the induction of peroxisomal enzymes when CL and NC were administered together showed the suppressive effect of NC on the CL-induced increase in the activity of an enzyme such as the cyanide-insensitive fatty acyl-CoA oxidizing system (FAOS) after 3—5 d treatment.[15] Thus, in the early phase of the treatment with CL there was no significant difference in the peroxisomal enzyme activities between the CL-group and the group treated with CL and NC, and the activity of both groups was increased by about 4-fold of the control. This finding clearly shows that NC does not effect the disposition, *i.e.* the pharmacokinetics of CL. Furthermore the suppressive effect of NC on CL-induced peroxisome proliferation was also found when CL was administered intraperitoneally (data not shown). From these results we concluded that the drug's suppressive effect on enzyme induction might be due to a modulation of the cellular response, *i.e.* the biosynthetic process of the enzymes. The finding that the suppression was present even after 2 weeks of treatment when the inductive potency of CL would be at its height supports this conclusion. The *in vitro* experiment also showed that the inhibitory effect of NC was not due to its direct action on the LH molecule itself (data not shown). Microsomal drug metabolizing enzymes such as AH and AD were not influenced by either CL or NC, and the hepatic content of cytochrome P-450 was slightly increased by both drugs. Compared with those enzymes, ω-oxidation is remarkably unique in character because its response to CL is quite similar to that to peroxisomal enzymes such as FAOS and CAT. Also, the induction was markedly suppressed by NC in a manner similar to that by FAOS and CAT. Although the mechanism of suppression of peroxisome proliferation by calcium antagonist has not been clarified, a similar mechanism might underlie the suppression of induction of ω-oxidation by NC. If this induction was mediated by a specific receptor (binding protein), the calcium antagonists could compete with the drugs, or affect the metabolism of the drugs, causing a decrease in their inducement activities. There might also be another possible explanation: that the induction process is mediated by a calcium-related mechanism. Thus, our present results suggest that a calcium-related mechanism including protein phosphorylation and/or a calmodulin-dependent process might participate during drug-induced ω-oxidation, as suggested in drug-induced peroxisome proliferation. Although the enzyme participating in ω-oxidation is housed in the microsomes, the biosynthesis may be regulated by a mechanism common to that contributing to the regulation of biosynthesis of peroxisomal enzymes.

**Acknowledgement** We are grateful to Miss Y. Senzaki of this department for her excellent technical assistance.

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# The Growth Inhibition of *Streptococcus mutans* by 5′-Nucleotidase Inhibitors from *Areca catechu* L.

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**New 5′-nucleotidase inhibitors named NF-86I, NF-86II were recently isolated from the seeds of *Areca catechu* L. NF-86I and NF86II showed inhibitory effects on the growth of *Streptococcus mutans* MT8148(c) and *Streptococcus mutans* MT6715(g), respectively. In addition, these inhibitors could inhibit insoluble glucan formation from sucrose. NF-86I and NF-86II were found to be polyphenolic substances. Some polyphenols such as tannic acid bind non-specifically to proteins (tannic activity). The 5′-nucleotidase inhibitors that we isolated did not show any such activity. However, the growth inhibitory activity and the inhibitory effect on water-insoluble glucan production were equal to tannic acid. It is therefore considered that these inhibitors bind specifically to the bacterial cell surface. Our findings suggest that the 5′-nucleotidase inhibitors NF-86I and NF-86II may be useful anti-plaque preventing agents.**

**Keywords** *Streptococcus mutans*; 5′-nucleotidase inhibitor; growth inhibition; glucan formation; polyphenol

Several strains of oral streptococci are able to initiate dental plaque formation in both experimental animals and humans.[1,2] *Streptococcus mutans*, one of the potent caries inducers, can colonize on the tooth surface and form dental plaque by water-insoluble glucan synthesized from sucrose.[3] At present, various antibiotics and chemical bacteriocides are being used to prevent bacterial infection, but these agents are too active to prevent dental plaque formation; they disturb the normal bacterial flora of the oral cavity and digestive tract. Therefore, we have been studying to find nontoxic growth inhibitors of *Streptococcus mutans* in plants. These substances are useful as a preventive medicine. Recently, we isolated new 5′-nucleotidase inhibitors, named NF-86I, NF-86II, NPF-86IA, NPF-86IB, NPF-86IIA and NPF-86IIB, from the seeds of *Areca catechu* L.[4] Betel-quid consists of the seeds of *Areca catechu* (betel nuts), and betel-quid chewing has been a common practice in South East Asia and Central Asia from time immemorial. It is well known that 5′-nucleotidase is located primarily in the plasma membrane[5–7] and some inhibitors of membrane-bound enzymes show antitumor and immunopotentiator activity.[8] We have already reported that the 5′-nucleotidase inhibitors showed antitumor activity[9] and were found to be polyphenolic substances. Some polyphenols such as tannic acid have been reported to have strong tannic activity[10] and antibacterial activity.[11] However, tannic acid did not use the growth inhibitor of *Streptococcus mutans* for its tannic activity and toxicity. Tannic acid could not inhibit 5′-nucleotidase activity from snake venom (*Crotalus atrox*) and rat liver membrane.[12] 5′-Nucleotidase inhibitory polyphenols that we have isolated showed almost no tannic activity and low toxicity.[9] In this report, we describe the growth inhibition of *Streptococcus mutans* by the 5′-nucleotidase inhibitory polyphenols.

## Materials and Methods

**Chemicals** 5′-Nucleotidase inhibitors, NF-86I and NF-86II, were prepared from the seeds of *Areca catechu* L. as described in a previous paper.[4] High performance liquid chromatography (HPLC) analysis revealed that NF-86I consisted of NPF-86IA and NPF-86IB at a ratio of 1 to 7 by weight, and NF-86II consisted of NPF-86IIA and NPF-86IIB at a 1 to 1 ratio. ASF medium 104 was purchased from Ajinomoto Co., Inc. Tannic acid was obtained from Dainippon Pharmacy Co., Ltd.

**Bacterial Strains and Growth Condition** *Streptococcus mutans* MT8148 (c) and *Streptococcus mutans* MT6715 (g) were kindly provided by Prof. S. Hamada, Osaka University. The organisms were grown in ASF medium 104 supplemented with 1% glucose at 30 °C.

**Growth Inhibitory Activity** The minimum inhibitory concentrations of the 5′-nucleotidase inhibitors for the bacterial strains used were determined by two-fold dilution in ASF medium 104 supplemented with 1% glucose. The tested compounds did not give turbidity in these media. A volume of 50 $\mu$l of the diluted culture ($OD_{660}=1.2$) of a fresh overnight culture of the test organism was applied to the test tube containing a 10 ml of serial two-fold dilutions of samples. The tubes were incubated at 30 °C for 24 h. The minimum inhibitory concentration was defined as the lowest concentration of the samples that prevented completely visible growth.

**Glucan Synthesis by *Streptococcus mutans*** The minimum inhibitory concentration for the formation of water-insoluble glucan by *S. mutans* was determined by two-fold dilution test in ASF medium 104 supplemented with 1% sucrose. One ml of the diluted culture ($OD_{660}=1.2$) of a fresh overnight culture of *Streptococcus mutans* was applied to a test tube containing 9 ml of serial two-fold dilutions of samples. The tubes were incubated at 30 °C for 24 h. The minimum inhibitory concentration was defined as the lowest concentration of the samples that prevented completely visible insoluble glucan formation.

## Results

The minimum inhibitory concentrations of the 5′-nucleotidase inhibitors on ASF medium 104 supplemented with 1% glucose are shown in Table I in comparison with that of tannic acid. Tannic acid is a typical compound of polyphenols and known to precipitate proteins (tannic activity). Under the above medium used, tannic acid did

TABLE I. The Minimum Growth Inhibitory Concentration ($\mu$g/ml) for Microorganisms

| Microorganisms | The minimum growth inhibitory concentration ($\mu$g/ml) | | |
|---|---|---|---|
| | NF-86I | NF-86II | Tannic acid |
| *Streptococcus mutans* MT8148 (c) | 12.5 | 6.25 | 12.5 |
| *Streptococcus mutans* MT6715 (g) | 50 | 12.5 | 25 |
| *Streptococcus faecium* ATCC 8043 | 25 | 12.5 | 12.5 |
| *Staphylococcus aureus* ATCC 6538P | 25 | 12.5 | 12.5 |
| *Bacillus subtilis* ATCC 6633 | 100 | 50 | 100 |
| *Proteus mirabilis* ATCC 9921 | >200 | >200 | >200 |
| *Proteus vulgaris* ATCC 6380 | >200 | >200 | >200 |
| *Escherichia coli* K-12 W3630 | >200 | >200 | >200 |
| *Candida albicans* ATCC E10231 | >200 | >200 | >200 |
| *Saccharomyces cerevisiae* ATCC 4226 | >200 | >200 | >200 |

TABLE II. The Minimum Inhibitory Concentration (μg/ml) of Water-Insoluble Glucan Formation by *Streptococcus mutans*

| Compounds | The minimum inhibitory concentration (μg/ml) | |
|---|---|---|
| | *S. mutans* MT8148 (c) | *S. mutans* MT6715 (g) |
| NF-86I | 400 | 50 |
| NF-86II | 400 | 50 |
| Tannic acid | 400 | 50 |

not give turbidity. The growth inhibitory activities of both NF-86I and NF-86II were equal to or somewhat better than that of tannic acid against *S. mutans* (c) and *S. mutans* (g). NF-86I and NF-86II inhibited moderately the growth of other gram-positive bacteria, but did not inhibit the growth of gram-negative bacteria and fungi at 200 μg/ml. Next, the inhibitory effect against glucan synthesis was carried out in ASF medium 104 supplemented with 1% sucrose. These compounds inhibited the water-insoluble glucan formation by *S. mutans* at the concentration of 50 to 400 μg/ml (Table II).

## Discussion

Some inhibitors of membrane-bound enzymes are known to show antitumor and immunopotentiator activity.[8] 5′-Nucleotidase is a typical membrane-bound enzyme and is used extensively as a marker for the plasma membrane in a variety of cells and tissues.[13] Inhibitors of 5′-nucleotidase are, therefore, expected to show these activities. Recently, we have isolated new 5′-nucleotidase inhibitors, NF-86I and NF-86II from the seeds of *Areca catechu* L.[4] These compounds were polyphenolic substances and showed antitumor activity, prolonging the life span of mice inoculated with Ehrlich ascites carcinoma.[9] The acute $LD_{50}$ values of these compounds in mice were more than 150 mg/kg after intraperitoneal injection and more than 500 mg/kg after oral administration. Some polyphenols, such as tannic acid, bind non-specifically to proteins and precipitate them (tannic activity). However, tannic acid did not show any inhibitory effect on 5′-nucleotidase activity at 200 μg/ml, and the 5′-nucleotidase inhibitors, NF-86I and NF-86II, did not show any significant tannic activity.[12] Therefore, 5′-nucleotidase inhibitors specifically bind to the enzyme protein. Tannic acid has been reported to have antibacterial activity.[11] In the present study, the growth inhibitory efficacy of 5′-nucleotidase inhibitors, NF-86I and NF-86II, was determined in ASF medium 104 supplemented with 1% glucose using *S. mutans* and other common microorganisms. Furthermore, we examined the inhibitory effect against glucan synthesis in a sucrose medium by *S. mutans*. As tannic acid non-specifically binds to proteins and gives turbidity, ASF medium 104 was selected in the present study. The 5′-nucleotidase inhibitors showed inhibitory effects on the growth of *S. mutans* MT8148 (c), *S. mutans* MT6715 (g) and other gram-positive bacteria (Table I). Hence, the 5′-nucleotidase inhibitors inhibited insoluble glucan formation from sucrose (Table II). The ability of glucan formation from sucrose by *Streptococcus mutans* MT8148 (c) is more potent than *Streptococcus mutans* MT6715 (g) (data not shown). It is of interest to note that the gram-positive bacteria tested were more sensitive than the gram-negatives and fungi. The difference in antibacterial effect may be due to the binding ability of the 5′-nucleotidase inhibitors to the outer membrane of the bacterial cell wall. Tannic acid is a typical polyphenolic compound and shows strong tannic activity.[10] However, tannic acid did not show any inhibitory effect on 5′-nucleotidase activity. The polyphenolic 5′-nucleotidase inhibitors showed almost no tannic activity.[12] Nevertheless, the antibacterial activities and inhibitory effects on glucan production of the 5′-nucleotidase inhibitors were somewhat better than those of tannic acid. It is likely, therefore, that the 5′-nucleotidase inhibitors, NF-86I and NF-86II, specifically bind to the bacterial cell surface. Although the relationships between the tannic activity and antibacterial activity of polyphenols is a complicated matter, it became obvious from our experimental results that the antibacterial activities and the inhibitory effects on glucan formation of the 5′-nucleotidase inhibitory polyphenols, which did not show tannic activity, are equal to those of tannic acid.

Dental caries is mainly initiated by plaque formation on the tooth surface caused by oral bacteria. *Streptococcus mutans*, one of a number of microorganisms that inhabit the oral cavity, form dental plaque by synthesizing water-insoluble glucan from sucrose. Therefore, inhibition of plaque formation due to the antibacterial action is an effective way of preventing dental caries. The inhibitors that we isolated from *Areca catechu* L. show moderate antibacterial activity toward *S. mutans* and repress the formation of the water-insoluble glucan. The seeds of *Areca catechu* are one of the most commonly consumed tobacco substances in Southeast Asia. From the above results, the 5′-nucleotidase inhibitors are promising as anti-plaque, preventive agents.

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# Formycin A Resistant Mutants Due to Defect in Adenosine Transport System in *Vibrio parahaemolyticus*

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**An antibiotic formycin A inhibited growth of *Vibrio parahaemolyticus* under certain conditions, which suggested that formycin A was taken up by cells under these conditions. We found that formycin A was transported *via* the adenosine transport system which we previously reported as a $Na^+$-coupled cotransport system. We isolated many formycin A resistant mutants, and about half of them grew very poorly on adenosine as a sole source of carbon. Judging from their reversion frequencies, these mutants seemed to have single mutations. Respiration driven uptake of $^{14}C$-adenosine was not observed in such mutants; also, $Na^+$ uptake induced by the addition of adenosine or formycin A to a cell suspension was completely abolished in them. Thus we conclude that these mutants possess a defect in the $Na^+$/adenosine cotransport system, and have become formycin A resistant.**

**Keywords** adenosine transport; formycin A; resistant mutant; *V. parahaemolyticus*

Formycin A is an antibiotic, an analog of adenosine, and has been reported to inhibit the growth of tumor cells,[1] viruses,[2] and *Xanthomonas oryzae*, but not of many kinds of bacteria such as *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and so on.[3]

*Vibrio parahaemolyticus* is a well-known food poisoning bacterium in Japan and a slightly holophilic marine bacterium. We found that although formycin A did not inhibit the growth of *V. parahaemolyticus* cells in rich medium, it did inhibit their growth under certain conditions. This implies that formycin A is transported by *V. parahaemolyticus* cells. Thus we analyzed the transport system for formycin A. In *Vibrio* it is known that an electrochemical potential of $Na^+$ across the cytoplasmic membrane is the driving force for transport of many substrates.[4] Cells of *V. parahaemolyticus* have a unique $Na^+$ pump coupled to respiration.[5] This $Na^+$ pump has been reported in many *Vibrio* species.[6] The electrochemical potential of $Na^+$ established by the $Na^+$ pump is utilized not only for active transport of nutrients but also for flagella rotation[7] and adenosine triphosphate (ATP) synthesis.[8,9] We reported previously that *V. parahaemolyticus* cells possessed a $Na^+$ coupled adenosine transport system[10] which was unique since even nucleotides such as adenosine monophosphate (AMP) could be transported.[11] It was not clear, however, whether formycin A was transported by this adenosine system or by another transport system(s) in *V. parahaemolyticus*.

In *E. coli*, mutants possessing reduced activity for nucleoside transport have been isolated from showdomycin (uridine analog) resistant mutants.[12] Analyses of these mutants have shown that there are at least two transport systems for nucleosides, NupG and NupC systems in *E. coli*[13] and that showdomycin resistant mutants have a defect in the NupC system. Thus nucleoside analog antibiotic is useful for analyses of nucleoside transport systems.

Here, we report that formycin A is transported *via* the adenosine transport system in *V. parahaemolyticus*. We isolated formycin A resistant mutants, and from them isolated adenosine (and formycin A) transport negative mutants.

## Experimental

**Bacterium and Growth** A wild type of *V. parahaemolyticus* AQ3334[5] was used. Unless otherwise stated, cells were grown in medium S[14] (pH 7.5) supplemented with 0.5% polypeptone and 3 mM adenosine under aerobic conditions at 37 °C. Solid media used were a glycerol plate (medium S containing 40 mM glycerol), a formycin A plate (glycerol plate containing 100 μM formycin A) and an adenosine plate (medium S containing 10 mM adenosine), each containing 1.5% agar.

**Isolation of Mutants** Mutants were isolated as follows. Cells mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine[15] (30 μg/ml) were divided into 10 tubes and inoculated into medium S supplemented with 40 mM glycerol, shaken at 37 °C for 2 h, followed by an addition of 100 μM formycin A, and again shaken for 24 h. Aliquots were then diluted with fresh medium S containing 40 mM glycerol and 100 μM formycin A, and shaken at 37 °C for 12 h. Aliquots were diluted with medium S $10^6$-fold, and spread on the glycerol plate containing 100 μM formycin A. The plates were incubated at 37 °C for 24 h. Fifteen large colonies were picked up from each plate (total 150 colonies). These formycin A resistant mutants were replica plated on the glycerol plate and the adenosine plate. About one half of the mutants grew very poorly on the adenosine plate, and others grew normally. Mutants which grew very poorly on adenosine were further analyzed.

**Measurement of $Na^+$ Uptake** Cells were harvested in the late exponential phase of growth, washed twice with 0.2 M 4-morpholinepropanesulfonic acid (Mops)–tetramethylammonium hydroxide (TMAH) (pH 7.5) and 5 mM $MgSO_4$, and suspended in the same buffer. Uptake of $Na^+$ was measured in assay mixture consisting of 0.2 M *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine)–TMAH (pH 8.5), 0.1 mM NaCl and cells (2—3 mg protein/ml), using a $Na^+$-selective electrode, as described previously.[16]

**Adenosine Transport** Cells were harvested in the late exponential phase of growth, washed twice with 0.2 M Mops–Tris (pH 7.5) and 10 mM $MgSO_4$, and suspended in the same buffer. Assay mixture for adenosine transport contained 0.2 M Mops–Tris (pH 7.5), 10 mM $MgSO_4$, 20 mM Tris–lactate, 2 mM NaCl and cells (about 0.1 mg protein/ml). Transport was initiated by addition of [U-$^{14}C$]adenosine (final 0.1 mM), and samples were taken at intervals, filtered, and counted.[10]

**Other Method** Protein contents were determined as described by Lowry *et al.*[17]

**Materials** [U-$^{14}C$]Adenosine was purchased from Amersham Co. $Na^+$-selective electrode was from Radiometer Co. (Copenhagen).

## Results

**Effects of Formycin A on Growth of *V. parahaemolyticus*** We examined effects of formycin A on the growth of *V. parahaemolyticus* cells with various carbon sources (Fig. 1). Although these cells grew well on glucose, adenosine or polypeptone even in the presence of 100 μM formycin A, they were unable to grow on glycerol (or succinate) in the presence of formycin A. Thus, the effects of formycin A on growth of *V. parahaemolyticus* depend on growth conditions. Inhibition of growth of cells by formycin A implies the presence of a transport system for

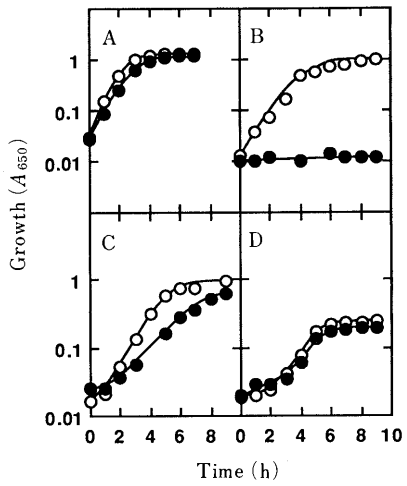


Fig. 1. Effects of Formycin A on Growth of *V. parahaemolyticus* with Various Carbon Sources

Cells were grown in medium S supplemented with 0.5% polypeptone (A), 40 mM glycerol (B), 20 mM glucose (C) or 12 mM adenosine (D) in the absence (○) or presence (●) of 200 μM formycin A.

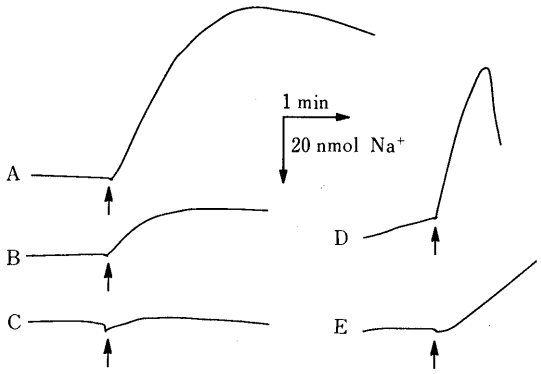


Fig. 2. Competitive Inhibition of $Na^+$ Uptake Induced by Addition of Formycin A or Adenosine

At the time points indicated by arrows, 0.1 mM formycin A (A, B and C) or 0.1 mM adenosine (D and E) was added to cell suspension. In some cases, cells were preincubated with 0.1 mM adenosine (B), 0.4 mM adenosine (C), or 1 mM formycin A (E). An upward deflection represents uptake of $Na^+$.

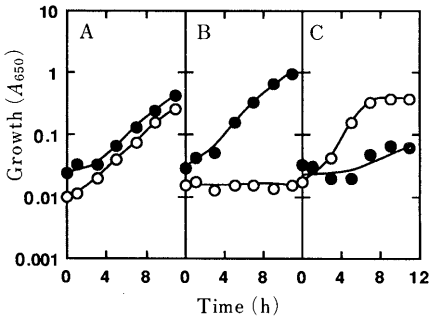


Fig. 3. Growth of the Wild Type and the Mutant FR15 under Various Conditions

The wild type cells (○) and the mutant FR15 cells (●) were grown on 40 mM glycerol (A), 40 mM glycerol in the presence of 200 μM formycin A (B) or 20 mM adenosine (C).

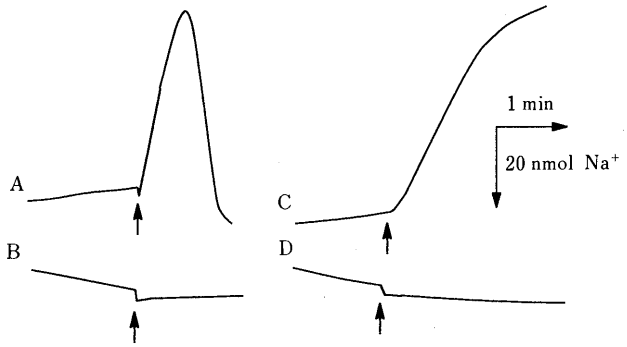


Fig. 4. $Na^+$ Uptake Induced by Addition of Adenosine or Formycin A in the Wild Type Cells and the Mutant FR15 Cells

At the time points indicated by arrows, 0.1 mM adenosine (A and B) or 0.1 mM formycin A (C and D) was added to the assay mixture containing the wild type cells (A and C) or the FR15 cells (B and D). An upward deflection represents uptake of $Na^+$.

formycin A in *V. parahaemolyticus* cells. Since we are interested in the transport of antibiotics, we investigated the transport system for formycin A.

**Transport of Formycin A** One possibility is that formycin A is transported *via* the adenosine transport system which is a $Na^+$-coupled cotransport system.[10] We measured $Na^+$ uptake by the cells, which would be induced by addition of formycin A to cell suspension, using a $Na^+$-selective electrode. As shown in Fig. 2, large $Na^+$ uptake was observed when formycin A was added to cell suspension, as well as when adenosine was added. We observed faster initial velocity of $Na^+$ uptake when adenosine was added than when formycin A was added. These results indicate that formycin A was transported by a mechanism of $Na^+$/formycin A cotransport, although the velocity was slower than $Na^+$/adenosine cotransport. The $Na^+$ uptake induced by an addition of 0.1 mM formycin A was strongly inhibited by the presence of adenosine in a concentration-dependent manner. Almost complete inhibition was observed in the presence of 0.4 mM adenosine. On the other hand, $Na^+$ uptake induced by the addition of 0.1 mM adenosine was partially inhibited by the presence of 1 mM formycin A. These results suggest that formycin A was transported by the $Na^+$/adenosine cotransport system, although affinity of the transport system is much higher for adenosine than for formycin A.

**Isolation and Properties of Mutants** The isolation of mutants and their characterization are good means to determine whether formycin A and adenosine are transported *via* the same carrier or not. Thus we attempted to isolate transport negative mutants. If formycin A is really transported by the $Na^+$/adenosine system, it should be possible to isolate adenosine transport negative mutants from formycin A resistant mutants. In fact, we did isolate many formycin A resistant mutants, and isolated many adenosine transport negative mutants which were among them. Figure 3 shows growth properties of mutant FR15, a representative of such mutants. FR15 cells grew normally on glycerol regardless of the absence or the presence of 100 μM formycin A, although wild type AQ3334 cells were unable to grow in the presence of 100 μM formycin A. The mutant cells grew very poorly with adenosine as a sole source of carbon, although wild type cells grew normally. We observed similar growth patterns with many other mutants.

We measured adenosine transport activity in these mutants. Both $Na^+$ uptake induced by addition of adenosine to cell suspension and that induced by formycin A

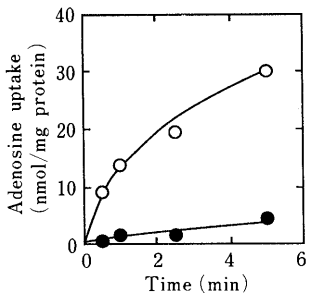


Fig. 5. Adenosine Uptake in the Wild Type Cells and the Mutant FR15 Cells

Transport was initiated by the addition of 0.1 mM [U-$^{14}$C]adenosine to the assay mixture containing the wild type cells (○) or the FR15 cells (●).

were completely abolished in the mutant FR15 (Fig. 4). We obtained similar results with other mutants (data not shown). Judging from the reversion frequencies of the mutants (in the order of $10^{-7}$), the mutations are thought to be single mutations. We observed restored $Na^+$ uptake when adenosine or formycin A was added to cell suspension of revertant cells (data not shown). Thus, we conclude that single mutations destroyed both $Na^+$/formycin A cotransport and $Na^+$/adenosine cotransport, indicating that formycin A and adenosine are transported *via* the same system.

We also tested $^{14}$C-adenosine uptake in the mutant FR15 cells and in the wild type AQ3334 cells (Fig. 5). Adenosine uptake activity in the mutant cells was reduced to a few percent of the wild type cells. Similar results were obtained with all mutants tested which showed very poor growth on adenosine (data not shown).

## Discussion

Formycin A has been reported to be an inefficient inhibitor for the growth of many bacteria. However, we found that the growth of *V. parahaemolyticus* was strongly inhibited by formycin A under certain conditions. The effects of formycin A depended on carbon source. For example, when glycerol was the sole source of carbon, formycin A strongly inhibited the growth. On the other hand, formycin A did not give significant effect on the growth when glucose, polypeptone or adenosine was the carbon source. There are three possible reasons for this difference in formycin effect on the growth: 1) difference in transport of formycin A, 2) difference in metabolism of formycin A, and 3) difference in sensitivity of target of formycin action. We observed differences in adenosine transport activity and differences in adenosine metabolism between cells grown on various carbon sources (data not shown). A combination of these factors could be the reason for the difference in the formycin-sensitivity.

We found that formycin A was transported with $Na^+$ *via* the adenosine transport system. Very recently we found that guanosine, inosine, uridine and cytidine were also substrates for this transport system (unpublished results). Thus substrate specificity in the adenosine (formycin A) transport system is rather broad.

We isolated negative mutants of the adenosine transport system. These mutants grew very poorly on adenosine as a sole source of carbon. It seems possible to clone the structural gene for the adenosine transport system using such mutants as host cells. Cloning and sequencing of the gene would be valuable for biochemical and molecular biological analyses of the adenosine (formycin A) transport system. Such investigations are now under way.

Judging from the sensitivity of adenosine transport to monensin, an ionophore which catalyzes exchange of $Na^+/H^+$, we suggested the possible existence of another adenosine transport system in *V. parahaemolyticus* cells.[10] We observed a complete loss of $Na^+$ uptake induced by adenosine in many mutants; however, all mutants showed some uptake of $^{14}$C-adenosine. Furthermore, in spite of the defect in the $Na^+$/adenosine cotransport system, FR mutants were able to grow on adenosine as a sole source of carbon although very slowly. These observations also suggest the existence of another adenosine transport system, although the activity of such system would not be high enough to support the normal growth of cells on adenosine.

**Acknowledgment** This research was supported by grants from the Ministry of Education, Science and Culture of Japan to Y.S.T. and T.T.

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# Evaluation of Low-Molecular Gelatin as a Pharmaceutical Additive for Rapidly Absorbed Oral Dosage Formulation

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**The dissolution behavior of ibuprofen from a kneaded mixture with low-molecular gelatin (LM gelatin) has been studied in comparison to kneaded mixtures prepared with other additives. Their *in vivo* absorption behaviors were also examined. The LM gelatin markedly enhanced the dissolution rate of ibuprofen compared to that with polyethylene glycol (PEG) 6000, dextran T10 or pullulan. Oral administrations of the kneaded mixture to beagle dogs showed the LM gelatin to be most effective in accelerating the absorption rate of ibuprofen among the additives used.**

**Keywords** low-molecular gelatin; pharmaceutical additive; dissolution; absorption; kneaded mixture

Very recently we reported that low-molecular gelatin (LM gelatin), a hydrolysate of common gelatin, enhanced the absorption rates of some drugs by improving their dissolution characteristics because of its high aqueous solubility.[1-3] In this continuing investigation, we have evaluated LM gelatin to be one of the most useful additives for rapidly absorbed solid drug formulation in comparison with other additives such as polyethyleneglycol (PEG) 6000, dextran T10 and pullulan which were used as references having approximately same molecular weights and high aqueous solubilities as the LM gelatin.

## Experimental

**Materials** Low-molecular gelatin (mean molecular weight (MW) = 6000) was kindly supplied by Nitta Gelatin Co., Ltd. (Osaka). Polyethylene glycol (PEG) #6000 (MW = 7800—9000) was obtained from Nacalai Tesque, Inc. (Kyoto). Dextran T10 (MW = 10200) was obtained from Pharmacia LKB (Sweden). Pullulan (MW = 5500) was kindly supplied by Hayashibara Co., Ltd. (Okayama). Ibuprofen (Nisshin Flour Milling Co., Ltd., Tokyo) was used as supplied. All other reagents and solvents were of analytical grade.

**Sample Preparation** A kneaded mixture of ibuprofen with each additive was prepared by the kneading method. Ibuprofen and additives in a weight ratio of 1 : 1 were weighed and placed in a mortar, and each mixture was kneaded with the same amount of water for 1 h. Drying was carried out *in vacuo* at room temperature for 48 h. The fraction that passed through a 100 mesh sieve was used in the following experiments.

**Dissolution Studies** Dissolution of ibuprofen from the samples was measured according to the paddle method (JP XI). A volume of 900 ml deionized water was kept at 37 °C and rotated at 100 rpm. Sample powders each equivalent to 80 mg of ibuprofen with or without filling in hard capsules were put into the test solution. An aliquot was withdrawn periodically and immediately filtered through a 1.0 $\mu$m membrane filter (Toyo Roshi Co., Ltd., Tokyo). The filtrate was extracted with organic solvent to remove additives. The drug concentration in the organic phase was determined spectrophotometrically at 220 nm. All studies were done in triplicate.

***In Vivo* Absorption Studies** Beagle dogs (10—13 kg, 2—3 years old) were given a liquid diet (Besvion®; Snow Brand Milk Co., Ltd., Tokyo) for 2 d before being fasted for 24 h. The powders (equivalent to 80 mg of ibuprofen) were put into hard capsules of size 0. Each preparation was given orally to the dogs together with 20 ml of water. Blood samples were taken at appropriate intervals from the forefoot vein and centrifuged for 15 min at 3000 rpm to obtain serum fractions. To 1 ml of serum was added 1 ml of 1 N HCl, and the serum was then extracted with 5 ml of cyclohexane containing 3 $\mu$g/ml of flurbiprofen as an internal standard. The organic phase was evaporated. The residue was dissolved in 100 $\mu$l of acetonitrile and assayed by reversed phase high-performance liquid chromatography (HPLC). The HPLC conditions were as follows: pump, Hitachi 655A-11 type; detector, Hitachi 655A-21 type; column, LiChrosorb RP-18 (250 × 4 mm i.d.); mobile phase, acetonitrile–water–phosphoric acid 275 : 225 : 0.5); flow rate, 1 ml/min; detection, 220 nm.

**Pharmacokinetic Analysis** Bioavailability parameters were calculated from the serum concentration–time curve up to 5 h post-administration. Moment analysis was employed to calculate the area under the serum concentration–time curve (*AUC*), the mean residence time (*MRT*) and the variance of residence time (*VRT*).[4]

## Results and Discussion

Figure 1 shows the dissolution profiles of ibuprofen from

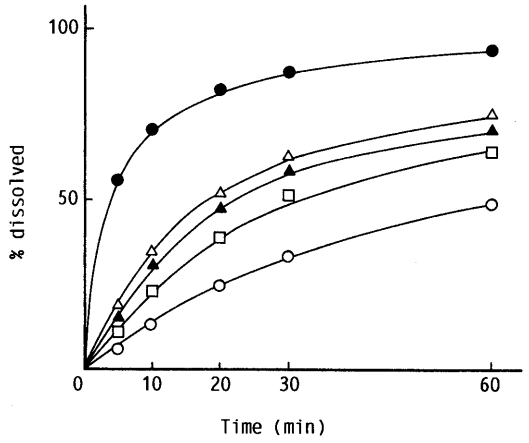


Fig. 1. Dissolution Profiles of Ibuprofen from Its Kneaded Mixture with Several Additives in Water at 37 °C

○, ibuprofen alone; ●, LM gelatin kneaded mixture; △, dextran T10 kneaded mixture; ▲, PEG 6000 kneaded mixture; □, pullulan kneaded mixture.

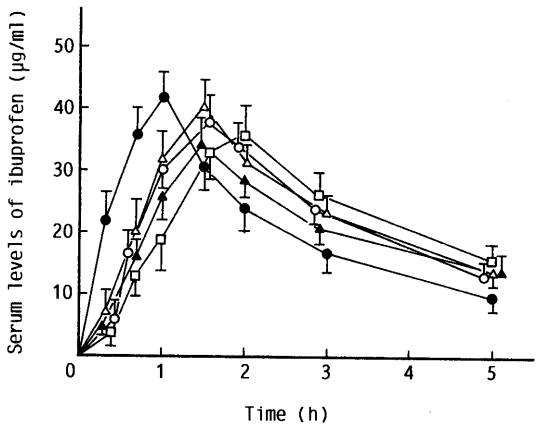


Fig. 2. Mean Serum Levels of Ibuprofen Following the Oral Administration of Ibuprofen Powder and Its Kneaded Mixture with Several Additives to Beagle Dogs

Each point represents the mean ± S.E. ($n = 4$). ○, ibuprofen alone; ●, LM gelatin kneaded mixture; △, dextran T10 kneaded mixture; ▲, PEG 6000 kneaded mixture; □, pullulan kneaded mixture.

TABLE I. Bioavailability Parameters Following Oral Administration of Ibuprofen Kneaded Mixtures

| Preparation | $AUC_{0-5h}$ (h·μg/ml) | $MRT$ (h) | $VRT$ ($h^2$) |
|---|---|---|---|
| Ibuprofen alone | 104.66±11.40 | 2.35±0.11 | 1.58±0.07 |
| Kneaded mixture | | | |
| LM gelatin | 103.55±16.86 | 1.99±0.16[a] | 1.62±0.07 |
| PEG 6000 | 115.41±20.31 | 2.34±0.13 | 1.58±0.06 |
| Dextran T10 | 105.64±10.08 | 2.43±0.28 | 1.57±0.08 |
| Pullulan | 110.34±15.60 | 2.51±0.17 | 1.40±0.21 |

Values represent the mean±S.D. ($n=4$). *a*) Significantly different from ibuprofen alone at $p<0.05$.

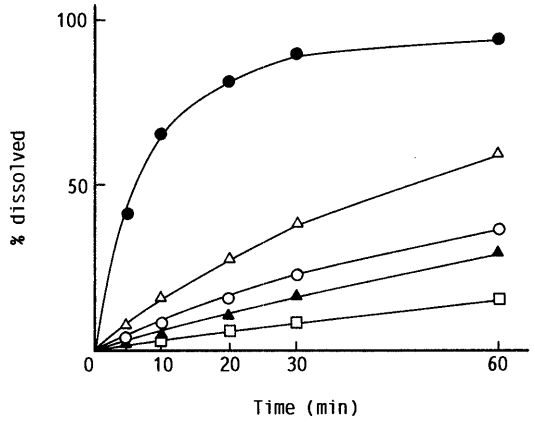


Fig. 3. Dissolution Profiles of Ibuprofen Powder and Its Kneaded Mixture with Several Additives from Gelatin Capsule in Water at 37 °C

○, ibuprofen alone; ●, LM gelatin kneaded mixture; △, dextran T10 kneaded mixture; ▲, PEG 6000 kneaded mixture; □, pullulan kneaded mixture.

its kneaded mixtures with several additives in water 37 °C. All kneaded mixtures exhibited faster dissolution patterns than ibuprofen alone, and that with LM gelatin showed a particularly higher dissolution rate than other additives. The surface of ibuprofen might become more hydrophilic through the dispersion in LM gelatin, compared with other additives.[1)]

Figure 2 shows the mean serum levels of ibuprofen following the oral administration of kneaded mixtures to the beagle dogs. It is evident that the LM gelatin significantly enhanced the initial serum concentration of ibuprofen and reduced the peak concentration time ($T_{max}$). The other additives affected the serum concentration of ibuprofen only insignificantly. These distinct differences of additive effects were confirmed from the bioavailability parameters (Table I). As shown in Table I, the mean residence time ($MRT$) value, the index of rate of bioavailability, for the ibuprofen–LM gelatin kneaded mixture following oral administration was significantly reduced in comparison with those of others. However, the $AUC$ was almost the same for all samples. The differences in the rate of bioavailability between the LM gelatin and other additives may be explained by the dissolution and absorption characteristics from the kneaded mixtures. That is, the enhanced dissolution of the kneaded mixtures with the other additives was less or cancelled out due to the negative effect imparted by the interaction such as complexation of the drug and the additives that retarded permeability, resulting in decrease in the apparent overall absorption rate of the drug. Moreover, the slow dissolution of the kneaded mixture from the capsule may be considered due to the interaction of the drug or the additive in the kneaded mixture with gelatin, the capsule component which causes less dissolution than drug alone. In fact, the dissolution of ibuprofen from the kneaded mixture in the gelatin capsule was slower than the ibuprofen alone (Fig. 3). However, the dissolution of drug from the capsule containing LM gelatin was markedly higher than the drug alone.

We have already reported that LM gelatin was very effective in accelerating the rate of absorption of a variety of drugs from the gastrointestinal tract after oral administration.[1,2)] The data presented here further emphasized that LM gelatin was very effective as an additive providing fast dissolution and absorption of a drug.

**Acknowledgement** The authors are grateful to Nitta Gelatin Co., Ltd. for supplying the low-molecular gelatin sample.

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# PHOTOCHEMICAL TRANSFORMATION OF BUFADIENOLIDE[1)]

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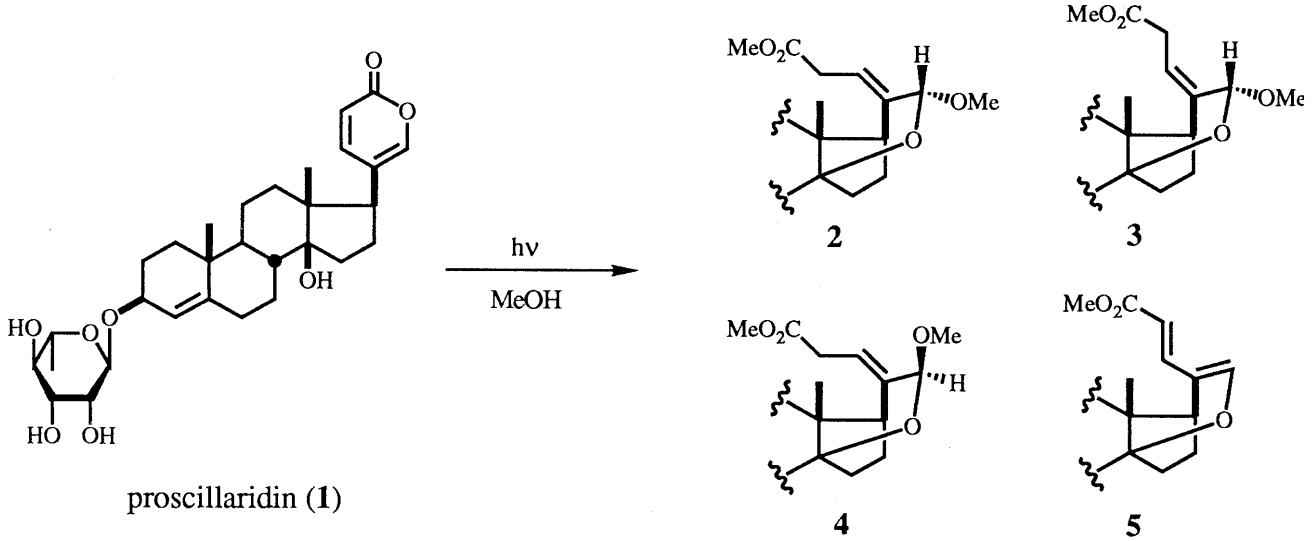
The photolysis of a bufadienolide glycoside, proscillaridin (**1**), in MeOH with a high-pressure mercury arc lamp afforded the three novel compounds (**2-4**) with a 14β,21-epoxy-21-methoxychola-4,20(22)-diene skeleton, and the known compound (**5**). The structures of **2**, **3**, and **4** were elucidated spectroscopically.

**KEYWORDS** bufadienolide; proscillaridin; cardiac glycoside; photolysis; photochemical transformation; methyl 14β,21-epoxy-21-methoxychola-4,20(22)-dienoate

During the course of our investigation on cardiac ingredients, proscillaridin (**1**), we found that the α-pyrone moiety of bufadienolide underwent several chemical modifications to give various functionalized compounds.[1,2)] In this communication, we would deal with the novel photochemical transformation of bufadienolide leading to 14β,21-epoxycholane derivatives (**2-5**).

A solution of proscillaridin (**1**) in methanol was irradiated in a Pyrex vessel with a high-pressure mercury arc lamp (400 W) at room temperature for 5 h under nitrogen atmosphere to afford a photoproduct exhibiting one spot on thin-layer chromatography. The photoproduct was subjected to column chromatography on $SiO_2$ using $CHCl_3$:MeOH=12:1 as eluate followed by high performance liquid chromatography (Develosil ODS-A-5, MeOH:$H_2O$=9:1) to furnish four compounds **2** (20%), **3** (17%), **4** (8%), and **5** (35%).

Compound **2**,[3)] a colorless crystalline powder, mp 67-69°C, $[\alpha]_D^{25}$ -71.2° (c=0.5, $CHCl_3$), was shown to have the molecular formula $C_{32}H_{48}O_9$ by its FAB and HRFAB-MS spectra. The IR spectrum of **2** showed a carbonyl absorption band due to an ester group (1740 $cm^{-1}$), and lacked a conjugated carbonyl absorption. Compared with proscillaridin (**1**), the $^1H$ NMR spectrum of **2** was devoid of the signals characteristic of α-pyrone moiety [δ 6.23 (1H, d, *J*=9.8 Hz, 23-H), 7.26 (1H, d, *J*=2.5 Hz, 21-H), 7.93 (1H, dd, *J*=2.5, 9.8 Hz, 22-H) ]. Instead, the spectrum had new signals due to one methine proton




proscillaridin (**1**) **2** **3** **4** **5**

Chart 1

Table I. $^{13}C$ NMR Data for **2**, **3**, **4**, and **5**

| | 2 | 3 | 4 | 5 |
|---|---|---|---|---|
| 1 | 32.2[a)] | 32.2[a)] | 32.2[a)] | 32.4[a)] |
| 2 | 29.1[b)] | 28.9[b)] | 28.6[b)] | 27.8 |
| 3 | 74.2 | 74.2 | 74.2 | 74.2 |
| 4 | 120.5 | 120.4 | 120.3 | 120.7 |
| 5 | 147.5 | 147.7 | 147.9 | 147.2 |
| 6 | 35.5 | 35.5 | 35.5 | 35.5 |
| 7 | 27.0 | 27.0 | 27.0 | 27.0 |
| 8 | 38.7 | 38.8 | 38.2 | 37.9 |
| 9 | 49.4 | 49.5 | 49.5 | 49.9 |
| 10 | 37.6 | 37.6 | 37.6 | 37.5 |
| 11 | 19.9 | 19.8 | 19.9 | 20.2 |
| 12 | 24.8 | 25.8 | 25.4 | 31.5 |
| 13 | 44.8 | 44.6 | 45.4 | 40.6 |
| 14 | 87.2 | 87.2 | 86.4 | 91.7 |
| 15 | 32.0[a)] | 31.8[a)] | 31.6[a)] | 31.9[a)] |
| 16 | 28.4[b)] | 28.4[b)] | 28.3[b)] | 30.7 |
| 17 | 46.9 | 54.1 | 47.0 | 43.4 |
| 18 | 16.1 | 16.6 | 15.9 | 15.4 |
| 19 | 18.8 | 18.8 | 18.9 | 18.9 |
| 20 | 142.9 | 142.7 | 141.8 | 120.8 |
| 21 | 101.9 | 97.2 | 98.4 | 150.0 |
| 22 | 118.0 | 117.7 | 113.2 | 143.6 |
| 23 | 32.3 | 32.4 | 32.4 | 109.3 |
| 24 | 172.0 | 172.1 | 172.4 | 168.5 |
| 1' | 99.2 | 99.1 | 99.1 | 99.2 |
| 2' | 71.5 | 71.5 | 71.5 | 71.5 |
| 3' | 71.8 | 71.8 | 71.8 | 71.8 |
| 4' | 73.0 | 73.5 | 73.5 | 73.2 |
| 5' | 68.3 | 68.0 | 68.0 | 68.2 |
| 6' | 17.6 | 17.5 | 17.5 | 17.5 |
| 21-$OCH_3$ | 54.9 | 54.9 | 57.0 | |
| 24-$OCH_3$ | 51.9 | 51.9 | 51.8 | 51.3 |

Assignments for these compounds were made with the aid of DEPT and $^{13}C$-$^{1}H$ COSY data. a), b) Assignment may be exchangeable within the same vertical column.

indicating the presence of a ketal moiety, and signals of two methoxyl protons [δ 3.38, 3.68 (3H, each, both s)] and one olefin proton [δ 5.55 (1H, dd, *J*=6.8, 7.9 Hz)]. Compound **2** also had a ketal moiety, as its $^{13}C$ NMR spectrum showed (δc 101.9). Wartburg et al. reported that bufogenin converted iso-bufogenic acid ester by alkaline hydrolysis and subsequent acidification.[4] In the view of this report and the spectroscopic features of **2**, the photoproduct (**2**) was assumed to have an isobufogenic acid ester skeleton, probably was formed *via* a photo-pyrone intermediate.[5] Thus, the plane structure of **2** was established as methyl 3β-[(6-deoxy-α-L-mannopyranosyl)oxy]-14β, 21-epoxy-21-methoxychola-4,20-(22)-dienoate. Compounds **3**[6] and **4**[7] also had the same molecular formula $C_{32}H_{48}O_9$ as **2**, as showen by their FAB and HRFAB-MS spectra. The $^{1}H$ and $^{13}C$ NMR of **3** and **4** are very similar to those of **2**, and they indicated that compound **3** and **4** had the same functional group as **2**. So compounds **3** and **4** were proved to be diastereomeric and/or geometrical isomers of **2**.

In order to determine the absolute stereochemistry of the photolysis products (**2**-**4**), difference NOE experiments were carried out. NOE enhancements between the ketal proton (21-H) and 22-H in **2** indicated the *Z*-configuration of $C_{20}$-$C_{22}$ double bond and the *R*-configuration on $C_{21}$.[8] In compound **3**, the NOEs appeared not only between 17-H and 22-H but also between 21-H and 23-$H_2$. Compound **4** showed NOE enhancements between 17-H and 23-$H_2$, but it exhibited no NOE in irradiating 21-H (Fig. 1). Based on these spectral properties, the structures of **2**, **3**, and **4** were elucidated to be as shown in Chart 1.

In conclusion, we have found the novel photochemical transformation of bufadienolide glycoside in which the α-pyrone moiety in **1** was cleaved to form three methyl 14β,21-epoxy-21-methoxychola-4,20(22)-dienoates (**2**-**4**), and methyl 14β,21-epoxychola-4,20,22-trienoate (**5**). It should be noted that this photochemical conversion of proscillaridin (**1**) requires no previous protection of the hydroxyl groups in the sugar moiety and afforded the photolysis products (**2**-**5**) in high total yield. Since compounds with a 14β,21-epoxy-21-methoxycholane skeleton have not been found before, compounds **2**, **3**, and **4** may be of interest for their biological and pharmacological activities.

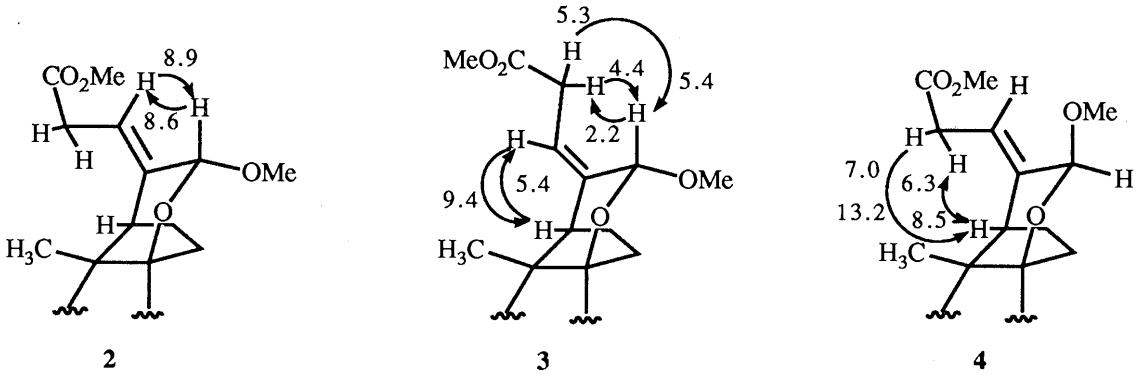



Fig. 1. NOEs Observed in NOE Difference Spectra of **2**, **3**, and **4** (%)

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3) FAB-MS (m/z): 583 $(M+Li)^+$. High-resolution FAB-MS: Calcd for $C_{32}H_{48}O_9Li$: 583.346; Found: 583.351. IR (KBr, $cm^{-1}$): 3425, 1740. $^1H$ NMR (δ, 400 MHz, $CDCl_3$): 0.86 (3H, s , 13-$CH_3$), 1.07 (3H, s 10-$CH_3$), 1.30 (3H, d, *J*=6.2 Hz, 5'-$CH_3$), 2.63 (1H, d, *J*=4.2 Hz, 17-H), 3.07 (1H, dd, *J*=6.8, 17.0 Hz, 23-H), 3.15 (1H, dd, *J*=7.9, 17.0 Hz, 23-H), 3.38 (3H, s, 21-$OCH_3$), 3.42 (1H, dd, *J*=9.3, 9.3 Hz, 4'-H), 3.68 (3H, s, 24-$OCH_3$), 3.70-3.80 (2H, m, 3'-H, 5'-H), 3.92 (1H, s, 2'-H), 4.12 (1H, dd, *J*=5.9, 9.9 Hz, 3-H), 4.88 (1H, s, 21-H), 4.96 (1H, s, 1'-H), 5.30 (1H, s, 4-H), 5.55 (1H, dd, *J*=6.8, 7.9 Hz, 22-H).

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6) **3**: a white crystalline powder. mp 70-72°C. $[\alpha]_D^{25}$ -109.9° (c=0.70, $CHCl_3$), FAB-MS (m/z): 583 $(M+Li)^+$. High-resolution FAB-MS: Calcd for $C_{32}H_{48}O_9Li$: 583.346; Found: 583.346. IR (KBr, $cm^{-1}$): 3425, 1740. $^1H$ NMR (δ, 400 MHz, $CDCl_3$): 0.87 (3H, s, 13-$CH_3$), 1.07 (3H, s, 10-$CH_3$), 1.30 (3H, d, *J*=6.2 Hz, 5'-$CH_3$), 2.30 (1H, d, *J*=5.5 Hz, 17-H), 3.02 (1H, dd, *J*=6.6, 17.6 Hz, 23-H), 3.11 (1H, dd, *J*=7.9, 17.6 Hz, 23-H), 3.40 (3H, s, 21-$OCH_3$), 3.44 (1H, dd, *J*=9.5, 9.5 Hz, 4'-H), 3.68 (3H, s, 24-$OCH_3$), 3.74-3.80 (2H, m, 3'-H, 5'-H) 3.93 (1H, br-s, 2'-H), 4.13 (1H, dd, *J*=5.9, 9.9 Hz, 3-H), 4.93 (1H, d, *J*=1.3 Hz, 1'-H), 5.11 (1H, d, *J*=0.7 Hz, 21-H), 5.30 (1H, s, 4-H), 5.43 (1H, ddd, *J*=0.7, 6.6, 7.9 Hz, 22-H).

7) **4**: a white crystalline powder. mp 88-90°C. $[\alpha]_D^{25}$ -31.5° (c=0.6, $CHCl_3$). FAB-MS (m/z): 583 $(M+Li)^+$. High-resolution FAB-MS: Calcd for 583.346; Found: 583.346. IR (KBr, $cm^{-1}$): 3440, 1745. $^1H$ NMR (δ, 400 MHz, $CDCl_3$): 0.92 (3H, s, 13-$CH_3$), 1.06 (3H, s, 10-$CH_3$), 1.30 (3H, d, *J*=6.0 Hz, 5'-$CH_3$), 2.30 (1H, d, *J*=5.3 Hz, 17-H), 3.02 (1H, dd, *J*=6.8, 15.4 Hz, 23-H), 3.10 (1H, dd, *J*=8.2, 15.4 Hz, 23-H), 3.45 (1H, dd, *J*=9.0, 9.0 Hz, 4'-H), 3.55 (3H, s, 21-$OCH_3$), 3.67 (3H, s, 24-$OCH_3$), 3.70-3.80 (2H, m, 3'-H, 5'-H), 3.92 (1H, s, 2'-H), 4.12 (1H, dd, 7.5, 8.1 Hz, 3-H), 4.95 (1H, s, 1'-H), 5.00 (1H, d, *J*=1.5 Hz, 21-H), 5.29 (1H, s, 4-H), 5.66 (1H, ddd, *J*=1.5, 6.8, 8.2 Hz, 22-H).

8) In the NOE experiment of **2**, irradiation of 17-H and 23-$H_2$ resulted in no NOE enhancements between them.


(Received January 28, 1991)

# THE CHEMICAL REPAIR REACTION OF THYMINE AND THYMIDINE DIOL DERIVATIVES, MODELS OF OXIDATIVELY DAMAGED NUCLEIC ACIDS, BY TRIVALENT PHOSPHORUS COMPOUNDS

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When treated with trivalent phosphorus compounds, thymine and thymidine diol derivatives, models of oxidatively damaged nucleic acids, were reductively repaired to regenerate the original thymine and thymidine derivatives. A possible mechanism is proposed.

KEYWORDS 1,3-dimethylthymine diol; thymidine diol derivative; phosphite; phosphine; reduction; repair reaction; damaged nucleic acid model; mechanism

It has been reported recently that the *cis* isomers of thymine and thymidine diols are released in human and rat urine as the result of excision repair of oxidatively damaged DNA.[1] Although the several kinds of excision repairs for the abnormal nucleic acids by endonucleases are known in biological systems, we considered that there would possibly be other types of repair mechanism for oxidatively modified nucleic acids. Thus, we have examined the repair reactions of thymine and thymidine diols as models of damaged nucleic acids, using a wide variety of reducing agents.[2] The results so far showed that only trivalent phosphorus compounds effectively repaired the diol derivatives to the original thymine and thymidine derivatives, as reported here.

A mixture of 1,3-dimethylthymine diol (*cis* **1** or *trans* **2**)[3] (1 mmol) with phosphorus compounds was treated under the conditions in Table I. After the reaction, the solution was concentrated *in vacuo* and the residue was purified by preparative T.L.C. with ether to give 1,3-dimethylthymine (**3**) in good yield.[4]

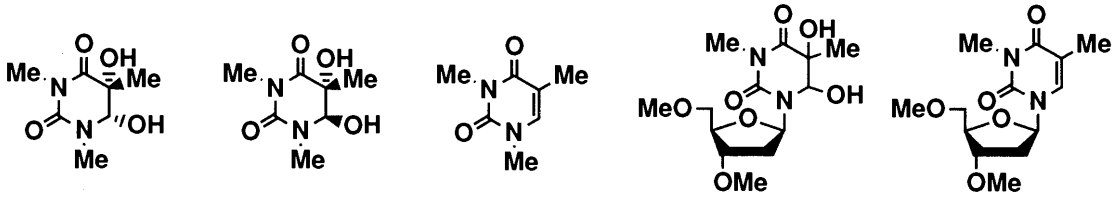


Chart 1

In both **1** and **2**, the reaction proceeded at the same rate to give almost the same yield of **3** in spite of their different stereochemistry, therefore the same reaction intermediate could be considered. The results using several tri and pentavalent phosphorus compounds, summarized in Table I, showed that only trivalent phosphorus compounds were effective for this repair reaction. The reaction of thymidine diol derivative (**4**) with triphenylphosphite gave β-thymidine derivative (**5**)[5] in 89% yield, and not α-thymidine isomer. Therefore it seemed reasonable to assume that the movement of the lone pair of $N_1$ followed by the scission of the $C_{1'}$-O bond in ribosyl moiety,[6] did not take place.

The nucleophilic attack on carbonyl carbon by trivalent phosphorus compounds at the initial stage is generally accepted as the mechanism of the Perkow reaction (reaction of α-halocarbonyl compound

with trivalent phosphorus compound), although the details are still not completely resolved.[7,8] Therefore, we would like to propose tentatively a Perkow type of reaction mechanism shown in Chart 2 for the reductive reapir reaction.

To our knowledge, natural and stable trivalent phosphorus compounds have not yet been reported in the field of biochemistry. However, if a trivalent phosphorus derivative is brought about transiently in living cells, this type of repair reaction for oxidatively modified nucleic acids might occur under certain conditions in live bodies.

Table I. The Results of Reaction of **1** and **2** with Phosphorus Compounds

| Compound | Reagent | Equivalent | Solvent | Conditions | | Yield of **3** (%) |
|---|---|---|---|---|---|---|
| **1** | $PBr_3$ | 10.0 | THF | R.T. | 20 h | 61.0 |
| **2** | | | | | | 74.4 |
| **1** | $PCl_3$ | 2.4 | THF | Reflux | 20 h | 73.2 |
| **2** | | | | | | 72.0 |
| **1** | $(PhO)_3P$ | Neat | — | 100 °C | 3 h | 86.7 |
| **2** | | | | | | 85.7 |
| **1** | $(MeO)_3P$ | Neat | — | 100 °C | 16 h | 86.0 |
| **2** | | | | | | 85.3 |
| **1** | n-$Bu_3P$ | Neat | — | 100 °C | 24 h | 20.0 |
| **2** | | | | | | 20.0 |
| **1** | $PCl_5$ | 2.4 | THF | Reflux | 20 h | 0 |
| **2** | | | | | | 0 |
| **1** | $POCl_3$ | 2.4 | THF | Reflux | 40 h | 0 |
| **2** | | | | | | 0 |

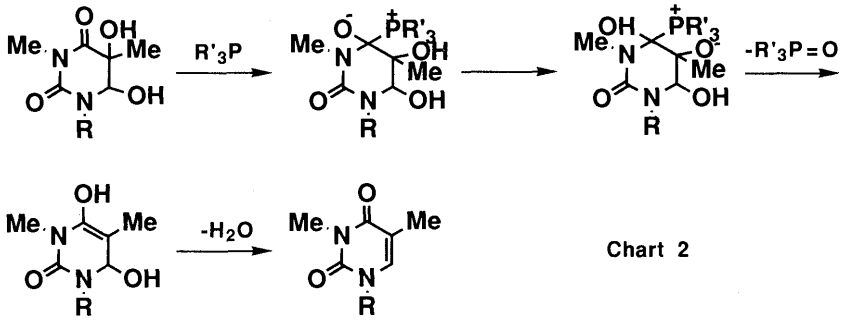


Chart 2

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(Received February 19, 1991)

# NEW SYNTHESIS OF 2-THIO-PAF AND RELATED COMPOUNDS AS SUBSTRATES OF PAF ACETYLHYDROLASE AND PHOSPHOLIPASE $A_2$

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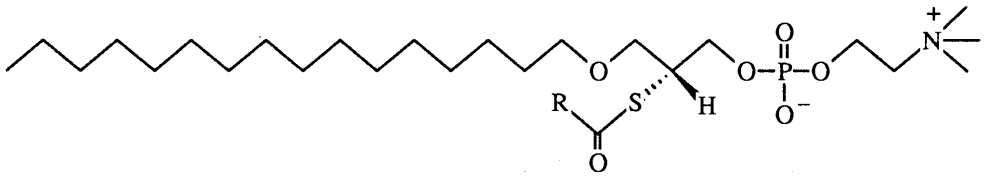
2-Thio-PAF and related compounds (**1a-c**) as substrates of PAF acetylhydrolase and phospholipase $A_2$ were synthesized stereoselectively from optically active 2-*O*-benzylglycerol monoacetate.

**KEYWORDS** 2-thio-PAF; PAF acetylhydrolase; phospholipase $A_2$; arachidonic acid; octanoic acid; stereoselective synthesis

The platelet-activating factor (PAF) is a potent lipid mediator with a wide spectrum of biological activities. The amount of PAF in tissues and body fluids is considered to be regulated by PAF acetylhydrolase, in addition to PAF-synthesize enzyme.[1] It has been reported that the level of PAF acetylhydrolase activity in blood/serum is correlated with respiratory symptoms in asthmatic children.[2] On the other hand, phospholipase $A_2$ is now thought to be a key enzyme to release arachidonate, which is a precursor of prostanoids, from membrane phospholipids after stimulation in various cells including neutrophils, macrophages and vascular endothelial cells.[3]

In this study, we developed a new and convenient method to synthesize 1-*O*-hexadecyl-2-thioacetyl-2-deoxy-*sn*-glycero-3-phosphocholine (2-thio-PAF, **1a**), 1-*O*-hexadecyl-2-thiooctanoyl-2-deoxy-*sn*-glycero-3-phosphocholine (**1b**) and 1-*O*-hexadecyl-2-thioarachidonyl-2-deoxy-*sn*-glycero-3-phosphocholine (**1c**). These were used in simple and reproducible methods for measuring PAF acetylhydrolase activity and phospholipase $A_2$ activity, respectively.[4]



**1a**: R= $CH_3$ (2-thio-PAF), **1b**: R=$CH_3(CH_2)_6$, **1c**: R=$CH_3(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_2$

Stereoselective synthesis of 2-thio-PAF from (*R*)-2,3-isopropylideneglycerol tosylate has been reported by Bhatia and Hajdu.[5] However, in their synthesis route, racemization at the 2-position and formation of 1-thioacetate were liable to occur *via* the formation of an epoxide during the introduction of the thioacetyl group. We developed a new synthesis route to prevent these side reactions. Our approach is as follows, (*S*)-1-*O*-acetyl-2-*O*-benzylglycerol, which is easily obtained by lipase-catalyzed esterification, is utilized as a chiral source, and a tetrahydropyranyl group is chosen to protect the primary hydroxyl group to prevent the formation of the epoxide and the S→O acyl migration.

Chart 1 shows the synthesis procedure for 2-thio-PAF and related compounds. Optically pure (*R*)-(-)-3-tosyloxy-2-benzyloxy-1-propanol (**3**) was obtained from a reaction of (*S*)-(+)-2-*O*-benzylglycerol monoacetate[6] (>90%ee **2**) with *p*-toluenesulfonyl chloride in pyridine, followed by chemoselective deacetylation using NaOH-MeOH and recrystallization of the product from isopropyl ether (90% from **2**). After protection of the hydroxyl group of **3** with dihydropyran and *p*-toluenesulfonic acid, the tosylate (**4**) was treated with hexadecanol in the presence of NaH in DMF to afford an alkylglycerol (**5**) (55% from **3**). Hydrogenation of **6** over 5% Pd-C in ethanol gave the alcohol (**6**) in a quantitative yield which was then treated with *p*-nitrobenzenesulfonyl chloride in the presence of triethylamine in $CHCl_3$. The resulting sulfonate (**7**) was treated with potassium thioacetate to give the thioacetate (**8a**). Reduction of **8a** with $LiAlH_4$ in THF gave the thiol which was then treated with octanoyl chloride or arachidonic acid / diethyl phosphorocyanidate[7] in the presence of triethylamine to give **8b** or **8c**. Deprotection of the tetrahydropyranyl group of **8a-c** using PPTS in ethanol at 55°C gave the alcohol. The crude alcohol was phosporylated with 2-bromoethyl phosphorodichloridate in the presence of triethylamine in $CHCl_3$ then

stirred with $H_2O$ to give the phosphorylate. The crude phosphorylate was heated at 65°C with excess trimethylamine in a sealed tube to afford 2-acylthiophospholipid (**1a-c**).[8] **1a**: $[a]_D$ -6.1°, **1b**: $[a]_D$ -2.7°, **1c**: $[a]_D$ -2.8° ($CHCl_3$/MeOH=4/1).

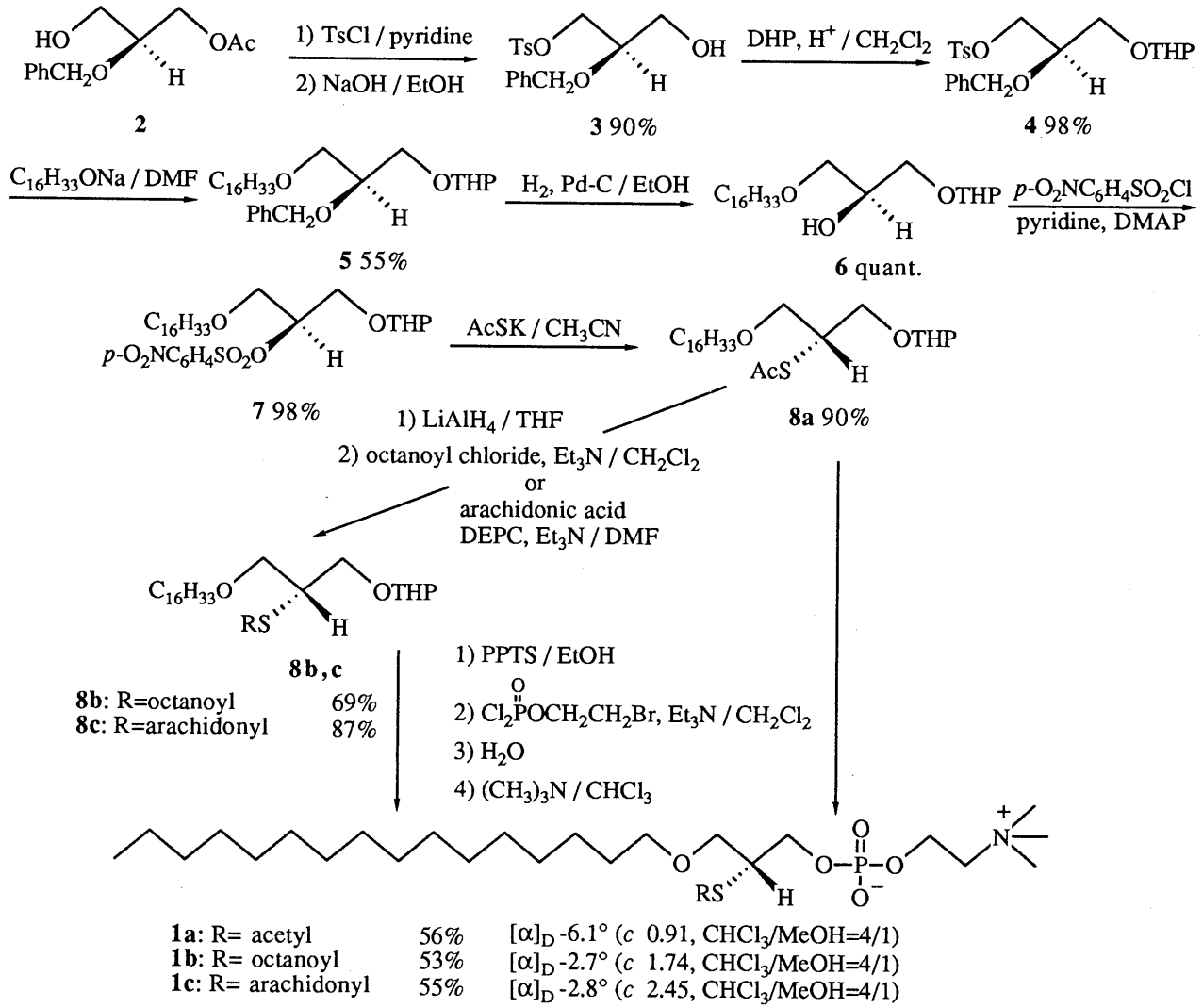



Chart 1

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(Received March 8, 1991)

# SYNTHESIS OF BIOLOGICALLY ACTIVE GALACTOSYL AND GLUCOSYL-GLYCEROL DERIVATIVES

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Galactosyl and glucosyl-glycerol derivatives which mimic the structure of the lipoteichoic acid of Staphylococcus aureus were synthesized by using a chiral glycerol derivative. Glucosyl-glycerol derivatives (14 and 22) had stronger anti-inflammatory activity than galactosyl-glycerol derivatives (13 and 21).

KEYWORDS lipoteichoic acid; galactosyl-glycerol derivative; glucosyl-glycerol derivative; anti-inflammatory activity; (S)-1-O-acetyl-2-O-benzyl-glycerol

Membrane teichoic acids or lipoteichoic acids are important components of the cell wall of most Gram-positive bacteria. They are located in the inner region of the cell membrane and the wall. The structure of a lipoteichoic acid of Staphylococcus aureus has been elucidated by Baddiley.[1] It consists of a glycolipid unit joined by a phosphodiester linkage to a glyceryl phosphate polymer (Chart 1).

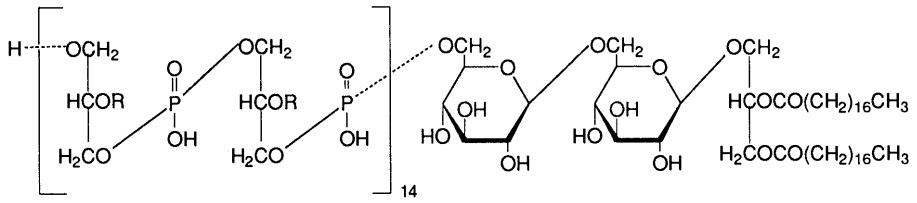


Chart 1

Recently, these glycerolipids (1 and 2) were isolated from the Okinawan marine sponge Phyllospongia foliacens by Kitagawa and coworkers.[2] A galactolipid (1) exhibits anti-inflammatory activity, and a sulfonoglycolipid (2) resists complement fixation reactions (Chart 2).

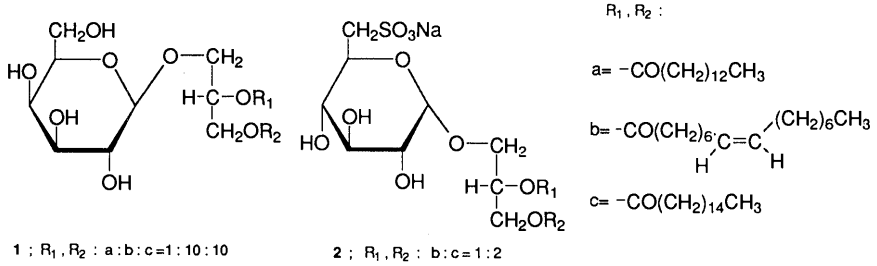


Chart 2

As a part of our synthesis studies of biologically active new compounds by modifying the cell wall structures of bacteria,[3] we report the stereoselective synthesis of (R)- and (S)-2,3-di-O-palmitoyl-1-O-galactopyranosyl-glycerol (13 and 21), (R)- and (S)-2,3-di-O-palmitoyl-1-O-glucopyranosyl-glycerol (14 and 22) in order to clarify the influence of such structural changes on anti-inflammatory activity.

First, (R)-glycerols, (R)-galactosyl-glycerol (13) and (R)-glucosyl-glycerol derivatives (14) were synthesized as shown in Chart 3. (S)-1-O-Acetyl-2-O-benzyl-glycerol (3)[4] was treated with 3,4-dihydro-2H-pyran and pyridinium p-toluenesulfonate (PPTS) in dichloromethane at 0℃, followed by deacetylation with $NH_4OH$-MeOH (1:10) at room temperature to give the tetrahydropyranylated compound (4) (83%, syrup, $[\alpha]_D$ +26.5°). The glycosylation of (R)-2-O-benzyl-1-O-tetrahydropyranyl-glycerol (4) with monosaccharide donors (5) and (6) was performed with $Ag_2O$, $I_2$, and powdered Molecular Sieves 4A (MS4A), followed by detetrahydropyranylation with PPTS in EtOH at 55℃ to give the glycosides (7) and (8) (7: 39%, syrup, $[\alpha]_D$ -17.8°, 8: 36%, syrup, $[\alpha]_D$ -16.6°). The configuration of the glycosidic linkage of 7 and 8 was assigned as $\beta$ from the $^1$H-NMR spectrum of their anomeric protons at $\delta$ 4.53 as a doublet with $J_{1',2}$=8.0Hz and $\delta$ 4.57 as a doublet with $J_{1',2}$=8.5 Hz, respectively. The benzyl groups in 7 and 8 were removed by hydrogenolysis with palladium-on-carbon (Pd-C) to give the 2,3-dihydroxyl compounds (9) and (10) in 94% and 99% yields, respectively. The acylation of 9 and 10 with palmitoyl chloride, trietylamine, and 4-dimethylaminopyridine (DMAP) gave the diacylated compounds (11) and (12) in 85% and 72% yields, respectively. Selective removal of the acetyl groups of 11 and 12 with hydrazine monohydrate[5] at reflux for 15 min in 85% EtOH gave the (R)-galactoglycerolipid (13) and (R)-glucoglycerolipid (14) (13; 18%, mp 83-84℃, $[\alpha]_D$ -6.8°, 14; 30%, mp 77-79℃, $[\alpha]_D$ -11.0°).

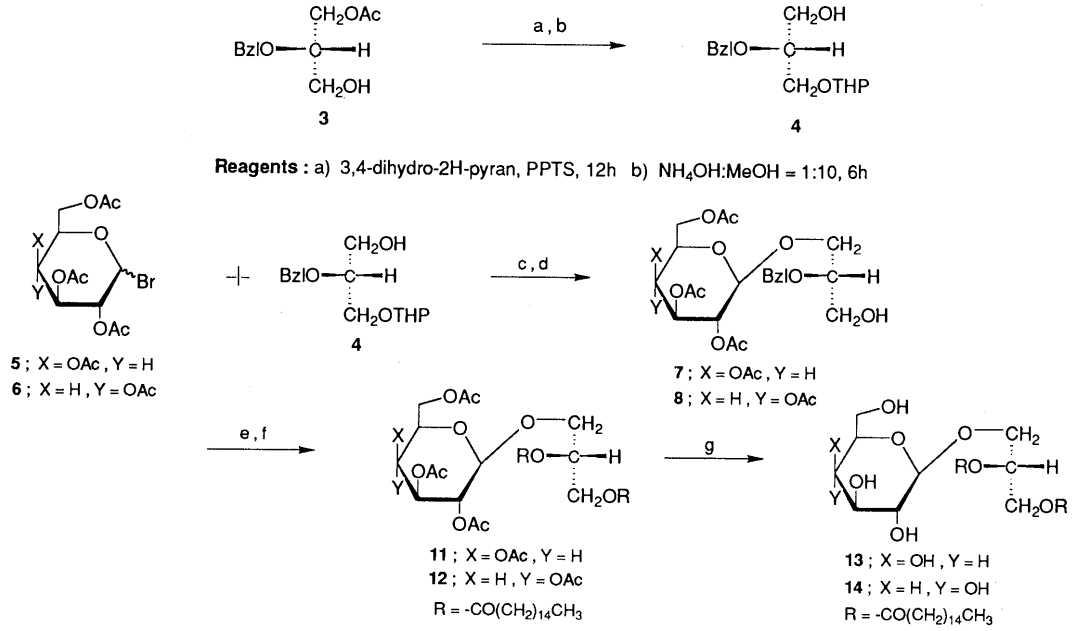


Chart 3

Next, (S)-glycerols, (S)-galactosyl-glycerol (21), and (S)-glucosyl-glycerol derivatives (22) were also synthesized as shown in Chart 4. The 3-hydroxyl group of 4 was protected with benzyl bromide, NaH, and n-$Bu_4NI$ to afford 15 in 96% yield. The removal of the tetrahydropyranyl group of 15 with PPTS in EtOH at 55℃ gave the glycosyl acceptor of (S)-1,2-di-O-benzyl-glycerol (16) (80%, syrup,

$[\alpha]_D$ -18.2°). Similarly, the glycosylation of 16 with monosaccharide donors (5) and (6) followed by debenzylation gave the 2,3-dihydroxyl compounds (17) and (18) in 67% and 65% yields, respectively. The β-configuration of the anomeric protons of 17 and 18 was confirmed by $^1$H-NMR spectrum data, which revealed a signal for H-1 at δ 4.53 (J=7.5 Hz) and at δ 4.56 (J=8.1 Hz), respectively. The acylation of 17 and 18 gave the diacylated compounds (19) and (20) in 66% and 58% yields, respectively. Finally, the selective deacetylation gave the (S)-galactoglycerolipid (21) and (S)-glucoglycerolipid (22) (21; 52%, mp 79-81℃, $[\alpha]_D$ -11.5°, 22; 22%, mp 82-84℃, $[\alpha]_D$ -11.7°). The structures of all compounds were characterized by $^1$H-NMR and $^{13}$C-NMR spectroscopy, as well as infrared (IR) spectroscopy and elemental analyses. The $^1$H-NMR spectrum of 21 was identical with that of 1c.

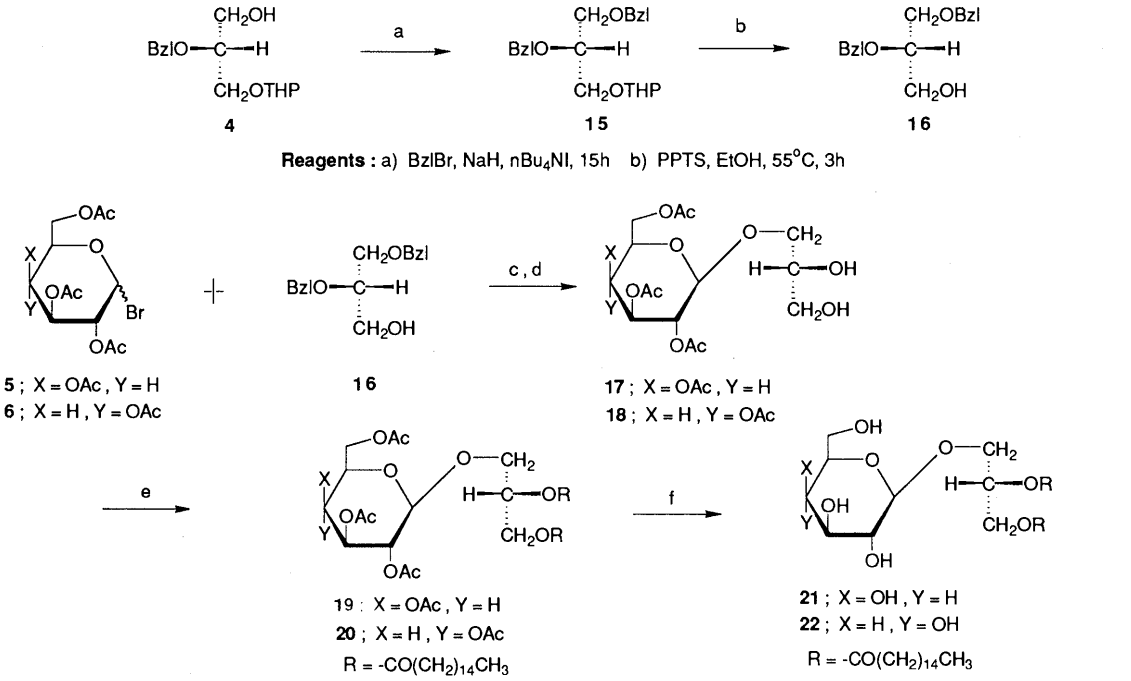


Chart 4

Preliminary biological examination revealed that glucosyl glycerolipids (14 and 22) had stronger anti-inflammatory activity than galactosyl glycerolipids (13 and 21).

ACKNOWLEDGEMENT We are grateful to Professor I. Kitagawa for providing the $^1$H-NMR spectrum of 1c.

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(Received March 8, 1991)

# AILANTHOIDINE, A NOVEL BENZO[C]PHENANTHRIDINE ALKALOID WITH A CYANOPYRIDINE PENDANT, FROM A XANTHOXYLUM SPECIES

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The structure of ailanthoidine isolated from the bark of *Xanthoxylum ailanthoides* Sieb. et Zucc. has been deduced as **1** on the basis of its spectral data including 2-D NMR spectroscopy and an X-ray analysis of **7B**.

**KEYWORDS** *Xanthoxylum ailanthoides*; benzo[c]phenanthridine; alkaloid; 2-D NMR; COLOC; X-ray analysis

In a previous paper,[1] we reported the isolation of a new alkaloid, ailanthoidine, along with many known components from the bark of *Xanthoxylum ailanthoides* Sieb. et Zucc.. Here we describe the structure elucidation of the new alkaloid (**1**).

Ailanthoidine (**1**), mp 260-263°C, $[\alpha]_D$ =0, shows a blue fluorescence on TLC and turns yellow on prolonged exposure to air or light, indicating that it is a dihydrobenzo[c]phenanthridine alkaloid.[2] Its IR spectrum shows a cyano group at 2235 $cm^{-1}$. Its $^1H$-NMR spectrum shows signals[2-4] characteristic of dihydrobenzo[c]phenanthridine with a substituent at $C_6$ (Table I), although $C_4$-H is shifted upfield by 1.11 ppm in comparison with dihydrochelerythrine (**2**) because of shielding by the $C_6$-substituent (*vide infra*).

An NOE analysis (Fig.1) confirmed the assignment. The EIMS of **1** showed a small molecular ion peak at *m/z* 479 corresponding to $C_{29}H_{25}N_3O_4$ and showed a diagnostic ion peak fragment at *m/z* 348 ($M^+$-131) due to the loss of the $C_6$-substituent ($C_8H_7N_2$, *m/z* 131) from the molecular ion. The $^1H$-NMR spectrum also showed five additional signals due to the $C_6$-substituent group (Table I). Decoupling experiments showed that the signal at δ 2.68 was coupled with $C_6$-H (δ 4.75) and a methyl group (δ 1.22), suggesting that the substituent group on $C_6$ has a secondary methyl moiety. Moreover, the coupling pattern (ABX system) of three aromatic protons indicates the presence of a 2,4-disubstituted pyridine ring containing a cyano group in the $C_6$-substituent. NOE enhancement occurred between $C_\alpha$-H and $C_{3'}$-H (12.8%) but not between $C_\alpha$-H and $C_{5'}$-H. This shows that a plane structure of ailanthoidine can be represented by formula **1**, as an NOE would exist between $C_\alpha$-H and both $C_{3'}$-H and $C_{5'}$-H if ailanthoidine (**1**) had a 2-cyanopyridine moiety. To obtain additional support for the structure (**1**) of ailanthoidine, the $^{13}C$-NMR spectra of **1**, **2**, 4-cyano-2-ethylpyridine (**3**),[5] and 2-cyano-4-ethylpyridine (**4**)[7] were measured. The $^{13}C$-NMR spectrum data are given in Tables II and III. Methyl, methylene, and methine carbons of **1** and **2** were assigned using $^1H$-$^{13}C$ COSY, and quarternary carbons were assigned by COLOC except the 2' and 4' carbons (δ 119.4 and 166.7) in the pyridine ring.(Fig 2). Table III shows that the chemical shifts of the carbons in the $C_6$-substituent of **1** are very similar to those of **3** (not **4**). This allowed us to assign these two unidentified carbons, as shown in Table III and indicates that the plane structure of ailanthoidine can be represented by formula **1** having a 4-cyanopyridine moiety. To determine the stereochemistry, we subjected **1** to X-ray analysis, but it did not provide crystals suitable for the structural determination.

In a synthesis study on ailanthoidine (**1**), treatment of chelerythrine chloride (**6**) with carbanion prepared from 4-ethylpyridine and LDA[9] in HMPA-THF afforded two diastereomeric adducts, A (**7A**), mp 217-219°C, and B (**7B**), mp 178-180°C, in 19 and 11% yields, respectively.[10] As shown in Table I, the signals due to $C_4$-H (δ 6.95) in **7A** and to $C_7$-$OCH_3$ (δ 3.50) in **7B** are shifted upfield by 0.72 and 0.38 ppm, respectively, in comparison with the corresponding signals in **2**. These observations could be reasonably explained by anisotropy due to a newly introduced pyridine ring.

Since $C_4$-H of ailanthoidine (**1**) also was shifted upfield, the findings definitely indicate that **7A** has the same stereochemical relationship as **1** with respect to $C_6$ and $C_\alpha$, although the position of the substituent group(s) on the pyridine ring was different. Unfortunately, a natural type of **7A** did not provide crystals suitable for X-ray analysis, so an artificial type of **7B** was analyzed by X-ray.[11] The molecular structure of **7B** is shown in Fig. 3, so the stereostructure of adduct A can be represented by formula **7A**. Consequently, we propose formula **1** as the stereostructure of ailanthoidine.[13]

Table I. $^1$H-NMR Data for Ailanthoidine (**1**), Dihydrochelerythrine (**2**), Adduct A (**7A**), and Adduct B (**7B**)[a]

| | 1[b] | 2 | 7A[b] | 7B[b] |
|---|---|---|---|---|
| NMe | 2.39 s | 2.60 s | 2.45 s | 2.62 s |
| $C_7$-OMe | 3.99 s | 3.88 s | 3.99 s | 3.50 s |
| $C_8$-OMe | 3.97 s | 3.93 s | 3.96 s | 3.80 s |
| $OCH_2O$ | 6.05, 6.06 d (1.4) | 6.04 s | 5.99, 6.04 d (1.3) | 6.06, 6.08 d (1.3) |
| 1-H | 7.11 s | 7.11 s | 7.08 s | 7.13 s |
| 4-H | 6.56 s | 7.67 s | 6.95 s | 7.68 s |
| 6-H | 4.75 d (10.5) | 4.29 s | 4.52 d (10.7) | 4.43 d (8.2) |
| 9-H | 7.02 d (8.5) | 6.94 d (8.5) | 7.00 d (8.4) | 6.91 d (8.6) |
| 10-H | 7.57 d (8.5) | 7.51 d (8.5) | 7.54 d (8.4) | 7.50 d (8.6) |
| 11-H | 7.74 d (8.5) | 7.70 d (8.5) | 7.68 d (8.6) | 7.74 d (8.6) |
| 12-H | 7.49 d (8.5) | 7.47 d (8.5) | 7.45 d (8.6) | 7.48 d (8.4) |
| CMe | 1.22 d (6.9) | --- | 1.12 d (7.2) | 1.21 d (7.1) |
| $C_\alpha$-H | 2.68 dq (10.5, 6.9) | --- | 2.56 dq (10.7, 7.2) | 2.71 dq (8.2, 7.1) |
| 3'-H | 6.41 br s | --- | --- | --- |
| 5'-H | 7.31 dd (5.0, 1.4) | --- | 6.82 d (5.9)[c] | 6.89 d (5.9)[c] |
| 6'-H | 8.79 dd (5.0, 0.6) | --- | 8.36 d (5.9)[d] | 8.35 d (5.9)[d] |

a) Chemical shifts are given in δ (ppm) and the values in parentheses are coupling constants in Hz.
b) Assignments were made with the aid of NOE analysis. c) Including 3'-H. d) Including 2'-H.

Table II. $^{13}$C-NMR Data for the Skeletal Carbons in **1** and **2**

| | 1 | 2 |
|---|---|---|
| NMe | 41.8 | 41.3 |
| OMe | 55.8, 61.0 | 55.8, 61.1 |
| $OCH_2O$ | 101.1 | 101.0 |
| 1 | 104.4 | 104.3 |
| 2 | 148.0 | 148.1 |
| 3 | 147.6 | 147.5 |
| 4 | 100.2 | 100.7 |
| 4a | 130.9 | 130.8 |
| 4b | 139.3 | 142.7 |
| 6 | 62.6 | 48.7 |
| 6a | 124.0 | 124.2 |
| 7 | 147.0 | 146.1 |
| 8 | 152.1 | 152.3 |
| 9 | 111.5 | 111.0 |
| 10 | 119.3 | 118.7 |
| 10a | 127.3 | 126.4 |
| 10b | 124.9 | 126.3 |
| 11 | 119.8 | 120.1 |
| 12 | 123.8 | 123.8 |
| 12a | 126.7 | 126.3 |

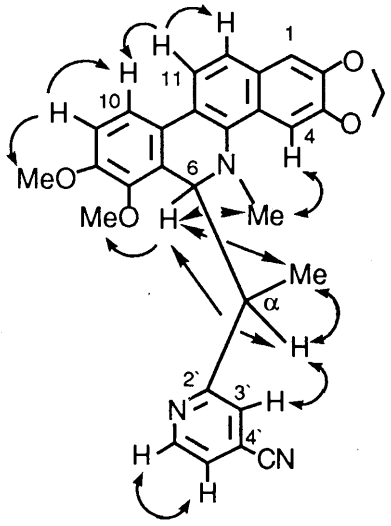



Fig. 1. NOEs Observed in **1**

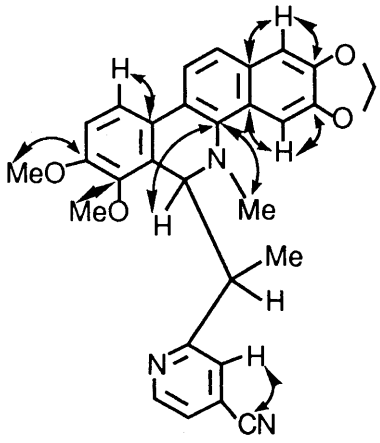
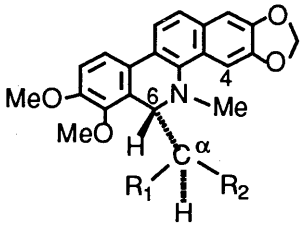
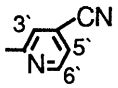



Fig. 2. Long - Range Coupling Observed in COLOC Experiment of **1**

Table III. $^{13}C$-NMR Data for the $C_6$ Side Chain of **1**, **3**, and **4**

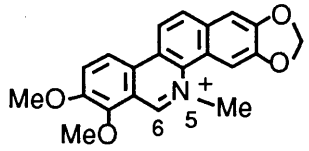
| | 1 | 3 | 4 |
|---|---|---|---|
| CMe | 16.9 | 13.4 | 14.0 |
| $C_\alpha$ or $CH_2$ | 44.9 | 31.3 | 28.0 |
| CN | 116.8 | 116.8 | 117.5 |
| 2' | 166.7 | 165.1 | 133.8 |
| 3' | 126.0 | 123.8 | 128.3 |
| 4' | 119.4 | 120.6 | 154.7 |
| 5' | 122.1 | 122.4 | 126.7 |
| 6' | 149.9 | 150.2 | 150.9 |



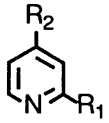
(1) $R_1$ = Me $R_2$ = 

(7A) $R_1$ = Me $R_2$ = 

(7B) $R_1$ =  $R_2$ = Me



(2) 5,6-dihydro (6)



(3) $R_1$ = Et $R_2$ = CN

(4) $R_1$ = CN $R_2$ = Et

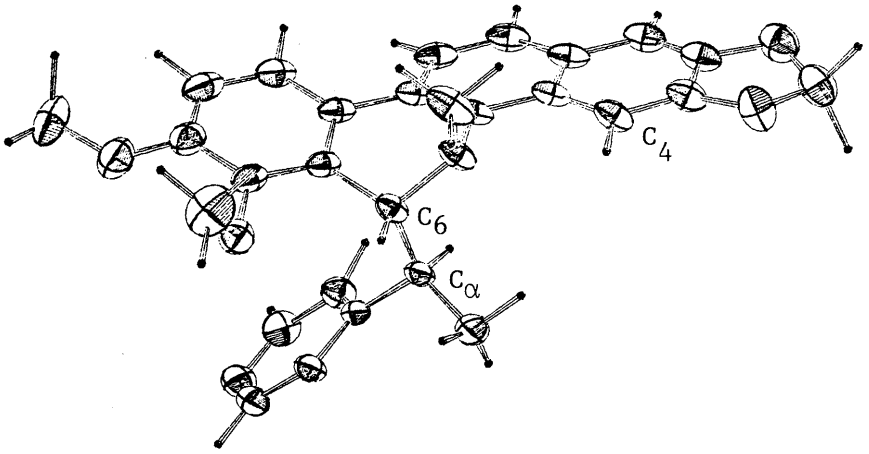



Fig. 3. Molecular Structure of Adduct B (**7B**)

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5) 4-Cyano-2-ethylpyridine (**3**) was prepared by dehydration of ethionamide with bis(tri-n-butyl)tin oxide[6] in 80.3% yield.
6) M.-I.Lim, W.-Y.Ren, and R.S.Klein, *J. Org. Chem.*, **47**, 4594 (1982).
7) 2-Cyano-4-ethylpyridine (**4**) was prepared in 85.2% yield by treating 4-ethylpyridine N-oxide[8] with trimethylsilyl cyanide.
8) M.Shimizu, T.Naito, G.Ohta, T.Yoshikawa, and K.Dohmori, *Yakugaku Zasshi*, **72**, 1474 (1952).
9) M.P.LaMontagne, M.S.Ao, A.Markovac, and J.R.Menke, *J. Med. Chem.*, **19**, 360 (1976).
10) Treating **6** with 2-ethylpyridine, **3** or **4** did not give isolable products under similar reaction conditions.
11) Crystal data for **7B**: $C_{28}H_{26}N_2O_4$, M=454.5, triclinic, space group $P_1$ with unit cell dimensions a=14.441(7), b=18.523(3), c=9.041(6) Å, $\alpha$=93.07(4)°, $\beta$=102.47(5)°, $\gamma$=86.63(2)°, $U$=2355.2 Å$^3$, $Z$=4, and $D_C$=1.282 g/cm$^3$. The reflection data were collected on a Rigaku AFC-5 diffractometer for 3°< 2$\theta$< 120° using Cu$K_\alpha$ radiation ($\lambda$=1.54 Å) and the $\omega$<30°<$\omega$–2$\theta$ scan method at a 2$\theta$ scan speed of 4°/min. The structure was solved by the direct method using MULTAN (UNICS-III system[12]) program and refined by full-matrix least-squares. The final $R$ value was 0.0623 for 5948 independent reflections [$F_O$>3$\sigma$($F_O$)].
12) T.Sakurai and K.Kobayashi, *Rep. Inst. Phys. Chem. Res.*, **55**, 69 (1979).
13) To our knowledge, this is the first alkaloid having a cyanopyridine pendant.


(Received March 11, 1991)

# SYNTHESIS OF SAFRAMYCINS. VI. THE USEFUL TRANSFORMATION OF (-)-SAFRAMYCIN A TO (-)-SAFRAMYCIN Mx TYPE COMPOUND[1)]

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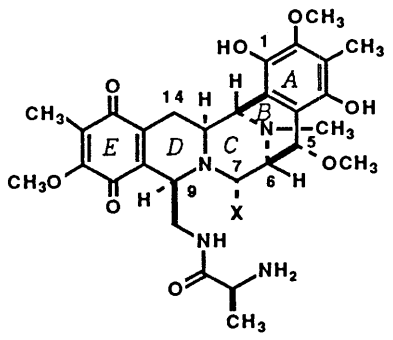
A transformation of (-)-saframycin A **3** to the saframycin Mx type compound **15** is described. Treating (-)-saframycin A **3** with selenium oxide in acetic acid afforded (-)-saframycin G **8**, followed by catalytic reduction and regioselective oxidation to provide the hydroquinone **15**.

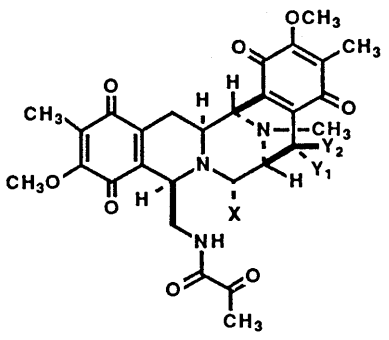
KEYWORDS saframycin; transformation; regioselective oxidation; hydroquinone

The saframycins Mx 1 **1** and Mx 2 **2** were discovered in the culture broth of the myxobacterium, *Myxococcus xanthus* strain Mx x48 in 1988.[2)] Their relative and absolute configurations were derived from NMR studies and by comparing the optical rotations with those of known safracins. These compounds were clearly similar to the saframycins which showed potent antitumor activities.[3)] Saframycins Mx were very sensitive to light and to oxygen, and above pH 7 were quickly oxidized to the bisquinone because of they have a hydroquinone group at the A ring. We recently reported the total synthesis of (±)-saframycin B **4**[4)] and the transformation of (±)-2 to (±)-saframycins C **5** and D **6**.[1)] We report here the transformation of (-)-saframycin A **3** to the saframycin Mx type hydroquinone **15** as a part of a synthesis endeavor in the area of saframycins.

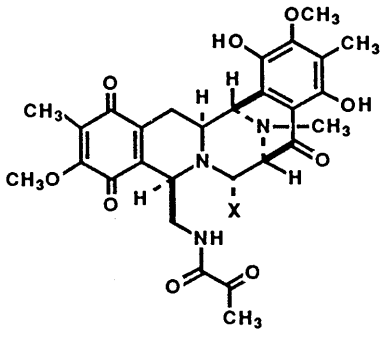
Treating (-)-saframycin A **3** with selenium oxide (10 eq) in acetic acid at room temperature for 64 h afforded (-)-saframycin G **8**,[5)] ($[\alpha]_D$ -46.0° (*c* = 1.0, MeOH)) in 30.4% yield (3.6% yield of **3** was recovered) along with (-)-saframycin G acetate **9**,[5)] ($[\alpha]_D$ -40.0° (*c* = 0.8, MeOH)), (+)-saframycin F **7**,[5)] ($[\alpha]_D$ +135.7° (*c* = 0.6, MeOH)), and (+)-5-*epi*-saframycin G **10**,[5)] ($[\alpha]_D$ +28.7° (*c* = 0.87, MeOH)) in 7.1%, 3.8%, and 7.9% yields, respectively. The hydroxy stereochemistry of **8** was assigned on the basis of a 0.5 Hz coupling between H-5 (δ 4.321) and H-6 (δ 3.459). The H-5 (δ 5.085) and H-6 (δ 3.481) coupling for the C-5 isomer **10** was 7.3 Hz. Acetylation of **8** with acetic anhydride in pyridine at room temperature for 1 h



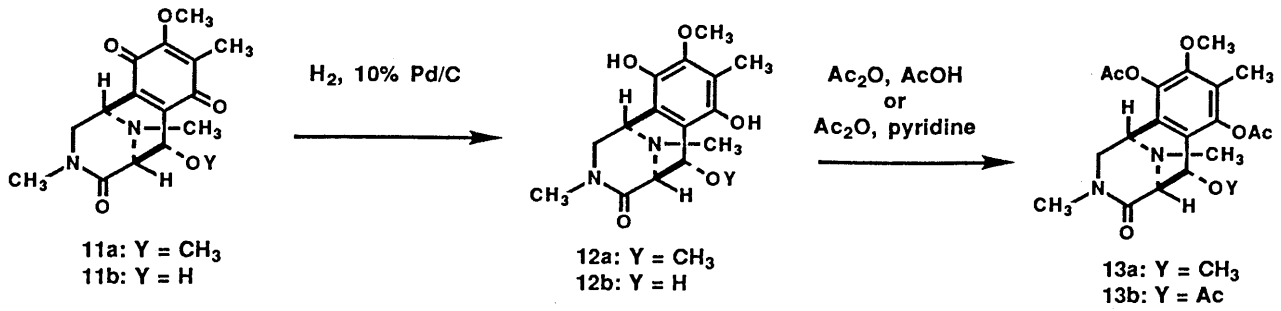
**saframycins**
**Mx 1 (1): X = OH**
**Mx 2 (2): X = H**



**saframycins**
**A (3): X = CN, $Y_1$ = $Y_2$ = H**
**B (4): X = $Y_1$ = $Y_2$ = H**
**C (5): X = $Y_2$ = H, $Y_1$ = $OCH_3$**
**G (8): X = CN, $Y_1$ = OH, $Y_2$ = H**
**9: X = CN, $Y_1$ = OAc, $Y_2$ = H**
**10: X = CN, $Y_1$ = H, $Y_2$ = OH**



**saframycins**
**D (6): X = H**
**F (7): X = CN**




11a: Y = $CH_3$
11b: Y = H

12a: Y = $CH_3$
12b: Y = H

13a: Y = $CH_3$
13b: Y = Ac

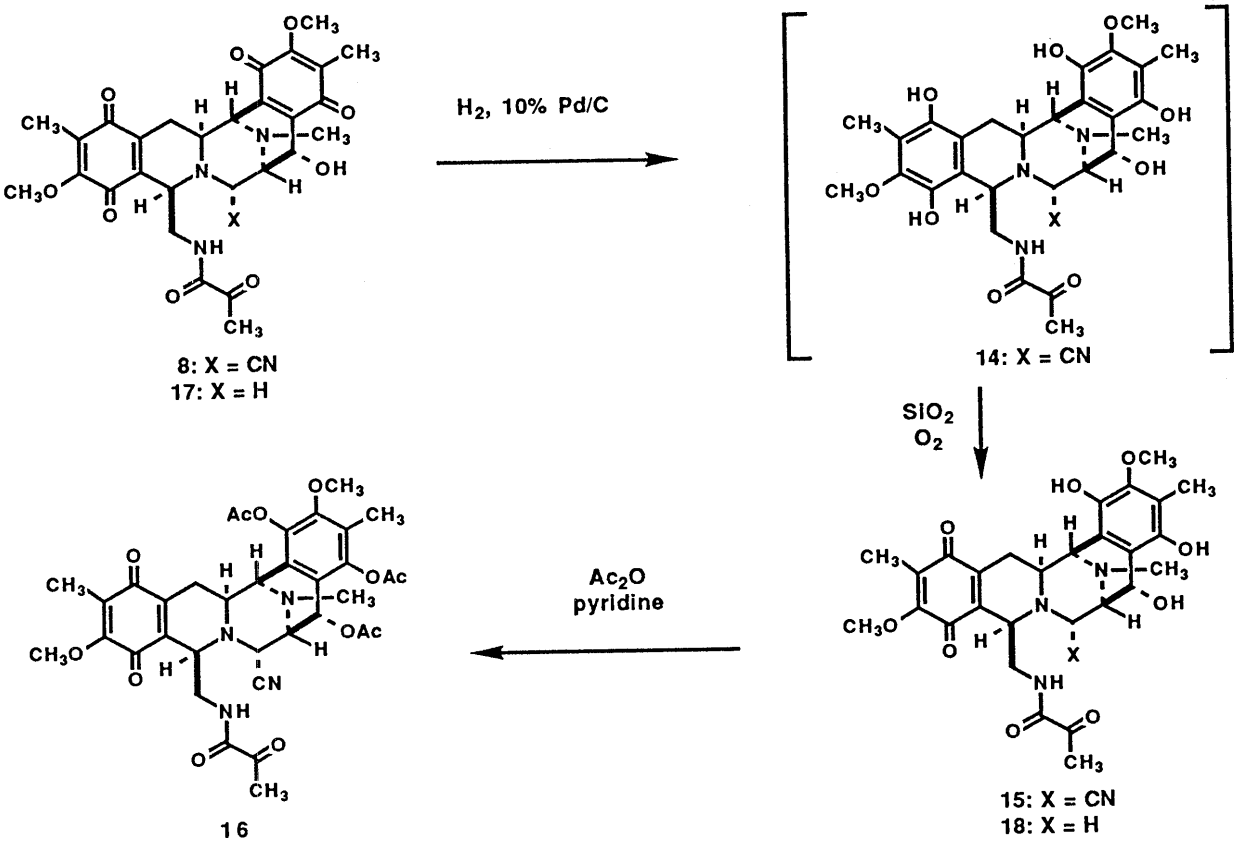
afforded **9** (65.8% yield), whose $^1$H NMR spectrum indicated a low-field shift of the signal of the H-5 proton (δ 5.395, $J$ = 0.5 Hz). The synthetic saframycins F and G were identical with the natural one when spectroscopic $^1$H NMR, $^{13}$C NMR, IR, UV, MS, and TLC data were compared.[6]

Subsequently we studied the regioselective reduction of the quinone at the A ring to the hydroquinone. A preliminary experiment was carried out using the readily available model compounds **11a** and **11b**.[1] Hydrogenation of the 5-methoxyquinone **11a** with 10% Pd/C in ethyl acetate gave the hydroquinone **12a**,[5] (mp 210-212°C) (69.2%), IR (KBr): 3510, 3400-3020, 1645, 1620 cm$^{-1}$; UV $\lambda_{max}$ nm (log ε): 237 (3.39), 293 (3.60); EI-MS (m/z); 322 ($M^+$, 67), 250 (100); $^1$H NMR (270 MHz, $CDCl_3$): 2.20 (3H, s, Ar $CH_3$), 2.61 (3H, s, $NCH_3$), 2.86 (3H, s, $NCH_3$), 3.20 (1H, d, $J$ = 12.2 Hz, H-14aα), 3.59 (3H, s, $OCH_3$), 3.78 (3H, s, $OCH_3$), 3.81 (1H, dd, $J$ = 0.7, 0.5 Hz, H-6), 3.93 (1H, dd, $J$ = 12.2, 5.0 Hz, H-14aβ), 4.23 (1H, dd, $J$ = 5.0, 0.5 Hz, H-15), 4.45 (1H, d, $J$ = 0.7 Hz, H-5), 5.66 (1H, s, OH), 6.24 (1H, s, OH). Treating **12a** with acetic anhydride in acetic acid at 100°C for 20 h gave **13a**[5] in 71.4% yield.[7] Hydrogenation of the 5-hydroxyquinone **11b** with 10% Pd/C in ethyl acetate gave the hydroquinone **12b**,[5] (mp 221-223°C (dec.)) (60.8%). Acetylation of **12b** with acetic anhydride in pyridine gave **13b**[5] (mp 232-234°C) (63.2% yield), whose $^1$H NMR spectrum indicated a low-field shift of the signal of the H-5 proton (δ 6.08, s). Thus we efficiently synthesized the hydroquinone **12a** and **12b**,[8] embodying all of the skeletal features of the "right half" of the saframycins Mx.

Encouraged by the results of these model studies, we successfully applied the transformation of (-)-saframycin G **8** to the saframycin Mx type compound **15**. Unlike the ABC model, the saframycin system has the potential to form the hydroquinone isomers, thereby creating a regiochemical problem in the reduction and/or oxidation steps. But we were able to anticipate that this oxidation might be highly regioselective in the chemistry of the saframycins Mx.[2] Hydrogenation of **8** with 10% Pd/C in ethyl acetate for 20 min gave the leuco compound **14**[8] in quantitative yield. This is not isolatable and after removing the solvent in vacuo, treating **14** with $SiO_2$ in ethyl acetate at room temperature for 24 h in the presence of oxygen afforded (-)-**15**[5] (mp 170-172°C (dec.)) (52% yield), $[\alpha]_D$ -27.8° ($c$ = 1.0, MeOH).[9] Assignment of **15** was made by 400 MHz $^1$H NMR analysis. The diagnostic homoallylic coupling (2.7 Hz) between H-9 (δ 3.93) and H-14β (δ 1.57) over five bonds was observed, together with the data of natural compounds.[10] Acetylation of **15** with acetic anhydride in pyridine gave the acetate **16**[5] (45.8% yield) ($[\alpha]_D$ -63.0° ($c$ = 1.0, $CHCl_3$)). The structure of the acetate **16** was supported by the $^{13}$C NMR spectrum, which showed seven carbonyl carbon peaks at δ 161.3, 168.5, 169.8, 170.4, 180.3, 185.8, and 195.7. In addition, the $^1$H NMR spectrum showed nine methyl peaks at δ 1.91, 2.09, 2.12, 2.19, 2.26, 2.43, 2.51, 3.81, and 4.08.

In summary, we have achieved a useful transformation of (-)-saframycin A **3** to (-)-saframycin Mx type compound **15** via (-)-saframycin G **8**. Efforts to apply it to the total synthesis of saframycins Mx are now being made.[11]

**ACKNOWLEDGMENTS** We are grateful to Professor Tadashi Arai of Chiba University for a sample of saframycin A and a copy of $^1$H NMR spectrum (100 MHz, pyiridine-d5) of (-)-saframycin E. We are also greatly indebted to Dr. N. Shimma (Nippon Roche Research Center) for measurement of FAB-MS.

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4) A. Kubo, N. Saito, H. Yamato, K. Masubuchi, and M. Nakamura, *J. Org. Chem.*, **53**, 4295 (1988); For an alternative total synthesis of (±)-saframycin B see: T. Fukuyama and R. A. Sachleben, *J. Am. Chem. Soc.*, **104**, 4957 (1982).
5) All structural assignments were confirmed by $^{1}H$ NMR, $^{13}C$ NMR, infrared, ultraviolet, and mass spectra.
6) Y. Mikami, K. Takahashi, K. Yazawa, C. Hour-Young, T. Arai, N. Saito, and A. Kubo, *J. Antibiot.*, **41**, 734 (1988).
7) In constract to the conversion of **12b** to **13b**, acetylation of **12a** with acetic anhydride in pyridine at room temperature did not yield the acetate **13a** but restored **12a** to **11a** in 81.3% yield.
8) For simplicity, the proper IUPAC names and numbering of saframycins are used in this paper.
9) In terms of mitomycin chemistry, the leuco form of mitomycin F reacts with silica gel in the presence of oxygen to afford 9-*epi*-mitomycin B see: M. Egbertson, S. J. Danishefsky, and G. Schulte, *J. Org. Chem.*, **52**, 4424 (1987) and references cited therein.
10) This coupling was negligible in the spectra of the pentacyclic methoxyarene **14**. $^{1}H$ NMR (270 MHz) δ 1.83 (1H, dd, H-14β), 2.18 (6H, s, Ar $CH_3$ x 2), 2.21 (3H, s, $COCH_3$), 2.40 (3H, s, $NCH_3$), 3.11 (1H, dd, H-14a), 3.16 (br d, H-14α), 3.36 (2H, br s), 3.58 (1H, m), 3.71 (3H, s, $OCH_3$), 3.78 (3H, s, $OCH_3$), 4.08 (2H, br s), 4.24 (1H, br s), 4.54 (1H, s, H-5), 6.52 (1H, br d, NH). However, due to serious line broadening in the $^{1}H$ NMR spectrum of the leuco compound in methanol-d4, a definitive assignment of the protons via decoupling is not possible. $^{13}C$ NMR δ ($CD_3OD$), 9.25 (q), 9.54 (q), 24.3 (q), 25.8 (t), 41.0 (t), 42.5 (q), 56.1 (d), 57.0 (d), 57.1 (d), 57.4 (d), 60.6 (q), 60.8 (q), 64.0 (d), 64.9 (d), 116.6 (s), 117.4 (s), 117.8 (s), 117.9 (s), 118.0 (s), 118.5 (s), 119.7 (s), 139.4 (s), 140.3 (s), 144.3 (s), 144.3 (s), 146.3 (s), 146.8 (s), 160.6 (s), 195.9 (s).
11) We carried out a similar transformation of (±)-**17**[1] to the hydroquinone **18**[5] (mp 189-191°C (dec.)) in 64.5% yield. Comparison of spectral data of (±)-**18** with those of reported natural saframycin E[12] showed that synthetic **18** is similar to natural saframycin E. However, complete identification was not possible, as the natural sample was not available.
12) Saframycin E is a novel antitumor antibiotic discovered in the culture broths of *Streptomyces lavendulae* along with saframycins A-D. The structure of saframycin E was not elucidated because none of the original sample remains, and repeated attempts to isolate the compound were unsuccessful. T. Arai, K. Takahashi, and A. Kubo, *J. Antibiot.* **30**, 1015 (1977).


(Received March 14, 1991)

# PERMEABILITY OF GLYCOSIDES THROUGH HUMAN ERYTHROCYTE MEMBRANE

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The permeability of glycosides (arbutin, salicin, glycyrritin, p-nitrophenyl- β -D-glucopyranoside, p-nitrophenyl- β -D-galactopyranoside, p-nitrophenyl- β -D-lactopyranoside, p-nitrophenyl- β -D-maltopyranoside) and their aglycons through human erythrocyte membrane was investigated. The glycosides permeated slowly, compared with their aglycons. Glycoside having disaccharide did not permeate the erythrocyte membrane. This suggested that the introduction of disaccharide to a drug significantly depresses the permeability of glycoside through erythrocyte membrane. The drug entrapped in erythrocytes was not released into the outer medium.

**KEYWORDS** transport; human erythrocyte; drug delivery system; glycoside; aglycon; disaccharide; targetting

As erythrocytes circulate for a long time (120 days is the average lifespan in humans), they may be accessible as a storage depot for the sustained release of drugs. The erythrocyte membranes have been used as drug delivery systems because they are biodegraded in the body. [1,2] As the mechanism of permeation was not elucidate completely, we need to study the matter further.

In our previous paper, [3] the regulation of the permeation rate and capacity through human erythrocyte membrane may be useful to control the release of drug from the erythrocyte carriers. The erythrocyte carriers-entrapped impermeative drugs can deliver selectively to the liver and spleen because most of the erythrocyte carriers are destroyed in them. The attempts to entrap the drug in the erythrocytes are carried out with protein and enzymes which are high-polymer. [1] If an anticancer medicine were entrapped in the erythrocyte carriers, it might be delivered selectively to the liver or spleen. But attempts to make the low molecular weight drugs permeative have not been successful. It is important to discover substituents impermeatized by drug bindings.

Here, we report on in vitro experiments on the permeation through human erythrocyte membrane of a glycoside (arbutin (ARB), salicin (SAL), glycyrritin (GLRI), p-nitrophenyl- β -D-glucopyranoside (NGLP), p-nitrophenyl- β -D-galactopyranoside (NGAP), p-nitrophenyl- β -D-lactopyranoside (NLP) and p-nitrophenyl- β -D-maltopyranoside (NMP)) and its aglycon (hydroquinone (HYD), saligenin (SALG), glycyrretinic acid (GLRE) and p-nitrophenol (NP)).

Erythrocytes were prepared according to a previous report. [3] All reagents used were high grade. The permeability of drugs through human erythrocyte membrane was investigated by the methods described previously. [3] The intracellular concentration of drugs was analyzed by high performance liquid chromatography (the detector was spectrofluorometer or spectrophotometer). The erythrocyte-entrapped GLRI, NLP or NMP was prepared according to preswollen ghost methods. [4] The dissociation constant of drugs was calibrated by the usual method. The partition coefficient of drugs (P) was measured by the octanol-water system at 37°C. P showed the ratio drug concentration in octanol divided by the concentration of the molecular-form drug in water.

Table I shows Tmax (the time at maximal intracellular concentration) and distribution of the drug in the suspending medium, the erythrocyte cytosol and the erythrocyte membrane. ARB and SAL, glycosides having monosaccharide and HYD, SALG and GLRE permeated human erythrocyte membrane although the drugs varied in the rate and capacity of the transport. On the other hand, the glycoside was markedly different from its aglycon in the rate of transport. In the aglycons, HYD, SALG and GLRE, the rate of transport was so rapid that the equilibrium was attained almost within one minute. In contrast to these drugs, the permeation of SAL was slower and it took 4 h before the equilibrium was attained. The

Table I. Distribution of Drugs in the Suspending Medium, the Erythrocyte Cytosol and the Erythrocyte Membrane

| Drug | Medium | Fraction | Percentage (the detected amount/the total amount added) of the drug detected in each fraction | | | Tmax |
|---|---|---|---|---|---|---|
| | | | Suspending medium | Cytosol components | Membrane | |
| SAL | PBS | | 55.9 ± 1.3 | 42.0 ± 2.3 | 0.58 ± 0.13 | 4 h |
| | plasma | | 57.0 ± 2.2 | 41.4 ± 3.4 | 0.87 ± 0.50 | |
| SALG | PBS | | 43.3 ± 1.8 | 58.3 ± 1.5 | 0.70 ± 0.20 | 1 min |
| | plasma | | 53.6 ± 2.4 | 46.4 ± 1.2 | 0.92 ± 0.59 | |
| ARB | PBS | | 68.8 ± 1.9 | 28.3 ± 1.2 | 0.86 ± 0.40 | > 6 h |
| | plasma | | 68.6 ± 2.3 | 28.8 ± 1.0 | 0.67 ± 0.55 | |
| HYD | PBS | | 41.5 ± 1.8 | 60.2 ± 1.4 | 0.63 ± 0.41 | 1 min |
| | plasma | | 43.7 ± 0.4 | 56.2 ± 1.0 | 0.79 ± 0.37 | |
| GLRI | PBS | | 98.3 ± 1.1 | 5.4 ± 0.1 | 0.18 ± 0.13 | ---- |
| | plasma | | 94.5 ± 1.2 | 4.7 ± 1.0 | 0.86 ± 0.40 | |
| GLRE | PBS | | 17.4 ± 0.4 | 76.9 ± 4.3 | 7.85 ± 1.59 | 1 min |
| | plasma | | 90.1 ± 0.5 | 3.1 ± 0.1 | 0.05 ± 0.01 | |
| NP | PBS | | 31.9 ± 1.1 | 66.3 ± 0.8 | 0.49 ± 0.29 | 1 min |
| | plasma | | 76.5 ± 0.9 | 20.2 ± 0.4 | 0.50 ± 0.13 | |
| NGLP | PBS | | 52.7 ± 1.1 | 46.6 ± 1.0 | 0.55 ± 0.12 | 1 h |
| | plasma | | 56.2 ± 0.9 | 44.1 ± 0.8 | 0.45 ± 0.22 | |
| NGAP | PBS | | 48.3 ± 0.3 | 52.9 ± 0.3 | 0.31 ± 0.10 | 1 h |
| | plasma | | 52.0 ± 0.8 | 49.7 ± 0.3 | 0.16 ± 0.09 | |
| NLP | PBS | | 95.6 ± 0.5 | 3.3 ± 0.4 | 0.35 ± 0.19 | ---- |
| | plasma | | 95.3 ± 0.4 | 2.8 ± 0.5 | 0.35 ± 0.02 | |
| NMP | PBS | | 95.1 ± 1.1 | 3.7 ± 0.2 | 0.49 ± 0.18 | ---- |
| | plasma | | 97.4 ± 0.6 | 3.0 ± 0.1 | 0.33 ± 0.08 | |

Erythrocytes (0.9 ml) were incubated with 1.1 ml of drug-containing PBS or plasma at pH 7.4 at 37°C until the concentration came to equilibrium (except ARB, 6 h) and then centrifuged at 2,900 x g for 15 min to give the sup. (the suspending medium containing the remaining drug) and the ppt. (erythrocytes). The erythrocytes were homogenized with one volume of distilled water and the resultant hemolysate was centrifuged at 725 x g or 16,260 x g for 15 min to give the hemolysate sup. (containing the cytosol component) and ppt. (the membrane). Data reported represent the means ± S.D. of three or four experiments.

permeation of ARB was still slower and the permeation increased fairly linearly during incubation for 6 h. The intracellular concentration was lower in permeation by ARB and SAL than HYD and SALG, respectively. GLRI, having disaccharide, barely permeated the human erythrocyte membrane, even though the ARB and SAL permeated, though the rate of transport was slow. The concentration of the permeated aglycon was higher in the permeation from PBS to erythrocytes than from the plasma to erythrocytes. In the glycoside, the rate and capacity of the transport were not influenced by the kind of suspending medium.

To reveal the origin of impermeability of GLRI having disaccharide, we examined the synthetic glycosides introduced monosaccharide or disaccharide to the NP as aglycon. The permeation of the glycosides with NP as aglycon showed tendencies similar to those above. The permeation of NP was rapid and equilibrium was attained almost within one minute. The permeation of the NGLP and NGAP with monosaccharide was slower. It took 1 h before the equilibrium was attained. The NLP and NMP with disaccharide scarcely permeated the erythrocyte membrane. The concentration of the permeating NP was appreciably higher in the permeation from PBS to erythrocytes than from plasma to erythrocytes. In the glycosides, NGLP, NGAP, NLP and NMP, the rate and capacity of the transport were not influenced by the kind of suspending medium used.

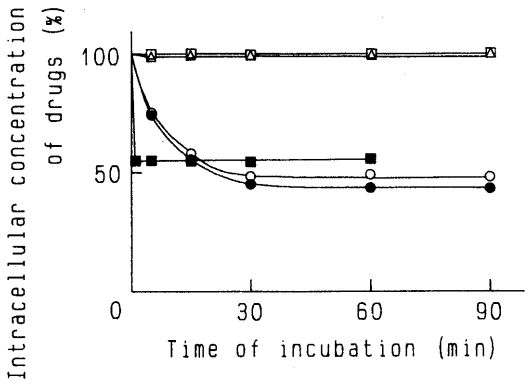


Fig. 1 Release of Glycoside and Its Aglycon from the Drug-Containing Preswollen Ghosts into the Suspending PBS. NP(■), NGLP (●), NGAP(○), NLP(□), NMP(△).

Table II. Dissociation Constant and Partition Coefficient of Drugs

| Drug | pKa | log P |
|---|---|---|
| SAL | N [a)] | -1.28 |
| SALG | 10.05 | 0.68 |
| ARB | 9.85 | -1.64 |
| HYD | 10.33 | 0.45 |
| GLRI | 4.93 | 2.80 |
| GLRE | 4.63 | 4.77 |
| NP | 7.07 | 1.90 |
| NGLP | N | -0.46 |
| NGAP | N | -0.66 |
| NLP | N | -1.64 |
| NMP | N | -1.46 |

a) N is nonelectrolytes.

As shown in Fig 1, a behavior similar to the inward transport was also observed in the outward transport of the drugs from preswollen ghosts to the suspending medium. The outward transport of HYD, SALG, GLRE and NP was also very rapid and almost completed within one minute. GLRI, NLP and NMP entrapped in the erythrocyte interiors according to the method of preswollen ghost released very little through the human erythrocyte membrane but the ARB, SAL, NGLP and NGAP with a monosaccharide were released, though the rate of transport was slow.

Table II shows the dissociation constant and partition coefficient of the drugs. Generally, the transport of drug is fundamentally related to the partition coefficient, as the drugs permeate lipid membrane.[5] The Tmax was related to the partition coefficient (correlation coefficient, $r = -0.98$) in glycosides, except the GLRI, NLP and NMP with disaccharide. The permeability was related to neither the dissociation constant nor the partition coefficient of drugs. It has been remarked that the permeability of a drug is related to the molecular weight (Mw). But it did not appear that the increase of Mw was related to the impermeation since GLRE (Mw, 470.69) permeates rapidly while NLP and NMP (Mw, 463.4) do not permeate at all. The impermeability may be caused by introducing disaccharide.

In this investigation, although there were various questions to consider, it was suggested that introducing disaccharide to drugs we want to deliver impermeatized the drug. An anticancer medicine applied by this mechanism may be entrapped in the erythrocyte carriers. Because most of the erythrocytes are destroyed in the liver and spleen, it may be possible that the anticancer medicine is delivered selectively in them. This investigation may be important for using erythrocyte-entrapped drugs in drug delivery systems.

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(Received March 18, 1991)

# A NEW SYNTHESIS OF THE APORPHINE ALKALOIDS (±)-GLAUCINE AND (±)-NANTENINE

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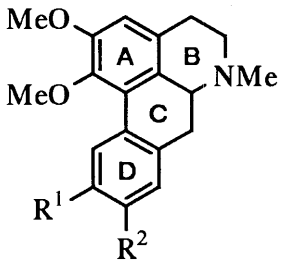
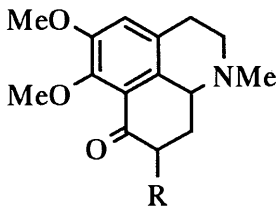
(±)-Glaucine and (±)-nantenine were newly synthesized by aromatization of cyclohexenone derivatives which were prepared by [3C+3C] condensation of two units, 1,1-bis(ethylthio)-2-propanone and the Mannich base.

**KEYWORDS** aromatic synthesis; [3C+3C] annulation; condensation; aporphine alkaloid; (±)-glaucine; (±)-nantenine

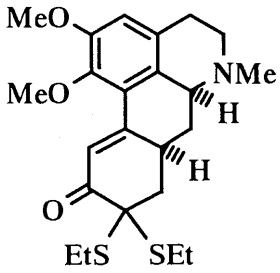
The total synthesis of the aporphine alkaloids has attracted much attention owing to their structural and pharmacological interest.[1] Most of the total syntheses of the bases were achieved, as a key step, by constructing the biphenyl frameworks *via* various intramolecular carbon-carbon bond formations between two benzene rings. On the other hand, annulations to construct aromatic rings are useful method in the synthesis of natural products and biologically active compounds.[2] We reported a new route to the 1,2-benzenediol congeners using [3C+3C] annulation in our previous papers.[3] Here we describe a synthesis of (±)-glaucine (**1**)[4] and (±)-nantenine (**2**)[5] having a 1,2-benzenediol moiety in the D-ring, which prompted us to apply our aromatic synthesis.



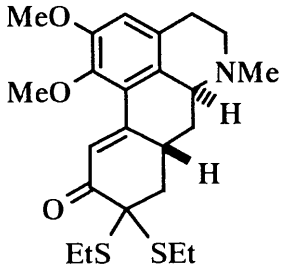

(**1**) $R^1=R^2=$ OMe
(**2**) $R^1, R^2=OCH_2O$
(**8**) $R^1=R^2=$ OH




(**3**) R= $CH_2NMe_2$
(**4**) R= H




(**6**)




(**7**)

The Mannich base (**3**) used as a three-carbon unit of [3C+3C] condensation was derived from the ketone (**4**)[6] on treatment with dimethylamine hydrochloride and formalin in MeOH. Another three-carbon unit was 1,1-bis(ethylthio)-2-propanone (**5**)[7] which has been used for aromatic annulation in our laboratory.[3,8] The Mannich base (**3**) was condensed with the ketone (**5**) in the presence of NaH in dimethoxyethane to give two isomeric cyclohexenones (**6**) and (**7**) (47% and 11% yields from compound **4**

respectively). These were separated by column chromatography. Inspection of a molecular model shows that the enone part (C=CH-C=O) of **6** is located on the same plane as the benzene ring, while that of **7** is twisted from the aromatic ring. These structural characteristics can be well explained by their IR, $^1$H-NMR, and UV spectral data.[9] The ketone (**6**) was dethioacetalized by NBS in MeCN / AcOH / $H_2O$ and isomerized by refluxing AcOH to give the diol (**8**) in 38% yield. The isomeric ketone (**7**) was also converted to **8** in the same manner (32%). Methylation of **8** with diazomethane in $Et_2O$ / MeOH gave (±)-glaucine (**1**) in 71% yield. The melting points of compound **1** (mp 134-136°C)[10] and its picrate [mp 192-194°C (dec.)][11] were the same as those of (±)-glaucine reported in the literature. The IR, $^1$H-NMR, and UV spectral data of **1** were superimposable over those of (+)-glaucine.

Methylenation of compound **8** with KOH and $CH_2Cl_2$ in DMSO gave (±)-nantenine (2) in 43% yield. Its melting point (mp 140-141°C)[12] and spectral data ($^1$H-NMR[5] and Mass[13]) were also identical with those reported in the literature.

In conclusion, (±)-glaucine (**1**) and (±)-nantenine (**2**) were prepared from both cyclohexenones (**6**) and (**7**), which were obtained from condensation of two 3C units, 1,1-bis(ethylthio)-2-propanone (**5**) and the Mannich base (**3**). The utility of the [3C+3C] annulation was shown by the aromatic synthesis of the aporphine alkaloids which have a 1,2-benzenediol moiety.

**ACKNOWLEDGMENT** The authors thank Professor Osamu Hoshino of the Science University of Tokyo for providing the authentic sample of natural glaucine.

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9) Compound **6**: IR ($CHCl_3$) 1646 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) δ 7.58 (1H, d, $J$=2.5 Hz, olefinic proton). UV (EtOH) $\lambda_{max}$ nm(ε): 315.4(17990); Compound **7**: IR ($CHCl_3$) 1652 $cm^{-1}$. $^1$H-NMR ($CDCl_3$) δ 6.50 (1H, d, $J$=2.5 Hz, olefinic proton). UV (EtOH) $\lambda_{max}$ nm(ε): 302.4(13008).

10) lit.[4b] mp 118-120°C; lit.[4c] mp 134-136°C; lit.[4f] mp 127-129°C; lit.[4g] mp 137-139°C.

11) lit.[4d] mp 190-193°C(dec.); lit.[4e] mp 191-193°C(dec.); lit.[4f] mp 199-200°C (dec.); lit.[4h] mp191-192°C(dec.); lit.[4i] mp 191-193°C (dec.); lit.[4k] mp 193-194°C.

12) lit.[4d] mp 138-139°C; lit.[5a] mp 140-142°C; lit.[5b] mp 141-142°C.

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(Received March 25, 1991)

# FORMATION OF SUPEROXIDE BY BENZOFURAZANS IN *ESCHERICHIA COLI* UNDER AEROBIC INCUBATION

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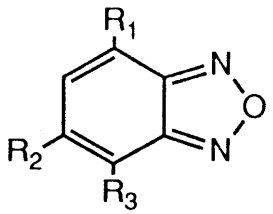
The superoxide ($O_2^{\cdot-}$) production in *Escherichia coli* through the action of benzofurazans (BZs) was examined using the cytochrome *c* (cyt. *c*) reduction method. Adding BZs to *E. coli* cell suspensions caused the cyt. *c* reduction, which was completely inhibited by superoxide dismutase (SOD). The effects of BZs on cyt. *c* reduction was in the order of benzofurazan (**1**) ≈ 4,7-dimethylbenzofurazan (**2**) ≈ 4,7-dibromobenzofurazan (**3**) < 4-bromo-6-cyanobenzofurazan (**4**) < 4,7-dicyanobenzofurazan (**5**). This was correlated with the toxicity of BZs against *E. coli* growth (**1** ≈ **2** ≈ **3** < **4** < **5**) and with the redox potentials of BZs (**1** ≈ **2** < **3** < **4** < **5**). The formation of compound **5** anion radical in the cell suspensions in the absence of dioxygen ($O_2$), was determined using ESR spectrum. The ESR signal of the anion radical disappeared with the addition of $O_2$. The BZs effected the $O_2^{\cdot-}$ production in *E. coli* cells.

**KEYWORDS** benzofurazan; oxygen toxicity; 4,7- dicyanobenzofurazan anion radical; superoxide; cytochrome *c* reduction

There has been considerable interest in redox active compounds such as paraquat (PQ),[1-3] plumbagin[4-6] and heteropentalenes[7-10] which increase the intracelluar flux of superoxide ($O_2^{\cdot-}$) by incubating aerobic *Escherichia coli*.

Recently, as part of our research on benzofurazans (BZs),[11-12] we have reported the toxicity of BZs in *E. coli*, which may be due to $O_2^{\cdot-}$ produced within *E. coli* in the presence of dioxygen ($O_2$).[13] BZs inhibit the growth of *E. coli*, induce the specific activity of superoxide dismutase (SOD), and greatly increase the rate of the cyanide-resistant respiration.

Here we present the results of the $O_2^{\cdot-}$ production in *E. coli* through the action of BZs (Chart 1) using the cytochrome *c* (cyt. *c*) reduction method and ESR spectra.



| Compound | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| **1** | H | H | H |
| **2** | $CH_3$ | H | $CH_3$ |
| **3** | Br | H | Br |
| **4** | Br | CN | H |
| **5** | CN | H | CN |

Chart 1. Structures of Benzofurazan and Its Derivatives

*E. coli* B $B_{12}^-$, ATCC 29682, was grown for 16 h in glucose minimal medium. *E. coli* cell suspensions were prepared as previously described.[13] The cell suspensions used were about 5.2 x $10^8$ cells/ml ($OD_{600}$ = 0.65). Reaction was started by adding BZs or PQ to the reaction mixture containing 25 μM cyt. *c* and the *E. coli* cell suspensions. The spectral change of the absorbance at 550 nm was measured at 25 °C.

Compounds **4** and **5** mediated markedly cyt. *c* reduction in *E. coli* (Fig. 1). As shown in Fig. 1, the effects of the BZs on cyt. *c* reduction were in the order of **1** ≈ **2** ≈ **3** < **4** < **5**, which was correlated with the toxicity of BZs against *E. coli* growth (**1** ≈ **2** ≈ **3** < **4** < **5**)[13] and with the redox potentials of BZs [**1** ($E_{1/2}$ = -1440mV vs SCE in $CH_3CN$) ≈ **2** (-1570mV) < **3**

(-1043mV) < **4** (-788mV) < **5** (-455mV)]. Compound **5**, which has a higher redox potential than other BZs, was the most potent mediator of the cyt. *c* reduction.

PQ also mediated the cyt. *c* reduction in *E. coli* cells aerobically, but much less effectively than compound **5** (Fig. 1, line 6). The rate of the cyt. *c* reduction mediated by PQ was less than one-tenth of that by compound **5** as shown in Fig. 1, lines 5 and 6.

Figure 2 shows that the cyt. *c* reduction depended on the concentration of compound **5**. Compound **5** induced this reduction catalytically in the presence of *E. coli* and $O_2$. SOD completely inhibited the reduction (Fig. 2, line 6), which indicates that the cyt. *c* reduction was caused by $O_2^{\cdot-}$.[14] The cyt. *c* reduction provoked by PQ was also inhibited by SOD (data not shown). BZs, like PQ, appear to act as a mediator in *E. coli* to produce $O_2^{\cdot-}$.

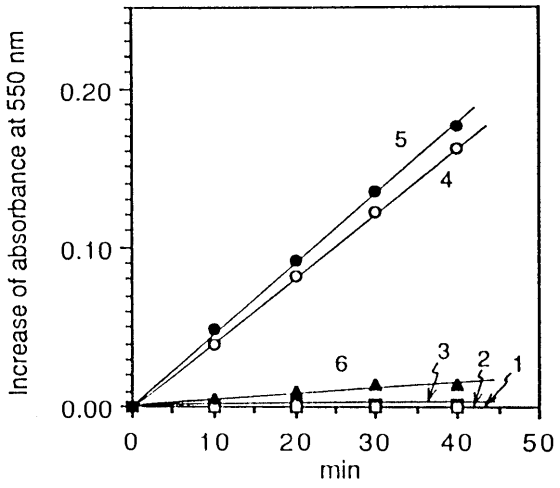



Fig. 1. Cyt. *c* Reduction Mediated by BZs in *E. coli*

Line 1 , 1 mM compound **1**; line 2 , 1 mM compound **2** ; line 3 , 1 mM compound **3** ; line 4 , 1 mM compound **4** ; line 5 , 10 μM compound **5** ; line 6 , 10 μM PQ.

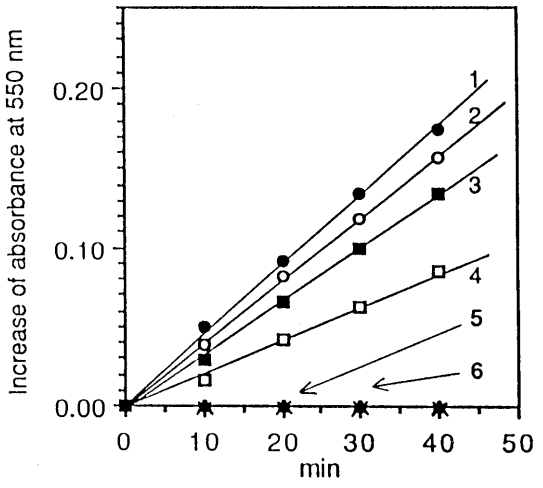



Fig. 2. Cyt. *c* Reduction Mediated by Compound **5** in *E. coli*

Line 1 , 10 μM compound **5** ; line 2, 7.5 μM compound **5** ; line 3 , 5 μM compound **5** ; line 4 , 1 μM compound **5** ; line 5 , 0 μM compound **5** ; line 6 , 10 μM compound **5** and SOD (2000U).

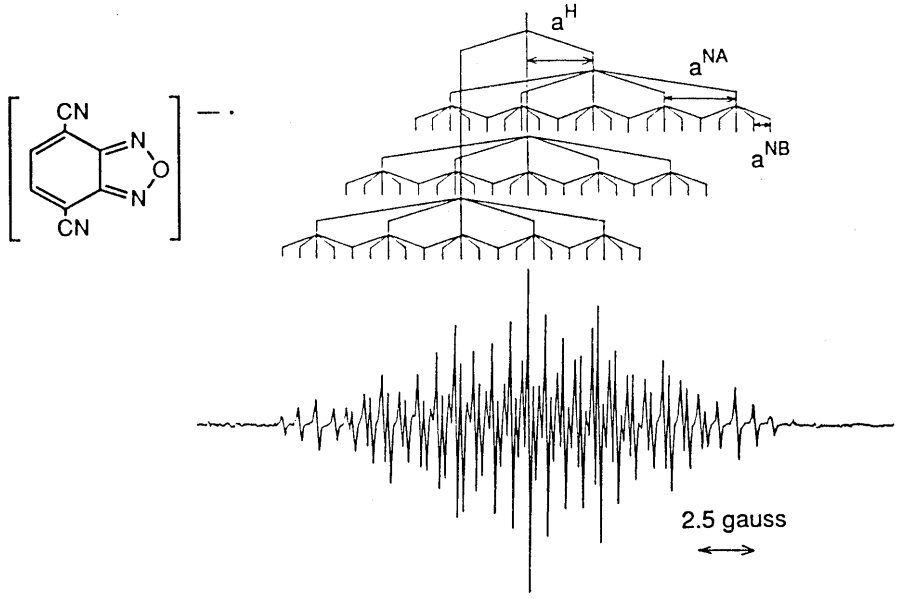
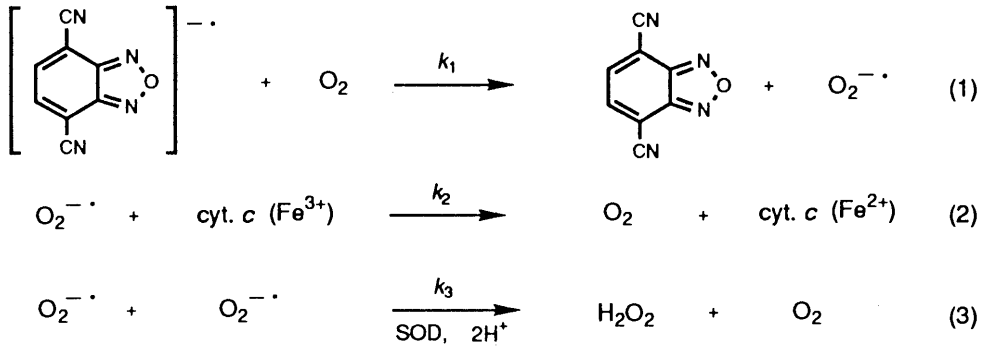



Fig. 3. ESR Spectrum of Compound **5** Anion Radical Produced by *E. coli* Cell Suspensions-Compound **5**-Glucose System at 25 °C under an Ar Atmosphere

Fig. 3 shows the ESR spectrum of the reduced form of compound **5** at 25 °C, which can be obtained by adding compound **5** to *E. coli* cell suspensions in the presence of glucose under an Ar atmosphere. The splitting constants were : $a^H$ = 2.9 gauss, $a^{NA}$ = 3.2 gauss, $a^{NB}$ = 0.8 gauss. The hyperfine couplings of this spectrum were fairly consistent with those ( $a^H$ = 2.4 gauss, $a^{NA}$ = 3 gauss, $a^{NB}$ = 0.8 gauss ) of 2,1,3-benzothiadiazole-4,7-dicarbonitrile anion radical generated electronically.[15] The signal of the spectrum in Fig. 3 disappeared on addition of a small amount of $O_2$. From these results, the reduced form of compound **5** can be assigned to compound **5** anion radical. The mechanism of the $O_2^{-\cdot}$ production in *E. coli* is proposed as follows.

Chart 2




BZs can be reduced to the corresponding anion radicals in *E. coli* and be reoxidized by $O_2$ with the production of $O_2^{-\cdot}$ and regeneration of the parent compound ( Chart 2, eq. 1 ). The reaction in Chart 2, eq. 2 can be inhibited by SOD, because $k_3$ ( = 1.8 ~ 3.5 x $10^9$ $M^{-1}s^{-1}$ : the rate constant of $O_2^{-\cdot}$ disproportionation by SOD ) is bigger than $k_2$ ( = 0.3 ~ 1.4 x $10^6$ $M^{-1}s^{-1}$ ). [16-18]

In this communication, production amounts of $O_2^{-\cdot}$ in *E. coli* through the action of BZs were examined with or without SOD using the cyt. *c* reduction method. That the activity was well correlated with the bacteriostatic activity of BZs should support the idea that the toxicity is due to $O_2^{-\cdot}$ production.

Further studies on the differences in the toxicities between BZs and PQ are in progress.

**ACKNOWLEDGEMENT** The authors are grateful for a research grant from the Ministry of Education, Science and Culture of Japan.

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(Received March 25, 1991)

# STRUCTURE OF MURRANIMBINE, A NOVEL DIMERIC CARBAZOLE ALKALOID FROM *MURRAYA EUCHRESTIFOLIA*[1)]

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In continuous studies of the constituents of *Murraya euchrestifolia* Hayata (Rutaceae) collected in Taiwan, a novel dimeric carbazole alkaloid named murranimbine was isolated and the structure was characterized as **1** by spectrometric analysis.

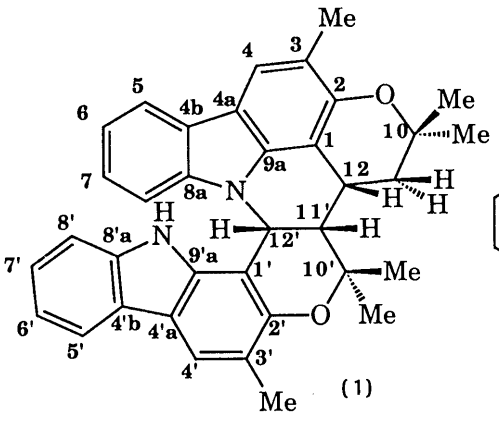
KEYWORDS *Murraya*; *Murraya euchrestifolia*; Rutaceae; carbazole alkaloid; murranimbine; girinimbine; dimer; HMBC

*Murraya euchrestifolia* Hayata, a shrub growing in the central and southern parts of Taiwan, has been reported to contain many kinds of carbazole[2)] and carbazolequinone[3)] alkaloids. Further studies of the components of the root bark of the plant gave a novel dimeric carbazole alkaloid. This paper deals with the spectrometric structural elucidation of this dimeric carbazole alkaloid named murranimbine (**1**).

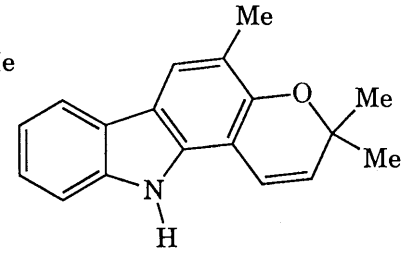
Murranimbine (**1**) was isolated as a racemic pale yellow oil[4)] from the acetone extract by repeated silica gel column and preparative thin layer chromatograhy. The molecular formula $C_{36}H_{34}N_2O_2$ [$M^+$ 526.2585. Calcd. 526.2617] was confirmed by high-resolution mass spectrometry . The UV absorption bands in MeOH at $\lambda_{max}$ 218, 240, 260, 306, and 336 nm and the IR bands in $CHCl_3$ at $\nu_{max}$ 3460 $cm^{-1}$ due to an N-H group indicated the presence of a carbazole nucleus in the molecule.[5)] An occurrence of a characteristic mass fragment ion at m/z 263 analyzed for $C_{18}H_{17}NO$ [$M^+$ 263.1338. Calcd. 263.1310] corresponding to a half of the molecule indicated a dimeric carbazole structure of murranimbine (**1**). In the $^1$H-NMR spectrum in acetone-$d_6$ (Table I), there were two aryl methyls at $\delta$ 2.30 and 2.32, four geminal methyls attached to oxygenated carbons at $\delta$ 0.60, 1.54, 1.44, and 1.60, and a $D_2O$ exchangeable N-H proton signal at $\delta$ 10.55. In the H-H COSY spectrum, there were two sets of four aromatic proton signals (each 1H), including the characteristic H-5[5,6)] and H-5' in the carbazole nuclei (see Table I), and two deshielded signals at $\delta$ 7.65 and 8.00 which had long-range couplings with the aryl methyls. Also there were NOE responses with the aryl methyls. These features showed the presence of a non-substituted aromatic A-ring and H-4, each in the carbazole nucleus. Further, the relationships of the remaining signals at $\delta$ 6.12 (1H, d, J=2.9 Hz, H-12'), 2.74 (1H, dd, J=2.9 & 5.1 Hz, H-11'), 4.07 (1H, m, H-12), 2.21 (1H, dd, J=5.1 & 12.8 Hz, H-11), and 2.08 (H-11, overlapped by the solvent signal) were shown by the H-H COSY spectrum. Among these, the doublet at $\delta$ 6.12 was assignable to a proton (H-12') on a benzylic methine attached to a nitrogen atom considering the lower chemical shift value of its proton. These data together with the result of an H-C long-range COSY (J=5 Hz) spectrum, shown by arrows in formula **3**, suggested that this alkaloid contained two girinimbine (**2**)[3,8)] units in the molecule. However, no information about the connection between the two girinimbine (**2**) units was obtained from this COSY spectrum. Next, we analyzed the HMBC spectrum (J=8 Hz) of murranimbine (**1**). Cross peaks of three-bond correlations related to the connectivity of two units (**2**) are shown by the lines in Fig 1. The proton (H-12', $\delta_H$ 6.12) of the benzylic methine attached to N-atom was related to both another benzylic methine carbon (C-12, $\delta_C$ 32.43) on upper unit and to the oxygenated quaternary carbon (C-10', $\delta_C$ 80.13) on the lower unit, which was

Table I.[9] $^{1}H$- and $^{13}C$-NMR Data for Murranimbine (1) in Acetone-$d_6$

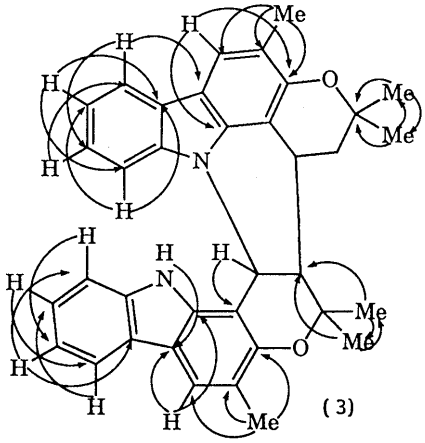
| NO[6] | $\delta_H$ | $\delta_C$ | NO[6] | $\delta_H$ | $\delta_C$ |
|---|---|---|---|---|---|
| 1 | - | 103.74 | 1' | - | 104.98 |
| 2 | - | 150.27 | 2' | - | 152.11 |
| 3 | - | 118.65 | 3' | - | 119.32 |
| 3-Me | 2.32* (3H, s) | 16.61 | 3'-Me | 2.30* (3H, s) | 16.61 |
| 4 | 7.65 (s) | 120.00 | 4' | 8.00 (s) | 122.95 |
| 4a | - | 113.24 | 4'a | - | 116.63 |
| 4b | - | 124.92 | 4'b | - | 124.69 |
| 5 | 7.84 (d, 7.7) | 119.84 | 5' | 8.08 (d, 7.3) | 120.09 |
| 6 | 6.87 (t, 6.7) | 118.98 | 6' | 7.19 (t, 7.0) | 119.94 |
| 7 | 6.61 (t, 7.0) | 124.18 | 7' | 7.30 (t, 7.0) | 124.96 |
| 8 | 6.17 (d, 8.4) | 111.03 | 8' | 7.44 (d, 7.7) | 111.51 |
| 8a | - | 140.51 | 8'a | - | 141.08 |
| 9 | - | - | 9' | 10.55** | - |
| 9a | - | 138.64 | 9'a | - | 139.72 |
| 10 | - | 76.51 | 10' | - | 80.13 |
| 10-Me | 1.44 (3H, s) | 24.64 | 10'-Me | 0.60 (3H, s) | 25.00 |
| 10-Me | 1.60# (3H, s) | 30.84 | 10'-Me | 1.54 (3H, s) | 34.31 |
| 11 | 2.08# | 37.10 | 11' | 2.74 (dd, 2.9, 5.1) | 45.50 |
| | 2.21 (dd, 5.1, 12.8) | | | | |
| 12 | 4.07 (m) | 32.43 | 12' | 6.12 (d, 2.9) | 52.78 |



(1)



(2)

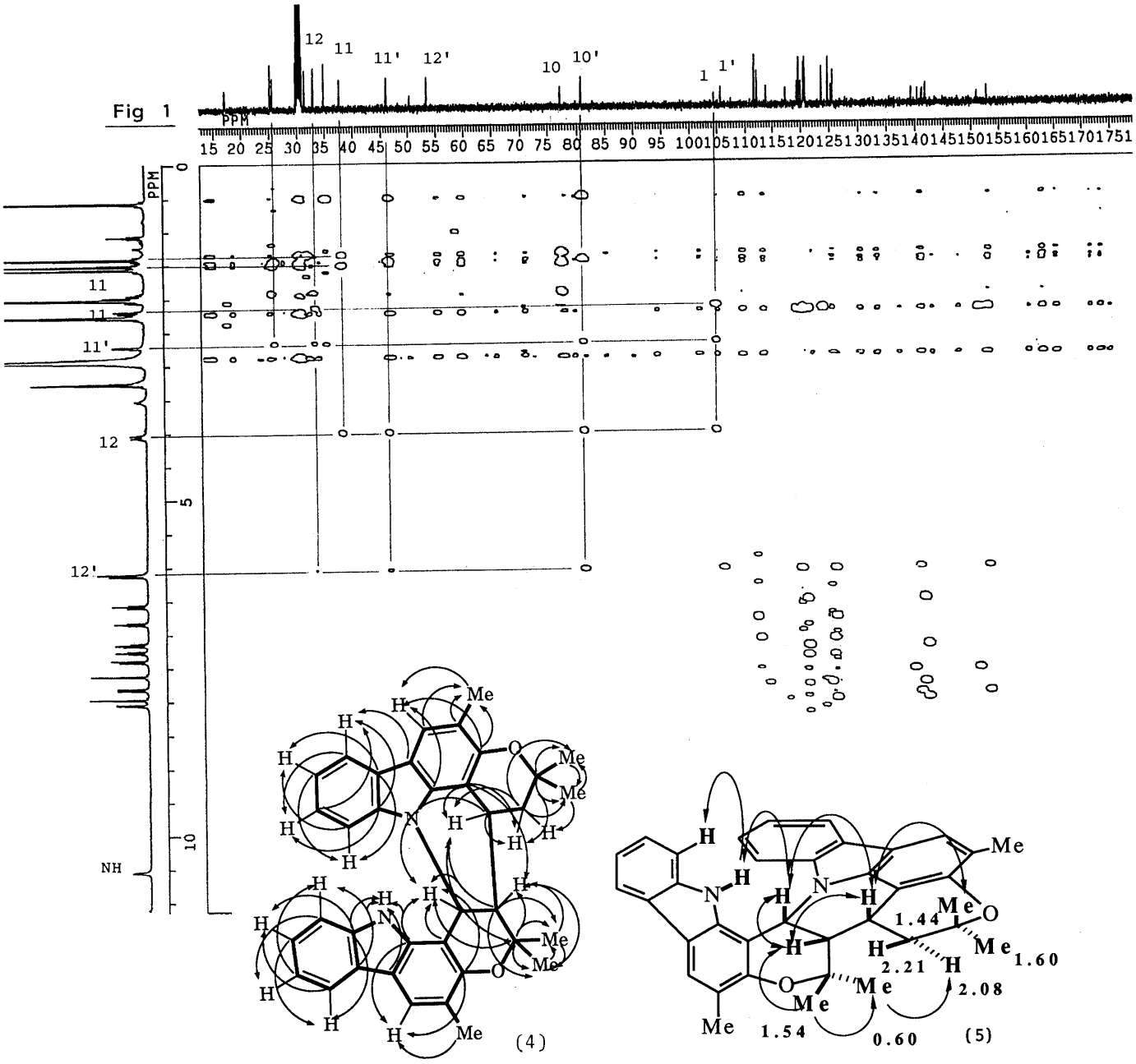


(3)

also related to the benzylic methine proton (H-12, $\delta_H$ 4.07) in the upper unit. The methine proton (H-11', $\delta_H$ 2.74) coupled with both of two benzylic protons [$\delta_H$ 6.12 (H-12') and $\delta_H$ 4.07 (H-12)] had a three-bond relationship with an aromatic carbon (C-1, $\delta_C$ 103.74), which was related to the methylene protons (H-11, $\delta_H$ 2.21) in the upper unit. The methylene carbon (C-11, $\delta_C$ 37.10) was also related to the geminal methyl protons at $\delta_H$ 1.44 and 1.60. The presence of these three-bond relationships in the HMBC spectrum as well as other relationships summarized in formula 4 indicated that the structure of murranimbine should be represented by 1. The relative stereochemistry was proposed by correlations through differential NOE experiments (formula 5). Irradiation of H-12' resulted in a 6% enhancement of the signal at $\delta$ 2.74 (H-11') and 4.07 (H-12) as well as a 7% enhancement of the N-H signal. In the irradiation of H-11', there were 7 and 8% area increases in the signals at $\delta_H$ 6.12 (H-12') and 4.07 (H-12), respectively. In the irradiation of H-12, 8 and 5% enhancements of the signal at $\delta_H$ 6.12 (H-12') and 2.74 (H-11'), respectively, appeared as well as a 10% NOE of the methyl signal at $\delta_H$ 1.44. These results led us to assign the structure 1 to murranimbine.

The structural component, girinimbine (2), has been known to occur in some plant sources.[3,5,8] However, isolation of the dimer 1 represents the first example from nature.

Fig 1




ACKNOWLEDGEMENTS We thank Professors T.-S. Wu (National Cheng Kung University, Taiwan) and C.-S. Kuoh (Chia-Nan Junior College of Pharmacy, Taiwan) for the collection and identification of the plant material, and for generous discussions.

REFERENCES AND NOTES

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3) H. Furukawa, T.-S. Wu, T. Ohta, and H. Furukawa, Chem. Pharm. Bull., 33, 4132 (1985).
4) $[\alpha]_D$ 0° ($CHCl_3$). No CD (in MeOH) absorption was observed in 210 - 400 nm range.
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6) Numbers were given tentatively as shown in formula 1.
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9) Values are in $\delta$ (ppm) from TMS. Each proton signal corresponds to 1 H, unless otherwise stated. Figures in parentheses are coupling constants (J) in Hz. All assignments were established by H-H, H-C, and H-C long-range COSY, and HMBC spectrometries and differencial NOE experiments. * Long-range coupled. ** Disappeared with $D_2O$. # Overlapped the solvent signal.

(Received April 3, 1991)